ORIGINAL ARTICLE

COMPARISON OF DIAGNOSTIC VALUES OF SERUM PCR AND SERUM GALACTOMANNAN ANTIGEN ASSAY FOR DIAGNOSIS OF ASPERGILLOSIS

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Abstract

Introduction: Aspergillosis is a major threat to immunocompromised hosts, but also to immunocompetent patients. Early diagnosis remains a diagnostic challenge, and there is a need for rapid and sensitive diagnostic methods.

Aim: The aim of the study was to compare the diagnostic value of serum PCR assay with serum galactomannan antigen test, in the diagnosis of both invasive and non-invasive aspergillosis.

Material and methods: During a period of two years (2014 – 2016), sera from 125 patients divided into 4 groups (group I-immune deficiency, 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 molecular (PCR) and serological methods (galactomannan.

Results: PCR in serum demonstrated the following 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 cystic fibrosis group. The sensitivity and specificity of galactomannan in serum were 64.29% and 57.14%, 40.91% and 62.5%, and 40.91% and 62.5% in groups I, II, and III, respectively.

Conclusion: Our results indicate that both PCR and galactomannan in serum could be useful adjunct tests for the diagnosis of both invasive and non-invasive aspergillosis, so an early antifungal treatment is initiated in order to achieve a more favorable clinical outcome.

Keywords: *Aspergillus; aspergillosis serum galactomannan; serum PCR.*

Introduction

Invasive fungal infections (IFIs) are severe systemic infections with high mortality rates, particularly in immunocompromised patients. Invasive pulmonary aspergillosis (IPA) is the most severe form of aspergillosis with the highest mortality rate. These infections are very common in patients with hematologic malignancies, chemotherapy-induced neutropenia, hematopoietic stem cell transplantation (HSCT), and solid organ transplantation (1,2). Many studies have recently demonstrated that IPA is more frequently registered in non-neutropenic, immunocompetent

patients without classic risk factors, who have been treated in the intensive care unit (ICU) (3). Most of these patients have chronic obstructive pulmonary disease (COPD), have received broad-spectrum antibiotic treatment or systemic corticosteroids, have impaired mucociliary clearance, diabetes, chronic renal disease, and liver failure; however, IPA can also be found in patients without underlying diseases (4). IPA is more frequently registered in critically ill patients treated in intensive care units (3). Chronic aspergillosis is a locally invasive form of aspergillosis and is seen mainly in patients with mild immunodeficiency or with chronic lung disease. Aspergilloma and allergic bronchopulmonary aspergillosis (ABPA) are noninvasive forms of aspergillosis (4).

Diagnosis of IPA remains a laboratory challenge, since clinical symptoms and signs are non-specific. The gold standard method for diagnosing IPA is the detection of fungi by histopathological examination of lung tissue. Standard mycological methods with culture on fungal media are time-consuming and insensitive. Diagnosis is also difficult because most of the diagnostic tools lack specificity or sensitivity in the early phase of the infection. Over the recent years, novel molecular and serological methods have been developed to improve diagnosis of IPA in patients at high risk. Rapid, noninvasive, culture-independent diagnostic methods have contributed towards faster and better detection of invasive IFI and are essential for timely antifungal treatment. Among these methods, serological diagnostic tests focus on detecting biomarkers such as galactomannan (GM) from *Aspergillus*, in serum or BAL, and detection of DNA from the fungus in primarily sterile specimens. These diagnostic approaches have gained importance in the mycology laboratory (5).

The aim of the study was to compare the diagnostic value of serum PCR assay with serum galactomannan antigen test, in the diagnosis of both invasive and non-invasive aspergillosis.

Material and Methods

Study design

The study was performed at the Laboratory for diagnosis of fungal infections at the Institute of Microbiology and Parasitology, Faculty of Medicine Skopje, Macedonia, during a 2-year period, as part of an ongoing PhD study during the 2014-2016 period.

Group of patients and mycological analyses

Sera from 125 patients divided into 4 groups (group I-immune deficiency, 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 molecular and serological methods, during a period of two years (2014-2016). Samples were frozen and stored at −70°C for retrospective testing. These groups included patients with primary immune deficiency, critically ill patients treated in ICUs, patients with chronic aspergillosis and cystic fibrosis patients. IPA was defined according to the revised definitions by the EORTC/MSG (European Organization for Research and Treatment of Cancer/Mycoses Study Group) consensus group (6).

