Available online at www.sciencedirect.com

ScienceDirect

journal homepage: www.elsevier.com/locate/mjafi

Original Article

β-D-Glucan and *Aspergillus* Galactomannan assays in the diagnosis of invasive fungal infections

Mahadevan Kumar [a,*], *M. Mugunthan* [b]

[a] *Professor, Department of Microbiology, Armed Forces Medical College, Pune 411040, India*
[b] *Resident, Department of Microbiology, Armed Forces Medical College, Pune 411040, India*

ARTICLE INFO

Article history:
Received 17 June 2016
Accepted 27 October 2017
Available online 28 November 2017

Keywords:
Invasive fungal infections
Antigen detection assays
β-D-Glucan
Galactomannan

ABSTRACT

Background: With an increase in the number of patients who are immunosuppressed or immunocompromised there has been an increase in invasive fungal infection (IFI) in the past few decades with its associated high morbidity and mortality, ranging from 60% to 90%. The critical problem is the identification of the causative fungus and initiation of appropriate therapy. Hence, there is a requirement for better diagnostic methods for IFI. Detection of markers for the presence of fungi during early stage of the infection, such as constituents of the cell wall or fungal DNA, is essential for timely diagnosis of IFI. Galactomannan (GM) which is a cell wall surface antigen is the most studied diagnostic marker, followed by 1,3 β-D-Glucan (BG) which is seen in deep layers of cell wall.

Methods: We have assessed the effectiveness of Galactomannan/β-D-Glucan for the early diagnosis of IFI in immunosuppressed patients in our tertiary care setting.

Results: The sensitivity, specificity, positive predictive value and negative predictive value of GM assay were 45%, 93%, 86% and 63% respectively, while the BG assay showed a sensitivity of 78%, specificity of 85%, Positive predictive value (PPV) 84% and Negative predictive value (NPV) 79%.

Conclusion: BG assay is better for detection of IFI in patients with immunosuppression. However, a combination of both BG and GM assays would be the best approach as BG assay is highly sensitive, while the GM assay is highly specific for diagnosing IFI.

Introduction

Invasive fungal infections (IFI) have increased in the past two decades owing to greater numbers of immunosuppressed or immunocompromised patients. These infections are associated with high morbidity and mortality in 60–90% of cases[1–6] while the prevalence of IFI in haematological malignancy ranges from 24 to 31%.[7–9] The fundamental issue is the identification of the pathogenic causative fungi and initiation of timely therapy. Hence, there is a necessity for better methods for diagnosis of IFI. Detection of markers for fungal disease in clinical samples, such as fungal DNA or cell wall

* *Corresponding author.*
E-mail address: dr.mkumar011@gmail.com (M. Kumar).
https://doi.org/10.1016/j.mjafi.2017.10.005
0377-1237/

components, is essential in order to achieve the above.[10,11] Galactomannan (GM) an antigen found on the surface of the fungal cell wall and 1,3 β-D-Glucan (BG) which is seen in the deeper layers of the cell wall have been used for this purpose.[12–14] These antigens are unique to fungi and the BG protein comprises 58–60% of the cell wall while the mannoproteins comprise 38–40% of the cell wall.[15] The GM assay, has shown a sensitivity ranging from 30% to 100% and a specificity ranging from 38% to 98%.[16] Similarly the BG assay has also shown variable results, however with a slightly increased sensitivity and specificity, ranging between 70% and 90%.[17–19] We have attempted to combine both the GM and BG assays in the diagnosis of IFI in immunosuppressed patients in our tertiary care setting.

Materials and methods

The study was carried out in a tertiary care hospital and Medical College in a time span of one and a half years. The sample size was worked out taking sensitivity as 70–90% with a confidence interval of 95% and a 7% error of margin: as per the calculation the minimum sample size required was 71 and 77 respectively for BG and GM.[20] Hence 80 patients were studied in each group with 80 controls. Consequently, a total of 80 patients suspected to be suffering from invasive fungal disease as per the criteria laid out by the European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group and the Mycoses Study Group (EORTC/MSG) were selected based on a combination of the following: host factors, clinical criteria (clinical manifestations and radiologic evidence) and mycologic criteria (direct microbiologic or pathologic evidence).[21] In addition, patient demographics along with clinical parameters including age, underlying disease, type of transplantation, steroid use, neutropenia, antibiotic use and antifungal therapy use were obtained. Two blood samples were collected from each patient one week apart. Two antigen detection assays were carried out simultaneously on these serum samples as follows.

