DIVISION OF SPECIAL PATHOGEN AND TRANSPLANT PRODUCTS

Biomarker Qualification

Microbiology Review

Detection of Galactomannan in Serum
by
Platelia™ *Aspergillus* Enzyme-linked Immunosorbent Assay
(BioRad Laboratories and Sanofi Diagnostics)

Sponsor:	Mycoses Study Group
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1. Executive Summary

The GM assay was approved by the Center for Devices and Radiological Health, Food and Drug Administration, in 2003 for the testing of serum samples as an aid to the diagnosis of Invasive Aspergillosis (IA). The test brochure recommends that results of the Platelia *Aspergillus* EIA be used in conjunction with other diagnostic procedures such as culture and histopathological findings. A serum sample is considered positive at a cut-off index of ≥ 0.5 based on testing of 2 aliquots of the same sample and another sample collected at a subsequent time point. The sponsor is seeking qualification of detection of galactomannan as a biomarker for enrollment of patients with hematologic disorders, in a clinical trial of invasive aspergillosis according to the European Organization for Research and Treatment of Cancer /Mycosis Study Group (EORTC/MSG) criteria of 2008. The EORTC/MSG guidelines recommend detection of galactomannan as a stand-alone microbiologic criterion for diagnosis of probable patients in association with clinical and host factors. This review analyses the findings from *in vitro*, animal and clinical studies.

The i*n vitro* studies show that binding with the monoclonal antibody EB-A2 (which is part of the Platelia *Aspergillus* EIA) is not uniform throughout all fungal structures (hyphae, spores, conidia) of the *A. fumigatus* mold and reacts strongly with non-germinating and young conidia. The use of ultrasound technology using colloidal gold labeled anti-rat immunoglobulin, mycelia from *A. fumigatus* and EB-A2 indicated that labeled substances within the cell were not specific to a particular fungal structure. Studies show cross-reactivity with several fungal and bacterial species *in vitro*.

The precision and reproducibility of Platelia *Aspergillus* E.I.A. were reviewed previously by CDRH and the assay was cleared for testing of serum samples. The analytical specificity relative to medical conditions unrelated to *Aspergillus* infections was within acceptable limits recommended by the CDRH. There were instances of cross-reactivity with *Penicillium spp*., *Paecilomyces spp.* and *Alternaria spp.*

Studies in an animal model of invasive aspergillosis show that there is a direct relationship between colony forming units (cfu) of *Aspergillus* in the lung of New Zealand White Rabbits and the GM titers obtained from the rabbit serum.

A total of 41 clinical studies were reviewed. Of these, the performance of the Platelia *Aspergillus* EIA at cut-off index of 0.5, 0.6 – 0.9, 1.0, or 1.5 was measured in 27 studies in neutropenic patients with hematological disorders. Based on the 27 studies in neutropenic patients with hematological disorders, the overall sensitivity and specificity of the Platelia *Aspergillus* EIA, irrespective of the cut-off index, ranged from 12 to 100% and 34 to 100%, respectively. Results showed that a high cut-off index, ≥ 1.5, increased the specificity and decreased sensitivity; a low cut-off increased the sensitivity and decreased specificity. Of the 27 studies, performance of the assay at more than one cut-off was measured in 12 studies (Table A). Overall, the results show a trend towards higher specificity as the cut-off is increased from ≥0.5 to ≥1.0. The specificity at a cut-off of ≥1.0 using two consecutive samples gives the best overall value in this parameter (Table B). Since the specificity is very important for enrollment of patients with aspergillosis in a clinical trial, a cut-off of ≥1.0 based on two consecutive positive samples would be optimal for enrollment of patients in the clinical trial.

Table A: Studies using the GM assay in which more than one cut-off indices

Reference	≥0.5 (2 samples) 5/6 studies				≥1.0 (1 sample) 9/10 studies				≥1.0 (2 samples) 6 studies				≥1.5 (1 sample) 8 studies			
	Sens	Spec	PPV	NPV	Sens	Spec	PPV	NPV	Sens	Spec	PPV	NPV	Sens	Spec	PPV	NPV
Maertens *et al*., 2001					92	95	72	99	90	98	88	98				
Maertens *et al*., 2002					95	85	59	99	94	99	94	99				
Maertens *et al*., 2004	97	99	97	99	93	100	100	97	79	100	100	93	83	100	100	94
Maertens *et al*., 2007	92	98	88	99	82	97	82	97					76	98	85	96
Becker *et.al*., 2003					59	75	48	83	47	93	73	82	18	84	30	73
Kawazu *et al*., 2004	100	84	35	100	100	86	38	100	64	98	70	97	82	90	41	98
Lai *et al.*, 2007	100												79	94	55	98
Ulusakarya *et al*., 2001					100	92	64	100					69	96	69	96
Herbrecht *et.al*., 2002 (adults only)					35	97	87	84					26	99	92	83
Suankratay *et al*., 2006	94	67	59	96					88	97	94	94				
Marr *et al*., 2004	82	74			54	100										
Marr *et al*., 2005					48	88	42	91					43	93	53	91

Sens = sensitivity; spec = specificity; PPV = positive predictive value; NPV = negative predictive value

Based on the 20 studies which reported different performance parameters (see Table 14 for details), the specificity ranges between 92 to 100% at a cut-off index of ≥1.0 based on testing of 2 samples ; based on testing of 1 sample the specificity ranged between 75 and 100%. However, the PPV is maximal when 2 samples are tested at a cut-off of ≥ 1.0.

Table B. Overall performance of the Platelia assay at different cut-off and testing of one or consecutive serum samples. Results are presented as mean (median)

Parameter	Mean (Median) Cut-off			
	≥0.5 (consecutive samples) (8 studies)	≥1.0 (1 sample) (10 studies)	≥1.0 (consecutive samples) (9 studies)	≥1.5 (1 sample) (9 studies)
Sensitivity	90 (93)	82 (93)	79 (88)	61 (69)
Specificity	87 (86)	91 (94)	97 (98)	94 (95)
PPV	66 (72)[1]	66 (64)[2]	80 (85)	65 (55)
NPV	98 (99)[1]	96 (97)[2]	96 (98)	91 (94)

1 = based on 6 studies; 2= based on 9 studies

Most of the studies collected specimens twice a week; however, the optimum interval between the sample collections was not determined. Most of the prospective studies tested samples in batches twice a week and 85% of the studies that reported storage temperatures stated that the samples were stored at -70^{o} or -80^{o} C. In one study (Penack *et al*., 2008), a correlation between GM levels and survival was shown; in patients that died there was a steep increase in GM levels and the index was higher compared to the GM indices of those who survived. In a study by Maertens *et al*., 2001, the patients with intermediate levels of GM were shown to survive longer than the patients with high levels of GM but that they subsequently died.

GM is found in many fungi and bacteria and can cause false positive results. The presence of other fungal infections was not systematically reported in the studies that were reviewed. It is, therefore, important that future studies employing the GM assay be designed appropriately to rule out the presence of cross-reacting species that can lead to false positive results and be systematically reported. In addition, cross-reactivity with some of the antimicrobial drugs such as β-lactam antibiotics (piperacillin – tazobactam, amoxicillin – clavulanate, amoxicillin and ampicillin) and plasmalyte was shown. Treatment with such antimicrobial agents and the use of plasmalyte should be documented and such patients either not be enrolled or excluded from analysis in clinical trials.

Recommendations:

Platelia *Aspergillus* EIA may be used at a cut-off index of ≥ 1.0 based on testing of 2 serum samples collected at an interval of 48 hours for enrollment of patients in clinical trials for the treatment of aspergillosis in patients with hematological malignancy. Efforts should be made to exclude patients with other infections. Also, patients treated with other antifungal drugs should be excluded. Additionally, patients receiving treatment with antibacterial agents such as piperacillin – tazobactam, amoxicillin – clavulanate, amoxicillin and ampicillin as well as plasmalyte should either not be enrolled or excluded from analysis. It is also recommended that a subset analysis should be done for patients who are diagnosed based on standard microbiologic criteria (culture, histopathology, etc.) and those that are only galactomannan positive.

2. Introduction and Background.

The diagnosis of invasive aspergillosis (IA) has posed major challenges to the medical community. The overall problem has stemmed from poor standardization and correlation between the microbiological, radiological and clinical findings of invasive aspergillosis. Platelia *Aspergillus* enzyme immunoassay (EIA; BioRad Laboratories), for detection of galactomannan (GM), has been cleared by the US FDA, Center for Devices and Radiological Health (CDRH) for diagnosis of aspergillosis in conjunction with culture or histopathological evidence. The primary purpose of this review is to qualify the detection of galactomannan (GM), in serum, by Platelia *Aspergillus* EIA as a biomarker for diagnosis of invasive aspergillosis (IA) in neutropenic patients with hematologic stem cell transplant (HSCT) and hematologic malignancy. The specific objectives are as follows:

- To investigate the utility of detection of galactomannan by the Platelia *Aspergillus* EIA, as a stand-alone microbiological test, for the purpose of upgrading patients categorized as possibly having invasive aspergillosis to the category of "probable" as recommended by the European Organization for Research and Treatment of Cancer /Mycosis Study Group (EORTC/MSG) in 2008. As mentioned above, the product brochure states that the results of the Platelia *Aspergillus* EIA "should be considered in conjunction with other diagnostic procedures such as microbiological culture, histological examination of biopsy samples and radiologic evidence."

- For the purpose of enrollment in clinical trials, determine a suitable cut-off index and whether this should be based on testing of one or more samples in conjunction with other clinical and host factors for diagnosis of invasive aspergillosis.

  The current product brochure for the Platelia *Aspergillus* EIA recommends a cut-off for positivity be ≥0.5 based on testing of 3 samples (2 aliquots of the same positive sample and testing of another sample collected at a different time point). The brochure recommends that when a positive result is obtained, an aliquot from the same sample be retested. Additionally, that a new sample should be collected and the assay repeated.

- To investigate possible sources and reasons for cross-reactivity, apart from those cited in the brochure. The product brochure specifies cross-reactivity with *Penicillium, Alternaria, Paecilomyces, Geotrichum, and Histoplasma,* and β-lactam antibiotics.

- To evaluate other relevant information with regard to the use of the Platelia *Aspergillus* EIA as a stand-alone microbiological marker for IA.

2.1 Galactomannan and Biology of Aspergillosis

Galactomannan is a hetero-polysaccharide composed of a mannan core and lactofuransyl side-chain (Figure 1) and found in the cell wall primarily of mold-like fungi especially in *Aspergillus spp.* and *Penicillium spp.* but is also found in other species of fungi.

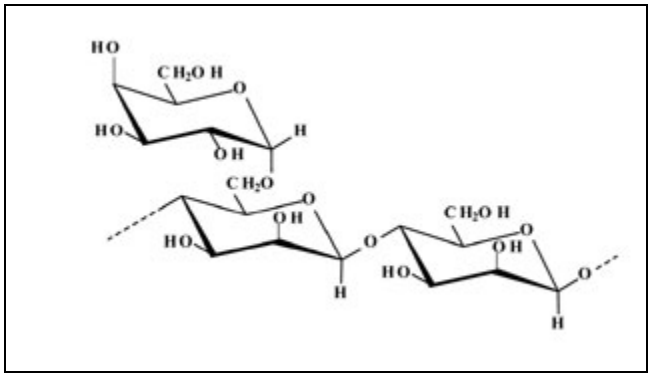

Figure 1 Chemical structure of galactomannan

The genus *Aspergillus* comprises more than 250 species. The species most commonly implicated in aspergillosis are *A. fumigatus, A. flavus, A. niger,* and *A. terreus* of which *A. fumigatus* is the most common accounting for over 50% of infections by this genus. Colonial morphology is an important characteristic in the classification of the species. Colonies of four species of *Aspergillus* are shown in Figure 2.

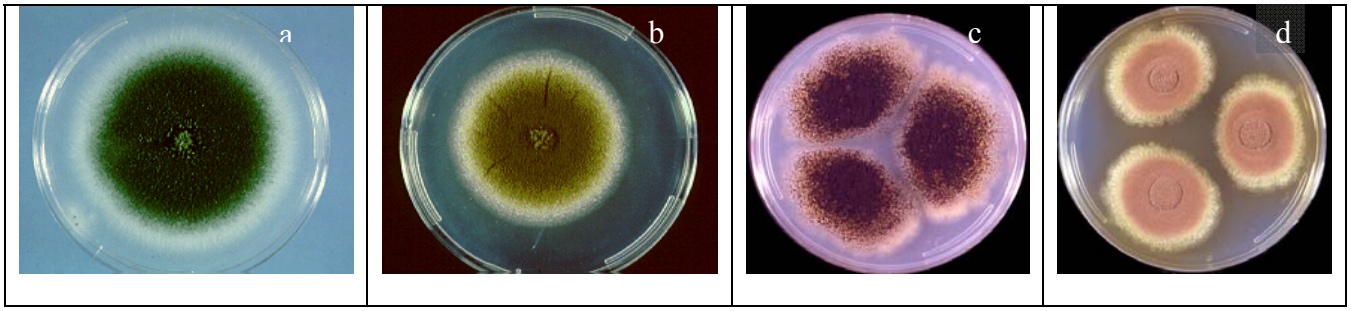

Figure 2: Colonies of 4 *Aspergillus spp*.: (a) *A. fumigatus,(b) A. flavus, (c) A. niger,* and *(d) A terreus* (Wikipedia)

Aspergillus spp. are opportunistic ascomycetes that can grow at temperatures ranging from 25^0C to 50^0C. The hyphae are septate and hyaline and the conidiophores produce numerous conidia that are approximately 2 - 3 µm in diameter (Figures 3 and 4). The septate hyphae of *Aspergillus spp.* branch at a characteristic 45^0 angle (Figure 5). The many spores are widely dispersed in the environment, the mean concentration of conidia in the air is 0.2 to 15 conidia/m^3 (Park et al.2009), the inhalation of these spores may result in aspergillosis when conditions in the host are optimal for fungal growth. The spores of *Aspergillus spp.* recognize transmembrane receptors such as Toll-like receptors to which they bind as a preliminary step to infection.

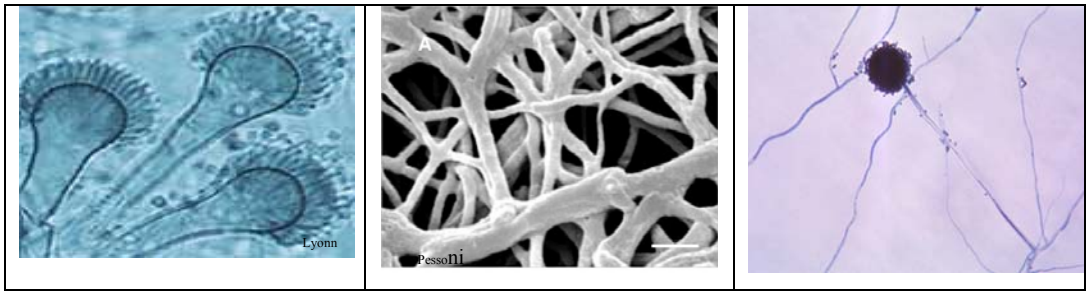

Figure 3: Columnar conidial heads with conidia. Figure 4: Mycelial mat of a mold-like fungal. Figure 5: Acute angle branching of *Aspergillus* spp. (Wikipedia)

Aspergillosis has become an important cause of morbidity and mortality in humans with a morbidity rate ranging from 30 - 70%. It is a spectrum of diseases governed by host factors. Dependent on the strength of the immune response of the host an encounter with *Aspergillus spp.* might be limited to colonization, develop an infection or develop a hypersensitivity illness. Figure 6 is a depiction of the aspergillosis spectrum (Park et al., 2009). Innate immunity, regulatory T cells and IL 17 are major barriers to infection by *Aspergillus spp*. A major innate immune mechanism that defends against fungal infections is immunity mediated by the neutrophils. Therefore, neutropenic patients are likely candidates for the development of aspergillosis. The immuno-compromised and neutropenic patients such as patients with Graft-versus host disease, cancer, recipients of HSCT and patients with other hematological disorders and critically ill patients are at high risk for infection with *Aspergillus spp.* Also at risk are patients, such as those with rheumatoid arthritis, who are being treated with other recognized T-cell suppressants. Neutropenia is defined as a decrease of polymorphonuclear leukocytes below the normal values (2.5 x 10^3 cells /µL to 7.0 x 10^3 cells/µL). Other cells in the hematopoetic system are also negatively affected when neutropenia occurs. Thrombocytopenia is a common feature in neutropenic patients and a significant decrease in the platelet count could result in abnormal bleeding in neutropenic patients.

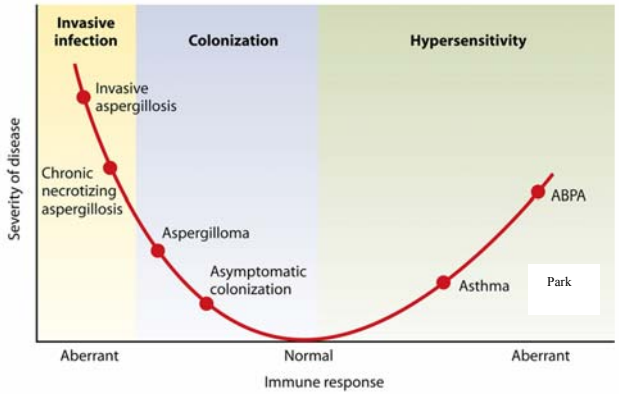

Figure 6 Diagram of the diseases caused by Aspergillus spp. as a result of immune response of the host.

The number of cases of IA has been rising. The fact that the number of transplant recipients has increased is a contributing cause of the increase in invasive aspergillosis. However, it should be pointed out that the development of new antifungal drugs has concomitantly increased and under ideal circumstances these two events should balance each other. Examples of some of the more recently approved antifungal agents are voriconazole and echinocandins. In spite of the availability of new therapeutic agents the invasive fungal infections (IFI) due to *Aspergillus spp.* continue to rise. It is felt that the increase could be attributed in part to the difficulty in establishing the diagnosis at an early stage of the infection. *Aspergillus spp* grows at a rate of 1 to 2 cm per 24 hours therefore as with most diseases, early diagnosis and early initiation of therapy are key elements in the improvement of the patient outcome.

A schematic by Wingard *et al*. (2007), postulates the progression of aspergillosis from colonization to full blown disease (Figure 7). The route of infection is usually inhalation of some of the numerous spores that are found widely dispersed in the environment. Once in the immuno-compromised host the numerous spores can travel to the alveoli of the

lung after which they can enter the blood stream and become disseminated to various organs of the body.

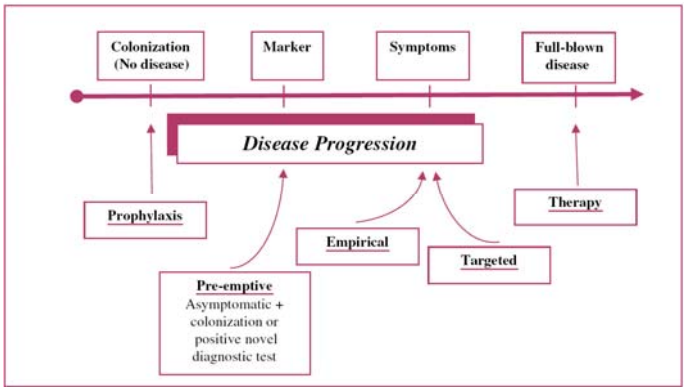

Figure 7: Schematic of the progression of *Aspergillus* infection to invasive fungal disease showing various points at which medical intervention could be made (Wingard *et al*., 2007).

The *Aspergillus* GM is shed in the blood stream and other tissues during acute infection as part of the growth process. GM is a soluble, heat stable antigen released during hyphal growth. Indirect immunofluorescence indicated that reactions to EB-A2 is not uniform throughout all fungal structures (hyphae, spores, conidia) of the *A. fumigatus* mold but reacted strongly with non-germinating and young conidia. Studies have shown that the amount of GM released varies according to the species of *Aspergillus* (Hackem *et al.,* 2008). The amount of GM released by *A. fumigatus* is less than that of other species. It has been speculated that the small quantity of the antigen is part of the limitations of the galactomannan EIA (GM EIA). However, it is well known that production and release of GM into circulation is dependent on the site of infection, growth of fungus and may be intermittent or absent. For example, a child with chronic granulomatous disease presented with multiple abscesses that had an intranodule GM concentration ranging from < 10 ng/mL to 70 ng/mL but evidence of GM was not detected in serum, urine, or buffy coat nor were the samples positive by nucleic acid and other tests (Minnik-Kersten *et al*., 2004).

