Effect of involved *Aspergillus* species on galactomannan in bronchoalveolar lavage of patients with invasive aspergillosis

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### Abstract

**Purpose.** The detection of galactomannan (GM) in bronchoalveolar lavage (BAL) fluid is an important surrogate marker for the early diagnosis and therapeutic monitoring of invasive aspergillosis (IA), regardless of the involved species of *Aspergillus*. Here, we utilized the Platelia *Aspergillus* GM enzyme immunoassay (Bio-Rad) to evaluate the GM index in BAL fluid samples from patients with proven, probable or putative IA due to *Aspergillusflavus* versus *Aspergillusfumigatus*.

**Methodology.** In a prospective study between 2009 and 2015, 116 BAL samples were collected from suspected IA patients referred to two university hospitals in Tehran, Iran.

**Key findings.** According to European Organization for Research and Treatment of Cancer and Mycoses Study Group and Blot criteria, 35 patients were classified as IA patients, of which 33 cases tested positive for GM above 0.5 and, among these patients, 22 had a GM index ≥1. Twenty-eight were culture positive for *A. flavus* and seven for *A. fumigatus*. The GM index for *A. flavus* cases was between 0.5–6.5 and those of *A. fumigatus* ranged from 1 to 6.5. The sensitivity and specificity of a GM index ≥0.5 in cases with *A. flavus* were 86 and 88 % and for *A. fumigatus* patients were 100 and 73 %, respectively.

**Conclusion.** Overall, the mean GM index in patients with *A. fumigatus* (3.1) was significantly higher than those of *A. flavus* (1.6; P-value=0.031) and the sensitivity of GM lower for *A. flavus* when compared to *A. fumigatus*. This finding has implications for diagnosis in hospitals and countries with a high proportion of *A. flavus* infections.

### INTRODUCTION

Invasive aspergillosis (IA) is one of the most significant and challenging invasive fungal infections, and is a major cause of morbidity and mortality in high-risk patients [1, 2]. This disease occurs mainly in patients with a markedly impaired immune system. After *A. fumigatus*, *A. flavus* is the second leading cause of invasive and non-invasive aspergillosis [3, 4]. In many tropical and sub-tropical countries, including India, Saudi Arabia, Pakistan, Iraq, Turkey and Iran, *A. flavus* has most frequently been reported as the predominant etiological agent causing mainly cerebral or sino-orbital aspergillosis, or ocular infections [5, 6]. Early diagnosis of IA remains a challenge, but it is important for better therapeutic outcomes [7]. Galactomannan (GM) antigen, a polysaccharide secreted from the *Aspergillus* spp., correlates with the growth phase of the fungus [8–11]. *Aspergillus* GM antigen detection in serum and...
bronchoalveolar lavage (BAL) fluid by the Bio-Rad Platelia sandwich enzyme immunoassay (EIA, Bio-Rad) has been studied extensively [12, 13]. Several previous studies have also evaluated the GM assay in BAL samples in various patient groups and conditions; they reported a higher parallel sensitivity and specificity in comparison to serum specimens [14–16]. According to the revised European Organization for Research and Treatment of Cancer and Mycoses Study Group (EORTC/MSG) diagnostic criteria, a positive BAL GM test is a mycological criterion of IA in association with host and clinical or radiological findings [17]. The GM assay has gained widespread acceptance as a sensitive method for the early diagnosis and therapeutic monitoring of IA by applying it directly to specimens from the target organ [18, 19]. In the present study, we evaluated the GM index in BAL samples from patients with proven, probable or putative IA due to A. flavus versus A. fumigatus using Platelia Aspergillus EIA.

METHODS
Patient
In a prospective study, 116 BAL fluid samples were collected from patients with pulmonary disorders who were referred to the bronchoscopy section of the Shariyat and Imam Khomeini Hospitals, Tehran University of Medical Sciences, Iran, from June 2009 to October 2015. Written informed consent was taken from all participants. We included all data on suspected patients of IA (host factors, clinical features and mycological data) as defined by EORTC/MSG [17]. In addition, we used the clinical algorithm suggested by Blot et al. [20], which includes the endotracheal aspirate culture to demonstrate proven or putative IA in the existence of matchable symptoms, unusual thoracic medical imaging and either host factors or BAL fluid positive for Aspergillus on direct examination and culture.

