MMTN
MEDICAL MYCOLOGY TRAINING NETWORK

Laboratory diagnosis of fungal infections

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ASIA FUNGAL WORKING GROUP
an ISHAM working group

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Laboratory Diagnosis of Fungal Infection

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Introduction

Clinically important mycoses

- Skin and mucosal infections: ~billion
- Chronic severe (including NTDs) :
  - —Chronic pulmonary aspergillosis
  - —Mycetoma (NTD)
  - —Chromoblastomycosis (NTD)
  - —Sporotrichosis (NTD)
- Acute invasive
  - —Candidiasis
  - —Aspergillosis
  - —Cryptococcosis
  - —Mucomycosis
  - — Pneumocystis jirovecii pneumonia
  - —Teleromycosis marneffei

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Prevalence of fungal diseases-estimate precision

>150 million people have serious fungal diseases

~3,000,000 cases of chronic pulmonary aspergillosis
~223,100 cases of cryptococcal meningitis complicating HIV/AIDS
~700,000 cases of invasive candidiasis
~500,000 cases of PJP
~250,000 casesof invasive aspergillosis

Number of deaths from fungal disease:

>1.6million≈tuberculosis

Bongomin F, Gago S, Oladele RO, et al. Global and multi-national prevalence of fungal diseases —estimate precision. J Fungi, 2017, 3(4): E57

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Main determinants on incidence and prevalence of fungal disease

- **Socio-economic, geo-ecological characteristics ;**
- **Increasing number of at-risk populations :**

—AIDS
—Tuberculosis
—COPD
—Asthma
—Cancers
—Organ transplantation
—Corticosteroid therapy

Bongomin F, Gago S, Oladele RO, et al. Global and multi-national prevalence of fungal diseases —estimate precision. J Fungi, 2017, 3(4): E57

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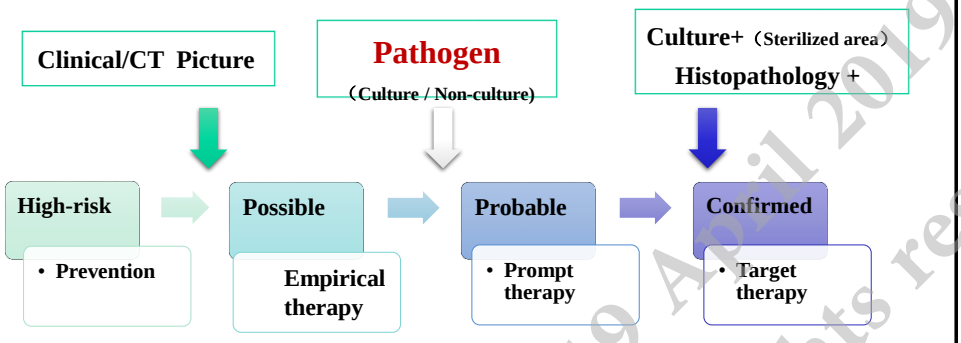
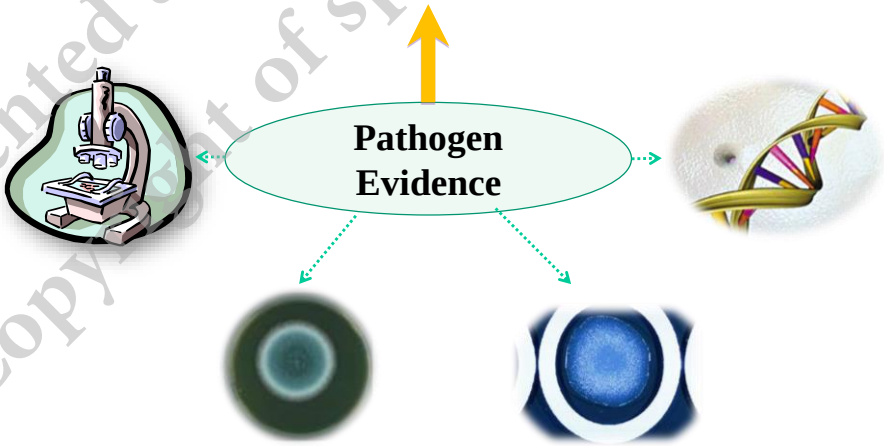
Diagnostic & Therapeutic Strategies of IFD



Ben, et al. Clin Infect Dis. 2008; 46:1813–21

Early diagnosis is the key to reduce the death!

Key of the Diagnosis



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Microbiological evidence-confirmed diagnosis

Microscopic exam.

- ☐Sterile area ☐Pathology

Culture：

- Positive in sterile area ：
- ☐CSF ☐Tissues ☐ Extract
- ☐Blood（Yeast、*Fusarium* etc., but not *Aspergillus*）

Antigen detection: Cryptococcus antigen

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Microbiological evidence-Probable Diagnosis

Microscopic：

- Filamentous fungi positive in either:
- ☐Sputum ☐BAL ☐Brancho-brush ☐Sinus extract

Culture：

- Filamentous fungi positive in either ：
- ☐ Sputum ☐BAL ☐Brancho-brush ☐Sinus extract
- Antigen detection：
- G test (Serum)
- GM test (Plasma、Serum、BALF、CSF）

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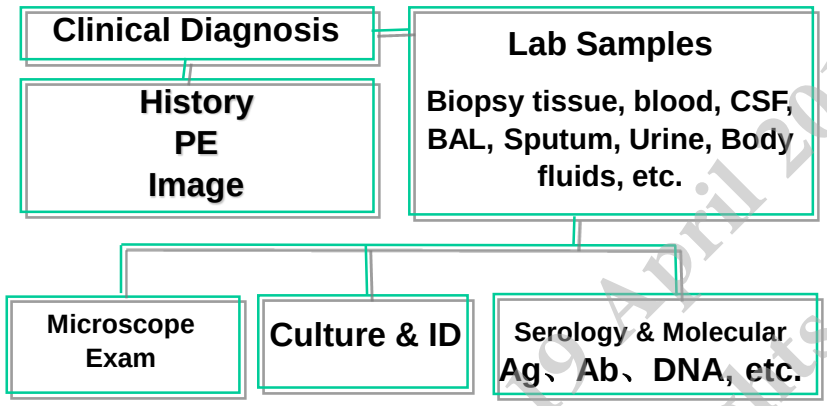
Diagnosis of Fungal Infection



