Culture and sensitisation in asthma, CF and COPD – what does it tell us?

Catherine Pashley
Background

• Worldwide prevalence estimates
  Asthma > 300 million
  Chronic obstructive pulmonary disorder (COPD) ~ 64 million
  Cystic fibrosis (CF) ~ 70,000

• Significant morbidity and mortality

• Filamentous fungal colonisation of the airways

• Allergic bronchopulmonary aspergillosis (ABPA)
  *Aspergillus fumigatus, A. niger, A. flavus, A. nidulans, A. oryzae, A. glaucus*
  Asthma 0.7% - 3.5%
  CF 7% - 9%
  COPD increasingly recognised

• Allergic bronchopulmonary mycoses (ABPM)
  *Penicillium, Candida, Curvularia, Drechslera, Fusarium, Geotrichum Helminthosporium, Schizophyllum, and Stemphylium*
Culture of fungi from respiratory samples

- Mycology laboratory accreditation programs are common
- No national standard guidelines for processing respiratory samples
  - USA, Canada and Australia
  - UK BSOP57
- Historically < 10%
- Much higher recovery - research protocol
- Media: PDA or SDA
  - *Alternaria alternata, Aspergillus fumigatus, Botrytis cinerea, Cladosporium herbarum, Epicoccum nigrum, Leptosphaeria cinerea, Penicillium chrysogenum*
- Antibiotics
  - Cloramphenicol 16 μg/ml and gentamicin 4 μg/ml (Dolan, *Appl Microbiol* 1971)
  - Cloramphenicol 50 μg/ml
- Fluconazole 5 μg/ml (Randhawa et al. *Curr Sci* 2005)
Sputum culture methodology

**Research approach**
- Plugs separated from saliva
  - (Pizzichini et al. *Eur Respir J* 1996)
  - 170 mg (± 80 mg)
    - (Bakare et al. *Mycoses*, 2003)
  - PGCF
- 37°C up to 7 days

**Microbiology**
- Add equal volume 0.1% DTT
  - 37°C for 15 min
- Diluted 1:500 sterile water
- 1-10 µl Sabouraud agar
- 37°C for 2 days

**BSOP57**
- Fungal culture
  - 1-10 µl
  - Sabouraud agar
  - 37°C for 5 days

**Mycology**
- 37°C for 2 days

- 41 patients with COPD
- 55 sputum samples
- Culture rates significantly higher using research approach compared to BSOP57

**Pashley et al. (2012) Medical Mycology 50:433-438**

**Graph:**
- **Red** indicates **Research**.
- **Blue** indicates **HPA mycology**.
- **Yellow** indicates **HPA microbiology**.

**Statistical significance:**
- **** $P < 0.01$
- *** $P < 0.001$
### Yeast culture from 55 sputum samples

<table>
<thead>
<tr>
<th></th>
<th>Diluted-homogenised</th>
<th>Homogenised</th>
<th>Plug</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10 µl 100 µL</td>
<td>10 µl 100 µL</td>
<td>170 (±80) mg</td>
</tr>
<tr>
<td>% YP PGCF plates</td>
<td>16.4 50.9</td>
<td>87.3 96.4</td>
<td>94.5</td>
</tr>
<tr>
<td>% YP SC plates</td>
<td>10.9 34.5</td>
<td>83.6 96.4</td>
<td>94.5</td>
</tr>
<tr>
<td>No. of YP samples on both media</td>
<td>2 17</td>
<td>45 52</td>
<td>51</td>
</tr>
<tr>
<td>No. of YP samples on PGCF only</td>
<td>7 11</td>
<td>3 1</td>
<td>1</td>
</tr>
<tr>
<td>No. of YP samples on SC only</td>
<td>4 2</td>
<td>1 1</td>
<td>1</td>
</tr>
</tbody>
</table>

- **Clear effect of diluting sputum**
  - PGCF, $Q=129.381$, df=4, $P<0.000$
  - SC, $Q=137.654$, df=4, $P<0.000$

- **Microbiological**
  - 9/55 versus 52/55, $\chi^2_{(1)}$ McNemar $=41.02$, $P<0.0001$

- **Mycological**
  - 48/55 versus 52/55, $\chi^2_{(1)}$ McNemar $=2.25$, $P=0.1336$

**Aspergillus fumigatus** n = 16 sputum samples

- Clear effect of diluting sputum
  - PGCF, $Q=33.667$, df=4, $P<0.000$
  - SC, $Q=13.867$, df=4, $P<0.008$

