EVALUATION OF BAL PCR FOR DIAGNOSIS OF ASPERGILLOSIS

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ABSTRACT

Introduction: Aspergillosis is a worldwide problem in both patients with compromised immune system and patients with comorbidities treated in intensive care units. Early diagnosis of aspergillosis is still a big challenge for both clinicians and laboratory workers. Standard mycological methods have low sensitivity, and therefore rapid and more sensitive methods for early diagnosis of aspergillosis are necessary.

Aim: The aim of this study was to determine the usefulness of a PCR assay performed in bronchoalveolar lavage fluid in comparison to the standard mycological method (BAL culture) for diagnosis of aspergillosis.

Material and Methods: Specimens of 125 patients divided into four groups and classified according to clinical diagnosis and EORTC/MSG criteria were analyzed at the laboratory for mycology at the Institute of Microbiology and Parasitology, with standard mycological (BAL culture) and molecular methods (BAL PCR), during a 2-years period (2014-2016).

Results: In our study, 71 isolates of *Aspergillus* were confirmed with BAL culture. Out of these, 63.33% were confirmed in patients with chronic aspergillosis, followed by 56.67% of cystic fibrosis patients, 51.43% of primary immune deficiency patients, and 43.33% patients with prolonged stay in ICU. BAL culture demonstrated the following percentages of sensitivity and specificity: 64.29% and 100%, 59.09% and 100%, 54.55% and 12.5%, 100% and 54.17%, in all four groups, respectively. *A. fumigatus* was confirmed in 53 out of 67 positive BAL specimens. Out of these, 17 isolates were detected in the group of chronic aspergillosis, followed by the group of immune deficiency and cystic fibrosis with 14 isolates each, and eight isolates were confirmed in the group of critically ill patients with prolonged stay in ICU. A. flavus were confirmed in 11 positive BAL cultures. Only 3 isolates of A. terreus were recovered from positive BAL cultures. PCR performed in BAL specimens yielded the following sensitivity and specificity percentages in all four groups: 67.86% and 85.71%, 72.73 % and 75%, 40.91% and 50%, 50% and 50% in all groups, respectively.

Conclusion: Results of our study indicate that PCR in BAL specimen is a valuable molecular diagnostic tool for early diagnosis of aspergillosis, especially if it is used along with the standard

mycological methods, like BAL culture, in order to provide valuable information for clinicians to initiate an early antifungal treatment that could enable more favorable clinical outcome.

Key Words: aspergillosis, *Aspergillus*, BAL culture, BAL PCR, diagnosis.

Introduction

Serious fungal infections continue to be significant cause of morbidity and mortality in patients with prolonged steroid treatment, acquired immune deficiency, patients with severe neutropenia, hematopoietic stem cell and solid organ transplantation patients (1). Aspergillosis usually affects the pulmonary system and can be presented as aspergilloma, chronic pulmonary aspergillosis, allergic bronchopulmonary aspergillosis and invasive aspergillosis, which is the leading cause of death, mainly among immunocompromised patients. A delay in initiation of an appropriate antifungal treatment, due to limitation of sensitive diagnostic methods, results in rapid progression of the fungal disease and increased mortality from these life-threatening infections (2). Diagnosis of invasive aspergillosis in the most laboratories still relies on conventional mycological methods, which require a lot of time for incubation of fungi on culture media, and are not sensitive enough (3). Criteria for diagnosis of invasive aspergillosis include clinical, radiological and mycological findings (4). DNA from *Aspergillus* has been previously detected in BAL specimens by means of real-time polymerase chain reaction (PCR). This method has been recently shown to provide valuable data for etiological diagnosis of invasive aspergillosis in both immunocompromised and non-neutropenic patients (5). A recent agreement between scientists enabled this method to be included in the EORTC/MSG criteria (6,7). Chronic pulmonary aspergillosis and allergic broncho-pulmonary aspergillosis (ABPA) are also problematic for diagnosis for both clinicians and laboratory workers (8). From recently, guidelines by the European Society of Clinical Microbiology and Infectious Diseases and the European Respiratory Society (ESCMID/ERS) recommend use of PCR in BAL specimens for the diagnosis of non-invasive aspergillosis with C-II grade (9). PCR-based diagnostic methods have been used in routine clinical practice for many years, and from recently they are used to diagnose or rule out suspected invasive fungal infections. For definitive mycological diagnosis of invasive aspergillosis, an accurate and reliable diagnostic method is specifically needed, due to the serious nature of this infection among immunocompromised patients.