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Mycological Diagnosis

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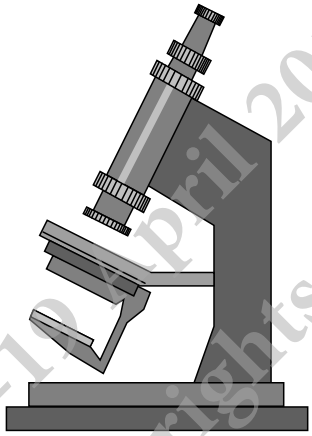
Direct Microscopic Examination

- KOH
- NS
- Calcofluor white (Fluorescent)
- India (Chinese) ink
- Gram
- Giemsa
- Histopathology



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Materials

- Sputum
- Bronchoalveolar lavage
- Blood cultures
- Bone marrow
- Tissues and surgical material
- Cerebrospinal fluid
- Urine
- Fluids :
  - Peritoneal
  - Pleural
  - Pericardic
  - Ascitic fluid
  - Joint fluids

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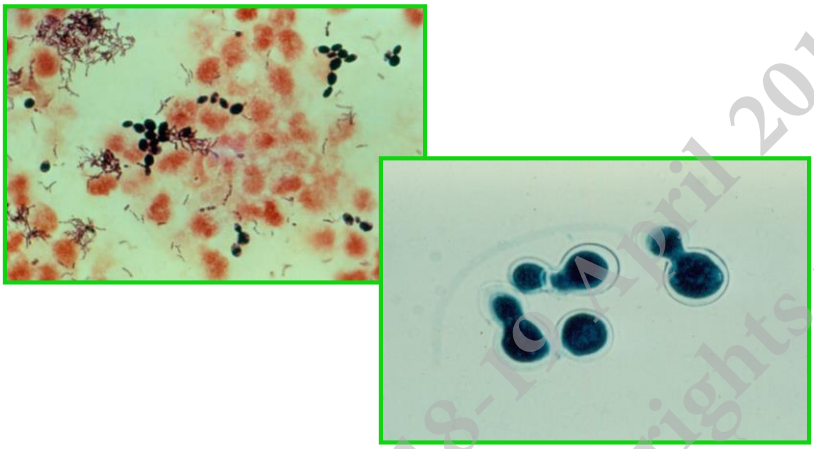
Direct Examination-Yeast cell



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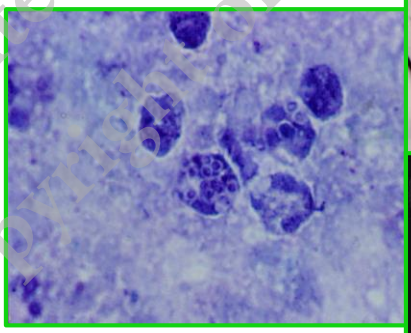
Teleromycosis marneffei



intracellular yeast cell

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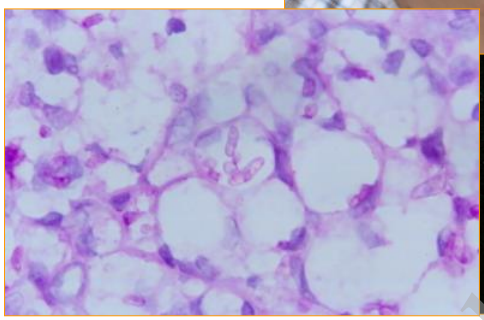
Teleromycosis marneffei



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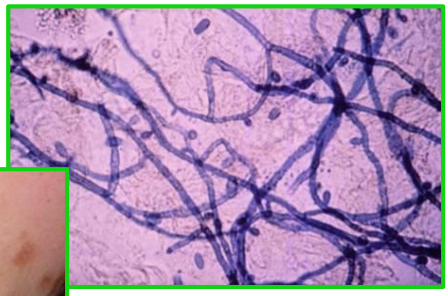
Candidiasis-direct examination



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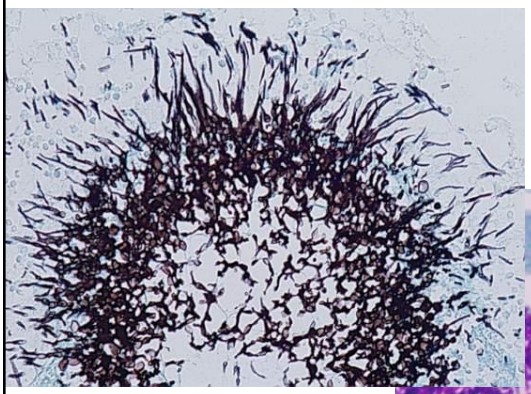
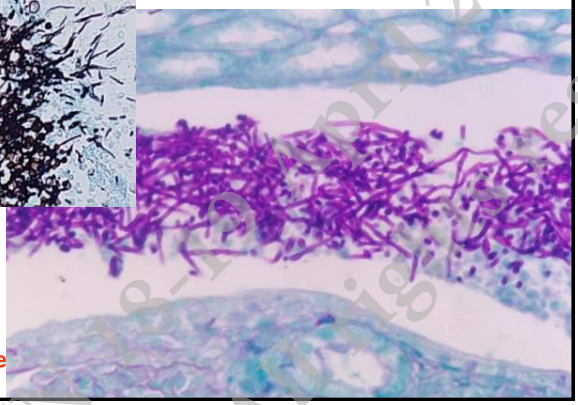
Histopathology



Candidiasis



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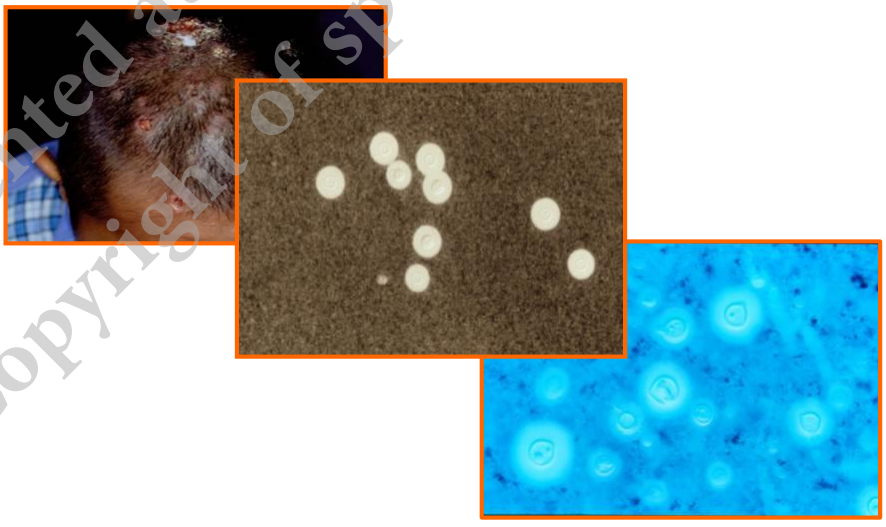
Cryptococcosis-direct examination



