ISSN: 2577 - 8455

Review Article

Archives of Infectious Diseases & Therapy

Diagnostic Significance of Serum and BAL Galactomannan (GM) Enzyme Immune Assay in Invasive Aspergillosis (IA) with Reference to EORTC/MSG - A Short Review

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Submitted: 24 Aug 2018; **Accepted**: 31 Aug 2018; **Published**: 24 Sep 2018

Abstract

Invasive aspergillosis is a life-threatening mycelial fungal infection in immunocompromised patients and is associated with high mortality and morbidity. Patients undergoing hematopoietic stem cell transplant (HSCT) and neutropenic patients are particularly at risk. The degree and duration of neutropenia is an independent risk factor for invasive fungal infections. Patients with prolonged and severe neutropenia (ANC less than 500cells/cumm) are more susceptible. The lung is the most common site of infection and vascular invasion by Aspergillus species is a common histopathological feature of invasive aspergillosis (IA). As there is a lack of adequate immune response, patients with IA fail to develop classical signs and symptoms of the disease making diagnosis of IA more difficult. The results of fungal cultures are often delayed and cytopathological examination, yields negative results as there is lack of sensitivity and specificity. Biopsy specimens may be unproductive if the sample is collected at an advanced stage of the disease. Galactomannan (GM) detection in serum and Broncho alveolar lavage fluid (BAL) seems to be useful in establishing or excluding the diagnosis of invasive aspergillosis. Multicentre studies reported that there was no conclusive benefit of determining serum and BAL GM levels in the diagnosis of invasive aspergillosis among immunocompetent hosts. A serum and BAL GM test should not be ordered routinely in non-immunocompromised hosts.

Keywords: Invasive Aspergillosis, Immunocompromised, Neutropenia, HSCT, Galactomannan.

Introduction

Invasive fungal infections (IFI) are known to be significant cause of morbidity and mortality in cancer patients with hematological malignancies and patients undergoing allogenic bone marrow transplantation [1-3]. Aspergillosis has become an important cause of localised and invasive fungal infections in immunocompromised patients with a morbidity ranging from 30 - 70%. It is a spectrum of disease governed by hosts immunity and environmental factors. Depending on the immune status of the host, response of the host with *Aspergillus* spp. might be limited to colonization, develop an infection with severe disseminated disease or develop a hypersensitivity illness in the form of allergic bronchopulmonary aspergillosis (ABPA). Galactomannan (GM) is a polysaccharide antigen that exists primarily in the cell walls of *Aspergillus* species. GM may be released into the blood, body fluids and tissues even in the early stages of *Aspergillus* invasion, and the presence of this antigen can be sustained for 1 to 8 weeks in the circulation or lungs [4].

Therefore, detection of the GM antigen level via enzyme linked immunosorbent assay (ELISA) can be useful in making an early diagnosis of Invasive Aspergillosis (IA). Currently, serum GM detection is considered a microbiological diagnostic criterion for fungus infection in neutropenic patients according to the guidelines of 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) [5].The cut-off value for serum GM detection to be positive is generally set at an index of 0.5. Recently, Broncho alveolar lavage fluid (BAL) GM detection has also been strongly recommended in the 2016 Infectious Disease Society of America guidelines as a test providing high quality evidence in neutropenic patients, but its clinical application in non-neutropenic patients lacks evidence and its optimal threshold has not been determined [6-8].

The *Aspergillus* GM is shed in the blood stream and other tissues during acute infection as part of the growth process. GM is a soluble, heat stable antigen released during hyphal growth. Indirect immunofluorescence indicated that reactions to EB-A2 is not uniform throughout all fungal structures (hyphae, spores, conidia) of the A. *fumigatus* but reacted strongly with non-germinating and young conidia. Studies have shown that the amount of GM released varies according to the species of *Aspergillus* [9]. The amount of GM released by A. *fumigatus* is less than that of other species. It has been speculated that the small quantity of the antigen is part of the limitations of the galactomannan enzyme immune assay EIA (GM EIA). However, it is well known that production and release of GM into circulation is dependent on the site of infection, growth of fungus and may be intermittent or absent [10]. The objective of this review article is to evaluate the diagnostic significance of serum and Broncho alveolar lavage fluid galactomannan (GM) detection in patients with clinically and radiologically suspected invasive aspergillosis with reference to EORTC/MSG classification for invasive fungal diseases.

