DIVISION OF SPECIAL PATHOGEN AND TRANSPLANT PRODUCTS

Biomarker Qualification

Detection of Galactomannan in Broncho-Alveolar Lavage Fluids
by
Platelia *Aspergillus* Enzyme Immunoassay
(BioRad Laboratories, USA and Sanofi Diagnostics, France)

Sponsor: On behalf of Mycoses Study Group:

Peter G. Pappas, M.D.
Division of Infectious Diseases
University of Alabama, Birmingham
Birmingham, AL 35294

Paul E. Verweij, M.D.
Department of Medical Microbiology
University Hospital Nijmegen
The Netherlands

Reviewers:
Microbiology: Simone M. Shurland Ph.D.,
Medical Officer: Elizabeth O'Shaughnessy, M.D.
Statistics: Cheryl Dixon, Ph.D.

Concurrence:
Microbiology Team Leader: Shukal Bala, Ph.D.
Statistics Team Leader: Karen Higgins, Ph.D.

Division Director: Renata Albrecht, M.D.

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1. Executive Summary

The sponsor is seeking approval to include a positive galactomannan assay in bronchoalveolar lavage (BAL) fluids as the only microbiologic criteria, in conjunction with clinical and radiological findings, for the diagnosis of patients with probable invasive aspergillosis in predisposed neutropenic patients with hematologic cancer or recipients of hematopoietic stem cell transplants (HSCT). This is for the purpose of enrollment in clinical trials for treatment of invasive aspergillosis according to the *European Organization for Research and Treatment of Cancer and Mycoses Study Group* (EORTC/MSG) criteria. The EORTC/MSG criteria were first published in 2002 and were used to classify patients with proven, probable, or possible invasive fungal infection and were used in a majority of the studies included in this review (Table 1A and 1B). The EORTC criteria were recently updated and published by de Pauw *et al*. (2008).

In 2003, the US FDA Center for Devices and Radiological Health (CDRH) approved the Platelia *Aspergillus* enzyme immunoassay (EIA; BioRAD Laboratories) for testing of serum samples. The brochure recommends that results of the Platelia *Aspergillus* EIA be used in conjunction with other standardized methods such as clinical, radiological, culture and histopathological findings for diagnosis of invasive aspergillosis. A serum sample is considered positive at a cut-off index ≥ 0.5 based on testing of two aliquots of the same sample and another sample collected at a different time. However, the performance of the galactomannan assay in biological samples other than serum for the diagnosis of invasive pulmonary aspergillosis (IPA) has not been evaluated by CDRH.

The sponsor proposes a positive galactomannan test, Platelia *Aspergillus* EIA (*hereforth referred to as galactomannan assay)*, in BAL as a "stand-alone" microbiological criterion to define "probable" invasive pulmonary aspergillosis (IPA) based on the EORTC/MSG criteria (2008). A positive galactomannan test will be based on testing of a single BAL sample at a cut-off of ≥ 1.0.

In Vitro Studies

In vitro studies show that the Platelia *Aspergillus* EIA in BAL fluids, spiked with known moderate and high concentrations of *Aspergillus* galactomannan, has good precision for detection of galactomannan (CV% range 2-12%). The galactomannan assay in BAL fluids also demonstrate good reproducibility (96%) between runs at a cut-off of ≥1.0, between days when retested on the same aliquot as well as when retested after freezing. However, caution may be needed in interpreting a single positive test at indices between 0.5 and 0.9 as the reproducibility was low (75%). It will be appropriate to test two aliquots of the same BAL specimen.

In vitro studies demonstrate that false positives can occur due to cross reactivity with *Histoplasma capsulatum* and other fungi such as *Penicillium* sp., *Paeliocymyces* sp. *Alternaria* sp., *Geotrichum* sp. (for details see Table 7) and with Plasmalyte®, a solution used during bronchoscopy for collection of BAL fluid samples.

Animal Studies

In experimentally induced neutropenic mice, rats, and rabbits with invasive *Aspergillus* infection, the galactomannan index in BAL fluids ranged from 0.75 to 4.0. The overall sensitivity of the galactomannan was variable and ranged from 25% to 100%, depending on the day post-infection and the cut-off index. However, the overall specificity (100%) and PPV (100%) remained consistent in all animal studies.

Becker *et al*. (2000), study in rats showed reduced sensitivity of the galactomannan assay in BAL at specific times during the course of the infection. For example, cultures of BAL fluids were more sensitive than blood cultures or the galactomannan assay in both BAL fluids and serum samples during the initial stages of infection (up to day 3 post intra-tracheal inoculation). In contrast, at a later stage of infection (at day 5 or 7 post intra-tracheal inoculation), the galactomannan assay in BAL and serum was more sensitive than cultures (BAL fluids or blood). The results suggest that performance of the galactomannan assay in BAL fluids may be a good indicator of IPA during later stages of infection.

A study by Francesconi *et al*. (2006), in NZW rabbits showed that antifungal treatment reduced sensitivity of the galactomannan assay in BAL fluids from 100% to 92% in rabbits treated with triazoles (ravuconazole) and polyenes (amphotericin B) compared to untreated rabbits (sensitivity 100%), while specificity remained at 100%. The results suggest that treatment with antifungal agents such as triazoles and polyenes lower the residual fungal load in lung tissue and also diminish the sensitivity of the galactomannan assay in BAL fluids with no overall effect on the specificity.

Three of the animal model studies of invasive aspergillosis also assessed the optimal cut-off index of the galactomannan assay in BAL. Based on receiver operator characteristic (ROC) curves, the study by Francesconi *et al*. (2006) in rabbits showed that the optimal cut-off index value in BAL fluids was ≥ 0.75. Studies in mice (Ahmad *et al*., 2007) and rats (Khan *et al*., 2008) were based on two cut-off indices and showed that increasing the cut-off value from ≥ 0.5 to ≥ 1.5 decreased the sensitivity between 26% to 52% while retaining the specificity at100%. Overall, the studies in animal models of invasive aspergillosis suggest that a cut-off between 0.5 and 1.0 may be appropriate for testing of BAL fluids.

Clinical Studies

A total of twelve clinical studies were reviewed, of which six studies were from patients with hematological malignancies and six studies were from non-hematological populations (Table 1). The EORTC/MSG (2002) criteria were used for a majority of the studies with the exception of two studies [Verweij *et al*. (1995) and Maertens *et al.* (2009)]. Verweij *et al*. (1995) study was published before the EORTC/MSG criteria were defined, however, the host criteria and radiological criteria, i.e., presence of a pulmonary infiltrate used in Verweij *et al*., (1995) study were similar to the EORTC/MSG criteria. Maertens *et al.* (2009) study defined the proven, probable and possible invasive aspergillosis patients based on the 2008 EORTC/MSG classification scheme. Datasets for

the study by Husain *et al*., 2008, which incorporated the results from two solid organ transplant studies (Clancy *et al*., 2007 and Husain *et al*., 2007), were available for independent analysis.

In the twelve studies under review, patients with hematologic malignancies had a higher **prevalence** of the IA compared to the non-hematological populations in BAL fluids (Tables 1A and 1B). The overall **sensitivity** (range 61 % to 100%) of the galactomannan assay in BAL fluids yielded variable results in different patient populations across the different clinical studies (Tables 1A and 1B). Becker *et al.* (2003) and Penack *et al*. (2008) studies in patients with hematological malignancies showed the highest sensitivity (92% - 100%) of the galactomannan assay. The low sensitivity reported among the hematological patients in one study (Musher *et al*., 2004) was possibly due to the fact that >75% of patients received systemic antifungal therapy. Similar results were reported in the non-hematological patient population (Meersseman *et al*., 2008). Thus, it is recommended that a BAL fluid sample be collected, if possible, before anti-fungal treatment is administered.

The **sensitivity** of the galactomannan assay was higher when a bronchoscopy was performed following positive CT findings resulting in a better prediction for early diagnosis of IPA in untreated patients. Though the sensitivity of the galactomannan assay in BAL was higher than in serum, the correlation of the galactomannan assay results between BAL and serum samples has been discordant. However, none of the studies reported positive results in serum with negative results in BAL. The sensitivity of culture of respiratory specimens for *Aspergillus* species was approximately 30% in patients with hematological malignancies and hematopoietic stem cell transplants. Detection of galactomannan in BAL fluid was more sensitive than culture and results of the galactomannan assay in BAL fluids were available faster (>72 hours) than culture. Musher *et al*. (2004) reported that for case patients who were culture-positive, using the galactomannan assay in real time would have yielded an earlier diagnosis in 24 patients, thus indicating the usefulness of galactomannan testing for an earlier diagnosis of IPA. The available data support the use of a positive galactomannan result in BAL fluid as an indicator for the diagnosis of "probable" invasive aspergillosis.

The **specificity** of galactomannan assay in BAL fluids varied (range, 79% - 100%) across the studies at a cut-off ≥1.0, which may reflect the heterogeneity of the source population. Increasing the cut-off index values from ≥0.5 to ≥1.0 had minimal effect on sensitivity, however, the specificity of the galactomannan assay improved by 4% in one study (Tables 1A and 1B). The **negative predictive value** was consistent across studies (96% -100%), suggesting that a negative result correlated with absence of disease. The **positive predictive value** of the galactomannan assay in BAL fluids ranged from 54% to 100% among the patients with hematological malignancies. However, as shown in the prospective and retrospective cohort studies by Becker *et al.* (2003), the galactomannan assay in BAL was a better predictor of IPA when used in conjunction with other diagnostic criteria based on the patient's underlying disease (host factors), clinical and

radiological diagnostic imaging, and when confirmed by culture or histopathology as described in the EORTC/MSG criteria of 2002.

False positive results should be considered in patients treated with antibiotics produced by *Penicillium* sp. (ampicillin, amoxicillin, and piperacillin-tazobactam) or electrolyte solutions such as Plasmalyte® used in bronchoscopy. In addition, clinical specimens from non-hematologic patients colonized with *Aspergillus* species or infected with *Histoplasma capsulatum,* or other rare invasive fungal infections such as those caused by *Penicillium* species or *Paeliocymyces* species have been reported to cross-react with the galactomannan assay. Although cases of *Penicillium* sp. and *Paeliocymyces* sp. have not been reported in the studies in patients with hematologic malignancies, it is not clear whether attempts were made to systematically detect fungal species other than *Aspergillus* for patients enrolled in the study.

Overall, the results from the eleven studies suggest that galactomannan assay in BAL fluid is a useful diagnostic tool provided that the study population is well defined using the EORTC/MSG criteria to identify the population at risk for developing IPA. There was a higher prevalence of IPA in patients with hematological malignancies and HSCT than in patients without hematological malignancies. The studies suggests that the galactomannan assay in BAL would have greater applicability in patient populations at greater risk for IPA infection, i.e. the hematological malignancies population, especially in identifying "probable" invasive aspergillosis patients based on EORTC/MSG criteria. In addition, the specificity and positive predictive value of the galactomannan assay was higher in neutropenic patients with hematological malignancies and HSCT when compared to non-hematologic patients, for example, solid organ transplant patients. Based on the clinical and animal model studies of invasive aspergillosis, a cut-off index ≥ 1.0 appears to be optimal in order to maintain relatively high specificity (low false positivity) with minimal effect on the sensitivity of the galactomannan assay in BAL fluids. From a clinical trial perspective, the specificity and the positive predictive value of the galactomannan assay are important in that only patients with invasive aspergillosis are enrolled in the trial. Most studies retested the BAL fluid sample using an aliquot of the same sample, thus it is recommended that the performance of the assay in BAL fluids be considered positive based on testing of two aliquots of the same sample.

Recommendations

Based on the clinical and animal model studies of invasive aspergillosis the following is recommended:

- An optimal cut-off index ≥ 1.0 should be used in order to maintain relatively high specificity (low false positivity) with minimal effect on the sensitivity of the galactomannan assay in BAL fluids.
- The performance of the assay in BAL fluids should be considered positive based on testing of two aliquots of the same sample.
- The positive galactomannan test results should be used in conjunction with clinical and radiological findings in neutropenic patients for the enrollment of "probable" invasive aspergillosis patients in clinical trials.
- The use of this assay should be restricted to patient populations with hematological malignancies or recipients of hematopoietic stem cell transplant as there is insufficient data to comment on other patient populations at this time.
- Investigators should comply with the standard practice of obtaining a fungal smear and culture on all BAL fluid samples according to EORTC/MSG criteria. Results of fungal smears and fungal cultures must be clearly documented on the case report form for each BAL sample so that the presence of *Aspergillus* species or other rare fungal pathogens (such as *Penicillium, Paecilomyces, Geotrichum,* and *Histoplasma)* that may cross react with the galactomannan assay is documented (for details see Platelia *Aspergillus* EIA test brochure). All positive and negative results should be documented on case report forms and included in the datasets and all efforts should be made to either exclude such patients from enrollment or excluded from analysis.
- A BAL fluid sample should be collected, if possible, before anti-fungal treatment is initiated as exposure to mold-active antifungal agents appears to affect the sensitivity of the galactomannan assay in BAL. Patients receiving antibiotics, produced by *Penicillium* species, such as piperacillin-tazobactam and amoxicillin-clavulanate should be excluded and prior use of antibiotics and the timing should be documented.
- Use of Plasmalyte should be excluded for bronchoscopies in patients being considered for enrollment in clinical trials for the treatment of aspergillosis.

Table 1A: Summary of five clinical studies of the performance of the assay in BAL fluids among neutropenic patients with hematological malignancies

Reference, Country [a]	Host factor	Total	Proven IPA	Probable IPA	Possible IPA	Without IPA	Estimated Prevalence [b]	BAL GMI ≥ 0.5				BAL GMI ≥ 1.0			
								Sn [c]	Sp	PPV	NPV	Sn [c]	Sp	PPV	NPV
Becker *et al*., 2003 Netherlands	Hematological malignancies (retrospective)	29	1	6	2	18	28%					100%	100%	100%	100%
	Hematological malignancies (prospective)	53	3	9	12	23	34%					92%	100%	100%	96%
Musher *et al*., 2004 United States	HSCT	99	49			50		76%	94%			61%	98%		
Verweij *et al*., 1995 Netherlands	Hematological malignancies	19		7	2	10						71%	90%		
Desai *et al*., 2009 United States	Hematological malignancies	85	9		37	39	19%	78%	84%			78%	92%	54% [d]	97%
Penack *et al.,* 2008 Belgium	Hematological malignancies	45	17			28	12%*					100%	79%	74%	100%
Maertens *et al.,* 2009 Belgium	Hematological malignancies	128	31	27	29	41	45%	97%	80%	68%	98%	91%	88%	76%	96%
Overall Mean (Median)							**23% (23%)**	**83% (78%)**	**86% (84%)**			**85% (91%)**	**92% (92%)**	**81% (76%)**	**98% (97%)**

Note: GMI = galactomannan index; HSCT = hematopoietic stem cell transplant recipients; Sn = sensitivity; Sp = specificity; PPV= positive predictive value; NPV = negative predictive value; Shaded regions = Not Stated

[a] EORTC/MSG (2002) criteria were used with the exception of the study by Verweij *et al*., 1995 and Maertens *et al.,* 2009;

[b] Estimated Prevalence = (proven + probable IPA)/(proven+probable+without IPA);

[c] Sensitivity = proportion with proven + probable IPA based on the galactomannan assay;

[d] Includes possible IPA patients and patients without IPA

Table 1B: Summary of six clinical studies of the performance of the assay in BAL fluids among immuno-compromised neutropenic patients

Reference, Country	Host factor	Total	Proven IPA	Probable IPA	Possible IPA	Without IPA	Estimated Prevalence[a]	BAL GM ≥ 0.5				BAL GM ≥ 1.0			
								Sn[b]	Sp	PPV	NPV	Sn[b]	Sp	PPV	NPV
Shahid *et al*., 2008 India	Bronchogenic carcinoma	69	6	17	13	33	41%					100%	100%	100%	100%
Clancy *et al*., 2007 United States	Solid organ transplants	81	2	3		76	6%	100%	84%	29%	100%	100%	91%	42%	100%
Husain *et al*., 2007, United States	Lung transplants	116	2	4		110	5%	67%	88%	23%	98%	67%	93%	36%	98%
Husain *et al*., 2008, United States	Solid organ transplants	196	11			185		82%	87%			72%			
Meersseman *et al*., 2008[c] Belgium	Intensive care unit[e]	72	26			46	36%	88%	87%	79%	93%				
Nguyen *et al*., 2007 United States	Non-immunocompromised	73	6			67	8%	100%	78%	29%	100%	100%	88%	43%	100%
Overall Mean (Median)							**19% (8%)**	**87% (88%)**	**85% (87%)**	**40% (29%)**	**98% (99%)**	**88% (100%)**	**93% (92%)**	**55% (43%)**	**99% (100%)**

Note: GMI = galactomannan index; Sn = sensitivity; Sp = specificity; PPV= positive predictive value; NPV = negative predictive value;
Shaded regions = Not Stated
[a]Estimated Prevalence = (proven + probable IPA)/(proven+probable+without IPA);
[b]Sensitivity = proportion with proven + probable IPA based on the galactomannan assay;
[c]Patient population included hematological malignancies:42%; solid cancer, organ transplant, cirrhosis, and steroids: 58%.

2. Introduction and Background

The purpose of this review is to qualify the detection of the galactomannan in broncho-alveolar lavage fluid (BAL) by Platelia *Aspergillus* enzyme immunoassay (EIA; BioRAD Laboratories) as a biomarker for the diagnosis of patients suspected to have an invasive infection with *Aspergillus* species. The sponsor is seeking approval to include a positive result of Platelia *Aspergillus* EIA test in BAL fluid specimens as a stand alone microbiologic criterion for the diagnosis of invasive aspergillosis in conjunction with clinical and radiological findings in a predisposed host i.e., patients with hematologic malignancies and neutropenia or hematopoietic stem cell transplant (HSCT) recipients. This is for the purpose of enrollment in clinical trials of antifungal drugs according to European Organization for Research and Treatment of Cancer and Mycoses Study Group (EORTC/MSG) criteria.

In 2003, the Center for Devices and Radiological Health, Food and Drug Administration (CDRH, FDA) approved the serum-based Platelia *Aspergillus* EIA (BioRad Laboratories) for use in the United States in conjunction with other diagnostic procedures such as clinical and radiological findings, culture and histo-pathological evaluation. The product brochure further specifies that a positive result should be based on testing of two serum samples (two aliquots of one sample and if positive another serum sample should be collected and tested) at a galactomannan cut-off index ≥ 0.5. Although the Platelia *Aspergillus* EIA has been validated for serum, galactomannan can be detected in several other body fluids including urine, cerebrospinal fluid, and bronchoalveolar lavage (BAL) (Klont *et al*., 2004). However, the performance and optimal interpretive cut-off index values of the Platelia *Aspergillus* EIA in biological samples other than serum for the diagnosis of IPA has not been validated.

In this submission, the sponsor proposes that a positive galactomannan result will be based on testing of one BAL fluid sample at a galactomannan index of ≥ 1.0. The use of assay as a biomarker for monitoring of disease and response to anti-fungal therapy in clinical trials is not the focus of this review.

The sponsor provided 23 published studies of galactomannan assay used in the testing of BAL fluid specimens. In addition, the sponsor provided a dataset from the study by Husain *et al*. (2008) which incorporated the results from two solid organ transplant studies (Clancy *et al*., 2007 and Husain *et al*., 2007), for an independent analysis by FDA. An independent search of the PubMed database using search terms "*Aspergillus",* "aspergillosis", "Platelia", "galactomannan" "animal studies", "bronchoalveolar lavage" and "BAL" and combinations of these terms were used to identify 20 additional relevant publications. Clinical studies were included if investigators used the EORTC/MSG criteria for diagnosis of invasive aspergillosis (IPA), provided data on sensitivity, specificity, and predictive values, included a hematological patient population or populations that overlapped, and studies that provided data on BAL specimens. One of the studies (Verweij *et al*., 1995), was published before the EORTC/MSG criteria were defined, however, the host criteria and radiological criteria, i.e. presence of a pulmonary infiltrate used in the study were similar to the EORTC/MSG criteria. Studies that assessed the performance of the galactomannan assay other than the Platelia *Aspergillus*

EIA were excluded. Twelve clinical studies fulfilled the criteria and were further analyzed. The review focuses on the following issues:

- Assessment of the use of the Platelia *Aspergillus* EIA in the detection of galactomannan in BAL fluid from different patient populations and correlation with IPA.
- Assessment of the performance characteristics including the sensitivity, specificity, predictive values, and cross-reactivity for testing of BAL fluid specimens.
- Evaluation of an optimal cut-off value in BAL specimens
- Assessment of the usefulness of detection of galactomannan by the Platelia *Aspergillus* EIA as a stand-alone microbiological test in patients with hematological malignancies and HSCT recipients, to classify patients as "probable" invasive aspergillosis based on the EORTC/MSG criteria.

2.1. Invasive Aspergillosis

2.1.1. Disease

Invasive aspergillosis is a life-threatening mold infection in certain populations of immunocompromised hosts and is associated with an unacceptably high mortality rate. The lung is the most common site of infection and vascular invasion by *Aspergillus* species is a common histopathological feature of invasive pulmonary aspergillosis (IPA). Poorly controlled infection may lead to extension to mediastinal and chest-wall structures and hematogenous dissemination that can involve virtually any organ including the brain. Neutropenic patients with hematological malignancies and recipients of hematopoietic stem cell transplant (HSCT) are particularly at risk. In patients with hematological malignancies and allogeneic HSCT recipients, the prevalence of IPA is generally 12-18%, though the prevalence varies between centers (Penack *et al*., 2008; Slobbe *et al*., 2008; Lefflang *et al*., 2008). The incidence of IPA has increased in parallel with the increase in immunosuppressed patients on new and effective myelosuppressive treatments (Latge, 1999; Oren and Goldstein, 2002; Seyfarth *et al*., 2001). Other populations at risk include solid organ transplants, advanced AIDS, inherited immunodeficienies such as chronic granulomatous disease (CGD), chronic steroid use, and treatment with T-cell immunosuppressants, (Husain *et al.,* 2008, Antinori *et al.,* 2009; Gupta *et al.,* 2009; Singh *et al.,* 2006). Invasive aspergillosis is increasingly observed in non-classic settings such as critically ill intensive care patients (Meersseman *et al.,* 2008).

2.1.2. Diagnosis

Diagnosis of IPA remains challenging because clinical signs and symptoms and radiologic presentations are not specific and fungal cultures by standard techniques are insensitive (~40%). Invasive aspergillosis has an unacceptably high mortality rate ranging from 30% -50% (Klont *et al*., 2004), therefore a laboratory test that provides faster results and is more specific and sensitive than culture in detecting *Aspergillus* species in clinical specimens would facilitate earlier diagnosis and treatment options, and improve clinical outcomes. Early diagnosis and treatment of IPA has been shown to improve survival (von Eiff *et al*., 1995). It is widely recognized that the diagnosis of IPA

is difficult largely due to the insensitivity of culture and histopathology which are considered to be the "gold standard" laboratory tests for evidence of invasive disease. Therefore, a test with a high negative predictive value would help to reduce the use of some potentially toxic antifungal drugs (von Eiff *et al*., 1995). A test with a high positive predictive value (high pre-test probability) and high specificity would be helpful for the diagnosis of IA in clinical trials to ensure that patients who actually have the disease are enrolled.

2.1.3. EORTC/MSG Criteria for Invasive Fungal Diseases

In 2002, the EORTC/MSG consensus group published standardized definitions of invasive fungal disease to strengthen the consistency and reproducibility of clinical studies (Ascioglu *et al*., 2002) [Table 2]. According to the EORTC/MSG criteria (Tables 3 and 4), which is based upon a multi-disciplinary evidence approach, patients can be classified as "proven", "probable" or "possible" IPA.

The category of **proven invasive fungal disease** (IFD) requires demonstration of fungal hyphal elements in tissue of a normally sterile site with evidence of tissue damage. **Proven IA** requires culture and identification of *Aspergillus spp.* from a tissue biopsy or fine needle tissue aspirate. However, culture of *Aspergillus spp.* from blood cultures invariably represents contamination. The EORTC/MSG considered positive histopathology or fungal culture from these sterile specimens as the gold standard for diagnosis of IPA.

The category of **probable IFD** requires a host factor, clinical features and mycological evidence to be present. The committee supported that mycological evidence was necessary for the category of probable IFD, however may not be sufficient for the definitive diagnosis of IFD. Specimens obtained from adjacent normal sites or sites normally colonized with resident commensal flora (e.g. sputum, BAL fluid or sinus aspirate) that showed the presence of hyphal elements are considered acceptable mycological evidence. A positive galactomannan result by Platelia *Aspergillus* EIA in BAL, CSF or ≥ two serum samples was listed as acceptable mycological evidence of invasive aspergillosis though its use in certain populations and the type of indirect tests used were not specified.

The committee supported that mycological evidence was not necessary for the category of "**possible"** IFD. If mycological evidence is used, the specimens used in the probable IPA were permitted. However, it was stated that this classification was included on the premise that these patients would not be included in the clinical trials.

Table 2. Criteria for the diagnosis of invasive aspergillosis based on 2002 EORTC/MSG definitions

Host factors

- Neutropenia (<500 neutrophils/mm^3 for more than 10 days
- Persistent fever for 196 h refractory to appropriate broad-spectrum antibacterial treatment in high-risk patients
- Body temperature either >38°C or <36°C and any of the following predisposing conditions:
  - Prolonged neutropenia (110 days) in previous 60 days,
  - recent or current use of significant immunosuppressive agents in previous 30 days,
  - proven or probable invasive fungal infection during previous episode of neutropenia, or
  - coexistence of symptomatic AIDS
- Signs and symptoms indicating graft-versus-host disease, particularly severe (grade ≥2) or chronic extensive disease
- Prolonged (>3 weeks) use of corticosteroids in previous 60 days

Clinical criteria

- Must be related to site of microbiological criteria and temporally related to current episode
- Lower respiratory tract fungal disease
  - Major
    - Any of the following new infiltrates on CT imaging: halo sign, air-crescent sign, or cavity within area
  - Minor
    - Symptoms of lower respiratory tract infection (cough, chest pain, hemoptysis, dyspnea);
    - Physical finding of pleural rub;
    - any new infiltrate not fulfilling major criterion;
    - pleural effusion
- Sinonasal infection
  - Major
    - Suggestive radiological evidence of invasive infection in sinuses (i.e., erosion of sinus walls or extension of infection to neighboring structures, extensive skull base destruction)
  - Minor
    - Upper respiratory symptoms (e.g., nasal discharge, stuffiness); nose ulceration or eschar of nasal mucosa or epistaxis; periorbital swelling; maxillary tenderness; black necrotic lesions or perforation of hard palate
- CNS infection,
  - Major
    - Radiological evidence suggesting CNS infection (e.g., mastoiditis or other parameningeal foci, extradural empyema, intraparenchymal brain or spinal cord mass lesion)
  - Minor
    - Focal neurological symptoms and signs (including focal seizures, hemiparesis, and cranial nerve palsies); mental changes; meningeal irritation findings; abnormalities in CSF biochemistry and cell count (provided that CSF is negative for other pathogens by culture or microscopy and negative for malignant cells)

Microbiologic criteria

- Positive result of culture for mold (including *Aspergillus, Fusarium*, or *Scedosporium* species or *Zygomycetes*) or *Cryptococcus neoformans* or an endemic fungal pathogen from sputum or bronchoalveolar lavage fluid samples
- Positive result of culture or findings of cytologic/direct microscopic evaluation for mold from sinus aspirate specimen
- Positive result for Aspergillus antigen in specimens of bronchoalveolar lavage fluid, CSF, or ≥2blood samples

Adapted from Ascioglu *et al*., Clin Infect Dis 2002; 34:7 - 14

Table 3: Differentiation between the 2002 and 2008 EORTC/MSG Criteria for Invasive Aspergillosis

Class	Diagnostic criteria 2008	Diagnostic criteria 2002
Proven	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 major (or 2 minor) clinical factors PLUS one mycological factor (cytology or direct microscopy, culture or galactomannan detection)
Possible	Presence of a host factor and 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)

Adapted from De Pauw B. *et al*. (Clin Infect Dis 2008; 46:1813-1821), Ascioglu *et al*., (Clin. Inf. Dis. 2002) and microbiology review by Dr. Berkeley on serum galactomannan

Table 4: Criteria for Invasive Aspergillosis based on recommendations from the EORTC and Mycoses Study Groups as defined by (Ascioglu *et al*., 2002)

Criteria	Proven	Probable	Possible
Host factors	Not required	At least 1 Host factor	At Least 1 Host Factor
Clinical Factors	Not required	One Major **OR** Two Minor	One major **OR** Two Minor
Mycological Factors	Histopathological or cytopathologic evidence of lung tissue invasion or damage demonstrating hyphae in specimens from needle aspiration, biopsy or autopsy **OR** Positive culture for *Aspergillus* species obtained by sterile procedure from normally sterile site	Cytologic or direct microscopy finding of hyphae from sinus aspirate specimen **OR** Positive culture result of *Aspergillus* species cultivated from sputum or BAL specimens **OR** Positive result for *Aspergillus* antigen from BAL, CSF or > 2 blood specimens*	**OR** Cytologic or direct microscopy finding of hyphae from sinus aspirate specimen **OR** Positive culture result of *Aspergillus* species cultivated from sputum or BAL specimens **OR** Positive result for *Aspergillus* antigen from BAL, CSF or > 2 blood specimens*

Note: Adapted from Ascioglu *et al*., Clin. Inf. Dis. 2002; CT = computed tomography
Major factors = CT evaluation "halo" OR "air-crescent" signs; **Minor factors** = lower respiratory tract infection (shortness of breath, cough, pleuritic chest pain, haemoptysis or dyspnoea), a physical finding of pleural rub and new pulmonary infiltrates not fulfilling major criterion, pleural effusion; * PCR and β-D-glucan test or Galactomannan assay

In 2008, the EORTC/MSG group (de Pauw *et al*., 2008) modified the criteria such that it reflected advances in diagnostic technology and defining patient populations for clinical

and epidemiological research. At the time of publication of the criteria, the Platelia *Aspergillus* galactomannan EIA was the only indirect test for testing of serum samples to be used mycological evidence of invasive aspergillosis in conjunction with clinical and radiological findings in a predisposed host. Like the 2002 criteria, the classification scheme was based on a combination of host factors, clinical or radiological features and mycological evidence (Tables 5 and 6).

Table 5. Criteria for the diagnosis of invasive aspergillosis based on 2008 EORTC/MSG definitions

Host factors

- Recent history of neutropenia (<500 neutrophils/mm^3 for more than 10 days) temporally related to the onset of fungal disease
- Receipt of an allogeneic stem cell transplant
- Prolonged use of corticosteroids (excluding among patients with allergic bronchopulmonary aspergillosis) at a mean minimum dose of 0.3 mg/kg/day of prednisone equivalent for 13 weeks
- Treatment with other recognized T cell immunosuppressants, such as cyclosporine, TNF-α blockers, specific monoclonal antibodies (such as alemtuzumab), or nucleoside analogues during the past 90 days
- Inherited severe immunodeficiency (such as chronic granulomatous disease or severe combined immunodeficiency)

Clinical criteria

- Lower respiratory tract fungal disease
  - The presence of 1 of the following 3 signs on CT:
    - Dense, well-circumscribed lesions(s) with or without a halo sign
    - Air-crescent sign
    - Cavity
  - Tracheobronchitis
    - Tracheobronchial ulceration, nodule, pseudomembrane, plaque, or eschar seen on bronchoscopic analysis
- Sinonasal infection
  - Imaging showing sinusitis plus at least 1 of the following 3 signs:
    - Acute localized pain (including pain radiating to the eye)
    - Nasal ulcer with black eschar
    - Extension from the paranasal sinus across bony barriers, including into the orbit
- CNS infection, 1 of the following 2 signs:
  - Focal lesions on imaging
  - Meningeal enhancement on MRI or CT

Mycological/Microbiologic criteria

- Direct test (cytology, direct microscopy, or culture)
  - Mold in sputum, bronchoalveolar lavage fluid, bronchial brush, or sinus aspirate samples, indicated by 1 of the following:
  - Presence of fungal elements indicating a mold
  - Recovery by culture of a mold (e.g., *Aspergillus*, *Fusariu*m, *Zygomycetes*, or *Scedosporium* species)
- Indirect tests (detection of antigen or cell-wall constituents)
  - Galactomannan antigen detected in plasma, serum, bronchoalveolar lavage fluid, or CSF

Adapted from De Pauw B. *et al*. Clin Infect Dis 2008; 46:1813-1821

Table 6: Criteria for the diagnosis of invasive aspergillosis based on 2008 EORTC/MSG definitions

Criteria	Proven	Probable	Possible
Host factors	Not required	At least 1 Host factor	At Least 1 Host Factor
Clinical Factors	Not required	Presence of 1 of the following 3 signs on CT • dense , well circumscribed with or without a halo sign • air crescent sign • cavity	Presence of 1 of the following 3 signs on CT • dense , well circumscribed with or without a halo sign • air crescent sign • cavity
Mycological Factors	Histopathological or cytopathologic evidence of lung tissue invasion or damage demonstrating hyphae in specimens from needle aspiration, biopsy or autopsy **OR** Positive culture for *Aspergillus* species obtained by sterile procedure from normally sterile site	Cytologic or direct microscopy finding of hyphae from sinus aspirate specimen **OR** Positive culture result of *Aspergillus* species cultivated from sputum or BAL specimens **OR** **Galactomannan antigen detected in plasma, serum, BAL or CSF**	**None**

Note: Adapted from DePauw *et al.,* Clin. Inf. Dis. 2008; CT = computed tomography; CSF = cerebrospinal fluid

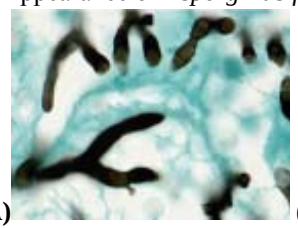
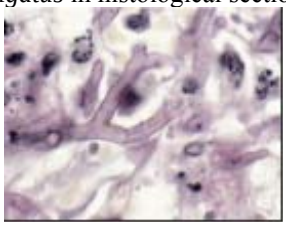
The studies in this review complied with the 2002 EORTC/MSG Ascioglu *et al*. (2002) as outlined in the Table 2 and 4, unless stated otherwise. Deviations from the 2002 EORTC/MSG guidelines and current understanding of the application of the galactomannan assay in BAL in patient populations as it applies to the revised definitions in 2008 by EORTC/MSG (de Pauw *et al*., 2008) will be discussed. In addition, the use of the galactomannan assay in BAL will be evaluated as a "stand-alone" mycological criterion in classifying patients with probable IPA.

2.1.4. Microbiology of Aspergillus species.

The genus *Aspergillus* is a ubiquitous environmental mold, found most often in the soil, water, decaying vegetation, or organic debris. *Aspergillus* species are highly aerobic, found in oxygen-rich environments and commonly grow on the surface of substrates. Approximately 200 species of *Aspergillus* have been described and less than 30 species are reported as pathogenic for humans. Some common pathogenic *Aspergillus* species that produce infection in humans include *A. fumigatus, A. flavus, A. niger, A. terreus, A. nidulans,* and *A. glaucans*. *A. fumigatus* is the most common species causing invasive aspergillosis in humans (Bodey and Vartivarian, 1989; Latge, 1999). *A. fumigatus* sporulates abundantly with each conidial head producing thousands of conidia. Until recent years, *Aspergillus* species were viewed as weak pathogens and inhalation of

conidia by an immunocompetent individual rarely has any adverse effect since the fungus is generally eliminated by innate immune mechanisms (Latge, 1999). The conidia released into the atmosphere have a diameter small enough (2-3 μm) to reach the lung alveoli. Environmental surveys indicate that humans can inhale at least several hundred *A. fumigatus* conidia per day. Lung tissues containing hyphal elements of *A. fumigatus* are shown in Figure 1.

Figure 1: Appearance of *Aspergillus fumigatus* in histological sections and culture specimens

(A)  **(B)**  **(C)** 

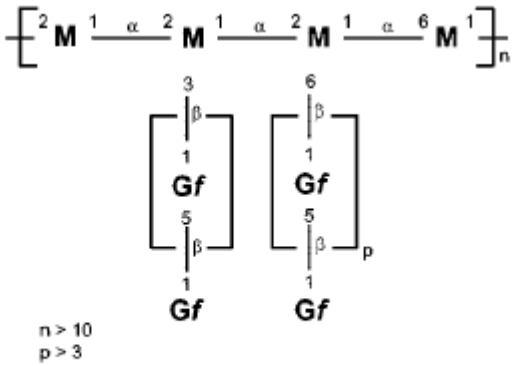
(A) Gomori methananmine silver (GMS) stain demonstrating appearance of hyphae in section of rabbit lung in experimental IPA (B) Similar section of rabbit lung using Periodic acid Schiff (PAS) stain highlighting background histological detail and hyphal morphology (Hope *et al*., 2005) C) *A. fumigatus* culture on Sabouraud agar

Galactomannan Structure

Aspergillus species are known to release exo-antigens, during growth, *in vitro* and *in vivo.* Low levels of galactomannan antigens present in the body fluids of patients with invasive IPA have been reported in serum, urine, BAL, and cerebrospinal fluid. Galactomannan is heat-stable and water soluble, cell wall polysaccharide that is released by *Aspergillus* species during fungal growth (Klont *et al*., 2004; Mennink-Kersten *et al*., 2004). When a fungal spore germinates it produces hyphae, which in turn grows by increasing in length through the addition of new material to the hyphal wall at or near the tip. During growth phase, galactomannan is incorporated into the fungal cell wall, but because of weakness at the tip, galactomannan is released as a result of the forward streaming of protoplasm. It is during the logarithmic phase of growth that the release of galactomannan antigens can be detected. Hope *et al*. (2007) reported a close temporal relationship between hyphal penetration of the endothelial layer and an increase in galactomannan levels.

The structure of galactomannan is composed of a linear core of mannose residues with $\alpha(1\rightarrow2)$- and $\alpha(1\rightarrow6)$-linked branching residues (Dalle *et al*., 2005; Stynen *et al*., 1992). This mannose core is non-immunogenic (Marr and Leisenring, 2005). The side chains are composed exclusively of $\beta(1\rightarrow5)$-galactofuranose or $\beta(1\rightarrow4)$-galactopyranose moieties at the C-6 and C-3 positions joined to the $\alpha(1\rightarrow2)$ - mannose units of the core (Figure 2). These side chains are antigenic and have an average degree of polymerization of four (Stynen *et al*., 1992).

Figure 2: Structure of galactomannan



Aspergillus fumigatus galactomannan

2.2. Platelia Galactomannan EIA.

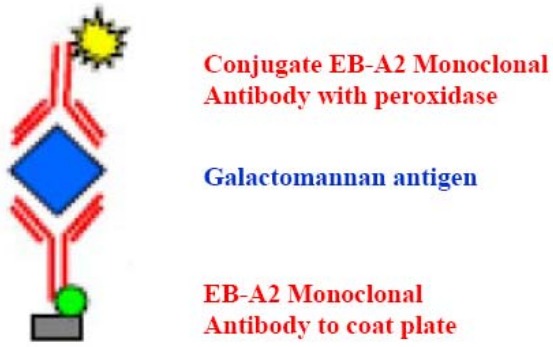
The galactomannan assay was developed by Stynen *et al*., (1995) using a rat monoclonal antibody, EB-A2, directed against the antigenic side chains (1→5)-β-D-galactofuranoside of the galactomannan molecule in *Aspergillus* species.