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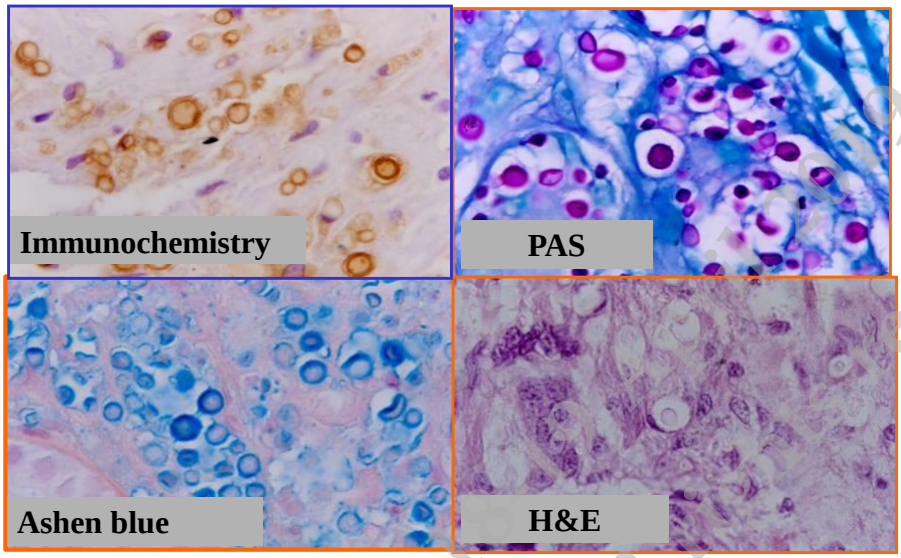
Cryptococcosis-histopathology



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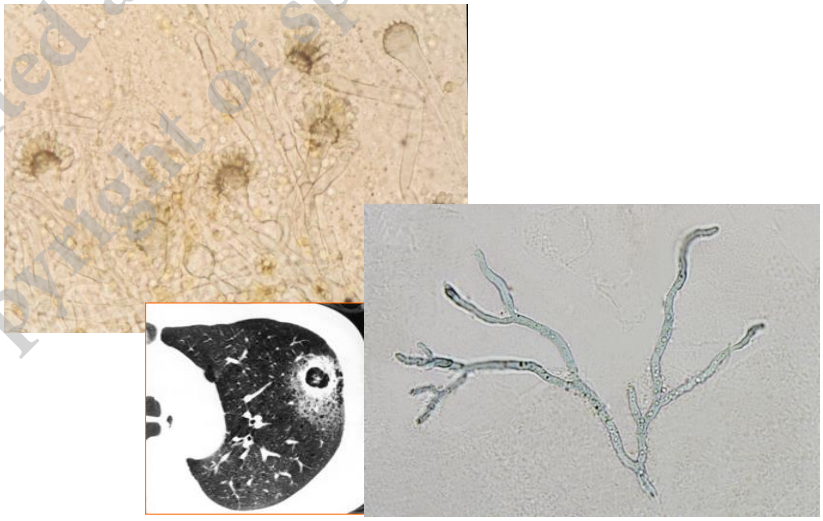
Aspergillosis-direct examination



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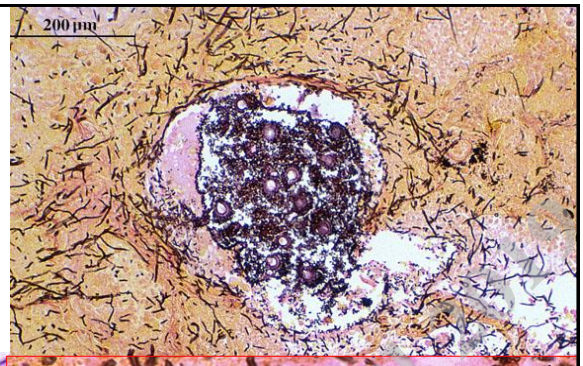
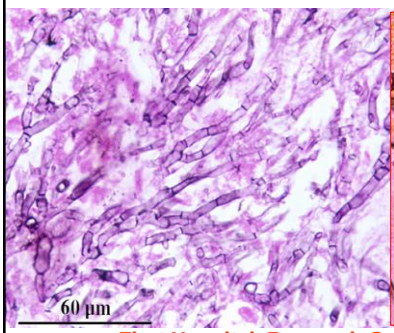
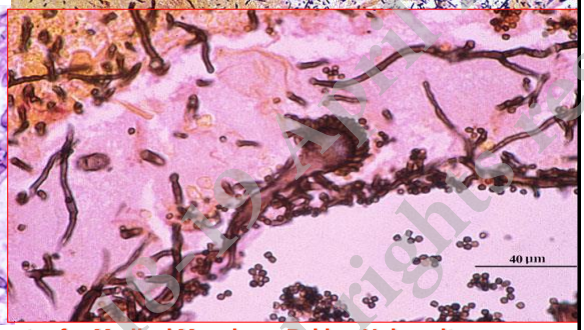
Aspergillosis-histopathology







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Mucomycosis-direct examination

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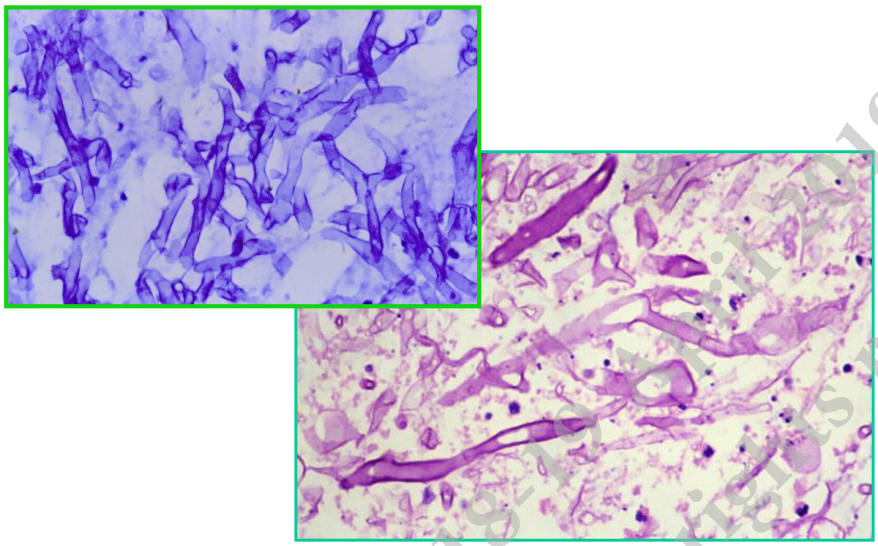
Mucomycosis-histopathology



