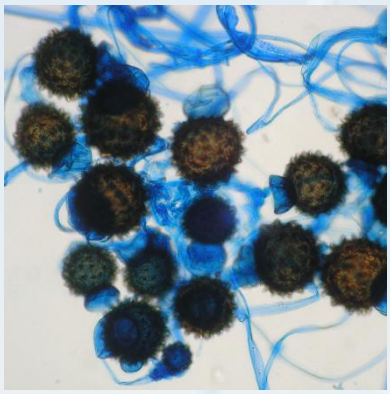
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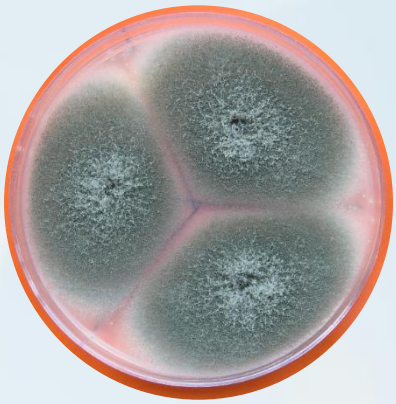
Standard Operating Procedures for Medical Mycology

Laboratory Diagnosis of Fungal Infections and Detection of Antifungal Resistance

Third Edition | 2026

Antimicrobial Resistance Surveillance and Research Network







Indian Council of Medical Research
New Delhi, India

General disclaimer: The Indian Council of Medical Research (ICMR) has taken all reasonable precautions to ensure the accuracy and reliability of the information presented in this SOP. The technical content has been provided by the ICMR Nodal Centre for Mycology and is intended for informational and scientific purposes. The procedures and recommendations should be implemented by appropriately trained personnel in accordance with applicable institutional protocols and biosafety requirements.

Standard Operating Procedures for Medical Mycology, 2026
ICMR - Antimicrobial Resistance Research & Surveillance Network (AMRSN)
Third edition

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Foreword

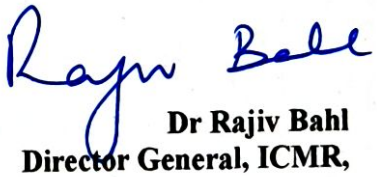
Fungal infections are an important public health concern in India, particularly among immunocompromised individuals and patients with serious underlying illnesses. Timely and accurate laboratory diagnosis, together with the detection of antifungal resistance, is essential for guiding appropriate treatment and improving patient outcomes.

Building upon the second edition published in 2019, the revised third edition of the Standard Operating Procedures for Medical Mycology, 2026, represents a substantial expansion of the earlier manual, reflecting advances in medical mycology and the evolving needs of clinical laboratories. It covers essential procedures for specimen collection, transport and processing; direct microscopy and staining; fungal culture and identification; and relevant serological, antigen-based and molecular diagnostic approaches. The manual also addresses antifungal susceptibility testing (AFST) and the detection of antifungal resistance, supporting the generation of reliable and reproducible laboratory results.

This edition incorporates updated information on medically important fungi, their diagnostic characteristics, alongside practical guidance on laboratory procedures, culture media, reagents and troubleshooting. The inclusion of supplementary information in annexures further enhances its practical utility as a reference for routine laboratory work and training. Continued adherence to standardised procedures, quality assurance practices and appropriate biosafety requirements will be essential for the effective implementation of this guidance.References to commercial methods, products or equipment are provided for informationalpurposesonly and do not implyendorsement by ICMR.

I am confident that the revised SOP will serve as a valuable reference for microbiologists, pathologists, laboratory professionals, researchers and other healthcare personnel across the country. I hope that its use will promote greater consistency in laboratory practices, strengthen diagnostic capacity and contribute to the timely and accurate diagnosis of fungal infections and improved patient care in the country.

I convey my best wishes to all users of this manual.



Dr Rajiv Bahl
Director General, ICMR,
Secretary, Department of Health Research

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Acronyms and Abbreviations

ACN – Acetonitrile
AFST – Antifungal Susceptibility Testing
AMR – Antimicrobial Resistance
ATCC – American Type Culture Collection
BACTEC – Becton Dickinson Automated Culture System
BAL – Bronchoalveolar Lavage
BHIA – Brain Heart Infusion Agar
BSA – Bird Seed Agar
BSL – Biosafety Level
CDC – Centers for Disease Control and Prevention
CFU – Colony-Forming Unit
CFW – Calcofluor White
CGB – Canavanine Glycine Bromothymol Blue
CHA – Cornmeal Heart Infusion Agar
CHCA – α-Cyano-4-hydroxycinnamic acid
CLSI – Clinical and Laboratory Standards Institute
CMA – Corn Meal Agar
CSF – Cerebrospinal Fluid
Ct – Cycle Threshold
CTAB – Cetyltrimethylammonium Bromide
CzA – Czapek-Dox Agar
DDW – Double-Distilled Water
DMSO – Dimethyl Sulfoxide
DRBC – Dichloran Rose Bengal Chloramphenicol (agar)
DTM – Dermatophyte Test Medium
DW – Distilled Water
ECV – Epidemiological Cut-Off Value
EDTA – Ethylenediaminetetraacetic Acid
EUCAST – European Committee on Antimicrobial Susceptibility Testing
GMS – Gomori Methenamine Silver
HPLC – High-Performance Liquid Chromatography
I – Intermediate
IA – Invasive Aspergillosis
ID – Identification
IFI – Invasive Fungal Infection
IU – International Unit
KT – Kwartler and Tajima (1964) Medium
LCB – Lactophenol Cotton Blue
MALDI-TOF MS – Matrix-Assisted Laser Desorption/Ionisation Time-of-Flight Mass Spectrometry
MCT – Microcentrifuge Tube
MEA – Malt Extract Agar
MEC – Minimum Effective Concentration
MIC – Minimum Inhibitory Concentration
MOPS – 3-(N-morpholino)propanesulfonic acid
NALC – N-acetyl-L-Cysteine
NWT – Non-wild-type

OD – Optical Density
PAS – Periodic Acid–Schiff
PCA – Potato Carrot Agar
PCR – Polymerase Chain Reaction
PDA – Potato Dextrose Agar
PVA – Polyvinyl Alcohol
QC – Quality Control
R – Resistant
RPMI – Roswell Park Memorial Institute
RSA – Rice Starch Agar
S – Susceptible
SDA – Sabouraud Dextrose Agar
SDD – Susceptible Dose Dependent
TAE – Tris-Acetate-EDTA Buffer
TAT – Turnaround time
TBE – Tris-Borate-EDTA Buffer
TE – Tris-EDTA Buffer
UV – Ultraviolet
VRC – Voriconazole
WT – Wild-type
YMA – Yeast Malt Agar
YMB – Yeast Malt Broth

Chapter 1: Introduction

The global burden of fungal infections has remarkably increased over the past two to three decades. Historically, human fungal infections were primarily attributed to pathogenic dimorphic fungi and agents causing superficial mycoses. However, since the 1980s, opportunistic fungi have emerged as increasingly common causes of infection, particularly among immunocompromised individuals. In recent years, a growing number of previously uncommon and emerging fungal pathogens have also been recognised as significant causes of infection in immunosuppressed hosts.

Approximately 160,000 fungal species are currently accepted, whereas the total global fungal diversity has been estimated at 2.2–3.8 million species; on this basis, only about 3–8% of extant fungi have been formally named (Wijayawardene *et al.*, 2024). Over 400 fungal species have been implicated in human infections, although many have been reported only sporadically, and fewer than 100 species are considered common human pathogens. Of these, 19 species have been designated by the World Health Organization as fungal priority pathogens on the basis of their public health importance and the unmet need for research and development (World Health Organization, 2022). Despite significant advances in molecular and biochemical methods for fungal identification and classification, morphological characteristics continue to play a central role in routine diagnostic mycology. Morphological assessment remains particularly important for the identification of moulds. For yeasts, evaluation of colony morphology and microscopic features on specialised media, such as cornmeal agar, also contributes substantially to accurate identification. Although molecular and biochemical techniques are increasingly being developed and validated, their integration into routine laboratory practice remains incomplete in many settings (Kozel and Wickes, 2014).

Chapter 2: Overview of Laboratory Diagnosis of Fungal Infections

2.1 The Diagnostic Approach

The diagnosis of fungal infections requires a multidisciplinary approach involving clinicians, microbiologists, pathologists, and radiologists. Since many fungi associated with human disease are saprophytic, close communication between the laboratory and treating physician is essential for interpreting microbiological findings. Histopathological demonstration of fungal elements in tissue remains a key criterion for establishing tissue invasion and distinguishing infection from colonisation or contamination (Donnelly et al., 2020).

Invasive fungal infections often present with non-specific clinical manifestations, making localisation challenging. Although advances in imaging have improved detection, obtaining tissue biopsies or aspirates from deep-seated lesions may be difficult, particularly in immunocompromised or thrombocytopenic patients. These challenges, together with the limited sensitivity and prolonged turnaround time of conventional culture methods, have increased interest in non-culture-based diagnostic approaches, including antigen detection, serological and molecular assays, although further validation is required for routine clinical application (Kozel and Wickes, 2014).

Conventional mycological methods **(Table 2.1)** rely largely on culture-based techniques, which may require several days to weeks. Consequently, direct microscopy and histopathological examination remain essential for rapid preliminary diagnosis, demonstrating fungal elements, identifying tissue invasion, and distinguishing clinically significant infection from contamination or colonisation.

A major challenge in fungal diagnosis is distinguishing true infection from colonisation, particularly with opportunistic fungi commonly found on skin, mucosal surfaces, and in the environment (de Hoog et al., 2020). Fungal isolation must be interpreted alongside clinical, radiological, and host-related factors. Evidence of tissue invasion, compatible clinical manifestations, and risk factors such as immunosuppression, malignancy, transplantation, and ICU admission support invasive infection. Isolation from sterile sites is generally more significant than recovery from non-sterile specimens. Repeated isolation and increasing fungal burden provide additional evidence. Biomarkers, including galactomannan, beta-D-glucan (BDG), and cryptococcal antigen, can further support diagnosis.

Table 2.1: Summary of techniques used in the laboratory diagnosis of fungal infections. Adapted from Westblade et al. (2023) and Kozel and Wickes (2014).

Culture-based (Conventional techniques)	Non-culture-based (Serology and Molecular techniques)
Direct microscopy (Chapters 3-6)	Detection of fungal-specific: ▪ Antigen ▪ Antibody ▪ Cell wall component (chitin, galactomannan, (1→3)-β-D-glucan) ▪ Metabolites ▪ Nucleic acids (Chapter 9)
Culture isolation (Chapter 3) ▪ Standard ▪ Lysis-centrifugation ▪ Automated detection systems (Uses radiometric or non-radiometric markers to identify growth e.g. BACTEC)	
Identification of the isolate (Chapters 4-6)	

Chapter 3: Sample Collection, Transport and Processing

The primary objectives of a clinical mycology laboratory are the efficient recovery of fungal pathogens from clinical specimens and their accurate identification. The success of laboratory diagnosis depends largely on the quality, adequacy, and timely processing of specimens submitted for examination.

Key factors influencing diagnostic yield include:

(1) Appropriate sample/ specimen collection
(2) Prompt transportation to the laboratory
(3) Correct processing of the specimen
(4) Use of appropriate culture media for specimen inoculation followed by incubation under appropriate conditions

3.1 Collection of Specimens

Specimens should be collected aseptically in clean, sterile, leak-proof containers and transported promptly to the laboratory, preferably within 2 hours or as per specimen-specific requirements. They should be processed and inoculated onto primary isolation media without delay to preserve fungal viability. Specimens, collection techniques and rejection criteria are detailed in **Table 3.1**.

Swabs are generally suboptimal due to limited sample volume and risk of desiccation but may be required for sites such as the external auditory canal, nasopharynx, throat, vagina and cervix, or for environmental sampling. Swabs from wounds and draining lesions require cautious interpretation due to possible contamination.

All specimens must be labelled with patient name, age, sex and unique identification number. Requisition forms should include specimen source, collection date and time, relevant clinical history, current antimicrobial or antifungal therapy, hospital/unit number and treating clinician's name.

Table 3.1: Summary of samples received in the mycology laboratory, their salient collection technique and rejection criteria (Evans et al., 1989; Murray *et al*., 2007; Larone *et al*., 2023)

Specimen	Collection	Unacceptable specimen
I. Superficial and cutaneous mycoses		
Skin	▪ Lesions decontaminated with 70% alcohol ▪ Small scales scraped off from the margin by dry scalpel blade or glass slide. Strongly macerated skin between toes can be removed by forceps. ▪ Collected on sterilised glass plate/paper envelope ▪ Vesicles collected by gentle deroofing with needle	Cotton swabs except moist skin areas
Hair	▪ Select hairs that fluoresce under a Wood's lamp ▪ Affected hairs should be removed completely using epilation forceps/ scraping with a blunt scalpel blade. Specimen should include hair stubs, plugged follicles and skin scales. ▪ Alternatively, brush is rubbed vigorously on the scalp. ▪ Adhesive tape may also be used	Clipped hair
Nail	▪ Infected site is cleaned with 70% alcohol ▪ Clippings from nail (discoloured, dystrophic or brittle) as far back as possible from the free edge ▪ Full thickness of the nail to be included ▪ In superficial white onychomycosis, collect scraping from white spots, discarding the uppermost layer	Superficial scrapings, swabs
Mucous membrane (mouth, throat, vagina, rectum)	▪ Ideally scraping with a blunt scalpel/ spatula ▪ Moist swabs to be collected in a sterile container	Dry swab
Ear	▪ Scrapings or moist swabs from ear canal ▪ Ear washings	Dry swab
Ocular	▪ To be collected by ophthalmologist only	Swabs/samples collected by

	▪ Scrapings from ulcerated, suppurative areas (scrapings from deeper layers of cornea preferable) ▪ Specimen directly inoculated at the bedside or can be transported to the laboratory between two sterile glass slides (one for microscopy, and the other for culture) ▪ Fluid specimen (aqueous/vitreous tap) collected in a sterile syringe	laboratory loop
II. Subcutaneous mycoses		
Pus	▪ Aseptically with needle and syringe from undrained abscess ▪ Pus expressed from abscess opened with scalpel / scrapings, crust collected from superficial lesion; transported to laboratory either in sterile container/syringe and needle	Swab or materials from open wound
Biopsy	▪ Ulcerated lesions of the skin are biopsied ▪ 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	Samples received in thioglycolate broth/ formol saline
Granules	▪ Collected by lifting the crust at the opening of sinus. Curette the draining sinus to get granules/grains ▪ Frequently found underneath the pus or collected from the removed bandages ▪ Aspirated from undrained sinuses	
III. Systemic mycoses		
Cerebrospinal fluid	▪ Obtained by lumbar puncture by a clinician / or by cisternal puncture ▪ ~3 mL in a sterile tube normally ▪ Occasionally 15–30 mL may be required	Insufficient quantity
Body fluids	▪ Sterile tube or in a heparinised syringe	Swabs
Bone Marrow	▪ 0.2–0.3 mL collected in a sterile heparinised syringe from iliac crest/ sternum ▪ Sterile cap is placed on heparinised syringe and transported ▪ immediately	Clotted bone marrow aspirate

Blood	▪ Using aseptic technique, collect venous 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. In children the volume must be adjusted to body weight so that no more than 1% of total blood volume is withdrawn: approximately 1–2 mL for neonates and infants under 10 kg, 2–4 mL for children 10–20 kg, and 5–10 mL above 20 kg, maintaining the same blood-to-broth ratio (CLSI M47, Principles and Procedures for Blood Cultures, 2nd edition) ▪ Multiple blood cultures at timed intervals to be collected ▪ Automated blood culture systems (e.g., BACTEC)/ lysis centrifugation technique may improve sensitivity	
Urine	▪ Early morning 25–50 mL of clean-catch midstream urine specimen ▪ Suprapubic aspirate, catheterised specimen ▪ Collected in sterile container	24-hour collection, from the bag of catheterised patients
Feces	▪ Not usually acceptable in the mycology laboratory ▪ Collected occasionally to assess *Candida* carriage in gastrointestinal tract as surveillance culture	
Sputum	▪ 5–10 mL; early morning prior to eating ▪ Use mouth rinse and brush before collection ▪ Induce sputum when unproductive ▪ Collected in sterile wide-mouthed container	Saliva, nasal secretion, throat swab, 24-hour collection
Bronchial brush/washing/bronchoalveolar lavage	▪ Collected in sterile container using fibre optic bronchoscopes	Dried specimen
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	

Serology	▪ Serum: 1–2 mL with 1:100 parts merthiolate ▪ 3–5 mL of spinal fluid with same concentration of merthiolate	Specimen collected after skin testing with histoplasmin antigen while performing serology for histoplasmosis

3.1.1 Collection of Environmental Samples (CDC, 2024)

Environmental sampling may be performed to assess fungal contamination in healthcare facilities and other settings. Appropriate collection, storage, and transportation of samples are essential to ensure the reliability of laboratory results.

1. Surface Swabs: Sterile swabs may be used to collect samples from environmental surfaces. Samples should be collected from an area of at least 1 square inch. Whenever feasible, at least two swabs should be obtained from each sampling site: one for direct microscopic examination and the other for culture. Swabs shall be maintained at room temperature and transported to the laboratory promptly.
2. Soil Samples and Contact (Touch) Plates: Soil samples shall be collected in sterile, leak-proof containers or sterile zippered bags and transported promptly to the laboratory. Contact plates used for surface sampling shall be handled aseptically and protected from contamination during transport. Samples shall be stored at room temperature until processing.
3. Culture Plates: Following sample collection, culture plates shall be sealed securely with laboratory sealing film or parafilm to prevent contamination and desiccation and shall be transported to the laboratory immediately.
4. Air Samples: Air sampling may be performed using a slit sampler or other validated air-sampling devices. Samples shall be collected in accordance with the manufacturer's instructions and promptly transported for laboratory processing.

3.2 Specimen Transport, Processing and Storage (de Hoog *et al*., 2020; Larone *et al*., 2023)

Transport Clinical specimens should be transported in sterile, leak-proof containers to prevent contamination and preserve integrity. Humidification may be required to prevent desiccation, except for skin, hair and nail specimens, which should be transported dry. Transport media should be used only when specimens can be completely recovered; anaerobic media are generally unsuitable for mycological investigations.

Specimens should be processed and inoculated promptly, ideally within a few hours, as delays may reduce fungal viability and culture yield. If processing is delayed, most specimens may be refrigerated at 4°C. However, blood and cerebrospinal fluid (CSF) should be maintained at 30–37°C and processed without delay, while dermatological specimens should be stored dry at

room temperature. Appropriate transport and storage conditions are essential to preserve fungal viability and minimise contamination or microbial overgrowth.

3.3 Processing of Specimens in the Laboratory

1. Specimens Each specimen shall be carefully examined prior to processing to identify the portions most likely to contain fungal elements. Areas that are caseous, purulent, haemorrhagic, necrotic, or otherwise abnormal should be preferentially selected for direct microscopy, culture, and other diagnostic procedures, as appropriate.
2. Specimens that are improperly collected, submitted in leaking or damaged containers, obtained from inappropriate sites, inadequately labelled, or accompanied by incomplete patient or specimen information should be rejected, and the concerned clinician should be notified.
3. When a specimen is deemed unsuitable for processing due to insufficient quantity, poor quality, contamination, or improper collection, a repeat specimen should be requested.
4. Specimens obtained from suspected cases of mycetoma should be examined with a dissecting microscope to identify and recover characteristic granules before processing.
5. Punch biopsy specimens should be carefully inspected to ensure that they have been obtained and sectioned vertically rather than horizontally, thereby allowing representation of all tissue layers.
6. Clinical specimens should be processed as soon as possible after receipt and inoculated onto appropriate primary isolation media under suitable incubation conditions. Recovery of viable fungal pathogens is essential for definitive identification, susceptibility testing, epidemiological investigations, and other downstream analyses.
7. Unless otherwise indicated, clinical specimens suspected of containing fungal pathogens other than dermatophytes should be handled in accordance with Biosafety Level 2 (BSL-2) practices. Additional containment measures may be required when handling specimens suspected of containing highly infectious dimorphic fungi or other high-risk pathogens, in accordance with institutional biosafety guidelines.
8. Large-volume fluid specimens should be concentrated prior to culture to improve the detection and recovery of fungal pathogens by centrifugation at 1,500–2,000 × g for 5 minutes, after which the sediment should be used for direct microscopy and inoculation onto appropriate isolation media.

3.3.1 Direct inoculation

1. Many clinical specimens may be inoculated directly onto primary isolation media without prior processing.
2. Specimens suitable for direct inoculation include abscess aspirates, bone marrow aspirates, CSF, swabs, sterile body fluids, hair, skin scrapings, nail specimens, bronchial washings, bronchial brushings, and other similar specimens.
3. Fluid specimens of up to approximately 0.5 mL may be inoculated directly onto culture media.
4. If a specimen contains clotted, membranous, or particulate material, the material should be aseptically minced using a sterile scalpel or forceps prior to inoculation to maximise

fungal recovery.

5. 3–5 drops of fluid should be inoculated into each culture tube or onto each culture medium, as appropriate.

Individual processing of samples is described as follows: (McGinnis, 1980; Evans *et al*., 1989; Procop *et al*., 2017; de Hoog *et al*., 2020)

3.3.2 Processing of Sputum Specimens (Figure 3.1)

Method: N-acetyl-L-cysteine (NALC) treatment

Principle: NALC is a mucolytic agent that liquefies respiratory secretions by cleaving disulfide bonds within mucoproteins, thereby facilitating the release and recovery of fungal elements from sputum specimens.

Stock solution to be prepared:

1. Sodium citrate solution (2.94%): Prepare 2.94% sodium citrate in distilled water and sterilise by autoclaving at 121°C for 15 minutes. To prepare 100 mL, dissolve 2.94 g of trisodium citrate dihydrate ($Na_3C_6H_5O_7 \cdot 2H_2O$, MW 294.10) in approximately 80 mL of distilled water and make up to a final volume of 100 mL; this is equivalent to 0.1 mol/L. Store at room temperature and discard if turbidity or precipitation develops.
2. NALC solution (0.5%): Dissolve 0.5 g of N-acetyl-L-cysteine in 100 mL of sterile sodium citrate solution. Prepare this solution fresh before use.
3. M/15 Phosphate Buffer (pH 6.8–7.1): Prepare phosphate buffer and adjust the pH to 6.8–7.1 before use. Sørensen phosphate buffer, pH 6.8–7.1: dissolve 9.47 g of anhydrous disodium hydrogen phosphate (Na_2HPO_4) in 1 L of distilled water (solution A) and 9.08 g of potassium dihydrogen phosphate (KH_2PO_4) in 1 L of distilled water (solution B). Mix approximately equal volumes of solutions A and B and adjust to pH 6.8–7.1 with either solution before use. Sterilise by autoclaving at 121 °C for 15 minutes.
4. Following NALC treatment, the specimen should be centrifuged, and the resulting sediment should be used for direct microscopic examination and inoculation onto appropriate fungal culture media.

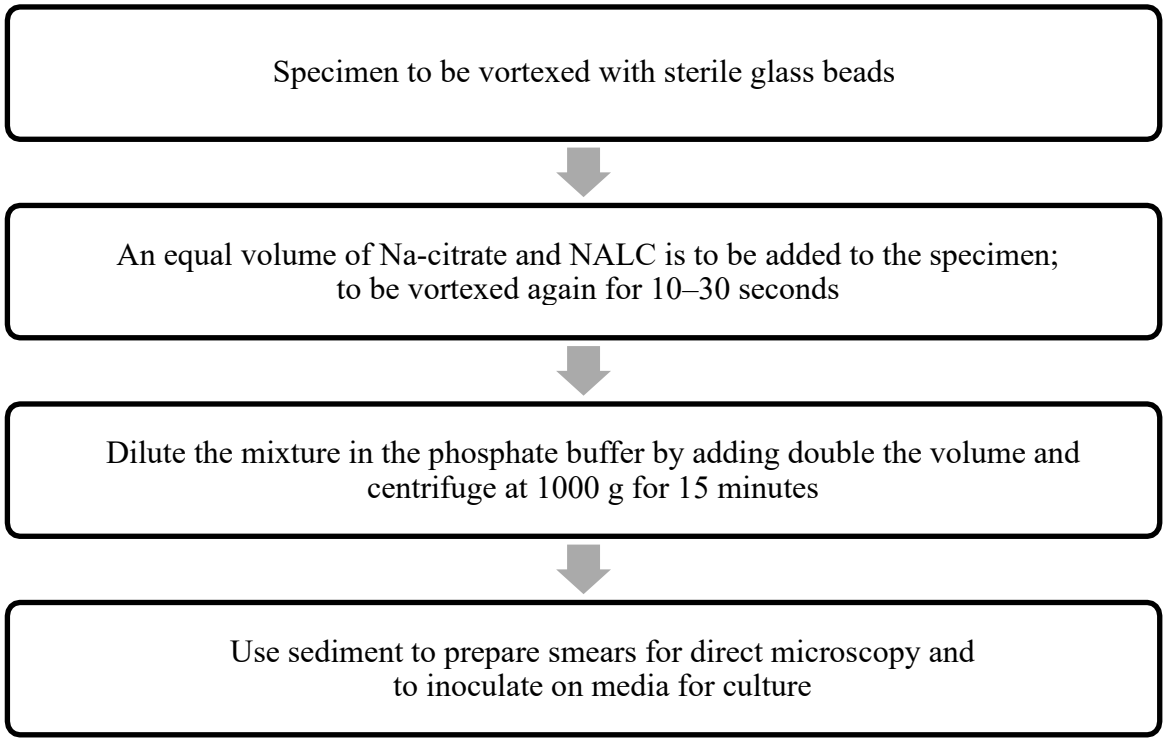


Figure 3.1: Flow diagram showing the processing of sputum samples in the mycology laboratory

3.3.3 Processing of Blood Specimens

Buffy coat preparation may be performed to detect intracellular organisms. Smears prepared from the buffy coat can be stained with PAS, Giemsa, or Calcofluor White (CFW).

Biphasic Medium

1. At least 5–10 mL of blood is inoculated into a biphasic culture medium for adults (a blood-to-broth ratio of 1:10 to 1:20 is recommended). In children, the volume must be adjusted to body weight so that no more than 1% of total blood volume is withdrawn: approximately 1–2 mL for neonates and infants under 10 kg, 2–4 mL for children 10–20 kg, and 5–10 mL above 20 kg, maintaining the same blood-to-broth ratio
2. Two culture bottles may be used; one can be incubated at 25°C and the other at 37°C.
3. After 48 hours, 5 days, and 7 days of incubation, tilt the bottle so that the liquid covers the entire agar surface without reaching the neck of the bottle. Maintain this position for approximately 1 hour before returning the bottle to the upright position.

Broth Culture (Primarily Used for Bacterial Culture)

1. This can also be used to isolate yeasts.
2. Blood is inoculated into the broth medium.
3. Subculture onto appropriate solid media after 1, 2, 3, and 7 days of incubation. Alternatively, examine the broth macroscopically for growth, prepare a smear if growth is suspected, and subculture onto solid media.
4. Incubate the solid media for at least 48 hours.

5. Examine the plates daily for yeast growth; initially, colonies may appear as tiny pinpoint colonies.

Membrane filter technique

1. A membrane filtration apparatus fitted with a 0.45 μm pore-size membrane filter is used.
2. Add 50 mL of sterile 0.2% Triton X-100 to the filtration apparatus.
3. Filter the specimen through the membrane, then wash as per laboratory protocol.
4. Place the membrane onto a Sabouraud Dextrose Agar (SDA) plate with the inoculated surface facing upward.
5. Seal the plate with laboratory sealing film and incubate at 30°C.
6. Examine the culture regularly for fungal growth.

Automated Continuous-Monitoring Blood Culture Systems

1. Several commercial automated blood culture systems are available for detecting bloodstream infections.
2. For adults, 8–10 mL of blood per culture bottle is recommended, with two to three sets of blood cultures collected from separate venipuncture sites.
3. The BACTEC™ Myco/F Lytic culture medium can be used to recover both mycobacteria and fungi from blood specimens.

3.3.4 Processing of Body Fluid and Urine Specimens

1. Process specimens by filtration or centrifugation at 2,000 × g for 10 minutes.
2. Inoculate the sediment onto appropriate fungal culture media and prepare direct microscopy smears as indicated.

3.3.5 Processing of Exudates, Pus, and Drainage Specimens

1. Examine the specimen macroscopically for granules or fungal elements.
2. If granules are present, wash them with sterile saline and crush gently before microscopic examination and culture.
3. Centrifuge liquid specimens when appropriate and culture the sediment on suitable fungal media.

3.3.6 Processing of Tissue specimens (Figure 3.2)

1. Upon receipt, determine the nature of the specimen (purulent/caseous material or tissue biopsy) - whether mucormycosis is clinically suspected, whether tissue is intended for histopathological examination.
2. Processing of purulent/caseous material: Perform a 10–20% KOH mount (or KOH with PAS/ CFW, if available) for direct microscopy. Inoculate appropriate fungal culture media. Send a portion for histopathology, if indicated.
3. Processing of tissue specimens when mucormycosis is NOT suspected: cut tissue into small pieces for microscopy, then process in a sterile tissue homogeniser for culture on appropriate media.

4. Processing of tissue specimens when mucormycosis is suspected. Do not grind or homogenise the tissue. Gently tease the tissue apart using sterile forceps and scissors. Use teased tissue for direct microscopy and fungal culture.
5. For histopathology, place a representative portion of the tissue in 10% neutral-buffered formalin (formalin-saline). Special fungal stains such as PAS and Gomori Methenamine Silver (GMS) should be requested whenever invasive fungal infection is suspected.

Note: Grinding or homogenisation should be avoided because the broad, aseptate hyphae of mucorales are fragile and may be damaged, thereby reducing culture yield.

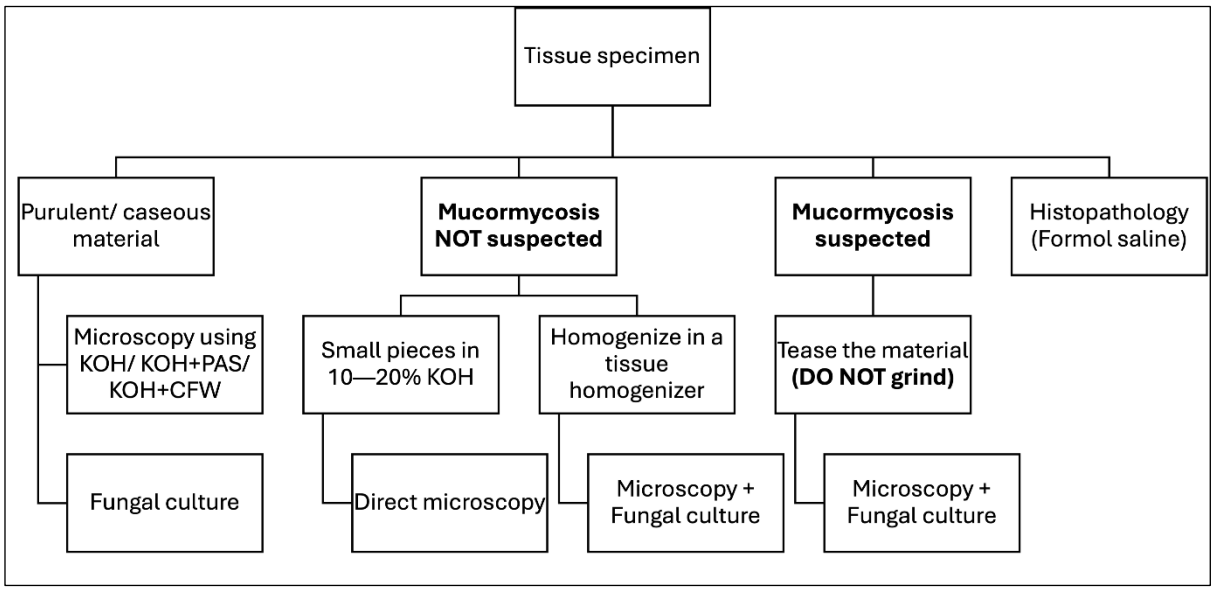


Figure 3.2: Flow diagram for the laboratory processing of tissue specimens, showing the divergent pathway when mucormycosis is clinically suspected. Adapted from Procop et al. (2017) and de Hoog et al. (2020). (KOH: Potassium hydroxide, PAS: periodic acid–Schiff)

3.3.7 Processing of Cerebrospinal Fluid (CSF) Specimens

CSF Findings in Fungal Meningitis

1. No characteristic pathognomonic CSF findings are observed.
2. Pressure may be normal or increased.
3. Glucose levels are usually normal to decreased (commonly 10–40 mg/dL).
4. Protein concentrations are usually elevated and may be markedly increased (approximately 50–1,000 mg/dL).
5. CSF lactate may be elevated; alcohol content is not routinely useful and is rarely assessed.
6. Pleocytosis is usually present and may range from approximately 20–1,000 cells/mm³, although counts commonly remain below 300 cells/mm³.
7. A predominance of lymphocytes is commonly observed in infections caused by *Cryptococcus* spp. and *H. capsulatum*.

8. A predominance of neutrophils (polymorphonuclear leukocytes) may be seen in infections caused by *Candida* spp. and *Aspergillus* spp.
    i. Microscopy: Direct microscopy may occasionally demonstrate fungal elements, although sensitivity is generally low for *Histoplasma* and *Aspergillus* infections.
    ii. India Ink staining shows round budding yeast cells with a surrounding halo, suggestive of *Cryptococcus* spp.
    iii. CSF examination may be negative in patients with intracerebral lesions.
    iv. Gram staining may aid in the detection of *Candida* spp. and *Actinomyces* spp., whereas modified acid-fast staining may be useful for the detection of *Nocardia* spp.

Culture

1. Isolation of fungi from CSF is often difficult, except for *Cryptococcus neoformans*, which is recovered more frequently.
2. CSF cultures may occasionally be positive in infections caused by *Coccidioides immitis*, *H. capsulatum*, and *Blastomyces dermatitidis*.
3. Where clinically appropriate, submission of a larger volume of CSF may improve the recovery of fungi, particularly when the fungal burden is low.
4. The culture yield for *Candida*, *Aspergillus*, and Mucorales (formerly Zygomycetes) is generally low.
5. Centrifuge the CSF specimen and perform microscopy and culture on the sediment; the supernatant may be used for antigen detection assays.
6. Repeated lumbar punctures may increase diagnostic yield when clinically indicated.
7. Fungal cultures may remain positive during the early phase of antifungal therapy; therefore, specimens collected after initiation of treatment (up to 2 weeks for *Cryptococcus* spp.) may still yield the organism (Rolfes *et al*., 2015).
8. In selected cases, alternative specimen collection methods (e.g., ventricular or cisternal CSF sampling) may be considered based on clinical indications and institutional protocols.

3.3.8 Processing of Catheter Tips for Isolation of Fungi

1. Carefully remove the external dressing using aseptic technique.
2. If required, clean the skin around the catheter insertion site with an appropriate antiseptic before removing the catheter.
3. Withdraw the catheter aseptically through the skin.
4. Sterile forceps may be used to handle and remove the catheter
5. For short intravascular catheters, aseptically cut the distal segment with sterile scissors and place it in a sterile container. The catheter segment should be approximately 2.5–5 cm in length.
6. For long-term indwelling catheters, aseptically collect the distal 5 cm of the catheter for culture.
7. If purulent exudate is present at the catheter exit site, collect a specimen for direct microscopy (e.g., Gram stain) and culture.
8. Using sterile forceps, roll the catheter tip across the surface of an appropriate culture

medium at least four times.

9. The laboratory should report the colony count from the catheter culture in accordance with established laboratory protocols.
10. When fungal isolates (e.g., *Candida* spp.) are recovered from catheter tips, they should be subcultured and appropriately stored for further identification and antifungal susceptibility testing, as indicated.

3.4 Difficulties Encountered in Isolating Fungi from Clinical Specimens

Isolation of pathogenic fungi from tissue specimens mixed with yeasts or bacteria: In some cases, fungal elements may be demonstrated by direct microscopy, but the causative fungus cannot be recovered in culture. One common reason is overgrowth by contaminating or colonising bacteria and/or yeasts. Specialised methods, such as acidification of SD broth or use of peptone-glucose-fluconazole agar, have been described to facilitate the recovery of filamentous fungi from such specimens.

3.4.1 Acidification of Sabouraud Dextrose Broth

1. Wash the tissue specimen with sterile distilled water and cut it into small pieces under aseptic conditions.
2. Prepare five tubes containing equal volumes (5–10 mL) of SD broth. Add increasing amounts of 1N HCl to the tubes (e.g., 1, 2, 3, 4, and 5 drops, respectively) to obtain varying levels of acidification.
3. Inoculate each tube with a small fragment of tissue and incubate at 30°C for 24 hours.
4. Subculture approximately 0.1 mL from each tube onto BHI agar. Incubate the inoculated media at both 30°C and 37°C.
5. Any fungal colonies recovered should be subcultured onto fresh media for purification and identification.

3.4.2 Use of Peptone-Glucose-Fluconazole Agar for isolation of mycelial fungi from specimens contaminated with yeast

1. Homogenise the specimen (e.g., sputum or bronchoalveolar lavage (BAL) fluid) as appropriate and inoculate onto Peptone-Glucose-Fluconazole Agar (peptone 10 gm/L, glucose 20 gm/L, agar 20 gm/L, chloramphenicol 40 mg/L, gentamicin 25 mg/L, fluconazole 5 mg/L, distilled water 1 L, final pH 6.8–7.0).
2. Incubate the inoculated medium at 25°C and 35–37°C.

Examine cultures regularly for the growth of filamentous fungi and subculture isolates for identification

3.5 Direct microscopy

Direct microscopy is of immense importance in the mycological investigation of clinical specimens. Unlike many bacteria, fungi may require prolonged incubation before growth becomes evident in culture. In addition, many fungi are environmental saprophytes and may represent colonisation or contamination rather than true infection. Therefore, culture results

should always be interpreted in conjunction with the clinical presentation, direct microscopic findings, histopathological evidence (when available), and other laboratory investigations. **(Figure 3.3)**.

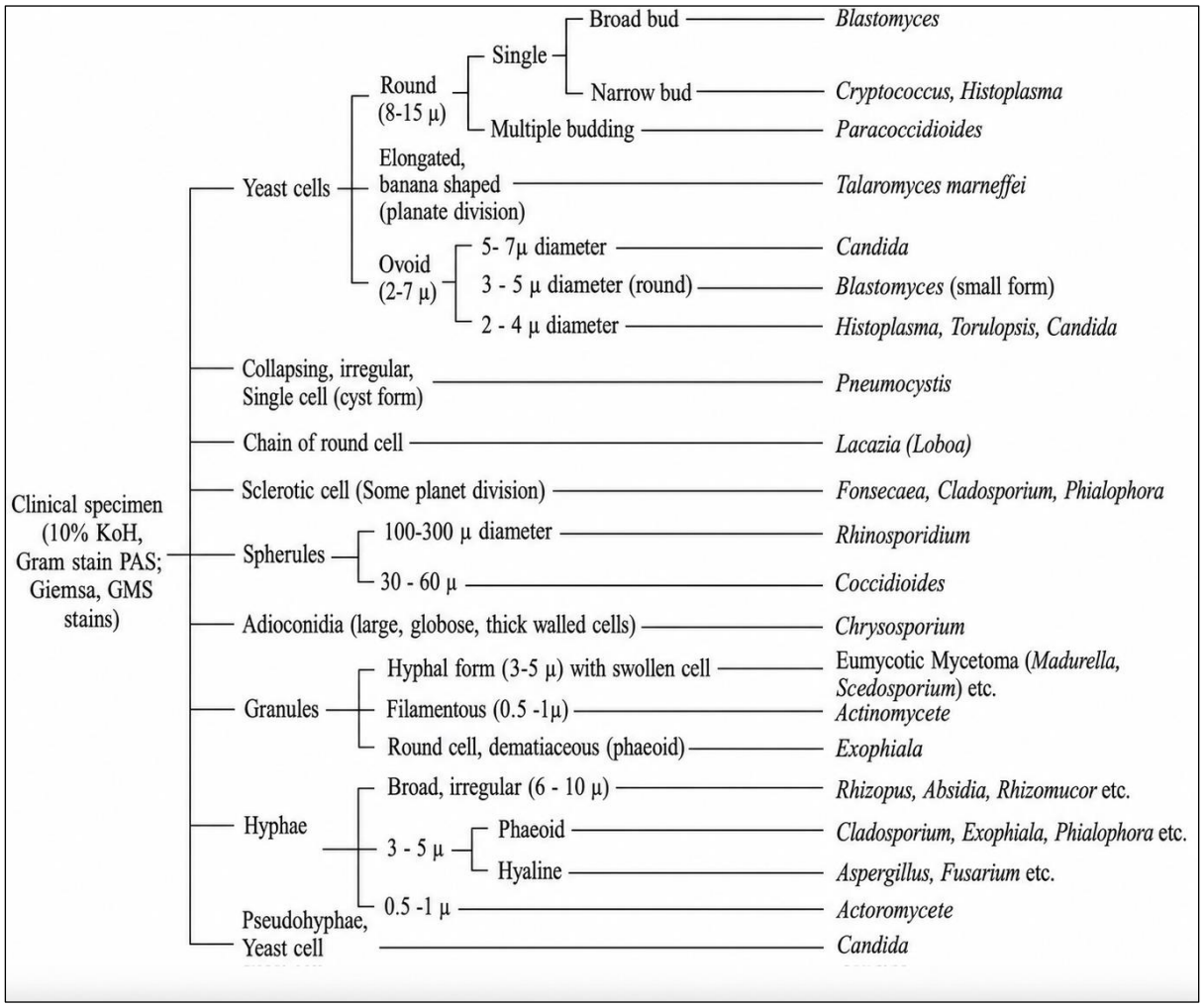


Figure 3.3: Morphological approach to the presumptive identification of medically important fungi in direct clinical specimens

A variety of wet mount preparations and histopathological stains are available for the direct detection and identification of fungi in clinical specimens. The choice of technique depends on the type of specimen, the suspected fungal pathogen, and the diagnostic objective, such as demonstrating fungal morphology, tissue invasion, capsule, or melanin production. While wet mount preparations provide rapid preliminary diagnosis, permanent staining methods enhance visualisation of fungal structures and improve diagnostic accuracy. The commonly used wet mount preparations and staining techniques in medical mycology are summarised in **Table 3.2**. The commonly used stains are described here under the following headings: Principle, Procedure, Observation, Modification, Advantages, Limitations, Precautions and Quality Assurance. For a detailed explanation on the preparation of reagents and stains used in mycology, see **Annexure A**.

Table 3.2: Wet Mount preparations and Stains used in mycology (Evans et al., 1989; Larone *et al*., 2023)

Wet Mount	Stains
1. Potassium hydroxide (KOH) (10–40%) 2. Calcofluor White (CFW) 3. India ink/ Nigrosin 4. Fluorescent antibody stain	1. Gram's stain 2. Romanowsky stains, e.g., Giemsa, Rapid Giemsa and Wright 3. Periodic acid–Schiff (PAS) 4. Methenamine Silver Stain (Gomori's, Grocott's modification and Rapid) (GMS) 5. Mayer mucicarmine stain 6. Masson–Fontana stain 7. Alcian Blue 8. Toluidine Blue 9. Modified Kinyoun acid-fast stain for *Nocardia*

3.5.1 Potassium Hydroxide (KOH) mount preparation

Principle:

- Potassium hydroxide (KOH) is a strong alkali used as a clearing agent for the direct microscopic examination of fungi in clinical specimens.
- KOH digests cellular material and debris while leaving the fungal cell wall intact, thereby facilitating visualisation of fungal elements.
- A 10–20% KOH solution is commonly used. Higher concentrations (up to 40% KOH) may be used for specimens that are difficult to clear.
- KOH mounts are commonly used for specimens such as sputum, pus, urine sediment, skin scrapings, nail clippings, hair, and homogenised tissue specimens.

Procedure:

1. Place a drop of KOH solution on a clean, grease-free glass slide.
2. Transfer a small quantity of the specimen into the KOH drop using a sterile loop, applicator stick, or scalpel blade, as appropriate.
3. Gently place a clean coverslip over the preparation, avoiding air bubbles.
4. Allow the preparation to stand at room temperature in a moist chamber until adequate clearing is achieved.
5. Tissue specimens generally require 20–30 minutes for clearing; in selected cases, prolonged exposure (including overnight incubation) may improve visualisation.
6. Gentle warming may accelerate clearing, but should be used cautiously to avoid artefacts and drying of the preparation.

Observation:

- Examine the preparation initially at low power, then systematically scan the entire

coverslip in a zigzag pattern.

- Suspected fungal elements should be examined under high-power magnification.
- Reduced illumination may improve visualisation of transparent fungal structures.
- Observe for:
    - Hyphae (septate or aseptate) **(Figure 3.4)**
    - Branching pattern and angle of branching
    - Hyphal width and pigmentation
    - Budding yeast cells
    - Pseudohyphae
    - Other fungal structures, as applicable

Modification:

For enhanced contrast, KOH may be combined with stains such as Methylene blue or Parker Blue-Black fountain ink. These stains provide a coloured background and improve the visibility of fungal elements. If present, fungal elements will appear as prominent, refractile objects.

Advantages:

Simple, rapid, inexpensive

Limitations:

- Artefacts may be present in pus and sputum, superficially resembling hyphal and budding forms of fungi. These artefacts may be produced by cotton or wool fibres, starch grains (in pleuritis) or cholesterol crystals.
- KOH mount demonstrates fungal elements but generally does not permit definitive identification of the fungal species.
- The preparation cannot be preserved indefinitely and gradually deteriorates on drying. Keeping the slides in a moist chamber prevents/delays drying.

Precautions:

- The KOH drop should be of an appropriate size; excessive volume may cause the coverslip to float.
- Preparations should be kept in a moist chamber to prevent drying and crystal formation, reducing the fungus's visibility.
- After adequate clearing, gentle pressure may be applied to the coverslip using folded filter paper or the handle of a teasing needle to ensure even distribution of the specimen.
- KOH solution should be stored in tightly closed containers and dispensed in small working aliquots to minimise contamination.

Quality Assurance:

- Fungal spores or hyphal fragments may contaminate the KOH solution and result in false-positive findings.
- A negative control preparation should be examined regularly (preferably daily when

the reagent is in use) to monitor contamination.

- Reagents should be prepared, stored, and replaced in accordance with laboratory quality management procedures.

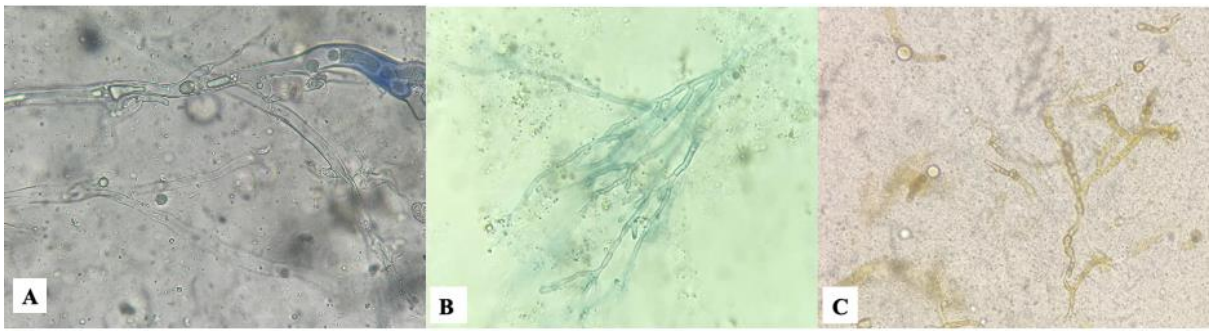


Figure 3.4: Light microscopy of KOH-CFW mount showing A) broad, aseptate hyphae with right-angled branching, B) hyaline, septate hyphae with acute angle branching, and C) phaeoid septate hyphae (original magnification, 400×) (KOH: Potassium hydroxide, CFW: Calcofluor white)

3.5.2 KOH-Calcofluor White (CFW) Preparation

Principle:

Calcofluor White (CFW) is a fluorescent stain used for direct microscopic examination of clinical specimens, often in combination with KOH, which clears host cellular material and debris. CFW binds to chitin and other fungal cell-wall polysaccharides, enhancing the visualisation of hyphae, pseudohyphae, yeasts and spores under UV or blue-violet excitation. Related fluorochromes include Uvitex 2B and Blankophor. CFW is also useful for detecting *Pneumocystis jirovecii* cysts, which exhibit characteristic wall fluorescence. However, as CFW is non-specific, artefacts such as mucus, cellular debris and fibres may also fluoresce, requiring cautious interpretation

Procedure:

1. Place the specimen to be examined on a clean, grease-free glass slide.
2. Add a drop of KOH-CFW solution to the specimen. Alternatively, KOH and CFW may be mixed in equal volumes before use.
3. Gently mix the specimen with the reagent and place a clean coverslip over the preparation, avoiding air bubbles.
4. Allow the preparation to stand at room temperature (approximately 25°C) for a few minutes until it clears adequately. Gentle warming of the slide may be used to accelerate the clearing process, if required.

Observation:

- Examine the preparation using a fluorescence microscope with appropriate UV or blue-violet excitation and barrier filters.
- Observe for bright fluorescent fungal elements, including hyphae, pseudohyphae, yeast

cells, and spores, against a dark background **(Figure 3.5).**

- If dematiaceous (melanised) fungi are suspected, the preparation should also be examined under a bright-field microscope, as these fungi may stain poorly with CFW.
- *C. glabrata* may exhibit only faint fluorescence and may therefore be difficult to detect.
- Note that non-fungal structures such as elastin and collagen fibres may also fluoresce, typically producing a yellow-green fluorescence, and should be distinguished from fungal elements.

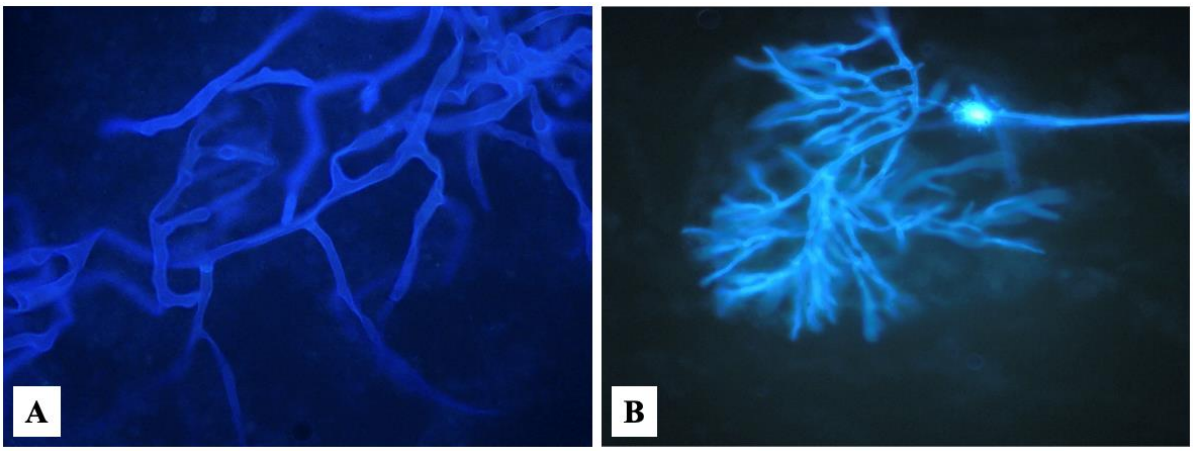


Figure 3.5: KOH-CFW mount using blue-white fluorescence showing the presence of A) broad, ribbon-like, aseptate hyphae with right-angled branching, and B) thin, septate hyphae with acute angle branching (original magnification, 400×) (KOH: Potassium hydroxide, CFW: Calcofluor white)

Modification:

Addition of Dimethyl Sulfoxide (DMSO), which accelerates keratin digestion, eliminating or reducing the need for heating. Brief warming of the preparation may be used to hasten clearing when DMSO is not added (avoid overheating).

Advantages:

- Rapid, simple, inexpensive screening test, with high sensitivity for detecting fungi directly from clinical specimens.
- Enables easy visualisation of fungal hyphae, pseudohyphae, yeasts, arthroconidia, and spherules.

Limitations:

- Fluorescence is not specific to fungi; cellulose-containing materials such as cotton fibres, plant debris, paper fibres, and some contaminants may also fluoresce, leading to false-positive interpretations.
- Poor-quality or scanty specimens may result in false-negative findings.

Precautions:

- Use freshly prepared or properly stored CFW reagent to maintain staining quality.
- Examine the preparation promptly after staining to minimise photobleaching.
- Ensure the fluorescence microscope is properly aligned with the correct excitation and barrier filters.

Quality assurance:

- Check the KOH-CFW reagent before initial use, at least weekly thereafter, and whenever a new batch of reagent is prepared.
- Use an aqueous suspension of actively growing *Candida albicans* as a positive control. The yeast cell walls should exhibit bright blue-white or green fluorescence, depending on the excitation source and filter combination used.
- A negative control should consist of the KOH–CFW reagent without any specimen or fungal elements and should show no fluorescent fungal structures.
- Ensure that the fluorescence microscope, light source, and filter sets are functioning properly and are maintained according to the manufacturer's recommendations.
- Record all quality control results and investigate any unexpected findings before reporting patient results.

