Diagnosis of Fungal Infections
Clinical Classification of the Fungi

For clinicians, dividing the fungi into four categories of mycoses, according to the type of infection, is much more useful. The fungi are categorized as follows:

- **Superficial (Cutaneous) mycoses (hair, skin & nails / no invasion).**
  - Dermatophytes such as agents causing Athlete’s foot, ring-worm & agents of infection such as tinea & piedra. They infect keratinized tissues.

- **Subcutaneous mycoses.**
  - Without dissemination to distant sites.
  - Infections include chromoplasmomycosis, mycetome, etc.

- **Systemic mycosis.**
  - Agents include the genera *Blastomyces, Histoplasma*, etc

- **Opportunistic.**
Diagnosis of Fungal Infection

• The fungi are saprophytic and parasitic eukaryotic microorganisms.
• Fungi were regarded as relatively insignificant causes of infection. It is now well documented that they are the common cause of infection, particularly in immunocompromised patients such as (Candida and Aspergillus species).
• Early and accurate diagnosis of fungal infections is important for several reasons, including timely institution of antifungal therapy and to decrease the unnecessary use of toxic antifungal agents.

Identification of Fungi:
• Hyphae or spores
  • Sepetate or nonseptate hyphae
    • Monomeric or diphormic
      • Types of spores produced
        • Arrangement of spores
        • Growth rate
• Specimens should be examined macroscopically and microscopically. The gross examination allows for selection of the proper portion of the specimen that will likely contain the fungus.

• Microscopic detection of a fungus in clinical specimens can alert the physician to the disease agent and alert the laboratory staff to select the appropriate media and inoculation techniques that will enhance the recovery of the fungus.

• Accurate diagnosis of fungal diseases is not straightforward. A new patient will be asked to give a history of the symptoms they have been experiencing. Depending on the history a number of laboratory tests may be ordered. These may well include the following tests.

• Newer more rapid molecular test are being developed to enhance the detection of different fungal species, many are still in the testing stage and research is needed to provide rapid and cost-effective diagnostic tests. Current methods for diagnosing fungal infections are slow and take too much time to process. The faster a positive diagnosis can be reached the better the outcome for the patient.
Collection and Transport of the Clinical Specimens

The diagnosis of fungal infections is dependent on the selection and collection of an appropriate clinical specimen for culture.

**SPECIMENS INCLUDE:**

- Skin scrapings.
- Hair and nails.
- Respiratory tract secretions.
- Cerebrospinal spinal fluid.
- Blood.
- Mouth and vagina.
- Urine.
- Pus.
- Ocular specimen.
- Tissue.
- Skin biopsy.

- **Moist swab from a mucosal surface (inside the mouth or vagina) in a special transport medium.**
- **A swab should be taken from pustules in case of secondary bacterial infection.**
- **They are transported in a sterile container or a black paper envelope.**
Rapid Methods of Examination

• Direct microscopic examination of the clinical specimen (potassium hydroxide preparation).
• Serodiagnosis (immunodiffusion testing, Enzyme immunoassay, etc).
• Cryptococcal antigen detection.
• Molecular Detection: **Nucleic acid probe for identification, Real-Time PCR identification, Nucleic Acid Sequencing.**
• MALDI-TOF (Matrix-Assisted laser Desorption Ionization) is a biophysical method that significantly reduces the time require specifically identify fungal aorganism.
• PNA-fish Probes (for the identification of Candida spp. From blood cultures).
Direct Examination of Clinical Specimen

Direct microscopic examination of fungal cells within the clinical specimen is a valuable diagnostic procedure for the following reasons:

- In many instances, a tentative or even a definitive diagnosis can be made \textit{before the growth of fungal cells would be apparent in culture}.

- Observing fungal cells in a clinical specimen may be more valuable as a criterion for diagnosis than isolating in a culture.

