Update on molecular diagnostic tests for yeast and mould infections: where do we stand?

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2nd ESCMID Conference on Invasive Fungal Infections 16-18 January 2013

Molecular diagnostic tests

- Huge diversity of applications
  - Identification
  - Typing
  - Resistance detection
  - …
  - Detection in clinical samples
- Two mains species
  - Aspergillus
  - Candida

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Diagnosis

- PCR data are always not included in the consensual criteria for definitions of invasive fungal diseases [1] although many PCR assays have been developed over the previous 20 years
- Large number of different PCR protocols published and the absence of a consensus on the optimal PCR technique to be used
  - DNA targets, although this is predominantly the rDNA gene
  - the chemistry of the probes (hydrolysis probes; hybridization probes; or molecular beacons)
  - the PCR apparatus used
- Pub Med interrogation with « aspergillus », « PCR », and « diagnosis » over the last 5 years gives 252 publications and 24 reviews in English

(1) De Pauw, B. et al. *Clin Infect Dis* **46**, 1813-21 (2008)

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Virology

- Different protocols are also published for viruses, but this does not hamper the acceptance of diagnostic PCR in this field.
- For viruses it is common to work with $>10^3$ copies. This is not the case with IA where the number of copies is at the limit of detection of the PCR which exacerbates the limits and pitfalls of PCR.

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Difficulties when developing a PCR assay

- Technical issues: PCR assay designs have evolved during these past 20 years.
  - Internal controls [1, 2] (should be mandatory [3], cannot be human DNA)
  - Enzymatic prevention of contamination [1]
  - Avoid nested PCR format [2, 5]
- Reach a consensual procedure based on the "Minimum Information for the publication of real-time Quantitative PCR Experiments" (MIQE) guidelines [2]

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(1) Bretagne, S. et al. *J Clin Microbiol* **33**, 1164-8 (1995)
(2) Bustin, S.A. et al. *Clin Chem* **55**, 611-22 (2009)
(3) Hoorfar, J. et al. *J Clin Microbiol* **41**, 5835 (2003)
(4) Burkardt, H.J. *Clin Chem Lab Med* **38**, 87-91 (2000)
(5) Bretagne, S. *Curr Infect Dis Rep* **12**, 430-6 (2011)

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A MIQE-Compliant Real-Time PCR Assay for *Aspergillus* Detection

Gemma L. Johnson[1*], David F. Bibby[3], Stephenie Wong[4], Samir G. Agrawal[1,2], Stephen A. Bustin[1]

1 Blizard Institute of Cell and Molecular Science, Queen Mary University, London, United Kingdom, 2 Department of Haemato-Oncology, St Bartholomew's Hospital, London, United Kingdom, 3 Division of Infection, Barts and the London NHS Trust, London, United Kingdom, 4 Queen Elizabeth Hospital, Kowloon, Hong Kong, Special Administrative Region, People's Republic of China

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End of the story?

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Table 3. Clinical evaluation of BAL and blood in 11 adults at high risk of IFD following intensive chemotherapy or allogeneic stem cell transplantation.

	EORTC/MSG score		BAL fluid		Blood/serum	
Episode	2002	2008	PCR result (mean C_q)	GM result	PCR result (mean C_q)	GM result[i]
1	Possible	Non classifiable	Negative	0.4	Negative	<0.5
2	Possible	Possible	Negative	0.1	Negative	<0.5
3	Possible	Non classifiable	Negative	0.2	Negative	<0.5
4	Possible	Possible	Negative	0.4	Negative	<0.5
5	Possible	Non classifiable	Negative	0.2	Negative	<0.5
6	Possible	Possible	Negative	0.3	Negative	<0.5
7	Possible	Possible	Negative	0.4	Negative	<0.5
8	Probable	Non classifiable	34.8	4.1	NA	NA
9	Possible	Non classifiable	27.8	6.4	A. Negative[ii], B. Negative, C. 37.6	A. Negative[ii], B. Negative, C. 2.2
10	Possible	Non classifiable	Negative	0.3	Negative	<0.5
11	Possible	Non classifiable	Positive[i]	1.41	36.8	0.15

NA = no sample available for testing.
i. <0.5 indicates that all samples from serial testing were negative.
ii. C_q not stated, as >1 µl was loaded, hence C_q is not comparable.
iii. A, B, C represent serial samples (see corresponding text).

doi:10.1371/journal.pone.0040022.t003

Risk of false positives

- Presence of environmental fungi (DNA), which can contaminate every step of the PCR process
- The standard precautions include
  - working in laminar flow hoods (efficient against fungal elements rather than DNA)
  - wearing gloves and gown
  - using a unidirectional workflow environment with physically separated laboratories for pre-, per-, and post-PCR analysis
  - the use of aerosol-resistant tips and specific pipettes
  - never open the qPCR plates (no amplicon in the environment)
  - difficult to maintain these precautions in a routine laboratory
- However, these measures are inadequate when the contamination is in the sampling tubes [1] or IV administrated to the patient [2]
  - Primer specificity

(1) Harrison, E. et al. Fungal DNA contamination of blood collection tubes. in *48th ICAAC*, Washington, DC, 2008
(2) Millon, L. et al. *Med Mycol* **48**, 661-4 (2010).

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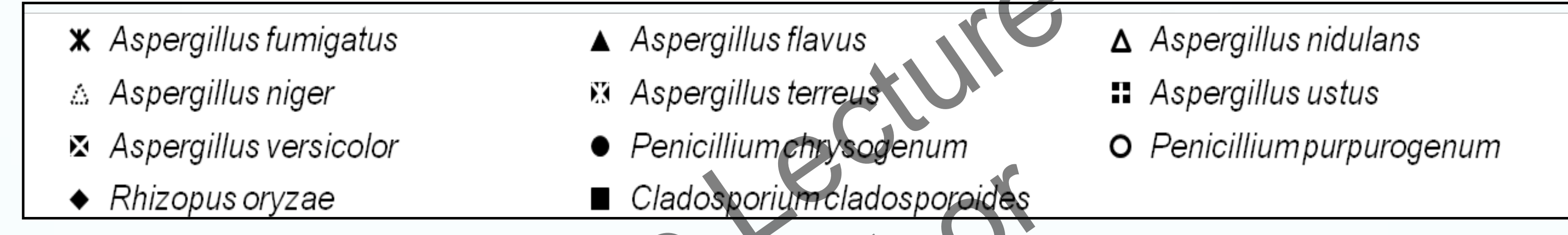
Primer specificity



