

PCR – PLATFORMS, STRENGTHS AND WEAKNESSES

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Abstract

As highlighted in the 1st Advances Against Aspergillosis Conference the development and use of molecular methods to aid in diagnosis of invasive aspergillosis is extensive, but consensus over an optimal method has not been achieved (1). With high sensitivities and specificities the benefits of *Aspergillus* PCR assays are clear and the use of real-time PCR platforms for quantifying fungal burden, reducing the sample turn around time and minimising the opportunity for contamination have further enhanced their clinical relevance. Apart from contamination (2) the other weaknesses of *Aspergillus* PCR methods are often less evident, albeit possibly widespread:

1) Choice of specimen (BAL, CSF, Serum or Whole Blood).

The wrong choice may limit target availability and/or introduce false positive and false negative results due to colonisation or contamination and PCR inhibition, respectively.

2) Extraction Procedure (Manual or Automated).

The performance of any PCR assay is dependent on the quality of the DNA extracted and the use of a robust and reproducible extraction procedure is paramount as the variation in the quality and quantity of DNA released by different methods can be great.

3) PCR assay design.

In designing a PCR assay time must be taken to minimise oligonucleotide cross hybridisation. Non-specific oligonucleotides may generate false positive results. However, the use of non-specific primers in a PCR assay utilising a specific probe may lead to the generation of false negative results.

4) PCR Platform

The choice of PCR platform is critical. The use of real-time platforms over block-based thermocyclers is widely preferred, although the choice of real-time platform can also be significant (3).

5) PCR results

Result interpretation in a clinical context is also important. It is generally accepted that two consecutive, reproducible positive PCR results are required in determining a true positive result. In a clinical setting this may be difficult with patients receiving antifungal therapy possibly reducing, already low, fungal burden below reproducible PCR thresholds.

In conclusion PCR is a useful tool to aid in the diagnosis of invasive aspergillosis, although it is essential that an optimal method be agreed to allow inclusion in future consensus diagnosis criteria. It should be used in conjunction with other methods (e.g. GM ELISA and HRCT) to enhance the opportunity for detection of this devastating infection.

References

- 1) Buchheidt D and Hummel M. Aspergillus polymerase chain reaction (PCR) diagnosis. *Med Mycol.* 2005; **43**(Suppl 1): 139-145.
- Loeffler J, Hebart H, Bialek R, Hagmeyer L, Schmidt D, Sertey FP, Hartmann M, Eucker J and Einsele H. Contaminations occurring in fungal PCR assays. J Clin Microbiol. 1999; 37(4): 1200-1202.
- 3) White PL, Barton R, Guiver M, Linton CJ, Wilson S, Smith M *et al.* on behalf of the UK Fungal PCR consensus group. A consensus on fungal PCR diagnosis? A UK-

Ireland evaluation of PCR methods for detection of systemic fungal infections. Submitted for Publication 2006.



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PCR – Platforms, Strengths and Weaknesses





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The clinical use of Aspergillus PCR

Widespread

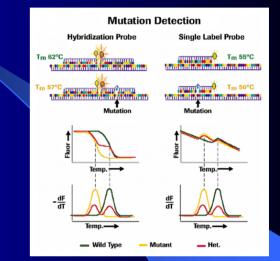
- Up to 2004: 45 papers¹
 - Sensitivities: 40 100%
 - Specificities: 64 100%
- 2004 to date: 19 papers
 - Sensitivities: 12 100%
 - Specificities: 75 100%
- Lack of standardisation
- A consensus PCR method is essential
 - Multi-centre collaboration
 - Evaluate DNA extraction and PCR amplification

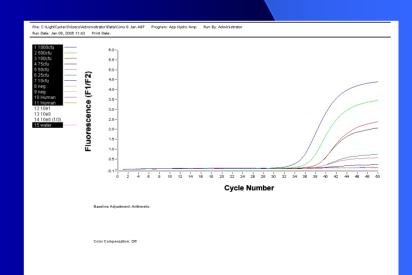
¹Buchheidt and Hummel, 2005 Med Mycol 43 S139-145

Benefits

Benefits of real-time PCR¹:

