Pathogenesis of *Aspergillus* in CF: a role for molecular diagnostics?

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Aspergillus in CF

- The incidence and diversity of fungal pulmonary infections in CF is becoming increasingly recognised.
- *Aspergillus fumigatus* is still the most clinically relevant fungal infection and causes significant morbidity.
- The US CFF registry indicates an approximate doubling of reported *A. fumigatus* cultured from CF sputum between 1995 to 2005.
- ? artificial due to improved methods of culture and increased reporting.
  ? true increase secondary to environmental and treatment factors (e.g. broad spectrum antibiotics and nebulised antibiotics).
- Many diseases caused by *A. fumigatus* in CF.
- Hypersensitivity reactions are the most common.
**Aspergillus hypersensitivity in CF**

- Viscous CF secretions allow *Aspergillus* spores to become trapped and colonize the airways. Spore germination releases allergens and proteases. A predominant type 1 hypersensitivity response is evoked when the immune system encounters these allergens - sensitization.

- Sensitization to *Aspergillus* is defined as immediate cutaneous reactivity to *Aspergillus* antigens or a ≥ class 2 specific IgE antibody response. There are usually no clinical symptoms.

- Persistent antigen exposure can cause a heightened allergic response - ABPA. Clinical symptoms include SOB, wheeze, cough and pulmonary infiltrates.

- Around 60% of CF patients demonstrate sensitization and 15% ABPA.

- Wide clinical and immunological spectrum of hypersensitivity ranging between sensitization and ABPA. There is no clear rationale for treatment in patients that do not meet ABPA diagnostic criteria.

- Natural history of colonization, sensitization and ABPA poorly understood.
Aspergillus hypersensitivity in CF

- The development of ABPA is thought to require continued antigen exposure in a susceptible host.
- Presence and quantity of *A. fumigatus* in airways may be important.
- To date, no correlation has been found between growth of *A. fumigatus* on routine culture and ABPA.
- However, sputum culture of *A. fumigatus* has a wide reported prevalence of 12-57%. Routine culture methods can be insensitive - optimal sampling, culture media and growth conditions are important. Routine culture only uses a small proportion of the sputum sample which may not be representative.
- The variable reported prevalence indicates a need to improve methods of detection.
- In the absence of clinical symptoms, the presence of *A. fumigatus* on routine culture is currently not an indication for antifungal treatment.
- Precise detection and early targeted treatment could prevent development of ABPA by removing antigen stimulus.
Research Objectives

To develop precise measures of *Aspergillus* load in CF sputum and examine its correlation to serological sensitization and clinical parameters.

Disease association study – is there an association between atopic tendency and/or allergy to other respiratory fungi and the development of ABPA/ABPM?
Comparative study of *A. fumigatus* precipitins and IgG immunoCAP

To document the frequency of azole resistance by a cross sectional observation study and assess the molecular mechanisms of resistance (CYP51A sequencing and microsatellites).

To investigate the changing fungal diversity during CF pulmonary exacerbations and treatment by using advanced parallel gene sequencing.
Aim 1 – measures of *Aspergillus* load

• Hypothesis – as *A. fumigatus* load increases the immune response increases.

• Key to this research therefore is **optimal** DNA extraction and **precise** Real-time PCR technology.

• Advent of commercial kits. Which technologies are best?
FXG™: RESP (Asp+), Myconostica Ltd

- Commercially available – under strict quality control.
- First **clinically approved** kit for detection of *Aspergillus* in respiratory secretions.
- FXG™: RESP (Asp+) kit is based on molecular beacon Real-time PCR chemistry. This is the most specific real-time chemistry – sensitivity to one base pair.
- Molecular beacon probes are complementary to a portion of the 18S rDNA of *Aspergillus*. 
Molecular beacon chemistry

- The FXG™: RESP (Asp+) kit also contains positive and negative controls and an internal amplification control (IAC). The IAC is a plasmid containing a fragment of DNA not present in *Aspergillus* that will be amplified unless PCR inhibitory substances are present.
- Previous studies of real time PCR for *Aspergillus* detection have used ‘in-house’ primer and probe design - non-standardized, non-comparable results.
- 2 other Real-time PCR kits for the detection of *Aspergillus* are commercially available but these have been designed for use with blood to diagnose IA and are not clinically approved.
- No previous studies have used molecular beacons for detection and quantification of *Aspergillus* in sputum.
- This is the first kit to have a standardized curve for post PCR quantification derived from AF293 DNA.
DNA extraction

• Optimal DNA extraction is also vital to evaluate the effect of *Aspergillus* load on clinical presentation.
• It has a major influence on sensitivity and specificity.
• The fungal wall must be digested and cell membrane lysed to release DNA.
• DNA must then be purified to remove PCR inhibitors (e.g. DNase).
• Must prevent external contamination – class 2 hood.
• It is important that methods are reproducible, have little operator variability, are fast and have relatively low cost.
• Applicable to high output laboratories.
DNA extraction

