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Detection of Galactomannan and (1-3)-β-D-glucan for Early Diagnosis of Invasive *Aspergillosis* in Hematological Cancer Patients

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Abstract: Invasive *Aspergillosis* (IA) is one of the most common life-threatening opportunistic invasive mycosis in hematological cancer patients. Early diagnosis of IA is difficult; the need for early clinical diagnosis and management presents the need for new noninvasive, culture-independent diagnostic tools. Galactomannan (GM) and (1-3)-β-D-glucan (BG) antigenemia can serve as useful markers for IA. The current study was conducted to prospectively evaluate the clinical significance of GM and BG detection in the diagnosis of IA in patients with hematological malignancies in China. In 378 patients with hematological malignancies, GM and BG were detected. Analysis of sensitivity, specificity, Positive Predictive Value (PPV) and Negative Predictive Value (NPV) of GM and BG assay were performed. Detection of BG for diagnosis of IA with plasma reported sensitivity, specificity, PPV and NPV values as 66, 92, 86 and 78%, respectively. The GM detection for diagnosis of IA in serum reported sensitivity, specificity, PPV and NPV values as 48, 97, 93 and 71%, respectively. Combination of the two tests improved the specificity (to 100%) and PPV value (to 100%) of each individual test without affecting the sensitivity and NPV. The study concluded that serum GM and plasma BG can serve as potential markers of *Aspergillosis*. The detection of GM and BG has shown high specificity and PPV for screening high-risk hematological patients. The combined measurement of GM and BG appeared to be useful for early diagnosis of IA.

Key words: (1-3)-β-D-glucan, galactomannan, hematological cancer, invasive *Aspergillosis*

INTRODUCTION

Immunocompromised patients, in particular those with haematological malignancies carry a high risk for invasive fungal infections (IFIs), which are mostly life-threatening (Mikolajewska *et al*., 2012). Invasive *Aspergillosis* (IA) is an increasingly common and often fatal opportunistic fungal infection among hematological cancer patients receiving cytotoxic chemotherapy (Pazos *et al*., 2005); with mortality rates ranging from 60~90% in patients with leukemia and recipients of hematopoietic stem cell transplants (HSCTs) (Chamilos and Kontoyiannis, 2006; Pagano *et al*., 2006; Fukuda *et al*., 2003; Barnes and Marr, 2006).

Effective management of IA includes strategies to optimize prevention, early antifungal treatment, immunomodulation; and in some cases, surgery if required. Prompt initiation of antifungal therapy in patients with IA is critical in improving the adversity caused by this disease. It has been noticed that sensitivity of conventional diagnostic methods for IA is unacceptably low, leading to very late detection of the infection. Therefore, rapid serological diagnostic methods appear to be most useful to screen high-risk patients prospectively (Jones and McLintock, 2003). In the past decade, these diagnostic methods have focused on the detection of surrogate markers such as the galactomannan (GM) antigen (Guinea *et al*., 2008; Lai *et al*., 2007; Foy *et al*., 2007) and (1-3)-β-D-glucan (BG) (Pickering *et al*., 2005; Ishizuka *et al*., 2004; Ellis *et al*., 2008). (1-3)-β-D-glucan (BG) is a fungal cell wall component circulating in the blood of patients with IA, Invasive Candidiasis (IC) and other IFIs. Galactomannan is a polysaccharide cell-wall component of *Aspergillus* spp. which is released into the host's circulation during fungal growth in tissues and can be detected in the serum of patients with IA (Chamilos and Kontoyiannis, 2006). The tests designed for these possess high sensitivity and include a double-sandwich enzyme-linked immunosorbent assay (ELISA) for GM antigen Platellia™ *Aspergillus* assay (Bio-Rad Laboratories, Hercules, California, USA) and Fungitell™

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1-3 beta-D-glucan chromogenic assay (Associates of Cape Cod Inc., East Falmouth, Massachusetts, USA) for BG antigen (De Pauw *et al*., 2008; Hope *et al*., 2005).

The present study aimed to assess prospectively the usefulness of serum GM and plasma BG measurements in the routine practice and surveillance of IA along with possible caveats in diagnosis and treatment of patients with hematological malignancies in China.

MATERIALS AND METHODS

A total of 378 adult patients with hematological disorders who had been admitted from November 2005 through May 2010, at the Affiliated Hospital, Academy of Military Medical Sciences, Beijing, China and stratified according to high risk for IA were enrolled in the study. These patients were classified either as proven IA (n = 42), probable IA (n = 101), possible IA (n = 45) and no IA (n = 190). Their levels of GM in serum and BG in plasma were analyzed weekly. If the patients were thought to be at high risk, serum GM levels and plasma BG levels were measured twice weekly. All patients in the study were on antifungal agents.

