# DEVELOPMENT OF NOVEL METHODS FOR THE DIAGNOSIS OF INVASIVE PULMONARY ASPERGILLOSIS

By

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## ABSTRACT

**Background:** Invasive pulmonary aspergillosis (IPA) is a common cause of mortality in haemato-oncology patients and early diagnosis is vital for improving outcomes. Since lung biopsy in this acute setting is rarely performed due to the associated risks, an empirical strategy remains the standard of care in many haematology units, but leads to overtreatment with antifungal drugs, which have significant side-effects. This project has developed novel approaches for detecting IPA, allowing early and specific treatment of genuine fungal infection.

**Methods:** A combination marker approach involving a new *Aspergillus* qPCR assay, an EORTC/MSG-endorsed GM ELISA and an *Aspergillus* LFD, was used to establish a robust diagnosis of IPA from clinical broncho-alveolar lavage (BAL) fluid samples. The inflammatory cytokine profile associated with IPA biomarker positive BAL fluid was also evaluated. Finally, antigen and qPCR detection were combined in a proximity ligation assay (PLA), to demonstrate proof-of-principle for a diagnostic assay for the earliest possible detection of fungal infections.

**Results:** A dual testing approach involving a novel MIQE-compliant *Aspergillus* qPCR assay and an *Aspergillus* LFD showed a sensitivity and specificity of 100% and 94%, respectively in BAL fluid, unlike in blood where this approach was not sensitive. Results confirmed previously published concerns over the repeatability of GM in serum, whereas BAL GM results appear stable. Biomarker detection results in exhaled breath condensate did not correlate well with results in BAL fluid samples. Respiratory samples did not identify a distinct inflammatory marker profile in IPA. Finally, antibodies raised against JF5 mannoprotein were used to develop a PLA test to detect active growth of *Aspergillus.*

**Conclusions:** The optimised qPCR is a very sensitive and highly specific aid in IPA diagnosis. A combination biomarker approach could be incorporated into a diagnostic-driven approach to patient management to direct antifungal treatment to patients with evidence of invasive fungal disease.

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## LIST OF ABBREVIATIONS

- 2PF — 2-Pentylfuran
- Ab — Antibody
- ABPA — Allergic broncho-pulmonary aspergillosis
- ALL — Acute lymphoblastic leukaemia
- AML — Acute myeloid leukaemia
- APC — Antigen presenting cell
- ARDS — Adult respiratory distress syndrome
- BAL — Broncho-alveolar lavage
- BG — Beta-D-glucan
- BHQ — Black hole quencher
- BLAST — Basic Local Alignment Search Tool
- Bp — Base pairs
- CBA — Cytokine bead array
- CF — Cystic fibrosis
- CFU — Colony forming units
- CGD — Chronic granulomatous disease
- COPD — Chronic obstructive pulmonary disease
- CPA — Chronic pulmonary aspergillosis
- Cq — Quantification cycle
- CRP — C-reactive protein
- CT — Computerised tomography
- CV — Coefficient of variation
- CXR — Chest x-ray
- DMSO — Dimethyl sulfoxide
- DNA — Deoxyribonucleic acid
- EAPCRI — European *Aspergillus* PCR Initiative
- EBC — Exhaled breath condensate
- EDTA — Ethylenediaminetetraacetic acid
- ELF — Epithelial lining fluid
- ELISA — Enzyme-linked immunosorbent assay
- EORTC/MSG — European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group and the National Institute of Allergy and Infectious Diseases Mycoses Study Group
- FACS — Fluorescence-activated cell sorting
- GM — Galactomannan
- GVHD — Graft-versus-host disease
- HIV — Human immunodeficiency virus
- HRCT — High resolution computerised tomography
- HSCT — Haematopoietic stem cell transplantation
- IA — Invasive aspergillosis
- IFD — Invasive fungal disease
- Ig — Immunoglobulin
- IL — Interleukin

- INF — Interferon
- IPA — Invasive pulmonary aspergillosis
- ITS — Internal transcribed spacer
- LFD — Lateral flow device
- LNA — Locked nucleic acid
- LOD — Limit of detection
- LOQ — Limit of quantification
- LT — Long-term
- MAb — Monoclonal antibody
- MALDI-TOF — Matrix-assisted laser desorption/ionization-time of flight
- MDS — Myelodysplastic syndrome
- MFI — Median fluorescence intensity
- MIQE — Minimum Information for Publication of Quantitative Real-Time PCR Experiments
- MPO — Myeloperoxidase
- MWCO — Molecular weight cut-off
- NADPH — Nicotinamide adenine dinucleotide phosphate
- NASBA — Nucleic acid sequence based amplification
- NCBI — National Center for Biotechnology Information
- NET — Neutrophil extracellular trap
- NPV — Negative predictive value
- NTC — No template control
- OD — Optical density
- pAb — Polyclonal antibody
- PA-EIA — Platelia *Aspergillus* enzyme immuno assay
- PBS — Phosphate buffered saline
- PE — Phycoerythrin
- PLA — Proximity ligation assay
- PPV — Positive predictive value
- qPCR — Quantitative polymerase chain reaction
- RNA — Ribonucleic acid
- SOT — Solid organ transplant
- ST — Short-term
- Th — T helper
- TNF — Tumour necrosis factor
- VOC — Volatile organic compound

## DECLARATION OF ORIGINALITY

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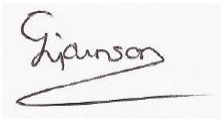

Gemma Johnson

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## LIST OF PUBLICATIONS ARISING FROM THIS THESIS

### Original research

- Proximity ligation assay for the sensitive, specific and early diagnosis of invasive fungal disease (2013). Johnson GL, Shannon M, Thornton CR, Agrawal SG, Bustin SA. *MYCOSES* 56:48-49 (Abstract).
- Significant decline in galactomannan signal during storage of clinical serum samples (2013). Johnson GL, Sarker S, Hill K, Tsitsikas DA, Morin A, Bustin S, Agrawal SG. *Int. J. Mol. Sci.* 14, 12970-12977.
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- A MIQE-Compliant Real-Time PCR Assay for Aspergillus Detection (2012). Johnson GL, Bibby DF, Wong S, Agrawal SG, Bustin SA. *PLoS ON*E 7(7): e40022.
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### Reviews

- Biomarkers for invasive aspergillosis: the challenges continue (2014). Johnson G, Ferrini A, Dolan SK, Nolan T, Agrawal S, Doyle S, and Bustin SA. *Biomarkers in Medicine*, Vol. 8, No. 3, P 429-451.

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# CHAPTER 1

# INTRODUCTION

## 1.1 Overview

The incidence and clinical severity of invasive fungal disease (IFD) have increased significantly in recent years, posing an increasingly important clinical problem. This challenge has become more acute as the use of cytotoxic treatments in patients with common disorders such as asthma, inflammatory bowel disease or rheumatoid arthritis is adding to the number of immunocompromised patients beyond the classic groups of patients with cancer, HIV-infection or undergoing transplants. Concomitantly, there has been a steady increase in the frequency of opportunistic IFDs in these patients, where they are a major cause of morbidity and mortality. There is substantial controversy concerning optimal diagnostic criteria for IFD, which is very difficult to detect at an early stage. In haemato-oncology patients, the most widely studied group, the usual scenario involves commencement of empirical treatment in the context of a febrile illness and neutropenia unresponsive to broad spectrum antibiotics. Frequently, antifungal agents are started in this setting without definite evidence of an IFD, with associated financial costs, significant toxicities and potential drug-drug interactions (1-3). Consequently, an improvement in diagnostic accuracy would lead to early, specific and appropriate treatment of genuine fungal infection, minimising the unwarranted application of expensive and potentially harmful therapy.

This chapter

- introduces the mould *Aspergillus*, the most common cause of IFDs in the severely immunocompromised population
- describes the range of infections caused by *Aspergillus*, and then focuses on invasive aspergillosis (IA), the clinical problem and the theoretical approaches to its diagnosis in the high risk haemato-oncology population

- discusses current options and emerging technologies and reviews the practical application of these technologies in the context of published findings and
- provides an overview of the state of the art, and the important questions that still remain.

## 1.2 *Aspergillus*

*Aspergillus* is the name given to a family of common moulds, first described by the Italian priest and botanist Pier Antonio Micheli in 1729 (4). Micheli named the mould after the 'Aspergillum'; an instrument used for sprinkling holy water, due to the similarity of its appearance under the microscope (Figure 1A and 1B).

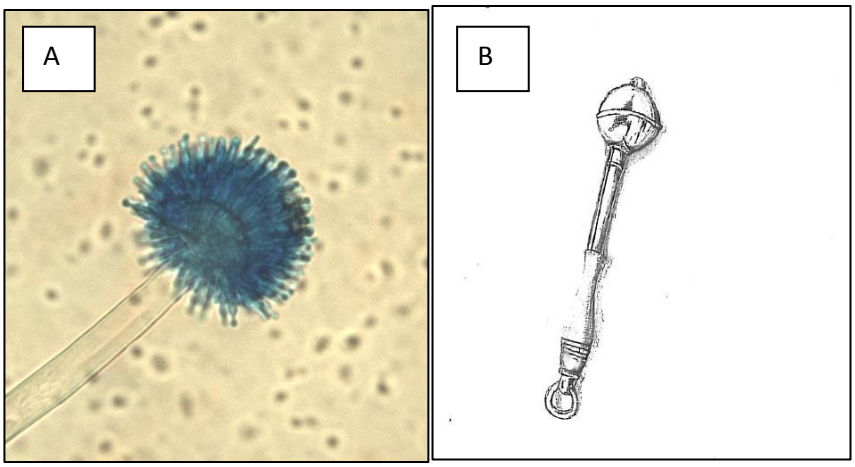



**Figure 1. A) Spore-forming structures (conidia) of the fungus *Aspergillus*, B) An aspergillum.**

A) Credit: Microbiology department, Royal London Hospital. B) Credit: Shaun Johnson, Ad Meliora Art and Design, UK.

The genus *Aspergillus* was originally divided into subgenera and groups (5). The current classification scheme (6), which conforms to the rules of the "International Code of Botanical and Mycological Nomenclature", divides the genus into 7 subgenera and sections that consist of related species. By the current code, different names can be assigned to the different asexual and sexual stages of a single species (7). Until recently, no *Aspergillus*

species were known to have a sexual cycle, though genes were identified that linked them to sexual reproduction (8-11). However, heterothallic reproductive cycles have recently been discovered in three medically important *Aspergillus* species, namely *Aspergillus fumigatus*, *A. parasiticus* and *A. flavus*.

*Aspergillus* is a saprotrophic fungus found in nutrient-rich soil or decaying vegetation and plays a significant role in carbon and nitrogen cycling by breaking down dead organic matter. However, it is also an opportunistic human pathogen, which can cause allergic and infectious diseases, depending on the immune status and pulmonary structure of the host. During the asexual cycle, haploid hyphae (mycelia) produce conidiophore vesicles, from which a series of mitotic budding events occur. Chains of haploid spores (conidia) are formed and released through a disruption in the environment. Conidia are dispersed in the air and found in both indoor and outdoor environments (12). Transmission of fungal conidia to human host occurs mainly via inhalation and although the average person inhales up to 200 conidia each day (13), this will cause most healthy individuals no ill effect. The conidia are removed by mucociliary clearance or by epithelial cells and alveolar macrophages if they travel as far as the lungs. Macrophages also initiate a proinflammatory response, to recruit neutrophils. However, when a host is immunocompromised, for example, during treatment for cancer, or when undergoing transplants, *Aspergillus* conidia are able to evade macrophage killing, colonise, germinate and cause disease.

Aspergillosis describes a range of diseases caused by *Aspergillus* species, including both invasive and allergic disease. There are four main types:

1. Allergic broncho-pulmonary aspergillosis (ABPA) – a condition where a patient develops an allergy to *Aspergillus* spores, predominantly affecting patients with asthma and cystic fibrosis.

2. Chronic pulmonary aspergillosis (CPA) – a cavitating process in the lungs due to invasion of the lung tissue by *Aspergilllus*, commonly associated with underlying lung disease or alcoholism.

3. Aspergilloma - a mycetoma or fungus ball, which exists in a body cavity such as the lung, usually developing in pre-existing lung cavities such as those caused by tuberculosis.

4. IA – primarily a pulmonary infection, but haematogenous spread can occur to any organ, most frequent in individuals with severely weakened immune systems.

## 1.3 Invasive Aspergillosis

IA is one of the leading causes of infectious mortality in patients with haematological malignancies receiving intensive chemotherapy and haematopoietic stem cell transplantation (HSCT) (14, 15), with a mortality rate as high as 90% in the immunocompromised population (16). Incidence of IA ranges from 5% to more than 20% in high risk groups and *A. fumigatus* is the most common species implicated, accounting for approximately 90% of all cases of life-threatening infections (17). However, other strains, particularly *A. flavus* and *A. terreus* are increasingly shown to be associated with IA (18-20). The reported frequencies of *A. fumigatus, A. flavus, A. terreus, A.nidulans* and *A.niger* isolated in IA range from 30-67%, 14-33%, 3-28%, 1-2.5%and 5-8% respectively (21-23). *A. fumigatus* has smaller conidia than other *Aspergillus* species, with an average conidia

diameter of 2-3.5µm. Upon inhalation, this enables it to travel deep into the lower respiratory tract of the host and into the lung alveoli.

## 1.4 Host immune defences against IA

### *1.4.1 First line defences*

The main portal of entry for *Aspergillus* is the airway, in the form of conidia inhalation (Figure 2A). The airway epithelium is the first line of defence of the lungs against invading microorganisms and defences include mucus-secreting cells, which trap inhaled conidia and ciliated cells, which transport the trapped conidia to the oropharyngeal junction to be swallowed. The cough and sneeze reflexes are added mechanical defences to aid in expelling particles. An additional defence is provided by the airway lining fluid, which contains antimicrobial compounds such as lysozyme and defensins. However, not all inhaled *Aspergillus* conidia will be removed in this way. Some move to the lung, where they must evade the first line immunity presented by Surfactant proteins A and D (SP-A, SP-D). These soluble receptors bind *Aspergillus* conidia, leading to increased phagocytic capacity of alveolar macrophages and neutrophils (Figure 2B).

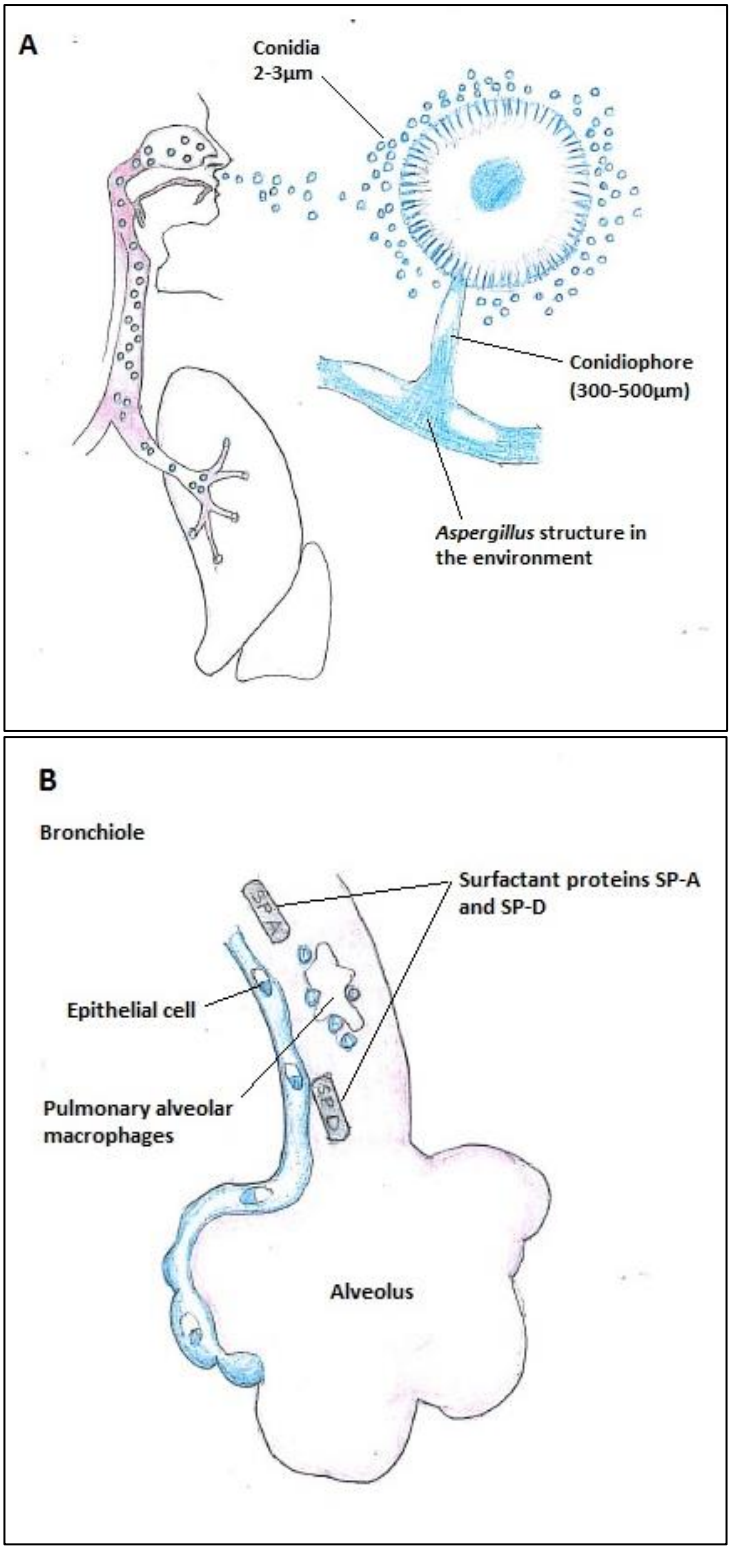



**Figure 2. The *Aspergillus* infection pathway.** A. *Aspergillus* conidia are inhaled into the lung and reach the alveoli. B. In the bronchiole the conidia meet surfactant proteins SP-A and SP-D, which trap conidia and promote phagocytosis by macrophages.

Credit: Shaun Johnson, Ad Meliora Art and Design, UK.

### *1.4.2 Cells of the innate immune response*

Once spores reach the lungs the host has two important defence needs:

- to restrict spores germinating into invasive hyphae
- to limit fungus-induced and inflammation-induced tissue damage

The binding and internalisation of *Aspergillus fumigatus* by respiratory epithelial cells has been demonstrated (24) and these intra-epithelial conidia may survive and germinate (25). This evidence suggests that *A. fumigatus* may use this internalisation step to evade immune cells and as a starting point for dissemination.

Macrophages used to be considered the first line of defence against spores, with neutrophils specifically targeting hyphae (26). But it has become evident that neutrophils have a much more complex role. For example, they have been shown to kill swollen (but not resting) ungerminated *Aspergillus* conidia after phagocytosis (27). Neutrophils exhibit phagosome proteolytic activity, initiate oxidative mechanisms of microbicidal activity and release granule proteins that have crucial roles in the innate immune response to *Aspergillus* infection. Two enzymes necessary for *Aspergillus* elimination are Myeloperoxidase (MPO), which catalyses the formation of reactive hypochlorites (ClO-) from $H_2O_2$ and Cl-, and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, which together with superoxide dismutase ensures formation of reactive oxygen species such as superoxide and hydroxyl radical (Figure 3). MPO is stored in neutrophil granules, whereas NADPH oxidase is a membrane bound complex and both are activated during a respiratory burst (28), The absence of MPO increases mortality rates in mice infected with *A. fumigatus* and the absence of NADPH oxidase makes mice more susceptible to *A. fumigatus* infection (29). This helps explain why patients with chronic granulomatous disease (CGD) are at such high risk of IA, as the disease is characterised by the absence of

NADPH oxidase from neutrophils and macrophages. Neutrophils, along with epithelial cells, also produce lactoferrin, which sequesters iron, inhibiting *Aspergillus* growth (30). Pentraxin 3 is a secreted pathogen recognition receptor, released from neutrophil granules, that has been shown to interact with *A. fumigatus*. In the presence of *Aspergillus* conidia, the binding of pentraxin 3 to the spore surface is important for opsonisation. It has been demonstrated that neutrophils take up pentraxin 3 –coated spores more efficiently than uncoated spores (31), with neutrophils forming extracellular traps (NET) upon encountering germinating spores and hyphae in the lung (32, 33). Binding of pentraxin-3 to conidia via galactomannan, also enhances uptake by alveolar macrophages and dendritic cells (34).

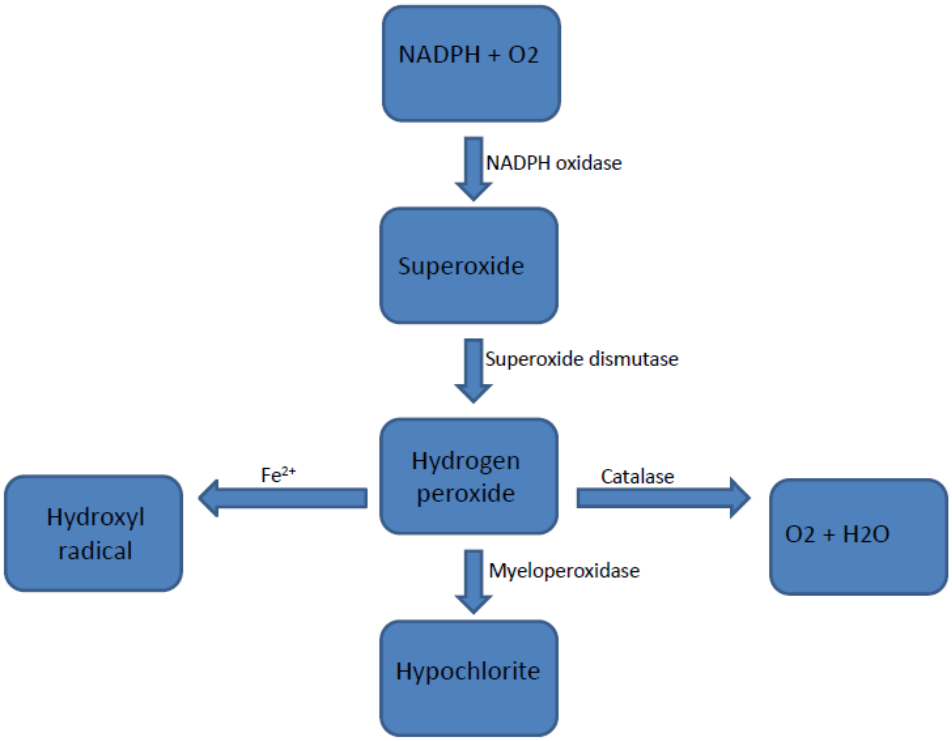



**Figure 3. Oxidative mechanisms of microbicidal activity initiated by neutrophils to destroy invading organisms.**

The enzyme NADPH oxidase acts together with superoxide dismutase to form reactive oxygen species such as superoxide, which then reacts to form hydrogen peroxide. The enzyme myeloperoxidase combines the hydrogen peroxide with chloride.

In response to infection, neutrophils are rapidly recruited to the alveolar spaces and cooperate to inactivate conidia. Together with alveolar macrophages they prevent conidial germination in the immunocompetent host. Hence, prolonged neutropenia is a major risk factor for IA.

### *1.4.3 The adaptive immune response*

Microbial pathogens are endocytosed by antigen-presenting cells, such as dendritic cells, processed and presented preferentially to CD4+ T helper (Th) cells. Dendritic cells act to initiate both innate and adaptive immunity, with control of infection largely dependent on Th cell selection and clonal expansion to direct effector cells against germinating *Aspergillus* conidia and growing hyphae (35). In the murine model, three patterns of Th activation have been shown to characterise the adaptive immune response. CD4+ Th cells can develop into Th1, Th2 or Th17 cells (36-38). The development of Th1 responses is influenced by cytokines such as INF-γ, IL-6, TNF-α and IL-12, stimulating macrophage and neutrophil activity and healing, in the relative absence of Th2 cytokines such as IL-4 and IL-10 (39) (Figure 4). Th2 responses are associated with decreased INF-γ, disease progression and poor outcome. An association has been demonstrated between unimpaired innate immune activity, producing high detectable levels of TNF-α and IL-12 in BAL fluid, and resistance to IA in a mouse model (40). In the same study, down-regulation of TNF-α and IL-12 production was seen in immunocompromised mice with defective innate immunity and resulted in impaired fungal clearance and increased mortality. Resistance was increased in these mice by IL-12 administration. At a later stage of infection, fungal antigen-loaded dendritic cells influence naïve T cells to mature into low- INF-γ-producing lymphocytes, via IL-23. This adaptive immune response counter-regulates IL-12 production, reducing INF-γ production and impairing host antifungal immunity, resulting in high fungal tissue burden (41).

Chemokines appear to enhance lymphocyte and macrophage recruitment, with MCP-1/CCL2 markedly induced in the lungs of neutropenic mice challenged with *A. fumigatus* conidia, playing a vital role in recruitment of immune cells to the lungs in early host defence (42).

More recently, a strong Th17 response has been documented in *Aspergillus* infection (43), with the pro-inflammatory cytokine IL-17A involved in the recruitment, activation and migration of neutrophils to the site of inflammation. In a single exposure model, the *A. fumigatus*-specific CD4 T-cell airway response has been demonstrated to consist of a dominant Th1 and a smaller Th17 population (44, 45). However, the involvement of the Th17 pathway in human IA remains unresolved. A study using human monocyte-derived macrophages reported a limited role of IL-17 in *Aspergillus* infection and low levels of IL-17 in serum and BAL fluid of haematology patients with IA (46). Immune response in the human haemato-oncology setting is further discussed in Section 5.2.

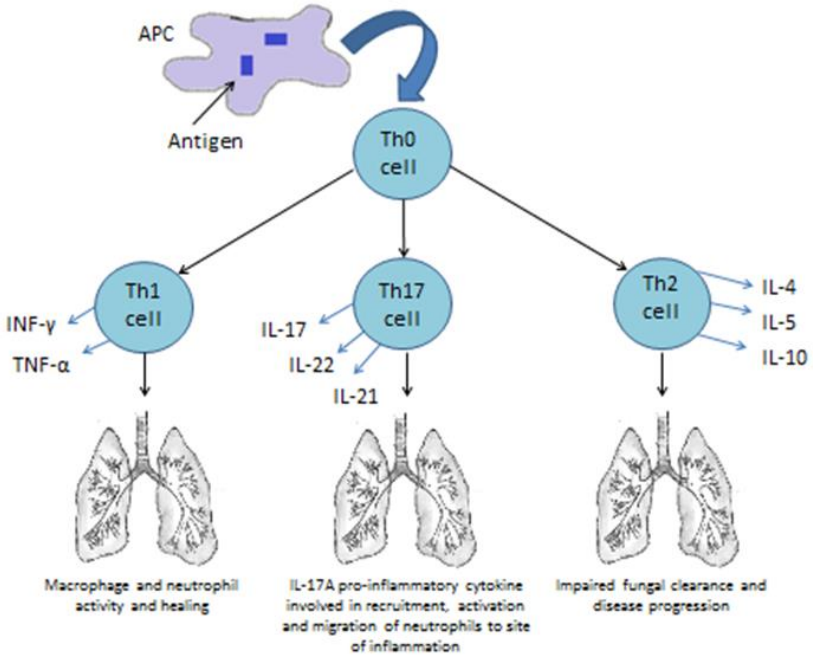



**Figure 4. Activation of the TH1, Th2 and Th17 CD4 T-cell responses in the lung**

APC = antigen presenting cell.

## 1.5 Clinical Diagnosis of IA

### *1.5.1 The haemato-oncology setting*

Early diagnosis of IA is important, as early, appropriate treatment has been shown to be a prognostic indicator. With early diagnosis, treatment may be initiated before the infection becomes irreversible (47, 48). Patients with IA suffer considerable morbidity (49) and often the management of the underlying malignancy is compromised, as treatment is delayed or cannot be given at all due to the high risk of relapse during subsequent therapy. IA diagnosis remains a challenge as the clinical manifestations are ambiguous. The most frequent clinical manifestation in this setting is invasive pulmonary aspergillosis (IPA) and the most frequent scenario is that of a neutropenic patient with non-specific symptoms, such as persistent fever, which is unresponsive to broad-spectrum antibiotics. Patients may also present with cough and sputum production, pleuritic chest pain due to vascular invasion leading to small pulmonary infarcts, upper respiratory tract symptoms and/or evidence of sinusitis. High-risk patients already have a number of co-morbidities and complicated drug treatment regimes. Current diagnostic tools are described in the literature with widely varying sensitivities and specificities (as discussed in section 1.6), with many requiring highly skilled operators and facilities that are not routinely available in many centres. To further complicate diagnosis, the diagnostic gold standards of a histological demonstration of IFD and/or recovery of the causative agent in culture from a normally sterile site are rarely feasible *ante-mortem* as patients are too unwell to undergo invasive procedures.

Due to this diagnostic uncertainty many patients are unnecessarily treated for presumed IA. This exposes them to associated drug toxicities and has major cost implications, particularly when antifungal therapy is usually initiated empirically, based upon fever and abnormal findings on chest computerised tomography (CT).

### *1.5.2 EORTC/MSG guidelines*

The European Organisation for Research and Treatment of Cancer and Mycoses Study Group introduced the EORTC/MSG guidelines, a set of consensus criteria, in 2002 (50) and published a revised version in 2008 (51). These were intended for use in clinical and epidemiological research and are used as a tool for the classification of IFD in immunocompromised patients. However, the guidelines act as a tool to standardise clinical research and were not intended to help with patient management. These criteria are used to classify potential cases according to the likelihood of underlying IFD into possible, probable and proven. In the original 2002 guidelines, 'proven' cases require histological evidence or isolation of the suspected etiological agent in culture from a normally sterile site, namely tissue. 'Possible' and 'probable' classifications are based on a combination of host, clinical and microbiological criteria, with the clinical criteria sub-divided into 'major' (i.e. specific radiological changes) and 'minor' (mostly clinical symptoms and signs).

The aim of the 2008 revisions was to improve specificity, partly by removing the sub-criterion of 'minor clinical' and restricting the clinical criteria for fungal lower respiratory tract infections to very specific CT scan findings (51). In addition, the classification of 'possible' now required a combination of host and clinical factors, with microbiological factors no longer scoring in the absence of clinical factors. This is a significant change which downgraded the importance of microbiology in defining likelihood of infection. Our own data (at Barts Health NHS Trust) highlighted the impact of this change, with a 75% reduction of cases previously classified as 'probable' IA, all downgraded to non-classifiable (52). This was due to the combination of host and microbiological factors no longer being sufficient factors in the establishment of possible infection. This change leads to difficulties

in interpreting previous studies where the 2002 definitions were used to evaluate the performance of microbiological tests. The limitations of the EORTC/MSG criteria in clinical studies are discussed further in section 1.9.

### *1.5.3 Antifungal patient management*

In terms of patient management the most suitable strategy is heavily dependent on the prevalence and epidemiology in the clinical setting, and the availability and turn-around time of suitable diagnostic tests. There are three approaches to prophylaxis: (i) no prophylaxis; (ii) mould-inactive prophylaxis (i.e. fluconazole) and (iii) mould-active prophylaxis (e.g. itraconazole, voriconazole, posaconazole), as shown in Figure 5 (53). An empiric approach to management remains the standard in many centres. Using this approach, antifungal treatment is commenced when there are clinical signs and symptoms of invasive mould disease, but in the absence of identification of a fungal pathogen, whilst carrying out investigations to confirm or exclude. There are varied definitions of 'signs and symptoms of invasive mould disease', but a recent suggestion by a working group defines it as a high risk patient who has persistent or relapsing fever refractory to broad spectrum antibiotics, without an identified cause for 72 hours or more (54). With persistent fever as a trigger for this management strategy, it is important to consider that fever may be absent if corticosteroids are administered. Empiric treatment is likely to lead to the treatment of patients who do not have IFD, as demonstrated by a retrospective study carried out at St Bartholomew's hospital in London, showing that 44% of high risk haemato-oncology patients received antifungal treatment for suspected IFD, whilst the EORTC/MSG revised criteria identified less than 5% of this cohort as probable/proven disease.

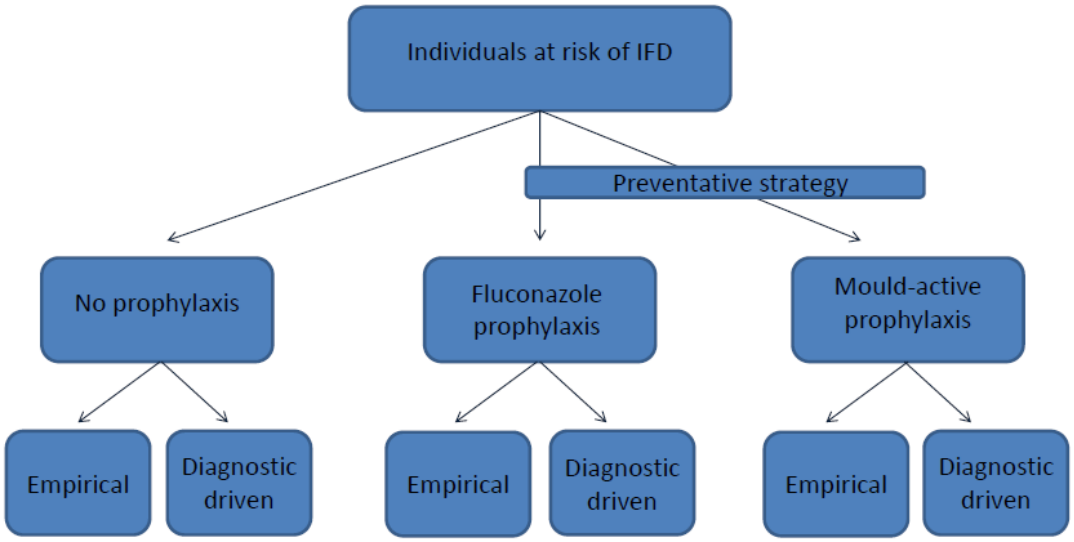



**Figure 5. Antifungal strategies for patients at risk of invasive fungal disease.**

Adapted from Agrawal *et al* (53).

In a diagnostic driven approach, biomarker detection and imaging techniques are combined to direct therapy and aim for a rapid assessment of the likelihood of IFD. The success of such an approach is heavily reliant on the early availability of biomarker assays and imaging services, and its efficacy has not yet been established. This is further complicated by the use of a sub-optimal reference standard in the evaluation of assay performance, as discussed in sections 1.5.2 and 1.9. Ultimately, to establish a diagnosis and identify the infective organism would be the optimal management strategy.

## 1.6 Laboratory-based diagnostic options

### *1.6.1 Traditional Techniques*

Histological examination of the infected tissue remains the "gold standard" for diagnosis, and is a requirement for a "proven" case, by EORTC/MSG definitions. However, obtaining tissue may be impractical due to the risks associated with the underlying disease.

Laboratory identification of *Aspergillus* species has long been based on morphology. Phenotype-based diagnostic criteria include the shape, size, colour and ornamentation of asexual or sexual structures. However, such characteristics are unstable and clinical isolates are sometimes atypical, with slow sporulation and aberrant conidiophore formation. A Gram's stain or Grocott's methenamine silver stain may be carried out to identify any fungal elements. Cells from bronchial washings can be concentrated by centrifugation and processed with 10% potassium hydroxide, Parker ink or calcofluor white mounts for examination (55). However, microscopy offers a low sensitivity and fungal elements cannot be identified to the species level. Structures can only be seen if they are present in abundance, usually at a later stage of infection (56). Molecular identification based on the sequencing of DNA targets has shown that classification by phenotypic characteristics is not always reliable, especially for less common species (57). DNA sequence variation in a number of genes, including the universal ribosomal DNA regions ITS and the large ribosomal subunit D1-D2, have been used to arrange fungi into molecular phylogeny groups. This has led to the identification of new fungal species that are morphologically similar to *A. fumigatus*, but are genetically distinct (58). Such species could easily be misidentified by clinical laboratories (59), which can have severe consequences as they include human pathogens such as *A. lentulus and N. pseudofischeri* (60, 61), that have been reported to be resistant *in vitro* to certain antifungal agents (62). Therefore, although direct visualisation is simple and rapid approach, identification of *Aspergillus* species by microscopy alone may be misleading. Confirmation by culture is desirable and often essential for definitive identification of the pathogen.

Culture of *Aspergillus* from an infected sterile site also provides definitive proof of disease and allows species determination and susceptibility testing, at a relatively low cost. However, these are rarely obtained in practice, due to inaccessibility of the lesion and the

underlying condition of the patient. Also, culture has the disadvantages of being a slow technique (it takes days) and being relatively insensitive (63). The failure to culture from a histopathologically confirmed case of IA may be due to the non-viability of the organism, as it is important to remember that antifungal treatment is often started empirically. Culture from a non-sterile site e.g. sputum and broncho-alveolar lavage (BAL) fluid, must be interpreted with caution, due to the ubiquitous nature of fungal spores. However, the isolation of *Aspergillus* species from a sputum or BAL sample is highly predictive of IPA in neutropenic patients (64, 65).

### *1.6.2 Imaging*

As IA in severely immunocompromised people will typically involve the lung, a chest x-ray (CXR) may reveal nodules, consolidation, or non-specific infiltrates. However, CXR often shows no abnormalities. CT scanning of the chest is more sensitive than CXR and is the preferred radiological method, as it may detect lung changes in the early stages of infection (66, 67) . Chest CT interpretation is pivotal to assigning a likelihood of IPA using the EORTC/MSG guidelines. Indeed, a key change between the EORTC/MSG 2002 and 2008 criteria was the elimination of minor clinical criteria and emphasis placed on specific CT findings.

Typical radiological signs of IA include the “halo” sign, which is a pulmonary nodule or mass surrounded by a zone of ground glass attenuation (67, 68). Although the halo sign is not specific to fungal infection, pulmonary aspergillosis is the most common cause of the halo sign in populations at high risk of fungal infection. Therefore in the appropriate clinical setting it is considered as early evidence of pulmonary aspergillosis (66). However, the presence of the halo sign is short-lived and early scanning is vital (69).

The air crescent sign occurs when air fills the space between devitalised tissue and surrounding parenchyma and is recognised as a crescent-shaped area of radiolucency within a parenchymal consolidation. The air crescent sign is not specific to *Aspergillus* infection, but is highly suggestive of pulmonary aspergillosis in the appropriate clinical setting. These lesions are considered to be characteristic of later-stage disease, marking the recovery phase of the infection (70).

The specificity of radiological features for IPA diagnosis has been assessed in patients with haematologic malignancy. A retrospective review demonstrated that radiological detection of at least 1 lesion with imaging suggestive of aspergillosis had 90% positive predictive value for IPA (71).

Smaller nodules (<1 cm), ground-glass opacities, and consolidation are non-specific features and do not necessarily suggest IPA. However, following the 2008 revisions to the EORTC/MSG guidelines, there is increasing evidence to support extending the radiological suspicion of IPA to less specific chest CT scan findings when supported by microbiological evidence in high-risk haematological patients (72).

Pulmonary macronodules, the halo sign, and the air-crescent sign have been best studied extensively in patients with IPA and haematological malignancy or stem cell transplantation (73, 74). Radiological features are not as well characterised in other settings with IPA (75, 76).

### *1.6.3 Serological assays*

A key trait of *Aspergillus* pathogenicity is angioinvasion. In the immunocompromised host this can lead to dissemination to other organs of the body (77). Angioinvasion results in the appearance of circulating surrogate markers in the blood, which can be tracked using

immunological methods. This has led to development of non-culture based methods using monoclonal antibodies (MAbs) to detect antigens that circulate in the body (78). Blood is usually tested, due to the minimally invasive sampling method and the potential to collect large volumes on a regular basis. However, most of the reported antigen detection tests have lacked the required sensitivity and specificity in the clinical setting (79). The serological detection of an IA marker has been most extensively investigated using the *Aspergillus* cell wall polysaccharide galactomannan.

#### 1.6.3.1 Galactomannan

Galactomannan (GM) is a polysaccharide component of the cell wall of *Aspergillus* and *Penicillium* species (80-82). GM antigen is released into the blood during hyphal growth. A rat MAb has been targeted to the galactofuranose moieties of GM and used to commercially develop two assays; the Pastorex kit (Sanofi Diagnostics Pasteur, Mames-La-Coquette, France) and the immunoenzymatic sandwich microplate Platelia ELISA (BioRad, Mames-La-Coquette, France). The Pastorex kit is now rarely used, whilst the Platelia ELISA is FDA approved and CE marked for both serum and BAL fluid samples, and recommended by the EORTC/MSG as one of the mycological criteria for IA diagnosis (51). The assay uses the EB-A2 MAb, directed towards the B(1,5)-linked galactofuranoside side-chain residues of the GM molecule, with 4 or more epitopes required for the antibody to bind (83).

Reported sensitivity and specificity values for the Platelia ELISA have been variable and it is clear that this is in part due to the variation in cut-off for assigning a positive result, patient population, relative prevalence of IA in the studied population, number of consecutive positive results required to confirm positivity and the inclusion of different combinations of proven/probable/possible cases in the assessment of assay performance. Maertens *et al*

(84) reported impressive sensitivity and specificity values of 92.6% and 95.4% respectively, when performing twice weekly screening of patients with haematological malignancy. Importantly, a cutoff of GM index 1.0 was used, which is higher than the recommended serum cutoff of 0.5, which is widely accepted in Europe and approved by the FDA. Also, 2 consecutive positive results were required to confirm positivity, as recommended by the kit manufacturer. These performance figures were taken from an analysis of 'proven' IA cases only, by histology. The authors state that if they included their 'probable'IA cases (similar to EORTC scoring criteria, but predating the criteria), which can be regarded as essentially unverified 'true' cases, then assay sensitivity would reduce to 82%.

Pazos *et al* (85) reported similar performance in patients with haematological malignancy, using a cut-off of 1.5. Though very limited in sample numbers (5 proven and 3 probable by EORTC criteria), they reported sensitivity and specificity of 100% and 97% respectively in proven cases and 88% and 98% respectively when considering both 'proven' and 'probable' as true cases.

In a large study by Herbrecht *et al* (86), again in patients with haematological malignancy, the results from serum assays were not as promising, with reported assay sensitivity of only 65% in proven cases. However, the threshold of positivity was set at 1.5, which is very high for a serum sample and may explain why some of the false negative results occurred. The authors go on to suggest a reduction in the cut-off in their discussion section. The authors also report a negative predictive value of 100% in patients undergoing HSCT. Results for patients with probable IFD (rather than proven) are less encouraging in this trial; with reported assay sensitivity of 16%. Similarly inadequate results have been reported by others (87, 88). Pinel *et al* report a very low positive predictive value (PPV), though it is important to note that reported IA prevalence is also very low. Maertens *et al* (84) report very high PPV and high prevalance. This is to be expected, as PPV has been shown to

increase with prevalence for both proven and probable cases. Reported performance is better in the haematological malignancy and bone marrow transplant (BMT) populations than solid organ transplant (SOT) populations, as reflected in a recent meta-analysis (89), in which the sensitivity of serum GM testing for patients with haematological malignancy was 70%, in BMT recipients was 82% and in SOT recipients was only 22%.

In addition, there are many reports that challenge the specificity of the GM-ELISA: it detects a soluble antigen produced during infection with *Geotrichum capitatum*, with no evidence of aspergillosis (90), reacts with *Candida* species (91), cross-reacts with other opportunistic fungi in some other fungal infections such as histoplasmosis (92) and may react with antigens from *Cryptococcus neoformans* in patients with cryptococcosis (93). There have also been consistent and continuous reports of false positive results having been obtained from patients receiving β-lactam antibiotics (94-97). There is some evidence that this particular problem is caused by manufacturing complications, possibly the presence of cross-reacting antigens from *Penicillium* (98). Fortunately, the most recent evidence suggests that these difficulties have been largely resolved (99) and the clinical problem can be minimised if batches are tested before use and/or if samples are collected prior to infusion (100). Further studies into the usefulness and application of GM testing may help to reduce the clinical uncertainty. Application to BAL fluid certainly appears very promising as a diagnostic tool, as discussed below.

#### 1.6.3.2 (1-3)-β-D-glucan

(1-3)-β-D-glucan (BG) is a component of the cell wall of most fungi, with the exceptions of *Cryptococcus* and *Zygomycetes (101)*. Assays make use of the ability of BG to activate a cascading series of serine proteases in the Limulus amoebocyte lysate coagulation cascade.

The presence of BG is detected either by a turbidimetric assay or via cleavage of a synthetic chromogenic substrate.

Fungitec-G was the original BG assay, made by Seikagaku Biobusiness (Tokyo, Japan). Performance of this assay in the diagnosis of IFD was assessed by Obayashi *et al* (102). Sensitivity and specificity of 90% and 100% respectively, were reported in cases of proven fungal and non-fungal infection; demonstrating serum testing as an effective approach to IFD diagnosis.

