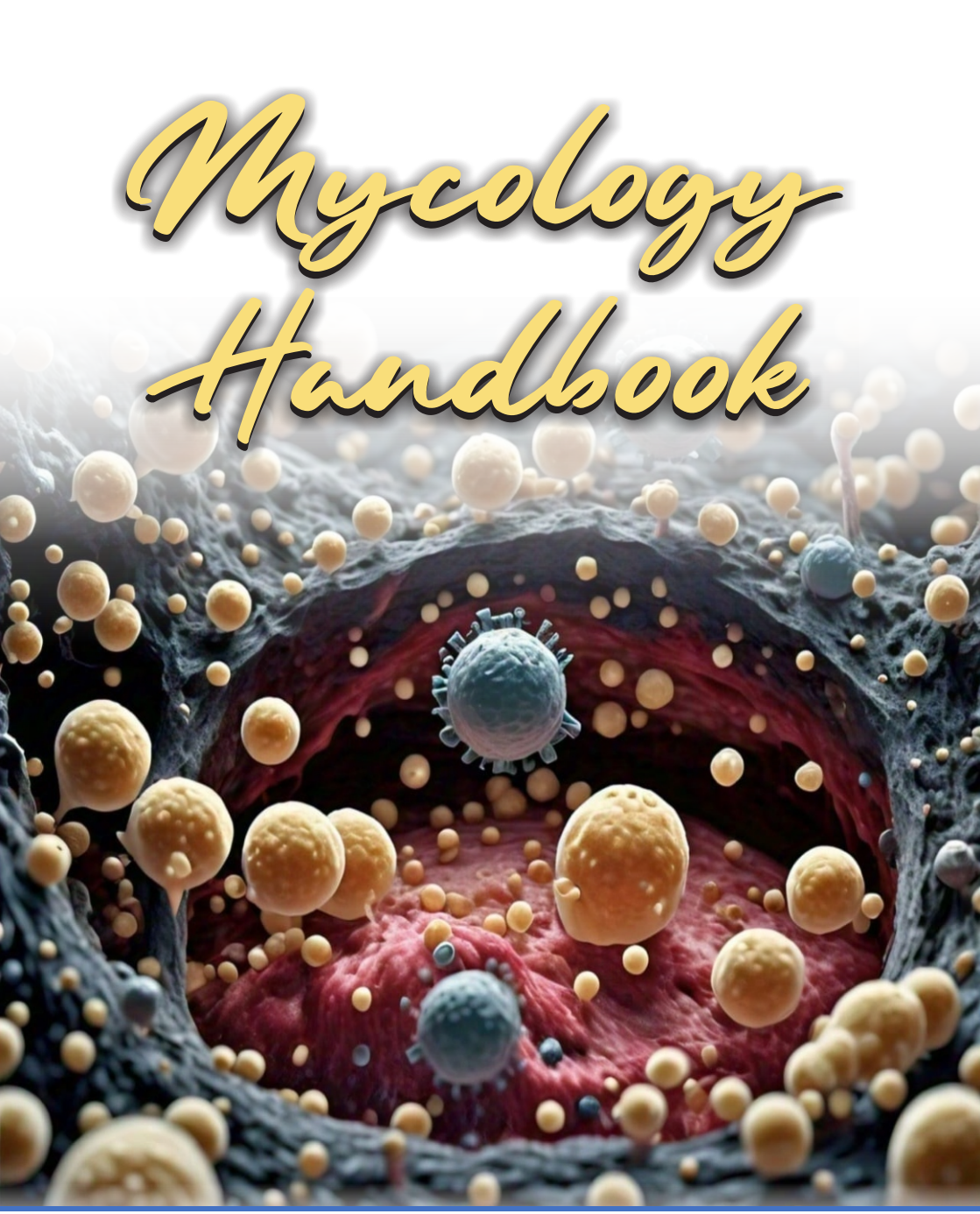
Mycology Handbook



National Institutes of Health Pakistan

Mycology Handbook

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National Fungal Disease Surveillance System

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Preface

Fungal pathogens are a significant threat to public health owing to increasing prevalence in hospitalized patients and increasing resistance to available antifungal agents. Fungal infections and diagnostics have received less attention leading to the burden of fungal diseases, their changing pathogenesis, and antifungal resistance being unknown. World Health Organization recommends three interventions against fungal infections including investing in research and development; improving laboratory capacity and infection surveillance; and improving public health interventions for fungal infection prevention and control. The WHO has issued Fungal Priority Pathogen List (2022) with Candida *albicans, Candida auris, Aspergillus fumigatus and Cryptococcus neoformans* included in critical priority group. Fungal diagnostics are a challenge globally. Most fungal pathogens lack rapid diagnostics, and those that exist are not widely available or affordable globally. The field of fungal diagnostics has evolved from microscopy and culturing to more advanced non-culture-based tools such as novel PCR assays, T2Candida, next generation sequencing and nanotechnology-based tools.

In Pakistan, the assessment of the laboratories capacity towards fungal diagnostics has shown that only few laboratories have fungal diagnostics capacity. The National Institutes of Health has initiated a project funded by Centers for Disease Control and Prevention USA with the aim to build fungal diagnostic and surveillance capacity of laboratories (particularly for *C. auris*) at tertiary care hospitals in Pakistan. *C. auris* is an emerging multidrug-resistant yeast that can colonize the skin and cause invasive infections. It can spread readily between patients in healthcare facilities, causing numerous outbreaks that have been difficult to control. Containment of *C. auris* spread largely depends on timely detection and implementation of appropriate IPC measures.

The Mycology Handbook has been developed to support the microbiologist and laboratorians for fungal diagnostics. The document include various sections covering the topics on basic mycology classification, specimen collection methods, microscopic techniques, various culture media,

Preface

biochemical identification methods, automated systems, antifungal susceptibility testing methods, antigen based detection of fungi etc.

The document has been developed under the stewardship of Dr. Muhammad Salman, CEO National Institutes of Health, whose leadership and critical review is extremely instrumental in finalizing the handbook. Technical oversight and development of the document is provided by Dr. Afreenish Amir, Dr. Rabia Tabassum, and Ms. Kiran Nisa. We thank Dr. Shawn Lockhart (CDC USA) for his critical review and technical guidance for the book.

The contributions of National Fungal Disease Surveillance Team at National Institutes of Health; Maj. Gen. Dr. Aamer Ikram, Dr. Mumtaz Ali Khan, Dr. Muhammad Amjad, Ms. Tarbia Amir, Dr. Amna Ali, Mr. Waleed Khan, Dr. Mahwish Bhatti, Mr. Muhammad Talha, Ms. Ayesha Zulfiqar, Mr Abdul Hanan for supporting the development process is gratefully acknowledged. We acknowledge the scientific overview and contributions of Maj Gen Dr. Irfan Ali Mirza, Col. Sakeenah Naqvi, Dr. Afia Zafar, Dr. Kausar Jabeen, Dr. Joveria Farooqi, Dr. Muhammad Usman, Dr. Summiya Nizamuddin, Dr. Amtul Latif, Dr. Asghar Javaid, Dr. Mumtaz Ahmad, Dr. Ambreen Fatima, Dr. Fouzia Zeeshan, Dr. Hina Bukhari, Dr. Yasmeen Lashari, Dr. Maria Khan, Dr. Sundas Shaukat, Dr. Shafqat Husnain, Dr. Muna Malik, Dr. Kiran Ahmed, Dr. Sana Anwar, Dr. Fazal Hanan, Dr Sabeen Arif, Mr. Noor Sade Khan and Mr. Muhammad Aslam.
Our gratitude to all those committed towards utilization of this guiding document.

Dr. Muhammad Salman
CEO National Institutes of Health

Contents

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Abbreviations

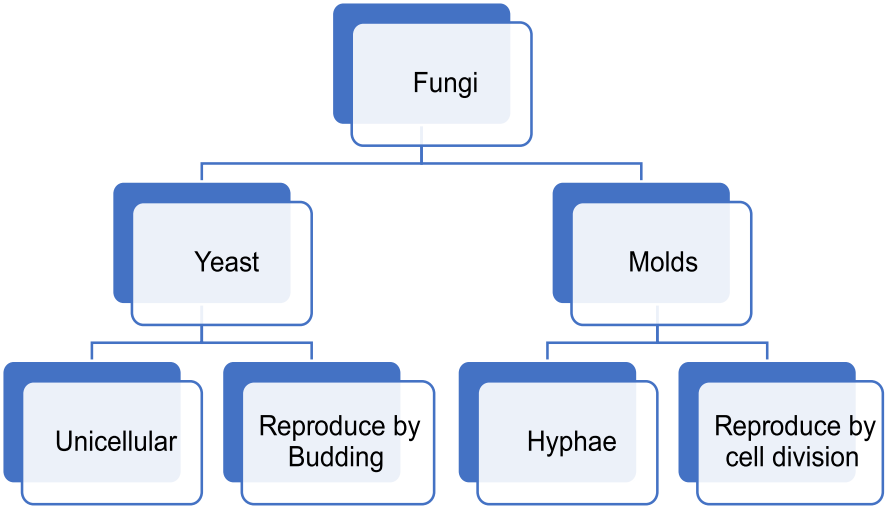
AFST	Antifungal susceptibility testing
API	Analytical profile index
AST	Antimicrobial susceptibility testing
ATCC	American Type Culture Collection
BDG	β-d-glucan
BMD	Broth micro dilution
BSC	Biological safety cabinet
BSL	Biosafety level
CDC	Centers for Disease Control and Prevention
CHCA	α-Cyano-4-hydroxycinnamic acid
CLSI	The Clinical and Laboratory Standards Institute
CSF	Cerebrospinal fluid
EIA	Enzyme immune assay
E-Test	Epsilometer test
EUCAST	European Committee on Antimicrobial Susceptibility Testing
FDA	Food and drug administration
GM	Galactomannan
GTT	Germ tube test
IA	Invasive aspergillosis
IC	Invasive candidiasis
ID-GN	Gram negative bacillus identification

Abbreviations

ID-GP	Gram positive cocci identification
ID-YST	Yeast identification
KOH	Potassium hydroxide
LCB	Lactophenol cotton blue
MALDI TOF MS	Matrix Assisted Laser Desorption Ionization Time-of-Flight
MHA	Mueller-Hinton agar
MIC	Minimum inhibitory concentration
PDA	Potato dextrose agar
QC	Quality control
RPMI	Roswell Park Memorial Institute
SBA	Sheep blood agar
SDA	Sabouraud dextrose agar
TMB	Tetramethylbenzidine

1. Introduction to Fundamental Mycology

Fungi are a group of eukaryotic microorganisms that exist in two basic forms yeasts and molds. Yeasts are microscopic fungi consisting of solitary cells that reproduce by budding. Molds, in contrast, occur in long filaments known as hyphae and reproduce by asexual/sexual spores such as conidiospores, sporangiospores and arthrospores.