Set 7
Set 6
Set 5
Set 4
Set 3
Set 2
Set 1

-10 -5 0 5 10 15 20 >25 ΔCq

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Odile Cabaret and Stéphane Bretagne, personal data

Risk of false positives

- Environmental DNA
  - Use probes to confirm diagnosis (no SYBER Green and melting curves)
  - Primer specificity
    - The more specific, the lower the risk of false positives with environmental DNA
    - The more specific, the more likely to miss non-*fumigatus* infection

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Risk of false negatives

- The problem is to detect minute concentrations of fungal DNA among huge amounts of undesirable human DNA for the detection of invasive fungal diseases.
- Example: ITS1 primer

Enter Query Sequence

BLASTN programs search

Enter accession number(s), gi(s), or FASTA sequence(s)

Clear

TCCGTAGGTGAACCTGCGG

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Download GenBank Graphics

Homo sapiens unplaced genomic scaffold, alternate assembly HuRef SCAF_1103279181758

Sequence ID: ref|NW_001839755.1| Length: 5613 Number of Matches: 1

Range 1: 826 to 844 GenBank Graphics Next Match Previous Match

Score	Expect	Identities	Gaps	Strand
38.2 bits(19)	0.059	19/19(100%)	0/19(0%)	Plus/Plus

```
Query  1       TCCGTAGGTGAACCTGCGG  19
               |||||||||||||||||||
Sbjct  826     TCCGTAGGTGAACCTGCGG  844
```

Download GenBank Graphics Sort by: E value

Homo sapiens unplaced genomic contig, GRCh37.p10 Primary Assembly

Sequence ID: ref|NT_167214.1| Length: 161802 Number of Matches: 2

Range 1: 110917 to 110935 GenBank Graphics Next Match Previous Match

Score	Expect	Identities	Gaps	Strand
38.2 bits(19)	0.059	19/19(100%)	0/19(0%)	Plus/Plus

```
Query  1       TCCGTAGGTGAACCTGCGG  19
               |||||||||||||||||||
Sbjct  110917  TCCGTAGGTGAACCTGCGG  110935
```

Range 2: 154889 to 154907 GenBank Graphics Next Match Previous Match First Match

Score	Expect	Identities	Gaps	Strand
38.2 bits(19)	0.059	19/19(100%)	0/19(0%)	Plus/Plus

```
Query  1       TCCGTAGGTGAACCTGCGG  19
               |||||||||||||||||||
Sbjct  154889  TCCGTAGGTGAACCTGCGG  154907
```

Download GenBank Graphics Sort by: E value

Homo sapiens chromosome 2 genomic scaffold, alternate assembly CHM1_1.0

Sequence ID: ref|NW_004078008.1| Length: 138392755 Number of Matches: 30

Range 1: 37531320 to 37531335 GenBank Graphics Next Match Previous Match

Score	Expect	Identities	Gaps	Strand
32.2 bits(16)	3.6	16/16(100%)	0/16(0%)	Plus/Minus

Features: 453557 bp at 5' side: uncharacterized protein LOC408029
50718 bp at 3' side: LOW QUALITY PROTEIN: zinc finger protein 285

```
Query  4         GTAGGTGAACCTGCGG  19
                 ||||||||||||||||
Sbjct  37531335  GTAGGTGAACCTGCGG  37531320
```

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Identification

- ITS1 designed for species identification[1]
- Starting material: DNA from pure culture
- ITS1 not suitable for diagnosis from human clinical samples

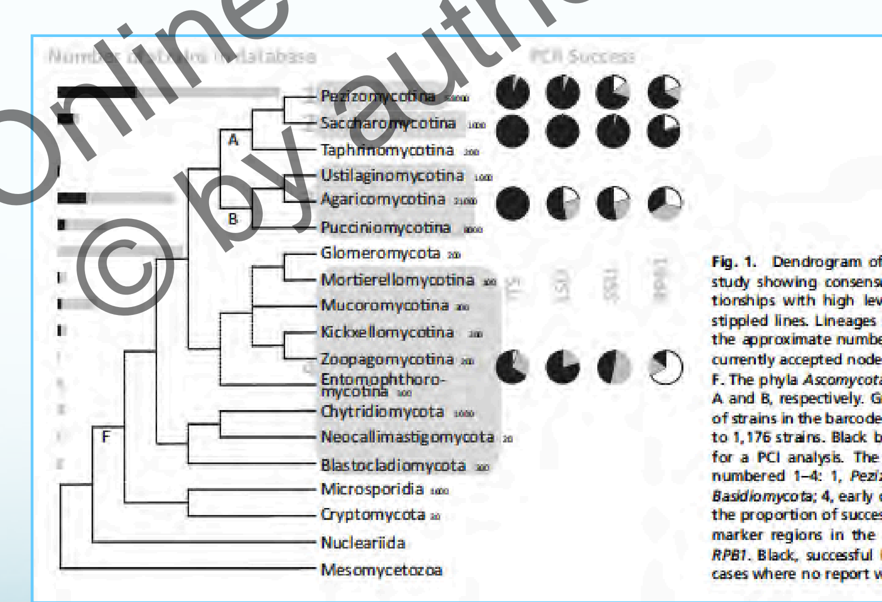

Fig. 1. Dendrogram of 17 fungal lineages sampled in this study showing consensus relationships and sampling. Relationships with high levels of uncertainty are indicated by stippled lines. Lineages are labeled and listed together with the approximate number of currently described species. The currently accepted node for delineating *Fungi* is indicated by F. The phyla *Ascomycota* and *Basidiomycota* are indicated by A and B, respectively. Gray bars to the left indicate numbers of strains in the barcode database, with the longest bar equal to 1,176 strains. Black bars indicate the proportions selected for a PCI analysis. The four datasets analyzed for PCI are numbered 1–4: 1, *Pezizomycotina*; 2, *Saccharomycotina*; 3, *Basidiomycota*; 4, early diverging lineages. Pie charts indicate the proportion of success from attempts to amplify the four-marker regions in the following order: ITS, LSU, SSU, and *RPB1*. Black, successful PCRs and sequences; gray, uncertain cases where no report was given; white, unsuccessful PCR.

(1) Schoch CL et al PNAS 2012; 6241-6

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Still unsolved issue: *Aspergillus* DNA origin?

