Classification and new diagnostic methods for invasive fungal infections

Stéphane Bretagne

National Reference Center for Mycoses & Antifungals
CNRS URA3012

INSTITUT PASTEUR

Tunis 24th May 2012

Classification
Invasive Fungal Diseases

- **Proven IFD**
- **Probable IFD**
- **Possible IFD**
- **EORTC/MSG criteria**

Ascioglu et al CID 2002; Ben de Pauw et al CID 2008

Classification
Invasive Fungal Diseases

- Proven IFD
  - Irrespective of host factors or clinical features
  - Demonstration of fungal elements in diseased tissue
  - If no culture, conclude to proven mold or yeast IFD
    - Except if histological appearance sufficiently distinctive (e.g. endemic mycoses such as Histoplasmosis, Coccidioidomycosis, Blastomycosis)
  - Individual IFD entities require culture and identification

Ben de Pauw et al CID 2008

Table 1: Criteria for proven mould infections

Analysis and specimen	Molds[a]
Microscopic analysis: sterile material	Histopathologic, cytopathologic, or direct microscopic examination[b] of a specimen obtained by needle aspiration or biopsy in which hyphae or melanized yeast-like forms are seen accompanied by evidence of associated tissue damage
Culture	
Sterile material	Recovery of a mold or "black yeast" by culture of a specimen obtained by a sterile procedure from a normally sterile and clinically or radiologically abnormal site consistent with an infectious disease process, excluding bronchoalveolar lavage fluid, a cranial sinus cavity specimen, and urine
Blood	Blood culture that yields a mold[d] (e.g., *Fusarium* species) in the context of a compatible infectious disease process
Serological analysis: CSF	Not applicable

Ben de Pauw et al CID 2008

Table 1: Criteria for proven mould infections

Analysis and specimen	Molds[a]
Microscopic analysis: sterile material	Histopathologic, cytopathologic, or direct microscopic examination[b] ... which hyphae or melanized yeast-like forms are seen accompanied by evidence of associated tissue damage
Culture	
Sterile material	Recovery of a mold or "black yeast" by culture of a specimen obtained by a sterile procedure from a normally sterile and clini- ... s ... al ... sinus cavity specimen, and urine
Blood	Blood culture that yields a mold[d] (e.g., *Fusarium* species) in the context of a compatible infectious disease process
Serological analysis: CSF	Not applicable

Biopsies and culture by a sterile procedure

[d] Recovery of *Aspergillus* species from blood invariably represents contamination

Ben de Pauw et al CID 2008

Table 1: Criteria for proven yeast infections

Analysis and specimen	Yeasts[a]
Microscopic analysis: sterile material	Histopathologic, cytopathologic, or direct microscopic examination[b] of a specimen obtained by needle aspiration or biopsy from a normally sterile site (other than mucous membranes) showing yeast cells—for example, *Cryptococcus* species indicated by encapsulated budding yeasts or *Candida* species showing pseudohyphae or true hyphae[c]
Culture	
Sterile material	Recovery of a yeast by culture of a sample obtained by a sterile procedure (including a freshly placed [<24 h ago] drain) from a normally sterile site showing a clinical or radiological abnormality consistent with an infectious disease process
Blood	Blood culture that yields yeast (e.g., *Cryptococcus* or *Candida* species) or yeast-like fungi (e.g., *Trichosporon* species)
Serological analysis: CSF	Cryptococcal antigen in CSF indicates disseminated cryptococcosis

Ben de Pauw et al CID 2008

Table 1: Criteria for proven yeast infections

Analysis and specimen	Yeasts[a]
Microscopic analysis: sterile material	Histopathologic, cytopathologic, or direct microscopic examination[b] of a specimen obtained by needle aspiration or biopsy from a site (including mucous membranes) showing yeast cells—for example, *Cryptococcus* species indicated by encapsulated budding yeasts or *Candida* species showing pseudohyphae or true hyphae[c]
Culture	
Sterile material	Recovery of a yeast by culture of a sample obtained by a sterile procedure (including a freshly placed [<24 h ago] drain) from a normally sterile site showing a clinical or radiological abnormality consistent with an infectious disease process
Blood	Blood culture that yields yeast (e.g., *Cryptococcus* or *Candida* species) or yeast-like fungi (e.g., *Trichosporon* species)
Serological analysis: CSF	Cryptococcal antigen in CSF indicates disseminated cryptococcosis

Biopsies and culture

Blood or sterile material culture

Ben de Pauw et al CID 2008

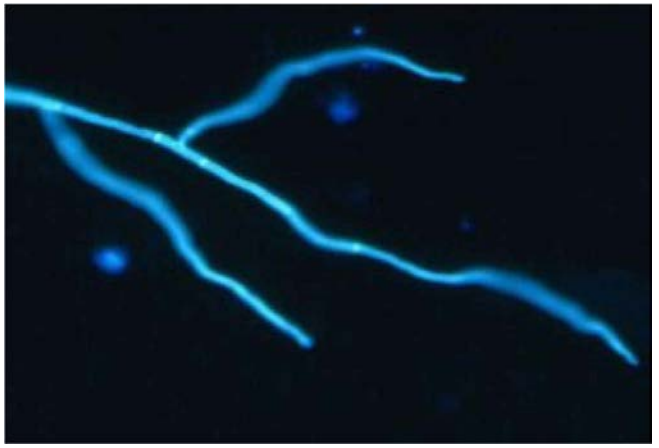
Table 1: Criteria for proven IFD
foot notes

[a] If culture is available append the identification at the genus or species level from the culture results

[b] Tissue and cells submitted for histopathologic or cytopathologic studies should be stained by Grocott-Gomori methenamine silver stain or by periodic acid Schiff stain to facilitate inspection of fungal structures. Whenever possible, wet mounts of specimens from foci related to IFD should be stained with a fluorescent dye (e.g. calcofluor or blankophor)

Direct examination using a fluorescent dye

- « Aspergillus » like



- « Mucormycosis » like

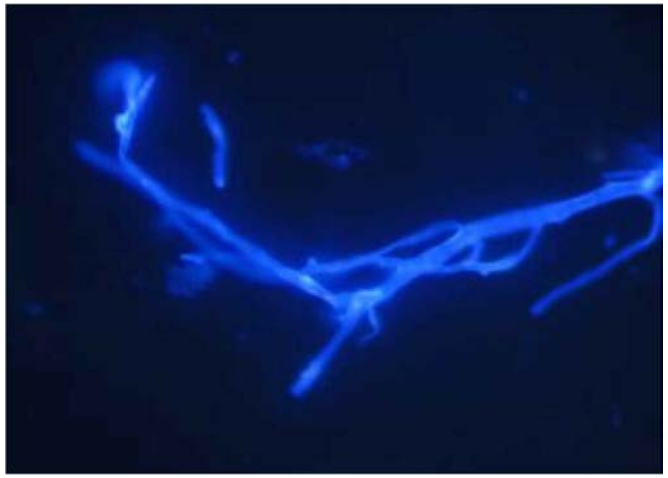


Table 2: Criteria for probable IFD

- Host factors
  - Neutropenia (< 500 neutrophils /mm3 for > 10 days) related to the onset of IFD
  - Allo-HSCT
  - Steroids (>0.3 mg/kg/d for >3 weeks)
  - Immunosuppressants (cyclosporine, anti-TNF, monoclonal Ab, nucleoside analogues) for the last 90 days
  - Inherited severe immunodeficiency

Table 2: Criteria for probable IFD

- Host fac

What about ICU patients?

  - Neutropenia (< 500 neutrophils /mm3 for > 10 days) related to the onset of IFD
  - Allo-HSCT
  - Steroids (>0.3 mg/kg/d for >3 weeks)
  - Immunosuppressants (cyclosporine, anti-TNF, monoclonal Ab, nucleoside analogues) for the last 90 days
  - Inherited severe immunodeficiency

Table 2: Criteria for probable IFD

- Clinical criteria[b]
  - Lower respiratory tract fungal disease[c]
    - The presence of 1 of the following 3 signs on CT:
      - Dense, well-circumscribed lesions(s) with or without a halo sign
      - Air-crescent sign
      - Cavity
  - Tracheobronchitis
    - Tracheobronchial ulceration, nodule, pseudomembrane, plaque, or eschar seen on bronchoscopic analysis
  - Sinonasal infection
    - Imaging showing sinusitis plus at least 1 of the following 3 signs:
      - Acute localized pain (including pain radiating to the eye)
      - Nasal ulcer with black eschar
      - Extension from the paranasal sinus across bony barriers, including into the orbit
  - CNS infection
    - 1 of the following 2 signs:
      - Focal lesions on imaging
      - Meningeal enhancement on MRI or CT
  - Disseminated candidiasis[d]
    - At least 1 of the following 2 entities after an episode of candidemia within the previous 2 weeks:
      - Small, target-like abscesses (bull's-eye lesions) in liver or spleen
      - Progressive retinal exudates on ophthalmologic examination

Table 2: Clinical criteria for probable IFD

Clinical criteria[b]

Lower respiratory tract fungal disease[c]

The presence of 1 of the following 3 signs on CT:

Dense, well-circumscribed lesions(s) with or without a halo sign

Air-crescent sign

Cavity

Tracheobronchitis

Tracheobronchial ulceration, nodule, pseudomembrane, plaque, or eschar seen on bronchoscopic analysis

Sinonasal infection

Imaging showing sinusitis plus at least 1 of the following 3 signs:

Acute localized pain (including pain radiating to the eye)

Nasal ulcer with black eschar

Extension from the paranasal sinus across bony barriers, including into the orbit

CNS infection

1 of the following 2 signs:

Focal lesions on imaging

Meningeal enhancement on MRI or CT

Disseminated candidiasis[d]

At least 1 of the following 2 entities after an episode of candidemia within the previous 2 weeks:

Small, target-like abscesses (bull's-eye lesions) in liver or spleen

Progressive retinal exudates on ophthalmologic examination

Table 2: Criteria for probable IFD

Mycological criteria
- Direct test (cytology, direct microscopy, or culture)
  - Mold in sputum, bronchoalveolar lavage fluid, bronchial brush, or sinus aspirate samples, indicated by 1 of the following:
    - Presence of fungal elements indicating a mold
    - Recovery by culture of a mold (e.g., *Aspergillus, Fusarium,* Zygomycetes, or *Scedosporium* species)
  - Indirect tests (detection of antigen or cell-wall constituents)[e]
    - Aspergillosis
      - Galactomannan antigen detected in plasma, serum, bronchoalveolar lavage fluid, or CSF
    - Invasive fungal disease other than cryptococcosis and zygomycoses
      - β-D-glucan detected in serum

Table 2: Criteria for probable IFD

Mycological criteria

Direct test (cyt

Cytology, direct microscopy and culture

Mold in sput dicated by 1 of the following:

Presence of fungal elements indicating a mold

Recovery by culture of a mold (e.g., *Aspergillus, Fusarium,* Zygomycetes, or *Scedosporium* species)

Indirect tests (detection of antigen or cell-wall constituents)[e]

Aspergillosis

Galactomannan antigen detected in plasma, serum, bronchoalveolar lavage fluid, or CSF

