Mycopathologia (2005) 159: 487–493
DOI: 10.1007/s11046-005-4996-9

The contribution of galactomannan detection in the diagnosis of invasive aspergillosis in bone marrow transplant recipients

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Received 30 September 2004; accepted in revised form 1 April 2005

Abstract

Until recently, accurate microbiological diagnosis of invasive aspergillosis (IA) was seldom established in HSCT recipients. Blood samples are rarely positive for Aspergillus species, the reliability of the cultures depends of the specimen (if taken from a normally sterile site or not) and biopsy samples require invasive procedures, rarely recommended in patients with severe thrombocytopenia. Implementing the international consensus defining the microbiological criteria for the diagnosis of Aspergillus infection, we retrospectively evaluated the role of serum galactomannan (GM) detection by EIA to diagnose IA among HSCT patients with proven invasive fungal infection (IFI) and the impact of serum storage in GM concentrations. The EIA assay allowed categorizing as "probable" 5 of the 10 cases of "possible" aspergillosis (50%). Considering a lower cut-off level for the reaction (1.0), 80% of the cases could be categorized as "probable" aspergillosis. Positive or undetermined results were detected one to 4 months before the diagnosis of IA in eight of the 11 patients (72.7%) with proven IFI. Retesting the stored samples after a second storage for four years, we could observe lower reactivity in 20% of the samples. The detection of galactomannan by the EIA test represents a major advance in the diagnosis of IA in HSCT recipients at high risk of IA. A better understanding of the kinetics of the GM in different clinical situations is necessary to maximize the benefit of the test in Aspergillus surveillance.

Key words: antigen detection, Aspergillosis, galactomannan, HSCT, Platelia, serum storage

Introduction

The diagnosis of invasive aspergillosis (IA) in hematopoietic stem cell transplant (HSCT) recipients has been hampered by the limited diagnostic yield of classical techniques such as culture, microscopy and histology. Microbiological diagnosis is seldom established since blood cultures are rarely positive for *Aspergillus* species, mainly in early stages of infection, when early diagnosis is crucial. Likewise, the reliability of the culture depends on the specimen, if taken of a normally sterile site or not. Although the presence of septate hyphae in tissue is most commonly due to *Aspergillus* species in this population [2], invasive procedures to obtain specimens of the infected site are not always possible due to the presence of severe thrombocytopenia [2, 8, 23]. Moreover, air crescent and halo sign as seen on high resolution CT scans, a useful diagnostic tool validated in neutropenic patients, lacks specificity and predictive value in non-neutropenic HSCT recipients.

As a consequence of the inability to reliably diagnose IA, a broad spectrum of case definition was used in the last decades by different authors, HSCT centers and countries. This lack of standardization hindered the interpretation and comparison of the results from studies evaluating the new techniques

developed for the diagnosis of aspergillosis, such as the sandwich EIA for detection of galactomannan (GM).

In 2002, the Invasive Fungal Infections (IFIs) Cooperative Group published the international consensus defining opportunistic IFIs in immunocompromised patients with cancer and HSCTs [2]. The consensus incorporated the detection of galactomannan antigen to define invasive *Aspergillus* infection. Thus, according to the new definitions, tissue demonstration of hyphae along with a positive culture for *Aspergillus* or with a positive result for *Aspergillus* antigen defines proven or probable IA, respectively.

As more studies evaluating the EIA assay were published, others factors affecting the sensitivity and specificity of the test have emerged. In the present study, implementing the microbiological criteria for the diagnosis of *Aspergillus* infection, we retrospectively evaluated the contribution of serum GM detection in establishing the diagnosis of aspergillosis in HSCT patients with proven IFIs. In addition, we evaluated the impact of serum storage on GM index values and the cut-off levels in the definition of possible or probable aspergillosis.

Materials and methods

Patients enrolled in the HSCT Program of the University of São Paulo Medical School have blood samples and oral swabs taken weekly from conditioning to day + 120 for viral surveillance. These serum samples are routinely stored at −20 °C in the Virology Laboratory of the Institute of Tropical Medicine. When the Platelia® *Aspergillus* test became commercially available in Brazil, we retrospectively reviewed the charts of HSCT recipients with fungal infections who underwent sinus and pulmonary biopsies and selected stored serum samples to evaluate the role of serum detection of galactomannan in the etiological definition of proven IFIs. The study was approved by the Ethical Committee of the University of São Paulo Medical School.

