The Role of Biomarkers for Diagnosis of and Therapeutic Decisions Related to Invasive Aspergillosis in Children

Brian T. Fisher[1,2]

[1]Division of Infectious Diseases, The Children's Hospital of Philadelphia, Philadelphia, PA

[2]Center for Pediatric Clinical Effectiveness, The Children's Hospital of Philadelphia, Philadelphia, PA

Abstract

Invasive aspergillosis (IA) is a feared opportunistic infection for many immuncompromised children resulting in significant morbidity and mortality. Timely diagnosis based on traditional testing modalities and clinical evaluation has proven to be challenging. Assays designed to measure IA biomarkers such as galactomannan (GM) and beta-D-glucan (BG) represent a potential significant improvement in the ability to diagnose pediatric IA. However, as with any new diagnostic tool, clinicians need to be aware of the operating characteristics of these assays in order to apply them in the appropriate settings. Published adult and pediatric data regarding GM and BG testing are reviewed and guidance regarding the appropriate utility of these assays in children is offered.

Keywords

Galactomannan; beta glucan; PCR; aspergillosis; pediatrics

Introduction

The list of pediatric conditions resulting in immunocompromised states continues to grow. These immunocompromised states include both primary immune deficiencies such as chronic granulomatous disease as well as acquired immune deficiency secondary to HIV. Additionally, there are those immune deficient states resulting from chemotherapeutic exposures for various malignancies and from life saving measures such as solid organ and hematopoietic stem cell transplantation (HCT). Finally, there is an ever-expanding population of patients receiving immune modulating therapies for autoimmune conditions.

Although the type and degree of immune dysfunction varies across each of these patient groups, for many of these immunocompromised children invasive aspergillosis (IA) is a feared opportunistic infection resulting in significant increases in healthcare expenditures and ending in mortality for more than half of those infected [1,2]. The reason for the high mortality rate is multifactorial including delays in diagnosis, limited therapeutic options and the difficulty of resolving these infections in children with compromised immune systems. Although limited, previous data suggest that outcomes for IA can be improved with initiation of therapeutic interventions at the earliest possible time point [3,4].

Corresponding Author: Brian T. Fisher, DO, MSCE, Division of Infectious Diseases, The Children's Hospital of Philadelphia, 34th Street and Civic Center Boulevard, CHOP North, Room 1515, Philadelphia, PA 19104, fisherbria@email.chop.edu, Telephone: (267) 426-7110, Fax: (215) 590-5705.

However, making a timely diagnosis of IA can be challenging. In 2002, a cooperative group of the European Organization for Research and Treatment of Cancer (EORTC) along with the Mycoses Study Group (MSG) published consensus recommendations for defining an invasive fungal infection (IFI) inclusive of IA [5]. Their definitions for proven or probable IA included various host factors, specific radiographic information (e.g., halo sign or air-crescent sign), clinical findings, and microbiologic data. The latter focused mostly on culture or histopathology data from biopsy specimens obtained via invasive procedures. Although presence of *Aspergillus* antigen in broncoalveolar lavage or blood specimens was included as supportive microbiologic evidence for an IA diagnosis, the cooperative group recognized that the availability of these antigen assays and clinical data supporting their use were sparse at the time.

In the ensuing years a number of large-scale adult prospective clinical observational studies were completed resulting in the FDA approval of two indirect assays, the galactomannan (GM) enzyme immunoassay (EIA) and $(1\rightarrow3)$ Beta-D-Glucan assay (BG), for diagnosing IFI and specifically of IA [6–11]. Not only did the data from these adult trials support the diagnostic capabilities of these assays, they also revealed the potential to shorten the window of time between onset and detection of IA [8,9]. Based on these and other studies, the revised EORTC/MSG definitions for all IFI and specifically IA incorporated GM and BG positive results as mycological criteria supportive of a probable diagnosis [12].

While the benefits of these indirect diagnostic tools are multiple (e.g., less invasive testing, earlier time to diagnosis) clinicians caring for immunocompromised pediatric patients at risk for IA must be careful in extrapolating the adult data as the kinetics of these tests can vary by age and patient population. The pediatric specific data for each of these assays will be reviewed and recommendations on appropriate testing will be offered.