The principle of the EIA procedure is the binding of the rat EB-A2 monoclonal antibody to galactomannan antigen in the sample. Serum samples are heat-treated at 100º C for 3 to 4 minutes in the presence of EDTA in order to dissociate immune complexes and to precipitate serum proteins that could possibly interfere with the test. The treated serum sample is centrifuged at 10,000 g for 10 minutes. The supernatant is placed in a microtitration plate coated with EB-A2, the monoclonal anti-galactomannan antibody. A reaction mixture containing conjugate antibody linked with peroxidase is added to the antibody coated wells and incubated for 90 minutes at 37°C. If galactomannan is present, a monoclonal antibody / galactomannan / monoclonal antibody-peroxidase complex is formed (Figure 3). The wells are washed to remove unbound materials. A substrate chromogen solution containing tetramethylbenzidine is added to the plate for 30 minutes in a dark room at room temperature. Tetramethylbenzidine reacts with peroxidase enzymes such as horseradish peroxidase resulting in a color change. The enzyme reaction is stopped by the addition of sulfuric acid. The resulting color changes are read on a spectrophotometer at a wavelength at 450 nm (reference OD at 620/630 nm). The presence or absence of *Aspergillus* galactomannan antigen in the test sample is determined by calculation of an index for each patient specimen. The calculated index value of the specimen is the ratio between the optical densities of the specimen to the threshold control. The optical density of the threshold (cut-off) control is recommended by the manufacturer to be between 0.3 and 0.8. The ratio between the negative control and threshold (cut-off) control was recommended to be ≤ 0.5 and that of the positive control to the threshold control to be ≥ 2. A study by Verweij *et al.* (1998) reported that the Platelia kit positive control contains 5 ng/mL of galactomannan, and the threshold

(cut-off) control contains a galactomannan concentration of 1 ng/mL; no galactomannan was present in the negative control.

In 2003, the galactomannan assay was validated and approved by FDA for testing in serum specimens only. For this, testing of three serum samples is recommended; two aliquots of one sample and if positive another serum sample should be collected and tested. An index value ≥ 0.5 is considered to be positive for galactomannan antigen.

Figure 3: EIA monoclonal antibody complex structure



The product brochure clearly specifies that positive galactomannan results for serum samples should be considered for diagnosis in conjunction with other diagnostic procedures such as microbiological culture, histological examination of biopsy samples and radiological evidence to aid in the diagnosis of IPA. The sandwich EIA is commercially available by BioRad Laboratories (Redmond, WA or Marnes-la-Coquette, France) and Sanofi Diagnostics Laboratories (Pasteur, Marnes-la-Coquette, France). The galactomannan assay results are generally available within three hours in comparison to standard culture methods results that are available after 3-7 days, facilitating rapid diagnosis (Aquino *et al*., 2007). The assay is very sensitive, with a lower limit of detection in serum of 0.5 ng/mL. Based on the product package insert, the reported sensitivity and specificity in serum ranges from 50% - 92.6% and 94% - 99.6%, respectively, in patients with hematologic malignancy (Stynen *et al.*, 1992; Dalle *et al*., 2005).

One difficulty with the galactomannan assay is that there are a number of factors that can lead to a false positive reading. Gut colonization by fungi, presence of chemotherapy induced mucositis, bacterial antigens, food sources and cottons swabs have been shown to interfere with the galactomannan assay and produce false positive readings. Several studies have shown that several non-*Aspergillus* fungal organisms show strong positive results by the galactomannan assay. Cross-reactivity has been demonstrated using culture supernatants and patients sera at index values ≥ 0.5 (Table 7 and Package Insert, Platelia *Aspergillus* EIA).

Table 7: Non-*Aspergillus* fungal organisms that cross react with the galactomannan EIA.

Fungus	GM Index ranges	Exoantigens tested in culture supernatants	Assay test in Serum	Reference #
Alternaria species	2.1	Yes	No	**Swanink *et al*., 1997
Blastomyces dermatitidis (mold form)	0.6 – 2.8	Yes	No	Cummings *et al*., 2007
Cladosporium species	0.5	Yes	No	**Swanink *et al*., 1997
Cryptococcus neoformans[*]	0.1 – 4	Yes	Yes	Dalle *et al*., 2005; De Jesus et al., 2007
Geotrichum capitum	1.2 - 5	Yes	Yes	Giacchino *et al*., 2006
Fusarium solani	0.6	Yes	No	**Swanink *et al*., 1997
Neosartorya pseudofischeri	NS	No	No	Jarv *et al*., 2004
Nigrospora oryzae	1.79 – 1.86	Yes	No	Cummings *et al*., 2007
Paecilomyces lilacinus	NG	Yes	No	Cummings *et al*., 2007
Paecilomyces variotii	5.7	Yes	No	**Swanink *et al*., 1997
Penicillium chrysogenum	6.9	Yes	No	**Swanink *et al*., 1997; Cummings *et al*., 2007
Penicillium digitatum	5.8	Yes	No	**Swanink *et al*., 1997
Rhodotorula rubra	0.7	Yes	No	**Swanink *et al*., 1997
Trichophyton rubrum	0.5	Yes	No	**Swanink *et al*., 1997
Trichothecium roseum	NG	Yes	No	Cummings *et al*., 2007

Note: NS = Galactomannan index not specified
** Platelia EIA BioRad Laboratories Package Insert;

Several authors have reported that therapeutic agents derived from *Penicillium* species cross react in the galactomannan assay. These agents include the semisynthetic penicillins such as piperacillin-tazobactam (Aubry *et al*., 2006; Walsh *et al*., 2004; *Package Insert,* Platelia *Aspergillus* EIA). More recent studies have shown treatment with other beta-lactam antibiotics (amoxicillin-clavulanic acid, amoxicillin, ampicillin or phenoxymethylpenicillin) also cross-react with the galactomannan assay in serum (Mattei *et al*., 2004; Bart-Delabesse *et al*., 2005; for more details see Microbiology review of serum galactomannan by Dr. Lynette Berkeley, dated 10/29/2009). Galactomannan index values ≥ 0.5 have been reported in patients during a treatment regimen with these antibiotics. However, the index values were reduced after discontinuation of antibacterial treatment.

The PATH (Prospective Antifungal Therapy) alliance registry (Neofytus D. *et al*., 2009) reported 250 episodes of invasive fungal infections (IFI) occurring in 234 patients who had received a HSCT. *Aspergillus* species accounted for almost 60% of all fungal infections, followed by *Candida* (almost 25%), Zygomyces (7.2%), and other molds (almost 7%). *Fusarium spp*. accounted for about a third of the infections in the "other molds" category. Endemic fungal infections were extremely rare; one patient had histoplasmosis.

In a report of 391 patients with hematologic malignancies who had proven or probable invasive mold infection (Table 8) (Pagano *et al*., 2001), *Aspergillus* species accounted for 75.7%, Zygomycetes for 11.5%, Hyalohyphomycetes in 10.2%, and other molds

(*Fusarium, Scedosporium, Scopulariopsis, Acremonium* species) in 2.3%. In another report of 121 patients who had received HSCT and experienced an invasive fungal infection, 25% were due to *Candida*, and 71% were due to *Aspergillus* species (Pagano *et al.,* 2007).

Table 8: Fungal infections in patients with hematologic malignancy or recipients of HSCT

Fungal Pathogen	**Incidence of Invasive Fungal Disease**
Yeast	**25%**
Candida	25%
Other yeast	< 1%
Mold	**75%**
Aspergillus species	60%
Zygomyces	7-8%
Fusarium/Scedosporium	3-4%
Other	2-5%

Source: Review of Serum Galactomannan, H. Shamsuddin, M.D.

These reports indicate that the fungi that cross react with *Aspergillus* in the galactomannan assay are clinically uncommon (less than 2% of invasive fungal infections, and less than 5% of all mold infections). Therefore, for the purpose of clinical trial enrollment for IA treatment development, the impact of the presence of these fungi is expected to be minimal.

3. Performance of the Platelia *Aspergillus* EIA

3.1. *In Vitro* Studies

3.1.1. Precision and Reproducibility

Precision and reproducibility was evaluated in two studies (for details *see Appendix-I*). In a study by Husain *et al*. (2008), galactomannan testing of BAL and serum samples showed comparable results (Table 9). There was low variability of the galactomannan assay (CV% range 2 - 12%) in testing of BAL samples compared to serum between runs, between days, and between duplicate aliquots of the same specimen. In another study (unpublished study report by the MSG group), low variability at moderate and high concentrations of galactomannan, between runs and days was reported (Table 10), thus suggesting good precision for testing of BAL fluid samples (for details see *Appendix-I*).

Table 9: Galactomannan detection in BAL and serum reconstituted using the kit positive and threshold controls

Controls	**Serum**		**BAL**	
	mean ± SD	**CV%**	**mean ± SD**	**CV%**
Positive				
Day 1	2.93 ± 0.35	12%	3.40 ± 0.20	6%
Day 2	3.23 ± 0.25	8%	3.61 ± 0.09	2%
Threshold				
Day 1	0.61 ± 0.12	19%	0.86 ± 0.05	6%
Day 2	0.59 ± 0.06	10%	0.91 ± 0.03	4%

Table 10: Comparison of the galactomannan indices at known concentrations of galactomannan in spiked serum and BAL samples between runs and between days.

Spiked specimen	Intra-assay (3 aliquots)** Mean ± SD (CV%)		Inter-assay (2 days)*** Mean ± SD (CV%)	
	Serum-GMI	BAL-GMI	Serum-GMI	BAL-GMI
High positive* (4.0 ng/mL)	2.01 ± 0.07 (3.3%)	4.39 ± 0.43 (9.8%)	2.05 ± 0.097 (4.7%)	4.50 ± 0.30 (6.8%)
Moderate positive* (2.0 ng/mL)	0.98 ± 0.85 (8.7%)	2.48 ± 0.31 (12.3%)	1.11 ± 0.16 (14.5%)	2.54 ± 0.26 (10.4%)
Negative* (0 ng/mL)	0.08 ± 0.03 (41.2%)	0.21 ± 0.03 (14.7%)	0.08 ± 0.03 (40.6%)	0.16 ± 0.06 (33.3%)

GMI = galactomannan index
* Spiked Samples
** Intra-assay = mean ± SD of same sample run at 3 different times;
***Inter-assay = mean ± SD of same sample run on 2 separate days

The studies by Husain *et al*. (2008) and an unpublished study report by the MSG group evaluated the galactomannan assay by retesting of positive clinical BAL specimens, using aliquots of the same sample. The galactomannan assay results were reproducible at indices ≥ 1.0 (96% - 100%), however, variability were greater at index values between 0.5 and 0.9 (75% - 93%), suggesting that caution may be needed in interpreting a single positive test at indices ≤ 0.9 (Table 11). Repeat testing performed on another aliquot of the same sample following storage at -20°C for up to two years was reproducible (80%) at index values ≥ 0.5 ($R^2 = 0.9051$).

Overall, the studies suggest that the assay for testing of BAL fluid samples was reproducible between runs, between days when retested on the same aliquot as well as when retested after freezing. However caution may be needed in interpreting a single positive test at indices between 0.5 and 0.9.

Table 11: Proportion of BAL and serum specimens positive upon retesting

Study	GMI range	Serum No./total (%)	BAL No./total (%)
Husain *et al*., 2008	0.5-0.9	NS	14/15 (93)
	≥ 1.0	NS	17/17 (100)
Unpublished report[2]	0.5-0.9	50/89 (56)[1]	57/76 (75)
	≥ 1.0	98/110(89)	80/83 (96)

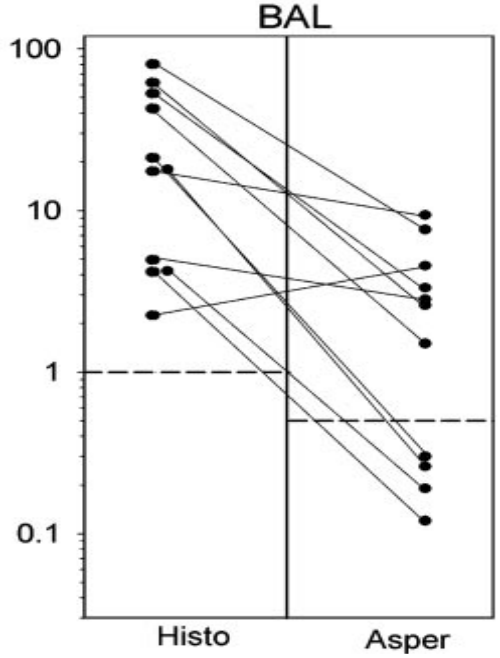
Note: GMI = galactomannan index; BAL = bronchoalveolar lavage; NS = not specified
[1]Based on testing of two aliquots of a sample and results must be reproducible to be reported as positive
[2]Study done in Miravista Laboratory (Dr. Joseph Wheat)

3.1.2 Cross-reactivity Studies

A study by Wheat *et al*. (2007), reported cross reactivity with the Platelia assay in BAL and serum specimens from patients with proven **histoplasmosis** (Figure 4) and from mice

infected with ***Histoplasma capsulatum*** yeast cells**.** The cross reactivity correlated with the level of positivity in the *Histoplasma* antigen EIA; BAL and serum specimens from patients with *Histoplasma* antigen levels of 40 units or more were positive in the galactomannan assay. Similar results were also observed in splenic tissues from mice experimentally infected with *H. capsulatum,* thus suggesting that false positives can occur with the assay in patients infected with *Histoplasma capsulatum* (for details see *Appendix-I*)*.*

Figure 4: Comparison of antigen levels in BAL fluids from 11 patients with histoplasmosis tested using the Platelia *Aspergillus* EIA and second-generation *Histoplasma* EIA (left) kits



Note: The vertical axis depicts antigen units for the Histoplasma EIA and Platelia Aspergillus EIA. The cut-offs for positivity are 1.0 unit for the second-generation *Histoplasma* EIA and 0.5 GMI for the Platelia *Aspergillus* EIA, as shown by broken horizontal lines. Results for the same specimens tested in both assays are connected by solid lines.

It is important to note that histoplasmosis is extremely rare in patients with hematological malignancies and HSCT (Neofytus *et al*., 2009); therefore cross reactivity with the galactomannan assay due to histoplasmosis in this population is not clinically relevant.

Plasmalyte solution used in the procedures performed for BAL washes during bronchoscopy have shown positive results in the galactomannan assay. The Plasmalyte solution includes the ingredient sodium gluconate. Sodium gluconate is formed by the fermentation process of *Aspergillus niger, Aspergillus flavus,* and *Penicillium* species. Hage *et al*. (2007), reported cross-reactivity in 19 patients that had Plasmalyte solution used in a bronchoscopy examination and reported index values ranging from 4.1 to 8.2 in the galactomannan assay. Testing of the different

lots of the plasmalyte solution showed index values ranging from 5.4 to 5.6. Thus use of plasmalyte for bronchoalveolar lavage should be excluded in clinical trials.

3.2. Animal Studies

Five animal studies evaluated the galactomannan assay for testing of BAL fluid from experimentally induced neutropenic animals at a known time of onset with *Aspergillus* infection (for details see *Appendix-II*). Four of the studies were conducted in rats and rabbits infected with *A. fumigatus* by intra-tracheal or intravenous route; one study was conducted in mice infected intravenously with *A. terreus* (Table 12). The overall specificity and PPV remained consistent in all animal studies, with a reported result of 100%, however, the overall sensitivity ranged from 60% to 100%.

Table 12: Summary of animal studies showing the BAL galactomannan sensitivity

Study (Year)	Animal Type	Route of infection	Inoculation size[a]	Cut-off index	N	GMI range	Sn[b]	Sp	PPV	NPV
A. fumigatus										
Becker *et al*., 2000	Rats	IT	2×10^4	≥ 1.0	23	≥ 1.0	60%	NS	NS	NS
Francesconi *et al*., 2006	Rabbits	IT	1×10^8	≥ 0.75	24	0.75 – 4.0	100%	100%	100%	100%
Khan *et al*., 2008	Rats	IV	1×10^8	≥ 0.5	30	0.82 – 2.32	77%	100%	100%	46%
De Jesus *et al*., 2007	Rabbits	IT	NS	≥ 0.5	NS	1.5 – 6.8	NS	NS	NS	NS
A. terreus										
Ahmad *et al*., 2007	Mice	IV	1×10^5	≥ 0.5	72	0.88 – 1.53	80%	100%	100%	52%

Note: N = Total number of animals tested; Sn = sensitivity, Sp = specificity, NPV = negative predictive value, PPV = positive predictive value; IT = intra-tracheal; IV = intravenous; NS = not stated;
[a]Results expressed as conidia concentration
[b]Overall sensitivity

Of the five studies, three studies followed the progression of the disease up to nine days post-infection and for two studies the time of evaluation was not specified. In the three studies that sampled BAL specimens during the course of the disease, the sensitivity of the galactomannan assay for testing of BAL varied from 25% to 100% (Table 13) depending on the day the BAL specimen was collected. Though, the inoculation size of *Aspergillus* species was similar across studies, there appears to be a reduced sensitivity in animals whose infection was initiated by intravenous inoculation of conidia compared to animals that were infected by the intra-tracheal route thereby suggesting that severity of disease may influence galactomannan levels. The authors also noted that the sensitivity and mean concentrations of circulating galactomannan in BAL appeared to increase over time in animals that were infected with *A. fumigatus* and *A. terreus.* The index values ranged from 0.82 to 2.32 over the nine days post infection, suggesting that the galactomannan positivity in BAL correlates with tissue fungal burden.

Table 13: Summary of animal studies showing sensitivity of the galactomannan assay in BAL over 9-day infection period

	Animal Type	Route of infection	Inoculation size[a]	Cut-off index	GMI range	Sensitivity of BAL GM Assay				
						Day1 %(n)	Day 3 %(n)	Day 5 %(n)	Day 7 %(n)	Day 9 %(n)
A. fumigatus										
Becker *et al*., 2000	Rats	IT	2 x 10^4	≥ 1.0	≥ 1.0	25% (8)	40% (5)	100% (5)	100% (5)	ND
Khan *et al*., 2008	Rats	IV	1 x 10^8	≥ 0.5	0.82 – 2.32	100% (6)	66% (6)	83% (6)	100% (6)	33% (6)
A. terreus										
Ahmad *et al*., 2007	Mice	IV	1 x 10^5	≥ 0.5	0.88 – 1.53	83% (12)	83% (12)	58% (12)	83% (12)	92% (12)

Note: IT = intra-tracheal; IV = intravenous; NS = not stated; [a]Results expressed as conidia concentration

Becker *et al*. (2000), study accounted for the reduced sensitivity of the assay in BAL to specific times during the course of the infection. The study in experimentally induced neutropenic rats infected with *Aspergillus fumigatus* by the intra-tracheal route showed that cultures of BAL fluid were more sensitive than detection of galactomannan by the Platelia assay in BAL or serum up to day 3 or during the initial stages of infection (Table 14). In contrast by days 5 and day 7 or later stages of infection, detection of galactomannan by the galactomannan assay in BAL and serum was more sensitive than cultures. Blood cultures were not positive at any of the time points tested. There appeared to be no specific trend in the serum galactomannan positivity relative to BAL galactomannan positivity over a nine day period. Overall, these findings demonstrate that galactomannan concentration in BAL and serum are associated with the later stages of the disease or disease severity. Thus the results suggest that detection of galactomannan in BAL or serum specimens by the galactomannan assay may be a good diagnostic indicator for diagnosis of IPA.

Table 14: PCR, EIA, and fungal culture analyses of BAL fluid and blood of rats with IPA after dissection.

Day	No. of animals	BAL fluid				Blood			
		PCR (no. of rats positive)	ELISA (no. of rats positive)	Median concn (ng/ml) of GM (range)	Culture (no. of rats positive [mean CFU/ml])	PCR (no. of rats positive)	ELISA (no. of rats positive)	Median concn (ng/ml) of GM (range)	Culture (no. of rats positive [mean CFU/ml])
1	8	2	2	<1 (<1–2.7)	8 (4)	0	0	<1 (<1)	0 (0)
3	5	1	2	<1 (<1–11)	3 (1)	1	3	4.4 (<1–6.2)	0 (0)
5	5	2	5	11.6 (5.4–157)	0 (0)	1	4	10.2 (<1–26.2)	0 (0)
7	5	1	5	8.8 (3.9–114)	1 (0)	2	5	19.2 (7.6–48)	0 (0)

[a] The in-house PCR method was used.

A study by Francesconi *et al*. (2006), showed that antifungal treatment can also lead to variation in the sensitivity of the galactomannan assay in BAL, by reduction in the fungal load in experimentally induced neutropenic rabbits infected with *Aspergillus fumigatus*, when treated for 12 days with antifungal agents. The galactomannan assay had a sensitivity and specificity of 100% using at cut-off of 0.75. In analyses of antifungal treatment, an overall decrease in sensitivity of the assay in BAL to 92% was reported with the specificity remaining the same at 100% compared to untreated rabbits. The authors also noted that the type of antifungal therapy administered prior to the time of

BAL evaluation had a significant effect on the sensitivity of the assay. The type of antifungal treatment differed in their effects on galactomannan levels. For example a triazole (ravuconazole), and polyene, (amphotericin B deoxycholate) caused a decline in galactomannan levels in BAL and showed an overall reduction in sensitivity of the galactomannan assay in BAL of 33% to 50% compared to untreated rabbits. Persistently elevated levels were found in rabbits treated with the echinocandin, micafungin, which may be due to the mechanism of action of echinocandins, i.e. disruption of hyphae without clearing the hyphae from tissue (Table 15). These findings suggest that mold-active antifungal agents, specifically the triazoles and polyenes, lower the residual fungal load in lung tissue and also diminish the sensitivity of the assay in BAL with no overall effect on the specificity.

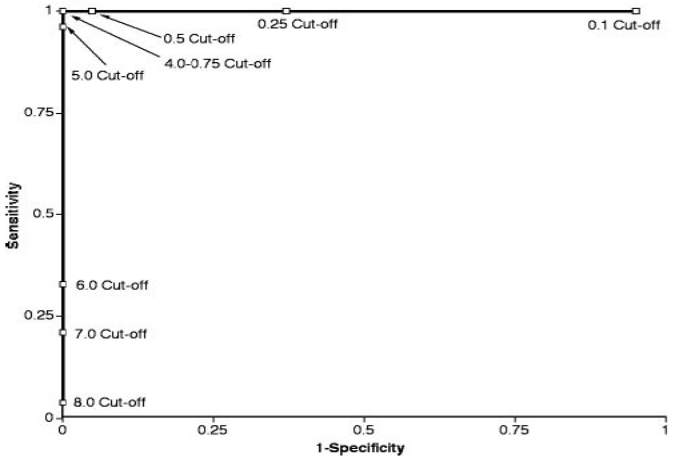
Table 15: Effect of therapy on the diagnostic yield of galactomannan assay and residual fungal burden in BAL in animals with experimentally induced IPA

Treatment group (no. of animals)	Diagnostic yield of[b]:		
	EIA (GMI)	PCR (log DNA/ml)	Culture (log CFU/ml)
Untreated controls (24)	6.0 ± 0.18[d,e]	3.3 ± 0.46[e,f]	0.70 ± 0.2[e]
Animals treated with:			
Ravuconazole (17)	3.2 ± 0.50[d]	1.6 ± 0.47[e]	0.42 ± 0.24
Micafungin (26)	6.8 ± 0.30	2.1 ± 0.37[e]	0.21 ± 0.10[e]
Deoxycholate amphotericin B (18)	4.7 ± 0.50[e]	1.2 ± 0.40[f]	0.16 ± 0.15[e]

[a] All *P* values (see below) represent comparisons of treatment groups to untreated controls.
[b] Data are means ± SEM.
[c] DNA data are expressed in femtograms.
[d] $P < 0.0001$.
[e] $P < 0.05$.
[f] $P = 0.003$.

Three animal studies also assessed the optimal cut-off index as an interpretive criteria based on the sensitivity of the galactomannan assay in BAL in the diagnosis of IPA. Francesconi *et al.* (2006), study in rabbits infected with *A. fumigatus* assessed the optimal performance of the assay in BAL by plotting a receiver operator characteristic (ROC) curve (Figure 5). The ROC curve assesses the relationship of the true positives (sensitivity) against the false positives (i.e., 1- specificity) at different cut-off index values. The results showed that the optimal cut-off index value in BAL based on a ROC curve was ≥ 0.75. The authors noted that increasing the cut-off value to more than 0.75 resulted in little or no effect on specificity but decreased the sensitivity.

Figure 5: Receiver operator curve (ROC) curve for the galactomannan assay in BAL fluids in NZW rabbits



Though a ROC curve was not performed, the studies by Ahmad *et al*. (2007), in mice infected with *A. terreus* and Khan *et al*. (2008), in rats infected with *A. fumigatus* showed results similar to the rabbit study by Francesconi *et al*. (2006), in that increasing the cut-off value from ≥0.5 to ≥ 1.5 decreased the sensitivity by as much as 26% to 52% while retaining the specificity (Table 16). Overall, the data suggests that the optimal cut-off index should be ≥0.5 but no more than 1.5 in order to retain the optimal sensitivity of the galactomannan assay.

Table 16: Effect of changing cut-off values in the BAL galactomannan from animal studies

Study (Year)	Animal Type	Route of infection	Cut-off value based on ROC analysis	BAL GM ≥ 0.5		BAL GM ≥ 1.5	
				Sn[a]	Sp	Sn[a]	Sp
A. fumigatus							
Francesconi *et al*., 2006	Rabbits	IT	0.75	100%	100%	ND	ND
Khan *et al*., 2008	Rats	IV	ND	76%	100%	50%	100%
A. terreus							
Ahmad *et al*., 2007	Mice	IV	ND	80%	100%	28%	100%

Note: Sn = sensitivity, Sp = specificity, IT = intra-tracheal; IV = intravenous; ND = not determined;
[a]Overall sensitivity

3.3. Clinical Studies

A total of twelve clinical studies were reviewed, of which six studies included patients with hematological malignancies. The remaining studies were conducted in non-hematologic patient populations; one in bronchogenic carcinoma patients, three in solid organ transplants, one in an intensive care unit (which included some patients with hematological malignancies) and one in a non-immunocompromised patient population (Table 17). Five studies included only adults with an age range, 18-77 years, and three studies (Musher *et al*., 2004, Meersseman *et al*., 2008 and Maertens *et al.,* 2009) reported mean age distribution of the population, four studies included pediatric patients (Nyguyen

et al., 2007; Clancy *et al*., 2007; Husain *et al*., 2008; Desai *et al*., 2009), of which the study by Desai *et al*. (2009) evaluated a pediatric population only (average age; 10.3 years).. Of twelve studies reviewed, eight were retrospective studies, three prospective studies and one study by Becker *et al*. (2003) was conducted in two parts that incorporated a retrospective and a prospective cohort. Husain *et al*. (2008), study evaluated a compilation of laboratory results conducted at the MiraVista Laboratory from two studies (Clancy *et al*., 2007 and Husain *et al*., 2007) and parts of the dataset were also available for an independent review. For complete summary of individual clinical studies please see *Appendix-III*.

Two commercially available galactomannan assays were used in the studies; the Platelia *Aspergillus* EIA by Sanofi Diagnostics was used mostly in European countries and BioRad Laboratories Platelia *Aspergillus* EIA was used in the United States. However, there was no difference in the two kits used as they were manufactured and distributed by the same company, i.e., Sanofi Diagnostics (Table 17). The performance of the galactomannan assay for testing of BAL fluids was performed according to the manufacturer's specifications (see *Section 2.4*) except that the data were analyzed using different cut-off galactomannan indices and for most studies one BAL sample was tested. Two studies, Becker *et al*. (2003) and Verweij *et al*. (1995) reported positive results based on a cut-off index ≥ 1.0 only. Three studies reported results based on a cut-off index ≥ 0.5 only (Meersseman *et al*., 2008, Penack *et al*., 2008, Husain *et al*., 2007); however, the remaining seven studies reported results at the two cut-off indices (≥ 0.5 or ≥ 1.0) and plotted a ROC curve to determine the optimal interpretive cut-off index.

Of the twelve studies, five studies reported that the galactomannan assay was performed in one central laboratory and the study by Verweij *et al*. (1995), reported that one technician performed the galactomannan assay on all BAL specimens tested. Nine of the eleven studies indicated that the galactomannan assay was performed using a single BAL specimen; however Shahid *et al*. (2008) reported the use of two consecutive samples, though the details of the collection of specimens was not specified (Table 17). Most studies batch-tested the BAL specimens and samples were stored at -20°C to -80°C until testing. However, three studies (Clancy *et al*., 2007; Penack *et al*., 2008; Becker *et al*., 2003) tested BAL specimens in real-time or as received. Seven of the twelve studies reported retesting of another aliquot of the same BAL fluid and showed improved specificity.

Table 17: Study comparison on the BAL galactomannan testing, storage and type of Platelia *Aspergillus* EIA used

Author (Year) Country	Host Factors	Age[a]	Study Design	Kit Used (Laboratory)	Cut-off index[b]	Repeat Testing	Testing Procedure	Storage Temp.
Becker *et al*., 2003 Netherlands,	Hematological malignancies	18 - 79 yrs	Retrospective	Sanofi	1.0	same sample*	Batch	- 20°C
			Prospective	Sanofi	1.0	same sample	As received	NA
Musher *et al*., 2004 United States	HSCT	Cases = 45 yrs Control = 41 yrs	Retrospective	BioRad	0.5*	-	Batch	- 70°C
Verweij *et al*., 1995 Netherlands	Hematological malignancies	20 - 72 yrs	Retrospective	Sanofi (Central Lab)*	1.0**	same sample	Batch	- 80°C
Desai *et al*., 2009 United States	Hematological malignancies	10.3 yrs*	Retrospective	BioRad	0.98*	Not retested	Batch	- 70°C
Penack *et al*., 2008 Belgium	Hematological malignancies	23 – 74 years	Prospective	BioRad	0.5	NS	As received	NA
Maertens *et al.,* 2009 Belgium	Hematological malignancies	50 years*	Retrospective	BioRad	NS*	NS	NS	NS
Shahid *et al*., 2008 India	Bronchogenic carcinoma	45 - 75 yrs	Retrospective	BioRad	0.5	same and new sample	Batch* (2)	- 20°C
Clancy *et al*., 2007 United States	Solid organ transplants	4 - 79 yrs	Retrospective	BioRad (Central Lab)	2.1*	same sample	As received	NA
Husain *et al*., 2007 United States	Solid organ transplants	18 - 65 yrs	Prospective	BioRad (Central Lab)	0.5*	Retested on same sample next day	Batch	- 80°C
Husain *et al*., 2008 United States	Solid organ transplants	4 - 79 yrs	Retrospective	BioRad (Central Lab)	NS	Retested on same sample next day	Batch	- 80°C
Meersseman *et al*., 2008 Belgium	Intensive care unit	60 yrs*	Prospective	Sanofi	0.5*	NS	NS	NS
Nguyen *et al*., 2007 United States	Non-immunocompromised	2 - 77 yrs	Retrospective	BioRad (Central Lab)	1.18*	NS	NS	NS

Note: NS = not specified; NA = not applicable

[a] *Mean age of the study population; [b]*Based on receiver operator characteristics (ROC) curve analysis

3.3.1. Sensitivity

The sensitivity of the galactomannan assay for testing of BAL fluids was variable and ranged from 50 % to 100 % across the eleven studies (Table 18). Factors that may explain the disparate results between studies include (1) study population, (2) prevalence of the disease in the population, (3) the EORTC/MSG criteria used to establish diagnosis (proven or probable IA), (4) the number of samples to be considered a true positive, (5) use of antifungal agents (6) presence of circulating antibodies, and (7) type of *Aspergillus* species.

(1) Study population

Of the twelve studies reviewed, six studies included neutropenic patients with hematological malignancies (Becker *et al*., 2003; Musher *et al*., 2004; Verweij *et al*., 1995; Penack *et al*., 2008; Desai *et al*., 2009; Maertens *et al.,* 2009) (Table 18). However, only two studies, in hematologic patients, actually reported the threshold for neutropenia to be ≤500 cells/μL (Becker *et al*., 2003; Penack *et al*., 2008). Similarly, in non-hematologic patients neutropenia threshold (<1000 cells /μL) was reported in only one (Nguyen *et al*., 2007) of the six studies (Table 18).

In the hematologic malignancy population, the sensitivity of the galactomannan assay ranged from 61% to 100% in patients with proven or probable IA (Table 18). Becker *et al*. (2003) and Penack *et al*. (2008), reported the sensitivity of 92% - 100% of the Platelia galactomannan assay in BAL specimens among patients with hematologic malignancies suggesting that well-defined criteria as specified by the EORTC/MSG group, is predictive of IPA. The lowest sensitivity of 61% at a cut-off index ≥ 1.0 was observed in a study of by Musher *et al*. (2004), which may be due to the fact that >75% of patients were receiving antifungal therapy at the time of bronchoscopy and also in part due to the case-control study design. The case-control study is not designed to follow patients based on disease progression, as such may have given an imprecise measurement of the true prevalence of the disease in the study population. The reported low sensitivity of 71% reported by Verweij *et al*. (1995) did not use the EORTC/MSG criteria to identify patients with IPA as the criteria were not published; however, patients were classified based on similar host criteria and radiographic findings (i.e. pulmonary infiltrates on chest x-ray). Chest x-rays are less sensitive than chest CT scans for diagnosis of lung abnormalities, therefore the patients in Verweij *et al*. (1995) study were probably diagnosed later in their disease course. A lower sensitivity was also observed in pediatric hematological patient population (Desai *et al*., 2009) compared to adult hematologic patients with IPA (Becker *et al.* 2003, Penack *et al*. 2008, Maertens *et al.* 2009).

In the six studies in non-hematologic proven or probable IPA patients, the sensitivity of the assay ranged from 67% to 100%. The variability in sensitivities could be reflective of the small number of patients with proven or probable IPA enrolled in each study. For example, in the study by Husain *et al*. (2007), of the 116 patients enrolled in the study only two patients were reported as proven IPA and four had probable IPA based on EORTC/MSG criteria.

Table 18: Overall sensitivity of the galactomannan assay in BAL fluids across the studies

Author (Year) Country	Host Factors[a] (neutrophil count/μL)	N[b]	Cut-off index Value[c]	Type of Aspergillus (No. positive)[d]	GMI range	Second BAL specimen collected	Antifungal Treatment[e]	Estimated Prevalence[f]	Sensitivity % (No. positive/total number in group)			
									Proven	Probable	Possible[g]	Proven + Probable
Becker *et al*., 2003 Netherlands,	Hematological malignancies (retrospective)†	29	1.0	Afm (5) Afl (1) An (1) Asp (2)	NS	Yes	AmpB	28%	100% (1/1)	100% (6/6)	50% (1/2)*	100% (7/7)
	Hematological malignancies (prospective)†	53	1.0	Asp (6)	NS	Yes	NS	34%	100% (3/3)	89% (8/9)	42% (5/12)**	92% (11/12)
Musher *et al*., 2004 United States	HSCT	99	0.5*	Asp (27)	NS	Yes	NS	NA	NS	NS	NS	61% (30/49)
Verweij *et al*., 1995 Netherlands	Hematological malignancies	19	1.0	Afm (3)	NS	No	NS	NA	NA	71% (5/7)	50% (1/2)	71% (5/7)
Desai *et al*., 2009 United States	Hematological malignancies	85	0.98*	Asp (9)	0.34	No	Vrc, Mn	19%	NS	NS	NS	78% (7/9)
Penack *et al*., 2008 Belgium	Hematological malignancies†	45	0.5	Asp (23)	0.7 – 23.5	No	NS	12%†	NS	NS	NS	100%
Maertens *et al.*, 2009 Belgium	Hematological malignancies	128	NS*	Afm (23) Afl (3) An (1) At(1) And (1)	0.1 – 8.4	No	Itr*,Vrc*, Psc*	45%	100% (31/31)	NS	NS	92% (53/58)
Shahid *et al*., 2008 India	Bronchogenic carcinoma	69	0.5	Afm (10) Afl (8) Asp (5)	NS	No	NS	41%	100% (6/6)	100% (17/17)	15% (2/13)	100% (23/23)
Clancy *et al*., 2007 United States	Solid organ transplants	81	2.1*	Afm (4) Asp (1)	2.1 - 10.2	Yes	AmpB, Vrc	6%	100% (2/2)	100% (3/3)	NS	100% (5/5)
Husain *et al*., 2007 United States	Solid organ transplants	116	0.5*	Afm (3) At (1) Afl (1) An (1)	0.2 - 10.1	No	Vrc, Itr	5%	50% (1/2)	75% (3/4)	NS	67% (4/6)
Husain *et al*., 2008 United States	Solid organ transplants	196	NA	NA	NA	Yes	NS	NA	NA	NA	NA	72% (8/11)
Meersseman *et al*., 2008 Belgium	Intensive care unit	69	0.5*	Asp (15)	0.6 - 7.9	Yes	Vrc, Cpn	36%	88% (23/26)	NS	NS	88% (23/26)
Nguyen *et al*., 2007 United States	Non-immunocompromised††	73	1.18*	Asp (6)	2.0 - 8.89	No	NS	8%	100% (6/6)	NS	NS	100% (6/6)

Note: GMI = galactomannan index; HSCT = hematopoietic stem cell transplant recipients; NS = not specified, NA = Not applicable; [a] EORTC/MSG (2002) criteria were used for all studies except the study by Verweij *et al*., 1995 and Maertens *et al., 2009*; †Neutropenia: neutrophils <500 cells/μL ; †† neutrophils< 1000 cells/μL [b]N= Total number of patients; [c] *Based on plotting an ROC curve; [d]Afm = *A. fumigatus;* Afl = *A. flavus;* An = *A. niger;* And = *A. nidulans* Asp = *Aspergillus* sp.; At = *A. terreus*; [e] AmpB=amphotericin B; Vrc = voriconazole; Mn = micafungin; Itr = itraconazole; Cpn = caspofungin Psc = posaconazole, *Given as prophylaxis; [f]Estimated prevalence = (Proven IPA + Probable IPA)/ (Proven IPA +Probable IPA + Patients without IPA); †Actual incidence based on study population; [g]*Suspected cases = 2/2 (100%); ** Suspected cases = 75% (6/8)

(2) Prevalence of disease in population
The estimated prevalence of IPA was determined as follows:

$$\text{Estimated prevalence} = \frac{\text{Proven IPA + Probable IPA}}{\text{(Proven IPA +Probable IPA + Patients without IPA)}}$$

Patients with hematological malignancies are known to have a higher prevalence of IPA compared to other immunosuppressed populations such as organ transplant patients; a prevalence of 12-18% was reported in published literature (Penack *et al*., 2008; Slobbe *et al.,* 2008; Lefflang *et al.,* 2008). The results of four of the six studies reviewed in the hematological malignancy population were consistent with other published literature, in that the estimated prevalence ranged from 12% to 45% (Table 18). In the remaining two studies (Musher *et al*., 2004; Verweij *et al*., 1995) the prevalence was indeterminate due to study design differences. Overall, the hematologic patient populations had a higher prevalence of the disease, and the sensitivity of the galactomannan assay was higher compared to non-hematologic patient populations. The high prevalence is related to severe neutropenia observed in patients with hematologic malignancies, suggesting that the performance of the galactomannan assay in BAL fluids would have greater applicability in patient populations at a greater risk for IPA infection.

