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Original article

EVALUATION OF (1,3)-β-D-GLUCAN ASSAY IN RESPIRATORY SAMPLES FOR DIAGNOSIS OF ASPERGILLOSIS

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Introduction: Aspergillosis is significant problem in immunocompromised and critically ill patients. Early diagnosis is still challenging, and therefore there is a need for rapid and more sensitive diagnostic methods.

Aim: the aim was to evaluate the performance, sensitivity and specificity of the panfungal (1,3)-b-D-glucan marker in respiratory samples, compared to conventional method, for early diagnosis of aspergillosis.

Material and methods: Samples of 125 patients divided into 4 groups, classified according to clinical diagnosis and EORTC/MSG criteria, were analysed at the Institute of Microbiology and Parasitology, with conventional methods and (1,3)-b-D-glucan marker in respiratory samples, during a period of two years.

Results: A total of 71 isolates of *Aspergillus* were confirmed in this study. Four isolates originated from bloodcultures. 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 panfungal (1,3)-b-D-glucan marker in respiratory samples, were: 71.43% and 85.71%, 72.73% and 62.5%, 68.18% and 0%, 50% and 50%, in all 4 groups, respectively.

Conclusion: Results of this study demonstrate that a positive (1,3)-b-D-glucan marker in respiratory samples highlights the value of this test as a diagnostic adjunct in diagnosis of aspergillosis, along with results from conventional mycological analyses, so timely antifungal treatment with a favorable clinical outcome, is achieved.

Keywords: *Aspergillus*, respiratory tract, aspergillosis, 1,3-ß-D-glucan panfungal marker

Introduction

The incidence of invasive fungal infections (IFI) has dramatically increased in recent decades. Despite availability and clinical use of new antifungal drugs, the mortality rate from IFI remains high, particularly in ICU patients[1]. Invasive aspergillosis is the second most common IFI in immunocompromised patients undergoing steroid treatment, chemotherapy resulting in severe neutropenia, hematopoietic stem cell and solid organ transplantation, and ICU treated patients on mechanical ventilation. Aspergillosis usually affects the respiratory system and manifests as a broad-spectrum of diseases including aspergilloma, chronic pulmonary aspergillosis, allergic

bronchopulmonary aspergillosis and invasive aspergillosis, which is the most aggressive form of infection, rapidly spreading to the brain, heart, liver, and kidneys, with a very high mortality rate[2]. Criteria for diagnosis of invasive aspergillosis have greatly benefited from the European Organisation for the Research and Treatment of Cancer (EORTC) and Mycoses Study Group (MSG) recommendations for defining IFI including aspergillosis[3].

To achieve a favorable prognosis of the life-threatening IFI, an early initiation of antifungal treatment is necessary. It relies on a timely and accurate diagnosis, which in turn is still a big laboratory challenge, because clinical symptoms and signs, as well as radiological signs, are often non-specific. Histopathologic demonstration of microorganisms in tissue samples or growth of fungi on culture media, is still the "gold standard". However, procedures for specimen collection are invasive and can be contraindicated in patients with profound respiratory insufficiency. Classical methods are time-consuming and relatively insensitive as they are positive in less than 30% of cases with aspergillosis.

Due to these limitations, a lot of work has been done in recent years to develop non-culture-based diagnostic assays for detection of invasive IFI, such as fungal biomarkers[4]. Detection of fungal cell wall components is a rapid and attractive tool to diagnose aspergillosis. (1,3)-β-D-glucan (BDG) is a panfungal marker, which is a cell wall polysaccharide, found in many pathogenic fungi, including *Aspergillus* species, that can be present early in the blood and body fluids of patients suffering from IFI. β-D-glucan concentrations show a constant rise even before clinical signs are manifested, then begin to decline, and eventually become negative if patients respond well to antifungal treatment. Conversely, patients who do not respond show no decrease or show a continuous rise of this fungal marker.