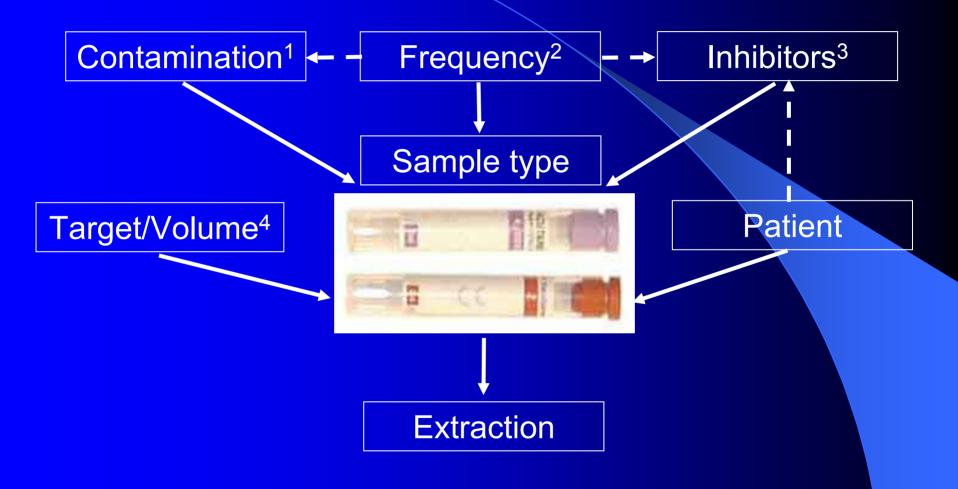
- Minimal contamination risk
- No post amplification handling
 - UDG treatment
- Fast turn-around time
- High Sensitivity and Specificity
 - Using fluorescently labelled probes
- Quantification
 - Monitor fungal burden
 - Therapeutic response





¹Bretagne and Costa, 2006 Clin Chim Acta 363 221-228

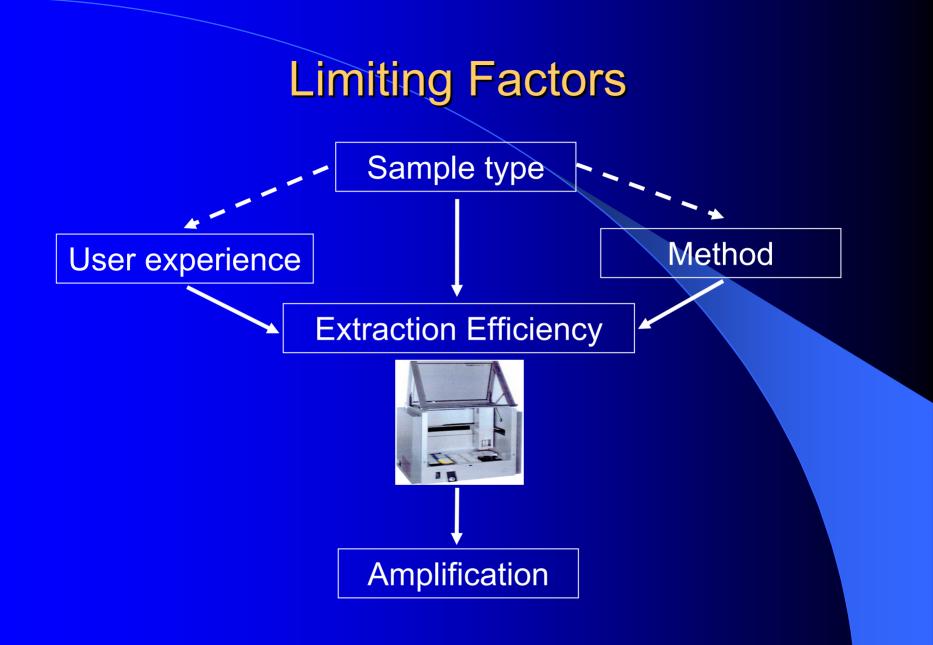
Limiting Factors



¹Williamson, 2001 MD Thesis; ²Verweij, 2005 Med Mycol 43 S121-4; ³Garcia *et al.*, 2002 J Clin Micro 40 1567-1568; ⁴Halliday *et al.* 2005 BJH 132 478-486

Choice of Specimen

- Choice of specimen:
 - BAL
 - Linked PCR positive BAL with IA
 - Inhalation of *Aspergillus* spores
 - Colonisation
 - Invasive
 - CSF
 - Limited studies
 - Invasive
 - Serum/Plasma
 - Extensive successful studies
 - Targets Circulating DNA
 - Whole Blood
 - Extensive successful studies
 - Targets DNA, fungal fragments
 - Extended extraction procedure



Comparison of extraction methods

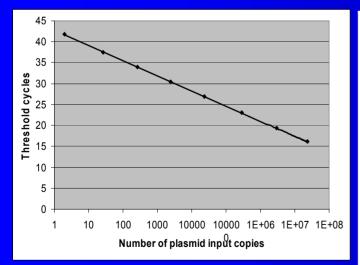
• **1997**¹

- Five commercial extractions kits
- DNA from clinical and EDTA-blood spiked with A. niger
- QIAamp Tissue kits: 1-10 cfu/ml
- 1998²
 - Six 'in-house' procedures
 - DNA extracted from A. fumigatus mycelial mat
 - Bead-beating
- 2002³
 - MagNA Pure Method and the QIAmp Tissue kit
 - DNA from culture, spiked and clinical bloods
 - MagNA Pure: Quick, sensitive with low levels of contamination

