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Original article

POLYMERASE CHAIN REACTION IN SERUM FOR DIAGNOSIS OF ASPERGILLOSIS

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Abstract

Introduction: Aspergillosis is a major threat for immunocompromised and critically ill patients. Early diagnosis and initiation of appropriate antifungal therapy are essential for favorable clinical outcome. Laboratory diagnosis is still challenging, since conventional methods are not sensitive enough. Therefore, there is a need for rapid and more sensitive methods for early diagnosis of aspergillosis. The polymerase chain reaction (PCR) offers great promise for faster diagnosis of fungal infections.

Aim: The aim of this study was to evaluate the performance of a PCR assay in serum, compared to conventional method, for the diagnosis of aspergillosis.

Material and methods: Specimens of 125 patients divided into 4 groups (group I - immunodeficiency, group II - prolonged stay in ICU, group III - chronic aspergillosis, group IV - cystic fibrosis), classified according to clinical diagnosis and EORTC/MSG criteria, were analysed at the Institute of Microbiology and Parasitology, Faculty of Medicine, Skopje, with conventional and molecular methods, during a period of two years (2014-2016).

Results: Seventy-one isolates of *Aspergillus* were confirmed in BAL culture. *Aspergillus* was detected in 63.33% of patients with chronic aspergillosis, followed by 56.67% of cystic fibrosis patients, 51.43% of patients with primary immunodeficiency, and 43.33% of patients with prolonged stay in ICU. 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 the I, II, III and IV group, respectively. PCR performed in serum yielded the following results for sensitivity and specificity: 53.57% and 100% in group I, 36.36% and 100% in group II, 9.09% and 100% in group III, respectively. Serum PCR was not performed in the group with cystic fibrosis.

Conclusion: Our results indicate that PCR in serum could be a useful adjunct test for diagnosis of aspergillosis, along with results from conventional mycological method, so a timely antifungal treatment with a favorable clinical outcome, is achieved.

Keywords: *Aspergillus, aspergillosis*, PCR, serum, diagnosis

Introduction

Aspergillosis is defined as an infection caused by *Aspergillus* species, and most often targets immunocompromised and critically ill patients. Over 250 species of *Aspergillus* exist in nature, but fewer than 40 are causing infections in humans[1]. Among these species, *Aspergillus fumigatus* is the most common cause of serious and invasive infections in humans[2]. Aspergillosis is associated with high morbidity and mortality[3]. It 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[4]. Timely

and accurate diagnosis is crucial for early initiation of antifungal treatment in order to improve the prognosis of the disease.

Conventional mycological diagnosis is very difficult and poses a big laboratory challenge. Conventional methods are time-consuming and insensitive, since they are positive in less than 30% of all invasive *Aspergillus* infections, and they depend on the quality of the specimen submitted[5]. In the absence of a single "gold standard" test for the diagnosis of aspergillosis, the clinical and radiological evidence, as well as the combination of mycological and histopathology findings, and host factors, are needed to obtain accurate and definite diagnosis. The European Organization for Research and Treatment of Cancer and the Mycoses Study Group Education and Research Consortium (EORTC/MSGERC) consensus definitions of invasive fungal diseases (IFDs) were last updated in 2008[6].

In the previous definitions[6], indirect tests for diagnosing invasive fungal disease were only included if there was sufficient evidence that they had been standardized and validated. While tests for galactomannan and panfungal BDG marker were incorporated, tests for detecting fungal nucleic acid were not. The successful use of PCR for the detection of *Aspergillus* in human specimens has been reported in many publications previously, but even though it has existed for almost 2 decades, the technique was not included in the European Organization for Research and Treatment of Cancer and the Mycoses Study Group (EORTC/MSG) consensus definitions for the diagnosis of invasive fungal diseases because of a lack of standardization. To contribute to inclusion of *Aspergillus* PCR in diagnosis of aspergillosis, the International Society of Human and Animal Mycology working group Fungal PCR Initiative, has made significant effort toward setting a standard for the technique after vigorous multicenter validation[7]. The inclusion of PCR in the European Organization for Research and Treatment of Cancer (EORTC) and the Mycosis Study Group (MSG) mycological criteria permitted the reclassification of nine patients treated for IA but classified as possible cases due to a lack of EORTC/MSG mycologic criteria. They were reclassified from possible to probable aspergillosis and thus sensitivity was increased to 71.7%. Combining the galactomannan index with serum PCR increased the detection rate of invasive aspergillosis with 88.2% of sensitivity[8].

