

The current management landscape: aspergillosis

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Diagnosing invasive aspergillosis (IA) has long been challenging due to the inability to culture the causal *Aspergillus* agent from blood or other body fluids. This shortcoming has fuelled an interest in non-culture-based diagnostic techniques such as the detection of galactomannan (GM) in blood and bronchoalveolar lavage fluid, the detection of 1,3- β -D-glucan (BDG) in blood and the detection of *Aspergillus* DNA by PCR-based techniques. Past decades have witnessed important improvements in our understanding of the strengths and limitations of antigen assays and in the standardization of PCR-based DNA techniques. These assays are now being incorporated into care pathways and diagnostic algorithms; they help us to steward and monitor antifungal therapies and to predict treatment outcomes.

Introduction

Invasive aspergillosis (IA), usually affecting the respiratory tract, occurs almost exclusively in patients with varying degrees of immunodeficiency, and produces a wide range of clinical manifestations. The nature and extent of the compromised immunity, as well as the prophylactic use of antifungals and accommodation in protective environments, predict the risk.¹ Patients at high risk include allogeneic stem cell transplant recipients, those being treated for acute myeloid leukaemia, myelodysplastic syndromes or aplastic anaemia, and subgroups of solid organ transplant recipients.¹ However, over the last decade several novel risk groups have been identified, including (but not restricted to) patients with chronic obstructive lung disease, liver cirrhosis, auto-immune disorders and influenza pneumonia. Many of these latter groups of patients are not managed in haemato-oncology or transplantation units but are hospitalized in intensive care facilities.^{2,3} Many cases of IA still remain undiagnosed or are only identified at autopsy because of difficulties in making an early diagnosis.⁴ This shortcoming has resulted in a widespread and well-accepted practice of starting antifungals prophylactically or empirically in the absence of confirmation of fungal infection or disease. Although this approach is considered 'standard of care' by many treating physicians, this strategy can also result in unnecessary antifungal drug treatment, adverse drug reactions and increased healthcare expenditure.⁵

Diagnostic tools targeting fungal biomarkers [e.g. galactomannan (GM), 1,3- β -D-glucan (BDG) and fungal DNA] have been developed over the past few decades. These assays display improved performance characteristics compared with culture

and microscopic examination, the more conventional diagnostic tools. In recent clinical practice, these novel tests have been increasingly used to determine treatment strategies and to influence patient management.⁶ However, the ability to understand test performance in different at-risk populations with different prevalence of disease and in different clinical specimens is required. Assessing the clinical utility of these tests and feeding back the interpretation of test results to treating physicians has become a key element of antifungal stewardship (AFS), especially in centres with a large population of immunocompromised patients.

Conventional diagnostic tools

Culture and microscopic examination have been the cornerstones of the microbiological diagnosis of IA. However, culture is time-consuming and requires considerable expertise. In addition, blood cultures are notoriously negative, even in disseminated disease, and culture from any respiratory specimen has only low to moderate sensitivity and predictive value.^{7–9} In an attempt to minimize the over-interpretation of the clinical significance of a positive culture for *Aspergillus* species, Bouza *et al.*¹⁰ developed a score based on easily obtainable clinical and microbiological information. This score included: (i) a sample obtained by invasive procedures (1 point); (ii) two or more positive samples from the same patient (1 point); (iii) underlying leukaemia (2 points); (iv) presence of neutropenia (5 points); and (v) corticosteroid treatment (2 points).¹⁰ Patients with a score of 0 had only a 2.5% probability of IA. Those with a score of 1 or 2 had an increased probability of 10.3%. The probabilities rose to 40% for patients

with a score of 3 or 4, and to 70% for those with a score of ≥ 5 . This score helps to rule out the probability of proven or probable aspergillosis in an unselected population and better defines the subpopulations that need more aggressive diagnostic work-up for the confirmation of disease. Similar scores have not yet been developed for other mould pathogens.

