Biomarker Applications in Diagnostics of Fungal Infections

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Abstract

Diagnosing invasive mold disease has long been problematic owing to the inability to culture the causal fungal agent from blood or other body fluids. This has fueled an interest in nonculture-based techniques such as the detection of galactomannan in blood and bronchoalveolar fluid, the detection of beta-D-glucan in blood, and the detection of fungal DNA by PCR-based platforms. The past decades have witnessed important improvements in our understanding of the strengths and limitations of the 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.

8.1 Introduction

Invasive fungal infections are usually caused by yeast or mold pathogens. Diagnosis of invasive yeast infections is often based on a positive culture from a sterile body site (e.g., blood culture positive for *Candida* spp.) or on a specific serological test (e.g., cryptococcal antigen test). Diagnosis of invasive mold infections is less

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straightforward; culture is frequently negative, and non-culture-based mycological tests exhibit major shortcomings, although advances have been made over the past two decades. Herein, we will focus on the availability and the use of biomarkers for diagnosing invasive mold disease with a particular emphasis on invasive aspergillosis.

Invasive mold infections, usually affecting the respiratory tract, occur almost exclusively in patients with varied degrees of immunodeficiency and produce a wide range of clinical manifestations. The risk is determined by the nature and the extent of the compromised immunity as well as the prophylactic use of antifungals and the accommodation in protective environments [1]. Patients considered at high-risk include allogeneic stem cell transplant recipients, those being treated for acute myeloid leukemia, myelodysplastic syndromes or aplastic anemia, and subgroups of solid organ transplant recipients [1]. However, over the past decade, several novel risk groups have been identified, including (but not restricted to) patients with chronic obstructive lung disease, liver cirrhosis, autoimmune disorders, and influenza pneumonia. Many of these latter patients do not reside in hemato-oncology or transplantation units but are being hospitalized in intensive care facilities [2, 3]. However, many cases of invasive mold disease 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 any confirmation of fungal infection or disease. Although this approach is considered "standard of care" by many treating physicians, this also results in unnecessary antifungal drug treatment, adverse drug reactions, and increased healthcare expenditure [5]. Diagnostic tools targeting fungal biomarkers (galactomannan, β-D-glucan, fungal DNA) have been developed over the past 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 are being increasingly used to determine a treatment strategy and to influence patient management [6]. However, understanding 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, especially in centers with a large population of immunocompromised patients.

8.2 Available Biomarkers

8.2.1 Conventional Tools

Culture and microscopic examination have always been the cornerstones for making a microbiological diagnosis of IFD. However, culture is time-consuming and requires considerable expertise. In addition, blood cultures are notoriously negative (with the exception of *Fusarium* spp.), 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 overinterpretation of the clinical significance of a positive culture for *Aspergillus* species, Bouza and colleagues developed a helpful score based on easily obtainable clinical and microbiological information, including (a) a sample obtained by invasive procedures (1 point), (b) two or more positive samples from the same patient (1 point), (c) underlying leukemia (2 points), (d) presence of neutropenia (5 points), and (e) corticosteroid treatment (2 points) [10]. Patients with a score of 0 had only a 2.5% probability of invasive aspergillosis. Those with a score of 1 or 2 had an increased probability of 10.3%. The probabilities rose to 40% and 70%, respectively, for patients with a score of 3 or 4 or 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 subpopulation which needs more aggressive diagnostic work-up for the confirmation of disease. Similar scores have not yet been developed for other mold pathogens.

However, 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.

8.2.2 Galactomannan

The fungal cell wall is almost exclusively composed of polysaccharides, including galactomannan (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. demonstrated that the kinetics of GM release and subsequent levels are closely related to the dynamics of angioinvasion, concluding that that GM is only released into the circulation after the fungus has invaded the endothelial compartment [12].