3.5.3 India Ink or Nigrosin Preparation

India Ink India Ink or Nigrosin staining is a negative staining technique used primarily to demonstrate capsules surrounding microorganisms, especially *Cryptococcus* in CSF and suspected *Cryptococcus* samples. The stain darkens the background, while the yeast cell remains unstained giving a negative contrast and allowing visualisation of encapsulated organisms without heat fixation or additional staining.

Procedure:

1. Prepare the mount in the centre of a clean, grease-free glass slide.
2. Place one drop of India ink or Nigrosin stain in the centre of the slide. Avoid excessive staining, as it may produce an overly dark background. If the preparation appears too dark during examination, a small amount of sterile water may be added at the edge of the coverslip to dilute the stain.
3. Add one loopful of the specimen, or preferably the centrifuged sediment from a fluid specimen (e.g., CSF, urine, or other body fluids), adjacent to the drop of stain.
4. Mix the specimen and stain thoroughly using a sterile loop or needle. If a loop is used, allow it to cool before use to prevent the stain from precipitating.
5. Hold a clean coverslip vertically and bring one edge into contact with the fluid mixture on the slide. The fluid will spread along the edge of the coverslip by surface tension.
6. Gently lower the coverslip onto the preparation, taking care to avoid trapping air bubbles. If air bubbles are present, they may be carefully moved toward the edge of the coverslip, although manipulation should be minimised.
7. Examine the preparation immediately under the microscope. If immediate examination is

not possible, keep the slide in a moist chamber to prevent drying.

Observation:

- Systematically scan the entire coverslip at low power in a zigzag pattern. Encapsulated yeast cells of *Cryptococcus neoformans* appear as bright, refractile cells surrounded by a clear, unstained halo (capsule) against a dark background. **(Figure 3.6)**
- Under high power, the cells can also be seen, containing refractile bodies and surrounded by the unstained capsule. Characteristic pinched-off budding, when observed, is confirmatory for diagnosis.

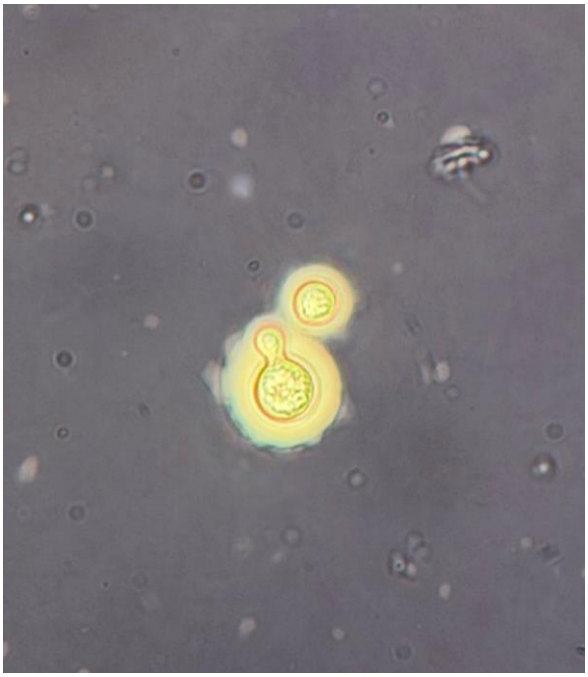


Figure 3.6: India ink mount of CSF sample showing the presence of budding yeast cells with a surrounding halo, suggestive of *Cryptococcus* spp. (original magnification, 400×)

It should be noted that:

1. In addition to the classical budding yeast form, *Cryptococcus neoformans* may exhibit atypical morphological forms, including elongated cells resembling pseudohyphae. Such forms are more commonly observed in patients with advanced HIV infection and may be associated with a high organism burden.
2. In advanced cryptococcal infection, particularly in patients with late-stage AIDS, the capsules of individual yeast cells may be difficult to distinguish because the organisms can become embedded within abundant extracellular capsular material.
3. Rarely, microcapsular or non-capsulated strains of *Cryptococcus* spp. may be encountered. In such cases, additional staining methods, such as Gram staining, may aid in demonstrating the yeast cells.
4. A negative CSF India ink preparation does not exclude cryptococcosis when clinical and radiological findings suggest a focal intracranial mass lesion. This is particularly recognised with *Cryptococcus gattii*, which preferentially causes cryptococcoma. In

such cases, brain biopsy is required for definitive diagnosis.

5. Attention should be paid to the periphery of the coverslip during microscopic examination, as yeast cells may accumulate at the edges due to fluid movement during coverslip placement. Therefore, excess fluid should not be removed from the margins of the preparation.
6. High protein concentrations in CSF may occasionally result in flocculation of India ink, making visualisation of the cryptococcal capsule more difficult.
7. Preparations made with Nigrosin stain tend to dry rapidly, particularly in warm climates; therefore, prompt microscopic examination is recommended.

Advantages:

Rapid and inexpensive techniques demonstrating the characteristic capsule of *Cryptococcus* species.

Limitations:

- The sensitivity of the India ink or Nigrosin preparation is influenced by the fungal burden. In HIV-associated cryptococcal meningitis, positivity rates may exceed 90%, whereas substantially lower sensitivities, <60% positivity, are observed in non-HIV patients and in specimens with low numbers of organisms.
- Capsule-deficient or poorly encapsulated strains may not be detected.

Precautions:

- India ink or Nigrosin stain should be mixed thoroughly before each use to ensure uniform distribution of stain particles.
- The stain should be examined periodically for contamination by preparing and microscopically examining a drop of stain alone. Any particulate matter, fungal elements, or other contaminants may lead to erroneous interpretations.
- False-positive interpretations may occur due to artefacts such as air bubbles, monocytes, neutrophils, or other cellular debris. Under high-power magnification, air bubbles appear as hollow, refractile structures and lack the characteristic yeast cell morphology. Monocytes and neutrophils may exhibit a partial halo-like appearance due to their irregular or crenated margins; however, they do not demonstrate the well-defined capsule, refractile yeast cell body, or characteristic budding seen in *Cryptococcus* spp.

Quality assurance:

- A known encapsulated *Cryptococcus neoformans* reference strain (e.g., ATCC 208821/H99) or a previously characterised encapsulated clinical isolate showing a distinct capsule by India ink.
- India ink should be homogeneous and free of clumps or precipitates.

3.5.4 Gram stain

Gram stain is generally not the preferred method for the direct detection of fungi in clinical specimens, as its sensitivity for fungal elements is limited compared with other mycological stains (Procop et al., 2017; Westblade et al., 2023). However, it may be useful for demonstrating yeasts and fungal elements in certain specimens. Gram staining is particularly useful for examining *Candida* spp., *Malassezia* spp., and *Sporothrix* spp., but is less reliable for demonstrating the yeast forms of other dimorphic fungi. Most medically important fungi stain Gram-positive and appear as purple-staining yeast cells, budding yeasts, pseudohyphae, or hyphae. The stain is especially useful for demonstrating fungal elements in sputum, vaginal secretions, purulent material, gastric washings, lung aspirates, and urine specimens.

Procedure:

1. Prepare a thin smear of the specimen on a clean, grease-free glass slide and allow it to air dry. Fix the smear by gentle heat fixation.
2. Flood the smear with crystal violet (gentian violet) and allow it to stand for 1 minute.
3. Gently rinse with water and cover the smear with Gram iodine solution for 1 minute.
4. Rinse again with water and decolourise with acetone for approximately 30 seconds. Immediately rinse with water and counterstain with safranin for 30 seconds.
5. Wash gently with water, allow the slide to air-dry, and examine under oil immersion.

Observation:

- Assess the Gram reaction (positive or negative), morphology, size, shape, budding pattern, presence of pseudohyphal or hyphal elements, and arrangement of fungal cells under an oil-immersion objective.
- Yeasts and most fungal elements typically appear Gram-positive, staining purple to violet. Filamentous fungal hyphae may stain variably with Gram stain and may be poorly visualised. *Nocardia* spp. appear as delicate, branching, Gram-positive filaments.
- Care should be taken to correlate the findings with clinical and culture results, as Gram stain alone is not sufficient for definitive fungal identification.

Modification:

- Hucker Gram stain for improved crystal violet stability.
- Using Gram stain in conjunction with fluorescent stains (e.g., CFW) for rapid screening.

Advantages:

- Rapid, simple, and inexpensive.
- Demonstrates budding yeast cells, pseudohyphae and Gram-positive filaments, aiding presumptive identification of *Candida* spp*., Cryptococcus* spp. or *Nocardia* spp.

Limitations:

- Not suitable for definitive identification of fungal species.
- Filamentous fungi may stain inconsistently, and their morphology is not optimally visualised.
- Does not differentiate *Candida* species.
- Cannot reliably distinguish viable from non-viable fungal cells.

Precautions:

- Use fresh yeast colonies (18–48 hours old) for optimal morphology.

Quality assurance:

- Use control organisms with each new batch of reagents or weekly, as required by laboratory policy. Control organism: *Candida albicans* ATCC 90028 is a Gram-positive budding yeast with pseudohyphae (purple).

3.5.5 Giemsa Stain

Giemsa stain is useful for demonstrating intracellular fungal elements, particularly the yeast forms of *Histoplasma capsulatum*, and may also be used to visualise the trophozoite forms of *Pneumocystis jirovecii* (Procop et al., 2017; de Hoog et al., 2020). It is a Romanowsky-type stain composed of methylene blue, azure dyes, and eosin. It differentially stains nucleic acids and cytoplasmic components, allowing visualisation of fungal cells within host tissues and inflammatory cells.

Procedure:

1. Homogenise the tissue specimen, if required, and prepare a thin, uniform smear on a clean glass slide.
2. Allow the smear to air dry.
3. Fix the smear with methanol (methyl alcohol) for 3–5 minutes.
4. Flood the slide with freshly prepared Giemsa stain and allow it to stain for approximately 30–45 minutes according to laboratory protocol.
5. Gently rinse the slide with running tap water.
6. Allow the slide to air dry or blot gently with absorbent paper.
7. Examine the preparation under oil immersion objective.

Observation:

- *H. capsulatum* appears as small (2–5 μm), oval, intracellular budding yeast cells, often located within macrophages.
- The yeast cells stain pale to dark blue and may be surrounded by a clear halo, which represents a staining artefact rather than a true capsule.
- Trophozoite forms of *P. jirovecii* may also be visualised.

Advantages:

Additionally, it helps demonstrate the host's inflammatory response.

Limitations:

- Morphological overlap with other intracellular organisms like *Leishmania* amastigotes.
- Poor sensitivity for *P. jirovecii* as compared to GMS stain.

Precautions:

Prepare the stain using phosphate buffer at pH 6.8, as incorrect pH affects staining quality.

Quality assurance:

Quality control is generally performed using previously confirmed positive patient smears or validated in-house reference slides.

3.6 Culture techniques

Clinical specimens from suspected fungal infections should be cultured even when direct microscopy is negative, as culture remains essential for pathogen recovery and identification. Using different media and incubation temperatures (25–30°C and, when indicated, 35–37°C) can improve fungal recovery and aid identification through characteristic growth patterns. Cycloheximide, commonly added to selective media, may inhibit several saprophytic, opportunistic and medically important fungi.

No single medium supports the isolation of all fungi. Sabouraud Dextrose Agar (SDA) and Brain Heart Infusion (BHI) agar are widely used for primary isolation. Cultures are generally incubated at 25–30°C, with additional incubation at 35–37°C when indicated. Media may be dispensed in screw-capped tubes or 100-mm Petri dishes.

Petri dishes provide a larger surface area, facilitating fungal isolation and dilution of inhibitory substances. Approximately 25 mL of medium per plate helps prevent dehydration during prolonged incubation. However, plates are more prone to contamination and drying and should be secured with gas-permeable tape or placed in sterile gas-permeable bags.

Cultures should be examined daily during the first week and at least twice weekly thereafter. Handling procedures that may generate infectious aerosols should be performed in a certified biological safety cabinet, following laboratory biosafety guidelines.

Culture tubes offer a smaller growth surface but reduce contamination, dehydration and exposure risks, making them useful for prolonged incubation.

As fungal colony morphology may vary across media, cultural characteristics should be assessed on a standard medium. SDA is commonly used for macroscopic morphological

assessment of medically important fungi and remains a principal primary isolation medium in clinical mycology laboratories.

3.6.1 Gross morphology

The gross (macroscopic) morphology of fungal colonies provides important preliminary information for fungal identification. Colony characteristics should be assessed systematically, including growth rate, surface appearance, texture, and pigmentation. These features should preferably be evaluated on a standard medium such as SDA.

Points to be noted (Kwon-Chung *et al*., 1992)

1. Growth Rate (Characteristic Colony Development)

    a. Visible growth within 2–5 days: rapid grower
    b. Visible growth within 6–10 days: intermediate grower
    c. Visible growth within 2–3 weeks: slow grower
    d. The growth rate may vary depending on the culture medium, incubation temperature, and incubation conditions

2. Surface: The colony surface should be examined for its overall configuration, which may be described as:

    a. Flat
    b. Hemispherical
    c. Raised
    d. Folded
    e. Verrucose (wart-like)
    f. Cerebriform (brain-like)

3. Texture: Colony texture is an important characteristic and may be described as:

    a. Yeast-like
    b. Glabrous (smooth)
    c. Powdery
    d. Granular
    e. Velvety
    f. Cottony

    The texture of the colony may be influenced by:

    a. Type of culture medium
    b. Incubation temperature
    c. Age of the colony

    In general, colonies with a more granular or powdery texture tend to produce larger numbers of spores than smooth or glabrous colonies.

4. Pigmentation: Pigmentation is an important but often variable characteristic. The colour

of a fungal colony should be assessed both on the surface (obverse) and the reverse side of the culture.

- Surface pigmentation is usually determined by the colour of conidia or spores and may not become apparent until sporulation is well developed.
- Reverse pigmentation results from soluble pigments diffusing into the medium and from pigmentation of the vegetative hyphae.

Pigmentation may vary considerably between strains of the same species and may also be influenced by the culture medium, incubation conditions, and colony age. Therefore, pigment production should be interpreted in conjunction with other macroscopic and microscopic characteristics.

In addition to routine fungal culture methods, selected fungal pathogens may require specialised media and processing protocols for successful isolation. The detection and identification of *Malassezia* spp. from CSF specimens using lipid-supplemented culture media is outlined below.

Chapter 4: Yeast Identification (Conventional Methods)

4.1 Identification of Yeasts

Yeasts are morphologically, physiologically and biochemically diverse fungi that grow predominantly as unicellular organisms and reproduce by budding or fission. True yeasts produce sexual spores (ascospores or basidiospores), whereas yeast-like fungi reproduce asexually. Conventional identification combines cultural, microscopic and biochemical characteristics (Haley *et al*., 1971; Evans *et al*., 1989; Kwon-Chung *et al*., 1992).

4.1.1 Culture Characteristics:

Colony morphology provides preliminary identification clues **(Table 4.1)**. Assess colour, shape, texture and growth rate. Colonies are typically smooth, ranging from moist and creamy to dry, and may be white, cream, tan, pink, salmon or orange. *Rhodotorula* spp. characteristically produce pink to coral-red colonies due to carotenoid pigments.

Table 4.1: Yeast morphology flow chart (Adapted from Kwon-Chung *et al*., 1992)

<table>
<tr><td rowspan="7">Colony colour and morphology</td><td colspan="3" rowspan="2">Brown to black colony</td><td rowspan="2">Direct Mount</td><td>Yeast only</td><td>Phaeococcomyces</td></tr>
<tr><td>Yeast and hyphae</td><td>Aureobasidium
Exophiala</td></tr>
<tr><td rowspan="2">Pink to red colony</td><td rowspan="2">Satellite colonies</td><td>Present</td><td colspan="2">Forcibly discharged conidia present</td><td>Sporobolomyces</td></tr>
<tr><td colspan="2">Absent</td><td>Purify</td><td>Rhodotorula</td></tr>
<tr><td colspan="3" rowspan="2">White colony</td><td rowspan="2">Germ Tube Test</td><td colspan="2">Positive</td><td>Candida albicans</td></tr>
<tr><td>Negative</td><td>Purify</td><td>Dalmau plate
YBC-VITEK®</td></tr>
<tr><td colspan="3" rowspan="2">Moist mycelial colony</td><td rowspan="2">Direct Mount</td><td colspan="2">Arthroconidia</td><td>Geotrichum
Trichosporon</td></tr>
<tr><td colspan="2">Other types of conidia</td><td>Hyphomycete</td></tr>
</table>

4.1.2 Microscopic Features

Microscopic examination evaluates cell morphology and reproductive structures, including:

- **Cell morphology:** Shape (round, oval, elongated or cylindrical), size, arrangement and presence or absence of a capsule.
- **Budding pattern:** Unipolar (*Malassezia* spp.), bipolar (*Hanseniaspora* spp.) or multipolar (*Candida* spp.); some yeasts, such as *Schizosaccharomyces*, reproduce by fission.
- **Asexual structures:** Blastoconidia, arthroconidia, ballistoconidia, germ tubes, pseudohyphae and true hyphae **(Figure 4.1)**.
- **Sexual structures:** Presence, arrangement, number, shape, size and wall characteristics of ascospores or basidiospores.

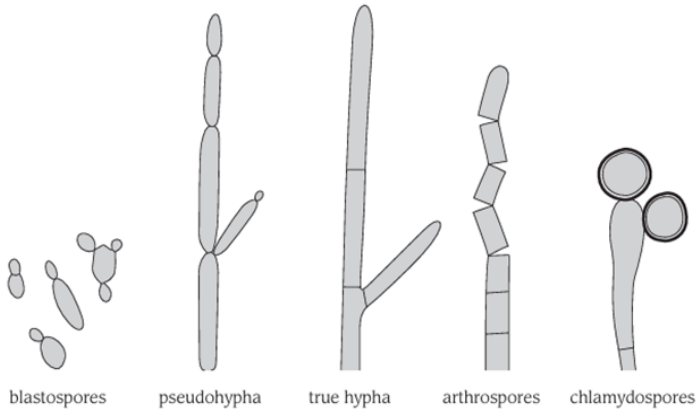


Figure 4.1: Asexual spores

4.1.3 Physiological and Biochemical Tests

These tests support species-level identification and include:

- Sugar fermentation and assimilation profiles
- Utilisation of different nitrogen sources
- Urea hydrolysis, particularly useful for presumptive identification of *Cryptococcus* spp.
- Temperature tolerance, including growth at 37°C or higher
- Gelatin liquefaction

The combined assessment of cultural, microscopic and physiological characteristics remains fundamental to conventional yeast identification, particularly in laboratories with limited access to molecular and proteomic diagnostic methods.

A summary of the overall workflow of yeast identification is provided in **Figure 4.2** below.

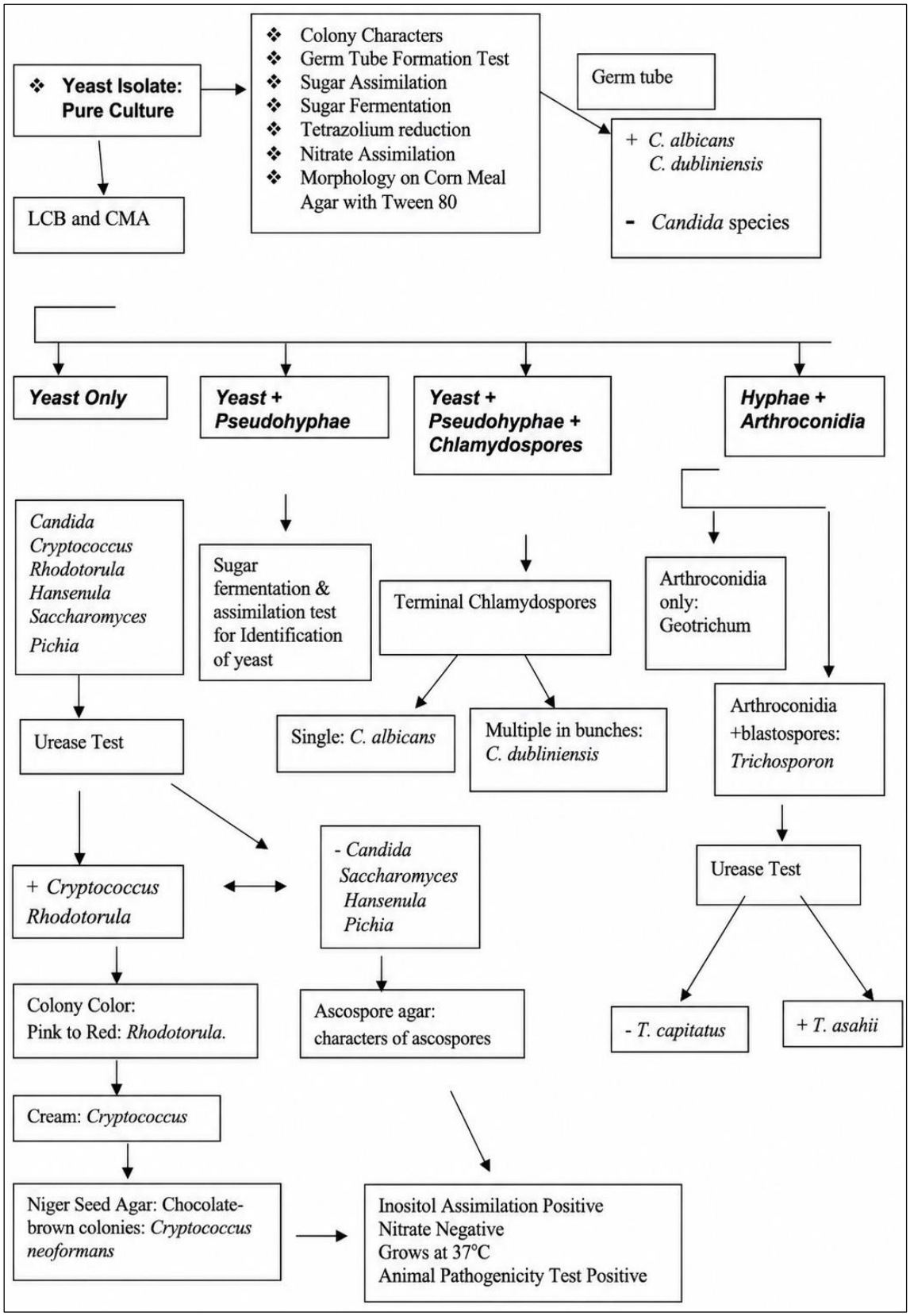


Figure 4.2: Yeast Identification Scheme (Kwon-Chung *et al*., 1992)

4.2 Identification Procedures

4.2.1 Isolation Techniques for Mixed Cultures

Before proceeding with yeast identification, the purity of the isolate should be confirmed, as primary cultures may contain bacterial contaminants or mixed yeast populations. Purification of yeasts may be achieved by the following methods:

Isolation of yeasts from cultures contaminated with bacteria (Larone *et al*., 2023)

i) Isolation on Sabouraud Dextrose Agar (SDA)

- Suspend a colony of the yeast isolate in sterile distilled water.
- Inoculate a loopful of suspension onto an SDA plate.
- Incubate at 25°C for 48 hours.
- Prepare a wet mount from an isolated colony and examine microscopically to assess purity.
- If the isolate remains contaminated, proceed to the next step.

ii) Isolation on selective media containing antibiotics

Suitable media include:

- SDA with chloramphenicol and gentamicin
- SDA with penicillin and streptomycin
- Brain Heart Infusion (BHI) agar containing 10% blood supplemented with chloramphenicol and gentamicin

Procedure:

- Prepare a suspension of a yeast colony in sterile distilled water.
- Inoculate a loopful of suspension onto one of the above media.
- Incubate at 25°C for 48 hours.
- Examine an isolated colony using a wet-mount preparation to confirm purity.
- If contamination persists, proceed to the next step.

iii) Acidification of SDA broth

- Suspend a colony of the yeast isolate in sterile distilled water.
- Prepare four tubes containing 10 mL of Sabouraud Dextrose Broth (SDB). 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.
- Inoculate each tube with 0.5 mL of the yeast suspension.
- Incubate at 25°C for 24 hours.
- Examine the cultures for purity and subculture onto appropriate solid media if required.

Isolation of yeasts from mixed yeast cultures

Mixed yeast cultures may contain more than one yeast species and should be purified before identification.

- Suspend a colony of the mixed yeast culture in sterile distilled water.
- Streak a loopful of the suspension onto an SDA plate to obtain well-isolated colonies.
- Incubate at 25°C for 48 hours.
- Examine the plate for differences in colony morphology and select well-isolated colonies for subculture. Assess the purity of the isolate by colony morphology and microscopic examination. If the culture remains mixed, repeat the isolation procedure until a pure culture is obtained.

Note: Some yeast species may exhibit both smooth and rough colony variants within a pure culture. Therefore, differences in colony morphology alone should not be interpreted as evidence of mixed culture and should be confirmed by microscopic examination and subsequent identification tests.

Isolation of yeasts from cultures contaminated with moulds

Mould contamination may interfere with the isolation and identification of yeasts. Purification can be achieved using the following methods:

i) Isolation on Yeast Malt Agar (YMA)

- Suspend a small portion of the mixed yeast-mould colony in a tube containing sterile distilled water
- Inoculate a loopful of the suspension onto a YMA plate.
- Incubate at 25°C for 4–6 days.
- Examine the culture for purity. If mould contamination persists, proceed to the next step.

ii) Isolation in Yeast Malt Broth (YMB)

- Suspend a small portion of the yeast-mould culture in 10 mL of YMB.
- Incubate the YMB at 25–30°C for 48 hours.
- Using a capillary pipette, remove a small portion of sediment carefully without disturbing the mycelial pellicle.
- Inoculate the sediment onto a YMA plate.
- Incubate at 25°C for 4–6 days.
- Assess the purity of the culture by wet mount examination. If contamination persists, proceed to shake culture as described in the next step.

iii) Isolation in Shake Culture

- Inoculate a small portion of the yeast–mould culture into approximately 100 mL of

YMB contained in an Erlenmeyer flask.

- Incubate the flask on a rotary shaker at 30°C for 4–6 days.
- Using a sterile capillary pipette, carefully remove a small amount of sediment while avoiding the uptake of mycelial aggregates.
- Inoculate the sediment onto a YMA plate.
- Incubate at 25–30°C for 4–7 days
- Examine the resulting colonies and prepare a wet mount to confirm purity.

Principle: These procedures exploit the differences in growth characteristics and sedimentation patterns of yeasts and moulds. Under broth culture and shake-culture conditions, yeast cells tend to remain suspended or settle as sediment, whereas moulds commonly form pellicles, mycelial mats, or mycelial aggregates, facilitating the selective recovery of yeast cells for subsequent identification.

4.2.2 Direct mounts

Direct mounts are prepared to examine the microscopic morphology of yeasts and to assess the purity of isolates obtained in culture. Microscopic examination provides valuable information regarding cell shape, size, budding pattern, and the presence of structures such as pseudohyphae, hyphae, arthroconidia, or blastoconidia. Lactophenol Cotton Blue (LCB) is commonly used to prepare direct mounts. The stain serves as both a mounting and staining medium, allowing clear visualisation of fungal structures. Examination of LCB mounts helps confirm the presence of a pure yeast culture and provides preliminary morphological characteristics that may assist in identification.

4.2.3 Germ tube (GT) Test (Larone *et al*., 2023)

A germ tube (GT) is a filamentous outgrowth arising from a yeast cell, typically measuring approximately half the width and three to four times the length of the parent cell. The germ tube test is a rapid screening method used for the presumptive identification of *Candida albicans*. Production of germ tubes within 2 hours of incubation in serum is considered indicative of *C. albicans*. Because certain other species, particularly *Candida dubliniensis*, may also produce germ tubes, positive results should be confirmed by additional identification methods, such as the Corn Meal Agar (CMA) test and other phenotypic or molecular methods.

Procedure

1. Ensure that the test is performed using a fresh growth from a pure culture.
2. Prepare a light suspension of the test organism in 0.5 mL of sterile serum (pooled human, rabbit, or fetal calf serum).
3. The optimal inoculum concentration is approximately 10^5–10^6 cells/mL.
4. Incubate the suspension at 37°C for exactly 2 hours.
5. After incubation, place a drop of the suspension on a clean glass slide and cover with a coverslip.
6. Examine microscopically for the presence of germ tubes. Germ tubes represent the

initiation of hyphal growth, arising directly from the yeast cell. They have parallel walls at their point of origin and are not constricted.

7. The test should be read after 2 hours of incubation. Delayed examination may result in pseudohyphal formation, leading to misinterpretation.

Interpretation:

- Positive test: Germ tubes are observed arising directly from the yeast cell without constriction at the point of origin and with parallel walls. Presence of germ tube formation indicates a positive test **(Figure 4.3)**.
- Negative test: No germ tubes are observed, or filamentous structures show constriction at the point of origin, indicating pseudohyphae rather than true germ tubes.

Notes and Precautions:

1. Germ tube formation is influenced by several factors, including the type of medium, inoculum size, incubation temperature, concentration of simple carbohydrates, pH, and microaerophilic conditions.
2. The concentration of the inoculum is critical for optimal germ tube production. Maximum germ tube formation occurs when the inoculum contains approximately 10^5–10^6 cells/mL. As the cell concentration increases beyond this range, the percentage of cells forming germ tubes decreases. A faintly turbid serum suspension is considered ideal.
3. Approximately 95% of clinical isolates of *C. albicans* produce germ tubes when incubated in serum at 37°C for 2–3 hours (Westblade et al., 2023).
4. A neutral pH (approximately 7.4) promotes optimal germ tube development. Bacterial contamination may inhibit or interfere with germ tube formation.
5. Antimicrobial agents carried over from the primary isolation medium may suppress germ tube production. In addition, certain inhibitory substances present in human serum, such as ferritin, may affect germ tube development. Older cultures may also exhibit reduced or absent germ tube formation despite belonging to germ tube-positive species.
6. False-positive germ tube reactions may occur with *C. dubliniensis* and, occasionally, with *C. tropicalis* and *Rhodotorula* spp., particularly when incubation exceeds the recommended 2-hour period. Therefore, prolonged incubation should be avoided.
7. Quality-control strains should be included in each batch of tests. A known strain of *C. albicans* should be used as a positive control, and *C. tropicalis* as a negative control.
8. Nearly 5% of *C. albicans* isolates may fail to produce germ tubes. In such cases, species identification should be confirmed using additional methods, such as CMA morphology, carbohydrate assimilation tests, or other biochemical and molecular identification techniques.



Figure 4.3: Microscopy demonstrating true germ tubes arising from yeast cells without constriction at their point of origin, consistent with a positive germ tube test (original magnification, ×400)

Differentiation between *C. dubliniensis* and *C. albicans*: Because *Candida dubliniensis* and *Candida albicans* share several phenotypic characteristics, including germ tube production and chlamydospore formation, additional tests are often required for accurate differentiation **(Table 4.2)**.

Table 4.2: Differential features of *C. dubliniensis* and *C. albicans*

Characteristics	*Candida dubliniensis*	*Candida albicans*
Colonies on CHROMagar *Candida* at 37°C	Typically dark green; colour is most distinct after 72 hours of incubation	Usually, light green or bluish green
Growth at 45°C (48 hours)	Absent or poor growth	Growth usually present (may be variable) (Gales et al., 1999)
Chlamydospores	Abundant; often arranged in pairs, chains, or small clusters	Usually single, occasionally in pairs or small clusters
Colonies on Staib agar	Rough colonies, often with a hyphal fringe	Smooth, shiny colonies
Assimilation (≤48 hours)	**Xylose**: Absent	**Xylose**: Present/ variable
	Methyl-α-D-glucoside: Absent	**Methyl-α-D-glucoside**: Present/ variable
	Trehalose: Absent	**Trehalose**: Present

4.2.4 Morphological Characterisation on Corn Meal Agar (Dalmau plate technique)

The Dalmau plate technique on Corn Meal Agar (CMA) supplemented with 1% Tween 80 is used to study the microscopic morphology of yeasts. It is particularly useful for demonstrating structures such as pseudohyphae, true hyphae, blastoconidia, chlamydospores, and other reproductive structures that aid in yeast identification.

Procedure:

1. Prepare a 90 mm CMA plate containing 1% Tween 80 and divide it into four quadrants for inoculation with different isolates.
2. Using a sterile needle or straight wire, lightly touch a yeast colony and make 2–3 parallel streaks, approximately 3.5–4 cm long and spaced 1.2 cm apart, on the agar surface.
3. Place a flame-sterilised and cooled 22 mm square coverslip over a portion of the streaks. The coverslip creates a partially anaerobic environment that promotes the development of characteristic morphological structures at its margins.
4. Incubate the plate at 25°C for 3–5 days.
5. After incubation, remove the Petri dish lid and place the plate directly on the microscope stage.
6. Examine the margins of the coverslip first at low power, then at high power.
7. Record the presence and arrangement of the following morphological structures: **(Tables 4.3 and 4.4)**
    - True hyphae
    - Pseudohyphae
    - Blastoconidia (blastospores)
    - Chlamydospores
    - Arthroconidia
    - Ascospores or basidiospores (when present)

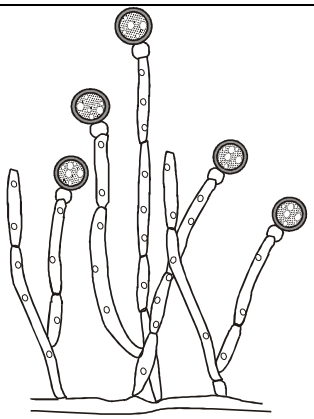
Interpretation: The morphology observed on CMA is an important characteristic for differentiating and identifying yeast species. Attention should be given to the presence, abundance, and arrangement of pseudohyphae, true hyphae, blastoconidia, and chlamydospores, as these features are valuable in distinguishing species of *Candida* and other clinically important yeasts.

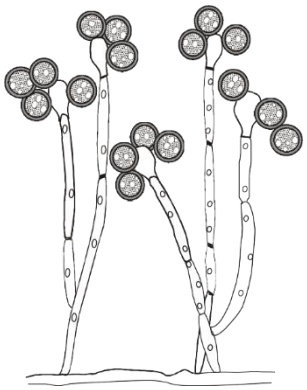
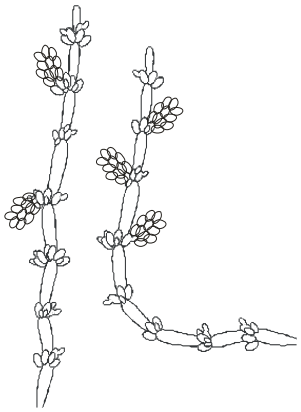
Note: Morphological observations obtained using the Dalmau plate technique should be interpreted alongside colony characteristics, physiological tests, and biochemical identification methods to ensure accurate species identification.

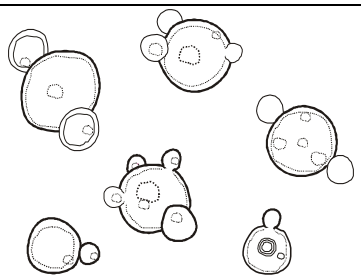
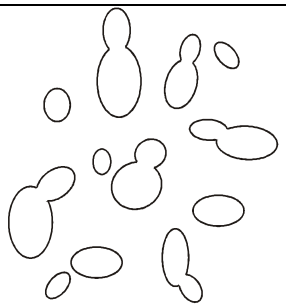
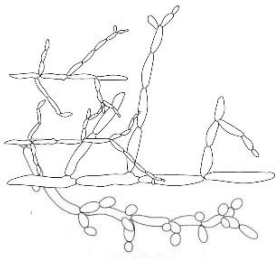
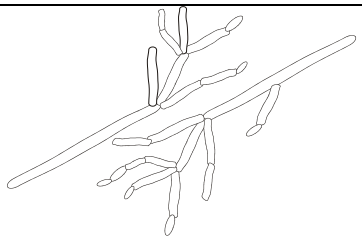
Table 4.3: Morphology of different *Candida* species on corn meal agar with Tween 80
(Adapted from de Hoog *et al*., 2020)

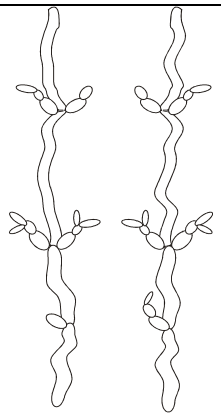
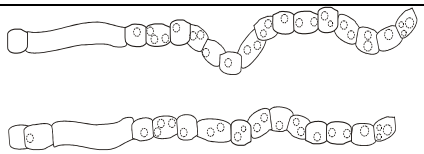
Morphology on CMA with Tween 80	Yeast
Terminal chlamydoconidia	*C. albicans,* *C. dubliniensis*
Abundant pseudohyphae, pine forest arrangement, blastoconidia formed at or in between septa.	*C. tropicalis*
Elongated yeasts, abundant pseudohyphae (matchstick-like appearance)	*C. krusei* (new name: *Pichia kudriavzevii*)
Giant hyphae, blastospores at nodes	*C. parapsilosis*
Scant pseudohyphae with chains of blastoconidia	*C. guilliermondii* (new name: *Meyerozyma guilliermondii*)
Yeasts only	*C. glabrata* (new name: *Nakaseomyces glabrata*), *C. famata* (new name: *Debaryomyces hansenii*), *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.
Abundant rectangular blastoconidia, criss-cross clusters, arrangement of logs in a stream	*C. kefyr (*new name*: Kluyveromyces marxianus)*

Table 4.4: Colony characteristics and microscopic morphologies of different Yeasts

Yeast	Colony characteristics (SDA)	Microscopic morphology (CMA)
Candida albicans	White to cream coloured, smooth, soft	

		Hyphae and pseudohyphae formed with clusters of blastospores (4–5 µm) at internodes. Terminal chlamydospores (8–12 µm) formed in most species.
Candida dubliniensis	White to cream coloured, smooth, soft	 Hyphae and pseudohyphae formed with clusters of blastospores (4–5 µm) at internodes. Terminal chlamydospores (8–12 µm) formed in clusters.
Candida guilliermondii	White to cream coloured, butyrous	 Budding cells spherical to sub-spherical to broadly ellipsoidal. Pseudomycelium may be present, but hyphae are not produced.

Candida famata	White to cream coloured, butyrous	 Budding cells are broadly ellipsoidal. 3.5–5 × 2–3.5 µm Pseudomycelium absent.
Candida glabrata	White to cream coloured, soft, glossy and smooth	 Pseudomycelium absent. Some strains may form a few branched chains of ovoidal cells.
Candida parapsilosis	Cream coloured to yellowish, glistening and soft, mostly smooth or partly or entirely wrinkled	 Fine and coarse pseudomycelium (giant forms). Blastospores (3.7 µm), single or in short chains.
Candida krusei	White to cream coloured, butyrous	 Budding cells broadly ellipsoidal to cylindrical (4–5 × 2.5 µm).

		Pseudomycelium often present, robust.
Candida lusitaniae	White to cream coloured, glistening, soft and smooth	 Budding cells ellipsoidal. Hyphae and germ tubes are absent. Pseudomycelium is often present and well developed.
Trichosporon species	Colonies restricted, white, farinose, irregularly folded at the centre, with a flat marginal zone	 Budding cells and lateral conidia are absent. Arthroconidia cylindrical.

4.2.5 Ascospore Production and Detection Test

Determination of ascospore production is an important step in the identification and classification of yeasts. Some yeasts produce ascospores readily on primary isolation media, whereas others require specialised sporulation media and prolonged incubation. Ascospore formation is generally induced under conditions of nutrient limitation.

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 agar plates containing ascospore-producing medium.
2. Incubate at 25°C under aerobic conditions.

3. Examine the culture in 3–5 days, then weekly for 3 weeks.
4. Prepare a wet mount of the yeast in distilled water.
5. Examine the wet mount using the high-power objective.
6. Observe for ascospore formation and record their surface topography, size, colour, margins and the number of ascospores per ascus **(Table 4.5).**
7. If the ascospores are not seen in a wet mount, perform a modified acid-fast stain (ascospores are acid-fast while vegetative yeast cells are not (Westblade et al., 2023).
8. *Saccharomyces cerevisiae* should be included as a positive control for production on media and staining procedure.

Table 4.5: Ascospore morphology on Malt Extract Agar (2–5-day growth)

Description of ascospores	Yeasts
1–4 hat shaped ascospores in asci	*Pichia anomala*, *Pichia norvegenesis, 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*

4.2.6 Ballistospore Formation

Forcibly discharged conidia or ballistoconidia are an important criterion for distinguishing *Sporobolomyces* from *Rhodotorula*. The presence of satellite conidia on the agar plate is the first indication of ballistoconidial formation.

Procedure

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

4.2.7 Sugar Fermentation Procedure

1. Prepare liquid fermentation medium containing peptone (1%), sodium chloride (0.5%), and Andrade's indicator (0.005%). Sterilise by autoclaving at 120°C for 15 minutes at

15 psi. Add filter-sterilised sugar to the medium at a concentration of 2%. Pour into sterile test tubes (~5 mL) and place a sterile Durham tube in each tube.

2. Plug the tubes with colour-coded cotton plugs.
3. Suspend a heavy inoculum of yeast grown on a sugar-free medium.
4. Inoculate each carbohydrate broth with approximately 0.1 mL of inoculum.
5. Incubate the tubes at 25°C up to 1 week. Examine the tubes every 48–72 hours for acid production (pink colour) and gas (in Durham's). Gas production in the tube is considered fermentation-positive, whereas only acid production may simply indicate that the carbohydrate is assimilated.

4.2.8 Sugar assimilation test (Auxanographic technique)

Sugar assimilation is performed by impregnating the sugar discs; the Pour plate auxanographic method of Wickerham and colleagues (Wickerham and Burton, 1948) is a commonly used method for carbohydrate assimilation testing.

Preparation of Yeast Nitrogen Base (YNB):

Yeast nitrogen base is prepared using the ingredients as follows:

Potassium dihydrogen orthophosphate (KH_2PO_4)	1.0 g
Magnesium sulfate ($MgSO_4$)	0.5 g
Ammonium sulfate [$(NH_4)_2SO_4$]	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, Becton Dickinson, Sparks, MD, USA)	6.7 g
Distilled water	100 mL
Sterilise by filtration and store at 4°C.	
2. Agar	20.0 g
Distilled water	980 mL

1. Dispense the agar in 18 mL quantities in 18 × 150 mm screw-capped tubes. Autoclave at 121°C and store at 4°C.
2. Prepare a yeast suspension from a 24–48-hour-old culture in 2 mL of YNB by adding heavy inoculum. 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 dish.
3. Allow the Petri dish to set at room temperature until the agar surface hardens.
4. Place the various carbohydrate-impregnated discs (sugar discs) onto the agar plate.
5. Sugar discs can be obtained commercially or can be prepared as follows:
   Punch 6 mm-diameter discs from Whatman No. 1 filter paper. Sterilise the discs by

placing them in a hot air oven for 1 hour. Alternatively, sterile discs can also be procured from the manufacturer. Add a drop of 10% filter-sterilised sugar solution to each disc. Dry the disc at 37°C and store at 4°C in an airtight container.

6. Incubate the plates at 25–30°C for 3–4 days.
7. The presence of growth around the disc is considered positive for that carbohydrate. Growth around the glucose disc is recorded first; this disc serves as a positive control (yeast viability).

4.2.9 Nitrate Assimilation Test (Auxanographic technique)

Preparation of Yeast Carbon Base:

Potassium dihydrogen orthophosphate (KH_2PO_4)	1 g
Magnesium sulfate ($MgSO_4$)	0.5 g
Glucose	20 g
Noble agar	25 g
Distilled water	1 L

Mix the reagents by boiling and autoclaving at 121°C at 15 psi for 15 minutes.

OR

Preparation of Yeast Carbon Base and Agar separately:

1. Yeast Carbon Base (Difco™) 11.7 g
   Distilled water 100 mL
   Sterilise by filtration and store at 4°C until use.
2. Agar 20 g
   Distilled water 980 mL

   1. Autoclave at 121°C at 15 psi for 15 minutes. Dispense in 18 mL quantities in 18 × 150 mm screw-capped tubes.
   2. Make a yeast suspension in molten Yeast Carbon Base agar, pour it into a 90 mm Petri dish, and allow it to cool.

OR

Prepare the suspension from a 24–48-hour culture in 2 mL of Yeast Carbon Base to a McFarland No. 1 standard. Add this suspension to 18 mL of molten agar cooled to 45°C. Mix well and pour into 90 mm Petri dishes.

1. Allow the plate to cool to room temperature.
2. Divide the plate into two halves and label them.
3. Place the potassium nitrate (KNO_3) and peptone discs on the agar surface in the corresponding labelled halves.

Potassium nitrate discs are prepared as follows:

Potassium nitrate	30 g
Distilled water	1 L

Mix the reagents and autoclave at 121°C at 15 psi for 15 minutes. Take 6 mm discs (punched from Whatman No. 1 filter paper), saturate them with the potassium nitrate solution, dry them in a sterile Petri dish, and store at 4°C until use.

The peptone disc is prepared similarly to the KNO_3 disc.

Peptone	30 g
Distilled water	1 L

Incubate the plate at 25–30°C for 7 days. Check for the growth around the disc. The test is considered valid if growth is present around the peptone. The growth around the KNO_3-impregnated disc, in the presence of growth around the peptone control disc, indicates a positive nitrate assimilation reaction.

4.2.10 Urea Hydrolysis

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

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

Rapid Urea Hydrolysis: The rapid urea hydrolysis test is done in microtitre plates and used to screen for *Cryptococcus neoformans.*

1. Freshly 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 a freshly isolated colony of yeast into the urea broth into a well.
4. Seal the plate with tape and incubate for 4 hours at 37°C.
5. Pink to red colour is a positive test.
6. Similarly, inoculate the controls, *C. neoformans* as a positive control and *C. albicans* or an uninoculated well as a negative control.

4.2.11 Temperature Studies

Another important step in identifying the yeast is determining its ability to grow at elevated temperatures. It can be used to distinguish *Cryptococcus neoformans* from other *Cryptococcus* species and *Candida dubliniensis* from *Candida albicans.*

Procedure:

1. Inoculate the culture on two tubes of Malt Extract Agar.
2. Incubate one tube at 37°C and the other at 25°C.
3. Examine the tube every day up to 4–7 days for the presence of growth.
4. Growth must be present in both tubes before concluding that the yeast could grow at 37°C.

4.2.12 Canavanine Glycine Bromothymol Blue Agar (CGB) Test

This test is used to differentiate between *C. neoformans* and *C. gattii*. The latter hydrolyses glycine to form ammonia, thereby shifting the medium towards alkalinity and changing its colour to blue, whereas the former does not. Canavanine acts as a selective agent.

Procedure:

1. Prepare CGB agar slants. **(See Annexure A for media preparation)**
2. Inoculate the surface of the slant using a minimum inoculum of *C. neoformans* or *C. gattii* with a straight wire loop.
3. Put a positive control (*C. gattii*) and a negative control (*C. neoformans*) simultaneously.
4. Incubate at 25°C for 1–5 days.
5. After 5 days, a positive result is indicated by a colour change from greenish yellow (pH 5.8) to cobalt blue (pH 7.0).

4.2.13 Phenol oxidase test

The test is performed using Bird seed agar (BSA), Staib's medium or Niger seed agar. The agar contains caffeic acid extracted from *Guizotia abyssinica* (Niger) seeds. *C. neoformans* produces the enzyme phenol oxidase (laccase), which oxidises the caffeic acid into melanin, producing distinct brown-black colonies within 3 to 5 days (up to 1 week). This medium is used as a selective and differential culture medium for the presumptive identification of *C. neoformans*.

Procedure:

1. Prepare the agar. **(See Annexure A for media preparation)**
2. Streak the yeast/*C. neoformans* culture on the medium.
3. Incubate the medium at 25–30°C and observe every day for up to 1 week.
4. *C. neoformans* produces dark brown or black colonies within 3–5 days of inoculation.

Note: Identification of medically important yeasts is based on a combination of morphological, physiological, and biochemical characteristics, as summarised in **Table 4.9** at the end of the chapter.

4.2.14 Chromogenic Media for *Candida* Identification (Bernal *et al*., 1996)

Chromogenic media are specialised agar-based media designed for the detection and preliminary identification of *Candida* species. These media contain chromogenic substrates that are broken down by species-specific enzymes, such as:

- β-N-acetylhexosaminidase (commonly present in *Candida* species).
- A secondary enzyme, like β-glucosidase or β-phosphatase (depending on the specific medium). The enzymatic reactions produce distinctive colony colours, enabling visual differentiation between species.

CHROMagar *Candida*: a widely used chromogenic medium specifically designed for identifying *Candida* species. It is highly effective in differentiating species based on the colours of colonies formed on the agar **(Table 4.6).**

Table 4.6: Colony colours of different *Candida* species on CHROMagar (Bernal *et al*., 1996)

***Candida* species**	**Colony Colour on CHROMagar**
Candida albicans	Green
Candida tropicalis	Blue to purple
Candida krusei	Pink with a rough texture
Candida glabrata	Pale pink to white
Candida parapsilosis	Cream to pale pink
Candida dubliniensis	Dark green
Candida auris (as per CDC)	Pink to red

4.2.15 Selective Auris Medium (SAM) for *Candida* (*Candidozyma*) *auris* Identification (Das *et al*., 2021)

Purpose: SAM is a cost-effective, in-house-preparable selective medium for the rapid isolation and presumptive identification of *C. auris* from clinical yeast isolates and yeast-positive blood culture broths, particularly useful in resource-limited settings where MALDI-TOF MS and molecular methods are unavailable (Das *et al*., 2021).

Principle: *C. auris* demonstrates a unique and clinically exploitable combination of stress tolerances that distinguishes it from virtually all other medically important yeasts, namely, the simultaneous ability to grow under high osmotic stress (12.5% NaCl), metal stress (9 mM

ferrous sulfate), and elevated temperature (42°C) (Das *et al*., 2021). The selective medium exploits this dual physiological stress tolerance: while most non-*C. auris* yeasts can individually tolerate high salt or metal stress, the combination substantially inhibits the growth of most non-*C. auris* yeasts. The molecular basis of this tolerance is thought to relate to higher expression of the *Hog1* stress-response pathway gene in *C. auris* compared with other *Candida* species (Das *et al*., 2021). Growth on SAM at 42°C within 48–72 h constitutes a presumptive identification of *C. auris*, which should be confirmed by MALDI-TOF MS or molecular sequencing.

Media components and preparation

Base medium:

- Yeast extract-peptone-dextrose (YPD) broth - prepared per manufacturer's directions
- Agar powder (ultra-pure) - 2.5% (w/v) (added to YPD broth before autoclaving)
- Sodium chloride - 12.5% (w/v) (added directly to the medium before autoclaving)

Selective agent (added after autoclaving):

- Ferrous sulfate ($FeSO_4$) — 9 mM final concentration

Preparation procedure:

1. Prepare YPD broth per the manufacturer's instructions; add agar powder (2.5%) and sodium chloride (12.5%).
2. Autoclave at 121°C for 15 minutes.
3. Prepare a 1 M ferrous sulfate stock solution freshly, within 10–15 minutes of use (to prevent aerial oxidation and precipitation). Sterilise by syringe filtration through a 0.2 µm membrane filter.
4. Allow the autoclaved medium to cool to approximately 80°C.
5. Add ferrous sulfate stock solution to achieve a final concentration of 9 mM (i.e., 9 mL of 1 M stock per litre of medium). Mix thoroughly by gentle swirling; do not vortex.
6. Pour into sterile 90 mm Petri dishes to a depth of 5–6 mm and allow to solidify at room temperature.
7. Sterility check: incubate two plates from each batch at 25°C and 37°C for 2 days before use.
8. Shelf life: 2–4 days at 4°C. Prepare fresh batches before use owing to the gradual oxidation and precipitation of iron on storage.

Inoculation and incubation:

1. Touch a single isolated colony with a sterile inoculation loop and streak directly onto SAM, using minimal inoculum (the primary streak area should not be visible to the naked eye).
2. For yeast-positive blood culture broths: inoculate one loopful of broth directly onto SAM.

3. Incubate at 42°C for 48 hours (read at 48 hours; if negative, re-examine at 72 hours before reporting negative).

Interpretation

- Presumptive positive**:** Moderate to heavy growth within 48 h; confirm by MALDI-TOF MS or molecular sequencing
- Delayed positive**-** Growth after 48–72 hours
- Negative- No growth after 72 hours

4.2.16 Commercial Yeast Identification System (Pincus *et al*., 2007; Morovati *et al*., 2023)

Principle:

Commercially available identification systems are described in the chart below. Among these, the most widely used systems are the API 20C Yeast Identification System (API Analytab Products, Plainview, NY) and the BioMérieux VITEK System (Hazelwood, MO). The API 20C system requires less reagent preparation. The VITEK system is automated **(Table 4.7).** Both systems are based on modifications to auxanographic assimilation techniques. When the organism can assimilate a particular carbohydrate (in a capsule of reconstituted API substrates or in wells containing the VITEK substrate), a colour change is observed. Both systems require morphological studies and the germ tube test to obtain a more complete profile for yeast identification.

