CLINICAL SCIENCE

EVALUATION OF (1,3)-β-D-GLUCAN ASSAY IN DIAGNOSIS OF INVASIVE FUNGAL INFECTIONS WITH ASPERGILLUS

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Abstract

Invasive fungal infections caused by *Aspergillus* are a significant problem in immunocompromised and critically ill patients and associated with increased morbidity and mortality. Early diagnosis of invasive aspergillosis is still a big clinical and diagnostic challenge. Conventional methods are not sensitive enough, and therefore, there is a need for rapid, more sensitive methods for early diagnosis of invasive fungal infections with *Aspergillus*. The aim of this study was to evaluate the diagnostic performance, sensitivity and specificity of serological panfungal (1,3)-β-D-glucan marker compared to conventional method for diagnosis of invasive fungal infections with *Aspergillus*. Material and methods: Specimens of 125 patients divided into 4 groups (group I - immune deficiency, group II - prolonged ICU stay, group III - chronic aspergillosis, group IV - cystic fibrosis), classified according to clinical diagnosis and EORTC/MSG criteria, were analyzed at the Institute of Microbiology and Parasitology, with conventional and serological methods, during a period of two years. Results: A total of 71 isolates of *Aspergillus* were confirmed in this study. Four isolates were recovered from bloodculture of patients with primary immune deficiency. With BAL culture, *Aspergillus* was detected in the group of chronic aspergillosis (63.33%), followed by the groups of cystic fibrosis (56.67%), primary immune deficiency (51.43%), and the group with prolonged ICU stay (43.33%). Sensitivity and specificity of BAL culture were: 64.29% and 100%, 59.09% and 100%, 54.55% and 12.5%, 100% and 54.17%, in I, II, III and IV group, respectively. In 79.1% (53/67) from positive BAL cultures in all groups, *A. fumigatus* was confirmed, of which, 32.1% (17/53) in group III, followed by group I – 26.42% (14/53) and group IV – 26.42% (14/53), and 15.1% (8/53) in group II. Other species confirmed in BAL were *A. flavus* 16.42% (11/67) and *A.terreus* 4.48% (3/67). Sensitivity and specificity of the serological panfungal (1,3)-β-D-glucan (BDG) marker were: 64.71% and 85.71%, 50% and 87.5%, 36.36% and 50%, in groups I, II and III, respectively. No positive findings of the panfungal (1,3)-β-D-glucan (BDG) marker were found in the group with cystic fibrosis. Conclusion: The results obtained in this study have demonstrated that a positive (1,3)-β-D-glucan assay highlights the value of this test as a diagnostic adjunct in the serodiagnosis of invasive fungal infections with *Aspergillus*, and along with the results from conventional mycological investigation, helped in reaching a timely antifungal treatment with a favorable clinical outcome.

