Diagnosis for systemic fungal infections – non-culture based methods

Arunaloke Chakrabarti
Professor & In-Charge
Center for Advanced Research in Medical Mycology
& WHO Collaborating Center
Professor & Head, Department of Medical Microbiology
Postgraduate Institute of Medical Education & Research
Chandigarh – 160012, India

Novel diagnostic techniques

- Rapid, sensitive, specific low turn-around time

- **Non-culture based**
  - Antibody/Antigen detection
  - β-D-glucan detection
  - Metabolite detection
  - PCR

Antibody detection

- Helps in endemic mycoses (histo, blasto, cocci, paracocci, sporo) in immunocompetent hosts
- But, not useful for opportunistic fungal infections in immunocompromised hosts
- Can be used in *Candida* endocarditis

Antigen detection

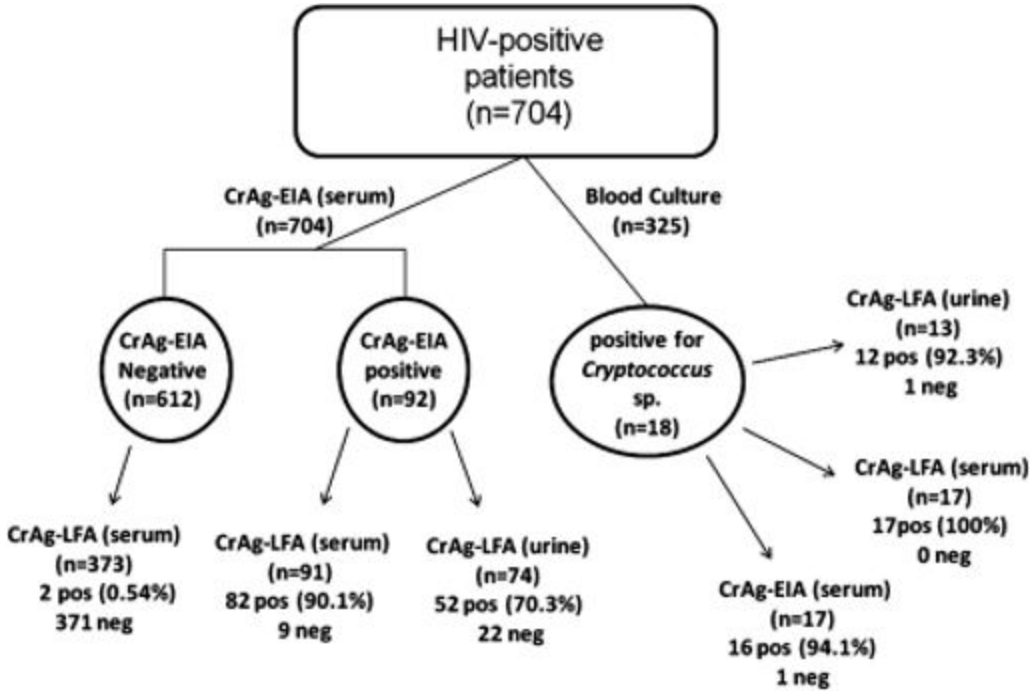
- Cryptococcosis (LA, ELISA) – excellent
- Histoplasmosis (RIA, ELISA) – very good
- Candidiasis
  - Mannan (LA, ELISA) – promising
  - Enolase (48kD, ELISA) – promising
  - 47kD (broken down product of HSP90) – variable result
  - Secretory aspartyl proteinase – variable result
  - Cand-Tec, Cand-Tec MT – sensitivity 33-71%
- Aspergillosis
  - Galactomannan (LA, ELISA) – promising

Ag detection in Cryptococcosis

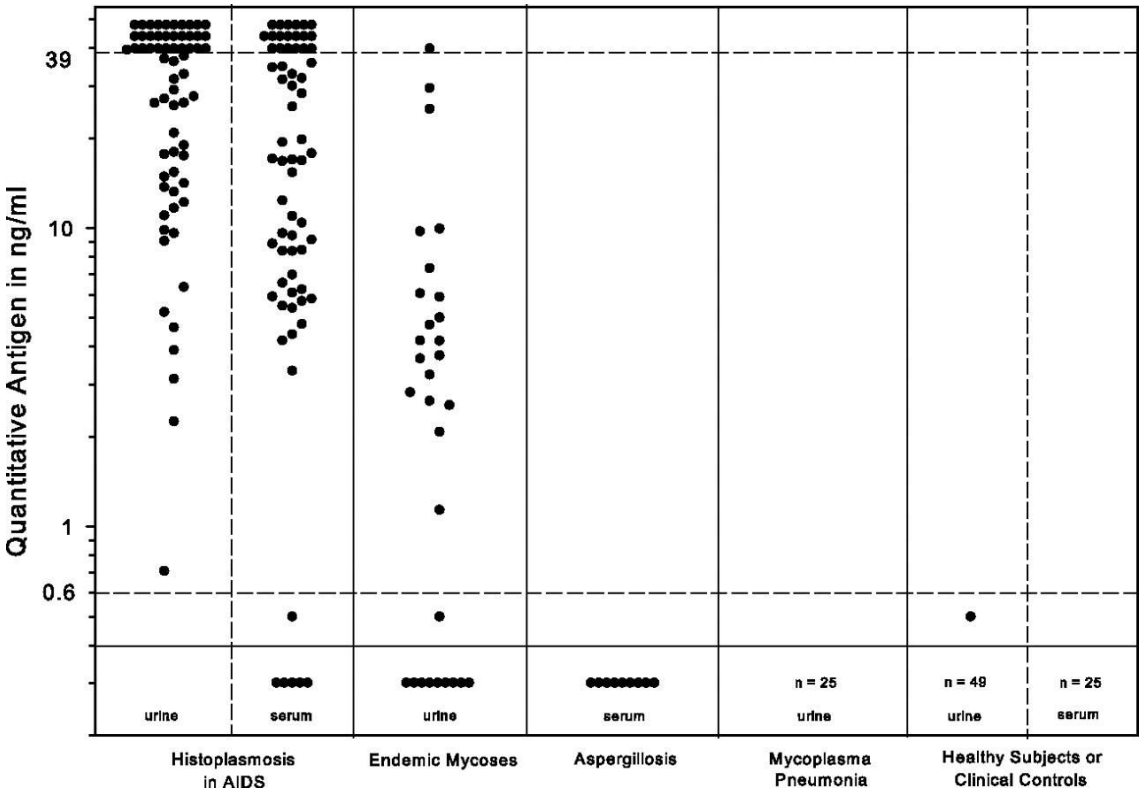
Evaluation of a Newly Developed Lateral Flow Immunoassay for the Diagnosis of Cryptococcosis

Mark D. Lindsley,[1] Nanthawan Mekha,[2] Henry C. Baggett,[3] Yupha Surinthong,[2] Rinrapas Autthateinchai, Pongpun Sawatwong,[3] Julie R. Harris,[1] Benjamin J. Park,[1] Tom Chiller,[1] S. Arunmozhi Balajee,[1] and Natteewan Poonwan[2]

Clinical Infectious Diseases 2011;53(4):321–325



Ag detection in histoplasmosis



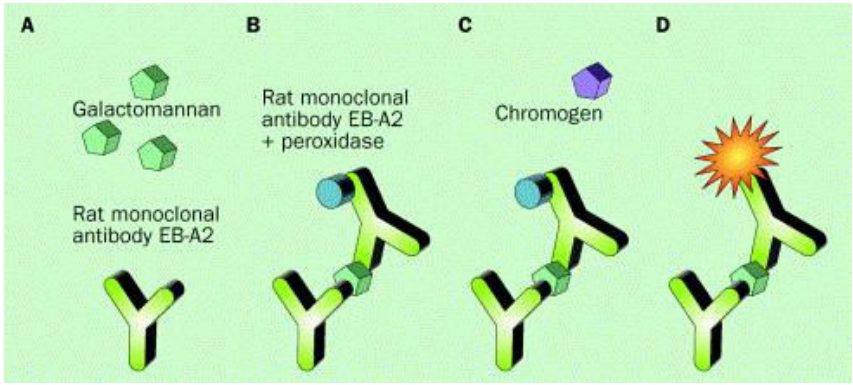
Type of disease	Sensitivity (%)
Disseminated	90-95
Acute pulmonary	80
Chronic pulmonary	20%

Connolly *et al.* Clin Vaccine Immunol 2007; 14: 1587-91

IC – mannan & anti-mannan detection

- **Combined mannan/anti-mannan** (Platelia, Bio-Rad) – meta-analysis of 14 studies – sensitivity (83%), specificity (86%) [Mikulska *et al*. Crit Care 2010; 14: R222]
- Sensitivity best for *C. albicans* (80-100%), intermediate for *C. tropicalis* & *C. glabrata*, and lowest for *C. parapsilosis* & *C. krusei* (40–50%) [Mikulska *et al.* Crit Care 2010; 14: R222]

