sektion für hygiene und medizinische mikrobiologie

Meet-the-Expert Session „How to best diagnose fungal infections“

Conventional methods

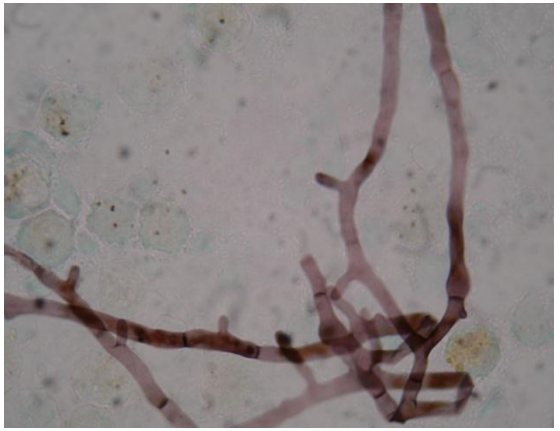
Cornelia Lass-Flörl

Laboratory methods used for diagnosis of invasive fungal infection

- Conventional microbiologic methods
  - Direct microscopy (Gram, Giemsa, and KOH/calcofluor stains)
  - Culture
  - Identification
  - Susceptibility testing
- Histopathologic methods
  - Conventional microscopy
  - Direct immunofluorescence
  - In situ hybridization
- Immunologic and biochemical methods
  - Histoplasma antigen test
  - Cryptococcal antigen test
  - Galactomannan test
  - (1→3)β-D-glucan test
- Molecular methods
  - Direct detection
  - Identification

Alexander BD, Pfaller MA 2006) Clin Infect Dis. 43:S15-S27

Spectrum of opportunistic fungal pathogens: Rising awarness!



[Bauder et al, Med Myc, 2000. 38: 249-253]

[Nucci et al, Clin Micro Rev 2005, Garbino et al, Trans Int 2005, Jossi et al, IJID 2010]

It is now clear that there are no truly nonpathogenic fungi and that virutally any fungus can cause a lethal mycosis in a sufficiently immunocompromised host.

Lass-Flörl C, Mycoses 2011

Conventional methods for the laboratory diagnosis of fungal infections

1. Identification of patients who are at the highest risk for fungal infection so that they can be monitored more closely;

2. Development of laboratory methods that will provide evidence of infection earlier and more reliably than has been possible historically.

Alexander BD , Pfaller MA (2006) Clin Infect Dis. 43:S15-S27

Direct microscopy

- Direct microscopic examination is a crucial first-line procedure in detecting the presence of fungal elements.
- It is a rapid, useful and cost-effective means of diagnosing fungal infections.
- Microscopy can usually yield preliminary information that a yeast or mould is present.
- Is a “must” for sterile specimens and BALs obtained from immunocompromised patients.

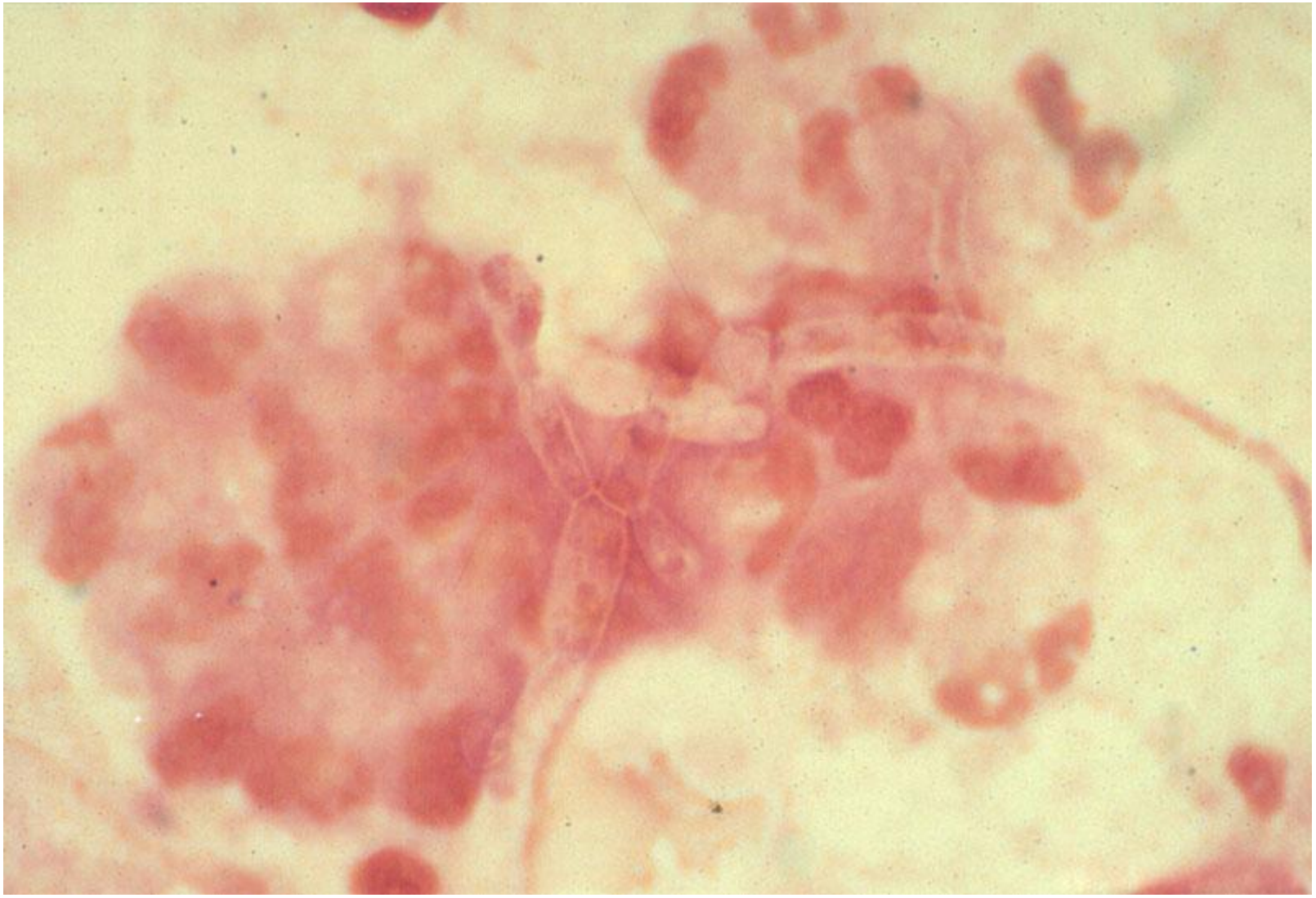
Lass-Flörl C, Mycoses 2011

Characteristic microscopic features of opportunistic and pathogenic fungi in clinical specimens

Fungi	Microscopic morphologic features in clinical specimens
Candida species	Oval budding yeasts 2–6 μm in diameter. Pseudohyphae and true hyphae may be present.
Cryptococcus neoformans	Spherical budding yeasts of variable size, 2–15 μm in diameter. Capsule may be present or absent. No hyphae or pseudohyphae.
Trichosporon species	Hyaline arthroconidia, blastoconidia, and pseudohyphae, 2–4 by 8 μm in size.
Malassezia species	Small oval budding yeasts. "Bowling pin" appearance with collarette. Both hyphal and yeast forms may be seen in skin scrapings.
Aspergillus species	Hyaline, septate, dichotomously branched hyphae of uniform width (3–6 μm). Conidial heads may be seen in cavitary lesions.
Other hyaline Hyphomycetes[a]	Hyaline, septate, dichotomously branching hyphae. Angioinvasion is common. Adventitious conidiation may be present. May be indistinguishable from *Aspergillus* species.
Zygomycetes	Broad, thin-walled, pausi-septate hyphae, 6–25 μm wide, with nonparallel sides and random branches.
Dematiaceous fungi	Pigmented (brown, tan, or black) hyphae, 2–6 μm wide. May be branched or unbranched. Often constricted at the point of septation.
Histoplasma capsulatum	Small (2–4 μm in diameter), intracellular, budding yeasts.
Coccidioides immitis/posadasii	Spherical, thick-walled spherules, 20–30 μm in diameter. Mature spherules contain small (2–5 μm in diameter) endospores. Released endospores may be mistaken for yeast. Arthroconidia and hyphae may form in cavitary lesions.
Blastomyces dermatitidis	Large (8–15 μm in diameter), thick-walled budding yeast cells. The junction between mother and daughter cells is typically broad based. Cells may appear multinucleate.
Sporothrix schenckii	Elongated or "cigar-shaped" yeast cells of varying size (rare). Tissue reaction forms asteroid bodies.
Penicillium marneffei	Oval, intracellular yeast cells bisected with a septum (fission yeast).
Pneumocystis jiroveci (carinii)	Cysts are round, collapsed, or crescent shaped. Trophozoites seen on staining with Giemsa or immunofluorescent stains.