The aim of this study was to evaluate the usefulness of a PCR-based method in BAL specimen, in comparison to the conventional mycological method (BAL culture) for diagnosis of aspergillosis.

Material and Methods

Study Design, Specimens and Methods

A prospective diagnostic study was performed at the Mycology laboratory at the Institute of Microbiology and Parasitology, Faculty of Medicine, Skopje, Republic of Macedonia, in the period of 2 years (2014-2016). BAL specimens from 125 patients divided in four groups,

according to clinical diagnosis and risk factors for development of invasive fungal infection, were analyzed at the mycology laboratory. Invasive aspergillosis was defined according to the EORTC/MSG revised definitions' consensus group (4). Analysis of the samples was performed with standard mycological methods (BAL culture) on Sabouraud and chromogenic CALB medium (Oxoid). BAL specimens were homogenized and divided in two parts. One part of the specimen (1-5ml) was centrifuged, and the suspended pellet (500ml) was directly inoculated on the media for growth of fungi (Sabouraud dextrose agar with chloramphenicol (40μg/ml), and incubated at 37°C during 48 hours, and the other part was frozen at -80°C, and then used for molecular analysis, without sending information to clinicians for the results.

Molecular detection of *Aspergillus* DNA. Extraction of DNA from BAL. BAL specimens were treated with N-acetil cystein-Na hydroxide. BAL specimens were centrifuged at 13.000rpm. The leucocyte pellet was resuspended in 300μl of 1×phosphate buffered saline solution and the mixture was incubated with 100-125 U lyticase, during 30 minutes at 37°C for fungal cells degradation. The residual material was further treated with 500-1000μg proteinase K and 0.5% SDS (Natrium dodecyl sulphate) at 55°C during 1 hour. The residual cell material was then lysed while incubated with additional 100μl 2×Aspergillus buffer for extraction, during 30 minutes at 65°C. Purification of DNA was performed with conventional phenol-chloroform extraction. The precipitation of DNA was performed with 0.7 volume of isopropanol, to obtain a pellet, which was further washed with 70% ethanol and dried on air. Analysis of the DNA concentration was performed with spectrophotometer at wavelength 260 and 280nm. The DNA extracts were frozen at -20°C until the PCR procedure (10).

Controls for Extraction

Negative controls were tubes with purified water without DNA for evaluation of the contamination during extraction. 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 500μl. To determine the total number of injected CFU, 100μl 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*

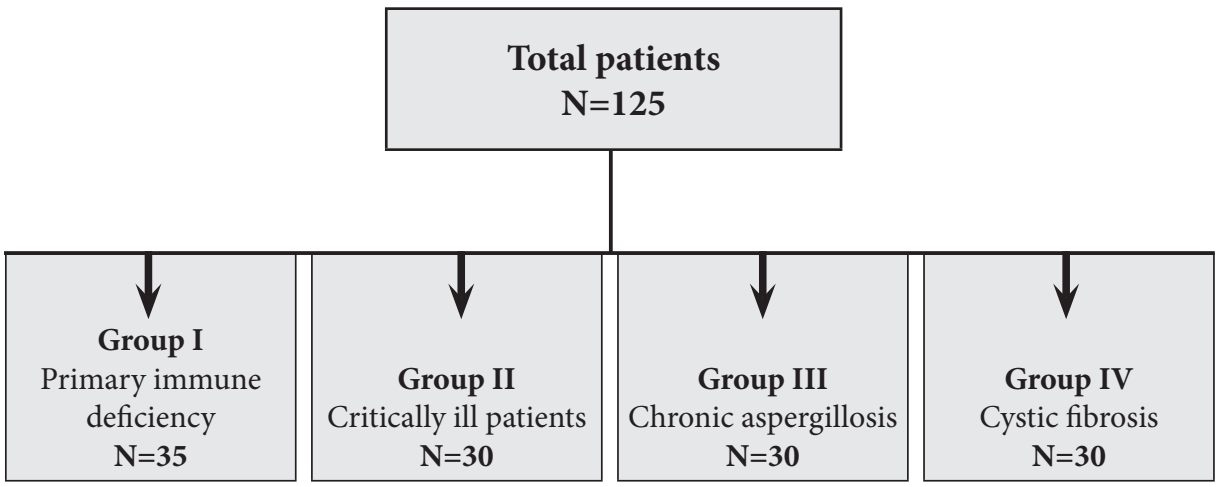
PCR was performed in 25ml mixture containing 50-150 nanograms of DNA like a template. This PCR mixture contained around 0.5 U Taq DNA polymerase, 6.25nmol DNTP, 10pmol primers (for the first PCR step – first set of primers; for the second PCR step – another set of primes). The PCR products were separated with 2.5% agarose gel electrophoresis 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 (11).