КЛИНИЧКИ ИСТРАЖУВАЊА

ЕВАЛУАЦИЈА НА (1,3)-β-D-ГЛИКАН ЕСЕЈ ВО ДИЈАГНОЗА НА ИНВАЗИВНИ ИНФЕКЦИИ СО ASPERGILLUS

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Извадок

Инвазивните фунгални инфекции со *Aspergillus* претставуваат сериозен проблем кај имунокомпромитираните лица и критично болните лица, и се асоцирани со зголемен морбидитет и морталитет. Рана дијагноза на инвазивната аспергилоза е сè уште голем клинички и дијагностички предизвик. Конвенционалните методи не се доволно сензитивни, и заради тоа, се наметнува потреба за брзи и посензитивни методи за рана дијагноза на инвазивни фунгални инфекции со *Aspergillus*. Целта на оваа студија беше да се евалуира дијагностичкиот перформанс, сензитивноста и специфичноста на серолошкиот панфунгален маркер (1,3)-β-D-гликан споредено со конвенционалниот метод за дијагноза на инвазивните фунгални инфекции со *Aspergillus*. Материјал и методи: Примероци од 125 пациенти, поделени во 4 групи (група I - имун дефицит, група II - пролонгиран престој во ЕИЛ, група III - хронична аспергилоза, група IV - цистична фиброза), и класифицирани според клиничката дијагноза и EORTC/MSG критериумите, беа анализирани на Институтот за микробиологија и паразитологија, со конвенционални и серолошки методи, во тек на две-годишен период. Резултати: Вкупно 71 изолат на *Aspergillus* беа потврдени во оваа студија. Четири изолати беа докажани во хемокултура, кај пациенти со примарен имун дефицит. Со култура на БАЛ, *Aspergillus* најчесто беше детектиран во групата на хронична аспергилоза (63,33%), по што следуваа групите со цистична фиброза (56,67%), примарен имун дефицит (51,43%), и групата лица со пролонгиран престој во единиците за интензивно лекување (43,33%). Сензитивноста и специфичноста на културите на БАЛ беа: 64,29% и 100%, 59,09% и 100%, 54,55% и 12,5%, 100% и 54,17%, во I, II, III и IV група, соодветно. Во 79,1% (53/67) од позитивните култури на БАЛ во сите групи, беше докажан *A.fumigatus*, од кои, 32,1% (17/53) од група III, потоа 26,42 % (14/53) од група I и 26,42% (14/53) од група IV, како и 15,1% (8/53) од група II. Други специеси потврдени во БАЛ беа *A.flavus* 16,42% (11/67) и *A.terreus* 4,48% (3/67). Сензитивноста и специфичноста на серолошкиот панфунгален (1,3)-β-D-гликан (BDG) маркер беа: 64,71% и 85,71%, 50% и 87,5%, 36,36% и 50%, во групите I, II и III, соодветно. Не беа детектирани позитивни наоди од панфунгалниот (1,3)-β-D-гликан (BDG) маркер во групата со цистична фиброза. Заклучок: Резултатите од оваа студија покажаа дека позитивен наод на (1,3)-β-D-гликан ја истакнува вредноста на овој тест како дијагностичко надополнување во серодијагнозата на инвазивните фунгални инфекции со *Aspergillus*, и заедно со резултатите од конвенционалните миколошки испитувања, помагаат во навремена примена на антифунгална терапија, и постигнување поволен клинички исход.

Introduction

Invasive fungal infections are significant causes of morbidity and mortality, especially in immunocompromised patients undergoing steroid treatment, chemotherapy resulting in severe neutropenia, hematopoietic stem cell and solid organ transplantation.[1] AIDS and malignant diseases can also contribute to development of this opportunistic fungal infection. 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 and rapidly spreading form of infection 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 invasive fungal infections including invasive aspergillosis.[3] To achieve a favorable prognosis of these life-threatening fungal infections, an early initiation of an antifungal therapy 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 specimens or growth of fungal agents in culture media is still the "gold standard" method.[4] However, invasive procedures for specimen collection may be sometimes contraindicated, especially in patients with profound respiratory insufficiency. Conventional methods are time-consuming and relatively insensitive, since they are positive in less than 30% of all invasive *Aspergillus* infections, and they depend on the quality of the specimen submitted. Also, some fungal pathogens require prolonged incubation, which could further delay the mycological diagnosis.[5]

Due to all these limitations, a lot of work has been done in recent years for development of alternative nonculture-based diagnostic assays for detection of invasive fungal infections, like detection of fungal biomarkers. Serum (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 in patients suffering from invasive fungal infections. Serum β-D-glucan concentrations show a constant rise even before manifestation of clinical signs, and then start to decrease, and eventually become negative if patients respond well to antifungal treatment.[6] Conversely, patients not responding do not show a decrease or show a continuous rise of this marker. The Fungitell test (Associates of Cape Cod) is a chromogenic kinetic test that was approved in 2003 by the U.S. Food and Drug Administration for the presumptive diagnosis of invasive fungal infections.[7] It may allow earlier diagnosis of invasive fungal infections than is otherwise possible with other conventional methods. The Fungitell BDG assay is a chromogenic, quantitative EIA based on the clotting cascade of the *Limulus* or horseshoe crab. Unlike most other standard ELISA tests, 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 have shown that in patients with a higher risk of development of invasive fungal infections, single positive β-D-glucan testing is associated with sensitivity and specificity generally ranging between 60 and 90%.[6] 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 invasive fungal infection.[8]

The aim of this study was to evaluate the diagnostic performance, sensitivity and specificity of serum (1,3)-β-D-glucan BDG marker in comparison with conventional methods (culture) for diagnosis of invasive infections with *Aspergillus* species.

Material and methods

Study design

A prospective diagnostic study was performed at the Institute of Microbiology and Parasitology, Faculty of Medicine, Skopje, Republic of Macedonia, during a 2-year period (2014-2016).