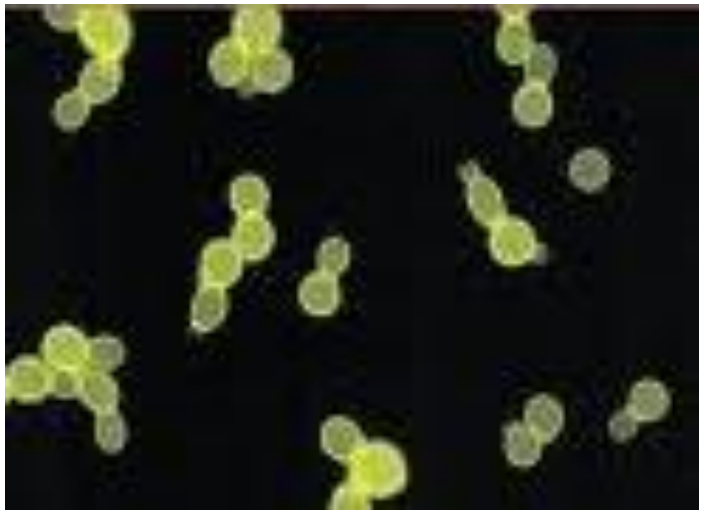
[a] Includes *Fusarium, Scedosporium, Acremonium,* and *Paecilomyces* species.

Alexander BD , Pfaller MA (2006) Clin Infect Dis. 43:S15-S27

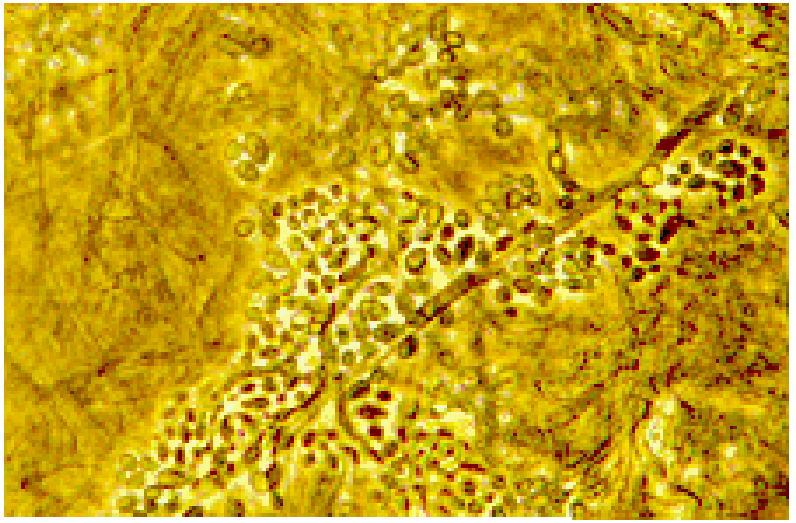


Microscopy/Histology

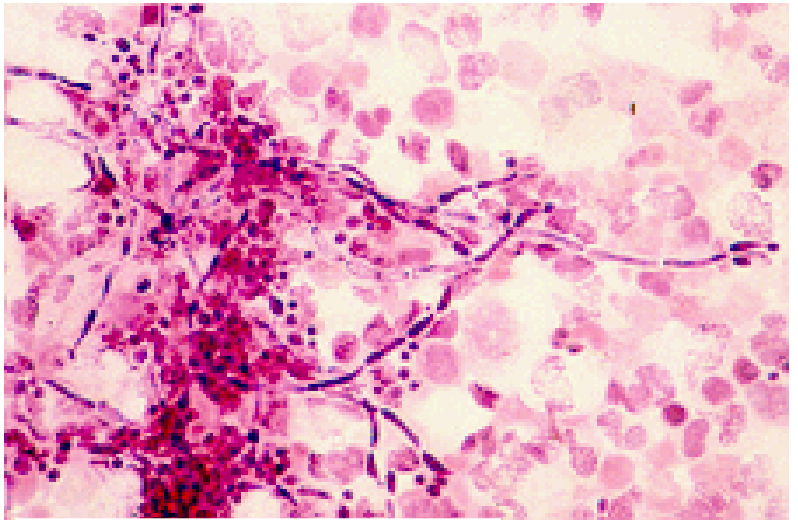
Yeasts/Candida sp.