- Impacts on DNA extraction
  - Blood [1] : fungal elements
    - Large volume
    - Stringent DNA extraction
    - Free DNA not useful for controlling DNA extraction
  - Serum [2,3,4] : circulating nucleic acids
    - Automated DNA extraction [2,3]
    - Large volume [4]
    - Avoid stringent DNA extraction
    - DNA (mouse, plant, virus, plasmid) as DNA extraction control

(1) White, P.L. et al. *J Clin Microbiol* **48**, 1231-40 (2010)
(2) Costa, C. et al. *J Clin Microbiol* **40**, 2224-7 (2002)
(3) Suarez, F. et al. *J Clin Microbiol* **46**, 3772-7 (2008)
(4) White, P.L. et al. *J Clin Microbiol* **49**, 3842-8 (2011)

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JOURNAL OF CLINICAL MICROBIOLOGY, Oct. 2011, p. 3596–3599 Vol. 49, No. 10
0095-1137/11/$12.00 doi:10.1128/JCM.00647-11
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Analysis of Performance of a PCR-Based Assay To Detect DNA of *Aspergillus fumigatus* in Whole Blood and Serum: a Comparative Study with Clinical Samples▽

Leticia Bernal-Martínez, Sara Gago, María J. Buitrago, Alicia Gomez-Lopez, Juan L. Rodríguez-Tudela, and Manuel Cuenca-Estrella*

- Both blood and serum samples available in 26 cases
- No significant differences observed
  - Cq= 37.6 for blood vs 37.4 for serum
- «We recommend serum samples as the most convenient hematological sample to use for *Aspergillus* DNA quantification when serial determinations are done»

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PCR in bronchoalveolar lavage

- Metaanalysis [1]
  - Sensitivity: 79% (72.8-83.1)
  - Specificity: 94% (92.1-95.0)
- Metaanalysis [2]
  - Sensitivity: 0.91 (0.79–0.96)
  - Specificity: 0.92 (0.87–0.96)
- Metaanalysis [3]
  - Sensitivity: 77.2% (62-87.6%)
  - Specificity: 93.5% (90.6-95.6%)

(1) Tuon, F.F. *Rev Iberoam Micol* **24**, 89-94 (2007)
(2) Sun, W. et al. *PLoS One* **6**, e28467 (2011)
(3) Avni T et al *J Clin Microbiol* **50**, 3652-8 (2012)

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Summary of the 15 manuscripts included in meta-analysis of *Aspergillus* PCR testing BAL (Tuon, 2007)

			Sensitivity (%)	
Manuscript	Extraction Protocol	Appropriate disease definitions	Tuon	Original
Spreadbury 1993	Bead-beating in liquid N_2 Phe/Chloro/Alc ppt	Unknown	100	100
Melchers 1994	Bead-beating, Phe/Chloro/Alc ppt	yes	100	100
Bretagne 1995	Hot detergent (10mM NaOH 100 C/10min)	yes	100	100
Jones 1998	PK digestion Phe/Chloro/Alc ppt	Proven yes prob/poss combined	100	100
Verweij 1995	Bead-beating, Phe/Chloro/IIAA ppt	yes	71	71
Tang 1993	Bead-beating in liquid N_2 Phe/Chloro/Alc ppt	Unknown	100	100
Einsele 1998	Lyticase, then PK/SDS Phe/Chloro/Alc ppt	yes	66	63 (100)
Skladny 1999	Lyticase, then PK/SDS Phe/Chloro/Alc ppt	yes	36	100*
Buchheidt 2001	Lyticase, then PK/SDS Phe/Chloro/Alc ppt	yes	76	100
Hayette 2001	Bead-beating in liquid N_2 Phe/Chloro/Alc ppt	Proven yes, probable needs CT	100	100
Raad 2002	SDS/PK/ Phe/Chloro/Alc ppt	yes	69	69
Sanguinetti 2003	DNeasy Plant mini kit	yes	90	90
Jalava 2003	Lyticase, bead-beating, Phe/Chloro/Alc ppt	yes	73	73
Musher 2003	Master Pure yeast kit	Proven yes, prob/poss combined	67	67
Spiess 2003	Lyticase, then PK/SDS Phe/Chloro/Alc ppt	Yes	100	100

*Note: Paper does not state that all the patients with proven probable disease had BAL specimens tested. It only presents PCR positive results. Sensitivity in the original manuscript was calculated using a combined whole blood/BAL approach

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Sanguinetti 2003	DNeasy Plant mini kit	yes	90	90
Jalava 2003	Lyticase, bead-beating, Phe/Chloro/Alc ppt	yes	73	73
Musher 2003	Master Pure yeast kit	Proven yes, prob/poss combined	67	67
Spiess 2003	Lyticase, then PK/SDS Phe/Chloro/Alc ppt	Yes	100	100

Lyticase = 5/15; sensitivity = 70%
Bead-beating = 5/15 sensitivity = 94%

*Note: Paper does not state that all the patients with proven probable disease had BAL specimens tested. It only presents PCR positive results. Sensitivity in the original manuscript was calculated using a combined whole blood/BAL approach

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Meaning of a PCR+ result

- *Aspergillus* DNA was detected:
  - in BAL from patients with culture or microscopy confirmed IPA (21/22, 95%)
  - in sputum in 15 of 19 (78.9%) patients with ABPA
  - in sputum in 30 of 42 (71.4%) patients with CPA
  - in BAL from normal volunteers (4/11, 36.4%)
- Define thresholds using quantitative PCR?
  - High heterogeneity of BAL procedures
- *In culture -/PCR+ samples, detection of triazole-resistance mutations in 55.1% of samples (ABPA and CPA patients)*

(1) Denning DW et al. *Clin Infect Dis* **52**, 1123-9 (2011)

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Evaluation of qPCR assay performance

