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Abstract

Fungi are an important but frequently overlooked cause of morbidity and mortality in humans. Life-threatening fungal infections mainly occur in immunocompromised patients, and are typically caused by environmental opportunists that take advantage of a weakened immune system. The filamentous fungus Aspergillus fumigatus is the most important and well-documented mould pathogen of humans, causing a number of complex respiratory diseases, including invasive pulmonary aspergillosis, an often fatal disease in patients with acute leukemia or in immunosuppressed bone marrow or solid organ transplant recipients. However, non-Aspergillus moulds are increasingly reported as agents of disseminated diseases, with Fusarium, Scedosporium, Lomentospora and mucormycete species now firmly established as pathogens of immunosuppressed and immunocompetent individuals. Despite well-documented risk factors for invasive fungal diseases, and increased awareness of the risk factors for life-threatening infections, the number of deaths attributable to moulds is likely to be severely underestimated driven, to a large extent, by the lack of readily accessible, cheap, and accurate tests that allow detection and differentiation of infecting species. Early diagnosis is critical to patient survival but, unlike Aspergillus diseases, where a number of CE-marked or FDA-approved biomarker tests are now available for clinical diagnosis, similar tests for fusariosis, scedosporiosis and mucormycosis remain experimental, with detection reliant on insensitive and slow culture of pathogens from invasive bronchoalveolar lavage fluid, tissue biopsy, or from blood. This review examines the ecology, epidemiology, and contemporary methods of detection of these mould pathogens, and the obstacles to diagnostic test development and translation of novel biomarkers to the clinical setting.

Key words: Aspergillus, aspergillosis, Fusarium, Lomentospora, Scedosporium, mucormycosis, monoclonal antibody, lateral-flow device, fungal diagnostics, mycoses

Abbreviations

AFB (acid-fast bacillus); AIDS (acquired immune deficiency syndrome); ABPA (allergic bronchopulmonary aspergillosis); ABPM (allergic bronchopulmonary mycosis); AML (acute myeloid leukaemia); AB (Aspergillus bronchitis); AS (Aspergillus sensitisation); BALf (bronchoalveolar lavage fluid); CCPA (chronic cavitary pulmonary aspergillosis); CF (cystic fibrosis); CFPA (chronic fibrosing pulmonary aspergillosis); CGD (chronic granulomatous disease); CNPA (chronic necrotising pulmonary aspergillosis); CNS (central nervous system); CPA (chronic pulmonary aspergillosis); COPD (chronic obstructive pulmonary disease); CT (computed tomography); ELISA (Enzyme-Linked Immunosorbent Assay); EORTC/MSG (European Organisation for Research and Treatment of Cancer/Mycology Study Group); EPS (extracellular polysaccharide); FDSC (Fusarium dimerum species complex); FOSC (Fusarium
oxysporum species complex); FSSC (Fusarium solani species complex); GM (galactomannan);

HSCT (haematopoietic stem-cell transplant); IPA (invasive pulmonary aspergillosis); ISHAM
(International Society for Human and Animal Mycology); LFA (lateral-flow assay); LFD
(lateral-flow device); MRI (magnetic resonance imaging); MAb (monoclonal antibody); MDS
(myelodysplastic syndrome); NPV (negative predictive value); NTM (non-tuberculosis
mycobacteriosis); PCR (polymerase chain reaction); PET (positron emission tomography);
POCT (point-of-care test); PPV (positive predictive value); PSC
(Pseudallescheria/Scedosporium species complex); PTB (pulmonary tuberculosis); SAIA (sub-
acute invasive pulmonary aspergillosis); TAFC (triacetylfusarinine C).

Introduction

In 1991, it was estimated that there were 1.5 million species of fungi on the planet
(Hawksworth, 1991). With the advent of high throughput sequencing, that estimate was up-
graded to as many as 5.1 million species (Blackwell, 2011). Despite this enormous number,
in which fungi outnumber plants by at least 6 to 1, only ~700 species have been reported as
causing infections in humans (Brown et al., 2012a), while the number that are regularly
reported as disease-causing agents in the clinical setting is limited to an even smaller
number of species. Nevertheless, that handful of species, which includes yeasts, yeast-like
fungi, and moulds, collectively cause enormous but largely unrecognised damage to human
health and well-being in the form of superficial infections of the mucosa, skin and nails
(Schwartz, 2004), deep infections of the bones and joints (Taj-Aldeen et al., 2015), asthma
and allergies (Denning et al., 2014), and life-threatening disseminated infections in already
seriously ill patients, amounting to billions of affected people each year (Brown et al.,
2012a,b).

Fungi that cause fatal disseminated infections are typically opportunistic, taking
advantage of immune systems weakened caused by primary immunodeficiencies (Lanternier
et al., 2013) or AIDS (Limper et al., 2017), haematological malignancies (Pergam, 2017), or
the use of potent immuno-suppressive therapies that deplete microbial immunity
(Gundacker and Baddley, 2015). The fungi regarded as the most important medically are
Aspergillus, Candida, Coccidioides, Cryptococcus, Histoplasma, Paracoccidioides,
Pneumocystis, and Talaromyces. Only one of these, Aspergillus, is a true filamentous fungus
or mould, the remainder being yeasts, yeast-like, or thermally dimorphic fungi. It is
estimated that Aspergillus diseases, caused principally by the single species Aspergillus
fumigatus, collectively affect around 9 million people worldwide, with a further 10 million
people estimated to be at risk of Aspergillus infection. However, because of under-diagnosis
and under-reporting of Aspergillus diseases, these figures are likely to be under-estimates
(Denning et al., 2013). Also under-diagnosed or under-reported, are the diseases caused by
other mould pathogens (Fusarium spp., species in the Pseudallescheria/Scedosporium
complex (PSC), Lomentospora prolificans, and the mucormycetes comprising, amongst
others, Mucor, Rhizomucor, and Rhizopus spp.), despite their increasing frequency as
infectious etiologies in the ever-expanding populations of immunocompromised and
diabetic patients, and their involvement in polymicrobial diseases following traumatic injury.

The aim of this review is to examine the ecology, epidemiology, and methods of
detection of these under-reported moulds in the context of human disease. It aims to
highlight the paucity of diagnostic tests for these pathogens that, despite repeated calls by
learned societies, opinion leaders, and action groups for better access to cheap diagnostics, remain pitifully inadequate, adding to the unacceptably high rates of morbidity and mortality caused by these fungi, and the added financial burden to healthcare providers consequent of delayed or incorrect diagnosis. The reasons for our repeated failure to develop cheap, accurate, and user-friendly diagnostic tests are discussed, as are the steps that might be taken to ‘release the brakes’ on bench-to-bedside translation of diagnostics to the clinical setting, enabling access to point-of-care tests, particularly in poor and developing countries that lack access to well-resourced and well-equipped diagnostic facilities.

**Aspergillus species pathogenic to humans**

The genus *Aspergillus* comprises between 260 and 837 species of ascomycete fungi, making it one of the most abundant groups of fungi on the planet (Hawksworth, 2011). Despite their abundance, only a small number of species are known to cause diseases in humans, with a single anamorphic species *Aspergillus fumigatus* (teleomorph *Neosartorya fischeri*) in the section *Fumigati* responsible for >80% of recorded infections in humans (Latgé, 1999). Other well-established species capable of causing infections include *A. flavus* (section *Flavi*), *A. terreus* (section *Terrei*), *A. niger* (section *Nigri*) and *A. nidulans* (section *Nidulante*) (Sugui et al., 2015), while a number of sibling species of *A. fumigatus* have emerged as human pathogens over recent years. These rarer species in the section *Fumigati* bare morphological similarities to *A. fumigatus*, but worryingly also exhibit intrinsic resistance to anti-fungal drugs (Sugui et al., 2015; Van der Linden et al., 2011), further complicating detection and treatment with the advent of azole-resistance in clinical strains of *A. fumigatus* (Abdolrasouli et al., 2018). Of these emerging species, *A. udagawae* (*Neosartorya udagawae*), *A. lentulus*, and *A. pseudofischeri* (*Neosartorya pseudofischeri*) are the most frequently reported as causing disease in humans. Notwithstanding these, *A. fumigatus* remains the most important pathogen in the *Aspergillus* genus in terms of prevalence and capacity to cause disease in humans. It is almost the most important mould pathogen of humans compared to all other mould species capable of causing life-threatening disseminated infections.

**Aspergillus diseases**

While *Aspergillus* spp. have recently emerged as the cause of deep-seated tissue infections following combat-related injuries in military personnel (Paolino et al., 2012; Warkentien et al., 2012; Tribble and Rodriguez, 2014), they are best known as opportunistic pathogens which cause life-threatening infections in immuno-compromised individuals whose impaired immunity is unable to eliminate the infectious propagules (air-borne spores) inhaled into the lungs. In patients with defective cell-mediated immunity due to cytotoxic chemotherapy or T-cell dysfunction due to corticosteroid or other immunosuppressive therapy, *Aspergillus* spores germinate to produce invasive hyphae which proliferate, leading to the often-fatal disseminated disease invasive pulmonary aspergillosis (IPA). These fungi can also cause allergic and chronic respiratory diseases as a result of an over-zealous host response to saprotrophic colonisation of the lung following inhalation of air-borne spores. The clinical consequences of this are most severe in the context of underlying respiratory disorders such as chronic obstructive pulmonary disease (COPD), post pulmonary tuberculosis (PTB), non-
tuberculosis mycobacteriosis (NTM), bronchiectasis, and cystic fibrosis (CF). The role of *Aspergillus* spp. in bronchiectasis in the non-CF lung is less well understood, but *Aspergillus* sensitisation and/or allergic bronchopulmonary aspergillosis (ABPA) is a cause and also a consequence of bronchiectasis (Chotirmall and McElvaney, 2014; Chotirmall and Martin-Gomez, 2018). The sequel to saprotrrophic colonisation is infection and the development of chronic pulmonary aspergillosis (CPA), a slowly progressive lung disease, with poor clinical outcome (Denning et al., 2011 and 2013; Takazono and Izumikawa, 2018). The sequel to aprotrophic colonisation is infection and the development of chronic pulmonary aspergillosis (CPA), a slowly progressive lung disease, with poor clinical outcome (Denning et al., 2011 and 2013; Takazono and Izumikawa, 2018).

The global burden of *Aspergillus* lung diseases is vast (Bongomin et al., 2017). There are an estimated 300,000 cases of IPA per year with approximately 10 million at risk annually (Brown et al., 2012b; Bongomin et al., 2017). The global burden of ABPA in asthma is approximately 4.8 million, ABPA in cystic fibrosis approximately 6,675, CPA approximately 3 million (including 1.6 cases post-tuberculosis), with CPA complicating ABPA approximately 400,000 (Denning et al., 2013). In the United Kingdom, there are estimated to be around 4,000 cases of IPA each year, 178,000 cases of ABPA in people with asthma, and 3600 patients with CPA, based on burden estimates post PTB and in sarcoidosis (Pegorie et al., 2017).

**Invasive Pulmonary Aspergillosis**

A fatality due to disseminated invasive pulmonary aspergillosis was first described in 1953 following autopsy of a patient with agranulocytosis disease (Rankin, 1953). At that time, IPA was a rare, albeit poorly recognised, disease of immunocompromised patients. It is perhaps ironic that modern advances in the treatment of haematological malignancies, especially remission-induction therapy for acute myeloid leukaemia (AML) or myelodysplastic syndrome (MDS), and the use of highly-effective immune modulating drugs (Kyi et al., 2014; Reinwald et al., 2016), have led to a dramatic increase in the incidence of IPA in immunodeficient patients, such that the mortality rate of IPA exceeds 50% in neutropenic patients, and reaches 90% in haematopoietic stem-cell transplant (HSCT) recipients (Segal, 2009; Kousha et al., 2011). The major risk factors for the disease include neutropenia, HSCT and solid organ transplantation, high-dose corticosteroids, haematological malignancy, cytotoxic therapy, advanced AIDS, and chronic granulomatous disease (CGD)(Latgé, 1999; Dutkiewicz and Hage, 2010).

