ORIGINAL ARTICLE

GALACTOMANNAN ANTIGEN TEST IN RESPIRATORY SAMPLES FOR ASPERGILLOSIS DIAGNOSIS

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Abstract

Introduction: Invasive aspergillosis is an important cause of mortality in patients with malignant diseases, and is an underestimated infection in critically ill patients. Early diagnosis is still challenging, therefore a rapid and more sensitive diagnostic methods could be beneficial.
Aim: the aim of the study was to evaluate the sensitivity and specificity of the galactomannan antigen test in respiratory samples, compared to conventional methods, for early diagnosis of aspergillosis.
Material and methods: Samples of 125 patients divided into 4 groups, classified according to diagnosis and EORTC/MSG criteria, were analysed at the Institute of Microbiology and Parasitology, with culture and galactomannan test in respiratory samples, during a period of two years.
Results: A total of 71 isolates of *Aspergillus* were confirmed in this study. Culture of respiratory samples revealed *Aspergillus* in the group of chronic aspergillosis (63.33%), followed by groups of cystic fibrosis (56.67%), primary immune deficiency (51.43%), and the group with prolonged ICU stay (43.33%). Sensitivity and specificity of respiratory samples' culture were: 64.29% and 100%, 59.09% and 100%, 54.55% and 12.5%, 100% and 54.17%, in all four groups, respectively. Sensitivity and specificity of galactomannan in respiratory samples, were: 75% and 57.14%, 86.36% and 62.5%, 81.82% and 0%, 50% and 70.83%, in all 4 groups, respectively.
Conclusion: The results of this study demonstrate that positive galactomannan test in respiratory samples could be a useful diagnostic adjunct in diagnosis of aspergillosis, along with results from conventional mycological analyses, so timely antifungal treatment is administered.

Key words: *Aspergillus, aspergillosis, galactomannan, respiratory tract*

Introduction

The incidence of invasive fungal infections (IFI) has dramatically increased in recent decades. Aspergillosis usually affects the respiratory system causing aspergilloma, chronic pulmonary aspergillosis, allergic bronchopulmonary aspergillosis and invasive aspergillosis (IA), which is the most aggressive form of the infection with these fungi (1,2). IA is mainly caused by

Aspergillus fumigatus. *Aspergillus* can invade the trachea and lungs, resulting in airway colonization, subsequent lung inflammation, and necrotizing pneumonia. They can affect distant organs through hematogenous spread. In the past, IA was recognized as occurring mainly in patients with haematological malignancies, solid organ or hematopoietic stem cell transplants, HIV infection, or in patients receiving long-term immunosuppressive therapy (3). However, it has been found that nonneutropenic patients, like chronic obstructive pulmonary disease (COPD) patients and patients with bronchiectasis, or previous tuberculosis, are also susceptible to pulmonary aspergillosis. Invasive aspergillosis may also be an underestimated infection in critically ill patients treated in intensive care units (ICUs). Despite availability and clinical use of new antifungal drugs, the mortality rate from IA in ICU patients remains high (4). The criteria for IA diagnosis have benefited from the European Organization for the Research and Treatment of Cancer (EORTC) and Mycoses Study Group (MSG) recommendations for defining IFI including aspergillosis (5). To achieve a more favorable prognosis of the life-threatening IA, an early initiation of antifungal treatment is mandatory. It relies on a timely and accurate diagnosis, which is still a big clinical and laboratory challenge, since clinical symptoms and radiological signs of IA are non-specific. Histopathological demonstration of molds in tissue samples or growth of molds on culture media, is still the “gold standard”. On the other hand, the procedures for specimen collection are invasive, which can be contraindicated in patients with profound respiratory insufficiency. Conventional mycological methods are also time-consuming and insensitive, since they are positive in less than 30% of cases with IA. Because of these limitations, a significant amount of work has been done in the past few decades regarding the development of non culture-based diagnostic assays for detection of IFI, like fungal biomarkers (5). Galactomannan (GM) is a polysaccharide antigen that exists in the *Aspergillus* cell walls, which is released into blood and other body fluids even in early stages of invasion (6,7). Levels of this antigen can be determined by enzyme-linked immunosorbent assay (ELISA), which can contribute towards earlier IA diagnosis. Currently, serum GM detection is considered a microbiological diagnostic criterion for fungal infection in neutropenic patients, according to the EORTC/MSG guidelines (5). Recently, GM detection in bronchoalveolar lavage was also strongly recommended in the 2016 Infectious Diseases Society of America guidelines (8) as a test providing high-quality evidence in neutropenic patients, however, its clinical application in nonneutropenic patients lacks evidence and its optimal threshold (9).