Galactomannan Testing

History of Galactomannan

In 1978 Lehmann and Reiss created a rabbit model of IA with a goal of devising a test capable of detecting a circulating antigen from *Aspergillus* [13]. They were successful at raising an anti-serum from these rabbits that was specific to a certain carbohydrate antigenic moiety. In subsequent studies they identified this antigen as GM [14]. Their efforts established a foundation for development of various assays to detect GM antigenemia. Galactomannan is a polysaccharide composed of a mannose backbone with galactose side groups, which compromises a major component of the cell wall of *Aspergillus* spp. During an IA infection, GM is released resulting in the potential for detection of this antigen in the serum or other body fluids of an infected patient. In the decades after the isolation of GM antigen, a number of techniques were explored to accurately detect BM from human specimens [15,16]. A sandwich ELISA assay using the monoclonal antibody, EB-A2, emerged as the leading technique as it was able to detect lower amounts of circulating GM [17,18]. The commercially manufactured Platelia™ *Aspergillus* EIA kit from Bio-Rad Laboratories has made this sandwich ELISA assay technique for detecting GM available for routine clinical use.

Adult data on Galactomannan testing

It has been over two decades since the first large prospective trial evaluated the use of serial serum GM testing to detect the onset of IA in adult patients with prolonged neutropenia using the EORTC/MSG IFI definitions as the gold standard [9]. Considering a GM index of 1.0 or greater as positive, this testing strategy resulted in 72.9 % sensitivity, 94.7 % specificity, 61.3 % PPV, and 96.8 % NPV for detection of IA. Results were improved when data from autopsy findings were included. Detection of GM preceded the time of definitive

IA diagnosis in 96 % of the proven IA cases by a median of 17 days (range of 2–110 days). Since this sentinel publication a number of similar studies have evaluated GM surveillance testing in various adult immunocompromised cohorts. These studies have been summarized in two separate meta-analyses each of which highlight the variable performance of GM testing across different patient types [10,11]. The meta-analysis by Pfeiffer et al., suggested that the GM assay was likely most useful in a population of hematologic malignancy patients or HCT recipients [10]. Importantly, the meta-analysis from the Cochrane Collaboration suggested that the sensitivity of the assay improved from 64 % to 78 % when the threshold for positivity was decreased from a GM index of 1.5 to 0.5. This decrease in the threshold to optimize sensitivity did compromise specificity from 95 % to 81 % [11]. Based on these data the manufacturer recommendation for a positive result is a galactomannan index of ≥0.5.

Pediatric data on Galactomannan testing

As is typical with many diagnostic tests, extrapolating results from primarily adult studies to pediatric patients can be misleading. Fortunately, there have been a number of prospective observational studies that inform the use of serum GM testing in children (Table 1) [19–24]. Taken collectively the operating characteristics are reassuring for the use of GM testing in children, however, as with any diagnostic test there needs to be caution when applying study results to specific clinical scenarios. Notably two studies [24,25] reported a 0 % sensitivity mainly because only one proven or probable case of IA was identified in these cohorts. Although each of these cohorts included patients considered at high risk for IFI, ultimately few patients developed IA limiting the potential number of true positive patients. This reinforces the fact that GM testing should not be applied to scenarios where there is a low likelihood of IA.

Galactomannan testing approach

Although the operating characteristics of GM in high-risk populations seem reasonable, a number of questions still remain as to what time is ideal to initiate testing. Recently, the first published pediatric specific guideline for fever and neutropenia supported the implementation of twice-weekly GM testing in children with fever and neutropenia at high-risk for IFI [26]. This approach is reasonable as arguably the pre-test probability of IA in a febrile neutropenic patient is higher than that of a patient with just neutropenia. It is important to note that the recommendation for GM testing was graded as weak based on moderate-quality evidence.

