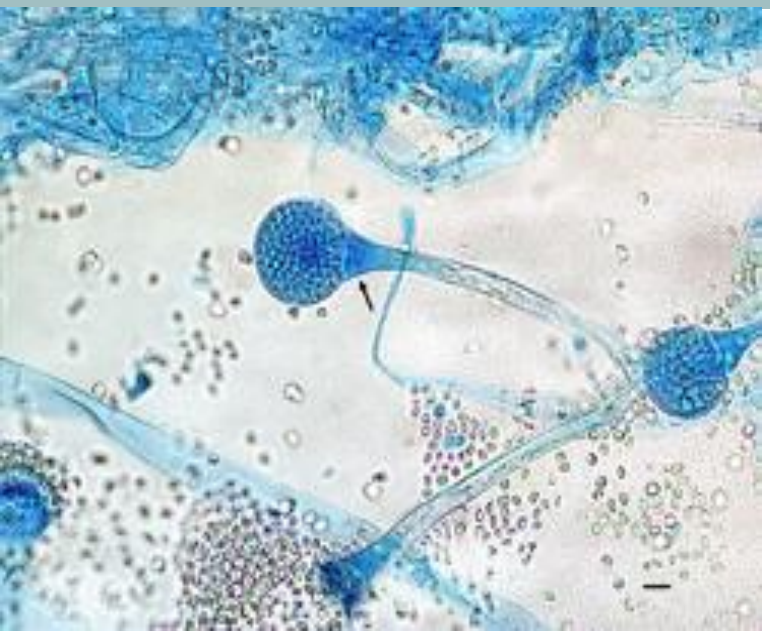
# Current Challenges in the Diagnosis of



# Fungal Infections

# Introduction

- Moulds and yeasts are widely distributed in the environment.
- Of the 100,000 fungal species present, only 300 have been linked to diseases in humans and animals.
- Human mycoses are caused by true or opportunistic fungal pathogens

Lass-Flörl C et al. Methods Mol Biol. 2017;1508:3-15.

# Fungal infections

- Vary from superficial to systemic and are an emerging medical concern.
- Account for 10 % of all nosocomial infections.
- Deep, systemic infections are less common but can affect any organ.
- Immunocompromised hosts are at an increased risk of suffering from fungal infections

  Eg. Organ transplant recipients

  Lung diseases.

Lass-Flörl C et al. Methods Mol Biol. 2017;1508:3-15.

# Health Concern

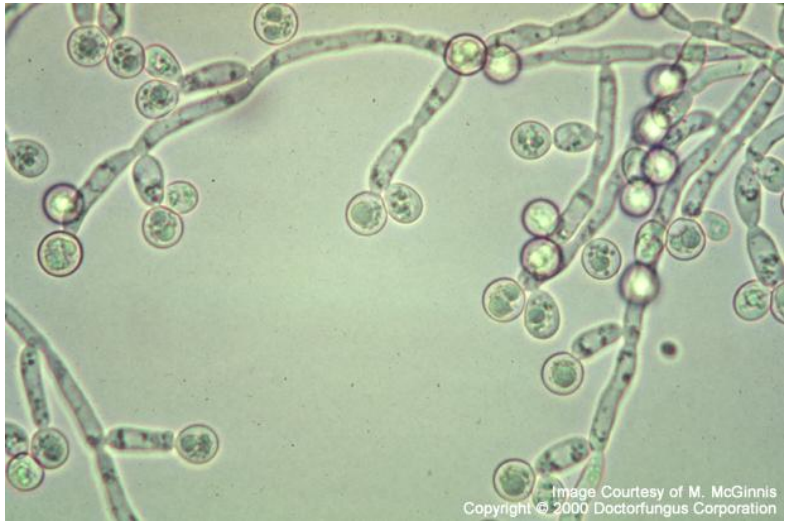
- Fungal diseases are a concern in the medical and public health community because of the
  - increasing number of people with weakened immune systems and
  - the advances in health care practices.

