Improvement in laboratory diagnosis & point-of-care testing

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What the clinicians require from lab?

- Diagnosis of invasive fungal infections rapidly
- Identification of causative fungus
- Antifungal susceptibility report in real-time
- Culture independent – if possible

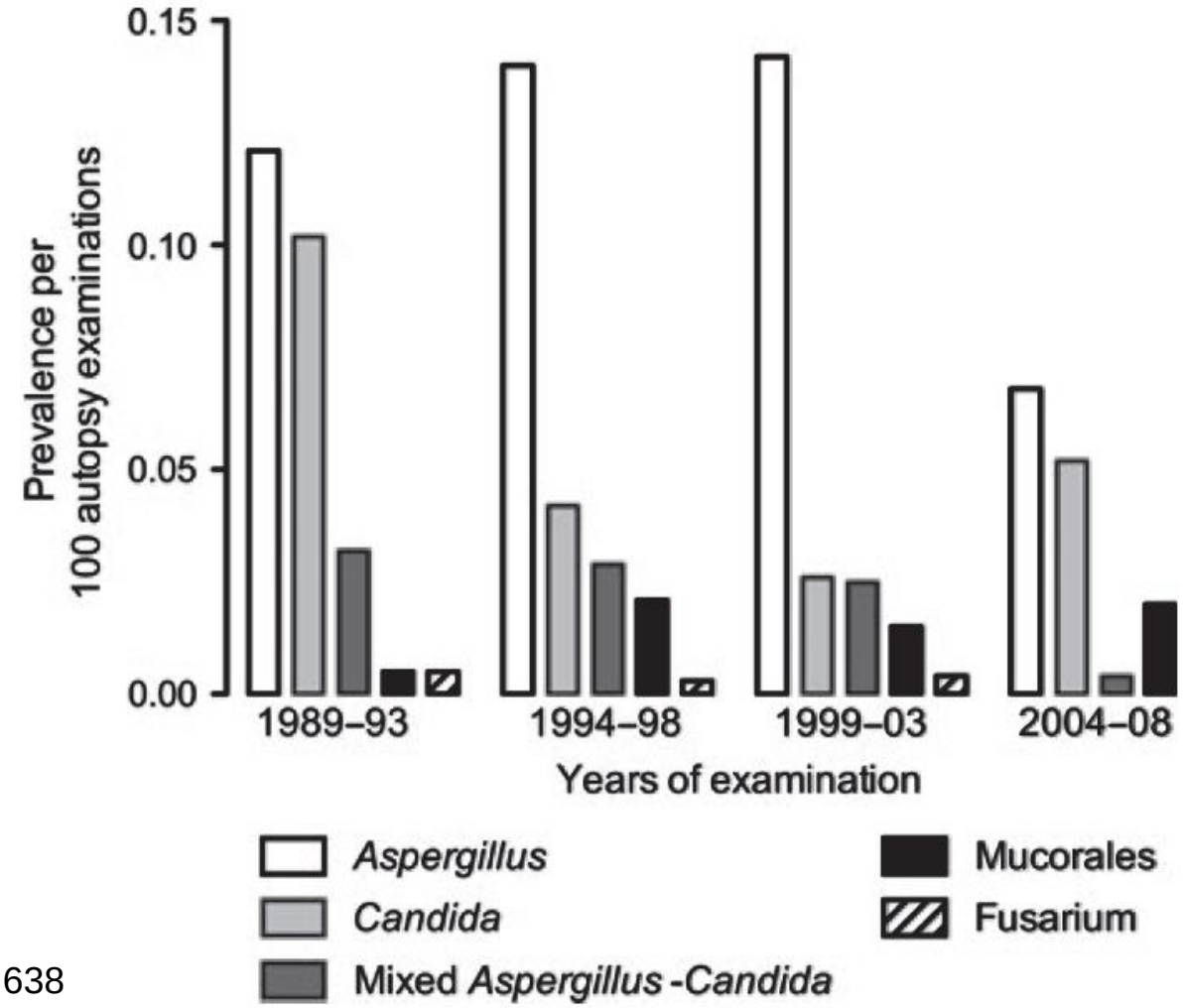
- Where do we stand today?

Hard facts

- Success of antemortem diagnosis **~50%**
- Most deaths from fungal diseases are **avoidable** if diagnosed early & prompt therapy instituted
- Clinical symptoms & signs are **non-specific**
- Manifestations on imaging seldom help in immunocompetent
- Many transformational improvement in **diagnostics** over last 10-15 years (MALDI-TOF, sequencing, galactomannan, β-glucan etc.)
- Access to diagnostics **patchy** in developing countries

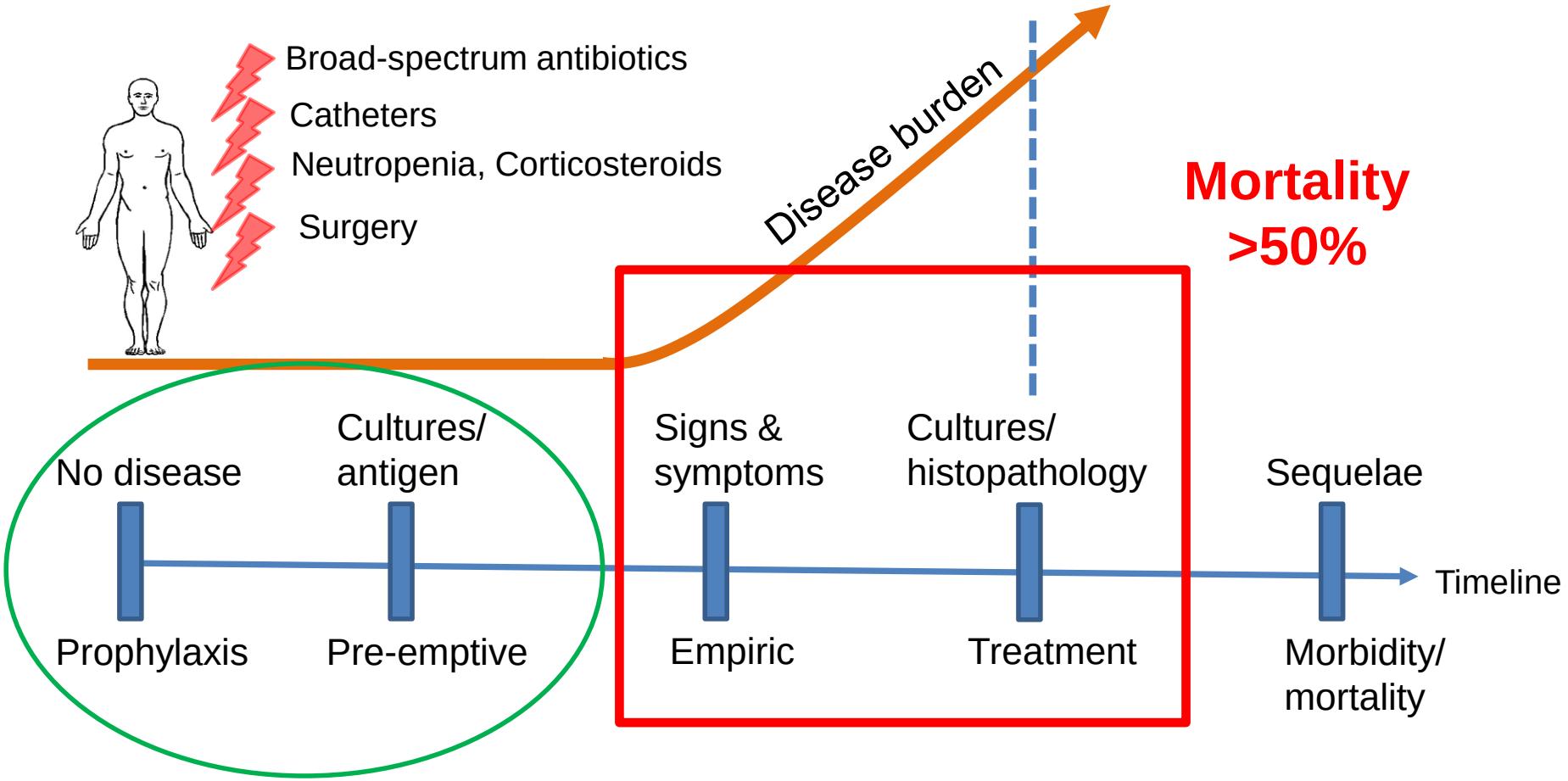
Improvement in diagnosis
(MD Anderson autopsy data on haematological malignancy)

- 84% of IFI were diagnosed post-mortem during 1989-93
- 49% of IFI were diagnosed post-mortem during 2004-08
- Improvement in aspergillosis diagnosis due to pre-emptive approach or introduction of molecular tests



