

Prospective Monitoring for Invasive Aspergillosis Using Galactomannan and Polymerase Chain Reaction in High Risk Pediatric Patients

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Background: The diagnosis of invasive aspergillus remains a challenge in the care of high-risk patients. Outcomes are improved when invasive aspergillus is diagnosed early, prompting the initiation of appropriate antifungal therapy. We evaluated the utility of prospective monitoring for invasive aspergillosis (IA) using biomarkers such as serum galactomannan (GM) and/or blood polymerase chain reaction (PCR) in high-risk pediatric patients.

Methods: Patients with high-risk leukemia (HRL) or allogeneic hematopoietic cell transplant (HCT) recipients were prospectively monitored twice weekly for IA using GM and PCR for *Aspergillus species*.

Results: Sixty-eight patients had collected ≥ 2 specimens. The 1086 specimens were collected; 627 from HRL (58%) and 459 (42%) from HCT recipients. Median specimens/patient was 11.0 (2 to 58), and median follow-up/patient was 98.5 days (14 to 437). Fifty-six percent of samples were obtained from patients receiving mold-active agents; 32% HRL and 89% HCT. There were no proven, 3 probable, and 20 possible episodes of IA. Thirteen specimens (1.2%) from 4 patients (5%) were GM+. None were positive by PCR.

Conclusions: The prospective use of GM and PCR in this high-risk pediatric population did not identify cases of proven IA. A high false positive rate was not detected. It is speculated that changes in clinical practice, such as early use of empiric and/or prophylactic mold-active agent and frequent imaging studies have impacted the epidemiology of IA. In a population with low incidence of IA, the use of these assays as a screening device on blood may not further enhance current outcomes.

Key Words: aspergillus, pediatrics, galactomannan, polymerase chain reaction, prospective screening

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Invasive aspergillosis (IA) infections continue to have a high morbidity and mortality in immunocompromised populations.^{1–4} Outcomes are improved when IA is diagnosed early, prompting the initiation of appropriate antifungal therapy.^{5–8} However, early diagnosis is hampered by nonspecific findings, rarity of positive blood cultures, and concerns about potential morbidity from invasive procedures, which are often necessary to establish tissue diagnosis.^{9,10} Surrogate biomarkers of IA, such as the galactomannan (GM) assay, and polymerase chain reaction (PCR) for conserved regions of the aspergillus genome, such as *Aspergillus species* specific 28S rRNA sequence, have become attractive candidates for evaluation.

Prospective monitoring for IA using GM and in adults has shown: (a) earlier diagnosis may be associated with improved outcome¹¹; (b) the practice can lead to more judicious use of prophylactic and empiric antifungal therapy¹²; (c) it may decrease the need for invasive diagnostic procedures.¹³ The sensitivity and specificity for the GM assay cited by these studies is encouraging; the best results are encountered in autopsy-controlled prospective evaluations of high-risk patient populations.^{14,15}

The use of PCR for the early diagnosis of IA shows promise, with reported specificity ranging from 85% to 100% and sensitivity of 29% to 100%.^{16–18} Differences in the efficacy of DNA extraction, primers, type of PCR reaction, and specimen (whole blood, plasma) have been shown to affect the sensitivity of the assay.^{19–21} Assays remain nonstandardized and there is no consensus on the role of PCR in prospective monitoring for IA.

Recent studies in pediatrics suggest that the incidence of IA is lower than in adults.^{22–24} Whether this is due to more aggressive empiric and/or prophylactic broad-spectrum antifungal therapy, increased use of imaging studies, or differences in host characteristics is not clear. Low incidence, coupled with previously reported higher rates of GM+ false positivity pose a challenge to the practice of prospective monitoring for IA.^{24,25} It is not clear whether the addition of PCR to prospective GM testing would improve early detection of IA in pediatric patients.

We report on the results of prospective monitoring for IA using GM and PCR in high-risk pediatric patients with hematologic malignancies and allogeneic HCT. Our study is one of the first to evaluate the concurrent use of GM and PCR diagnosis of IA in the pediatric population. The aims of our study were to: identify the incidence of IA in a large population of high-risk pediatric patients; evaluate the feasibility of prospective monitoring for IA using GM and PCR; and correlate prospectively collected data with

clinical, radiographic, microbiological, and histopathologic evidence of IA.