Galactomannan for invasive aspergillosis

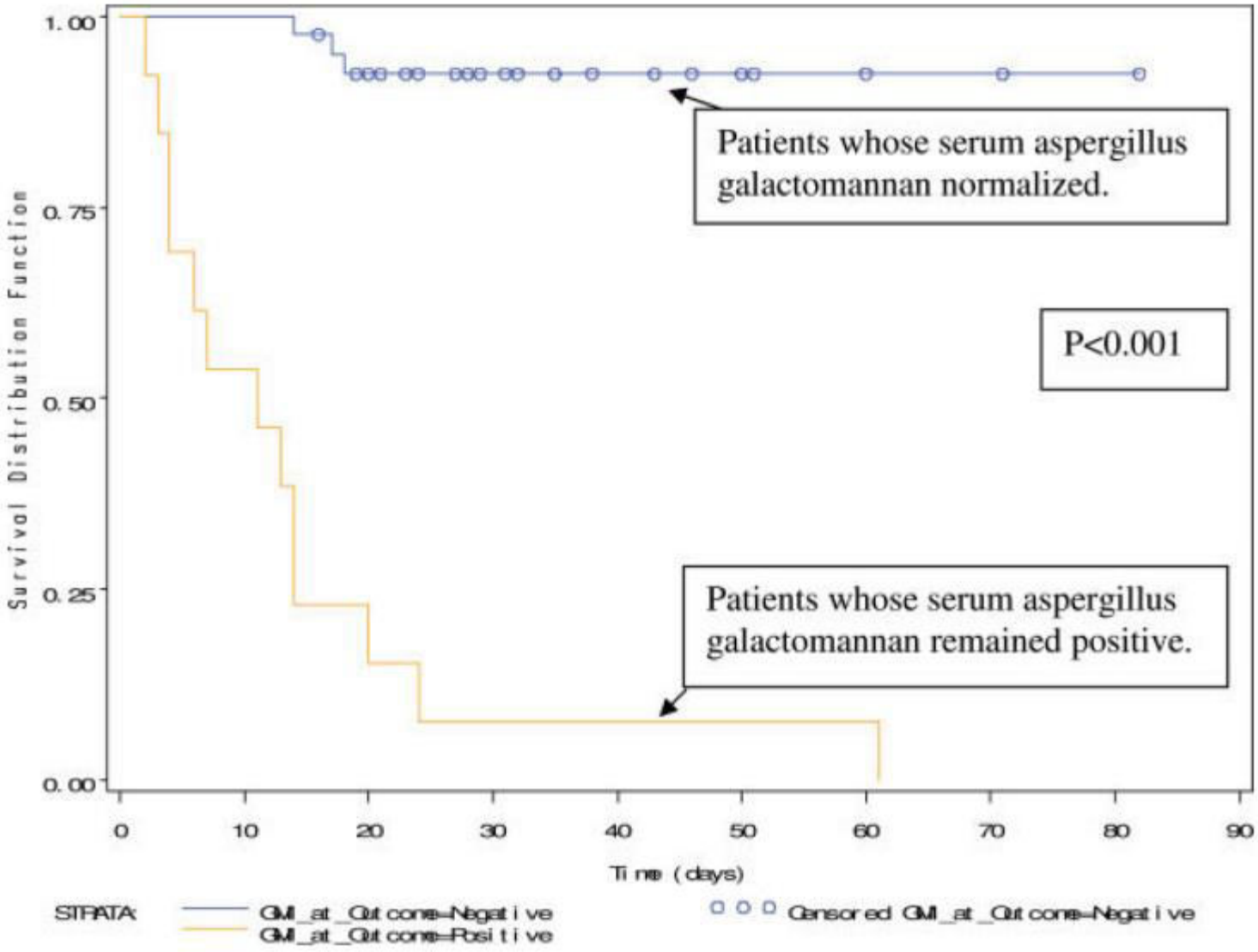


- Cell wall component of *Aspergillus* spp., though present in other fungi
- Microbiological criterion for probable IFI in EORTC/MSG
- Cut-off value of GMI - ?1.5 or 0.5
- **May be utilized to exclude IA, rather than confirming it**
- **May be detected 5-8d before clinical/radiological findings**
- Meta-analysis (27 studies during 1966-2005) – sensitivity-71%, specificity-89% (CID 2006; 42: 1417)

Galactomannan detection in BAL

- In hematological malignancy patients. Marteans *et al.* (2009) & Niguyen *et al.* (2010) showed sensitivity – 91% (cut-off – 0.85) & 70% (cut-off-1.0), specificity - >90%
- GM detection in serum little value in non-neutropenic, as neutrophil clears GM by mannose-binding receptor (Mannink-Kersten et al. Lancet Infect Dis 2004; 4: 349-57)
- Meersseman *et al.,* 2008 evaluated 72 non-neutropenic ICU patients, BAL sample with GM cut off 0.5 – sensitivity 88%, specificity 87%
- Standardization of BAL protocol is an issue
- GM recommended in serial samples, problem in BAL

GMI predicts outcome



Cancer 2007; 110: 880

Galactomannan test

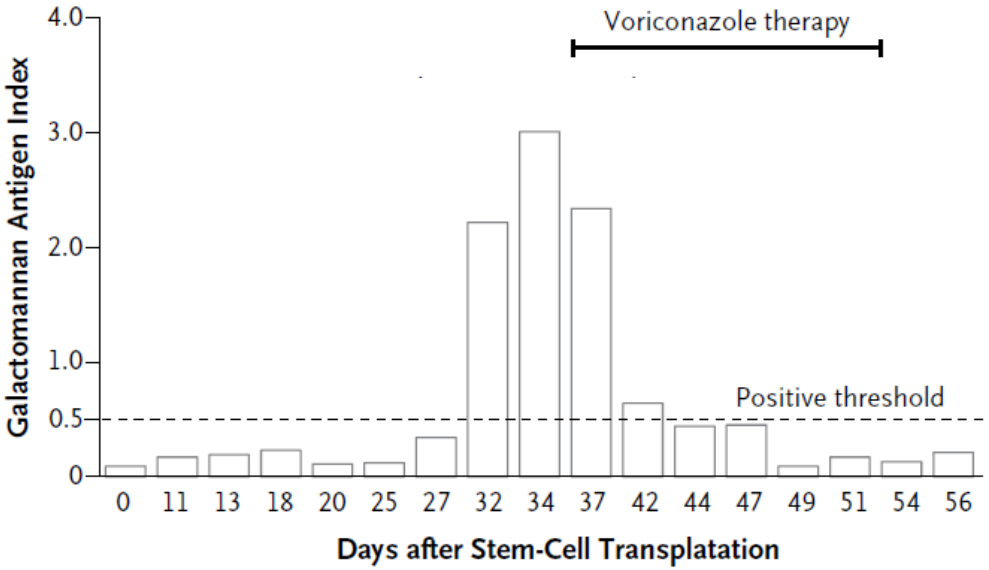
False-negative

- Previous antifungal exposure
- Current antifungal therapy
- Inappropriate diagnostic criteria for IA
- Low frequency of testing
- Cut-off value too high
- Disease of low severity
- Low volume of sampling
- Long storage of samples
- Non-neutropenic patients

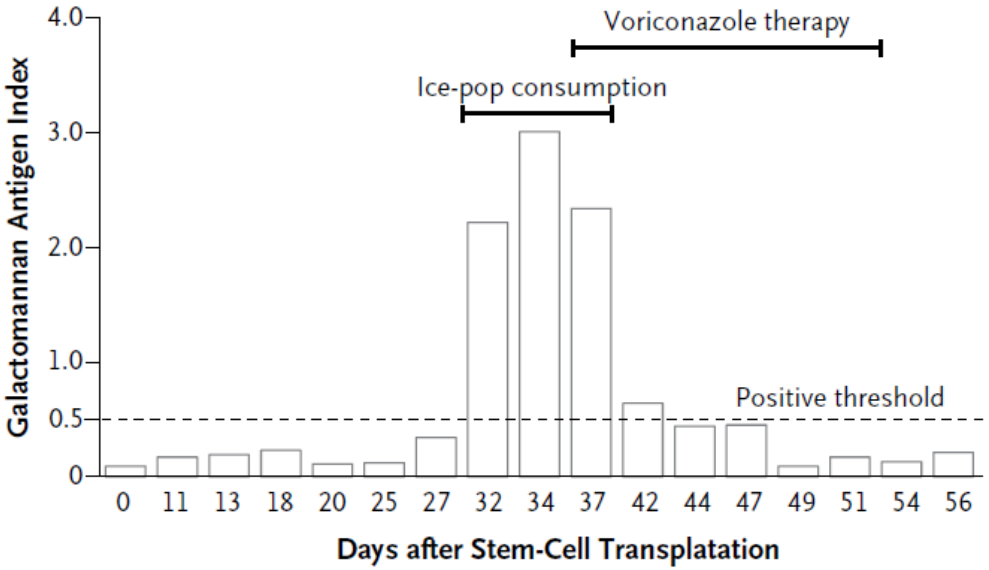
False-positive

- **Use of antibiotics**
- Pediatrics & neonates
- Infection by certain fungi
- Dialysis
- Autoantibodies
- Bacteraemia
- Plasmolyte (sodium gluconate)
- Contamination with cotton swab
- Multiple myeloma

- 42y-F – HLA matched HSCT from unrelated donor for myeloproliferative disorder
- Serum GM accessed twice weekly from Day 0 of Tx
- GM index increased to 2.22 & 3.01 on D32 & D34
- At that time she had GVHD
- But, she was afebrile with no pulmonary/sinus symptoms
- CT scan of brain, sinus, abdomen – normal
- Voriconazole started on D35



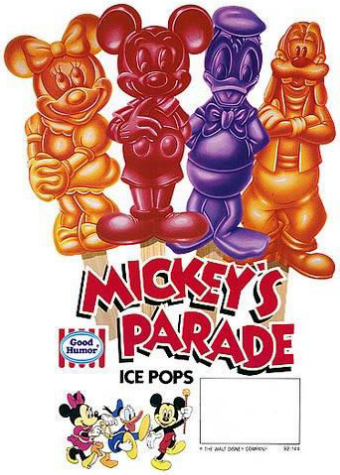
- 42y-F – HLA matched HSCT from unrelated donor for myeloproliferative disorder
- Serum GM accessed twice weekly from Day 0 of Tx
- GM index increased to 2.22 & 3.01 on D32 & D34
- At that time she had GVHD
- But, she was afebrile with no pulmonary/sinus symptoms
- CT scan of brain, sinus, abdomen – normal
- Voriconazole started on D35