Invasive fungal disease other than cryptococcosis and zygomycoses

β-D-glucan detected in serum

Indirect tests: galactomannan and ß-D-glucan

Definition: probable or possible IFD

PROBABLE =

HOST FACTOR

+ CLINICAL CRITERION

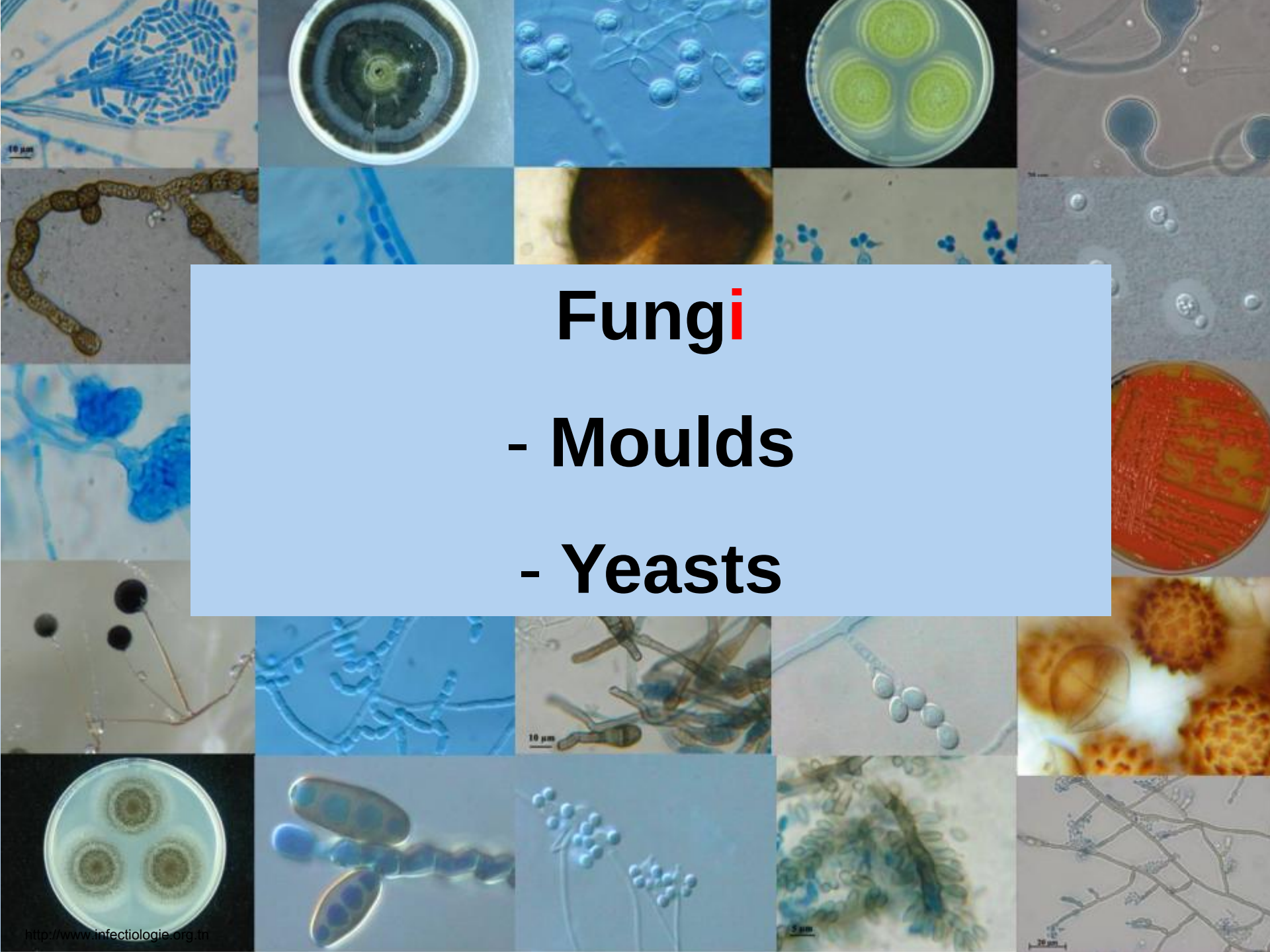
+ MYCOLOGICAL CRITERION

POSSIBLE =

HOST FACTOR

+ CLINICAL CRITERION

“New” diagnostic tools

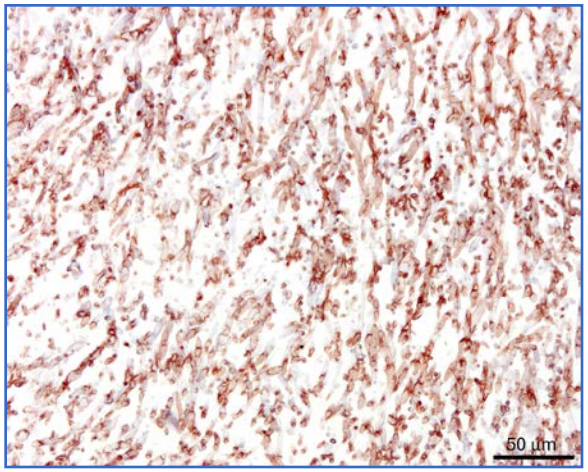


Fungi

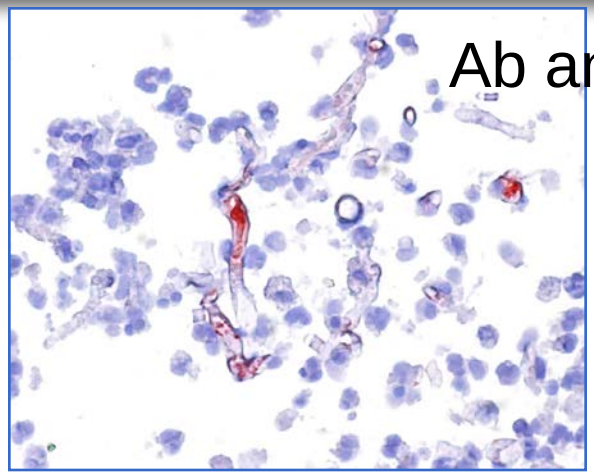
- Moulds

- Yeasts

Biopsies: Immunostaining

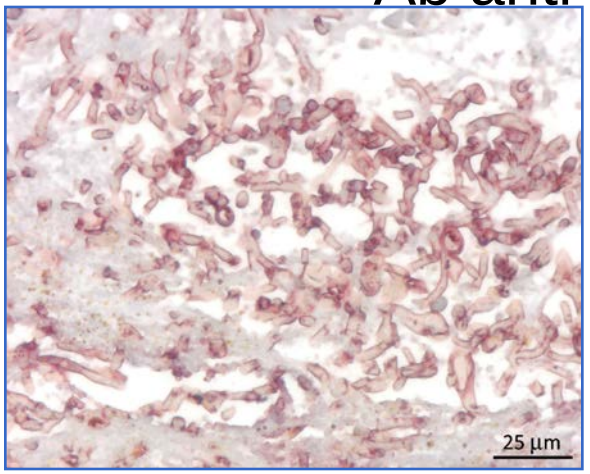


Ab anti-*Aspergillus*



Ab anti-*Rhizomucor*

Ab anti-*Candida*



G. Jouvion, F. Chrétien
Institut Pasteur
Human Histopathology & Animal Models
Infection and Epidemiology Department

Guarner &Brandt Clin Micro Rev 2011

Molecular ID from biopsies

- Common practice
  - DNA extraction
  - Amplification of ITS regions
- Pitfalls
  - Poor DNA quality from formalin fixed tissues (ask for -80° C)
  - At least 40% of false identification in public data base (GenBank)
  - Hybridization of primers with human DNA (hence low sensitivity)

- ITS1 (TCCGTAGGTGAACCTGCGG)
- 19 nucleotides
- Identities = 19/19 (100%), Gaps = 0/19 (0%)
- Query 1 TCCGTAGGTGAACCTGCGG
- |||||||||||||||||||
- Sbjct 110917 TCCGTAGGTGAACCTGCGG

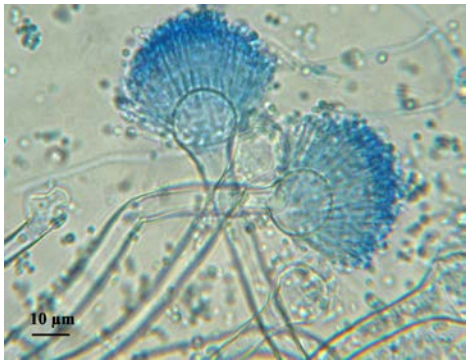
- > Homo sapiens unplaced genomic contig, alternate assembly HuRef

White T, et al In PCR-protocols a guide to methods and applications. Academic press: 1990:315-322

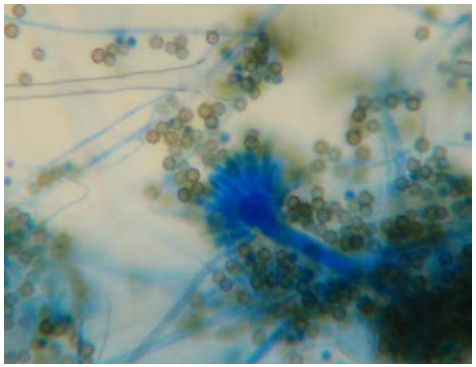
Importance of fungal culture for species identification

Species	% (n=246)
A. fumigatus	85
A. flavus	4
A. nidulans	3
A. terreus	2
A. niger	4
A. ustus	0.5
A. versicolor	0.5
Others	1

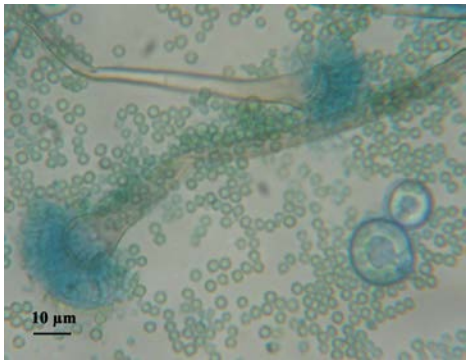
Lortholary et al CMI 2011



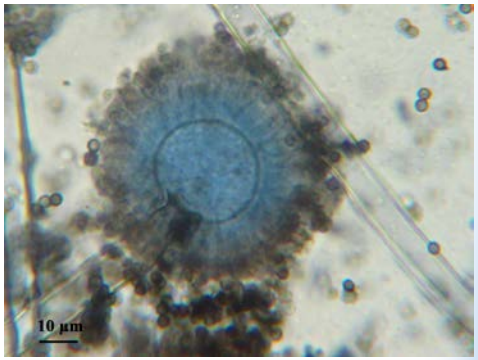
A. terreus



A. ustus



A. nidulans

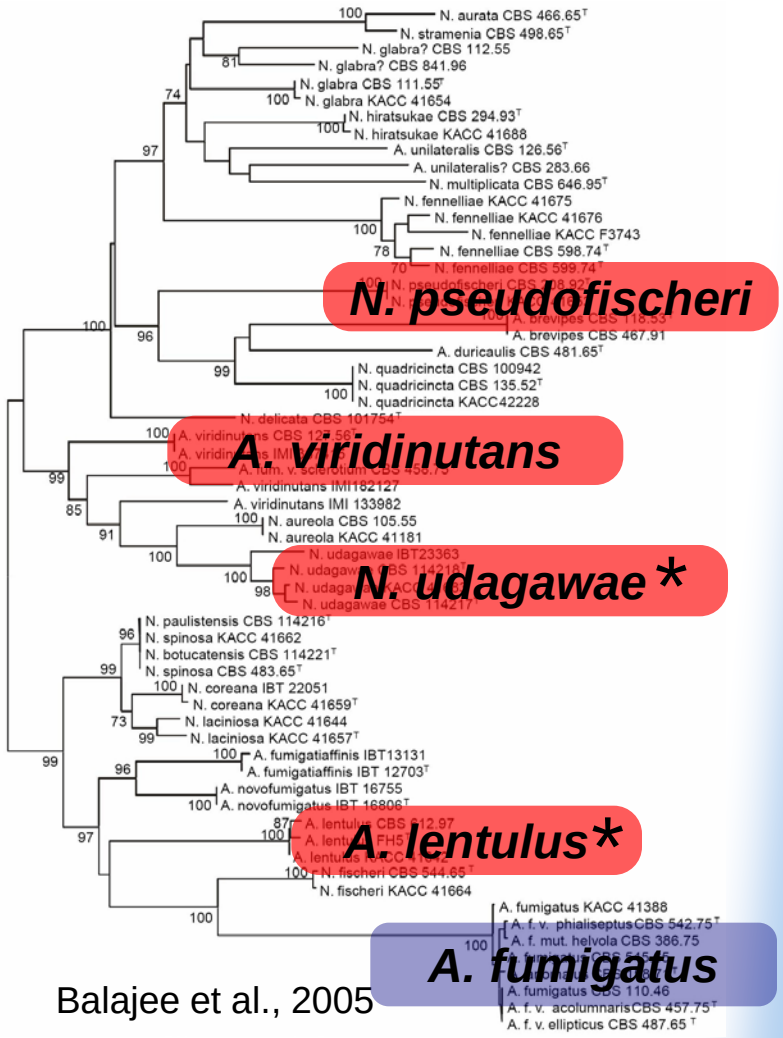


A. niger

Aspergilli taxonomy

Innate resistance to azoles

Innate susceptibility to azoles



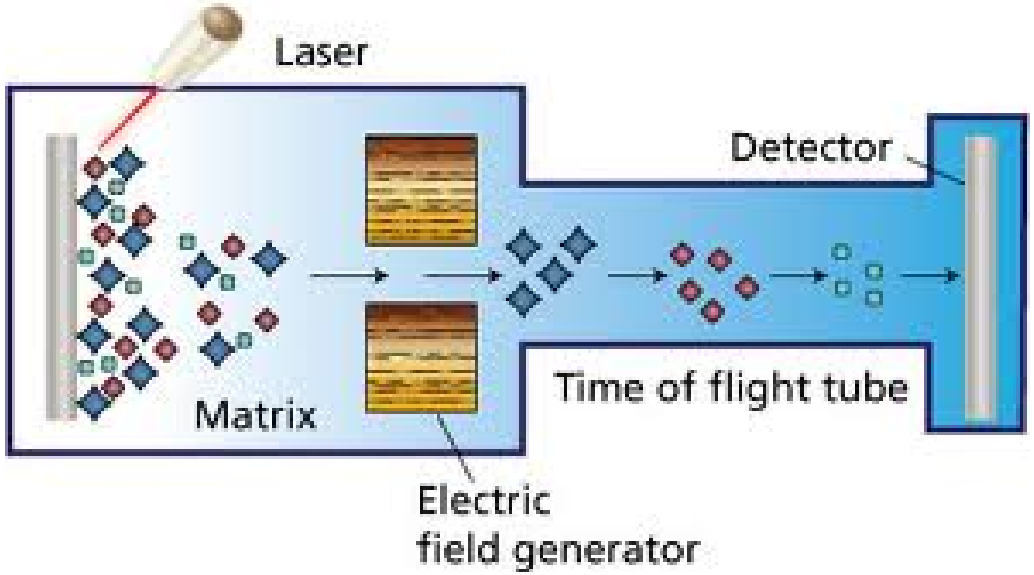
Balajee et al., 2005

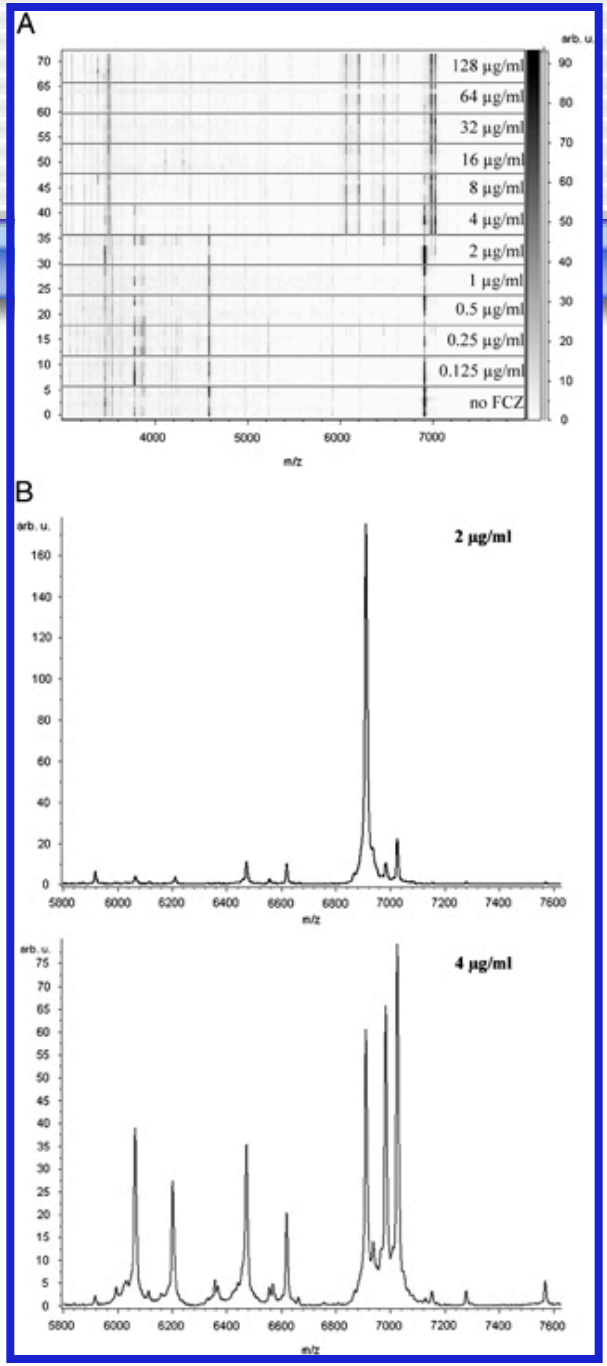
Innate resistance

species	Section	AmB	Azoles	Candines
A. fumigatus	*Fumigati*			
A. lentulus				
A. fumigatiaffinis				
A. viridinutans				
A. fumisynnematus				
N. fischeri				
N. pseudofischeri				
N. udagawae				
N. fennelliae				
N. hiratsukae				
N. spinosa				