**Allergic Bronchopulmonary Aspergillosis**

Of the different yeasts and filamentous fungi able to cause allergic bronchopulmonary mycosis (ABPM) in humans (Chowdary et al., 2014), *A. fumigatus* and allergic bronchopulmonary aspergillosis (ABPA) are the most extensively studied. Allergic bronchopulmonary aspergillosis is one of the most severe *Aspergillus*-related diseases, with the potential to progress into pleuropulmonary fibrosis and respiratory failure (Carsin et al., 2017). It occurs almost exclusively in cystic fibrosis or asthmatic patients, and is characterised by a hypersensitivity reaction to *Aspergillus* spp., with diverse clinical and radiological manifestations.
Bronchiectasis

Bronchiectasis is a chronic respiratory disease characterised by progressive and irreversible dilation of the bronchial lumen, with impaired mucociliary clearance, recurrent infection, and chronic inflammation. *Aspergillus* species are the most common filamentous fungi cultured from respiratory secretions of CF, non-CF, and bronchiectasis patients (Aogán et al., 2018; Máz et al., 2015, 2018). *Aspergillus fumigatus* is the most frequently recovered species and the species most likely to cause infections, followed by *A. flavus, A. niger* and *A. terreus* which tend to colonise, rather than infect, the lung (Máz et al., 2018). These species also differ in their geographical distributions, with a higher prevalence of *A. niger* and *A. terreus* in Japan, and *A. flavus* in India and China (Máz et al., 2018).

Chronic Pulmonary Aspergillosis

Chronic pulmonary aspergillosis (CPA) is a semi-invasive disease (Chan et al., 2016), lying somewhere in between allergic and invasive forms of aspergillosis (Sehgal et al., 2018). It comprises a number of lung diseases that are long-term sequelae to pulmonary tuberculosis (PTB), namely *Aspergillus* nodule, simple pulmonary aspergilloma, chronic cavitary pulmonary aspergillosis (CCPA; presence of one or more cavities with or without aspergilloma, accompanied by pulmonary and systemic symptoms), chronic fibrosing pulmonary aspergillosis (CFPA; cavitation and fibrosis involving two or more lobes), and subacute invasive pulmonary aspergillosis (SAIA)(Denning et al., 2016) also called chronic necrotising pulmonary aspergillosis (CNPA)(Kaymaz et al., 2016) leading to progressive destruction of the lung (Ohba et al., 2012). CCPA is the most common form of CPA (Denning et al., 2003), with most cases of CPA occurring in South-East Asia, Western Pacific, and Africa, where prevalence of PTB is the highest. In high-income settings, COPD appears to be the most important pre-disposing condition for the disease (Smith and Denning, 2011). Unlike IPA, CPA occurs in immunocompetent patients after treatment for TB, leading to weight loss, intense fatigue, severe shortness of breath and life-threatening haemoptysis. *Aspergillus fumigatus* is the most frequently identified species in negative acid-fast bacillus (AFB) sputum smears from sub-Saharan Africans following treatment for PTB. Indeed, CPA is an important differential diagnosis for what appears to be smear-negative tuberculosis (Denning et al., 2011).

Detection of *Aspergillus* diseases

Detection of *Aspergillus* lung diseases remains a significant clinical challenge. Clinical manifestations of *Aspergillus* lung infection or colonisation are non-specific, as are radiological abnormalities seen on X-rays or on computed tomograms of the chest (chest-CT). Despite this, anomalies on a chest-CT are used in some centres to initiate antifungal drug treatment in at-risk patients. However, the burgeoning costs of antifungal treatments (Schumock et al., 2016) and prolonged hospitalisation for patients with suspected, but often unproven, pulmonary mycosis, has turned the spotlight on diagnostics and the need for improved detection methods that enable the implementation of antifungal stewardship programmes which have the potential deliver significant savings in antifungal drug expenditure (Nwankwo et al., 2018).
Diagnostic-driven approaches to antifungal treatment have been shown to be more
effective than empirical treatment, driving down costs, and improving outcomes for
patients (Barnes, 2013). The emergence of triazole resistance in clinical strains of A.
fumigatus and other Aspergillus spp. (Rivero-Menendez et al., 2016; Abdolrasouli et al.,
2018; Zoran et al., 2018), driven by a multiplicity of factors including widespread use of
azole fungicides in agriculture (Fisher et al., 2018; Mortensen et al., 2010; Verweij et al.,
2009), anti-fungal prophylaxis (Fisher et al., 2018), evolution of resistance in infecting strains
in the lung during or after long-term (sometimes life-long) therapy with the same antifungal
drug (Howard et al., 2009; Verweij et al., 2016), and sub-therapeutic dosing of patients (van
der Elst et al., 2015), is a serious cause for concern. Up to 12% of Aspergillus infections are
estimated to be resistant to antifungal drugs (Rivero-Menendez et al., 2016), with up to 7%
of Aspergillus strains from stem cell and solid organ transplant patients found to be
resistant (Baddley et al., 2009; Kontoyiannis et al., 2010; Pappas et al., 2010).

Diagnostics has an important part to play in guiding patient treatment, by restricting
the use of antifungal drugs to those that truly need them. It is important to note that a
combination approach to diagnostics is more likely to be effective than a single test
approach, despite pressures to cut healthcare costs. No single test fits all, especially where
an organism such as A. fumigatus is capable of causing a number of different diseases of the
lung. For this reason, abnormalities in a chest-CT that raise the suspicion of a fungal lung
infection in an at-risk patient should be accompanied by complementary biomarker tests
that add additional layers of proof. Biomarker tests such as ‘pan-fungal’ assays for fungal
(1→3)-β-d-glucan, and the Bio-Rad Platelia® ELISA for Aspergillus galactomannan (GM-
ELISA) in serum are FDA-approved and are included in the EORTC/MSG and ESCMID-ECMM-
ERS guidelines for disease detection (De Pauw et al., 2008; Ullmann et al., 2018), while
others such as the point-of-care Aspergillus lateral-flow device (AspLFD) have been recently
CE-marked and are now available commercially for IPA detection. These assays, and other
commercial or experimental tests currently in development, are discussed in the context of
allergic (ABPA), semi-invasive (CPA), and invasive (IPA) Aspergillus lung diseases.

Invasive Pulmonary Aspergillosis

Invasive diagnostic procedures

Bronchoscopy and culture

Diagnosis of IPA at the onset of disease is of paramount importance as prognosis worsens
significantly over time. Detection of circulating biomarkers in blood such as Aspergillus
galactomannan, fungal (1→3)-β-d-glucan, and Aspergillus DNA would indicate that the
disease is already well progressed. Early detection of Aspergillus lung infection is only
achievable by using tissue biopsy or bronchoalveolar lavage fluid (BALf) recovered during
invasive bronchoscopy, which allow culture of the pathogen or detection of biomarkers of
infection, respectively. The ‘gold standard’ test for diagnosis of IPA is culture of Aspergillus
from a sterile lung biopsy, with associated necrosis of tissues due to presence of hyphal
elements. There are a number of problems with culture, including the need for invasive
tissue sampling, slow growth or no growth of isolates from cultured material, and difficulties
in differentiating Aspergillus spp. from other agents of hyalohyphomycoses (e.g. other
hyaline septate moulds such as Fusarium and Scedosporium species) in the absence of
characteristic spore-bearing structures. This leads to lengthy turnaround times, and overall
poor sensitivity of culture-based detection compared to biomarker tests (Prattes et al.,
2014). A major benefit of culture is the recovery of isolates for antifungal susceptibility
testing and molecular detection of resistance mutations (Postina et al., 2018), especially
given the increasing prevalence of triazole resistance in Aspergillus fumigatus isolates (Abdolrasouli
et al., 2018).

Biomarker detection in BALf

Lateral-flow technology

The advantage of assaying BALf for Aspergillus biomarkers, is that it allows increased
sensitivity and speed of detection compared to culture. The Aspergillus lateral-flow device
(AspLFD), a point-of-care test (POCT) that incorporates a monoclonal antibody (mAb), JF5,
which detects a biomarker of active growth (Thornton, 2008; Thornton, 2014), allows rapid
near-patient diagnosis of IPA, with confirmatory detection of disease using laboratory-based
GM-ELISA, (1→3)-β-d-glucan, or polymerase chain reaction (PCR) tests of BALf samples.
Combination biomarker testing adds considerable power to IPA diagnosis, with a number of
studies demonstrating improvements in diagnostic sensitivity when these assays are used in
combination (Hoennigl et al., 2014; Johnson et al., 2015; Eigl et al., 2017). There have been
numerous studies conducted with the prototype AspLFD test, with two meta-analysis
studies (Pan et al., 2015; Zhang et al., 2019) evaluating its diagnostic performance in
combination with GM-ELISA or (1→3)-β-d-glucan tests. The more recent study by Zhang et
al. (2019), which undertook a meta-analysis of 13 previously-published studies of the
prototype AspLFD, showed that a positive GM-ELISA and a positive (1→3)-β-d-glucan or
AspLFD test provided a confirmatory diagnosis of IPA.

The AspLFD was CE-marked in May 2018 for use with both serum and BALf, with
Mercier et al. (2019a) reporting the performance of the CE-marked test in a retrospective
evaluation of BALf samples from haematology patients across four centres in the
Netherlands and Belgium. In this multicentre study, the CE-marked test had a good
performance for the diagnosis of proven IPA versus controls (no IPA), with a specificity of
86% and sensitivity of 82%. Two follow-up studies (Jenks et al., 2019; Mercier et al., 2019b)
have compared the AspLFD with the newly developed sōnā galactomannan lateral-flow
assay (GM-LFA) produced by IMM. In the study by Jenks et al. (2019), both the AspLFD and
GM-LFA were highly sensitive and specific for probable/proven IPA versus no IPA in patients
with haematological malignancies, with specificities of close to 90% for both the AspLFD and
GM-LFA when read at 15 min, and with 100% sensitivity for the AspLFD when read at 25
min. In patients at risk for IPA but without neutropenia or underlying haematological
malignancy, the GM-LFA and AspLFD tests showed sensitivities ranging from 58% to 69%,
with specificities between 68% and 75% (Jenks et al., 2018). When the two tests were
combined, sensitivity increased to 81% while specificity remained around 60%, making this a
reasonable approach for IPA diagnosis in non-neutropenic patients.

In the study by Mercier et al. (2019b), which retrospectively tested 235 BALf samples of
adult haematology patients from four centres on behalf of the Dutch-Belgian Mycosis Study
Group (DB-MSG), performance of the AspLFD and GM-LFA for proven IPA were similar. In
cases of proven and probable IPA, the tests had identical specificity, but a higher sensitivity
and a better NPV for the GM-LFA. However, a very faint test line is discernible in the GM-
LFA with BALf samples from patients with no IPA, and is also present with the sample running buffer (i.e. strictly negative tests). This means that a comparator is required in cases where a weak test line is evident in order to determine whether this is due to non-specific binding (NSB), or due to an actual but weak line. Because of this ambiguity, a reading card is included in the IMMY test against which test line intensities are compared. Any test line considered fainter than a line of semi-quantitative intensity 1 is considered to be negative. Alternatively, plain running buffer can be used to create a negative control LFA against which clinical samples can be compared. Any test line more intense than the negative control can be considered positive. However, this procedure introduces additional uncertainty, variability, and cost, since a negative control must be included for every sample run.