Calcofluor-white-stain



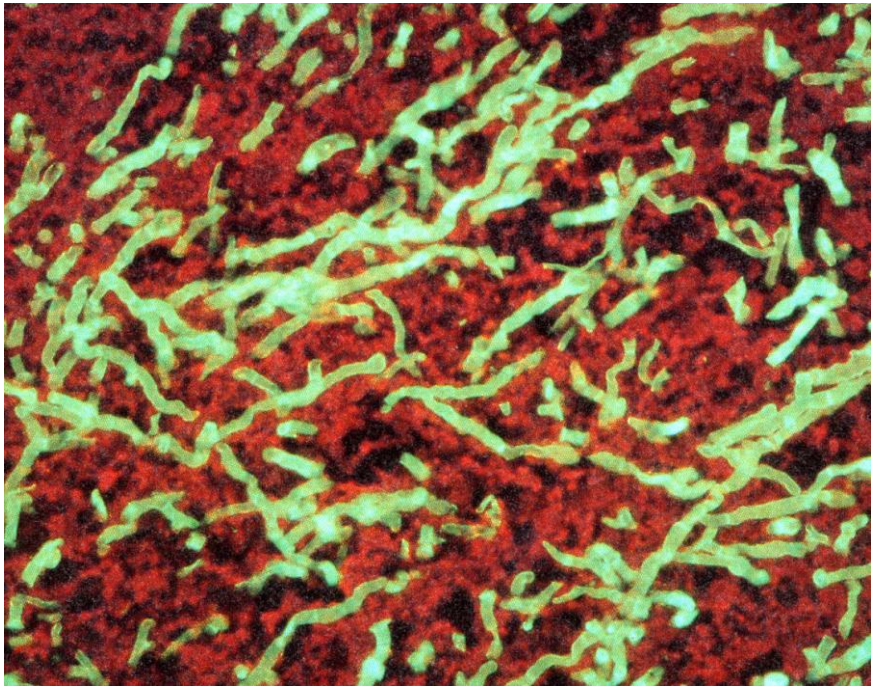
KOH-specimen



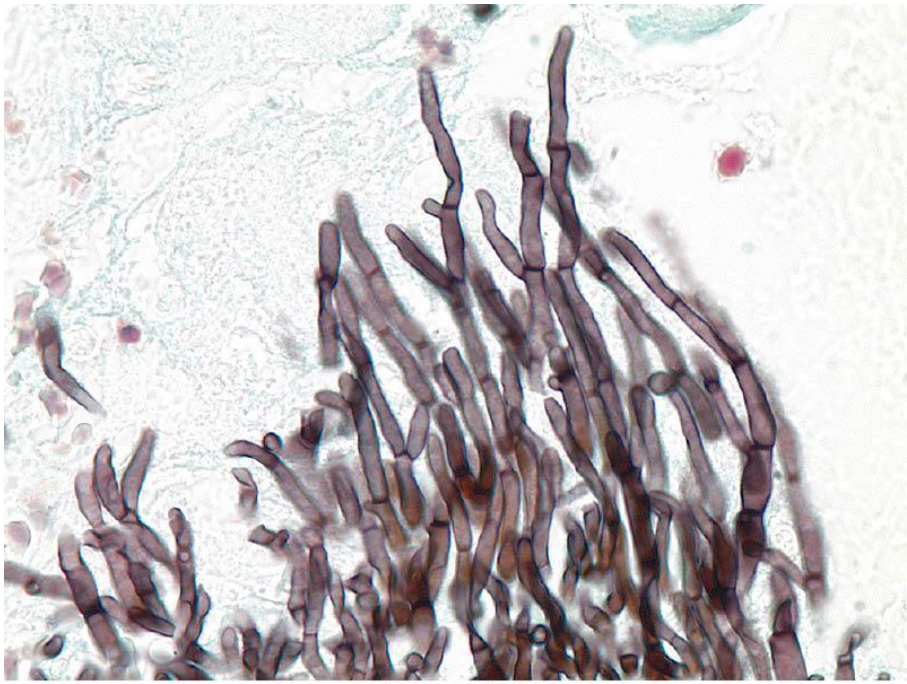
PAS-stain

Microscopy/Histology

Aspergillus like sp.