Lewis RE *et al*. Mycoses 2013; 56: 638

Management of invasive fungal infections

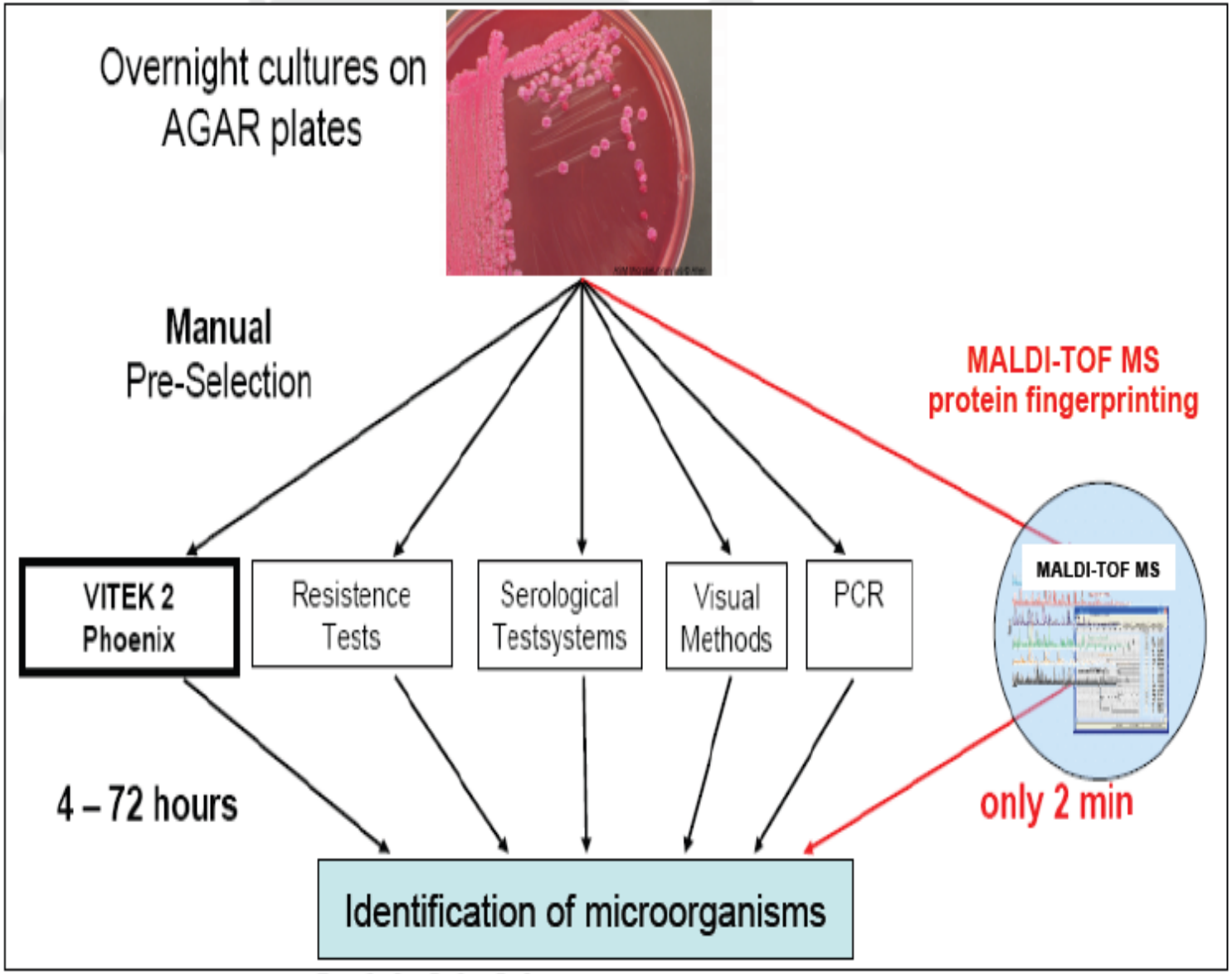


Improvement diagnosis – Proteomic approach? Genomic?

Proteomic based approach – improvement in conventional method

- Identification – yeast & mycelial fungi
- Susceptibility testing
- Molecular typing

MALDI TOF – rapid microbial identification

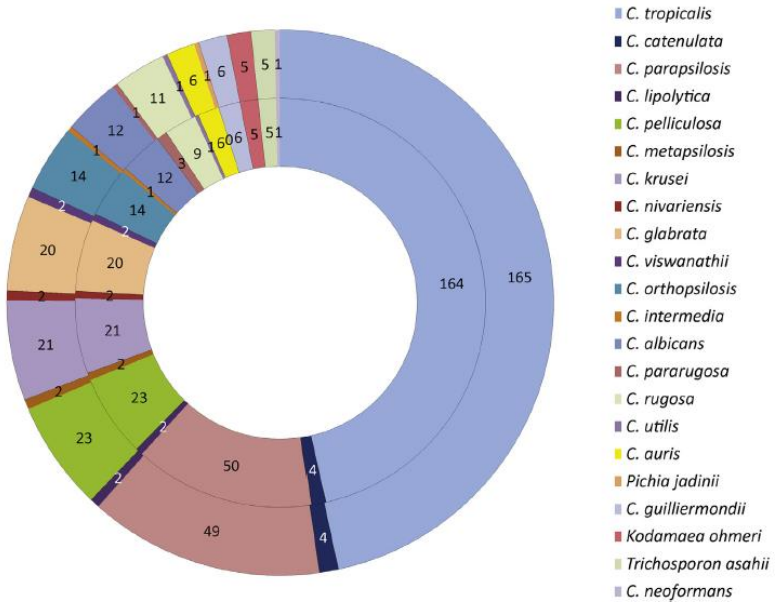


Matrix-assisted laser desorption ionization time-of-flight mass spectrometry for the rapid identification of yeasts causing bloodstream infections

A. K. Ghosh, S. Paul, P. Sood, S. M. Rudramurthy, A. Rajbanshi, T. J. Jillwin and A. Chakrabarti

Clin Microbiol Infect 2015; 21: 372–378

- 354 sequence yeast (standardization)
- 367 blind clinical yeast (validation)
- Database updated for *Candida auris, C. viswanathii, Kodamaea ohmeri* etc.



- **Inner ring – MALDI**
- **Outer ring – sequence identified**

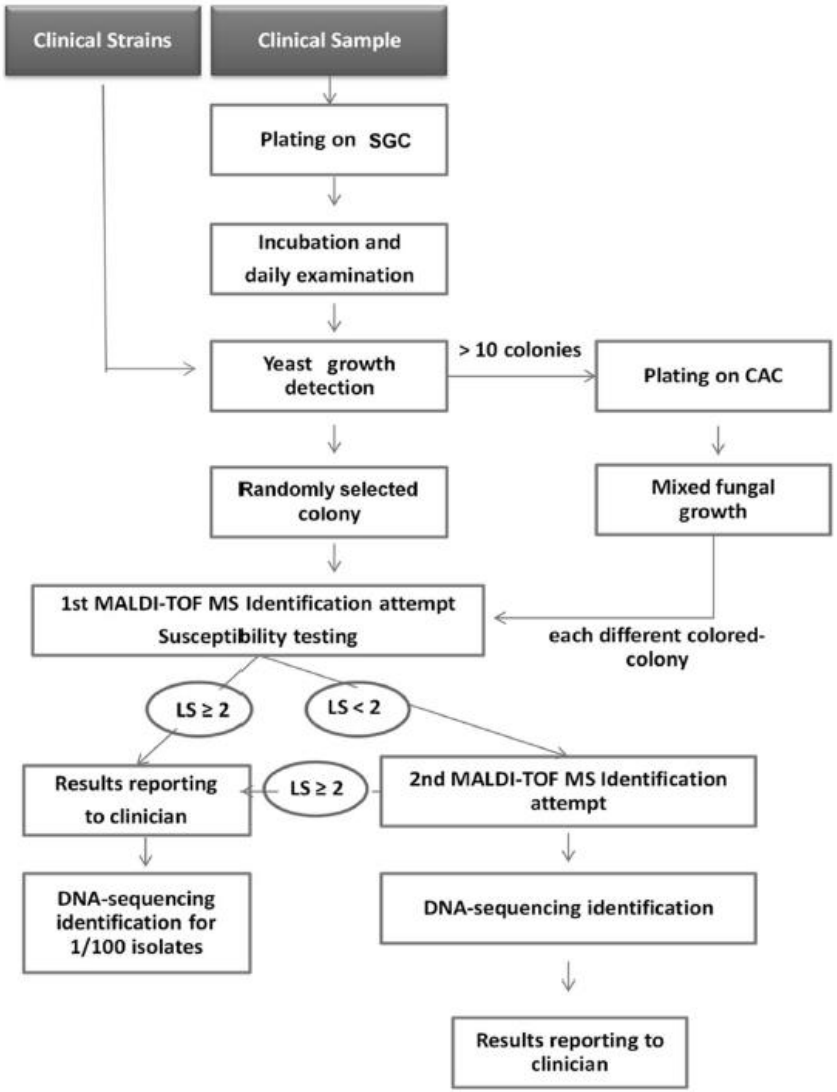
MALDI-TOF correctly identified 98.9% as compared to PCR-sequencing

Routine identification and mixed species detection in 6,192 clinical yeast isolates

Carole Cassagne[1,2,*], Anne-Cécile Normand[2], Lucas Bonzon[1], Coralie L'Ollivier[1,2], Magali Gautier[1], Fakhri Jeddi[1,2], Stéphane Ranque[1,2] and Renaud Piarroux[1,2]

Medical Mycology, 2016, 54, 256–265

- 6,192 yeast of 42 species evaluated
- First or second MALDI identified 98.79% yeast
- Average turnaround time – 0.346 d
- 8.78% mixed infection handled with additional chromogenic media



Mycelial fungi identification

Mycelial Fungi

Standardisation of protein extraction

Aspergillus flavus (ATCC-204304),
Rhizopus arrhizus (CBS-131512),
Fusarium solani (NCCPF-58A0057)

