# New fungal diagnostics

***Invasive fungal disease is a significant cause of morbidity and mortality in the immunocompromised patient. Early and accurate diagnosis is essential to improve outcome. This article explores non-culture based methods as aids to diagnosis as well as a means to risk stratify patients in order to determine the need for targeted therapy.***

Advances in medicine, particularly within the field of transplantation, have resulted in a growing number of immunosuppressed individuals who are at increased risk of invasive fungal disease (Cohen, 2000). This article reviews the use of non-culture based tests for diagnosis of invasive fungal disease. *Candida* and *Aspergillus* are the most commonly encountered invasive fungal diseases in the UK but exact epidemiological data are lacking. Invasive fungal diseases are not notifiable diseases and surveillance data are largely derived from routine microbiological reports.

Data on the incidence of invasive candidosis are likely to be most robust owing to the relative ease of microbiological diagnosis, which is predominantly through culture of the pathogen from blood, although not all cases of invasive candidosis will present with candidaemia. *Candida* spp. accounted for 1.8% of monomicrobial bloodstream infections in 2011, making it the tenth most common bloodstream infection-causing organism (Public Health England, 2013).

The prevalence of invasive aspergillosis is much more difficult to estimate as diagnosis is challenging, relying on specific radiological changes supported with mycology or histological evidence within affected tissue. Routine laboratory reports are therefore likely to be under-representative and epidemiological trends for invasive aspergillosis have instead been inferred from studies of specific high-risk population groups (*Table 1*).

**Table 1. Invasive fungal infection – estimated prevalence, 2002**

| Patient group | Invasive candidosis risk estimates | Invasive aspergillosis risk estimates |
|---|---|---|
| Allograft bone marrow transplant | 4% | 10% |
| Solid organ transplant | 5% | 1.9% |
| Leukaemia | 3% | 6% |
| Solid organ tumour | 3% | 2% |
| Advanced cancer | 1% | 1.5% |
| Intensive care unit | 1% | 0.2% |
| Burns | 5.6% | 1.9% |
| Renal dialysis | 0.2% | 0.02% |
| HIV/AIDS | 0.2% | 4% |

Adapted from Health Protection Agency (2006)

Invasive fungal disease has a high attributable mortality and there is therefore great interest in both its prevention and timely diagnosis in order to facilitate prompt therapy. Mortality rates for invasive candidosis are estimated to be approximately 30% (Kibbler et al, 2003), whereas those for invasive aspergillosis are 60% or higher if the diagnosis is delayed (von Eiff et al, 1995). High attributable mortality and difficulty in detecting early disease have led to the widespread use of prophylactic antifungals in high-risk populations and liberal empirical use in febrile immunosuppressed patients. Rising antifungal costs (*Table 2*) and emerging resistance have made it a priority to address rational use of antifungal drugs in routine practice.

## Diagnosis

The standardized diagnosis of fungal disease is based on a combination of clinical observation and laboratory investigation. In 2002 the European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group and the National Institute of Allergy and Infectious Diseases Mycoses Study Group (EORTC/MSG) published standard definitions for inva-

**Table 2. Antifungal drug costs**

| Antifungal agent | Dose per day | Cost per day (route) |
|---|---|---|
| Fluconazole | 400 mg | £10.80 (IV) |
| Itraconazole | 200 mg | £63.77 (IV) |
| Posaconazole | 800 mg | £93.56 (PO) |
| Voriconazole | 8 mg/kg | £215.99 (IV)* |
| Caspofungin | 50 mg | £327.67 (IV) |
| Liposomal amphotericin B | 3 mg/kg | £406.10 (IV)* |
| Flucytosine | 100 mg/kg | £84.92 (IV)* |

IV = intravenous; PO = oral. Costing as per British National Formulary September 2013–March 2014. * assuming patient's weight is 70 kg

**Dr M Backx** *is Specialist Registrar in Infectious Diseases and Medical Microbiology and* **Dr PL White** *is Principal Clinical Scientist in the Department of Medical Microbiology, Mycology, Molecular Biology and Wales Centre for Mycobacteria, Public Health Wales, Cardiff and* **Professor RA Barnes** *is Professor of Medical Microbiology and Honorary Consultant in the Department of Medical Microbiology and Infectious Diseases, Institute of Infection and Immunity, School of Medicine, Cardiff University, Cardiff CF14 4XN*

*Correspondence to: Professor RA Barnes (barnesra@cardiff.ac.uk)*

sive fungal infections. These were developed to facilitate the identification of a homogenous group of patients for clinical and epidemiological research (Ascioglu et al, 2002). The definitions assign three levels of probability to the diagnosis of invasive fungal disease – 'proven', 'probable' and 'possible' – and rely on the identification of host factors as well as clinical and microbiological criteria. Subsequently, in 2008, revised definitions were developed which aimed to reduce the overrepresentation of dubious possible cases while capturing more patients with proven or probable disease (De Pauw et al, 2008). To facilitate this, specific radiographic changes that occur relatively late in the disease process were incorporated (*Table 3*).