Chronic pulmonary aspergillosis is also a big diagnostic challenge for both clinicians and laboratory workers. From recently, guidelines edited jointly by the European Society of Clinical Microbiology and Infectious Diseases and the European Respiratory Society (ESCMID/ERS) recommend the use of *Aspergillus* PCR in BAL for the diagnosis of non-invasive aspergillosis[9,10].

Allergic bronchopulmonary mycoses are complex pulmonary disorders caused by immune reactions against fungi, most often *A. fumigatus*, which colonize the airways of patients with chronic lung disease, most commonly asthma or cystic fibrosis. The diagnostic criteria proposed by the International Society for Human and Animal Mycology (ISHAM)-ABPA working group (AWG) in 2013 are still widely used for diagnosing ABPA. Since the inception of these guidelines more than a decade ago, newer evidence has emerged concerning diagnostic test performance for ABPA, which required an update of these recommendations due to advances in diagnostics and therapeutics[11].

The aim of this study was to evaluate the usefulness of an *Aspergillus* PCR assay performed in serum, compared to conventional method, for diagnosis of both invasive and non-invasive aspergillosis.

Material and methods

Study design

A diagnostic study was performed at the Institute of Microbiology and Parasitology,

Faculty of Medicine, Skopje, Republic of Macedonia, during a 2-year period, as part of an ongoing PhD study during the period 2014-2016.

Group of patients and standard mycological investigations

Clinical specimens (from mucosal surfaces of respiratory tract and blood cultures) from 125 patients divided into 4 groups (group I - immunodeficiency, group II - prolonged stay in ICU, group III - chronic aspergillosis, group IV - cystic fibrosis), according to clinical diagnosis and risk factors for invasive aspergillosis, were analyzed at the laboratory for mycological diagnosis of fungal infections at the Institute of Microbiology and Parasitology, Faculty of Medicine, Skopje, Republic of Macedonia. 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[5].

Conventional mycological methods

The specimens were analyzed with standard mycological methods, by inoculation of samples on culture media for support of fungal growth (Sabouraud and chromogenic CALB medium (Oxoid)). Specimens from the respiratory tract were homogenized and divided in two parts. One part of the specimen (1-5 ml) was centrifuged, and the suspended pellet (500 μl) was directly inoculated on the media for growth of fungi (Sabouraud dextrose agar with chloramphenicol (40 μg/ml), and incubated at 35°C during 48 hours, and the other part was frozen at -80°C, and then used for molecular analysis.

Identification of *Aspergillus*. Final identification of *Aspergillus* species was performed with macroscopic analysis of mold colonies and further microscopic analysis of the reproductive elements (conidia) with lactophenol cotton blue method.