The Fungitell test (Associates of Cape Cod) is a chromogenic kinetic test that was approved in 2003 by the U.S. FDA for the presumptive diagnosis of IFI[5]. It may allow earlier diagnosis of IFI comparing to conventional methods. The Fungitell BDG assay is a chromogenic, quantitative EIA based on the clotting cascade of the *Limulus* or horseshoe crab. This assay is a kinetic ELISA, meaning that each well for each patient sample, which is run in duplicate, is read, and optical density values recorded every 30 seconds over a 40-minute period. Findings from 4 different meta-analyses performed over the years show that in patients with higher risk for development of IFI, single positive β-D-glucan test is associated with a sensitivity and specificity ranging between 60 and 90%. Other studies, performed primarily in patients with hematologic malignancies, have shown that the presence of two consecutively positive β-D-glucan results increase specificity of the assay to almost 99%, suggesting that these results may be used as a diagnostic marker for the presence of an IFI[6].

The aim of this study was to evaluate the diagnostic performance, sensitivity and specificity of the panfungal (1,3)-β-D-glucan marker 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, Macedonia, during a 2-year period, as part of an ongoing PhD study during the period 2014-2016.

Group of patients and mycological analyses

Samples from respiratory tract (sputum, tracheal aspirate and BAL) from 125 patients divided into 4 groups, according to clinical diagnosis and risk factors for aspergillosis, were analyzed at the Laboratory for diagnosis of fungal infections, at the Institute of Microbiology and Parasitology, Faculty of Medicine, Skopje, Macedonia. These groups included patients with primary immune deficiency, critically ill patients treated in ICUs, patients with chronic aspergillosis and cystic fibrosis patients. IFI was defined according to the revised definitions by the EORTC/MSG (European Organization for Research and Treatment of Cancer/Mycoses Study Group) consensus group, with the necessary modification that (1,3)-β-D-glucan panfungal marker was not included in the microbiological criteria.

Conventional mycological methods

The samples were investigated with conventional mycological methods, by inoculation of specimens on fungal media (Sabouraud and chromogenic CALB medium (Oxoid)). Identification of *Aspergillus* at the species level was performed with macroscopic analysis of grown mold colonies and further microscopic analysis of the reproductive elements (conidia) using the lactophenol cotton blue method. After inoculating specimens for culture, all samples were frozen and stored at -70°C for retrospective BDG panfungal marker testing.

(1,3)-β-D-glucan panfungal marker

A commercially available Fungitell assay (Cape Cod Diagnostics, USA) was used according to manufacturer's instructions[6]. Respiratory samples were centrifuged at 1000 rpm for 10 min, and supernatant was used for (1,3)-β-D-glucan panfungal marker detection. Thereafter, the respiratory samples were equally treated as sera samples. Interpretation of the panfungal BDG marker values was as follows: < 60 pg/ml were negative for the BDG marker; 60-79 pg/ml were indeterminate; and a positive test result was defined as a sample with cut-off level ≥ 80 pg/ml. A detailed methodology of BDG assessment in serum and BAL has been previously described[7,8].
Statistical analysis was performed 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 samples from 125 patients were divided in 4 groups [patients with primary immune deficiencies, critically ill patients treated in intensive care units (ICUs), patients with chronic aspergillosis and cystic fibrosis (CF)] according to clinical diagnosis and EORTC/MSG (European Organization for Research and Treatment of Cancer/Mycoses Study group) criteria (Figure 1).