The **aim of this study** was to evaluate the sensitivity and specificity of galactomannan antigen test in respiratory samples, compared to conventional method, for early diagnosis of aspergillosis.

Material and Methods

Study Design

A diagnostic study was performed at the Institute of Microbiology and Parasitology, Faculty of Medicine, Skopje, North Macedonia, during a 2-year period, as part of an ongoing PhD study during the 2014-2016 period.

Patient Group and Mycological Analyses

Respiratory tract samples (sputum, tracheal aspirate and BAL) of 125 patients, divided into 4 groups, according to clinical diagnosis and IA risk factors, were analyzed at the Laboratory for diagnosis of fungal infections, at the Institute of Microbiology and Parasitology, Faculty of Medicine, Skopje, North Macedonia. These groups included patients with primary immune deficiency, critically ill patients treated in ICUs, patients with chronic aspergillosis and cystic fibrosis patients. IA was defined according to the revised definitions by the EORTC/MSG (European Organization for Research and Treatment of Cancer/Mycoses Study Group) consensus group (5).

Conventional Mycological Methods

Lower respiratory tract specimens (BAL, tracheal aspirate and sputum) were analyzed with conventional mycological methods, by their inoculation on fungal media (Sabouraud and chromogenic CALB medium (Oxoid)). Identification of *Aspergillus* on species level was performed with macroscopic analysis of grown mold colonies and further microscopic analysis of fungal conidia with lactophenol cotton blue method. After the specimens were inoculated for culture, all samples were frozen and stored at −70°C for retrospective galactomannan testing.

Galactomannan Antigen Detection

A commercially available sandwich ELISA test for galactomannan antigen detection of *Aspergillus* species was performed according to the manufacturer's instructions (Platelia *Aspergillus* protocol: Bio-Rad Laboratories, France) (10). Each sample was tested in duplicate, and the mean value was determined. Three hundred microliters of each respiratory sample and control were pipetted into individual 1.5 ml polypropylene tubes; 100 μL of sample treatment solution was added to each tube, and the tubes were mixed by vortexing. The tubes were then heated for 3 minutes at 100 degrees in a water bath. They were then centrifuged at 10,000xg for 10 minutes. The supernatant was used for detection of the galactomannan antigen. Fifty microliters of the treated respiratory sample supernatant were added to each well. The plate was incubated in a dry microtiter plate incubator for 90±5 minutes at 37^0C, after which it was washed 5 times. Two hundred microliters of the chromogenic solution were added to each well, avoiding exposure to strong light. The plate was incubated in the dark at room temperature (18-25^0C) for 30±5 minutes. One hundred microliters of the stop solution were added to each well, in the same order as the chromogenic solution was added. The optical density of each well was read at 450 nm (reference filter 620/630 nm). The microtiter plates were read within 30 minutes after the addition of the stop solution. The optical density (OD) was determined spectrophotometrically with a microplate reader (Bio-Rad, France). The results were interpreted based on the index calculated from the measured OD at a wavelength of 450 nm. Indices ≥ 0.5 were considered positive.