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

BAL specimens from 125 patients were analyzed in 4 groups of patients, according to clinical diagnosis and EORTC/MSG criteria (Figure 1).

Figure1. *Classification of patients' groups according to clinical diagnosis and EORTC/MSG (European Organization for Research and Treatment of Cancer/Mycoses Study group) criteria*



According to the data of analysis of our participants' gender, men were more frequently distributed in primary immune deficiency group, chronic aspergillosis and cystic fibrosis group (60%, 60%, 53.33% respectively), whereas in the critically ill patients' group, treated in ICU, both genders were equally distributed. Analysis of our participants' age demonstrated that average age was: 40.8±17.7, 59.7±13.3, 64.7±6.3, and 28.9±8.5 years (Table 1).

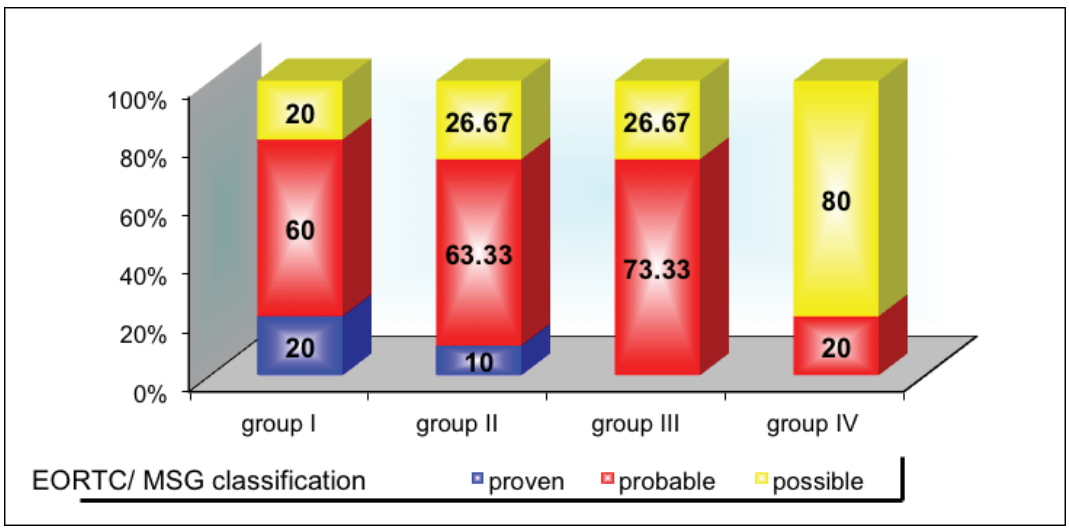
Table 1. *Characteristics of patients according to gender and age*

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 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)

In Figure 2 the distribution of the participants according to clinical diagnosis is presented for proven, probable and possible fungal infection, following the EORTC/MSG criteria. Only a small percentage of patients had proven infection with *Aspergillus* according to these criteria. Twenty percent of these patients had some form of primary deficiency and 10% had a prolonged stay in an intensive care unit.

Figure2. *Distribution of fungal infections according to EORTC/MSG criteria in all group*



Differences in distribution of proven, probable and possible fungal infection were statistically significant between primary immune deficiency versus chronic aspergillosis and cystic fibrosis group, and between critically ill patients' group versus chronic aspergillosis and cystic fibrosis group (Table 2).