• The difficulty of cell wall lysis has led to many complex and expensive methods that vary between studies.
• Griffiths et al 2006 found that enzyme digestion plus bead beating is the most sensitive and reproducible method of DNA extraction. Griffiths LJ, J Med Microbiol, 2006. 55 (pt 9): p1187-91
• Now number of commercial kits available.
MycXtra™

• MycXtra™ DNA extraction kit (Myconostica Ltd). This kit has been designed for the extraction of fungal DNA from respiratory samples.
• The MycXtra™ kit is based on an enzyme lysis solution and bead beating followed by several DNA purification steps.
• Only kit shown to remove the substances DNase, TOBI, HTS and Promixin – advantage over QIAmp DNA minikit.
MycXtra™ efficiency

- The MycXtra™ and FXG™ kits can be used together and have been shown to successfully extract *Aspergillus* DNA from BAL samples.

- In order to test this kit with CF sputum, 6 CF sputum samples were collected. All were negative for *Aspergillus* on culture. Each sample was homogenized with sputasol and then
  - 2 spiked with AF 293 spores $10^5$
  - 2 with AF293 mycelia grown in SAB broth
  - 2 with AF293 DNA 50µl of 4ng/ml.

- The Ct threshold is 38.1 for this assay. Any value below this indicates a positive result.
All Ct values in this experiment were between 25-31.
Early challenges and patient results
Sample collection

- Patients for this study are currently being recruited from the Manchester Adult CF Centre (MACFC).
- To date 54 patients have been recruited. Aim 200.
- Each patient has submitted a sputum sample, had blood taken for serology and had skin prick tests.
- Sputum samples are initially processed in a microbiology category 3 containment laboratory.
- SAB culture at 30, 37 and 45°C for 14 days.
- Remaining sputum sample is homogenized with NaOH-NALC (BBL™ MycoPrep) and heated at 80°C for 20 minutes. This decontaminates samples allowing safe removal from the CL3 laboratory to a molecular laboratory.
Experiment 1

• The 54 sputum samples - MycXtra™ DNA extraction kit.
• Real-time PCR was then run on a Smartcycler® (Cepheid) platform with the FXG™:RESP (Asp+) kit.
• 9 of these samples were known to be culture positive on SAB agar.
• First run - all 54 samples were negative.
Why no positive PCR signal?

- ? Effect of heat to sterilize samples prior to removal from CL3 laboratory. Outside normal research protocol.
- Spiking samples probably does not represent the existence of fungal spores/mycelia in CF lungs.
- ? Tougher fungal cell wall – adaptation to CF lung environment.
- Viscoelasticity of purulent CF secretions – spores/hyphae in complex matrix.
- ? Aspergillus biofilm.


Fungal Research Trust www.aspergillus.org.uk
? Heat

• 9 culture positive CF samples were processed through the protocol minus the initial heat step.
• All remained PCR negative.
• Unlikely to be this step alone.
Improving DNA extraction

1) Looxster® (SIRS labs) – fungal DNA enrichment by removal of eukaryotic DNA post extraction.

2) Sonication prior to DNA extraction – improve homogenization.

3) High velocity bead beating (Precellys®) – break down fungal cell walls.
• The use of the Looxster® DNA purification system allowed our culture positive samples (and some culture negative samples) to register positive in the Real-time PCR reaction.
Sonication

• The use of sonication prior to DNA extraction made samples much easier to handle as all were completely homogenized.
• This is likely to lead to improved reproducibility.
• The optimal time is 60 seconds. Longer than this appears to degrade DNA.
• The use of 60 seconds sonication improves Ct values 2-fold.
Precellys

• High velocity bead beating which can effectively break down most tissues.

• No improved DNA extraction found to date but optimal time for this method of bead beating under further investigation.
Aspergillus quantification

• Methods to improve DNA extraction must be applicable to high throughput laboratories and costs must be kept low if this is to be built into routine clinical practice.

• The additional use of sonication and fungal DNA concentration appears to improve extraction efficiency.

• This research is in its early stages. Extraction and reproducibility must still be optimized. However this initial data shows *A. fumigatus* quantification is possible from CF sputum.
Fungal allergy - SPTs

- Atopy to *A. fumigatus* most common – 75% patients.
- 52% had more than 1 positive fungal SPT.
- Patients with atopy to common allergens are more likely to be atopic to fungal allergens.
Fungal allergy - serology

- 69% demonstrated a ≥ class 2 response to *A. fumigatus* – ABPA risk.
- 46% demonstrated a > class 2 response to more than 1 fungal allergen – risk of ABPM.
- Good correlation between total IgE and specific IgE *Aspergillus*. Coefficient 0.869, p=<0.01.
Fungal allergy - IgG

- Patients were subdivided by serology and clinical features into 3 groups – ABPA, sensitization and control. A ROC curve was produced to identify if IgG level can be used in the diagnosis of ABPA.
- The area under the curve was 0.527 indicating an approximate 50% sensitivity and 50% specificity. Levels of specific IgG are not helpful in the diagnosis of ABPA.
- There was also very poor correlation between positive IgG and positive precipitins.
## Antifungal resistance

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<td>ITRA</td>
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