MATERIALS AND METHODS

Study Population

The study population was comprised of consecutive patients treated at Children's Hospital Los Angeles (CHLS) between March 2006 and July 2007 and deemed to be at increased risk of developing IA secondary to expected prolonged neutropenia > 10 days, steroid use (> 2 wk) and/or allogenic hematopoietic stem cell transplantation.^{8,26} Two cohorts were included in our study, separated by type of therapeutic exposure and extent of expected immunosuppression. High-risk leukemia (HRL) included patients with new diagnosis high-risk acute lymphoblastic leukemia (ALL), acute myelogenous leukemia (AML), or any relapsed leukemia (ALL or AML). Hematopoietic cell transplantation (HCT) recipients included individuals with hematologic malignancy and primary immune deficiency. Patients who underwent HCT after their initial diagnosis of acute leukemia were included again, as they were subject to new, increased risk of developing IA, if the period from initial enrollment to HCT was greater than 2 months. The Committee on Clinical Investigations at CHLA (Institutional Review Board) approved the conduct of this study. Informed consent was obtained from all subjects according to the Declaration of Helsinki.

Data Collection and Definitions

Prospective, twice weekly, collection of serum GM and once weekly whole blood PCR was performed on study participants during IA risk periods. For HRL patients, specimens were collected during hospitalization for fever and neutropenia (absolute neutrophil count < 500 cells/ μ L). For HCT recipients, when possible a specimen was collected before initiation of conditioning therapy and then twice weekly during periods of neutropenia and episodes of graft versus host disease (GvHD) treated with immunosuppressive therapy. Specimen collection continued on all patients until recovery from neutropenia, completion of treatment with immunosuppressive therapy for GvHD, or death. Data regarding antimicrobial prophylaxis and/or treatment, radiographic imaging, relevant invasive procedures, culture results, and outcome were collected. Assay results were not reported to treating clinicians.

IA was diagnosed according to standards proposed by a consensus committee of the European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative group and National Institute of Allergy and Infectious Disease Mycoses Study Group (EORTC/MSG).²⁷ Patients were categorized as: proven, probable, possible, and no evidence of IA. To be considered proven, individuals need to have histopathologic evidence of a deep-tissue mold infection or positive culture from a specimen obtained from the affected site or blood. For probable, there needs to be 1 host factor criterion (prolonged neutropenia, persistent fever, signs, and symptoms of GvHD); and 1 microbiological criterion (positive culture from sputum, bronchoalveolar lavage fluid, sinus aspirate specimen, or GM+ from ≥ 2 blood samples); and 1 major (or 2 minor) clinical criteria [radiographic evidence of invasive infection (major), or signs and symptoms suggestive of infection such as new radiographic infiltrate not fulfilling major criteria, or clinical symptoms such as cough,

hemoptysis, and chest pain (minor)]. Possible includes presence of at least 1 host factor criterion; and 1 microbiological or 1 major (or 2 minor) clinical criteria from abnormal site consistent with infection.

GM Assay and PCR

Once collected, all specimens for GM testing were immediately processed, by centrifugation; serum was stored frozen (-70°C) until batch testing was performed. For PCR, blood specimens stored frozen (-30°C) until processed. Laboratory personnel performing the assay were blinded to patient disease status.

GM Assay

One-stage immunoenzymatic sandwich microplate assay (Platelia *Aspergillus* EIA; BioRad Laboratories) was performed at CHLA, according to manufacturer instructions. GM index ≥ 0.5 was considered positive.²⁸ First GM+ was confirmed by repeat testing using a new aliquot of the same specimen, and with a new specimen. Subsequent GM+ were confirmed by repeat testing with a new aliquot of the same sample. Positive control (index > 2.0), negative control (index < 0.4), and cutoff controls in duplicate [optical density (OD) range: 0.3 to 0.8] were included with each batch tested. For the purposes of this study, a subject was considered GM+ only if they had a GM index ≥ 0.5 on more than 2 separate consecutive samples.

