PCR for Diagnosis in Mycology

Marie-Pierre Hayette
University Hospital of Liège
NRC Mycosis

SBMHA symposium 2015

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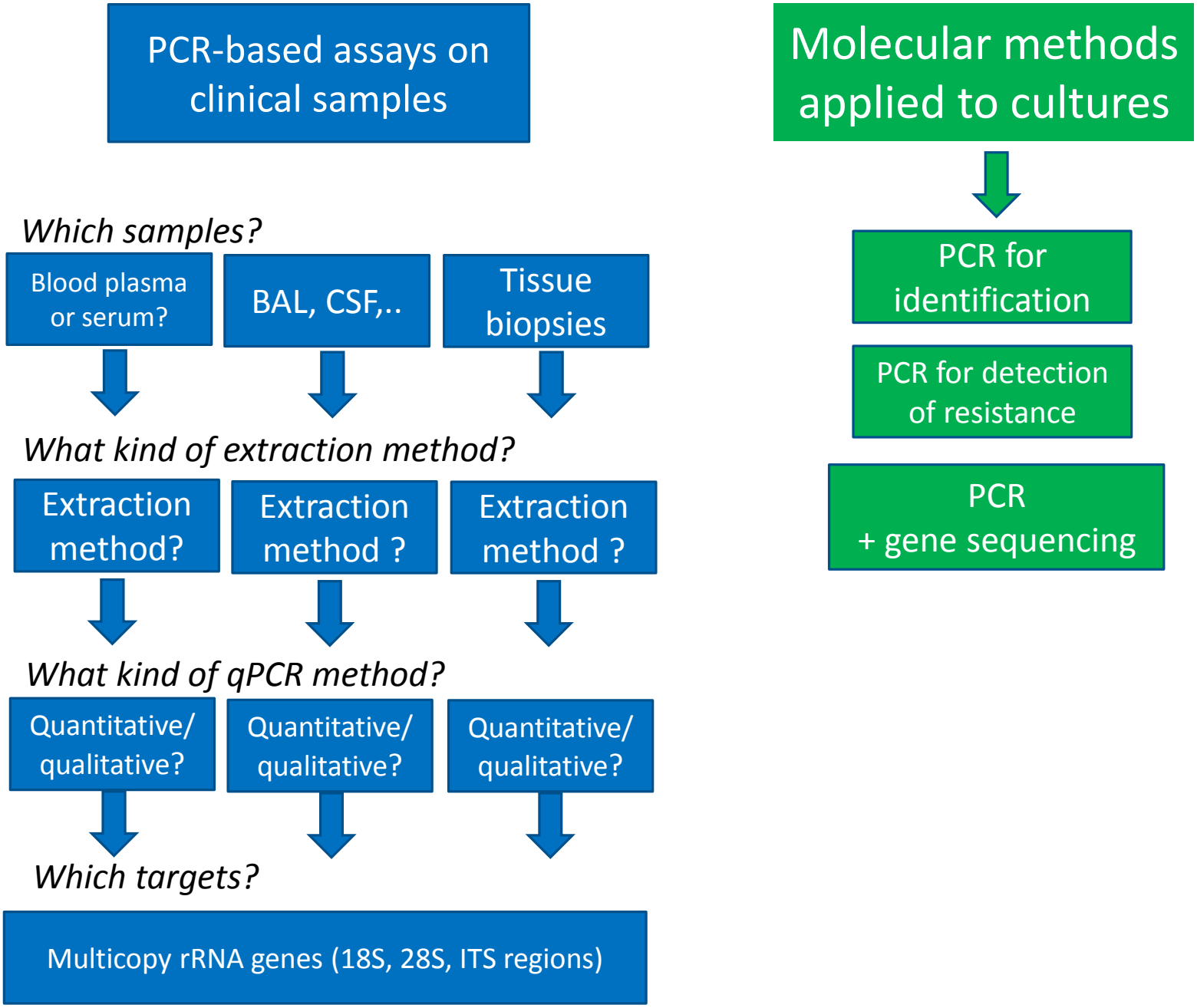

PCR-based assays on clinical samples
Which samples?
Blood plasma or serum?
BAL, CSF,..
Tissue biopsies
What kind of extraction method?
Extraction method?
Extraction method ?
Extraction method ?
What kind of qPCR method?
Quantitative/ qualitative?
Quantitative/ qualitative?
Quantitative/ qualitative?
Which targets?
Multicopy rRNA genes (18S, 28S, ITS regions)
Molecular methods applied to cultures
PCR for identification
PCR for detection of resistance
PCR + gene sequencing

Pan-fungal PCR

- Only in patients at high risk of fungal infection
- In sterile fluids or biopsies (with or whithout positive microscopy)
- Multiplex PCR assays or panfungal with high conserved regions of fungal DNA.
- In house tests or commercial kits

Lagrou K. et al. ASM Book 2015
Buitrago MU JCM 2014

Panfungal PCR: *in house assays*

Targets ITS1 and ITS2

Gene sequencing

Retrospective study

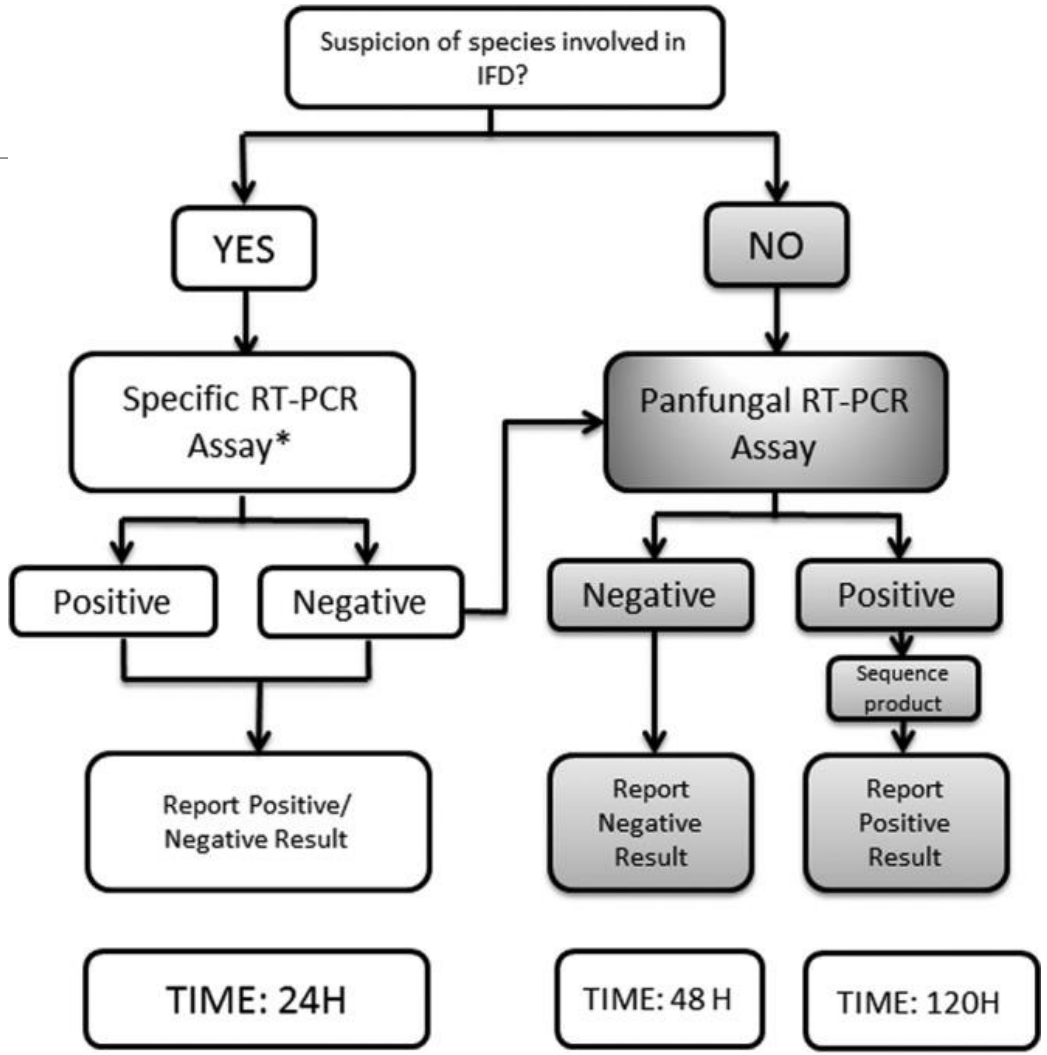
151 biopsies (fresh and paraffin-embedded tissues