Group of patients and mycological investigations

In this study, clinical specimens (from mucosal surfaces of respiratory tract and blood cultures) from 125 patients divided into 4 groups, according to clinical diagnosis and risk factors for invasive aspergillosis, were analyzed at the Laboratory for diagnosis of fungal infections of the Institute of Microbiology and Parasitology, Faculty of Medicine, Skopje, Republic of North Macedonia. These groups included patients with primary immune deficiency, critically ill patients treated in intensive care units, patients with chronic aspergillosis and cystic fibrosis patients. Invasive fungal infection 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.[3] The specimens were investigated with conventional mycological methods, by inoculation of specimens on culture media for support of fungal growth (Sabouraud and chromogenic CALB medium (Oxoid)). Blood culture was performed with automated BacT/Alert system (*bioMerieux*, France), Gram stain and culture on Sabouraud and selective chromogenic CALB medium (Oxoid). Identification of *Aspergillus* on species level was performed with macroscopic analysis of grown mold colonies and further microscopic analysis of the reproductive elements (conidia) with lactophenol cotton blue method. Detection of (1,3)-β-D-glucan panfungal marker was made by Fungitell assay (Associates of Cape Cod).[7] A total of 5 µl of serum were briefly pretreated with 20 µl alkaline reagent solution (0.125 M KOH/0.6 M KCl) for 10 min at 37°C and then 100 µl reconstituted Fungitell reagent was added to the sample placed into triplicate wells of a 96-well microtiter plate. The reaction was incubated for 40 minutes

at 37°C and the optical density was measured at 405/490 nm with spectrophotometer. The mean rate of optical density change was determined for each well, and the BDG marker concentration was determined by comparison to a standard curve. Interpretation of BDG marker values was as follows: <60 pg/ml, negative; 60 to 79 pg/ml, indeterminate; ≥80 pg/ml, positive. The test results of the BDG marker assay were not available for the clinicians' decision on treatment (BDG results were not used for the management or classification of IFI). Proven and probable IFI were considered to be true-positive cases for analysis. Patients with possible invasive fungal infection were considered to be true-negative cases.

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

Specimens from mucosal surfaces of respiratory tract and blood cultures from 125 patients were divided in 4 groups (patients with primary immune deficiencies, critically ill patients treated in intensive care units, patients with chronic aspergillosis and cystic fibrosis) according to clinical diagnosis and EORTC/MSG (European Organization for Research and Treatment of Cancer/Mycoses Study group) criteria (Fig. 1).

Gender analysis of study patients revealed that men were more frequently distributed in I, III and IV group (60%, 60%, 53.33% respectively), whereas in group II, both genders were equally distributed. The 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, respectively (Table 1).

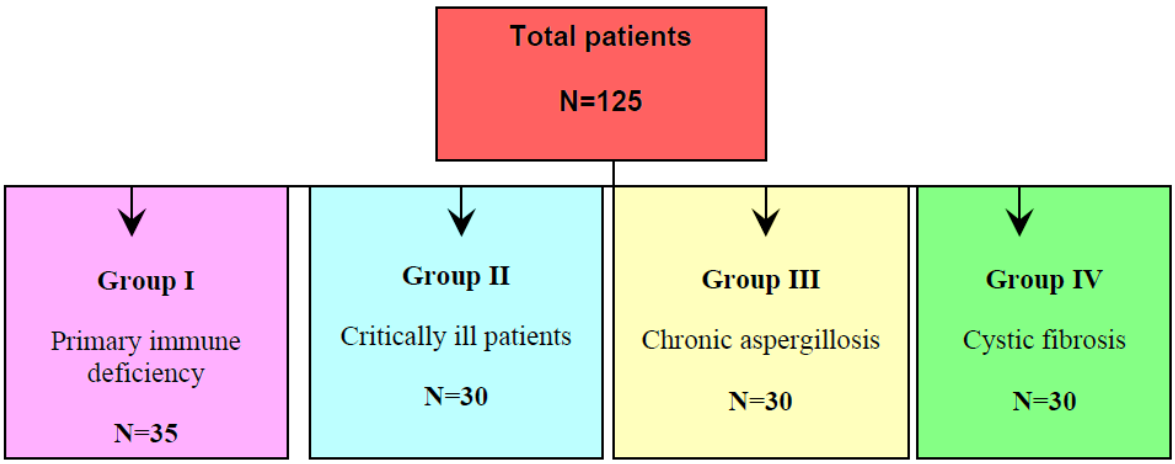

Fig. 1. Classification of patient groups according to clinical diagnosis and EORTC/MSG (European Organization for Researsh and Treatment of Cancer/Mycoses Study group) criteria

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%)
	ap = 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)

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) 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) of patients had some type of primary deficiency, and 10% (3/30) had a prolonged stay in an intensive care unit.

