

Utility of the *Aspergillus* galactomannan antigen testing for neutropenic paediatric patients

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SUMMARY

Invasive aspergillosis (IA) is an increasingly important cause of morbidity and mortality particularly in paediatric patients. Early diagnosis and the initiation of efficacious antifungal treatments could affect the prognosis of these patients. The aim of this study was to determine the clinical contribution of Galactomannan (GM) screening in paediatric patients. We reviewed the records of all in-patients, and followed up, in the various units at the Medical Faculty Children's Hospital of Erciyes University (Kayseri, Turkey), those who had at least one GM assay result from August 2009 to April 2012. Paediatric patients were classified as proven, probable or possible, according to the European Organization for Research and Treatment of Cancer/

Mycoses Study Group (EORTC/MSG). Twenty-five patients, with proven IA (n=3), probable IA (n=9) and possible IA (n=13) were included in the study. The GM antigen assay results were analysed in 158 blood samples from 47 patients. At the cut-off value of 0.5 ng/ml, the sensitivity was 68% [95% confidence interval (CI); 47-85]; specificity, 77% (95% CI; 55-92). To obtain more accurate results with GM testing, the diagnosis of IA should be confirmed by clinical investigation and the factors causing false positivity of the test should also be considered.

Keywords: paediatric patients, invasive aspergillosis, galactomannan antigen.

INTRODUCTION

Paediatric patients undergoing cytotoxic chemotherapy for leukemia or stem cell transplantation commonly develop neutropenia, a serious complication characterized by an abnormally low neutrophil count that causes patients to become immunocompromised, and hence vulnerable to opportunistic infections. *Aspergillus* species represent one of the groups of pathogens associated with such opportunistic infections, with *Aspergillus* spores being able to enter the body through the nose following inhalation,

travel down the respiratory tract, and then enter the bloodstream, causing fungemia and systemic infection in individuals with weakened immune systems [1, 2]. Given the immunocompromised nature of paediatric patients receiving chemotherapy or stem cell transplants, opportunistic *Aspergillus* infections, or invasive aspergillosis (IA), is associated with significantly high morbidity and mortality rates in these patients, with rates between 69% and 85% being reported in various studies [3-5]. Despite the great risk it represents for the well-being and life of paediatric patients, diagnosis of IA can still not be performed timely or reliably due to issues related with currently available diagnostic methods. Paediatric patients with neutropenia generally have various hematological and coagulation-related disorders, which complicate or exclude the use of invasive

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diagnostic methods for these patients. Consequently, there has been an increasing interest in recent years in the use of non-culture methods for diagnosing IA, with the Platelia *Aspergillus* (Bio-Rad, Marnes La-Coquette, France) serological test representing one such method [6]. This potential method is the galactomannan (GM) assay, which enables the detection and identification of galactomannan molecules, one of the components of *Aspergillus* cell walls synthesized especially by growing and dividing cells [7, 8]. This assay has received FDA approval in 2003 and various studies have been conducted to date for determining the performance as well as the GM cut-off index (CI) of the GM assay among adult patients for the diagnosis of IA [9-11].

In this context, the aim of our study was to carry out a validation of GM CI values reported in previous studies, and also to assess the prognostic value and utility of the GM assay for paediatric neutropenic patients diagnosed with IA.

■ PATIENTS AND METHODS

We reviewed the records of all inpatients, followed up them in the various units at the Erciyes University, Medical Faculty, Children's Hospital (Kayseri, Turkey), who had at least one GM assay result from August 2009 to April 2012. The patients were selected among the paediatric population aged under 18 with neutropenia. Demographic and clinical characteristics were registered. Inclusion criteria were as follows: antibiotic use for more than five days, fever (axillary 38°C or >37.5°C for one hour at least), unexplained neutropenia (<500/neutrophils/mm³), radiological findings of pulmonary infiltrates or fungus ball, ≥1 risk factor for IA, growing of *Aspergillus* spp. in the absence of non-fungal infection in the lower respiratory tract samples or the presence of clinical and radiological infection findings in sterile body parts, and growing of *Aspergillus* spp. in sterile specimens. The GM samples were collected twice a week from the patients who met the aforementioned criteria. Based on the European Organization for Research and Treatment of Cancer/Mycoses Study Group (EORTC) criteria, the patients were classified into three categories including proven, probable, and possible, regardless of GM test results [12]. Control patients have not