¹Loeffler *et al.* 1997 J Clin Micro 35 3311-12; Van Burik *et al.* 1998 36 299-303; ³Loeffler *et al.*, 2002 J Clin Micro 40 2240-43

The Importance of efficient extraction

- In a clinical scenario IA = <1cfu/ml</p>
 - Typical sample 2ml = <2cfu</p>
- Targeting a single copy gene = 2 copies in 2ml
- rRNA genes = 10² copies/organism ≥ 2x10² copies in 2ml



where Y = -1.5705ln(X) + 42.71¹

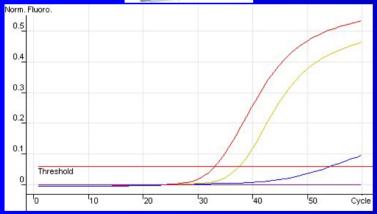
Sample	Est. copies	Result	Ср	copies
1000cfu	10 ⁵	Pos	34.9	. 144
500cfu	5x10⁴	Pos	36.4	56
100cfu	1x10 ⁴	Pos	37.6	25
75cfu	7.5x10 ³	Pos	37.8	23
50cfu	5x10 ³	Pos	38.1	19
10cfu	1x10 ³	Pos	38.0	20
0cfu	0	Neg	-	-

¹White *et al.* 2006, CID 42 479-86

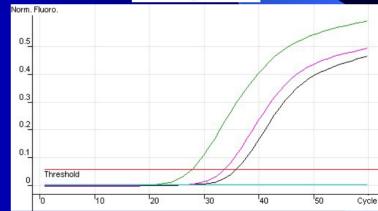
Comparison of automated methods

- A. fumigatus (1000, 100 and 20conidia)
 - Bead-beaten
 - Extracted using the MagNA Pure (Total NA) or Easy Mag



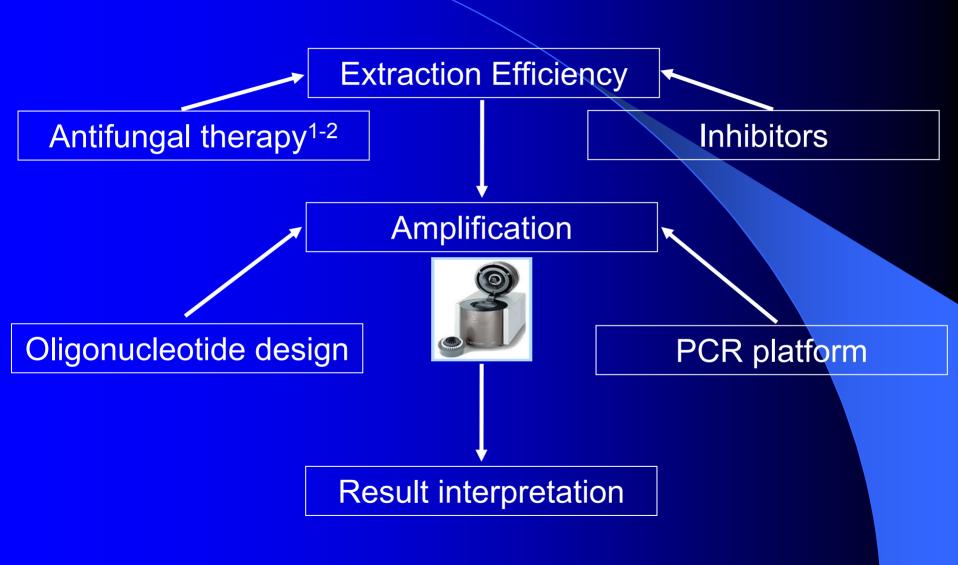






Sample	Result	Ср	Copies	Sample	Result	Ср	Copies
1000cfu	Pos	33.3	226	1000cfu	Pos	28.1	6 <mark>512</mark>
100cfu	Pos	37.8		100cfu	Pos	34.0	6
20cfu	Pos	54.5		20cfu	Pos	36.2	
0cfu	Neg	-		0cfu	Neg	-	

Limiting Factors



1Halliday et al. 2005 BJH 132 478-486; ²Buchheidt et al. 2004 BJH 196-202

Oligonucleotide design

HUMAN185.SEC 18ALB4.SEC FUM18.SEC

HUMAN18S.SEC 18ALB4.SEC

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HUMAN185.SEQ FUM18.SEQ HUMAN185.SEQ 18ALB4.SEQ FUM18.SEQ