132 patients with proven IFI

Sensitivity

89% (overall)

80% in case of negative culture results



Buitrago MU JCM 2014

Panfungal PCR: *commercial kits*

Kit provider	Type of targets	Validated samples	Technology
Septi-fast ® Test MGRADE (Roche, Switzerland)	19 bacteria+6 fungi (Af+5 Candida sp.)	Whole blood	qPCR
Vyoo ® Test (Analytik, Germany	34 bacteria + 7 fungi (Af, 6 Candida)	Whole blood	Conventional PCR
RenDx ® Fungiplex assay (Renishaw, UK)	**Aspergillus sp (3 species)+ Candida sp+ C. glabrata and C. krusei**	Blood, serum or plasma	PCR+ raman technology
Sepsitest ® (Molzym, US, CE IVD kit)	Panbacterial + panfungal	Blood+biopsies+ liquids	PCR+gene sequencing (16S or 18S)
Provit® (Mobidiag, Finland)	60 bacteria+13 fungi+bacterial resistance gene	Blood cultures	Microarray
Magicplex™ Sepsis Real-time Test, Seegene, Korea	85 bacteria+6 fungi+bacterial resistance genes	Whole blood	Multiplex qPCR

Clinical evaluation of commercial nucleic acid amplification tests in patients with suspected sepsis

Lars Ljungström[1], Helena Enroth[2], Berndt EB Claesson[2], Ida Ovemyr[3], Jesper Karlsson[2], Berit Fröberg[2], Anna-Karin Brodin[2], Anna-Karin Pernestig[3], Gunnar Jacobsson[1], Rune Andersson[4] and Diana Karlsson[3*]

Comparison of Magicplex sepsis (multiplex PCR) vs Provit kit (microarry on blood cultures
382 episodes of sepsis
Whole blood/blood cultures

Results : % of positivity

Blood cultures as reference (11%)
Magicplex (9,7%)
Provit (8,4%)

Conclusion → **Low performance of both tests**

Ljungström BMC Infect. Dis 2015

Biomarkers and Molecular Analysis to Improve Bloodstream Infection Diagnostics in an Emergency Care Unit

Anne J. M. Loonen[1,6], Cornelis P. C. de Jager[2], Janna Tosserams[2], Ron Kusters[3], Mirrian Hilbink[4], Peter C. Wever[5], Adriaan J. C. van den Brule[1,6]*

Comparison of SepsiTest (Molzym)
Vs Magicplex sepsis (multiplex PCR) vs
Blood cultures