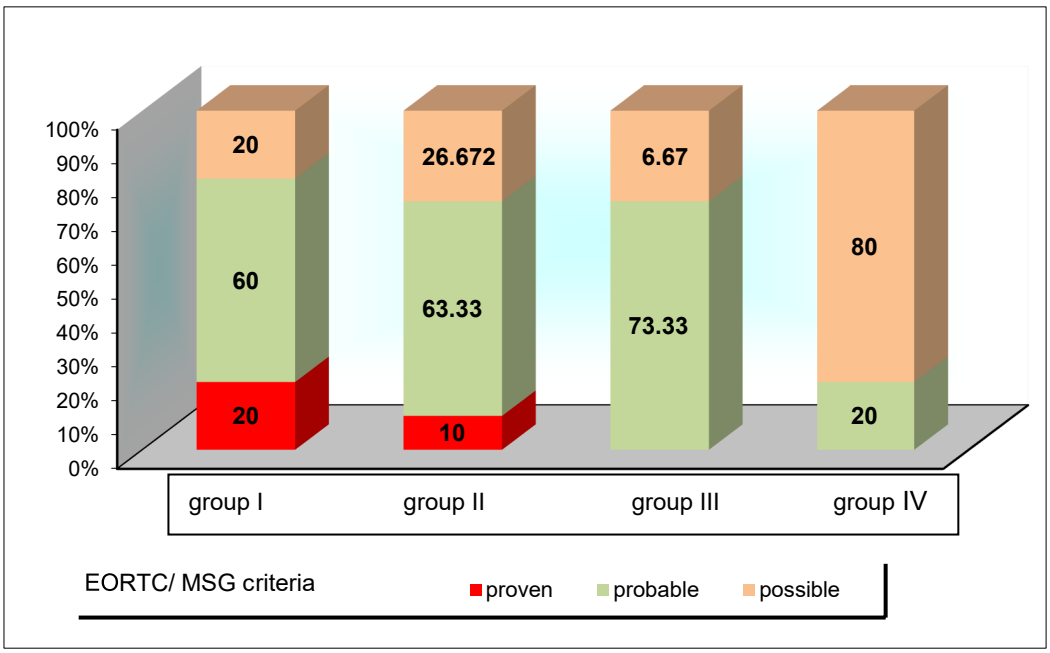

Fig. 2. Distribution of fungal infections according to EORTC/MSG criteria in all groups

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	**Group I N=35**	**Group II N=30**	**Group III N=30**	**Group IV N=30**
Gender	**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

Mycological investigation of blood cultures in our patients demonstrated positivity only in 4 patients. All positive blood cultures were discovered from patients with primary immune deficiency. *A. fumigatus* was identified as an etiological agent in all positive blood cultures (Table 3).

Table 3. Positive blood cultures in four groups of patients

Aspergillus	**Group I N=35**	**Group II N=30**	**Group III N=30**	**Group IV N=30**
Blood cultures	**n (%)**	**n (%)**	**n (%)**	**n (%)**
Negative 121 (96.8%)	31 (88.57%)	30 (100%)	30 (100%)	30 (100%)
Blood cultures – species				
***A. fumigatus* n=4**	4	0	0	0

Differences in positivity of blood cultures were insufficient for analysis of the statistical significance (p=0.46).

With cultural analysis of bronchoalveolar lavage (BAL), presence of *Aspergillus* was most frequently found in the group of chronic aspergillosis (63.33%), followed by the CF group (56.67%), the group with primary immune deficiency (51.43%), and 43.33% of patients hospitalized in ICU.

Regarding the presence of fungi in positive BAL specimens, the most frequently identified species (79%) was *A. fumigatus* (53/67). Thirty-two percent of the isolates (17/53) of *A. fumigatus* originated from specimens of patients with chronic aspergillosis, and 26% (14/53) were identified in specimens from patients with primary deficiency and cystic fibrosis (Table 4).

Table 4. Bronchoalveolar lavage (BAL) culture and identified fungal species

	Group I N=35	Group II N=30	Group III N=30	Group IV N=30
Blood cultures (%)	**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 in BAL				
A. fumigatus n=53	14	8	17	14
A. flavus n=11	2	4	2	3
A. terreus n=3	2	1	0	0

[a]p(Chi-square test)

Investigation of presence of panfungal marker (1,3)-beta-D-glucan (BDG) in serum was performed in parallel with blood culture and BAL culture. Positive findings of panfungal marker, in parallel with positive blood culture and BAL culture, were detected in 19 (54.29%) patients in group I, and 12 patients (40%) in both II and III group (Table 5). The differences in the distribution of positive and negative specimens were confirmed as statistically significant between the group with primary immune deficiency and cystic fibrosis group (p=0.0000016).