The lack of efficient diagnostic tools has led to the development of surrogate markers, based on the detection of fungal cell wall components or fungal DNA in clinical specimens.

The role of biomarkers

Galactomannan

The fungal cell wall is almost exclusively composed of polysaccharides, including GM, a molecule composed of mannose residues with side chains of β -(1-5)-linked galactofuranosyl units. During the initial phase of logarithmic fungal growth, GM is incorporated into the cell wall, but as apical growth continues the hyphal tip becomes weaker and releases GM.¹¹ Using an *in vitro* model of the human alveolus, Hope *et al.*¹² demonstrated that the kinetics of GM release and its subsequent levels are closely related to the dynamics of angio-invasion. They concluded that GM is only released into the circulation after the fungus has invaded the endothelial compartment.

GM can be detected in various body fluids by a commercially available sandwich ELISA (Platelia *Aspergillus*[®], Bio-Rad, Marnes-La-Coquette, France). This test uses EB-A2, a rat monoclonal antibody that specifically binds to four galactofuranosyl residues, as both a capture and a detecting antibody.¹³ Antibody-coated microplates are read using an optical reader that calculates the ratio of the optical density, relative to a control provided by the manufacturer (the 'optical density index').¹⁴ The test is included as a mycological criterion within the European Organisation for Research and Treatment of Cancer/Mycoses Study Group (EORTC/MSG) consensus definitions and has become the mainstay for diagnosing probable IA.¹⁵ This simple ELISA can be performed at the local laboratory level; however, no external quality control exists yet. The assay has been extensively evaluated and is the subject of meta-analyses and systematic reviews.^{16–18} Sensitivities of 17%–100% have been reported depending on the index cut-off used to determine positivity and on the nature of the population at risk. Indeed, the test performs best in adult and paediatric neutropenic patients (frequently undergoing intensive chemotherapy for leukaemia) and less well in non-neutropenic patients, including organ transplant recipients and stem cell transplant recipients with graft-versus-host disease.^{16–18} This probably reflects differences in immunopathogenesis of disease and fungal burden, and represents a serious limitation of the assay when used as a screening tool in unselected immunosuppressed patients.^{19,20} Earlier studies used an index of ≥ 1.5 to define positivity, as initially recommended by the manufacturer. More recently, the US FDA has approved a cut-off index value of ≥ 0.5 based on testing of two separate blood samples, or a single sample with a value of ≥ 1.0 (restricted to patients with haematological malignancies or recipients of haematopoietic stem cell transplants).²¹ European Conference on Infections in Leukaemia (ECIL) guidelines recommend a single value of ≥ 0.7 or multiple (consecutive) values of ≥ 0.5 for blood specimens.²² Notably, the 2008 EORTC-MSG revised consensus

document has not specified a cut-off, but refers to the manufacturer's instructions.¹⁵ However, improved sensitivity with the use of lower cut-offs usually comes with a loss of specificity.²³

Although fairly specific for *Aspergillus* species, cross-reactivity with non-*Aspergillus* fungi (including but not limited to *Fusarium* species, *Penicillium* species, *Acremonium* species, *Alternaria* species and *Histoplasma capsulatum*) may occur. In addition, galactofuranosyl residues are also present in other macromolecules, resulting in false-positive test results. Table 1 summarizes established causes of false positivity and false negativity.

GM testing can also be applied to other types of specimens, including bronchoalveolar lavage (BAL) fluid.^{24,25} Diagnostic bronchoscopy with lavage is performed when radiographic abnormalities of the lung have been detected, usually by pulmonary CT scan. In this setting, the pre-test probability of (fungal) disease is much higher than when screening a blood sample from an asymptomatic patient; hence specificity becomes crucial and a higher threshold of positivity is needed. Cut-off values of 1.0 have been recommended (and approved by the US FDA²¹), although it is likely that even higher thresholds may be necessary.²⁶ Recently, an index cut-off of 1.0 has also been suggested for analysing CSF samples from patients with (suspected) cerebral aspergillosis.²⁷ Stringent criteria still need to be developed for use with other body fluids (urine, abscesses, pleural fluid, ascites, and so on).