125 samples analysed
Emergency department

SEN. SPE.PPV.NPV.
SepsiTest 11%, 96%, 43%, 80%,
Magicplex 37%, 77%, 30%, 82%

Conclusion → **Poor performance of both tests**

Loonen Plos One 2014

PCR coupled with ESI-Mass Spectrometry

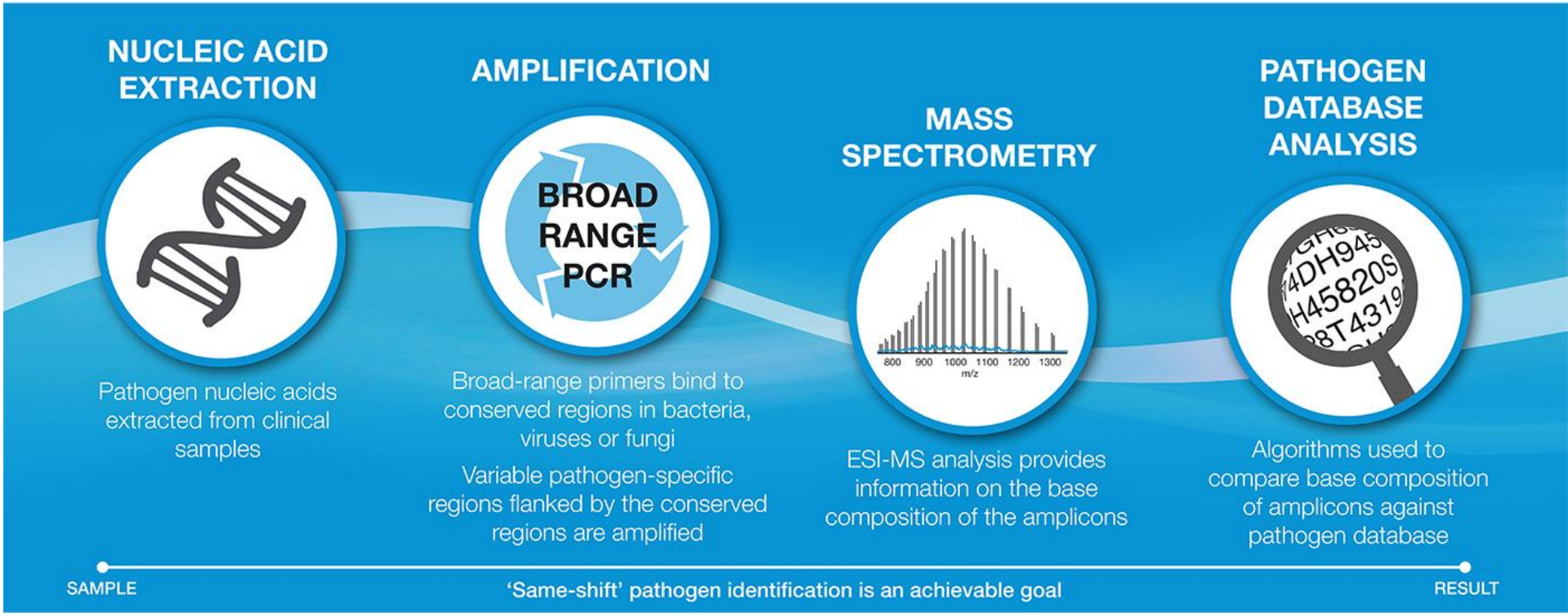
Broad Assay Menu and Sample Types

ASSAY	COVERAGE	SAMPLE TYPE
BAC BSI	**750+ Bacteria**, Candida and 4 Antibiotic Resistance Markers: mecA, vanA, vanB and kpc	5ml EDTA whole blood
BAC SFT		Sterile fluids and tissues
BAC LRT	Identical coverage as BAC BSI and BAC SFT with additional **semi-quantitative threshold**	BAL and ETA
Fungal	**200+** fungi	BAL and Isolates
Viral IC	**130+** viruses in 13 reporting groups	Plasma

CE Marked. For In Vitro Diagnostic Use.

Principle

PCR/ESI-MS: Polymerase chain reaction/electrospray ionization-mass spectrometry



Ecker et al. Expert Rev Mol Diagn 2010;10:399–415

Abbott
A Promise for Life

Evaluation of the Broad-Range PCR/ESI-MS Technology in Blood Specimens for the Molecular Diagnosis of Bloodstream Infections

Elena Jordana-Lluch[1,2,5], Montserrat Giménez[1,2], Mª Dolores Quesada[1], Belén Rivaya[1], Clara Marcó[1], Mª Jesús Domínguez[3], Fernando Arméstar[4], Elisa Martró[1,5,6]*, Vicente Ausina[1,2,5]

410 Whole blood specimen
Emergency unit and ICU patients
"suspicion of sepsis"

Table 1. Agreement between methods according to the two gold standards used by microorganisms isolated by conventional microbiological methods and detected by IRIDICA.

	Global		Emergency Room		Intensive Care Unit	
	BC gold standard	Clinical infection criterion	BC gold standard	Clinical infection criterion	BC gold standard	Clinical infection criterion
Matched positives (*n*)	176	217[a]	147	152[a]	29	65 [a]
Matched negatives (*n*)	143	143	n.a.	n.a.	143	143
IRIDICA overcalls (*n*)	80	39	21	16	59	23
IRIDICA misses (*n*)	64	64	56	56	8	8
Overall agreement (%)	68.9	77.8	n.a.	n.a.	72.0	87.0
Positive agreement (%)	73.3	77.2	72.4	73.1	78.4 [b]	89.0 [b]
Negative agreement (%)	64.1	78.6	n.a.	n.a.	70.8 [b]	86.1 [b]

CONCLUSION

Sensitivity, specificity, positive and negative predictive values compared with blood culture were 83.3%, 78.6%, 33.9% and 97.3%respectively,
and 90.5%, 87.2%, 64.4% and 97.3% respectively, in comparison with the clinical infection criterion.

Jordana-Lluch J PlosOne 2015

More recent concept: the syndromic approach

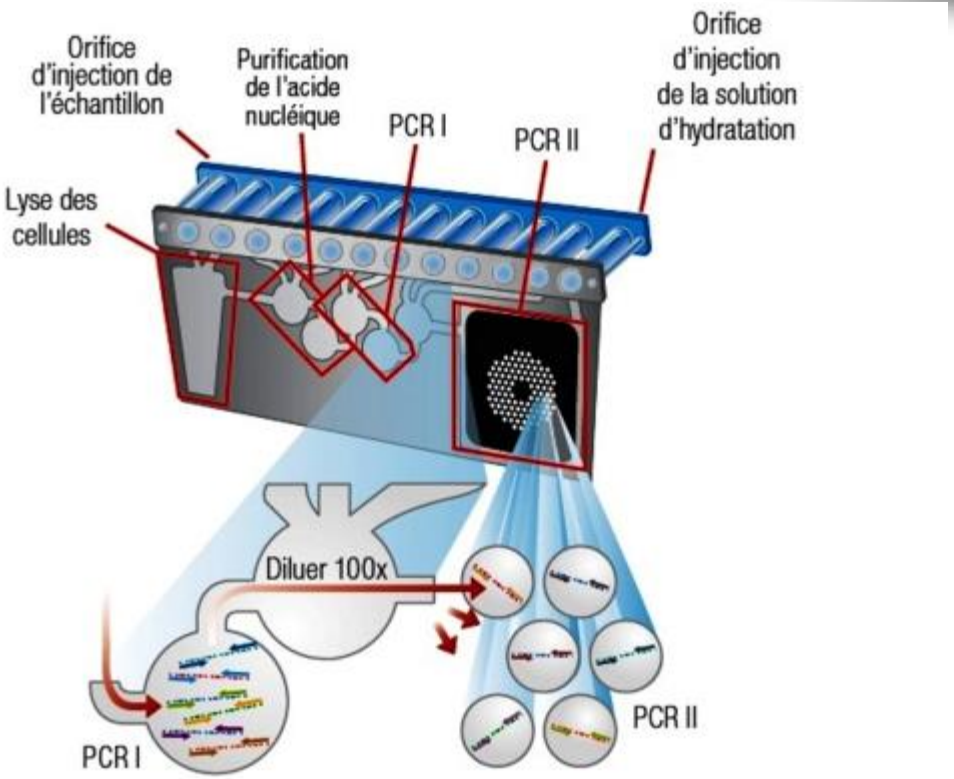
- Application ofr fungal diagnosis: meningitis
  - Very few fungal targets: only in CSF with Cryptococcus for Film array (BioFire, bioMérieux)
  - Genmark (not yet)

ePlex, GENMARK

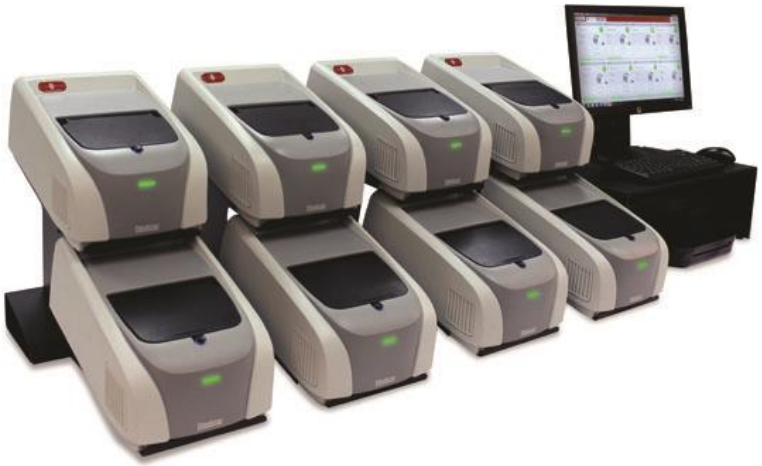
award winning

Technology

Click here to learn more about GenMark's proprietary eSensor® technology



BIOFIRE, bioMérieux



Molecular methods=targeted PCR-based assays

	Pneumocystis	Candida	Aspergillus	Mucorales
Used in routine?	Yes	Rarely	Rarely	In development
Which technology?	Real-time PCR (quantitative or qualitative)			
Commercial kits?	Yes mostly in house	Yes	Yes	In development
Kit providers	FTD *P. jirovecci* (fast- track Diagnostics)	Sacace Renishaw	Myconostics Fast-Track PathoNostics Renishaw	In house only

xtraction methods

Isolation (Bacteria & Fungi)