Table 5. Detection of BDG marker in serum

	Group I N=35	Group II N=30	Group III N=30	Group IV N=30
BDG	**n (%)**	**n (%)**	**n (%)**	**n (%)**
negative 82 (65.6%)	16 (45.71%)	18 (60%)	18 (60%)	30 (100%)
positive 43 (34.4%)	19 (54.29%)	12 (40%)	12 (40%)	0
I vs II vs III [a]p=0.25 I vs IV [a]p=0.0000016**				

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

Results of the descriptive statistics for the concentration of the BDG marker are presented in Table 6. Along with blood culture and BAL culture, a statistically significantly lower concentration of panfungal BDG marker was measured in the group of cystic fibrosis compared to all other groups (p<0.0001). The average concentration of BDG panfungal marker was highest in the first group (93.17±55.3 pg/ml), followed by II, III and IV group (70.1±50.0, 68.6±48.1, 4.2±1.1 pg/ml respectively). The median value of con-

centration of BDG panfungal marker in all four groups was 112 pg/ml (range 36-133), 44 pg/ml (range 33–96), 42.5 pg/ml (range 34–96), and 4 pg/ml (range 4–5), respectively.

Table 6. Descriptive statistics for the concentration of the BDG marker in serum

	Aspergillus **BDG concentration (pg/ml)**			
	mean ± SD	min-max	median (IQR)	p-value
BDG				
group I	93.17±55.3	32–254	112 (36–133)	H=7.34 [d]p<0.0001
group II	70.1±50,0	17–211	44 (33–96)	I vs IV [c]p<0.0001
group III	68.6±48.1	29–199	42.5 (34–96)	II vs IV [c]p<0.0001
group IV	4.2±1.1	1–6	4 (4–5)	III vs IV [c]p<0.0001

[c]p (Mann0Whitney U test) [d]p (Kruskal-Wallis test)

Comparative diagnostic performances of conventional (blood culture and BAL culture) and panfungal BDG marker for diagnosis of invasive infections with *Aspergillus* in the group with immune deficiency are presented in Table 7.

Table 7. Diagnostic performances of conventional (blood culture and BAL culture) and serological methods in the group with immune deficiency

Method	Se(%)	Sp(%)	PPV(%)	NPV(%)	LR+(%)	LR-(%)
Blood culture	14.29	100	100	22.58	/	0.86
BAL culture	64.29	100	100	41.18	/	0.36
BDG in serum	64.71	85.71	94.74	37.5	4.5	0.42

Comparative diagnostic performances of conventional (BAL culture) and serological methods for diagnosis of invasive infections with *Aspergillus* in the group with prolonged ICU stay in critically ill patients are presented in Table 8.

Table 8. Diagnostic performances of conventional (BAL culture) and serological methods in the group with prolonged ICU stay

Method	Se(%)	Sp(%)	PPV(%)	NPV(%)	LR+(%)	LR-(%)
BAL culture	59.09	100	100	47.06	/	0.41
BDG in serum	50	87.5	91.67	38.89	4	0.57

Comparative diagnostic performances of conventional (BAL culture) and serological methods for diagnosis of invasive infections with *Aspergillus* in the group with chronic aspergillosis are presented in Table 9.

Table 9. Diagnostic performances of conventional (BAL culture) and serological methods in the group with chronic aspergillosis

Method	Se(%)	Sp(%)	PPV(%)	NPV(%)	LR+(%)	LR-(%)
BAL culture	54.55	12.5	63.16	9.09	0.62	3.64
BDG in serum	36.36	50	66.67	22.22	0.73	1.27

In the group with cystic fibrosis, only BAL culture was analyzed, and this method had the following diagnostic performances: sensitivity 100%, specificity 54.17%, positive predictive value 35.29%, negative predictive value 100%, likelihood ratio for positive finding was 2.18%, likelihood ratio for negative finding was 0.

Discussion

Invasive fungal infections present an increasing global burden in immunocompromised and critically ill patients. Early mycological diagnosis with adequate detection and identification of the etiological agent and antifungal susceptibility profile is critical for favorable clinical outcome.[1]

