Improved Detection of Circulating *Aspergillus* Antigen by Use of a Modified Pretreatment Procedure[▽]

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Detection of circulating galactofuranose (galf) antigens, including galactomannan (GM), by the Platelia *Aspergillus* (PA) enzyme-linked immunosorbent assay (ELISA) is an important tool in the early diagnosis of invasive aspergillosis (IA). We used a modified pretreatment technique (MT) on consecutive negative PA ELISA plasma samples from IA patients in order to improve the detection of the fungal components present. Plasma samples (52) were collected from healthy donors, and 174 plasma samples with a galactomannan index (GMI) below 0.5 were collected from 25 unclassifiable and 23 IA patients. The PA ELISA reactivity of pretreated samples was determined before (conventional technique [CT]) and after (MT) filtration using a Microcon filter with a 50-kDa cutoff (Millipore). For the MT, the sensitivity of the PA ELISA increased from 42.9% (CT) to 78.6% (MT) using a cutoff for the GMI of 1.5 in the probable and proven group, whereas specificity slightly decreased from 98.7% to 96.1% in the control group. The 10-fold concentration step increased the GMI as high as 121-fold. The MT resulted not only in positive reactivity in samples that tested negative with the CT but also in the earlier detection of antigen by 2 to 17 days.

Invasive aspergillosis (IA) has become the most common fungal infection among patients treated for hematological malignancy and those that undergo solid-organ or allogeneic hematopoietic stem cell transplantation (HSCT) (17, 31, 34). The high mortality, with rates approaching 100% in severely immunocompromised populations, is partly due to the difficulty in establishing a diagnosis at an early stage of infection, since presenting symptoms are nonspecific and the sensitivity of cultures is low (7, 33, 37). Techniques to improve timely diagnosis have focused on the detection of circulating *Aspergillus* markers, including DNA, galactofuranose (galf) antigens (e.g., galactomannan [GM]), and 1,3- β -D-glucan (BG) (1, 3, 10, 19, 22, 39). The galf antigen enzyme-linked immunosorbent assay (ELISA) (Platelia *Aspergillus* [PA]; Bio-Rad, France) is the most widely used assay because it is standardized and commercially available, unlike DNA detection assays. Furthermore, circulating *Aspergillus* antigen can be detected at an early stage of infection in patients with IA (6, 13, 15), whereas the release of DNA might depend on the immune status of the host and is likely to be negligible during the early course of infection in severely immunocompromised patients (21). The Fungitell BG test (Associates of Cape Cod) has recently been approved by the FDA and seems quite promising. However, the assay is not specific for *Aspergillus* species, and the value of the test for early diagnosis of IA has yet to be determined (8, 25–27, 29).

The PA ELISA is based on the rat immunoglobulin M monoclonal antibody EB-A2, which binds the β -(1–5)-galactofuranosyl (galf) side chains of the *Aspergillus* GM molecule (12, 35, 36). In

addition to GM, the culture supernatant of *Aspergillus fumigatus* contains different glycoproteins of 35 to 94 kDa and a large complex of more than 100 kDa, all of which also react with the EB-A2 antibody (12, 32). Other enzymes, including phospholipase C, a phytase, and an alkaline phosphatase, have been shown to contain galf and to react with EB-A2 (24). These findings strongly suggest that the GM antigen is not, in fact, a single molecule but is rather a family of molecules for which expression can be modulated by the immediate fungal environment (24). However, the actual galf antigens that circulate in the body fluids of patients have not been characterized.