Culture grown in solid media

Culture grown in liquid media

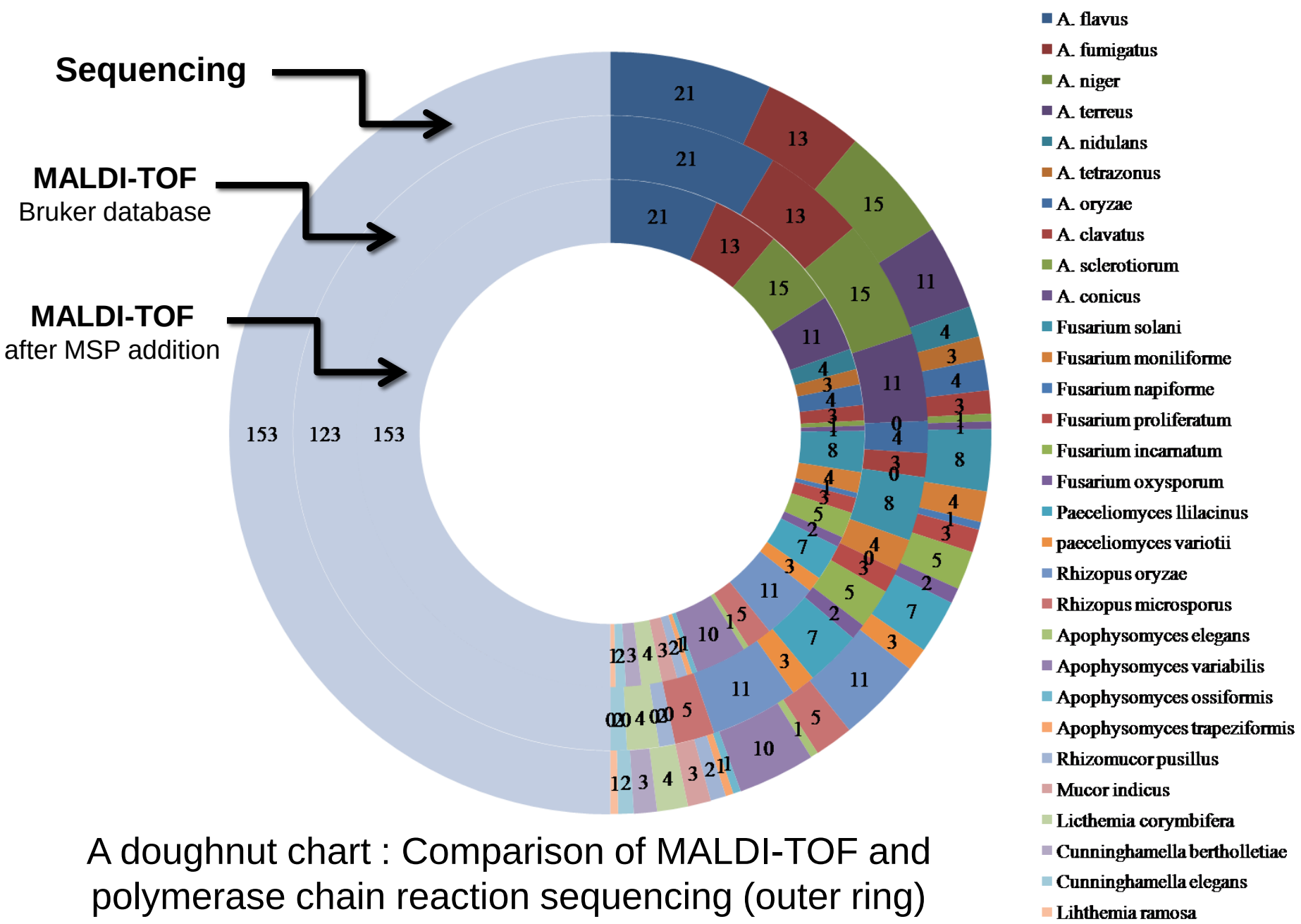
153 sequence proved strain included

Identify by MALDI TOF MS standardized method

MALDI TOF identified 123 (80%), 30(20%) remained unidentified

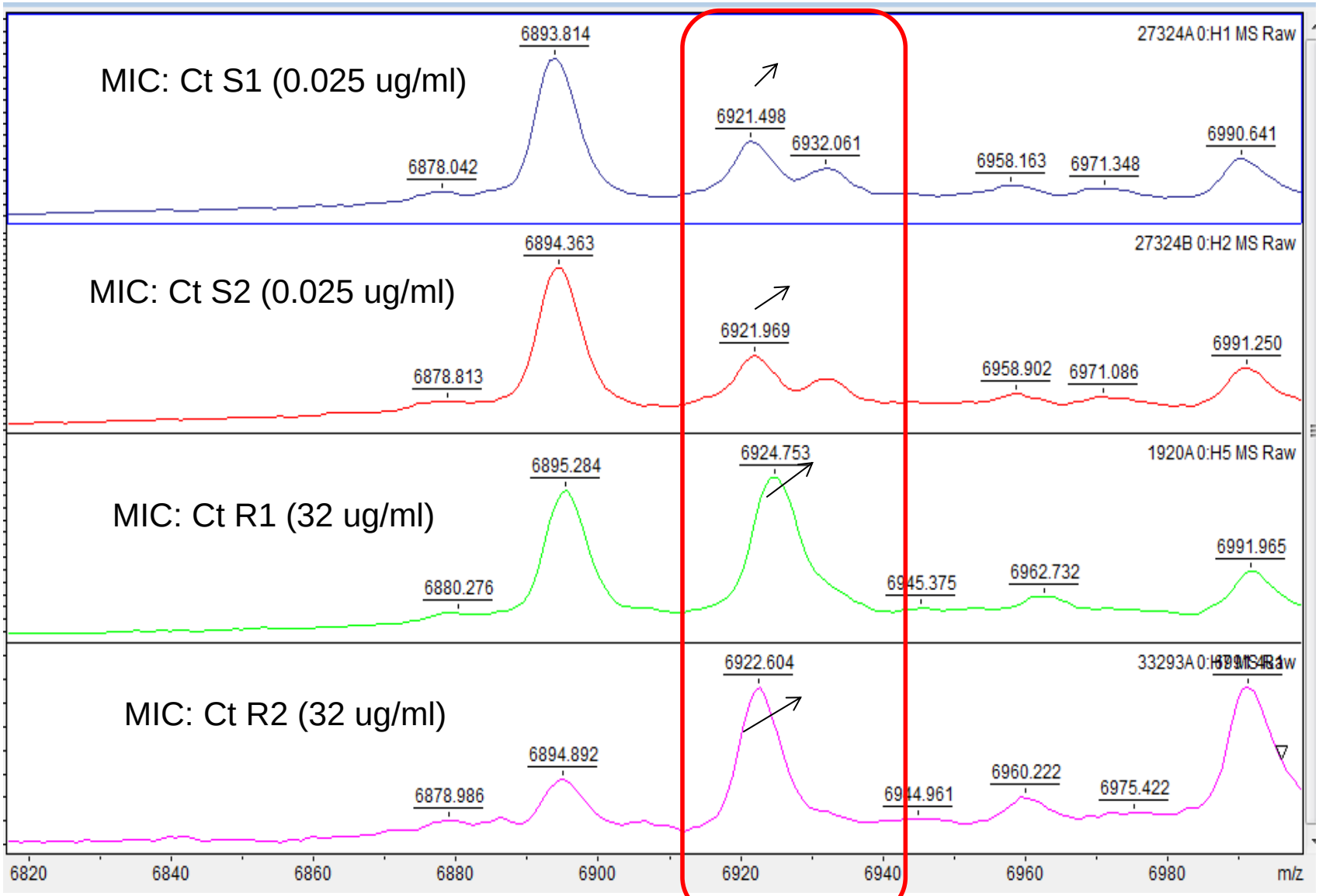
A total of 12 species (5-genus) included in the existing database

Sl no	Name of the organism	No included in the database
1	Aspergillus conicus	1
2	Aspergillus nidulans	2
3	Aspergillus sclerotiorum	1
4	Aspergillus tetrazonus	3
5	Apophysomyces elegans	1
6	Apophysomyces ossiformis	1
7	Apophysomyces trapeziformis	1
8	Apophysomyces variabilis	3
9	Cunninghamella bertholletiae	1
10	Cunninghamella elegans	1
11	Fusarium napiforme	1
12	Mucor indicus	1



A doughnut chart : Comparison of MALDI-TOF and polymerase chain reaction sequencing (outer ring)

MALDI TOF based detection of resistant *C. tropicalis*



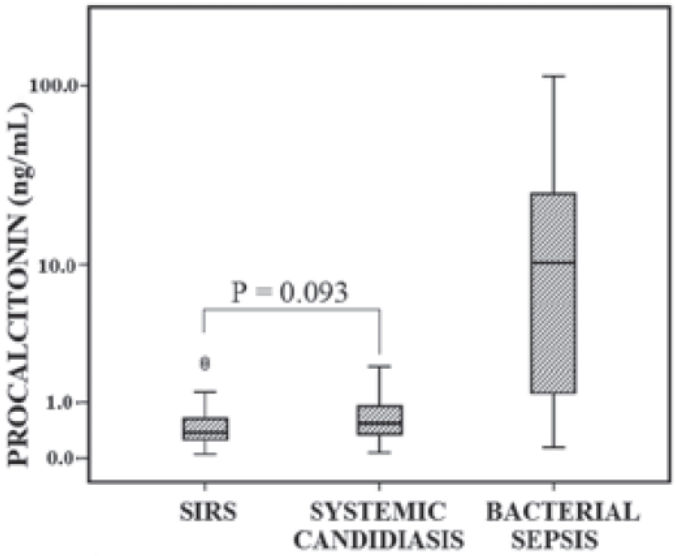
Culture independent methods – proteomic approach

- Detection in clinical sample – promising, but success limited
- Limitation
  - presence of biomarker in pg
  - No scope of prior amplification before detection
- **Biomarkers**
  - Procalcitonin & CRP
  - β-D-glucan detection
  - Galactomannan

Procalcitonin, C-reactive protein and serum lactate dehydrogenase in the diagnosis of bacterial sepsis, SIRS and systemic candidiasis