While the GM-LFA and AspLFD are both relatively easy to use, the AspLFD is somewhat simpler, and does not suffer from NSB unlike the IMMY test. For the AspLFD, no sample pre-treatment is required with non-bloody, non-viscous BALf, which can be applied directly to the test, with results available within 15 minutes. In the case of bloody or viscous BALf, heating with a buffer (3 minutes) followed by centrifugation (5 minutes) is required, with a total test time of approximately 25 minutes. For the GM-LFA, all samples must be pre-treated with heating for 6-8 minutes and centrifugation for 5 minutes, bringing the total test time to roughly 45 minutes. Notwithstanding this, Mercier and co-workers (2019a,b) conclude that both tests show a good performance for diagnosis of IPA in haematology patients using BALf. Both tests can be used for fast screening of patients, although confirmation using an adjunct test such as GM-ELISA, PCR or culture is merited.

**Cytokines**

An important limitation of biomarker tests is that sensitivity may decrease during antifungal prophylaxis or empirical therapy of patients with haematological malignancies (Eigl et al., 2015; Prattes et al., 2015). To mitigate this loss of test sensitivity, alternative signature molecules in BALf have recently been explored which might act as adjunct biomarkers of the disease. Gonçalves et al. (2017) investigated the alveolar cytokine profiles of patients at high risk of developing IPA. The cytokines IL-1β, IL-6, IL-8, IL-17A, IL-23, and TNF-α were significantly increased among patients with the disease. However, IL-6, IL-8, and IL-23 best differentiated between cases of IPA and controls, whereas the remaining cytokines displayed an inconsistent contribution to discrimination. The cytokine IL-8 was the dominant discriminator, with alveolar levels of ≥904 pg/ml predicting IPA with a sensitivity of 90%, a specificity of 73%, and a negative predictive value of 88%.

**Siderophores**

Iron is essential for the growth of *Aspergillus fumigatus*, and targeting its acquisition blocks infection by the pathogen (Leal et al., 2013). In iron-poor environments such as serum, the pathogen produces low-molecular-weight siderophores to scavenge ferric iron from the host (Haas, 2003; Hass et al., 2015). One of these, the extracellular siderophore triacyltufusarine C (TAFc), is produced following spore germination, and is detectable in serum from patients with proven or probable IPA (Carrol et al., 2016). The siderophore is also detectable in BALf, and an evaluation by Orasch et al. (2017) of BALf samples from patients with haematological malignancies (of which 73% received antifungal prophylaxis or
empirical treatment at the time of bronchoscopy) showed that diagnostic sensitivities of the GM-ELISA and AspLFD tests could be increased when used in combination with TAFc detection, in line with previous BALf GM-ELISA/AspLFD combination testing (Hoenigl et al., 2014; Prattes et al., 2015).

Polymerase chain reaction

*Aspergillus* polymerase chain reaction (PCR) has been shown for many years to be a promising diagnostic procedure for IPA detection, especially when combined with other diagnostic biomarker tests (White et al., 2013; Buchheidt et al., 2017). Despite the widespread use of ‘in-house’ tests, PCR was not included in the 2008 EORTC/MSG diagnostic criteria for IPA (De Pauw et al., 2008) due to a lack of assay standardisation and validation. However, PCR will be included in the latest iteration of the guidelines, following systematic review of the evidence for its clinical use in comparison with antigen testing (White et al., 2015). The availability of commercial PCR tests has aided assay standardisation (Rath and Steinmann, 2018), with three PCR assays specifically tailored to *Aspergillus* detection, and which have been evaluated using BALf or respiratory samples. The MycAssay Aspergillus® assay is a real-time PCR which detects *A. fumigatus* and other *Aspergillus* spp., with a sensitivity of 80% to 94.1%, and a specificity of 87.6% to 98.6%, in non-haematology and mixed patient groups (Torelli et al., 2011; Guinea et al., 2013; Orsi et al., 2015). The AsperGenius® test comprises two PCR assays, one for the detection and differentiation of *Aspergillus* spp. (AsperGenius Species multiplex®), and the other (AsperGenius Resistance multiplex®) for the detection of four azole resistance markers within the Cyp51A gene of *A. fumigatus* (L98H, TR34, T289A, Y121F). The AsperGenius Species multiplex® assay contains a probe for the *A. fumigatus* complex (*A. fumigatus* and the emerging sibling pathogens *A. lentulus*, *A. udagawae*, and *A. viridinutans*), and a probe for *Aspergillus* spp. (the *A. fumigatus* complex and *A. flavus*, *A. terreus*, and *A. niger*). Using BALf, the AsperGenius® test has a sensitivity of 68.4% to 84%, and a specificity of 80% to >92% (Chong et al., 2016; Guegan et al., 2018). In a study of 91 patients with GM-ELISA positive BALf samples, 79% were positive for *A. fumigatus* or *Aspergillus* spp. DNA, with theazole resistance mutation TR34/L98H detected in 8 of the cases, and T289A/Y121F mutations in a further 3 cases (Schauwvlieghe et al., 2017). The MycoGenie® assay detects *A. fumigatus* and the TR34/L98H mutation, and has a test sensitivity of 71.1% and specificity of >92% when used with BALf samples (Guegan et al., 2018). Notwithstanding improvements in PCR standardisation, the Infectious Diseases Society of America (ISDA) currently recommends that *Aspergillus* PCR be used in individual cases if combined with other diagnostic and clinical data (Misch and Safdar, 2016; Patterson et al., 2016; Lass-Flörl, 2019).

Semi-invasive and non-invasive diagnostic procedures

Biomarker detection in serum

*ELISA, PCR, (1→3)-β-D-glucan, and LFD tests*

The literature on the relative specificities and sensitivities of fungal (1→3)-β-D-glucan tests, Bio-Rad *Platelia*® GM-ELISA, in-house PCR assays, and prototype AspLFD test, for serum-based diagnosis of *Aspergillus* diseases is capacious, and has been reported extensively
elsewhere (Held et al., 2013; White et al., 2013; Pan et al., 2015; Held and Hoenigl, 2017; Rath and Steinmann, 2018; Ruhnke et al., 2018; Patterson and Donnelly, 2019).

To date, there have been no studies evaluating the diagnostic performance of the CE-marked AspLFD when used with serum samples. However, an ELISA (GP-ELISA) which employs the same mAb (JF5) used in the AspLFD (and also in the experimental Aspergillus proximity ligation assay (Mercier et al., 2018)) has recently been developed and CE-marked by Euroimmun Medizinische Labordiagnostika AG, and its performance compared to the Platelia® GM-ELISA for IPA diagnosis using serum samples from immunocompromised patients (Dichtl et al., 2019). While the specificities of the GM-ELISA and GP-ELISA were 99% and 96%, respectively, both assays demonstrated low sensitivities. When tests were extended to all sera available in the time frame of 7 days before to 7 days after the day of proven diagnosis, 47% and 56% of the cases were detected by the GM-ELISA and GP-ELISA, respectively. The low sensitivities of both tests underline the need for serial tests when using patient serum.

Molecular imaging using mAb JF5 and siderophores

Abnormalities in a chest-CT as diagnostic indicators of IPA (defined as dense, welldelineated, nodular infiltrates with and without ground glass attenuation (the so-called ‘halo sign’)) (De Pauw et al., 2008) are not disease-defining, with other pathologies (non-Aspergillus lung infections, and neoplastic and inflammatory processes) giving similar anomalies. Furthermore, these radiological indicators are transient in neutropenic patients, or are rare in non-neutropenic patients (Prattes et al., 2014). While magnetic resonance imaging (MRI) provides unrivalled spatial resolution and soft tissue contrast, and can be used as a detection aid for Aspergillus cerebral and central nervous system infections (Starkey et al., 2014; Marzolf et al., 2016), its utility as an imaging modality for lung infections is limited due to the lack of detectable protons in air-filled spaces and potential artefacts between air-tissue interfaces.

For these reasons, attempts have been made to improve the accuracy of radiology for diagnosis of IPA by combining positron emission tomography (PET) with CT and MRI (Thornton, 2018). The Aspergillus-specific mAb, JF5, when conjugated to the radionuclide ⁶⁴Cu, has been used in immunoPET/MRI as a disease-specific tracer (⁶⁴Cu[NODAGA-JF5]), and allows accurate and sensitive non-invasive detection of A. fumigatus lung infections in vivo (Rolle et al., 2016; Davies et al., 2017). Furthermore, humanisation of JF5 (Davies et al., 2017) has allowed translation of the imaging technology to the clinic. A first-in-human clinical trial commenced in 2018, with successful detection of IPA in a neutropenic haematology patient, corroborated by BALf detection using the AspLFD, GM-ELISA, and culture (Thornton, pers. comm.).

Alternative approaches to the molecular imaging of IPA include the use of Aspergillus siderophores in PET. When coupled to ⁶⁸Ga, a positron emitter with complexing properties similar to those of Fe(III), the iron-chelating siderophore TAFc showed accumulation in the lungs of immunosuppressed rats with IPA, with uptake correlating with severity of Aspergillus infection (Petrik et al., 2010 & 2012a,b). While still at the pre-clinical stage of development, a [⁶⁸Ga]TAFc tracer for molecular imaging of Aspergillus lung infections in vivo, holds enormous diagnostic potential, alongside antibody-guided PET/MR imaging (immunoPET/MRI), for non-invasive detection of IPA in humans.
Evidence for the presence of *Aspergillus* carbohydrate biomarkers in urine was first shown in counter-immunodiffusion assays with urine from rabbits experimentally infected with *A. fumigatus* (Lehman and Reiss, 1978). Antiserum used in the assay was prepared from an immunosuppressed and infected rabbit (rather than an animal immunised with an *A. fumigatus* antigen preparation), and showed that a carbohydrate antigen was detected in the urine of the experimentally infected rabbits. Detection of the antigen was not found in uninfected animals or in animals infected with *Candida albicans*. While human urinary antigen was not investigated, and the identity of the carbohydrate was not established, this early study was the first to demonstrate the excretion of *A. fumigatus* carbohydrate antigen(s) into the urine of animals with IPA.

The identity of a carbohydrate antigen in the urine of experimentally infected rabbits, and in the urine of patients with IPA, was subsequently demonstrated by Dupont and co-workers (Dupont et al., 1987). Galactomannan (GM) was detected in animal and human urine samples using a sandwich ELISA employing rabbit antiserum raised against purified GM. Using this assay, urinary GM was detectable throughout the course of lethal aspergillosis in all 16 experimentally infected animals, in concentrations of 24 to 1,900 ng GM/ml urine, with antigen excretion roughly paralleling the extent of disease. Galactomannan was detected in the urine of 7 of 13 patients with IPA, with concentrations of 1 to 83 ng GM/ml urine. A contemporaneous study by Bennett et al. (1987) showed that within 24 hours after intravenous injection, 35% of galactomannan was excreted into the urine of immunocompetent rabbits. Galactomannan detection in urine was also investigated as a means of diagnosing experimental and spontaneous aspergillosis in cattle (Jensen et al., 1993a). An inhibition ELISA using a GM-specific mAb EB-A1 (Stynen et al., 1992), reacted positively with urine samples from normal cattle, and so could not be used to detect systemic bovine aspergillosis. The same mAb was used by Haynes et al. (1990) to detect GM in the urine of neutropenic patients undergoing bone marrow transplantation or remission induction therapy for leukemia. In immunoblotting studies, EB-A1 showed diffuse staining (indicative of carbohydrate) in urine samples from patients with IPA, but no staining with urine from patients without evidence of the disease.