Patients

The group of patients with proven IFI consisted of: (1) One allogeneic HSCT patient with proven *Aspergillus* sinusitis with bone and tissue necrosis. Surgical debridement of the sinuses demonstrated the presence of hyphae and positive cultures confirmed *Aspergillus* sp infection. (2) Ten allogeneic HSCT recipients with clinical symptoms and tomographic images compatible with invasive pulmonary aspergillosis with lung biopsies showing septate hyphae. (3) Four patients with *Candida parapsilosis* fungemia. (4) One patient with disseminated *Fusarium* sp infection as demonstrated by positive blood cultures and histological necropsy findings. (5) One patient with a mycetoma-like *Phialemonium curvatum* infection diagnosed by histology and positive cultures from biopsy samples. To better evaluate the occurrence of false-positive galactomannan detections, we further included serum samples from 22 HSCT patients who did not have any fungal infection during follow-up. Thus, serum samples from 39 patients were analyzed. Patients' characteristics are shown on Table 1.

Sample selection

The serum samples for GM detection were selected within a 15–60 days interval, before and during the period when diagnosis of IFI was established. In patients without any fungal infection during follow-up, the samples were selected at random. The samples selected for this study had been stored at −20 °C for 1 to 5 years. One hundred and seventy one serum samples (median 4, ranging from 3 to 11 per patient) were selected and analyzed.

Impact of serum storage on GM concentration

To evaluate if the serum storage represented an impact on the concentration of GM, after testing, the samples were stored again at the Mycology Laboratory IMTSP-USP, and retested after 4 years.

Antifungal policies

During the study period, no antifungal prophylaxis was routinely adopted among HSCT recipients. Excepted by oral itraconazole, other antifungal agents with activity against molds were not available in Brazil at that time. Prophylaxis was introduced according to the clinician's discretion. Thus, during this period, only one patient

Table 1. Patient characteristics

Variable	Cases (%) $N = 11$	Controls (%) $N = 28$	Total $N = 39$
Gender			
Male	6 (54.5)	14 (50)	20 (51.3)
Female	5 (45.4)	14 (50)	19 (48.7)
HSCT type			
Allogeneic	10 (90.9)	24 (85.7)	34 (87.2)
Autologous	01 (9.1)	04 (14.3)	05 (12.8)
Age (years)			
<10	01 (9.1)	0	01 (2.5)
11–40	09 (81.8)	26 (92.8)	35 (89.7)
>40	01 (9.1)	02 (7.2)	03 (7.7)
Underlying diseases			
CML	05 (45.4)	08 (28.5)	13 (33.3)
AML	0	05 (17.8)	05 (12.8)
ALL	0	04 (14.3)	04 (10.2)
SAA	03 (27.2)	07 (25)	10 (25.6)
MM	01 (9.1)	02 (7.2)	03 (7.7)
NHL	02 (18.2)	02 (7.2)	04 (10.2)
Conditioning			
Bus + Mel	07 (63.6)	19 (67.8)	26 (66.6)
Bus + CIC	03 (27.2)	08 (28.5)	11 (28.2)
TBI + CIC	01 (9.1)	01(3,6)	2 (5.1)
GVHD prophylaxis[a]			
MTX + CYA	03 (30)	09 (37.5)	12 (35.3)
MTX + CYA + PRD	06 (60)	12 (50)	18 (52.9)
CYA + PRD	01 (10)	03 (12.5)	04 (11.7)

[a] In 34 alloHSCT recipients.

who had *Aspergillus* infection before HSCT, received amphotericin B as a secondary prophylaxis. Excepted by cases 1 and 4, the patients were receiving empirical doses of Amphotericin B introduced for the treatment of FUO. Doses were adjusted after tissue biopsies showed the presence of hyphae (Figure 1).

Sandwich EIA

The sandwich EIA *Platelia Aspergillus* (Bio-Rad Laboratories, Marnes La Coquette, France) was performed at The Mycology Laboratory, IMTSP – USP, as described by the manufacturer. Each plate contained a positive control (5 ng of galactomannan per ml), a threshold control (1 ng of galactomannan per ml), and a negative control (no galactomannan). The ratio between the optical density of the test sample and of the threshold control was calculated for each serum sample. A ratio of less than 1.0 was considered negative, a value greater than 1.5 was positive, and those between 1.0 and 1.5 were considered undetermined (gray zone), according to manufacturer recommendations. All positive samples were retested to confirm the result.