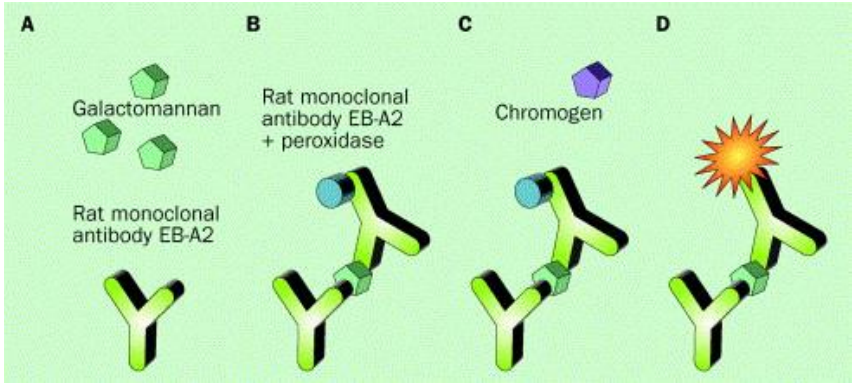
Fabio Miglietta[1], Maria Letizia Faneschi[1], Giambattista Lobreglio[2], Claudio Palumbo[1], Adriana Rizzo[1], Marco Cucurachi[3], Gerolamo Portaccio[4], Francesco Guerra[2], Maria Pizzolante[2]

Le Infezioni in Medicina, n. 3, 230-237, 2015



Variables	*All patients (n=145)*	*SIRS (n=42)*	*P*	*Bacterial sepsis (n=70)*	*P*	*Systemic candidiasis (n=33)*
PCT (ng/mL)						
day 0	0.9 (0.4-9.4)	0.38 (0.26-0.64)	<0.001	10.2 (1.2825.3)	<0.001	0.55 (0.360.9)
day 2	0.6 (0.3-4.9)	0.28 (0.12- 0.5)	<0.001	4.9 (0.711.9)	0.001	0.5 (0.20.6)
CRP (mg/L)						
day 0	91.7 (55.7164)	68.6 (48.5139)	<0.001	128.6 (77-254.7)	<0.001	60.5 (54.4-96.5)
day 2	69.8 (52.3117)	58.5 (47.183)	0.001	99.9 (58-180.2)	0.046	67.4 (50-78.8)
PLT (x 10^9/L)						
day 0	175 (102-243)	197 (158-254)	0.006	152 (91-225)	0.146	172 (110-258)
day 2	162 (79-248)	207 (114-265)	0.014	143 (57-210)	0.166	146 (123-272)
LDH (U/L)						
day 0	489 (391-637)	430 (361-575)	0.004	572 (404-776)	0.103	486 (401-582)
day 2	516 (385-659)	451 (372-539)	0.182	542 (390-731)	0.923	526 (440-591)
WBC (x 10^9/L)						
day 0	11.5 (8.4-15.4)	11.2 (9.4-15.1)	0.204	13.1 (8.8-17.4)	0.034	9.6 (5.8-15.6)
day 2	10 (7.8-13.1)	9.6 (7.5-12)	0.154	11.3 (8.1-15.6)	0.415	9.8 (6.5-13.5)

Galactomannan for invasive aspergillosis



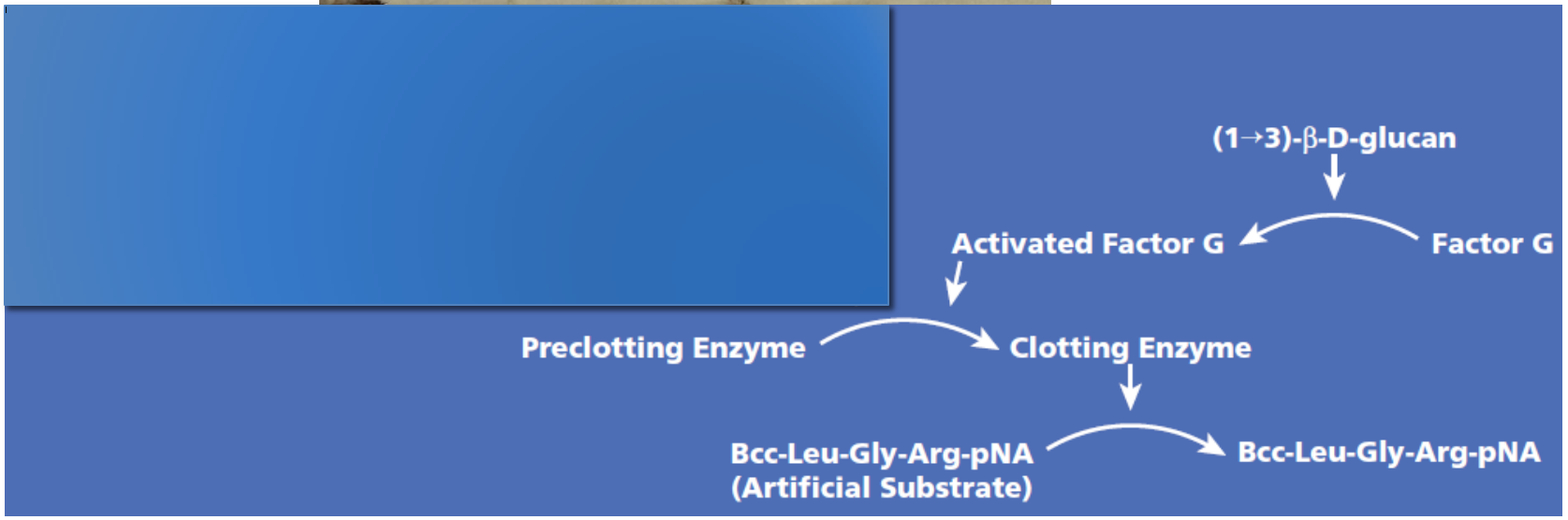
- Cell wall component of *Aspergillus* spp., though present in other fungi
- Microbiological criterion for probable IFI in EORTC/MSG
- Cut-off value of GMI - ?1.5 or 0.5
- Meta-analysis (27 studies) – sens.-71%, spec.-89% (CID 2006; 42: 1417)
- **May be utilized to exclude IA, rather than confirming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

Pros & Cons of GM test

Amsden JR. Curr Fung Infect Rep 2015; 9:111

- FDA approved GM test in serum & BAL
- Detectable GM precedes clinical infection
- BAL GM precedes serum GM
- Good positive & negative predictive value in Haematology-Oncology
- Limitation
  - Standardization required in other clinical conditions
  - Cross-reaction with some fungi (*Geotrichum, Penicillium, Histoplasma* etc.)
  - Variable turnaround time depending of number of specimens
  - False positive tests
  - Well-equipped laboratories & trained staff to perform the test

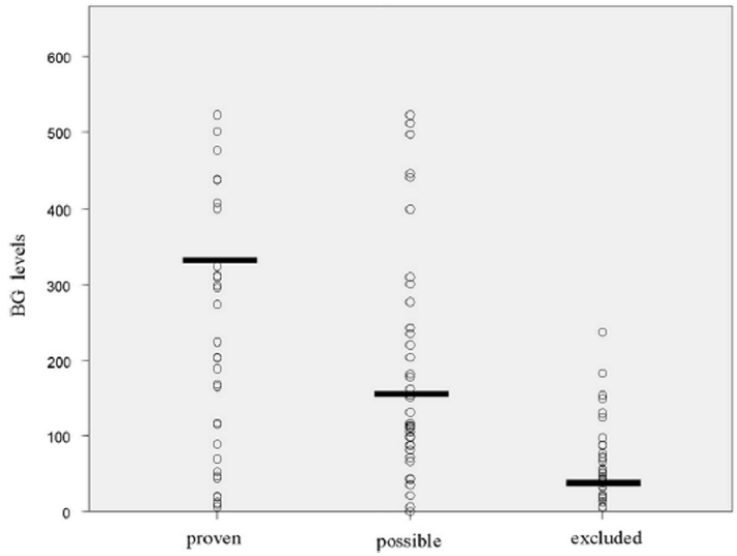
1,3-β-D-glucan detection



The performance of BDG as per meta-analysis

Furfaro E, *et al*. Curr Fung Infect Rep 2015; 9: 292

Reference	Patient population	No. of studies (no. of patients)	Type of fungal infection	Sensitivity (95 % CI)	Specificity (95 % CI)
Karageorgopoulos et al., 2011	HM, SOT	16 (2979)	IA, IC, PJP	0.77 (0.67–0.84)	0.85 (0.80–0.90)
Lu et al., 2011	HM, SOT, ICU	13 (1708)	IA, IC	0.76 (0.67–0.83)	0.85 (0.73–0.92)
					Higher for 2 consecutive samples
Onishi et al., 2012	HM, HIV, ICU, SOT	36 (5453)	IA, IC, PJP	0.80 (0.77–0.82)	0.82 (0.81–0.83)
				0.96 (0.92–0.98)	0.84 (0.83–0.86)
Karageorgopoulos et al., 2012	HIV, HM, HSCT, SOT	14 (2800)	PJP	0.95 (0.91–0.97)	0.86 (0.82–0.90)
Lamoth et al., 2012	HM, HSCT	6 (1771)	IA, IC	0.50 (0.34–0.65) for 2 consecutive samples	0.99 (0.97–0.99) for 2 consecutive samples



- Pan-fungal marker except Mucor & possibly Cryptococcosis
- Positive before clinical symptoms
- Helps to monitor therapy
- False positivity, difficulty to test, cost limit its test