Preparations for direct examination of clinical specimen include:

- Potassium hydroxide (KOH) preparation
- India ink
- in addition, a few staining techniques such as Giemsa.
- Unstained wet-mount
- Stained dried smear
- Histopathology of biopsy with special stains, e.g., periodic acid-Schiff (PAS).
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)
- Spores inside a hair (endothrix) or outside a hair (ectothrix).
- Fungal elements are sometimes difficult to find, especially if the tissue is very inflamed, so a negative result does not rule out fungal infection.

A yeast infection can be identified by the presence of:

- Yeast cells, which may be dividing by budding
- Pseudohyphae (branched filaments similar to those of a dermatophyte) forming a pseudomycelium.
WET SMEARS-

1- PREPARATION WITH Potassium Hydroxide (KOH) (UNSTAINED): Patches from the mucous membrane of the mouth, vagina, skin, or nails scraping, sputum are collected in a sterile container.

These are examined in a KOH wet mount. Yeast cells with budding mixed pseudohyphae are seen. The presence of pseudohyphae shows colonization and tissue invasion and so their demonstration is significant.

For detection of Candida, wet smear microscopy has been positive in the majority, but not in all cases.

2- STAINED PREPARATIONS- PREPARATION with KOH:

The specimen should be first examined microscopically for necrotic, purulent, bloody areas. Because these areas are most likely to yield evidence of fungal growth, they are selected for direct examination.

Preparation with KOH clears the tissue and cellular debris from all types of specimens without damaging the fungal cells.

This clearing process requires only 5 to 10 min, after which one can observe the fungal morphology as well as the pigment of the fungal cell wall. The slide is examined under bright-field microscope.
The disadvantage of using KOH:

• its reaction with pus, sputum, and skin; in these instances.
• KOH can damage the microscope stage if the slide overflows.
• In addition, crystals can form on standing so that reading of smear becomes difficult.

Tentative diagnosis can be derived from the presence of fungal elements compatible to the etiologic agents of aspergillosis, mucormycosis, dermatophytosis, candidiasis, sporotrichosis, or cryptococcosis.

To confirm such a diagnosis, however, cultural proof is necessary.
TKOH preparation of candida showing **pseu**do**hyphae**

KOH preparation showing hyphae
3- Preparation of a WET MOUNT.

- Pick up the sample and place a small drop of on the slide.
- Try to put a chunk of material you can see by eye on the slide.
- Place a cover glass over the sample and observe it under the microscope first using the 10X objective.
4- Preparation with Calcoflour White and KOH:
The dye is useful for demonstrating the presence of fungal cells in clinical specimens because it binds to polysaccharides. A fluorescence microscope is needed for detecting fungal cells prepared with Calcoflour White. Yeast cells, pseudohyphae and hyphae display a chalk-white or brilliant apple-green fluorescence.

- The disadvantages of using Calcoflour are the need for a fluorescence microscope- inability of the dye to detect the endospores, and the difficulty in interpreting vaginal secretions.

5- Preparation with India Ink:
India ink is useful for indicating the presence or absence of extracellular polysaccharide capsules of fungal cells. Because India ink serves as a negative stain, the encapsulated yeast cells can readily be detected against the dark back ground. The presence of encapsulated yeast cells in CSF in almost always an indicator of Cryptococcal meningitis.

6- Preparation with Periodic Acid-Schiff (PAS) Stain:
The PAS stain one of the most widely used stains for fungal histopathology. In a direct examination of clinical specimen, PAS stain is sometimes used when a KOH preparation do not reveal fungi that are suspected to be present.

PAS preparation requires 20 to 25 minutes. Light green is preferred as a counter stain because the fungus appears deep purplish red against the contrasting background color. For the counter staining process the slides should be placed in light green stain for 5 sec and washed for 5 to 10 sec between steps. The PAS reaction stains fungal polysaccharide.

For good results, both the periodic acid solution and the sodium Meta bisulphate solution should be fresh and protected from light. For laboratories with a fluorescent microscope, calcoflour white is preferred over PAS.
PAS stain of *Aspergillus*  
Preparation with Calcoflour White and KOH  
India Ink Preparation
7- Gormori`S Methenamine Silver Stain (GMS):

It is based on the liberation of aldehyde groups and their subsequent identification by the reduced silver method.

