Turkish Journal of Medical Sciences

http://journals.tubitak.gov.tr/medical/

Research Article

Turk J Med Sci
(2016) 46: 335-342
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doi:10.3906/sag-1408-100

Comparison of galactomannan, beta-D-glucan, and *Aspergillus* DNA in sera of high-risk adult patients with hematological malignancies for the diagnosis of invasive aspergillosis

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Received: 25.08.2014 • **Accepted/Published Online:** 28.06.2015 • **Final Version:** 17.02.2016

Bckground/aim: Invasive aspergillosis (IA) is a fatal infection that is difficult to diagnose in immunocompromised patients. In this study, *Aspergillus*-specific DNA was searched using real-time PCR (RT-PCR) in serum samples. Galactomannan (GM) and/or beta-D-glucan (BDG) tests were previously performed on these samples for 70 neutropenic patients with hematological malignancy.

Materials and methods: The patients were categorized according to the criteria of the European Organization for Research and Treatment of Cancer/Mycoses Study Group (EORTC/MSG). Among the patient serum samples, the first positive GM or BDG test sample and the median sample of GM or BDG test for negative patients were used to detect DNA levels by RT-PCR method (Light Cycler 480, Roche Molecular Biochemicals, Meylan, France) using a commercial kit (Way2Gene Fungi; Genmar, İzmir, Turkey).

Results: When the proven and probable IA group were considered as real patients, sensitivity of *Aspergillus*-specific DNA test was 90%, specificity was 73.3%, positive predictive value was 81.8%, and negative predictive value was 84.6%.

Conclusion: This study found that searching for specific DNA by RT-PCR method has a sensitivity as high as the GM test. Although specificity was rather low, it was concluded that it can be used jointly with GM and BDG tests after decreasing contamination by severe laboratory applications.

Key words: Invasive aspergillosis, galactomannan test, beta-D-glucan test, specific DNA

1.Introduction

Aspergillus species are mainly soil saprophytes and are commonly found in nature. They cause different clinical situations in humans, ranging from toxic-allergic disorders to invasive diseases. Invasive diseases are the most dangerous, and all cancer patients with hematological malignancies, patients undergoing bone marrow and organ transplantation, patients with cystic fibrosis, and patients with autoimmune and chronic granulomatosis diseases are at high risk. Sinonasal and pulmonary infections are the most common forms of invasive aspergillosis (IA) and may result in disseminated infection by spreading from these foci (1–3). Its frequency accounts for 40%–60% of cases (4,5). *A. fumigatus* is the predominant etiological agent, followed by *A. flavus* and *A. terreus* (6,7).

Early initiation of effective antifungal treatment is essential for improving the outcomes in infected patients. Clinical findings are not specific, and conventional diagnostic methods, such as direct microscopic examination and cultivation, are not sufficient to detect the disease. The utility of standard blood cultures for *Aspergillus* species is limited because of a high percentage of false negative results, even in patients with disseminated aspergillosis. Therefore, new diagnostic approaches, such as searching for galactomannan (GM) and 1,3-beta-D-glucan (BDG) antigens and specific DNA in samples, came into question (8).

Galactomannan is a molecule that is present in the cellular walls of *Aspergillus* species and can be secreted extracellularly. Several studies have shown that the determination of GM in serum, bronchoalveolar lavage

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(BAL) fluid, and other samples by a commercial kit using the sandwich enzyme immunoassay (EIA) method (Bio-Rad Laboratories, Marne-La-Coquette, France) is beneficial for the diagnosis of IA (9–12). The results are read by a spectrophotometer and expressed as optical density index. The European Conference on Infections in Leukaemia (ECIL) has strongly recommended that it be performed as a screening test in bone marrow transplants, and in patients with hematological malignancies with high incidence of IA. An optical density index of >0.7 in a single sample and of >0.5 in two consecutive samples suggests a diagnosis of IA (13).

BDG is not specific to *Aspergillus* species; it is a component in the cellular walls of many fungi, except for *Cryptococcus neoformans* and the fungi of the order Mucorales (14–19). It is one of the promising nonculture-based early diagnostic tests for the diagnosis of invasive fungal infections (IFIs). This test is based on the reaction of BDG with factor G of the horseshoe crab coagulation cascade, and there are multiple commercial assays available for BDG antigen detection. The Fungitell assay (Beacon Diagnostics, Falmouth, MA, USA) is the most utilized in the United States and Europe. With this assay, at least two consecutive results greater than 80 pg/mL are optimal for diagnosis of IFIs (15).