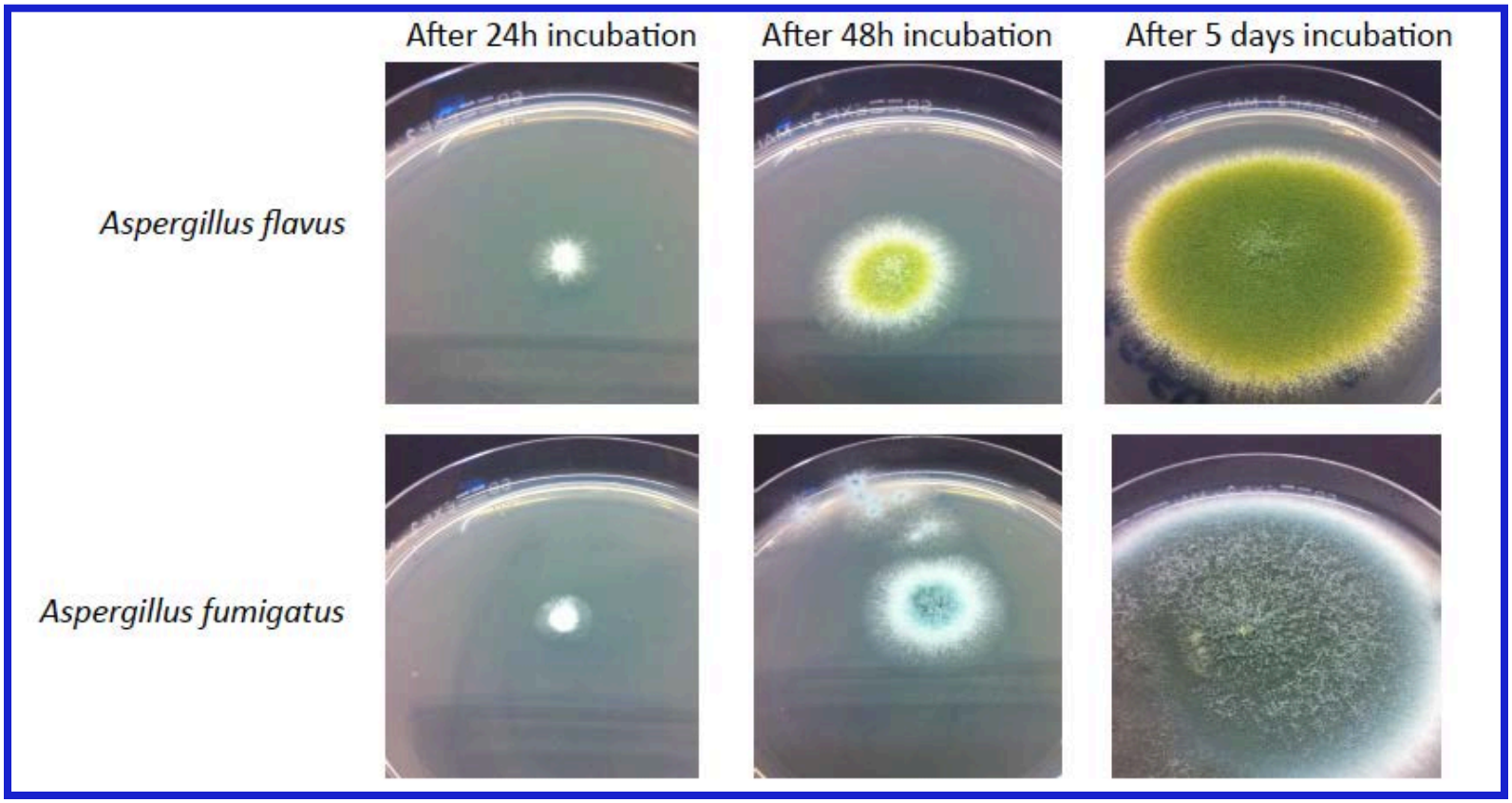
Alcazar-Fuoli et al. 2008, Balajee et al. 2006

MALDI-TOF

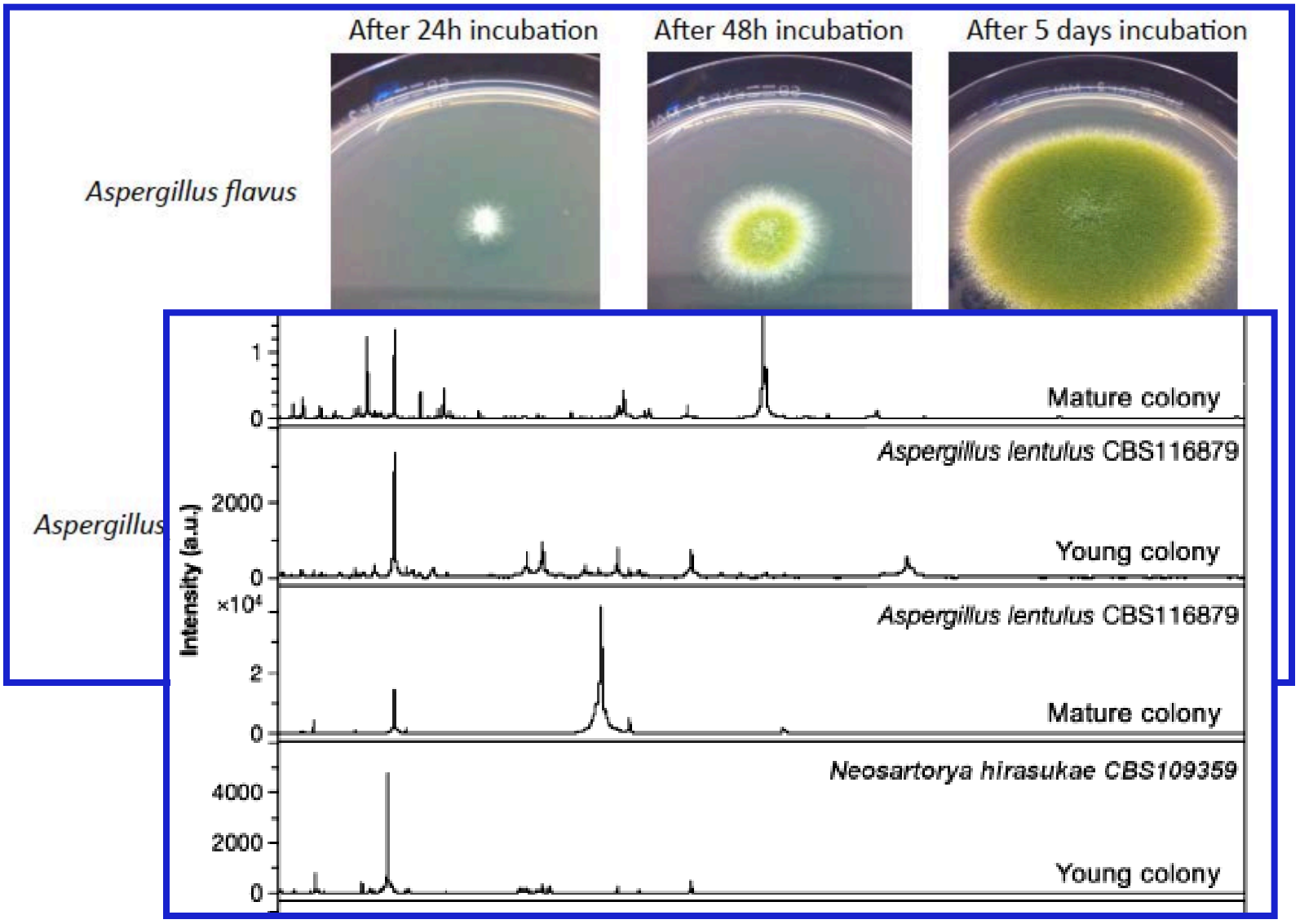




Phenotypic method



Schrenzel ECCMID 2012, London



Design specific age-dependent data banks

Alanio et al CMI 2010

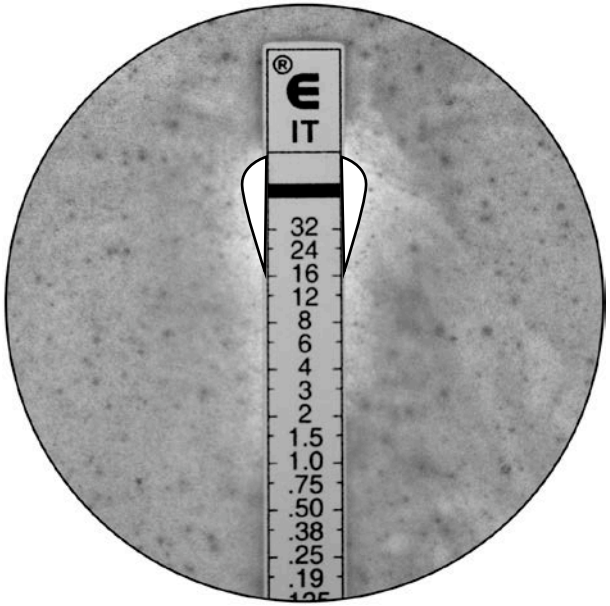
MALDI-TOF

- Easier for yeast ID
- Direct ID in positive blood culture

Ferroni et al JCM 2010; Spanu etal JCM 2012

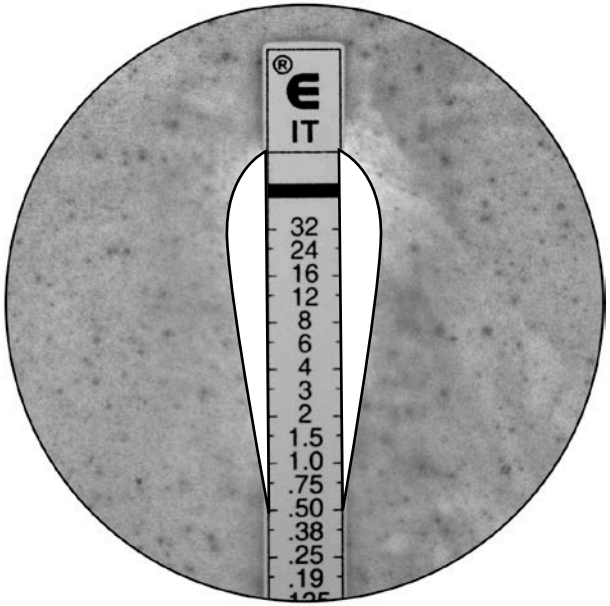
MIC (Etest®) for molds

Resistant



CMI =16 mg/l

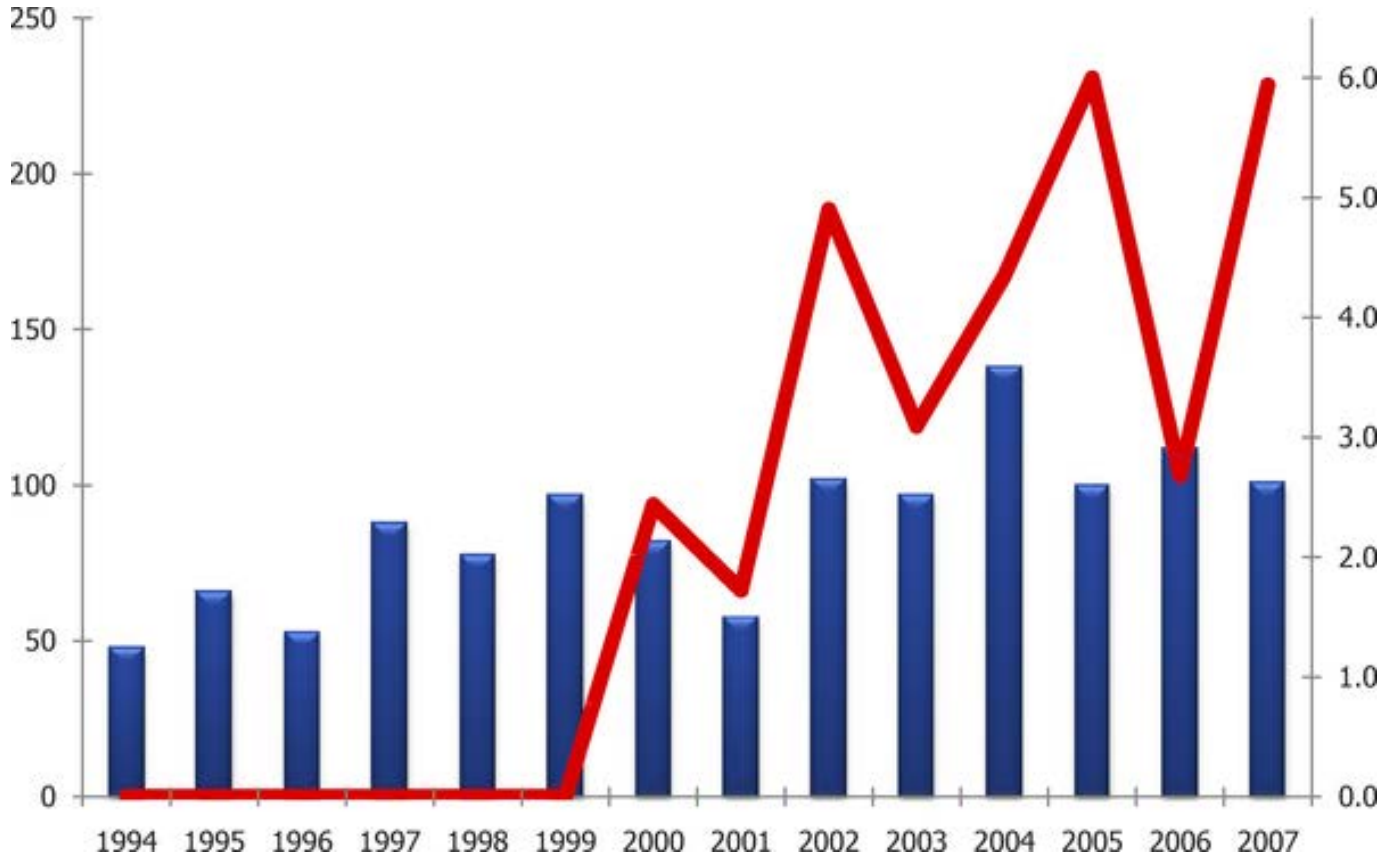
Susceptible



CMI =0.5 mg/l

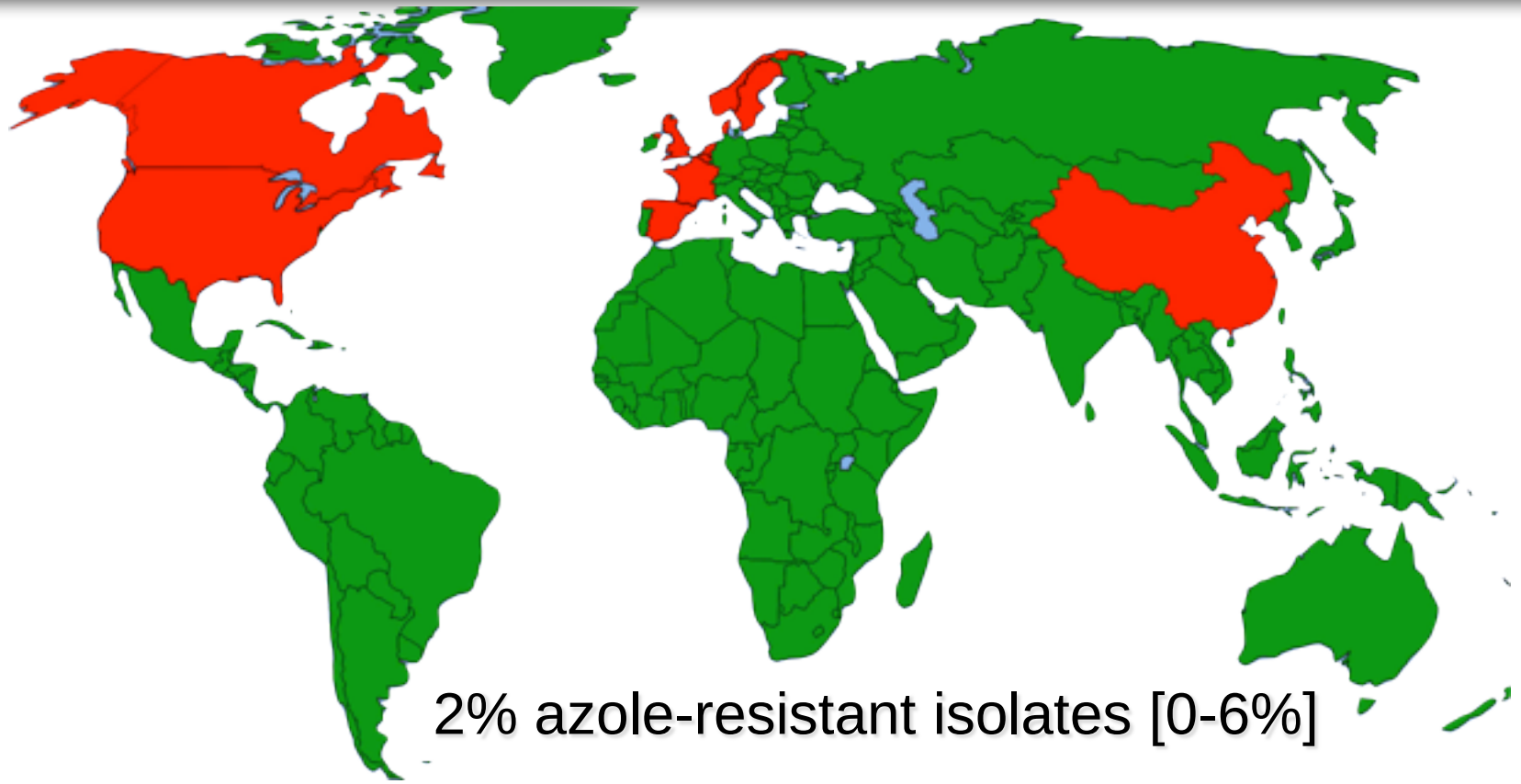
EUCAST values: Arendrup et al Clin Microbiol Inf March 2012