Polymerase Chain Reaction

Whole blood specimens were processed using the QIAamp DNA Blood Mini Kit (Qiagen) with an initial 30 seconds bead-beating step in a FastPrepFP120A instrument using 1 mm beads. Each specimen run incorporated a negative control, which entailed concurrent processing of 200- μ l sterile water. Processing of the specimens resulted in a 3 to 5-fold concentration of the purified DNA. Each specimen was set-up in duplicate. Real-time amplification reactions were run on iCycler with MyiQ Single-Color Real-Time PCR Detection System (Bio-Rad), using primers and probe for detection of aspergillus specific 28S rRNA sequence and protocol adapted from White et al.²¹ Each real-time PCR set-up included 3 to 4 positive control reactions (run in duplicate) derived from a dilution series of purified *Aspergillus fumigatus* DNA representing between 6 and 3750 genome copies per reaction.

Clinical Practice and Prophylaxis for IA

HRL patients admitted for fever and neutropenia were housed on a separate floor of the hospital, in rooms equipped with portable HEPA filters. Neutropenic patients with fever of unknown origin were managed with broad-spectrum antimicrobials for the first 72 hours of their hospitalization. Mold-active agents (MAA; lipid formulation of amphotericin B, voriconazole, or an echinocandin) were added by day 5 if there was persistence of fever or if there was clinical and/or radiographic suspicion for invasive fungal infection. Computed tomography (CT) scans of the chest, abdomen, pelvis, and sinuses/brain (when appropriate) were performed on patients who remained febrile and neutropenic after 5 days. Serum GM could be submitted at the discretion of the attending physician.

HCT recipients were housed in the bone marrow transplant unit with high-efficiency particulate air filtered rooms or laminar airflow. Each patient had weekly blood,

stool, and urine surveillance cultures sent for fungal, viral, and bacterial pathogens. Broad-spectrum antimicrobials (usually meropenem and vancomycin) were initiated after day 0 with first fever > 38.0. Patients were placed on antifungal prophylaxis on day -6, and continued until engraftment. Those transplanted before September 2006 received fluconazole (N = 7), whereas individuals transplanted afterward received micafungin (N = 13) prophylaxis. The change was instituted as part of a unit-wide attempt to increase antimold coverage. Those with persistent fevers before engraftment or with clinical suspicion or evidence of IA were placed on empiric voriconazole.

RESULTS

There were 69 patients enrolled on study. All except one had at least 2 GM specimens collected during the study period. Sixty-eight patients were included in the final analysis. Ten patients with a primary diagnosis of ALL or AML subsequently underwent HCT. These patients were considered to be at new, increased risk of IA and are included in both groups of patients (HRL and HCT). Median time from study entry to HCT for these patients was 2.6 months.

Patient Characteristics

Table 1 provides details of the demographics and diagnoses between HRL and HCT patients. For both groups, the most common underlying disease was ALL, followed by AML. Among HRL patients, the distribution of ALL patients was as follows: high risk (N = 26), relapse (N = 20), and infantile (N = 3). HCT recipients were younger, had shorter follow-up, and greater median number of samples when compared with HRL patients. About 94.8% of HRL and 100% of HCT patients had a documented period of neutropenia (< 500 cells/ μ L) lasting greater than 10 days. Median duration of longest episode of neutropenia was 20.1 days versus 24.6 days for HRL and

HCT patients, respectively. Sex distribution was equal in both groups.

Among HCT recipients (N = 20), 4 patients underwent matched-sibling, 14 matched-unrelated, and 2 matched-cord blood transplants. Varying combinations of agents were used for conditioning and GvHD prophylaxis, depending on primary disease and source of progenitor stem cell. The most common agents used for conditioning were busulfan or alemtuzumab (55% each), followed by cyclophosphamide (50%), fractionated total body irradiation (TBI; 45%), etoposide (35%), antithymocyte globulin (30%), fludarabine (25%), or melphalan (15%). GvHD prophylaxis regimens included tacrolimus for 95% of patients, followed by methotrexate (50%), corticosteroids (40%), and cyclosporine/mycophenolate (20%). Fifteen patients (75%) undergoing HCT received corticosteroids for a median duration of 40 days (range: 7 to 135 d), as prophylaxis and/or treatment of GvHD.