In another trial, published the same year, the assay performance was assessed in immunodeficient mycosis patients with Candidemia, Aspergillosis and Cryptococcosis (103). They reported BG levels in healthy volunteers to be <10pg/ml, with significant elevation observed patients with Candidemia (>300pg/ml) and IA (>25pg/ml, rising rapidly over time). However, little or no rise was seen in patients with Cryptococcosis (<17pg/ml).

Performance of the Fungitell assay (formally called Glucatell, Associates of Cape Cod, USA) as a diagnostic tool for IFD was evaluated by Odabasi *et al* (101). This assay differs from the Fungitec-G assay in that it is derived from a different species of horseshoe crab. Measurement from healthy volunteers and non-neutropenic patients with Candidemia was used to select a cut-off of ≥60pg/ml in the Fungitell assay, as opposed to the cut-off of >20pg/ml previously assigned to the Fungitec-G assay (102). In a test panel of 30 healthy volunteers and 30 patients with proven Candidemia, sensitivity and specificity values of 97% and 93% respectively, were calculated. In the same report, the authors present results from serum BG testing in a trial involving 283 neutropenic adults undergoing chemotherapy. Using a cut-off of 60pg/ml and requiring ≥1 positive serum sample, an NPV of 100% was reported when looking at the EORTC proven and probable cases. Specificity was reported as 90% when a single positive result was required, 96% when 2 sequential

positive results were required and 99% when 3 were required. All patients were receiving antifungal prophylaxis.

There is a paucity of data addressing the clinical sensitivity of the BG assay when looking specifically for *Aspergillus* species. Kami *et al* (104) reported performance figures from BG plasma testing in 16 cases of proven IA (host evidence of tissue invasion and culture positive sputum/lung), 14 suspected IA (CT changes and persistent fever/histopathological evidence of IA) and 185 cases of no IA. The Fungitec-G assay was used for once weekly plasma screening, with a single positive result required. Patient-based analysis gave sensitivity and specificity of 63% and 76% respectively. However, it is noted that sensitivity was 88% in cases of disseminated IA and only 38% in cases of localised IA (lung only). This suggests that blood screening is not suitable for early diagnosis of IPA. In sample-based analysis, BG assay sensitivity was only 16%, which emphasises the need for repeated blood sampling for diagnosis.

Pazos *et al* (105) reported performance figures from BG serum testing in 40 neutropenic patients. The Fungitell assay was used for twice weekly serum screening, with a single positive result required and a cut-off of 120pg/ml. When considering true positives to be both EORTC/MSG proven and probable cases and true negatives to be those with no evidence of IFD, sensitivity and specificity were reported as 87.5% and 89.6% respectively, with a false positive rate of 10.3% (3 positive results from 29 cases with no evidence of IFD). When looking at the five proven IA cases only, sensitivity was 100%. In the three probable cases, sensitivity was 66%. The use of the BG assay as an adjunct in the diagnosis and management of IA is suggested by recent data that showed a sensitivity and specificity of 63 and 93%, respectively, in patients with acute leukaemia using two consecutive samples with a cut-off value of 7pg/ml (106). The test gave similar results for IA and candidiasis, although the time interval between onset of fever and BG result was shorter

for IA. Positive BG test results were obtained before the results of any other conventional diagnostic method, including cultures, histopathology or radiological criteria, which may make it useful for early evaluation and pre-emptive initiation of appropriate antifungal treatment. A recent meta-analysis of 16 studies concluded that serum BG measurement has a good diagnostic accuracy for IFD diagnosed in accordance with the EORTC/MSG criteria, with a pooled sensitivity of 76.8% and a specificity of 85.3% (107). However, there was marked statistical heterogeneity among the studies and significant technical differences between the studies, extending to the method of measurement, the type of BG used as standard, the pre-treatment method and the cut-off levels. Similar results were reported by another meta-analysis that included 17 studies where IA was specifically targeted as a subgroup for analysis; here sensitivity and specificity were 77 and 83%, respectively, and heterogeneity was equally significant (108). In addition, the timing and frequency of BG testing for patients at risk has not been standardised, and neither have the criteria for defining a positive test result. A study focusing on routine use of BG for panfungal screening of IFD in patients with haematological malignancy revealed limited usefulness of this test in such a setting, with low sensitivity combined with an extremely low positive-predictive value (11.8%) being the major limitations (109). There are other factors that could generate a positive BG result. False positive BG results have been documented in haemodialysis (110) and exposure to glucan-containing gauze such as used in major surgery (111), as well as in patients receiving intravenous immunoglobulin (112). Therefore, results should always be interpreted in the context of the clinical procedure. This is particularly important because BG detection lacks mycological specificity for IA diagnosis. However, it could be used to exclude IA in some scenarios and increase certainty of IA when GM is positive (105).

### *1.6.4 Real time PCR*

Real-time quantitative PCR (qPCR) is firmly established as the method of choice for the detection of pathogen-derived nucleic acids in routine clinical diagnostics (113). Several features make qPCR well suited to the clinical environment:

- Speed: assay reaction times are typically measured in tens of minutes
- Convenience: the homogenous assay format obviates the need for post-amplification processing
- Simplicity: the assay requires two primers, an optional probe and a single enzyme
- Sensitivity: single copy targets can be detected, if not quantified
- Specificity: a well-designed assay is specific for a single target, but mismatch-tolerant assays are easily designed
- Robustness: a well-designed assay will yield results across a wide range of reaction conditions
- High throughput: thousands of reactions can be carried out on a single run
- Quantification: the dynamic range is typically huge (eight to nine orders of magnitude)
- Familiarity: PCR has been around for many years and its advantages and disadvantages are well understood
- Cost: assay reagents are inexpensive; together with the trend towards smaller reaction volumes the costs per assay are low.

There are three main drawbacks to qPCR assays: (i) their sensitivity to environmental inhibitors that are concentrated along with pathogens during sample processing can lead to false-negative results – this is important in fungal qPCR where large volumes of starting material are often tested, increasing the opportunity for concentration of inhibitors, as highlighted by the European *Aspergillus* PCR Initiative (EAPCRI) recommendations to extract from at least 3 ml of whole blood and elute DNA into less than 100 µl (114), (ii) assays determine only total pathogen number and do not provide information about whether a pathogen has the ability to establish an infection or not and (iii) variable assay conditions and ill-defined assay designs can generate significant inter-laboratory variation, leading to unreliable and often contradictory results obtained from the same samples.

PCR has been extensively used as an aid in the diagnosis of IA (115). However, a lack of technical standardisation and poor understanding of the kinetics of *in vivo* fungal DNA release has resulted in the development of numerous assays amplifying diverse target regions and reporting a wide range of PCR sensitivities and specificities (114). A recent systematic review of *Aspergillus* PCR methods using whole blood samples (116) highlighted the variation in methods across 16 studies that included three extraction and four disruption methods, three different starting volumes, three different specimen types, three different target genes and four different PCR methods. Only four qPCR assays were included in this review and there was sufficient information to determine PCR efficiency for only two – both suboptimal at 91.6% and 86%. This, together with the inadequate reliability of amplification from blood and serum has led to attempts to reach a consensus on PCR diagnosis. This was pioneered by EAPCRI, which aims to standardise *Aspergillus* PCR (114). The authors have thus far focused on the extraction procedure, which they suggest is the limiting step in achieving reliable detection of *Aspergillus*. However, closer examination of their data obtained by analysing the results from their PCR performance

panels reported by various laboratories leads to a different conclusion: the amplification results obtained using the range of assays tested are highly variable. Excluding one assay that gives no result at all, the detection threshold in different centres ranges from 0.27 to 270 copies of target/ml, a considerable 1,000-fold difference that can easily account for the difference between a positive and a false-negative result. This analysis demonstrates that there is a maximum difference of 100-fold when the same PCR strategy is carried out in different centres, readily explaining the variability commented on by the authors of the meta-analyses (117). The data also confirm that assay performance is greatly influenced by the amplification platform used, as previously identified by a UK and Ireland multi-centre study of PCR methods for *Aspergillus* species (118). Consequently, far from leading to the conclusion made by the EAPCRI, the appropriate conclusion is that the PCR assays are highly variable, with different PCR efficiencies resulting in significantly different sensitivities.

With such a lack of technical standardisation, it is not surprising that the EORTC/MSG do not endorse the routine use of PCR in the diagnosis of IA (50). There are several additional reasons for this, which are discussed in depth in Chapter 2, where the quality of broad spectrum *Aspergillus* qPCR assays published between December 2000 and December 2011 is reviewed.

### *1.6.5 Lateral flow device (LFD)*

The use of hybriboma technology recently led to the generation of the *Aspergillus*-specific MAb JF5 and the development of an immuno-chromatographic lateral flow device (LFD) for point of care diagnosis of IA (119). Importantly, this MAb targets an extracellular glycoprotein that is only released from the tip of actively growing *Aspergillus* hyphae and so

does not detect quiescent or moribund spores. Cross-reactivity is seen with some closely related *Penicillium* and *Paecilomyces* species, but not with clinically relevant *Candida albicans, Cryptococcus neoformans, Fusarium solani or Rhizopus oryzae* IA (119). The LFD has been evaluated in a guinea pig model of IA and became positive earlier than GM and BG assays (120). However, this study did not take place in the presence of antifungal therapy, which may reduce assay sensitivity. The same group addressed the influence of antifungal therapy on the performance of the LFD (121) in a more recent report. They found reduced serum sensitivity in animals treated with antifungals. However, marker levels remained elevated in BAL fluid samples from the same animals. The LFD was tested in a small number of BAL and serum samples from patients with haematological malignancies (122). Strong agreement was reported between the LFD, an in-house qPCR and GM testing in BAL fluid. Hoenigl *et al* evaluated the LFD using 39 BAL specimens for IPA diagnosis in 37 haematological malignancy and SOT patients (123). All 12 EORTC probable patients had LFD positive BAL samples. All 18 patients that did not have evidence of IFD (scored 'No') had LFD negative BAL samples, giving a sensitivity and NPV of 100%. Reported assay specificity was 81%, due to LFD positive BAL samples from 5 patients scored as possible. However, these samples are not necessarily 'false positive' LFD results. It is worth noting that in 3 of the 5 samples, GM Index was 0.6-0.7. Although this falls below the cut-off recommended in the literature (124, 125), making the results negative, this index value is much higher than the value in LFD negative cases. All 5 of these patients had received antifungal treatment at the time of BAL sampling, which could explain GM negative results. It is also worth noting that *A. fumigatus* growth was reported in 4 of the 10 BAL cultures from SOT patients, but only in 1 of 27 BAL cultures from haematological malignancy patients. This culture negativity is typical of the haematological malignancy

setting, in which early, empirical systemic antifungal use is common, further highlighting the difficulties in diagnosis in this clinical setting.

The LFD appears to be a very promising new technology in the targeting of surrogate biomarkers of IA. It is a rapid, easy to use device, but is limited by its subjective interpretation. Also, the test has recently undergone optimisation for serum testing, in terms of the pre-treatment needed to remove serum proteins that can affect the reliability of the result. The performance of the LFD in serum samples has been evaluated from EORTC/MSG proven/probable versus no-IPA cases in a haematological population (126). LFD sensitivity and specificity were 81.8% and 84.8% respectively when a single positive result was required to assign patient positivity and 59.1% and 98.0% respectively when 2 or more positive results were required to assign positivity. In another study, the performance of serum LFD testing was evaluated in patients during and after allogeneic HSCT, with comparable specificity values of 86.8% (single positive required) and 97.8% (two or more positives required) (127). Reported sensitivity was much lower, at 40% (single positive required) and 20% (two or more positives required).

The LFD test may prove very useful in combination with detection of another marker, for example molecular detection. It is worth further investigation, to look at the level of concordance between different assays.

## 1.7 Selection of clinical specimens

Blood sampling allows non-invasive serial monitoring of biomarkers of IA. Angioinvasion makes it possible to detect *Aspergillus* in the bloodstream, though little is known about the exact form of the fungal components present in the blood, when they appear nor their persistence or clearance. The GM assay has been most extensively tested using serum,

with variable performance reported, as already discussed in section 1.6.3.1. Although not validated for plasma testing, some studies have evaluated performance in these samples. White *et al* found comparable results when performing serum and plasma testing in the haematology population (128). The authors found that index values tended to be slightly higher in plasma than serum from proven/probable/possible cases, but this didn't reach statistical significance.

Due to the possibility of non-invasive serial sampling for monitoring, blood has been the focus of detection of *Aspergillus* DNA for many studies. There has been extensive discussion as to the nature of positive PCR results in blood specimens. Viable fungi are extremely rare, as evidenced by poor blood culture results (129). Nonviable *Aspergillus* cells may be present inside phagocytic cells though many patients will be profoundly neutropenic. It is not clear whether free *Aspergillus* DNA might circulate in blood, but it has been shown that *in vitro Aspergillus* DNA is stable in serum (130). EAPCRI have published protocols for DNA extraction from serum (131) and whole blood (132). One important consideration is that these protocols were developed and validated using blood spiked with *Aspergillus* conidia. DNA extraction from conidia, combined with the presence of a complex, sturdy cell wall, requires extensive and harsh extraction methods that can damage DNA and RNA to the extent that amplification becomes unreliable (133). Arguably, such harsh extraction steps are not required *in vivo* since *Aspergillus* present in blood is highly unlikely to be conidial as *Aspergillus* rarely sporulates *in vivo* (134).

Marker detection in blood, serum and BAL fluid has been investigated in a guinea pig model of IA (135). The authors found BAL fluid to be the most suitable sample for early diagnosis of IPA by day 3 (post conidia inhalation) using PCR, GM and BG assays. Marker levels remained elevated throughout the study. Despite high fungal burden within tissue and BAL samples, it was found that serum and whole blood may remain negative with all 3 assays,

even at an advanced stage of disease. This study, however, did not include any antifungal treatment, which may interfere with marker detection in the clinical setting. Another study looked at LFD, GM and BG testing in the guinea pig model with antifungal treatment (121). The authors found that serum marker levels were significantly reduced in the presence of antifungal treatment, whilst all marker levels remained elevated in BAL fluid.

The BioRad Platelia GM assay has recently been validated for BAL fluid testing. As with serum testing, reported performance is variable due to inconsistency in assay cut-off value, inadequate negative controls and lack of standardisation of BAL fluid collection; such as the volume of saline instilled. However, GM testing from BAL fluid has been reported to be more sensitive and specific than serum GM testing in immunocompromised patients (125, 136, 137) with significantly higher index values suggesting much greater fungal load in these respiratory samples than in the blood. A combination of PCR, PCR-ELISA and GM testing in the BAL fluid of patients with haematological malignancies was shown to enhance routine laboratory diagnosis of IA (138). This is probably not surprising considering that IA is primarily a pulmonary infection.

Detection of *Aspergillus* markers from BAL fluid samples represents one of three scenarios: (1) current disease, (2) true colonisation, or (3) a marker for future development of invasive disease. Whilst the detection of *Aspergillus* from respiratory samples does not prove IA, the specificity of *Aspergillus* isolation from the respiratory tract in immunocompromised patients – such as those with neutropenia—is very high (64).

*Aspergillus*-PCR positive BAL fluids have higher human DNA levels (evaluated by human beta-globin gene DNA quantification) than *Aspergillus*-negative BAL fluids (139). This may reflect increased cellular content and perhaps reflects the presence of phagocytosed conidia and hyphae in macrophages and neutrophils.

## 1.8 Novel body fluid target: Exhaled breath condensate

Use of breath analysis in the aiding of clinical diagnosis is not a new idea. It has long been known that a pungent, fruity smell on a patient's breath, caused by acetone, is a warning sign for diabetes. Exhaled breath condensate (EBC) was first reported as a human bodily fluid in 1980 (140).

Exhaled breath contains mostly water vapour, but also water-soluble volatile gases and aerosolised non-volatile particles, including ions and proteins (141). There is much discussion about the origin of non-volatile markers in EBC; they have a cellular source, but is it the airways or the alveoli? Flow dependence may indicate whether a marker originates from the airways or alveoli (142), with the marker concentration dependence on expiratory rate suggesting that it is mainly derived from the airway and lack of flow dependence suggesting the alveolar region as the source of the marker. Droplets of epithelial lining fluid (ELF), which are the source of non-volatiles in EBC, could be formed in the airways, upper respiratory tract or upper gastrointestinal tract. However, they are most likely to be formed in the airways, as this is where air turbulence is greatest.

The technology to collect and cool exhaled air in a condenser system is now available, with several custom collection techniques and commercially available kits reported in the literature. The EcoScreen I (Viasys, USA, Europe) device has a mouth piece and one-way air valve, to prevent contamination with saliva. When the patient breathes into the mouthpiece, the valve block separates inspiratory and expiratory air. This valve block is connected to the collecting system, which consists of a condenser and a collection vial. The collecting system is inside a cooling cuff, which is kept cold by a refrigerator. The water vapour in breath condenses and drops into the vial. The EcoScreen II allows the

measurement of respiratory parameters during sample collection. This system is well described in the literature, but is very large and so lacks portability and requires thorough cleaning between uses.

The R-Tube (Respiratory Research, USA) is small, lightweight and portable. It also has a disposable polypropylene collecting system, so cleaning is not required between uses. The exhalation valve serves as a syringe-style plunger to enable the user to pull the fluid from inside the condenser wall. This device does not require a power supply. An aluminium cooling sleeve is placed over the tube; however, since this sleeve will inevitably warm up during sample collection, sample collection temperature is neither constant nor known, though it could be addressed. Although the portability and ease-of-use make this device suitable for collection in the home, the sample still needs to be brought to the laboratory for testing.

There is evidence to suggest that the choice of collection device influences the type of molecules detected. Rosias *et al* (143) hypothesised that the adhesive properties of different condenser coatings would interfere with the measurement of icosanoids and proteins. They found that the concentration of 8-isoprostane (an icosanoid) detected was significantly higher using a silicone coating on the inside of the condenser, compared to glass, aluminium, polypropylene or Teflon. A glass coating also resulted in detection of significantly higher concentration than using aluminium, polypropylene or Teflon. The group also found that detected albumin concentration was higher with silicone or glass than with the other coatings.

Liu *et al* (144) compared glass, siliconised glass, EcoScreen and R-Tube condensers for the collection and measure of nitrogen oxides, total protein, mucin and pH in healthy volunteers. Measured nitrogen oxide levels were significantly higher in EcoScreen than in

siliconised glass or R-Tube devices. However, nitrogen oxides can increase from the leaching of NO out of plastics (including Teflon). Indeed, the authors demonstrated $NO_x$ contribution from the EcoScreen device to aqueous media. This did not occur with a glass device. Total protein concentration was higher with EcoScreen than glass or R-Tube devices. There was, however, no significant difference in mucin levels or pH between the devices. It is important to note that $NO_x$ stability is associated with temperature. Using the R-Tube device, temperature gradually increases during sampling, and a constant temperature cannot be maintained. Therefore, this device may be better suited to the monitoring of markers that are not affected by temperature.

EBC biomarker investigations have been performed in the setting of inflammatory conditions and connective tissue disorders, including chronic obstructive pulmonary disease (COPD), adult respiratory distress syndrome (ARDS), asthma and pneumonia. Studies have revealed changes in patterns in NO, hydrogen peroxide ($H_2O_2$), cytokines and inflammatory proteins (145).

### *1.8.1 Volatile organic compounds (VOCs)*

There is much variability in the measurement of breath gas VOCs, due to the inability to standardise flow rate and volume of exhalation. These volatiles are variably in equilibrium with gaseous components. It is important to optimise the sampling procedure where possible. The American Thoracic Society/European Respiratory Society (ATS/ERS) created a task force to determine guidelines for the use of EBC. They highlighted the importance of the choice of condensing equipment, the breathing pattern during collection, the consumption of food and drink prior to sampling and the use of medication prior to sampling, as just a few of the variables that may affect the ability to detect desired target

biomarkers in EBC (145). It is vital that all of these variables are recorded and sampling techniques are made as consistent as possible.

The most studied volatile marker is exhaled NO and this is considered a good marker of airway inflammation (146). The study of lung inflammation currently involves invasive methods such as collection of BAL fluid and semi-invasive sputum induction. Plasma is also used for the measuring of inflammatory biomarkers, but these are likely to reflect systemic rather than lung inflammation. EBC collection would certainly be preferable to these invasive sampling methods, offering the potential for non-invasive and repeatable monitoring of airway biomarkers.

### *1.8.2 Non-volatiles*

Epithelial lining fluid is massively diluted by water vapour in an EBC sample. Dilution estimates have been made, but the assays used for assessment of dilution are themselves a source of variability. There is currently no gold standard EBC dilution marker, just as is the case in BAL sampling. However, ratios of non-volatile constituents, such as cytokines in the Th1 and Th2 profile, can be calculated and then deviation compared with the underlying condition.

There have been attempts within studies to standardise against an additional EBC component, such as total protein or urea content. Urea is not volatile and it is neither produced nor destroyed in significant amounts by intact lungs. It rapidly equilibrates across the barriers between blood and air spaces (147). Therefore, it is likely that the concentration of urea in plasma and ELF are similar. It has been attempted previously to use urea to measure the dilution of respiratory fluid in BAL sampling. However, urea

rapidly enters the instilled fluid during the lavage, so this is not a reliable marker. EBC collection, however, does not involve instilled fluid, so this is not a problem.

Detection of non-volatiles has thus far been at the lower limits of accuracy for most assays. When coupled with the unknown degree of variability in sampling and in assay performance, there is a definite need for the use of appropriate assay controls, with the use of a dilution marker or ratios of different biomarkers measured concurrently.

It is not always necessary to use a dilution marker or calculate the ratios of biomarkers, for example, when the result of the assay serves as an indicator of abnormality. Detection of rhinovirus RNA in a human respiratory sample by reverse transcription-PCR requires simply a yes/no answer. It is not necessary to calculate detection levels/concentrations. However, the assay must be reliable, with no false positivity.

### *1.8.3 Pathogen detection*

The reliable and early detection of pathogens in EBC would constitute a major diagnostic breakthrough. There have been attempts to look for volatiles to identify bacteria in EBC. But this is a complex area and the high degree of variability in the composition of human breath makes it hard to identify diagnostic biomarkers (148).

Detection of *Pseudomonas aeruginosa* has been attempted in cystic fibrosis (CF) patients (149), through the detection of 2-aminoacetophenone (2AA) in the breath of patients colonised with *P. aeruginosa*. However, it was found that environmental factors can greatly affect the reliability of the biomarker detection.

Chambers *et al* (150) published limited data on the detection of 2-Pentylfuran (2PF); a volatile produced by *Aspergillus* species, as well as *Fusarium* and *Scedosporium*. Using gas

chromatography with mass spectrometry (GC/MS) they demonstrated 2PF detection in the breath of 16/32 patients at risk of *Aspergillus* colonisation or infection (due to chronic lung disease, asthma, ABPA, CF, COPD or SOT). Results of the 2PF breath tests were compared with recurrent isolation of *Aspergillus* from sputum or BAL fluid over 2 months, with a sensitivity and specificity of 77% and 78%, respectively. 2PF was not detected in breath samples from any of the 14 healthy controls. The group report 2 cases of 2PF detection from the breath of severely immunocompromised patients with IA, with 2PF levels becoming undetectable following effective treatment for IA. Similarly, volatiles have been identified that are specific to *M. tuberculosis* and *M. bovis*. Further testing is needed for validation of the monitoring of these volatiles as a marker of disease.

Genomic DNA has been detected in EBC (151), with detection of the beta actin gene fragment reported in 65.7% of EBC samples. The authors found extracted DNA and neat EBC samples equally suited for detection. In another report (152) the human beta actin gene was detected in all DNA extracts from EBC samples collected by EcoScreen.

Attempts have been made to detect pathogen nucleic acids using qPCR. However, recent literature suggests that detection is at the lower limit of detection. Costa *et al* (153) used the R-Tube to collect EBC from patients with confirmed respiratory viruses. They extracted DNA from EBC samples, with an internal control (beta globin) added and found no correlation between BAL fluid virus levels and EBC detection.

St George *et al* (154) used qPCR to look for Influenza virus in EBC samples, in comparison with nasopharyngeal swabs. A single positive EBC was recorded, with very low viral load. This sample corresponded to a swab with the highest recorded viral load (lowest Cq), suggesting very low sensitivity of EBC sampling. Vogelberg *et al* (155) concluded that EBC is unsuitable for the detection of DNA from *P. aeruginosa*, and *Burkholderiacepacia* in CF

patients with sputum positive samples. The authors state that the qPCR used had a demonstrated detection limit of as low as 10fg DNA (in a dilution series of positive control DNA), but failed to detect pathogen DNA in EBC samples.

Fabian *et al* (156) reported low sensitivity when using qPCR to detect *Influenza A* or B from breath of infected patients. These reports together suggest that non-volatiles are massively diluted in EBC samples and enrichment from EBC is likely to be required to achieve a more reliable result.

With reported detection of nucleic acids in EBC thus far being at the lower limits of detection for most assays, together with the unknown degree of variability in sampling and the variability in assay performance, there is a definite need for standardisation where possible and the use of appropriate assay controls.

## 1.9 Clinical use of existing diagnostics – performance analysis

A major obstacle in the performance analysis of any of these tests is the wide variation of conditions in which they are used. It is clear that the wide variation in reported sensitivity and specificity values are in part due to the variation in patient population, relative prevalence, clinical specimen type tested, method of handling, pre-treating and storing clinical specimens, time delay in performing sampling (in relation to antifungal administration) and the number of consecutive positive results required to confirm positivity.

Another major factor in the performance analysis of diagnostic tests for IA is the rarity of confirmation by a 'gold standard'. In the absence of the diagnostic gold standard of a histological study and/or recovery of the etiologic agent in culture from a normally sterile site, diagnosis is heavily reliant on a strong degree of suspicion. How then are the

performances of new diagnostic tests/combinations of tests to be evaluated in the clinical setting? In reality, the EORTC/MSG criteria are often used as a reference standard, in order to evaluate the clinical performance of a test/combination of tests for IA. It is important to consider the errors can occur in classification by a reference standard. For example, important changes in the lungs can be missed due to the delay in obtaining a CT scan (69). Changes in a particular biomarker may be caused by the presence of an alternative condition in patients (157), or by drug treatment (94), leading to misclassification. When evaluating the performance of the GM assay there is obvious incorporation bias, as the test is part of the EORTC/MSG reference standard for assigning a 'probable' score, but this method of evaluation is still used (89). Review of the literature also highlights the variation in the use of the EORTC/MSG guidelines as a reference standard for evaluating assay performance. When evaluating the performance of new diagnostic tests, some studies use proven/probable/possible as true positive results and EORTC/MSG non-classifiable as true negatives. Other studies use proven/probable as true positives and EORTC/MSG possible/non-classifiable as true negatives. Whilst this provides a means to evaluate new tests and the performance of tests in parallel, it is far from ideal. Only EORTC/MSG proven cases fulfil the criteria for a definitive diagnosis of IA. Probable and possible cases are assigned as estimations of the likelihood of infection and are therefore not a definitive diagnosis. Likewise, an EORTC/MSG non-classifiable case is not a definitive true negative. It simply means that it doesn't fulfil the EORTC/MSG risk criteria for suspicion of an IFD. Consequently, a positive molecular biomarker result will be interpreted as a false positive result in an EORTC/MSG non-classifiable case, yet we cannot be sure that this is the clinical reality. It is also important to note that the modified 2008 EORTC/MSG criteria elevated the importance of specific CT scan findings, with microbiological factors no longer scoring in the absence of clinical factors. However, a negative CT scan of the chest in the presence

of positive biomarker results could be due to the timing of the scan itself, as previously discussed, and/or due to IA in another organ, such as the brain. A recent retrospective analysis carried out at St Bartholomew's Hospital in London revealed two 'proven' cases of IA in which no major clinical criteria were met (52), one of which was only discovered *post-mortem*. This highlights the limitations of the EORTC/MSG criteria as a reference standard for evaluating the performance of new tests for IA diagnosis.

### 1.9.1 Conclusions

Diagnosis of IA in the haemato-oncology setting presents a very unique combination of challenges. There are co-morbidities to consider, the likely presence of antimicrobials in the body that have the potential to affect biomarker detection, the need for minimally invasive sampling in very sick individuals or the rarity of definitive diagnosis and the absence of clearly-defined 'outcomes'. As a result, most patients receive antifungal treatment and will either recover from infection or die, without proof of whether they had an infection or not, let alone whether it was *Aspergillus*.

Whilst the EORTC/MSG criteria form the only reference standard available, they have limitations that require consideration. The development of new diagnostic tools may highlight new diagnostic possibilities or stages of disease that have not been previously perceived. This is likely to change the way in which patients are classified with respect to IA and influence decisions as to what patients are targeted for antifungal drug treatment.

It is unlikely that a single test could or would ever be used for the clinical diagnosis of IA in the high risk haemato-oncology setting. It makes sense, in this difficult clinical setting, to employ a combination of tests, looking for different markers of *Aspergillus*, to increase diagnostic certainty. For example, the diagnostic yield from BAL fluid for IPA is potentially

optimised with application of a combination of tests; as recently demonstrated in a study that reported improved sensitivity with the concomitant application of galactomannan and PCR (158). However, tests that have the potential to change our definition of a disease require more than consideration of their accuracy; they also require assessment of the clinical consequences of the changes that they bring (159).

### 1.9.2 Thesis Aims

The major aim of this study was to use novel approaches to make significant progress in the ability to detect IPA, allowing early and specific treatment of genuine fungal infection.

1. The first aim of this study was to develop, optimise and validate a robust and reliable quantitative pan-*Aspergillus* qPCR assay. To do this, a critical review of published *Aspergillus* qPCR assays was performed and assay limitations were highlighted. This information was used, together with strict adherence to published qPCR design and reporting guidelines, to design a new qPCR assay.

2. The second aim of this study was to develop and validate a combination biomarker detection approach in BAL fluid, for the early diagnosis of IPA. To do this, an existing GM enzyme immuno-assay and a newly-developed antibody-based test utilising an LFD were compared with the new *Aspergillus* qPCR assay to evaluate their effectiveness for the detection of *Aspergillus* from the BAL fluid of patients with IPA. Results were used to establish the optimal parameters for achieving a definitive and consistent diagnosis of IPA using clinical samples from an ongoing study, in comparison with EORTC/MSG criteria.

3. The third aim of this study was to determine whether respiratory samples are useful for identifying a distinct inflammatory marker profile in IPA. To do this, BAL fluid samples from

combination biomarker-positive and –negative patients were evaluated by fluorescence-activated cell sorting (FACS) analysis, to quantify selected immune cytokines.

4. The fourth aim of this study was to evaluate the potential of EBC as a pioneering sample for non-invasive diagnosis of lung pathogens by providing proof-of-principle for the reliable and rapid detection of *Aspergillus*. To do this, EBC clinical samples from an ongoing study were tested using an LFD/qPCR/GM combination biomarker detection approach and results compared to those obtained from respective BAL fluid samples.

5. The fifth and final aim of this study was to bring together antigen and real-time qPCR detection in a proximity ligation assay (PLA), to demonstrate proof-of-principle for a diagnostic assay for the earliest possible detection of fungal infections. To do this, the JF5 MAb and a polyclonal serum raised against the same target antigen were combined with qPCR to develop a new PLA application for the detection of an *Aspergillus*-specific N-linked glycoprotein antigen that is secreted constitutively at the hyphal apex in actively growing organisms.

# CHAPTER 2

# MATERIALS AND METHODS

## 2.1 Patient selection and sample collection

### *2.1.1 Barts Health NHS Trust Clinical specimens*

This study was a retrospective evaluation of biomarker performance for IPA diagnosis, with no impact on patient management. The Study cohort of patients were a subset of those recruited in an observational study of IPA diagnosis in the Division of Haemato-Oncology, St Bartholomew's Hospital, based on the availability of BAL fluid samples and full clinical data. 25 BAL fluid samples from 24 adults at high risk of IFD following intensive chemotherapy or allogeneic stem cell transplantation were obtained between August 2005 and February 2011. After 72 hours of persistent fever unresponsive to antibiotics, a CT scan of the chest was performed and, if abnormal, followed by a bronchoscopy and BAL. Two physicians independently scored each episode using the EORTC/MSG 2008 criteria (51).

#### 2.1.1.1 Ethics Statement

The work was ethically approved by the East London & the City Local Research Ethics Committee 1. Participants were recruited from Barts and the London. Study title: Early diagnosis of invasive Aspergillosis in a high risk group of patients using serum and bronchoalveolar lavage fluid real time PCR and galactomannan ELISA.

REC reference number: 05/Q0603/68.

Ethics amendment dated 28/10/2009.

Approval number ReDA : 003933 QM.

### 2.1.1.2 Patient Selection

This was a single centre, prospective, observational study of haemato-oncology patients at high risk of developing IA at Barts Health NHS Trust.

Inclusion criteria

- Age greater than 18
- Able to understand the implications of participation in the Trial and give written informed consent
- Patients with acute myeloid leukaemia (AML), myelodysplastic syndrome (MDS) and acute lymphoblastic leukaemia (ALL) undergoing intensive chemotherapy (predicted neutropenia of $<0.5 \times 10^9/L$ for > 10 days) and/or receiving high dose steroids
- Patients undergoing allogeneic HSCT
- Patients requiring high dose steroids for graft versus host disease (GVHD) post HSCT
- Patients with a history of probable or proven invasive aspergillosis and having chemotherapy, regardless of their underlying haematological malignancy

Exclusion criteria

- Inability to give informed consent
- Patients aged less than 18
- Pre-existing chest disease

### 2.1.1.3 Study Interventions

Study interventions began prior to the start of chemotherapy/immunosuppression and continued until recovery of the neutrophil count to > 1.0 x $10^9$/L. If fungal infection occurred, testing continued until discharge.

Clinicians responsible for daily care of patients included in the trial were not informed of the results from the marker detection package.

Study number and sample date were provided, without patient identifiers such as name or hospital number. Samples were analysed in batches.

A febrile episode is defined as any of the following:

- Temperature > 38°C for more than 4 hours
- Temperature > 38°C on two occasions > 4 hours apart within a 24 hour period
- Temperature > 38.5°C on one occasion

### 2.1.1.4 Sampling

#### *2.1.1.4.1 Bronchoscopy with BAL*

A bronchoscopy was performed in all patients in whom a HRCT chest was found to be abnormal, as per recommended clinical practice. Each patient was assessed immediately prior to the procedure. Fibre-optic bronchoscopy and BAL were performed in the lobe most affected radiologically by lesions suggestive of invasive fungal infection. BAL was performed using 3 x 60 ml aliquots of 0.9% normal saline. Each aliquot was injected and aspirated after 5 seconds. The fluid was examined by Gram stain, silver stain, bacterial and fungal cultures and viral PCR.

10ml of the fluid obtained was utilised in the study, aliquoted and stored at -80°C, to be analysed using the marker detection package. Inflammatory cytokines were also to be measured on this fluid.

### *2.1.1.4.2 Exhaled breath condensate*

Exhaled breath condensate (EBC) was collected from each patient at entry to the study and throughout the course of treatment as indicated, for example by an unexplained fever. Collection of EBC was achieved by patients breathing at normal frequency and tidal volume for 15 minutes through a mouthpiece and a two-way non-rebreathing valve (Respiratory Research, Charlottesville, VA, USA), which also traps saliva (Figure 6). The expired air was passed through a tube and cooled by an aluminium sleeve at –80°C that is completely enclosed by thermal insulation. Samples were aliquoted and stored at -80°C, to be assessed by the marker detection package.

The EBC was performed:

i) On entry into the study, prior to all treatment

ii) When the neutrophil count fell to < 0.5 x $10^9$/L or 24 hours after finishing chemotherapy (whichever is later)

iii) At the time of request for HRCT for suspected IFD,

iv) When the neutrophil count recovered to > 1 x $10^9$/L and, in case of IFI, at the time of discharge

**Figure 6. Collection of exhaled breath condensate.**

Collection is achieved using a condensation device designed to exclude gross salivary contamination. Patients sitting comfortably breathe at tidal volumes into a mouthpiece attached to a cold condenser for approximately 15 minutes.

### *2.1.1.4.3 Blood*

Twice weekly whole blood (collected in BD Vacutainer 4ml plastic whole blood tubes with spray-coated K2EDTA) and serum (collected in BD Vacutainer SST 4ml tubes with silica clot activator, polymer gel, silicone-coated interior) were collected, to be analysed using the marker detection package. Inflammatory cytokines were also to be measured on this fluid. Samples were aliquoted and stored at -80°C.

### *2.1.1.4.4 Histological material*

Histological material from three patients with haematological malignancies was provided by the Pathology department at the Royal London Hospital. These samples consisted of a post-mortem lung biopsy (sample A) and an antemortem brain biopsy (sample B), from two patients with proven IA and a control post-mortem lung biopsy with no evidence of fungal

infection (sample C). All three samples were provided as shavings, taken from paraffin wax embedded tissue blocks.

#### 2.1.1.5 Data from Other Clinical Investigations

Data from radiological (chest x-ray [CXR], HRCT, CT sinuses/brain) and microbiological investigations (cultures) were included in the study.

#### 2.1.1.6 Data Collection

Simple clinical data were collected for each patient by members of the research team blinded to all the results above.

#### 2.1.1.7 Anti-fungal management

Patients followed the existing antifungal protocol of the Department of Haemato-Oncology (see Appendix 1).

#### 2.1.1.8 Radiology Review

All CXRs, HRCTs and other radiological investigations pertaining to possible fungal infection were assessed by an expert radiologist blinded to all laboratory and clinical data.

### 2.1.1.9 Diagnosis of Invasive fungal infection

Published EORTC/MSG diagnostic criteria for fungal infections were used to ascertain likelihood of IFD (see Appendix 2).

### 2.1.2 Innsbruck clinical specimens

BAL samples from the Innsbruck University Hospital (Austria) were obtained during routine diagnostic investigations and provided anonymously. The following cohorts were obtained;

'Gold standard' proven IPA cohort was obtained at routine diagnostic work-up at the Innsbruck University Hospital between January 2008 and November 2012 (10 patients with 11 BAL samples). Proven cases were defined by positive histology and *Aspergillus* culture from a lung tissue biopsy sample.

Negative control cohort - 16 BAL samples from 16 adults without any EORTC/MSG risk factors for IFD, in whom routine diagnostic procedures were negative for IPA (microscopy, culture and local GM testing and *Aspergillus* PCR).

## 2.2 Isolation of total DNA from clinical specimens and fungal culture

### *2.2.1 DNA extraction from histological material*

Shavings were obtained from the three wax embedded histological blocks [Section 2.1.1.4.4] using disposable blades. Extractions were carried out in a Class 2 biosafety cabinet, which was cleaned with BioCleanse wipes and DNA Away (VWR, UK) before and after use. Extraction was carried out as follows and summarised in the workflow chart (Figure 7). 1 ml xylene (Sigma Aldrich) was added to a tube containing a 5mm$^2$ section of paraffin wax embedded tissue. This was incubated with shaking for 5 minutes at room temperature, centrifuged 10,000 x g for 2 minutes, followed by removing and discarding

the supernatant. This entire process was repeated, with the shaking incubation time increased to 20 minutes. 1ml absolute ethanol (Sigma Aldrich) was added, and the tube centrifuged at 10,000 x g for 3 minutes. The supernatant was discarded, and the ethanol step repeated. Samples were air dried for 10 minutes in a laminar flow hood. Tissue pellets were resuspended in 180µl of ATL buffer and 20µl proteinase K (both from QIAamp tissue kit, Qiagen, Germany) by pulse vortexing, incubated at 56°C for 2 hours, and boiled (in a 125°C heating block) for 5 minutes. After cooling to room temperature, the Qiagen QIAamp tissue kit column extraction method was followed according to the manufacturer's instructions, eluting into 50 µl buffer AE. Extracted DNA was quantified using a NanoDrop 2000 spectrophotometer (Thermo Scientific). DNA-free water was run as a negative extraction control in each batch of extractions to monitor contamination. DNA extracts were stored at -20°C prior to PCR analysis.

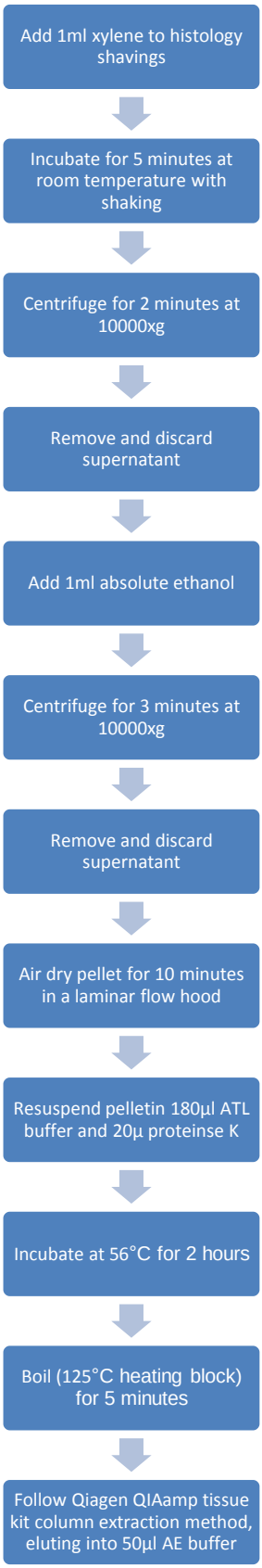



**Figure 7. Work flow chart for DNA extraction from histological material.**

### *2.2.2 DNA extraction from fungal culture*

The following fungal isolates were selected for use in this study;

1. *A. fumigatus* (clinical isolate, confirmed by the Microbiology department at the Royal London Hospital, using MALDI-TOF)
2. *A. flavus* (NCPF 2008)
3. *A. terreus* (NCPF 2729)
4. *A. niger* (NCPF 2275)
5. *A. nidulans* (NCPF 2181)
6. *Candida albicans* (NCPF 3939)
7. *C. dubliniensis* (NCPF 3949)
8. *Fusarium solani* (CBS 224.34)
9. *Scedosporium prolificans* (CBS 100391)
10. *Rhizopus oryzae* (CBS 112.09)

Fungal strains were grown on Sabouraud agar (Oxoid Limited, UK) at 37°C. Genomic DNA was extracted according to the in-house molecular microbiology standard operating procedure for extraction from fungal culture (MM-1.3 version 4, 30/9/2007) in the Medical Microbiology Laboratory of the Royal London Hospital. 0.3-1g of 0.5 mm diameter glass beads (BioSpec products) were added to an apex tube with an O-ring screw cap. 180µL ATL buffer and 20µl proteinase K (both from QIAamp DNA extraction kit, Qiagen, Germany) were added to the tube. A 1µl inoculation loop was used to add 2 loopfuls of biomass from the culture plate. The tube was shaken in a mini bead-beater for 30 seconds on the 'homogenise' setting (BioSpec products), before being incubated at 56°C for 2 hours, with vortexing every 30 minutes for 10 seconds on a medium setting. A QIAamp DNA mini kit (Qiagen, Germany) was used to complete the extraction, following the manufacturer's

protocol from the step where AL buffer is added. Elution comprised two 100µl volumes of AE, with centrifuging between. Extracted DNA was quantified using the Implen Nanophotometer and diluted in Qiagen kit AE buffer to a concentration of 2ng/µl for PCR testing. A negative extraction control of RNase free water was included with each batch of extractions to monitor contamination. DNA extracts were stored at -20°C prior to PCR analysis.

### *2.2.3 DNA extraction from BAL fluid, EBC and whole blood*

200µl of clinical sample were added to a 2ml sample tube with 10µl proteinase K (both from Qiagen EZ1 DNA tissue kit) and mixed by vortex for 10 seconds on a medium setting. This was incubated at 56°C for 15 minutes and spun down to remove condensation from lid. Extraction was performed on the EZ1 robot (Qiagen, Germany), using EZ1 DNA tissue card programme and tissue kit, eluting into 50µl. DNA-free water was run as a negative (fungal free) extraction control in every batch of extractions to monitor for contamination during the extraction process. DNA extracts were stored at -20°C prior to PCR analysis.

## **2.3 qPCR assay design tools**

### *2.3.1 CLC Sequence Viewer*

A search of the National Center for Biotechnology Information (NCBI) GenBank sequence database (http://www.ncbi.nlm.nih.gov/genbank/) was performed to obtain accession numbers for the target organisms. Sequences were imported into the CLC Sequence Viewer (http://www.clcbio.com) and aligned to identify suitable target sequences

### *2.3.2 Beacon Designer*

Primers and a probe with locked nucleic acid (LNA) modifications were designed by Beacon Designer, version 7.2 (Premier Biosoft), selecting for a primer annealing temperature of 55°C and amplicon length of ≤100 base pairs (bp). LNA probe modification was selected for increased thermal stability, providing a more rigid structure for greater sensitivity and discrimination of the target amplicon. The dual-labelled probe was selected to incorporate a FAM fluorophore and a black hole quencher (BHQ-1), for detecting and quantifying the specific target sequence.