(3) EORTC/MSG classification of proven or probable IPA
The 2002 EORTC/MSG criteria were used for ten of the studies with the exception of Maertens *et al.* (2009) which used 2008 EORTC/MSG criteria and Verweij *et al*. (1995) which was published before the EORTC/MSG criteria were defined (Table 18). There was considerable variability in the sensitivity results of the galactomannan assay based on classification of proven, probable, and possible IPA.

Becker *et al*. (2003), was the only study that reported the sensitivity of the galactomannan assay for proven and probable IPA among patients with hematologic malignancies, while the remaining studies of hematologic patients reported the sensitivity of the galactomannan assay as combined proven and probable IPA. Based on Becker *et al*. (2003), retrospective and prospective study, the sensitivity of the galactomannan assay in BAL was often higher among proven IPA patients compared to probable or possible IPA, thus suggesting that using a well defined set of criteria (host factors, clinical and radiological observations, and mycological factors) was associated with high sensitivities of the galactomannan assay (Table 18). Patients classified as probable IPA had sensitivities of 89-100% similar to proven cases of 100% (Table 18), suggesting that there was a greater concordance of the mycological criteria specified by the EORTC/MSG group for "proven" and "probable" IPA patients. It is important to note that galactomannan testing was not used as part of the criteria used for "probable" IA patients in this study. The data suggests that outcomes in these two groups are similar and thus likely represent comparable groups. Furthermore, the published EORTC/MSG guidelines emphasize the importance of managing proven and probable IPA patients similarly.

The combined sensitivity of the galactomannan assay in patients with proven and probable cases of IPA ranged from 61% - 100% (Musher *et al*., 2004; Penack *et al*., 2008; Desai *et al*., 2009; Maertens *et al.,* 2009). The lower sensitivities were possibly due to the small number of patients with IPA and the fact that > 75% of patients in one study (Musher *et al*., 2004) received systemic antifungal therapy.

Becker *et al*. (2003) retrospective and prospective studies in "possible" IPA patients reported sensitivities of 42% and 50% for the galactomannan assay, respectively. The results demonstrate the relatively reduced applicability and poor diagnostic potential of the galactomannan assay in BAL fluids in classifying patients as "possible" IPA. The 2002 EORTC/MSG criteria recommended that patients classified as "possible" should be excluded from clinical trials, since this group may include an over representation of doubtful IPA cases.

The modified EORTC/MSG guidelines (de Pauw *et al*., 2008), however recommend that "possible" patients be included in clinical trials and re-defined the "possible" classification as patients with appropriate host factor that had sufficient clinical evidence consistent with IPA but for which there was no culture or histopathological evidence. Becker *et al*. (2003), study, defined these patients as "suspected" cases that included patients with hematologic malignancies that had one major clinical criterion and negative histopathology, cytology and fungal cultures. It is important to note that galactomannan detection was not used as a microbiologic criterion in the study. The sensitivity of the galactomannan assay in BAL among the "suspected" cases in the study population was 75%. Although the "suspected" case sensitivity were lower than the sensitivity of the assay in proven (100%) and probable (89 -100%) IPA patients, the sensitivity in "suspected" cases were significantly higher than the 42-50% reported for the "possible" patients. These findings suggest a well defined set of criteria (host factors, clinical and radiological observations) are associated with high sensitivities of the galactomannan assay and are important in determining "possible" IPA patients especially as culture-based methods are insensitive.

Penack *et al*. (2008) study was the only study that used galactomannan positive results as the only microbiologic diagnostic test and the patients were classified as possible IPA and not probable IPA. The authors identified six (26%) patients with similar host factors (i.e., hematological malignancies) who had sufficient clinical and radiological evidence consistent with IPA but for which there was no microbiological support. A limitation of the Penack *et al*. (2008), study is that there was no long-term follow-up in these six patients and it was unclear whether the positive galactomannan index in these patients correlated with IPA. As is the standard practice, all efforts should be made to either confirm the presence of *Aspergillus* species or exclude patients with other infections.

(4) Number of samples considered to be a true positive

Two of the six studies in the hematologic malignancy patient population (Becker *et al*., 2003; Musher *et al*., 2004) reported reproducible results based on retesting of the BAL specimen using an aliquot of the same sample (Table 17). Additionally, the study by Husain *et al*. (2008), although in a non-hematologic population, showed reproducibility of

results on retesting of a second aliquot of the BAL specimen. BAL specimens that were re-tested, 93% were reproducible at low galactomannan indices (0.5 – 0.9) and 100% were reproducible at higher galactomannan indices (≥ 1.0); see Table 11. The results suggest that if the galactomannan assay is performed correctly, a single positive test, based on testing of two aliquots of BAL at an index ≥ 1.0, is indicative of a true positive result.

Becker *et al*. (2003), and Musher *et al*. (2004), reported that a second BAL specimen was collected in few patients (2 -5 patients/study). Both studies reported negative results and lower sensitivity of the galactomannan assay upon retesting of a second BAL specimen (Table 18). Similar to the hematological population, Clancy *et al*. (2007), Husain *et al*. (2008), and Meersseman *et al*. (2008), reported a lower sensitivity of the galactomannan assay in non-hematological patients that had a second BAL specimen tested. This was probably due to the fact that antifungal treatment had been administered prior to the second BAL.

Given that bronchoscopy is an invasive procedure, obtaining a second BAL specimen is usually impractical in critically ill patients with hematological malignancies or in recipients of HSCT because many patients are intolerant of the procedure and are at increased risk of bleeding due to thrombocytopenia or coagulation abnormalities The results suggest that the performance of the galactomannan assay based on the testing of two aliquots of one sample appears adequate in determining galactomannan positivity as a biomarker of IPA.

(5) Use of antifungal agents

Exposure to mold-active antifungal agents appears to affect the sensitivity of the galactomannan assay in BAL. As was shown in the animal model studies of invasive aspergillosis, certain antifungal agents can potentially lower the sensitivity of galactomannan assay for the detection of *Aspergillus* species in BAL by lowering the residual fungal burden. Three of the six studies in the hematological malignancy patient population (Becker *et al*., 2003; Desai *et al*., 2009; Maertens *et al.,* 2009) and three of the six studies in the non-hematological patient population (Clancy *et al*., 2007; Husain *et al*., 2008; Meersseman *et al*., 2008) reported the effect of mold-active antifungal agents in patients classified as proven or probable IPA (Table 18). No one particular antifungal agent was associated with a decrease in sensitivity, though the number of patients with IPA treated with antifungal agents was small. However, the overall effect of antifungal use was related to a decreased sensitivity when a second BAL specimen was available for analysis. Becker *et al*. (2003), study showed that all five patients who had a second bronchoscopy performed and were on treatment with mold-active antifungal agents at the time of BAL collection were negative by the galactomannan assay (Figure III-1 in *Appendix III*). Patients who received more than two days of antifungal treatment tended to be negative for galactomannan in BAL. Most patients were treated with deoxycholate amphotericin B or the lipid formulation of amphotericin B. Maertens *et al.* (2009) 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.

Similar to the studies in patients with hematologic malignancies, in the non-hematologic patients who had multiple BAL fluids collected had decreased galactomannan levels in BAL fluids tested after the start of antifungal therapy. Although, the results were based on a small number of patients, overall the findings suggests that exposure to mold- active antifungal agents is an important variable that reduces the sensitivity of the galactomannan assay in BAL fluid.

Thus it is recommended that collection of a BAL fluid specimen for galactomannan testing should be obtained, if possible, before systemic anti-fungal treatment is administered. Otherwise, patients on antifungal treatment should be excluded from analysis.

(6) Presence of circulating antibodies

None of the studies in hematological patients evaluated the effect of circulating human serum antibodies against *Aspergillus* on the detection of galactomannan by the assay. However, Shahid *et al*. (2008), in a non-hematological patient population, measured circulating serum antibodies at the time when the BAL specimen was collected. All proven IPA patients and approximately 82 - 85% of the probable IPA patients were positive for circulating anti-*Aspergillus* antibodies by the EIA, double immunodiffusion, and dot-blot assay methods. However, only two of the possible IPA patients were weakly positive for circulating antibodies. Patients without IPA and healthy controls were negative for circulating anti-*Aspergillus* antibodies by any method. The sensitivity of the galactomannan assay in BAL among definite (proven), probable, and possible IPA cases with circulating antibodies were 100%, 100%, and 15%, respectively. Circulating antibodies do not appear to affect the sensitivity of the galactomannan assay in BAL, though confirmatory prospective studies are needed.

(7) Type of *Aspergillus* species

Among the hematologic malignancy population, three of the six studies (Verweij *et al*., 1995; Becker *et al*., 2003, Maertens *et al.,* 2009), reported specific *Aspergillus* species; the remaining studies did not specify the species. *A. fumigatus* was reported as the pathogen in the majority of patients. Becker *et al*. (2003) and Maertens *et al.* (2009) also reported IPA caused by *A. flavus, A. niger, A. nidulans,* and *A. terreus*. Similar results were reported in the non-hematological patient population. The overall galactomannan index values reported for *Aspergillus* sp. ranged from 0.2 to 23.5 (Table 18). However, the number of patients infected with any type of *Aspergillus* species was small, thus no inference can be made on the type of species in relation to sensitivity of the assay. It is unclear if attempts were made to identify the *Aspergillus* or other fungal species in all patients enrolled.

3.3.2. Specificity

The specificity of galactomannan assay in BAL fluids varied (range, 74% - 100%) across the eleven studies depending on the patient population (Table 19). The variable specificity could be due to factors such as (1) selection of the cut-off index to define positivity (2) colonization with *Aspergillus* species, (3) cross reactivity with pathogens other than *Aspergillus*, (4) cross-reactivity with antibiotics, and (5) cross-reactivity with Plasmalyte® solution

Table 19: Summary of patients without IPA

Author (Year) Country	Host Factor[a]	N[b]	Cut-off index Value[c]	No. of Patients without IPA (BAL positive)	Specificity	Classification of patients without IPA	GMI range	Colonizing species[d]	Concomitant Antifungal Used[e]	Concomitant Antibacterial Use[f]
Becker *et al*., 2003 Netherlands,	Hematological malignancies (retrospective)	29	1.0	18 (0)	100%	Similar host factors as patients with IPA	NS	NS	NS	NS
	Hematological malignancies (prospective)	53	1.0	21 (0)	100%		NS	NS	NS	NS
Musher *et al*., 2004 United States	HSCT	99	0.5*	50 (2)	96%	Patients with other diagnosis (pneumonia caused by other fungi, viral or bacteria)	NS	NS	NS	NS
Verweij *et al*., 1995 Netherlands	Hematological malignancies	19	1.0**	10 (1)	90%	Similar host factors as patients with IPA	NS	NS	NS	NS
Desai *et al*., 2009 United States	Hematological malignancies	85	0.98*	39 (7)	82%	Similar host factors as patients with IPA	NS	NS	NS	Zosyn (8)
Penack *et al*., 2008 Belgium	Hematological malignancies	45	0.5	28 (6)	74%	Similar host factors as patients with IPA	NS	NS	NS	NS
Maertens *et al.*,2009 Belgium	Hematological malignancies	128	NS*	41	88%	Similar host factors as patients with IPA	0.1 – 5.5	NF	None	None
Shahid *et al*., 2008 India	Bronchogenic carcinoma	69	0.5	33 (0)	100%	Similar host factors as patients with IPA and healthy controls	NS	NS	NS	NS
Clancy *et al*., 2007 United States	Solid organ transplants	81	2.1*	76 (12)	84%	Similar host factors as patients with IPA	0.5 -8.1	Afm (1) Afl (1) An (1) Pnsp (1)	L-AmB (2) Vrc (3)	Zosyn (0)
Husain *et al*., 2007 United States	Solid organ transplants	116	0.5*	110 (13)	88%	Similar host factors as patients with IPA	0.5 -6.3	Afm (1) Afl (1) At (1) Av(2) Pnsp (3) Pasp (1)	Itr (5) Vrc (2)	Zosyn (2)
Husain *et al*., 2008 United States	Solid organ transplants	196	0.5	185 (23)	88%	Similar host factors as patients with IPA, HIV healthy patients, surveillance controls	NS	NS	NS	NS
Meersseman *et al*., 2008 Belgium	Intensive care unit	69	0.5*	46 (6)	86%	Deceased patients	NS	NS	Not specified (4)	Zosyn (4) AMC (0)
Nguyen *et al*., 2007 United States	Non-immunocompromised	73	1.18*	67 (9)	87%	healthy controls	1.0 - 5.0	A. fm (1) An (1) At (1)	NS	Zosyn (0) AMC (0)

Note: GMI = galactomannan index; HSCT = hematopoietic stem cell transplant recipients; NS = not specified; NF = none found.
[a] EORTC/MSG (2002) criteria were used for all studies except the study by Verweij *et al*., 1995 and Maertens *et al.,* 2009; [b]N= Total number of patients; [c] *Based on plotting an ROC curve; [d]Afm = *A. fumigatus;* Afl = *A. flavus;* An = *A. niger;* Asp = *Aspergillus* sp.; At = *A. terreus*; Pnsp = *Penicillium* species; Pasp = *Paecilomyces* sp; [e] AmB=amphotericin B; L-AmB = liposomal amphotericin B; Vrc = voriconazole; Itr = itraconazole; [f] Zosyn = piperacillin-tazobactam; AMC = amoxicillin/clavulanic acid;

(1) Optimal cut-off index value

Of the five studies in the hematologic patients that used the EORTC/MSG criteria to classify patients, one study (Becker *et al*., 2003) reported the specificity based upon a cut-off index ≥ 1.0 only; whereas another (Penack *et al*., 2008) reported based upon a cut-off index ≥ 0.5. The remaining three studies (Musher *et al*., 2004; Desai *et al*., 2009, Maertens *et al.,* 2009) determined galactomannan positivity in BAL by evaluating at both thresholds and determined the optimal cut-off value based on graphing ROC curves. Raising the cut-off value from 0.5 to 1.0, improved the specificity of the galactomannan assay in the studies by 4% to 8% with minimal effect on the sensitivity. The findings suggest that a cut-off index of 1.0 may be appropriate for testing in BAL fluids among the hematological malignancy patient population (Table 20).

Similar to the studies in hematologic patients, three of six studies in the non-hematologic patients (Clancy *et al*., 2007; Husain *et al*., 2008; Nguyen *et al*., 2007) showed that increasing the cut-off index values from 0.5 to 1.0 had a significant effect on the specificity of the galactomannan assay in BAL specimens (Table 20). The relative improvement in specificity (5% - 10%) was observed in studies that had a low prevalence of IPA such as solid organ transplants and non-neutropenic patient populations.

The overall results suggest an optimal cut-off index be ≥1.0 in order to retain the optimal specificity of the galactomannan assay with minimal effect on sensitivity.

Table 20: Summary of ROC curve analysis and comparison of cut-off values of proven and probable IPA cases (combined) at 0.5 and 1.0

Author (Year) Country	Host Factor[a]	N[b]	Estimated Prevalence[c]	Cut-off index[d]	BAL GM ≥ 0.5				BAL GM ≥ 1.0			
					Sn[e]	Sp	PPV	NPV	Sn[e]	Sp	PPV[f]	NPV
Becker *et al*., 2003 Netherlands,	Hematological malignancies (retrospective)	29	30%	ND	NS	NS	NS	NS	100%	100%	100%	100%
	Hematological malignancies (prospective)	53	36%	ND	NS	NS	NS	NS	92%	100%	100%	96%
Musher *et al*., 2004 United States	HSCT	99	NA	0.5	76%	94%	NS	NS	61%	98%	NS	NS
Verweij *et al*., 1995 Netherlands	Hematological malignancies[!]	19	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Desai *et al*., 2009 United States	Hematological malignancies	85	19%	0.98	78%	84%	NS	NS	78%	92%	54%**	97%
Penack *et al*., 2008 Belgium	Hematological malignancies	45	12%	0.5	100%	79%	100%	74%	NS	NS	NS	NS
Maertens *et al.,* 2009 Belgium	Hematological malignancies	128	45%	NS	97%	80%	68%	98%	91%	88%	76%	96%
Shahid *et al*., 2008 India	Bronchogenic carcinoma	69	41%	ND	NS	NS	NS	NS	100%	100%	100%	100%
Clancy *et al*., 2007 United States	Solid organ transplants	81	6%	2.1	100%	84%	29%	100%	100%	91%	42%	100%
Husain *et al*., 2007 United States	Solid organ transplants	116	5%	0.5	67%	88%	23%	98%	67%	93%	36%	98%
Husain *et al*., 2008 United States	Solid organ transplants	196	NA	ND	82%	87%	NS	NS	NS	NS	NS	NS
Meersseman *et al*., 2008 Belgium[h]	Intensive care unit	69	36%	0.5	88%	87%	79%	93%	NS	NS	NS	NS
Nguyen *et al*., 2007 United States	Non-immunocompromised	73	8%	1.18	100%	78%	29%	100%	100%	88%	43%	100%

Note: Sn = sensitivity; Sp = Specificity; PPV = positive predictive value; NPV = Negative predictive value; HSCT = hematopoietic stem cell transplant recipients; NS = not specified; ND = Not determined; NA = Not applicable
[a]EORTC/MSG (2002) criteria were used for all studies except the study by Verweij *et al*., 1995 and Maertens *et al.,* 2009;
[b]N= Total number of patients;
[c]Estimated prevalence = (Proven IPA + Probable IPA)/ (Proven IPA +Probable IPA + Patients without IPA);
[d]Based on ROC curves
[e]Sensitivity = (No of proven and probable IPA positive)/(Total No. of IPA patients in proven and probable Population)
[f]**Includes possible IPA patients and patients without IPA
[h] 33% of the patients had a hematological malignancy

(2) Patients colonized with *Aspergillus* species,

In the six studies in patients with hematological malignancies, none of the authors reported patients without proven or probable IPA colonized with *Aspergillus* species. In the studies with non-hematologic patients, three of the six studies (Clancy *et al*., 2007; Husain *et al*., 2007; Nguyen *et al*., 2007) reported patients who were colonized with *Aspergillus* species and had similar BAL galactomannan indices ranges (0.86 – 8.1) to that of patients with IPA (range 2.1 – 10.1) (Table 19). Clancy *et al*. (2007), study reported that three of the 76 lung transplant patients without clinical evidence of IPA had cultures that yielded *Aspergillus*

species (*A. terreus*, *A. niger*, *A. flavus*) and the BAL galactomannan assay index ranged from 3.0 – 8.1. It is important to note that lung transplant patients accounted for approximately 42% of the false positives galactomannan results reflecting colonization of the airways in these patients. Three patients who had BAL cytology that revealed hyphal elements had BAL galactomannan range of 0.86 -8.1.

Husain *et al*. (2007), study reported that seven of 110 lung transplant patients were colonized with *Aspergillus* species at the time of BAL galactomannan testing (BAL galactomannan range, 0.61 – 1.45).

Nguyen *et al*. (2007), study in non-immunocompromised patients, reported that two of the 67 patients without IPA were reported as colonized with *Aspergillus*; culture results showed *A. fumigatus* and *A. niger* (galactomannan index, 1.04) in one patient and *A. terreus* (galactomannan index, 2.67) in the second patient.

None of these patients, in all three studies, were reported to have developed evidence of aspergillosis infection (Table 19). These observations imply that the galactomannan assay performed in BAL is not able to distinguish between invasive disease and colonization in non-hematologic patients. Follow-up is necessary in determining whether a positive galactomannan assay result is correlated with progression to IPA disease.

(3) Cross reactivity with pathogens other than *Aspergillus*
Galactomannan is also found on the fungal cell membranes of non-*Aspergillus* fungal organisms. False positive galactomannan results may occur due a variety of causes as listed in Table 7 above. A number of studies have reported index values that had galactomannan index values ≥ 0.5 in the sera of patients infected or colonized with these fungal species. None of the six studies in the hematological malignancies patient population reported colonization with non-*Aspergillus* organisms.

Colonization with *Penicillium* and *Paecilomyces* species were reported in two of the six non-hematological study populations (Clancy *et al*., 2007; Husain *et al*., 2007) among the patients without proven or probable IPA (Table 19). Clancy *et al*., (2007) study in lung transplant patients reported one patient without IPA that had a culture that yielded *Penicillium sp.* and galactomannan index result in BAL of 1.62. Husain *et al*., (2007) reported two patients without IPA colonized with *Penicillium sp.* that had a galactomannan index value in BAL of 2.4 and 6.34 and one patient colonized with *Paecilomyces sp.* with an index of 4.67. The results suggest that screening for non-hematological patients colonized or infected with these organism by culture or histopathological evaluation is important, since they are likely to contribute to the false positive results observed in the assay in BAL specimens. As stated previously, cross reactivity with the galactomannan assay in BAL and serum specimens can occur in patients with histoplasmosis (Wheat *et al*., 2007). However, none of the clinical studies reviewed in either the hematologic or non-hematologic patients, reported patients colonized or infected with *H. capsulatum*. It is also noted that the incidence of *Penicillium* and *Paecilomyces, Histoplasma* species is extremely low in the hematological malignancy and HSCT populations. Therefore the

cross reactivity of galactomannan assay with fungal species other than *Aspergillus* is not expected to be clinically relevant.

It is recommended, however, for the enrollment of clinical trials that investigators comply with current standard practice of obtaining a fungal smear and culture on all BAL samples. Results of fungal smears and fungal cultures should be clearly documented on the case report form for each BAL sample so that the presence of *Aspergillus* species or other rare fungal pathogens that could cross react with the galactomannan assay is documented.

(4) Cross-reactivity with antibiotics
Cross reactivity of certain beta-lactam antibiotics, namely piperacillin-tazobactam and amoxicillin-clavulanate is well described (Mennink-Kersten *et al.,* 2004; Wheat *et al.,* 2006, Aubry *et al.,* 2006; Weisser *et al.,* 2005; Herbrecht *et al.,* 2002). Of the six studies in patients with hematological malignancies, two studies (Desai *et al*., 2009; Penack *et al*., 2008) reported the use of piperacillin-tazobactam and amoxicillin. Desai *et al*. (2009), study reported that eight patients had documented use of piperacillin-tazobactam or amoxicillin, however, the authors did not report any change in galactomannan detection in BAL specimens (Table 19); the relevance of the finding is unknown. Penack *et al*. (2008), study reported that patients with neutropenia and fever of unknown origin were treated immediately with piperacillin-tazobactam (3 x 4.5 g/day), though no correlation was made between galactomannan index levels and time of treatment with piperacillin-tazobactam in these patients.

In the studies of non-hematologic patients, two of the six studies reported positive galactomannan assay results in patients treated with piperacillin-tazobactam. Husain *et al*. (2007), study reported one patient on piperacillin-tazobactam with a positive galactomannan result of 2.45. Meersseman *et al*. (2008), reported four of 33 patients in the study population treated with piperacillin-tazobactam had positive galactomannan result (range: 1.3 – 5.8). The authors stated that these patients treated with piperacillin – tazobactam remained positive until four days after the antibiotic had been stopped. Thus, a positive galactomannan result by the galactomannan assay in BAL specimens in patients treated with antibiotics such as piperacillin-tazobactam, ampicillin and amoxicillin should be interpreted with caution, since it has been shown to cause false positives. It is recommended that in a clinical trial for development of antifungal drugs, patients receiving these antibiotics should be excluded and prior use of antibiotics should be documented.

(5) Cross-reactivity with Plasmalyte® solution
The cross reactivity with Plasmalyte is an issue for BAL galactomannan testing because plasmalyte may be used to perform BAL washes during bronchoscopy. Only one study reported that Plasmalyte was not used in bronchoscopy examination (Husain *et al*., 2007). Other wash fluids can be substituted, e.g. saline; therefore it is recommended that Plasmalyte should be avoided for bronchoscopies in patient populations at risk of invasive aspergillosis.

3.3.3. Predictive Values

The negative predictive value (97% to 100%) of the galactomannan EIA assay was relatively high and consistent across the hematologic and non-hematologic populations (Table 20). These findings suggest that a negative result is often associated with the absence of disease.

The positive predictive value (PPV) in the studies with hematologic patients, ranged from 54% -100% at galactomannan assay index cut off ≥1.0. The lowest PPV of 54% was reported in the pediatric study (Desai *et al*., 2009) compared to PPV of 100% in an adult population (Becker *et al.*, 2003) 100% (Table 20).

In the non-hematological population, using a GM index cut-off ≥ 1.0, the PPV ranged from 36% -100% (Table 20). Three of the six non-hematologic patient population studies (Clancy *et al*., 2007; Husain *et al*., 2007; Nguyen *et al*., 2007) reported PPV ≤ 43%; this may be due to the low prevalence (5-8%) of IPA in these patient populations. Increasing the cut-off index values from 0.5 to 1.0 increased the PPV by 13% - 14% (Table 20). The change in the cut-off had no effect on the sensitivity. The results suggest an optimal cut-off index ≥ 1.0 will increase the overall PPV of the galactomannan EIA assay in BAL specimens.

3.3.4. Comparison with other diagnostic methods
A critical question is the diagnostic applicability of the galactomannan assay in BAL in comparison with other diagnostic procedures. In other words, whether, the galactomannan assay will help in the diagnosis before or in the absence of other investigative procedures.

(1) Comparison with clinical and diagnostic radiology
A high resolution thoracic CT scan is an important tool in making an early diagnosis of IPA. Chest x-rays are less sensitive than thoracic CT scans for evaluation of suspected IPA and high resolution thoracic CT scans are now used to follow patients for IPA. Three studies followed patients based upon abnormal radiographic findings on chest X-rays. The study by Verweij *et al*. (1995), consisted of 19 patients with hematological malignancies who had chest X-rays performed. The results show that patients with abnormal chest X-rays showing focal non-anatomical infiltrates or any cavitating lesions were more likely to have a positive BAL galactomannan result (71% of patients) compared to 10% of patients with diffuse infiltrates distributed throughout the lung fields or anatomical focal infiltrates not characteristic of IPA. Similar findings were reported by Musher *et al*. (2004), in patients with hematological malignancies; the sensitivity of the galactomannan assay in BAL in patients with nodular or cavitating lesions (82%) were higher than patients with diffuse infiltrates (60%) at a cut-off index of 1.0. Decreasing the cut-off index to ≤0.5 did not decrease the sensitivity of the galactomannan assay in patients with nodular or cavitating lesions.

The EORTC/MSG guidelines specify that typical lesions such as the “halo sign” and “air-crescent” signs identified as nodular or cavitating lesions based on radiological or CT

imaging procedures are more likely associated with infection. The thought is that early CT scan will reduce the time to diagnosis of IPA which will facilitate early initiation of anti-fungal treatment. Becker *et al*. (2003), study evaluated these characteristic lesions in neutropenic hematological-oncological patients. The "halo" signs were more often observed early in the course of disease and "air-crescent" or "wedge-shaped or nodular abnormalities" often appeared after antifungal therapy or bone marrow recovery. All patients had characteristic "halo" signs observed before or at the time of BAL galactomannan positivity. However, other signs such as the "crescent or cavitation" or "wedge-shaped or nodular abnormalities" varied between patients and were not specific to any of the group classification (i.e. proven, probable or possible IPA) based on the EORTC/MSG criteria. Becker *et al*. (2003), also noted on an average, from the start of the neutropenic episode characterized as a spike in fever, patients showed characteristic abnormalities based upon CT scans at 4.0 ± 0.8 days and had a positive galactomannan result in BAL at 6.9 ± 1.1 days. The results do suggest that when a CT evaluation is used at an early stage (i.e., observation of the "halo" sign) and performed systematically, the sensitivity of the galactomannan assay was higher when a bronchoscopy is performed following positive CT findings resulting in a better predictor for diagnosing IPA early in untreated patients.

(2) Comparison with serum galactomannan

Unlike BAL specimens, serum specimens are easier to obtain and serial sampling can be performed at different time intervals. However, a correlation between presence of galactomannan in serum and BAL was shown to be discordant. Five studies evaluated detection of galactomannan in serum collected at the same time as a BAL specimen was obtained (Table 21). The sensitivity (range; 67% - 100%) of galactomannan assay in BAL specimens was higher than the sensitivity (range; 25% -78%) in serum specimens (Table 21).

Three studies reported that over half of patients with invasive aspergillosis have a positive result in BAL and negative result in serum at the time of BAL collection (Husain *et al.*, 2008; Becker *et al.,* 2003; Meersseman *et al.*, 2008). None of the studies reported positive results in serum with negative results in BAL, but that pattern is possible.

Table 21: Sensitivity of BAL galactomannan assay in comparison to other conventional methods in proven and probable IPA patients

Author (Year) Country	Host factor[a]	N[b]	Sensitivity %[c]			
			BAL GM at cut-off index ≥ 0.5	BAL GM at cut-off index ≥ 1.0	Serum GM at cut-off index ≥ 0.5	Culture or Direct Microscopy
Becker *et al*., 2003 Netherlands,	Hematological malignancies (retrospective)	29	ND	100%	25%	60%
	Hematological malignancies (prospective)	53	ND	92%	ND	ND
Musher *et al*., 2004 United States	HSCT	99	76%	61%	ND	55%
Verweij *et al*., 1995 Netherlands	Hematological malignancies	19	NA	NA	NA	NA
Desai *et al*., 2009 United States	Hematological malignancies	85	ND	78%**	78%	ND
Penack *et al*., 2008 Belgium	Hematological malignancies	45	100%	NA	71%	ND
Maertens *et al*., 2009 Belgium	Hematological malignancies	128	97%	91%	ND	74%
Shahid *et al*., 2008 India	Bronchogenic carcinoma	69	ND	100%	ND	100%
Clancy *et al*., 2007 United States	Solid organ transplants	81	100%	100%	25%	ND
Husain *et al*., 2007 United States	Solid organ transplants	116	67%	67%	ND	ND
Husain *et al*., 2008 United States	Solid organ transplants	196	72%	ND	ND	ND
Meersseman *et al*., 2008 Belgium	Intensive care unit	69	88%	ND	42%	58%
Nguyen *et al*., 2007 United States	Non-immunocompromised	73	100%	100%	60%	100%

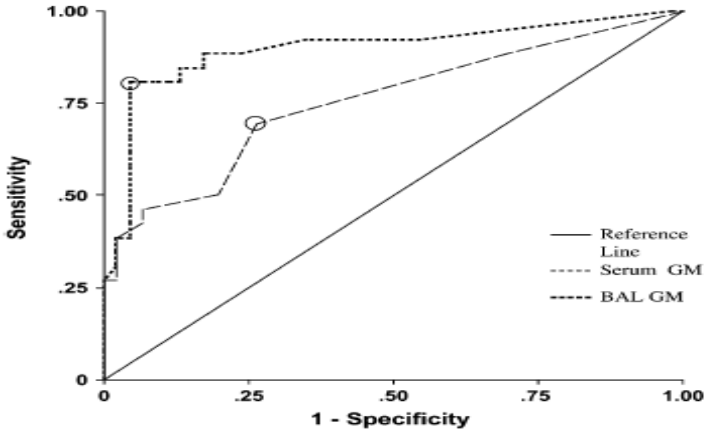
Note: HSCT = hematopoietic stem cell transplant recipients; ND = Not determined; NA = Not applicable
[a]EORTC/MSG (2002) criteria were used for all studies except the study by Verweij *et al*., 1995 and Maertens *et al*., 2009
[b]N= Total number of patients;
[c]Sensitivity = (No of proven and probable IPA positive)/(Total No. of patients in proven and probable IPA population)

The study by Verweij *et al*. (1995), suggests the timing between serum sampling and BAL sampling may also explain the differences in the sensitivities. Patients had a positive serum galactomannan before the appearance of the first clinical or radiological feature that led to suspicion of invasive *Aspergillus* infection. In their study population (n=19), serum samples were obtained between six weeks before and two weeks after the BAL fluid

sample was obtained. Among the five probable IPA patients, four patients had serum samples that were positive at three and 30 days before the BAL specimen was obtained and tested positive. These findings are different from those reported by Penack *et al*. (2008), and Becker *et al*. (2003), that serum specimens were often negative before and after BAL collection. Becker *et al*. (2003), noted that sera taken up to 3- 4 weeks before and at least two months after BAL positivity were negative.

In the studies with non-hematologic patients, the sensitivity of the galactomannan assay for testing in BAL was higher (range, 67% - 100%) than serum (range, 25% - 60%) for detection of galactomannan; however, serum had less false positive results compare to BAL. A higher false positive result in BAL could be due to colonization of the airways with *Aspergillus* for example in lung transplant patients. Two studies (Nguyen *et al*., 2007 and Clancy *et al*., 2007) in organ transplant patients reported that there was an increased likelihood of obtaining false-positive results with BAL galactomannan at a cut-off value of ≥ 0.5 (PPV 29% and 29%, respectively) or ≥ 1.0 (PPV 42% and 43%, respectively) compared to sera-based galactomannan (PPV 50 % and 75%, respectively). A similar observation in hematologic patients was not available from any of the studies reviewed. Meersseman *et al*. (2008), study in intensive care patients showed that the sensitivity of serum galactomannan at an index value ≥ 0.5 in the diagnosis of proven IA was much lower (42%) than in BAL (88%). The median value of the assay in serum (galactomannan index = 0.3) for the proven cases was significantly lower than in BAL (galactomannan index = 4.1) ($p < 0.005$). However, the specificity of the assay at an index value ≥ 0.5 in serum (93%) was higher than BAL (87%). Using the ROC curve to assess the performance of the galactomannan assay in BAL in the diagnosis of IPA at different cut-off values is illustrated in Figure 6. Qualitatively, the ROC curve showed that at a cut-off level of ≥ 0.5 corresponding to the galactomannan assay in BAL is displaced further toward the upper left-hand corner of the box than the curve for serum. Quantitatively, the AUC values for the assay in BAL and serum are 0.898 and 0.755, respectively. Thus, both qualitatively and quantitatively ROC analysis demonstrates that the assay in BAL provides better discrimination than in serum in diagnosing patients with proven IA.

Figure 6: Receiver operator characteristic (ROC) curve for galactomannan detection in BAL at a cut-off index ≥ 0.5



Overall, the results suggest that detection of galactomannan in BAL has a higher sensitivity than serum specimens. None of the studies reported positive results in serum with negative results in BAL. However, these observations were based on relatively few patients and results should be interpreted cautiously.

Comparison with culture methods

Six studies evaluated positivity based on culture or direct microscopy at the same time the BAL specimen was obtained and tested (Table 21), three from a hematological malignancy population (Becker *et al*., 2003; Musher *et al*., 2004, Maertens *et al.,* 2009) and three from non-hematological population (Nguyen *et al*., 2007; Shahid *et al*., 2008; Meersseman *et al*., 2008). The sensitivity of culture or direct microscopy methods were variable and ranged from 55% to 100%. Four studies (Becker *et al*., 2003; Musher *et al*., 2004; Maertens *et al.,* 2009; Meersseman *et al*., 2008) reported that the galactomannan assay for testing of BAL was more sensitive than culture or direct microscopy combined, whereas two studies (Nguyen *et al*., 2007; Shahid *et al*., 2008), reported no difference in the sensitivity of culture or direct microscopy compared to galactomannan positivity in BAL fluid. In these two studies, the reported sensitivity for BAL galactomannan and culture or direct microscopy was 100%.

Musher *et al*. (2004), showed that the sensitivity of the galactomannan assay in BAL in IPA case patients that were culture positive had higher median galactomannan indices (4.31) than case patients that were culture negative (0.75). Patients that were culture positive for *Aspergillus* species, the sensitivity of the galactomannan assay in BAL at a cutoff index ≥ 0.5 was 89% compared to patients that were culture negative (59%). However, the sensitivity of the galactomannan assay decreased by 11% when the index cutoff was increased to ≥1.0 in both patients that were culture positive for *Aspergillus* species or patients that were culture negative. The results suggest that if the galactomannan assay was performed in real time, 89% of the confirmed cases would have yielded an earlier diagnosis. The results highlight the main problems with fungal cultures as a diagnostic tool in its limited sensitivity and delay in diagnosis of IPA.

Nguyen *et al*. (2007), showed that the sensitivity based on direct microscopy and culture results alone in proven and probable IPA patients with bronchogenic carcinoma were 100% and 71%, respectively. BAL galactomannan testing at a cut-off ≥ 1.0 was more sensitive in probable IPA patients than using either direct microscopy or culture alone. However, detection of galactomannan in BAL was no more sensitive than the combined sensitivity of direct microscopy and culture, suggesting no additional benefit of the assay in BAL for the diagnosis of IPA in patients with bronchogenic carcinoma. The results of the galactomannan assay in BAL fluids are available faster than by culture, thus indicating the usefulness of galactomannan testing for an earlier diagnosis of IPA.

3.3.5 Limitations

The limitations noted in this review is the small number of studies (n =11) that evaluated the use of galactomannan in BAL in different patient populations at varying risk of invasive aspergillosis. The performance of the assay was evaluated in six studies in hematologic patients, five studies in non-hematological patients, and one study had a mixed population of ICU patients which included hematological patients. The studies in non hematological populations consisted of solid organ transplant patient populations, including lung and liver transplants. Of note, the prevalence, specificity, and PPV were notably low in the non-hematologic patient population. This may be reflective of the differing risks for development of IPA in non hematological patients compared to a hematologic patient population. The galactomannan assay is limited in that the assay is unable to discriminate between colonization and infection with *Aspergillus* species which is a particular problem in lung transplant where colonization of airways with *Aspergillus* species needs to be taken into account. Bronchoscopy is more commonly performed in solid organ transplants with pulmonary infiltrates compared to hematologic patients, therefore, BAL galactomannan could be a useful adjunctive test for diagnosis of IPA. However, to define an appropriate galactomannan cut-off index in non-hematologic patients (e.g. solid organ transplants) would require further research using well-designed prospective clinical trials to incorporate the use of the galactomannan assay in BAL fluids along with the conventional approach and newer molecular diagnostic methodologies.