Both GM and BDG tests are now incorporated in the diagnosis of probable IFIs in patients with appropriate host and clinical factors, as defined by the revised European Organization for Research and Treatment of Cancer/Mycoses Study Group (EORTC/MSG) criteria (20). The major limitation of these tests is their variable performance characteristics, which are influenced by the prevalence of the disease, the testing strategy used, age, underlying condition, concomitant therapy with certain antimicrobials, and even food consumption (8,21).

Another approach that can be used in the diagnosis of IA involves searching for *Aspergillus*-specific DNA due to its advantages, such as the sensitivity and rapid availability of the results. Although searching for *Aspergillus*-specific DNA has been subject to numerous studies, it was not included in the EORTC/MSG diagnostic criteria (20,22–30). The main concern about the molecular detection of IA is the lack of standardization and validation (8). In research, various samples, extraction methods, primary probes, and polymerase chain reaction (PCR) formats were used, all of which are factors that can potentially affect the results (8,21,31).

In this study, *Aspergillus* DNA was searched using real-time PCR (RT-PCR) in stored serum samples, in which GM and/or BDG results were previously determined for 70 neutropenic patients with hematological malignancy. We aimed to compare the diagnostic values of these tests and provide new data for the literature. The rapid, sensitive, and high-throughput properties of PCR testing for diagnosing IA prompted us to investigate its performance.

2.Materials and methods

A total of 70 adult patients, who were followed at the Hospital Hematology Clinic of Uludağ University, were included in the study. None received mold active prophylaxis. The inclusion criteria were the presence of risk factors for IA (neutropenia is defined as an absolute neutrophil count below 0.5×10^9/L, recent or current use of immunosuppressive agents or corticosteroids, and/or persistent fever under broad-spectrum antibiotherapy). Clinical data and microbiological and radiological results were reviewed to classify cases according to the EORTC/MSG 2008 criteria. Proven IFI requires that a fungus be detected by histological analysis, culture of a tissue specimen, or sterile body fluid taken from a site of the disease. Probable IFIs require the presence of a host factor, a clinical criterion, and a mycological criterion. Cases that meet the host factor criteria and a clinical criterion, but not the mycological criteria, are considered possible IFIs. (20).

Stored serum samples (–80 °C) of the study group were used, and these were taken twice a week. Among these samples, the first GM or BDG positive sample and the median sample of GM or BDG for serially negative patients were used to detect DNA level with the RT-PCR (Light Cycler 480 Instrument; Roche Molecular Biochemicals, Meylan, France) method (25). DNA extraction from the samples was carried out using the ZR Fungal/Bacterial DNA MiniPrep Kit (Zymo Research Corp., Irvine, CA, USA), according to the recommendations of the manufacturer. The presence of *Aspergillus* DNA was detected using a commercial kit (Way2 Gene Fungi; Genmar, İzmir, Turkey). In accordance with the recommendations of the manufacturer, 15 µL of enzyme and primary-probe mixture (LightCycler 480 Probes Master; Roche Molecular Biochemicals) was mixed with 5 µL of extracted DNA and distributed to 96-well plates (LC480 96-well plate white; Roche Molecular Biochemicals). The denaturation was achieved with 10 min of preincubation at 95 °C. After preprocessing, the device was set to 45 cycles of 5 s at 95 °C, 10 s at 55 °C, and 20 s at 72 °C, and distilled water was used as a negative control. Before cycle 40, the samples in which exponential fluorescent increase was detected compared to the negative control were considered as positive (25–28). As positive standards were not used, quantification of DNA amount was not performed, and an internal control was used as the PCR control.

Results of direct microscopic examination and cultivation of sputum, BAL, bronchial lavage (BL), and tracheal aspirate (TAS) were evaluated retrospectively from the laboratory records. Growth in all samples with

positive direct microscopy was considered significant, whereas in cases with negative microscopy, growth in more than one sample was required for significance (32).

GM (Platelia *Aspergillus*) and BDG (Fungitell assay) results of the patient group were obtained from the laboratory records. No patient with positive BDG was suspected of having PCP and candidemia. Optic density (OD) index of ≥0.5 for GM and levels of ≥80 pg/mL for BDG in two consecutive serum samples obtained from a patient were considered positive.

Cases were grouped as proven, probable, possible, and non-IA according to EORTC/MSG criteria using direct microscopic examination, cultivation, and GM and BDG results. The specificity, sensitivity, positive predictive value (PPV), and negative predictive value (NPV) of tests were calculated according to these groups (20).

We investigated the results using SPSS 20 (IBM Corp., Armonk, NY, USA).