Molecular detection of Aspergillus DNA

Extraction of DNA from serum. The blood obtained with venipuncture in containers with EDTA was used for molecular analysis. A total of 3-5 ml of peripheral blood was mixed with 5 volumes of buffer for lysis of erythrocytes (0.155 M NH_4Cl, 0.01 M NH_4HCO_3, 0.1 mM EDTA (pH 7.4)), and this mixture was incubated during 10 minutes at 4°C. After lysis of erythrocytes, the specimen was centrifuged at 300×g during 10 minutes. The supernatant was discarded, and leukocytes were washed once 1×PBS solution (1.4 M NaCl, 50 mM KCl, 90 mM $Na_2PO_4 \cdot 2H_2O$, 20 mM KH_2PO_4 (pH 7.4)) and it was centrifuged again. The sediment from blood was further treated with the following procedure: the leukocyte pellet was resuspended in 300 μl of 1×PBS solution and the mixture was incubated with 100-125 U lyticase (lyticase-50,000 U; Sigma) during 30 minutes at 37°C, for degradation of fungal cells. The residual material of human and fungal cells was treated with 500-1,000 μg proteinase K (Boehringer) and 0.5% SDS (Natrium dodecyl sulphate) (Sigma) at 55°C during 1 hour. The residual cell material was then lysed while incubated with additional 100 μl 2×*Aspergillus* buffer for extraction (400 mM Tris-Cl, 1 M NaCl, 20 mM EDTA, 2% Natrium dodecyl sulphate) during 30 minutes at 65°C[12]. Purification of fungal and human DNA was performed with conventional phenol-chlorophorm extraction[13]. The precipitation of DNA was performed with addition of 0.7 volume of isopropanol, so a pellet can be obtained, which was washed with 70% ethanol, and afterwards it was dried on air. The concentration of DNA was analyzed with spectrophotometer at 260 and 280 nm. The DNA extracts were frozen at -20°C until the PCR procedure.

Controls for extraction. Negative controls were tubes with purified water without DNA, for evaluation of contamination during extraction process. Positive controls were included for every extraction and verification of efficacy, with inoculation of saline solution with approximately 150 CFU of *A. fumigatus* conidial suspensions, in a volume of 500 *μl.* To

determine the total number of injected CFU, 100 μl of the suspension containing around 30 CFU, was inoculated on the surface of the Sabouraud dextrose agar, which was incubated for 72 hours at 30°C.

PCR for *Aspergillus*

PCR reaction was performed in a volume of 25 μl mixture containing approximately 50-150 nanograms of total DNA as a template. This PCR mixture contained around 0.5 U Taq DNA polymerase, 6.25 nmol DNTP, 10 pmol primers (for the first PCR step - first set of primers: AFU 7S-AFU 7AS; for the second PCR step - another set of primers AFU 5S-AFU 5AS), which derived from sequences of *A. fumigatus* 18S rRNA gene). PCR was performed using an automated thermocycler, with the following protocol: for the first PCR step, denaturation for 2 minutes at 94°C, followed by 23 cycles of 40 seconds at 94°C, annealing for 1 minute at 65°C, and 1 minute at 72°C, with the final step performed for 5 minutes at 72°C, after which the mixture was kept at 4°C; for the second PCR step, 2 minutes at 94°C, followed by 35 cycles of 40 seconds at 94°C, 1 minute at 65°C, and extension of 1 minute at 72°C, with the final step performed for 5 minutes at 72°C, after which the mixture was kept at 4°C. For the second PCR, approximately 1-2 μl of the first PCR product was used. The PCR products were separated with 2.5% agarose gel electrophoresis, further dyed with ethidium bromide, and visualized with UV light. Control specimens included all components of the reaction mixture, except genomic DNA. As positive and negative controls for PCR, DNA of a human cell line T47D and diluted solution of *A. fumigatus* were used as templates[12].

PCR primers for Aspergillus

Primers	Sequences (5'→ 3')	Amplicons
AFU 5S	AGG GCC AGC GAG TAC ATC ACC TTG	1436-1459
AFU 5AS	GG G (AG)GT CGT TGC CAA C(CT)C (CT)CC TGA	1648-1771
AFU 7S	CGG CCC TTA AAT AGC CCG	1296-1313
AFU 7AS	GA CCG GGT TTG ACC AAC TTT	1681–1700

Statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS) for Windows. The results of the 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 immunodeficiency, 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[6] (Figure 1).