Table 4.7: Commercial yeast identification kits categorised by diagnostic scope (Group I: Single species; Group II: Multiple species; Group III: Multiple genera)

Group I (for single species)	
Albicans – Sure (*C. albicans*)	Germ tube (*C. albicans*)
Albicans ID (*C. albicans*)	Rapidec *albicans* (*C. albicans*)
Albistrip (*C. albicans*)	*C. albicans* screen (Murex CA 50) (*C. albicans*)
Bacticard *Candida* (*C. albicans*)	Bichrolate krusei (*C. krusei*)
Fluoroplate (*C. albicans*)	Caffeic acid disk (*C. neoformans*)
Group II (for several species)	
API 20C AUX system	BBL Minitek Yeast Set
CandiSelect	*Candida* Check
CHROMagar *Candida*	Fungiscreen H
Group III (for multiple genera)	
AMS – Yeast biochemical Card	Microbial Identification System (MID)
Microscan Rapid Yeast ID	Quantum II

RapID Yeast Plus System	

Specimen: A pure culture of yeast cells 24–48 hours old.

Reagents and materials required but not supplied with the kit.

a. For the API 20C yeast identification system:

1. Sterile wooden applicator stick
2. Sterile Pasteur pipettes
3. Squeeze bottle
4. Incubator (30°C)
5. Water bath
6. Sabouraud Dextrose Agar plates.

b. For the VITEK Yeast identification system:

1. Sterile wooden applicator stick
2. Sterile tubes containing 1.8 mL
3. Colourimeter (VITEK colourimeter)
4. Incubator (30°C)
5. SDA plates

Procedure:

a. API 20C yeast identification system:

1. Melt the basal broth medium in the ampoules by keeping them in the autoclave or by boiling in a 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, then place the sample in the incubation tray.
4. Open the ampoule as per the manufacturer's instructions and inoculate the molten medium using an applicator stick. Adjust the density to < 1+ on the Wickerham card.
5. Inoculate 0.2 mL yeast suspension into 20 capsules by using a Pasteur pipette and place the lid on the tray.
6. Incubate the tray at 28–30°C for 72 hours. Read the results after 24, 48 and 72 hours.

b. VITEK Yeast Identification System:

1. Prepare yeast suspension in tubes containing 1.8 mL of saline. Adjust the density to the McFarland No.2 standard using the VITEK colourimeter.
2. Label the yeast cards and place them 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 the sealer module and incubate at 30°C for 24 hours or 48 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 one to three different yeast identifications for an individual isolate.
4. It takes 3 days to obtain 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.
3. Results are usually obtained after 24 hours, and a few isolates may require additional incubation.

4.2.17 Identification of *Candida auris* (CDC, 2026)

C. auris can be misidentified as several different organisms when using traditional phenotypic methods for yeast identification. **Table 4.8** summarises common misidentifications by identification method.

Table 4.8: Common misidentifications of *C. auris* noted with commercial yeast identification systems

Identification methods	Organism *C. auris* misidentified as
VITEK® 2 YST#	*Candida haemulonii* *Candida duobushaemulonii*
API 20C	*Rhodotorula glutinis* (characteristic red colour not present) *Candida sake*
API ID 32C	*Candida intermedia* *Candida sake* *Saccharomyces kluyveri*
BD Phoenix yeast identification system	*Candida haemulonii* *Candida catenulata*
MicroScan	*Candida famata#* *Candida guilliermondii** *Candida lusitaniae#** *Candida parapsilosis**
RapID Yeast Plus	*Candida parapsilosis**

There have been reports of *C. auris* being misidentified as *Candida lusitaniae* and *Candida famata* on VITEK® 2. A confirmatory test, such as cornmeal agar, may be warranted for these species.

* On cornmeal agar, *C. guilliermondii*, *C. lusitaniae*, and *C. parapsilosis* generally make pseudohyphae, and *C. auris* generally does not make hyphae or pseudohyphae. If hyphae or pseudohyphae are not present on cornmeal agar, any *C. guilliermondii, C. lusitaniae*, and *C. parapsilosis* isolates identified on MicroScan or any *C. parapsilosis* isolates identified on RapID Yeast Plus should be submitted for further identification.

Diagnostic devices based on Matrix-assisted Laser Desorption/Ionisation Time-of-Flight Mass Spectrometry (MALDI-TOF MS) can differentiate *C. auris* from other *Candida* species. However, not all reference databases used in MALDI-TOF devices enable detection. Accurate identification of *C. auris* can be performed using:

- Bruker Biotyper brand MALDI-TOF MS using the updated Bruker FDA-approved MALDI Biotyper sirus/smart platform under MBT Compass software, using the current IVD reference library together with the separately licensed MBT Filamentous Fungi library.
- BioMérieux VITEK® (MALDI-TOF) MS uses the current IVD Knowledge Base (and, for moulds, the RUO/SARAMIS or Mycology database as applicable).

Table 4.9 The biochemical properties of medically important yeast (*-variable, F-fermented, W-weak)

Species	Growth at 37°C	Pellicle in broth	Pseudo-/true hyphae	Chlamydospores	Germ tubes	Capsule, India ink	Assimilation of:												Fermentation of:						Urease	KNO_3 Utilisation	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*	-	-	-	-	-	-	-	-	-
C. dubliniensis	+	-	+	+	+	-	+	+	+	-	+	-	-	-	+*	-	+	-	F	F	-	-	F	F	-	-	-	-
C. famata	+	-	-	-	-	-	+	+	+	+*	+	+	+	-	+	+	+	+*	W	-	W	-	-	W	-	-	-	-*
C. glabrata	+	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	+	-	F	-	-	-	-	F	-	-	-	-
C. guilliermondii	+	-	+	-	-	-	+	+	+	-	+	+	+	-	+	+	+	+	F	-	F	-	F*	F	-	-	-	-*
C. kefyr	+	-	+	-	-	-	+	-	+	+	+	-	+*	-	+*	+	-	-	F	-	F	F*	F	-	-	-	-	-
C. krusei	+	+	+	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	F	-	-	-	-	-	+*	-	-	-*
C. lambica	+*	+	+	-	-	-	+	-	-	-	-	-	-	-	+	-	-	-	F	-	-	-	-	-	-	-	-	-*
C. lipolytica	+	+	+	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-*
C. lusitaniae	+	-	+	-	-	-	+	+	+	-	+	-	+	-	+	-	+	-	F	-	F	-	F	F	-	-	-	-*
C. parapsilosis	+	-	+	-	-	-	+	+	+	-	+	-	-	-	+	-	+	-	F	-	-	-	-	-	-	-	-	-
W. anomalus	W	-*	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	F	F*	F	-	F*	-	-	-	-	+
C. rugosa	+	-	+	-	-	-	+	-	-	-	+	-	-	-	+*	-	-	-	-	-	-	-	-	-	-	-	-	-
C. tropicaliss	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	+	+	-	-	-	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	+	-	-	-

Chapter 5: Mould Identification (Conventional Methods)

The general scheme for the conventional identification of moulds is depicted in **Figure 5.1**. Culture media commonly used to study the morphology of moulds include Sabouraud Dextrose Agar (SDA), Malt Extract Agar (MEA), Potato Dextrose Agar (PDA), Potato Carrot Agar (PCA), Oatmeal Agar (OMA) and Czapek–Dox Agar (CzA). Gross examination of colonies on these media provides valuable information on growth rate, colony colour, texture, topography, production of diffusible pigments and exudates, and the characteristics of aerial and submerged hyphae (Barnett *et al*., 1998). Macroscopic fungal structures, including ascocarps, pycnidia, sclerotia, sporodochia, and synnemata, are best examined using a dissecting (stereo) microscope, which allows detailed assessment of their morphology and arrangement (de Hoog *et al*., 1995).

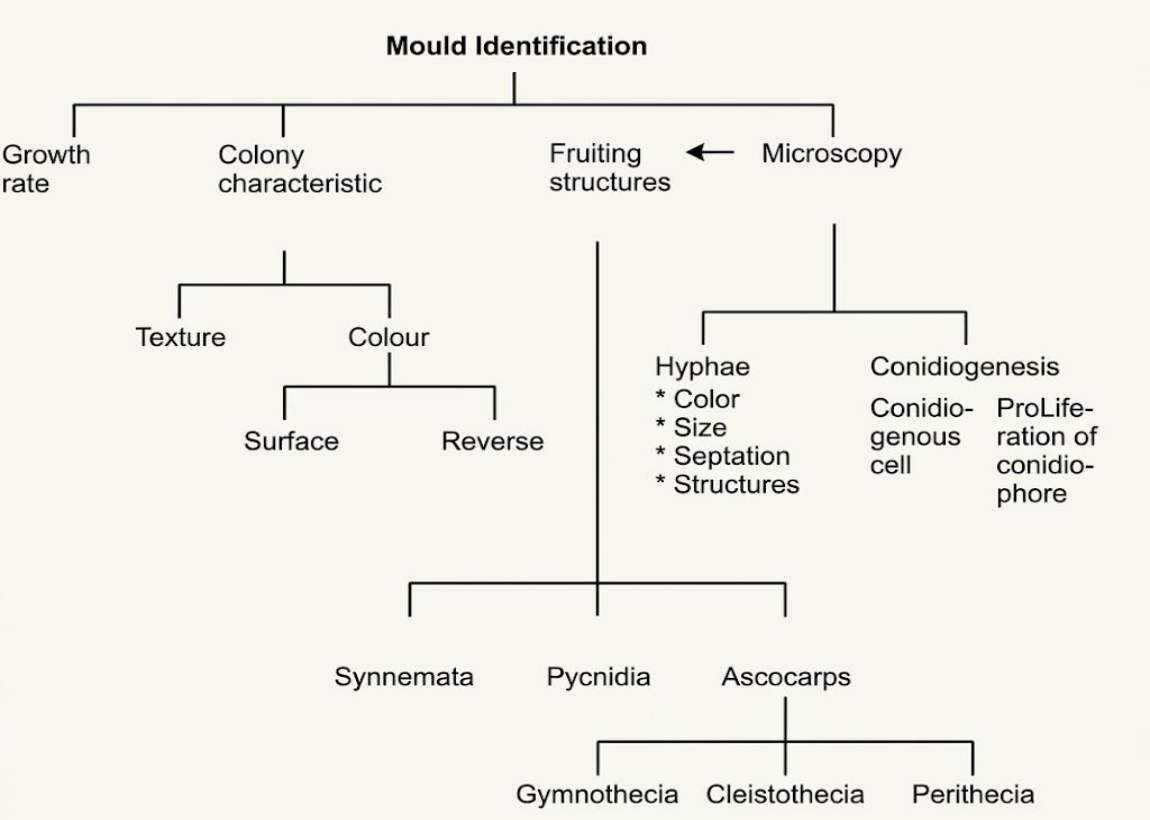


Figure 5.1: Mould Identification Scheme

5.1 Techniques Used for Mould Identification

5.1.1 Tease Mount

The tease mount is a method for mounting fungi for microscopic examination and is the most commonly used technique in mycology laboratories. However, interpretation may be difficult, as conidia and spores are frequently dislodged from the conidiogenous cells during preparation, disrupting their characteristic arrangement (Koneman *et al*., 2006).

Procedure

Standard Tease Mount (Figure 5.2)

1. Place a small drop of lactophenol cotton blue (mounting medium) on a clean, grease-free microscope slide.
2. Remove a small portion of growth, aseptically, midway between the colony centre and edge using an L wire. Place the removed colony on a drop of lactophenol cotton blue (LCB) on a slide.
3. Gently tease the fungal fragment apart with the dissecting needles to produce a thin, even spread.
4. Carefully lower a coverslip onto the edge of the drop, allowing the mounting fluid to spread evenly beneath it and avoiding the entrapment of air bubbles.
5. Remove any excess lactophenol cotton blue by blotting gently with filter/blotting paper.
6. For preserving the mount, seal the edges of the coverslip with nail varnish.

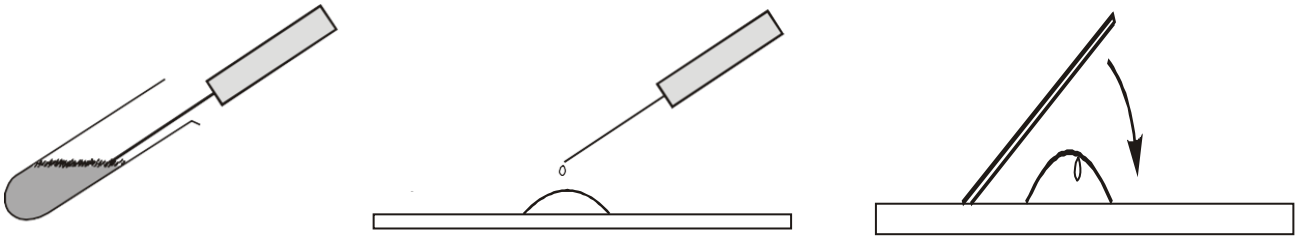


Figure 5.2: Standard Tease Mount

5.1.2 Tape Technique (Figure 5.3)

1. Place a drop of mounting fluid on the slide.
2. Take a 2-cm piece of transparent adhesive tape. Hold one end of the tape with forceps, then lightly touch the adhesive surface on the fungal colony to collect the fungal structure.
3. Place the tape with the fungal material facing downward onto the mounting medium on the slide. Detach the tape from the forceps.
4. Examine the preparation microscopically, first at low power and then at higher magnification as required (Koneman *et al*., 2006).

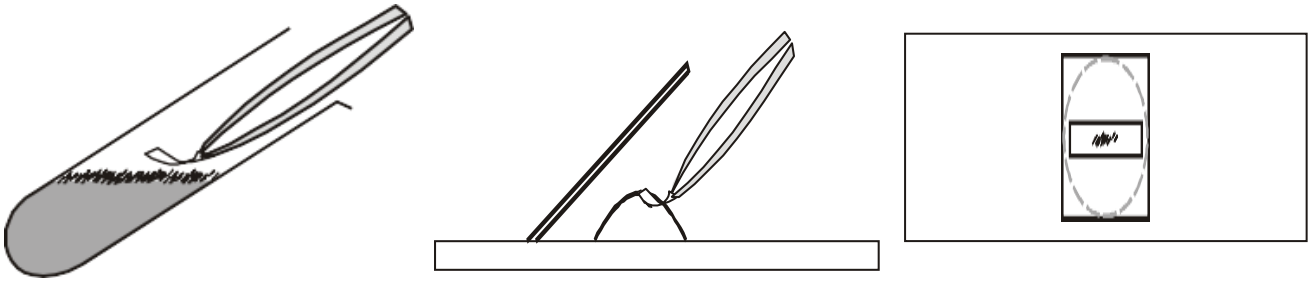


Figure 5.3: Tape Technique

5.1.3 Mounting Mucorales and Fruiting Structures

Sporangia, ascocarps, and other fruiting bodies trap air bubbles during slide preparation, making slides unsatisfactory for microscopic study. To minimise air entrapment, the following technique is recommended:

1. Place a drop of 95% ethanol, ethyl acetate or water with 0.05% Tween 80 on a clean slide. These wetting agents prevent the formation of air bubbles around fruiting structures.
2. Transfer the fungal culture containing fruiting bodies to the drop of wetting agent. Add a drop of the mounting fluid and examine as in the standard technique (Koneman et al., 2006).

5.2 Confirmation of Dimorphic Fungi

- Dimorphic fungi exhibit distinct morphological forms at different temperatures and nutritional conditions. Many thermally dimorphic fungi grow predominantly in a mould form at approximately 25°C and develop a tissue-associated yeast or spherule phase at approximately 37°C, although the precise morphological response varies among species.
- *Blastomyces dermatitidis, Histoplasma capsulatum, Paracoccidioides brasiliensis, Talaromyces marneffei* and *Sporothrix schenckii* complex grow as yeast in tissue and at 37°C. *Coccidioides immitis* produces spherules and endospores at 37°C in tissue and when grown on special medium.

The conversion from the mould phase to the tissue phase is influenced by several factors, including temperature, culture medium composition, and carbon dioxide (CO_2) concentration. In *P. brasiliensis* and *H. capsulatum,* nutrition seems extremely important, whereas elevated CO_2 concentrations enhance the development of the yeast form in *S. schenckii* and the spherule form in *C. immitis*.

5.2.1 Mould to Yeast form conversion of dimorphic fungi: precautions and quality control

- Perform all procedures within the biological safety cabinet.
- Do not prepare slide cultures for dimorphic fungi.
- For quality control, inoculate a known culture simultaneously under identical conditions to verify successful mould-to-tissue phase conversion.

- Conversion from the mould to the tissue (yeast or spherule) phase can be induced using the enriched media and incubation conditions listed in **Table 5.1**, which also indicates the look-alike genera that may cause diagnostic confusion.

Table 5.1: Mould to yeast form conversion of dimorphic fungi (Adapted from Larone *et al*., 2023

Fungus	Media and conditions	Similar-looking fungi at 25°C
Blastomyces dermatitidis	Kelley's agar, 37°C; KT medium	*Chrysosporium* spp.
Coccidioides immitis	Modified converse medium, 40°C, 5 to 10% CO_2	*Malbranchea* spp., *Uncinocarpus* spp., *Auxarthron* spp., *Arachniotus* spp.
H. capsulatum	BHIA with glutamine, 37°C, Pine's Agar Medium	*Sepedonium* spp.
P. brasiliensis	BHIA, 37°C	*Chrysosporium* spp.
Sporothrix spp.	BHIA, 37°C, 5–10% CO_2	*Acrodontium* spp.
T. marneffei	5% sheep BA, BHIA, 37°C	*Penicillium* spp.

5.3 Cycloheximide Resistance

The following fungi exhibit resistance to cycloheximide and can grow in its presence when incubated at 30°C.

1. *B. dermatitidis*
2. *S. schenckii*
3. *C. immitis*
4. *H. capsulatum*
5. *P. brasiliensis*
6. Most of the dermatophytes

The following fungi are inhibited by cycloheximide:

1. *Lichtheimia* spp.
2. Mucorales (*Rhizopus*)
3. *Scedosporium* spp.
4. Some strains of *Candida* spp. and *Aspergillus* spp.
5. *Cryptococcus* spp.

5.4 Slide Culture Technique

When moulds are difficult to identify from tease mounts, the slide culture technique may be employed. It allows the fungal structures to be examined in situ, with the conidia and their arrangement preserved and undisturbed. Relatively nutrient-poor media, such as cornmeal agar (CMA) and Potato Dextrose Agar (PDA), are preferred, as they promote sporulation (Koneman *et al*., 2006).

5.4.1 Prepare the 'Setup'

1. In a 100 mm glass Petri dish, place a filter paper, a V-shaped glass rod, a microscope slide and a coverslip.
2. Autoclave the entire assembly at 121°C and 103 kPa (15 psi) for 15 minutes
3. The sterilised assembly may be stored in a Petri dish canister for up to 2 weeks until required.

Procedure

1. Aseptically cut 1-cm square agar blocks from a CMA or PDA agar plate.
2. Transfer the agar block onto the slide within the assembly.
3. Using a sterile inoculating loop, place a small amount of the colony onto each of the four sides (vertical faces) 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; a humid atmosphere prevents the agar block from drying out.
6. Addition of 10% glycerine to sterile water helps prevent condensation on the slide.
7. Place the slide culture in a Petri dish and incubate at room temperature (or 30°C) in the dark.
8. Slide culture is ready to be taken down when mature conidia or spores are observed **(Figure 5.4)**.

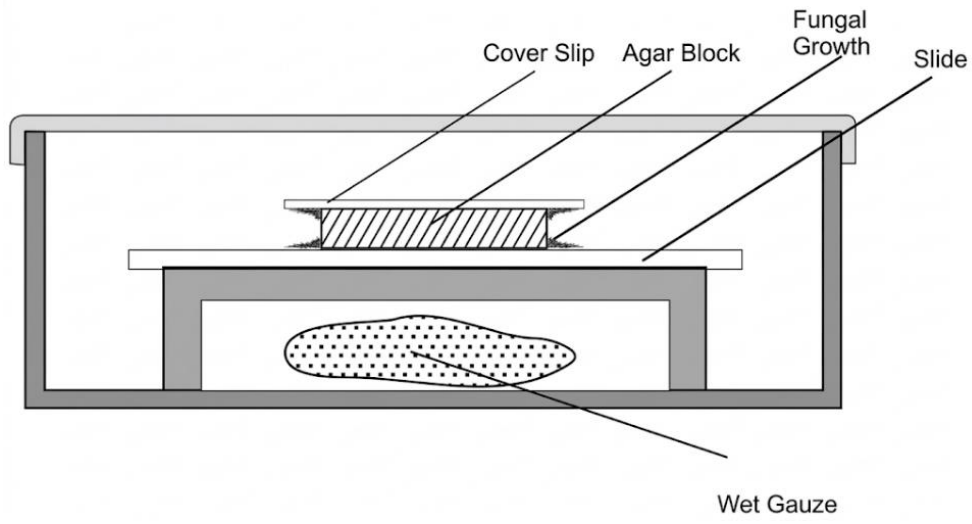


Figure 5.4: Slide culture technique

5.4.2 Taking down the Slide Culture

1. Take a small drop of mounting medium (LCB) on a microscope slide.
2. With the forceps, carefully remove the coverslip from the agar block. Do not push or pull the coverslip.
3. Pass the coverslip through the blue portion of the flame very quickly. This will heat-fix the fungus and its spores. Do not overheat, as it may collapse the hyphae.
4. Carefully place the coverslip on the mounting medium, avoiding any air entrapment.
5. Wipe off the excess mounting medium. Seal the edges of the coverslip with nail polish.
6. The second mount can also be prepared from the microscope slide of the slide culture assembly by removing the agar block. Put a drop of lactophenol on a coverslip. Seal the coverslip with nail varnish.
7. A 95% alcohol or ethyl acetate drop may be used to soak the colony before using lactophenol blue to avoid air bubbles.
8. After removing the coverslip, aseptically transfer the agar block onto a fresh, suitable culture medium to preserve the fungal isolate for future use.

5.5 Temperature Tolerance

Thermotolerance is a useful characteristic that can aid in identifying several medically important moulds. **Tables 5.2** and **5.3** show upper growth limits and key techniques used for identification of selected medically important fungi (de Hoog *et al*., 2020).

Table 5.2: Thermotolerance of some Medically Important Fungi (adapted from de Hoog *et al*., 2020, Larone *et al*., 2023**)**

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

Table 5.3: Techniques used for the identification of a few fungi (adapted from de Hoog *et al*., 2020, Larone *et al*., 2023**)**

Suspected mould	Technique Utilised/Recommended
A. fumigatus	Temperature tolerance, 48°C
B. dermatitidis	Exoantigen, mould to yeast conversion, Gen probe technique

Basidiobolus spp.	Forcibly discharged primary conidia
Cladophialophora carrionii	Temperature tolerance, 35°C
Conidiobolus spp.	Forcibly discharged primary conidia
H. capsulatum	Exoantigen, mould to yeast conversion, DNA probe-based technique
P. brasiliensis	Mould-yeast conversion
Rhizomucor pusillus	Temperature tolerance, 55°C
Sepedonium spp.	Morphology and negative exoantigen for *Histoplasma*
Sporobolomyces spp.	Forcibly discharged primary conidia
Exophiala dermatitidis	Temperature tolerance, 40°C
Cladophialophora bantiana	Temperature tolerance, 42°C

5.6 Identification of Mucorales

Mucorales are a group of filamentous fungi that generally produce broad coenocytic (non-septate) or rarely septate hyphae, which branch irregularly and have a 10–25 µm diameter. Sexual reproduction is characterised by the formation of thick-walled zygospores, while asexual reproduction typically occurs through a sac-like structure, the sporangium, which contains the sporangiospores. Historically grouped together as the "zygomycetes," two orders are of interest in medical mycology: Entomophthorales, in which the spores are forcibly discharged; and the Mucorales, in which the sporangiospores arise by cleavage of the sporangial cytoplasm and are passively liberated. Under the current classification, these two orders are placed in separate phyla: Mucorales in Mucoromycota and the Entomophthorales in Zoopagomycota **(**Larone *et al*., 2023)**.**

General Features

1. Cultures show rapid growth with a woolly texture. Colour may be white to grey to black depending on the species and culture age.
2. Some Mucorales have vegetative hyphae in the form of horizontal runners, called stolons. A stolon that touches the substrate is called a node, while the segment between two adjacent nodes is called an internode.
3. Rhizoids develop at the node, while sporangiophores might arise at the node or the internode. The relationship between the stolons, rhizoids, and sporangiophores is an important morphological characteristic used to differentiate genera within the order Mucorales.
4. Asexual structure: sporangium may have its sporangiospores in a row termed merosporangium or a reduced sporangium with few sporangiospores called sporangiola.
5. Columella is the sterile dome-shaped area at the apex of the sporangiophore. Apophysis is the swelling in the sporangiophore which merges with the columella. Collarettes are

the remnants of the sporangial wall remaining attached to the junction of the columella and the sporangiophore after the rupture of the sporangium **(Figure 5.5)**.

6. The identification key for common medically important Mucorales is given in **Figure 5.6**.

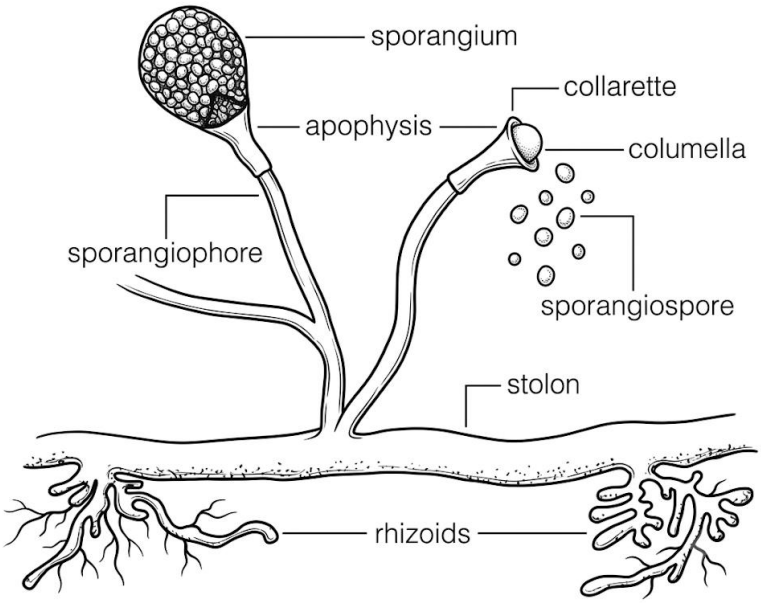


Figure 5.5: General morphological features of Mucorales (de Hoog *et al*., 2016)

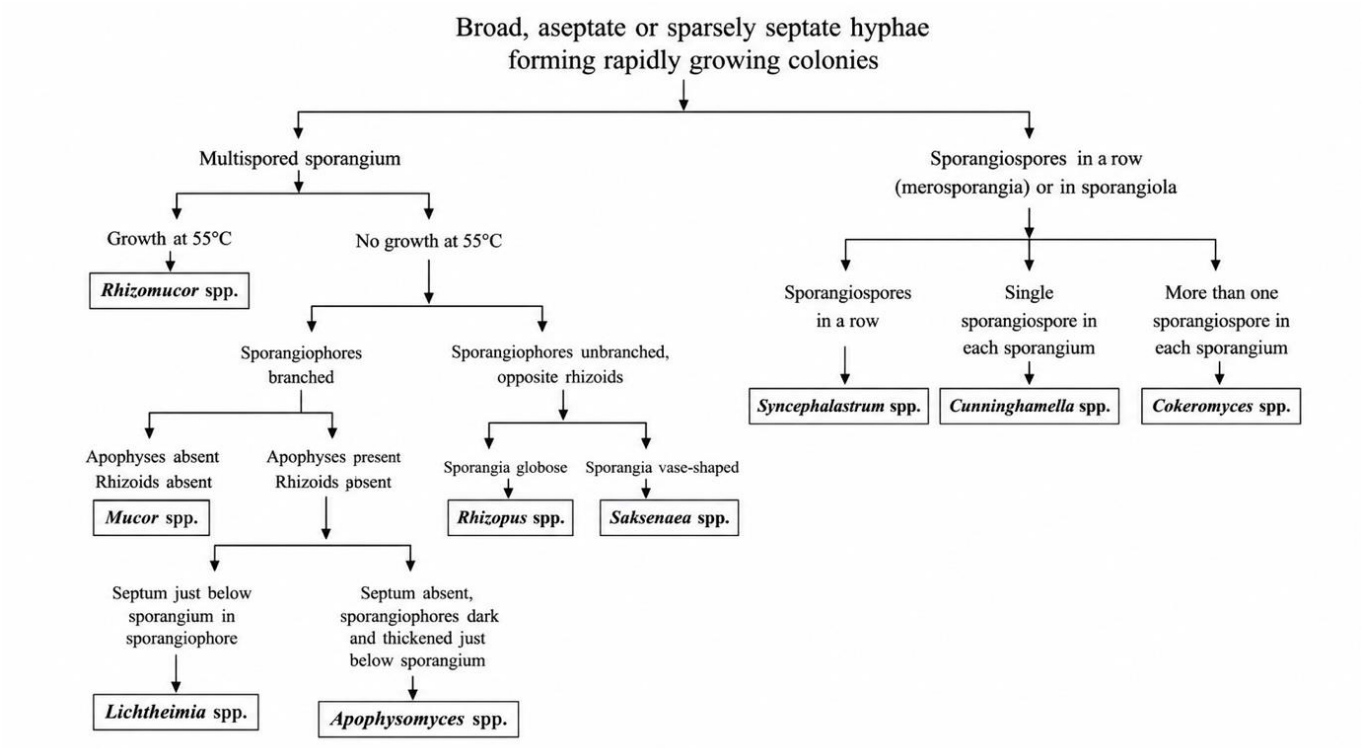


Figure 5.6: Key to identify medically important Mucorales (Compiled by Dr Arvind A. Padhye from de Hoog *et al*., 2020, Larone *et al*., 2023)

5.7 Macroscopic and Microscopic Identification of Medically Important Moulds (de Hoog *et al*., 2020)

The medically important moulds have been divided into Mucormycetes, Hyaline hyphomycetes, Dematiaceous moulds and Thermally Dimorphic fungi. Their growth characteristics on various media (SDA, MEA, PDA, PCA, OMA, and CzA) at 25 or 37°C and the microscopic findings observed on Lactophenol Cotton Blue (LCB) mounts are described below in **Table 5.4**. Representative images are shown in **Annexure B**.

Table 5.4: Macroscopic and Microscopic Identification of Medically Important Moulds

Organism	Colony Characteristics	Microscopic Characteristics
Mucormycetes		
Rhizopus arrhizus (formerly *Rhizopus oryzae*)	Grown on SDA, 30°C**:** Rapidly growing, woolly, tall colonies; whitish to greyish brown in colour	Hyaline stolon (15–25 µm thick) is slightly brown at the nodes where rhizoids and sporangiophores are produced. Sporangiophores are 12 µm high and 18 µm wide, brown, unbranched, and arranged singly or in tufts. Sporangia (50–250 µm) are spherical, brownish-grey to black; the columella comprises 50–70% of the sporangium, ellipsoidal; the apophysis is short. Rhizoids are sparingly branched, brownish sporangiospores (6–8 × 4.5–5 µm), rhomboidal or lemon-shaped with longitudinal striae. Chlamydospores are single or in chains, spherical to ovoidal and smooth-walled.
Rhizopus microsporus (formerly *R. microsporus var. rhizopodiformis)*	Grown on MEA, 30°C**:** Expanding, dark greyish brown colonies	Sporangiophores are 500 µm high and 8 µm wide, produced singly or in groups of four; macro- and micro-sporangiophores can be distinguished. Sporangia (100 µm) are spherical, bluish or greyish black; the columella is pyriform, up to 80% of the sporangium. Sporangiospores (6 µm) are finely spinulose.
Rhizopus homothallicus	Grown on SDA, 30°C**:** Rapidly growing, low, cottony, greyish to greyish-brown colonies. Markedly	Broad, aseptate (coenocytic) ribbon-like hyphae bearing stolons and rhizoids; the rhizoids are poorly developed and sparingly branched. Sporangiophores are short, brownish and poorly developed, produced in tufts of two to five opposite the rhizoids; asexual sporulation is often sparse or absent on routine media. When formed, sporangia are globose, greyish-black and apophysate, with a hemispherical columella

	thermotolerant, grows at 46–48°C	that collapses to an umbrella-like form after spore release; sporangiospores are subglobose to ovoid, one-celled and small. The key distinguishing feature is that it produces abundant zygospores in a single (unmated) culture. The zygospores are globose, golden-brown, spiny/ornamented, and supported by unequal (uneven-sized) opposed suspensor cells.
Mucor racemosus	Grown on MEA, 30°C**:** Expanding, pale, greyish-brown colonies	Hyaline sporangiophores, up to 20 mm high, 14–17 µm wide, sympodially and monopodially branched. Sporangia brownish, up to 90 µm in diameter; columella subspherical to pyriform, often with truncate bases, light brown, with collars. Smooth-walled sporangiophores, spherical to broadly ellipsoidal, 8–10 µm diameter.
Mucor indicus	Grown on MEA, 30°C**:** Expanding, deep yellow, aromatic colonies	Sporulation is strongly influenced by light. Sporangiophores hyaline to yellowish, up to 10 mm high and 14 µm wide, repeatedly sympodially branched, with long branches. Sporangia yellow to brown, up to about 75 × 40–50 µm, with diffluent, transparent walls; columellae subspherical to applanate, rarely elongated, up to 56 × 53 µm. Sporangiospores are smooth-walled, subspherical to ellipsoidal, 5.4–5.7 × 4.4 µm. Chlamydospores abundant in light. Heterothallic. Suspensors unequal. Zygospores spherical to slightly compressed, up to 100 µm in diameter, with stellate spines, black.
Mucor irregularis (formerly *Rhizomucor variabilis*)	Grown on MEA, 30°C**:** expanding, lanose to hairy, whitish to ochraceous, with buff-ochre reverse	Sporangiophores arising from hyphae or from stolons; rhizoids abundant. Sporangiophores hyaline, up to 2 mm long, 9–23 µm wide, simple or once branched, with branches terminating at a higher level than the main stems; branches all ending in a sporangium. Sporangia (sub)spherical, up to 100 µm diameter; membrane diffluent; columella spherical, ellipsoidal to cylindrical, about 40 µm wide, sometimes lobed, with or without apophysis. Sporangiospores hyaline, smooth-walled, very variable, mostly subspherical to ellipsoidal, 3–11 × 2–7 µm. Chlamydospores abundant, heterothallic.
Rhizomucor pusillus	Grown on MEA, 30°C**:** Rapidly growing dark brown colonies. The fungus grows up to 55°C	Sporangiophores (11–15 µm wide), brownish, intensively monopodially branched, apical branches terminating with a sporangium, sporangiophores anchored to the substrate by short rhizoids. Sporangia (up to 100 µm) spherical, diffluent membrane;

		columella spherical to pyriform without apophysis. Sporangiospores (3–4 µm) are hyaline and smooth-walled.
Lichtheimia corymbifera **(**formerly *Absidia corymbifera***)**	Grown on SDA, 30°C**:** Floccose, light olive grey colony; mycelium profusely branched, with stolons and rhizoids	Sporangiophores are solitary or in groups and branch repeatedly to form corymbs. Sporangia (100–150 µm) spherical to pyriform; columella hemispherical to ovoidal, with 1–2 nipple-like projections and a long conical apophysis. Sporangiospores are smooth and yellowish to greenish. Sporulation induced on water agar.
Apophysomyces variabilis	Grown on SDA, 30°C**:** Rapidly growing, white to pale yellow in colour. The fungus grows up to 42°C	Sporangiophores (300–540 µm) are generally single, unbranched, tapering toward the apex; topped by pyriform sporangia with funnel or bell-shaped apophysis (10–46 × 11–40 µm). Columella hemispherical. Sporangiospores (5.4–8 × 4.5–7 µm) cylindrical, smooth-walled. Sporulation induced on water agar and Dichloran Rose Bengal Chloramphenicol (DRBC) agar.
Cunninghamella bertholletiae	Grown on SDA, 30°C**:** Rapidly growing greyish floccose colony	Sporangiophores erect, with a whorl of short lateral branches at the apical region, each branch ending in a swollen vesicle (15–40 µm). The vesicles are covered with spine-like denticles, each supporting a round to oval sporangiolum (5–8 × 6–14 µm; each sporangiolum yields one spore). Sporangiospores (7–11 µm) oval to tear-shaped.
Saksenaea vasiformis	Grown on OMA, 30°C**:** Expanding grey. Sporulation needs to be induced using water agar	Sporangiophore (900 × 6–9 µm), single, unbranched, with rhizoids dichotomously branched. Sporangia (50 × 20 µm) are single, terminal, flask-shaped, multispored; the columella is hemispherical. Sporangiospores (3–4 × 1.5–2 µm) are smooth and elongated.
Cokeromyces recurvatus	Grown on SDA, 30°C**:** Slow to moderate growth, light grey	Sporangiophores (100–500 × 9 µm) are unbranched without rhizoids; they produce vesicles at the apical end of the sporangiophore. Sporangioles (8–11 µm) with few spores, with a columella, and without an apophysis are borne at the tips of long, recurved and twisted stalks that arise from the terminal vesicle of sporangiophores. Sporangiospores (2.5 × 4.5 µm) are smooth-walled, spherical to ovoid. At 35–37°C in 5–7% CO_2, thin-walled, round yeast cells (15–90 µm in diameter) develop, with

		single or multiple budding that may superficially resemble the ship's-wheel arrangement characteristic of *Paracoccidioides brasiliensis.*
Syncephalastrum racemosum	Grown on MEA, 30°C**:** expanding, greyish, abundant aerial mycelium	Sporangiophores (10–25 µm wide) arise from rhizoids, irregularly branched, each branch bearing a terminal vesicle (80 µm), merosporangia over the entire surface of the terminal vesicle. Merosporangia (33 × 4 µm) are cylindrical and contain 3–18 spores in a single row. Merospores (3–7 µm) are smooth-walled, spherical to ovoidal.
Entomophthorales		
Conidiobolus coronatus	Grown on PDA, 30°C: Colonies expanding, hyaline, with radial blooms	Hyphae 6–15 µm wide, Conidiophores 60–90 µm tapering at the tip. Primary conidia (40 µm diameter), prominent papillate base, hair-like appendages (villous conidia). Conidia are forcibly discharged.
Basidiobolus ranarum	Grown on SDA, 30°C: Colonies yellowish to grey, glabrous and waxy, with many radial folds. The ability to grow at 37°C is variable, but all grow at 30°C	Zygospores within ten days are globose, with smooth to undulate thick walls, and a prominent beak at one side of the zygospore. The apical portion of the conidiophore enlarges, and the globose conidium is blown out from the tip of the swelling, carrying with it the top of the vesicle as a residual attachment. The subsporangial swelling or vesicle then emits a stream of fluid that propels the spore.
Hyaline Hyphomycetes		
Aspergillus flavus	Grown on SDA, 30°C: Rapidly growing, velvety, yellowish-green, zonate colonies	Conidiophore (2–2.5 × 10–20 µm), fine to coarsely rough. Vesicles are elliptical or subglobose; sterigmata cover either the entire or three-fourths 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 (3.5 µm) echinulate, (sub)spherical. Sclerotia may be present.
Aspergillus fumigatus	Grown on SDA, 30°C: The surface is velvety or powdery, with various	Conidiophores are smooth, relatively short (usually 300 µm long), and 5–10 µm in diameter. The phialides are uniseriate, close together (compact), forming only on the upper two-thirds of the vesicle, parallel to the axis of the conidiophore (known as

	shades of green when young, with a narrow white border; the colony develops a darker grey colour with age	columnar formation). Conidia are round, smooth, or slightly rough and 2–3.5 µm in diameter.
Aspergillus niger	Grown on SDA, 30°C**:** Colonies grow rapidly, appear velvety or slightly flocculent and sporulate heavily; colonies have a black or brownish-black appearance	Conidiophores (1.5–3.0 × 15–20 µm) have a thick, smooth wall. A vesicle is typically globose, producing sterigmata on its entire surface. The sterigmata develop in a double series. Conidia (3.5–5.0 µm) are brown, ornamented with warts and ridges.
Aspergillus terreus	Grown on SDA, 30°C**:** Colonies are velvety with a brown cinnamon or tan colour	Conidiophores (100–250 µm × 4.5–6 µm) are smooth and hyaline, producing dome-shaped or hemispherical vesicles. Vesicles bear sterigmata on the upper half of their surface. The sterigmata are doubled. Conidia (1.8 × 2.4 µm) are smooth-walled, spherical to broadly ellipsoidal.
Aspergillus nidulans (sexual morph formerly named *Emericella nidulans*)	Grown on CzA, 30°C**:** Rapidly growing, green, cream-buff or honey yellow. Reverse dark purplish	Conidial heads short, columnar, up to 80 µm long. Conidiophore stipes brownish, 60–130 × 2.5–3.0 µm. Vesicles hemispherical, 8–10 µm diameter. Conidiogenous cells biseriate, 5–9 × 2–3 µm. Metulae 5–6 × 2.3 µm. Conidia spherical, rugulose, subhyaline, green in mass, 3–4 µm diameter. Ascomata spherical, purple, 100–200 µm diameter, surrounded by a yellowish to cinnamon layer of scattered hyphae bearing a dense aggregation of pale yellow, thick-walled, spherical to subspherical Hülle cells. Asci 8-spored, spherical to subspherical, 7–12 µm diameter. Ascospores purple-red, smooth-walled, with two equatorial crests, lenticular, 3.8–4.5 × 3.5–4.0 µm (excluding the crests).
Aspergillus candidus	Grown on CzA, 30°C**:** growing slowly, white to pale yellow colonies	Conidial heads radiate, white. Conidiophore stipes 200–500 µm in length, smooth-walled to finely roughened, hyaline. Vesicles spherical to subspherical, 10–40 µm diameter. Conidiogenous cells are biseriate in larger heads, occasionally uniseriate in smaller heads. Metulae cover the entire surface of the vesicle. Conidia spherical to

		subspherical, 2.5–4.0 µm diameter, smooth-walled, hyaline. Sclerotia, when present, are reddish-purple to black.
Aspergillus clavatus	Grown on CzA, 30°C**:** growing rapidly, bluish-green, consisting of a dense felt of conidiophores	Conidial heads radiate, later splitting into several columns. Conidiophores 2–4 mm in length; stipes smooth-walled, hyaline. Vesicles clavate, 40–60 µm diameter. Conidiogenous cells uniseriate. Conidia smooth-walled, pale green, ellipsoidal, 7–8 × 2–3 µm.
Aspergillus versicolor	Grown on CzA, 30°C**:** Yellow, orange-yellow to yellow-green or pale pink, often poorly sporulating colonies	Conidial heads are radiate. Conidiophores are smooth-walled and hyaline. Vesicles are subspherical to ellipsoidal, 12–16 µm in diameter. Conidiogenous cells are biseriate, with phialides longer than or as long as the metulae. Conidia echinulate, spherical, 2–3 µm in diameter. Hülle cells may be present in some isolates.
Fusarium solani	Grown on OMA, 30°C**:** Colonies are white to cream-coloured, reverse green to bluish-brown. Sporodochia present	Conidiophores arise laterally from hyphae. Monophialides with a distinct collarette. Macroconidia on shorter, branched conidiophores, which soon form sporodochia, moderately curved with short, blunt apical and indistinctly pedicellate basal cells, mostly 3-septate. Microconidia are usually abundant and are produced on elongate conidiophores. Chlamydospores are frequent, smooth or rough-walled, terminal or intercalary.
Fusarium oxysporum	Grown on OMA, 30°C**:** Colonies are white, usually becoming purple; orange sporodochia in some strains; reverse hyaline to dark blue or dark purple	Conidiophores are short, single, lateral monophialides present and arranged in densely branched clusters. Macroconidia fusiform, slightly curved, pointed at the tip, 3–5-septate, basal cells pedicellate. Microconidia abundant, never in chains, mostly non-septate, ellipsoidal to cylindrical. Chlamydospores terminal to intercalary.
Fusarium incarnatum	Grown on OMA, 30°C**:** Rapidly growing colonies; aerial mycelium floccose, at first whitish, later becoming	Conidiophores scattered in the aerial mycelium, loosely branched; polyblastic conidiogenous cells abundant. Sporodochial macroconidia slightly curved, with a foot-cell, 3–7-septate, 20–46 × 3.0–5.5 µm. Conidia on aerial conidiophores (blastoconidia) are usually borne singly on scattered denticles, fusiform to falcate,

	avellaneous to buff-brown; reverse pale, becoming peach-coloured	mostly 3–5-septate, 7.5–35 × 2.5–4.0 µm. Microconidia sparse or absent. Chlamydospores are sparse, spherical, becoming brown, intercalary, single or in chains.
Fusarium lichenicola (formerly *Cylindrocarpon lichenicola*)	Grown on OMA, 30°C**:** Colonies are growing rapidly, purplish-red to pale brown, velvety to floccose; reverse dark brown	Conidiophores are rather long, simple or poorly branched. Phialides formed terminally on lateral branches, which are forked once or twice, and taper towards the apex. Conidia straight, sometimes slightly curved, hyaline, smooth-walled, cylindrical to fusiform with rounded tip and distinctly truncate base, usually 3-septate but occasionally up to 5-septate, 18–40 × 5–7 µm; microconidia absent. Chlamydospores abundant, hyaline to brown, spherical, 6–9 µm diameter, mostly terminally on short lateral branches but occasionally intercalary, single, in chains or in compact clusters.
Purpureocillium lilacinum (formerly *Paecilomyces lilacinus*)	Grown on MEA, 30°C**:** Colonies floccose, vinaceous to violet	Conidiophores (400–600 × 3–4 µm) arising from hyphae, form tufts, yellow to purple, rough-walled, with densely clustered phialides, consisting of a swollen basal part that tapers into a thin neck. Conidia ellipsoidal to fusiform, in divergent chains.
Sarocladium kiliense (formerly *Acremonium kiliense*)	Grown on MEA, 30°C**:** Colonies are smooth, greyish-orange or ochraceous; reverse brown	Hyphae are narrow and fragile. Phialides are thin-walled, cylindrical and slightly tapering or intercalary. Conidia (3–6 × 1.5 µm) are ellipsoidal to short cylindrical. Unicellular chlamydoconidia present.
Schizophyllum commune	Grown on OMA, 30°C**:** Colonies are woolly, pale greyish brown, forming macroscopically visible fruit bodies (kidney-shaped) in concentric zones	Hyphae are hyaline and of diverse structure, often with clamp connections. Conidia absent. Basidia are arranged in dense *palisades* on the lower sides of fruit bodies, each bearing 4 basidiospores on erect sterigmata. Basidiospores (6–7 × 2–3 µm) are smooth, thin-walled, and elongated with lateral scars.
Lomentospora prolificans (formerly	Grown on OMA, 30°C**:** Colonies are moist, flat, olivaceous-grey to blackish	Conidiogenous cells locally aggregated in small brushes, flask-shaped, often bearing a long, inconspicuously annellated zone. Inflated conidia may arise alongside hyphae. Conidia (3–7 × 2.5 µm) are smooth, thick-walled, and olivaceous brown.

Scedosporium prolificans)		
Scopulariopsis brevicaulis (sexual morph formerly named *Microascus brevicaulis*)	Grown on MEA, 30°C: Colonies whitish, powdery to felty, soon becoming pinkish brown; reverse cream coloured to brownish	Conidiogenous cells are single or in small brush-like groups on undifferentiated hyphae; conidiogenous cells are cylindrical with a slightly swollen base and an equally wide annellated zone of variable length. Conidia (5–8 × 5–7 µm) are spherical to obovoidal, rough-walled with a truncated base.
Microascus cirrosus	Grown on PCA, 30°C**:** Colony restricted, brownish-grey	Ascomata spherical with a relatively long cylindrical or tapering beak, bearing a whorl of apical hairs. Asci obovoid to spherical, 8-spored. Ascospores reniform, straw-coloured, 5–6 × 3.4 µm with an inconspicuous germ pore.
Scedosporium apiospermum	Grown on PDA, 30°C**:** Expanding, cottony, occasionally lanose, brownish at the centre, with a whitish, lobate or irregular and fimbriate margin	*Scedosporium* synanamorph: conidiogenous cells percurrent, lateral or terminal, subhyaline, smooth-walled, usually cylindrical, producing subhyaline, obovoidal or ellipsoidal, 5–14 × 3–5 um, smooth-walled conidia. Graphium synanamorph: erect, dark synnemata with dark olivaceous black stalks, producing subhyaline, cylindrical or claviform conidia, 5–13 × 2–4 um, with a truncate base, from percurrently elongating conidiogenous cells. Conidia are also borne directly on undifferentiated hyphae, abundant, sessile or on short protrusions, brown, usually spherical or subspherical, 5–7 × 4–6 um, thick-walled.
Scedosporium boydii (formerly *Pseudallescheria boydii*)	Grown on SDA, 30°C**:** Floccose colonies that are dark grey to brownish grey	Brown cleistothecia (100–200 µm) towards the periphery of the culture; spherical, light brown to black. Asci are ovate to subglobose and contain 8 ascospores. Ascospores (6–7 × 4–4.5 µm) are lemon-shaped, smooth-walled, pale yellow to golden brown, with 2 germ pores.
Dematiaceous moulds		
Alternaria alternata	Grown on SDA, 30°C**:** colonies olivish black	Conidiophores (50 × 3–6 µm) mostly unbranched, smooth, with one or a few conidial scars. Conidia (20–63 × 9–18 µm) are ellipsoidal with a short cylindrical beak, rugose with muriform septation, single scar at the tip, produced in long chains.