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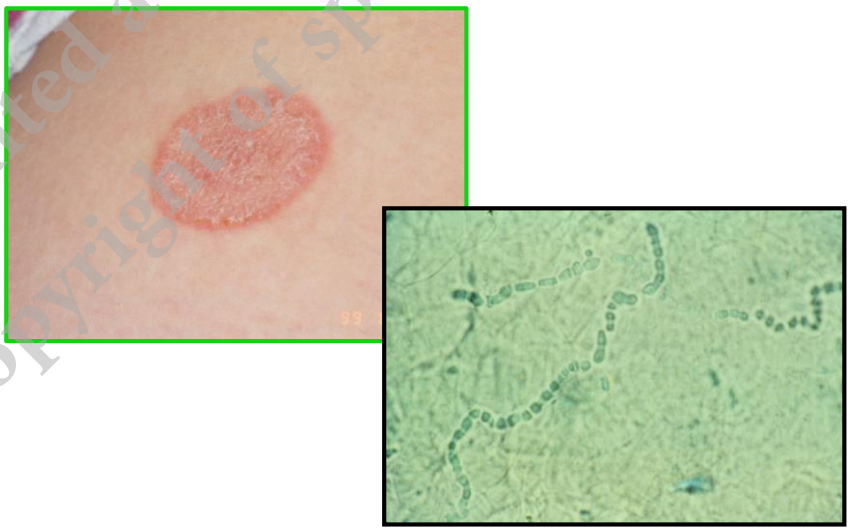
Dermatophytosis-direct examination



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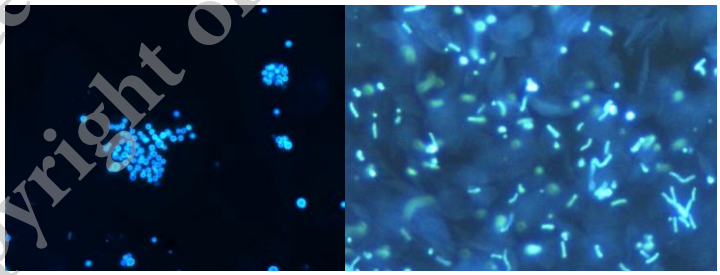
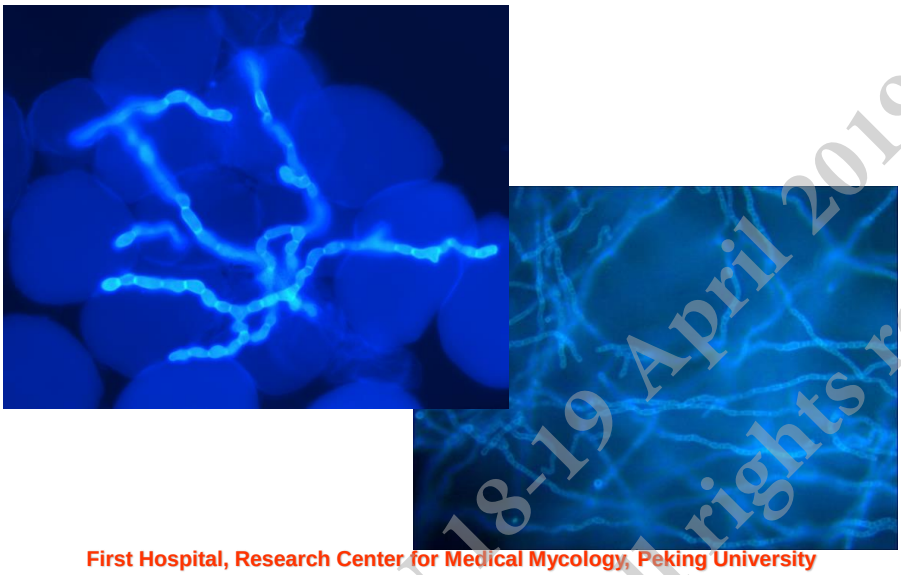
CFW (Florescent) Staining

- Binding with polysaccharides on the chitin ring to develop fluorescence
- Combined with KOH dissolution
- Easier to find fungal elements
- Show the fungal structure clearly

Calcofluor white stain of *Malassezia*



Calcofluor white stain of dermatophytes



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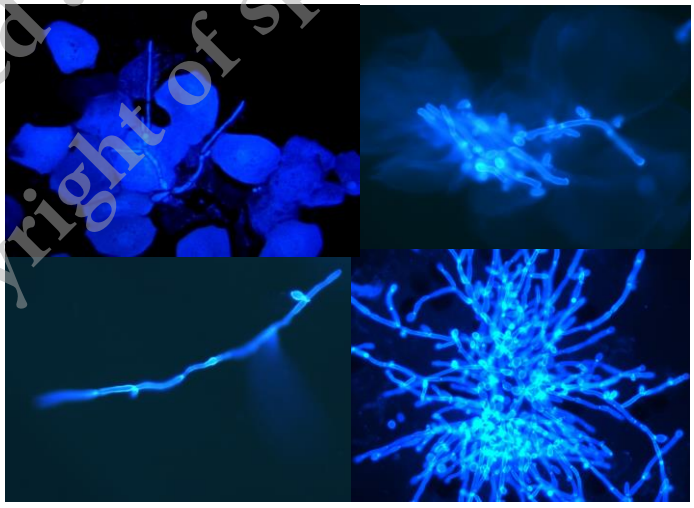
CFW staining of *Candida* species