Certainly, additional data in various patient settings are necessary to refine the optimal testing window. For instance, receipt of prophylactic antifungal therapy has been shown to reduce the sensitivity of GM testing in predominantly adult cohorts [6,27]. The reduction in sensitivity while on antifungals is less pronounced when the threshold GM index of 0.5 is applied. Additionally, the vast majority of pediatric data are derived from populations of children with neutropenia secondary to chemotherapy for malignancy or conditioning for HCT.

Galactomannan testing in non-neutropenic populations

As noted previously, there are a number of additional populations at risk for IA. However, data are limited on the utility of GM testing in immuncompromised patients that are not neutropenic. Multiple case reports have documented repeatedly negative GM assays in patients with chronic granulomatous disease and proven IA suggesting limited utility in this patient group [28–30]. Furthermore, data from solid organ transplant recipients suggest variable effectiveness of serum GM testing in this population during active IA [31–35].

Galactomannan testing on non-serum specimens

There have been a number of published reports evaluating the diagnostic utility of detecting GM in other body fluids. An adult report including 99 patients with hematologic disorders suggested that GM testing of specimens obtained at the time of a bronchoalveolar lavage fluid (BAL) was diagnostically useful [36]. A similar pediatric study retrospectively reported on GM testing of BAL specimens from children with immunocompetent and compromised conditions. The latter included primary immune deficiencies, various malignancies and HCT and solid organ transplant recipients. Among these immuncompromised patients, GM testing of BAL specimens seemed to be useful with sensitivity, specificity, positive and negative predictive values of 78 %, 100 %, 58 %, and 96 %, respectively.[37] Serum GM testing has been found to be less useful for solid organ transplant recipients. Some reports have noted improvement in the diagnostic capabilities of the assay when applied to BAL specimens in this patient population 38–40]. These data should be interpreted with some caution as these studies were dominated by adult patients. Additionally, there is a real risk for false positive results in this setting especially among lung transplant patients where *Aspergillus* pulmonary colonization is known to exist.

The evidence to support GM testing on cerebral spinal fluid (CSF) is limited to case reports and case series that included patients where the suspicion of an IA infection was high [41,42]. At this time, GM testing on BAL and CSF specimens should be limited to patients where suspicion for an active IA infection is high and routine testing on all obtained BAL and CSF should be discouraged as false positive results can be misleading and result in unnecessary additional investigations. Finally, Fisher et al., recently reported on the simultaneous surveillance of serum and urine GM levels in children with prolonged neutropenia [20]. The results were promising and suggested that urine may be a reasonable specimen for early detection of GM. However, the increased number of false positive results dictates refinement of the assay with urine specific thresholds for positivity before it can be endorsed in this manner.

Galactomannan levels to monitor response to therapy

Recent publications have investigated the predictive value of baseline GM levels for IA outcomes and also evaluated the utility of following serum GM testing to monitor effectiveness of antifungal therapy in patients with IA [43–45]. Bergeron et al., showed that baseline elevation of the GM index was associated with mortality at 60 days (Hazard ratio of 1.25 (95 % CI: 1.1. to 1.43) per one unit increase in the GM index) and that follow-up GM levels after initiating therapy were accurately correlated with both improvement and lack of improvement of IA [44]. Nouer et al., compared two definitions for successful antifungal therapy in 115 adults with IA. The first definition was based on GM testing results and the second definition relied on EORTC criteria for establishing improvement [43]. They found a 91 % agreement for successful response to antifungal therapy for the two definitions. Finally, Chai et al., focused on the kinetics of GM assay results in the first two weeks after initiation of IA treatment in 202 patients [45]. A GM index reduction of >35 % predicted a good response to therapy by week 12, likewise an increase in the GM index from negative to positive within 2 weeks of starting antifungal therapy predicted a poor response. The majority of patients in each of these trials were adults and thus the generalizability of these results to children is not clear. However, it is reasonable to consider follow-up GM assay results as an adjunctive measure to monitoring the effectiveness of IA therapy in children.

Beta-D-Glucan Testing

History of Beta-D-Glucan

Beta glucans are a group of heterogeneous glucose polysaccharides linked by beta glycosidic bonds. Contrary to GM, BG is found in the cell wall of various pathogenic fungi including but not limited to species of *Aspergillus, Candida, Fusarium*, and *Scedosporium*. It should be noted that BG is also found in *Pneumocystis jiroveci* but notably absent from *Cryptococci* and organisms of the Mucorales family (such as *Absidia, Mucor* and *Rhizopus*) [46].