In our study, we detected only 4 positive blood cultures caused by *A. fumigatus*, and all of them were from patients with primary immune deficiencies. Blood culture, as a diagnostic test for invasive aspergillosis, with aspergillemia, according to EORTC/MSG classification, had 14.29% sensitivity and specificity 100%. The significance of positive blood culture with *Aspergillus* species varies depending on the patient population. In the study of Kontoyiannis et al, positive blood cultures with *Aspergillus* species represented pseudofungemia in all 12 patients with solid tumors, whereas proven or probable aspergillosis was registered in 12 of 24 patients with hematological malignancies.[9] In another study, which analyzed patients with pulmonary aspergillosis, aspergillemia was registered in 10.1% of patients of 89 patients examined.[10] Transplantation of hematopoetic stem cells was the main predisposing condition for the development of invasive aspergillosis.[11] According to literature, there are no studies investigating the significance or importance of positive blood cultures with *Aspergillus* in this high-risk group of patients. In a retrospective study of Simoneau et al., of a total of 525 patients with transplantation of hematopoetic stem cells, 377 received allogenic, and 148 autologous transplantations. Aspergillemia was registered 23 times in 21 patients. According to Simoneau, positive blood cultures with *Aspergillus* are very rare and usually clinically insignificant, despite the capability of this fungus to cause invasion of vascular compartments in immunocompromised patients. *Aspergillus* fungemia in this study was represented with 17% of all fungemia cases (23/131) during a 23-year-follow-up of all fungemia cases in this medical center.[12] In a similar medical center, during a 17-year follow-up, fungemia with *Aspergillus* was registered in 4% of all cases with fungemia. Still, in this study, non-transplant patients with hematological malignancies were also included.[10] In the study of Simoneau and collaborators, only one of 19 cases of fungemia with *Aspergillus* was confirmed as true fungemia. All cases of aspergillemia were detected during a period of 11 years, with a system based on lysis–centrifugation.[12] Out of 23,000 blood cultures analyzed, only 0.2% demonstrated positivity with growth of *Aspergillus*. Despite the fact that all blood cultures were investigated with a biosafety cabinet, still, contamination with conidia of filamentous fungi couldn't have been prevented. During recent years, many studies have analyzed true aspergillemia with automated systems, and none of these documented aspergillemia.[12,13] In the study of Simoneau,

experimental inoculation of blood culture bottles was performed, with BacT/Alert system, and growth with *Aspergillus* was confirmed, which additionally adds to the capability of the system to support growth of filamentous fungi.[12] According to Lopes-Bezerra, vascular endothelial cells exposed in vitro to kill hyphae of Aspergillu were continuously destroyed.[14] Probably, viability of endocytosed hyphae of *Aspergillus* species is deeply compromised, which contributes to small chances for recovery of fungi by blood culture. Although A. fumigatus can grow in blood culture bottles, still, blood cultures from patients with invasive aspergillosis are usually negative, and reasons for this are still unclear.[15] Girmenia et al. presented a small number of positive blood cultures (10%) in patients with invasive aspergillosis, which contributed to the general perception of a very low sensitivity of blood cultures for diagnosis of invasive aspergillosis.[10] Most scientists agree that positive blood cultures with *Aspergillus* are very rare, even in high-risk patients, like transplant patients with hematopoetic stem cells, hence most positive blood cultures are actually pseudofungemia, and are not connected with real invasive aspergillosis. Also, some studies suggest that DNA of *Aspergillus* is free in the blood, so most likely that is the reason for the low sensitivity of blood cultures for diagnosis of invasive aspergillosis.[16] As previously discuissed, clinical and radiological presentation, as well as the number of positive blood cultures and the system of blood cultures used, should be taken into consideration when analyzing the significance of positive blood cultures with *Aspergillus*. Ussully only one positive blood culture with the automated system means pseudofungemia.

In our study, the culture of BAL specimens demonstrated growth of *Aspergillus* most frequently in the group of chronic aspergillosis (63.33%), followed by 56.67% of patients with cystic fibrosis, 51.43% of patients with primary immune deficiency, and 43.33% of patients with prolonged ICU stay. Sensitivity and specificity of BAL culture was: 64.29% and 100%, 59.09% and 100%, 54.55% and 12.5%, 100% and 54.17%, in I, II, III and IV group respectively. In the study of Tashiro *et al*., 165 isolates of *Aspergillus* species were detected in culture of respiratory tract of 139 patients. Of these, 62 (45%) were colonized with *Aspergillus*, but didn't demonstrate clinical symptoms of aspergillosis, and the other 77 patients (55%) had some type of pulmonary aspergillosis classified as chronic (48%), aspergilloma (29%), invasive (13%), or ABPA (10%). In the study of Tashiro, patients with chronic necrotizing aspergillosis or aspergillom, most frequently had COPD, tuberculosis or cancer of the lungs. Some of them had received systemic immunosupresive drugs for a prolonged period, or had some chronic diseases like diabetes, cancer or hepatic chirrosis.[17] In patients with invasive aspergillosis, the main predisposing factor had been hematological malignancy, and they were subsequently treated with immunosupresive drugs. Patients with ABPA frequently demonstrated signs of bronchial asthma (88%) or other atopic diseases (63%). In our study regarding the distribution of species from positive BAL cultures, in all four groups, *A. fumigatus* was identified in 79.1% (53/67), and from these, 32.1% (17/53) in patients with