In clinical practice, patients at risk are screened by obtaining plasma or serum twice-weekly or more, which is then tested with the PA ELISA using a GM index (GMI) of 0.5 as the cutoff value. The assay is also used to monitor the response to therapy with antifungal agents (16). The performance of the PA ELISA is most favorable among allogeneic HSCT recipients, as circulating antigen can be detected in approximately two-thirds of cases before diagnosis is made by other means (13). While the specificity is generally high (85 to 100%), the sensitivity varies from 33% to 100% for proven and probable cases of IA as defined by the European Organization for Research and Treatment of Cancer-Mycoses Study Group (EORTC-MSG) consensus guidelines for fungal definitions (2, 19, 28). One of the causes for variability is false-negative reactivity. Cases have been described in which, despite other proof of IA and repeated sampling, circulating GM was not detected (4, 14, 30, 40). This might be explained by levels of circulating GM that are below the detection limit of the PA ELISA as a consequence of the encapsulation of infection or the presence of *Aspergillus* antibodies (9, 40). The use of antifungal agents may also reduce circulating GM as has been shown in a recent study (18).

We recently showed that PA reactivity was retained above 50

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kDa after the filtration of plasma samples from patients with IA (23). Consequently, the PA reactivity of plasma samples might possibly increase if the filtration step is employed as a concentration method. In order to test our hypothesis, we included the filtration step in the PA ELISA and tested this new technique on plasma samples from healthy donors and on consecutive negative PA ELISA plasma samples that had been obtained from patients with possible, probable, or proven IA based on the EORTC-MSG consensus definitions for invasive fungal infection as well as those considered unclassifiable (2). Our aim was to increase the lower detection limit of the PA ELISA and to reduce the number of false-negative results too often found with IA patients (4, 14, 40).

MATERIALS AND METHODS

Study design and patient description. The patients included in this retrospective study were at high risk of developing IA because of prolonged neutropenia that resulted from intensive chemotherapy for underlying hematologic disease or for HSCT. Patients received no mold-active antifungal prophylaxis, and amoxicillin-clavulanate and piperacillin-tazobactam were not given routinely. Plasma samples that had been obtained prospectively were collected from 52 healthy donors (52 samples), and plasma samples with a GMI below 1.0 were collected from 25 unclassifiable patients (51 samples), 9 patients with possible IA (38 samples), 9 with probable IA (78 samples), and 5 with proven IA (27 samples). Three of these patients with proven IA were children (ages 4 to 14). All other subjects were adults (age ≥ 16). Possible, probable, and proven diagnoses were based on the EORTC-MSG consensus definitions (2), and patients that did not meet the criteria for possible IA were considered unclassifiable.

PA ELISA. The PA ELISA (Bio-Rad, Marnes-la-Coquette, France) reactivity of plasma samples was determined according to the manufacturer's instructions. Briefly, 50 μ l of a reaction mixture containing horseradish peroxidase-conjugated anti-GM monoclonal antibody EB-A2 was added to each well of a microtiter plate coated with the same monoclonal antibody, EB-A2, and then 50 μ l of pretreated sample was added. After 90 min of incubation at 37°C, the plates were washed five times with washing buffer before 200 μ l of buffer containing tetramethylbenzidine solution was added. Then the plates were incubated for another 30 min in the dark at room temperature, and then 100 μ l of 1.5 N sulfuric acid was added to stop the reaction. The optical density (OD) was read at 450 and 620 nm. Results were recorded as a GMI relative to the OD₄₅₀ of the cutoff sample (i.e., 1.0 ng GM) obtained in the same run. A plasma sample was considered positive when the GMI was ≥ 0.5 .

Pretreatment (conventional technique [CT]). Pretreatment of samples is necessary to dissociate immune complexes and to precipitate proteins. According to the manufacturer's instructions, pretreatment of the R3, R4, and R5 calibration samples was done by mixing 300 μ l of each sample with 100 μ l of treatment solution (4% EDTA), and the mixture was subsequently boiled for 3 min. After centrifugation (10,000 $\times$ g, 10 min), 50 μ l of the supernatant was used for further testing. For plasma samples, the same procedure was followed; however, 750 μ l of each sample was mixed with 250 μ l of treatment solution in order to obtain 50 μ l of supernatant for the conventional test and an amount of 500 μ l for an additional step (modified technique [MT]).