β-D-Glucan assay

Blood samples from the patients were collected in sterile, BG-free plain vacutainer (BG-free) (BD India). Serum after separation was stored at −80 °C until testing. Fungitell BG assay kit (Associates of Cape Cod, USA) was used to determine the BG levels in the samples. As per the manufacturer's instructions a standard curve was plotted using five standard concentrations namely 500, 250, 125, 62.5, 31.25, and 0 pg/mL and the assays were carried out in sets of 20 serum samples each. The cutoff for positivity was taken as 80 pg/ml. Briefly, each sample was assayed in triplicate with 5 μl of serum in each well and incubated with a pretreatment solution of 20 μl of 0.125 M KOH–0.6 M KCl for 10 min at 37 °C in order to inactivate inhibitors present in the serum. Following this the Fungitell BG reagent was added to each well. An ELISA reader (VICTOR X3, *Perkin Elmer*, USA) was used to carry out the kinetic analysis of the reaction and read every minute at 405 nm (reference filter 560 nm) for 40 mins at a temperature of 37 °C.

GM assay

GM assay was carried out using the Platelia *Aspergillus* enzyme immunoassay test kit (Bio-Rad Laboratories, France). Steps of a routine ELISA were carried out except for the initial heating of the 300 μl of the serum at 120 °C after addition of 100 μl of serum treatment solution. Optical density of the wells was read at 450 nm (reference filter 620 nm) in an ELISA reader (BioRad, India). Cutoff values were calculated as per manufacturer's instructions and the samples that qualified the validity criteria and had an optical density index of >0.5 were labelled positive.

Results

Of the 80 patients, 39 were cases of haematological malignancy followed by solid organ tumours (10), immunesurveillance (10), febrile neutropenia (7), organ transplant recipients (2), and the rest were cases of suspected mycosis with varied clinical backgrounds. The haematological cases were as follows: acute leukaemia (22), Hodgkin's lymphoma (1), non-Hodgkin's lymphoma (1), multiple myeloma (6), chronic leukaemia (3) and autologous bone marrow transplant (6). When classified using the EORTC criteria, of the 80 cases, 13 were categorized as proven cases, 34 as probable and the remaining 33 samples were categorized as possible cases. Sixty two and 36 patients tested positive for BG and GM antigen respectively on both occasions. Of the healthy controls 12 and 6 tested positive for BG and GM antigen respectively (Table 1). 29 out of these 80 patients were on β lactam antibiotics.

Discussion

Diagnosis of IFI is a challenging task for the clinicians and the microbiologist. Culture has a long turnaround time and is not sensitive in the identification of invasive infection by filamentous fungi and lacks specificity for invasive candidiasis.[1,22] Thus, histopathological examination of tissue biopsy specimen is considered the reference standard for establishing the diagnosis.[11] As the IFI's are deep seated the methods for obtaining specimens for histopathological examination are invasive in nature and are a cause for increased morbidity especially in the setting of immunosuppression. Thus serological assays as a less invasive method have greater utility in the diagnosis of IFI.

In our study, the BG assay was positive in 62 out of 80 cases and in 12 out of 80 healthy controls with a sensitivity of 78%, specificity of 85%, PPV of 84% and an NPV of 79% along with a

Table 1 – Results of BG (β-D-Glucan assay) and GM (Galactomannan assay) in cases and controls.

Assay	Cases		Control	
	Positive	Negative	Positive	Negative
BG	62	18	12	68
GM	36	44	6	74

Table 2 – Sensitivity, specificity, PPV, NPV and positive likelihood ratio of 1,3 β-D-Glucan assay.

Study	Sensitivity	Specificity	Positive predictive value	Negative predictive value
Ostrosky-Zeichner et al., 2005 USA	70%	87%	–	–
Pazos et al., 2005 USA	87.5%	89.6%	–	–
Persat et al., 2008 Japan	77.8%	92.5%	–	–
This study[a]	78%	85%	84%	79%
	CI 66.79–86.09%	CI 75.26–92.00%	CI 75.16–89.82%	CI 71.34–85.15%

[a] Positive likelihood ratio = 5.17 (CI 3.03–8.82).

Table 3 – Sensitivity, specificity, PPV, NPV and positive likelihood ratio of Galactomannan assay.