Benefits:

- Removal of human DNA
- Removal of PCR inhibitors
- Sensitivity increase up to factor 40,000
- Broad-range lysis of Gram-positive, Gram-negative bacteria and fungi
- DNA-free reagents
- Low hands-on-time

MolYsis™ is a pre-analytical tool serving the molecular detection of bacteraemia, fungemia and other infections. M
constitutes of a unique system for the targeted isolation of microbial DNA from clinical samples. By the selective l
human cells and the degradation of non-target human DNA, PCR analysis of pathogens is extremely enhanced th
increased sensitivity and specificity.

Complete automation

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

NPHS Microbiology, Cardiff, United Kingdom[1]; Henri Mondor Hospital, Créteil, France[2]; Karolinska University Hospital, Stockholm, Sweden[3]; Radboud University Nijmegen Medical Centre, Nijmegen, Netherlands[4]; Royal Hospital for Sick Children, Glasgow, United Kingdom[5]; Charite Hospital, Berlin, Germany[6]; Cepheid AB, Toulouse, France[7]; University of Padua, Padua, Italy[8]; Cardiff University, UHW, Cardiff, United Kingdom[9]; and Wuerzburg University, Wuerzburg, Germany[10]

Received 8 September 2009/Returned for modification 6 November 2009/Accepted 4 February 2010

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]

Clinical Performance of *Aspergillus* PCR for Testing Serum and Plasma: a Study by the European *Aspergillus* PCR Initiative

P. Lewis White,[a] Rosemary A. Barnes,[b] Jan Springer,[c] Lena Klingspor,[d] Manuel Cuenca-Estrella,[e] C. Oliver Morton,[f] Katrien Lagrou,[g] Stéphane Bretagne,[h] Willem J. G. Melchers,[i] Carlo Mengoli,[j] J. Peter Donnelly,[i] Werner J. Heinz,[c] Juergen Loeffler,[c] the EAPCRI

Public Health Wales Microbiology Cardiff, Cardiff, United Kingdom[a]; Cardiff University, UHW, Cardiff, United Kingdom[b]; University of Würzburg Medical Center, Würzburg, Germany[c]; Karolinska University Hospital, Stockholm, Sweden[d]; Spanish National Centre for Microbiology, Instituto de Salud Carlos III, Madrid, Spain[e]; University of Western Sydney, Sydney, NSW, Australia[f]; National Reference Centre for Mycosis, University Hospitals Leuven, Leuven, Belgium[g]; Department of Microbiology and Immunology, Paris Diderot University, Saint Louis Hospital-APHP, Paris, France[h]; Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands[i]; University of Padua, Padua, Italy[j]

White L. JCM 2010, 2013, 2015

Clinical Performance of *Aspergillus* PCR for Testing Serum and Plasma: a Study by the European *Aspergillus* PCR Initiative

P. Lewis White,[a] Rosemary A. Barnes,[b] Jan Springer,[c] Lena Klingspor,[d] Manuel Cuenca-Estrella,[e] C. Oliver Morton,[f] Katrien Lagrou,[g] Stéphane Bretagne,[h] Willem J. G. Melchers,[i] Carlo Mengoli,[j] J. Peter Donnelly,[i] Werner J. Heinz,[c] Juergen Loeffler,[c] the EAPCRI

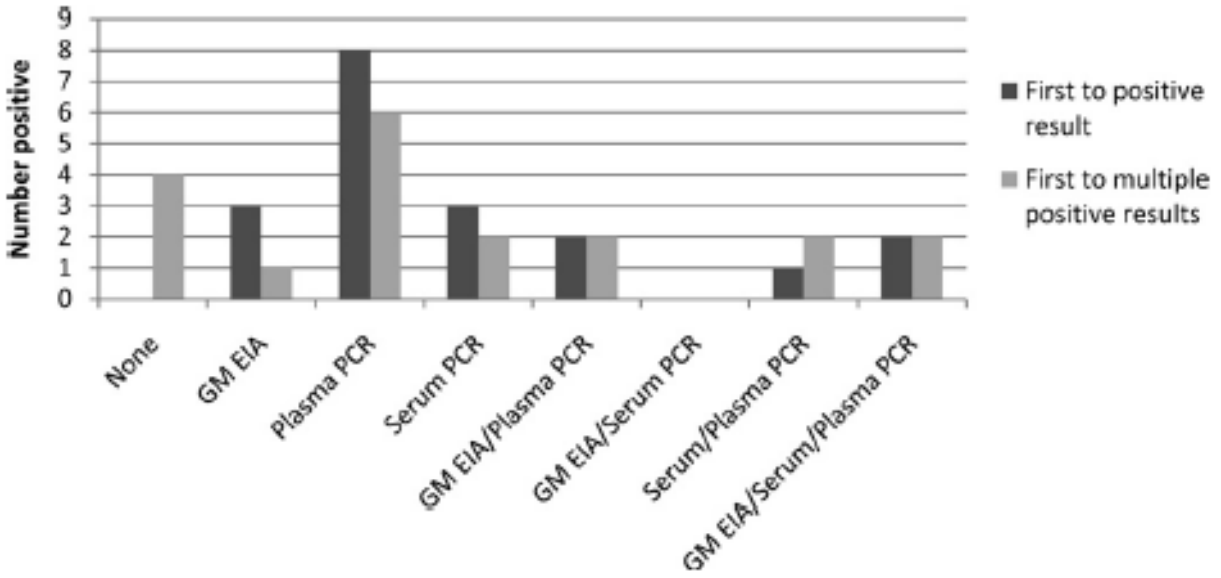
Comparative evaluation of Aspergillus PCR on serum and plasma
In population of haematological patients
Proven/probable IA
In two centers with standardized qPCR
Automated DNA extraction

Free DNA is more abundant in plasma than in serum

Poor agrement between plasma PCR and GM in serum 85%

Clinical performance PCR plasma: 95% vs 68% serum

Clinical treshold >=2 PCR positive tests



White L. JCM 2015

Validation of a New *Aspergillus* Real-Time PCR Assay for Direct Detection of *Aspergillus* and Azole Resistance of *Aspergillus fumigatus* on Bronchoalveolar Lavage Fluid