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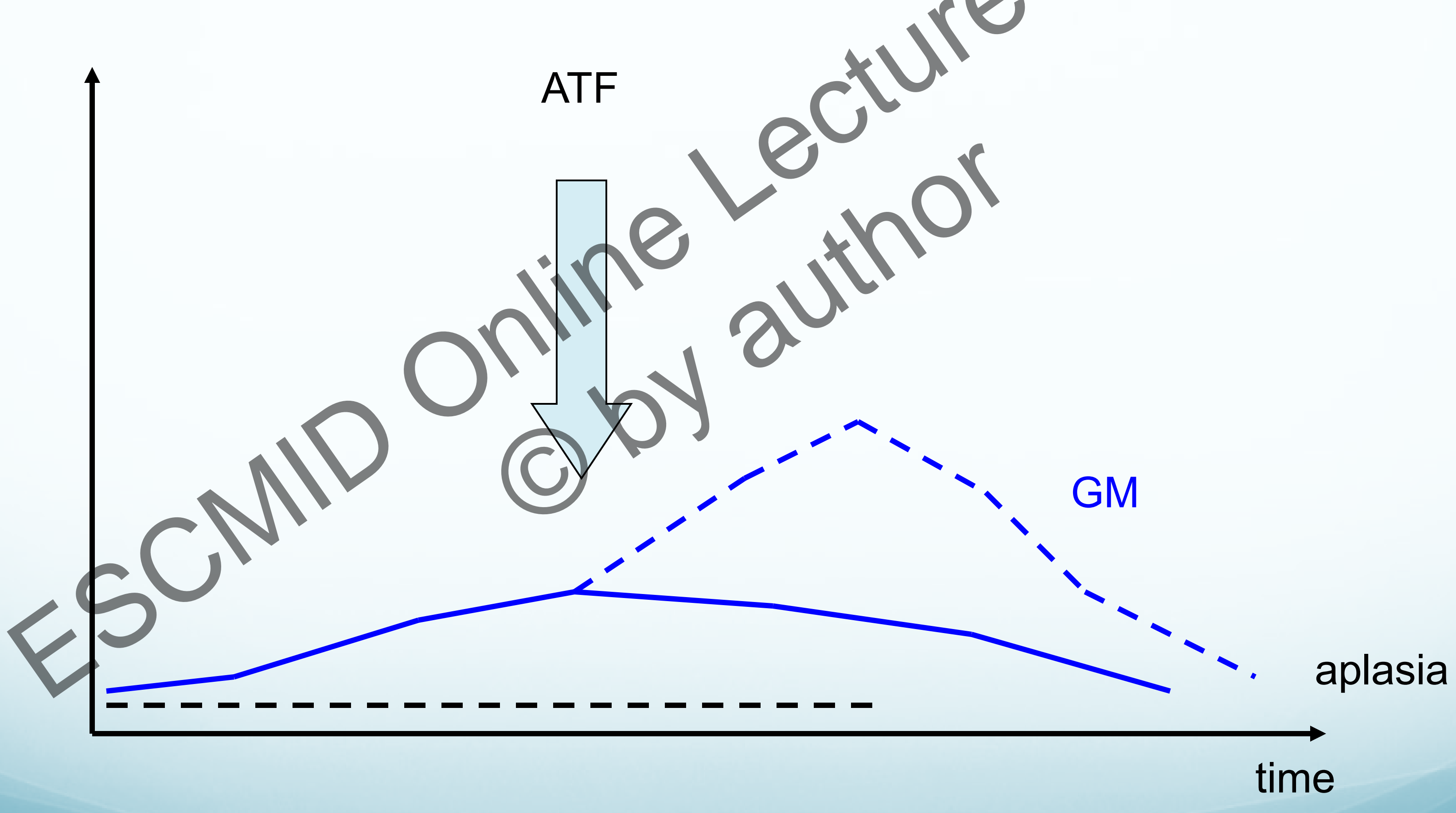
Development of an aspergillosis

qPCR combined use with galactomannan[1]
Anti-fungal therapy decreases GM sensitivity[2]



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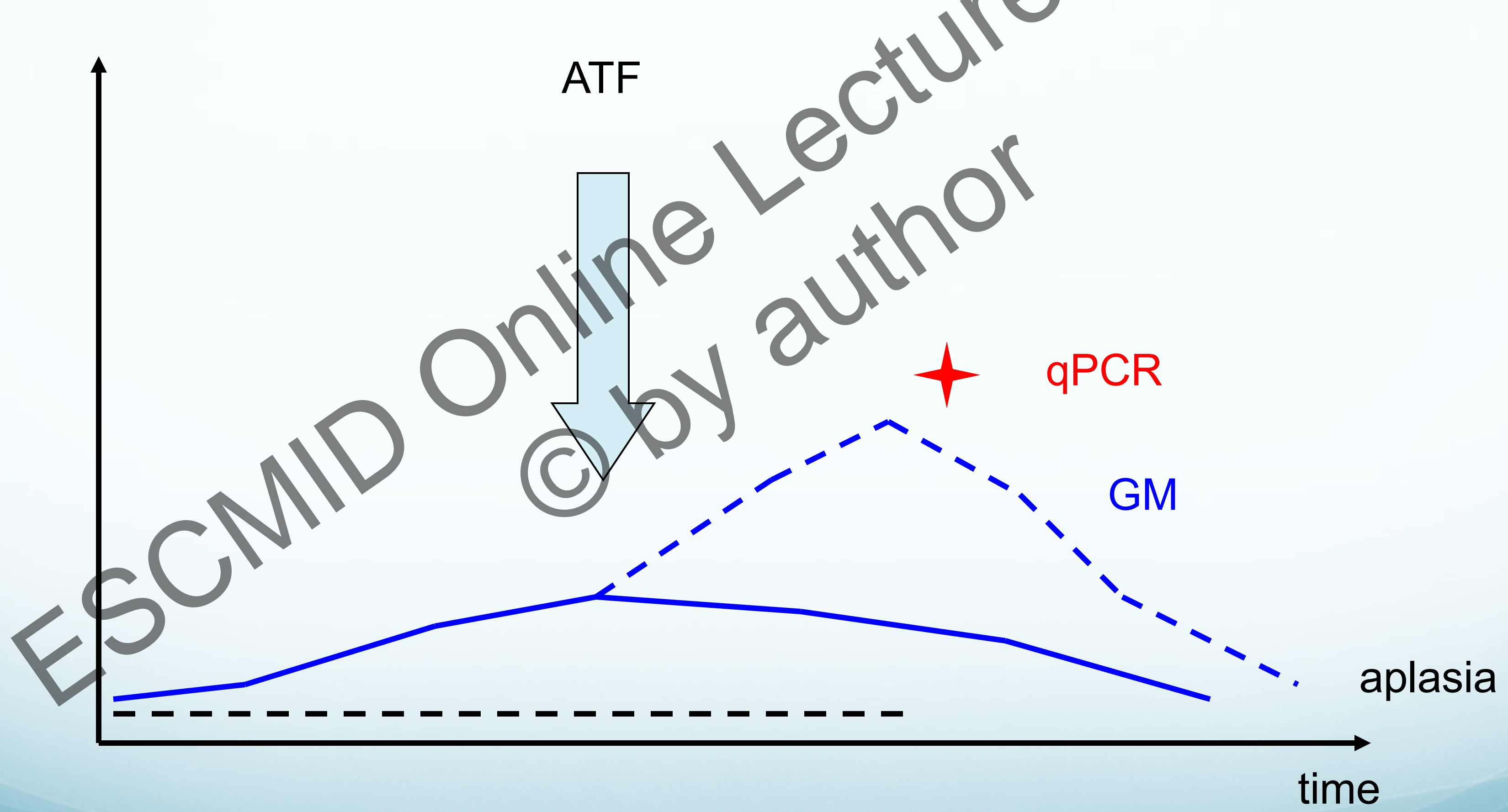
(1) Millon, L. et al. *J Clin Microbiol* **43**, 5097-101 (2005)
(2) Marr KA et al, *Clin Infect Dis* **40**,1762-9 (2006)