GM can be detected in various body fluids by a commercially available sandwich enzyme-linked immunosorbent assay (ELISA; Platelia *Aspergillus*®, Bio-Rad, Marnes-la-Coquette, France). This test uses EB-A2, a rat monoclonal antibody which specifically binds to four galactofuranosyl residues, both as capture and detecting antibody [13]. In the presence of antigen in a clinical specimen, a monoclonal antibody-antigen-monoclonal antibody complex is formed. A chromogenic substrate is added to reveal the presence of such complexes by turning blue. Microplates are read using an optical reader that calculates the ratio of the optical density relative to a control provided by the manufacturer (the so-called optical density index) [14]. The test is included as a mycological criterion within the EORTC/MSG consensus definitions and has become the mainstay for diagnosing probable invasive aspergillosis [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 between 17% and 100% have been reported

depending on the index cutoff used to determine positivity and on the nature of the population at risk. Indeed, the test performs best in adult and pediatric neutropenic patients (frequently undergoing intensive chemotherapy for acute leukemia) 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 United States Food and Drug Administration (US FDA) has approved a cutoff 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 hematological malignancies or recipients of hematopoietic stem cell transplant) [21]. The European Conference on Infections in Leukemia (ECIL) guidelines recommend a single value of ≥0.7 or multiple (consecutive) values of ≥0.5 for blood specimens [22]. Of note, the 2008 EORTC-MSG revised consensus document has no specified cutoffs for positivity, but refers to the manufacturer's instructions [15]. However, improved sensitivity with the use of lower cutoffs comes with a loss of specificity.

Although fairly specific for *Aspergillus* species, cross-reactivity with non-*Aspergillus* molds (including but not limited to *Fusarium* spp., *Penicillium* spp., *Acremonium* spp., *Alternaria* spp, and *Histoplasma capsulatum*) may occur. In addition, galactofuranosyl residues are also present in other macromolecules, resulting in false-positive test results. Table 8.1 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 [23, 24]. Diagnostic bronchoscopy with lavage is performed when radiographic abnormalities of the lung have been detected, usually by pulmonary CT-scanning. In this setting, the pretest probability of (fungal) disease is much higher than when screening a blood sample from an asymptomatic patient; hence specificity becomes crucial such that a higher threshold of positivity is needed. Cutoff values of 1.0 have been recommended (and approved by the US FDA [21]), although it is likely that even higher thresholds are needed [25]. Recently, an index cutoff of 1.0 has also been suggested for analyzing cerebrospinal fluid (CSF) samples from patients with (suspected) cerebral aspergillosis [26]. Stringent criteria still need to be developed for use with other body fluids (urine, abscesses, pleural fluid, ascites, etc.)

8.2.3 Beta-1,3-D-glucan

Unlike GM, β-D-glucan (BDG) is a polysaccharide component of the cell wall of many pathogenic fungi including *Candida* spp., *Fusarium* spp., and *Pneumocystis*. The main exceptions are *Mucorales* and some *Cryptococcus* species [27]. 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

Table 8.1 Limitations of antigen assays in diagnosing fungal disease

	Galactomannan (GM)	Beta-D-glucan (BDG)
Reactivity with fungal species	*Aspergillus* sp., *Fusarium* sp., *Paecilomyces* sp., *Acremonium* sp., *Penicillium* sp., *Alternaria* sp., *Histoplasma capsulatum*, *Blastomyces dermatitidis*, *Cryptococcus neoformans*, *Emmonsia* sp., *Wangiella dermatitidis*, *Prototheca*, *Myceliophthora*, *Geotrichum capitatum*, *Chaetomium globosum*	*Pneumocystis jiroveci*, *Aspergillus* sp., *Fusarium* sp., *Histoplasma capsulatum*, *Candida* sp., *Acremonium* sp., *Trichosporon* sp., *Sporothrix schenckii*, *Saccharomyces cerevisiae*, *Coccidioides immitis*, *Prototheca*
False-positive test results	Semisynthetic β-lactam antibiotics[a]	Semisynthetic β-lactam antibiotics
	Multiple myeloma	Human blood products, including immunoglobulins, albumin, plasma, coagulation factor infusions, filtered through cellulose membranes
	Blood products collected using Fresenius Kabi bags	Cellulose hemodialysis/hemofiltration membranes
	Gluconate-containing plasma expanders (e.g., Plasmalyte)	Exposure to (surgical) gauze
	Flavored ice pops/frozen dessert containing sodium gluconate	Bacterial bloodstream infections (e.g., *Pseudomonas aeruginosa*)
	Bifidobacterium sp. (gut)	
	Severe mucositis or gastrointestinal graft-versus-host disease	
	Enteral nutritional supplements	
False-negative test results	Concomitant use of mold-active antifungal agents	Concomitant use of antifungal agents
	Mucolytic agents (BAL) such as Sputasol or SLsolution	