2.2 Diagnosis.

Major impediments to early diagnosis of IA are that the clinical signs and symptoms are for the most part non-specific. Early clinical manifestations include signs and symptoms of pneumonia, such as cough, sputum production, hemoptysis, pleuritic pain, or pleural friction rub, or signs and symptoms of sinusitis, such as nasal discharge, nasal bleeding, nasal eschar, pain, or orbital swelling. The clinical symptoms of invasive *Aspergillus* infection (IA) can mimic tuberculosis and other infections.

Conventional outcome evaluation of aspergillosis frequently relies on subjective and nonspecific variables. Some studies have shown that high-resolution computerized tomography (CT) might result in early diagnosis in high-risk IA patients but the distinctive lesions that are visible by radiologic methods such as the ‘halo’ and the ‘air-crescent’ signs, are not specific for *Aspergillus* species. The ‘halo’ sign is not pathognomonic for fungal infection. The ‘halo’ sign is also seen in mucormycosis and in non-fungal pulmonary diseases (Won *et al*., 1998). Additionally, these signs are not usually seen in solid organ transplant recipients with invasive aspergillosis. Lung

transplant recipients frequently lack a characteristic radiographic appearance and present most often as focal areas of patchy consolidation (De Pauv *et al*., 2008).

The gold standards for diagnosis are histological examination and fungal culture of tissues. Figure 8 shows a stained histological section with growing *Aspergillus* structures. Isolation of the fungus by culture is both time consuming and insensitive and fails to aid in the detection of between 30–50% of invasive aspergillosis cases (Hussain *et al*., 2000; Herbrecht et. al. 2002). Furthermore, cultures for fungi and cytopathological examination of respiratory specimens often yield negative results and lack sensitivity for detecting the fungus in an early stage of the infection. Repeated microbiologic and histopathologic samplings are difficult to obtain in these critically ill patients. Additionally, biopsy specimens may be unproductive if the sample is collected at an advanced stage of the disease.

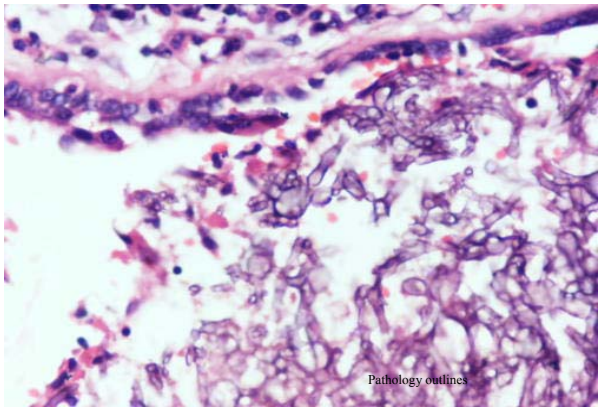

Figure 8: Cells of *Aspergillus spp*. in tissue

Although, more definitive indicators in the diagnosis of IA can possibly be had from histological and bronchiolar lavage (BAL) samples for culture, the procedures required to obtain such samples are invasive and might lead to excessive bleeding and other complications in the patient. The availability of a rapid, non-invasive method for the diagnosis of invasive aspergillosis that has a reproducible, measurable, and quantitative endpoint is clearly needed.

The EORTC, invasive fungal infections cooperative group (IFICG) and the MSG have for the purpose of clinical and research activities, classified the certainty of the presence of IA into three groups based on clinical manifestations, signs and symptoms, and microbiologic parameters (Table 1). Host factors such as patients with cancer, treated or untreated HSCT recipients who were suspected of having an invasive fungal infection and mycology test results especially positive results from normally sterile sites are important considerations. The patients with **proven** aspergillosis are those who have definitively been diagnosed with *Aspergillus spp.* as demonstrated histologically and cytologically by the presence of septate hyphae in diseased tissues and microbiologically by microscopy and/or culture, histopathological or cytological methodology. Table 2 outlines the criteria for diagnosis of proven IA. The **probable** patients are those who demonstrate the presence of a host factor, a clinical feature and a mycological element as evidence of the disease. Table 3 lists broad categories for some of the host factors, clinical and mycological criteria identified by the EORTC/MSG. The category of

possible IFD was defined more strictly in 2008 than 2002 to include only those cases with the appropriate host factors and with sufficient clinical evidence consistent with IFD but for which mycological evidence was absent.

Table 1 EORTC/MSC Criteria for invasive aspergillosis

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

Table 2 Criteria for classifying proven invasive aspergillosis

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

Modified from EORTC/MSG Revised Definition of Invasive Fungal Disease, 2008

Table 3 Major categories of host, clinical and mycological criteria for classifying invasive fungal diseases

Host factors	Clinical criteria	Mycological criteria
Recent history of neutropenia	Lower respiratory tract fungal diseases	Direct test (cytology, direct microscopy, or culture)
Receipt of an allogenic stem cell transplant	Tracheobronchitis	Aspergillosis Galactomannan antigen detected in plasma, serum, bronchoalveolar lavage fluid, or CSF
Prolonged use of corticosteroids	Sinonasal infection	Invasive fungal disease other than cryptococcosis and zygomycoses β-D-glucan detected in serum
Treatment with other recognized T cell immunosuppressants	CNS infection	Cryptococcal antigen in CSF indicates disseminated cryptococcosis
Inherited severe immunodeficiency	CNS infection	

2.3 Galactomannan EIA

The galactomannan assay was developed in the Netherlands by Stynen *et al*., 1992, and was later marketed in Europe by Sanofi Diagnostics Pasteur. The Sanofi Diagnostics Pasteur company was purchased in 1999 by BioRad Laboratories. The Platelia™ *Aspergillus* EIA was approved for use in the United States by the CDRH in 2003. In Europe, the cut-off for the Sanofi Diagnostic Pasteur test, prior to approval of the test in the USA, was an index of ≥1.5 with an indeterminate zone of 1.0 (≥1.0 and ≤ 1.5). When the FDA approved the Platelia™ *Aspergillus* EIA in 2003 for testing of serum samples in adults a cut-off index of ≥ 0.5 was adopted. In 2006, the cut-off index for the Platelia *Aspergillus* EIA kits sold in Europe was changed to ≥0.5 the same as the index in the U.S.A and in July 2008, the European countries switched completely to a cut-off value of ≥0.5. At present the major difference between the kits sold in Europe and those sold in the U.S. is that of language. The European insert is translated into five languages while the U.S. kit is in English.

Each GM molecule has as many as ten epitopes. Both capture and detector antibodies specific for the epitopes can be attached to the molecule. The Platelia *Aspergillus* EIA comprises of a rat monoclonal antibody (MAb) EB-A2 that reacts with the specific epitope of GM. It is an IgM antibody with an avidity constant of $2x10^9$ to $5x10^9$ M and binds to an epitope located on the β (1→5) galactofuranose-containing side chain of the GM molecule. A similar epitope seems to be present in other fungi. The epitope recognized by the EB-A2 MAb, is a common oligosaccharide moiety of a wide range of intracellular and extracellular glycoproteins of *Aspergillus* species (Stynen *et al*., 1992) and therefore, detection of GM can possibly be used as a biomarker for the diagnosis of IA. Figure 9 is a graphic presentation of the principle of the GM EIA test.

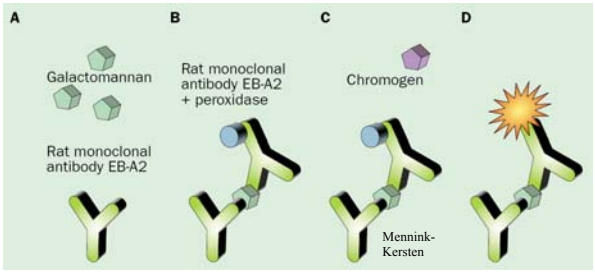

Figure 9. Mechanism of action of Platelia GM assay

BioRad Platelia *Aspergillus* EIA
The principle of the Platelia™ *Aspergillus* EIA, currently in use, is the same as the original assay. EB-A2 monoclonal antibody directed against *Aspergillus* GM is absorbed on the inner surface of a 96 well micro-titer plate. There are two major phases to the assay (a) the extraction phase and (b) the test phases. Inappropriate treatment in either phase could lead to inaccurate results. Following is an overview of the current assay.

- ***Extraction phase:*** With the use of separate pipette tips 300 µL of each control or test serum are pipetted into separate polypropylene tubes, then 100 µL of ethylenediamine-tetraacetic acid (EDTA) solution, the serum treatment solution, are added and the two solutions are mixed vigorously then heated at 120^0C for six

minutes. The heated mixture is then centrifuged at 10,000 x g for 10 minutes. The supernatant of the mixture is used for GM testing. It can be used immediately or stored at 2-8^{0}C for up to 48 hours.

- ***Testing phase of EIA:*** With all reagents at room temperature and mixed appropriately 50 µL of conjugate (antibody label) are added to each test /control designated well. This is followed by the addition of 50 µL of treated sample. The plate is covered and incubated at 37^{0}C for 90 (±5) minutes. After incubation, the contents of each well are aspirated separately and the antigen-antibody complexes at the bottom of each well are washed five times with a washing solution containing tris NaCl buffer, tween 20 and thimerosal. The wells are drained and then 200 µL of Substrate-Chromogen reagent reaction solution are added to each well and the mixture is incubated at room temperature in the dark for 30 minutes. The reaction is stopped by the addition of a stop solution. The contents of the wells are mixed and the optical density of each well is read at 450 nm.

The assay uses one negative, one positive (> 4 ng/mL GM) and a cut-off control (1 ng/mL). The cut-off control is run in duplicate and the mean OD is used to calculate the sample index. The classification of a sample as to the presence or absence of GM is determined by its index which is calculated by dividing the OD of the sample by the mean OD of the cut-off control. The test brochure suggests cut-off index for serum GM as ≥0.5. Samples with an index ≥ 0.5, i.e., positive for GM, should be repeated using another aliquot of the same sample as well as testing of another sample collected at a different time. Sera with an index < 0.5 are considered negative for GM but it is recommended that if the symptoms suggest IA the test should be repeated. The Platelia *Aspergillus* EIA brochure states that the test be used only as an aid to the diagnosis of IA and should be used in conjunction with either culture or histological examination and radiological imaging.

The test validity criteria state that the OD of each cut-off control must fall between ≥ 0.3 and ≤ 0.8. The index for the positive control must be >2 and that for the negative control must be < 0.4. Additional details of testing methodology precautions and test interpretation can be found in the Platelia *Aspergillus* EIA package insert (BioRad Laboratories, 2009).

The sandwich-enzyme immunoassay (EIA) for the detection of galactomannan has been evaluated *in vitro*, in animals, and in immunocompetent and immunocompromised subjects. Some of the performance characteristics of the assay developed by BioRad for the detection of galactomannan in a serum matrix such as the reproducibility, cross reactivity, sensitivity, specificity, and predictive values are summarized below:

Reproducibility Studies*:* Inter-assay and intra-assay variability were determined using a panel of 6 pooled patient serum samples (one negative, one low positive, two positives, and two high positives) obtained from actual clinical trial sites. Each of the 6 panel members was tested at 3 sites [at 2 of the 3 sites, testing was done in triplicate (x3) on 3 different days, using 1 reagent lot (total number of replicates at each site = 9). At the third site, each of the 6 panel members was tested in duplicate (x2) on 3 different days, using the same reagent lot (total number of replicates at the third site = 6)]. One operator performed all precision testing at each site. The data were analyzed according to the

National Committee for Clinical Laboratory Standards (NCCLS; now renamed as Clinical Laboratory Standards Institute, CLSI). The mean optical density (OD) and mean index value, standard deviation (SD), percent coefficient of variation (%CV), within lot precision (intra-assay) and within site (inter-assay) precision for each panel member at each site are illustrated below in Table 4. The results show intra-assay variability (%CV) to be low (<15%) at 2 sites. Inter-assay variability (%CV) was also <15 % at 2 sites. Site 2 showed higher intra-assay and inter-assay variability results than did the other two sites.

Table 4. Results of reproducibility tests performed at three sites for the BioRad Platelia® *Aspergillus* E.I.A.

Site 1

	Neg		Low Pos		Pos #1		Pos #2		High Pos #1		High Pos #2	
	OD	Index	OD	Index	OD	Index	OD	Index	OD	Index	OD	Index
N=	9	9	9	9	9	9	9	9	9	9	9	9
Mean=	0.052	0.09	0.445	0.74	0.702	1.17	0.931	1.56	1.227	2.06	2.887	4.83
Within Run (intra-assay)[1] SD=	0.002	0.00	0.022	0.03	0.059	0.09	0.044	0.08	0.051	0.09	0.089	0.17
%CV=	N/A	N/A	4.8%	4.4%	8.4%	7.6%	4.7%	5.1%	4.2%	4.4%	3.1%	3.6%
Total (inter-assay)[2] SD=	0.036	0.04	0.051	0.08	0.070	0.14	0.044	0.25	0.058	0.29	0.169	0.58
%CV=	N/A	N/A	11.5%	10.4%	10.0%	11.6%	4.7%	15.7%	4.7%	14.3%	5.9%	11.9%

Site 2

	Neg		Low Pos		Pos #1		Pos #2		High Pos #1		High Pos #2	
	OD	Index	OD	Index	OD	Index	OD	Index	OD	Index	OD	Index
N=	9	9	9	9	9	9	9	9	9	9	9	9
Mean=	0.040	0.10	0.280	0.70	0.364	0.89	0.602	1.49	0.801	2.01	1.361	3.43
Within Run (intra-assay)[1] SD=	0.006	0.01	0.041	0.09	0.023	0.07	0.045	0.11	0.046	0.10	0.047	0.11
%CV=	N/A	N/A	14.5%	13.0%	6.4%	7.6%	7.5%	7.1%	5.7%	4.8%	3.5%	3.2%
Total (inter-assay)[2] SD=	0.006	0.03	0.058	0.19	0.083	0.18	0.057	0.28	0.042	0.53	0.079	1.00
%CV=	N/A	N/A	20.8%	27.0%	22.7%	19.8%	9.5%	18.7%	5.3%	26.5%	5.8%	29.2%

Site 3

	Neg		Low Pos		Pos #1		Pos #2		High Pos #1		High Pos #2	
	OD	Index	OD	Index	OD	Index	OD	Index	OD	Index	OD	Index
N=	6	6	6	6	6	6	6	6	6	6	6	6
Mean=	0.049	0.10	0.388	0.81	0.652	1.36	0.830	1.73	1.158	2.41	2.378	4.96
Within Run (intra-assay)[1] SD=	0.003	0.01	0.009	0.02	0.082	0.17	0.068	0.14	0.094	0.20	0.126	0.25
%CV=	N/A	N/A	2.4%	2.4%	12.5%	12.2%	8.2%	8.2%	8.1%	8.2%	5.3%	5.1%
Total (inter-assay)[2] SD=	0.012	0.03	0.078	0.13	0.068	0.15	0.104	0.25	0.082	0.15	0.111	0.34
%CV=	N/A	N/A	20.0%	15.8%	10.5%	11.1%	12.5%	14.3%	7.1%	6.2%	4.7%	6.8%

N/A = not applicable **Same as Table 1 of the test brochure**

Cross Reactivity

A study to evaluate the effect of potentially interfering medical conditions unrelated to invasive aspergillosis was performed with one lot of the Platelia(R) *Aspergillus* EIA kit. The serum samples listed in Table 5 were tested for cross-reactivity with the Platelia(R) *Aspergillus* EIA. A total of 151 sera was tested and showed no cross reactivity against samples isolated from patients with these diseases.

Table 5. Results of Platelia *Aspergillus* EIA tests on serum samples from patients with medical conditions unrelated to aspergillosis for validation tests for cross reactivity.

Pathology	# Samples Tested	# Positives
Rheumatoid Factor	10	0
ANA Positive	10	0
IgG Hypergammaglobulinemia	10	0
IgM Hypergammaglobulinemia	10	0
Cancer*	13	0
Non-Viral Cirrhosis (primary biliary; alcohol induced; drug induced)	10	0
Multiple Transfusions	10	0
Multiparous Females	10	0
HAV	10	0
HCV	10	0
Rubella	10	0
CMV	10	0
Syphilis (RPR+)	10	0
Toxoplasmosis	10	0
Mycoplasma	10	0

* One each of adenocarcinoma, bladder, breast(2), colon, endometrial, lung, melanoma (metastatic), prostate, renal, and squamous(3).

Same as Table 2 of the test brochure

Clinical Studies*:* Clinical testing to evaluate the sensitivity, specificity, and predictive values of the Platelia(R) *Aspergillus* EIA was conducted at three sites located in the U.S. and Canada. The study was conducted retrospectively using a total of 1724 (1262 controls and 462 from patients with aspergillosis) serum samples collected from 172 adult patients (143 controls and 29 with aspergillosis) with bone marrow transplant or leukemia from the following populations diagnosed according to EORTC criteria of 2002:

- patients without signs of invasive aspergillosis (control patients)
- patients with probable invasive aspergillosis
- patients with proven invasive aspergillosis

Sensitivity: Sensitivity testing was conducted at three sites on a combined total of 29 patients diagnosed with proven or probable invasive aspergillosis. Results in Table 6 show an overall sensitivity of 79% for both probable and proven aspergillosis patients.

Table 6. Performance of the assay for the diagnosis of aspergillosis

Diagnosis	Number of Patients	Sensitivity	95% Confidence Interval
Proven Aspergillosis	11	81.8% (9/11)	52.3 – 94.0%
Probable Aspergillosis	18	77.8% (14/18)	54.8 – 91.0%
Combined Proven and Probable Aspergillosis	29	79.3% (23/29)	61.6 – 90.2%

Same as Table 4 of the test brochure

In pediatric patients the sensitivity was low (53%).

Specificity: Specificity testing was conducted at three sites on a combined total of 1262 samples obtained from 143 bone marrow transplant (BMT) and leukemia patients without signs of invasive aspergillosis (control patients). The results in Table 7 show a combined specificity of 89%.

Table 7. Performance of the Platelia Aspergillus EIA for the diagnosis of aspergillosis

Site	Number of Patients	Specificity	95% Confidence Interval
1	28	78.6% (22/28)	60.5 – 89.8%
2	77	93.4% (71/77)	84.0 – 96.4%
3	38	89.5% (34/38)	75.9 – 95.8%
Combined Sites	143	88.8% (127/143)	92.6 – 93.0%

Same as Table 7 of the test brochure

Specificity was higher when analyzed by the number of samples tested and was over 80% at all three sites (Table 8).

Table 8. Performance of the Platelia Aspergillus EIA for the diagnosis of aspergillosis

Site	Number of Samples	Specificity	95% Confidence Interval
1	349	98.0% (342/349)	95.9 – 99.0%
2	560	98.6% (552/560)	97.2 – 99.3%
3	353	98.9% (349/353)	97.1 - 99.6%
Combined	1262	98.5% (1243/1262)	97.7 - 99.0%

Same as Table 8 of the test brochure

Predictive value: Positive and negative predictive values were analyzed for the patient population in this study based on the actual prevalence rate observed in this study. The results in Table 9 show the PPV of 27% in adult patients and 17% in pediatric subjects at a prevalence rate of 5%. The NPV was >97% in both adult and pediatric patients at the same prevalence rate.

Table 9. Predictive values for adult and pediatric patients at various population prevalence using the Platelia *Aspergillus* EIA for galactomannan.