Ga-Lai M. Chong,[a] Wendy W. J. van de Sande,[b] Gijs J. H. Dingemans,[c] Giel R. Gaajetaan,[c] Alieke G. Vonk,[b] Marie-Pierre Hayette,[d] Dennis W. E. van Tegelen,[c] Guus F. M. Simons,[c] Bart J. A. Rijnders[a]

37 BAL from hematology patients +40 ICU
Population classified as IA, probable, unclassifiable
GM LBA ≥1
PCR AsperGenius®
Identification+detection of azole resistance

(TR_{34}/L98H, TR_{46}/T289A, Y121F mutations on CYP51A gene

PCR *Aspergillus*	Hematology	ICU
Sensitivity	88.9%	80%
Specificity	89.3%	93.3%
PPV	72.7%	80%
NPV	96.2%	93.3%

Cuenca-Estrella CMI 2012

ESCMID guidelines

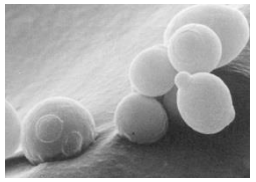


TABLE 2. Summary of recommendations by *Candida* disease, specimen and test evaluated

Disease	Specimen	Test	Recommendation	Level of evidence
Candidaemia	Blood	Blood culture	Essential investigation[a]	NA
	Serum	Mannan/anti-mannan	Recommended	II
		B-D-glucan	Recommended	II
		Other antibodies	No recommendation	No data
		Septifast PCR kit	No recommendation	No data
		In-house PCR	No recommendation	No data
Invasive candidiasis	Blood	Blood culture	Essential investigation	NA
	Serum	Mannan/anti-mannan	No recommendation	No data
		B-D-glucan	Recommended	II
		Septifast PCR kit	No recommendation	No data
		In-house PCR	No recommendation	No data
	Tissue and sterile body fluids	Direct microscopy and histopathology	Essential investigation	NA
		Culture	Essential investigation	NA
		Immuno-histochemistry	No recommendation	No data
		Tissue PCR	No recommendation	No data
		In situ hybridization	No recommendation	No data
Chronic disseminated candidiasis	Blood	Blood culture	Essential investigation	NA
	Serum	Mannan/anti-mannan	Recommended	II
		B-D-glucan	Recommended	II
		Septifast PCR kit	No recommendation	No data
		In-house PCR	No recommendation	No data
	Tissue and sterile body fluids	Direct microscopy and histopathology	Essential investigation	NA
		Culture	Essential investigation	NA
		Immuno-histochemistry	No recommendation	No data
		Tissue PCR	No recommendation	No data
		In situ hybridization	No recommendation	No data
Oropharyngeal and oesophagic candidiasis	Swab	Culture	Essential investigation	NA
		In-house PCR	No recommendation	No data
	Biopsy[b]	Direct microscopy and histopathology	Essential investigation	NA
		Culture	Essential investigation	NA
		In-house PCR	No recommendation	No data
Vaginal candidiasis	Swab/vaginal secretions	Direct microscopy	Essential investigation	NA
		Culture	Essential investigation	NA
		Commercial tests	Use validated test only	NA
		In-house PCR	No recommendation	No data

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

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

Medicine E[1] and Unit of Infectious Diseases,[2] Rabin Medical Center, Beilinson Hospital and Sackler Faculty of Medicine, Tel-Aviv University, Tel Aviv, Israel

54 studies included

4694 patients→963 proven/probable or possible invasive candidiasis

Perfect sensitivity+ specificity calculated when ***candidemia+ healthy controls***

Positivity rates = 85%

Vsblood cultures = 38%

Conclusion of the meta-analysis

- Best results if WB samples, rRNA or P450 genes andif limit of detection is >10 cfu/ml

Avni T. JCM 2011

Performance of *Candida* Real-time Polymerase Chain Reaction, β-D-Glucan Assay, and Blood Cultures in the Diagnosis of Invasive Candidiasis

M. Hong Nguyen,[1] Mark C. Wissel,[2] Ryan K. Shields,[1] Martin A. Salomoni,[2] Binghua Hao,[1] Ellen G. Press,[1] Ryan M. Shields,[2] Shaoji Cheng,[1] Dimitra Mitsani,[1] Aniket Vadnerkar,[1] Fernanda P. Silveira,[1] Steven B. Kleiboeker,[2] and Cornelius J. Clancy[1,3]

[1]Department of Medicine, University of Pittsburgh, Pennsylvania; [2]Viracor-IBT Laboratories, Lee's Summit, Missouri; and [3]Department of Medicine, VA Pittsburgh Healthcare System, Pennsylvania

Fungitell® serum or plasma
Population with invasive candidiasis and controls
RT-PCR ITS1 & ITS2 on WB, plasma et/or serum
C. albicans+C. tropicalis,
C. glabrata+C.krusei, C. parapsilosis complex