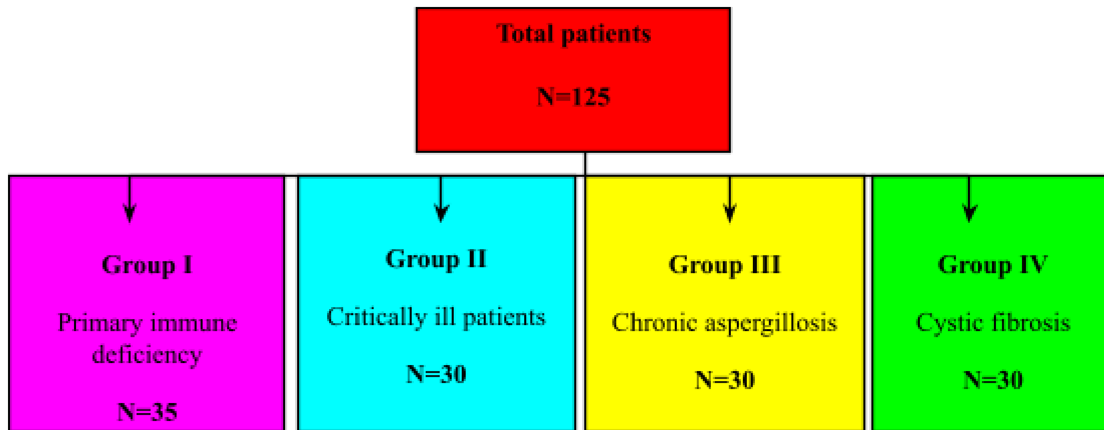
A statistical analysis was performed by using the Statistical Package for the Social Sciences (SPSS) for Windows. The results of our study are presented as numbers and percentages. Differences in distribution of proven, probable and possible fungal infections with *Aspergillus*

were compared by Pearson Chi square test. P value less than 0.05 was considered statistically significant.

Results

Respiratory tract samples from 125 patients were divided in 4 groups (first group of patients with primary immune deficiencies, second group with critically ill patients treated in ICUs, third group of patients with chronic aspergillosis and fourth group of patients with cystic fibrosis (CF)). The four groups classification was performed according to primary clinical diagnosis and EORTC/MSG criteria (European Organization for Research and Treatment of Cancer/Mycoses Study group) (Fig. 1).

Figure 1: *Patient groups classification according to clinical diagnosis and EORTC/MSG (European Organization for Research and Treatment of Cancer/Mycoses Study group) criteria*



The gender analysis of the study participants revealed that men were more frequently distributed in I, III and IV group (60%, 60%, 53.33% respectively), whereas in the II group, both genders were equally distributed. Average age of patients in all groups were: 40.8±17.7, 59.7±13.3, 64.7±6.3, and 28.9±8.5 years (table 1).

Table 1. *Characteristics of patients according to gender and age*

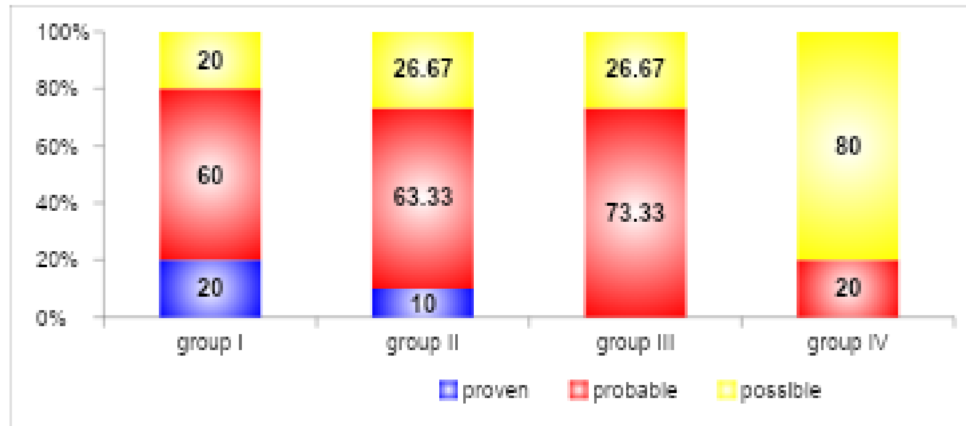
Aspergillus				
	Group I N=35	**Group II N=30**	**Group III N=30**	**Group IV N=30**
Gender	**n (%)**	**n (%)**	**n (%)**	**n (%)**
Men 70 (56%)	21 (60%)	15 (50%)	18 (60%)	16 (53.33%)
Women 55 (44%)	14 (40%)	15 (50%)	12 (40%)	14 (46.67%)