**Table 3. EORTC/MSG criteria for proven, probable and possible invasive fungal disease**

| | | |
|---|---|---|
| Proven invasive fungal disease | Microscopic analysis of normally sterile material | By histopathological, cytopathological or direct microscopic means demonstrating fungal infection and in the case of mould infection accompanied by evidence of tissue damage |
| | Culture of normally sterile material | Including blood culture demonstrating fungal growth |
| | Serological analysis of CSF | Positive for Cryptococcal antigen |
| Probable invasive fungal disease | Host factors | Neutropenia >10 days temporally associated with onset of invasive fungal disease |
| | | Recipient of an allogeneic stem cell transplant |
| | | Steroids (>0.3 mg/kg/day for >3 weeks) |
| | | Treatment with T-cell immunosuppressants, e.g. tumour necrosis factor-α blocker |
| | | Inherited severe immunodeficiencies, e.g. chronic granulomatous disease |
| | Clinical criteria | Lower respiratory tract disease – air crescent, cavity, lesion and/or halo on computed tomography |
| | | Tracheobronchitis – ulcer, nodule, plaque, pseudomembrane or eschar on bronchoscopy |
| | | Sinonasal disease – acute localizing pain radiating to eye, nasal ulcer with black eschar or extension of lesion across bony barriers |
| | | Disseminated candidiasis – small target-like abscesses in liver or spleen, or progressive retinal exudates |
| | | CNS disease – focal lesions or meningeal enhancement |
| | Mycological criteria | Direct test (cytology, direct microscopy, culture) on sputum, bronchoalveolar lavage, bronchial brush or sinus aspirate samples showing fungal elements or positive culture for fungus |
| | | Indirect test (biomarkers) – galactomannan (*Aspergillus*) in plasma, serum, bronchoalveolar lavage, CSF or β-glucan (fungal infection other than zygomycoses or cryptococcosis) in serum |
| Possible invasive fungal disease | Cases that meet the criteria for a host factor and a clinical criterion but for which mycological criteria are absent | |

EORTC/MSG = European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group and the National Institute of Allergy and Infectious Diseases Mycoses Study Group. Adapted from De Pauw et al (2008)

Despite being developed as a tool to facilitate research, the definitions have now been widely adopted in clinical practice. It should be borne in mind that some patients may have invasive fungal disease and yet not fulfil these diagnostic criteria. It should also be noted that the required EORTC/MSG host criteria may preclude this framework from being used in the critical care setting where non-immunocompromised patients are nonetheless at risk of developing invasive fungal disease. In fact, the critical care population account for approximately 45% of reported candidaemias (Kibbler et al, 2003). Aspergillosis is also emerging as a significant problem in the critically ill (Meersseman and Van Wijngaerden, 2007).

## Biomarkers

Within the mycological criteria of the EORTC/MSG definitions are included indirect tests for the detection of fungal antigen or fungal cell-wall constituents, namely: galactomannan antigen, glucan and cryptococcal antigen (*Table 3*). Other biomarkers which are currently not used within the definitions include mannan antigen/anti-mannan antibody, *Candida albicans* germ tube antibody and polymerase chain reaction.

### Galactomannan

Galactomannan is a fungal wall component of *Aspergillus* and circulating galactomannan may be detected in serum, bronchoalveolar lavage fluid and CSF, as well as other fluids, using an enzyme-linked immunosorbent assay (ELISA) (Mennink-Kersten et al, 2004). Galactomannan detection is included in the EORTC/MSG criteria for identifying probable invasive fungal disease (De Pauw et al, 2008). The performance of this test for the diagnosis of invasive aspergillosis has been reviewed in a number of meta-analyses with differing results in different population groups (Pfeiffer et al, 2006; Leeflang et al, 2008). This is likely related to the pathogenesis of *Aspergillus* infection (Cordonnier et al, 2009a).