Aureobasidium pullulans	Grown on MEA, 30°C**:** colonies rapidly growing, smooth, covered with slimy exudate, cream coloured or pink, later becoming brown or black	Vegetative hyphae (3–12 µm), hyaline, locally converting into blackish-brown chlamydospores; expanding hyphae with irregularly dichotomous branching. Conidiogenous cell undifferentiated, intercalary in hyaline hyphae. Conidia produced synchronously in groups from small denticles; variable in shape and size, 1-celled. Budding is frequently observed. Endoconidia present in intercalary cell.
Bipolaris australiensis	Grown on PDA, 30°C**:** Initially white, soon becoming grey to black with a black reverse. Rapid growth. Texture is woolly to cottony.	Hyphae are septate and dark. Conidiophores may be up to 150 µm in length, are sympodial, geniculate, simple or branched, bearing conidia through pores or openings (poroconidia). Conidia have 2 to 5 transverse distosepta or pseudosepta (septa that do not extend to the cell wall, with cells enclosed within sacs) and 3 to 6 cells. They measure approximately 14–40 × 6–11 µm. A flattened hilum or point of attachment is seen on the basal cell. Conidia germinate from both poles (bipolar).
Chaetomium globosum	Grown on PCA, 30°C**:** Colony growing rapidly with pale or olivaceous mycelium and often with yellow, greyish green, or green to red exudates	Conidia absent. Ascomata spherical, ovoidal or obovoidal, 175–280 µm in diameter. Peridium brown, composed of *textura intricata*. Ascomal hairs numerous, usually unbranched, flexuose, undulate or coiled, septate, brownish, up to 500 µm long. Asci clavate, 30–40 × 11–16 µm, 8-spored. Ascospores limoniform in face view, bilaterally flattened, usually brownish, 9–12 × 8–10 × 6–8 µm, with an apical germ pore.
Achaetomium strumarium (formerly *Chaetomium strumarium*)	Grown on SDA, 30°C**:** Colonies are white to cream, slightly raised in the centre, and cottony, with the reverse developing an amber, purplish, or reddish-brown pigment after 14 days	Ascocarps (100–250 µm), subglobose, pale brown and ostiolate with a cell wall consisting of interwoven hyphae or irregularly shaped cells. Ascocarps bear many thin-walled, hypha-like appendages (setae 200 µm × 3–4 µm). Setae are straight or flexuose, verruculose, septate, pale brown and lightly thickened at the base. Asci cylindrical, 8-spored. Ascospores are initially hyaline and become dark brown at maturity. They have a single apical pore.
Curvularia lunata (sexual morph formerly	Grown on PCA, 30°C**:** colonies expanding, black,	Conidiophore unbranched, erect, flexuose in the apical part with dark brown scars. Conidia (21–31 × 8.5–12 µm) smooth-walled, olivaceous brown with polar end cell;

named *Cochliobolus lunatus*)	hairy, stromata occasionally present.	conidia 3-septate, the terminal cell swollen and distinctly larger than the remaining cells; conidia obovoidal to broadly clavate.
Curvularia spicifera (formerly *Bipolaris spicifera*, earlier *Drechslera spicifera*)	Grown on PCA, 30°C**:** colonies expanding, appearing glassy with sooty powder of conidia or hairy if poor sporulation	Conidiophore (250 × 4–8 µm) unbranched, regularly zig-zagged in the apical part with dark brown scars on the edges. Conidia (20–40 × 9–14 µm) brown, smooth, cylindrical with rounded ends, protruding, truncate dark hilum, small hyaline area just above the hilum, consistently with three distosepta (pseudosepta).
Cladophialophora carrionii (formerly *Cladosporium carrionii*)	Grown on PCA, 30°C**:** colonies are slow-growing, dry, velvety, and olivaceous black	Conidia formed in densely branched acropetal chains, which contain up to 6 conidia; chains on branched or unbranched denticles. Conidia olivaceous brown, smooth-walled, lemon-shaped, narrow towards both ends.
Cladophialophora bantiana (formerly *Cladosporium bantianum*)	Grown on PCA, 30°C**:** Moderately expanding, velvety to hairy, powdery, when sporulating abundantly, olivaceous green; reverse olivaceous black	Conidia formed in long, strongly coherent, poorly branched, sessile, lateral or terminal chains on undifferentiated hyphae, pale olivaceous, ellipsoidal to fusiform or nearly cylindrical, 6–11 × 2.5–5 µm. Large chlamydospores occasionally present.
Colletotrichum gloeosporioides (sexual morph formerly named *Glomerella cingulata*)	Grown on OMA, 30°C**:** Colonies extremely variable, effuse, grey to brown, with pinkish patches; reverse dark brown with vinaceous stains	Stromata are generally absent in culture. Conidia borne on elongated phialides in acervular conidiomata, or, in early stages of development, on solitary fertile hyphae and appressoria. Conidia straight, cylindrical, obtuse at the apex, 9–24 × 3.0–4.5 µm. Appressoria 6–20 × 4–12 µm, clavate or irregular. Ascomata may be present.
Colletotrichum truncatum (formerly *Colletotrichum capsici*)	Grown on OMA, 30°C**:** Colonies are moderately expanding (2 mm/day), flat, without aerial mycelium,	Stromata formed from brown hyphae. Setae hyaline to pale brown, 80–150 µm long. Conidia borne on elongated phialides in acervular conidiomata. Conidia smooth-walled to verruculose, sickle-shaped, 20–24 × 3.5–4.5 µm. Appressoria brown, ellipsoidal and somewhat lobed, 6.5–13.0 × 5.5–7.5 µm.

	buff, covered with olivaceous grey to iron-grey acervuli; reverse buff to pale olivaceous-grey	
Exophiala jeanselmei (formerly *Phialophora jeanselmei*)	Grown on SDA, 30°C**:** Colonies restricted, initially moist, soon become velvety, with olivaceous green aerial mycelium	Yeast-like growth, when present, consists of subspherical cells, each with an annellidic but producing conidia, often cohering into long chains and changing over into hyphae. Conidiogenous cells on hyphae are intercalary or rocket-shaped. With inconspicuous annelated zones, the latter are slightly tapering, nearly smooth and produce narrow ellipsoidal conidia.
Exophiala spinifera (formerly *Phialophora spinifera*)	Grown on SDA, 30°C**:** colonies restricted, olivaceous black, with slimy centre due to yeast-like growth	Yeast cells are abundant. Conidiophores erect, cylindrical, multicelled with apical and intercalary, narrow, cylindrical annelated zones up to several millimetres long. Conidia obovoidal**.**
Exophiala dermatitidis (formerly *Wangiella dermatitidis*)	Grown on SDA, 30°C**:** Colonies are smooth and waxy, often with brown pigment exuded into the agar	Yeast cells with abundant conidiogenous cells intercalary or free, flask-shaped; annelated zones relatively wide, often in small groups, producing broadly ellipsoidal conidia. Spherical phialides with large, fragile collarettes may be present; phialoconidia are small, ellipsoidal to subcylindrical.
Exserohilum rostratum (formerly *Drechslera rostrata*)	Grown on PCA, 30°C**:** Colonies spreading, brown	Conidiophores up to 230 µm long, 5–8 um wide, erect to flexuose, often geniculate, olivaceous brown, pale towards the apex. Conidia straight or slightly curved, ellipsoidal to fusiform or rostrate, smooth-walled to finely roughened, brown to olivaceous brown, 30–128 × 9–23 µm, with 4–14 distosepta, with dark bands at both ends.
Fonsecaea pedrosoi (formerly *Hormodendrum pedrosoi*)	Grown on OMA, 30°C**:** Colonies, lanose to velvety, olivaceous, reverse olivaceous black	Conidiogenous cells pale olivaceous, cylindrical, intercalary or terminal, with clusters of prominent denticles. Conidia (3.5–5 × 1.5–2 µm) in short chains, smooth, clavate. Locally ampulliform, dark olivaceous-brown; phialides with deep, funnel-shaped collarettes may be present.

Neoscytalidium dimidiatum (formerly *Scytalidium dimidiatum*)	Grown on SDA, 30°C: White fluffy mycelial growth turns to dark grey to blackish brown within 14–21 days	Septate, branched, dematiaceous and subhyaline hyphae with a variable width ranging from 2–6 µm. The narrow and broad hyphae, both pigmented and non-pigmented, fragmented to form thick-walled cylindrical, one- or two-celled arthospores not separated by disjuncture cells.
Phaeoacremonium krajdenii	Grown on MEA, 30°C**:** Flat, mostly felty, greyish-brown, in reverse dark brown	Aerial structures: Mycelium consisting of branched, septate hyphae that occur singly or in bundles of up to 8; hyphae strongly tuberculate with warts, verrucose, dark to medium brown and 2–3 µm wide. Conidiophores are short and usually unbranched, occasionally constricted at the basal septum. The bases of older, percurrently rejuvenating cells are often inflated, up to 5-septate, and often bear, besides the terminal phialide, a second one at the apical septum. Phialides terminal or lateral, often polyphialidic, sparsely tuberculate to verruculose, rarely smooth-walled, pale brown to hyaline; collarettes slightly flaring, 1–3 × 1–2 µm. Conidia subhyaline to hyaline, oblong-ellipsoidal or allantoid. On surface or submerged in the agar: phialides hyaline, cylindrical. Conidia hyaline, oblong-ellipsoidal to allantoid, some cylindrical, 4–12 × 1–2 µm.
Phaeoacremonium rubrigenum	Grown on MEA, 30°C**:** Flat, felty, greyish ruby to greyish rose towards the edge, in reverse dark ruby to greyish rose towards the edge.	Aerial structures: Mycelium consisting of branched, septate hyphae that occur singly or in bundles of up to 5; hyphae tuberculate with warts up to 1 µm diameter, verruculose, orange to pale brown and 1.5–3.0 µm wide. Conidiophores mostly short and usually unbranched, 0–4-septate, often bearing 2 lateral phialides next to the terminal phialide. Percurrent rejuvenation occurs occasionally, and each newly proliferated segment is swollen at the base. Phialides terminal or lateral, mostly monophialidic, verruculose, sparsely tuberculate, rarely smooth, pale brown to hyaline; collarettes present. Conidia oblong-ellipsoidal, obovoid, or occasionally reniform to allantoid. On the surface or submerged in the agar, phialides are hyaline and cylindrical. Conidia hyaline, allantoid, some oblong-ellipsoidal, 4–7 × 1–1.5 µm.

Phialophora verrucosa	Grown on SDA, 30°C**:** Colonies are slow-growing, brownish grey to nearly black	Phialides (3–4 µm × 4–7 µm) formed laterally or on the tip of hyphae; flask- or vase-shaped with flared mouth. Appear singly or occasionally in clusters with or without stalks. Conidia (2.5 × 3 µm) sub-hyaline, thin-walled, broadly ellipsoidal
***Phoma* spp.**	Grown on OMA, 30°C**:** Slow-growing, pigmented, greenish-grey to dark brown, with reddish pigments noted in some species	Colonies with dark pycnidia which are spherical, solitary or aggregated, usually each with a single ostiole, sometimes multi-ostiolate. Conidiogenous cells phialidic, hyaline, lining the inner wall of the pycnidium, producing abundant conidia which are extruded in slimy masses. Conidia hyaline to pale coloured, small, mostly unicellular, ellipsoidal, cylindrical, fusiform, pyriform or spherical. Chlamydospores with muriform septation, resembling the conidia of *Alternaria*, are produced in some species. *Phoma* is a classical genus of coelomycetes with small pycnidial fruit bodies**)**. *Phoma* spp. has discrete conidiogenous cells lining the pycnidial cavity, whereas conidiogenous cells of *Pleurophoma* species are part of multicellular filaments.
Rhinocladiella mackenziei (formerly *Ramichloridium mackenziei*, earlier *R. obovoideum*)	Grown on OMA, 30°C**:** 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 prominent, 1 µm wide basal scar.
Verruconis gallopava (formerly *Ochroconis gallopava*, earlier *Dactylaria gallopava*)	Grown on OMA, 30°C: Colourless, smooth to felty, dry flat, tobacco brown to brownish; dark brown pigment is exuded into agar	Hyphae are brown with thick walls. Conidiophores are flexible, cylindrical to acicular, bearing a few conidia at the tip. Denticles are fragile, frills remain on the denticle and the conidial base. Conidia 2-celled, smooth-walled to verruculose, subhyaline, clavate, constricted at septum (2.5–4.5 × 11–18µm). Apical cell wider than the basal cell.
Dactylaria constricta *(*formerly *Ochroconis constricta)*	Grown on OMA, 30°C: Colonies grow slowly, velvety brown with rust brown reverse	Hyphae pale olivaceous, smooth; aerial hyphae strongly flexose. Conidiophores arise laterally on hyphae, poorly differentiated, ovoidal to cylindrical, usually non-septate, and bear 1–6 conidia on long, open denticles. Conidia (5–12 × 25–40 µm) are 2-celled, broadly ellipsoidal, constricted at the septum, and have rounded ends.

Thermally Dimorphic fungi		
Histoplasma capsulatum	Grown on SDA, 25°C**:** colonies are granular to cottony initially white, later becoming brownish. Reverse initially cream-coloured, becoming brownish with age. On Brain Heart Infusion (BHI) agar supplemented with 5% blood, smooth, yeast-like colonies are noted on incubation at 37°C	Micro- and macroconidia are produced. Microconidia (sub)hyaline, sessile or arising on short stalks from undifferentiated hyphae, hyaline, smooth- and thin-walled, 1-celled, pyriform to clavate, 1–4 × 2–6 µm. Chlamydospore-like macroconidia brown, arising from short conidiophores, thick-walled, tuberculate or with cylindrical projections, 1-celled, spherical, 8–14 µm diameter. At 37°C on BHI, a yeast phase with cells up to 7 µm in diameter forms. Gymnothecia are yellowish to tan, initially spherical, and later stellate due to continuing growth of spirally twisted appendages. Peridial hyphae are smooth and thin-walled, branched, composed of cells with parallel walls, with spirally twisted appendages originating from the centre of the ascoma, having firm walls. Asci sub-spherical, evanescent. Ascospores subspherical, 1.2–1.5 µm diameter. Heterothallic.
Talaromyces marneffei (formerly *Penicillium marneffei*)	Grown on SDA, 25°C**:** colony grows rapidly, greyish floccose colonies with soluble pigment that is brownish red to wine coloured. At 37°C, soft white to tan, dry, yeast-like colonies (has prominent central septum) with smooth to convoluted surface	Hyphae in part spirally twisted. Conidiophores creeping or fasciculate, 70–150 × 2.5–3.0 µm; penicilli generally biverticillate but also irregularly monoverticillate or more complex. Metulae 7–11 µm long, in whorls of 3–5. Phialides in whorls of 4–7, ampulliform to acerose, 6–10 × 2.5–3.0 µm. Conidia smooth-walled, ellipsoidal, 2.5–4.0 × 2–3 µm, often with prominent scars, borne in short, disordered chains. An arthroconidial form ('yeast phase') is produced at 37°C.
Sporothrix globosa	Grown on MEA, 25°C: Colonies are moist and have wrinkled or folded surfaces. Colony initially white, later	At 25°C, conidiophores arise at right angles from the sides of the hyphae. Conidiophores are slender and taper from a 1–2 µm diameter at the base to 0.5 µm. The tip of the conidiophore expands to form a small vesicle with sympodially arranged denticles. Each denticle bears conidia, resulting in a flower-like

	brown or nearly black; reverse grey to brownish-black. Colony loses colour with repeated subculturing	arrangement. Often, hyaline or brown conidia arise alongside the hyphae. At 37°C, multilaterally budding yeast forms develop.
Blastomyces dermatitidis	Grown on SDA, 25°C**:** Colonies are whitish, fluffy, reverse cream coloured to brownish. On BHI at 37°C, yeast colonies are butyrous, off-white to cream-coloured	Conidia (2–10 µm) are produced on lateral, morphologically non-specialised conidiophores or on the hyphal tips. Conidia are hyaline, spherical to oval, or sometimes dumbbell-shaped with smooth walls. The yeast cells (8–15 µm, rarely up to 30 µm) are hyaline, smooth and thick-walled with a broad base. 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 a small cluster.
Coccidioides immitis	Grown on SDA, 25°C**:** Fast-growing colonies with variable texture and pigments. Texture may vary from powdery, cottony, velvety, to granular. Isolates may be grey, buff, yellow or brown. The reverse side may vary from orange to brown	Fertile hyphae at 25°C usually arise at right angles. Arthroconidia are hyaline, 1-celled, short cylindrical to barrel-shaped, smooth-walled, moderately thick-walled, 3–8 × 3.5–4.5 µm, alternating with empty disjunctor cells; conidia, after liberation, have frill-like remains of the adjacent cells at both ends. At 37°C in vitro on BHI agar, the yeast phase is absent.
Emergomyces pasteurianus (formerly *Emmonsia pasteuriana*)	Grown on SDA, 25°C**:** Moderate growth, white, woolly to powdery with smooth sectors; reverse brownish. On BHI agar at 37°C, growth is butyrous	Conidia at 25°C on slender stalks alongside hyphae or in groups on inflated cells, subhyaline, slightly verruculose, thin-walled, 1-celled, spherical, 2–3 × 3–4 µm. Additional sessile, broad-based, verrucose conidia 6–7 × 4.0–4.5 µm are present. At 37°C on BHI, yeast cells are ellipsoidal, 2–4 µm in length, budding at narrow bases, intermingled with larger yeast cells budding at broad bases.

Chapter 6: Identification of Dermatophytes

Dermatophytes are keratinophilic fungi capable of invading the keratinised tissues of skin, hair, and nails. Under the current phylogenetic classification, they are grouped into seven genera: *Trichophyton*, *Epidermophyton*, *Microsporum*, *Nannizzia*, *Paraphyton*, *Lophophyton*, and *Arthroderma* (de Hoog *et al*., 2020. Differentiation between these genera is based primarily on colony characteristics on Sabouraud Dextrose Agar (SDA) together with the microscopic morphology of the macroconidia and microconidia. Accurate identification of certain species requires supplementary tests, including growth on specialised media to enhance pigmentation or sporulation, and physiological tests that assess specific nutritional requirements, the ability to perforate hair, urease production, and thermotolerance.

Colony characteristics: Examination of the colony characteristics on SDA is one of the key criteria for the identification of dermatophytes. Each colony should be observed for surface pigmentation and texture (cottony, woolly, velvety, powdery, cerebriform, granular, or glabrous) and topography (elevation, margin, and folding).

Microscopic characteristics: The appearance and arrangement of the macroconidia and microconidia aid in the identification of most dermatophytes. The features of the macroconidia, in particular their size, shape, number of septa, wall characteristics (thickness and surface), and mode of attachment to the hyphae are especially useful for assigning the genus (**Table 6.1**). Several additional, non-specific structures may support identification when considered alongside the characteristic conidia, including chlamydospores, spiral hyphae, nodular organs, pectinate bodies, arthroconidia, and racquet hyphae (Larone *et al*., 2023).

6.1 Wood's Lamp identification

The Wood's lamp emits long-wave ultraviolet light and can detect hair fluorescence, a feature of infection by certain dermatophytes. It is particularly useful for detecting inconspicuous scalp lesions. Apart from its use in diagnosis, the Wood's lamp can be used to select infected hairs for laboratory investigation.

The Wood's filter transmits ultraviolet radiation in the 320 and 400 nm range, with a peak at 365 nm. When tissue or infected hair is exposed to this long-wavelength ultraviolet radiation, certain metabolites produced by the infecting dermatophyte absorb the radiation and re-emit it at a longer, visible wavelength, producing fluorescence (Gupta *et al*., 2004).

6.1.1 Precautions

The lamp should be allowed to warm up for about 1 minute. The examination room should be completely dark. Black occlusive shades or a windowless room are preferred. The examiner should also allow sufficient time for dark adaptation to see contrasts clearly.

6.2 Urea hydrolysis (Urease test)

Several dermatophytes can hydrolyse urea; therefore, a test that detects urease production can be utilised to differentiate them (Larone *et al*., 2023).

6.2.1 Procedure

- Prepare urea agar medium using Difco™ Urea Agar Base with 0.4% glucose and dispense into tubes.
- After inoculation, incubate the test isolate at 25–30°C for up to 7 days.
- Examine for the change in colour from straw yellow to red, which indicates the production of urease.

6.2.2 Results

T. interdigitale - positive; *T. rubrum* - negative; *T. mentagrophytes* - positive; *T. erinacei* - negative*; T. megninii* - positive.

Note: *Trichophyton indotineae*, an emerging species within the *T. mentagrophytes/T. interdigitale* complex and an important cause of terbinafine-resistant dermatophytosis in India, may be urease-negative or weakly positive. A negative urease test in a clinically aggressive *T. mentagrophytes*-like isolate should therefore not be considered definitive for species identification. Molecular identification, including Internal Transcribed Spacer (ITS) sequencing and/or detection of relevant resistance-associated markers such as Squalene Epoxidase (SQLE) mutations, may be required. Urease does not identify to species within the *T. rubrum* complex and must not be used for that purpose (Su *et al*., 2019).

6.3 In vitro hair perforation test

In vitro hair invasion by dermatophyte differs from *in vivo* invasion. The fungus does not reproduce the same pattern of hair invasion seen in natural infection. Instead, as the fungus grows in culture alongside the hair, certain dermatophytes produce characteristic perforations, wedge-shaped tunnels or holes penetrating the hair shaft. This test is particularly useful for differentiating *T. mentagrophytes* from *T. rubrum*, two species that are difficult to distinguish on morphological grounds alone (Ajello *et al*., 1957, Kidd *et al*., 2022).

6.3.1 Procedure

1. Collect a short length of human hair (approximately 1-cm long), preferably blond prepubertal hair, place it in a Petri dish and autoclave at 121°C at 15 psi for 10 minutes.
2. To it, add 25 mL of sterile distilled water and 2–3 drops of filter-sterilised 10% yeast extract.
3. Inoculate the plate with a small fragment of test fungus.
4. Incubate the plates at room temperature (25°C).

5. At regular intervals, remove individual hair segments, mount them in lactophenol cotton blue, and examine microscopically for evidence of perforation. The plates should be examined for up to 4 weeks before considering the test as negative.

6.3.2 Result

Positive: *Trichophyton mentagrophytes* and *T. interdigitale* (within 14 days), *Microsporum canis* (14 days), *Nannizzia gypsea*, *N. nana*, *N. persicolor* and *Paraphyton cookei*.

Negative: *T. rubrum* (both the downy and the granular forms), *M. audouinii* and *M. ferrugineum*, all read at 28 days (Kidd et al., 2022).

6.4 Morphological Characteristics of Dermatophytes

The morphological characteristics of macroconidia across the three major traditionally recognised dermatophyte genera are summarised in **Table 6.1**. The corresponding characteristics of microconidia are summarised in **Table 6.2**.

Table 6.1: Characteristics of macroconidia (Compiled from de Hoog *et al*., 2017 and Larone *et al*., 2023)

Genus	Size (µm)	Septations	Wall thickness	Wall surface	Occurrence	Attachment
Trichophyton	8–86 × 4–14	1–12	Thin–thick	Smooth	Usually rare	Single/clusters
Microsporum	12–110 × 6–25	5–15	Thick	Rough	Usually numerous (rare in *M. audouinii*)	Single
Epidermophyton	20–40 × 7–12	1–6	Thin/ Moderately thick	Smooth	Numerous	Single/clusters of 2–3

Table 6.2: Characteristics of microconidia

Genus	Shape	Occurrence	Attachment
Trichophyton	Spherical, pyriform or clavate; species-dependent	Numerous (except *T. schoenleinii*)	Single/clusters
Microsporum	Pyriform/clavate	Rare/numerous	Single
Epidermophyton	Not produced	Absent	Not applicable

6.5 Characteristics of clinical material in dermatophytosis, correlated with Wood's lamp findings and macroscopic and microscopic characteristics

The pattern of hair invasion observed on Wood's lamp examination and KOH microscopy, together with the corresponding dermatophyte species, is summarised in **Table 6.3** (Larone *et al*., 2023). The colony morphology, microscopic features, and results of ancillary physiological tests (urease, hair perforation) for the clinically important dermatophyte species are summarised in **Table 6.4** and **Figures in Annexure B**.

Table 6.3: Characteristics of clinical materials in dermatophytosis

Wood's lamp findings on scalp examination	**Examination of KOH mount (skin scraping)**	**Examination of KOH mount (Hair)**	**Dermatophyte implicated**
Bright yellow to green fluorescence	Hyphae and chains of arthroconidia	Small spores, arthroconidia 2–5 µm in diameter, easily dislodged from hair	*M. audouinii; M. canis; M. ferrugineum*
No fluorescence / dull fluorescence	Hyphae with arthroconidia in mass and chains	Large spores (5–8 µm) arranged in chains or masses	*N. gypsea; N. nana; M. vanbreuseghemii*
No fluorescence	Hyphae and chains of arthroconidia	Chains of spores on surface of hair	*T. mentagrophytes; T. verruccosum*
	Hyphae and chains of arthroconidia	Chains of arthroconidia inside the shortened hair stubs	*T. soudanense; T. tonsurans; T. violaceum*
	Cup-like clusters with chains of arthroconidia	Hyphae with air bubbles or tunnels and fat globules within the hair shaft	*T. schoenleinii*

Table 6.4: Cultural and other characteristics of dermatophytes (Compiled from de Hoog *et al*., 2017; Larone *et al*., 2023; Kidd *et al*.,2022)

Dermatophyte spp.	Colony morphology on SDA	Microscopic morphology	Other tests	Comments
Epidermophyton floccosum **(Annexure B)**	Slow growing, flat, fluffy, soon becomes velvety or powdery; turns 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	Not applicable	Do not invade hair
M. audouinii	Slow growing, flat, velvety with whitish tan to brown surface, reverse salmon to pale brown	Macroconidia and microconidia usually rare. When present, macroconidia 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
M. canis **(Annexure B)**	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 - *Arthroderma otae*. Grows confluently on Rice grain medium with yellow pigment
N. gypsea	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

Dermatophyte spp.	Colony morphology on SDA	Microscopic morphology	Other tests	Comments
M. 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
N. nana	Rapid growing, flat, powdery to fluffy with sandy surface. Reverse brownish red to dark red	Macroconidia numerous, ellipsoidal, thin walled, rough surface, with 1–3 cells. Microconidia pear shaped and sessile	Urease test positive. Hair perforation test positive	Poor growth at 37°C. Teleomorph - *Arthroderma obtusum*
Paraphyton cookei	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. cajetani*
M. vanbreuseghemii	Rapid growing, flat, powdery with yellowish tan to pink surface. Reverse no pigmentation or yellowish	Macroconidia numerous. Cylindrical to fusiform, rough, thick-walled with 7–10 celled	—	Teleomorph - *A. grubyi*
N. persicolor	Rapid growing, flat, fluffy to powdery, yellowish to pinkish buff	Macroconidia often absent, spindle shaped, smooth thin walled ~6 celled.	Urease test positive. Hair perforation test positive	Teleomorph - *A. persicolor.* Spiral hyphae resembling *T.*

Dermatophyte spp.	Colony morphology on SDA	Microscopic morphology	Other tests	Comments
	surface. Reverse peach to rose	Microconidia subglobose to club shaped in clusters		*mentagrophytes* produced
Trichophyton mentagrophytes complex (zoophilic) [*T. mentagrophytes sensu stricto* (formerly *T. mentagrophytes* var. *quinckeanum*)], animal-adapted form of *T. interdigitale* and *Trichophyton* anamorph of *A. benhamiae*	Rapid growing, flat, powdery to granular, 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*
T. mentagrophytes **complex** (anthropophilic) **(Annexure B)**	Rapid growing, powdery to fluffy, cream to buff white surface; reverse pale to red brown	Macroconidia rare, club shaped, smooth. Microconidia abundant, spherical/drop shaped in clusters. Spiral hyphae present	Urease test positive. Hair perforation positive	Macroconidia induced on SDA + 3–5% NaCl. Teleomorph - *A. vanbreuseghemii , A. benhamiae*
T. 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; yellow nodular bodies. Spiral hyphae rarely seen	Urease test positive. Hair perforation test positive	—

Dermatophyte spp.	Colony morphology on SDA	Microscopic morphology	Other tests	Comments
T. rubrum (cosmopolitan variant)	Slow growing, flat/heaped, velvety or powdery becoming 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 absent	Urease test negative. Hair perforation test negative	—
T. rubrum (Afro-Asiatic variant, formerly *T. raubitschekii*) **(Annexure B)**	Slow growing, powdery to velvety, cream to deep red surface. Reverse wine red	Macroconidia abundant, club shaped with rare rat tail extension. Microconidia drop shaped to round with many chlamydospores	Urease test positive. Hair perforation negative	—
T. violaceum	Slow growing, heaped, glabrous, cream to lavender deep purple surface	Macroconidia rare. Microconidia usually not found on SDA; enhanced on thiamine medium	Urease test positive/weak. Hair perforation negative	—
T. tonsurans **(Annexure B)**	Slow growing, powdery turning velvety; surface tan/yellow/rose. Reverse yellowish to mahogany red	Macroconidia rare, small pencil/club shaped. Microconidia abundant, tear shaped; balloon forms seen	Urease test positive. Hair perforation mostly negative	—
T. verrucosum	Very slow growing, folded, white or yellow powdery.	Macroconidia rare, string bean shaped, 3–5-celled. Microconidia	Urease test negative. Hair perforation negative	Thiamine essential for sporulation

Dermatophyte spp.	Colony morphology on SDA	Microscopic morphology	Other tests	Comments
	Reverse no pigment	seen on enriched media; chlamydospores on SDA		
T. schoenleinii	Slow growing, heaped, leathery, white to tan surface	Macroconidia absent. Microconidia very rare. Hyphae knobby, favic chandeliers. Chlamydospores abundant	Urease test variable. Hair perforation negative	—

6.6 Growth on Dermatophyte Test Medium

Dermatophyte Test Medium is a selective medium used to isolate pathogenic fungi from cutaneous specimens. This medium is intended to be used as a primary isolation medium (Taplin *et al*., 1969).

6.6.1 Principle

Dermatophyte Test Medium contains papain digest of soybean meal as an amino acid source, along with other nitrogenous substances necessary for fungal growth. Dextrose provides an energy source; phenol red, a colourimetric indicator, is used to visualise the pH shifts in the medium; and cycloheximide suppresses the growth of many non-dermatophytic fungi, while antibiotics, like chlortetracycline and gentamicin, suppress bacterial contaminants.

6.6.2 Procedure

1. Inoculate the specimen as soon as possible after it is received in the laboratory.
2. Place the specimen at the centre of the agar with sterile forceps.
3. Press carefully to ensure firm contact with the agar surface.
4. Incubate the inoculated media at 22–25°C for up to 14 days. Do not incubate cultures at 33–37°C.
5. Examine the culture daily for a change in the colour of the medium and evidence of fungal growth.

6.6.3 Results

Table 6.5: Growth on Dermatophyte Test Medium (DTM)

Control Organisms (ATCC® Strains)	Growth Characteristics on DTM
Trichophyton mentagrophytes (ATCC® 9533)	White cotton-like growth with pink to red colour change
Aspergillus niger (ATCC® 16404)	Inhibition, partial to complete
Candida albicans (ATCC® 10231)	White/off-white growth, no colour change
Escherichia coli (ATCC® 25922)	Inhibition, partial to complete

This medium is intended to be used as a primary isolation medium. Dermatophytes produce typical morphology and a pink-to-red colour in the medium surrounding the colony within 10 to 14 days of incubation. Occasionally, a bacterial contaminant may cause a colour change in the medium during this period, but it can be distinguished by its colonial morphology. Colour changes occurring after 14 days of incubation should be disregarded, as these are more likely to represent contaminating (non-dermatophyte) fungi rather than a true positive result. Confirmation of dermatophyte identification requires isolation in pure culture, followed by morphological examination and, where necessary, biochemical and/or serological testing.

6.6.4 Precautions

The ability to detect microorganisms by culture-based techniques may be adversely affected by several pre-analytical and analytical factors, including improper specimen collection, storage, or inoculation; initiation of anti-infective therapy prior to specimen collection; incorrect incubation temperature or atmosphere; inadequate incubation duration; and improper storage or handling of culture media.

6.7 Hair bait technique

The hair-bait technique was introduced to isolate keratinophilic fungi, including dermatophytes (Vanbreuseghem *et al*., 1952).

6.7.1 Procedure

1. Fill half of a Petri dish (90–100 mm) with moistened soil, using sterile distilled water. The amount of water varies from sample to sample, depending on each sample's moisture content.
2. Bait this with strands of autoclaved horsehair.
3. After one or two weeks of incubation at room temperature, examine the growth on the hair and culture it on SDA containing cycloheximide and chlortetracycline.
4. The resulting isolates can then be studied in detail and identified to the species level.

Chapter 7: Preservation of Isolates and Slides

7.1 Storage Techniques

Various technologies have been found useful for maintaining culture collections. Although storage in liquid nitrogen and lyophilisation are considered ideal methods, some simple and inexpensive alternatives are also available (Evans *et al*., 1989; Stockdale PM *et al*., 1989).

7.1.1 Periodic subculture and storage at room temperature or refrigeration

Periodic subculture is the simplest method of maintaining fungal isolates by transferring actively growing cultures onto fresh culture media at regular intervals. Although widely practised in routine diagnostic laboratories, this method is suitable only for short-term preservation, as repeated subculturing may lead to contamination, phenotypic variation, genetic drift, or loss of sporulation.

- Fungi can be routinely subcultured on an agar slope (a low-nutrient medium is preferred).
- Subculturing intervals vary according to the organism, with cultures generally transferred/subcultured every 3–6 months when maintained at room temperature and at longer intervals (approximately 6–12 months) when refrigerated, if appropriate.
- Transfer each culture onto at least two new slopes.
- Screw-capped or cotton-plugged tubes may be used. The risk of mite infestation is higher with cotton-plugged tubes. For mucorales it is advisable to use either cotton plugs or loosely screwed tubes to facilitate better air exchange (Vellanki, S et al., 2018).

Quality Control

- Verify purity before every subculture.
- Examine colony morphology and microscopic characteristics after each transfer.
- Record the date of each subculture.
- Limit the number of sequential subcultures whenever possible.

Advantages

- Simple and inexpensive.
- Requires minimal laboratory infrastructure.
- Suitable for routine laboratory use.
- Allows rapid recovery of viable cultures.

Limitations

- Not suitable for long-term preservation.
- Increased risk of contamination.
- Repeated subculturing may alter morphology, sporulation, virulence, and antifungal susceptibility (Smith and Onions, 1994).

7.1.2 Water Culture Technique (Castellani's Water Method)

It is one of the simplest and least expensive methods for preserving fungi.

Method A: Preservation of Mycelial Fragments

- Dispense 2 mL of sterile distilled water in small screw-cap bottles and sterilise by autoclaving.
- Remove mycelia from the fungal culture and aseptically transfer to the screw-capped bottle.
- Store at room temperature.

Method B: Preservation of Conidial Suspension

- To a sporulating culture, aseptically add 2 mL of sterile distilled water.
- Dislodge the conidia using a sterile inoculating loop.
- With a sterile pipette, remove the suspension and transfer it into a sterile screw-capped bottle.
- Take care not to transfer the agar.
- Store at room temperature, away from light.

Quality Control

- Use only pure, uncontaminated fungal cultures.
- Ensure strict aseptic technique during transfer.
- Confirm culture viability periodically by subculturing onto appropriate fungal media.
- Examine stored cultures periodically for contamination and evaporation.

Advantages

- Simple and inexpensive.
- Does not require specialised equipment.
- Suitable for long-term preservation of many filamentous fungi.
- Minimal maintenance after storage.

Limitations

- Not all fungal species survive equally well.
- Periodic viability testing is required.
- Contamination during preparation can compromise culture integrity.
- Some delicate or fastidious fungi may lose viability over prolonged storage.

7.1.3 Storage under mineral oil

- Autoclave liquid paraffin twice (double autoclaving) at 121°C, 15 psi for 15 minutes.
- Grow fungal culture on a short agar slant.

- Add mineral oil to the culture to a depth of 10 mm above the highest point of the agar slants.
- Store at room temperature.

Quality Control

- Use only pure fungal cultures.
- Confirm sterility of the mineral oil before use.
- Periodically examine cultures for contamination, evaporation, or drying.
- Verify viability by subculturing at recommended intervals.

Advantages

- Simple and inexpensive.
- Suitable for long-term preservation of many fungi (Several years and up to around 30 years in some cases (Smith & Onions, 1983)).
- Reduces desiccation and may reduce the risk of contamination.
- Minimal maintenance after storage.

Limitations

- Not suitable for all fungal species.
- Recovery may require careful removal of excess oil.
- Some isolates may show altered sporulation after prolonged storage.
- Regular viability assessment is recommended.

7.1.4 Storing at −20°C or −70°C

Method A: Agar Slant Preservation

1. Grow the isolate on slopes of a suitable agar medium.
2. Cover the culture with a cryoprotectant appropriate to the organism — 10% glycerol is the standard choice for most fungi; 10% DMSO may be used as an alternative for strains that are glycerol-sensitive or show poor recovery with glycerol (ATCC, n.d.).
3. Screw the caps firmly and store at −20°C or −70°C.

Method B: Mycelial Suspension in Glycerol

1. Take 10% glycerol in a small screw-capped bottle. Sterilise at 121°C at 15 psi for 10 minutes.
2. Transfer mycelia from the fungal culture to a bottle containing glycerol.
3. Store at −20°C or −70°C.

Quality Control

- Use only pure, uncontaminated fungal cultures.

- Ensure the cryoprotectant is sterile before use.
- Monitor and maintain the required storage temperature throughout the storage period.
- Assess viability periodically by subculturing representative stored isolates.
- Avoid repeated freeze–thaw cycles**.**

Advantages

- Excellent long-term preservation method.
- Maintains genetic and phenotypic stability.
- High recovery rate after storage.
- Suitable for most medically important fungi.

Limitations

- Requires dedicated freezer facilities.
- Power interruptions may compromise viability.
- Some isolates may require optimisation of cryoprotectant concentration.
- Repeated thawing can reduce culture viability.

7.1.5 Freeze-drying

- This technique is used for both moulds and yeasts. There are many methods for achieving freeze-drying, depending on the equipment available.
- The technique of centrifugal freeze-drying is used successfully; though a technique allowing variation of cooling rate, such as a shelf freeze-dryer, is preferred (Ryan and Smith, 2007).

Quality Control

- Use only pure fungal cultures.
- Confirm adequate drying before sealing.
- Ensure vacuum integrity of sealed ampoules.
- Perform periodic viability testing by rehydration and subculture.
- Store ampoules in a moisture-free environment.

Advantages

- Excellent method for very long-term preservation.
- High stability of fungal isolates.
- Minimal maintenance after storage.
- Easy transportation of preserved cultures.
- Preserves morphological and genetic characteristics.

Limitations

- Requires specialised lyophilisation equipment.
- Relatively expensive compared to other preservation methods.
- Some delicate fungal species may show reduced survival.
- Recovery requires careful rehydration of the culture.

7.1.6 Mounting Medium for Permanent Preparations of Microfungi

The slide culture method described by Riddell (1950), using lactophenol cotton blue as the mounting fluid, is the most widely used technique among medical mycologists to study the microscopic morphology of pathogenic and related fungi. Although this technique provides satisfactory, temporarily stained preparations of fungal growth with minimal conidial dislodgement, these preparations are generally not permanent. The slow evaporation of lactophenol from the edges of the coverslips eventually distorts conidia due to their loss of turgor. Such preparations have to be discarded and redone for demonstration or teaching.

- Sealing the coverslip edges with a clear lacquer may prolong the stability of the preparation; however, the suitability of the sealing medium should be validated before routine use. As noted by McGinnis (1974; 1980), different nail lacquer brands exhibit varying levels of resistance to cracking and adhesion to glass.
- Several alternative mounting fluids, such as polyvinyl alcohol (Carmichael, J.W., 1955; Carmichael, 1962), Hoyer's fluid (Cunningham, 1972), and modified Papanicolaou's staining procedure (Miller *et al*., 1973), have been used to make permanent slide preparations of various groups of fungi. Here are a couple of mounting media for permanent preparations of fungi that have proven satisfactory.

Lactophenol cotton blue with Polyvinyl Alcohol (PVA) (Huber's PVA Mounting Medium, modified) (Larone, 2002)

Reagents:

- Polyvinyl alcohol (PVA): molecular weight 70,000–100,000 (Sigma Chemical Co. catalogue no. P-1763)
- Phenol: purified grade (Sigma no. P-5566 or Fisher no. A911-500)
- Lactic acid: ACS reagent (Sigma no. L-1893)
- Aniline blue: certified (Aldrich Chemical Co., catalogue no. 86, 102-2). This is analogous to cotton blue.

Preparation:

- Add 7.5 g of PVA powder to 50 mL of cold deionised water in a beaker.
- Place the beaker on a heated stirring plate and add a magnetic stirring rod.
- Place a thermometer in the beaker to monitor temperature.
- Add 22 g of lactic acid (before adding phenol).

- Add 22 g of phenol crystals (22 mL of melted phenol). CAUTION: phenol is corrosive and causes severe skin burns and is readily absorbed through the skin. Weigh and melt phenol only in a fume hood, wearing nitrile gloves, a laboratory coat and eye protection, and keep polyethylene glycol 300/400 or a proprietary phenol spill kit immediately available for skin decontamination.
- Add 0.05 g aniline blue.
- Heat and stir the solution until the temperature reaches 90°C. Do not boil or exceed 100°C. Remove from the hot plate.
- Dispense into small dropper bottles (thoroughly washed and dried ones can be used). Tighten dropper bottle caps and store at room temperature.

Procedure

- Before mounting slide cultures, it is important to note that many medically important fungi, such as *Aspergillus*, *Penicillium*, and *Paecilomyces* species, produce dry chains of conidia. It is therefore necessary to wet the growth on the slide culture by adding a wetting agent so that the conidia become wet and release trapped air. Any preparation containing trapped air bubbles beneath the coverslip will allow slow evaporation of the mounting fluid, thereby distorting conidia and hyphal growth due to loss of turgor.

Wetting agent

- 95% ethanol 50 mL, acetone 25 mL, and 85% lactic acid 25 mL

Place a drop of the wetting agent gently onto the fungal growth on the prepared slide and the coverslip to be mounted. Gently roll the slide or coverslip to ensure that all fungal growth is wetted. Drain the excess wetting agent on clean filter paper. The preparation is now ready to be mounted in the PVA mounting fluid. Place 1 drop of PVA mounting fluid on the slide with the fungal growth obtained from the slide culture. Apply the coverslip gently so that no air bubbles are trapped. Allow to dry on a flat surface. Similarly, prepare a second slide using the fungal growth on the coverslip.

Chapter 8: MALDI-TOF MS-based Fungal Identification

Matrix-assisted Laser Desorption/Ionisation-Time of Flight Mass Spectrometry (MALDI-TOF MS) for fungal identification is based on generating a protein mass spectrum (primarily ribosomal proteins) from microbial cells and comparing it with a reference database (Singhal *et al*., 2015). **Figure 8.1** shows a schematic diagram of MALDI-TOF MS.

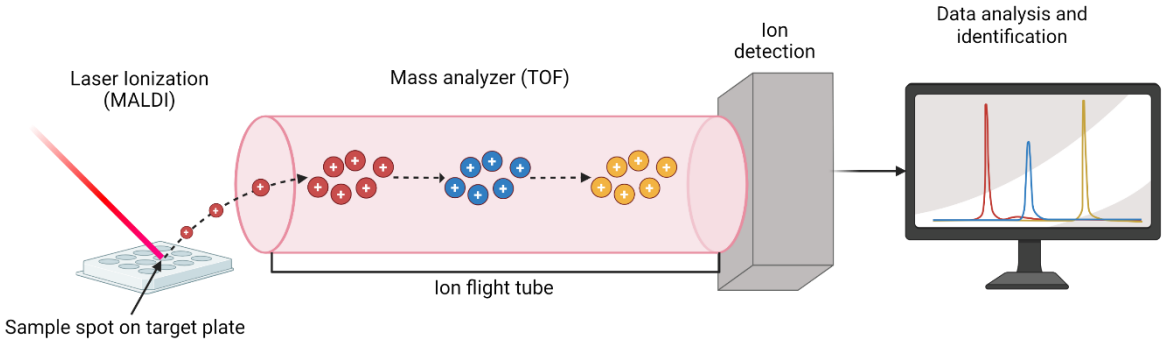


Figure 8.1: Schematic diagram of MALDI-TOF MS (Created in BioRender. Rudramurthy, S. (2026) https://BioRender.com/l8vj3fn)

Two commonly used commercially available platforms for fungal identification in clinical laboratories are VITEK® MS (bioMérieux) and MALDI Biotyper® (Bruker Daltonics):

8.1 VITEK® MS MALDI-TOF (bioMérieux) (Westblade LF *et al*., 2013, Rychert J *et al*., 2018)

8.1.1 Preparation of the MALDI Matrix Solution

VITEK MS α-cyano-4-hydroxycinnamic acid (CHCA) matrix is provided as a ready-to-use prepared reagent.

8.1.2 Preparation of the VITEK® MS-DS Target Slide

1. The VITEK® MS-DS (disposable slide) is a single-use target on which the sample and the matrix are co-crystallised before the slide is loaded into the instrument. It replaces the reusable steel target plate used by the MALDI Biotyper, and because it is disposable it removes the plate-cleaning step described in Section 8.2.2.
2. The VITEK® MS-DS target slides are designed for single-use only and must be stored in a dust-free environment.
    - Do not touch the upper face of the VITEK® MS-DS target slides before use.
    - Handle the VITEK® MS-DS target slides by the edges or barcode end.
    - Take care not to contaminate the VITEK® MS-DS target slides and avoid spilling liquid on them.
    - Do not write or mark on the VITEK® MS-DS target slides.
3. Before adding samples to the VITEK® MS-DS target slide, place the VITEK® MS-DS target

slide on a flat surface, such as a silicone mat, that will prevent it from sliding.

The VITEK® MS-DS target slide for use with the VITEK® MS instrument is the disposable VITEK® MS-DS target slide with 48 sample positions. The sample positions are divided into three sections. Each section is called an acquisition group. The three small spots in the centre of each acquisition group must not be used for identifying microorganisms. These three small spots (xA1, xB1, xC1) are used for calibration **(Figure 8.2)**.

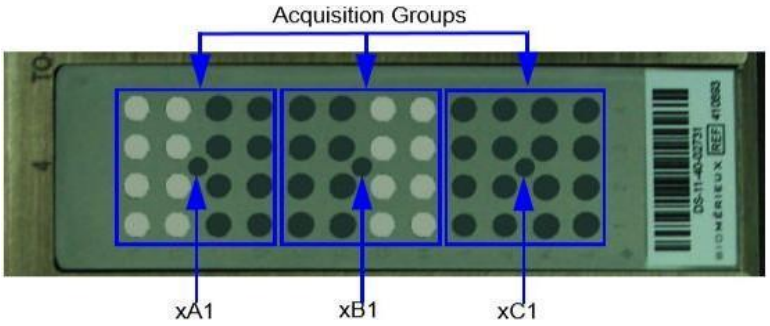


Figure 8.2: VITEK® MS-DS target slide showing the acquisition groups

8.1.3 Preparation of the calibration strain

- The *E. coli* ATCC® 8739™ strain must be deposited at positions xA1, xB1, and xC1, depending on the number of samples tested (1 calibrator per acquisition group of 16 spots).
- The *E. coli* ATCC® 8739TM strain culture should be 18–24 hours old.

1. Using a toothpick, collect and smear a thin layer of *E. coli* ATCC® 8739™ strain cells onto the calibration spot for each acquisition group used on the VITEK® MS-DS target slide.
2. Immediately add 1 µL of VITEK® MS-CHCA matrix to each calibration spot using a pipette and replace the pipette tip after each addition.
3. Allow each spot to dry completely.

8.1.4 Sample Preparation

The microorganisms to be identified must first be isolated on a suitable culture medium using established protocols. It is preferable to use colonies approximately 3 mm in diameter. However, it is also possible to use colonies that are considerably smaller or larger.

8.1.5 Sample Preparation of Yeasts (Westblade LF *et al*., 2013)

1. Collect a portion of an isolated colony using a sterile toothpick.
2. Apply the sample to the centre of the spot. Take special care not to apply too much. The appropriate amount on a spot is shown in the picture below **(Figure 8.3)**. If enough colony is not deposited, take a fresh sterile toothpick and add another portion of the colony.



Figure 8.3: Appropriate amount of sample to be deposited on the slide

3. Smear a thin layer of the sample onto the spot using the toothpick, then discard the toothpick.
4. Add 1 µL VITEK® MS formic acid (FA) to the sample.
5. Allow the spot to dry completely.
6. Add 1 µL of VITEK® MS-CHCA matrix to the centre of the spot using a pipette, then discard the pipette tip.
7. Repeat the procedure using fresh toothpicks and pipette tips to deposit other samples (if any).
8. After 5 minutes, check for crystal formation on the spots.

Inexperienced users may find using a magnifying glass helpful. Successful measurement generally requires adequate matrix crystallisation, visible as a yellowish film. Ideally, most of the spot's surface will be coated with crystals **(Figure 8.4)**.

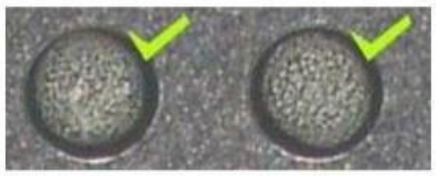


Figure 8.4: Matrix crystal formation after addition of the matrix solution

Run in the VITEK® MS instrument.

Note: It is possible to prepare samples for both bacteria and yeasts on the same VITEK® MS-DS target slide.

8.1.6 Sample preparation of Moulds (Rychert J *et al*., 2018)

Requirements (according to manufacturer instructions)

VITEK® MS MOULD KIT: 2mL round-bottomed colourless microtubes, Ethanol 70% (HPLC or ACS grade), Formic acid 70% (HPLC or ACS grade), Acetonitrile (HPLC or ACS grade)

- API suspension medium or (deionised water) 2 mL
- Sterile cotton swab
- Microcentrifuge capable of 10,000–14,000 × g
- Biosafety cabinet
- Micropipette & tips
- Vortex

Method (according to manufacturer instructions)**:**

1. Wet a sterile cotton swab using suspension medium or sterile deionised water.
2. Press the swab against the side of the tube to remove excess liquid.
3. Collect approximately a circle of 1 to 2 cm diameter of mould from the agar plate using the swab, selecting spores (conidia) and hyphae if possible.
4. Suspend the collected material in a 2 mL round-bottomed tube containing 900 µL of 70% ethanol.
5. Mix the contents of the round-bottomed tube using a vortex mixer.
6. Centrifuge the round-bottomed tube for at least 2 minutes at 10,000–14,000 × g.
7. Discard all the supernatant using a pipette. Use caution to avoid dislodging the pellet.
8. Add 40 µL of 70% formic acid and mix with a vortex mixer until completely resuspended.
9. Add 40 µLof acetonitrile and mix using a vortex-type mixer.
10. Centrifuge for at least 2 minutes at 10,000–14,000 × g.
11. Following this step, the manufacturer's protocol considers the sample inactivated. All preceding steps, from collection of the mould from the agar plate to this point, must be performed in a biological safety cabinet.
12. Spot immediately 1 µL of the supernatant on a target slide.
13. Allow the spot to dry completely.
14. Add 1 µL of VITEK® MS-CHCA matrix to each target slide spot using the pipette and replace the pipette tip after each addition of matrix.
15. Allow the spot to dry completely.
16. Run in the VITEK® MS instrument (All steps are illustrated in **Figure 8.5**).



Figure 8.5: Sample processing method for identification of moulds using VITEK® MS MALDI-TOF (bioMérieux) (Source: VITEK MS Mould kit insert)

Note:

1. Control strains for yeast and moulds to be included with the test isolates: *C. krusei* ATCC® 6258; *C. parapsilosis* ATCC® 22019; *A. flavus* ATCC® 204304; *A. fumigatus* ATCC® 204305.
2. Once the VITEK® MS-DS target slide is prepared, it must be tested within 72 hours.
3. Before spectra acquisition, it must be stored at room temperature in its original packaging.
4. If the sample deposit is of optimum quality, acquisition can be performed up to 3 times on the same spot.

8.1.7 Spectra acquisition and interpretation

1. Log in to the VITEK® MS Prep Station software.
2. Scan the VITEK® MS target slide barcode or enter the barcode using the keyboard to fill in the ‘Enter Slide ID’ field**.**
3. For each sample, enter the ‘Lab ID’ on the plate using the keyboard.
4. Select the organism type as ‘Fungi’.

8.1.8 Acquiring the Spectrum

1. Double-click on the **VITEK® MS acquisition station icon** on the desktop.
2. Enter the Username and Password in the Login dialogue box.
3. Click 'Validate' to start the application.

Inserting the Adapter

1. Ensure that the VITEK® MS target slide spots are dry.
2. Click Open. Part of the vacuum system is vented, and the application displays the vacuum level.
3. After about 1 minute, the stage is repositioned, the front door opens, and the carrier emerges.
4. Place the VITEK® MS target slide into the adapter.
5. Using the barcode reader, scan the barcodes on the VITEK® MS target slides. As the barcode is scanned, the application displays the sample positions.
6. Verify the barcode shown in the diagram.
7. Slide the adapter into the carrier unit until it can go no further. The acquisition can now start.
8. Click START.
9. The adapter retracts into the VITEK® MS equipment and the vacuum starts.
10. Acquisition starts as soon as the vacuum system reaches the operating pressure.
11. The spectrum being acquired is displayed.
12. At the end of the acquisition, the VITEK® MS target slide can be removed and another run performed by clicking OPEN.

8.1.9 Results and Interpretation

- A confidence value is calculated to express the similarity between the spectrum obtained from the unknown organism and reference spectra in the knowledge base. Then thresholds are applied to retain only the more significant organisms or groups.
- A single identification is displayed with a confidence value of 60–99.9 when a single significant organism or organism group is retained.
- Low Discrimination identifications are displayed when more than one significant organism or organism group are retained, but not more than 4. In this case, the sum of the confidence values is 100.
- When more than 4 organisms or groups are found, the organisms are considered non-identified. In this case, a list of possible organisms is displayed, and the sum of confidence values is less than 100.
- When no match is found, the organism is considered as non-identified (**Table 8.1**).

Table 8.1: Interpretation of VITEK® MS identification results by confidence value (Source: VITEK MS Mould kit insert)

Confidence level	Choice	% Probability	Comments
Good	1	60 to 99.9	
Low discrimination	2 to 4	Sum = 100	Separate by further testing
No ID	NA	NA	No significant choice
	>4	Sum<100	Inconclusive identification

8.2 MALDI Biotyper® Sirius

8.2.1 Required Chemicals and Accessories (according to manufacturer instructions)

- Double-distilled water (DDW)
- Absolute ethanol (EtOH)
- Acetonitrile (ACN)
- 98% Formic acid (FA)
- Trifluoroacetic acid (TFA)
- Matrix: α-cyano-4-hydroxycinnamic acid (HCCA). Saturated solution prepared by dissolving the HCCA in 100 µL of standard solvent made up of 50 µL acetonitrile, 47.5 µL sterile deionised water and 2.5 µL trifluoroacetic acid — that is, acetonitrile: water: trifluoroacetic acid in the ratio 50:47.5:2.5 v/v.
- Wooden application sticks, pipette tips or plastic inoculation loops to apply material
- 50–1000 µL pipette tips and a suitable pipette
- 2–200 µL pipette 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

8.2.2 Instrumental procedure

1. Open Flex control software.
2. Click the arrow to move the MALDI target plate in or out.
3. Open MALDI Biotyper Realtime Classification software.
4. Click on ‘File’.
5. Select ‘New Classification’.
6. Enter the project name.
7. Click ‘New’.
8. Select the position of the target samples and right-click and select ‘Add Analytes’.
9. Enter 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. Review the project summary and click ‘Finish’ to initiate sample identification.

8.2.3 Type of Target plate

Plate Type	Features	Usage
Disposable (MBT Biotarget 96) (Figure 8.6)	96-position polymer plate. AnchorChip™ effect: hydrophobic coating rings each hydrophilic spot, concentrating sample/matrix and preventing cross-contamination between adjacent spots. Individually barcoded for sample traceability.	Routine bacterial/fungal identification runs. Single-use — discarded after one run; eliminates cleaning and re-validation between runs.
Reusable (MSP 96 polished/ ground steel) (Figure 8.7)	96-position stainless-steel plate. Polished-steel finish preferred for samples in volatile organic solvents; ground-steel finish for general/broader application. Withstands repeated cleaning cycles.	Used across multiple identification runs. Requires a validated cleaning/decontamination protocol and a contamination check before each reuse.