- Fungal infections are difficult to diagnose because clinical symptoms, signs, and radiographic manifestations are unspecific.

Sahoo AK et al. Indian Dermatol Online J. 2016 Mar-Apr; 7(2): 77–86

# Common Fungal Pathogens

- The most important fungal pathogens are
  - *Aspergillus species,*
  - *Candida species*
  - *Cryptococcus species,*
  - *Pneumocystis jirovecii , and*
  - *Mucormycetes (formerly* Zygomycetes ), with *Rhizopus species, Mucor species, and Lichtheimia species*

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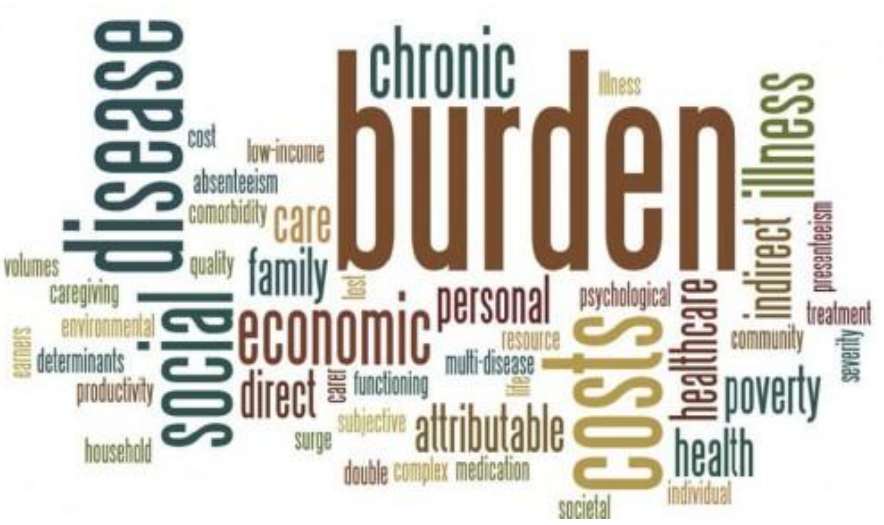
# Burden

- Superficial fungal infections are common diseases worldwide.
- They can affect a variety of body locations and are named accordingly.
- 20–25 per cent of the world population suffer with fungal infections.




Tyagi S et al. Intl J Basic Appl Sc 2014; 3 (1):26-29

# Characteristics of main fungal infections worldwide

| Body location | Pathogen type | Organ | Most frequent genus | Estimated incidence of infection* |
|---|---|---|---|---|
| superficial | primary | Skin and hair | *Malassezia* | ~140,000,000 cases/year |
| cutaneous | primary | Skin and nails | Trichophyton<br>*Epidermophyton*<br>*Microsporum* | ~1,500,000,000 cases/year |
| mucosal | opportunistic | Vagina, digestive tract, urinary tract and | *Candida* | ~75,000,000 cases/year<br>~9,500,000 cases/year |
| | | eye | *Aspergillus, Fusarium* | ~1,000,000 cases/year |
| systemic | opportunistic | any organ (lungs, brain, bloodstream etc.) | *Candida*<br>*Aspergillus*<br>*Cryptococcus*<br>*Histoplasma*<br>*Pneumocystis*<br>*Coccidioidomyces*<br>and so on | ~300,000 cases/year<br>~350,000 cases/year<br>~1,000,000 cases/year<br>~500,000 cases/year<br>>200,000 cases/year<br>up to 300,000 cases/year |

Lass-Flörl C et al. Methods Mol Biol. 2017;1508:3-15.
Vandeputte P et al. Intl J Microbiol 2012, Article ID 713687, 26

