Standard Operating Procedures for Fungal Identification and Detection of Antifungal Resistance

Antimicrobial Resistance Surveillance and Research Network

2nd Edition, 2019

Indian Council of Medical Research
New Delhi, India

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Foreword

The fungal infections have become important to Indian context, and this manual details the procedures of identification and detection of antifungal resistance for medically important fungi. This Standard Operating Procedures (SOP) is intended to assist as a reference material for routine activities of a clinical mycology laboratory of ICMR Antimicrobial Resistance Surveillance and Research Network (AMRSN). This edition of SOP embodies the continued efforts of ICMR to provide clinically relevant and practical document for the standardized in vitro susceptibility testing of fungal pathogens.

Important topics such as procedures for the sample collection, transport and processing of specimens, taxonomy and characteristics of important fungi, and techniques for the culture and identification of fungal pathogens, antifungal susceptibility testing (AFST) are elaborated in the SOP. The second edition of SOP is updated with the new topics such as cultural and microscopic features (phenotypic identification) of medically important moulds, and MALDI-TOF based fungal identification. The manual is provided with the latest essential information regarding the antifungal drug susceptibility testing methods such as broth micro dilution method and disk diffusion testing for yeasts and moulds including revised breakpoints. The breakpoints for the control isolates are also included in the manual. The manual has updated information for molecular testing of antifungal drug resistance mechanism especially for azole resistance in yeasts. Reference to any commercial method or equipment does not mean endorsement of ICMR, this is only for the purpose of this research study.

The editors have done a commendable job in compiling and presenting the manual in easy and precise manner. I applaud the diligent efforts of all the contributors and ICMR team. I hope that users will find it impactful and will be benefited immensely from this manual. I am optimistic that this manual will meet its intended objectives and it will further evolve for the clinical as well as research purposes through periodic revisions and updates.

I convey my best wishes to all.

Prof. Balram Bhargava
Secretary, DHR & Director General, ICMR

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ABBREVIATIONS

ACN	Acetonitrile
ATCC	American Type Culture Collection
BA	Blood Agar
$BaSO_4$	Barium Sulphate
BDT	Big dye terminator
BHIA	Brain Heart Infusion Agar
BSA	Birdseed agar
CFU	Colony Forming Unit
CFW	Calcofluor white
CGB	Canavanine Glycine Bromothymol Blue Agar
cm	Centimetre
CMA	Corn Meal Agar
CSF	Cerebro-spinal fluid
CZ	Czapek Dox agar
DDW	Double distilled water
DEPC	Diethyl pyrocarbonate
dL	Decilitre
DMSO	Dimethyl Sulphoxide
dNTP	Deoxyribonucleotide triphosphate
DRBC	Dichloro-Rose-Bengal-Chloramphenicol
DW	Distilled water
ECV	Epidemiological Cut-Off Value
EDTA	Ethylenediaminetetraacetic acid
EtOH	Ethyl alcohol
FA	Formic acid
GI	Gastrointestinal
GMS	Gomori's Methenamine Silver Stain
GT	Germ Tube
HCCA	α-Cyano-4-hydroxycinnamic acid
HCl	Hydrochloric acid

HIV	Human-Immunodeficiency Virus
KH_2PO_4	Monopotassium phosphate
KNO_3	Potassium Nitrate
KOH	Potassium Hydroxide
LCB	Lactophenol Cotton Blue
MALDI-MS	Matrix-assisted laser desorption/ionization Mass spectroscopy
MEA	Malt Extract Agar
MEC	Minimal Effective Concentration
mg	Milligram
MIC	Minimum Inhibitory Concentration
mL	Millilitre
Na_2HPO_4	Disodium Phosphate
NALC	N-acetyl L- cysteine
OD	Optical Density
PAS	Periodic acid Schiff
PCR	Polymerase chain reaction
PDA	Potato Dextrose Agar
QC	Quality Control
RPM	Rotation per minute
RPMI	Roswell Park Memorial Institute
SDA	Sabouraud dextrose agar
TAE buffer	Tris-Acetate-EDTA buffer
TE buffer	Tris-EDTA buffer
TFA	Trifluoroacetic acid
UV	Ultra-Violet
YCB	Yeast carbon base
YMA	Yeast malt agar
YNB	Yeast Nitrogen Base
µg	Microgram

Sample Collection, Transport and Processing of Specimens

CHAPTER 1

Specimen Collection, Transport and Processing

The principal goals of a sound clinical mycology laboratory are to isolate efficiently and to identify accurately the suspected etiological agents of fungal infection. Success depends much on the quality of clinical specimens sent to the laboratory.

The following points need to be emphasized:

a. Appropriate sample/specimen collection
b. Prompt transportation
c. Correct processing of the specimen
d. Inoculation of specimens onto appropriate culture media and incubation at suitable temperature

Specimens should be collected aseptically, placed in sterile containers, delivered to the laboratory within 2 hours, processed, and then inoculated to primary isolation media within a few hours of collection. Viability may decrease with prolonged specimen storage.

Swabs are not encouraged; however, specimens from the environment or certain body sites such as the ear canal, nasopharynx, throat, vagina and cervix are not readily collected by other means. Swabs for collection of material from open wounds or draining lesions are frequently contaminated with environmental microorganisms.

All specimens sent to mycology laboratory must be clearly labeled with the patients' name, age, sex, unit number, date and time of collection, source of specimen, antimicrobial therapy together with a brief relevant clinical history and the name of the attending physician.

1. Collection of specimen

Table 1.1: Collection of specimens for diagnosis of fungal infections

S. No	Specimen	Collection	Unacceptable specimen
1	Pus	• Aseptically with needle and syringe from undrained abscess. • Pus expressed from abscess opened with scalpel; transported to laboratory either in sterile container/ syringe and needle	Swab or materials from open wound
2	Biopsy	• Place between two sterile gauze pads, sterile petri dish/ tube (containing 2-3 mL of sterile normal saline/ brain heart infusion broth) • Tissue is collected from centre and edge of the lesion.	Swabs, samples collected in thioglycolate broth or normal saline

3	Grains	• Collected by lifting the crust at the opening of a sinus. • Grains frequently found underneath the pus or collected from the removed bandages • Aspirated from undrained sinuses	
4	Cerebrospinal fluid	• 3 mL in a sterile tube	Insufficient quantity
5	Body fluids	• Sterile tube or in a heparinized syringe	Swabs
6	Bone marrow	• 0.2-0.3 mL collected in a sterile heparinized syringe • Sterile cap is placed on heparinized syringe and transported immediately	Clotted bone marrow
7	Blood	• 5-10 mL in yellow Vacutainer/syringe or in biphasic media containing brain heart infusion broth and agar; blood: broth ratio should be maintained at 1:10. • Multiple blood cultures at timed intervals to be collected • BACTEC/lysis centrifugation technique may improve sensitivity	
8	Urine	• Early morning 25-50 mL of clean catch midstream urine specimen • Suprapubic aspirate, catheterized specimen • Collected in sterile container	24 hours collection is unacceptable
9	Faeces	• Not usually acceptable in the mycology laboratory. • Sometime collected to access *Candida* carriage in GI tract.	
10	Sputum	• 5-10 mL; early morning prior to eating • Use mouth rinse and brush before collection • Collected in sterile wide mouthed container.	Saliva, nasal secretion, throat swab, 24 hour collection
11	Bronchial brush/washing/ broncho alveolar lavage	• Collected in sterile container using fiber optic bronchoscopes	Dried specimen
12	Lung biopsy	• Collected by bronchoscope, • fluoroscope guided trans-thoracic needle aspiration or open lung biopsy • Best specimen is open lung biopsy but it is hazardous	
13	Serology	• Serum: 1-2 mL • 3-5 mL of spinal fluid	Specimen collected after skin test with histoplasmin while performing serology for histoplasmosis

2. Transport of specimens

- Specimens should be transported in sterile, humidified, leak-proof container. Dermatological specimens, however, should be transported in a dry container. Transport medium should not be used unless the specimen can be easily and completely retrieved from the medium. Although fungi can be recovered at times from specimens submitted in anaerobic transport media, such media should be avoided.
- Specimens should be processed and inoculated to primary isolation media as soon as possible after collection, ideally within few hours. It should not be presumed that successful methods for storage of fungal cultures are suitable for temporary storage of clinical specimens that harbor relatively few fungal cells.
- The effect of refrigeration on fungal specimens has not been well studied, but if processing is to be delayed for more than several hours, it is recommended that specimens be stored under refrigeration at 4°C with the following exceptions: blood and cerebrospinal fluid are stored at 30-37°C (some fastidious organism may not survive at 4°C) dermatological specimens are stored at 15-30°C (dermatophytes survival is best at this temperature).

3. Processing of specimen in the laboratory

- Specimens should be first examined carefully. The examination allows for selection of the proper portion of the specimen that will likely contain the fungus. Caseous, purulent or bloody areas and necrotic materials are processed.
- Specimens from cases of mycetoma are examined with the dissecting microscope for the presence of granules before processing.
- Punch biopsies should be examined carefully to ensure that they are divided vertically and not horizontally so that each layer of tissue is represented in each specimen.
- Clinical specimens must be processed as soon and as carefully as possible on the appropriate isolation media and temperature. Recovery of pathogens is necessary for their identification and evaluation against the antifungal agents.
- Most specimens suspected of having fungi other than dermatophytes should be handled according to practices outlined for Biosafety Level 2**.**

Direct inoculation

- Many specimens (up to 0.5 mL) can be directly inoculated to media; specimens like abscess aspirate, bone marrow aspirates, cerebrospinal fluid, swabs, body fluid, hair, skin scraping, nail, bronchial washing or brushing etc. can be directly inoculated.
- If the fluid has a clot or membranous material, mince with sterile scalpel and inoculate to media. 3-5 drops of fluid should be inoculated to each tube of media.

Concentration

Large volume of fluids should be concentrated by centrifugation (1500-2000 X g for 5 minutes) before inoculation to isolation media as a means to enhance the detection and recovery of fungi.

3.1. Sputum

Method: N-acetyl L- cysteine (NALC) treatment
Principle: NALC is mucolytic agent due to ability to split the disulfide bonds in the mucoprotein.

Stock solution to be prepared:
1. 2.94% of Na-citrate in distilled water – autoclaved at 121^{O}C x 15 minutes
2. 0.5 g of NALC in 100 mL of Na-citrate (to be freshly prepared)
3. M/15 phosphate buffer (pH 6.8-7.1)

Procedure

a. Specimen to be vortexed with sterile glass beads.
b. Equal volume of Na-citrate and NALC to be added to the specimen; to be vortexed again for 10-30 seconds.
c. Dilute mixture in the phosphate buffer by adding double the volume and centrifuge at 1000 g for 15 minutes.
d. Use sediment to prepare smears for direct microscopy and to inoculate on media for culture.

3.2. Blood

Biphasic medium

- At least 3-5 mL blood is cultured in biphasic medium (a ratio of 1 : 10 to 1 : 20 blood to broth is utilized).
- Two bottles can be utilized. One can be incubated at 25^{0}C and the other at 37^{0}C. After 48 hours, 5 days, and 7 days incubation, tilt the bottle in such fashion that fluid covers the whole agar surface; but does not reach the neck of the bottle. Keep the bottle in this position for one hour.

Lysis centrifugation system

- The Isolator (Wampole Laboratories, Cranbury, NJ, USA) or manual lysis centrifugation system appears to be most sensitive method for recovery of *Histoplasma capsulatum,* other dimorphic fungi and filamentous fungi.
- Isolator specimen should remain at room temperature until processing, optimally within 16 hours of collection.
- The sediment is streaked on varieties of media without cycloheximide.
- On chocolate agar, all fungi can grow well except *H. capsulatum*. For isolation of *H. capsulatum* BHIA with glutamine and Pine's agar medium can be used.
- For *H. capsulatum* plates should be incubated at least for 3 weeks

Automated, continuously monitoring blood culture system

- ESP system (Trek Diagnostics, Westlake, Ohio, USA), Bac T/Alert system (BioMérieux, Marcy-l'Étoile, France) and Bactec system (Becton Dickinson Microbiology system, Sparks, MD, USA).
- 5 mL of blood is sufficient.
- Bactec Myco/F Lytic medium can be used both for *Mycobacterium* & fungi.

3.3. Body fluid, urine

Filtration or centrifugation at 2000 X g for 10 minutes.

3.4. Exudates, pus, drainage

Washing, centrifugation and crushing of granules.

3.5. Cerebrospinal fluid

a. No characteristic pathognomic feature seen
b. Pressure - normal or increased
c. Glucose - normal or slightly decreased (10-40 mg/dL)
d. Protein - usually elevated, sometimes very high (50-1000 mg/dL)
e. Alcohol content - rarely helpful
f. Pleocytosis - rarely exceeds 300 (range 20-1000/mm^3)
g. Predominant lymphocytes (*Cryptococcus, Histoplasma*)
    - ♦ Predominant polymorphs (*Aspergillus, Candida*) Organisms
    - ♦ Positive in *Cryptococcus* (~50% in India ink)*, Candida*
    - ♦ Rarely in *Histoplasma, Aspergillus*
    - ♦ Negative in intracerebral lesion
    - ♦ Gram stain help in *Candida, Nocardia* & *Actinomyces*

e. Difficult to isolate fungi from CSF except *Cryptococcus neoformans*
    - ♦ *C. immitis, H. capsulatum, B. dermatitidis* infection occasionally positive
    - ♦ *Candida, Aspergillus*, Zygomycetes – very low yield.

To improve isolation of fungi

- Always culture 10-30 mL of CSF.
- Centrifuge CSF - culture & microscopy of sediment; supernatant may be used for antigen detection.
- Repeat lumbar puncture at least 3 times.
- May collect sample even after start of therapy as fungi can be isolated till 3rd day of therapy.
- Consider cisternal puncture if lumbar puncture is negative.

Catheter tip for isolation of fungi

- External dressing carefully removed.
- Alcohol used to remove blood, or antibiotic ointment from the site of catheter insertion. Catheter is withdrawn through the skin.
- Sterile forceps may be used to remove the catheter.

Short intra-vascular catheters: Shaft cut off from the catheter hub with sterile scissor and dropped into a sterile vessel or cup (the catheter segment length should be 2.5 - 5 cm).

Larger indwelling catheters: Distal 5 cm should be aseptically collected.

Purulent exudates: If present at the catheter exit site, a sample should be collected for Gram stain and culture.

Catheter tip culture: Catheter tip rolled across the surface of the culture plate at least four times. The laboratory shall report the colony count for the catheter culture. Sub-cultures of *Candida* from line tips stored at 4^{O}C and sent to central lab.

3.6. Tissue specimen (Biopsy)

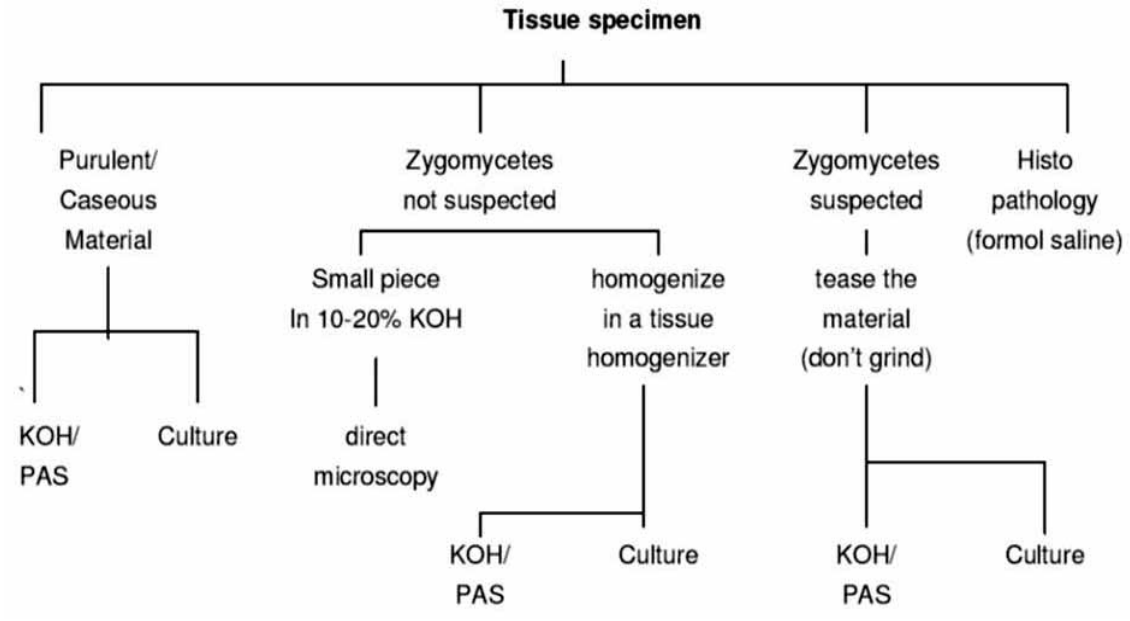

Figure 1: Processing of tissue specimens

4. Direct microscopic examination and staining of clinical specimens

Direct microscopy is of immense importance for mycological investigation of clinical specimens. Unlike bacteria, fungi may take pretty long time to grow in culture. Moreover, many fungi are saprophytic. Therefore, clinical situation should be correlated with direct microscopy findings and culture (Figure 1 & 2).

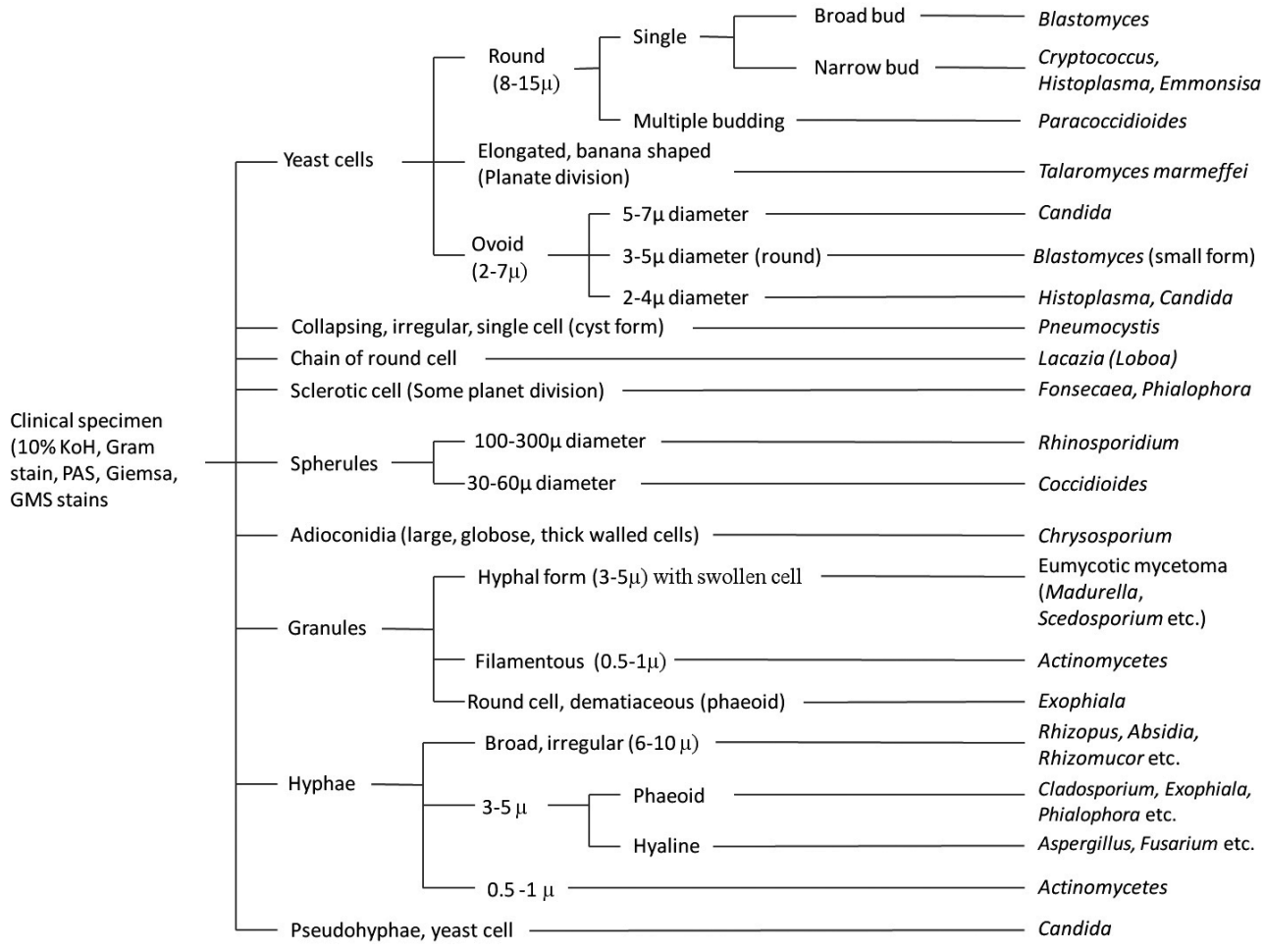

Figure 2: Direct microscopy examination of clinical samples

4.1. Potassium hydroxide mount preparation

Tables 1.2: Potassium hydroxide (KOH-mounting fluid)

(a) With Dimethyl Sulphoxide (DMSO)

Distilled water	60 mL
Potassium hydroxide	20 g
DMSO	40 mL

(b) Without DMSO

Distilled water	80 mL
Potassium hydroxide (KOH)	20 g
Glycerine	20 mL

- Addition of DMSO or glycerine prevents rapid drying of the fluid and permits observations of slide for up to 48 hours.
- DMSO also acts as an excellent cleansing agent.

1. Potassium hydroxide is a strong alkali used as a clearing agent to observe fungi in the specimen in a wet mount preparation.
2. 10-20% KOH is usually used depending on the specimen; occasionally 40% may be used when not cleared by 10-20% KOH.
3. Used for specimen such as sputum, pus, urine sediment, homogenate from biopsy tissue to clear cell debris.

Preparation of the mount

1. Take a clean grease-free glass slide.
2. Place a large drop of KOH solution with a Pasteur pipettete.
3. Transfer small quantity of the specimen with a loop or the tip of a scalpel into the KOH drop.
4. Put a clean cover slip over the drop gently so that no air bubble is trapped.
5. Place the slide in a moist chamber and keep at room temperature.
6. Tissue usually takes 20-30 minutes; sometimes overnight contact with KOH is useful for getting a positive result.
7. Clearing can be hastened by gentle heating of the slide, but it is best avoided.

Observation

1. Examine the clear specimen under low power (10X or 20X objective). Scan the entire cover slip from end to end in a zigzag fashion.
2. If any fungal elements are suspected, examine under high power (40X objective).
3. Reduce the light coming into the condenser while examining at high power.
4. Look for branching hyphae, type of branching, the colour, septation and thickness of hyphae, budding yeast cells.

Modification

For more distinction, stains like methylene blue or Parker blue-black fountain ink may be used along with KOH. This will impart a colored background and fungal elements, if present, will show as prominent refractile objects.

Advantage of KOH preparation

- Simple, cheap and rapid

Disadvantage

- Pus and sputum may contain artifacts, which may superficially resemble hyphal and budding forms of fungi. These artifacts may be produced by cotton or wool fibers, starch grains (in pleuritis) or cholesterol crystals.
- It gives an idea about the presence of hyphal element but cannot distinguish different fungi.
- Preparation cannot be kept for too long; but drying can be prevented /prolonged by keeping the slides in a moist chamber.

Precautions

1. The drop of KOH should not be so large that the cover slip floats.
2. If kept outside a moist chamber, the KOH dries and crystals form that restricts the visibility of the fungus.
3. After clearing, pressure is to be gently applied on the top of the cover slip with a fold of filter paper or the handle of a teasing needle. This ensures even spreading of the material onto the slide.
4. KOH should be kept in a closed container in small aliquots ready to use on the workbench.

Quality assurance

Fungal spores or hyphae may contaminate the KOH solution kept in the laboratory and may give false positive results. So, a negative control should be put up every day.

4.2. KOH-Calcofluor white (CFW) solution mixture

Calcofluor white: This substance is available commercially, under a variety of trade names.

Stock solution: Calcofluor white: 1 g in 100 mL distilled water

Working solution: 1:10 dilution of stock solution in 0.05% Evans blue. Always store the stock and working solution in the refrigerator at 4^0C.

Principle: CFW stain may be used for direct examination of most specimens using fluorescent microscopy. The cell wall of the fungi (b-1,3 and b-1,4 polysaccharides, specifically cellulose & chitin of cell wall in fungi) binds the stain and fluorescent blue-white or apple green depending on the filter combination used. The CFW and its related compounds like Uvitex 2B and Blankophor are non-specific flourochromes, which are used in the textile industry and with the addition of KOH will enhance the visualization of fungal element in specimens for microscopic examination. Fungal elements appear bluish-white against a dark background when excited with UV or blue-violet radiation. Optimal fluorescence occurs with UV excitation. A barrier filter 510, 520 or 530 nm should be used for eye protection. Typical *Pneumocystis jiroveci* cysts are 5-8 μm in diameter, round and uniform in size and they exhibit characteristic peripheral cyst wall staining with an intense internal double parenthesis like structure. Yeast cells are differentiated from *Pneumocystis jiroveci* by budding and intense internal staining. Human cellular materials and cotton fibers may exhibit non-specific fluorescence. KOH–CFW preparations may be preserved for several days at 4°C in a humid chamber.

Procedure

1. Place the material to be examined onto a clean glass slide
2. Add a drop of KOH-Calcofluor solution or mix in equal volumes before processing.
4. Mix and place a cover glass over the material
5. If necessary, allow the KOH preparation to sit at room temperature (25°C) for a few minutes until the material has been cleared. The slide may be warmed to speed the clearing process.

6. Observe the preparation by UV microscopy. Calcofluor may not stain strongly dematiaceous fungi. If such is suspected, the preparation should be examined by bright field microscope. *Candida glabrata* may fluoresce only very faintly. Elastin and collagen will also fluoresce, but with a yellow-green fluorescence.

Quality control

1. Check the reagent prior to use, weekly, and with each new batch of calcofluor prepared.
2. Using an aqueous suspension of actively growing *Candida albicans*, the yeast cell walls will be bright green or blue white depending upon ultraviolet filters used.
3. Negative control consists of KOH and calcofluor combined.

4.3. India ink or Nigrosin preparation for identification of *Cryptococcus species*

Nigrosin Staining Solution: 10.0 g Nigrosin (granular) in 100 mL of Formalin (10%). Place the solution in a boiling water bath for 30 minutes. Add 10% formalin lost by evaporation. Filter twice through double filter paper (Whatman No. 1).

Procedure

1. The preparation is to be made in the centre of a clean, grease-free, glass slide.
2. Put one drop of India ink or Nigrosin on the centre of the slide. Too much stain makes the background too dark. (Upon examination, if the staining appears too dark, a little amount of water may be applied on the edge of the cover-slip and the cover-slip gently tapped. This dilutes the stain to some extent).
3. Put one loopful of the specimen or preferably centrifuge sediment from the fluid specimen to be tested (e.g. CSF, spinal fluid, urine, and other body fluids) close to the drop of the stain.
4. Mix the two well with the loop, or preferably a sterile needle. The loop should be cooled before use; otherwise, the stain tends to precipitate.
5. Hold the cover-slip vertically such that one edge just touches the fluid on the slide. The fluid will spread on the edge by surface tension.
6. Keeping that edge in contact with the fluid surface, drop the cover-slip gently on the fluid, so that no air bubble is trapped inside. If there are air bubbles, the surface of the cover slip may be gently tapped by the needlepoint, so as to move the bubbles towards the edge. But this should be avoided as far as practicable.
7. Examine slide immediately under the microscope. Since the stain tends to dry fast in air, if immediate examination is not feasible, the slide should be kept in a moist chamber (covered petri dish with a wet filter paper on which a triangular glass rod is placed).
8. Scan the entire cover slip from end to end in a zigzag fashion. Encapsulated yeast (*Cryptococcus neoformans)* is seen under low power as luminous dots in an otherwise dark background. Under high power, the cells can also be seen, containing refractile bodies, and surrounded by the unstained thickness of the capsule. Characteristic pinched-off budding, when observed, is confirmatory for diagnosis.

It should be noted specially that

a. Besides the classical budding-yeast form, various unusual forms can also be seen including elongated forms that look like pseudohyphae; this is mainly due to a very high multiplication rate of the organism in HIV/AIDS patients.
b. In the very late stage in progressive AIDS, it may be difficult to differentiate the capsules of individual cells; the organism may remain enmeshed in a matting of the capsular material.
c. Micro- or non-capsulated strains of the organism are also reported on rare occasions. In such cases, Gram stain of the sample is helpful in identification.
d. The edges of the cover-slip should be specially examined. While placing the cover-slip, the yeast cells tend to move towards the periphery along with the fluid. For this reason, the common practice of draining the extra fluid from the sides should be avoided.
e. If the protein content of the CSF is too high, India ink sometimes may form floccules, which make it difficult to demonstrate the capsule.
f. In case of Nigrosin stain the preparation dries up quite fast, which is a problem in hot climatic conditions. So, quick examination is essential.
g. In case of HIV positive patients, > 90% of cases may be positive by the India ink/Nigrosin test, whereas in non-HIV cases, < 60 % positivity is seen.

Precautions

1. The India ink or Nigrosin should be shaken well before every preparation.
2. The stain should be regularly checked for contamination by checking only the stain under microscope.
3. False positive readings may occur with air bubbles or monocytes or neutrophils. Air bubbles, under the high power, will be hollow and will not show the typical cell with characteristic nuclei. Monocytes and neutrophils have a crenated margin (and not the entire margin seen in cryptococcal cell) and will not show the characteristic refractive cell inclusions and the luminous halo around the cell is not well demarcated.

4.4. Gram's stain

Table 1.3: Gram's stain reagents

1.	Crystal Violet Reagents	
	Crystal Violet (85% dye)	2 g
	Ethyl alcohol (95%)	10 mL
	Distilled water	100 mL
2.	Grams Iodine Solution	
	Iodine	1 g
	Potassium iodide	2 g
	Distilled Water	300 mL
3.	Counter Stain	
	Safranin O	1 g
	Ethyl alcohol (95%)	40 mL
	Distilled Water	400 mL
4.	Decolorizer	
	Acetone or Alcohol	

Gram's stain is usually a poor stain to use when examining a specimen for a fungus. Gram's stain may be used when examining smears of *Candida, Malassezia*, and *Sporothrix* but should not be relied upon to demonstrate the yeasts of the other dimorphic fungi. All fungi are gram positive. It is most useful for demonstrating mycelial elements and budding yeasts cells in sputa, vaginal secretions, purulent material, gastric washing, lung aspirates and urine.

Procedure

1. Make a very thin smear of the material on a clean grease-free glass slide.
2. Dry in air.
3. Fix the smear by flaming the slide.
4. Add gentian violet to cover the smear and leave undisturbed for 1 minute.
5. Drain off the gentian violet by tilting the slide and rinse in flowing tap water taking care that the water flow does not directly fall on the smear.
6. Add Gram's iodine solution to cover the smear and leave for 1 minute.
7. Rinse with water in the same way as above.
8. Flood with acetone for about 30 seconds.
9. Rinse again with water.
10. Counter stain with safranin for 30 seconds, and rinse in water.
11. Dry in air and observe.

Observation

Gram reaction (positive or negative), size, shape and arrangement of elements should be observed under the oil immersion field.

4.5. Giemsa stain

Giemsa stain preparation

Table 1.4: Compound stain formation by interaction of methylene blue and eosin

Giemsa Powder	600 mg
Methyl alcohol	50 mL
Glycerine	50 mL

- Grind Giemsa powder in mortar. Pour methyl alcohol and glycerine and decant from top.
- Grind it again with glycerine till whole stain is dissolved.
- Keep the stain at 55°C for 2 hours; shaking gently at 30 minutes interval.
- Keep it for two weeks for maturation.

Table 1.5: Buffer solution for giemsa stain

Na2HPO4	6.77 g
KH2PO4	2.50 g
Distilled water	1000 mL
pH	7.2

- Dilute the stain with buffer for use.
- Fix smear with absolute methyl alcohol.
- Put stain on smear for 15 minutes.
- Blot dry and examine.