EORTC/MSG

Invasive fungal infections (IFI) have emerged as an important cause of morbidity and mortality in cancer patients. Patients with hematological malignancies and that undergoing bone marrow transplantation are at high risk of invasive mycoses. Aggressive chemotherapeutic protocols for treatment resulting in prolonged and profound neutropenia are the most important contributory factors. Blood culture lacks the sensitivity but with the availability of modern serological techniques, the diagnosis of systemic fungal infections has significantly improved. A standard definition of IFI was developed by members of the European Organization for Research in the Treatment of Cancer–Invasive Fungal Infection Cooperative Group (EORTC) and the National Institute of Allergy and Infectious Diseases-Mycoses Study Group (MSG). These definitions were developed to facilitate

1. The identification of reasonably homogeneous groups of patients for clinical and epidemiologic research
2. To help design clinical trials
3. To evaluate new drugs and management strategies for invasive fungal infections
4. To foster communication between international researchers

EORTC/MSG recommend detection of GM as a standalone microbiological assay for diagnosis of invasive fungal infections in association with clinical and host factors. The EORTC, invasive fungal infections cooperative group (IFICG) and the MSG, classified invasive aspergillosis (IA) into three groups based on clinical manifestations, signs and symptoms, and microbiologic parameters for the purpose of clinical and research and epidemiological activities. Host factors with underlying immunocompromised conditions such as cancer and other hematologic malignancies, patients undergoing autologous and allogenic transplantations with clinical and/or radiological suspicion of invasive fungal infection and mycology test results (microbiological and histopathological) from normally sterile body sites are taken into considerations. A patient can be labelled as proven case of aspergillosis when *Aspergillus* spp. has been isolated or demonstrated by microbiological techniques either by microscopy or by culture and demonstration of septate hyphae in diseased tissues by histologically and cytological evaluation in suspected patients. Patients who demonstrate mycological element either by microscopy or histological evaluation as evidence of the disease along with the presence of a host factors (cancer, transplantation) a clinical feature suggestive of IFI are labelled as probable case of aspergillosis. Possible IFI was defined as only those cases with the appropriate host factors and with sufficient clinical evidence consistent with IFI but for which mycological evidence was absent. This criteria was defined more appropriately in the year 2008 than 2002.

Table 1: EORTC/MSG Criteria for invasive aspergillosis adapted from reference 5

Class	Diagnostic criteria 2008	Diagnostic criteria 2002
Proven	Proof by demonstration of fungal elements in tissues	Demonstration of fungus in tissue histopathology or positive culture of tissues obtained by invasive procedure
Probable	Presence of a host factor, a clinical criterion and a mycological criterion (cytology or direct microscopy of sputum, culture or galactomannan detection)	One host factor plus one clinical feature plus one mycological factor (cytology or direct microscopy, culture or galactomannan detection)
Possible	Presence of a host factor, a clinical criterion but absence of mycological criteria.	One host factor plus two minor clinical features or one major clinical factor or mycological criteria (cytology or direct microscopy of sputum, culture or galactomannan detection)

Table 2: Criteria for classifying proven invasive aspergillosis adapted from reference 5. Galactomannan Enzyme Immune Assay (GM EIA)

Analysis and specimen	Aspergillus species
Microscopic analysis: sterile material	Histopathologic, cytopathologic, or direct microscopic examination of a specimen obtained by needle aspiration or biopsy in which hyphae forms are seen accompanied by evidence of associated tissue damage
Culture Sterile material	Recovery of *Aspergillus* spp. by culture of a specimen obtained by a sterile procedure from a normally sterile and clinically or radiologically abnormal site consistent with an infectious disease process, excluding bronchoalveolar lavage fluid, a cranial sinus cavity specimen, and urine
Blood	Blood culture that yields *Aspergillus* spp. in the context of a compatible infectious disease process

Galactomannan is a hetero-polysaccharide consisting of a mannan core and lactofuransyl side chains. This circulating antigen is found in the cell wall primarily of mycelial fungi especially in *Aspergillus spp*. and *Penicillium spp*. Other species of fungi likr Geotrichum also demonstrate GM antigen. More than 250 species of *Aspergillus spp*. Have been identified so far. The most common clinically isolated species of *Aspergillus* are *A. fumigatus*, *A. flavus*, *A. niger*, and *A. terreus*. *A. fumigatus* is the most common accounting for over 50% of infections by this genus.

Stynen et al developed galactomannan assay in Netherlands for diagnosis of invasive aspergillosis [11]. Each GM molecule has as many as ten epitopes. Both capture and detector antibodies specific for the epitopes can be attached to the molecule. The *Aspergillus* Enzyme Immune Assay basically consists of a rat monoclonal antibody (MAb) EB-A2 that reacts with the specific epitope of GM. It is an IgM antibody with an avidity constant of 2x109 to 5x109 M and binds to an epitope located on the β (1→5) galactofuranose containing side chain of the GM molecule. A similar epitope seems to be present in other fungi. The epitope recognized by the EB-A2 MAb, is a common oligosaccharide moiety of a wide range of intracellular and extracellular glycoproteins of *Aspergillus* species and therefore, detection of GM can possibly be used as a biomarker for the diagnosis of IA.