3. Results

In this study, 57 of 70 neutropenic patients with hematological malignancy had clinical findings (lower or upper respiratory tract symptoms such as cough, chest pain, hemoptysis, dyspnea, nasal discharge, stuffiness, and epistaxis; lower or upper respiratory physical examination findings such as pleural rub, eschar of nasal mucosa, periorbital swelling, and black necrotic lesions; lower or upper respiratory imaging findings such as dense, well-circumscribed lesions with or without a halo sign, air-crescent sign, cavity or erosion of sinus walls, and extension of infection to neighboring structures) of IA, while there were no clinical findings in 13 patients. Positive histopathology, wet-smear examination, and culture in 6 (10.5%) biopsy specimens and 15 (26.3%) respiratory tract samples (sputum, BAL) were obtained from patients with clinical findings. The *Aspergillus* species that were isolated from 21 patients (36.8%) are summarized in Table 1.

Serial galactomannan antigen screening was done in all patients in the study group. It was positive in two consecutive serum samples in 18 of 21 (85.7%) patients who were direct microscopy- and culture-positive. Bronchoscopy was performed in the remaining three patients with negative serum GM levels, since clinical findings and imaging suggested IA, and both *Aspergillus* growth and GM positivity was detected in BAL samples (Table 1). Serum GM positivity was detected in 18 symptomatic patients whose samples did not display any growth, whereas no positivity was noted in patients without clinical findings (Table 1). In Table 2, GM results and culture outcomes are compared in 57 IA-symptomatic patients.

BDG was screened in a total of 46 patients, 35 with clinical findings and 11 without clinical findings. BDG could not be analyzed in patients who had growth in biopsy samples. BDG was positive in 8 of 12 (66.7%) patients who had growth in respiratory tract samples (Table 1). While growth and GM positivity were detected in all BAL samples obtained from 4 patients with negative serum BDG level, BAL BDG was positive in 3 samples. BDG was positive in 5 more patients who had no growth but displayed clinical findings. GM was also positive in 4 of these patients, while one remained negative (Table 1). Positivity in terms of direct microscopic examination and cultivation in patients (35 patients) with clinical findings who underwent both GM and BDG tests are shown in Table 3.

Specific DNA was found in the samples of all 6 patients in whom tissue diagnosis was made and was positive in 12 of 15 patients with growth in the respiratory tract samples. In two patients who remained negative, GM and BDG were positive, and in one patient both GM and BDG and the median serum sample specific DNA was negative, although *A. terreus* growth occurred in the BAL sample. While GM and BDG remained negative in a patient who had *A. fumigatus* growth, only DNA was detected (Table 1). DNA was positive in 18 of 19 patients who had clinical findings and GM and/or BDG positivity but no growth. DNA positivity was detected in 4 (23.5%) of 17 symptomatic patients who had no growth and remained GM/BDG-negative. Furthermore, DNA positivity was detected in 4 (30.8%) of 13 patients without clinical findings (Table 1). Since there was no significant difference between these percentages ($P = 0.7$; χ^2 test), positivity found in the possible IA group was not considered in favor of IA. GM/BDG, DNA, and culture positivities of the 57 symptomatic patients are compared in Table 4.

The grouping of patients according to the EORTC/MSG criteria is shown in Table 5 (20). DNA findings were not included in the grouping, since DNA detection was not among the criteria. When proven and probable IA patients (40 patients) were considered as the real patient group, sensitivity of DNA detection was 90%, specificity was 73.3%, positive predictive value was 81.8%, and negative predictive value was 84.6%.

4. Discussion

Aspergillus species are common saprophytic, heat-resistant molds that grow in various environmental conditions. Although hundreds of *Aspergillus* spores are inhaled every day, disease rarely occurs. Various clinical situations can develop, ranging from allergic diseases in atopic individuals to invasive diseases in immunosuppressed people (1,2,4). The most important challenge is the difficulty in diagnosis of invasive diseases. Thus, a comparison of various methods used in the diagnosis of IA was carried out in this study.

Table 1. Direct microscopic examination, culture, GM, BDG, and DNA test results of the study patients.