[a]Include ampicillin, amoxicillin-clavulanate, and piperacillin-tazobactam (currently this problem seems largely abated compared to previous reports)

and carries the European CE label for the presumptive diagnosis of invasive fungal infection [28]. The remainder are only marketed in Japan. Fungitell detects BDG through a pathway in the *Limulus* amebocytes lysate (LAL), an aqueous extract of blood cells from the horseshoe crab, *Limulus polyphemus*. Bacterial endotoxins and BDG can activate different coagulation cascades in the LAL; bacterial endotoxins specifically activate factor B and C, whereas BDG activates factor G. The Fungitell assay uses a modified pathway in the LAL by removing factor C. Thus, in the absence of factor C, the coagulation cascade is activated only in the presence of BDG [14]. Also this test has been included in the EORTC/MSG definitions of invasive fungal disease [15]. Of note, cutoff values for determining positivity differ markedly between these assays [14].

Any systematic review of this 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 [29–32]. Most studies report good sensitivity, but specificity and positive predictive value for diagnosing mold infections is poor due to a high rate of false-positive results (Table 8.1), regardless of the specimen [33]. However, the negative predictive value is around 80–90%. Unfortunately, the BDG assay is not pathogen specific and therefore cannot differentiate fungal species. In addition, pretest preparations may limit its routine applicability.

8.2.4 Polymerase Chain Reaction

Polymerase chain reaction (PCR)-based methods have been developed for the diagnosis of fungal diseases. The main advantage is the very high sensitivity for the 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, lack of standardization due to the use of in-house assays using varied protocols involving different specimens, extraction techniques, molecular targets, amplification platforms, and detection techniques 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), established to remedy this situation for diagnosing invasive aspergillosis, has made tremendous progress in standardizing protocols for efficient DNA extraction and amplification [34–40]. Clinical validation in multicentre prospective studies is ongoing.

Commercially available as well as in-house platforms using genus-/species-specific genes and panfungal targets have been developed, and the usefulness of PCR for diagnosing invasive fungal disease has been recently reviewed [41, 42]. Superior performance compared to the serological biomarkers has been suggested and high negative predictive values have been consistently documented, despite all methodological variabilities [43–45]. 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 time point.

8.2.5 Lateral-Flow Device for Invasive Aspergillosis

A lateral-flow device (LFD) was developed for a point-of-care diagnosis of invasive aspergillosis. This assay uses a monoclonal antibody that is highly specific to growing *Aspergillus* species (but different from the one used in the Platelia assay) [46, 47]. Compared to GM and BDG assays, the LFD test is quick (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 reaction in the GM and BDG tests have not (yet) been seen. A recent meta-analysis of seven studies (with mainly solid organ transplant recipients) yielded a pooled sensitivity, specificity, and 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% when using serum samples (in which case a heating step is required) [48]. More data on the impact of antifungal prophylaxis or therapy on the performance are needed [49]. Of note, similar lateral-flow devices have proven to be very successful for the diagnosis of cryptococcal disease and are currently being developed for diagnosing non-*Aspergillus* mold infections (including *Fusarium* and *Scedosporium* species).