### *2.3.3 BLAST*

Primer specificity was evaluated *in silico* using the Basic Local Alignment Search Tool (BLAST) (http://blast.ncbi.nlm.nih.gov/) to find regions of local similarity between sequences.

### *2.3.4 MFold*

Target secondary structures and primer/template accessibility were assessed using the MFOLD web server (http://mfold.bioinfo.rpi.edu/) using corrections for ionic conditions of 50 nM Na+ and 3 mM Mg2+, and a folding temperature of 55°C (160).

## ***2.4 Aspergillus* qPCR assay development**

All qPCR assays were performed on a Bio-Rad CFX Real-time system (BioRad, Hercules, CA, USA) using a 96-well plate format.

### *2.4.1 Assay optimisation using SYBR Green I chemistry*

Using SYBR Green I chemistry, concentrations of forward and reverse primer were optimised. Optimal primer concentrations (50–400nM) were determined by identifying conditions resulting in the lowest quantification cycle (Cq) combined with absence of primer dimer formation, with each concentration run in four replicate wells. Reaction volumes were set at 10µl. SYBR Green assays contained 1X BioRad iQ SYBR Green Supermix, 200nM each primer, and 1µl of DNA extract.

SYBR Green I chemistry and melt curve analyses were used to determine optimal primer annealing temperatures using the CFX's temperature gradient function (50°C–60°C). Initial thermal cycling conditions were 1 cycle of 95°C for 3 minutes, followed by 40 cycles of denaturation at 95°C for 10 seconds and annealing/polymerisation with a temperature gradient from 50–60°C for 30 seconds, which allowed selection of the optimal annealing temperature.

### *2.4.2 Hydrolysis probe reactions*

Once optimal conditions were established, hydrolysis probe reactions were run comprising 1X BioRad iQ Supermix, 200nM of each primer, 100nM probe, and 1µl of DNA. Thermal cycling conditions were 1 cycle of 95°C for 3 minutes, followed by 40 cycles of denaturation at 95°C for 10 seconds and annealing/polymerisation at 59°C for 30 seconds.

### *2.4.3 PCR Efficiency/Analytical Sensitivity*

2ng of genomic DNA, corresponding to $6x10^4$ genome copies of *A. fumigatus* (clinical isolate [Section 2.2.2]) was serially diluted over 6 orders of magnitude in 200ng of *Aspergillus*-free human DNA per dilution. Using qPCR with hydrolysis probe, standard

curves were constructed to calculate PCR efficiency, the linear range of the assay and the limits of detection and quantification. PCR efficiencies were calculated from the slopes of eight standard curves that were run in duplicate or triplicate on separate plates, incorporating 91 data points. Quantification was determined on the basis of one *A. fumigatus* genome containing 30fg of DNA, calculated from a genome size of $4.29 \times 10^6$ bp.

### *2.4.4 PCR Setup Controls*

Multiple negative no template controls (NTCs) were included with every assay and amplification of a single NTC well invalidated the entire qPCR run, leading to a repeat run. A positive control containing six genome copies of *A. fumigatus* (clinical isolate) was also included with each run, to monitor inter-assay consistency. Amplification had to be reproducible, occurring in all replicate wells, for a sample to be considered 'PCR positive'.

### *2.4.5 Carryover Contamination Controls*

All DNA preparations were carried out in a pre-PCR facility that was physically separate from the qPCR laboratory. A unidirectional workflow pattern (pre- to qPCR) was enforced, with physically separate laboratories utilised for pre- and qPCR analysis. Reaction set-up took place in a laminar-flow biosafety cabinet, following thorough cleaning of the cabinet, pipettes and tip boxes with DNA-Away (VWR, UK). Plates were never opened in the qPCR facility post-PCR.

### *2.4.6 Inhibition Testing*

The SPUD inhibition assay (161) was used as an exogenous amplification control. This is a control qPCR assay in which a synthetic SPUD amplicon is the only amplifiable target in the absence of any added nucleic acid. This generates a reference Cq value for the SPUD amplicon, characteristic of an uninhibited assay. If DNA from a sample is added, an increase in Cq of more than one cycle and a reduction in amplification efficiency indicate the presence of PCR inhibitors in the sample. Each qPCR reaction comprised 1X BioRad iQ Supermix, 400nM of SPUD forward and reverse primers, 200nM of FAM-labelled SPUD probe quenched with a BHQ, 1µl of SPUD amplicon, and 1 µl of sample extract. Thermal cycling conditions were 1 cycle of 95°C for 3 minutes, followed by 40 cycles of 95°C for 10 seconds, 61°C for 15 seconds and 70°C for 30 seconds.

### *2.4.7 Monitoring human DNA content of DNA extracts*

A β-globin hydrolysis probe assay was used to monitor the relative human DNA content of clinical sample DNA extracts (162). This assay consisted of the amplification primers β -globin-354F, 5-GTG CAC CTG ACT CCT GAG GAG A-3, β -globin-455R, 5-CCT TGA TAC CAA CCT GCC CAG-3 and a dual-labelled fluorescent TaqMan probe β -globin-402T, 5-(FAM)AAG GTG AAC GTG GAT GAA GTT GGT GG(BHQ1)-3. Each qPCR reaction comprised 1X BioRad iQ Supermix, 300nM of both forward and reverse primers, 100nM probe and 1 µl of sample extract. Thermal cycling conditions were 1 cycle of 95°C for 3 minutes, followed by 40 cycles of 95°C for 10 seconds and 59°C for 30 seconds.

### *2.4.8 Analysis of PCR products*

PCR products were analysed on the Lab901 ScreenTape D1200 (Agilent) microfluidics system, which consists of an acrylamide based matrix that has been optimised to separate double stranded DNA fragments between 125 and 1200 base pairs. The ScreenTape D1200 was used in conjunction with the D1200 Ladder and D1200 loading buffer in the Lab901 TapeStation®.

PCR products were also sequenced to confirm amplification of the target sequence. Sequencing of PCR products was carried out by the Genome Centre Sanger sequencing service, using BigDye 3.1 chemistry with visualisation on the ABI 3730 capillary sequencer.

## 2.5 GM assay

GM detection was performed by Platelia *Aspergillus* enzyme immunoassay (PA-EIA, Bio Rad, France). The GM assay is an immunoenzymatic sandwich microplate assay for the detection of *Aspergillus* galactomannan antigen in serum and BAL fluid. The assay uses rat monoclonal MAb EBA-2, directed against *Aspergillus* galactomannan. The MAbs are used to coat the wells of the microplate and bind the antigen, and to detect the antigen bound to the sensitised plate.

Techniques were carried out as recommended by the manufacturer. Results were expressed as a GM index value – the ratio of the sample optical density (OD) divided by the mean OD of the 2 threshold controls. Serum samples were scored as positive if the GM index was ≥0.5. BAL fluid samples were evaluated using a cut-off of ≥0.8 and/or ≥1.0, for performance analysis. EBC samples were not scored; instead the GM index alone was recorded. This is due to there being no approved cut-off for this sample type.

### *2.5.1 Sample selection for signal loss investigations*

Short-term (ST) storage at +4 °C: Microbiology laboratory records from February 2009 to March 2010 were filtered to exclude GM data (i) for non-serum samples and (ii) samples repeat-tested after greater than 48hours. A total of 109 serum samples (from 65 different patients) and 8 BAL fluid samples were identified.

Long-term (LT) storage at −80 °C: A total of 54 serum samples (from 35 different patients) and 12 BAL fluid samples were collected from July 2005 to September 2007, GM tested and sample aliquots were stored at −80 °C. These samples were repeat tested after 2–4 years storage at −80 °C in 2009. In total, 183 clinical samples were included in this study.

## 2.6 LFD for *Aspergillus*

LFD testing was performed on BAL fluid and serum clinical samples as per manufacturer's recommendations. Briefly, samples were thawed and mixed thoroughly by vortexing for 10 seconds on a medium setting and centrifugation for 1 minute at 14,000rpm. For testing of clinical serum samples, serum was diluted 1:1 (v/v) with tissue culture medium and 100µl of diluted serum applied directly to the LFD. For BAL fluid and EBC samples, 100µl of neat sample was applied directly to the LFD. If the *Aspergillus* antigen is present in the sample, the test line appears within 15 minutes of application (Figure 8). Each LFD device was independently checked by two users and assessments were always concordant.


**Figure 8. Antibody-based detection of *Aspergillus* antigen.**

The LFD showing a positive result for *Aspergillus* detection, indicated by the two bands, one of which serves as a positive assay control and the other being specific for the presence of antigen recognised by the monoclonal antibody JF5.

## 2.7 Cytokine profiling

### *2.7.1 Assay selection*

The CBA Human Th1/Th2/Th17 Cytokine Kit and Human Chemokine Kit (BD Biosciences, USA) were selected, in order to analyse five human chemokines and seven cytokines involved in the differentiation of/secreted by each Th1/Th2/Th17 cell type.

### *2.7.2 BD CBA kit protocol*

The BD CBA system (BD Biosciences, USA) was used on the BD FACS Canto II to quantitate multiple analytes in serum, BAL fluid and EBC samples. The assay uses antibody-coated beads, each with its own unique fluorescence intensity, to capture specific antigens and form unique populations on a flow cytometer. The detection reagent is a mixture of phycoerythrin (PE) labelled antibody. This binds the capture bead-bound antigen and

provides a fluorescent signal proportional to the amount of bound antigen. FCAP Array version 1.0.1 analysis software identifies each bead population, generates a standard curve, and calculates the concentration of unknown samples.

BD CBA Human Th1/Th2/Th17 kit was used to measure IL-2, IL-4, IL-6, IL-10, IL-17A, IFN-γ and TNF in clinical samples.

BD CBA Human chemokine kit was used to measure IL-8, CCL5 (RANTES), CXCL9 (MIG), CCL2 (MCP-1) and CXCL10 (IP-10).

All samples were run in duplicate. Standard curves for each analyte (cytokine/chemokine) were generated using the lyophilised standards provided in the kit. Mean analyte concentrations in clinical samples were determined from the appropriate standard curve.

#### 2.7.2.1 Cytometer set up

Analyte bead arrays were acquired on a FACS Canto II with standard laser and filter configuration (BD Biosciences, UK). The instrument was calibrated daily using CS&T beads (BD Biosciences, UK). BD FACS Diva clinical software (version 6.1.3) was used for data acquisition and analysis to determine the median fluorescence intensity of the bead population.

#### 2.7.2.2 BD CBA Human Th1/Th2/Th17 kit - Preparation of assay standards

Assay standards were prepared as recommended by the manufacturer. A vial of lyophilised standards was opened and the beads transferred to a 15ml conical tube, labelled 'top standard'. 2ml of assay diluent was added to reconstitute the standards, with gentle mixing using the pipette. This was left at room temperature to equilibrate for 15 minutes. 12 BD Falcon 12 × 75-mm sample acquisition tubes were labelled and arranged in the

following order: 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128, and 1:256. 300µl of Assay Diluent was pipetted into each tube. 300µl was taken from the Top Standard and transferred to the 1:2 dilution tube, and mixed thoroughly by pipette. Serial dilutions were made by continuing to transfer 300µl from the 1:2 tube to the 1:4 tube and so on to the 1:256 tube. The standard curve for each cytokine covers a concentration range from 20 to 5000pg/ml. A single tube was prepared containing only Assay Diluent to serve as the 0pg/ml negative control. This resulted in 9 standard tubes and 1 negative control.

### 2.7.2.3 BD CBA Human chemokine kit - Preparation of assay standards

Assay standards were prepared as recommended by the manufacturer. A vial of lyophilised standards was opened and the beads transferred to a 15ml conical tube, labelled 'top standard'. 4ml of assay diluent was added to reconstitute the standards, with gentle mixing using the pipette. The protocol is as described in section 2.7.2.2. The standard curve for each chemokine covers a concentration range from 10 to 2500pg/ml.

### 2.7.2.4 Assay procedure (both kits)

The number of assay tubes for a given experiment was determined (9 standards + 1 negative control + X unknowns = Y assay tubes). Each capture bead suspension bottle was thoroughly mixed by vortex and Y x 10µl volume of each capture bead was added to a single tube labelled 'mixed Capture Beads'. This tube was vortexed to thoroughly resuspend the contents.

For the BD CBA Human Th1/Th2/Th17 kit and for serum sample testing ONLY: Capture Beads were resuspended in Serum Enhancement Buffer by the following procedure. The

mixed capture bead preparation was centrifuged at 200 x g for 5 minutes. The supernatant was aspirated and discarded. The pellet was resuspended in Serum Enhancement Buffer (equal to the volume removed after centrifugation) and mixed by vortex to resuspend. This tube was incubated for 30 minutes at room temperature, protected from light.

Samples and standards were prepared for analysis by vortexing the mixed capture beads and 50µl were added to all assay tubes. 50µl of the Standard dilutions were added to the labelled control tubes and 50µl of each clinical sample was added to the appropriately labelled sample tube. 50µL of PE Detection Reagent was added to all assay tubes. All sample tubes were gently mixed by pipette and incubated for 3 hours at room temperature, protected from light. Following the incubation, 1ml of Wash Buffer was added to every assay tube and the tubes were centrifuged at 200 x g for 5 minutes. Supernatant was aspirated and discarded from each assay tube. 300µl of Wash Buffer was added to each assay tube to resuspend the bead pellet.

Samples were taken to the flow cytometer to analyse. The appropriate acquisition template was opened and the number of events to be counted was set to 2100 of R1 gated events, to ensure that the sample file contained approximately 300 events per Capture Bead. The number of events to be collected was set to 'all events', to ensure that no true bead events were lost due to incorrect gating.

The negative control tube was run first, followed by the Standard tubes from highest dilution to Top Standard, and the unknown clinical samples.

The analyte data were analysed using FCAP Array software.

## 2.8 Proximity Ligation Assay

The Proximity Ligation Assay (PLA) uses qPCR as a detection method to quantify antibody-antigen binding. Here a modified method is described, making use of TaqMan Protein Assay reagents (Applied Biosystems, Life Technologies).

The technology is shown in Figure 9 and described below.

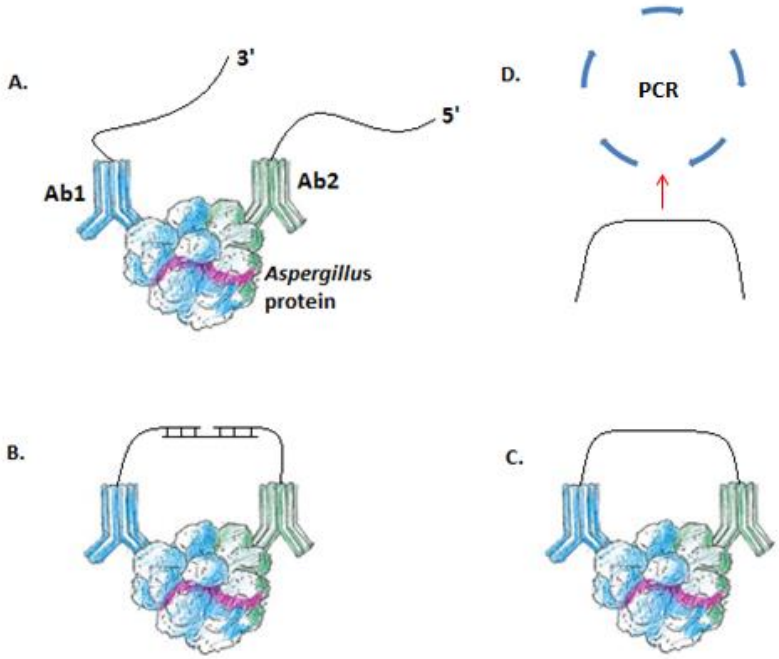



**Figure 9. The steps involved in the TaqMan Protein Assays: Proximity ligation with real-time PCR.**

Two antibodies (Ab1 and Ab2), which bind to 2 different epitopes or multiple identical epitopes on the same target protein, are conjugated to 2 different oligonucleotides through a biotin-streptavidin linkage. Antibodies can be a single MAb, two different MAbs, two polycolonal antisera or a combination of MAb and polyclonal sera. One of the oligonucleotides has a free 3' end and the other a free 5' end. When conjugated, these form the two assay probes. The free ends of the oligonucleotides are brought into proximity when both probes bind to their respective epitopes (Figure 9A). The 3' and 5'

ends are connected by a 'linker' oligonucleotde, that is complementary to the end of the 3' oligo and the start of the 5' oligo (Figure 9B). A ligation reaction seals the gap to generate a molecule that can be amplified by PCR (Figure 9C). Amplification is detected by fluorescence in real-time (Figure 9D).

### *2.8.1 Assay control demonstration*

The Applied Biosytems TaqMan Protein Expression kit was run, using ready-biotinylated polyclonal antibody (pAb) to human IL-6 and a purified recombinant protein target, supplied by Life Technologies. The control assay was run using the kit designated TaqMan Protein Assay II (TPA II) – unpublished and commercially not yet available improvements to the original assay, supplied by Life Technologies under a confidentiality agreement.

#### 2.8.1.1 Preparation of Assay Probe A and Assay Probe B

The 200nM biotinylated antibody was briefly centrifuged to spin the liquid to the bottom of the tube (700 x g for 30 seconds), and placed on ice. The 3' oligonucleotide was also placed on ice. 5µl of the 200nM biotinylated antibody was combined with 5µl of 200nM 3' oligonucleotide in a tube, mixed gently by tapping the tube and briefly centrifuged (700 x g for 30 seconds). This tube was incubated at room temperature for 60 minutes.

The Assay Probe Storage Buffer was allowed to come to room temperature, before 90µl was added to the Ab-oligonucleotide tube and mixed gently by tapping the tube. The tube was incubated at room temperature for 20 minutes. Assay Probe A could be stored at –20°C for up to 6 months.

*Assay Probe B was prepared using the same protocol, but with the 5' oligonucleotide instead of the 3'.*

### 2.8.1.2 Preparation of Assay Probe solution

Assay Probe Dilution Buffer and pre-made Assay Probes A and B were placed on ice to thaw.

The reagents were combined as follows;

| **Components** | **Volume for 96 reactions (full plate)** |
|---|---|
| Assay probe dilution buffer | 216µl |
| Assay probe A (20x) | 12µl |
| Assay probe B (20x) | 12µl |
| **Total volume** | **240µl** |

This tube was gently mixed by tapping and centrifuged (700 x g for 30 seconds), before being placed on ice.

### 2.8.1.3 Preparation of recombinant protein target dilution plate

IL-6 recombinant protein target and Serum Dilution Buffer were thawed on ice. A 96-well reaction plate was placed on ice and a dilution series of the protein target was prepared in column 1 as follows;

a. 45µL of serum dilution buffer was pipetted into well A1 and 48µl into wells B1-H1.

b. 5µL of recombinant protein target (20,000pg/mL) was pipetted into well A1 and pipetted up and down several times to mix the sample.

c. 12µL was transferred from well A1 to B1 and pipetted up and down several times to mix. This was continued, transferring 12µL from the previous dilution well to the next dilution well, pipetting up and down several times to mix, continued down column 1 of the plate

until recombinant protein was added to well G1 (Figure 10). Well H1 did not contain any recombinant protein, only serum dilution buffer (no protein control).

The plate was sealed with MicroAmp clear adhesive film and briefly centrifuged (700 x g for 30 seconds) to remove bubbles and placed back on ice.

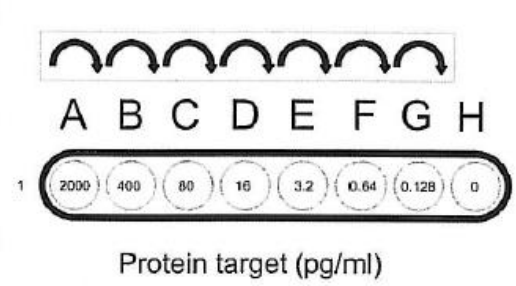



**Figure 10. Serial dilution of recombinant protein target in row 1 of the dilution plate.**

### 2.8.1.4 Performing the binding reaction

A 96-well plate was placed on ice and 2µl of Assay Probe solution was pipetted into wells 1-4 of rows A to H. Recombinant protein dilutions were transferred to the binding reaction plate as follows;

a. 2µl of the diluted recombinant protein was transferred from well A1 of the dilution plate to wells A1-4 of the binding plate (Figure 11)

b. Step **a** was repeated for rows B through to H of the binding reaction plate

The binding reaction plate was sealed using a MicroAmp Clear Adhesive Film and briefly centrifuged to bring the liquid to the bottom of the wells (700 x g for 30 seconds). The plate was incubated at room temperature for 90 minutes. The plate was put on ice and the ligation reaction set up within 15 minutes.

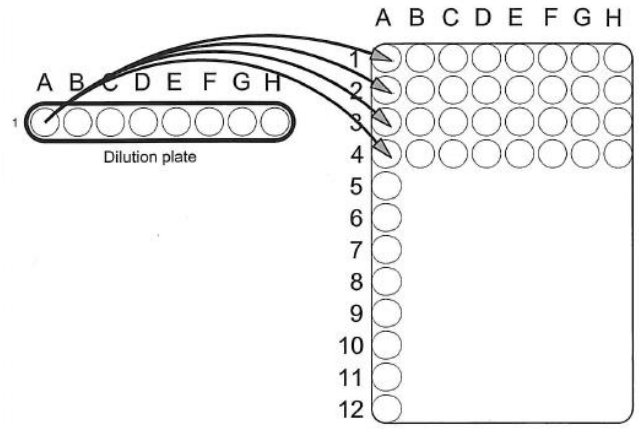



**Figure 11. Preparation of the binding reaction plate.**

### 2.8.1.5 Performing the ligation reaction

DNA Ligase II (250x), TaqMan Protein Expression Fast Master Mix II (2x), nuclease-free water and Universal PCR Assay II (20x) were put on ice to defrost. Tubes were gently flicked to mix and briefly centrifuged (700 x g for 30 seconds). A MicroAmp Optical 96-well Reaction Plate was also put on ice.

The ligation mix was prepared as follows and inverted to mix;

| **Ligation/PCR solution** | **Volume for 1 reaction** |
|---|---|
| Fast Master Mix II (2x) | 10µl |
| DNA Ligase II (250x) | 0.076µl |
| Universal PCR Assay II (20x) | 1µl |
| Nuclease-Free Water | 4.92µl |
| **Total Volume** | **16µl** |

16µl of this master mix was pipetted into columns 1-4 of the Reaction Plate. The plate was sealed with a MicroAmp Optical Adhesive Cover and briefly centrifuged (700 x g for 30 seconds). The reaction plate was loaded into a StepOne real-time system (Life

Technologies). Thermal cycling conditions were 1 hold at 25°C for 15 minutes (ligation step), 1 hold at 95°C for 2 minutes (ligase inactivation), followed by 40 cycles of denaturation at 95°C for 1 second and annealing/polymerisation at 60°C for 20 seconds. Data from the TaqMan protein expression experiment was analysed using a threshold setting of 0.2 with automatic baseline. The Cq data was exported for comparative analysis.

### *2.8.2 Aspergillus-specific probe development*

#### *2.8.2.1 Aspergillus*-specific antibody production

Monoclonal antibody (Ab) JF5 was prepared by Dr Christopher Thornton (University of Exeter), as previously described (119). Mouse MAb JF5 was raised against an extracellular glycoprotein secreted by actively growing *Aspergillus* hyphae, with mice challenged with lyophilised mycelium of *A. fumigatus* AF293. The JF5 hybridoma cell line was produced. Ig subclass and target epitope characterisation revealed the MAb belonged to the IgG3 class and binds a protein epitope that does not contain carbohydrate moieties. Dr Thornton also provided antiserum (pAb) raised in rabbits against purified *A. fumigatus* mannoprotein antigen (119) suspended in phosphate buffered saline (PBS). Three New Zealand white male rabbits were each given four sub-cutaneous injections at 2-week intervals. Each injection contained a total of 1.0mg of antigen plus 25µg of Quil A adjuvant (Superfos a/s, Denmark) with each injection. Test bleeds were performed prior to immunisation and 7 days after the third injection. The final bleed was made 1 week after the fourth injection.

### *2.8.2.2 Aspergillus* culture filtrate preparation

A crude culture filtrate (CF) of *A. fumigatus* AF293 was provided by Dr Thornton. This CF acted as a positive control samples for *Aspergillus* PLA development, as it contained the target glycoprotein. This CF was prepared by Dr Thornton as follows;

Three replicate sterile tissue culture flasks (Greiner Bio-One) containing 15ml tissue culture medium (TCM; RPMI-1640 medium containing 10% (v/v) fetal calf serum, 10mM L-glutamine and penicillin-streptomycin) were inoculated with $10^3$ washed spores per ml TCM and incubated at 37°C with shaking (50 rpm). Washed spore suspensions were prepared from malt extract agar cultures. After 48 hours growth, the contents of flasks were centrifuged at 4,000 rpm for 10 minutes and the cell-free culture fluids stored at -20°C prior to assay by PLA.

### 2.8.2.3 Purified glycoprotein target preparation

Purified glycoprotein target was prepared by Dr Thornton as previously described (119). Antigen was purified from PBS extracts of lyophilised mycelium by affinity chromatography with a Protein A IgG Plus Orientation kit (Pierce Biotechnology, Rockford, IL) containing immobilised MAb JF5. Ascitic fluid was prepared from JF5 hybridoma cells in female BALB/c mice (Eurogentec, Belgium). The mice were injected with $10^6$ hybridoma cells washed in PBS. After 3 weeks approximately 5ml of ascitic fluid was recovered from each mouse and stored at -20°C prior to use. For preparation of the affinity column, 15µl of ascitic fluid was mixed with 2ml of binding buffer and the solution was applied to the protein A-agarose matrix. Crude PBS antigen extract was incubated with the immobilised antibody and bound antigen was eluted with 0.1M glycine-HCl (pH 2.8) buffer.

### 2.8.2.4 Preparation of biotinylated antibodies

This step required the following reagents;

a. Monoclonal antibody JF5 (1–2µg/µl)

b. Polyclonal serum (1–2µg/µl)

c. Life Technologies Probe/buffer kit

d. Life Technologies Core reagents base kit/mastermix

e. APEX Biotin-XX Ab labelling kit (Invitrogen) or EZ-Link Sulfo-NHS-LC-Biotin, No-Weigh Format Biotinylation kit (Thermo Scientific)

f. Thermo Slide-A-Lyzer MINI Dialysis Unit, 7K MWCO

Biotinylation was performed using two commercial kits, for comparison.

Biotinylation of the selected Abs was performed using the APEX Biotin-XX Ab labelling kit (Invitrogen), as follows:

The APEX antibody labelling tip was gently tapped on a hard surface to settle all resin at the bottom of the tip. Both caps were removed from the labelling tip and the tip was placed into a microcentrifuge tube. The resin was hydrated by applying 100µl of wash buffer (Component C) to the resin in the tip. 10µl of antibody solution was applied to the top of the resin. The antibody solution was gently pushed onto the resin using the elution syringe (Component H). Any drops that eluted from the tip were discarded as waste.

To the vial of reactive dye (Component A), the following were added;

a. 2µl Dimethyl sulfoxide (DMSO, Component D); pipetted up and down to dissolve

b. 18µl Labelling buffer (Component E); pipetted up and down to dissolve

10µl of this was added to the top of the resin, and the solution was gently pushed through. Any dye that eluted from the tip was discarded as waste. The tip was incubated at room

temperature for 2 hours. The APEX antibody labelling tip was washed twice with 50µl of wash buffer (Component C) by applying 50µl to the top of the resin, pushing through the tip into the microcentrifuge tube. 10µl of neutralisation buffer (Component F) was added to a clean microcentrifuge tube and the APEX antibody labelling tip was transferred to this tube. 40µl of elution buffer (Component G) was applied to the top of the resin. This was pushed through the tip to elute the labelled antibody into the microcentrifuge tube containing neutralisation buffer. The 50µl of eluted solution was mixed to ensure neutralisation and the tube placed on ice.

The biotin-labelled Ab solution was extensively dialysed in cold PBS (pH 7.4) using the Thermo Slide-A-Lyzer MINI Dialysis Unit.

Biotinylation of the selected Abs was performed using the EZ-Link Sulfo-NHS-LC-Biotin, No-Weigh Format Biotinylation kit (Thermo Scientific), as follows:

a. A vial of Sulfo-NHS-LC-Biotin was removed from the freezer and brought to room temperature.

b. The antibody was prepared in PBS (dialysed in cold PBS (pH 7.4) using the Thermo Slide-A-Lyzer MINI Dialysis Unit).

c. A 10mM solution of the biotin reagent was prepared by adding 180µl of water to the 1mg microtube.

d. The appropriate volume of 10mM biotin reagent solution was added to the antibody solution.

e. The tube was incubated on ice for two hours

f. With antibody labelling completed, excess biotin was removed by extensive dialysis in cold PBS (pH 7.4) using the Thermo Slide-A-Lyzer MINI Dialysis Unit.

### 2.8.2.5 Performing the Forced Proximity Probe Test

The Forced Proximity Probe Test is performed to determine whether or not the biotinylated antibody can bind to the oligonucleotide. The average Cq value is calculated for each biotinylated antibody and negative control. The ΔCq value is calculated for each biotinylated antibody: AvgCq(negative control) – AvgCq(Forced Proximity Probe). ΔCq≥8.5 is required for the antibody to pass the Forced Proximity Probe Test. This confirms that the biotinylated antibody is suitable for use in TaqMan Protein Assays experiments.

The biotinylated antibody will fail this test if there is an excess free biotin in the preparation or if the biotinylation step has been unsuccessful.

The biotinylated antibody was first diluted to 200nM (30µg/mL) using Antibody Dilution Buffer as the diluent. This dilution was briefly centrifuged to spin the liquid to the bottom of the tube (700 x g for 30 seconds) and placed on ice. The oligonucleotide mix was prepared in a clean tube by combining 5µl of 200nM 3' oligonucleotide with 5µl of 200nM 5'oligonucleotide. The tube was gently mixed by tapping and briefly centrifuged (700 x g for 30 seconds) and placed on ice.

Two tubes were labelled: Forced Proximity Probe and Negative Control. Working on ice, the following mixes were made in the appropriate tube;

| **Component** | **Forced Proximity Probe Tube** | **Negative Control Tube** |
|---|---|---|
| 200nM Prox-Oligo mix | 2µl | 2µl |
| 200nM biotinylated Ab | 2µl | ---- |
| Ab dilution buffer | ---- | 2µl |
| **Total Volume** | **4µl** | **4µl** |

The tubes were mixed gently by tapping and centrifuged (700 x g for 30 seconds). The tubes were incubated at room temperature for 60 minutes. 396µl of Assay Probe Dilution Buffer was added to each tube and the tubes were incubated at room temperature for 30 minutes, before being placed on ice.

DNA Ligase (500x), Ligase Dilution Buffer (1x) and Ligation Reaction Buffer (20x) were thawed on ice. The DNA Ligase was diluted with Ligase Dilution Buffer as follows;

a. The DNA ligase tube and Ligase Dilution Buffer were gently flicked to mix and briefly centrifuged (700 x g for 30 seconds)

b. 2µl DNA Ligase (500x) was combined with 198µl Ligase Dilution Buffer in a new tube, gently flicked to mix and put on ice.

The ligation solution was prepared as follows and inverted to mix;

| **Ligation solution** | **Volume** |
|---|---|
| Ligation Reaction Buffer (20x) | 50µl |
| Deionised water | 908µl |
| Diluted ligase | 2µl |
| **Total** | **960µl** |

A MicroAmp Optical 96-well Reaction Plate was put on ice. 4µl of the Forced Proximity Probe mix was added to wells A1-4. 4µl of the Negative Control mix was added to wells A5-8. 96µL of the ligation solution was pipetted into each of the 8 reaction wells and pipetted up and down once to mix. The plate was sealed with a MicroAmp Clear Adhesive Film, and briefly centrifuged (700 x g for 30 seconds). Using a thermal cycler with a heated cover, the sealed plate was incubated at 37 °C for 10 minutes. The plate was returned to ice.

TaqMan Protein Expression Fast Master Mix (2x), Universal PCR Assay (20x) were put on ice to defrost. Both tubes were gently flicked to mix and briefly centrifuged (700 x g for 30 seconds). A MicroAmp Optical 96-well Reaction Plate was also put on ice.
The PCR reaction mix was prepared in a tube as follows, briefly mixed by tapping the tube and centrifuged (700 x g for 30 seconds);

| **Real-time PCR mastermix** | **Volume** |
|---|---|
| Fast Master Mix (2x) | 100µl |
| Universal PCR Assay (20x) | 10µl |
| **Total** | **110µl** |

11µl of this master mix was pipetted into wells A1-12 of the new Optical 96-well Reaction Plate. 9µl of ligation product was pipetted from the original reaction plate into the corresponding wells of the Optical 96-well Reaction Plate. The wells were pipetted up and down once to mix. The plate was sealed with a MicroAmp Optical Adhesive Cover and briefly centrifuged (700xg for 30 seconds).
The PCR plate was loaded into a Bio-Rad CFX Real-time system (BioRad, Hercules, CA, USA). Thermal cycling conditions were 1 hold at 95°C for 20 seconds, followed by 40 cycles of denaturation at 95°C for 3 seconds and annealing/polymerisation at 60°C for 30 seconds. Data from the TaqMan protein expression experiment was analysed using a threshold setting of 0.2 with automatic baseline. $\Delta Cq \geq 8.5$ was required for the antibody to pass the Forced Proximity Probe Test.

Once biotinylated Abs had passed the Forced Proximity Probe Test they could be used to prepare PLA probes. Assay Probes were prepared as described in section 2.8.1.1.

### 2.8.2.6 *Aspergillus* PLA – original TPA I protocol - method using TaqMan® Protein Assays Open Kit

Preparation of Assay Probe Solution was performed as described in section 2.8.1.2.

**Binding reaction** - A 96-well plate was placed on ice and 2µl of Assay Probe solution was pipetted into the appropriate number of reaction wells. 2µl of *Aspergillus* culture filtrate (dilutions made in 1xPBS as appropriate) was added to appropriate wells in the binding plate. The binding reaction plate was sealed using a MicroAmp Clear Adhesive Film and briefly centrifuged to bring the liquid to the bottom of the wells. The plate was incubated at 37°C for 60 minutes inside a thermal cycler with a heated cover. The plate was put on ice and the ligation reaction set up within 15 minutes.

**Ligation reaction** - DNA Ligase (500x), Ligase Dilution Buffer and Ligation Reaction Buffer (20x) were placed on ice to defrost. The DNA Ligase was diluted with Ligase Dilution Buffer as follows;

a. The DNA ligase tube was gently flicked to mix and briefly centrifuged (700 x g for 30 seconds)

b. The Ligase Dilution Buffer was briefly mixed by vortex and briefly centrifuged (700 x g for 30 seconds)

c. 2µl DNA Ligase (500x) was combined with 998µl Ligase Dilution Buffer in a new tube, gently flicked to mix and put on ice.

The ligation solution was prepared as follows and inverted to mix;

| **Ligation solution** | **Volume for 1 reaction** |
|---|---|
| Ligation Reaction Buffer (20x) | 5µl |
| Deionised water | 90.9µl |
| Diluted ligase | 0.1µl |
| **Total** | **96µl** |

96µL of the ligation solution was pipetted into each binding reaction well, on ice. Each well was pipetted up and down once to mix. The plate was re-sealed with a MicroAmp Clear Adhesive Film, and briefly centrifuged (700 x g for 30 seconds). Using a thermal cycler with a heated cover, the sealed plate was incubated at 37 °C for 10 minutes. The plate was returned to ice.

**Real-time PCR reaction -** TaqMan Protein Expression Fast Master Mix (2x) and Universal PCR Assay (20x) were put on ice to defrost. Both tubes were gently flicked to mix and briefly centrifuged (700 x g for 30 seconds). A MicroAmp Optical 96-well Reaction Plate was also put on ice.

The PCR reaction mix was prepared in a tube as follows, briefly mixed and centrifuged (700 x g for 30 seconds);

| **Real-time PCR mastermix** | **Volume for 1 reaction** |
|---|---|
| Fast Master Mix (2x) | 10µl |
| Universal PCR Assay (20x) | 1µl |
| **Total** | **11µl** |

11µl of this master mix was pipetted into the appropriate number of wells in the new Optical 96-well Reaction Plate. 9µl of ligation product was pipetted from the original reaction plate into the corresponding wells of the Optical 96-well Reaction Plate. The wells were pipetted up and down once to mix. The plate was sealed with a MicroAmp Optical Adhesive Cover and briefly centrifuged. The PCR plate was loaded into a Bio-Rad CFX Real-time system (BioRad, Hercules, CA, USA). Thermal cycling conditions were 1 cycle of 95°C for 20 seconds, followed by 40 cycles of denaturation at 95°C for 3 seconds and annealing/polymerisation at 60°C for 30 seconds.

Data from the TaqMan protein expression experiment was analysed using a threshold setting of 0.2 with automatic baseline. The Cq data was exported for comparative analysis.

### 2.8.2.7 *Aspergillus* PLA – TPA II KIT

Using a simplified work flow and modified reagents, Life Technologies provided the following PLA protocol, as shown in Figure 12.

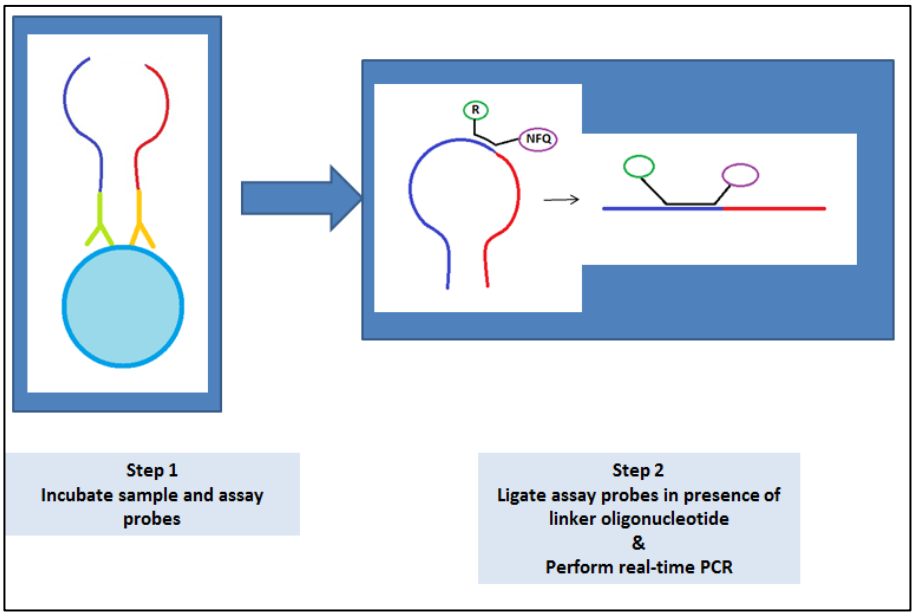



**Figure 12. TaqMan Protein Assay II work flow.**

R = fluorescent reporter. NFQ = non-fluorescent quencher.

Preparation of Assay Probe Solution was performed as described in section 2.8.1.2.
The binding reaction was performed as described in section 2.8.1.4, with 2µl of the Assay Probe solution and 2µl of *Aspergillus* culture filtrate dilutions added to each well.
The ligation reaction was performed as described in section 2.8.1.5, with addition to appropriate wells. Real-time PCR was performed as described in section 2.8.1.5, but on a Bio-Rad CFX Real-time system (BioRad, Hercules, CA, USA).

#### 2.8.2.8 Antibody affinity purification

The Thermo Scientific NAb Protein A Plus spin kit was used for rapid affinity purification of the *Aspergillus* IgG antibodies. Protein A is a bacterial protein that binds with high specificity to mammalian IgG and is generally preferred for affinity purification of rabbit, pig, dog and cat IgG. Protein A purification was performed as recommended by the manufacturer.

## 2.9 Statistical Analyses

### *2.9.1 Significant decline in galactomannan signal during storage of clinical serum samples*

#### 2.9.1.1 Analysis of clinical data

Data from serum testing were presented on a scatter plot, with fractional polynomial regression lines for each storage type. As the relationship between the two test results was found not to be linear, fractional polynomial regression was the best choice to capture the exact relationship between them. Due to routine twice-weekly screening of high-risk

patients, more than one sample was collected for most of the patients. As first and second test values are likely to be correlated, due to sampling from the same patient, the Wilcoxon Signed Rank Sum Test was used to test the GM signal loss both in the ST as well as LT serum samples based on the first sample taken from each patient. Probability values <0.05 were defined as significant based on a 2-tailed test. All calculations were carried out by Dr Shah-Jalal Sarker, using the statistical software package Intercooled STATA 10.1 (Stat Corp, College Station, TX, USA, 2008).

#### 2.9.1.2 Assay Repeatability

In order to determine the intra- and inter-assay variability, the OD value of the threshold control, loaded in duplicate, was recorded for 40 randomly selected assay runs. The mean OD was calculated, together with range. Inter-assay repeatability of the threshold control was calculated by using the Jones and Payne (163) coefficient of variation (CV) for duplicate measures.

### *2.9.2 Combination biomarker diagnosis of IA in BAL fluid*

#### 2.9.2.1 Pairwise agreement

Cohen's kappa coefficient (including 95% confidence intervals) was calculated to measure the agreement between any two biomarkers (LFD/qPCR/GM). Kappa values were interpreted using the method of Landis and Koch (164), with values greater than 0.8 representing almost perfect agreement.

#### 2.9.2.2 Diagnostic performance of EORTC/MSG criteria

The EORTC/MSG criteria (51) were evaluated against the clinical biomarker diagnosis of IPA, both with and without GM results included – excluding GM data removes incorporation bias, as GM is itself a criterion in the EORTC/MSG scoring. The sensitivity, specificity, positive predictive values (PPV) and negative predictive values (NPV) of EORTC/MSG scoring were determined using i) proven/probable/possible as positive results and EORTC/MSG none as negatives and ii) proven/probable as positives and EORTC/MSG possible/none as negatives.

#### 2.9.2.3 Diagnostic performance of the biomarker assay, using EORTC/MSG criteria as the gold standard

Diagnostic performance of the individual and combination biomarker assays was evaluated in the Study cohort. Combined assay results were treated as a single entity (positive or negative) and discrepant results excluded from analysis. Sensitivity, specificity, PPV and NPV were determined using the EORTC/MSG (excluding GM) proven/probable IA as true positives and those with possible/none as true negatives, with 95% confidence intervals.

### *2.9.3 Evaluation of lung and serum inflammatory cytokine profile associated with IPA biomarker positive BAL fluid*

FCS 2.0 data files were exported from the BD CBA assay for analysis using FCAP Array software. This software reads the FCS 2.0 data files from an experiment, locates clusters (with assigned analytes) and determines the median fluorescence intensity (MFI) of the detector antibody for each analyte. The software fits a standard curve to the data from the

concentration standards. The 5-parameter logistic fitting equation was used to fit the data, as recommended by BD. The standard curve for each analyte was used to calculate concentration values in each Study sample.

Cytokine and chemokine distributions between defined populations were compared using the non-parametric Mann Whitney test, with 2 tailed P values and 95% confidence intervals calculated (GraphPad Prism 5).

# CHAPTER 3

# DESIGN AND DEVELOPMENT OF A NEW, ROBUST AND RELIABLE PAN-*ASPERGILLUS* qPCR ASSAY

## 3.1 Background

qPCR is the most widely used molecular technology for diagnostic applications designed to detect and quantify pathogens (113).

Its accessibility, characteristic sensitivity, specificity, and wide linear dynamic range make qPCR well suited to use within the clinical environment.

However, there are several drawbacks to qPCR assays:

(i) need to extract DNA which can result in contamination of sample and false positive results

(ii) sensitivity to environmental inhibitors, which can cause false-negative results

(iii) inability to provide information about whether a detected pathogen has the ability to establish an infection or not and

(iv) inadequately designed and controlled qPCR assays can generate significant inter-laboratory variation, leading to unreliable and often contradictory results obtained from the same samples

As a consequence, although qPCR is widely used, the peer-reviewed literature contains numerous publications reporting inconsistent data from poorly designed assays, resulting in unreliable conclusions and diagnoses (133, 165).

PCR has been widely used in attempts to establish a diagnosis of IA. DNA samples commonly analysed are extracted from various fractions of blood (serum, plasma, whole blood), BAL fluid specimens as well as tissue, including paraffin-embedded sections. However, a lack of technical standardisation and relatively poor understanding of the kinetics of *in vivo* fungal DNA release continues to hamper the broad applicability of this technique. Hence qPCR has not yet been included in the EORTC/MSG mycological criteria for defining IA (51).