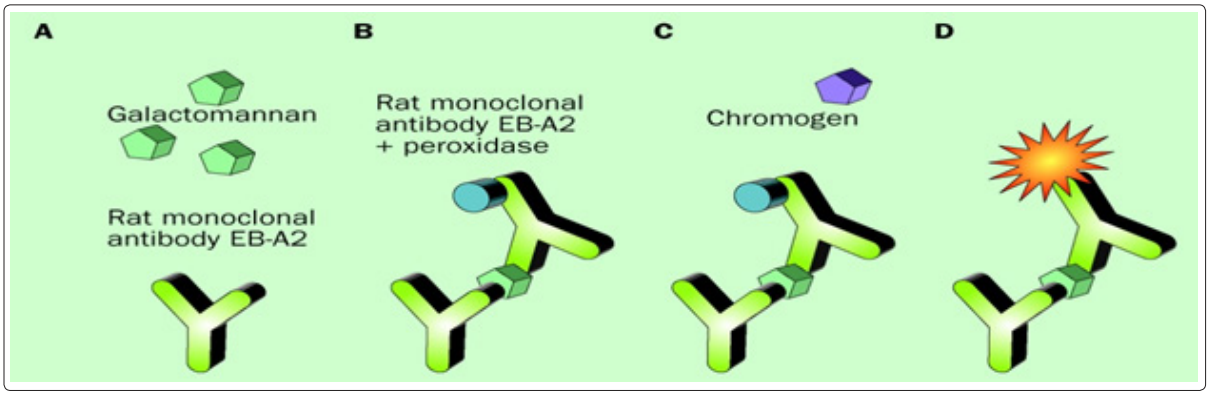

Figure 1: Principle of the Galactomannan enzyme immune assay in a graphical re-presentation. A serum ratio is calculated by dividing the optical density of the patient's serum sample by the mean optical density of the two threshold control samples that contain 1µg/L of galactomannan.

Comparative Studies

Cancer patients and patients undergoing allogenic and autologous organ transplantations are susceptible to develop invasive mycelial fungal infections with significant mortality and morbidity. Malignancies are known to cause immune deficiencies. Patients with cancer require specialized treatment which involves the use of chemotherapeutic drugs, radiotherapy and surgical procedures. These treatment modalities tend to seriously impair the immune mechanisms of patients. Recent advances in the treatment modalities, such as bone marrow and hematopoietic stem cell transplantation and the use of more intensive chemotherapeutic regimens, have added substantially to the number of patients who are able to survive the malignancy but with weakened immune systems. Vascular invasion by *Aspergillus* species is a common histopathological feature of invasive aspergillosis (IA) lung are the most common site of infection. High degree of immune suppression leads to extension of disease to mediastinal and chest-wall structures and hematogenous dissemination that can involve virtually any organ including the brain occurs in uncontrolled disease with poor outcome. Severely neutropenic patients with hematological malignancies and recipients of hematopoietic stem cell transplant (HSCT) are high risk individuals. The prevalence of IA is around 12%, in patients with hematological malignancies and allogeneic HSCT recipients but varies from centre to centre [12-14]. With the increase in number of immunocompromised patients and with availability of on newer and highly effective myelosuppressive agents, the incidence of IA has increased in recent times [15, 16]. Patients with advanced AIDS, inherited congenital immunodeficiency syndromes, chronic granulomatous diseases (CGD), long standing use of systemic corticosteroids, and patient's undergoing solid organ transplants with with T-cell immunosuppressants are other IA susceptible individuals [17, 18]. Critically ill intensive care patients are also susceptible to Invasive aspergillosis [19]. Nicolas C. et al. evaluated the performance of galactomannan and (1-3) β-D-glucan in 29 serum samples from patients with multiple myeloma and Waldenstrom's macroglobulinemia without invasive fungal disease to address issues of false positivity. Galactomannan and (1-3) β-D-glucan assays were not falsely elevated in any patient. (1-3) β-D-glucan assay results were uninterpretable in 24% of patients. Patients with IgG levels of >2,000 mg/dl had higher odds of uninterpretable (1-3) β-D-glucan results [20].