# Nature, target, mode of action, and fungal resistance mechanisms of the major antifungal drugs used in human therapy

| Antifungal agent | Mode of action and cellular target | Mechanism of resistance |
|---|---|---|
| polyenes | binding to ergosterol | absence of ergosterol (loss of function mutation in *ERG3* or *ERG6*)<br>decrease of ergosterol content in cells |
| azoles | inhibition of cytochrome p450 function: 14$\alpha$-lanosterol demethylase (*ERG11*) sterol $\Delta^{22}$ desaturase (*ERG5*) | efflux mediated by multidrug transporters<br>decrease of affinity in Erg11p by mutations<br>upregulation of *ERG11*<br>alterations in the ergosterol biosynthetic pathway |
| allylamines | inhibition of squalene epoxidase (*ERG1*) | unknown |
| morpholines | inhibition of sterol $\Delta^{14}$ reductase (*ERG24*) and the $\Delta^{7-8}$ isomerase (*ERG2*) | unknown |
| 5-fluorocytosine | inhibition of nucleic acids synthesis | defect in cytosine permease<br>deficiency or lack of enzymes implicated in the metabolism of 5-FC<br>deregulation of the pyrimidine biosynthetic pathway |
| echinocandins | inhibition of $\beta$-1,3 glucan synthase (*FKS1&2*) | alteration of affinity of echinocandins for $\beta$(1,3)-glucan synthase |

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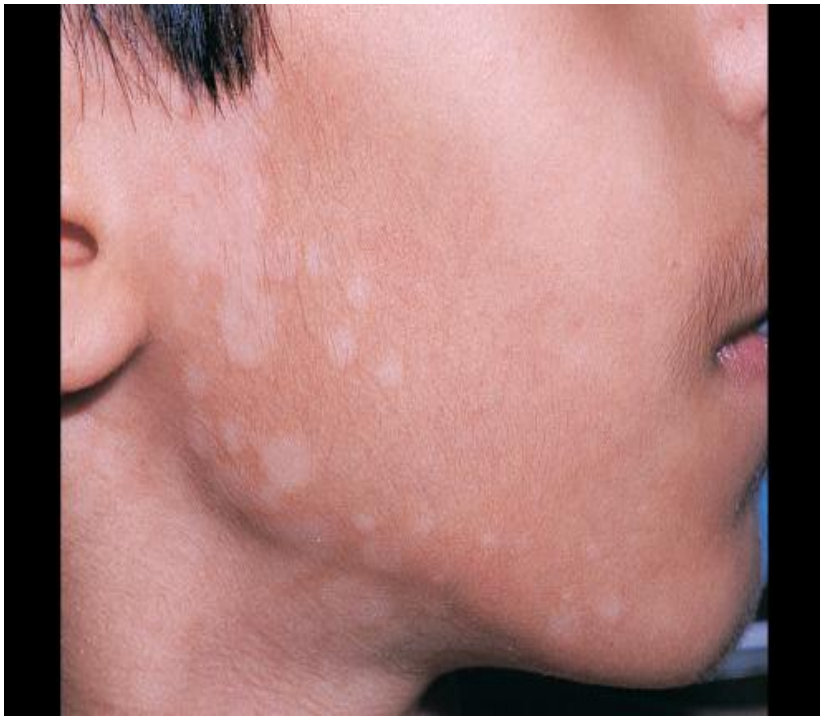
# Superficial Fungal Infection

- Superficial mycoses are limited to the outermost layers of the skin and hair.
- Eg: Tinea versicolor, a fungus infection that commonly affects the skin of young people.



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# Superficial Fungal Infection

- Dermatophyte infection
- Pityriasis versicolor
- Cutaneous candidiasis

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# *Dermatophytes*

- Commonly called “ringworm” infection.
- Causative organism
  - Epidermophyton
  - Trichopyton and
  - Microsporum.
- Made of septate hypae which branch out into mycelium.