Uses of Giemsa stain

This stain is used when intracellular structures are to be examined like the yeast cells of *Histoplasma capsulatum*. The intracellular cells of *H. capsulatum* stain light to dark blue and have a hyaline halo. The halo is not a capsule, rather a staining artifact. This stain can also be used to visualize trophozoite stage of *Pneumocystis jiroveci*. It is a compound stain formed by the interaction of methylene blue & eosin.

Procedure

1. Homogenize tissue section and make a thin uniform smear.
2. Flood the slide with methyl alcohol and leave for 3-5 minutes for fixation.
3. Add prepared Giemsa stain for 45 minutes.
4. Wash slide thoroughly with running tap water.
5. Blot dry with absorbent paper.
6. Observe under oil immersion lens.

Observation: Intracellular budding yeast.

4.6. Periodic Acid Schiff (PAS) stain

PAS stain preparation

(a) Formal ethanol mixture: 10 mL of 40 % formaldehyde in 90 mL of absolute alcohol.
(b) Periodic acid solution 1%: 1.0 g periodic acid in 100 mL distilled water.
(c) Basic fuchsin Solution: 0.1 g Basic fuchsin in 200 mL distilled water.
- Warm to 50°C and filter
- Add 20 mL 1 N HCl (83 mL concentrated HCl / 1000 mL distilled water) cool to 25°C.
- Add sodium bisulphate 1 g, store in a screw top bottle in the dark for two days
- Add activated charcoal 0.5 g; shake intermittently for one hour
- Filter, store in dark – colored tightly closed bottle in refrigerator (5 years). Solution can be used till it turns pink.

(d) Light green working solution
- 0.2% light green (stock solution): Light green, SF yellowish 0.2 g in 100 mL distilled water
- 10 mL of stock solution to 50 mL distilled water

Applications: It is histopathological stain used to detect fungi in clinical specimens, especially yeast cells and hyphae in tissues. Fungi stain bright pink-magenta or purple against an orange background if picric acid is used as the counter stain or against a green background if light green is used. The procedure is a multistep method combining hydrolysis and staining.

Principle: The periodic acid oxidizes, 1, 2 – glycol groupings of the fungus polysaccharides to aldehyde groups. The reactive aldehyde groups' combine with the basic fuchsin in such a manner that it cannot be bleached out when treated with sodium metabisulphite.

Precautions: A slide of either skin or nail scrapings containing a dermatophyte should be stained along with slides of the specimen as positive control. Periodic acid may deteriorate and no longer oxidize the hydroxyl groups. This should be suspected when fungal elements on the control slide appear unstained. The Periodic acid solution should be kept in a dark bottle. The stock of Periodic acid (a white powder) should be kept in a dark bottle. The sodium metabisulphite solution is unstable. Deterioration of this reagent is suspected when the control slides show no evidence of fungi after having been subjected to a bleaching process e.g. background stains as intensely as the do the fungal elements.

Procedure

1. Fix slide by flaming.
2. Immerse in ethanol for 1 minute.
3. Place in 5% periodic acid for 5 minutes.
4. Wash gently in running tap water for 2 minutes.
5. Place in basic fuchsin for 2 minutes.
6. Wash gently in running tap water for 2 minutes.
7. Place in sodium metabisulphite (0.5%) for 3-5 minutes.
8. Wash gently in running tap water for 2 minutes.
9. Counter stain with dilute aqueous light green (0.2%) if for 2 minutes.
10. Dehydrate in 70%, 80%, 95%, 100% ethanol and xylene: each for 2 minutes.
11. Mount the slide.

Observation: Fungi stain a bright pink-magenta or purple against a green background when light green is used as a counter stain.

Interpretation: Occasionally bacteria as well as polymorphonuclear neutrophils may retain the basic function but, in neither case, should there be any difficulty in differentiating these structures from mycotic elements. PAS stain used in clinical microbiology laboratory is a modification of Hotchkiss-McManus PAS stain used in the histopathology laboratory. The modification of this stain as used in the clinical laboratory may occasionally stain some cocci and white cell granules as well as diphtheroid, *C. minutissimum*, Differentiation of fungus & tissues is enhanced when counter stained as in Gridley's modification.

4.7. Gomori's Methenamine Silver Nitrate Stain (GMS)

Composition of GMS Stain

- **10% chromic acid:** 10 g chromic acid in 100 mL distilled water
- **1% sodium metabisulfite:** 1.0 g sodium bisulfate in 100 mL distilled water
- **5% Silver nitrate:** 5.0 g silver nitrate in 100 mL distilled water. Store in black colored bottle at 4°C.
- **3% aqueous methamine (hexaethyltetramine):** Methanamine 3.0 g in distilled water 100 mL
- **5% aqueous borax:** 5.0 g borax (sodium borate) in 100 mL distilled water
- **1% aqueous gold chloride:** 15 grain vial gold chloride in 100 mL distilled water
- **5% sodium thiosulfate solution:** 5.0 g sodium thiosulfate in 100 mL distilled water

- **Light green working solution:** Prepare stock solution of 0.2% light green (Light green, SF yellowish 0.2 g in distilled water 100 mL). Then add 10 mL of stock solution in 50 mL distilled water to get working solution.
- 70% alcohol
- 95% alcohol
- Absolute alcohol
- Xylol, used from dropper bottle
- Mounting medium

Applications: Gomori's methenamine silver stain is perhaps the most useful stain for visualizing fungi in tissue. Fungal elements are sharply delineated in black against a pale green or yellow background. They are, however, specialized stains that are more often used in the histopathology laboratory rather than the microbiology laboratory. Grocott's modification of Gomori's methenamine silver stain is commonly used for the histopathological examination of de paraffinized tissue for fungi.It is especially useful as a histopathological tool and for the detection of *Pneumocystis jiroveci*.

Principle: The alcohol groups present in the cell wall of the fungus gets oxidized by the oxidizing agent (chromic acid) to aldehyde group. The latter acts as a reducing agent which reduces silver nitrate to metallic silver and in turn stain the fungal cell wall. GMS (Grocott's modification) delineates fungal elements sharply in black against a pale green background.

Procedure

1. Dry the smear and then fix in absolute methanol for 5 minutes.
2. Wash in distilled water.
3. Dip slide in coplin jar containing 4% chromic acid for 45 minutes.
4. Wash in distilled water.
5. Add 1% sodium / potassium metabisulphite for 1 to 2 minutes.
6. Wash in distilled water.
7. Dip slide in working solution of hexamine (which is preheated in a water bath to 56°C) for one hour (smear becomes dark brown).
8. Wash with distilled water or if smear turns black, wash with 0.1% $FeCl_3$.
9. Wash with 5% sodium thiosulphate for 2 minutes.
10. Wash with distilled water.
11. Wash with 1% light green solution for 1 minute.
12. Dry and see under oil immersion.

Observation: The control slide with fungal elements stains black; background stained green. *Pneumocystis jiroveci* cysts and fungi may appear very much alike. Look for various cyst forms, including those that show dark centers, cup shaped crescents, and cysts with fold like lines (look like punched in ping pong balls). If dark staining organisms appear more oval, look carefully for budding forms, which may differentiate the organisms.

A. *P. jiroveci* cysts – 70% should have delicately staining walls, usually brown or gray. They will appear somewhat transparent with structures described as "parenthesis" staining black; these curved structures are usually thick (much thicker than the cyst wall).
B. Fungi and Actinomycetes – grey to black.
C. Glycogen, Mucin and red blood cells – rosy taupe to gray.
D. Background is pale green.

Yeast Identification

CHAPTER 2

Yeast Identification

Yeasts are heterogeneous fungi that superficially appear as homogenous. Yeasts grow as unicellular form and divide by budding, fission or a combination of both. The true yeasts are those fungi that reproduce sexually, developing ascospores or basidiospores. In contrast, yeast- like fungi or imperfect fungi reproduce only by asexual means. The various yeasts are distinguished from each other based upon a combination of morphological and biochemical criteria. Morphology and the methods of asexual reproduction are primarily used to identify genera, whereas biochemical tests are used to differentiate the various species.

Approaches for the identification of the yeasts include:

1. Culture characteristics : Colony color, shape and texture.
2. Asexual structures
   - Shape and size of cells.
   - Type of budding : unipolar (Malassezia), bipolar (Hansaniaspora kloekera), multipolar (Candida), fission (Schizosaccharomyces).
   - Presence or absence of arthroconidia, blastoconidia, ballistoconidia, clamp connections, germ tubes, hyphae, pseudohyphae, sporangia or sporangiospores.
3. Sexual structures : arrangement, cell wall, ornamentation, number, shape and size of ascospores or basidiospores.
4. Physiological studies:
   - Sugar fermentation
   - Sugar assimilation
   - Nitrogen utilization
   - Urea hydrolysis
   - Temperature studies
   - Gelatin liquefaction

Identification Procedures

1. Isolation techniques for mixed cultures

Before proceeding for the identification of the yeast it is necessary to confirm the purity of the yeast because initially isolated yeast may be contaminated or in mixed culture.

Purification of the yeasts can be done by the following techniques.

A. For bacterial contamination

i) Isolation on Sabouraud dextrose agar (SDA)

a. Suspend a colony of the yeast in sterile distilled water.
b. Inoculate a loopful of suspension on SDA plate.
c. Incubate at 25°C for 48 hours.
d. Prepare a wet mount from an isolated colony and verify the purity of the colony.
e. If the culture is not pure further steps are necessary (step ii).

ii) Isolation on SDA agar plus chloramphenicol and gentamicin / SDA plus penicillin and streptomycin / BHI plus 10% blood plus chloramphenicol and gentamicin.

a. Suspend a colony of the yeast in sterile distilled water.
b. Inoculate a loopful of suspension on any one of the above media.
c. Incubate at 25°C for 48 hours.
d. Prepare a wet mount from an isolated colony and verify the purity of the colony.
e. If the culture is not pure further steps are necessary (step iii)

iii) Acidification of SDA broth

a. Suspend a colony of the yeast in sterile distilled water.
b. To each of the 4 tubes containing 10 mL of the SDA broth, add one drop of 1 N HCl to the first tube, 2 drops to the second tube, three drops to the third tube and 4 drops to the fourth tube.
c. Add 0.5 mL of yeast suspension to each tube.
d. Incubate at 25°C for 24 hours. Check for the purity.

B. For mixed yeasts

1. Suspend a colony of the yeast in sterile distilled water
2. Streak a loopful of the suspension onto a SDA agar plate.
3. Incubate at 25°C for 48 hours.
4. Check for the purity. If the yeasts are not pure the procedure must be repeated. Note: some strains may have both rough and smooth colony types in pure culture.

C. For mould contamination

i) Isolation on yeast malt agar (YM)

a. Suspend a small portion of yeast-mould colony in a tube containing sterile distilled water
b. Inoculate a loopful of the suspension on YM agar plate.
c. Incubate the plates at 25°C for 4-6 days.
d. Check for the purity of the colony. If not pure, further steps are necessary (Step ii).

ii) Isolation in yeast malt broth

a. Suspend a small portion of the yeast -mould isolate to a tube containing 10 mL of broth.
b. Incubate the YM broth at 25-30°C for 48 hours. Using a capillary pipettete, remove a small portion of sediment carefully without disturbing the mycelial pellicle.
c. Inoculate the sediment on YM agar.

d. Incubate at 25°C for 4-6 days.
e. Check for the purity of the culture by examining the wet mount. If the culture is not pure further steps are necessary (Step iii).

iii) Isolation in shake culture

a. Streak a small portion of the yeast - mould isolate into YM broth in an Erlenmeyer flask (approx. 100 mL).
b. Incubate the flask in a rotary shaker at 30°C for 4-6 days.
c. Remove a small amount of the sediment with a sterile capillary pipettete taking care not to take the mycelial balls by accident.
d. Inoculate the sediment onto YM agar
e. Incubate at 25-30°C for 4-7 days and prepare a wet mount using a small portion of the yeast colony.

2. Direct mounts

Direct mounts are made to study yeast microscopic morphology and to determine the purity of the isolates. Direct mounts are made using Lactophenol Cotton Blue (LCB).

3. Germ tube test

Germ tube test is used for presumptive identification of *Candida albicans*. It is a rapid screening test where the production of germ tubes within two hours in contact with the serum is considered as indicative of *Candida albicans or C. dubliniensis*. This test must be validated with Corn Meal Agar (CMA) test.

Procedure

1. Ensure that the test starts with a fresh growth from a pure culture.
2. Make a very light suspension of the test organism in 0.5 mL of sterile serum (pooled human serum or fetal calf serum). The optimum inoculum is 10^5- 10^6 cells per mL.
3. Incubate at 37°C for exactly two hours.
4. Place one drop from the incubated serum on a slide with a cover slip. Observe under the microscope for production of germ tubes. Germ tubes represent initiation of hyphal growth, arising directly from the yeast cell. They have parallel walls at their point of origin and are not constricted.
5. To record a positive, about 30% of the cells should show germ tube production.
6. Suitable controls should be kept with each test; a known strain of *Candida albicans* should be tested with each new batch of serum.

NOTE:

1. The medium, inoculum size, temperature of incubation, concentration of simple carbohydrates and microaerobic conditions influence GT formation.
2. Increased concentration of inoculum causes a significant decrease in the percentage of cells forming germ tubes. Maximum percentage of GT formation occurs when 10^5 to 10^6 cells/mL are used as inoculum. As the concentration of cells increases, the percentage of GT formation decreases. A faintly turbid serum suspension is ideal for maximum GT development.

3. Approximately 95% of clinical isolates of *C. albicans* produce germ tubes when incubated in serum at 37°C for 2-3 hours.
4. A neutral pH (7.4) facilitates maximum development of germ tubes. Bacterial contamination may interfere with production of germ tubes.
5. Antimicrobial substances in the isolation medium may interfere with this test; human serum may contain inhibitory substances (such as ferritin) that suppress GT development.
6. Controls: *C. albicans* and *C. tropicalis* are run with each group of GT determinations to serve as positive and negative controls respectively.

4. Morphological characters on Corn Meal Agar (Dalmau plate)

1. Prepare Cornmeal agar containing 1% Tween 80 in a 90-mm plate. Divide the plate into 4 quadrants and label each quadrant.
2. Using a sterile needle or straight wire, lightly touch the yeast colony and then make 2-3 streaks of approximately 3.5 – 4 cm long and 1.2 cm apart.
3. Place a flame sterilized and cooled 22 mm square cover glass over the control part of the streak. This will provide partially anaerobic environment at the margins of the cover slip.
4. Incubate the plates at 25°C for 3-5 days.
5. Remove the lid of the petri plate and place the plate in the microscope stage and observe the edge of the cover glass using low power objective (10X) first and then high-power objective (40X).
6. Morphological features like hyphae, pseudohyphae, blastospores, ascospores, chlamydospores, basidiospores or sporangia are noted.

5. Ascospore production & detection test

Identification of yeast also involves determining whether or not the isolate has the ability to form ascospores. Some yeast will readily form ascospores on primary isolation medium whereas others require special media. Ascospores are produced under limited nutrients in the media.

The commonly used media are:
- Malt extract agar (5% malt extract and 2% agar)
- Acetate agar (0.5% sodium acetate trihydrate and 2% agar pH 6.5 - 7.0)
- V-8 juice agar (commercially available)

Procedure

1. Inoculate the yeast onto ascospores producing agar plates.
2. Incubate aerobically at 25°C.
3. Examine the culture in 3-5 days and weekly thereafter for 3 weeks.
4. Prepare wet mount of the yeast in distilled water.
5. Examine the wet mount under oil-immersion objective.
6. Observe for ascospores form, surface topography, size, color, brims and number of ascospores per ascus.
7. If the ascospores are not seen in a wet mount, perform modified acid-fast stain (ascospores are acid fast).
8. *Saccharomyces cerevisiae* should be included as positive control for production on media and staining procedure.

Table 2.1: Morphology on Corn Meal Agar with Tween 80

Terminal chlamydoconidia	*C. albicans,* *C. dubliniensis*
Abundant pseudohyphae, pine forest arrangement, blastoconidia formed at or in between septa	*C. tropicalis*
Elongated yeasts, abundant pseudohyphae (matchstick like appearance)	*C. krusei*
Giant hyphae, blastospores at nodes	*C. parapsilosis*
Scant pseudohyphae with chains of blastoconidia	*C. guilliermondii*
Yeasts only	*C. glabrata, C. famata, Pichia anomala,* *P. augusta, Cryptococcus neoformans*
Short, distinctly curved pseudohyphae with occasional blastoconidia at septa	*C. lusitaniae*
Arthroconidia with blastoconidia	*Trichosporon spp.*
Arthroconidia without blastoconidia	*Geotrichum spp.*

Table 2.2: Ascospore morphology on Malt Extract Agar, (2-5 days growth)

1-4 hat shaped ascospores in asci	*Pichia anomala*, *Pichia norwegenesis*, *C. utilis* and *C.cifferi*
1-4 spherical or short ellipsoidal ascospores	*Saccharomyces cerevisiae*
1-2 / ascus, spherical with warts	*C. famata*
4/ ascus, ellipsoidal with slimy sheath	*Blastoschizomyces capitatus*
1-2/ ascus, spherical	*C. krusei*
1-4/ ascus, spherical to hat shaped, protuberances on one or two edges	*C. lipolytica*
1-4/ ascus, clavate	*C. lusitaniae*

6. Ballistospore Formation

Forcibly discharged conidia or ballistoconidia formation is an important criterion to distinguish *Sporobolomyces* from *Rhodotorula*. Presence of satellite conidia on the agar plate is the first indication for the formation of ballistoconidia.

Procedure

1. Prepare the plates by pouring thin layer of malt extract agar or yeast extract agar on the top and bottom of the petri plate.
2. Inoculate the bottom part of the plate with the yeast. Cover the inoculated part with the top part containing agar.
3. Incubate the plate at 25°C for 2-3days so that the inoculated surface is on the top.
4. Formation of the ballistospores can be seen by new growth in the form of mirror image of the original colony on the uninoculated bottom surface.

7. Sugar Fermentation

1. Prepare liquid fermentation medium containing peptone (1%), sodium chloride (0.5%), Andrade's indicator (0.005%). Sterilize by autoclaving at 120°C for 15 minutes at 15 pounds pressure.
2. Add filter sterilized sugar at the concentration of 2% to the medium. Pour into the sterile test tubes (approx. 5 mL) and place sterile Durham's tube into each tube.
3. Plug the tubes with colour coded cotton plugs.
4. Inoculum preparation is done by suspending heavy inoculum of yeast grown on sugar free medium.
5. Inoculate each carbohydrate broth with approximately 0.1 mL of inoculum.
6. Incubate the tubes at 25°C up to 1 week. Examine the tubes every 48-72 hours interval for the production of acid (pink color) and gas (in Durham's). Production of gas in the tube is taken as fermentation positive while only acid production may simply indicate that carbohydrate is assimilated.

8. Sugar assimilation test (Auxanographic technique)

The Disc impregnation - pour plate auxanographic method of Wickerham and colleagues has with stood the test of time and are easier to perform than the liquid auxanographic technique (which were claimed to be more sensitive and specific but were later not found so).

Preparation of yeast nitrogen base using following constituents:

- Potassium dihydrogen orthophosphate (KH_2PO_4) - 1.0 g
- Magnesium sulfate ($MgSO_4$) - 0.5 g
- Ammonium sulfate (NH_4SO_4) - 5.0 g
- Noble agar - 25.0 g
- Distilled water - 1 L
- Autoclave at 115°C for 15 minutes

OR

Prepare YNB and agar separately as follows:

1. YNB (Difco) - 6.7 g in 100 mL distilled water. Sterilize by filtration and store at 4°C.
2. Agar – 20.0 g in 980 mL distilled water. Dispense in 18 mL quantities in 18 x 150 mm screw-capped tubes. Autoclave at 121°C and store at 4°C.

Procedure

1. Prepare a yeast suspension from a 24-48 hours old culture in 2 mL of YNB by adding heavy inoculum.
2. Add this suspension to the 18 mL of molten agar (cooled to 45°C) and mix well. Pour the entire volume into a 90-mm petri plate.
3. Allow the petri plate to set at room temperature until the agar surface hardens.
4. Place the various carbohydrate-impregnated discs onto the surface of the agar plate.

5. Sugar discs can be obtained commercially or can be prepared as follows: Punch 6 mm diameter disc from Whatman no. 1 filter paper. Sterilize the disc by placing them in hot air oven for 1 hour. Add a drop of 10% filter sterilize sugar solution to each disc. Dry the disc at 37°C and store at 4°C in airtight container.
6. Incubate the plates at 37°C for 3-4 days.
7. The presence of growth around the disc is considered as positive for that particular carbohydrate.
8. Growth around glucose disc is recorded first which serves as positive control (viability of yeast).

9. Nitrate assimilation test (Auxanographic technique)

Preparation of Yeast Carbon base

- Potassium dihydrogen orthophosphate (KH_2PO_4) – 1.0 g
- Magnesium sulfate ($MgSO_4$) - 0.5 g
- Glucose – 20.0 g
- Noble agar – 25.0 g
- Distilled water – 1 L
- Mix the reagents by boiling and autoclave at 121°C for 15 minutes.

OR

Prepare yeast carbon base and agar separately:

1. Yeast carbon base (Difco) - 11.7 g in 100 mL distilled water. Sterilize by filtration and store at 4°C until use.
2. Agar – 20.0 g in 980 mL distilled water. Autoclave at 121°C for 15 minutes.
   - ♦ Dispense in 18 mL quantities in 18 X 150 mm screw-capped tubes.
   - ♦ Make a yeast suspension in molten YCB agar and pour into 90 mm diameter petri plate and allow cooling.

OR

1. Prepare the suspension from a 24-48 hours culture in 2 mL of YCB equal to Mc Farland No. 1 standard. Add this suspension to 18 mL of molten agar cooled at 45°C. Mix well and pour into 90 mm petri plates.
2. Allow the plate to cool to room temperature.
3. Divide the plate into two halves and label them.
4. Place the potassium nitrate disc (KNO_3) and peptone disc on the surface of the agar on corresponding labelled halves.
5. Prepare potassium nitrate discs (30.0 g potassium nitrate in 1 L distilled water)
6. Mix the reagents and autoclave at 15 psi for 15 minutes. Take 6 mm disc (punched from Whatman No. 1 filter paper) and saturate with the potassium nitrate solution and dry the discs in sterile petri plate and store at 4°C until use.

Peptone disc is prepared similarly as KNO_3 disc.

- Peptone – 30 g
- Distilled water - 1 L

Incubate the plate at 37°C for 7 days. Check for the growth around the disc. The test is considered valid if the growth is present around the peptone. The growth around the KNO3 impregnated disc is considered positive.

10. Urea hydrolysis

The Christensen's urea agar is recommended and is prepared according to the manufacturer instructions. Using a loop inoculate a small amount of the yeast colony on the agar surface.

- Inoculate appropriate control (*C. albicans* - negative control, *C. neoformans* - positive control).
- Incubate the slant at 25°C for 2-5 days. Read the test and the control tubes.
- A deep pink color indicates a positive test.

11. Rapid urea hydrolysis

The rapid urea hydrolysis test is done in microtitre plates and used to screen for *C. neoformans.*

1. Reconstitute each vial of Difco urea® broth with 3 mL of sterile water on the day to be used.
2. Dispense 3-4 drops into each well of the microtitre plate.
3. Transfer a heavy inoculum of freshly isolated colony of yeast into urea broth into a well.
4. Seal the plate with a tape and incubate for 4 hours at 37°C.
5. Pink to red color is positive test.
6. Similarly inoculate the controls*, C. neoformans* as positive control and *C. albicans* or uninoculated well as negative control.

12. Temperature studies

Another important step in the identification of the yeast is by determining the ability to grow at an elevated temperature. It can be used to distinguish *C. neoformans* from other species of *Cryptococcus* and *C. dubliniensis* from *C. albicans*.

Procedure

1. Inoculate two tubes of malt extract agar with the isolate.
2. Incubate one tube at 37°C and other at 25°C.
3. Examine the tube everyday up to 4-7 days for the presence of growth.
4. Growth must be present in both the tubes before concluding that the yeast has the ability to grow at 37°C.

13. Canavanine Glycine Bromothymol Blue Agar (CGB) Test

This test is used to differentiate between *C. neoformans* from *C. gattii*. The latter hydrolyses glycine to form ammonia to alter the pH of the medium towards alkalinity and thus change its color to blue while the former does not. Canavanine acts as a selective agent.

Procedure

1. Prepare CGB agar slants in tubes.

2. Inoculate surface of the slant using minimum inoculum of *C. neoformans* or *C gattii* with a straight wire loop.
3. Put a positive control (*C gattii*) and negative control (*C. neoformans*) simultaneously.
4. Incubate at 25°C for 1 – 5 days.
5. At the end of 5 days positive result is shown by color change from greenish yellow (pH 5.8) to cobalt blue (pH 7.0).

14. Phenoloxidase test

Cryptococcus neoformans when grown on the medium containing bird seed agar produces dark brown or black colonies containing melanin.

Procedure

1. Prepare birdseed agar (BSA) (*Guizotia abyssinica* / Niger seeds) or Caffeic acid agar in the test tube.
2. Streak the culture of yeast/ *C. neoformans* on the media.
3. Incubate the media at 25 - 30°C and observe every day up to one week.
4. *C. neoformans* produces dark brown or black colonies with in 3-5days of inoculation.

15. Commercial yeast identification system

Commercially available identification systems are described in the chart below. Most commonly used systems are: API 20C Yeast Identification Systems (API Analytab Products, Plainview, NY) and Biomerieux Vitek System (Hazelwood, MO) .The API 20C system requires less preparation of reagents. The Vitek system is an automated system. Both the systems are based on the modification of the auxanographic assimilation techniques.

Principle: When the organism is able to assimilate particular carbohydrate (in a capsule of reconstituted substrates of API systems/ in the wells containing the substrate of Vitek, change in color is observed. Both the systems require morphological studies and germ tube test in order to obtain more complete profile of yeast identification.

Specimen: A pure culture of 24-48 hours old yeast cells growing on non-selective agar (e.g. -SDA) is required.

Reagents and material required but not supplied with the kit.

a). For API20C yeast identification system:

- Sterile wooden applicator stick
- Sterile Pasteur pipettetes
- Squeeze bottle
- Incubator (30°C)
- Waterbath
- Sabouraud's Dextrose Agar plates

b). For Vitek yeast identification system:

- Sterile wooden applicator stick
- Sterile tubes containing 1.8 mL
- Colorimeter (Vitek colorimeter)
- Incubator (30°C)
- Sabouraud's Dextrose Agar plates.

Procedure

a. API 20C yeast identification system

1. Melt the basal broth medium in the ampoules by keeping in the autoclave or by boiling in water bath
2. Allow to cool to 48-50°C.
3. Prepare an incubation tray. Use a squeeze bottle to dispense 20 mL of water into the tray and then place the sample into the incubation tray.
4. Open the ampoule as per the manufacturer's instructions and inoculate the molten medium using applicator stick. Adjust the density to < 1+ on the wickerham card.
5. Inoculate 0.2 mL yeast suspension into 20 capsules by using Pasteur pipettete and place the lid on the tray.
6. Incubate the tray at 28- 30°C for 72 hours. Read the results after 24, 48, 72 hours.

b. Vitek Yeast Identification system

1. Prepare yeast suspension in tubes containing 1.8 mL of saline. Adjust the density to McFarland No.2 standard using Vitek colorimeter.
2. After labeling the yeast cards, place the card in the filling stand with a transfer tube that is in yeast suspension.
3. Inoculate the card using the filling nodule.
4. Seal the card with sealer module and incubate at 30°C for 24 hours or 58 hours depending on the readings provided in the instrument.

Results

a. API 20C yeast identification system

1. It does not include rhamnose and urea.
2. Germ tube test and morphological studies should be included.
3. Sometimes, it gives 1-3 different type of yeast identification for an individual isolate.
4. It takes 3 days to obtain final results.

b. Vitek Yeast Identification system

1. Yeasts with abundant capsules and isolates with extensive mycelial growth are sometimes difficult to suspend.
2. Morphological studies and other tests may be required when some strains react similarly in the test system.

Results obtained usually after 24 hours and few isolates may require additional incubation.

