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Application of diagnostic markers to invasive aspergillosis in children

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Early mycological detection of *Aspergillus* species is the cornerstone for a prompt diagnosis, appropriate treatment strategies, and improved survival of patients with invasive aspergillosis (IA), irrespective of age. However, the currently available laboratory tests for the diagnosis of IA include culture with direct microscopy, histology, and antigenic markers, such as galactomannan and β -1, 3-D-glucan, *Aspergillus* spp. DNA detection by PCR, and imaging studies, such as high-resolution CT scan. However, all need further validation, especially in children. In this review we focus on the diagnosis of IA emphasizing the current perspectives, difficulties in interpretation, and the need of further evaluation from a pediatric point of view.

Keywords: invasive aspergillosis; β -1, 3-D-glucan; galactomannan; children

Introduction

Invasive aspergillosis (IA) is an increasing problem in children and is associated with high attributable morbidity and mortality rates, as well as early and late onset complications.¹ There are significant differences reported in the epidemiology patterns of IA among different pediatric populations and adults.² Children with either congenital or acquired immunodeficiency may suffer from IA. Previous studies have emphasized the increased risk for IA in children with acquired immunodeficiencies after immunosuppressive therapy for cancer (especially for hematological malignancies), in children with advanced human immunodeficiency virus infection, bone marrow failure syndromes or allogeneic hematopoietic stem cell or solid organ transplantation.^{3–5} Furthermore, children with inherited defects of phagocytic host defenses, including chronic granulomatous disease and those with cystic fibrosis are at increased risk of presenting IA.^{6,7} Additionally, low-birth weight and premature infants are susceptible to IA due to reduced chemotactic, phagocytic, and microbicidal activity, skin breakdown, prior antibiotic therapy, or prolonged use of steroids.⁸

Early mycological detection of *Aspergillus* species is the cornerstone for a prompt diagnosis, appropriate treatment strategy, and improved survival of patients with IA, irrespective of age. On the other hand, current laboratory examinations for IA detection need further validation, especially in children. Routine methods for rapid specific identification of *Aspergillus* species are generally not available. The current diagnostic markers for IA include conventional and more recent methods under evaluation. Conventional methods of diagnosis include direct microscopy and histology, and culture of respiratory and various fluids and tissues. Recently, more rapid and sensitive methods have been developed, for example, the detection of antigenic markers, such as galactomannan and β -1, 3-D-glucan, the detection of molecular markers of *Aspergillus* DNA by polymerase chain reaction, and improved imaging studies such as high-resolution CT scans.

Various problems characterize the older and the more recently introduced diagnostic markers, especially in pediatric patients. A few examples of these problems include *in vitro* culture lacks sensitivity, histological diagnosis requires invasive methods that are often difficult in children and are nonspecific to speciation, galactomannan lacks sensitivity in children compared with adults,

imaging lacks specificity, and fungal DNA detection requires standardization, especially for samples from children.⁹ In this short review we focus on the diagnostic panel used for the diagnosis of IA, emphasizing the current perspectives, interpretation difficulties, and the need of further evaluation from a pediatric point of view. Also, we briefly mention the standard methods in which no differences in performance exist between adults and children.

Conventional methods

Conventional methods include direct microscopy and histology and culture of respiratory and other fluids and tissue. However, culture is insensitive and is time consuming.¹⁰ The high percentage of culture-negative results may be attributed to the atypical appearances of *Aspergillus* spp. or to the lack of expertise for species identification. Additionally, the time required to obtain culture results delays the onset of antifungal treatment, leading to deterioration of survival outcomes. Tissue diagnosis also requires invasive procedures that are complicated with serious side effects, especially in patients with thrombocytopenia.

Molecular markers

The polymerase chain reaction (PCR) represents one of the most investigated rapid diagnostic methods with clinical utility for IA. A recent meta-analysis of the use of PCR from blood, serum, or plasma samples for the detection of IA reported that the sensitivity and specificity for two consecutive positive samples were 0.75 (95% CI 0.54–0.88) and 0.87 (95% CI 0.78–0.93), respectively.¹¹ A similar meta-analysis evaluating PCR on bronchoalveolar lavage fluid revealed a sensitivity of 0.91 (95% confidence interval (CI), 0.79–0.96) and specificity of 0.92 (95% CI, 0.87–0.96).¹² However, PCR has a number of drawbacks that include the lack of standardization and uniformity of methods and results obtained among laboratories. Moreover, the difficulty of interpretation of the findings obtained may result in inconsistencies and unreliable results in both adult and pediatric populations. To overcome these problems and to optimize PCR findings, a real-time quantitative PCR (qPCR) assay based on minimum information for the publication of real-time quantitative PCR experiments (MIQE) guidelines has been recently proposed to detect *Aspergillus* spp., with promising results.¹³