BAL sampling
Fiberoptic bronchoscopy (Olympus BF20D) with BAL was performed if feasible. The sampling area was selected based on the infiltrate location on the chest radiograph. Then, 50 ml sterile normal saline was injected through the device. The suction channel of the bronchoscope was used to aspirate 25–30 ml fluid, yielding clinical samples, which were collected into sterile tubes and immediately transferred to the Medical Mycology Laboratory at Mazandaran University of Medical Sciences.

Processing of BAL fluid specimens
All BAL samples were centrifuged for 5 min at 5000 r.p.m. and then the supernatants were stored at −80°C. The remaining sediment was used for direct microscopic examination and culture. The smear from each sample was mounted with 20% potassium hydroxide (KOH) and Calcofluor White to detect the hyphal elements. Sabouraud dextrose agar supplemented with chloramphenicol (40 µg ml⁻¹) was used for the isolation of fungi. The plates were incubated at 30°C for 5 days. Aspergillus species were identified by morphological characteristics and molecular sequencing of beta tubulin [21].

GM Platelia Aspergillus assay
The Platelia Aspergillus GM EIA (Bio-Rad Laboratories, Marnes-la-Coquette, France) was used to detect GM on centrifuged BAL fluid specimens, according to the manufacturer’s manual, using 300 µl of the BAL sample. Positive and negative control ELISA kits were included in each test. The results were recorded as GM indexes ≥0.5 and ≥1.

Data analysis
The mean age, standard deviation, sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), receiver operating characteristic curve (ROC) and interactive dot diagram were calculated for each value using MedCalc software version 14.8. Positive and negative likelihood ratios were computed by an online website (http://araw.medec.uic/cgi-bin/testcalc.pl). The t-test was applied to compare the GM index of A. flavus and A. fumigatus among positive IA patients. The estimated prevalence of invasive pulmonary aspergillosis (IPA) was determined by the following formula: (proven IPA+probable IPA)/(proven IPA+probable IPA+patients without IPA).

RESULTS
The mean age of the 116 studied patients was 46±15.4 ranging from 12 to 78 years. Seventy-three (62.9%) patients were male. Table 1 shows clinical characteristics of all studied patients in the current study. Of the 116 BAL samples, 26/116 (22.4%) and 38/116 (32.7%) were positive by direct microscopy and culture, respectively. Out of 116 patients, 35 cases were classified as IA. According to EORTC/MSG criteria [17], two cases were classified as proven IA and 16 cases as probable IA. In addition, using the clinical algorithm suggested by Blot et al. [20], 17 cases were categorized as putative IA (Table S1, available with the online Supplementary Material). Three cases were also defined as possible IA based on the underlying host factor and radiological features, however these cases were excluded from the final analysis since mycological criteria were lacking.

Using polymerase chain reaction (PCR)-sequencing targeting the beta-tubulin region of rDNA, A. flavus (28, 80%) and A. fumigatus (7, 20%) were identified. Out of 35 patients with IA, 10 (28.6 %) had underlying haematologic malignancy. Seventeen patients had evidence of invasive airway infection with nodules, ulceration, pseudomembrane and mass obstruction of the airway; therefore invasive Aspergillus tracheobronchitis is an alternative or additional disease classification to IPA for these patients. There were three patients with cavities and prior tuberculosis or a lung injury from chemical warfare who may be better classified as having chronic pulmonary aspergillosis (CPA), but Aspergillus IgG detection was not done to confirm the diagnosis. Five patients with lung cancer probably had IA, although this is not one of the host groups in the EORTC/MSG criteria. Likewise, there were five patients with COPD,
two of whom had *Aspergillus* tracheobronchitis, in whom the precise and complete *Aspergillus* diagnosis was unclear.