The difference between yeast and mold is given below.

Yeast

Molds

(Source: https://microbiologyinfo.com/sabouraud-dextrose-agar-sda-composition-principle-uses-preparation-and-colony-morphology/)

1. Introduction to Fundamental Mycology

Criteria	Yeast	Molds
Definition	Unicellular oval shaped	Multicellular filamentous
Cellular structure	Unicellular	Multicellular
Shape	Oval or round shaped	Thread like
Types	1500	400,000
Color	Creamy white and orange	Black, Green, White and Orange
Common method of Reproduction	Budding/ binary fission	Asexual & sexual spore
Colony morphology	Soft, opaque & cream colored	Filamentous with aerial/vegetative hyphae
Incubation conditions	35-37°C	25-28°C
Aerobic/Anaerobic	Yeast can grow in both conditions	Mostly grow in aerobic conditions
Asexual spores	Blastospores Chlamydospores	Sporangiospores, conidia & arthrospores
Sexual spores	Ascospores	Zygospores, Ascospores, Oospores & Basidiospores

Criteria	Yeast	Molds
Diagnosis/Identification	Identification based on physiological tests and morphological differences	Identification based on microscopic examination & morphology of asexual spores
Energy Production	Convert carbohydrates to alcohol and CO_2 through fermentation under anaerobic conditions	Secrete hydrolytic enzymes that degrade biopolymers such as cellulose, starch and lignin
Examples	*Candida, Cryptococcus*	*Penicillium, Rhizopus, Mucor, Aspergillus, Epidermophyton*

Classification of Fungi

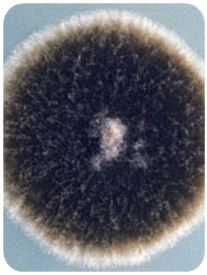
The four basic classifications of fungi are as follows

Mucormycetes	Ascomycetes (Sac Fungi)	Basidiomycetes (Club Fungi)	Deutromycetes (Imperfect Fungi)
• Rhizopus	• Mycosphaerella	• Agaricus	•Cercospora
• Mucor	• Aspergillus	• Polyporus	•Collectotrichum
• Absidia	• Yeast	• Puccinia	•Trichoderma
• Saksenaea	• Pencillium	• Ustilago	•Pyricularia
• Rhizomucor	• Neurospora	• Lycoperdon	•Alternaria
	• Peziza		

Rhizopus

Neurospora

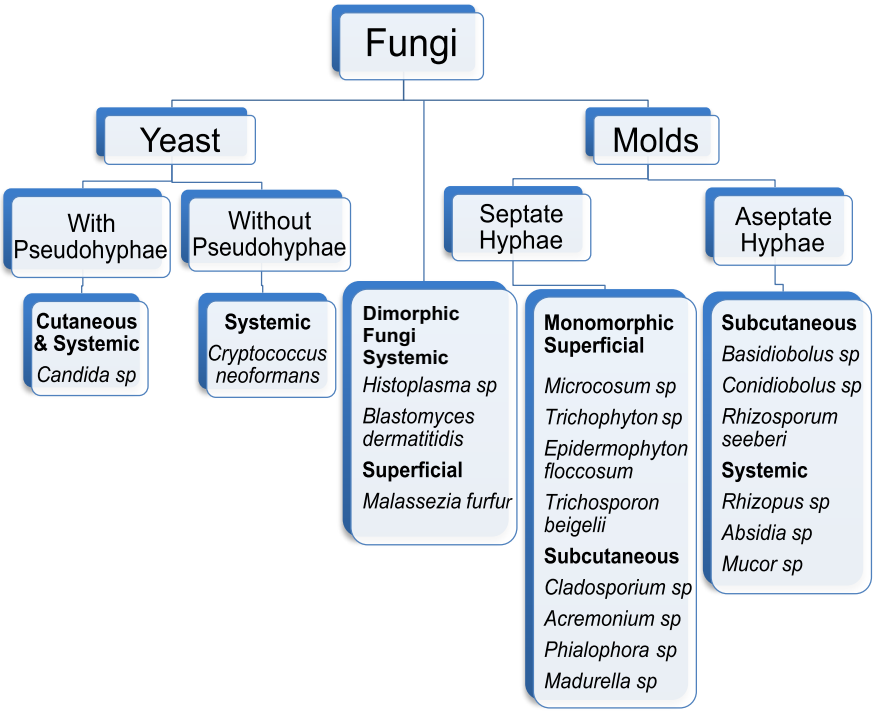
Agaricus



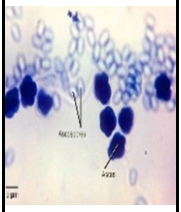
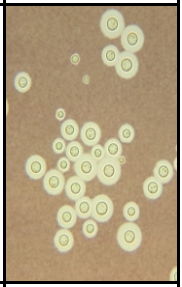
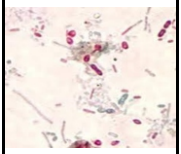
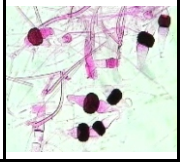
Alternaria

(Source: https://microbenotes.com/classification-of-fungi/)

Clinical Classification of Medically Important Fungi

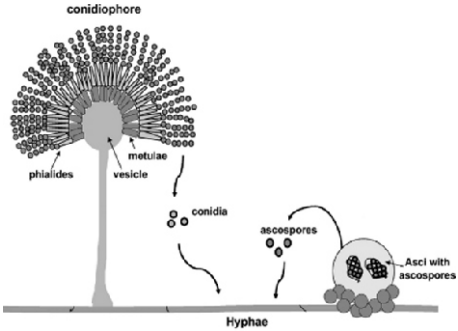


Characteristics of Specific Group of Fungi

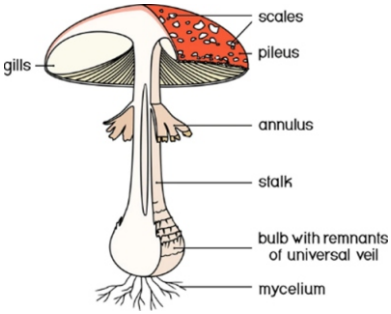
Groups of fungi				
Group	**Characteristics**	**Examples**	**Medically important Species**	**Images**
Ascomycota	Septate hyphae Ascus with ascospores in ascocarp Conidiospores	*Cup fungi* *Edible Mushrooms* *Morels* *Truffles* *Neurospora* *Penicillium*	*Aspergillus spp.* *Trichophyton spp.* *Microsporum spp.* *Epidermophyton spp.* *Blastomyces dermititidis* *Histoplasma capsulatum*	
Basidiomycota	Basidia produce basidiospores in a basidiocarp	*Club fungi* *Rusts* *Stinkhorns* *Puff balls* *Mushrooms* *Cryptococcus neoformans* *Amanita phalloides*	*Cryptococcus neoformans*	
Microsporidia	Lack mitochondria, peroxisomes, centrioles, Spores produce a polar tube	*Enterocystozan bieneusi*	*Enterocystozan bieneusi*	
Mucormycota	Mainly saprophytes Coenocytic hyphae Haploid nuclei Zygospores	*Mucor* *Rhizopus* *Mucorales*	*Mucor spp.*	

(Source: https://www.labxchange.org/library/items/lb:LabXchange:4d924996-98e3-3868-bff6-591322425a74)

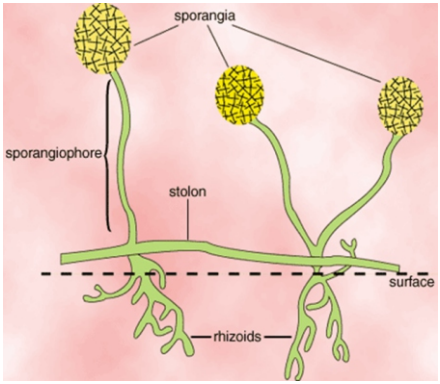
Structure of Fungi



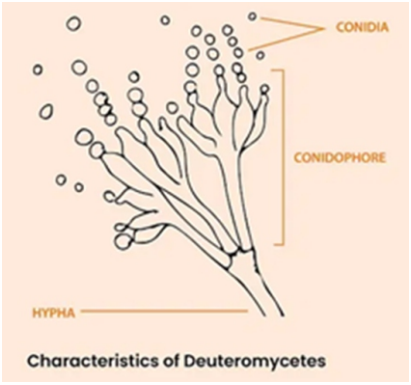
Structure of Ascomycetes



Structure of Basidiomycetes



Structure of Mucormycetes

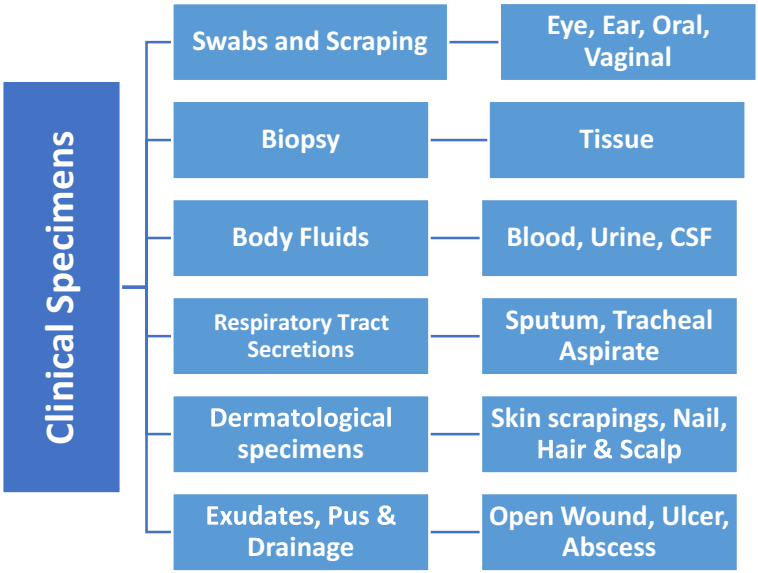


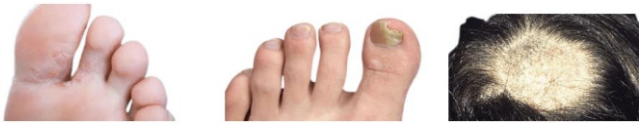
Structure of Deuteromycetes

(Source: https://www.sparknotes.com/biology/microorganisms/fungi/section1/)

2. Clinical Specimens Collection, Transport and Processing

Hair, nail clippings, skin scrapings, CSF, blood, and urine are the most common clinical specimens for the diagnosis of fungal infections. Clinical specimens for the diagnosis of fungal infection includes:



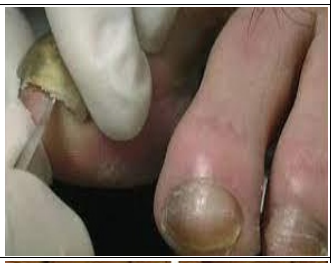
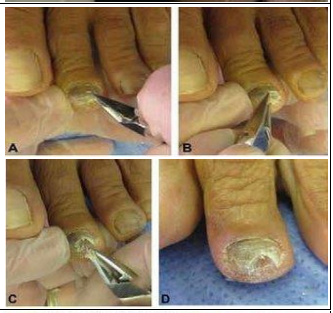


Specimens for the diagnosis of Fungal Infections

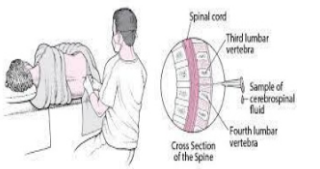
(Source:https://microbeonline.com/sample-collections-for-laboratory-diagnosis-of-fungal-infections/)

2. Clinical Specimens Collection, Transport and Processing

Specimen collection for the diagnosis of fungal infection

No of Obs	Specimens	Collection	Procedure
1.	Skin Scrapings	• Clean skin with 70% alcohol. • Scrape edges of lesion with a blunt scalpelblade. • Collect skin scales in a sterile petri dish. • If a skin scraping does not yield sufficient material, then a swab or Scotch tape could be pressed on the lesion.	
2.	Nail Clippings	• Clean nail with 70% alcohol. • Examine for damaged, discolored, brittle or dystrophic area. • Entire thickness of the damaged nail should be cut as far back as possible. Any crumbly material or material under the nail should be collected and sent in a sterile container. • If skin lesions are present they should be scraped and the material collected should be sent separately.	
3.	Hair and Scalp	• No cleaning of scalp is needed. • Select infected areas and with the forceps, epilate at least 10 hairs. • Hair can be obtained by plucking, brushing or with a sticky tape. • A Woods lamp can be helpful in locating infected areas. • In piedra infections, cut infected hair with scissors. • Submit specimen to laboratory in sterile petri dish.	

2. Clinical Specimens Collection, Transport and Processing

4.	CSF	• With the patient lying on his side, a single sample (1-10 ml CSF) is drawn by a trained physician after giving local anesthesia and sterile preparation of aspiration site (usually between lower lumbar spines). • Sample is sent immediately to the laboratory.	
5.	Blood	• A physician or nurse can collect an intravenous sample after skin disinfection. • In adults, two samples (10 ml each) are submitted in an aerobic and anaerobic blood culture bottle. • In children, a single sample (4-10 ml) is submitted in a pediatric blood culture bottle.	
6.	Urine	• Collect 10-20 ml midstream urine in a sterile container during the first morning urination. • Void the first portion of the urine, and then catch the rest in the container without stopping the stream. • Ensure hand hygiene prior to collection by washing hands thoroughly with soap and water, and then drying with a paper towel. Females should spread labia and clean the urethral meatus in a front-to- back direction. • Catheter sample is collected by a physician or nurse. • Clamp hub of Foley's catheter distally. • Clean hub sequentially with pyodine and 70% alcohol. • Aspirate collected urine from the hub with a sterile needle and syringe. • A 24-hourine collection and Foley's catheter tip samples are not acceptable.	

7.	Tissue	• Surgical collection and punch biopsies may be used for skin lesions. • If grains are visible to the naked eye, collect aseptically for smear and culture. • Tissue is collected from centre and edge of the lesion. • Sterile normal saline should be added to the tissue to prevent drying (just enough to keep tissue moist).	
8.	Abscess	• Clean skin with 70% alcohol and palpate point of highest fluctuation. • Insert sterile syringe needle and aspirate fluid from the most fluctuant point. • If needed, obtain sample from base of lesion and abscess wall (scrapings, punch biopsy).	
9.	Sputum	• 5-10 ml; early morning prior to eating. • Use mouth rinse and brush before collection. • Collected in sterile wide mouthed container	

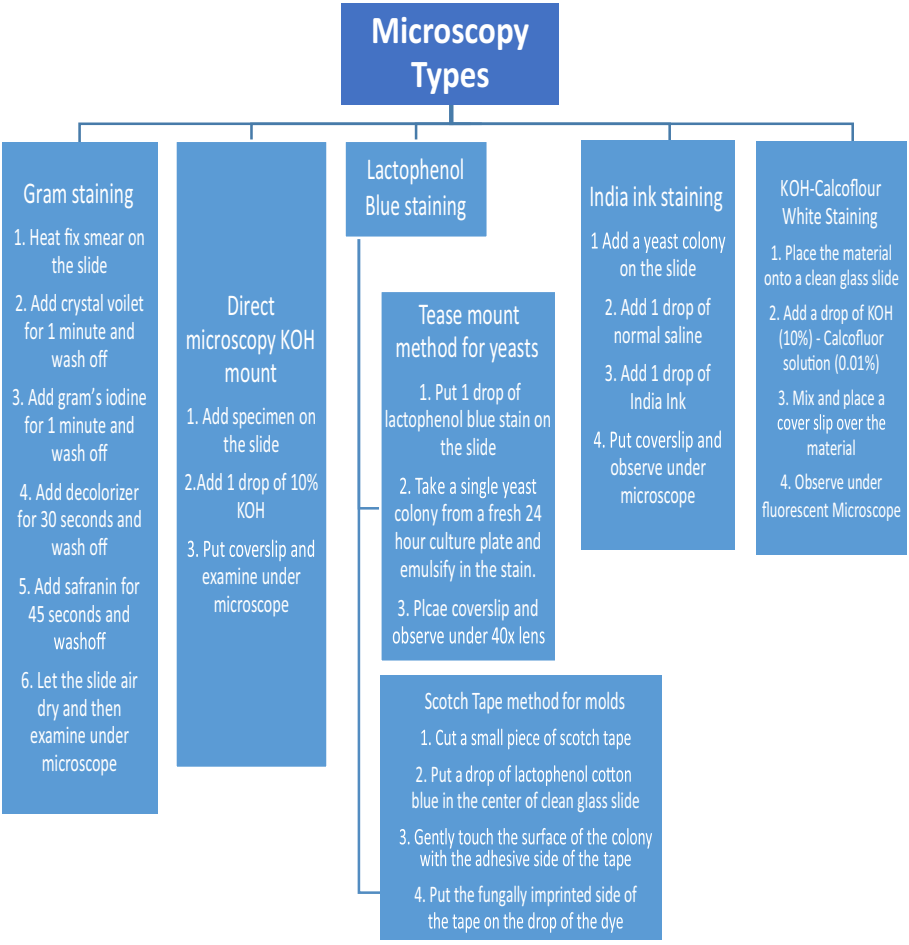
Transport and Processing of Specimen for the Diagnosis of Fungal Infections

No of Obs	Specimen	Transport Device	Recommended Volume	Optimal Transport	Stability	Processing
1.	Abscess	Sterile container 	0.5- 1 ml	Ambient temperature	≤ 72 hours ambient temperature	Direct inoculation or centrifugation; process within 2 hours, refrigerate specimen prior to processing
2.	Blood	Isolator tube 	10 ml	Ambient temperature Don't refrigerate	24 hours room temperature Don't refrigerate	Incubate 2 bottles at 37°C in an automated system. Perform microscopy and subculture once Positive.
3.	Bone Marrow	Isolator tube 	1-3 ml	Within 2 hours of collection at room temperature	≤ 24 hours, Room temperature Don't refrigerate	Direct inoculation, Process within 2 hours, maintain specimen at 37ºC prior to processing
4.	Body Fluids (Abdominal, joint, Pleural, synovial, pericardial etc.)	Sterile container, Swab, Dual swab with Amies transport medium 	≥ 3 ml sputum or fluid 1-5 gm tissue in swab or Dual swab set	Ambient temperature	≤ 72 hours ambient temperature ≤ 72 hours refrigerated temperature	Direct inoculation
5.	Catheter tips	Sterile container 		Ambient temperature	≤ 24 hours, Room temperature	
6.	Cerebrospinal fluid (CSF)	Sterile container 	2 ml	≤ 6 hours, Room temperature	48 hours, ambient (room) temperature	Centrifuge CSF in sterile tube at 2000-2500 rpm for 10 minutes. Pour the supernatant back into the collection container and store for antigen detection. The pellet is used for smear and wet mount and for subsequent culture.
7.	Dermal Sites (Hair, nail, Skin Scalp)	Sterile container	Nail Clippings Hair with Shaft Skin scraping	Ambient temperature	2 weeks, Ambient temperature	**Skin scrapings** are directly inoculated on agar surface by using sterile forceps.

		24 hours, Room temperature  Place in envelop for transport				**Nails** are cut into small pieces with the scalpel or sterile blade. Place directly on the agar surface using sterile forceps. Press lightly in to agar surface. Cut strands of **hair** into 1 mm lengths with a scalpel or sterile blade. Press lightly into selective media with sterile forceps.
8.	Ear culture	eSwab and Dual Swab with Amies transport medium 	0.1 ml aspirate 1 swab or dual swab test	24 hours, Room temperature	≤ 72 hours ambient temperature ≤ 72 hours refrigerated	Direct inoculation onto a media
9.	Nasal/ Sinus	Sterile container, eSwab and Dual Swab with Amies transport medium		Ambient temperature	≤ 72 hours, ambient (room) temperature	Direct inoculation onto a media
10.	Eye culture	Sterile container, eSwab and Dual Swab with Amies transport medium	1-5 ml 1 swab or dual swab set	24 hours, Room temperature	≤ 72 hours ambient temperature ≤ 72 hours refrigerated	Direct inoculation onto a media
11.	Genital specimen for Candida	eSwab and Dual Swab with Amies transport medium	1 swab or dual swab set	Ambient (room) temperature	1 week ambient (room) temperature 1 week, refrigerated	Direct inoculation onto a media

						
12.	Urine	Sterile container, Culture and sensitivity Urine Transport Kit  	1 ml in sterile cup 3 ml in preservative	Ambient temperature or refrigerated	Preserved: ≤ 48 hours at either ambient (room) temperature or refrigerated Unpreserved: ≤ 2 hours at ambient (room) temperature; ≤ 24 hours, refrigerated	Centrifuge the urine for 10-15 minutes at 2000 rpm. Decant the supernatant and pool the sediment if necessary Direct inoculation onto a media
13.	Wound	Sterile container, eSwab and Dual Swab with Amies transport medium	1-2 ml of fluid 1 swab or Dual swab test	Ambient temperature	≤ 72 hours ambient (room) temperature	Direct inoculation onto media
14.	Respiratory Lower	Sterile container 	≥ 2 mL	Ambient (room) temperature or refrigerated	≤ 72 hours ambient (room) temperature ≤ 72 hours refrigerated	Direct inoculation onto media
15	Tissue (Biopsy)	Swabs, sterile screw-cap container	Small piece	Ambient (room) temperature	≤15 min	Mince using a sterile blade after placing tissue in a sterile petri plate Inoculate tissue on culture plates