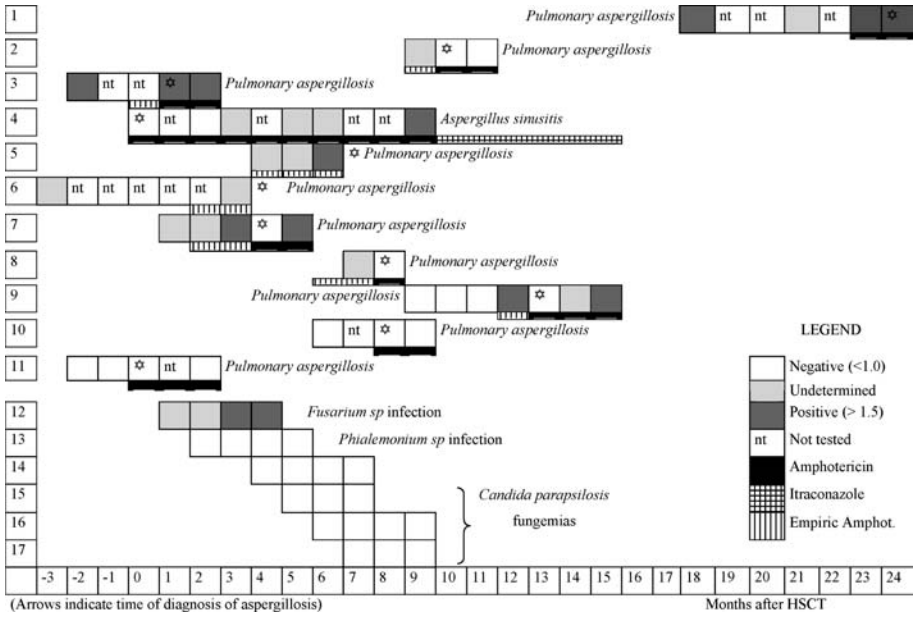

Figure 1. Galactomannan detection and follow-up of patients with proven invasive fungal infection.

Statistics

Data were analyzed for statistical significance by Fisher exact test or by the χ^2 test. Values of $P \leq 0.05$ were considered significant.

Results

Galactomannan detection

Seven patients had at least one sample with positive galactomannan detection during follow-up: One patient had proven *Aspergillus* sinusitis and five had clinical symptoms and tomographic images compatible with invasive pulmonary aspergillosis with a lung biopsy showing hyphae. The remaining one had disseminated *Fusarium* infection confirmed by blood cultures and necropsy histopathologic and mycological findings that also revealed two species of Candida in multiple organs (Table 2).

Three patients had undetermined galactomannan detection according to manufacturer criteria. All had clinical symptoms and tomographic images compatible with invasive pulmonary aspergillosis and a lung biopsy showing hyphae.

Twenty-nine of the 39 patients (74.3%) had galactomannan detection persistently negative. Two of these patients had clinical symptoms and tomographic images compatible with invasive pulmonary aspergillosis. One of these patients was receiving prophylactic amphotericin B uninterruptedly, because of Aspergillus infection before transplantation (Table 2). The remaining 27 belonged to the control group: Twenty-two patients without any fungal infection during follow-up, four patients with candidemia and one patient with Phialemonium curvatum infection. The results of galactomannan detection according to patients' follow-up are shown in Figure 1. All patients with tomographic images suggestive of invasive pulmonary aspergillosis as well as the patient with fusariosis died few weeks after the diagnosis. The patient with *Aspergillus* sinusitis and those with *Candida parapsilosis* fungemia and *Phialemonium curvatum* infection survived.

Table 2. Galactomannan detection according to clinical and mycological diagnosis

Diagnosis	Galactomannan detection (cut-off)			
	(> 1.5)	(1.0–1.5)	(< 1.0)	Total
Invasive fungal infection				
Aspergillus sinusitis	1	0	0	1
Possible pulmonary aspergillosis	**5**	**3**	2	10
Fusarium infection	1	0	0	1
Candidemia	0	0	4	4
Phialemonium infection	0	0	1	1
No fungal infection	0	0	22	22
Total	7	3	29	39

(Number in bold indicate cases of "possible" pulmonary aspergillosis that could be categorized as "probable" by the GM assay with cut-off level at 1.0).