At a GM index ≥0.5, of 116 patients, 33 (28.4%) with IPA and five (4.3%) cases without IPA had positive results. However, at a GM index ≥1, of these studied patients, 22 (19.0%) cases had positive results. Ten patients with IA had a GM index ≥2, including two cases with proven IA. In IA patients with positive GM, the GM index ranged from 0.5 to 6.5. In positive GM cases with a positive BAL fluid culture, the mean GM index (1.9) was higher than those with negative cases with a negative culture (0.34). The mean of the GM index in IA patients with *A. fumigatus* (3.1) was significantly higher than those of *A. flavus* (1.6; *P* = 0.031).

Table 2 shows the sensitivity and specificity, positive and negative predictive values of GM detection in all BAL samples for GM indexes ≥0.5 and ≥1. The sensitivity and specificity of a GM index ≥0.5 in cases with *A. flavus* were 86 and 88% and for *A. fumigatus* patients were 100 and 73%, respectively.

The area under the ROC curve (AUC) for a GM index ≥0.5 in all BAL samples was 0.91 (95% CI, 92.1–99.8, *P* < 0.0001) while at a GM index ≥1 it was 0.86 (95% CI, 94.5–96.6, *P* < 0.0001). On the other hand, the AUC using the standard cut-off GM index ≥0.5 for *A. flavus* culture-positive BAL samples was 0.89 (95% CI, 91.0–99.5, *P* = 0.0001) and for *A. fumigatus* it was 0.94 (95% CI, 95.1–100, *P* < 0.0001).

**Table 1.** Clinical characteristics of the 116 patients studied

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>All patients</th>
<th>Patients with IA</th>
<th>Patients without IA</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of patients</td>
<td>116</td>
<td>35</td>
<td>81</td>
</tr>
<tr>
<td>Men/women</td>
<td>73/43</td>
<td>23/12</td>
<td>30/31</td>
</tr>
<tr>
<td>Age, mean±SD, year</td>
<td>46±15.4</td>
<td>44.7±16.2</td>
<td>46.6±15.2</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>IA classification</th>
<th>No. of patient</th>
<th>EORTC/MSG criteria</th>
<th>Blot et al. criteria</th>
<th>Non-IA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Underlying disease</td>
<td>No. of proven IA</td>
<td>No. of probable IA</td>
<td>No. of putative IA</td>
<td>No. of non-IA</td>
</tr>
<tr>
<td>Haematological malignancies</td>
<td>29</td>
<td>0</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>COPD</td>
<td>16</td>
<td>0</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>Lung cancer</td>
<td>10</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Pulmonary and respiratory disorders</td>
<td>21</td>
<td>0</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Organ transplantation</td>
<td>16</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Autoimmune diseases</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Renal failure</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Tuberculosis with cavity</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>Breast cancer</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>ICU patients</td>
<td>6</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>CGD</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total cases</td>
<td>116</td>
<td>2</td>
<td>16</td>
<td>17</td>
</tr>
</tbody>
</table>

IA, invasive aspergillosis; No., number; SD, standard deviation; COPD, chronic obstructive pulmonary disease; ICU, intensive care unit; CGD, chronic granulomatous disease.

**Table 2.** Sensitivity, specificity and predictive values for two cut-off GM indexes ≥0.5 and ≥1 for 116 collected BAL samples

<table>
<thead>
<tr>
<th>Criteria</th>
<th>GM index</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>PPV</th>
<th>NPV</th>
<th>95% CI</th>
<th>AUC value</th>
<th>Cost value*</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>A. flavus</em></td>
<td>≥0.5</td>
<td>86</td>
<td>88</td>
<td>52</td>
<td>97</td>
<td>91.0–99.5</td>
<td>0.89</td>
<td>0.137</td>
<td><em>P</em> &lt; 0.05</td>
</tr>
<tr>
<td>≥1</td>
<td>57</td>
<td>89</td>
<td>47</td>
<td>92</td>
<td>94.2–96.3</td>
<td>0.82</td>
<td>0.157</td>
<td><em>P</em> &lt; 0.05</td>
<td></td>
</tr>
<tr>
<td><em>A. fumigatus</em></td>
<td>≥0.5</td>
<td>100</td>
<td>73</td>
<td>40</td>
<td>100</td>
<td>95.1–100</td>
<td>0.94</td>
<td>0.226</td>
<td><em>P</em> &lt; 0.05</td>
</tr>
<tr>
<td>≥1</td>
<td>86</td>
<td>88</td>
<td>56</td>
<td>97</td>
<td>91–99.5</td>
<td>0.92</td>
<td>0.123</td>
<td><em>P</em> &lt; 0.05</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>≥0.5</td>
<td>91</td>
<td>82</td>
<td>47</td>
<td>98</td>
<td>92.1–99.8</td>
<td>0.91</td>
<td>0.166</td>
<td><em>P</em> &lt; 0.05</td>
</tr>
<tr>
<td>≥1</td>
<td>57</td>
<td>86</td>
<td>44</td>
<td>92</td>
<td>94.5–96.6</td>
<td>0.86</td>
<td>0.172</td>
<td><em>P</em> &lt; 0.05</td>
<td></td>
</tr>
</tbody>
</table>