The detection of BG is based on the use of factor G prepared from the amebocytes of either *Limulus polyphemus* (American horseshoe crab) or *Tachypleus tridentatus* (Japanese horseshoe crab) [46]. The discovery of this horseshoe crab lysate extract dates back to 1968, when Levin and Bang first reported on the ability to detect bacterial endotoxin using horseshoe crabs amebocytes. Their original assay required the activation of the ambocytes' factor C by bacterial endotoxin resulting in activation of a clotting cascade [47]. It was eleven years later that Lehmann and Reiss isolated both GM and BG from the serum of rabbits infected with *Aspergillus fumigatus* [13]. The BG was found to be non-antigenic in these early animal studies and thus not initially pursued as a biomarker. A short time later Morita et al., discovered that BG interacted with factor G that triggered the coagulation cascade of the horseshoe crab amebocytes but via an alternate pathway to that of bacterial endotoxin [48]. Obayashi et al., subsequently created lysate from the amebocytes that retained factor G but was lacking in factor C establishing an assay for the detection of BG that did not also respond to bacterial endotoxin [49].

There are currently two commercially made BG assay kits, the Fungitec G Test MK® manufactured by the Japanese company Seikagaku Biobusiness Corporation and the Fungitell® Assay (formerly Glucatell) from the Associates of Cape Cod, Inc. Each kit relies on the use of amebocyte lysate from either Japanese or American horseshoe crabs. The Fungitell® Assay has received FDA approval and thus will be the focus of the remainder of this section. It should be noted that the two kits have been shown to be comparable in the identification of BG and do not cross react with other non-(1→3) beta- D-glucans [7].

Adult data on Beta-D-Glucan testing

The operating characteristics of the BG assay for identifying all IFI were initially elucidated in two adult cohorts. The first included twice weekly testing during the neutropenic periods of 283 adult patients with newly diagnosed AML or myelodysplastic syndrome undergoing initial induction chemotherapy and receiving fungal prophylaxis with either caspofungin or itraconazole [7]. The resultant sensitivity, specificity, PPV and NPV for diagnosing proven or probable IFI of 100 %, 90 %, 43 %, and 100 %, respectively. Only four of the IFI events were specifically IA. For all four IA cases the BG result was positive and for 3 of the 4 it became positive before IA was clinically apparent.

The second study evaluated 95 adult patients with fever and neutropenia down to 16 years of age [8]. Thirty-eight patients with proven, probable or possible IFI were identified, 24 of which were thought to have possible or probable IA. The resultant sensitivity, specificity, PPV and NPV were 97.4 %, 28.6 %, 55.2 %, and 92.3 %. Likely the specificity reported was reduced because of inclusion of patients with a "possible" IFI designation as this designation is much less definitive. Operating characteristics specific to IA could not be elucidated given the data published.

Although the Fungitell® Assay can detect BG levels down to 1 pg/mL the manufacturer recommended cutoff for a positive value is 80 pg/mL with an indeterminate range of 60–

79 pg/mL and a negative result being <60 pg/mL. The indeterminate range was created to account for normal human serum levels of BG ranging from 10–40 pg/mL. These baseline human levels are thought to result from the presence of yeast in the normal flora of the alimentary and gastrointestinal tract.

Pediatric data on Beta-D-Glucan testing

A study evaluating this BG assay in children without risk for IFI was performed to establish pediatric baseline BG levels. The results suggested that children can have substantially elevated levels of BG in the absence of IFI resulting in significant concern in how to interpret BG assay results in children [50]. To date, there are limited data regarding BG testing in at-risk pediatric immunocompromised patients [51,52] making it impossible to make any recommendations for employing BG testing for detection of IA in children. Fortunately, large pediatric prospective studies in both the United States and Europe are currently ongoing. The results of these studies in the coming years should help to inform the utility or lack thereof for BG testing in children.

