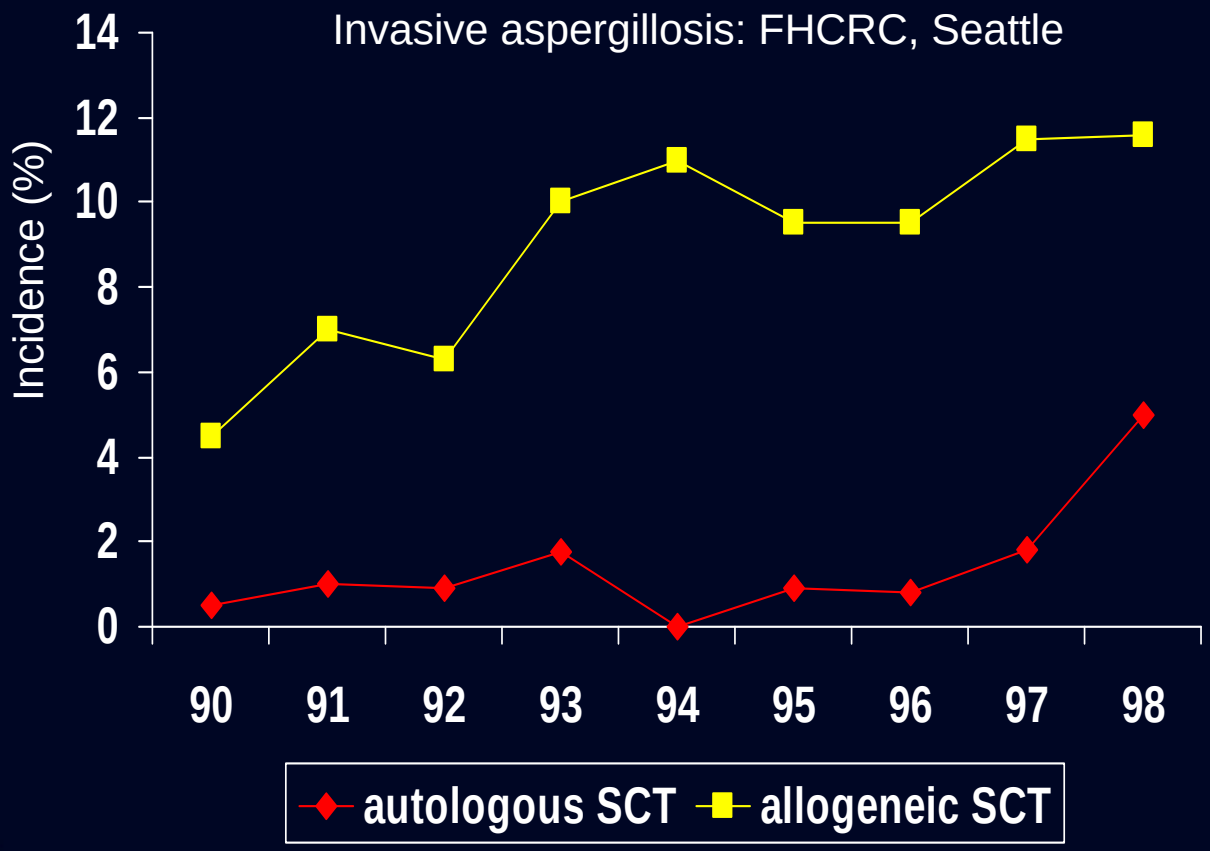
A Consensus Method for Molecular Diagnostic testing

Dr P Lewis White
NPHS Microbiology Cardiff

Background

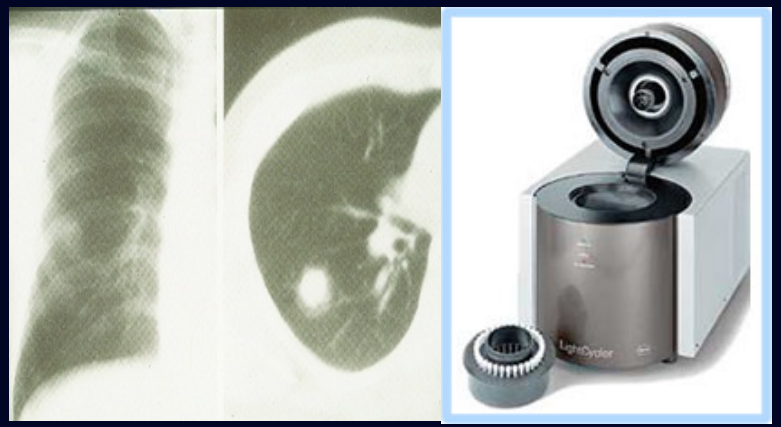
- Invasive fungal infections (IFI) are increasing:



Marr et al Clin Infect Dis 2002:34;909-17

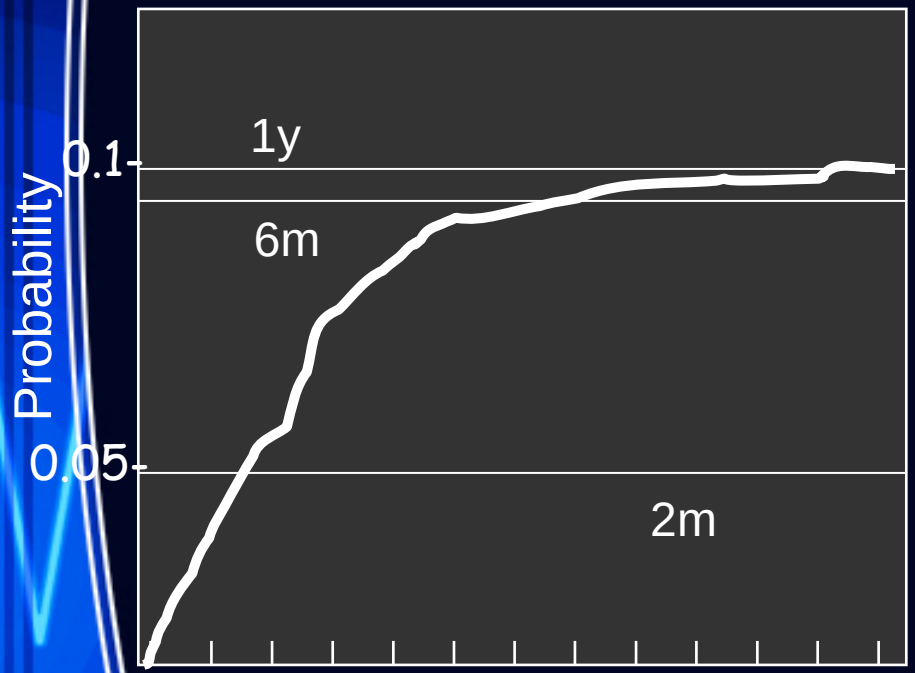
Background

- Invasive fungal infections (IFI) are increasing:
  - Increased “at-risk” population
  - Awareness
  - Detection techniques
    - Radiological
    - Serological
    - Molecular



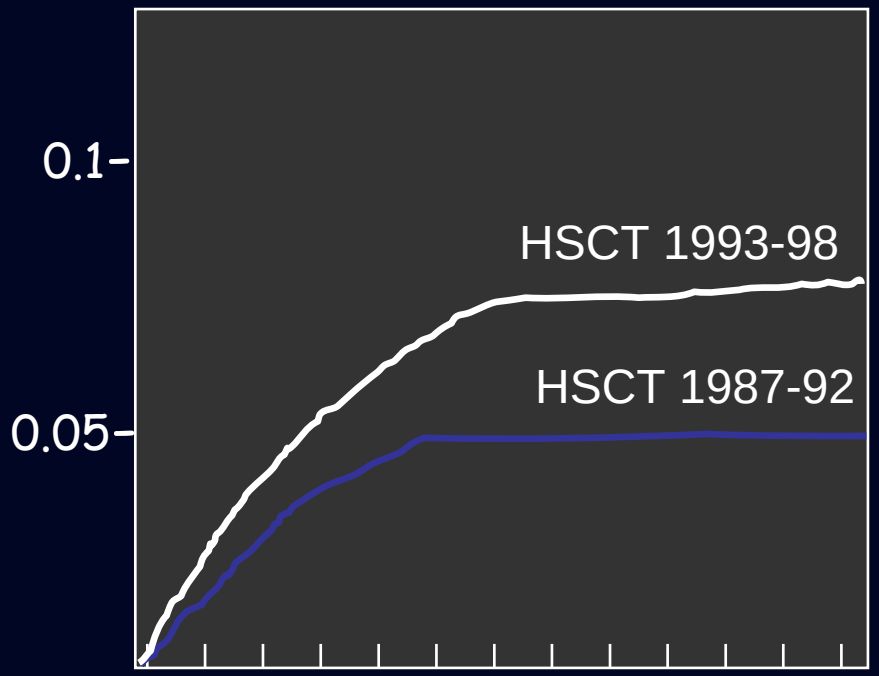
  - Changes in practice

Probability of developing proven/probable IA
187 patients 1993-98



Months 1-12 after transplantation

Probability of developing IA among patients alive at day 40



Days 40-365 after transplantation

Marr *et al* Blood 2002; 100: 4358-66

Changes in SCT practice

- Non-myeloablative transplantation
- Unrelated / mismatched transplantation
- Umbilical cord grafts
- Haplotype mismatched transplantation
- Increased transplant population
  risk of GVHD
  intensive immunosuppression
  risk of CMV
- Delayed haemopoietic recovery
  immune reconstitution