Two recent reviews have examined the quality of PCR-based studies in the diagnosis of IA (116, 166). Khot and Fredricks (166) examined the quality of PCR-based studies in fungal diagnostics using 42 variables from the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines to analyse the impact of analytical variables on diagnostic performance. The focus of their review was the diagnostic performance of the assays. Therefore, the majority of MIQE variables used in the analysis focused on aspects of study design, patient population selection, sampling, criteria for assigning likelihood of IFD and evaluation of assay diagnostic performance. The focus of the review was not on the quality of the PCR assays themselves, with only 13 of the 42 selected analytical variables specifically addressing the PCR assay design, control and validation. The review does however highlight that a significant number of studies fail to use or report the use of negative extraction controls to monitor contamination during the nucleic acid extraction stage. Also, only just over half of the studies evaluated using the MIQE guidelines reported the use of some form of inhibition or amplification control, even though it is well known that PCR inhibitors can reduce product yield and even result in complete failure of the PCR. Mengoli *et al* (116) report a systematic review and meta-analysis on the use of PCR tests for the diagnosis of IA, focusing on the practical application of PCR assays and their diagnostic performance. They highlight the variation in methods across 16 studies, including PCR targets and chemistry, concluding that formal validation of this technology for the diagnosis on IA in specific patient populations will require the design of a standard for *Aspergillus* PCR. Attempts to reach a consensus on PCR diagnosis have been coordinated by EAPCRI, who have thus far focused on the extraction procedure (114). However, closer examination of their data reveals the high variability of PCR assays, with different PCR efficiencies resulting in significantly different sensitivities [Section 1.6.4].

Taken together, these reports emphasise the need to examine the quality of published assays. A development that may improve the reliability of PCR-based assays for the diagnosis of IA was the publication of the MIQE guidelines (167). These provide a blueprint for good assay design and aim to restructure qPCR methods into a more consistent format that will encourage detailed auditing of experimental detail, data analysis and reporting principles (168). MIQE has become acknowledged as the defining event in the maturing of qPCR technology and its implementation is essential if qPCR is to remain the benchmark technology for molecular diagnosis (133).

This chapter first describes a critical literature review of *Aspergillus* qPCR assays, focusing on the analysis of the parameters that define good assay design and examining the quality of broad spectrum *Aspergillus* qPCR assays published between December 2000 and December 2011. Next, a series of design steps and experiments is described in the development, optimisation and validation of the first MIQE-compliant pan-*Aspergillus* qPCR assay.

## 3.2 RESULTS: Critical review of the use of qPCR for the detection of Aspergillus-derived nucleic acids

### *3.2.1 Search strategy*

The review excludes case studies, reviews and non-English language papers and limits itself to an analysis of the first publication of any particular assay. Assays that target *A. fumigatus* only were also excluded. Whilst *A. fumigatus* accounts for approximately 90% of all cases of life-threatening IA (17), there has been an increase in the frequency of other *Aspergillus* species associated with IA, with reported frequencies of *A. flavus, A. terreus, A. nidulans* and *A. niger* of 14-33%, 3-28%, 1-2.5% and 5-8%, respectively (21-23). This is of

relevance to the design of a reliable diagnostic assay for IA, which must be sufficiently specific to detect all clinically-relevant *Aspergillus* species whilst not amplifying other, closely related non-*Aspergillus* targets, such as *Candida spp*.

Nested PCR assays were also excluded. Nested PCR assays have been used to enhance analytical sensitivity (169). However, it is arguable whether analytical sensitivity increases assay sensitivity (170), whereas its reduced specificity has been documented (171, 172). Also, the use of nested-PCR assays is considered too prone to false-positive results and unlikely to attain widespread acceptance outside specialist molecular centres (131).

In addition, alternative nucleic acid-based tests such as fluorescence *in situ* hybridization and isothermal amplification techniques such as nucleic acid sequence-based amplification (NASBA) were excluded. Although there are a few reports describing the use of NASBA, an isothermal technique for the amplification of RNA (173-176), the theoretical advantage of higher sensitivity and reduced likelihood of carry-over contamination must be balanced by the complexity of this technology. The review presented in this chapter focuses on real-time qPCR, the most commonly used nucleic acid-based test.

A PubMed search was performed for articles published over a period of 12 years between December 2000 and December 2011 using the search criteria 'aspergillosis [Title/Abstract] OR aspergillus [Title/Abstract] AND PCR [Title/Abstract] AND diagnosis [Title/Abstract]'. The search resulted in 223 publications, which were reduced to ten after applying the exclusions discussed above, (Table 1).

| Study | Author | Type of PCR assay | Target | Targeted *Aspergillus* species | Amplicon size (bp) | Secondary structures at primer binding location? | Type of Standards | Efficiency[a] | Limit of detection |
|---|---|---|---|---|---|---|---|---|---|
| 1 | **Khot, 2008(177)** | Hydrolysis probe | 18S rDNA | *Aspergillus* spp | 114 | No | Genomic DNA | No information | 1fg (single target gene copy) |
| 2 | **Schabereiter-Gurtner, 2007(178)** | SYBR Green with bi-probes for *Aspergillus* species-specific melt point analysis | ITS2 | *A. fumigatus, A. flavus, A. nidulans, A. niger, A. terreus* | 242-251 | Yes | Conidia spiked in blood and genomic DNA | No information | 0.45-0.9 CFU/PCR reaction |
| 3 | **Musher, 2004(158)** | Molecular beacon | 18S rDNA | *Aspergillus* spp | 219 | No | Genomic DNA | No information | No information (lowest standard 1pg) |
| 4 | **Sanguinetti, 2003(179)** | Hydrolysis probe | 18S rDNA | *Aspergillus* spp | 124 | Yes | Recombinant DNA | 118%[b] | 10 target gene copies |
| 5 | **Costa, 2002(180)** | Hybridisation probes | Mit | *A. fumigatus* and *A. flavus* | 91 | Yes | Genomic DNA | 77.1% | 3fg genomic DNA (less than a single genome copy) |
| 6 | **Kami, 2001(181)** | Hydrolysis probe | 18S rDNA | *Aspergillus* spp | 156[c] | No | Recombinant DNA | 91.6%[a] | 10 target gene copies |
| 7 | **Faber, 2008(182)** | Hybridisation probes for *Aspergillus* species-specific melt point analysis | 18S rDNA | *A. fumigatus, A. flavus, A. niger* and *A. terreus* | 504[c] | No | Conidia spiked in blood | No information | *A. fumigatus* 5 CFU/ml, others 1-5 CFU/ml |
| 8 | **White, 2006(183)** | Hydrolysis probe | 28S rDNA | *Aspergillus spp* | 184 | Yes | Recombinant DNA | 86%[b] | 5 gene copies/<br>10CFU/mL |
| 9 | **Springer,** | a) Hybridization probes | ITS1 | a) 3 groups; i) *A. fumigatus, A. versicolor, A.* | 153 | Yes | Conidia spiked in | No | 20 CFU/ml |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | **2011(184)** | for melt point analysis<br><br>b)Hydrolysis probe | | *nidulans,* ii) *A. terreus, A. niger,* iii) *A. flavus*<br><br>b) *Aspergillus* spp | | | blood | information | |
| 10 | **Walsh, 2011(185)** | a) Hydrolysis probe and primers targeting specific *Aspergillus* species<br><br>b) Hydrolysis probe | a) ITS1<br><br>b) 18S rDNA | a) *A. fumigatus, A. flavus, A. niger, A. terreus*<br><br>b) *Aspergillus* spp. | a) *A. fumigatus* 136bp*, A. flavus* 89bp, *A. niger* 79bp, *A. terreus* 70bp<br><br>b) 153 | a) Yes<br><br>b) No | Recombinant DNA | a) *A. fumigatus* 99.6%, *A. terreus* 93.5%<br><br>b) 95.8% | 5 target gene copies |

**Table 1. Comparisons of real-time PCR studies for detection of multiple *Aspergillus* species.**

[a] Efficiency, if not quoted in the original paper, is derived from the formula: **PCR efficiency = 10 $^{-1/slope}$ -1** (where slope refers to the slope of the standard curve given in the study. However, this is frequently not provided by the authors)

[b] Information on efficiency or slope of standard curve not available in the paper, the efficiency of the assay is estimated from standard curve or amplification curve shown in the paper. Refer to Appendix 3 for an example of calculation in detail.

[c] Information on size of the amplicon were not given in the paper, the size were deduced by mapping the primers on an *A. fumigatus* 18S ribosomal RNA gene sequence. Abbreviations: rDNA: ribosomal DNA; Mit: mitochondrion; ITS: internal transcribed spacer; spp: species; CFU: Colony forming units.

### *3.2.2 Type of PCR assays*

The ten publications emphasise the variation in strategies used for the detection of *Aspergillus* species. There are two broad approaches:

- A simple and common approach is the use of broad-range PCR in which a universal set of primers and a probe are designed from consensus regions in the genome of *Aspergillus* species (Figure 13). The main advantage of using genus-specific sets of primers and probe is that all *Aspergillus* species including rare ones would be detected in a single reaction. However, identification of specific species would not be possible. Examples of this approach are the assays reported in studies 1-4,6-8,10b)
- An alternative approach is to use different sets of primers targeting specific *Aspergillus* species. Examples are study 5, where multiplex PCR identifies two *Aspergillus* species (*A. fumigatus* and *A. flavus*) along with some *Candida* species. It is important to note that multiplex PCR assays are more difficult to design, as it is vital to avoid complementarity between all primers. Also, competition for reagents can reduce assay efficiency, especially if one of the targets is present at much lower levels than the others. In study 9a, different primer and probe sets were designed to target four *Aspergillus* species, run as individual assays. This would be an expensive method. A slightly different method is to combine broad range primers with species-specific probes (studies 2, 7 and 9). Using species-specific probes can be clinically useful, as knowing the exact species may be important for clinical management (e.g. *A. terreus* strains are known to be more drug resistant to amphotericin B deoxycholate). The rather serious disadvantage is that infection with other *Aspergillus* species will be missed.

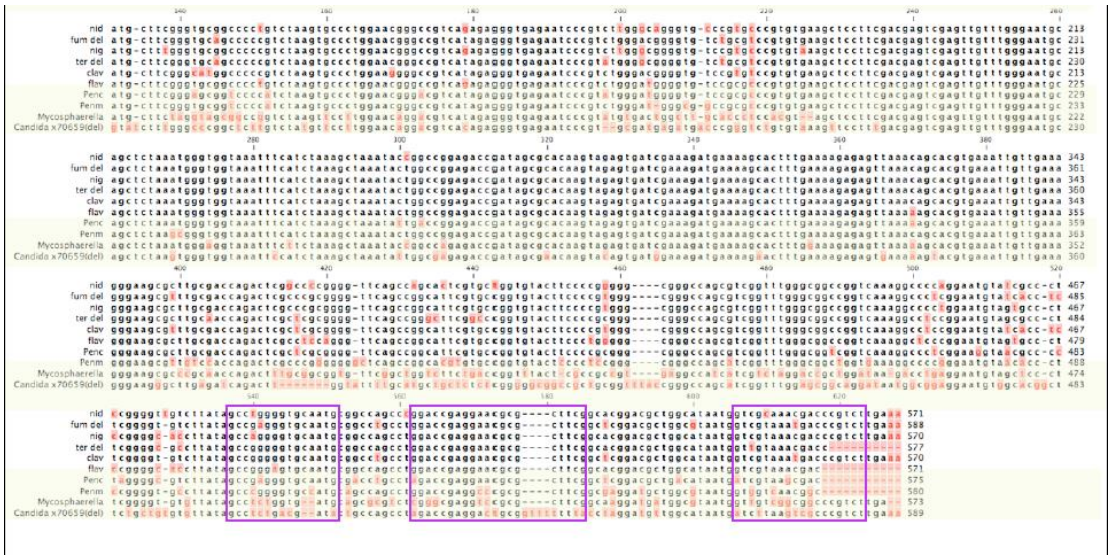


**Figure 13. Sequence alignment of 28S rDNA region for six *Aspergillus* and four non-*Aspergillus* species.**

Example primer and probe target regions for *Aspergillus* species specific amplification are highlighted by the purple boxes

Additional variation includes the use of different types of chemistries and different targets.

- The different chemistries that have been used to detect *Aspergillus*, include hydrolysis probes (studies 1,4,6, 8, 9b,10a, 10b ), hybridisation probes (studies 5, 7 and 9a), molecular beacons (study 3) and SYBR Green (study 2), even though this approach, which relies on a non-specific double stranded DNA-binding dye, lacks the specificity essential for use in clinical diagnostic assays (186). Hence that study increased the specificity of the SYBR green assay with biprobes (sequence specific hybridisation probes) labeled with fluorophore Cy5. Confirmation of the identity of the amplified product was achieved by subsequent melting temperature determination of the biprobe dissociating from the target sequence. However, quantification of the target was not possible.

- There are four major targets for qPCR-based assays: the 18S and 28S rRNA genes, the internal transcribed spacer (ITS) and mitochondrial DNA. Studies 1, 3, 4, 6, 7, 10b target the 18S rDNA (or nuclear small subunit) ribosomal gene. Study 8 targets the 28S rDNA ribosomal gene. These regions are highly conserved among *Aspergillus* species, making them ideal targets for a broad range assay. Also, the ribosomal gene is present in multiple copies within the genome, which increases the sensitivity of the assay (187, 188). However, although constant in the same strain, the ribosomal gene copy numbers are variable among different *A. fumigatus* strains, and range from 38-91 in the eight strains analysed (189). This will complicate attempts to quantify fungal load with PCR assays targeting ribosomal gene regions, and may make it more difficult to compare fungal load between patients. It will also affect the apparent LOD. The ITS regions, targeted in studies 2, 9 and 10a, are more heterogeneous (190, 191) and are therefore suitable targets for species-specific primers and probes. Study 5 targeted a mitochondrial gene, which is another multiple copy gene target (192).

### *3.2.3 Assay specificity*

Studies differ considerably in terms of the methods and extent to which analytical specificity is determined, as there are no standard techniques or criteria. Although primer targets are generally identified by aligning sequences retrieved from public databases, this *in silico* approach is insufficient for establishing the analytical specificity of a PCR assay. Ideally, amplicons should be sequenced and that sequence should be used to verify that the intended target has been amplified. Furthermore, it is good practice to challenge the assay with organisms that have a high likelihood of cross-

reacting with the target; in the case of *Aspergillus spp*, genera that are close phylogenetic relations—eg, *Penicillium* and *Paecilomyces* spp are especially important to consider. A further consideration is that sequences are being continuously deposited in public databases; a unique sequence at the time of primer design may subsequently align with a sequence from an unrelated species or genus deposited at a later date. Also, some of the deposited sequences or sequence assignments may simply be incorrect.

After designing oligonucleotide primers, a Primer BLAST search (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) should be performed to determine the specificity. A Primer-BLAST search was performed for the primer pairs used in each study. Unsurprisingly, since there is 100% sequence identity across the 18S subunits of *Aspergillus* and *Penicillium,* the primers of studies 1, 2, 3, 4, 6, 7 and 10b amplify *Penicillium* and *Paecilomyces* species. Of these, only studies 2 and 7 ensure *Aspergillus* specificity of detection, through biprobe melt analysis. The probes in studies 1, 3, 6 and 10b do not provide the necessary specificity to exclude Penicillium and Paecilomyces species. An example is shown in Figure 14, in which 18S ribosomal RNA gene partial sequences from two *Penicillium* strains and an *A. terreus* strain are aligned, with the primer and probe binding sites highlighted. This shows 100% homology across the three sequences, indicating that the *Penicillium* strains will also be amplified and detected in this assay.

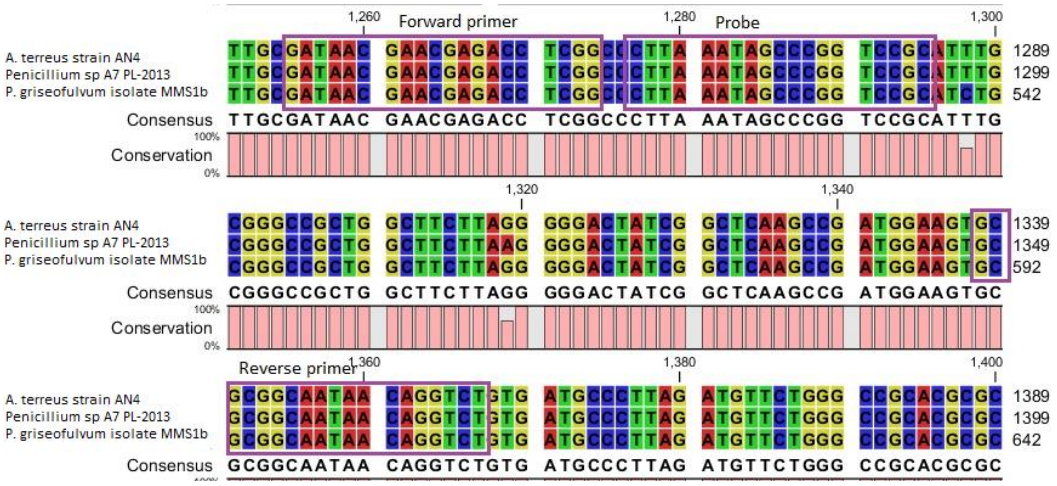



**Figure 14. 18S ribosomal RNA gene partial sequences aligned in CLC Sequence Viewer.**

Primer and probe binding sites for study 1(177) highlighted.

The primers of studies 3, 6, 7 and 10b also amplify *Candida* species, generating an amplicon of approximately the same size as the target *Aspergillus* species, however the probe probably adds the necessary specificity to exclude *Candida* detection, due to base mismatches within the sequence. An example is shown in Figure 15, in which 18S ribosomal RNA gene partial sequences from two *Candida* strains and an *Aspergillus terreus* strain are aligned, with the primer and probe binding sites highlighted. This shows 100% homology across the forward and reverse primer binding sites for three sequences, indicating that the assay primers would bind to and amplify *Candida* species. However, there are 4 single base pair mismatches between the *Candida* sequences and the assay probe, so the probe will probably add the necessary specificity to exclude *Candida* species from detection. This would need to be confirmed empirically. However, if the primers amplify more than one target, PCR efficiency could suffer and the assay's sensitivity could be reduced.

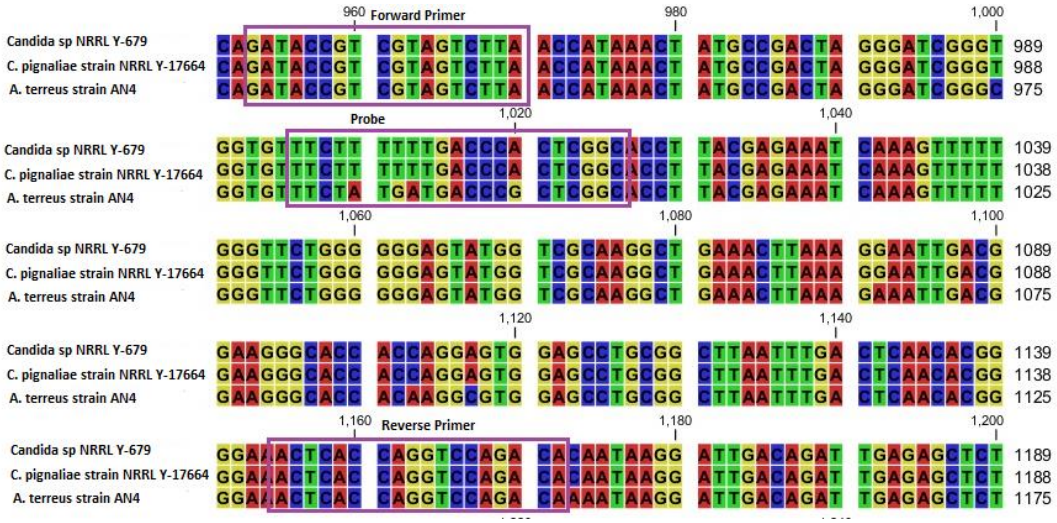



**Figure 15. 18S ribosomal RNA gene partial sequences aligned in CLC Sequence Viewer.**

Primer and probe binding sites for study 3 (158) highlighted.

The primers from study 8 have only a single base mismatch with *Candida* at the 5' end of each primer and therefore the assay is also likely to amplify *Candida* species, however, the probe adds the necessary specificity to exclude *Candida* detection, although again this must be confirmed empirically. Study 3 targets *Aspergillus* species, but the sequence alignment analysis presented in Figure 16 shows that there are several mismatches to *A. nidulans* and *A. niger* in the molecular beacon. Therefore the assay may not detect these species efficiently.

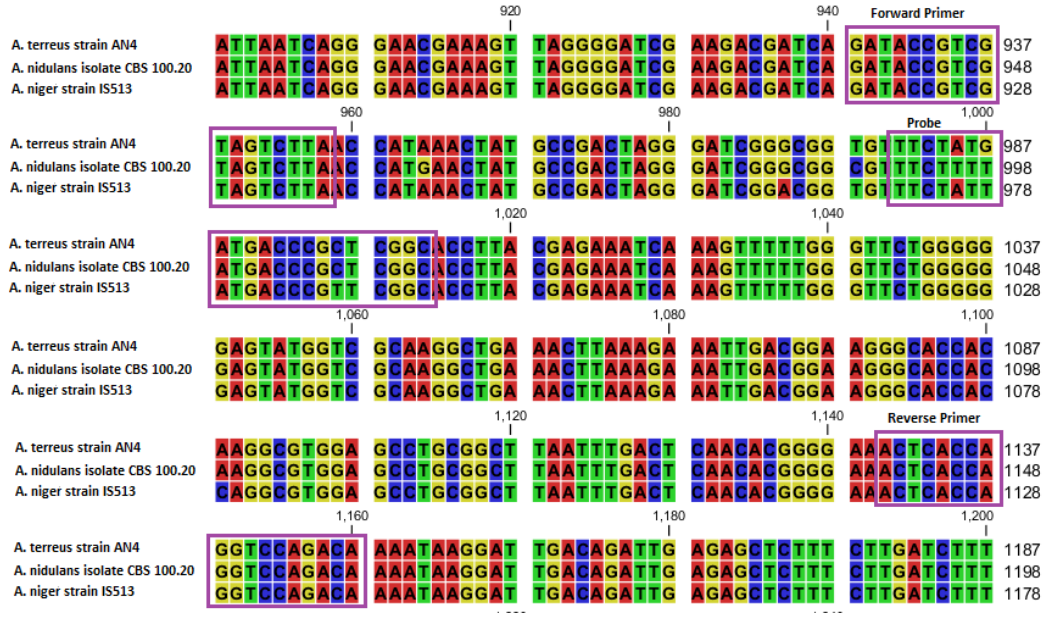



**Figure 16. 18S ribosomal RNA gene partial sequences aligned in CLC Sequence Viewer.**

Primer and probe binding sites for study 3 (158) highlighted.

Study 4 also targets *Aspergillus* species, but primer analysis shows there is 1 mismatch to *A. flavus* at the 3' end of the reverse primer and 2 mismatches to *A. nidulans* in the reverse primer, therefore the assay may also not amplify these species efficiently. Crucially, sequence analysis reveals that the probe in study 4 does not bind between the forward and reverse primer binding sites (Figure 17). The probe does not bind the 124bp target amplicon at all, hence from a technical point of view it is therefore not possible for that assay to detect any amplicons. A correction of the probe sequence was published online in July 2005, two years after the publication of the original article. However, the publication of the original article in a peer-reviewed journal, with incorrect probe sequence, highlights the importance of the rigorous auditing and reporting of experimental detail that the MIQE guidelines promote.


**Figure 17. *A. fumigatus* strain MJ-X6 18S ribosomal RNA gene partial sequence.**

Primer and probe binding sites for study 4 (179) highlighted.

Study 8 also targets *Aspergillus* species, but primer analysis shows there is 1 mismatch to *A. nidulans* in the reverse primer and 2 mismatches in the probe, therefore the assay may not amplify and detect these species efficiently. The authors of study 5 state that their assay targets *A. fumigatus* and *A. flavus* mitochondrial DNA. However, analysis of the primer pair reveals that they do not amplify *A. flavus* at all.

Study 6 is one of two assays deemed to be optimal by the United Kingdom Fungal PCR Consensus Group (118). However, the reverse primer from study 6 anneals to human DNA and the forward primer anneals with a single base mismatch at the 3' end, therefore the assay may amplify human DNA, generating a 163bp product (Figure 18). Although the probe adds the necessary specificity to exclude detection of human DNA, this would be expected to reduce the sensitivity of the assay. This was indeed acknowledged by the United Kingdom Fungal PCR Consensus Group (118), who reported that the detection threshold of this assay was significantly reduced when using blood

extracts, and speculated that this was possibly as a result of amplification of human DNA. They also sequenced the amplicons and revealed the high sequence similarity with the human 18S rRNA gene. This cross homology with human DNA makes the assay sub-optimal.

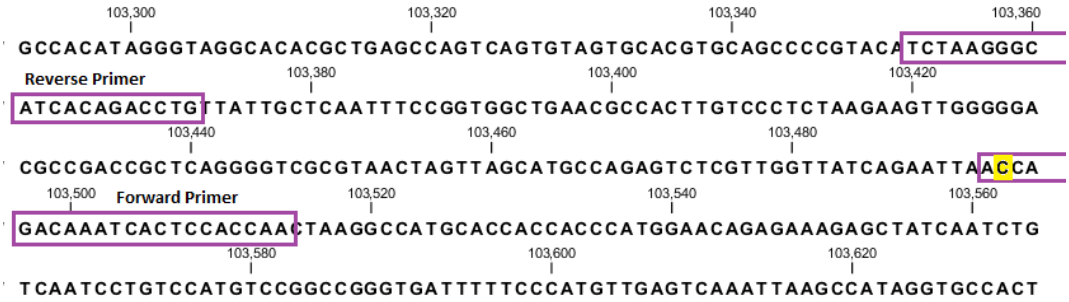



**Figure 18. *Homo sapiens* BAC clone RP11-237A9 partial sequence.**

Primer binding sites for study 6 (181) highlighted. A single base mismatch in the forward primer is highlighted in yellow.

Study 9 targets *Aspergillus* species using broad range primers and adds species specificity by using hybridization probes for melt point analysis, to detect i) *A. fumigatus, A. versicolor, A. nidulans,* ii) *A. terreus, A. niger*, iii) *A. flavus*. However, primer-BLAST analysis shows that the primers are in fact *A. fumigatus*-specific and do not amplify any other *Aspergillus* species.

Study 10a uses hydrolysis probes and primers targeting *A. fumigatus, A. flavus, A. niger* and *A. terreus* specifically in individual assays. However, inspection reveals that the primers and probe of the '*A. fumigatus*-specific assay' will also bind *A. lentulus*, with only a single mismatch in the reverse primer (Figure 19). Therefore, this may be amplified and detected. This would need to be tested empirically. The '*A. flavus*-specific assay'

will also amplify and detect *A. oryzae*, as the primers and probe bind with 100% homology (Figure 20). The '*A. niger*-specific assay' will also amplify and detect *A. tubingensis* (Figure 21). *A. tubingensis* belongs to the *Aspergillus* section Nigri, which includes species that morphologically resemble *Aspergillus niger*. However, sequencing identifies it as a separate species in its own right. Study 10 highlights the difficulty in species-specific assay design, with sequencing identifying new, genetically distinct species that are often morphologically indistinguishable from their relatives.

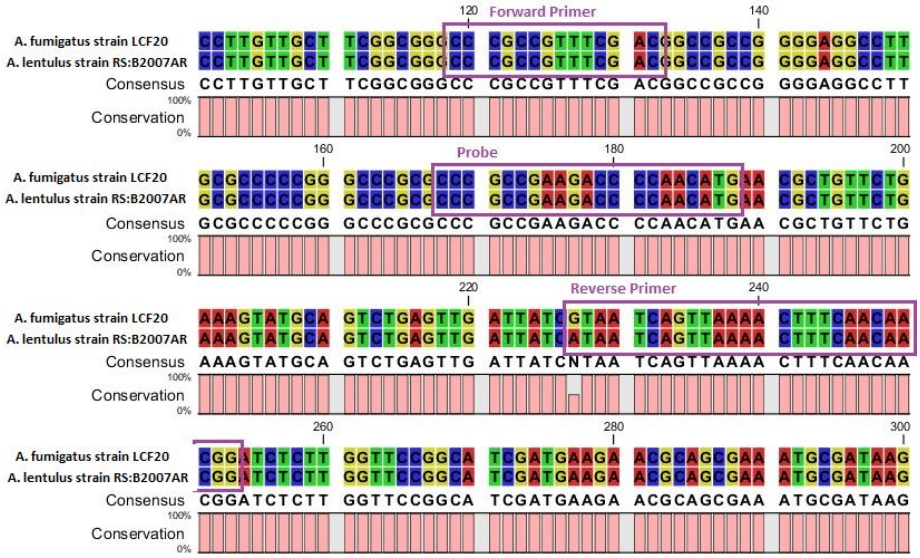



**Figure 19. ITS1 sequences aligned in CLC Sequence Viewer.**

Primer and probe binding sites for the '*A. fumigatus*-specific assay' in study 11(185) highlighted. A single base mismatch in the reverse primer is highlighted.

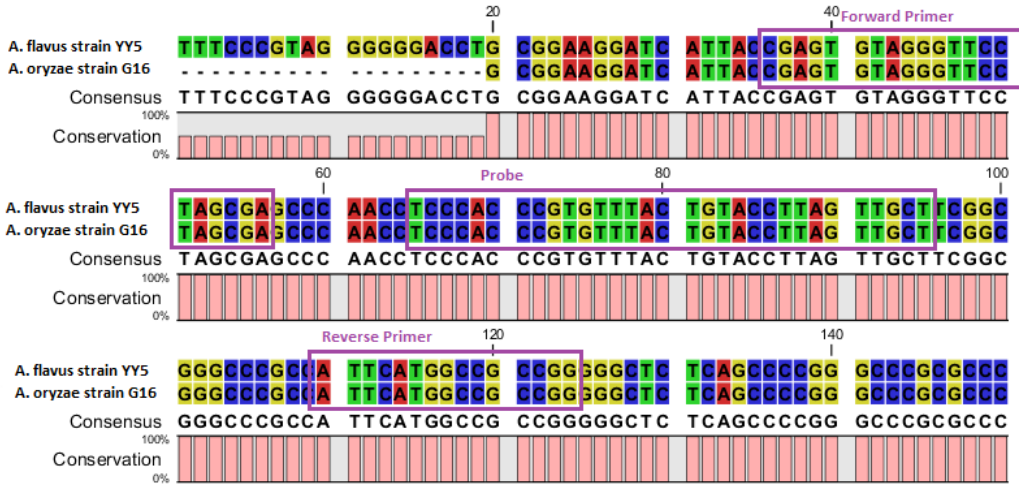



**Figure 20. ITS1 sequences aligned in CLC Sequence Viewer.**

Primer and probe binding sites for the '*A. flavus*-specific assay' in study 11(185) highlighted.

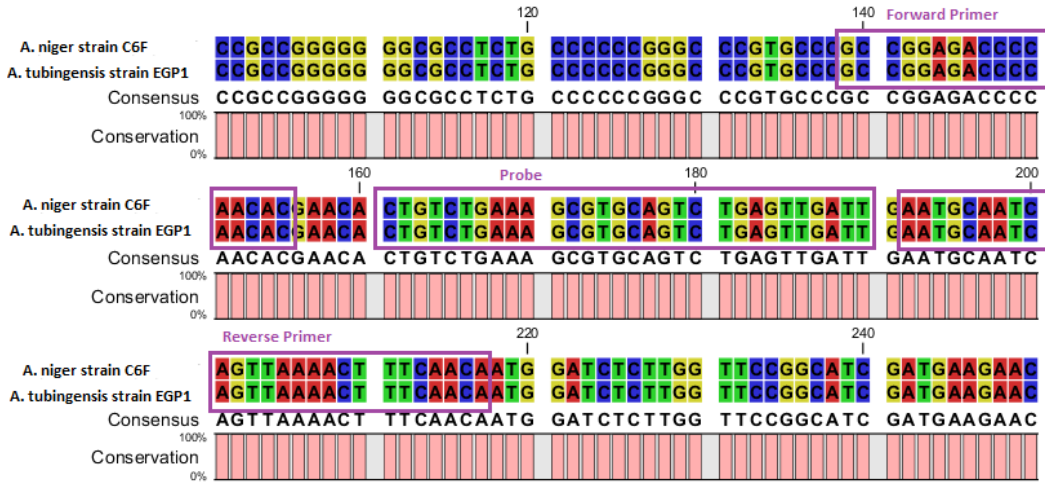



**Figure 21. ITS1 sequences aligned in CLC Sequence Viewer.**

Primer and probe binding sites for the '*A. niger*-specific assay' in study 11(185) highlighted.

### *3.2.4 Primers characteristics and performance*

Efficiency of a PCR reaction determines analytical sensitivity and reliability in quantification of the assay. A good PCR assays should have an efficiency of 90% or more, with optimisation making an efficiency of close to 100% fairly easy to achieve for most assays.

Efficiency of a PCR assay depends on a number of factors. The most important factors are size of amplicon and absence of primer and amplicon secondary structures (193).

Shorter amplicons generally result in a more efficient PCR, with amplicon sizes of below 100-150bp optimal and 60-70bp amplicons increasingly common. A comparison of amplicon size was made between the reviewed studies. If the size of the amplicon or the exact location on the targeted sequence was not given in the paper, it was deduced from mapping the primers set on an *A. fumigatus* 18S ribosomal RNA gene sequence. 8 of the 10 studies reported results from an assay with an amplicon size over 150bp. A frequently used strategy in *Aspergillus* qPCR assay design is to adopt previously designed primers from conventional PCR assays, as used in study 7. This approach leads to suboptimal qPCR as the amplicons are relatively large (greater than 500bp in assay 7), since the primary means of detection by "legacy" PCR is to separate amplicons by gel electrophoresis according to their size.

Another reason for avoiding the use of "legacy" primers is that they have not been designed to avoid areas of target secondary structure. Target accessibility to primer annealing is an important parameter that is best be checked *in silico* as efficient qPCR assays require primers annealing to areas of minimal secondary structure. Useful analysis tools for determination of secondary structures of RNA or DNA molecules are found on the MFOLD web server (http://mfold.bioinfo.rpi.edu/). MFOLD was used to determine the likelihood of primer binding at sites of secondary structure (160). Folding of the amplicons from studies 2, 4, 5, 8, 9 and 10a (*A. flavus, A. fumigatus* and *A. terreus*) shows that primer binding is likely to occur at sites of secondary structure, as shown in Figure 22.

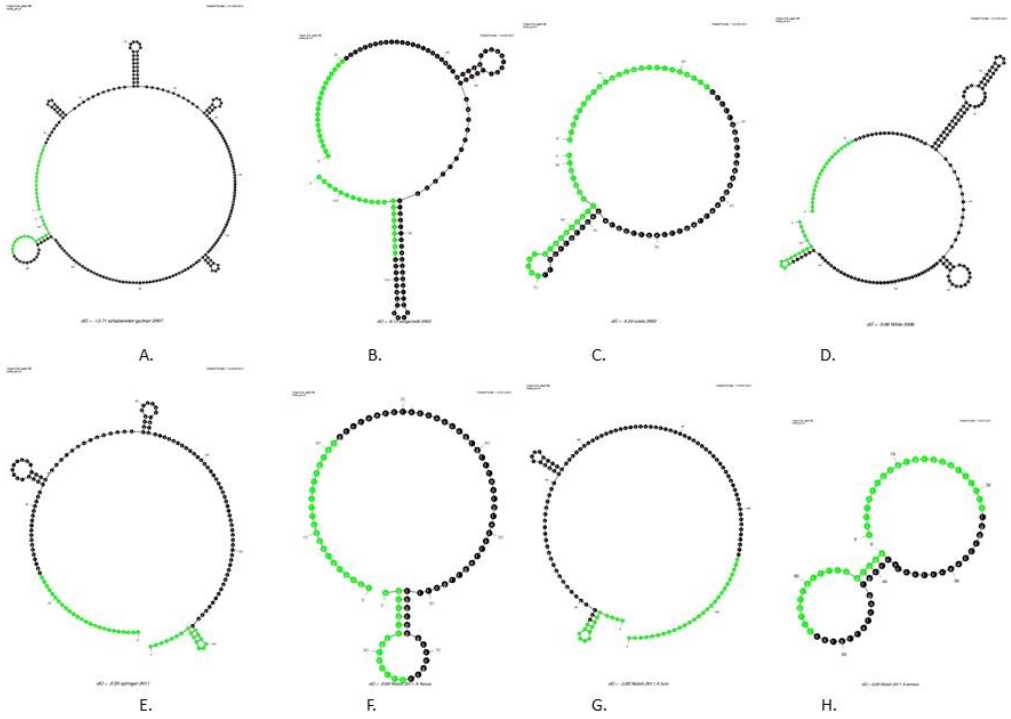



**Figure 22. MFOLD analysis results of analysed papers.**

Results display amplicons with primer binding sites at locations of secondary structure. Primer binding locations shown (green). A. Study 2; B. Study 4; C. Study 5; D. Study 8; E. Study 9; F. Study 10a (*A. flavus*); G. Study 10a (*A. fumigatus*); H. Study 10a (*A. terreus*).

Study 5 quotes a PCR efficiency of 77%, despite amplifying a short region of DNA (91bp); however, structure analysis of the assay reveals a large stem structure at the 3'-end of the amplicon. This secondary structure incorporates 15 bases of the 22 base reverse primer binding site. Formation of this secondary structure could reduce the ability of the primer to bind its target sequence, resulting in decreased product yield or a failed reaction. It is likely that inefficient primer annealing at the site of secondary structure is reflected in the low PCR efficiency reported by this assay.

### *3.2.5 Type of standards*

Assay efficiency can be calculated from the slope of the standard curve using the following equation:

$$\textbf{PCR efficiency} = 10^{-1/\text{slope}} - 1$$

The preparation of reliable standards is essential for qPCR, when the standard curve is used as a method for absolute quantification in unknown samples. Accurate pipetting is essential for performing a dilution series and aliquots should be stored in low volume to minimize freeze-thawing and ensure the stability of the standards. Genomic DNA and plasmids containing cloned target sequences are commonly used as standards in qPCR. Studies 4, 6, 8 and 10 use recombinant DNA, with dilutions of the purified plasmid target gene. Extreme caution should be exercised when handling plasmid sequences, as they are present in a very high concentration and therefore pose a high risk for PCR contamination. Studies 1, 3 and 5 use genomic DNA, extracted from pure *Aspergillus* culture. Concentration of the target gene is calculated by identifying the genome size of the organism of interest, identifying the mass of DNA per genome using the equation:

**m = (n) (1.096e-21g/bp)** **where n = plasmid size (bp), m = mass, e-21 = x10$^{-21}$**

Then dividing the mass of the genome by the copy number of the gene of interest per haploid genome, calculating the mass of genomic DNA containing the copy numbers of interest.

A limitation of the use of genomic DNA or plasmid DNA in standard curve preparations is that they only reflect the analytical sensitivity of the qPCR itself. As the DNA and plasmid dilutions are added directly to the PCR reaction, they do not control for issues of extraction efficiency or the effect of potential inhibitors in the clinical samples.

Studies 7 and 9 use standards prepared by spiking known amount of *Aspergillus* conidia into human blood. DNA is extracted prior to running the qPCR assay. This method will assess the analytical sensitivity of the DNA isolation method in combination with the qPCR assay. It will reflect the analytical sensitivity of the DNA isolation method in releasing *Aspergillus* DNA from fungal cells. Hence, it is difficult to compare the performance of assays that use different type of standard, unless they are using identical DNA isolation protocols. Study 2 uses both genomic DNA dilutions to assess qPCR assay sensitivity and conidia spiking dilutions in blood to assess extraction efficiency.

### *3.2.6 Assay efficiency and limit of detection (LOD)*

It is often impossible to ascertain the reliability of published data, as (i) NTC data are frequently omitted, (ii) detailed information about LOD is lacking or obscured to allow for so-called 'background amplification' and (iii) PCR efficiencies are not reported. Despite being an essential piece of information about the performance of the assay, only studies 5, 6, 8 and 10 had included their assay efficiency or the slope of the standard curve in their article. An attempt was made to estimate the efficiency of Study 4, using the amplification plot (see Appendix 3).

Study 4 had an estimated efficiency of 118%, which is theoretically impossible, rising to 130% if the neat sample is excluded. This may be due to non-specific amplification (e.g.

primer dimers or secondary products), poor pipetting or both. The broad range *Aspergillus* assays from studies 6 and 10 reported acceptable efficiencies of 91.6% and 95.8% respectively.

Study 1 had the lowest reported LOD of 1 target gene copy. However, this is reported at Cq 41, which is very late and would suggest sub-optimal amplification efficiency. Studies 2, 7, 8 and 9 determined the LOD with conidia spiked in blood. The LOD ranged from 5-20 colony forming units (CFU) per ml of blood, which was equivalent to 0.5-6 CFU per PCR reaction.

An efficient and reliable probe-based qPCR assay does not detect amplification in NTC wells. Hence, the LOD is determined by the highest dilution of a standard curve that results in reliable amplification. This can be as low as one copy of DNA, in which case Poisson's law of small numbers predicts that six out of ten amplification replicates should be positive. The theoretical threshold should not be reported if it is greater than the results can actually demonstrate. NTCs are essential to monitor contamination during the extraction stage. Amplification detected in these wells is either due to primer-dimers or contamination with target DNA. Alternatively, the increase in fluorescence that is seen in the reaction may not be due to actual amplification; instead it may reflect degradation of the probe. Despite NTC data being an essential piece of information about the performance of the assay, only 5 of the 10 reviewed studies mention the use of NTCs. Of these, only two studies actually report the results of the NTC reactions. Study 7 reports that there was no amplification in the NTC wells. Study 1 reports that all NTC reactions were negative. However, in the context of their paper, this does not mean that there was no amplification in the NTC wells. This study ran their qPCR assay for 45 cycles and assigned a positive cut-off of Cq 41, which they calculated to be the LOD of the assay and approximately equivalent to a single copy of

the target 18S rRNA gene. The authors did not claim that the NTCs did not amplify, merely that they crossed threshold at Cq>41. Ideally, signal amplification should not be observed at all in the NTC wells. If the NTCs do cross the threshold, their Cqs should be at least five cycles from the Cqs of a 'positive' sample (194). Any amplification in an NTC well will complicate interpretation of results, particularly when fungal load in a clinical sample is low and detection occurs at the limits of sensitivity of the assay.

### *3.2.7 Summary*

qPCR has become the most widely used molecular technology for diagnostic applications designed to detect and quantify pathogens. Key reasons include its accessibility, sensitivity, specificity, and wide linear dynamic range. One potential application is its use for the detection of IA, caused by *Aspergillus* fungi. Although the high mortality rate associated with IA and complications in surviving patients make early diagnosis and treatment essential for adequate therapeutic management, its definitive diagnosis is hampered by the limited reliability of current diagnostic tools.

A wide variety of methods, targeting different DNA regions, have been reported, each with its own merits. In the setting of IA, it is vital to maximise assay efficiency for single target copy detection, with sufficient controls to monitor for contamination. It is apparent from the reviewed studies that assays are falling short of their potential sensitivity and specificity due to inadequate design and controls. Furthermore, it has become increasingly clear that PCR-based assays in general are compromised by their variable performance as well as lack of transparency in reporting experimental details. These variables hinder the interpretation of diagnostic performance and so impede the widespread acceptance of

qPCR-based technology in clinical diagnostics. These variables are addressed by the MIQE guidelines (195, 196).

Together with optimization and ideally standardisation of sample collection and nucleic acid extraction methods, improved qPCR assay design and reporting are critical parameters to address, if endorsement by the EORTC/MSG is to be achieved for the diagnosis of IA.

## 3.3 RESULTS: A MIQE-compliant real-time PCR assay for *Aspergillus* detection

### *3.3.1 Assay characterisation*

#### 3.3.1.1 Primers and amplicon

Accession numbers were obtained for the target organisms *A. fumigatus* (Accession no FJ 840490), *A. flavus* (Accession no: D63696), *A. terreus* (Accession no. DQ173743) and *A. niger* (Accession no. GQ338836), and aligned to identify suitable target sequences [Section 2.3.1]. Primers and a probe with LNA modifications were designed by Beacon Designer, version 7.2 [Section 2.3.2]. Selected primer and probe sequences and binding sites are shown in Figure 23. *In silico* analysis of primer binding sites was performed using Primer-BLAST [Section 2.3.3]. Unsurprisingly, since there is 100% sequence identity across the 18S subunits of *Aspergillus* and *Penicillium*, the primers of the new assay amplify *Paecilomyces* and *Penicillium* species, including the clinically significant *Talaromyces marneffei*. The assay probe does not provide sufficient specificity to exclude these species. There is no significant similarity with other clinically relevant fungal pathogens and no similarity to the human genome.