Increasing acquired resistance in *A. fumigatus*



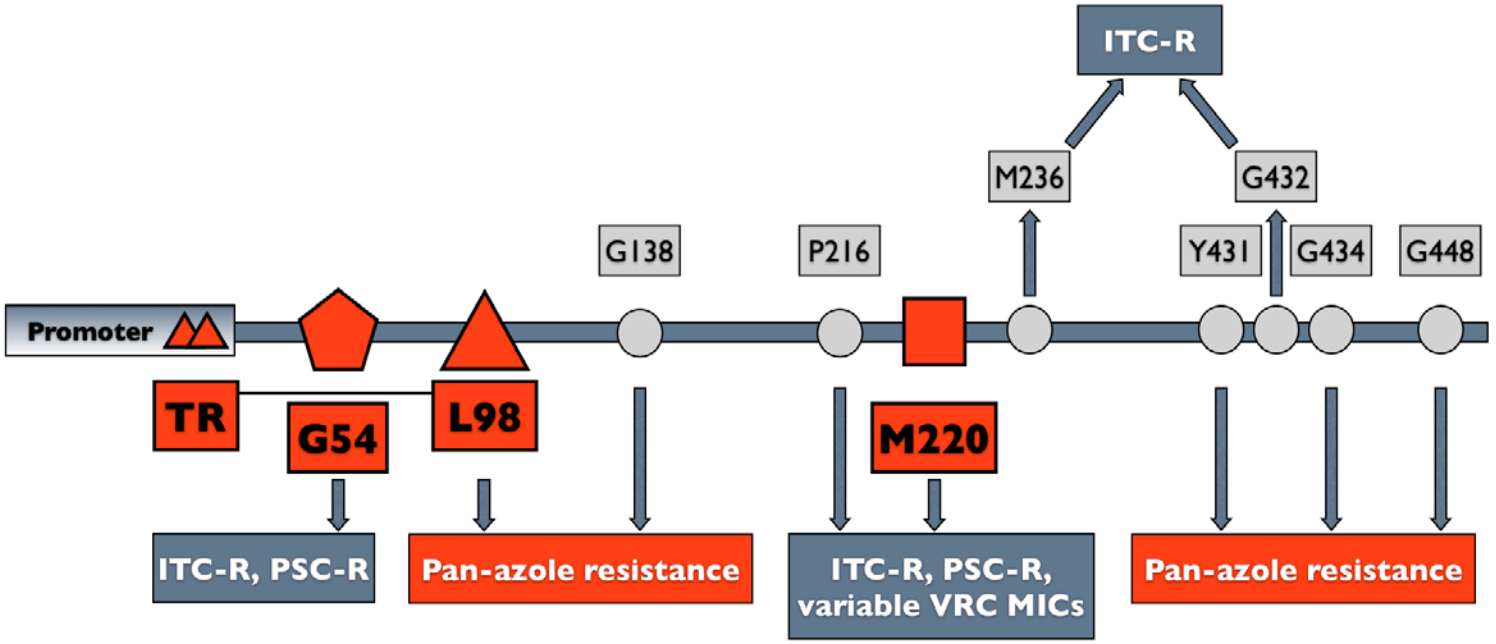
Snelders et al, Plos Medicine 2008

Epidemiological data



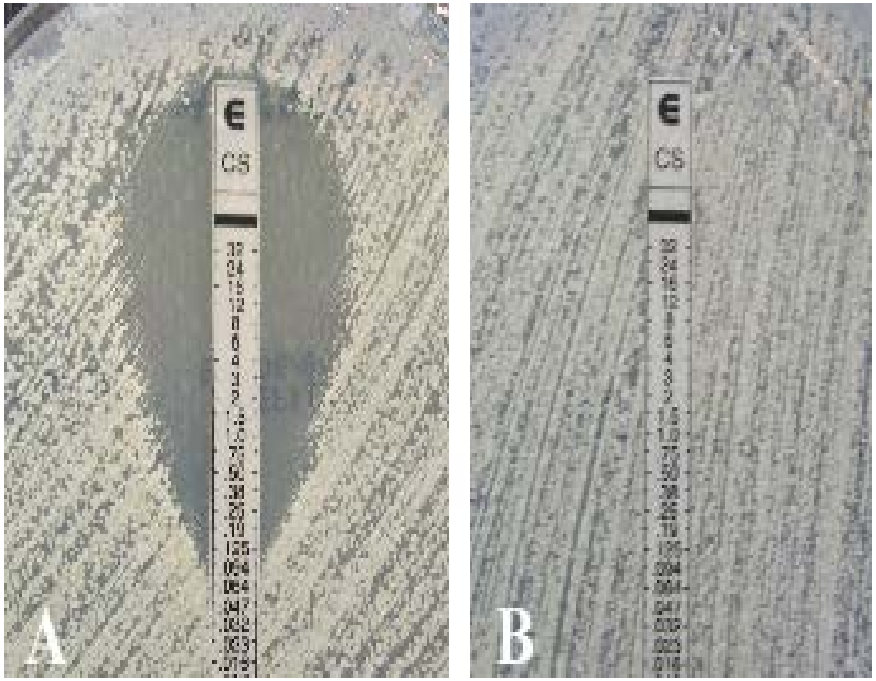
Alanio et al. J. Antimicrob. Chemother. 2010; Bueid et al. J. Antimicrob. Chemother. 2010
Howard et al. Med Mycol. 2011, Lockhart et al. AAC. 2011; Van der Linden et al. EID 2011

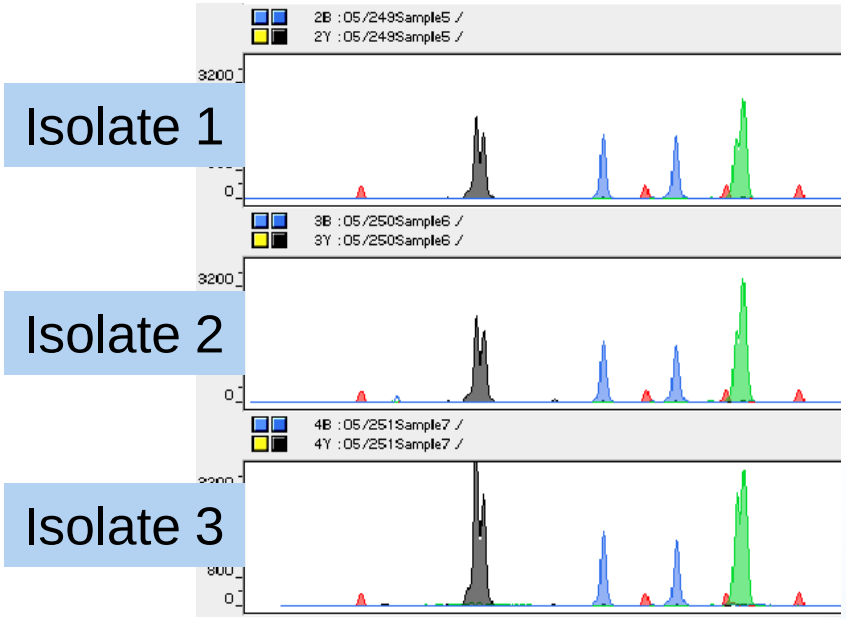
Resistance mechanisms in *A. fumigatus*



Alanio et al. Curr Fungal Infect Rep 2011; Howard et al. Med Mycol 2011; Denning et al. CID 2011

MIC (Etest®) for yeasts



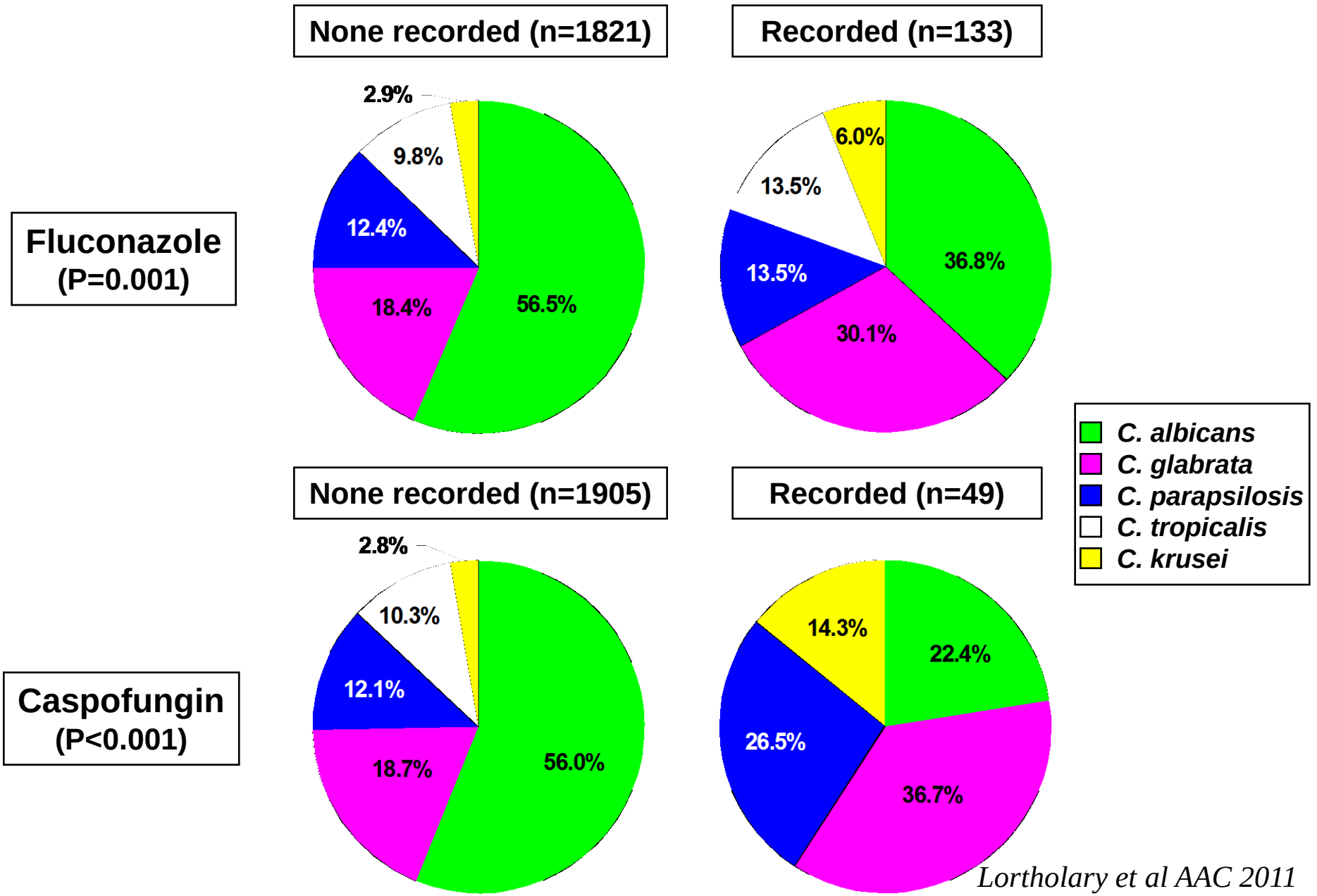


Acquired resistance under antifungal pressure

Microsatellite genotyping

Baixench et al JAC 2007

Recent (within 30 previous days) exposure



Lortholary et al AAC 2011

Current biomarkers

- Ag
  - GM
  - ß-D-glucan
  - Mn
  - GMX cryptococcus
  - *Histoplasma* sp.
- DNA
  - *Aspergillus*
  - *Candida*

Galactomannan: ELISA (BioRad)