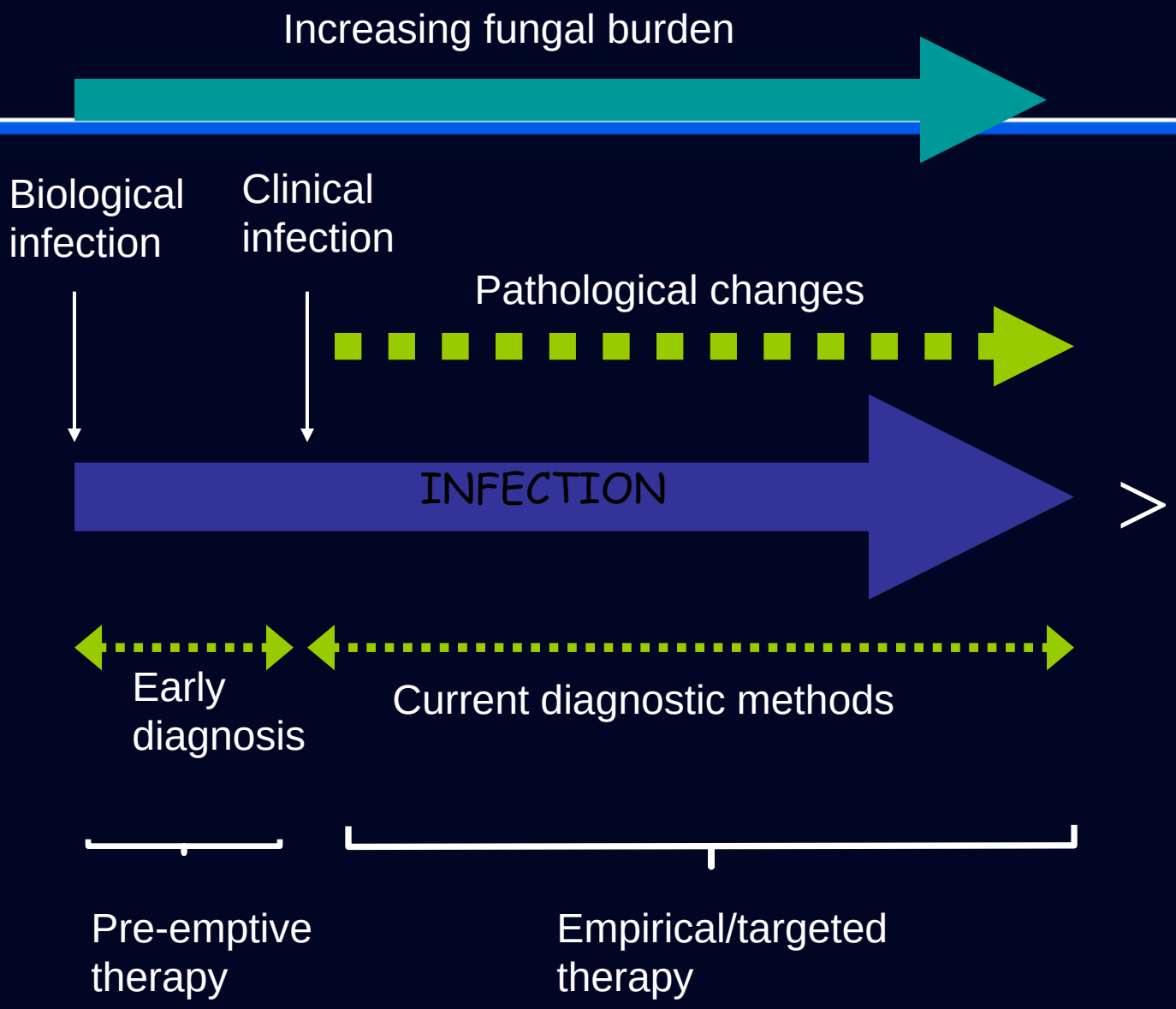
Early initiation of therapy critical

Rx within 96h	- 3 complete resolution
	- 3 partial response
Rx delayed >2w	11/11 died

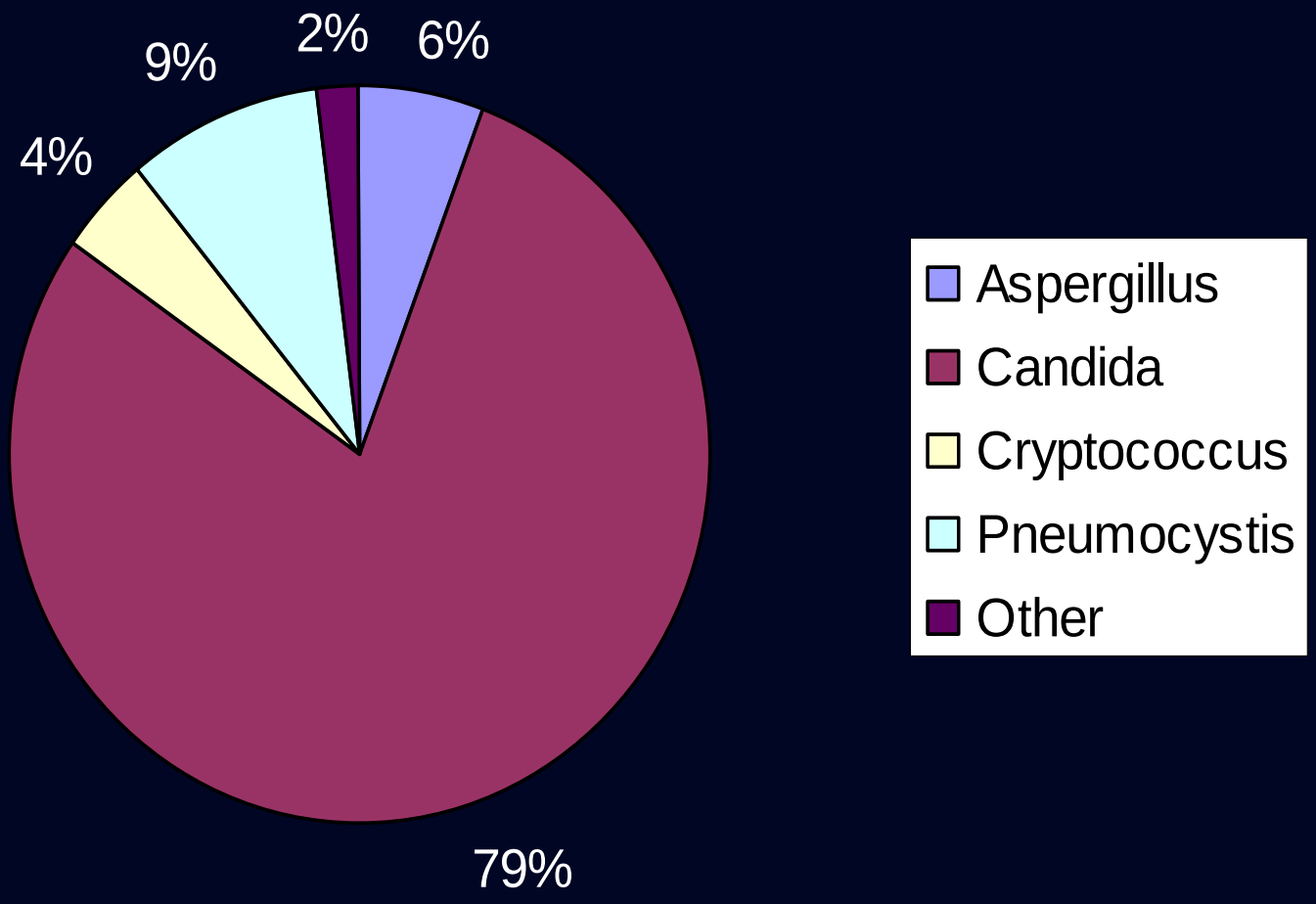
Aisner Ann Intern Med 1977; 86: 539-43

Time from onset of pneumonia to start of Rx	**<10d**	**>10d**
Mortality	**41%**	**90%**