PPV, positive predictive value; NPV, negative predictive value; CI, confidence interval; AUC, area under the ROC curve.

*The average cost resulting from the use of the diagnostic test at that decision level. Note that the cost reported here excludes the ‘overhead cost’, i.e. the cost of doing the test, which is constant at all decision levels.
Fig. 1 shows the GM index based on culture results in four different groups of patients, including all culture-negative BAL samples, *A. flavus* culture-positive BAL samples, *A. fumigatus* culture-positive BAL samples and other *Aspergillus* species culture-positive BAL samples.

According to our results on the prevalence of IA, the positive and negative likelihood ratios were calculated as 15 and 0.06, respectively (Fig. S1). The value cost of 0.166 at a GM index ≥0.5 was the best cut-off point for diagnosis of IA, while this value at a GM index ≥1 was 0.172.

**DISCUSSION**

In clinical practice, GM index evaluation in BAL has been encouraged as a suitable assessment for early diagnosis of IA in patients with various underlying disorders [22–25].

Several studies reported the value of GM antigen detection in BAL fluid as a promising tool for diagnosis of IA due to *A. fumigatus* [26–29]. Two GM indexes ≥0.5 [16, 30, 31] and ≥1.0 [7, 28, 32] for BAL fluid have been proposed for the assessment of GM in diagnosis of IA due to *A. fumigatus*; however, to the best of our knowledge, data on *A. flavus* is scarce.

In the present study, we therefore compared the Platelia GM index in BAL fluid from patients with proven, probable or putative pulmonary aspergillosis due to *A. flavus* versus *A. fumigatus*. Importantly, we found an increased index of GM in seven cases with *A. fumigatus* with critical host factors in comparison to patients with *A. flavus*. A GM index of ≥0.5 has shown reliable sensitivity and specificity for all tests. On the other hand, applying a GM index ≥1, 11 cases with positive *A. flavus* in BAL fluid culture had negative GM test results. Therefore, it seems that a GM index ≥0.5 would be an appropriate option for early diagnosis of IA, especially due to *A. flavus* and subsequently to proceed the rapid antifungal therapy in patients at high risk for IA. The ROC curve analysis indicated AUC values of 0.91 (95% CI, 92.1–99.8) for GM testing using a cut-off ≥0.5, which is in agreement with the previous report by He et al. [33] who reported a similar ROC value for the GM index in BAL fluid (AUC=0.91; 95% CI).

In the current study, the overall sensitivity and specificity of the GM index considering a cut-off value of ≥0.5 were 91 and 82%, respectively. The sensitivity results were in line with previous reports of Maertens et al. [7], D’Haese et al. [28] and Luong et al. [34], and specificity was similar to the findings of Maertens et al. [7], Desai et al. [35] and Clancy et al. [36].

However, in contrast to our results, [32], Luong et al. [37] and Reinwald et al. [38] reported sensitivities of 100, 100, 67% and specificities of 73, 78, 96%, respectively [32, 37, 38]. In our study, the overall sensitivity and specificity were 57 and 86% for a GM index of ≥1.0, respectively, which is somewhat different from the previous studies by Husain et al. [39], Musher et al. [40] and Khodavaysi et al. [41]. These differences might be due to different underlying conditions of the IA patients tested, various sizes of investigated populations, antifungal treatment, the different guidelines or diagnostic criteria for definition of proven or probable IA, the applied GM index as the diagnostic criteria and the species of *Aspergillus* involved in IA.