Other issues, such as appropriate sample timing, the frequency of prospective consecutive sampling, and the number of positive PCR results required to initiate antifungal treatment remain to be established.¹⁴ A useful practice to improve the sensitivity of the assay is to obtain the samples before the introduction of antifungal therapy. A recent report on an *in vivo* model revealed that the sensitivity of both qPCR and GM in the early diagnosis phase of IA was significantly impacted by the use of posaconazole and caspofungin.¹⁵ Furthermore, the definition of an episode of IA as PCR positive, with two positive results within 14 days in patients with hematopoietic stem cell transplantation and acute leukaemia, raised the sensitivity, specificity, positive predictive value, and negative predictive value of IA (100%, 75.4%, 46.4%, and 100%, respectively).¹⁶

As far as pediatric patients are concerned, data concerning DNA detection of *Aspergillus* spp. with different PCR techniques are lacking. Only one recent multicenter study conducted by Hummel *et al.* has evaluated with nested PCR assay 291 clinical samples from 71 pediatric and adolescent patients with suspected IA during the period 2000–2007.¹⁷ Samples were obtained mainly from blood but cerebrospinal fluid and bronchoalveolar lavage samples were also included. According to their results, the sensitivity and specificity were high, with rates of 80% and 81%, respectively. Additionally, the reported positive and negative predictive values were 40% and 96%, respectively. A large multicenter study that is currently on-going to investigate the role of PCR in IA does not include children.

Novel molecular markers

The proteomic signature of specific *Aspergillus* spp. is thought to be paramount to widen future diagnostic panels and to develop more personalized therapeutic regimens for IA. Recent immunoproteomic studies have focused on the detection of new more accurate, novel immunodiagnostic markers of *Aspergillus* spp.^{18–20} Several extracellular proteins of *Aspergillus* are highly immunogenic and may serve as specific diagnostic antigens in humans in the future. Among immunoactive proteins of *Aspergillus* spp., efforts have been undertaken to identify the one with the best immunogenic potential. A recent study by Shi *et al.*, based on immunoproteomic data, reported that 17 different extracellular proteins of

A. fumigatus represent novel candidate biomarkers for the future detection of IA.¹⁸ In particular, thioredoxin reductase GliT (TR) presented the best immunogenic potential for *A. fumigatus*, with analysis showing low homology to other *Aspergillus* spp. and no homology to any human proteins.¹⁹ In another study, a panel of three novel proteins could serve as potential allergens with specific IgE immunoreactivity in patients suffering from allergic bronchopulmonary aspergillosis (ABPA).²⁰ Essential virulent factors, such as siderophores, represent candidate markers for *Aspergillus* detection and simultaneously for developing therapeutic targeted inhibitors as novel antifungal agents.²¹

Galactomannan assay

Serum

The galactomannan assay (GM) appears to be an attractive serological diagnostic marker for detecting IA. Recent data have demonstrated that GM seems to be predictive of outcome in hematological patients with invasive pulmonary aspergillosis. In particular, high baseline serum GM antigen levels on day 45 posttransplantation were significantly associated with poor outcome.²²

Among the GM tests developed, the double-sandwich ELISA is found to be the most accurate, irrespective of patient age. To date, no formal recommendations have been published for GM testing in serum specifically in the pediatric population. For that reason cut-off values are mainly extrapolated from adult populations. In addition, false-positivity of the GM test in children might be attributed to several factors, such as concomitant administration of various antibiotics (i.e., piperacillin/tazobactam), cross-reactivity with environmental moulds such as *Penicillium marneffei* or *Cryptococcus neoformans*, milk-based diet, and the presence of *Bifidobacterium bifidum*.²³

The results of previously published studies have to be interpreted with caution since they suffer from heterogeneity of cut-off values, of definitions of assay positivity, and of the analyses performed (e.g., analyzing patients, episodes or some single sample results). Among 10 studies conducted (7 prospective) evaluating serum GM in children, the number of pediatric patients included in each study varied between 20 and 347, and the number of samples from 413 to 2376 (Table 1).^{24–34} Furthermore, the number of patients with proven/probable invasive

fungal diseases (IFD), and of controls, also varied widely (median 9.5 (range, 1–28) and 63 (range 8–338), respectively). True positive GM results are additionally variable, ranging from 0 to 100% (4 studies with ≥ 10 patients with proven/probable IFD: 28–92% (median, 71.5%)), while true negative results fluctuated from 22 to 100% (7 studies with ≥ 10 controls: 49–100% (median, 88.5%)).