The development of a commercial latex agglutination test for *Aspergillus* GM (Pastorex *Aspergillus*, Sanofi Diagnostics Pasteur, France) using another GM-reactive rat mAb, EB-A2, also developed by Stynen et al. (1992), enabled standardised testing of GM antigenemia in the serum of IPA patients as a means of diagnosing IPA. The test was also investigated as a diagnostic tool for IPA using GM antigenuria in bone marrow transplant recipients (Ansorg et al., 1994). After modification of the assay for urine testing, it had a detection limit in native urine of approximately 20 ng GM/ml. Antigen was found in 79 (36.4%) of 217 serial urine samples, compared to 40 (11.8%) of 340 serum samples. Overall, antigenuria preceded antigenemia, and was more persistent. The sensitivity, specificity, PPV and NPV of antigenuria for autopsy-proven aspergillosis and clinically-suspected *Aspergillus* infection were 57%, 53%, 31% and 77%, respectively, while those of antigenemia were 43%, 53%, 25% and 71%, respectively. It was therefore concluded that urine testing was more reliable than serum testing for the detection of *Aspergillus* GM.
Refinement of the latex agglutination test led to the development of a quantitative sandwich ELISA for GM detection in patient serum, and which forms the basis of the commercial Bio-Rad Platelia® ELISA for GM detection in patient serum and BALf samples. The ELISA, which detects less than 1ng GM/ml sample, was also investigated as a urine test for GM in patients with IPA (Stynen et al., 1995). When GM was detected in urine, it was always lower than in matched serum samples. In addition, GM was always detected earlier in serum than in urine. The maximal number of days separating patient death and the first detection of GM in patient serum and urine were ≥39 and ≤12, respectively. Therefore, in contrast to a previous report (Rogers et al., 1990), it was recommended that serum rather than urine be used for the detection of GM, because of the later diagnosis obtained with urine samples.

Since the pioneering work in the 1990s, there has been limited interest in GM antigenuria as a detection method for IPA due to reports of its limited utility and high rates of false positivity, which vary between 8% and 47% (Klont et al., 2004). However, since the introduction of the AspLFD POCT, which has revolutionised point-of-care testing for the disease, there has been renewed interest in GM detection in urine, due to its non-invasive nature and the applicability of lateral-flow technology to urine biomarker testing. Dufresne et al. (2012) recently reported the development of a new GM-reactive mAb (mAb476), and demonstrated the feasibility of using the mAb in an immunochromatographic assay for detection of GM-like antigens in urine. Further development of the assay into a dip-stick format has demonstrated the potential of the test to detect proven or probable IPA in patients at high-risk for the disease (patients with haematological malignancy, and recipients of solid organ or HSCT). Per-patient sensitivity and specificity in the overall cohort was 80% and 92%, respectively, and the urine assay correlated with serum galactomannan indices (Marr et al., 2018). While the test uses non-invasive urine samples for GM detection, the requirement for sample pre-treatment means that it is unlikely to be a point-of-care test for IPA. Nevertheless, it demonstrates the applicability of lateral-flow technology to urine testing for Aspergillus diagnostic biomarkers, and for urine-based detection of other fungal pathogens of humans such as Cryptococcus (Drain et al., 2019) and Histoplasma (Libert et al., 2018).

**Urine siderophores**

In addition to cell wall carbohydrates such as GM, A. fumigatus and other Aspergillus species produce proteins that are detectable in urine. These include the iron-scavenging siderophores TAFC and ferricrocin. In a rat model of experimental aspergillosis, TAFC and ferricrocin (FC) were quantified in urine and serum with matrix-assisted laser desorption ionisation (MALDI)(Luptáková et al., 2017). The limits of detection of the ferri-forms of TAFC and FC in rat serum were 0.28 and 0.36 ng/ml, respectively, while in urine the limits of detection were 0.02 and 0.03 ng/ml, respectively. The mean concentrations of TAFC and FC in urine were 0.37 and 0.63 µg/ml, respectively. In a rat model of IPA, ferricrocin and TAFC detection in urine using mass spectrometry therefore appeared to provide a non-invasive means of detecting Aspergillus lung infections.

The siderophore TAFC has also been evaluated as a urine biomarker of IPA in humans using mass spectrometry (Hoenigl et al., 2019). Urine TAFC, normalised to creatinine, was determined in 44 samples from 24 patients with underlying haematological malignancies and probable, possible or no IPA, and compared to GM antigenuria using the Bio-Rad
Platelia® ELISA. Matched blood samples were also tested for GM, and for (1→3)-β-D-glucan using the Fungitell test. The TAFC/crea index determination in urine samples showed a promising performance for diagnosis of IPA, with sensitivities and specificities comparable to those reported for GM in serum and BALf, and superior to GM antigenuria determinations. Compared to TAFC detection in human BALf and serum samples (Carroll et al., 2016; Orasch et al., 2017), the diagnostic performance of TAFC levels in urine seems to be superior, which may be due to accumulation in the bladder as shown in recent animal models (Petrik et al., 2014 and 2015).

Allergic Bronchopulmonary Aspergillosis (ABPA)

Diagnosis of ABPA is extremely difficult, reflected in a number of different diagnostic definitions of the disease. To unify diagnosis and therapy of ABPA, the International Society for Human and Animal Mycology (ISHAM) produced consensus-based guidelines in 2013 (Agarwal et al., 2013). Isolation of A. fumigatus from sputum samples can sometimes be useful, but is not included in the guidelines due to its poor sensitivity and specificity. As with other Aspergillus lung diseases, consolidation seen in a chest-CT is non-pathognomonic and may indicate another pulmonary infection such as pulmonary tuberculosis. Nevertheless, radiographic pulmonary opacities consistent with ABPA (Agarwal et al., 2013) are included under ‘other criteria’, of which there should be at least two of three present in a patient with bronchial asthma or cystic fibrosis (the other two being presence of precipitating IgG antibodies against A. fumigatus in serum, or total eosinophil count >500 cells/µl in steroid naïve patients). These additional criteria should accompany both of the ‘obligatory criteria’ which are (1) a positive Type I skin test (immediate cutaneous hypersensitivity to Aspergillus antigen) or elevated IgE levels against A. fumigatus, and (2) elevated total IgE levels (>1000 IU/ml).

The heavy reliance on serology to include or exclude ABPA has led to efforts to improve test reproducibility since production of IgG and IgE antibodies to Aspergillus spp. is usually determined using a crude antigen extract with inherent variability and cross-reactivity with other fungal antigens (Reed, 1978; Crameri et al., 2009). The cloning of genes that encode allergenic proteins of A. fumigatus enabled improvements in the reliability of serological diagnosis of ABPA using recombinant antigens (Crameri et al., 1998a,b; Fricker-Hidalgo et al., 2010). A recent study by Alghamdi et al. (2019) of CF patients with ABPA, Aspergillus sensitisation (AS), or Aspergillus bronchitis (AB) showed that patients with ABPA had significantly greater IgE reactivity to the allergenic proteins Asp f1, f2, f3, and f4 compared to patients with AS. Patients with AB expressed higher IgG positivity to Asp f1 and Asp f2 compared with those with ABPA. There were very low IgE antibody levels against all recombinant antigens in AS patients. Asp f1 IgG reactivity in ABPA patients correlated with positive sputum culture. It was concluded that the use of multiple recombinant allergens may improve the diagnostic accuracy in CF complicated with ABPA or AB. Furthermore, Asp f1 reactivity might be a useful marker for guiding antifungal therapy in ABPA.

The role of fungi in bronchiectasis not due to CF is poorly defined. Aogán et al. (2018) characterised for the first time the mycobiome in bronchiectasis, assessing its clinical relevance in two geographically distinct cohorts from the CAMEB study, an international multicentre cross-sectional Cohort of Asian and Matched European Bronchiectasis patients. The mycobiome was determined in 238 patients by targeted amplicon shotgun sequencing of the 18S-28S rRNA internal transcribed spacer regions ITS1 and ITS2. The bronchiectasis...
mycobiome profiles were dominated by *A. fumigatus* in Singapore/Kula Lumpur, and by *A. terreus* in Dundee, the latter associated with exacerbations. High frequencies of *Aspergillus*-associated disease including sensitisation and ABPA were detected. This study showed that the mycobiome is of clinical relevance in bronchiectasis, and screening for *Aspergillus*-associated disease should be considered even in apparently stable patients.

**Chronic Pulmonary Aspergillosis (CPA)**

Symptoms of CPA (taken here as CCPA, CFPA, and SAIA/CNPA) are non-specific and, as with other *Aspergillus* lung diseases, diagnosis of the disease based on culture of fungi from respiratory specimens lacks sensitivity, with culture positivity as low as 11.8% (Takazono and Izumikawa, 2018). Radiological findings on a chest-CT are variable and overlapping and depend on the state and stage of the disease. Diagnosis of a simple pulmonary aspergilloma in an immunocompetent individual is based on detection of a single lung cavity with no progression over at least 3 months of observation, while an *Aspergillus* nodule is defined by the presence of one or more nodules without cavitation (Denning et al., 2016; Muldoon et al., 2016). The diseases CCPA and SAIA share the similar characteristics of one or more cavities with or without fungal ball, accompanied by radiological progression such as expanding thick-walled cavities and pericavitary infiltration (Denning et al., 2016). They differ in that SAIA also involves hyphal invasion of the lung parenchyma (Hope et al., 2005). This subtle difference creates a diagnostic dilemma, especially when histopathology is lacking. The time course of radiological progression (CCPA ≥3 months; SAIA 1-3 months) and process of cavitary formation (CCPA typically occurs in pre-existing cavities, while SAIA cavities are formed during *Aspergillus* infection) are therefore needed for disease differentiation, but are reliant on serial radiography. The syndrome CFPA is defined by cavitation and fibrosis involving two or more lobes leading to a major loss of lung function, and is generally regarded as the end result of untreated CCPA (Denning et al., 2003 and 2016).

Galactomannan-ELISA of serum has limited diagnostic value for CPA detection, with low sensitivity and specificity even when the threshold index value for test positivity is reduced from ≥1.5 (the manufacturer’s recommended threshold index value for serodiagnosis of IPA (Maertens et al., 2007)) to ≥0.5 (Kitasato et al., 2009). Galactomannan-ELISA of BALf showed relatively higher sensitivity (77.2%) and specificity (77.0%), when the index threshold value for test positivity was reduced further to ≥0.4 (Izumikawa et al., 2012). In a more recent study (Salzer et al., 2018), the GM-ELISA was evaluated alongside the AspLFD and levels of cytokines for diagnosis of CPA (Salzer et al., 2018). Sensitivity and specificity of the GM-ELISA with a cut-off of ≥0.5 was 41% and 100%, respectively. When the cut-off was increased to ≥1.0, the sensitivity decreased to 30% while specificity remained at 100%. The AspLFD was positive with only 7% of patients. Therefore, based on test sensitivities, both the GM-ELISA and AspLFD showed insufficient performance for diagnosing CPA. However, their high specificities provide high positive predictive values, which may help identify semi-invasive or invasive disease. In this study, the cytokine profiles of CPA patients did not differ significantly from patients with other respiratory disorders, but showed significantly higher levels of IFN-γ, IL-1b, IL-6, IL-8, and TNF-α compared to healthy controls.

Further evaluation of the AspLFD test using serum and BALf samples from CPA patients has shown improved diagnostic performance (Takazono et al., 2019). In this study, the diagnostic performance of the AspLFD was compared to the GM-ELISA and (1→3)-β-D-
glucan tests using samples from patients with SAIA, simple pulmonary aspergilloma, and respiratory disease controls (PTB, NTM, COPD, interstitial pneumonia, prior lung surgery, lung cancer, and bacterial infection (pneumonia, lung abscess)). The sensitivity and specificity of the AspLFD using serum were 62.0% and 67.7%, respectively, while serum GM-ELISA (cut-off index value of ≥1.5) showed a sensitivity of 22% and a specificity of 92.3%, respectively. Plasma (1→3)-β-D-glucan tests (cut-off value of 19.3 pg/ml), had a sensitivity of 48% and a specificity of 90.8%, respectively. Using BALf samples, the sensitivity and specificity of the AspLFD were 66.7% and 69.2%, respectively, while the GM-ELISA (cut-off index value of ≥0.6) had a sensitivity of 72.7% and specificity of 83.1%. *Aspergillus* precipitating antibody had a test sensitivity of 70%. Given these results, it was concluded that the performance of the AspLFD serum test was acceptable for the diagnosis of CPA as a POCT.