Prevalence	PPV	95% Confidence Interval	NPV	95% Confidence Interval
Adult Patients:				
16.9%	59.0%	43.4 – 72.9%	95.5%	90.5 – 97.9%
5%	27.2%	13.7% - 46.7%	98.8%	95.4 – 99.7%
Pediatric Patients:				
13.6%	39.1%	22.2 – 59.2%	92.2%	85.3 – 96.0%
5%	17.6%	6.5 – 13.8%	97.2	92.1 – 99.1%

Adapted from the description in the test brochure

3. Performance of the Platelia® *Aspergillus* EIA in the literature.

The sandwich enzyme immunoassay (EIA) for the detection of galactomannan has been evaluated *in vitro*, in animals, and in immunocompetent and immunocompromised subjects.

This review is based on all studies submitted by the sponsor and additional studies, identified by an independent literature search, through Pub Med, within last five years,

using the terms serum GM, and Platelia assay. Of the 39 publications identified, 11 were found relevant to the subject of this review.

3.1 *In vitro* Studies

Several studies support the usefulness of detection of galactomannan by the Platelia *Aspergillus* EIA. Studies also show cross-reactivity with fungal species other than *Aspergillus* as well as bacteria (for details see Appendix – I). For example, Stynen *et al*. (1992), reported cross-reactivity of EB-A2 with a number of fungal species. The avidities of EB-A2 with extract preparations from different fungi are listed in Table 10. The avidities between EB-A2 and *Aspergillus fumigatus*, *Penicillium digitatum* and *Trichophyton rubrum* were similar.

Table 10: Avidities of EB-A1* and EB-A2 with the extracts of various fungi determined by ELISA

Fungus	Avidity constant (1/M)	
	EB-A1	**EB-A2**
Aspergillus fumigatus	2×10^9	5×10^9
Penicillium digitatum	3×10^9	5×10^9
Trichophyton rubrum	1×10^9	5×10^9
Trichophyton interdigitalis	4×10^8	2×10^9
Botrytis tulipae	1×10^9	4×10^9
Wallemia sebi	5×10^9	3×10^9
Cladosporium cladosporioides	8×10^8	2×10^8
Fusarium solani	$<10^{7a}$	$<10^7$
Trichodermna viride	$<10^77$	$<10^7$
Candida albicans[b]	$< 10^7$	$< 10^7$
Saccharomyces cerevisiae[b]	$< 10^77$	$< 10^7$
Cryptococcus neoformans[c]	$<10^7$	$<10^7$

a $<10^7$, the avidity constant was too low to be calculated. (Stynen et al.)
h Purified mannan.
'Purified glucuronoxylomannan
***EB-A1 represents another clone of monoclonal antibody originally screened by** Stynen *et al., 1992*

A study by Swanink *et al*., 1997, evaluated the cross-reactivity with bacterial and fungal species using the Platelia™ *Aspergillus* EIA. Twenty-nine fungal strains isolated from clinical samples, and bacterial isolates from 40 blood cultures of febrile neutropenic patients were tested. For the purpose of this test the reaction with *A. fumigatus* was designated 100% (Table 11). At the dilution of 1:1,000 all *Aspergillus spp.* showed strong reactivity ranging from 100% to 122%. *Penicillium chrysogenum* showed the strongest reaction of 128%. Strong positive reactions were also obtained from *Penicillium digitatum* (107%) and *Paecilomyces variotii* (106%). All other fungi had GM concentrations below the index of 1.0. The one *C. albicans* isolate tested was positive. Of the 27 gram-positive bacterial isolates, five gave positive results and two of the 12 gram negative bacteria gave positive results (Table 12).

Table 11. Reactivity of fungal cultures to the sandwich ELISA tested for GM

Fungus	Concn by ELISA (ng/ml)	Reactivity compared with that of *Aspergillus fumigatus*[a]
Aspergillus fumigatus	5.4	100
Aspergillus flavus	5.8	107
Aspergillus niger	6.4	119
Aspergillus terreus	6.6	122
Paecilomyces variotii	5.7	106
Penicillium chrysogenum	6.9	128
Penicillium digitatum	5.8	107
Alternaria species	2.1	39
Rhodotorula rubra	0.7	13
Fusarium solani	0.6	11
Fusarium oxysporum	0.3	6
Trichophyton mentagrophytes	0.4	7
Trichophyton rubrum	0.5	9
Rhizopus oryzae	0.4	7
Absidia corymbifera	0.4	7
Scopulariopsis brevicaulis	0.4	7
Exophilia species	0.4	7
Cladosporium species	0.5	9
Pseudallescheria boydii	0.4	7
Trichoderma viride	0.4	7
Acremonium species	0.4	7
Candida albicans	0.3	6
Candida glabrata	0.3	6
Candida krusei	0.3	6
Candida parapsilosis	0.3	6
Candida tropicalis	0.3	6
Geotrichum candidum	0.4	7
Saccharomyces cerevisiae	0.4	7
Cryptococcus neoformans	0.3	6

[a] The reactivity for *Aspergillus fumigatus* is set at 100.

` `Table 12. Reactivity of recent isolates from blood cultures by Platelia ELISA

Microorganism cultured from the blood	No. of serum samples positive/no. of samples tested
Gram-positive organisms	
Staphylococcus epidermidis	2/5
Staphylococcus aureus	0/1
Streptococcus sanguis	0/3
Streptococcus mitis	0/5
Streptococcus oralis	0/2
Streptococcus pneumoniae	0/1
Enterococcus faecalis	2/3
Micrococcus spp.	0/1
Corynebacterium jeikeium	1/6
Gram-negative organisms	
Pseudomonas aeruginosa	1/4
Pseudomonas cepacia	0/1
Escherichia coli	1/4
Enterobacter cloacae	0/2
Achromobacter xylosoxidans	0/1
Candida albicans	1/1

3.2 Animal Studies

(1) A study by Marr *et al*., 2004 measured the GM levels in 20 neutropenic New Zealand White rabbits infected intra-tracheally with 2 (high of 1 x 10^8 or low of 5 x 10^7) concentrations of conidia of *A. fumigatus*. Neutropenia was induced by intravenous administration of cytarabine (Ara-C; Pharmacia-Upjohn). Ten uninfected rabbits were included as controls. Antibiotics were administered to prevent opportunistic infections. Surviving animals were treated with an antifungal agent for ≤ 12 days. Serum samples were collected from the rabbits which were then sacrificed the following day and the lung tissue cultured. The determination of GM positivity was made on the basis of a cut-off index of ≥ 0.5 and the testing of a second aliquot of the positive serum sample. Of the 30 rabbits, 17 were true positives and 9 true negatives. There were one false positive and three false negative results. Rabbits that received higher concentrations of inoculum had higher indices of GM in their serum samples compared to those from infected with lower concentration of spores (Figure 10) which, not surprisingly, suggests that the fungal burden influences directly the level of the GM index. Figure 11 shows a positive correlation between the log colony forming units of lung tissue obtained after the animal was sacrificed and the GM indices from the sera of the infected rabbits collected the day before euthanasia.

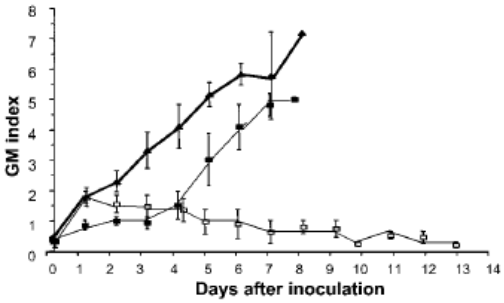

Figure 10: GM index in rabbits infected with 10^8 (black triangles and white squares) and 5 x 10^7 (black squares) coni-dia of *A. fumigatus*. A subset of rabbits was treated at day 1 after inoculation with amphotericin B (0.5 mg/kg/day; white squares)

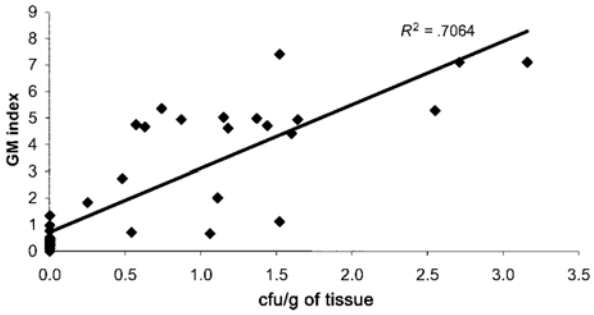

Figure 11: GM indices and colony forming units from 20 infected rabbits (1 X 10^8) *A. fumigatus* conidia and 10 uninfected rabbits

Another recent study by Wheat *et al*., 2007 measured GM in 32 serum samples from neutropenic New Zealand White rabbits infected intra-tracheally with 1.25 x 10^8 spores of *A. fumigatus*. The time of collection of serum samples was not specified. The serum samples had been stored two years previously at -70^0C. All serum samples were positive by GM Platelia *Aspergillus* EIA but negative by the *Histoplasma* EIA. The results of the rabbits infected with A*spergillus* spores support the high level of sensitivity of the test in identifying the presence of GM in serum.

3.3 Clinical Microbiology Studies

Of the 30 clinical studies reviewed to evaluate the performance of the Platelia *Aspergillus* EIA, 27 studies were in neutropenic patients undergoing HSCT or with hematologic malignancy, two in liver transplant patients and one in lung transplant patients (Table 13). For details please see Appendix – II.

3.3.1 Specificity and Sensitivity of the Assay

The overall specificity and sensitivity of the assay in serum samples yielded variable results across the different clinical studies at different cut-off points of the galactomannan index (Table 13). The results obtained from testing of serum from hematological and HSCT neutropenic patients by the Platelia *Aspergillus* EIA were measured by one or more cut-off indices ranging from ≥ 0.5 to ≥1.5. There were 9 studies at a cut-off of 1.5 based on testing of 1 sample; there were 10 studies each at cut-off 1.0 based on testing of 1sample or 2 samples; 9 studies at a cut-off of 0.5 tested 2 samples; 12 studies evaluated the results at more than one cut-off value ; see Table 14. Using two or more consecutive samples at a cut-off index of ≥ 0.5 and testing of 2 samples (based on the recommendations in the test brochure) the specificity and sensitivity varied from 67% to 99% and 71% to 100%, respectively; PPV and NPV varied from 33% to 97% and 96% to 100%, respectively. At a cut-off index of 1.0, specificity and sensitivity varied from 92 to 100% and 47% to 100%, respectively; the PPV and NPV varied from 29% to 100% and 82% to 100%, respectively. In one study (Maertens *et al*., 2004), performance was measured at five cut-off ranges, ≥ 1.5, 1.0, 0.6 to 0.8, and 0.5 using two consecutive samples and a single sample. . Overall, the results suggest an improvement in specificity at a cut-off of ≥ 1.0 compared to ≥ 0.5 with minimal change in sensitivity.

Two studies in liver transplant recipients and 1 study in lung transplant recipient reported the performance of the assay at a cut-off of ≥ 0.5 based on testing of 2 or more samples. Specificity (76%) was low in lung transplant patients (Husain *et al*., 2004). In a study by Kwak *et al*., 2004, 1,594 samples from 154 liver transplant patients were tested. One patient was classified as probable and only that patient gave positive results, in fact there were three positive samples for this patient. Sera from 20 patients without IFI gave 23 false positive results, seven of these patients were being treated with piperacillin-tazobactam; four died but in autopsy were shown not to have IA. The reproducibility of test results by sample in this study was 98.5%. The observations are very limited to support the use of galactomannan assay for enrollment of liver or lung transplant patients in the clinical trials for the treatment of aspergillosis.

An overall analysis of 20 studies in hematologic neutropenic patients, included testing at a cut-off of 0.5, 1.0 and 1.5 (Table 14). Based on median values, the highest sensitivity and NPV were obtained by consecutive specimens at a cut-off index of ≥ 0.5. This NPV was only slightly better than that obtained with the use of 1 sample at a cut-off ≥ 1.0. The highest specificity and PPV were achieved by testing consecutive specimens at a cut-off of ≥1.0.

Table 13: Summary of clinical studies for the performance of the galactomannan assay in serum

No.	Reference (Study site)	Pre-disposing Condition	Diagnosis	*Aspergillus* species Isolated	PMN/µL	Parameter	Cut-off indices							Comments
							≥1.5 (2X)	≥ 1.5 (1X)	≥1.0 (2X)	≥1.0 (1X)	0.6-0.9	≥ 0.5 (2 X)	≥ 0.5 (1 X)	
	Test Brochure	NA	NA	NA	NA	Sensitivity%						81		
						Specificity %						89		
						PPV %						55		
						NPV%						97		
A. Hematological/HSCT patients														
1	Maertens *et al*., 1999 Prospective study	Hematological malignancy adults (n=186)	Possible = 61 Probable = 6 Proven = 27 Controls = 92	NS	< 500	Sensitivity %			93					single positive samples 2 times / wk; all were autopsy samples and number represents episodes
						Specificity %			95					
						PPV %			93					
						NPV%			95					
2	Maertens *et al*., 2001 (Belgium)	Hematologic disorders BMT,HSCT adults (n=253)	Probable IA = 29 Proven IA = 7 Possible IFI = 83 Probable IFI = 2 No IFI = 132	*A fumigatus* *A flavus*	< 500	Sensitivity %				92				single sample ≥ 1.0. ≥ 2 times/wk; results based on proven and probable cases (autopsy)
						Specificity %				95				
						PPV %				72				
						NPV%				99				
						Sensitivity %			90					Final results based on 30 proven, 9 probable, and 264 no IFA
						Specificity %			98					
						PPV %			88					
						NPV%			98					
3	Maertens *et al*., 2002	HSCT adult (n=100)	Possible IA= 0 Probable IA= 0 Proven IA = 18 Proven IFI = 3 Possible IFI = 6 No IFI= 73			Sensitivity %			94	95				consecutive and single positive samples
						Specificity %			99	85				
						PPV %			94	59				
						NPV%			99	99				
4	Maertens *et al*., 2004 (Germany)	Hemato-oncological malignancy, neutropenic, allogenic HSCT adult (n= 108)	Probable IA= 13 Proven IA=16 PossibleIFI=20 No IFI=59			Sensitivity %	62	83	79	93		97	97	Single and consecutive samples at ≥ 1.5, 1.0, 0.9 - 0.6 ≥2 times/wk
						Specificity %	100	100	100	100		99	85	
						PPV %	100	100	100	100		97	72	
						NPV%	87	94	93	97		99	98	
5	Maertens *et al*., 2007 (Belgium) Retrospective	Hematological disorder, neutropenia (n=239) episodes	Possible IA = 0 Probable IA =19 Proven IA = 19 No IA = 201	NS	NS	Sensitivity %		76		82		92	97	Using single samples for ≥ 1.5, ≥1.0 and 2X ≥ 0.5 ≥ 2 times / wk. Calculations based on episodes
						Specificity %		98		97		98	91	
						PPV %		85		82		88	66	
						NPV %		96		97		99	100	
6	Becker *et.al*., 2003 (Netherlands)	Hematological disorders and neutropenic adults (n=160)	Possible = 18; Suspected=4 Probable = 11 Proven = 2 Other IFI = 4 No IFI=121	NS	<500	Sensitivity %	12	18	47	59	59			Only 44 patients tested including 4 treated empiric Amp B. 121 no IFI patients were not tested by GM assay
						Specificity %	95	84	93	75	61			
						PPV %	50	30	73	48	36			
						NPV%	74	73	82	83	80			
7	Kawazu *et al*., 2004 (Japan) Prospective study	Hematological disorders **(**n=96**)**	Possible = 13 Probable = 2 Proven = 9 No IFI = 72	*A. flavus* *A fumigatus* *A. spergillus*	< 500	Sensitivity %	45	82	64	100	100	100	100	≥ 1 .5, 1.0, 0.5 single sample or any 2 consecutive positive samples; Cut-off ≥0.6 3 times/wk
						Specificity %	98	90	98	86	93	84	34	
						PPV %	63	41	70	38	55	35	12	
						NPV%	95	98	97	100	100	100	100	

No.	Reference (Study site)	Pre-disposing Condition	Diagnosis	*Aspergillus* species Isolated	PMN/µL	Parameter	Cut-off indices							Comments
							≥1.5 (2X)	≥ 1.5 (1X)	≥1.0 (2X)	≥1.0 (1X)	0.6-0.9	≥ 0.5 (2 X)	≥ 0.5 (1 X)	
8	Yoo *et. al.*, 2005 (Korea) Prospective study	Hematological disease HSCT adults (n=128)	Possible = 0 Probable = 12 Proven = 2 Controls = 114	NS	NS	Sensitivity %						86		≥0.5 in ≥ 2 consecutive. 2 times /wk
						Specificity %						78		
						PPV %						33		
						NPV%						98		
9	Yoo *et. al.*, 2007 (Korea)	Hematological disease Neutropenic adult (n=78)	Possible = 15 Probable = 7 Proven = 0 Controls = 56	NS	NS	Sensitivity%						71		consecutive positive samples excludes possible in calculations of parameters
						Specificity						98		
						PPV %						83		
						NPV%						97		
10	Lai *et al.*, 2007 (Taiwan) Prospective study	Hematological malignancy & Other IA at risk adults (n=189)	Possible = 26 Probable = 9 Proven = 5 Controls =149	NS	NS	Sensitivity %		79				100		Variable 1 or ≥ 2 positive ,index ≥ 1.5 Excludes possible from calculations of parameters
						Specificity %		94						
						PPV %		55						
						NPV%		98						
11	Buchheidt *et al*., 2004	Hematological malignancy (n=165) Calculated as patient episodes (n=205)	Possible = 90 Probable = 3 Proven = 8 No IA = 104			Sensitivity %	14							≥ 2 consecutive samples > 1.5; excludes possible from calculations of parameters and 2 probable cases based on GM results
						Specificity %	99							
						PPV %	50							
						NPV%	94							
12	Ulusakarya *et al*., 2001 (France)	Hematological Neutropenic - adults (n=135)	Possible = 2 Probable = 6 Proven = 10 No IFI s= 117	NS	<500	Sensitivity %		69		100				> 1.5, , ≥1.0 in single samples 1 time/wk excludes possible in calculation
						Specificity %		96		92				
						PPV %		69		64				
						NPV%		96		100				
13	Herbrecht *et.al*., 2002 Prospective study – 4 cohorts of adults and children with Hematological malignancy	Cohort 1: Fever of unknown origin (n=220; 261 episodes)	Possible = 1 Probable = 0 Proven = 0 Controls = 260	NS	<500	Sensitivity %		100						Random single testing ≥ 0.8. At suspicion and 3 consecutive days after, then 1/wk until resolution Possible results not included in the calculation Sensitivity, Specificity, PPV & NPV
						Specificity %		95						
						PPV %		7						
						NPV%		100						
		Cohort 2: Suspected Pulmonary Infection (n=274; 297 episodes)	Possible = 53 Probable = 67 Proven = 25 Controls = 152	NS	<500	Sensitivity %		28						
						Specificity %		99						
						PPV %		95						
						NPV%		58						
		Cohort 3: Suspected extra-pulmonary infection (n=28; 28 episodes)	Possible = 0 Probable = 0 Proven = 5 Controls = 23	NS	<500	Sensitivity %		40						
						Specificity %		100						
						PPV %		100						
						NPV%		88						
		Cohort 4: Transplant recipients (n=206; 211 episodes)	Possible = 1 Probable = 0 Proven = 1 Controls = 209	NS	<500	Sensitivity %		100						
						Specificity %		91						
						PPV %		10						
						NPV%		100						
		Cumulative (adults and children) [Average of cohorts 1, 2, 3 and 4] (n=728; 797 episodes)	Possible = 55 Probable = 67 Proven =31 Controls = 644	NS	<500	Sensitivity %		32						
						Specificity %		95						
						PPV %		48						
						NPV%		90						