β -1,3-D-Glucan

Unlike GM, BDG is a polysaccharide component of the cell wall of many pathogenic fungi, including *Candida* species, *Fusarium* species and *Pneumocystis*. The main exceptions are Mucorales and some *Cryptococcus* species.²⁸ Four assays are now commercially available, of which the Fungitell[®] assay (Associates of Cape Cod, Inc., East Falmouth, MA, USA) has been approved by the US FDA and carries the European CE label for the presumptive diagnosis of invasive fungal infection (IFI).²⁹ The remainder are only marketed in Japan. Also, the Fungitell test has been included in the EORTC/MSG definitions of invasive fungal disease (IFD).¹⁵ Notably, cut-off values for determining positivity differ markedly between these assays.¹⁴

Any systematic review of the Fungitell BDG test is hampered by significant heterogeneity among the patient populations, testing strategies and the inclusion of retrospective and case-controlled studies alongside prospective cohort studies with low numbers of documented fungal diseases.^{30–33} Most studies report good sensitivity, but specificity and positive predictive values for diagnosing mould infections are poor due to a high rate of false-positive results (Table 1), regardless of the specimen.³⁴ However, the negative predictive value is around 80%–90%. Unfortunately, the BDG assay is not pathogen specific and therefore cannot differentiate between fungal species. In addition, pre-test preparations may limit its routine applicability.

Polymerase chain reaction

PCR-based methods have been developed for the diagnosis of IA. The main advantage is the very high sensitivity for real-time detection of fungal DNA. In addition, PCR-based methods can be applied to any specimen type, including whole blood, serum, plasma, BAL fluid, CSF and tissue samples. However, the

Table 1. Limitations of antigen assays in the diagnosis of invasive fungal disease

	Galactomannan	β -D-glucan
Reactivity with fungal species	<i>Aspergillus</i> spp., <i>Fusarium</i> spp., <i>Paecilomyces</i> spp., <i>Acremonium</i> spp., <i>Penicillium</i> spp., <i>Alternaria</i> spp., <i>Histoplasma capsulatum</i> , <i>Blastomyces dermatitidis</i> , <i>Cryptococcus neoformans</i> , <i>Emmonsia</i> spp., <i>Wangiella dermatitidis</i> , <i>Prototheca</i> , <i>Myceliophthora</i> , <i>Geotrichum capitatum</i> , <i>Chaetomium globosum</i>	<i>Pneumocystis jirovecii</i> , <i>Aspergillus</i> spp., <i>Fusarium</i> spp., <i>Histoplasma capsulatum</i> , <i>Candida</i> spp., <i>Acremonium</i> spp., <i>Trichosporon</i> sp., <i>Sporothrix schenckii</i> , <i>Saccharomyces cerevisiae</i> , <i>Coccidioides immitis</i> , <i>Prototheca</i>
False-positive test results	Semi-synthetic β -lactam antibiotics ^a Multiple myeloma Blood products collected using Fresenius Kabi bags Gluconate-containing plasma expanders (e.g. Plasmalyte) Flavoured ice-pops/frozen dessert containing sodium gluconate <i>Bifidobacterium</i> spp. (gut) Severe mucositis or gastrointestinal graft-versus-host disease Enteral nutritional supplements	Semi-synthetic β -lactam antibiotics Human blood products, including immunoglobulins, albumin, plasma, coagulation factor infusions, filtered through cellulose membranes Cellulose haemodialysis/haemofiltration membranes Exposure to (surgical) gauze Bacterial bloodstream infections (e.g. <i>Pseudomonas aeruginosa</i>)
False-negative test results	Concomitant use of mould-active antifungal agents Mucolytic agents (bronchoalveolar lavage) such as Sputasol or SL solution	Concomitant use of antifungal agents