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

Aspergillus	**group I N=35**	**group II N=30**	**group III N=30**	**group IV N=30**
n (%)	**n (%)**	**n (%)**	**n (%)**	**n (%)**
proven 10 (8%)	7 (20%)	3 (10%)	0	0
probable 68 (54.4%)	21 (60%)	19 (63.33%)	22 (73.33%)	6 (20%)
possible 47 (37.6%)	7 (20%)	8 (26.67%)	8 (26.67%)	24 (80%)
	[b]p < 0.001 I vs II p=0.3 II vs III p = 0.345 III vs IV p < 0.001 I vs III p = 0.03* II vs IV p < 0.001 I vs IV p < 0.001			

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

Analysis of BAL culture demonstrated presence of *Aspergillus* in 63.33% in chronic aspergillosis group (19/30), followed by 56.67% in the cystic fibrosis group (17/30), 51.43% primary immune deficiency group (18/35) and 43.33% in critically ill patients' group (13/30). The most frequently encountered species was *A. fumigatus* (79%). Seventeen out of fifty- three isolates of *A. fumigatus* originated from chronic aspergillosis patients' specimens and fourteen isolates were confirmed in both primary deficiency and cystic fibrosis patients' specimens. Other positive findings of *A. fumigatus* isolates were confirmed in ICU patients' specimens (15.1%; 8/53) (Table 3). Three isolates of *A. flavus* were demonstrated in the CF patients' specimens. Two isolates of *A. terreus* were also confirmed in BAL specimens from primary immune deficiency and one isolate in critically ill patients' group.

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

	group I N=35	**group II N=30**	**group III N=30**	**group IV N=30**
BAL culture	**n (%)**	**n (%)**	**n (%)**	**n (%)**
negative 58 (46.4%)	17 (48.57%)	17 (56.67%)	11 (36.67%)	13 (43.33%)
positive 67 (53.6%)	18 (51.43%)	13 (43.33%)	19 (63.33%)	17 (56.67%)
	Chi-square: 2.59 p = 0.46			
Identified mold species 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)

PCR in BAL confirmed 20 (57.14%) positive specimens with *Aspergillus* in patients with primary immune deficiency, 18 (60%) in patients with prolonged stay in ICU, 13 (43.33%) positive specimens in patients with chronic aspergillosis and 15 (50%) in patients with cystic fibrosis. *A. fumigatus* was confirmed as etiological agent in all positive specimens (Table 4).

Table 4. *PCR in BAL and Aspergillus DNA recovered from BAL in all groups*

group *Aspergillus*				
	group I N=35	**group II N=30**	**group III N=30**	**group IV N=30**
BAL PCR n (%)	**n (%)**	**n (%)**	**n (%)**	**n (%)**
no 59 (47.2%)	15 (42.86%)	12 (40%)	17 (56.67%)	15 (50%)
yes 66 (52.8%)	20 (57.14%)	18 (60%)	13 (43.33%)	15 (50%)
	Chi-square: 2.06 p = 0,6			
BAL PCR – fungus species				
A. fumigatus n=66	20	18	13	15

p(Chi-square test)

Comparative diagnostic performances of conventional (BAL culture) and molecular methods, for diagnosis of infections with *Aspergillus* in the group with immune deficiency are presented in Table 5.

Table 5. *Diagnostic performances of conventional (BAL culture) and molecular methods in the group with immune deficiency*

Test	Se(%)	Sp(%)	PPV(%)	NPV(%)
BAL culture	64.29	100	100	41.18
BAL PCR	67.86	85.71	95	40

Comparative diagnostic performances of conventional (BAL culture) and molecular methods, for diagnosis of invasive infections with *Aspergillus* in the group with prolonged ICU stay, in critically ill patients are presented in Table 6.

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

Test	Se(%)	Sp(%)	PPV(%)	NPV(%)
BAL culture	59.09	100	100	47.06
BAL PCR	72.73	75	88.89	50

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 chronic aspergillosis*

Test	Se(%)	Sp(%)	PPV(%)	NPV(%)
BAL culture	54.55	12.5	63.16	9.09
BAL PCR	40.91	50	69.23	23.53

Comparative diagnostic performances of conventional (BAL culture) and molecular methods, for diagnosis of invasive infections with *Aspergillus* in the cystic fibrosis group, are presented in Table 8.

Table 8. *Diagnostic performances of conventional (BAL culture) and molecular methods in cystic fibrosis*

Test	Se(%)	Sp(%)	PPV(%)	NPV(%)
BAL culture	100	54,17	35,29	100
BAL PCR	50	50	20	80

Discussion

Early diagnosis of invasive aspergillosis still remains a big clinical and laboratory challenge. It should be based on integration of clinical, mycological and radiological data, as well as analysis of risk factors in different patient populations. It should provide early detection and laboratory testing of the antifungal susceptibility pattern of the isolated fungus. Bronchoalveolar lavage (BAL) is a specimen that could enable diagnosis based on microbiologic culture when patients are unable to provide sputum. This possibility also allows application of PCR, which could help in faster identification of particular microbes, especially of a great interest for immunocompromised patient population. This approach enables sooner diagnosis than conventional methods (12).