The primers generate a 76bp amplicon. Target secondary structures and primer/template accessibility were assessed using the MFOLD web server [Section 2.3.4]. Folding of the amplicon shows that the forward primer binding site is predicted to be free of secondary structure, as shown in Figure 24. The program predicts secondary structure in the form of a stem loop at the 3' end of the reverse primer binding site. However, ΔG is -1.06, suggesting that formation of this double stranded state is unlikely to occur.

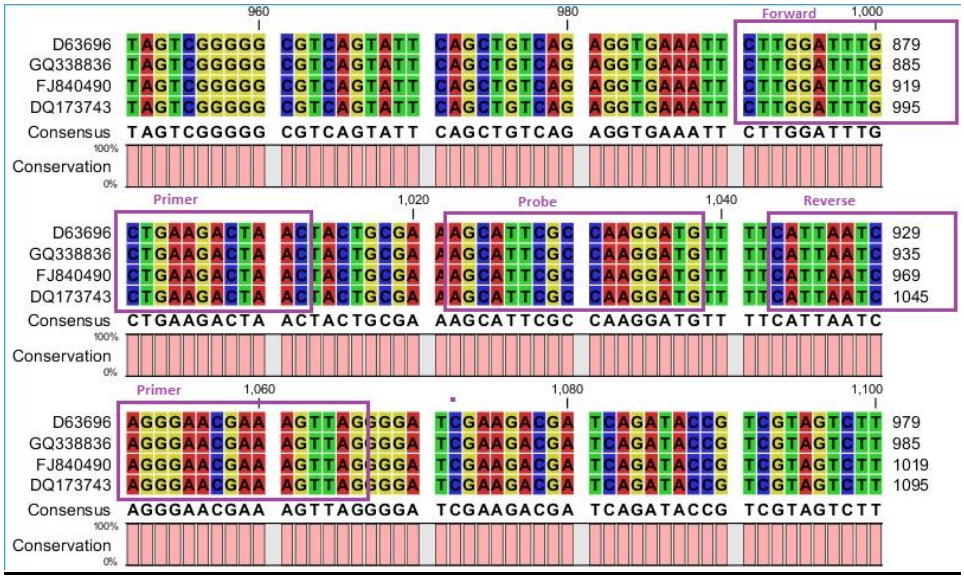
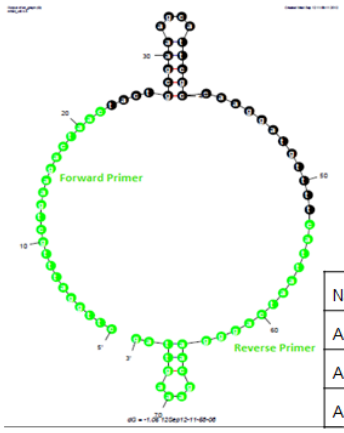



**Figure 23. 18S ribosomal RNA gene partial sequences aligned in CLC Sequence Viewer.**

Primer and probe binding sites for the new assay design highlighted.




| Name | Purpose | Sequence 5' to 3' |
|---|---|---|
| Asp-rv | Reverse primer | 5'-CTAACTTTCGTTCCCTGATTAATG-3' |
| Asp-fw | Forward primer | 5' –CTTGGATTTGCTGAAGACTAAC-3' |
| Asp-LNA | LNA fluorescent probe | 5'FAM- catCctTggCgaAtgct-3'BHQ-1 |

**Figure 24. MFOLD analysis results of the new assay.**

Results display amplicon with primer binding sites shown (green). Primer and probe sequences are shown. Upper case letters of probe sequence indicate position of LNA-incorporated oligonucleotides.

### 3.3.1.2 Annealing temperature and primer concentration

Optimal primer annealing temperature and primer concentration was investigated [Section 2.4.1] using *A. fumigatus* DNA template [Section 2.2.2] and established a wide optimum primer annealing temperature range of 55-61.4°C (Table 2). The annealing temperature was set at 59°C for subsequent reactions. Optimal primer concentration was calculated to be 200nM of each primer.

| Annealing temperature (°C) | Cq values (cycles) |
|---|---|
| 65 | 29.5 |
| 64.5 | 27.2 |
| 63.3 | 25.1 |
| 61.4 | 24.2 |
| 59 | 24.1 |
| 57 | 24.2 |
| 55.7 | 24.5 |
| 55 | 24.2 |

**Table 2. Temperature gradient analysis of the primers with *A. fumigatus* DNA template.**

Analysis performed using SYBR Green.

### 3.3.1.3 Analytical Specificity

DNA was extracted from clinically relevant fungal isolates [Section 2.2.2] and qPCR performed under optimised conditions for hydrolysis probe reactions [Section 2.4.2], to evaluate the analytical specificity of the qPCR assay. The assay detects *A. fumigatus*, *A. flavus. A. niger, A. terreus* and *A. nidulans* DNA (Figure 25); it does not detect *C. albicans, C. dubliniensis*, *S. prolificans, F. solani* and *R. oryza*e DNA. No amplification was seen with human DNA (study blood samples [Section 2.1.1.4.3], DNA extracted [Section 2.2.3], tested negative by *Aspergillus* qPCR [Section 2.4.2] and positive by human β-globin qPCR]) or in any NTC well [Section 2.4.4].

Assay specificity was validated using SYBR Green I chemistry and melt curve analysis [Section 2.4.1] of *A. fumigatus* genomic DNA, indicating a melting temperature of 77°C for the target amplicon (Figure 26). PCR products were analysed [Section 2.4.8], suggesting amplification of the appropriate target sequence.

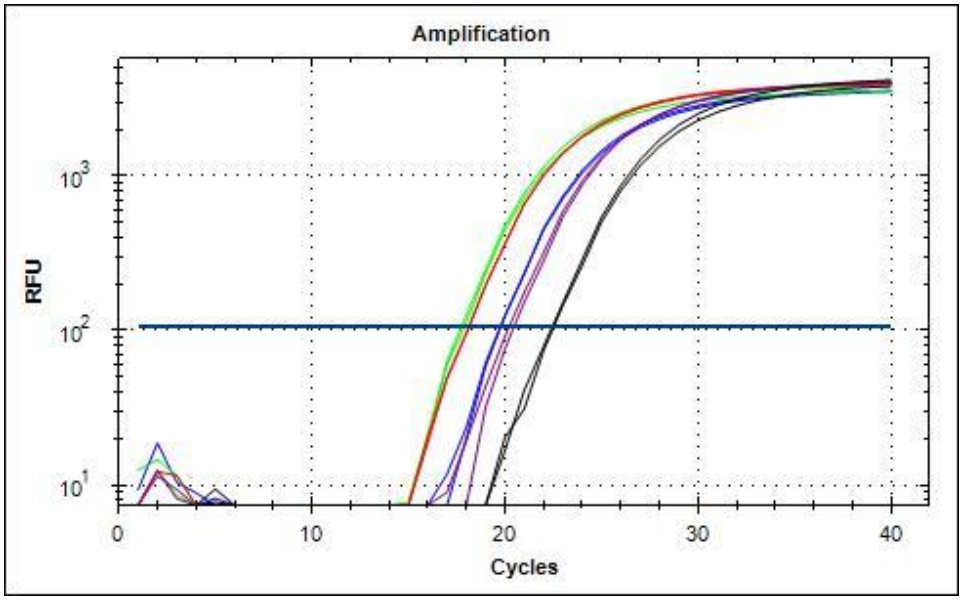



**Figure 25. 18S assay amplification curve for *Aspergillus* species.**

*A. flavus* (green trace), *A. terreus* (red trace), *A. nidulans* (purple trace), *A. fumigatus* (blue trace) and *A. niger* (black trace). *A. flavus* and *A. terreus* were assayed at similar concentration ($2x10^5$ genomes load). In order to make the amplification plots more visible, *A. nidulans* and *A. fumigatus* were run at $6x10^4$ and *A. niger* at $1.5x10^4$ genomes loaded.

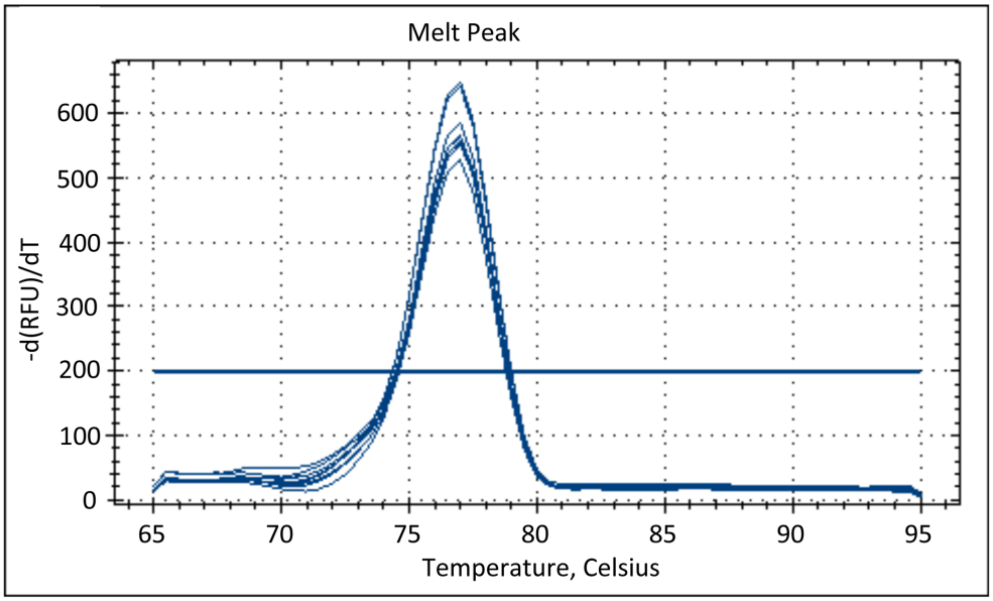



**Figure 26. Dissociation (melt) curve analysis of amplification products.**

*A. fumigatus* genomic DNA dilutions of 2 ng ($6x10^4$genomes), 200 pg ($6x10^3$ genomes) and 20 pg ($6x10^2$ genomes). Melting temperature of 77°C.

### *3.3.2 PCR efficiency/ analytical sensitivity*

*A. fumigatus* DNA was extracted from a clinical isolate [Section 2.2.2] and qPCR performed under optimized conditions for hydrolysis probe reactions [Section 2.4.2], to calculate PCR efficiency, the linear range of the assay and the limits of detection and quantification [Section 2.4.3]. PCR efficiency range was 95-107% (Figure 27). Dilutions spanned six orders of magnitude from 2ng ($6x10^4$ genomes) to 20fg (0.6 genomes). 0.6 genomes give rise to an appropriate amplification product in every assay.

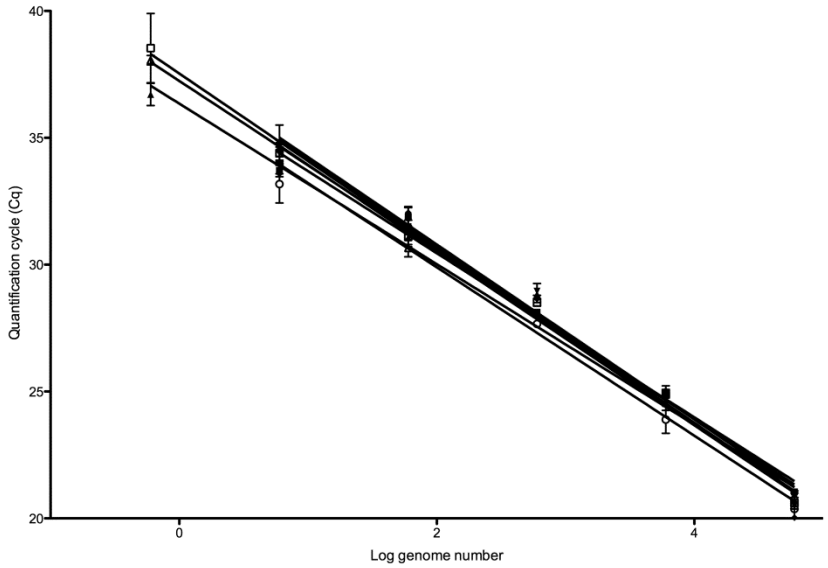



**Figure 27. Eight standard curve plots, generated using *A. fumigatus* genomic DNA.**

### *3.3.3 Reproducibility*

Reproducibility was high across all six orders of magnitude, with an inter-run standard deviation that ranged from 0.37 at an average Cq of 20.66 (for $6x10^4$ genomes load) to 0.97 at an average Cq of 37.77 (for 0.6 genome load). Limits of quantification and detection were calculated as 6 and 0.6 *A. fumigatus* genomes, respectively.

### *3.3.4 Clinical Evaluation*

#### 3.3.4.1 Histological material

Histological material was obtained from three patients with haematological malignancies [Section 2.1.1.4.4]. Total DNA was extracted from these samples [Section 2.2.1] and used to evaluate the *Aspergillus* qPCR assay [Section 2.4.2], with all extracts loaded in duplicate wells (Table 3). A post-mortem lung biopsy (sample A) and an ante-mortem brain biopsy

(sample B), from two patients with proven IA, amplified with mean Cqs of 24.6 and 31.5, respectively. Referring to the standard curve, this equates to 35 and two *A. fumigatus* genome equivalents/ng DNA extract, respectively. A control post-mortem lung biopsy with no evidence of fungal infection (sample C), did not amplify.

| Tissue sample | Diagnosis | Mean Cq | DNA load of extract (ng/µl) | *A. fumigatus* genome equivalents/ng DNA extract |
|---|---|---|---|---|
| A | Proven IA | 24.6 | 83 | 35.5 |
| B | Proven IA | 31.5 | 13 | 1.8 |
| C | No fungal infection | Negative | 172 | 0 |

**Table 3. qPCR results when testing DNA extracts from histological samples.**

A (post-mortem lung biopsy), B (ante-mortem brain biopsy) and C (post-mortem lung biopsy). Genome equivalent of PCR result calculated from *A. fumigatus* standard curve plot.

### 3.3.4.2 Inhibition testing

Each of the DNA extracts from histological material was included in a SPUD assay [Section 2.4.6] and results indicated that no inhibition was present in these samples (Figure 28).

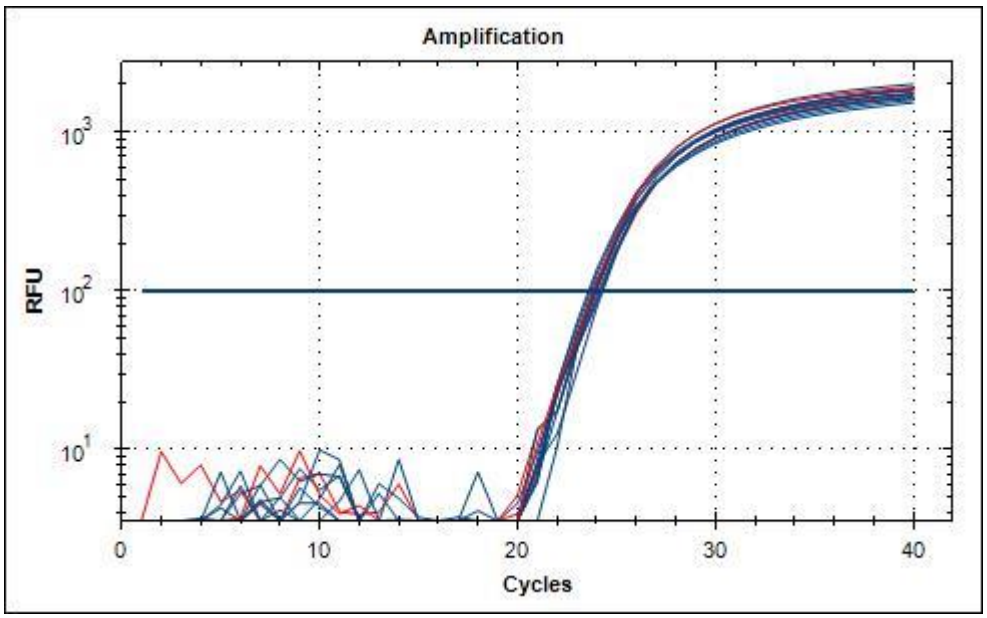



**Figure 28. SPUD assay amplification curve for histological samples.**

Samples A, B and C (blue traces). Reference Cq value for the SPUD amplicon, characteristic of an uninhibited assay (red trace).

## 3.4 DISCUSSION

This chapter describes 3 key findings;

- the lack of rigorous standards of practice has resulted in widespread publication of poor qPCR data, resulting in inappropriate conclusions
- In the context of reliable detection of pathogens in general, and *Aspergillus* in particular, the major limitation of qPCR is the lack of standardisation at every stage of the molecular process from sample type and processing to interpretation of results
- when optimised, qPCR is an extremely sensitive and highly specific tool to aid in IA diagnosis

PCR is widely used as a diagnostic tool in clinical laboratories and is particularly effective for detecting and identifying infectious agents for which routine culture and microscopy methods are inadequate. *Aspergillus*-specific qPCR assays have been proposed as alternatives to conventional diagnostic procedures for IA, where early diagnosis and treatment are critical (114, 177, 197). However, the EORTC/MSG do not endorse the routine use of PCR in the defining of IA (50). There are several reasons for this: (i) disparate chemistries that include the use of SYBR Green (198), although it lacks the required specificity for use in clinical diagnostic assays (186), hydrolysis probes (179, 181, 199), hybridisation probes (180) and molecular beacons (175, 200); (ii) variability of targets (28S, 18S and ITS2) (114, 181, 201); (iii) sample-specific instrument-dependent variability (116, 118), including contamination-prone nested PCR methods aimed at enhancing analytical sensitivity (169, 180).

*Aspergillus* qPCR assay design has also been hampered by the use of primers designed for conventional PCR assays (180, 182, 201). This can lead to suboptimal qPCR as the amplicons are relatively large and no structural analyses of primers or primer binding sites have been carried out. These parameters have a strong influence on PCR efficiency and determine analytical sensitivity and reliability of quantification (193). Hence it is not surprising to note that one report quotes a PCR efficiency of 77% (180), despite amplifying a short region of DNA (91bp). Structure analysis (160) of the amplicon revealed a large stem structure at its 3'-end. In a setting that requires low copy number detection, it is vital to have contamination assessment at the DNA extraction stage. Negative extraction controls (no DNA template present) are extracted alongside clinical samples, to detect any cross contamination or environmental contamination. A significant number of studies fail to use or report the use of negative extraction controls to monitor contamination during the extraction stage and, even if they do, they are usually water-only, as opposed to

controls that have similar properties to the clinical sample but without target nucleic acid (166). Ideally, biological (sample) replicates should also be included, especially when the qPCR results suggest very low levels of fungal DNA. Even though it is well known that PCR inhibitors can reduce product yield and even result in complete failure of the PCR, only just over half of the studies evaluated by Khot et al (166) reported the use of some form of an inhibition control, also referred to as an amplification control.

With any assay targeting *Aspergillus*, the risk of procedural contamination is high, and false-positive results may arise due to the ubiquitous nature of fungi in the environment, as well as product carryover from positive samples or from cross-contamination by PCR products from earlier experiments (166). Therefore, rigorous procedures were instituted for the monitoring of contamination during sample handling, DNA extraction and PCR set up [Sections 2.2, 2.4.4 and 2.4.5]. Reagents were tested for contamination on a regular basis and a separate biosafety cabinet was used for the reaction setup. Multiple negative controls were included, i.e. samples that are as similar to the test samples as possible but exclude the target. The SPUD assay (161) was used, as inhibition assessment is mandatory for all clinical diagnostic qPCR assays. The inclusion of such rigorous experimental controls has enabled the avoidance of procedural false positivity and false negativity.

This chapter describes the development of an assay that is not just rapid, sensitive and specific, but that rigorously complies with the MIQE guidelines, both in design and in its reporting. The resulting assay satisfies all of these criteria essential for a clinically useful molecular assay:

A. Speed: Following extraction of DNA, results from the qPCR assay are available within 60 min. The development of fast PCR reagents, together with the use of smaller reaction volumes and novel thermocyclers has the potential to reduce this to below 5 minutes. Indeed, the fastest PCR currently completes 30 cycles in 17 seconds (Carl Wittwer, pers. commun.)

B. Sensitivity: analytical sensitivity is a critical parameter of any diagnostic qPCR assay since the fungal load in the blood of patients with IA is believed to be very low (30-100fg/µl or less) (180, 199, 202, 203). Analytical sensitivity refers to the smallest number of nucleic acid molecules that can be detected and distinguished from a zero result. This is best done using a standard curve, which defines the range of the assay and hence the upper and lower target concentration that can be reported. The new assay design presented in this chapter targets an amplicon devoid of secondary structure, has been extensively optimised, is linear over at least six logs of template concentration and has an efficiency close to 100%. Its limit of quantification is 6 *Aspergillus fumigatus* genomes, whereas its limit of detection is 0.6 genome copies. Since a single *A. fumigatus* genome contains between 38 and 91 target rDNA sequence copies (189), this suggests the new assay can detect as few as 23 target copies (189).

C. Specificity: analytical specificity is determined by identifying the percentage of samples without the target sequence that generate a positive result. If a well-designed assay is used, in the absence of contamination, this will be zero. In a qPCR experiment all detectable products, be they specific or non-specific, contribute to the final

amplification plot and hence any qualitative or quantitative result. Post reaction melt analysis using SYBR Green I dye confirmed the specificity of the assay, since it resulted in a single peak and sequencing of the amplicons confirmed the amplification of *Aspergillus* target sequences. The assay designed in this chapter is a pan-*Aspergillus* assay since *A. fumigatus* is implicated in only up to two thirds of cases of IA. Importantly, the assay does not amplify human DNA or fungal DNA extracts from *Candida, Fusarium, Scedosporium* or *Rhizopus* species, well-characterised pathogens of immunocompromised (and immunocompetent) patients. Unsurprisingly, since there is 100% sequence identity across the 18S subunits of *Aspergillus* and *Penicillium*, *Penicillium* DNA is also amplified.

D. Cost: Excluding the expense of a thermocycler, the cost of running this *Aspergillus*-specific assay including the use of spin column DNA extraction, qPCR reagent and plastics cost is less than USD 500 per 96 reactions. In comparison, the Platelia *Aspergillus* galactomannan antigen test (Bio-Rad Laboratories), which is included in the EORTC/MSG criteria for the likelihood of IFD (50) has a list price of approximately USD 770 for 96 reactions.

The new qPCR assay was evaluated on histological material from two cases of proven IA with *A. fumigatus* (see Table 3).

All relevant parameters have been reported, clearly demonstrating the huge potential qPCR has as a tool for the early diagnosis of IA. However, reliable diagnosis of IA depends on at least two factors in addition to the quality of the qPCR. The first is sample quality:

sample collection, preparation and transport as well as nucleic acid extraction methods are critical parameters in test performance and must be optimised and, ideally, standardised. In principle, extraction of fungal nucleic acids, especially if present in a cell-free state, from BAL fluid, blood and serum is relatively straightforward; however, it is easy to co-purify inhibitors of the PCR that will generate inconsistent and unreliable results. Hence dilution curves are recommended for every sample to highlight contamination. Secondly, regular calibration of the real-time instrument is crucial for obtaining consistent and accurate results. Quantification cycle (Cq) values are neither absolute nor invariant, but vary between assays carried out on different days with different reagents, different users or on different instruments. This is because the Cq depends on the instrument's threshold setting, which in turn depends on background fluorescence, which varies with different probes, chemistries, instruments and assay protocols. Finally it is important to emphasise that a definitive identification of *Aspergillus* in clinical samples relies on histology, as this is the only direct evidence for invasion of the tissue (50, 51). Therefore, an assessment of the clinical utility of any molecular assay, for detection of *Aspergillus*, is challenging (204) and will require prospective studies.

# CHAPTER 4

# DEVELOPMENT OF A COMBINATION BIOMARKER DETECTION APPROACH TO IPA DIAGNOSIS

## 4.1 BACKGROUND

Invasive fungal disease (IFD) is a significant risk to haematology patients, due to their underlying disease and its treatment, which lead to periods of prolonged immunosuppression (205). IA is the most common cause of mortality due to infectious disease in patients with haematological malignancy (206, 207) and early diagnosis and treatment are vital for improving outcomes (208). However, early diagnosis is hampered by the limitations of current biomarker assays, together with a lack of consensus on the best sampling site for early detection; both in terms of practicability and the sensitivity of detection. For these reasons, together with the high morbidity and mortality associated with delayed diagnosis, an empiric treatment strategy has become the standard of care in many haematology units, in the setting of persistent, unexplained fever despite antibiotic therapy (209). This approach exposes many patients who do not have IFD to antifungal drugs, with the associated drug toxicities, a potential impact on their subsequent management once labelled as having had an IFD; while healthcare systems are exposed to spiralling drug costs (210).

These considerations have prompted attempts to optimise and standardise the use of existing assays (50, 51, 89, 114, 117) and develop new antigen-based technologies (119) to serve as additional tools for rapid and early diagnosis of IA.

*Aspergillus* detection has been most extensively investigated in blood; a preferred sample due to minimally invasive sampling and potential for serial monitoring. Detection of circulating serum GM has generated variable results, with a recent meta-analysis of 27 studies concluding that GM is moderately useful for surveillance of patients with IA (89). However, significant heterogeneity was observed between studies, which depended largely

on the patient population and the type of reference standard used. Sensitivity and specificity of 71% and 89% were reported when considering only proven cases as 'true positive' for IA. Positive predictive value (PPV) was calculated for a range of prevalence estimations, with the highest PPV calculated as a disappointing 62%, at a prevalence estimation of 20%. With IA prevalence in the setting of patients with haematological malignancy ranging from 1-15% (211), this assay is best for ruling out disease, rather than making a positive diagnosis. Two further reviews (212, 213) confirmed the reported heterogeneity between studies, with a wide range of reported assay sensitivities. Reasons for this included the effects of antifungal prophylaxis and variations in testing protocol, including frequency of testing, the cut off used to assign positivity and the number of positive sera results required to define IA. A further reason is the lack of reproducibility from repeat testing of the same serum sample, including long term storage (214), but also short term storage of under 7 days (215, 216). These reports raise doubts about the interpretation of data from stored samples, as well as real-time testing for patient management using GM. However, a recent report claims the long term stability of GM in serum and BAL fluid samples stored at -20°C (217).

Attempts to improve diagnostic sensitivity have led to investigation of respiratory samples, as *Aspergillus* infections are primarily air-borne pulmonary infections, with secondary haematogenous dissemination. BAL fluid from an established guinea pig model gave a GM positive result 2 days sooner than serum (218). GM levels remained elevated in another guinea pig model of IA, in the presence of antifungal therapy, whilst assay sensitivity in serum was reduced (121). These findings are consistent with reports of reduced GM assay sensitivity in patients receiving antifungal therapy (219). A recent meta-analysis of 30 studies evaluating BAL GM for IA diagnosis reported a pooled sensitivity and specificity of 87% and 89% using a cut off of 0.5 (220). Increasing the cut off to 1.0 gave a higher

specificity of 95%, whilst maintaining a superior sensitivity to serum testing. However, the included studies varied widely in the chosen cut off value, definition of a 'true positive' (Proven/probable VS proven only), likelihood scoring (EORTC 2002 VS 2008 criteria, with or without modifications) and the underlying disease of the study population. This resulted in significant variation in reported sensitivity (from 33-100%) and specificity (from 50-100%).

The *Aspergillus*-specific mAb JF5 has been used in an immunochromatographic LFD for point of care diagnosis of IA (119). Diagnostic utility of the LFD was demonstrated in a guinea pig model of infection, showing superior sensitivity and specificity when compared to serum GM (120). Limited clinical evaluation of the LFD in serum has given variable results, with sensitivity reported as 82% in a haematological malignancy population (126) and 40% in an HSCT population (127). An evaluation of the LFD in 39 BAL fluids from a mixed haematology and SOT population reported sensitivity and NPV of 100%, and specificity of 81% (123).

Real time PCR has been extensively used in the development of IA detection assays. However, there are no externally validated methods for PCR-based diagnosis and the lack of standardisation of protocols has been highlighted and extensively discussed in Chapter 3.

Evaluation of these assays in combination has revealed improved sensitivity and PPV, without significant loss of specificity (126, 138, 221, 222). The EORTC/MSG published definitions for IFD to facilitate clinical studies and research (50, 51); however, these definitions were not designed for clinical management. Due to the difficulty in establishing the gold standard diagnosis of proven IPA, the EORTC/MSG criteria have been used extensively to evaluate the performance of diagnostic methods (123, 126, 127, 220, 223). Publications have highlighted the poor diagnostic performance of the original EORTC/MSG

definitions when compared to autopsy examination (224, 225), as well as the revised definitions (52, 226). This raises doubts as to the use of the EORTC/MSG criteria, an imperfect reference standard, to assess new diagnostic approaches.

Whilst there is considerable heterogeneity in the reported diagnostic performance of individual assays , there is a general consensus that i) BAL fluid is a suitable sample for early IA diagnosis due to its superior sensitivity to serum testing, ii) a combination of biomarker assays provides an optimal testing strategy.

This chapter first describes a series of experiments that were performed to clarify the effects of sample storage on the repeatability of GM levels using the Platelia *Aspergillus* enzyme immunoassay (PA-EIA, Bio Rad, Marnes-la-Coquette, France). Next, a series of experiments is described that assessed the feasibility of a combination biomarker testing approach in BAL fluid from CT-guided bronchoscopy, to diagnose IPA. The best biomarker combination was then selected as a proposed diagnostic-driven testing approach to patient management, to direct antifungal treatment to patients with evidence of invasive fungal disease and used to evaluate the diagnostic performance of the EORTC/MSG criteria, using BAL fluid from patients with haematological malignancy. In order to highlight the inappropriate use of the EORTC/MSG criteria to evaluate the performance of diagnostic methods, the performance of the biomarker assays, individually and in combination, was evaluated using the EORTC/MSG criteria as the reference standard. Finally, the diagnostic performance of the biomarker assays was evaluated in corresponding blood samples.

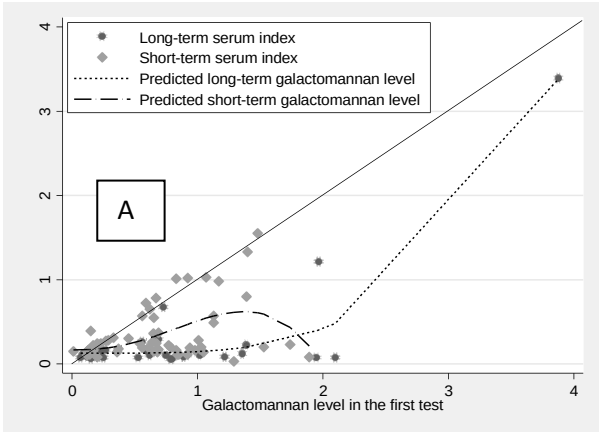
## 4.2 RESULTS

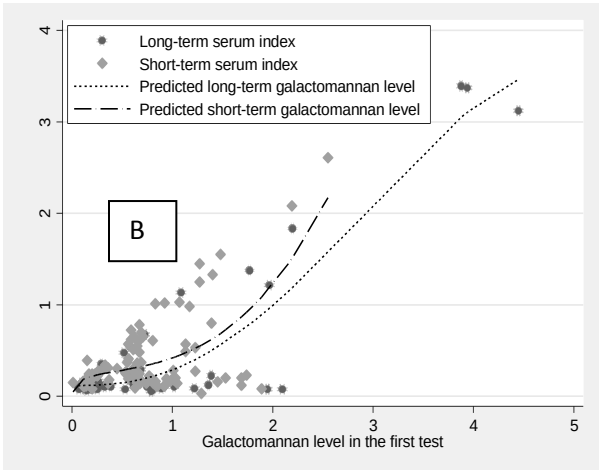
### *4.2.1 Significant decline in galactomannan signal during storage of clinical serum samples*

Serum and BAL fluid samples were collected from patients in the Division of Haemato-Oncology at St Bartholomew's Hospital undergoing serum GM monitoring in a screening strategy throughout their episode of intensive chemotherapy [Section 2.1.1]. Samples were selected as described in Section 2.5.1 to form the Long-term (LT) storage and Short-term (ST) storage test groups. GM detection was performed by PA-EIA [Section 2.5].

#### 4.2.1.1 Scatter Plot

Data from serum testing were presented on a scatter plot and analysed as described in section 2.9.1.1. Figure 29 shows that there was a clear loss of GM signal in both the ST and LT serum, with the signal loss more apparent in the plot including only the first sample taken from each patient (Figure 29A) than in the plot including all serum data (Figure 29B). Repeated samples from the same patient may mask the loss of signal in both ST and LT stored sera. Loss of GM signal was higher in LT than ST stored samples. BAL fluid samples did not show GM signal loss following either in ST or LT storage (Figure 29C). Importantly, the removal of the two outlier values from Figures 29A and 29C does not change the shape of the relationships significantly.



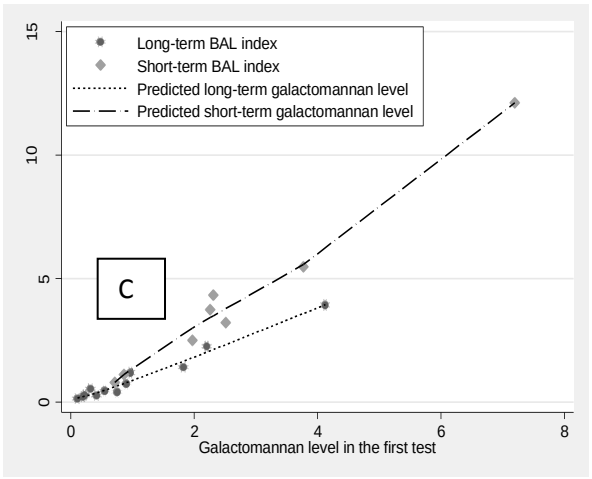




**Figure 29. Scatter plot with fractional polynomial lines for reduction in GM level.**

Diagonal solid lines indicate the line of equality. (A) GM serum level excluding repeat sera from patients with multiple samples; (B) GM serum level including multiple samples from the same patient; (C) GM BAL level (no repetition) during short-term and long-term storage.

#### 4.2.1.2 Statistically significant reductions in GM signal

**Serum samples (without repetition):** The Wilcoxon Signed Rank Sum Test [Section 2.9.1.1] applied to the first sample from each patient, showed statistically significant reductions in GM signals both in the ST storage (n = 65, median GM1 index: 0.65, median GM2 index: 0.19; $p < 0.001$) and in the LT storage (n = 35, median GM1 index: 0.56, median GM2 index: 0.10; $p < 0.001$), as shown in Table 4. In total, 44/65 (68%) ST and 31/35 (89%) LT serum samples showed a reduction in GM signal upon repeat testing.

In the ST serum group, 65.2% of samples that were initially GM positive became GM negative on repeat testing; with an average GM signal reduction of 50.2% in samples that were initially GM positive. In the LT serum storage group, 67.9% of GM positive samples became GM negative on repeat testing; with an average GM signal reduction of 49.3%.

To ascertain the stage at which GM signal loss occurred, repeat testing in a subset of GM positive samples (n = 14) was performed following ST storage from the original serum sample or using the EDTA pre-treated sample. All EDTA pre-treated samples remained positive. In contrast 9/14 (64%) of repeats from the original serum sample became negative.

| | **Sample size** | **Samples with GM reduction** | **Median GM1 index** | **Median GM2 index** | ***p*-value** |
|---|---|---|---|---|---|
| Short-Term Serum | 65 | 44 | 0.65 | 0.19 | <0.001 |
| Long-Term Serum | 35 | 31 | 0.56 | 0.10 | <0.001 |

**Table 4. Results of the Wilcoxon Rank Sum Test.**

Analysis was applied to both ST and LT stored serum populations. GM1 = first GM assay run. GM2 = repeat GM assay run.

### 4.2.1.3 Assay Repeatability

The OD value of the threshold control, loaded in duplicate, was recorded for 40 randomly selected assay runs (Figure 30) and used to determine the intra- and inter-assay variability [Section 2.9.1.2]. The duplicate values of the OD were repeatable as most of them are close to the line of equality and all measurements were within the recommended limits (OD 0.3–0.8). The mean OD of the 40 threshold control measurements was 0.49, with range 0.33–0.69. The coefficient of variation for duplicate measures was 7.09, which confirms repeatability.

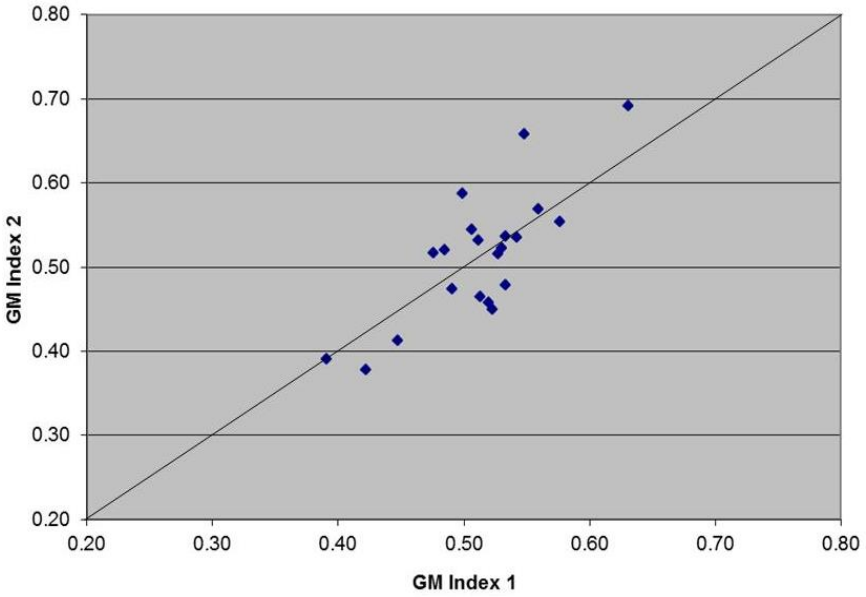



**Figure 30. Scatter plot for repeatability of the threshold control.**

Controls provided with the PA-EIA kit were loaded in duplicate wells (GM Index 1 and GM Index 2) over 40 randomly selected assay runs. Diagonal solid line indicates the line of equality.

### 4.2.1.4 Summary

The international EORTC/MSG mycological criteria for defining IA include serum and BAL fluid GM results as key diagnostic criteria (51). These are routinely obtained using the PA-EIA, which provides non-invasive, quantitative *Aspergillus*-specific detection for serum GM.

Recent reports have raised concerns with regard to the repeatability of GM levels. The results presented in this chapter confirm these doubts and broaden these findings by establishing a lack of repeatability of serum GM testing of the same sample. A clinically significant reduction in GM reactivity was recorded, with two thirds of GM positive sera becoming negative following ST or LT storage. This phenomenon was not seen with repeat GM testing of BAL fluid following ST or LT storage. We have published our rate of IFD in haemato-oncology at Barts Health, using the EORTC/MSG criteria of probable/proven IFD, which are usually regarded as representing true cases, as only 8/589 episodes (1.4%) by 2008 and 20/589 (3.5%) by 2002 criteria (52). It has been previously reported (227) that the decrease in GM values after retesting was much more pronounced in those samples with initially false positive GM results. Clearly, in the results presented in this chapter, the phenomenon of GM signal loss cannot be correlated to the EORTC/MSG criteria of IFD given the much higher frequency GM signal loss in both ST and LT data sets.

The OD value of the threshold control is critical in the calculation of the GM index value of clinical samples. Repeatability of the OD of the threshold control was confirmed by the CV for duplicate measures. These data indicate that technical issues per se cannot explain the observed GM signal loss in clinical samples. Furthermore, extensive review of the technical performance of the assay by different operators was undertaken, in conjunction with Bio-Rad, including monitoring of heating block temperature and plate washer performance. No concerns were identified. Although environmental contamination is a risk during testing, the presented data are not consistent with contamination, which would lead to random increases or decreases with repeat testing. In contrast, the data show a consistent pattern of signal loss, with the first reading being higher than the repeat. In addition, sporadic repeat testing of GM negative serum samples never gave a positive result.

The repeatability of the OD of the threshold control suggests that the observed GM signal loss was specific to clinical serum samples. Interestingly, following ST storage of sera, the GM signal loss was not seen if the repeat was performed using EDTA pre-treated samples from the first step of the testing protocol, all of which remained positive. Although the mechanisms of GM decline in clinical serum samples are unclear, they appear to occur in the original serum sample prior to the EDTA pre-treatment stage. The data do not permit specification of an acceptable storage time without major signal loss, but it can be concluded that serum signal loss is associated with storage, and that it is therefore important to minimise the time from phlebotomy to the EDTA stage of processing. Further work needs to be done to determine whether short term storage without major signal loss is possible.

### *4.2.2 Combination biomarker diagnosis of IA in BAL fluid*

This study was a retrospective evaluation of biomarker performance for IPA diagnosis, with no impact on patient management. The Study cohort of patients were a subset of those recruited in an observational study of IPA diagnosis in the Division of Haemato-Oncology, St Bartholomew's Hospital [Section 2.1.1], based on the availability of BAL samples and full clinical data. 25 BAL fluid samples from 24 adults at high risk of IFD following intensive chemotherapy or allogeneic stem cell transplantation were included in this study.

The performance of three biomarker assays were evaluated; the *Aspergillus* LFD [Section 2.6], GM assay [Section 2.5] and the new MIQE-compliant *Aspergillus* qPCR [Chapter 3, section 3.3]. Total DNA was extracted for qPCR analysis, as described in section 2.2.3.

Assay sensitivity was ascertained using BAL samples from a 'gold standard' proven IPA cohort of 11 BAL fluids from 10 patients, obtained at routine diagnostic work-up at the Innsbruck University Hospital between January 2008 and November 2012 [Section 2.1.2]. Specificity was calculated from BAL samples from a negative control cohort from the same institution - 16 BAL samples from 16 adults without any EORTC/MSG risk factors for IFD, in whom routine diagnostic procedures were negative for IPA (microscopy, culture and local GM testing and *Aspergillus* PCR).

### 4.2.2.1 Assay sensitivity

Results of qPCR, LFD and GM testing in the gold standard proven IPA cohort are shown in Table 5. The sensitivity of the qPCR assay and the LFD were both 100%. The sensitivity of the GM assay was 81.8% (GM Index ≥0.8) or 72.7% (GM Index >1.0). There was 100% agreement between the qPCR and LFD assays. The calculation of agreement with the GM assay is not possible as all samples are positive based on LFD and qPCR.

| Sample | LFD result | GM result (0.8) | GM result (1.0) | qPCR result |
|---|---|---|---|---|
| Proven 1 | Positive | Positive | Positive | Positive |
| Proven 2 | Positive | Positive | Positive | Positive |
| Proven 3 | Positive | Positive | Positive | Positive |
| Proven 4 | Positive | Positive | Positive | Positive |
| Proven 5 | Positive | Positive | Positive | Positive |
| Proven 6 | Positive | Negative | Negative | Positive |
| Proven 7* | Positive | Positive | Positive | Positive |
| Proven 8* | Positive | Negative | Negative | Positive |
| Proven 9 | Positive | Positive | Positive | Positive |
| Proven 10 | Positive | Positive | Positive | Positive |
| Proven 11 | Positive | Positive | Negative | Positive |
| **Assay Sensitivity** | **100%** | **81.82%** | **72.73%** | **100%** |

**Table 5. Testing results and sensitivity of assays in the gold standard proven IPA cohort.**

(N=11). *samples from the same patient.

### 4.2.2.2 Assay specificity

Results of qPCR, LFD and GM testing in the negative control cohort are shown in Table 6. The specificity of all 3 assays was 93.75%. The pair-wise agreements of three assays were 100%.

| Sample | LFD result | GM result (0.8) | GM result (1.0) | qPCR result |
|---|---|---|---|---|
| Negative 1 | Negative | Negative | Negative | Negative |
| Negative 2 | Negative | Negative | Negative | Negative |
| Negative 3 | Positive | Positive | Positive | Positive |
| Negative 4 | Negative | Negative | Negative | Negative |
| Negative 5 | Negative | Negative | Negative | Negative |
| Negative 6 | Negative | Negative | Negative | Negative |
| Negative 7 | Negative | Negative | Negative | Negative |
| Negative 8 | Negative | Negative | Negative | Negative |
| Negative 9 | Negative | Negative | Negative | Negative |
| Negative 10 | Negative | Negative | Negative | Negative |
| Negative 11 | Negative | Negative | Negative | Negative |
| Negative 12 | Negative | Negative | Negative | Negative |
| Negative 13 | Negative | Negative | Negative | Negative |
| Negative 14 | Negative | Negative | Negative | Negative |
| Negative 15 | Negative | Negative | Negative | Negative |
| Negative 16 | Negative | Negative | Negative | Negative |
| **Assay Specificity** | **93.75%** | **93.75%** | **93.75%** | **93.75%** |

**Table 6. Testing results and specificity of assays in the negative control cohort.**

(N=16).

