Notes

52

LABORATORY DIAGNOSIS OF FUNGI

52.1 INTRODUCTION

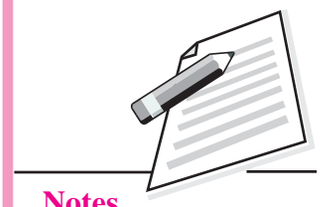
We have learned in earlier chapters about various fungal infections. This chapter deals with the diagnosis and in particular laboratory diagnosis of fungal infection

As processed with bacterial infection, laboratory diagnosis of fungal infection starts with appropriate specimen collection & transport. And in most fungal infections the identifications are based primarily on the assessment of colony morphology & microscopic features. Key biochemical tests may be required to differentiate between the genes & species. Also molecular techniques like Nucleic acid probe assays are being used with increased frequency to provide early confirmation in suspected cases of deep seated mycoses. Serological studies are required in some instances to establish differential diagnosis. Non culture methods & automated system too are available for diagnosis of fungal infections.

OBJECTIVES

After reading this lesson, you will be able to:

- list the steps involved in the diagnosis of fungal infection
- describe the Specimen collection and transport
- explain the Direct examination and mount preparation
- describe the Selection & innoculation of culture media
- explain Incubation of fungal cultures
- describe the Presumptive diagnosis of fungal isolates



52.2 SPECIMEN COLLECTION AND TRANSPORT

For laboratory diagnosis of fungal infections various specimens can be received in the laboratory; Physicians, Nurses, ward personnel & Laboratory technologists needs to work together in developing protocols that ensure the proper collection and prompt collection of specimen.

Fig. 52.1: Sterile container for collection of specimen for fungal culture

The selection of appropriate collection devices & transport containers, labeling of the specimen & complete requisition forms are important considerations in ensuring the correct diagnosis of fungal infections as followed in Table 1

Table 52.1: Transport condition for diagnosis of fungal infection

Specimen	Transport condition
Sputum	Sterile Screw capped container
Bronchoscopy Fluid	Sterile Screw capped container
CSF	If delay anticipated, specimen should be left at room temperature
Urine	If delay beyond 2hrs is anticipated, refrigerate at 4°C
Blood	Biphasic agar broth bottles designed especially for fungal cultures
Tissue biopsy	the specimen should not be frozen or allowed to dehydrate prior to culture

In all cases the specimen should be transported as early as possible to the laboratory. In general the specimen that are not processed immediately are held at room temperature (for urine if delay more that 2 hrs refrigerate at 4°C). Cryptococcus neoformans, cystoplasma capsulatum & Blastomyces dermatitidis do not survive well in frozen or iced specimen.

Notes

Criteria for specimen rejections

1. Absence of patient identification on the container or discrepancy between the information
2. Sputum specimen with >25 squamous epithelial cells as per low power field
3. A dried out swab or if the material collected is insufficient
4. The sample submitted in an improper container
5. The 24hr sputum or urine specimen for fungal culture is received

52.3 SPECIMEN PROCESSING

On receiving the specimen, it should be promptly processed. The direct wet mounts or smears are prepared and for culture the specimen is inoculated on culture media.

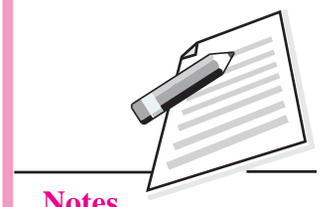
52.3.1 Direct Examination

Almost all the specimens are processed for direct microscopic examination. This provides the presumptive diagnosis for the physician and also aid in the selection of appropriate culture media.