No.	Reference (Study site)	Pre-disposing Condition	Diagnosis	*Aspergillus* species Isolated	PMN/µL	Parameter	Cut-off indices							Comments
							≥1.5 (2X)	≥ 1.5 (1X)	≥1.0 (2X)	≥1.0 (1X)	0.6-0.9	≥ 0.5 (2 X)	≥ 0.5 (1 X)	
		Cumulative (adults only) [Average of Cohorts 1, 2, 3 and 4] (n= NS; 797 episodes)	Possible = 47 Probable = 61 Proven = 26 Controls =	NS		Sensitivity %		26		35				
						Specificity %		99		99				
						PPV %		92		87				
						NPV%		83		84				
14	Pinel *et al*., 2003 (France) Prospective study	High risk patients from hematological department and ICUs (n=807)	Possible = 22 Probable = 31 Proven = 3 No IA = 751	*A. fumigatus* *A. flavus* *A. terreus*		Sensitivity %			50					2 consecutive positive samples index ≥ 1.0.Excludes possible in calculations
						Specificity %			100					
						PPV %			85					
						NPV%			98					
15	Steinbach *et al.*, 2007 (U.S.A.) Prospective study	Hematological disease GVHD or Neutropenia pediatric (n=64)	Possible = 0 Probable= 1 Proven = 0 non IA=55 no IFI=8	NS	NS	Sensitivity %						NS		2 separate consecutive new aliquot of first positive sample retested 2 times / wk
						Specificity %						87		
						PPV %						NS		
						NPV%						NS		
16	Bretagne *et al*., 1997 (France) Prospective study	Hematological malignancy-adult (n=50)	Possible IA= 0 Suspected IA=9 Probable IA= 3 Proven IA = 3 Other fungal infections=14 No fungal infection = 21	*A. fumigatus* *A. flavus* *A. niger* *A. ustus*	< 500	Sensitivity %					100			≥ 2 consecutive samples ≥ 1 g/mL (OD=0.8) 1 time / wk. Excludes suspected in calculations
						Specificity %					76			
						PPV %					60			
						NPV%					100			
17	Rohlich *et al,* 1996 (France)	Hematological disorder BMT- pediatric (15) Neutrapenic pediatric (22) Total -37	Possible IA = ? Probable IA = 10 Proven IA =? No IA = 27	NS	200	Sensitivity %			100					2 consecutive positive. Each sample tested twice.
						Specificity %			93					
						PPV %			83					
						NPV%			100					
18	Penack *et al*., 2008 (Belgium) Prospective study	Hematological malignancy Neutropenia or HSCT adult (n=200)	Possible = 26 Probable = 11 Proven =12 Control = 151	N S	NS	Sensitivity %							100	Samples tested 2 times/ wk. Not using GM to define- 31 possible, 5 probable, 12 proven, 151 controls. Values based on proven and probable not using GM in definition of IA
						Specificity %							94	
						PPV %							61	
						NPV%							100	
19	Busca *et al.*, 2006 (Italy) Prospective study	HSCT recipients (n=71) Solid organ transplant (n=3) Total=74	Possible =7 Probable = 0 Proven = 2 Controls = 65	NS	NS	Sensitivity %			100					2 consecutive positive samples
						Specificity %			92					
						PPV %			29					
						NPV%			100					
20	Suankratay *et al*., 2006 (Thailand) Prospective study	Hematological malignancy, prolonged neutropenia, HSCT recipients, adults (n=44); treatment episodes = 50	Probable = 12 Proven = 5 possible & no IA = 33	NS	<500	Sensitivity %	77		88		94	94		2 consecutive index ≥1.0 33 possible and no IA does not specify the number of patients but refer to episodes
						Specificity %	100		97		79	67		
						PPV %	100		94		70	59		
						NPV%	89		94		96	96		

No.	Reference (Study site)	Pre-disposing Condition	Diagnosis	*Aspergillus* species Isolated	PMN/µL	Parameter	Cut-off indices							Comments
							≥1.5 (2X)	≥ 1.5 (1X)	≥1.0 (2X)	≥1.0 (1X)	0.6-0.9	≥ 0.5 (2 X)	≥ 0.5 (1 X)	
21	Pazos *et al*., 2005 (Spain) Prospective study	Hematological malignancy adult (n=40)	Possible = 3 Probable = 3 Proven = 5 Controls = 29	*A. fumigatus* *A. flavus*	NS	Sensitivity % Specificity % PPV % NPV%	88 90 70 96							2 consecutive positive samples.. including retest of first positive , index ≥ 1.5. 2 times/wk
22	Marr *et. al*., 2005 (U.S.A.)	Hematological malignancy HSCT-adult (n=315)	Possible = 0 Probable = 26 Proven = 20 Controls = 269	NS	NS	Sensitivity % Specificity % PPV % NPV%		43 93 53 91		48 88 42 91			70 70 28 93	One positive test -14 to + 14 days of diagnosis. 2 times /wk
23	Marr *et al*., 2004 (U.S.A.) Prospective study	Bone marrow transplant HSCT recipients (n=67)	Possible = 8 Probable = 11 Proven = 13 Controls = 35	*A. fumigatus*	NS	Sensitivity % Specificity % PPV NPV				54 100 NS NS		82 74 NS NS		≥ 2 consecutive positive samples repeat testing 1 time /wk. Includes possible in calculation of parameters
24	Rovira *et al*., 2004 (Spain) Prospective study	Hematological malignancy-;Allo-HSCT recipients adults (n=74)	Possible IA= 2 Probable IA= 5 Proven IA = 1 Control = 66	*A. fumigatus* *A. flavus* *A. terreus*	NS	Sensitivity % Specificity % PPV % NPV%		67 100 100 97						1 sample with index > 1.5. 1-2 times /wk
25	Sulahian *et al*., 1996 (France)	Hematological Malignancy, BMT adults (n= 211)	Possible = 8 Probable = 15 Proven = 25 Controls = 163	*A. fumigatus* *A. flavus* *A nidulans*	NS	Sensitivity % Specificity % PPV % NPV%					83 81 54 95			Tested in duplicate.≥ 0.7 When suspected samples taken 1/day
26	Sulahian *et. al*., 2001 (France)	Hematological malignancy BMT Adults/children (n=450)	Possible = 0 Probable = 22 Proven = 22 Control = 406	*A. fumigatus* *A. flavus* *A.niger*	NS	Sensitivity % Specificity % PPV % NPV%	89 98 79 99							≥ 2 consecutive positive & retest of first positive Index ≥ 1.5 2 times/wk
		Hematological malignancy ASCT children (347)	Possible =0 Probable =4 Proven = 5 Control = 338	*A. fumigatus* *A. flavus*	NS	Sensitivity % Specificity % PPV % NPV%	100 90 21 100							≥ 2 consecutive positive & retest of first positive Index ≥ 1.5 2 times/wk
27	Machetti *et al.,* 1998 (France)	Hematological malignancy Bone Marrow (HSCT) Transplant Adults (n=22)	Possible = 1 Probable = 3 Proven = 1 Control = 17	*A. fumigatus*	NS	Sensitivity % Specificity % PPV % NPV%	75 82 50 93							3 times/week in 1 st. month 1 time /week in 2nd and 3 rd month consecutive positive samples excludes possible in calculation

No.	Reference (Study site)	Pre-disposing Condition	Diagnosis	*Aspergillus* species Isolated	PMN/µL	Parameter	Cut-off indices							Comments
							≥1.5 (2X)	≥ 1.5 (1X)	≥1.0 (2X)	≥1.0 (1X)	0.6-0.9	≥ 0.5 (2 X)	≥ 0.5 (1 X)	
B: Liver transplant														
28	Fortun *et.al*., 2009 (France)	Liver transplant Adults (n= 88)	Possible = 0 Probable =1 Proven = 2 No IA = 85	NS	NS	Sensitivity %						67		2 consecutive serum samples ≥ 0.5 1 time / wk
						Specificity %						67		
						PPV %						NS		
						NPV%						NS		
29	Kwak *et al*., 2004 (U.S.A.)	Liver disease Liver transplant Adults (n=154)	Possible = 0 Probable = 1 Proven = 0 Controls = 153	*A. fumigatus*	NS	Sensitivity %								≥0.5 in repeat of positive sample 2 times/wk
						Specificity %						87		
						PPV %								
						NPV%								
C. Lung Transplant														
30	Husain *et. al*., 2004 (U.S.A.) Prospective Study	Lung transplant Adults (n=70)	Possible = 0 Probable = 3 Proven = 9 Controls = 58	*A. fumigatus* *A. flavus* *A.niger*		Sensitivity %						25		≥ 0.5 2 consecutive samples with repeat of first positive sample. 2 times/wk
						Specificity %						76		
						PPV %						18		
						NPV%						83		

NS = not stated.

Table 14: Performance of the assay at a cut-off index of ≥ 0.5, ≥ 1.0, and ≥ 1.5

No	Reference	≥ 0.5 (2 samples)				≥1.0 (1 sample)				≥ 1.0 (2 samples)				≥ 1.5 (1 sample)			
		Sens	Spec	PPV	NPV	Sens	Spec	PPV	NPV	Sens	Specs	PPV	NPV	Sens	Specs	PPV	NPV
1	Maertens *et al*., 1999					93	95	93	95								
2	Maertens *et al*., 2001					92	95	72	99	90	98	88	98				
3	Maertens et.al., 2002					95	85	59	99	94	99	94	99				
4	Maertens *et al*., 2004	97	99	97	99	93	100	100	97	79	100	100	93	83	100	100	94
5	Maertens *et al*., 2007	92	98	88	99	82	97	82	97					76	98	888555	968696
6	Becker *et.al*., 2003					59	75	48	83	47	93	73	82	18	84	30	73
7	Kawazu *et al*., 2004	100	84	35	100	100	86	38	100	64	98	70	97	82	90	41	98
8	Yoo *et. al*., 2005	86	78	33	98												
9	Yoo *et. al*., 2007	71	98	83	97												
10	Lai *et al*., 2007	100												79	94	55	98
11	Ulusakarya *et al*., 2001					100	92	64	100					69	96	69	96
12	Herbrecht *et.al*., 2002 (adults only)					35	97	87	84					26	99	92	83
13	Pinel *et al*., 2003									50	100	85	98				
14	Steinbach *et al*., 2007		87														
15	Rohlich *et al*, 1996 (France)									100	93	83	100				
16	Busca *et al*., 2006									100	92	29	100				
17	Suankratay *et al*., 2006	94	67	59	96					88	97	94	94				
18	Marr *et. al*., 2005					48	88	42	91					43	93	53	91
19	Marr *et al*., 2004	82	74			54	100										
20	Rovira *et al*., 2004													67	100	100	97
Overall Mean (Median)		90 (93)	87 (86)	66 (72)	98 (99)	77 (93)	92(94)	69(64)	95 (97)	79 (88)	97 (98)	80 (85)	96 (98)	60 (69)	95 (95)	68 (55)	91 (94)

Sens = sensitivity; Spec = specificity; PPV = positive predictive value; NPV = negative predictive value

A few of the individual studies need to be highlighted at this point. In a study by Maertens *et al*., 2004 that tested a total of 1642 serum samples at a cut-off index of ≥0.5, 848 (97.9%) sera were negative. However, when the cut-off limit was raised to 1.5 or 1.0 the specificity was 100%. In the same study based on the analysis of 74 episodes, the specificity per episode at a cut-off ≥ 0.5 was 85.1% for a single positive sample but increased to 98.7 % when evaluated based on testing of two consecutive positive samples. It is noteworthy that a patient whose serum was consistently negative died of rapidly progressive veno-occlusive disease and the autopsy showed the presence of fungal hyphae consistent with *Aspergillus spp*.

3.3.2 Acceptable cut-off for positivity

The Platelia assay was first utilized as an aid to the diagnosis of IA in Europe. The cut-off index for positivity at that time ranged from 1.0 to ≥ 1.5 until 2006 when European countries adopted the FDA approved cut-off of ≥ 0.5. Some of the options used to obtain an accurate cut-off are listed below.

- When the results were based on one result of a single sample the cut-off of 1.0 to ≥ 1.5 resulted in a higher specificity but a lower sensitivity than the cut-off index of ≥ 0.5. The lower cut-off index of ≥ 0.5 also resulted in a greater number of false positive results than did the higher cut-off values for a single test.
- Samples resulting in an index ≥ 0.5 were repeated using a different aliquot of serum from the same sample. The sample was classified as positive if the result of the repeat aliquot was also ≥ 0.5. However, if the volume of the sample was insufficient to repeat the test the original value was accepted as the final result.
- If there was disagreement between the results of two different aliquots, a third aliquot of the same sample was repeated in another run (Steinbach *et al.,* 2007).

Some studies defined 'true' positive GM antigenemia as having two consecutive samples from the same patient with a result of an index of ≥ 0.5 (Asano-Mori *et al*., 2007). For the purpose of diagnosis, it is critical that as few positive samples as possible be missed because the fungus grows so rapidly that early detection might make the difference between life and imminent death. With a ≥ 0.5 cut-off it is possible to identify from 95 – 100% of the positive cases. However, for the purpose of enrollment into clinical trials, to evaluate the efficacy of therapeutic agents for the treatment of aspergillosis, it is critical that false positives should be avoided. The results indicate that there were two ways to increase the specificity of the test (1) to increase the cut-off and (2) to increase the number of samples per patient. At a cut-off of ≥ 1.0, and testing of 2 consecutive serum samples, the studies showed that the sensitivity and specificity varied from 48% to 100% and 75% to 100%, respectively; the PPV and NPV varied from 42% to 100% and 83% to 100%, respectively.

Three cohorts (patients treated with itraconazole vs fluconazole, fluconazole prophylaxis + amphotericin B therapy, and serotoxin + fluconazole) of hematological patients were used to study the effect of mold–active drugs on the sensitivity and cut-off indices of the Platelia *Aspergillus* EIA (Marr *et al*., 2005). Three hundred and fifteen patients were tested. There were 20 proven, 26 probable and 269 control patients. Patients considered as possible IA were excluded from the study. The results indicate that when the cut-off index was decreased from ≥ 1.5 to ≥ 0.5 the time interval between the day of first positive GM result and the day of the diagnosis of IA by traditional methods increased from 1.0 to 2.5 days prior to diagnosis i.e., diagnosis could be made earlier by about 1.5 days. Further, that the sensitivity of the test samples taken during the week of diagnosis had increased when the cut-off was ≥ 0.5, and that administration of anti-fungal therapy during the week of specimen collection resulted in a sensitivity of 59%, and specimens collected on the day of treatment decreased the sensitivity of the assay even more to 52%. In patients who were not receiving a mold-active agent the sensitivity was 89%. Therefore a low cut-off index is important in patients who are receiving mold-active anti-fungal therapy.

3.3.3 Reproducibility

Becker *et al*., 2003, in a prospective blinded study tested inter-laboratory reproducibility using 200 randomly collected serum samples from 160 patients with and without invasive pulmonary aspergillosis (13 patients with proven or probable IPA, 22 with possible or suspected IPA, four other IFI and 121 without IFI). The samples were split and tested at another laboratory (Erasmus Medical Center was the primary laboratory and University Medical Center Nijmegen, the comparator laboratory. The criterion for positivity was the same in the comparator laboratory as it was in the primary laboratory i.e., two positive samples at a cut-off of ≥ 1.0. The results from both laboratories were the same for 188 (94%) serum samples. In the comparator laboratory, 2 samples showed results that were discordant from that of the original laboratory. The results were based on single test of a one serum sample.

3.3.4 Sample collection, preservation and testing

In most of the studies the serum samples used to determine the level of GM were collected within a week of testing. One study utilized animal sera (Sulahian *et al*., 1996). Studies that were conducted retrospectively utilized serum samples that were stored at either – 70^0C or – 80^0C, some studies stored samples at -20^0C (Becker *et al.*, 2003, Maertens *et al.,* 2004). The test brochure for the Platelia *Aspergillus* EIA states that serum samples could be subjected to as many as four freeze/thaw cycles.

Some studies have made a diagnosis on the basis of the results of one sample (Lai *et al.*, 2007, Penack *et al.,* 2008, Rovira *et al* 2004). One study (Becker *et al.*, 2003) examined the accuracy of the results of split samples and a significant number of studies used multiple samples that were sometimes specified in a particular order such as consecutive as opposed to random samples.

For the majority of tests the researchers stated that the manufacturer's instructions in performing the Platelia *Aspergillus* assay were followed. Most researchers tested the samples in batches. Generally, testing was done twice weekly (Penack *et al*., 2008) on specified days. Samples were preserved by freezing. Samples were stored at either -20^{0}C (Maertens *et al*., 2004; Sulahian et al., 1996) or -70/-80^{0}C (Marr *et al.,* 2004; Bretagne *et al,* 1997); 71 % of the samples were stored at the latter temperature. The details described in the brochure are appropriate and no changes are recommended to sample collection and storage.

3.3.5 The effect of heat treatment of samples

Heat treatment of sample decreases the GM index. In a study by Dale *et al*., 2005, cross-reactivity with *Cryptococcus neoformans* using the Platelia *Aspergillus* EIA was evaluated and the results show that heat treated samples resulted in lower indices than those that were untreated. Table 15 is a comparison of purified Cryptococcal antigen treated with and without heat then tested with the Platelia *Aspergillus* EIA. The indices of the heat treated sample are much lower than the untreated sample. The reaction with heat labile material might be significant enough to cause the result of a sample to be called negative when it is in fact positive.

Table15: Index of reactivity of heated and unheated purified components of *Cryptococcus neoformans* in Platelia *Aspergillus* assay.

Concn of carbohydrate	Index of reactivity					
	GXM serotype A		GXM serotype D		GalXM	
	No heat	Heat	No heat	Heat	No heat	Heat
10 μg/ml	0.14	0.07	0.13	0.06	>5	>5
1 μg/ml	0.14	0.05	0.11	0.07	>5	3.4
100 ng/ml	0.09	0.05	0.12	0.03	1.25	0.39
10 ng/ml	0.11	0.04	0.19	0.04	0.33	0.08
1 ng/ml	0.12	0.05	0.19	0.07	0.13	0.06

[a] "No heat" and "heat" indicate that there was not and was heat treatment prior to the enzyme immunoassay, respectively.

3.3.6 Time of collection of 2 consecutive samples

As discussed in section 3.3.1, it is important to characterize a positive result based on testing of 2 samples. The time difference in collection between the two consecutive samples has not been clearly defined. In a study by Stynen *et al*., 1992, the MAB EB-A2 reacted most strongly with non-germinating and young conidia. This is an indication that the time difference should not be too great. Two to three days would be adequate.

3.3.7 Time interval between infection and positivity

It would be difficult to determine the time of *Aspergillus* infection of a patient, since infection could occur prior to transplant or diagnosis of the underlying disease process. Data were not presented that could assist in an accurate estimation of the time interval between infection and the appearance of a positive GM EIA test. GM tended to increase

at the onset of fever; a few studies reported the time interval between a febrile attack and the appearance of a positive GM test. Busca *et al*., 2006, showed that the time interval between a positive Platelia *Aspergillus* EIA and a positive CT scan ranged from -12 to + 9 days. This study also showed that the median time of CT abnormality was three days after the febrile episode.

3.3.8 Galactomannan levels in survivors versus deceased

Penack *et al*., 2008, in the study of neutropenic hematologic ally malignant patients, showed that there was generally no correlation in GM concentrations between the probable and proven stages of IA in different patients. However, within a single patient the GM indices correlated with the clinical course of IA. Further, in the Penack study the medium indices for patients who recovered and who died were 1.7 and 9.5, respectively. This study used 76 patients who were febrile for three days and did not improve with the treatment of broad spectrum antibiotics. Eighteen of those patients received positive GM results and were classified as either proven or probable IA. Three of the 58 negative patients subsequently developed IA. Another set of 55 patients was tested after six days of persistent fever and non responsiveness to broad spectrum antibiotics. Of these 21 patients were positive and were classified as proven or probable IA. Two of the 34 negative patients subsequently developed IA. Further, it was shown that all of the patients who died had GM indices > 2.5. Only 3/18 GM positive patients who survived had an index > 2.5. It was found that in most patients the serum GM level tended to rise at the onset of fever and that an increase in GM index tended to rise faster in the patients who died than in those who survived (Figure 12). The GM level of the recovered patients ranged from 0.6 to 3.9 whereas the level in those who died ranged from 2.9 to 22.8. As the patient responded positively to therapy the GM level decreased. Figure 13 shows the difference in the increase of GM related to patients who died as indicated by a steep incline compared to the more gradual increase in the patients who recovered. The study showed that in febrile patients with no response to broad spectrum antibiotics for ≥ 6 days the Platelia *Aspergillus* EIA was highly accurate in detecting GM. The additional time allowed the fungus more time to grow and therefore the concentration of GM in the blood was high.