It is also interesting to note that in most studies serial GM testing was assessed in children with hematological malignancies and after allogeneic HSCT (screening performed once or twice weekly); the results show a sensitivity and specificity profile of GM testing that is similar to that observed in adults. However, in patients with inherited immunodeficiencies (i.e., chronic granulomatous disease) or other nonhematologic categories, there is usually lack of angioinvasion and presence of dysfunctional neutrophils and monocytes. In these cases, serum GM is often negative.^{35,36}

The comparison of five studies, which use European Organization for Research and Treatment of Cancer (EORTC)/Mycosis Study Group (MSG) criteria and give adequate information for individual patients, with results of a formal meta-analysis of adult data conducted by Pfeiffer *et al.*, revealed that GM sensitivity in children is 0.76 (95% CI 0.62–0.87) compared to 0.73 (95% CI 0.46–0.61) in adults, while GM specificity reached 0.86 (95% CI 0.68–0.95) in children compared to 0.90 (95% CI 0.88–0.92) in adults, respectively.³⁷ In 2011 these studies were comprehensively analyzed and discussed by the European Conference on Infections in Leukemia (ECIL) 4 Group and its recommendations are reported electronically.³⁸ According to published data, prospective monitoring of GM every 3–4 days in children at high risk for IFD is reasonable for early diagnosis of IA, despite the number of limitations in the available pediatric data mentioned above (i.e., wide variations among the studies regarding cut-off, definition of positivity, etc.).^{38,39} In addition, although the optimal cut-off value of GM in the serum of children is not well defined, ECIL 4 recommendations support the use of a threshold of an optical density index 0.5.³⁸

Bronchoalveolar lavage and cerebrospinal fluid

The presence of galactomannan in bronchoalveolar lavage (BAL) fluid (BAL GM) is an alternative

Table 1. Serum galactomannan (GM) assay in studies with pediatric patients

Author Ref. no.	No. of ped. pts.	No. of GM samples	Definition of GM positivity	Type of collection	Cut-off values (ng/mL)	IFD definition	Results
Rohrlich <i>et al.</i> ²⁴	37	413	Two consecutive samples	Screening 2 × wk: during immunosuppression	≥0.93	Guiot CID 1994	PPV: 83%, FP: 5.7% Clinical + radiol. signs occurred at a mean of 13.4 days after GM (+)
Sulahian <i>et al.</i> ²⁵	347	2376	Two consecutive samples	Screening 2 × wk: during immunosuppression	≥1.5	EORTC/MSG	SP: 89.9% SN: 10% FP: 10.1%
Herbrecht <i>et al.</i> ²⁶	NR**	540	Per sample	Screening 2 × wk: during immunosuppression	≥1.5	EORTC/MSG	SP: 47.6% PPV: 15.4% NPV: 96.7% FP results more frequently in Chil. [44.0%] than in adults [0.9%]; <i>P</i> = 0.0001. Most FP occurred in HSCT pts.
Challier <i>et al.</i> ²⁷	20	207	NR	NR	≥1	EORTC/MSG	SN and SP about 90% with clinical and radiological symptoms and 50% in absence
El Mahallawy <i>et al.</i> ²⁸	91	NR	NR	NR	NR	EORTC/MSG	SN: 79% SP: 61% PPV: 54% NPV: 83%
Hovi <i>et al.</i> ²⁹	98	932	NR	Screening 1 × wk: during immunosuppression	NR	EORTC/MSG	NR
Steinbach <i>et al.</i> ³⁰	64	826	Per sample	Screening 2 × wk: during immunosuppression	≥0.5	EORTC/MSG	SP: 97.5%§ Exclusion of pts. receiving PIP/TAZ = SP: 98.4%
Hayden <i>et al.</i> ³¹	56	990	Per sample	Screening 1 × wk: during immunosuppression	≥0.5	EORTC/MSG	SN: 65.7% GM may precede clin. + microbial. + radiolog. evidence of IA.
Armenian <i>et al.</i> ³²	68	1086	Two consecutive samples	Screening 2 × wk: during immunosuppression	≥0.5	EORTC/MSG	13 samples (1.2%) from 4 patients (5%) were GM+.
Castagnola <i>et al.</i> ³³	119	1798	Per sample or two consecutive samples	Not specified At least 2 × week	≥0.7 for single test or 0.5-0.72 for consecutive tests	EORTC/MSG	SP: 98%, SN: 32% PPV: 70%, NPV: 92% Better GM performance after chemother. than after HSCT
de Mol <i>et al.</i> ³⁴	41	41	Per sample		≥0.5	EORTC/MSG	Among 13 pts with a pos. serum GM, 11 had a pos. BAL GM (Spearman's Coeff. 0.719). BAL GM SN: 82.4% SP: 87.5%. PPV: 82.4% NPV: 87.5%

*NR: not reported, § SN: sensitivity, SP: specificity, PPV: positive predictive value, NPV: negative predictive value, FP: false positive.