Various methods for direct examinations are

- Direct wet mount of specimen
- India Ink
- KOH/calcoflurol mounts
- Lactophenol cotton blue (LPCB) mounts
- Frozen section of tissue biopsies
- Modified Kinyoun Acid Fast Stain for Nocardia

Direct Microscopic Observations	Presumptive Identification
Hypae relatively small (3-6 μm) and regular in size, dichotomously branching at 45° angles with distinct cross-septa	Aspergillus spp
Hypae irregular in size (6-50 μm), ribbonlike, and devoid of septa.	Zygomycetes (Phycoycetes) rhizopus-Mucor
Hypae small (2-3 μm) and regular, some branching with rectangular arthrospores sometimes seen, found only in skin, nail scrapings and hair	Dermatophyte group Microsporum spp Trichophytoon spp Epidermophyton spp



Hyphae regular in diameter (3-6 µm), parallel walls, irregular branching, septate, dark yellow, brown or hyaline.	Phaeohyphomyces spp Hyalohyphomyces spp
Hyphae, distinct points of constriction simulating link sausages (pseudohyphae), with budding yeast forms (blastospores) often seen.	Candida spp
Yeast forms, cell spherical and irregular in size (5-20 µm) classically with a thick polysaccharide capsule (not all cells are encapsulated), with one or more buds attached by a narrow constriction	Cryptococcus neoformans Cryprococcus spp, nonencapsualted
Small budding yeast, relatively uniform in size (3-5 µm) with a single bud attached by a narrow base, extracellular or within macrophages	Histoplasma capsulatum
Yeast forms, large (8-20 µm) with cells appearing to have a thick, double-contoured wall, with a single bud attached by a broad base	Blastomyces dermatitidis
Large, irregularly sized (10-50 µm) thick walled spherules, many of which contain small (2-4 µm) round endospores	Coccidiodes immitis

52.3.2 Preparation of Mounts

The tease mount, transparency tape method and microslide techniques are commonly used methods for microscopic examination.

The mold colony is mounted in a drop of Lactophenol cotton blue stain on a glass slide and examined microscopically

The specimen are directly mounted in 40% & 10% KOH for skin and nail specimens respectively. The skin and nail samples are mounted on the glass slide to which two drops of KOH preparation is added and kept for sometime. KOH helps in dissolving the epithelial cells and thus aid in fungal visibility.

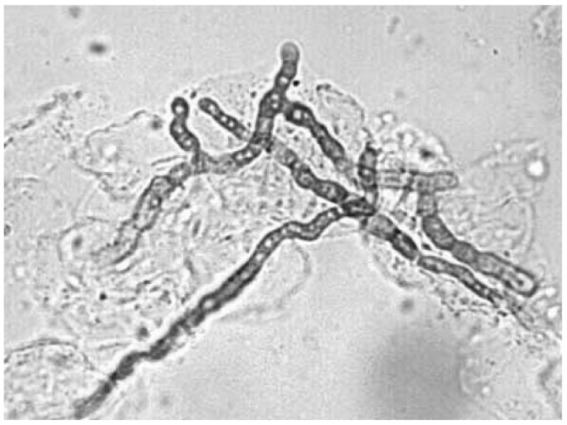


Fig. 52.2: KOH mount of infected skin scales showing typical dermatophyte hyphae breaking up into arthroconidia.

India Ink - India ink can be added to specimens such as spinal fluids or exudates to provide a dark background that will highlight hyaline yeast cells and capsular material (halo effect). Hence, it should be used to examine specimens suspected of containing Cryptococcus neoformans. White blood cells may be distinguished from Cryptococcus neoformans because of the irregular edge of the halo and the pale cell wash. The India ink preparation is not routinely offered by the laboratory. If a request is received for it, the laboratory should call the physician and offer a Cryptococcus Antigen Test instead. The procedure will be performed only in particular instances with the approval of the director or supervisor.