been evaluated with the same inclusion criteria. Neutropenic or steroid-treated patients without any clinical clue and with no radiological abnormalities and no microbiological isolation of *Aspergillus* species were interpreted as a control group [8]. The patients who were diagnosed with chronic granulomatosis were excluded.

GM assay

Serum GM was estimated to be used in the Platelia *Aspergillus* EIA test (Bio-Rad Laboratories, Marnes, France) assay according to the manufacturer's instructions. All serum samples were taken from patients in their neutropenic period and were stored at -80°C. The GM index value was calculated for the positive and negative controls, as well as for each patient specimen (a mean GM index value was calculated for samples run in duplicate). The GM index values were calculated by dividing the optical density of the sample by the mean optical density of the 2 cut-off control replicates. The positive control was expected to have a GM index value of greater than 2 for a run to be considered valid. Negative control samples were expected to have a GM index value of <0.4, and the cut-off control values were acceptable when ≥0.3 and ≤0.8. Patient samples with a calculated GM CI value of ≥0.5 ng/mL were considered positive for GM. After all samples had been analyzed, data were combined with the clinical data, which had been collected independently. The study was approved by the ethics board of the medical faculty.

Culture Method

In the mentioned period, bronchoalveolar lavage (BAL), peritoneal, abscess and tissue samples were inoculated onto Sabouraud dextrose agar (Oxoid, UK) with and without antibiotics after microscopic examination; they were incubated at 37°C and 25°C. Conventional methods were used for the identification of isolates; colony color and morphology, growth rate, microscopic characteristics (shape and color of heads with conidia, number of sterigmata, shape of vesicle, structure of conidiophores, presence and shape of hulle cells) were considered [13].

Statistical analysis

The Shapiro Wilk's test was used to check the normality of data. The Mann-Whitney U test was

used for between-group comparisons. ROC analysis is used in clinical epidemiology to quantify how accurately medical diagnostic tests (or systems) can discriminate between the states of two patients, typically referred to as “diseased” and “non diseased”. Receiver operating characteristic (ROC) curves were plotted and the area under the ROC curves was calculated with a 95% CI to identify IA disease. A cut-off value was obtained by the highest positive and negative predictive values. Using this value, sensitivity, specificity, positive predictive rate, negative predictive rate and accuracy rate statistical diagnostic measures were calculated with 95% CIs. To validate the diagnostic reliability, the Kappa test was also used. To display the GM distribution among groups, an interactive dot graph was plotted. Analysis was performed by using MedCalc software (Version 9.2.0.1) by considering $p < 0.05$ as statistically significant.

■ RESULTS

A total of 47 patients were included in the study. The median age of the patients was 10 years (in a range 0.3-18). Twenty-five of the 47 patients were classified as proven, probable or possible IA, according to EORTC/MSG definition, independently from of GM results. A total of 3/25 (12%) of the