3.4 General Statistical Implications

The goal of most clinical trials is to determine whether an investigational drug is effective for the indication claimed i.e. the treatment of invasive aspergillosis. Future clinical trials for the treatment of invasive aspergillosis will most likely be non-inferiority trials. If in a non-inferiority trial an excessive number of patients with a diagnosis of invasive aspergillosis who actually do not have invasive aspergillosis are enrolled into the trial, the true difference in treatment effects would be biased toward showing non-inferiority. In other words, the trial could lead to the conclusion that the investigational treatment regimen was non-inferior to an active comparator when in fact the investigational treatment was not non-inferior (possibly inferior) in patients who actually had invasive aspergillosis. Therefore it is important to enroll patients in a clinical trial with a non-inferiority design who have the disease in question. This is not as great of an issue for superiority trials because enrollment of subjects without the disease would most likely bias the study against showing a difference and would tend to be conservative on the type I error of a superiority trial. However, the power of the study whould be reduced and this would be of concern given the difficulty in enrolling subjects with invasive aspergillosis.

It is imperative that the performance characteristics of a diagnostic test are adequate for the intended patient population. Since invasive aspergillosis is a serious fungal disease, it is important in clinical practice for a diagnostic test to have high sensitivity so that true cases of invasive aspergillosis will not be missed and left untreated. For clinical trials, however, it is important that subjects without the disease are not enrolled into the trial. In this case, specificity is of greater interest. Given only those subjects who test positive with the test

will be enrolled into a clinical trial, the primary diagnostic measure that is of concern is the percentage of patients without invasive aspergillosis among those diagnosed as having invasive aspergillosis based on the positive test result. This percentage is 1 minus the positive predictive value (PPV). PPV is not intrinsic only to the test (sensitivity and specificity) but also depends on the prevalence of the disease in the population considered. Thus, to minimize enrolling patients without invasive aspergillosis into the trial based on a positive diagnostic test result, 1-PPV is the probability that should be minimized (or maximize PPV) when selecting an appropriate diagnostic test.

Analyses were conducted on behalf of the MSG to investigate the impact of false positives on the inflation of type I error in a non-inferiority study (i.e. concluding that the investigational treatment is non-inferior to the active comparator when the investigational treatment is truly worse than the active comparator by a magnitude that is greater than the non-inferiority margin selected for the trial). The sponsor's main points of these analyses are summarized herein. Assuming sensitivities and specificities in the range of 0.85 to 0.97, if the prevalence of the disease in the population considered for the trial is greater than 0.3 then even in the worst case scenario, the positive predictive value (PPV) is greater than 0.7. If subjects can be chosen in such a way that the prevalence is at least 0.50, the PPV is 0.9 or greater. The results of the analyses further show, as would be expected, that false non-inferiority conclusions can arise as the positive predictive value decreases. Specifically, as the PPV falls below 0.7, for studies with a power of 0.8 and a non-inferiority margin of 0.1, the risk of falsely concluding non-inferiority rises to levels that may be unacceptable. However, the results do suggest that if the PPV is in the range of 90% then the risk of falsely concluding non-inferiority when the investigational treatment is actually inferior is generally small.

There are limitations to the calculations of type I error performed by the sponsor. Firstly, it is only possible to consider a limited number of scenarios, though it is believed that the assumptions considered by the sponsor are reasonable. Secondly, the scenarios are simplified. For example, the sponsor considers scenarios in which all subjects are enrolled based on the results of the galactomannan assay. The galactomannan assay is only one of three possible mycological criteria that may be used for classifying a subject as having probable invasive aspergillosis for the population of subjects with hematologic malignancies or recipients of HSCT and is not used at all for the diagnosis of proven invasive aspergillosis. Thirdly, it assumes that the values of sensitivity and specificity are accurately measured. The sensitivity and specificity of the galactomannan are based on comparisons to culture results which are known to have low sensitivity. Lastly, it does not consider the other two components that are considered in the assessment of assay sensitivity of a non-inferiority study, namely historical evidence of sensitivity to drug effect and the constancy assumption. If there is information that there is historical evidence of sensitivity of drug effect in a population similar to that used for future non-inferiority trials using the galactomannan as one possible enrollment criteria, then the constancy assumption would have been met in terms of the enrollment criteria and the concern with the use of the galactomannan assay causing a bias towards non-inferiority

will be far less of concern. For further discussion of the principles of non-inferiority trials, refer to the draft Guidance for Industry: *Non-Inferiority Clinical Trials* (www.fda.gov).

Though the sponsor's calculation of type I error helps to explore the inter-relationship between positive predictive value (through the false positive rate) and type I error, it is understood that these calculations are overly simplistic. For any future non-inferiority study, a non-inferiority margin justification will be needed and that justification needs to consider the assay sensitivity of the non-inferiority study. It is believed that there is adequate historical evidence of drug effect and that this drug effect is robust over varying levels of the diagnosis of invasive aspergillosis infection (refer to the Statistical Review written by Cheryl Dixon and Karen Higgins for full discussion on this matter). A test with a high positive predictive value and high specificity would be most reliable for making the diagnosis of invasive aspergillosis in clinical trials and thereby more likely to ensure that patients who actually have the disease are enrolled.

4. Conclusions

Animal studies demonstrate that the galactomannan assay in BAL fluids correlates with tissue fungal burden as well as with the course of the disease, as galactomannan concentrations tend to be higher in the later stages of the disease or in more severe disease. Clinical results also correlate with results from animal studies in that the sensitivity of the assay is higher among proven and probable patients with IPA, thus suggesting good diagnosis potential. Variables such as the prevalence of the disease in different populations i.e., hematological malignancy versus non-hematologic populations and concomitant use of antifungal agents affect the sensitivity of the assay.

Overall, the evidence from animal and clinical studies indicate that the galactomannan is a sensitive and specific test in BAL specimens for diagnosis of invasive aspergillosis in patients with hematologic malignancy and it is this population that is the main focus of clinical trials of invasive aspergillosis. The strength of the galactomannan assay is that a negative result often correlates with absence of disease. Increasing the cut-off index ≥ 1.0, increases the specificity and PPV value of the galactomannan assay and reduces the number of false positives, with little effect on sensitivity. It is recommended that the galactomannan assay in BAL fluids be used in conjunction with clinical and radiological findings as outlined in the EORTC/MSG criteria for enrollment of "probable" IPA patients in clinical trials. Following enrollment, every effort should be made to comply with the current standard practice of obtaining a fungal smear and culture on all BAL samples, so as to minimize false positive results. Results of fungal smears and fungal cultures must be clearly documented on the case report form for each BAL sample so that the presence of *Aspergillus* species or rare fungal pathogens such as *Penicillium, Paecilomyces, Geotrichum,* and *Histoplasma* that may cross react with the galactomannan assay is documented (for details see Platelia *Aspergillus* EIA test brochure). Patients receiving these antibiotics produced by *Penicillium* sp (ampicillin, amoxicillin and piperacillin-

tazobactam) should be excluded and prior use of antibiotics should be documented. The use of plasmalye solution should also be an exclusion criterion.

It is recommended that galactomannan can be qualified for use as biomarker for diagnosis of in "probable" invasive aspergillosis patients used in conjunction with clinical and radiological findings in neutropenic patients with hematological malignancies or recipients of HSCT. It is suggested that the galactomannan assay in BAL fluid be considered positive, based on testing of two aliquots of the same sample, at a cut-off index ≥ 1.0 for enrollment of "probable" patients into clinical trials of antifungal agents for treatment of invasive aspergillosis.

5. References

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APPENDIX I - *In vitro* Studies

I.1. Reproducibility studies

1. Husain *et al*. (2008), study is a compilation of laboratory results conducted at MiraVista Laboratory from two studies (Clancy *et al*., 2007 and Husain *et al*., 2008) (See *Appendix III*). Parts of the dataset were also available for an independent review.

   The authors assessed the reproducibility of the Platelia *Aspergillus* EIA (BioRad Laboratories) in BAL and serum. BAL specimens that were negative by Platelia *Aspergillus* EIA, at a cut off of ≥ 0.5, were reconstituted with the kit positive and threshold controls. Reconstituted positive and cut-off controls in normal human serum were used as comparators as described in the test brochure. However, the details of the methods were not specified. A total of five aliquots of the positive controls and cut-off control were tested in BAL and serum on two consecutive days. The average, standard deviation and coefficient of variation (CV%) was calculated for the positive and cut-off controls. Overall, there was minimal degree of variability of the samples performed in the same analytical run or on different days in serum as well as in BAL (Table I-1). The CV% of the galactomannan assay for testing of serum appears to be higher compared to BAL (CV <10%). The reason for a higher positivity for both controls in BAL than in serum is unknown but could suggest that there may be some variability based on the type of specimen.

   Overall, the results suggest that there was low variability (CV%; 2-6%) of the performance of the galactomannan assay in BAL specimens to known concentrations of galactomannan indicating that the galactomannan assay in BAL has good precision.

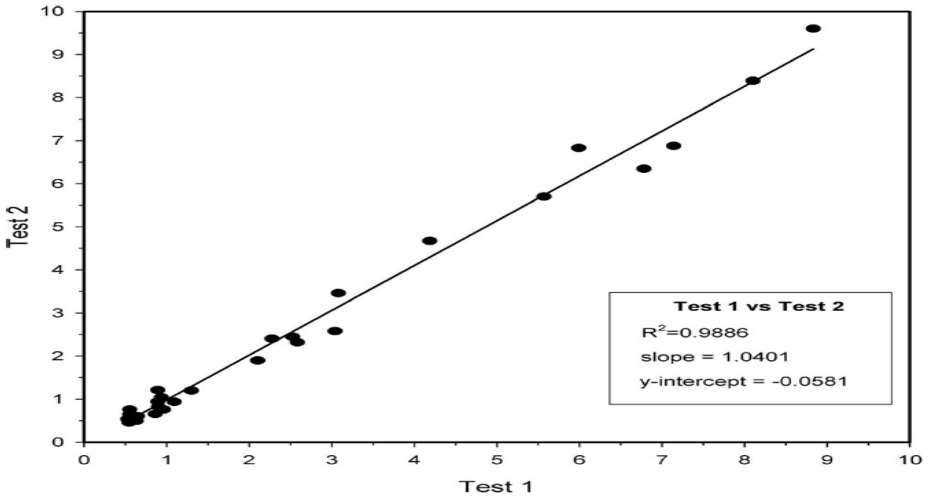
   Table I-1: Galactomannan detection in BAL and serum reconstituted using the kit positive and threshold controls

Controls	Serum GM index		BAL GM index	
	mean ± SD	CV%	mean ± SD	CV%
Positive				
Day 1	2.93 ± 0.35	12%	3.40 ± 0.20	6%
Day 2	3.23 ± 0.25	8%	3.61 ± 0.09	2%
Threshold				
Day 1	0.61 ± 0.12	19%	0.86 ± 0.05	6%
Day 2	0.59 ± 0.06	10%	0.91 ± 0.03	4%

   BAL specimens were obtained from the two studies in solid organ transplant patients that were performed at MiraVista Diagnostics laboratory. Specimens from patients based on surveillance, routine monitoring following transplantation or diagnosis of suspected invasive *Aspergillus* infection were collected and stored until testing. The timing between storage and testing was not specified. A positive result was based on index value ≥ 0.5. All positive samples were verified by repeat testing of another aliquot of the same sample the following day. The authors evaluated the reproducibility of the galactomannan assay by comparing the result in the first and second test in patients that had a positive result. A total of 23 patients had a positive BAL galactomannan result at a cut-off ≥ 0.5. The reproducibility was 93.3% for

specimens between 0.5 and 0.9 (n = 15) and 100% for those ≥1.0 (n = 17) (Figure I-1). The results suggest that a repeat test using a different aliquot of the same BAL specimen was reproducible at low (0.5 – 0.9) and at higher (≥1.0) levels of galactomannan.

Figure I-1: Evaluation of the galactomannan assay in positive BAL specimens performed on consecutive days



Note: The horizontal axis represents the galactomannan assay results of the initial testing (test 1) and the vertical axis represents the galactomannan assay result of the repeat testing (test 2)

2. An unpublished study report by the Dr LJ Wheat, Miravista Diagnostics, evaluated the precision, reproducibility, and the limit of detection in BAL in specimens. Known concentrations of the *Aspergillus* galactomannan from the assay's positive controls and purified *Aspergillus* galactomannan were diluted in BAL specimens. Preparation of the purified *Aspergillus* galactomannan was based on a previously published method. BAL and serum specimens were collected from patients known to be negative by the Platelia *Aspergillus* EIA. Saline was used as a comparator. The number of measurements per concentration was not specified. The results were expressed as galactomannan index values (Table I-2). The galactomannan indices in BAL were similar to saline. However, there was a trend that the galactomannan levels in serum were lower than BAL though the significance of this finding is unknown. The sponsor stated that the limit of detection in both BAL and serum was 0.5 ng/mL. The results suggest that the constituents of BAL did not alter the detection of galactomannan.

Table I-2. Comparison galactomannan detection in serum, saline, and BAL

Material	Serum-GMI	Saline-GMI	BAL-GMI
Kit positive control	2.97	4.14	4.19
Kit cut off control	0.92	1.27	1.27
GM 10 ng/ml	1.40	2.33	2.26
GM 5 ng/ml	1.44	1.62	1.98
GM 1 ng/ml	0.91	0.79	1.24
None	0.08	0.07	0.16

The sponsor also assessed the variability of the galactomannan assay between lots and runs and between days to known concentrations of *Aspergillus* galactomannan in BAL specimens. The variability of the galactomannan assay between aliquots and runs was evaluated by testing of BAL and serum samples spiked with high and moderate concentrations of *Aspergillus* galactomannan and a negative control. The variability of the assay was also assessed by testing the same specimen in BAL and serum on two separate days (Table I-3). The coefficient of variation between lots and days were low for BAL and serum samples that were positive compared with high CV% observed for the negative control specimen values. In general, the variability of the galactomannan assay was low as measured by the CV% (range 7% - 12%) for moderate to high concentrations of galactomannan between aliquots and between days, suggesting that the galactomannan assay has precision in BAL specimens.

Table I-3: Comparison of the galactomannan indices at known concentrations of galactomannan in spiked serum and BAL samples between runs and between days.

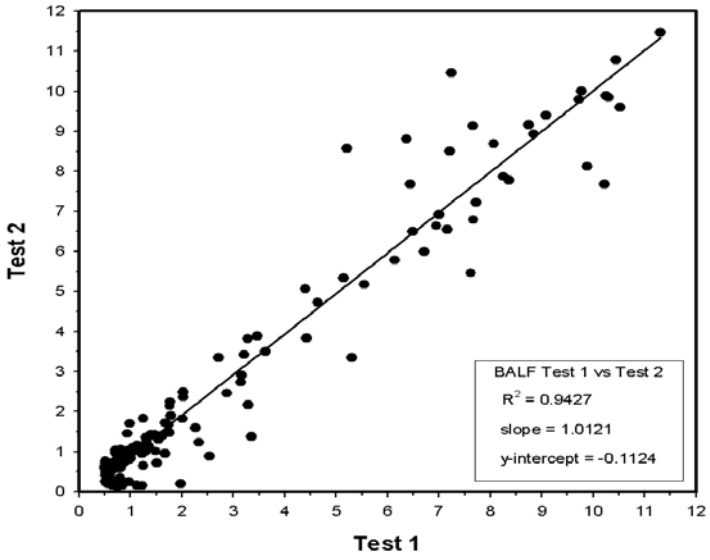
Spiked specimen	Intra-assay (3 aliquots)** Mean ± SD (CV%)		Inter-assay (2 days)*** Mean ± SD (CV%)	
	Serum-GMI	BAL-GMI	Serum-GMI	BAL-GMI
High positive* (4.0 ng/mL)	2.01 ± 0.07 (3.3%)	4.39 ± 0.43 (9.8%)	2.05 ± 0.097 (4.7%)	4.50 ± 0.30 (6.8%)
Moderate positive* (2.0 ng/mL)	0.98 ± 0.85 (8.7%)	2.48 ± 0.31 (12.3%)	1.11 ± 0.16 (14.5%)	2.54 ± 0.26 (10.4%)
Negative* (0 ng/mL)	0.08 ± 0.03 (41.2%)	0.21 ± 0.03 (14.7%)	0.08 ± 0.03 (40.6%)	0.16 ± 0.06 (33.3%)

GMI = galactomannan index
*Spiked samples
**Intra-assay = mean ± SD of same sample run at 3 different times;
***Inter-assay = mean ± SD of same sample run on 2 separate days

The sponsor also assessed the results of the first and second test on stored specimens that were positive at MiraVista Diagnostics in 2007. The patient population from which the BAL specimens were collected was not specified. A positive result was based on index value ≥ 0.5. The sponsor stated that all positive samples were retested based on when a specimen had an index value ≥ 0.5, the test was repeated on another aliquot of the same sample. Therefore, a confirmed positive on repeat testing was based on testing of two samples and both results yielded an index of ≥ 0.5. Results of test 1 and test 2 in BAL were evaluated by linear regression. The linear regression results showed that the galactomannan assay results were reproducible in BAL specimens that

were retested ($R^2 = 0.9427$) (Figure I-2), indicating that a positive single test in BAL specimen when retested is more likely to give similar results.

Figure I-2: Linear Regression of samples retested on the same aliquot in BAL specimens in determining positivity



Results of test 1 and test 2 in BAL were also evaluated based on categories , that is, specimens with low levels (indices 0.5 – 0.9) and higher levels (indices ≥ 1.0) of galactomannan. The proportion of BAL specimens on repeat testing that had lower reproducible results at indices from 0.5 to 0.9 (75%) than the at indices ≥ 1.0 (96%) (Table I-4). The results suggest that caution is needed when interpreting a single test in BAL with an index value between 0.5 and 0.9. Though overall the reproducibility in BAL was higher than in serum, similar to BAL, repeat testing of serum specimens showed lower reproducible at indices between 0.5 and 0.9 than at indices ≥ 1.0. A single positive test in BAL at cut-off index ≥ 1.0 was reproducible at 100%. The results suggest that repeat testing using a different aliquot of the same BAL specimen were reproducible at higher levels (index ≥ 1.0) of galactomannan and may be variable at low levels (0.5 – 0.9) of galactomannan.

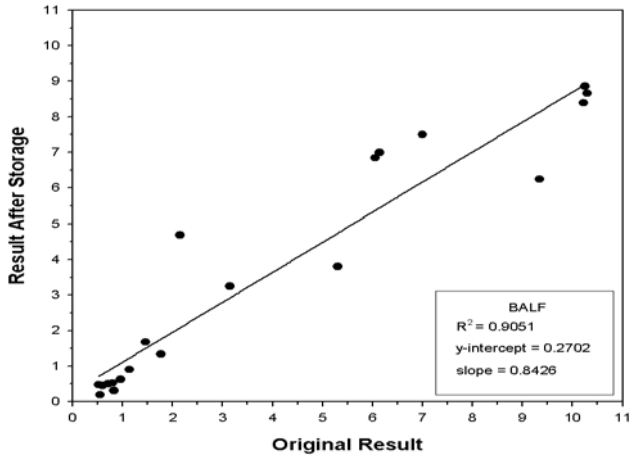
Table I-4: Proportion of BAL and serum specimens positive upon retesting

GMI range	Serum No./total (%)	BAL No./total (%)
0.5-0.9	50/89 (56)[1]	57/76 (75)
≥ 1.0	98/110(89)	80/83 (96)

Note: GMI = galactomannan index; BAL = bronchoalveolar lavage;
[1]Must be reproducible to be reported as positive

Repeat testing was performed another on aliquot of the same sample following storage at -20°C for up to two years. A total of 20 clinical BAL specimens were stored for up to two years at -20°C. The results showed that, the galactomannan assay for testing of BAL specimens that were retested after freezing, 80% (16/20) of the samples had galactomannan index ≥ 0.5 (R^2 = 0.9051) (Figure I-3) thereby suggesting that the results of the galactomannan are reproducible when retested after freezing.

Figure I-3: Linear Regression of samples retested on the same aliquot in BAL specimens following storage at -20°C for 2 years in determining positivity



Overall, the data suggest that the galactomannan assay demonstrates reproducibility between runs, between days, and when retested on the same aliquot as well as when retested after freezing. However, there was some variation in results from samples that had low index values, with particularly high CV% and in samples that were positive between 0.5 and 0.9, suggesting caution in the interpretation of results at low index levels (i.e. 0.5 – 0.9).

I.2. Cross Reactivity of BAL specimens

(1) Wheat *et al*. (2007), evaluated the *Aspergillus* galactomannan EIA from BAL fluid specimens submitted to a local laboratory. No description of patients' signs and symptoms of disease were specified. The specimens were tested for the presence of *Histoplasma capsulatum* and *Aspergillus* galactomannan antigens. The second-generation *Histoplasma* antigen EIA test (MiraVista Diagnostics, Indianapolis, IN) was used and results of ≥ 1 unit, on retested samples, were reported as positive for histoplasmosis. The Platelia *Aspergillus* EIA, performed according to manufacturer's specification, was used and results with an index value ≥ 0.5 were reported as positive in BAL specimens. A total of 39 BAL specimens were evaluated of which 11 were positive *for H. capsulatum* and 28 were negative for *H. capsulatum*. Of the 11 BAL specimens that were positive for *H. capsulatum* (Table I-5), seven (63%) were positive for galactomannan (Figure I-4). The *Histoplasma* antigen EIA results from these seven patients ranged from 2.2 - 61.7

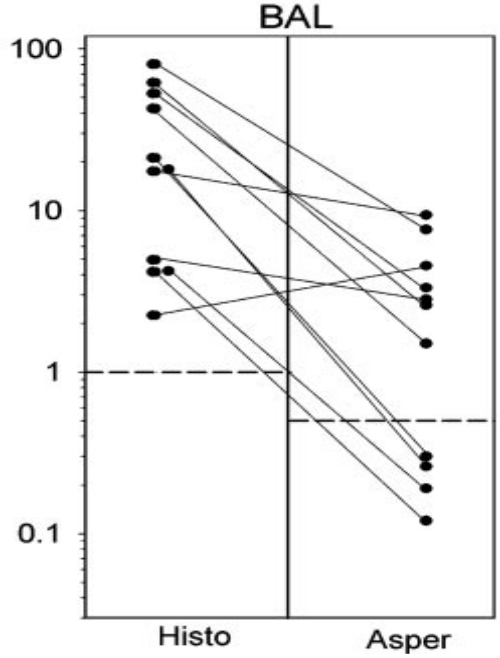
units compared to a range of 4.2-21.2 units in the remaining four patients. The galactomannan results for each of these seven patients were not specified. In contrast, of the 28 BAL specimens that were negative for *H. capsulatum*, 18 (64%) had positive galactomannan index values ranging from 0.84 to 9.29 and the remaining10 BAL specimens were negative with index value < 0.5, however, the range was not specified. Fungal cultures were not reported for the BAL specimens. The results indicate that false positive results in the galactomannan assay can occur for BAL specimens from patients with histoplasmosis.

Table I-5: Results of the Histoplasma EIA and galactomannan assay in 39 BAL specimens

BAL Fluid specimens (n=39)	No. GM positive/ No. tested	Second generation *Histoplasma* EIA result
Histoplama EIA positive, (n=11)	**7/11**	**2.2 – 61.7**
Histoplama EIA negative, (n=28)	**18/28**	**-**

Note: GM = galactomannan tested using the Platelia *Aspergillus* EIA

Figure I-4: Comparison of antigen levels in BAL fluids from 11 patients with histoplasmosis tested using the Platelia *Aspergillus* EIA and second-generation *Histoplasma* EIA (left) kits

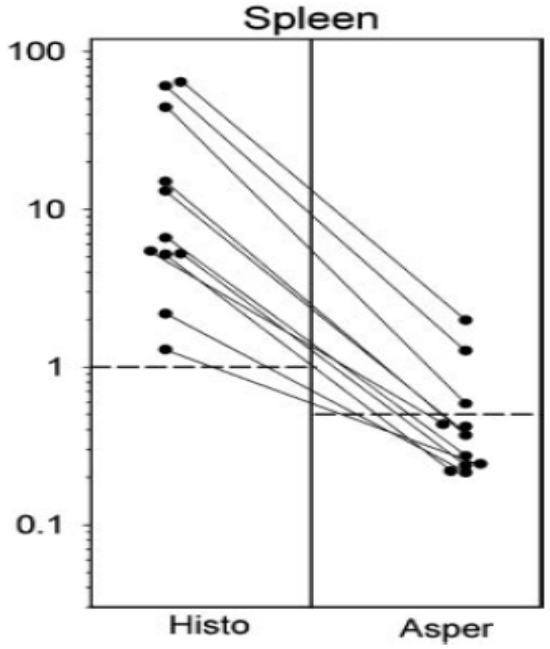


Note: The vertical axis depicts antigen units for the Histoplasma EIA and Platelia Aspergillus EIA. The cut-offs for positivity are 1.0 unit for the second-generation *Histoplasma* EIA and 0.5 GMI for the Platelia *Aspergillus* EIA, as shown by broken horizontal lines. Results for the same specimens tested in both assays are connected by solid lines.

In another experiment, Wheat *et al*. (2007), evaluated the Platelia *Aspergillus* EIA (BioRad Lab) galactomannan assay in experimental animal models of histoplasmosis.

Non-immunosuppressed mice (n = 11) were infected intranasally with 10^6 organisms of *H. capsulatum* yeast cells. Mice were sacrificed at day 10 post-infection. The spleen was harvested from each mouse and homogenized in 2.0 mL of sterile RPMI medium. The 11 splenic homogenate suspensions were diluted to a 1:10 dilution and tested using the *Histoplasma* antigen EIA and 1:1 dilution was used in the Platelia *Aspergillus* EIA. Three splenic homogenates had positive galactomannan index values ≥0.5 and the Histoplasma antigen EIA results for each of the homogenates were 44.4 units, 60.5 units and 64.0 units, respectively (Figure I-5). The splenic homogenates from the remaining eight mice were negative for galactomannan with galactomannan index values < 0.5 and had *Histoplasma* antigen EIA results ranging from 1.3 to 15.0 units. These results indicate that false positive galactomannan results can occur in patients infected with *Histoplasma capsulatum.*

Figure I-5: Comparison of antigen levels from spleen tissues of mice with histoplasmosis



(2) Hage *et al*. (2007), tested the plasmalyte solutions for the presence of galactomannan by the Platelia *Aspergillus* galactomannan EIA (BioRad Laboratories). Plasmalyte is an intravenous solution used to perform bronchoscopy. A sample of the instilled fluid is usually re-collected and sent for analyses by centrifuging the BAL fluid to form a pellet for culture and the supernatant is sent for galactomannan testing. Plasmalyte contains sodium gluconate (503 mg/100 mL) produced by fermentation in mold cultures such as *Aspergillus niger, Aspergillus flavus,* and *Penicillium* and therefore can cross react with the galactomannan assay. The authors noted that 19 consecutive BAL specimens collected using Plasmalyte solution from 19 patients at a single institution, were positive with the *Aspergillus* galactomannan EIA. The galactomannan index values ranged from 4.1 to 8.2 (Table I-6). Of the 19 samples,

17 were culture negative, one specimen had 1+ growth of *A. fumigatus* and another had 2+ growth of *Paecilomyces* species. Four different lots of the plasmalyte solution showed positive results (range 5.4 - 5.6). Five patients had their bronchoscopy repeated using normal saline and retested using the *Aspergillus* galactomannan EIA; all five patients were negative for galactomannan (Table I-6). The timeline between the original and repeat BAL specimens was not specified.

Table I-6: Galactomannan testing in BAL fluid using Plasmalyte compared to normal saline

Test specimen	No. positive/no. tested	Galactomannan index
Human BAL fluid with Plasmalyte for lavage	19/19	4.1–8.2
Human BAL fluid with normal saline for lavage	0/5	0.24–0.34
Plasmalyte solution (four different lots)	4/4	5.4–5.6

The authors also tested tenfold dilutions (range from 10^3 to 10^6) of Plasmalyte solution used to obtain the BAL specimens. The galactomannan assay was performed according to the manufacturer's instructions (BioRad Laboratories). Results showed that a 1 in 10 dilution of Plasmalyte yielded a result comparable to the cut-off control (1 ng/mL). The authors estimated that each bottle of plasmalyte contained about 10 ng/mL galactomannan. The authors' further estimated that approximately100 mL of plasmalyte solution (used during bronchoscopy) is sent for BAL galactomannan testing which would contain approximately 1 μg of galactomannan. The use of Plasmalyte should not be used for bronchoscopies in immunocompromised patients at risk for IPA. If Plasmalyte solution is used, it should be clearly documented and those patients should be excluded from clinical trials of IPA.

APPENDIX II - Animal Studies

II.1. Infection with *Aspergillus fumigatus*

1. Becker *et al*. (2000), evaluated the Platelia *Aspergillus* EIA (Sanofi Diagnostics) in female RP albino rats after inoculation with *Aspergillus* species. The polymerase chain reaction (PCR) for detection of genomic DNA of *A. fumigatus* and culture for the presence of fungal organism were used as comparators. Rats were rendered neutropenic by intraperitoneal administration of cyclophosphamide (75 mg/kg) five days before inoculation, followed by repeated doses (60 mg/kg) after inoculation at day 3 and day 7. Animals were treated daily with ciprofloxacin (660 mg/L) and polymyxin B (100 mg/L) in their drinking water and intramuscular doses of amoxicillin (40 mg/kg/day) for bacterial prophylaxis for the duration of the experiment. An infection was initiated by intra-tracheal route at day 0 with 2 x 10^4 of the conidia of *Aspergillus fumigatus*. Control animals were inoculated with phosphate buffered saline (PBS). On days 1, 3, 5 and 7 after inoculation, bronchoscopy was performed using PBS. In addition, serum specimens were processed for fungal cultures, galactomannan assay, and PCR. The presence of disseminated fungal infection was determined by harvesting and culturing the lungs, liver, spleen, and brain of rats (n = 4 per group) sacrificed on days 1, 3, 5 and 7 after inoculation. The authors stated that the majority of rats had disseminated disease as determined by positive fungal cultures and presence of genomic DNA by the PCR method; however, the culture results in different tissues were not included. The Platelia galactomannan assay in BAL and serum was performed according to manufacturer's instructions. Each plate was calibrated using normal rat serum spiked with galactomannan at concentrations of 0, 1, 1.5, 2, 3, 4, 6, 8, and 12 ng. Results for the EIA were considered positive if the optical density at 450 nm was higher than the optical density of the threshold control (i.e., 1.0 ng/mL), equivalent to a galactomannan index value ≥ 1.0. The PCR method used was an experimental procedure with primers amplifying the 18s rRNA gene and analyzed by Southern blot analysis using an *Aspergillus* specific DNA probe. Fungal cultures of the BAL fluids for all rats were positive on day 1 after inoculation, with the number of CFUs decreasing over time and were negative by days 5 to 7 (Table II-1). However, the number of animals positive by the galactomannan assay in BAL increased over time. The sensitivity of the galactomannan assay in BAL increased from 25% on day 1 (galactomannan concentration range, 1 - 2.7 ng/mL) to 100% by day 5 (galactomannan concentration range, 5.4 - 157 ng/mL). PCR showed inconsistent results in BAL with only one or two rats positive on different days.

Table II-1: PCR, EIA and fungal culture analyses of BAL fluid and blood of rats with IPA after dissection.

Day	No. of animals	BAL fluid				Blood			
		PCR (no. of rats positive)	ELISA (no. of rats positive)	Median concn (ng/ml) of GM (range)	Culture (no. of rats positive [mean CFU/ml])	PCR (no. of rats positive)	ELISA (no. of rats positive)	Median concn (ng/ml) of GM (range)	Culture (no. of rats positive [mean CFU/ml])
1	8	2	2	<1 (<1–2.7)	8 (4)	0	0	<1 (<1)	0 (0)
3	5	1	2	<1 (<1–11)	3 (1)	1	3	4.4 (<1–6.2)	0 (0)
5	5	2	5	11.6 (5.4–157)	0 (0)	1	4	10.2 (<1–26.2)	0 (0)
7	5	1	5	8.8 (3.9–114)	1 (0)	2	5	19.2 (7.6–48)	0 (0)

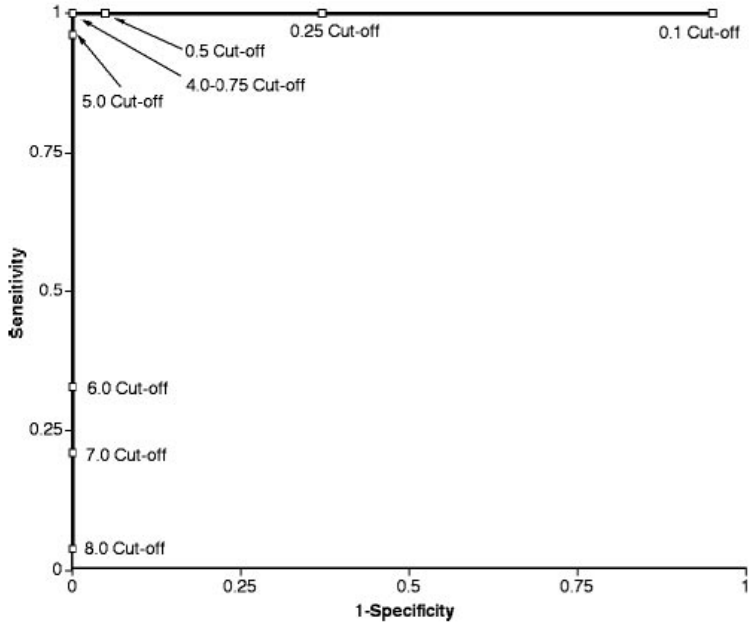
[a] The in-house PCR method was used.

Fungal blood cultures were negative on all days. Similar to the galactomannan assay positivity in BAL samples, the number of animals positive for galactomannan in serum increased over time. The sensitivity of the galactomannan assay in serum increased from 0% on day 1 (galactomannan concentration < 1 ng/mL) to 100% on day 7 (galactomannan concentration range: 7.6 - 48 ng/mL). Similar to BAL, the PCR results in serum were inconsistent with only one or two rats positive on different days.

Overall, the results suggest that during the initial stages of infection (up to day 3) culture-based methods in BAL were more sensitive than blood cultures or galactomannan detection in BAL or serum. However, by day 5 and day 7 of the infection, detection of galactomannan in BAL and serum was more sensitive than cultures. Blood cultures were not positive at any of the time points tested. These findings suggest that galactomannan concentration in BAL and serum are associated with the later stages of the disease or disease severity.

2. Francesconi *et al*. (2006), evaluated the Platelia *Aspergillus* EIA (BioRad Laboratories) galactomannan assay for testing of BAL fluid in neutropenic NZW rabbits after inoculation with strain ATCC MYA-1163 of *A. fumigatus*. A total of 24 neutropenic NZW rabbits were infected by endotracheal inoculation with 1 x 10^8 conidia. Nineteen healthy rabbits served as controls. Rabbits were considered to have IPA when lung tissues showed pulmonary lesions and had positive histology and quantitative cultures demonstrating high titers of *Aspergillus* organisms. Though, the time of necropsy and the fungal load (viable counts) were not specified, a BAL fluid specimen was obtained from each rabbit at the time of postmortem examination by infusing a resected lung preparation with normal saline . The galactomannan assay was performed according to manufacturer's instructions. The authors stated that the sensitivity and specificity was 100% with galactomannan index values ranging from 0.75 to 4.0. A receiver operating characteristic (ROC) curve that graphs the changes in the true positive fraction to the false positive fraction at different index values was plotted. The results in Figure II-1 suggest that the optimal performance of the galactomannan assay in BAL was achieved at an index value ≥ 0.75. Lowering the index value to less than 0.75 resulted in lower specificity while retaining sensitivity (100%). However, a higher index ≥ 4.0 decreased the sensitivity while retained the specificity. In corresponding serum samples, the galactomannan EIA had 100% sensitivity at an optical density cut-off ratio of 0.5.

Figure II-1: Receiver operator curve (ROC) analysis for BAL galactomannan assay in NZW rabbits



In another experiment, the authors examined the effect of antifungal treatment on the performance characteristics of Platelia *Aspergillus* EIA (BioRad Laboratories) in BAL fluid from experimentally induced IPA in neutropenic NZW rabbits. Culturing of fungal organisms from lung tissues was used as a comparator. Neutropenic rabbits were infected by endotracheal inoculation with 1 x 10^8 conidia of *A. fumigatus*, ATCC MYA-1163 strain. Antifungal treatment was administered for 12 days starting at 24 hours after endotracheal inoculation of *A. fumigatus* conidia. Antifungal drugs from each of the major classes were examined: a triazole (ravuconazole: 5 and 10 mg/kg), an echinocandin (micafugin: 0.5 to 2 mg/kg) and a polyene (amphotericin B deoxycholate: 1 mg/kg). A BAL was performed as described above. At an index value ≥ 0.75, the sensitivity of the galactomannan assay, in BAL from rabbits with IPA after treatment with antifungal therapy, was lowered from 100% to 92% with no change in the specificity and the area under the curve was reduced by 1% (Figure II-2). Similar to BAL, the sensitivity of the galactomannan assay in serum and culture samples declined to 90% and 16%, respectively (Table II-2).

Figure II-2: Receiver operator curve (ROC) analysis in BAL in (A) untreated and (B) treated animals with experimentally induced IPA

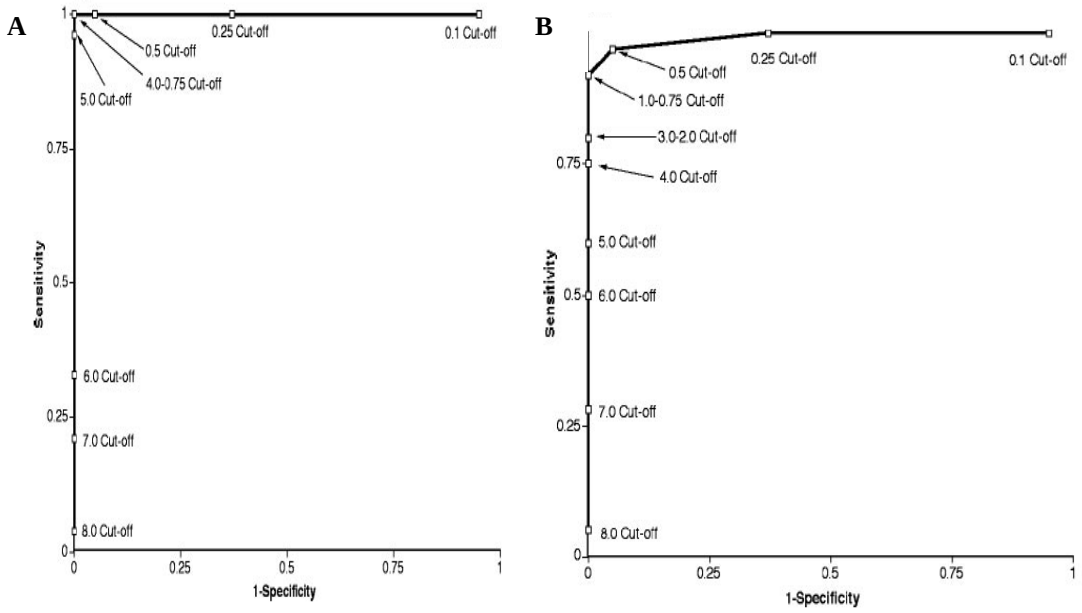


Table II-2: Sensitivity of galactomannan EIA and residual fungal burden in BAL in animals with experimentally induced IPA

Treatment group	% Sensitivity (no. of animals)					
	BAL fluid culture	BAL GM EIA	Serum GM EIA	BAL qPCR	GM EIA in culture-negative BAL fluid	qPCR in culture-negative BAL fluid
Untreated (controls)	42 (24)[b,c]	100 (24)[b]	100 (17)[b]	80 (24)[c]	100 (13)	70 (13)
Treated	16 (61)[b,d]	92 (61)[b]	90 (60)[b]	50 (61)[d]	90 (51)	47 (51)

[a] Data are presented as percentages of sensitivity of assays for animals with microbiologically and histologically documented aspergillosis.
[b] $P < 0.0001$; all P values represent comparisons of EIA and qPCR methods to BAL fluid culture.
[c] $P = 0.02$.
[d] $P = 0.0002$

The authors also evaluated the galactomannan assay based on the type of antifungal drugs administered. The mean galactomannan index in BAL in rabbits treated with ravuconazole and amphotericin B were lower than in the untreated controls, though the mean galactomannan results in BAL in rabbits treated with micafungin were similar to that of untreated rabbits (Table II-3). The mean colony forming units of *A. fumigatus* was significantly lower in BAL fluid cultures from rabbits treated with micafungin and amphotericin B than in the untreated controls. However, in animals treated with ravuconazole though the fungal load was reduced, it was not statistically significant. Antifungal treatment reduced the sensitivity of the galactomannan assay to 92% at an index cutoff of ≥ 0.75, with no change to the specificity (100%). These findings suggest that mold-active antifungal agents, specifically the triazoles and polyenes, lower the residual fungal load in lung tissue and the sensitivity of the galactomannan assay in BAL with no overall effect on the specificity.