Pretreatment (MT). For this additional step, 500 μ l of supernatant obtained with the standard PA ELISA procedure was introduced into a 1.5-ml tube containing a filter with a 50-kDa cutoff (Microcon filter; Millipore, Bedford, MA). The tube was then centrifuged at 14,000 $\times$ g for about 4 to 6 min until a retentate (which is the part above 50 kDa) of 50 μ l was obtained. The 50- μ l concentrated supernatant was then added to the wells of the microtiter plate. The R3, R4, and R5 calibration samples were pretreated according to the CT. Samples from both techniques can be applied to the same microtiter plate, and ratios are all calculated using the CT.

RESULTS

Determination of a cutoff for the modified pretreatment technique. The mean GMI of healthy donor plasma increased from 0.126 (CT) to 0.632 (MT) due to the concentration step (Table 1; Fig. 1). The mean GMI of the plasma from patients with unclassifiable invasive fungal infection increased from

TABLE 1. Summary of PA ELISA findings using the CT and the MT on plasma samples from negative control groups and patients with IA^a

Patient group	No. of donors/ patients	No. of samples	CT, per sample analysis				CT, per patient analysis				MT, per sample analysis				MT, per patient analysis			
			GMI range	Mean GMI $\pm$ SD	95% CI	No. of positive samples (%)	Mean GMI $\pm$ SD	95% CI	No. of positive patients (%)	GMI range	Mean GMI $\pm$ SD	95% CI	No. of positive samples (%) ^a	Mean GMI $\pm$ SD	95% CI	No. of positive patients (%) ^b		
Healthy donor	52	52	0.043–0.569	0.126 $\pm$ 0.075	0.106–0.147	1 (1.9)							0.136–5.676	0.632 $\pm$ 0.786	0.414–0.851	2 (3.8)		
Unclassifiable	25	51	0.042–0.481	0.134 $\pm$ 0.088	0.109–0.159	0 (0)							0.210–1.857	0.611 $\pm$ 0.321	0.520–0.701	2 (3.9)		
Possible IA	9	38	0.052–0.558	0.196 $\pm$ 0.148	0.148–0.245	2 (5.3)							0.166–5.484	1.257 $\pm$ 1.276	0.837–1.676	11 (28.9)		
Probable IA	9	78	0.039–0.959	0.245 $\pm$ 0.209	0.198–0.292	10 (12.8)							0.158–6.377	1.595 $\pm$ 1.459	1.266–1.924	31 (39.7)		
Proven IA	5	27	0.065–0.938	0.370 $\pm$ 0.316	0.245–0.495	8 (29.6)							0.067–7.376	2.319 $\pm$ 2.332	1.397–3.242	14 (51.9)		

^a CI, confidence interval. SD, standard deviation. Positive samples/patients were ≥ 0.500 for the CT. Positive samples/patients were ≥ 1.500 for the MT.

^b The cutoff value of the MT ELISA was randomly set at 1.5 in order to be just above the one-time standard deviation of the mean GMI.

FIG. 1. Effect of modified pretreatment technique (MT) on GMI of negative control plasma (healthy donors and unclassified IA patients) and on GMI of plasma from IA patients. Horizontal bars indicate the mean GMI values in each group. Horizontal lines indicate the cutoff values (CV) for the two different methods, i.e., the cutoff of the CT is 0.5, and the cutoff for the MT was set at 1.5.

0.134 (CT) to 0.611 (MT) (Table 1; Fig. 1). Both groups were used as negative controls, and an arbitrarily chosen cutoff value of 1.5 was selected for the MT in order to keep the false positivity rate below 4%. One of the healthy donor samples that was positive with the MT was also positive with the CT. The two samples in the unclassifiable group that became positive with the MT were from the same patient.

Samples from possible, probable, and proven IA cases. The mean plasma GMI values of the possible, probable and proven IA groups all increased, from 0.196, 0.245, and 0.370 to 1.257, 1.595, and 2.319, respectively (Table 1; Fig. 1). The concentration step increased the GMI on average 6.4-fold for the samples of possible IA cases, 6.5-fold for those of the probable IA cases, and 6.3-fold for those of the proven IA cases. The GMI values increased, ranging from 1.8- to 41.4-fold, 1.1- to 121.3-fold, and 0.7- to 48.9-fold for the samples from the cases of possible, probable, and proven IA, respectively.