Positive findings of *Aspergillus* in BAL culture in our study were as follows: chronic aspergillosis (63.33%), cystic fibrosis (56.67%), primary immune deficiency (51.43%) and critically ill patients with prolonged stay in ICU (43.33%), respectively. Sensitivity of BAL culture was 64.29%, 59.09%, 54.55% and 100%, and specificity of BAL culture was 100%, 100%, 12.5% and 54.17%, in all four groups respectively. Data from the study of Tashiro and coworkers show that 165 isolates of *Aspergillus* species were detected in BAL culture of 139 patients. Less than 50% (45%) of these patients were positive for *Aspergillus* without clinical manifestations of aspergillosis. Other patients presented with some clinical form of pulmonary aspergillosis (chronic aspergillosis (48%), aspergilloma (29%), invasive (13%) or ABPA (10%)). In our study, *A. fumigatus* was identified in 79.1% (53/67) of all positive BAL specimens. Seventeen patients out of fifty-three (32.1%) had chronic aspergillosis. In the group with primary immune deficiency and cystic fibrosis, *A. fumigatus* was identified in 14 patients in both groups (26.42%). Eight patients treated in ICUs were also positive for *A. fumigatus* (15.1%). Second the most frequent species identified in our patients was A. flavus, which was confirmed in eleven out of sixty-seven patients (16.42%). A. terreus was detected in three out of sixty-seven patients (4.48%). Four out of eleven isolates of A. flavus were detected in critically ill patients treated in ICU, and 27.3% in the group with cystic fibrosis. Two severely immunocompromised patients (with AIDS) and one patient with metastatic tumor of the brain treated in ICU, were positive for A. terreus. Mennink-Kersten and coworkers showed the following distribution of *Aspergillus* among 165 confirmed isolates in BAL cultures in their study: 41% A. fumigatus, 32% A. niger, A. versicolor (12%), A. terreus (6%), A. flavus (5%), A. nidulans (2%), A. sydowii (1%) and unidentified Aspergillus species (0,6%) (13). A. fumigatus was also confirmed as a predominant species in patients with invasive aspergillosis (82%), aspergilloma (68%) and chronic aspergillosis (54%), while A. niger was on the second place. *A. flavus*, *A. niger* and one case with mixed infection with two species (*A. flavus*/*A. niger*) were detected in the study of Zarrinfar and coworkers (23 %) (14). The most frequent agent in this study was A. flavus, compared to the results of our study, which demonstrated predominance of A. fumigatus.

Laboratory findings of many mycology laboratories show that *A.* fumigatus can sometimes only colonize the respiratory tract, without causing any clinical signs or symptoms of aspergillosis in patients. Demonstration of presence of Aspergillus species in more specimens without appropriate pharmacological response to antibiotic treatment, especially in high-risk patients, should be suspicious to clinicians for a possible development of invasive aspergillosis and a need for prompt

implementation of antifungal treatment (15,16,17). Colonization in the respiratory tract can be transient, but could also indicate development of an invasive infection with Aspergillus (18). Our patients with chronic aspergillosis demonstrated *Aspergillus* in BAL culture in 63.33%, all due to A. fumigatus. Results from the study of Tashiro and coworkers showed similar data to our study. A. fumigatus was also the predominant species in their study (54%) (19). A. fumigatus (69%) was also demonstrated as the most frequent isolate in positive BAL cultures in the study of Perfect and coworkers, followed by *A*. niger (13%), A. flavus (2%) and other species (5%) (20). The allergic form of aspergillosis due to hypersensitivity reactions of the human body to chronic presence of Aspergillus (ABPA), is usually present in patients with cystic fibrosis. In these patients, the predominant cause is A. fumigatus (21). Our patients with cystic fibrosis were the most often colonized with A. fumigatus - 82.4% (14/17), but 10% demonstrated presence of A. flavus (17.6%). These results show that timely identification of *Aspergillus* would allow clinicians to initiate an adequate antifungal treatment depending on the clinical parameters of the patient.