Molecular Detection of *Aspergillus* DNA

Extraction of DNA from serum. 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 for 10 minutes at 4°C. After lysis of erythrocytes, the specimen was centrifuged at 300×g for 10 minutes. The supernatant was discarded, and leucocytes were washed once in 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 centrifuged again. The leucocyte 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) for 30 minutes at 37°C. 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 for 1 hour. The residual cell material was then lysed while incubated with an additional 100 µl 2×*Aspergillus* buffer for extraction (400 mM Tris-Cl, 1 M NaCl, 20 mM EDTA, 2% Natrium dodecyl sulphate) for 30 minutes at 65°C. Purification of fungal and human DNA was performed with phenol-chloroform extraction. The precipitation of DNA was performed with the addition of 0.7 volume of isopropanol, further washed with 70% ethanol, and afterwards dried in air. The concentration of DNA was analyzed with a spectrophotometer at 260 and 280 nm. The DNA extracts were frozen at -20°C until the PCR procedure (7).

Controls for extraction. Negative controls were purified water without DNA. Positive controls were included for every extraction and verification, with inoculation of saline solution containing 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*

The PCR reaction was performed in a 25 µL mixture containing approximately 50-150 nanograms of total DNA. 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 were derived from sequences of *A. fumigatus* 18S rRNA gene). The PCR was performed in an automated thermocycler. The PCR products were separated by 2.5% agarose gel electrophoresis, dyed with ethidium bromide, and visualized under UV light. The control 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 a diluted solution of *A. fumigatus* were used as templates (7).

Detection of galactomannan

A commercially available sandwich ELISA test for the detection of GM antigen was performed according to the instructions of the manufacturer (8). Each sample was tested in duplicate, and the mean value was determined. The optical density of each well was read at 450 nm. The microtiter plates were read within 30 minutes after the addition of a stop solution. The optical density was determined spectrophotometrically using a microplate reader. The results were interpreted based on the index calculated from the measured OD at a wavelength of 450 nm.

Although the threshold for a positive GM test result is still controversial, we used an optical density index cut-off of 0.5 for serum samples.

Statistical Analysis

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. The sensitivity, specificity, positive predictive value, and negative predictive value were evaluated. Differences in the distribution of proven, probable, and possible fungal infections with *Aspergillus* were compared by the Pearson Chi square test. A p-value less than 0.05 was considered statistically significant.

Results

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

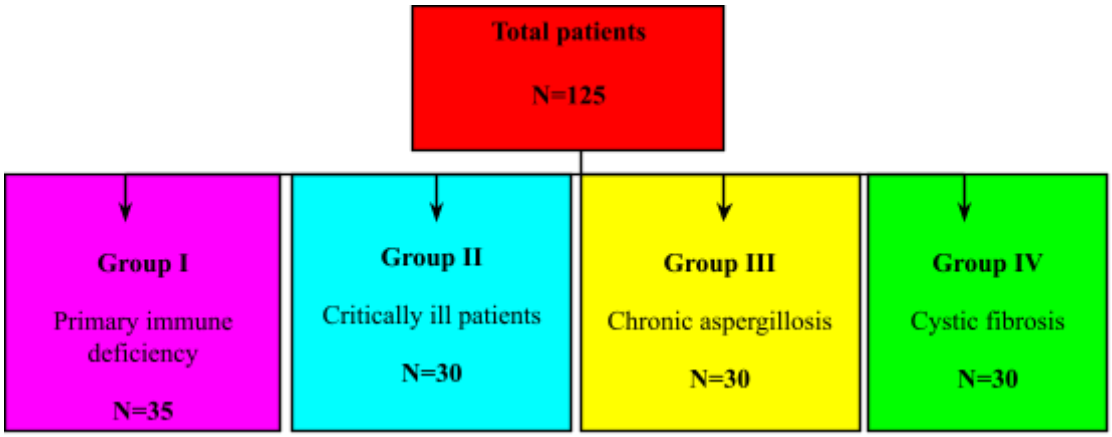

Fig.1: Classification of patient groups according to EORTC/MSG (European Organization for Research and Treatment of Cancer/Mycoses Study group) criteria

According to the participants' gender, men were more frequently distributed in I, III and IV group (60%, 60%, 53.33% respectively), whereas in the II group, both genders were equally distributed. Average age of all patients were: 40.8±17.7, 59.7±13.3, 64.7±6.3, and 28.9±8.5 years in all four groups, respectively (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	21 (60%)	15 (50%)	18 (60%)	16 (53.33%)

70 (56%)				
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 the participants, according to diagnosis for proven, probable, and possible fungal infection, with EORTC/MSG criteria (European Organization for Research and Treatment of Cancer/Mycoses Study group), is presented in Figure 2. According to the EORTC/MSG criteria, only a small percentage of patients had a proven *Aspergillus* infection. Of these, 20% (7/35) patients had some type of primary deficiency, and 10% (3/30) patients had a prolonged stay in the ICU.