As with ABPA, detection of *Aspergillus*-specific IgG plays and important role in the diagnosis of CPA (Richardson and Page, 2018). There are a number of *Aspergillus*-specific IgG tests available commercially (Page et al., 2016; Takazona et al., 2019). In a study comparing six of these assays (Page et al., 2016), the automated ImmunoCAP fluoroenzyme immunoassay and Immunolite ELISA variant (Page et al., 2015) were significantly superior to the other assays (Dynamiker, Genesis, and Serion) in diagnosing CPA, with identical specificities (both 96%) and specificities (both 98%). Precipitin testing performed poorly, as did a recently introduced quantitative serum *Aspergillus fumigatus*-specific IgM assay (Yao et al., 2018a). Questions remain concerning the optimum cut-off values for IgG test positivities, with values provided by manufacturer’s appearing to be sub-optimal for diagnosis of the disease (Page et al., 2016, 2019; Sehgal et al., 2018). Despite this, it appears that serial serum testing for *Aspergillus*-specific IgG may provide a useful means of monitoring treatment response and outcome since serum IgG levels decrease during antifungal treatment and resolution (Yao et al., 2018b).

The limitations of the commercial *Aspergillus*-specific IgG tests are their specificity for *A. fumigatus*, their expense, and their reliance on automation. Approximately 40% of CPA cases are caused by non-*fumigatus* spp. (e.g. *A. niger* and *A. versicolor*), which these tests would not detect (Tashiro et al., 2011). The cost of the tests and their reliance on automated procedures makes them unsuitable for resource-limited settings. While most CPA (and ABPA) patients are diagnosed in high-income countries, prevalence of these diseases is believed to be higher in low- to middle-income countries (Denning et al., 2011). Consequently, a test for CPA that requires minimal laboratory equipment, akin to the AspLFD test for IPA, is needed. This need appears to have been met by a novel anti-*Aspergillus* antibody LFA manufactured by LDBIO Diagnostics, with other LFAs for *Aspergillus* immunoglobulins in development (Richardson and Page, 2018). The LDBIO Diagnostics LFA or immuno-chromatographic test (ICT) has recently been evaluated in a multicenter comparison of assays for CPA diagnosis (Piarroux et al., 2019). The sensitivity and specificity of the ICT were 88.9% and 96.3%, respectively, compared to 93.1% sensitivity and 94.3% specificity for an anti-*Aspergillus* IgG immunoblot assay. Since the ICT displays good diagnostic performance and complies with the ASSURED (Affordable, Sensitive, Specific, User-friendly, Equipment-free, and Delivered) criteria, it is appropriate for diagnosis of CPA in resource-limited settings. Marked improvements in IPA detection have been met through combination diagnostic testing. As advocated previously (Kobayashi and Thornton, 2014), a similar approach may prove beneficial for CPA diagnosis. A combination of serological and
biomarker tests such as the LDBIO ICT and OLM AspLFD might allow rapid, simple, cheap, but accurate diagnosis of the disease.

**Fusarium species pathogenic to humans**

The genus *Fusarium* comprises plant, human, and animal pathogenic species with worldwide distribution (Jain et al., 2011; Muraosa et al., 2017). As a group of organisms, they are a major constraint to food security, not only as pathogen of crop plants, but also as producer of mycotoxins contaminating the food chain (Thornton and Wills, 2015). In humans, *Fusarium* spp. cause a spectrum of diseases including nail infections (onychomycosis) (Arrese et al., 1996; Westerberg and Voyack, 2013), bone and joint infections (Koehler et al., 2014), infections of the skin and of burn wounds (Nucci and Annaissie, 2002; Latenser, 2003; Gurusidappa and Mamatha, 2011; Muhammed et al., 2013; Nucci et al., 2013; van Diepeningen et al., 2014), mycotic eye infections (fungal keratitis) (Jurkunas et al., 2009; He et al., 2011), and deep tissue infections following combat-related blast injury (Paolino et al., 2012; Warkentien et al., 2012; Tribble and Rodriguez, 2014). Up until the 1980s, most reported *Fusarium* infections were to the nails and eyes, and were locally invasive infections. Since then, there has been an increase in the number of cases of life-threatening disseminated disease (fusariosis) in patients with haematological malignancies (García-Ruiz et al., 2015), in HIV patients (Esnakula et al., 2013), in patients with diabetes and liver cirrhosis (Chen et al., 2017), in solid organ transplant recipients (Muhammed et al., 2013; Mohanty and Sahu, 2014), and most particularly in allogeneic HSCT recipients (Fanci et al., 2013; Scheel et al., 2013; Al-Hatmi et al., 2016), reflecting their greater immunosuppression and profound and prolonged neutropenia. Fusariosis is now the second most common mould disease of humans after aspergillosis (Guarro, 2013), multidrug-resistant and refractory to treatment (Fanci et al., 2013; Al-Hatmi et al., 2016), with poor prognosis (Tortorano et al., 2014), and with mortality rates reaching 75%.

At least 70 species of *Fusarium* are able to cause infections in humans, and are grouped into species complexes (Guzzo, 2013). Most infections in humans are caused by only four species; *F. petrophilum* and *F. keratoplasticum* in the *Fusarium solani* species complex (FSSC) (Short et al., 2013), an unnamed species in the *Fusarium dimerum* species complex (FDSC), and a further unnamed species in the *Fusarium oxysporum* species complex (FOSC). Members of these species complexes are readily isolated from environmental samples, including soil and plant material (Thornton and Wills, 2015), air (Scheel et al., 2013), and water systems and plumbing fixtures (Dogget, 2000; Anaissie et al., 2002; Mehl and Epstein, 2008; Anaissie et al., 2011; Short et al., 2011). Indeed, contaminated water systems appear to be an important environmental source of pathogenic fusaria in community-acquired and nosocomial outbreaks of fusariosis, with a recent study showing that hospital and communal sink biofilms are heavily colonised by pathogenic species in the FSSC, FDSC, and FOSC complexes (Al-Maqtoofi and Thornton, 2016), and thus a potential source of infectious propagules.
Detection of *Fusarium* diseases

Radiology, histology and culture

In countries with high rates of fungal keratitis, confocal microscopy of the eye has proved useful in the non-invasive detection of fungal structures (hyphae, pseudo-hyphae, and yeast-like structures) in the infected cornea (Brasnu et al., 2007). However, direct microscopy is non-specific, and so is unable to discriminate between the various different fungi (*Aspergillus*, *Candida*, *Fusarium*) capable of causing mycotic infections of the cornea.

Radiological findings in CT may be useful in raising the suspicion of a *Fusarium* lung infection, but are non-specific, with findings including alveolar and interstitial infiltrates, nodules and cavities that are typical of other mould infections (Marom et al., 2008). In chest CT, nodules and masses were the most common findings, with a halo sign being absent in 80% of cases.

The ESCMID and ECMM joint guidelines for diagnosis of hyalohyphomycosis strongly recommend histopathology for the diagnosis of fusariosis (Tortorano et al., 2014). However, in tissue, the hyphae of *Fusarium* typically resemble those of *Aspergillus*, with hyaline and septate filaments that dichotomise at acute and right angles (Nucci and Anaissie, 2007). Attempts have been made to improve the accuracy of *in situ* detection of *Fusarium* in tissues, but these remain experimental, relying on immunohistochemical procedures that employ cross-reactive antisera (Fukuzawa et al., 1995; Kaufman et al., 1997; Saito et al., 1999), or on molecular detection using in-house PCR assays (Salehi et al., 2016). Definitive diagnosis of invasive *Fusarium* infections therefore relies on culture of the fungus from skin biopsies and other infected tissues (sinuses, lungs) or from the blood. Fusariosis is perhaps unique amongst the invasive mould diseases, in that the pathogens’ infectious propagules are spread haematogenously, and can be recovered from the bloodstream in up to 82% of cases (Grossman et al., 2012; Muhammed et al., 2013). In contrast, *Aspergillus* is rarely detected in the blood. Haematogenous spread of *Fusarium* spores accounts for the multiple necrotic skin lesions that develop in 75-90% of disseminated infections (Van Diepeningen et al., 2015a), a clinical manifestation uncommon in disseminated aspergillosis. Culture from tissue and blood samples enables identification of *Fusarium* based on the crescent or banana-shaped macroconidia characteristic of this genus. However, identification to species level, which is essential for guiding clinical management (Muhammed et al., 2011), is difficult and relies on nucleic acid-based tests of tissue specimens or axenic cultures.

Nucleic acid tests

Ribosomal RNA gene sequences, and sequencing of the internal transcribed spacer (ITS) region, a procedure widely used for the identification of other mould species, is of limited value for identifying and bar-coding *Fusarium* species due to the presence of duplicated divergent alleles in this region. For this reason, molecular identification of clinical isolates relies on three loci; *TEF-1α* (translation elongation factor-1α) (García-Ruiz et al., 2015; Zarrin et al., 2016; Muhammed et al., 2018), *RPB1* (the largest subunit of RNA polymerase), and *RPB2* (the second largest subunit of RNA polymerase) (Al-Hatmi et al., 2016). Based on these loci, DNA sequence databases have been constructed, and can be interrogated at FUSARIUM-ID ([http://isolate.fusariumdb.org/guide.php](http://isolate.fusariumdb.org/guide.php)) and at CBS-KNAW ([http://www.westerdijkinstitution.nl/fusarium/](http://www.westerdijkinstitution.nl/fusarium/)), for the identification of environmental and
clinical isolates. Using a newly generated *Fusarium*-specific monoclonal antibody (ED7) that detects an extracellular antigen (a 200kDa water-soluble carbohydrate), Al-Maqtøofi and Thornton (2016) were able to track pathogenic fusaria in hospital and communal sink biofilms, with FSSC sequence type (ST) 1-a, FOSC ST 33, and FDSC ST ET-gr isolates identified using TEF-1α sequencing of recovered strains. This study demonstrated the specificity of mAb ED7, which might find use in the detection of *Fusarium* infections in humans based on detection of the 200kDa biomarker in patient samples. Multi-locus sequence typing (MLST) is currently regarded as the most robust method for differentiating *Fusarium* species complexes (Wang et al., 2011) or identifying isolates to species level (Tortorano et al., 2014), with TEF-1α and RPB2 employed in many MLST diagnostic regimens (Van Diepeningen et al., 2015b).

There are currently no nucleic acid-based detection systems CE-marked or FDA-approved for the diagnosis of human fusariosis, but a number of experimental polymerase chain reaction (PCR) tests have been reported for the detection and quantification of *Fusarium* DNA in murine models of fusariosis, and in human tissue specimens (Bernal-Martinez et al., 2012; Muraosa et al., 2014; Salehi et al., 2016). A duplex RT-PCR using two specific molecular beacon probes targeting a highly conserved region of the rDNA gene was developed for *F. solani* and non-*Fusarium solani* DNA detection, and validated in two mouse models of invasive infection (Bernal-Martinez et al., 2012). Specificity of the assay was 100%. While sensitivity of the assay in a *F. solani* infection model was 93.9% for lung tissues and 86.7% for serum samples, its sensitivity in a *F. oxysporum* infection model was 87% for lung tissues and 42.8% for serum samples. A *Fusarium* genus-specific and FSSC-specific RT-PCR system has also been developed, which targets the 28s ribosomal RNA gene (Muraosa et al., 2014). To apply the RT-PCR system to the molecular diagnosis of fusariosis, performance was evaluated using plasma and whole blood samples from a mouse model of invasive *F. solani* infection. The sensitivity of the RT-PCR system was found to be 100% in plasma, but no amplification was detected in whole blood. In a retrospective multicentre study, Salehi et al. (2016) reported the discrimination of fusariosis, aspergillosis, mucormycosis, and scedosporiosis in formalin-fixed paraffin-embedded human tissue specimens using multiple real-time quantitative PCR assays. The qPCR assays, targeting the ITS2 region of ribosomal DNA using fluorescently labelled primers, was used to identify clinically important genera and species of *Fusarium*, *Aspergillus*, *Scedosporium*, and mucormycetes, and the molecular identification compared to results from histological examination. *Fusarium oxysporum* and *F. solani* DNA was amplified from five specimens from patients initially diagnosed by histopathology as having aspergillosis. This study demonstrates that histopathological features of moulds may be easily confused in tissue sections, and that qPCR can be used for the rapid and accurate identification of fungal pathogens to the genus and species levels directly from FFPE tissues. Despite this, molecular tests should be used only to supplement conventional laboratory tests (Tortorano et al., 2014).