Serum IA Testing

A total of 1086 GM specimens were collected (627 HRL, 459 HCT) during a median follow-up of 98.5 days per patient (range: 14 to 437 d). Median number of specimens per patient was 9.0 (2 to 36) for HRL and 16.5 (9 to 58) for HCT recipients. As seen in Figure 1, 610 (56.2%) samples were obtained from patients on MAA: 200 of 627 (31.9%) HRL, and 410 of 459 (89.3%) HCT recipients. Thirty (2.8%) samples were obtained from patients receiving piperacillin/tazobactam (Zosyn) therapy, and all were GM -. Real-time PCR analysis was applied to 554 blood specimens isolated from 52 patients; including all patients with > 1 GM+ result.

Clinical Diagnosis of IA

There were 20 separate episodes of possible IA in 19 patients (18 with 1 episode, 1 with 2 episodes), per EORTC/MSG criteria. In all instances, patients met at least 1 host factor criterion, and 2 minor clinical criteria. Three patients had at least 2 GM+ specimens and were deemed probable

TABLE 1. Patient Characteristics

Variables	HRL (N = 58)	HCT (N = 20)	Combined (N = 78)
Age, years			
Median (SD)	11.4 (4.6)	9.6 (5.6)	11.1 (4.9)
Range	0.4-22.2	0.5-17.8	0.4-22.2
Sex			
Male, number (%)	36 (62.1)	12 (60.0)	48 (61.5)
Diagnosis, number (%)			
Acute lymphoblastic leukemia	49 (84.5)	8 (40.0)	57 (73.1)
Acute myelogenous leukemia	9 (15.5)	5 (25.0)	14 (17.9)
Primary immune deficiency	0	3 (15.0)	3 (3.8)
Congenital marrow failure	0	2 (10.0)	2 (2.6)
Other	0	2 (10.0)	2 (2.6)
Neutropenia			
> 10 d, number (%)	55 (94.8)	20 (100.0)	75 (96.1)
Samples per patient			
Median (SD)	9.0 (8.7)	16.5 (14.5)	11.0 (11.6)
Range	2-36	9-58	2-58
Days on study			
Median (SD)	107.0 (101.2)	85.0 (100.6)	98.5 (100.7)
Range	14-384	35-437	14-437
Outcome			
Overall survival, number (%)	42 (72.4)	11 (55.0)	53 (67.9)

HCT indicates hematopoietic cell transplantation; HRL, high-risk leukemia.

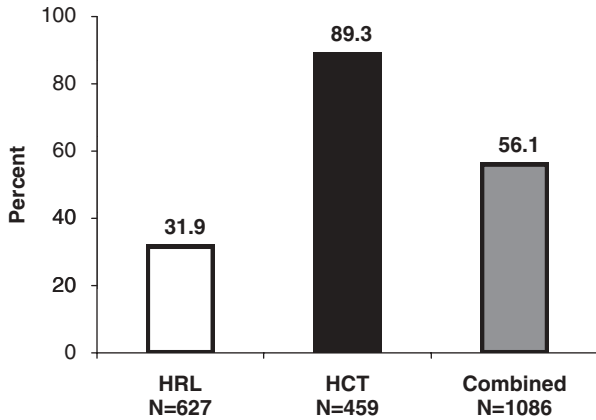


FIGURE 1. Proportion of samples collected from patients already on mold-active agents.

IA, per EORTC/MSG criteria. In addition, one patient had a false positive GM (index 0.65), which was negative on repeat testing. The patient never developed clinical or radiographic evidence of IA. There were no proven IA episodes.

Nineteen out of 22 patients with a diagnosis of probable or possible IA during the course of the study were tested for *Aspergillus* DNA by PCR (217 of 554 specimens). Fourteen out of 217 (6.5%) specimens were PCR positive. Only 3 of these specimens from 2 patients coincided with or immediately preceded (ie, ≤ 2 wk) an episode of probable/possible IA. Conversely, there were 11 PCR positive specimens that were believed to be unrelated to IA.