von Eiff Respiration 1995

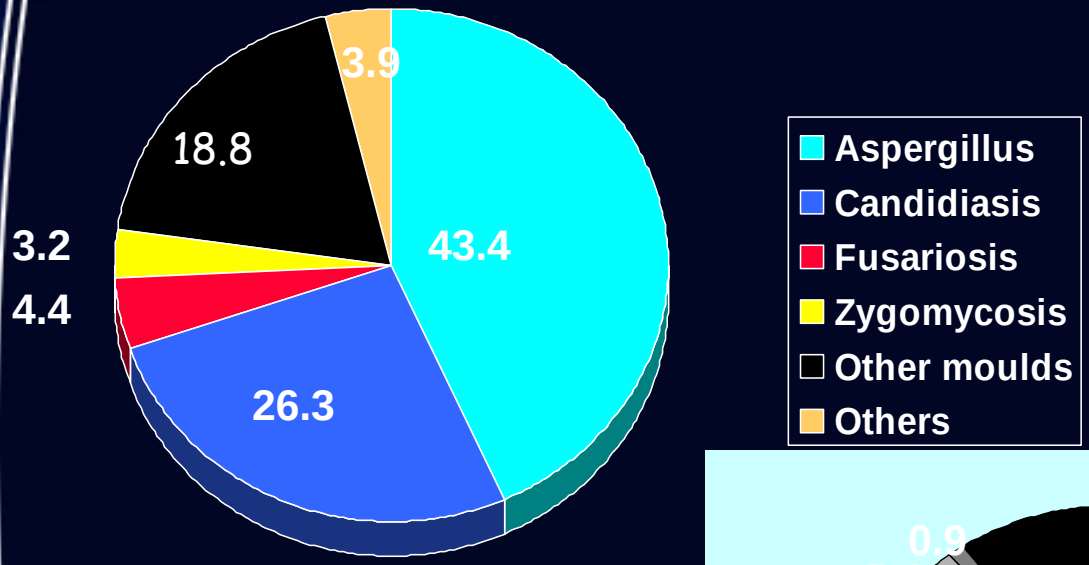

Increasing fungal burden
Biological infection
Clinical infection
Pathological changes
INFECTION
Early diagnosis
Current diagnostic methods
Pre-emptive therapy
Empirical/targeted therapy

Incidence of fungal infection in England and Wales (1990-9)



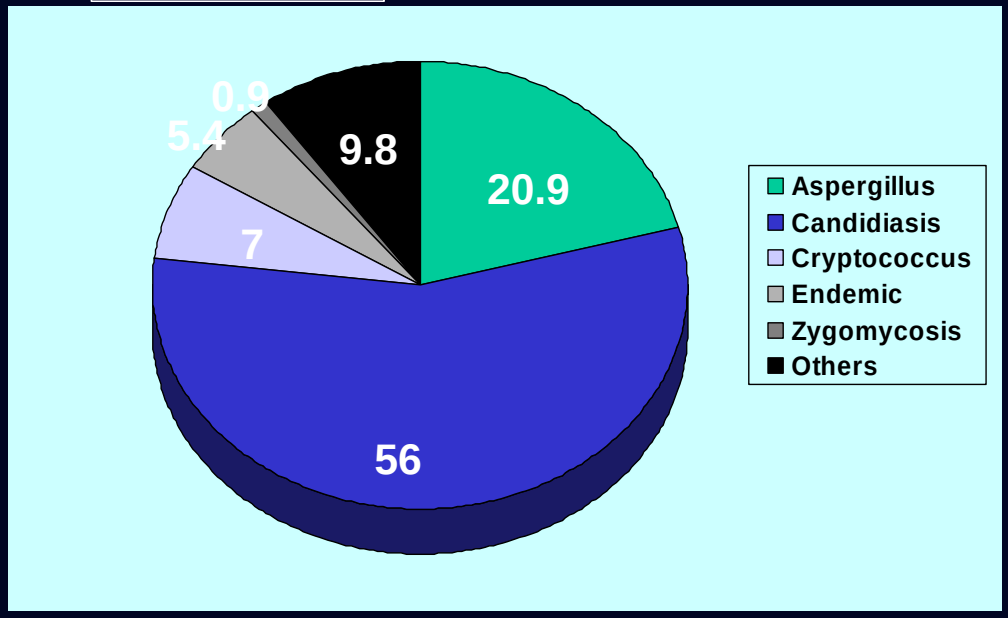
Lamagni *et al.* Epidemiol. Infect. 2001; 126: 397-414

IFD in different hospital settings



251 cases IFI in SCT recipients

316 cases of IFI in SOT recipients



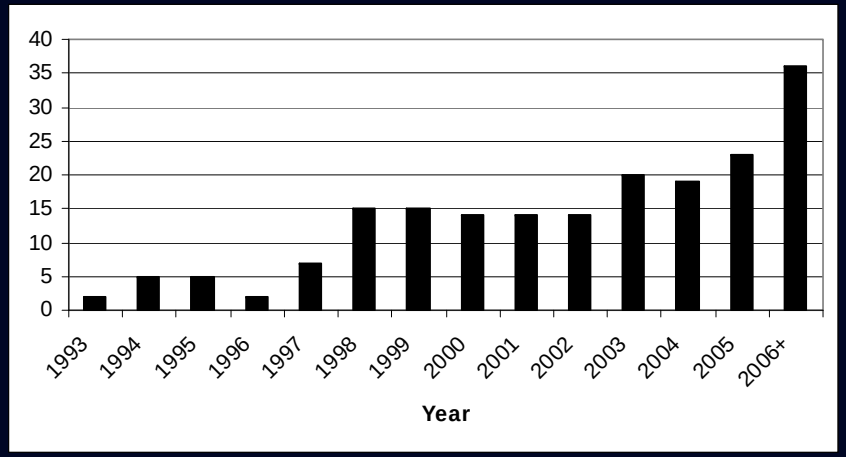
Pappas ICAAC 2003 abst. M-1010

Current Focus of Fungal PCR

- Mainly *Aspergillus* and *Candida*
- Mostly *Aspergillus*
  - Higher mortality rate
  - Greater difficulty in diagnosis
    - 50% invasive candidal infections will be BC positive
- Early diagnosis paramount
- *Aspergillus* PCR

The History of *Aspergillus* PCR

- **1990s**
- **1993 – June 2007 almost 200 published articles**
- **1998 – June 2007 > 20 reviews**
  > One new manuscript per month
- **No large scale evaluation**
- **Very little standardisation**
  - **Specimen (type and volume)**
  - **DNA Extraction**
  - **PCR amplification**
  - **Result Interpretation**



The influence of the Specimen

Contamination[1]

Frequency[2]

Inhibitors[3]

Sample type

Target/Volume[4]

Patient

White AAA 2006

Extraction

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

Choice of Specimen

BAL

- Linked PCR positive BAL with IA
- Inhalation of *Aspergillus* spores
- Colonisation
- Invasive