Development of an aspergillosis



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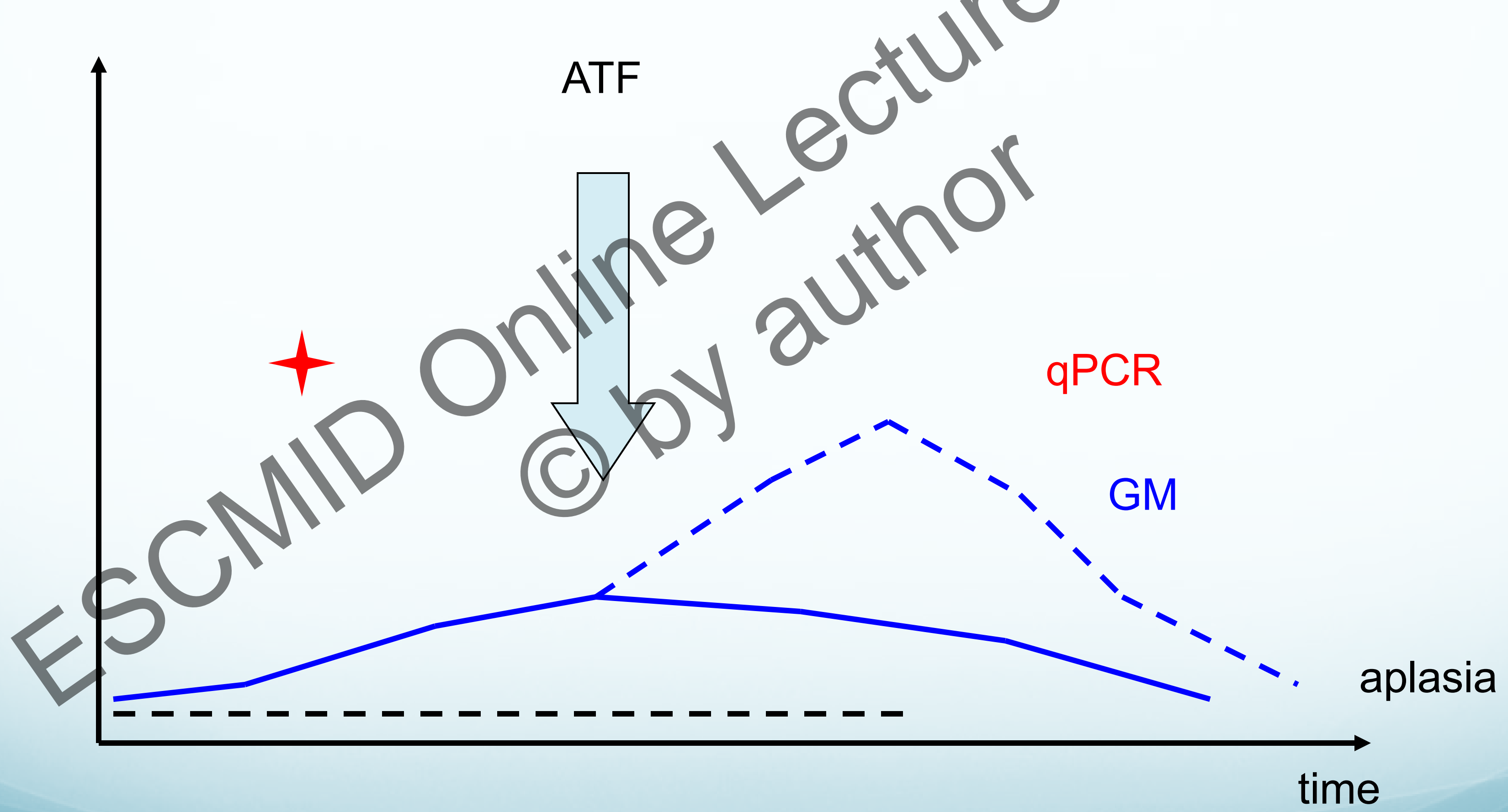
Development of an aspergillosis