A study conducted by Marisa H. et all at, University of Arkansas for Medical Sciences, Little Rock, overall, 257 patients fulfilled criteria for proven or probable aspergillosis and were eligible for outcome evaluation. Correlation between GM (within ≤1 week before outcome) and define outcomes was excellent, with correlation coefficient of 0.8737 and 0.9123 for survival and global outcome, respectively. The correlation coefficient for all outcomes was comparable across age groups (paediatric and adult patients) and treatment modalities, including allogeneic transplantation. This strong correlation is also supported by extensive preclinical data and recent clinical reports. Marisa H. et al. concluded that serum GM is a good marker for invasive aspergillosis outcome [21]. A meta-analysis conducted by Christopher D. et all at University of Wisconsin Medical School, evaluated twenty-seven studies from 1966 to 28 February 2005 were included. Overall, the galactomannan assay had a sensitivity of 0.71 (95% confidence interval [CI], 0.68–0.74) and specificity of 0.89 (95% CI, 0.88–0.90) for proven cases of invasive aspergillosis. Subgroup analyses showed that the performance of the test differed by patient population and type of reference standard used with conclusion that the galactomannan assay has moderate accuracy

for diagnosis of invasive aspergillosis in immunocompromised patients. Extensive studies with attention to the impact of antifungal therapy, rigorous assessment of false-positive test results, and assessment of the utility of the test under nonsurveillance conditions are needed [22].The prospective single center study conducted by Wouter Meersseman et al. to investigate the role of GM in Broncho alveolar lavage (BAL) fluid as a tool for early diagnosis of invasive aspergillosis in the ICU. All patients with risk factors identified in the study were evaluated. BAL for culture and GM detection, serum GM levels, and computed tomography scan were obtained for all included patients with signs and symptoms of pneumonia. Patients were classified as having proven, probable, or possible invasive fungal infections as per EORTC MSG classification. There were 26 proven invasive aspergillosis cases. Using a cut-off index of 0.5, the sensitivity and specificity of GM detection in BAL fluid was 88 and 87%, respectively. The sensitivity of serum GM was only 42%. In 11 of 26 proven cases, BAL culture and serum GM remained negative, whereas GM in BAL was positive. Wouter Meersseman et al. concluded that GM detection in BAL fluid seems to be useful in establishing or excluding the diagnosis of invasive aspergillosis in the ICU setup [19]. Another study by M. Hong Nguyen, et all, at University of Florida College of Medicine, by use of bronchoalveolar lavage to detect galactomannan for diagnosis of pulmonary aspergillosis among non-immunocompromised hosts. Researchers assessed the role of Broncho alveolar lavage (BAL) in detecting galactomannan (GM) for diagnosing pulmonary aspergillosis in 73 non-immunocompromised patients with pulmonary infiltrates. Six patients had pulmonary aspergillosis, two each with acute invasive pulmonary aspergillosis, chronic necrotizing pulmonary aspergillosis, and aspergilloma. All six patients had a BAL GM level of >1.18. The sensitivity, specificity, and negative predictive value (NPV) for a BAL GM level of >1.0 were 100%, 88.1%, and 100%, respectively. Notably, the positive predictive value (PPV) was only 42.9%, likely reflecting the low prevalence of pulmonary aspergillosis among non-immunosuppressed patients. The combination of BAL microscopy and culture had a sensitivity and NPV similar to those of BAL GM detection but a higher specificity and PPV (92.5% and 54.6%, respectively). Moreover, a BAL GM test did not identify any cases that were not diagnosed by conventional methods like microscopy and culture. M. Hong Nguyen et al. came to the inference that there was no conclusive benefit of determining BAL GM levels in the diagnosis of pulmonary aspergillosis among non-immunocompromised hosts. Given the likelihood of false positive results, a BAL GM test should not be ordered routinely in non-immunocompromised hosts [23]. A multicentre prospective observational study by Brian T. Fisher et al. in children with anticipated prolonged neutropenia was performed. Serum specimens were collected twice weekly, and urine was collected once weekly during neutropenic periods. Operating characteristics were calculated using the GM EIA optical density index cut-offs of 0.5 and 1.0 for both serum and urine specimens. At least one serum or urine specimen was tested from 198 patients. Ten patients had one or more repeatedly positive serum specimens, while 37 patients had one or more repeatedly positive urine specimens. The specificity of serum and urine testing was 95% and 80%, respectively. Although the urine test resulted in a higher false positivity rate, it successfully identified the only case of probable invasive aspergillosis. Data suggest that the serum GM EIA does not provide frequent false positive results. Screening for galactomannan, or a related antigen in urine, needs to be further evaluated as it may be amenable to development of surveillance strategies [24].