Table 2.3: The biochemical properties of medically important yeast (*-variable, F-fermented, W-weak)

Species	Growth at 37^{0}C	Pellicle in broth	Pseudo-/true hyphae	Chlamydospores	Germ tubes	Capsule, India ink	Assimilation of:												Fermentation of:							Urease	KNO_3 Utilization	Phenol Oxidase	Ascospore
							Glucose	Maltose	Sucrose	Lactose	Galactose	Melibiose	Cellobiose	Inositol	Xylose	Raffinose	Trehalose	Dulcitol	Glucose	Maltose	Sucrose	Lactose	Galactose	Trehalose				
C. albicans	+	-	+	+	+	-	+	+	+	-	+	-	-	-	+	-	+	-	F	F	-	-	F	F	-	+	-	-	-
C. catenulata	*+	-	+	-	-	-	+	+	-	-	+	-	-	-	+	-	-	-	F*	-	-	-	F	-	-	+	-	-	-
C. dubliniensis	+	-	+	+	+	-	+	+	+	-	+	-	-	-	*+	-	+	-	F	F	-	-	F	F	-	+	-	-	-
C. famata	+	-	-	-	-	-	+	+	+	*+	+	+	+	+	+	+	+	*+	W	-	W	-	-	W	-	+	-	-	*-
C. glabrata	+	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	+	-	F	-	-	-	F	F	-	+	-	-	-
C. guilliermondii	+	-	+	-	-	-	+	+	+	-	+	+	+	+	+	+	+	+	F	-	F	-	F*	F	-	+	-	-	*-
C. kefyr	+	-	+	-	-	-	+	-	+	+	+	-	*+	-	*+	+	-	-	F	-	F	F	F	-W	-	-	-	-	-
C. krusei	+	+	+	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	F	-	W	-	W	W	-	+	-	+	-
C. lambica	*+	-	+	-	-	-	+	-	-	-	-	-	-	-	+	-	+	-	F	-	-	-	-	-	-	+	-	-	-
C. lipolytica	+	-	+	-	-	-	+	-	-	-	-	-	-	-	+	-	+	-	-	-	-	-	-	-	-	+	-	-	-
C. lusitaniae	+	-	+	-	-	-	+	-	-	-	-	-	-	-	+	-	+	-	F	-	F	-	F	F	-	-	-	-	*-
C. parapsilosis	+	-	+	-	-	-	+	-	-	-	-	-	-	-	+	-	+	-	F	-	-	-	-	-	-	-	-	-	-
W. anomalus	W	*-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	F	F*	F	-	F*	-	-	+	-	-	-
C. rugosa	+	-	+	-	-	-	+	-	-	-	+	-	*+	-	*+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
C. tropicalis	W	+	+	-	-	-	+	+	+	-	+	-	+	-	+	-	+	-	F	F	F	-	F*	F	-	-	-	-	-
K. ohmeri	+	+	+	-	-	-	+	W	+	-	+	-	+	-	V	+	+	-	-	W	+	-	+W	-W	-	-	-	-	-
C. auris	+	-	-	-	-	-	+	+	+	-	-	-	-	-	-	-	-	-	F	-	W	-	-	W	-	-	-	-	-
C.neoformans	+	-	-	-	-	+	+	+	+	-	+	-	+	+	+	*+	+	+	-	-	-	-	-	-	+	-	-	-	-
P. kudriavzevii	+	+	+	-	-	-	+	-	-	-	+	*+	-	+	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-
C. laurentii	*-	-	-	-	-	+	+	+	+	+	+	-	+	+	+	*+	+	+	-	-	-	-	-	-	-	+	+	-	-
C. tereus	+	-	-	-	-	+	+	+	-	*+	*+	-	-	+	+	+	+	+	-	-	-	-	-	-	-	+	+	-	-
R. glutinis	+	-	-	-	-	*-	+	+	+	-	*+	+	+	-	+	+	+	+	-	-	-	-	-	-	-	+	-	-	-
R. rubra	+	-	-	-	-	*-	+	+	+	-	+	+	*+	-	+	+	+	+	-	-	-	-	-	-	-	+	-	-	-
S. cerevisiae	*+	-	*-	-	-	-	+	+	+	-	+	-	-	-	*+	+	*+	-	F	F	F	-	F	F*	-	-	-	+	*-
G. candidum	+	+	+	-	-	-	+	+	-	-	+	+	+	-	+	-	-	-	-	-	-	-	-	-	-	+	-	-	-
T. asahi	+	+	+	-	-	-	+	-	*+	+	+	-	+	*+	*+	-	*+	-	-	-	-	-	-	-	-	+	+	-	-
T. mucoides	*+	-	+	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	+	-	-	-
T. ovoides	W	+	+	-	-	-	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	+	-	-	-

Table 2.4: Phenotypic differentiation of *Malassezia* species

	M. furfur	***M. pachydermatis***	***M. sympodialis***	***M. obtusa***	***M. restricta***	***M. slooffiae***	***M. globosa***	***M. dermatis***	***M. japonica***	***M. nana***	***M. yamatoensis***	***M. arunalokei***
Colony morphology texture	Umbonate usually smooth, soft, friable	Pale, convex, smooth soft, friable	Flat smooth, shiny, soft	Smooth, flat, sticky	Dull smooth, hard and brittle	Finely folded brittle	Rough, coarse, brittle	Convex entire or lobed margin	Dull wrinkled entire lobed edge	Dull smooth, convex, soft, viscous	Wrinkled folded with entire lobbed margin	Flat to raised, moderately convex with entire margins
Colony color	Cream	Cream	Cream to buff	Cream	Cream	Cream to buff	Cream to buff	Yellow to white	Pale yellow	Cream to yellow	Yellowish white	Dull, whitish to cream-color
Cell shape and size	Elongated oval or sphereical 6µm	Cylindrical 2.5 -4.0µm long	Ovoid to globose 2.5-5µmlong	Cylindri-cal 4-6µm	Spherical oval 2-4 µm	Cylindri-cal 1.5-3.5µm long	Spherical 6-8µm diameter	Spherical oval 2.0-10µm	Spherical oval 2.0-7.0µm	Ovoid or globose 2.5-3µm long	Ovoid ellipsoidal 2.0-7.0µm	Ovoid, globose, 2.18-6.05µm long
Budding pattern	Broad base	Broad bud base, pronounced bud scar	Some sympodial budding	Broad bud base	Narrow bud base	Broad bud base	Narrow bud base	Narrow bud base	Some sympodial budding	Narrow bud base	Narrow bud base	Monopolarly on a narrow base
G+C content (%)	66.4	55.655	62.2	60.7	59.9	68.8	53.3	60.4	60.4	ND	ND	ND
Catalase reaction	+	Variable	+	+	_	+	+	+	+	+	+	-
Urease reaction	+	+	+	+	+	+	+	ND	ND	ND	ND	
Growth at 37°C	Good	Good	Good	Poor	Poor	Good	Poor	Good	Present	Good	Good	+
Maximum growth temp. (°C)	40.41	40.41	40.41	38	38	40.41	38	40.41	38	Variable	38	37
Tween 20	+	+	-	-	-	+	-	+	-	Variable	+	-, ppt
Tween 40	+	+	+	-	-	+	-	+	Weak	+	+	-, ppt
Tween 60	+	+	+	-	-	+	-	+	+	+	+	-, ppt

	M. furfur	*M. pachydermatis*	*M. sympodialis*	*M. obtusa*	*M. restricta*	*M. slooffiae*	*M. globosa*	*M. dermatis*	*M. japonica*	*M. nana*	*M. yamatoensis*	*M. arunalokei*
Tween 80	+	Variable	+	-	-	-	-	+	-	Variable	+	Variable
Crem EL	+	Variable	-	-	-	-	-	+	ND	-	ND	-, ppt
Aesculin hydrolysis	Weak	Variable	+	+-	-	-	-	+	ND	-	ND	-
Assimilation of glycine	+		-	-	-	-	-	ND	ND	ND	ND	ND
Precipitate on Dixon's agar	-	ND	+	-	ND	-	-	+	ND	+	ND	ND

Techniques Used for Identification of Moulds

CHAPTER 3

Techniques Used for Identification of Moulds

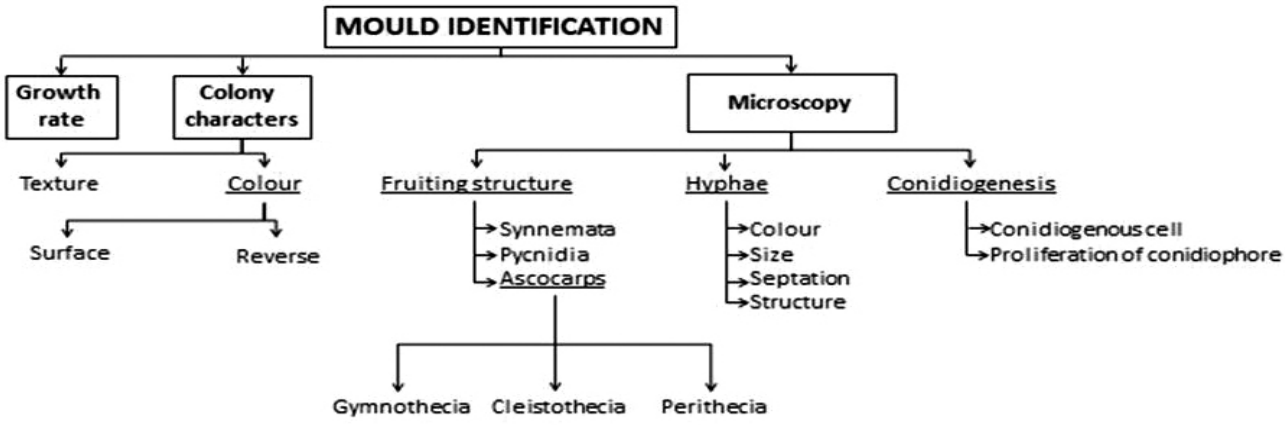

Figure 3: Mould identification scheme

I. Colony characteristic

- Media routinely used for studying the morphology are Sabouraud dextrose agar, malt extract agar, potato dextrose agar or Czapek-dox agar. Important data like growth rate, color, texture, diffusible pigment, exudates, aerial and submerged hyphae, and colony topography can be obtained by gross examination.
- Structures like ascocarps, pycnidia, sclerotia, sporodochia and synnemata can be studied with the help of dissecting microscope.

II. Tease mount

It is the quickest way to mount fungi for microscopic examination. Though being the most common technique used in mycology laboratory; identification is often difficult because of the dislodgement of conidia and spores from the conidiogenous cell.

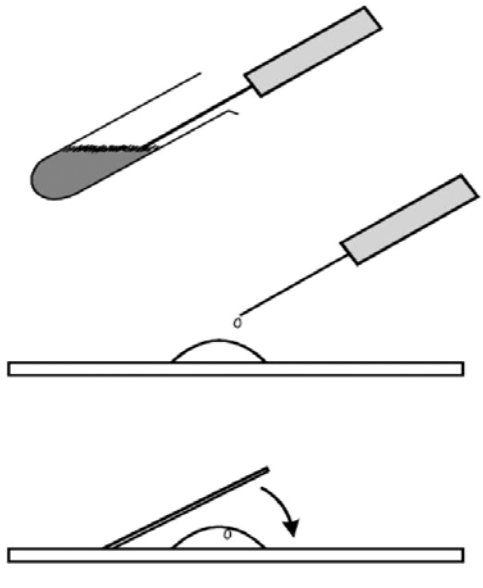

Figure. 4: Standard Tease Mount

Procedure

a) Standard tease mount

1. Place a small drop of lactophenol cotton blue (mounting medium) on a clean microscopic slide.
2. Remove aseptically a small portion of growth midway between the colony center and edge. Place the removed colony on a drop of lactophenol cotton blue on a slide.

3. Tease the fungus using a pair of dissecting needles so as to have a thin spread out.
4. Gently place a cover slip at the edge of the drop of mounting fluid.
5. Avoid trapping air bubbles. Excess of lactophenol can by wiped out using a blotting paper.
6. For preserving the mount, seal the edges of coverslip with nail polish/varnish.

b) Tape technique

1. Place a drop of mounting fluid on the slide.
2. Take 2 cm long tape from a tape roll. Touch one end of the tape to a forceps/ stick and then lightly touch the colony with the stick/forceps.
3. Lay the tape with the surface containing the fungus face down into the mounting medium on the slide. Detach the tape from the stick.
4. Examine the mount directly.

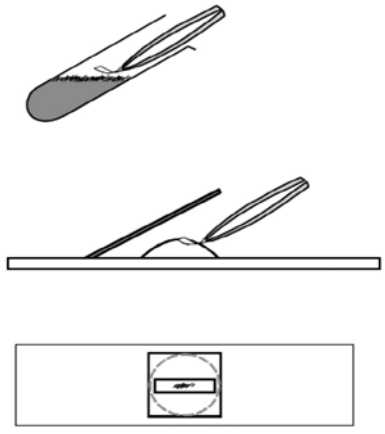

Figure 5: Tape Technique

c) Mounting Zygomycetes and fruiting structures

Sporangia, ascocarps and other fruiting bodies trap air bubbles making preparations unsatisfactory for microscopic study. Thus, following techniques are recommended.

1. Take a drop of 95% ethanol, ethyl acetate or water with 0.05% Tween 80 on a slide. All these are wetting agents, preventing formation of air bubbles around fruiting structures.
2. Transfer fungus from the culture to the drop of fluid. Add a drop of the mounting fluid and examine as in the standard technique.

III. Confirmation of dimorphic fungi

Dimorphic fungi exist in different morphological forms at different temperatures and under separate nutritional conditions. They grow as moulds at 25°C on routine media while in the tissue or on special media at 37°C they grow either as yeasts or spherules.

Blastomyces dermatidis, Histoplasma capsulatum, Paracoccidioides brasiliensis, Penicillium marneffei and *Sporothrix schenckii* grow as yeast in tissue and at 37°C. *Coccidioides immitis* produce spherules and endospores at 37°C in tissue and when grown on special medium.

Temperature, medium composition and CO2 concentration can all affect the mould to tissue form conversion. For *P. brasiliensis* and *H. capsulatum* nutrition seems to be extremely important while in *S. schenckii* and *C. immitis* yeast and spherule form respectively is enhanced by atmospheric CO2.

Table 3.1: Mould to tissue form conversion of dimorphic fungi

S. No	Fungus	Media and conditions	Similar looking fungi at 25°C
1	*Blastomyces dermatitidis*	Kelley's agar, 37°C; KT medium	*Chrysosporium spp.*
2	*Coccidioides immitis*	Modified convers medium, 40°C, 5 to 10% CO_2	*Malbranchea spp., uncinocarpus spp., Auxarthron spp., Archniotus spp.*
3	*H. capsulatum*	BHIA with glutamine, 37°C, Pine's Agar Medium	*Sepedonium spp.*
4	*P. brasiliensis*	BHIA, 37°C	*Chrysosporium spp*
5	*S. schenckii*	BHIA, 37°C, 5-10% CO_2	*Acrodontium spp.*
6	*T. marneffei*	5% sheep BA, BHIA, 37°C	*Penicillium spp. other than T. marneffei*

- Carry out all procedures within biological safety cabinet.
- Do not set up slide cultures for dimorphic fungi.
- For quality control a known culture can be inoculated simultaneously to check for conversion conditions.

IV. Cylcoheximide resistance

- The following fungi are resistant to cycloheximide at 30°C i.e. All of them grow in the presence of cycloheximide, e*g., B. dermatitidis, S. schenckii, C. immitis, H. capsulatum, P. brasiliensis* and most of the dermatophytes.
- While the following fungi are inhibited by cycloheximide: *Absidia, Rhizopus, Scedosporium,* Some strains of *Candida* & *Aspergillus*, *Mucor and Cryptococcus.*

V. Slide culture technique

Whenever it is difficult to identify moulds with tease mounts, slide cultures can be put up. Nutritionally deficient media like corn meal agar, potato dextrose agar etc. are good for enhancing sporulation.

a) Prepare the 'Setup"

- In a 100 mm glass petri dish, place a filter paper, V-shaped glass rod, a microscopic slide and a coverslip.
- Autoclave the whole setup at 121°C for 15 minutes.
- Store the setups in a petri dish canister for a maximum period of 2 weeks.

b) Procedure

1. Aseptically cut 1 cm square agar blocks from CMA or PDA.
2. Transfer the agar block on to the slide in the setup.
3. Transfer very small amount of colony to the four sides of the agar block.
4. With sterile forceps, place the coverslip on the inoculated agar block.
5. Add 1 – 1.5 mL, of sterile water to the petri dish; humid atmosphere does not allow the agar block to dry out.
6. 5-20% glycerine can be added to the sterile water to prevent condensation of moisture on the slide.

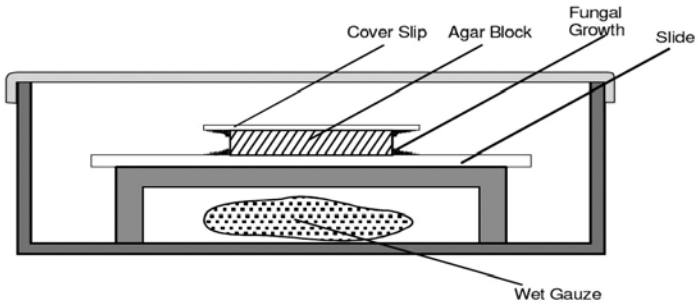

Figure 6: Slide Culture Technique

7. Place the slide culture in a petridish canister and incubate in the dark.
8. Slide culture is ready to be taken down when mature conidia or spores are observed.

c) "Taking – down" the slide culture

- Take a small drop of mounting medium (LCB) on a microscopic slide.
- With the forceps, carefully remove the coverslip from the agar block. Do not push or pull the coverslip.
- Pass the coverslip through the blue portion of flame very quickly. This will heat fix the fungus and its spores (overheating can result in collapse of the hyphae).
- Carefully place the coverslip on the mounting medium so as to avoid trapping any air bubbles.
- Wipe off the excess of mounting medium. Seal the edges of coverslip with nail polish.
- Second mount can also be prepared from the microscope slide of the slide culture setup, by removing the agar block. Put a drop of lactophenol and a coverslip. The coverslip can be sealed with nail polish.
- A drop of 95% alcohol or ethyl acetate may be used to soak the colony before using lactophenol blue to avoid air bubble.
- The block can be dropped aseptically on a suitable media for the growth. This would help to maintain the fungus in the laboratory.

VI. Temperature tolerance

Thermotolerance is a useful characteristic that can be used as an aid in the identification of several medically important moulds.

Table 3.2: Thermotolerance of some medically important fungi

Fungus	Upper Growth Limits (°C)
Aspergillus fumigatus	48 – 50
Cladophialophora bantiana	42 – 43
Cladophialophora carrionii	35 – 36
Fonsecaea pedrosoi	38
Rhizomucor pusillus	50 – 55
Trichophyton mentagrophytes	37
Exophiala dermatitidis	40

VII. Techniques used for identification of few fungi (Table 3.3)

Table 3.3: Techniques for the identification of fungi

S. No	Suspected mould	Technique utilized / Recommended
1	*A. fumigatus*	Temperature tolerance 48°C
2	*B. dermatitidis*	Exoantigen, mould to yeast conversion, Gen probe technique
3	*Basidiobolous sp.*	Forcibly ejected sporangiola
4	*Cladophialophora carrionii*	Temperature tolerance, 35°C
5	*Conidiobolus spp*	Forcibly ejected conidia
6	*H. capsulatum*	Exoantigen, mould to yeast conversion Gen probe technique
7	*P. brasiliensis*	Mould – yeast conversion
8	*Rhizomucour pusillus*	Temperature tolerance, 55°C
9	*Sepedonium sp.*	Morphology and negative exoantigen for *Histoplasma*
10	*Sporobolomyces*	Forcibly ejected conidia
11	*Exophiala dermatitidis*	Temperature tolerance 40°C
12	*Cladophialophora*	Temperature tolerance 42°C

VIII. Techniques to enhance sporulation in moulds

- **Slide culture technique** as explained above.

- **Micro culture technique**: Same procedure as in slide culture technique but it is simple & more beneficial.

  Procedure: Use any nutrient deficient medium. Cut 3-4 small blocks of medium from corner of the plate & place these blocks on the plate at a distance. Now inoculate all the four corners of the block with the fungus. Place the cover slip on each block. Seal the petri plate with parafilm or leucoplast and incubate at 25°C for a week to month with frequent examination of the cultures for any sign of sporulation.

- **Water Agar Technique**

  This technique is used to enhance sporulation in *Mucorales*. Pour approximately 18-20 mL of distilled water (DW) in a sterile petri plate with or without 0.2 mL of 10% filter sterilized yeast extract. Now place 4-5 blocks of fungal growth in DW water. Care should be taken that the growth on the block should be on obverse position. Seal the plate with parafilm & keep gently at 25°C for one to two weeks. After one week, if growth is seen in whole plate above the water, then make LCB using L shaped wire. If sporulation is not sufficient, then incubate for one more week.

- **Dichloro-Rose-Bengal-Chloramphenicol (DRBC) Agar**

  This medium is used especially for the growth of *Mucorales*. This medium is available commercially in powder form. Suspend 31.6 g of the powder in 1 litre of purified water and mix thoroughly. Heat with frequent agitation & boil for 1 minute for the complete dissolution of powder. Autoclave the mixture at 121°C for 15 minutes. Pour in plates or in tubes for solidification. Inoculate & keep at 25°C for one to two weeks till the fungus sporulates.

- **Use of wooden twigs**

  Take some small pieces of wooden twigs (twigs of any plant can be used as a trial and error for those not sporulating using above mentioned methods) and autoclave at 121°C for 15 minutes to sterilize them. While pouring the medium (any nutrient deficient medium) in plates, put these sterile twigs in plates in such a way that half of the part is submerged inside the medium & half remains above the medium. Solidify & check the sterility. Inoculate the fungi where twig is submerging inside the medium. Incubate at 25° C for one week or more.

- **Use of special media**

  Some fungi fail to sporulate on routine media and require special media for sporulation. *Aspergillus spp.* grow and produce fruiting bodies on malt extract agar (MEA) & Czapek Dox (CZ) agar. Similarly, most of the black fungi sporulate on oat meal agar (OMA) and dermatophytes on MEA and potato dextrose agar (PDA).

- **Soil extract medium**

  This medium helps in the development of cleistothecia in *Blastomyces dermatitidis* & *Histoplasma capsulatum,* in addition to sporulation in *Mucorales*. Take garden soil and autoclave at 121°C for 3 hours. Filter through Whatman No. 2 filter paper. Take the filtrate and add tap water (500 gm/ 1000 mL), Yeast extract 1g, dextrose 2 g, agar 1.5 g, KH_2PO_4 0.5 g. Adjust the pH to 7.0 before autoclaving at 121°C for 20 minutes.

Table 3.4: Cultural and microscopic characteristics of hyaline fungi

Organism	Cultural Characteristics	Microscopic characteristics
Aspergillus flavus	Rapidly growing, velvety, yellowish-green, zonate colonies	Conidiophore (2-2.5 x 10-20 µm) fine to coarsely rough. Vesicles elliptical or subglobose; sterigmata cover either the entire or three fourth of the vesicle surface. In small vesicles, only the primary series of sterigmata is produced, and in large vesicles, the sterigmata develop in a double series. Conidia (3-5µm) echinulate, spherical sclerotia may be present.
Aspergillus fumigates	Colony grows rapidly, usually have dark-blue green colour and velvety texture	Conidial heads are typically radiate, later splitting to form loose columns (mostly 300-400 µm in diameter), biseriate but having some heads with phialides borne directly on the vesicle (uniseriate). Conidiophore stipes are hyaline and coarsely roughened, often more noticeable near the vesicle. Conidia are globose to subglobose (3-6 µm in diameter), pale green and conspicuously echinulate. Some strains produce brownish sclerotia.
Aspergillus niger	Colonies consist of a compact white or yellow basal felt covered by a dense layer of dark-brown to black conidial heads.	Conidial heads are large (up to 3 mm by 15 to 20 µm in diameter), globose, dark brown, becoming radiate and tending to split into several loose columns with age. Conidiophore stipes are smooth-walled, hyaline or turning dark towards the vesicle. Conidial heads are biseriate with the phialides borne on brown, often septate metulae. Conidia are globose to subglobose (3.5-5 µm in diameter), dark brown to black and rough-walled.
Aspergillus terreus	Colonies are typically suede-like and cinnamon-buff to sand-brown in colour with a yellow to deep dirty brown reverse.	Conidial heads are compact, columnar (up to 500 x 30-50 µm in diameter) and biseriate. Metulae are as long as the phialides. Conidiophore stipes are hyaline and smooth-walled. Conidia are globose to ellipsoidal (1.5-2.5 µm in diameter), hyaline to slightly yellow and smooth-walled.
Fusarium dimerum complex	Colonies growing slowly; surface usually orange to deep apricot due to confluent conidial slime; aerial mycelium sometimes floccose and whitish.	Conidiophores loosely branched, with short, often swollen phialides, 10-18 x 4-5 µm. Macroconidia strongly curved and pointed at the apex, mostly one-(some up to three)-septate, 5-25 (-32) x 1.5-4.2 µm. Microconidia absent. Chlamydospores mostly intercalary, exceptionally terminal, spherical to ovoidal, 6-12 µm diam, smooth-walled, single or in chains.
Fusarium incarnatum equiseti complex	Colonies growing rapidly; aerial mycelium floccose, at first whitish, later becoming avellaneous to buff-brown; reverse pale, becoming peach-coloured.	Conidiophores scattered in the aerial mycelium, loosely branched; polyblastic conidiogenous cells abundant. Sporodochial macroconidia slightly curved, with foot-cell, three to seven-septate, 20-46 x 3.0-5.5 µm. conidia on aerial conidiophores (blastoconidia) usually borne singly on scattered denticles, fusiform to falcate, mostly three to five-septate, 7.5-35 x 2.5-4.0 µm. Microconidia sparse or absent. Chlamydospores sparse, spherical, 10-12 µm diameter, becoming brown, intercalary, single or in chains.

Organism	Cultural Characteristics	Microscopic characteristics
Fusarium oxysporum	Colonies growing rapidly, 4.5 cm in four days, aerial mycelium white, becoming purple, with discrete orange sporodochia present in some strains; reverse hyaline to dark blue or dark purple.	Conidiophores are short, single, lateral monophialides in the aerial mycelium, later arranged in densely branched clusters. Macroconidia are fusiform, slightly curved, pointed at the tip, mostly three septate, basal cells pedicellate, 23-54 x 3-4.5 µm. Microconidia are abundant, never in chains, mostly non-septate, ellipsoidal to cylindrical, straight or often curved, 5-12 x 2.3-3.5 µm. Chlamydospores are terminal or intercalary, hyaline, smooth or rough walled, 5-13 µm. In contrast to *F. solani* complex, the phialides are short and mostly non-septate.
Fusarium solani	Colonies growing rapidly, 4.5 cm in four days, aerial mycelium white to cream, becoming bluish-brown when sporodochia are present.	Macroconidia are formed after 4-7 days from short multiple branched conidiophores which may form sporodochia. They are three to five-septate (usually three-septate), fusiform, cylindrical, often moderately curved, with an indistinct pedicellate foot cell and a short blunt apical cell, 28-42 x 4-6 µm. Microconidia are usually abundant, cylindrical to oval, one to two-celled and formed from long lateral phialides, 8-16 x 2-4.5 µm. Chlamydospores are hyaline, globose, smooth to rough-walled, borne singly or in pairs on short lateral hyphal branches or intercalary, 6-10 µm.
Lomentospora prolificans (formerly Scedosporium prolificans)	Colonies are rapid growing, spreading, olive-grey to black and have a suede-like to downy surface texture.	Conidia are borne in small groups on distinctive basally swollen, flask-shaped conidiophores, which occur singly or in clusters along the vegetative hyphae. Conidia are aggregated in slimy heads, single-celled, hyaline to pale-brown, ovoid to pyriform, 3-7 x 2-5 µm, and have smooth thick walls.
Pseudallescheria boydii	Colonies are fast growing, greyish-white, suede-like to downy with a greyish-black reverse.	Numerous single-celled, pale-brown, broadly clavate to ovoid conidia, 4-9 x 6-10 µm, rounded above with truncate bases are observed. Conidia are borne singly or in small groups on elongate, simple or branched conidiophores or laterally on hyphae. Conidial conidiophores or laterally on hyphae. Cleistothecia (non-ostiolate ascocarps) are yellow-brown to black, spherical, 50-200 µm in diameter, and are mostly submerged in the agar and are composed of irregularly interwoven brown hyphae. When crushed cleistothecia release numerous, faintly brown, ellipsoidal ascospores, 4-5 x 7- 9 µm in size. Erect synnemata may be present in some isolates. *P. boydii* is homothallic and is recognised by smaller cleistothecia (50-200 µm).
Scedosporium apiospermum	Colonies are fast growing, greyish-white, suede-like to downy with a greyish-black reverse.	Numerous single-celled, pale-brown, broadly clavate to ovoid conidia, 4-9 x 6-10 µm, rounded above with truncate bases are observed. Conidia are borne singly or in small groups on elongate, simple or branched conidiophores or laterally on hyphae. Conidial development can be described as annellidic, although the annellations (ring-like scars left at the apex of an annellide after conidial secession) are extremely difficult to see. Erect synnemata may be present in some isolates. Whereas S. apiospermum is heterothallic (requires mating of two strains and hass larger cleistothecia 140-480 µm. Optimum temperature for growth is 30-37°C.

Table 3.5: Cultural and microscopic characteristics of dematiaceous fungi

Organism	Cultural Characters	Microscopic characteristics	Growth temperature
Alternaria alternate	Colonies are fast growing, black to olivaceous-black or greyish, and are suede-like to floccose.	Microscopically, branched acropetal chains (blastocatenate) of multicellular conidia (dictyoconidia) are produced sympodially from simple, sometimes branched, short or elongate conidiophores. Conidia are obclavate, obpyriform, sometimes ovoid or ellipsoidal, often with a short conical or cylindrical beak, pale brown, smooth-walled or verrucose	Temperature: optimum 25-28°C; maximum 31-32°C
Cladophialophora bantiana	Colonies are moderately fast growing, olivaceous-grey, suede-like to floccose and grow at temperatures up to 42-43°C.	Conidia are formed in long, sparsely branched, acropetal chains from undifferentiated conidiophores. Conidia are one-celled (very occasionally two-celled), pale brown, smooth-walled, ellipsoid to oblong-ellipsoid and are 2-3 x 4-7 µm in size.	Cultures of C. *bantiana* represent a potential biohazard to laboratory personnel and must be handled with extreme caution in class II Biological Safety cabinet (BSCII). *C. bantiana* may be distinguished from *Cladosporium* species by the absence of conidia with distinctly pigmented hila, the absence of shield cells and by growth at >40°C (compared with *C. carrionii* which has a maximum growth temperature of 35-37°C, and *Cladosporium* species which have a maximum of <35°C). *C. bantiana* may be further distinguished from *C. carrionii* by the formation of very long, sparsely branched chains of conidia. ITS sequencing is recommended.
Cladophialophora carrionii	Colonies are slow growing, reaching 3-4 cm in diameter after one month, with a compact suede-like to downy surface and are olivaceous-black in colour.	Microscopy shows ascending to erect, olivaceous-green, apically branched, elongate conidiophores producing branched acropetal chains of conidia. Conidia are pale olivaceous, smooth-walled or slightly verrucose, limoniform to fusiform, 1.5-3.0 x 2.0-7.0 µm in size. Bulbous phialides with large collarettes and minute, hyaline conidia are occasionally formed on nutritionally poor media. Maximum growth temperature 35-37°C.	Conidia are smaller and comprise heavily branched chains which fall apart much more easily than in the other *Cladophialophora* species. ITS sequencing is recommended

Organism	Cultural Characters	Microscopic characteristics	Growth temperature
Curvularia hawaiiensis *Synonymy: Bipolaris hawaiiensis*	Colonies powdery to hairy, black.	Conidiophores erect, unbranched, septate with conidial scars on the edges, up to 80 µm long. Conidia smooth- and rather thick-walled, brown, with (3-) 5 (-7) septa, cylindrical to cigar-shaped, 18-35 × 6-9 µm.	
Curvularia lunata	Colonies black, downy.	Conidiophores erect, unbranched, septate with dark brown scars. Conidia smooth-walled, olivaceous brown, end cells somewhat paler; conidia obovoidal to broadly clavate, curved at the subterminal cell, 21-31 × 8.5-12.0 µm, 3-septate, the subterminal cell swollen and distinctly larger than the remaining cells.	
Curvularia spicifera *Synonymy: Bipolaris spicifera*	Colonies appearing glassy with sooty powder of conidia, or hairy if sporulation is poor.	Conidiophores erect, unbranched, septate, up to 250 µm long and 4-8 µm wide, regularly zig-zagged in the apical part, with dark brown scars on the edges. Conidia brown, cylindrical with rounded ends, medium brown except for narrow subhyaline spots at the extremities, 20-40 × 9-14 µm, with 3 septa.	
Exophiala dermatitidis	Colonies are slow growing, initially yeast-like and black, becoming suede-like, olivaceous-grey with the development of aerial mycelium with age. A brown pigment is often produced in the agar.	The initial yeast-like phase is characterised by unicellular, ovoid to elliptical, budding yeast-like cells. The yeast like cells are hyaline and thin-walled when young becoming darkly pigmented (dematiaceous) and thick-walled when mature. With the development of mycelium, flask-shaped to cylindrical annellides are produced. Conidia are hyaline to pale brown, one-celled, round to obovoid, 2-4 x2.5-6 µm, smooth-walled and accumulate in slimy balls at the apices of the annellides or down their sides.	Cultures grow at 42°C and on media containing 0.1% cycloheximide.

Organism	Cultural Characters	Microscopic characteristics	Growth temperature
Exophiala jeanselmei	Colonies are initially smooth, greenish-grey to black, mucoid and yeast-like, becoming raised and developing tufts of aerial mycelium with age, often becoming dome-shaped and suede-like in texture. Reverse is olivaceous black.	Numerous ellipsoidal, yeast-like, budding cells are usually present, especially in young cultures. Scattered amongst these yeast-like cells are larger, inflated, subglobose to broadly ellipsoidal cells (germinating cells) which give rise to short torulose hyphae that gradually change into unswollen hyphae. Conidia are formed on lateral pegs either arising apically or laterally at right or acute angles from essentially undifferentiated hyphae or from strongly inflated detached conidia. conidiogenous pegs are 1-3 µm long, slightly tapering and imperceptibly annellate. Conidia are hyaline, smooth, thin walled, broadly ellipsoidal, 3.2-4.4 x1.2-2.2 µm, and with inconspicuous basal scars.	Cultures grow at 37°C but not at 40°C.
Exophiala spinifera	Colonies are initially mucoid and yeast-like, black, becoming raised and developing tufts of aerial mycelium with age, finally becoming suede-like to downy in texture. Reverse is olivaceous-black.	Conidiophores are simple or branched, erect or sub-erect, spine-like with rather thick brown pigmented walls. Conidia are formed in basipetal succession on lateral pegs either arising apically or laterally at right or acute angles from the spine-like conidiophores or from undifferentiated hyphae. Conidiogenous pegs are 1-3 µm long, slightly tapering and imperceptibly annellate. Conidia are one-celled, subhyaline, smooth, thin-walled, subglobose to ellipsoidal, 1.0-2.9 x1.8-2.5 µm, and aggregate in clusters at the tip of each annellide. Toruloid hyphae and yeast-like cells with secondary conidia are typically present.	Cultures will grow on media containing 0.1% cycloheximide. No growth at 40°C
Exserohilum rostratum	Colonies are grey to blackish-brown, suede-like to floccose in texture and have an olivaceous-black reverse.	Conidia are straight, curved or slightly bent, ellipsoidal to fusiform and are formed apically through a pore (poroconidia) on a sympodially elongating geniculate conidiophore. Conidia have a strongly protruding, truncate hilum and the septum above the hilum is usually thickened and dark, with the end cells often paler than other cells, walls often finely roughed. Conidial germination is bipolar.	