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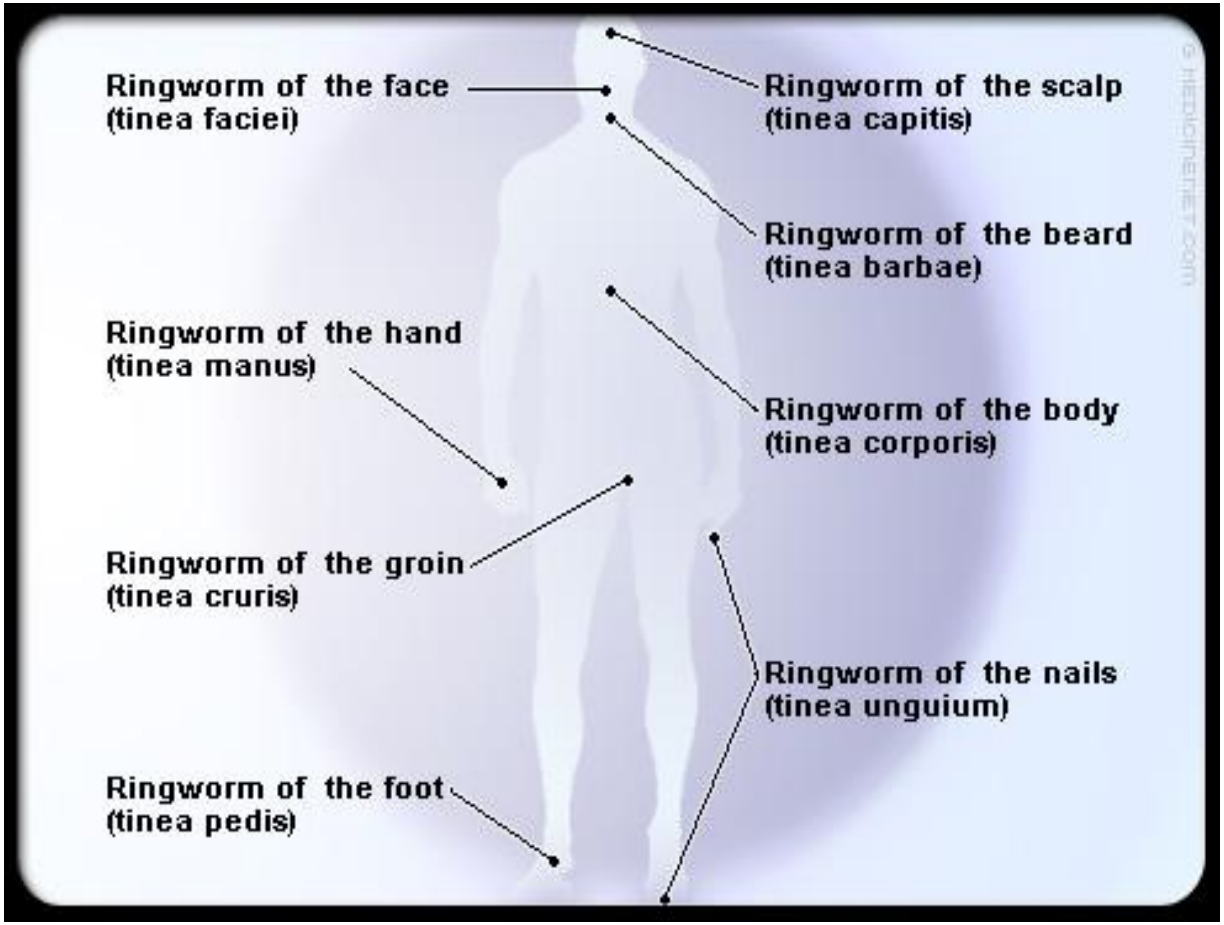
# Dermatophyte infection

- Tinea capitis
- Tinea corporis
- Tinea cruris
- Tinea pedis
- Tinea manuum
- Tinea unguium

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# Dermatophytic onychomycosis

- Categorised into several distinct forms:
  - distal-lateral subungal,
  - proximal subungal,
  - superficial white,
  - endonyx,
  - Total dystrophic and
  - mixed-type onychomycosis.