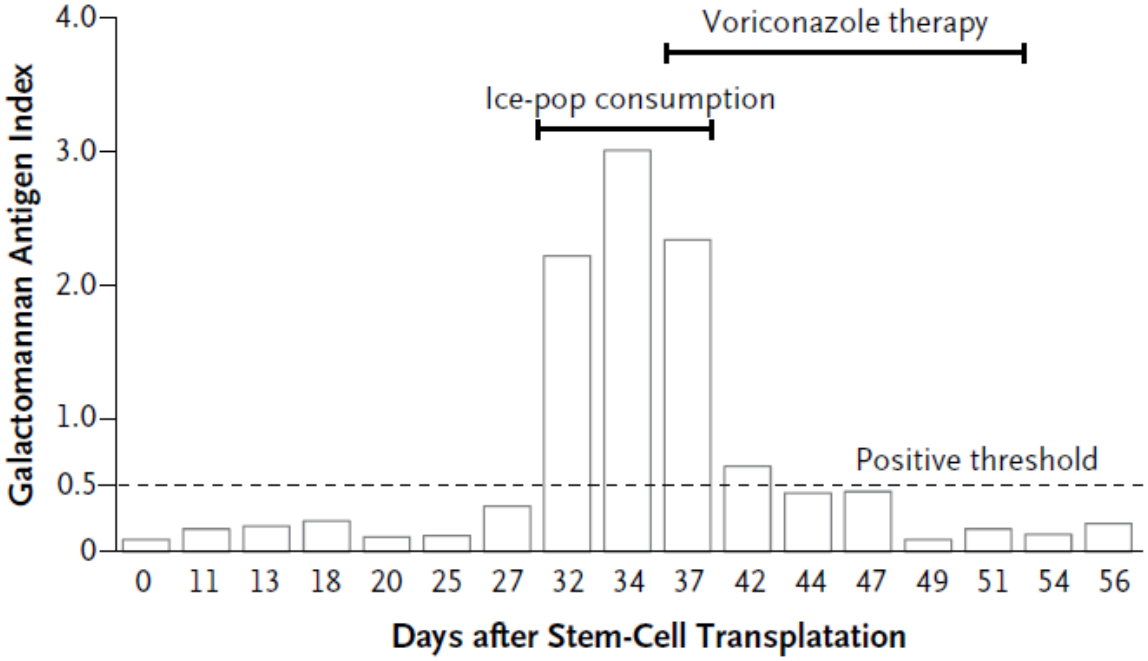


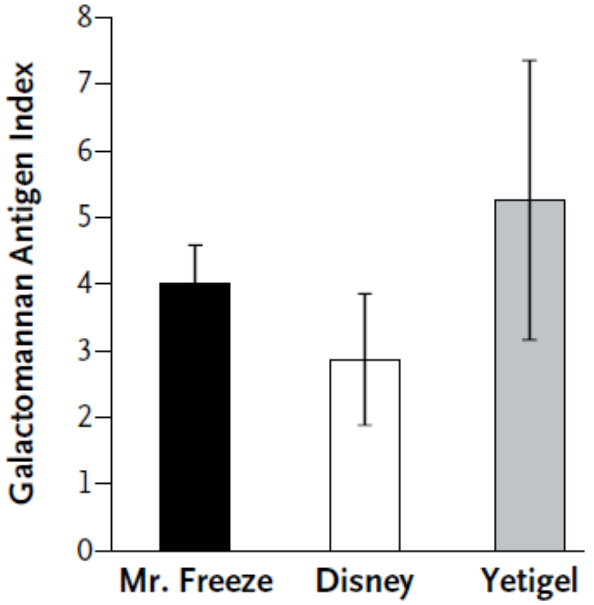
False Positive Galactomannan Test after Ice-Pop Ingestion

nicolas.guigue@sls.aphp.fr

N ENGL J MED 369;1 NEJM.ORG JULY 4, 2013







Galactomannan Testing for Early Diagnosis of *Exserohilum rostratum* Infection

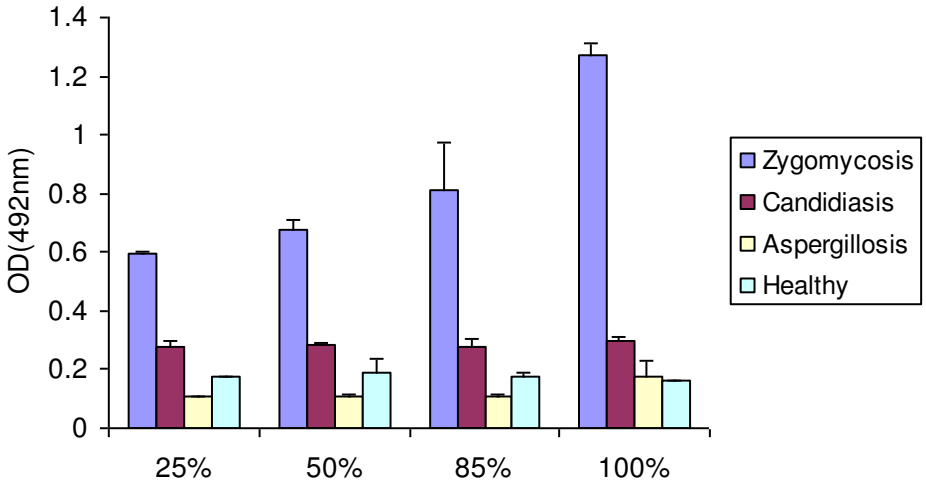
Maya Korem, Itzhack Polacheck, Ayelet Michael-Gayego, Jacob Strahilevitz

J. Clin. Microbiol. **2013, 51(8):2800.**

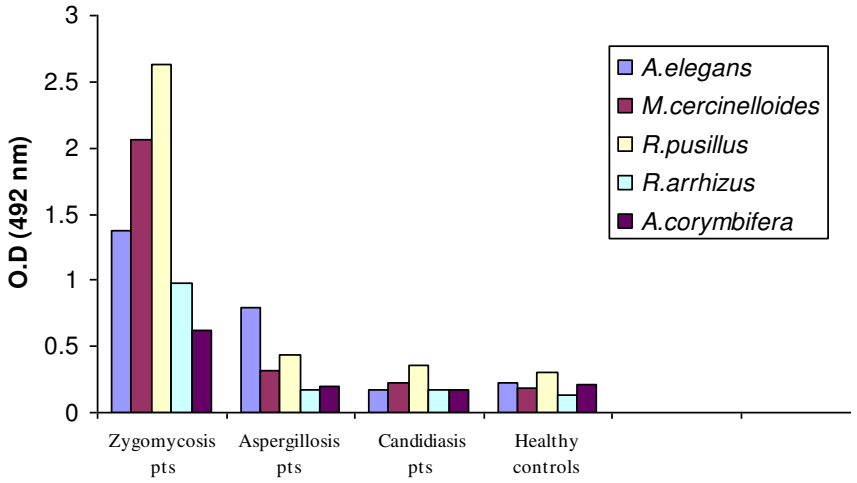
Sample	Galactomannan ODI[a] for sample with:	
	No dilution	10^{-3} dilution
Serum		
Upon diagnosis	2.25	
After 2 wk of treatment	1.5	
After 3 wk of treatment	0.95	
4 wk following neutrophil recovery	0.52	
Fungal extract		
Exserohilum rostratum (our case)	5.08	2.01
Aspergillus fumigatus (ATCC 64026)	5.02	1.85

[a] ODI, optical density index.

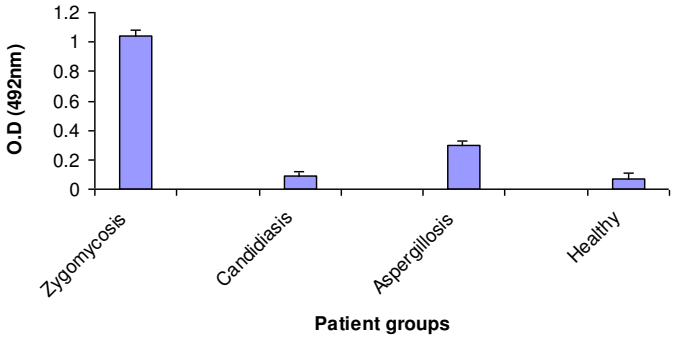
Some attempt in mucormycosis



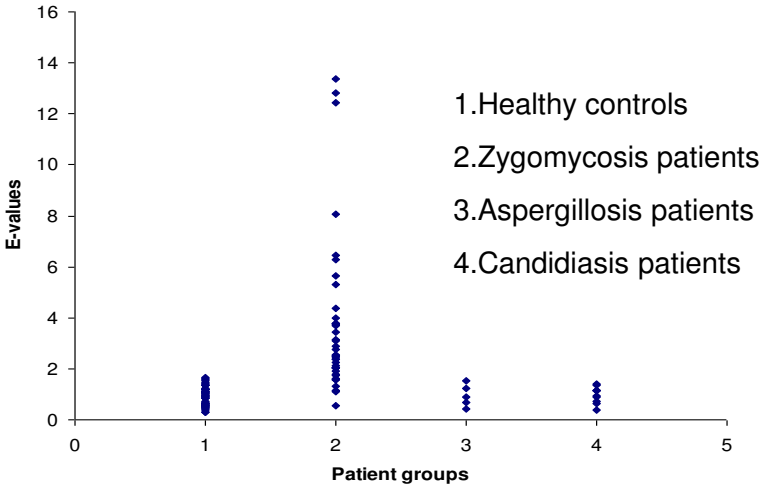
Different ammonium sulfate precipitated fraction against sera



85-100% fraction of different species against sera



Sera raised in rabbit against 85-100% fraction

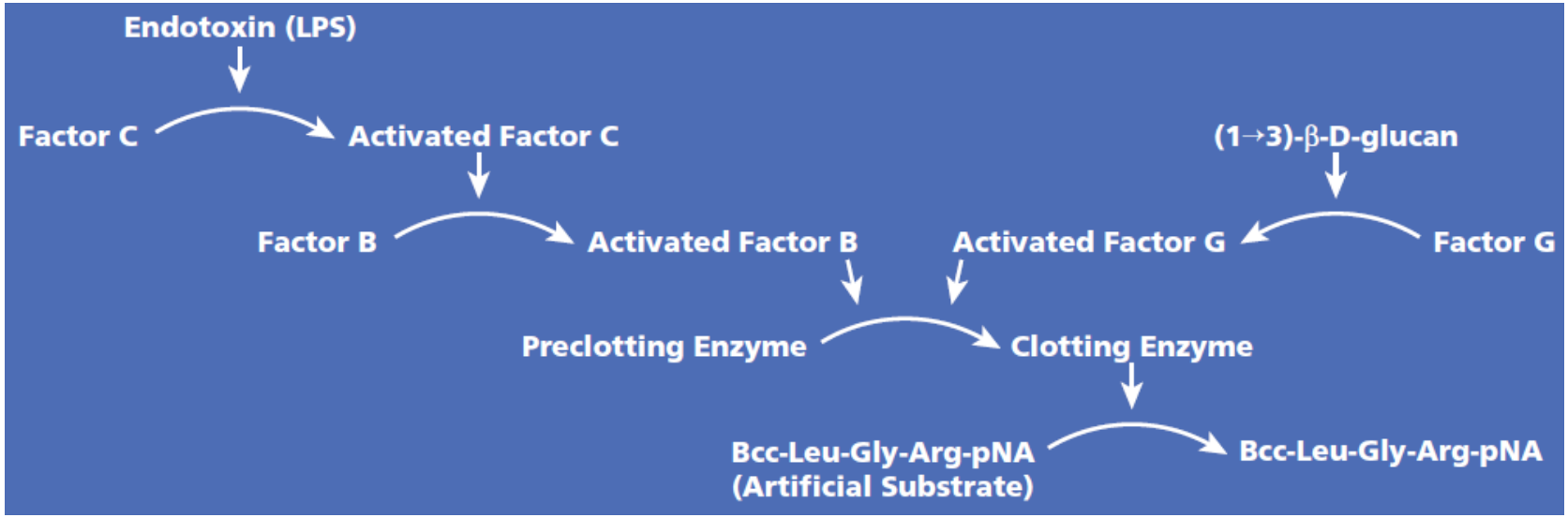


Antibody detection using purified Ag

1,3-β-D-glucan detection

Fungitec G-test (Seikagaku Corp., Japan)