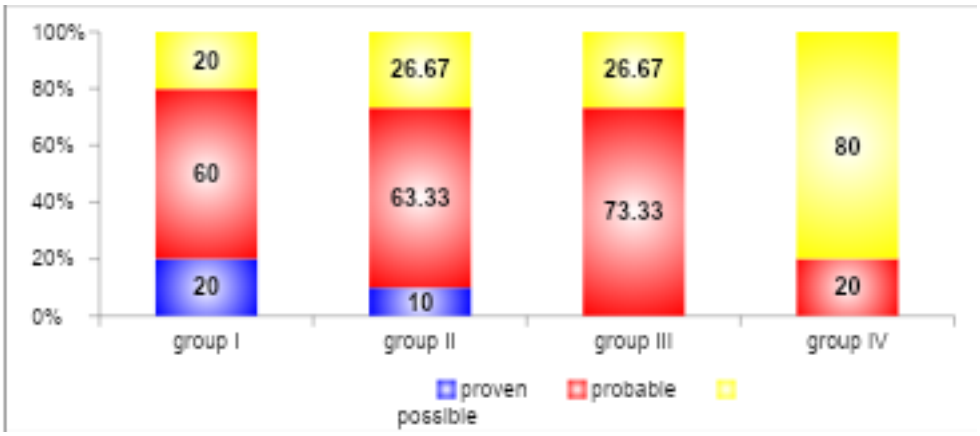

Fig.2. Distribution of fungal infections according to EORTC/MSG criteria in all groups

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

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

Aspergillus	**group I** **N=35**	**group II** **N=30**	**group III** **N=30**	**group IV** **N=30**
n (%)	**n (%)**	**n (%)**	**n (%)**	**n (%)**
proven 10 (8%)	7 (20%)	3 (10%)	0	0

probable 68 (54.4%)	21 (60%)	19 (63.33%)	22 (73.33%)	6 (20%)
possible 47 (37.6%)	7 (20%)	8 (26.67%)	8 (26.67%)	24 (80%)
	[b]p < 0.001			
	I vs II p=0.3	II vs III p = 0.345	III vs IV p < 0.001	
	I vs III p = 0.03*	II vs IV p < 0.001		
	I vs IV p < 0.001			

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

Regarding the application of PCR in serum, presence of *Aspergillus* DNA was confirmed in 42.86% patients with primary deficiency, 26.67% patients with prolonged ICU stay, and in 6.67% 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 3).

Table 3. PCR in serum

Ggroup *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)

The results from comparative diagnostic performance of serum PCR test and serum galactomannan antigen test in the group of patients with immunodeficiency are shown in Table 4. The sensitivity, specificity, positive and negative predictive values of serum PCR test were: 53.57% / 100% / 100% / 35%. The sensitivity, specificity, positive and negative predictive values of serum galactomannan antigen test were: 64.29 % / 57.14% / 85.71% / 28.57%, in group I, respectively.

Table 4. Diagnostic performances of serum PCR test and serum galactomannan antigen test in the group of patients with primary immunodeficiency

Test	**Se(%)**	**Sp(%)**	**PPV(%)**	**NPV(%)**
Serum PCR	53.57	100	100	35

Galactomannan in serum	64.29	57.14	85.71	28.57

The results of the comparative diagnostic performance of the serum PCR test and the serum galactomannan antigen test in the group of critically ill patients and prolonged hospital stay in ICU are presented in Table 5. The sensitivity, specificity, positive and negative predictive values of the serum PCR test were: 36.36% / 100% / 100% / 36.36%. The sensitivity, specificity, positive and negative predictive values of the serum galactomannan antigen test were: 40.91% / 62.5% / 75% / 27.78%, in group II, respectively.

Table 5. Diagnostic performances of serum PCR test and serum galactomannan antigen test in the group of patients with prolonged hospital stay in the ICU

Test	**Se(%)**	**Sp(%)**	**PPV(%)**	**NPV(%)**
Serum PCR	36.36	100	100	36.36
Galactomannan in serum	40.91	62.5	75	27.78

The results of the comparative diagnostic performance of serum PCR test and serum galactomannan antigen test in the group of patients with chronic aspergillosis are presented in Table 6. The sensitivity, specificity, positive and negative predictive values of the serum PCR test were: 9.09% / 100% / 100% / 28.57%. The sensitivity, specificity, positive and negative predictive values of the serum galactomannan antigen test were: 40.91% / 62.5% / 75% / 27.78%, in group III, respectively.