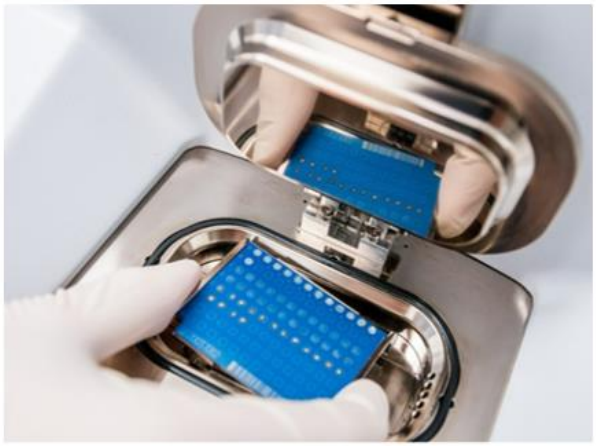


Figure 8.6: Disposable MBT Biotarget 96

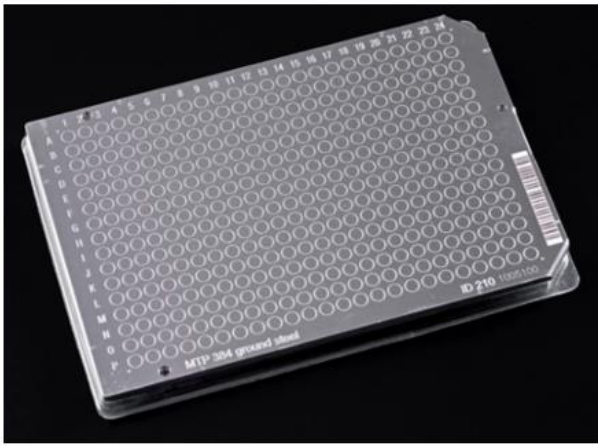


Figure 8.7: Reusable Target plate

8.2.4 On-Plate Formic Acid Extraction Method for Yeast (Ghosh *et al.*, 2015)

1. Emulsify one loopful of fresh culture in 1 mL of sterile DDW in a 1.5 mL microcentrifuge tube.
2. Centrifuge the suspension at 13,000 rpm for 2 minutes.
3. Discard the supernatant and repeat steps 1 to 4 twice.
4. Resuspend 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, then air-dry them again.
7. Add 0.8 µL of matrix solution to each spot.

8.2.5 Off-plate Formic Acid Extraction Methods for Moulds (Shao J *et al.*, 2018):

1. Inoculate fungal growth in 5 mL of Sabouraud Dextrose Broth in a 15 mL centrifuge tube.
2. Incubate the tubes on 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 into a microcentrifuge tube using blunt-end pipette tips.
5. Centrifuge at 13,000 rpm for 5 minutes.
6. Completely remove the medium.
7. Add 300 µL of sterile DDW to wash the pellet.
8. Centrifuge at 13,000 rpm for 5 minutes.
9. Remove 1.2 mL of supernatant and replace with 900 µL absolute ethanol and keep for 5 minutes.
10. Remove the ethanol by pipetting, followed by drying in a vacuum concentrator.
11. Emulsify the dried pellet in 50–80 µL 70% 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 using 0.5 mm acid-washed glass beads to ensure adequate lysis.

15. Centrifuge at 10,000 rpm for 5 minutes.
16. Spot 4–6 µL of the supernatant (1.5–2 µL each time, 3–4 times) onto the surface of the 96-target polished steel plate and allow to dry.
17. Overlay the dry spot with 1 µL matrix solution.

Both on-plate and off-plate extraction methods are depicted in **Figure 8.8** below.

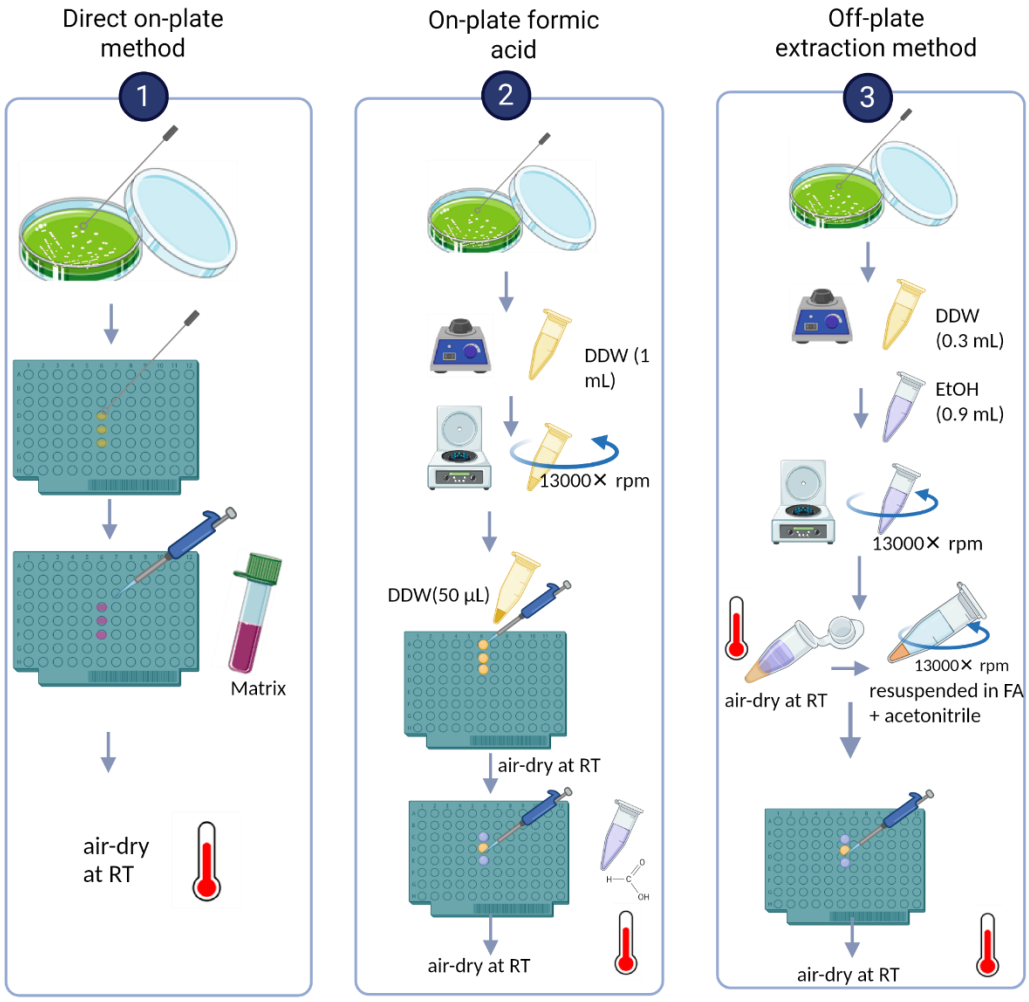


Figure 8.8: MALDI Workflow: Direct, On-Plate and Off-plate Formic Acid Extraction Methods (Created in BioRender. Sharma, V. (2026) https://BioRender.com/4uh30c9)

8.2.6 Off-plate Formic Acid Extraction Method for Melanised Fungi (Paul S *et al*., 2019):

1. Culture the isolates on SDA and incubate for 3 to 7 days at 28°C.
2. Harvest very small pieces of mycelial growth using a teasing needle and place them in 1.5 mL DDW in a 2 mL microcentrifuge tube.
3. Vortex well to disperse the mycelial clump and produce a thin and smaller structure.
4. Centrifuge it for 10 minutes at 13,000 rpm.
5. Completely remove the supernatant and replace it with 1 mL of 70% ethanol.
6. Incubate at room temperature for 5 minutes.

7. Centrifuge the suspension at 13,000 rpm and discard 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) onto the surface of the 96-target polished steel plate and allow to dry.
15. Overlay the dry spot with 1 µL matrix solution**.**

8.2.7 Formic Acid Extraction Method for Dermatophytes (L'Ollivier *et al*., 2013)**:**

1. Culture the isolates on SDA and incubate for 3 to 7 days at 28°C.
2. Vortex well to disperse the mycelial clump and produce a thin and smaller structure.
3. Centrifuge it for 10 minutes at 13,000 rpm.
4. Completely remove the supernatant and replace it with 700 µL of 70% ethanol.
5. Centrifuge the suspension at 13,000 rpm and discard the supernatant.
6. Resuspend in 300 µL of distilled water.
7. Centrifuge it for 10 minutes at 13,000 rpm and discard the supernatant.
8. Emulsify the dried pellet in 50–80 µL of 98% FA.
9. Incubate at room temperature for 5 minutes.
10. Add 50–80 µL ACN.
11. Add 0.5–0.75 mm sterile acid-washed glass beads and vortex vigorously for 20–30 minutes.
12. Spot 3–5 µL of the supernatant (1.5–2 µL each time for 3–4 times) onto the surface of the 96-target polished steel plate and allow it to dry.
13. Overlay the dry spot with 1 µL matrix solution.

After spotting on the MALDI target plate, the following steps are to be followed for the interpretation of identification scores:

- Air-dry the plate prior to the Biotyper analysis
- The spectra will be acquired using the FLEX control software and matched against the existing database in the MALDI Biotyper software.

According to Bruker, if the score is <1.7, the identification is not reliable; if the score is between 1.7 and 1.99, the identification is confirmed up to the genus level; if the score is ≥2, the identification is confirmed up to the species level.

8.2.8 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 lint-free tissue, clean the MALDI target plate thoroughly with 70% aqueous ethanol.
6. Rinse the MALDI target plate with tap water and wipe it with a lint-free tissue.
7. Cover the MALDI target plate with 100 µL of 80% aqueous TFA.
8. Thoroughly wipe all target positions with a lint-free tissue.
9. Rinse the MALDI target plate with deionised water and wipe it dry with lint-free tissue.
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.

8.3 Instrument and database versions

8.3.1 VITEK® MS

Fungal identification by VITEK® MS is performed using IVD Knowledge Base (KB) version 3.2, covering 1,316 microbial taxa in total, of which 207 are yeast and mould species/groups combined. Filamentous fungi are processed separately using the dedicated VITEK® MS Mould reagent kit prior to spectral acquisition (Magrì C *et al*.,2026).

8.3.2 MALDI Biotyper® Sirius

Fungal identification by the MALDI Biotyper® Sirius is performed using the MBT Compass HT reference library (research-use-only: 2026 version, 4,842 species across 768 genera; IVD: 2023 version, 4,239 species across 686 genera), together with the dedicated MBT HT Filamentous Fungi Module for mould identification (research-use-only: 2026 version, 295 species/groups; IVD: 225 species/groups, CE-IVD) (as per manufacturer specifications at https://www.bruker.com/en/products-and-solutions/microbiology-and-diagnostics/microbial-identification/maldi-biotyper-reference-libraries.html).

Note: Database and knowledge-base versions are current as of 2026; laboratories should verify the installed version against manufacturer documentation at the time of testing, as these libraries are updated periodically.

Chapter 9: Molecular Identification of Fungi

Sanger sequencing is the most common standard method for the identification of yeast and mould. This chapter describes the procedure for the identification of fungi by Sanger sequencing.

9.1 DNA Extraction (Karakousis *et al*., 2006)

Fungal DNA extraction is challenging because of the rigid cell wall, requiring enzymatic, mechanical or chemical disruption. Enzymes such as zymolase and lyticase facilitate cell wall digestion, with zymolase more effective for yeasts (*Candida* and *Cryptococcus*) and lyticase for moulds. Mechanical methods include glass-bead vortexing, freeze–thaw cycles, sonication and liquid-nitrogen grinding, often combined with enzymatic treatment to improve DNA yield. Chemical lysis uses detergents and alkaline agents. Commercial silica column-based kits, such as QIAamp DNA Mini Kit, have been used for isolates as well as clinical samples (Mohapatra *et al*., 2022). CTAB-based lysis followed by phenol–chloroform purification is another approach (Dar *et al*., 2025; Mülhardt and Beese, 2007).

9.2 Target Selection

Targets for molecular diagnostic tests for fungal infections include single- and multicopy nuclear and mitochondrial genes and RNA. Multicopy gene targets exhibit higher sensitivity.

9.2.1 Multicopy gene targets (Ferrer *et al*., 2001)

Ribosomal DNA gene cluster: rDNA is a tandem array of 50–100 copies in the haploid genome of all fungi. It comprises the small subunit SSU rDNA (18S), the 5.8S gene and the large subunit LSU (28S) gene. Separating 18S and 5.8S is the ITS1 region, and separating 5.8S and 28S is the ITS2 region, both called internal transcribed spacer regions, as shown in **Figure 9.1**. Between each transcript is an intergenic spacer region (IGS). Some basidiomycetes may harbour a fourth gene, 5SrRNA. Within a single repeat, regions exhibit different rates of evolutionary change, which can be used to examine the evolutionary histories of a group of taxa. The coding regions are highly conserved; the ITS regions are moderately variable (the divergent domain); and the IGS region contains highly variable sequences in rDNA. So, this enables the design of universal primers targeting conserved regions and species-specific probes targeting variable regions, which can be used to identify species. Universal primers ITS1 and ITS2, designed from conserved regions flanking the internal transcribed spacer (ITS) of the fungal rRNA gene cluster, are widely used for broad-range amplification and identification of fungal pathogens.



Figure 9.1: Ribosomal gene cluster

Pan-fungal primers used for the identification of yeasts and moulds. A combination of ITS1 (5' TCCGTAGGTGAACCTGCGG) and ITS4 (5'TCCTCCGCTTATTGATATGC) or a combination of ITS5 (5' GGAAGTAAAAGTCGTAACAAGG 3') and ITS4 are preferred targets.

9.3 Preparation of solutions

9.3.1 Storage, Stability and Handling of Reagents

In general, solutions are prepared with molecular-biology-grade reagents and nuclease-free water, dispensed into single-use aliquots, and labelled with the name, concentration, date of preparation, expiry date and preparer's initials.

- Buffers (TE, TAE, TBE, sodium acetate) are prepared and stored at room temperature for up to six months.
- Ethidium bromide solutions are stored at 2–8 °C protected from light.
- Enzyme solutions (RNase A, Proteinase K, lyticase, Taq DNA polymerase)
- dNTP and primer solutions are stored in aliquots at -20 °C, thawed on ice and added to the reaction immediately before use; repeated freeze-thaw cycles must be avoided.
- 2-mercaptoethanol-containing buffers and any solution containing a labile enzyme are prepared fresh on the day of use.

Any solution showing turbidity, precipitation, discoloration or microbial growth is discarded.

9.3.2 Safety Precautions

- Phenol and Tris-equilibrated phenol are corrosive and cause severe skin burns; handle only in a fume hood wearing nitrile gloves, a laboratory coat and eye protection, and keep polyethylene glycol or a proprietary phenol spill kit available.
- Chloroform and acetonitrile are volatile and toxic by inhalation and must be handled in a fume hood.
- Trifluoroacetic acid and formic acid are corrosive and volatile. 2-mercaptoethanol is toxic and malodorous and must be handled in a fume hood.
- Ethidium bromide is a mutagen; handle with gloves, use a designated work area, and collect all contaminated gels, tips and buffer as chemical waste for disposal in accordance with institutional policy.
- Ultraviolet transilluminators require a UV-blocking face shield.
- All fungal cultures are handled in a biological safety cabinet: a Class II cabinet is adequate

for routine moulds and yeasts, whereas cultures of the thermally dimorphic fungi (*Histoplasma capsulatum*, *Coccidioides* spp., *Blastomyces* spp., *Talaromyces marneffei* and *Paracoccidioides* spp.) are Risk Group 3 agents and must be manipulated only in a Class II cabinet within a BSL-3 facility, with sealed plates and no procedure that may generate infectious aerosols performed on the open bench.

Solution compositions and preparation methods are given in **Tables A-I** below.

A. Lysis Buffer for Mycelial DNA Extraction

Component	Stock Conc.	Working Conc.	Volume/Amount per 100 mL	Purpose
Tris-HCl, pH 8.0	1 M	100 mM	10 mL	Buffers pH, stabilizes DNA
EDTA, pH 8.0	0.5 M	20 mM	4 mL	Chelates Mg^{2+}/Ca^{2+}; inhibits nucleases
NaCl	5 M	250 mM	5 mL	Ionic strength; aids downstream protein/polysaccharide precipitation
SDS	10% (w/v)	2%	20 mL	Anionic detergent; lyses membranes, denatures proteins
Proteinase K	20 mg/mL	100–200 µg/mL	0.5–1 mL (added fresh)	Digests wall/membrane-associated proteins, inactivates nucleases

Preparation: Combine Tris, EDTA and NaCl in water and autoclave; at room temperature, slowly mix in the SDS to avoid excessive foaming; bring to volume with Nuclease-free water. Store base buffer (without Proteinase K) at RT. Add Proteinase K (stored at -20 °C) fresh immediately before use.

B. Lysis Buffer for Yeast DNA Extraction

Component	Stock Conc.	Working Conc.	Volume/Amount per 100 mL	Purpose
Tris-HCl, pH 8.0	1 M	50 mM	5 mL	Buffers pH
EDTA, pH 8.0	0.5 M	10 mM	2 mL	Chelates cations; inhibits nucleases
NaCl	5 M	100 mM	2 mL	Ionic strength
SDS	10% (w/v)	1%	10 mL	Denatures proteins, lyses membranes

Component	Stock Conc.	Working Conc.	Volume/Amount per 100 mL	Purpose
Proteinase K	20 mg/mL	100–200 µg/mL	0.5–1 mL (added fresh)	Digests residual wall/membrane proteins and nucleases

Preparation: Combine Tris, EDTA and NaCl in water and autoclave; at room temperature, slowly mix in the SDS to avoid excessive foaming; bring to volume with Nuclease-free water. Store base buffer (without Proteinase K) at RT. Add Proteinase K (stored at -20 °C) fresh immediately before use.

C. TAE Buffer (Tris-Acetate-EDTA)

Component	Stock Conc.	Working Conc.	Volume/Amount per 100 mL (1×)	Purpose
Tris base	50× stock: 242 g/L	40 mM	2 mL of 50× stock	Buffers pH
Glacial acetic acid	50× stock: 57.1 mL/L	20 mM (acetate)		Provides acetate counter-ion, conductivity
EDTA, pH 8.0	50× stock: 100 mL of 0.5M/L	1 mM		Chelates divalent cations, protects DNA from nuclease activity

Preparation of 50× stock (per 1 L): Dissolve 242 g Tris base in ~700 mL water; add 57.1 mL glacial acetic acid and 100 mL 0.5 M EDTA (pH 8.0); adjust to 1 L with water. For working 1× buffer: dilute 2 mL of 50× stock into 98 mL water per 100 mL. Store at RT.
Note: Preferred for larger fragments, and longer and faster runs; lower buffering capacity than TBE.

D. TBE Buffer (Tris-Borate-EDTA)

Component	Stock Conc.	Working Conc.	Volume/Amount per 100 mL (0.5×)	Purpose
Tris base	10× stock: 108 g/L	45 mM	5 mL of 10× stock	Buffers pH
Boric acid	10× stock: 55 g/L	45 mM		Forms borate complexes with DNA backbone;

Component	Stock Conc.	Working Conc.	Volume/Amount per 100 mL (0.5×)	Purpose
				higher buffering capacity
EDTA, pH 8.0	10× stock: 40 mL of 0.5M/L	1 mM		Chelates divalent cations

Preparation of 10× stock (per 1 L): Dissolve 108 g Tris base and 55 g boric acid in ~800 mL water; add 40 mL 0.5 M EDTA (pH 8.0); adjust to 1 L. For working 0.5× buffer: dilute 5 mL of 10× stock into 95 mL water per 100 mL. Store at RT.
Note: Better resolution for small fragments and longer electrophoresis runs; more stable for reuse than TAE.

E. Phenol:Chloroform:Isoamyl Alcohol (25:24:1)

Component	Stock/Form	Ratio in Mix	Volume per 100 mL Total Mix	Purpose
Phenol (Tris-buffered, pH 8.0)	Redistilled, equilibrated	25 parts	50 mL	Denatures and precipitates proteins at aqueous-organic interface
Chloroform	Reagent grade	24 parts	48 mL	Enhances phase separation; removes residual lipids and denatures proteins
Isoamyl alcohol	Reagent grade	1 part	2 mL	Reduces foaming; stabilizes clean interface between phases

Preparation: Equilibrate phenol to pH ≥ 7.8 (Tris-saturated) before use — unequilibrated acidic phenol partitions DNA into the organic phase. Combine equilibrated phenol, chloroform, and isoamyl alcohol in a 25:24:1 ratio. Store in an amber glass bottle at 4°C, overlaid with a thin layer of Tris buffer to prevent oxidation; discard if the mixture turns pink/yellow (indicates oxidation).

F. Proteinase K

Parameter	Value
Stock concentration	20 mg/mL (reconstituted in nuclease-free water or 50% glycerol/TE for long-term storage)
Working concentration in lysis buffers	50–400 µg/mL (application-dependent; see buffers 1–3 above)
Storage	Aliquot and store at −20°C; stock at 4°C short-term

Function: Broad-spectrum serine protease; digests histones, structural, and wall/membrane-associated proteins, and inactivates co-purifying nucleases (DNases/RNases), freeing intact genomic DNA**.**

Preparation: Reconstitute lyophilised powder in nuclease-free water to 20 mg/mL; aliquot to avoid repeated freeze-thaw. Always add to lysis buffers fresh, immediately before use - never autoclave or heat-sterilise with the buffer, as heat inactivates the enzyme.

G. Gel Loading Buffer (6×) for DNA

Component	Stock/Form	Working Conc. (6×)	Amount per 100 mL	Purpose
Glycerol	Neat (100%)	30% (v/v)	30 mL	Increases sample density so it sinks into the gel well
Bromophenol blue	Solid	0.25% (w/v)	0.25 g	Tracking dye; co-migrates at ~300 bp in standard agarose gels
Xylene cyanol FF	Solid	0.25% (w/v)	0.25 g	Tracking dye; co-migrates at ~4000 bp, tracks slower migration front
EDTA, pH 8.0 (optional)	0.5 M	10 mM	2 mL	Chelates cations; optional stabiliser

Preparation: Dissolve dyes and EDTA in a portion of water, add glycerol, then bring to final volume with water. Mix thoroughly (dyes dissolve slowly); store at RT or 4°C, protected from light. Dilute 1 µL per 5 µL DNA sample (6× → 1× final).

H. Ethidium Bromide Solution (DNA staining)

Parameter	Stock Conc.	Working Conc.	Volume per 100 mL	Purpose
Ethidium bromide	10 mg/mL (in water)	0.5 µg/mL	5 µL of stock per 100 mL buffer/gel volume	Intercalates between DNA base pairs; fluoresces under UV, enabling band visualisation

Preparation: Dissolve EtBr powder in water to 10 mg/mL; stir several hours (light-sensitive — wrap container in foil); store at 4°C in a dark bottle. Add to molten agarose or to a post-run staining bath at 0.5 µg/mL final.
Note: Potent mutagen — always handle with gloves and dedicated equipment; dispose of via institutional hazardous waste protocol, not down the drain.

I. Primer Solutions

Parameter	Stock Conc.	Working Conc.	Preparation Detail	Purpose
Primer stock	100 µM	—	Reconstitute lyophilised primer in nuclease-free water or TE (pH 8.0) based on nmol yield stated on the synthesis/vendor report	Concentrated stock for long-term storage
Primer working solution	100 µM stock	10 µM	Dilute 1:10 (e.g., 10 µL stock + 90 µL nuclease-free water)	Ready-to-pipette dilution for routine PCR/qPCR setup
Final in PCR/qPCR reaction	10 µM working	0.1–0.5 µM (assay-dependent)	Add per master mix protocol	Forward/reverse primers flank the target sequence, providing free 3′-OH ends for polymerase-mediated

Parameter	Stock Conc.	Working Conc.	Preparation Detail	Purpose
				extension and defining amplicon specificity

Preparation: Briefly centrifuge lyophilised primer tubes before opening to collect material at the bottom. Resuspend to 100 µM stock, vortex, aliquot into single-use volumes to minimise freeze-thaw cycles, and store at −20°C. Prepare 10 µM working dilutions in nuclease-free water for bench use; store working dilutions at −20°C.

9.4 DNA isolation protocols

9.4.1 Isolation of Genomic DNA from Yeast (Radford, 1991)

1. Take a loopful of overnight-grown yeast culture and place it in a 1.5 mL microfuge tube.
2. Wash the pellet twice with sterile distilled water, centrifuging at 7,500 rpm for 4 minutes after each wash.
3. Resuspend the pellet in 400 µL of breaking buffer (100 mM Tris-HCl, pH 8.0; 50 mM EDTA; 1% SDS).
4. Add 0.3 g of sterile acid-washed glass beads (425–600 µm) to the cell suspension, then add 400 µL of phenol:chloroform (1:1).
5. Vortex vigorously for 5 minutes, then cool on ice for 30 seconds.
6. Centrifuge at 13,000 rpm, room temperature, for 5 minutes. Transfer the aqueous (upper) phase to a fresh sterile microfuge tube and add an equal volume of chloroform:isoamyl alcohol (24:1).
7. Centrifuge at 10,000 rpm for 7 minutes.
8. Collect the aqueous phase; add 1/10 of the volume of 3 M sodium acetate (pH 5.2) and an equal volume of chilled isopropanol. Mix and incubate at −20°C for 2 hours to precipitate DNA.
9. Pellet the DNA at 10,000 rpm for 5 minutes and discard the supernatant.
10. Wash the pellet with 0.5 mL of 70% ethanol; vortex briefly, centrifuge at 10,000 rpm for 5 minutes, carefully aspirate the ethanol, and air-dry the pellet at 37°C for ~1 hour (or until visibly dry).
11. Resuspend the dried pellet in an appropriate volume of TE buffer. Allow the DNA to dissolve for at least 6 hours at room temperature with intermittent gentle mixing.
12. Assess DNA quality and quantity by electrophoresis on a 0.8% agarose gel and/or by microvolume spectrophotometry (e.g., NanoDrop).
13. Store the purified DNA at −20°C until further use.

9.4.2 Isolation of Genomic DNA from Mycelial Fungi (Möller et al., 1992; Raeder and Broda, 1985)

1. Grow the mycelial isolate on a Sabouraud Dextrose Agar (SDA) slant for 3–4 days at 25°C. Harvest and wash conidia using an L-shaped wire into a 1.5 mL microfuge tube containing sterile normal saline.
2. Inoculate into Sabouraud Dextrose Broth and incubate at 25 -30°C on a rotary shaker (150 rpm) for approximately 3 days.
3. Recover mycelia by filtration through a 50 mL syringe barrel packed with sterile glass wool. Wash the resulting fungal mat with acetone to accelerate dehydration and remove residual pigments/lipids (this step is specific to phaeoid fungi).
4. Transfer the mat onto Whatman No. 1 filter paper and air-dry at 37°C.
5. Transfer the thoroughly dried mycelial mat (0.3–0.5 g) to a clean, chilled mortar. Add a small volume of liquid nitrogen and grind quickly to a fine powder with a pestle, replenishing liquid nitrogen as needed to keep the tissue frozen throughout grinding.
6. Immediately add 2–3 mL of lysis buffer (100 mM Tris-HCl, pH 8.0; 50 mM EDTA; 4–5% SDS), pre-warmed to 65°C, and mix to form a slurry. Transfer 0.7 mL of this suspension to a 1.5 mL microfuge tube.
7. Add 1.4 µL of Proteinase K (20 mg/mL stock) and incubate at 56°C for 1 hour in a dry/water bath with occasional mixing.
8. Add 700 µL of buffered phenol: chloroform (1:1), briefly vortex, and centrifuge at room temperature for 5 minutes at 10,000 rpm.
9. Transfer the upper aqueous phase to a fresh sterile microfuge tube without disturbing the interface. Add an equal volume of chloroform:isoamyl alcohol (24:1), repeat centrifugation, and collect the upper aqueous phase into a fresh tube.
10. Add 1/10th volume of 3 M sodium acetate and two volumes of ice-cold ethanol. Mix and incubate at −20°C for 2 hours to precipitate DNA. Visible DNA threads should appear.
11. Centrifuge at 10,000 rpm for 2–5 minutes at room temperature. Without disturbing the pellet, discard the supernatant and wash the pellet twice with 500 µL ice-cold 70% ethanol.
12. Air-dry the pellet at 37°C until all residual ethanol has evaporated.
13. Resuspend the pellet in an appropriate volume of TE buffer. Allow the DNA to dissolve for at least 6 hours at room temperature with intermittent gentle mixing.
14. Assess DNA quality and quantity by electrophoresis on a 0.8% agarose gel or using a microvolume spectrophotometer (eg. NanoDrop).

9.4.3 Quantitative and qualitative determination of DNA

There are two methods to check the quality of extracted DNA:

i. **Gel electrophoresis:**

Agarose gel preparation

1. To check the quality of the DNA, a 0.8% agarose gel should be prepared in 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 in a microwave or on a hot plate for 1-2 minutes. (Note: Uniform clear solution should be seen)

4. Allow it to cool to room temperature (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 the 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 dye and mix it with 8 µL of genomic DNA.
2. Mix it without bubble formation and load into the 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 gel's progress by observing dye movement.
6. When electrophoresis has completed, turn off the power supply and remove the lid of the gel box.
7. 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.
8. Remove the gel from the gel tray and expose it to UV light. This is most commonly done using a gel documentation system. DNA bands should show up as fluorescent bands. Take a picture of the gel.
9. Properly dispose of the gel and running buffer in accordance with institutional regulations.

ii) **Microvolume Spectrophotometer:**

1. Measure 1µl of DNA Sample at 340 nm wavelength.
2. Note the OD and 260/280 ratio.
3. 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.
4. 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.
5. 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.

9.5 PCR protocol (Kramer and Coen, 2006; Lorenz, 2012)

Optimising a specific PCR can be time-consuming and complex due to the numerous parameters involved. These parameters include the following:

- Quality and concentration of DNA template.
- Design and concentration of primers.
- Concentration of Mg^{2+} ions.

- Concentration of four dNTPs.
- PCR buffer system.
- Selection and concentration of DNA polymerase.
- PCR thermal cycling conditions.
- Addition and concentration of PCR additives and co-solvents.

9.5.1 Materials

- Template DNA
- Forward and reverse primers
- $MgCl_2$
- dNTPs (dATP, dCTP, dGTP, dTTP)
- 10× PCR buffer having 500 mM KCl, 100 mM Tris-HCl, pH 8.0
- DNA Polymerase (Taq, Pfu, Tsg polymerase)
- PCR additives (glycerol, DMSO, formamide) – Optional

9.5.2 Setting up PCR

The common volume of PCR used per reaction mixture is usually 10, 25 or 50 µL. All reaction components can be mixed in a 0.5 mL PCR tube in any sequence, except for DNA polymerase, which should be added last. It is recommended to mix all the components right before PCR cycling.

For each PCR, the master mix is prepared by mixing the components shown in **Table 9.2**.

Table 9.2 Components of PCR master mix (for 50 µL reaction mixture)

PCR Buffer with $MgCl_2$ (10×)	5 µL
Primers (forward and reverse; 10 µM)	2 µL of each
Each dNTP (2.5 mM)	4 µL
DNA polymerase (3U)	0.5 µL
Template DNA (200–500 ng)	2–10 µL
Nuclease-free water	Make up to 50 µL

9.5.3 PCR Cycling (Kramer and Coen, 2006):

A typical PCR program starts with an initial denaturation step at 95°C for 2–5 minutes, followed by 30 cycles of denaturation at 94°C for 1 minute, primer annealing at 45–65°C (decided by primer Tm) for 1 minute, primer extension at 72°C for 1 minute and a final extension step at 72°C for 5 to 10 minutes.

9.5.4 Verifying PCR Amplification

About 8-10 µL of PCR product is loaded onto a 1–2% agarose gel to check for the presence of PCR bands. The gel is visualised by staining with ethidium bromide. A successful PCR amplification should yield a specific band of the expected size, without nonspecific bands or smears.

- If there is a single sharp band, dilute the amplicon with nuclease-free water in a 1:10 ratio.
- Gel extraction method: If there are more than one bands in the electrophoresis, it is recommended to cut the specific band by visualising on the UV transilluminator using scalpel blade. Transfer the cut band into a 1.5 mL microfuge tube and weigh it on the weighing balance. Purify the DNA using a Gel Extraction Kit (eg., Qiagen Gel Extraction Kit).

9.5.5 Sequencing PCR amplicons

Sequencing of PCR amplicons can be performed using the genetic analyser available in the set-up.

Table 9.3. The amount of template to use in a cycle sequencing reaction is given below:

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.

Steps in sequencing (Table 9.4)

For DNA sequencing, the following reagents are required

A. 5X sequencing buffer (ThermoFischer)
B. Big dye terminator (version 3.1, ThermoFischer)
C. 6 picomole primer.
D. MilliQ (Deionised double distilled water)

Table 9.4. DNA Sequencing: Master Mix preparation

Reagents	Quantity in µl
H_2O	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 the mixture.

Sequencing protocol

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

Step	Cycles
1.	T=96˚C for 1 minute.
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
6.	4°C Hold

d. Start the run in the thermal cycler

Note:

- Within 12 hours of run completion – store at 0 to 4°C.
- After 12 hours of run completion – store at -20°C for 2 to 3 days.
- After storage and before opening the tube, briefly centrifuge to bring the contents to the bottom.

9.5.6 Purification of sequencing reaction:

Ethanol /EDTA/Sodium Acetate Precipitation

1. Mix the sequencing PCR product by gentle tapping and briefly spin the tube in the centrifuge.
2. Add 2 µL 3 M sodium acetate pH 4.6 + 2.0 µL 125mM EDTA (Freshly prepared) + 10 µL Milli-Q water in 10 µL of sequencing PCR reaction,
3. Mix well by tapping and briefly centrifuge.
4. Add 80 µL of absolute ethanol and mix properly by inverting the tube 10 times Important: Do not centrifuge at this step

5. Incubate the tube for 15-20 minutes at -20°C
6. Centrifuge at13,000 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.
9. Centrifuge at13,000 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
    Important: There should be no ethanol left in this step.
13. Add 10 µL HiDi (Formamide provided by ABI), mix the pellet properly, short spin, and incubate the mix at 37°C for 15 minutes; then mix the pellet by tapping and quick spin the mix. Heat the reaction at 95°C for 5 minutes and quickly chill on ice for 10 minutes.
    Note: Sample can be stored at 4 °C at this step.
14. Load the sample in the Genetic analyzer for capillary electrophoresis as per the machine protocol.

9.6 Sequence editing and BLAST analysis

After sequencing, the obtained chromatogram files are first viewed in FinchTV to assess the quality of nucleotide peaks and ensure accurate base calling. The sequence files are then imported into SeqMan for editing, where low-quality ends are trimmed, and clean consensus sequences are generated. Generate consensus sequences from forward and reverse reads. Once high-quality sequences are obtained, they will be subjected to BLAST analysis for identification. Use the DNA sequences for identification using nucleotide BLAST search of NCBI (http: //blast.ncbi.nlm.nih.gov/blast) or Westerdijk Fungal Biodiversity Institute (http://www.westerdijkinstitute.nl/).

Chapter 10: Antifungal Susceptibility Testing

Antifungal susceptibility testing (AFST) is important for guiding antifungal therapy, monitoring emerging resistance, and supporting antifungal stewardship. Standardised reference methods developed by the Clinical and Laboratory Standards Institute (CLSI) and the European Committee on Antimicrobial Susceptibility Testing (EUCAST) have enabled reproducible and comparable susceptibility testing across laboratories. The CLSI broth microdilution reference methods include the M27 (M27, 4th ed., 2017) document for susceptibility testing of yeasts, the M38 (M38 3rd ed., 2017) document for filamentous fungi, and the M44 (M44, 3rd ed., 2018) document for antifungal disk diffusion testing of selected yeast species with breakpoints, ECVs and QC ranges published in the current supplements CLSI M27M44S (4th ed., 2026), M38M51S (4th ed., 2026) and M57S (4th ed., 2022). These documents provide standardised recommendations for inoculum preparation, test conditions, endpoint interpretation, quality control, and, where available, clinical breakpoints or epidemiological cutoff values. Similarly, EUCAST has developed standardised broth microdilution methodologies for both yeasts and moulds (EUCAST E.Def 7.4, 2025; E.Def 9.4, 2022; E.Def 11.0, 2025).

Although broth microdilution remains the reference standard, commercially available gradient diffusion methods are also available. These include the Etest (bioMérieux), M.I.C. Evaluator (Oxoid), and Ezy MIC™ Strip (HiMedia), which provide minimum inhibitory concentration (MIC) values by using predefined antifungal concentration gradients on agar media (Tan *et al*., 2024; Mushtaq *et al*., 2010).

10.1 Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeasts: M27, 4th edition, CLSI, USA. (The method described here is incorporated from the reference document)

10.1.1 Introduction

The method described here is intended for testing yeasts that cause invasive infections. These yeasts encompass *Candida* species and *Cryptococcus neoformans*. The method has not been validated in studies of the yeast form of dimorphic fungi, such as *Blastomyces dermatitidis* or *Histoplasma capsulatum* var. *capsulatum*.

M27-4th ed. is a "reference" standard that provides a consensus-developed method to facilitate agreement among laboratories in measuring yeast susceptibility to antifungal agents. An important use of a reference method is to provide a standard basis for developing other methods, thereby achieving interlaboratory agreement within specified ranges. For example, broth microdilution methods that use an indicator dye to facilitate breakpoint determination have been designed to produce results that parallel those obtained with the broth microdilution reference method.

10.1.2 Preparing Antifungal Agents

Source

Antifungal standards or reference powders can be obtained commercially, directly from the drug manufacturer, or from commercial sources. 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 [usually expressed in micrograms (µg) or International Units per mg of powder], and its expiration date. The powders should be stored as recommended by the manufacturers, or at -20°C or below in a desiccator (preferably under vacuum). When the desiccator is removed from the freezer, it should be allowed to come to room temperature before opening (to avoid condensation).

Weighing Antifungal Powders

All antifungal agents are assayed for standard units of activity. The assay units can vary widely from the powder's actual weight and often differ within a drug production lot. Thus, a laboratory must standardise its antifungal solutions based on assays of the antifungal powder lots used.

Either of the following formulae may be used to determine the amount of powder or diluent needed for a standard solution:

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

Or

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

The antifungal agent powder should be weighed on an analytical balance calibrated with approved reference weights from a national metrology organisation. Usually, it is advisable to accurately weigh a portion of the antifungal agent greater than the required amount and to calculate the volume of diluent needed to obtain the desired concentration.

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:

$$\text{Weight (mg)} = \frac{100 \text{ mL} \times 1280 \text{ µg/mL}}{750 \text{ µg/mg}} = 170.7 \text{ mg} \quad (3)$$

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

$$\text{Vol. (mL)} = \frac{182.6\ \text{mg} \times 750\ \mu\frac{\text{g}}{\text{mg}}}{1280\ \mu\frac{\text{g}}{\text{mL}}} = 107.0\ \text{mL}$$

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

Preparing Stock Solutions

Antifungal stock solutions are to be prepared at concentrations of at least 1280 µg/mL or ten times the highest concentration to be tested, whichever is greater. However, some antifungal agents with limited solubility may need lower concentrations. In all cases, information provided by the drug manufacturer should be considered when determining solubility.

Use of Solvents Other than Water

Some drugs must be dissolved in solvents other than water. 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 sulfoxide (DMSO), ethyl alcohol, polyethylene glycol, and carboxymethyl 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 the final strength in the test medium. This procedure avoids dilution artefacts caused by the precipitation of low-solubility compounds in aqueous media.

For example, to prepare a broth microdilution test series containing a water-insoluble drug that can be dissolved in DMSO, with the highest desired test concentration of 16 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 1,600 µg/mL. Next, prepare further dilutions of this stock solution in DMSO. The solutions in DMSO will be diluted 1:50 in test medium and further diluted 2-fold upon inoculation, resulting in a final DMSO concentration of 1%. At this concentration (without drug), DMSO should be used as a dilution control in the test. **Tables 10.1** and **10.2** are depicting the dilution schemes of water-insoluble and water-soluble antifungal agents respectively.

The example above assumes 100% potency of the antifungal powder. If the potency differs, the calculations mentioned above should be applied.

Table 10.1: Scheme for preparing dilutions 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 Add DRUG Amount (ml) +	From Tube 1 -	From Tube 1 0.5 +	From Tube 1 0.5 +	From Tube 1 0.5 +	From Tube 4 0.5 +	From Tube 4 0.5 +	From Tube 4 0.5 +	From Tube 7 0.5 +	From Tube 7 0.5 +	From Tube 7 0.5 +	Step 1 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	

Table 10.2: Scheme for preparing dilution series 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
Tube #	TUBE 1	2X TUBE 2	4X TUBE 3	2X TUBE 4	4X TUBE 5	8X TUBE 6	2X TUBE 7	4X TUBE 8	8X TUBE 9	2X TUBE 10	
Source Add DRUG Amount (ml) +	From Stock 1.0	From Tube 1 1.0 +	From Tube 1 1.0 +	From Tube 3 1.0 +	From Tube 3 0.5 +	From Tube 3 0.5 +	From Tube 6 1.0 +	From Tube 6 0.5 +	From Tube 6 0.5 +	From Tube 9 1.0 +	Step 1 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 5X (1:4)
RPMI 1640 (ml)	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	
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	

Filtration

Normally, stock solutions do not support the growth of contaminating microorganisms, so they can be considered sterile. If additional sterility assurance 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, the absence of adsorption should be confirmed by checking and then documenting the results of the appropriate QC assays.

Storage

Sterile stock solutions should be dispensed in small volumes into sterile polypropylene or polyethene vials, carefully sealed, and stored (preferably at -60°C or below, but never at a temperature greater than -20 °C). Vials should be removed from storage as needed and used the same day. Any unused antifungal agent should be discarded at the end of the day. Most antifungal agent stock solutions can be stored at or below -60°C for 6 months or more without significant loss of activity. In all cases, any instructions provided by the drug manufacturer need to be considered and supersede these general recommendations. Any significant deterioration in antifungal agents may be determined by reviewing susceptibility testing results using QC strains.

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; anidulafungin, 0.015 to 8 µg/mL; caspofungin, 0.015 to 8 µg/mL; fluconazole, 0.125 to 64 µg/mL; flucytosine, 0.125 to 64 µg/mL; isavuconazole, 0.0313 to 16 µg/mL; ketoconazole, 0.0313 to 16 µg/mL, itraconazole and new triazoles 0.0313 to 16 µg/mL; micafungin, 0.015 to 8 µg/mL; and ravuconazole 0.0313 to 16 µg/mL.

10.1.3 Test Procedures

Preparing Growth Medium

A completely synthetic medium should be used for antifungal susceptibility testing. Roswell Park Memorial Institute (RPMI) 1640 culture medium, with glutamine, without bicarbonate, and with phenol red as a pH indicator, has been found to be at least as satisfactory as several other synthetic media and has been used to develop the standard.

Preparing Buffers

Media should be buffered to a pH of 7.0 ± 0.1 at 25°C. A buffer should be selected that does not antagonise antifungal agents. Tris buffer is unsatisfactory because it antagonises flucytosine's activity. Zwitterionic buffers are preferable to buffers that readily traverse the cell membrane, such as phosphate buffers, because the latter can theoretically produce unexpected interactions with antifungal agents. One buffer that has been found to be satisfactory for antifungal testing is MOPS (3-(N-morpholino)propanesulfonic acid) (final concentration of 0.165 mol/L at pH 7.0). The pH of each batch of medium should be measured with a pH meter when prepared; it should be between 6.9 and 7.1 at room temperature (25°C). MIC performance characteristics for each batch of broth are evaluated using a standard set of quality-control organisms.

Preparing Diluted Antifungal Agents

The conditions for preparing and storing diluted antifungal agents include:

- Using sterile 12 × 75 mm plastic test tubes to prepare drug dilutions and sterile, disposable, multiwell microdilution plates (96 U-shaped wells made with untreated polystyrene) to perform the tests.
- Using a growth control tube containing RPMI 1640 culture medium without antifungal agents (but with nonaqueous solvent when necessary) for each organism tested.

Note: Some antifungal agents may be affected by the use of treated plasticware; therefore, glassware or untreated plasticware should be used for preparing drug dilutions and microtiter trays.

Water-Soluble Antifungal Agents

For water-soluble antifungal agents, twofold dilutions may be prepared by volumetric dilution in broth. The procedure for antifungals that are not water-soluble differs from that for water-soluble agents and is described below.

The total volume of each prepared dilution depends on the number of tests performed. Because 0.1 mL of each antifungal drug dilution is used per test, 1 mL is sufficient for about 9 tests, allowing for pipetting. A single pipette should be used to measure all diluents and to add the stock antifungal agent solution to the first tube. A separate pipette should be used for each remaining dilution in that set. Because the drugs will be diluted 1:10 when combined with the inoculum, the working antifungal agent solutions are 10 times more concentrated than the final concentrations. Many people find working with 1:10 dilutions easy and convenient. However, some automated pipettes deliver only 1.0 or 0.1 mL volumes; therefore, a 1:11 ratio 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 of drug are obtained.

Water-insoluble Antifungal Agents

For water-insoluble antifungal agents, an initial dilution series should be prepared at 100 times the final concentration in an appropriate solvent.

The broth microdilution test is performed using sterile, disposable, multiwell microdilution plates (96 U-shaped wells). Each nonaqueous solution should then be diluted 50-fold in RPMI 1640 culture medium while adding into the respective wells of the multi-well microdilution plate, to achieve two-fold strength needed for the test. The drug concentrations are dispensed into the wells of rows 1–10 of the microdilution plates at 0.1 mL volumes using a multichannel pipette. Column 1 contains the highest drug concentration, and column 10 contains the lowest drug concentration. These plates may be sealed in plastic bags and stored frozen at -70°C for up to 6 months without deterioration of drug potency.

10.1.4 Preparing the Inoculum

The steps for preparation of the 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 must be maintained at 35°C throughout.
2) The inoculum should be prepared by picking five colonies of approximately 1 mm in diameter from a 24-hour-old culture of *Candida* species or a 48-hour-old culture 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) or sterile water.
3) The resulting suspension should be vortexed for 15 seconds, and the cell density adjusted with a spectrophotometer by adding sufficient sterile saline to increase the transmittance to that produced by a 0.5 McFarland standard at 530 nm wavelength. This procedure yields a yeast stock suspension containing 1×10^6 to 5×10^6 cells per mL. A working suspension is prepared by diluting the stock suspension 1:20, then diluting 1:50 in RPMI 1640 medium, yielding 1×10^3 to 5×10^3 cells per mL.

10.1.5 Inoculating and Incubating Susceptibility Tests

Each well of a microdilution tray is inoculated on the day of the test with 100 µL of the corresponding 2× diluted inoculum suspension.

The (two-fold) inoculum prepared as above is diluted 1:1 when the drug-wells are inoculated, and the desired final inoculum size is achieved (0.5×10^3 to 2.5×10^3 CFU/mL).

The growth control wells contain 100 µL of sterile, drug-free medium and are inoculated with 100 µL of the corresponding diluted (2×) inoculum. The QC organisms are tested in the same manner and are included each time a batch of isolates are tested. Sterility control (drug-free medium only) can be performed in column 12 of the microdilution plate.

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

For isolates in which clumping hinders the application of these definitions, dispersion of the yeast suspension by pipetting, vortexing, or other techniques can help.

Except for *C. neoformans*, tubes are incubated (without agitation) at 35°C for 24 hours (or up to 48 hours for isolates that are not growing well at 24 hours). When testing *C. neoformans*, incubate the tubes for 70 to 74 hours before determining results.

10.1.6 Reading Minimum Inhibitory Concentration Results

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

Amphotericin B: For amphotericin B, endpoints are typically easily defined, and the MIC is read as the lowest drug concentration that prevents any discernible growth. Trailing endpoints with amphotericin are uncommon.

Flucytosine and azole antifungals: For flucytosine and, especially, for azoles such as fluconazole and ketoconazole, endpoints are typically less sharp and may be a significant source of variability. A less stringent endpoint (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 the same across all drug concentrations above the MIC. The allowable turbidity can be estimated by diluting 0.2 mL of drug-free control growth with 0.8 mL of media to produce an 80% inhibition standard. Even dispersion of clumps, which can become evident after incubation, can improve the reproducibility of endpoint determination. Reference strains of defined susceptibility can also be used to train new personnel.

Echinocandin Antifungal Agents: For echinocandins, MIC endpoints should be determined after 24 hours of incubation and read as the lowest drug concentration to produce a prominent decrease in turbidity (≈ 50% reduction). This corresponds to approximately a 50% reduction in growth relative to the antifungal agent–free growth control.

10.1.7 Interpretation of Results

Interpretive breakpoints have been established only for some organism-drug combinations at present. The clinical relevance of testing other organism-drug combinations remains uncertain, but the relevant information can be summarised 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 the detection of such isolates, and all that can be concluded at present 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.

For *Candida* spp. without breakpoints, epidemiological cutoff values (ECVs) that define the wild-type (WT) distribution limit have been established for common *Candida* spp. and amphotericin B and may be useful for distinguishing between WT and non-wild-type (NWT) isolates (i.e., those with intrinsic or acquired known resistance mechanisms)

Fluconazole: Species-specific breakpoints for *C. albicans*, *Candida glabrata*, *Candida parapsilosis*, and *Candida tropicalis* and fluconazole have been established. These breakpoints are not applicable to *Candida krusei*; therefore, identification to the species level is necessary to accurately interpret and report MICs. For *C. glabrata* isolates with MICs in this range, the clinical appropriateness of fluconazole and the dosing regimen should be determined by the treating clinician in accordance with current clinical guidance. The utility of testing *C. neoformans* isolates is unknown, but data suggest a correlation between elevated MICs and clinical failure.

Itraconazole: Previously published CLSI breakpoints for itraconazole were established with limited clinical data, and emerging data now suggest those breakpoints were incorrect. Therefore, the previously published breakpoints should not be used.

For *Candida* spp. without breakpoints, ECVs that define the WT distribution limit have been established for *Candida* spp. and itraconazole and may be useful for distinguishing WT from NWT isolates.

Voriconazole: Breakpoints for *C. albicans, C. parapsilosis, C. tropicalis, and C. krusei* and voriconazole have been established. These data are drawn from in vitro tests, animal models, and six clinical trials, with the majority taken from a clinical trial in non-neutropenic patients with candidemia. In other settings, the clinical relevance of breakpoints is uncertain.

Posaconazole, Ravuconazole, and Isavuconazole: Experience with testing posaconazole and ravuconazole using the broth dilution methodology indicates that *Candida* and *Cryptococcus* spp. MICs vary between 0.03 and 16 µg/mL, with the majority of isolates inhibited by ≤ 1 µg/mL for these antifungal agents. For isavuconazole, yeast MICs range from 0.008 to 8 µg/mL, with the majority (90% of tested isolates) inhibited at 0.5 µg/mL. However, data are not yet available to indicate a correlation between MIC and treatment outcome with these agents.

Ketoconazole: Experience to date with the procedures described in this standard indicates that yeast MICs range from 0.0313 to 16 µg/mL. However, data are not yet available to indicate a correlation between MIC and treatment outcome with ketoconazole.

Echinocandins (Anidulafungin, Caspofungin, and Micafungin): Using procedures described in this standard, MIC survey data for > 2500 patient isolates of *Candida* spp. indicate that MICs vary between 0.007 and 8 µg/mL, with ≥ 99% of isolates inhibited by ≤ 2 µg/mL. However, isolates harbouring *FKS* mutations, the major resistance mechanism to echinocandins, have been identified with MICs that fall below 2 µg/mL. Therefore, species-specific breakpoints have been established to discriminate between potentially resistant mutants and susceptible strains.

Note: For *Candida* spp. without breakpoints, ECVs defining the WT distribution limits have been established, and anidulafungin and micafungin may be useful for distinguishing WT from NWT isolates. Caspofungin susceptibility testing in vitro has been associated with significant

interlaboratory variability, contributing to reports of false resistance when using the reference method described in this chapter. The cause of the variability is unclear. For this reason, no ECVs have been established.

Flucytosine: Based largely on historical data and partially on the drug's pharmacokinetics, interpretive breakpoints for *Candida* spp. and flucytosine have been established. Previously published CLSI breakpoints for flucytosine were established with limited clinical data, and emerging data now suggest those breakpoints were incorrect. Therefore, the previously published breakpoints should not be used.

For *Candida* spp. without breakpoints, ECVs that define the WT distribution limit have been established, and some antifungal agents may be useful for distinguishing WT from NWT isolates.

For *C. auris,* tentative breakpoints given by CDC are used (Fluconazole≥32 µg/mL, Amphotericin B ≥2 µg/mL, Anidulafungin and Micafungin ≥4 µg/mL and Caspofungin ≥2 µg/mL).

10.1.8 Impact of Time Reading: 24 hours versus 48 hours

For broth microdilution, QC limits are established for all antifungal agents at 24 hours. Broth microdilution MICs should be read at:

• 24 hours for the echinocandins, amphotericin B, fluconazole, flucytosine, itraconazole, isavuconazole, posaconazole, ravuconazole, and voriconazole

• 72 hours for *Cryptococcus* spp. isolates against all antifungal agents referenced above

Current breakpoints are defined only for the 24-hour readings.

Agitating the plates is optional and may simplify endpoint reading. Microdilution wells should be scored using a reading mirror, and the growth in each well compared with the growth control (antifungal agent–free) well.

Pipetting, vortexing, or other mixing techniques should be tried when clumping of an isolate hinders scoring. The MIC for amphotericin B is defined as the lowest concentration in which there is no growth (optically clear). For the azoles, echinocandins, and flucytosine, the MIC is defined as the lowest concentration at which there is a 50% decrease in growth.