Glucatell test (Associates of Cape Cod, USA)



Comparison of β-D-glucan assay kits

Kit	Fungitec G test-MK	BG STA β-glucan test	β-glucan test WAKO	Endosafe-PTS-gulcan	Fungitell
Manufacturer	Seikagaku corp. (Japan)	Maruha (Japan)	WAKO pure chemicals (Japan)	Charles River Lab. (USA)	Associates of Cape Cod Inc. (USA)
Lysate	*Tachypleus tridentatus*	*Tachypleus tridentatus*	*Limulus polyphemus*	*Limulus polyphemus*	*Limulus polyphemus*
Method	Kinetic chromogenic	Kinetic chromogenic	Kinetic turbidometric	Kinetic chormogenic	Kinetic chromogenic
Detection range (pg/mL)	3.9-500	10-1000	6-600		31.25-500
Cutoff value (pg)	20	20	11	11	60-80
FDA approval	No	No	No	No	Yes

1,3-β-D-glucan detection

Advantages

- Non-invasiveness of test
- Possibility of early diagnosis
- High sensitivity & specificity
- High –ve predictive value

  (eliminate IFIs)

1,3-β-D-glucan detection

Advantages

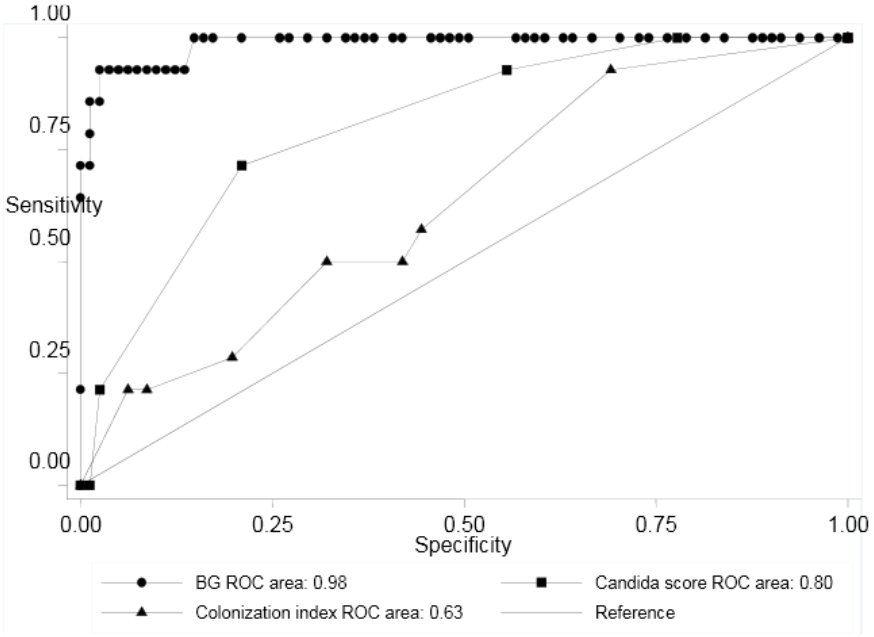
- Non-invasiveness of test
- Possibility of early diagnosis
- High sensitivity & specificity
- High –ve predictive value (eliminate IFIs)

Disadvantages

- Non-specificity, cannot identify pathogen
- Proneness to false-+ve results (contamination with cellulose-based dialysates, certain antibiotics, drug containing glucan, gauze, serious bacterial infections, immunoglobulins or albumin, environmental fungi)
- User unfriendliness –send out to reference lab
- Many clinicians are not convinced with the results

Comparison of BDG, CI, CS in ICU

Parameters	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
BG cut off value, 80pg/ml	92.9	93.7	72.2	98.7
Candida score ≥3	85.7	88.6	57.1	97.2
Colonization index ≥0.5	64.3	69.6	27.3	91.7

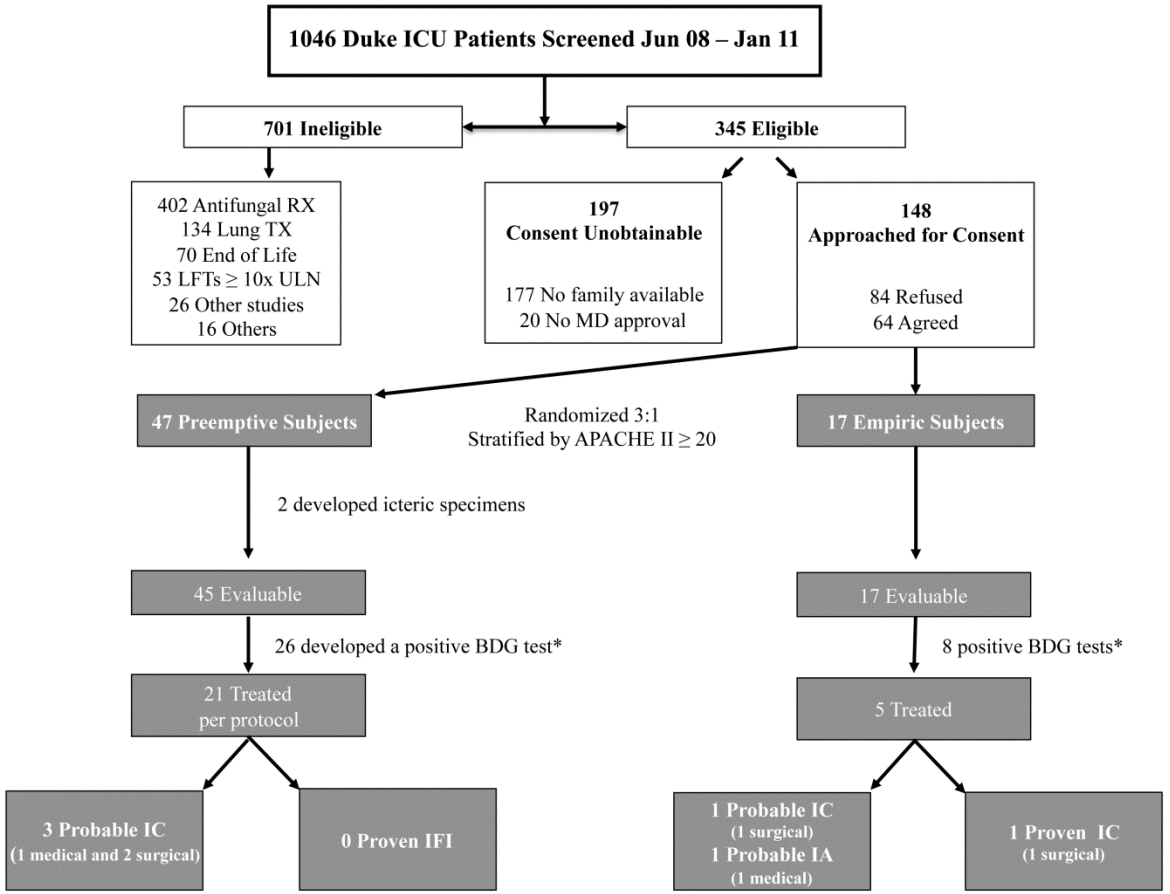


BDG detected IC 1-3d before blood culture positive

Posteraro *et al*. Critical Care 2011; 15: R 249

BDG test in ICU

- Single test with >80pg/mL – sensitivity-97%, specificity-20% (Tissot *et al.* ICCAC 2010, Boston, 2010)
- Low specificity due high prevalence of bacterial sepsis, albumin infusion, dialysis, contact with gauze
- Two tests with >150pg/mL – sensitivity-73%, specificity-78%
- BDG rise at least 2 days before clinical suspicion



Pre-emptive arm
- Less antifungal use
- 6% proven/probable IC

Empiric arm
- More antifungal use
- 18% proven/probable IC

Hanson KE *et al.* PLoS One 2012; 7: e42282

Metabolite detection

- Diagnostic & prognostic significance
- D-arabinitol in invasive candidiasis, D-mannitol in cryptococcosis & invasive aspergillosis
- Methods

  -Gas liquid chromatography(GC)

  -GC-mass spectrometry (GC-MS)

  -Enzymatic - fluorometric

  -Enzymatic – colorimetric

  -Spectrofluorometrically in COGAS FARA II auto-analyzer (Roche)