Organism	Cultural Characters	Microscopic characteristics	Growth temperature
Fonsecaea pedrosoi	Colonies are slow growing, flat to heaped and folded, suede-like to downy, olivaceous to black with black reverse.	Polypoidal, at least two types of condidiation (cladosporium, rhinocladiella, phialophora). Conidiogenous cells pale olivaceous, arranged in loosely branched systems, with prominent denticles. Conidia pale olivaceous, clavate to ellipsoidal, in short chains, subhyaline, smooth and thin walled, 3.5-5 x1.5-2 µm.	
Madurella mycetomatis	Colonies are slow growing, flat and leathery at first, white to yellow to yellowish-brown, becoming brownish, folded and heaped with age, and with the formation of aerial mycelia. A brown diffusible pigment is characteristically produced in primary cultures. Grains of *Madurella mycetomatis* (tissue microcolonies) are brown or black.	Although most cultures are sterile, two types of conidiation have been observed, the first being flask-shaped phialides that bear rounded conidia, the second being simple or branched conidiophores bearing pyriform conidia (3-5 µm) with truncated bases. The optimum temperature for growth of this mould is 37°C. Grains are 0.5-1.0 mm in size, round or lobed, hard and brittle, composed of hyphae which are 2-5 µm in diameter, with terminal cells expanded to 12-15 µm in diameter.	*M. mycetomatis* can be distinguished from *Trematosphaeria grisea* by growth at 37°C and its inability to assimilate sucrose.
Neoscytalidium dimidiatum (Formely Scytalidium dimidiatum)	Cultures are effuse, hairy, dark grey to blackish-brown, or white to greyish, with a cream-coloured to deep ochraceous-yellow reverse. Colourless mutants often occur.	Arthroconidia are typically present in chains of one to two-cells, darkly pigmented, 3.5-5 x 6.5-12 µm, produced by theholothallic fragmentation of undifferentiated hyphae. Pycnidia, only occasionally formed in older cultures are black, ostiolate and contain numerous hyaline, flask-shaped phialides. Phialoconidia are at first one-celled and hyaline, later becoming three-celled, brown, with the centre cell darker than the end cells and are ovoid to ellipsoidal in shape.	ITS and D1/D2 sequencing may be used for accurate species identification.

Organism	Cultural Characters	Microscopic characteristics	Growth temperature
Phialophora verrucosa	Colonies are slow growing, initially dome-shaped, later becoming flat, suede-like and olivaceous to black in colour.	Phialides are flaskshaped or elliptical with distinctive funnel-shaped, darkly pigmented collarettes. Conidia are ellipsoidal, smooth-walled, hyaline, mostly 3.0-5.0 x1.5-3.0 µm, and aggregate in slimy heads at the apices of the phialide.	
Pyrenochaeta romeroi	Colonies restricted, flat, evenly velvety or floccose, greyish-sepia to fuscous black; reverse olivaceous-black	Pycnidia submerged, ostiolate, black, spherical to pyriform, 85-100µm diameter, with thick walls, often covered with stiff, dark hyphae. Condia produced from ampulliform phialides lining the innermost pycnidial wall, oozing out in slimy drops, hyaline, ellipsoidal to bacilliform, 2.5 x 1.3-1.5 µm	Confirmation by molecular method
Rhinocladiella mackenziei	Colonies growing moderately rapidly, velvety, olivaceous brown.	Conidiophores arising at right angles from creeping hyphae, stout, thick-walled, brown, 3.0-4.5 µm wide, 10-25 µm long, apically with short-cylindrical denticles. Conidia brown, ellipsoidal, 8.5-12.0 × 4-5 µm, with a prominent, wide basal scar.	Cultures of *R. mackenziei* represent a potential biohazard to laboratory personnel and must be handled with extreme caution in a class II Biological Safety cabinet. ITS and D1/D2 sequencing is recommended for accurate species identification
Trematosphaeria grisea (formerly M. grisea)	Colonies are slow growing, dark, leathery, folded with radial grooves and with a light brown to greyish surface mycelium. With age, colonies become dark brown to reddish-brown and have a brownish-black reverse. Grains are brown.	Microscopically, cultures are sterile, although hyphae of two widths have been described, thin at 1-3 µm in width or broad at 3-5 µm in width.	The optimum temperature for growth of *T. Grisea* is 30°C; this fungus does not grow at 37°C. It can be distinguished from *Madurella mycetomatis* by the inability to grow at 37°C and to assimilate lactose.

Organism	Cultural Characters	Microscopic characteristics	Growth temperature
Verruconis gallopava	Colonies are smooth to suede-like, dry, flat, tobaccobrown to brownish-black with dark brown diffusible pigment.	Hyphae are brown with relatively thick walls. Conidiophores are mostly cylindrical to acicular, sometimes poorly differentiated, bearing a few conidia at the tip. Conidia are two-celled, subhyaline to pale brown, smooth-walled to verrucose, cylindrical to clavate, constricted at the septum, 11-18 x2.5-4.5 µm in size, with the apical cell wider than the basal cell. A remnant of a denticle may also be been at the conidial base.	Optimum growth at 35°C, tolerant to 40°C.

Table 3.6: Cultural and microscopic characteristics of *Mucorales*

Organism	Culture Characteristics	Microscopic Characteristics	Additional features
Saksenaea vasiformis	Colonies are fast growing, downy, white with no reverse pigment, and made up of broad, non-septate hyphae.	Sporangia are typically flask-shaped with a distinct spherical venter and longneck, arising singly or in pairs from dichotomously branched, darkly pigmented rhizoids. Collumellae are prominent and dome-shaped. Sporangiospores are small, oblong, 1-2 x 3-4 µm, and are discharged through the neck following the dissolution of an apical mucilaginous plug.	Laboratory identification of this fungus may be difficult or delayed because of the mould's failure to sporulate on primary isolation media or on subsequent subculture onto potato dextrose agar. Sporulation may be stimulated by using the agar block method. Failure to sporulate also prohibits antifungal susceptibility testing.
Rhizopus microsporus	Colonies are dark greyish-brown, up to 10 mm high producing simple rhizoids.	Sporangiophores are brownish, up to 400 µm high and 10 µm wide, and may be produced in groups of one to four, usually in pairs. Sporangia are greyish-black, spherical, up to 100 µm in diameter. Columellae are subglobose to globose to conical comprising 80% of the sporangium. Sporangiospores are angular to broadly ellipsoidal or subglobose, up to 5-9 µm in length and are distinctly striate. Chlamydospores may be present. Zygospores are dark red–brown, spherical, up to 100 µm in diameter, with stellate projections and unequal suspensor cells.	

Organism	Culture Characteristics	Microscopic Characteristics	Additional features
Rhizopus homothallicus	Colonies on SDA are fast growing, white, floccose, and devoid of pigmentation on the reverse side. Within 10 to 14 days of incubation at 28 to 30°C, colonies turn grayish.	Broad, hyaline, aseptate, branching hyphae producing very few sporangiophores opposite poorly developed rhizoids. The sporangiophores measure 5 to 27 µm in diameter and 50 to 150 µm in length bearing globose to subglobose sporangia measuring 50 to 150 µm in diameter. The sporangiospores are angular-globose, grayish, 3.5 to 5.0 µm in length, and 4.0 to 6.5 µm in width. The striking feature is abundant homothallic, thick-walled zygospores that were reddish/ golden brown in colour measuring 40 to 100 µm in diameter, including stellate spines. Suspensor cells are uneven in size, the larger ones being globose.	Thermotolerant (46 to 48°C)
Rhizopus arrhizus	Colonies are very fast growing, about 5-8 mm high, with some tendency to collapse, white cottony at first becoming brownish grey to blackish-grey depending on the amount of sporulation.	Sporangiophores up to 1500 µm in length and 18 µm in width, smooth-walled, non-septate, simple or branched, arising from stolons opposite rhizoids usually in groups of three or more. Sporangia are globose, often with attenuated base, greyish black, powdery in appearance, up to 175 µm in diameter and many spored. Columellae and apophysis together are globose, subglobose or oval, up to 130 µm in height collapsing to an umbrella-like form after spore release. Sporangiospores are angular, subglobose to ellipsoidal, with striations on the surface, and up to 8 µm in length.	
Rhizomucor pusillus	Cultures are characterised by compact, low growing (2-3 mm high), grey to greyish brown coloured mycelium There is positive assimilation of sucrose and no thiamine dependence.	Development of typical sympodially branched, hyaline to yellow-brown sporangiophores (8-15 µm in diameter), always with a septum below the sporangium. Sporangia are globose (40-60 µm in diameter), each possessing an oval or pear-shaped columella (20-30 µm), often with a collarette. Sporangiospores are hyaline, smooth-walled, globose to subglobose, occasionally oval (3-5 µm), and are often mixed with crystalline remnants of the sporangial wall. Chlamydospores are absent. Zygospores are rough walled, reddish brown to black, 45-65 µm in diameter and may be produced throughout the aerial hyphae in matings between compatible isolates.	Temperature optimum 35-55°C maximum 55°C.

Organism	Culture Characteristics	Microscopic Characteristics	Additional features
Rhizomucor pusillus	Cultures are characterised by compact, low growing (2-3 mm high), grey to greyish brown coloured mycelium There is positive assimilation of sucrose and no thiamine dependence.	Development of typical sympodially branched, hyaline to yellow-brown sporangiophores (8-15 µm in diameter), always with a septum below the sporangium. Sporangia are globose (40-60 µm in diameter), each possessing an oval or pear-shaped columella (20-30 µm), often with a collarette. Sporangiospores are hyaline, smooth-walled, globose to subglobose, occasionally oval (3-5 µm), and are often mixed with crystalline remnants of the sporangial wall. Chlamydospores are absent. Zygospores are rough walled, reddish brown to black, 45-65 µm in diameter and may be produced throughout the aerial hyphae in matings between compatible isolates.	Temperature optimum 35-55°C maximum 55°C.
Apophysomyces variabilis	Colonies are fast growing, whitish with scarce aerial mycelium and no reverse pigment. .	Sporangiophores are erect, generally arising singly, unbranched, slightly tapering towards the apex, up to 100-400 µm long, 2-3.5 µm in width near the apophysis, hyaline when young but developing a light greyish brown pigmentation with age. Sporangia are multispored, small (15-50 µm diameter), typically pyriform in shape, hyaline at first, sepia-coloured when mature, with distinct apophyses and columellae. Columellae are hemispherical in shape and the apophyses are short and distinctly funnel-shaped. Sporangiospores are smooth-walled, variable in shape, trapezoid, ellipsoidal, sub-triangular or claviform, (5-14 x 3-6 µm), subhyaline to sepia in mass.	Laboratory identification of this fungus may be difficult or delayed because of the mould's failure to sporulate on primary isolation media or on subsequent subculture onto potato dextrose agar. Sporulation may be stimulated by the use of nutrient deficient media, like cornmeal-glucose-sucrose-yeast extract agar, Czapek Dox agar, or by using the agar block method.
Cunninghamella bertholletiae	Colonies are very fast growing, white at first, but becoming dark grey and powdery with sporangiola development	Sporangiophores up to 20 µm wide, straight, with verticillate or solitary branches. Vesicles subglobose to pyriform, the terminal ones up to 40 µm and the lateral ones 10-30 µm in diameter. Sporangiola are globose (7-11 µm diameter), or ellipsoidal (9-13 x6-10 µm), verrucose or short-echinulate, hyaline singly but brownish in mass.	

Organism	Culture Characteristics	Microscopic Characteristics	Additional features
Mucor circinelloides	Colonies are floccose, pale greyish-brown and grow poorly at 37°C (maximum growth temperature 37°C).	Sporangiophores are hyaline and mostly sympodially branched with long branches erect and shorter branches becoming circinate (coiled). Sporangia are spherical, varying from 20-80 µm in diameter, with small sporangia often having a persistent sporangial wall. Columellae are spherical to ellipsoidal and are up to 50 µm in diameter. Sporangiospores are hyaline, smoothwalled, ellipsoidal, and 4.5-7 x3.5-5 µm in size. Chlamydospores are generally absent. Zygospores are only produced in crosses of compatible mating types and are reddish brown to dark-brown, spherical with stellate spines, up to 100 µm in diameter and have equal to slightly unequal suspensor cells.	
Mucor indicus	Colonies are characteristically deep yellow, aromatic and have a maximum growth temperature of 42°C	Sporangiophores are hyaline to yellowish, erect or rarely circinate and repeatedly sympodially branched, with long branches. Sporangia are yellow to brown, up to 75 µm in diameter, with diffluent membranes. Columellae are subglobose to pyriform, often with truncate bases, up to 40 µm high. Sporangiospores are smooth-walled, subglobose to ellipsoidal, and 4-5 µm in diameter. Chlamydospores are produced in abundance, especially in the light. Zygospores are black, spherical up to 100 µm in diameter, with stellate spines and unequal suspensor cells.	
Mucor ramosissimus	Colonial growth is restricted, greyish and does not grow at 37°C (maximum temperature for growth is 36°C).	Sporangiophores are hyaline, slightly roughened, tapering towards the apex and are erect with repeated sympodial branching. Sporangia are grey to black, globose or somewhat flattened, up to 80 µm in diameter and have very persistent sporangial walls. Columellae are aplanate (flattened), up to 40-50 µm in size and are often absent in smaller sporangia. Sporangiospores are faintly brown, smooth-walled, subglobose to broadly ellipsoidal, 5-8 x4.5-6 µm in size. Oidia may be present in the substrate hyphae. Chlamydospores and zygospores are absent.	

Table 3.7: Cultural and microscopic characteristics of dimorphic fungi

Organism	Culture characteristics	Microscopic characteristics	Additional features to remember
Blastomyces dermatitidis	SDA (25°C): Colonies whitish fluffy, reverse cream coloured to brownish. BHI (37°C): Colonies butyrous, off white to cream in colour.	(25°C): Conidia 2-10 µm are produced on lateral, morphologically non specialized conidiophores or on the hyphal tips. Conidia are hyaline, spherical to oval, or sometimes dumbbell shaped with smooth walls. BHI(37°C) :Yeast cells (8-15 µm, rarely upto 30 µm) are hyaline, smooth and thick walled. Cells produce buds through a wide pore. Daughter cells do not separate from the mother cells until they are nearly the same size. Continuous budding without separation of the early bud may result in small cluster of cells	Cultures of *B. dermatitidis* represent a biohazard to laboratory personnel and must be handled in a class II Biological Safety cabinet. In the past, conversion from the mould form to the yeast form was necessary to positively identify this dimorphic pathogen from species of *Chrysosporium* or *Sepedonium*. However, culture identification by exoantigen test and/or molecular methods is now preferred to minimise manipulation of the fungus.
Coccidioides immitis	SDA (25°C) Fast growing colonies with variable textures and pigments. Texture may vary from powdery, cottony, velvety to granular. Isolates may be gray, buff, yellow or brown. Reverse side may vary from orange to brown	SDA (25°C) Hyphae produce spore bearing branches that have a diameter twice that of vegetative hyphae and branch at right angles. Arthroconidia (2.5 x 4.3 µm) are 1-celled, short cylindrical to barrel shaped, smooth walled, alternate with empty disjunctor cells. Liberated conidia carry a portion of the walls of the intervening sterile segments. BHI (37°C): No yeast phase produced on BHI agar.	Cultures of *Coccidioides immitis* represent a severe biohazard to laboratory personnel and must be handled with extreme caution in class II Biological Safety cabinet. Culture identification by either exoantigen test or DNA sequencing is preferred to minimise exposure to the infectious propagule.
Histoplasma capsulatum	SDA (25°C): Colonies granular to cottony initially white, later becoming brownish. Reverse initially cream coloured, becoming brownish with age. BHI with Blood (37°C): Smooth, yeast like colonies.	SDA(25°C): Micro as well as macro conidia are produced. Microconidia (1-4 x 2-6 µm) sessile or arising on sort stalks from undifferentiated hyphae, hyaline, smooth, thin walled, 1-celled, pyriform to clavate. Macroconidia (8-14) brown, arise from short conidiophores, thick walled tuberculate or with cylindrical projections, spherical. BHI (37°C) :Yeast phase with budding cell, upto 7 µm in diameter	Cultures of *Histoplasma capsulatum* represent a severe biohazard to laboratory personnel and must be handled with extreme caution in a class II Biological Safety cabinet.

Organism	Culture characteristics	Microscopic characteristics	Additional features to remember
Emmonsia pasteuriana	Colony white coloured with tan on the reverse.	Thin (1–2µm diameter) hyaline septate hyphae, perpendicular slender conidiophores with terminal conidia or1–3 conidia clustered in a flower-like arrangement. Occasional intercalary chlamydoconidia noticed. The yeast conversion on brain heart infusion agar with blood at 37°C after 21 days shows small cells (2.5µm in diameter) with narrow base budding.	
Talaromyces marneffei (formely Penicillium marneffei)	Colonies at 25°C are fast growing, suede-like to downy, white with yellowish-green conidial heads. Colonies become greyish-pink to brown with age and produce a diffusible brownish-red to wine-red pigment. On brain heart infusion (BHI) agar containing blood incubated at 37°C, colonies are rough, glabrous, tan-coloured and yeast-like.	Conidiophores generally biverticillate and sometimes monoverticillate; hyaline, smooth-walled and bear terminal verticils of three to five metulae, each bearing three to seven phialides. Phialides are acerose to flask-shaped. Conidia are globose to subglobose, 2-3 µm in diameter, smooth-walled and are produced in basipetal succession. Microscopically, yeast cells are spherical to ellipsoidal, 2-6 µm in diameter, and divide by fission rather than budding. Numerous short hyphal elements are also present.	Cultures of *Talaromyces marneffei* may represent a biohazard to laboratory personnel and should be handled with caution in a class II Biological Safety cabinet.
Sporothrix schenckii	Colonies at 25°C, are slow growing, moist and glabrous, with a wrinkled and folded surface. Some strains may produce short aerial hyphae and pigmentation may vary from white to cream to black. On brain heart infusion (BHI) agar containing blood at 37°C colonies are glabrous, white to greyish-yellow and yeast-like consisting of spherical or oval budding yeast cells.	Conidiophores arise at right angles from thin septate hyphae and are usually solitary, erect and tapered toward the apex. Conidia are formed in clusters on tiny denticles by sympodial proliferation at the apex of the conidiophore, their arrangement often suggestive of a flower. As the culture ages, conidia are subsequently formed singly along the sides of both conidiophores and undifferentiated hyphae. Conidia are ovoid or elongated, 3-6 x 2-3 µm, hyaline, one-celled and smooth-walled. In some isolates, solitary, darkly-pigmented, thick- walled, one-celled, obovate to angular conidia may be observed along the hyphae.	DNA sequencing using ITS, D1/D2, β-tubulin, calmodulin and chalcone synthase genes is recommended for species identification.

Identification of Dermatophytes

Dermatophytes are the keratinophilic fungi capable of invading keratinous tissue. All dermatophytes are classified into three Genera namely *Epidermophyton, Microsporum* and *Trichophyton*. Differentiation into three Genera is based on the colony characteristics on Sabouraud dextrose agar and microscopic morphology. The accurate identification of some dermatophytes requires additional tests such as growth on special media for enhancement of pigment production or sporulation, physiological tests to determine special nutritional requirements/capability to perforate hair/ to hydrolyze urea/ to tolerate high temperature.

Colony characteristics: examination of the colony characteristics on the Sabouraud dextrose agar is one of the important criteria for the identification of dermatophytes. The colony should be observed for the pigmentation on the surface and reverse, texture on the surface (cottony, wooly, velvety, powdery, ceribriform, granular or glabrous) and topography (elevation, margin and folding).

Microscopic characteristics: on microscopic examination, the appearance and arrangement of the macroconidia and/microconidia aid in the identification of most of the dermatophytes. The characteristics of the macroconidia especially the size, shape, number of septations, characters of their walls and the way they are borne on the hyphae are important to describe the Genus of the dermatophyte (**Table 3.8**). Various other non-specific structures that are helpful in identification in conjunction to characteristic macroconidia are chlamydospores, spirals, nodular organs, pectinate organs, arthroconidia and racquet mycelium.

Table 3.8: Characteristics of macroconidia in dermatophytes

Genus	Size (µ)	Septation	Wall thickness	Wall surface	Occurrence	Attachment
Trichophyton	8-86/ 4-14	1-12	Thin-thick	Smooth	Usually rare	Single/clusters
Microsporum	6-160/ 6-25	1-15	Thick	Rough	Usually numerous (rare in *M. audouinii)*	Single
Epidermophyton	20-40/ 7-12	1-6	Thick	Smooth	Numerous	Single/clusters of 2/3

Urea hydrolysis (see section 10, Chapter 2)

Urease test

A number of dermatophytes have the ability to hydrolyze urea and, therefore, a test that demonstrates the production of urease can be utilized in their differentiation.

Procedure

- Prepare urea agar medium using Difco Urea Agar Base with the addition of 0.4% glucose and dispense in tubes.
- After inoculation, incubate at 26°C for 7 days.
- Examine for the change in colour from straw to red which indicates the production of urease.

Results

Positive: *T. interdigitale*; *T. mentagrophytes*; *T. megninii*
Negative: *T. rubrum*; *T. erinacei*

Table 3.9: Cultural and microscopic characteristics of dermatophytes

Dermatophyte species	Colony morphology on SGA	Microscopic morphology	Other tests	Comments
Epidermophyton floccosum	Slow growing, flat, fluffy, soon becomes velvety or powdery; turn tan to olive-brown/ green; reverse yellowish tan	Microconidia absent, numerous macroconidia broad, blunt to clavate with smooth thin wall and 2-6 celled. Chlamydospores are usually plenty	Urease positive. Hair perforation negative	Do not invade hair
Microsporum audouinii	Slow growing, flat, velvety with whitish tan to brown surface, reverse salmon to pale brown	Macroconidia and microconidia usually rare. Macroconidia if present are large with beak and constriction in the middle and smooth or echinulate surface	Urease negative. Hair perforation negative	Poor growth on Rice grain media
Microsporum canis	Rapid growing, initially white with pale to yellow pigment; later tan, cottony with central knob. Reverse yellow to orange brown	Macroconidia numerous, large, spindle shaped, thick echinulate wall and 8-15 celled. Microconidia few, sessile and drop shaped	Urease positive. Hair perforation positive	Teleomorph- *Arthrodermaotae* Grows confluently on Rice grain media with yellow pigment.
Microsporum cookie	Rapidly growing, powdery granular to velvety, yellowish to greenish brown surface. Reverse wine red	Macroconidia few, broader thick-walled and rough. Microconidia abundant and drop shaped	Urease test positive. Hair perforation test positive	Teleomorph- *A. cajetan*i
Microsporum ferrugineum	Slow growing, waxy with many folds and yellowish to deep rust colour surface. Reverse yellow to orange	Macroconidia and microconidia absent. Hyphae well formed with arthroconidia	Urease negative. Hair perforation test negative.	Geographically restricted

Dermatophyte species	Colony morphology on SGA	Microscopic morphology	Other tests	Comments
Microsporum gypseum	Rapid growing, powdery to granular with cinnamon to rosy buff. Reverse pale yellow to brownish	Macroconidia numerous, ellipsoid, thin, echinulate wall and 3-9 septa. Microconidia are rare, drop shaped and sessile.	Urease positive. Hair perforation test positive	Good growth on Rice grain media
Microsporum nanum	Rapid growing, flat, powdery to fluffy with sandy surface. Reverse brownish red to dark red	Macroconidia numerous, ellipsoidal, thin walled, rough surface, 1-celled. Microconidia pear shaped and sessile.	Urease test positive. Hair perforation test positive	Poor growth at 37^0C. Teleomorph- *Arthrodermaobtusum*
Microsporum persicolor	Rapid growing, flat, fluffy to powdery, yellowish to pinkish buff surface. Reverse peach to rose	Macroconidia often absent, spindle shaped, smooth thin walled and around 6 celled. Microconidia subglobose to club shaped and seen in clusters.	Urease test positive. Hair perforation test positive	Teleomorph- *A. persicolor*. Spiral hyphae, resembling *T. mentagrophytes* is produced
Microsporum vanbreuseghemii	Rapid growing, flat, powdery with yellowish tan to pink surface. Reverse no pigmentation or yellowish	Macroconidia numerous. Cylindrofusiform, rough, thick-walled with 7-10 celled		Teleomorph- *A. grubyi*
Trichophyton mentagrophytes complex (anthropophilic) [human adapted form of *T. interdigitale*]	Rapid growing, powdery to fluffy, cream to buff white surface; reverse pale to red brown	Macroconidia rare, club shaped, smooth, microconidia abundant, spherical or drop shaped and seen in clusters. Spiral hyphae present	Urease test positive. Hair perforation positive	Macroconidia can be induced on SGA + 3-5% NaCl. Teleomorph- *A. vanbreuseghemii A benhamiae*
Trichophyton mentagrophytes (nodular variant and morphotype of *T. interdigitale*) [earlier *T. krajdenii*]	Slow growing, cottony with white to cream surface and yellow margin. Reverse deep yellow	Macroconidia rare, microconidia abundant, drop shaped and sometime round coiled yellow nodular bodies and yellow granular. Spiral hyphae rarely seen	Urease test positive. Hair perforation test positive	

Dermatophyte species	Colony morphology on SGA	Microscopic morphology	Other tests	Comments
Trichophyton mentagrophytes complex. (Zoophilic) [*T. mentagrophytes sensu strict* (former *T. mentagrophytes* varquinckeanum)], animal adapted form of *T. interdigitale* and *Trichophyton* anamorph of *A. Benhamiae*	Rapid growing, flat, powdery to granular, with cream to buff or tan surface. Reverse pale to red brown	Macroconidia rare, club shaped, smooth, microconidia abundant, spherical, arranged in clusters	Urease test positive. Hair perforation positive	Teleomorph- *A. vanbreuseghemii, A benhamiae*
Trichophyton rubrum (Afro-Asiatic variant)	Slow growing, powdery to velvety, cream to deep red surface. Reverse wine red	Macroconidia abundant, club shaped and rarely rat tail extension. Microconidia drop shaped to round with many chlamydospores	Urease test positive. Hair perforation - negative	
Trichophyton rubrum (cosmopolitan variant)	Slow growing, flat/ heaped, velvety or powdery which becomes highly folded, surface white to cream to deep rose. Reverse wine red; rarely yellowish orange	Macroconidia rare; pencil shaped. Microconidia drop shaped abundant, scanty or not formed.	Urease test positive. Hair perforation test- negative	
Trichophyton schoenleinii	Slow growing, heaped up, leathery, white to tan, glabrous or white to powdery surface	Macroconidia absent. Microconidia very rare. Hyphae appear knobby and clubbed at ends. (Favic chandeliers). Chlamydospores abundant.	Urease test variable. Hair perforation test- negative	

Dermatophyte species	Colony morphology on SGA	Microscopic morphology	Other tests	Comments
Trichophyton tonsurans	Slow growing, powdery surface which turn to velvety. Surface becomes tan or bright yellow or rose. Reverse yellowish to mahogany red.	Macroconidia rare, small pencil or club shaped, thin and smooth wall. Microconidia abundant tear shaped produced on undifferentiated hyphae or simple condiophores. Some microconidia enlarge and appear like balloons.	Urease test positive. Hair perforation test- mostly negative	
Trichophyton veruccosum	Very slow growing, folded, white or yellow powdery. Reverse no pigment	Macroconidia rare, string bean shaped, thin and smooth walled 3-5 celled. Microconidia abundant on media supplemented with thiamine and inositol. They are small and borne on hyphae. On SGA chlamydospores are seen	Urease test negative. Hair perforation test- negative	Thiamine essential for sporulation
Trichophyton violaceum	Slow growing, heaped, glabrous, cream to lavender deep purple surface	Macroconidia rare. Microconidia usually not found on SGA. Production of microconidia enhanced on thiamine medium	Urease test positive/ weak. Hair perforation - negative	

MALDI-TOF-MS Based Fungal Identification

CHAPTER 4

MALDI-TOF-MS based Fungal Identification

Bruker's MALDI-Biotyper

Required chemicals and accessories

- Double distilled water (DDW)
- Absolute ethanol (EtOH)
- Acetonitrile (ACN)
- 98% Formic acid (FA)
- Trifluoroacetic acid (TFA)
- Matrix HCCA (100 µL of matrix solution containing: 50 µL acetonitrile, 2.5 µL trifluoroacetic acid, 1 mg α-cyano-4-hydroxycinnamic acid and 47.4 µL sterile deionized water)
- Wooden application sticks, pipette tips or plastic inoculation loop to apply material
- 50–1000 µL pipettete tips and a suitable pipette
- 2–200 µL pipettete tips and a suitable pipette
- 0.5–10 µL pipette tips and a suitable pipette
- Microcentrifuge tube, PCR clean 2 mL
- MALDI target plate
- Test tube rotator
- 0.5-0.75 mm sterile acid washed glass beads (Sigma-Aldrich, Saint Quentin Fallavier, France)
- Benchtop centrifuge
- Vortex

MALDI target plate cleaning procedure

1. Transfer the target into a petri dish
2. Cover the surface of the MALDI target plate with 70% aqueous ethanol
3. Incubate for 5 minutes at room temperature
4. Remove the MALDI target plate and rinse it thoroughly under running tap water
5. Using a Kimwipe, clean the MALDI target plate intensively with 70% aqueous ethanol
6. Rinse the MALDI target plate with tap water and wipe it with a Kimwipe
7. Cover the MALDI target plate with 100 µL of 80% aqueous TFA
8. Intensively wipe all target positions with a Kimwipe
9. Rinse the MALDI target plate with deionized water and wipe it dry with a Kimwipe.
10. Allow the target to dry at room temperature for at least 15 minutes
11. If the cleaned MALDI target plate is not immediately used, store it in the plastic storage box.

Instrumental procedure

1. Open Flex control software
2. Click the arrow to In or Out the MALDI target plate

3. Open MALDI Biotyper Realtime Classification software
4. Click on 'File'
5. Select 'New Classification'
6. Give project name
7. Click on New
8. Select position of target samples and right click and select 'Add Analytes'
9. Give the ID of each sample and click on 'Next'
10. Select the MALDI Biotyper Methods (MSPs from Libraries/ MSPs from Taxonomy Trees) and click 'Next'
11. Check the project summary and finally click 'Finish' for automatically identify the samples.

On-Plate Formic Acid Extraction Method for Yeast

1. Emulsify one loop-full of fresh culture in 1 mL sterile DDW in 1.5 mL microcentrifuge tube
2. Centrifuge the suspension at 13,000 rpm for 2 minutes
3. Discard the supernatant and repeat steeps 1 to 4 twice
4. Re-suspend the pellet in 50 µL sterile DDW
5. Spot 1 µL of this suspension in duplicate on the MALDI plate and dry in air
6. Overlay the spots with 0.5 µL of 98% formic acid and again air dry it
7. Added 0.8 µL of matrix solution on each spot
8. Identify each dried spot by MALDI-TOF MS.
9. The spectra will be acquired by FLEX control software and the spectra will be matched against the existing database present in MALDI Biotyper software.
10. According to Brucker, if the score is <1.7 the identification is not reliable; score is in between > 1.7 to 1.99 the identification is confirmed up-to genus level; score is in between >2 the identification is confirmed up-to species level.

Formic Acid Extraction Method for Molds

1. Inoculate fungal growth in 5 mL of Sabouraud dextrose broth in 15 mL centrifuge tube
2. Spin the tubes in a test tube rotator at 50 rpm for 12–24 hours at 28ºC
3. Keep the tubes in static condition for 10 minutes for sedimentation of hyphal flakes
4. Transfer 1.5 mL of suspension in to a microcentrifuge tube by using blunt-end pipettete tips
5. Centrifuge at 13,000 rpm for 5 minutes
6. Completely remove the media
7. Add 1.5 mL sterile DDW for washing the pellet
8. Centrifuge at 13,000 r.p.m. for 5 minutes
9. Remove 1.2 mL supernatant and replace with 700 µL absolute ethanol and keep for 5 minutes
10. Remove the ethanol content by pipetteting and vacuum concentrator
11. Emulsify the dried pellet in 50–80 µL formic acid (FA)
12. Incubate at room temperature for 5 minutes
13. Add 50–80 µL acetonitrile (ACN)
14. Vortex the emulsion for 5–10 minutes with 0.5 mm acid-washed glass beads for proper lysis
15. Centrifuge at 10,000 rpm for 5 minutes
16. Spot 4-6 µL of the supernatant (1.5-2 µL each time for 3-4 times) on to the surface of 96-target polished steel plate and allow to dry
17. Overlay the dry spot was with 1 µL matrix solution

18. Air dry prior to MALDI-TOF MS analysis
19. The spectra will be acquired by FLEX control software and the spectra will be matched against the existing database present in MALDI Biotyper software.
20. According to Bruker, if the score is <1.7 the identification is not reliable; score is in between 1.7 to 1.99 the identification is confirmed up-to genus level; score is in between >2 the identification is confirmed up-to species level.