Table II-3: Effect of therapy on the diagnostic yield of galactomannan assay and residual fungal burden in BAL in animals with experimentally induced IPA

Treatment group (no. of animals)	Diagnostic yield of[b]:		
	EIA (GMI)	PCR (log DNA/ml)	Culture (log CFU/ml)
Untreated controls (24)	6.0 ± 0.18[d,e]	3.3 ± 0.46[e,f]	0.70 ± 0.2[e]
Animals treated with:			
Ravuconazole (17)	3.2 ± 0.50[d]	1.6 ± 0.47[e]	0.42 ± 0.24
Micafungin (26)	6.8 ± 0.30	2.1 ± 0.37[e]	0.21 ± 0.10[e]
Deoxycholate amphotericin B (18)	4.7 ± 0.50[e]	1.2 ± 0.40[f]	0.16 ± 0.15[e]

[a] All *P* values (see below) represent comparisons of treatment groups to untreated controls.
[b] Data are means ± SEM.
[c] DNA data are expressed in femtograms.
[d] $P < 0.0001$.
[e] $P < 0.05$.
[f] $P = 0.003$.

3. Khan *et al*. (2008), evaluated the Platelia *Aspergillus* EIA (BioRad Laboratories) galactomannan assay in BAL fluid from experimentally induced IPA in neutropenic rats. Rats were ***immunosuppressed*** with a single intraperitoneal injection of cyclosphamide (70 mg/kg) given 2 days before inoculation. Thirty-six rats were infected intravenously with 1 x 10^6 conidia of *A. fumigatus* CBS 113.26 strain. Six healthy rats selected randomly were used as controls. Animals were killed in groups of six on 1, 3, 5, 7 and 9 days post-infection and BAL and blood collected. BAL fluid specimen was obtained using phosphate buffer saline and centrifuged at 10,000 g for 10 minutes. The supernatant was used for testing by the galactomannan assay and the sediment was used for culture. For the galactomannan assay in both serum and BAL, an index value ≥ 0.5 was considered as a positive test. The latex agglutination test for the detection of the β-D-glucan antigen (BDG) and a nested-PCR using primers specific for rDNA region of *A. fumigatus* were used as comparators. Lungs from all infected rats (n=30) were cultures positive, however none of the BAL fluid or blood specimens were culture positive (Table II-4). Twenty-three rats had an index value ≥ 0.5 and 15 rats at an index value ≥ 1.5 for the testing of BAL and serum samples (Table II-5 and II-6). The number of rats positive for the assay in BAL specimens at various index values over 9 days post-infection are shown in Table II-5. The sensitivity of the galactomannan assay in BAL at an index ≥ 0.5 ranged from 33% to 100% over the 9 days post-infection with mean index values of 0.82 to 2.32. The specificity of the galactomannan assay in BAL was 100%, since none of the six uninfected control rats yielded positive galactomannan results. A ROC curve was not performed to determine the optimal index value, though increasing the index value ≥ 1.5 resulted in an overall lower sensitivity of 50% with no effect on the overall specificity. The results suggest that an index value in BAL between 0.5 and 1.5 may be optimal in diagnosis rats with IPA.

Table II-4: Individual line data of results of culture, galactomannan, latex agglutination and PCR specimens of *A. fumigatus* infected rats.

Animal group	Post infection (days)	Culture		Serum			BAL	
		Lung tissue	BAL	BDG	GM	PCR	GM	PCR
I (n = 6)	1	+	−	+	+	+	+	−
		+	−	−	+	+	+	+
		+	−	+	+	−	+	+
		+	−	+	+	−	+	+
		+	−	+	−	−	+	+
		+	−	+	+	+	+	+
II (n = 6)	3	+	−	+	+	+	−	+
		+	−	+	+	+	+	+
		+	−	+	+	−	−	−
		+	−	+	+	+	+	+
		+	−	+	+	−	+	+
		+	−	+	+	+	+	+
III (n = 6)	5	+	−	+	+	+	+	+
		+	−	+	+	+	+	+
		+	−	−	+	+	+	−
		+	−	+	+	+	−	+
		+	−	+	+	+	+	+
		+	−	+	+	+	+	+
IV (n = 6)	7	+	−	+	−	−	+	+
		+	−	+	+	+	+	+
		+	−	+	−	−	+	−
		+	−	+	+	+	+	+
		+	−	+	+	+	+	−
		+	−	+	+	−	+	+
V (n = 6)	9	+	−	−	−	+	+	+
		+	−	+	+	−	−	−
		+	−	+	+	+	−	−
		+	−	−	−	−	+	−
		+	−	−	−	−	−	−
		+	−	−	−	+	−	+
		30(100)	0(0)	24(80)	23(76)	19(63)	23(76)	21(70)

Note: GM = galactomannan a cut-off value of 0.5 was taken as positive; BDG = latex agglutination results for 1-3-β-D-glucan antigen a cut-off value of 80 pg/mL was taken as positive; PCR = polymerase chain reaction;

Table II-5: Galactomannan levels in BAL of 30 rats infected with *A. fumigatus* and sacrificed at different time points

Post infection (days)	No. rats	GM index values (mean + SD*)	No. rats at different cut-off index		
			<0.5	≥0.5–1.5	>1.5
1	6	2.26 + 0.91	0	1	5
3	6	2.32 + 1.50	2	1	3
5	6	1.80 + 1.40	1	3	2
7	6	1.65 + 1.35	0	1	5
9	6	0.82 + 0.47	4	2	0

*Mean GM value in normal rats = 0.24+0.09.

Table II-6: Galactomannan levels in serum of 30 rats infected with *A. fumigatus* killed at different time points

Post infection (days)	No. rats	GM index values (mean+SD*)	No. rats at different cut-off index		
			<0.5	≥0.5–1.5	>1.5
1	6	1.61 + 1.45	1	3	2
3	6	3.35 + 0.09	0	0	6
5	6	3.45 + 0.16	0	4	2
7	6	2.08 + 1.12	2	0	4
9	6	0.97 + 1.66	4	1	1

*Mean GM in normal rats = 0.24 + 0.13.

The combination of the testing for galactomannan in BAL and serum samples at an index value ≥ 0.5 showed that 18 were positive in both BAL and serum, five rats were positive in BAL and negative in serum, five rats were negative in BAL and positive in serum and two rats were both negative for BAL and serum (Table II-7). The combined number of animals positive by the galactomannan assay in BAL and serum appears to be at the highest between days 1 and 7, with an overall combined sensitivity of 100% at a cut-off index ≥0.5. However, the combined sensitivity of the galactomannan assay at a cut-off index ≥0.5 in serum and BAL decreases to 67% on day 9. The clinical significance of this finding is unknown.

Table II-7: Sensitivity of BAL galactomannan assay for diagnosis of IPA at different time periods and cut-off values

	Sensitivity% (no. with indicated result/total) on Days				
Test	**1**	**3**	**5**	**7**	**9**
BAL GM					
Cutoff ≥ 0.5	100% (6/6)	67% (4/6)	83% (5/6)	100% (6/6)	33% (2/6)
Cutoff ≥ 1.5	83% (5/6)	50% (3/6)	33% (2/6)	83% (5/6)	0% (5/6)
Serum GM					
Cutoff ≥ 0.5	83% (5/6)	100% (6/6)	100% (6/6)	67% (4/6)	33% (2/6)
Cutoff ≥ 1.5	33% (2/6)	100% (6/6)	33% (2/6)	67% (4/6)	17% (1/6)
BAL or serum GM					
Cutoff ≥ 0.5	100% (6/6)	100% (6/6)	100% (6/6)	100% (6/6)	67% (4/6)

4. De Jesus *et al*. (2007), evaluated the Platelia *Aspergillus* EIA (BioRad Laboratories) in New Zealand White rabbits (n = 7) infected by intra-tracheal route with *A. fumigatus* ATCC MYA-1163 strain. BAL specimens were collected, though the time of collection post infection was not specified. The authors stated that sections of the lung tissues were harvested and homogenized using ice-cold phosphate buffered saline and were processed for fungal culture, though the details of the methods were not specified.

Lung tissues of all infected rabbits yielded *Aspergillus* in culture. All rabbits were positive for galactomannan with index values ranging from 0.8 to 4.2 in plasma and 1.5 to 6.8 in BAL fluid (Table II-8). The results in Table II-8 also show mice infected with *C. neoformans* were positive for the galactomannan assay in serum.

Table II-8: Results of Platelia *Aspergillus* EIA in BAL and liver homogenates specimens in rabbits infected with *A. fumigatus*

Specimen source	Assay[f]	No. of specimens positive for antigen/ no. tested	
		Aspergillus	*Cryptococcus*
Infected patients	Af GalM EIA	20/20[a]	0/25[b]
	Cn CPS LA	0/20[a]	24/25[b]
Mice experimentally infected with *C. neoformans*	Af GalM EIA	5/5[a]	0/8[c]
	Cn CPS LA	0/5[a]	8/8[c]
Rabbits experimentally infected with *A. fumigatus*	Af GalM EIA	7/7[d]	0/8[e]
	Cn CPS LA	0/7[d]	8/8[e]

[a] Serum.
[b] Serum or CSF.
[c] Spleen tissue.
[d] BAL fluid.
[e] Lung tissue.
[f] Af, *A. fumigatus*; Cn, *C. neoformans*.

II.2. Infection with *Aspergillus terreus*

Ahmad *et al*. (2007), evaluated the Platelia *Aspergillus* EIA (BioRad Laboratories) in BAL fluid from immunosuppressed mice. BALB/c mice were immunosuppressed with 4 intraperitoneal injections of cyclophosphamide (200 mg/kg) given 4 days and 1 day before inoculation, on the day of inoculation and 3 days after inoculation. Seventy-two mice were infected intravenously with 1 x 10^5 conidia of *A. terreus* CBS 106.25 strain. Twelve mice selected randomly were used as controls. Mice were killed in groups of 12 on days 1, 3, 5, 7 and 9 post-infection. Blood was collected from each animal at the time of sacrifice. BAL fluid was collected by exposing the trachea and lavaging the lung four times with phosphate buffered saline. The BAL specimens were centrifuged and the supernatant was used for galactomannan antigen detection by EIA. Fungal cultures were performed on the homogenized lung tissues, the sediment of BAL specimen after centrifugation and blood cultured on Sabouraud dextrose agar supplemented with chloramphenicol for the inhibition of bacterial flora. The authors stated that the lung tissues of all infected mice were culture positive, but the blood and BAL specimens were negative. Forty-eight mice were positive for galactomannan in BAL by EIA at galactomannan index value ≥ 0.5 and 17 mice at galactomannan index value ≥ 1.5 (Table II-9). The sensitivity of BAL galactomannan ranged from 58% to 92% at an index value ≥ 0.5 with mean galactomannan index values of 0.88 to 1.53 over the 9 days post-infection. There appears to be a decrease in number of mice which are positive by the galactomannan assay in BAL at an index value ≥ 0.5 between days 3 and 7. Increasing the cutoff value to ≥ 1.5 in BAL resulted in an overall lower sensitivity of 28% with no effect on specificity and PPV, however the overall NPV increased. A ROC analysis was not performed to determine the optimal performance of the

galactomannan assay in BAL. The author stated that the combined specificity, sensitivity, PPV and NPV in BAL specimens of 60 mice infected with *A. terreus* at a cutoff value of ≥ 0.5 was 80%, 100%, 100% and 52% respectively (Table II-10). In the corresponding serum samples, 47 mice were positive for galactomannan in sera by EIA using a cut-off value of ≥ 0.5 for galactomannan (Table II-11). The sensitivity and NPV for sera galactomannan was 78% and 48% respectively, with no change in the specificity and PPV (100%). The sensitivity of the assay in serum was lower than in BAL suggesting that the assay in BAL provides better discrimination than in serum in the diagnosis of IPA.

Table II-9: Galactomannan results in BAL of 60 mice infected with *A. terreus* and sacrificed at different time points

Postinfection (days)	No. of mice	GM (mean ± SD[a])	No. of mice with index values of			nPCR
			<0.5	≥0.5 to <1.5	≥1.5	
1	12	0.88 ± 0.51	2	9	1	9
3	12	0.97 ± 0.50	2	8	2	10
5	12	1.50 ± 1.07	5	1	6	12
7	12	1.53 ± 0.78	2	5	5	10
9	12	1.01 ± 0.55	1	8	3	8
Total	60		12 (20)	31 (52)	17 (28)	49 (82)

Data in parentheses indicate percentage.
[a] Mean index value in normal mice is 0.34 ± 0.06.

Table II-10: Comparison of the combined sensitivity, specificity, PPV and NPV in BAL and serum specimens of 60 mice infected with *A. terreus*

Diagnostic markers	Sensitivity	Specificity	%	
			PPV	NPV
Cumulative				
Serum				
GM	78	100	100	48
BDG	43	100	100	26
nPCR	73	100	100	42
BAL				
GM	80	100	100	50
nPCR	81	100	100	52
Combined				
Serum				
GM + BDG	83	100	100	54
GM + *A. terreus* DNA	95	100	100	80
BDG + *A. terreus* DNA	83	100	100	54
GM + BDG + *A. terreus* DNA	95	100	100	75
BAL				
GM + *A. terreus* DNA	98	100	100	92

Note: GM = galactomannan; BDG = 1-3-β-D glucan latex agglutination assay; nPCR = nested PCR
BAL = broncho-aveolar lavage

Table II-11: Galactomannan results in sera of 60 mice infected with *A. terreus* and sacrificed at different time points

Postinfection (days)	No. of mice	GM (mean ± SD[a])	No. of mice with index value of			nPCR
			<0.5	≥0.5 to <1.5	≥1.5	
1	12	1.06 ± 0.63	1	9	2	9
3	12	1.51 ± 1.15	3	4	5	8
5	12	1.15 ± 0.87	4	4	4	10
7	12	1.90 ± 0.93	1	3	8	8
9	12	1.01 ± 0.55	4	5	3	9
Total	60		13 (22)	25 (42)	22 (37)	44 (73)

Data in parentheses indicate percentage.
[a] Mean index value in normal mice is 0.19 ± 0.04.

APPENDIX III - Clinical Microbiology Studies

Eleven clinical studies were available in which BAL fluid samples were tested by the Platelia *Aspergillus* EIA. Unless specified otherwise, the EORTC/MSG criteria published in 2002 were used for diagnosis of patients with proven, probable or possible IPA (See *Section 2.2*). These EORTC/MSG criteria were recently updated in 2008 by DePauw *et al*. The galactomannan assay for testing of BAL samples was performed according to the manufacturer's specifications (See *Section 2.4*) for testing of serum samples as the Platelia *Aspergillus* EIA is not FDA approved for use in BAL fluid samples.

III.1. Hematological malignancies (*unless specified otherwise*)

1. Becker *et al*. (2003), tested BAL specimens from hematological-oncological patients with neutropenia by the Platelia *Aspergillus* EIA (Sanofi Diagnostics) galactomannan assay. The study, conducted in the Netherlands, was performed in two parts. The authors included a retrospective and prospective cohort in their study and it was the only study among the five studies in hematological patients that followed all patients based on disease progression. In the first part of the study, BAL samples were collected based upon a computed tomography (CT) scan of the chest that showed radiographic abnormalities consistent with IPA. The study was retrospective in that eligible patients had BAL specimens collected and stored at - 20°C. The authors stated that patients received oral ciprofloxacin (500 mg twice daily) for selective bowel decontamination and either fluconazole (200 mg/d) or itraconazole (200 mg twice daily) as antifungal prophylaxis at the time of enrollment, though the duration of prophylaxis was not specified. The 2002 EORTC/MSG guidelines were used to classify proven, IFI, and possible with the exception that the "possible" category was divided into two groups. The first group termed as "suspected" included patients that had one major clinical criterion with negative histopathology, cytology and fungal cultures. The other group termed as "possible" patients included patients with either (1) a positive culture or cytology or histological staining demonstrating *Aspergillus* species or (2) at least 2 minor clinical criteria with negative bacterial and viral cultures from lower respiratory tract specimens. Patients without IPA were defined as patients not classified in any IPA category and did not receive empirical antifungal treatment. Laboratory personnel were blinded to the identity and clinical status of the patient. All patients had bronchoscopy performed during the neutropenic phase of the disease and prior to antifungal treatment. If multiple BAL specimens were obtained, galactomannan positivity was based on the first BAL specimen obtained. The galactomannan assay results in BAL were reported as positive based on cut-off index ≥ 1.0. The authors stated that all doubtful or positive samples were retested. A total of 29 patients, age 18 to 79 years, had BAL specimens collected based on CT scan findings, of which one had proven IPA, six probable, two suspected, two possible, and 18 patients were without IPA. All patients with proven, probable, and suspected IPA were positive for the galactomannan assay in BAL; one of the two possible patients was positive (Table III-1). None of the 18 patients classified as non-IPA were positive for galactomannan in BAL. The overall specificity, PPV, and NPV were 100%. The results suggest that the galactomannan assay tested in BAL may be predictive of IPA in proven, probable, and suspected IPA patients using well defined criteria that include clinical and radiological observations.

Table III-1: Galactomannan detection in serum and BAL from neutropenic hematological patients in first study

		GM detection in serum		GM detection in BAL fluid	
	No. patients	No. patients tested	No. patients positive (2 × index > 1·0)	No. patients tested	No. patients positive (1 × index > 1·0)
Proven IPA	2	2	1 (50%)	1	1 (100%)
Probable IPA	11	11	5 (45%)	6	6 (100%)
Suspected IPA	4	4	2 (50%)	2	2 (100%)
Possible IPA	18	18	4 (22%)	2	1 (50%)
Empiric ampho B	4	4	1 (25%)	1	0 (0%)
Other IFI*	4	4	0 (0%)	2	0 (0%)
No IFI	117	44	5 (11%)	16	0 (0%)

*Invasive pulmonary fungal infections other than IPA, proven and probable.

The authors followed the nine cases of IPA (one proven IPA, six probable IPA and two suspected IPA) that were BAL galactomannan positive. Patients with possible IPA were excluded from the analysis as per EORTC/MSG guidelines. Patients were followed until the patient was discharged from hospital or reached the end of the neutropenic episode. The follow-up period in patients ranged from five weeks to three months. Clinical, radiologic, and microbiologic observations including detection of galactomannan in BAL specimens and in serum, and the results of fungal cultures in combination with histopathology or microscopy were documented (Figure III-1).

Additional BAL specimens were obtained and tested either after the start of antifungal treatment or during another neutropenic episode. Five of the nine patients had a second BAL specimen collected. In four patients, a second BAL specimen was collected more than one week after the start of antifungal treatment (amphotericin B or lipid formulation of amphotericin B) and one patient had a second BAL specimen collected during another neutropenic episode. All five patients were negative for the galactomannan assay in the second BAL specimen; suggesting that antifungal treatment reduces the sensitivity of the galactomannan assay in BAL specimens.

Serum samples were collected at the beginning and twice weekly up until the patient was discharged from hospital or at the end of the neutropenic episode. Specimens were collected at least 1- 3 weeks prior and up to two months after the index BAL specimen was galactomannan positive. The galactomannan assay was considered positive on two consecutive serum samples that had an optical density index ratio ≥ 1.0 .The testing of consecutive serum specimens was not explicitly defined. Three patients were serum galactomannan positive (2 of 6 probable IPA cases and 1 of 2 suspected IPA patients). None of the serum specimens from the proven IPA patient or the remaining five patients were positive for galactomannan (Figure III- 1). In the three patients, sera were galactomannan positive prior to and in the same week that the BAL was positive. The galactomannan assay was positive in serum specimens no longer than one week

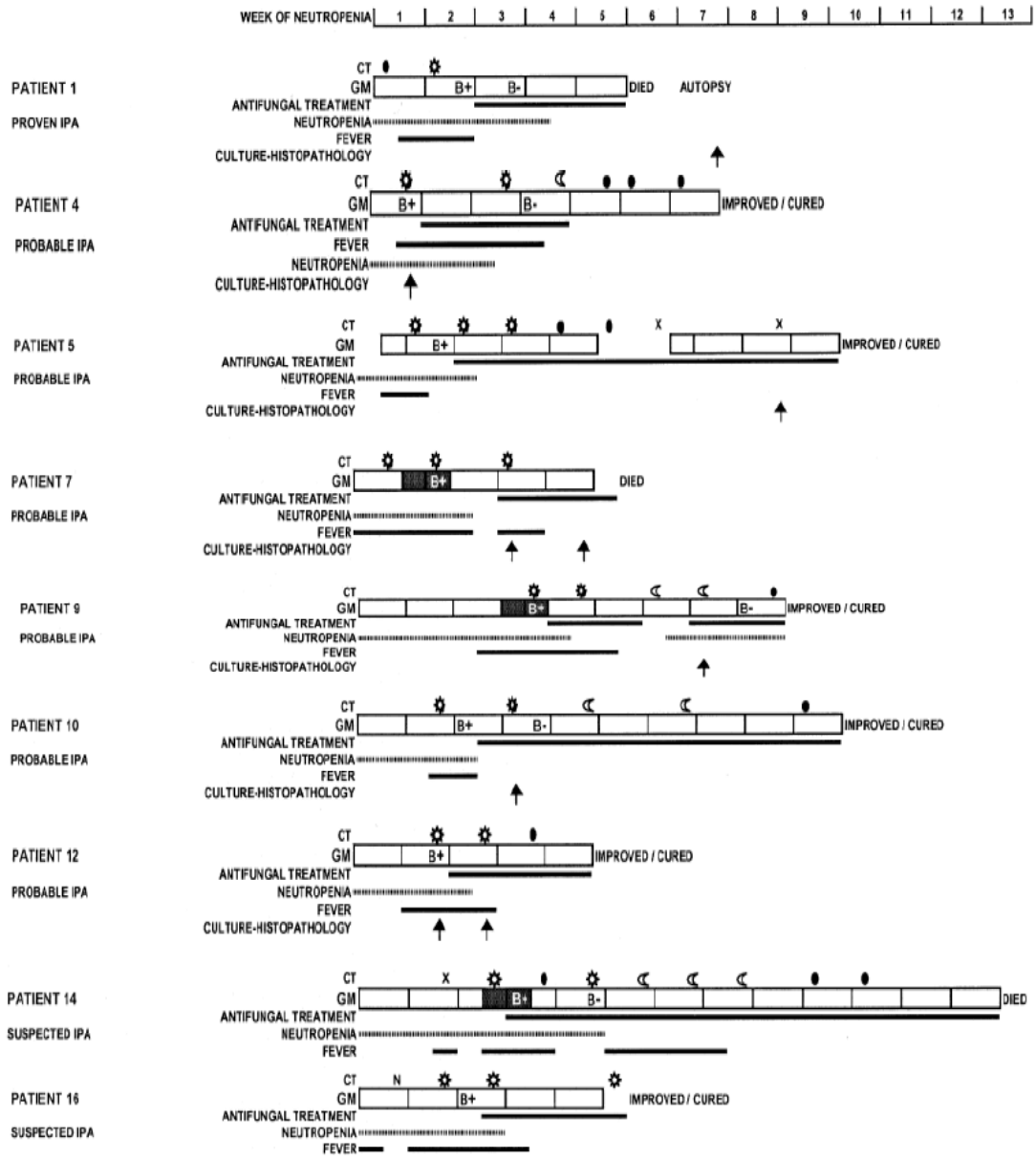
after antifungal regimen was initiated which was possibly due to a reduced fungal load leading to undetectable galactomannan levels in serum.

Characteristic radiological lesions on CT scan of the nine IPA patients were documented as major or minor abnormalities. The major abnormalities include the "halo" and "air crescent" or cavitations on CT scan (Figure III-1). Minor abnormalities were new infiltrates documented as "wedge-shaped or nodular" or non-specific abnormalities. All patients showed the characteristic "halo" signs on CT evaluation at the time of and 2- 3 weeks after the BAL galactomannan was positive. In four of the nine patients multiple "air-crescent" signs on CT evaluation around the 3rd week of illness or during anti-fungal treatment were reported. The authors also noted that the "air-crescent" signs were observed during the patients' bone-marrow recovery. Multiple nodular, wedge-shaped lesions and non-specific abnormalities were noted after several weeks of antifungal treatment. Since the criterion for performing a bronchoscopy in the proven, probable, and suspected IPA population was based on the presence of CT abnormalities, the results do suggest that when a CT evaluation is used at an early stage (i.e. observation of the "halo" sign) it is predictive of IPA in untreated patients.

Fungal cultures in combination with histopathology and microscopy were performed in patients with proven or probable IPA. By definition, fungal cultures were negative in suspected cases. The causative organism in most patients was *A. fumigatus*, however in one patient *A. flavus*, and in another patient *A. niger* was identified. Two patients had acutely branched septated hyphae in sputum and lung biopsy highly suggestive of *Aspergillus* species. Three of the nine patients had fungal cultures that were positive around the time of BAL positivity. The remaining patients had fungal cultures positive 3-4 weeks after BAL galactomannan positivity. Three patients died during hospitalization (1 patient had proven IPA, 1 probable IPA and 1 suspected IPA), however only one patient (i.e. proven IPA patient) had fungal confirmation at time of autopsy.

The results suggest that if the galactomannan assay was performed in real time, 67% of the confirmed cases would have yielded an earlier diagnosis.

Figure III-1: Summary of galactomannan results in BAL given CT findings and culture or histopathology in patients with proven, probable or suspected IPA of hemato-oncological patients with neutropenia



NOTE: Timing of CT, GM detection, antifungal treatment, fever and culture/histopathology in patients with IPA. Symbols: ✲ = halo sign, ☾ = crescent sign or cavitation, ● = wedge-shaped or nodular abnormalities, × = non-specific abnormalities, N = no abnormalities. GM detection: □ = serum negative, ■ = serum positive, B+ = BAL fluid positive, B– = BAL fluid negative. Arrows: positive findings in culture/histopathology.

The time to galactomannan positive results in the nine IPA cases relative to the onset of fever are shown in Table III-2. The authors also noted that on an average from the start of the neutropenic episode characterized as a spike in fever, patients showed characteristic abnormalities based upon CT scans at 4.0 ± 0.8 days and positive galactomannan result in BAL at 6.9 ± 1.1 days. However, detection of galactomannan in BAL may reflect the time the specimens were collected based on CT evaluation. However, results should be interpreted with caution since the number of patients tested was small.

Table III-2: Comparison of days to positive results from start of fever during a neutropenic episode in proven and probable patients

Test	Days to positivity (mean ± SD)
Clinical and radiological findings	4.0 ± 0.8
Culture	14.8 ± 5.0
Detection of galactomannan	
Serum	4.6 ± 1.6
BAL	6.9 ± 1.1

In the second part of the study, BAL samples were prospectively collected from patients with abnormalities on CT scan. BAL specimens were tested within three days of the sample collection; the methods for storage between testing and sampling were not specified. Patients were classified as proven, probable, possible, suspected or non-IPA as described previously. BAL specimens were obtained during the neutropenic episode and prior to antifungal treatment. If multiple BAL specimens were obtained, galactomannan positivity was based on the first BAL specimen. A total of 53 patients had BAL specimens collected based on CT findings, of which 3 had proven, 9 probable, 8 suspected, 12 possible IPA, and 21 patients without invasive fungal infection (Table III-3). The authors stated that of the 20 BAL specimens that were positive by the galactomannan assay, only six were culture positive for *Aspergillus* species, though the authors did not specify whether the positive cultures were from individual patients. Of the 21 patients without invasive fungal infections, none were positive for galactomannan in BAL. The overall sensitivity of the galactomannan assay based upon EORTC/MSG criteria, had the highest sensitivity observed among the proven IPA patients (3/3; 100%) and the lowest sensitivity in the possible IPA group (5/12; 42%) (Table III-3). The number of patients in each group was small and therefore, the results should be interpreted with caution; however, the overall specificity of galactomannan in BAL specimens was 100%.

Table III-3: Galactomannan detection in BAL fluid from neutropenic patients

	GM detection in BAL fluid		
	No patients	No patients tested	No patients positive (1 × index > 1·0)
Proven IPA	3	3	3 (100%)
Probable IPA	10	9	8 (89%)
Suspected IPA	9	8	6 (75%)
Possible IPA	13	12	5 (42%)
Empiric ampho B	2	2	0 (0%)
Other IFI*	3	2	0 (0%)
No IFI	158	21	0 (0%)

*Invasive pulmonary fungal infections other than IPA, proven and probable.

The authors have stated that in only one patient, the second BAL specimen was positive instead of the first specimen. No further information was provided on this patient. The authors also state that all BAL specimens obtained after more than two days of antifungal treatment with amphotericin B deoxycholate or the lipid formulation of amphotericin B were negative for galactomannan. None of the BAL samples had a positive cytology or culture result.

Overall, the two parts of the study (retrospective and prospective) suggests that when a CT scan evaluation of the lung is used at an early stage and used systematically with the performance of a bronchoscopy, the galactomannan assay in BAL specimens has a high sensitivity and predictive value for diagnosing IPA early in untreated patients. BAL specimens should be obtained before the start of antifungal treatment as specimens tend to be negative after treatment. Detection of galactomannan in BAL and serum specimens can occur at the same time, however, this was observed in relatively few patients.

2. Musher *et al*. (2004), evaluated the Platelia *Aspergillus* EIA galactomannan assay (BioRad Laboratories) in BAL specimens from hematopoietic stem cell transplant patients. The study was conducted in Washington State in the US. The 2002 EORTC/MSG guidelines were used to classify patients as proven or probable IPA patients. Cases were a random selection of patients diagnosed with proven or probable IPA that had BAL specimens collected within 30 days of IPA diagnosis. If multiple samples were collected, the sample closest to the diagnosis date was selected. The date of IPA diagnosis was defined as the first day on which the infection was confirmed by culture or histopathological examination of respiratory specimen such as BAL, tissue biopsy, or sputum. A random selection of patients with diagnoses other than invasive aspergillosis (e.g. bacterial pneumonia, viral pneumonia, pneumonia caused by other fungi) and that had BAL specimens collected were included as controls. BAL specimens were collected and stored at -70°C until testing. The galactomannan assay was performed according to the manufacturer's instructions as described previously.

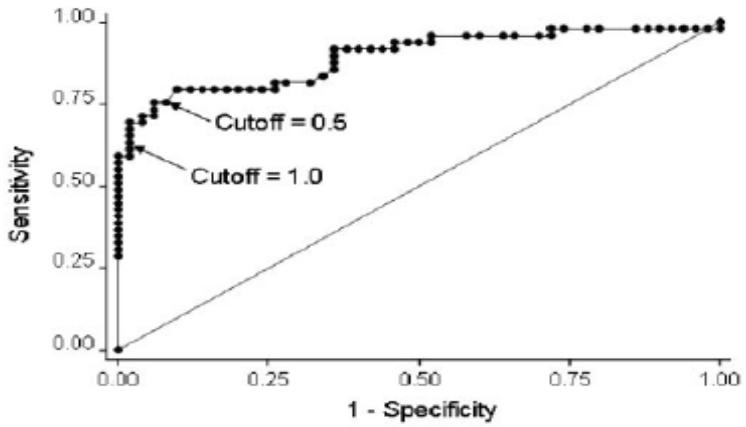
Results were evaluated based on the optical density of the specimen relative to the threshold control. A total of 49 case patients with proven or probable aspergillosis and 50 controls had BAL specimens collected. The number of IPA patients classified as proven or probable were not specified. Of the 49 cases, 27 patients were culture positive for *Aspergillus* species in BAL specimens and the remaining 22 patients had a lung biopsy, autopsy or culture of other respiratory samples to establish a diagnosis. For the control patients, the BAL specimens, by definition, were culture negative for *Aspergillus* species. Thirty case patients (61%) were galactomannan positive in BAL compared to only one control patient, at an index value ≥1.0 (Table III-4). The false positivity rate among the controls was 2%. When the index cut-off was lowered from ≥ 1.0 to ≥ 0.5, the percentage of cases that were galactomannan positive by the galactomannan assay in BAL increased to 76%, as well as the false positivity fraction to 4%. The ROC curve showed that lowering the cut-off index to ≥ 0.5 improved the sensitivity by 15% ($p < 0.01$) but compromised the false positive fraction by 4% ($p = 0.16$) (Figure III-2). The ROC curve demonstrates that lowering the index cut-off further would considerably compromise the false positive rate for only a small increase in the sensitivity. Optimal performance of the galactomannan assay in the study population was achieved by using an index cut-off value of ≥ 0.5.

Table III-4: Performance characteristics of the galactomannan assay.

Test	No. of results				% Sensitivity (95% CI)	% Specificity (95% CI)
	True positive	True negative	False positive	False negative		
qPCR assay	31	47	0	15	67 (52–81)	100 (93–100)
GM EIA with index of 1.0	30	49	1	19	61 (46–75)	98 (89–100)
GM EIA with index of 0.5	37	47	3	12	76 (61–87)	94 (84–99)

[a] BAL fluid analysis results from 46 case and 47 control patients for qPCR and 49 case and 50 control patients for GM EIA are shown.

Figure III-2: ROC curve for galactomannan assay with a decreasing index value to define positivity



The performance of the galactomannan assay in BAL was also examined in case patients on antifungal therapy compared to case patients that were not on antifungal therapy. Among the case patients alone, case report forms were available for 44 of the

49 case patients at the time of bronchoscopy; of the 44 patients 35 patients had antifungal therapy and nine patients were not on antifungal treatment. The type of antifungal therapy and whether antifungal therapy was treatment or prophylaxis and the period between start of antifungal therapy and collection of the BAL specimen was not specified. The authors noted that the sensitivity of the BAL galactomannan assay in patients receiving antifungal therapy was higher than patients not receiving antifungal therapy (Table III-5). The low sensitivity of 61% (cut-off index ≥ 1.0) may be due to the fact that >75% of patients in the study were treated with antifungal therapy at the time of bronchoscopy which presumably reduced the fungal burden in the lung. The results should be interpreted with caution, since there were a small number of patients (25%) that did not receive antifungal therapy. In addition, the authors did not specify the period between start of antifungal therapy and collection of the BAL specimen.

Table III-5: Sensitivity of galactomannan EIA relative to antifungal therapy

Test	Not on antifungal therapy			On antifungal therapy			*P* value[b]
	No.	Sensitivity (%)	95% CI	No.	Sensitivity (%)	95% CI	
GM EIA with index of 0.5	9	67	30–95	35	80	63–92	0.40
GM EIA with index of 1.0	9	44	14–79	35	66	48–81	0.28
qPCR assay	7	43	10–82	34	77	59–89	0.17
qPCR or GM EIA with index of 0.5	7	57	18–90	33	91	76–98	0.06

[a] Antifungal therapy data were not available for five patients.
[b] *P* value from Fisher's exact test comparing sensitivities between patients receiving antifungal therapy and patients not receiving antifungal therapy.

The performance of the galactomannan assay in BAL was also examined among case patients with nodular or focal radiographic abnormalities and compared with case patients with bilateral radiographic abnormalities. Twenty-two patients had nodular or focal infiltrates and 10 patients had bilateral infiltrates. At a cut-off index of 1.0, the sensitivity of the galactomannan assay in BAL was higher in patients with nodular or focal infiltrates (82%) than those with bilateral infiltrates (60%) (Table III-6). Decreasing the cut-off index to ≥0.5, did not alter the increased sensitivity in patients with nodular or focal infiltrates (73%) compared to those with bilateral infiltrates (40%). The results suggest the galactomannan assay has a higher sensitivity for testing of BAL fluids from patients with nodular or focal infiltrates than in patients with bilateral radiographic abnormalities. Dense, well-circumscribed lesions(s) with or without a halo sign, air-crescent sign or cavity on chest CAT scan are more suggestive of IPA than are diffuse infiltrates in the hematologic patient population and are part of the clinical criteria in the current EORTC/MSG criteria [de Pauw *et al*., 2008]

Table III-6: Performance of the BAL galactomannan assay in case patients with radiographic appearance of cavitary lesions.

Test	Nodular vs focal infiltrate[a]			Bilateral infiltrate			*P* value[b]
	No.	Sensitivity (%)	95% CI	No.	Sensitivity (%)	95% CI	
GM EIA with index of 0.5	22	82	60–95	10	60	26–88	0.22
GM EIA with index of 1.0	22	73	50–89	10	40	12–74	0.12
qPCR	22	73	50–89	9	56	21–86	0.42
qPCR or GM EIA with index of 0.5	21	91	70–99	9	56	21–86	0.05

[a] Included four patients with cavitary lesions.
[b] *P* value from Fisher's exact test comparing sensitivities between patients with nodular or focal infiltration and those with bilateral infiltration.

The performance of the galactomannan assay in BAL was also compared in case patients that were BAL culture positive with patients that were BAL culture negative. Twenty-seven case patients were culture positive for *Aspergillus* species and 22 case patients were culture negative that had additional procedures including lung biopsy, autopsy or culture of other respiratory samples to establish a diagnosis. Overall, whether the galactomannan assay cut-off was at ≥0.5 or increased to ≥1.0, the sensitivity of the galactomannan assay for testing of BAL samples was higher among case patients whose BAL cultures were positive compared with case patients whose BAL fluid was culture negative. The median galactomannan EIA index value in culture-positive BAL specimens was 4.31 compared to 0.75 in culture-negative BAL specimens (Table III-7). The results suggest that for case patients that were culture-positive, using the galactomannan assay in real time would have yielded an earlier diagnosis in 24 patients (sensitivity, 89%). The authors also noted that of the 22 case patients that were BAL culture negative, 18 patients had more than one additional procedure performed to establish a diagnosis. Of the 18 patients, 12 had a second bronchoscopy and 13 patients had surgical lung biopsy performed (video assisted or open). In other words, if the galactomannan assay was performed in real time using a cut-off index of ≥ 0.5, in the 8 (66%) of 12 patients who had a second bronchoscopy performed and in 8(62%) of 13 patients who had a lung biopsy, these invasive procedures with their accompanying risks such as bleeding could have been avoided.