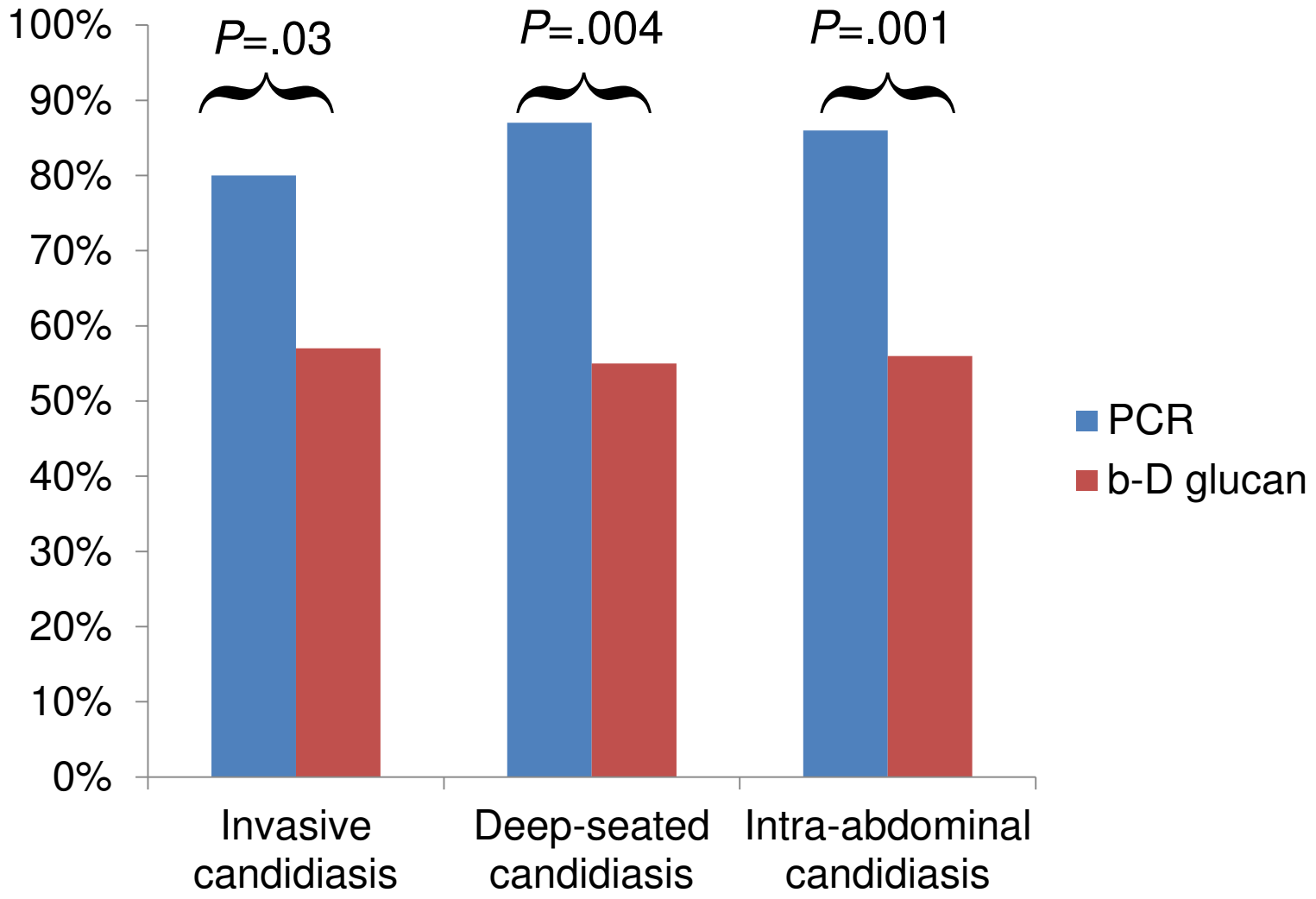
Nucleic acid based detection tools

- RNA based detection assay
  - NASBA – Nucleic Acid Sequence Based Amplification (LOD – 1cfu)
- Isothermal reaction
  - RCA – Rolling Circle Amplification
    - Padlock probe & two primer pairs, used on isolates only
    - Differentiate closely related species, can detect SNPs
    - Can be moved into microarray assay functioning at constant temperature
  - LAMP – Loop mediated isothermal amplification
    - Used in detection of *P. brasiliensis, O. gallopava, P. marneffei* in tissue

Nucleic acid based detection tools

- PCR based detection assay
  - Real time PCR or qPCR or RTQ-PCR
  - High resolution melting curve experiment with new fluorescent dye (EvaGreen, less toxic on polymerase)
    - Melting point & melting behaviour detected
    - Detect SNPs
- Several issues – low fungal DNA, contamination, validation
- Serious attempt only in IA by European Aspergillus Initiative
- No serious attempt yet for IC and mucormycosis

Sensitivity of PCR & β-D-glucan detection



Nguyen *et al.* Clin Infect Dis 2012; 54: 1240-8

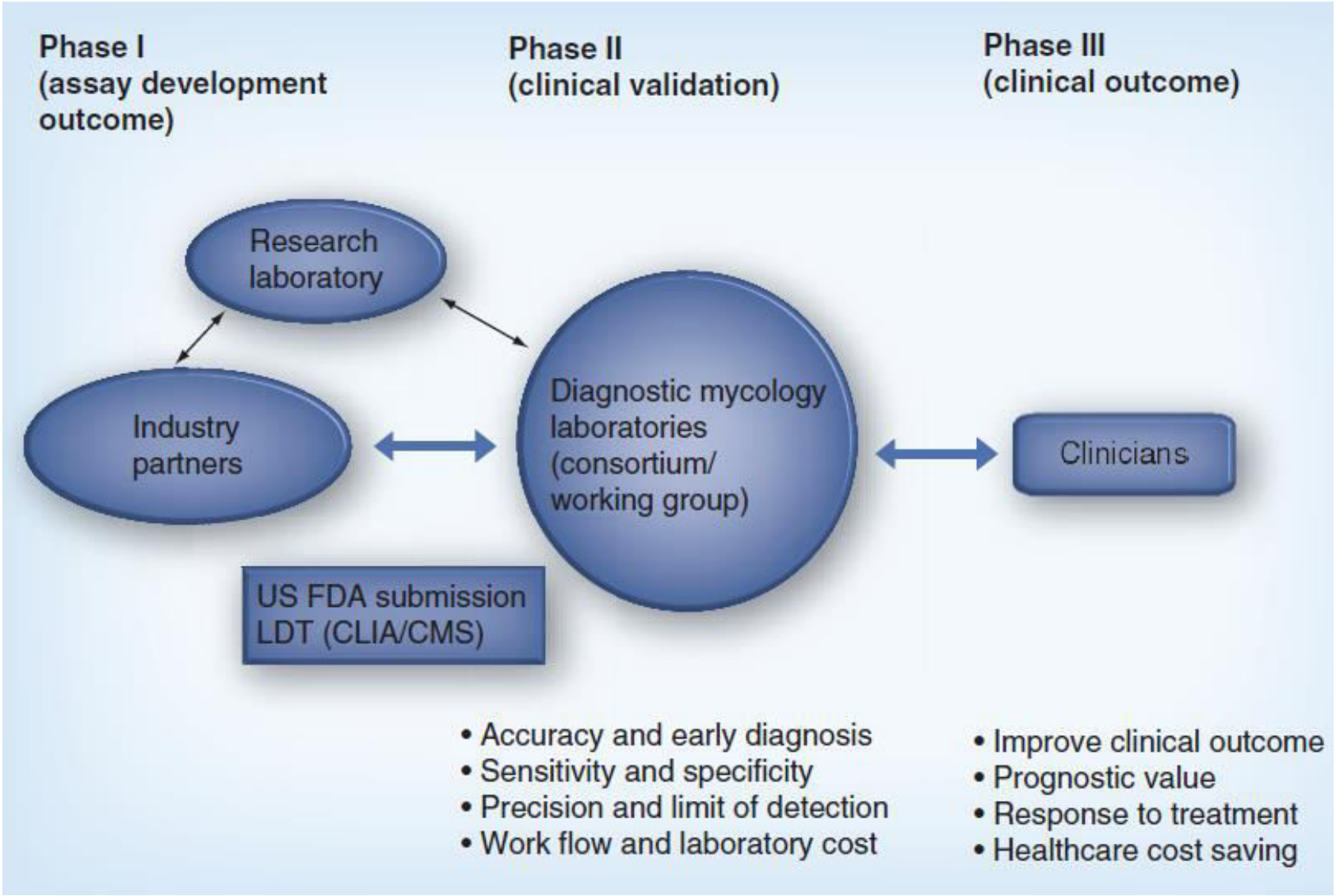
DNA detection – technical issues

- Set up a PCR – need to know DNA sequence to be amplified
- **Implementation for routine use – much more difficult**
- Two major issues – fungal DNA load low, contamination
- To avoid contamination (**fungal spore & DNA in environment & reagents**)
  - Uracyl-N-glycosylase can cut previously amplified product
  - Use real time quantitative PCR
  - **Manipulation under laminar flow** – avoid spore but not DNA
  - Lot of commercial enzymes are produced by fungi
    - Limit use of unnecessary reagents
    - **Commercial tubes containing anticoagulants may have fungal DNA (18%)**
    - Negative extraction control

DNA detection – technical issues

- **Control of amplification yield (avoid PCR inhibitors)** – same result in every test
  - Commercial DNA extraction kits remove residual PCR inhibitors
  - **10-20% tubes used for blood collection may have PCR inhibitors**
  - Amplification performance monitored by internal control
- **Human DNA yield is much higher than the fungal DNA**
  - Low amplification yield may give positive signal
  - Specific control for each primer set
  - Use heterogeneous DNA (plasmid, virus, mouse DNA) as internal control
    - As quantity of control known – identical result in absence of PCR inhibition