chronic aspergillosis. *A. fumigatus* was also identified in an equal number of patients in I group - 26.42% (14/53) and IV group - 26.42% (14/53), and 15.1% (8/53) in the group of critically ill patients. Other species confirmed in our study, in positive BAL cultures, were *A. flavus* (16.42% (11/67) and *A. terreus* 4.48% (3/67)). Of these, 36.4% (4/11) were due to isolates of *A. flavus*, 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, in our study, *A. fumigatus* was a dominant fungus in AIDS patients (4/6), who had their CD4 numbers below 50/mm3 and 10/mm3. Similar data were presented in the study of Meyohas *et al.*, who confirmed CD4 numbers below 50/mm3 in their patents with positive BAL culture.[18] In the study of Lortholary, 28 out of 33 patients (84.8%) had a positive BAL culture for *Aspergillus*.[19] In the study of Menninkx-Kersten, distribution of *Aspergillus* among 165 confirmed isolates in BAL cultures, demonstrated presence of 41% of *A. fumigatus* and 32% 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%).[20] In this study, A. fumigatus was the predominant species in patients with invasive aspergillosis (82%), aspergilloma (68%), and chronic aspergillosis (54%), while A. niger was on the second place. Zarrinfar et al. demonstrated presence of A. flavus, A. niger and one case with mixed infection with two species (A. flavus/A. niger) in positive (23 %) BAL cultures.[21] In contrast to our study, where A. fumigatus was predominant species, the most frequent agent in the study of Zarrinfar was A. flavus.[21] In our study, we did not isolate A. niger in BAL cultures of our patients. Although *A. fumigatus* is considered as the most pathogenic species, still this species can frequently be a colonizer of the respiratory tract without any clinical manifestation of invasive aspergillosis, which was also registered in our study, especially in those patients categorized as possible infections according to EORTC/MSG criteria. Diagnostic value of *Aspergillus* identification in respiratory specimens is sometimes questionable, since it is very difficult for the clinican to differentiate between colonization and infection. According to Ader, discovery of the same species of *Aspergillus* in more specimens during an antibiotic treatment, without favorable pharmacological response, in patients with a high risk, should raise a concern for the development of invasive aspergilosis.[22] Therefore, isolation of *Aspergillus* from respiratory tract specimens in critically ill patients with high risk and clinical signs of pneumonia requires a faster decision for a prompt initiation of antifungal treatment.[23,24] Although in some cases colonization is transient in the respiratory tract, still it could present as a serious warning sign of an infection with *Aspergillus*.[25] In 63.33% of our patients with chronic aspergillosis, BAL culture confirmed presence of *Aspergillus*, and all were due to *A. fumigatus*. Similar data were found in the study of Tashiro, where *A. fumigatus* was the predominant species (54%), followed by *A. niger* (24%), *A. terreus* (10%), *A. versicolor* (6%), *A. flavus* (4%), and A. *nidulans* (2%) (17). Perfect *et al.* also confirmed *A. fumigatus* (69%) as the

most frequent isolate in positive BAL cultures, followed by *A. niger* (13%), *A. flavus* (2%), and other species (5%) among their patients[26]. ABPA is an allergic form of aspergillosis due to hypersensitivity to *Aspergillus*, where the predominant cause is *A. fumigatus*.[27] In our study, all isolates of CF specimens were positive for *A. fumigatus* - 82.4% (14/17), and only 10% due to *A. flavus* (17.6%).

The serological diagnosis of infection with *Aspergillus* species was performed with detection of the panfungal (1,3)-beta-D-glucan (BDG) marker in patients' sera. The concentration of BDG marker in all four groups was 112 pg/ml (range 36–133), 44 pg/ml (range 33–96), 42.5 pg/ml (range 34–96), and 4 pg/ml (range 4–5), respectively. BDG panfungal marker in serum from immune deficiency patients demonstrated sensitivity of 64.71% and specificity 85.71%. In contrast to our results, with median values of this marker 112 pg/ml, Lahmer et *al*. demonstrated much higher concentrations of BDG marker in 22 out of 30 critically ill patients with hematological malignancies (median value 306 pg/ml).[28] According to values of BDG marker and mycological evidence, 10 patients were classified as probable invasive aspergillosis (34%) and 12 patients (40%) as possible aspergillosis. The overall sensitivity of the assay was 90% and specificity 85% in patients with invasive aspergillosis, in contrast to our results, where we demonstrated a lower sensitivity (64.71%) and specificity (85.71%).