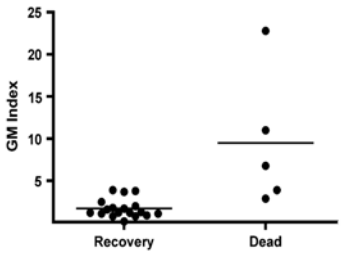

Figure 12. Comparison of Serum GM levels
of patients who recovered as compared to those who died

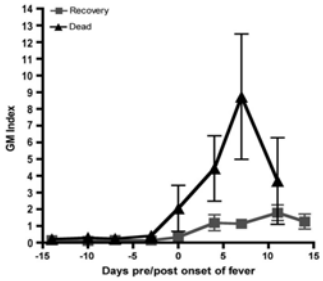

Figure 13. The GM concentrations increase in
Patients who died ▲ vs who recovered ■

Additionally, it was shown that the GM levels appeared earlier in bronchoalveolar lavage (BAL) fluid as compared to serum (for details see Appendix – II and Microbiology review by Dr Shurland for bronchoalveolar lavage).

Results of the study by Maertens *et al*., 2001, support the results by Penack *et al*., 2008. Figure 14 presents a graphic representation of the findings of Maertens with regard to the GM levels of patients who died compared to those who survived. Further, Maertens' study suggests that the patients whose GM levels were intermediate survived longer than those who died but they too subsequently died.

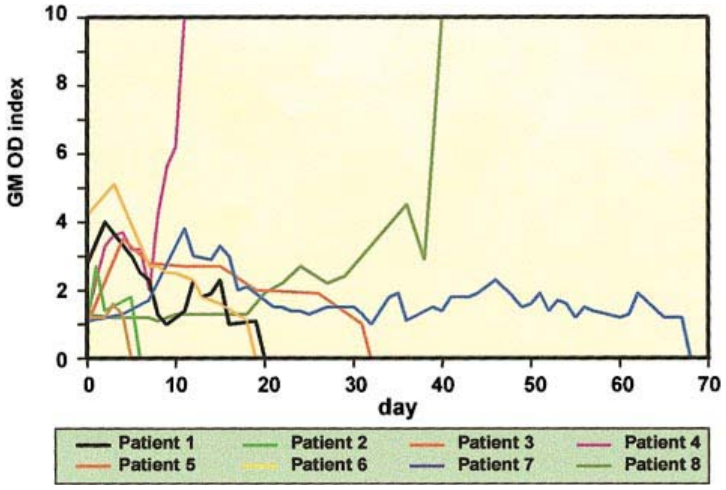

Figure 14: Quantification of GM ELISA results. Time course of antigenemia in 8 selected patients. Six patients, including 4 survivors (give patients no?), cleared GM. Patients nos. 4 and 8 represent a larger group of patients with rising antigen titers; they all died of or with IA.

3.3.9 Pre-disposing conditions

There was a wide range of predisposing conditions for IA (Table 16) reported in the clinical studies reviewed. Decreased leukocyte number and function are the most common risk factors for IA. The predominant predisposing condition and the focus of this review is hematological malignancies.

Table 16. List of Predisposing hematological conditions

Hematologic malignancies
Acute myeloblastic leukemia
Non-Hodgkins Lymphoma
Acute lymphoblastic leukemia
Chronic myelogenous leukemia
Multiple myeloma
Chronic lymphocytic leukemia
Aplastic anemia
Myelodysplasic syndrome
Allogenic hematopoietic stem cell transplantation
Autologous hematopoietic stem cell transplantation
Bone marrow transplant recipients

The country in which the patients resided and the type of assays performed are important in determining whether the results are relevant to the U.S. population. Two of the characteristics in determining relevance of *Aspergillus* testing are the climate of the countries and the health practices of the population. Most of the studies were done in developed European countries with climatic changes similar to those in the U.S.A. The standard of health care of these countries does not differ significantly from that of the USA; the European results could be relevant to the U.S. populations.

3.3.10 Cross-reactivity

The Platelia *Aspergillus* EIA shows a significant amount of cross-reactivity with fungal species, chemotherapeutic agents, antibiotic and foods. The known cross-reacting substances were avoided where possible and if unavoidable were taken into consideration in the evaluation of the test results. The exclusion of cross-reacting agents positively affected the specificity of the results.

Cross reactivity with pathogens
The use of ultrasound technology comprising colloidal gold labeled anti-rat immunoglobulin, mycelia from *A. fumigatus* and EB-A2 indicated that labeled substances within the cell were not specific to a particular fungal structure.

The extent of the cross-reactivity varied both with the species and with strains. In a study by Cummings *et al*., 2007, a small degree of cross-reactivity between the mold-like fungi was reported. In other studies cross-reactivity from other fungi sometimes yielded higher indices than *Aspergillus spp.* A study by Cummings *et al*., 2007, showed that the cell wall of *Blastomyces spp*. contains varying amounts of galactose and mannose. Also, the cell wall of *Penicillium spp.* is comprised of glucose, galactose and mannose. There was not as much cross-reactivity with yeast cells although they contain both of the two major monosaccharides.

The difference in cross-reactivity within strains and species of fungi might be influenced by the conditions under which the fungi are grown both *in vitro* and i*n vivo.* Growth media might influence the composition and or relative proportion of the components of the cell wall. In some studies the fungal extracts were diluted but since the initial concentration of the fungal exo-antigen was not stated, the actual working concentration of antigen in each dilution was unknown. Generally, GM was detected in the lower dilutions but tended to taper off in higher dilutions. These results suggest that the detection of GM in fungi is concentration dependent.

Like other fungi, the structure of *Cryptococcus spp*. contains both galactose and mannose. The structure of *Cryptococcus neoformans* GM is similar to the GM of *Aspergillus spp*. Figure 15 shows the structure of the galactomannan contained in both *Cryptococcus spp.* and *Aspergillus spp.*

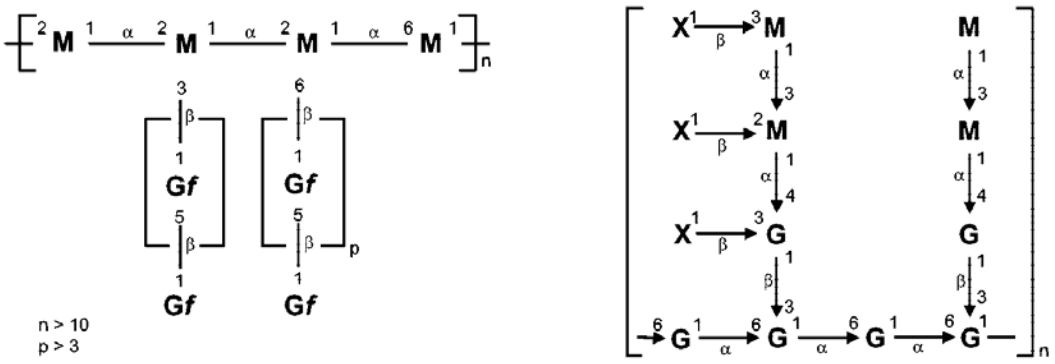

Figure 15. Structure of Galactomannan (L) *Aspergillus fumigatus* and ® *Cryptococcus neoformans*

Aspergillus fumigatus GM comprises a linear core of α-1,2- and α-1,6-linked mannan residues. The side chains which are used in the *Aspergillus* EIA consist of α -1,5 galactofuranosyl units linked to the C-3 and C-6 positions of the α-1,2-linked mannose units. The repeating unit of the *Cryptococcus neoformans* galactoxylomannan consists of a backbone of α-1,6-linked galactose units, with side chains composed of galactose, mannose, xylose, and O-acetyl residues branched on alternate galactose residues. The MAb EB A2 recognizes the β-(1→5)-linked galactofuranoside side chain of *Aspergillus* GM. The similarity in structure of the GM molecules of *Cryptococcus neoformans* and *Aspergillus fumigatus* provides epitopes that are also similar thus the monoclonal antibody EB-A2 also recognizes and binds to the epitopes of species in both genera. In fact the binding sites are so similar that when the sample is diluted the values of both tests are similarly reduced.

The cross-reactivity of other fungal species that cause concern in the Platelia *Aspergillus* assay is repeated in the case of *G. capitatum.* The structure of the GM of *Geotrichum spp*. is similar to that of *Aspergillus spp*. (Figure 16).

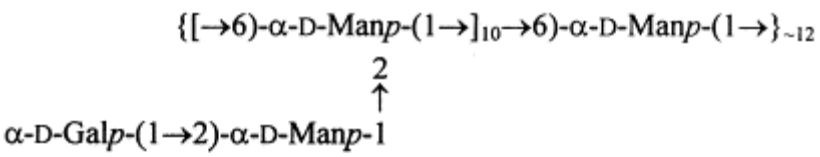

Figure 16: The chemical structure of Geotrichum spp.

One of the greatest causes of cross – reactivity is *Penicillium spp.* The cross-reactivity with *Penicillium spp.* had been identified from the initial experiments of Stynen *et al*., 1992. As with some strains of *Cryptococcus species* the indices of *Penicillium species* in the Platelia *Aspergillus* EIA rises and falls directly with that of *Aspergillus spp*. This problem with *Penicillium spp.* poses the greatest challenge to the use of galactomannan as a biomarker for IA because GM could also be a biomarker for penicilliosis. One author suggested that the high index obtained from *Penicillium marneffei* in the Platelia *Aspergillus* assay might be used to detect the presence of this fungus in HIV patients. It is important that whatever criteria are used for positivity ensures that they result in a high PPV and NPV for the test. A list of non-*Aspergillus* fungi causing false positive results at the GM index of ≥ 0.5 is presented in Table 17.

Table 17: List of non *Aspergillus* /*Neosartorya pseudofischeri* *fungi with GM indices ≥ 0.5 in Platelia *Aspergillus* EIA

Fungus	Site of isolation	GM index	Microbiological confirmation	Study
Cryptococcus neoformans	BAL, Serum CSF,, sputum, urine, Reference strains (NIH, ATCC)	3.5 - >5.0	Yes	Giacchino *et a*l., 2006 ; Dalle *et al*. 2005 ; Huang *et al*., 2007
Cryptococcus laurenti	Toe nail	> 5	Yes	Dalle *et al*. 2005,
Geotrichum capitatim	Serum Respiratory tract , Urine	1.2 - >5	Yes	Giacchino *et al*., 2006
Penicillium marneffei	Serum	1.6 - 20	Yes	Huang *et al*., 2007
Penincillium. chrysogenum	isolate	6.9	Yes	Cummings *et al*., 2007
Penicillium. lilacinus	isolate	1.3 - 7.5	Yes	Cummings *et al*., 2007
Penicillium digitatum	isolate	5.8	Yes	Swanink *et al*., 1996
Histoplasma capsulatum	Serum	0.5 – 9.8	Yes	Wheat *et al*., 2007,
B. dermatitidis (mould isolates)	isolate	0.6 – 2.8	Yes	Cummings *et al*., 2007
Nigrospora oryzae	serum	1.8 – 1.9	Yes	Cummings *et al*., 2007
Trichothecium roseum	isolate	1.3 – 7.5	Yes	Cummings *et al*., 2007
Paecilomyces variotii	isolate	5.8	Yes	Swanink *et al*., 1996
Alternaria species	Isolate	2.1	Yes	Swanink *et al*., 1996
Candida albicans	Serum	1.0 – 5.8	yes	Swanink *et al*., 1996

(1) *Cryptococcus* spp, *Penicillium* spp. , *Alternaria*, *Paecilomyces, Geotrichum* spp. *Histoplasma* spp identified in the manufacturer's brochure (2) *Neosartorya pseudofischeri* is the sexual phase of *Aspergillus fumigatus* therefore not included in this list

In a study by Wheat *et al*. (2007), forty eight serum samples from patients with proven histoplasmosis and 12 control samples negative for histoplasmosis were tested by the Platelia™ *Aspergillus* EIA (BioRad Laboratories). The reference standard supplied by the manufacturer was 1.0 ng/mL. The cut-off limit for a positive test was the index ≥ 0.5 and was based on the result of a single sample. Of the 48 samples positive for *Histoplasma capsulatum* 50% were also positive by the Platelia™ *Aspergillus* assay. All of the negative samples were negative for *Aspergillus*. Figure 17 is a comparison of the antigen levels between the *Histoplasma* and *Aspergillus* EIA. It can be noted the samples in which the level of *Histoplasma* antigen were low as measured by the *Histoplasma* EIA were also low in the *Aspergillus* EIA. The data indicate that the positive results for *Aspergillus* were obtained when the results of the sample tested by the *Histoplasma* EIA were high e.g. 70 % of the GM positives results occurred when the *Histoplasma* EIA results were ≥40 units (p-0.043) by chi-squared test. Conversely, 27 serum samples that were positive for *Aspergillus* GM by Platelia™ were negative for *Histoplasm*a antigen by EIA. The GM indices of these samples ranged from 0.54 to 9.08. The study shows that cross-reactivity with *Histoplasma capsulatum* will occur when using the Platelia *Aspergillus* assay.

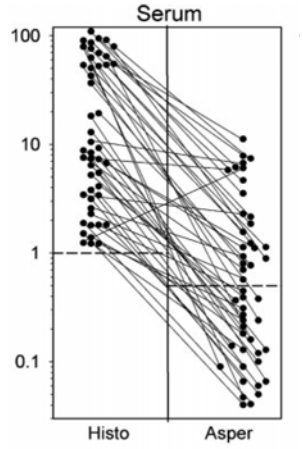

Figure 17: Comparison of detectable Antigen levels in *Histoplasma* and *Aspergillus in the same serum sample*

The sensitivity of the Platelia™ *Aspergillus* EIA in this study was 45%, the specificity was 100%, the positive predictive value (PPV) 100%, and the negative predictive value (NPV) 60%.

Candida albicans, a yeast that is a major cause of fungal infections, has a lower avidity with EB-2 by 10^2 and cross-reacts very little in the Platelia™ *Aspergillus* EIA. *Cryptococcus neoformans,* another yeast, poses a problem of cross-reactivity in the Platelia *Aspergillus* EIA. When the test was first designed the avidity of *Cryptococcus neoformans* was as low as that of *C. albicans* but more recently there have been problems with *Cryptococcus neoformans* cross-reactivity both in serum samples and with cultures of isolates. Cross-reactivity with *C. neoformans* from analysis of studies cited above appears to be concentration dependent. In the U.S.A., *Histoplasma capsulatum* causes disseminated and pulmonary disease only in the Eastern half of the country (Ohio and Mississippi river valley) and could therefore be misinterpreted primarily in those parts of the country. Infections due to *Penicillium marneffei are*

usually confined to Southeast Asia. However, it should be kept in mind that cases infected with these fungi could travel from the endemic to other areas where the fungi are less prevalent (for details see review by Dr. Hala Shamsuddin).

Table 18 lists bacteria that gave false positive results. However, the symptoms of bacteremia are unlikely to be mistaken for those of IA. It is felt that the concentration of bacterial species in the blood must be very high to cause cross-reactivity in the Platelia *Aspergillus* EIA.

Table 18 List of bacteria with GM indices ≥ 0.5 in Platelia *Aspergillus* EIA

Bacteria	**Site of isolation**	**GM index**	**Microbiological confirmation**	**Study**
Staphylococcus epidermidis	serum	0.6 – 2.5	yes	Swanink *et al*., 1996
Enterococcus faecalis	serum	1.8 – 2.4	yes	Swanink *et a*l., 1996
Corynebacterium jeikeium	serum	0.8 – 1.8	yes	Swanink *et al*., 1996
Corynebacterium jeikeium	serum	1.1 – 3.7	yes	Swanink *et al*., 1996
Escherichia coli	serum	1.3 – 1.8	yes	Swanink *et a*l., 1996

Bacteria unlikely to be misdiagnosed for aspergillosis no evidence of clinical, radiological or microbiological evidence

Table 19 shows the results obtained by retesting of sequential samples collected approximately 7 days before and 7 days after a positive serum sample was obtained (Cummings *et al*., 2007). It should be noted that except for one patient with *C. albicans* infection the samples were obtained from patients with bacteremia. The sensitivity for the fungal cultures GM detection was 100% and the specificity was 88%. The positive predictive index was 57% and the negative predictive index was 100%. For the recent blood culture (1995-1996) isolates which were predominantly bacteria gave a sensitivity of 0, specificity of 80%, PPV of 0% and the NPV 94%.

Table 19: Results of the ELISA reactivity using sequential serum samples from eight patients with positive ELISA results during bacteremia or fungemia

Patient no.	Blood culture result	*Aspergillus* infection[a]	ELISA reactivity with the positive serum sample[b]	ELISA reactivity on retesting	ELISA reactivities of sequential serum samples collected between 7 days before and 7 days after the positive sample was obtained[c]
1	*S. epidermidis*	Unlikely	2.5	2.1	0.7, 0.6, **2.5**, 1.0, 1.4
2	*S. epidermidis*	Unlikely	2.9	2.3	0.7, 2.9, **2.9**, 1.9, 0.5, 0.5
3	*E. faecalis*	Possible	2.4	1.6	2.2, 2.5, **2.4**, 1.8, 2.5
4	*E. faecalis*	Unlikely	2.2	2.2	0.8, 0.8, **2.2**
5	*C. jeikeium*	Unlikely	1.8 (0.9)[d]	2.1 (0.8)	0.2, 0.2, **1.8**, 0.8, 1.2
6	*P. aeruginosa*	Unlikely	3.7	3.9	1.1, **3.7**, 2.0
7	*E. coli*	Unlikely	1.8	1.5	0.4, **1.8**, 0.4, 1.3
8	*C. albicans*	Proven	3.3	3.5	0.4, 1.0, **3.3**, 5.8

[a] Unlikely infection; no clinical, radiological or microbiological evidence of invasive aspergillosis; possible infection, clinical and radiological evidence of invasive aspergillosis, but negative culture results; proven infection, histopathological evidence of tissue invasion by hyphae and tissue culture yielding *Aspergillus* species.
[b] The values represent a ratio calculated by dividing the optical density of the serum sample by that of the control serum spiked with 1 ng of galactomannan per ml Ratios greater than 1.5 were considered to indicate a positive result.
[c] Boldface values represent the reactivities of the serum samples that were obtained during the episode of bacteremia or fungemia.
[d] Values in parentheses indicate the reactivities of a second serum sample that was obtained on the same day.

Therapeutic agents causing cross-reactivity.

Plasmalyte:

Surmont and Stockman, 2007 showed that sodium gluconate (plasmalyte solution) gave a false positive reaction with the Platelia *Aspergillus* EIA test. The patient, a 68 year old female previously diagnosed with breast cancer was infused with plasmalyte solution. When serum from this patient was tested because of a possible lung infiltrate, the test resulted in an index of 1.85 in an assay using a cut-off index of 0.7. When the intravenous plasmalyte solution was discontinued the serum samples became negative.

In order to identify any interfering factor causing a false-positive galactomannan result, the authors tested samples of plasmalyte. A sample from the plasmalyte lot that was used to infuse patients resulted in an index of 3.85. To obtain confirmation of the identity of the interfering substance, single serum samples of five cardiac surgery patients in whom there were no indications of fungal infection but who had received plasmalyte for one day after surgery, were tested. A control group of four patients who did not receive plasmalyte were also tested. The GM tests for the patients to whom plasmalyte was administered resulted in indices of 0.82, 0.94, 0.70, 0.79, and 1.11; all samples were positive under test evaluation criteria. The sera of four control patients who did not receive the plasmalyte resulted in indices ranging between 0.05 and 0.46. All of the tests were performed in the same run as the samples from patients on plasmalyte.

Cross reactivity with antimicrobial agents

Olivier *et al*., 2004 showed cross reactivity with **piperacillin-tazobactam**, by Platelia *Aspergillus* EIA. Piperacillin-tazobactam treatment was significantly associated with the occurrence of false-positive results. Additionally, it was shown that the indices of false positive results increased with the duration of treatment with this antibiotic. For example, an index was 0.92 after one to three days compared to 21.2 if the treatment was administered for 13 days. Piperacillin-tazobactam is a combination of an ureidopenicillin and a β-lactamase inhibitor. Tazobactam is a penicillanic acid sulfon derivative.