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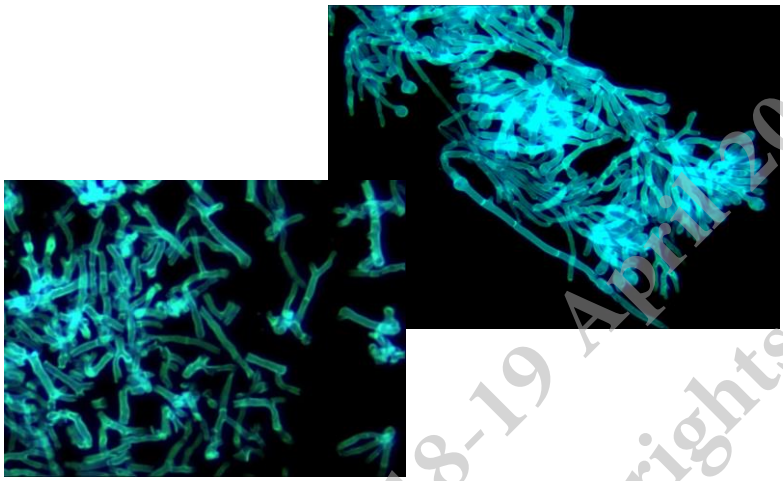
CFW staining of *Aspergillus* species



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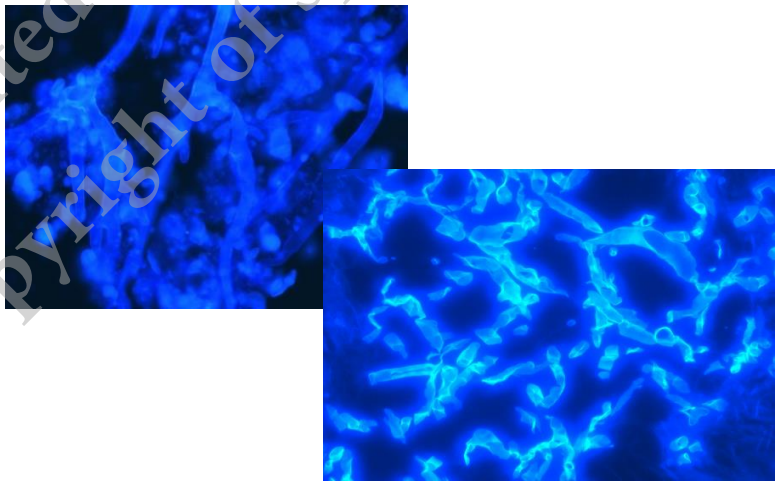
CFW staining of *Mucor* species



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Summary of direct examination

- Yeast cells: yeast;
- Yeast cells and pseudohyphae: *Candida* spp.;
- Yeast cells with capsules: *Cryptococcus* spp.;
- Transparent septate hyphae, about 45˚ branches: *Aspergillus* spp.;
- Transparent, non-septate hyphae, wide, about 90˚ branches: *Mucor* spp.;
- Brown or black hyphae or conidia: dematiaceous fungi

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Different culture media

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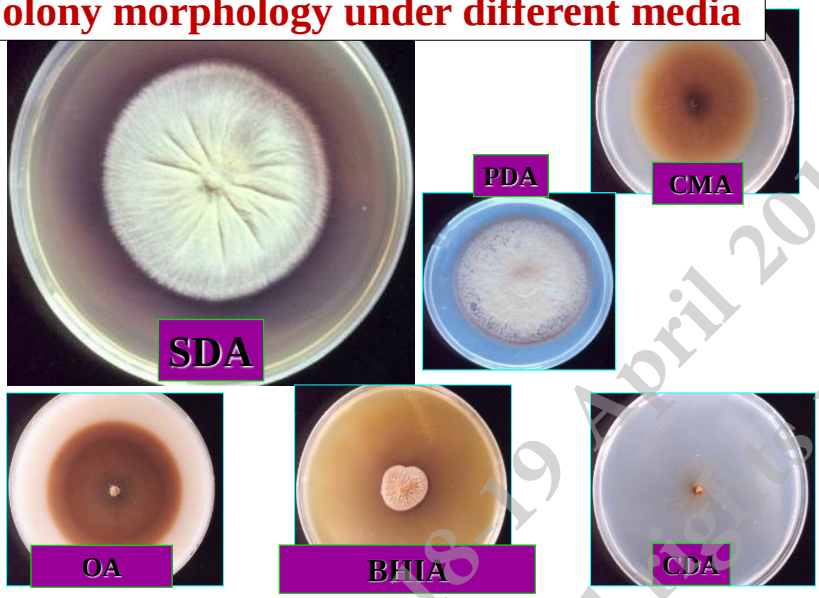
Colony morphology under different media



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Explanation of the Culture Results

- ***Cryptococcus* spp., *P.marneffei*: confirmed diagnosis;**
- ***Candida* spp., *Aspergillus* spp.: combine with clinical and other reference;**
- **Sterile area, blood and CSF: confirmed diagnosis;**
- **Pus, sputum or urine: carefully explanation, repeat the culture if necessary;**
- **Combination the direct exam and culture result is very important.**

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Which isolates need to identify into species level

- Isolates from the sterile area, blood, CSF, body fluid, BAL etc.;
- Isolates from ICU, burn pts., organ transplantation pts.,
- Exact ID is necessary to help the selection