	$^{a}p = 0.81$			
Age (years) mean±SD, min-max				
	40.8±17.7 5-69	59.7±13.3 4-78	64.7±6.3 52-76	28.9±8.5 18-52

[a]p(Chi-square test)

The participants' distribution, according to clinical diagnosis for proven, probable and possible fungal infection, with EORTC/MSG criteria (European Organization for Research and Treatment of Cancer/Mycoses Study Group), are presented in figure 2. According to EORTC/MSG criteria, only a small percentage of patients had proven infection with *Aspergillus*. Of these, 20% (7/35) patients had some type of primary deficiency, and 10% (3/30) patients had a prolonged stay in ICU.

Figure 2. *Distribution of fungal infections according to EORTC/MSG criteria in all groups*



Differences in distribution of proven, probable and possible fungal infection with *Aspergillus* were statistically significant between group I versus groups III and IV, and between group II versus groups III and IV (Table 2).

Table 2. *Distribution of proven, probable and possible fungal infections according to EORTC/MSG criteria*

Aspergillus	**group I** **N=35**	**group II** **N=30**	**group III** **N=30**	**group IV** **N=30**
n (%)	**n (%)**	**n (%)**	**n (%)**	**n (%)**
proven 10 (8%)	7 (20%)	3 (10%)	0	0
probable 68 (54.4%)	21 (60%)	19 (63.33%)	22 (73.33%)	6 (20%)

possible 47 (37.6%)	7 (20%)	8 (26.67%)	8 (26.67%)	24 (80%)
	[b]$p < 0.001$			
	I vs II p=0.3	II vs III p = 0.345	III vs IV $p < 0.001$	
	I vs III p = 0.03*	II vs IV $p < 0.001$		
	I vs IV $p < 0.001$			

[a]p(Chi-square test) [b](Fisher exact test) *p<0.05 **p<0.01

The highest rate of *Aspergillus* in respiratory samples culture was registered in the chronic aspergillosis group (63.33%), followed by 56.67% detected in the CF group, 51.43% in the group with primary immune deficiency, and 43.33% in patients hospitalized in ICU. However, the differences in positive respiratory cultures among all groups were insufficient for statistical significance (p=0.46).

The most frequent species (79%) identified in respiratory samples was *A. fumigatus* (53/67). Thirty-two percent of *A. fumigatus* isolates (17/53) originated from samples of patients with chronic aspergillosis, and 26% (14/53) were identified in samples from patients with primary deficiency and cystic fibrosis (Table 3).

Table 3. *Culture of respiratory tract samples and identified fungal species*

	group I N=35	**group II N=30**	**group III N=30**	**group IV N=30**
Respiratory culture	**n (%)**	**n (%)**	**n (%)**	**n (%)**
negative 58 (46.4%)	17 (48.57%)	17 (56.67%)	11 (36.67%)	13 (43.33%)
positive 67 (53.6%)	18 (51.43%)	13 (43.33%)	19 (63.33%)	17 (56.67%)
	Chi-square: 2.59 p = 0.46			
Identified mold species				
A. fumigatus n=53	14	8	17	14
A. flavus n=11	2	4	2	3
A. terreus n=3	2	1	0	0

p(Chi-square test)