Some *Candida* spp. isolates, typically *C. albicans* and *C. tropicalis*, exhibit significant trailing growth (partial growth inhibition over an extended antifungal concentration range) for azoles, especially fluconazole and voriconazole. In about 5% of isolates with fluconazole, trailing growth can be so extensive that it makes a susceptible isolate appear completely resistant. Two independent in vivo investigations of this phenomenon using murine models of disseminated candidiasis showed that isolates with this behaviour should be categorised as "susceptible" rather than "resistant." This concept has been corroborated by a clinical demonstration in which

oropharyngeal candidiasis caused by such isolates responds to low doses of fluconazole, typically used to treat susceptible isolates

10.1.9 Other Modifications

In addition to ongoing efforts to simplify the procedures described in this standard, some more fundamental modifications to the method have been developed to address specific problems and are described in **Table 10.3**. These modifications are not part of the current methodology, but interested laboratories may wish to explore their clinical relevance.

Table 10.3: Modifications for special circumstances (CLSI M27-S3, Third International Supplement)

Drug	Organism	Modification
Amphotericin B	*Candida* spp.	Use of antibiotic Medium 3 may enhance detection of resistance, but this medium is not standardised and substantial lot-to-lot variability is possible (Lozano-Chiu et al., 1997)
All drugs	*C. neoformans*	Use of Yeast Nitrogen Base may enhance the growth of *C. neoformans* and improve the clinical relevance of antifungal MICs
All drugs	All organisms	Supplementation of the test medium so that it contains glucose at a final concentration of 20 g/L may simplify endpoint determination

10.1.10 Quality Control

Growth Control: Each broth microdilution series should include a growth control of basal medium without an antifungal agent to assess the viability of the test organisms. In the broth tests, the growth control also serves as a turbidity control for endpoint readings.

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 minimise variation in MIC interpretation among observers. All laboratory personnel who perform these tests should independently read a selected set of dilution tests. The results are recorded and compared with those 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.

Table 10.4 shows the MIC ranges for quality control and reference strains, and the expected ranges for strains found acceptable as quality control strains. Also shown are additional strains that can be useful for conducting reference studies.

Table 10.4: Recommended 24-hour MIC limits for quality control strains for broth microdilution procedures (CLSI-M27M44S 4th Edition)

Organism	Antifungal Agent	MIC QC Range, µg/mL	MIC QC Mode, µg/mL	MICs Within Range, %
Candida albicans ATCC®[a] 90028	Manogepix	0.004–0.016	0.008	100
	Oteseconazole[b]	0.002–0.016	0.004	95.3
Candida krusei ATCC® 6258	Amphotericin B	0.5–2	1	100
	Anidulafungin	0.03–0.12	0.06	97.9
	Caspofungin[c]	0.12–1	0.5	98.8
	Fluconazole	8–64	16	100
	Flucytosine	4–16	8	97.5
	Ibrexafungerp[d]	0.25–1	0.5	100
	Isavuconazole	0.06–0.5	0.25	95.2
	Itraconazole	0.12–1	0.5	95.8
	Ketoconazole	0.12–1	0.5	95.4
	Micafungin	0.06–0.25	0.12–0.25	99.6
	Posaconazole	0.06–0.5	0.25	100
	Rezafungin	0.016–0.12	0.03	99.5
	Voriconazole	0.06–0.5	0.12	98.3
Candida parapsilosis ATCC® 22019	Amphotericin B	0.25–2	0.5	97.1
	Anidulafungin	0.25–2	1	95
	Caspofungin	0.25–1	0.5	96.7
	Fluconazole	0.5–4	2	98.2
	Flucytosine	0.06–0.25	0.12	99.2
	Ibrexafungerp[d]	0.06–0.25	0.12	99.0
	Isavuconazole	0.016–0.06	0.06	90.5
	Itraconazole	0.06–0.5	0.25	95.8
	Ketoconazole	0.03–0.25	0.06/0.12	97.5
	Manogepix	0.008–0.03	0.016	100
	Micafungin	0.5–2	0.5–1	100
	Oteseconazole[b]	0.008–0.06	0.03–0.25	99.6
	Posaconazole	0.03–0.25	0.12	96.7
	Rezafungin	0.25–2	0.5	99.2
	Voriconazole	0.016–0.12	0.016–0.03	100
C. parapsilosis ATCC® 90018	Oteseconazole[b]	0.001–0.008	0.004	99.2

Footnotes

a. ATCC® is a registered trademark of the American Type Culture Collection.

b. For oteseconazole, any of the QC strains are appropriate for routine QC testing.

c. The QC ranges were established using data generated in 2010 from 15 reference laboratories. Since then, caspofungin susceptibility testing has shown significant variation. Therefore, misclassification of susceptible isolates may occur despite acceptable performance of the QC strains within the range shown in this table

d. The 48-hour microdilution MIC QC range for ibrexafungerp is available for *C. krusei* ATCC® 6258: 0.25 to 1 µg/mL. No 48-hour MIC QC range for ibrexafungerp is currently available for *C. parapsilosis* ATCC® 22019.

10.1.11 Result Interpretation as per Clinical Breakpoints and Epidemiological Cutoffs Given by CLSI

Interpretation of antifungal susceptibility testing (AFST) results is based on standardised criteria established by the Clinical and Laboratory Standards Institute (CLSI). Clinical breakpoints (CBPs) are available for selected antifungal agent–species combinations and categorise isolates according to their likelihood of clinical response **(Table 10.5).** Where CBPs are unavailable, epidemiological cutoff values (ECVs) are used to differentiate wild-type (WT) isolates from those with acquired or emerging resistance mechanisms (non-wild-type, NWT). ECVs for *Candida* species where CBPs are not available, and other ascomycetous yeasts are given in **Table 10.6**. ECVs for *Cryptococcus* and basidiomycetous yeasts are presented in **Table 10.7**.

Table 10.5: Minimal inhibitory concentration breakpoints for in vitro broth dilution susceptibility testing of *Candida* spp. and select antifungal agents after 24-hour incubation (CLSI-M27M44S 4th Edition)

Antifungal Agent	Species	MIC Breakpoints and Interpretive Categories, µg/mL			
		S	I	SDD	R
Anidulafungin[a]	*C. albicans*	≤ 0.25	0.5	–	≥ 1
	C. glabrata[b]	≤ 0.12	0.25	–	≥ 0.5
	C. guilliermondii[b]	≤ 2	4	–	≥ 8
	C. krusei[b]	≤ 0.25	0.5	–	≥ 1
	C. parapsilosis[c]	≤ 2	4	–	≥ 8
	C. tropicalis	≤ 0.25	0.5	–	≥ 1
Caspofungin[a,d]	*C. albicans*	≤ 0.25	0.5	–	≥ 1
	C. glabrata	≤ 0.12	0.25	–	≥ 0.5
	C. guilliermondii[b]	≤ 2	4	–	≥ 8
	C. krusei[b]	≤ 0.25	0.5	–	≥ 1
	C. parapsilosis[c]	≤ 2	4	–	≥ 8
	C. tropicalis	≤ 0.25	0.5	–	≥ 1
Fluconazole[e,f]	*C. albicans*	≤ 2	–	4	≥ 8
	C. glabrata[b]	–	–	≤ 32	≥ 64
	C. krusei[b,g]	–	–	–	–
	C. parapsilosis[c]	≤ 2	–	4	≥ 8
	C. tropicalis	≤ 2	–	4	≥ 8
Micafungin[a]	*C. albicans*	≤ 0.25	0.5	–	≥ 1
	C. glabrata[b,h]	≤ 0.06	0.12	–	≥ 0.25
	C. guilliermondii[b]	≤ 2	4	–	≥ 8
	C. krusei[b]	≤ 0.25	0.5	–	≥ 1
	C. parapsilosis[c]	≤ 2	4	–	≥ 8
	C. tropicalis	≤ 0.25	0.5	–	≥ 1
Rezafungin[i]	*C. albicans*	≤ 0.25	–	–	–
	C. auris	≤ 0.5	–	–	–

	C. dubliniensis	≤ 0.12	–	–	–
	C. glabrata	≤ 0.5	–	–	–
	C. krusei[b]	≤ 0.25	–	–	–
	C. parapsilosis[c]	≤ 2	–	–	–
	C. tropicalis	≤ 0.25	–	–	–
Voriconazole[a]	*C. albicans*	≤ 0.12	0.25–0.5	–	≥ 1
	C. glabrata[b,j]	–	–	–	–
	C. krusei[b]	≤ 0.5	1	–	≥ 2
	C. parapsilosis[c]	≤ 0.12	0.25–0.5	–	≥ 1
	C. tropicalis	≤ 0.12	0.25–0.5	–	≥ 1

Abbreviations: ECV, epidemiological cutoff value; I, intermediate; MIC, minimal inhibitory concentration; R, resistant; S, susceptible; SDD, susceptible-dose dependent.

Footnotes

a. For these antifungal agents, the data are based largely on experience with non-neutropenic patients with candidemia. The clinical relevance of the antifungal agents in other settings is uncertain.

b. These *Candida* spp. are also recognised under the following alternate taxonomic names:
   - *C. auris: Candidozyma auris*
   - *C. glabrata: Nakaseomyces glabratus*
   - *C. guilliermondii: Meyerozyma guilliermondii*
   - *C. krusei: Pichia kudriavzevii*

c. When no further species determination can be performed, *C. parapsilosis* breakpoints may be applied in areas where the prevalence of the cryptic species (*C. orthopsilosis* or *C. metapsilosis*) is low (e.g. North America). However, if further species determination identifies one of the cryptic species within the complex, *C. parapsilosis* breakpoints should not be applied. Instead, the laboratory report should indicate that no breakpoints are available for interpretation and that ECVs should be considered (see CLSI document M57S).

d. Caspofungin susceptibility testing in vitro has shown significant interlaboratory variability, leading to reports of likely false resistance when the reference method described in the CLSI document M27 is used. The exact cause of the variability is unclear. When caspofungin is tested, susceptible results may be reported as "susceptible." However, laboratories should confirm "intermediate" or "resistant" results with one of the following options:
   - Perform additional susceptibility testing with micafungin or anidulafungin
   - DNA sequencing of the FKS genes to identify resistance hot-spot mutations in FKS1 (all *Candida* spp.) and FKS2 (*C. glabrata* only) is also an option, although it is currently not widely available and is performed only for research purposes.
   - Send the isolate to a reference laboratory for confirmation

Candida spp. that are resistant to anidulafungin or micafungin or that possess characteristic FKS hot-spot mutations are considered resistant to all echinocandins, including caspofungin, and should be reported as such.

e. Susceptibility depends on achieving the maximum possible blood level. For fluconazole, doses higher than the standard dosing amount (6 mg/kg/day) may be needed in adults with normal renal function and body habitus.

f. For fluconazole, these breakpoints are based on extensive experience with mucosal and invasive infections due to *Candida* spp. When an isolate is identified as *C. glabrata,* and the MIC is ≤ 32 µg/mL, the clinician should determine whether fluconazole is appropriate in the specific clinical context. If so, patients should receive the maximum fluconazole dosage regimen. Expert consultation on selecting a maximum dosage regimen may be useful.

g. Isolates of *C. krusei* are intrinsically resistant to fluconazole. MIC testing is therefore unnecessary, and isolates should always be reported as resistant or intrinsically resistant

h. The selected breakpoints have been established to distinguish resistant variants from susceptible isolates. Differences in breakpoints reflect methodological issues. Due to methodological issues in vitro, the micafungin breakpoint against *C. glabrata* is lower than that of other echinocandins, which does not reflect any inherent clinical differences in efficacy. True differences in antifungal activity among the echinocandins are rare.

i. At this time, only susceptible breakpoints have been set for rezafungin. Once more data are available, intermediate and resistant breakpoints will be added.

j. For *C. glabrata* and voriconazole, current data are insufficient to demonstrate a correlation between in vitro susceptibility testing and clinical outcome.

Table 10.6: Epidemiological cutoff values for in vitro susceptibility testing of *Candida* spp. and other ascomycete yeasts with no breakpoints[a] (CLSI-M57S 4th Ed.)

Antifungal Agent	Species	ECV, µg/mL[b,c,d]
Amphotericin B	*C. albicans*	2
	C. dubliniensis	0.5
	C. glabrata[e]	2
	C. guilliermondii[e]	2
	C. kefyr[e]	2
	C. krusei[e]	2
	C. lusitaniae[e,f]	2
	C. metapsilosis	1
	C. orthopsilosis	2
	C. parapsilosis	1
	C. pelliculosa[e]	1
	C. tropicalis	2
	Saccharomyces cerevisiae	2
Anidulafungin	*C. auris*	1
	C. dubliniensis	0.12
	C. duobushaemulonii	1
	C. haemulonii	0.5
	C. kefyr[e]	0.25
	C. lusitaniae[e]	1
	C. metapsilosis	0.5
	C. orthopsilosis	2
	S. cerevisiae	1
Caspofungin	*C. auris*	0.5
	C. duobushaemulonii	0.25
	C. lusitaniae[e]	1
	C. metapsilosis	0.25
	C. orthopsilosis	1
	S. cerevisiae	2
Fluconazole	*C. dubliniensis*	0.5
	C. duobushaemulonii	32
	C. guilliermondii[e]	8
	C. haemulonii	128[g]
	C. kefyr[e]	1
	C. lusitaniae[e]	1
	C. metapsilosis	4
	C. orthopsilosis	2

	C. pararugosa[e]	16
	C. pelliculosa[e]	8
	C. rugosa[e]	8
	S. cerevisiae	32[h]
Isavuconazole	*C. duobushaemulonii*	0.25
Itraconazole	*C. dubliniensis*	0.25
	C. duobushaemulonii	1
	C. glabrata[e]	4
	C. guilliermondii[e]	2
	C. kefyr[e]	0.5
	C. krusei[e]	1
	C. lusitaniae[e]	1
	C. metapsilosis	1
	C. orthopsilosis	0.5
	C. parapsilosis	0.5
	C. pelliculosa[e]	1
	C. tropicalis	0.5
	S. cerevisiae	2
Micafungin	*C. auris*	0.5
	C. dubliniensis	0.12
	C. duobushaemulonii	0.5
	C. kefyr[e]	0.12
	C. lusitaniae[e]	0.5
	C. metapsilosis	1
	C. orthopsilosis	1
	C. pelliculosa[e]	0.12
	S. cerevisiae	0.5
Posaconazole	*C. albicans*	0.06
	C. dubliniensis	0.12
	C. duobushaemulonii	1
	C. glabrata[e]	1
	C. guilliermondii[e]	0.5
	C. haemulonii	1
	C. kefyr[e]	0.5
	C. krusei[e]	0.5
	C. lusitaniae[e]	0.06
	C. metapsilosis	0.25
	C. orthopsilosis	0.25
	C. parapsilosis	0.25
	C. pelliculosa[e]	2
	C. tropicalis	0.12
	S. cerevisiae	2
Voriconazole	*C. duobushaemulonii*	0.5
	C. glabrata[e]	0.25
	C. haemulonii	2
	C. metapsilosis	0.06
	C. orthopsilosis	0.12
	C. pelliculosa[e]	0.25
	S. cerevisiae	0.5

Footnotes

a. All *Candida* spp. listed are sensu stricto except when stated otherwise.

b. The ECVs in this document were established using broth microdilution as outlined in CLSI document M27. If another methodology is used for susceptibility testing, that method must be validated against broth microdilution before the ECVs are used, just as other methods must be validated before breakpoints established using broth microdilution are used.
c. ECVs capture ≥ 97.5% of the statistically modelled population. Because ECVs are defined by phenotypic rather than genotypic data, in rare instances, ECVs may not identify some potentially resistant isolates (non-wild-type).
d. If the 24-hour growth control is insufficient, it should be incubated for an additional 24 hours.
e. These *Candida* spp. are also recognised under the following alternate taxonomic names:
   - *C. glabrata: Nakaseomyces glabrata*
   - *C. guilliermondii: Meyerozyma guilliermondii*
   - *C. kefyr: Kluyveromyces marxianus*
   - *C. krusei: Pichia kudriavzevii*
   - *C. lusitaniae: Clavispora lusitaniae*
   - *C. pararugosa: Wickerhamiella pararugosa*
   - *C. pelliculosa: Wickerhamomyces anomalus*
   - *C. rugosa: Diutina rugosa*
f. *C. lusitaniae* is not intrinsically resistant to amphotericin B. However, it may develop in vivo resistance to amphotericin B during therapy. When phenotypic resistance was observed in studies, the phenotype was detected only with agar gradient strips and was not detected by broth microdilution methods.
g. The fluconazole ECV for *C. haemulonii* is very high and may be an indication of intrinsic resistance or of limited susceptibility to this agent. A minimal inhibitory concentration (MIC) value lower than the ECV does not imply that the isolate is susceptible to fluconazole.
h. The fluconazole ECV for *S. cerevisiae* is high and may be an indication of intrinsic resistance or of limited susceptibility to this agent. An MIC value lower than the ECV does not imply that the isolate is susceptible to fluconazole.

Table 10.7: Epidemiological cutoff values for in vitro susceptibility testing of *Cryptococcus* spp. and other basidiomycete yeasts with no breakpoints (CLSI-M57S 4th Ed.)

Antifungal Agent	Species (genotype)	ECV, µg/mL[b,c]
Amphotericin B	*C. gattii* (VGI)	0.5
	C. deuterogattii (VGII)	1
	C. neoformans (VNI)	0.5
	Rhodotorula mucilaginosa	2
	Trichosporon asahii	1
Fluconazole	*C. gattii* (VGI)	16
	C. deuterogattii (VGII)	32
	C. neoformans (VNI)	8
	T. asahii	8
Flucytosine	*C. gattii* (VGI)	4
	C. deuterogattii (VGII)	32
	C. neoformans (VNI)	8
Itraconazole	*C. gattii* (VGI)	0.5
	C. deuterogattii (VGII)	1
	C. neoformans (VNI)	0.25
	R. mucilaginosa	4
	T. asahii	1
Posaconazole	*C. neoformans* (VNI)	0.25
	R. mucilaginosa	4

	T. asahii	1
Voriconazole	*C. gattii* (VGI)	0.5
	C. deuterogattii (VGII)	0.5
	C. neoformans (VNI)	0.25
	R. mucilaginosa	16

Footnotes

a. The ECVs for *Cryptococcus* were established for the distinct molecular types. The phylogeny of *Cryptococcus* is currently undergoing revision. VGI and VGII are molecular genotypes of *C. gattii* and *C. deuterogattii* (formerly *C. gattii*), respectively, that can be recognised only by molecular typing of isolates using polymerase chain reaction- or DNA sequencing–based methods, including multilocus sequence typing and amplified-fragment length polymorphism. The molecular types may or may not correspond to unique species, and future editions of this document may name them differently in accordance with accepted taxonomic changes. Globally, VNI is the most common molecular genotype of *C. neoformans*.

b. The ECVs in this document were established using broth microdilution as outlined in CLSI document M27.6. If another method is used for susceptibility testing, it must be validated against broth microdilution before ECVs are used, just as other methods must be validated before breakpoints established by broth microdilution are used.

c. ECVs capture ≥ 97.5% of the statistically modelled population. Because ECVs are defined by phenotypic rather than genotypic data, in rare instances, ECVs may not identify some potentially resistant isolates (non-wild-type).

10.2 Reference Method for Broth Dilution Antifungal Susceptibility Testing of Filamentous Fungi; Approved Standard; M38, 3rd edition CLSI, USA (The method described here is incorporated from the reference document)

10.2.1 Introduction

CLSI document M38, 3rd edition, is a "reference" standard developed through a consensus process to facilitate agreement among laboratories in measuring the susceptibility of moulds to antifungal agents. An important use of a reference method is to provide a standard basis for developing other methods.

The advantages which a standardised method provides are ease of performance, economy, and more rapid results. Any method that produces concordant results with this reference method would be considered in conformity with CLSI document M38, 3rd edition.

The method (M38, 3rd edition) described here is intended for testing common filamentous fungi, including the dermatophytes (*Trichophyton* spp., *Microsporum* spp., and *Epidermophyton* spp.), more prevalent *Aspergillus* spp. and species complexes, *Fusarium* spp. and species complexes, *Rhizopus* spp. and other Mucorales, *Lomentospora prolificans* (*Scedosporium prolificans*), the mycelial form of species included in the *S. schenckii* complex, and dematiaceous (phaeoid [black]) moulds.

10.2.2 Antifungal Agents

For details on the source of antifungals, weighing antifungals, preparing stock solutions, using solvents other than water (appendix), storage of stock solutions, and the number of concentrations tested, see the M27, 4th edition explained above.

10.2.3 Test Procedure

The preparation of broth medium and buffers in this protocol is the same as mentioned above in M27, 4th edition.

10.2.4 Preparing Diluted Antifungal Agent

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

- Use sterile, plastic test tubes to prepare drug dilutions and sterile, disposable, multiwell microdilution plates (96 U-shaped wells) to perform the tests.
- Use a growth control well containing RPMI 1640 broth without antifungal agents (but with a nonaqueous solvent, if necessary) for each organism tested.
- When two-fold dilutions of a water-soluble antifungal are to be used, they may be prepared volumetrically in broth. The procedure for insoluble antifungals differs from that for water-soluble agents and is described below. **Tables 10.8** and **10.9** present the dilution scheme for preparing water-soluble and insoluble antifungal agents, respectively.
- 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 per test, 1.0 mL will be sufficient for about 8 tests (one microdilution tray), allowing for pipetting. A single pipette is used to measure all diluents and then add the stock antifungal solution to the first tube. A separate pipette is used for each remaining dilution in that set. Because the drugs will be diluted 1:2 when combined with the inoculum, the working antifungal solutions are twice as concentrated as the final concentrations.
- For antifungal agents that cannot be prepared as stock solutions in water, such as ketoconazole, amphotericin B, or itraconazole, a dilution series should first be prepared at a 100x final strength in an appropriate solvent. Each of these nonaqueous solutions should now be diluted 1:50 in RPMI 1640 medium.
- For example, if a dilution series with final concentrations ranging from 16 µg/mL to 0.0313 µg/mL is desired, a concentration series from 1,600 to 3.13 µg/mL should first be prepared in DMSO. To prepare 5 mL volumes of diluted antifungal agent (sufficient for 45 tests), first pipette 4.9 mL volumes of RPMI 1640 broth into each of 10 sterile test tubes. Now, using a single pipette, add 0.1 mL of DMSO alone to 4.9 mL of broth (control medium), then the lowest (3.13 µg/mL) drug concentration in DMSO, then the 6.25 µg/mL concentration and continue in sequence up the concentration series, each time adding 0.1 mL volumes to 4.9 mL broth. These volumes can be adjusted according to the total number of tests required. Because the drugs will be diluted 1:2 with the inoculum, the working antifungal solutions are twice as concentrated as the final concentrations.

Table 10.8: Scheme for preparing dilution series 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 5X (1:4)
RPMI 1640 (ml)	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	
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 10.9: Scheme for preparing dilutions 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
		2X	4X	8X	2X	4X	8X	2X	4X	8X	
Tube #	TUBE 1 (Stock) (100 X)	TUBE 2	TUBE 3	TUBE 4	TUBE 5	TUBE 6	TUBE 7	TUBE 8	TUBE 9	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	

10.2.5 Inoculum Preparation

When the risk of substantial spatter or aerosolisation is present, the manipulation should be performed in a Class IIA or IIB biological safety cabinet.

Filamentous fungi that pose a risk for respiratory infection (e.g., *Blastomyces dermatitidis*, *Coccidioides* spp., *Histoplasma capsulatum*, *Paracoccidioides brasiliensis*, *Cladophialophora*

bantiana, *T. marneffei*, and *Rhinocladiella mackenziei*) should be cultivated only by experienced personnel under biosafety level 3 conditions, with stringent room access control and respiratory protection according to applicable biosafety regulations.

Most fungi should be grown on Potato Dextrose Agar for seven days at 35°C or until good conidiation or sporulation is obtained. Initial work demonstrated that reliable non-germinated conidial or sporangiospore suspensions could be prepared by a spectrophotometric procedure. A concentration of viable conidial or sporangiospore test inocula in the range of approximately 0.4×10^4 to 5×10^4 CFU/mL yields the most reproducible MIC data. The steps for preparing the non-dermatophyte mould inoculum are as follows

To induce conidium and sporangiospore formation, most fungi (*Aspergillus* species, *P. boydii*, *R. arrhizus* and *S. schenckii*) should be grown on Potato Dextrose Agar for 7 days at 35°C. *Fusarium* species should be incubated for 18 to 72 hours at 35°C, then for an additional 7 days at 25 to 28°C. Cover seven-day-old 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 pipette. The addition of one drop (approximately 0.01 mL) of polysorbate 20/Tween 20 will facilitate the inoculum preparation of all non-dermatophyte fungi, especially those with hydrophobic spores. 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 the suspension is vortexed for 15 seconds. (Caution: remove the cap carefully as liquids adhering to the cap may produce aerosols upon opening.) The densities of the conidial or sporangiospore suspensions are read and adjusted to an optical density (OD) that ranges from 0.09 to 0.11 (80–82% transmittance) for *Aspergillus* species and *S. schenckii* and 0.15 to 0.17 (68–70 % transmittance) for *Fusarium* species, *P. boydii* and *R. arrhizus*. These suspensions will be diluted 1:50 in the standard medium. Inoculum suspensions of *P. boydii* may require a lower (50%) dilution factor. The 1:50 inoculum dilutions correspond to 2× the required density, approximately 0.4×10^4 to 5×10^4 CFU/mL. The test inoculum will be made in sufficient volume to directly inoculate each well with 0.1 mL of the corresponding diluted inoculum suspension.

Inoculum quantification can be performed by plating 0.01 mL of a 1:10 dilution of the adjusted inoculum onto Sabouraud glucose agar to determine the viable CFU/mL. The plates will be incubated at 28–30°C and observed daily for the presence of fungal colonies. Colonies should be counted as soon as possible after growth becomes visible, especially for *R. arrhizus isolates*. The incubation times will range from 24 hours or less (*R. arrhizus*) to 5 days (*P. boydii*).

10.2.6 Inoculating Broth

Inoculate each well on the day of the test with 0.1 mL of the 2× conidial or sporangiospore inoculum suspension. This step will dilute the drug concentrations, inoculum densities, and, if used, the solvent to the final desired test concentrations. The growth control wells will contain 0.1 mL of the drug diluents (2%) without an antifungal agent. QC and reference organisms are also tested in the same manner and should be included each time an isolate is tested.

10.2.7 Incubation

All microdilution trays are to be incubated (without agitation) at 35°C. Trays containing *Rhizopus* species should be examined after 21 to 26 hours of incubation before MIC results are determined. Most other opportunistic filamentous fungi, including *Fusarium* spp., *Aspergillus* spp., and *Sporothrix schenckii*, are evaluated after 46 to 50 hours of incubation. *P. boydii* is examined after 70 to 74 hours **(Table 10.10)**.

Table 10.10: Recommended incubation times for determining MICs or MECs for non-dermatophyte moulds (CLSI M38 3rd edition)

Antifungal Agent	Fungal Organism	Recommended incubation in hours*
All except echinocandins (amphotericin B, fluconazole, triazoles)	*Rhizopus* spp. and other Mucorales	21–26
	Aspergillus spp. and species complexes *Fusarium* spp. and species complexes *Sporothrix schenckii* species complex *Cladophialophora bantiana* *Exophiala dermatitidis* *Purpureocillium lilacinum* *Paecilomyces variotii*	46–50
	Lomentospora prolificans (*Scedosporium Prolificans*)	70–74
Echinocandins	*Aspergillus* spp. and species complexes *P. variotii*	21–26
	L. prolificans (S. prolificans)	46–72

*Alternative: MIC or MEC results can be read on the first day when sufficient growth (i.e., confluent growth covering the bottom of the well) is present in the growth control well (i.e., contained drug-free medium).

Abbreviations: MEC, minimal effective concentration; MIC, minimal inhibitory concentration.

10.2.8 Reading Results

The MIC is the lowest concentration of an antifungal that substantially inhibits the organism's growth as visually detected. In the conventional microdilution procedure, growth in each MIC well is compared with that of the growth control using a reading mirror.

When testing echinocandin antifungal agents, MEC evaluation provides more consistent and reproducible susceptibility data than the conventional MIC reading. The MEC is the lowest drug concentration at which small, rounded, compact hyphal forms form, compared with the hyphal growth seen in the growth control well. To evaluate the MEC, the growth in each well should be compared with the growth control (antifungal agent–free medium) using a reading mirror or a microscope. Plates should never be agitated (vortexed) before reading, because it will prevent accurate determination of MIC or MEC values. Reference strains of defined susceptibility can be used to train new personnel. The guidelines for reading the results for the various drugs are described below.

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 (100% inhibition). Trailing end points with amphotericin B are not usually encountered, and it is possible that such a pattern could reflect clinically relevant drug resistance.

Flucytosine, fluconazole, and ketoconazole: For flucytosine and especially for azoles, such as fluconazole and ketoconazole, end points are typically less well defined than those described for amphotericin B, which may contribute to a significant source of variability. A less stringent endpoint (slight turbidity is allowed above the MIC) has improved interlaboratory agreement. For this class, turbidity allowed corresponds to approximately a 50% (or greater) reduction in growth compared to the control well (drug–free medium). When this turbidity persists, it is often the same across all drug concentrations above the MIC.

Itraconazole, Posaconazole, Voriconazole, and Isavuconazole: For non-dermatophyte mould isolates, the MIC is read as the lowest drug concentration that prevents any discernible growth (100% inhibition). Trailing growth endpoints with these agents against *Aspergillus* spp. and most other opportunistic mould pathogens are uncommon. Such a pattern could reflect clinically relevant antifungal resistance, as demonstrated for *A. fumigatus* strains that are clinically resistant to itraconazole. However, when testing dermatophyte isolates against voriconazole, posaconazole, and itraconazole, the turbidity observed corresponds to approximately 80% or more reduction in growth compared with the control well (antifungal agent–free medium).

Echinocandins (Anidulafungin, Caspofungin, Micafungin): For echinocandins, endpoints are typically less well defined than those described for amphotericin B, and applying the MEC endpoint improves reproducibility. The MEC is defined as the lowest concentration of an antifungal agent that results in the growth of small, rounded, compact hyphal forms compared with the hyphal growth observed in the growth control well.

Ciclopirox: For ciclopirox, dermatophyte endpoints are typically less well defined than those described for amphotericin B. Applying a less stringent endpoint (allowing some turbidity above the MIC) has improved interlaboratory agreement. For this antifungal agent class, the turbidity allowed corresponds to approximately 80% or more reduction in growth compared with growth in the control well (antifungal agent–free medium).

Griseofulvin: For griseofulvin, dermatophyte endpoints are typically less well defined than those described for amphotericin B. Applying a less stringent endpoint (allowing some turbidity above the MIC) has improved interlaboratory agreement. For this antifungal agent class, the allowed turbidity corresponds to approximately an 80% or greater reduction in growth compared with growth in the control well (antifungal agent–free medium).

Terbinafine: For terbinafine, dermatophyte endpoints are typically less well defined than those described for amphotericin B. Applying a less stringent endpoint (allowing some turbidity above the MIC) has improved interlaboratory agreement. For this antifungal agent class, the allowed turbidity corresponds to approximately an 80% or greater reduction in growth compared with growth in the control well (antifungal agent–free medium).

10.2.9 Interpretation of Results

Although breakpoints for mould testing have not been established or approved by CLSI or any regulatory organisation, ECVs have been defined for some *Aspergillus* spp. and species complexes vs amphotericin B, caspofungin, and four triazoles (isavuconazole, itraconazole, posaconazole, and voriconazole) **(Table 10.11)**. These ECVs may help identify non-wild-type (NWT) *Aspergillus* spp. isolates (i.e., isolates for which the antifungal agent being evaluated appears to have reduced activity and may harbour resistance mechanisms).

Amphotericin B: Experience with the procedures described in this standard indicates that amphotericin B MICs for most non-dermatophyte opportunistic mould isolates cluster between 0.5 and 2 µg/mL, with modes (most frequent MICs) for *Aspergillus* spp. ranging between 0.5 and 1 µg/mL, except for *A. terreus* species complex (mode, 2 µg/mL). However, amphotericin B MICs for some other species (e.g., species complexes of *A. flavus, A. nidulans,* and *A. versicolor*, some members of the *Aspergillus* section *Fumigati*, *Acremonium strictum, Purpureocillium lilacinum, Scedosporium apiospermum,* and *L. prolificans* [*S. prolificans*]) can be above 2 µg/mL and as high as 16 µg/mL. Although little data are available regarding the correlation between MIC and treatment outcome with amphotericin B for filamentous fungi, MICs above 2 µg/mL have been associated with treatment failures, and MICs below 2 µg/mL with clinical cure.

Flucytosine: Filamentous fungi are not usually susceptible to flucytosine, and most MICs are >64 µg/mL for these isolates. The exceptions are some isolates of *Aspergillus* species and phaeoid (dematiaceous) fungi.

Fluconazole: Filamentous fungi are usually not susceptible to fluconazole, and most MICs are >64 µg/mL for these isolates, excepting some isolates of the dimorphic fungi and dermatophytes.

Ketoconazole: Experience to date with the procedures described in this standard indicates that mould MICs range from 0.0313 to 16 µg/mL. However, data are not yet available to indicate a correlation between MIC and the treatment outcome with ketoconazole.

Itraconazole, Posaconazole, Voriconazole, and Isavuconazole: The importance of proper preparation of drug dilutions for this insoluble compound cannot be overemphasised. Using incorrect solvents or deviating from the dilution scheme can lead to substantial errors due to dilution artefacts. As for ketoconazole, experience to date with the procedures described in this standard indicates that MICs for moulds range from 0.0313 to 16 µg/mL. However, preliminary data indicate that high itraconazole MICs (>8 µg/mL) are associated with clinical resistance to this agent when measured by the M38-A3 microdilution method after 48 hours of incubation. Data are not yet available to indicate a correlation between MIC and treatment outcome with the new triazoles.

Table 10.11: Epidemiological cutoff values for in vitro susceptibility testing of various *Aspergillus* spp. with no breakpoints[a] (CLSI-M57S 4th Ed.)

Antifungal Agent	Species	ECV, µg/mL[b,c]
Amphotericin B	*A. flavus*	4
	A. fumigatus	2
	A. niger	2
	A. terreus[d]	4
	A. versicolor	2
Caspofungin[e]	*A. flavus*	0.5
	A. fumigatus	0.5
	A. niger	0.25
	A. terreus	0.12
Isavuconazole	*A. flavus*	1
	A. fumigatus	1
	A. niger	4
	A. terreus	1
Itraconazole	*A. flavus*	1
	A. fumigatus	1
	A. niger	4
	A. terreus	2
Posaconazole	*A. flavus*	0.5
	A. niger	2
	A. terreus	1
Voriconazole	*A. flavus*	2
	A. niger	2
	A. terreus	2

Footnotes

a. ECVs for *Aspergillus* spp. are determined after 48 hours of incubation for testing triazoles and amphotericin B and after 24 hours of incubation for testing caspofungin (see CLSI document M38).

b. The ECVs in this document were established using broth microdilution as outlined in CLSI document M38. If another method is used for susceptibility testing, it must be validated against broth microdilution before the ECVs are used, just as other methods must be validated before breakpoints established using broth microdilution are used.

c. ECVs capture ≥ 97.5% of the statistically modelled population. Because ECVs are defined by phenotypic rather than genotypic data, in rare instances, ECVs may not identify some potentially resistant isolates (non-wild-type).

d. Low minimal inhibitory concentrations for *A. terreus* do not correlate with positive clinical outcomes, and testing is not recommended. Use of this antifungal agent is not recommended according to published treatment guidelines.

e. The caspofungin minimal effective concentration is the lowest concentration of caspofungin that leads to the growth of small, rounded, compact hyphal forms compared with the hyphal growth seen in the growth control well (see CLSI document M38).

10.2.10 Epidemiological cutoff values for Mucorales (Espinel-Ingroff *et al.*, 2015)

Epidemiological cutoff values (ECVs) for amphotericin B, posaconazole, and itraconazole and four Mucorales species obtained in 8 to 14 laboratories by the CLSI M38-A2 broth microdilution method are given in **Table 10.12.**

Table 10.12: Epidemiological cutoff values (ECVs) for amphotericin B, posaconazole, and itraconazole and four Mucorales species (Espinel-Ingroff *et al.*, 2015)

Species	Antifungal agent[a]	MIC (µg/mL)		Calculated statistical ECV (µg/mL) (% of MIC above the ECV or non-WT)[b]	
		Range	Mode[c]	≥95%	≥97.5%
L. corymbifera	AMB	0.06–16	0.5	1 (2.9)	2 (0.7)
	POS	0.06–4	0.5	1 (1.8)	2 (0.9)
	ITR	0.06–8	0.25	ND	ND
M. circinelloides	AMB	0.03–4	0.25	1 (0)	2 (0)
	POS	0.06–16	1	4 (5)	4 (5)
	ITR	0.25–16	4	ND	ND
R. arrhizus	AMB	0.03–4	1	2 (1.2)	4 (0)
	POS	0.03–32	0.5	1 (10.9)	2 (3.2)
	ITR	0.06–16	0.5	2 (5.1)	2 (5.1)
R. microsporus	AMB	0.06–4	0.5	2 (2.1)	2 (2.1)
	POS	0.06–16	0.5	1 (5.1)	2 (2.2)
	ITR	0.25–32	1	ND	ND

Footnotes

a. AMB, amphotericin B; POS, posaconazole; ITR, itraconazole.

b. Calculated ECV comprising ≥95 or ≥97.5% of the statistically modelled population. ND, not determined due to insufficient number of laboratories or isolates/species.

c. MIC is most frequently obtained for each distribution.

10.2.11 Dermatophyte susceptibility Interpretation

In the absence of established clinical breakpoints for dermatophytes, AFST results for the *Trichophyton mentagrophytes–Trichophyton interdigitale* complex may be interpreted using the upper limit of wild-type MIC distribution (UL-WT) reported by Shaw et al (Shaw *et al.*, 2020). MIC values above the respective UL-WT are considered non-wild-type (NWT) and should not be interpreted as clinical resistance. These values were established for the *T. mentagrophytes–T. interdigitale* complex of Indian origin and should be used accordingly. UL-WT MIC values determined were 0.06 µg/mL for miconazole, luliconazole, and amorolfine; 0.25 µg/mL for voriconazole; 0.5 µg/mL for itraconazole, ketoconazole, and ciclopirox

olamine; 1 µg/mL for clotrimazole and sertaconazole; 8 µg/mL for terbinafine; 16 µg/mL for naftifine; 32 for fluconazole; and 64 for griseofulvin (Shaw *et al.*, 2020).

10.2.12 Quality Control

The goals of a quality control program are to monitor the following:

- The precision and accuracy of the susceptibility test procedure.
- The performance of reagents, testing conditions, and instructions used in the test.
- The performance of persons who conduct the tests and read the results.

The goals are best realised by, but are not limited to, the use of quality control and reference strains selected for their genetic stability and usefulness in the particular method being controlled.

Quality control parameters include the following:

Growth Control: Each broth microdilution series should include a growth control of basal medium without an antifungal agent to assess the viability of the test organisms. In the broth tests, the growth control also serves as a turbidity control for endpoint readings.

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 minimise variation in MIC interpretation among observers. All laboratory personnel who perform these tests should independently read a selected set of dilution tests. The results are recorded and compared with those obtained by an experienced reader. Specific reference strains with predetermined MICs are particularly helpful for this purpose, especially with itraconazole.

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. **Table 10.13** lists the expected ranges for strains found acceptable as quality control strains. Also shown are additional strains that can be useful for conducting reference studies.

Table 10.13: Recommended 24- and 48-hour minimal inhibitory concentration or minimal effective concentration limits for quality control and reference strains using broth microdilution antifungal susceptibility testing procedures *(*M38M51S, 4th ed*)*

Organism	Purpose	Antifungal Agent	MIC/MEC Range, µg/mL	MIC/MEC Mode, µg/mL	MICs/MECs Within Range, %
Candida albicans ATCC®[a] 90028	QC	Manogepix	0.004–0.016	0.008	100
		Oteseconazole[b]	0.002–0.016	0.004	95.3
Candida krusei ATCC® 6258	QC	Amphotericin B	0.5–2	1	100
		Anidulafungin	0.03–0.12	0.06	97.9
		Caspofungin[c]	0.12–1	0.5	98.8
		Fluconazole	8–64	16	100
		Flucytosine	4–16	8	97.5
		Isavuconazole	0.06–0.5	0.25	95.2
		Itraconazole	0.12–1	0.5	95.8
		Ketoconazole	0.12–1	0.5	95.4
		Micafungin	0.06–0.25	0.12–0.25	99.6
		Posaconazole	0.06–0.5	0.25	100
		Rezafungin	0.016–0.12	0.03	100
		Voriconazole	0.06–0.5	0.12	98.3
Candida parapsilosis ATCC® 22019	QC	Amphotericin B	0.25–2	0.5	97.1
		Anidulafungin	0.25–2	1	95
		Caspofungin[c]	0.25–1	0.5	96.7
		Fluconazole	0.5–4	2	98.2
		Flucytosine	0.06–0.25	0.12	99.2
		Isavuconazole	0.016–0.06	0.06	90.5
		Itraconazole	0.06–0.5	0.25	95.8
		Ketoconazole	0.03–0.25	0.06/0.12	97.5
		Manogepix	0.008–0.03	0.015	100
		Micafungin	0.5–2	0.5–1	100
		Oteseconazole[b]	0.008–0.06	0.03–0.25	99.6
		Posaconazole	0.03–0.25	0.12	96.7
		Rezafungin	0.25–2	0.5	100
		Voriconazole	0.016–0.12	0.016–0.03	100
C. parapsilosis ATCC® 90018	QC	Oteseconazole[b]	0.001–0.008	0.004	99.2
Aspergillus flavus ATCC® 204304 (48 hours)	QC	Isavuconazole	0.5–4	1	95.8
		Manogepix	0.008–0.06	0.008	94.3
	Reference	Amphotericin B	0.5–4	ND	100
		Itraconazole	0.25–0.5	ND	100
		Posaconazole	0.06–0.5	ND	100
		Ravuconazole	0.5–4	ND	100
		Voriconazole	0.5–4	ND	100
Aspergillus fumigatus ATCC® MYA-3626 (48 hours)	QC	Manogepix	0.008–0.06	0.03	98.6
		Olorofim	0.008–0.06	0.03	98.2
	Reference	Amphotericin B	0.5–4	2	98.7
		Itraconazole	0.25–2	1	95.7
		Voriconazole	0.25–1	0.5	100
	Reference (MEC)	Anidulafungin	≤ 0.016	N/A	100

Abbreviations: ATCC®, American Type Culture Collection; MEC, minimal effective concentration; MIC, minimal inhibitory concentration; N/A, not applicable; QC, quality control.

a. ATCC® is a registered trademark of the American Type Culture Collection.
b. For oteseconazole, any of the QC strains are appropriate for routine QC testing.
c. The QC ranges were established using data generated in 2010 from 15 referral laboratories. Since then, caspofungin susceptibility testing has been associated with significant variation for some yeast species. Therefore, misclassification of susceptible yeast isolates might occur despite acceptable performance of the QC strains according to the range in this table.

10.3 Method for Antifungal Disk Diffusion Susceptibility Testing of Yeasts; Approved Guideline M44, 3rd edition, CLSI, USA (The method described here is incorporated from the reference document)

10.3.1 Introduction

Although CLSI broth dilution reference methods are available for antifungal susceptibility testing, there remains a need for an alternative, easy, quick, and cost-effective method to make antifungal susceptibility testing more readily available in the clinical microbiology laboratory. CLSI document M44-A2 provides an established methodology for disk diffusion testing of *Candida* spp.; zone interpretive criteria for caspofungin, fluconazole, and voriconazole; and recommended quality control ranges for caspofungin, fluconazole, posaconazole, and voriconazole.

The method described in this document is intended for testing *Candida* spp. 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*. Moreover, testing of filamentous fungi (i.e., moulds) is not addressed in the current procedure.

The method described herein must be followed exactly to obtain reproducible results. When new problems are recognised, or improvements in these criteria are developed, changes will be incorporated into future editions of this guideline and also distributed in periodic informational supplements.

10.3.2 Definitions

Antimicrobial susceptibility test interpretive category – 1) a classification based on an in vitro response of an organism to an antimicrobial agent at levels of that agent corresponding to blood or tissue levels attainable with usually prescribed doses of that agent; 2) susceptible antimicrobial susceptibility test interpretive category – a category that implies that an infection due to the isolate may be appropriately treated with the dosage of an antimicrobial agent recommended for that type of infection and infecting species, unless otherwise contraindicated; 3) susceptible-dose dependent (S-DD) antimicrobial susceptibility test interpretive category – a category that includes isolates with antimicrobial agent minimal inhibitory concentrations (MICs) that approach usually attainable blood and tissue levels and for which response rates may be lower than for susceptible isolates; 4) intermediate (I) antimicrobial susceptibility test interpretive category – a category that 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 categorised as either “susceptible” or “resistant”; NOTE: This category also includes a buffer zone, which should prevent small, uncontrolled, technical factors from causing major discrepancies in interpretations; 5) resistant antimicrobial susceptibility test interpretive category – resistant isolates that are not inhibited by the usually achievable concentrations of the agent with normal dosage schedules or where clinical efficacy has not been reliable in treatment studies; 6) nonsusceptible (NS) test interpretive category – a category used for organisms that currently

have only a susceptible interpretive category, but not susceptible-dose dependent, intermediate, or resistant interpretive categories (i.e., susceptible-only interpretive category); NOTE: This category is often given to new antimicrobial agents for which no resistant isolates have yet been encountered.

10.3.3 Selection of Antimicrobial Agent Disks for Routine Testing and Reporting

Although interpretive criteria are now available for a limited number of organism-drug combinations, routine testing is not generally recommended. However, some institutions may find it useful to systematically test selected drug-organism combinations (eg, fluconazole vs *Candida* from sterile sites). At each institution, the decision to test yeast isolates is best made collaboratively among infectious disease practitioners, the pharmacy and therapeutics committee, clinical microbiology personnel, and the infection control committee.

10.3.4 Reagents for the Disk Diffusion Test

Mueller-Hinton Agar + 2% Glucose and 0.5 µg/mL Methylene Blue Dye (GMB) Medium:

Among the many agar media available, supplemented Mueller-Hinton agar is a good choice for routine yeast susceptibility testing.

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 of 7.2–7.4 at room temperature after gelling. The 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.

10.3.5 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.

10.3.6 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 nonfrost-free freezer until needed. The disks may retain greater stability if stored frozen until the day of use. Always refer to the product insert for instructions. The unopened disk containers should be removed from the refrigerator or freezer 1 to 2 hours before use to allow them

to equilibrate to room temperature before opening. This procedure minimises condensation when warm air comes into contact with 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 colour.
- 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.

10.3.7 Turbidity Standard for Inoculum Preparation

To standardise the inoculum density for a susceptibility test, a BaSO4 suspension with turbidity equivalent to a 0.5 McFarland standard or its optical equivalent should be used.

10.3.8 Procedure for Performing the Disk Diffusion Test

Inoculum Preparation: Direct Colony Suspension Method

Steps for preparation of the inoculum are as follows:

(1) All organisms need to be subcultured onto blood agar or Sabouraud Dextrose Agar to ensure purity and viability. The incubation temperature must be maintained at 35°C (±2°C) throughout.
(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 containing 1×10^6 to 5×10^6 cells per mL and should produce semiconfluent growth with most *Candida* species isolates.

Inoculation of Test Plates

(1) 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.
(2) 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 agar rim is swabbed.

(3) 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 unstandardised inocula for streaking plates.

Application of Disks to Inoculated Agar Plates

(1) 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 centre to centre. 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.
(2) The plates are inverted and placed in an incubator set to 35°C (± 2°C) within 15 minutes after the disks are applied.

10.3.9 Reading Plates and Interpreting Results

Examine each plate after 20–24 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 semiconfluent 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. 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 of incubation.

Interpretation of Disk Diffusion Test Results

Zone Diameter Interpretive Standards

Table 10.14 provides zone-diameter interpretive criteria to accurately categorise the susceptibility levels of organisms to caspofungin, fluconazole, micafungin and voriconazole. These categories were developed by first comparing zone diameters to minimal inhibitory concentrations (MICs) for a large number of isolates, including those with known mechanisms of resistance relevant to the particular drug. Minimal inhibitory concentrations and corresponding zone sizes were analysed in relation to the drug's pharmacokinetics from normal dosing regimens. Of note, interpretive breakpoints for fluconazole are not applicable to *Candida krusei*; thus, identification to the species level is required in addition to MIC determination.

10.3.10 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 usually approach 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 interpretation, especially for drugs with narrow pharmacokinetic-toxicity margins.

Resistant (R): Resistant strains are those that are not inhibited by the usually achievable concentrations of the agent with normal dosage schedules, or that have zone diameters within a range where clinical efficacy has not been reliable in treatment studies.

10.3.11 Zone Diameter Interpretive Criteria

Disk diffusion zone diameters correlate inversely with MICs from standard dilution tests. These criteria are based on zone diameter vs MIC comparisons for the MIC interpretive criteria defined in the CLSI document M27.

Table 10.14: Zone diameter and equivalent minimal inhibitory concentration breakpoints for select antifungal agents against *Candida* spp. after 24-hour incubation (CLSI-M27M44S 4th Edition)

Antifungal Agent[a]	Species	Zone Diameter Breakpoints and Interpretive Categories, mm				Equivalent MIC Breakpoints and Interpretive Categories, µg/mL			
		S	I	SDD	R	S	I	SDD	R
Caspofungin	*C. albicans*	≥ 17	15–16	–	≤ 14	≤ 0.25	0.5	–	≥ 1
	C. glabrata	–	–	–	–	≤ 0.12	0.25	–	≥ 0.5
	C. guilliermondii	≥ 13	11–12	–	≤ 10	≤ 2	4	–	≥ 8
	C. krusei	≥ 17	15–16	–	≤ 14	≤ 0.25	0.5	–	> 1
	C. parapsilosis	≥ 13	11–12	–	≤ 10	≤ 2	4	–	≥ 8
	C. tropicalis	≥ 17	15–16	–	≤ 14	≤ 0.25	0.5	–	> 1
Fluconazole	*C. albicans*	≥ 17	–	14–16	≤ 13	≤ 2	–	4	≥ 8
	C. glabrata	–	–	≥ 15	≤ 14	–	–	≤ 32	≥ 64
	C. krusei[b]	–	–	–	–	–	–	–	–
	C. parapsilosis	≥ 17	–	14–16	≤ 13	≤ 2	–	4	≥ 8
	C. tropicalis	≥ 17	–	14–16	≤ 13	≤ 2	–	4	≥ 8
Micafungin	*C. albicans*	≥ 22	20–21	–	≤ 19	≤ 0.25	0.5	–	≥ 1
	C. glabrata	≥ 30	28–29	–	≤ 27	≤ 0.06	0.12	–	≥ 0.25
	C. guilliermondii	≥ 16	14–15	–	≤ 13	≤ 2	4	–	≥ 8
	C. krusei	≥ 22	20–21	–	≤ 19	≤ 0.25	0.5	–	≥ 1
	C. parapsilosis	≥ 16	14–15	–	≤ 13	≤ 2	4	–	≥ 8
	C. tropicalis	≥ 22	20–21	–	≤ 19	≤ 0.25	0.5	–	≥ 1
Voriconazole	*C. albicans*	≥ 17	15–16	–	≤ 14	≤ 0.12	0.25–0.5	–	≥ 1
	C. glabrata[c]	–	–	–	–	–	–	–	–
	C. krusei	≥ 15	13–14	–	≤ 12	≤ 0.5	1	–	≥ 2
	C. parapsilosis	≥ 17	15–16	–	≤ 14	≤ 0.12	0.25–0.5	–	≥ 1
	C. tropicalis	≥ 17	15–16	–	≤ 14	≤ 0.12	0.25–0.5	–	≥ 1

Abbreviations: I, intermediate; MIC, minimal inhibitory concentration; R, resistant; S, susceptible; SDD, susceptible-dose dependent.

Footnotes

a. Breakpoints may also be used for 48-hour readings if the 24-hour growth control is insufficient.
b. Isolates of *C. krusei* are intrinsically resistant to fluconazole, so their MICs should not be interpreted using this scale (see Appendix B).
c. For *C. glabrata* and voriconazole, current data are insufficient to demonstrate a correlation between in vitro susceptibility testing and clinical outcome.

10.3.12 Quality Control

Part of quality management focused on fulfilling quality requirements, which includes operational techniques and activities used to fulfil 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:

- *Candida albicans* ATCC 90028;
- *Candida parapsilosis* ATCC 22019;
- *Candida tropicalis* ATCC 750; and
- *Candida krusei* ATCC 6258.