In non-neutropenic patients a local inflammatory response limits the angio-invasive process and it is cleared rapidly from the circulation making it less detectable in the serum. However, in neutropenic patients, a poor immune response results in invasion of blood vessels, reduced clearance of galactomannan by the immune system and therefore higher levels of galactomannan antigenaemia. The development of invasive aspergillosis is a progressive process starting with local subclinical infection and progressing to overt disease. Galactomannan detection can be used to pre-emptively treat individuals who display clinical signs of infection before the development of overt disease and radiographic changes, e.g. the

febrile neutropenic patient (Maertens et al, 2005; Cordonnier et al, 2009b).

The positive predictive value of the test (the ability to rule in a diagnosis of infection) is heavily influenced by the prevalence of invasive aspergillosis in the test population. Using galactomannan as a screening test to determine whether pre-emptive therapy is indicated is therefore limited to populations with a greater than 5–10% risk of developing invasive aspergillosis such as neutropenic patients with acute myeloid leukaemia, patients with myelodysplastic syndromes undergoing intensive chemotherapy and patients receiving allogeneic stem cell transplants during the early engraftment phase. The negative predictive value (the ability to rule out a diagnosis) is less affected by prevalence such that the test may be used to exclude invasive aspergillosis in patients where empiric antifungal drugs are traditionally used (e.g. in febrile neutropenia). However, the use of concurrent antifungals either as prophylaxis or empiric therapy needs to be considered as they will impact on performance of the assay (Marr et al, 2005).

## β-Glucan

1-3-β-D-glucan is a cell wall component of the majority of fungal species with the exception of zygomycetes and *Cryptococcus* spp., where it is absent or limited. A number of different assays are available with differing reactivity to β-glucan and different cut-offs for defining positivity. A systematic review of the literature pertaining to the use of these tests within haematological patients within the EORTC/MSG framework showed some variability of the performance of these tests (Marchetti et al, 2012). This is likely as a result of differences in patient populations, study design and interpretation of the EORTC/MSG criteria. Studies requiring two consecutive positive tests demonstrated a specificity approaching 100% for the diagnosis of invasive candidiasis and aspergillosis (Kawazu et al, 2004; Ellis et al, 2008).

The detection of β-glucan has been included in the EORTC/MSG criteria for the diagnosis of probable invasive fungal disease (De Pauw et al, 2008). It has also shown potential for diagnosing *Pneumocystis* pneumonia which is associated with high levels of β-glucan in the circulation, potentially removing the need for obtaining deep respiratory samples (Onishi et al, 2012). Owing to the low prevalence of the less common fungi, the utility of this test for their diagnosis cannot be assessed. There remain a number of unanswered questions requiring a well-designed randomized controlled trial exploring the optimum cut-off values and number of tests required to define a positive test and the utility of this test for use in pre-emptive therapy.

## Cryptococcal antigen

The polysaccharide capsule of *Cryptococcus neoformans* can be detected in serum and CSF using latex agglutination, ELISA and immunochromatographic lateral flow devices (Babady et al, 2009; Binnicker et al, 2012). The sensitivity of these tests ranges from 62% to 97% depending on the site of infection, being highest for cryptococcal meningitis (Pappas et al, 2001; Dromer et al, 2007). The specificity of the tests for the diagnosis of invasive cryptococcal disease approaches 100% (Tanner et al, 1994; Jaye et al, 1998). Serum and CSF cryptococcal antigen testing are recommended for the diagnosis of disseminated fungal infection and meningitis respectively and are included in the EORTC/MSG criteria for the diagnosis of invasive cryptococcal disease (De Pauw et al, 2008). However, it should be noted that data within the haematology oncology population are lacking with the majority of data derived from studies involving HIV-positive patients and solid organ transplant recipients (Antinori et al, 2001; Dromer et al, 2007; Chuang et al, 2008).

## *Aspergillus* lateral flow device

The lateral flow device is an immunochromatographic device that incorporates a monoclonal antibody, JF5, that binds to an extracellular glycoprotein found on *Aspergillus* (Thornton, 2008). The monoclonal antibody is highly specific for *Aspergillus* spp. and does not cross react with other clinically relevant fungi. When compared to a galactomannan assay it demonstrated comparable sensitivity and specificity in discriminating proven or probable invasive aspergillosis from patients without invasive aspergillosis (White et al, 2013). However, unlike the galactomannan assay, the test is cheap, quick and easy to perform and may represent an alternative for laboratories that do not possess the necessary hardware and expertise, and could be used as an out of hours alternative.