IA categories (number of patients)	Specimen type (number of specimens)	Direct microscopic examination	Aspergillus species	No. of patients with positive GM	No. of patients with positive BDG	No. of patients with positive DNA
Proven IA (6)	Lung biopsy tissue (3) Nasal biopsy tissue (2) Pleural fluid (1)	Positive (6)	*A. flavus* (5) *A. fumigatus* (1)	6	Not done	6
Probable IA (5)	Sputum (2 P) BAL (2 P) Sputum (1 N)	Positive (4) Negative (1)	*A. flavus* (3) *A. terreus* (1) *A. fumigatus* (1)	5	5	5
Probable IA (3)	TAS (1) BAL (1) Sputum (1)	Positive (3)	*A. flavus* (2) *A. fum + A. flav* (1)	3	Not done	3
Probable IA (2)	BAL (1) BAL (1)	Positive (1) Negative (1)	*A. fum + A. ter* (1) *A. flavus* (1)	2	2	N
Probable IA (2)	BAL (2)	Positive (2)	*A fumigatus* (1) *A. flavus* (1)	2	N	2
Probable IA (1)	BAL (1)	Positive (1)	*A. niger* (1)	N	1	1
Probable IA (1)	BAL (1)	Positive (1)	*A. fumigatus* (1)	N	N	1
Probable IA (1)	BAL (1)	Negative (1)	*A. terreus* (1)	N	N	N
Probable IA (4)	N		N	4	4	4
Probable IA (2)	N		N	2	N	2
Probable IA (1)	N		N	N	1	1
Probable IA (11)	N		N	11	Not done	11
Probable IA (1)	N		N	1	Not done	N
Possible IA (13)	N		N	N	N	N
Possible IA (1)	N		N	N	Not done	1
Possible IA (3)	N		N	N	N	3
Non-IA (8)	N		N	N	N	N
Non-IA (1)	N		N	N	Not done	N
Non-IA (3)	N		N	N	N	3
Non-IA (1)	N		N	N	Not done	1
TOTAL (70)	21 specimens	Positive (18)	*Aspergillus* spp. (21)	36	13	44

IA: invasive aspergillosis; BAL: bronchoalveolar lavage; GM: galactomannan; BDG: beta-D-glucan; N: negative.

Table 2. Comparison of culture and GM results in symptomatic patients.

	DME and culture (+)	DME and culture (–)	Total
GM (+)	18	18	36 (63.2%)
GM (–)	3	18	21
Total	21 (36.8%)	36	57

DME: direct microscopic examination; GM: galactomannan.

Table 3. Culture positivity in samples of symptomatic patients who underwent GM and BDG tests (35 samples).

	DME and culture (+)	DME and culture (–)	Total
GM and BDG (+)	7	4	11
GM (+) BDG (–)	2	2	4
GM (–) BDG (+)	1	1	2
GM and BDG (–)	2	16	18
Total	12	23	35

DME: direct microscopic examination; GM: galactomannan; BDG: beta-D-glucan.

Table 4. GM/BDG, DNA, and culture positivity in symptomatic patients.

	DME and culture (+)	DME and culture (–)	Total
GM/BDG (+) DNA (+)	17	18	35
GM/BDG (+) DNA (–)	2	1	3
GM/BDG (–) DNA (+)	1	4	5
GM/BDG (–) DNA (–)	1	13	14
Total	21	36	57

GM: galactomannan; BDG: beta-D-glucan.

Table 5. Grouping of the patients according to EORTC/MSG criteria and positivity data.

Patient group	Clinical finding	Histopathology (+)	DME and culture (+)	GM (+)	BDG (+)[a]
Proven IA (6)	6	6	6	6	-[b]
Probable IA (34)	34	0	15	30	13[c]
Possible IA (17)	17	0	0	0	0[d]
Non-IA (13)	0	0	0	0	0[e]
Total (70)	57	6	21	36	13

[a]: Beta-D-glucan (BDG) was analyzed in a total of 46 patients; [b]: not analyzed, [c]: analyzed in 19 patients; [d]: analyzed in 16 patients; [e]: analyzed in 11 patients.
DME: direct microscopic examination; GM: galactomannan.

Microscopic examination of patient samples and cultivation are indispensable classical diagnostic methods. Although blood and tissue biopsy cultures are the most valuable samples in the diagnosis of invasive diseases, the sensitivity of blood samples is rather low (<5%), and obtaining tissue sample is difficult due to thrombocytopenia. In this study, diagnosis could be made in only 6 of 57 symptomatic patients (10.5%) by tissue biopsy. Therefore, most samples used for diagnosis were obtained from lower and upper respiratory tract specimens. In the diagnosis of IA in neutropenic patients with hematologic malignancy, sensitivity of respiratory tract samples ranged between 15% and 77%, whereas positive predictive value was considered as approximately 70%–80% (33–39). In this study, growth occurred in 26.3% of the respiratory samples, which is mostly BAL and considered significant, since direct microscopic examination was also revealed to be positive in 85.7%.