### 4.2.2.3 Combination testing approach to clinical diagnosis of IPA

Results of qPCR, LFD and GM testing in the Study cohort are shown in Table 7. Microscopy, including calcofluor staining, and mycological culture of all BAL fluids were negative for evidence of *Aspergillus*. However, 5/25 samples were positive for all three biomarkers,

while 15/25 were negative with all three assays. Based on the high sensitivity and specificity of triple testing in the gold standard proven IPA and negative control cohorts, the following test interpretation and clinical action are suggested (see Table 7): a positive result for all 3 tests indicates IPA and antifungal treatment is advised; if all 3 tests are negative, IPA is excluded and antifungal treatment should be withheld in a stable patient. However, a negative result does not rule out non-*Aspergillus* IFD and further investigation is required if IFD is still suspected.

Pairwise comparison of tests [Section 2.9.2.1] showed that the highest level of agreement was between qPCR and LFD, with a Kappa value of 0.88 (almost perfect agreement, see Table 8). All other pairs of biomarkers, regardless of the GM threshold used for positivity (≥0.8 or >1.0), showed significantly less agreement. Based on this result, it is suggested that agreement between LFD and qPCR tests determines the clinical interpretation in the face of a discrepant GM result. This is reflected in the results of Study samples 7-10, in which an LFD negative/qPCR negative/GM positive result is interpreted as negative for IPA and withholding of antifungals is advised. Disagreement between LFD and qPCR tests was seen in Study sample 6 only and interpreted clinically as inconclusive and further clinical investigation would be advised.

24 of the 25 Study BAL samples were collected after commencing antifungal therapy. Median time from antifungal start to bronchoscopy was 5.5 days in combination test negative samples (range 1-69 days) and 6 days in combination test positive samples (range 4-8 days).

| Sample | LFD result | GM result (0.8) | GM result (1.0) | qPCR result | Test interpretation | Suggested action |
|---|---|---|---|---|---|---|
| 1 | Positive | Positive | Positive | Positive | Positive | Treat for IPA |
| 2 | Positive | Positive | Positive | Positive | Positive | Treat for IPA |
| 3 | Positive | Positive | Positive | Positive | Positive | Treat for IPA |
| 4 | Positive | Positive | Negative | Positive | Positive | Treat for IPA |
| 5 | Positive | Positive | Negative | Positive | Positive | Treat for IPA |
| 6 | Positive | Negative | Negative | Negative | Inconclusive | Repeat tests/further clinical investigation required |
| 7 | Negative | Positive | Positive | Negative | Negative | Withhold/stop antifungal treatment* |
| 8 | Negative | Positive | Positive | Negative | Negative | Withhold/stop antifungal treatment* |
| 9 | Negative | Positive | Positive | Negative | Negative | Withhold/stop antifungal treatment* |
| 10 | Negative | Positive | Negative | Negative | Negative | Withhold/stop antifungal treatment* |
| 11 | Negative | Negative | Negative | Negative | Negative | Withhold/stop antifungal treatment* |
| 12 | Negative | Negative | Negative | Negative | Negative | Withhold/stop antifungal treatment* |
| 13 | Negative | Negative | Negative | Negative | Negative | Withhold/stop antifungal treatment* |
| 14 | Negative | Negative | Negative | Negative | Negative | Withhold/stop antifungal treatment* |
| 15 | Negative | Negative | Negative | Negative | Negative | Withhold/stop antifungal treatment* |
| 16 | Negative | Negative | Negative | Negative | Negative | Withhold/stop antifungal treatment* |
| 17 | Negative | Negative | Negative | Negative | Negative | Withhold/stop antifungal treatment* |
| 18 | Negative | Negative | Negative | Negative | Negative | Withhold/stop antifungal treatment* |
| 19 | Negative | Negative | Negative | Negative | Negative | Withhold/stop antifungal treatment* |
| 20 | Negative | Negative | Negative | Negative | Negative | Withhold/stop antifungal treatment* |
| 21 | Negative | Negative | Negative | Negative | Negative | Withhold/stop antifungal treatment* |
| 22 | Negative | Negative | Negative | Negative | Negative | Withhold/stop antifungal treatment* |
| 23 | Negative | Negative | Negative | Negative | Negative | Withhold/stop antifungal treatment* |
| 24 | Negative | Negative | Negative | Negative | Negative | Withhold/stop antifungal treatment* |
| 25 | Negative | Negative | Negative | Negative | Negative | Withhold/stop antifungal treatment* |

**Table 7. Testing results and suggested clinical interpretation in the Study cohort.**

(N=25).

*unless a non-*Aspergillus* IFD is still suspected.

| Test | Cohen's Kappa (95% CI) |
|---|---|
| LFD+qPCR | 0.88 (0.66-1.0) |
| LFD+GM (0.8) | 0.53 (0.18-0.88) |
| LFD+GM (1.0) | 0.34 (-0.08-0.76) |
| qPCR+GM (0.8) | 0.62 (0.30-0.93) |
| qPCR+GM (1.0) | 0.42 (-0.004-0.84) |

**Table 8. Pairwise agreement of tests in the Study cohort.**

(N=25).

### 4.2.2.4 Diagnostic performance of EORTC/MSG criteria

The EORTC/MSG criteria were evaluated against the LFD+qPCR clinical biomarker diagnosis of IPA as described in section 2.9.2.2. The EORTC/MSG 2008 scores, with and without GM, in the Study cohort are shown in Table 9, along with the results of the dual LFD/qPCR combination as the best biomarker pairing. Performance parameters for the EORTC/MSG scoring criteria (with and without GM) were evaluated against the dual LFD/qPCR approach as the proposed standard, to direct antifungal treatment to patients with evidence of IPA (Table 10). The low sensitivity values suggest that the EORTC/MSG definitions are inappropriate for IPA diagnosis.

| Sample | Dual LFD/qPCR interpretation | EORTC/MSG 2008 | EORTC/MSG 2008 (excluding GM) |
|---|---|---|---|
| 1 | Positive | None | None |
| 2 | Positive | None | None |
| 3 | Positive | None | None |
| 4 | Positive | Probable | Possible |
| 5 | Positive | Probable | Probable |
| 6 | Inconclusive | None | None |
| 7 | Negative | None | None |
| 8 | Negative | None | None |
| 9 | Negative | None | None |
| 10 | Negative | Probable | Probable |
| 11 | Negative | None | None |
| 12 | Negative | None | None |
| 13 | Negative | None | None |
| 14 | Negative | None | None |
| 15 | Negative | None | None |
| 16 | Negative | None | None |
| 17 | Negative | None | None |
| 18 | Negative | None | None |
| 19 | Negative | None | None |
| 20 | Negative | Possible | Possible |
| 21 | Negative | Possible | Possible |
| 22 | Negative | Possible | Possible |
| 23 | Negative | Possible | Possible |
| 24 | Negative | Possible | Possible |
| 25 | Negative | Probable | Probable |

**Table 9. EORTC/MSG 2008 classification in the Study cohort (N = 25).**

| Test | % Sensitivity (95% CI) | % Specificity (95% CI) | PPV (95% CI) | NPV (95% CI) |
|---|---|---|---|---|
| EORTC (Probable vs Possible/None) | 40.00 (6.49-84.60) | 89.47 (66.82-98.39) | 50.00 (8.30-91.70) | 85.00 (62.08-96.62) |
| EORTC excluding GM (Probable vs Possible/None) | 20.00 (3.30-71.19) | 89.47 (66.82-98.39) | 33.33 (5.47-88.45) | 80.95 (58.08-94.44) |
| EORTC (Probable/Possible vs None) | 40.00 (6.49-84.60) | 63.16 (38.38-83.65) | 22.22 (3.47-59.94) | 80.00 (51.91-95.43) |
| EORTC excluding GM (Probable/Possible vs None) | 40.00 (6.49-84.60) | 63.16 (38.38-83.65) | 22.22 (3.47-59.94) | 80.00 (51.91-95.43) |

**Table 10. Performance parameters for the EORTC/MSG criteria (with and without GM included), in the Study cohort (N=25), using dual LFD/qPCR testing as the gold standard for clinical diagnosis of IPA.**

### 4.2.2.5 The use of EORTC/MSG scores as a reference standard to evaluate the performance of biomarker assays

Diagnostic performance of the individual and combination biomarker assays was evaluated in the Study cohort, using the EORTC/MSG (excluding GM) criteria as the gold standard [Section 2.9.2.3]. Results are shown in Table 11. The small sample size together with the disagreement between the assay results and the EORTC/MSG definitions are reflected in the low assay sensitivity results. These poor performance statistics are in stark contrast to the high sensitivity and specificity obtained in the gold standard and negative control cohorts [Sections 4.2.2.1 and 4.2.2.2], suggesting that the EORTC/MSG definitions are inappropriate for the clinical diagnosis of IPA.

| Assay | N | Samples excluded | % Sensitivity (95% CI) | % Specificity (95% CI) | % PPV (95% CI) | % NPV (95% CI) |
|---|---|---|---|---|---|---|
| LFD+PCR+GM (0.8) | 25 | 5 | 50.00 (8.17-91.83) | 77.78 (52.36-93.45) | 20 (3.3-71.19) | 93.33 (67.98-98.89) |
| LFD+PCR+GM (1.0) | 25 | 6 | * | 82.35 (56.55-95.99) | * | 87.5 (61.62-98.08) |
| LFD+PCR | 25 | 1 | 33.33 (5.47-88.45) | 80.95 (58.08-94.44) | 20 (3.3-71.19) | 89.47 (66.82-98.39) |
| LFD+GM (0.8) | 25 | 5 | 50.00 (8.17-91.83) | 77.78 (52.36-93.45) | 20 (3.3-71.19) | 93.33 (67.98-98.89) |
| LFD+GM (1.0) | 25 | 6 | * | 82.35 (56.55-95.99) | * | 87.5 (61.62-98.08) |
| PCR+GM (0.8) | 25 | 4 | 50.00 (8.17-91.83) | 78.95 (54.43-93.82) | 20 (3.3-71.19) | 93.75 (69.69-98.96) |
| PCR+GM (1.0) | 25 | 5 | * | 83.33 (58.56-96.23) | * | 88.24 (63.52-98.20) |
| GM (0.8) | 25 | 0 | 66.67 (11.55-94.53) | 68.18 (45.13-86.08) | 22.22 (3.47-59.94) | 93.75 (69.69-98.96) |
| GM (1.0) | 25 | 0 | * | 72.73 (49.78-89.20) | * | 84.21 (60.40-96.43) |
| LFD | 25 | 0 | 33.33 (5.47-88.45) | 77.27 (54.62-92.09) | 16.67 (2.76-63.90) | 89.47 (66.82-98.39) |
| PCR | 25 | 0 | 33.33 (5.47-88.45) | 81.82 (59.70-94.70) | 20 (3.3-71.19) | 90 (68.26-98.47) |

**Table 11. Performance parameters for the biomarker assays, individually and in combination, in the Study cohort (N = 25).**

Analysis performed using EORTC/MSG 2008 criteria (excluding GM) as the gold standard for clinical diagnosis of IPA. GM cut-off is shown in brackets. *Calculation was not possible due to the absence of any 'true positives' from the data set.

### 4.2.2.6 Biomarker detection in blood

Study patients underwent twice weekly blood sampling [Section 2.1.1.4.3] during the course of study participation. Retrospective testing of LFD and GM was performed on serum samples and qPCR on DNA extracts from EDTA blood samples. Results are shown in Table 12. In the 5 cases with combination biomarker positive BAL fluid, 4 had consistently negative serum GM results during the period of neutropenia (samples 1 and 3-5). Only one case (sample 2) was serum GM positive and also had one blood qPCR positive result – interestingly, the BAL qPCR indicated this patient had the highest respiratory fungal load in Study cohort. Study case 4 had two consecutive LFD positives in serum, but GM and qPCR were negative. Study case 3 had one qPCR positive result, but LFD and GM testing were negative. In the other 20 Study cohort episodes that were not combination biomarker

positive in BAL fluid, 115 blood/serum samples were analysed and all were negative by LFD, GM and qPCR.

| | BAL fluid | Blood | | |
|---|---|---|---|---|
| Sample | Test interpretation | LFD | GM (0.5) | PCR |
| 1 | Positive | Negative | <0.5 | NA |
| 2 | Positive | NA | 2.2 | Positive |
| 3 | Positive | Negative | <0.5 | Positive |
| 4 | Positive | Positive | <0.5 | Negative |
| 5 | Positive | Negative | <0.5 | NA |
| 6 | Inconclusive | Negative | <0.5 | NA |
| 7 | Negative | Negative | NA | NA |
| 8 | Negative | Negative | <0.5 | NA |
| 9 | Negative | NA | NA | NA |
| 10 | Negative | Negative | <0.5 | Negative |
| 11 | Negative | Negative | <0.5 | Negative |
| 12 | Negative | Negative | <0.5 | Negative |
| 13 | Negative | Negative | <0.5 | Negative |
| 14 | Negative | Negative | <0.5 | Negative |
| 15 | Negative | Negative | <0.5 | Negative |
| 16 | Negative | Negative | <0.5 | Negative |
| 17 | Negative | Negative | <0.5 | Negative |
| 18 | Negative | Negative | <0.5 | Negative |
| 19 | Negative | Negative | <0.5 | Negative |
| 20 | Negative | Negative | <0.5 | Negative |
| 21 | Negative | Negative | <0.5 | Negative |
| 22 | Negative | Negative | <0.5 | Negative |
| 23 | Negative | Negative | <0.5 | Negative |
| 24 | Negative | Negative | <0.5 | NA |
| 25 | Negative | Negative | <0.5 | NA |

**Table 12. Biomarker testing results in blood samples from the Study cohort (N=25).**

NA = no sample available for testing

< 0.5 indicates that all samples from serial testing were negative

## 4.3 DISCUSSION

IPA is the most common manifestation of IFD in haemato-oncology patients (15) and its diagnosis and treatment remain a challenge.

The poor repeatability of serum sample GM testing presented in this chapter suggest that: (i) caution should be exercised when testing serum samples after a period of storage, including samples stored at −80 °C; (ii) the interpretation of GM negative results obtained after delays in processing may be problematic; (iii) careful coordination is required between the laboratory and clinical teams to minimise the time from sampling to testing; (iv) a better understanding of GM kinetics is necessary to address the impact of sample processing and storage times on detectable GM concentrations. Furthermore, given a wide range of sensitivity and specificity data plus multiple causes of false-positive results for the PA-EIA reported in the literature, a prudent approach to clinical utilisation of GM testing would be to combine the PA-EIA with other markers of *Aspergillus* in a diagnostic algorithm (53).

This chapter demonstrates that a triple biomarker approach - LFD, GM and qPCR – in BAL fluid has a very high sensitivity and specificity for IPA. In the Study cohort of haemato-oncology patients at high-risk of IFD, the LFD and qPCR combination showed almost perfect agreement (Cohen's kappa 0.88), which is then proposed as a diagnostic-driven testing approach to patient management, to direct antifungal treatment to patients with evidence of IPA, in the absence of direct examination or culture of pulmonary tissue. Having defined the sensitivity of triple biomarker testing in the proven IPA cohort, it was possible to show the low sensitivity of the EORTC/MSG criteria using the biomarker approach as the reference standard in the Study cohort, i.e., in patients in whom a definitive, proven diagnosis has not been made. This data, and other reports (52, 224-226), highlight that the

EORTC/MSG criteria, developed for clinical research, should be used with caution both when evaluating new diagnostic approaches for IFD and when used in clinical algorithms defining management on "probable/possible" classification. To highlight this, the diagnostic performance of the individual and combination biomarker assays was also evaluated in the Study cohort, using the EORTC/MSG (excluding GM) criteria as the reference standard. Results suggested individual biomarker assay performance to be very poor (33-66% sensitivity) in this small sample population. Of the five Study episodes that tested GM, LFD and PCR positive in BAL, three of these episodes were classified as having no risk factors for IA (none) by EORTC-scoring, and would therefore be regarded as 'false positive' test results. Given the documented limitations of the EORTC criteria, it could be argued that these cases are in fact true positives and that the sensitivity of these assays is much greater than suggested in comparison to the EORTC-scored, which is an imperfect reference standard.

In clinical practice, two factors may significantly impact on our proposed biomarker approach for IPA diagnosis in BAL fluid: the use of mould-active drugs (as prophylaxis or treatment) and the availability of rapid bronchoscopy. There are very limited and conflicting reports about the effect of antifungal therapy on the performance of assays in BAL fluid. Antifungal therapy has been reported to both decrease (228) and increase (158) the diagnostic performance of qPCR in BAL fluid from high risk patients. In a guinea pig model of IPA, levels of LFD and GM remained elevated in the BAL fluid of animals treated with antifungals, whereas serum sensitivity was reduced (121). In the Study cohort presented in this chapter, the median interval between starting mould-active antifungal treatment and bronchoscopy was 5.5 days in the dual LFD/qPCR negative cases and 6 days in the positive cases. Therefore, neither the use of anti-fungal drugs nor the delay in bronchoscopy is likely to explain the biomarker negative results. Furthermore, the five

triple biomarker positive BAL fluids were obtained after four to eight days of mould-active antifungal therapy, which highlights the utility of biomarker testing in this setting, whereas microscopy and culture of BAL fluid were uninformative.

Another key clinical issue is whether the proposed biomarker approach could be used in the blood and avoid the need for bronchoscopy. In the Study cohort, blood was sampled twice weekly and retrospective testing of LFD, GM and qPCR was performed. Overall, the data indicate the insensitivity of combination biomarker detection in blood compared to respiratory sampling. Furthermore, concerns over the repeatability of GM in serum (215, 216) have been confirmed in this chapter, whilst also demonstrating that BAL GM results appear stable (229).

A positive BAL GM result would support the use of antifungal drugs. The GM assay showed 72.7% sensitivity in the proven IPA cohort when a cut-off index of 1.0 was used, as deemed 'optimal' by a recent meta-analysis of GM-BAL studies (220), but 81.8% using a lower cut-off of 0.8. The latter value may be more relevant to our clinical practice, where mould-active antifungal therapy was started in the Study cohort patients prior to bronchoscopy. Furthermore, the combination biomarker approach revealed 4 BAL samples in the Study cohort with GM positivity that was discrepant with dual LFD/qPCR negativity. The near perfect agreement between LFD and qPCR would suggest that these cases should be interpreted as having false positive GM results and no evidence of IPA. Disagreement between the LFD and qPCR tests was seen in a single Study cohort sample (GM was negative). As the two tests performed identically in the gold standard proven IPA and negative control cohorts with 100% agreement, in cases of discrepancy the results should be interpreted as invalid and repeat testing and further clinical investigation recommended.

The EORTC/MSG criteria (50, 51) are often used as a diagnostic reference standard because of the rarity of proven IPA cases. However, misclassification by these criteria can occur for many reasons: lung lesions may be missed due to delays in CT scanning (69); biomarker changes may be caused by an alternative condition to IPA (157) or by drug treatment (94). Furthermore, when evaluating the performance of the GM assay there is incorporation bias, as the test is part of the EORTC/MSG criteria for assigning a 'probable' score, but this method of evaluation is still used (89). In this chapter, the EORTC/MSG 2008 criteria were used (51), with and without GM results included, to assign likelihood of IFD in the Study cohort and the results were evaluated against the dual LFD/qPCR combination as a reference standard for clinical diagnosis of IPA. Depending on whether an EORTC/MSG 'possible' score was deemed a negative or positive result, sensitivity of the EORTC/MSG criteria was between 20-40%. The performance of the EORTC/MSG criteria has also been evaluated in autopsy studies : 8 of 22 (225) and 78 of 314 (224) cases of autopsy-proven IFD were diagnosed as proven or probable by EORTC/MSG 2002 criteria; sensitivities of 36% and 25%, respectively; while the EORTC/MSG 2008 modifications identified 6 of 40 cases of autopsy-proven IFD - a sensitivity of 15%. These sensitivity values for EORTC/MSG criteria in proven autopsy cases are comparable to the data presented in this chapter, using dual LFD/qPCR testing as the reference standard and further support this combination testing approach. However, it is important to note that in the study by Chamilos *et al* (224) there is very limited information provided about the tests performed and results used in EORTC/MSG scoring. It is not clear whether any imaging was performed, or antigen testing, such as GM ELISA. This could have a significant impact on an analysis of the diagnostic performance of the EORTC/MSG classification system. In the study by Subira *et al* it is stated that no antigen testing was performed (225). Also, although imaging was available,

less than half of the patients in this study actually had CT scans performed. Again, this absence of test results limits the utility of the EORTC/MSG classification in this study.

The study presented in this chapter has a number of limitations. Selection of adult patients at high risk of developing IPA, who have had a bronchoscopy, leads to a high pre-test probability of IFD. The timing of the bronchoscopy in relation to the course of IFD may also introduce bias, as collection can occur at various stages of disease. However, the Study cohort patients followed a standardised protocol [Section 2.1] and in clinical practice such bias could be minimised by using integrated care pathways (53). BAL sampling is also not standardised, raising issues about the interpretation of biomarker thresholds and quantification.

In conclusion, the data presented in this chapter suggest that a dual LFD/qPCR testing approach in BAL fluid could be a new diagnostic-driven testing approach to patient management, to direct antifungal treatment to patients with evidence of IPA in the absence of direct examination or culture of pulmonary tissue. This proposed combination test would require validation. Importantly, biomarkers remain informative even if antifungals are commenced before bronchoscopy. A systematic approach to bronchoscopy combined with dual LFD/qPCR testing could make a significant difference to IFD management. We envisage LFD testing as a near-patient assay in the bronchoscopy suite, allowing an immediate decision on antifungal drug usage, with qPCR results within 24 hours for a definitive diagnosis.

# CHAPTER 5

# INNOVATIVE APPROACHES TO IPA DIAGNOSIS

## 5.1 EVALUATION OF EXHALED BREATH CONDENSATE AS A PIONEERING SAMPLE FOR NON-INVASIVE DIAGNOSIS OF LUNG PATHOGENS

### *5.1.1 Background*

The analysis of exhaled breath condensate (EBC) provides a non-invasive method for identifying biomarkers associated with lung pathology, making it especially useful for longitudinal assessments of patients. Although its use was first reported in 1980 (140), a number of technical limitations have limited its value for clinical applications. EBC is believed to reflect the composition of the airway lining fluid and alveoli and has been shown to contain bioaerosols (bacteria, fungi and viruses) derived from the respiratory tract (230). The reopening of closed bronchioles and alveoli creates turbulence in the airways, which results in non-volatile compounds being aerosolised (231). There is still dispute as to whether EBC contains aerosol particles derived mainly from the respiratory lining fluid of the lower airways (232), or from the central airways (233), and despite the commercial development of devices for EBC collection, it is still impossible to standardise flow rate and the volume of exhalation. Despite this unknown degree of sampling variability and uncertainly as to the origin of EBC content, this biological sample may prove useful in the study of inflammatory pulmonary disorders. In theory, the proximity of the sample to the initial site of infection, together with the relative ease of sample collection in comparison to BAL fluid (non-invasive and minimal discomfort, with the potential to even sample from ventilated patients) makes EBC an attractive option for diagnosing respiratory infections such as IPA.

In practice, the small number of studies that have investigated the potential of EBC sampling for nucleic acid detection of respiratory pathogens have recorded discouraging results, describing a lack of sensitivity (154-156) and poor correlation with BAL fluid (153),

nasopharyngeal swabs (154) and sputum (155). There have been a limited number of attempts to detect specifically non-nucleic acid-based markers of IPA. One encouraging report recorded 2-pentyl-furan detected in the breath of two IPA patients, but not following treatment or in healthy controls (234, 235). Two conference papers have reported GM positive results in EBC from patients at high risk of IPA, with corresponding GM positive serum (236, 237)**;** however, neither was followed up with a full publication.

Despite the generally discouraging results and negative conclusions published regarding the usefulness of EBC, the development of the combination biomarker testing approach described in Chapter 4 makes it worthwhile investigating this clinical sample in the diagnosis of IPA.

This section (Section 5.1) describes a series of experiments evaluating the potential of EBC as a pioneering sample for non-invasive diagnosis of IPA. First, the feasibility of qPCR/LFD/GM testing for *Aspergillus* biomarker detection is assessed in EBC samples from a Study cohort of haemato-oncology patients at high-risk of IFD. These results are correlated with *Aspergillus* qPCR/LFD/GM testing results in corresponding BAL fluid from CT-guided bronchoscopy.

### *5.1.2 Results*

This study was a retrospective evaluation of biomarker performance for IPA diagnosis, with no impact on patient management. The Study cohort of patients were a subset of those recruited in an observational study of IPA diagnosis in the Division of Haemato-Oncology, St Bartholomew's Hospital [Section 2.1.1], based on the availability of BAL samples, EBC

samples and full clinical data. 37 EBC samples from 12 adults at high risk of IFD following intensive chemotherapy or allogeneic stem cell transplantation were included in this study.

The performance of three biomarker assays were evaluated: (i) the *Aspergillus* LFD [Section 2.6], (ii) GM assay [Section 2.5] and (iii) the new MIQE-compliant *Aspergillus* qPCR [Chapter 3, section 3.3]. Total DNA was extracted for qPCR analysis, as described in section 2.2.3. EBC testing results were compared to biomarker testing results obtained in corresponding BAL fluid samples [Chapter 4, section 4.2.2.3]. Results are shown in Table 13.

All EBC samples gave an instant 'weak positive' test line within minutes of being added to the LFD. EBC from 11 of the 12 episodes were consistently GM index <0.5. Only EBC sample 14 had a positive GM result, with GM index 1.3. However, the corresponding qPCR result was negative, and the BAL fluid from this patient was negative by all 3 biomarker assays.

EBC from 2 episodes tested PCR positive for *Aspergillus* DNA. A single EBC from sample 2 amplified with Cq39 (Figure 31) and corresponding BAL fluid tested positive by all 3 assays. Two consecutive EBC samples from sample 4 amplified with Cqs of 36-39 (Figure 32) and corresponding BAL fluid tested positive by all three assays. Positive PCR results were consistent across triplicate wells and also when the assay was repeated. Twenty-five percent of wells in every assay were NTCs and no amplification products were detected in these wells. There was insufficient sample to perform a repeat extraction. All EBC extracts were tested in the β-globin hydrolysis probe assay to monitor the relative human DNA content [Section 2.4.7]. All extracts were negative for human DNA.

| | BAL fluid | | | EBC | | |
|---|---|---|---|---|---|---|
| Sample | LFD result | GM Index | qPCR result | LFD result | GM Index | qPCR result |
| 2 | Positive | 6.4 | Positive | Positive | missing | Cq 39 |
| 4 | Positive | 0.8 | Positive | Positive | missing | Cq 36-39 |
| 8 | Negative | 1.8 | Negative | Positive | <0.5 | negative |
| 10 | Negative | 0.8 | Negative | Positive | <0.5 | negative |
| 11 | Negative | 0.4 | Negative | Positive | <0.5 | negative |
| 13 | Negative | 0.2 | Negative | Positive | <0.5 | negative |
| 14 | Negative | 0.2 | Negative | Positive | 1.3 | negative |
| 15 | Negative | 0.3 | Negative | Positive | <0.5 | negative |
| 16 | Negative | 0.2 | Negative | Positive | <0.5 | negative |
| 20 | Negative | 0.1 | Negative | Positive | <0.5 | negative |
| 22 | Negative | 0.3 | Negative | Positive | <0.5 | negative |
| 23 | Negative | 0.4 | Negative | Positive | <0.5 | negative |

**Table 13. Testing results for EBC samples and corresponding BAL fluid in the Study cohort (N=12).**

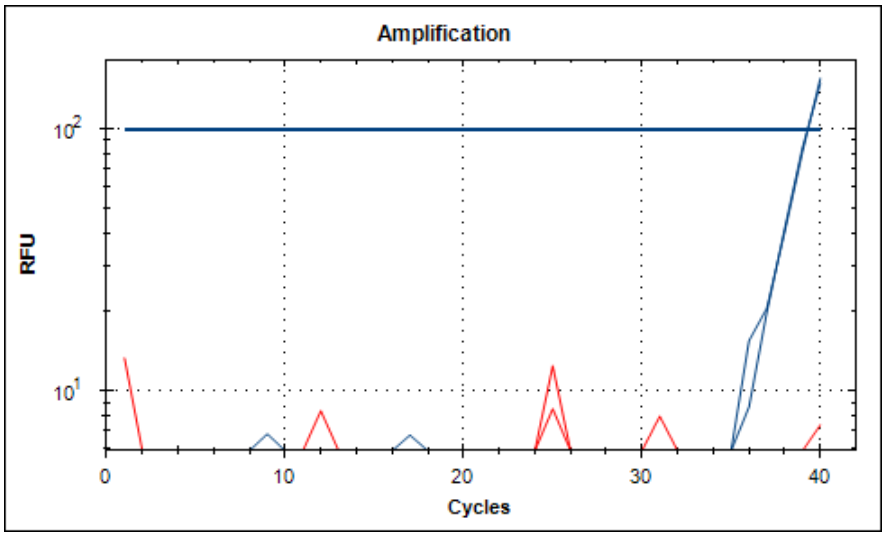



**Figure 31. qPCR assay amplification curve for an EBC from Study sample 2.**

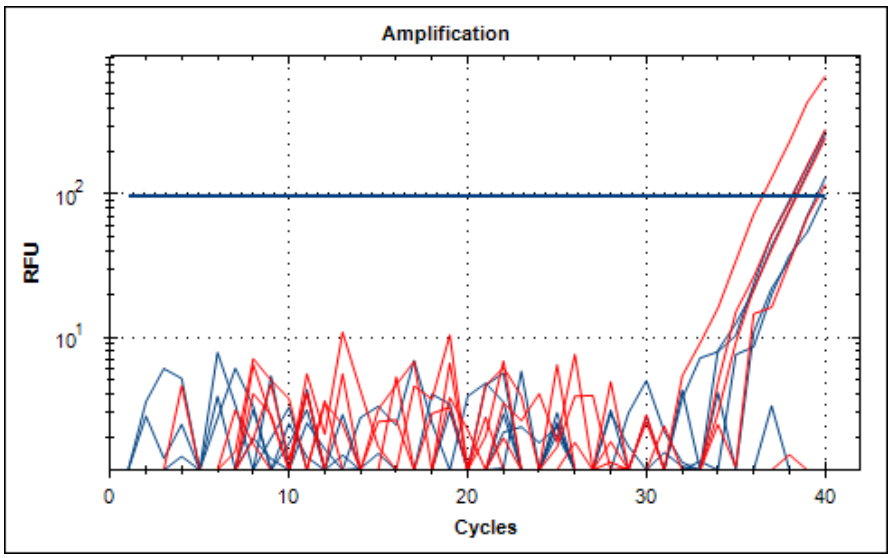



**Figure 32. qPCR assay amplification curves for 2 consecutive EBC samples from Study sample 4.**

### *5.1.3 DISCUSSION*

Although amplification was very late (at the limits of detection), three EBC samples were PCR positive for *Aspergillus* DNA. Amplification at such a high Cq must be interpreted with caution. However, the results were consistent across triplicate wells and also when the assay was repeated. With consistent amplification between Cq 36 and 39, and no amplification of the negative control wells, these EBC samples are very likely to be positive for *Aspergillus* DNA, albeit in very low copy number. This allows no conclusions to be drawn with regards to the clinical relevance of such extremely low target copy numbers. However, this is this first report of the detection of *Aspergillus* DNA from EBC samples. In addition, the PCR results for EBC samples were in full agreement with results from BAL fluid. The two patients that had PCR positive EBC samples also had corresponding PCR positive BAL fluid samples. Furthermore, these BAL samples were positive by GM and LFD assays, inferring that these two patients should be treated for IPA (see Chapter 4). The other 10 patients were PCR negative in both EBC and BAL samples. GM and LFD testing of

EBC did not appear to be useful in this limited data set. Interestingly, all EBC samples were negative for human DNA, using a β-globin qPCR assay. Previous studies reported human DNA in 65% (151) and 100% (152) of tested EBC samples. However, a different EBC sampling device was used in these cases. Therefore, the results presented in this thesis section may reflect the limitations of the sampling technique itself.

This study is limited by the small population size, together with the limited EBC sample volume available for testing. This prevented repeat DNA extraction from the original sample, confirmation testing with an alternative assay and also prevented any investigation into the optimisation of EBC sample handling, such as a step to concentrate the sample prior to testing. EBC is a highly dilute, low protein sample. Therefore, biomarkers will be highly diluted and attempts to detect them will push assays to their lower limits of accuracy. Whilst the results of this study demonstrate detection of *Aspergillus* DNA from EBC, the amplification threshold of detection in these samples was not reached until 36-39 cycles. In Study sample 2 the EBC sample amplified at Cq 39, whilst the corresponding BAL fluid sample amplified at Cq 27.8, a 4,000-fold difference. This suggests that enrichment from EBC is required to get a more reliable result. There are several ways in which this can be accomplished, most readily by enriching for *Aspergillus* nucleic acids using magnetic beads coated with *Aspergillus* specific probes. These could be added to EBC and allow several thousand-fold enrichment (*e.g.* from 50ml to 5μl).

Despite the limitations of this study, the results provide encouraging data regarding the possible use of EBC samples in an early detection method for IPA.

## 5.2 EVALUATION OF LOCAL (LUNG) AND SYSTEMIC (SERUM) INFLAMMATORY PROFILE ASSOCIATED WITH IPA BIOMARKER POSITIVE BAL FLUID IN IMMUNOCOMPROMISED PATIENTS

### *5.2.1 Background*

IPA is the most common cause of mortality due to mould disease in haemato-oncology patients (206, 207). Their underlying disease and its treatment lead to periods of prolonged immunosuppression (205), leaving them lacking sufficient innate immune effector cells (neutrophils and macrophages) to protect the lungs from spores. The lung is regularly challenged by microbial pathogens and the local immune response must be rapid and efficient, to prevent potentially lethal infections. Neutrophils and macrophages are crucial in the early defence steps against *Aspergillus* infection [Section 1.4.2]. Phagocytes residing in the lungs at the time of infection will attack the fungi, whilst additional effector cells are recruited to the site of infection by inflammatory cytokines and chemokines. Impaired recognition and phagocytosis and extracellular killing mechanisms by effector cells in haemato-oncology patients allow inhaled spores to survive and germinate to produce hyphae that can become angioinvasive (13).

The adaptive immune response has been extensively investigated in the murine model, as described in Section 1.4.3. Immune response is difficult to investigate in the human haemato-oncology setting, due to the presence of other co-morbidities, neutropenic episodes and corticosteroid treatment. This is reflected in the paucity of publications profiling immune response to IPA in this setting. The tissue lining the respiratory tract is fragile and easily damaged by excessive or inappropriate inflammation. Immune response to pathogens must be regulated to rapidly clear infection, whilst not exposing the tissue to chronic inflammation. The immune response to IPA in the lung has not been directly

investigated *in vivo* in immunocompromised humans. However, an evaluation of the local and systemic inflammatory response associated with pulmonary complications in immunocompromised patients did include 16 cases of fungal pneumonia attributed to *Aspergillus* species (238). Patients with haematological malignancies treated with chemotherapy, HSCT recipients and patients who had received SOT were demonstrated to generate cytokine responses to pneumonia. Increased BAL levels of TNF-α, IL-6, IL-8 and IL-10 and serum TNF-α and IL-6 were found in immunocompromised patients when compared to healthy controls. Within the immunocompromised population, significantly higher BAL fluid levels of IL-6 and IL-8 were found in patients with pulmonary infiltrates of an infectious aetiology than in those with non-infectious complications. The authors observed a trend towards a more intense inflammatory response in those with bacterial infections, as opposed to fungal or viral. Chai *et al* (239) studied serum IL-6, IL-8, IL-10, IFN-γ, and CRP trends in the setting of immunocompromised patients with IA, over the first 4 weeks of therapy and found that circulating IL-6 and CRP levels were high at the start of therapy and generally decreased with antifungal treatment. Persistence of elevated IL-6 and CRP levels after 1 week of therapy was associated with an increased likelihood of treatment failure and death ($P = 0.02$, for both IL-6 and CRP). However, it is likely that these patients were receiving multiple antibiotics/antiviral treatment, making interpretation very difficult. The generation of cytokine responses by immunocompromised patients has also been demonstrated in *Pneumocystis jirovecii* infection (240), in which higher BAL fluid level of IL-6 was found in immunocompromised patients with *P. jirovecii* infection than in non-infected immunocompromised or immunocompetent controls. IL-17, IL-10 and TNF-α levels were no different between the groups. Though very limited in number, these reports suggest that patients with haematological malignancy, receiving cytotoxic chemotherapy, still mount an immune response to respiratory pathogens in the lung. Innate lung defences

have also been shown to be highly inducible in mice receiving myeloablative chemotherapy when infected with *Streptococcus pneumoniae* (241, 242) and *A. fumigatus* conidia (243). Innate defence did not depend on recruited neutrophils or resident alveolar macrophages. Instead results support a hypothesis that the principal means of protection is a localised innate response by lung epithelium.

Western blotting and ELISA are traditional techniques for cytokine analysis. However, both are limited by their ability to report only a single antigen at a time, requiring up to 100µl of sample for analysis of each required antigen. Multiplexed bead arrays are a very efficient alternative to these traditional methods (244, 245), quantifying multiple cytokines at once, in a single 50µl sample, in a relatively short time. This multiplexing technique is more suited to studies in which clinical sample volumes are limited.

Two commonly used multiplex bead arrays are the BD Biosciences CBA kit (San Diego, CA) and the Luminex system. Both use immobilised antibodies on spherical beads to catch soluble antigens from a sample in suspension. Fluorescence systems are used to detect binding of a secondary antibody to the soluble antigen. Whilst the Luminex system must be run on a specific Luminex machine, the BD CBA assay will run on any flow cytometer.

Limited sample volume, together with local flow cytometer availability, made the BD Biosciences CBA kit the most suitable option for use in this study.

This section first describes a series of experiments in which fluorescence-activated cell sorting (FACS) analysis was used to quantify 5 human chemokines and 7 cytokines involved in the differentiation of/secreted by each Th1/Th2/Th17 cell type in clinical samples from cases of proven IPA and patients with haematological malignancy at high risk of IA. These results were analysed to determine whether a respiratory cytokine profile could be used as

a tool in the early diagnosis of respiratory infection, reflecting initial response to infection. Finally, respiratory sample results are compared to circulating cytokine and chemokine levels detected in serial serum samples over the course of antifungal therapy in two haemato-oncology patients.

## 5.2.2 Results

### 5.2.2.1 Cytokine and chemokine concentration in BAL fluids from 'gold standard' proven IPA cohort and haemato-oncology Study cohort

This study was a retrospective evaluation of inflammatory cytokine profiling for IPA diagnosis, with no impact on patient management. The 'gold standard' proven IPA cohort of 11 BAL fluids came from 10 patients, obtained at routine diagnostic work-up at the Innsbruck University Hospital between January 2008 and November 2012 [Section 2.1.2]. The Study cohort of patients were a subset of those recruited in an observational study of IPA diagnosis in the Division of Haemato-Oncology, St Bartholomew's Hospital [Section 2.1.1], based on the availability of BAL samples and full clinical data. 20 BAL fluid samples from adults at high risk of IFD following intensive chemotherapy or allogeneic stem cell transplantation were included in this study. Sample numbers correspond to those investigated in Chapter 4. Corresponding biomarker test results for *Aspergillus* are taken from Chapter 4, defining BAL fluids as combination biomarker positive or negative.

The CBA Human Th1/Th2/Th17 Cytokine Kit and Human Chemokine Kit (BD Biosciences, USA) were selected, in order to quantify 5 human chemokines (IL-8, CCL5 (RANTES), CXCL9 (MIG), CCL2 (MCP-1) and CXCL10 (IP-10)) and 7 cytokines (IL-2, IL-4, IL-6, IL-10, IL-17A, IFN-γ and TNF) in BAL fluid. Testing was performed on the BD FACS Canto II [Section 2.7.2].

Assay standards were prepared for the Th1/Th2/Th17 cytokine kit [Section 2.7.2.2] and the chemokine kit [Section 2.7.2.3] and used to generate a standard curve covering a range from 20 to 5000pg/ml and 10 to 2500pg/ml, respectively. Clinical samples were tested [Section 2.7.2.4] and data files exported for analysis using FCAP Array software, to calculate cytokine and chemokine concentrations (pg/ml) in each clinical sample [Section 2.9.3].

LFD+PCR combination test positive vs negative: The mean concentration of each cytokine and chemokine (pg/ml) is shown in Table 14, for BAL fluid from LFD+PCR dual test (DT) positive and negative samples [biomarker test results from chapter 4, sections 4.2.2.1 and 4.2.2.3]. All variables were non-normally distributed. Median concentrations of each cytokine and chemokine are shown for DT+ and DT- samples, together with the concentration range. The Mann-Whitney U Test was conducted to compare cytokine and chemokine distributions between patients that were DT+ and DT-, with 2-tailed P values (Table 14), to determine whether there was a significant difference in the median concentration of any cytokines and chemokines between the two populations.

Concentrations of IL-2, IL-4, IL-10, TNF-α, INF-γ and IL-17A were <100pg/ml in both DT+ and DT- patients. There was no significant difference in the median concentration of these analytes between the DT+ and DT- populations. Concentration of INF-γ is calculated to be significantly different in distribution between the 2 populations (P = 0.018). However, there are only 5 non-zero values, all in the DT+ population, all below the lowest assay standard concentration (<20pg/ml).