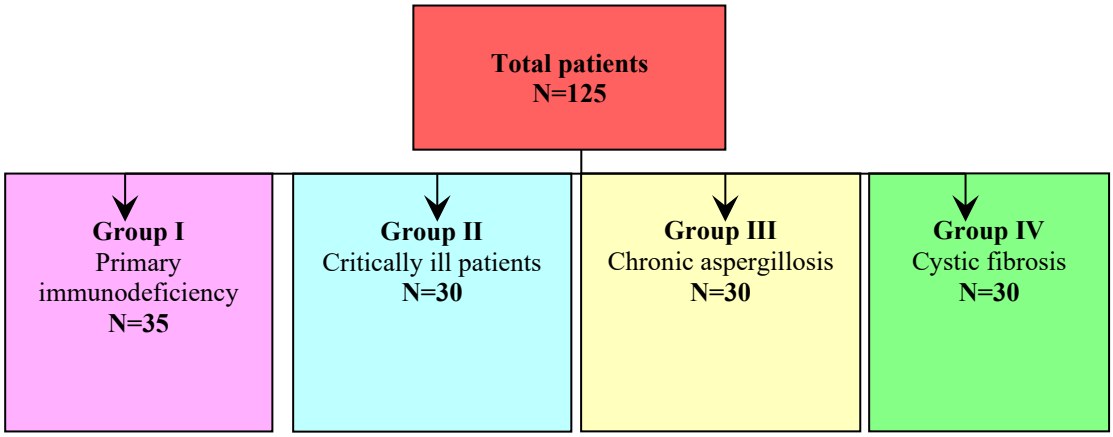

Fig. 1. Classification of patient groups according to clinical diagnosis and EORTC/MSG (European Organization for Research and Treatment of Cancer/Mycoses Study group) criteria

Analysis of patients' gender showed that men were more frequently represented in groups I, III and IV (60%, 60%, 53.33%, respectively), whereas in the second group, 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 (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)

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. 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 an intensive care unit.

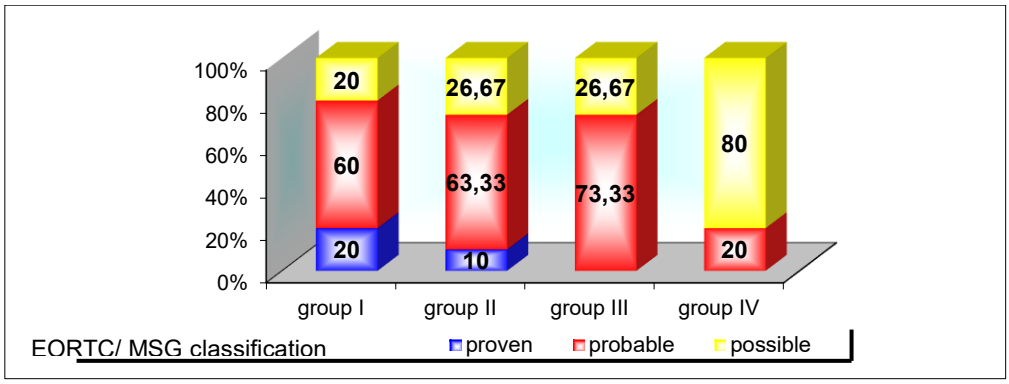

Fig. 2. Distribution of fungal infections according to EORTC/MSG criteria in all groups

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 **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

BAL cultures most frequently demonstrated the presence of *Aspergillus* in the group of chronic aspergillosis (63.33%), followed by the specimens from the CF group (56.67%), the group with primary immunodeficiency (51.43%), and patients hospitalized in ICU (43.33%). However, the differences in positive BAL cultures among the four groups were insufficient for statistical significance (p=0.46). The most frequent species (79%) identified in positive BAL specimens 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 or cystic fibrosis (Table 3).