Table 6. Diagnostic performances of serum PCR test and serum galactomannan antigen test in the group of patients with chronic aspergillosis

Test	**Se(%)**	**Sp(%)**	**PPV(%)**	**NPV(%)**
Serum PCR	9.09	100	100	28.57
Galactomannan in serum	40.91	62.5	75	27.78

In the group with cystic fibrosis, only galactomannan in serum was performed, therefore, the comparison of the two methods was not analyzed.

Discussion

Aspergillus is considered one of the leading common causes of death in immunocompromised patients, with mortality rates up to 40% to 50% in patients with acute leukemia and recipients of hematopoietic stem cell transplantation. Recently, immunocompetent patients without any risk factors for invasive fungal infections, have reportedly suffered from IPA as well. Early diagnosis of IPA can significantly improve patient prognosis and increase the chances of the patient's

survival, provided the treatment is started earlier. This is still a significant challenge for both clinicians and laboratory workers (1).

Non-invasive and nonculture-based methods diagnostic strategies could offer a major benefit for early aspergillosis diagnosis. Screening for circulating *Aspergillus* DNA in the diagnosis of aspergillosis has been the subject of many studies for many years (8), and has demonstrated a potential in the definitive diagnosis of aspergillosis, especially when combined with other biomarkers. The detection of galactomannan (GM) can also contribute to early mycological diagnosis (10).

We compared the diagnostic value of the serum PCR assay with the serum galactomannan antigen test, in the diagnosis of both invasive and non-invasive aspergillosis. We have demonstrated the highest sensitivity of *Aspergillus* PCR in serum in the first group, with selected high-risk patients with immunodeficiencies. In our study, the sensitivity of the galactomannan antigen test was 64.29%, and compared with the molecular method, the serum PCR demonstrated slightly lower sensitivity (53.57%). The sensitivity of PCR, ranging from 72 to 88%, and the specificity ranging from 75 to 98.7%, were demonstrated by other studies. Some studies have reported sensitivity as low as 26% (11). A meta-analysis of 16 studies including 1618 patients, demonstrated an overall sensitivity of 88% and a specificity of 75%. If two consecutive tests are used to define positivity, the sensitivity and specificity would be 75% and 87%, respectively. The results of this study concluded that two positive tests are necessary to confirm the diagnosis, while one negative PCR result is sufficient to exclude it (12). As a screening tool, a negative PCR result can help rule out invasive aspergillosis. Most previous studies have focused on the sensitivity of serum PCR testing in high-risk patients, such as hematological malignancies or hematopoietic stem cell transplants (13). Raad has demonstrated a sensitivity of 100% for proven IPA infections, but only 57% for probable or possible invasive aspergillosis, in a study analyzing solid cancers (14). Studies that included patients with solid organ transplants or cancers have demonstrated lower sensitivity of serum PCR. In a meta-analysis of the diagnostic performance of *Aspergillus* PCR, the presence of at least two positive whole-blood PCR specimens in a high-risk patient should be considered very indicative, if not confirmatory, of IA (15). A similar sensitivity of 58% of serum GM test was demonstrated in the study of Pfeiffer (16). Leeflang registered a sensitivity of 78% (17) and Arvanitis demonstrated a sensitivity of 92% with the serum GM test (11). It has been demonstrated that the detection of galactomannan levels in serum is inferior to BAL GM detection, which is considered to be a better way for distinguishing IPA from other infectious lung diseases.

The Aspergillus PCR in serum showed lower sensitivity (36.36%) in the group of critically ill patients with prolonged ICU stay. Bocci and coworkers also demonstrate that sensitivity is lower in serum samples than in respiratory specimens (1). According to the 2019 meta-analysis published by Cruciani, the pooled sensitivity and specificity of *Aspergillus* PCR from blood are reported to stand between 79% and 80% for a single positive result, and 60% and 94% for two consecutive positive test results in immunocompromised people (18). However, the performance of PCR in blood decreases during systemic mold-active prophylaxis or treatment, and in

non-neutropenic patients (13). According to Boch and coworkers, serum PCR has a sensitivity as low as 11% in ICU patients, although it improved to 56% in BAL specimens (19). In a retrospective single-center comparative analysis of galactomannan, PCR, and mycologic analysis of pulmonary samples in both neutropenic and non-neutropenic patients, the PCR sensitivity tended to be better in neutropenic patients (82.1%) than in non-neutropenic patients (62.5%). Sensitivity of the serum GM test in our group of critically ill patients with prolonged stay in the ICU was 40.91%. According to Eigl, galactomannan is often absent in the serum of non-neutropenic patients, in whom airway-invasive growth is more typical (20). Thus, GM testing in BAL is preferred in this setting. Some studies reported results on the combined diagnostic capabilities of the GM and PCR assays in non-hematological patients, which is significantly improved compared with the use of either method alone. In the study by Lahmer and co-workers, serum GM levels have been elevated above the cutoff of >0.5, in 3 patients only (10%) (21). The sensitivity of the serum GM test was lower than in our study (30%). One of the problems with serum GM determination is the occurrence of false positive findings, which lowers its specificity, because critically ill patients are sometimes treated for bacteraemia, that could be an additional reason for false positive results.