The concentration of CCL5 was low in both populations, <100pg/ml in all 16 DT+ samples and in 13/15 DT- patients, with no significant difference in median concentration between the 2 populations.

| | Mean concentration (pg/ml) | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Sample | IL-8 | CCL5 (RANTES) | MIG (CXCL9) | CCL2 (MCP-1) | IP-10 (CXCL10) | IL-2 | IL-4 | IL-6 | IL-10 | TNF | INF-g | IL-17A | LFD+qPCR result |
| **Proven 1** | 33721.6 | 50.2 | 1209.3 | 4079.7 | 412.9 | 0.0 | 0.0 | 838.1 | 0.0 | 1.7 | 0.0 | 12.3 | Positive |
| **Proven 2** | 6495.9 | 0.0 | 951.0 | 1153.7 | 0.0 | 0.0 | 0.2 | 2275.0 | 9.9 | 81.2 | 0.0 | 2.0 | Positive |
| **Proven 3** | 26928.1 | 3.6 | 500.7 | 2830.5 | 12.2 | 0.0 | 0.0 | 312.8 | 0.0 | 0.3 | 0.0 | 1.5 | Positive |
| **Proven 4** | 18178.3 | 3.2 | 3214.5 | 687.2 | 0.0 | 0.0 | 0.0 | 181.5 | 2.1 | 9.7 | 0.2 | 0.5 | Positive |
| **Proven 5** | 6667.1 | 74.8 | 3133.3 | 10574.2 | 4052.8 | 0.0 | 0.1 | 347.3 | 1.0 | 16.1 | 3.4 | 6.4 | Positive |
| **Proven 6** | 1006.7 | 0.2 | 77.4 | 1860.7 | 76.4 | 0.0 | 0.1 | 108.8 | 0.0 | 0.0 | 0.0 | 4.5 | Positive |
| **Proven 7** | 2715.4 | 5.3 | 1.7 | 9.8 | 3.6 | 0.0 | 0.0 | 0.0 | 0.1 | 1.3 | 0.1 | 15.7 | Positive |
| **Proven 8** | 4520.2 | 0.0 | 0.0 | 2.2 | 0.0 | 0.0 | 0.0 | 0.4 | 0.2 | 58.5 | 0.0 | 5.5 | Positive |
| **Proven 9** | 900.9 | 1.3 | 0.4 | 4.8 | 0.0 | 0.0 | 0.0 | 0.1 | 0.0 | 0.0 | 0.0 | 0.0 | Positive |
| **Proven 10** | 11834.2 | 5.7 | 171.2 | 2872.5 | 329.6 | 0.0 | 0.1 | 1723.6 | 1.0 | 0.1 | 0.0 | 0.0 | Positive |
| **Proven 11** | 25237.1 | 8.9 | 111.5 | 1361.4 | 19.3 | 0.0 | 0.0 | 957.7 | 0.5 | 6.8 | 0.0 | 2.3 | Positive |
| **Study 1** | 2411.6 | 18.2 | 163.7 | 558.3 | 817.5 | 0.0 | 0.0 | 267.5 | 1.3 | 1.3 | 0.0 | 63.2 | Positive |
| **Study 2** | 3213.0 | 4.9 | 80.2 | 6192.5 | 865.5 | 6.1 | 5.4 | 4067.6 | 9.9 | 5.3 | 4.2 | **error** | Positive |
| **Study 3** | 1297.5 | 25.5 | 131.7 | 222.9 | 173.7 | 0.0 | 0.8 | 41.5 | 0.0 | 1.5 | 0.0 | 0.0 | Positive |
| **Study 4** | 2595.5 | 19.3 | 17912.6 | 710.7 | 3489.2 | **error** | **error** | **error** | **error** | **error** | **error** | **error** | Positive |
| **Study 5** | 401.0 | 2.5 | 103.4 | 90.3 | 162.1 | 1.1 | 1.0 | 95.6 | 0.6 | 0.0 | 1.8 | 0.0 | Positive |
| **Study 7** | 32.7 | 0.0 | 10.1 | 43.5 | 5.8 | 1.8 | 0.0 | 2.0 | 0.0 | 1.7 | 0.0 | 0.0 | Negative |
| **Study 8** | 7391.9 | 1324.6 | 5965.2 | 10242.6 | 4237.8 | 0.0 | 2.6 | 286.0 | 3.9 | 1.6 | 0.0 | 0.0 | Negative |
| **Study 9** | 1274.0 | 32.8 | 1141.3 | 746.3 | 1398.5 | 0.0 | 0.8 | 83.1 | 0.0 | 0.7 | 0.0 | 0.0 | Negative |
| **Study 10** | 250.8 | 8.1 | 17.3 | 208.2 | 23.2 | 0.0 | 0.0 | 20.5 | 1.3 | 1.3 | 0.0 | 0.0 | Negative |
| **Study 12** | 3308.3 | 15.2 | 381.5 | 95.3 | 381.7 | 0.0 | 0.0 | 13.9 | 0.6 | 1.2 | 0.0 | 0.0 | Negative |
| **Study 13** | 1411.0 | 121.2 | 13361.5 | 121.3 | 1842.1 | 2.3 | 2.1 | 71.0 | 0.7 | 2.8 | 0.0 | 11.3 | Negative |
| **Study 15** | 100.2 | 4.6 | 5.9 | 35.0 | 11.9 | 0.0 | 1.8 | 20.1 | 0.0 | 1.9 | 0.0 | 0.0 | Negative |
| **Study 16** | 718.2 | 5.6 | 36.5 | 7227.2 | 83.1 | 0.0 | 1.2 | 266.3 | 1.3 | 1.6 | 0.0 | 18.2 | Negative |
| **Study 17** | 34.2 | 0.0 | 1.5 | 23.2 | 2.2 | 0.0 | 0.0 | 3.1 | 0.0 | 0.6 | 0.0 | 0.0 | Negative |
| **Study 18** | 1184.1 | 2.2 | 6.2 | 3200.0 | 59.4 | 0.0 | 0.9 | 854.5 | 0.7 | 3.0 | 0.0 | 84.1 | Negative |
| **Study 19** | 68.1 | 16.8 | 69.7 | 36.6 | 27.1 | 0.0 | 1.1 | 18.3 | 0.0 | 0.0 | 0.0 | 18.2 | Negative |
| **Study 20** | 129.2 | 4.5 | 64.0 | 122.8 | 98.7 | 0.7 | 0.0 | 5.8 | 0.0 | 0.0 | 0.0 | 13.1 | Negative |
| **Study 23** | 71.3 | 1.7 | 2.9 | 62.5 | 4.7 | 0.8 | 1.1 | 8.3 | 1.4 | 0.6 | 0.0 | 17.2 | Negative |
| **Study 24** | 3700.1 | 47.2 | 22.5 | 516.7 | 62.1 | 0.0 | 1.0 | 400.6 | 0.0 | 1.1 | 0.0 | 10.8 | Negative |
| **Study 25** | 24.6 | 1.6 | 6.1 | 26.3 | 11.8 | 0.0 | 0.0 | 0.0 | 0.0 | 1.9 | 0.0 | 0.0 | Negative |
| **DT+ MEDIAN** | 3866.6 | 5.1 | 147.7 | 932.2 | 119.2 | 0.0 | 0.0 | 267.5 | 0.5 | 1.5 | 0.0 | 2.1 | |
| **DT+ RANGE** | 401.0-33721.6 | 0.0-74.8 | 0.0-17912.6 | 2.2-10574.2 | 0.0-4052.8 | 0.0-6.1 | 0.0-5.4 | 0.0-4067.6 | 0.0-9.9 | 0.0-81.2 | 0.0-4.2 | 0.0-63.2 | |
| **DT- MEDIAN** | 250.8 | 5.6 | 22.5 | 121.3 | 59.4 | 0.0 | 0.9 | 20.1 | 0.0 | 1.3 | 0.0 | 0.0 | |
| **DT- RANGE** | 24.6-7391.9 | 0.0-1324.6 | 1.5-13361.5 | 23.2-10242.6 | 2.2-4237.8 | 0.0-2.3 | 0.0-2.6 | 0.0-854.5 | 0.0-3.9 | 0.0-3.0 | 0.0-0.0 | 0.0-84.1 | |
| **DT +/- P value** | 0.001 | 0.737 | 0.161 | 0.244 | 0.921 | 0.458 | 0.166 | 0.068 | 0.357 | 0.480 | 0.018 | 0.946 | |

**Table 14.** Dual LFD+qPCR biomarker test results are shown. Cytokine and chemokine distributions between patients that were DT+ and DT-, with 2-tailed P values calculated.

**Table 14. Mean cytokine and chemokine concentrations detected in BAL fluid samples from the gold standard proven IPA (N=11) and study cohorts (N=20).**

The concentrations of IL-8, CXCL9, CCL2, CXCL10 and IL-6 were very variable within the 2 populations, with a concentration range of 2-4 logs. A highly significant difference was calculated between the median IL-8 concentration of DT+ (median= 3866.6pg/ml) and DT- samples (median = 250.8pg/ml) with a P value of 0.001 (Figure 33).

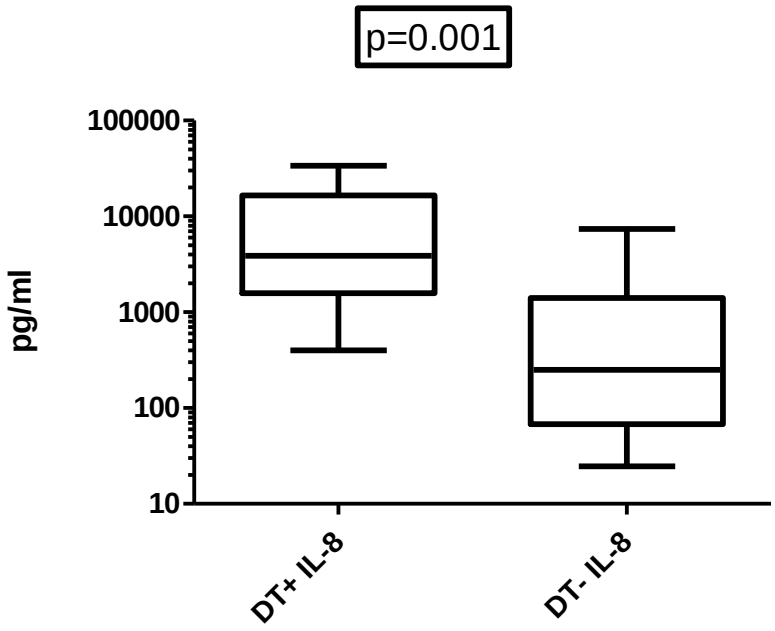



**Figure 33. Box plot showing IL-8 concentrations detected in dual test positive (DT+) and negative (DT-) BAL fluids.**

The upper hinge of the boxes represents the 75th percentile. The middle horizontal line of the boxes represents the median value. The lower hinge of the boxes represents the 25th percentile.

Exclusion of patients that do not meet EORTC/MSG inclusion criteria: In a separate analysis, samples were excluded from patients not meeting the EORTC/MSG 2008 (51) host factor inclusion criteria. Only immunocompromised patients with cancer, hematopoietic stem cell transplant recipients, solid-organ transplant recipients, patients with hereditary immunodeficiencies, connective tissue disorders, and receiving immunosuppressive agents (such as corticosteroids or T cell immunosuppressants) were included in this analysis. With this exclusion, proven 1-4 and 9-10 were removed from analysis. The difference between

the median IL-8 concentration of DT+ patients meeting EORTC/MSG host criteria and DT- samples remains significant, with a P value of 0.016 (Figure 34).

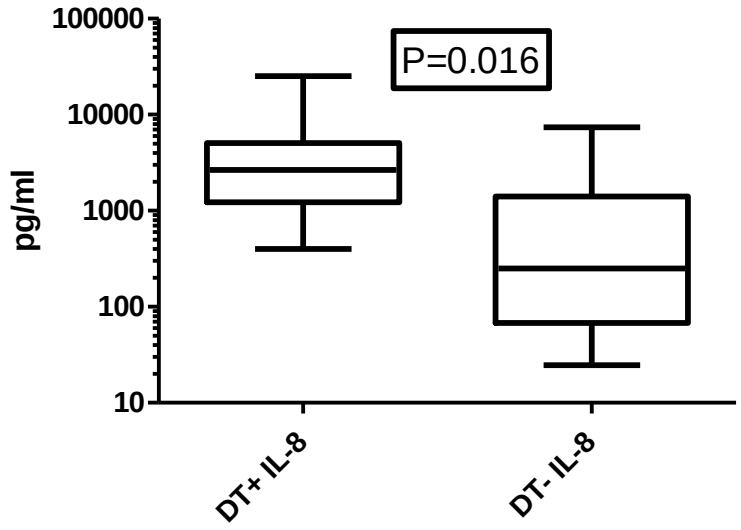



**Figure 34. Box plot showing IL-8 concentrations detected in dual test positive (DT+) and negative (DT-) BAL fluids, with the exclusion of patients not meeting EORTC/MSG host factor inclusion criteria.**

The upper hinge of the boxes represents the 75th percentile. The middle horizontal line of the boxes represents the median value. The lower hinge of the boxes represents the 25th percentile.

### 5.2.2.2 Cytokine and chemokine concentration in serum samples from the haemato-oncology Study cohort

The Study episodes included in this chapter had EDTA blood and serum samples collected twice weekly for the duration of their time in the study [section 2.1.1.4.3]. However, previous investigations, including the combination biomarker testing described in chapter 4, exhausted the supply of the majority of these samples. Consequently, only Study samples 5 and 10 had serum samples available for evaluation of inflammatory cytokine profiling for IPA diagnosis. Five serum samples from Study 5 (from seven days prior to

starting antifungal treatment to 13 days after starting antifungal treatment) and four serum samples from Study 10 (from three days prior to starting antifungal treatment to seven days after starting antifungal treatment) were included in this study. The serum samples were analysed using the CBA Human Th1/Th2/Th17 Cytokine Kit and Human Chemokine Kit (BD Biosciences, USA) as described in section 5.2.2.1.

Circulating serum levels of IL-8, CCL5, MIG (CXCL9), CCL2 and IP-10 (CXCL10) from seven days prior to starting antifungal treatment to 13 days after starting antifungal treatment are shown for haemato-oncology Study sample 5 (Figure 35). The cytokine levels for the BAL fluid sample, collected 4 days after commencing antifungal treatment, are shown for comparison. Results show that IL-8 concentration is 10-fold higher in BAL fluid than in serum, with serum levels remaining stable over the testing period. CCL5 level is high in serum and remains elevated throughout the study period (400-580pg/ml). Circulating serum concentrations of MIG (CXCL9), CCL2 and IP-10 (CXCL10) increase from seven days prior to antifungal start, until they all peak at seven days after antifungal treatment start, then all decline with time.

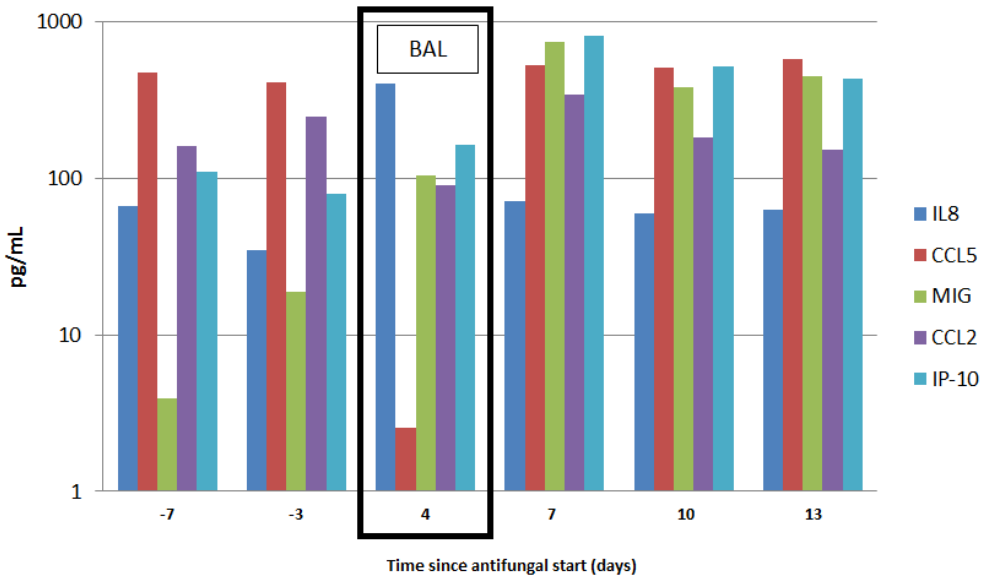



**Figure 35. Bar graph showing circulating serum levels of IL-8, CCL5, MIG (CXCL9), CCL2 and IP-10 (CXCL10) in Study sample 5, from 7 days prior to starting antifungal treatment to 13 days after starting antifungal treatment.**

Levels in BAL fluid, sampled four days after starting antifungal treatment, are shown for comparison.

Circulating serum levels of IL-2, IL-4, IL-6, IL-10, TNFα and INF-γ from seven days prior to starting antifungal treatment to 13 days after starting antifungal treatment are shown for haemato-oncology Study sample 5 (Figure 36). IL-17A detection was not included in this test. Results show that IL-6 concentration is elevated in circulating serum and remains elevated 13 days after the start of antifungal treatment.

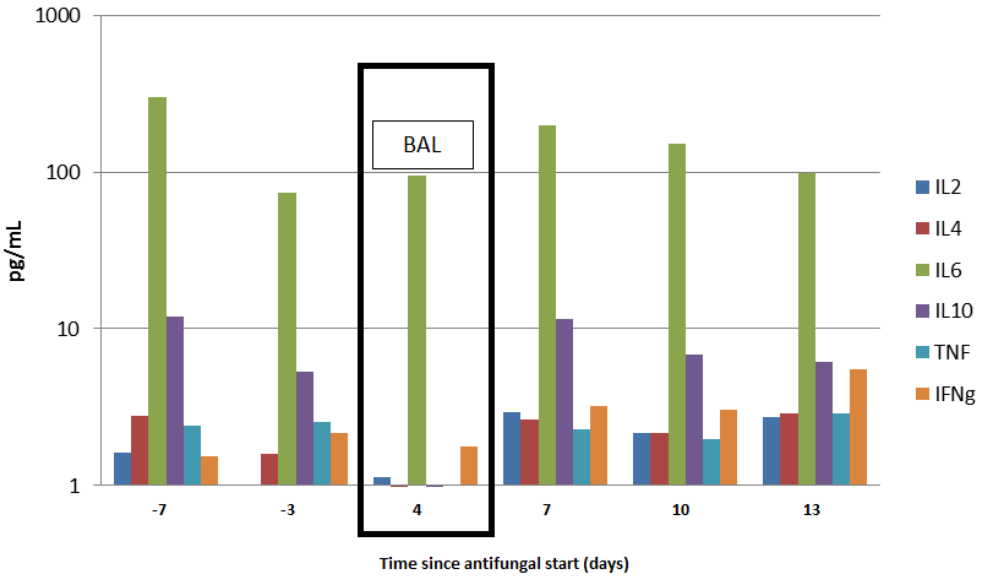



**Figure 36. Bar graph showing circulating serum levels of IL-2, IL-4, IL-6, IL-10, TNF and INF-γ in Study sample 5, from 7 days prior to starting antifungal treatment to 13 days after starting antifungal treatment.**

Levels in BAL fluid, sampled four days after starting antifungal treatment, are shown for comparison.

Circulating serum levels of IL-8, CCL5, MIG (CXCL9), CCL2 and IP-10 (CXCL10) from three days prior to starting antifungal treatment to seven days after starting antifungal treatment are shown for haemato-oncology Study sample 10 (Figure 37). The cytokine levels for the BAL fluid sample, collected 3 days after commencing antifungal treatment, are shown for comparison. Results show circulating serum concentrations of IL-8 and CCL2 increase from 3 days prior to antifungal start, until they all peak at 3 days after antifungal treatment start, then both decline. CXCL9 and CXCL10 concentrations remain low in serum throughout the

testing period. CCL5 concentration increases in serum throughout the testing period.

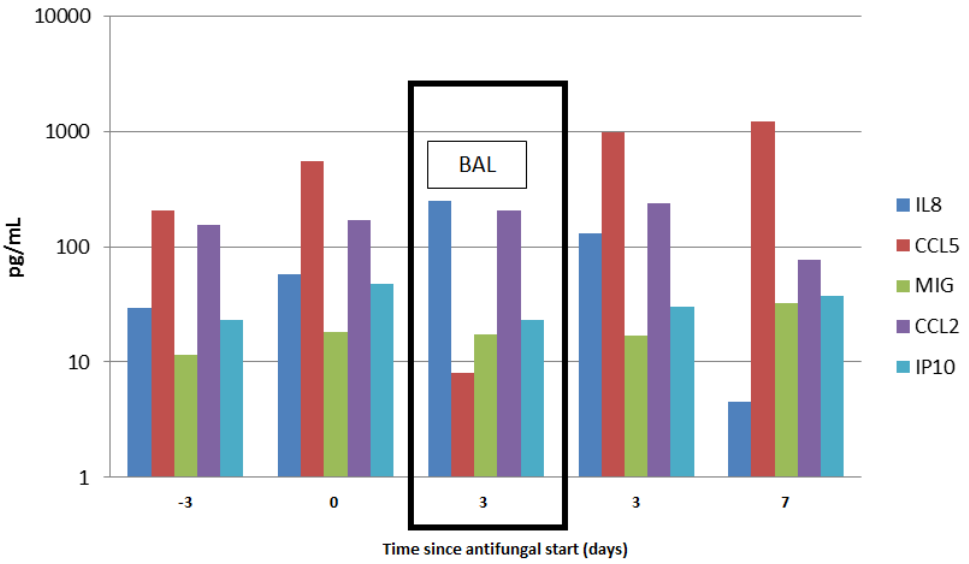



**Figure 37. Bar graph showing circulating serum levels of IL-8, CCL5, MIG (CXCL9), CCL2 and IP-10 (CXCL10) in Study sample 10, from 3 days prior to starting antifungal treatment to 7 days after starting antifungal treatment.**

Levels in BAL fluid, sampled 3 days after starting antifungal treatment, are shown for comparison.

Circulating serum levels of IL-2, IL-4, IL-6, IL-10, TNFα, INF-γ and IL-17A from 3 days prior to starting antifungal treatment to 7 days after starting antifungal treatment are shown for haemato-oncology Study sample 10 (Figure 38). Results show that IL-6 concentration is elevated in circulating serum and remains elevated 7 days after the start of antifungal treatment. IL-6 concentration is approximately 40-fold higher in circulating serum than BAL fluid at 3 days after antifungal treatment start.

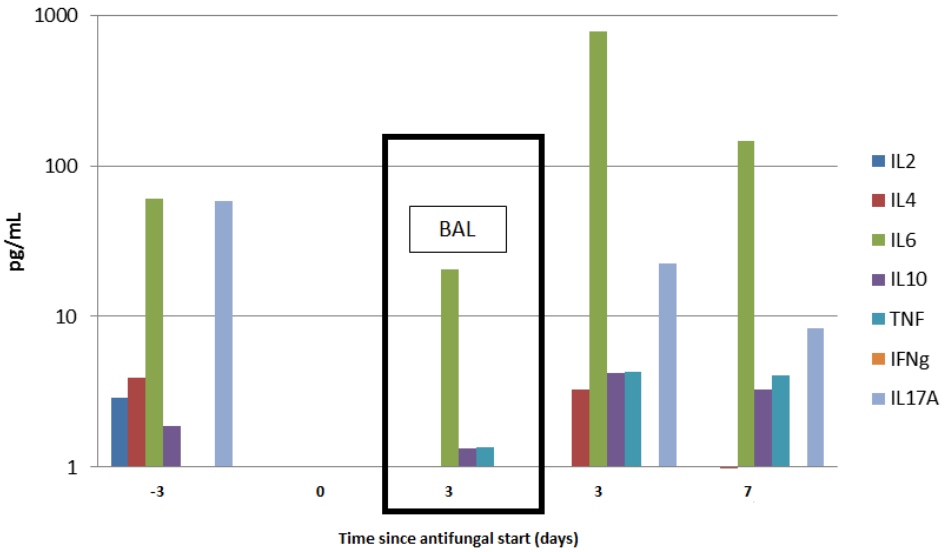



**Figure 38. Bar graph showing circulating serum levels of IL-2, IL-4, IL-6, IL-10, TNF and INF-γ in Study sample 10, from 3 days prior to starting antifungal treatment to 7 days after starting antifungal treatment**

Levels in BAL fluid, sampled 3 days after starting antifungal treatment, are shown for comparison.

### *5.2.3 Discussion*

IA is primarily a pulmonary pathology in the haemato-oncology setting. The main aim of this series of investigations was to determine whether a respiratory cytokine profile could be used as a tool in the early diagnosis of IPA, reflecting initial response to infection.

This study identified that INF-γ levels were very low in all tested BAL fluids, undetectable in 84% of samples. Low INF-γ levels have previously been observed in BAL fluids from patients with IPA (239) and may be the result of immune suppressive treatments (246) received by these patients. However, a limitation of this study is the absence of healthy control samples. Therefore, it is not possible to know for certain that the assay is efficiently detecting INF-γ.

The median IL-8 concentration in the BAL fluid of DT+ patients was 3866.6pg/ml and was significantly higher than the median concentration in the BAL fluid of patients with

suspected non-*Aspergillus* infection (DT-) (P=0.001). Data was also analysed with the exclusion of patients not meeting EORTC/MSG host inclusion criteria. The 5 excluded samples were from cases of histologically proven IPA, from patients whose underlying disease did not leave them profoundly immunocompromised and who were not receiving immune suppressive agents. With the exclusion of these patients, IL-8 concentration remained significantly higher in the DT+ patients (P=0.016).

The high levels of IL-8 detected in the BAL fluid are likely to reflect a localised immune response in the lung. This finding is consistent with results obtained *in vitro* (247), in which human respiratory epithelial cells produced IL-8 when stimulated with proteases released by *A. fumigatus* or by fungal filaments. Neutrophils stimulated with *A. fumigatus* antigens also produce IL-8, via TLR2 and TLR4 (248). However, it is unlikely that neutrophils and monocytes are the main source of IL-8 during febrile episodes and neutropenia (249).

IL-6 concentration is higher in the DT+ (median = 267.5pg/ml) than the DT- (median = 20.1pg/ml) BAL fluids, though the difference in the median values is not statistically significant. The inflammatory response detected in these Study samples, characterised by high BAL fluid levels of IL-6 and IL-8, are in agreement with a previous report (238), however the IL-8 concentrations detected in this study using cytokine bead array chemistry are considerably higher than those reported previously (using ELISA detection).

Although the results of this study indicate a higher concentration of IL-8 in the BAL fluid of suspected *Aspergillus* versus non-*Aspergillus* infection, the data set is small. None of the inflammatory mediators evaluated was specific enough to clearly distinguish between suspected *Aspergillus* and non-*Aspergillus* infections.

The availability of corresponding serum samples was very limited in the haemato-oncology Study cohort. Serial serum samples from two Study episodes were identified and the inflammatory cytokine profiles were evaluated. The main difference between local (lung) and systemic (serum) inflammatory response was in detected CCL5 concentration, which was higher in serial serum samples than in BAL fluid. CCL5 is chemotactic for T-cells and is required for T cell infiltration and activation in the adaptive immune response. IL-6 levels were also high in serial serum samples, as reported in previous studies (238, 239). However, this data set is too small to draw any conclusions about systemic inflammatory response in the Study cohort. Analysis of routine laboratory screening results shows that coagulase negative *Staphylococci* was cultured from the blood of 18 of the 25 haemato-oncology Study episodes on at least one occasion during the study period. *Enterococcus* (including *E. faecium*), *Enterobacter, Escherichia coli, Klebsiella pneumoniae* and *Corynebacterium* were also reported as isolated from the blood of DT- Study patients during the study period.

The isolation of many pathogens from blood cultures, together with the multiple antibiotics/antiviral treatment that the patients receive, and the absence of defined outcome (fever is often resolved without a clear indication of the pathogen responsible) make it very difficult to investigate immune response to IPA in the haemato-oncology setting. Although their underlying disease and its treatment lead to periods of prolonged immunosuppression, leaving them with insufficient neutrophils and macrophages to protect the lungs, the results of this study support previous reports that patients with haematological malignancy, receiving cytotoxic chemotherapy, still mount an immune response to respiratory pathogens in the lung. The analysis of this mechanism may identify new prognostic markers and/or therapeutic targets.

## 5.3 PROXIMITY LIGATION ASSAY FOR THE SENSITIVE, SPECIFIC AND EARLY DIAGNOSIS OF INVASIVE FUNGAL DISEASE

### *5.3.1 Background*

IPA occurs predominantly in immunocompromised patients, with an attributed mortality rate as high as 90% (250). During IPA pathogenesis, conidia adhere to the pulmonary epithelial cells that line the alveoli and are internalised. In immunocompromised hosts these conidia germinate to form hyphae, which exit the pulmonary epithelial cells and penetrate endothelial cells to access the blood vessel lumen. The bloodstream carries hyphal fragments to distal sites where they adhere to and penetrate the luminal surface of endothelial cells and invade deep organs (251). Therefore, rapid recognition and early, appropriate treatment are vital whilst fungal burden is still relatively low and localised to the lungs.

qPCR holds significant promise for the sensitive and specific identification of *Aspergillus* in clinical samples. However, as already discussed, there are significant issues with the practical application of this technology. Although I have identified major flaws in current qPCR methods and introduced a new qPCR assay, optimised and validated according to best practice as per the MIQE guidelines [Chapter 3, (117)], there remains the fact that the presence of a DNA sequence does not prove active infection. Pathogen DNA detection is uninformative with respect to whether the fungus is actively growing and so one cannot unequivocally associate its presence with infection.

The *Aspergillus* LFD uses a MAb (JF5) that is highly specific for an epitope expressed only by actively growing hyphae (119). This assay provides a rapid and reliable assessment of the presence of actively growing *Aspergillus*. However, the main drawback of any pure

antibody-based strategy is the relative lack of sensitivity compared with nucleic acid-based assays such as qPCR.

Data presented in chapter 4 has demonstrated a very high level of agreement between qPCR and LFD detection in BAL fluid of high risk Haematology patients. Whilst positive qPCR results were always supported by positive LFD in this small study population, the relative performance parameters of the technologies indicate that PCR positivity would pre-date LFD positivity in an early testing/screening approach (126). With a high risk of assay contamination from *Aspergillus* spores that are ubiquitous in the environment, and DNA detection not proving active infection, an unsupported qPCR positive result could be false positive for IA.

As a consequence there is a need for an assay that combines the specificity of hyphal-specific antibody detection with the sensitivity of qPCR. The proximity ligation assay (PLA), invented by Fredriksson *et al* (252) could in theory be used to achieve this, using two antibodies to detect adjacent epitopes on the same protein [Section 2.8], in this case proteins expressed exclusively at the growing tips of *Aspergillus* hyphae. PLA is a quantitative assay, allowing quantification of fungal burden in clinical samples. Compared to traditional ELISA it is more sensitive (10- to 100-fold), its dynamic range is broader (4 logs vs 2.5 logs), its workflow is simpler (no wash steps), its time to result is faster (2.5 vs 5 hours) and it only requires a few microlitres of clinical sample. Together with enrichment methods already discussed, this provides the potential to increase further the sensitivity of this assay. PLA detection of actively growing *Aspergillus* would provide a ground-breaking new approach to early detection of IA.

This section describes the development of a new PLA application for the early and reliable detection of IA in high risk patients, using the JF5 MAb and a polyclonal serum raised

against the same *Aspergillus*-specific N-linked glycoprotein antigen that is secreted constitutively at the hyphal apex in actively growing organisms.

### *5.3.2 Results*

#### 5.3.2.1 IL-6 expression in a Control Assay

In order to gain familiarity with the work flow for TaqMan Protein Assays, a control assay was performed using antibodies and assay conditions that had been optimised at Life Technologies, to demonstrate the performance of PLA technology.

A ready-biotinylated polyclonal antibody (pAb) to human IL-6 and a purified recombinant protein target were used in a positive control assay, as described in section 2.8.1. The pAb was split into two pools and used to prepare assay probe A and assay probe B [Section 2.8.1.1]. Assay probe solution was prepared [Section 2.8.1.2] and a dilution plate was prepared from recombinant protein target [Section 2.8.1.3]. Assay probe solution and recombinant protein dilutions were combined in a binding plate [Section 2.8.1.4] reaction. Following binding of the oligo probes to their protein targets, a ligation reaction was performed to seal the gap to generate a molecule that was amplified and detected by qPCR [Section 2.8.1.5]. Data from the TaqMan protein expression experiment was analysed using a threshold setting of 0.2 with automatic baseline. The Cq data was exported for comparative analysis and used to generate a standard curve of 5-fold serial dilutions from 2,000 to 0.128pg/mL. The result at 0pg/mL represents the no protein control (NPC). A dynamic range of 0.128-2000pg/mL was demonstrated (Figure 39), with linearity over 4 logs in this assay.

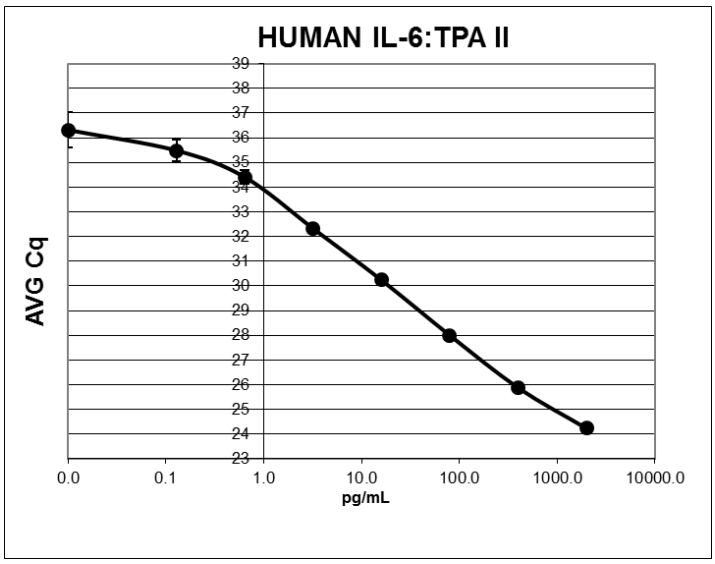



**Figure 39. Detection of IL-6 recombinant protein dilutions 0.128-2000pg/mL, by pAb probes in a PLA control assay.**

The result at 0pg/mL represents the no protein control (NPC).

### 5.3.2.2 *Aspergillus* PLA

#### 5.3.2.2.1 Antibody selection

Life Technologies recommend the simplest way to perform PLA is to use a single pAb and split it into two pools to prepare 3' and 5' probes. pAbs must be immunogen affinity purified prior to use, to remove non-specific antibodies, as their presence diminishes the sensitivity and specificity of the assay (253). The performance of unpurified or protein A purified pAbs have not been well characterised.

MAbs have been shown to perform well in PLAs (254, 255). Life Technologies advise that a single MAb is not suitable for generating a pair of assay probes because it binds to a single epitope and therefore cannot form the required proximity binding pair. However, the JF5 MAb developed by Dr Christopher Thornton (119) binds an *Aspergillus*-specific mannoprotein antigen by recognising multiple repeat epitopes on the same antigen (Figure

40). Therefore, I reasoned that this single MAb could be used to generate both PLA probes. Dr Thornton provided JF5 MAb and antiserum (pAb) raised in rabbits against purified *A. fumigatus* mannoprotein antigen [Section 2.8.2.1].

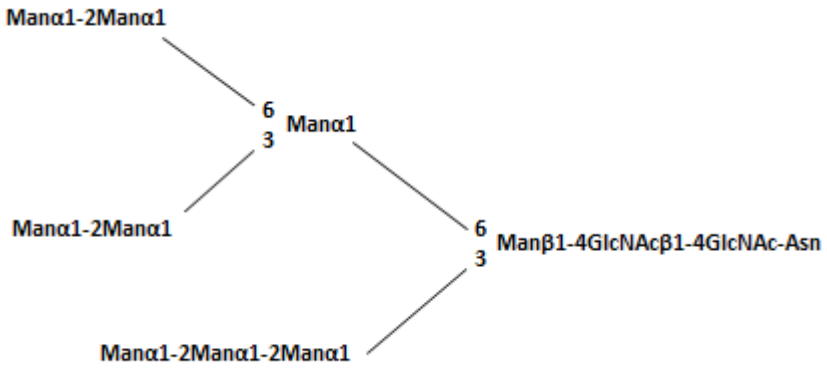



**Figure 40. Structure of the Aspergillus-specific mannoprotein antigen recognised by MAb JF5.**

α1,3 and α1,6 linked high mannose glycoprotein. Structural detail kindly provided by Dr Christopher Thornton (Exeter, UK).

### 5.3.2.2.2 Probe development – Part 1

The crude pAb antiserum and the JF5 MAb were biotinylated using the APEX antibody labelling kit [Section 2.8.2.4]. The biotinylated antibodies were extensively dialysed against PBS, to remove any free, unbound biotin. A forced proximity test was performed for each antibody to determine whether or not the biotinylated antibody was suitable for making proximity probes [Section 2.8.2.5]. The results of the first forced proximity tests are shown in Figure 41. The ΔCq value was calculated for each biotinylated antibody: Average Cq (negative control) – Average Cq (Forced Proximity Probe). ΔCq≥8.5 was required for the antibody to pass the Forced Proximity Probe Test.

Figure 41A shows the results of the pAb assay. ΔCq is <8.5, therefore the pAb fails the test. Figure 41B shows the results of the MAb assay. ΔCq is <8.5, therefore the MAb also fails the test. This could have been caused by the presence of free biotin in the preparation, if dialysis was insufficient, or by under-biotinlyation of the antibodies. A third explanation for failing the forced proximity test is that the antibody concentration was lower than expected, as the Apex kit indicates that recovery is typically between 40-80%.

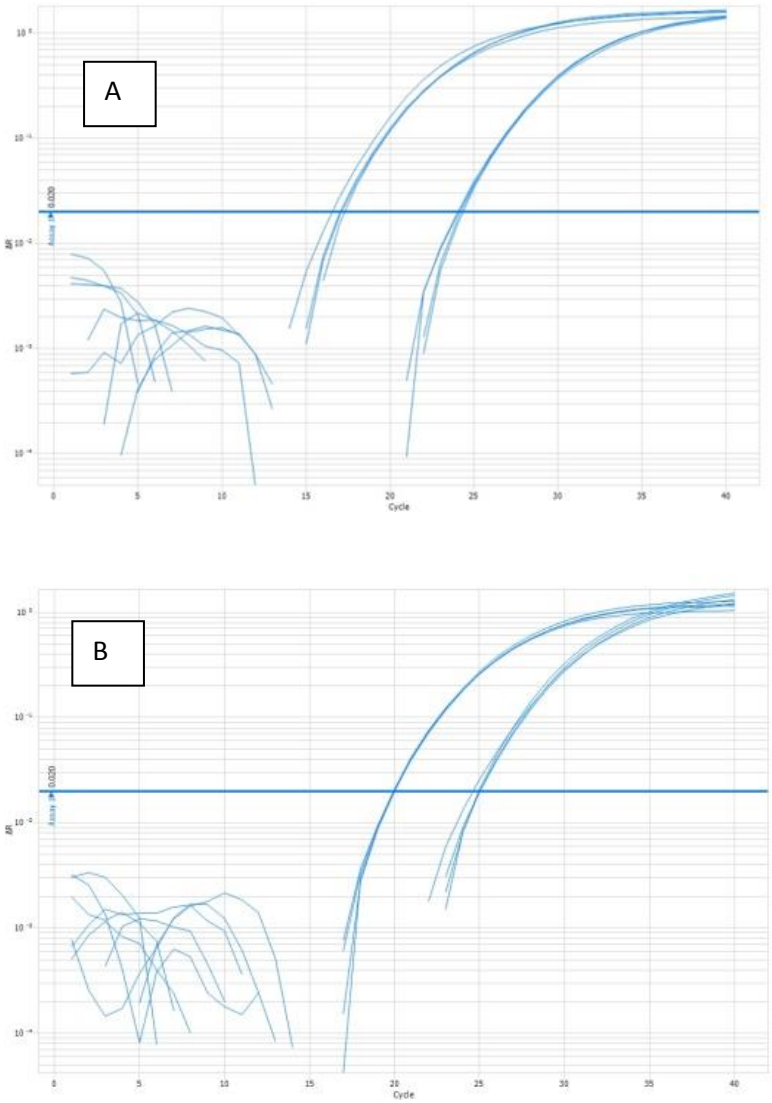



**Figure 41. Forced proximity test amplification curve for a) pAb forced proximity probe (left trace) and negative control (right trace) and b) MAb forced proximity probe (left trace) and negative control (right trace).**

### 5.3.2.2.3 TaqMan Protein Assay – TPA I protocol – Part 1

Despite failing the forced proximity test, the decision was made to use the biotinylated pAb pool and MAb to make an initial set of proximity probes. The purpose was to test whether or not the probes could generate a signal even with sub-optimal probe labelling. These probes were used together with the TaqMan Protein Assays Open Kit [Section 2.8.2.6] in the first attempt at *Aspergillus* PLA.

The 3' and 5' pAb probes were incubated with 10-fold serial dilutions of a crude preparation of purified target glycoprotein [Section 2.8.2.3] or with buffer (Negative control) in the binding step, followed by a ligation reaction to seal the gap to generate a molecule that was amplified and detected by qPCR [Section 2.8.2.6]. Increasing Cqs were seen with serial 10-fold dilutions of purified glycoprotein antigen (A to D), with a 70-fold difference between the highest dilutions (D) and the antigen-negative sample (Negative C) (Figure 42). Results successfully demonstrate the ability of the PLA to detect its target antigen.

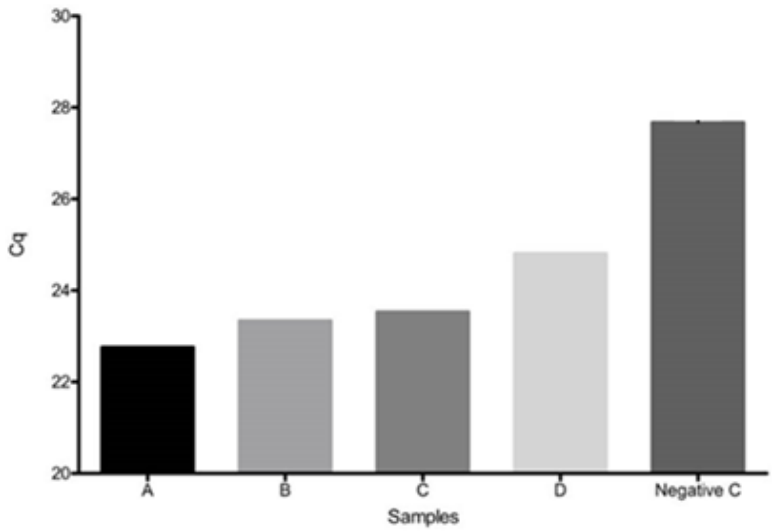



**Figure 42. Detection of Aspergillus-specific N-linked glycoprotein antigen by TPA I in serial 10-fold dilutions of purified glycoprotein antigen (A to D), and antigen-negative sample (Negative C).**

In a second PLA, combinations of 3' (A) and 5' (B) probes were incubated with a 1:1000 dilution of purified glycoprotein antigen or with buffer (Negative control). The results for pAb A/pAb B (Figure 43A), MAb A/pAb B (Figure 43B) and pAb A/MAb B (Figure 43C) are shown. In all cases, the ΔCq was <2.5 between an antigen positive and negative sample. This is a very small difference, but demonstrates a successful PLA run.

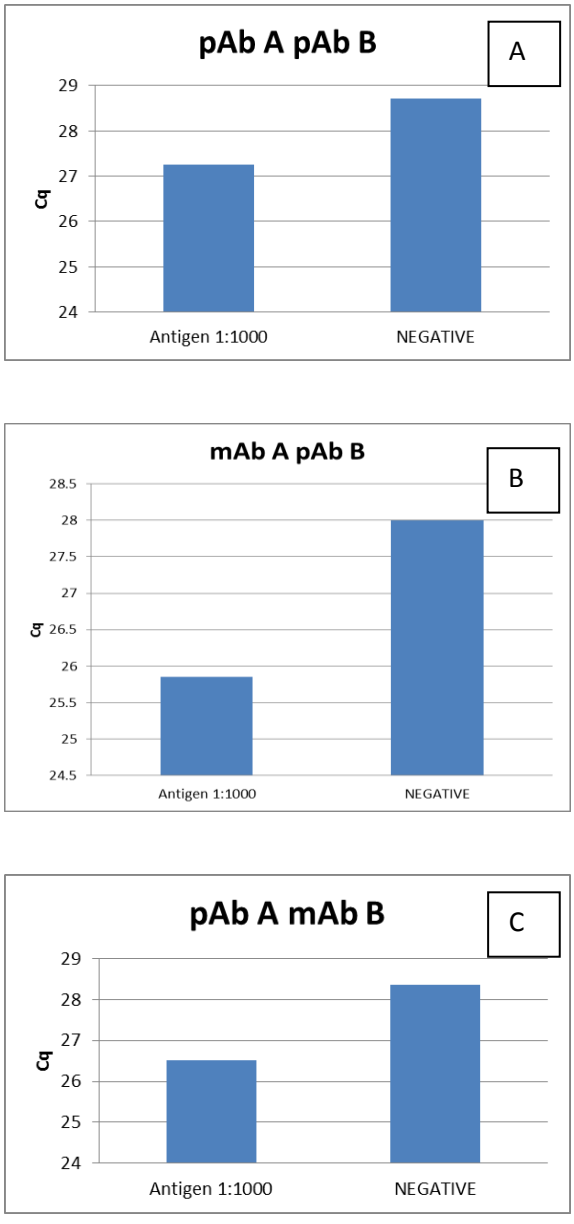



**Figure 43. Bar graph representation of PLA result for probes A) pAb A/pAb B, B) MAb A/pAb B and C) pAb A/MAb B PLA.**

Cq (y axis) is shown for a 1:1000 dilution of purified glycoprotein antigen and negative control (buffer).

### 5.3.2.2.4 Probe development – Part 2

The crude pAb antiserum and the JF5 MAb were biotinylated using the APEX antibody labelling kit.

The biotinylation step was repeated in the pAb serum and MAb, using the APEX antibody biotinylation kit, but with the addition of a dialysis step prior to biotinylation as well as after, in order to remove any contaminants that may interfere with the biotinylation process. Results for the pAb serum are shown in Figure 44. ΔCq is 11, meaning that the antibody pool passes the test and is suitable for making proximity probes.

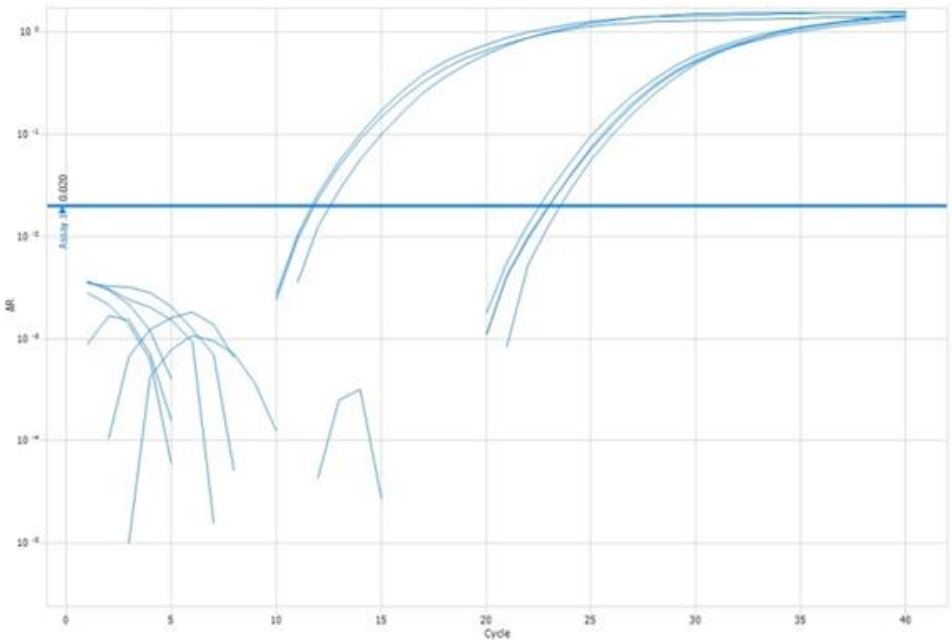



**Figure 44. Repeat forced proximity test amplification curve for pAb forced proximity probe (left trace) and negative control (right trace).**

The pAb biotinylation process was also performed using an alternative kit; Thermo EZ link Biotinylation kit [Section 2.8.2.4], as recommended in the TaqMan Protein Assays Chemistry Guide. The results of the forced proximity test were disappointing, as shown in

Figure 45, with a ΔCq of only 3.5. Therefore, this biotinylated Ab pool failed the forced proximity test.