In the present series, only the patient with sinusitis could be considered as proven Aspergillus infection before the EIA assay, according to the international consensus. The use of galactomannan antigen detection added substantial power to the diagnosis of IA categorizing as "probable" 5 of the 10 cases of "possible" aspergillosis (50%). Considering the cut-off level at 1.0, three more cases would be upgraded to "probable" aspergillosis (80%).

Impact of serum storage

Of the 171 serum samples tested, 42 were retested after storage for 4 years. Eight samples (19%) show different results as compared to first detection, always trending to*ward* lower reactivity: one sample that tested positive turned undetermined when retested, and 4 undetermined and 3 positive samples turned negative when retested. Although a decrease in serum reactivity could be observed after storage, statistical analysis did not show significance.

Discussion

A considerable increase in the incidence of IA has been reported in the last decades [3, 5, 6, 16]. Unfortunately, mortality rates have been persistently high in HSCT recipients despite early diagnosis by CT scan followed by early introduction of antifungal therapy.

In the late nineties, some studies have investigated the role of galactomannan detection by EIA in early diagnosis of IA. However, at that moment, the information provided by those studies could be

easily misinterpreted as a consequence of the lack of an international consensus defining IFIs in immunocompromised patients. As a consequence, the reported sensitivity and specificity of the test have varied from 57–100% to 66–100%, respectively [7, 13, 18].

Implementing the definitions of IFIs in immunocompromised hosts established by Ascioglu et al. [2] in 2002, and using the galactomannan detection by EIA, we could categorize as probable aspergillosis 50% of the cases of proven IFI. If we consider a lower cut-off for the reaction (an undetermined result as a true positive) as suggested by some authors [8, 10, 12, 21, 24], this percentage rises to 80%. These data suggest that in patients with tomographic images compatible with fungal infection, *Aspergillus* is more likely to be the causative agent and argue the need for lung biopsies in patients with such radiological images and galactomannan assay positive in consecutive samples.

In this retrospective study, we selected serum samples within a 15-day interval, a periodicity dictated by the CMV surveillance. The need of two consecutive positive samples was not clearly established when the study was initiated. More positive results would probably be obtained if a greater number of serum samples were included in the analysis. However, the economical situation of the different hospitals also should be taken into account when defining the periodicity of the test. Since a good performance of the test has been observed in studies using single assay with cut-offs at 0.8 [10], the cost-effectiveness of galactomannan surveillance at different intervals should be evaluated in larger prospective trials.

Concerning to the cut-off levels, other authors have observed similar findings and suggested a different interpretation of the results independently of manufacturer's recommendations. Verweij et al. [21] suggested that the samples should be considered negative for values between 0.8 and 1.0 and positive for those above 1.0. Martens et al. [8] suggested that results above 1.0 in two consecutive samples should be considered positive. However, other authors observed a decrease in the specificity with cut-off values of 1.0 with no improvement in the sensitivity of the assay [20]. Mennink-Kersten et al. suggested that a cut-off of 0.7 would be the best, according to a receiver operating characteristic analysis. Moreover, the authors stressed that increasing antigen serum levels strongly indicates the presence of IA even if the cut-off level is not achieved and should lead to a diagnostic work up immediately [13]. Maertens et al. observed a good performance of the test using a "static" cut-off at 0.8 (single assay), or a "dynamic" cut-off at 0.5 (two sequential sera) [10]. In 2003, the test was cleared by the FDA for diagnostic use in the US, with a cut-off at 0.5 [24]. More recently, Marr et al. confirmed the results found by the authors mentioned above and further suggested that the cut-off could be safely decreased to 0.5 [12].

In the present study, improved sensitivity without loss in specificity was observed with cut-off index at 1.0. However, lowering the cut-off at 0.5, a greater number of false-positive results would be obtained, probably because the interval between samples was longer than recommended for this lower cut-off.

Some authors have suggested that the freeze-taw step in retrospective studies may decrease the levels of galactomannan concentrations [12]. Considering that the samples were stored from 1 to 5 years before tested, lower reactivity due to prolonged storage could explain those three cases of gray zone and those two negative detections in patients with tomographic images compatible with IPA and lung biopsy showing septate hyphae. The decrease in reactivity after storage at −20 °C has been consistently reported for the latex agglutination test [22] but no data is currently available for the EIA assay.