Sources of false positivity

Clinicians utilizing either the GM or BG assays should be aware of factors that have been sighted as possible sources for false positive results. Table 2 denotes a number of medications, medical interventions, and other organisms that have been suggested as possible causes of false positive results for BG and GM testing [46,53,54]. Concomitant piperacillin-tazobactam exposure has often been cited as a possible cause of a false positive GM result.[24] This is concerning institutions where this medication is used in the setting of fever and neutropenia. However, recent data has suggested that false positive results secondary to piperacillin-tazobactam are not specific to the antibiotic but rather the manufacturer of the antibiotic [55,56]. Similarly, recent studies have exonerated other beta lactam antibiotics such as ampicillin-sulbactam as a cause of false positive GM or BG testing [57]. *Pneumocystis jiroveci* is also listed as a cause of a "false positive" BG test. Technically this is not a true false positive as the BG assay is detecting beta glucan from the cell wall of *Pneumocystis jiroveci* as it would for other fungal infections. The BG assay has been successfully used to identify *Pneumocystis jiroveci* in various immunocompromised populations [58]. However, in this instance the implications for therapeutic management would of course be different.

Galactomannan and Beta-D-Glucan Assays as adjunctive tools

Most of the studies evaluating the effectiveness of GM and BG testing for detecting IA have done so in isolation during a specific clinical scenario such as neutropenia or neutropenia with fever. Alternatively, the results of these assays can be used as adjuncts to clinical and radiographic results to guide clinicians not just in the diagnosis of IA but also in the appropriate time to initiate antifungal therapy. Cordonnier et al., took this approach and compared the use of GM testing in conjunction with other clinical and radiographic findings to guide pre-emptive initiation of antifungal therapy versus empiric antifungal therapy in adults with persistent fever and neutropenia despite broad-spectrum antibiotics [59]. Although IFI was ultimately diagnosed more frequently in the pre-emptive arm, there was no difference in IFI-related mortality and there was a noted reduction in antifungal use. Based on these and other similar studies, the 2010 IDSA update for IA endorsed this pre-emptive approach that leverages non-invasive biomarkers in conjunction with clinical and radiographic data as an alternative option to routine empiric antifungal therapy for prolonged fever and neutropenia [60]. Subsequent to the release of the IDSA guidelines Pagano et al., published a prospective adult observational trial that revealed an increased rate of IFI and attributable mortality in patients managed in a pre-emptive approach resulting in

questions regarding the safety of pre-emptive therapy [61]. Thus far no such study has been performed in a pediatric specific cohort. Pediatric studies that combine data from these biomarkers with data from clinical, radiographic and other traditional laboratory results to guide both the diagnosis of IA and guidance on initiating antifungal therapy are urgently needed. Until such studies are completed, it is difficult to endorse such approaches in children.

Conclusion

For many decades researchers and clinicians have yearned for diagnostic tools that would improve upon the abilities of traditional diagnostic testing to identify IA. The primary goal of improved diagnostic modalities is to identify the presence of IA at its earliest point of onset so that important therapeutic measures can be enacted and thus better outcomes achieved. Galactomannan, and BG assays are two non-invasive biomarkers that are bringing us closer to this goal. However, in isolation none of these laboratory tools are perfect for detecting IA. Based on the reviewed data it is reasonable to employ serum GM testing in at risk pediatric patients. Although the data are less convincing, GM testing in BAL or CSF specimens is reasonable in patients with a high suspicion for IA. Comparatively, the data for BG testing in pediatric patients are much less and the lack of information on an appropriate threshold for this assay in children should limit its application in pediatric patients until further evidence is available.

To date, both GM and BG assays have received the most attention in the medical literature as biomarkers for the diagnoses of IA. The time from initial discovery of these two biomarkers to their application in clinical settings required multiple decades of investigation. Fortunately, other testing modalities for early detection of IA are in development or are on the horizon. *Aspergillus* PCR testing of the blood has received significant attention in recent years [62]. Most recently, Badiee et al., reported reassuring operating characteristics of a commercially available nested-PCR for *Aspergillus* in 62 pediatric patients with hematologic disorders [63]. Although the results of PCR testing were favorable, it should be noted that the incidence rate of IA was extremely high in this cohort (27.4 %) and thus the results may not be generalizable. Before routine use of an *Aspergillus* PCR can be supported in children, there needs to be further investigations to better define the operating characteristics in at risk populations, to establish optimal testing strategies, and to standardize testing processes. In addition to PCR testing, various novel proteomic techniques have been employed to more efficiently identify a number of candidate proteins that may not only serve as new biomarkers to aide in the diagnosis of IA but that also have the potential to correlate with *Aspergillus* pathogenicity and stage of infection [64,65].