Prospective study
April 2009-2011
Pittsburg, USA

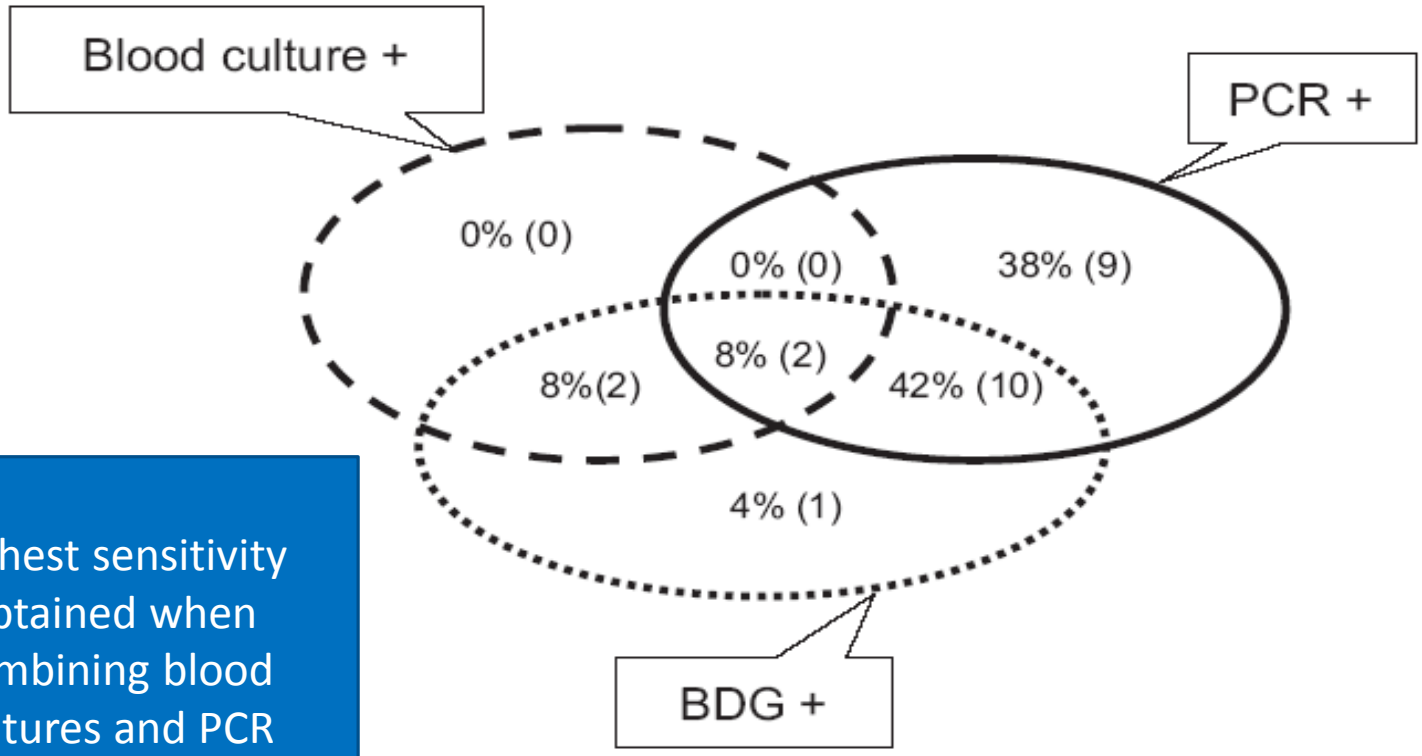
Nguyen MH,CID 2012

Performance of *Candida* Real-time Polymerase Chain Reaction, β-D-Glucan Assay, and Blood Cultures in the Diagnosis of Invasive Candidiasis

Assay	Invasive Candidiasis (n = 55)	Candidemia[a] (n = 22)	Deep-Seated Candidiasis[a,b] (n = 38)	Intra-abdominal Candidiasis (n = 34)
PCR[c]				
Sensitivity	80% (44/55)	59% (13/22)	89% (34/38)	88% (30/34)
Specificity	70% (51/73)			
BDG (positive ≥80 pmol/mL)				
Sensitivity	56% (31/55)	68% (15/22)	53% (20/38)	56% (19/34)
Specificity	73% (53/73)			
BDG (positive ≥60 pmol/mL)				
Sensitivity	69% (38/55)	81% (18/22)	66% (25/38)	65% (22/34)
Specificity	63% (46/73)			
P value[d]				
PCR vs BDG (positive ≥80 pmol/mL)	.03	.77	.004	.0015
PCR vs BDG (positive ≥60 pmol/mL)	.31	.23	.04	.06

Nguyen MH,CID 2012

PCR performance



Highest sensitivity obtained when combining blood cultures and PCR

Nguyen MH,CID 2012

Evaluation of a Commercially Developed Semiautomated PCR–Surface-Enhanced Raman Scattering Assay for Diagnosis of Invasive Fungal Disease

P. Lewis White,[a] Samantha J. Hibbitts,[b] Michael D. Perry,[a] Julie Green,[c] Emma Stirling,[c] Luke Woodford,[c] Graeme McNay,[c] Ross Stevenson,[c] Rosemary A. Barnes[b]

Public Health Wales, Microbiology Cardiff, UHW, Cardiff, United Kingdom[a]; School of Medicine, Cardiff University, UHW, Cardiff, United Kingdom[b]; Renishaw Diagnostics Ltd., Glasgow, United Kingdom[c]

In vitro testing of spiked blood samples and clinical assessment on stored blood samples.
Sensitivity: 1 -10cfu/ml

Only 28 samples
Sensitivity 80%

- better in case of candidemia)
- Sensitivity vary with sample type =serum<whole blood<plasma

Specificity 87,5%

Conclusion → **Nee of further evalutation in a prospective study**

Real-time PCR *Pneumocystis jirovecii*

Meta-analysis (*Summah H.. Chin Med J 2013)*

- 10 studies were included 1990-2010
- Sensitivity 98%
- Specificity 94%
- Better performance on BAL than other samples

RT-PCR semi-quantitative: definition of cut-off® *(Fillaux J. et Berry A. Methods Mol Biol. 2013;943:159-70)*

- **Ct ≥ 28** : *probable colonisation*
- **22 ≤ Ct ≥ 28** : possible PcP
- **Ct < 22**: PcP

Proposed algorithme for PcP diagnosis

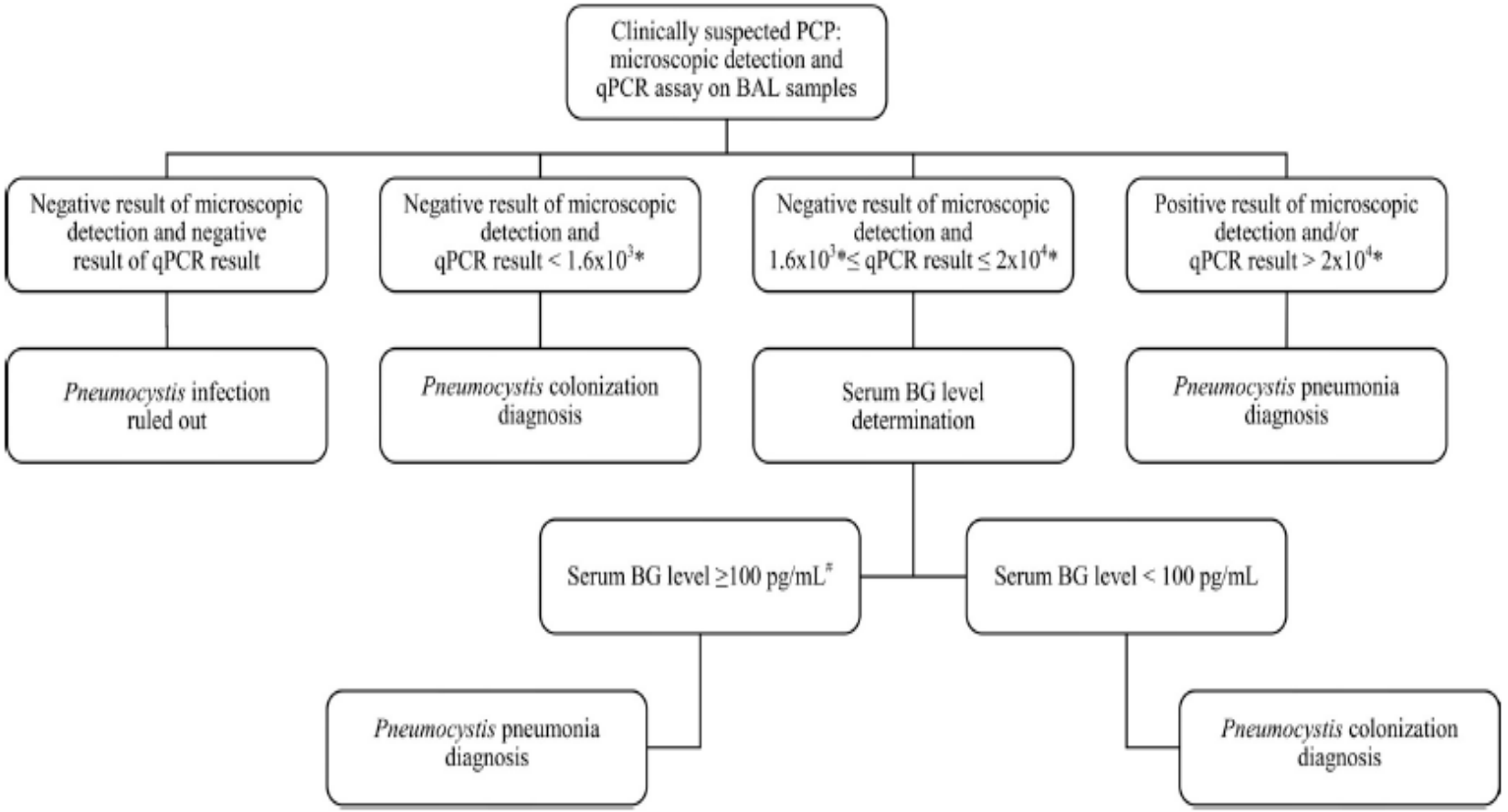
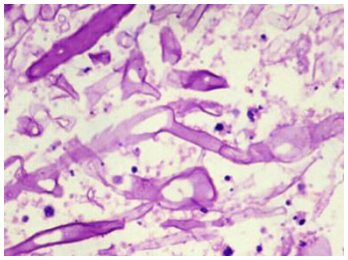