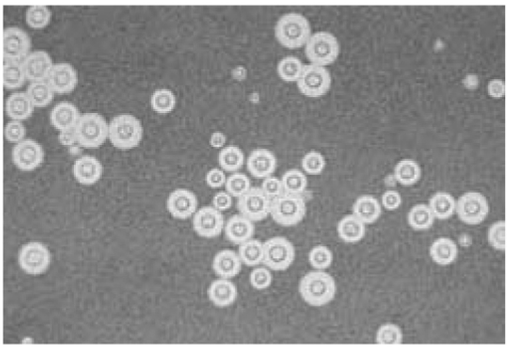
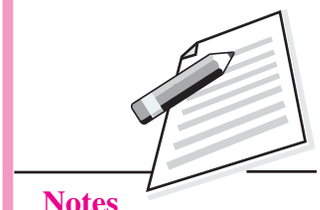


Fig. 52.3: *Cryptococcus neoformans* using a light India ink staining preparation

Gram Stain

Gram stain is usually a poor stain to use when examining a specimen for a fungus. Gram stain may be used when examining smears of Candida, Malassezia, and Sporothrix but should not be relied upon to demonstrate the yeast of the other dimorphic fungi. A gram stain will demonstrate the filaments of Nocardia and Actinomyces which may produce clinical signs resembling mycotic infections.



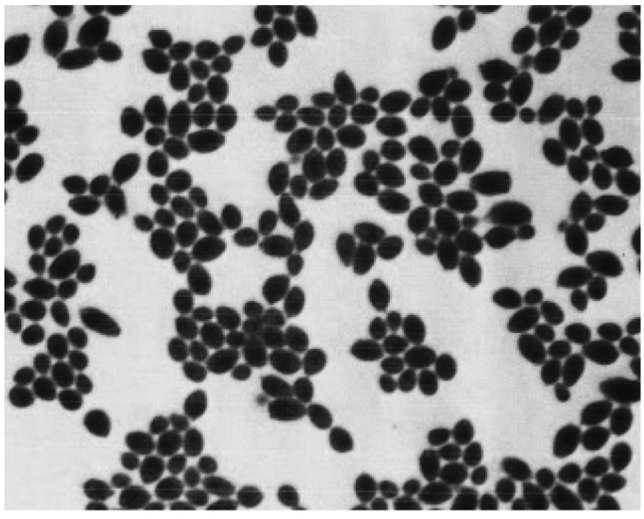


Fig. 52.4: Gram stain of Candida albicans

Modified Kinyoun Acid Fast Stain for Nocardia

a. Make a thin smear of the specimen to be stained; fix in methanol. A positive control smear (Nocardia asteroides) and a negative control smear (Streptomyces sp.) must be included.

b. Kinyoun carbolfushcin; 5 minutes, no heat.

c. Rinse with water.

d. 50% ethanol rinse; flood and pour off until excess carbolfuchsin is removed.

e. Rinse with water.

f. Decolorize with 0.5% (aqueous) H SO ; 3 minutes. 2 4

g. Rinse with water

h. 1% (aqueous) methylene blue; 1 minute.

i. Rinse with water.

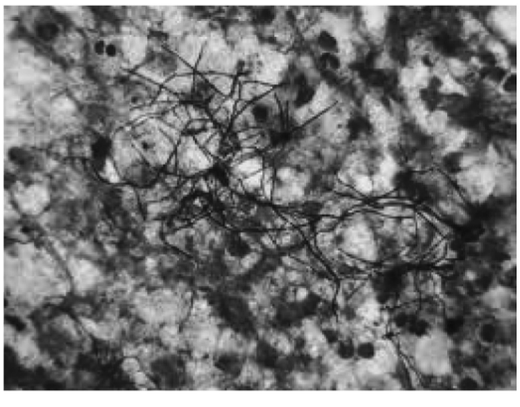


Fig. 52.5: Modified AFB staining for Nocardia spp.

Selection and Inoculation of culture media

Generally two types of culture media are used, nonselective (such as brain heart infusion heart) it permits growth of virtually all clinically relevant fungi. The use of sabourauds dextrose agar as primary recovery medium is discouraged as it is insufficiently rich to recover certain fastidious pathogenic species, particularly dimorphic fungi. Rather, the use of Potato flake agar (PFA), inhibitory mold agar (IMA), or combination of sabouraud's dextrose agar with heart infusion (SABHI) agar is recommended.