Table III-7: Sensitivity of galactomannan EIA relative to BAL fluid culture positivity for *Aspergillus* species

Test	BAL fluid culture positive			BAL fluid culture negative			*P* value[b]
	No.	Sensitivity (%)	95% CI	No.	Sensitivity (%)	95% CI	
GM EIA with index of 0.5	27	89	71–98	22	59	36–79	0.02
GM EIA with index of 1.0	27	78	58–91	22	41	21–64	0.02
qPCR	24	96	79–100	22	36	17–59	<0.001
qPCR or GM EIA with index of 0.5	23	100	85–100	22	64	41–83	0.001

[a] Among patients with proven or probable aspergillosis.
[b] *P* value from Fisher's exact test comparing sensitivities between patients with BAL fluid cultures that revealed growth of *Aspergillus* species (positive) and those with culture-negative BAL fluid cultures.

3. Verweij *et al*. (1995) evaluated the Platelia *Aspergillus* EIA (Sanofi Diagnostics) for testing of BAL specimens from patients with hematological malignancies between the ages of 20 to 72 years. The study was conducted in the Netherlands. Patients were classified as probable, possible and without IPA based on radiological abnormalities of pulmonary infiltrates by chest x-ray only. Chest x-rays are less sensitive than chest CT scans for diagnosis of lung abnormalities therefore the patients were probably diagnosed later in their disease course. Probable IPA patients had a radiographic chest X-ray showing focal non-anatomical infiltrates or any cavitating lesions. Patients without IPA had chest X-rays that showed diffused infiltrates characterized by nodular, reticular or reticulo-nodular lesions that were distributed throughout both lung fields or observed as anatomical focal infiltrates. Possible IPA patients had infiltrates on the chest X-ray that could not be classified into probable or without IPA based upon radiographic abnormalities. BAL specimens were obtained using saline and cultured for

the presence of bacteria, viruses, fungi and parasites. BAL specimens were stored at -80°C until testing. Each test included a negative control BAL specimen and each BAL specimen was tested in duplicate by one technician. A total of 54 BAL specimens were collected from 19 patients with hematological malignancies and 35 non-neutropenic patients. The EIA was calibrated using culture negative and PCR negative BAL fluid samples from the 35 non-neutropenic patients and spiked with a range of dilutions of galactomannan (0.25 – 10 ng/mL). However, the threshold control was calculated based on a Gaussian distribution of the mean optical density values of culture negative BAL specimens (mean, 0.09; range 0.056 to 0.146) plus 4 standard deviations. The optical density of the threshold in BAL was determined as 0.17. Though the optical density in the threshold is lower than the recommended optical density value, that is, between 0.3 and 0.8. The variation may not affect the validity of the galactomannan assay, since results were reported as positive for BAL galactomannan based on optical density cut-off ≥ 0.17, equivalent to a cut-off index value ≥ 1.0.

Of the 19 patients with hematological malignancies, seven were classified as probable IPA, two as possible IPA and ten patients without IPA (Table III-8). Five of the seven probable IPA patients had a positive BAL galactomannan result, with optical density values ranging from 0.24 to 0.58 and three patients had positive BAL cultures for *A. fumigatus*. One of the two possible patients and one of the ten patients without IPA had positive BAL galactomannan results. The sensitivity of BAL galactomannan assay among probable IPA patients was 71%. The results suggest that the galactomannan assay is relatively a good indicator of IPA based on focal non-anatomical infiltrates or any cavitating radiological abnormalities in patients with hematological malignancies. However, the sensitivity in possible patients was 50%, though these results were based on a very small number of patients. Based on the testing of BAL in the galactomannan assay, 90% of the patients without these radiological abnormalities among patients with hematological malignancies were truly negative.

Table III-8: Characteristics of 19 patients with hematological malignancies

Category[a]	Patient no.	Sex,[b] age (yr)	Underlying disease and/or condition(s)[c]	BAL fluid analysis			No. of samples positive by serum ELISA/ total no. of samples	Time between first positive serum and BAL[d]	Granulocyte count (10^9/liter)	Outcome
				Culture	PCR	ELISA[e]				
A	1	M, 43	NHL	*Aspergillus fumigatus*	+	0.581 (+)	3/3	−4	3.6	Deceased
	2	M, 36	NHL	*Aspergillus fumigatus*	+	0.278 (+)	3/4	−3	5.8	Deceased
	3	M, 48	HL	*Aspergillus fumigatus*	+	0.128 (−)	0/3		2.5	Survived
	4	M, 54	AML	*Stenotrophomonas maltho-philia*	+	1.322 (+)	1/1		<0.1	Survived
	5	M, 22	AA, BMT	*Citrobacter freundii, Kleb-siella pneumoniae*	+	0.370 (+)	25/28	−30	<0.1	Survived
	6	M, 58	MDS, BMT	Negative	−	0.247 (+)	3/11	−11	2.1	Survived
	7	M, 20	Myelofibrosis	*Pseudomonas aeruginosa, Candida albicans*	−	0.095 (−)	NA[f]		<0.1	Deceased
B	1	M, 59	AML	Negative	+	0.736 (+)	NA		<0.1	Survived
	2	M, 68	NHL	*Candida glabrata*	−	0.113 (−)	NA		9.2	Deceased
C	1	M, 48	AML, BMT	*Candida glabrata*	+	0.152 (−)	0/1		<0.1	Survived
	2	F, 36	AML	Negative	+	0.122 (−)	NA		<0.1	Deceased
	3	M, 34	CML, BMT	*Candida glabrata*	−	0.220 (+)	2/7		<0.1	Survived
	4	F, 52	MM	Negative	−	0.093 (−)	0/1		<0.1	Deceased
	5	F, 43	ALL, BMT	*Candida albicans*	−	0.075 (−)	0/5		3.8	Deceased
	6	F, 40	AML	Negative	−	0.077 (−)	0/1		2.1	Survived
	7	F, 44	MM	Herpes simplex virus	−	0.087 (−)	0/1		3.5	Survived
	8	F, 62	CLL	*Pneumocystis carinii*[g]	−	0.079 (−)	NA		2.0	Deceased
	9	F, 56	AML	*Candida albicans*	−	0.114 (−)	0/2		<0.1	Deceased
	10	F, 72	AML	Negative	−	0.098 (−)	0/2		<0.1	Deceased

[a] Categories: A, probable IPA, with the chest roentgenogram showing focal nonanatomical infiltrates or any cavitating lesion; B, possible IPA in patients with infiltrates on the chest roentgenogram who could not be classified into group A or C; C, IPA unlikely, with the chest roentgenogram showing diffuse infiltrates characterized by nodular, reticular, or reticulonodular lesions which were distributed throughout both lung fields or anatomical focal lesions.
[b] M, male; F, female.
[c] NHL, non-Hodgkin's lymphoma; HL, Hodgkin's lymphoma; AML, acute myeloid leukemia; AA, aplastic anemia; BMT, bone marrow transplantation; MDS, myelodysplastic syndrome; CML, chronic myeloid leukemia; MM, multiple myeloma; CLL, chronic lymphoblastic leukemia.
[d] Patients from whom at least one serum sample was taken within 6 weeks before the BAL fluid sample was obtained. Values are numbers of days between the time the first serum sample was found to be positive by ELISA and the date of bronchoscopy.
[e] Optical density value; plus and minus signs indicate interpretation of results.
[f] NA, not available.
[g] Microscopic detection with Giemsa staining.

Comparison of serum and BAL galactomannan results: The sensitivity of the detection of galactomannan in BAL specimens was compared with serum and culture methods. For this, serum samples were obtained between six weeks before and two weeks after the BAL fluid sample collection. Serum samples were tested in duplicate by one technician. The optical density of control serum spiked with 1 ng of galactomannan per mL was used as cutoff value for serum (optical density value, 0.17). Results in serum were reported as positive based on optical density value ≥ 0.17 (cutoff index ratio $\geq$ 1.0). Of the 19 patients with hematological malignancies, only 14 patients had serum tested for detection of galactomannan; six were probable IPA and eight without IPA. No serum samples in patients with possible were available for galactomannan testing (Table III-8).

Serum galactomannan results highly corresponded with the BAL galactomannan results, in that the five 5 probable IPA patients that were positive in BAL were also serum positive, and the one patient that was negative in BAL was also negative in serum. The authors stated that galactomannan was detected in the serum even before the BAL was performed. Four of the five probable IPA patients had serum samples that were positive between 3 to 30 days before the BAL sample was obtained and was positive. The results suggest that galactomannan may be detected in serum before

infiltrates become visible on the radiographic observations. However, one patient without IPA was positive for galactomannan in both BAL and sera. In this patient, two of the seven serum samples obtained were positive for galactomannan on the day that the BAL was performed and 1 day thereafter, the remaining five serum samples obtained during two weeks following the BAL were negative for galactomannan. The authors stated that this patient was treated with amphotericin B (1 dose of 10 mg) at time of BAL and serum galactomannan positivity and with amphotericin B (1 mg/kg/day) and ciprofloxacin which resulted in resolution of pulmonary infiltrates after two weeks.

Comparison of culture to galactomannan results: When the BAL galactomannan results were compared to culture results, only three of the probable IPA patients had positive culture results that grew *A. fumigatus*, whereas the possible patients or patients without IPA were not culture positive for *Aspergillus* species. The results highlight the main problem with fungal cultures as a diagnostic tool in its limited sensitivity and the delay in diagnosis of IPA even in patients with radiographic abnormalities.

4. Desai *et al*. (2009), evaluated the Platelia *Aspergillus* EIA (BioRad Lab) in BAL specimens from pediatric patients with a mean age of 10.3 years. The study population included patients admitted to the Children's Hospital in Los Angeles. BAL specimens were collected and stored at -70°C and specimens were batched and tested for galactomannan by EIA according to manufacturer's instructions. A positive result was based on galactomannan index ≥ 0.5. Serum specimens were also tested for comparison. The 2002 EORTC/MSG guidelines were used to classify patients with proven, probable and possible IPA, with the exception that detection of galactomannan in BAL was not used as microbiologic evidence of IA in the classification of probable patients. However, a positive serum galactomannan was included as *stand alone* microbiological criteria for characterization of patients as "probable" IPA. BAL specimens were not retested and specimens were considered positive if the clinical sample had galactomannan index ≥ 0.5. A total of 85 patients (59 immunocompromised and 25 immunocompetent) had a bronchoscopy performed in which BAL specimens were collected. Three patients had proven IPA, 6 probable IPA, 37 possible IPA and 39 patients with no evidence of IPA. The authors combined proven and probable IPA categories and stated the mean BAL galactomannan index was 3.4. The BAL galactomannan index in possible IPA patients was 0.72 and in patients with no IPA was 0.34. An ROC curve analysis indicated that the optimal performance of the BAL galactomannan assay was achieved at a cut-off index ≥ 0.98 (Figure III-3). At this cut-off, the sensitivity for proven and probable IA was 78% and specificity 92% (Table III-9). As suggested by the relatively high specificity, the false positives in this population were relatively low. However, the relatively low sensitivity may be hampered by the small number of patients with IA.

The positive and negative predictive values in all patients were 54% and 97%, respectively. It appears that the patients without the disease are patients characterized as possible and without IPA, which would explain a low PPV. By excluding the

possible IPA patients from the patients without the disease, the PPV could increase by 46% thereby suggesting that the test is useful in the diagnosis of IA if used selectively in a well defined population of patients, especially in the pediatric population. The authors stated that lowering the BAL galactomannan cut-off value to ≥ 0.5 had no effect on the sensitivity, but decreased the specificity from 92% to 84%. Restricting the population to immunocompromised pediatric patients only, based on ROC curve analysis the optimal index cut-off value reduces to 0.87, the specificity increases to 100%, though has no effect on the sensitivity (78%).

The results suggest that the galactomannan assay in BAL samples may be useful at a cut-off index between 0.87 and 0.98 in the diagnosis of proven and probable IA in immunocompromised pediatric patients. The results also indicate that the galactomannan BAL is not a useful test in immunocompetent pediatric patients and this has also been demonstrated in immunocompetent adults (*See* Nguyen *et al.*, 2007)

Figure III-3: ROC curve for BAL galactomannan assay in pediatric patients

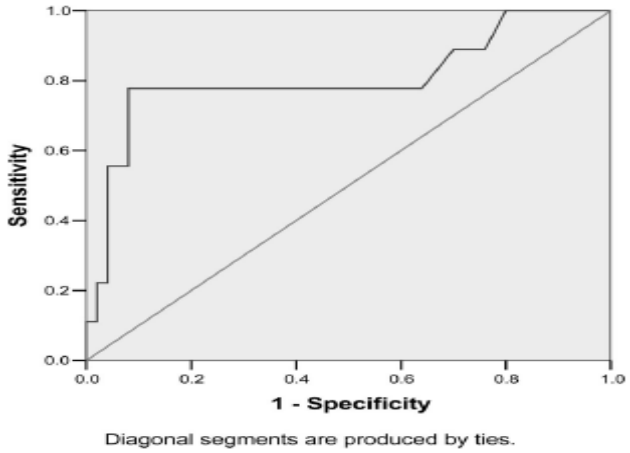


Table III-9: Summary of performance characteristics of BAL galactomannan assay for the diagnosis of IPA in pediatric patients

Test	Sensitivity	Specificity	PPV	NPV
BAL GM				
Cutoff ≥ 0.5	**78%**	**84%**	-	-
[a]Cutoff ≥ 0.87	**78%**	**100%**	**58%***	**96%***
[b]Cutoff ≥ 0.98	**78%**	**92%***	**54%***	**97%***
Serum GM (2 samples)				
Cutoff ≥ 0.5	**78%**	**100%**		
Both serum and BAL GM[c]				
All patients	**89%**	**90%**		
Immunocompromised patients only	**89%**	**92%**		

Note: [a]Immunocompromised patients only
[b]All patients
[c]Serum GM ≥ 0.5 and BAL GM ≥0.98
*Includes possible IPA patients and patients without IPA

A total of 38 patients had serum galactomannan performed at the time of BAL collection. The authors stated that in patients with serum galactomannan value ≥0.5 that had no prior positive the testing was repeated on the same serum patient sample and on a new serum patient sample. However, the collection of serum samples in patients with prior positives was not specified. The authors also performed a ROC curve analysis and determined that the optical cut-off value was ≥ 0.5, though the ROC curve should be interpreted with caution since proven and probable IPA patients were pre-selected on positive galactomannan index values ≥0.5. The sensitivity and specificity of the assay in serum at a cut-off index ≥ 0.5 in diagnosis of IPA was 78% and 100% respectively which is similar to BAL at a cut-off of 0.98 (Table III-9).

The combination of the assay in serum (cutoff value ≥0.5) and BAL (cutoff value ≥0.98) showed a sensitivity and specificity of 89% and 90%, respectively, irrespective of the immune status. Restricting the population to immunocompromised patients only, retained the sensitivity and increased the specificity by 2% (Table III-9); thus suggest no added benefit of the galactomannan assay for testing of serum samples in the diagnosis of IPA among pediatric patients.

Semisynthetic agents derived from molds, such as piperacillin, have been associated with false positives in detection of galactomannan in BAL. Of the 85 pediatric patients, 8 patients were treated with piperacillin-tazobactam within 3 days of obtaining the BAL specimen. The authors did not specify whether treatment with piperacillin-tazobactam ended before the BAL specimen was collected. The authors stated that treatment with piperacillin-tazobactam had no significant effect on the detection of galactomannan in BAL.

Treatment with anti-fungal agents has also been associated with decrease in the detection of galactomannan in BAL. The authors evaluated the patient population that was treated with anti-fungal agents. Of the 85 pediatric patients 37 patients had received at least one dose of mold-active agent during the seven days prior to BAL specimen collection. Three of the 26 immunocompetent patients had BAL collected while the patient was on antifungal agents, though no further information was given about galactomannan index values in these patients. The authors also noted that eight probable IPA patients were on mold active agents at least seven days prior to bronchoscopy. One probable IPA patient had been on mold active therapy (voriconazole and micafungin) for 24 days up to the week before the bronchoscopy was performed. This patient's galactomannan index in BAL was 0.14 and in serum 0.24, suggesting that treatment with anti-fungal agents at least one week prior to BAL testing may decrease the sensitivity of the galactomannan assay in BAL, though the occurrence was based on a small number of patients. No further information was specified for the period of anti-fungal treatment in the patients and galactomannan index levels that were positive in the galactomannan assay in BAL.

Overall, the study suggests that the galactomannan assay for testing of BAL may be useful at a cut-off index between 0.87 and 0.98 in the diagnosis of proven and probable

IA in immunocompromised pediatric patients. The galactomannan assay has a low sensitivity when tested in BAL; this may be limited by the low prevalence of IPA in this pediatric patient population in which 30% of the population were immunocompetent. Based on testing of both serum and BAL specimens, from 38 patients, did not improve the sensitivity. As shown in other studies, antifungal treatment reduces the sensitivity of the assay in BAL, thus suggesting that BAL samples should be collected prior to antifungal treatment.

5. Penack *et al*. (2008), evaluated the Platelia *Aspergillus* EIA (BioRad Laboratories) galactomannan assay in BAL specimens in patients with hematological malignancies at a tertiary hospital in Germany. The authors stated that patients received oral levofloxacin (500 mg/day) for selective bowel decontamination and amphotericin B solution (4 x 200 mg/day) as antifungal prophylaxis as standard therapy. A chest x-ray was carried out at least 1 - 3 day before neutropenia (neutrophil count, $< 500/mm^3$ for > 10 days) and at onset of fever. BAL specimens were collected based on high resolution computed tomography (CT) scan of the chest that showed atypical infiltrates. The 2002 EORTC/MSG guidelines were used to classify proven, probable and possible IPA patients. The galactomannan assay was performed according to manufacturer's specifications as described previously. Results with an index value ≥ 0.5 were considered positive. Serum specimens were also tested for comparison. A positive test based on the galactomannan assay was included as a stand-alone microbiological criterion for characterization of patients as "probable" IPA. Twelve patients had proven IPA, 11 probable IPA, 26 possible IPA and 51 patients without IPA. However, a total of 45 patients, age 23 to 74 years, had BAL specimens collected based on pulmonary infiltrates, which included the 23 proven and probable IPA patients and 22 patients without IPA. All of the proven and probable IPA patients were galactomannan positive in BAL and the 22 patients without IPA were negative by the assay (Table III-10). The median galactomannan index for proven or probable IPA patients was 5.3 (range 0.7 – 23.5). When the galactomannan assay was used without reference to EORTC/MSG criteria to define IPA, the sensitivity, specificity, PPV, and NPV of the test in BAL specimens was 100%, 78.6%, 100% and 73.9%, respectively. However, by including the galactomannan test results according to the 2002 EORTC/MSG criteria the PPV and specificity increased to 100%. The authors stated that the additional 6 patients that were positive by the assay in BAL had negative fungal cultures. The galactomannan indices in these 6 patients were not specified.

   Overall, the results of the galactomannan assay in BAL is predictive of IPA in proven or probable patients using well defined criteria that include clinical and radiological observations and confirmed with standard microbiological methods. However, when the galactomannan assay in BAL is used in real-time in accordance with the 2002 EORTC/MSG criteria, there was a potential to miss at least 26% (6/23) of the IPA population with similar host factors (i.e., hematological malignancies) that had sufficient clinical and radiological evidence consistent with IPA but for which there was no microbiological support. As such these results should be interpreted with caution since there was no long-term follow-up i.e., culture or autopsy, in these six

patients and it is unclear whether the positive galactomannan index in these patients correlated with the definitive evidence of IPA.

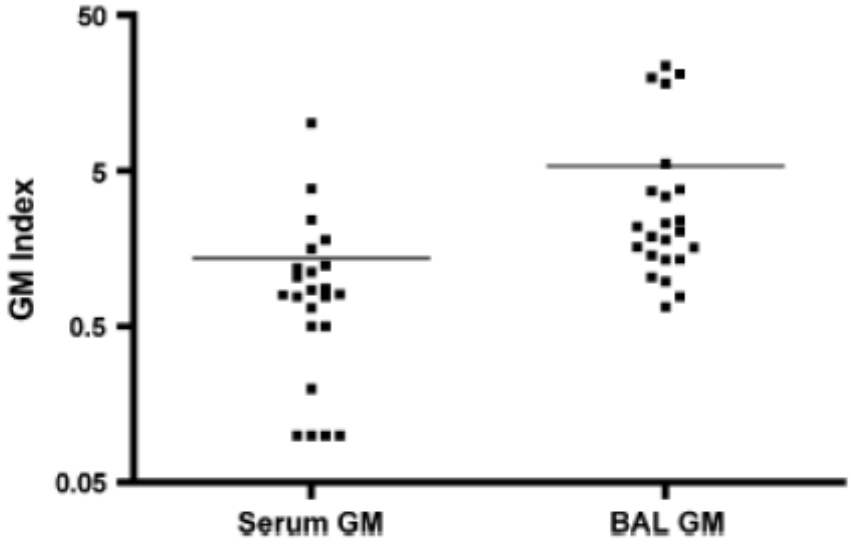
Table III-10: Performance of the galactomannan assay in BAL to predict proven and probable IPA patients

	According to EORTC/MSG criteria[a]	**Exclusion of the assay in BAL[b]**
Incidence of proven/probable IPA	23 (11.5%)	17 (8.5%)
Sensitivity	100%	100%
Specificity	100%	78.6%
PPV	100%	73.9%
NPV	100%	100%

Note: PPV = positive predictive value; NPV = negative predictive value
EORTC = European Organization for Research and Treatment of Cancer
MSG = Mycoses Study Group
[a]Using the EORTC/MSG criteria to define IPA that includes the galactomannan assay
[b]Excluding the galactomannan assay to define IPA

Serum samples were collected twice weekly during the neutropenic episode. The assay was performed as described by the manufacture for serum specimens and was considered positive at an index value ≥ 0.5. The authors stated that all proven and probable IPA patients had a positive serum galactomannan during the course of the disease. However, 18 of the 23 patients (71%) were positive in serum at the same time as the BAL was positive. The period between time of serum and BAL fluid positivity, in the remaining patients, was not specified. The galactomannan index was higher in BAL (median 5.3, range 0.7 – 23.5) than in serum (median 1.4, range 0.2 – 10.5) (Figure III-4). The results suggest that the results of the galactomannan assay in serum when performed at the same time as BAL do not increase the overall sensitivity of the galactomannan assay.

Figure III-4: Comparison of the galactomannan EIA indices results in BAL with serum



NOTE: Galactomannan enzyme immuno assay (GEI) test results in broncho-alveolar lavage (BAL) were higher as compared with results in serum at diagnosis of pneumonia in patients with probable or proven invasive aspergillosis ($n = 23$, $P = 0.02$). In five patients, serum GEI tests were negative [galactomannan (GM) index < 0.5] and BAL GEI tests were positive (GM index ≥ 0.5) in samples collected at the same day.

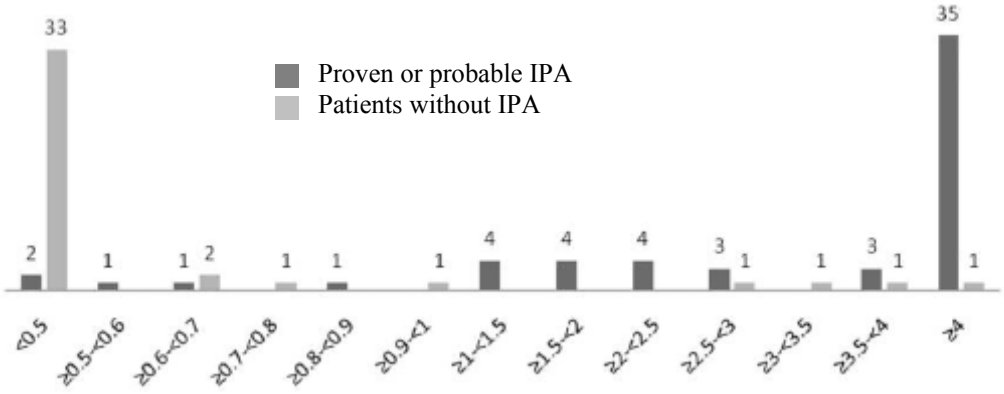
The authors stated that patients with neutropenia and fever of unknown origin immediately received broad spectrum antibiotics that included piperacillin-tazobactam (3 x 4.5 g/day). However, no further information was provided of the galactomannan index levels in patients treated with these antibiotics.

6. Maertens *et al.* (2009) evaluated the Platelia *Aspergillus* EIA (BioRad Laboratories) to detect galactomannan in the BAL fluids from patients with hematological disorders. The study population included patients admitted to a hospital in Leuven, Belgium. The study was retrospective and patients included had undergone a diagnostic bronchoscopy with BAL for evaluating new pulmonary infiltrates while receiving broad spectrum antibiotics. The authors stated that none of the patients were treated with piperacillin-tazobactam or amoxicillin-clavulanate. In addition, samples from patients were included in the study if the BAL fluid specimens were obtained before the start of systemic antifungal therapy. However, 19 patients received oral prophylaxis with an antifungal drug (14 received itraconazole, 4 received voriconazole, and 1 received posaconazole) at the time of sampling that could affect the galactomannan index. The authors stated that plasmalyte solution was not used to perform BAL. BAL sampling was performed by infusion of a total of two 20 mL aliquots of normal saline, installed sequentially and aspirated in sterile containers of the area of the lung that showed an infiltrate or consolidation location based on thoracic imaging. BAL fluid samples were

submitted for bacterial, fungal, mycobacterial cultures and for galactomannan testing. The galactomannan assay was performed according to the manufacturer's specifications as described previously. However, retesting of the BAL specimens was not specified. No interpretive cut-off values were used; results of the galactomannan assay in BAL fluid were reported as a galactomannan index.

The 2008 EORTC/MSG guidelines were used to classify proven, probable and possible IFI patients including IPA. Briefly, proven IPA was defined as hyphae compatible with *Aspergillus* species observed in tissue specimens together with a positive culture result. Probable IPA was defined as the presence of clinical criteria for lower respiratory tract fungal disease with dense, well circumscribed lesions with or without a halo, an air-crescent sign or a cavity and mycologic evidence of fungal infection as defined as positive cytology, microscopy, culture or serum galactomannan result (serum galactomannan results were not available for review). Although BAL specimens were tested, the detection of galactomannan in BAL fluid was not included as one of the mycologic criteria for diagnosis of patients. Possible IPA was defined as the presence of sufficient clinical evidence consistent with IPA but without mycologic support. Patients that died and showed no histologic signs of IPA on autopsy were classified as having no IPA. Recent history of neutropenia (<500 neutrophils/μL) is one of the host factor important for diagnosis of patients according to EORTC criteria of 2008. However, it is unclear whether all patients included in the study were neutropenic (it appears that at least 34 patients were stated to be non-neutropenic; also, the neutrophil count used for characterizing neutropenia was not specified). A total of 128 hematology patients with pulmonary infiltrates or nodules had a BAL fluid tested using the galactomannan assay. Thirty-one patients had proven IPA, 27 probable IPA, 29 possible IPA and 41 patients without IPA. The authors excluded patients with possible IPA from the analysis because the true nature of their pulmonary lesions could not be adequately determined. The BAL galactomannan index values of the remaining 99 patients, who included proven, probable and patients without IPA, are shown in the histogram (Figure III-5). The mean BAL galactomannan index value for patients with proven and probable IPA was 4.3 (range, 0.1 – 8.4) compared to patients without IPA that had a mean galactomannan index value of 0.6 (range, 0.1 – 5.5). It is important to note that 19 of the 99 patients received antifungal prophylaxis at the time a BAL was collected. The patients on antifungal prophylaxis could not be excluded from analysis as the raw data were not available for review.

Figure III-5. Histogram of the 99 bronchoalveolar samples from hematologic patients



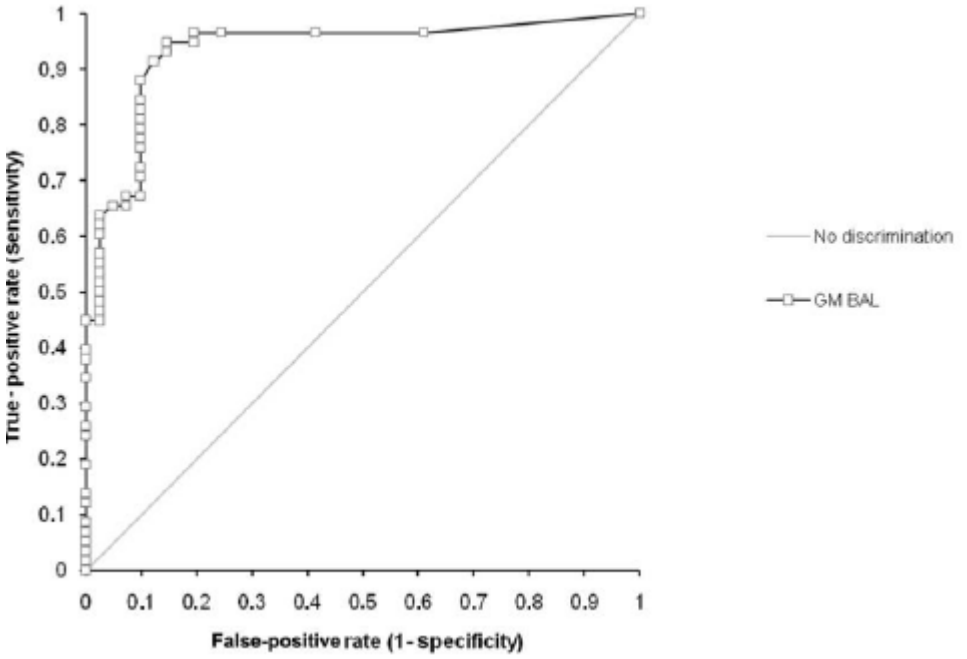
The performance of the galactomannan assay in BAL fluid for the diagnosis of IPA was evaluated at various cut-off values (Table III-11). The results showed that when the cut-off index value was lowered from ≥ 1.5 to ≥1.0, the sensitivity increased from 84% to 91%, while the specificity decreased from 90% to 88%. The estimated positive and negative predictive value of the galactomannan assay in BAL fluid was 76% and 96% at a cut-off index value ≥ 1.0. However, a further decrease in the cut-off value to ≥ 0.5 showed a greater decrease in specificity (80%) and positive predictive value (68%) with a small increase in sensitivity (96%). The ROC curve analysis showed an area under the ROC curve of 0.93, suggesting good diagnostic accuracy within the population (Figure III-6). The findings suggest that a cut-off between 1.0 and 1.5 may be appropriate for testing in BAL fluids among the hematological malignancy patient population, in order to maintain a high specificity and sensitivity.

Table III-11. Performance of the Platelia EIA in BAL fluid among hematologic patients

Comparison, EIA cutoff	Sensitivity (95% CI), %	Specificity (95% CI), %	κ coefficient	Positive likelihood	Negative likelihood	Diagnostic odds ratio	PP accuracy, %	NP accuracy, %
Proven IA vs no IA								
⩾0.5	100	80.4 (68.4–92.6)	0.71	5.1	0	∞	68	100
⩾1.0	96.7 (90.6–100)	87.8 (77.8–97.8)	0.78	7.9	0.04	216	77.3	98.4
⩾1.5 and ⩾2.0	93.5 (84.9–100)	90.2 (81.2–99.3)	0.80	9.6	0.07	134	80.4	97
Proven + probable IA vs no IA								
⩾0.5	96.5 (91.9–100)	80.4 (68.4–92.6)	0.69	4.9	0.04	115	68	98.2
⩾1.0	91.3 (84.2–98.6)	87.7 (77.8–97.8)	0.75	7.5	0.1	76	76.3	96
⩾1.5	84.4 (75.2–93.8)	90.2 (81.2–99.3)	0.73	8.6	0.1	50	78.8	93.1
⩾2.0	79.3 (68.9–89.7)	90.2 (81.2–99.3)	0.69	8.1	0.2	35	77.7	91.1
Proven + probable + possible vs no, ⩾1.0	93.4 (87.2–99.7)	55.2 (43.3–67.1)	0.48	2.0	0.1	17.5	65.5	90.2
Proven + probable vs possible + no, ⩾1.0	86.9 (78.4–95.4)	92.5 (86.2–98.8)	0.79	11.6	0.14	82.1	91.4	88.6

NOTE. Data are for bronchoalveolar lavage fluid from patients with hematologic conditions, assuming a 30% prevalence rate for invasive pulmonary aspergillosis. IA, invasive aspergillosis; NP, negative predictive; PP, positive predictive.

Figure III-6. Receiver operating characteristic (ROC) curve analysis of the galactomannan assay in BAL fluid among hematologic patients



The performance of the galactomannan assay for testing BAL fluid samples was compared with other microbiological methods, i.e. culture, microscopy or serum galactomannan. Cultures for fungi were performed by inoculating specimens onto Sabouraud glucose agar at 37°C for 48 hours. In plates in which there was no growth at 48 hours, were kept up to 19 days at 30°C. *Aspergillus* species were identified on the basis of their cultural characteristics and the morphologies of their conidiophores and conidia. Twenty-nine (50%) of the 58 patients that were diagnosed as proven or probable IPA and were culture positive for *Aspergillus* species (23 *A. fumigatus,* 3 *A. flavus,* 1 *A. niger*, 1 *A. terreus* and 1 *A. nidulans*). Microscopic analysis was performed only on 77 of the patients (45 of the proven or probable IPA patients and 32 patients without IPA). For direct microscopy examination, a smear was made on a slide and stained using the Gomori stain to detect the hyphal elements. Septated hyphae exhibiting angular dichotomous branching were regarded as *Aspergillus* species. Twenty four (53%) of the 45 proven or probable IPA patients tested had a positive smear for fungal hyphae. By definition, patients without IPA had negative smears and cultures for *Aspergillus* species. The overall sensitivity of either culture or microscopy positive was 74%. The authors stated that for 20 of the 58 proven or probable IPA patients who were BAL culture and microscopy negative, galactomannan positivity was the only microbiologic finding for IPA. The results suggests that the galactomannan testing in BAL may be more sensitive than culture-based methods.

Of the 19 patients on antifungal prophylaxis, 14 had proven or probable aspergillosis. The authors noted that among the 58 proven or probable patients with galactomannan

index values <1.0, false negative results were documented in 5 patients, of whom 3 patients received antifungal prophylaxis. However, of the remaining 53 proven or probable IPA patients who had galactomannan index values ≥ 1.0, 11 received antifungal prophylaxis.

A total of 24 patients with proven or probable IPA had pre-existing neutropenia compared to 34 proven or probable IPA patients who were non-neutropenic at the time of BAL galactomannan testing. However, the definition and duration of neutropenia was not specified. The authors noted that the mean galactomannan index value for the 24 neutropenic patients (3.9) was similar to the mean BAL galactomannan index (4.5) in the 34 non-neutropenic patients. The clinical relevance is unknown, since the timing of the bronchoscopic evaluation in relation to the neutropenia was not specified.

III.2. Cancer patients

7. Shahid *et al*. (2008), evaluated the Platelia *Aspergillus* EIA (BioRad Laboratories) galactomannan assay in BAL specimens in patients with and without bronchogenic carcinoma. The study population included patients admitted in the outpatient department or the tuberculosis and chest hospital in India. The 2002 EORTC/MSG guidelines were used to classify patients as proven, probable and possible IPA, however, incorporated the results of the galactomannan assay in BAL, CSF or ≥ 2 blood samples as part of the only microbiological factors. The authors stated that two consecutive BAL specimens were obtained from each patient; however, the period between sampling was not specified. BAL specimens were stored at -20°C and the galactomannan assay was performed according to manufacturer's instructions. Results with a cutoff value of ≥ 1.0 were considered as positive. A total of 85 patients had a bronchoscopy performed, 69 patients had confirmed diagnosis of bronchogenic carcinoma and had not received any type of antifungal therapy; 16 healthy volunteers controls matched to the cases based on age and sex that had no history of lung disease. Of the 69 patients with bronchogenic carcinoma, 6 patients had proven IPA, 17 probable, 13 possible and 33 patients without IPA. The 6 proven IPA patients, the 17 probable IPA and 2 of 13 possible patients were BAL galactomannan positive (Table III-12). None of the non-IPA patients or healthy controls was positive for galactomannan. However, the range of galactomannan index values was not specified for the different groups. The sensitivity of BAL galactomannan assay in proven, probable and possible IPA was 100%, 100% and 15% respectively. The overall specificity and PPV were each 100%, since both the non-IPA and healthy controls in this population were negative. The high sensitivity of the galactomannan assay in BAL indicates good diagnostic potential in lung cancer patients with proven and probable IPA.

Table III-12: Comparison of galactomannan assay with direct microscopy, culture and PCR method on BAL specimens from patients with bronchogenic carcinoma

	No. of patients						
			Serology			BAL	
Aspergillosis types	Direct	Culture	ELISA	DID	DBA	PCR	GM-EIA
Definite IPA, n=6	6	6*	6	6	6	6†	6
Probable IPA, n=17	1	12‡	15	14	14	17§	17
Possible IPA, n=13	0	0	2 (Weak)‖	0	0	8¶	2
Non-IPA, n=33	0	0	0	0	0	1#	0
Healthy controls, n=16	0	0	0	0	0	0	0
Total	7	18	23	20	20	32	25

BAL indicates bronchoalveolar lavage; ELISA, enzyme-linked immunosorbent assay; DID, double immunodiffusion; DBA, dot blot assay; PCR, polymerase chain reaction; GM-EIA, galactomannan-enzyme immunosorbent assay

*Culture showed growth of *A. fumigatus* (n=4) and *A. flavus* (n=2).

† PCR amplicons corresponded with *Aspergillus* species isolated in culture from respective patients.

‡ Culture showed growth of *A. fumigatus* (n=6) and *A. flavus* (n=6).

§ PCR amplicons corresponded with *Aspergillus* species isolated in culture from 12 respective patients; however, in the remaining 5 patients, who did not show growth of Aspergilli in culture, 4 patients had amplicons of *A. fumigatus*, and 1 patient had amplicons of *A. flavus*.

‖ These 2 patients with initial weak positive ELISA results had positive PCR results and, later on during follow-up, had positive results for ELISA, DID, and DBA.

¶ Seven patients were positive for *A. fumigatus* DNA, and 1 patient was positive for *A. flavus* DNA.

An amplicon of *A. fumigatus* was detected.