It is used for demonstration of polysaccharide content on the fungus in tissue. The aldehydes reduce the methenamine silver nitrate complex, resulting in brown black staining fungal cell wall.

The GMS is better than other fungal stains as:

It stains both live and dead fungi in contrast to PAS which stains only live fungi.

The GMS highlights fungal hyphae.
Culture Medias

Culture identifies which organism is responsible for the infection:

- To find out the source of infection
- To select the most suitable treatment

Common media for primary fungal isolation include

- **Sabouraud dextrose agar**
- **Brain-Heart Infusion agar (BHI)** Either in petri dishes or screw top tubes.

The media may be enriched with **5% to 10% sheep blood** to support the growth of certain fungi. Specimens that are likely to be contaminated with other microorganisms, such as urine or sputum, are set up on agar media containing antimicrobials. Chloramphenicol, streptomycin, or penicillin are incorporated into the agar to inhibit the growth of bacteria.

Most fungi also thrive on **Potato Dextrose Agar (PDA)**, but this can be too rich for many fungi, so that excessive mycelial growth is obtained at the expense of sporulation.
The most commonly used cultures media used for fungal growth are as follows:

- **Sabouraud agar**
- **Brain Haert infusion agar**
- **Potato dextrose agar & broth** >rich medium for growing a wide range of fungi.<
- **Yeast Agar and broth**
  - Mycological Agar
  - Malt Extract Broth
  - Soy Peptone Yeast Extract Agar
  - **Water Cornmeal Agar (CMA)** >use for isolating fungi from surface-sterilized substrates
  - **Potato Carrot Agar (PCA)** >relatively weak medium, good for some Fungi imperfecti
  - Antibiotic Agar>isolating fungi from substrates not readily sterilized, or to clean a culture bacterial contaminated.

- Growing the fungus in culture may take several weeks, incubated at 25-30⁰C.
A negative culture may arise because:
The condition is not due to fungal infection.
The specimen was not collected properly.
Antifungal treatment had been used prior to collection of the specimen.
There was a delay before the specimen reached the laboratory.
The laboratory procedures were incorrect.
The organism grows very slowly.
Special Tests

1- Chlamydospore Formation for Candida

Inoculate suspected Candida on plates or slides with Chlamydospore agar. Incubate at room temperature for 48 to 72 h.

Examine under microscope for presence of Chlamydospore and characteristic growth of mycelia. *Candida albicans* produces abundant chlamydospires in the lateral.

Chlamydospores formation of the yeast *Candida albicans*. 
Special Tests

2- GERM tube formation (Reynauld-Braude Phenomenon):

rapid “presumptive test” for *Candida albicans*. culture of yeast is incubated in bovine serum for 2-3 h at 37°C.

The test is positive if there are short hyphae without a constriction where the hypha joins the parent cell. Not all *C. albicans* isolates are germ tube positive.

Old cultures, heavy inoculum and cultures from SAB can give negative results. It is important to read the test within 3 hrs as other Candidal species will also form germ tubes in serum after this time period.

Germ tube appears to be the cheapest test, but it is time consuming and laborious.
Biochemical tests:

Carbohydrate Fermentation (Carbohydrate Assimilation):

This test is used for definite speciation of Candida and few other fungal. Carbohydrate assimilation test (Modified Wickerham Method): The carbohydrates used are Glucose, Maltose, Lactose, Sucrose, Galactose, Xylose, Trehalose, Cellibiose.

Rapid Urease Test:

This test is used to detect presence of urease enzyme produced by different Candida species. Urea agar slants are used. Conversion of the yellow slope to pink or red is considered positive. A negative test is reported when there is no color change observed.

OTHER TECHNIQUES:

- Microslide Culture
- Hair Perforation test
- In vitro conversion of Dimorphic molds
- antigen test
- Nucleic acid probe testing.