Analysis per patient, in which only the highest plasma GMI value was taken, showed that the mean GMI values increased from 0.269, 0.512, and 0.466 to 1.948, 2.857, and 3.755 for the samples of possible, probable, and proven IA cases, respectively, resulting in 7.2-, 5.6-, and 8.1-fold increases due to the concentration step (Table 1; Fig. 2).

Effect of the modified pretreatment technique on control groups and IA patients using the new cutoff. The sensitivity of the PA ELISA increased using the MT and a cutoff for the GMI of 1.5, as shown by the number of negative plasma samples (i.e., the GMI was <0.5) that turned positive after using the MT. Whereas 1 (2%) additional sample and 2 (3.9%) additional samples turned positive in the healthy group of unclassifiable cases of IA (Table 1; Fig. 1), in the possible, probable, and proven IA cases, 9 (25%), 21 (30.9%), and 6 (31.6%) additional plasma samples turned positive (Table 1; Fig. 1). Analysis per patient by use of the highest GMI of each

control showed that one sample (2%) turned positive in the healthy donor group and that one (4%) sample turned positive in the group of unclassifiable IA cases (Table 1). In the possible, probable, and proven IA groups, the results from two (28.6%), two (40%), and three (100%) additional patients, respectively, turned positive (Table 1; Fig. 2). Percentages were calculated using the total number of negative samples or patients.

The sensitivity increased from 42.9% (6/14) to 78.6% (11/14) with the MT (per patient analysis) when only the probable and proven cases were considered true cases of IA (true-positive group). By contrast, the specificity decreased slightly from 98.7% (76/77) to 96.1% (74/77) when using the healthy controls and patients with unclassifiable IA as the true-negative group.

Effect of the modified pretreatment technique on individual patients with possible, probable, or proven IA. The samples from 10 patients were further analyzed in order to follow the course of the level of antigen in consecutive plasma samples (Table 2). Circulating antigen was detected 13 days earlier in one patient with possible IA (Fig. 3A) whose high-resolution computed tomography (HRCT) showed a nonspecific pulmonary infiltrate. The patient was wrongly diagnosed with lung carcinoma after 16 days of antifungal treatment with amphotericin B followed by liposomal amphotericin B, and IA became apparent only after oral treatment with itraconazole and the slow resolution of the infiltrates on HRCT. One patient with a persistently negative GMI using the CT was positive with the MT 8 days before probable IA was diagnosed and antifungal therapy was started (Fig. 3B). Nine months earlier when the patient underwent an HSCT, plasma samples also became GM positive with the MT, but the patient could not be classified as IA. Circulating antigen was detected 2, 3, and 17 days earlier in three patients with probable IA after using the

FIG. 2. Effect of modified pretreatment technique (MT) on GMI of plasma from IA patients. Analysis was performed per patient, which means that only the highest GMI value was taken. Horizontal bars indicate the mean GMI values in each group. Horizontal lines indicate the cutoff values (CV) for the two different methods, i.e., the cutoff of the CT is 0.5, and the cutoff for the MT was set at 1.5.

modified method (Table 2; Fig. 3C and D). In patient 3 (Fig. 3C), there was an initial decrease in the GMI after starting antifungal treatment, followed by an increase in the GMI during liposomal amphotericin B treatment shortly before the patient died. Patient 4 showed an increase in the GMI, which was closely followed by fever, thoracic pain, and a pleural rub. The start of antifungal treatment after the development of dyspnea and nonspecific infiltrates on HRCT resulted in a decrease of the GMI (Fig. 3D). Two patients with proven IA were positive 3 and 2 days earlier than with the CT (Fig. 3e, Table 2). Patient 6 was a case of hepatic IA, which has recently been published (38). The MT GMI pattern coincided with the CT GMI pattern and was out of range when the CT GMI increased above 1.0. Furthermore, a broncho-alveolar lavage (BAL) sample taken 2 weeks after HSCT showed an increase of the GMI from 0.3 to 2.9 after using the MT for plasma samples. Circulating antigen was also detected in the samples of three patients with proven IA after the MT was used, whereas those tested with the CT were persistently negative

(Table 2, patients 8 to 10). The plasma samples with the highest GMI values (MT) increased from 0.327 (CT) to 1.549 (patient 8), from 0.080 (CT) to 3.910 (patient 9), and from 0.360 (CT) to 1.929 (patient 10).