Formic Acid Extraction Method for Melanized Fungi

1. Culture the isolates on SDA and incubate for 3 to 7 days at 28°C
2. Harvest very small pieces of mycelial growth by using teasing needle and place in 1.5 mL DDW in 2 mL microcentrifuge tube
3. Vortex well to disperse the mycelial clump and produce thin and smaller structure
4. Centrifuge it for 10 minutes at 13,000 rpm
5. Completely remove the supernatant and replace with 1 mL of 70% ethanol
6. Incubate at room temperature for 5 minutes
7. Centrifuge the suspension at 13,000 rpm and discarded the supernatant
8. Dry the pellet properly in a vacuum concentrator (Savant™ DNA SpeedVac™ concentrator, Thermo Fisher Scientific, USA)
9. Add 50-80 µL of 98% FA to resuspend the pellet
10. Incubate for 5 minutes at room temperature
11. Add an equal amount of ACN
12. Add 0.5-0.75 mm sterile acid washed glass beads (Sigma-Aldrich, Saint Quentin Fallavier, France) and vortex vigorously for 10-15 minutes
13. Centrifuge the suspension at 10,000 rpm for 5 minutes
14. Spot 4-6 µL of the supernatant (1.5-2 µL each time for 3-4 times) on to the surface of 96-target polished steel plate and allow to dry
15. Overlay the dry spot was with 1 µL matrix solution
16. Air dry prior to MALDI-TOF MS analysis
17. The spectra will be acquired by FLEX control software and the spectra will be matched against the existing database present in MALDI Biotyper software.
18. According to Bruker, if the score is <1.7 the identification is not reliable; score is in between 1.7 to 1.99 the identification is confirmed up-to genus level; score is in between >2 the identification is confirmed up-to species level.

(Alternative system available: Vitek MS, BioMérieux)

Molecular Identification of Fungi

CHAPTER 5

Molecular Identification of Fungi

I. DNA Extraction

1. Yeast

1. Sub culture the yeast on SDA (Sabouraud Dextrose agar, Himedia) or PDA (Potato Dextrose agar) with antibiotic (chloramphenicol 0.05 g/L)
2. Incubate the culture at 37°C for 24-48 hours.
3. Add a loopful of culture in 50 mL of yeast extract-phosphate-dextrose broth in 200 mL flask.
4. Incubate overnight at 30˚C.
5. Pellet down growth in 1.5 mL of microfuge tube at 7500 rpm for 2 minutes.
6. Suspend the pellet in 200 µL of Lysis buffer (100 mM Tris HCl [pH8.0], 50 mM EDTA, 3% SDS).
7. Add 1µl of RNase (10 mg/mL, Thermo Scientific) & incubate at 37˚C for 30 minutes.
8. Add 1µl of Proteinase (20 mg/mL, Thermo Scientific) & incubate at 56˚C for 2 hours.
9. Add 0.3 g of sterile glass beads.
10. Mix well by vortexing (or use a bead beater) and then add 200 µl of 1:1 of Phenol: chloroform to the suspension.
11. Vortex this mixture vigorously for one minute followed by cooling on ice for 30 seconds.
12. Add 200 µl of TE buffer & again vortex briefly.
13. Centrifuge the mixture at 13000 rpm at room temperature for 5 minutes.
14. Take aqueous phase in clear sterile microfuge tube and 1:1 chloroform: Iso-amyl alcohol.
15. Collect aqueous phase and add 1/10th volume of 3M sodium acetate. Add equal volume of chilled isopropanol. Mix and incubate at -20° C for 2 hours for DNA precipitation.
16. Collect the DNA precipitate at 10000 rpm for 5 minutes and decant the supernatant.
17. Add 0.5 mL of 70% ethanol, vortex to wash the pellet.
18. Centrifuge at 10000 rpm for 5 minutes, aspirate out the supernatant carefully and allow the pellet to dry at 37° C for one hour.
19. Re-suspend the pellet in appropriate volume of TE Buffer.
20. Allow DNA to dissolve at room temperature with intermittent mixing.

Note: After DNA extraction, if proteinase K and RNase treatment was given, first treat the DNA with RNase, incubate it at 37° C followed by proteinase K. To denature the residual proteinase K heat the DNA at 95°C for 10 minutes. (Don't heat the DNA for more than 10 minutes at 95°C, DNA will degrade).

2. Moulds

1. Grow mycelial isolate on Sabouraud dextrose agar (SDA) slope for three to four days at 25°C. Collect the conidia and wash with normal saline.
2. Inoculate into Sabouraud dextrose broth and incubate at 37°C in rotary shaker at 150 rpm for about 2 days.

3. Recover mycelia by filtration through 50 mL syringe which is packed with sterile glass wool. The fungal mat formed in syringe is washed with acetone.
4. Transfer mat to a Whatman filter paper no. 1 and let it dry at 37°C.
5. Transfer mat to a clean mortar and add a small volume of Liquid nitrogen (-196°C).
6. Grind the frozen mat quickly to make fine powder using a pestle and adding more liquid nitrogen.
7. To this powder immediately add 2-3 mL of lysis buffer preheated to 65°C and mix well to make slurry. Transfer 0.7 mL of this suspension to 1.5 mL microfuge tube.
8. Add 1µL of proteinase K (20 mg/mL) and incubate the tube at 55°C for one hour with occasional mixing.
9. To this add 700 µl of buffered Phenol: chloroform (1:1), vortex briefly and centrifuge at room temperature for 5 minutes at 11000 rpm.
10. Transfer the top aqueous phase to another sterile microfuge tube without disturbing the interface and add equal volume of chloroform: isoamyl alcohol (24:1) to the supernatant. Repeat the centrifugation and collect the upper aqueous phase into a fresh tube.
11. Add 1/10th volume of 3M sodium acetate and add two volumes of ice chilled ethanol to allow the DNA to precipitate. Gently invert the tubes to mix contents and to see the threads of DNA precipitation.
12. Centrifuge at 10000 rpm for 2-5 minutes at room temperature. Without disturbing the pellet decant the supernatant and wash the pellet twice with 500 µl of ice cold 70% ethanol.
13. Allow the pellet to dry at 37°C till the ethanol evaporates completely.
14. Resuspend the pellet in appropriate amount of TE buffer. The DNA is allowed to dissolve at room temperature with intermittent mixing.
15. 1 µl of RNase is added and incubated at 37°C for 30 minutes.

II. Quantitative and qualitative determination of DNA

The following two methods can be used to check the quality of extracted DNA

a) Qualitative method (Agarose gel electrophoresis)
b) Quantitative method (Nanodrop spectrophotometer) (2000 Thermo).

a) Gel electrophoresis

Agarose gel preparation

1. To check the quality of the DNA, 0.8% agarose gel should be prepared in 50 mL volume of 1X TAE buffer.
2. Weigh out 0.4 g of agarose and add 50 mL of 1X TAE Buffer, swirl to mix.
3. Melt the agarose by microwave or by heat plate for 1-2 minutes. (Note: Uniform clear solution should be seen)
4. Allow it to cool at room temperature till 60°C.
5. Add 1 µL of ethidium bromide (10 mg/mL) and swirl to mix
6. Pour the gel slowly into the gel casting tray without formation of air bubbles. Insert the comb and double check that it is correctly positioned.
7. Allow the gel to solidify for 30 minutes, preferably 1 hour, with the lid on if possible.
8. After solidification of agarose, place the gel in the electrophoresis apparatus containing 1X TAE buffer. (Note: the gel should be submerged completely in the buffer).

Loading of samples

1. Take 2 µL of loading buffer and mix it with 8 µL of PCR Product.
2. Mix it without bubble formation and load into appropriate well in the gel
3. Close the gel tank, switch on the power-source and run the gel by applying current (100 Volts, 400 mA for 30 minutes).
4. Check that a current is flowing
5. Monitor the progress of the gel by loading dye movement.
6. Switch off and unplug the gel tank and carry the gel (in its holder if possible) to the dark-room to look at on the UV light-box.
7. Observing separated DNA fragments
8. When electrophoresis has completed, turn off the power supply and remove the lid of the gel box.
9. Remove gel from the gel box. Drain off excess buffer from the surface of the gel. Place the gel tray on paper towels to absorb any extra running buffer.
10. Remove the gel from the gel tray and expose the gel to UV light. This is most commonly done using a gel documentation system. DNA bands should show up as orange fluorescent bands. Take a picture of the gel.
11. Properly dispose of the gel and running buffer as per institution regulations.

Note: If the sample shows smearing in agarose gel it means DNA degradation. In this case check reagents (buffers, Phenol: chloroform: iso amyl alcohol solution).

b) Quantification of DNA using spectrophotometer Nanodrop 2000

Take 1µl of the DNA sample and measure OD and 260/280 ratio at 340 nm wavelength.

Troubleshooting in DNA extraction

- The minimum DNA concentration for accurate PCR amplification should be in the range of 100-200 ng/µl with 260/280 ratio (quality) must be 1.70-1.90.
- In case the 260/280 ratio comes >2.00 it indicates RNA contamination. Treat the DNA with RNase enzyme by adding 1µl and incubate it at 37°C for 2 hours.
- If the 260/280 ratio comes <1.70 it indicates protein contamination. Treat the DNA with 1 µL Proteinase enzyme by adding 1µL of proteinase K to extracted DNA and incubate it at 56°C for 2 hours. To inactivate, proteinase K activity, heat the DNA at 95°C for 10 minutes.

III. STEP 2: PCR amplification

Amplify DNA of large ribosomal subunit (LSU; rDNA) gene or internal transcribed spacer (ITS) in a thermal cycler (Eppendorf master cycler gradient, Germany) using pan- fungal primers for rDNA, NL-1 (5'-GCATATCAATAAGCGGAGGAAAAG-3) and NL-4 (5'-GGTCCGTGTTTCAAGACGG-3) targeting D1/D2 region of 26/28S rDNA of large ribosomal subunit; and ITS-1 (5'-TCCGTAGGTGAACCTGCGG-3) and ITS-4 (5'TCCTCCGCTTATTGATATGC-3') for the internal transcribed spacer regions (ITS-1 and ITS-2).

Table 5.1: Reaction mixture for amplication of rDNA of large ribosomal subunit (LSU) gene or internal transcribed spacer (ITS)

PCR Reaction Mixture (100 µL)				
S.No.	**Reagent**	**Initial Conc.**	**Final Conc.**	**Volume used**
1	PCR Buffer	10X	1X	10 µL
2	Forward primer	10 picomole	0.2	2 µL
3	Reverse primer	10 picomole	0.2	2 µL
4	dNTP mix	10 millimolar	0.2	2 µL
5	Taq polymerase	1000 units/mL	0.5 units	0.5 µL
6	Milli Q water			83.5 µL

Template: Use 0.5-1.0 µL DNA as template according to the concentration.

PCR program

For NL-1 and NL-4 set of primers

1. T=95^{O}C for 5 minutes.
2. T=94^{O}C for 1 minute.
3. T=55^{O}C for 30 seconds.
4. T=72^{O}C for 2 minutes.
5. GO TO STEP 2, REPEAT 35 CYCLES.
6. T = 72 ^{O}C for 5 minutes.
7. 20^{O}C HOLD.

For ITS-1 and ITS -4 set of primers

1. T=95^{O}C for 5 minutes.
2. T=94^{O}C for 1 minute.
3. T=55^{O}C for 30 seconds.
4. T=72^{O}C for 1 minutes.
5. GO TO STEP 2 REPEAT 40 CYCLES
6. T = 72^{O}C for 7 minutes.
7. 20^{O}C HOLD

Electrophorese the amplified products on 1% agarose gel and view the size and quality of PCR products in UV transilluminator or gel documentation system.

IV. Gel extraction of PCR products

Excise the amplified products from the gel using QIAquick Gel Extraction Kit (Qiagen, Hilden) or other kits as per manufacturer instructions.

V. Sequencing PCR

1. DNA quantitation (Pre requisite step)

Quantify the purified PCR product by measuring OD at 260 nm (Thermo Nanodrop 2000) or by agarose gel (1.5%) electrophoresis.

2. Template quantity

Table 5.2: Template concentrations for sequencing reaction

Template	Quantity
PCR Product:	
100-200 bp	1-3 ng
200-500 bp	3-10 ng
500-1000 bp	5-20 ng
Plasmid:	150-300 ng

Note:

- Higher DNA quantities give higher signal intensities.
- Too much template makes data appear too heavy, with strong peaks at the beginning of the run that fade rapidly
- Too little template or primer reduces the signal strength and peak height. The template quantities given above will work with all primers.

3. Reagents for DNA sequencing

a. 5X sequencing buffer (Invitrogen, USA)
b. Big dye terminator (3.1/1.1 version, Invirogen USA)
   - ♦ BDT version 3.1 should be used for sequencing 300-700bp size.
   - ♦ BDT 1.1 version should be used for 100-250 bp product size.
c. 6 picomole primer
d. MilliQ (deionized double distilled water)

Table 5.3: Master mix preparation for sequencing reaction

Reagents	Quantity
H_20	5.75 µl
5X sequencing buffer	1.75 µl
Big dye terminator (1:16 dilution)	0.50 µl
6 picomole primer	1.00 µl
Template	1.00 µl
Total reaction volume	10 µl

Note: Gently mix the reaction mixture and centrifuge, to pellet down the mixture.

4. Steps in sequencing PCR

- Place the tube in a thermal cycler.
- Set the volume to 10 µl and select 0.5 mL tube.
- Run on PCR machine using following conditions:

Step	Cycles
1.	T=96˚C for 1 minutes.
2.	T=96˚C for 10 seconds.
3.	T=50˚C for 05 seconds.
4.	T=60˚C for 4 minutes.
5.	Go to step 2 Repeat 25 Cycles
7.	4˚C Hold

- Start the run in the thermal cycler (Ependorff Master Cycler Gradient) as
- Switch on the thermal cycler
- Go to files press enter then load and select the SEQ PCR.
- After the run is complete, centrifuge the tube briefly to bring the contents to the bottom of the wells.
- Store the tube according to when you are continuing the protocol. (**Note:** Store at 0-4°C for 12 hours; Store at -20°C for 2-3 days after 12 hours).
- After storage and before opening the tube, centrifuge the tube briefly to bring the contents to the bottom.

VII. Purification of sequencing reaction

A. Ethanol /EDTA/Sodium Acetate Precipitation

1. Mix the sequencing PCR product by gentle tapping and short spin the tube in centrifuge.
2. Add 2 µL 3M Acetate pH 4.6 + 2.0 µL 125mM EDTA (Freshly prepared) + 10 µL MilliQ water in 10 µL of Sequencing PCR reaction,
3. Mix well with tapping and briefly centrifuge.
4. Add 80 µL of absolute ethanol and mix properly by inverting the tube 10 times. (**Note:** Do not centrifuge at this step).
5. Incubate the tube for 15-20 minutes at -20°C
6. Centrifuge at13000 rpm for 30 minutes placing the tube in the spin column in the upward position.
7. Aspirate the supernatant immediately without disturbing the pellet
8. Add 180 µL of 70% ethanol (freshly prepared) and wash the pellet by inverting the tube 50 times gently
9. Centrifuge at13000 rpm for 15 minutes placing the tube in the spin column in the upward position.
10. Aspirate the supernatant immediately without disturbing the pellet.
11. Repeat steps 8 to 10 once.
12. Dry the pellet at room temperature. (**Note**: There should be no ethanol left in this step).
13. Add 10 µL HiDi (Formamide provided by ABI) and mix the pellet properly and short spin and incubate the mix at 37°C for 15 minutes and again mix the pellet by tapping and quick spin the mix. Heat the reaction at 95°C for 5 minutes and quick chill in ice for 10 minutes.

Note: Sample can be stored in this step at 4°C before loading in to the automated DNA Sequencer (Genetic Analyser 3130, ABI).

VIII. Capillary electrophoresis

- Analyze the reaction products on ABI Prism 3130 automated DNA analyser (Applied Biosystems, California, USA). Load the sample in 96 well plate according to the well description and make the entry of the sample in hard copy as well as in the sequencer data collecting software according to the well description
- Use the DNA sequences so obtained for identification using nucleotide Blast search of NCBI (http: //blast.ncbi.nlm.nih.gov/blast) or Westerdijk Fungal Biodiversity Institute **(**http://www.westerdijkinstitute.nl/).

Antifungal Susceptibility Testing

CHAPTER 6

Antifungal Susceptibility Testing (AFST)

A. Yeast

1. Broth dilution antifungal susceptibility testing

The method of antifungal susceptibility testing described here is intended for testing yeasts isolated from disseminated invasive infections. These yeasts comprise mainly *Candida* species, and *Cryptococcus neoformans*. This method has not been approved and used to test the yeast form of dimorphic fungi.

Broth microdilution method should be performed for amphotericin B, fluconazole, itraconazole, voriconazole, posaconazole, caspofungin, anidulafungin, micafungin following M27/A3 protocol of CLSI.

1.1. Antifungal agents

Source

Antifungal standards or reference powders can be obtained commercially, directly from the drug manufacturer or from reputed company as pure salt. Pharmacy stock or other clinical preparations/ formulations **should not** be used. Acceptable powders bear a label that states the drug's generic name, its assay potency [measure of drug activity expressed in terms of the amount required to produce an effect of given intensity; usually expressed in micrograms (µg) or International Units per mg of powder], and its expiration date. The powders are to be stored as recommended by the manufacturers, or at -20°C or below in a desiccator (preferably in a vacuum). When the desiccator is removed from the freezer, it is to be allowed to come to room temperature before it is opened (to avoid condensation of water).

Weighing antifungal powders

All antifungal agents are assayed for standard units of activity. The assay units can differ widely from the actual weight of the powder and often differ within a drug production lot. Thus, a laboratory must standardize its antifungal solutions based on assays of the lots of antifungal powders that are being used. Either of the following formulae may be used to determine the amount of powder or diluent needed for a standard solution:

$$\text{Weight (mg)} = \frac{\text{Volume (mL)} \times \text{Concentration (µg/mL)}}{\text{Assay Potency (µg/mg)}} \qquad \text{(Formula: 1)}$$

or

$$\text{Vol. (mL)} = \frac{\text{Weight (mg)} \times \text{Assay Potency (µg/mg)}}{\text{Concentration (µg/mL)}} \qquad \text{(Formula: 2)}$$

The antifungal powder should be weighed on an analytical balance that has been calibrated with Standards. Usually, it is advisable to accurately weigh a portion of the antifungal agent in excess of that required and to calculate the volume of diluents needed to obtain the concentration desired.

Example: To prepare 100 mL of a stock solution containing 1280 µg/mL of antifungal agent with antifungal powder that has a potency of 750 µg/mg, use the first formula to establish the weight of powder needed:

$$\text{Weight (mg)} = \frac{\text{100 mL} \quad \text{1280 µg/mL}}{\text{750 µg/mg}} = \frac{\text{(Target Vol.) X (Desired Conc.)}}{\text{(Potency)}} = 170.7 \text{ mg} \quad (3)$$

Because it is advisable to weigh a portion of the powder in excess of that required, powder was deposited on the balance until 182.6 mg was reached. With that amount of powder weighed, formula (2) above is used to determine the amount of diluent to be measured:

$$\text{Volume (mL)} = \frac{\text{182.6 mg} \quad \text{750 µg/mg}}{\text{1280 µg/mL}} = \frac{\text{(Powder Weight) X (Potency)}}{\text{(Desired Concentration)}} = 107.0 \text{ mL}$$

Therefore, the 182.6 mg of the antifungal powder should be dissolved in 107.0 mL of diluent.

Preparing stock solutions

Antifungal stock solutions should be prepared at concentrations of at least 1280 µg/mL or ten times the highest concentration to be tested, whichever is greater. There are some antifungal agents, however, of limited solubility that can require lower concentrations. In all cases, information provided by the drug manufacturer should be considered as part of determining solubility.

Use of solvents other than water

Some drugs must be dissolved in solvents other than water (see Table 6.1). Information on the solubility of an antifungal compound should be included with the drug. Such drugs should be dissolved at concentrations at least 100 times higher than the highest desired test concentration. Commonly used agents include: dimethyl sulfoxides (DMSO), ethyl alcohol, polyethylene glycol, and carboxy methyl cellulose. When such solvents are used a series of dilutions at 100 times the final concentration should be prepared from the antifungal stock solution in the same solvent. Each intermediate solution should then be further diluted to final strength in the test medium. This procedure avoids dilution artifacts that result from precipitation of compounds with low solubility in aqueous media.

For example, to prepare for a broth macrodilution test series containing a water-insoluble drug that can be dissolved in DMSO for which the highest desired test concentration is 6 µg/mL first weigh 4.8

mg (assuming 100% potency) of the antifungal powder and dissolve it in 3.0 mL DMSO. This will provide a stock solution at 1600 µg/mL. Next, prepare further dilutions of this stock solution in DMSO (See Tables 6.2 and 6.3). The solutions in DMSO should be diluted 1:50 in test medium and a further two-fold when inoculated, reducing the final solvent concentration to 1% DMSO at this concentration (without drug) should be used in the test as a dilution control.
The example above assumes 100% potency of the antifungal powder. If the potency is different, the calculations as mentioned above should be applied.

Filtration

Normally, stock solutions do not support contaminating microorganisms and they can be assumed to be sterile. If additional assurance of sterility is desired, they are to be filtered through a membrane filter. Paper, asbestos, or sintered glass filters, which may adsorb appreciable amounts of certain antifungal agents, are not to be used. Whenever filtration is used, it is important that the absence of adsorption by appropriate assay procedures is documented.

Storage

Small volumes of the sterile stock solutions are dispensed into sterile polypropylene or polyethylene vials, carefully sealed, and stored (preferably at -60°C or below but never at a temperature greater than -20°C). Vials are to be removed as needed and used the same day. Any unused drug is to be discarded at the end of the day. Stock solutions of most antifungal agents can be stored at -60°C or below for six months or more without significant loss of activity. In all cases, any directions provided by the drug manufacturer are to be considered as a part of these general recommendations and should supersede any other directions that differ. Any significant deterioration of an antifungal agent may be ascertained by comparing with the quality control strains.

1.2. Number of concentrations tested

The concentrations to be tested should encompass the breakpoint concentrations and the expected results for the quality control strains. Based on previous studies, the following drug concentration ranges should be used: amphotericin B, 0.0313 to 16 µg/mL; flucytosine, 0.125 to 64 µg/mL.; ketoconazole, 0.0313 to 16 µg/mL.; itraconazole and new triazoles 0.0313 to 16 µg/mL; fluconazole 0.125 to 64 µg/mL; echinocandins (anidulafungin, caspofungin, and micafungin), 0.0313 to 16 µg/mL.

1.3. Test procedures

Broth medium

A completely synthetic medium is RPMI 1640 (with glutamine, without bicarbonate, and with phenol red as pH indicator) was found as satisfactory for testing the filamentous fungi and has been used to develop the proposed standard.

Buffers

Media should be buffered to a pH of 7.0 ± 0.1 at 25°C. A buffer should be selected that does not antagonize antifungal agents. Tris buffer is unsatisfactory because it antagonizes the activity of

flucytosine. Zwitterion buffers are preferable to buffers that readily traverse the cell membrane, such as phosphate buffers, because, theoretically, the latter can produce unexpected interactions with antifungal agents. One buffer that has been found to be satisfactory for antifungal testing is MOPS (3-(N-morpholino) propane sulfonic acid] (final concentration 0.165 mol/L for pH 7.0). The pH of each batch of medium is to be checked with a pH meter when the medium is prepared; the pH should be between 6.9 and 7.1 at room temperature (25°C). MIC performance characteristics of each batch of broth are evaluated using a standard set of quality control organisms

Preparing diluted antifungal agents

A list of antifungal agents and their diluents is given in Table 6.1:

Table 6.1: Solvents and diluents for preparation of stock solutions of antifungal agents

S. No.	Antifungal Agent	Solvent (Full Strength and Intermediate Solutions)	Diluent (Final concentrations)
1	Amphotericin B	DMSO	Medium
2	Anidulafungin	DMSO	Medium
3	Caspofungin	Water	Medium
4	Ciclopirox	DMSO	Medium
5	Fluconazole	Water	Medium
6	Flucytosine	Water	Medium
7	Griseofulvin	DMSO	Medium
8	Itraconazole	DMSO	Medium
9	Ketoconazole	DMSO	Medium
10	Micafungin	Water	Medium
11	Posaconazole	DMSO	Medium
12	Ravuconazole	DMSO	Medium
13	Terbinafine	DMSO	Medium
14	Voriconazole	DMSO	Medium

DMSO = Dimethyl sulfoxide

The steps for preparation and storage of diluted antifungal agents are as follows:

1. Use sterile, 12 x 75 mm plastic test tubes to perform the tests
2. Use a growth control tube containing RPMI 1640 broth without antifungal agents (but with nonaqueous solvent where necessary) for each organism tested.
3. Close the tubes with loose screw-caps, or plastic or metal caps.

Water soluble antifungal agents

When two-fold dilutions of a water-soluble antifungal are to be used, they may be prepared volumetrically in broth (Table 6.2). The procedure for antifungals that are not soluble in water is different from that for water-soluble agents and is described below.

The total volume of each antifungal dilution to be prepared depends on the number of tests to be performed. Because 0.1 mL of each antifungal drug dilution will be used for each test, 1.0 mL should be adequate for about nine tests, allowing for pipetteting. A single pipettete is used for measuring all diluents and then for adding the stock antifungal solution to the first tube. A separate pipettete is

used for each remaining dilution in that set. Because there will be a 1:10 dilution of the drugs when combined with the inoculum, the working antifungal solutions are ten times more concentrated than the final concentrations.

Many persons find working with 1:10 dilutions (as shown in Table 6.2) easy and convenient. However, some automated pipettetes deliver only 1.0 or 01 mL volumes; therefore, a ratio of 1:11 would be preferable. It is unimportant whether the final test volume is 1.0 mL or 1.1 mL. If 1:11 dilutions are made, the dilution scheme should be altered so that the same final concentrations or drug are obtained.

Water insoluble antifungal agents

For antifungal agents that cannot be prepared as stock solutions in water, such as ketoconazole, amphotericin B, or itraconazole, a dilution series of the agent should be prepared first at 100 x final strength in an appropriate solvent. Each of these nonaqueous solutions should now be diluted tenfold in RPMI 1640 broth. (Table 6.3)

For example, if a dilution series with final concentrations in the range 16 µg/mL to 0.0313 µg/mL is desired, a concentration series from 1,600 to 3.13 µg/mL should have been prepared first in DMSO. To prepare 1 mL volumes of diluted antifungal agent (sufficient for 10 tests), first pipettete 0.9 mL volumes of RPMI 1640 broth into each of 11 sterile test tubes. Now, using a single pipettete, add 0.1 mL of DMSO alone to one 0.9 mL lot of broth (control medium), then 0.1 mL of the lowest (3.13 µg/mL) drug concentration in DMSO, then 0.1 mL of the 6.25 µg/mL concentration and continue in sequence up the concentration series, each time adding 0.1 mL volumes to 0.9 mL broth. These volumes can be adjusted according to the total number of tests required. Because there will be a 1:10 dilution of the drugs when combined with the inoculum, the working antifungal solutions are tenfold more concentrated than the final concentrations.

Table 6.2: Scheme for preparing dilutions of water soluble antifungal agents to be used in broth dilution susceptibility tests

Drug - Starting Concentration (µg/ml)	Stock 5120	2	4 160	8	16	32 20	64	128	256 2.5	511	Remarks
		2X	4X	2X	4X	8X	2X	4X	8X	2X	
Tube #	TUBE 1	TUBE 2	TUBE 3	TUBE 4	TUBE 5	TUBE 6	TUBE 7	TUBE 8	TUBE 9	TUBE 10	
Source	From Stock 1.0	From Tube 1	From Tube 1	From Tube 3	From Tube 3	From Tube 3	From Tube 6	From Tube 6	From Tube 6	From Tube 9	Step 1
Add DRUG Amount (ml) +		1.0 +	1.0 +	1.0 +	0.5 +	0.5 +	1.0 +	0.5 +	0.5 +	1.0 +	Row 1
Add Solvent RPMI (ml)	7.0	1.0	3.0	1.0	1.5	3.5	1.0	1.5	3.5	1.0	
Intermediate Drug Concentration (µg/ml)	640	320	160	80	40	20	10	5	2.5	1.25	
Add Drug from Tube Row 1 Above (ml) +	1.0 +	1.0 +	1.0 +	1.0 +	1.0 +	1.0 +	1.0 +	1.0 +	1.0 +	1.0 +	Step 2 Row 2
RPMI 1640 (ml)	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	5X (1:4)
Final Concentration at 1:5 (µg/ml)	128	64	32	16	8	4	2	1	0.5	.25	2X
From Row 2 add Drug to Micro titer plate (ml)	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	Step 3 (1:1)
Add Inoculum to Plate (ml)	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	Step 4 (T.Vol 0.2ml)
FINAL DRUG CONC in Well at 1: 100 (µg/ml)	64	32	16	8	4	2	1	0.5	0.25	0.125	

Table 6.3: Scheme for preparing dilution series of water-insoluble antifungal agents to be used in broth dilution susceptibility tests

Drug - Starting Concentration (µg/ml)	1,600	2	4	8 200	16	32	64 25	128	256	511	Remarks
Tube #	TUBE 1 (Stock) (100 X)	2X TUBE 2	4X TUBE 3	8X TUBE 4	2X TUBE 5	4X TUBE 6	8X TUBE 7	2X TUBE 8	4X TUBE 9	8X TUBE 10	
Source	From Tube 1	From Tube 1	From Tube 1	From Tube 1	From Tube 4	From Tube 4	From Tube 4	From Tube 7	From Tube 7	From Tube 7	Step 1
Add DRUG Amount (ml)	-	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	Row 1
+		+	+	+	+	+	+	+	+	+	
Add Solvent DMSO (ml)	-	0.5	1.5	3.5	0.5	1.5	3.5	0.5	1.5	3.5	
Intermediate Drug Concentration (µg/ml)	1,600	800	400	200	100	50	25	12.5	6.25	3.13	
Add Drug from Tube Row 1 Above (ml)	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	Step 2 Row 2 (1:50)
+	+	+	+	+	+	+	+	+	+	+	
RPMI 1640 (ml)	4.9	4.9	4.9	4.9	4.9	4.9	4.9	4.9	4.9	4.9	
Final Concentration at 1:50 (µg/ml)	32	16	8.0	4.0	2.0	1.0	0.5	0.25	0.125	0.0625	(2X)
From Row 2 add Drug to Micro titer plate (ml)	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	Step 3 (1:1)
Add Inoculum to Plate (ml)	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	Step 4 (T.Vol 0.2ml)
FINAL DRUG CONC in Well at 1: 100 (µg/ml)	16	8	4	2	1	0.5	0.25	0.125	0.0625	0.0313	

Inoculum preparation

The steps for preparation of inoculum are as follows:

1. All organisms should be sub-cultured from sterile vials onto Sabouraud dextrose agar or peptone dextrose agar and passaged at least twice to ensure purity and viability. The incubation temperature throughout must be 35°C.
2. The inoculum should be prepared by picking five colonies of ~ 1 mm in diameter from 24-hour old culture of *Candida* species or 48 hours old cultures of *C. neoformans*. The colonies should be suspended in 5 mL of sterile 0.145 mol/L saline (8.5 g/L NaCl; 0.85% saline).
3. The resulting suspension should be vortexed for 15 seconds and the cell density adjusted with a spectro-photometer by adding sufficient sterile saline to increase the transmittance to that produced by a 0.5 McFarland standard (see Appendix) at 530 nm wavelength. This procedure will yield a yeast stock suspension of 1 x 10^6 to 5 x 10^6 cells per mL. A working suspension is made by a 1:100 dilution followed by a 1:20 dilution of the stock suspension with RPMI 1640 broth medium which results in 5.0 x 10^2 to 2.5 x 10^3 cells per mL.