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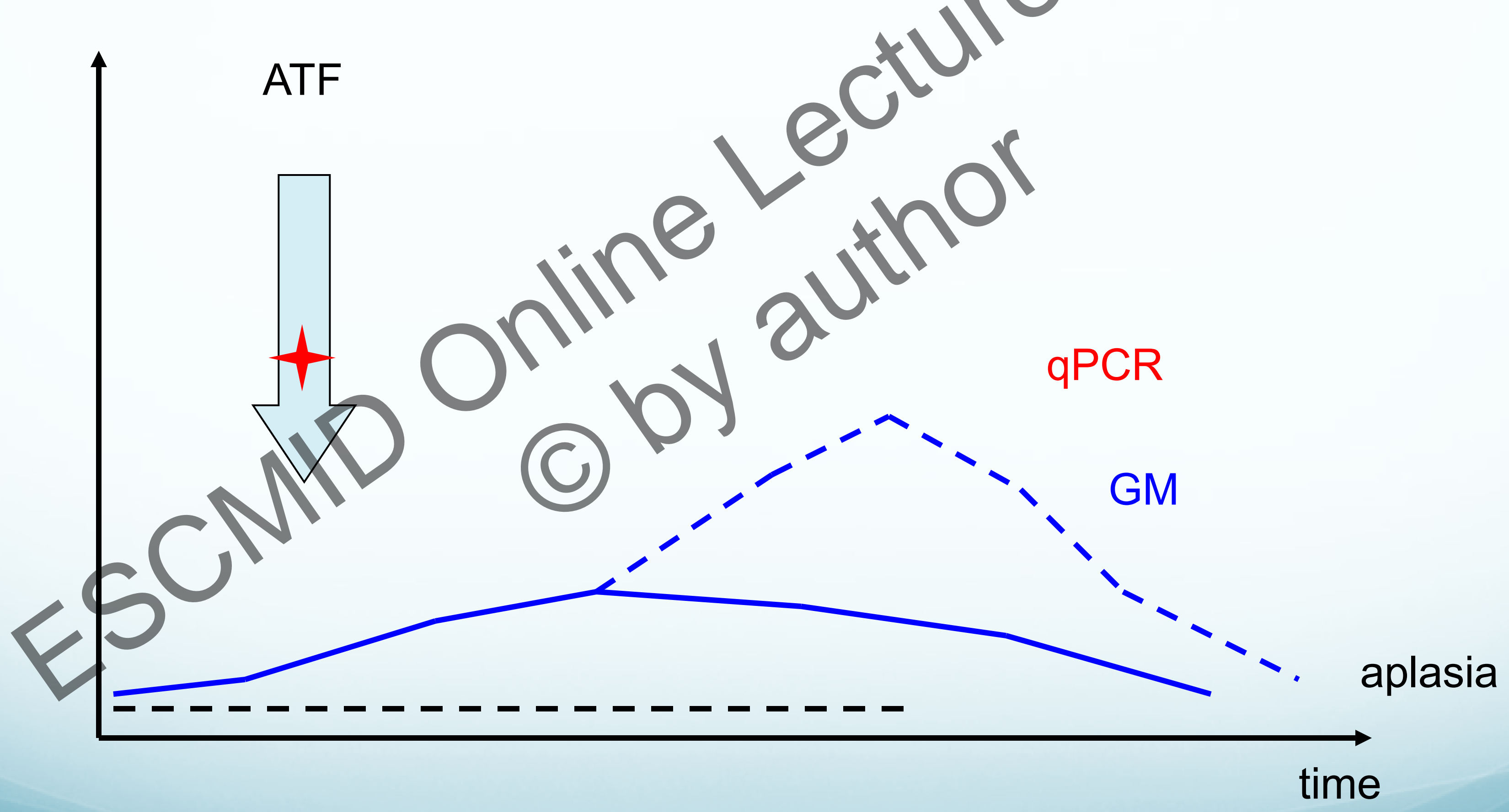
Development of an aspergillosis



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Performance of qPCR overall and according to sample selection

Definition of a patient tested positive for IA	No. patients tested positive / No. patients with IA	Sensitivity % (95% CI)	No. patients tested negative / No. patients without IA	Specificity % (95% CI)	Youden index[a]
Overall performance: all serum samples collected during study enrollment (n=1,534)					
≥1 positive test with ß-glucan	9/11	82 (48-98)	79/174	45 (38-53)	0.27
≥1 positive test with qPCR	8/11	73 (39-94)	79/174	45 (38-53)	0.18
≥1 positive test with ß-glucan or qPCR	11/11	100 (72-100)	39/174	22 (16-29)	0.22
Early diagnosis of IA: selection of serum samples before early antifungal therapy (n=747)					
≥1 positive test with ß-glucan	5/11	45 (17-77)	113/172	66 (58-73)	0.11
≥1 positive test with qPCR	4/11	36 (11-69)	113/172	66 (58-73)	0.02
≥1 positive test with ß-glucan or qPCR	7/11	64 (31-89)	76/172	44 (37-52)	0.08

Cordonnier, C. et al. *Clin Infect Dis*; **48:**1042-51 (2009)

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Cordonnier, C. et al. *Clin Infect Dis*; **48:**1042-51 (2009)

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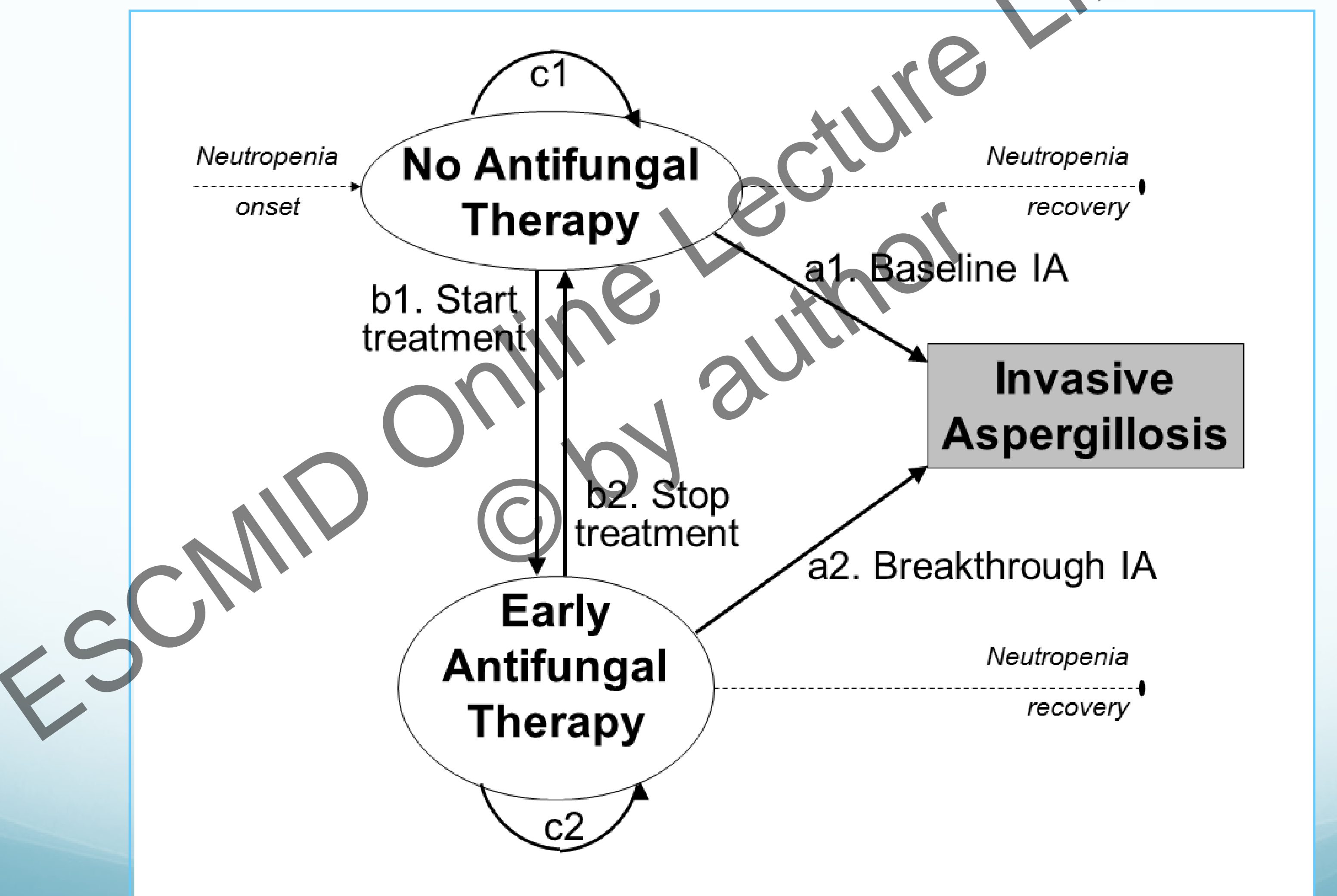
Event History Analysis