Storing Quality Control Strains

- The quality control strains should be tested by the standard disk diffusion test procedure described herein using the same materials and methods that are used to test clinical isolates.
- Quality control strains are stored in a way that minimises the possibility of mutation in the organism.
- There are several methods for prolonged storage of reference strains. For example, yeasts may be grown on Potato Dextrose Agar slants and then frozen at -70°C. Alternatively, strains can be preserved by suspending yeasts in vials containing 10% glycerol solution for freezing and storage at -70°C.
- Working quality control cultures are stored on blood agar or Sabouraud Dextrose Agar at 2 to 8°C and subcultured each week for no more than three successive weeks. New working cultures should be prepared at least monthly from frozen, freeze-dried, or commercial cultures.
- Frozen or freeze-dried cultures should be subcultured at least twice prior to testing.
- A quality control strain can be used to monitor the precision (repeatability) and accuracy (trueness) of the disk test as long as there is no significant change in the mean zone diameter that cannot be attributed to a faulty methodology. If an unexplained result suggests a change in the organism's inherent susceptibility, a fresh new stock culture of the control strain should be obtained.

10.3.13 Zone Diameter Quality Control Limits

Acceptable zone diameter quality control limits for quality control strains are listed in **Table 10.15**. 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, weekly.

10.3.14 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 fall outside the acceptable range (based on 95% confidence limits, 1 out of 20 random results may be out-of-control result). 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 10.15.**

Table 10.15: Recommended quality control zone diameter (mm) ranges after 24-hour incubation (CLSI-M27M44S 4th Edition)

Antifungal Agent	Disk Content, µg	*Candida albicans* ATCC® 90028*	*Candida krusei* ATCC® 6258	*Candida parapsilosis* ATCC® 22019	*Candida tropicalis* ATCC® 750
Caspofungin	5	18–27	19–26	14–23	20–27
Fluconazole	25	28–39	–#	22–33	26–37
Manogepix	5	32–44	–	26–34	33–43
Micafungin	10	24–31	23–29	14–23	24–30
Posaconazole	5	24–34	23–31	25–36	23–33
Rezafungin$	5	13–20	14–20	9–16	14–20
Voriconazole	1	31–42	16–25	28–37	–#

Abbreviation: ATCC®, American Type Culture Collection.

Footnotes

* ATCC® is a registered trademark of the American Type Culture Collection.

\# QC ranges have not been established for these fungal strain–antifungal agent combinations, owing to their extensive interlaboratory variation during initial QC studies.

$ QC ranges for rezafungin were established using data from only one disk manufacturer. Disks from other manufacturers were not available at the time of testing.

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

10.3.16 Corrective Action

Out-of-Control Result Due to an Obvious Error

Obvious reasons for out-of-control results include:

- Use of the wrong disk.
- Use of the wrong control strain.
- Obvious contamination of the strain; or
- 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 10.15,** 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 continue until the problem is fully resolved.

Additional Corrective Action: When immediate corrective action does not resolve the problem, the issue is likely due to a system error rather than a random one. The following common sources of error should be investigated:

- Zone diameters were measured and transcribed correctly.
- The turbidity standard has not expired, is stored properly, meets performance requirements, and was adequately mixed prior to use.
- All materials used were within their expiration date and stored at the proper temperature.
- The incubator is at the proper temperature and atmosphere.
- Other equipment used (e.g., pipettes) are functioning properly.
- Disks are stored desiccated and at the proper temperature.
- The control strain has not changed and remains uncontaminated.
- Inoculum suspensions were prepared and adjusted correctly.
- Inoculum for the test was prepared from a plate incubated for the appropriate duration, and in no case was it 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 that uses 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.

10.3.17 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.

10.3.18 Limitations of Disk Diffusion Methods

The disk diffusion method described here has been standardised for *Candida* species only; for other yeasts, consultation with an infectious disease specialist is recommended to determine the need for susceptibility testing and interpret 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.

10.3.19 Verification of Patient Results

Multiple test parameters are monitored in accordance with the quality control recommendations described in this standard. However, acceptable results from testing quality control strains do not guarantee accuracy when testing patient isolates. It is important to review all results from all drugs tested on a patient's isolates before reporting. 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.

10.3.20 Preparation of common media used in AFST

Preparation of Mueller-Hinton Agar + 2% Glucose and 0.5 µg/mL Methylene Blue Dye

The medium can be prepared and poured as the complete medium with supplements, or the supplements can be added to commercially prepared Mueller-Hinton agar plates. Using the latter technique enables the use of routine Mueller-Hinton agar plates from the bacteriology laboratory.

Preparation of Supplemented Mueller-Hinton Agar:

(1) Mueller-Hinton agar should be prepared from a commercially available dehydrated Mueller-Hinton agar base according to the manufacturer's instructions.
(2) Dissolve 0.1 g of methylene blue dye in 20 mL of distilled water and warm gently to dissolve. Do not overheat. Add 100 µL of this solution to 1 L of agar suspension.
(3) Add 20 g of glucose per litre of agar suspension.
(4) Autoclave as directed by the manufacturer's instructions.
(5) Immediately after autoclaving, allow the agar solution to cool in a 45 to 50°C water bath.
(6) Pour the freshly prepared and cooled medium into plastic, flat-bottomed Petri dishes on a level, horizontal surface to give a uniform depth of approximately 4 mm. This corresponds to 67 to 70 mL of medium for plates with diameters of 150 mm and 28 to 30 mL for plates with a diameter of 100 mm.
(7) The agar medium should be allowed to cool to room temperature and, unless the plate is used on the same day of preparation, stored at refrigerator temperature (2 to 8°C). The agar medium should have a pH between 7.2 and 7.4 at room temperature.
(8) Plates should be used within seven days after preparation unless adequate precautions, such as wrapping in plastic, have been taken to minimise drying of the agar.
(9) A representative sample of each batch of plates should be examined for sterility by incubating at 30 to 35°C for 24 hours or longer. Plates should undergo quality control testing.

McFarland 0.5 Barium Sulfate Turbidity Standard

To standardise the inoculum density, a $BaSO_4$ turbidity standard (0.5 McFarland) is used.

The procedure consists of the following steps:

(1) Prepare this turbidity standard by adding 0.5 mL of 0.048 mol/L $BaCl_2$ (1.175% w/v $BaCl_2$ H_2O) to 99.5 mL of 0.18 mol/L H_2SO_4 (1% v/v) with constant stirring to maintain a suspension.
(2) Verify the correct density of the turbidity standard by using a spectrophotometer with a 1-cm light path and a matched cuvette to determine the absorbance. The absorbance at 625 nm should be 0.08–0.10 for the 0.5 McFarland standard.
(3) Distribute 4 to 6 mL into screw-cap tubes of the same size as those used in growing or diluting the broth culture inoculum.
(4) Tightly seal these tubes and store them in the dark at room temperature.
(5) Vigorously agitate this turbidity standard on a mechanical vortex mixer just before use.
(6) The barium sulfate standards should be replaced, or their densities verified monthly.

RPMI 1640 medium buffered with 0.165 M MOPS, 1 L.

10.4 g powdered medium

34.53 g MOPS buffer

Dissolve the powdered medium in 900 mL of distilled water. Add MOPS to a final concentration of 0.165 mol/L, then stir until fully dissolved. While stirring, adjust the pH to 7.0 at 25°C using 1 mol/L sodium hydroxide. Add additional water to bring the medium to a final volume of 1 L. Filter sterilise and store at 4°C until use **(Table 10.16)**.

Table 10.16: Composition of RPMI 1640 Medium

Constituent	g/L water	Constituent	g/L water
L-arginine (free base)	0.200	Biotin	0.0002
L-asparagine (anhydrous)	0.050	D-pantothenic acid	0.00025
L-aspartic acid	0.020	Choline chloride	0.003
L-cystine, 2HCl	0.0652	Folic acid	0.001
L-glutamic acid	0.020	Myo-inositol	0.035
L-glutamine	0.300	Niacinamide	0.001
Glycine	0.010	PABA	0.001
L-histidine (free base)	0.015	Pyridoxine HCl	0.001
L-hydroxyproline	0.020	Riboflavin	0.0002
L-isoleucine	0.050	Thiamine HCl	0.001
L-leucine	0.050	Vitamin B12	0.000005
L-lysine HCl	0.040	Calcium nitrate, H_2O	0.100
L-methionine	0.015	Potassium chloride	0.400
L-phenylalanine	0.015	Magnesium sulfate (anhydrous)	0.04884
L-proline	0.020	Sodium chloride	6.000
L-serine	0.030	Sodium phosphate, dibasic (anhydrous)	0.800
L-threonine	0.020	D-glucose	2.000
L-tryptophan	0.005	Glutathione, reduced	0.001
L-tyrosine, 2Na	0.02882	Phenol red Na	0.0053
L-valine	0.020		

10.4 Antifungal Susceptibility Testing Using Commercial Gradient Diffusion Strips

Commercial gradient diffusion antifungal susceptibility strips (Etest (bioMérieux), M.I.C. Evaluator (Oxoid), Ezy MIC™ Strip (HiMedia), etc.) are available and may be used for selected yeasts and moulds.

10.4.1 Principle

- These strips contain a predefined continuous gradient of antifungal concentrations immobilised on a plastic strip. After placement on an inoculated agar surface, the antifungal diffuses into the medium, and an inhibition ellipse develops. The MIC is read at the point where the edge of the inhibition ellipse intersects the scale on the strip.

10.4.2 Procedure

1. Prepare a fresh (18–24 hours) pure culture.
2. Prepare an inoculum equivalent to 0.5 McFarland standard.
3. Swab the inoculum uniformly onto RPMI 1640 agar with 2% glucose (or medium recommended by the manufacturer).
4. Allow the agar surface to dry for approximately 10–15 minutes.
5. Remove the strip aseptically from its package.
6. Place the strip on the agar surface with the concentration scale facing upward, ensuring complete contact with the agar.
7. Incubate plates in an inverted position at 35 ± 2°C.
8. Read results after 24 hours (extend to 48 hours if growth is insufficient).
9. Determine the MIC at the point where the inhibition ellipse intersects the strip scale.
10. Interpret MIC values according to current CLSI or EUCAST clinical breakpoints.
11. The exact reading instructions should follow the strip-specific package insert.
12. Always apply quality control strains, i.e. *C. krusei* ATCC 6258 and *C. parapsilosis* ATCC 22019, with each batch.

Note: Commercial gradient diffusion strips should be performed according to the latest manufacturer's Instructions for Use (IFU).

Annexure A: Media and Stains

This annexure describes the preparation of the culture media and staining techniques used in a diagnostic mycology laboratory. The formulations and preparation methods for the culture media described in this section are adapted primarily from standard textbooks, including Larone's Medically Important Fungi: A Guide to Identification (7th edition), Medical Mycology by Kwon-Chung and Bennett, Koneman's Colour Atlas and Textbook of Diagnostic Microbiology (7th edition) and Medical Mycology: A Practical Approach (Evans & Richardson).

MEDIA

Culture media are specialised nutrient preparations used for the isolation, cultivation, identification, and maintenance of fungi from clinical specimens. In medical mycology, the appropriate selection and use of culture media are essential for the recovery and identification of pathogenic fungi. Culture media provide the nutrients and environmental conditions necessary for fungal growth while allowing observation of characteristic colony morphology, pigmentation, and sporulation, which are important for species identification. A variety of culture media are available for mycological investigations, each formulated for a specific purpose. General-purpose media support the growth of a broad spectrum of fungi, whereas selective and differential media contain inhibitory agents or specialised substrates that facilitate the isolation and identification of specific fungal pathogens.

1) Ascospore Agar

Component	Quantity
Potassium acetate	10.0 g
Yeast extract	2.5 g
Dextrose	1.0 g
Agar	30.0 g
Distilled water	1.0 L

Procedure:

1. Mix thoroughly to give a uniform suspension.
2. Heat to a boil for 1 minute with agitation.
3. Dispense 8.0 mL in screw-top tubes.
4. Autoclave at 121°C at 15 psi for 15 minutes.
5. Cool in a slanted position.
6. The pH is to be kept at 6.4.
7. Store at 2–8°C.
8. Protect from dehydration.

2) Brain Heart Infusion Agar (BHIA)

Component	Quantity
Calf brain infusion	200.0 g
Beef heart infusion	250.0 g
Peptone	10.0 g
Glucose	2.0 g

Component	Quantity
Sodium chloride	5.0 g
Disodium phosphate	2.5 g
Agar	15.0 g
Distilled water	1.0 L

OR

Component	Quantity
Commercially available Brain heart infusion agar (dehydrated)	52.0 g
Distilled water	1.0 L

1. Dissolve ingredients by boiling; dispense in tubes.
2. Distribute 25.0 mL in 3 Oz bottles.
3. Autoclave at 121°C at 15 psi for 15 minutes.
4. Cool in a slanting position with a one-inch butt.
5. Shelf life is 30 days in test tubes and 14 days in Petri dishes.
6. The medium can be enriched by the addition of 5.0% sheep blood
7. Antibiotics, cycloheximide (0.5 mg/mL) and chloramphenicol (0.05 mg/mL) may be added to the medium prior to autoclaving to prevent growth of saprophytic fungi and bacteria, respectively.

3) Biphasic medium for blood culture

a) Brain heart infusion agar as described above

b) Brain heart infusion broth.

Component	Quantity
Brain heart infusion (commercially available)	52.0 g
Distilled water	1.0 L

1. Suspend and dissolve brain heart infusion in 1.0 L of water.
2. Autoclave at 121°C at 15 psi for 15 minutes.
3. Add 30.0 mL to each bottle aseptically.
4. The final pH is to be kept at 7.4.

4) Bird Seed Agar (BSA) [modified by Paliwal and Randhawa, J Clin Microbiol 1978; 7:346-8]

Niger seeds are scientifically known as *Guizotia abyssinica*.

Component	Quantity
Bird seed	70.0 g
Distilled water	1.0 L

Boil for 30 minutes. Filter through gauze and add the following:

Component	Quantity
Agar	15.0 g

Component	Quantity
Diphenyl	1.0 g
Chloramphenicol	50.0 mg

1. Autoclave at 121°C at 15 psi for 10 minutes.
2. Dispense in tubes as slants.
3. Shelf life is 2 weeks.
4. Black coloured colonies of *Cryptococcus neoformans* are due to the caffeic acid extracted from birdseed.

5) Canavanine glycine bromothymol – blue agar (CGB)

Solution A

Component	Quantity
Glycine	10.0 g
Potassium dihydrogen orthophosphate (KH_2PO_4)	1.0 g
Magnesium sulfate ($MgSO_4$)	1.0 g
Canavanine sulfate	30.0 mg
Thiamine hydrochloride	1.0 mg
Distilled water	100.0 mL

Adjust to pH 5.6 and filter-sterilise.

Solution B

Component	Quantity
Bromothymol blue	0.4 g
Sodium hydroxide (NaOH) 0.01 M	64.0 mL
Distilled water	36.0 mL

1. Add 2.0 mL of solution B to 88.0 mL of distilled water.
2. Add 2.0 g of agar, autoclave at 121°C and 15 psi for 15 minutes, then cool to about 55°C.
3. Add 10.0 mL of solution A, mix well, and pour into tubes or plates.

6) Caffeic acid agar

Component	Quantity
Glucose	5.0 g
Ammonium sulfate	5.0 g
Yeast extract	2.0 g
Potassium phosphate	0.8 g
Magnesium sulfate	0.7 g
Caffeic acid	0.18 g
Ferric citrate solution	4.0 mL
Agar	20.0 g
Distilled water	1.0 L

1. Add 20.0 mg of ferric citrate to 4.0 mL of distilled water to make the ferric citrate solution just before adding it to the medium.

2. Mix reagents.
3. Bring all the reagents to a boil.
4. Autoclave for 12 minutes at 121°C.
5. Dispense into Petri dishes or tubes.
6. Shelf life is 14 days.

7) Christensen's urea agar (Isenberg, 2004)

Component	Quantity
Peptone	1.0 g
Dextrose	5.0 g
NaCl	5.0 g
KH_2PO_4	2.0 g
Agar	20.0 g
Distilled water	895.0 mL
Phenol red (2.0%)	5.0 mL

1. Sterilise by autoclaving at 121°C at 15 psi for 10 minutes.
2. Cool the autoclaved base (900.0 mL) to 50–55 °C, then add 100.0 mL of 20.0% (w/v) urea solution that has been sterilised by membrane filtration (0.22 μm; Seitz filtration in the original method). This gives a final volume of 1.0 L and a final urea concentration of 2.0% (w/v). Do not autoclave the medium after the urea has been added, as urea decomposes on heating.

8) Corn Meal Agar (CMA)

Component	Quantity
Corn Meal	50.0 g
Distilled water	1.0 L
Agar	15.0 g

1. Mix cornmeal in 500.0 mL of distilled water.
2. Heat for 1 hour or autoclave for 10 minutes at 15 psi.
3. Filter the suspension through a gauze.
4. Bring the volume to 1.0 L and add the agar. Bring this to a boil.
5. Autoclave at 121°C at 15 psi for 15 minutes.
6. Dispense in sterile Petri dishes.
7. Shelf life is 14 days.

9) Corn Meal Tween 80 Agar

1. To plain Corn Meal Agar, add 10.0 mL of Tween 80 for 1.0 L of medium.
2. Pour in sterile Petri dishes.

10) Corn Meal Tween 80 and 0.2% dextrose

Component	Quantity
Powdered corn meal agar with 0.2% dextrose (Difco™)	17.0 g
Distilled water	1.0 L
Tween 80	10.0 mL

1. Add powdered cornmeal agar with 0.2% dextrose to the distilled water and mix.
2. Heat to a boil with frequent agitation till the medium is completely dissolved.

3. Remove from heat and add 10.0 mL Tween 80; mix thoroughly.
4. Sterilise by autoclaving at 121°C at 15 psi for 15 minutes.
5. Dispense in Petri dishes.
6. The final pH is to be kept at 6.2 at room temperature.

11) Converse liquid medium (Levine modification) for *Coccidioides* [Converse, 1955]

Component	**Quantity**
Ammonium acetate	1.23 g
Glucose	4.0 g
Dipotassium phosphate	0.52 g
Potassium phosphate	0.4 g
Magnesium sulfate	0.4 g
Zinc sulfate	0.002 g
Sodium chloride	0.014 g
Sodium carbonate	0.012 g
Tamol	0.5 g
Calcium chloride	0.002 g
Agarose (purified)	10.0 g
Distilled water	1.0 L

1. Autoclave at 121°C at 15 psi for 15 minutes.
2. Dispense in Petri dishes.

12) Czapek-Dox Solution Agar (Isenberg, 2004)

Component	**Quantity**
Magnesium sulfate	0.5 g
Potassium chloride	0.5 g
Dipotassium phosphate	1.0 g
Ferrous sulfate	0.01 g
Sodium nitrate	3.0 g
Glucose	30.0 g
Agar	15.0 g
Distilled Water	1.0 L

1. Mix reagents and bring to a boil.
2. Autoclave at 121°C at 15 psi for 15 minutes and pour in Petri dishes.

13) Czapek solution Agar for *Aspergillus*

Component	**Quantity**
Sodium nitrate	3.0 g
Dipotassium phosphate	1.0 g
Magnesium sulfate	0.5 g
Potassium chloride	0.5 g
Ferrous sulfate	0.01 g
Glucose	30.0 g
Agar	15.0 g
Distilled water	1.0 L

1. Mix the reagents by boiling.
2. Autoclave at 121°C at 15 psi for 15 minutes.

3. Dispense in Petri dishes or test tubes.
4. Shelf life: 30 days in tubes / 14 days on plates.

14) Dermatophyte test medium (DTM)

Component	Quantity
Phytone peptone	10.0 g
Dextrose	10.0 g
Phenol red solution	40.0 mL

(0.5 gm in 15.0 mL of 1N NaOH made up to 100.0 mL with distilled water).

Component	Quantity
0.8 M HCl	6.0 mL
Cycloheximide	500.0 mg
Gentamicin	100.0 mg
Agar	20.0 g
Distilled water	1.0 L

1. Suspend phytone, dextrose and agar in 100.0 mL of distilled water and mix thoroughly.
2. Heat to boiling till all ingredients are dissolved; remove from heat.
3. Add phenol red, HCl, cycloheximide and gentamicin.
4. Autoclave at 121°C at 15 psi for 15 minutes.
5. Final pH is adjusted to 5.5.
6. Pour in test tubes.

15) Fermentation broth

Component	Quantity
Powdered yeast extract	4.5 g
Peptone	7.5 g
Bromothymol blue	0.04 g
Distilled water	1.0 L

1. Dissolve bromothymol blue in 3.0 mL of 95.0% ethanol.
2. Mix reagents and add bromothymol blue.
3. Adjust pH to 7.0.
4. Dispense 2.0 mL into each tube.
5. Autoclave at 121°C at 15 psi for 15 minutes.
6. Allow the medium to cool.
7. To each tube, aseptically add 1.0 mL of 6.0 % aqueous solution of carbon source to be tested, which has been filtered through a 0.22 µm filter.
8. Shelf life: 30 days when stored at 4°C.

16) 12.0% gelatin for demonstration of proteolytic activity

Component	Quantity
Heart infusion broth (Difco™)	25.0 g
Gelatin	120.0 g

1. Suspend powdered broth and gelatin in distilled water and heat to a boil.
2. Dispense into screw-capped tubes.
3. Autoclave at 121°C for 10 minutes.
4. Final pH is to be maintained at 7.2–7.4.

17) Glucose-Yeast Extract Medium

Component	Quantity
Glucose	10.0 g
Yeast extract	5.0 g
Agar	15.0 g
Distilled water	1.0 L

1. Mix the reagents in distilled water and bring to a boil.
2. Autoclave at 121°C at 15 psi for 15 minutes.
3. Slant the tubes.
4. Shelf life is 30 days.

18) Gorodkowa's medium for ascospores

Component	Quantity
Glucose	0.63 g
Sodium chloride	1.3 g
Beef extract	2.5 g
Agar	2.5 g
Distilled water	250.0 mL

1. Mix reagents and bring to a boil.
2. Autoclave at 121°C at 15 psi for 15 minutes.

19) KT Medium [Kwartler and Tajima, 1964]

Component	Quantity
Tween 80	0.2 mL
Potassium sulfate	0.5 g
Magnesium citrate	1.5 g
L-Asparagine	5.0 g
Casamino Acids	3.0 g
Glycerol	20.0 mL
Agar	15.0 g
Albumin-Dextrose Solution, Ster	100.0 mL
Albumin, Fraction 5 (Pentex)	5.0 g
Dextrose	7.0 g
Sodium Chloride (NaCl)	0.85 g
ETF water	9.0 mL

1. Dissolve magnesium citrate in 900.0 mL of ETF water

2. While stirring this solution, add the following components and dissolve sequentially: potassium sulfate, asparagine, casamino acids, Tween 80, and glycerol. Adjust pH to 6.6.
3. Add the agar and heat to boiling to dissolve.
4. Autoclave at 121°C at 15 psi for 15 minutes. Cool to 56°C in a water bath.
5. Prepare the sterile solution by dissolving albumin first, then dextrose and NaCl in 100.0 mL of ETF water.
6. Adjust the pH to 6.6. Inactivate by incubating for 30 minutes at 56°C.
7. Sterilise by filtering using a 0.22 µm filtration system.
8. Add albumin solution to agar aseptically. Mix well.
9. Dispense aseptically.

Note: When preparing a sterile solution, dissolve albumin completely before adding dextrose and sodium chloride, then adjust pH. The albumin used must be Fraction 5.

20) *Histoplasma* Mould to Yeast Form Conversion Medium

Component	Quantity
Brain heart infusion agar base	5.2 g
Glutamine (200.0 mM stock)	1.0 mL
Sheep blood	5.0 mL
Distilled water	100.0 mL

1. Prepare brain heart infusion agar base.
2. Autoclave at 121°C at 15 psi for 15 minutes.
3. Add glutamine and sheep blood.
4. Dispense in a test tube.
5. Slant the test tubes.
6. Shelf life is 30 days at 4°C.

21) Kelley's Agar

Component	Quantity
Glucose	10.0 g
Bactopeptone	10.0 g
Sodium chloride	5.0 g
Beef extract	3.0 g
Haemoglobin solution	20.0 mL
Agar	15.0 g
Distilled water	980.0 mL

1. Add 5.0 mL of citrated sheep blood to 15.0 mL of distilled water. This is the haemoglobin solution.
2. Dispense in tubes and autoclave at 121°C for 10 minutes.
3. Slant the test tubes.

22) Malt Extract Agar

Component	Quantity
Malt extract	20.0 g

Component	Quantity
Peptone	1.0 g
Glucose	20.0 g
Agar	15.0 g
Distilled water	1.0 L

1. Mix reagents and bring to a boil.
2. Autoclave at 121°C for 15 minutes.
3. Shelf life: 30 days in a test tube/ 14 days in Petri dishes.

23) Oatmeal Agar (OMA)

Component	Quantity
Tomato paste	10.0 g
Oatmeal	10.0 g
Magnesium sulfate	1.0 g
Potassium phosphate	1.0 g
Sodium nitrate	1.0 g
Agar	15.0 g
Distilled water	1.0 L

1. Adjust pH to 5.6 with sodium hydroxide.
2. Autoclave at 121°C for 20 minutes.
3. Dispense in Petri dishes.

24) Pine's Agar Medium for Yeast Phase/*H. capsulatum* Pine's Citrate Medium
Solution 1

Component	Quantity
Potassium phosphate monobasic	8.00 g
Ammonium sulfate	8.00 g
Magnesium sulfate septahydrous	0.86 g
Calcium chloride	0.08 g
Zinc sulfate (for yeast phase only)	0.05 g
Water QS	1.0 L

Note: Filter sterilise stock solution

Solution 2

Component	Quantity
Ferrous sulfate, heptahydrous	5.70 g
Manganese chloride hexahydrous	0.80 g
Sodium molybdate, dihydrate	0.15 g
Hydrochloric acid, conc.	1.00 mL
Water QS	1.0 L

Solution 3

Component	Quantity
Casein hydrolysate (ICN)	40.00 mL

Solution 4

Component	Quantity
Inositol	0.20 g
Thiamine hydrochloride	0.20 g
Calcium pantothenate	0.20 g
Riboflavin	0.20 g
Nicotinamide (Niacinamide)	0.10 g
Biotin	0.01 g
Water QS	1.0 L

Solution 5

Component	Quantity
Hemin	0.200 g
ETF-water QS	1.0 L

Solution 6

Component	Quantity
DL-thioctic acid	0.01 g
Ethyl alcohol, 95.0%	10.00 mL

Solution 7

Component	Quantity
Coenzyme A	0.01 g
Water	10.00 mL

Pine's Citrate Medium, Yeast Phase, Part 1

Component	Quantity
Solution 1 (Salts)	250.00 mL
Solution 2 (Minor elements)	10.00 mL
Solution 3 (Casein hydrolysate)	40.00 mL
Solution 4 (Vitamins)	10.00 mL
Solution 5 (Hemin)	10.00 mL
Solution 6 (Thioctic Acid)	1.00 mL
Solution 7 (Coenzyme A)	1.00 mL
Glucose	10.00 g
Ketoglutaric acid	1.00 g
L-Cysteine hydrochloride	1.00 g
Glutathione (Reduced)	0.50 g
Asparagine	0.10 g
L-Tryptophan	0.02 g
Citric Acid (For Yeast Phase only)	10.00 g

Component	Quantity
Water, QS	500.00 mL

Combine the 7 solutions in 80.0% of the water, then dissolve the remaining components. Adjust the pH to 6.5 with 20.0% potassium hydroxide, QS to the full volume, and filter-sterilise.

Note: The citric acid is for the yeast phase medium only.

Note: This part is added to Pine's Citrate medium, Part 2

Pine's Citrate Medium, Yeast Phase, Part 2

Component	Quantity
Oleic Acid solution, Liquid:	0.10 mL
Oleic Acid	0.10 g
ETF water	100.00 mL
Starch solution, Liquid:	500.00 mL
Starch, Soluble	1.00 g
ETF Water	500.00 mL

Agar Formula

Component	Quantity
Oleic Acid solution, Agar	1.00 mL
Oleic Acid	0.10 g
ETF Water	100.00 mL
Starch solution, Agar	500.00 mL
Starch, Soluble	2.00 g
Agar	10.00 g
ETF Water	500.00 mL

Note: Filter and sterilise stock solutions

For final medium: While part 2 is still hot, add part 1. Mix thoroughly and dispense aseptically.

25) Potato Dextrose Agar (PDA)

Component	Quantity
Potatoes	250.0 g

(Peel, slice, and boil potatoes in 100.0 mL of distilled water for 1 hour, filter and add the following)

Component	Quantity
Dextrose	20.0 g
Agar	20.0 g

Distilled water to make the final volume up to 1 L.

1. Bring to a boil to dissolve agar.
2. Autoclave at 121°C for 15 minutes.

26) Potato Carrot Agar (PCA)

Component	Quantity
Potatoes	150.0 g
Carrot	150.0 g

(Peel, slice and boil potatoes and carrots in 150.0 mL of D.W. for 1 hour, filter and add the following)

Component	Quantity
Agar	15.0 g

Distilled water to make the final volume up to 1 L

1. Bring to a boil to dissolve agar.
2. Autoclave at 121°C for 15 minutes.

27) Rice Starch Agar (RSA)

Component	Quantity
Rice grains, uncooked, white, unenriched	10.0 g
Tween 80	10.0 mL
Agar	15.0 g
Distilled water	1.0 L

1. Add rice to boiling water and continue boiling for 30 seconds.
2. Add Tween 80 and Agar.
3. Restore volume to 1.0 L.
4. Autoclave at 121°C for 15 minutes.
5. Leave in a water bath at 80°C overnight.
6. Decant clear portion, refilter and again autoclave (pH 6.2).
7. Pour in Petri dishes.

28) Sabouraud Dextrose Agar (SDA)

Component	Quantity
Glucose	40.0 g
Peptone	10.0 g
Agar	20.0 g
Distilled water	1.0 L

1. Mix reagents by boiling.
2. Dispense into tubes.
3. Autoclave at 121°C for 15 minutes.
4. Final pH of 5.5 to 5.6.
5. Allow tubes to cool in a slanted position.
6. Store at 4°C.
7. Shelf life of 30 days in test tubes and 14 days in Petri dishes.

b. Neutral (Emmon's Modification) Sabouraud Dextrose Agar

Component	Quantity
Glucose	20.0 g

Component	Quantity
Peptone	10.0 g
Agar	20.0 g
Distilled water	1.0 L

1. Procedure of preparation is similar to SDA.
2. Final pH of 6.8 to 7.0.

c. Sabouraud Dextrose Agar with Cycloheximide, Chloramphenicol, and Gentamicin

To the molten SDA, add

Component	Quantity
Cycloheximide	0.5 mg/mL
Chloramphenicol	0.05 mg/mL
Gentamicin	0.02 mg/mL

1. Add 500.0 mg of cycloheximide to 10.0 mL of acetone in the boiling medium. Mix well. Add 50.0 mg of chloramphenicol and 20.0 mg of gentamicin to 10.0 mL of 95.0% alcohol in the boiling medium.
2. Mix the medium thoroughly. Tube and autoclave at 121°C for 10 minutes.

d. Modifications of SDA

i) SDA with thiamine

Component	Quantity
Thiamine	1.0 mg

ii) SDA with yeast extract (Growth of Dermatophytes)

Component	Quantity
Yeast extract	5.0 g/L

iii) SDA without Dextrose

For maintaining stock cultures

29) Selective *Auris* Medium

Component	Quantity
Yeast extract–peptone–dextrose (YPD) broth	Per manufacturer's directions
Agar powder (ultra-pure), added to YPD broth before autoclaving	2.5% (w/v)
Sodium chloride, added directly to the medium before autoclaving	12.5% (w/v)
Ferrous sulfate ($FeSO_4$), added after autoclaving	9.0 mM (final concentration)

1. Prepare YPD broth per the manufacturer's instructions; add agar powder (2.5%) and sodium chloride (12.5%).
2. Autoclave at 121°C for 15 minutes.

3. Prepare a 1.0 M ferrous sulfate stock solution freshly, no more than 10–15 minutes before use (to prevent aerial oxidation and precipitation). Sterilise by syringe filtration through a 0.2 µm membrane filter.
4. Allow the autoclaved medium to cool to approximately 80°C.
5. Add ferrous sulfate stock solution to achieve a final concentration of 9.0 mM (i.e., 9.0 mL of 1.0 M stock per litre of medium). Mix thoroughly by gentle swirling; do not vortex.
6. Pour into sterile 90 mm Petri dishes to a depth of 5–6 mm and allow to solidify at room temperature.
7. Sterility check: incubate two plates from each batch at 25°C and 37°C for 2 days before use.
8. Shelf life: 2–4 days at 4°C. Prepare fresh batches before use owing to gradual oxidation and precipitation of iron on storage.

30) Soil Extract Agar

Component	Quantity
Soil	500.0 g
Glucose	2.0 g
Yeast extract	1.0 g
Potassium phosphate	0.5 g
Agar	15.0 g
Tap water	1.0 L

1. Mix 500.0 g of garden soil and 1.0 L of tap water.
2. Autoclave at 121°C at 15 psi for 15 minutes.
3. Filter through Whatman No. 2 filter paper. This is the soil infusion.
4. Add reagents and bring the volume up to 1.0 L.
5. Dispense in test tubes.

31) Water Agar

Component	Quantity
Agar	20.0 g
Distilled water	1.0 L

1. Dispense in test tubes.
2. Autoclave at 121°C for 15 minutes.

32) Xanthine agar

Component	Quantity
Nutrient agar	23.0 g
Xanthine	4.0 g
Distilled water	1.0 L

The method of preparation is the same as tyrosine agar except that tyrosine is replaced with xanthine.

33) Yeast extract phosphate medium with ammonia for selective isolation of *H. capsulatum* and *B. dermatitidis*

Component	Quantity
Yeast extract	1.0 g

Component	Quantity
Phosphate buffer	1.0 g
Bacto agar	20.0 g
Chloramphenicol	0.5 g
Gentamicin	0.2 g
Distilled water	1.0 L

Phosphate buffer preparation

1. Dissolve 40.0 g Na_2HPO_4 in 300.0 mL distilled water.
2. Add 60.0 g of KH_2PO_4.
3. Adjust pH with HCl or NaOH.
4. Adjust volume to 400.0 mL.
1. Suspend ingredients in distilled water, heat to a boil with frequent agitation.
2. Sterilise by autoclaving at 121°C for 15 minutes.
3. Final pH at 6.0.
4. Use with ammonia.
5. Specimens are inoculated onto medium in Petri dish by spreading 0.5 mL to 1.0 mL on the surface.
6. Put one drop (~0.5 mL) on the agar surface.
7. Allow ammonia to diffuse without being spread (Higher concentration of ammonia will inhibit other pathogenic fungi).

34) Yeast peptone Dextrose agar

Component	Quantity
Bacto peptone	1.0 g
Glucose	2.0 g
Yeast extract	1.0 g
Distilled water	100.0 mL

Sterilise at 121°C for 15 minutes.

STAINS

1) Calcofluor white (CFW)

Stock Solution

Component	Quantity
Calcofluor white	1.0 g
Distilled water	100.0 mL

Working solution

1:10 dilution of stock solution in 0.05% Evans blue.

2) Gram Stain (Hucker modification)

Crystal Violet Reagents

Component	Quantity
Crystal violet (85.0% dye)	2.0 g

Component	Quantity
Ethyl alcohol (95.0%)	10.0 mL
Distilled water	100.0 mL

Gram Iodine Solution

Component	Quantity
Iodine	1.0 g
Potassium iodide	2.0 g
Distilled Water	300.0 mL

Counter Stain

Component	Quantity
Safranin O	1.0 g
Ethyl alcohol (95.0%)	40.0 mL
Distilled Water	400.0 mL

Decolouriser

Acetone or Alcohol

3) Giemsa Stain

Component	Quantity
Giemsa powder	600.0 mg
Methyl alcohol	50.0 mL
Glycerine	50.0 mL

1. Grind Giemsa powder in a mortar. Pour methyl alcohol and glycerine, then decant from the top.
2. Grind it again with glycerine till whole stain is dissolved.
3. Keep the stain at 55°C for 2 hours; shaking gently at 30-minute intervals.
4. Keep it for two weeks for maturation.

Buffer Solution

Component	Quantity
Na_2HPO_4	6.77 g
KH_2PO_4	2.50 g
Distilled Water	1.0 L
pH	7.2

Dilute the stain with buffer for use.

4) Lactophenol Cotton Blue Stain (LCB)

Principle

Phenol kills fungal elements, lactic acid preserves morphology, glycerol prevents drying, and cotton blue binds to chitin in the fungal cell wall, staining fungal structures blue.

Component	Quantity
Phenol	20.0 g
Lactic acid	20.0 g

Component	Quantity
Glycerine	40.0 g
Cotton blue	0.05 g
Distilled water	20.0 mL

Phenol should be melted by placing the container in warm water before weighing.

Add in order: Lactic acid → glycerine → distilled water → phenol.
Then dissolve over even heat.

Procedure for preparation of LCB mounts

1. Place one drop of LCB on a clean slide.
2. Transfer a small portion of the fungal colony.
3. Tease gently using sterile needles.
4. Place the coverslip carefully.
5. Examine under low- and high-power objectives.

Observation

1. Hyphae stain blue.
2. Conidia, spores, conidiophores and sporangia are clearly visualised.
3. Characteristic fungal morphology can be identified.

Precautions

1. Use young fungal cultures.
2. Avoid thick mounts.
3. Prevent air bubbles beneath the coverslip.

5) Nigrosin Staining Solution

Component	Quantity
Nigrosin (granular)	10.0 g
Formalin (10.0%)	100.0 mL

1. Place the solution in a boiling water bath for 30 minutes. Add 10.0% formalin lost by evaporation.
2. Filter twice through double filter paper (Whatman No.1).

6) Potassium hydroxide (KOH-mounting fluid)

(a) With DMSO

Component	Quantity
Distilled water	60.0 mL
Potassium hydroxide	20.0 g
DMSO	40.0 mL

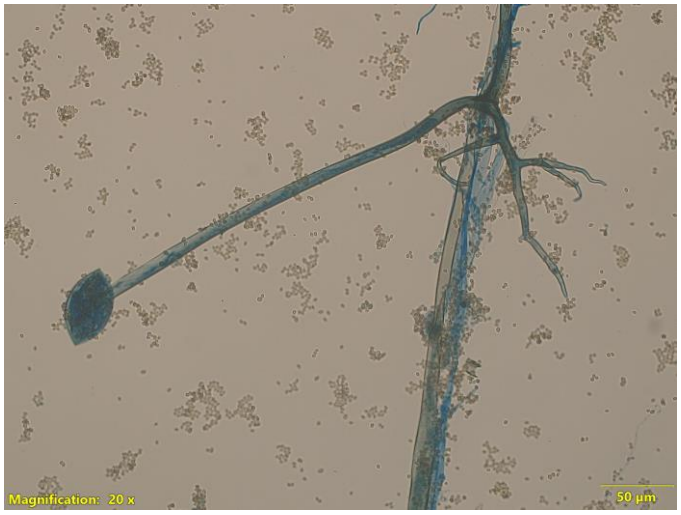
(b) Without DMSO

Component	Quantity
Distilled water.	80.0 mL
Potassium hydroxide	20.0 g

Component	Quantity
Glycerine	20.0 mL

1. Addition of DMSO or glycerine prevents rapid drying of the fluid and permits observations of the slide for up to 48 hours.
2. DMSO also accelerates keratin digestion/clearing of the specimen, permitting immediate microscopic examination without the need for slide heating - particularly useful for thickly keratinised specimens such as nails and hair.

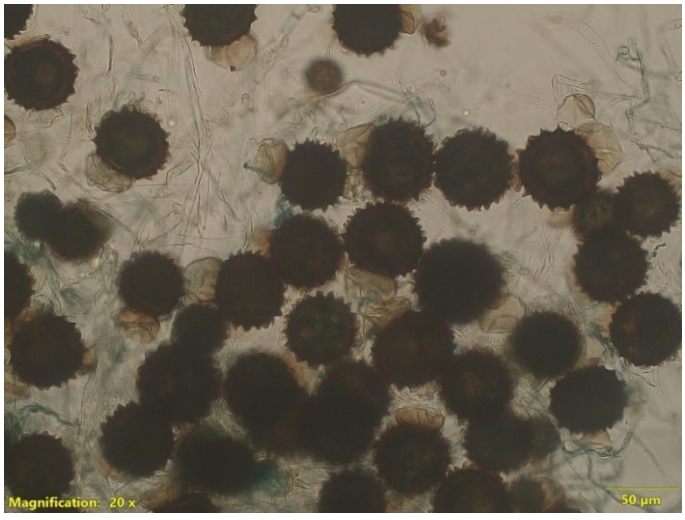
Annexure B: Representative Images of Medically Important Fungi



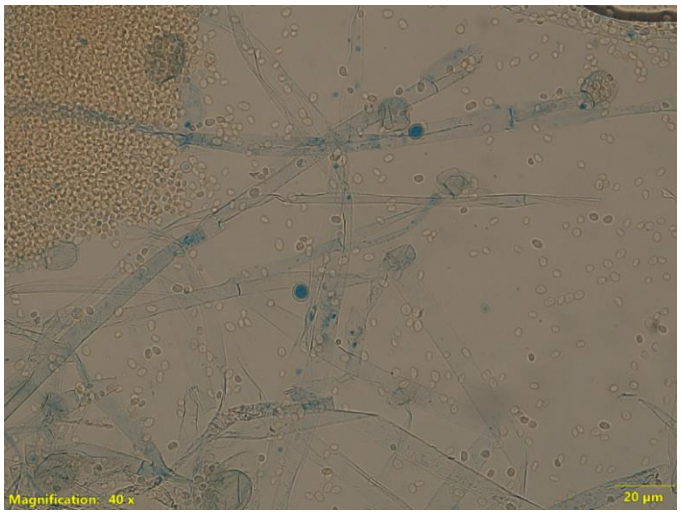
Lactophenol Cotton Blue (LCB) mount of *Rhizopus arrhizus* (grown on SDA, 30°C)



LCB mount of *Rhizopus microsporus* (grown on MEA, 30°C)



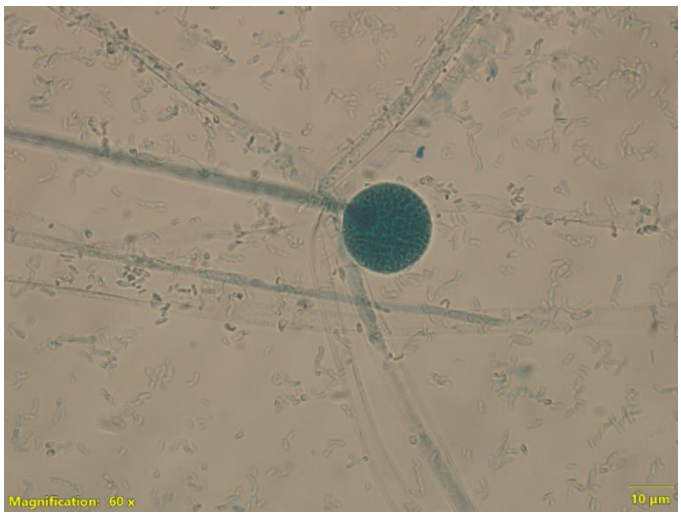
LCB mount of *Rhizopus homothallicus* depicting the golden-brown, spiny/ ornamented zygospores and suspensor cells (grown on SDA, 30°C)



LCB mount of *Mucor racemosus* (grown on MEA, 30°C)



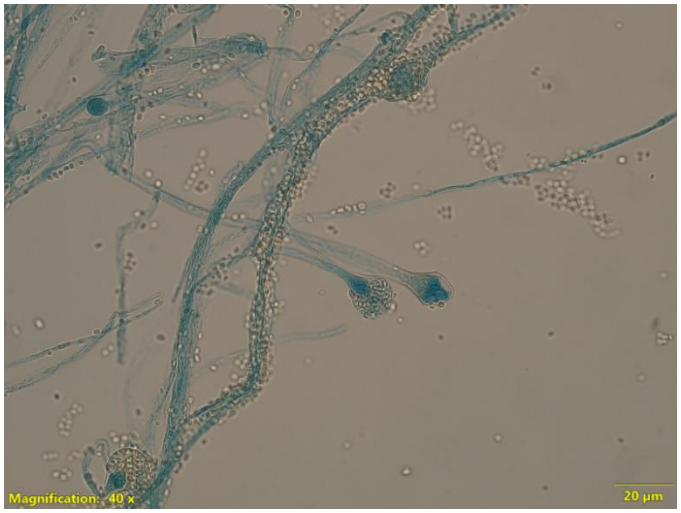
LCB mount of *Mucor indicus* (grown on MEA, 30°C)



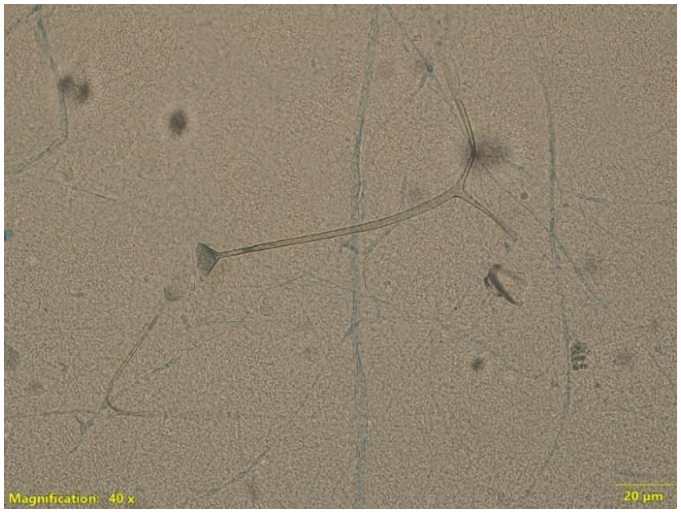
LCB mount of *Mucor irregularis* (grown on MEA, 30°C)



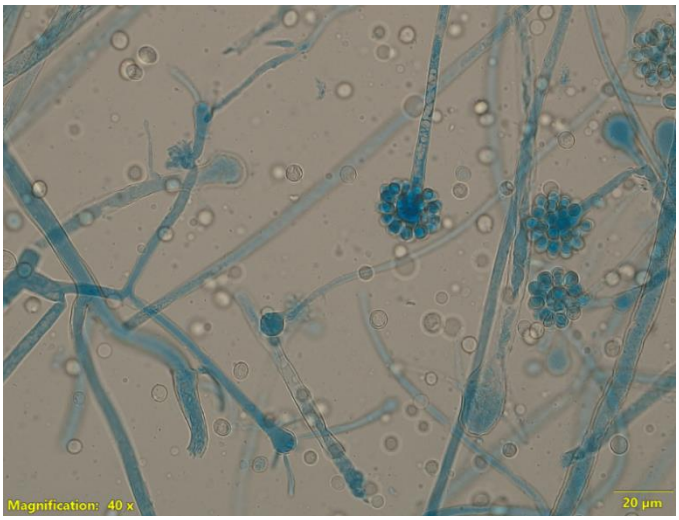
LCB mount of *Rhizomucor pusillus* (grown on MEA, 30°C)



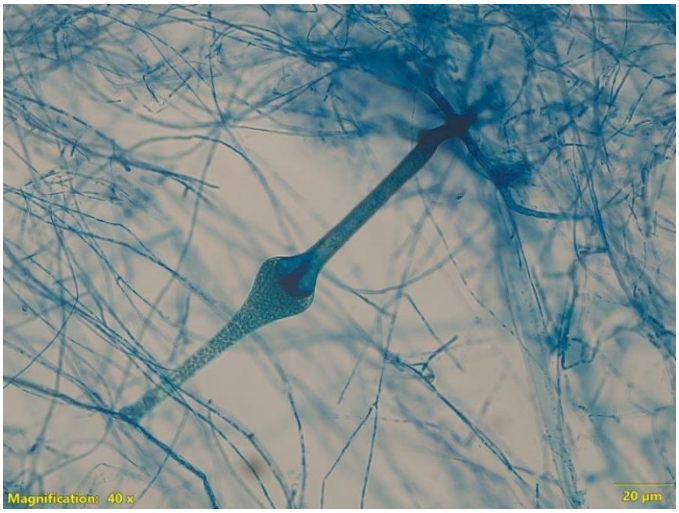
LCB mount of *Lichtheimia corymbifera* (grown on SDA, 30°C)



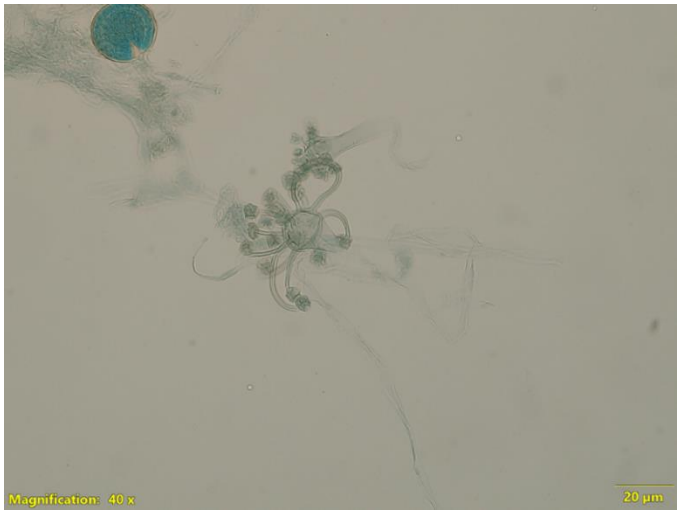
LCB mount of *Apophysomyces variabilis* (grown on DRBC at 30°C for sporulation**)**



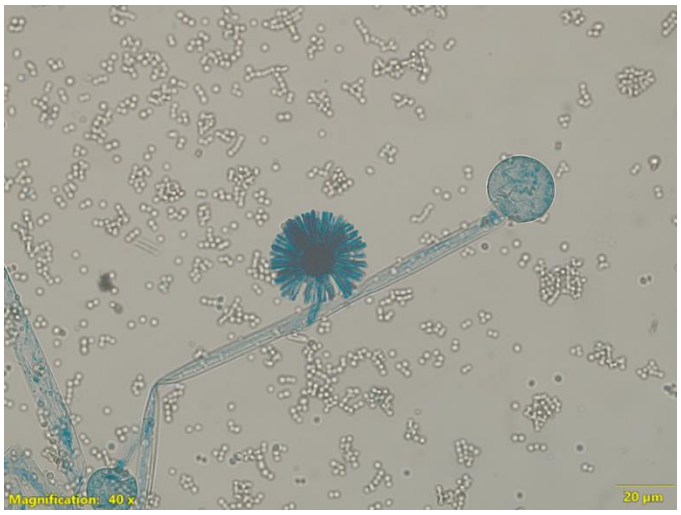
LCB mount of *Cunninghamella bertholletiae* (grown on SDA, 30°C**)**



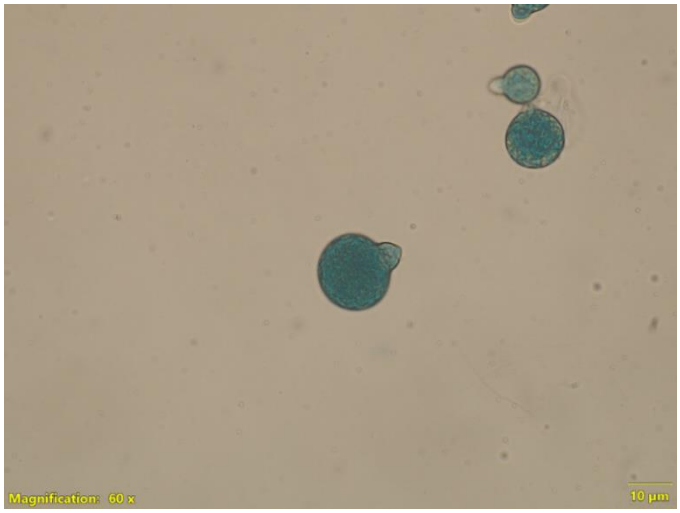
LCB mount of *Saksenaea vasiformis* (grown on OMA, 30°C**)**



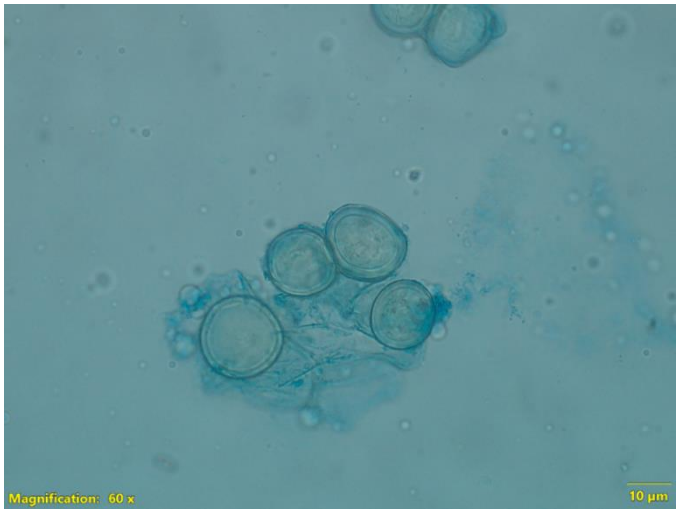
LCB mount of *Cokeromyces recurvatus* (grown on SDA, 30°C**)**