3. Microscopy of Yeast and Molds



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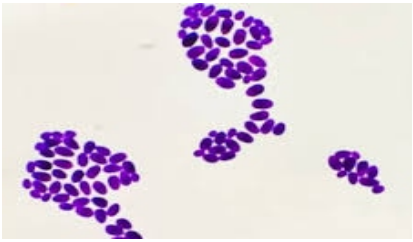
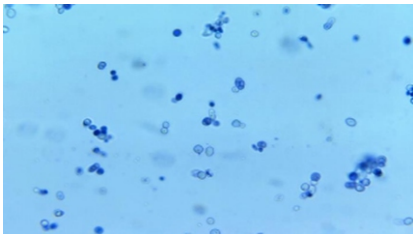
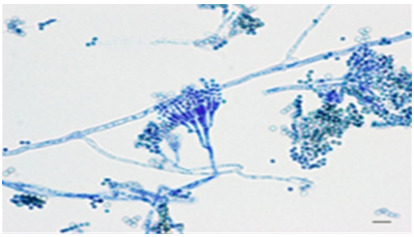


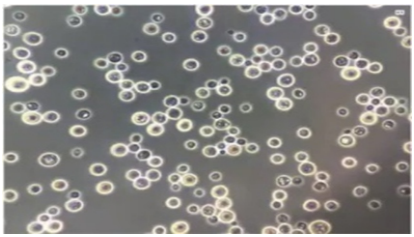
Gram staining of *Candida albicans*



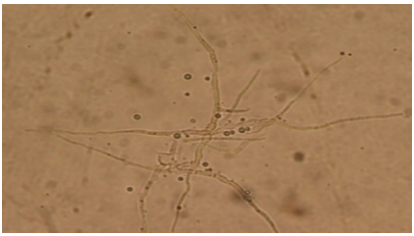
Lactophenol cotton blue staining of Yeast



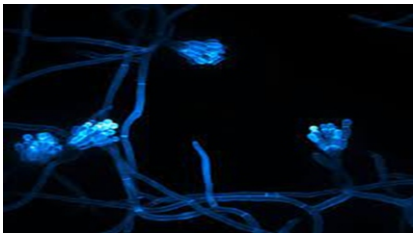
Lactophenol cotton blue staining of Molds



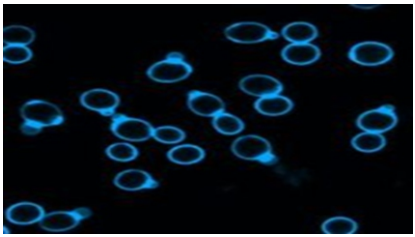
India Ink Staining



KOH Mount showing Fungal Hyphae



KOH-Calcofluor white staining of Aspergillus



KOH-Calcofluor white staining of Yeast

3. Microscopy of Yeast and Molds

Direct microscopy of skin scrapings and nail clippings

The material is examined by microscopy by one or more of these methods:

- Potassium hydroxide (KOH) preparation
- An unstained wet-mount

Microscopy can identify a dermatophyte by the presence of:

- Fungal hyphae (branched filaments) making up a mycelium
- Arthrospores (broken-off spores)
- Arthroconidia (specialised external spores)

Fungal elements are sometimes difficult to find, especially if the tissue is very inflamed, so a negative result does not rule out fungal infection.

Wet mount of fungal cultures

The direct examination of fungal cultures is done through wet mount. It provides identification of the fungal hyphae which shows the patient has a fungal infection. Wet mount is done for all samples received in the mycology laboratory.

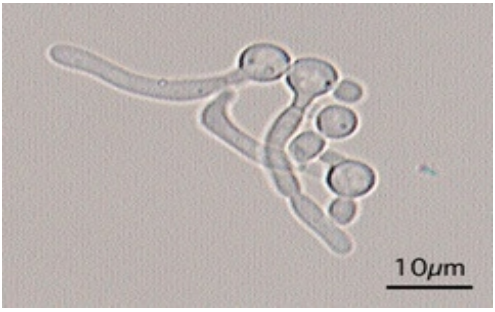
Germ Tube Test

It is a screening test which is used to differentiate *Candida albicans* from other yeast. Germ tube (GT) formation was first reported by Reynolds and Braude in 1956. When Candida is grown in human or sheep serum at 37°C for 3 hours, they form germ tubes, which can be detected with a wet KOH films as filamentous outgrowth extending from yeast cells. It is

positive for *Candida albicans* and *Candida dubliniensis*. Approximately 95 - 97% of Candida albicans isolates develop germ tubes when incubated in a proteinaceous media. Germ Tube solutions contains tryptic soy broth and fetal bovine serum, essential nutrients for protein synthesis. It is lyophilized for stability.

Procedure of Germ Tube Test

- Put 0.5 ml of sheep or human serum into a small tube.
- Using a Pasteur pipette, touch a colony of yeast and gently emulsify it in the serum.
- Incubated the tube at 37°C for 2 to 3 hours.
- Transfer a drop of the serum to a slide for examination.
- Place coverslip and examine microscopically under low and high power objective lens.

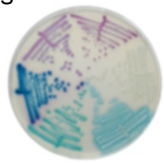
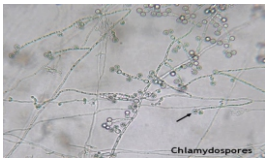


Germ tubes of *Candida albicans*

(Source: https://en.wikipedia.org/wiki/Germ_tube)

4. Identification of Yeast

Recommended Culture media for isolation of yeasts

S#	Type of Medium	Properties/Purpose
1.	Sabouraud Dextrose Agar (SDA)	• Universal primary medium for fungal culture • Antibiotics can be added for the inhibition of bacterial growth.  *Candida albicans* on SDA
2.	CHROMagar TM Candida plus	• Selective chromogenic culture medium for use in the qualitative direct detection, differentiation and presumptive identification of *Candida* species including *C. auris*.  *Candida* spp. on CHROMagar TM Candida plus Source:https://www.chromagar.com /en/product/chromagar-candida-plus/
3.	Cornmeal Tween20 agar	• Used for cultivation of fungi as well as to study chlamydospores production by Candida species and other yeasts.  Chlymydospore production in *Candida albicans* Source: https://www.medical-labs.net/induce-chlamydospore-formation-of-candida-albicans-using-corn-meal-agar-3019/

4. Identification of Yeast

Sabouraud Dextrose agar

Sabouraud's dextrose agar (SDA) is a universal primary medium for fungal culture. Sabouraud agar is commercially available and contains peptone, glucose and agar. Pancreatic digest of casein and peptic digest of animal tissue provide amino acids, nitrogen, carbon, vitamins and minerals for organism's growth. Dextrose is an energy source. Agar is the solidifying agent. The high concentration of dextrose and the acidic pH of the medium permit selectivity of fungi. The medium can be supplemented with chloramphenicol to increase bacterial inhibition.

(Source: © Liofilchem® - Sabouraud Dextrose Agar - Rev.0 / 20.11.2015)

CHROMagar TM Candida plus

CHROMagar™ Candida Plus is a selective chromogenic culture medium intended for use in the qualitative direct detection, differentiation and presumptive identification of *Candida* species including *C. auris.* The test is performed with swabs from skin, throat, ears and vaginal specimens as well as sputum, urine and stools samples, in parallel to cultures on Sabouraud agar, to aid in the Candidiasis diagnosis. Results can be interpreted after 24-48 h of aerobic incubation at 30-37 °C.

(Source:https://www.chromagar.com/en/product/chromagar-candida-plus/)

4. Identification of Yeast

Cornmeal Tween20 agar

Corn Meal Agar is a general-purpose medium for the cultivation of fungi, in particular for the production of chlamydospores by *Candida albicans*. In this medium, Corn Meal Infusion provides all the nutrients required for fungal growth and Agar is the gelifying agent. The medium can be supplemented with Tween 80 to enhance chlamydospore formation. The addition of Glucose enhances the chromogenesis of *Trichophyton* spp. while Trypan Blue provides a contrasting background that facilitates the observation of morphological features.

(Source: https://alphabiosciences.com/corn-meal-agar-c03-115/)

5. Identification of Molds

Recommended culture media for primary isolation of molds

Type of Medium	Properties/Purpose
Sabouraud Dextrose Agar (SDA)	• Classic pigment & morphology
Potato Dextrose Agar	• Enhances reproductive structure production • Enhances colony color
Brain Heart Infusion Agar	• Enhances growth of dimorphic fungi
Mycosel Agar	• Contains both chloramphenicol (inhibit bacterial growth) and cycloheximide (inhibits saprobic fungi and some opportunistic pathogens)
Dermatophyte Test Medium (DTM)	• Partially differential and selective for dermatophytes • With antibiotic to inhibit bacteria • With cycloheximide to inhibit non - dermatophytes

Mycosel Agar

Mycosel agar, a selective medium principally formulated for the isolation of dermatophytes but also used for the isolation of other pathogenic fungi from specimens contaminated with saprophytic fungi and bacteria. Soy peptone and glucose support good growth of both bacteria and fungi. The incorporation of two antibiotics, chloramphenicol and cycloheximide, makes the medium selective: chloramphenicol inhibits bacterial growth while cycloheximide inhibits the growth of saprophytic fungi. Specimen is directly inoculated on

Mycosel medium. Plates are incubated at 25-30°C in incubator for 7-14 days. Results are interpreted on the basis of growth of fungus and its colony color and morphology.