Michaël Schwarzinger et al submitted

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Dynamic Performance of Serum Biomarkers for the Early Detection of Invasive Aspergillosis in Febrile, Neutropenic Patients: an Event History Analysis

- The event history analysis confirmed that the risk of IA is a complex time function of neutropenia duration and risk management.
- The quantitative PCR assay accelerated the early detection of IA (*P*=.010), independently of other diagnostic information used to treat, while (1->3)-beta-D-glucan assay did not (*P*=.53)

Michaël Schwarzinger et al submitted

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Clinical validation

- Hebart, H. et al. A prospective randomized controlled trial comparing PCR-based and empirical treatment with liposomal amphotericin B in patients after allo-SCT. *Bone Marrow Transplant* **43**, 553-61 (2009)
  - At day 100, no difference was observed in the incidence of IFI (primary end point) and survival between the two arms
- Barnes, R.A. et al. Clinical impact of enhanced diagnosis of invasive fungal disease in high-risk haematology and stem cell transplant patients. *J Clin Pathol* **62**, 64-9 (2009)
  - No excess morbidity or mortality was seen in patients in whom empiric antifungal treatment was withheld, and there were substantial savings in antifungal drug expenditure
- Blennow, O. et al. Randomized PCR-based therapy and risk factors for invasive fungal infection following reduced-intensity conditioning and hematopoietic SCT. *Bone Marrow Transplant*, 45(12):1710-8 (2010)
  - *Aspergillus* PCR tests performed on clinical suspicion after day 100 were insufficiently sensitive to be diagnostically useful
- Impact of prophylaxis?

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Candida

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0095-1137/11/$12.00 doi:10.1128/JCM.01602-10
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PCR Diagnosis of Invasive Candidiasis: Systematic Review and Meta-Analysis

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

- Metaanalysis:
  - pooled sensitivity = 0.95 (95% CI: 0.88-0.98)
  - pooled specificity = 0.92 (95% CI: 0.88-0.95)
  - " direct PCR offers an attractive method for early diagnosis of specific *Candida* spp. Its effects on clinical outcomes should be investigated »
- Same technical issues as for *Aspergillus*
- Performance: comparison with blood culture

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JOURNAL OF CLINICAL MICROBIOLOGY, Aug. 2009, p. 2405–2410 Vol. 47, No. 8
0095-1137/09/$08.00+0 doi:10.1128/JCM.00491-09
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Utility of a Commercially Available Multiplex Real-Time PCR Assay To Detect Bacterial and Fungal Pathogens in Febrile Neutropenia▽

Marie von Lilienfeld-Toal,[1,2]†* Lutz E. Lehmann,[3]† Ansgar D. Raadts,[3] Corinna Hahn-Ast,[1] Katjana S. Orlopp,[1] Günter Marklein,[4] Ingvill Purr,[4] Gordon Cook,[2] Andreas Hoeft,[3] Axel Glasmacher,[1] and Frank Stüber[5]

JOURNAL OF CLINICAL MICROBIOLOGY, Oct. 2010, p. 3510–3516 Vol. 48, No. 10
0095-1137/10/$12.00 doi:10.1128/JCM.00147-10
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Multiplex Blood PCR in Combination with Blood Cultures for Improvement of Microbiological Documentation of Infection in Febrile Neutropenia▽

F. Lamoth,[1] K. Jaton,[2] G. Prod'hom,[2] L. Senn,[1] J. Bille,[2] T. Calandra,[1] and O. Marchetti[1]*

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Journal of Clinical Microbiology, Aug. 2009, p. 2405–2410 Vol. 47, No. 8
0095-1137/09/$08.00+0 doi:10.1128/JCM.00491-09
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Utility of a Commercially Available Multiplex Real-Time PCR Assay To Detect Bacterial and Fungal Pathogens in Febrile Neutropenia

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Multiplex Blood PCR in Combination with Blood Cultures for Improvement of Microbiological Documentation of Infection in Febrile Neutropenia

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- Study one:
  - Blood culture prior to ATB in febrile neutropenia cannot be replaced by SeptiFast
  - In contrast, the PCR technique results in a positivity rate of 15% during antimicrobial therapy
- Study two:
  - This data set does not allow the estimation of the test's diagnostic performance
  - Discrepancy between BC and SeptiFast is consistent with the identification of the circulation of unviable fungal components

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Comparison of Whole Blood, Serum, and Plasma for Early Detection of Candidemia by Multiplex-Tandem PCR

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- The sensitivity, specificity, positive predictive value, and negative predictive value of the assay with whole blood were 75%, 97%, 95%, and 85%, respectively
- Fungal DNA was not detected by MT-PCR in 6/24 (25%) whole blood samples
- *Candida* DNA was detected more often in serum (71%) and plasma (75%) than in whole blood (54%)

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Use of Quantitative Real-Time PCR To Study the Kinetics of Extracellular DNA Released from *Candida albicans*, with Implications for Diagnosis of Invasive Candidiasis