Table 3. Bronchoalveolar lavage (BAL) culture and identified fungal species

BAL 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 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

p(Chi-square test)

Table 4. PCR in serum

	Group *Aspergillus*			
	group I N=35	**group II N=30**	**group III N=30**	**group IV N=30**
Serum PCR	**n (%)**	**n (%)**	**n (%)**	**n (%)**
No 100 (80%)	20(57.14%)	22(73.33%)	28(93.33%)	30(100%)
Yes 25 (20%)	15(42.86%)	8(26.67%)	2(6.67%)	0
	Chi-square: 23.09 [a]p < 0.000039			
	I vs II [a]p=0.17 II vs III [b]p= 0.08 III vs IV [b]p= 0.5			
	I vs III [b]p= 0.0014** II vs IV [b]p= 0.0046**			
	I vs IV [b]p<0.001			

[a]p(Chi-square test), [b](Fisher exact test)

Regarding the application of PCR in serum, presence of *Aspergillus* DNA was confirmed in 42.86% of patients with primary deficiency, 26.67% of patients with prolonged ICU stay, and in 6.67% of patients with chronic aspergillosis. Statistically significant differences were confirmed between group I *versus* group III (p=0.0014) and group IV (p<0.0001), and between group II *versus* group IV (p=0.0046) (Table 4).

Results from comparative diagnostic performance of conventional (BAL culture) and PCR in serum in the group with immunodeficiency are presented in Table 5.

Table 5. Diagnostic performances of BAL culture and PCR in serum in the group with immunodeficiency

Test	**Se (%)**	**Sp (%)**	**PPV (%)**	**NPV (%)**
BAL culture	64.29	100	100	41.18
Serum PCR	53.57	100	100	35

Results from the comparative diagnostic performance of conventional (BAL culture) and molecular methods for diagnosis of aspergillosis in the group of critically ill patients with prolonged ICU stay are presented in Table 6.

Table 6. Diagnostic performances of conventional (BAL culture) and molecular methods in the group with prolonged ICU stay

Test	**Se (%)**	**Sp (%)**	**PPV (%)**	**NPV (%)**
BAL culture	59.09	100	100	47.06
Serum PCR	36.36	100	100	36.36

Comparative diagnostic performances of conventional (BAL culture) and molecular methods for diagnosis of invasive infections with *Aspergillus* in the group with chronic aspergillosis are presented in Table 7.

Table 7. Diagnostic performances of conventional (BAL culture) and molecular methods in the group with chronic aspergillosis

Test	**Se (%)**	**Sp (%)**	**PPV (%)**	**NPV (%)**
BAL culture	54.55	12.5	63.16	9.09
Serum PCR	9.09	100	100	28.57

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%, and likelihood ratio for negative finding was 0.

Discussion

Aspergillus has emerged as one of the most common causes of death in severely immunocompromised patients, with mortality rates up to 40% to 50% in patients with acute leukemia and recipients of hematopoietic stem cells transplantation. Invasive aspergillosis is also the most frequent fungal infection in critically ill patients. Despite the clinical use of highly efficient new antifungal drugs, morbidity and mortality from aspergillosis remains as high as 50 to 70%[14,15]. Early initiation of effective antifungal treatment is essential in order to achieve favorable outcome. Therefore, high-risk patients could greatly benefit from efficient, noninvasive diagnostic strategies allowing earlier diagnosis of aspergillosis. Screening for circulating *Aspergillus* DNA by PCR has shown potential in the definitive diagnosis of aspergillosis, especially in combination with other biomarkers and conventional mycological methods[16].

In our study, BAL culture confirmed presence 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 immunodeficiency, and 43.33% of patients with prolonged stay in ICU. 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 by 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 did not demonstrate clinical symptoms of aspergillosis, and the other 77 patients (55%) had some type of pulmonary aspergillosis that was classified as chronic (48%), aspergilloma (29%), invasive (13%), or ABPA (10%). Patients with chronic necrotizing aspergillosis or aspergilloma most frequently had COPD, tuberculosis or lung cancer. Some of them had received systemic immunosuppressive drugs for a prolonged period, or had some chronic diseases such as diabetes, cancer or hepatic cirrhosis[17]. In patients with invasive aspergillosis, the main predisposing factor was hematological malignancy, and subsequent treatment with immunosuppressive drugs. Patients with ABPA frequently exhibited signs of bronchial asthma (88%) or other atopic diseases (63%).

Regarding the distribution of species from positive BAL cultures in our study, *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 equal number of patients in group I - 26.42% (14/53) and group IV-26.42% (14/53), and 15.1% (8/53) in the group of critically ill patients. Other species confirmed in positive BAL cultures in our study, 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.