The performance of the galactomannan assay for testing of BAL samples was compared with conventional microbiological methods (culture and direct microscopy). BAL specimens for direct microscopy and cultures were processed as received. Direct microscopy was performed using 10% potassium hydroxide and lactophenol Cotton Blue mounts. BAL fluids were also cultured on Sabouraud agar containing chloramphenicol. Biopsy specimens for histopathology were collected by bronchial biopsy from 55 patients that presented with intra-luminal growth. Among the remaining 14 patients, 12 patients underwent needle biopsies and 2 had percutaneous lung biopsies. All histopathologic results were reviewed by experienced pathologists. Eighteen patients had documented IPA of which 6 were proven cases [*A. fumigatus* (n=4) and *A. flavus* (n=2)] and 12 were probable cases, *A. fumigatus* (n=6) and *A. flavus* (n=6). Thirty-three patients were classified as without IPA. All of the proven IPA patients were positive by culture and direct microscopy examination. One probable IPA patient had a positive direct microscopy; 12 of the 17 probable IPA had positive culture results. No patients classified as possible IPA had positive culture or direct microscopy results. The sensitivity based on direct microscopy alone in proven and probable IPA patients were 100% and 6%, respectively. The sensitivity based on culture alone in proven and probable IPA patients were 100% and 71%, respectively. The overall specificity based on direct microscopy and culture results were 100%.

BAL galactomannan testing at a cut-off ≥ 1.0 was more sensitive in probable IPA patients than using either direct microscopy or culture alone. However, detection of galactomannan in BAL was no more sensitive than the combined sensitivity of

direct microscopy and culture, suggesting no additional benefit of the galactomannan assay in BAL for the diagnosis of IPA. However, the results of the galactomannan assay in BAL are available faster than fungal culture results, thus indicating the advantage of galactomannan assay over culture for earlier diagnosis of IPA.

It has been speculated that the presence of circulating anti-*Aspergillus* antibodies might interfere with the galactomannan assay leading to false negative results. In this study, serologic evaluation to detect anti-*Aspergillus* antibodies was performed by the double immunodiffusion (DID), enzyme-linked immunosorbent assay (EIA) and dot blot assay (DBA). The DID and DBA methods were in-house laboratory methods and the EIA method was based on a previously published method. All proven IPA patients were positive for circulating anti- *Aspergillus* antibodies by the EIA, DID and DBA methods (Table III-12). Approximately 82-85% of the probable IPA patients were positive for circulating anti- *Aspergillus* antibodies by either EIA, DID or DBA. However, only two possible IPA patients were weakly positive by EIA whereas the DID and DBA methods did not detect antibodies in any possible IPA patients. None of the non-IPA and healthy controls had circulating anti-*Aspergillus* antibodies by any method. The results suggest that presence of anti-*Aspergillus* antibodies do not interfere with the galactomannan assay in BAL.

III.3. Solid Organ Transplants

8. Clancy *et al*. (2007), evaluated the Platelia *Aspergillus* EIA (Bio-Rad Laboratories) in BAL specimens from solid organ transplant recipients at a teaching hospital in Florida. The authors stated that a modified EORTC/MSG guideline (2002) was used to categorize patients as proven, probable, and possible or no IPA, however the modifications to the EORTC/MSG criteria were not specified. BAL fluid samples were tested as received at a central laboratory (MiraVista Diagnostics); however, the period between BAL collection and testing was not specified. The galactomannan assay was performed according to the manufacturer's instructions as previously described and results were reported as positive based on an optical density index value ≥ 0.5. All positive samples were retested using the same aliquot of the BAL sample and considered positive only if the repeat test was also positive.

   A total of 81 transplant (24 heart, 22 kidney, 19 liver and 16 lung) patients had a bronchoscopy performed. The patient ages ranged from 4 years to 79 years. Two patients were diagnosed as proven IPA, three had probable IPA and 76 were without IPA. No patient was classified as possible IPA. The five patients with proven or probable IPA were galactomannan positive with index values ≥ 2.1 (range 2.1 to 10.2). Of the 76 patients without IPA, 12 were BAL galactomannan positive at a cut-off index ≥ 0.5 (range 0.55 – 8.1) and seven patients were BAL galactomannan positive at a cut-off index values ≥ 1.0 (range 1.09 – 8.1) (Figure III-7). The sensitivity, specificity, PPV and NPV for BAL testing at a galactomannan index value ≥ 0.5 were 100%, 84%, 29% and 100% respectively.

Increasing the cut-off to ≥ 1.0, had no effect on the sensitivity and NPV (100%), however, was associated with improvement in the performance of the galactomannan assay by eliminating five false positives thus increasing the specificity from 84 to 91% and increasing the PPV from 29 to 42% (Table III-13). The ROC curve analysis in this study population indicated that the optimal performance of the galactomannan assay was achieved by using an index cut-off of 2.1 (Figure III-8). However, this should be interpreted with caution because all proven and probable IPA cases had galactomannan levels ≥ 2.1. Furthermore, the number of proven/probable IPA cases (n=5) in the study population was low.

Figure III-7: Distribution of BAL galactomannan results

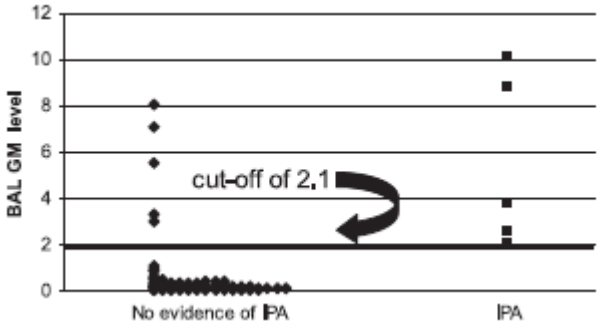
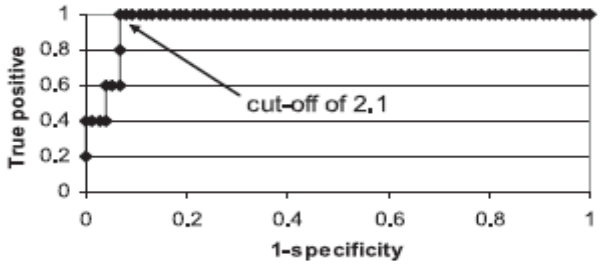


Table III-13: Performance of BAL galactomannan assay compare to conventional methods for diagnosis of IPA

Test	% (no. with indicated result/total)			
	Sensitivity	Specificity	PPV	NPV
BAL GM				
Cutoff, ≥0.5	100 (5/5)	84.2 (64/76)	29.4 (5/17)	100 (64/64)
Cutoff, ≥1	100 (5/5)	90.8 (69/76)	41.7 (5/12)	100 (69/69)
Cutoff, ≥1.5	100 (5/5)	92.1 (70/76)	45.4 (5/11)	100 (70/70)
Cutoff, ≥2	100 (5/5)	93.4 (71/76)	50 (5/10)	100 (71/71)
Cutoff, ≥2.5	80 (4/5)	93.4 (71/76)	44.4 (4/9)	98.6 (71/72)
Serum GM (single value, ≥0.5)	25 (1/4)	97 (33/34)	50 (1/2)	91.7 (33/36)
Positive cytology	50 (2/4)	93.2 (69/74)	28.6 (2/7)	97.2 (69/71)
Positive culture for *Aspergillus* sp.	40 (2/5)	93.4 (71/76)	28.6 (2/7)	95.9 (71/74)
Positive cytology or culture	60 (3/5)	90.8 (69/76)	30.0 (3/10)	97.2 (69/71)
Cavity seen on chest CT	80 (4/5)	100 (73/73)	100 (4/4)	98.7 (73/74)

Figure III-8: Receiver operator characteristic (ROC) curve for BAL galactomannan assay



Serum specimens were collected within three days of BAL specimens. Serum specimens were tested as received according to manufacturer's instructions and considered positive at optical density index ≥ 0.5. All serum samples were retested and considered positive if the repeat test was also positive. Of the 81 transplant patients, 38 patients (4 of the 5 patients with IPA and 34 of the 76 patients without IPA) had galactomannan testing in serum and BAL performed (Table III-14). The galactomannan assay sensitivity in serum (25%) was considerably lower compared to BAL (100%). Of the four IPA patients tested, only one patient had a positive serum galactomannan (index = 0.93). The BAL galactomannan index value for this patient was 10.12. Detection of galactomannan in the remaining three IPA cases showed that in BAL the galactomannan results ranged from 2.58 to 8.83 whereas the sera galactomannan was ≤ 0.5 (Table III-15). These results suggest that the level of galactomannan detection was higher in BAL than in serum. Despite a higher sensitivity of the galactomannan assay in BAL than serum, the specificity of the galactomannan assay in serum (97%) was higher than in BAL at any of the cut-off values (range 84% to 93%). The results suggest that there was an increase likelihood of obtaining false-positive results with BAL compared with sera in this study population. Seven patients had false positive BAL compared to one patient with a false positive serum galactomannan (Table III-14).

Table III-14: Concordance between serum and BAL galactomannan levels

Serum GM level	Total no. of patients (no. with IPA)		
	BAL GM, <0.5	BAL GM, 0.5–0.9	BAL GM, ≥1.0
<0.5	26 (0)	4 (0)	6 (3)
≥0.5	1 (0)	0	1 (1)

Among the five IPA patients, only the two proven had serial bronchoscopies performed after initial BAL galactomannan result (Table III-15). The authors stated that patients were treated with either voriconazole or amphotericin B lipid complex after a positive BAL galactomannan result. However, one proven IPA patient received prophylaxis with amphotericin B lipid complex (5 mg/kg) for 3 weeks prior to the time of BAL collection; the galactomannan index for BAL sample was positive (index = 8.83) and the serum was negative (index = 0.07). The patient was treated for a period of one year with voriconazole and amphotericin B lipid complex. Within the year the patient had three BAL specimens collected, and at 21 days after initiation of therapy the

galactomannan assay in BAL remained positive (7.72), however at 5 months and 9 months after initiation of antifungal therapy the BAL galactomannan had decreased to 0.19 and 0.26, respectively (Table III-15). By contrast, in this patient the serum galactomannan was negative up to day 3 following the initiation of therapy. No further information was given for testing of sera. In the second proven IPA patient, at two months following the initiation of antifungal treatment with voriconazole, the BAL galactomannan decreased from an initial result of 2.58 to 0.13. The galactomannan assay remained negative (0.23) up to one month after treatment ended at month 8. The serum galactomannan in this patient was negative at six days after the initiation of antifungal therapy. The results suggest that two to five months of antifungal therapy was associated with a decline in BAL galactomannan to levels ≤0.5. However, negative results in serum were observed during antifungal therapy, and no information was given on antibody levels.

Table III-15: Clinical characteristics of patients with IPA

Patient (age [yr], sex)	Transplant status[a]	Reason(s) for BAL	CXR/CT scan	Antibiotic(s) prior to or at the time of BAL[b]	BAL GM result[c]	Serum GM result[d]	Cytology result	Transbronchial biopsy of BAL fluid result	BAL fluid culture result	Diagnosis	Treatment[e]	Outcome, follow-up period
1 (54, male)	3rd liver transplant; 3 wk; Sol (10 mg/day), FK506	Fever, sepsis	Multifocal consolidations	AmBLC (3 wk), Lvx, Van	8.83*	0.07*	ND	ND	No fungus	Proven IPA (disseminated aspergillosis by brain and thyroid biopsies)	Vrc added to AmBLC after BAL result (treated for 1 yr); resection of the brain and thyroid lesions	Survived, 1.2 yr
2 (40, male)	Kidney; 5 yr; Pred (60 mg/day)	Cough, pleurisy, hemoptysis	Multiple bilateral cavities with surrounding ground glass opacities	Tim, Azm	2.58†	0.04†	Acute inflammation; no hyphae	Acute inflammation; no hyphae	Negative	Proven IPA (culture of pleural fluid, *A. fumigatus*)	Vrc (8 mo)	Survived, 2 yr
3 (64, male)	Heart; 2 yr; Siro, Pred (10 mg/day)	Fever	Cavity with surrounding infiltrates	Mxf	2.1	ND	Inflammation and hyphae	Necrotic tissues with hyphae	*Candida* sp.	Probable IPA	AmBLC plus Vrc immediately after BAL, then Vrc after GM result (6 mo)	Survived, 10 mo
4 (58, male)	Kidney; 3 mo; ATG, FK506, Myco, Pred (10 mg/day)	Fever, chest pain	Multiple bilateral cavities/nodules	Cro, Azm, Sxt	10.12	0.93‡	Inflammation; hyphae	Acute and chronic inflammation; no hyphae	*A. fumigatus*, *A. flavus*	Probable IPA	AmBLC immediately after BAL, then Vrc after GM result (3 mo)	Survived, 1.3 yr
5 (42, male)	3rd kidney transplant; 5.3 yr; FK506, Myco, Pred (10 mg/day)	Fever, cough	Multiple bilateral cavities and nodular densities	None	3.77	0.13	No hyphae	Focal acute/chronic inflammation; no hyphae	*Penicillium* sp., *A. fumigatus*	Probable IPA	Vrc (1.25 yr)	Survived, 1.25 yr

[a] Transplant status: transplanted organ; time from last transplant to BAL; immunosuppressive regimen. Abbreviations: Sol, methylprednisolone (Solu-Medrol); FK506, tacrolimus; Pred, prednisone; Siro, sirolimus; ATG, anti-thymocyte globulin; Myco, mycophenolate.

[b] Abbreviations: AmBLC, amphotericin B lipid complex; Lvx, levofloxacin; Van, vancomycin; Tim, ticarcillin-clavulanic acid (Timentin); Azm, azithromycin; Mxf, moxifloxacin; Cro, ceftriaxone; Sxt, trimethoprim-sulfamethoxazole.

[c] Symbols: *, follow-up BAL GM results during antifungal therapy, 7.72 (21 days), 0.19 (5 months), 0.26 (9 months); †, follow-up BAL GM results during antifungal therapy, 0.13 (2 months), 0.23 (9 months).

[d] Symbols: *, follow-up serum GM results over the next 4 days, 0.07, 0.07, 0.06; †, follow-up serum GM results over the next 6 days, 0.44, 0.06, 0.06, 0.12; ‡, follow-up serum GM results during antifungal therapy, 0.11 (4 days), 0.06 (1 month), 0.07 (1 month), 0.07 (2 months). ND, not done.

[e] Abbreviations: Vrc, voriconazole; AmBLC, amphotericin B lipid complex.

The performance of the galactomannan assay in BAL was compared with other conventional methods such as cytology, culture and biopsy. The sensitivity of the galactomannan assay in BAL (100%) was higher than cytology (50%), culture (40%) and transbronchial biopsy (25%) (Table III-13). Furthermore, the assay in BAL resulted in an earlier time to diagnosis of IPA patients than any of the conventional methods (Table III-15). In the two proven IPA patients, the galactomannan assay was first positive in one patient, 1 week before the brain biopsy showed *Aspergillus* in a resection of brain and thyroid lesions, and in the other patient at four weeks before the pleural fluid culture was positive for *A. fumigatus*. Similarly, in one probable IPA patient, the BAL was positive for galactomannan several days before the culture was positive for *A. fumigatus*. In the remaining two probable IPA patients, the BAL galactomannan was positive within two days of the cytology findings that revealed hyphae.

Twelve of the 76 patients without IPA had a positive BAL galactomannan result with an index ≥ 0.5 (range from 0.55 to 8.1) (Table III-16). None of the 12 patients received piperacillin-tazobactam. One patient without IPA had a BAL culture that yielded *Penicillium* species with BAL galactomannan optical density index of 1.62. Five of the 12 patients without IPA were lung transplant recipients, of which three had cultures that yielded *Aspergillus* species and the BAL galactomannan optical density index ranged from 3.0 to 8.1. The authors noted if lung transplant recipients were excluded, the PPV increased in patients receiving other solid organ transplants from 29% to 62% at a cutoff of ≥ 0.5. Though the PPV of the galactomannan assay in serum remained unchanged (83%) when lung transplant recipients were excluded, the PPV in serum was higher than in BAL (62%). Thus further emphasizing the increase likelihood of false-positive results with BAL compared with sera in the solid organ transplants population.

It is important to note that there were no cases of proven/probable IPA in lung transplant patients in this study. Lung transplant patients accounted for 3/7(43%) of the false positive BAL galactomannan results at a cut off ≥ 1.0. This is expected as airway colonization with *Aspergillus* is relatively common in lung transplant patient and *Aspergillus* species can be found in airway cultures in 25 to 30% of patients. When lung transplant patients were excluded from the analysis of this study, using a cut off ≥ 1.0, the specificity improved from 91 to 93% and the PPV improved from 42 to 65%.

Bronchoscopy is more commonly performed in solid organ transplants with pulmonary infiltrates compared to hematologic patients; therefore, the BAL galactomannan could be a useful adjunctive test for diagnosis of IPA. However, more studies are needed to define an appropriate galactomannan cut-off index in this population. The galactomannan test in BAL specimens is probably less useful for diagnosis of IPA in lung transplant than in other organ transplant patients because of higher rates of false positive results.

Table III-16: Characteristics of 12 patients without IPA that with BAL galactomannan results ≥ 0.5

Patient (age [yr], sex)	Transplant status[a]	Reason(s) for BAL	CXR/CT scan	Antibiotic(s) prior to or at the time of BAL[b]	BAL GM result	Serum GM result[c]	Cytology result	Transbronchial biopsy of BAL fluid result	BAL fluid culture result	Diagnosis	Treatment[d]	Outcome, follow-up period (cause of death)
1 (57, male)	Heart; 2.5 yr; Pred (10 mg/day), FK506	Fever, cough, wt loss, diabetic ketoacidosis	Large nodular mass extending to chest wall; mediastinal adenopathy	Fep, Van	0.55	0.1	No hyphae	Focal lymphocytic inflammation; yeast but no hyphae	*C. albicans*	Pneumonia due to *Rhodococcus equi*	No antifungal	Survived, 1.5 yr
2 (40, male)	3rd liver transplant; 3 wk; Sol (20 mg/day), FK506	Hypotension, multisystem organ failure	Multiple bilateral nodules	Flc, Fep, Metro	0.56	0.1, 0.33	No hyphae (+) yeast	ND	*C. glabrata*	Bacteremia and pneumonia due to *Pseudomonas aeruginosa*	AmBLC (2 days), then Vrc (3 wk) (until death)	Died, 3 wk (bowel perforation and multisystem organ failure)
3 (48, female)	Single lung; 5.5 yr; CyA, Aza, Pred (10 mg/day)	Fever, shortness of breath, cough	Focal mild consolidation	Sxt, Fep	0.63	ND	No hyphae	Mild acute cellular rejection	*Candida*	Rejection	No antifungal	Survived, 1.3 yr
4	Bilateral lung; 4 yr; FK506, Siro, Pred (5 mg/day)	Fever, hypoxemia	Ground glass opacification (diffuse)	Sxt	0.86	0.2	PCP	Interstitial lymphocytic infiltrate	No fungus	Pneumocystis pneumonia	No antifungal	Survived, 4 mo
5 (65, male)	Kidney; 4 yr; CyA, Myco, Pred (10 mg/day)	Fever, chills, rigors, weakness	Patchy airspace disease and ground glass opacity (focal)	Fep, Van	0.96	0.06	No hyphae; normal cells	Mild interstitial edema; no hyphae	No fungus	Community-acquired pneumonia	No antifungal	Survived, 1.1 yr
6 (63, female)	Heart; 1.5 mo; Sol (50 mg/day), FK506	Septic shock	Consolidation (multifocal)	Fep	1.09	0.11, 0.24	ND	ND	No fungus	Refractory pulmonary valve endocarditis due to *Enterococcus faecalis*	Vrc (2.5 days) (until death)	Died, 3 wk (multisystem organ failure and refractory enterococcal bacteremia)
7 (55, male)	Kidney; 10 yr; CyA, Myco, Pred (10 mg/day)	Fever, chills, shortness of breath, weak	Consolidation (focal); hilar adenopathy	None	1.62, 0.10 (2 days later)	ND	No hyphae	Squamous cell cancer; no hyphae	*Penicillium*	Lung cancer with postobstructive pneumonia	No antifungal	Survived, 4 mo
8 (51, female)	Bilateral lung; 5 wk; FK506, Aza, Pred (10 mg/day)	Bronchial stenosis due to lung reperfusion lung injury	Focal consolidation	Van, Gat, Dapsone	3.04, 1.5 (7 days later)	ND	Acute inflammation cells; hyphae	ND	*A. terreus*	Bacterial pneumonia	Inhaled AmB (3 days) (to "eradicate colonization")	Survived, 1.4 yr
9 (55, male)	Heart; 3 wk; OKT3, FK506, Myco, Sol (100 mg/day)	Post-transplant sepsis	Extensive focal consolidation; pleural effusion	Fep, Van	3.35, 0.12 (2 days later), 0.13 (3 days later), 0.10 (4 days later)	0.12, 0.13, 0.10	ND	ND	No fungus	Nosocomial bacterial pneumonia due to *Klebsiella pneumoniae*	Vrc (2.25 mo) (until death)	Died, 2.25 mo (sepsis due to multidrug-resistant *Acinetobacter*; cardiopulmonary arrest)
10 (60, male)	Heart; 2 mo; CyA, Myco, Sol (150 mg/day)	Fever, cough, hemoptysis	Focal consolidation with reticulonodular component	Fep, Van, Azm, Metro, Sxt	5.57	0.11	No hyphae; acid-fast organisms	ND	No fungus	Pneumonia due to *Nocardia asteroides*	Vrc (7 days) (until death)	Died, 8 days (multisystem organ failure, sepsis)
11 (59, female)	Single lung; 2.5 mo; FK506, Aza, Pred (20 mg)	Fever, cough, chills	No infiltrates (X-ray only)	Sxt	7.14	ND	Acute inflammation; hyphae	Acute pneumonia; no hyphae	*A. flavus*, *A. niger*	Pneumonia due to *Pseudomonas aeruginosa*; mild rejection	No antifungal	Survived, 1.3 yr
12 (33, female)	Single lung; 2 yr; FK506, Pred (10 mg/day)	Shortness of breath, nausea	Ground glass consolidation (focal) and pleural effusion	Sxt	8.1	ND	Many macrophages; (+) hyphae	Mild lymphocytic bronchitis with unattached hyphae	*A. flavus*	MRSA pneumonia	Itraconazole (1 wk)	Died, 2 mo (disseminated MRSA infection [autopsy])

[a] Transplant status: transplanted organ; time from last transplant to BAL; immunosuppressive regimen. Abbreviations: Cya, cyclosporin A; Aza, azathioprine; OKT3, muromonab-CD3; Sol, methylprednisolone (Solu-Medrol); FK506, tacrolimus; Pred, prednisone; Siro, sirolimus; Myco, mycophenolate.
[b] Abbreviations: Fep, cefepime; Van, vancomycin; Azm, azithromycin; Metro, metronidazole; Gat, gatifloxacin; Sxt, trimethoprim-sulfamethoxazole.
[c] ND, not done.

9. Husain *et al*. (2007) evaluated the Platelia *Aspergillus* EIA (Bio-Rad Laboratories) in BAL specimens from lung transplant recipients. The 2002 EORTC/MSG guidelines were used to classify patients as proven or probable IPA. BAL specimens were stored at -80°C until testing. The galactomannan assay was performed at a central laboratory (MiraVista Diagnostics). Results were reported as positive based on an optical density index value ≥ 0.5. All positive samples were retested using the same aliquot and considered positive only if the repeat test was also positive. The authors noted that sterile saline and not Plasmalyte (Baxter Healthcare Corporation Deerfield, IL) was used for bronchoscopy evaluation and BAL sampling. The patients' ages ranged from 18 years to 65 years. A total of 333 BAL specimens were evaluated from116 patients. Two patients were diagnosed as proven IPA, four probable IPA and 110 patients without IPA. For the six IPA patients, the BAL galactomannan index ranged from 0.22 to 6.83, of which four patients were positive at a cut-off value ≥ 0.5 (Table III-17).

Table III-17: Clinical characteristics and galactomannan values in lung transplant patients with IPA

Patient	Age/Sex	Underlying disease	Onset posttransplant (months)	Time elapsed from positive GM to onset of symptoms (days)	Site of infection	Definite/ probable	Prior *Aspergillus* colonization	Pathogenic *Aspergillus* species	GM index in BAL	Concomitant antifungal prophylaxis	Outcome
1	46/M	Emphysema	20	30	Pulmonary	Probable	No	*A. fumigatus*	1.21	No	Alive
2	41/F	Cystic fibrosis	25	5	Pulmonary	Probable	No	*A. terreus*	6.83	No	Alive
3	42/F	Alpha-1 antitrypsin deficiency	22	Symptoms preceded by 16 days	Pulmonary	Definite	No	*A. fumigatus*	3.48	No	Died
4	42/M	Cystic fibrosis	23	NA	Pulmonary	Probable	No	*A. flavus*	0.22	Voriconazole	Alive
5	44/F	Alpha-1 antitrypsin deficiency	70	0	Pulmonary	Probable	Yes	*A. fumigatus*	3.74	No	Alive
6	44/M	Alpha-1 antitrypsin deficiency	3	NA	Tracheobronchitis	Definite	No	*A. niger*	0.24	Itraconazole	Alive

GM, galactomannan; NA, not applicable.

Of the 110 non IPA patients, 13 were positive by the galactomannan assay in BAL at a cut-off index ≥ 0.5 and seven patients were positive at a cut-off index ≥ 1.0 (Figure III-9 and Table III-18). Seven of the 13 patients were colonized with *Aspergillus* species at the time of BAL galactomannan testing and four of these seven patients subsequently developed IPA. In two patients, BAL galactomannan was positive at 60 days prior to the diagnosis of IPA. In one patient (#4; Table III-18) BAL galactomannan index increased from 0.61 to 6.83 at the time of IPA diagnosis. It is also important to note that patient #4 was also colonized with *A. terreus* at the time of testing of the second BAL sample for presence of galactomannan. In another patient (#2), BAL galactomannan increased from 0.54 to 1.21 at the time of IPA diagnosis (Table III-18). The remaining two patients, patients #5 and #10 (Table III-18), had positive BAL galactomannan indices for more than a year prior to diagnosis of IPA. Patient #5 (Table III-18), was colonized with *A. versicolor* and received antifungal prophylaxis with voriconazole for six months at the time of BAL galactomannan testing; the galactomannan index value decreased from 1.20 to negative indices (not specified). Patient #10 (Table III-18) had an endobronchial stent placement, was colonized with *A. fumigatus* and was placed on long-term prophylaxis with itraconazole for bronchomalacia in the transplanted lung.

The BAL galactomannan index was initially 1.45 and nine months later the patient was still colonized with *A. fumigatus,* however the BAL galactomannan result had decreased to 0.7. The time to IPA diagnosis in patients #5 and #10 was not specified.

Figure III-9: Histogram of 305 BAL samples from patients who did not develop invasive aspergillosis

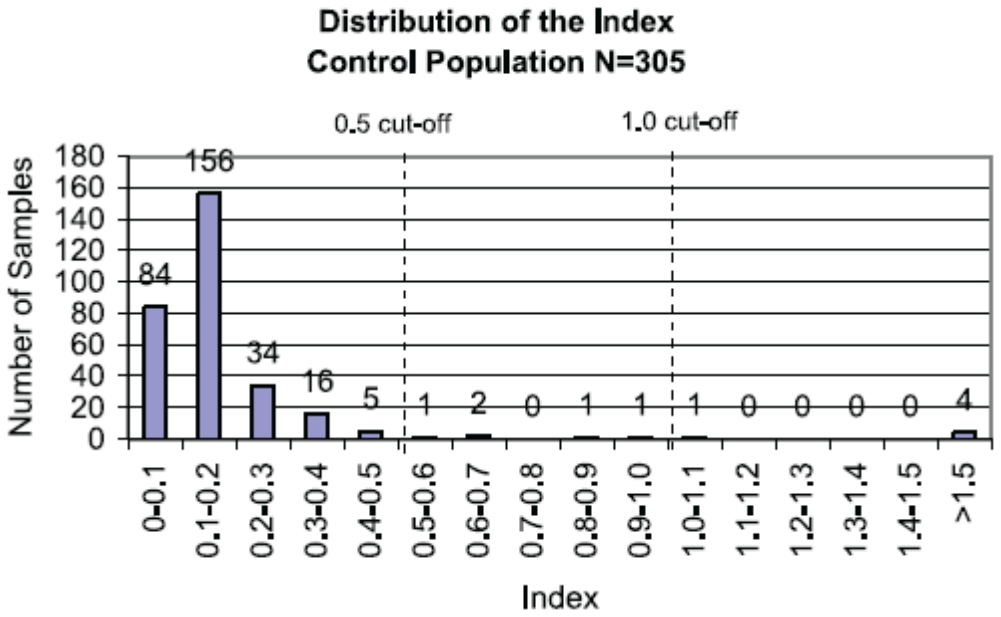


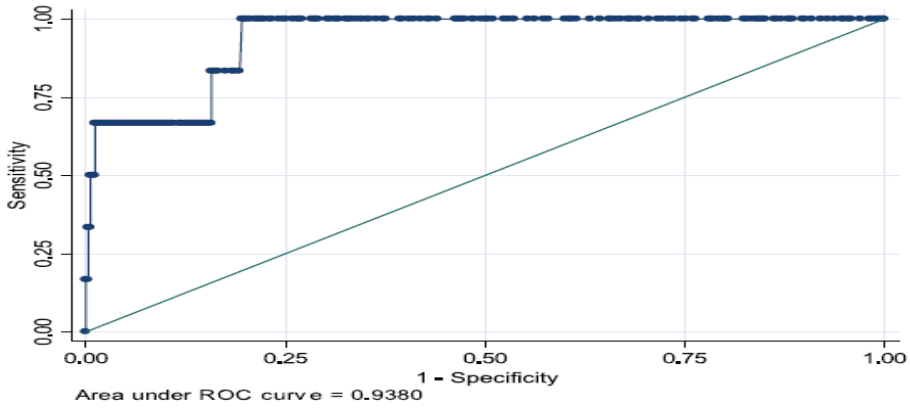
Table III-18: False positive results in 13 lung transplant patients without IPA.

Patient	Time posttransplant (months)	Colonizing species	Concomitant antifungal agent use	Concomitant piperacillin-tazobactam use	Galactomannan index
1	12	*A. flavus*	None	None	1.04
2	20	None	None	None	0.54
3	29	*A. flavus*	Itraconazole	None	0.95
4	24	*A. terreus*	None	None	0.61
5	12	*A. versicolor*	Voriconazole	None	1.20
6	20	None	None	None	0.65
7	22	*Penicillium*	Voriconazole	None	2.40
8	34	None	None	None	0.83
9	32	*A. versicolor*	Itraconazole	None	0.652
10	50	*A. fumigatus*	Itraconazole	None	1.45
10	59	*A. fumigatus*	Itraconazole	None	0.70
11	4	*Penicillium*	Itraconazole	Yes	0.58
11	6	*Paecilomyces*	No	None	4.67
12	5	*Penicillium*	No	None	6.34
13	8	None	No	Yes	2.45

The sensitivity, specificity, PPV and NPV for BAL testing at an index value ≥ 0.5 were 67%, 88%, 23% and 98%, respectively. Increasing the cut-off to ≥ 1.0, had no effect on the sensitivity (67%) and NPV (98%), but increased the specificity (93%) with little change in PPV (36%). The ROC curve using a generalized estimation equation regression analysis suggests that the optimal performance of the galactomannan assay was achieved by using an index cut-off of 0.5. Increasing the cut-

off index from ≥ 0.5 to ≥ 1.0 retained the sensitivity (67%) and improved the specificity from 88% to 93% (Figure III-10).

Figure III-10: Receiver operative characteristics (ROC) curve for the galactomannan assay in BAL



Of the six patients with IPA, two patients received antifungal prophylaxis. One patient with proven IPA received oral itraconazole for more than one month prior to the diagnosis of IPA for colonization due to *A. niger*. Another patient with probable IPA received voriconazole prophylaxis for more than one year prior to IPA diagnosis for persistent *Aspergillus* colonization and later developed new pulmonary nodules on CT scan and BAL fluid culture that yielded *A. flavus*. The BAL samples were galactomannan negative (galactomannan index 0.22 and 0.24) in the two patients (Table III-17).

Colonization with *Penicillium* species was reported in three patients without IPA at the time of BAL galactomannan testing (Table III-18). Patients # 7, #12, and patient #11 were positive with galactomannan index values of 2.4, 6.34, and 0.58, respectively. These were probably false positives as patient # 7 was colonized with *Penicillium* species, patient #12 was on antifungal therapy with itraconazole and patient #11 was treated with piperacillin-tazobactam at the time of initial bronchoscopy result. Two months later the BAL galactomannan result increased to 4.67 in patient #11, however at the time of second BAL galactomannan testing the patient was colonized with *Paecilomyces* species which is known to cross react with the assay. One other patient was on piperacillin-tazobactam and had a positive BAL galactomannan result of 2.45.The period between initial BAL specimen that was negative and development of nodules on CT scan and positive culture results in this patient was not specified. No correlation between antifungal use and the galactomannan index could be established due to a small number of subjects in the study.

10. Husain *et al*. (2008), is a compilation of laboratory test results conducted at MiraVista Laboratory from two studies (Clancy *et al*., 2007 and Husain *et al*., 2008) reviewed above. Parts of the dataset were also available for an independent review.

    The authors evaluated the galactomannan assay in BAL specimens from solid organ transplant recipients. The classification scheme used to diagnose patients with IPA was similar to that described previously for Clancy *et al*. (2007), and Husain *et al*. (2007). BAL specimens collected as part of clinical care and were tested as received. However, BAL specimens from patients based on surveillance or routine monitoring following transplantation were stored for later testing. The timing between storage of specimens and testing was not specified. A positive result was based on cut-off index ≥ 0.5. All positive samples were verified by repeat testing of another aliquot of the same sample.

    A total of 196 solid organ transplant patients had BAL specimens obtained of which 11 were IPA patients and 185 controls. Of the 185 controls, 119 were lung transplant patients who had BAL specimens collected for surveillance, suspected rejection, or possible infection. The remaining 66 controls were patients who had BAL specimens obtained for diagnostic reasons included 26 lung transplant patients, 15 kidney transplant patients, 13 liver transplant patients, and 12 heart transplant patients. Additional BAL specimens were collected from asymptomatic individuals including 44 HIV patients and 12 patients with no underlying disease; the data from these patients were not available for review. Of the 11 patients with IPA, four had proven IPA and seven had probable IPA (based on an independent data analysis). No patients were classified as possible IPA. Although the authors did not provide the data, an independent analysis showed that the BAL galactomannan index in 11 IPA patients ranged from 0.2 -10.1. Nine patients were positive at cut-off index ≥ 0.5 and eight patients were positive at a cut-off index ≥ 1.0. Of the 185 diagnostic and surveillance controls, 23 were BAL galactomannan positive at a cut-off index value ≥ 0.5 and 10 were BAL galactomannan positive at a cutoff index value ≥ 1.0.

    The specificity of the galactomannan assay in BAL ranged from 73% to 100% at a cut-off index ≥ 0.5 (Table III-19). The highest specificity was in the healthy controls with HIV infection (100%) and least specificity in colonized controls (78%) and diagnostic controls (73%) populations. Increasing the cut-off value from ≥ 0.5 to ≥ 1.0 had a minimal effect on the specificity in surveillance controls and HIV controls, however the specificity increased by 6% to 14% in the remaining populations, thus suggesting that a cut-off ≥ 1.0 reduces the number of false positives even if the control population is not well defined. The distribution of BAL galactomannan results in patients with IPA and controls are shown in Figure III-11. In contrast, changing the cut-off index value to ≥ 0.5 had minimal effect on the sensitivity (8/11) compared at a cut-off index ≥ 1.0 (9/11), because of the low prevalence of IPA in the solid organ transplant population (11/185).

Figure III-11: Distribution of BAL galactomannan detection in patients with and without IPA.

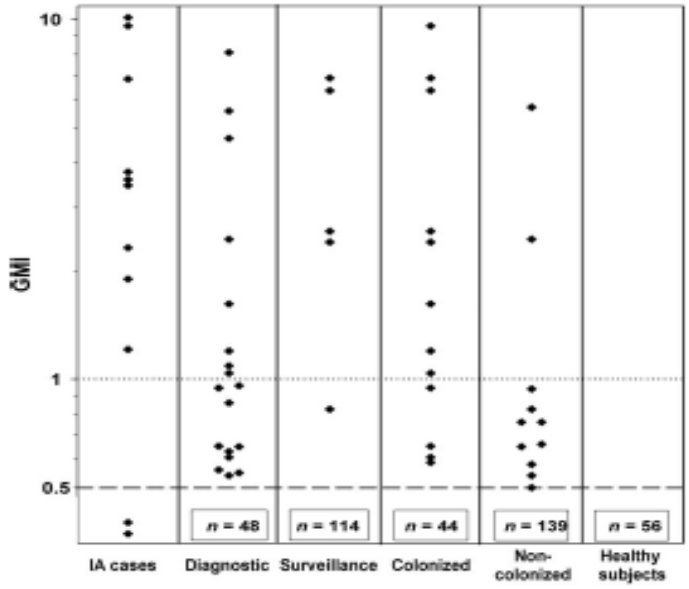


Table III-19. Specificity of the galactomannan assay in BAL at various cut-offs and in different controls

Group	Specificity % (no. with negative result/total)	
	≥ 0.5	≥ 1.0
Surveillance control	96% (114/119)	97% (115/119)
Diagnostic control	73% (48/66)	87% (58/66)
Surveillance and diagnostic controls	86% (162/185)	93% (173/185)
Non-colonized controls	93% (139/150)	99% (148/150)
Colonized Controls	78% (44/56)	86% (48/56)
Healthy controls with HIV infection	100% (56/56)	100% (56/56)

Of the 11 patients with IPA, three patients were treated with mold-active antifungal agents. One patient who was treated with amphotericin B had a positive BAL galactomannan index of 8.3. Two patients who received antifungal agents (itraconazole and voriconazole) had negative BAL galactomannan results at 0.22 and 0.24, respectively. The duration of antifungal treatment in relation to period of IPA diagnosis was not specified. Of the 185 controls without IPA, 25 patients were colonized with *Aspergillus* species at time of BAL galactomannan testing, of which 8 patients [*A. flavus* (n=4), *A. terreus* (n=2), and *A. versicolor* (n=2)] were positive [index range 0.53 to 8.1] for BAL galactomannan and galactomannan index ranged from. All positive BAL galactomannan samples from patients colonized with *Aspergillus* were from lung transplant patients. Twenty-seven patients without IPA were colonized with species other than *Aspergillus* at the time of BAL galactomannan testing, three with *Penicillium* species and one with *Paecilomyces* species. The BAL galactomannan results ranged from 1.62 to 6.78. Among the

patients without IPA, four were treated with piperacillin-tazobactam, of which three had a positive BAL galactomannan result ranging from 0.56 to 4.2.