Study	Sensitivity	Specificity	Positive predictive value	Negative predictive value
Hachem RY et al., 2009, USA	49%	99%	95%	62%
Boutboul et al., 2002, France	44%	87%	94%	–
Foy PC et al., 2007, USA	50%	94%	46%	94%
This study[a]	45%	93%	86%	63%
	CI 33.85–56.53%	CI 84.39–97.20%	CI 72.81–93.08%	CI 57.74–67.43%

[a] Positive likelihood ratio = 6.0 (CI 2.68–13.44).

positive likelihood ratio of 5.17 (CI 3.03–8.82) (Table 2). Other researchers have demonstrated a similar trend. Ostrosky-Zeichner et al.[17] showed sensitivity and specificity of 70% and 87% while Pazos et al.[23] showed a sensitivity of 87.5% and Specificity of 89.6% and Persat et al.[24] reported 77.8% sensitivity and 92.5% specificity. Moreover, the specificity of the assay was about 85% thus making BG a good diagnostic marker for detecting most fungi. The other studies did not report – PPV and NPV of this assay. Use of cotton gauze during specimen collection may lead to false positive results.[25,26] This may account for the false positive results in the healthy controls seen in our study. The BG assay requires an incubating ELISA reader as the assay is to be carried out at a temperature of 37 °C, In addition other requirements are strict assay conditions like a laminar air flow cabinet and BG free glassware/plasticware and a well-trained technician in order to avoid error free and false positive results. Fungi such as *Aspergillus* spp., *Candida* spp., *Fusarium* spp., *Blastomyces dermatitidis*, *Coccidioides* spp., *Histoplasma capsulatum*, *Pneumocystis jirovecii* and *Trichosporon* spp., liberate BG into the plasma during infection and this circulating antigen can be detected by the BG assay whereas fungi such as Fusarium, Zygomycetes, and Cryptococcus cannot be detected as they lack BG in their cell wall.[27–29] A baseline level of 10–40 pg/mL of BG is seen normally in human serum. Thus, a value more than 80 pg/mL is considered significant. This test therefore acts as a surrogate marker for invasive fungal infection but cannot discriminate among fungal species. It is a rapid presumptive screen for invasive fungal infection. As with any serologic test, sera that are haemolysed or lipaemic can interfere with the testing procedure thereby reducing the sensitivity of the test. Bacteremic patient sera can give false-positive reactions.

Aspergillus GM assay was positive in 36 out of 80 cases and 6 out of 80 healthy controls. Sensitivity, specificity, PPV and NPV obtained in our study were 45%, 93%, 86% and 63% respectively while the positive likelihood ration was 6.0 (CI 2.68–13.44), which is similar to studies done by Hachem et al.[30] Boutboul et al.[31] and Foy et al.[32] (Table 3). In our study 29 out of the 80 cases were on β-Lactam antibiotics which could have given false positives in the assay. Positivity in patients receiving β lactam antibiotics should be interpreted cautiously and confirmed by other diagnostic methods.[33] Several studies have reported that the impact of antifungal agents in reducing the antigenemia by decreasing the fungal load make the test less sensitive in patients receiving antifungal agents.[16,34] This may explain the lowered sensitivity of the GM assay in this study and emphasizes the importance of collection of serum sample prior to the administration of antifungals. Humanized milk frequently contain high concentrations of Galactomannan, therefore the interpretation of a positive test must take into account this dietary factor in young children. Galactomannan (*Aspergillus*) antigens are rapidly cleared from the circulation and thus require frequent monitoring during the period of high risk. This is an important factor more so because of the fact that the ELISA formats operate at the lower limit of detection. Antigen detection in blood or other samples like BAL is minimally invasive and can serve as an useful adjunct n the diagnosis of IFI.

All the patients who tested positive by the GM test were also positive by the BG test, thus the sensitivity remained unchanged when these assays were combined for diagnosis. Therefore, though the BG assay is more sensitive as compared to the Galactomannan assay a combination of both tests would possibly be the best way to diagnose IFI in high risk patients on antifungal therapy as BG testing is highly sensitive, while GM antigen testing is highly specific for diagnosing IFI.

Conflicts of interest

The authors have none to declare.