DISCUSSION

Timely diagnosis of IA is essential for the survival of patients suffering from this fungal infection. However, invasive procedures are often precluded; hence, current diagnostic techniques are based on the detection of circulating biological markers early in the disease course. The PA ELISA is the most widely used assay, but there are several problems related to antigen detection using this assay. Circulating antigen is not detected in the samples of some patients with proven IA despite repeated sampling (false negative) (40). Also, the performance of antigen detection might be reduced in patients receiving antifungal agents for prophylaxis or treatment. Other causes for failure to detect antigenemia could be the reduced

TABLE 2. Patient characteristics and effects of the MT on first day of positive GMI result

Patient no.	Age (yr), sex	Underlying disease ^a	Site of infection	EORTC-MSG classification	Figure/reference	No. of days MT GMI was positive before CT GMI was positive or comparison of discordant results
1	47, F	AML	Lung	Possible IA	Fig. 3A	13
2	48, M	ALL	Lung	Probable IA	Fig. 3B	Negative (CT) to positive (MT)
3	60, F	AA	Lung	Probable IA	Fig. 3C	3
4	44, M	AML	Lung	Probable IA	Fig. 3D	17
5	66, M	ALL	Lung	Probable IA		2
6	53, F	Lymphoma	Liver	Proven IA	Fig. 3E; 38	3
7	29, M	MDS	Lung	Proven IA	11	2
8	4, M	CGD	Lung	Proven IA	40	Negative (CT) to positive (MT)
9	14, M	AML	Skin	Proven IA		Negative (CT) to positive (MT)
10	5, M	ALL	Lung	Proven IA		Negative (CT) to positive (MT)

^a AA, aplastic anemia; ALL, acute lymphatic leukemia; AML, acute myeloid leukemia; CGD, chronic granulomatous disease; MDS, myelodysplastic syndrome.

FIG. 3. Levels of circulating GM detected by CT (□) compared with the levels detected by MT (■) in plasma from IA patients. Horizontal lines indicate the cutoff values for the two different methods, i.e., the cutoff for the CT (dashed line) is 0.5, and the cutoff for the MT (solid line) was set at 1.5. OR, out of range; ITZ, itraconazole; VOR, voriconazole; AMB, liposomal amphotericin B; AmpB, amphotericin B. (A) Patient 1, possible IA; (B) patient 2, probable IA; (C) patient 3, probable IA; (D) patient 4, probable IA; (E) patient 5, proven IA.

release of *galf* antigens due to environmental factors, such as pH and available nutrients at the site of infection (19).

In order to reduce the number of false-negative PA ELISA results, we tested a modified pretreatment technique to see if we could increase the concentration of circulating antigen that might be present in low amounts in negative plasma samples. The filtration of plasma improved the recovery of *galf* antigens by the PA ELISA. The background ELISA reactivity also increased, although the GMI values of >90% of negative samples from healthy donors and unclassifiable patients remained below 1.0 after filtration, indicating that false-positive reactivity does not significantly increase when using the modified method. Some increase in the background noise is inevitable, since other blood components are also concentrated with this filtration step. In addition, two samples that turned positive were from a patient with unclassified infections that were later defined as probable IA during a second episode 9 months later (patient 2). This might suggest transient antigenemia during the first neutropenic episode. In order to compensate for the increased background reactivity, a cutoff value of 1.5 was chosen for the modified pretreatment method. This value is probably too high, and a new cutoff should be determined using receiver operator characteristic curve analysis. However, the samples of the patients with possible, probable, and proven IA used in this pilot study were selected based on their negative GM reactivity. In order to determine the optimal cutoff, patients and samples should be selected prospectively as described previously in a study from Maertens et al. (16).

