The European PCR standardisation process

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Aspergillus PCR through the ages!!

White and Barnes, 2009 Chapter 29 In *A.fumigatus* and Aspergillosis
"We're almost fully automated now."
Aspergillus PCR through the ages!!

White and Barnes, 2009 Chapter 29 In A.fumigatus and Aspergillosis
Consensus?

You can agree with me or you can be wrong.
UK Standardisation of Aspergillus PCR

• 2006 – First with multi-centre comparison of methods
• Distribution of QCMD panels
• Extraction based variation
  – Bead-beating in combination with Automated extraction
• Two optimal PCR methods – multi-centre testing
  – One for TaqMan
  – One for Light Cycler
• Sample type effect
  – Platform
• Amplification of human DNA

\[\text{White et al. 2006 J Mol Diag}\]
Sunday afternoon
25th June

Contact
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European Collaboration

- ISHAM: International Society for Human and Animal Mycology
- EAPCRI: European Aspergillus PCR Initiative
- EBMT: European Group for Blood and Marrow Transplantation
- EORTC: European Organisation for Research and Treatment of Cancer
- PCR
- HSCT
- chemotherapy
Interested parties

- 86 participants
- 69 centres
- 24 countries
Structure of the EAPCRI

- Steering committee
  - LAB Working Party
  - CLIN Working Party
  - STAT Working Party
  - Commercial Section
EAPCRI 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

- Improve the diagnosis of IA
Aim

- To define a standard for - not a standard - PCR for Aspergillus in blood specimens
### Options for Aspergillus PCR

**Whole Blood**
- Red cell lysis
  - White cell lysis
  - Proteinase K/SDS digestion
  - Fungal lysis – Bead beating
  - Cellular material
  - Hyphae
  - Conidia
  - Hypha

**Blood Clot**
- Fungal lysis – Bead beating
  - DNA purification/ppt/elution

**Serum/Plasma**
- DNA purification/ppt/elution

**CSF**
- PCR amplification
  - Real-time PCR
  - Conventional PCR
  - Pan-fungal RT PCR
  - Genus/species specific RT PCR
  - Pan-fungal PCR
  - Broad range multiplex PCR

**Tissue biopsy**
- Tissue Invasion

**Tissue Invasion**
- IPA, Invasive Sinusitis, Localised respiratory disease

**Cerebral Disease**
- IPA, Invasive Sinusitis, Localised respiratory disease

**Fungaemia, Vascular Invasion, Tissue Invasion**
- Combination?

**IPA, Invasive Sinusitis, Localised respiratory disease**
- Respiratory specimens

**Antifungal Therapy**
- Pan-fungal PCR
  - Sequencing/Micro Array
  - Luminex/SERRS technology
Principles behind testing blood – inhalation

Insufficient host defences

Germination within respiratory tract

Colonisation

Localised disease*

Aveolar macrophage translocation

Inhalation of spores

Insufficient host defences

Epithelial uptake

Germination

Tissue invasion

Angioinvasion

Blood

Clot

Serum/plasma

Whole blood

*Chronic, allergic or fungal ball

Acute disease

Haematogenous dissemination

Sufficient host defences

Elimination
Format for EAPCRI evaluation

- Distribution of QC panels
- Evaluate PCR amplification alone
- Evaluate DNA extraction in combination with PCR amplification
- Identify common parameters that enhance performance
Results from 1st distribution

- **DNA panel**
  - 85.7% of centres achieved required threshold (equivalent to DNA extracted from 50 *A. fumigatus* conidia)

- **Whole blood panel**
  - Sensitivity: 64.5% (95% CI: 49.0-77.5)
  - Specificity: 89.6% (95% CI: 79.1 – 95.2)
  - DOR: 15.1 (95% CI: 4.4 – 52.2)
  - Only 50% of centres achieving PCR threshold maintained this cut-off when extracted DNA from WB

- Centres maintaining threshold used entire specimen and bead-beating
## 2nd Distribution – with recommendations

<table>
<thead>
<tr>
<th>Protocol</th>
<th>sensitivity</th>
<th>95% CI</th>
<th>specificity</th>
<th>95% CI</th>
<th>DOR</th>
<th>95% CI</th>
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<tbody>
<tr>
<td>All</td>
<td>80.6%</td>
<td>68.2 – 88.9</td>
<td>86.3%</td>
<td>76.1 – 92.6</td>
<td>39.8</td>
<td>12.4 – 127.3</td>
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<tr>
<td>Compliant</td>
<td>88.7%</td>
<td>79.8 – 94.0</td>
<td>91.6%</td>
<td>79.1 – 96.9</td>
<td>119.9</td>
<td>44.9 – 319.9</td>
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<tr>
<td>Non-compliant</td>
<td>57.6%</td>
<td>37.9 – 75.2</td>
<td>77.2%</td>
<td>61.2 – 87.9</td>
<td>8.9</td>
<td>1.7 – 45.5</td>
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### Bivariate meta-regression analysis between logit sensitivity and the additional covariates

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<tr>
<th>logit sensitivity</th>
<th>All centres</th>
<th>Centres with 100% specificity</th>
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<tr>
<td></td>
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<tr>
<td>WCLB</td>
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<tr>
<td>NaOH</td>
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<td>NS</td>
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<td>28S</td>
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Multivariate meta-regression analysis between logit sensitivity and the additional covariates

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<td>Elution volume</td>
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Invasive fungal disease

Specimens available

Literature review/Expert discussion

Determine preferred specimens

Evaluate existing methodology

Determine optimal protocol by analytical validity

Multi-centre evaluation of recommendations by QC

Distribution of quality control (QC) panels

Analysis of results

Provide methodological recommendations

Animal model validation

Multi-centre clinical evaluation

Define clinical validity and utility

Inclusion in disease defining criteria
The current EAPCRI recommendations are:

All recommendations apply to EDTA whole blood.

1. A minimum of 3 ml of blood needs to be extracted
2. Bead-beating is required for lysis of fungal cells
3. A real time PCR platform using a multi-copy target and species / genus-specific hybridization probes
4. Analysis of all specimens in duplicate, if discrepancy occurs, repeat on identical DNA extract
5. An Internal control PCR is essential (preferably non-human)
6. The use of a negative control for DNA extraction and PCR assay is required
7. Elution volume <100μl
8. EDTA is the only anticoagulant to be used, sodium citrate and heparin should not be used
9. Some commercial products have been linked with fungal contamination. All batches of reagents should be screened for possible contamination prior to use
Acknowledgements

The EAPCRI LWP:

- Juergen Loeffler (Chair), Wuerzburg
- Stephane Bretagne, Paris
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- Lewis White, Cardiff
- Lena Klingspor, Stockholm
- Elaine Mc Culloch, Glasgow
- Bettina Schulz, Berlin

- Website: www.eapcri.eu