*Distallateral subungal is the most common and presents with onycholysis and subungal hyperkeratosis*

Salmon N et al. Prescriber 2013 April; 24(8): 31–37

# Features of different types of onychomycosis

| Type | Clinical features | Most frequently found dermatophyte |
|---|---|---|
| *Distal lateral subungal* (DLSO) | onycholysis<br>subungal hyperkeratosis | *T. rubrum* |
| *Superficial white* (SWO) | white chalky patches on the surface of the nail | *T. mentagrophytes* |
| *Proximal subungal* (PSO) | leukonychia<br>onycholysis<br>starts at the proximal nail fold | *T. rubrum* |
| *Endonyx* (EO, rare) | invasion via skin with direct invasion of the nail plate | *T. soudanense* or *T. violaceum* |
| *Mixed types* (MO) | DLSO + SWO<br>DLSO + PSO | as above |
| *Total dystrophic* (TDO) | end result of all types of onychomycosis | as above |

Salmon N et al. Prescriber 2013 April; 24(8): 31–37

# Overview of laboratory methods available for the diagnosis of fungal diseases

- **Conventional methods**
  - Direct microscopy (KOH/Calcofl uor white stains, Giemsa)
  - Histopathology
  - Culture
- **Serology**
  - Galactomannan test for *Aspergillus species*
  - Mannan test for *Candida species*
  - Antibody test for aspergillosis in immunocompetent hosts
  - ß-1-3 D glucan panfungal test
  - Lateral device fl ow assays for *Aspergillus and Cryptococcus species*
  - Antigen and antibody based assays for non-European mycoses
- **Molecular methods**
  - Fluorescence *in situ hybridization test*
  - PCR assays

Lass-Flörl C et al. Methods Mol Biol. 2017;1508:3-15.

# Specimen collection

- Specimen collection depends on the infection site involved and may include
  - skin scrapings,
  - body fluids,
  - blood, and/or
  - tissue biopsies

- Test interpretation often requires experience in mycology

- Results must be carefully considered along with signs and symptoms as well as the medical history

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Lass-Flörl C et al. Methods Mol Biol. 2017;1508:3-15.

# Specimens applied for diagnosing fungal infections

| Assay | Specimens | Goal | Results | Time |
|---|---|---|---|---|
| Potassium hydroxide solution (KOH) | Skin scrapings, hair or nail clippings, tissue, vaginal secretions, body fluids, sputum | KOH dissolves non-fungal elements and reveals fungi on the microscope slide | Screening tool which detects fungal elements; does not inform about genus or species | Rapid, 40 min |
| Calcofluor white stain (CWS) | Skin scrapings, hair or nail clippings, body fluids, sputum, BALs | CWS binds to fungal elements and fluoresces under ultraviolet light; allows visualization sensitive visualizing fungal elements | Screening tool which detects fungal elements; does not inform about genus or species | Rapid, 40 min |
| Cultures | Skin, nail, hair, body fluids, tissue, swabs, sputum, blood | The specimen is placed on culture media | Tool to diagnose fungal infections, to perform species identification and antifungal susceptibility testing | Days to weeks |

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# Specimens applied for diagnosing fungal infections

| Assay | Specimens | Goal | Results | Time |
|---|---|---|---|---|
| Antifungal susceptibility testing | Fungal cultures | Follow-up to fungal culture; susceptibility testing may be ordered to guide antifungal therapy | To guide antifungal treatment, to monitor fungal epidemiology | 3–4 days |
| Antigen testing | Blood, urine, CSF, body fluids | Detects antigens associated with specific fungi, a variety of tests is available | To diagnose and monitor infection | Rapid |
| Antibody testing | Blood, CSF, other body fluids | Detects immune response to a specific fungus; may be ordered on a single sample or on acute and convalescent samples collected 2–3 weeks apart | Diagnose current or recent infection by specific fungus; to monitor treatment | Days |
| Molecular tests for DNA and RNA | Fungus isolated in culture, or blood, CSF, body fluids, tissue | Detects genetic material of a specific fungus | Detects fungal DNA or RNA; not yet widely available, not yet validated, preferable in research settings | Days |

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# A Little Fungal ABC

- The microscopic presence of fungal elements in tissue is indicative of a fungal infection.