IA were proven, 9/25 (36%) were probable, and 13/25 (52%) were possible. There were no clinical findings of IA in 22 patients. Subtype diseases of the study group are shown in Table 1. Three proven patients were diagnosed with acute lymphoblastic leukemia (ALL). The direct examination of these patients’ tissues (two lungs and one brain) revealed hyphae and the analysis of the culture showed growth of *Aspergillus spp.* In the proven patient group, three had a serum GM level of ≥ 0.5 ng/mL at least once. The probable patient group included nine patients with neutropenic ALL, acute myeloblastic leukemia (AML), and non-Hodgkin lymphoma (NHL) disease. In all patients, radiological examination demonstrated nodular lesions, while culture and direct examination of dense defined respiratory tract samples showed growth of *Aspergillus spp.* Seven out of nine patients had a serum GM level of ≥ 0.5 ng/mL at least once. The possible patient group included 13 neutropenic ALL, AML, Hodgkin lymphoma (HL), aplastic anemia and haematopoietic stem cell transplantation. Eleven patients had nodular lesions, while two had the halo sign, as confirmed by computed tomography. Six patients had a serum GM level of ≥ 0.5 ng/mL at least once. Of the patients, six were on amphotericin B, whereas eight received secondary anti-fungal prophylaxis with voriconazole. Four patients received empiri-

Table 1 - Demographic characteristics and underlying diseases of patients with aspergillosis.

Demographic Variable	Proven IA (n=3)	Probable IA (n=9)	Possible IA (n=13)	Controls (n=22)	Total (n=47)
Gender					
Female	1	2	10	5	18
Male	2	7	3	17	29
Age					
Mean	13.0	10.3	10.0	9.9	10.2
Range	7.0-17.0	3.0-16.0	4.0-16.0	0.3-18	0.3-18
Disease					
ALL	3	5	3	7	18
AML	-	2	3	3	8
HL	-	-	1	9	10
NHL	-	2	-	-	2
Aplastic Anaemia	-	-	1	1	2
HSCT recipients	-	-	5	2	7

ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; HL, Hodgkin’s lymphoma; NHL, non Hodgkin’s lymphoma; HSCT, haematopoietic stem cell transplant.

cal antifungal treatment for persistent fever: three patients; liposomal amphotericin B, one patient; caspofungin, corticosteroid therapy was administered to 48% of all patients, and 67% of those received corticosteroid therapy for ≥ 3 days.

The GM antigen assay results were analyzed in 158 blood samples from 47 patients. The median number of tests per patient was three. At least one sample was tested as positive in 4 of the 22 control patients and age was not associated with false-positive results. Two of the four patients received carbapenem and one patient received Plasmalyte for treatment. The other patient had intestinal perforation, causing secondary peritonitis. The ROC curve was constructed to define the optimal serum GM cut-off value for the diagnosis of IA (Figure 1).

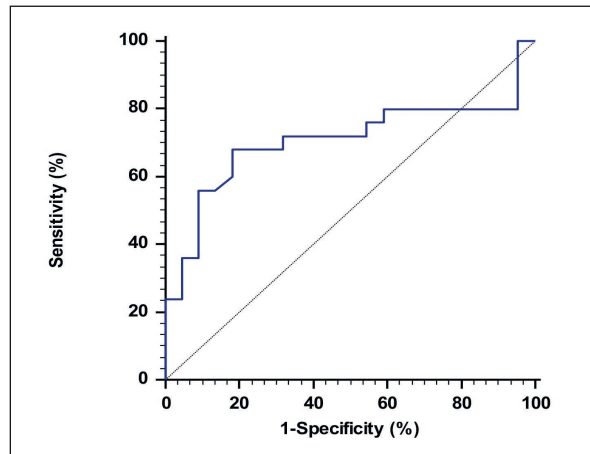


Figure 1 - Receiver operating characteristic (ROC) curve of the galactomannan assay for all patients.

Figure 2 - A dot diagram displaying the GM distribution among groups for the cut-off point of 0.5 ng/ml.