Inoculating broth

Before adjusting the inoculum, 0.1 mL of the various antifungal concentrations are placed in 12 x 75 mm tubes. The growth control receives 0.1 mL of drug diluent without antifungal agent. Within 15 minutes after the inoculum has been standardized (up to two hours if inoculum is kept at 4°C), 0.9 mL of the adjusted inoculum is added to each tube in the dilution series and mixed. This results in 1:10 dilution of each antifungal concentration and 10% dilution of the inoculum.

Incubation

With the exception of *C. neoformans*, tubes are incubated (without agitation) at 35°C for 46 to 50 hours in ambient air. When testing *C. neoformans*, tubes should be incubated for a total of 70 to 74 hours before determining results.

1.4. Reading results

The MIC is the lowest concentration of an antifungal that substantially inhibits growth of the organism as detected visually. The amount of growth in the tubes containing the agent is compared with the amount of growth in the growth control tubes (no antifungal agent) used in each set of tests as follows:

- **Amphotericin B:** For amphotericin B end points are typically easily defined and the MIC is read as the lowest drug concentration that prevents any discernible growth. Trailing end points with amphotericin B are not usually encountered.

- **Flucytosine and azoles antifungal**: For flucytosine and especially for azoles, such as fluconazole and ketoconazole, end points are typically less sharp and may be a significant source of variability. A less stringent end point (slight turbidity is allowed above the MIC) has improved interlaboratory agreement and also discriminates between putatively susceptible and resistant isolates. When turbidity persists, it is often identical for all drug concentrations above the MIC. The amount of allowable turbidity can be estimated by diluting 0.2 mL of drug-free control growth with 0.8 mL of media, producing an 80% inhibition standard. Even dispersion of clumps that can become evident after incubation can make end-point determination more reproducible. Reference strains of defined susceptibility can also be used in the training of new personnel.

1.5. Interpretation of results

Interpretive breakpoints have been established at present only for some organism-drug combinations. The clinical relevance of testing other organism-drug combinations remains uncertain, but the relevant information can be summarized as follows:

- **Amphotericin B:** Experience to date indicates that amphotericin B MICs for *Candida* spp. isolates are tightly clustered between 0.25 and 1.0 μg/mL. When isolates that appear resistant to amphotericin B in animal models are tested by M27, MIC values greater than 1 μg/mL may be obtained. Unfortunately, the M27 methodology does not consistently permit detection of such isolates and all that can at present be concluded is that if an M27 amphotericin B MIC of > 1 μg/mL is obtained for a *Candida* spp. isolates, then that isolate is likely resistant to amphotericin B. Current work suggests that testing with Antibiotic Medium-3 supplemented with 2% glucose permits more reliable detection of resistant isolates. However, the reproducibility of this method is still under study and laboratories that choose to do this testing must carefully compare their results with those obtained for isolates with known responses to amphotericin B.
- **Flucytosine:** Based largely on historical data and partially on the drug's pharmacokinetics, interpretive breakpoints for *Candida* spp. and flucytosine have been established.
- **Fluconazole:** Based on a large data package presented by flucoanzole's manufacturer, interpretive breakpoints for *Candida* spp. and fluconazole have been established. These data are principally drawn from studies of oropharyngeal candidiasis and of invasive infections due to *Candida* spp.

in non-neutropenic patients and their clinical relevance in other settings is uncertain (in recent meta- analysis, the breakpoint is confirmed). In addition, these interpretive breakpoints are not applicable to *C. krusei*, and thus identification to the species level is required in addition to MIC determination. The utility of testing isolates of *C. neoformans* is currently under intense study, and recent data do suggest a correlation between elevated MIC and clinical failure.

- **Ketoconazole:** Experience to data using the procedures described in this standard indicates that yeast MICs vary between 0.0313 and 16 μg/mL. However, data are not yet available to indicate a correlation between MIC and outcome of treatment with ketoconazole.
- **Itraconazole:** Based on a large data package presented by itraconazole's manufacturer, interpretive breakpoints for *Candida* spp. and itraconazole have been established. This data is entirely from studies of oropharyngeal candidiasis, and their clinical relevance in other settings is uncertain. In addition, the importance of proper preparation of drug dilutions for this insoluble compound can not be over emphasized. Use of the incorrect solvents or deviation from the dilution scheme suggested in Table 6.3 can lead to substantial errors due to dilution artefacts.
- **New Triazoles:** Experience to date with posaconazole-, ravuconazole-, and voriconazole-using procedures described in this standard indicates that yeast MICs vary between 0.03 and 16 μg/mL with the majority of isolates inhibited by <1 μg/mL of all three agents. However, data are not yet available to indicate a correlation between MIC and outcome of treatment with these agents.

1.6. Broth microdilution modifications

A substantial body of data has now been presented that documents excellent concordance between results obtained by the broth macrodilution methodology described above and a broth microdilution adaptation. The ease of performance of broth microdilution tests is very attractive, and most clinical laboratories will probably choose to implement this method rather than the broth macrodilution method.

The steps and testing conditions that are relevant to the broth microdilution test are discussed in detail.

The 10-fold drug dilutions described for the broth macrodilution procedure should be diluted 1:5 with RPMI to achieve the two times strength needed for the broth microdilution test. The stock inoculum suspensions are prepared and adjusted, as described, under the broth macrodilution test. The stock yeast suspension is mixed for 15 seconds with a vortex diluted 1:50 and further diluted 1:20 with medium to obtain the two times test inoculum (1×10^3 to 5×10^3 CFU/mL). The (two-fold) inoculum is diluted 1:1 when the wells are inoculated and the desired final inoculum size is achieved (0.5×10^3 to 2.5×10^3 CFU/mL). The broth microdilution test is performed by using sterile, disposable, multiwell microdilution plates (96 U shaped wells). The 2x drug concentrations are dispensed into the wells of rows 1 to 10 of the microdilution plates in 100 μL volumes with a multichannel pipettete. Row 1 contains the highest (either 64 or 16 μg/mL) drug concentration and row 10 contains the lowest drug concentration (either 0.12 or 0.03 μg/mL). These trays may be sealed in plastic bags and stored frozen at -70°C for up to 6 months without deterioration of drug potency. Each well of a microdilution tray is inoculated on the day of the test with 100 μL of the corresponding 2x diluted inoculum suspension, which brings the drug dilutions and inoculum densities to the final concentrations mentioned above. The growth control wells contain 100 μL of sterile drug-free medium and are inoculated with 100 μL of the corresponding diluted (2X) inoculum suspensions. The QC organisms are tested in the same manner and are included each time an isolate is tested. (Table 6.4) Row 11 of the microdilution plate can be used to perform the sterility control (drug-free medium only). The microdilution plates are

incubated at 35°C and observed for the presence or absence of visible growth. The microdilution wells are scored with the aid of a reading mirror, the growth in each well is compared with that of the growth control (drug-free) well.

A numerical score, which ranges from 0 to 4, is given to each well using the following scale: 0, optically clear; 1, slightly hazy; 2, prominent decrease in turbidity; 3, slight deduction in turbidity; and 4, no reduction of turbidity.

For isolates in which clumping hinders applying these definitions, dispersion of the yeast suspension by pipetteting, vortexing or other techniques can help. The MIC for amphotericin B is defined as the lowest concentration in which a score of 0 (optically clear) is observed and for 5-FC and the azoles, as the lowest concentration in which a score of 2 (prominent decrease in turbidity) is observed. Prominent decrease in turbidity corresponds to approximately 50% inhibition in growth as determined spectrophotometrically. The microdilution MICs read at 48 hours (72 hours for most *C. neoformans*) provide the best agreement with the reference broth macrodilution method.

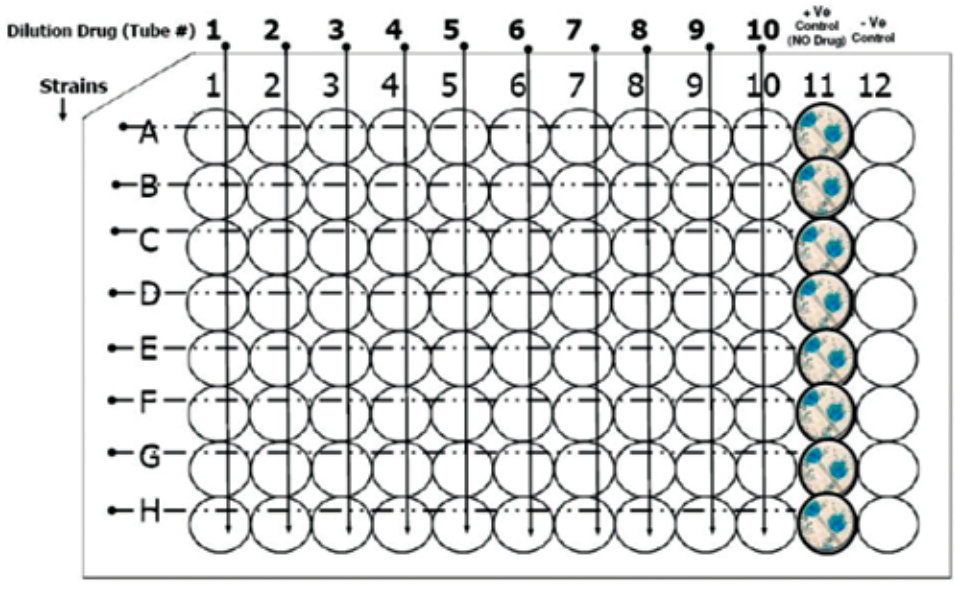

Figure 7: Plating scheme recommended for broth microdilution

1.7. Quality control

- **Growth contro**l: Each broth macrodilution series should include a growth control of basal medium without antifungal agent to assess viability of the test organisms. With the broth tests, the growth control also serves as a turbidity control for reading end points.
- **Purity control:** A sample of each inoculum is streaked on a suitable agar plate and incubated overnight to detect mixed cultures and to provide freshly isolated colonies in the event retesting proves necessary.
- **End point interpretation control:** End point interpretation is monitored periodically to minimize variation in the interpretation of MIC end points among observers. All laboratory personnel who perform these tests should read a selected set of dilution tests independently. The results are recorded and compared to the results obtained by an experienced reader.

- **Quality control strains**: Ideal reference strains for quality control of dilution tests have MICs that fall near the middle of the concentration range tested for all antifungal agents; e.g. an ideal control strain would be inhibited at the fourth dilution of a seven-dilution series, but strains with MICs at either the third or fifth dilution would also be acceptable.

1.8. Impact of time reading: 24 hours versus 48 hours

The M27-A2 methodology for *Candida* recommends an end point reading at 48 hours. For most isolates, the difference between readings at 24 hours versus 48 hours is minimal and will not alter the interpretative category (i.e., does not change whether the isolate would be categorized as "susceptible" or "resistant"). However, recent work has begun to include 24-hour readings, because (a) MICs can often be read at 24 hours; and (b) readings taken at 24 hours may be more clinically relevant for some isolates. Isolates for which the earlier reading is important show a dramatic rise in MIC between 24 hours and 48 hours due to significant trailing growth (partial inhibition of growth over an extended range of antifungal concentrations).

Estimated as occurring in about 5% of isolates, this trailing growth can be so great as to make an isolate that appears susceptible after 24 hours appear completely resistant at 48 hours. Two independent in vivo investigations of this phenomenon that employed murine models of disseminated candidiasis, have shown that isolates with this behavior should be categorized as "susceptible" rather than "resistant." This concept has been corroborated by a demonstration that trailing growth can be eliminated by lowering the pH of the test medium to 5 or less and by a clinical demonstration that oropharyngeal candidiasis due to such isolates respond to a low dose of fluconazole used to treat typical susceptible isolates. In light of these observations, both 24-hour and 48-hour microdilution expected MIC ranges are provided for the QC strains and eight systemic antifungal agents (Table 6.4). Also shown are additional strains that can be useful for conducting reference studies.

Table 6.4: Recommended MIC or MEC limits for QC and reference strains for broth dilution procedure

Organism	Purpose	Antifungal Agent	MIC Range (µg/mL)	Mode	% of MICs Within Range	Incubation Time
Paecilomyces variotii ATCC® MYA-3630 (see note 1)	QC	Amphotericin B	1-4	2	100	48 hours
		Itraconazole	0.06-0.5	0.12	100	48 hours
		Voriconazole	0.015-0.12	0.06	100	48 hours
		Posaconazole	0.03-0.25	0.06	99.5	48 hours
	Reference (MEC)	Anidulafungin	<0.015	N/A	100	24 hours

Organism	Purpose	Antifungal Agent	MIC Range (µg/mL)	Mode	% of MICs Within Range	Incubation Time
Candida parapsilosis ATCC® 22019	QC	Amphotericin B	0.5-4.0	2	91.7	24 hours
		5FC	0.12-0.5	0.25	97.9	24 hours
		Fluconazole	1.0-4.0	2	98.1	24 hours
		Itraconazole	0.12-0.5	0.25	97.5	24 hours
		Ketoconazole	0.06-0.5	0.12	98.3	24 hours
		Voriconazole	0.03-0.25	0.06	100	24 hours
		Ravuconazole	0.03-0.25	0.06	98.3	24 hours
		Posaconazole	0.06-0.25	0.12	98.8	24 hours
		Anidulafungin	0.5-2.0	1	95	24 hours
		Caspofungin	0.5-4.0	1	92.9	24 hours
		Micafungin	0.5-4.0	1	100	24 hours
Candida krusei ATCC® 6258	QC	Amphotericin B	1.0-4.0	2	100	24 hours
		5FC	8.0-32	16	99.6	24 hours
		Fluconazole	16-128	32	100	24 hours
		Itraconazole	0.25-1.0	0.5	100	24 hours
		Ketoconazole	0.25-1.0	0.5	99.6	24 hours
		Voriconazole	0.12-1.0	0.5	100	24 hours
		Ravuconazole	0.25-1.0	0.5	100	24 hours
		Posaconazole	0.12-1.0	0.5	99.6	24 hours
		Anidulafungin	0.03-0.12	0.06	97.5	24 hours
		Caspofungin	0.25-1.0	0.5	97.5	24 hours
		Micafungin	0.12-0.5	0.25	99	24 hours
Aspergillus flavus ATCC® 204304 (see note 2)	Reference	Amphotericin B	0.5-4	ND	100	48 hours
		Itraconazole	0.25-0.5	ND	100	48 hours
		Voriconazole	0.5-4	ND	100	48 hours
		Ravuconazole	0.5-4	ND	100	48 hours
		Posaconazole	0.06-0.5	ND	100	48 hours
Aspergillus fumigatus ATCC® MYA-3626 (see note 1)	Reference	Amphotericin B	0.5-4.0	2	98.7	48 hours
		Itraconazole	0.25-2.0	1	95.7	48 hours
		Voriconazole	0.25-1.0	0.5	100	48 hours
	Reference (MEC)	Anidulafungin	<0.015	N/A	100	24 hours
Aspergillus fumigatus ATCC® MYA-3627	Reference	Amphotericin B	0.5-4.0	2	99.2	48 hours
		Itraconazole	>16	>16	95	48 hours
		Voriconazole	0.25-1.0	0.5	99.2	48 hours
Aspergillus flavus ATCC® MYA-3631	Reference	Amphotericin B	1.0-8.0	2	98.8	48 hours
		Voriconazole	0.5-2.0	1	98.3	48 hours
		Posaconazole	0.12- 1.0	0.5	97.1	48 hours

Organism	Purpose	Antifungal Agent	MIC Range (µg/mL)	Mode	% of MICs Within Range	Incubation Time
Aspergillus terreus ATCC® MYA- 3633 (see note 1)	Reference	Amphotericin B	2.0-8.0	4	98.3	48 hours
		Voriconazole	0.25-1.0	0.5	99.2	48 hours
	Reference (MEC)	Anidulafungin	<0.015	N/A	99.6	24 hours
Fusarium moniliforme ATCC® MYA-3629 (see note 1)	Reference	Amphotericin B	2.0-8.0	4	99.6	48 hours
		Itraconazole	>16	>16	97.9	48 hours
		Voriconazole	1.0-4.0	2	100	48 hours
		Posaconazole	0.5-2.0	1	98.1	48 hours
	Reference (MEC)	Anidulafungin	>8	N/A	97.5	48 hours
F. solani ATCC® 3636 (see note 1)	Reference (MEC)	Anidulafungin	>8	N/A	96.7	48 hours
Scedosporium apiospermum ATCC® MYA-3635	Reference	Amphotericin B	4.0-16	8	98.8	72 hours
		Voriconazole	0.5-2.0	1	100	72 hours
		Posaconazole	1.0-4.0	2	98.3	72 hours
Scedosporium apiospermum ATCC® MYA-3634 (see note 1)	Reference (MEC)	Anidulafungin	1.0-4.0	2	96.7	48 hours
Trichophyton mentagro-phytes MRL 1957 ATCC® MYA- 4439 (see note 3)	Reference	Ciclopirox	0.5-2	1	97.5	4 days
		Griseofulvin	0.12-0.5	0.25	96.3	4 days
		Itraconazole	0.03-0.25	0.06	96.2	4 days
		Posaconazole	0.03-0.25	0.06	95.2	4 days
		Terbinafine	0.002-0.008	0.004	97.9	4 days
		Voriconazole	0.03-0.25	0.06	95.2	4 days
T. rubrum MRL 666 ATCC® MYA- 4438 (see note 3)	Reference	Ciclopirox	0.5-2	1	97.5	4 days
		Fluconazole	0.5-4	1	95.2	4 days
		Voriconazole	0.008-0.06	0.015	96.1	4 days

NOTE 1: Although some of the anidulafungin MEC (various moulds) and > 50% inhibition MIC (*Fusarium* isolates only) ranges are off-scale, these isolates could aid in the identification of potential resistance or the determination of the novel MEC end point. The anidulafungin concentration range in the study was 0.015 µg/mL to 32 µg/mL, but off-scale MICs of >32 from that study are reported in Table 4 as >8 to be consistent with the recommended routine testing range for this compound.

NOTE 2: The MIC ranges for *A. flavus* ATCC® 204304 are based on data from a collaborative study that were not obtained according to the CLSI/NCCLS document M23 process. However, this is the only mould for which reproducible reference limits were established for ravuconazole and it is included in the table for this reason.

NOTE 3: Four days or until good growth (confluent hyphal growth covering the bottom of the well) is obtained in the growth control well

2. Method for antifungal disk diffusion susceptibility testing of Yeasts

The method described here is intended for testing *Candida* species. This method does not currently encompass any other genera and has not been used in studies of the yeast form of dimorphic fungi, such as *Blastomyces dermatitidis* or *Histoplasma capsulatum.* The method described herein must be followed exactly to obtain reproducible results.
Zone interpretation criteria as per M44/A2 protocol of CLSI are available for fluconazole, voriconazole, and caspofungin and recommended quality control ranges for caspofungin, fluconazole, posaconazole and voriconazole.

2.1. Reagents for the disk diffusion test

Mueller-Hinton Agar + 2% Glucose and 0.5 µg/mL Methylene Blue Dye (GMB) Medium - Of the many agar media available, supplemented Mueller-Hinton agar to be a good choice for routine susceptibility testing of yeasts.

pH of Mueller-Hinton Agar + 2% Glucose and 0.5 µg/mL Methylene Blue Dye Medium - The pH of each batch of prepared Mueller-Hinton agar should be checked. The agar medium should have a pH between 7.2 and 7.4 at room temperature after gelling. pH can be checked by one of the following means:

- Macerate a sufficient amount of agar to submerge the tip of a pH electrode.
- Allow a small amount of agar to solidify around the tip of a pH electrode in a beaker or cup.
- Use a properly calibrated surface electrode.

Moisture on agar surface: If excess surface moisture is present, the agar plates should be dried in an incubator or laminar flow hood with the lids ajar until the excess moisture has evaporated (usually 10 to 30 minutes). The surface should be moist, but with no droplets on the agar surface or the petri dish cover.

2.2. Storage of antimicrobial disks

Cartridges containing commercially prepared paper disks specifically for susceptibility testing are generally packaged to ensure appropriate anhydrous conditions. Disks should be stored as follows:

- Refrigerate the containers at 8°C or below, or freeze at -14°C or below, in a non-frost-free freezer until needed. The disks may retain greater stability if stored frozen until the day of use. Always refer to instructions in the product insert.
- The unopened disk containers should be removed from the refrigerator or freezer 30 to 60 minutes before use so they may equilibrate to room temperature before opening. This procedure minimizes the amount of condensation that occurs when warm air contacts cold disks.
- Once a cartridge of disks has been removed from its sealed packaging, it should be placed in a tightly sealed, desiccated container.

- A disk-dispensing apparatus should be fitted with a tight cover and supplied with an adequate desiccant. The dispenser should be allowed to warm to room temperature before opening. The desiccant should be replaced when the indicator changes color.
- When not in use, the dispensing apparatus containing the disks should always be refrigerated.
- Only disks within their valid shelf life may be used. Disks should be discarded on the expiration date.

Turbidity standard for inoculum preparation: To standardize the inoculum density for a susceptibility test, a $BaSO_4$ suspension with turbidity, equivalent to a McFarland standard or its optical equivalent should be used.

2.3. Procedure for performing the disk diffusion test

Inoculum preparation: Direct colony suspension method

1. All organisms need to be subcultured onto blood agar or Sabouraud dextrose agar to ensure purity and viability. The incubation temperature throughout must be 35°C (±2°C)
2 Inoculum is prepared by picking **five distinct colonies** of approximately 1 mm in diameter from a 24-hour-old culture of *Candida* species. Colonies are suspended in 5 mL of sterile 0.145 mol/L saline (8.5 g/L NaCl; 0.85% saline).
3. The resulting suspension is vortexed for 15 seconds and its turbidity is adjusted either visually or with a spectrophotometer by adding sufficient sterile saline or more colonies to adjust the transmittance to that produced by a 0.5 McFarland standard at 530 nm wavelength. This procedure will yield a yeast stock suspension of 1 x 10^6 to 5 x 10^6 cells per mL and should produce semi-confluent growth with most *Candida* species isolates.

Inoculation of test plates

- Optimally, within 15 minutes after adjusting the turbidity of the inoculum suspension, a sterile cotton swab is dipped into the suspension. The swab should be rotated several times and pressed firmLy against the inside wall of the tube above the fluid level. This will remove excess fluid from the swab.
- The dried surface of a sterile Mueller-Hinton + GMB agar plate is inoculated by evenly streaking the swab over the entire agar surface. This procedure is repeated by streaking two more times, rotating the plate approximately 60° each time to ensure an even distribution of inoculum. As a final step, the rim of the agar is swabbed.
- The lid may be left ajar for three to five minutes, but no more than 15 minutes, to allow for any excess surface moisture to be absorbed before applying the drug-impregnated disks.

NOTE: Variations in inoculum density must be avoided. Never use undiluted overnight broth cultures or other unstandardized inocula for streaking plates.

Application of disks to inoculated agar plates

Antimicrobial disks are dispensed onto the surface of the inoculated agar plate. Each disk must be pressed down to ensure its complete contact with the agar surface. Whether the disks are placed individually or with a dispensing apparatus, they must be distributed evenly so that they are no closer than 24 mm from center to center. Ordinarily, no more than 12 disks should be placed on a 150-mm

plate, or more than five disks on a 100-mm plate. Because the drug diffuses almost instantaneously, a disk should not be moved once it has come into contact with the agar surface. Instead, place a new disk in another location on the agar. Disk should be placed no less than 10 mm from the edge of the petri dish.The plates should inverted and placed in an incubator set to 35°C (± 2°C) within 15 minutes after the disks are applied.

2.4. Reading plates

Examine each plate after 20 to 24 hours of incubation. If the plate was satisfactorily streaked and the inoculum was correct, the resulting zones of inhibition will be uniformly circular and there will be a semi confluent lawn of growth. The plate is held a few inches above a black, non-reflecting background illuminated with reflected light. Measure the zone diameter to the nearest whole millimetre at the point at which there is a prominent reduction in growth. This is highly subjective, and experience results in greater accuracy (trueness). Pinpoint microcolonies at the zone edge or large colonies within a zone are encountered frequently and should be ignored. If these colonies are subcultured and retested, identical results are usually obtained, i.e., a clear zone with microcolonies at the zone edge or large colonies within the zone. Read at 48 hours only when insufficient growth is observed after 24 hours incubation.

2.5. Interpretation of disk diffusion test results

Tables 6.5 - 6.10 provide zone diameter interpretive criteria to categorize accurately the levels of susceptibility of organisms to antifungal agents.

Interpretive categories

- **Susceptible (S):** The susceptible category implies that an infection due to the strain may be appropriately treated with the dose of antimicrobial agent recommended for that type of infection and infecting species, unless otherwise contraindicated.
- **Susceptible-Dose Dependent (S-DD):** The susceptible-dose dependent category includes isolates with antimicrobial agent MICs that approach usually attainable blood and tissue levels and for which response rates may be lower than for susceptible isolates. Susceptibility is dependent on achieving the maximal possible blood level. This category also includes a buffer zone, which should prevent small, uncontrolled, technical factors from causing major discrepancies in interpretations, especially for drugs with narrow pharmacotoxicity margins.
- **Intermediate (I):** The intermediate category includes isolates with antimicrobial agent MICs that approach usually attainable blood and tissue levels and for which response rates may be lower than for susceptible isolates and/ or available data do not permit them to be clearly categorized as either “susceptible” or “resistant”. This category also includes a buffer zone, which should prevent small, uncontrolled, technical factors from causing major discrepancies in interpretations.
- **Resistant (R):** Resistant strains are those that are not inhibited by the usually achievable concentrations of the agent with normal dosage schedules or when zone diameters have been in a range where clinical efficacy has not been reliable in treatment studies.
- **Nonsusceptible (NS):** The nonsusceptible category includes organisms that currently have only a susceptible interpretive category, but not intermediate, susceptible-dose dependent, or resistant interpretive categories. This category is often given to new antimicrobial agents for which no resistant isolates have yet been encountered.

Zone diameter interpretive criteria

Disk diffusion zone diameters correlate inversely with MICs from standard dilution tests. Tables 6.5 - 6.10 list the zone diameter interpretive criteria.

2.6. Quality control

Part of quality management focused on fulfilling quality requirements, which includes operational techniques and activities used to fulfill these requirements.

Reference strains for quality control: To control the precision (repeatability) and accuracy (trueness) of the results obtained with disk diffusion test procedure, several quality control strains should be obtained from a reliable source. The recommended quality control strains include:

1. *Candida albicans* ATCC 90028;
2. *Candida parapsilosis* ATCC 22019;
3. *Candida tropicalis* ATCC 750; and
4. *Candida krusei* ATCC 6258.

Zone diameter quality control limits: Acceptable zone diameter quality control limits for quality control strains are listed in Table 6.5. The overall performance of the test system should be monitored using these ranges by testing the appropriate control strains each day the test is performed or, if satisfactory performance is documented, testing may be done weekly.

Frequency of quality control testing

- **Daily Testing:** When testing is performed daily, for each antimicrobial agent/organism combination, 1 out of every 20 consecutive results may be out of the acceptable range (based on 95% confidence limits, 1 out of 20 random results may be out of control). Any more than 1 out-of-control result in 20 consecutive tests requires corrective action.

- **Weekly testing:** demonstrating satisfactory performance for conversion from daily to weekly quality control testing. Test all applicable control strains for 20 consecutive test days and document results.

To convert from daily to weekly quality control testing, no more than 1 out of 20 zone diameters for each antimicrobial agent/organism combination may be outside the acceptable zone diameter limits in Table 6.5.

Implementing weekly quality control testing

Weekly quality control testing may be implemented once satisfactory performance has been documented Perform quality control testing once per week and whenever any reagent component of the test (e.g., a new lot of agar plates or a new lot of disks from the same or a different manufacturer) is changed.
If any of the weekly quality control results are out of the acceptable range, corrective action is required.
If a new antimicrobial agent is added, it must be tested for 20 consecutive test days and satisfactory performance documented before converting to a weekly schedule. In addition, 20 days of consecutive

testing are required if there is a major change in the method of reading test results, such as conversion from manual zone measurements to an automated zone reader.

Corrective action

Out-of-Control result due to an obvious error

Obvious reasons for out-of-control results include:

1. Use of the wrong disk;
2. Use of the wrong control strain;
3. Obvious contamination of the strain; or
4. Inadvertent use of the wrong incubation temperature or conditions.

In such cases, document the reason and retest the strain on the day the error is observed. If the repeated result is within range, no further corrective action is required.

Out-of-Control result not due to an obvious error

- Immediate Corrective Action: If there is no obvious reason for an out-of-control result, immediate corrective action is required.
- Test the antimicrobial agent/organism combination for a total of five consecutive test days. Document all results in question.
- If all five zone diameter measurements for the antimicrobial agent/organism combination are within acceptable ranges, as defined in Table 6.5, no additional corrective action is necessary.
- If any of the five zone diameter measurements are outside the acceptable range, additional corrective action is required.
- Daily control tests must be continued until final resolution of the problem can be achieved. Additional Corrective Action When immediate corrective action does not resolve the problem; it is likely due to a system error versus a random error.

The following common sources of error should be investigated:

1. Zone diameters were measured and transcribed correctly.
2. The turbidity standard has not expired, is stored properly, meets performance requirements, and was adequately mixed prior to use.
3. All materials used were within their expiration date and stored at the proper temperature.
4. The incubator is at the proper temperature and atmosphere.
5. Other equipment used (e.g., pipettetes) are functioning properly.
6. Disks are stored desiccated and at the proper temperature.
7. The control strain has not changed and is not contaminated.
8. Inoculum suspensions were prepared and adjusted correctly.
9. Inoculum for the test was prepared from a plate incubated for the correct length of time and in no case, was more than 24 hours old.

It may be necessary to obtain a new quality control strain (either from freezer stock or a reliable source) and new lots of materials (including new turbidity standards), possibly from different manufacturers.

If the problem appears to be related to a commercial product, the manufacturer should be contacted. It is also helpful to exchange quality control strains and test materials with another laboratory using the same method. Until the problem is resolved, an alternative test method should be used. Once the problem is corrected, documentation of satisfactory performance for another 20 consecutive days is required before returning to weekly quality control testing.

Reporting patient results when out-of-control tests occur

Whenever an out-of-control result or corrective action is necessary, careful assessment of whether to report patient results should be made on an individual basis, taking into account if the source of error, when known, is likely to have affected relevant patient results. Options that may be considered include suppressing the results for an individual antimicrobial agent; retrospectively reviewing individual patient or cumulative data for unusual patterns; and using an alternate test method or a reference laboratory until the problem is resolved.

2.7. Limitations of disk diffusion methods

Application to various organism groups

The disk diffusion method described in this document has been standardized for *Candida* species only. For other yeasts, consultation with an infectious disease specialist is recommended for guidance in determining the need for susceptibility testing and interpretation of results. Published reports in the medical literature and current consensus recommendations for therapy of uncommon microorganisms may obviate the need for testing. If necessary, a reference dilution method may be the most appropriate alternative testing method, and this may require submitting the organism to a reference laboratory.

Verification of patient results

Multiple test parameters are monitored by following the quality control recommendations described in this standard. However, acceptable results derived from testing quality control strains do not guarantee accurate results when testing patient isolates. It is important to review all of the results obtained from all drugs tested on a patient's isolates prior to reporting the results.

Unusual or inconsistent results should be verified by checking for the following:
1) transcription errors;
2) Contamination of the test (recheck purity plates); and
3) Previous results on the patient's isolates.

If a reason for the unusual or inconsistent result cannot be ascertained, repeat the susceptibility test, verify the species identity, or request a new clinical specimen. Each laboratory must develop its own policies for verification of unusual or inconsistent antimicrobial susceptibility test results.

Table 6.5: Recommended quality control zone diameter (mm) ranges

Antifungal agents	Disk content (µg)	*C. albicans* ATCC90028	*C. parapsilosis* ATCC 22019	*C. tropicalis* ATCC 750	*C. krusei* ATCC 6258
Caspofungin	5	18-27	14-23	20-27	19-26
Fluconazole	25	28-39	22-33	26-37	-*
Voriconazole	1	31-42	28-37	-*	16-25
Posaconazole	5	24-34	25-36	23-33	23-31

* Quality control ranges have not been established for these strain/antimicrobial agent combinations, due to their extensive interlaboratory variation during initial quality control studies.