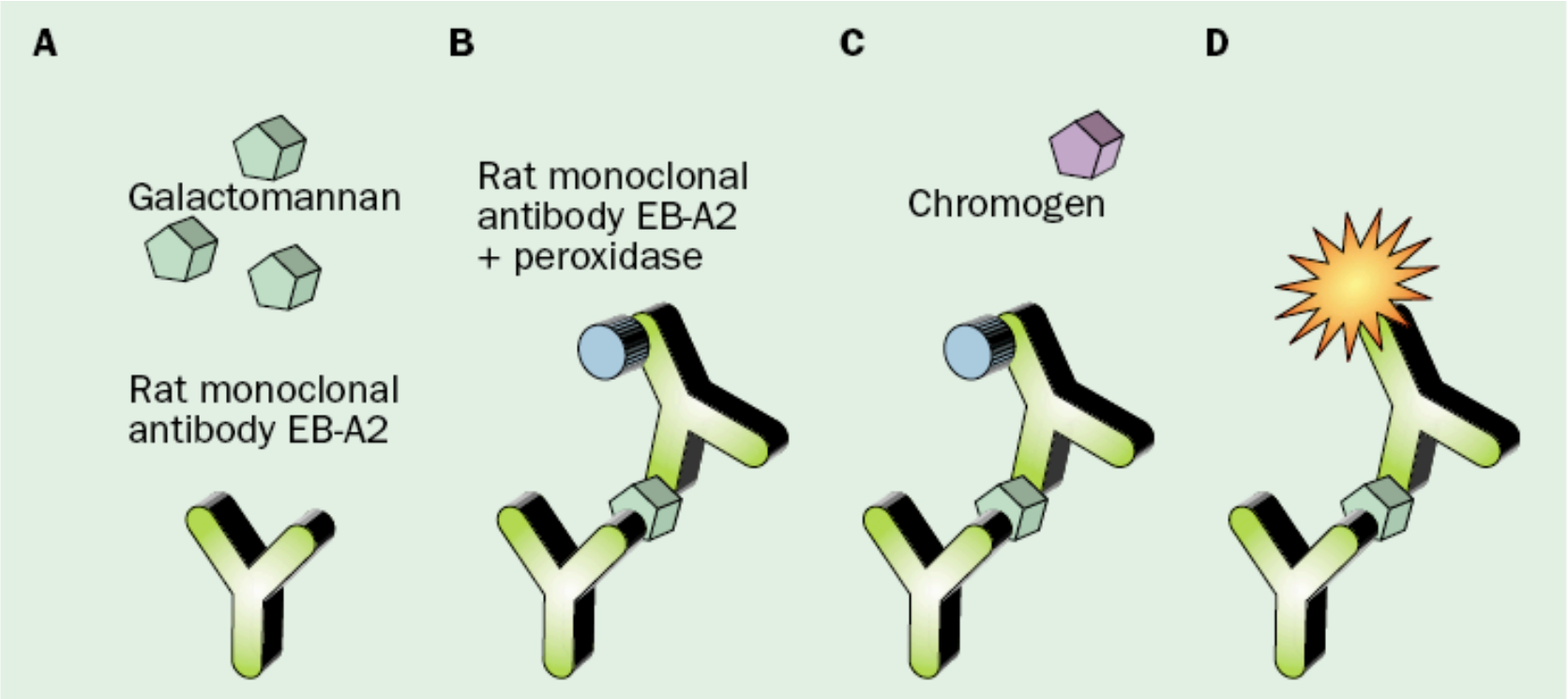
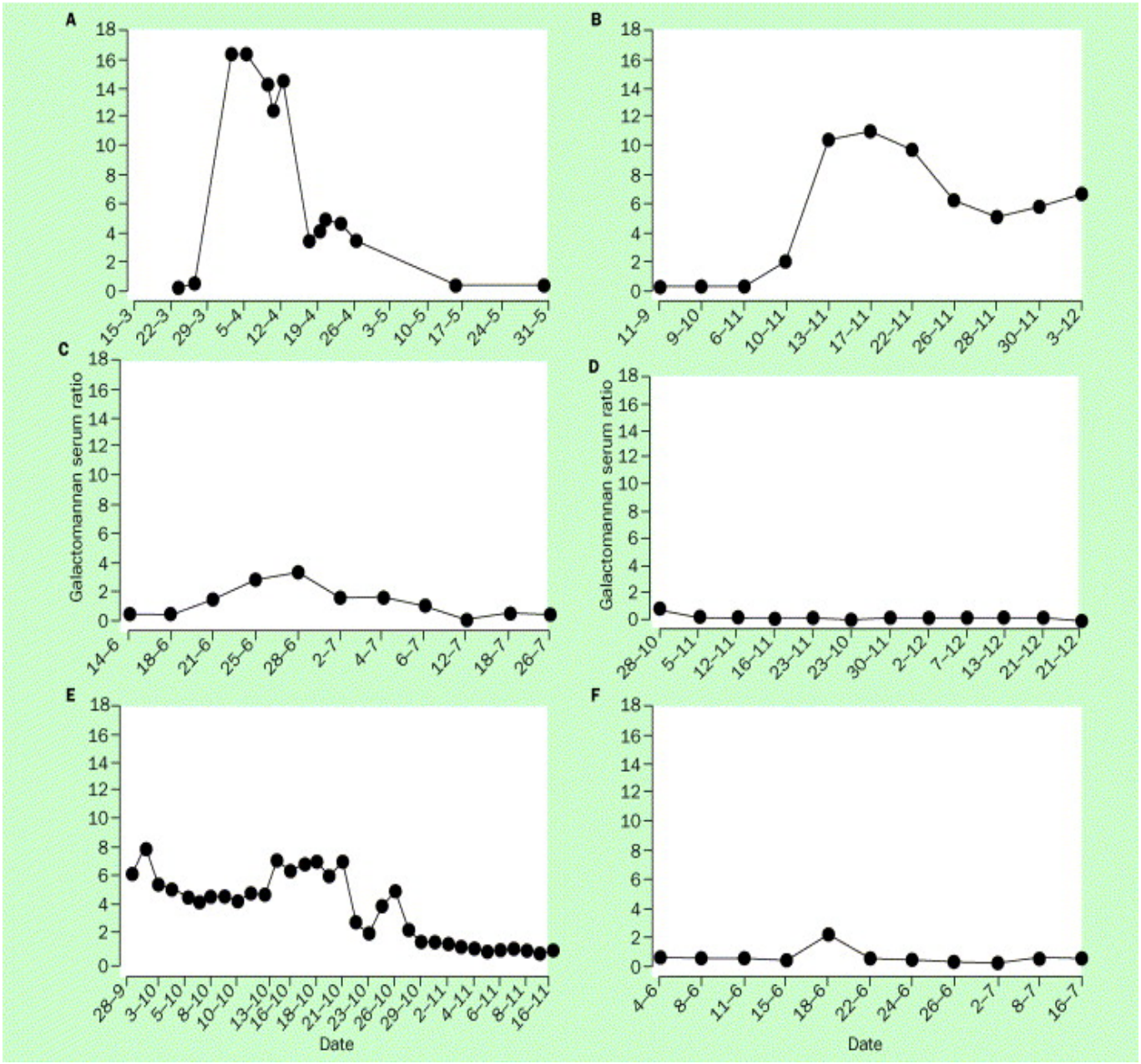


Figure 1. The Platelia Aspergillus ELISA technique. A serum ratio is calculated by dividing the optical density of the patient's serum sample by the mean optical density of two threshold control samples that contain 1 μg/L of galactomannan.

Mennink-Kersten et al, Lancet Inf Dis, 2004



Mennink-Kersten et al, Lancet Inf Dis, 2004

GM recommendations for strategy in adults (ECIL3)

Prospective monitoring of serum* is a feasible approach in adult neutropenic patients undergoing intensive chemotherapy for leukemia or receiving an allogeneic stem cell transplantation for the early diagnosis of invasive aspergillosis (AII)

GM monitoring is recommended every three to four days in admitted patients (AII)

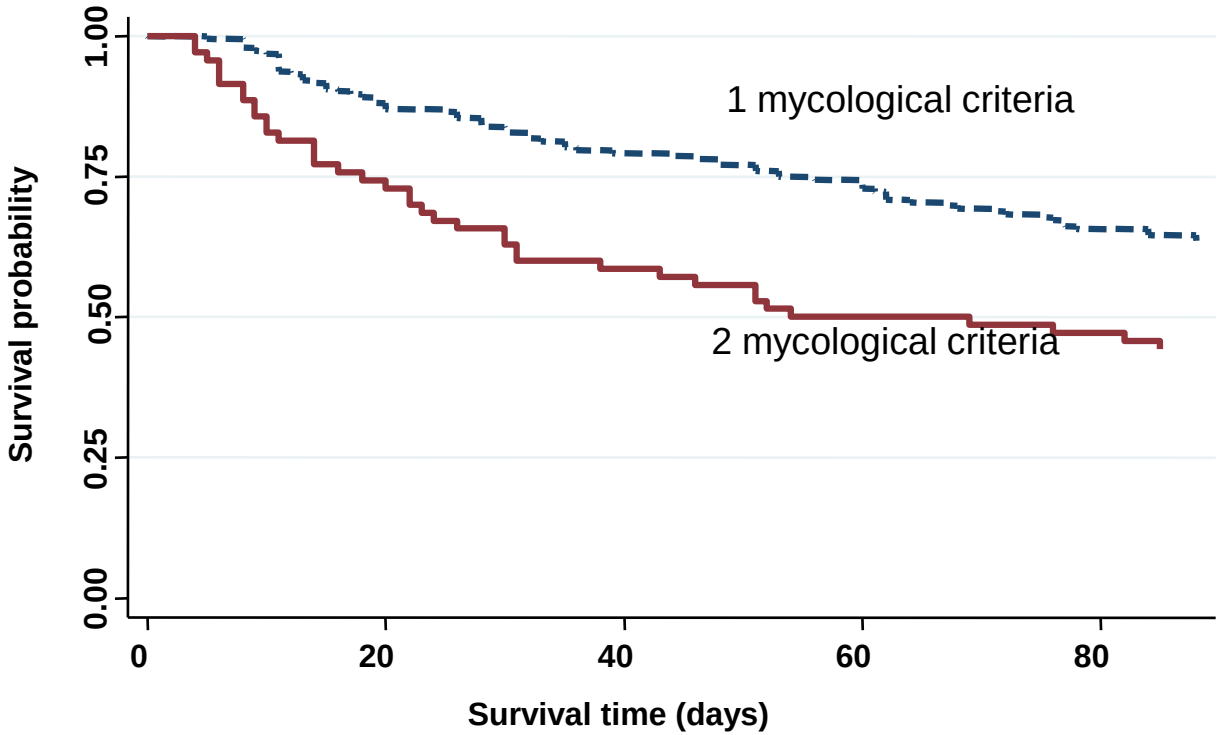
In GM positive patients persistent GM antigenemia during therapy is a poor prognostic sign and should prompt a reassessment of the management of the patient (BII)

A diagnostic driven strategy that incorporates GM monitoring should be combined with high resolution CT imaging, appropriate clinical and microbiological evaluation to early diagnose invasive aspergillosis. A single positive GM index of ≥0.7 or 2 consecutive samples of ≥0.5 should prompt a diagnostic work-up (AII)

* Plasma may also be used (CIII)

Marchetti et al BMT 2011
European Conference on Infections in Leukaemia

Prognosis value of combined diagnostic means



National Reference Centre for Mycology and Antifungals
Lortholary et al CMI 2011

Factors that influence GM performance

Table 2 Biological and epidemiological factors that influence the performance of GM detection in invasive aspergillosis[3]

Biological factors	*Epidemiological factors*
Site of infection	Patient population
Aspergillus species causing infection	Sampling strategy
Microenvironment at the site of infection: nutrients, oxygen level, pH	Definition of a positive result
Exposure to antifungal agents	Definition of an IFD
Molecular structure of released galactomannan	Prevalence of IFD
Underlying condition/neutropenia/ level of immunosuppression	Cutoff for positivity
Renal clearance, hepatic metabolism	Laboratory experience
Circulating galactomannan antibodies	Nutritional factors (galactomannan-containing food)
Storage of clinical sample	Treatment with semi-synthetic β-lactam antibiotics
Pre-analytical treatment procedure	

Marchetti et al BMT 2011

Factors that influence GM performance

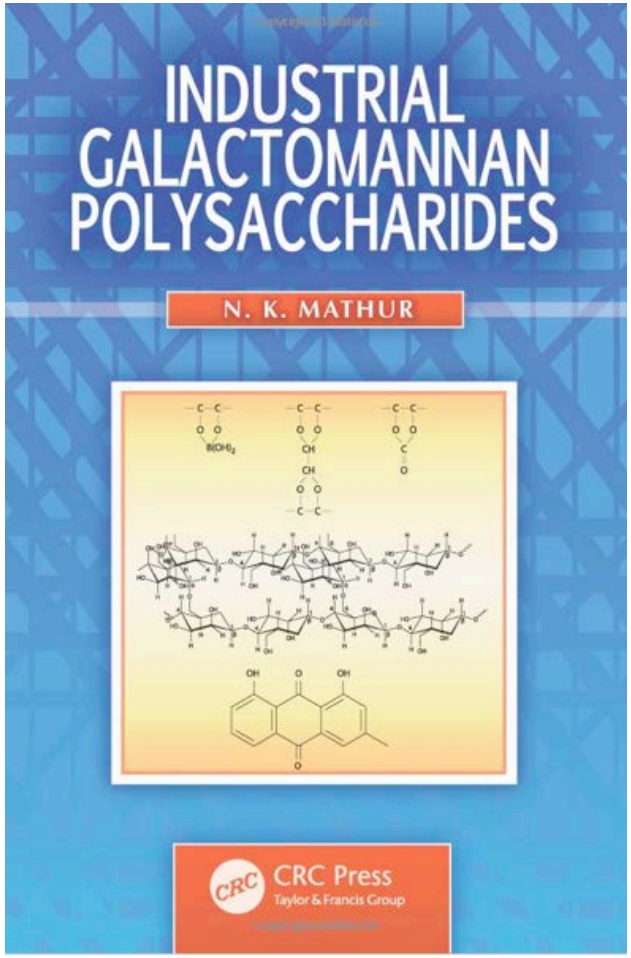
Table 2 Biological and epidemiological factors that influence the performance of GM detection in invasive aspergillosis[3]

Biological factors	*Epidemiological factors*
Site of infection	Patient population
Aspergillus species causing infection	Sampling strategy
Microenvironment at the site of infection: nutrients, oxygen level, pH	Definition of a positive result
Exposure to antifungal agents	Definition of an IFD
Molecular structure of released galactomannan	Prevalence of IFD
Underlying condition/neutropenia/ level of immunosuppression	Cutoff for positivity
Renal clearance, hepatic metabolism	Laboratory experience
Circulating galactomannan antibodies	Nutritional factors (galactomannan-containing food)
Storage of clinical sample	Treatment with semi-synthetic β-lactam antibiotics
Pre-analytical treatment procedure	

Marchetti et al BMT 2011

Galactomannan

Food-processing using galactomannan to modify food texture



Factors that influence GM performance

Table 2 Biological and epidemiological factors that influence the performance of GM detection in invasive aspergillosis[3]

Biological factors	*Epidemiological factors*
Site of infection	Patient population
Aspergillus species causing infection	Sampling strategy
Microenvironment at the site of infection: nutrients, oxygen level, pH	Definition of a positive result
Exposure to antifungal agents	Definition of an IFD
Molecular structure of released galactomannan	Prevalence of IFD
Underlying condition/neutropenia/ level of immunosuppression	Cutoff for positivity
Renal clearance, hepatic metabolism	Laboratory experience
Circulating galactomannan antibodies	Nutritional factors (galactomannan-containing food)
Storage of clinical sample	Treatment with semi-synthetic β-lactam antibiotics
Pre-analytical treatment procedure	

Marchetti et al BMT 2011

GM specificity

- Numerous GM-producing fungal species

Fungus
Acremonium species
Alternaria alternata
Botrytis tulipae
Cladosporium cladosporioides
Cladosporium herbarum
Cryptococcus neoformans
Fusarium oxysporum (but not *Fusarium solani*)
Geotricum capitatum
Paecilomyces variotii
Penicillium chrysogenum
Penicillium digitatum
Penicillium marneffei
Rhodotorula rubra
Trichophyton interdigitalis
Trichophyton rubrum
Wallemia sebi
Wangiella (Exophiala) dermatitidis

Aquino, VR et al, Mycopathologia, 2007

When facing a positive GM result

- Disease
  - To gather and to analyze EORTC/MSG criteria
- In parallel, explore the other possible sources
  - Antibiotics (test batches if necessary)
  - Other species than *Aspergillus* spp.
  - Mucites, gastrointestinal diseases
  - Intravenous products