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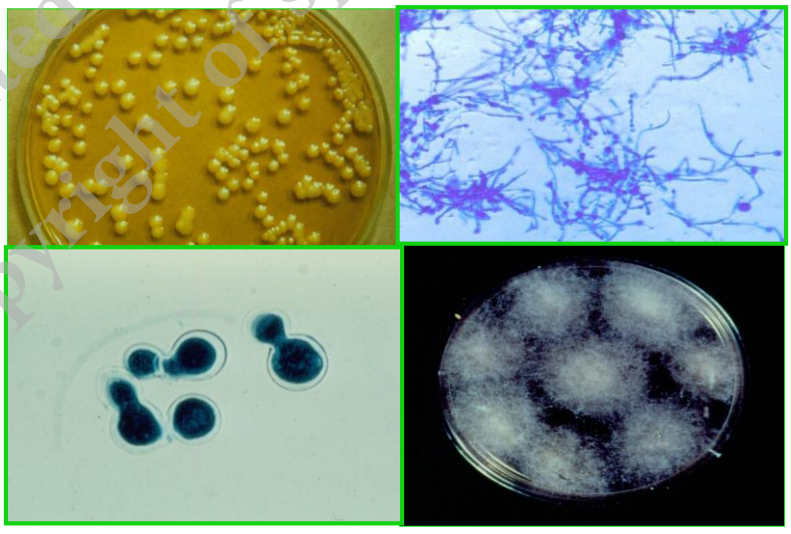
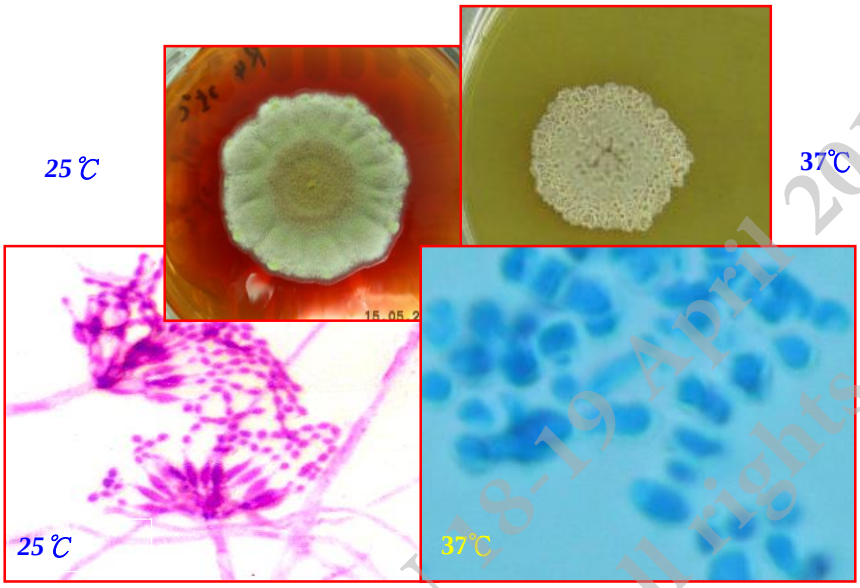
Fungal ID-Differentiate yeast or mold



Dimorphic fungi: *T. marneffei*



Principle of Yeast identification

- "Yeast" is not a formal taxa, but is a widespread form of growth in ascomycetes and basidiomycetes;
- Identification needs to combine morphological, physiological and biochemical characteristics as well as molecular and mass spectrometry characteristics;

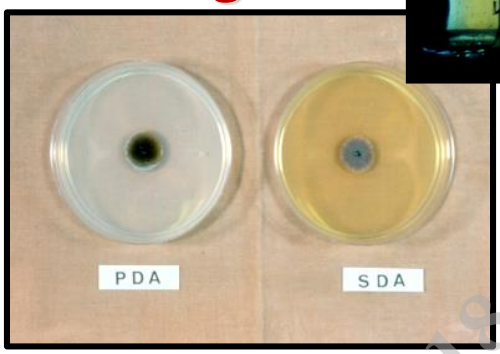
Identification of molds

- Most molds can be identified by culture;
  -colony form, surface color, and growth rate
  -culture medium, temperature, etc.
- Transmitted culture to low nutrient medium to promote sporulation
  -PDA and CMA
- The characteristic of condiogenesis under the microscope was observed by the technique of slide culture

Culture ID-Morphology

- **The ID were determined by the morphology of colonies, microscopic characteristic of fungal sporulation;**
- **Try to identify under the microscope;**
- **Need to see sporulation (needle picking, tape or slide culture)**
- **Stimulate sporulation media: PDA and CMA**

Dematiaceous fungi



Red pigment

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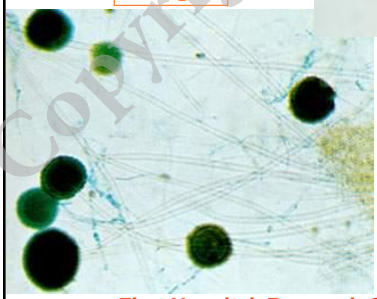
Three common pathogenic *Aspergillus* spp.

A. niger



A.flavus

A.fumigatus

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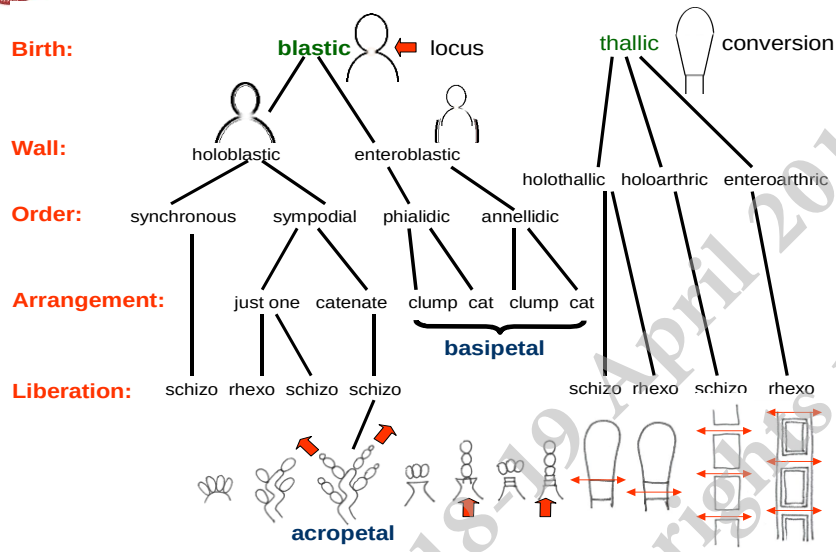
Conidiogenesis



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Culture ID-biochemical method

- CHROMagar Candida(48hr)
- API 20C AUX(48-72hr)
- RapID Yeast Plus System(4-5hr)
- Automatic system
  - ID 32C strip system(24-48hr)
  - Vitek Yeast Biochemical Card system
  - Vitek 2 ID-YST card system (24hr)
  - Quantum II
  - Biolog YT MicroPlate system (Biolog, USA)

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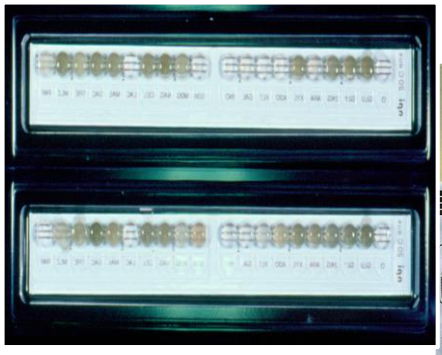
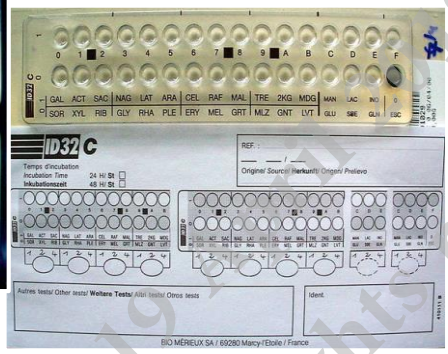
Biochemical ID