- **AFST Break Points**

Table 6.6: List of antimicrobial agents, zone diameters and MIC break points for *Candida albicans, Candida tropicalis* and all other yeasts not listed in following tables.

Anti Microbial Agent	Disc concent (µg)	Disc Diffusion Zone diameter (mm)			MIC (µg/mL)		
		S	I	R	S	I	R
Anidulafungin	-	-	-	-	≤ 0.25	0.5	≥ 1
Caspofungin	5	≥ 17	15-16	≤ 14	≤ 0.25	0.5	≥ 1
Fluconazole	25	≥ 17	14-16*	≤ 13	≤ 2	4*	≥ 8
Micafungin	-	-	-	-	≤ 0.25	0.5	≥ 1
Voriconazole	1	≥ 17	15-16*	≤ 14	≤ 0.125	0.25-0.5*	≥ 1

Note: S : Susceptible, I : Intermediate, R : Resistant, *SDD : Susceptible Dose Dependent

Table 6.7: List of Antimicrobial Agents, Zone diameters and MIC break points for *Candida glabrata*

Anti Microbial Agent	Disc concent (µg)	Disc Diffusion Zone diameter (mm)			MIC (µg/mL)		
		S	I	R	S	I	R
Anidulafungin	-	-	-	-	≤ 0.125	0.25	≥ 0.5
Caspofungin	-	-	-	-	≤ 0.125	0.25	≥ 0.5
Fluconazole	25	≥ 16	15*	≤ 14	≤ 16	32*	≥ 64
Micafungin	-	-	-	-	≤ 0.06	0.125	≥ 0.25
Voriconazole@	1	≥ 17	15-16*	≤ 14	≤ 0.125	0.25-0.5*	≥ 1

Note: S: Susceptible, I : Intermediate, R : Resistant, *SDD : Susceptible Dose Dependent, @: Presumptive CLSI Break Points

Table 6.8: List of Antimicrobial Agents, Zone diameters and MIC break points for *Candida guilliermondii*

Anti Microbial Agent	Disc concent (µg)	Disc Diffusion Zone diameter (mm)			MIC (µg/mL)		
		S	I	R	S	I	R
Anidulafungin@	-	-	-	-	≤ 0.25	0.5	≥ 1
Caspofungin	5	≥ 13	11-12	≤ 10	-	-	-
Fluconazole@	25	≥ 17	14-16*	≤ 13	≤ 2	4*	≥ 8
Micafungin@	-	-	-	-	≤ 0.25	0.5	≥ 1
Voriconazole@	1	≥ 17	15-16*	≤ 14	≤ 0.125	0.25-0.5*	≥ 1

Note: S: Susceptible, I : Intermediate, R : Resistant.@: Presumptive CLSI Break Points, *SDD : Susceptible Dose Dependent

Table 6.9: List of Antimicrobial Agents, Zone diameters and MIC break points for *Candida krusei*

Anti Microbial Agent	Disc concent (µg)	Disc Diffusion Zone diameter (mm)			MIC (µg/mL) (24 hours)		
		S	I	R	S	I	R
Anidulafungin	-	-	-	-	≤ 0.25	0.5	≥ 1
Caspofungin	5	≥ 17	15-16	≤ 14	≤ 0.25	0.5	≥ 1
Fluconazole@	25	≥ 17	14-16*	≤ 13	≤ 2	4*	≥ 8
Micafungin	-	-	-	-	≤ 0.25	0.5	≥ 1
Voriconazole@	1	≥ 15	13-14*	≤ 12	≤ 0.5	1*	≥ 2

Note: S: Susceptible, I: Intermediate, R : Resistant, *SDD : Susceptible Dose Dependent, @: Presumptive CLSI Break Points

Table 6.10: List of Antimicrobial Agents, Zone diameters and MIC break points for *Candida parapsilosis*

Antimicrobial Agent	Disc concent (ug)	Disc Diffusion Zone diameter (mm)			MIC (µg/mL)		
		S	I	R	S	I	R
Anidulafungin	-	-	-	-	≤ 2	4	≥ 8
Caspofungin	5	≥ 13	11-12	≤ 10	≤ 2	4	≥ 8
Fluconazole	25	≥ 17	14-16*	≤ 13	≤ 2	4*	≥ 8
Micafungin	-	-	-	-	≤ 2	4	≥ 8
Voriconazole	1	≥ 15	13-14*	≤ 12	≤ 0.125	0.25-0.5*	≥ 1

Note: S : Susceptible, I : Intermediate, R : Resistant, *SDD : Susceptible Dose Dependent

B. Moulds

1 Broth dilution antifungal susceptibility testing

This document describes method for testing the susceptibility of filamentous fungi (moulds) that cause invasive (*Aspergillus* spp., *Fusarium* spp., *Rhizopus oryzae*, *Pseudallescheria boydii*, *Scedosporium apiospermum*, *Sporothrix schenckii*, and other pathogenic moulds) and cutaneous (the dermatophytes *Trichophyton*, *Microsporum*, and *Epidermophyton* spp.) fungal infections to antifungal agents.
Refer to SOP for broth dilution antifungal susceptibility testing of yeasts for drug stock solution preparation and dilution procedures. Please follow all the precautions mentioned earlier for weighing, storing and preparing antifungal drugs.

1.1. Procedure

1.1.1. Drug concentrations

The concentrations to be tested should encompass the expected results for the available QC strains. Based on previous studies for non-dermatophyte moulds, the following drug concentration ranges may be relevant: amphotericin B, 0.0313 to 16 µg/mL; flucytosine, 0.125 to 64 µg/mL; ketoconazole, 0.0313 to 16 µg/mL; itraconazole, posaconazole and voriconazole, 0.0313 to 16 µg/mL; fluconazole, 0.125 to 64 µg/mL; and echinocandins (anidulafungin, caspofungin, and micafungin), 0.0313 to 16 µg/mL. Suitable drug concentration ranges for testing dermatophytes are: ciclopirox, miconazole and luliconazole, 0.016 to 16 µg/mL; griseofulvin, 0.25 to 128 µg/mL; itraconazole, amorolfine and voriconazole, 0.0078 to 4 µg/mL; fluconazole, 0.0625 to 32 µg/mL; ketoconazole, clotrimazole and naftifine, 0.0312 to 16 µg/mL; and terbinafine and sertaconazole, 0.0156 to 8 µg/mL.

The media, buffers remain the same as for broth microdilution testing of yeasts (Please refer the Standard Operating Procedures for Mycology Laboratories)

1.1.2. Inoculum preparation

Non-dermatophyte species

- Non-germinated conidial or sporangiospore suspensions in a range of approximately $0.4x10^4$ to $5x10^4$ CFU/mL provide the most reproducible MIC data.
- To induce conidium and sporangiospore formation, grow fungi on potato dextrose agar for seven days at 35°C or until good sporulation is obtained. In *Mucorales* and *Aspergillus* spp., good sporulation may be obtained after 48 hours of incubation. *Fusarium* spp. may need to be incubated for 48 to 72 hours at 35°C and then until day seven at 25°C to 28°C to induce sufficient conidiation.
- Cover conidiation colonies with approximately 1 mL of sterile 0.85% saline and prepare a suspension by gently probing the colonies with the tip of a transfer pipettete. Addition of one drop (approximately 0.01 mL) of Tween 20 will facilitate the preparation of *Aspergillus* spp. inocula.
- The resulting mixture of conidia or sporangiospores and hyphal fragments is withdrawn and transferred to a sterile tube. After allowing heavy particles to settle for three to five minutes, transfer the upper homogeneous suspension to a sterile tube, tighten the cap, and mix with a

vortex mixer for 15 seconds. (CAUTION: Remove the cap carefully, as liquid adhering to the cap may produce aerosols upon opening.)

- Read and adjust the densities of the conidial or sporangiospore suspensions to an optical density (OD) at 530 nm that ranges from 0.09 to 0.13 for *Aspergillus* spp., *Purpureocillium lilacinum*, *P. variotii*, *Exophiala dermatitidis*, and *S.schenckii*; 0.15 to 0.17 for *Fusarium* spp., *S. apiospermum*, *Ochroconis gallopava*, Cladophialophora *bantiana*, *R. oryzae*, and other *Mucorales* species; and 0.25 to 0.3 for *Curvularia* spp. and *Alternaria* spp. Dilute these suspensions 1:50 in the standard medium. Inoculum suspensions of *S. apiospermum*, *Curvularia* spp., and *Alternaria* spp. may require a lower (50%) dilution factor. The 1:50 inoculum dilutions will be 2X (twofold) more concentrated than the density needed or approximately 0.4×10^4 to 5×10^4 CFU/mL.
- Make the test inoculum in sufficient volume to directly inoculate each well with 0.1 mL of the corresponding diluted inoculum suspension.

Dermatophyte species

- Most dermatophyte isolates produce sufficient conidia on potato dextrose agar. However, conidium formation by *Trichophyton rubrum* is very poor on standard fungal media including potato dextrose agar. Thus, the use of oatmeal agar has been recommended as the optimal growth medium for inducing conidium formation in *T. rubrum* isolates.
- Dermatophyte isolates should be grown on potato dextrose agar or oatmeal agar (*T. rubrum* isolates only) at 30°C for four to five days or until good conidial growth is present.
- Cover colonies with approximately 1 mL of sterile 0.85% saline, and prepare a suspension by gently probing the colonies with the tip of a transfer pipettete or sterile swab.
- Allow the resulting suspension to settle for five to 10 minutes, count conidia with a hemocytometer, and adjust the concentration as needed. The final suspension should be made 2x more concentrated than the density needed for testing; 1×10^3 to 3×10^3 CFU/mL.

1.1.3. Inoculating RPMI-1640 medium

Inoculate each well on the day of the test with 0.1 mL of the 2x conidial or sporangiospore inoculum suspension. The growth control wells will contain 0.1 mL of the corresponding diluted inoculum suspension and 0.1 mL of the media. Test QC and reference organisms (Reference strains used; *Paecilomyces variotii* ATCC MYA-3630, *Candida parapsilosis* ATCC 22019, *Aspergillus flavus* ATCC 204304, *Aspergillus flavus* ATCC MYA-3631, *Aspergillus fumigatus* ATCC MYA-3627, *Aspergillus fumigatus* ATCC MYA-3626 and *Candida krusei* ATCC 6258) in the same manner and include each time an isolate is tested.

1.1.4. Incubation

Incubate all microdilution trays at 35°C without agitation. For *Alternaria* spp. incubation at 30°C is recommended. Examine *Rhizopus* spp. after 21 to 26 hours of incubation to determine MIC results. Evaluate most other opportunistic filamentous fungi, including *Fusarium* spp., *Aspergillus* spp., and *S. schenckii*, after 46 to 50 hours of incubation. Examine *Scedosporium* spp. after 70 to 74 hours. For the echinocandins, evaluate isolates after 21 to 26 hours (eg, *Aspergillus* spp.) and 46 to 72 hours (*Scedosporium* spp.), or the first day when sufficient growth (confluent growth covering the bottom of the well) is present in the growth control well (drug-free medium) for MEC determination. Evaluate trays containing dermatophyte isolates after four days of incubation.

1.2. MIC and MEC reading results

The MIC is the lowest concentration of an antifungal agent that substantially inhibits growth of the organism, as detected visually when testing most antifungal agents. For the conventional microdilution procedure, compare the growth in each MIC well with that of the growth control with the aid of a reading mirror. When testing echinocandin antifungal agents, evaluate the MEC, which is the lowest concentration of drug that leads to the growth of small, rounded, compact hyphal forms as compared to the hyphal growth seen in the growth control well. For evaluating the MEC, compare the growth in each well with that of the growth control (drug-free medium).

- **Amphotericin B:** For amphotericin B, MIC is read as the lowest drug concentration that prevents any discernible growth (100% inhibition). Trailing end points with amphotericin B are usually not encountered. Such a pattern may reflect clinically relevant drug resistance.
- **Fluconazole and Ketoconazole:** For this drug class, the turbidity allowed corresponds to approximately 50% or more (non-dermatophyte isolates) to 80% or more (dermatophyte isolates) reduction in growth compared to the growth in the control well (drug-free medium).
- **Itraconazole, Posaconazole, and Voriconazole:** For these azoles, end points are typically easily defined and the MIC is read as the lowest drug concentration that prevents any discernible growth (100% inhibition). However, when testing dermatophyte isolates against voriconazole, sertaconazole, miconazole, luliconazole, clotrimazole, amorolfine and itraconazole, the turbidity allowed corresponds to approximately 80% or more reduction in growth compared to the growth in the control well (drug-free medium).
- **Echinocandins (anidulafungin, caspofungin, micafungin):** The MEC is read as the lowest concentration of drug that leads to the growth of small, rounded, compact hyphal forms as compared to the hyphal growth seen in the growth control well.
- **Ciclopirox:** For ciclopirox, end points are typically less well defined than that described for amphotericin B. Application of a less stringent end point (allowing some turbidity above the MIC) has improved interlaboratory agreement. For this drug class, the turbidity allowed corresponds to approximately 80% or more reduction in growth compared to the growth in the control well (drug-free medium).
- **Griseofulvin:** For griseofulvin, end points are typically less well defined than that described for amphotericin B. Application of a less stringent end point (allowing some turbidity above the MIC) has improved interlaboratory agreement. For this drug class, the turbidity allowed corresponds to approximately 80% or more reduction in growth compared to the growth in the control well (drug-free medium).
- **Terbinafine and naftifine:** For terbinafine, end points are typically less well defined than that described for amphotericin B. Application of a less stringent end point (allowing some turbidity above the MIC) has improved inter laboratory agreement. For this drug class, the turbidity allowed corresponds to approximately 80% or more reduction in growth compared to the growth in the control well (drug-free medium). However, when testing dermatophytes; MIC will be defined as the lowest concentration showing 100% growth inhibition for terbinafine and naftifine.

Proceed for QC as mentioned previously. The interpretation of the results can be referenced from the guidelines as mentioned in the SOP and recent update provided (Table 6.11-6.15).

2. Antifungal disk diffusion susceptibility testing of non-dermatophyte filamentous fungi

The method described is for testing moulds that cause invasive disease (*Alternaria* spp., *Aspergillus* spp., *Bipolaris* spp., *Fusarium* spp., *Paecilomyces* spp., *Rhizopus oryzae* and other *Mucorales* species, the *Pseudallescheria boydii* species complex, and *Lomentospora prolificans*). This method does not currently encompass the yeast or mould form of endemic dimorphic fungi or the dermatophytes.

2.1. Procedure

Refer to SOP for antifungal disk diffusion susceptibility testing of yeasts for media preparation and other precautions. Please follow all the precautions mentioned earlier for weighing, storing and preparing antifungal drugs.

2.1.1. Inoculum preparation

- Non-germinated conidial or sporangiospore suspensions in a range of approximately $0.4x10^6$ to $5x10^6$ CFU/mL provided the most reproducible MIC data. Proceed with the reference strains as for the test isolates (Reference strains; *Paecilomyces variotii* ATCC MYA-3630 and *Candida krusei* ATCC 6258).
- To induce conidium and sporangiospore formation, most fungi should be grown on potato dextrose agar for 7 days at 35 ± 2°C or until good sporulation is obtained; good sporulation may be obtained after 48 hours of incubation for some isolates (eg, *Mucorales* moulds and *Aspergillus* spp.). *Fusarium* spp. may need to be incubated for 48 hours to 72 hours at 35 ± 2°C and then until day 7 at 28 to 30 ± 2 °C.
- Cover sporulating colonies with approximately 1 mL of sterile 0.85% saline and prepare a suspension by gently probing the colonies with the tip of a transfer pipettete. Adding one drop (approximately 0.01 mL) of Tween 20 may facilitate the preparation of *Aspergillus* inocula, in particular.
- The resulting mixture of conidia or sporangiospores and hyphal fragments is withdrawn and transferred to a sterile tube. After heavy particles are allowed to settle for 3 to 5 minutes, the upper homogeneous suspension is transferred to a sterile tube, the cap is tightened, and it is mixed with a vortex mixer for 15 seconds.
- The densities of the conidial or sporangiospore suspensions are read on a spectrophotometer with a 1 cm light path at 530 nm wavelength and adjusted to an optical density that ranges from 0.09 to 0.13 for *Aspergillus* spp., *Purpureocillium lilacinum*, and *Paecilomyces variotii*; 0.15 to 0.17 for *Fusarium* spp., *R. oryzae*, and other *Mucorales* spp., the *Pseudallescheria boydii* species complex, and *L. prolificans*; and 0.25 to 0.3 for *Alternaria* and *Curvularia* spp.
- These suspensions are used undiluted.

2.1.2. Inoculation of test plates

Optimally, within 15 minutes after adjusting the turbidity of the inoculum suspension, dip a sterile cotton swab into the undiluted inoculum suspension. Rotate the swab several times and press firmLy against the inside wall of the tube above the fluid level. This removes excess fluid from the swab. Inoculate the dried surface of a sterile non-supplemented Mueller-Hinton agar plate by evenly streaking the swab over the entire agar surface. This procedure is repeated by streaking two more times, rotating the plate approximately 60° each time to ensure an even distribution of inoculum. As a final step, swab the rim of the agar.

2.1.3. Application of disks to inoculated agar plates

Dispense antimicrobial disks onto the surface of the inoculated agar plate. Each disk must be pressed down to ensure its complete contact with the agar surface. Disks should be no closer than 32 mm from center to center when testing amphotericin B, caspofungin, and itraconazole; and no closer than 55 mm from center to center when testing posaconazole and voriconazole, especially against *Alternaria* spp., *Aspergillus* spp., *Curvularia* spp., and *Paecilomyces* spp. Ordinarily, no more than four to six disks should be placed per 150 mm plate, nor more than one disk per 100 mm plate. Invert the plates and place them in an incubator set to 35 ± 2°C within 15 minutes after the disks are applied.

2.2. Reading plates

Examine each plate after 20 to 24 hours of incubation. If the plate was satisfactorily streaked and the inoculum was correct, the resulting zones of inhibition should be uniformly circular and there should be a semi confluent lawn of growth. The plate is held a few inches above a black, non-reflecting background illuminated with reflected light. Measure the zone diameter to the nearest whole millimetre at the point at which there is a prominent reduction in growth. This is highly subjective, and experience results in greater accuracy (trueness). Pinpoint microcolonies at the zone edge or large colonies within a zone are encountered frequently and should be ignored. If these colonies are subcultured and retested, identical results are usually obtained, i.e., a clear zone with microcolonies at the zone edge or large colonies within the zone. Read at 48 hours only when insufficient growth is observed after 24 hours' incubation.

2.3. Zone diameter epidemiological cutoff values

Clinical breakpoints have not been established for mould testing. However, tentative epidemiological cutoff values (ECVs) are available to detect moulds with reduced susceptibility (non–wild type) to amphotericin B, caspofungin, itraconazole, posaconazole, and voriconazole. ECVs can be used as a measure of the emergence of strains with reduced susceptibility to a given agent. They are not clinical breakpoints. Although organisms whose MICs/MECs/zone diameters fall outside the ECV show reduced susceptibility as compared to the wild-type population, and may exhibit one or more acquired resistance mechanisms, they may yet respond to clinical treatment because the MIC/MEC may lie below (zone diameter above) the true (and as yet undetermined) clinical breakpoint.
Proceed for QC as mentioned in Standard Operating Procedures for Mycology Laboratories. The interpretation of the results can be referenced from the guidelines as mentioned in the SOP and recent update provided (Table 6.11-6.15).

Table 6.11: List of antimicrobial agents, zone diameters and MIC break points for *Aspergillus flavus* and *Aspergillus fumigatus*

Antimicrobial Agent	Disc concent (µg)	Disc Diffusion Zone diameter (mm)			MIC (µg/mL)		
		S	I	R	S	I	R
Amphotericin B	10	≥ 15	-	≤ 14	≤ 2	-	≥ 4
Caspofungin	5	≥ 17	-	≤ 16	≤ 0.06	-	≥ 0.125
Itraconazole	10	≥ 17	-	≤ 16	≤ 1	-	≥ 2
Posaconazole	5	≥ 17	-	≤ 16	≤ 0.5	-	≥ 1
Voriconazole	1	≥ 17	-	≤ 16	≤ 1	-	≥ 2

Note: S : Susceptible, I : Intermediate, R : Resistant

Table 6.12: List of Antimicrobial agents, zone diameters and MIC break points for *Aspergillus nidulans*

Antimicrobial Agent	Disc concent (µg)	Disc Diffusion Zone diameter (mm)			MIC (µg/mL)		
		S	I	R	S	I	R
Amphotericin B	10	≥ 15	-	≤ 14	-	-	-
Caspofungin	5	≥ 17	-	≤ 16	-	-	-
Itraconazole	10	≥ 17	-	≤ 16	≤ 1	-	≥ 2
Posaconazole	5	≥ 17	-	≤ 16	≤ 1	-	≥ 2
Voriconazole	1	≥ 17	-	≤ 16	≤ 2	-	≥ 4

Note: S : Susceptible, I : Intermediate, R : Resistant

Table 6.13: List of antimicrobial agents, zone diameters and MIC break points for *Aspergillus niger*

Antimicrobial Agent	Disc concent (µg)	Disc Diffusion Zone diameter (mm)			MIC (µg/mL)		
		S	I	R	S	I	R
Amphotericin B	10	≥ 15	-	≤ 14	≤ 2	-	≥ 4
Caspofungin	5	≥ 17	-	≤ 16	≤ 0.06	-	≥ 0.125
Itraconazole	10	≥ 17	-	≤ 16	≤ 2	-	≥ 4
Posaconazole	5	≥ 17	-	≤ 16	≤ 1	-	≥ 2
Voriconazole	1	≥ 17	-	≤ 16	≤ 2	-	≥ 4

Note: S : Susceptible, I : Intermediate, R : Resistant

Table 6.14: List of Antimicrobial agents, zone diameters and MIC break points for *Aspergillus terreus*

Antimicrobial Agent	Disc concent (µg)	Disc Diffusion Zone diameter (mm)			MIC (µg/mL)		
		S	I	R	S	I	R
Amphotericin B	10	≥ 15	-	≤ 14	-	-	-
Caspofungin	5	≥ 17	-	≤ 16	≤ 0.06	-	≥ 0.125
Itraconazole	10	≥ 17	-	≤ 16	≤ 1	-	≥ 2
Posaconazole	5	≥ 17	-	≤ 16	≤ 0.5	-	≥ 1
Voriconazole	1	≥ 17	-	≤ 16	≤ 1	-	≥ 2

Note: S : Susceptible, I : Intermediate, R : Resistant

Table 6.15: List of antimicrobial agents, zone diameters and MIC interpretive criteria for *Aspergillus versicolor*

Antimicrobial Agent	Disc concent (µg)	Disc Diffusion Zone diameter (mm)			MIC (µg/mL)		
		S	I	R	S	I	R
Amphotericin B	10	≥ 15	-	≤ 14	≤ 2	-	≥ 4
Caspofungin	5	≥ 17	-	≤ 16	≤ 0.125	-	≥ 0.25
Itraconazole	10	≥ 17	-	≤ 16	≤ 2	-	≥ 4
Posaconazole	5	≥ 17	-	≤ 16	≤ 4	-	≥ 8
Voriconazole	1	≥ 17	-	≤ 16	≤ 2	-	≥ 4

Note: S : Susceptible, I : Intermediate, R : Resistant

Molecular detection of azole resistance

CHAPTER 7

Molecular Detection of Azole Resistance

C. albicans* and *C. tropicalis

C. albicans and *C. tropicalis* with fluconazole MIC ≥8 µg/mL (resistant to fluconazole), SDD isolates (MIC = 4 µg/mL) and three isolates with MIC = 0.12 µg/mL (sensitive to fluconazole) should be selected for screening of azole resistance mechanisms. The two major mechanisms responsible for azole resistance; mutations in lanosterol-14 alpha-demethylase (erg11) and the up regulation of efflux pumps, *CDR-1*, *CDR-2*, *erg-11* and *MDR-1* should be evaluated.

1.1. Screening for SNP's in *erg11*

1.1.1. DNA extraction

- Sub-culture the yeast on SDA (Sabouraud Dextrose agar) or PDA (Potato Dextrose agar) with antibiotic (chloramphenicol 0.05 g/litre)
- Incubate the culture at 37^{O}C for 24-48 hours.
- Add a loopful of culture in 50 mL of yeast extract-phosphate-dextrose broth in 200 mL flask.
- Incubate overnight at 30^{O}C.
- Pellet down growth in 1.5 mL microfuge tube at 7500 rpm for 2 minutes.
- Suspend the pellet in 200 µl of lysis buffer (100 mM Tris HCl [pH 8.0], 50 mM EDTA, 3% SDS).
- Add 1µL of RNase (10 mg/mL, Thermo Scientific) and incubate at 37^{O}C for 30 minutes.
- Add 1µL of proteinase (20 mg/mL, Thermo Scientific) and incubate at 56^{O}C for 2 hours.
- Add 0.3 g of sterile glass beads.
- Mix well by vortexing and then add 200 µL of equal amount of phenol: chloroform to the suspension.
- Vortex this mixture vigorously for one minute followed by cooling on ice for 30 seconds.
- Add 200 µL of TE buffer & again vortex briefly.
- Centrifuge the mixture at 13000 rpm at room temperature for 5 minutes.
- Take aqueous phase in clear sterile microfuge tube and add equal volume of chloroform: isoamyl alcohol. Collect aqueous phase, add 1/10th volume of 3M Sodium acetate and add equal volume of chilled isopropanol. Mix and incubate at -20^{O}C for 2 hours.
- Spin the microfuge tube at 10000 rpm for 5 minutes and decant the supernatant.
- Wash the pellet with 0.5 mL of 70% ethanol.
- Centrifuge at 10000 rpm for 5 minutes, carefully aspirate the supernatant and allow the pellet to dry at 37^{O}C for one hour.
- Re-suspend the pellet in appropriate volume of TE Buffer.
- Allow DNA to dissolve at room temperature with intermittent mixing.
- Note: After DNA extraction, if proteinase K and RNase treatment was given, first treat DNA with RNase, incubate it at 37^{O}C followed by proteinase K treatment. To denature the residual proteinase K, heat the DNA at 95^{O}C for 10 minutes.

1.1.2. Quantitative and qualitative determination of DNA

Following methods can be used tocheck the quality of DNA.

- Agarose gel electrophoresis
- Spectrophotometric (Nanodrop) method

1.1.2.1 Gel electrophoresis

Agarose gel preparation

- To check the quality of the DNA, prepare 0.8% agarose gel in 50 mL of 1X TAE buffer.
- Weigh out 0.5 g of agarose and add 50 mL of 1x TAE Buffer, swirl to mix.
- Melt the agarose by using microwave or by heating plate for 1-2 minutes (Note: Uniform clear solution should be seen).
- Allow it to cool at room temperature till 60°C.
- Add 1 µL of ethidium bromide (10 mg/mL) and swirl to mix.
- Pour the gel slowly into the gel casting tray without any air bubbles. Insert the comb and double check to ensure that it is correctly positioned.
- Allow the gel to solidify.
- After solidification, place the gel in the electrophoresis apparatus containing 1X TAE buffer.
- Note: the gel should be submerged completely in the buffer

Loading of samples

- Take 10 µL of loading buffer and mix it with 2 µL of isolated DNA.
- Mix and load into appropriate well in the gel.
- Run the gel by applying current 100 volts, 400 mA for 30 minutes.
- Monitor the progress of the electrophoresis by noting the position of loading dye.
- View the gel under UV light.
- Gel picture can also be taken to document the results. Expose the gel to UV light in a gel documentation system. DNA bands should show up as orange fluorescent bands. Take a picture of the gel.
- Note: If the sample shows smearing in agarose gel, it indicates DNA degradation. In this case check reagents (buffers, phenol: chloroform: isoamyl alcohol solution).
- Ethidium bromide is carcinogenic, therefore properly dispose the gel and running buffer as per institution regulations.

1.1.2.2. Checking quality and quantity of DNA using Nanodrop 2000

Measure DNA sample (1 µL) at 340 nm wavelength. Note OD & 260/280 ratio.

Notes:

- The minimum DNA concentration for accurate PCR amplification should be in the range of 100-200 ng/µL with 260/280 ratio between 1.70-1.90.
- In case the 260/280 ratio is >2.00, it indicates RNA contamination. Add 1 µL RNase enzyme to DNA and incubate it at 37°C for 2 hours.

- If the 260/280 ratio is <1.70, it indicates protein contamination. Treat the DNA with 1 µL Proteinase enzyme by adding 1 µL of proteinase K to extracted DNA and incubate it at 56°C for 2 hours. To inactivate proteinase K activity, heat the DNA at 95°C for 10 minutes.

1.1.3 Amplification

After extraction of DNA, amplification of the gene of interest is carried by thermocycler. The full length erg11 gene from fluconazole resistant and sensitive *C. albicans* and *C. tropicalis* will be amplified by using a number of primers where 3-4 fragments of 600-700 bp portions will be amplified.

Table 7.1: Primers to amplify *erg11* from *C. tropicalis*

S No.	Type	Sequence (5'->3')	Length	Tm	Amplicon
1	F	CCGCCGTCGTTCCTTTAATC	20	59.35	698
	R	AAGCCTGTGGGATATTCTTCAA	22	57.41	
2.	F	ACGACGCGAAATTAAACGATGA	22	59.34	645
	R	AGCAGAACCAAACCATGGGA	20	59.52	
3	F	CACCATCTTGGTTGTTTTCCCA	22	59.30	677
	R	CAGCGTCACGTCTCCAGTAA	20	59.76	
4	F	GGAAAGAGACCATGGTGTTGC	21	59.46	698
	R	TGGTATGAGCATAACCGGCA	20	59.17	
5	F	GCACATGCCATTGCATTCTA	20	57.12	681
	R	GGTGGGTCGACTGAAACGTA	20	59.69	
6	F	TGCTGAAATCGTTTGGGAAAAG	22	58.02	682
	R	AAACGGAACTGTACCATCTCCTTC	24	60.56	
7	F	GCATTTACTATTAAAGTCCCAGCA	24	57.31	500
	R	GTTTGAGTTAATTCGACATCCTTGT	25	58.10	

Table 7.2: Primers to amplify *erg11* from *C. albicans*

S. No.	Type	Sequence (5'->3')	Length	Tm	Amplicon
1	F	AGACAAAGAAAGGGAATTCAATCGT	25	64.08	660
	R	ACATTGGCAACCCCATGAGT	20	65.05	
2	F	TGATGTTTCTGCTGAAGATGCT	22	62.87	627
	R	AACCAAGCAGAAGTAGAAGCAGA	23	64.42	
3	F	ACTGAGAAGAGAACGTGGTGA	21	63.92	516
	R	GCTTTGGCAGCAGCAGTATC	20	63.89	
4	F	AGGTGGTGATTTGAATGATTTGACT	25	64.10	595
	R	ACAATCTGAACACTGAATCGAAAGA	25	63.80	

PCR should be put up in the presence of 200 µM of each dNTP (Fermentas, India), 0.25 µM of each primer (Sigma St. Louis, MO), 0.2 µL of 3U (stock) of *Taq* polymerase (Bangalore Genie), Taq buffer (It is available as 10X, bring to 1X) (Bangalore Genei, Bangalore, India), 50-100 ng of DNA and use

molecular grade water to make final volume of 20 µL. The amplification reactions will be performed in a Thermocycler (Eppendorf Mastercycler, Hamburg, Germany) as mentioned in Table 7.3.

Table 7.3: Conventional PCR cycling conditions

Step		Temperature	Time	
Denaturation		95°C	10 min	
Amplification	Denaturation	95°C	30 sec	Repeat for 35 cycles
	Annealing	As per primers Tm	30 sec	
	Extension	72°C	1 min	
Extension		72°C	10 min	
Hold		20°C		

1.1.4 Sequencing PCR

For DNA sequencing of *erg-11*, following reagents are required

a. 5X sequencing buffer (Invitrogen, USA)
b. Big dye terminator (3.1/1.1 version, Invitrogen USA)
   - ♦ BDT version 3.1 should be used for sequencing 300-700 bp size
   - ♦ For 100-250 bp product size, BDT 1.1 version should be used
c. 6 picomole primers
d. Molecular grade water

Table 7.4: Master mix preparation for DNA sequencing

Reagents	Quantity in µL
Molecular grade water	5.75
5X sequencing buffer	1.75
Big dye terminator (1:16 dilution)	0.50
6 picomole primer	1.00
Template	1.00
Total reaction volume	10

Note: Gently mix the reaction mixture and centrifuge at 2000 rpm for 10 seconds, to pellet down the mixture.