In the study by Mennink-Kersten, the distribution of *Aspergillus* among 165 confirmed isolates in BAL cultures demonstrated the 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%)[18]. In their study, *A. fumigatus was the predominant species in patients with invasive aspergillosis* (82%), aspergilloma (68%), and chronic aspergillosis (54%). 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[19]. In contrast to our study, where *A. fumigatus was predominant species, the most frequent agent in the study by Zarrinfar was A. flavus.*

Although A. fumigatus is considered the most pathogenic species, still, it can frequently colonize the respiratory tract without any clinical manifestation of invasive aspergillosis. This finding was also registered in our study, particularly in those patients categorized as having possible infections according to EORTC/MSG criteria. The diagnostic value of identification of *Aspergillus* in respiratory specimens is sometimes questionable, as it is very difficult to differentiate between colonization and infection. According to Ader, discovery of the same

species of *Aspergillus* in more specimens during antibiotic treatment, without favorable pharmacological response, in high-risk patients should raise suspicion for the development of invasive aspergillosis[20]. Therefore, isolation of *Aspergillus* from respiratory tract specimens in critically ill patients with high risk and clinical signs of pneumonia need faster decision for a prompt initiation of antifungal treatment. Although in some cases, colonization is transient in the respiratory tract, still, it could present a serious warning sign of an infection with *Aspergillus*.

In our patients with chronic aspergillosis, BAL culture confirmed presence of *Aspergillus* in 63.33%, and all were due to *A. fumigatus*. Similar data were found in the study by 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[21]. ABPA is an allergic form of aspergillosis due to hypersensitivity to *Aspergillus*, where the predominant cause is *A. fumigatus*[11]. 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%).

Microbiological diagnosis of invasive aspergillosis is still a big diagnostic challenge, and relies upon detection of fungus in specimens from lower respiratory tract. This requires prolonged incubation and delays the mycological diagnosis. Therefore, early diagnosis of infections with *Aspergillus* can provide clinicians with timely and useful information for prompt initiation of appropriate antifungal treatment. With the development of molecular methods, researchers have tried to find more reliable methods for detection of DNA of *Aspergillus* in clinical specimens in high-risk patients[5]. *Aspergillus* DNA detection for the diagnosis of aspergillosis has been the subject of many studies for more than 20 years[22].

BAL culture, although considered a gold standard, has a low sensitivity for diagnosis of pulmonary aspergillosis. Also, obtaining BAL specimens for culture via bronchoscopy can sometimes be difficult, but, still, BAL remains the most representative specimen for mycological diagnosis, and very important for detecting pulmonary fungal infections[25]. Many studies have tried to give answer about sensitivity and specificity of PCR for diagnosing aspergillosis compared to conventional methods. In our study, we evaluated the *Aspergillus* PCR method for early detection of DNA in patients with increased risk for invasive fungal infections. Sensitivity and specificity of PCR in serum were 53.57% and 100% in group I, 36.36% and 100% in group II and 9.09% and 100% in group III. In our study, the highest sensitivity of *Aspergillus* PCR in serum was obtained in the first group, which included high-risk patients such as those with hematological malignancies or hematopoietic stem cell transplants. Lower sensitivity of *Aspergillus* PCR in serum was demonstrated in the group with critically ill patients. Other studies have demonstrated sensitivities ranging from 72 to 88% and specificities from 75 to 98.7%. Some studies revealed even lower sensitivity (as low as 26%)[23,24].