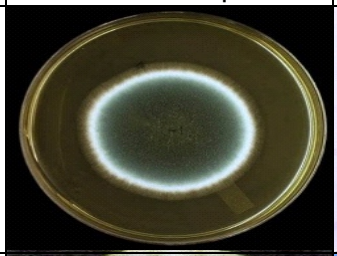
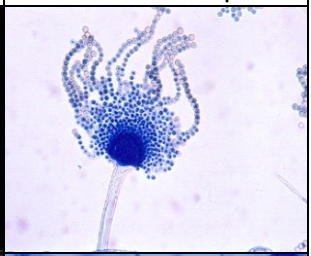
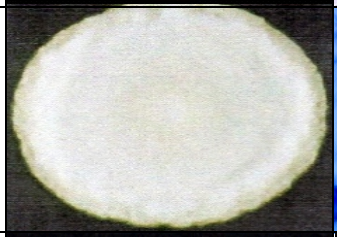
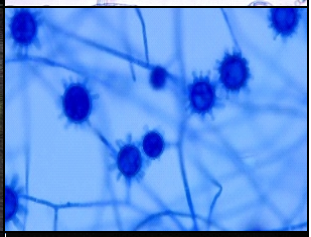
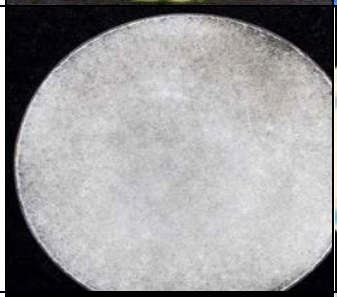
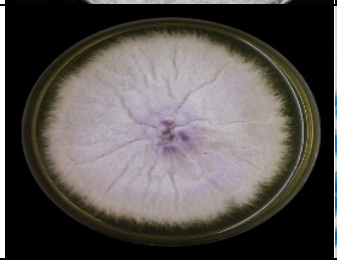
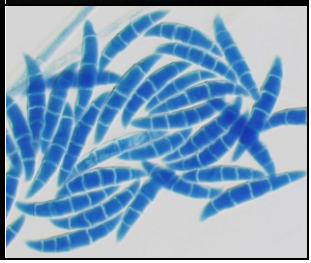
Dermatophyte Test Medium

This medium turns red in color with dermatophytes and is useful for distinguishing dermatophytes from other pathogenic fungi. It is based on Sabouraud's dextrose agar with added cycloheximide to inhibit saprotrophic growth, antibiotic to inhibit bacterial growth, and phenol red a pH indicator. The pH indicator is useful in distinguishing a dermatophyte fungus, which utilizes nitrogenous material for preferred metabolism, producing alkaline by-products, imparting a red color change to the medium. Incubate the plate at 28°C for 2 weeks. Examine plates daily for the presence of growth and pigment.

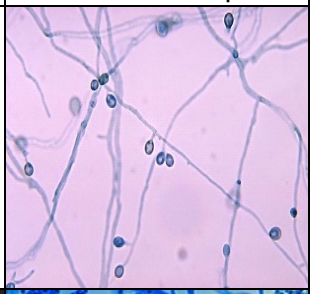
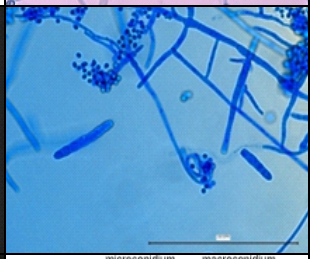
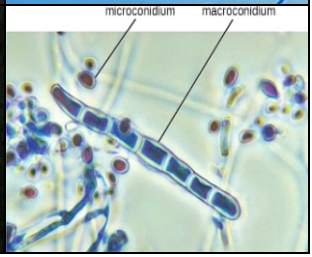
Potato Dextrose Agar

Potato Dextrose Agar (PDA) is used for the cultivation of fungi. Potato Dextrose Agar (PDA) is a general purpose medium for yeasts and molds that can be supplemented with acid or antibiotics to inhibit bacterial growth. Potato Dextrose Agar (PDA) contains dextrose as a carbohydrate source which serves as a growth stimulant and potato infusion that provides a nutrient base for luxuriant growth of most fungi. Starch in PDA induces sporulation better than on SDA. Agar is added as the solidifying agent. Chloramphenicol acts as a selective agent to inhibit bacterial overgrowth of competing microorganisms from mixed specimens, while permitting the selective isolation of fungi.

5. Identification of Molds

Species	Macroscopic	Microscopic
Aspergillus fumigatus		
Histoplamsa		
Rhizopus		
Fusarium		

5. Identification of Molds

Species	Macroscopic	Microscopic
Scedosporium		
T.mentagrophytes		
T.rubrum		

(Source: WHO Fungal Priority pathogen list Oct 2022.pdf)

6. Biochemical identification of Fungi

Biochemical Identification of Yeast

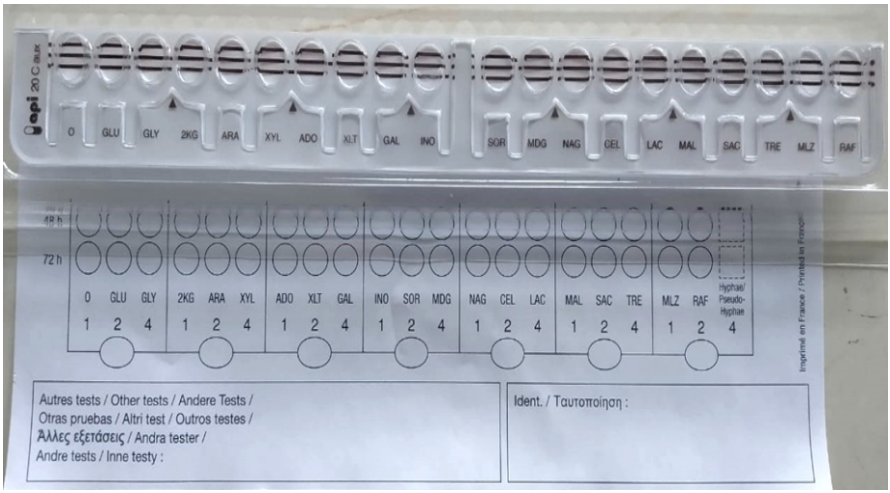
Species	Growth at 370C	Pellicle in broth	Pseudo-/True	Chlamydospore	Germ tubes	Capsule, India	Fermentation of:						Urease	KNO, Utilization	Phenol Oxidase	Ascospore
							Glucose	Maltose	Sucrose	Lactose	Galactose	Trehalose				
C.albicans	+	–	+	+	+	–	F	F	–	–	F	F	–	–	–	–
C.catenulata	+*	–	+	–	–	–	F*	–	–	–	–	–	–	–	–	–
C.dubliniensis	+	–	+	+	+	–	F	F	–	–	F	F	–	–	–	–
C.famata	+	–	–	–	–	–	F	–	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	–	–	–	–	–	–	–	–	–
C.pintolopesii	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
C.rugosa	+	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–
C.tropicalis	+	+	+	–	–	–	F	F	F	–	F*	F	–	–	–	–
C. zeylanoides	–	–*	+	–	–	–	–	–	–	–	–	–	–	–	–	–
C.neoformans	+	–	R	–	–	+	–	–	–	–	–	–	±	–	+	–
C.albidus	–	–	–	–	–	+	–	–	–	–	–	–	±	+	–	–
c. laurentii	+	–	–	–	–	+	–	–	–	–	–	–	+	–	–	–
C.luteolus	–	–	–	–	–	+	–	–	–	–	–	–	+	–	–	–
C.Tereus	–*	–	–	–	–	+	–	–	–	–	–	–	±	+	–	–
C.uniguttulatus	–	–	–	–	–	+	–	–	–	–	–	–	+	–	–	–
R.glutinis	+	–	–	–	–	–*	–	–	–	–	–	–	+	+	–	–
R.rubra	+	–	–	–	–	–*	–	–	–	–	–	–	+	–	–	–
S.cerevisiae	+	–	–*	–	–	–	F	F	F	–	F	F	–	–	–	+
P.anomala	+*	–	–	–	–	–	F	F*	F	–	F	–	–	+	–	+
G.candidum	–*	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–
B.capitatus	+	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–
P. wickerhamii	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
S.salmonicolor	+*	–	+*	–	–	–	–	–	–	–	–	–	+	+	–	–
T.asahii	+		+	–	–	–	–	–	–	–	–	–	+	+	–	–
T.mucoides	+		+	–	–	–	–	–	–	–	–	–	+	–	–	–

(Source: Yeasts: characteristics and identification By J. A. Barnett, R. W. Payne, D. Yarrow,2000)

6. Biochemical identification of Fungi

API (Analytical Profile Index) 20C Aux

API 20 C AUX is a system for the precise identification of the most frequently encountered yeasts. Yeasts are inoculated into basal medium in test strips containing dehydrated substrates for assimilation testing. The API 20 C AUX system consists of a strip that contains 20 microcupules; all but two contain dehydrated substrates for determining substrate utilization profiles. As the yeasts utilize various substrates, the microcupules will appear cloudy. The reactions are compared to the first reaction cupule, which does not contain a carbohydrate substrate. The test strips are incubated at 30°C for 24–72 h. The results of assimilation reactions are read and converted to a seven-digit biotype profile number.

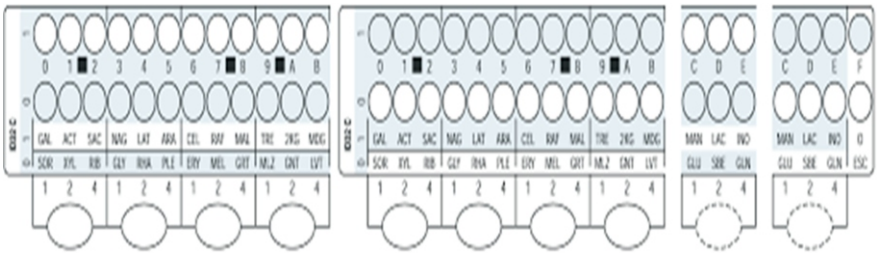


(Source: BioMérieux. API 20 C AUX Yeast identification system)

6. Biochemical identification of Fungi

ID 32C Yeast Identification System

This system consists of a single-use disposable plastic strip with 32 wells containing substrates for 29 assimilation tests (carbohydrates, organic acids, and amino acids), one susceptibility test (cycloheximide), one colorimetric test (esculin), and a negative control. Results are available after 24-48 hours.



Profile of ID32 Yeast Identification System

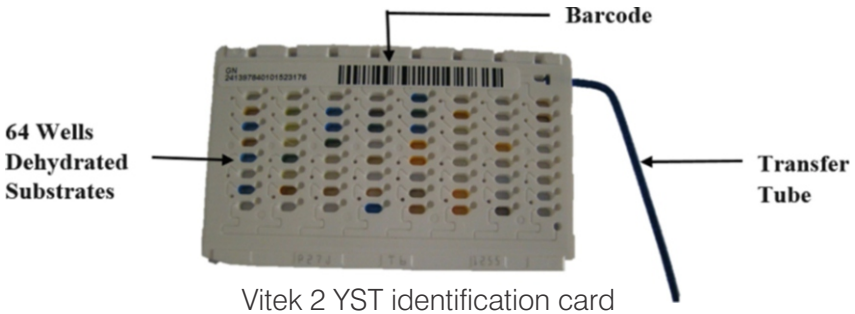
(Source: https://www.mediray.co.nz/media/15793/om_biomerieux_test-kits_ot 32200_package_insert-32200.pdf)

7. Identification of yeast and molds by using automated instruments

VITEK 2 Compact

The VITEK 2 Compact (30/60 card capacity) system uses a fluorogenic methodology for organism identification and a turbidimetric method for susceptibility testing using a 64 well card that is barcoded with information on card type, expiration date, lot number and unique card identification number. Test kits available include ID-GN (gram negative bacillus identification), ID-GP (gram positive cocci identification), AST-GN (gram negative susceptibility) and AST-GP (gram positive susceptibility), ID-YST (Yeast identification), AST-YST (Yeast susceptibility).