Very low sensitivity of *Aspergillus* PCR in serum (9.09%) was demonstrated in the group with chronic aspergillosis. In the study by Imbert and coworkers, among 16 patients with noninvasive forms of aspergillosis, 10 were immunocompetent, 3 had metastatic malignancy, 2 had solid organ transplants and 1 had alcoholic liver cirrhosis. Clinical forms were simple aspergilloma ($n = 7$), colonization ($n = 4$), chronic cavitary aspergillosis ($n = 4$) and chronic bronchitis ($n = 1$). None of these patients had positive serum GM or PCR (22). Many studies have demonstrated low sensitivity for detecting *Aspergillus* DNA in patients with chronic aspergillosis. This has been explained by the hypothesis that *Aspergillus* DNA can be more easily detected in respiratory specimens from the site of infection than in blood or serum. A possible explanation for the lower sensitivity or a negative PCR in serum is that the specimen is not taken from the site of infection, and the transient DNAemia.

The utility of the serum GM antigen detection test for the diagnosis of pulmonary aspergillosis other than IPA is controversial. In our group of patients with chronic aspergillosis, the GM antigen test in serum demonstrated a sensitivity of 40.91%, similar to the findings in the study by Park and collaborators, who demonstrated a sensitivity of 38%. Hemoptysis has been suggested as a possible explanation for the high rate of serum GM positivity (9), probably due to bleeding from bronchial artery, allowing GM to reach the circulation. Patients with slowly progressive pulmonary aspergillosis are likely to demonstrate serum GM positivity because of the release of fungal antigens into the bloodstream due to angioinvasive growth of *Aspergillus* (23). Kono and co-workers demonstrated sensitivity of serum GM levels as low as 14.3% for the diagnosis of chronic aspergillosis and ABPA (24). Park and co-workers found that the sensitivity was only 38% for serum GM (25). Kitasato and co-workers reported a sensitivity of serum GM of 21.4% at a cut-off ≥1.5 and 50% at a cut-off ≥0.5 for chronic aspergillosis (26). Fujiuchi et al. reported that at a cut-off level of ≥0.5, the sensitivity for CNPA was 63.4% (27). In contrast to these

results, Sehgal and coworkers demonstrated poor sensitivity of serum GM for diagnosing chronic aspergillosis. According to Sehgal, the poor performance of the serum GM test is likely due to the less invasive nature of chronic aspergillosis (28). In a recent study, the sensitivity of serum GM was only 23%. Therefore, the serum GM antigen test cannot be used for the diagnosis of CPA (29).

Our study demonstrates that PCR is useful both in non-neutropenic patients and non-hematologic populations. As previously reported, our result indicates that performing both the GM antigen test and PCR on the same serum, increases the sensitivity of the diagnostic approach. Our study has few limitations, the major one being a single-center study. Additionally, we used only a single serum sample for investigation, which didn't allow assessment of reproducibility of the methods. Financial constraints prevented this analysis to be performed twice.

Conclusion

The results of this study indicate that a single method, either molecular or serological, cannot provide definite diagnosis of invasive or non-invasive aspergillosis. As these methods detect different biomarkers of the disease, combining them is likely more useful.

Implementation of different microbiological methods, as well as appropriate interpretation of results, in collaboration with clinicians, is the most important aspect for accurate and precise etiological diagnosis of aspergillosis and early initiation of antifungal treatment, leading to achieve a favorable clinical outcome.

Ethical approval: protocol number and date of approval: This study was approved by the Institutional Review Board of the Faculty of Medicine, University "Ss Cyril and Methodius", Skopje, Republic of North Macedonia (at the 10th regular session of the Institutional Review Board) (Decision No. dated 27 October 2011).

Consent for publication: Written informed consent was obtained from all patients prior to inclusion in the study.

Conflict of interest: The authors report no financial or personal conflicts of interest.

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Author Contributions: Gordana Mirchevska is the sole author of this work and was responsible for all aspects of the study, including conception, design, data acquisition, analysis, interpretation, manuscript drafting, and critical revision. The author approved the final version and takes full responsibility for the integrity and accuracy of this work.

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