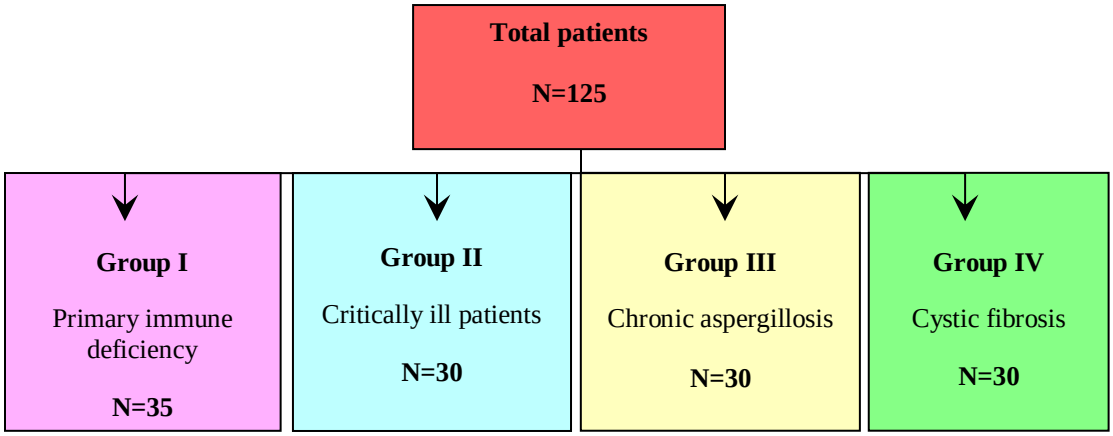

Fig. 1. Classification of patient groups according to clinical diagnosis and EORTC/MSG (European Organization for Research and Treatment of Cancer/Mycoses Study group) criteria

Gender analysis of study participants revealed that men were more frequently represented in groups I, III and IV (60%, 60%, 53.33%, respectively), whereas in group II both genders were equally represented. The average age of patients in all groups was: 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	59.7±13.3	64.7±6.3	28.9±8.5
	5-69	4-78	52-76	18-52

[a]p(Chi-square test)

Distribution of patients, 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), is 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.

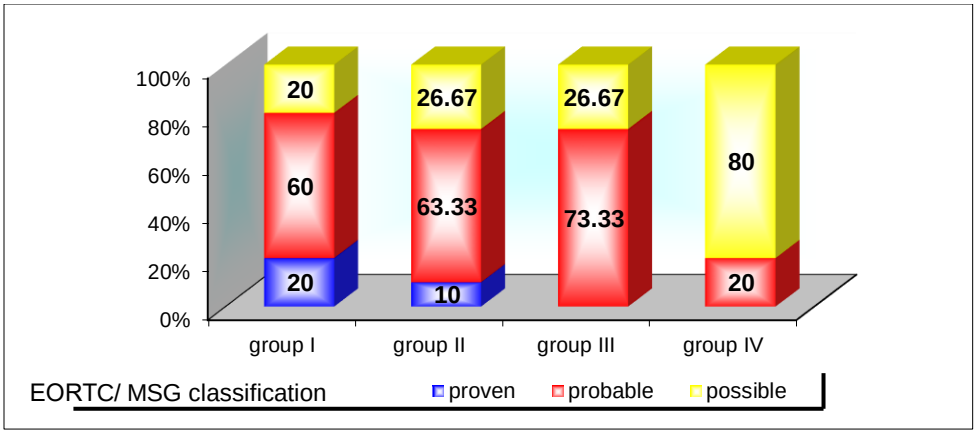

Fig. 2. Distribution of fungal infections according to EORTC/MSG criteria in all groups

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 **n (%)**	**group I N=35 n (%)**	**group II N=30 n (%)**	**group III N=30 n (%)**	**group IV N=30 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

Culture of respiratory samples demonstrated presence of *Aspergillus* most frequently in the group of chronic aspergillosis (63.33%), followed by the specimens of the CF group (56.67%), 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 the four 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

Respiratory culture	**group I N=35 n (%)**	**group II N=30 n (%)**	**group III N=30 n (%)**	**group IV N=30 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)

Panfungal β-D-glucan (BDG) marker in respiratory samples in the group with primary immunodeficiencies was positive in 21 (60%) cases. Of these, 6 out of 7 (85.71%) were classified as proven, 14 out of 21 (66.67%) as probable, and 1 out of 7 (14.29%) as possible infections, according to the EORTC/MSG criteria. The concentration of the BDG marker ranged from 94-462 pg/ml, with an average concentration of 253.05±113.2 pg/ml (Table 4).