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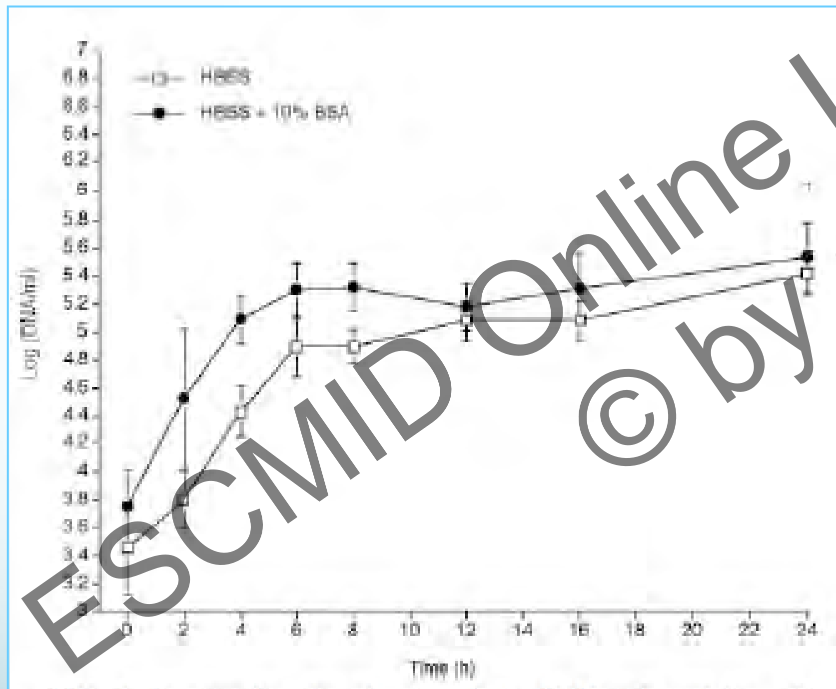

FIG. 3. In vitro kinetics of extracellular DNA released from *C. albicans* cultured in HBSS alone and HBSS plus 10% BSA over a 24-h period. †, $P = 0.001$ in comparison to DNA levels at $T = 0$ h. The figure represents results from nine replicate experiments. The concentration of DNA is expressed in femtograms.

- Rabbits with disseminated candidiasis showed a steady increase of detectable DNA levels in plasma as disease progressed
- Plasma cultures showed minimal growth of *C. albicans*
- Cell-free *C. albicans* DNA is released into the bloodstream of hosts with disseminated candidiasis

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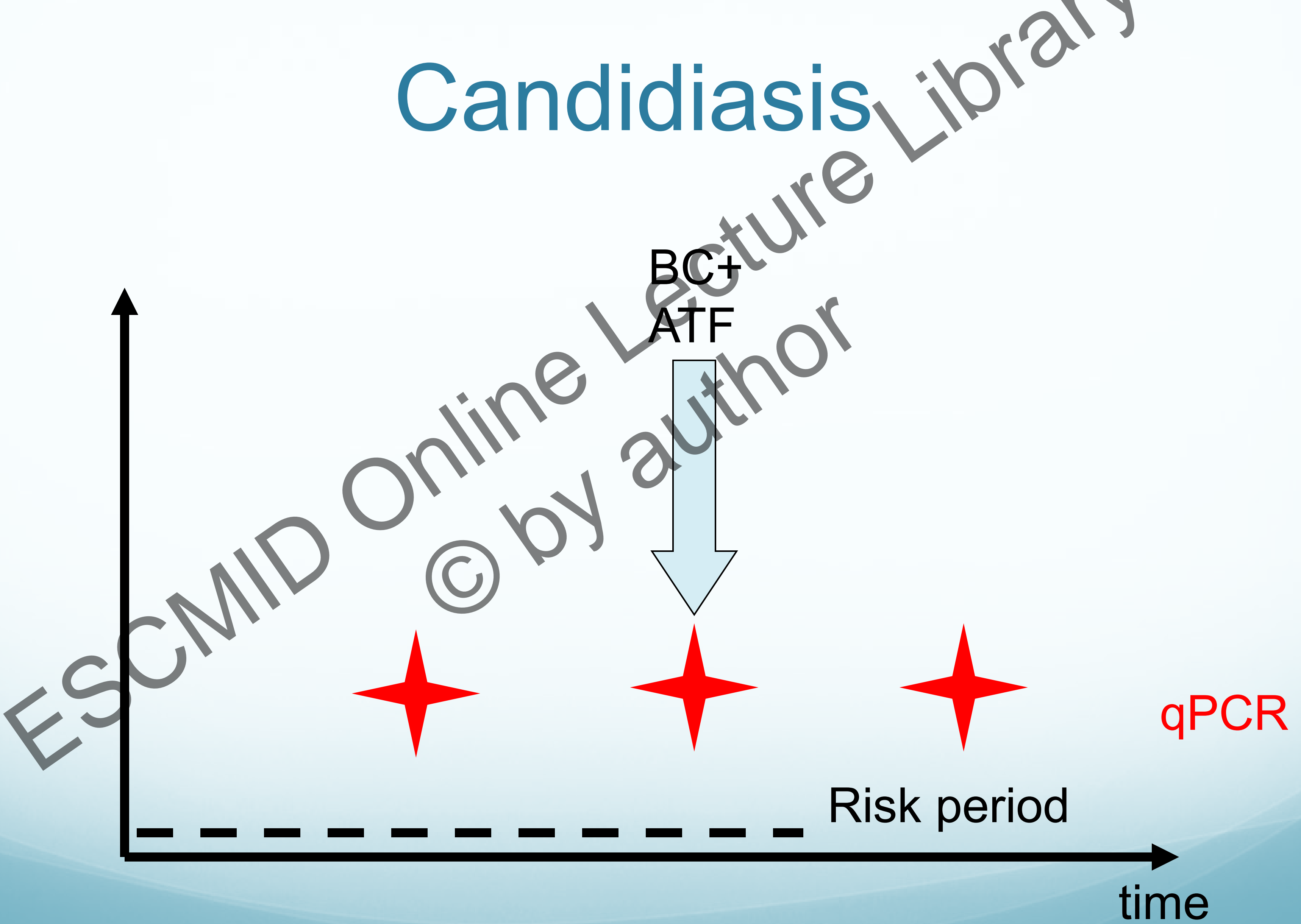
Candidiasis



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

- Testing Blood [1,2]
  - 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
- Testing serum [3]
  - Less standardization

*The European *Aspergillus* PCR Initiative

(1) White, P.L. et al. *J Clin Microbiol* **48**, 1231-40 (2010)
(2) White, P.L. et al. *J Clin Microbiol* **48**, 3753-5 (2010)
(3) White, P.L. et al. *J Clin Microbiol* **49**, 3842-8 (2011)

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Conclusion

- MIQE for experimental validation
  - qPCR format, internal and negative controls
- Primer choice: still controversial
  - Species specific vs. pan-fungal strategy
  - No access to primer sequence in commercial kits
- Clinical specimens: adapt DNA extraction
  - Serum / Plasma (simple, automated methods)
  - Blood (commercial and automated methods)
  - BAL fluid (variability of clinical samples)
- Clinical validation: prospective studies
  - Depends on prevalence of the screened disease
  - Fungal diseases are a complex time function of neutropenia duration and risk management

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