DNA Sequencing: Sequencing PCR amplification protocol

- Place the tube in a thermal cycler.
- Set the volume to 10 µL and select 0.5 mL tube.
- Run on PCR machine using following conditions:

Step	Cycles
1.	T=96˚C for 1 minute
2.	T=96˚C for 10 seconds
3.	T=50˚C for 5 seconds
4.	T=60˚C for 4 minutes
5.	Go to step 2 Repeat 25 Cycles
6.	Hold at 20˚C

- Start the run in the thermal cycler (Eppendorf Mastercycler Gradient); Switch on the thermal cycler; Go to files, press enter and then load and select the SEQ PCR
- After the run is complete, centrifuge the tube briefly to bring the contents to the bottom of the wells.
- Store the tube according to when you are continuing the protocol. (**Note:** store at 0 to 4°C for 12 hours; store at -20°C for 2 to 3 days after 12 hours).
- After storage and before opening the tube, centrifuge the tube briefly to bring the contents to the bottom.

Purification of sequencing reaction

A. Ethanol /EDTA/Sodium acetate precipitation

- Mix the sequencing PCR product by gentle tapping and a short spin in centrifuge.
- Add 2 µL 3M Acetate pH 4.6 + 2 µL 125mM EDTA (Freshly prepared) + 10 µL molecular grade water in 10 µL of Sequencing PCR reaction
- Mix well with tapping and short spin in centrifuge.
- Add 80 µL of absolute ethanol and mix by inverting the tube 10 times
  Important: Do not centrifuge at this step
- Incubate the tube for 15-20 minutes at -20°C
- Centrifuge at 13,000 rpm for 30 minutes placing the tube in the spin column in the upward position
- Aspirate the supernatant immediately without disturbing the pellet
- Add 180 µL of 70% ethanol (freshly prepared) and wash the pellet by inverting the tube 50 times
- Centrifuge at 13,000 rpm for 15 minutes placing the tube in the spin column in the upward position
- Aspirate the supernatant immediately without disturbing the pellet
- Repeat step 8 to 10 once
- Dry the pellet at room temperature
  Important: There should be no ethanol left after this step
- Add 10 µLHiDi (Formamide provided by ABI) and mix the pellet properly. Spin the samples for a short time and incubate at 37°C for 15 minutes. Again mix the pellet by tapping and quick spin. Heat the reaction at 95°C for 5 minutes and quick chill on ice for 10 minutes.
  Note: Sample can be stored at this step (4°C)
- Load the sample in the automated DNA Sequencer (Genetic Analyser 3130, ABI).

Criteria of loading samples into Sequencer (Genetic Analyser 3130, ABI; Will vary with instrument**)**

- Open Data collection software
- Go to plate manager

- Click New (for new plate record formation)
- Enter detail
  - PLATE ID: Date on which samples are running,
  - PLATE TYPE: 96 well,
  - Sequencing analysis program,
  - Operator name:
  - Owner name:
- Press OK (Then new window will open, enter the details of the samples).
- Results group:
- Instrument protocol:
- Analysis protocol:
- Click enter after verifying the details.
- Open Sequencing analysis software v2.5 to analyse the sequence data.

1.1.5. Sequence analysis

The forward and reverse sequences should be imported to seqman software. The consensus sequences will be generated and should be saved in FASTA format. Compare the sequences from resistant and susceptible isolates using Clustal X and identify SNP's in the *erg-11* gene.

2.1 Evaluation of upregulation of efflux pumps in fluconazole resistant and sensitive *C. albicans* and *C. tropicalis*

Suspend a colony of the fluconazole sensitive and resistant *C. albicans* and *C. tropicalis* into SD broth and incubate at 37°C till the log phase is reached. From this growing culture inoculate SD flasks supplemented with sub-optimal MIC concentration of fluconazole and flasks containing media without fluconazole with the organisms in triplicates. Grow the organisms till mid-log phase and proceed as mentioned below.

2.1.1 RNA isolation

- Wash the cell pellet twice with sterile deionized water. Add 1 mL TRIzol (Sigma St. Louis, MO) reagent and 500 mg glass beads (180 µm in diameter, sterile) to cell pellet.
- Vortex the suspension for ten minutes and chill on ice after every 30 seconds during vortexing.
- Incubate for 2-3 minutes at room temperature (20-30ºC).
- Add 0.2 mL of chloroform per 1 mL of TRIzol (Thermo-Fischer). Transfer samples to microfuge tube and mix tube by inverting for 15-20 seconds.
- Next, incubate samples for 2-3 minutes at room temperature and centrifuge for 15 minutes at 4°C (at no more than 12,000 g).
- Transfer the aqueous phase (top layer) to a fresh microfuge tube.
- Precipitate the RNA from the aqueous phase by mixing equal volume of chilled isopropanol.
- Incubate the samples overnight at -20ºC.
- Centrifuge the samples at 12,000 g for 5 minutes at 4°C. Aspirate the supernatant and add 0.25 mL of 75% ethanol (in DEPC-treated water). Mix the microfuge tube by inverting gently and spin at 12000 g for 5 minutes at 4°C.
- Discard the supernatant and air dry the pellet on ice.

- Finally, dissolve the pellet in DEPC-treated water and leave on ice for 10 minutes and tap to dissolve. Check the RNA quantity and quality by measuring the absorbance in a nanodrop (Thermo Fisher Scientific) and note down A260/A280 ratio.

2.1.2. cDNA synthesis

Synthesize the cDNA as per the protocol in Table 7.5. The cycling conditions are as mentioned in Table 7.6.

Table 7.5: cDNA synthesis

S.No.	Reagent	Volume
1	5X cDNA synthesis buffer	4 µL
2	dNTP Mix	2 µL (500 µM)
3	RNA Primer	1 µL
4	RT Enhancer	1 µL
5	Verso Enzyme	1 µL
6	Molecular grade water	Variable
7	Template RNA	1-5 µL (1 ng)
8	Total Volume	20 µL

Table 7.6: Reverse transcription cycling program

	Temp.	Time	No of Cycles
cDNA synthesis	42°C	30 min	1 cycle
Inactivation	95°C	2 min	1 cycle

2.1.3 Relative quantification for gene expression

A relative quantification analysis compares two ratios:

a. The ratio of a target DNA sequence to a reference DNA sequence in an unknown sample
b. The ratio of the same two sequences in a standard sample called a “calibrator”

Target was the nucleic acid of interest (cDNA generated from fluconazole resistant and sensitive isolates), while the reference was a nucleic acid that is found at constant copy number in all samples (Actin) and serves as endogenous control. The reference can be used for normalization of sample-to-sample differences.

Real time PCR should be put up for all the samples in triplicates. The reaction mixture should be as mentioned in Table 7.7 and cycling conditions are as mentioned in Table 7.8.

Table 7.7: Preparation of reaction mixture for SYBR green real time PCR

Reagents	Volume (µL)
SYBERGREEN Master Mix, 2X	5
Primer (forward), 10 pm	0.5
Primer (Reverse), 10 pm	0.5l
cDNA (50-100 ng/µl)	1
Molecular grade water	3

Table 7.8: Primers to amplify cdr-1, cdr-2, erg-11, mdr-1 from *C. albicans* and *C. tropicalis*

C. tropicalis	**Sequence (5'->3')**	**Tm**
CDR1-F-RT	TCGCCGTTTGCTGAAGAAGA	60.25
CDR1-R-RT	GCAATCCCCAATTTCGATGGT	59.24
CDR2-F-RT	AAGGTGCAATCCAAAAGGGTG	59.31
CDR2-R-RT	CTCAATATCACTGGGTGCTCCA	59.83
ERG11-F-RT	TTGCCATTCGGTGGTGGTAG	60.32
ERG11-R-RT	ACATCTGGAACCTTATCACCGTT	59.74
MDR1-F-RT	GCAGTTACCTCATCTGGAGCA	59.79
MDR1-R-RT	GCACCAAACAATGGGAACACA	59.86
ACTIN1-F	CGTCGGTAGACCAAGACACC	60.11
ACTIN1-R	CCCAGTTGGAGACAATACCGT	59.72

C. albicans	**Sequence (5'->3')**	**Tm**
CDR1-F-RT	CCAACAATACAAGACCAGCATCTCC	57.8
CDR1-R-RT	AATCGACGGATCACCTTTCATACG	56.9
CDR2-F-RT	CGAGGTGGAGCACTTTTCTTTTCAG	58.2
CDR2-R-RT	AGCTAATGCATCAGCAGATGGAC	57.3
ERG11-F-RT	TGGAGACGTGATGCTG	51.5
ERG11-R-RT	AGTATGTTGACCACCCATAA	50.8
MDR1-F-RT	GGGTAGTTCCGTGTTGGGTTTC	58.1
MDR1-R-RT	TGCTCTCAACTTTGGTCCGTTC	57.5
ACTIN1-F	CCAGCTTTCTACGTTTCC	53.06
ACTIN1-R	CTGTAACCACGTTCAGAC	52.72

Table 7.9: Thermal profile for SYBR green Real time PCR amplification

		Temperature	Time
Denaturation		95°C	10 min
Amplification	Denaturation	95°C	10 sec
	Annealing	Depends on primers Tm	10 sec
	Extension	72°C	10 sec
Melting		95°C	0.05 sec
		Depends on primers Tm	1 min
		97°C	0.11 ramp rate
Cooling		40°C	30 sec

The result is expressed as a normalized ratio,

Normalized Ratio = $(\text{Conc.}_{\text{Target}}/\text{Conc.}_{\text{Reference}})_{\text{sample}} / (\text{Conc.}_{\text{Target}}/\text{conc.}_{\text{Reference}})_{\text{Calibrator}}$

3. *A. flavus* and *A. fumigatus*

3.1 Screening of *A. flavus* and *A. fumigatus* for azole resistance

- Prepare Sabouraud's dextrose agar plates supplemented with 1 µg/mL voriconazole and 4 µg/mL itraconazole.
- Grow isolates on SDA for a period of three to four days.
- Harvest the spores and plate 10 µL inoculum of each isolate (0.09-0.13 OD) onto supplemented Sabouraud's dextrose agar plates (1 µg/mL voriconazole or 4 µg/mL itraconazole)
- Use SDA plates without supplemented antifungal as control.
- Incubate for 48 hours at 35°C. Observe growth at the inoculated spots and record. The growth will indicate the isolates which can grow in the presence of azoles.

3.2 PCR and sequencing analysis of Lanosterol 14-alpha demethylase coding genes

3.2.1 DNA extraction

- Grow isolates on SDA for a period of three to four days.
- Harvest the spores and inoculate into Sabouraud's dextrose broth (HiMedia) and incubate the flask at 37°C in a rotary shaker (New Brunswick scientific, USA) at 120 rpm for three to five days.
- Recover the mycelia by filtration and transfer the fungal mat (0.3-0.5 g) into a clean mortar.
- Add a small volume of liquid nitrogen to the fungal mat and grind to a fine powder using a pestle.
- To the fine powder, add 2-3 mL of lysis buffer.
- Transfer 700 µl of suspension into microfuge tube. To this, add 1.4 µL of proteinase K (Fermentas, USA) at a concentration of 20 mg/mL and incubated at 56°C for one hour. Add RNase at a final concentration of 10 µg/mL and incubated at 37°C for 30 minutes.
- To the above suspension, add 600 µL of phenol: chloroform: isoamyl alcohol (25:24:1) mixture and centrifuge at 12,000 rpm for 10 minutes.

- Transfer aqueous phase to a micro centrifuge tube, add 1/10th volume 3 M sodium acetate and precipitate DNA with equal volume of isopropanol.
- Wash the pellet with 70% alcohol and air dry.
- Dissolve genomic DNA in 50 µL of 1X TE buffer.
- Quantify DNA using Nanodrop (Thermo) and store at -20^{0}C (Refer section **1.1.2.2** for more details)

3.2.2.1. Amplification of *cyp51A, cyp51B* and *cyp51C*in *A. flavus*

After extraction of DNA, amplification of the *cyp51A, cyp51B* and *cyp51C* should be carried out by thermocycler. Amplify full length gene from resistant and sensitive *A. flavus* using a number of primers (Table 7.10) where different fragments of 600-700 bp portions will be amplified.

Table 7.10: Primers to amplify *cyp51A, cyp51B* and *cyp51C* from *A. flavus*

CYP51 Homologue	Primer Name	5'-3' Sequence	Position (bases)
CYP51A	AflaCYP51A F1	CAAGAACAGCCTGCACAGAG	324
	AflaCYP51AR1	GGGTGGATCAGTCTTATTA	1126
	AflaCYP51AF2	GCAATCATCGTCCTAAATC	1066
	AflaCYP51AR2	CTGTCCATTCTTGTAGGTA	1899
	AflaCYP51AF3	GCATGAGGGAGATCTATATG	1791
	AflaCYP51AR3	CCTATAATTGCTGGTTTCG	2649
	AflaCYP51AF4	TGAAGCTATTCAATGTAGAC	2480
	AflaCYP51AR4	ACTGCTGATGGTGTGCTAAG	3358
CYP51B	AflaCYP51B F1	AACACGACTAGGAGCTACAC	4182
	AflaCYP51BR1	CACCAATCCACTCTATC	5082
	AflaCYP51BF2	GATCAGGGAAATGTTCTTC	4948
	AflaCYP51BR2	ACGATCGCTGAGATTAC	5620
	AflaCYP51BF3	GTTCAGCAAATGTCGAG	5550
	AflaCYP51BR3	CCTTTCGTCTACCTGTT	6344
	AflaCYP51BF4	AGTGGAGAGCATCCATAGTGA	6231
	AflaCYP51BR4	ACAACCCGTTCAAGATATCGG	7339
CYP51C	AflaCYP51CF1	CTGTTGCAGAGCCGTTGATG	33
	AflaCYP51CR1	CAAAGAGCGACACATAAG	860
	AflaCYP51CF2	GGTAATGTCTGGTCATAGG	751
	AflaCYP51CR2	ATGAGCTTGGAATTGGG	1453
	AflaCYP51CF3	CGAATTCATCCTCAATGG	1336
	AflaCYP51CR3	GTCTCTCGGATCACATT	2137
	AflaCYP51CF4	GGAACTCTACCAAGAGCA	2018
	AflaCYP51CR4	CCTAGATACAGCTAGATACCC	2819
	Y319H-F	GCGGTTCTCTACCACGATTTG	677
	Y319H-R	AGGGTCTCTCGGATCACATTT	1120

Put PCR in the presence of 200 µM of each dNTP (Fermentas, India), 0.25 µM of each primer (Sigma St. Louis, MO), 0.2 µL of 3U (stock) of *Taq* polymerase (Bangalore Genie), Taq buffer (It is available

as 10X, bring to 1X) (Bangalore Genei, Bangalore, India), 50-100 ng of DNA and use molecular grade water to make final volume of 20 µL. Amplification reactions should be performed in a Thermocycler (Eppendorf Mastercycler, Hamburg, Germany) as mentioned in Table 7.11.

Table 7.11: Conventional PCR cycling conditions

Denaturation		**95°C**	**10 min**	
Amplification	Denaturation	95°C	30 sec	Repeat for 35 cycles
	Annealing	Depends on primers Tm	30 sec	
	Extension	72°C	1 min	
Extension		72°C	10 min	
Hold		20°C		

Next proceed for sequencing PCR (Refer section **1.1.4** for more details). After sequencing the resistant and sensitive isolates, compare the sequences to identify any relevant genomic changes.

3.2.2.2. Screening of azole resistance related SNPs in *A. fumigatus*

The most commonly found azole target site (*cyp51A*) mutations should be screened by amplifying the hot spot regions of *cyp51A* followed by sequencing.

Table 7.12: Primers to detect polymorphisms in *cyp51A*

Primer Name	**5'-3' Sequence**	**Amplified product (bp)**	**Target codon with hotspots underlined**
CYP51G54_L98HF1	GACGGCAATCTTGCTCAATG	445 *	**GGG** (G54) **CTC** (L98H)
CYP51G54_L98HR1	GAGTCAAGCCGTACTTGATG		
CYP51BG138F2	CGGACGTGGTGTATGATTGT	272	**GGC** (G138)
CYP51BG138R2	ATAGAGGTCAGCGAACTCAG		
M220F3	CGGCAATGGCTGAGATTACC	228	**ATG** (M220)
M220R3	GACGGCGCTGATTGATGATG		
G438(B)F4	CACTCTATCATGCGCAAGGT	324	**GGT** (G438)
G438(B)R4	CACAATGGTCGCCAGAATCA		

*The amplified product will cover the region where both the G54 & L98 SNPs are found.

PCR should be put up using primers mentioned in Table 7.12 and conditions as detailed above. The PCR products should be sequenced as described in section 1.1.4. The sequences should be analysed for the polymorphisms as mentioned in the above table. The details of the SNPs position are as mentioned in the *cyp51A* map as shown in the Figures 7.1 and 7.2 below.

ATGGTGCCGATGCTATGGCTTACGGCCTACATGGCCGTTGCGGTGCTGACGGCAATCTTGCTCAATGTTGTTTATCAATTAT
TCTTTCGGCTTTGGAACCGAACAGAACCGCCAATGGTCTTTCATTGGGTCCCATTTCTGGGTAGTACCATCAGTTACGGG(
G54)ATTGATCCCTACAAGTTCTTCTTTGCGTGCAGAGAAAAGGCAAGTCTCAAGATTGTAGTTTGACATTCATTCCTGGGC
GCATTGCTGAGTATTGCTTTCTTAACCGGCAGTATGGCGATATCTTCACTTTTATACTGTTGGGTCAAAAAACCACAGTCTAC
CTGGGCGTTCAGGGGAACGAGTTTATTCTCAACGGCAAGCTC(L98H)AAGGATGTCAATGCGGAAGAGGTCTATAGTCCAT
TGACGACCCCCGTTTTCGGATCGGACGTGGTGTATGATTGTCCCAATTCCAAGCTGATGGAGCAGAAAAAGTTCATCAAGT
ACGGC(G138)TTGACTCAGTCTGCGTTAGAGTCTCATGTGCCACTTATTGAGAAGGAGGTTTTGGACTATCTGCGCGATTC
ACCGAACTTTCAAGGCTCGTCCGGCCGGATGGACATCTCTGCGGCAATGGCTGAGATTACCATTTTTACCGCTGCTCGAG
CCCTCCAAGGCCAGGAAGTTCGTTCCAAACTCACGGCTGAGTTCGCTGACCTCTATCATGACCTGGACAAGGGCTTTACT
CCCATCAATTTTATG(M220)CTACCGTGGGCCCCATTGCCGCATAACAAGAAGCGAGATGCTGCTCATGCGCGCATGAGGT
CAATCTACGTTGACATCATCAATCAGCGCCGTCTTGACGGTGACAAGGACTCTCAGAAATCAGACATGATATGGAACCTGA
TGAACTGCACATACAAAAACGGCCAGCAAGTGCCTGATAAAGAGATTGCGCACATGATGATAACCCTGTTGATGGCTGGTC
AGCATTCGTCTTCGTCCATCAGCGCCTGGATTATGCTGAGACTGGCCTCACAGCCAAAAGTCCTCGAAGAGCTGTATCAG
GAACAGCTGGCCAATCTTGGCCCCGCCGGGCCAGACGGCAGTCTTCCTCCGCTCCAGTACAAGGATCTTGACAAACTTC
CCTTCCATCAACATGTTATTCGTGAAACCTTACGGATTCACTCCTCTATTCACTCTATCATGCGCAAGGTGAAAAGCCCCTT
GCCCGTTCCCGGGACCCCTTACATGATTCCTCCCGGTCGCGTGCTCCTTGCTTCACCTGGAGTGACAGCCCTCAGCGAC
GAACACTTCCCCAATGCTGGGTGCTGGGATCCCCATCGCTGGGAGAACCAGGCTACTAAGGAGCAGGAGAACGACGAG
GTTGTCGACTACGGTTACGGCGCCGTCTCCAAGGGCACGTCAAGTCCCTATCTTCCGTTTGGT(G438)GCTGGCCGACAC
CGCTGTATCGGCGAGAAATTCGCTTATGTCAACCTTGGTGTGATTCTGGCGACCATTGTGCGCCACCTGCGACTTTTCAAC
GTGGATGGAAAGAAAGGAGTCCCTGAAACTGACTATTCATCCCTCTTTTCGGGCCCCATGAAGCCAAGCATCATCGGCTG
GGAGAAGCGGTCGAAAAACACATCCAAGTGA

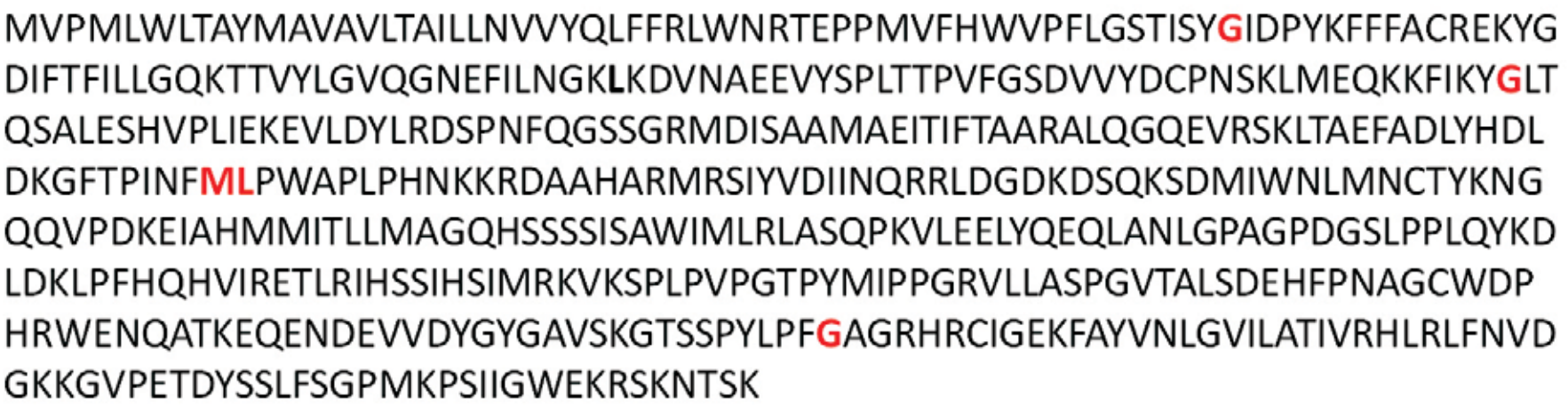

Figure 7.1 and 7.2: *cyp51A* map showing the hotspots where the mutations have been reported and the corresponding amino acids highlighted in red in the second figure.

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Glossary

Acid-fast	A property of cell walls that, during a staining reaction retain basic dyes when decolorized with mineral acids.
Acropetal	Having the youngest conidia at the apex of a chain
Aerial hyphae	Hyphae that grow above the agar surface
Aerobic	Having the ability to grow in the presence of oxygen
Annellide	A conidiogenous cell that gives rise to successive conidia in a basipetal manner. The apex of an annellide becomes longer and narrower as each subsequent conidium is formed and released. An apical ring composed of outer cell wall material, remains as each conidium is released.
Annelloconidium (pl. annelloconidia)	A conidium formed by an annellide
Annular frill	A ring or skirt like portion of cell wall material at the base of a conidium that remains when the conidium separates from its conidiophores.
Apex (pl. apices)	The tip
Arthroconidium (pl. arthroconidia)	A conidium formed by the modification of a hyphal cell(s) and then released by the fragmentation-lysis of a disjunctor cell or by fission through a thickened septum.
Arthrospore	See arthroconidium
Ascospore	A haploid sexual spore that is formed by free-cell formation in an ascus following karyogamy and meiosis.
Ascus (pl. asci)	A saclike cell that gives rise to ascospores. Asci are characteristic of the Ascomycetes.
Assimilation	The utilization of nutrients for growth, with oxygen serving as the final electron acceptor. $C_6H_{12}O_6+6O_2 \rightarrow 6CO_2+ 6H_2O$.
Ballistoconidium (pl. ballistoconidia)	A forcibly discharged conidium
Balloon form	Pertaining to a large globose conidium formed by some dermatophytes, especially *Trichophyton tonsurans.*
Basidiospore	A haploid sexual spore formed on a basidium following the process of karyogamy and meiosis.
Basidium (pl. basidia)	A specialized cell that gives rise to spores. Basidia are characteristic of the Basidiomycetes.
Basipetal	Having the youngest conidia at the base of a chain
Bipolar budding	The development of conidia at both ends of parent cell
Biseriate	Having phialides arising from metulae on the vesicles in species of *Aspergillus*
Black yeast	A dematiaceous, unicellular, budding fungus that typically forms a black, pasty colony.

Blastoconidium (pl. blastoconidia)	A conidium that is blown out from part of its parent cell and is typically released by fission through thickened basal septum.
Blastospore	See blastoconidium
Budding	A sexual formation of small rounded outgrowths from parent cell. These will become conidia.
Capsule	A gelatinous covering around a cell
Carry-over	Indigenous substances stored within the cells of *inoculum* nutrients in the original culture medium, or both. These substances support growth of the test isolate in an assimilation study.
Chlamydoconidium (p. chlamydoconidia)	A rounded, enlarged conidium that usually has a thickened cell wall and functions as a propagule.
Chlamydospore	See chlamydoconidium
Circinate	Coiled into a complete or partial ring
Clamp connection	A specialized hyphal bridge involved with nuclear division in the Basidiomycetes
Clavate	Club shaped
Cleistothecium (pl. Cleistothecia)	An enclosed fruiting body that contains randomly dispersed asci
Collarette	A small collar.
Columella (pl. columellae)	A sterile domelike expansion at the apex of a sporangiophore
Conical	Cone shaped
Conidiogenous cell	A cell that gives rise to conidia
Conidiophore	A specialized hypha upon which conidia develop
Conidium (pl. conidia)	An asexual, nonmotile, usually deciduous-propagule that is not formed by cytoplasmic cleavage, free-cell formation or by conjugation.
Cottony	See floccose
Denticle	A peg
Dimorphic	Having two different morphologic forms
Disjunctor cell	A cell that releases a conidium by its fragmentation or lysis
Echinulate	Having a delicate, spiny wall
Endospore	A spore formed within a spherule by a cleavage process following karyogamy and mitosis
Erect	Upright
Exudate	Droplets of fluid formed on the surface of a colony
Fermentation	The ability to utilize nutrients for growth, with organic compounds serving as the final electron acceptors. $C_4H_{12}O_6 \rightarrow 2C_2H_5OH + 2CO_2$.
Filament	A threadlike element of a bacterium; a hypha of a fungus
Fission	To split into two portions or cells
Fission arthroconidium	An arthroconidium that is released by fission through a double septum

Floccose	Having a cottony texture
Fragmentation	Separation of a hypha into conidia
Fungus (pl. fungi)	A eukaryotic, unicellular to filamentous, achlorophyllous organism having absorptive nutrition. A fungus reproduces by sexual, asexual, or both means.
Fusiform	Tapering at both ends; spindle shaped
Geniculate	Bent like a series of knees
Germ pore	An unthickened spot in a spore or conidial wall through which a germ tube may form
Germ tube	A hypha initially developing from a conidium or spore
Glabrous	Smooth
Hemispheric	Half of a sphere
Hilum (pl. hila)	A scar at the base of a conidium.
Hyaline	Without color
Hypha (pl. hyphae)	An individual filament of a fungus
Intercalary	Occurring within a hypha
Internode	That portion of a hypha that is between two nodes
Karyogamy	Fusion of two nuclei
Lanose	Having a wooly texture
Lysis	Dissolution
Macroconiduim (pl. macroconidia)	The larger of two conidia of two different sizes that are produced in the same manner by a single fungus.
Merosporangium (pl. merosporangia)	A sporangium having its sporangiospores in a single row
Metula (pl. metulae)	A sterile branch upon which phialides of some species of *Aspergillus* and *Penicillium* develop
Microconiduim (pl. microconidia)	The smaller of two conidia of two different sizes that are produced in the same manner by a single fungus
Moniliform	Having swellings
Mould	A filamentous fungus
Multiple budding	The development of several series of blastoconidia around a parent yeast cell
Muriform	Having vertical and horizontal septa
Mycelium	The aggregated mass of hyphae making up a fungus
Mycology	The branch of biology that deals with the study of fungi
Node	Where a stolon touches a surface
Nodular organ	A knot of hyphae that is often produced by dermatophytes
Obclavate	Club shaped in reverse
Obovoid	Egg shaped in reverse

Olivaceous	Having an olive shade of color
Oval	Egg-shaped
Papilla (pl. papillae)	A small nipple-shaped elevation
Penicillus	A brush like conidial head produced by members of the genus *Penicillium*
Percurrent	Developing through a previous apex
Perithecium (pl. perithecia)	A fruiting body having asci in a basal group or as a layer. Perithecia are usually flask shaped, with an opening through which the asci or ascospores escape
Phialide	A type of conidiogenous cell that gives rise to successive conidia from a fixed site in a basipetal manner. A phialide does not increase in length as the conidia are formed, and its apex does not become smaller in diameter. A collarette is often present at the apex of the phialide
Phialoconidium (pl. phialoconidia)	A conidium produced by a phialide
Pleomorphic	Having several forms. The term is also applied to dermatophyte colonies that become irreversibly sterile
Polymorphic	Having several forms
Pseudohypha (pl. pseudohyphae)	A series of blastoconidia that have remained attached to each other forming a filament. The blastoconidia are often elongated with the points of attachment between adjacent cells being constricted.
Pseudomycelium	A large amount of pseudohyphae.
Pycnidium (pl. pycnidia)	A saclike fruiting body that gives rise to conidia within its central area
Pyriform	Pear shaped
Rachis	An extension of a conidiogenous cell-bearing conidia
Racket hyphae (also spelled racquet)	A hypha having a series of cells that are swollen at one end
Radiating	Spreading from a common center
Rhizoid	Pertaining to a root like group of hyphae
Septum (pl. septa)	A crosswall
Simple	Of one piece; unbranched.
Solitary	Separate: alone.
Spherule	A sporangium like structure containing endospores that is produced by *Coccidioides immitis* or *Rhinosporidium seeberi.*
Sporangiolum (pl. sporangiola)	A sporangium that contains a small number of sporangiospores. Some sporangiola may contain only one sporangiospore
Sporangiophore	A specialized hypha that gives rise to a sporangium
Sporangiospore	A spore that is formed by a cleavage process following karyogamy and mitosis in sporangium

Sporangium (pl. sporangia)	An asexual sac like cell that has its entire content cleaved into sporangiospore
Spore	A reproductive propagle that forms either following meiosis or asexually by a cleavage process
Sterigma (pl. sterigmata)	A pedicel bearing a basidiospore
Stolon	A runner
Subglobose	Almost round
Submerge	Within the nutrient agar
Sympodial	Pertaining to the growth of conidiophore in which new successive lateral, subterminal apices of growth occur following successive conidium formation, Sympodial conidiophores are typically geniculate in appearance.
Synnema (pl. synnemata)	An erect macroscopic structure consisting of united conidiophores that bear conidia terminally, laterally, or in both ways.
Truncate	Ending abruptly
Tuberculate	Having fingerlike or wart like projections
Unipolar budding	The development of conidia at one end of the parent cell
Uniseriate	Having phialides that arise directly from the vesicle in species of *Aspergillus*
Verrucose	Having warts
Verticil	A whorl of conidiogenous cells or conidiophores arising from a common point
Verticillate	Having verticals
Vesicle	A swollen cell; the swollen apices of some conidiophore or sporangiophore
Villose	Bearing long, hairlike appendages
Yeast	A unicellular budding fungus that reproduces by sexual, asexual, or both means
Yeastlike	Pertaining to a unicellular budding fungus that reproduces by asexual means only
Zygospore	A resting spore in which meioses will occur. Zyospores result from the fusion of two similar hyphal elements. They are characteristic of the *Mucorales.*

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Standard Operating Procedures for Fungal Identification and Detection of Antifungal Resistance

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