^aIncluding ampicillin, amoxicillin/clavulanate and piperacillin/tazobactam (currently this problem seems largely abated compared with previous reports).

development of in-house assays using varied protocols involving different specimens, extraction techniques, molecular targets, amplification platforms and detection techniques has led to a lack of standardization, which has hampered the acceptance of these diagnostic assays. For this very reason, PCR has not yet been included in the EORTC/MSG consensus definitions as a reliable microbiological marker.¹⁵ Fortunately, over the past decade the European Aspergillus PCR Initiative (EAPCRI) has aimed to remedy this situation for diagnosing IA, and has made tremendous progress in standardizing protocols for efficient DNA extraction and amplification.^{35–41} Clinical validation in multicentre prospective studies is ongoing.

Commercially available as well as in-house platforms, using genus-/species-specific genes and pan-fungal targets, have been developed and the usefulness of PCR for diagnosing IFD has recently been reviewed.^{42,43} Superior performance compared with the serological biomarkers has been suggested and high negative predictive values have been consistently documented, despite all the methodological variabilities.^{44–46} Whereas two positive results seem to be required to rule in disease, it has been suggested that a single negative PCR result is sufficient to exclude *Aspergillus* disease at that timepoint.

Lateral-flow device for invasive aspergillosis

A lateral-flow device (LFD) was developed for a point-of-care diagnosis of IA. This assay uses a monoclonal antibody that is highly specific for growing *Aspergillus* species (but different from the one used in the Platelia *Aspergillus* assay).^{47,48}

Compared with the Platelia GM and Fungitell BDG assays, the LFD test is quick (taking 15 min to perform) and does not require expensive equipment or specific laboratory facilities to be run. Furthermore, cross-reactions with drugs or contaminants that have been shown to cause false-positive reactions in the GM and BDG tests have not (yet) been seen. A recent meta-analysis of seven studies (mainly with solid organ transplant recipients) yielded a pooled sensitivity, specificity and diagnostic odds ratio (DOR) for proven/probable versus no aspergillosis cases of 86%, 93% and 65.9%, respectively, when using BAL fluid and 68%, 87% and 11.9%, respectively, when using serum samples (in which case a heating step is required).⁴⁹ More data on the impact of antifungal prophylaxis or therapy on the performance of the LFD are needed.⁵⁰

Biomarkers in development

Despite significant recent advances, the available tools for diagnosing IA are far from perfect and clinicians often still struggle to make a timely diagnosis. Therefore, the search for novel targets and platforms that may further improve diagnostic capabilities continues. A number of electronic noses (eNoses) that have been developed over the past few years can discriminate between various lung diseases through an analysis of exhaled volatile organic compounds (VOCs). An eNose is cheap and non-invasive, and yields results within minutes. A number of prototypes have been designed to detect *Aspergillus* species VOCs.⁵¹ A proof-of-principle study showing that neutropenic patients with aspergillosis

have a distinct exhaled breath profile or signature ('breath print') that can be discriminated with an eNose has recently been published. This study showed a sensitivity of 100% and a specificity of 83%.⁵²

Using gas chromatography and mass spectrometry, US researchers were able to measure fungal volatile metabolites in breath samples of patients with IA. Detection of α -trans-bergamotene, β -trans-bergamotene, a β -vatenene-like sesquiterpene, or trans-geranylacetone identified these patients with 94% sensitivity and 93% specificity.⁵³ Although both techniques perform well for diagnosing IA, more extensive validation is needed.