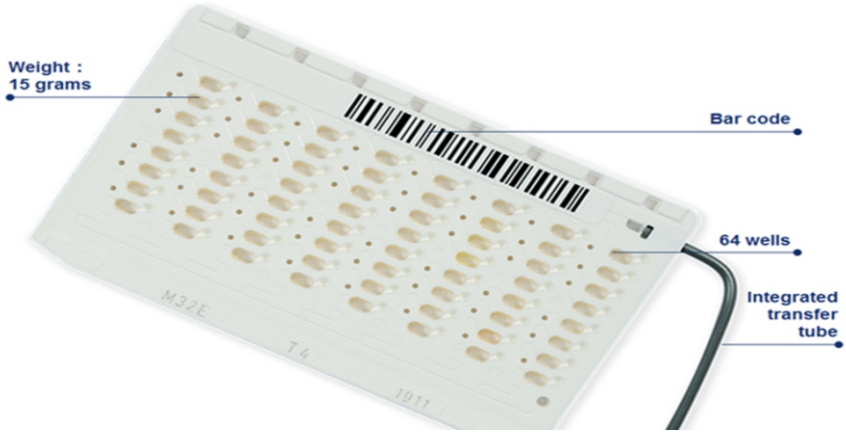
VITEK 2 Compact Machine



Vitek 2 YST identification card

(Source: https://www.mdgsb.com.my/products/biomerieux-vitek-2-system)

7. Identification of yeast and molds by using automated instruments



VITEK 2 Yeast AST Card

(Source: https://www.biomerieux-microbio.com/how-does-vitek-2-generate-mic-values/)

The densitometry of the culture is essential for the VITEK2 card so, the VITEK DensiCHEK makes it easy to accurately measure the optical density of a microorganism suspension with high quality and increased traceability.

7. Identification of yeast and molds by using automated instruments

7. Identification of yeast and molds by using automated instruments

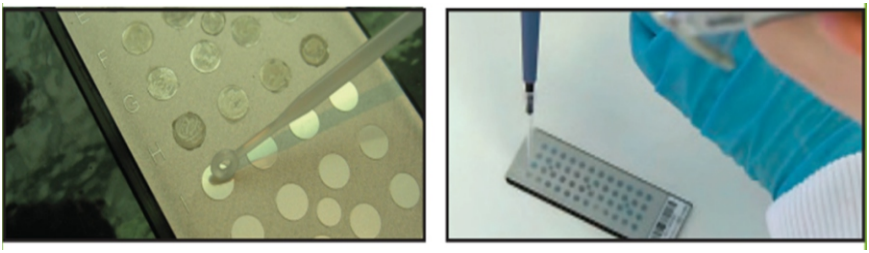
MALDI TOF MS (VITEK MS)

The VITEK MS system is a mass spectrometer using Matrix Assisted Laser Desorption Ionization Time-of-Flight (MALDI-TOF) technology for the identification of microorganisms cultured from clinical specimens. A portion of a bacterial or yeast colony from an agar plate, or a prepared Mycobacterium, Nocardia or molds extraction from a culture grown on a solid medium or a prepared Mycobacterium extraction from a culture grown in a liquid medium is applied to a spot on a VITEK MS-DS target slide. After matrix is applied, the VITEK MS-DS target slide is dried and then loaded into the VITEK MS instrument. The sample is subjected to multiple laser shots inside the VITEK MS instrument. The matrix absorbs the laser light and vaporizes, along with the sample, in the process gaining an electrical charge (ionization). Electric fields then guide the ions into the vacuum tube which separates them according to their mass (smaller molecules flying faster than larger ones) i.e. time of flight and displays the results as a series of lines or peaks (spectra) which corresponds to different fragments that have broken away from the original molecules in the sample. By analyzing the pattern of fragments, it is possible to deduce the structure of the molecules. The sample spectra are compared to a database spectrum developed from a number of microbial species.

7. Identification of yeast and molds by using automated instruments

VITEK MS machine

(Source: https://www.biomerieux.com/nl/en/our-offer/clinical-products/vitek-ms.html)

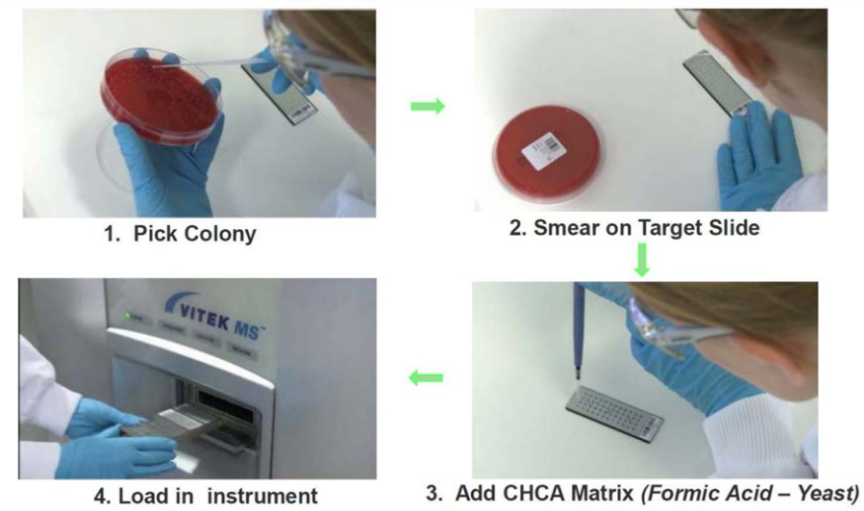


VITEK MS Target slide

(Source: https://www.biomerieux-usa.com/vitek-ms-reagents-accessories)

7. Identification of yeast and molds by using automated instruments

Workflow



(Source: https://www.biomerieux.co.uk/product/vitekr-ms)

8. Antifungal Susceptibility Testing

Kirby-Bauer Disk Diffusion Method

Purpose

The purpose of the Kirby-Bauer disk diffusion susceptibility test is to determine the sensitivity or resistance of pathogenic aerobic and facultative anaerobic bacteria or yeast to various antimicrobial compounds in order to assist a physician in selecting treatment options for patients. The pathogenic organism is grown on Mueller- Hinton agar in the presence of various antimicrobial impregnated filter paper disks. The presence or absence of growth around the disks is an indirect measure of the ability of that compound to inhibit that organism.

Requirement

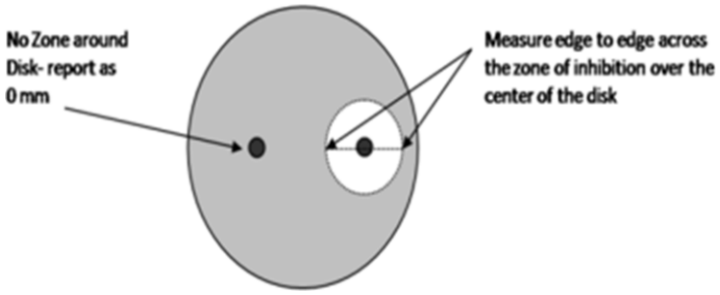
- Muller-Hinton agar plate supplemented with 0.5 μg/ml methylene blue and 2% dextrose.
- Glass tube with 0.85% sterile saline solution.
- Isolated yeast colonies from a 20-24 hours old culture grown on an antibiotic-free medium (SBA, SDA or PDA)

Procedure

- Make a suspension of 0.5 McFarland turbidity in 0.85% saline with a few colonies from a 20–24 hour old culture of Candida species on an antibiotic-free medium.
- Soak a sterile cotton swab in the suspension, rolling it along the glass wall of the tube to get rid of the excess moisture.
- Make lawn on Muller Hinton agar supplemented with methylene blue and 2% dextrose.

8. Antifungal Susceptibility Testing

- Using sterile forceps place antifungal discs on the agar surface and incubate the plates at 35+1°C for 20-24 hours in ambient air.
- Measures zone diameter for each antifungal:
    - For azoles measure zones from where there is 50-80% inhibition of growth.
    - For caspofungin measure zones from where there is complete inhibition of growth.
- Interpret readings according to the specific Candida species Compare with QC ranges for the recommended ATCC strains.
- Using the published CLSI guidelines, determine the susceptibility or resistance of the organism to each drug tested.
- For each drug, indicate on the recording sheet whether the zone size is susceptible (S), intermediate (I), or resistant (R) based on the interpretation chart.
- The results of the Kirby-Bauer disk diffusion susceptibility test are reported only as susceptible, intermediate, or resistant.



(Source: CLSI M44-A2 Ed 3. Method for antifungal disk diffusion susceptibility testing of yeast)

8. Antifungal Susceptibility Testing

Zone Diameter & Equivalent MIC Breakpoints for selected Antifungal agents against Candida Spp. after 24-Hour Incubation

Antifungal Agent	Species	Zone diameter Breakpoints and interpretive categories, mm				Equivalent MIC Breakpoints and interpretive Categories, ug/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	12-Nov	—	≤10	≤2	4	—	≥8
	C.krusei	≥17	15-16	—	≤14	≤0.25	0.5	—	>1
	C.parapsilosis	≥13	12-Nov	—	≤ 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	—	—	—	—	—	—	—	—
	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.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

Abbrevations: MIC, Minimal inhibitory Concentration, S, Sensitive, I,intermediate, SDD,Susceptible dose dependent, R, Resistant

(Source CLSI. Performance Standards for Antifungal Susceptibility Testing of Yeasts. 2nd ed. CLSI supplement M60. Wayne, PA: Clinical and Laboratory Standards Institute; 2020)

8. Antifungal Susceptibility Testing

Broth Microdilution

Broth micro-dilution is the standard method used in most reference laboratories worldwide. The method typically tests twofold dilutions of multiple antimicrobial agents in 96-well disposable plastic trays. This is considered gold standard in antifungal susceptibility testing.