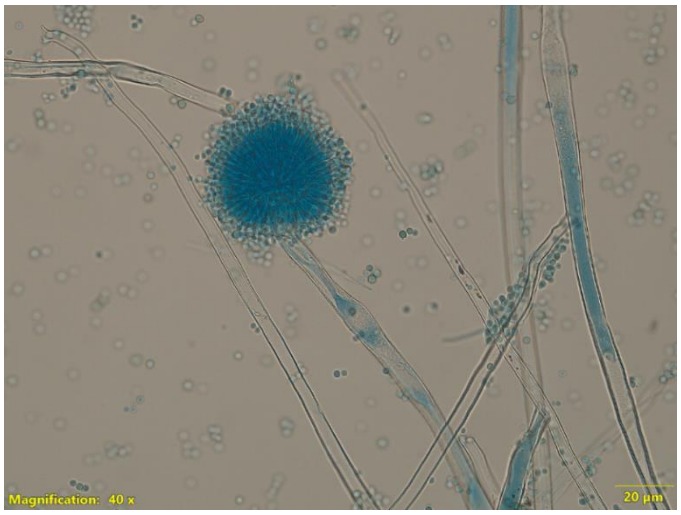
LCB mount of *Syncephalastrum racemosum* (grown on MEA, 30°C**)**



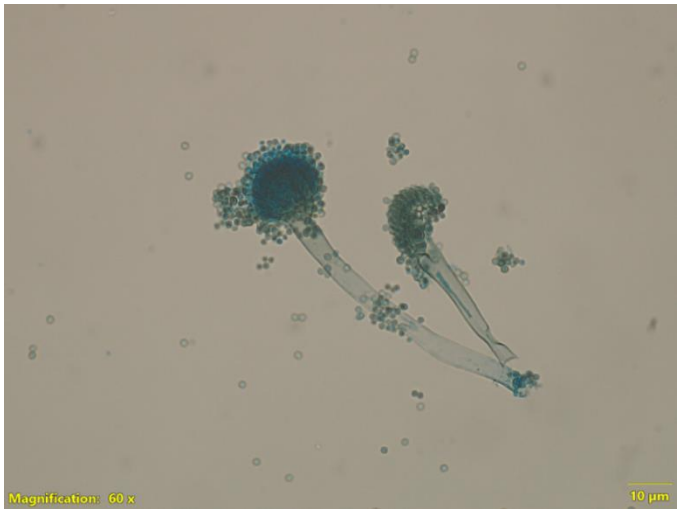
LCB mount of *Conidiobolus coronatus* (grown on PDA, 30°C)



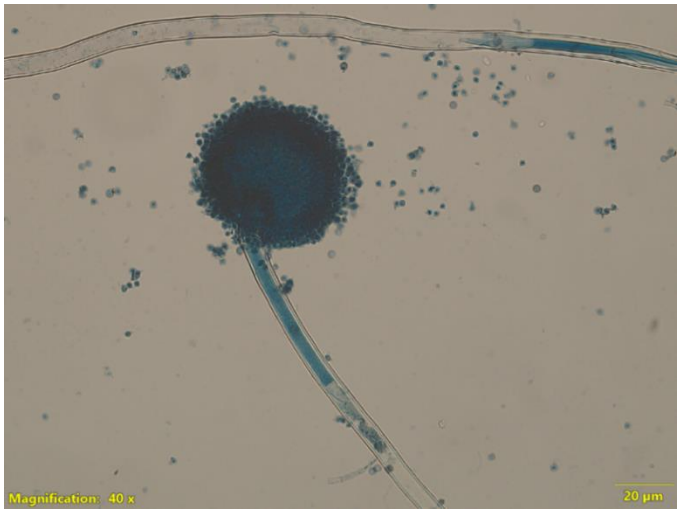
LCB mount of *Basidiobolus ranarum* (grown on SDA, 30°C**)**



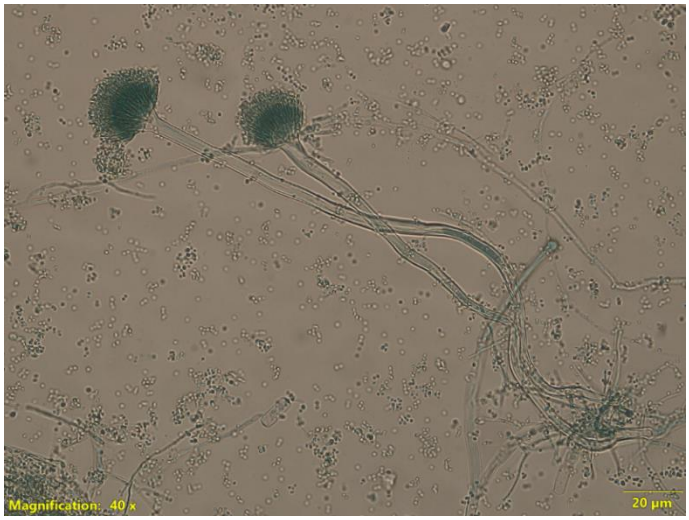
LCB mount of *Aspergillus flavus* (grown on SDA, 30°C**)**



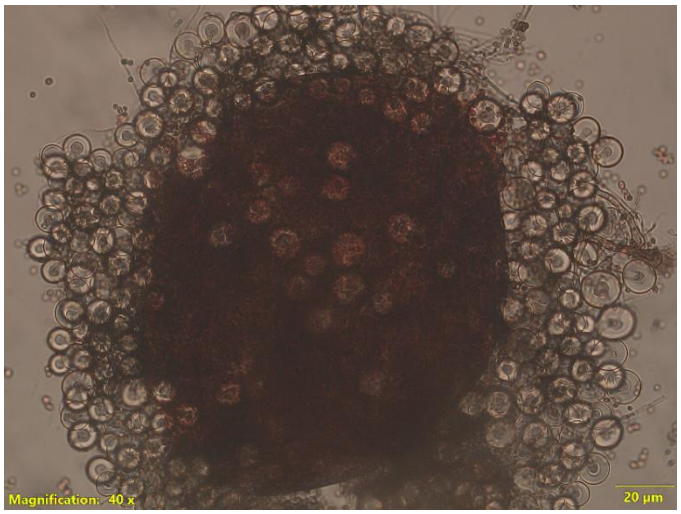
LCB mount of *Aspergillus fumigatus* (grown on SDA, 30°C**)**



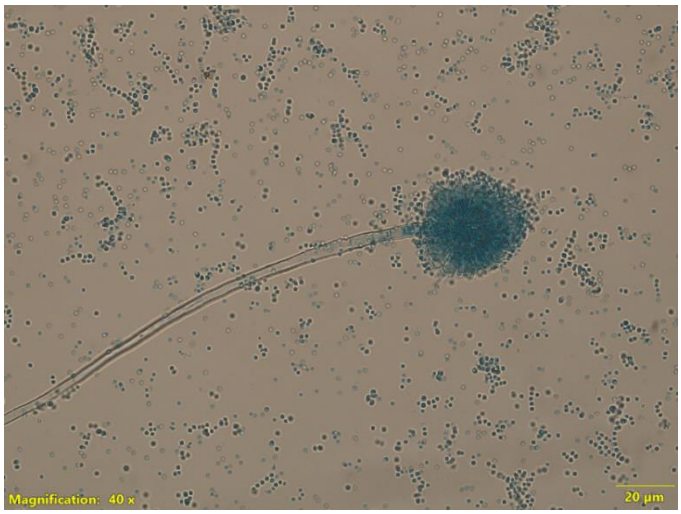
LCB mount of *Aspergillus niger* (grown on SDA, 30°C**)**



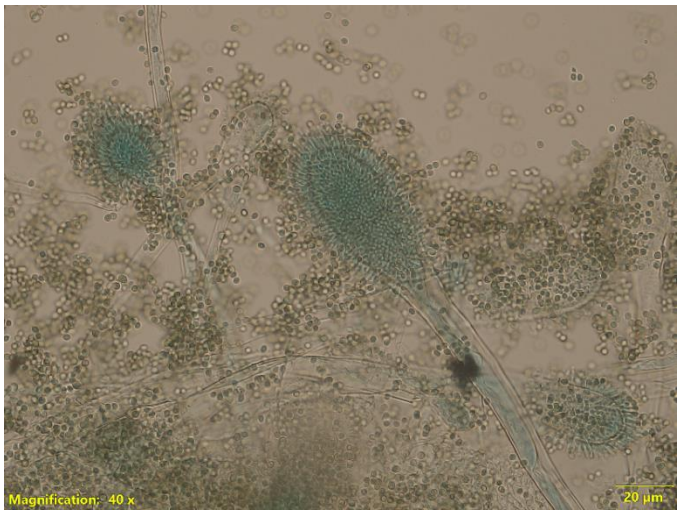
LCB mount of *Aspergillus terreus* (grown on SDA, 30°C**)**



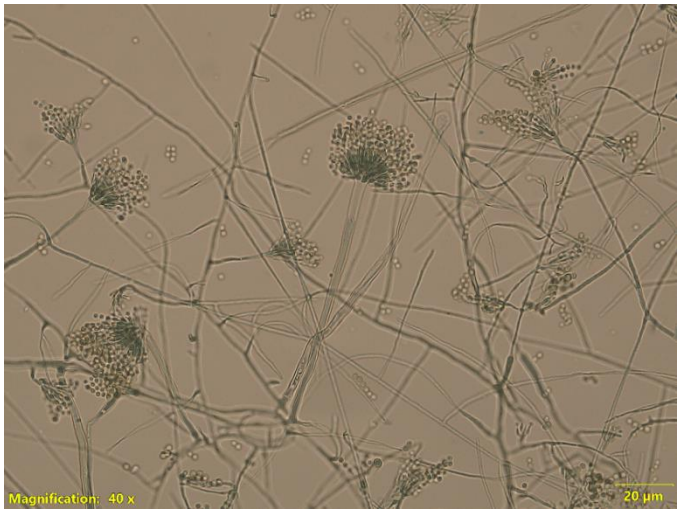
LCB mount of *Aspergillus nidulans* showing ascomata surrounded by Hülle cells (grown on CzA, 30°C)



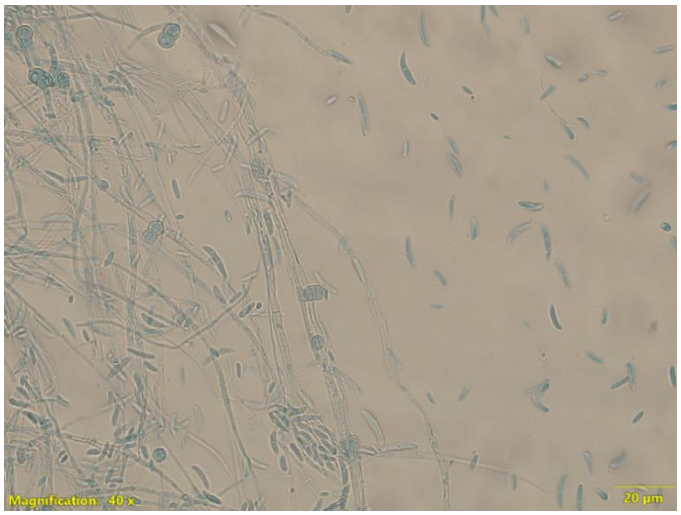
LCB mount of *Aspergillus candidus* (grown on CzA, 30°C)



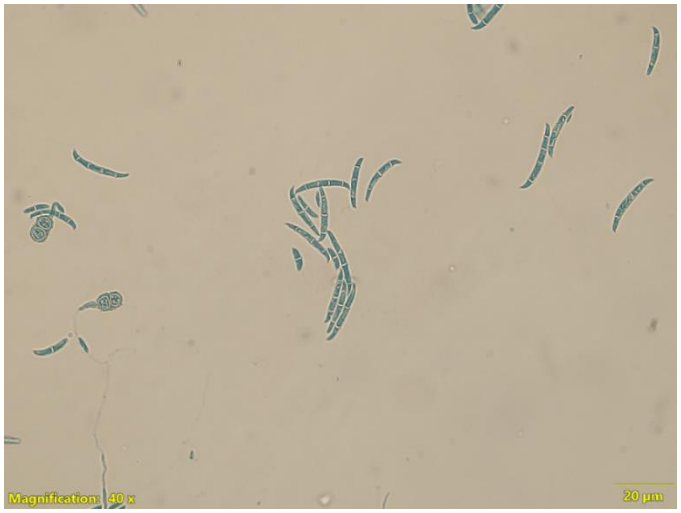
LCB mount of *Aspergillus clavatus* (grown on CzA, 30°C)



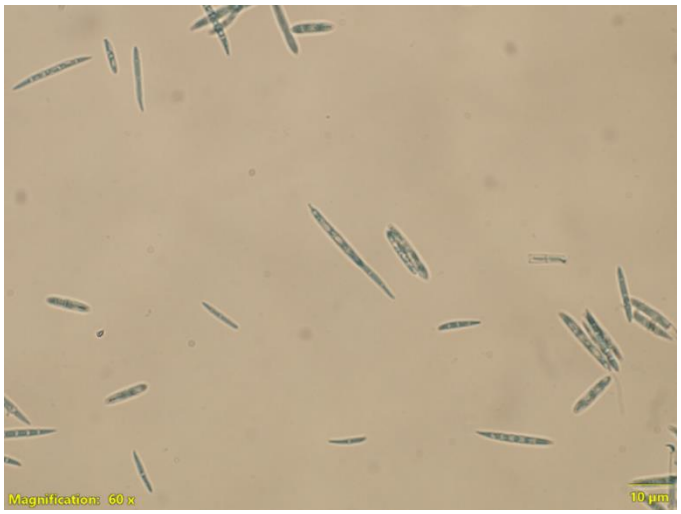
LCB mount of *Aspergillus versicolor* (grown on CzA, 30°C)



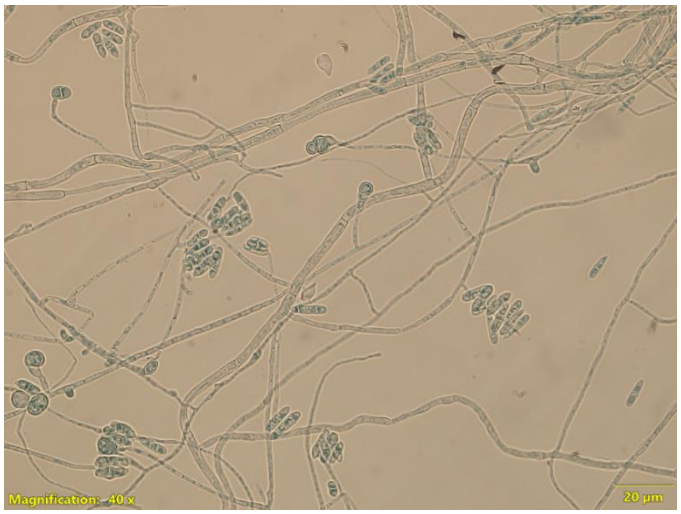
LCB mount of *Fusarium solani* showing chlamydospores and sporodochia (grown on OMA,



LCB mount of *Fusarium oxysporum* 3–5 septate fusiform macroconidia and chlamydospores (grown on OMA, 30°C)



LCB mount of *Fusarium incarnatum* showing macroconidia with 3–7 septa (grown on OMA, 30°C)



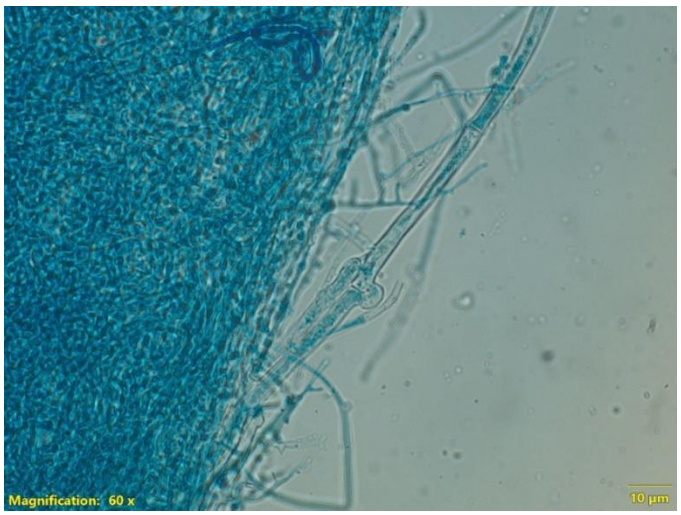
LCB mount of *Fusarium lichenicola* (grown on OMA, 30°C)



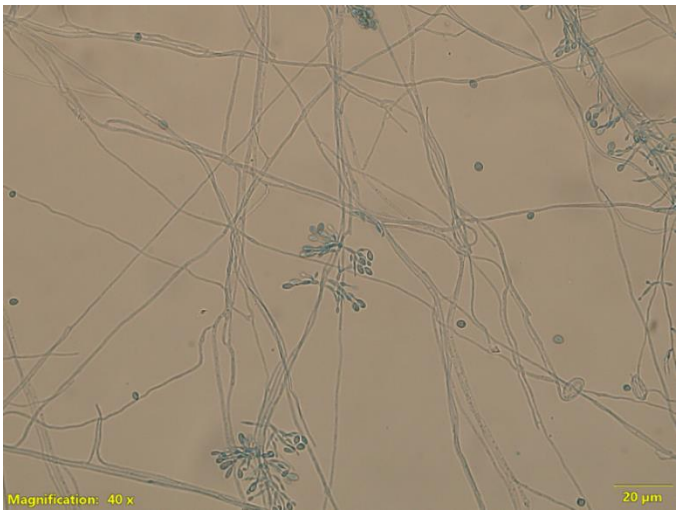
LCB mount of *Purpureocillium lilacinum* (grown on MEA, 30°C)



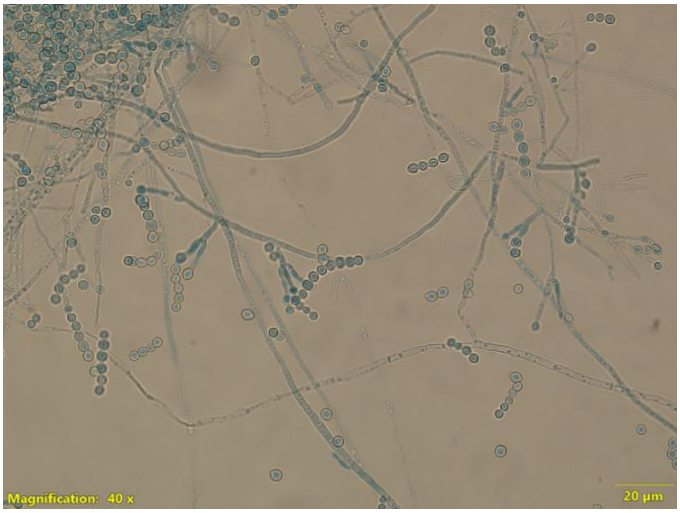
LCB mount of *Sarocladium kiliense* (grown on MEA, 30°C)



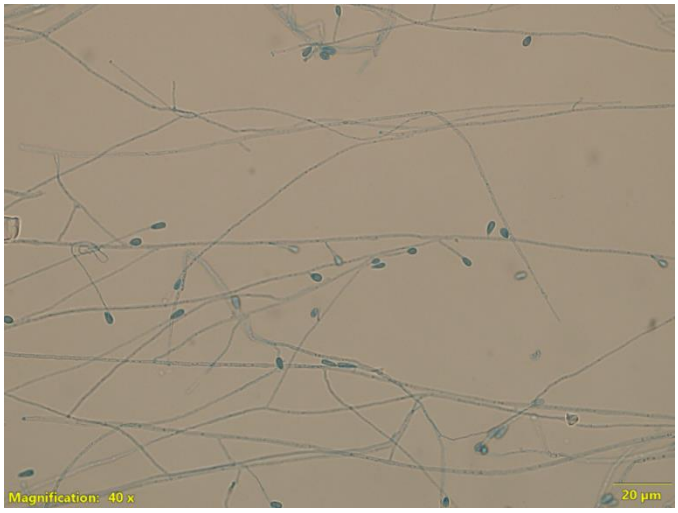
LCB mount of *Schizophyllum commune* showing clamp connections (grown on OMA, 30°C)



LCB mount of *Lomentospora prolificans* (grown on OMA, 30°C)



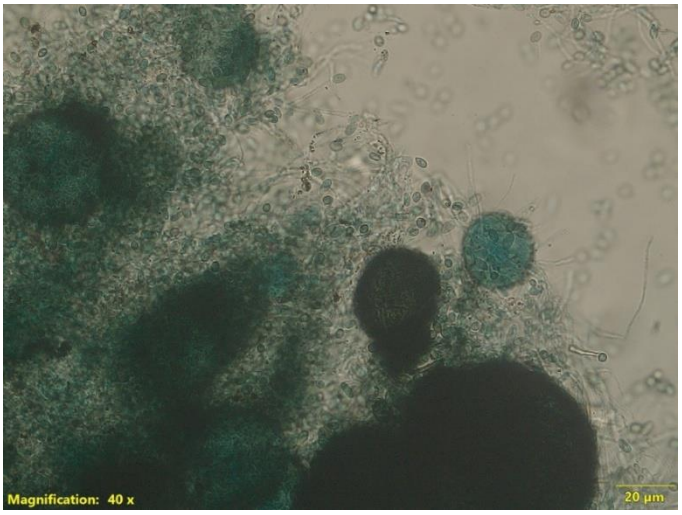
LCB mount of *Scopulariopsis brevicaulis* (grown on OMA, 30°C)



LCB mount of *Scedosporium apiospermum* (*Scedosporium* synanamorph) (grown on PDA, 30°C)



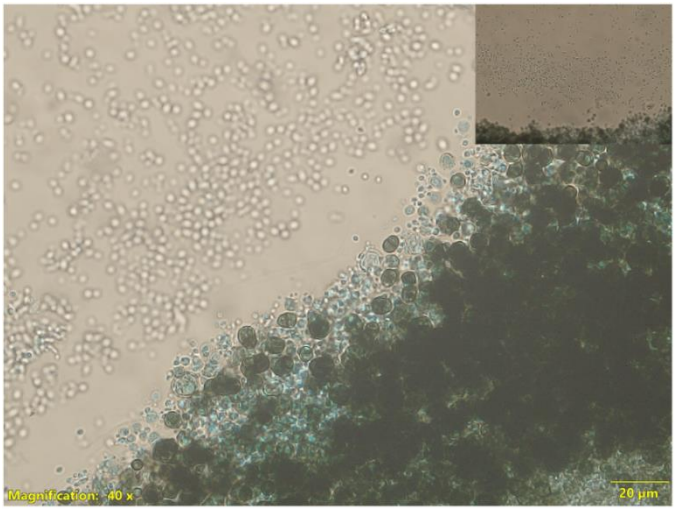
LCB mount of *Scedosporium apiospermum* (*Graphium* synanamorph) (grown on PDA, 30°C)



LCB mount of *Scedosporium boydii* (grown on SDA, 30°C)



LCB mount of *Alternaria alternata* (grown on SDA, 30°C)



LCB mount of *Aureobasidium pullulans* mature didymoconidia (Inset: new conidia) (grown on MEA, 30°C)



LCB mount of *Bipolaris australiensis* (grown on PDA, 30°C)



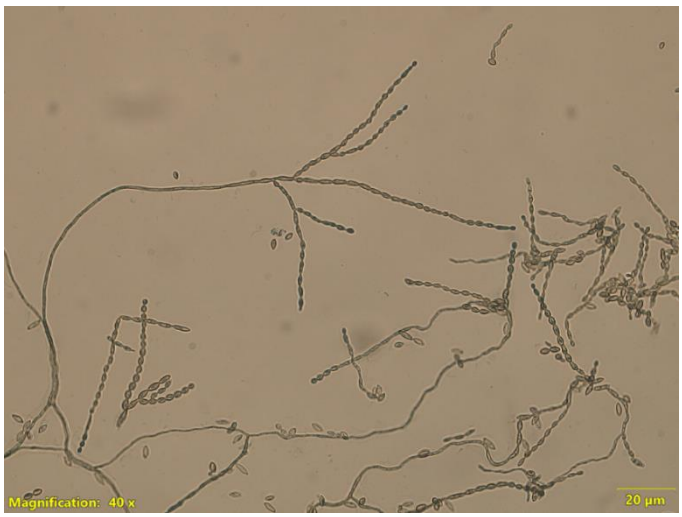
LCB mount of *Curvularia lunata* (grown on PCA, 30°C)



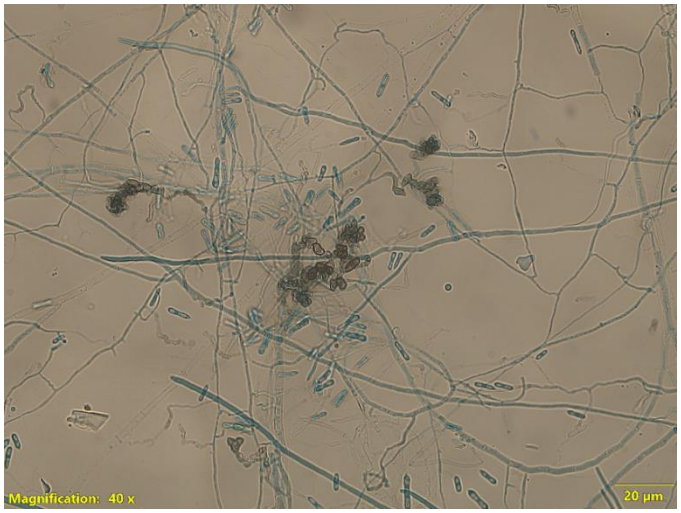
LCB mount of *Curvularia spicifera* (grown on PCA, 30°C)



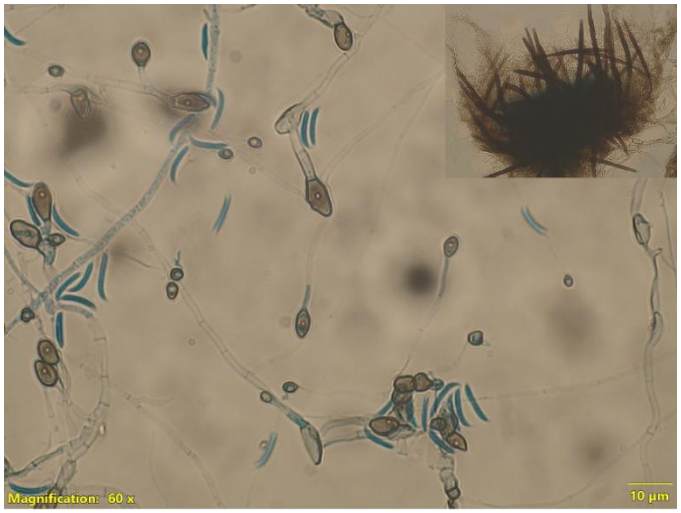
LCB mount of *Cladophialophora carrionii* (grown on PCA, 30°C)



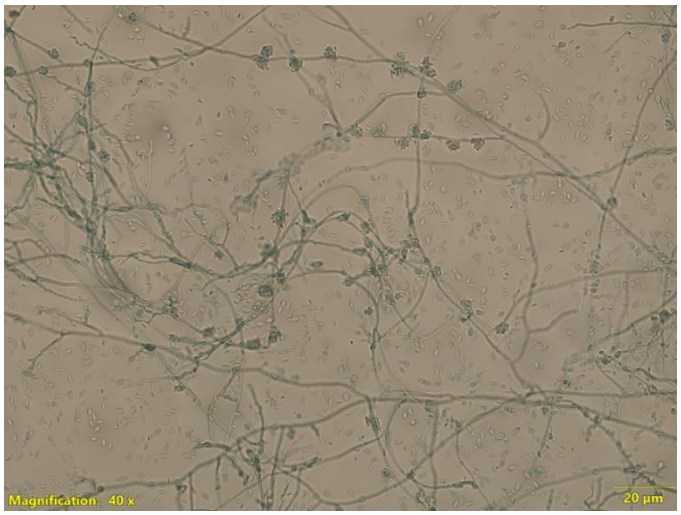
LCB mount of *Cladophialophora bantiana* (grown on PCA, 30°C)



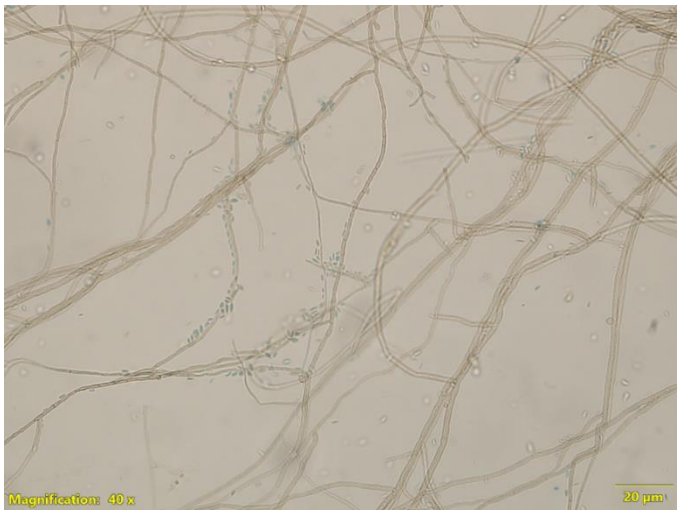
LCB mount of *Colletotrichum gloeosporioides* depicting appressoria (grown on OMA, 30°C)



LCB mount of *Colletotrichum truncatum* depicting appressoria; Inset: showing asexual fruiting structure, acervulus (grown on OMA, 30°C)



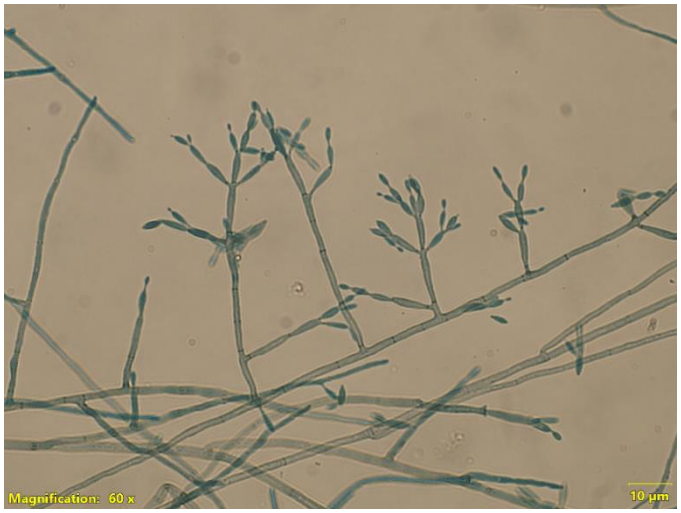
LCB mount of *Exophiala jeanselmei* (grown on SDA, 30°C)



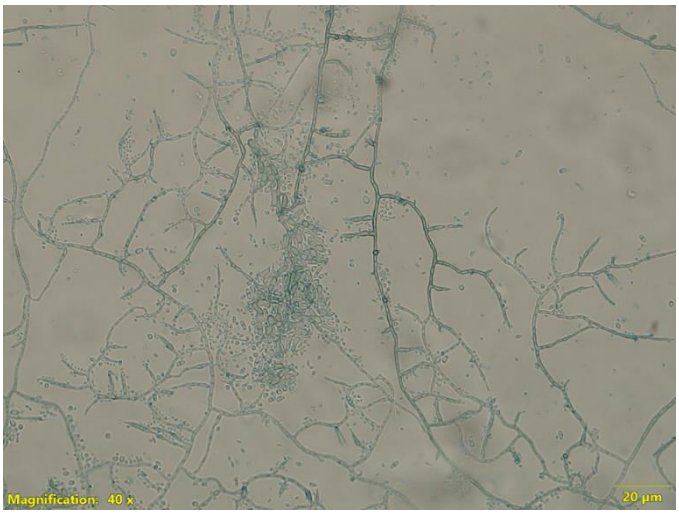
LCB mount of *Exophiala spinifera* (grown on SDA, 30°C)



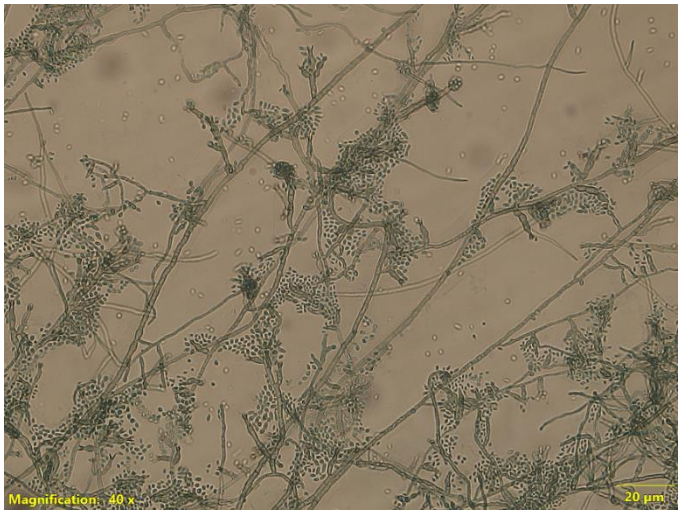
LCB mount of *Exserohilum rostratum* (grown on PCA, 30°C)



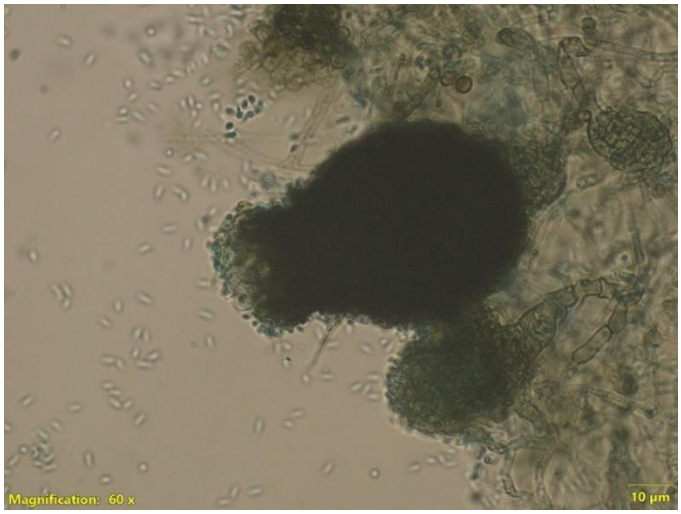
LCB mount of *Fonsecaea pedrosoi* (grown on OMA, 30°C)



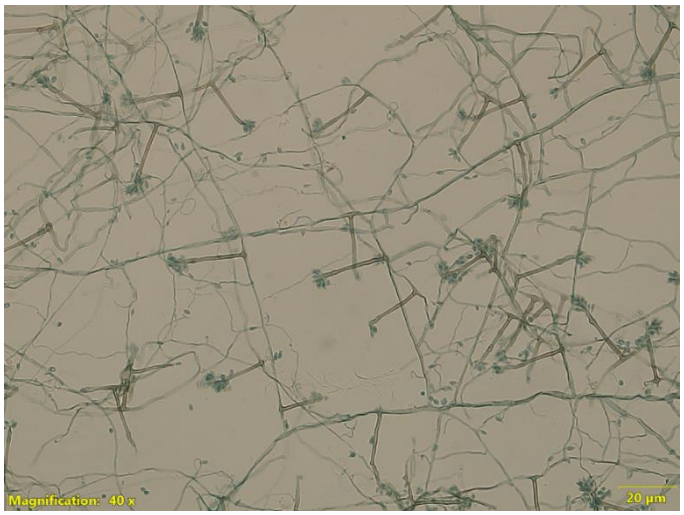
LCB mount of *Phaeoacremonium rubrigenum* (grown on MEA, 30°C)



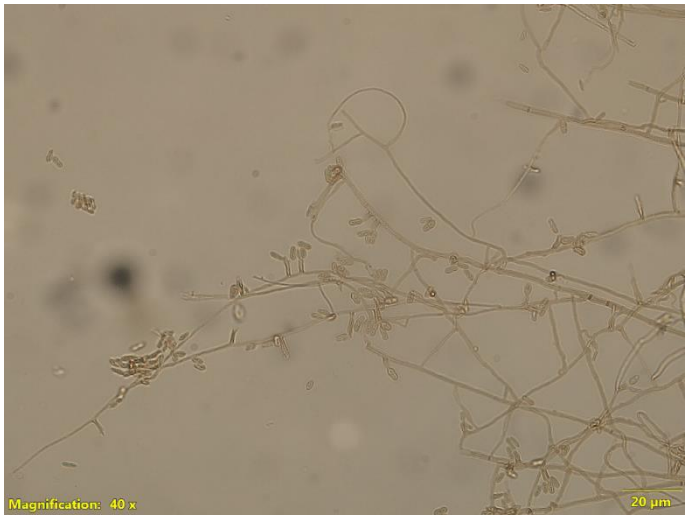
LCB mount of *Phialophora verrucosa* (grown on SDA, 30°C)



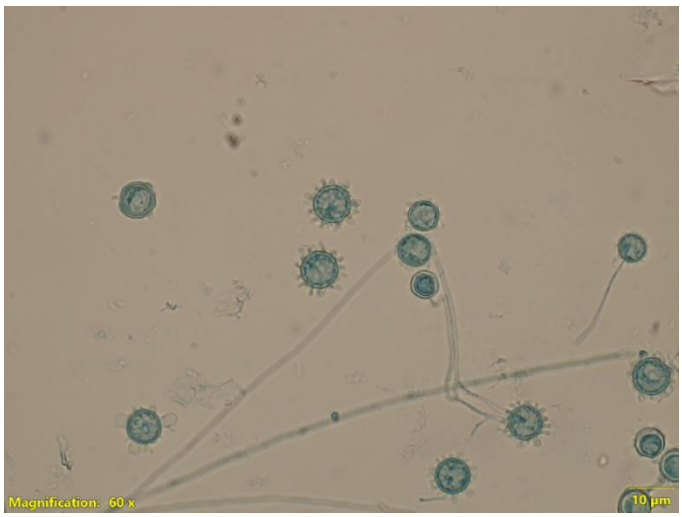
LCB mount of pycnidial fruiting bodies of *Phoma* spp. (grown on OMA, 30°C)



LCB mount of *Rhinocladiella mackenziei* (grown on OMA, 30°C)



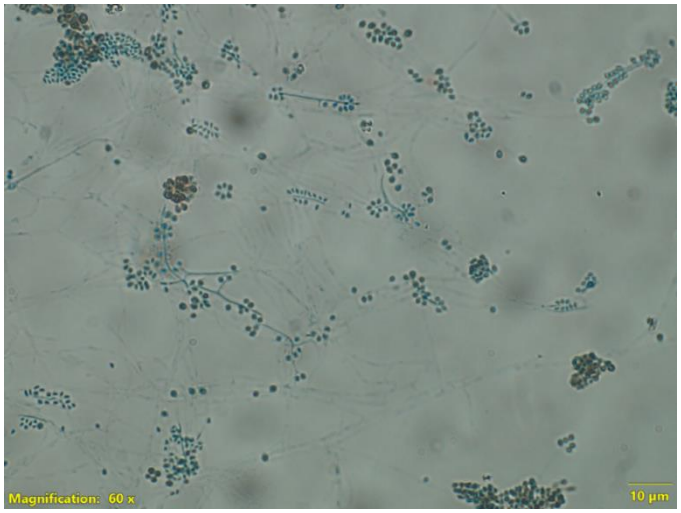
LCB mount of *Verruconis gallopava* (grown on OMA, 30°C)



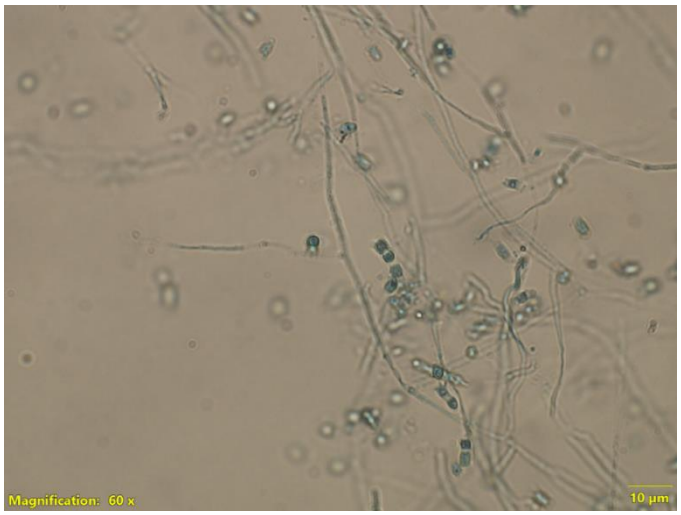
LCB mount of *Histoplasma capsulatum* made from growth (mycelial phase) showing the tuberculate macroconidia (grown on SDA, 25°C)



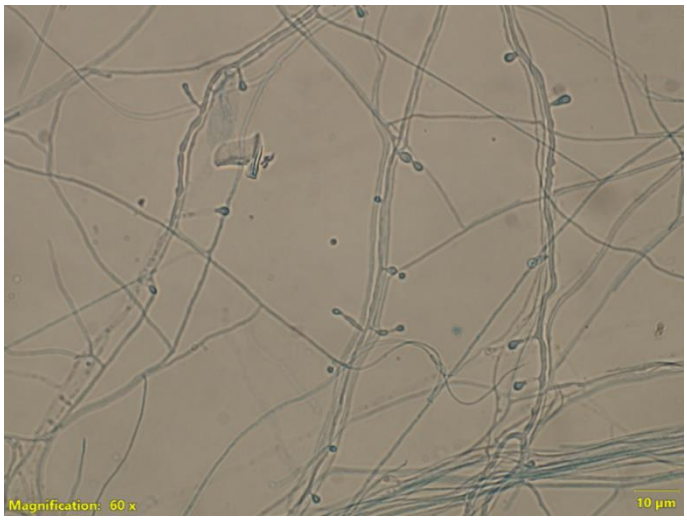
LCB mount of *Talaromyces marneffei* mycelial phase (grown on SDA, 25°C)



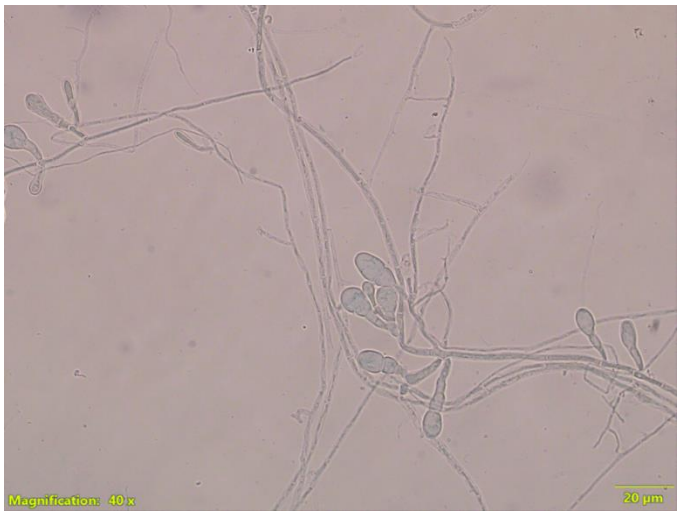
LCB mount of *Sporothrix globosa* mycelial phase (grown on MEA, 25°C)



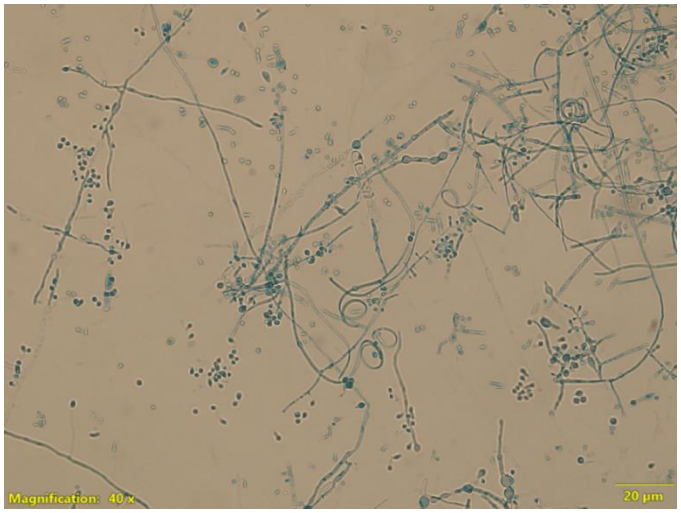
LCB mount of *Coccidioides immitis* mycelial phase depicting the barrel-shaped arthroconidia alternating with empty disjunctor cells (grown on SDA, 25°C)



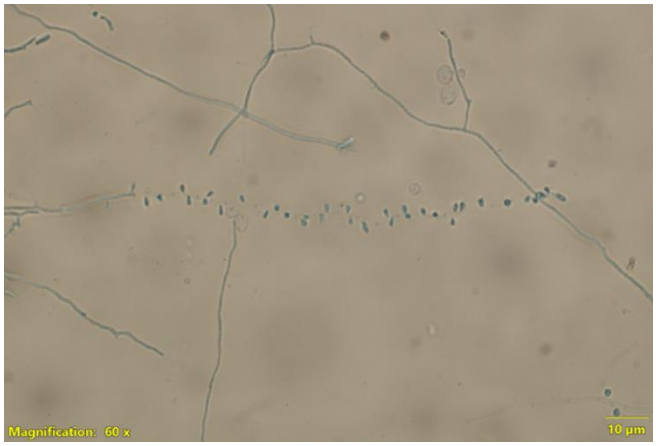
LCB mount of mycelial phase of *Emergomyces pasteurianus* (grown on SDA, 25°C)



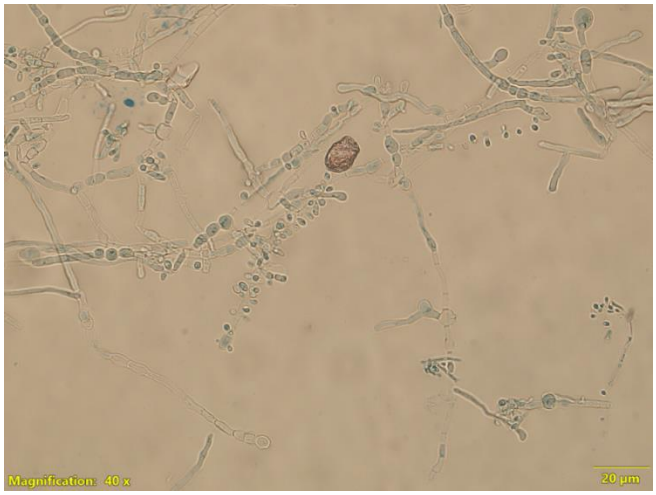
LCB mount of *Epidermophyton floccosum* (grown on SDA, 30°C)



LCB mount of *Trichophyton mentagrophytes*/*interdigitale* complex (grown on SDA, 30°C)



LCB mount of *Trichophyton rubrum* (grown on SDA, 30°C)



LCB mount of *Trichophyton tonsurans* (grown on SDA, 30°C)

Glossary

Glossary is adapted primarily from standard textbooks including Larone's Medically Important Fungi: A Guide to Identification (7th edition), Koneman's Colour Atlas and Textbook of Diagnostic Microbiology (7th edition), de Hoog's Atlas of Clinical Fungi (4th edition) and Medical Mycology: A Practical Approach (Evans & Richardson).

Acid-fast	A property of cell walls that, during a staining reaction, retains basic dyes when decolourised 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 conidiophore
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 incorporation and utilization of nutrients, such as carbon or nitrogen sources, by a microorganism for cellular growth and biosynthesis.
Ballistoconidium (pl. ballistoconidia)	A forcibly discharged conidium

Balloon form 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 specialised 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*

Blastoconidium (pl. blastoconidia) Holoblastic conidia produced by budding (blowing out) from a conidiogenous cell, singly, synchronously or in acropetal chains

Blastospore See 'Blastoconidium'

Budding An asexual formation of small, rounded outgrowths from a parent cell that develop into daughter cells/conidia

Capsule A gelatinous covering around a cell

Carry-over Substances carried over from the original culture medium in the inoculum that may support growth of the test isolate in an assimilation study

Chlamydoconidium (pl. 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 specialised hyphal bridge involved with nuclear division in the Basidiomycetes

Clavate Club-shaped

Cleistothecium (pl. Cleistothecia) A completely closed ascoma (fruiting body) that lacks a natural opening (ostiole). It contains randomly dispersed asci and releases its enclosed ascospores only upon the rupture or decay of its outer wall (peridium)

Collarette A small collar

Columella (pl. columellae)	A sterile domelike expansion at the apex of a sporangiophore
Conidiogenous cell	A cell that gives rise to conidia
Conidiophore	A specialised hypha upon which conidia develop
Conidium (pl. conidia)	An asexual, nonmotile, usually deciduous propagule formed externally by a fungus and usually capable of being dispersed
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
Exudate	Droplets of fluid formed on the surface of a colony
Fermentation	Metabolic process in which microorganisms obtain energy by converting organic substrates, such as carbohydrates, into simpler products without the use of an external electron acceptor such as oxygen; $C_6H_{12}O_6 \rightarrow 2C_2H_5OH + 2CO_2$
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 structure
Fragmentation	Separation of a hypha into conidia
Fungus (pl. fungi)	A eukaryotic, unicellular to filamentous, achlorophyllous organism having absorptive nutrition. A fungus may reproduce sexually, asexually, or by both means
Fusiform	Tapering at both ends; spindle-shaped
Geniculate	Bent like a series of knees
Germ pore	A thin-walled 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
Hemispherical	Shaped like half a sphere
Hilum (pl. hila)	A scar at the base of a conidium
Hyaline	Colourless/transparent
Hypha (pl. hyphae)	An individual filament of a fungus
Intercalary	Occurring within a hypha
Internode	The portion of a hypha between two nodes
Karyogamy	Fusion of two nuclei
Lanose	Having a woolly texture
Macroconidium (pl. macroconidia)	A relatively large conidium produced by a fungus that forms two morphologically distinct conidial types
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
Microconidium (pl. microconidia)	A relatively small conidium produced by a fungus that forms two morphologically distinct conidial types
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	Inversely club-shaped, with the broader end toward the base
Obovoid	Egg-shaped with the broader end toward the apex
Olivaceous	Having an olive shade of colour
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	Presence of several asexual forms of reproduction in addition to sexual
Pseudohypha (pl. pseudohyphae)	A chain of elongated blastoconidia that remain attached, forming a filament-like structure with constrictions at the points of attachment
Pseudomycelium	A large amount of pseudohyphae
Pycnidium (pl. pycnidia)	A sac-like fruiting body that gives rise to conidia within its central area
Pyriform	Pear-shaped
Rachis	An extension of a conidiogenous cell that bears conidia
Racket hyphae (also spelled racquet)	A hypha having a series of cells that are swollen at one end
Rhizoid	A root-like group of hyphae
Septum (pl. septa)	A crosswall
Spherule	A sporangium-like structure containing endospores that is produced by the dimorphic fungi *Coccidioides immitis* and *Coccidioides*

	posadasii. (Note: Spherules are also produced by *Rhinosporidium seeberi*, an organism traditionally studied in mycology but currently classified as a mesomycetozoean protist)
Sporangiolum (pl. sporangiola)	A sporangium that contains a small number of sporangiospores. Some sporangiola may contain only one sporangiospore
Sporangiophore	A specialised hypha that gives rise to a sporangium
Sporangiospore	Asexual spores formed within a sporangium by cleavage of its cytoplasm
Sporangium (pl. sporangia)	An asexual sac-like structure in which sporangiospores are formed by cleavage
Spore	A reproductive propagule that forms either following meiosis or asexually by a cleavage process
Sterigma (pl. sterigmata)	A pedicel bearing a basidiospore
Stolon	A horizontal aerial hyphae/runner, characteristic of *Rhizopus* species
Subglobose	Almost round
Sympodial	Pertaining to the growth of a conidiophore in which successive lateral, subterminal apices develop 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 wart-like or tubercle-like projections
Unipolar budding	The development of a bud 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	Arranged in a whorl; having structures arising from a common point
Vesicle	A swollen cell; the swollen apices of some conidiophore or sporangiophore
Villose	Bearing long, hair-like appendages
Yeast	A unicellular budding fungus that reproduces by sexual, asexual, or both means
Yeast-like	Pertaining to a unicellular budding fungus that reproduces by asexual means only
Zygospore	A resting spore in which meiosis will occur. Zygospores result from the fusion of two similar hyphal elements. They are characteristic of the Mucorales

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Cover page

Image credit: Dr Shivaprakash M Rudramurthy, PGIMER

Image descriptions (left to right):

1. ***Rhizopus homothallicus* – Zygospores**
   Lactophenol cotton blue (LPCB) preparation of *Rhizopus homothallicus* (isolated from bronchoalveolar lavage sample) observed under 40× magnification, showing multiple large, globose, dark brown to black zygospores. The zygospores characteristically exhibit rough, spiny to stellate surface projections. Hyaline, swollen, unequal suspensor cells are seen attached to the zygospores.

2. ***Aspergillus fumigatus* – Colony morphology**
   Colony morphology of *Aspergillus fumigatus* on Czapek-Dox (CZ) agar after 4 days of incubation at 28°C, showing characteristic greyish-blue colonies.
3. ***Schizophyllum commune* – Basidiocarps**
   Basidiocarps (fruiting bodies) of *Schizophyllum commune* observed following 6 weeks of incubation on malt extract agar (MEA) at 25°C. The basidiocarps represent the characteristic sexual fruiting structures of this basidiomycetous fungus.