API 20C AUX



ID 32C

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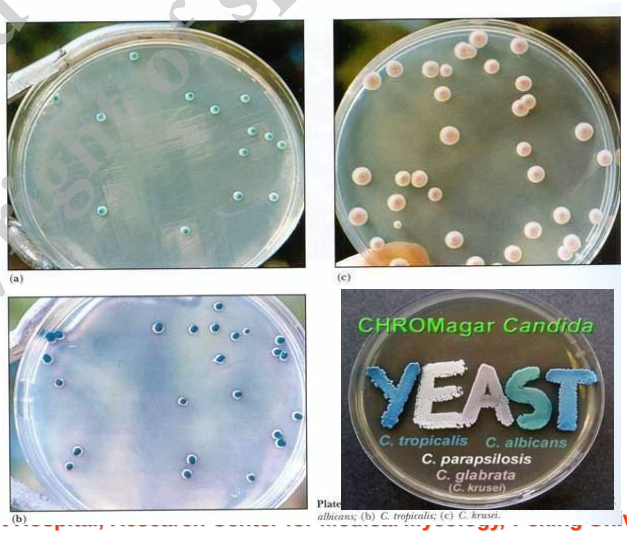
CHROMagar Candida



albicans; (b) C. tropicalis; (c) C. krusei.

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Vitek 2 ID-YST card system

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Culture ID—Molecular Way

- **PCR-EIA**
- **RLB, AFLP, SSCP, RAPD**
- **Gene Chips**
- **PNA-FISH (fluorescein-labeled peptide nucleic acid fluorescent in-situ hybridization)**
  - **26S rRNA, *Candida albicans, C.glabrata***
  - **sensitivity :100%, specificity: 100%**

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Common gene target for molecular ID

- rRNA(rDNA)gene
  - -Small Subunit (SSU) rDNA (18S)
  - -Large Subunit (LSU) rDNA (26S, 28S)
  - -Internal Transcribed Spacer (ITS)
  - -Intergenic Spacer (IGS)

Others:

- elongation factor
- ß-tubulin
- Calmodulin

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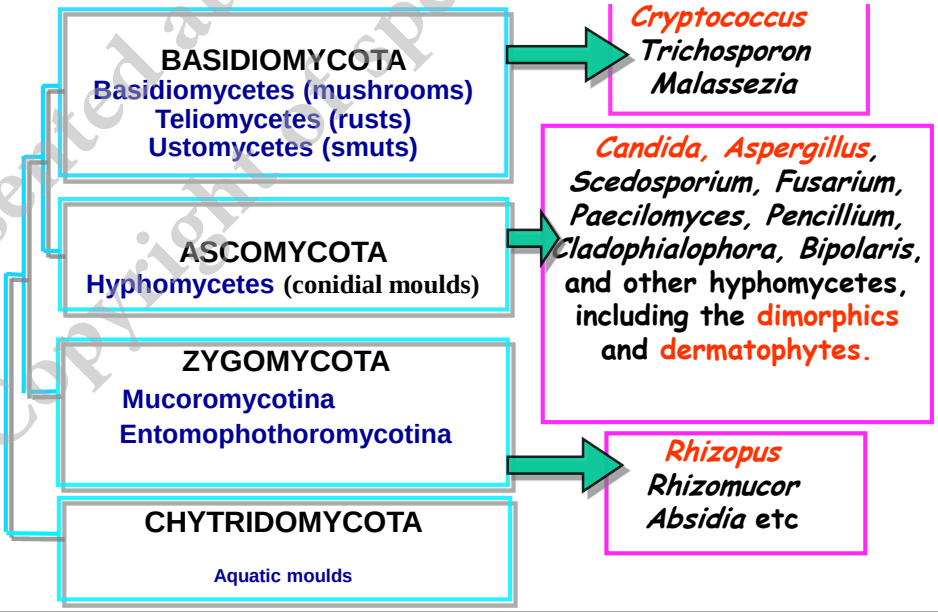
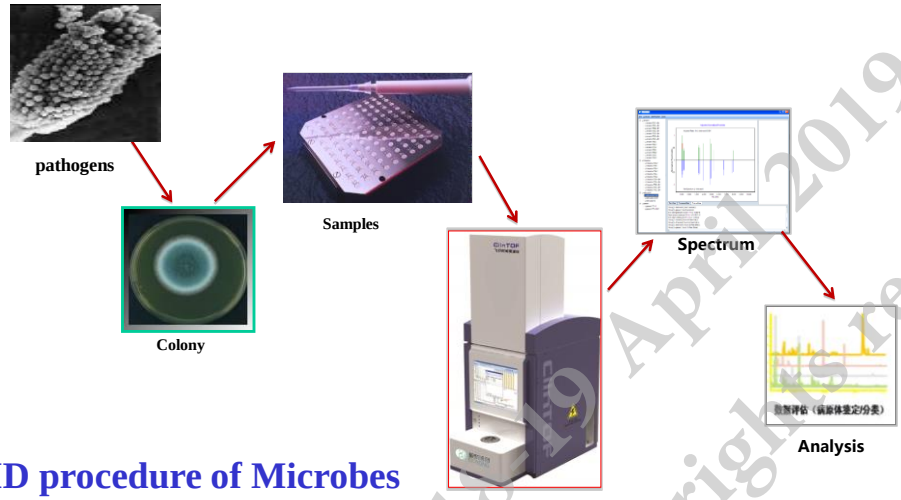
Classification of pathogenic fungi based on 18S-rRNA



MALDI-TOF MS



ID procedure of Microbes

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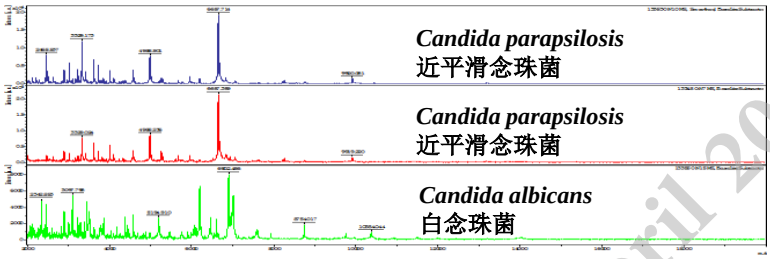
MALDI-TOF MS and other techniques

	Methods	Time	Cost (Yuan/sample)	Difficulties	Throughout
Conventional method	Phenotypic	7-14d	200	Complicated	low
	rDNA	1-2d	80	Complicated	low
	DNA-DNA hybridization	1-2d	1000	Complicated	5/day
	Lipid acid analysis	1-2h	1000	Special trainning	10/day
MALDI-TOF MS		3-5min	4	Simple, automatically	400/day

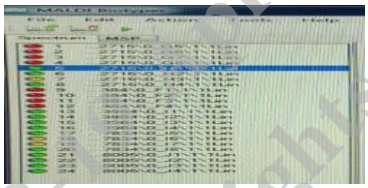
"Proteomic phenotyping is revolutionizing diagnostic mycology as fully reflecting species/morph varieties but often overcoming taxonomic hindrance."