Sensititre YeastOne

Among the commercially available BMD antifungal testing systems, only the Sensititre Yeast One system offers an echinocandin (caspofungin) on a dried 96-well BMD panel. The Sensititre YeastOne system is available in a dry-form 96-well panel with the colorimetric growth indicator Alamar Blue and has a shelf life of 24 months at ambient temperature. It has been used widely with excellent results in terms of accuracy and reproducibility. This product is user-friendly, and results are comparable to the standard method. Growth of yeast in the wells changes the color of alamar blue to pink, making MIC reading easier.

Growth of yeast in the wells changes the color of alamar blue to pink

(Source: https://www.thermofisher.com/order/catalog/product/YO9)

8. Antifungal Susceptibility Testing

Broth Microdilution of Yeasts

- Broth microdilution is a widely used method for antifungal susceptibility testing (AFST) of yeasts.
- The CLSI and EUCAST have established standards for broth microdilution testing of yeasts, with some differences in protocols.
- The CLSI guidelines recommend the use of 96-well microdilution plates with untreated polystyrene and RPMI 1640 culture medium buffered to pH 7.
- The EUCAST guidelines suggest the use of flat-bottomed microdilution plates and recommend avoiding low-evaporation lids.
- The inoculum size for yeasts in broth microdilution testing is typically $0.5x10^3$ to $2.5x10^3$ cells/ml.
- The endpoint of the assay is determined as the concentration of the antifungal drug that inhibits the growth of the yeast to a specified degree.
- Both CLSI and EUCAST use similar criteria to develop clinical breakpoints and interpretive categories for antifungal resistance and susceptibility in yeasts

Broth Microdilution of Molds

- Broth microdilution is a commonly used method for antifungal susceptibility testing (AFST) of molds.
- The CLSI and EUCAST have established standards for broth microdilution testing of molds, with some variations in protocols.
- The CLSI protocol uses 96-well microdilution plates with untreated polystyrene and RPMI 1640 culture medium buffered to pH 7.

8. Antifungal Susceptibility Testing

- The EUCAST protocol recommends the use of flat-bottomed microdilution plates and tissue-treated plates, with specific recommendations for avoiding low-evaporation lids.
- The inoculum size for molds in broth microdilution testing varies depending on the species, ranging from $0.4x10^4$ to $5x10^4$ conidia per ml for non dermatophytes and $1x10^3$ to $3x10^3$ conidia per ml for dermatophytes.
- Incubation times for molds in broth microdilution testing range from 46 to 50 hours, with exceptions for certain species.
- The endpoint for azoles and amphotericin B in broth microdilution testing of molds is complete growth inhibition, while for echinocandins, it is the minimal effective concentration (MEC) that leads to the growth of small, round, compact hyphal forms.

Antifungal clinical breakpoints and interpretive categories for yeasts and molds via CLSI and EUCAST standards of broth Microdilution

Antifungal agent	Species	CLSI breakpoint (µg/ml)				EUCAST breakpoint (mg/liter)			
		S	I	SDD	R	S	I	SDD	R
Anidulafungin	*C. albicans*	≤0.25	0.5	—	≥1	≤0.032	—	—	>0.032
	C. glabrata	≤0.12	0.25	—	≥0.5	≤0.064	—	—	>0.064
	C. guilliermondii	≤2	4	—	≥8	—	—	—	—
	C. krusei	≤0.25	0.5	—	≥1	≤0.064	—	—	>0.064
	C. parapsilosis	≤2	4	—	≥8	≤0.002	—	—	>4
	C. tropicalis	≤0.25	0.5	—	≥1	≤0.064	—	—	>0.064
Caspofungin	*C. albicans*	≤0.25	0.5	—	≥1	—	—	—	—
	C. glabrata	≤0.12	0.25	—	≥0.5	—	—	—	—
	C. guilliermondii	≤2	4	—	≥8	—	—	—	—
	C. krusei	≤0.25	0.5	—	≥1	—	—	—	—
	C. parapsilosis	≤2	4	—	≥8	—	—	—	—
	C. tropicalis	≤0.25	0.5	—	≥1	—	—	—	—
Micafungin	*C. albicans*	≤0.25	0.5	—	≥1	≤0.016	—	—	>0.016
	C. glabrata	≤0.06	0.12	—	≥0.25	≤0.032	—	—	>0.032
	C. guilliermondii	≤2	4	—	≥8	—	—	—	—
	C. krusei	≤0.25	0.5	—	≥1	—	—	—	—
	C. parapsilosis	≤2	4	—	≥8	≤0.002	—	—	>2
	C. tropicalis	≤0.25	0.5	—	≥1	—	—	—	—
Voriconazole	*C. albicans*	≤0.12	0.25–0.5	—	≥1	≤0.064	—	—	>0.25
	C. krusei	≤0.5	1	—	≥2	—	—	—	—
	C. parapsilosis	≤0.12	0.25–0.5	—	≥1	≤0.125	—	—	>0.25
	C. tropicalis	≤0.12	0.25–0.5	—	≥1	≤0.125	—	—	>0.25
	C. dubliniensis	—	—	—	—	≤0.064	—	—	>0.25
	A. fumigatus	—[b]	—[b]	—[b]	—[b]	≤1	—	—	>2

8. Antifungal Susceptibility Testing

Antifungal agent	Species	CLSI breakpoint (µg/ml)				EUCAST breakpoint (mg/liter)			
		S	I	SDD	R	S	I	SDD	R
Fluconazole	*C. albicans*	≤2	—	4	≥8	≤2	—	—	>4
	C. glabrata	—	—	≤32	≥64	≤0.002	—	—	>32
	C. parapsilosis	≤2	—	4	≥8	≤2	—	—	>4
	C. tropicalis	≤2	—	4	≥8	≤2	—	—	>4
	Non-species specific	—	—	—	—	≤2	—	—	>4
Itraconazole	*C. albicans*	—	—	—	—	≤0.064	—	—	>0.064
	C. dubliniensis	—	—	—	—	≤0.064	—	—	>0.064
	C. parapsilosis	—	—	—	—	≤0.125	—	—	>0.25
	C. tropicalis	—	—	—	—	≤0.125	—	—	>0.25
	A. flavus	—	—	—	—	≤1	—	—	>2
	A. fumigatus	—	—	—	—	≤1	—	—	>2
	A. nidulans	—	—	—	—	≤1	—	—	>2
	A. terreus	—	—	—	—	≤1	—	—	>2
Isavuconazole	*A. fumigatus*	—	—	—	—	≤1	—	—	>1
	A. nidulans	—	—	—	—	≤0.25	—	—	>0.25
	A. terreus	—	—	—	—	≤1	—	—	>1
Posaconazole	*C. albicans*	—	—	—	—	≤0.064	—	—	>0.064
	C. dubliniensis	—	—	—	—	≤0.064	—	—	>0.064
	C. parapsilosis	—	—	—	—	≤0.064	—	—	>0.064
	C. tropicalis	—	—	—	—	≤0.064	—	—	>0.064
	A. fumigatus	—	—	—	—	≤0.125	—	—	>0.25
	A. terreus	—	—	—	—	≤0.125	—	—	>0.25
Amphotericin B	*C. albicans*	—	—	—	—	≤1	—	—	>1
	C. glabrata	—	—	—	—	≤1	—	—	>1
	C. krusei	—	—	—	—	≤1	—	—	>1
	C. parapsilosis	—	—	—	—	≤1	—	—	>1
	C. tropicalis	—	—	—	—	≤1	—	—	>1
	A. fumigatus	—	—	—	—	≤1	—	—	>2
	A. niger	—	—	—	—	≤1	—	—	>2

(Reference: Berkow EL, Lockhart SR, Ostrosky ZeichnerL.2020. Antifungal susceptibility testing: current approaches. Clin Microbiol Rev 33:e00069-19. https://doi.org/10.1128/CMR .00069-19)

8. Antifungal Susceptibility Testing

Minimum Inhibitory Concentration Method E-Test

The concentration gradient strip technique is a combination of an agar-based diffusion method with a dilution method that determinates a minimal inhibitory concentration (MIC). A predefined exponential gradient of antifungal drug is immobilized on a plastic (E-test, bioMerieux, France) or impregnated on a paper (MTS, Liofilchem, Italy) strip. After homogenous inoculation of an agar plate, the strip is applied onto the agar surface and the drug is immediately released from the carrier to produce a continuous drug gradient in the agar medium. After incubation, an ellipse of growth inhibition is obtained, and the MIC is determined at the intersection of the ellipse with the scale on the upper side of the strip. The medium essentially used for testing is RPMI 1640 MOPS supplemented with 2% glucose and the incubation time is variable depending on the tested species.

(Source:https://www.cdc.gov/fungal/lab-professionals/afst-yeasts.html)

Requirement

- RPMI agar with phenol red and 2% dextrose. For RPMI media preparation, refer to EUCAST guidelines on antifungal susceptibility testing.
- Glass tube with 0.85% sterile saline solution.
- Isolated yeast colonies from a 20-24 hours old culture grown on an antibiotic-free medium (SBA, SDA or PDA)

Procedure

- Make a suspension of 0.5 McFarland turbidity with a few colonies

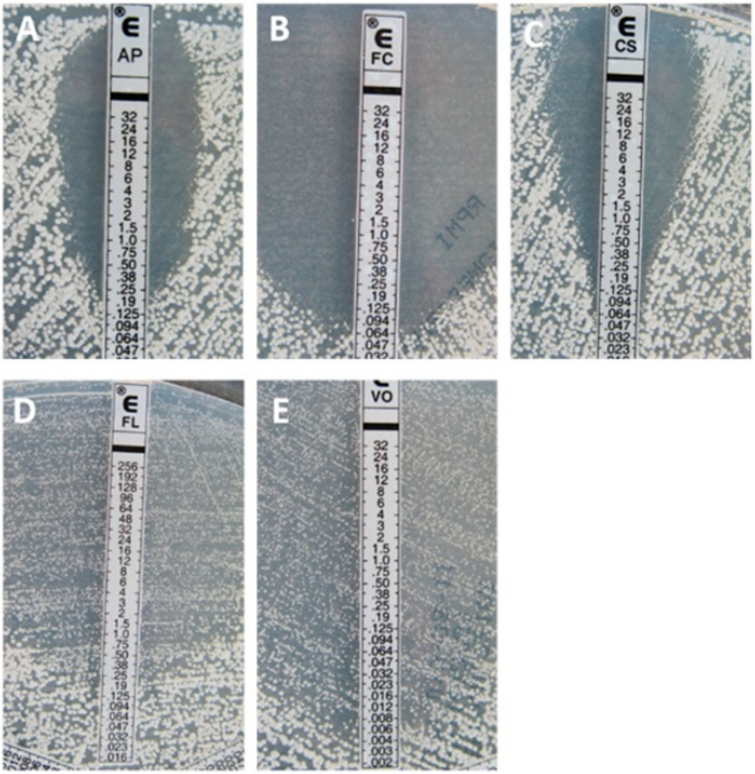
from a 20-24 hour old culture of Candida species on an antibiotic- free medium in 0.85% saline.