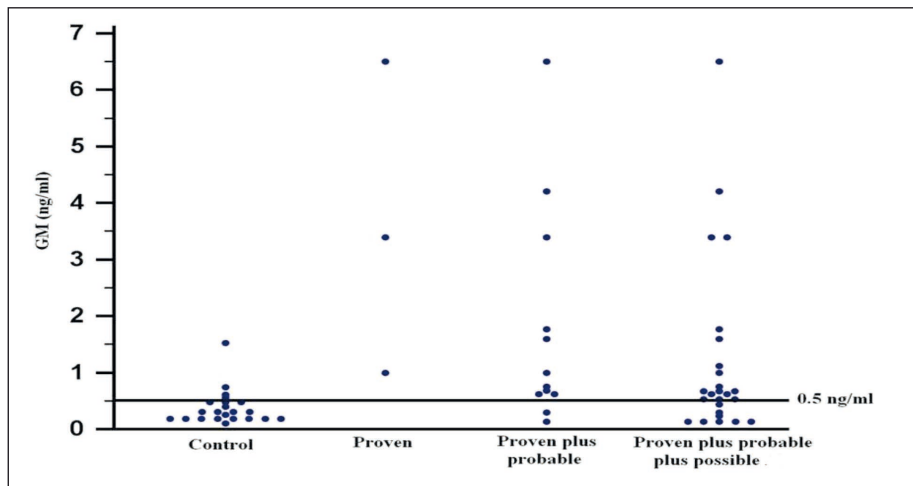


Table 2 - Diagnostic performance of galactomannan test at different cut-off values.

value		Sensitivity +LR		95% CI -LR	Specificity	95% CI
>0.21	1.26	80.00	0.55	59.3-93.1	36.36	17.2-59.3
>0.30	1.58	72.00	0.51	50.6-87.9	54.55	32.2-75.6
>0.40	2.26	72.00	0.41	50.6-87.9	68.18	45.1-86.1
>0.50	2.99	68.00	0.41	46.5-85.0	77.27	54.6-92.1
>0.51	3.74	68.00	0.39	46.5-85.0	81.82	59.7-94.7
>0.61	6.16	56.00	0.48	34.9-75.6	90.91	70.8-98.6
>0.71	3.96	36.00	0.70	18.0-57.5	90.91	70.8-98.6
>1	6.16	28.00	0.75	12.1-49.4	95.45	77.1-99.2

GM, galactomannan; IA, invasive aspergillosis; CI, confidence interval; +LR, positive likelihood ratio; -LR, negative likelihood ratio.

The area under the ROC curve was 0.85 (95% CI 0.68-0.95; $p < 0.001$). An interactive dot diagram that displays the distribution of GM values for these groups and the control group with a cut-off point of 0.5 ng/ml is presented in Figure 2.

Based on the CI ≥ 0.5 , the sensitivity and specificity of the GM test were 68% (95% CI: 47-85) and 77% (95% CI: 55-92), respectively. Receiver operating characteristic (ROC) curves were used to determine the optimal cut-off values for GM detection in serum.

The sensitivity and specificity with variable GM CI values are shown in Table 2.

■ DISCUSSION

Diagnosing IA among paediatric patients is complicated by the fact that such infections display rather general and non-specific clinical signs and symptoms that render any differential diagnosis difficult.

The GM assay, associated with various technical limitations and issues that adversely affects its diagnostic utility, has not yet received an approval for use in the diagnosis of IA in paediatric patients. Based on these considerations, the aim of our study was to provide data and information regarding the applied test false positive rate of the GM assay, and to determine applicable GM CI values for paediatric patients with neutropenia in a hospital setting.

Within the scope of the study, 47 paediatric patients with neutropenia undergoing treatment at our hospital were examined, and 25 of these paediatric patients were diagnosed as IA. Based on the study results, we have determined that the GM assay has a sensitivity of 68% (95% CI: 47-85) and a specificity of 77% (95% CI: 55-92) when applied to paediatric patients with neutropenia. The ROC analysis we have performed suggested an optimal GM CI value of ≥ 0.5 ng/mL in several instances. It was also observed that higher GM CI values were associated with lower sensitivity and specificity values for the GM assay.