The recommendation for BDG test

Furfaro E, *et al*. Curr Fung Infect Rep 2015; 9: 292

References	Guidelines	Patients	Type of infection	Strength of recommendation
Marchetti et al. BMT 2012	The third European Conference on Infections in Leukemia (ECIL-3)	Hemato-oncological patients	Invasive fungal infections	B II
Ruhnke et al. Annals of Oncology 2012	Infectious Diseases Working Party of the German Society of Haematology and Oncology (AGIHO)	Hemato-oncological patients	Invasive fungal infections	B
Cuenca-Estrella et al. Clin Microbiol Infect 2012	European Society of Clinical Microbiology and Infectious Diseases (ESCMID)	ICU patients	Invasive candidiasis	II
Dellinger et al. Crit Care Med 2013	Surviving Sepsis Campaign for ICU patients	ICU patients	Invasive candidiasis	B II

Good performance in suspected *Pneumocystis* & *Candida* infection

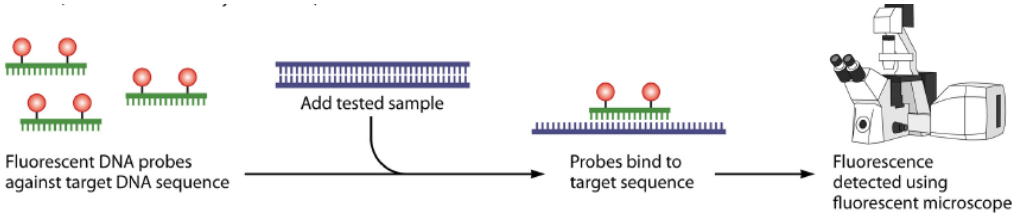
Nucleic acid based approach

Usefulness of Nucleic acid based techniques

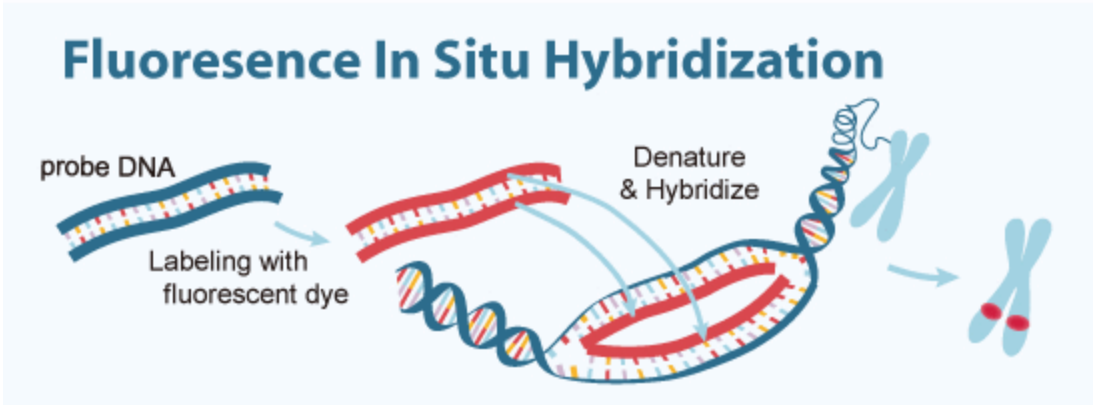
- **Pre-amplification possible**
- **Higher sensitivity & specificity**
- **Low turn around time**
- **GM released in active growth, PCR better in prophylaxis**
- Identify the organism that not grown in culture – NGS
- NGS in epidemiology – outbreak
- NGS in metagenomics & mycobiota
- Identify the organism present on histopathological sections
- **Challenge - diagnosis from clinical samples**

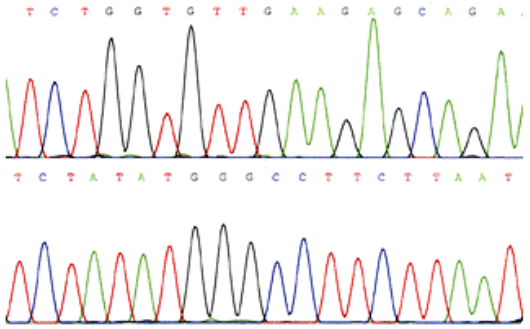
Identification of fungus in tissue

- Immunohistochemistry



- Extraction of DNA from tissue & sequencing





- Success – fresh tissue – 90%, formalin fixed – 70%

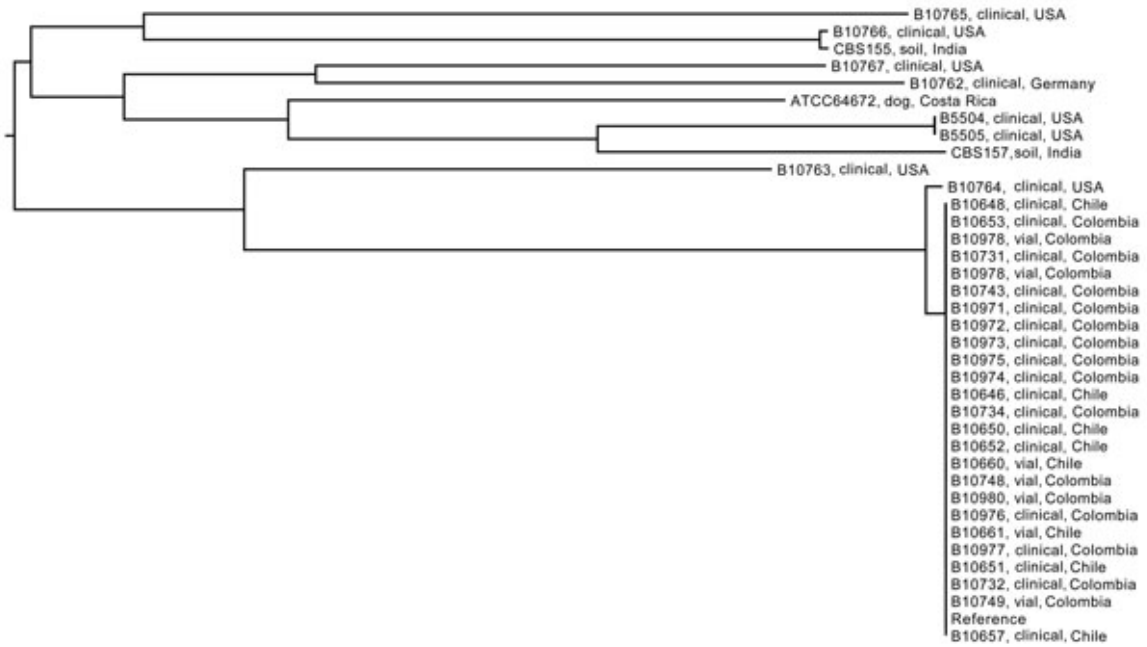
Whole-Genome Sequencing to Determine Origin of Multinational Outbreak of *Sarocladium kiliense* Bloodstream Infections

Kizee A. Etienne✉, Chandler C. Roe, Rachel M. Smith, Snigdha Vallabhaneni, Carolina Duarte, Patricia Escadon, Elizabeth Castaneda, Beatriz L. Gomez, Catalina de Bedout, Luisa F. López, Valentina Salas, Luz Maria Hederra, Jorge Fernandez, Paola Pidal, Juan Carlos Hormazabel, Fernando Otaiza, Fredrik O. Vannberg, John Gillece, Darrin Lemmer, Elizabeth M. Driebe, David M. Englethaler, and Anastasia P. Litvintseva

EMERGING INFECTIOUS DISEASES®

Volume 22, Number 3—March 2016

- 67 cases of **blood stream infection** due to *Sarocladium kiliense* in 8 different hospital in Chile
- As environmental source eliminated, medications of all patients reviewed
- Common medication – heparin, saline, potassium, **ondansetron (antinusea drug, from a single company, Colombia)**



Real challenge in clinical sample

- PCR based detection assay - Real time PCR or qPCR
- Large number of PCR protocols published over 20 years, but absence of consensus standardized technique
- PCR is not included in EORTC/MSG guideline

Comparison with virology

- Different protocol published for viruses, but this does not hamper acceptance of PCR in diagnostic virology
- For viruses – we deal with $>10^3$

Challenges in fungal PCR

- Too few fungal DNA in sample
- PCR inhibitors – heparin, haemoglobin, lactoferrin
- Standardization in each step – not considered yet in EORTC/MSG guideline

Standardization at each step

Factors	Possible solution
Choice of samples	•Tissue best, but difficult to get •Blood, serum, plasma, BAL
DNA extraction	•Use large sample volume •Low elution volume •Good fungal cell wall lysis method •Care for contamination

The MIQE Guidelines:
Minimum Information for Publication of Quantitative Real-Time PCR Experiments

Stephen A. Bustin,[1*] Vladimir Benes,[2] Jeremy A. Garson,[3,4] Jan Hellemans,[5] Jim Huggett,[6] Mikael Kubista,[7,8] Reinhold Mueller,[9] Tania Nolan,[10] Michael W. Pfaffl,[11] Gregory L. Shipley,[12] Jo Vandesompele,[5] and Carl T. Wittwer[13,14]

Clin Chem 2009; 55: 211

Co-infection	Broad range of PCR with post-amplification identification (SeptiFast PCR)

Challenges in fungal PCR

- Which sample to use – whole blood, plasma, serum, BAL?
- Contamination is a big issue - environment
  - 10-20% tube may have *Aspergillus* DNA contamination
  - 18% commercial tubes with anticoagulant have fungal DNA

Recommendation EAPCRI

- Blood volume of 3ml offer a better yield
- Serum sample can be used
  - High initial volume (>1ml), elution in small volume
  - Mechanical lysis better than enzymatic lysis of cell wall
  - Internal control, ITS target