The European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group and the National Institute of Allergy and Infectious Diseases Mycoses Study Group (EORTC/MSG) was used to classify IA episodes (Fukuda *et al*., 2003). According to Pazos *et al*. (2005), only proven IA and probable IA patients were considered true positive and patients with no IA were considered true negative.

Blood samples (about 2 mL of whole blood) were collected by venipuncture twice weekly until the high-risk condition for developing IFI had subsided. Serum GM testing was performed and reported according to the manufacturer's instructions (Platelia™ *Aspergillus* EIA test, Bio-Rad Laboratories, France). The result was considered true positive only when two consecutive samples for a patient tested positive, including the re-testing of the first sample (an index of ≥0.5 was considered positive). Glucatell™ test kit (Associates of Cape Cod, Falmouth, Massachusetts, US) was used for detecting BG. If the level of BG was ≥80 pg mL^{-1} in at least one serum sample, patients were considered positive. The sensitivity, specificity and predictive value of diagnostic tests were calculated according to their standard definitions. Sensitivity can be defined as the proportion of people with the target disorder in whom the test result is positive and specificity is the proportion of people without the target disorder in whom the test result is negative (Jaeschke *et al*., 1994; Reitsma *et al*., 2005).

All patients included should meet atleast one of the below mentioned criteria: neutrophils count less than 0.5×10^9/L lasting above 10 days, body temperature 38.5°C, lasting for 4 with sufficient broad spectrum antibiotics proves to be invalid with neutrophils count $<1.0\times10^9$/L, after accepting autologous or allogenic hematopoietic stem cell transplantation for more than 3 days, new lung lesions should be reported in chest radiographic inspection and history of fungal infections. Patients less than 18 years of age were excluded

The diagnosis standard included: The experimental objects were divided into four groups: diagnosed IA, highly suspicious IA, suspicious IA, non-invasive *Aspergillosis*, according to diagnosis standards (Ascioglu *et al*., 2002) of invasive *Aspergillosis* in patients with cancer or immunosuppression of hematopoietic stem cell transplantation, which are jointly formulated by the European cancer treatment organization (EORTC), invasive fungal infection group (IFICG) and the fungal disease group (MSG) of the National institute of allergy and infectious diseases (NIAD). Specific standards are as below:

- **Confirmed IA:** Patients having symptoms for fungal infection, including the host factors (neutropenia, fever, immunosuppressive therapy, application of glucocorticoids, graft versus host disease), histopathology (spaces reported in lung tissue microscopy, 45° branch of hypha or aspergillus cultured out of lung tissue), as well as microorganism culture (aspergillus cultivated from sterile parts of the specimen, with bronchoalveolar lavage fluid excluded)
- **Highly suspicious IA:** Patients have host factors with symptoms for fungal infection and with at least one microbiological standard (GM detection and (1, 3)-beta D glucan ((1, 3)-beta D-glucan, BG) excluded from the microbiological evidences after test), as well as clinical manifestations of a main or two secondary affected parts
- **Host factors:** (1) neutrophilic granulocyte count$<0.5\times10^9$/L lasting for 10 days; (2) body temperature higher than 38°C and or lower than 36°C with a risk factor for any of the following: a. neutropenia before 60 days, (above 10 days); b. powerful immunosuppressant was used or was being used within last 30 days; c. history of invasive fungal infections; d. AIDS occurrence at the same time; e. symptoms and signs of inhibitor resistance to host disease exist; f. steroid hormone used a for long term (3 weeks or more)

- **Clinical manifestations:** (1) Major clinical manifestations include: any of the following seepage signs found in lung CT detection: halo sign; crescent air sign; cavity appearing in real variable area. (2). Secondary clinical manifestations include: lower respiratory infection symptoms (cough, stethalgia, hemoptysis, dyspnea, etc.), persistent fever lasting for more than 96 h with reasonable treatment of broad spectrum antibiotics invalid
- **Microbiological standard:** (1) Sputum and bronchoalveolar lavage culture presents fungoid, (2) Presence of *Aspergillus* in sputum and bronchoalveolar lavage via direct microscopy or cytological examination, (3) Bronchoalveolar lavage fluid, cerebrospinal fluid or two servings of blood sample presents aspergillus antigen positive, (4) Sterile body fluids found in aspergillus through direct microscopy or cytology, (5) Fungi positive reported in blood culture and (6) Lung abnormalities and specimen related to lower respiratory tract infection (blood, sputum and bronchoalveolar lavage fluid, etc.) unable to cultivate any pathogenic bacteria
- **Suspicious IA:** Patients have host factors supporting fungal infection and presence of at least one microbiological standard or the clinical manifestations of one major or two secondary affected parts
- **Non-invasive *Aspergillosis* group:** No evidence of diagnosis of invasive *Aspergillosis* reported in this group