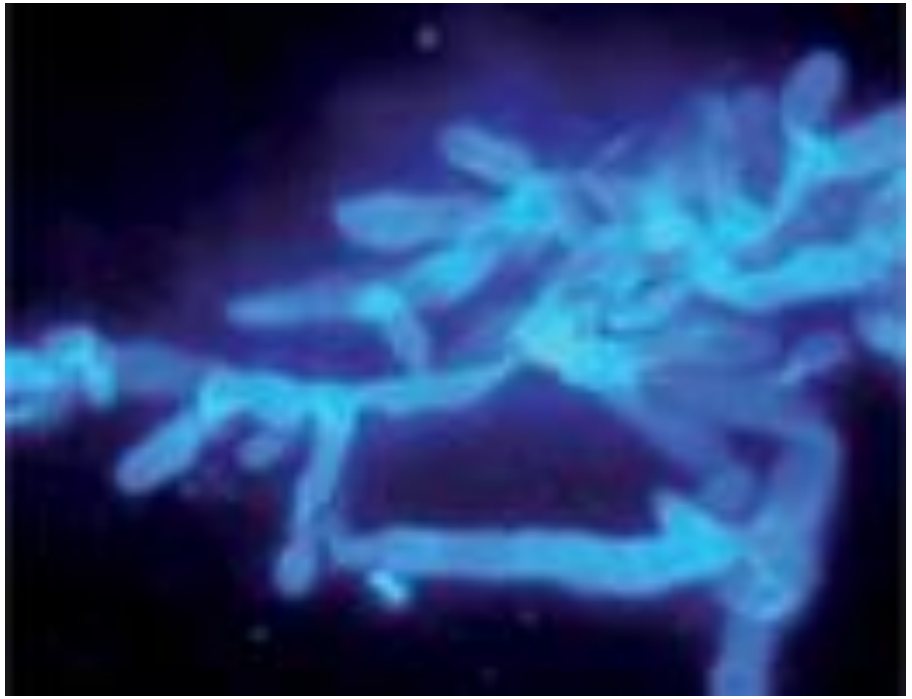
Calcofluor-white-staining



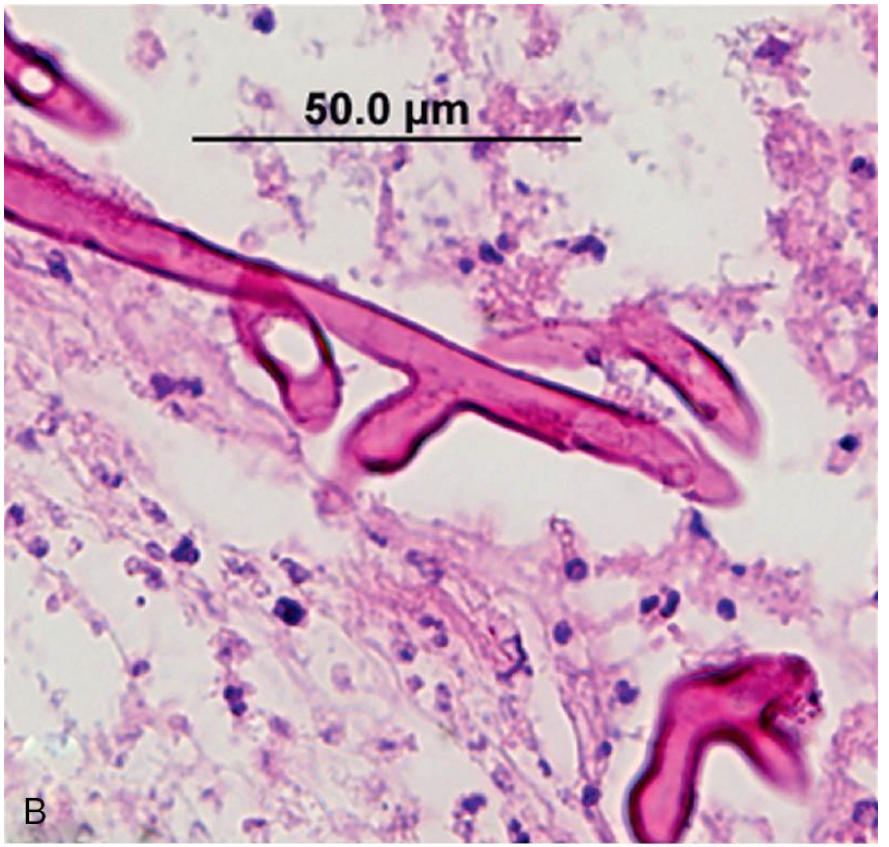
Methenamin-Silber-staining

Microscopy/Histology

Mucomycetes

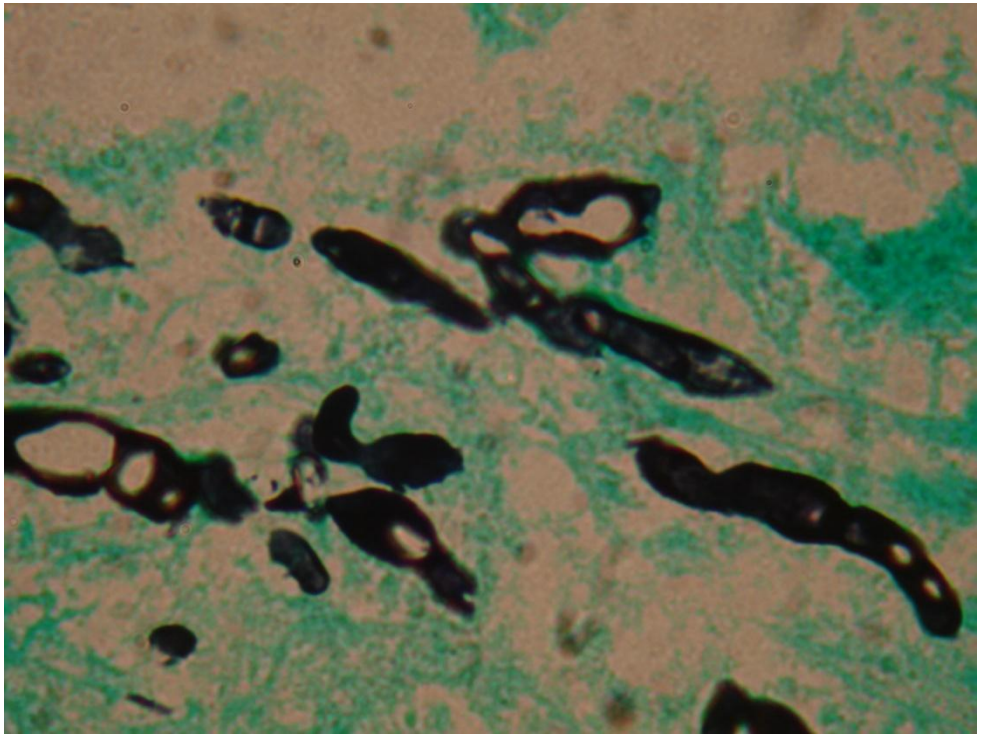


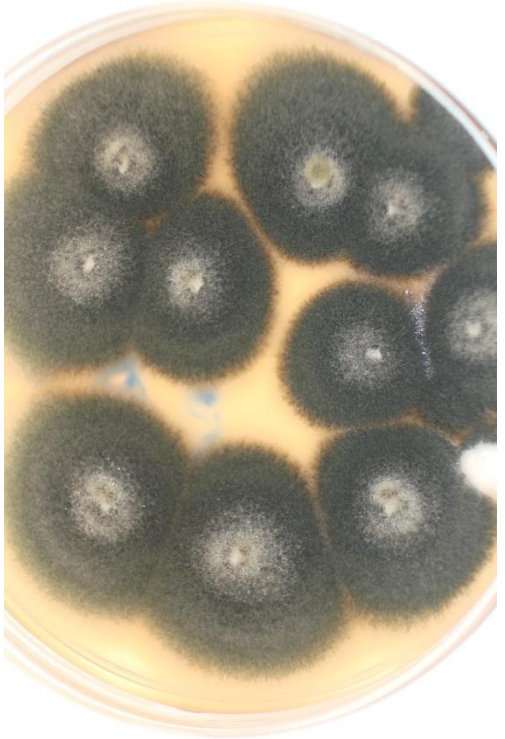
Calcofluor-white-staining



H&E-staining

How do we know it's *Aspergillus*?





***Aspergillus terreus*?**

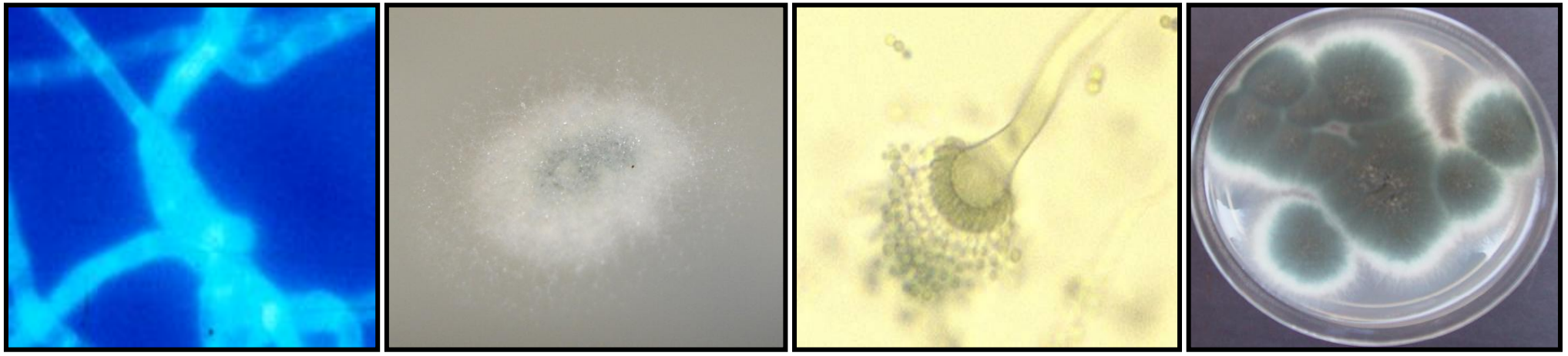
Direct microscopy

However, methods of direct examination might be less sensitive than culture and negative results of direct examination of a clinical specimen never rule out a fungal infection [1].

[1] Merz WG, Roberts GD (2003) Manual of clinical microbiology. Washington DC: Americal Society for Microbiology: 1668-85.

Rapid Identification of
A. fumigatus

±1 hr → 24-48 hours at 35-37° C → 3-7 days



Culture

Fungi have longer generation times than those of most bacteria, which results in slower recovery of the organisms from specimens.

2-10 days

Diagnosing candidaemia

Specimen	Test	Considerations	Remarks/Recommendations
Blood	Blood culture	• Number of blood cultures: 3 (2 to 4) • Total volume: Children <2kg, 2 to 4 mL, between 2 and 12 kg, 6 mL, between 12 and 36 kg, 20 mL and for adults 40 to 60 mL • Timing: Obtain blood cultures, one right after the other, from different sites following the clinical events that precipitated the blood culture • Site: Venipuncture remains the technique of choice. Blood obtained through an indwelling line is twice as likely to yield a contaminant than blood obtained through a properly prepared skin site • Frequency: Daily when candidaemia is suspected • Technique: Validated systems • Incubation time: At least five days • Performance: 50-75% S	• Essential investigation • Separate 20-ml blood samples obtained within a 30- min period, each divided equally between an aerobic and anaerobic blood culture vial in 10-ml aliquots, were considered to represent a single culture • A blood culture set compromising 60 mL blood obtained in a single session and divided in 10 mL aliquots among 3 aerobic and 3 anaerobic bottles • Lower sensitivity in neutropenic patients and under antifungal treatment • Sensitivity varies depending on the species and system (e.g. lower for BACTEC and *C. glabrata*) • ID is mandatory • Caution: Yeast in BC is not always *Candida* • Lysis-centrifugation showed efficacy when older systems of BC were used as comparators

Culture

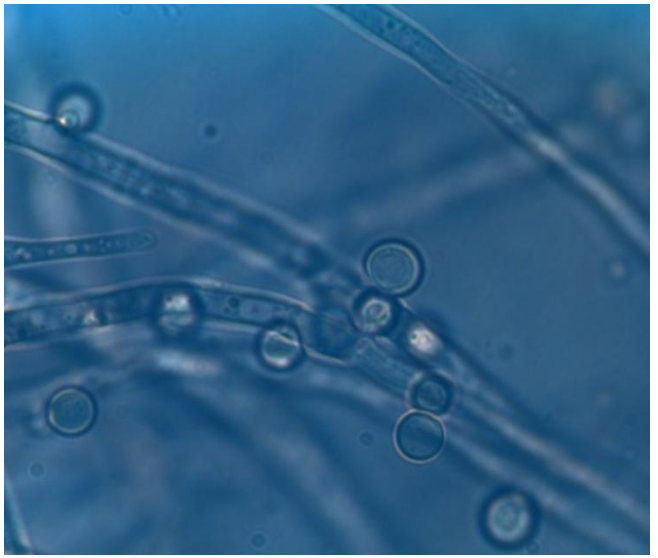
Among the 4 automated, continuous-monitoring blood culture systems that have been developed, the Bactec (Becton-Dickinson) and BacT/Alert (BioMérieux) systems are superior in their capacities for the recovery of yeast from blood.

Alexander BD , Pfaller MA (2006) Clin Infect Dis. 43:S15-S27

Pay attention to positive blood cultures in certain fungi!

Scedosporium prolificans
Fusarium species
Aspergillus terreus
Acremonium species
Paecilomyces species

produce additional forms in tissue/fluids



bloodstream

[Lass-Flörl & Richardson, CMI 2009]

Culture
Can we do better ?