Although considered a gold standard, culture of respiratory samples for diagnosis of aspergillosis is still a major diagnostic challenge. In recent years we have witnessed the development of new diagnostic methodologies and biomarkers that would enable easier detection of patients with aspergillosis. With molecular techniques, scientists tried to find more reliable methods for detection of DNA of Aspergillus in clinical specimens in high-risk patients (7). Aspergillus DNA detection for etiological diagnosis of aspergillosis has been the subject of many studies for more than 20 years (22). Numerous authors have previously demonstrated sensitivities ranging from 72 to 88% and specificities from 75 to 98.7%. Some studies revealed even lower sensitivity (as low as 26%) (23-25).

In our study, we evaluated a PCR method for early detection of DNA in patients with increased risk for invasive fungal infections with *Aspergillus*. Sensitivity and specificity of PCR in our study differ to some previously published studies. Several authors suggest the sensitivity of *Aspergillus* PCR in BAL to range between 80% and 100%, but other authors described much poorer results (26). The values for sensitivity and specificity of PCR in BAL in our study in all four groups were as follow: group I - 67.86% and 85.71%, group II – 72.73% and 75%, group III – 40.91% and 50%, and group IV - 50% and 50%, respectively. In the study of Aydogan and coworkers, in neutropenic mouse, DNA of *Aspergillus* was detected in 7/12 BAL specimens (58.3%), 7/19 blood specimens (36.8%) and 4/22 pulmonary specimens (18%) with Rt-PCR method (27). In an experimental study of Khan and coworkers, 60 immunosuppressed Wistar rats (with cyclophosphamide), were experimentally infected with conidia of *A. fumigatus* (28). After development of invasive aspergillosis, DNA was detected in BAL with nPCR. It was confirmed that sensitivity of nPCR was 70% in BAL. Higher sensitivity of PCR in BAL (90%) compared to serum (60%) was confirmed in the study of Zhang and coworkers, who demonstrated that in non-neutropenic patients, BAL provided higher sensitivity for detection of DNA. These data confirm the hypothesis that DNA of *Aspergillus* can be detected more easily in specimens of the site of infection (like BAL) compared to blood or serum (29). Skladny and coworkers report a sensitivity of 43% for proven and 33% for probable invasive fungal infection, using BAL PCR

(30). But, false negative results in proven invasive fungal infections have been also described in several studies. Buchheidt and coworkers detected negative PCR results in the BAL of two out of three patients with histological proof for invasive aspergillosis and Khot and coworkers reported a negative PCR result from one patient with a positive biopsy (26,31).

The slightly lower sensitivity of the PCR in our study, compared to other studies, might have several explanations. Some studies included only patients with acute myeloid leukemia and hematopoietic stem cell transplant recipients, while others included a large variety of immunocompromised patients. Other studies incorporated only patients suspected to have invasive pulmonary aspergillosis, whereas others included patients under screening surveillance. Several studies selected patients by definite radiographic results of pneumonia, thus improving the pre-test probability of the method, in contrast to our study, where this clinical information was lacking in the most of the patients (31). The impact of active anti-mold treatment or prophylaxis received by some patients at the time of BAL sampling could not be ruled out as well. We also had data that few our patients (insufficient data to be presented in this study), especially in the immune deficiency group, were on chronic anti-fungal therapy at the time of bronchoscopy, which might have decreased the number of organisms present in the BAL fluid, thus impairing identification by BAL PCR. Lastly, despite all our efforts, we couldn't definitively exclude the possibility of a technical failure in the assay procedure, which was still in a process of standardization during the time this study was performed. While our results suggest a lower sensitivity of the *Aspergillus* PCR in the BAL, the specificity of the test, especially for the first and the second group, is in line with previously reported data. Data from previous studies have shown specificities ranging between 84% and 100%, which can be compared to data in our study (26,31,32).

Conclusion

This study demonstrated that PCR in bronchoalveolar lavage highlights the value of the molecular assay as a diagnostic support in the etiological diagnosis of aspergillosis, along with results from conventional mycological investigation (culture), which may have significant implications for its treatment and prophylaxis. Molecular assays also have higher sensitivity and enable detection of fungal DNA during an antifungal treatment, when the possibility to detect viable fungi is much lower with culture.

Results of our study show that PCR assay could help in earlier initiation of an antifungal treatment in order to achieve more favorable clinical outcome, especially in patients with increased risk for development of invasive aspergillosis.

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