- Microscopy does not permit identifying the fungal species, but may provide information whether yeasts or moulds are present;

- In some cases an etiologic diagnosis may be possible,
  - e.g., for *Histoplasma capsulatum , Blastomyces dermatitidis , Coccidioides immitis/posadasii , Paracoccidioides brasiliensis , Pneumocystis jirovecii , Penicillium marneffei , or Sporothrix schenckii.*

- *Because the presence of* fungi in tissue is diagnostic for proven infection, biopsy should be attempted whenever possible

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# Cultures

- Cultures represent the gold standard in diagnosing fungal diseases

- Positive cultures must be interpreted with caution, as the clinical significance depends on the type of fungus detected and the type of specimen investigated.

  (a) For non-sterile sites, such as the skin, fungi identified may be disease-causing (pathogenic) or may be part of the normal skin flora; a mixture may be present.

  (b) Sterile samples, such as blood or tissues that are properly collected will not be contaminated with normal flora, and a positive culture identifies the causing pathogen

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# A Little Fungal ABC…

- A positive antigen test is suspicious for the presence of fungal disease, however, at least two positive results should be obtained.
- A positive antibody test of a single blood sample indicates exposure to a specific fungus, but does not indicate whether the exposure occurred recently or in the past.
- A rise in the level of antibody-titer between two serum samples collected over 2 or 3 weeks indicates an active fungal infection.
- Individuals with weakened immune systems may not produce antibodies as expected.

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# A Little Fungal ABC…

- Several molecular tests are not validated yet; a positive signal is indicative of a likely infection when the test was performed on a sterile body specimen such as blood or tissue biopsy .

- Some molecular tools have been developed for the detection and identification of fungi, but several assays need to overcome major limitations, as

  (a) tests are not commercialized,

  (b) restricted in their availability,

  (c) lacking clinical validation,

  (d) hardly tested in prospective clinical studies,

  (e) applied only on pure cultures,

  (f) showing cross-reactivity, and

  (g) displaying a limited spectrum of species detection

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# Serological Tests

- Noninvasive diagnostic tests include the detection of fungal cell wall components and antibodies.
- However, the detection of serum antibodies is ineffective, as immunocompromised patients lack specific antibody response.

| Method | Indication | Advantages | Disadvantages |
|---|---|---|---|
| Galactomannan (GM) Lateral flow assay | Early detection of invasive aspergillosis<br>2 Serum samples/ week<br>Positive cutoff index > 0.5<br>BAL, positive cutoff index > 0.5<br>Detection of invasive aspergillosis<br>Immunochromato-graphic dipstick assay for the qualitative and semiquantitative detection of *Aspergillus* antigen | A screening test to accompany conventional diagnostic methods in patients at high risk of IA:<br>For neutropenic adults and children<br>Consistent serum value > 1 is a sign of therapeutic failure<br>Easy to handle, sensitivity and specificity is similar to GM tests | No good data for non-neutropenic patients<br>Mould-active antifungal drugs have an impact on sensitivity<br>Persistent GM antigenemia during therapy is a poor prognostic sign and should prompt a reassessment<br>In some cases the stick is not easy to read, hence lack of result |