Lai *et al*., 2007 showed that nine of 189 patients with hematological disorder tested GM positive but were not thought to have a fungal infection; four of the nine patients were being treated with piperacillin-tazobactam, one with amoxicillin-clavulanate and two were receiving hemodialysis.

Mattei *et al*., 2004, showed that patients treated with **amoxicillin**, **amoxicillin-clavulanate** and piperacillin-tazobactam were likely to give false positive GM results. The results from this study and the study by Fortun *et al*., 2009 showed the effect of **ampicillin** on the Platelia *Aspergillus* EIA test results indicating that patients on current treatment with β- lactamase antibiotics should not be tested by the Platelia *Aspergillus* assay. Figure 18 shows the GM level of a patient treated with amoxicillin from day 47 to day 58 and Figure 19 (Bart- Delabesse *et al.*, 2005) depict the kinetics of the results of patients treated with β-lactam antibiotics, amoxicillin (AMX), amoxicillin-clavulanate (AMC), ampicillin (AMP) and piperacillin-tazobactam (TZP).Three

patterns emerged: the index increased from >2 at the start of treatment and declined 24 to 48 and 48 to 120 hours after treatment was discontinued [Figure 19(a)]; GM indices of >0.5 and ≤ 1.5 throughout the antibiotic therapy but became negative 24 - 48 hours after the discontinuation of treatment [Figure 19(b)]. Variable GM levels coincided with a change from intra-venous to oral amoxicillin – clavulanate, the accumulation of ampicillin infusions or the use of many batches of piperacillin-tazobactam. Examples of the levels are depicted in Figure 19(c).

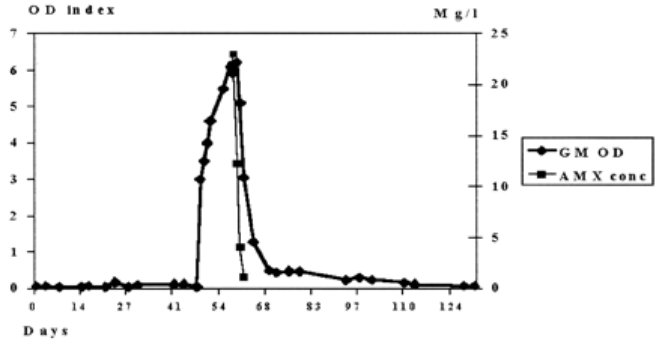

Figure 18: Correlation between plasmatic amoxicillin concentration (AMX) and GM OD treatment began on Day 47 and was discontinued on Day58

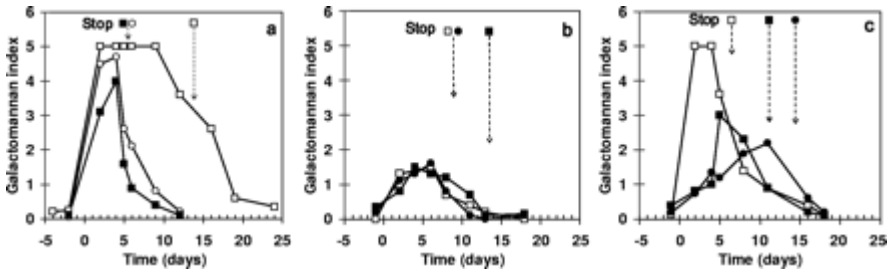

Figure 19. Representative kinetic patterns of GM in serum of patients receiving AMC (□), AMX (●), AMP (○), or TZP (■) therapy. Broken arrows indicate the time of discontinuation of antibiotic treatment. (a) High GM levels as exhibited in most patients treated with AMC, AMX, or TZP. (b) Low GM levels exhibited in a few patients treated with AMC, AMP, or TZP. (c) Both high and low GM levels exhibited in three patients as a result of a switch from infusion to oral AMC, variable dosage of AMX, and the use of different batches of TZP.

Cross reactivity with foods

Study by Gangneux *et al.*, 2003, in a short communication, reported cross reactivity with non-human produced milk using the Platelia *Aspergillus* EIA. By another assay, Pastorex *Aspergillus* latex agglutination tests, Ansorg *et al*., 1997 reported false positive GM results to be caused by the uptake of soluble GM from the environment. Among the objects tested from the environment were a series of foods. GM antigen was detected in 15 of 19 samples from meals cooked in a hospital that include five of six samples of canned vegetables from the supermarket and six samples of pasta and rice from health shops. GM was also detected in the feces of four BMT patients. The same study lists the foods in which GM was found at significant concentrations were pasta, rice, peas, carrots, mushroom and spinach. These results suggest that diet should be considered in the interpretation of the GM test results.

Cross reactivity due to immunologic reaction

Kwak *et al*., 2004, in liver transplant recipients demonstrated that patients with autoimmune liver disease were more likely to have false-positive tests than other liver transplant patients. A false-positive test, due likely to auto reactive antibodies or paraproteins, has been reported in other conditions associated with autoimmune phenomenon.

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Appendix - I

(Cross-reactivity)

Page

A. Cross Reactivity with Other Pathogens (Fungi and Bacteria):

B. **Cross Reactivity with Plasmalyte:**

C. **Cross Reactivity with Antimicrobial Agents:**

A. Cross-reactivity with other Pathogens (fungal and bacteria):

(1) Stynen *et al.* 1995: Cross reactivity of EB-A2 with a number of fungal species was reported (Table I-1. The avidities of EB-A2 with extract preparations from different fungi are listed in Table 1-1. The avidities between EB-A2 and *Aspergillus fumigatus* and *Penicillium digitatum* and *Trichophyton rubrum* were similar.

Table I-1: Avidities of EB-A1* and EB-A2 with the extracts of various fungi determined by ELISA

Fungus	Avidity constant (1/M) for MAb:	
	EB-A1	EB-A2
Aspergillus fumigatus	2×10^9	5×10^9
Penicillium digitatum	3×10^9	5×10^9
Trichophyton rubrum	1×10^9	5×10^9
Trichophyton interdigitalis	4×10^8	2×10^9
Botrytis tulipae	1×10^9	4×10^9
Wallemia sebi	5×10^9	3×10^9
Cladosporium cladosporioides	8×10^8	2×10^8
Fusarium solani	$<10^7$[a]	$<10^7$
Trichoderma viride	$<10^7$	$<10^7$
Candida albicans[b]	$<10^7$	$<10^7$
Saccharomyces cerevisiae[b]	$<10^7$	$<10^7$
Cryptococcus neoformans[c]	$<10^7$	$<10^7$

[a] $<10^7$, the avidity constant was too low to be calculated.
[b] Purified mannan.
[c] Purified glucuronoxylomannan.

***EB-A1 represents another clone of monoclonal antibody originally screened by** Stynen *et al.*

(2) Swanink *et al.* 1997: The study was conducted in the Netherlands for the purpose of testing the cross-reactivity with bacterial and fungal species using the Platelia™ *Aspergillus* EIA. Twenty-nine fungal strains isolated from clinical samples, and bacterial isolates from 40 blood cultures of febrile neutropenic patients were used in the study. Each fungal specimen had previously been stored at -80^{0}C but for this study they were sub-cultured on to sabouraud's dextrose agar and incubated at 30^{0}C for 48 hours. One loopful of the biomass from the culture was inoculated into Sabouraud's broth and re-incubated at 30^{0}C on a shaker rotating at 20 rpm for 48 hours for fast growing yeasts, or 96 hours for slower growing fungi. After incubation the broths were inspected for growth and then centrifuged at 20,000 x g for 5 minutes. The supernatants were filtered through a 0.45µm pore sized filter (Millipore). Ten-fold dilutions were made from the filtrate ranging from 1:1,000 to 1:1,000,000 in distilled water. The bacterial isolates were plated on sheep blood agar and each isolate was diluted to the turbidity equivalent to a 0.5 McFarland standard. The samples were tested using the Platelia™ *Aspergillus* EIA. The cut-off limit for this test was an index of 1.5 and the standard was 1 ng/mL.

For the purpose of this test the reaction with *A. fumigatus* was designated 100% (Table I.2). At the dilution of 1:1,000 all *Aspergillus spp* showed strong reactivity ranging from 100% to 122%. *Penicillium chrysogenum* showed the strongest reaction of 128%. Strong positive reactions were also obtained from *Penicillium digitatum* and *Paecilomyces variotii* (106%). All other fungi had GM concentrations below the index of 1.0. The one *C. albicans* species tested was positive. Of the 27 gram positive bacteria

only four gave positive results and three of the 12 gram negative bacteria gave positive results (Table I.3

Table I-2: reactivity of fungal cultures to the sandwich ELISA tested by GM Platelia ELISA

Fungus	Concn by ELISA (ng/ml)	Reactivity compared with that of *Aspergillus fumigatus*[a]
Aspergillus fumigatus	5.4	100
Aspergillus flavus	5.8	107
Aspergillus niger	6.4	119
Aspergillus terreus	6.6	122
Paecilomyces variotii	5.7	106
Penicillium chrysogenum	6.9	128
Penicillium digitatum	5.8	107
Alternaria species	2.1	39
Rhodotorula rubra	0.7	13
Fusarium solani	0.6	11
Fusarium oxysporum	0.3	6
Trichophyton mentagrophytes	0.4	7
Trichophyton rubrum	0.5	9
Rhizopus oryzae	0.4	7
Absidia corymbifera	0.4	7
Scopulariopsis brevicaulis	0.4	7
Exophilia species	0.4	7
Cladosporium species	0.5	9
Pseudallescheria boydii	0.4	7
Trichoderma viride	0.4	7
Acremonium species	0.4	7
Candida albicans	0.3	6
Candida glabrata	0.3	6
Candida krusei	0.3	6
Candida parapsilosis	0.3	6
Candida tropicalis	0.3	6
Geotrichum candidum	0.4	7
Saccharomyces cerevisiae	0.4	7
Cryptococcus neoformans	0.3	6

[a] The reactivity for *Aspergillus fumigatus* is set at 100.

Table I.-3: Reactivity of recent isolates from blood cultures

Microorganism cultured from the blood	No. of serum samples positive/no. of samples tested
Gram-positive organisms	
Staphylococcus epidermidis	2/5
Staphylococcus aureus	0/1
Streptococcus sanguis	0/3
Streptococcus mitis	0/5
Streptococcus oralis	0/2
Streptococcus pneumoniae	0/1
Enterococcus faecalis	2/3
Micrococcus spp.	0/1
Corynebacterium jeikeium	1/6
Gram-negative organisms	
Pseudomonas aeruginosa	1/4
Pseudomonas cepacia	0/1
Escherichia coli	1/4
Enterobacter cloacae	0/2
Achromobacter xylosoxidans	0/1
Candida albicans	1/1

(3) Cummings *et al*., 2007: Cross-reactivity against a wide variety of fungi such as dematiaceous fungi, hyaline molds, thermally dimorphic fungi and some species of yeasts at the St. Jude Children's Research Hospital (Memphis, Tenessee, U.S.A). Fifty-three fungal isolates comprising of 11 species of yeasts, 33 species of molds and three species of dimorphic fungi were tested using the Platelia™ *Aspergillus* EIA (BioRad Laboratories). Two strains each from the thermally dimorphic species *Blastomyces dermatitidis*, *Sporothrix schenkii*, and *Histoplasma capsulatum* were tested in both the yeast and mold phases. The extraction treatment of the isolates deviated from the usual serum samples and therefore the manufacturer's recommendations. The isolates were sub-cultured from sterile distilled water to sabouraud's glucose agar (SGA) plates and incubated at 30^0C for 48 hours. Growth from the first plate was sub-cultured to another SGA plate and re-incubated under the previous conditions. One loopful of the growth was transferred to sabouraud's broth and incubated at 30^0C. The yeasts were incubated for 48 hours whereas the molds were incubated for 96 hours. After incubation, the broth cultures were centrifuged at 2000 x g for 5 minutes and filtered through 0.45µm millipore filter. The supernatants were diluted to 10^3, 10^4, 10^5 in normal saline. After extraction, testing was conducted according to the manufacturer's instructions. An isolate was classified as positive based on testing of duplicate samples and a cut-off limit ≥ 0.5. Table I- 4 shows the GM reactions to various dilutions of fungi.

Table 1- 4: Galactomannan reactions to various dilutions of fungal species.

	Testing results		
	Dilution		
	10^3	10^4	10^5
Aspergillus sp.			
Aspergillus flavus (+ control)	+	+	–
Aspergillus fumigatus	+	–	–
Aspergillus niger	+	+	–
Aspergillus terreus	+	+	–
Yeasts			
Candida albicans	–	–	–
Candida glabrata	–	–	–
Candida krusei	–	–	–
Candida lusitaniae	–	–	–
Candida parapsilosis	–	–	–
Candida tropicalis	–	–	–
Cryptococcus neoformans	–	–	–
Geotrichum candidum	–	–	–
Rhodotorula mucilaginosa	–	–	–
Saccharomyces cerevisiae	–	–	–
Trichosporon beigelii	–	–	–
Dimorphic molds			
B. dermatitidis (mould isolates 1–6)	+	–	–
B. dermatitidis (yeast isolates 1–6)	–	–	–
H. capsulatum (mould)	–	–	–
H. capsulatum (yeast)	–	–	–
S. schenckii (mold)	–	–	–
S. schenckii (yeast)	–	–	–

	Dilution		
	10^3	10^4	10^5
Dimorphic molds			
B. dermatitidis (mould isolates 1–6)	+	–	–
B. dermatitidis (yeast isolates 1–6)	–	–	–
H. capsulatum (mould)	–	–	–
H. capsulatum (yeast)	–	–	–
S. schenckii (mold)	–	–	–
S. schenckii (yeast)	–	–	–
Dematiaceous molds			
Acremonium spp.	–	–	–
Alternaria spp.	–	–	–
Chaetomium globosum	–	–	–
Cladosporium carrionii	–	–	–
Cladosporium spp.	–	–	–
Curvularia spp.	–	–	–
Epicoccum nigrum	–	–	–
W. dermatitidis	–	–	–
Exophiala jeanselmei	–	–	–
Exserohilum rostratum	–	–	–
Fonsecaea pedrosoi	–	–	–
N. oryzae	+	–	–
Phialophora verrucosa	–	–	–
Phaeoannellomyces werneckii	–	–	–
Phoma herbarum	–	–	–
Pseudallescheria boydii	–	–	–
Hyaline molds			
Beauveria bassiana	–	–	–
Cunninghamella bertholletiae	–	–	–
Fusarium moniliforme	–	–	–
F. oxysporum	–	–	–
F. solani	–	–	–
Microsporum nanum	–	–	–
Mucor plumbeus	–	–	–
P. lilacinus	+	–	–
P. chrysogenum	+	+	–
Rhizopus spp.	–	–	–
Syncephalastrum ramosum	–	–	–
Trichophyton tonsurans	–	–	–
T. roseum	+	–	–

Of the 50 fungal species tested (1 strain each), including the two phases of the three dimorphic fungi, none were positive at the 10^5 dilution. Only 4 species were positive at the 10^4 dilution three of which were *Aspergillus spp*. *Aspergillus fumigatus* was negative and *Penicillium chrysogeniun* was positive at that concentration. At the lowest concentration 10^3 nine strains were positive. These included four *Aspergillus spp*, one B*lastomyces dermatidis* (mold phase), three hyaline molds *Phycomyces lilacinu*, *Penicillium chrysogenum* and *Trichothecium roseum* and one dematiaceous mold *Nigrospora oryzae.* The indices for *Aspergillus spp.* ranged from 0.81 – 7.54, *Blastomyces dermatitis* 0.6 – 2.8, *Nigrospora oryzer* 1.79 – 1.86, and the three hyaline molds *Trichothecium roseum, Penicillium chrysogenum, Phylomyces lilacinus* had index values of 1.30 -7.51.

All of the species that should have been GM positive were positive at 10^3 dilution, 75% of them were positive at 10^4 dilution, and none at 10^5 dilution. This included the positive control *Aspergillus flavus* which showed positivity at both of the two higher concentrations. On the other hand 11% of those that should have been negative at the highest concentration tested were positive and only 2% (1/46) of those that should have been negative were positive at the 10^4 dilution. Test reproducibility could not be determined because the relevant data of the repeat tests for each isolate were not available. However, the test protocol stated that for positivity at least two tests must be ≥ 0.5 and to be called negative both must be <0.5. Only to this extent could repeatability be assessed. The sensitivity and specificity of the Platelia test in this instance were 100% and 90% respectively. The PPV was 44% while the NPV was 100%.

(4) Wheat *et al.*, 2007: Forty eight serum samples from patients with proven histoplasmosis and 12 control samples negative for histoplasmosis were tested by the Platelia™ *Aspergillus* EIA (BioRad Laboratories). The reference standard supplied by the manufacturer was 1.0 ng/mL. The cut-off limit for a positive test was the index ≥ 0.5 and was based on a single sample result of ≥ 0.5. Of the 48 samples positive for *Histoplasma capsulatum,* 50% were also positive by the Platelia™ *Aspergillus* assay. All of the negative samples were negative for *Aspergillus*. Figure 1-1 is a comparison of the antigen levels between the *Histoplasma* and *Aspergillus* EIA. It can be noted the samples in which the level of *Histoplasma* antigen were low as measured by the *Histoplasma* EIA were also low in the *Aspergillus* EIA. The data indicate that the positive results for *Aspergillus* were obtained when the results of the sample tested by the *Histoplasma* EIA were high e.g. 70 % of the GM positives results occurred when the *Histoplasma* EIA results were ≥40 units (p-0.043) by chi-squared test. Conversely, 27 serum samples that were positive for *Aspergillus* GM by Platelia™ were negative for *Histoplasm*a antigen by EIA. The GM indices of these samples ranged from 0.54 to 9.08. The study shows that cross-reactivity with *Histoplasma capsulatum* will occur by the Platelia *Aspergillus* assay.

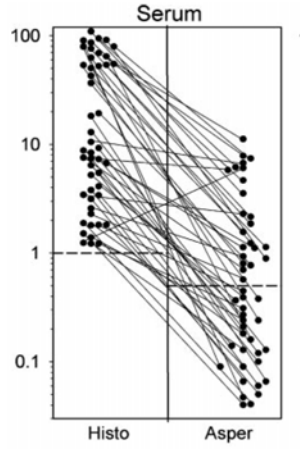


Figure 1-1: Comparison of detectable Antigen levels in *Histoplasma* and *Aspergillus in the same serum sample*

The sensitivity of the Platelia™ *Aspergillus* EIA in this study was 45%, the specificity was 100%, the positive predictive value (PPV) 100%, and the negative predictive value (NPV) 60%.

(5) Dalle *et al.*, 2005: Study was conducted at four medical institutions in France including the Institut Pasteur, the observations were made in patients with pulmonary cryptococcosis whose sera were positive by the Platelia™ *Aspergillus* EIA but who exhibited no signs of aspergillosis. The investigators hypothesized that epitopes of *Cryptococcus spp.* shed during cryptococcosis were cross-reacting with epitopes in *Aspergillus spp*. The authors presented a case study in a 43 year old male infected with H.I.V. who received treatment including amoxicillin-clavulanate because of unexplained fever to support the hypothesis. After day 6, blood cultures were positive

for yeasts which were subsequently identified as *Cryptococcus neoformans* (API32C, Biomurieux, France). The cryptococcal antigen test (CALAIS; Meridian Bioscience) was positive with a titer of 1: 20 on days 7 and 8. The GM indices by the Platelia *Aspergillus* assay (BioRad Laboratories, France) in serum samples collected on days 5, 7 and 9 were 4, 0.5 and 2.2, respectively. None of the cultures grew *Aspergillus spp*.; histopathological sections revealed the presence of only encapsulated yeast cells.