Antigen tests

At present, there are no antigen tests available commercially which are specific for *Fusarium* detection. While (1→3)-β-D-glucan detection can be used to diagnose fusariosis both in animal models (Khan et al., 2008) and in humans, its pan-fungal reactivity means that it lacks specificity. Disconcertingly, cross-reaction of the *Aspergillus* GM-ELISA with *Fusarium*
spp. has been reported, with roughly half of patients with fusariosis giving a positive test result with the assay (Tortorano et al., 2012). The reason for this cross-reactivity is uncertain, but may be due to the presence of the £1,5-linked Galf moieties recognised by the GM-reactive mAb EB-A2 being present in polysaccharide antigens from fungi other than Aspergillus, including Fusarium spp (Tortorano et al., 2012). Certainly, galactomannan has been found in Fusarium culture supernatants (Tortorano et al., 2012; Wiedemann et al., 2016). The Platelia Candida mannan ELISA has also been reported to cross-react with F. verticillioides, F. solani and F. oxysporum (Rimek et al., 2003). These findings call into question the validity of these tests for the specific diagnosis of aspergillosis and candidiasis, respectively. Despite this, the GM-ELISA has been evaluated in humans as a means of diagnosing fusariosis and as a monitoring tool for treatment response (Nucci et al., 2014), but there is little obvious legitimacy for using this assay for fusariosis detection in the clinical setting given the similar patient groups susceptible to candidiasis, aspergillosis and fusariosis.

**Pseudallescheria/Scedosporium species complex and Lomentospora prolificans**

Previously, Pseudallescheria boydii was considered synonymous with Pseudallescheria angusta, Pseudallescheria ellipsoidea, and Pseudallescheria fusoidea (Rainer et al., 2000), with a subsequent taxonomic study suggesting that P. boydii is a complex comprising six species (P. boydii, P. angusta, P. ellipsoidea, P. fusoidea, P. minutispora, and Scedosporium aurantiacum) (Gigado et al., 2005). Scedosporium apiospermum along with Graphium spp. (G. capitatum, G. eumorphum, and G. penicilloides) were considered to be synanomorphs of P. boydii (Rainer et al., 2006), with S. apiospermum then shown to be a species distinct from P. boydii (Gigado et al., 2008), challenging previous doctrine that this fungus was the asexual or anamorphic state of P. boydii. Currently, Pseudallescheria and Scedosporium are regarded as a composite of species, the Pseudallescheria/Scedosporium complex (PSC) (Luplertlop, 2018), with the genus Scedosporium now comprising S. aurantiacum, S. desertorum, S. minutisporum, S. cereisporum, and S. dehoogii, in addition to the S. apiospermum complex consisting of S. angustum, S. apiospermum, S. boydii, S. ellipsoidea, and S. fusoideum (Ramirez-Garcia et al., 2018). Prior to the advent of molecular taxonomy, the dematiaceous fungus Scedosporium prolificans (formerly S. inflatum) was believed to be a member of the genus Scedosporium, but has since been shown to be unrelated to Scedosporium, and has been re-classified as Lomentospora prolificans (Hennebert and Desai, 1974) with the genus Lomentospora being re-instated for this species (Lackner et al., 2014).

The species S. apiospermum, S. aurantiacum, and S. boydii are pathogenic to humans, causing eumycetoma, localised cutaneous infections, muscle, joint and bone infections, and fatal disseminated infections (Cortez et al., 2008; Taylor et al., 2014; Kondo et al., 2018; Ramirez-Garcia et al., 2018), and are consequently the most studied species in the genus. These fungi are soil saprotrophs that appear to have a proclivity for habitats impacted by human activity (Kalteis et al., 2009; Rougeron et al., 2015). However, the evidence for this association is questionable, since these organisms are also readily isolated from habitats such as brackish waters and estuarine muds (Thornton, 2009). Notwithstanding this, the numerous reports of these fungi as agents of disease in near-drowning events and following natural disasters such as tsunamis due to aspiration of soil-laden water (Garzoni et al., 2005;
Leroy and Smismans, 2007; Linscott, 2007; Nakamura et al., 2011, 2013; Angelina et al., 2013; Nakamura et al., 2013), demonstrates their abundance in the natural environment, and their ability to cause life-threatening central nervous system (CNS) and cerebral infections following traumatic implant of infectious propagules in the lungs (Ramirez-Garcia et al., 2017). Less is known about the ecology of *Lomentospora prolificans*, but it is a soil- and compost-borne fungus (Hennebert and Desai, 1974; Grantina-levina et al., 2013), and is present, alongside members of the PSC, in estuarine muds (Thornton et al., 2015).

Pathogens in the PSC complex and *L. prolificans* are persistent colonisers of the lungs of cystic fibrosis (CF) patients, and are the second most frequent moulds after *A. fumigatus* in the sputum of CF patients and those with underlying respiratory diseases (Cimon et al., 2000; Cooley et al., 2007; Blyth et al., 2010a,b; Sedlacek et al., 2015; Schwarz et al., 2015; Schwarz et al., 2017; Tomazin and Matos, 2017; Bouchara et al., 2018; Schwarz et al., 2018). While chronic airway colonisation of the lung by these fungi can lead to fatal outcomes in CF patients (Borghi et al., 2010), the role of these fungi in CF exacerbation, other than causing acute obstruction (Padoan et al., 2016), is not fully understood. Notwithstanding this, a trait common to all strains isolated from the CF lung is their high resistance to antifungal drugs, with *L. prolificans* demonstrating multi-drug resistance (Borghi et al., 2010; Sedlacek et al., 2015), and successful treatment of lung infections requiring triple antifungal drug regimes in several cases (Schwarz et al., 2018). Important risk factors for fatal disseminated infections (scedosporiosis and lomentosporiosis) include neutropenia and haematological malignancy (Maertens et al., 2000; Rodriguez-Tudela et al., 2009; Song et al., 2009), advanced AIDS (Tammer et al., 2011), chronic granulomatous disease (Santos et al., 2000), allogeneic stem cell transplantation (Tamaki et al., 2016), and solid organ transplantation (Husain et al., 2005). Malignancy, fungemia, central nervous system (CNS) and lung involvement are prognostic factors predicting worse outcome for scedosporiosis and lomentosporiosis (Seidel et al., 2019). Disseminated infections following lung transplantation are particularly problematic (Vagefi et al., 2005; Morio et al., 2010; Rolfe et al., 2013), with a *S. apiospermum* and *L. prolificans* mixed disseminated infection reported recently (Balandin et al., 2016).

**Detection of *Scedosporium* and *Lomentospora* diseases**

Radiology, histology and culture

*Scedosporium* and *Lomentospora* infections are often clinically indistinguishable from other invasive mould infections, with radiology based on chest CT providing little diagnostic worth due to non-specific indicators of fungal lung infection (Dabén et al., 2008; Holmes et al., 2013; Nakamura et al., 2013; Schwarz et al., 2015). In contrast, brain MRI is an important diagnostic modality for detecting disseminated CNS infections in immunosuppressed stem cell and solid organ transplant recipients, and in victims of near-drowning, particularly in cases where antifungal treatment fails to resolve disease, or where a clear spreading focus is absent (Morio et al., 2010; Bhuta et al., 2012; Holmes et al., 2013; Nakamura et al., 2013; Sharma and Singh, 2015; Tamaki et al., 2016; Ramirez-Garcia et al., 2018).

Histopathological examination of biopsy material has limited diagnostic utility as it is difficult to distinguish *Scedosporium/Lomentospora*-infected tissues from those infected with other hyaline fungi such as *Aspergillus* and *Fusarium* species (Ramirez-Garcia et al., 2018), although the dematiaceous (melanised) hyphae of *L. prolificans* may provide an
opportunity for species differentiation. Immunohistological procedures using polyclonal fluorescent antibodies have attempted to improve the accuracy of in situ detection (Kaufman et al., 1997), but cross-reaction with antigens from other fungi such as Aspergillus hamper their usefulness. For these reasons, diagnosis of scedosporiosis and lomentosporosis relies on the recovery of the etiological agent from clinical specimens and identification using macroscopic and microscopic features (Ramsperger et al., 2014; Luna-Rodriguez et al., 2019) in vitro. As with Fusarium species, accurate differentiation of species is critical for guiding clinical management due to variable antifungal susceptibilities (Gilgado et al., 2006; Cortez et al., 2008; Box et al., 2018). While Scedosporium and Lomentospora species can be recovered from sterile sites such as blood, bone and tissue biopsy specimens using standard mycological media, for example Saboraud’s dextrose agar (SDA), their frequent occurrence in sputum samples of CF patients (Sedlacek et al., 2015; Chen et al., 2017) necessitated the development of selective media that prevent overgrowth by faster growing Aspergillus, Candida and Fusarium species in polymicrobial clinical samples (Rainer et al., 2008; Blyth et al., 2010; Hong et al., 2016). One of the most effective media for selective isolation of Scedosporium species is Scedosporium Selective agar (SceSel+)(Rainer et al., 2008; Blyth et al., 2010) containing the antifungal agents benomyl and dichloran that limit the growth of Aspergillus and Candida. Scedosporium species and L. prolificans can then be further differentiated in culture with the inclusion of cycloheximide that is inhibitory to L. prolificans (Ramsperger et al., 2014).

Nucleic acid tests

Speciation of isolates within the Pseudallescheria/Scedosporium complex and their discrimination from L. prolificans based on microscopic features requires considerable expertise and can be problematic. For this reason, a number of molecular methods have been developed for the detection and differentiation of these fungi in axenic culture, sputum, and tissue samples including multiplex PCR (Harun et al., 2011); repetitive sequence-based PCR (rep-PCR)(Steinmann et al., 2011); qPCR, loop-mediated isothermal amplification (LAMP) and PCR-based reverse line blot (PCR-RLB)(Lu et al., 2011a,b); oligonucleotide arrays (Bouchara et al., 2009); DNA sequencing and restriction fragment length polymorphisms (RFLP)(Delhaes et al., 2008; Gilgado et al., 2005, 2008), and Rolling Circle Amplification (RCA)(Lackner et al., 2012). These techniques and their different capabilities are comprehensively reviewed elsewhere (Ramsperger et al., 2014; Ramirez-Garcia et al., 2018). Despite these myriad methods for pathogen detection and speciation, no single technique has been universally adopted nor are any commercial tests currently recommended for this purpose. Current guidelines recommend that nucleic acid-based assays be used in combination with conventional laboratory tests (Tortorano et al., 2014).

Proteomics and antigen tests

Matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF/MS) has been investigated as an alternative strategy for identification of fungi within the PSC (Coulibaly et al., 2011), and other pathogenic moulds (Santos et al., 2010; Cassagne et al., 2011; Bader, 2013; Lau et al., 2013). This technique currently lacks the requisite standardisation needed for clinical acceptability in medical mycology, but it nevertheless has the potential to replace gene-sequencing methods for mould identification, as it is
accurate, comparatively inexpensive and relatively quick compared to sequencing methods. However, it does require robust online reference databases constructed using similar fungal
growth and extraction procedures for interrogation of MS spectra from unknown species.
Commercially available MALDI-TOF/MS identification solutions are apparently inadequate
for *Scedosporium/Lomentospora* discrimination at present (Ramirez-Garcia et al., 2018).