Mimic physiologic temperature and decreased oxygen environment:
35°C, 6% O_2 - 10% CO_2

→ significant increase of *Aspergillus* spp
from autopsy tissue and various clinical samples (+ 31%)

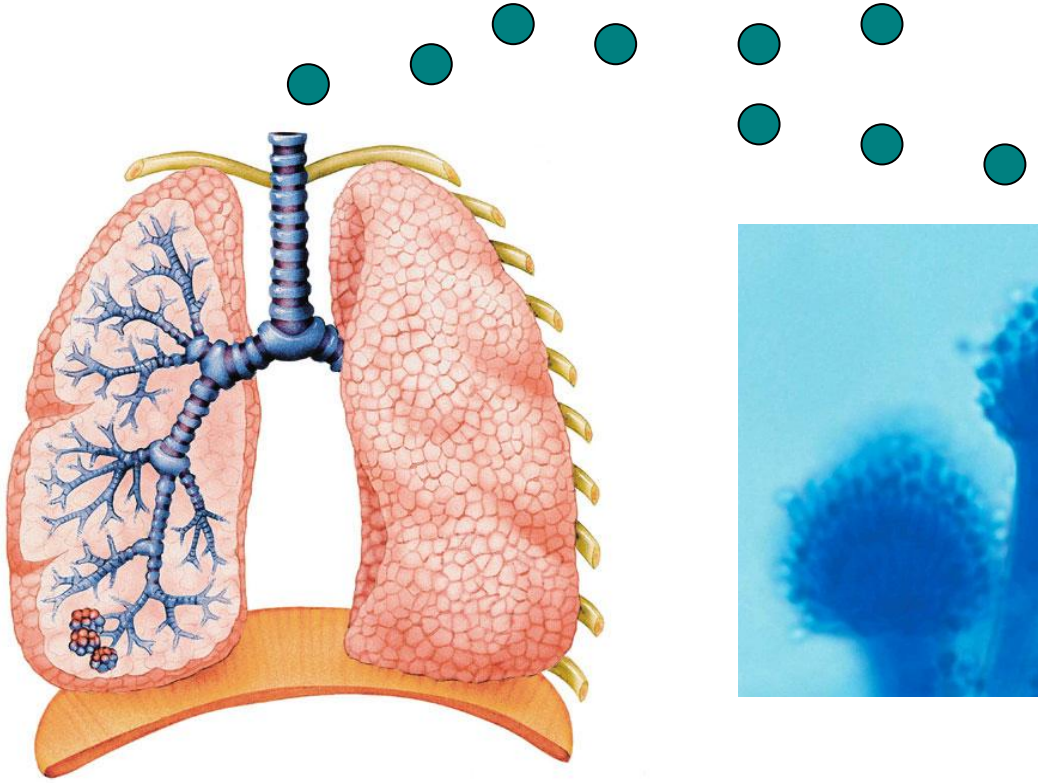
Tarrand JJ et al., *J Clin Microbiol* 2005; 43: 382

Predictive value of culture detection and identification

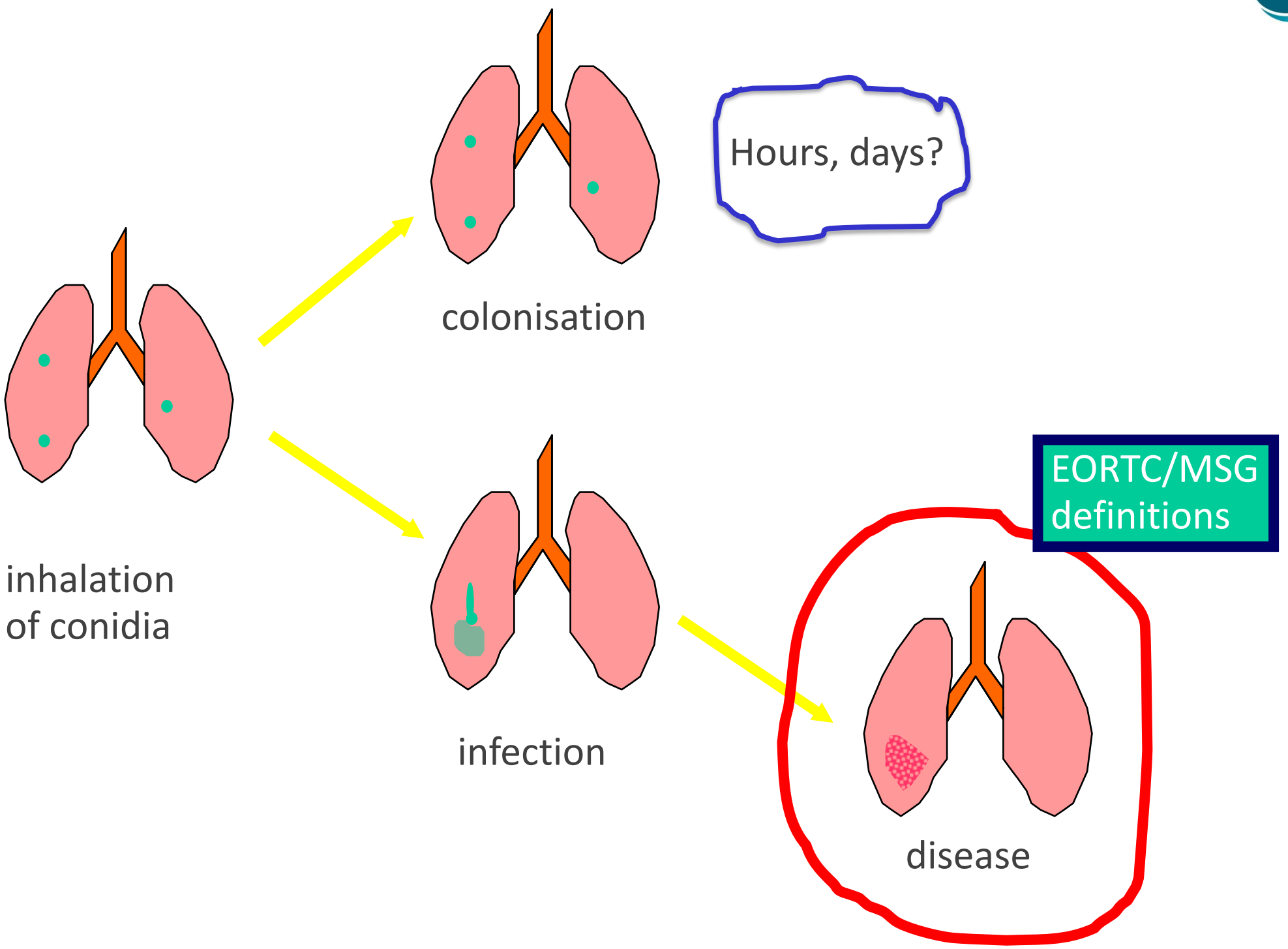
Most isolates of *Candida* species, *C. neoformans*, *H. capsulatum*, and *Fusarium* species obtained from blood cultures are clinically significant, others, such as *Aspergillus* species (not *A. t*erreus) and *Penicillium* species (not *P. marneffei*), are not [1, 2].

Isolation of *Aspergillus* species from cultures of respiratory tract specimens is especially problematic, because this organism is common in the environment and can colonize the respiratory tract of any individual without actually causing disease.