ELISA GM test performed in respiratory samples of patients from the group with primary immundeficiencies was positive in 24 (68.57%) cases. Of these, 6 out of 7 (85.71%) in proven, 15 out of 21 (71.43%) in probable, and 3 out of 7 (42.86%) in possible infections, according to EORTC/MSG criteria. The concentration of GM antigen, in 24 positive samples taken from respiratory samples, ranged from 1.1 to 4.3 pg/ml, with a mean concentration of 2.61±1.1 pg/ml. ELISA GM test in respiratory samples was characterized by 21 true positives, 3 false positives, 4 true negatives, and 7 false negatives; 7 respiratory samples with proven infection were labeled negative by the ELISA GM test, and 3 samples categorized as possible infection were labeled

positive by the test. The sensitivity, specificity, PPV and NPV of the GM test in respiratory samples were 75%, 57.14%, 87.5%, 36.36%, respectively.

Results from the comparative diagnostic performance of the conventional method and GM in respiratory samples in the immunodeficiency group are presented in Table 4.

Table 4. *Diagnostic performances of culture from RT samples and GM in respiratory samples in the group with primary immunodeficiency*

Test	Se(%)	Sp(%)	PPV(%)	NPV(%)
Culture from RT samples	64.29	100	100	41.18
Galactomannan in RT samples	75	57.14	87.5	36.36

The ELISA GM respiratory samples test performed to the patients with prolonged hospital stay in ICU from the critically ill group was in 22 (73.33%) of them. GM antigen was detected in all 3 cases categorized as proven infections, 16 of 19 (84.21%) probable, and 3 out of 8 (37.5%) possible, according to EORTC/MSG criteria. The concentration of GM in positive samples ranged from 1.2 to 4.7 pg/ml, with a mean concentration of 2.78±1.04 pg/ml. The ELISA GM respiratory samples test from patients with prolonged hospital stay in ICU was characterized by 19 true positive results, 3 false positives, 5 true negatives, and 3 false negative results. The calculated diagnostic performances were: sensitivity 86.36%, specificity 62.5%, positive predictive value 86.36%, negative predictive value 62.5%.

The results from the conventional method comparative diagnostic performance and GM in respiratory samples in the critically ill patients group with prolonged hospital stay in ICU are presented in Table 5.

Table 5. *Diagnostic performances of culture from RT samples and galactomannan in respiratory samples in the group of critically ill patients with prolonged ICU stay*

Test	Se(%)	Sp(%)	PPV(%)	NPV(%)
Culture from RT samples	59.09	100	100	47.06
Galactomannan in RT samples	86.36	62.5	86.36	62.5

In patients with chronic aspergillosis, GM performed simultaneously with respiratory samples culture detected 26 (86.67%) cases of *Aspergillus* infection, of which, according to the EORTC/MSG criteria, 18 were classified as probable and 8 as possible aspergillosis. The minimum concentration of GM measured in respiratory samples, simultaneously with respiratory samples culture, was 0.8, and the maximum was 2.8, the average concentration was 1.52±0.5. GM as a diagnostic test for *Aspergillus* infection in the chronic aspergillosis group, according to

the EORTC/MSG classification, had the following diagnostic performance: sensitivity 81.82%, specificity 0%, positive predictive value 69.23%, negative predictive value 0.82%. The specificity of this test in the chronic aspergillosis group of patients was 0%, i.e., all 8 patients from this group who had a possible *Aspergillus* infection according to the EORTC/MSG had a positive test for GM, i.e., they were marked as false positive results. The test had 4 false negative results, i.e., 4 patients who had a probable infection according to the EORTC/MSG had a negative result for GM in respiratory samples.

The results of the comparative conventional method diagnostic performance and GM in respiratory samples in the chronic aspergillosis group are presented in Table 6.