## Mannan antigen and anti-mannan antibody

Mannan is a component of the *Candida* cell wall and is one of the main antigens that circulate during invasive candidosis (Klis, 1994). A number of ELISAs are available for the detection of mannan antigen and anti-mannan antibody. A review of studies using mannan antigen and anti-mannan antibody for the diagnosis of invasive fungal disease as per the EORTC/MSG criteria revealed heterogeneity with regards to both study populations and study design (Marchetti et al, 2012). The combination of the mannan antigen and anti-mannan antibody test revealed the highest sensitivity and specificity for invasive candidosis, 83% and 86% respectively. Although the combination test may offer a diagnostic aid, insufficient data from randomized controlled trials with regards to test performance and cost-effectiveness were available in haematology oncology patients to include this in the EORTC/MSG criteria.

## *Candida albicans* germ tube antibody

Germ tubes are structures generated during *Candida albicans* growth and antibodies to these structures have been evaluated in the critical care setting for their diagnostic capabilities. In a multi-centre observational study

of patients with complicated abdominal conditions the combination of a negative *Candida albicans* germ tube antibody and β-glucan had a negative predictive value of 93.4% for invasive candidosis (Leon et al, 2012).

### Polymerase chain reaction

The majority of data for the use of polymerase chain reaction for the diagnosis of invasive fungal disease come from the field of invasive aspergillosis. Multiple polymerase chain reaction assays are reported in the literature differing in their sample type, gene targets and the platforms used (Bretagne and Costa, 2005). A meta-analysis of studies using different polymerase chain reactions testing blood showed that the sensitivity and specificity for two consecutive tests was high at 75% and 87% respectively (Mengoli et al, 2009). Screening using combination galactomannan and polymerase chain reaction testing has been successfully used as part of a pre-emptive approach in high-risk haematology patients (Barnes et al, 2009; Morrissey et al, 2013; Rogers et al, 2013). A meta-analysis of data looking at the diagnosis of invasive aspergillosis using polymerase chain reaction of bronchoalveolar lavage fluids showed a sensitivity and specificity of 79% and 94% respectively (Tuon, 2007).

Despite its performance, polymerase chain reaction was not included in the current EORTC/MSG criteria because of lack of standardization and inter-laboratory reproducibility. A process for standardization of laboratory protocols to allow for implementation of polymerase chain reaction in clinical practice has now been initiated through the European *Aspergillus* PCR Initiative (White et al, 2010). In addition, the use of polymerase chain reactions for the diagnosis of invasive candidosis, using blood samples, and *Pneumocystis* pneumonia, using bronchoalveolar lavage samples, provides rapid diagnostic tests with sensitivities and specificities in excess of 90% (Avni et al, 2011; Fan et al, 2013).

Polymerase chain reaction is also in development for the detection of mucormycosis. In a retrospective analysis using serum samples of patients with proven mucormycosis it demonstrated high sensitivity and specificity and is currently undergoing prospective evaluation (Millon et al, 2013). *Candida* polymerase chain reaction has been combined with T2 magnetic resonance in an attempt to improve the test's sensitivity, specificity and turnaround time. Whole-blood polymerase chain reaction products were combined with nanoparticles with complementary capture probes. In spiked samples containing *Candida* cells, even at very low levels, nanoparticles clustered together allowing for detection by magnetic resonance within 3 hours (Neely et al, 2013).

## Host factors

The current EORTC/MSG criteria define host factors for proven, probable and possible invasive fungal disease (*Table 3*). These have also been used to identify patients who may benefit from anti-fungal prophylaxis. However, these are not specific and may result in the unnecessary use of antifungal agents. Additionally, the EORTC/MSG criteria preclude the use of this framework in non-immunocompromised populations that are nonetheless at risk of invasive fungal disease. Further host factors have been identified and some of these have been incorporated in risk prediction scores to address these issues.