REFERENCES

1. Andriole VT. Infections with *Aspergillus* species. *Clin Infect Dis.* 1993;17(suppl 2):S481–S486.
2. Hope WW, Walsh TJ, Denning DW. Laboratory diagnosis of invasive aspergillosis. *Lancet Infect Dis.* 2005;5(10):609–622.

3. Grau S, Solano C, Garcia-Vidal C, et al. Pharmacoeconomic analysis of the use of voriconazole. Posaconazole and micafungin in the primary prophylaxis of invasive fungal infections in recipients of allogeneic hematopoietic stem cell transplants in Spain. *Value Heal J Int Soc Pharmacoecon Outcomes Res*. 2015;18(7):A581.
4. Burghi G, Lemiale V, Azoulay E. Invasive aspergillosis in critically ill hematology patients: outcomes and prognostic factors associated with mortality. *Crit Care*. 2011;15(suppl 1):P242.
5. Nihtinen A, Anttila V-J, Richardson M, et al. Invasive Aspergillus infections in allo-SCT recipients: environmental sampling, nasal and oral colonization and galactomannan testing. *Bone Marrow Transplant*. 2010;45(2):333–338.
6. Pagano L, Stamouli M, Tumbarello M, et al. Risk of invasive fungal infection in patients affected by acute promyelocytic leukaemia. A report by the SEIFEM-D registry. *Br J Haematol*. 2015;170(3):434–439.
7. Chamilos G, Luna M, Lewis RE, et al. Invasive fungal infections in patients with hematologic malignancies in a tertiary care cancer center: an autopsy study over a 15-year period (1989–2003). *Haematologica*. 2006;91(7):986–989.
8. Donhuijsen K, Petersen P, Schmid WK. Trend reversal in the frequency of mycoses in hematological neoplasias: autopsy results from 1976 to 2005. *Dtsch Arztebl Int*. 2008;105(28–29):501–506.
9. Hammond SP, Marty FM, Bryar JM, DeAngelo DJ, Baden LR. Invasive fungal disease in patients treated for newly diagnosed acute leukemia. *Am J Hematol*. 2010;85(9):695–699.
10. Falagas ME, Tassios PT. Enhanced and earlier detection of bacteremia and fungemia by multiplex polymerase chain reaction: how much enhanced, how much earlier, and at what cost? *Crit Care Med United States*. 2008;36:1660–1661.
11. Reiss E, Obayashi T, Orle K, Yoshida M, Zancope-Oliveira RM. Non-culture based diagnostic tests for mycotic infections. *Med Mycol*. 2000;38(suppl 1):147–159.
12. Cordonnier C, Botterel F, Ben Amor R, et al. Correlation between galactomannan antigen levels in serum and neutrophil counts in haematological patients with invasive aspergillosis. *Clin Microbiol Infect*. 2009;15(1):81–86.
13. Herbrecht R, Bories P, Moulin J-C, Ledoux M-P, Letscher-Bru V. Risk stratification for invasive aspergillosis in immunocompromised patients. *Ann N Y Acad Sci*. 2012;1272(1):23–30.
14. Bhaskaran A, Singer L, Mazzulli T, Prochnow T, Rotstein C, Husain S. Performance of (1,3)-β-D-Glucan in bronchoalveolar lavage of lung transplant recipients (LTRs) for the diagnosis of invasive pulmonary aspergillosis (IPA). *J Hear Lung Transplant*. 2016;33(4):S152.
15. Reiss E, Shadomy HJ, Lyon GM. Introduction to fundamental medical mycology. In: *Fundamental Medical Mycology*. 1st ed. New Jersey: John Wiley & Sons, Inc.; 2012:22–23.
16. Klein CN, Pfeiffer CD. Diagnosis of invasive aspergillosis: use of the galactomannan assay. *Curr Treat Options Infect Dis*. 2015;7(3):163–175.
17. Ostrosky-Zeichner L, Alexander BD, Kett DH, et al. Multicenter clinical evaluation of the (1→3) beta-D-glucan assay as an aid to diagnosis of fungal infections in humans. *Clin Infect Dis*. 2005;41(5):654–659.
18. Acosta J, Catalan M, del Palacio-Peréz-Medel A, et al. A prospective comparison of galactomannan in bronchoalveolar lavage fluid for the diagnosis of pulmonary invasive aspergillosis in medical patients under intensive care: comparison with the diagnostic performance of galactomannan and of (1→3)-beta-D-glucan. *Clin Microbiol Infect*. 2011;17(7):1053–1060.