- No growth from diluted-homogenised sputa (10µl or 100µl)

- 19%, 44% and 94% (10 µl, 100 µl homogenized sputum, neat plug)

- Mycological 3/16 versus 13/16, $\chi^2_{(1)} \text{ McNemar} = 9.09$, $P = 0.0026$

- Multiple logistic regression
  - media ($B=-0.528$, SE=0.204, $P=0.010$)
  - dilution ($B=1.403$, SE=0.108, $P=0.000$)
  - organism ($B=-5.333$, SE=0.331, $P=0.000$)

Independently had a significant effect, after correction for each other, on whether positive cultures were obtained

Volume dependency for culture of fungi from respiratory secretions and increased sensitivity of *Aspergillus* quantitative PCR

Marcin G. Fraczek, Marie B. Kirwan, Caroline B. Moore, Julie Morris, David W. Denning and Malcolm D. Richardson

- Five aspergillosis patients
- Sputum, bronchial aspirate & bronchoalveolar lavage (BAL) n = 23
- BSOP57, MRCM (higher volume undiluted), *Aspergillus* QPCR

- Recovery rate MRCM (87%) versus BSOP57 (8.7%) $P<0.001$
  - Sputum samples MRCM 44% versus BSOP57 0%
  - Bronchial aspirates MRCM 75% versus BSOP57 0%
  - BAL samples MRCM 20% versus BSOP57 10%

- MRCM 5/5 patients +ve versus BSOP57 1/5
• CF worldwide comparisons of fungal culture studies
  Fairly conserved - key fungal species
  Relative prevalence of individual species varies considerably

  Borman et al. Medical Mycology 2010: 48: S88-S97

Lack of standardization in the procedures for mycological examination of sputum samples from CF patients: a possible cause for variations in the prevalence of filamentous fungi

Andrew M. Borman*, Michael D. Palmer*, Laurence Delhaes†, Jacqueline Carrère‡, Loïc FavenneC§, Stéphane Ranque#, Jean-Pierre Gangneux^, Régine Horré$ & Jean-Philippe Bouchara†

• Several CF centres and mycology laboratories UK and France
• Semi-selective media, improve isolation of certain fungal pathogens associated with CF
  Not employed routinely

A. fumigatus; 8.6% to 88.9% of patients
Exophiala dermatitidia; 1.8% to 15.6% - selective media & prolonged incubation
Scedosporium apiospermum; 0.01% to 8.3% - semi-selective culture media
A standardised approach is required for all studies of fungal colonisation of the respiratory tract.

A more sensitive approach is needed for culture-based studies.

Transparency
Sensitisation methodology

- Skin prick testing (SPT); simple, safe, quick, cheap
- Intradermal tests; more sensitive but higher false positive
- In vitro measurement of specific IgE antibodies – more sensitive, more costly
  - Radioallergosorbent testing (RAST)
  - Immunoassay capture (ImmunoCAP) - higher sensitivity, comparable specificity
- Accuracy & reliability – very dependent on quality of fungal extracts
- Quality can vary dramatically between commercial suppliers in Europe and the USA
  - Inconsistencies in preparation of fungal extracts (fungal mycelia or spores)
  - Inter-strain variability
• SPT and specific IgE  (O'Driscoll et al. Clin Exp Allergy 2009)
  Aspergillus fumigatus, Alternaria alternata, Candida albicans, Cladosporium herbarum, Penicillium chrysogenum, Botrytis cinerea
  Agreement (positive or negative) in 93/121 patients (77%)
  Discordant in 28 patients (23%)
  Concordance rates; Botrytis (14%) to Alternaria (56%) – average 40%
  Aspergillus, IgE alone – missed 19%, SPT alone – missed 28%

• Recommendation: SPT and specific-IgE

• Newer diagnostic approaches
  Component resolved IgE testing

  IgE reactivity to commercially available fungal enzymes (Horner et al. Allergy Asthma Proc 2008)

Fluorescent halogen immunoassay (fHIA) (Green et al. J Mycol Med 2009)
Cross-reactivity among fungal allergens

  Clinical practice: allergen = single allergenic source or extract thereof
  Molecular biologist: allergen = single protein, encoded by single mRNA, able to induce a switch to IgE production in B cells

• >75% of mould-sensitised patients monosensitised
  Alternaria is the most common one (Alt a1)
  Monosensitisation to *A. fumigatus, Cladosporium herbarum, P. chrysogenum*, and *Saccharomyces cerevisiae* is very rare (<1%)