[1] Magadia R, Weinstein MP (2001) Infect Dis Clin North Am 15: 1009-24
[2] Duthie R, Denning DW (1995) Clin Infect Dis 20: 598-605

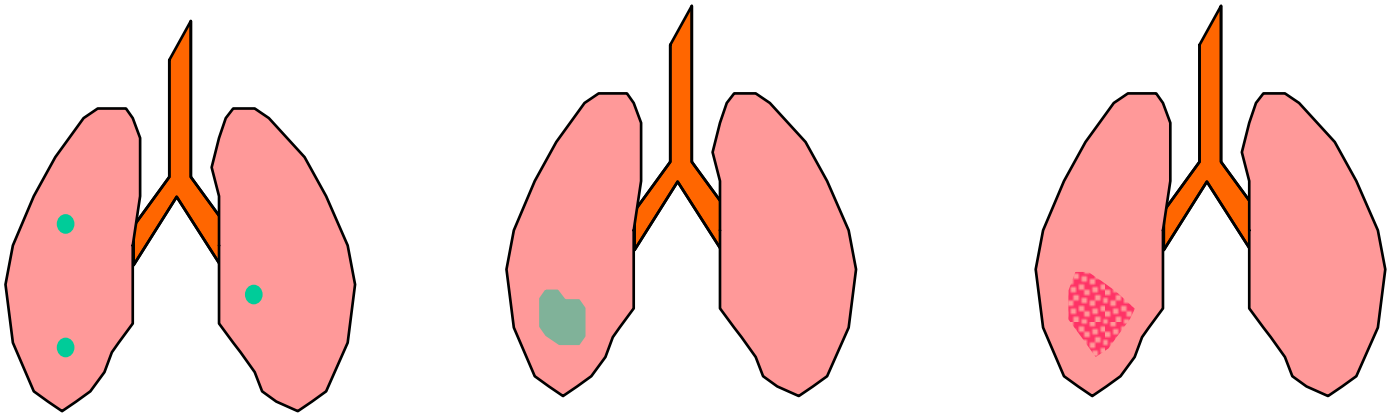


Because fungi are ubiquitous in the environment, their isolation from clinical specimens often represents transient colonization rather than invasive disease.


Hours, days?
colonisation
inhalation
of conidia
infection
EORTC/MSG
definitions
disease

Invasive opportunistic mycoses (IM)

Host: high risk, “sterile” specimen;



	colonisation	infection	disease
Class.:	No IM (?)	IMI	IMI
Culture:	positive	positive	positive
Microscopy:	negative	positive	positive

News

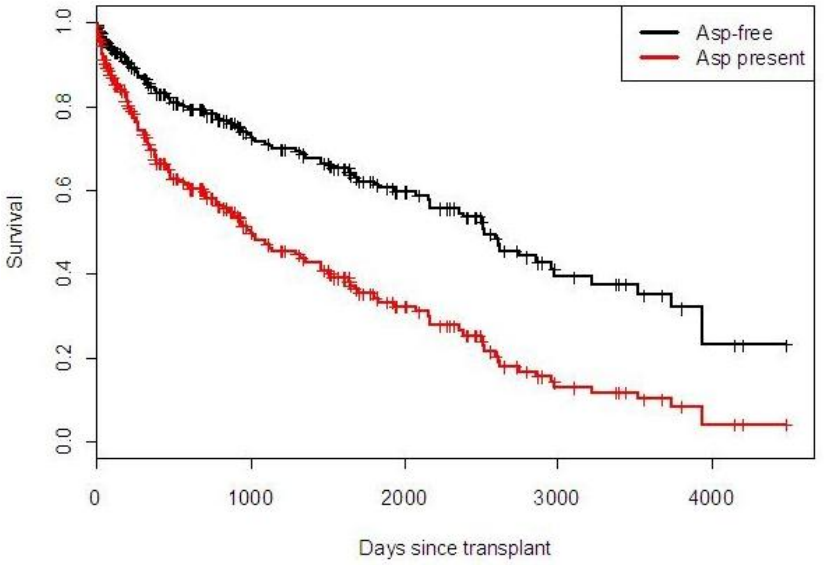
1. Isolation of Aspergillus spp. from patients.
2. Colonization following lung transplantation is associated with a significant increase in mortality.
3. Novel preventative strategies are required to minimize Aspergillus spp.

Goal

Investigation of the relationship between the isolation of Aspergillus spp. from the respiratory tract of lung transplant recipients and their risk of mortality.

Titel/ Author

Isolation of Aspergillus species from the respiratory tract of lung transplant recipients is associated with increased mortality.
Tim Felton et al. (Manchester, UK)



Risk of invasive aspergillosis for patients with cultures positive for Aspergillus species, by study and risk category

Risk category	Study, percentage risk (no. of patients with positive culture/total no. of patients)		
	Yu et al [1]	Horvath et al. [2]	Perfect et a [3]
High[a]	100 (17/17)	72 (34/47)	57 (117/206)
Intermediate[b]	37 (20/54)	58 (14/24)	15 (228/1510)
Low[c]	0 (0/9)	14 (1/7)	<1 (1/155)

[a] Includes allogeneic HSCT recipients, patients with neutropenia and patients with hematologic cancer.
[b] Includes autologous HSCT and solid-organ transplant recipients, patients receiving therapy with corticosteroids, HIV-infected patients and patients with malnutrition, diabetes, underlying pulmonary disease, or solid-organ cancer.
[c] Includes HIV-infected patients, patients with cystic fibrosis or connective tissue disease and other non-immunosuppressed patients

Alexander BD , Pfaller MA (2006) Clin Infect Dis. 43:S15-S27
[1] Yu VL et al. (1986) Am J Med 81: 249-254
[2] Horvath JA, Dummer S (1996) Am J Med 100: 171-178
[3] Perfect JR et al. 2001 Clin Infect Dis 33: 1824-1833

Invasive aspergillosis : update on conventional diagnosis

How to increase the PPV for IA of a positive sputum culture

- **at (high) risk patient**
- **multiple positive samples** [1]
- **quantitative culture** [1, 2]
- **use of a score** [2, 3]

[1] Nalesnik et al., J Clin Microbiol 1980; 11: 370
[2] Greub and Bille, Clin Microbiol Infect 1998; 4: 710
[3] Bouza and Muñoz, J Clin Microbiol 2005; 43: 2075

Invasive aspergillosis : update on conventional diagnosis

Prospective assessment of the clinical significance of isolating *A.fumigatus* by culture

404 *A.fumigatus* positive cultures (260 patients)
90 (22.3%) from 31 (12%) patients with IA

6%	if	1+ cult.
18%		2+
38%		≥3+
10%	if	1-2 score
40%		3-4
70%		≥ 5

Criteria	Score points
Invasive procedure	1
≥ 2 + cultures	1
Leukemia	2
Corticosteroids	2
Neutropenia	**5**

Bouza et al., *J Clin Microbiol* 2005; 43: 2075

Predictive value of culture detection and identification

The specific identification of the fungus isolated from culture specimens can also help in determining clinical significance;
Aspergillus niger is rarely a pathogen, whereas *A. terreus* and *Aspergillus flavus* have been shown to be statistically associated with invasive aspergillosis when isolated from cultures of respiratory tract specimens [1].

[1] Perfect JR et al. (2001) Clin Infect Dis 33: 1824-33

Risk factors for invasive Candidiasis

- ≥3 antibiotics
- Antibiotics ≥4 d
- Time ≥4 d in ICU
- Mechanical ventilation >48
- High APACHE II score
- Abdominal surgery
- CVC
- TPN
- Age

- Neutropenia
- Immunosuppression (chemotherapy, steroids,
  other therapies)
- Concomitant infection
- Diabetes mellitus
- *Candida* colonization ≥2 sites
- Candiduria (>100,000 colonies)

APACHE = Acute Physiology and Chronic Health Evaluation.