Table 6. *Diagnostic performances of culture from RT samples and galactomannan in respiratory samples in the group of chronic aspergillosis*

Test	**Se(%)**	**Sp(%)**	**PPV(%)**	**NPV(%)**
Culture from RT samples	59.09	100	100	47.06
Galactomannan in RT samples	81.82	0	69.23	0

The GM in the respiratory samples in the cystic fibrosis group was positive in 10 (33.33%) patients. Positive findings for GM were obtained in 3 out of 6 (50%) samples in the probable infections group, according to EORTC/MSG, and 7 out of 24 (29.17%) possible infections, according to these criteria. The concentration of GM in the positive samples ranged from 1.3 to 2.4 pg/ml, with a mean concentration of 1.9±0.4 pg/ml. The GM test of the respiratory samples from the cystic fibrosis patients was characterized with 3 true positive results, 7 false positives, 17 true negatives, and 3 false negatives. The calculated diagnostic performances were: sensitivity 50%, specificity 70.83%, positive predictive value 30% and negative predictive value 85%.

The results of the comparative conventional method diagnostic performance and GM in respiratory samples in the cystic fibrosis group are presented in Table 7.

Table 7. *Diagnostic performances of culture from RT samples and GM in respiratory samples in the group of cystic fibrosis*

Test	**Se(%)**	**Sp(%)**	**PPV(%)**	**NPV(%)**
Culture from RT samples	59.09	100	100	47.06
Galactomannan in RT samples	50	70.83	30	85

Discussion

Invasive fungal infections present an increasing global burden in immunocompromised and critically ill patients. Early fungi identification is crucial for better clinical outcome. Mycological diagnosis of invasive aspergillosis still presents a significant clinical and laboratory challenge (5).

In our study, respiratory samples culture demonstrated growth of *Aspergillus* most frequently in the chronic aspergillosis group (63.33%), followed by 56.67% patients with cystic fibrosis, 51.43% patients with primary immune deficiency, and 43.33% patients with prolonged stay in intensive care units (ICUs). The sensitivity and specificity of the respiratory samples culture were: 64.29% and 100%, 59.09% and 100%, 54.55% and 12.5%, 100% and 54.17%, in all groups respectively. Lower sensitivity than in our study was demonstrated in the study of Tashiro and coworkers, where 165 isolates of *Aspergillus* species were detected in RT sample culture of 139 patients, of which 45% were colonized with *Aspergillus* but haven't demonstrated clinical symptoms of aspergillosis. Other patients (55%) had some type of pulmonary aspergillosis, which was classified as chronic aspergillosis (48%), aspergilloma (29%), IA (13%) or ABPA (10%).

Due to conventional model's low sensitivity, we evaluated the GM potential in respiratory samples for diagnosis of aspergillosis. ELISA GM test in respiratory samples in the first group demonstrated sensitivity and specificity of 75% and 57.14%, respectively. Similar results have been demonstrated in the study of Lahmer and colleagues, who registered a 70% sensitivity, and higher specificity of 94%, with BAL GM Platelia assay (11). The negative predictive value of the BAL GM Platelia assay was 90%. In a study by Lahmer involving 49 immunosuppressed and ICU-treated patients, 26% had probable invasive aspergillosis, and a positive BAL galactomannan could be detected in 12 of 13 probable cases.

ELISA GM test in respiratory samples in the second group of ICU-treated patients demonstrated a GM test sensitivity and specificity of 86,36% and 62,5%. Different studies of detection of BAL GM have used different cut-off values (from 0.5–1.0), and have demonstrated sensitivities and specificities of 73%–100%, mainly in hematological patients (12), solid organ transplant recipients (13), and non-immunocompromised patients (14). They demonstrated sensitivity in lung transplant recipients (61%), which is significantly lower than the one in other populations (15). All of these studies demonstrate a high negative predictive value, and similar results were obtained in our study, especially among subjects in the first group (NPV 87.5%), however, they do not provide data for analysis between BAL GM results. An exception to this is the study by Maertens and collaborators (16), who reported results from 10 neutropenic and 19 non-neutropenic patients with proven aspergillosis, and a demonstrated higher sensitivity for the detection of GM in BAL. However, comparison between different studies is very difficult, because of differences in the sampling's timing, and because the effect of antifungal treatment has not been defined. Also, the amount of saline administered during the bronchoscopy procedure is different in different studies (12,13).