The BDG marker test in respiratory samples, as a diagnostic test for aspergillosis, provided the following diagnostic performances: sensitivity of 71.43%, specificity of 85.71%, positive predictive value of 95.24%, and negative predictive value of 42.86%. In 8 of 28 patients with primary deficiency and proven or probable *Aspergillus* infection, according to the EORTC/MSG classifications, the BAL BDG marker was negative, i.e. there were 8 false-negative results. The test had one false-positive result, i.e. one BAL sample from this group classified only as possible aspergillosis, tested positive with the BDG marker.

The results from comparative diagnostic performance of conventional method and panfungal BDG marker in respiratory samples in the group with immunodeficiency are presented in Table 4.

Table 4. Diagnostic performances of culture from RT samples and panfungal BDG marker in respiratory samples in the group with primary immunodeficiency

Test	**Se (%)**	**Sp (%)**	**PPV (%)**	**NPV (%)**
Culture from RT samples	64.29	100	100	41.18
BDG marker in RT samples	71.43	85.71	95.24	42.86

Panfungal β-D-glucan (BDG) marker in respiratory samples in the group with ICU treated patients was positive in 19 (63.33%) samples, of which 3 were categorized as proven infection, 13 of 19 (68.42%) as probable, and 3 of 8 (3755%) as possible infections, according to the EORTC/MSG criteria.

The concentration of the panfungal BDG marker in the positive samples ranged from 135-468 pg/ml, with a mean concentration of 270.53 ± 100.1 pg/ml. The BDG marker in respiratory samples provided the following diagnostic performances: sensitivity of 72.73%, specificity of 62.5%, positive predictive value of 84.21%, and negative predictive value of 45.45%. In 6 of 22 patients with prolonged broad-spectrum antibiotic therapy and proven or probable *Aspergillus* infection according to the EORTC/MSG classifications, The BDG marker in respiratory samples was negative, i.e. there were 6 false-negative results. The test had 3 false-positive results; that is, 3 respiratory samples classified only as possible infection were positive for the panfungal BDG marker.

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

Table 5. Diagnostic performances of culture from RT samples and panfungal BDG marker 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
BDG marker in RT samples	72.73	62.5	84.21	45.45

The panfungal B-D-glucan marker test in respiratory samples was positive in 23 (76.67%) cases from the chronic aspergillosis group. Of these, 15 out of 22 (68.18%) in the group of probable infections according to the EORTC/MSG criteria, and all 8 in the group of possible *Aspergillus* infections.

Among 23 positive respiratory samples, the concentration of the panfungal BDG marker ranged from 88 to 317 pg/ml, with an average concentration of 156.78 ± 69.8 pg/ml. The panfungal BDG marker in respiratory samples, as a diagnostic test for IFI, was characterized by 7 false-negative results, i.e. 7 out of 22 patients with probable *Aspergillus* infection, according to the EORTC/MSG criteria, were negative with the BDG marker test. The test had 8 false-positive results, all 8 patients with possible infection according to EORTC/MSG criteria, had a positive result for the BDG.

The results from the comparative diagnostic performance of conventional method and panfungal BDG marker in respiratory samples in the group with chronic aspergillosis are displayed in Table 6.

Table 6. Diagnostic performances of culture from RT samples and panfungal BDG marker in respiratory samples in the group of critically ill patients with chronic aspergillosis

Test	Se (%)	Sp (%)	PPV (%)	NPV (%)
Culture from RT samples	54.55	12.5	63.16	9.09
BDG marker in RT samples	68.18	0	65.22	0

The panfungal β-D-glucan (BDG) marker in respiratory samples in CF group was positive in 5 (16.67%) patients. According to EORTC/MSG criteria, positive findings of the panfungal marker were found in half of the samples from the group of probable infections, and in 2 out of 24 (8.44%) from the group of possible infections. The concentration of the panfungal BDG marker in the positive samples ranged from 6-459 pg/ml, with a mean concentration of 133.0±187.2 pg/ml. β-D-glucan marker in respiratory samples as a diagnostic test for IFI demonstrated: sensitivity of 50%, specificity of 91.67%, positive predictive value of 60%, and negative predictive value of 80%. According to the EORTC/MSG criteria, in 3 of 6 patients with cystic fibrosis and probable *Aspergillus* infection, β-D-glucan marker in respiratory samples was negative, i.e. there were 3 false-negative results. The test had 2 false positive results; that is, 2 respiratory samples from this group, classified only as possible infection, were positive for β-D-glucan.