8.2.6 Biomarkers in Development

Despite significant recent advances, the available tools for diagnosing invasive fungal disease are far from perfect and clinicians still struggle to make a timely diagnosis. Therefore, the search for novel targets and platforms that may further improve our diagnostic capabilities continues. An electronic nose (eNose) can discriminate various lung diseases through an analysis of exhaled volatile organic compounds. An eNose is cheap and noninvasive and yields results within minutes. A proof-of-principle study showing that neutropenic patients with aspergillosis have a distinct exhaled breath profile (or "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% [50].

Using gas chromatography and mass spectrometry, US researchers were able to measure fungal volatile metabolites in breath samples of patients with invasive aspergillosis [51]. Detection of α-trans-bergamotene, β-trans-bergamotene, a β-vatirenene-like sesquiterpene, or trans-geranylacetone identified these patients with 94% sensitivity and 93% specificity. Although both techniques perform well for diagnosing invasive aspergillosis, more extensive validation is needed.

In recent years, gliotoxin (GT), a virulence factor during hyphal growth, has been proposed as a diagnostic biomarker of invasive aspergillosis. *Aspergillus fumigatus* is the most important GT-producing fungal pathogen, although also non-*fumigatus Aspergillus* species can produce GT, as well as less common opportunistic pathogens such as *Penicillium* spp., *Gliocladium* spp., and *Pseudallescheria* spp. [52]. 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 [52]. Preliminary work demonstrated that bmGT is produced by a higher percentage of isolates of *A. fumigatus* than GT. A recent prospective study comparing the diagnostic accuracy of bmGT detection (by high-performance thin layer chromatography) with GM detection (Platelia assay) in 79 patients at risk for invasive aspergillosis suggests a higher sensitivity and positive predictive value for bmGT than GM and similar specificity and negative predictive value [53]. Importantly, combining both tests increased the predictive value of the individual biomarkers. Although promising, additional analysis with larger cohorts

of patients, as well as the development of an immunochemical method, are needed before this test can be implemented in clinical management.

8.3 Clinical Validity of Available Biomarkers

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

Based on factors related to host, underlying disease and condition, and fungal exposure, patients can generally be stratified into three risk groups for IFD (high, intermediate, and low), and risk-adapted antifungal strategies can be applied accordingly [54]. One generally considers a prevalence of ≥10% as being at high risk and ≤5% as being at low risk with intermediate lying in between. Importantly, risk assessment is a dynamic process and patients may gradually move to higher- or lower-risk categories (e.g., patients with refractory initially low-risk disease in need of intensive chemotherapy may become high-risk patients) [54]. Adequate risk assessment is an important element for the interpretation of test results. In clinical practice, physicians don't usually rely on the clinical sensitivity and specificity but rather on the positive and negative predictive values. These latter are influenced by the prevalence of disease in a population which determines the pretest probability of disease. Hence a diagnostic test for IFD with a sensitivity of 71% and a specificity of 89% will have a positive predictive value of only 12% in a population with a pretest probability of 2% (e.g., a kidney transplant recipient or a patient with first-line lymphoma therapy) [55]. 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 pretest probability of 15% increases the positive predictive value to almost 60% (or a six out of ten chance that the patient has IFD), while the negative predictive value remains high at 94%. Unlike predictive values, likelihood ratios (LR) are not influenced by prevalence; they inform us on how more likely the patient is to have IFD after the test results have become available, allowing us to calculate posttest probabilities (using Fagan's nomogram). 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 IFD are 90%. Conversely, for a test with a negative LR of 0.1, the chances of a patient with a negative result having IFD 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 pretest prevalence is further evidenced by the impact of the use of mold-active antifungal drugs, either as prophylaxis or as treatment, on the performance characteristics of diagnostic tests [56, 57]. Biomarker assays remain frequently negative (or falsely positive) in the presence of drugs that reduce the pretest probability of IFD to less than 5% (e.g., posaconazole prophylaxis during remission-induction therapy of acute myeloid leukemia) [58].