Diagnosis of aspergillosis – comparison GM/BDG/PCR

Characteristic	GM-EIA	B-D-glucan	PCR
Methodological recommendation	Single commercial assay with SOP: **Platelia Aspergillus antigen** (BioRad)	5 commercial assays: **Fungitell** (Associates of Cape Cod) **Fungitec G-Test MK** (Seikagaku Corporation) **B-G Star** (Maruha Corporation **B-Glucan Test Wako** (Wako Pure Chemicals) **Dynamiker Fungus** (1–3)-β-D-Glucan Assay (Dynamiker Biotechnology Co, Ltd)	**Pathonostics Aspergenius, Roche Septifast, Myconostica MycAssay, Ademtech Mycogenie, Renishaw Fungiplex,** Procedural recommendations for DNA extraction **(EAPCRI)**
Quality control	Internal – BioRad Proficiency panel	No	Independent – QCMD and EAPCRI panels
Sensitivity %	Blood: 79.3 BAL: 83.6–85.7	Blood: IA: 56.8–77.1	Blood: 84–88 BAL: 76.8–79.6
Specificity %	Blood: 80.5–86.3 BAL: 89.0–89.4	Blood: 81.3–97.0	Blood: 75–76 BAL: 93.7–94.5
False positive	Yes	Yes	Yes
False negative	Yes	Yes	Yes
Clinical utility	Yes	Limited	yes

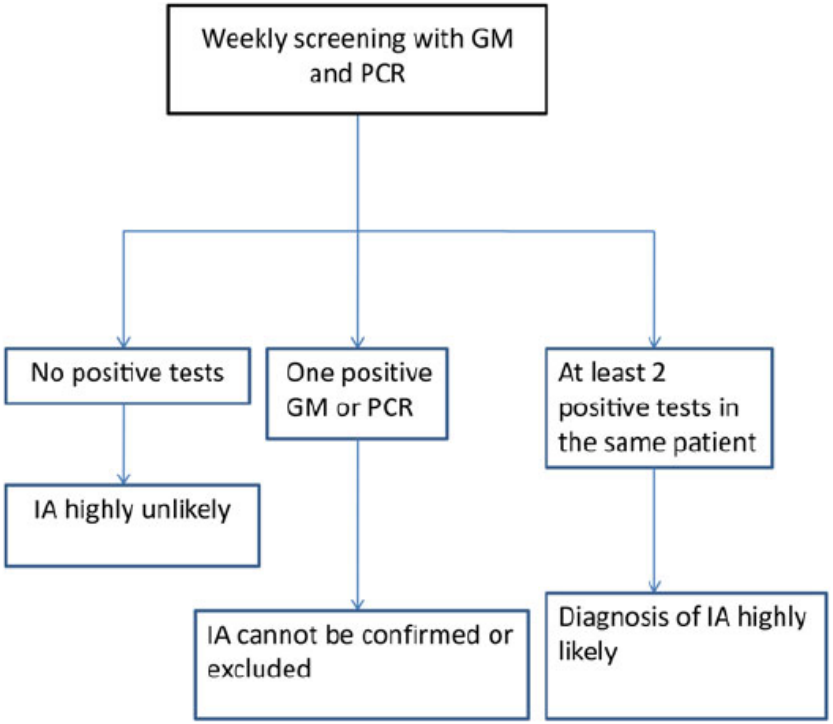
White PL *et al.* Clin Infect Dis 2015; 61: 1293

Galactomannan and Polymerase Chain Reaction–Based Screening for Invasive Aspergillosis Among High-Risk Hematology Patients: A Diagnostic Meta-analysis

Marios Arvanitis,[1,2,3] Theodora Anagnostou,[1,2] and Eleftherios Mylonakis[1,2]

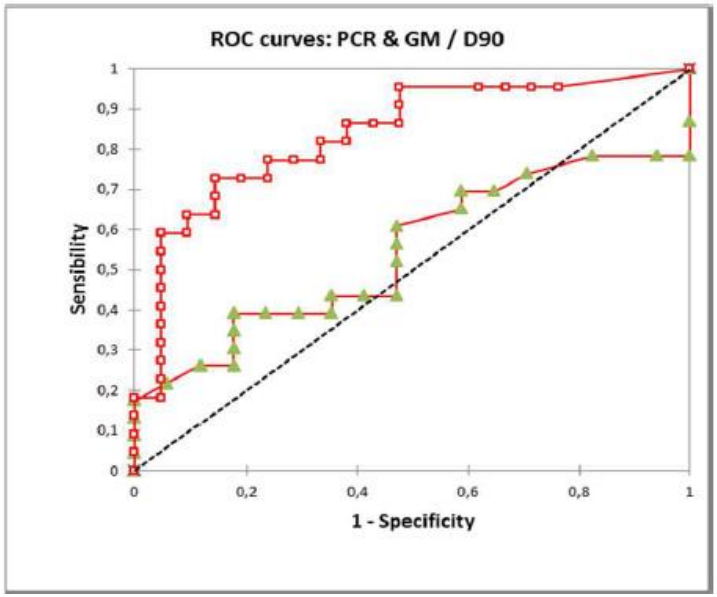
Clin Infect Dis 2015; 61: 1263

Test	Sensitivity, % (95% CI)	Specificity, % (95% CI)
PCR	84 (71–92)	76 (64–85)
2 PCRs	57 (40–72)	93 (87–97)
GM	92 (83–96)	90 (81–95)
2 GMs	62 (48–74)	95 (91–97)
GM or PCR	99 (96–100)	64 (49–77)
GM and PCR	68 (54–80)	98 (94–100)

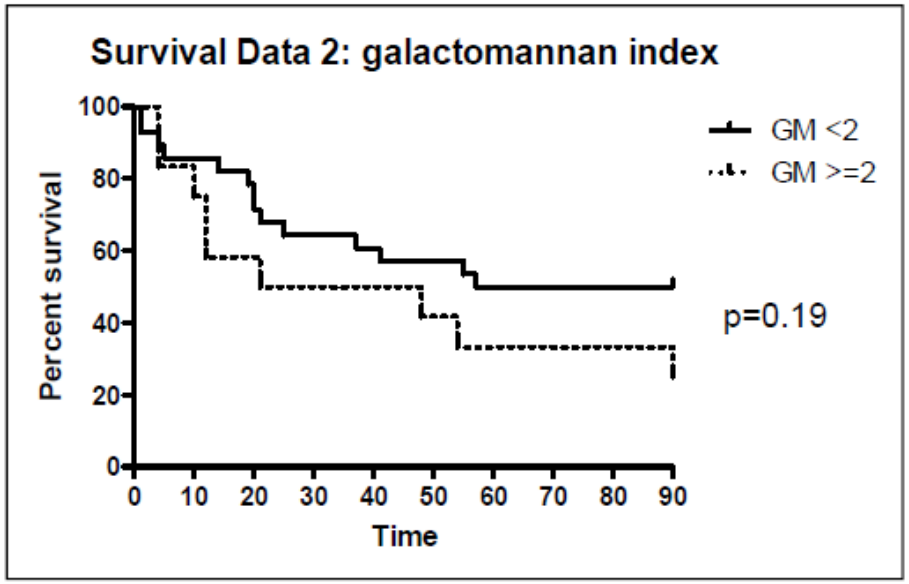


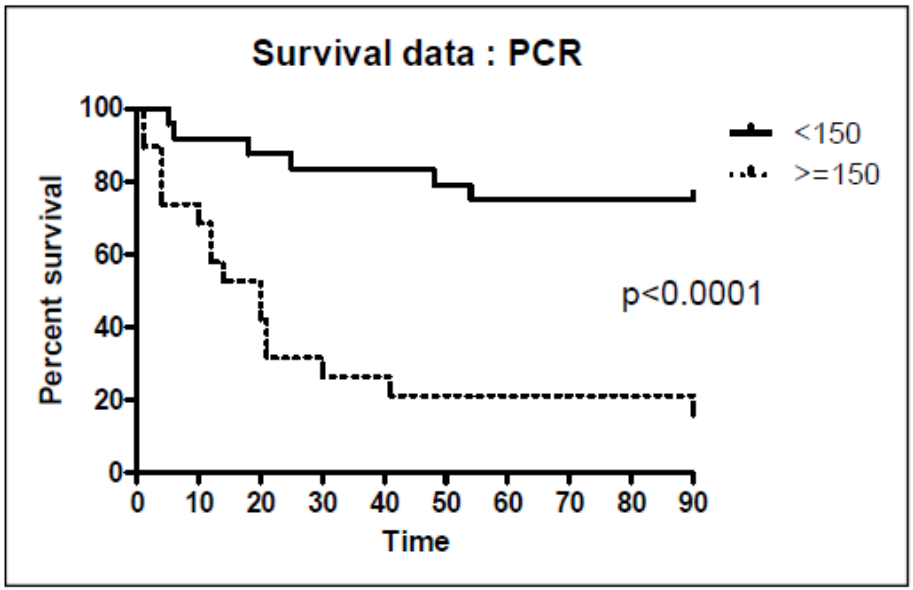
Aspergillus PCR is highly predictive of 90d mortality

- 941 patients, 5146 serum samples
- 51 patients – proven/probable IA
- PCR – 66.7% sens., 98.7% spec.
- GM – 78.4% sens., 87.5% spec.
- PCR+GM – 88.2% sens.



Imbert *et al.* Clin Microbiol Infect 2016, Feb 16 (on line)





Interpretation of non-culture diagnostic tests

- If blood culture is negative due to low level of candidemia, beta-glucan & PCR assays unlikely to make diagnosis reliably
- If a patient in low-risk group (ICU admission), positive result does not help, but negative result excludes the disease
- If a patient in high-risk group (repeated ileal leak or pancreatitis), a positive result increases the likelihood of invasive candidiasis
- Temptation – shorter turn around time & early therapy
- We tend to believe - non-culture diagnostic tests can identify blood culture negative primary or secondary deep-seated candidiasis
- Two high positive results are compelling
- Similarly multiple negative results are compelling

New techniques

POCT tests

How to go for POC test?