The results from the comparative diagnostic performance of conventional method and panfungal BDG marker in respiratory samples in the group with cystic fibrosis are shown in Table 7.

Table 7. Diagnostic performances of culture from RT samples and panfungal BDG marker in respiratory samples in the cystic fibrosis group

Test	Se (%)	Sp (%)	PPV (%)	NPV (%)
Culture from RT samples	100	54.17	35.29	100
BDG marker in RT samples	50	91.67	60	88

Discussion

IFI are an increasing global burden in immunocompromised and critically ill patients. Early identification of the fungus is critical for favorable clinical outcome. Mycological diagnosis of aspergillosis still presents a big clinical and laboratory challenge[4].

In our study, the conventional method with culture of respiratory samples demonstrated growth of *Aspergillus* most frequently in the group of patients with chronic aspergillosis (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 ICU. 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 groups respectively. Lower percentage was reported in a study by Tashiro *et al.*, where 165 isolates of *Aspergillus* species were detected in RT sample culture of 139 patients, of which 45% were colonized with *Aspergillus*, but did not manifest clinical symptoms of aspergillosis, and the remaining 55% had some type of pulmonary aspergillosis - they were classified as chronic aspergillosis (48%), aspergilloma (29%), invasive aspergillosis (13%) or ABPA (10%).

Regarding the distribution of *Aspergillus* species in our study, the most frequently identified agent was *A. fumigatus* (79.1% of the positive RT sample cultures), and from these, 32.1% were confirmed in patients with chronic aspergillosis. Other *Aspergillus* species in respiratory sample cultures were *A. flavus* (16.42%) and *A. terreus (*4.48%). Of these, 36.4% of *A. flavus were* confirmed in patients treated in ICU, and 27.3% in the group with cystic fibrosis. Two isolates of *A. terreus* (66.7%) were confirmed in patients with AIDS, and one isolate in a patient with metastatic tumor of the brain, treated in ICU. Still, A. *fumigatus* was a dominant fungus in AIDS patients in our study (4/6), who had their CD4 numbers below 50/mm^3 and 10/mm^3. Similar data were presented in a study by Meyohas *et al.*, who confirmed CD4 numbers below 50/mm^3 in their patents with positive RT sample culture[9]. In the study by Mennink-Kersten, the distribution of *Aspergillus* among 165 confirmed isolates in BAL cultures showed presence of 41% of *A. fumigatus* and 32% of *A. niger*, but also *A. versicolor* (12%), *A. terreus* (6%), *A. flavus* (5%), *A. nidulans* (2%), *A. sydowii* (1%) and unidentified *Aspergillus* species (0.6%)[10]. Zarrinfar *et al.* demonstrated the presence of *A. flavus*, *A. niger* and one case with mixed infection involving two species (*A. flavus*/*A. niger*) in positive BAL cultures (23%). In contrast to our study, where *A. fumigatus* was the predominant species, the most frequent agent in the study by Zarrinfar was A. flavus[11].