To further test the hypothesis, culture supernatants containing soluble antigens from 19 clinical and control strains of *Cryptococcus* isolates, 18 of which were *Cryptococcus neoformans,* were tested by the Platelia *Aspergillus* assay. Five samples each came from CSF and blood, and one each from a toe nail, urine, bronchiolar lavage and sputum specimens. There were also five control strains of serotypes A, B, C and D and one non-typable *Cryptococcus neoformans* strain. The standard sample was provided by the manufacturer and contained 1 ng/mL. Negative controls consisted of negative serum samples and distilled water. Cultures were plated on malt agar and incubated at 30^0C for five days. Five to 10 colonies of the growth were suspended in 1 mL of distilled water mixed for one minute and then centrifuged for five minutes at 10,000 x g. The supernatant was tested for both cryptococcal and *Aspergillus* antigens by the Platelia *Aspergillus* assay. Tests for *Aspergillus spp.* were performed in two batches. The sample from one batch was not heat treated but in the second batch the samples were both heat treated and untreated. Table I- 5 shows the results of the Platelia *Aspergillus* assays of two batches of cryptococcal antigen supernatants using both thermally treated and untreated specimens. The cut-off limit used was that recommended by the manufacturer's ≥0.5.

All of the samples showed high index of > 5.0 with the lowest index being 2.2. It is important to note that generally the heat treated samples resulted in lower indices. Table I- 6 shows a comparison of purified cryptococcal antigen treated with and without heat. For example, sample 2500 was diluted and the index decreased from 0.977 to 0.142 but so too did the cryptococcal antigen.

Table 1- 5: Index of activity of heat-treated and non heat-treated soluble antigens from suspensions of clinical and reference strains of *Cryptococcal spp.* in the Platelia *Aspergillus* assay.

Strain designation	Site of isolation	Identification	Index of reactivity in expt:		
			1 (no heat treatment)	2 (no heat treatment)	3 (heat treatment)
44	CSF	*C. neoformans*	4.8	>5	3.4
101	Blood culture	*C. neoformans*	4.7	>5	3.1
294	Blood culture	*C. neoformans*	>5	>5	>5
545	CSF	*C. neoformans*	3.8	>5	3.3
590	Urine	*C. neoformans* serotype A	>5	>5	>5
648	Blood culture	*C. neoformans* serotype A	4.1	>5	>5
681	CSF	*C. neoformans* serotype A	3.6	>5	>5
861	CSF	*C. neoformans*	4.8	>5	3.4
1451	CSF	*Cryptococcus sp.*	5	>5	3.4
1782	BAL	*C. neoformans* serotype D	>5	>5	>5
1829	Blood culture	*C. neoformans* serotype A	3.5	>5	>5
2323	Toe nail	*C. laurentii*	>5	>5	>5
2500[b]	Blood culture	*C. neoformans* serotype A	4.9	>5	3.5
2501[b]	Sputum	*C. neoformans* serotype A	4	>5	>5
NIH 68	Reference strain	*C. neoformans* serotype A	ND	3.1	2.6
NIH 112B	Reference strain	*C. neoformans* serotype B	ND	>5	>5
NIH 52D	Reference strain	*C. neoformans* serotype D	ND	2.8	2.2
CDC B.238	Reference strain	*C. neoformans* serotype C	ND	>5	>5
Cap 67	Reference strain	*C. neoformans* non typable	ND	>5	3.5

[a] CSF, cerebrospinal fluid; BAL, bronchoalveolar lavage; ND, not done. Experiments 2 and 3 were performed on the same antigenic extracts.
[b] Isolates 2500 and 2501 were obtained from the patient described in this paper.

Table I -6: Index of reactivity of heated and unheated purified components of the *Cryptococcal neoformans* capsule in Platelia *Aspergillus* assay.

Concn of carbohydrate	Index of reactivity					
	GXM serotype A		GXM serotype D		GalXM	
	No heat	Heat	No heat	Heat	No heat	Heat
10 μg/ml	0.14	0.07	0.13	0.06	>5	>5
1 μg/ml	0.14	0.05	0.11	0.07	>5	3.4
100 ng/ml	0.09	0.05	0.12	0.03	1.25	0.39
10 ng/ml	0.11	0.04	0.19	0.04	0.33	0.08
1 ng/ml	0.12	0.05	0.19	0.07	0.13	0.06

[a] "No heat" and "heat" indicate that there was not and was heat treatment prior to the enzyme immunoassay, respectively.

The study also reports cross-reactivity with *Cryptococcus neoformans* in a 43 year old male infected with H.I.V when tested by the Platelia™ *Aspergillus* EIA (BioRad). The patient had received anti-retroviral treatment, and treatment with amoxicillin-clavulanate because of an unexplained fever and fluconazole because of oral candidiasis, but the patient did not improve, In fact the *Aspergillus* GM results on days 5, 8, and 9 were 4.0 , 0.5 and 2.2 respectively. On day 6 a blood culture was taken and grew *Cryptococcus neoformans*. The cryptococcal antigen test, microscopy and culture of CSF were negative but the sputum grew *C. neoformans* and the biopsy showed encapsulated yeast consistent with *C. neoformans.* The patient died on day 14. The researchers found no satisfactory explanation for the positive Platelia *Aspergullus* assay but cross reactivity with *C. neoformans.*

(6) Giacchino *et al.* 2006: Cross reactivity with *Geotrichum capitatum* is documented based on 3 cases aged 7, 9 and 49 years of age with acute leukemia. Study was conducted in Rome. Patients were infected with disseminated *Geotrichum capitatum.* Although the patients did not show any signs and symptoms of aspergillosis, when serum samples obtained from these patients were tested by the Platelia *Aspergillus* assay (BioRad Laboratories) the results were positive for GM. *Geotrichum capitatum*

was isolated and identified from clinical samples of all three patients by the VITEC (Bio Merieux). At the time of sample collection the patients had not been treated with piperacillin-tazobactam or amoxicillin-clavulanate.

Because of the preceding cases the authors decided to investigate whether the antigens in *G. capitatum* and *Aspergillus spp.* cross-reacted. This follow-up study included 13 clinical isolates of *G. capitatum* comprising nine from blood, three respiratory and one from urine. There were four ATCC reference strains of *G. capitatum*, two isolates of *C. neoformans*, two isolates of *A. fumigatus,* one of *A niger,* and one ATCC reference strain each from *C. albicans* and *C. parapsilosis.* The cultures were plated on sabouraud's dextrose agar and incubated for five days at 30^0C. Five to ten colonies were suspended in 1 mL of distilled water, mixed and then centrifuged at 10,000 x g for 5 minutes. The supernatants were heat treated and tested according to the manufacturer's instruction on the Platelia *Aspergillus* EIA (BioRad Laboratories). The cut-off limit for positivity was ≥ 0.5. The index was calculated as directed by the manufacturer and is included in the description of the assay in section 1.

Of the 17 strains of *G capitatum* tested all resulted in an index >0.5 with a range from 1.2 to > 5.0. The two *C. neoformans* strains resulted in an index of 2.9 and 3.4, respectively. The three *Aspergillus spp* had indices > 5 whereas the indices of the two *Candida spp*. were < 0.5. Table I-7 shows the results of testing using the Platelia *Aspergillus* GM assay for 24 fungal strains.

Table I.- 7: Results of soluble antigen suspensions of clinical and reference strains of *Geotrichum capitatum* and other fungi in the Platelia Aspergillus GM assay.

Species and strain	Site of isolation[b]	Index of reactivity in the Platelia *Aspergillus* assay
A. fumigatus Rom	BAL	>5
A. fumigatus Mor	Sputum	>5
A. niger Tri	Ear	>5
C. albicans ATCC 90028	Reference strain	<0.5
C. parapsilosis ATCC 22019	Reference strain	<0.5
C. neoformans		
Sob	CSF	3.4
Imp	CSF	2.9
G. capitatum		
469	Urine	1.2
448	Blood	1.8
E202	Blood	2.2
PE2	Blood	1.9
BG2	Blood	1.8
Pisa	Respiratory tract	3.2
116A	Respiratory tract	1.9
434	Blood	1.2
114A	Blood	1.5
Monica	Respiratory tract	1.8
Palermo Dr	Blood	1.9
Palermo Ma	Blood	2.5
E299[a]	Blood	>5
ATCC 62963	Reference strain	2.3
ATCC 62964	Reference strain	1.9
ATCC 200927	Reference strain	2.7
ATCC 200925	Reference strain	1.7

[a] Isolate from patient 3.
[b] BAL, bronchoalveolar lavage fluid; CSF, cerebrospinal fluid.

(7) Huang *et al*., 2007: This was a prospective cohort study in Taiwan to compare the GM levels between *Penicillium marneffe*i and *Cryptococcus neoformans* in H.I.V. patients. The patients and controls were hospitalized between January, 2000 and December, 2006. Forty-eight patients were included in the study. Fifteen patients were infected with *P. marneffei*, 22 with *Cryptococcus neoformans* and 11 controls. The fungi

were identified by microscopy and culture of blood, CSF, lung aspirates, bone marrow, biopsy of lymph nodes, sputum culture, and the use of cryptococcal latex agglutination tests (Immuno mycologics). The Platelia *Aspergillus* test was performed on serum samples that had been stored at -80^{0}C after the patients were diagnosed with H.I.V. and an accompanying infection with an opportunistic fungus. Internal negative controls were obtained from HIV positive persons who showed no signs of opportunistic infections. Patients who had been treated with piperacillin-tazobactam and amoxicillin-clavulanate three days or less prior to the sample collection were excluded from the study. The Platelia *Aspergillus* EIA (BioRad Laboratories) was performed according to the manufacturer's instructions. Only one serum sample was tested for each person. The cut-off limits used were >0.5, >1.0 and >1.5.

Nine of the 10 patients with penicilliosis had GM indices > 0.5, eight of which were > 1.5. Two of the penicilliosis patients with fungemia had indices > 1.5 and four patients had indices < 0.5. Fourteen of the *Cryptococcus* infected patients had fungemia and of these 13 had indices < 0.5, one had an index > 1.0 and no patient in this category had an index > 1.5. Of the HIV-*Cryptococcus* infected patients without fungemia six out of a total of eight patients recorded indices < 0.5, three >0.5, two > 1.0 and one > 1.5. One of the negative control samples had an index >1.0. The time between the first positive GM test and the diagnosis of penicilliosis ranged from -7 to 0 days (average 0 days) and for *Cryptococcus* 0 to six days (average 1.5 days). Table 1- 8 shows the OD of GM at the various cut-off indices.

Table I.- 8: Serum GM OD and indices from HIV patients with penicilliosis, cryptococcus and controls

Patient group	GM OD index		No. (%) with a GM OD index of:			
	Range	Median	>1.5	>1.0	>0.5	<0.5
Penicilliosis						
With fungemia (n = 10)	0.401–>20	10.628[a]	8 (80.0)	8 (80.0)	9 (90.0)	1 (10.0)
Without fungemia (n = 5)	0.158–4.419	0.378[b]	2 (40.0)	2 (40.0)	2 (40.0)	3 (60.0)
All (n = 15)	0.158–>20	4.419[c]	10 (66.7)	10 (66.7)	11 (73.3)	4 (26.7)
Cryptococcosis						
With fungemia (n = 14)	0.112–1.168	0.231[a]	0 (0.0)	1 (7.1)	1 (7.1)	13 (92.9)
Without fungemia (n = 8)	0.115–3.849	0.263[b]	1 (12.5)	1 (12.5)	2 (25.0)	6 (75.0)
All (n = 22)	0.112–3.849	0.247[c]	1 (4.5)	2 (9.1)	3 (13.6)	19 (86.4)
Control (n = 11)	0.15–1.024	0.234	0 (0)	1 (9.1)	1 (9.1)	10 (90.9)

[a] $P < 0.001$ for comparison of GM OD indices for *Penicillium* fungemia and cryptococcal fungemia.
[b] $P = 0.464$ for comparison of GM OD indices for nonfungemic penicilliosis and nonfungemic cryptococcosis.
[c] $P < 0.001$ for comparison of GM OD indices for all cases of *Penicillium marneffei* infection versus all cases of cryptococcosis.

(8) Ja¨rv *et al,*. 2004: Cross-reactivity with *Neosartorya pseudofischeri* was evaluated in the Netherlands. *Neosartorya pseudofischeri* is the sexual stage (teleomorph) of the fungus *Aspergillus thermomutatus* (*A. fischerianus*). This is a case report of a 17 year old patient with stage IV Hodgkin's disease. The patient was discharged after eight days of chemotherapy for Hodgkin's disease. Five days later he returned with neutropenia. Growth resembling *Aspergillus spp.* was detected in a blood culture on Day 14. The growth in blood was sub-cultured on to malt extract and oatmeal agars and incubated at 25^{0}C. Abundant ascomata were produced and identified by scanning electron microscopy and sequencing of the internal transcribed spacer region. Comparison of the sequence in the GenBank showed that the sequence was 98% similar

to an isolate with GenBank accession number AF459729. The GenBank accession number AF459729 refers to a partial sequence of the 18S ribosomal RNA gene for *Neosartorya pseudofischeri.* The patient sera were tested for GM by the Platelia EIA (BioRad Laboratories, Marnes-la-Coquette, France). The test was carried out according to the manufacturer's instructions except for the cut-off index which the manufacturer, at that time, had recommended to be >1.5. The results of patient sample were 0.7, 1.63, 0.8 and 0.5 on days 6, 19, 20 and 33, respectively. The fungus was identified as *Neosartorya pseudofischeri*. After treatment with amphotericin B the patient's blood sample was found to be culture negative.

(9) Zhuang *et al*., 2008: Study was conducted at Mira Vista laboratory as described above and showed no cross-reactivity between the *Histoplasma* galactomannan and the glucuronoxylomannan of *Cryptococcus* when using both the *Histoplasma* EIA and Cryptococcal antigen latex agglutination system (CALAS) test (Meridian Bioscience), respectively. The *Histoplasma* assay is the same one used in the previous study to test for cross-reactivity between the GM of *Histoplasma spp.* and *Aspergillus spp.* The relevance of this study to the Platelia *Aspergillus* assay is that there appears to be cross reactivity between the *Histolpasma* GM and *Aspergillus* GM when using the Platelia *Aspergillus* assay. Perhaps the defect lies in the composition of the Platelia *Aspergillus* assay in the choice of MAb for the Platelia™ *Aspergillus* EIA. The results of the two studies show that the Platelia *Aspergillus* GM assay is not very specific for aspergillosis.

B. Plasmalyte:

(1) Surmont and Stockman, 2007: Sodium gluconate (plasmalyte solution) gave a false positive reaction with the Platelia *Aspergillus* EIA test. The patient, a 68 year old female previously diagnosed with breast cancer was infused with plasmalyte solution. When serum from this patient was tested because of a possible lung infiltrate the test resulted in an index of 1.85 in an assay using a cut-off index of 0.7. When the intravenous plasmalyte solution was discontinued the serum samples became negative.

In order to identify any interfering factor causing a false-positive galactomannan result, the authors tested samples of plasmalyte. A sample from the plasmalyte that was used to infuse patients resulted in an index of 3.85. To obtain confirmation of the identity of the interfering substance, single serum samples of five cardiac surgery patients in whom there were no indications of fungal infection but who had received plasmalyte for one day after surgery, were tested. A control group of four patients who did not receive plasmalyte was also tested. The GM tests for the patients to whom plasmalyte was administered resulted in indices of 0.82, 0.94, 0.70, 0.79, and 1.11, all positive under test evaluation criteria. The sera of four control patients who did not receive the plasmalyte resulted in indices ranging between 0.05 and 0.46. All of the tests were performed in the same run as the samples from patients on plasmalyte.

C. Cross-reactivity with antimicrobial drugs:

(1) Oliver *et al*., 2004: Sera from 26 patients, who had undergone HSCT and treated with piperacillin-tazobactam, were tested by Platelia™ *Aspergillus* EIA. **Piperacillin-tazobactam** treatment was significantly associated with the occurrence of false-positive results. Additionally, it was found that the indices of false positive results increased with the duration of this antibiotic treatment. For example, an index of 0.92 after one to three days compared to 21.2 if the treatment was administered for 13 days.

Piperacillin-tazobactam is a combination of an ureidopenicillin and a β-lactamase inhibitor. Tazobactam is a penicillanic acid sulfon derivative. Several investigators (Stynen *et al., 1995* Swanink *et al*., 1997, Huang *et al*., 2007 Cummings *et al*., 2007) have reported that the GM of *Penicillium species* is similar to that of *Aspergillus spp.* and this is responsible for the false positive results demonstrated in many studies.

(2) Fortun *et al*., 2009: In a prospective study, GM antigenemia was tested in samples from liver transplant patients. The results show that patients who received **ampicillin** and cefotaxime within the first 2-5 days after transplantation resulted in a positive GM index based on a cut-off of ≥ 0.5. However, the GM test was negative in other high risk patients who were allergic to β-lactam antibiotics and who had been previously colonized with microorganisms that were resistant to β-lactams received caspofungin. Aliquots from vials of ampicillin, piperacillin tazobactam, and cefotaxime and negative controls of 0.9% saline solution were tested in the GM assay. None of the negative controls or cefotaxime gave an index of ≥0.5; 67% of the ampicillin samples were positive and so were 50% of the piperacillin-tazobactam aliquots tested.

(3) Lai *et al*., 2007: Nine of 189 patients with hematological disorder tested GM positive but were not thought to have a fungal infection; 4 of the 9 patients were being treated with piperacillin-tazobactam, one with **amoxicillin-clavulanate** and two were receiving hemodialysis.

(4) Mattei *et al*., 2004: A 55 year old female treated with immunosuppressive drugs for myelodysplastic syndrome was admitted to the hospital in Italy because of pneumonia. The Platelia *Aspergillus* EIA (BioRad) tests done twice weekly were negative. The patient received empirical treatment with ceftazidime and amikacin, and later imipenem. She also received prophylaxis with itraconazole. Treatment with amoxicillin- clavulanate and amoxicillin were later begun because of a painful skin nodule on the leg. The treatment with **amoxicillin** began on from Day 47 and was discontinued on Day 58. The GM index suddenly increased from 0.06 to 4.01 two days later to 6.1 a week later. When amoxicillin-clavulanate was discontinued the GM index decreased to < 0.7 in 10 days. High-performance liquid chromatography assay analysis of serum samples resulted in concentrations of 23, 12.3, 4.1 and 1.1 mg/L of amoxicillin between February 13 and 16 and the sample collected on the same days gave GM indices of 5.9, 6.2, 5.1 and 3.2 respectively. Figure 1 - 2 shows the correlation between the blood concentration of amoxicillin and the optical density of the

corresponding serum samples with GM. The GM index rises rapidly between days 45 and 54 when the patient received amoxicillin.

In order to prove whether or not false positive results were obtained by amoxicillin-clavulanate samples were taken from three different batches of amoxicillin used at the same time to treat the patient, diluted and tested by Platelia *Aspergillus* assay The indices obtained were 6, 12.9 and 0.74 respectively. Samples from the 0.9% NaCl diluent were negative . Blood samples from healthy neutropenic patients were mixed with amoxicillin clavulanate from the same batches and were tested by PCR. The DNA probes hybridized with *A. fumigatus, A flavus* and *A versicolor.* The PCR results were negative for all three samples tested. Therefore, intravenous amoxicillin-clavulanate could possibly cause false positive results.

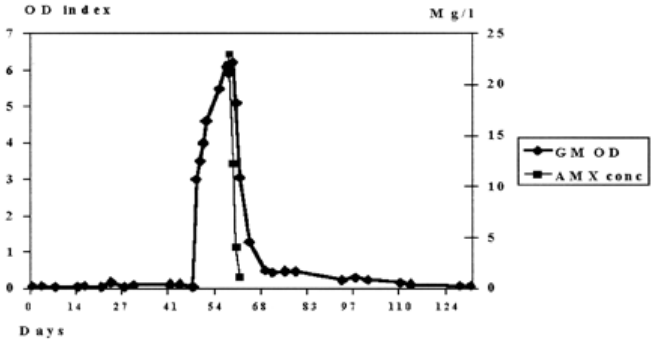


Figure I - 2: Correlation between plasmatic amoxicillin concentration (AMX) and GM OD treatment began on Day 47 and was discontinued on Day 58

Appendix - II

(Clinical Microbiology Studies)

A. Hematological malignancy and HSCT: **Page**

B. Liver Transplant

C. Lung Transplant

A. Hematological malignancy and HSCT:

(1) Maertens *et al*. (1999): This was one of the first prospective studies conducted to assess the effectiveness of the GM *Aspergillus* EIA (Sanofi Diagnostics Pasteur, France) in hematologic neutropenic (< 500 cells/μL) patients specifically bone marrow transplant patients. The patients with proven, probable and possible IA were enrolled and the proven category defined according to EORTC/MSG criteria. Additionally, the testing of one versus consecutive samples was compared for identifying positive results. The study comprised of 2,172 samples from 186 patients and 243 episodes. Of the 243 episodes, 27 were from proven IA, 6 probable, 77 possible and 133 no IA; sera from 121 episodes from the non-IA group were negative for IA (Table II-1). Of the 12 episodes that identified positive patients, eight positive episodes were identified utilizing one serum sample and four utilized multiple consecutive positive samples. The GM positivity was highest in patients with proven IA (61%) compared to probable (23%) and possible (3 %). The sensitivity and specificity of the assay with a cut-off index of ≥1.0 were 92.6% and 95.4% respectively. The Platelia test results were positive before (median 6 days, range -1 to -23 days) clinical suspicion of IA in 18 of 25 patients with proven IA.