Mass spectrometry (MS) has also recently been used by Thornton et al. (2015) to identify the
antigen bound by a mAb (CA4) specific to *L. prolificans*. This IgG1 mAb is highly specific,
binding to an intracellular protein from *L. prolificans*, but not with proteins extracted from
members of the PSC or from other unrelated moulds. CA4-reactive peptides in western
blots of cell extracts following two-dimensional electrophoresis were identified using MS as
fragments of the melanin-biosynthetic enzyme tetrahydroxynaphthalene reductase (4HNR).
This enzyme is one of a number of enzymes involved in DHN-melanin production of
dematious moulds such as *L. prolificans* (Al-Laaeiby et al., 2016). Confirmation of the CA4
antigen as the enzyme 4HNR was established using genome sequencing and targeted
disruption of the 4HNR-encoding gene. Mutants lacking the gene, and with abnormal
pigmentation (orange-brown sporation compared to the black spores of the wild type strain),
were enzyme-deficient and no longer reactive with mAb CA4.

The study of Thornton et al. (2015) further demonstrates the power of hybridoma
technology to develop mAbs specific to pathogenic moulds, but the generation of a species-
specific mAb to an intracellular antigen is unusual. The majority of genus or species-specific
mAbs generated against cell extracts from pathogenic yeasts, yeast-like fungi, and moulds,
bind to carbohydrate moieties on extracellular carbohydrate or glycoprotein antigens
(Thornton et al., 2002; Thornton, 2009; Davies and Thornton, 2014; Al-Maqtoofi and
Thornton, 2016; Davies et al., 2017; Morad et al., 2018). This is the case of mAbs specific to
members of the PSC. Thornton (2009) reported the development of IgG1 (clone HG12) and
IgM (clone GA3) mAbs specific to a heat-stable, water-soluble, 120kDa carbohydrate
present on the surface of conidia and hyphae of *S. apiospermum*, *S. aurantiacum*, and *S.
boydii*. The antigen is also readily detectable as an extracellular antigen that is produced in
abundance during active growth of these pathogens. The mAbs do not react with the
related fungus *S. dehoogii*, with *L. prolificans*, or other unrelated human pathogenic fungi.
The copious production of the 120kDa antigen and the availability of highly specific mAbs
for antigen detection, make this an ideal system for the development of a diagnostic test for
members of the PSC. To this end, we have developed a lateral-flow assay (Sced-LFA) for PSC
detection that comprises the IgG1 mAb HG12 (Figure 1), and which demonstrates the
feasibility of developing a POCT for rapid detection of pathogens in the PSC. In addition, we
have developed a quantitative sandwich ELISA that combines both mAbs HG12 and GA3.
This ELISA was recently used by Box et al. (2018) to determine the pharmacodynamics of
voriconazole in *in vitro* models of invasive pulmonary scedosporiosis. Given the recent CE-
marking and commercial availability of the AspLFD (OLM Diagnostics) and *Aspergillus GP-
ELISA* (Euroimmun AG) which both employ the *Aspergillus*-specific mAb JF5 (Thornton,
2009), the development of complementary qualitative LFA and quantitative ELISA tests for
the diagnosis of scedosporiosis, based on detection of the 120kDa in human BALf and serum
samples, is technically feasible.
Mucormycete species pathogenic to humans

Mucormycetes are thermotolerant, fast-growing saprotrophic moulds of the phylum Zygomycota order Mucorales, which are abundant in a wide range of ecological niches including soil and compost (Richardson, 2009), food (e.g. cheese where they cause spoilage (Hymery et al., 2014)), edible crops where they cause post-harvest storage rots (Saito et al., 2016), and household dust (Al-Humiany, 2010). The order Mucorales includes species in the genus Apophysomyces, Cunninghamamella, Lichtheimia, Mucor, Rhizomucor, Rhizopus, and Saksonaea, which are separated from the entomophthoromycetes Basidiobolus and Conidiobolus, zygomycete fungi that are also capable of causing disease in humans (Binder et al., 2014; Ribes et al., 2000). While mucormycetes such as Rhizopus oryzae (syn. Rhizopus arrhizus) and Lichtheimia corymbifera can cause allergic reactions in humans (Sircar et al., 2015; Rognon et al., 2016), it is the ability of these fungi to cause osteoarticular mycosis (Taj-Aldeen et al., 2017), and rhino-orbital-cerebral (Arndt et al., 2009; Son et al., 2016), oral (Hingad et al., 2012), pulmonary (Hung et al., 2015; Wang et al., 2016; Iqbal et al., 2017), gastrointestinal (Adhikari et al., 2019), cutaneous (Li et al., 2013; Arruebarrena et al., 2016; Zahoor et al., 2016) or disseminated infections (Walsh et al., 2012) in pre-disposed adult and paediatric patients (Dabritz et al., 2011) that is of the greatest concern, with a single species, Rhizopus oryzae responsible for >80% of all life-threatening infections (Richardson, 2009; Richardson and Page, 2018; Raghunath et al., 2019). This fungus and other pathogenic Mucorales (predominantly Lichtheimia, Mucor, and Rhizomucor) differ from other opportunistic moulds in their ability to infect a broader, more varied population of human hosts (Richardson, 2009; Gomes et al., 2011).

Cutaneous and deep tissue infections typically occur by direct inoculation during trauma, such as following combat-related blast injury (Paolino et al., 2012; Warkentien et al., 2012; Tribble and Rodriguez, 2014; Kronen et al., 2017), natural disasters (Andresen et al., 2005; Fanfair et al., 2012), or traffic accidents (Lelievre et al., 2014), but are also reported in patients with diabetes or haematological malignancies (Arnáz-García et al., 2009). Reports of disseminated mucormycosis, while rare, are increasing in incidence particularly in patients with uncontrolled diabetes, iron overload, and ketoacidosis (Binder et al., 2014; Rammaert et al., 2012), and also in patients with haematological malignancies and allogeneic stem cell transplants (Petrikkos et al., 2012; Sugui et al., 2011).

Detection of mucormycosis

Radiology, histology and culture

The aggressive and rapid course of mucormycete infections makes early detection critically important for the management both of localised cutaneous, deep-seated, and disseminated infections (Castrejón-Pérez et al., 2017). This is especially important in the context of their intrinsic resistance to short-tailed mould-active azole drugs (voriconazole (VCZ) and fluconazole) due to an evolutionary conserved amino acid substitution in the enzyme lanosterol 14α-demethylase responsible for ergosterol biosynthesis (Caramalho et al., 2017). This has led to an increased incidence of breakthrough infections amongst immunocompromised and high-risk patients receiving VCZ prophylaxis, with the suggestion
that VCZ prophylaxis should be thought of as one of the risk factors for mucormycosis (Ustun et al., 2007).

As with aspergillosis, fusariosis, and scedosporiosis, abnormalities observed in a chest-CT consistent with angio-invasive mould infections (nodules, halo signs, cavities, wedge-shaped infiltrates, and pleural effusions) are not pathognomonic of pulmonary mucormycosis, and so can only raise the suspicion of mucormycosis in the context of a patient’s clinical history. Despite this, the reversed halo sign in neutropenic patients with haematological malignancies appears to have high predictive value for mucormycosis (Legouge et al., 2014; Walsh et al., 2014). Computed tomography has also proven useful in the differential diagnosis of rhino-orbital-cerebral mucormycosis (ROCM) and bacterial orbital cellulitis, where the early symptoms of both diseases are similar (Son et al., 2016). However, it is unable to discriminate between ROCM and aspergillosis in cases of invasive fungal rhinosinusitis (Arndt et al., 2009). Culture of mucormycetes from biopsy samples (Hong and Park, 2017b) is problematic due to the fragile nature of mucormycete hyphae that are aseptate or pauci-septate (Skiada et al., 2013). Growth of mucormycetes in culture is rapid, but identification to the level of species and even genus requires considerable mycological expertise (Ziaee et al., 2016). As with other pathogenic moulds, MALDI-TOF/MS can be used to differentiate species once in axenic culture (Cornely et al., 2014; Cassagne et al., 2011; De Carolis et al., 2012; Schrodl et al., 2012), but more data are needed to authenticate this technique, and the robustness of reference databases needs to be fully evaluated.

In situ detection of mucormycetes in tissue samples relies on the microscopic observation of characteristic broad ribbon-like hyphae that can be stained with optical brighteners such as calcofluor white or with Grocott-Gomori methamine silver (Lass-Flörl, 2009; Antonov et al., 2015; Liao et al., 2015). Sputum and BALf samples can also show the characteristic broad aseptate hyphae (Glazer et al., 2000), which is a first indicator of mucormycosis (Al-Abbadi et al., 1997). However, in one case study, only 25% of sputum or BALf samples were positive pre-mortem (Kontoyiannis et al., 2000). Attempts have been made to improve the specificity and sensitivity of microscopy by using mucormycete-reactive antibodies in immunohistochemistry. The production of rabbit hyper-immune antisera and mAbs to immuno-dominant intracellular antigens of the Mucorales was reported by Jensen et al. (1994, 1996). Jensen et al. (1994) demonstrated that heterologous absorption rendered antisera, raised against Rhizopus oryzae somatic antigens, monospecific by indirect immunofluorescence, whereas heterologous absorption of Lichtheimia corymbifera antiserum did not abolish reactivity with R. oryzae. The reactivity of the heterologously absorbed antisera, and a murine IgG1 mAb (1A7B4) raised against L. corymbifera, enabled mucormycetes within bovine lesions to be identified by immunohistochemistry. In a subsequent study, Jensen et al. (1996) showed that a mAb (WSSA-RA-1), raised against water-soluble somatic antigens from R. oryzae, similarly bound to intracellular homologous antigens of between 14kDa and 110kDa. The high degree of specificity exhibited by mAb WSSA-RA-1 allowed its use in immunohistochemistry to detect the pathogen in situ in placenta, lymph node and gastrointestinal tract samples of cows with suspected systemic bovine zygomycosis. In a subsequent study (Jensen et al., 1997), the same mAb (WSSA-RA-1) was used in immunohistochemistry to improve the sensitivity and specificity of mucormycosis detection in patients with haematological malignancies.
Identification of mucoralean fungi in culture based on morphological characteristics is notoriously difficult, particularly with strains that fail to sporulate. For this reason, nucleic acid-based procedures have been developed that enable discrimination of Mucorales at both genus- and species-level, although evidence that identification beyond genus-level aids treatment is lacking. Unlike *Fusarium*, sequencing of the internal transcribed spacer regions ITS1 and ITS2 of ribosomal RNA gene (rDNA) has been shown to be a reliable method for species differentiation in the mucormycetes (Gade et al., 2017), with ITS sequencing recommended by ISHAM as a first-line method of identification (Balajee et al., 2009). Numerous in-house molecular assays have been developed for direct detection of mucoralean DNA in clinical samples (Skiada et al., 2018), including BALf (Lengerova et al., 2014), serum (Millon et al., 2016), and biopsy (skin, lung, stomach mediastine, nose, parotid gland) samples (Bernal-Martínez et al., 2012). However, their current lack of standardisation means that they are only moderately supported in the clinical guidelines for the diagnosis of mucormycosis (Cornely et al., 2014; Skiada et al., 2018). Despite this, PCR has proved useful in the detection of mucormycete infections, especially in infections that are culture-negative (Hammond et al., 2011). Lengerova et al. (2014) used PCR followed by high-resolution melt analysis (PCR/HRMA) to detect *Rhizopus* spp., *Rhizomucor pusillus*, *Lichtheimia corymbifera*, and *Mucor* spp. in BALf samples from immunocompromised patients who were at risk of invasive fungal disease. Real-time qPCR assays specific to the species identified in the PCR/HRMA test were used to validate the test. Of the 99 BALF samples collected from 86 patients with pulmonary abnormalities, 91% were negative and 9% were positive in the PCR/HRMA test, with a sensitivity and specificity 100% and 93%, respectively. By combining the postive PCR/HRMA results with positive real-time qPCR results, the specificity was increase to 98%. Due to its high NPV of 99%, the PCR/HRMA test is a fast and reliable technique for differential diagnosis of pulmonary mucormycosis in immunocompromised patients caused by the four most clinically important mucormycetes.