Thirty-three patients without an episode of probable/possible IA were tested by PCR (337 of 554 specimens). Ten out of 337 (3.0%) specimens from 7 patients were PCR positive. Thus, considering all patients tested by PCR, there were 21 out of 24 specimens that were PCR-positive, which did not correspond to episodes of probable/possible IA, yielding a false PCR-positivity rate of 87.5%. Mean quantitated value for positive specimens was 11.2 ± 3.2 *Aspergillus* genomes per PCR [mean threshold cycle (Ct): 44.2 ± 0.5], which was equivalent to approximately 200 genomes/ml blood; suggesting that the assay was prone to false positivity despite the implementation of fastidious specimen processing and PCR set-up procedures.

Figure 2 details the clinical course of the 3 patients with at least 2 GM+ samples (probable IA). Patient no. 1 was an 8.4-year-old female undergoing HCT for Diamond-Blackfan anemia, and was on study for 86 days. Four out of 20 specimens were GM+ and all were PCR negative. The patient was initially on fluconazole prophylaxis at first GM+ (day 51). Voriconazole was substituted because of clinical and radiographic suspicion of invasive fungal disease (day 52). Micafungin was added because of concern of progression of disease (day 59). The patient had a decrease in GM index soon after change in antifungal coverage, but had persistent abnormal findings on CT. Open lung biopsy performed on day 62 failed to demonstrate evidence of fungal disease, by culture or histopathology.

Patient no. 2 was an 8.5-year-old male with relapsed ALL, and was on study for 305 days. Seven out of 36 serum specimens were GM+ and 3 blood specimens were PCR positive, although only at 1 time point (during the episode of probable IA) was the patient concurrently GM and PCR positive. There was a previous suspicion for pulmonary fungal disease, and the patient was placed on lipid

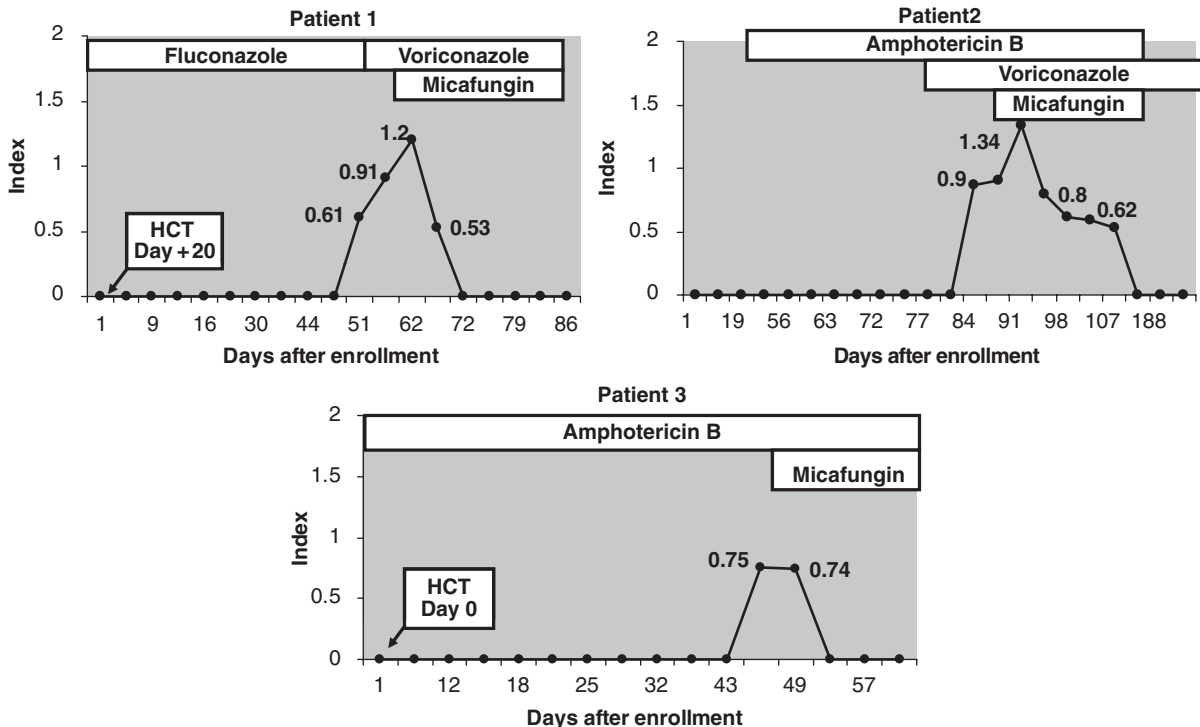


FIGURE 2. Clinical course of 3 patients with ≥ 2 GM+ (galactomannan) samples.