CSF

- Limited studies
- Invasive

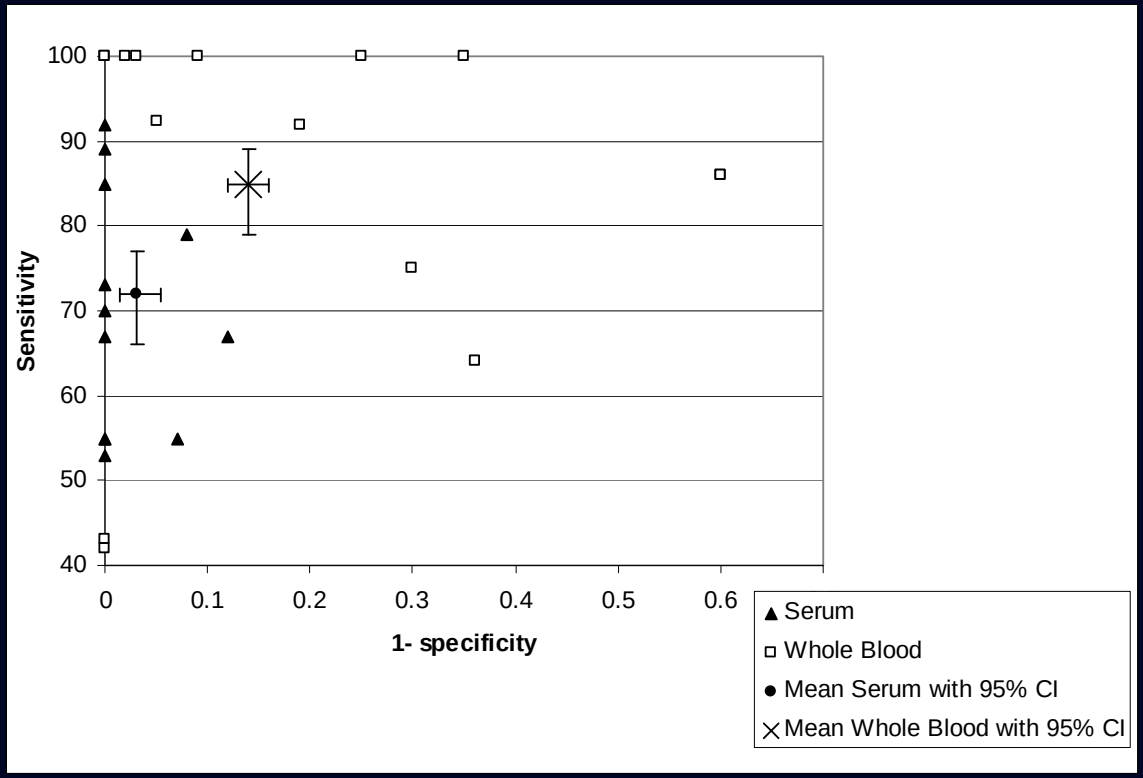
Serum/Plasma

- Extensive successful studies
- Targets Circulating DNA

Whole Blood

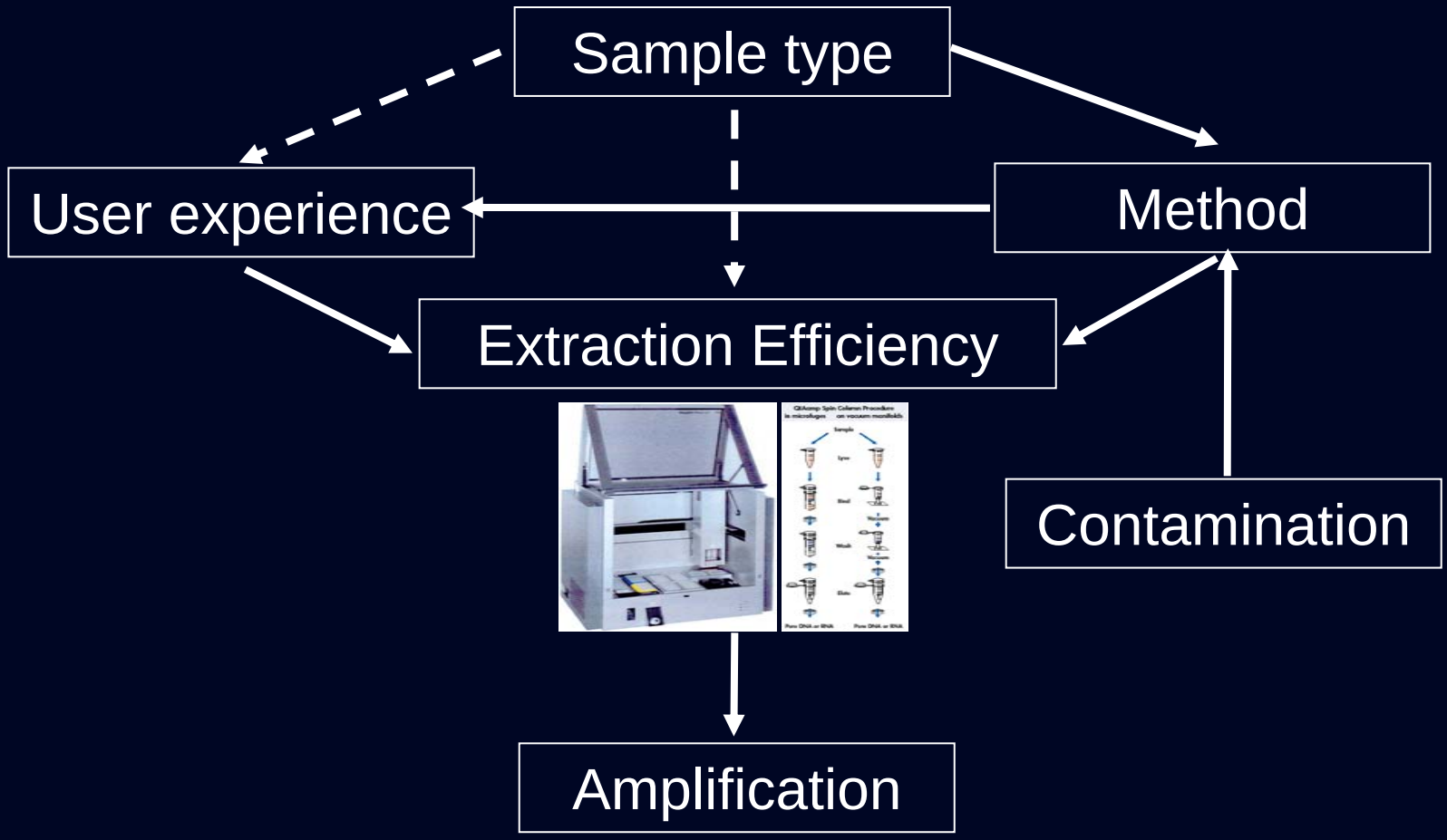
- Extensive successful studies
- Targets DNA, fungal fragments
- Extended extraction procedure

PCR using serum versus whole blood



White and Barnes Chapter 29 in Aspergillus fumigatus and Aspergillosis

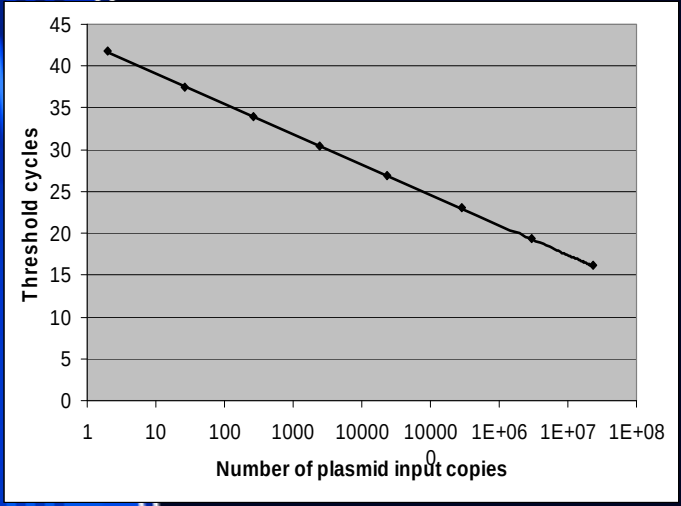
The Extraction Protocol



White AAA 2006

The Importance of efficient extraction

- In a clinical scenario IA = <1conidia/ml (equivalents)
  - Typical sample 2ml = <2conidia
- Targeting a single copy gene = 2 copies in 2ml
- rRNA genes = 10^2 copies/organism $\geq 2x10^2$ copies in 2ml



where Y = -1.5705ln(X) + 42.71[1]

Sample	Est. copies	Result	Cp	Calc. copies
1000cfu	10^5	Pos	34.9	144
500cfu	$5x10^4$	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	-	-

[1]White *et al.* 2006, CID 42 479-86

PCR Amplification

Extraction Efficiency

Antifungal therapy[1-2]