In the group of patients with possible, probable, and proven IA, the increase of the plasma GMI was 6.3 to 6.5 on average. This includes samples that showed only a minor increase, since they were probably true negatives. The sample volume was reduced from 500 μ l to 50 μ l by filtration, so the concentration was 10-fold. The concentration of R4 calibration samples, which contain 1 ng/ml of GM, resulted in a mean GMI of 7.3. However, the dilution of these concentrated samples resulted in a calculated GMI of 10 to 11 (i.e., $10 \times$ GMI 1 or GMI 1.1). Like other semiquantitative immunoassays, samples with a high OD are underestimated with respect to true levels of GM as measured in ng/ml, since the coated anti-GM antibodies show saturation kinetics, better known as the prozone effect. In order to use the PA ELISA quantitatively, samples have to be diluted to be within range of the cutoff value. The concentration of patient plasma samples showed the same underestimation. Most of the time, the expected 10-fold increase in the GMI was not observed. However, the increase could be as high as 121-fold, which was shown in a sample from a probable IA case that increased from 0.040 to 4.850 with the MT. The reason for this might be the fact that in the course of infection, different *galf*-containing molecules circulate in the patient's plasma and contribute differently to PA ELISA reactivity. Furthermore, *galf* molecules containing a lipid residue, like the glycosylphosphatidylinositol-anchored GM found by Costachel et al., might form micellar structures with increased reactivity (5, 20).

The actual size and conformation of circulating *galf* antigens remain to be determined. The circulating molecule(s) in vivo is larger than the 20 kDa of the purified GM molecule because this molecule is not contained above 50 kDa (unpublished results). These results confirm the findings of Morelle et al.,

who showed that sera from patients with aspergilloma contained antibodies against two galactomannoproteins of 55 to 65 kDa (24). However, membrane filtration is not an accurate way of determining molecular mass. Molecules might attach to the membrane itself, or the molecular conformation might be linear and not globular, which could mean that a molecule of 35 kDa is retained above a 50-kDa filter. In addition, R4 and R5 standard ELISA samples are also retained above 50 kDa. So it seems that serum spiked with purified GM results in the binding of the GM molecule to serum components. We also tested 30-kDa Microcon filters for the concentration procedure, but the results were comparable to the 50-kDa filtrations (results not shown). We chose to use the 50-kDa filter in the present study, as the filtration time depends on the pore size of the filter.

The MT in the PA ELISA resulted in earlier detection of circulating *galf* antigen (e.g., GM) as well as positive reactivity in samples that tested negative with the CT. These findings indicate that low levels of fungal antigen(s) are present in some patients with IA, including patients that are treated with antifungals and nonneutropenic patients. The patient with chronic granulomatous disease showed consistently negative GMI values in 26 plasma samples over a 6-month period (40). Unfortunately, only one plasma sample (obtained on 16 November 1998) was available for this study; however, the sample was positive after concentration, which clearly shows the advantage of the MT. Furthermore, *Aspergillus* DNA was undetectable in the plasma 19 and 4 days before and 7 days after (40) the concentrated plasma sample became positive for *Aspergillus* antigen.

Preliminary results showed that the MT might also be applicable to other body fluids, e.g., BAL or cerebrospinal fluid, and other polysaccharide antigens, e.g., mannan (Platelia *Candida*; Bio-Rad, France). One BAL sample (patient 6) was positive using the concentration step, suggesting that this rare case of primary hepatic IA (38) might also have originated from the lung.

In conclusion, the filtration of plasma samples clearly improved the sensitivity of *Aspergillus* antigen detection without a significant decrease in specificity. The filtration technique also allowed earlier detection of GM, suggesting that this modification might prove valuable for the screening and diagnosis of IA among patients at risk.

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