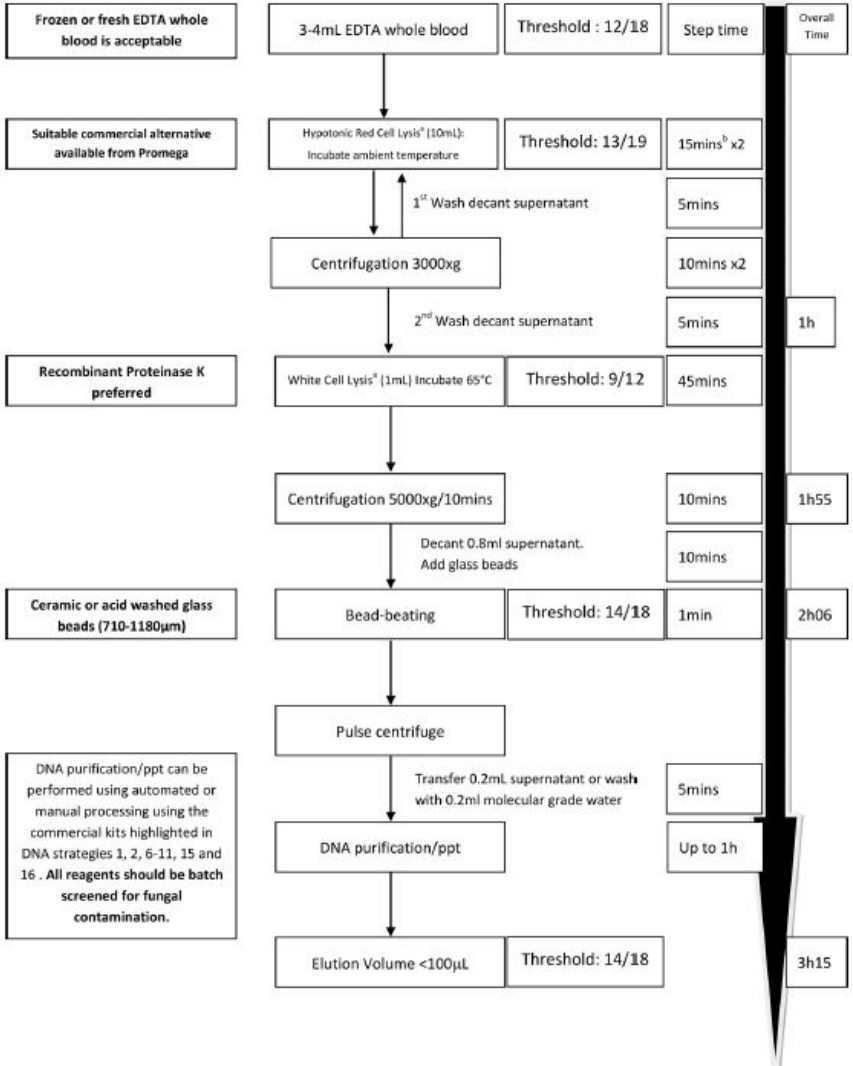
Development of molecular test

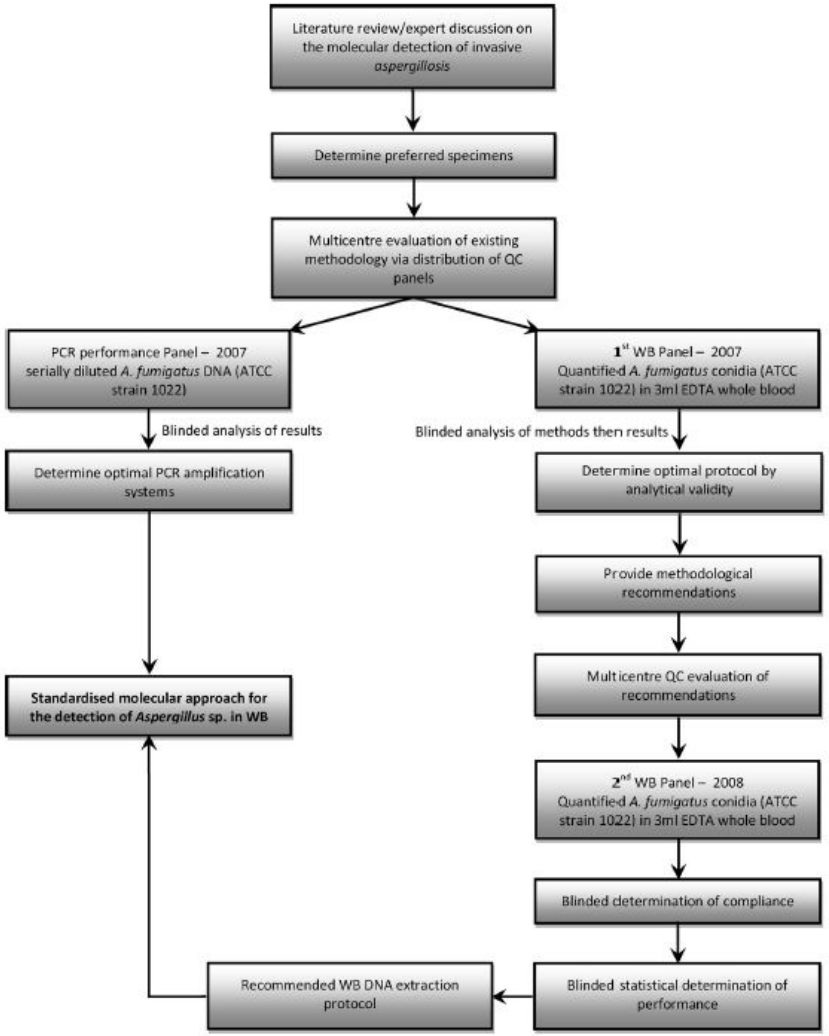


JOURNAL OF CLINICAL MICROBIOLOGY, Apr. 2010, p. 1231–1240 Vol. 48, No. 4
0095-1137/10/$12.00 doi:10.1128/JCM.01767-09

Aspergillus PCR: One Step Closer to Standardization▽†

P. Lewis White,[1]* Stéphane Bretagne,[2] Lena Klingspor,[3] Willem J. G. Melchers,[4] Elaine McCulloch,[5] Bettina Schulz,[6] Niklas Finnstrom,[7] Carlo Mengoli,[8] Rosemary A. Barnes,[9] J. Peter Donnelly,[4] and Juergen Loeffler[10] on behalf of the European *Aspergillus* PCR Initiative





Multicenter Comparison of Serum and Whole-Blood Specimens for Detection of *Aspergillus* DNA in High-Risk Hematological Patients

Jan Springer,[a] C. O. Morton,[b*] Michael Perry,[c] Werner J. Heinz,[a] Melinda Paholcsek,[a*] Mona Alzheimer,[a] T. R. Rogers,[b] Rosemary A. Barnes,[d] Hermann Einsele,[a] Juergen Loeffler,[a] P. Lewis White[c]

J. Clin. Microbiol. 2013, 51(5):1445.

Patient characteristic[a]	Value(s)
No. of patients	78
Male/female ratio	53:25
Median age (yr) of males (range)	53 (18–75)
Median age (yr) of females (range)	51 (20–72)
Mean no. of specimens per patient (range)	10.4 (3–32)
No. of AML patients	36
No. of ALL patients	10
No. of patients with other underlying diseases[b]	32

Assay	Performance value (% [95% CI])[a]				LR^{+b}	LR^{-}	DOR[c]
	Sensitivity	Specificity	PPV	NPV			
GM	80.9 (67.5–89.6)	96.8 (83.8–99.4)	97.4 (86.8–99.6)	76.9 (61.7–87.4)	25.3	0.20	126.5
WB PCR	85.1 (72.3–92.6)	64.5 (47.0–78.9)	78.4 (65.4–87.5)	74.1 (65.4–87.5)	2.4	0.23	10.4
Serum PCR	78.7 (65.1–88.0)	83.9 (67.4–92.9)	88.1 (75.0–94.8)	72.2 (56.0–84.2)	4.9	0.25	19.6
Combination testing[d]							
GM/GM	48.9 (35.3–62.8)	100 (89.0–100)	100 (85.7–100)	56.4 (43.3–68.7)	>489	0.51	>958.9
GM/WB	68.1 (53.8–79.6)	100 (89.0–100)	100 (89.3–100)	67.4 (53.0–79.1)	>681	0.32	>2,128.1
GM/serum	59.6 (45.3–72.4)	100 (89.0–100)	100 (87.9–100)	62.0 (48.2–74.1)	>596	0.40	>1,490
WB/WB	46.8 (33.3–60.8)	93.5 (79.3–98.2)	91.7 (74.2–97.7)	53.7 (40.6–66.3)	7.2	0.57	12.6
WB/serum	57.4 (43.3–70.5)	96.8 (83.8–99.4)	96.4 (82.3–99.4)	60.0 (46.2–72.4)	17.9	0.44	40.7
Serum/serum	53.2 (39.2–66.7)	100 (89.0–100)	100 (86.7–100)	58.5 (45.1–70.7)	>532	0.47	1,131.9

Commercial molecular assay

Assay	Vendor (location)	Method	Target	Specimen type
Yeast Traffic Light®	AdvanDx (MA, USA)	PNA–FISH	26S rRNA; *Candida* sp.	Blood (from positive blood-culture bottles)
MycAssay™ Pneumocystis	Trinity Biotech (County Wicklow, Ireland)	Real-time PCR	mlSU; *Pneumocystis jirovecii*	BAL; sputum
MycAssay™ *Aspergillus*	Trinity Biotech	Real-time PCR	18S rRNA; *Aspergillus* sp.	BAL; serum
Luminex xTAG® fungal assay	Luminex Molecular Diagnostics (ON, Canada)	Multiplex PCR coupled with bead probe fluid array	Up to 23 fungi	BAL; blood
IBIS Plex-ID fungal spectrum assay	IBIS Biosciences (CA, USA)	Multiplex PCR coupled with electrospray ionization mass spectrometry	Large-subunit rRNA; up to 75 fungi	BAL; blood
Seeplex® ACE PCR system	Seegene Diagnostics (Seoul, South Korea)	Multiplex PCR coupled with electrophoresis separation with fluorescence detection	*Candida* sp.	Blood
RenDx™ Fungiplex panel	Renishaw Diagnostics (Glasgow, UK)	Multiplex PCR coupled with surface-enhanced resonance Raman scattering detection	Up to 50 fungi	Blood
ICEPlex 16-plex fungal panel	PrimeraDx (MA, USA)	Multiplex PCR coupled with sequential separation of amplicons by capillary electrophoresis and multicolor quantitative detection	Multiple fungi	Blood
Prove-it™ sepsis assay	Mobidiag (Helsinki, Finland)	Multiplex PCR coupled with microarray	ITS; *Candida* sp.	Blood
T2*Candida*®	T2 Biosystems (MA, USA)	Nanoparticles and T2 magnetic resonance detection platform	ITS2	Whole blood