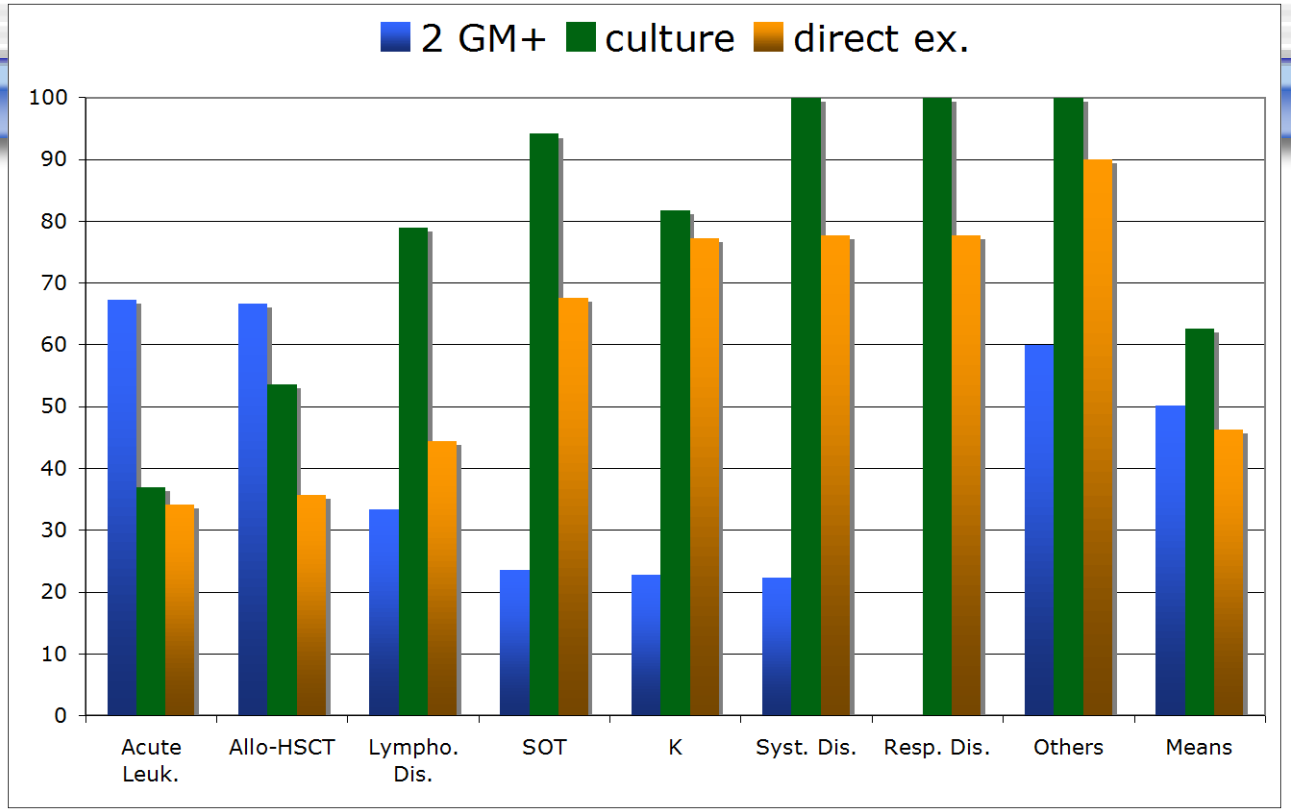
Factors that influence GM performance

Table 2 Biological and epidemiological factors that influence the performance of GM detection in invasive aspergillosis[3]

Biological factors	*Epidemiological factors*
Site of infection	Patient population
Aspergillus species causing infection	Sampling strategy
Microenvironment at the site of infection: nutrients, oxygen level, pH	Definition of a positive result
Exposure to antifungal agents	Definition of an IFD
Molecular structure of released galactomannan	Prevalence of IFD
Underlying condition/neutropenia/ level of immunosuppression	Cutoff for positivity
Renal clearance, hepatic metabolism	Laboratory experience
Circulating galactomannan antibodies	Nutritional factors (galactomannan-containing food)
Storage of clinical sample	Treatment with semi-synthetic β-lactam antibiotics
Pre-analytical treatment procedure	

Marchetti et al BMT 2011

Diagnostic means



Decrease of GM yield in Lymphoprolif. Dis. and other categories[1]

- poor performances in SOT [2]
- neutropenia/steroid ratio [3]
- diagnostic attitude for not performing culture when GM+?

(1) Lortholary et al CMI 2011 (2) Pfeiffer et al, CID, 2006 ; (3) Cordonnier et al, CMI 2009

Galactomannan indices in neutropenia (group 1; PMN < 100/mm3) vs non-neutropenia (groups 2+3; PMN≥100/mm3)

Galactomannan index	Group 1 (n = 17) PNN <100	Groups 2 + 3 (n = 81) PNN ≥ 100	p value
GM index ≥ 1	8 (44.4%)	8 (9.9%)	.001 [b]
GM index ≥ 0.7	8 (44.4%)	12 (14.8%)	.009 [b]
GM index ≥ 0.5	11 (61.1%)	15 (18.52%)	.001 [b]
GM index, mean ± SD	1.71 ± 1.99	.44 ± .75	.01 [a]
Steroid administration			
Yes	4.95 ± .64	.39 ± .44	.001 [a]
No	.72 ± .79	.47 ± .88	.19 [a]
Potentially GM contaminated antibiotic(s)			
Yes	2.6. ± 2.97	.64 ± 1.11	.76 [a]
No	1.44 ± 1.66	.33 ± .41	.003 [a]
Anti-mold therapy			
Yes	.76 ± .99	.43 ± .66	.694 [a]
No	2.1 ± 2.20	.44 ± .77	.006 [a]

NOTE. Data are no (%) of episodes
[a] p value of the non parametric Kruskall Wallis test
[b] p value of Fischer's exact test

C. Cordonnier et al, CMI 2009; Balloy et al Infect Immunity 2005; Stergiopoulou et al Am J Clin Pathol 2007

Galactomannan indices in neutropenia (group 1; PMN < 100/mm3) vs non-neutropenia (groups 2+3; PMN≥100/mm3)

Galactomannan index	Group 1 (n = 17) PNN <100	Groups 2 + 3 (n = 81) PNN ≥ 100	p value
GM index ≥ 1	8 (44.4%)	8 (9.9%)	.001 [b]
GM index ≥ 0.7	8 (44.4%)	12 (14.8%)	.009 [b]
GM index ≥ 0.5	11 (61.1%)	15 (18.52%)	.001 [b]
GM index, mean + SD	1.71 ± 1.99	.44 ± .75	.01 [a]
Steroid administration			
Yes	4.95 ± .64	.39 ± .44	.001 [a]
No	.72 ± .79	.47 ± .88	.19 [a]
Potentially GM contaminated antibiotic(s)			
Yes	2.6. ± 2.97	.64 ± 1.11	.76 [a]
No	1.44 ± 1.66	.33 ± .41	.003 [a]
Anti-mold therapy			
Yes	.76 ± .99	.43 ± .66	.694 [a]
No	2.1 ± 2.20	.44 ± .77	.006 [a]

NOTE. Data are no (%) of episodes
[a] p value of the non parametric Kruskall Wallis test
[b] p value of Fischer's exact test

C. Cordonnier et al, CMI 2009; Balloy et al Infect Immunity 2005; Stergiopoulou et al Am J Clin Pathol 2007

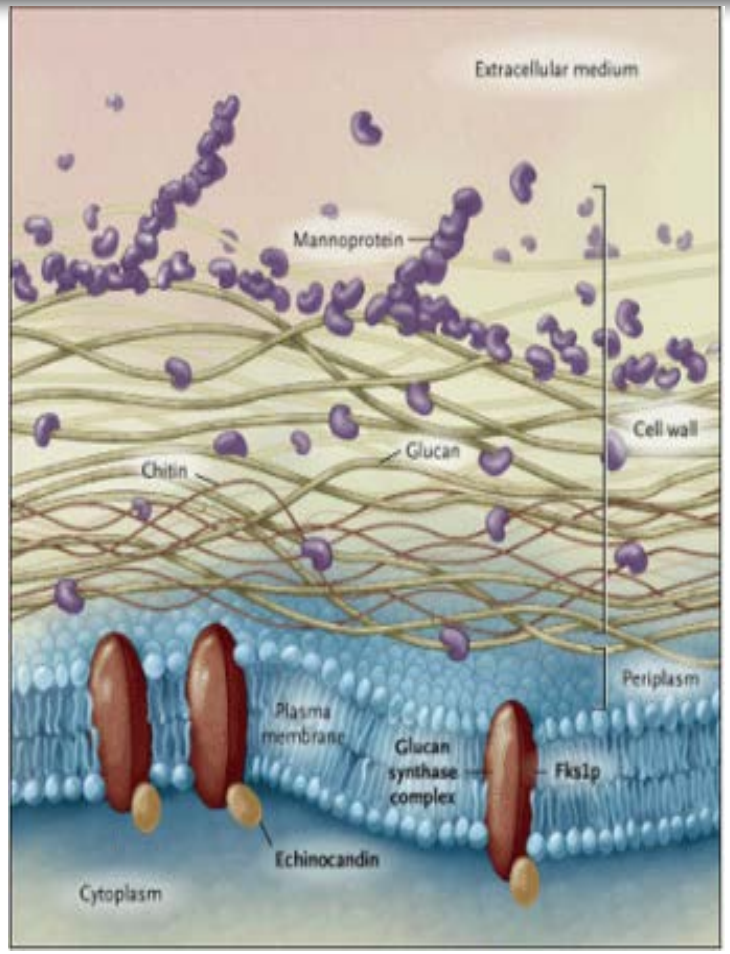
GM in BAL

- GM > 0.5 23/26 1st BAL
  - BAL procedure
  - 2 x 20 ml
- 6 false GM+ (13%)
  - Standards?
  - Colonization or infection?
- 15/26 positive direct examination or positive culture (58%)
  - direct examination or positive culture
- GM > 0.5 42% serum samples
  - Useful for follow-up
  - Less disputable meaning
- Reproducibility? Transfer in other centres?

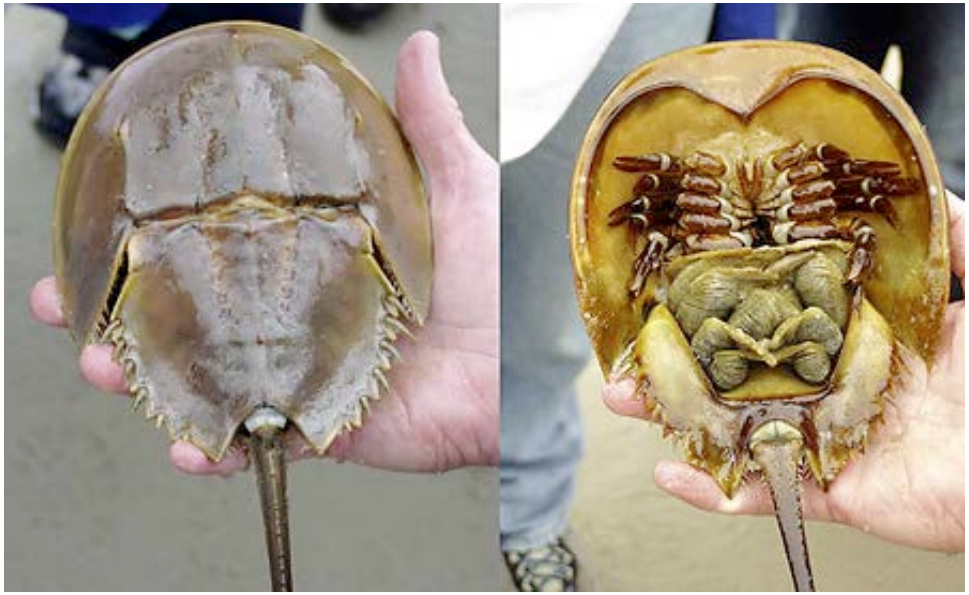
Meersseman W et al, Am J Resp Crit Care Med 2008

(1,3)beta-D-Glucan

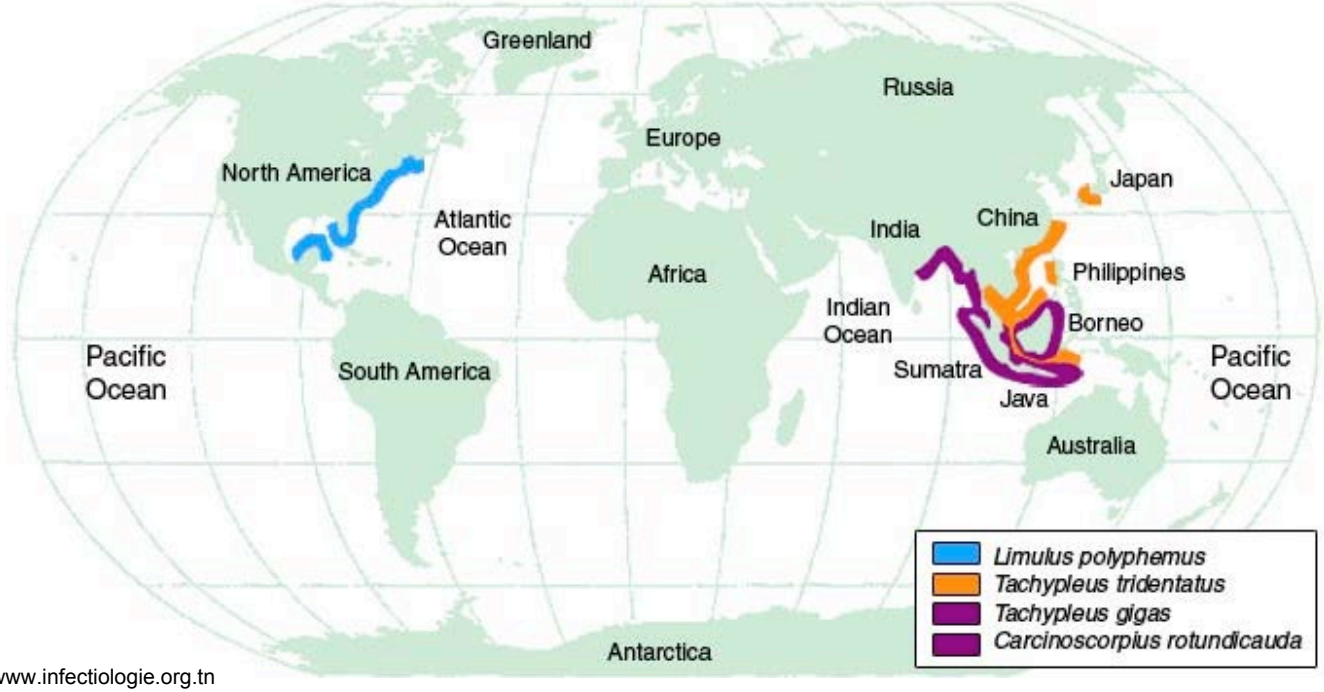
- Therapeutic target of echinocandins
- Ag common to most of the fungal species (excepted *Cryptococcus* spp and Mucorales)
  - *Candida, Saccharomyces, Aspergillus, Fusarium, Acremonium, …*
  - *Pneumocystis jirovecii*