Sabouraud's agar is sufficient for the recovery of dematophytes from cutaneous samples or yeasts from vaginal culture. Czepak's agar can be used for the subculture of aspergillus species if colony morphology is an important identifying criteria for any given unknown isolate. For more fastidious dimorphic fungi such as Blastomyces dermatiditis & soistoplasma capsulatum an enrich agar like IMA or SABHI is used and in particular for Histoplasma capsulatum media with the addition of 5-10% sheep blood is recommended. Cryptococcus neoformans, aspergillus fumigatus may be partially or totally inhibited by cycloheximide, therefore a nonselective media must always be used in parallel.

Fig. 52.6: Uninoculated Sabouraud's agar

Incubation of fungal culture

Each sample is cultured in two set of culture media and is incubated at two different temperatures at 30°C (Room temperature) and at 35°C

All fungal cultures are incubated for a minimum of 30 days before discarding as negative.

The choice between the use of culture tubes or plate is optional. For tube, the media is poured in thick slants to prevent dehydration during prolonged incubation period. After the medium is inoculated, do not screw down the cap too tightly because fungi require breathing.

Culture media in tube have advantage of ease of transport, while limitation is difficult to prepare stained mounts for microscopic examination and petridishes have the advantages of providing larger surface for growth resulting in better colony separation and making the cultures easier to examine and sub culture. Also tease mounts or transparency tape preparations are effectively made from plate cultures. The disadvantage being the plates may become dehydrated during prolonged incubation to prevent drying the plates may be placed into a sealed, moisturized polyester bad or the edges are sealed by oxygen permeable tape.

52.4 LABORATORY APPROACH TO PRESUMPTIVE IDENTIFICATION OF FUNGI

After the culture plates reveal that growth of probable fungi, the identification of the colonies is done by the characteristic colony morphology. Also a LPCB mount is prepared from the growth and observed under microscope for the details.

Fig. 52.7: LPCB mount of Aspergillus Spp.

Colonies with smooth, creamy, viscous or pasty appearance, a yeast must be considered. Dematiaccous molds produces colonies that are dark. Gray to black mycelium growth and reverse of the colony is black. For molds that grow within 3-5 days have a distinct border, and are white or patel on the surface. For molds that grow in 7-14 days or that have a cobweb aerial mycelium, one of the dimorphic species should be considered. See the table for details.

52.5 AUTOMATED SYSTEMS

The only current commercially available system is the applied Biosystems, Microseq OZ large subunit r DNA fungal sequencing kit. Micro scan also offer candida species identification kit but not for other fungi isolates.

Notes

52.6 MOLECULAR TECHNIQUES

Nucleic acid probe assays are being used with increasing frequency to provide early culture confirmation, especially in deep mycoses infection.

Looking on to future, it must be mentioned that nucleic acid sequencing has become the standard method for fungal identification especially in reference laboratories

INTEXT QUESTIONS 52.1

1. Urine specimen if delayed in transportation to laboratory needs to be refrigerated at ..... °C
2. CSF should be stored at ..... temperature if delay in transportation to laboratory is anticipated
3. Tissue biopsy should be ..... before sending it to laboratory
4. Fungal visibility is aided by adding ..... preparation
5. ..... preparation may be used to examine specimens suspected of Cryptococcal Infections
6. ..... culture media is used for diagnosis of fungi
7. All fungal cultures are incubated fro a minimum of ..... days before confirming as negative.

WHAT YOU HAVE LEARNT

- Appropriate specimen collection play a major role in laboratory diagnosis of fungal infection. The samples are collected in appropriate manner and in appropriate container and are transported to laboratory promptly. If delay is anticipated, the relevant consideration are observed. The first step in the processing starts with the direct microscopic examination of the specimen. In case of skin 40% KOH and for nail sample 10% KOH is used to dissolve the epithelial cells. After the direct examination the specimens are cultured on appropriate culture media and they are incubated at 30^0c and 35^0c. Separately they are observed regularly for growth and negative report is dispatched not before 30 days of complete incubation.