Retrospective diagnosis standard: Conformed IA, suspicious IA, eliminating IA can be diagnosed according to evidences of patients' therapeatics, clinical symptoms and inspection results. Specific standards are as follows:

- **Confirmed IA:** Patients have signs including fungal infection risk factors, clinical manifestations or a history of aspergillus infection in groups of the confirmed IA, highly suspicious IA and suspicious IA, of which are accompanied by pulmonary halo sign, air crescent sign, with no growth of pathogenic bacteria and mold in the sputum and swabs culture and with fluconazole treatment invalid and itraconazole, amphotericin B or a combination treatment of caspofungin effective
- **Eliminating IA:** Patients with signs of eliminating IA including deep fungal infection of non-invasive *Aspergillosis* and having no effect of antifungal therapy or antibiotics but affected by immuno-inhibitor treatment. Patients not meeting the microbiological diagnosis standard (GM and BG detection excluded) with pathogenic bacteria detected in sputum culture, swabs, blood culture
- **Suspicious IA:** Patients between the category of confirmed or eliminating IA fall into this category

RESULTS

Patient characteristics: A total of 3124 serum samples were collected and tested with the GM and BG assay. Demographic characteristics and underlying diseases of the patients are listed in Table 1. All patients in the IA group and no IA group had hematological malignancy as their underlying disease, with approximately 70% having undergone bone marrow transplantation. Overall, 143 patients fulfilled the criteria for proven (n = 42) and probable (n = 101) *Aspergillosis* and were eligible for outcome evaluation.

BG detection: Considering true positives as only the results obtained for patients with proven and probable IA and true negatives as the results in the no IA group of patients; the sensitivity, specificity and positive and negative predictive values of GM testing were 66, 92, 86 and 78%, respectively (Fig. 1).

GM detection: Considering true positives as the only results obtained for patients with proven and probable IA and true negatives as the results in the no IA group of

Table 1: Characteristics of patients at risk for invasive *Aspergillosis* (IA)

Characteristic	Proven IA		Probable IA		Possible IA		No IA	
No. of patients	42		101		45		190	
Median age, years (range)	36 (28-68)		40 (25-75)		42 (23-65)		39 (20-76)	
Male gender (%)	30 (71.4)		78 (77.2)		32 (71.1)		156 (82.1)	
	Proven IA		Probable IA		Possible IA		No IA	
Disease	No.	%	No.	%	No.	%	No.	%
Hematopoitic stem cell transplantation	28	66.7	72	71.3	30	66.7	124	65.3
Acute lymphocytic leukemia	3	7.1	13	12.9	4	8.9	20	10.5
Acute myeloid leukemia	3	7.1	6	5.9	6	13.3	26	13.7
Chronic lymphocytic leukemia	4	9.5	7	6.9	2	4.4	12	6.3
Myelodisplastic syndrome	2	4.8	2	2.0	1	2.2	3	1.6
Multiple syeloma	2	4.8	0	0.0	1	2.2	3	1.6
Severe aplastic anemia	0	0.0	1	1.0	1	2.2	2	1.1

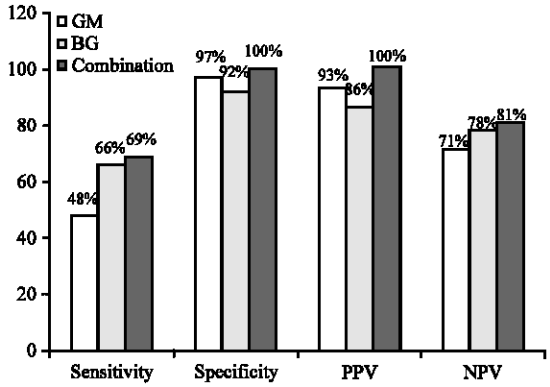

Fig. 1: Performances of the GM and BG assays for patients with IA (n = 143), PPV: Positive predictive value, NPV: Negative predictive value

patients; the sensitivity, specificity and positive and negative predictive values of GM testing were 48, 97, 93 and 71%, respectively (Fig. 1).

Combined detection of GM and BG: The results of combination of GM and BG detection for patients with IA are shown in Fig. 1. Most patients without IA who were false positive for one of the seromarkers tended to be negative for the other one. When both markers were used in combination, there was an improvement in diagnostic specificity. A combination of the two tests improved the specificity (to 100%) and PPV value (to 100%) of each individual test to detect IA without affecting the sensitivity and NPV. However, the sensitivity and NPV were 69 and 81%, respectively.