What we want?

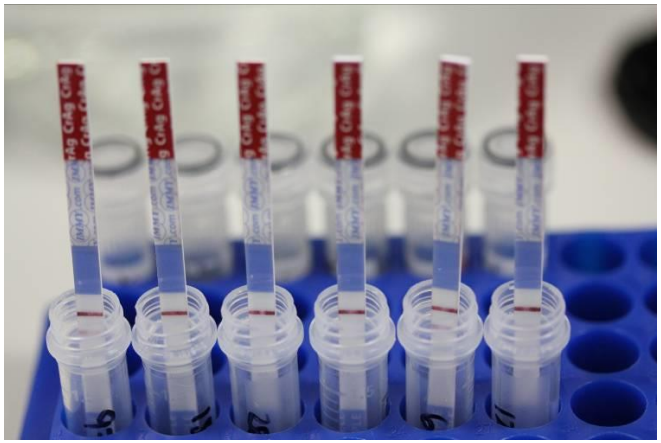
- Integration
- Automation
- Miniaturization
- Packaging
- Cheaper platform
- Easy disposability
- Economically feasible

Which technology can help us?

- Nanotechnology
- Micro/nanofabrication (biochips)
- microfluidics

Cryptococcal meningitis

- CrAg Lateral flow assay (Immy, Norman)
- No equipped lab or skill staff required
- Can identify disease before symptoms
- Temperature stable, rare cross-reaction
- Takes 10 minutes; Costs $2, cost-effective
- Cannot monitor therapy – clearance slow



Study in Uganda, Suspected cryptococcal meningitis

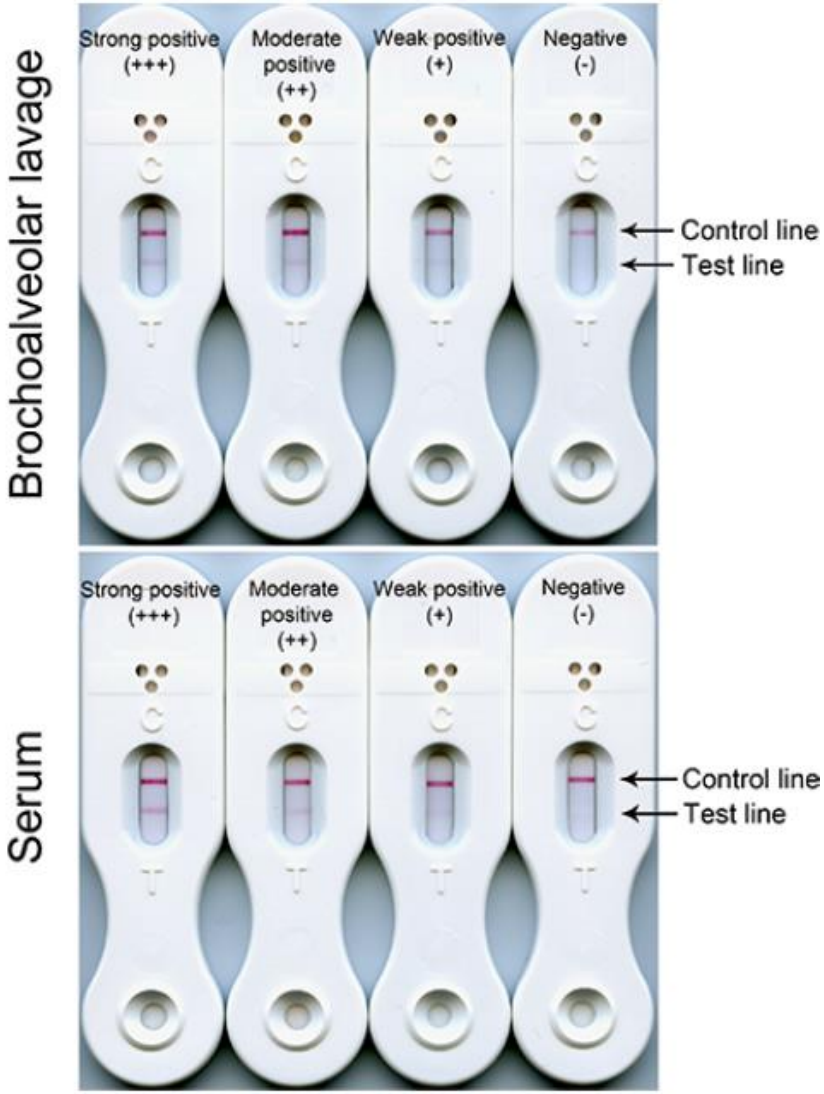
Diagnostic test	Number	Number positive/number tested (%)			
		Sensitivity	Specificity	PPV	NPV
CSF culture	806	459/510 (90.0)	296/296 (100.0)	459/459 (100.0)	296/347 (85.3)
100-µL volume	524	309/328 (94.2)	196/196 (100.0)	309/309 (100.0)	196/215 (91.2)
10-µL volume	282	150/182 (82.4)	100/100 (100.0)	150/150 (100.0)	100/132 (75.8)
India ink microscopy	805	438/509 (86.1)	288/296 (97.3)	438/446 (98.2)	288/359 (80.2)
CrAg LFA	666	435/438 (99.3)	226/228 (99.1)	435/437 (99.5)	226/229 (98.7)
CrAg latex (Meridian)	279	176/180 (97.8)	85/99 (85.9)	176/190 (92.6)	85/89 (95.5)
CrAg latex (IMMY)	749	452/466 (97.0)	283/283 (100.0)	452/452 (100.0)	283/297 (95.3)

Nalintya E, *et al.* Curr Fungal Infect Rep 2016; 10: 62

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
- Lot of variability in sensitivity & specificity among the laboratories

Aspergillosis diagnosis – BALF *Aspergillus* LFD

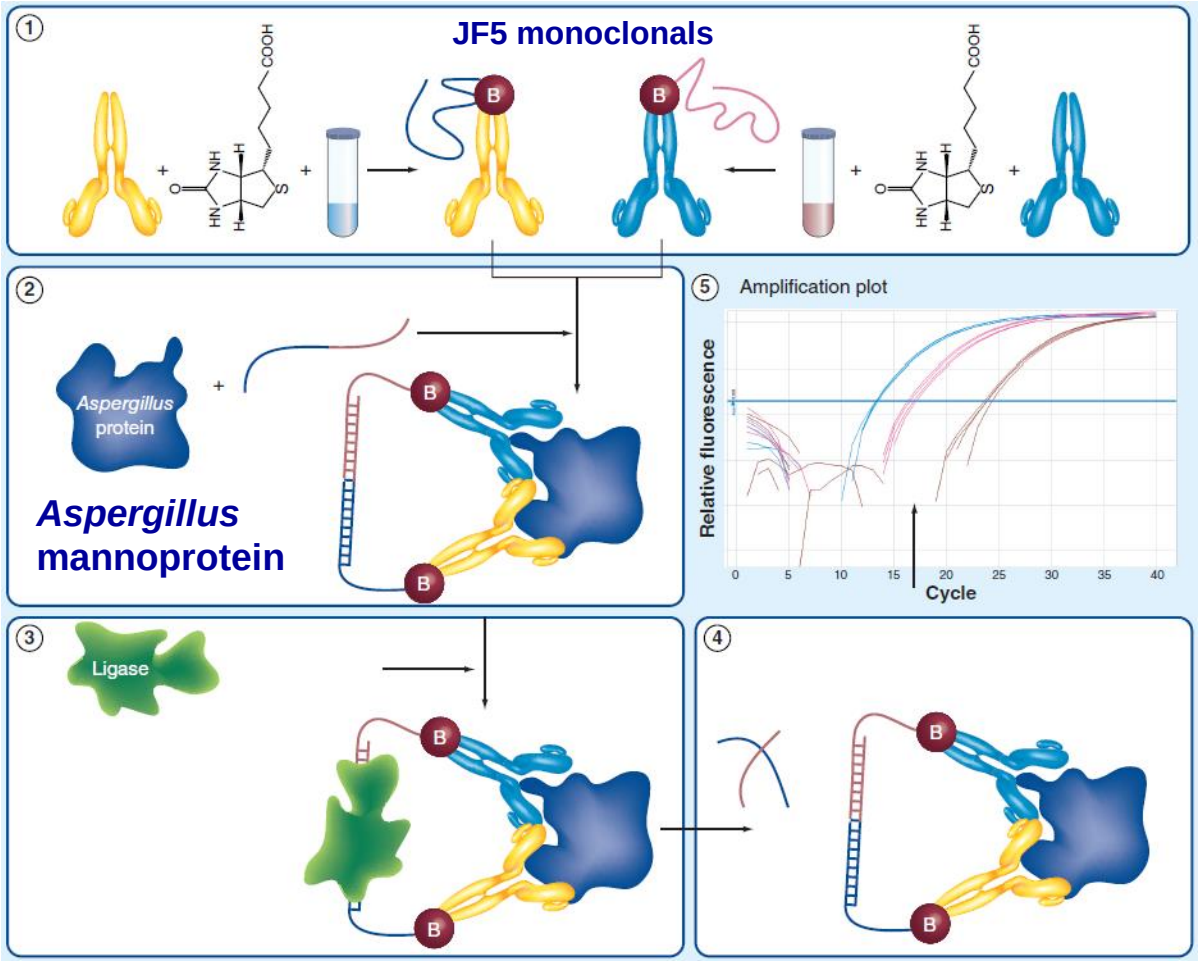
Patient group	Sensitivity	Specificity	PPV	NPV
Solid organ transplantation	94 % (15/16)	92 % (89/97)	65 % (15/23)	99 % (89/90)
Intensive care unit	79 % (26/33)	85 % (176/206)	57 % (26/46)	96 % (176/183)
Respiratory diseases	77 % (24/31)	92 % (195/211)	60 % (24/40)	97 % (195/202)
Hematological malignancies	65 % (30/47)	89 % (88/99)	73 % (30/41)	84 % (88/105)