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# Serological Tests

| Method | Indication | Advantages | Disadvantages |
|---|---|---|---|
| Mannan plus anti-mannan | Candidemia | Good sensitivity and specificity when combined in ICU patients<br>Early diagnosis prior to blood culture results<br>ESCMID Diagnostic & Management Guideline for Candida Diseases 2012 recommend this test-combination because of high negative PV | Limited experience<br>Non-mycological criterion<br>The sensitivity and specificity were 87.5 and 85.5% for (1 → 3)-β-D-glucan and 89.3 and 63.0% for mannan antigen plus anti-mannan antibody<br>*C. parapsilosis* and *C. guilliermondii* fungemias were not detected by the Platelia *Candida* Ag Plus assay |
| Molecular methods (polymerase chain reaction; PCR)<br>Most experience of in-house tests | DNA detection mainly of *Aspergillus* species less experience for Candida species<br>Suitable for blood, BAL, tissue | Early diagnosis (rapid techniques) with a high NPV<br>High sensitivity (multicopy genes), capacity for rapid speciation and ability to quantitate fungal burden<br>Low burden of organisms during bloodstream infections: <10 CFU/mL (in 25% <1 CFU/mL) and intermittent nature of candidemia due to hepatic clearance of fungal cells and/or periodic release of cells from deep organ sites into circulation | Non-mycological<br>Limited to reference laboratories (low availability)<br>High costs, improve technical equipment<br>Technical difficulties of efficient fungal DNA extraction from complex clinical samples |

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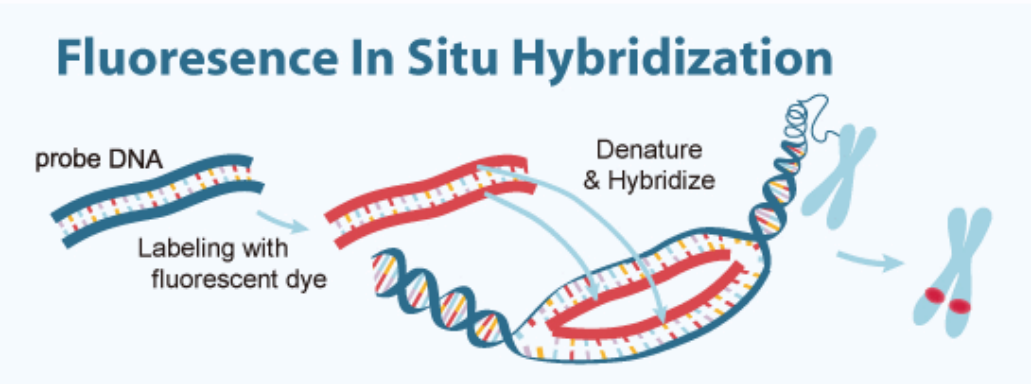
# Molecular Detection of Fungi in Clinical Specimens

## Fluorescence *In Situ* Hybridization ( FISH ) Test

- The FISH method uses PNA probes for the identifi cation of C. albicans directly from positive blood culture.
- The C. albicans PNA FISH test (Advandx) is based on a fl uorescein-labeled PNA probe that targets C. albicans 26S rRNA.
- The FISH results are unaffected by the type of blood culture system or broth formulation.
- This approach may provide a time saving of 24–48 h, compared with conventional laboratory identification methods used

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# Polymerase Chain Reaction (PCR)

- Detection of fungal DNA by means of PCR has been studied in detail for candidemia and invasive aspergillosis.

- The potential usefulness of DNA detection is evident, given that many opportunistic fungal pathogens grow slowly or are difficult to isolate.

- Clinical specimens include serum or whole blood for *Candida and Aspergillus species,* and respiratory samples
  (e.g., sputum, BAL fluid or tissue)
  for invasive aspergillosis.


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# Overview of commercially PCR test assays available