In recent years, gliotoxin (GT), a virulence factor released during hyphal growth, has been proposed as a diagnostic biomarker of IA. *Aspergillus fumigatus* is the most important GT-producing fungal pathogen, although non-*fumigatus* *Aspergillus* species can also produce GT, as well as less common opportunistic pathogens such as *Penicillium* species, *Gliocladium* species and *Pseudallescheria* species.⁵⁴ Unfortunately, GT is hard to detect in body fluids. Bis(methylthio)gliotoxin (bmGT), the inactive derivative of GT, is more stable and appears to be a more reliable indicator of infection than GT.⁵⁴ A recent prospective study compared the diagnostic accuracy of bmGT detection (by high-performance thin layer chromatography) with GM detection (Platelia assay) in 79 patients at risk of IA. The study suggested a higher sensitivity and positive predictive value for bmGT than GM and a similar specificity and negative predictive value.⁵⁵ Importantly, combining both tests increased the predictive value of the individual biomarkers.⁵⁵ Although promising, additional analysis with larger cohorts of patients and the development of an immunochemical method are needed before this test can be implemented in clinical management.

Clinical validity of available biomarkers

Assessing the clinical utility of a diagnostic test, i.e. how the result will determine a treatment strategy and potentially influence patient management and outcome, has become a key element of AFS programmes.

Based on factors related to the host, underlying disease and condition, and fungal exposure, patients can generally be stratified into three risk groups for IA (high, intermediate and low) and risk-adapted antifungal strategies can be applied accordingly.⁵⁶ A prevalence of $\geq 10\%$ is generally considered as being at high risk, $\leq 5\%$ as being at low risk and intermediate as being between these risk levels. It is important to note that risk assessment is a dynamic process and patients may gradually move to higher or lower risk categories (e.g. patients that initially have refractory, low-risk disease in need of intensive chemotherapy may become high-risk patients).⁵⁶ Adequate risk assessment is an important element for the interpretation of test results. In clinical practice, physicians often do not rely on clinical sensitivity and specificity but rather on the positive and negative predictive values. These values are influenced by the prevalence of disease in a population, which determines the pre-test probability of disease. Hence a diagnostic test for IA with a sensitivity of 71% and a specificity of 89% will have a positive predictive value of only 12% in a population with a pre-test probability of 2% (e.g. a kidney transplant recipient or a patient receiving first-line lymphoma

therapy).⁵⁷ However, the negative predictive value of 99.3% enables the fungal disease to be ruled out with a high degree of confidence. Using the same test in a population with a pre-test probability of 15% increases the positive predictive value to almost 60% (or a 6 out of 10 chance that the patient has IA) while the negative predictive value remains high at 94%. Unlike predictive values, likelihood ratios (LR) are not influenced by prevalence; they show how much more likely the patient is to have IFD after the test results have become available, allowing us to calculate post-test probabilities (using Fagan's nomogram).⁵⁸ For instance, if the prevalence of disease is 15% and the test has a positive LR of 50, then the chances of a patient with a positive result having IA are 90%. Conversely, for a test with a negative LR of 0.1, the chances of a patient with a negative result having IA are only 1.7%. Such a probability increase from 15% to 90% or decrease to 1.7% is clinically meaningful and should be used to guide antifungal management.

The importance of pre-test prevalence is further evidenced by the impact of the use of mould-active antifungal drugs, either as prophylaxis or as treatment, on the performance characteristics of diagnostic tests.^{59,60} Biomarker assays frequently remain negative (or falsely positive) in the presence of drugs that may successfully reduce the pre-test probability to $<5\%$ (e.g. posaconazole prophylaxis during remission-induction therapy of acute myeloid leukaemia).⁶¹

Finally, the results of all these assays should not be interpreted in isolation. An adequate and rapid diagnosis of IFD relies heavily on a few well-defined radiological features on the pulmonary CT scan (nodules with or without a halo, cavities and/or air crescent signs, as defined by the EORTC/MSG consensus criteria).⁶² Unfortunately, these abnormalities are time-dependent, largely restricted to profoundly neutropenic patients, and non-specific for invasive pulmonary mould disease. Moreover, non-specific radiological abnormalities may precede these classical signs, especially in less immunocompromised patients and in those with moderate or transient neutropenia.⁶³ Biomarkers can improve the specificity of these radiological features.