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rRNA operon

- 18S rRNA gene
- Panfungal primers
- Genus sp. probe

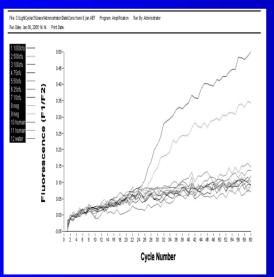
Block-based/Sybr Green

- False positives
- Probe based assay
 - False negatives

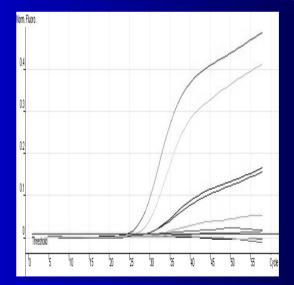
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PCR platforms

- UK Fungal PCR Consensus Group
 - Evaluated Aspergillus PCR methods¹
 - TaqMan Assay²
 - Most centres used Light Cyclers
 - Evaluate the method on other platforms
 - DNA extracted from spiked whole blood



Roche LightCycler



Corbett Rotor-Gene

¹White *et al*. In press; ²Kami *et al*. 200, CID 31 1504-12

PCR platforms

UK Fungal PCR Consensus Group

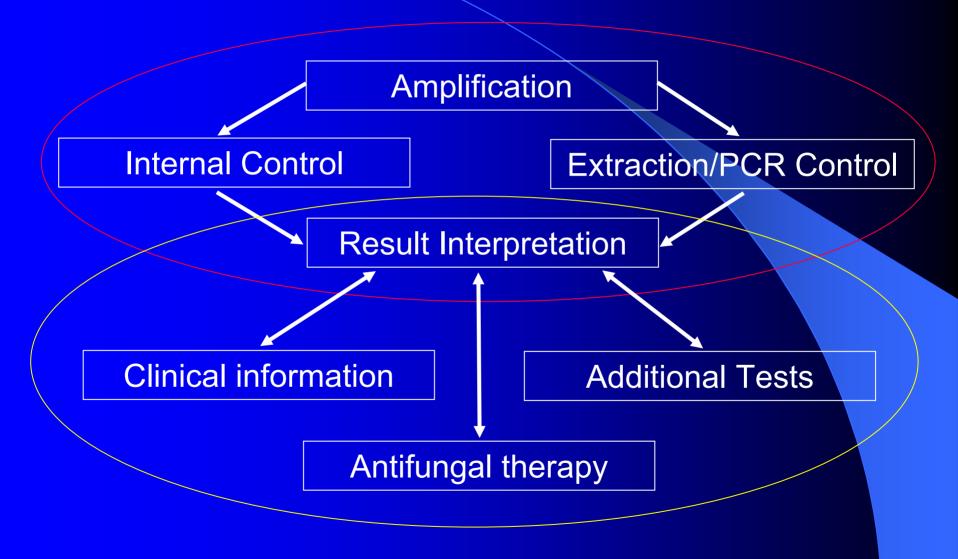
– Light Cycler Assay¹

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1 1000-0 2 300-0 4 75-0 6 25-0 0 Human 1 100-0 1 10

Sensitivity(%)								
Sample	Light Cycler	Rotorgene	Taqman					
100cfu	100	100	100					
75cfu	86	100	100					
50cfu	86	100	100					
25cfu	29	100	1 <mark>00</mark>					
10cfu	57	67	10 <mark>0</mark>					

¹White *et al.* 2006, CID 42 479-86;

Limiting Factors



Result Interpretation

- Differentiation of false positive result from a true positive¹
 - PCR enhanced sensitivity?
 - Early indicator of IFI?
 - Sub-clinical infection or colonisation?
- PCR positive patients²
 - DNA concentrations were very low
 - Below 100% reproducible limits
 - Positive sample: 1/2 replicates Cp <43</p>
 - >DNA concentrations = more probable diagnosis of IA
 - GM ELISA + PCR positive = poor prognosis

• Result interpretation guidelines³

¹Jordanides *et al.* 2005 BMT 35 389-95; ²Millon *et al.* 2005 J Clin Micro 43 5097-101; ³Halliday *et al.* 2005 BJH 132 478-86

Conclusions

- PCR has shown its usefulness in a number of studies
- It is an adjunctive test
 - ELISA and CT
 - Neutropenic Care Pathway
- Lack of a consensus method
 - Sample type
 - Extraction
 - Amplification
 - Interpretation
- UK Fungal PCR Consensus Group
 - Expand?
- EORTC accepted method