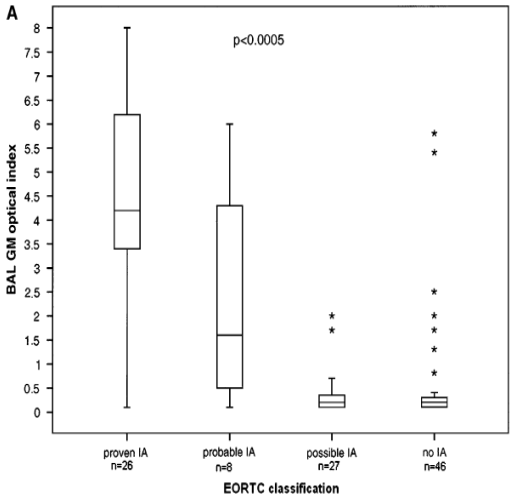
Raising the cut-off index from ≥ 0.5 to ≥1.0, increased the specificity of the galactomannan assay in BAL by as much as 6% to 14% depending on the type of controls used, with minimal effect on the sensitivity. The results indicate that the cut-off index of ≥1.0 reduces the number of false positives, especially in a solid organ transplant patient population. Antifungal treatment with itraconazole and voriconazole reduced the sensitivity of the galactomannan assay in BAL. In addition, patients colonized with *Aspergillus* species, colonized or infected with fungal species other than *Aspergillus (Penicillium* sp. and *Paecilomyces* sp.) or treated with antibiotics such as piperacillin-tazobactam result in false positives.

III.4. Intensive Care Unit

11. Meersseman *et al*. (2008), evaluated the Platelia *Aspergillus* EIA galactomannan assay (Sanofi Diagnostics Pasteur) in BAL specimens from patients in an intensive care unit (ICU) in Belgium. Patients included in the study population had at least one host factor i.e., hematologic malignancy, cancer, solid organ transplant recipient, HIV, Child C cirrhosis, steroid use or receipt of other immunosuppressive treatment. The 2002 EORTC/MSG guidelines were used to classify patients as proven, probable or possible IPA, however, detection of galactomannan in serum and BAL was not used as a microbiological criterion for diagnosis.

    BAL specimens were obtained upon inclusion into the study. BAL specimens were tested as received according to the manufacturer's instructions. Galactomannan index value ≥ 0.5 was considered positive. A total of 110 patients had a bronchoscopy performed. The ICU mortality rate was 66% and autopsies were performed in 95% of the fatal cases. The sensitivity and specificity was calculated from proven IPA cases as well as patients in which biopsy or an autopsy was performed. Sixty-one patients had signs indicative of IPA, of which 26 had proven, 8 probable, and 27 possible IPA. Forty-nine patients without IPA included three patients with proven non-*Aspergillus* mold infection, three patients with *Aspergillus* colonization and 43 patients without IPA. The BAL galactomannan results among the three groups of patients with IPA and the patients without IPA on day 1 are shown in Figure III-12. The median galactomannan index in BAL was the highest among patients with proven (index = 4.1) compared to probable (index = 1.5) and possible IPA (≤ 0.5).

Figure III-12: Distribution of galactomannan results on first BAL performed in ICU patients



Patients with probable and possible IPA were excluded from the analysis and only patients with proven based on an autopsy or other documented evidence of IPA were included thereby excluding patients colonized with *Aspergillus* . As with proven IPA cases, patients without IPA were confirmed by autopsy; however, the analysis included only the 43 patients without IPA and the three patients with proven non-*Aspergillus* mold infection. Patients colonized with *Aspergillus* were excluded. Twenty-three of the 26 proven IPA patients had a positive BAL galactomannan index ≥ 0.5 (range 0.6 -7.9) on the first BAL sample, thus the sensitivity was 88%. Of the 46 patients without IPA, six patients were galactomannan positive in BAL at a cut-off index ≥ 0.5 (Table III-20). There was a loss of specificity (87%) due to a larger number of false positives in this population. The ROC analysis suggests the optimal performance of the BAL galactomannan assay at a cut-off optical density index ≥ 0.5 (Figure III-13).

Table III-20: Patient Characteristics and Documentation of proven IPA

	Patient characteristics								Culture and/or Microscopic			ELISA (*ng/ml*)					
Patient No.	Age	LOS	Predicted Mortality (%)	Host Factor	Admission Diagnosis	Neutropenia (<500/mm^3)	Outcome	Di	Examination. (BAL)*	HRCT Lesions	Macro Lesions†	BAL D1	Serum D1	BAL D8	Serum D8	Antifungal R/‡	Histo Evidence
1	50	17	60	AML, GVHD	Encephalopathy	No	Death	4	No	NA	No	4.3	0.1	NA	NA	No	Autopsy
2	35	13	41	CML, GVHD	ARDS	No	Death	6	No	ARDS	No	0.3	0	3.9	0,7	No	Autopsy
3	74	7	77	CLL	ARDS	Yes	Death	4	Yes	ARDS	Yes	6.9	0.7	NA	NA	Caspo	Biopsy + aut
4	67	7	48	Cirrhosis	Peritonitis	No	Survived	6	Yes	NA	Yes	3.9	0	NA	NA	Ampho	Biopsy
5	79	4	95	ALL	Pneumonia	Yes	Death	2	Yes	NA	No	4,1	1.7	NA	NA	No	Autopsy
6	49	17	41	Steroids	Encephalopathy	No	Death	4	Yes	NA	No	3.4	0.5	NA	NA	Vorico	Autopsy + LP
7	77	15	70	Steroids, ILD	ARDS	No	Death	2	No	ARDS	No	0.1	0.1	2.2	0.1	No	Autopsy
8	53	68	55	Kidney Tx, steroids	Renal failure + peritonitis	No	Survived	6	Yes	Necrotizing	Yes	6.2	0.1	5.7	0.1	Caspo	Biopsy
9	64	14	60	AML	Pneumonia	Yes	Death	4	No	NA	No	0.1	0.6	2.7	0.1	Vorico	Autopsy
10	74	25	37	Steroids	Stevens-Johnson	No	Death	10	Yes	Nodular	No	3.4	0.1	6.2	0.1	No	Autopsy
11	47	21	87	Solid cancer, neutropenia	Enterocolitis	Yes	Death	8	Yes	Nodular	Yes	5.3	1.7	NA	NA	Caspo + Vorico	Autopsy
12	68	12	74	Solid cancer, neutropenia	Enterocolitis	Yes	Death	9	Yes	NA	No	5.7	3.2	NA	NA	Ampho	Autopsy
13	74	6	48	Liver Tx, steroids	Pneumonia	No	Death	3	Yes	Necrotizing	No	4.1	0.1	NA	NA	Liposomal ampho	Autopsy
14	78	19	8	Steroids	COPD	No	Death	4	Yes	Necrotizing	No	3.5	0.2	1.2	0.2	Caspo	Autopsy
15	48	8	53	Solid cancer, steroids	Cardiac arrest	No	Death	3	No	Necrotizing	No	0.6	0.1	NA	NA	no	Autopsy
16	48	5	53	AML	ARDS	Yes	Death	2	No	ARDS	No	3.6	0.1	NA	NA	No	Autopsy
17	76	11	94	CLL	Septic shock	Yes	Death	2	No	NA	Yes	7.4	0.2	NA	NA	No	Autopsy
18	60	9	20	Steroids (myasthenia)	Pneumonia	No	Death	3	Yes	Necrotizing	No	8.0	7.5	NA	NA	Vorico	Autopsy
19	70	3	37	Steroids, COPD	COPD	No	Death	4	Yes	NA	No	6.8	0.1	NA	NA	No	Autopsy
20	61	7	81	Cirrhosis	Septic shock, peritonitis	No	Death	4	Yes	NA	No	6.6	5	NA	NA	Caspo	Autopsy
21	74	8	46	Solid cancer	Pneumonia	No	Death	3	No	Necrotizing	No	4.6	0.1	NA	NA	No	Autopsy
22	60	7	72	Cirrhosis	Hepatorenal syndrome	No	Death	6	Yes	NA	No	5.6	0.1	NA	NA	No	Autopsy
23	60	6	44	CLL, GVHD	GVHD lung, liver	Yes	Death	3	Yes	Necrotizing	Yes	5.8	2.6	NA	NA	Caspo	Autopsy
24	44	16	99	AML, GVHD	GVHD liver, skin	Yes	Death	8	No	ARDS	No	7.9	0.6	NA	NA	Caspo	Autopsy
25	60	4	27	AML	Pneumonia	Yes	Death	5	No	Nodular	No	1.0	0.4	0.4	0.4	No	Autopsy
26	66	13	64	AML, GVHD	GVHD, liver, skin, lung	No	Death	5	No	NA	Yes	2.6	1.4	1.4	0.4	Vorico	Autopsy

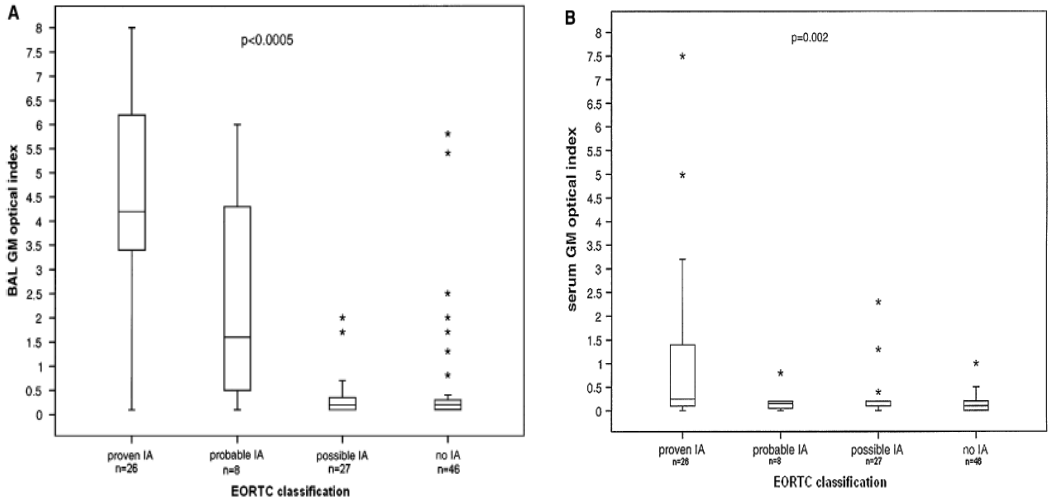
Definition of abbreviations: ALL = acute lymphoblastic leukemia; AML = acute myeloid leukemia; ampho = Amphotericin B; ARDS = adult respiratory distress syndrome; Aut = autopsy; BAL = bronchoalveolar lavage; Caspo = caspofungin; CLL = chronic lymphatic leukemia; COPD = chronic obstructive pulmonary disease; D1 = day of inclusion; D8 = 8 days after inclusion; Di = inclusion on Day *x* after admission; F = female; GVHD = graft-versus-host disease; HRCT = high-resolution computed tomography; ILD = interstitial lung disease; LOS = length of stay; LP = lumbar puncture; M = male; NA = not available,; Tx = transplant recipient; Vorico = voriconazole.

* Yes/no: denotes the presence/absence of *Aspergillus* in culture or branching hyphae visible through Gomori stain.

† Macro lesions: lesions seen during bronchoscopy and compatible with invasive aspergillosis.

‡ Antifungal R/ = antifungal treatment.

Figure III-13: Comparison of the distribution of galactomannan results in (A) BAL and (B) serum performed on Day 1 in ICU patients



Serum samples were collected twice weekly; however, galactomannan results were recorded only on Day 1 and Day 8 of the study. Like BAL, serum specimens were tested as received and considered positive at optical density index ≥ 0.5. The distribution of sera galactomannan index results among the three patient groups performed on day 1 is shown in Figure III-13. Twelve of the 26 proven IPA had a positive serum galactomannan positive index ≥ 0.5. The sensitivity of serum galactomannan in patients with proven IA was much lower (42%) than in BAL (88%) (Table III-21). The median value of galactomannan in serum on Day 1 (index = 0.3) for the proven cases was significantly lower than in BAL (index = 4.1) ($p < 0.005$). However, the specificity of the assay at an index value ≥ 0.5 in serum (93%) was higher than in BAL (87%).

A ROC curve to assess the ability of the galactomannan assay in BAL and serum in the diagnosis of IPA at different cut-off values is illustrated in Figure III-14. Qualitatively, the ROC curve corresponding to the galactomannan assay in BAL is displaced further toward the upper left-hand corner of the box than the curve for serum at a cut-off level of ≥ 0.5. Quantitatively, the AUC values for the galactomannan assay in BAL and serum are 0.898 and 0.755, respectively. Thus, both qualitatively and quantitatively ROC analysis demonstrates that the galactomannan assay in BAL provides better discrimination than in serum in diagnosing patients with proven IA.

Table III-21: BAL galactomannan and culture results of proven pathology-controlled cases compared with autopsied patients with no IPA.

	No. of Patients		
	Invasive Aspergillosis (*n* = 26)	No Invasive Aspergillosis† (*n* = 46)	Total
Serum galactomannan, no.‡			
Positive	11	3	14
Negative	15	43	58
Total	26	46	72
BAL galactomannan, no.‡			
Positive	23	6	29
Negative	3	40	43
Total	26	46	72
BAL culture, direct examination, no.§			
Positive (%)	15 (58)	14 (30)	29
Negative (%)	11 (42)	32 (70)	43
Total	26	46	72

Definition of abbreviation: BAL = bronchoalveolar lavage.

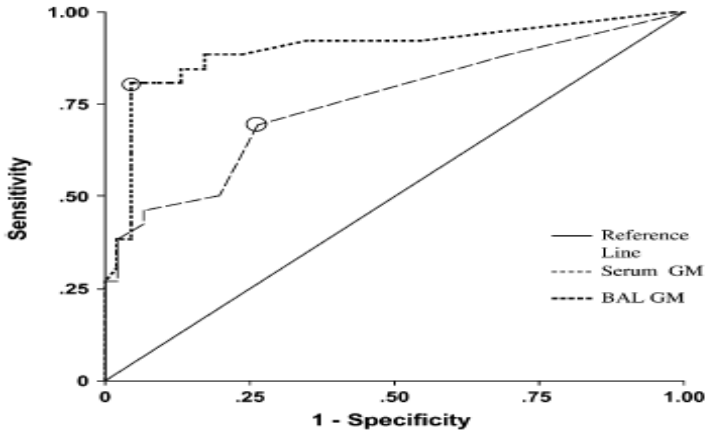
* Seventy-two cases subdivided in 69 deceased patients who underwent an autopsy (24 proven invasive aspergillosis [IA] cases, 2 proven zygomycosis cases, and 43 "no IA" cases) and three survivors with a lung biopsy (2 proven IA cases and 1 proven zygomycosis).

‡ Cutoff value for positivity: 0.5 ng/ml, only the galactomannan value of the first bronchoscopy and the serum value at the same day was taken into account.

† No invasive aspergillosis = no hyphal tissue invasion with *Aspergillus* spp or tissue invasion with non-*Aspergillus* molds.

§ Fourteen patients in the proven "no IA" category had positive culture and/or direct examination results. Among those were two patients in which *Zygomycetes* were seen on direct microscopic examination.

Figure III-14: Receiver operator characteristic (ROC) curve for galactomannan detection in BAL at a cut-off index ≥ 0.5



Of the 26 proven IPA, eight patients had a second BAL sample collected at 8 days after the initial BAL specimen. The reasons for collection of a second BAL specimen were not specified. Seven of the eight patients' second BAL specimen had galactomannan levels ≥ 0.5 (Table III-20); four patients were administered antifungal treatment (voriconazole or caspofungin). Three patients had a second BAL specimen that yielded a positive value while the first BAL was negative. Antifungal treatment was associated

with a decrease in galactomannan levels in 75% of the patients treated, though the period between BAL collection and start of antifungal treatment was not specified. The eight patients also had the galactomannan assay performed in serum (Table III-20). Only one patient had serum galactomannan levels ≥ 0.5. In this patient, the second BAL specimen was also positive; however, both the first BAL and serum specimens were negative. The patient was not treated with any antifungal agents. The remaining seven patients had negative second serum specimens; in six patients the second BAL specimen was positive. Four of these six patients were treated with antifungal agents (caspofungin and voriconazole). Thus the results suggest detection of galactomannan in serum may be transient and tends to be negative after antifungal treatment has been initiated. Of the 46 cases without IPA, 6 patients had galactomannan index values ≥ 0.5. Four of these six patients were treated with antifungals before death and the galactomannan index in these patients was higher in BAL than in serum. The authors did not specify the type of antifungal administered or whether these patients were later colonized or infected with *Aspergillus* or other fungal species. Exposure to mold-active antifungal agents appears to decrease the sensitivity of the galactomannan assay in both BAL and serum in patients leading to increased as false negative results. In addition, patients classified as false positives could be due to undiagnosed sub-clinical infection.

A total of 33 patients in the study were treated with piperacillin-tazobactam which included four patients without IPA who had both positive serum (range, 0.7 – 2.8) and BAL (range, 1.3 – 5.8) galactomannan results. The galactomannan results remained positive until four days after the antibiotic had been stopped. No patients were treated with amoxicillin-clavulanate acid. These results suggest that cross-reaction of the galactomannan assay in patients without IPA treated with piperacillin-tazobactam can occur.

The performance of the galactomannan assay in BAL was also compared in patients who had cultures or direct microscopic examination performed on the BAL specimen. Fifteen of the 26 proven cases had a positive culture and/or direct examination; of the 15 subjects, 10 subjects were positive by culture and direct examination and four were positive on direct examination only and one was positive for *Aspergillus* species culture only (Table III-20). The sensitivity and specificity based on direct microscopic examination or culture results was 58% and 70%, respectively. The mean galactomannan index was 5.0 in BAL from culture positive patients was higher than in BAL culture negative specimens (index = 3.3). The results suggest that for proven IPA patients that were culture positive, using the galactomannan assay in BAL would have yielded an earlier diagnosis.

The performance of the galactomannan assay for testing of BAL samples was also examined in neutropenic and non-neutropenic patients. Of the 26 proven IPA patients, 10 had neutropenia and the remaining 16 patients were on steroid treatment. The sensitivity of the galactomannan assay in BAL specimens was similar in neutropenic (90%) and non-neutropenic patients (88%). However, the sensitivity of the galactomannan assay for testing of sera was higher in the neutropenic patients (70%)

compared to the non-neutropenic patients (25%). Among the 10 neutropenic patients, 5 (50%) were positive by mycological culture or direct microscopy of fungal organisms; 10 of 16 non-neutropenic patients (63%) had positive cultures (Table III-21). Among patients who had a thoracic CT scan that displayed characteristic halo or air-crescent signs, 6 of the 10 proven neutropenic cases had positive CT findings (60%) compared to 9 of the 16 proven non-neutropenic patients without CT findings (56%).

Table III-22: Clinical characteristics of neutropenic and non-neutropenic patients with proven IPA

Characteristics	Neutropenic Patients (*n* = 10)	Nonneutropenic Patients (*n* = 16)	All Proven IA Cases (*n* = 26)
No. of males (%)	4 (40)	10 (63)	14 (54)
Age, mean yr	62	62	62
Clinical characteristics			
Fever, no. of patients (%)	8 (80)	10 (63)	18 (69)
Respiratory failure requiring MV, no. (%)	10 (100)	15 (94)	25 (96)
Length of stay, no. of days (range)	7 (4–21)	13 (3–68)	13 (3–68)
Macroscopic lesions,* no. of patients (%)	4 (40)	3 (19)	7 (27)
CT rate, no. of patients (%)	6 (60)	9 (56)	15 (58)
Necrotizing pneumonia on CT scan† (%)	1 (17)	6 (67)	7 (47)
Halo sign on CT scan (%)	0 (0)	13 (0)	0 (0)
Steroids, no. of patients (%)	2 (20)	3 (81)	15 (58)
Cirrhosis (%)	0 (0)	55 (19)	3 (12)
SAPS II‡	60	55	57
Predicted mortality, %	71	62	
Outcome			
Survival, no. of patients (%)	0 (0)	2 (12)	2 (8)
Lung autopsy (n = 25) and/or biopsy results			
Strong inflammation, low fungal burden	1/10 (10)	13/16 (81)	14/26 (54)
Scant inflammation, high fungal burden	9/10 (90)	3/16 (19)	12/26 (46)
Sensitivity of test, *n*/N (%)§			
BAL culture or direct examination positive	5/10 (50)	10/16 (63)	15/26 (60)
Aspergillus GM BAL	9/10 (90)	14/16 (88)	23/26 (88)
Aspergillus GM serum	7/10 (70)	4/16 (25)	11/26 (42)
Median GM value in BAL Day 1 (range)	5.5 0.1–7.9	4.0 0.1–8.0	4.3 0.1–8.0
Median GM value in serum Day 1 (range)	0.7 0.1–5.0	0.1 0.0–7.5	0.3 0.0–7.5

Definition of abbreviations: BAL = bronchoalveolar lavage; CT = computed tomography; GM = galactomannan; IA = invasive aspergillosis; MV = mechanical ventilation; SAPS II = Simplified Acute Physiology Score II.

* Macroscopic lesions were defined as ulcerative or pseudomembranous lesions in the trachea or the bronchi visible during the bronchoscopy.

† *See* online supplement for CT and chest X-ray illustrations of some studied patients.

‡ SAPS II is a score of severity of illness in critically ill patients (range between 12 and 163, with predicted mortalities between 1.3 and 100%, respectively).

§ Applying an optical density index cut off = 0.5.

III.5 Non-immunocompromised patients

12. Nguyen *et al*. (2007), evaluated the Platelia *Aspergillus* EIA galactomannan assay (Bio-Rad Lab) in BAL specimens from non-immunocompromised patients from a Florida hospital. The study was retrospective in patients undergoing bronchoscopy in which BAL specimens were collected. Patients were excluded if they were immunosuppressed with absolute neutrophil count < 1000 neutrophils/mm^3, had a history of hematologic malignancy, hematopoietic stem cell transplantation, solid organ transplant recipients, HIV infection and receipt of corticosteroids or other immunosuppressive agents within six months of diagnosis of pulmonary aspergillosis. Thus in this study population, the underlying diseases were not in accordance with the host factors listed in the 2002 EORTC/MSG guidelines. Patients were grouped as proven or presumed IPA. Proven IPA was based on radiological findings and confirmed culture or histopathology findings for *Aspergillus* species from BAL or sputum. Proven IPA was further defined as acute IPA if duration of symptoms was ≤ 1 month or chronic if the duration of symptoms was > 1 month. Presumed IPA was based on radiological findings and negative cultures or histopathology for *Aspergillus* species and other infectious or non-infectious causes for pulmonary findings had been ruled out.

    BAL specimens were sent on dry ice overnight and the galactomannan assay performed at a central laboratory (MiraVista Laboratories), though the details of the procedure of the galactomannan assay in BAL were not specified. Of the 73 patients, six patients had IPA, of which two had acute proven IPA, two had chronic proven IPA and two had presumed IPA (Table III-23). All six IPA patients had BAL galactomannan index levels ≥ 1.0 (range 1.18 - 8.89). In three patients (though not specified which category), BAL was collected from multiple segments of the lung and the galactomannan index level was ≥ 1.5 for each sample. Of the 67 patients without IPA, 15 patients had BAL galactomannan index levels ≥ 0.5 and eight patients had BAL galactomannan results ≥ 1.0 (Table III-24). The performance of the galactomannan assay in BAL in this population was highly sensitive (100%), and NPV (100%) at an index value ≥ 0.5. However, the specificity (78%) and predictive value (29%) was low, which may reflect the low prevalence of IPA and an increase number of false positives identified by the galactomannan assay in this population. Increasing the cutoff to ≥ 1.0, had no effect on the sensitivity and NPV (100%), the specificity increased from 78% to 88% and the PPV increased from 29% to 43%. The ROC curve analysis indicated that the optimal performance of the galactomannan assay was achieved by using an index cutoff of 1.18 (Figure III-15).

Table III-23: Characteristics of the patients with pulmonary aspergillosis

Age (yr) (sex)	Underlying disease(s)	Reason(s) for BAL	Antibiotic(s) used prior to or at the time of BAL	CXR/chest CT scan result	BAL GM level(s)	Serum GM level(s)	Microscopy result, TBBX result	Culture	Diagnosis	Treatment	Outcome at follow-up
66 (M)	CAD, DM, and obesity; admitted with acute pneumococcal sepsis	Fever, septic shock	CFP, Levo, Vanc	Bil parenchymal opacity, diffuse GGO, nodule	1.18	1.41 (+1 day)[b]	Inflammatory cells (yeasts), not done	BAL, *A. flavus*; sputum (−1 days),[b] *A. flavus*	Proven IPA[c]	No antifungal	Died, 4 days[b]
61 (M)	Previously healthy; admitted with severe upper GI bleed	Shock	CFP, Metro, Vanc, Tim	Nodular infiltrates	8.44	2.58 (−4 days),[b] 2.69 (−2 days)[b]	Not done, not done	BAL, *A. fumigatus*; sputum (−1 days, −2 days),[b] *A. fumigatus*	Presumed IPA	VORI until death	Died, 10 days[b]
67 (F)	COPD; cavitary lung lesion was found on admission CXR	Respiratory symptoms for 4 mo and cavitary lung lesion	Cipro, Metro	Consolidation with cavitation	2.0; after antifungal treatment, −0.62 (+19 days)[b]	0.05 (+5 days)[b]	Hyphae, inflammation and necrosis; hyphae seen in tissue	BAL, *Candida* and *A. fumigatus*	Proven CNPA	VORI and antibiotics until death	Died, 2 mo[b] (from ruptured AAA)
56 (M)	Healthy, admitted after a stroke; cavitary lung lesion was found on admission CXR	Wt loss, cough, hemoptysis, abnormal CXR	None	Cavitary lesion with surrounding consolidation	7.41, 7.34[d]	0.98 (−2 days)[b]	Hyphae, chronic and granulomatous inflammation, presence of eosinophils (no hyphae)	BAL, *A. fumigatus*; sputum (−3 days),[b] *A. fumigatus*	Probable CNPA	VORI for 1.3 yr	Lived (F/u, 1.3 yr)
51 (M)	Lung cancer, in remission for 5 yr; admitted for hemoptysis	Fever, respiratory complaints, and hemoptysis	Gati	Cavitary lesion with fungus ball; surrounding consolidation	1.43, 1.15[d]	0.08 (+3 days),[b] 0.11 (+4 days),[b] 0.08 (+5 days)[b]	No hyphae, not done	BAL, *Candida* and *A. fumigatus*	Proven aspergilloma; MAI pneumonia and cavitary lesion	VORI for 1 year and Rx for MAI	Lived (F/u, 1 yr)
46 (M)	Crohn's colitis, in remission, MAI pneumonia on treatment; admitted with hemoptysis	Fever, respiratory complaints, and hemoptysis	Mero and meds against MAI	Cavitary lung lesion with fungus ball	8.89 (LLL), 8.64[d] (RL)	Not done	Inflammatory cells (no hyphae), chronic and acute inflammation (no hyphae)	*A. fumigatus*	Proven aspergilloma; MAI cavitary lung lesion	VORI for 6 mo and Rx for MAI	Lived (F/u, 6 mo)

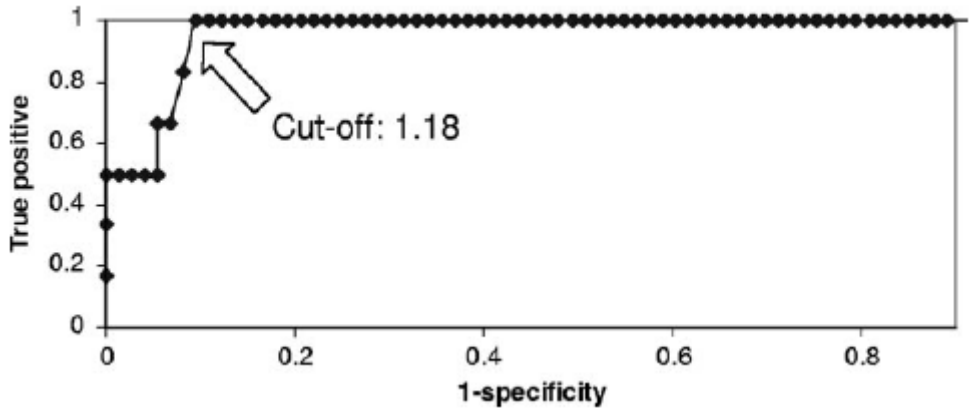
[a] Abbreviations: TBBX, transbronchial biopsy; M, male; F, female; CAD, coronary artery disease; DM, diabetes mellitus; COPD, chronic obstructive lung disease; AAA, abdominal aortic aneurysm; CXR, chest X ray; Bil, bilateral; GGO, ground glass opacification; MAI, *Mycobacterium avium-Mycobacterium intracellulare*; RLL, right lower lobe; LLL, left lower lobe; RL, right lung; F/u, follow-up time; Rx, treatment; meds, medications; CFP, cefipime; Levo, levofloxacin; Vanc, vancomycin; Cipro, ciprofloxacin; Gati, gatifloxacin; Metro, metronidazole; Tim, timentin; Mero, meropenem; VORI, voriconazole.
[b] Date given relative to the initial BAL (e.g., +1 day indicates 1 day after BAL, and −1 day indicates 1 day before BAL).
[c] Fungal hyphae were found within the lung parenchyma and blood vessels upon autopsy.
[d] BAL was performed on several lung specimens.

Table III-24: Performance characteristic of BAL galactomannan test in comparison to conventional tests

Test and cutoff	Sensitivity (%) (no. of positive samples/total no. of samples) (range)	Specificity (%) (no. of positive samples/total no. of samples) (range)	PPV (%) (no. of positive samples/total no. of samples) (range)	NPV (%) (no. of positive samples/total no. of samples) (range)
BAL GM				
≥0.5	100 (6/6) (54.1–100)	77.6 (52/67) (65.8–86.9)	28.6 (6/21) (11.3–52.2)	100 (52/52) (93.2–100)
≥1.0	100 (6/6) (54.1–100)	88.1 (59/67) (77.8–94.7)	42.9 (6/14) (17.1–71.1)	100 (59/59) (93.9–100)
≥1.5	66.7 (4/6) (22.3–95.7)	91 (61/67) (81.5–96.6)	40 (4/10) (12.2–73.8)	96.8 (61/63) (89.3–99.6)
≥2.0	66.7 (4/6) (22.3–95.7)	94 (63/67) (85.4–98.4)	50 (4/8) (15.7–84.3)	96.9 (63/65) (89.3–99.6)
≥2.5	50 (3/6) (11.8–88.2)	95.5 (64/67) (87.5–99.1)	50 (3/6) (11.8–88.2)	95.5 (64/67) (87.5–99.1)
Serum GM[a]				
≥0.5	60 (3/5) (14.7–94.7)	91.7 (11/12) (61.5–99.8)	75 (3/4) (19.4–99.4)	84.6 (11/13) (54.6–98.1)
≥1.0	40 (2/5) (5.3–85.3)	91.7 (11/12) (61.5–99.8)	66.7 (2/3) (9.4–99.2)	78.6 (11/14) (49.2–95.4)
BAL culture	66.7 (4/6) (22.3–95.7)	94 (63/67) (85.4–98.4)	50 (4/8) (15.7–84.3)	96.9 (63/65) (89.3–99.6)
BAL microscopy[b]	80 (4/5) (28.4–99.5)	96.9 (63/65) (89.3–99.6)	66.7 (4/6) (22.3–95.7)	98.4 (63/64) (91.6–100)
BAL culture or microscopy	100 (6/6) (54.1–100)	92.5 (62/67) (83.4–97.5)	54.5 (6/11) (23.4–83.2)	100 (62/62) (94.2–100)

[a] The serum GM test was performed for only 17 patients.
[b] BAL microscopy was performed for only 70 patients.

Figure III-15: Receiver operative characteristics (ROC) curve model for BAL galactomannan test



Direct microscopic evaluation of BAL or sputum specimens was performed in 70 of the 73 patients. Four of the five IPA patients and two of the 65 patients without IPA showed hyphae suggestive of *Aspergillus* under direct microscopic examination (Table III-24). The sensitivity, specificity, PPV and NPV based on direct microscopic evaluation was 80%, 97%, 68% and 98%, respectively. Of the 73 patients that had BAL fluid cultured, four of the six IPA patients and four of the 67 patients without IPA were culture positive. The sensitivity, specificity, PPV, and NPV based on BAL culture results alone was 67%, 94%, 50% and 97%, respectively. However, combining the results of cultures and direct microscopy demonstrating hyphae increased the sensitivity and NPV to 100% but resulted in a lowering the PPV (54%) than just BAL microscopy alone (67%). In this population, at a cut-off value at ≥ 0.5 or ≥ 1.0 the sensitivity of the galactomannan assay in BAL (100%) was similar to the combination of BAL microscopy and culture (100%), suggesting no added benefit of the assay in BAL for the diagnosis of IPA in this patient population. Furthermore, there was an increased likelihood of obtaining false-positive results in this population with the galactomannan assay at a cut-off value at ≥ 0.5 for testing of BAL (PPV =

28%) compared to the combination of microscopy and culture from BAL or sputum specimens (PPV = 54%).

Serum samples from 17 of the 73 patients (five of the six IPA patients and 12 of the 67 patients without IPA) were tested for galactomannan. The authors did not specify the timing of the collection of serum specimens in relation to the collection of BAL. As with BAL specimens, serum were sent and testing performed at a central laboratory (MiraVista Laboratories), though the details of the procedure of the galactomannan assay in serum were not specified. Of the five IPA patients that had serum tested three IPA patients (two acute IPA and one chronic IPA; range, 0.98 – 2.58) were positive for sera galactomannan at a cut-off index ≥ 0.5 (Table III-23). Of the 12 patients without IPA, one patient had serum galactomannan index levels ≥ 0.5 (Table III-24). At a cut-off value at ≥ 0.5 or ≥ 1.0, testing was more sensitive in BAL (100%) than in serum (70%). However, there was increased likelihood of obtaining false-positive results in BAL (PPV = 28%) than in serum (PPV = 75%) (Table III-24).

The authors examined factors that may influence the detection of galactomannan in BAL in patients with no evidence of IPA. Of the 67 patients without IPA, nine patients had BAL galactomannan index ≥ 1.0 (Table III-25) with a range of 1.04 to 5.89. Two of the nine patients were treated with antifungal agents after the positive BAL galactomannan result, one patient was admitted with signs and symptoms suggestive of pulmonary disseminated tuberculosis and, was treated with voriconazole for five days; the patient later died from tuberculosis. The other patient was diagnosed with advanced metastatic lung cancer and was treated with voriconazole for three months; the patient later succumbed to lung cancer. The remaining seven patients were not treated with antifungal agents. Two of the nine patients were culture positive for *Aspergillus* species (one patient *A. fumigatus* plus *A. niger* and one patient with *A. terreus)*; the authors stated that neither of the two patients developed any further evidence of aspergillosis, suggesting that the performance of the galactomannan assay in BAL samples is unable to distinguish between invasive disease and colonization. No patients received piperacillin-tazobactam or amoxicillin-clavulanate.

Table III-25: Characteristics of patients without evidence of pulmonary aspergillosis in BAL at galactomannan levels ≥ 1.0

Age (yr) (sex)	Underlying disease(s)	Reason(s) for BAL	Antibiotic(s) prior to or at the time of BAL	Chest X-ray/ chest CAT scan result	BAL GM level(s)[b]	Serum GM level(s)[b]	Microscopy result, TBBX result	Culture result	Diagnosis	Treatment (duration)	Outcome
52 (M)	Healthy	Fever, respiratory symptoms	CTX, AZI	Micronod IF, air space disease, med LN	1.04	Not done	Hyphae, chronic inflammation	*Aspergillus fumigatus*, *Aspergillus niger*	CAP	No antifungal, antibiotics	Lived (F/u, 6 mo)
48 (F)	COPD, pulmonary HTN	SOB	CTX, AZI	Multiple nodules, diffuse GGO, hilar LN	1.33, 0.11[c]	0.11 (+3 days), 0.05 (+2 m)	No hyphae, bronchiolitis (interstitial lung disease)	No fungus	Pulmonary HTN	No antifungal, antibiotics and steroid	Died (2 mo)
22 (F)	Healthy with miliary TB	Respiratory symptoms	CTX, AZI	New cavitary lung lesion and tib	0.32 (−12 days), 1.57	0.07 (−12 days), 0.11	No hyphae, necrotizing granuloma	No fungus	Miliary TB	VORI, 5 days, TB medications	Died (19 days) (from disseminated tuberculosis)
77 (M)	HTN	Fever, respiratory symptoms	CTX, AZI	Airspace disease, hilar and med LN	1.58	0.06 (+3 days)	No hyphae, BOOP	*Candida*	BOOP	No antifungal, steroid	Lived (F/u, 1 yr)
52 (M)	ICM	Respiratory symptoms	None	New cavitary lesion with surrounding infiltrate, hilar LN	2.38, 0.15[c]	0.15 (+10 days), 0.09 (+12 days), 0.07 (+13 days)	No hyphae, nondiagnostic	No fungus	Lung cancer (adenocarcinoma)	VORI, 3 mo (based on BAL GM), antibiotics	Died (4 mo) (lung cancer)
57 (M)	CAD, HTN	Respiratory symptoms	CTX, AZI	New cavitary lesion, med LN	2.67	Not done	No hyphae, acute and organizing pneumonia	*Aspergillus terreus*	CAP, TB	No antifungal, antibiotics	Lived (F/u, 3 mo)
2 (M)	Healthy	ARDS, fever	CTX, Vanc	Consolidation	2.89, 0.98[c]	0.09 (+3 days), 0.14 (+5 days)	No hyphae, not done	No fungus	MRSA pneumonia on viral pneumonia	No antifungal, antibiotics	Lived (alive at d/c; no F/u available)
64 (M)	COPD	Chest pain, s/p falls	None	Mass with necrotic center	4.98, 6.13[c]	0.12 (+4 days)	Yeast, not done	*Candida albicans*	Disseminated community-acquired MRSA infection	No antifungal, antibiotics	Died (9 days)
54 (F)	Hepatitis C virus with ESLD	Weakness, encephalopathy, respiratory failure	Tim, Vanc	Consolidation (focal), diffuse GGO	1.26, 0.37[c]	Not done	Hyphal elements and yeasts, not done	No fungus	Diffuse alveolar hemorrhage	No antifungal	Died (8 days) (hepatorenal syndrome and sepsis)

[a] TBBX, transbronchial biopsy; M, male; F, female; Micronod IF, micronodular infiltrates; COPD, chronic obstructive pulmonary disease; SOB, shortness of breath; s/p, status post; HTN, hypertension; TB, tuberculosis; ICM, ischemic cardiomyopathy; CAD, coronary artery disease; ESLD, end-stage liver disease; BOOP, bronchiolitis obliterans with organizing pneumonia; LN, lymphadenopathy; GGO, ground glass opacity; med, mediastinal; CAP, community-acquired pneumonia; d/c, discharge; F/u, follow-up; CTX, ceftriaxone; AZI, azithromycin; Tim, timentin; Vanc, vancomycin; VORI, voriconazole.

[b] Date given relative to the initial BAL (e.g., +1 day indicates 1 day after BAL, and −1 day indicates 1 day before BAL).

[c] BAL was performed from several lung segments.