When comparing sensitivity of serum to sensitivity of BAL PCR, the study by Zhang demonstrated better sensitivity for BAL PCR (90%) compared to serum PCR (60%), concluding that in non-neutropenic patients BAL provided higher sensitivity for detecting *Aspergillus* DNA[26]. This confirms the hypothesis that DNA of *Aspergillus* could be more easily demonstrated in specimens from the site of infection than in blood or serum. A possible explanation for the lower sensitivity or a negative PCR in serum may lie in the fact that the specimen is not taken from the site of infection. Additionally, DNA-emia may be transient and *Aspergillus* DNA present below the detection threshold of PCR. Many researchers agree that knowledge about DNA release and DNA-emia kinetics during invasive infections is not sufficient, and the optimal timing for obtaining blood sample for PCR is not known[25]. The testing of serum with PCR is based on detection of free circulating DNA of *Aspergillus*, and whole blood enables detection of conidia, hyphae, and free circulating DNA. Also, little is known about the pathogenesis of invasive aspergillosis, and there is no consensus on the origin

of nucleic acid in blood or serum/plasma, or even leukocytic pellets during invasive aspergillosis. Some researchers believe that blood culture rarely allows growth of *Aspergillus*. On the other side, dissemination of live fungal cells of *Aspergillus* happens via blood flow to internal organs, as *Aspergillus* can be cultured from heart and brain biopsy material[27]. Loeffler *et al.* found that whole blood was a better specimen for extraction of DNA of these fungi, compared to plasma[28], and Costa *et al.,* presented similar results obtained from serum, plasma and leukocyte pellet[29].

Based on these findings, it may be recommended to collect whole blood in EDTA tubes in order to have access to both free and cell-associated DNA. EDTA is also known to inhibit DNA in blood, without interfering with the PCR assay that often occurs in the presence of citrate, and heparin should be avoided because it inhibits Taq polymerase[30]. All of this ultimately raises the question of whether blood or serum is better for *Aspergillus* PCR? Two clinical studies comparing PCR assays for *Aspergillus* species in serum and whole blood failed to show a statistically significant difference in the accuracy of this assay[31].

Given that our results support a high sensitivity of serum for DNA detection, and taking into account the fact that PCR in serum is faster and easier to perform, with DNA extraction from serum being an easier procedure, requiring noanticoagulants that might interfere with PCR, serum may have the potential to be superior in the sample selection for PCR for diagnosing invasive aspergillosis.

Regarding the population, most studies have focused on high-risk patients, such as those with hematological malignancies or hematopoietic stem cell transplants[32]. Some studies have demonstrated lower sensitivity, particularly those involving patients with solid organ transplants or solid organ cancers. Raad has demonstrated a sensitivity of 100% for proven pulmonary invasive aspergillosis infections, but only 57% for probable or possible invasive aspergillosis, in a study analyzing solid cancers in the subjects[33].

A meta-analysis performed in 2009, including 16 studies with 1618 patients, showed an overall sensitivity of 88% and a specificity of 75%. However, if two consecutive tests are used to define positivity, the sensitivity and specificity would be 75% and 87%, respectively (34). The results of this study revealed that two positive tests were necessary to confirm the diagnosis, while one negative PCR result was sufficient to exclude the diagnosis[34]. As a screening tool, a negative PCR result can help to rule out invasive aspergillosis. Also, for confirmation of the diagnosis, two or more positive results can be used to strengthen the likelihood of invasive aspergillosis, for example, from possible to probable.

Conclusion

In our study, by BAL culture the most commonly detected was *Aspergillus* species in the chronic aspergillosis group, followed by the cystic fibrosis group, the primary immunodeficiency group, and the prolonged ICU stay group. *A. fumigatus* was the most frequently detected agent in positive BAL cultures. The sensitivity and specificity of MT-PCR for *Aspergillus* in serum, were: 53.57%/100%, 36.36%/100%, 9.09%/100%, in I, II and III groups, respectively.

The results of this study indicate that no single method could provide definite etiological diagnosis of invasive aspergillosis. When using a conventional method, it is necessary to provide more specimens from each patient, in frequent time intervals, and cautiously interpret results, 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.

In conclusion, the implementation and analysis of different microbiological methods, as well as appropriate interpretation of results, in collaboration with clinicians, are the most important aspect for establishing an accurate and precise etiological diagnosis of invasive

aspergillosis and early start of antifungal treatment in order to achieve favorable clinical outcome.

Conflict of interest statement. None declared.

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