The panfungal BDG marker in sera of critically ill patients in our study showed lower sensitivity compared to the group with primary immune deficiency (50%), and specificity was 87.5%. Similar results were obtained by Cai *et al*., who demonstrated lower sensitivity of BDG marker in their study, with sensitivity of 48.1% and specificity of 78.8%.[29] In the study of Lahmer *et al*., 49 immunosupressed patients with respiratory insufficiency and treated in ICU were analyzed. Thirteen of these patients (26%) had probable invasive aspergillosis. The BDG marker assay in these patients demonstrated much higher concentrations compared to patients without probable invasive aspergillosis (375 [103-1000 pg/mL; P<.001] in contrast to 64 [30-105 pg/mL; P<.001]).[30] Data from literature on BDG marker concentrations in serum in critically ill patients treated in ICU are very few and insufficient, since they show that serum concentrations of BDG marker do not always correlate with invasive aspergillosis and are not specific (if cut-off is 20 pg/mL).[31]

BDG in serum in the group with chronic aspergillosis showed sensitivity of 36.36% and specificity of 50%. In the study of Kami *et al*. 10/16 patients with proven aspergillosis, 8/14 with probable aspergillosis, and 44/185 control patients demonstrated positive findings with BDG panfungal marker in serum. Three of eight patients with localized invasive aspergillosis, and 7/8 patients with disseminated aspergillosis were positive for the BDG panfungal marker. Sensitivity and specificity of the panfungal BDG assay were 63% and 76%, respectively. Sensitivity was 88% in patients with disseminated aspergillosis, but only 38% in those patients with localized invasive aspergillosis.[32] Similar results were obtained in our study, with sensitivity of 36.36% and specificity of 50%, in patients with localized invasive aspergillosis. Sensitivity was

lower in patients with localized aspergillosis compared to patients with disseminated infections, and there was a statistically significant difference (p=0.0406). In another study, 29/178 patients with proven invasive aspergillosis, 33/210 probable cases of aspergillosis and 117/1877 specimens from patients without invasive aspergillosis were positive for BDG marker. Three of 99 specimens from patients with localized invasive aspergillosis and 26 of 79 specimens from patients with disseminated invasive aspergillosis were positive for BDG marker. In this analysis, sensitivity and specificity of the BDG assay was 16% and 94%, respectively. Sensitivity was 33% in patients with disseminated aspergillosis, but only 3% in patients with localized infection. Lower sensitivity of the assay was registered among patients with localized infection with *Aspergillus* compared to those with invasive form. This difference was statistically significant (p<0.0001).[32] In the group of cystic fibrosis, no elevated values of the panfungal marker was registered. Theel *et al*., evaluated the performance of the BDG assay in serum, for identification of invasive fungal infections in immunocompromised patients with proven, probable and possible aspergillosis according to EORTC/MSG criteria.[33] Among 109 patients, the BDG assay demonstrated a low positive predictive value for serological diagnosis of invasive fungal infections with serum analysis of BDG marker (26.7%). Still, the negative predictive value of the assay was much higher (84.8%). Mutschlechner *et al*. evaluated the BDG assay with sera obtained from non-selected transplant patients with solid organs suffering from proven and probable aspergilosis according to EORTC/MSG criteria. In 109 sera from 135 patients with proven, probable aspergillosis or without evidence of invasive aspergillosis, with cut-off of 100 pg/mL, sensitivity, specificity, positive and negative predictive value of the BDG assay were 79.2%, 81.8%, 69.2%, and 83.1%, respectively.34 Ahmad *et al*. evaluated diagnostic value of the BDG marker in immunocompromised mice, with intravenous injected conidia of *A. terreus*. The culture of lung specimens showed growth of *A. terreus*. Positivity of the BDG assay in serum was 43%.[35]

Conclusions

The results of this study have indicated that no single method could provide definite etiological diagnosis of invasive fungal infection caused by *Aspergillus*. When using a conventional method, it is neccessery to provide more specimens from each patient, in frequent time intervals, and cautiously interpret the results obtained, since colonisation with fungi without clinical signs of infection is possible. Still, clinicians should be aware that these methods are time-consuming, with low sensitivity, and depend on the quality of the specimen submitted.

Analysis of the serological panfungal (1,3)-beta-D-glucan marker has demonstrated that this assay could be an additional useful diagnostic tool for screening of invasive fungal infections, but results should be interpreted alongside other clinical and laboratory findings.

In conclusion, implementation and analysis of different microbiological methods, as well as appropriate interpretation of results, in collabora-

tion with clinicians, is the most important aspect towards accurate and precise etiological diagnosis of invasive aspergillosis and earlier start of antifungal treatment in order to achieve favorable clinical outcome.

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