Because of the difficulty in obtaining fungal DNA from human fluids, the possibility of detecting mucormycete DNA in paraffin-embedded and fresh tissues has been investigated in both an experimental model of disseminated mucormycosis (Dannaoui et al., 2010), and also in humans (Gade et al., 2017). The universal primers ITS1 and ITS2 were used in PCR to amplify the ITS1 region from mucormycete DNA in formalin-fixed paraffin-embedded kidney and brain tissues from mice infected with one of five species (*Rhizopus oryzae*, *Rhizopus microsporus*, *Lichtheimia corymbifera*, *Rhizomucor pusillus*, and *Mucor circinelloides*). Amplicons were then sequenced for species identification. Using this procedure, identification of major mucormycete species from formalin-fixed paraffin-embedded could reach 100%, provided sufficient starting material was available for DNA extraction. Gade et al. (2017) investigated the utility of a PCR targeting a 200-300 bp region of the extended 28S region of rDNA for molecular identification of DNA from mucormycetes and other fungi in formalin-fixed paraffin-embedded and fresh tissues. They demonstrated this region could be used to identify all genera and some species of clinically relevant mucormycetes. They also demonstrated that PCR amplification and direct sequencing of the extended 28S region of rDNA was more sensitive compared to targeting the ITS2 region, since they were able to detect and identify mucormycetes and other fungal pathogens in tissues from patients with histopathological and/or culture evidence of fungal infections that were negative in PCR using ITS-specific primers.
Antibody and antigen tests

While there are no routine serological tests for diagnosis of disseminated mucormycosis, ELISA has been used to study the relationship between antibody levels and exposure to Rhizopus microsporus in sawmill workers with allergic alveolitis (Sandven and Eduard, 1992), while recombinant antigens have been developed for the serodiagnosis of farmer’s lung caused by Lichtheimia corymbifera (Rognon et al., 2016). Detection of disseminated mucormycosis is not possible using tests for (1→3)-β-D-glucan since mucormycetes lack this carbohydrate in their cell walls (Cornely et al., 2014), but it can be used to rule out IPA, the most frequent differential diagnosis, or combined Aspergillus and Mucorales infections (Skiada et al., 2013). Early attempts to detect disseminated disease serologically were based on immunodiffusion (Jones and Kaufman, 1978), electrophoresis and immunoblotting (Wysong and Waldorf, 1987), and ELISA (Kaufman et al., 1989) of homogenate antigens challenged with sera from patients with mucormycete infections. However, non-specific reactivity with the antigen preparations was observed with sera from patients with aspergillosis and candidiasis also, which meant that the tests were unable to generically or specifically identify the etiologic agents of mucormycosis.

The immunological determinant of mucoralean intracellular glycans has been shown to comprise linear (1→6)-α-linked mannopyrasonyl residues (Miyazaki et al., 1979), with the cell walls of yeast and hyphal phases of mucoralean fungi containing fucomanopeptide and mannoprotein (Yamada et al., 1982, 1983). While these antigens have provided useful targets for direct visualisation of mucoralean hyphae in tissue samples using immunohistochemistry, their intracellular nature means that they have limited applicability as circulating biomarkers of mucormycosis in BALf and serum samples. Approaches have been used to identify extracellular antigens specific to the Mucorales (Hessian & Smith 1982), which might circulate in the bloodstream and therefore be suitable targets for detection by, for example, ELISA. Extracellular polysaccharides (EPS) of the Mucorales that appear, using polyclonal IgG antibodies, to be specific to this group of fungi, have been isolated and characterised (De Ruiter et al., 1991; 1992a,b, 1993, 1994; Notermans and Soentoro, 1986), and their immunodominant residues shown to comprise 2-O-methyl-D-mannose (De Ruiter et al., 1994).

Despite these extensive early studies, and despite our improved understanding of the polysaccharide cell wall architecture of mucoralean fungi (Lecointe et al., 2019), no genus- or species-specific mAbs are currently available that target immunogenic EPS, and which might prove suitable for the development of immunoassays for diagnosis of mucormycosis in humans. Notwithstanding this, somatic antigens diagnostic of systemic L. corymbifera infections have been detected in the urine of cattle (Jensen et al., 1993b), indicating that biomarkers of mucormycosis circulate in the bloodstream and are excreted. This raises the possibility of developing a non-invasive test for mucormycosis based on mucormycete antigenuria, provided that suitable targets can be identified and specific mAbs can be generated.

Conclusions and Future Prospects

One of the main priorities of the Global Action Fund for Fungal Infections (GAFFI) is availability and access of cheap and effective diagnostic tests for all, particularly those in
poorly-resourced countries. A commendable priority, but one littered with obstacles.

Technically, as this review has shown, it is possible to develop highly specific and sensitive assays for the detection of some of the most problematic of human pathogens, the filamentous fungi or moulds. Furthermore, it is possible to demonstrate the clinical utility of these tests in retrospective or semi-prospective studies. However, the vast majority of these tests remain at the experimental stage in the laboratory, with little prospect of translation to the clinic as commercially-available CE-marked or FDA-approved assays. One of the biggest obstacles to bench-to-bedside translation is the escalating costs for the regulatory approvals required at each of the different technical and commercial phases of a test’s development, and during its registration as an in vitro medical device (IVD).

As Figure 1 and the accompanying text demonstrates, the generation of an immunoassay, a lateral-flow assay (LFA), that encompasses many of the desirable qualities of a near-patient point-of-care test for a human pathogenic fungus (short-time to result; inexpensive equipment/instrumentation; suitable for use by personnel with limited training; minimal number of steps; uncomplicated interpretation (Kozel and Wickes, 2014; Wickes and Wiederhold, 2018)) is relatively simple given the availability of a high quality monoclonal antibody (in this case a well-characterised mAb that detects a water-soluble, heat-stable, carbohydrate antigen specific to pathogens in the Pseudallescheria/Scedosporium complex (Thornton, 2009)), and the technical know-how of how to apply it to LFA technology. Production of this demonstration test took a month to complete, including antibody purification and conjugation, LFA formatting, and determination of specificity and analytical sensitivity. The limit of detection of purified antigen is 3.75ng/ml (Figure 1), which is well within the level of analytical sensitivity required for clinical detection of a circulating antigen in human samples. Despite this, the demand for a PSC-specific test is insufficient to warrant further commercial development of the Sced-LFA, despite the importance of this group of fungi in human disease.

To take a test such as the Sced-LFA from the demonstration phase through to prototyping and then into test validation and verification with an FDA-approved manufacturer, requires investment beyond the capabilities of many diagnostic companies, certainly beyond a small to mid-size enterprise (SME), without a cast-iron guarantee of financial return and profit within a short period of time post CE-marking or FDA approval. This guarantee is unlikely to be met with tests for emerging or rare pathogens such as Fusarium, Lomentospora, Scedosporium, or the mucormycetes, especially given the investment and time required for CE-marking and FDA approval of an IVD, and subsequent integration of a novel test into hospital diagnostic workstreams. With every hospital or regional healthcare authority demanding in-house validation of a test’s performance compared to its own established diagnostic procedures, it can typically take several years before a test is widely accepted, and is purchased in sufficient numbers so as to justify the developer’s costs for R&D, manufacture, marketing, sales and distribution of the test. These same principles apply to any diagnostic test, whether it be an antigen test such as the Sced-LFA, or a molecular diagnostic test for fungal DNA (Wickes and Wiederhold, 2018).

If GAFFI’s desire for cheap and effective diagnostic tests for all is to be met, there needs to be a major re-think in the way that we, as a community of medical mycologists, diagnosticians, and medical practitioners, work together to better support the diagnostics sector to facilitate the development and translation of novel fungal diagnostics to the clinical setting. Without this community support, and without much-needed financial assistance from government funding agencies who have traditionally paid insufficient
attention to human mycoses and their detection, the availability of cheap, easy-to-use, and
effective diagnostic tests will be limited to those pathogens which infect sufficiently large
number of individuals, such as *Aspergillus*, *Cryptococcus*, and *Pneumocystis*, to warrant
diagnostic test development. An exemplar of where community and funding agency support
worked to the patient’s advantage was the Aspergillus Technology Consortium (AsTeC) for
aspergillosis clinical laboratory diagnostics. A similar approach needs to be adopted for
other invasive mould diseases.

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**References**

Abdolrasouli, A., Scourfield, A., Rhodes, J., Shah, A., Elborn, J. S., Fisher, M. C., Schelenz, S.,

 gastric mucormycosis - a rare but important differential diagnosis of upper
gastrointestinal bleeding in an area of *Helicobacter pylori* endemcity. *Wellcome Open
Research* **4**, 5.

W., and For the ABPA complicating asthma ISHAM working group. (2013). Allergic
bronchopulmonary aspergillosis: review of literature and proposal of new diagnostic


molecular epidemiology and genetic diversity of *Fusarium*, a significant emerging group

Al-Humiany, A. A. (2010). Opportunistic pathogenic fungi of the house dust in Turubah,

melanin biosynthesis genes in the human pathogenic fungus *Lomentospora prolificans*

species in hospital and communal sink biofilms by using a highly specific monoclonal


Prattes, J., Flick, H., Prüller, F., Koidl, C., Raggam, R. B., Palfner, M., Egl, S., Buzina, W.,
Rainer, J., and de Hoog, G. S. (2006). Molecular taxonomy and ecology of Pseudallescheria,
Petriella and Scedosporium prolificans (Microascaceae) containing opportunist agents
isolation procedure for members of the Pseudallescheria boydii complex. Antonie
Leeuwenhoek 93, 315-322.


Figure and Legend

Thornton Figure 1.

A.

1

2

3

B. T C

Antigen concentration (ng/ml)

5760
2880
1440
720
360
180
60
30
15
7.5
3.75

Limit of detection

Negative control

0.5 cm
Figure 1. Demonstration lateral-flow assay (Sced-LFA) for the detection of pathogens within the *Pseudallescheria/Scedosporium* species complex. A. Schematic diagram showing the internal workings of the Sced-LFA test. The monoclonal antibody HG12 (Thornton, 2009) is immobilized in the test zone (T) on a porous nitrocellulose membrane. Anti-mouse immunoglobulin immobilised to the membrane in a separate zone serves as an internal control (C). On addition of 100µl of analyte (for example bronchoalveolar lavage) to the release pad (1), mAb HG12 which is conjugated to NanoAct cellulose nanobeads (CNB) in the release pad (Y-shaped structures with yellow spheres), binds to the diagnostic 120kDa antigen (red spheres) and the antibody-CNB/antigen complex then moves along the porous membrane by capillary action (2). The same mAb immobilized in the test zone (T) binds to the complex (3), resulting in a positive test line. Any mAb-CNB conjugate that migrates unbound to the target antigen, binds to the internal control (C) indicating that the assay has run correctly. Where the 120kDa diagnostic antigen is present in the analyte, 2 lines appear (T and C) indicating infection, but samples that are antigen negative (no infection) result in a single control line (C) only. B. Test results using filtrates from an actively growing culture of *S. apisopermum*, and with mAb HG12 conjugated to red CNB particles. Positive tests results (T and C both present) are visible for antigen concentrations ≥3.75ng/ml filtrate (the limit of detection). The negative control (antigen-negative culture medium) produces a single internal control line only. The single-step Sced-LFA test is highly specific, quick (taking only 10 minutes to perform from addition of sample to interpretation of result), and is stable for 18 months at room temperature, making it ideal for use in resource-limited settings. The image shown in B is courtesy of the *Sced-LFA* contract manufacturer LateralDx. Scale bar = 0.5cm.