NEWER DIAGNOSTIC TECHNIQUES:

- Immune Diagnostic Methods
- PCR Technique
- Serological tests
- Latex Agglutination tests
- Immunoflorescence
Cheap Way to Make a Microslide Culture

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Microslide Culture

Slide Culture - Simple Humidity Chamber

Inoculation of Agar Plugs
*C. albicans* on Sabouraud Dextrose Agar at 48h - 30C

*C. albicans* in a vaginal swab (pseudohyphae & a few individual oval cells. (Gram Stain)
Mould growth

Pure or predominant mould

- Examine potential pathogen by source

Mixed culture (Mould/yeast/bacteria)

- Sterile source
  - Work up all

- Non-sterile source
  - No work up, report as mixed*

Nail
- Dermatophyte
- Scopulariopsis
- Fusarium
- Yeast

Skin scraping
- Dermatophyte
- Dematiaceous-Zygomycete
- Dimorphic

Hair/scalp
- Dermatophyte
- Malassezia
- Yeast

Sputum
- Dimorphic
- Pseudallescheria
- Aspergillus

Nasal, sinus, wound, lesion, sterile site, deep respiratory
- Work up all

*For certain sources and isolates, a call to the physician may be necessary to guide further workup.
<table>
<thead>
<tr>
<th>Pathogen</th>
<th>Clinical Characteristics</th>
<th>Mycologic Features</th>
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<tbody>
<tr>
<td>Aspergillus species</td>
<td>Most common invasive mold; appears most frequently as invasive pulmonary aspergillosis; disseminated infection, including skin in about 20% of patients; primary skin inoculation (necrotic lesion) in about 5% of patients</td>
<td>Blood cultures often not positive; non-culture-based methods (tests for galactomannan and 1,3-β-D-glucan) may facilitate diagnosis; polycyty resistance in some species (e.g., A. terreus); preferred therapy is with voriconazole</td>
</tr>
<tr>
<td>Fusarium species</td>
<td>Disseminated infection with pulmonary nodules and characteristic erythematous, nodular skin lesions, which may have a necrotic center; initial portal of entry often onychomycosis or nail lesion</td>
<td>Blood cultures positive in up to 50% of cases; clinical and microbiologic resistance to polycytyes common; better susceptibility to voriconazole and posaconazole; poor outcomes without reversal of underlying immune defect or cancer</td>
</tr>
<tr>
<td>Zygomyces</td>
<td>Sinus and pulmonary involvement common; primary skin lesion more common than skin lesions resulting from hematologic dissemination; associated with use of voriconazole as immunosuppressive therapy</td>
<td>Characteristic ribbonlike appearance, with rare appearance of cross-walls on histopathological examination; cultures of homogenized tissues may be negative; preferred therapy is with high-dose liposomal amphotericin B or posaconazole</td>
</tr>
<tr>
<td>Scedosporium species</td>
<td><em>S. apiospermum</em> common in respiratory tract, brain abscesses, and skin nodules; associated with near-drowning; <em>S. prolificans</em> associated with disseminated infection in patients with severe immunosuppression</td>
<td><em>S. apiospermum</em> resistant to polycytyes, more susceptible to extended treatment with broad-spectrum azoles; <em>S. prolificans</em> is dermataceous; multiantifungal drug resistance; blood cultures positive in about 50% of patients</td>
</tr>
<tr>
<td>Phaeohyphomycosis</td>
<td>Associated with disseminated and central nervous system infection in immunosuppressed patients; pulmonary, sinus, and cutaneous lesions from dissemination or primary infection</td>
<td>Melanin-containing hyphae seen with use of specific stains (e.g., Masson–Fontana) on histopathological examination; irregular hyphal shape; fungemia may be present in some species (e.g., exophiala); preferred therapy is with newer azoles or polycyclic</td>
</tr>
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Table 2. Clinical and Mycologic Characteristics of the Most Common Invasive Molds in Hematologic Cancers.
Serological tests

• Serologic markers such as fungal antigens and glucan are becoming increasingly important for early diagnosis of invasive fungal infections (IFI).

• Fungal antigens detected with commercially available kits.

• Measurement of glucan in blood may be useful as a primary screening tool for IFI.