Prattes J, *et al.* Curr Fungal Infect Rep 2016; 10: 43

Proximity ligation assay for the early detection of invasive aspergillosis

G. Johnson[1], M. Shannon[1], C. Thornton[1], S. Agrawal[2], C. Lass-Flörl[3], W. Mutschlechner[3], S. Bustin[1]

- PLA 10-100 fold higher sensitivity to GM
- 1000 fold higher sensitivity to lateral flow assay (LFD)
- No cross reaction with other fungal species

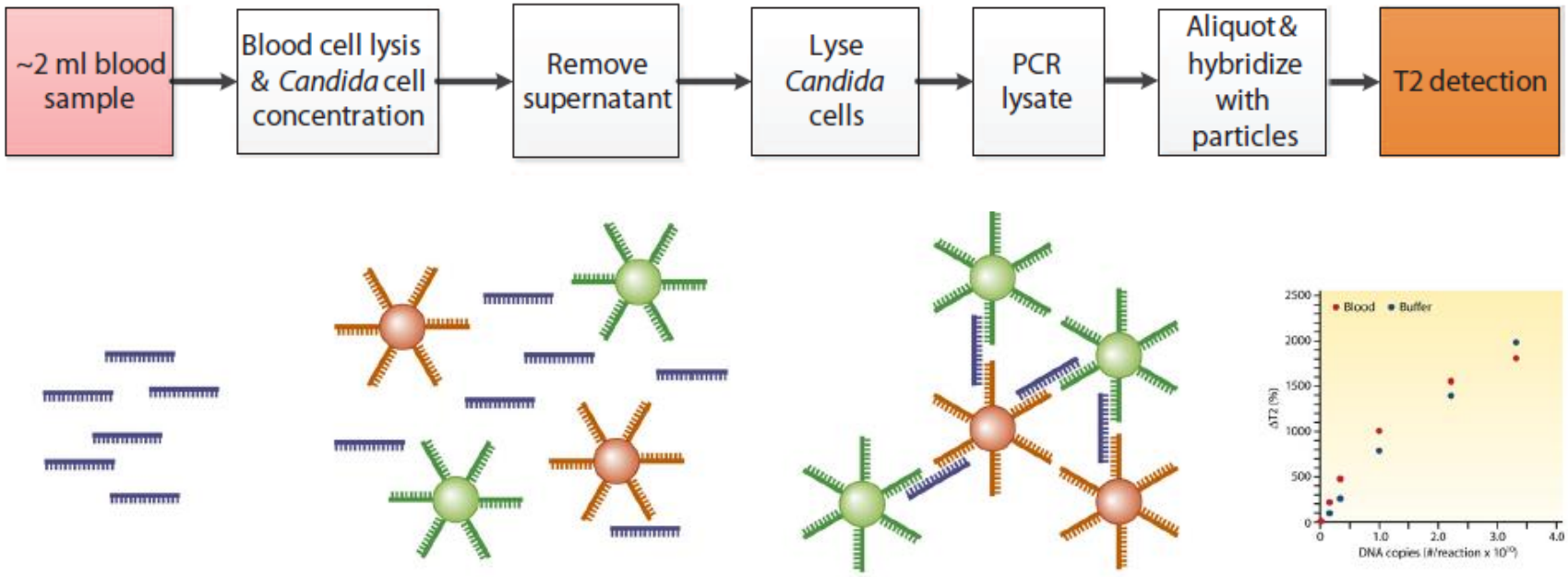


Biomark Med 2014; 8: 429-51; 25th ECCMID congress, Copenhagen, 2015

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]



- **Detection limit – 1-10CFU/ml compared to Multiplex PCR - >30CFU/ml**
- **Improve the time to detect – BACTEC – 2.6d, T2 – 3-4h**
- Detect only five common *Candida* species (95%); chance of contamination
- No antifungal susceptibility test performed & cannot replace blood culture

Mylonakis *et al*. Clin Infect Dis 2015; 60: 892

Histoplasmosis – LAMP assay

- LAMP uses alternate polymerase (*Bst*) compared to *Taq* in traditional PCR
- Requires less expensive equipment, cheaper, field study
- *Hcp100* - locus of *H. capsulatum* targeted in this assay
- This assay can help in identification of *H. capsulatum* isolate
- Can also detect *H. capsulatum* in urine samples of progressive disseminated histoplasmosis (sensitivity 67%)

Prattes J, *et al.* Curr Fungal Infect Rep 2016; 10: 43

MycoDx™ Invasive Fungal Assay

IMMUNETICS
CLINICAL LABORATORY SERVICES

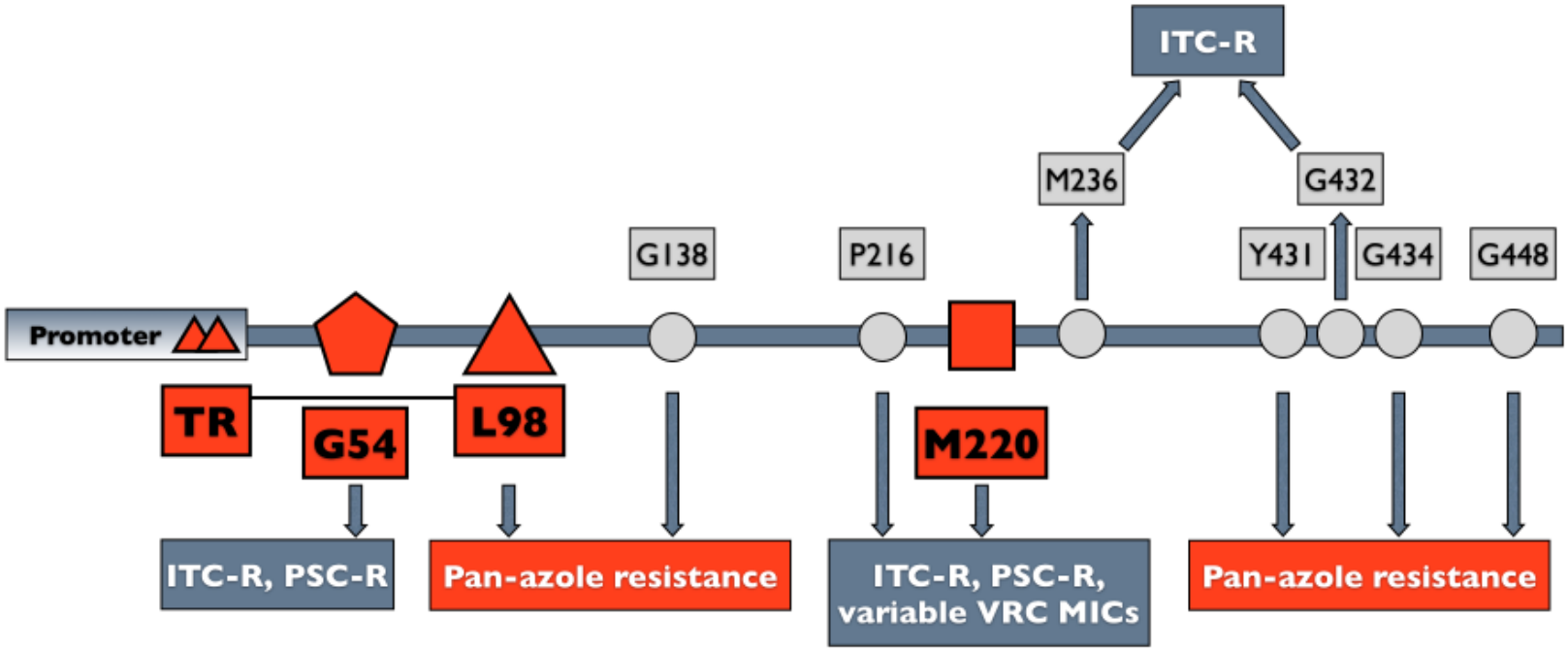
- **PCR ⟹ hybridization on microarray** with species specific probe
- 21 fungal targets at present
- Results directly from whole blood
- Turnaround time ~5h for all targets

MycoDx™ detects 21 Fungal Targets – with additional targets in development	
Candida albicans	*Aspergillus nidulans*
Candida dubliniensis	*Aspergillus niger*
Candida glabrata	*Aspergillus terreus*
Candida krusei	*Coccidioides immittis*
Candida guillermondii	*Coccidioides posadasii*
Candida lusitaniae	*Cryptococcus gattii*
Candida parapsilosis	*Cryptococcus neoformans*
Candida rugosa	*Cunninghamella* genus
Candida tropicalis	*Fusarium oxysporum*
Aspergillus flavus	*Lichtheimia corymbifera*
Aspergillus fumigatus	

Direct detection of *cyp*51*A* mutations

Chong *et al*. J Clin Microbiol 2015; 53: 868

- BAL, Sputum, tissue biopsies
  - How many mutants among the wild type isolates?
- Serum
  - Low amount of DNA (single copy) => low sensitivity



Summary

Thank you!

- Areas of interest – detection of fungi in blood, in formalin-fixed tissue, rapid identification of fungi, & antifungal drug resistance directly in clinical samples
- Proteomic approach – MALDI, biomarkers - promising
- Genomic approach – more promising, but majority tests are in house & not standardized
- EAPCRI is a bold initiative, but commercial closed system required
- New initiatives – genetic susceptibility, POCT (lateral flow, proximity ligation assay, microarray, nano technology, T2)