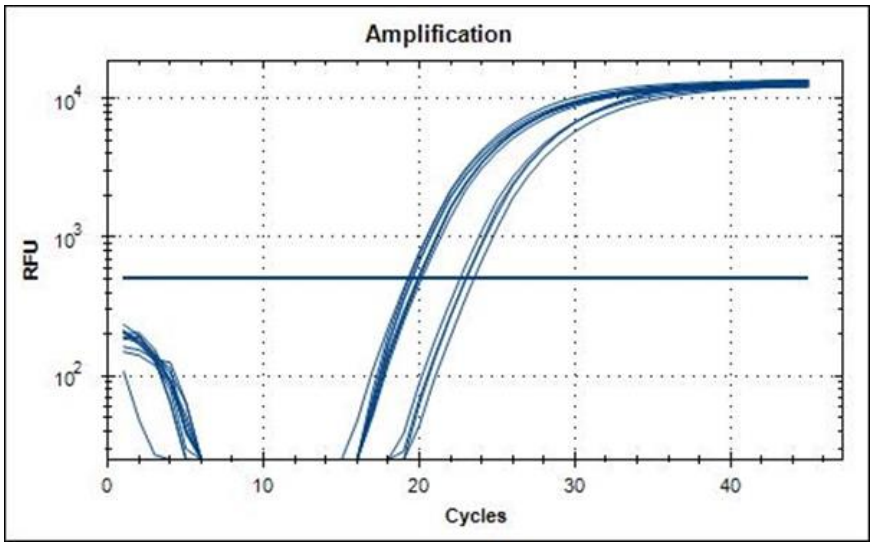



**Figure 45. Forced proximity test amplification curve for pAb forced proximity probe (left trace) and negative control (right trace).**

The decision was made to continue using the APEX biotinylation kit and proceed to prepare a biotinylated stock of the JF5 MAb.

### 5.3.2.2.5 TaqMan Protein Assay – TPA I protocol – Part 2

Once they had passed the forced proximity test, the biotinylated stocks of pAb and MAb were used to prepare new 3' (A) and 5' (B) proximity probes [Section 2.8.1.1].

Combinations of 3' and 5' probes were incubated with neat, 1:100 and 1:200 dilutions of a crude *Aspergillus* culture filtrate (CF) preparation [Section 2.8.2.2] or with buffer (negative control). The results for pAb A/pAb B (Figure 46A), MAb A/pAb B (Figure 46B) and pAb A/MAb B (Figure 46C) are shown. In all cases, the ΔCq was <2. This is a very small

difference, but demonstrates that PLA works in principle. However, further optimisation is needed.

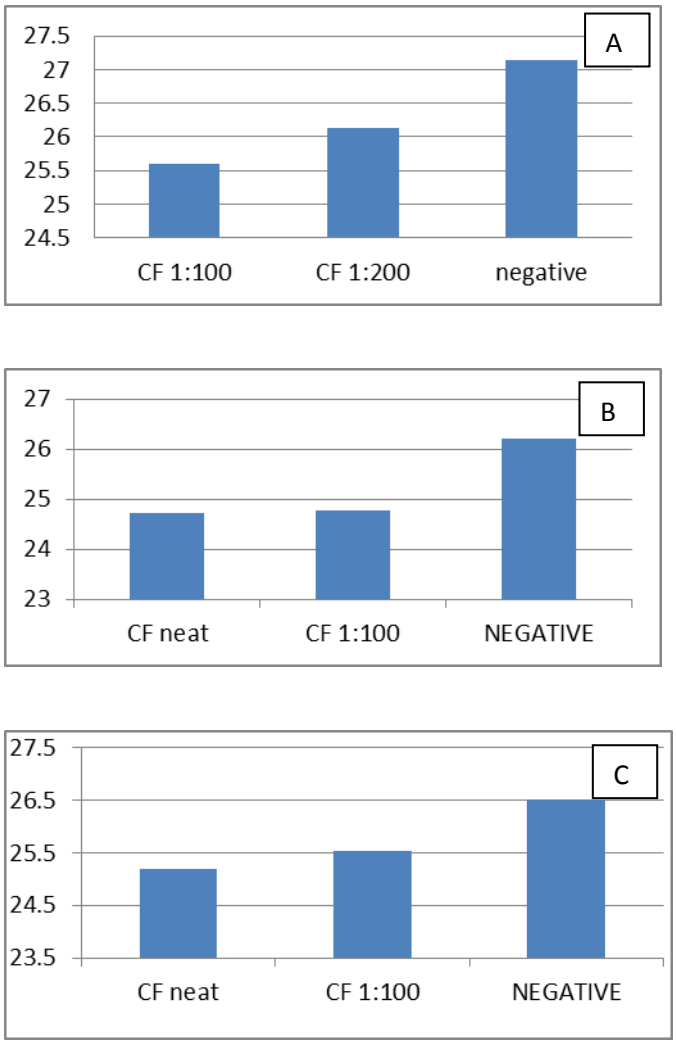



**Figure 46. Bar graph representation of PLA result for probes A) pAb A/pAb B, B) MAb A/pAb B and C) pAb A/MAb B PLA.**

Cq (y axis) is shown for dilutions of *Aspergillus* culture filtrate (CF) preparation and negative control (buffer).

### 5.3.2.2.6 TaqMan Protein Assay – TPA II protocol

A simplified work flow and modified reagents (designated TPA II) were provided by Life Technologies [Section 2.8.2.7]. The main difference between the original TPA I and the new TPA II protocols is the move from a 3 step process, consisting of i) the binding reaction,

ii) ligation and iii) PCR amplification, to a 2 step process in which ligation and PCR mixes are combined and take place in one step, on-board the thermocycler. Using the TPA II protocol, combinations of 3' and 5' probes were incubated with crude *Aspergillus* culture filtrate preparation or with buffer (Negative control). The results for pAb A/MAb B (Figure 47A) and MAb A/MAb B (Figure 47B) are shown. In both cases, the ΔCq was 5. Results demonstrate a higher ΔCq and a greater sensitivity compared to the TPA I protocol.

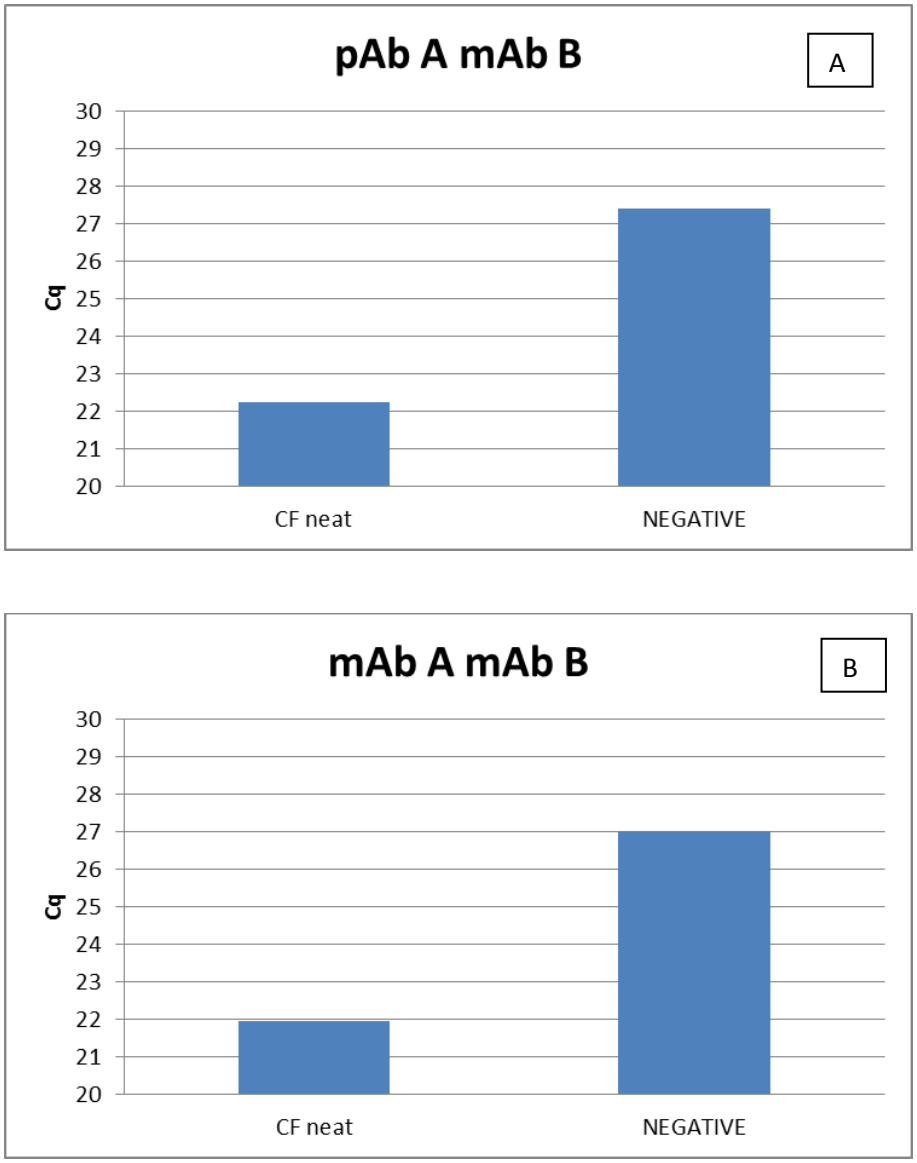



**Figure 47. Bar graph representation of PLA result for probes A) pAb A/MAb B and B) MAb A/MAb B.**

Cq (y axis) is shown for dilutions of Aspergillus culture filtrate (CF) preparation and negative control (buffer).

### 5.3.2.2.7 Further probe development

The MAb and pAb stocks were purified using the Thermo Scientific Nab protein A Plus spin kit [Section 2.8.2.8]. This kit purifies IgG antibody from other antibody classes that may be present. This purification step is used to remove non-specific antibodies that would otherwise be biotinylated along with the target antibodies in the next stage. The results of the forced proximity test for the protein A purified antibodies are shown for the MAb (Figure 48A) and pAb (Figure 48B). ΔCq is >8.5. Therefore, both antibodies pass the forced proximity test.

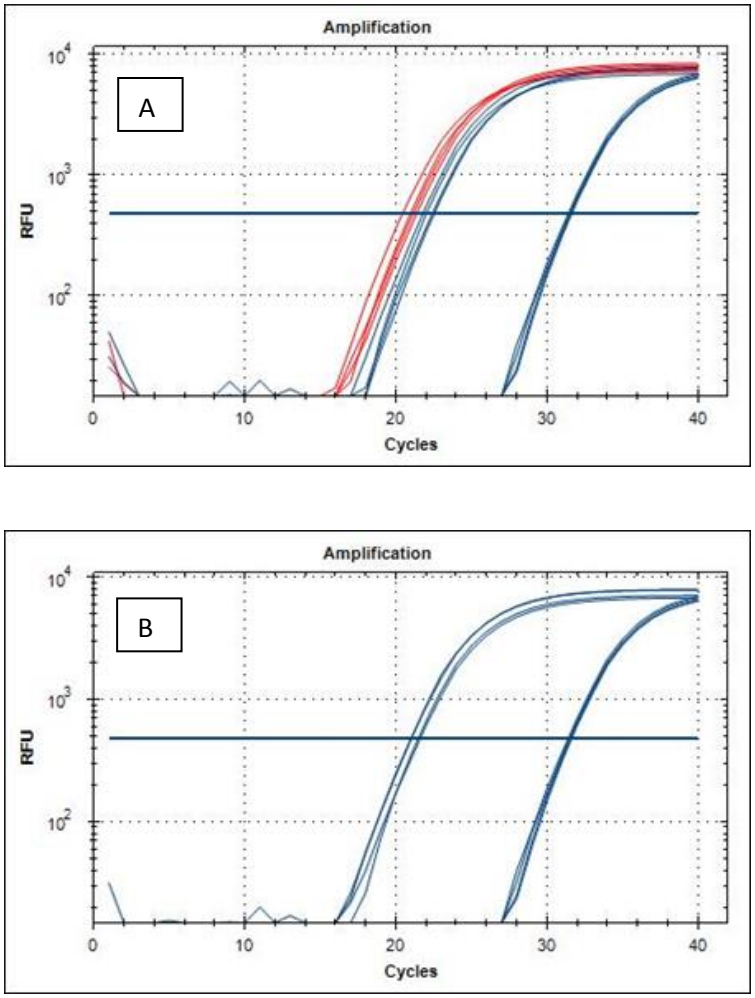



**Figure 48. Forced proximity test amplification curve for protein A purified A) MAb and B) pAb.**

Forced proximity probe (left trace) and negative control (right trace).

5.3.2.2.8 TaqMan Protein Assay – TPA II protocol – Part 2

Once they had passed the forced proximity test, the protein A purified and biotinylated stock of MAb was used to prepare new 3' (A) and 5' (B) proximity probes [Section 2.8.1.1].

These probes were incubated with crude *Aspergillus* culture filtrate preparation or with buffer (negative control). The results are shown in Figure 49. ΔCq was 4.40.

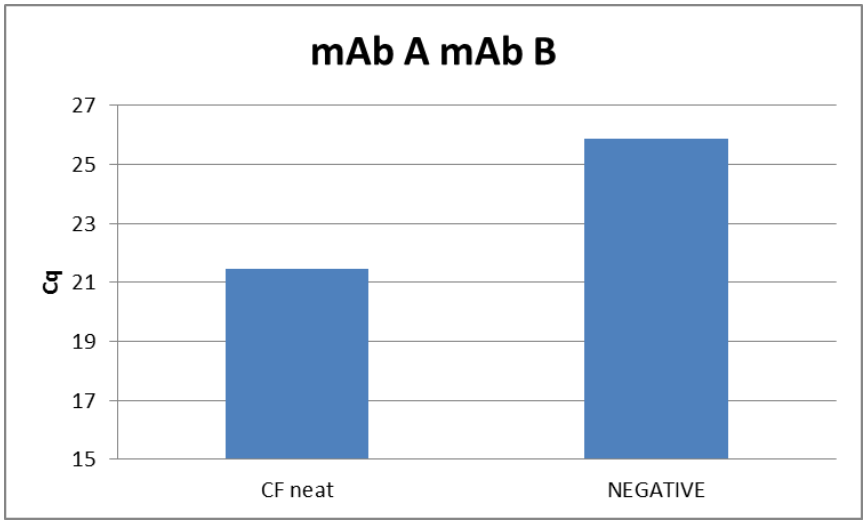



**Figure 49. Bar graph representation of PLA result for probes MAb A/MAb B.**

Cq (y axis) is shown for dilutions of *Aspergillus* culture filtrate (CF) preparation and negative control (buffer).

## *5.3.3 Discussion*

Molecular tools such as qPCR and ELISA are providing sensitive and specific detection of nucleic acids and proteins, respectively. This is enabling the earlier diagnosis and treatment of human diseases. These tools are being combined in innovative ways, as exemplified by the PLA, to push the limits of biomarker detection. PLA has previously been used to directly quantify proteins in biological samples such as plasma and serum (252, 253). To date, only a single publication has explored the use of PLA for microbial pathogen

detection (255), demonstrating proof of principle for MAb detection of porcine parvovirus and a bacterium.

This section describes three firsts:

1. the first ever application of PLA to the detection of fungal protein. The key criterion that will determine the success of PLA diagnostic assay for IA is the availability of PLA-compatible antibodies that target *Aspergillus*-specific proteins, ideally proteins that are secreted only during the active growth of the fungus. To this end, a MAb and pAb antiserum preparation raised against an *Aspergillus*-specific N-linked glycoprotein antigen (119), have been demonstrated to be capable of detecting antigen present in culture filtrate. Firstly, Life Technologies' commercially available TPA I open kit was used, together with a non-optimised antibody preparation for the target glycoprotein to show the feasibility of using PLA as a diagnostic assay. Results demonstrate the successful biotinylation and oligonucleotide attachment of the MAb and pAb, the ability of the PLA to detect its target antigen and an observed difference between the Cq of an *Aspergillus* antigen-positive and an antigen-negative sample. Unlike standard qPCR, the negative control in a PLA generates a Cq due to ligation of PLA probes being brought into proximity by chance. This background can be minimised through further optimisation of the assay parameters.

   Access was given to Life Technologies' new, improved TPA II open kit (not commercially available). This has increased the ΔCq and the sensitivity of the assay. However, Cq of the negative control remains <30. Further assay optimisation is needed to decrease background signal, by reducing non-specific

ligations. Altering the antibody to oligonucleotide ratio in the preparation of assay probes may achieve this by minimising free oligonucleotide.

2. This section describes the first PLA detection of a target protein with probes that are not an immunogen affinity purified pAb. The expectation is that further pAb purification will result in even better assay performance in terms of it sensitivity and dynamic range. An additional antibody purification step, in the form of protein A purification, was performed in this study, but did not result in a marked improvement in assay performance. In some respects, this could seem counter-intuitive. However, because the assay is very sensitive, antibody concentration and handling are very important and can have an impact on assay performance. If one considers that with each step, the antibody can be 'lost' or denatured, then it is possible for performance to decrease with these additional steps.

3. This section also demonstrates the first use of a single MAb for generating a pair of assay probes, binding multiple repeat epitopes on the same antigen. This significantly improves the potential of this assay, since it removes the requirement for two compatible antibodies, which had been thought an essential prerequisite for a successful PLA.

The results presented in this thesis section demonstrate the proof-of-concept for a PLA-based non-invasive diagnostic test that allows early detection of actively growing *Aspergillus* infection. However, several factors require improvement and optimisation in order to evaluate create an assay of suitable a standard for evaluation with clinical samples. This work should include the optimisation of both the antibody-oligonucleotide ratio in the probe preparation step and the probe concentration in the PLA assay. These steps will minimise free oligonucleotides in the PLA and minimise background signal

(increase Cq of the negative control). Optimisation of binding incubation and ligation incubation times may further improve assay performance. Optimisation of antibody preparation and purification steps may improve the sensitivity and dynamic range of the assay, however any additional steps need careful evaluation to ensure that the potential gain is not outweighed by the risk of antibody denaturation with each additional antibody handling step. The sensitivity and dynamic range of the assay will need to be established using purified target protein as well as *Aspergillus* supernatants and assay specificity will need to be established by testing supernatants from related and unrelated fungal species. It would then be necessary to evaluate assay performance using appropriate biological samples such as blood and BAL fluid, spiked with known number of *Aspergillus* targets. Normalisation against chromosomal DNA present in the sample should permit translation of qPCR quantification cycles into a quantitative PLA assay that determines proliferating fungal load within a patient sample. It is important to consider that blood and BAL samples could inhibit the PCR assay and may require additional purification. Building on the proof-of-concept demonstrated in this chapter, further optimisation and validation steps could result in a unique assay that allows more sensitive, specific and earlier detection of IA and so will translate immediately into more targeted clinical decision-making, helping reduce the mortality rate for high-risk individuals. It will also open the way for the development of additional assays that will assist with early and appropriate initiation of systemic antimicrobial therapy, leading to improved clinical outcomes, patient satisfaction and reduced associated costs. In the future, the combined testing of DNA and protein targets from the same sample on the same analytical platform (i.e. qPCR) may further improve the sensitivity and specificity of disease diagnosis. Finally, further technical improvements that make possible the multiplexing of PLAs will enable the detection of multiple infectious

organisms (i.e. different fungal species) in a single test, which ultimately may have the greatest positive impact on the health of at-risk patients.

# CHAPTER 6

# GENERAL DISCUSSION

Molecular diagnostics has had a huge impact on our ability to detect diseases and pathogens early, speedily and with great certainty. Unfortunately its contribution to more accurate and early diagnosis of fungal diseases has been somewhat peripheral, mainly due to the many limitations inherent in the techniques used and discussed throughout this thesis. The rising incidence of IFD, especially IA, remains an important cause of morbidity and mortality in the haemato-oncology setting (77, 256), where it is primarily a pulmonary pathology, with inhaled *Aspergillus* conidia reaching the lungs and germinating to produce invasive hyphae (207). The lack of definitive diagnostic criteria has a significant impact on survival in these patients (257), since it precludes early and targeted initiation of systemic antifungal therapy.

Culture from a normally sterile site is rarely feasible ante-mortem as patients are too unwell to undergo invasive procedures and viable fungi are extremely rare, as evidenced by poor blood culture results (129). Therefore, antifungal prophylaxis and empirical therapy have become commonplace in the haemato-oncology setting (54). This approach to patient management is likely to lead to treatment of patients who do not have IFD, exposing them to unnecessary drug toxicities and potential complication in the management of their underlying disease. By contrast, a diagnostic-driven approach combines imaging techniques and biomarker detection to direct antifungal therapy to those with evidence of IFD, withholding therapy in patients with negative diagnostic biomarker results. The success of this approach relies heavily on the early availability of imaging and biomarker detection assays. Respiratory specimens such as BAL fluid can be tested for biomarkers of infection, but the utility of a combination biomarker detection approach in BAL fluid for the early diagnosis of IPA has not been extensively studied. Although the introduction of new non-invasive tests, combined with more effective and better-tolerated antifungal agents, has resulted in lower mortality rates associated with

IPA, this disease continues to have substantial attributable mortality combined with a major impact on hospital resource use (258).

## 6.1 Findings

Nucleic acid-based tests, such as qPCR, are readily adaptable for use in the fungal diagnostic laboratory. However, the EORTC/MSG has not yet recommended the use of qPCR in the diagnosis of IA (51), mainly because of the absence of standardised protocols (187). Together with the inadequate reliability of amplification from blood and serum, this has led to attempts to reach a consensus for qPCR diagnosis. This was pioneered by EAPCRI, which aims to provide optimal standardised protocols for diagnostic *Aspergillus* PCR (114). Thus far its members have focused on blood samples and asserted that the efficiency of *Aspergillus* PCR is limited by the DNA extraction procedure and not by the PCR amplification. However, in this thesis it is shown that analysis of their own data demonstrates that PCR assays are in fact highly variable, with different PCR efficiencies resulting in significantly different sensitivities.

Increasing acceptance of the MIQE guidelines is a development that may further improve the reliability of qPCR-based assays for IA (167), by offering a strategy for reproducibility and quality control to improve practices in qPCR experiments. This thesis describes the development of the first qPCR assay targeting *Aspergillus* species designed, optimised and validated in strict compliance with the MIQE guidelines. This thesis also describes the evaluation of this new qPCR assay, alongside the existing EORTC/MSG-endorsed GM ELISA and the new *Aspergillus* LFD, for diagnosis of IPA. A new finding demonstrated in this thesis is that a dual *Aspergillus* LFD and qPCR testing approach applied to BAL fluid is a very

sensitive and highly specific aid in IPA diagnosis. A combination biomarker approach could be incorporated into a diagnostic-driven approach to patient management to direct antifungal treatment to patients with evidence of invasive fungal disease. These results support the use of LFD/qPCR testing as a clinical tool to aid in IPA diagnosis, in the absence of histological proof.

Clinical studies have relied on the EORTC/MSG classification of IFD to attribute a possible/probable likelihood of IFD in the absence of definitive proof. Instead, this thesis details an investigation into the use of a triple biomarker strategy (qPCR, LFD, GM) on BAL fluid and defines the performance characteristics of the assays in a gold standard cohort of proven cases of IPA and a cohort of patients with no EORTC/MSG risk factors nor evidence of IPA. The application of the triple testing in a study cohort shows near perfect agreement between qPCR and the LFD in BAL fluid, but disappointing assay performance with blood/serum in BAL positive cases. Importantly, while microscopy and culture are not informative in the face of mould-active anti-fungal therapy, biomarkers remain positive in BAL fluid. Using the LFD/qPCR biomarker data as a reference standard, the EORTC/MSG criteria have a sensitivity of less than 40%, in keeping with previous post-mortem comparisons. This highlights that the EORTC/MSG criteria should be used with caution when evaluating new diagnostic approaches for IFD and when used in clinical algorithms to define management of patients in unproven cases.

To date, there is a single report in the peer-reviewed literature in which the use of a triple biomarker strategy (PCR, LFD, GM) on BAL fluid has been evaluated (259). The PCR assay used in the study was a nested, two-step PCR (260), with traditional end-point detection achieved by separating products using agarose gel electrophoresis, with ethidium bromide staining. Therefore, quantification is not possible. The report is very recent (March 2014)

and although it included 78 BAL fluids, only three (4%) samples were from cases of histologically proven IPA. The other 96% were unproven and classified by EORTC/MSG likelihood. Assay sensitivity was calculated using an imperfect reference standard of EORTC/MSG proven/probable cases.

Biomarker detection in blood has failed to transform IFD management in haemato-oncology. There are conflicting reports about the clinical utility of serum-positive GM results in the diagnosis of IA. Although early reports indicated both high sensitivity and specificity, more recent studies have reported a lower sensitivity of between 40 and 50%, particularly in the setting of mould-active antifungal drugs and in the absence of serial sampling (261). The findings of this thesis indicate the insensitivity of combination biomarker detection in blood compared to respiratory sampling. Furthermore, this thesis presents data that confirm previously published concerns over the repeatability of GM in serum (215, 216), whilst also demonstrating that BAL GM results appear stable (229).

In the final results chapter of this thesis, three small-scale investigations are described, introducing novel approaches to IPA diagnosis. Findings are reported from a series of experiments evaluating the potential of EBC as a clinical sample for non-invasive diagnosis of IPA. The findings of this thesis indicate that biomarker detection results in EBC do not correlate well with results in BAL fluid samples. Although *Aspergillus* DNA was detected in the EBC of two patients with corresponding positive BAL fluid, detection was at the lower limits of assay accuracy and sample volume limitations prevented confirmation testing or attempts to optimise sample handling. From this small data set it is fair to conclude that EBC is currently unsuitable for clinically relevant detection of *Aspergillus* biomarkers in the respiratory tract. However, changes to sampling technique (262) or appropriate fractionation of EBC (263) may lead to improved results. Therefore, the translation of the

potential of EBC into practical diagnostic use may be possible with a greater understanding of the technical problems and subsequent optimisation of sample handling and processing steps.

This thesis also reports findings from an evaluation of the inflammatory cytokine profile associated with IPA biomarker positive BAL fluid. The findings reported are from a small sample population and do not identify any inflammatory markers that can distinguish between suspected *Aspergillus* and non-*Aspergillus* infections. The findings do, however, agree with previous reports that patients with haematological malignancy, undergoing cytotoxic therapy, still mount an immune response to respiratory pathogens in the lung (238-240).

In a final investigation into novel approaches to IPA diagnosis, this thesis details the first ever application of a PLA test to the detection of an *Aspergillus* diagnostic antigen. The findings presented in this thesis show that pAbs and a mAb raised against the JF5 mannoprotein (119) can be used to develop a PLA test to detect actively growing *Aspergillus* species. Further optimisation and normalisation against chromosomal DNA will allow the translation of Cq values into a quantitative assay to determine the proliferating fungal load in a clinical sample.

## 6.2 Future Directions

The findings presented in this thesis support the use of a dual LFD/qPCR biomarker detection approach in BAL fluid, to direct antifungal treatment for IPA in the absence of direct examination or culture of pulmonary tissue.

The empirical strategy of administering antifungal therapy in the setting of persistent or relapsing neutropenic fever despite antibiotics for 3-7 days, with no alternative microbiological aetiology identified, remains the standard approach to patient management in many centres, although there may be variations to the formal definition of empirical management. The widespread use of early CT scanning of the chest to help in the management of IFD is handicapped by the not insignificant limitation that it cannot identify the pathogen. However, with the emergence of new technologies, more informative biomarkers and a wider choice of antifungal drugs, a more rational approach may be to devise a diagnostic-driven management strategy, with the aim of withholding antifungal treatment in those who have biomarker negative results. Drives for this include the documented overtreatment of patients and the impact on hospital resource use. Indeed, the findings reported in this thesis are the basis for a new Public Health England (PHE)-led service, offering my *Aspergillus* qPCR together with LFD and GM combination testing at the Royal London Hospital as a diagnostic service that can be requested for BAL and blood samples within Barts Health NHS Trust, as well as from outside. Within Barts Health NHS Trust, the Haemato-Oncology department is changing the patient management algorithm, based on the results of this thesis, to incorporate my *Aspergillus* qPCR, LFD and GM combination testing, in order to move from the current CT/GM strategy to a diagnostic-driven approach incorporating the triple testing. In a setting where rapid CT scanning is available (within 24 hours of request), with direct bronchoscopy for timely BAL fluid sampling, it is possible to envisage a scenario in which antifungal treatment is withheld in a stable patient whilst awaiting biomarker test results. There are, however, some important caveats to such an approach. Firstly, if a patient is not stable, antifungal treatment will not be withheld whilst awaiting biomarker test results. Secondly, negative biomarker results

do not rule out a non-*Aspergillus* IFD. Therefore, if an IFD is still suspected further investigations are required.

Although the data presented in this thesis indicate the insensitivity of combination biomarker detection in blood compared to respiratory sampling, attempts were not made to optimise the DNA extraction method prior to qPCR analysis. For the purpose of direct comparison between respiratory and systemic sampling, an automated DNA extraction platform was used to extract total DNA from 200µl of EDTA whole blood or BAL fluid. EAPCRI have published protocols for DNA extraction from serum (131) and whole blood (132), recommending extraction from ≥3 ml EDTA blood specimens. In order to fully evaluate the use of blood samples it will be necessary to compare blood fractions and optimise the extraction protocol. In addition, the manufacturer-recommended protocol for LFD testing of serum samples has undergone revision since the generation of the data presented in this thesis. An evaluation of serum testing will be necessary using the revised protocol. Full evaluation of combination biomarker detection in blood samples will be incorporated into the forthcoming study at Barts Health NHS Trust. A combination of marker detection applied to blood may serve as a useful screening tool to further improve the identification of patients at high risk of developing IFD. A positive result may enable the start of early targeted antifungal therapy, while expensive and potentially toxic antifungal drugs can be withheld with persistently negative results. An alternate diagnostic strategy is to reserve testing for situations in which clinical and radiological data are suggestive of IA; in this scenario, marker detection applied to BAL, serum, and other tissues and fluids, may enable a definitive diagnosis of IA to be secured. It is possible to envisage a situation in which LFD testing is performed as a near-patient assay in the bronchoscopy suite, allowing an immediate decision on antifungal drug usage, with qPCR results within 24 hours for a definitive diagnosis. Although this approach does not facilitate early antifungal

therapy, as it relies on CT-guided bronchoscopy in patients who are suspected of having IFD, a more definitive diagnosis would enable the administration of specific antifungal therapy and would be of considerable benefit for future diagnostic and therapeutic research. As the need for rapid molecular diagnostics becomes more prominent, the requirement for tests to translate into diagnostics for point-of-care testing becomes more important. Enormous technological improvements have demonstrated that qPCR is an adaptable molecular tool and complex molecular analysis is no longer confined to the laboratory. With the availability of compact, handheld PCR devices (www.ahrambio.com, www.quantumdx.com) (264) and microfluidic chips to integrate DNA extraction and qPCR on a single platform (265), 40 qPCR cycles can now be completed in as little as ten minutes. Prototype devices can complete 30 cycles and record fluorescent output in 17 seconds. There is the real potential for qPCR assays to provide point-of-care diagnostics, with results generated in real-time to directly influence decisions in patient management. If this technology was incorporated into PLA development, there is the potential to detect actively growing pathogens in a matter of minutes.

## 6.3 Conclusions

In the immunocompromised setting of haemato-oncology, the diagnostic challenges of IFD remain a major clinical problem, resulting in the widespread use of antifungal drugs for prophylaxis and empirical treatment. The incidence of IA is rising, but its early diagnosis remains challenging. There is an urgent need to improve existing diagnostic tools and develop new technologies. An improved awareness of this problem has accelerated the pursuit of robust, rapid and reliable diagnostic tests. Each approach has its own limitations and it is likely that a combination of methods will be necessary to achieve optimal

performance for IA diagnosis. A combination biomarker approach could be incorporated into a diagnostic-driven approach to patient management, combining biomarker detection and imaging techniques to direct antifungal treatment to patients with evidence of IFD. The impact that these new tests have in the clinical setting will greatly depend on education and cooperation across departments and specialties, to ensure awareness of the tests available, turnaround time and practical limitations. A multi-disciplinary approach to the management of these high risk patients will require microbiologists, haematology consultants, registrars, radiologists, respiratory physicians and pharmacists to work together, ensuring that the appropriate diagnostic tests are requested and the results of these tests are acted upon. The data presented in this thesis, together with the study and procedural changes that it has set in motion, take this field forward. Progress will require a paradigm shift in thinking, suggesting that agreement between a combination of biomarker assays, detecting different markers of *Aspergillus* in BAL fluid, together with the clinical picture, is sufficient to make a clinical diagnosis of IPA and guide patient management.

One last thought……..

*"A paradigm shift is a distinctly new way of thinking about old problems…… the problem is that you can't embrace the new paradigm unless you let go of the old."*

(New Age Confusion, David A Lewis)

## Appendix 1

**Antimicrobial Protocol of Barts Health Haemato-oncology Unit**

**Prophylaxis for high-risk patients (AML, ALL, Auto/Allo,)**

- Start on Day 1 of chemotherapy, stop when neutrophil count > 0.5 x $10^9$/l
- Ciprofloxacin 750 mg bd
- Fluconazole 400 mg od
- Aciclovir 200 mg qds

**1st line antimicrobials**

- Stop ciprofloxacin prophylaxis
- Piptazobactam (Tazocin) 4.5 g tds
- Tobramycin 7 mg/kg od (use ideal body weight)
- Tobramycin to be stopped if BC negative for Gram negative rod after 48 hours
- Trough levels at 24 hours and daily thereafter (aiming for < 1 mg/l)
- If patient defervesces, stop antibiotics after five days
- Dosing intervals in renal impairment should be adjusted.

| **Creatinine clearance** | **Dosage interval** |
|---|---|
| > 60ml/min | 24 hours |
| 40 to 60 ml/min | 36 hours |
| 20 to 40ml/min | 48 hours |

Adjust antibacterials according to BC sensitivities when they are made available, particularly of importance in continuing fever

**Appendix 1 continued**

**2nd line antimicrobials (ongoing fever after 48 hours)**

- Imipenem 500 mg qds
- Vancomycin 1 g bd
- Trough levels before 3rd dose (aiming for through level <10 mg/l)
- Adjust Vancomycin dosing according to levels and Renal function

**3rd line antimicrobials (ongoing fever after another 48 hours)**

- Add in amphotericin 1 mg/kg daily
- Ambisome to be used only if uncontrollable infusional reactions, creatinine > 170 mol/l or doubling of baseline Creatinine.
- Decision to start amphotericin / ambisome to be made by attending consultant
- Can be considered at 48 hours

**Management of 'β-lactam allergy'**

- Remember that most patients (approx 70%) who report this are not allergic
- Low rate of cross-sensitivity with cephalosporins
- Do not give β-lactams if prior Type I reaction (anaphylaxis)
- Consider trial of β-lactams if uncertain history or skin rash only with antihistamine prophylaxis

**1st line antimicrobials with β-lactam allergy**

- Ceftazidime 2g tds
- Tobramycin 7 mg/kg od

1. To be stopped if BC negative for Gram negative rod after 48 hours
2. Trough levels at 24 hours and daily thereafter (aiming for < 1 mg/l)

**Appendix 1 continued**

**2nd line antimicrobials with β-lactam allergy (ongoing fever after 48 hours)**

- Ceftazidime 2g tds
- Tobramycin 7 mg/kg od
    1. To be stopped if BC negative for Gram negative rod after 48 hours
    2. Trough levels at 24 hours and daily thereafter (aiming for < 1 mg/l)
- Vancomycin 1 g bd
    1. Trough levels before 3rd dose (or second dose in renal impairment) (aiming for through level <10 mg/l)
    2. Teicoplanin if significant renal impairment

**3rd line antimicrobials with β-lactam allergy (ongoing fever after another 48 hours)**

- Add in amphotericin 1 mg/kg daily
- Ambisome to be used only if uncontrollable infusional reactions, creatinine > 170 mol/l or doubling of baseline Creatinine.
- Decision to start amphotericin / ambisome to be made by attending consultant
- Can be considered at 48 hours

## Appendix 2

**EORTC/MSG Criteria for the Diagnosis of Invasive Fungal Infection**

| *CATEGORY* | *DESCRIPTION* |
|---|---|
| **PROVEN** | **Histo/cytopathological demonstration of fungal hyphae or yeast cells** in tissue sample with associated tissue damage (microscopically or radiological)<br><br>**Positive culture from usually sterile tissue** with clinical or radiological sign of infection<br><br>**Positive blood culture for candida** accompanied by temporally related clinical signs/symptoms |
| **PROBABLE** | **≥ 1 Host Factor** *AND*<br><br>**≥ 1 Microbiological criterion** *AND*<br><br>**1 Major or 2 Minor Clinical criteria** from abnormal site consistent with infection |
| **POSSIBLE** | ≥ **1 Host Factor** *AND*<br><br>**1 Microbiological** *OR*<br><br>**1 Major or 2 Minor Clinical criteria** from abnormal site consistent with infection |

**Appendix 2 continued**

| *TYPE OF CRITERIA* | *CRITERIA* |
|---|---|
| **HOST FACTORS** | **Neutropenia** (<0.5 for >10 days)<br><br>**Fever** for >96 hours despite broad spectrum antibiotic therapy in high-risk patients<br><br>**Temperature > 38˚C or < 36˚C** WITH<br><br>i) >10 days neutropenia in previous 60 days<br><br>ii) Recent/current immunosuppressive therapy in last 30 days<br><br>iii) Proven/probable IFI during previous episode of neutropenia<br><br>iv) Coexistence of symptomatic AIDS<br><br>**> 3 weeks of corticosteroids** in last 60 days<br><br>**Signs/symptoms of GVHD** (particularly grade≥2 or chronic extensive disease) |
| **MICROBIOLOGICAL** | **Positive culture** for fungus on **sputum /BAL /sinus aspirate**<br><br>**Positive cytology/microscopy** for fungus on **sputum /BAL /sinus aspirate**<br><br>**Positive Aspergillus Antigen** on BAL /CSF />2 blood samples<br><br>**Positive blood culture** for candida<br><br>**Positive cytology /microscopy** for fungal elements in sterile body fluid samples<br><br>**2 positive urine cultures for yeasts or candida casts** in absence of catheter |
| **CLINICAL** | |

| | |
|---|---|
| **LRTI: Major** | **New CT signs**: halo; air-crescent; cavitating consolidation (in absence of other organisms) |
| **LRTI: Minor** | Symptoms of LRTI: cough, chest pain, haemoptysis, dyspnoea<br>Pleural rub<br>Pleural effusion<br>Any new infiltrate not fulfilling major criteria |
| **SINONASAL INFECTION:**<br>**Major**<br>**Minor** | **Radiological evidence of invasive infection in sinuses**<br>URT symptoms, nose ulceration, periorbital swelling, maxillary tenderness, necrosis or perforation of hard palate |
| **CNS INFECTION**<br>**Major**<br>**Minor** | **Radiological evidence suggesting CNS infection**<br>Focal neurological symptoms/signs<br>Cognitive change<br>Abnormalities in CSF biochemistry and cell count |
| **DISSEMINATED FUNGAL INFECTION** | Unexplained papular /nodular skin lesions<br>Intraocular evidence of chorioretinitis or endophthalmitis |
| **CHRONIC DISSEMINATED CANDIDIASIS** | Radiological demonstration of small target-like abscesses and ↑ALP |

## Appendix 3

### Estimation of efficiency of PCR assays from DNA standard amplification plot

**A.**

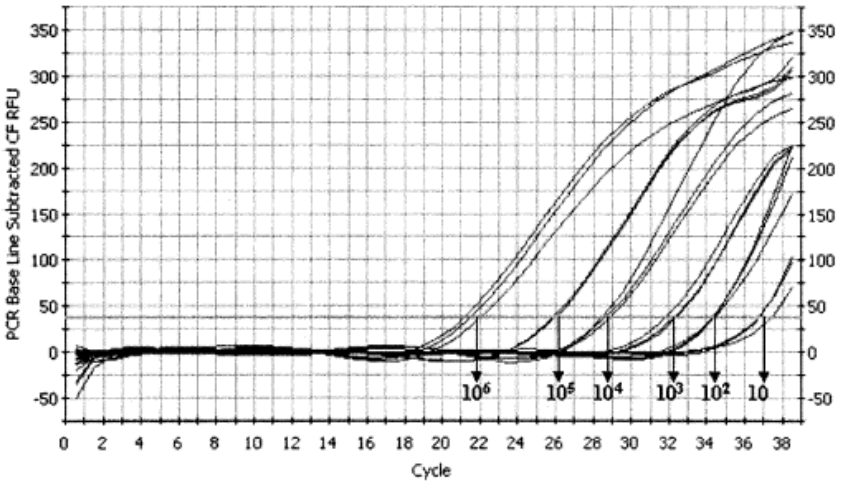



Figure.A1: Amplification plot of standard ***A. fumigatus*** DNA dilution series from Sanguinetti et al (179).

Estimated $C_q$ value from the above plot:

| **log concentration (x)** | 6 | 5 | 4 | 3 | 2 | 1 |
|---|---|---|---|---|---|---|
| **$C_q$ (y)** | 22 | 26 | 29 | 32 | 34.5 | 37 |

The equation for the slope of the regression line is calculated using the following formula:

$$b = \frac{\sum (x-\bar{x})(y-\bar{y})}{\sum (x-\bar{x})^2}$$

where x and y are the sample means AVERAGE (known_x's) and AVERAGE (known_y's) and the derived slope is **-2.957**

Using the formula(167)

**PCR efficiency = $10^{-1/slope} - 1$**

= $10^{-1/-2.957} - 1$

= **118 %**

**Therefore, the estimated efficiency of the assay is 118%.**

**Appendix 3 continued**

**B.** **Estimation of efficiency of PCR assays from DNA standard curve**

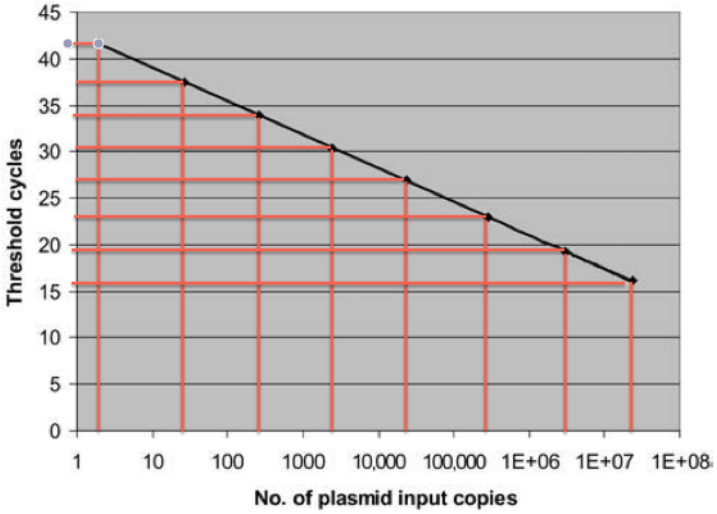
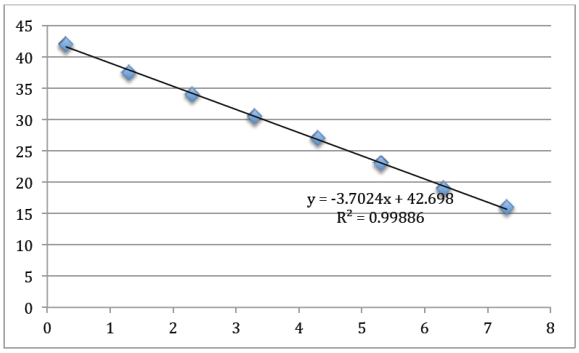



Figure.A2: Standard curve of ***A. fumigatus*** DNA dilution series from White et al (183).

| Copy No | Log | Cq |
|---|---|---|
| 2.00E+07 | 7.301029996 | 16 |
| 2.00E+06 | 6.301029996 | 19 |
| 200000 | 5.301029996 | 23 |
| 20000 | 4.301029996 | 27 |
| 2000 | 3.301029996 | 30.5 |
| 200 | 2.301029996 | 34 |
| 20 | 1.301029996 | 37.5 |
| 2 | 0.301029996 | 42 |




Efficiency = $[10^{(-1/slope)}] - 1$ =86%

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