Inhibitors

Amplification

Oligonucleotide design

PCR platform

White AAA 2006

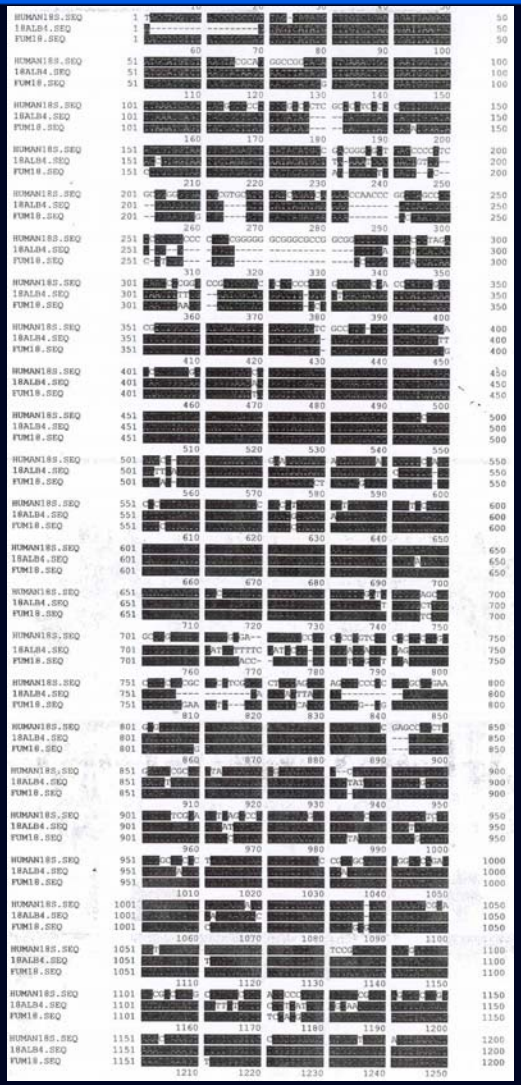
Result interpretation

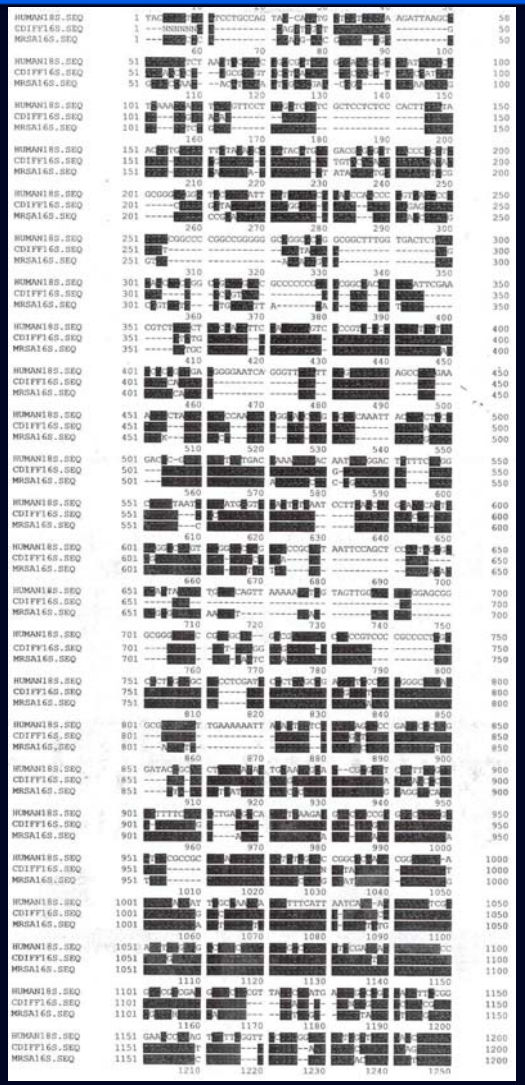
1Halliday *et al*. 2005 BJH 132 478-486; [2]Buchheidt *et al.* 2004 BJH 196-202

Oligonucleotide design

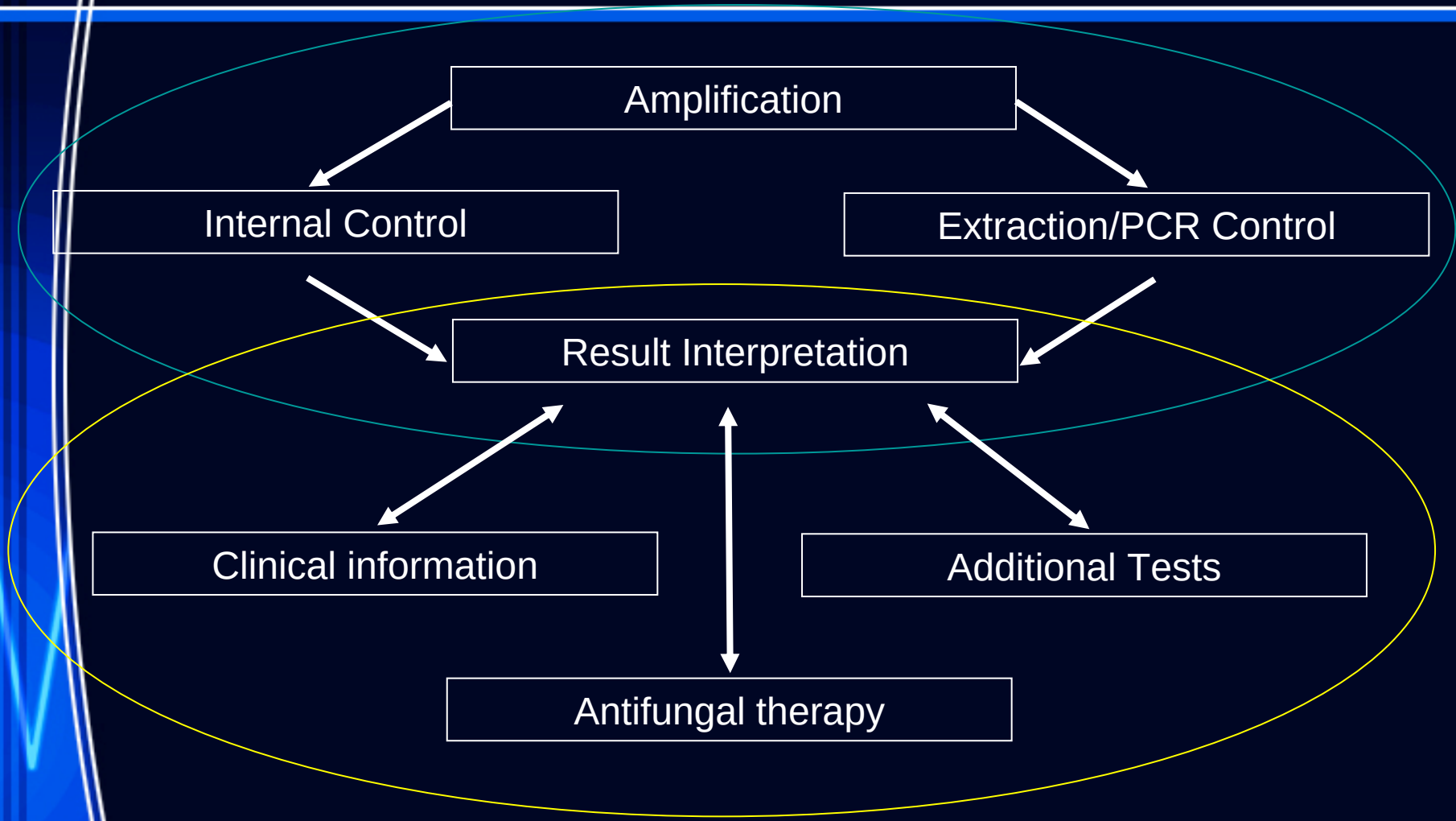
- rRNA operon
  - 18S rRNA gene
  - Panfungal primers
  - Genus sp. probe

- Block-based/Sybr Green
  - False positives
- Probe based assay
  - False negatives





