Rev. Inst. Med. Trop. Sao Paulo
55(3):145-147, May-June, 2013
doi: 10.1590/S0036-46652013000300001

VARIABILITY IN GALACTOMANNAN DETECTION BY PLATELIA *Aspergillus* EIA™ ACCORDING TO THE *Aspergillus* SPECIES

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SUMMARY

Here we investigate the extent to which different *Aspergillus* species release galactomannan (GM) *in vitro*. Marked variability was observed in GM reactivity between and within *Aspergillus* species, with *A. terreus* strains showing the highest GM indexes. The *in vivo* significance of these findings remains to be determined.

KEYWORDS: Invasive aspergillosis; Galactomannan; Diagnosis.

INTRODUCTION

Galactomannan (GM) is a termostable polysaccharide that is a component of the cell wall of a diverse range of fungi, including those belonging to the genus *Aspergillus*[1]. Since GM is released during fungal hyphal growth, its detection in circulation or in other body fluids allows for an early diagnosis of invasive aspergillosis (IA)[4]. However, when interpreting a positive GM result in a patient with suspected IA, one might consider the variety of factors that can potentially interfere with the test, including antibiotic use, dietary factors and cross-reactivity with non-*Aspergillus* fungi[3,9,10]. Although early investigations have suggested that GM levels may vary among *Aspergillus* species[7], there is still only very limited data to support this. Here we report that marked inter-species and intra-species variation occurs in the *Aspergilli*, considering GM release *in vitro*.

MATERIALS AND METHODS

Exoantigen testing was based on the method described by SWANINK *et al.*[8], in 12 well-characterized *Aspergillus* strains. All strains were kindly provided by Myconostica (National Aspergillosis Centre, UK). These included *A. fumigatus* (n = 4) strains Af294, Af10, Af71, and Af13073; *A. flavus* (n = 2), Afl5, and Afl16883; *A. niger* (n = 3), An9029, An1015, and An186; *A. terreus* (n = 2), At10071, and At49; and *A. nidulans* (n = 1), strain NEQAS UK. In brief, strains were subcultured to obtain pure young cultures in Sabouraud at 25 ºC for 48 h (*A. fumigatus, A. flavus* and *A. niger*) or 96 h (*A. terreus* and *A. nidulans*). One loop of each strain was used to prepare the inoculum in liquid Sabouraud medium, which was adjusted by spectrophotometry (530 nm) to 80-82% T ($2x10^5$ to $2.5x10^6$ FC/mL). Strains were incubated at 35 ºC for 48-96 h, centrifuged for five min with 2500 rpm and then filtered (Millipore 0.45 µm). Tenfold dilutions were applied successively, and reactivity of the sandwich ELISA to GM detection was determined in duplicate at the 10^{-6} dilution, using Platelia *Aspergillus* EIA kit (BioRad). The Platelia *Aspergillus* test was performed according to manufacturer's instructions. In short, 100 µL of Platelia treatment solution (4% ethylenediaminetetraacetic acid solution) was added to 300 µL of the adjusted innoculum, homogenized, and heated to 120 °C for six min in a heat block, followed by centrifugation at 10,000*g* for 10 min. Next, 50 µL of the supernatant and 50 µL of the horseradish peroxidase labeled monoclonal antibody (EBA-2) were incubated in antibody precoated microplates for 90 min at 37 °C. The plates were washed five times and incubated with 200 µL of substrate chromogen reaction solution for 30 ± 5 min in the dark at room temperature. The reaction was stopped with 1.5 N sulfuric acid solution, and the plates were read at an optical density (OD) of 450 nm, with a reference filter of 620/630 nm. Positive, negative, and cut-off controls were incorporated in each assay. GM results were expressed as optical densities (OD) – samples were considered positive when the ratio between the OD observed for the sample and the mean cut-off OD was > 0.5. Strains resulting in negative GM readings were again tested in duplicate at the previous dilution (10^{-5}).

RESULTS

Median GM indexes (range) at the 10^{-6} dilution were: *A. terreus*, 3.82 (1.30 to > 6.35); *A. nidulans*, 1.3; *A. fumigatus*, 0.88 (0.17-1.50); *A. niger*, 0.28 (0.24-0.79), and *A. flavus*, 0.16 (0.14-0.18). Considering *A. fumigatus* as the comparator, GM reactivity for the other *Aspergillus* species was 434% for *A. terreus*, 148% for *A. nidulans*, 32% for *A. niger*, and 18% for *A. flavus*. At the 10^{-6} dilution 50% of the *A. fumigatus* strains

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Table 1
Marked variations are observed in galactomannan reactivity when different *Aspergillus* species and strains are tested *in vitro*. All experiments were performed in duplicate at the 10^{-6} dilution using Platelia *Aspergillus* EIA kit (Bio-Rad). Optical densities represent mean values