• The clinical sensitivity and specificity of serologic assays for diagnosing IFI are somewhat variable.
Currently Available Molecular Diagnosis For Fungi

✔️ **Isolates growing in cultures:**
  - Nucleic Acid hybridization probes.
  - DNA sequencing
  - PCR (laboratory-developed)
  - PNA FISH for *Candida* spp. (blood only).

✔️ **Directly from patient specimens:**
  - PCR (Laboratory-developed)
Molecular Tools

1- Nucleic Acid Hybridization Probe:

- Add labeled DNA probe with complimentary sequence

- Label is hydrolyzed on unbound probe
- Chemiluminescence used to detect bound probe
1- Fungal nucleic acid hybridization probes

- Repid (2 hrs after growth in culture)
- Sensitive & specific
- Only available of 3 dimorphic fungi
1- Fungal nucleic acid hybridization probes:

<table>
<thead>
<tr>
<th>Organism</th>
<th>Sensitivity</th>
<th>Specificity</th>
</tr>
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<tbody>
<tr>
<td><em>Blastomyces dermatitidis</em></td>
<td>98.1%</td>
<td>99.7%</td>
</tr>
<tr>
<td><em>Coccidioides immitis</em></td>
<td>98.8%</td>
<td>100%</td>
</tr>
<tr>
<td><em>Histoplasma capsulatum</em></td>
<td>100%</td>
<td>100%</td>
</tr>
</tbody>
</table>

*a*cross-react with a few other fungi including *Emmonsia* species, *Paracoccidioides brasiliensis*, and *Gymnascella* species.

*b*does not distinguish between *Coccidioides immitis* and *Coccidioides posadasii*.
Ribosomal Structure: Fungi

commonly used targets for fungal sequencing in the clinical laboratory today are the D1-D2 region of the 28S subunit and the ITS region.

**2- DNA Sequencing of Fungi:**

**Molecular Tools**

major RNA transcript

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Molecular Tools

2- DNA Sequencing of Fungi:

Traditional Sequencing Workflow
2- DNA Sequencing of Fungi:

- Compare the DNA sequence from the patient-derived isolate sequence to known sequence libraries
  - MicroSeq library
  - laboratory-specific custom library
  - NCBI GenBank database

- Turnaround time can be fast as 8 hours after an organism has grown in culture.
Molecular Tools

3- Rapid, Direct Identification of Yeast from Blood Cultures:

3-1- PNA FISH Probe Method

peptide nucleic acid fluorescence in situ hybridization, or PNA FISH, probes for the direct identification of yeast from positive blood culture bottles.

*Candida glabrata*/ *C. Krusei* RNA Probe

*C. albicans*/ *C. parapsilosis* RNA Probe

*C. Tropicalis* RNA Probe
Molecular Tools

3- Rapid, Direct Identification of Yeast from Blood Cultures:

PNA FISH for *Candida* spp—Direct Detection from Blood
Traditional methods for fungal diagnosis

- Culture to solid media
- Wait for growth
- Wait for sporulation
- Subcultures (maybe a few times)
- Identification is based on macroscopic and microscopic morphology.
  - And perhaps a few biochemical tests for yeast.

- Slow
- Labour intensive
- Require highly experienced laboratory staff for visual recognition of the morphology.
Advantages of fungal molecular diagnosis

• Time
  • can safe days to weeks of identification
• Improved patient care
  • appropriate antifungal can be selected
  • appropriate care can be started sooner
• Improved safety for lab. Workers
  • Reduced manipulation of growing cultures (e.g. *Coccidioides* spp.).
Advantages of the rapid detection

- Improve patients safety
  - Identify the right yeast (approx. 90% of species covered w/traffic light)
  - Select the right drug
  - Rapid TAT

- Decrease hospital length of stay

- Cost savings
  - Fluconazole (400 mg, oral, 1 dollar/day - IV, 8 dollar/day)
  - Capsofungin (IV, 110 dollar/day)
Summary

• Molecular Diagnosis
  • Significantly reduce turnaround time for detection & identification of fungi in the clinical laboratory.

• Reliable identification of the fungi from the clinical specimen remains challenging.
  • But progress is occurring.