Chierico FD *et al.* *J of Proteomics* 2012;75:3314-30.

MALDI-TOF ID of *Candida* spp.



分值范围	说 明	符号	颜色
2.300 ... 3.000	highly probable species identification 完全可靠地鉴定到种的水平	(+++)	绿色
2.000 ... 2.299	secure genus identification, probable species identification 鉴定到种的水平	(++)	绿色
1.700 ... 1.999	probable genus identification 鉴定到属的水平	(+)	黄色
0.000 ... 1.699	not reliable identification 没有可信的鉴定结果	(-)	红色



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Non-culture diagnosis

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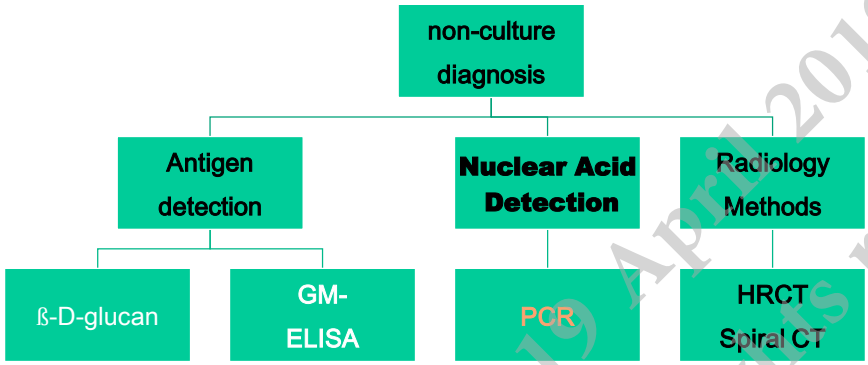
Non-culture Diagnostic Methods



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Comparation of Different Methods

	G test	GM test	LA test
Pathogen	Can. Asp. Pneumocystis, etc.	Asp.	Crypto.
Methods	Agglutination	ELISA	Latax
Samples	plasma	serum	serum、CSF
Time	4hrs.	6hrs.	30mins.
Sensitivity	60-90%	71%	90-100%
Specificity	60-90%	89%	≈100%

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The advantage of serology diagnosis

- Fast report
- Increased sensitivity
- Easy to get sample
- The examined makers are usually diagnostic
- Could be used for the evaluation of severity and treatment effect

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Cryptococcus antigen detection -agglutination test

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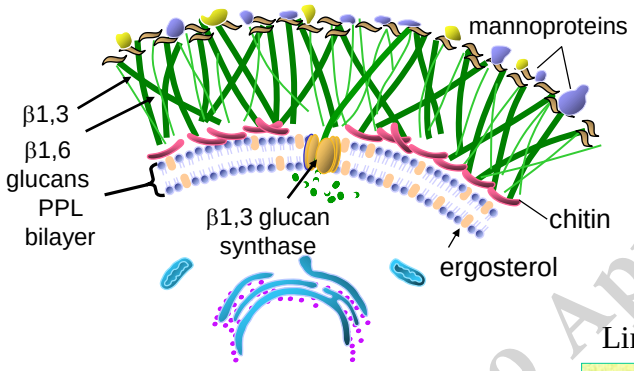
β -1,3-D-glucan



Limulus-G

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β-1,3-D-Glucan: Indication

- **Not only for *Candida* infection**
  - ***Aspergillus*、*Fusarium* and other molds (Miyazaki, J Clin Micro 33:3115, '95)**
  - ***Cryptococcus* negative (a-glucan in CW)**
- **Positive result could not confirm the fungal species**
- **May be reduced by glucanase within 5 days, the monitor period should be 2/week**

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GM test：EIA （Platelia）

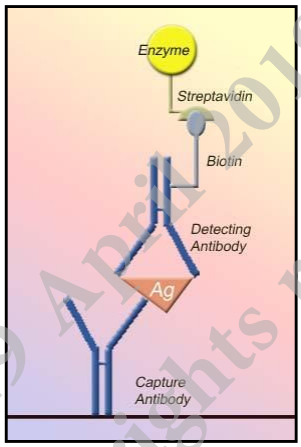
Detecte galactomannan,（GM）

3. Detection – avidin-enzyme + substrate

2. Detecting Ab – anti-galactomannan [biotin conjugated]

Ag – blood, urine, BAL

1. Capture antibody – monoclonal anti - galactomannan



Specificity = >90%　sensitivity = 75%

Summary of GM test

- **Screening for GM = aid for the early (pre-emptive?) diagnosis of IA in prolonged neutropenic patients:**
  - **Cutoff = 0.5**
  - **Frequency: 2x/week**
  - **Caveats**
- **Serum、BALF、CSF samples**
- **GM + CT: high predictive value**
- **GM + ß-D-glucan/PCR: increased specificity and sensitivity**

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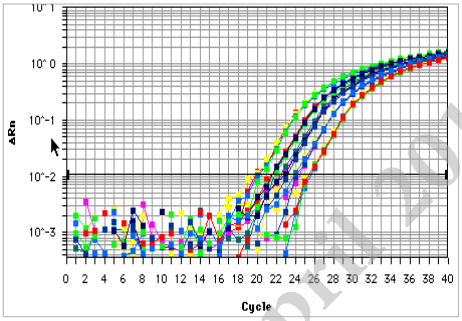
Real-time PCR for detecting *Candida* spp.



Design
- **Species selection**
- **DNA target selection**
- **Primers design**
- **Species/groups specific probe design**

Set up of multiplex real-time PCR

Clinical material
- **DNA extraction method**

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Thank you for your attention !