Result Interpretation



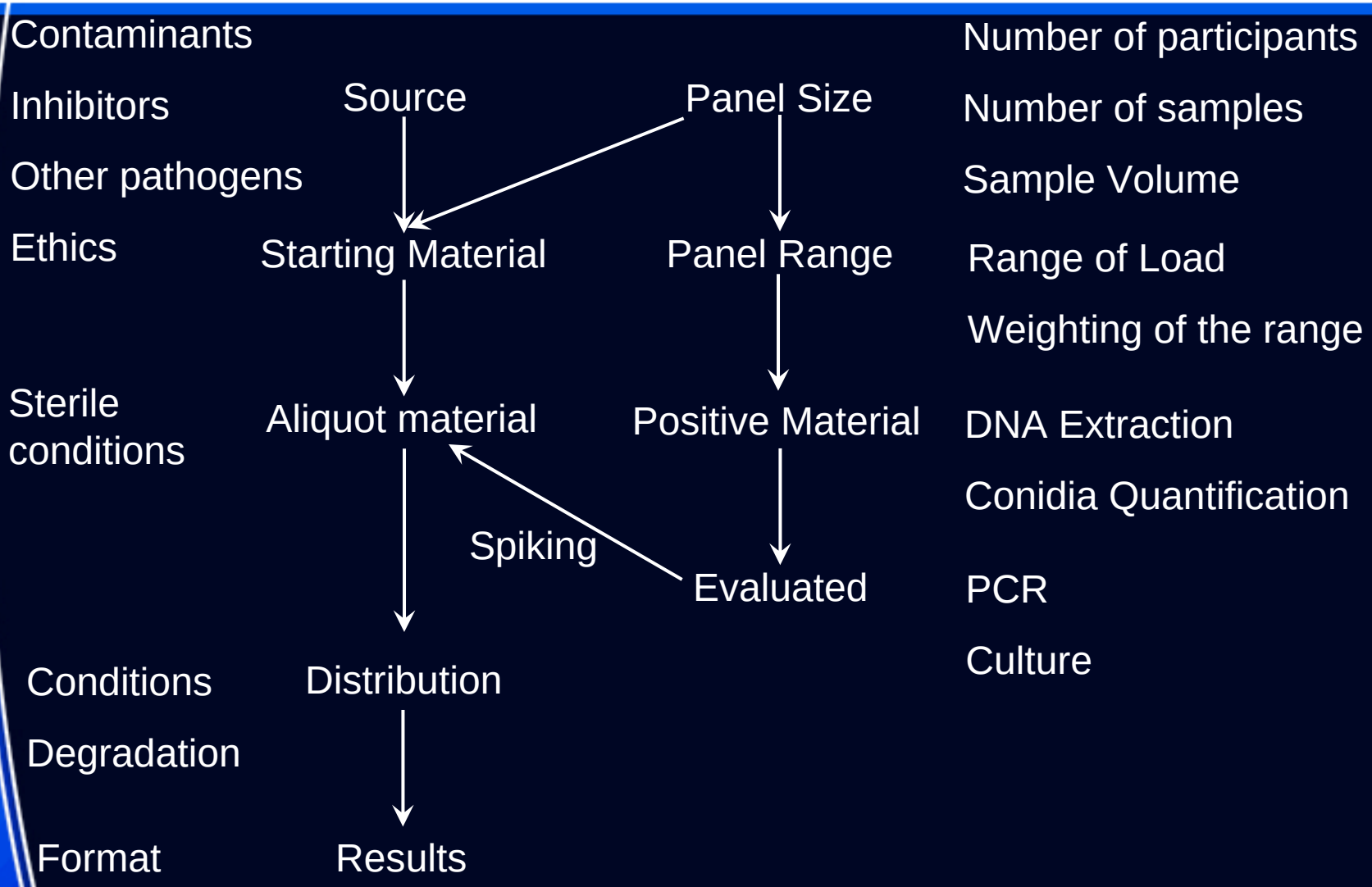
White AAA 2006

Standardisation

- Between 1993 - 2005 over 150 published articles
- 2006 – First with extensive comparison of methods[a]
  - UK-Ireland based.
  - Limited numbers.
- Bead-beating in combination with Automated extraction
-
- Two optimal PCR methods
  - One for TaqMan
  - One for Light Cycler
- Lead to the formation of the European *Aspergillus* PCR Initiative

[a]White *et al.* J Mol Diag 2006

Developing a QC panel



The UK Scenario – The First Panel

- **In 2002:**
  - **blood spiked with *Candida* and *Aspergillus***
  - **Evaluating extraction and amplification methods**

  - **Variation in Quality of results**
    - **Different extraction procedures**
    - **Different amplification procedures**
    - **Genus dependent**

  - ***Candida* assays**
    - **Less variation**
    - **10^1cfu**
    - **No false positive results**

  - ***Aspergillus* assays**
    - **Variation in sensitivity (10^5 – 10^1cfu)**
    - **1 nested assay = 10^1cfu**
    - **2 groups reported 1 false positive result**

- **7 groups:**
  - **Birmingham HPA**
  - **Bristol HPA (Mycology Ref. Lab)**
  - **Cardiff NPHS/UWCM**
  - **Dulwich HPA**
  - **Glasgow Royal Infirmary**
  - **Leeds HPA (Regional Mycology Lab)**
  - **Manchester HPA**

[a]White *et al.* J Mol Diag 2006

- **Extraction procedures**
  - **Wide variation in methods**
  - **Laborious**
  - **Variation in quality and quantity of DNA released**
    - **2 methods (1spin column, 1 semi-automated MGP)**
  - **To reduce labour, time and possible contamination**
    - **Semi automated MGP**

- ***Candida* assays**
  - **Consensus ?**

- ***Aspergillus* assays**
  - **Generally less sensitive**
  - **Variation in both sensitivity and specificity**
    - **Function of extraction/amplification methods**

[a]White *et al.* J Mol Diag 2006

The UK Scenario – The Second Panel

- **Concentrate on *Aspergillus* only**
- **Remove the extraction method variable**
  - **Evaluating amplification methods only**
- **In 2003:**
  - ***Aspergillus* DNA serially diluted in water**
  - **5 amplification methods tested**
    - **2 assays tested in duplicate**
- **Results**
  - **Variation in sensitivity and specificity**
  - **2 assays performed optimally**

[a]White *et al.* J Mol Diag 2006

- **Further tests needed:**
  - **2 optimal methods**
    - **Test laboratory reproducibility**
    - **DNA extracted from known *Aspergillus* quantities**
    - **Oligonucleotides to be distributed**
- **Include additional centres (Total = 10)**

The UK Scenario – The Third Panel

- **The DNA Distribution (2004):**
  - **Consisted of 16 samples:**
  - **8 positive**
    - **DNA extracted from known quantities of *Aspergillus fumigatus***
      - **6 extracted in water**
      - **2 extracted in blood**
    - **Range 5000 to 10cfu**
    - **Sample size: 1ml**
  - **8 negative**
    - **Roche molecular grade water dispensed in a clean cabinet**
    - **Cabinet or pipettes never exposed to *Aspergillus* DNA**