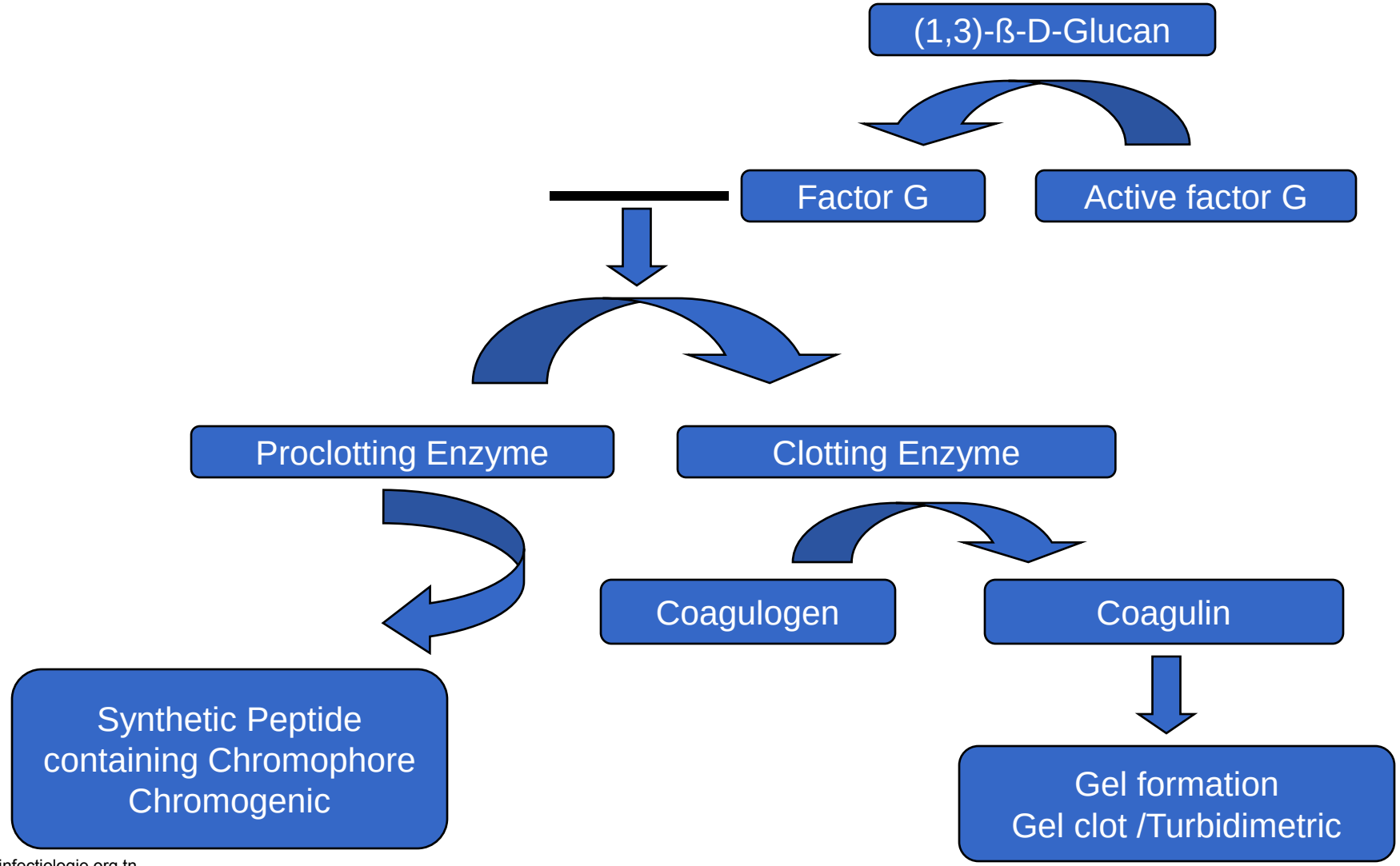
Bennet, NEJM, 2006

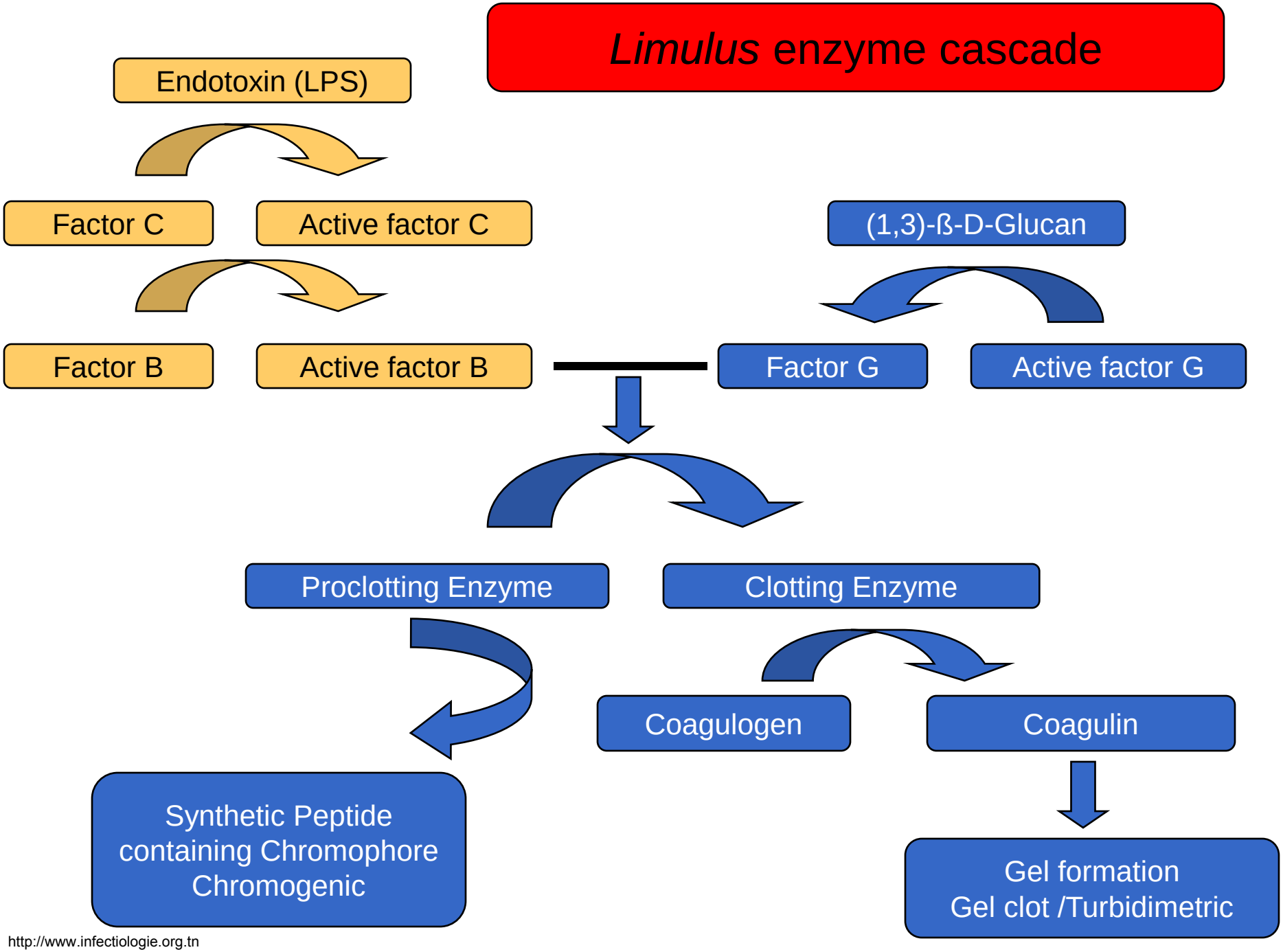


“horse-shoe crab”



Limulus enzyme cascade




Limulus enzyme cascade
Endotoxin (LPS)
Factor C
Active factor C
Factor B
Active factor B
(1,3)-ß-D-Glucan
Factor G
Active factor G
Proclotting Enzyme
Clotting Enzyme
Coagulogen
Coagulin
Synthetic Peptide
containing Chromophore
Chromogenic
Gel formation
Gel clot /Turbidimetric

4 different commercial kits

Table 3. Comparison of 4 commercial kits for the serum (1→3)-β-D-glucan (β-glucan) assay.

Variable	Fungitec G-Test MK	β-glucan Test Wako	B-G Star	Fungitell
Manufacturer	Seikagaku Corporation	Wako Pure Chemical	Maruha Corporation	Associates of Cape Cod
Country	Japan	Japan	Japan	USA
Approval year	1995	1996	2001	2004
Assay method	Kinetic chromogenic	Kinetic turbidimetry	Endopoint chromogenic	Kinetic chromogenic
Sample	Serum or plasma	Serum or plasma	Serum or plasma	Serum
Pretreatment	Alkali	Dilution and heating	Dilution and heating	Alkali
Standard β-glucan	Pachyman	Carboxymethyl-curdlan	Lentinan	Pachyman
Origin of lysate	*Tachypleus tridentatus*	*Limulus polyphemus*	*Tachypleus tridentatus*	*Limulus polyphemus*
Cutoff value, pg/mL	20	11	11	60 or 80
Measurable range, pg/mL	3.9–500	6–600	1.2–120	31.25–500
Turn-around time, min	30	90	30	40

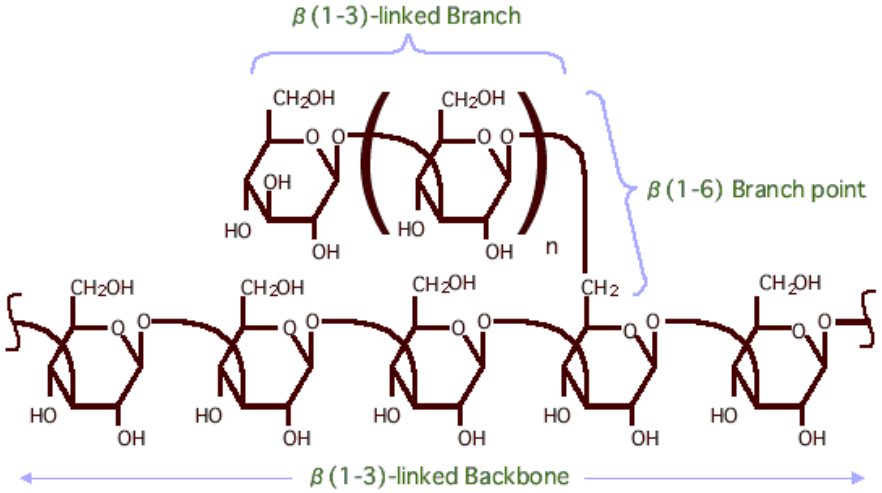
Obayashi et al CID 2008

False positives

Treatments	Immunoglobulines Albumin Coagulation factors Antibiotics (piperacillin – tazobactam) Others? (chemotherapies …)
Patient cares	Hemodialysis with cellulose membranes Gauze or other materials that contain glucans Tubes handling
Bacterial infections	Gram negative bacteria Some streptococci
Patient linked	Mucosal damages (yeast colonization) Hemolytic or lipemic samples …

Pickering JW et al, JCM 2005, 43 : 5957-62

Glucan



- β-glucans occur most commonly as cellulose in plants, the bran of cereal grains, the cell wall of baker's yeast, certain fungi, mushrooms and bacteria

- Some forms of beta glucans are useful in human nutrition as texturing agents and as soluble fiber supplements

Conclusions (EORTC / MSG)

- Included in EORTC/MSG criteria

De Pauw et al CID 2008

Candida versus *Aspergillus*

Table 3. Sensitivity of (1→3)-β-D-Glucan (BDG) Testing to Detect Proven or Probable Systemic *Candida* Infection in Comparison with Invasive Aspergillosis As Reported in Different Studies

Study	Cutoff(pg/mL)	Systemic *Candida* infections, proportion (%)	Invasive aspergillosis, proportion (%)
Hachem et al 2009 [27]	80 (2 consecutive values)	13/21 (62)	14/21 (67)
Koo et al 2009 [28]	80	26/41 (63)	24/32 (75)
Obayashi et al 2008 [19]	30	3/3 (100)	28/28 (100)
Persat et al 2008 [31]	80	22/26 (85)	48/70 (69)
Senn et al 2008 [32]	7 (2 consecutive values)	10/17 (59)	9/15 (60)
Akamatsu et al 2007 [33]	40	7/14 (50)	5/5 (100)
Ostrosky-Zeichner et al 2005 [36]	80	83/107 (78)	8/10 (80)
Odabashi et al 2004 [20]	80	9/11 (82)	4/4 (100)
Mori et al 1997 [43]	1000	11/12 (92)	4/4 (100)
Mitsutake et al 1996 [44]	60	27/32 (84)	5/5 (100)
Miyazaki et al 1995 [45]	10	11/11 (100)	3/3 (100)
Total from all studies[a]	...	222/295 (75)	152/197 (77)

[a] Total represents cumulative data

No difference between *Candida* and *Aspergillus* [1]

Performance similar to Galactomannan for invasive aspergilosis [2,3]

[1] *Karageorgopoulos et al CID 2011;* [2,3] *Leeflang MM et al Cochrane 2008; Pfeiffer CD et al CID 2006.*

β-D-glucan in ICU

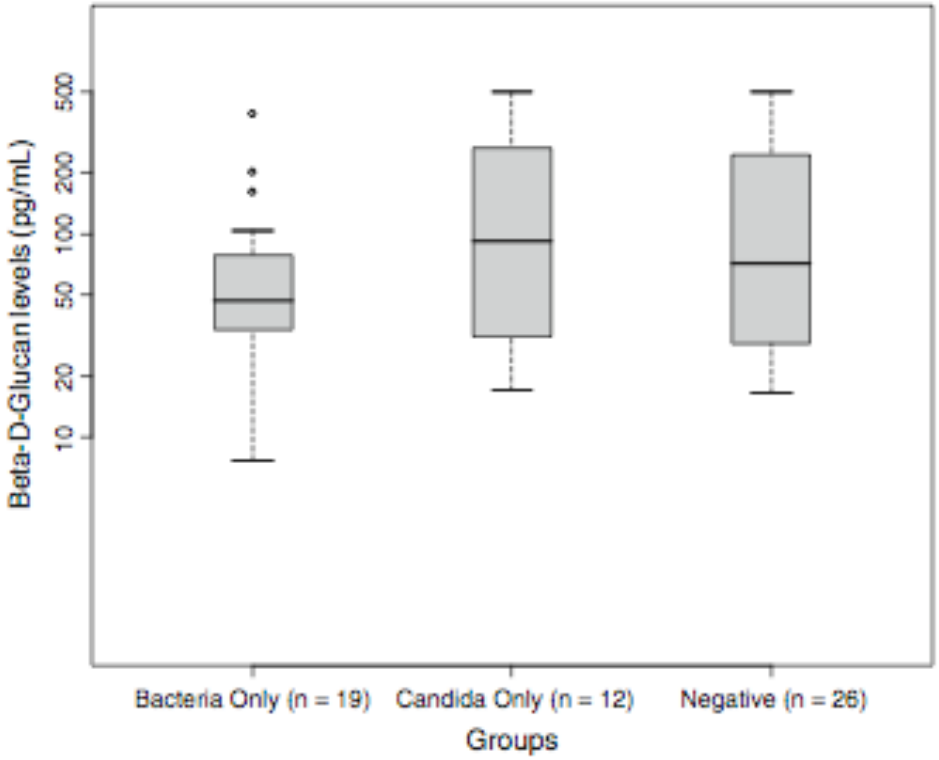


Fig. 2 Results of BG levels by culture group. Box-and-whisker plots of BG levels by culture groups. There were no significant differences across groups ($P = .57$ from the Kruskal-Wallis test).