Retesting the stored samples after a second storage for 4 years, we could observe lower reactivity in 20% of the samples. Although no statistical difference could be observed for the samples tested, these findings may have had clinical significance, in an individual basis. Thus, the prolonged storage itself could have represented a limitation of the present study in the evaluation of the EIA galactomannan assay.

Likewise, the use of amphotericin prophylaxis (and theoretically sustained amphotericin B serum levels) could have limited fungal dissemination through blood stream, reducing the concentration soluble galactomannan antigen in serum [23]. Marr et al. have recently demonstrated a decrease in sensitivity rates from 87.5% to 20% in HSCT recipients with proven IA receiving empirical or prophylactic antifungal compounds [12]. In the present study, one of the two patients with suspected pulmonary aspergillosis and persistently

negative galactomannan detection had received prolonged antifungal prophylaxis during follow-up. In 31 consecutive HSCT recipients receiving prophylactic amphotericin B and followed up from 2 to 8 months, Pereira (2001) did not detected galactomannan in any of the 135 serum samples tested [14]. Unfortunately, the effect of prophylaxis could not be evaluated in the present study due to the small number of patients included.

Other factors affecting the sensitivity of the test, such as the *Aspergillus* species, patient population, patient age, site of the infection, neutropenia, presence of anti-galactomannan antibodies, storage, among others, have recently been reviewed [13]. Of great interest in the setting of HSCT is the observation that the performance of the test may be lower late after transplant, in non-neutropenic, but immunosuppressed hosts [12]. The infection in patients with neutropenia is characterized by intense hyphae growth and high fungal burden, with release of galactomannan. This fact must be taken into account when using the test in non-neutropenic patients.

Excepted by the case of Fusarium infection, no other fungal infection showed cross-reaction with the EIA galactomannan assay. MAERTENS et al. [8] have initially reported 8% of false-positive reactions with the EIA – Platelia Aspergillus. This rate went up to 14% when autopsy findings were included and data reanalyzed [9]. It is important to stress that those studies have been published before the International Consensus defining IFI and false-positive rates can result different of previously published if data is revaluated under current definition of IA.

Actually, the real meaning of these apparently ''false-positive'' results is poorly understood. Reiss et al. [15] have associated these findings to a variety of conditions, such as asymptomatic infection or interference of drugs such as cyclophosphamide. Other authors have considered these ''false-positive'' as true positive results, especially in HSCT recipients. The galactomannan soluble antigen detected in serum could be up taken from some foods (pasta or rice) or antibiotics (amoxicillin, piperacillin), and reached bloodstream through the injured intestinal mucosae by the irradiation, cytotoxic therapy or GVHD [1]. Intravenous administration of piperacillin-tazobactam has also been associated with EIA reactivity in patients without evidence of aspergillosis [19]. Since this antibiotic combination was not available in Brazil when the study was carried out, this possibility was not considered.

Recently, Hamaki et al. [4], observed elevated galactomannan optical density ratio in a HSCT recipient who developed extensive chronic GVHD. In the present series, irrespective to the presence of GVHD, we did not detect the presence of galactomannan in 22 HSCT recipients who did not develop fungal infection during follow-up. Only one of the 39 patients (2.5%) included in this study had a ''false-positive'' result, demonstrating a good specificity of the test.

We also observed that galactomannan surveillance could be used for early introduction of *Aspergillus* pre-emptive therapy in transplant population, as recommended by some authors [11, 17]. Positive or undetermined results were detected one to 4 months (median one month) before the diagnosis of IA in eight of the eleven patients (72.7%) with proven or possible IA (Figure 1).

We conclude that the detection of galactomannan by the EIA test represents an advance in the early diagnosis of aspergillosis in high-risk HSCT recipients. Thus, the introduction of the test in the routine surveillance of these patients seems prudent. However, as with all biological tests, the limits and usefulness must be well known. A better understanding of the kinetics of the GM in different clinical situations is necessary to maximize the benefit of the test in *Aspergillus* surveillance.

Acknowledgements

We thank Dr. C.S. Pannuti and L.S. Vilas Boas, from the Virology Laboratory of the Instituto de Medicina Tropical de São Paulo, for providing the serum samples used in this study. The authors also thank Dr. RX Menezes for statistical analysis, and Drs F.L. Dulley, M.C.A. Macedo, R.L. Silva and R.S. Saboya, from the BMT Program, for providing excellent patient care and without whose support this study would not have been possible.

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