Antifungal preemptive therapy was given to the patients in case that GM = 0.5 is taken as starting point: Taking GM = 0.5 as the critical value, serum GM were tested in patients who did not respond to antibiotic therapy. These patients had two successive results and reported that GM≥0.5 was treated by antifungal preemptive therapy. There were 74 patients who had results of GM≥0.5 and of whom 64 patients were treated by antifungal preemptive therapy as a starting point, 55 patients were effective. Nine patients failed to respond to treatment and died, the fatality rate was 14.1%. Four patients with Aspergillus sputum culture positive were given antifungal therapy, in two patients it was effective, two patients were not valid and the fatality rate was 50.0%. All 68 patients were given antifungal therapy for more than two weeks and were tested for serum GM every 3 to 4 days after treatment. The GM value of 55 patients who responded to treatment decreased from 1.48±0.42 before treatment to 0.24±0.09 after treatment. The GM value of invalid cases was rising or sustained at a higher level.

Comparison of GM test and traditional test method: Taking 0.5 as positive starting point, antigen was tested in 61 of 143 patients who had been diagnosed and highly suspected. This was done earlier than sputum culture and radiology by 2-15 days. Positive GM test was reported after the Aspergillus sputum culture was positive in four patients for 2-4 days. Positive GM test was reported after the typical CT signs in three patients for 1-4 days, which may be related to GM test intervals. No positive culture in the sputum was seen for Aspergillus in 188 patients and their positive rate of GM was obviously higher than blood culture.

DISCUSSION

Despite the availability of active antifungal agents, invasive *Aspergillosis* among immunocompromised patients especially with hematologic malignancies continues to cause high morbidity, mortality and resource utilization (Woods *et al*., 2007). The diagnosis of IA poses a challenge for the clinician, due to poor prognosis and limited efficacy of currently available antifungal drugs. Traditional microbiological studies, such as direct microscopy and culture of respiratory specimen, have low sensitivity and appear positive only in the later stage of IA (Pazos *et al*., 2005). Invasive procedures relying on tissue biopsy or histopathological specimens are still considered the "gold standard" for establishing the diagnosis. However, in practice, these procedures are rarely performed. In recent years, the detection of different circulating surrogate markers such as fungal cell wall components (BG and GM) and genomic fungal DNA have emerged and improved the diagnosis of IA (Pazos *et al*., 2005; Hachem *et al*., 2009).

The Platelia™ *Aspergillus* kit for the detection of GM has been widely used in China for several years. In our study, we demonstrated that for a population of high-risk hematologic malignancy already receiving antifungal therapy, BG and GM assay can demonstrate a high specificity for IA. The sensitivity of galactomannan assay for IA has been variably reported among studies; from as low as 30% to as high as 100% (Hachem *et al*., 2009; Pfeiffer *et al*., 2006; Penack *et al*., 2008). This variability in the assay may be related in part to the hosts, their exposure to antifungal agents and cut-off value of a positive GM result. In Europe, manufacturers recommend a cut-off of 1.5 ng mL^{-1}, while in the United States, 0.5 ng mL^{-1} is the recommended cut-off value (Pazos *et al*., 2005). However, controversy exists about the interpretation of the index for the GM assay, which was originally set at 1.5 and was applied in Europe but which was lowered to 0.5 after review by the FDA. This

cutoff value has been recently shown to improve the overall performance of the test for adult hematology patients (De Pauw *et al*., 2008). In our study, a sample was considered positive if the index was = 0.5 (De Pauw *et al*., 2008; Maertens *et al*., 2007).

Several studies reported the impact of antifungal agents in lowering the antigen levels by decreasing the fungal load (Pfeiffer *et al*., 2006; Marr *et al*., 2005). All patients in our study were on antifungal agents, which may explain the lower sensitivity of the GM assay (48%). Furthermore, several studies reported a paradoxical increase in circulating GM after caspofungin treatment for proven IA was administered (Hachem *et al*., 2009; Klont *et al*., 2006). In our study, it was noticed that patients receiving caspofungin had a slightly higher sensitivity as compared to patients not receiving this drug.

The other noninvasive diagnostic method used in our study was the BG assay, which is widely used in China in the diagnosis of systemic mycosis. For patients with IA, the sensitivity and specificity of BG testing were 66 and 92%, respectively. Our results are in line with the study by (Persat *et al*., 2008) which reported that for overall IFIs, the BG assay had 77.8% sensitivity and 92.5% specificity. In our study, BG assay was shown to have a better sensitivity than that of GM testing in detecting IA. Moreover, the specificity of the test was above 90% for IA. Hence, BG might be beneficial marker for detecting IA. BG testing is associated with a high sensitivity, while GM testing is associated with a high specificity. Therefore, for high-risk patients with hematologic malignancy on antifungal therapy, combination of both tests may be the best approach.

CONCLUSION

IA poses a major threat to patients with underlying hematological malignancies. Our study demonstrates that the detection of GM and BG can be useful for the early diagnosis of IA in high-risk patients with hematological malignancies. However, a combination of the two tests may be the most promising approach to improve the specificity and PPV in China.

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