**48 episodes in children.

serological diagnostic marker, especially for invasive pulmonary aspergillosis, which constitutes the most common presentation of IA. The reported sensitivity rate of BAL GM is in general higher than that in serum due to the increased fungal burden in the bronchi of patients with pulmonary IA.³⁹ However, the role of BAL GM in pediatric IA has not been extensively evaluated. A recent retrospective study conducted by Desai *et al.* suggests that BAL GM is a valuable adjunctive diagnostic tool.⁴⁰ According to their results from 85 pediatric patients, 59 of whom were immunocompromised (9 with proven/probable invasive pulmonary aspergillosis), the receiver operating characteristic curves for the immunocompromised patients demonstrated an optimal BAL GM OD cut-off value of 0.87, that yielded a sensitivity for probable/proven IA of 78% and a specificity of 100%. At 0.87, the positive and negative predictive values among immunocompromised patients were 58% and 96%, respectively. According to the recommendations of the 2011 ECIL 4 Group, the limited published data support the value of GM in the diagnosis of pulmonary aspergillosis (BAL GM cut-off value 1) in children.³⁸ Nevertheless, systemic mold-active antifungal prophylaxis may decrease the performance of the test.³⁸

Although invasive pulmonary aspergillosis is the most frequent presentation of IA, cerebral aspergillosis also may occur, especially in severely immunocompromised patients undergoing HSC transplantation. GM can be found in the cerebrospinal fluid (CSF) but the data are very limited and based on only a few case reports or case series. A recent case series study by Viscoli *et al.* evaluated GM in CSF samples of five patients with probable cerebral aspergillosis and of 16 control patients.⁴¹ According to their results the median CSF GM index for the five patients with probable cerebral aspergillosis (eight samples) was 10.52 (mean, 81.93; standard deviation (SD), 200.96; range, 0.55 to 578.50). In contrast, the median CSF GM index from the 16 control patients (33 samples) was 0.29 (mean, 0.28; SD, 0.13; range, 0.09 to 0.75). A previous case report described a rare pediatric case of an 18-month infant suffering from corticosteroid-resistant nephrotic syndrome, which was complicated by invasive pulmonary and cerebral aspergillosis.⁴² The reported GM value was 4 ng/mL in CSF and 3.5 ng/mL in serum (normal value <1 ng/mL). Although further studies on larger patient popu-

lations and especially in children are needed, the recommendations of the ECIL 4 Group support the value of GM in the diagnosis of central nervous system aspergillosis (CSF GM cut-off 0.5).³⁸

Beta-D-glucan assay

The β -1, 3-D-glucan assay (BDG) is also thought to have clinical utility, both for surveillance and as a diagnostic tool for IA. The BDG is a nonspecific diagnostic marker that can be detected by two commercial assays (Fungitell[®], Associates of Cape Cod; Fungitec G[®], Seikagaku) in fungal infections attributed to *Aspergillus* and *Candida* spp., as well as those due to *Fusarium*, *Trichosporum*, *Saccharomyces*, and *Pneumocystis jirovecii*. Furthermore, bacteria, such as *Streptococcus pneumoniae* and *Pseudomonas aeruginosa*, and sometimes healthy individuals, might present positive BDG test results.⁴³ On the other hand, BDG is absent in cryptococcosis and mucormycosis. False-positive BDG has been associated with the concomitant use of antibiotics, such as cefepime, piperacillin/tazobactam, or meropenem.⁴³

According to the revised EORTC/MSG definitions, BDG is included as a mycological criterion of invasive fungal disease. In adult populations especially, the BDG test presents a satisfactory diagnostic for early IFD.^{43–45} Based on data in 2979 adult patients (594 with proven or probable IFD) the pooled sensitivity of BDG reached 76.8% (95% CI 67.1%–84.3%) and the pooled specificity of 85.3% (95% CI, 79.6%–89.7%), respectively. However, data regarding pediatric populations for IA are extremely limited. In a recent study conducted by Mularoni *et al.* the BDG test was evaluated in four children with proven IFD (three patients with candidemia, one patient with probable aspergillosis) by positive culture from a sterile site and/or the demonstration of fungal elements in diseased tissues.⁴⁶ The BDG test was performed using the Fungitell assay with a positive cut-off of 60 pg/mL. For all four patients BDG levels were >523 pg/mL. A major problem in performing a BDG test on samples from children is the lack of baseline levels for uninfected pediatric patients. For that reason, Smith *et al.* tried to evaluate the BDG test levels in 120 immunocompetent and uninfected pediatric patients;⁴⁷ the median age was 9.2 years (range, 7 months to 8 years). The median BDG level was 32 pg/mL and the mean value was 68 pg/mL. The mean values did not vary