FIG 5 Flow diagram for the diagnosis of *Pneumocystis* infections in patients with clinically suspected *Pneumocystis* pneumonia. Microscopic examination using methanol-Giemsa staining and an immunofluorescence assay (Bio-Rad, Marnes la Coquette, France) and a qPCR assay targeting the mitochondrial large subunit rRNA gene of *P. jirovecii* were used. BAL, bronchoalveolar lavage fluid; BG, (1→3)-β-D-glucan; PCP, *Pneumocystis* pneumonia; *, results expressed in DNA copies/μl; #, without factors that interfere with (1→3)-β-D-glucan level determinations, particularly concurrent invasive fungal infections.

Damiani C, JCM, 2013

Quantitative Polymerase Chain Reaction Detection of Circulating DNA in Serum for Early Diagnosis of Mucormycosis in Immunocompromised Patients



Laurence Millon,[1,2] Fabrice Larosa,[3] Quentin Lepiller,[2,4] Faezeh Legrand,[3] Steffi Rocchi,[1] Etienne Daguindau,[3] Emeline Scherer,[1,2] Anne-Pauline Bellanger,[1,2] Joel Leroy,[5] and Frederic Grenouillet[1,2]

[1]CNRS-Université de Franche-Comté, UMR 6249 Chrono-environnement, and Departments of [2]Parasitology-Mycology, [3]Clinical Hematology, [4]Virology, and [5]Infectious Diseases, University Hospital, Besançon, France

10 Patients with confirmed mucormycosis

Combinaison of 3quantitative RT-PCR, 18S RNA genes

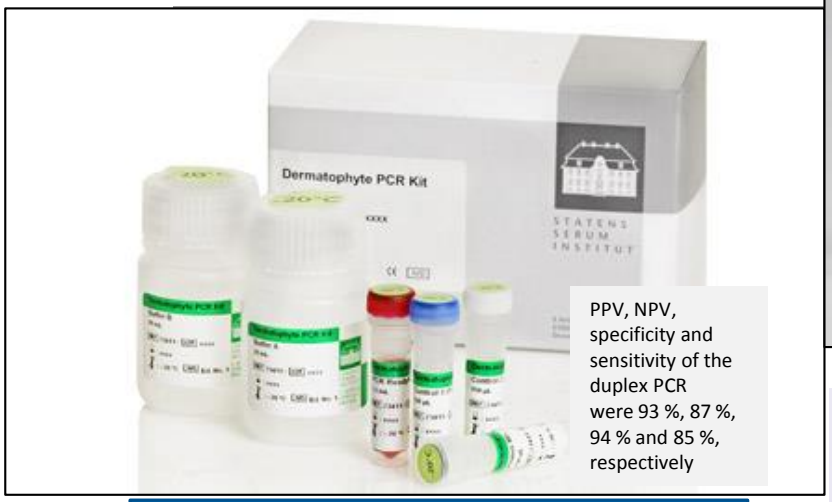
Targets: Mucor/Rhizopus, Lichtheimia, Rhizomucor

9/10 PCR positive serum

3-68 days before diagnosis confirmation

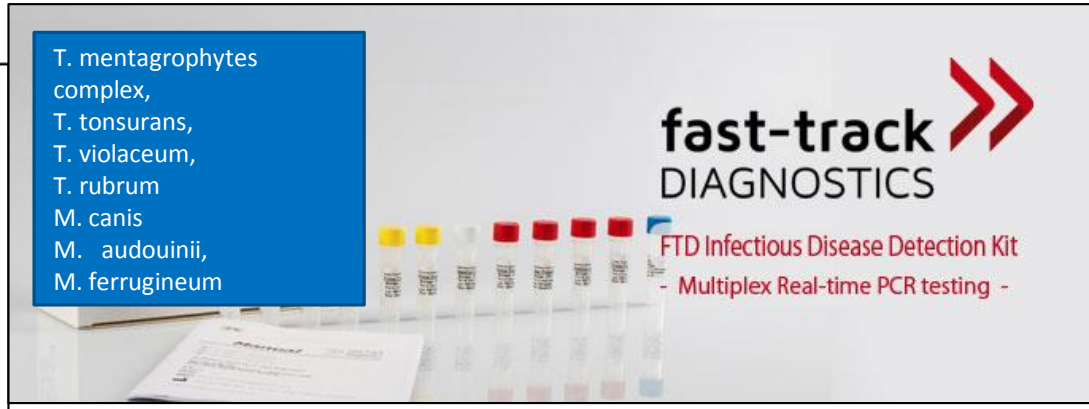
Millon L CID 2013

Superficial mycosis: commercial PCR kits for detection/identification of dermatophytes

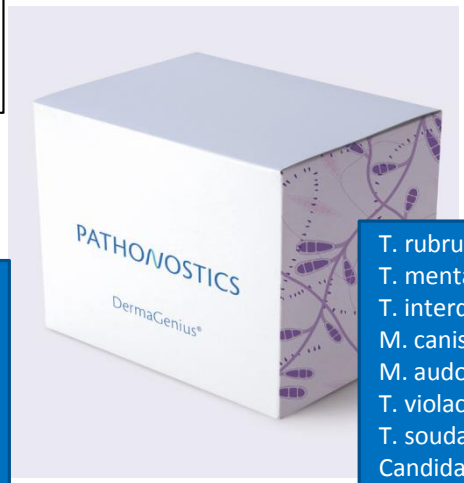


PPV, NPV, specificity and sensitivity of the duplex PCR were 93 %, 87 %, 94 % and 85 %, respectively