The most common species isolated in IA cases is *A. fumigatus*, followed by *A. flavus* (4). *A. flavus* accounted for more than half of the isolated strains in our study. However, this study was not an epidemiological trial, and, additionally, high prevalence of *A. flavus* was mentioned in other studies in which regional differences may have occurred (40).

Research has shown that the EIA kit used for searching for GM antigen has a wide range of sensitivity at 29%–100% (9). The most important reason for this difference is searching within various types of patient groups. In deeply neutropenic patients with hematologic malignancy, sensitivity is as high as 90%–100%. Similarly, in this study, 85.7% of 21 patients with positive culture results also showed GM positivity. Sensitivity is diminished in other cancer patients, solid organ transplants, cases with chronic aspergillosis, and local infections. It has been suggested that blood neutrophils clear GM antigen and decrease the sensitivity of the test in these patient populations (41,42).

In the literature, the sensitivity of BDG test has been reported as 70%–100% and specificity as 75%–90% (14–19). In this study, 66.7% of 21 patients with positive culture results also showed BDG positivity, slightly lower than in other studies. However, lack of BDG testing in all samples, especially those with histopathologic diagnosis, was considered as a limitation, and low sensitivity was attributed to this.

As mentioned above, GM and BDG tests were included in the EORTC/MSG criteria for use in the diagnosis of IA. In this study, 40 symptomatic patients were identified as having proven and probable IA by including GM and/or BDG positivity. In contrast, 21 patients were evaluated as having proven and probable IA using only direct microscopic examination and culture. It was clearly demonstrated that by adding new methods to existing ones and by using multiple methods simultaneously, the chance of diagnosis would increase. Recently, Hoenigl et al. (43) investigated 78 patients at risk for IA and found that *Aspergillus* PCR, GM, and lateral flow device tests are the most useful methods for diagnosing the disease by using BAL. They also showed that the combination of PCR and GM tests allows for sensitive and specific diagnosis of IA, which is in concordance with our study.

In this study, the sensitivity of *Aspergillus* DNA was 90% and the specificity in patient sera was 73.3%. It is possible to find numerous studies that compare GM, BDG, and specific DNA tests (22–30). In most of these studies, standard methods were used for GM and BDG, whereas different DNA search methods were used, as mentioned above. Therefore, it is possible to reach various results and new data that can contribute to the literature that underpins this study. In these comparative studies, sensitivity of different formats of PCR methods ranged between 41% and 100%. The highest levels of sensitivity were achieved by using >1 mL of whole blood samples and RT-PCR. In this study, however, 90% sensitivity was captured with the RT-PCR method using a 200-µL serum sample. We suggest that rather than type and amount of sample or extraction method used, primary probes and PCR format are more important in terms of sensitivity. Similar to sensitivity, various PCR formats had a specificity of 40%–100% (22–30). Nevertheless, due to decrease in contamination, highest specificity was achieved by RT-PCR. In this study, DNA was searched in samples where the previous analyses were performed for GM and BDG. Thus, this might be the reason for the low specificity (73.3%) obtained in this study compared to other studies that also used RT-PCR. There is a risk of contamination in each step, including obtaining blood samples from patients, transferring, and processing. In order to decrease false positivity, rigid laboratory regulations should be followed in all PCR test formats.

An experimental study from Turkey that compared the diagnostic performance of GM, BDG, and PCR showed that excellent specificity and PPV (both 100%) were obtained by PCR, although decreased sensitivity (41.2%) and NPV (16.7%) were disappointing (44). The study was an experimental rat study and perhaps the decrease in sensitivity findings is due to host differences. There is only one clinical study from Turkey about the diagnostic performance of GM and PCR. This study found that sensitivity values obtained by a commercial real-time PCR kit and GM were 65% and 23%, respectively. Aslan et al. (45) argued that the sensitivity of the PCR kit may be increased by using GM in combination in diagnosis. This finding is in accordance with our study, and the sensitivity reached by combining PCR and GM (90%) is higher than that obtained in studies from Turkey by using either GM (60%) or BDG (66%) (46,47).

In conclusion, despite these improvements, IA is still a disease that is difficult to diagnose. There is no single method for reaching a complete diagnosis. However, combined use of diagnostic tests can improve sensitivity. Although the low number of patients without IA is a limitation for this study, successful sensitivity and specificity were achieved by RT-PCR, and this can be used alongside GM and BDG tests after decreasing the contamination by tight laboratory applications.

Acknowledgment
This study was supported by a Scientific Research Program of the Faculty of Medicine of Uludağ University (Project No: KUAP(T) 2012/28).

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