- The identification of isolated fungi is basically based on the morphology of the colony. Also LPCB mount of the colony is prepared to observe the microscopic features. In some case biochemical test's may be required to differentiate the genes and species. Also nucleic acid probe assays are being used and also serologic studies are required in some instances to establish definitive clinical diagnosis.

TERMINAL QUESTIONS

1. Enlist the steps involved in the laboratory diagnosis of Fungi.
2. Describe the specimen collection and transport
3. Why we do not refrigerate the specimen in case of anticipated delay
4. Describe the methods of direct examination of fungiPresumptive diagnosis of Fungal infection

ANSWERS TO INTEXT QUESTIONS 52.1

52.1

1. 4
2. Room
3. Frozen
4. KoH
5. Indian Ink
6. Brain heart infusion
7. 30

HYALINE		MOLD COLONIES		DEMATIACEOUS		YEAST COLONIES	YEAST-LIKE COLONIES
Growth < 3days	Growth 3-5 days	Growth 3-5 days	Growth > 5 days	Growth 3-5 days	Growth > 5 days	Growth 2-5 days	Growth 2-5 days
Hyphae broad and aseptate	Hyphae hyaline and septate	Colonies often granular and pigmented, hyphae septate, hyaline	Hyphae	Dark colony, black reverse, hyphae yellow-pigmented and septate	Dark colony, black reverse, hyphae yellow-pigmented and septate	Smooth, pasty or macoid colonies	Yeastlike colonies with low aerial mycelium
Suspect Zygomyces Rhizopax Abxidia Syncephalasirum Circinella Cunninghamella Mucor	**Suspect agents of Hyalohyphomycocisis** **Conidia in Chains:** Aspergillas Pencillium Paecilomyces Scopulanopsis **Conidia in Clusters** Acremonium Fusarium Trichoderma Gliocladium **Conidia Borne Singly:** Scedosporium apiospermum Scedosporium Prolifercans (inflatum) Chrysosporium Sepedonium	**Suspect Dermatophyte** **Genus Microsporisia common:** Microsparum …….. **Uncommon:** Microsporium …….. Microsporum adnum **Genus Trichophytom common:** Trichophytom rabum Trichophyton mentogrophytes Trichophyton tonsutum Trichophyton Verracosum **Uncommon:** Trichophytom Violaceum Trichophyton Schoenleinii **Genus Epidermophyton:** Epidermophyton Floccosum	**Suspect Dimorphic fungi**	**Suspect Agent of Pharophyomycosis** **Conidia Muriform:** Shermaria Ulocladium Steaphylium Epicoccum **Conidia divided by Transverse Septa only:** Curvularia Bipolaris (Drechslera) Exterohilum **Pycnindia produced** Phoma Chaetomium	**Suspect Agent of Chromomycosis or mycetoma** **Caldosprium-Type Sporulation:** Clodophtalophora carrunil Cladophiolophora bantianum **Phialophora – type Sporulation:** Phialophora verrucosa Phialophora richardsiae Exophiala jeanselmei **Acrotheca-Type sporulation:** Fonsecaea pedrosoi Fonsecaea compacta	**Suspect Yeast** **Common:** Cadida albicans Candida Cryptococcus neoformans Cryptococcus Rhodotorida **Uncommon:** Hansenula anomala Malasseria furfur Malassezia furfur Saccharomyces cerevisiae **Rare:** Basidiobolus species Conidiobolus species **Black yeasts:** Pullalaria pullalans Phaeococcomyces species Yeast forms of dimorphic fungi	**Anthrocomidia Produced** **Suspect:** Geatrichum candidum Trichosporom beigelil coraplex Blastoschizomyces capitus