formulation amphotericin B (41 d) and voriconazole (5 d) before his first GM+ result. Micafungin was added 5 days after the first GM+ result (day 90) because of worsening clinical course. There was eventual improvement of radiographic and clinical findings, and it coincided with the decrease in GM index. CT-guided needle biopsy performed on day 91 (6 d after the first GM+ result) failed to demonstrate evidence of fungal disease, by culture or histopathology.

Patient no. 3 was a 16-year-old male undergoing HCT for relapse AML, and was on study for 57 days. He had been on lipid formulation amphotericin B therapy for 45 days because of history of gastrointestinal colonization with *Mucor species* at the time of first GM+. Antifungal coverage was not changed during the study-monitoring period, and GM became negative despite worsening respiratory distress. All blood specimens tested were negative by PCR. This Patient eventually died as a result of multiorgan failure secondary to a number of post-HCT complications. Incidentally, Bronchoalveolar lavage (BAL) had been performed 13 days before first GM+ result (day 33) because respiratory distress. BAL culture results were negative for *Aspergillus species* and GM index was 0.68.

DISCUSSION

During the past several decades, there has been an increase in the frequency of invasive fungal infections such as IA in adult immunocompromised patients, with substantial associated morbidity and mortality.^{29,30} Factors contributing to the increased incidence include greater number of susceptible hosts, intensification of immune suppression, and more aggressive antimicrobial prophylaxis strategies.^{31,32} Early diagnosis is felt to be critical to improving outcome of patients with IA, especially with the increased availability of more active therapeutic agents and the potential of combination therapy.^{6,33,34} Controlled trials in adults using twice-weekly GM testing screening have shown high sensitivity and specificity, and have facilitated the diagnosis of IA before clinical and radiographic findings in some high-risk patients.^{11,28} As result, noninvasive techniques using molecular diagnostic approaches, such as GM and PCR, have been increasingly considered for use in monitoring of patients at risk for IA. To date, however, no studies have documented a survival advantage to patients with early diagnosis with GM testing.

Hayden and colleagues³⁵ recently reported on their results of prospective monitoring for IA using serum GM alone in high-risk pediatric patients. Incidence of proven or probable IA in this population was 30.3%. Serum GM had a sensitivity of 65.7% and specificity of 87.2%, and GM was detected a median 10 days before clinical symptoms of IA in 7 of 11 patients with available clinical and radiographic correlates. El-Mahallawy and colleagues³⁶ examined whether the addition of blood PCR to prospective GM monitoring would enhance early detection of IA in a high-risk pediatric population with similarly high-IA incidence (30.8% probable or proven IA). The net sensitivity for detecting IA using this simultaneous testing was nearly 95%, whereas net specificity was 61%. Positive predictive value of serum GM was only 54%, as there were a significant number of false positive results. Furthermore, it was not clear whether this simultaneous approach of blood PCR and serum GM testing facilitated the early detection of IA.

Recent studies in pediatrics suggest that the incidence of IA may be lower than in adults with reported rates ranging from 1.5% to 3.2%.^{22,23} The reportedly high incidence in the above mentioned pediatric experiences^{35,36} may be owing to differences in institutional MAA prophylaxis, IA screening practices, or sampling bias. A previous study at our institution found the incidence of IA (proven or probable) to be 5.2% among allogeneic HCT recipients over 5 years (1996 to 2001).³⁷ In this study, we found a low incidence of both proven/probable IA and infrequent GM+ false positivity over a prolonged period of monitoring. The lower incidence of proven/probable IA in this study is in agreement with the more recent reports in pediatric populations that have found a trend toward decreased incidence of IA during the last decade.^{22,23,38} This may explain, in part, the low yield of such prospective monitoring in this presumably at risk population.