No discrimination between bacterial sepsis, candida and others

Heyland et al, J Crit Care 2011

β-D-glucan metaanalyses

β-D-Glucan Assay for the Diagnosis of Invasive Fungal Infections: A Meta-analysis

Drosos E. Karageorgopoulos,[1,2] Evridiki K. Vouloumanou,[1] Fotinie Ntziora,[1,2] Argyris Michalopoulos,[1,3] Petros I. Rafailidis,[1,4] and Matthew E. Falagas[1,4,5]

[1]Alfa Institute of Biomedical Sciences; [2]Department of Medicine, Laikon General Hospital, and [3]Intensive Care Unit and [4]Department of Medicine, Henry Dunant Hospital, Athens, Greece; and [5]Department of Medicine, Tufts University School of Medicine, Boston, Massachusetts

β-Glucan Antigenemia Assay for the Diagnosis of Invasive Fungal Infections in Patients With Hematological Malignancies: A Systematic Review and Meta-Analysis of Cohort Studies From the Third European Conference on Infections in Leukemia (ECIL-3)

Frédéric Lamoth,[1,a] Mario Cruciani,[2,a] Carlo Mengoli,[3] Elio Castagnola,[4] Olivier Lortholary,[5,6,7] Malcolm Richardson,[8] and Oscar Marchetti,[1] on behalf of the Third European Conference on Infections in Leukemia (ECIL-3)

Karageorgopoulos et al CID 2011; Lamoth et al CID 2012

Warning: false+ and false-

- False +
  - Blood products (immunoglobulines, albumin)
  - Hemodialysis with cellulose membrane
  - Antibiotics (amoxicillin-clavulanate, piperacillin-tazobactam)
  - Bacterial sepsis
  - Gauze (surgery)
  - Severe mucitis
- False -
  - Antifungals (empirical, prophylaxis)
  - Glucan non-producing fungi (mucorales, *Cryptococcus*)
- **Skilled technicians**
  - Risk of contamination from the bed-side to the lab
  - Not easy-to-perform test

Karageorgopoulos et al CID 2011; Marchetti et al ECIL3

β-D-glucan and pneumocystosis

Blood (1→3)-β-D-Glucan as a Diagnostic Test for HIV-Related *Pneumocystis jirovecii* Pneumonia

Paul E. Sax,[1] Lauren Komarow,[2] Malcolm A. Finkelman,[3] Philip M. Grant,[4] Janet Andersen,[2] Eileen Scully,[1] William G. Powderly,[5] and Andrew R. Zolopa[4] for the AIDS Clinical Trials Group Study A5164 Team

[1]Division of Infectious Diseases, Department of Medicine, Brigham and Women's Hospital and Harvard Medical School, and [2]Harvard School of Public Health, Boston, [3]Associates of Cape Cod, East Falmouth, Massachusetts; [4]Division of Infectious Diseases and Department of Medicine, Stanford University School of Medicine, Stanford, California; and [5]School of Medicine, University College Dublin, Ireland

Sax et al CID 2011

ß-D-glucan titers according to microscopy and *P. jirovecii* qPCR BAL results

	Group 1 Microscopy-positive qPCR-positive BAL n=10 (%)	Group 2 Microscopy-negative qPCR-positive BAL n=26 (%)	Group 3 Microscopy-negative qPCR-negative BAL n=34 (%)	*P* values
Number with §-glucan >500 pg/mL (%)	10 (100)	6 (23)	2 (6)	<0.0001
Number with §-glucan >80 and ²5 00 pg/mL (%)	0	19 (73)	8 (24)	<0.0001
Number with §-glucan ² 80 pg/mL (%)	0	1 (4)	24 (71)	<0.0001

JM Costa et al, CID 2012

ß-D-glucan performance

- Thus, ß-glucan specificity decreased from 70.6% to 41.7% depending on whether the microscopy-negative and qPCR-positive BALs were considered as true-positives or false-positives, respectively.

JM Costa et al, CID 2012

A systematic literature review on the diagnosis of invasive aspergillosis using polymerase chain reaction (PCR) from bronchoalveolar lavage clinical samples

Felipe Francisco Tuon

- 15 articles out of 45
- Criteria:
  - Data for Ss and Sp
  - >10 BAL
  - EORTC criteria
  - Inclusions of control patients
- No condition on PCR assays and DNA extraction
- Mean Sensitivity: 79% (95% CI: 72.8-83.1)
- Mean Specificity: 94% (95% CI: 92.1-95.0)

Rev Iberoam Micol 2007; 24 89-94

A systematic literature review on the diagnosis of invasive aspergillosis using polymerase chain reaction (PCR) from bronchoalveolar lavage clinical samples

Felipe Francisco Tuon

- 15 articles out of 45
- Criteria:
  - Data for Ss and Sp
  - >10 BAL
  - EORTC criteria
  - Inclusions of control patients

Aspergillus DNA detected in BAL from 4/11 volunteers (36%)

- Mean Sensitivity: 79% (95% CI: 72.8-83.1)
- Mean Specificity: 94% (95% CI: 92.1-95.0)

Denning et al CID 2011

Use of PCR for diagnosis of invasive aspergillosis: systematic review and meta-analysis

Carlo Mengoli, Mario Cruciani, Rosemary A Barnes, Juergen Loeffler, J Peter Donnelly

- 16 publications (>10,000 samples; 1618 patients)
  - EORTC criteria
  - Prospective design
- Sensitivity: 75% ≥ 2+ PCR (95% CI: 54-88)
  88% ≥ 1+ PCR (95% CI: 75-95)
- Specificity: 87% ≥ 2+ PCR (95% CI: 78-93)
  75% ≥ 1+ PCR (95% CI: 63-84)
- Prerequisite on PCR assays
  - Previously “validated”
  - No constraint on PCR itself

C. Mengoli, Lancet Inf Dis, 2009

Use of PCR for diagnosis of invasive aspergillosis: systematic review and meta-analysis

Carlo Mengoli, Mario Cruciani, Rosemary A Barnes, Juergen Loeffler, J Peter Donnelly

	Sample type	Sample volume	Cell wall disruption*	DNA extraction methods*	PCR method†	Target gene	Appropriate controls		Minimum samples needed for positive PCR	Methods used (refs)
							Negative‡	Positive§		
Hebart et al (2000)[28]	Whole blood	5 mL	Zymolyase	QIAamp	PCR-ELISA	18S rRNA	Yes	Serial dilutions	1 and 2	36
Hebart et al (2000)[29]	WH								1	37
Williamson et al (2000)[36]	Ser								1	37
Buchheidt et al (2001)[21]	WH and								1	38
Ferns et al (2002)[25]	WH								1	39,40
Raad et al (2002)[33]	WH								1 and 2	39
Buchheidt et al (2004)[22]	WH and								1 and 2	38
Kawazu et al (2004)[31]	Pla								1 and 2	41
Lass-Floerl et al (2004)[32]	WH								1	37
Halliday et al (2006)[27]	WH								2	42
Jordanides et al (2005)[30]	WH								1	37,42
Scotter et al (2005)[34]	WH								1 and 2	38
El Mahallawi et al (2006)[24]	Ser								2	36
Florent et al (2006)[26]	Serum	..	QIAamp DNA Mini Kit	QIAamp	PCR-ELISA	mtDNA	Yes	*A fumigatus* DNA (10 conidia) or inhibition control (*S pyogenes*)	2	39
White et al (2006)[35]	Whole blood	2 mL	Mechanical, glass beads	MagNA Pure	RT-PCR with TaqMan and nested PCR	28S rRNA	Yes	Serial dilutions or cloned PCR products in serial dilutions	1	37
Cesaro et al (2008)[23]	Whole blood	3 mL	Zymolyase	QIAamp	RT-PCR with FRET	18S rRNA	..	..	1 and 2	42

A fumigatus=*Aspergillus fumigatus*. BALF=bronchoalveolar lavage fluid. mtDNA=mitochondrial DNA. SDS=sodium dodecyl sulphate buffer. *S pyogenes*=*Streptococcus pyogenes*. ..=not reported. *QIAamp, QIAGEN; MagNA Pure, Roche. Zymolyase, ICN or Sigma; Lyticase, Sigma; Phenol-chloroform, Sigma; glass beads, Sigma. †TaqMan uses hydrolysis probe for real-time (RT)-PCR; FRET (fluorescent resonance energy transfer) uses hybridisation probe for RT-PCR. ‡Sterile water, sterile buffer, or blood from healthy individuals. §Serially diluted *Aspergillus* DNA, serially diluted conidia, or internal control (eg, plasmid).

Table 2: **Technical details of the PCR methods used in the studies included**

Whole blood vs serum= 12/16

Volume = 10 ml-200 µl

Zymolyase or Lyticase = 13/16

Nested-PCR = 6/16

DNA target 18S rRNA = 11/16

…

No requirement for PCR test

Metaanalysis

JOURNAL OF CLINICAL MICROBIOLOGY, Feb. 2011, p. 665–670 Vol. 49, No. 2
0095-1137/11/$12.00 doi:10.1128/JCM.01602-10

PCR Diagnosis of Invasive Candidiasis: Systematic Review and Meta-Analysis[▽]†

Tomer Avni,[1]* Leonard Leibovici,[1] and Mical Paul[2]

- pooled sensitivity = 0.95 (95% CI: 0.88-0.98)
- pooled specificity = 0.92 (95% CI: 0.88-0.95)
- No requirement for the PCR itself

Table 1. MIQE checklist for authors, reviewers, and editors.[a]

Item to check	Importance	Item to check	Importance
Experimental design		qPCR oligonucleotides	
Definition of experimental and control groups	E	Primer sequences	E
Number within each group	E	RTPrimerDB identification number	D
Assay carried out by the core or investigator's laboratory?	D	Probe sequences	D[d]
Acknowledgment of authors' contributions	D	Location and identity of any modifications	E
Sample		Manufacturer of oligonucleotides	D
Description	E	Purification method	D
Volume/mass of sample processed	D	qPCR protocol	
Microdissection or macrodissection	E	Complete reaction conditions	E
Processing procedure	E	Reaction volume and amount of cDNA/DNA	E
If frozen, how and how quickly?	E	Primer, (probe), Mg^{2+}, and dNTP concentrations	E
			E
			E
			D
			E
			D
			E
			D
			E
			D
			E
			E
			E
			D
			E
			E
			E
			D
			E
			E
			E
			E
			E
			E
			D
			D
			E
Location of each primer by exon or intron (if applicable)	E	Software (source, version)	E
What splice variants are targeted?	E	C_q or raw data submission with RDML	D

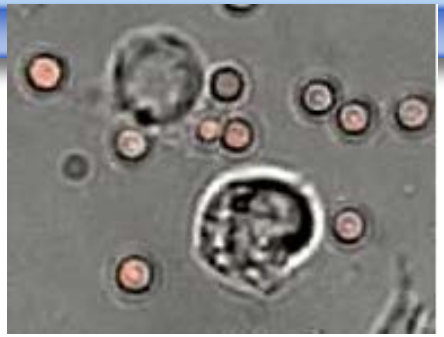
[a] All essential information (E) must be submitted with the manuscript. Desirable information (D) should be submitted if available. If primers are from RTPrimerDB, information on qPCR target, oligonucleotides, protocols, and validation is available from that source.
[b] FFPE, formalin-fixed, paraffin-embedded; RIN, RNA integrity number; RQI, RNA quality indicator; GSP, gene-specific priming; dNTP, deoxynucleoside triphosphate.
[c] Assessing the absence of DNA with a no–reverse transcription assay is essential when first extracting RNA. Once the sample has been validated as DNA free, inclusion of a no–reverse transcription control is desirable but no longer essential.
[d] Disclosure of the probe sequence is highly desirable and strongly encouraged; however, because not all vendors of commercial predesigned assays provide this information, it cannot be an essential requirement. Use of such assays is discouraged.

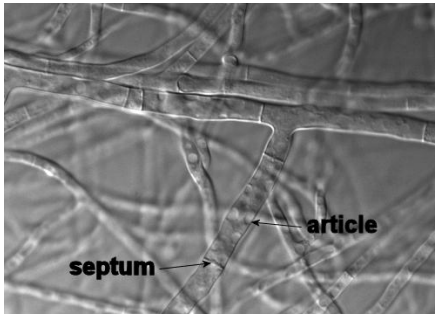
qPCR validation	
Evidence of optimization (from gradients)	D
Specificity (gel, sequence, melt, or digest)	E
For SYBR Green I, C_q of the NTC	E
Calibration curves with slope and *y* intercept	E
PCR efficiency calculated from slope	E
CIs for PCR efficiency or SE	D
r^2 of calibration curve	E
Linear dynamic range	E
C_q variation at LOD	E
CIs throughout range	D
Evidence for LOD	E
If multiplex, efficiency and LOD of each assay	E

Bustin SA, Clinical Chemistry 2009

Why these technical aspects so important?

- Origin of DNA
  - Conidia, hyphae, cell free DNA?
  - No rationale for DNA extraction and specimens
    - Fungus itself -> stringent DNA extraction
    - Cell-free DNA -> automated DNA extraction, simple protocols
- Numerous sources of false positives AND false negatives
  - Always very low DNA burdens





EAPCRI*: “Towards an European Standard for Aspergillus-PCR”

- P. Donnelly, J. Löffler, L. White
  - 2006-2007: 24 laboratories (all of them use qPCR)
- PCR amplification methods are very consistent in their performance
  - 95% of methods detected the predicted 100% threshold
  - *Aspergillus* gene target, PCR platform does not seem to matter
- Wide variation in the performance of extraction methods
  - Use of larger volumes of blood correlated with better performance: at least 4 ml EDTA blood should be used
  - Bead-beating methods performed optimally when testing QC panel
  - Specimen validation using animal models
  - Performance in clinical specimens?

*The European *Aspergillus* PCR Initiative

Lewis White et al, JCM 2010 and 2011

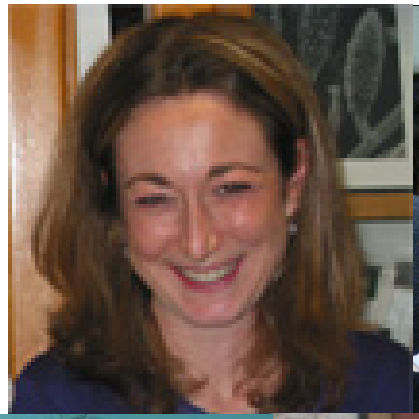
Goals for biomarkers

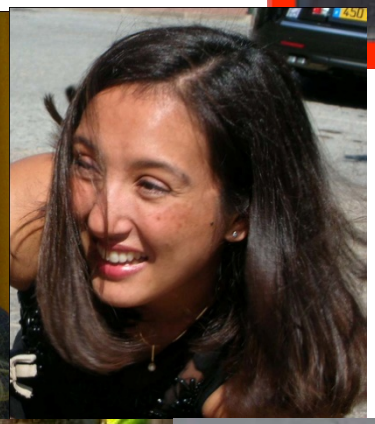
- Two ways of using biomarkers
  - Diagnostic tools
    - Low sensitivity, meaning for opportunistic diseases
  - Defining an optimal risk-based strategy minimizing the risks of both invasive fungal diseases and over-treatment

Conclusion

- Direct examination and culture far to be outdated
  - Identification
  - MIC
  - Resistance mechanisms
  - Prognosis (with GM)
- GM
  - Define your objectives (Diagnostic or screening)
  - If screening, prevalence of the disease should be >5%
- Glucan
  - *Pneumocystis*
- To program regular reappraisals
  - epidemiological trends, new treatments, new markers …







Institut Pasteur