Finally, the results of all these assays should not be interpreted in isolation. Nowadays an adequate and rapid diagnosis of IFD relies heavily on a few

well-defined radiological features on pulmonary CT scan (nodules with or without a halo, cavities, and/or air crescent signs, as defined by the EORTC/MSG consensus criteria) [59]. Unfortunately these abnormalities are time-dependent, largely restricted to profoundly neutropenic patients, and nonspecific for invasive pulmonary mold disease. Moreover, nonspecific radiological abnormalities may precede these classical signs, especially in less immunocompromised patients and in those with moderate or transient neutropenia [60]. Biomarkers have the capacity to improve the specificity of these radiological features.

8.4 Clinical Application of Biomarkers

8.4.1 Low-Risk Patients

Mold-active prophylaxis is not justified for low-risk patients (incidence $\leq 5\%$) as the number need to harm will exceed the number need to treat to prevent a fungal infection [61]. Also screening for biomarkers is unlikely to be clinically useful and certainly not cost-effective [58]. Only testing for biomarkers in patients with a clinical picture suggestive of an invasive mold infection, usually a new lung infiltrate, appears to be appropriate.

8.4.2 High-Risk Patient on Mold-Active Drugs

As evidenced by a recent Spanish study, the clinical utility of twice weekly biomarker (GM) screening on blood samples of high-risk patients is severely compromised when mold-active prophylaxis is given or empirical therapy has been started [58]. Because of a relative high number of false-positive GM assays and the low incidence of IFD in effectively prophylaxed asymptomatic patients, the positive predictive value was only 11.8%. However, biomarkers may still be useful to confirm a diagnosis in the event of failure of prophylaxis or breakthrough cases of invasive aspergillosis. Indeed, when used to diagnose invasive aspergillosis in case of clinical suspicion, the positive predictive value increased to 89.6%. For these patients, an efficient co-positioning of effective prophylaxis and diagnostic strategies seems feasible. For instance, empirical antifungal therapy could be replaced with a diagnostic strategy that employs early pulmonary CT scan and serum/plasma and BAL GM detection [62]. Given its high sensitivity and specificity, PCR detection of fungal DNA might be used as well.

8.4.3 High-Risk Patient Not Receiving Mold-Active Drugs

In a high-risk population not receiving mold-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 noninvasive diagnostic tests (GM, BDG, PCR) can be used for this [63]. 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 mean 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 [41, 64, 65]. Of note, antifungal therapy should not be initiated for patients with a single positive biomarker who have no clinical signs of invasive fungal disease; 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 [66]. Of course, such an approach will inevitably result in more documented cases of probable invasive fungal disease.

8.5 Can Biomarkers Be Used for Early Response Assessment?

Serum GM kinetics has been proposed as a good marker for predicting the outcome of patients with invasive aspergillosis, due to the excellent correlation observed in recent studies [67, 68]. In general, GM normalization after the initial 2 weeks of antifungal therapy is more prevalent in responders than in nonresponders (although the kinetics may depend upon the antifungal treatment), whereas persistently positive GM is associated with higher mortality [69, 70]. Given the performance characteristics of the assay, the correlation between GM values and patient outcome has predominantly been observed in studies composed of hematological patients only [71]. However, at present, no data suggest that the duration of antifungal therapy should be adjusted to the kinetics of biomarkers, including GM. Finally, whether a high baseline serum GM value or persistently positive assays supports the use of combination antifungal therapy, as carefully suggested in a recent study, remains to be determined [72]. The kinetics of BDG have been less vigorously studied, but preliminary data shows that prolonged persistence of BDG can occur despite resolution of the fungal infection.

8.6 Conclusion

Patients at risk for IFD constitute a heterogenous 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 useful way.

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