Reported sensitivity and specificity values of the GM assay show a certain amount of variation, since the assay has been used in different centers employing different GM CI values. Although GM CI values have been determined and reported for adult patients (GM CI ≥ 0.5 ng/mL) in accordance

with the manufacturer's recommendations, similar values have not been determined for paediatric patients, especially for those who are neutropenic [9]. A recent study reported excellent sensitivity and specificity values (82.2% and 82.5%) when the GM CI value was accepted as ≥ 0.7 ng/mL, while another study conducted with 62 paediatric patients identified sensitivity and specificity values of 90% and 92%, respectively, when the GM CI value was accepted as ≥ 0.5 ng/mL [14, 15]. In a different study, GM assay sensitivity and specificity values for cases suspected with IA was determined as 65.7% and 87.2%, respectively when a GM CI value of ≥ 0.5 ng/mL was employed as positive result for galactomannan [16]. In case CI values for the GM assay were determined for paediatric patients, it would then be possible to eliminate the variability observed in GM assay sensitivity and specificity values.

Agents and substances derived from fungi might lead to a decrease in the assay's sensitivity for GM, and thereby contribute to false-positive results [17, 18]. We have obtained false-positive results for four of our patients within the scope of this study, two of whom had received carbapenem for the treatment of infections caused by *Pseudomonas* bacteria. However, as emphasized by various studies, it is also possible for blood-derived products to contribute to false-positive results, since there is a possibility of blood antigens reacting with the galactomannan test and causing invalid results to be obtained [19]. In addition, it is known that plasmalyte containing minute amounts of *Aspergillus* galactomannans can also trigger false-positive results in GM assays, thus leading to inaccurate IA diagnoses [20].

This was the case for one of the patients in our study who had received plasmalyte treatment, with a false-positive result being observed for this paediatric patient.

Other possible scenarios that might lead to false-positive results with the GM assay include the ingestion of galactomannan-containing beverages and foods such as tea, milk, pasta, rice and pepper, and the ingestion of fungi-contaminated foods that contain fungal galactomannans [21]. The ingestion of such beverages and foods may thus contribute to the erroneous diagnosis of IA when using the GM assay. A previous study reported that damage to the intestinal mucosa

could also lead to false-positive results; however, a prospective study performed on a combined adult and paediatric population did not confirm such an observation or relationship [14, 22]. Within the context of our study, one of our paediatric patients who displayed a false-positive result had mucosa damage caused by intestinal perforation; there is a possibility that this condition and the patient's false-positive result were associated.

In our published case, nodular lesion was identified in the frontal lobe of the paediatric patient with acute lymphoblastic leukemia and a sample was obtained from the lesion and hyphae with septa hyphae was seen on microscopic examination. If galactomannan serum values were sequential, they were determined as 3.39 ng/ml vs 0.72 ng/ml [23]. Two paediatric studies, analysing twice weekly screening periods in 119 and 64 high-risk patients, respectively, suggest that the GM test may be useful for the diagnosis of IA in children receiving aggressive antineoplastic chemotherapy or undergoing allogeneic HSCT [24, 17]. Fisher et al. studied serum and urine samples in GM test and they established specificity as 95% and 80%, respectively. In the evaluation of urine GM samples, false positive rate was found high level [25].

Our study was limited by small number of patients, but we concluded that early diagnosis of IA is of vital importance for the treatment of paediatric patients with neutropenia. Furthermore, since our study was planned in 2009, validated aspergillus colonization (putative or proven IA) categorization could not be utilized in the invasive aspergillus patient classification [26].

If different centers were to conduct efforts for identifying GM CI values, it would be possible to eventually identify a common and generally accepted value for confirming the presence of IA. With the appropriate GM CI values, and the proper interpretation and identification of possible false-positive results, the GM assay has the potential to become an effective tool for diagnosing IA among paediatric patients with neutropenia. In this context, it is necessary to conduct further studies that will illustrate and support the diagnostic value of the GM assay.

Conflict of interest. The authors have no conflicts of interest to disclose.

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