Notably, we also found that five of the non-IA patients who were receiving beta-lactam antibiotics, had a GM index ≥0.5, which is comparable with the Meersseman et al. [42] study which reported four of 33 intensive care unit patients with a false-positive GM index in BAL samples. The mechanism of beta-lactam giving a false positive BAL GM test has not been well determined, however certain domains of the β-lactam drugs show cross-reactivity to *Aspergillus* GM molecules at different concentrations. The interfering molecules in serum probably diffuse into the epithelial lining fluid to exceed the cut-off threshold, leading to a false positive BAL GM test [43, 44].

In our study, the high frequency of positive GM tests in IA patients due to *A. flavus* is similar to results from Tunisia.
However, the GM result in BAL fluid for *A. flavus* in our study (28/116, 24.1 %) is not comparable to the results from The Netherlands (3.44 %) [47], Italy (4.5 %) [48], USA (0.8 and 1.2 %) [36, 39], Belgium (2.3 %) [7], France (2 %) [49], UK (0.6 %) [50], China (4 %) [33], Japan (0.6 %) [22], Czech and Slovak Republics (4.2 %) [51], Turkey (3.3 %) [52] and India (11.6 %) [46]. To the best of our knowledge, none of the previous studies reported a two-fold mean of the GM index in IA patients with *A. flavus* in comparison to IA patients with *A. fumigatus* [53] in an in vitro study found that the quantity of scattered GM varies according to the species of *Aspergillus*. They demonstrated that among different species of *Aspergillus*, *Aspergillus terreus* has the maximum GM index, closely followed by *A. fumigatus* in comparison to *Aspergillus niger* and *A. flavus* [53]. Another study showed a higher secretion of GM by *A. niger* and *A. flavus* in comparison to *A. fumigatus* [54]. In general, the reasons for differences in GM indexes between different strains of *Aspergillus* are not yet well known. However, the higher amount of in vivo GM released by *A. fumigatus* strains, in comparison to *A. flavus*, may be due to a higher germination rate (logarithmic phase) at 37 °C by *A. fumigatus*. On the other hand, the high β-D-galactofuranosidase produced by *A. fumigatus* might respond more avidly in the GM assay than *A. flavus* [55]. Further study is needed to clarify the reason for the differences in GM indexes in IA patients with different species of *Aspergillus*.

Although *A. fumigatus* is reported as the most prevalent etiologic agent of IA and CPA compared with other *Aspergillus* species [56, 57], in the present study *A. flavus* was the most common isolated species among *Aspergillus* spp. In Table 3 we also summarized the reported etiologic agents of IA in different countries and highlighted the GM index and mycological criteria used. Of note, *A. flavus* has been frequently reported as the leading cause of invasive aspergillosis in certain tropical and sub-tropical countries [58]. A limitation of the current study is that we did not study invasive rhinosinusitis, which is more often caused by *A. flavus* than other species.

Overall, our findings emphasized that a GM index ≥0.5 is the best cut-off value for screening patients suspected of IA. On the other hand, we observed that using a GM index ≥0.5 in patients with positive BAL fluid culture with *A. flavus* can help to avoid missing IA cases. In a patient group selected for a high likelihood of IPA, the sensitivity of GM was lower for *A. flavus* compared with *A. fumigatus*, which has implications for diagnosis in hospitals and countries with a high proportion of *A. flavus* infections.

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Conflicts of interest
P. E. V. has served as a consultant to and has received research grants from Astellas, Basilea, Gilead Sciences, Merck and Pfizer. S. S. has received a research grant from Astellas Pharma B.V. D. W. D. is President of the Global Action Fund for Fungal Infections (www.GAFFI.org) which aims to improve the outcome of patients with serious fungal infections across the world, including Invasive aspergillosis. All other authors have no conflicts of interest.

Ethical statement
The Ethics Committee of Mazandaran University of Medical Sciences (code: 1392/12/14) approved this research and written informed consent was obtained from the patients or next of kin.

References
32. Hsu LY, Ding Y, Phua J, Koh LP, Chan DS et al. Galactomannan testing of bronchoalveolar lavage fluid is useful for diagnosis of...