The change in incidence may be, in part, due to increasing use of empiric and/or preemptive antifungal therapy, which not only affects the prevalence of IA, but has been reported to decrease sensitivity of the GM assay,³⁹ potentially contributing to false negative results. Nearly, 60% of our samples were obtained while patients were on MAA, and the number approached 90% in individuals undergoing HCT. Preemptive initiation of antifungal therapy combined with radiographic imaging likely contributed to the lower incidence of proven IA reported in this study.

False positive results in the tests for *Aspergillus* GM antigen have been reported especially in children.^{24,25} Sulahian et al²⁴ found a false positivity rate of 2.5% in adults compared with 10.1% in pediatric patients with hematologic malignancies, whereas others have reported rates as high as 44% in children with neutropenic fever of unknown origin.²⁵ Highest rates have been observed in neonates (83%), and is thought to be due to cross-reactivity with *Bifidobacterium species*, which comprises up to 91% and 75% of the total fecal microflora in breast-fed and formula-fed infants, respectively.⁴⁰⁻⁴² Additional reports of false positive tests have been linked to concomitant piperacillin-tazobactam (Zosyn) therapy.^{43,44} Unlike previous studies, our cohort did not have a high-false positive GM rate. One specimen was presumably false elevated, whereas the remaining 12 were positive on at least 2 separate occasions, and corresponded with clinical and radiographic suspicion for IA. Furthermore, all samples obtained from patients receiving piperacillin/tazobactam (Zosyn) therapy were GM –.

The value of PCR for diagnosing invasive fungal infections has yet to be established. Although it is reported to have good specificity (>85%), its sensitivity has been varied (29% to 100%).^{16,17} Efficacy of DNA extraction, quality of primers (dimer formation and/or nonspecific amplification), type of PCR reaction block-based PCR, nested PCR, or real-time PCR, the size of population studied, and sample type (plasma, whole blood, or BAL) have all been shown to affect the sensitivity of the assay.¹⁹⁻²¹ Our experience in this study was that PCR surveillance for *Aspergillus* was confounded by the high proportion of false positives. *Aspergillus* (or other *Ascomycota*) are ubiquitous in hospital and laboratory environments and even with fastidious processing and set-up procedures, completely eliminating the possibility of air-borne conidia is difficult without sophisticated room air-filtration systems.

Another concern with a PCR-based approach is that we estimate that blood would need to contain > 100 genome equivalents of *Aspergillus* DNA/ml to be PCR positive. This threshold may explain why only 2 of 19 patients with 1 or more episodes of probable/possible IA were PCR positive concurrently with an episode. Clearly, this threshold could be reduced if the DNA in blood could be concentrated greater than the 3 to 5-fold achieved in this study. However, a significant factor limiting the magnitude of achievable concentration is the volume of blood available, which can be restricting in pediatric patients. Furthermore, Lass-Flörl et al⁴⁵ found that during antifungal therapy, patients with previously positive specimens became PCR negative. The aggressive use of empirical MAA in our patient population would potentially decrease likelihood that circulating blood levels of DNA-containing fungal material, conidia, or hyphal debris, would reach the threshold for a positive PCR.

A limitation of this study was that it was a single-center experience, and may not reflect the treatment and prophylaxis practices advocated at other centers. In particular, our findings may not be applicable to centers with more limited use of empiric MAA in high-risk pediatric patients. However, our reported low incidence of proven IA is in agreement with more recent studies that have demonstrated a similar downward trend over time.^{22,23} Our study was composed of a heterogeneous population of high-risk pediatric patients with median follow-up of nearly 100 days. We believe the diversity of patient population and prolonged follow-up adequately represented the epidemiology of IA at our institution.

These findings suggest that at present, IA is relatively infrequent in the setting of aggressive radiographic monitoring and preemptive MAA use. It is speculated that changes in clinical practice, early use of empiric and/or prophylactic MAA and frequent imaging studies have impacted the epidemiology of IA in this high-risk pediatric population. In an era of rising medical expenditures and cost-containment concerns, it is equally important to report on screening and diagnostic approaches, which are beneficial to the clinical setting, as it is to report on those, which may be less useful. Our study found that the use of GM and PCR assays, as a prospective screening device on blood may not further enhance current clinical outcomes. Their role in this patient population remains undefined.

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