Pappas PG et al. *Clin Infect Dis* 2004;38:161-189;
Ostrosky-Zeichner L et al. *Crit Care Med* 2006;34:857-63

Predictive value of culture detection and identification Candida colonization in ICU

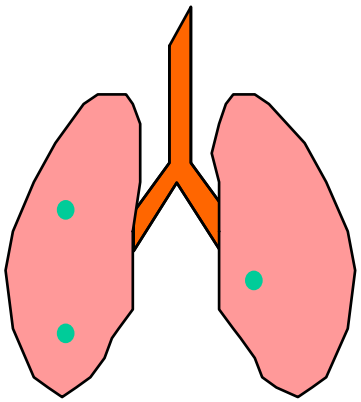
Colonization parameters	Sensitivity	Specificity	PPV	NPV
>2 sites	100%	22%	44%	100%
>2 sites	73%	56%	50%	77%
>3 sites	45%	72%	50%	68%
CCI*	100%	69%	66%	100%
cCCI**	100%	100%	100%	100%

* CCI = Candida colonization index;
** cCCI = corrected Candida colonization index

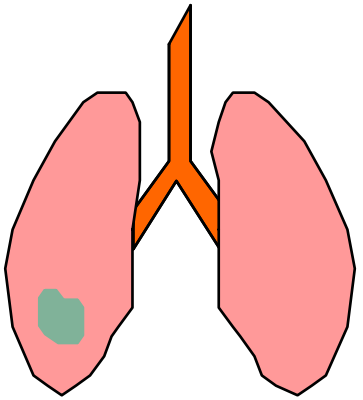
Pittet et al. (2004) Ann Surg

Invasive opportunistic mycoses (IM)

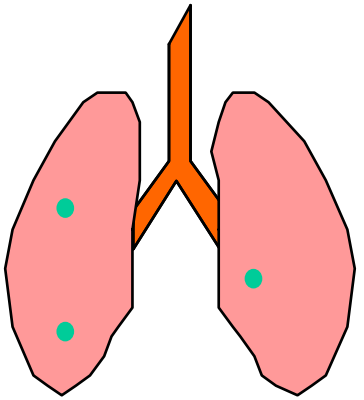
Host: high risk, “unsterile” specimen: BAL



Fungus



Infection



Colonisation

Class.:	No IM	Likely IMI	No IFI
Culture:	positive	Positive: Aspergillus	Positive: Candida
Microscopy:	negative	positive	positive

AST recommendation

Isolated from	FOR patient management	FOR Epidemiology
Blood and other deep sites	All isolates and particularly: 1. Strains from patients exposed to antifungal agents 2. Clinical failures 3. Rare and emerging species 4. Species that are known to be resistant or less susceptible to antifungal drug(s) in clinical use	• All isolates should be tested using a reference method or a validated commercial method
Superficial sites	• Failed to respond or relapsing infection • Surveillance cultures from patients exposed to antifungal agents	• Periodical epidemiological studies should be done

AST: Antifungal Susceptibility Testing

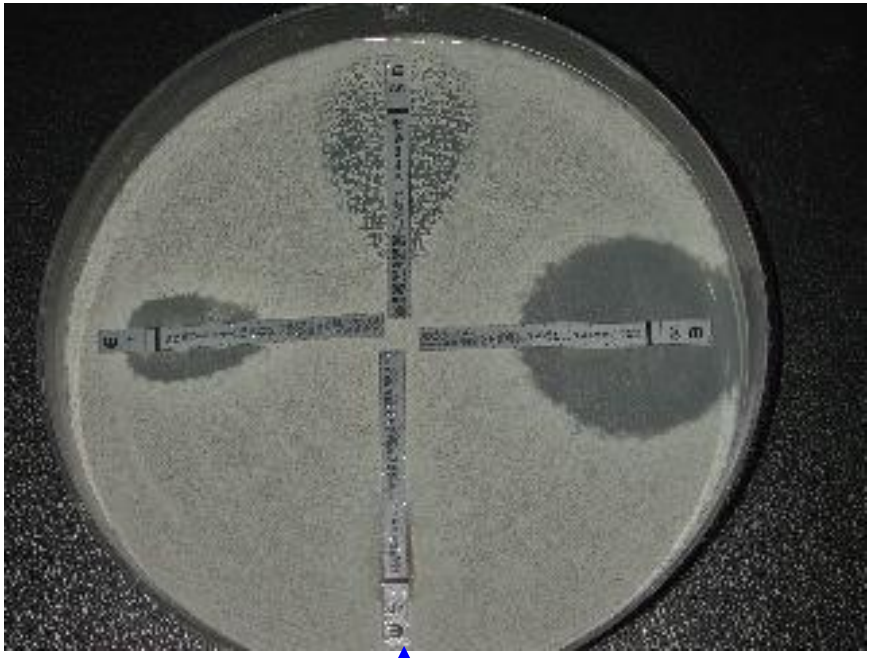
1) CLSI M27-A3, M27-S3, M44-A2
2) EUCAST Discussion Document E.Dis 7.1
3) Pfaller et al. J Clin Microbiol 1995;33:1104-7
4) EUCAST-AFST. Clin Microbiol Infect 2008;14:193-95
5) EUCAST-AFST. Clin Microbiol Infect 2008; 14:985-987
6) Alexander et al. J Clin Microbiol 2007;45: 698-706
7) Dannaoui et al. Clin Microbiol Infect 2010;16: 863-9
8) Cuenca-Estrella et al. J Clin Microbiol 2010;48:1782-6
9) Arendrup MC et al. Antimicrob Agents Chemother 2010;54:426-39

Detection of antifungal resistance in fungi => MIC determination

A. fumigatus

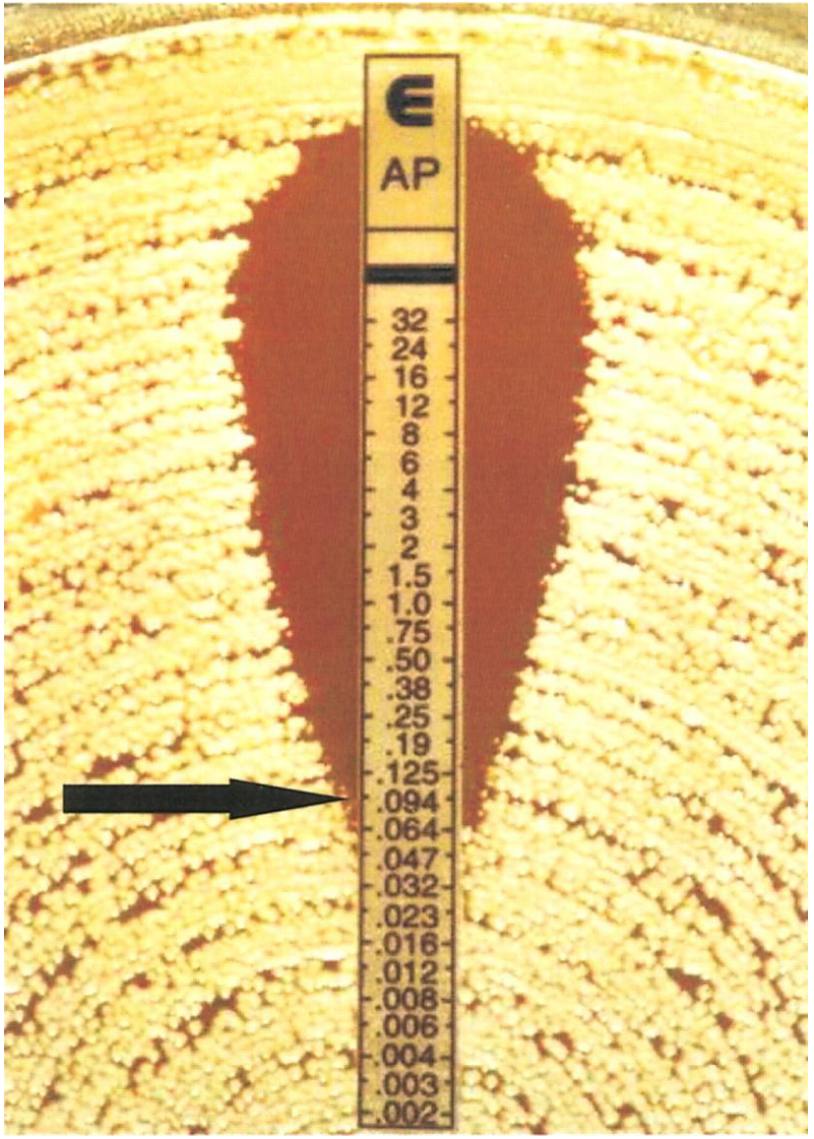
AmB : S

A. terreus



AmB : R

Etest (AB Biodisk) for determination of MICs of amphotericin B to Candida species



Alexander BD , Pfaller MA (2006) Clin Infect Dis. 43:S15-S27

Antifungal susceptibility testing: interpretive breakpoints with use of Eucast

Candida spp.