| Name | Affigene *Aspergillus* tracer | MycAssay *Aspergillus* | *Aspergillus* spp. Q-PCR alert kit | MycoReal *Aspergillus* |
|---|---|---|---|---|
| Company | Cepheid | Myconostica | Nanogen | Inogenetix |
| IVD accredited | Yes | Yes | Yes | No |
| Detection | Genus *Aspergillus* | Genus *Aspergillus* | Genus *Aspergillus* | *A. fumigatus, A. terreus, A. flavus, A. niger, A. nidulans*, and others |
| Species identification | No | No | No | Yes |
| Sample materials | Full blood, serum, plasma | BAL, sputum | BAL, sputum | Blood, liquor, BAL, puncture specimen, tissues, paraffin |
| DNA extraction kits | No kit/protocol | MycXtra | EXTRAcell | Protocol |
| Technology | Scorpions (FAM) amplification graphs | Molecular Beacons (FAM) amplification graphs | TaqMan-MGB (FAM) amplification graphs | HybProbes (LC640, 705) amplification and melting graphs |
| Target | 18S-rRNA-gene | 18S-rRNA-gene | 18S-rRNA-gene | ITS-region |
| Internal control | Plasmid (ROX) | Plasmid (HEX) | Beta-globin-gene (VIC) | Plasmid (LC610) |
| PCR-plattform | Mx3000P and Mx300P iQ and iQ5 Rotor-Gene 3000 | Light Cycler 2.0 ABI 7500, smart cycler MX3000 | ABI 7500 | Light Cycler 2.0 |
| Analysis | Automatically | Automatically | Manually | Manually |
| Sensitivity | 0.54 Genome equivalents/µl | 1.3 Copies of genome/PCR | 10 Copies of target/PCR | 3CFU/PCR |
| Specificity | Cross reaction *(Penicillium)* | Cross reaction *(Penicillium)* | Cross reaction *(Penicillium)* | Specific for *Aspergillus* |

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# PCR and GM antigen testing

- PCR and GM antigen testing has recently been shown to improve sensitivity and specificity, when compared to either test alone.
- A systematic review and meta-analysis was done on the use of PCR tests for the diagnosis of invasive aspergillosis (IA).
- Data from more than 10,000 blood, serum, or plasma samples obtained from 1618 patients at risk for IA were retrieved from 16 studies.
- A single PCR-negative result is thus sufficient to exclude diagnosis of proven or probable IA.
- However, two positive tests are required to confirm the diagnosis because the specificity is higher than attained from a single positive test.

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# PCR and GM antigen testing

- Efforts are underway to devise a standard for *Aspergillus PCR for screening which will help*
  - enabling formal validation of the PCR, and
  - identify patients who are most likely to benefit from its implementation.

- For diagnosis of pneumocystis pneumoniae, PCR may be helpful, and is more sensitive tha staining methods

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# Summary

## Diagnostic performance based on fungal diseases present

| Fungal diseases | Diagnostic performance | | | | | |
|---|---|---|---|---|---|---|
| | Microscopy | Culture | Xray/CT scan | Antigen | Antibody | DNA detection |
| *Candida* bloodstream | – | +++ | + | + | + | +++ |
| Cryptococcal meningitis | ++ | +++ | + | +++ | – | – |
| Invasive (pulmonary) aspergillosis | + | + | +++ | ++ | – | ++ |
| Chronic aspergillosis | + | + | +++ | – | +++ | ++ |
| Allergic aspergillosis | + | + | ++ | – | +++ | + |
| Coccidioidomycosis | + | ++ | ++ | – | +++ | – |
| Histoplasmosis | + | ++ | + | ++ | – | – |
| Mucormycosis | +++ | + | ++[a] | – | – | – |
| *Pneumocystis jirovecii* | +++ | – | ++ | – | – | ++ |
| Unknown fungi in various specimens | +++ | +++ | – | – | – | –[b] |

[a]Usually no differentiation between *Aspergillus* and other moulds
[b]A panfungal PCR may help in fungus identification

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# Conclusion

- A precise mycological diagnosis is only possible with the help of laboratory testing.
- In previous years, only a positive culture could reliably define a fungal infection.
- With currently available improved diagnostics, confirmation of the cause of fungal infection is possible by serological and molecular testing.
- Conventional microbiological and microscopic techniques remain the cornerstone of diagnosis but lack sensitivity.
- Combined implementation of other available tools is therefore mandatory for proper diagnosis
- DNA and RNA-based methods hold promise for improved sensitivity and specificity, but these methods will require extensive validation in clinical studies

# Thank You