• Fungi ~100 allergens including enzymes, backbone and cell wall proteins
  Two classes: species-specific, and cross-reactive allergens
  Species-specific major allergens: *A. fumigatus* (Asp f1), *A. alternata* (Alt a1), *Coprinus comatus* (Cop C1), and *Malassezia sympodialis* (Mala s1)
  Majority represent cross-reactive structures covering different protein families
  Many individuals with allergen-specific IgE do not develop allergy symptoms
Sensitisation to, and culture, of *A. fumigatus*

**Asthma**
- n=79 patients with mod-severe asthma
  - *A. fumigatus* IgE (n = 40) 63%
  - *A. fumigatus* IgG-only (n = 13) 39%
  - Non-sensitised asthmatics (n = 26) 31%
  - Healthy controls (n = 14) 7%

**COPD**
- n=128 patients with COPD
  - *A. fumigatus* cultured 47/128 (37%)
  - Healthy controls n=22 (9%)

Lung function significantly worse in *A. fumigatus* IgE-sensitised patients
Manchester, severe asthma n=133 (Menzies et al. *Allergy*, 2011)

Bafadhel *et al.* (2013) ERJ 43(1):64-71
### Sputum culture, sensitisation and lung function

<table>
<thead>
<tr>
<th>Asthma patients (n=126)</th>
<th>No fungi cultured (n=58)</th>
<th>Any fungi (n=68)</th>
<th>p &lt;0.01</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>FEV&lt;sub&gt;1&lt;/sub&gt;% of predicted, post bronchodilator</strong></td>
<td>82.8 (24.8)</td>
<td>70.8 (25.4)</td>
<td></td>
</tr>
<tr>
<td>Fungal sensitisation, (any)</td>
<td>38%</td>
<td>56%</td>
<td>0.08</td>
</tr>
<tr>
<td><em>Aspergillus fumigatus</em> (positive/n)</td>
<td>17/58</td>
<td>35/68</td>
<td>0.02</td>
</tr>
<tr>
<td><em>Penicillium chrysogenum</em></td>
<td>5/36</td>
<td>17/48</td>
<td>0.04</td>
</tr>
<tr>
<td><em>Botrytis cinerea</em></td>
<td>3/31</td>
<td>8/41</td>
<td>0.30</td>
</tr>
<tr>
<td><em>Alternaria alternata</em></td>
<td>6/39</td>
<td>11/58</td>
<td>0.80</td>
</tr>
<tr>
<td><em>Cladosporium herbarum</em></td>
<td>7/38</td>
<td>13/57</td>
<td>0.80</td>
</tr>
</tbody>
</table>

Culture positive & fungal sensitised asthmatics have significantly worse lung function than non-sensitised, culture negative 22% drop in post bronchodilator FEV<sub>1</sub>

Agbetile *et al.* (2012) *CEA* 42:782-791
Culture & ID of filamentous fungi from sputum

A. fumigatus complex
A. niger complex

Nuclear ribosomal operon

SSU (18S)  5.8S  LSU (28S)

ITS1  ITS2

Asthma n=126
A. fumigatus, 44%
Any fungi, 54%

COPD n=128
A. fumigatus, 37%
Any fungi 49%

Bafadhel et al. (2013) ERJ 43(1):64-71
Fungal infections and treatment in cystic fibrosis – review (Middleton et al. Curr Opin Pulm Med 2013)

• Previous dismissal of fungi in airway secretions as ‘oral contamination’ may have been inappropriate; recent studies finding correlations between the presence of fungi and worse clinical outcomes

• Newer fungal-specific culture media and molecular techniques show much higher prevalence of fungi than simple standard cultures

• Longitudinal studies needed to assist in determination of effect of fungal colonization/ infection on lung function decline

• Intervention studies to examine effect of antifungal agents are currently planned to determine whether suppression or eradication of these fungi improves clinical outcomes
Summary

Culture methodology

A standardised approach is required for all studies of fungal colonisation of the respiratory tract
A more sensitive approach is needed for culture-based studies
Transparency

Sensitisation methodology

Accuracy & reliability – very dependent on quality of fungal extracts
Recommendation: SPT and specific-IgE
Majority allergens represent cross-reactive structures covering different protein families
Many individuals with allergen-specific IgE do not develop allergy symptoms

Sensitisation to *A. fumigatus* associated with worse lung function in asthma, COPD and CF

Culture of filamentous fungi associated with worse lung function in asthma and CF, less clear in COPD
Acknowledgments

- Prof Andy Wardlaw
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- Dr Josh Agbetile
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- MRC
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