EUCAST Antifungal Clinical Breakpoint Table v. 4.0, valid from 2012-01-01

Antifungal agent	MIC breakpoint (mg/L)														Notes
	C. albicans		*C. glabrata*		*C. krusei*		*C. parapsilosis*		*C. tropicalis*		*C. guillermondii*		Non-species related breakpoints[1]		
	S ≤	R >	S ≤	R >	S ≤	R >	S ≤	R >	S ≤	R >	S ≤	R >	S ≤	R >	
															1. Non-species related breakpoints have been determined mainly on the basis of PK/PD data and are independent of MIC distributions of specific species. They are for use only for organisms that do not have specific breakpoints.
Amphotericin B	1	1	1	1	1	1	1	1	1	1	IE	IE	IE	IE	
Anidulafungin	0,03	0,03	0,06	0,06	0,06	0,06	--	--	0,06	0,06	IE[2]	IE[2]	IE	IE	**2.** The ECOFFs for these species are in general higher than for *C. albicans*.
Fluconazole	2	4	IE[2]	IE[2]	--	--	2	4	2	4	IE[2]	IE[2]	2	4	
Itraconazole	IP	IP	IP	IP	IP	IP	IP	IP	IP	IP	IP	IP	IP	IP	
Posaconazole	0,06	0,06	IE[2]	IE[2]	IE[2]	IE[2]	0,06	0,06	0,06	0,06	IE[2]	IE[2]	IE	IE	
Voriconazole	0.12[3]	0.12[3]	IE	IE	IE	IE	0.12[3]	0.12[3]	0.12[3]	0.12[3]	IE[2]	IE[2]	IE	IE	**3.** Strains with MIC values above the S/I breakpoint are rare or not yet reported. The identification and antimicrobial susceptibility tests on any such isolate must be repeated and if the result is confirmed the isolate sent to a reference laboratory. Until there is evidence regarding clinical response for confirmed isolates with MIC above the current resistant breakpoint (in italics) they should be reported resistant.

Antifungal susceptibility testing: interpretive breakpoints with use of Eucast

Aspergillus spp.

EUCAST Antifungal Clinical Breakpoint Table v. 4.0, valid from 2012-01-01

Antifungal agent	MIC breakpoint (mg/L)												Notes
	A. flavus		*A. fumigatus*		*A. nidulans*		*A. niger*		*A. terreus*		Non-species related breakpoints[1]		
	S ≤	R >	S ≤	R >	S ≤	R >	S ≤	R >	S ≤	R >	S ≤	R >	
													1. Non-species related breakpoints have been determined mainly on the basis of PK/PD data and are independent of MIC distributions of specific species. They are for use only for organisms that do not have specific breakpoints.
Amphotericin B	IE[2]	IE[2]	1	2	Note[3]	Note[3]	1	2	--	--	IE	IE	**2. T**he ECOFFs for these species are in general one step higher than for *A. fumigatus*. **3.** There are too few MIC data to establish ECOFFs and hence to suggest any breakpoints.
Anidulafungin	IE	IE	IE	IE	IE	IE	IE	IE	IE	IE	IE	IE	
Fluconazole	--	--	--	--	--	--	--	--	--	--	--	--	
Itraconazole[4]	1	2	1	2	1	2	IE[2,5]	IE[2,5]	1	2	IE[5]	IE[5]	**4.** Monitoring of itraconazole trough concentrations in patients treated for fungal infection is recommended. **5.** The MIC values for isolates of *A. niger* and *A. versicolor* are in general higher than those for *A. fumigatus*. Whether this translates into a poorer clinical response is unknown.
Posaconazole	IE[2]	IE[2]	0.12[6]	0.25[6]	IE[2]	IE[2]	IE[2]	IE[2]	0.12[6]	0.25[6]	IE	IE	**6.** Provided adequate drug exposure has been confirmed using therapeutic drug monitoring (TDM). There remains some uncertainty regarding cut-off values for posaconazole concentrations that separate patients with a high probability of clinical success from those with a low probability of clinical success. In some circumstances (e.g. patients with persistent and profound neutropenia, large lesions, or those with other features associated with a poor clinical outcome) a relatively high trough concentration should be sought. Preclinical and clinical data suggest this value should be >1 mg/L at steady state. For other patient groups a lower trough concentration may be acceptable. For prophylaxis a target concentration of >0.7 mg/L has been suggested.
Voriconazole	IP	IP	IP	IP	IP	IP	IP	IP	IP	IP	IP	IP	

Invasive fungal infections : update on conventional diagnosis

Conventional diagnosis

- direct examination
  of tissue
  of indirect clinical specimens (sputum, BAL)

	sputum/BAL	tissue
unstained	wet prep ± KOH	
routine stains	Gram	Gram
fungal stains	GMS, PAS	HE, GMS, PAS
fluorescent dyes	Calcofluor white Uvitex 2B Blankophor	Calcofluor white

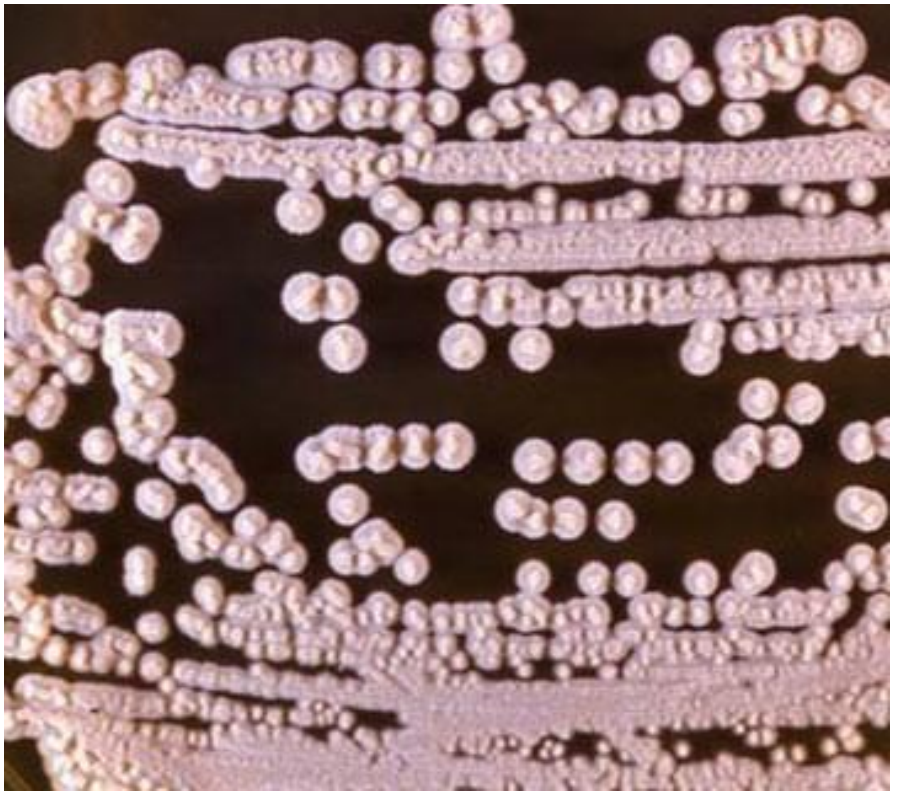
- cultures

Invasive fungal infections : update on conventional diagnosis

Conclusions

Conventional diagnosis of IFI is :

- suboptimal
- indispensable
  - ⇨ **genus, species**
  - ⇨ **AFST**
- perfectible



Thank you for your attention!