### Single nucleotide polymorphisms

The recognition of pathogen-derived structures by the pattern recognition receptors of the innate immune system is an integral part of the host defence against fungi. Genetic defects as well as single nucleotide polymorphisms affecting the pattern recognition receptors have been shown to be associated with an increased risk of developing invasive candidosis and invasive aspergillosis. A retrospective analysis of probable or proven invasive aspergillosis, as per the EORTC/MSG guidelines, showed that single nucleotide polymorphisms in the pattern recognition receptor Dectin-1 and DC-SIGN were associated with a significant increased risk of developing disease (Sainz et al, 2012). Additionally, single nucleotide polymorphisms in the TLR1, TLR2 and TLR4 genes are associated with an increased susceptibility to invasive candidosis (Smeekens et al, 2013).

### Risk prediction scores

There is increasing evidence that invasive fungal disease is more likely to occur in the critical care setting even in the absence of neutropenia and that the majority of these infections are caused by *Candida* spp. (Lamagni et al, 2001; Kauffman, 2006).

A number of randomized controlled trials have demonstrated that antifungal prophylaxis in the critical care setting reduces the risk of proven invasive fungal disease and may reduce mortality (Playford et al, 2006). A health technology assessment reviewed risk factors for the development of invasive fungal disease in patients admitted to UK NHS adult general care units in order to generate and internally validate risk models for the identification of patients who would benefit from anti-fungal prophylaxis (Harrison et al, 2013). Overall the incidence of invasive fungal disease was low (0.6%), with the vast majority caused by *Candida*.

The patients who developed invasive fungal disease, in line with previous studies (Leon et al, 2006; Ostrosky-Zeichner et al, 2007), had increased attributable mortality and length of stay. A number of host criteria were found to be associated with an increased risk of developing invasive fungal disease and included the number of central venous catheters present and the number of samples positive for fungal colonization. Analysis showed that adoption of the generated risk models would likely reduce mean hospital costs if prophylaxis were to be introduced when the risk of developing invasive fungal disease exceeded 1–2%. However, this would require 5–12% of the intensive care population to receive prophylaxis.

## Discussion

A number of tests are now available to the clinician to help in the diagnosis of proven and probable invasive fungal disease (*Table 3*). The introduction of matrix-assisted laser desorption/ionization time of flight (MALDI TOF) technology has allowed for the rapid identification of the more common fungal pathogens from clinical specimens and may obviate the need for more time-consuming identification methods (Lau et al, 2013). In addition, biomarkers have been used outside the EORTC/MSG framework in an attempt to shift away from the indiscriminate empiric use of potentially expensive antifungal agents to a more targeted approach.

For invasive aspergillosis in the haematology-oncology population the negative predictive value of *Aspergillus* specific biomarker (galactomannan, polymerase chain reaction) is such that it allows for a screening strategy, where antifungal agents can be safely withheld from the febrile neutropenic patient with negative biomarkers. Such an approach could also be adopted for invasive candidosis in the critical care population. However, the population at risk of invasive candidosis is poorly defined and *Candida* specific biomarkers (polymerase chain reaction, mannan antigen or anti-mannan antibody, β-glucan, *Candida albicans* germ tube antibody) have not been validated for pre-emptive therapy. Further studies are therefore required looking at risk prediction scores, possibly incorporating single nucleotide polymorphisms, and the performance of biomarkers within this population. Proteomics, genomics and single nucleotide polymorphism identification allow protein and gene expression of hosts and pathogens to be explored in order to gain further insight into the human response to invasive fungal disease (Tierney et al, 2012). Proteomics and genomics offer the exciting prospect of personalised medicine whereby screening and treatment could be tailored to the individual. **BJHM**

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## KEY POINTS

- Invasive fungal disease is a significant cause of morbidity and mortality in the immunocompromised patient.
- Early and accurate diagnosis of invasive fungal disease is essential to improve clinical outcome.
- The detection of fungal antigens (galactomannan, β-glucan, cryptococcal antigen) are included in standard definitions for the diagnosis of invasive fungal disease published by the European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group and the National Institute of Allergy and Infectious Diseases Mycoses Study Group.
- The detection of fungal DNA by polymerase chain reaction, antibodies directed against fungal cell wall components and novel fungal antigens, although not included in the standard definitions, provide further aids for the diagnosis of invasive fungal disease.
- Screening for galactomannan and *Aspergillus* DNA in populations at risk of invasive aspergillosis has been successfully used to determine the need for targeted therapy but further studies are required to determine whether this can be used for populations at risk of invasive candidosis.
- Proteomics, genomics and single nucleotide polymorphism identification offer the exciting prospect of personalized medicine whereby screening and treatment could be tailored to the individual.

51
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