Summary of molecular tests

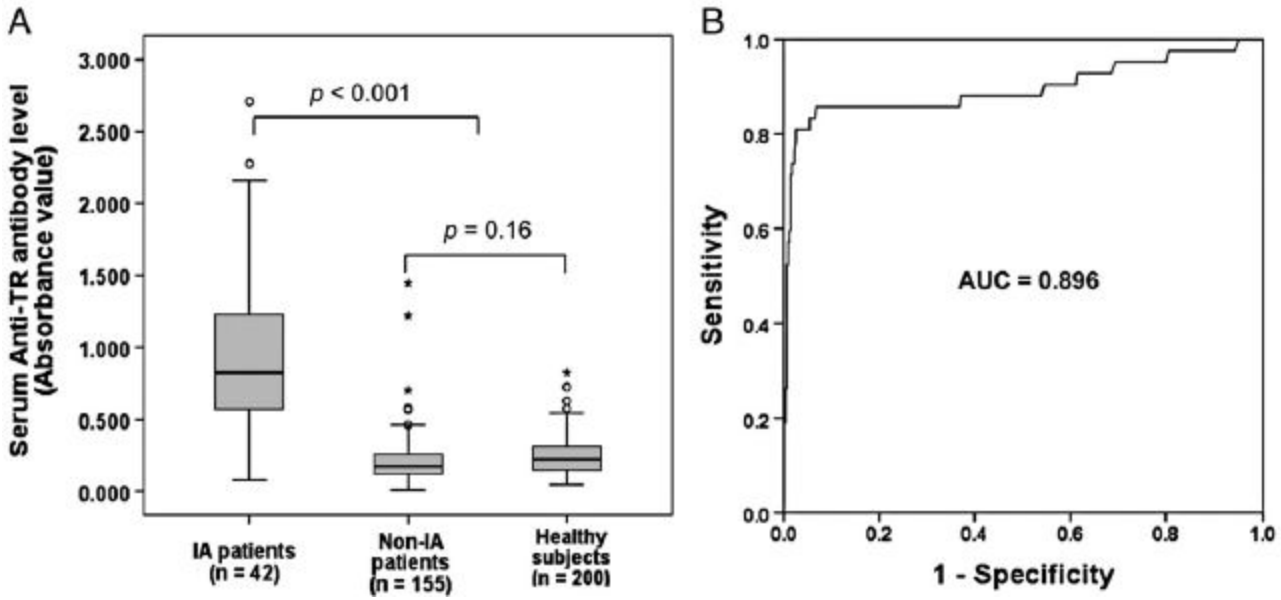
- Most molecular tests are in house with variable results
- Even evaluation of these tests are limited & controversial
- Data on cost-effective analysis is further limited
- Standardization of molecular tests is a big issue – only initiative EAPCRI for aspergillosis
- Commercial molecular tests much used for fungal identification
- Need of development of more consortia research
- Areas of interest – detection of fungi in blood, in formalin-fixed tissue, & identification of antifungal drug resistance directly in clinical samples

New techniques yet to be standardized in multi-centers

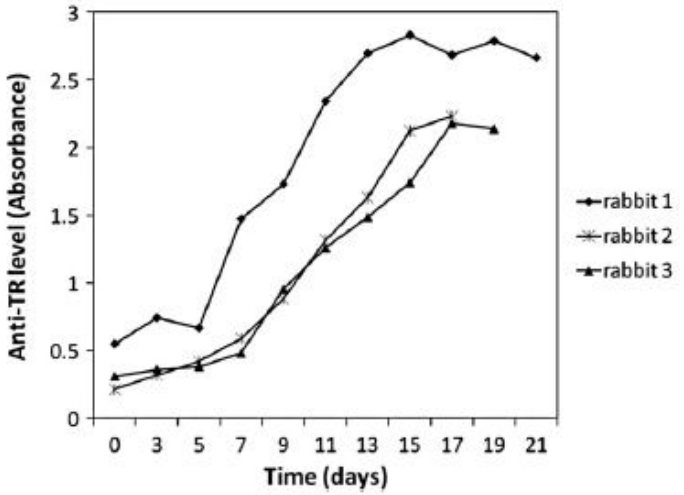
Antibody specific to thioredoxin reductase as a new biomarker for serodiagnosis of invasive aspergillosis in non-neutropenic patients

Li-ning Shi [a,b], Fang-qiu Li [b], Jing-fen Lu [b], Xiao-xiang Kong [b], Shi-qin Wang [b], Mei Huang [b], Hai-feng Shao [b], Shi-he Shao [a,*]

Clin Chim Acta. 2012 May 18;413(9-10):938-43.



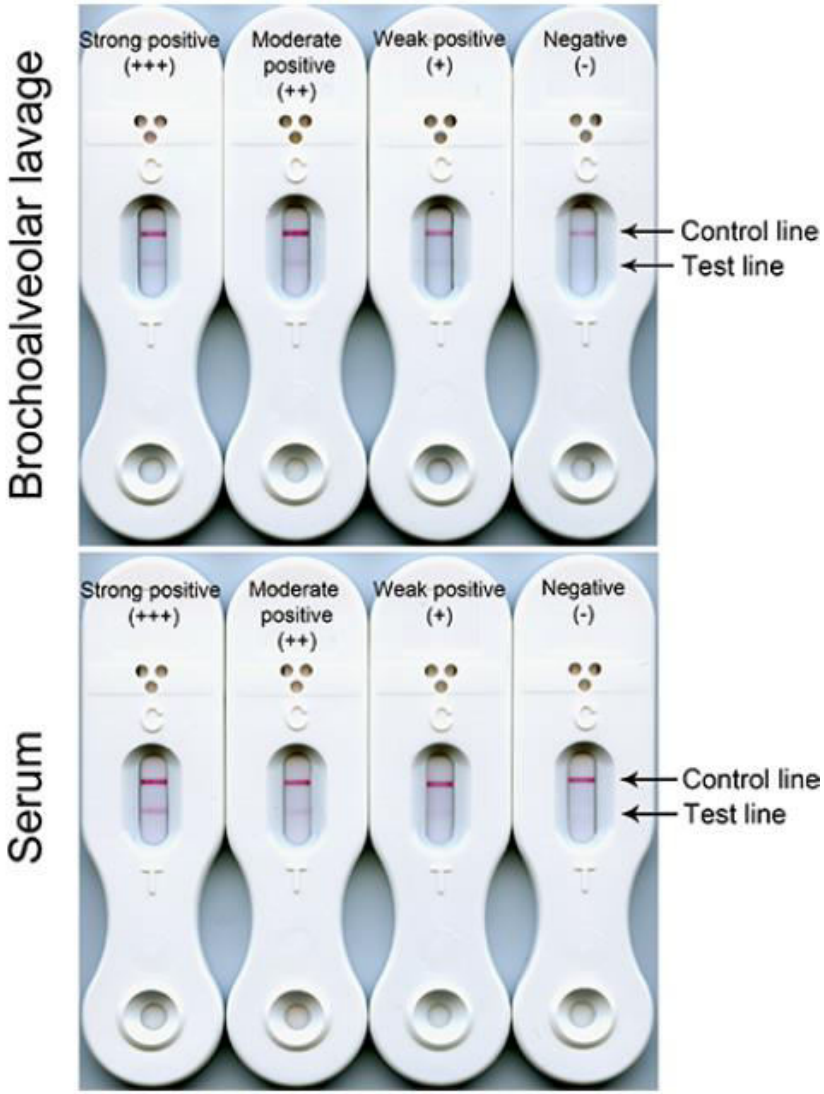
- Sensitivity – 96% (poor in neutropenic – 43.5%)
- Sensitivity significantly higher than GM (80.9% vs. 52.3%; p<0.01) – combining two sensitivity – 88.1%
- Negative in other fungal & bacterial infections, negative even in *A. flavus* & *A. niger* infections
- Antibody appears within 7-9 days of infection



Detection of Invasive Pulmonary Aspergillosis in Haematological Malignancy Patients by using Lateral-flow Technology

Christopher Thornton[1], Gemma Johnson[2], Samir Agrawal[3]

J. Vis. Exp. (61), e3721, DOI : 10.3791/3721 (2012).



- *Aspergillus* specific extracellular glycoprotein Ag
- Secreted during active growth of fungi
- Mab (JF5) developed
- Lateral-flow device (point of care)
- Useful in BAL

Evaluation of Real-Time PCR, Galactomannan Enzyme-Linked Immunosorbent Assay (ELISA), and a Novel Lateral-Flow Device for Diagnosis of Invasive Aspergillosis