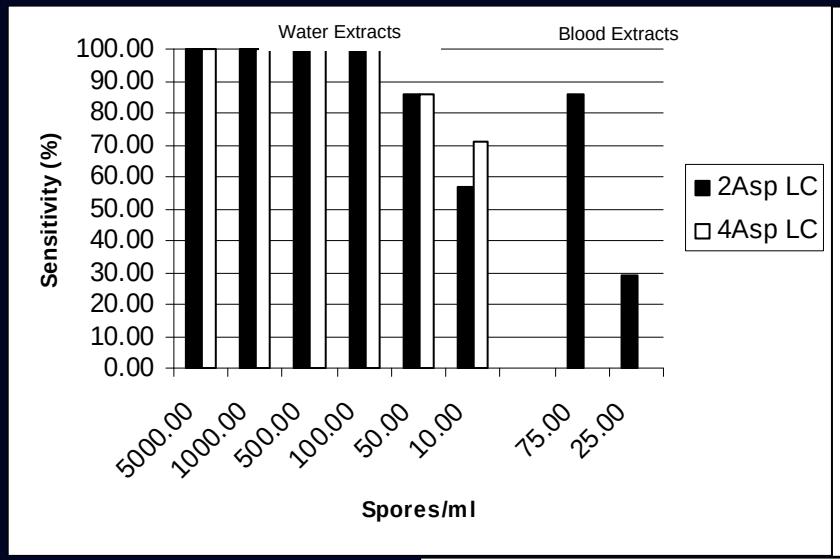
[a]White *et al.* J Mol Diag 2006

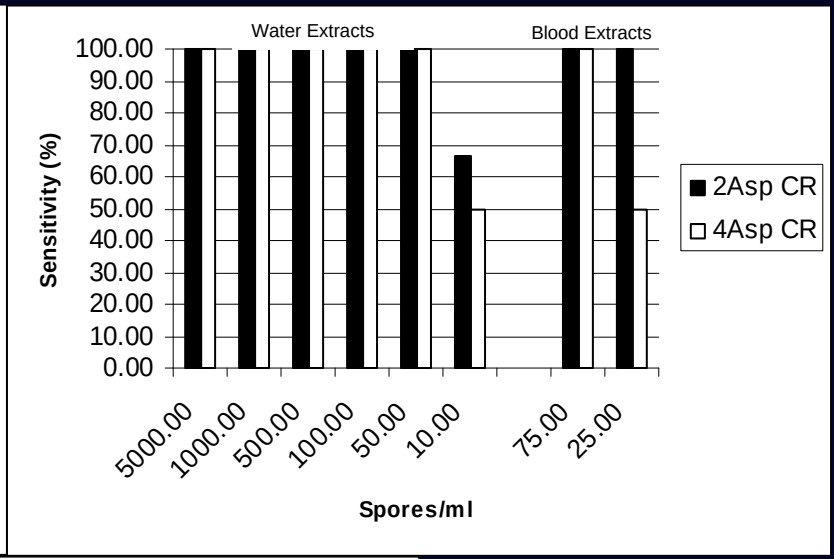
Assay Performance

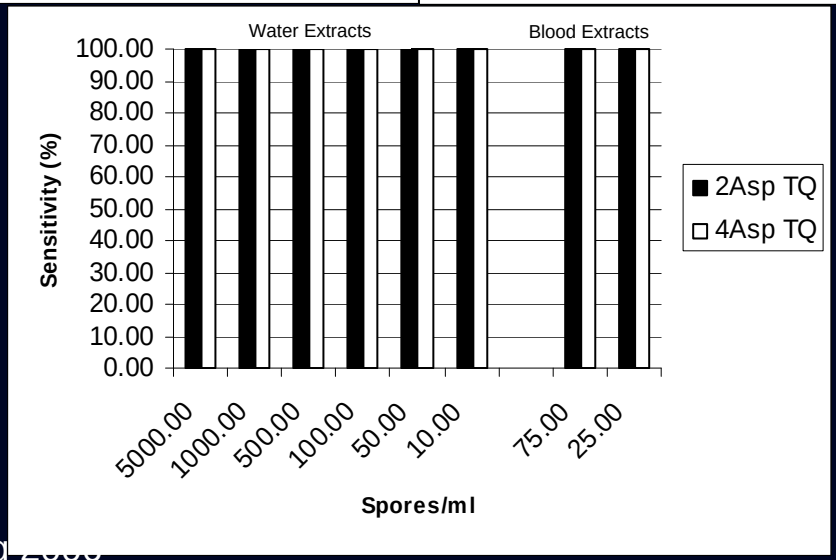
Platform		2Asp (95% CI)	4Asp (95% CI)	Difference (2Asp – 4Asp, 95% CI)
LightCycler	Sensitivity (%)	82.1 (70.1-90.0)	69.6 (56.7-80.1)	12.5 (1.8-23.3)
(n= 7 centres)	Specificity (%)	91.1 (80.7-96.1)	80.4 (68.2-88.7)	10.7 (0.3-21.9)
	PPV (%)	90.2 (79.0-95.7)	78.0 (64.8-87.3)	12.2
	NPV (%)	83.6 (72.4-90.8)	72.6 (60.4-82.1)	11.0
Rotor-Gene	Sensitivity (%)	95.8 (79.8-99.3)	87.5 (64.0-96.5)	8.3 (-10.1-32.1)
(n = 3 centres)	Specificity (%)	100 (86.2-100)	87.5 (64.0-96.5)	12.5 (-4.0-36.0)
	PPV (%)	100 (85.7-100)	87.5 (64.0-96.5)	12.5
	NPV (%)	96.0 (80.5-99.3)	87.5 (64.0-96.5)	8.5
TaqMan	Sensitivity (%)	100 (67.6-100)	100 (80.6-100)	0 (-32.4-19.4)
(n= 2 centres)	Specificity (%)	87.5 (52.9-97.8)	81.3 (57.0-93.4)	6.2 (-30.4-32.6)
	PPV (%)	88.9 (56.5-98.0)	84.2 (62.4-94.5)	4.7
	NPV (%)	100 (64.6-100)	100 (78.5-100)	0