Limitations of the Galactomannan EIA

As per the definition by EORTC/MSG in 2008, the use of galactomannan, as the sole microbiological criterion for diagnosis of IA is restricted to patients with hematologic malignancies or recipients of hematopoietic stem cell transplant (HSCT) who also have clinical and/or radiologic features consistent with invasive fungal infection. Infusion of solutions containing sodium gluconate such as Plasma Lyte, and administration of the antibacterial drugs like amoxicillin-clavulanic and Piperacillin-tazobactam may give false positive serum and BAL galactomannan assay. As the galactomannan antigen is also released into circulation by other fungi like, *Penicillium, Paecilomyces*, *Geotrichum*, and *Histoplasma capsulatum*, presence of co-existing infection with these fungi yields false positive results by serological cross-reactivity. Antifungal agents act by inhibiting fungal multiplication; this can potentially lower the sensitivity of galactomannan assay for the detection of *Aspergillus* species by lowering the residual fungal burden. The effect of antifungal agents in patients classified as proven or probable IA has been reported in three of the six studies in the hematological malignancy patient population and two of the six studies in the non-hematological patient population [19, 25-28]

A clinical specimen which is subjected to antifungal agents, especially BAL has shown decreased sensitivity for estimation of GM antigen, this concludes, the specimens for GM estimation should collected before administration of antifungal and antibacterial agents. Maertens et al. retrospective study showed that false negative results were noted in five patients, of whom three received mold active triazole antifungal prophylaxis, suggesting that mold active antifungals given prophylactically may negatively affect the sensitivity of the galactomannan assay in BAL fluid samples [25]. A study conducted by Becker *et al*. in 2003 showed that all five patients who had a second bronchoscopy performed and were on treatment with antifungal agents at the time of BAL collection were negative by the galactomannan assay. Administration of antifungal agents more than two days tended to be negative for galactomannan in BAL. Most of these patients were treated with conventional amphotericin B or the lipid formulation (Liposomal) of amphotericin B [27].

Diagnostic Difficulties in Aspergillosis

Invasive aspergillosis usually presents with non-specific clinical signs and symptoms making diagnosis more difficult. In addition, cancer and other immunocompromised patients fail to develop classical clinical presentation due to lack of adequate immune response. Depending on the site or system affected, early clinical manifestations include signs and symptoms of pneumonia, such as cough, sputum production, hemoptysis, pleuritic chest pain, or pleural friction rub, or signs and symptoms of sinusitis, such as nasal discharge, nasal bleeding, nasal eschar, pain, or orbital swelling. The clinical symptoms of invasive *Aspergillus* infection (IA) can mimic tuberculosis and other infections. Studies have shown that high-resolution computerized tomography (CT) might result in early diagnosis in high-risk IA patients but the distinctive lesions that are visible by radiologic methods such as the 'halo' and the 'air crescent' signs, are not specific for *Aspergillus* species. The 'halo' sign is not pathognomonic for aspergillosis but also seen in Mucormycosis and in non-fungal pulmonary diseases [29]. Additionally, these signs are not usually seen in cancer patients and patients undergoing solid organ transplantation with invasive aspergillosis. Lung transplant recipients frequently lack a characteristic radiographic appearance and present most often as focal areas of patchy consolidation. The

gold standards for diagnosis are histological examination and fungal culture of tissues. Isolation of the fungus by culture is both time consuming and insensitive and fails to aid in the detection of between 30–50% of invasive aspergillosis cases [30, 31]. Cultures for fungi and cyto-pathological examination of respiratory specimens often yield negative results and lack sensitivity for detecting the fungus in an early stage of the infection. Repeated microbiologic and histopathologic samplings are difficult to obtain in these critically ill patients. Additionally, biopsy specimens may be unproductive if the sample is collected at an advanced stage of the disease.

Conclusion

Early diagnosis of IA is of utmost importance to improve the prognostic outcome and challenging in patients with cancer and other hematological malignancies and patients undergoing organ transplantation. Specific diagnosis is rarely established before overwhelming fungal proliferation develops as the characteristic signs and symptoms are not seen always. The usefulness of radiological examination like high-resolution computed tomographic scan for diagnosis of IA is limited. Histopathological evaluation of tissue biopsies as a means of making a definite diagnosis is not without risk in the critically ill patient, and its sensitivity and specificity is unknown. Conventional diagnostic tests, such as culture and microcopy are time consuming and have only a sensitivity and specificity of around 50%. GM detection in serum and BAL fluid seems to be useful in establishing or excluding the diagnosis of IA in immunocompromised patients.

Conflict of Interest

Author declares no conflict of interests

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