P. Lewis White,[a] Christian Parr,[a,b] Christopher Thornton,[c] Rosemary A. Barnes[b]

J. Clin. Microbiol. 2013, 51(5):1510.

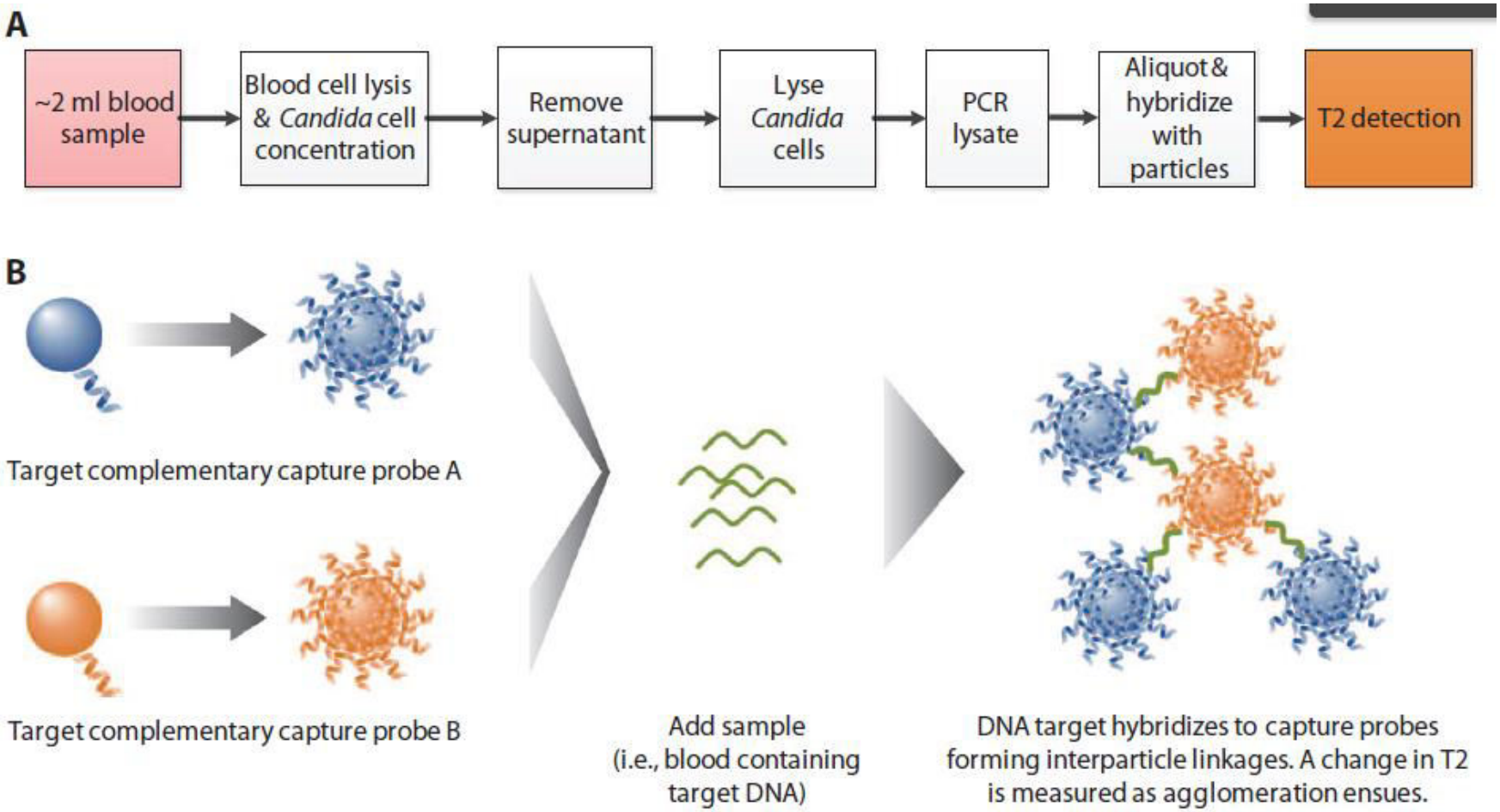
Demographic characteristic	Proven IA ($n = 8$)	Probable IA ($n = 14$)	Possible IA ($n = 22$)	No IFD ($n = 59$)
No. male/no. female	5/3	10/4	15/7	43/16
Mean age (yr)	48.3	45.1	53.6	55.9
Hematological malignancy (type, no.)	AML/MDS, 6; lymphoma, 1; CLL, 1	AML/MDS, 6; ALL, 3; CML, 2; lymphoma, 1; CLL, 1; SAA, 1	AML/MDS, 15; lymphoma, 4; ALL, 2; CML, 1	AML/MDS, 21; lymphoma, 16; myeloma, 12; SAA, 3; ALL, 2; CML, 2; CLL, 1; other, 2
HSCT (type, no.)	Allo, 2	Allo, 9; Auto, 1	Allo, 5	Allo, 13; Auto, 17
Disease manifestation (type, no.)	IPA, 3; IPA/sinusitis, 3; cerebral, 1; IPA/Dissem, 1	IPA, 10; IPA/sinusitis, 1; cerebral/sinusitis, 1; IPA/Dissem, 1; IPA/cerebral/sinusitis, 1	IPA, 16; sinusitis, 5; cerebral/sinusitis, 1	None

	% assay sample positivity[a] (% difference; 95% CI; *P*)		
Patient population	GM vs PCR	GM vs LFD	PCR vs LFD
Proven IA	**14.9 vs 28.2 (13.3; 0.43–26.1; 0.0501)**	14.9 vs 17.2 (2.3; −8.8–13.3; 0.8389)	28.2 vs 17.2 (11.0; 24.0–−2.1; 0.1240)
Proven/probable IA	31.0 vs 27.9 (3.1; 12.1–−6.0; 0.5050)	31.0 vs 26.6 (4.4; 13.1–−4.4; 0.3807)	27.9 vs 26.6 (1.3; 10.2–−7.6; 0.8192)
Proven/probable/possible IA	**17.8 vs 29.1 (11.3; 4.9–17.5; 0.0007)**	17.8 vs 20.1 (2.3; −3.5–8.0; 0.5019)	**29.1 vs 20.1 (9.0; 2.5–15.4; 0.0072)**
No IFD	9.7 vs 10.8 (1.1; −5.3–8.1; 0.7236)	9.7 vs 5.7 (4.0; 9.9–−1.7; 0.2289)	10.8 vs 5.7 (5.1; 11.6–−5.1; 0.1128)

T2 Magnetic Resonance Enables Nanoparticle-Mediated Rapid Detection of Candidemia in Whole Blood

www.ScienceTranslationalMedicine.org 24 April 2013 Vol 5 Issue 182 182ra54

Lori A. Neely,[1] Mark Audeh,[1] Nu Ai Phung,[1] Michael Min,[1] Adam Suchocki,[1] Daniella Plourde,[1] Matthew Blanco,[1] Vasiliki Demas,[1] Lynell R. Skewis,[1] Theodora Anagnostou,[2] Jeffrey J. Coleman,[2,3] Parris Wellman,[1] Eleftherios Mylonakis,[2,3] Thomas J. Lowery[1]*



Genetic susceptibility to IFIs

- Invasive aspergillosis
  - Genetic variability in plasminogen pathway (Zaas *et al.* Plos Genet 2008; 4: e1000101)
  - Toll-like receptor 4 polymorphisms (Bochud *et al.* NEJM 2008; 359: 1766)
- Invasive candidiasis
  - Variations in Dectin-1/CARD9 recognition pathway (Rosentul *et al*. J Infect Dis 2011; 204: 1138)
  - Cytokine gene polymorphisms (Panichakul *et al*. Am J Trip Med Hyg 2002; 67: 443)
  - Toll-like receptor 1 polymorphisms (Johnson *et al*. CID 2012; 54:502)
  - *CASPASE*- 12 alleles (Rosentul *et al*. Eur J Clin Microbiol Infect Dis 2012; 31: 277)

"Sequencing? No, this baby tells us how much we can charge for genome data."

Sequencing will tell us
Whether you may
Have aspergillosis
Or candidiasis!

Summary of diagnosis of IFI by non-culture methods

Method	Turnaround time	Sensitivity (%)	Specificity (%)	Reference
1,3-β-D-glucan detection	<24h	73	78	Tissot, 2010
		64	84	Koo, 2009
		56	73	Nguyen, 2012
Mannan & anti- mannan detection	<24h	79	84	Prella, 2005
		83	86	Mikulska, 2010
Galacto-mannan	<24h	71%	89%	CID, 2006
PCR	6 h	91	100	McMullan, 2008
		80	70	Nguyen, 2012

Mortality of opportunistic fungal infections

97%

22%

Variation due to:

- **timing of intervention (timely diagnosis)**
- **patients' defense system**

Screening improved?

High resolution CT scan

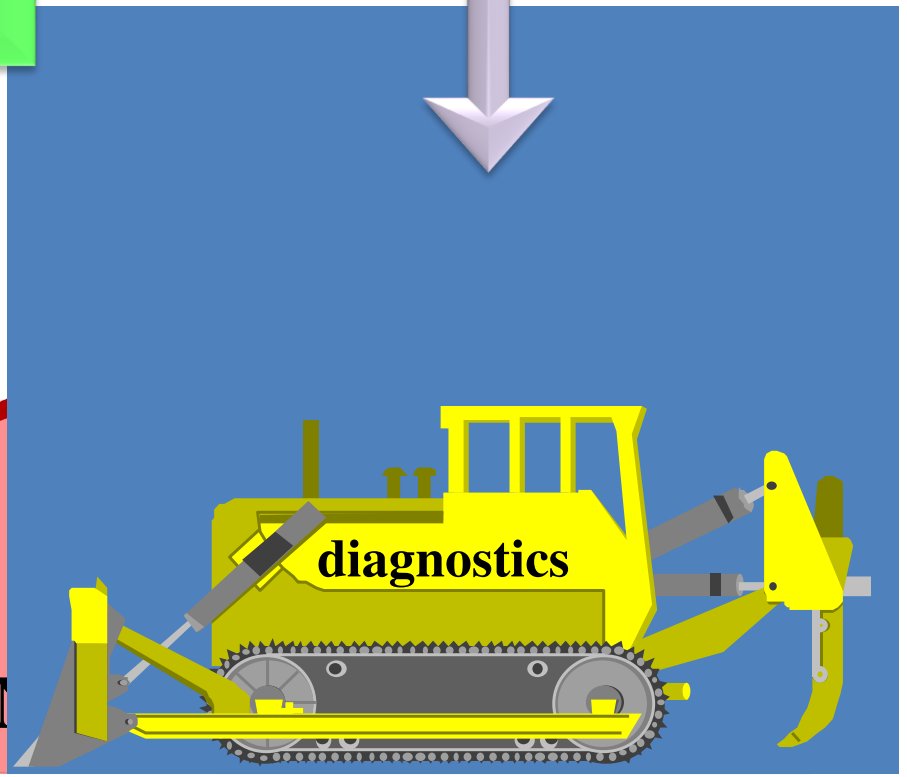
Galactomannan
β-D-Glucan

PCR

22%

New tools

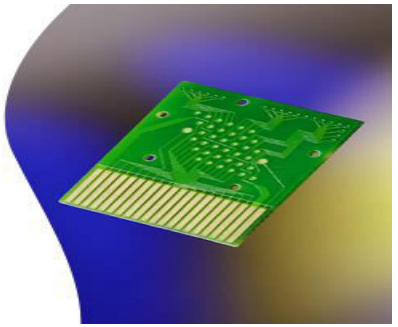
Traditional diagnosis



FUNGAL BURDE

Future direction

- Need of *in-vitro* biomarker for point of care
- May think about *in-vivo* biomarkers
- MS for identification of isolate – still a challenge
- Mass spectrometry (MS) of tissue for identification of fungi, but limited due to difficulty to acquire good tissue
- Carbohydrate MS need to be evaluated, as carbohydrate surface of fungi differ from species to species
- Chip technology



Thank you!

- **Any solution to a problem changes the problem.**

  — R. W. Johnson

- ***Life would otherwise be boring, no?***