Dermatophytes + T. rubrum



T. mentagrophytes complex,
T. tonsurans,
T. violaceum,
T. rubrum
M. canis
M. audouinii,
M. ferrugineum



T. rubrum,
T. mentagrophytes
T. interdigitale
M. canis
M. audouinii
T. violaceum
T. soudanense
Candida albicans
E. floccosum

Specificity and sensitivity of the PCR are 85 %, 87 %, 94 % and 100 %,

IDEXX BioResearch

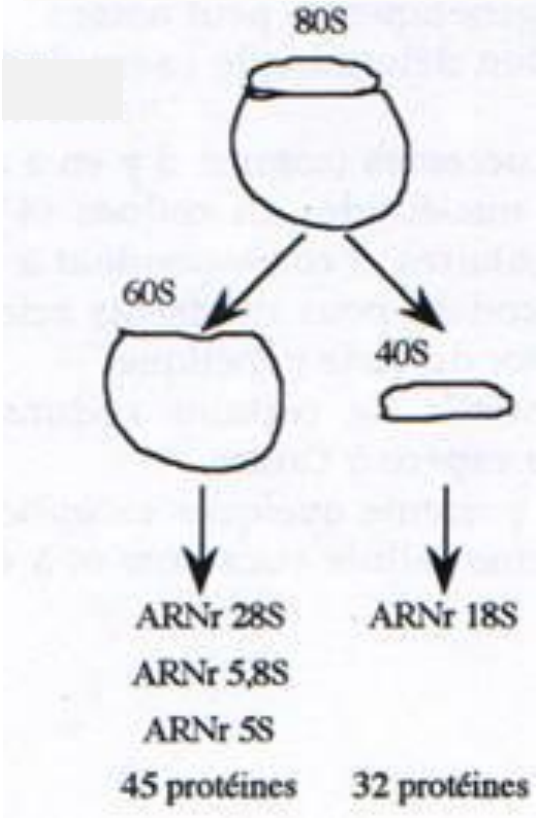
95% sensitivity
99 specificity

Microsporum spp.
M. canis
M. gypseum
M. ferrugineum
M. audouinii
Trichophyton spp.
T. mentagrophytes
T. tonsurans • T. rubrum
T. megninii
T. violaceum
T. schoenleinii

Hayette MP & Sacheli R Curr Fungal Infect Rep 2015
Hayette MP TIMM congress 2015

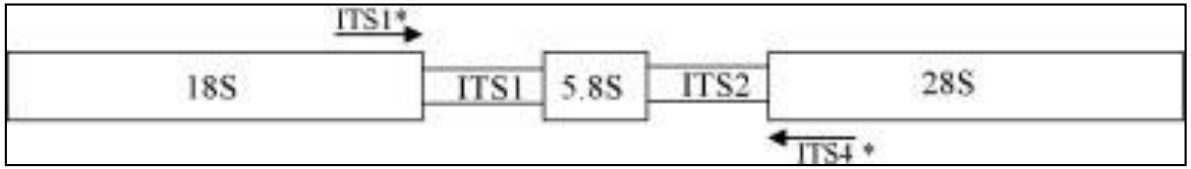
Molecular sequencing

EUCARYOTES



ITS/ARNr

- Internal Transcribed Spacer
- About 1500 pb
- rARN multicopy gene



Chronic granulomatosis
Multiples hepatic lesions
-hepatic puncture: mucorales
-positive blood culutre: mucorales
ITS sequencing: *Mortierella wolfii*

Layios N. CMI 2014

ITS primers: choose the right one

Primer name	Sequence (5'-3')	Reference
ITS1 (f)	TCCGTAGGTGAACCTGCGG	White *et al.* 1990
ITS1F (f)	CTTGGTCATTTAGAGGAAGTAA	Gardes et Bruns 1993
ITS2 (r)	GCTGCGTTCTTCATCGATGC	White *et al.* 1990
ITS3 (f)	GCATCGATGAAGAACGCAGC	White *et al.* 1990
ITS4 (r)	TCCTCCGCTTATTGATATGC	White *et al.* 1990
ITS86F (f)	GTGAATCATCGAATCTTTGAA	Turenne *et al.* 1999
SR6R (f)	AAGTAAAAGTCGTAACAAGG	Gräser *et al.*1999
LR1 (r)	GGTTGGTTTCTTTTCCT	Gräser *et al.*1999

Lagrou K. et al. ASM book 2015

Conclusion

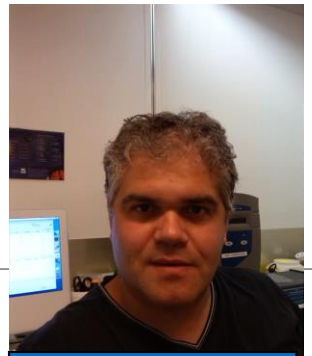
Need to define the place of fungal PCR and the methods to use for PCR in invasive fungal diagnosis: Aspergillus /Candida/mucorales→screening blood?

Need to have available PCR assays easy to use, not expensive and fully automated

Get the best DNA extraction method and you have >50% to succeed.

Giuseppina

Caroline



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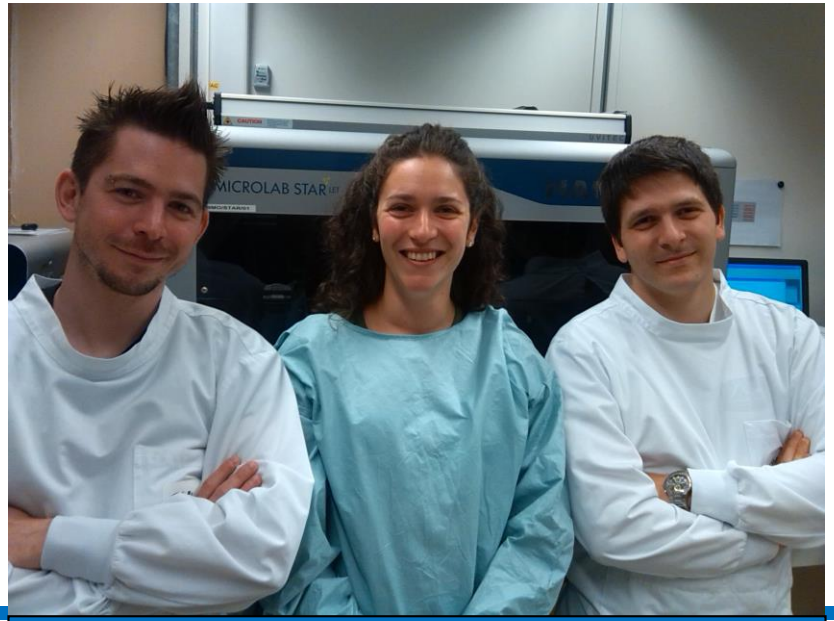
The team of the molecular biology plateform (microbiology division) in CHULg



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