The diagnostic value of confirmation of *Aspergillus* in respiratory samples can be questionable, as differentiating between colonization and infection is very difficult and requires additional clinical and laboratory analysis. Discovery of *Aspergillus* in more specimens during an antibiotic treatment in high-risk patients should raise concern for the development of aspergillosis [12]. Therefore, isolation of *Aspergillus* from RT samples in high-risk critically ill patients with signs of pneumonia should be a serious warning sign of an infection with *Aspergillus, requ*iring *fast* decision for early start of antifungal treatment. The sensitivity of respiratory sample cultures is unacceptably low, even in histopathology-proven cases, and invasive procedures with a higher

yield are infrequently performed due to patient comorbidities. Lass-Flörl *et al.* confirmed a sensitivity of 34% for IFI using conventional mycological method (21/61 positive samples)[13].
Due to the low sensitivity of the conventional mycological method, we evaluated the potential of the panfungal (1,3)-beta-D-glucan (BDG) marker in respiratory samples for diagnosis of aspergillosis.

The sensitivity/specificity of beta D-glucan marker in the respiratory samples were 71.43%/85.71%, 72.73%/62.5%, 68.18%/0%, 50%/50%, in all four groups respectively. A study by Bhaskaran *et al.*, for performance of the BAL BDG in lung transplant patients for diagnosing invasive aspergillosis, analyzed the BDG marker in fresh and frozen BAL samples with Fungitell test. Out of 195 samples, they confirmed 10 episodes of invasive aspergillosis. The sensitivity/specificity of the test were 80%/53%, and 60%/70% at 41 pg/ml and 108 pg/ml cut-offs, respectively. When 52 BAL samples were excluded due to anti-*Aspergillus* antifungal treatment during sampling process, the sensitivity/specificity were 75%/91%, respectively, with cut-off of 524 pg/ml. This study demonstrated moderate sensitivity and specificity of the BAL BDG assay[14].

Theel *et al.* evaluated the performance of the BDG assay in BAL for the identification of IFI in immunocompromised patients with proven, probable, and possible invasive aspergillosis according to EORTC/MSG criteria[15]. Among 109 subjects, Fungitell test showed a low positive predictive value for the identification of IFI from analysis of the BAL BDG marker (20.0%). However, the negative predictive value of Fungitell was significantly higher for BAL BDG marker (83.0%). Mutschlechner *et al.* evaluated the Fungitell assay with BAL obtained from unselected solid organ transplant recipients suffering from probable and proven aspergillosis according to EORTC/MSG criteria. In 233 BAL samples from 135 patients with proven, probable, or no invasive aspergillosis, with a cut-off of 100 pg/mL, the sensitivity, specificity, PPV and NPV were 79.2%, 38.5%, 27.6%, and 86.3% with BAL BDG assay[16]. Ahmad *et al*. evaluated the diagnostic value of the BAL BDG marker in immunosuppressed mice infected with intravenously administered conidia of *A. terreus*. Culture of lung homogenate allowed growth of *A. terreus*. The positivity of BDG marker was 43%[17]. According to Hoenigl, the sensitivity of the BAL BDG marker test was high (for the 80 pg/ml cutoff), but specificity was the major limitation of this test compared to culture[8]. The specificity of the BAL BDG marker test was lower than that of serum BDG marker, which could be a result of frequent *Candida* colonization of the upper and lower respiratory tract in critically ill patients.

Conclusions

In our study, *Aspergillus* species was most commonly detected by culture of respiratory samples in the group of patients with chronic aspergillosis, followed by the cystic fibrosis group, the primary immunodeficiency group, and the prolonged ICU stay group. *A. fumigatus* was the most frequently detected fungal agent in positive respiratory cultures.

The sensitivity and specificity of beta D-glucan marker in the respiratory samples were 71.43%/85.71%, 72.73%/62.5%, 68.18%/0%, 50%/50% in all four groups, respectively.
The results of our study indicate that no single method could provide definite etiological diagnosis of aspergillosis, but it highlights that a positive (1,3)-β-D-glucan marker test in respiratory samples is useful diagnostic adjunct in the diagnosis of aspergillosis.

The application of conventional methods and iimplementation of fungal biomarker tests, as well as appropriate interpretation of results in collaboration with clinicians, is the most important aspect for accurate and precise etiological diagnosis of aspergillosis and for early initiation of antifungal treatment to achieve a favorable clinical outcome.

Conflict of interest statement. None declared.

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