Pediatric clinicians electing to employ these diagnostic modalities need to do so judiciously in clinical scenarios where the pre-test probability for detecting IA is relatively high. Indiscriminate use of these tests will result in unnecessary medical expenditures and the potential to misinterpret their results. Researchers need to continue to establish the operating characteristics of each of these tests across various patient cohorts and under various clinical conditions. As noted previously fungal biomarkers present the possibility of identifying the presence of IA many days before clinical symptoms are apparent. While it is reasonable to theorize that an earlier diagnosis could translate into improved outcomes, no study to date has shown that biomarker testing has the ability to reduce the morbidity and mortality of IA. Therefore, additional efforts need to focus not just on determining the operating characteristics of novel fungal biomarkers but also to define their ability to reduce the morbidity and mortality of IA. Ultimately, it is likely that integrating these and similar future diagnostic tests with other clinical and traditional diagnostic data will result in the

greatest benefit for identifying IA at its earliest point of onset so that the morbidity and mortality of this feared infection can be reduced.

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Table 1

*Prospective pediatric observational trials evaluating the operating characteristics of Galactomannan testing for diagnosis of proven or probable aspergillosis

Author Year	Total # patients/episodes in study	Testing Indication	IA Definition[a]	Proven or probable aspergillosis	Sensitivity	Specificity	TP	FP	TN	FN	False positive rate [b]
Rohrlich 1996	37	Neutropenia	Guiot 1994 Criteria	10	100%	92.6%	10	2	25	0	7.4%
Sulaihan 2001	347	Neutropenia	Locally defined	9	100%	89.9%	9	34	304	0	10.1%
Steinbach 2007	64	Neutropenia/acute GVHD	EORTC/MSG 2002	1	0%	87%	0	8	55	1	12.7% [d]
Hovi 2007	117	Neutropenia	EORTC/MSG 2002	1	100%	93%	1	11	105	0	9.5%
Armenian 2009	78	Neutropenia +/− fever/GVHD	EORTC/MSG 2008	3 [e]	100%	98.7%	3⁴	1	74	0	1.3%
Fisher 2012	195	Neutropenia	EORTC/MSG 2002	1	0%	95%	0	10	184	1	5.2%

*
Table adapted with permission from Fisher et al. J Ped Inf Dis Soc 2012;1(2):103-11

[a]
The definition of proven or probable aspergillosis varied depending on the publication

[b]
Defined as the number of false positive results per total number of patients without IA

[c]
Case-control study design may inflate the calculated false positive rate for this study

[d]
After accounting for piperacillin-tazobactam exposure, the false positive rate was 8.5%.

[e]
All 3 probable cases required inclusion of positive GM EIA results to qualify for this designation

Table 2

Potential factors resulting in false positive testing for Beta-D-Glucan and Galactomannan Assays

	(1→3) Beta-D-Glucan Assay	Galactomannan EIA Assays
Medications	Intrvenous amoxicillin-clavulanate or ampicillin-sulbactam	*Piperacillin-Tazobactam *other beta lactam antibiotics
Infusions	*Intravenous immune globulin *Cellulose filters for IV infusion *Albumin	*Plasmalyte (electrolyte infusion) *Intravenous solution with sodium gluconate
Medical interventions	*Hemodialysis with cellulose filter *GuazeGauze packing on serosal surfaces	*Enteral feeding with soybean proteins
Other infections	*Pneumocystis jiroveci*	*_Penicillium_ spp. *_Histoplasma capsulatum_ *_Geotrichum_ *_Neosartoria_ *_Bifidobacterium_