The GM ELISA test sensitivity and specificity in respiratory samples in chronic aspergillosis patients were of 81.82% and 0% respectively. The specificity of this test was 0, i.e., all 8 patients

from this group who were classified as possible *Aspergillus* infection, according to EORTC/MSG criteria, had a positive ELISA GM test, therefore they were marked as false positive results. Our study also demonstrated a high sensitivity of the GM test in respiratory samples (81.82%), as has been previously demonstrated by Park Seong and colleagues, who obtained a sensitivity of BAL GM test of 92% (17), which suggests that BAL GM test is an effective test for pulmonary aspergillosis diagnosis. However, it should not be forgotten that the GM antigen test is a method for measuring the cell wall antigen of these fungi, and not a test for determining the invasion by these fungi. Nguyen and coworkers (14) compared the BAL GM performance with the GM serum assay in 4 cases of non-invasive pulmonary aspergillosis and found that the BAL GM assay demonstrated higher sensitivity than the GM serum. They obtained a 100% sensitivity for the BAL GM test at a cutoff ≥1.0 (all patients had BAL GM concentrations above 1.18), high specificity (88.1%), and excellent negative predictive value (100%). However, a limitation of the test was a low positive predictive value of 43%, reflecting the low prevalence of pulmonary aspergillosis in this population. Kono and collaborators demonstrated a 85.7% sensitivity for BAL GM test for the chronic aspergillosis diagnosis and ABPA (18). Park and colleagues found that the BAL GM test sensitivity was 92%, suggesting that BAL GM test is a more useful method for the pulmonary aspergilloma diagnosis (17).

The ELISA GM test in respiratory samples in the fourth group of our cystic fibrosis patients demonstrated a GM test sensitivity and specificity of 50% and 70.83%. Studies in the literature on utility of galactomannan in respiratory samples suggest that the lungs of CF individuals contain abnormally thick mucus that traps *Aspergillus* conidia and allows them to germinate in large numbers. As a result, large amounts of antigen are released during its growth, which likely contributes to the development of ABPA (19). Although defects in the anatomic barrier make these patients prone to enhanced colonization of the lungs with these fungi, intact immune cell function likely prevents further invasion by conidia. Despite this, it is still possible to assume that GM concentrations in patients colonized with *Aspergillus* are intermittent, and may only be positive when there is growth of *Aspergillus* in BAL or sputum culture. We also believe that galactomannan antigenemia may be more frequently present in patients with more frequent positive BAL or *Aspergillus* sputum cultures over a longer period of time, in contrast to one or two positive intermittent cultures during the year. In the study performed by Baxter and colleagues, 27% (39/146) of the sputa tested were *Aspergillus* positive by a standard culture. Over a 6-month period, these tests were repeated in 30 patients. Forty-seven percent (68/146) of the samples tested positive for GM. Over a 6-month period, all positive patients remained positive, and additional five patients who were negative became positive. GM demonstrated good reproducibility in CF sputum and therefore can be used to monitor response to antifungal therapy (20).

Despite the possibility for false positive and false negative detection and despite other available biomarkers, such as β-1,3-glucans or the development of a PCR method, the GM remains an unavoidable and useful biomarker for the IA diagnosis and has led to the recent development of new monoclonal antibodies and the lateral flow assay (LFA)-GM technology (21).

Conclusion

Sensitivity and specificity of galactomannan antigen marker detected by ELISA galactomannan test indicate that detection of galactomannan in respiratory samples could be a valuable tool for early IA diagnosis in immunodeficient and nonneutropenic patients.

Although no single method could provide definite etiological aspergillosis diagnosis, ELISA galactomannan test highlights that confirmation of galactomannan in respiratory samples is useful diagnostic adjunct in the aspergillosis diagnosis.

Application of conventional methods and implementation of fungal biomarker tests, as well as appropriate interpretation of results, in collaboration with clinical doctors, is the most important aspect towards accurate and precise etiological aspergillosis. diagnosis and early start of antifungal treatment, to achieve favorable clinical outcome.

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