19. Mohr JF, Sims C, Paetznick V, et al. Prospective survey of (1→3)-β-D-Glucan and its relationship to invasive candidiasis in the surgical intensive care unit setting. *J Clin Microbiol*. 2011;49(1):58–61.
20. Mayo Clinic (https://www.mayomedicallaboratories.com/test-catalog/Clinical+and+Interpretive/84356). ASPAG, Aspergillus (Galactomannan) Antigen, Serum. 2014 [cited 24.08.17]. pp. 8–10.
21. Cao JH, Li GH, De Pauw B, et al. Revised definitions of invasive fungal disease from 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) C. *Clin Infect Dis*. 2008;46(12):1813–1821.
22. Vazquez R, Vazquez-Guillamet MC, Suarez J, Mooney J, Montoya JG, Dhillon GS. Invasive mold infections in lung and heart-lung transplant recipients: Stanford University experience. *Transpl Infect Dis*. 2015;17(2):259–266.
23. Pazos C, Palacio AD, Pai U, Ponton J, Del Palacio A. Contribution of (1→3)-beta-D-glucan chromogenic assay to diagnosis and therapeutic monitoring of invasive aspergillosis in neutropenic adult patients: a comparison with serial screening for circulating galactomannan. *J Clin Microbiol*. 2005;43(1):299–305.
24. Persat F, Ranque S, Derouin F, Michel-Nguyen A, Picot S, Sulahian A. Contribution of the (1→3)-beta-D-glucan assay for diagnosis of invasive fungal infections. *J Clin Microbiol*. 2008;46(3):1009–1013.
25. Sims CR, Jaijakul S, Mohr J, Rodriguez J, Finkelman M, Ostrosky-Zeichner L. Correlation of clinical outcomes with beta-glucan levels in patients with invasive candidiasis. *J Clin Microbiol*. 2012;50(6):2104–2106.
26. Kanamori H, Kanemitsu K, Miyasaka T, et al. Measurement of (1-3)-beta-D-glucan derived from different gauze types. *Tohoku J Exp Med*. 2009;217(2):117–121.
27. Cornillet A, Camus C, Nimubona S, et al. Comparison of epidemiological, clinical, and biological features of invasive aspergillosis in neutropenic and nonneutropenic patients: a 6-year survey. *Clin Infect Dis*. 2006;43(5):577–584.
28. Obayashi T, Negishi K, Suzuki T, Funata N. Reappraisal of the serum (1→3)-beta-D-glucan assay for the diagnosis of invasive fungal infections – a study based on autopsy cases from 6 years. *Clin Infect Dis*. 2008;46(12):1864–1870.
29. Ostrosky-Zeichner L, Kullberg BJ, Bow EJ, et al. Early treatment of candidemia in adults: a review. *Med Mycol*. 2011;49(2):113–120.
30. Hachem RY, Kontoyiannis DP, Chemaly RF, Jiang Y, Reitzel R, Raad I. Utility of galactomannan enzyme immunoassay and (1,3) beta-D-glucan in diagnosis of invasive fungal infections: low sensitivity for *Aspergillus fumigatus* infection in hematologic malignancy patients. *J Clin Microbiol*. 2009;47(1):129–133.
31. Boutboul FF, Alberti C, Leblanc T, et al. Invasive aspergillosis in allogeneic stem cell transplant recipients: increasing antigenemia is associated with progressive disease. *Clin Infect Dis*. 2002;34(7):939–943.
32. Foy PC, van Burik J-A, Weisdorf DJ. Galactomannan antigen enzyme-linked immunosorbent assay for diagnosis of invasive aspergillosis after hematopoietic stem cell transplantation. *Biol Blood Marrow Transplant*. 2007;13(4):440–443.
33. Gorton RL, White PL, Bagkeris E, et al. Improved standardization of the bio-rad platelia aspergillus galactomannan antigen sandwich enzyme immunoassay using the DS2 (Dynex) Enzyme-Linked Immunosorbent Assay (ELISA) processing system. *J Clin Microbiol*. 2015;53(7):2072–2078.
34. Fisher CE, Stevens AM, Leisenring W, Pergam SA, Boeckh M, Hohl TM. Independent contribution of bronchoalveolar lavage and serum galactomannan in the diagnosis of invasive pulmonary aspergillosis. *Transpl Infect Dis*. 2014;16(3):505–510.