Clinical application of biomarkers

Low-risk patients

Mould-active prophylaxis is not justified for low-risk patients (incidence $\leq 5\%$) as it would result in an exceedingly high number needed to treat to prevent a fungal infection.⁶⁴ Also, screening for biomarkers is unlikely to be clinically useful and certainly not cost-effective.⁶¹ Only testing for biomarkers in patients with a clinical picture suggestive of an invasive mould infection, usually a new lung infiltrate, appears to be appropriate.

High-risk patients on mould-active drugs

As evidenced by a recent Spanish study, the clinical utility of twice-weekly biomarker (GM) screening of blood samples of high-risk patients is severely compromised when mould-active prophylaxis is given.⁶¹ Due to a relatively high number of false-positive GM assays and the low incidence of IFD in asymptomatic patients receiving effective prophylaxis, the positive predictive value was only 11.8%.⁶¹ However, biomarkers may still be useful to confirm a diagnosis in the event of failure of prophylaxis or breakthrough

cases of IA. Indeed, when used to diagnose IA in cases of clinical suspicion, the positive predictive value increased to 89.6%.⁶¹ For these patients, an efficient co-positioning of effective prophylaxis and diagnostic strategies seems feasible. For instance, empirical antifungal therapy could be safely replaced with a diagnostic strategy that employs an early pulmonary CT scan, serum/plasma and BAL GM detection without a need for GM surveillance in asymptomatic patients.⁶⁵ Given its high sensitivity and specificity, PCR detection of fungal DNA might be used as well.

High-risk patients not receiving mould-active drugs

In a high-risk patient population not receiving mould-active prophylaxis (fluconazole is permitted), a biomarker screening strategy using assays with high sensitivity and high negative predictive value can identify patients who do not have fungal infection and do not need antifungal therapy. All currently available non-invasive diagnostic tests (GM, BDG, PCR) can be used for this.⁶⁶ Of course, the rather low prevalence of fungal disease (even in high-risk patients not receiving prophylaxis) and the ubiquitous nature of contaminating fungal pathogens means that false-positive assays will be seen. This will overestimate the need for antifungal therapy, albeit at a much lower rate than when empirical therapy would be initiated. Moreover, this drawback can be largely overcome by more frequent testing (twice or thrice weekly) or by combining different biomarkers.^{42,67,68} Of note, antifungal therapy should not be initiated for patients with a single positive biomarker who have no clinical signs of IFD; however, this should trigger repeat sampling and further intensive diagnostic work-up that includes imaging and, if needed, bronchoscopy with lavage. This approach can be used without excess morbidity or mortality.⁶⁹ Of course, such an approach will inevitably result in more documented cases of probable IFD.

Can biomarkers be used for early response assessment?

Serum GM kinetics have been proposed as a suitable marker for predicting the outcome of patients with IA.^{70,71} In general, GM normalization after the initial 2 weeks of antifungal therapy is more prevalent in responders than in non-responders (although the kinetics may depend upon the antifungal treatment), whereas persistently positive GM is associated with higher mortality.^{72,73} Given the performance characteristics of the assay, the correlation between GM values and patient outcome has predominantly been observed in studies composed of patients with haematological disorders.⁷⁴ However, at present, no data suggest that the duration of antifungal therapy should be adjusted based on the kinetics of biomarkers, including GM. Finally, whether a high baseline serum GM value or persistently positive assays support the use of combination antifungal therapy, as carefully suggested in a recent study, remains to be determined.⁷⁵ The kinetics of BDG have been less vigorously studied, but preliminary data show that prolonged persistence of BDG can occur despite resolution of the fungal infection.

Conclusion

Patients at risk of IA constitute a heterogeneous group and are frequently subjected to preventative strategies. Hence, different

approaches may need to be used in different patient groups to maximize diagnostic accuracy. Understanding test performance in specific patient populations as well as in different clinical specimens, and acknowledging the strengths as well as the limitations of testing strategies, is imperative to maximize clinical benefit in an economically valuable way.

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