[a]White *et al.* J Mol Diag 2006

Sample type effect



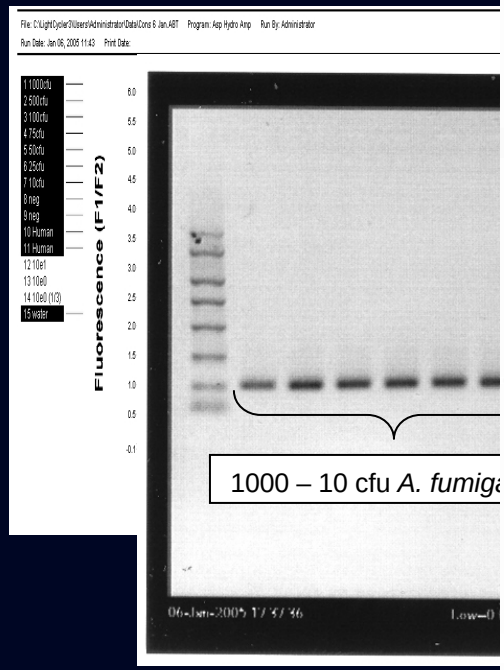


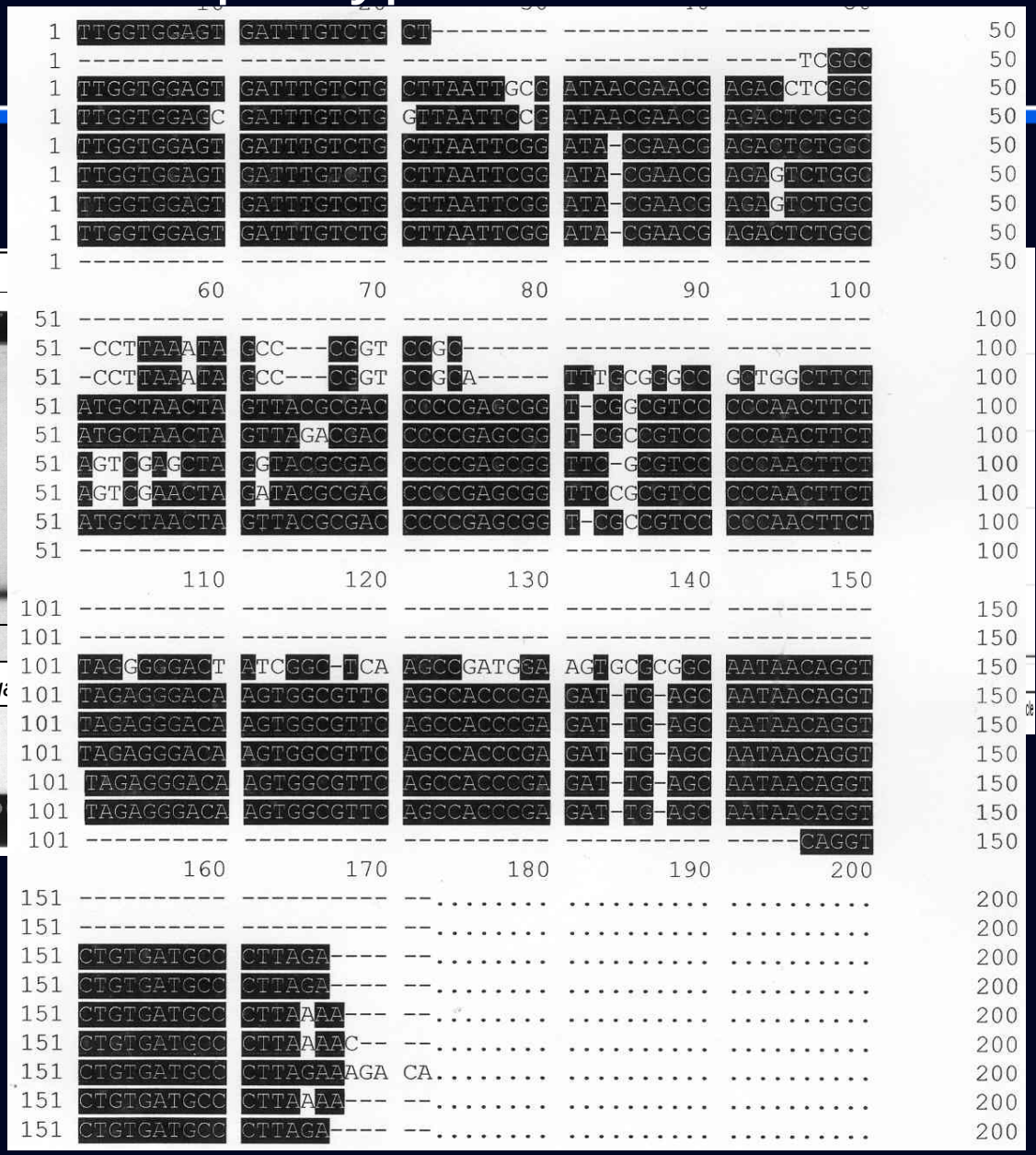


[a]White *et al.* J Mol Diag 2008

Investigating the sample-type effect

2Asp – 10 conidia

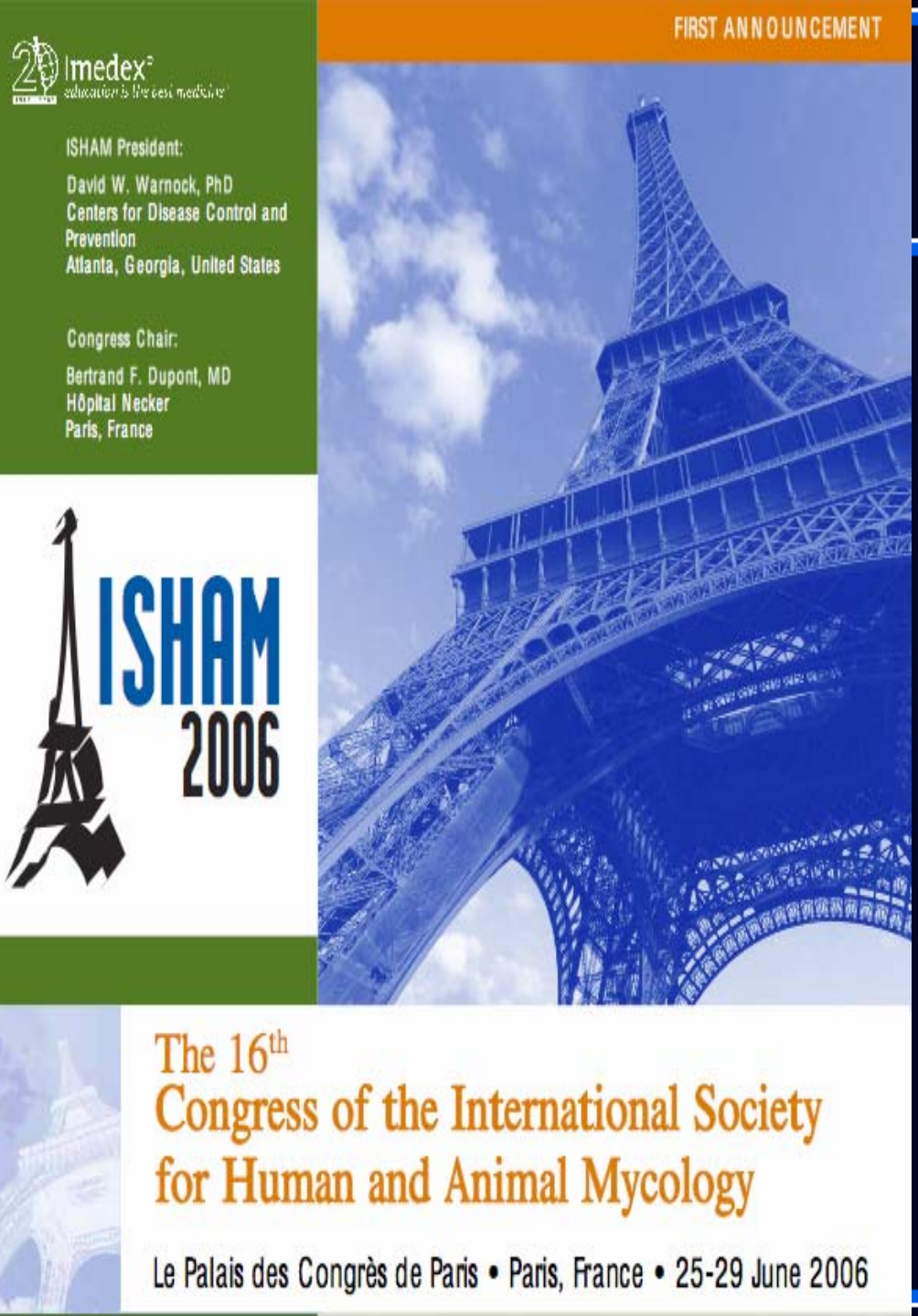




[a]White *et al.* J Mol Diag 2006

Summary of the UK Fungal PCR Work

- Most *Candida* assays are comparable
- Variation in the performance of *Aspergillus* PCR
  - Extraction technique
  - PCR system
- Two preferred assays
  - PCR platform dependent
- Platform performance varies
- Cross reaction with human DNA leading to false negative results



Sunday afternoon 25th June

Contact

p.donnelly@usa.net

The European *Aspergillus* PCR initiative

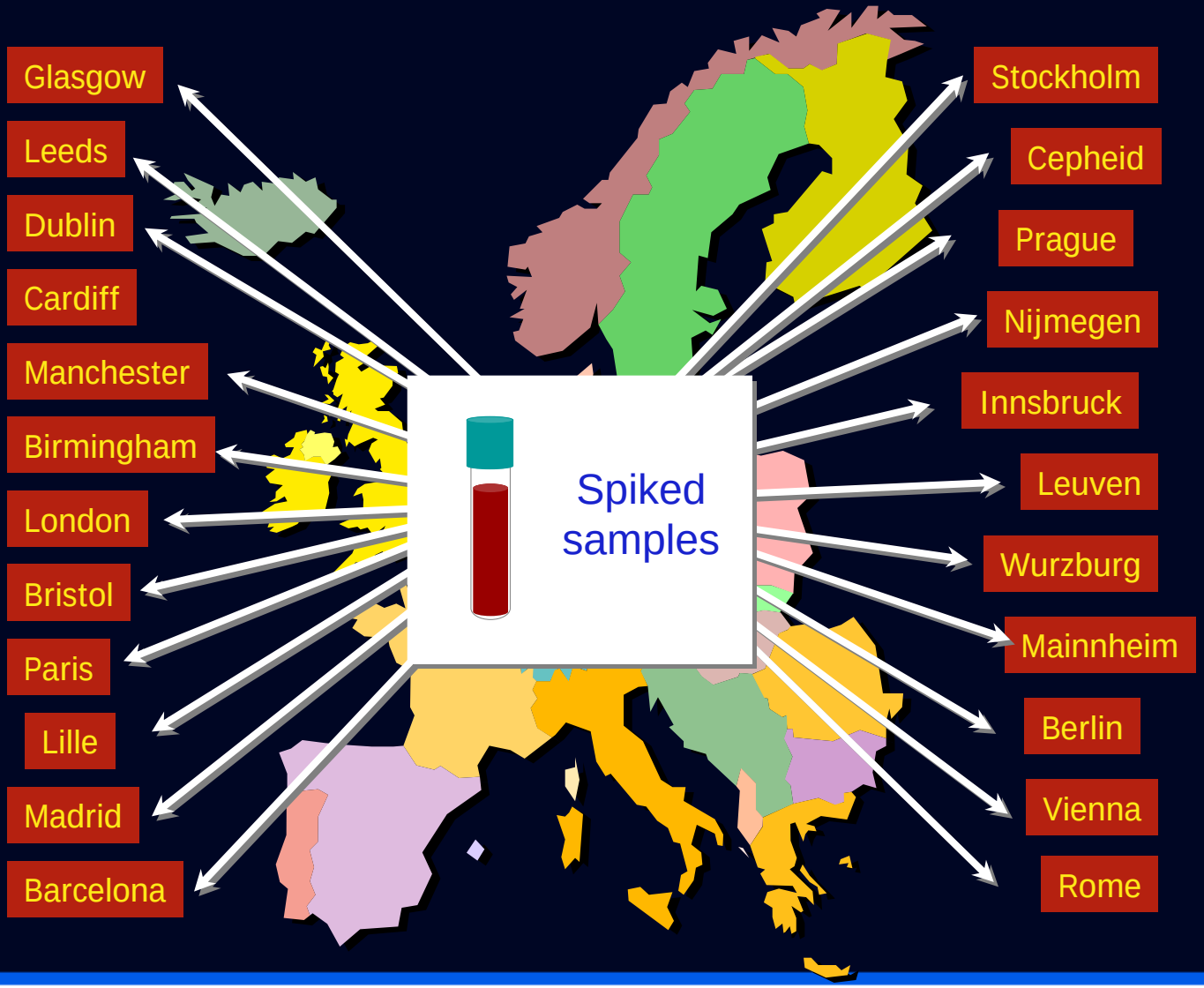
1st Meeting of the Laboratory Working Group

- Frankfurt – September 2006
- Lab Working Group Members
  - Juergen Loeffler (Chair of Group, Lab representative on Steering Group)
  - Stephane Bretagne
  - Niklas Finnstrom (Sangtec, commercial representative)
  - Willem Melchers
  - Lena Klingspor
  - Elaine McCulloch
  - Bettina Schulz
  - Lewis White
- 24 centres
- Key Points:
  - Initial sample type
  - Distribution
  - Extraction procedures
  - PCR amplification
  - Internal control

Working Group Objective

- Provide optimal methodology for inclusion in a multi-centre clinical trail to evaluate the performance and impact of PCR diagnosis
- Lead to inclusion in future consensus criteria for defining disease

Laboratory exercise



Watch this Space

M16 : the Eagle nebula Nik Szymanek