significantly by age or gender. These observations suggested that normal mean BDG values in children are in general higher than those from adults tested previously (68 pg/mL versus 48 pg/mL for children and adults, respectively), raising questions about the critical cut-off values of BDG in children. These two parameters—lack of data and possible higher normal BDG values in children—have led to the absence of recommendations of BDG testing in children.

Imaging studies

To date, the standard imaging technique for the detection of IA remains the CT scan. Characteristic CT findings for IA in adults include pulmonary nodules, halo sign, air crescent sign, and cavitation.^{48,49} In adult series studies, approximately 50% of cases show cavitation, with air crescent formation in 40%. These findings constitute basic clinical criteria in the revised EORTC/MSG definitions of invasive fungal disease.³⁸ However, the main drawbacks of CT scan are the limited specificity and predictive value. Previous papers reported that CT scans do not always allow the differentiation of pathogenic fungi. PET scan with CT imaging has been proposed as an alternative diagnostic procedure, which increases the specificity of detecting IA, since it provides a detailed and more functional information of findings; MRI findings are helpful in detecting cases of *Aspergillus* osteomyelitis and CNS involvement.⁵⁰ High T2 signal found in the cortex or subcortical white matter and, sometimes, the presence of hemorrhage representing infarcts are indicative of cerebral aspergillosis. Furthermore, up to 30% of adult patients with pulmonary aspergillosis may present multiple organ involvement, and for that reason multisystem radiological evaluation of high risk patients is required.

The main problem in the pediatric setting regarding imaging techniques is primarily the lack of specific lesions for the detection of IA.^{51–55} Radiographic findings in children are often nonspecific, especially in the younger age group (<5 years). Second, the limited data on imaging studies are focused only on immunocompromised children with underlying malignancies; information in other pediatric patient groups is lacking.⁵⁵ In a 10-year study evaluating radiographic findings of IA in children, a large variation of the lesions, which were often nonspecific, was reported.⁵² Furthermore, the cavi-

tation rate was found to be less common in children than in adult patients. Finally, the utility of a CT scan was limited to detecting multiple lesions and involvement of the chest wall.

According to the 2011 ECIL 4 Group recommendations, in high-risk children with febrile neutropenia >96 h or with focal clinical findings, imaging studies (lung CT scan or adequate imaging of the symptomatic region) should be performed.³⁸ Even atypical pulmonary infiltrates (e.g., fluffy masses) may support the diagnosis of invasive pulmonary aspergillosis in pediatric high risk patients. At least, a further diagnostic work-up (e.g., BAL, biopsy) should be considered in this patient group and mold-active antifungal treatment should be promptly initiated.

Novel imaging studies

In the recent years, novel sensitive radiotracers have been identified for the imaging of IA, such as antimicrobial peptides, antifungal agents, and chitin-specific agents.⁵⁶ Two siderophores, triacetylfusarinine (TAFC) and ferrioxamine E (FOX E), have been labeled with gallium-68 and used for PET imaging of *A. fumigatus* infection in rats. Based on results, both tracers revealed high metabolic stability and a favorable biodistribution.⁵⁷ A peptide (c(CGGRGLGPFC)-NH₂) labeled with indium-111 was also specifically bound on *Aspergillus* hyphae.⁵⁸ These novel sensitive radiotracers constitute future candidate technologies for IA imaging, but further investigation is required to assess the *in vivo* biodistribution and safety in human clinical trials.

Conclusions

Prompt diagnosis of IA in the pediatric setting remains a major challenge. Awareness of the optimal diagnostic procedures solely or in combination is mandatory for prompt detection of the etiologic fungal species. Standard and newer diagnostic methods of IA are extensively evaluated in adults, but these data cannot be simply extrapolated to pediatric patients.

In summary, the GM test can be used for children with caution, the utility of the β -1, 3-D-glucan needs further research for children, and imaging, particularly the CT scan, is a useful but nonspecific tool, whereas molecular markers such as PCR present the same problems and difficulties as in adults. While progress has been achieved in terms of GM and

certain recommendations have been made, further research is needed for the validation of newer diagnostic procedures in pediatric patients.

Conflicts of interest

The authors declare no conflicts of interest.

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