- Soak a sterile cotton swab in the suspension, rolling it along the glass wall of the tube to get rid of excess moisture.
- Make lawn on RPMI agar.
- Use sterile forceps to place E-test strips on the surface of the agar taking care not to move the strip and ensuring there are no air bubbles between the strip and the agar surface.
- Incubate the plates at 35 ± 1°C for 20-24 hours in ambient air

E-Test results reading and interpretation

- After 24 hours of growth at 35°C the gradient diffusion strips are ready to read.
- Gradient diffusion strips are read visually by opening the plate and observing how the growth intersects with the testing strip.
- If a sufficient lawn of growth is not obtained after 24 hours, the plate can be incubated for up to another 24 hours. Note: Only in rare occasions and with certain species would this occur if the steps are performed correctly.
- An ellipse where growth is not present may be seen in the lawn of growth, with the growth concentrated near the MIC endpoint. Isolates which are entirely resistant to that antifungal drug will show no ellipse.
- Always read the value on the side of the strip with the higher of the MIC values. If growth is inhibited between two MIC values, use the greater of the two values as the MIC.
- The interpretation of the MIC value is dependent upon the antifungal drug class. For polyenes (amphotericin B), the MIC is interpreted as

the value where there is 100% growth inhibition. For azole and echinocandin antifungals, the MIC is interpreted as the value where there is 80% growth inhibition.



(Source: Dannaoui, E., & Espinel-Ingroff, A. (2019). Antifungal susceptibly testing by concentration gradient strip Etest method for fungal isolates: a review. *Journal of Fungi*, *5*(4), 108)

Antifungal Susceptibility Testing and Interpretation for *Candida auris* (CDC Breakpoints)

All *Candida auris* isolates should undergo antifungal susceptibility testing according to CLSI guidelines. Although *C. auris* is commonly multidrug resistant, levels of antifungal resistance can vary widely across isolates.

There are currently no established *C. auris* specific susceptibility breakpoints. Therefore, breakpoints are defined based on those established for closely related Candida species and on expert opinion.

The correlation between microbiologic breakpoints and clinical outcomes is not known at this time. For this reason, the information below should be considered as a general guide and not as definitive breakpoints for resistance. Please note that a finding of an elevated minimum inhibitory concentration (MIC) for an antifungal drug should not necessarily preclude its use, especially if the use of other antifungal drugs for the patient has been ineffective.

Triazoles Breakpoints for *C. auris*

Triazole Class Drugs	Tentative MIC Breakpoints (µg/mL)	Comment
Fluconazole	≥32	Modal minimum inhibitory concentration (MIC) to fluconazole among isolates tested at CDC was ≥256; isolates with MICs ≥32 were shown to have a resistance mutation in the*Erg11* gene, making them unlikely to respond to fluconazole.
Voriconazole and other second generation triazoles	N/A	Consider using fluconazole susceptibility as a surrogate for second generation triazole susceptibility assessment. However, isolates that are resistant to fluconazole may respond to other triazoles occasionally. The decision to treat with another triazole will need to be made on case-by-case basis.

(Source: https://www.cdc.gov/fungal/candida-auris/c-auris-)

Polyenes Breakpoints for *C. auris*

Polyene Class Drugs	Tentative MIC Breakpoints (µg/mL)	Comment
Amphotericin B	≥2	Recent pharmacokinetic/pharmacodynamic analysis of *C. auris* in a mouse model of infection indicates that under standard dosing, the breakpoint for amphotericin B should be 1 or 1.5, similar to what has been determined for other *Candida* species. Therefore, isolates with an MIC of ≥2 should now be considered resistant. If using E-test for amphotericin B and an MIC of 1.5 is determined, that value should be rounded up to 2.

(Source: https://www.cdc.gov/fungal/candida-auris/c-auris-)

Echinocandins Breakpoints for *C. auris*

Echinocandins Class Drugs	Tentative MIC Breakpoints (µg/mL)	Comment
Anidulafungin	≥ 4	Tentative breakpoints are based on the modal distribution of echinocandin MICs of approximately 100 isolates from diverse geographic locations.
Caspofungin	≥ 2	
Micafungin	≥ 4	

Based on these MIC breakpoints, many isolates are resistant to multiple classes of drugs. Some U.S. *C. auris* isolates have been found to be resistant to all three classes of antifungal drugs. We have received reports of pan-resistance found in other countries as well. In the United States, about 90% of *C. auris* isolates have been resistant to Fluconazole, about 30% have been resistant to amphotericin B, and less than 5% have been resistant to Echinocandins. These proportions may include multiple isolates from the same individuals and may change as more isolates are tested.

(Source: https://www.cdc.gov/fungal/candida-auris/c-auris-)

9. Antigen Base Detection of Fungi

Galactomannan Detection

Galactomannan (GM) is an antigen found in the cell wall of several fungal species. Its detection by EIA has been in use for over a decade now as a non-invasive method to improve diagnostic sensitivity of invasive aspergillosis (IA). The test should never be used alone to confirm IA, and it should be interpreted in conjunction with clinical, radiological and culture findings as well as patient risk stratification. It is a double sandwich enzyme immunoassay that utilizes a rat monoclonal antibody EBA-2 directed against the galactofuranoside side chain of the GM antigen.

Procedure

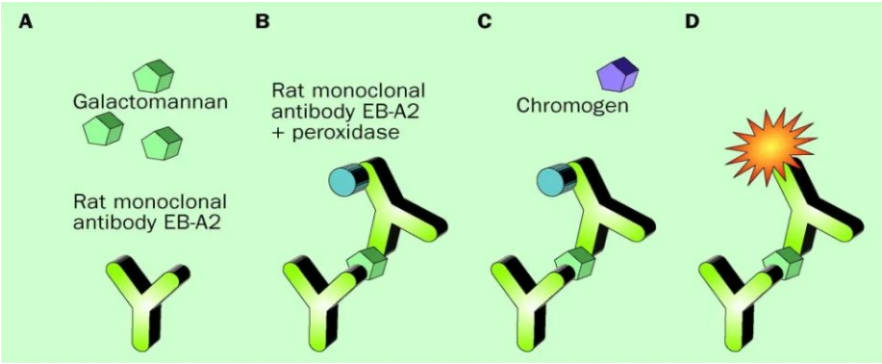
The monoclonal antibodies are used:

- To coat the wells of the microplate and bind the antigen.
- To detect the antigen bound to the sensitized microplate (conjugate reagent: peroxidase-linked monoclonal antibodies)

In the presence of galactomannan antigen, a monoclonal antibody-galactomannan-monoclonal antibody/peroxidase complex is formed. TMB (tetramethylbenzidine) Chromogen solution is added, which will react with the complexes bound to the well to form a blue color reaction. The enzyme reaction is stopped by the addition of acid, which changes the blue color to yellow. The absorbance of specimens and controls is determined with a spectrophotometer set at 450 and 620/630 nm wavelength

Interpretation of Results

The presence of clumping is indicative of the presence of cryptococcal antigen.



Aspergillus Galactomannan Enzyme Immunoassay
(Source: https://microbeonline.com/galactomannan-test-for-invasive-aspergillosis/)

β-D-Glucan Detection

1,3-β-d-glucan (BDG) is a polysaccharide that is a predominant and specific constituent of the cell wall in most fungi. BDG can be detected in serum during invasive fungal infections (IFI), serving as a biomarker for diseases like invasive aspergillosis (IA) and invasive candidiasis (IC). Mucorales and some basidiomycetous yeasts, such as Cryptococcus spp., are not usually detected by BDG testing because the polysaccharide is not a major cell wall component of these fungal species.

An enzyme immunoassay (EIA) based technique is used to detect the antigen in patient samples, serum is the only specimen type cleared by the FDA for this assay, and methodology is according to manufacturer instructions.

Fungi tell (formerly called Glucatell) is a US Food and Drug Administration–approved quantitative assay used to aid in the detection of invasive fungal infections and it is described below.The Fungi tell assay is a highly sensitive, microplate-based test that detects (1-3)-b-

9. Antigen Base Detection of Fungi

D-Glucan in serum. (1-3)-b-D-Glucan is a cell wall constituent of most medically important fungi including Candida and Aspergillus.

Procedure

1. The sample/pretreatment mixture is incubated in a 37°C ± 1°C microplate reader or heat block for 10 minutes.
2. 100µl of reagent is then added to the sample and standard curves.
3. The microplate is then placed in a microplate reader at 37°C ± 1°C.
4. The plate reader incubates and collects kinetic data for 40minutes.

Results

Results	(1,3)-β-D-Glucan Levels
Negative	(1,3)-β-D-Glucan values < 60 pg/ml
Intermediate	(1,3)-β-D-Glucan from 60-79 pg/ml
Positive	(1,3)-β-D-Glucan values ≥ 80 pg/ml

(Source: https://www.fungitell.com/pdfs/Fungitell_Brochure_PR18-016web.pd)

10. Biosafety

Safety in the mycology laboratory is crucial because of the potentially pathogenic fungi being studied as well as the hazardous nature of reagents, flames, glassware, and procedures. For safety and protection, senior supervisory staff should develop biosafety guidelines and ensure that all workers are well-versed with them. Ideally, biosafety guidelines should be science-based, made available broadly and be updated based on real-time feedback. Individual labs should further tailor these recommendations based on their own experiences.

Risk assessment

Five basic steps of risk assessment

- Identify potential hazards
- Decide who is at risk
- Assess likelihood of harm
- Implement adequate precautions
- Manage risks and hazardous situations

(Source: https://www.who.int/docs/default-source/medicines/norms-and-standards/guidelines/production/trs981-annex2-who-quality-risk-management.pdf)

Biosafety Cabinet

- Mycology laboratories involved in culturing filamentous fungi and handling these organisms should ideally be distinct and secluded from the primary microbiology laboratory. The isolated area should maintain negative air pressure relative to the main laboratory.
- Direct access to a Class II biological safety cabinet (BSC) is imperative for these activities, regardless of whether mycology work is

performed in a separate room or within a segregated portion of the primary laboratory.

- The majority of mycology diagnostic procedures can typically be conducted within a biosafety level (BSL)-2 laboratory.

(https://www.biobase.cc/Class-II-A2-Biological-Safety-Cabinet-pd46386305.html)

Waste Management

- The laboratory must have a waste management plan that identifies and categorizes waste as infectious and non-infectious at the site of waste generation.
- Strategies must be in place to differentiate between categories of waste such as the use of color-coded waste bags.
- All infectious waste must be either autoclaved or disinfected before it leaves the laboratory.

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National Institutes of Health Pakistan