Aspergillus strain	Galactomannan optical density
A. fumigatus (Af 294)	1.50
A. fumigatus (Af 10)	0.33
A. fumigatus (Af 71)	0.17
A. fumigatus (Af 13073)	1.43
A. flavus (Afl 5)	0.18
A. flavus (Afl 16883)	0.14
A. niger (An 9029)	0.28
A. niger (An 1015)	0.79
A. niger (An 186)	0.24
A. terreus (At 10071)	1.30
A. terreus (At 49)	> 6.35
A. nidulans (NEQAS UK)	1.30

(Af10 and Af71) were GM negative, as well as the two *A. flavus* strains and two out of the three *A. niger* strains tested (An186 and An9029). These were all positive at 10^{-5} dilution, with GM indexes varying from 0.51 to 1.02.

DISCUSSION

This study showed that marked variations occurred in GM release among *Aspergillus* species, with the highest GM indexes being observed for *A. terreus*. On the other hand, *A. niger* and *A. flavus* showed less reactivity in the GM ELISA test, in comparison to *A. fumigatus*. Previous studies had already suggested that *in vitro* GM release may vary according to the *Aspergillus* species being tested[3,8]. However, varied results have been observed among studies. For instance, two previous investigations found that *A. niger* isolates produced more GM than *A. fumigatus*[7,8]. Even though limited GM release by *A. flavus* was documented in our study as well as in the study by MENNINK-KERSTEN *et al.*[7] (which was presented in abstract form only), SWANINK *et al.*[8] showed that GM reactivity was 7% higher in *A. flavus*, in comparison to *A. fumigatus*. Common to all studies was the observation that *A. terreus* produces more GM than *A. fumigatus*[7,8].

One explanation for the lower amount of *in vitro* GM release in *A. niger* and *A. flavus* strains, in comparison to *A. fumigatus*, is the fact that *A. fumigatus* clearly demonstrates a higher germination rate at 37 °C[2], a temperature that was similar to the incubation temperature used in our experiment (35 °C). However, considering that both *A. terreus* and *A. nidulans* showed higher GM release in comparison to *A. fumigatus*, although they had been submitted to a higher period of incubation (96 h *versus* 48 h), these two species grew slower than *A. fumigatus* at 37 °C[2], and therefore germination rate by itself does not seem to explain the observed difference in GM indexes. Another potential explanation for such variability in GM levels is related to the quantity of β-D-galactofuranosidase produced by different *Aspergillus* strains. By degrading GM epitopes, the enzyme may interfere with the immunoassay by blocking the antigen-antibody complex formation. The demonstration that *A. fumigatus* produces 2-20% of the β-D-galactofuranosidase produced by *A. niger*[6] suggests that *A. fumigatus* could react more avidly in the GM EIA test than *A. niger*, as found in the current study. The influence of the β-D-galactofuranosidase on GM levels is not completely clear, since similar enzyme activity has been observed for patients with IA caused by *A. fumigatus* in spite of serum GM levels[5]. The impact of other variables, such as medium pH, glucose concentration and development fungal stage could not be addressed in the present study since all strains were submitted to the same test condition.

If the results of our study are confirmed *in vivo*, then differences in serum GM levels observed among patients with IA may also be related to the *Aspergillus* species causing the infection. As a result, caution would be required when diagnosing IA based on the use of a universal cut-off for serum GM detection (i.e., 0.5). In fact, a clinical study involving haematological patients showed higher sensitivity of the Platelia *Aspergillus* EIA test in the diagnosis of IA when the infection was caused by *Aspergillus* species other than *A. fumigatus* (49% *vs.* 13%, in comparison to *A. fumigatus*, respectively)[5]. One interesting finding of our study was the observation that variability in GM levels also occurred within each of the *Aspergillus* species evaluated. For instance, the magnitude of such differences was as high as ninefold, and this could potentially explain the difference in results obtained among studies.

In conclusion, this study showed that marked variations occurred in GM levels among distinct *Aspergillus* species, as well as among different strains belonging to the same species. It remains to be determined whether such differences could affect the determination of GM concentrations *in vivo*.

RESUMO

Variabilidade na detecção de galactomanana pelo Platelia *Aspergillus* EIA® de acordo com a espécie de *Aspergillus*

O estudo objetivou investigar a liberação *in vitro* de galactomanana (GM) em distintas espécies patogênicas de fungos do gênero *Aspergillus*. Grande variabilidade foi detectada tanto intra quanto inter espécies, sendo as cepas da espécie *A. terreus* relacionadas aos maiores índices de GM detectados. O significado *in vivo* destes achados permanece em aberto, porém merece investigação.

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Received: 10 July 2012
Accepted: 3 October 2012