Table II -1: Prospective evaluation of serum GM in patients at risk for IA

Parameter	Value for patients with:				Total
	Proven IA	Probable IA	Possible IA	No IA	
No. of episodes	27	6	77	133	243
No. of neutropenic episodes	24	6	74	120	224 (92%)
No. of patients	27	6	61	92	186
Underlying disorder (no. of episodes)					
Acute myelogenous leukemia	8	1	35	18	62 (25%)
Acute lymphocytic leukemia	2	1	6	7	16 (6.5%)
Chronic myelogenous leukemia			1	1	2 (0.8%)
Aplastic anemia	1		2	7	10 (4%)
Myelodysplastic syndrome		1	10	17	28 (11.5%)
Multiple myeloma	1	1	5	4	11 (4.5%)
Non-Hodgkin's lymphoma	9		6	3	18 (7.4%)
Autologous transplantation			1	54	55 (22.5%)
Chronic granulomatous disease			1		1 (0.4%)
Allogeneic transplantation (inpatient)	2	2	9	9	40 (16%)
Allogeneic transplantation (outpatient)	4		1	13	
No. of episodes of corticosteroids	15 (55%)	1 (16%)	20 (26%)	20 (15%)	56 (23%)
Neutropenia					
Mean duration (days)	28.8	16.5	21.2	12.4	17
Range (days)	0–180	0–38	0–60	0–75	
No. of episodes of antifungal therapy	22 (81%)	5 (83%)	69 (89%)	6 (4.5%)	102 (42%)
No. of samples (total)	276	66	644	1,186	2,172
Mean no. of samples/episode	10	11	8	9	9
No. of positive samples	168 (61%)	15 (23%)	21 (3.2%)	39 (3.2%)	243 (11%)

Autopsy was done in 71 cases of which 27 were proven and 44 possible IA. The results show high correlation between GM positive result based on a cut-off of 1.0 and correlation and autopsy findings (Table II -2).

Table II-2: ELISA results in 71 pathology-controlled studies

ELISA result	No. of patients		
	Invasive aspergillosis (n = 27)	No invasive aspergillosis (n = 44)	Total
Positive	25	2	27
Negative	2	42	44
Total	27	44	71

(2) **Maertens *et al*., 2001:** In a prospective study of patients with hematological disorders with ≤ 500 neutrophils/μL from 362 consecutive high-risk treatment episodes from 253 patients (7 proven IA, 30 probable IA, 2 proven IFI, and 97 probable IFI, and 226 no IFI) diagnosed according to EORTC/MSG criteria of 2002. Please note that there is some discrepancy between the numbers in the table and text. Antifungal prophylaxis with itraconazole or amphotericin B was administered during the period of neutropenia; itraconazole was continued through the period of immunosuppression. A positive GM was based on a cut-off index of consecutive ≥1. 0 and testing of each sample only once by Platelia *Aspergillus* (Sanofi Diagnostics Pasteur, Marnes-La-Coquette, France). In addition, serum samples were collected once weekly. Based on testing of 2 or more consecutive samples, all seven proven IA patients from the 12 episodes repeatedly tested positive. The results for sensitivity, specificity, PPV and NPV based on autopsy findings of 30 proven, 9 probable and 264 aspergillosis negative patients were 89.7%, 98.1%, 87.5% and 98.4%, respectively. Results in Table II-3 shows that testing of consecutive samples increases the specificity and PPV.

Table II-3: Comparison of results between a single sample and two or more consecutive positive samples

	1 positive sample, %				2 or more positive samples, %			
	Sens	Spec	PPV	NPV	Sens	Spec	PPV	NPV
Proven IA	100	94.6	68.1	100	100	98.1	85.7	100
Probable IA	66.6	94.6	30	98.8	55.5	98.1	50	98.4
Proven + probable IA	92.3	94.6	72	98.8	89.7	98.1	87.5	98.4
Possible IFI	11.1	94.6	30	83.8	7.4	98.1	44.4	83.8

Proven IFI and probable IFI were excluded from the analysis.
Sens indicates sensitivity; spec, specificity; for other abbreviations, see Tables 1 and 2.

The authors state that the detection of GM (cut-off ≥ 1.5) preceded the definitive diagnosis of IA in 96% (29/30) of proven cases, by a median of 17 days (range 2 to 110 days). There were no transient positive or any false negative episodes. Twenty-four of the patients whose GM indices continued to rise died of IA. Of the six patients with GM indices less than 0.9 in at least three consecutive serum samples 4 survived and 2 died. Included among the survivors was one patient with cerebral aspergillosis. GM was detected prior to initiation of broad spectrum antifungals in 62% (18 of 29 treated) of the patients by a median of six days (range 1-27 days) and coincided with the commencement of antifungal therapy in four patients. The EIA was positive before the sampling of the first positive culture with a mean of 10.5 days, range 1 -100 days. GM positivity occurred in 68% of the patients before new pulmonary infiltrates were seen on chest X-rays. The results in Figure II-1 show the time course of GM indices in 8 select patients. Patients 4 and 8 with rapidly increasing GM indices succumbed to IA in

the episode. Four of the patients with negative results survived and the remaining two patients (number 3 and 7) died of IA in subsequent episodes.

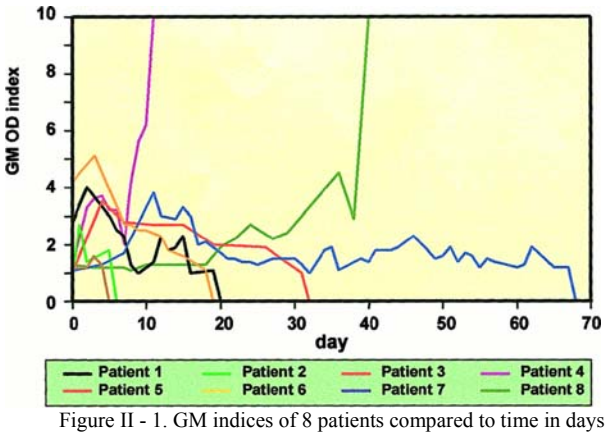


Figure II - 1. GM indices of 8 patients compared to time in days after start of antigenemia

(3) **Maertens *et al.* (2002):** One hundred adult neutropenic (<500 neutrophils/µL for 7 to 45 days) adult patients with hematological disorders undergoing allogeneic hematopoietic stem cell transplantation were tested in Belgium. Antifungal prophylaxis (itraconazole, or amphotericin B) was administered during the neutropenic phase. Diagnosis (Figure II-2) was according to EORTC criteria of 2002. Blood cultures were performed daily until defervescence. Serum samples were collected prospectively at least two times a week, from admission until death or discharge, and tested by Platelia *Aspergillus* EIA assay (BioRad laboratories). A positive GM was based on an index of ≥1.0 and testing of 2 serum samples. Serum samples were stored at -70°C and tested weekly. Eighteen patients (248 samples) tested positive by EIA assay.

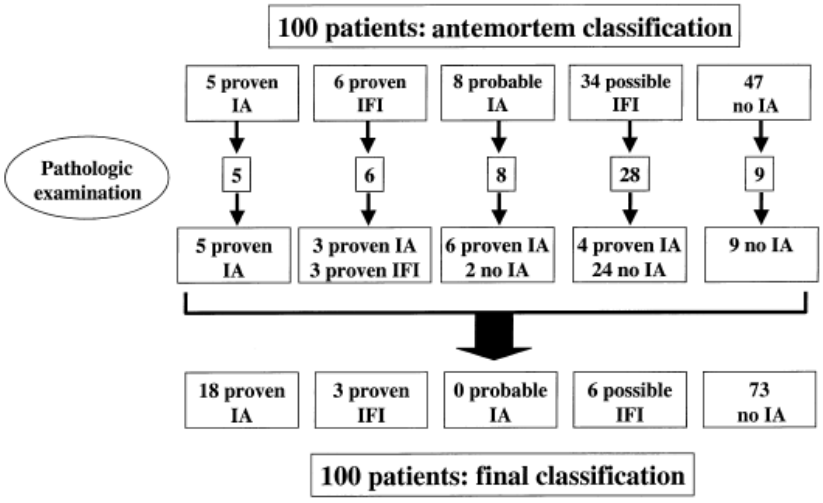


Figure II-2: Classification of patients at study entry (all patients) and postmortem examination (n=59)

Sensitivity and specificity of GM detection was 94.4% and 98.8%, respectively; PPV and NPV was 94.4% and 98.8%, respectively. Detection of GM preceded radiological findings by 8 to 9 days in 80% of the subjects and culture results in 89% patients (Table II- 4). Detection of GM antigenemia was useful in patients receiving steroids.

Table II-4: Temporal onset on GM antigenemia in patients with proven IA

Time point	No. of evaluable patients	No. (%) of patients with antigen[a]	Days between antigen detection and time point, median (range)
First day of fever	11[b]	6 (54.5)	3.5 (0–19)
First indication on chest x-ray	15[c]	12 (80)	8 (4–22)
High-resolution pulmonary CT scan	15[d]	12 (80)	6 (1–12)
Collection of first sample with positive culture results	18	16 (88.8)	9 (2–96)
Initiation of antifungal therapy	18	16 (88.8)	6 (0–14)
Definite diagnosis of IA	18	16 (88.8)	14 (5–106)
Death	18	17 (94.4)	14 (2–106)

NOTE. CT, computed tomography.
[a] At or before time point.
[b] Seven patients remained afebrile.
[c] One patient had residual lesions from a previous episode of IA, and 2 patients were not evaluable because of preexisting abnormalities visible on chest x-ray (pulmonary non-Hodgkin lymphoma and bronchiolitis obliterans).
[d] A high-resolution pulmonary CT scan was not performed for 3 patients receiving mechanical ventilatory support.

(4) **Maertens *et al*., 2004**: The study was conducted in Belgium between July 2001 and December, 2002. It was a prospective, blinded study and its objective was to evaluate the feasibility of lowering the cut-off index in the Platelia *Aspergillus* EIA in neutropenic patients with high pretest probability of IA. The patients were also undergoing chemotherapy for myeloid leukemia, or undergoing myeloablative allogenic HSCT. Patients with acute lymphoblastic leukemia were receiving high doses of steroids as part of their remission-induction chemotherapy. The study included serum samples from 124 treatment episodes from 104 patients (108 episodes- 16 proven IA, 13 probable IA, 20 possible IFI, 59 no IFI). Other criteria for enrollment were as follows:

- Consecutive serum GM levels in adult patients ≥16 years old that had hemato-oncological conditions and were at risk of developing invasive aspergillosis (IA). The age of the subjects ranged from 16-79 years with a median of 49 years.
- The patients were receiving chemotherapy for acute myeloid leukemia or myelolodysplasic syndrome with an expected neutropenia (< 500 cells/µL) for at least 14 days.

All of the patients were hospitalized in high efficiency particulate filter room until the neutrophil count was >500 cells/µL. The patients were diagnosed as proven, probable or possible aspergillosis as recommended by EORTC/MSG criteria. The Platelia *Aspergillus* EIA (BioRad) was used to test the serum samples. A total of 1642 samples (mean 13.2 samples/ episode) were tested. Eight hundred and sixty-six consecutive

serum samples were analyzed from 74 episodes (mean 11.7 sera/episode) taken from patients without clinical or radiological signs of IA. Collection of the serum samples commenced at the start of the cyto-reduction therapy and was terminated when either neutropenia normalized, the fungal infection cleared or at the end of hospitalization or death.

The samples were stored at -20^{0}C and tested twice weekly by a technician who was blinded to the status of the patients. The laboratory procedure followed the manufacturer's recommendation up to the point at which the sample was added to the microtiter plate. The in-house testing methodology differed from that of the manufacturer from the incubation phase to the test completion. This meant that after the sample was added to the plate, the test was performed automatically on a BEP III, Dade Behring, Marburg, Germany a semi automatic analyzer. The instrument was programmed to perform processing of the sample and the OD reading (450/620 nm) as prescribed by the manufacturer of the Platelia *Aspergillus* assay. The cut-off index was ≥1.0. However, the researchers analyzed the results using a number of different cut-off values ranging from 0.3 to 1.5. At the ≥0.5 range the effect of using the results of a single sample as well as the use of two consecutive samples was analyzed.

Table II.-5 shows the parameters of the various cut-off indices. At the cut-off values of indices 1.5 and 1.0, all of the samples of episodes without clinical and radiographic signs of IA were negative. At the ≥0.5 cut-off value using two consecutive samples, the sensitivity was 96.5%, the specificity was 98.6%, the positive predictive value 96.5% and the negative predictive value 98.6%. The results in table II- 5 appear to be inaccurate and perhaps corrected for some parameters. The second best cut–off was a single sample at 0.9 which has a slightly lower PPV.

Table II-5: Indices of samples at various cut-off values

	Static									Dynamic
OD index cut-off:	1·5	1·0	0·9	0·8	0·7	0·6	0·5	0·4	0·3	2 × ≥0·5
Sensitivity (%)	82·7	93·1	96·5	96·5	96·5	96·5	96·5	96·5	100	96·5
Specificity (%)	100	100	98·6	97·3	93·2	86·4	85·1	71·6	60·8	98·6
PPV (%)	100	100	96·5	93·3	84·8	73·7	71·8	57·1	50	98·6
NPV (%)	93·7	97·4	98·6	98·6	98·6	98·5	98·4	98·1	100	98·4
Efficacy (%)	95·1	98	98	97	94·2	89·3	88·3	78·6	71·8	98

*Excluding possible cases of fungal infection.
PPV, positive predictive value; NPV, negative predictive value.

A comparison of the performance of the assay at a cut-off value of ≥ 0.5 based on testing of a single sample as well as testing of two consecutive samples at a cut-off of ≥ 0.5 and 1.0 is shown in Figure II-3 . Both the one sample and two consecutive samples at cut off ≥ 0.5 give a similar proportion of positive results for proven (93.7%) and probable (100%) IA patients. At a ≥ 1.0 cut-off, however, approximately 2/3 of the cases were missed. Based on testing of two serum samples and a cut-off ≥ 1.0 approximately 25% of the proven and 20% of the probable cases were missed. There were no false positive cases When two consecutive samples at ≥ 0.5 cut-off were tested there was one false positive result. However when one sample at ≥ 0.5 was tested the false-positivity rate was 14.8%.

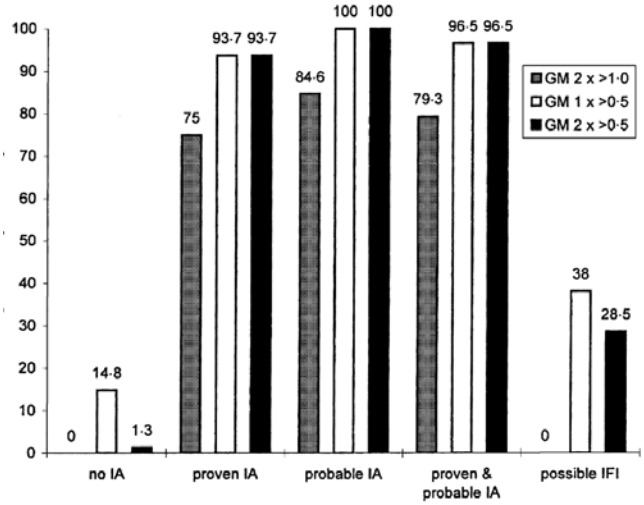


Figure II-3: Results of three different Cut-offs for patients classified by EORTC/MSG criteria as proven, probable and possible IA infections

A time course of GM in 3 selected patients is shown in Figure II-4. Two surviving patients (proven and probable) cleared GM while receiving antifungal therapy. The 3rd patient with proven IA a continuous increase in GM index was reported, despite adequate antifungal therapy; the authors also state that this patient GM results are a representation of 16 patients and they all died of IA. The authors also state that only one patient was negative at all time points tested and died of rapidly progressive veno-occlusive disease in the early post-transplant period; however, at autopsy; the patient was reported to have a necrotic pulmonary lesion with fungal hyphae compatible with *Aspergillus* (yet the culture was negative).

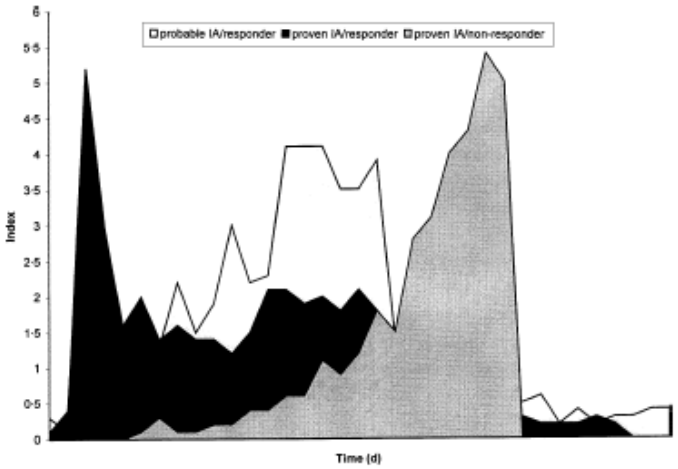


Figure: II-4: Time course of GM in 3 selected patients

(5) Maertens *et al*. 2007: This was a retrospective study in neutropenic patients, tested 4484 serum samples from 239 episodes in 203 patients (16 proven (19 episodes), and 19 probable and 168 negative IA (201 episodes)) with underlying HSCT at two centers in Belgium and Netherlands. Of the 3704 samples from the 201 control episodes, 99.4 % (3682) of the samples were negative and 0.6% (22) positive by Platelia™ *Aspergillus* EIA at a cut-off of ≥0.5. When the cut-off value decreased from ≥ 1.5 to ≥ 0.5, the

sensitivity increased by 21% (from 97.4% to 76.3%) but the specificity decreased by only 7% (from 90.5% to 97.5%). The PPV using the single test > 0.5 index was 66.1% and NPV 99.4%. The PPV with the ≥0.5 cut-off increased to 87.5% when two consecutive samples were tested without greatly affecting the sensitivity, specificity and NPV (Table II -6).

Table II- 6: Sensitivity and specificity of the Platelia Aspergillus EIA, according to selected cut-off OD indices and EORTC/MSG classification of episodes of IA

OD index cutoff value, episode classification	No. of episodes with positive results/no. of episodes tested	Sensitivity, % (95% CI)	No. of episodes with negative results/no. of episodes tested	Specificity, % (95% CI)
OD index ⩾1.5				
Proven IA	19/19	100 (85.4–100)	...	...
Probable IA	10/19	52.6 (28.9–75.5)	...	...
Overall	29/38	76.3 (59.8–88.6)	...	...
Control group	...	...	196/201	97.5 (94.3–99.2)
OD index ⩾1.0				
Proven IA	19/19	100 (85.4–100)	...	...
Probable IA	12/19	63.2 (38.4–83.7)	...	...
Overall	31/38	81.6 (65.7–92.3)	...	...
Control group	...	...	194/201	96.5 (93.0–98.6)
OD index ⩾0.5				
Proven IA	19/19	100 (85.4–100)	...	...
Probable IA	18/19	94.7 (74.0–99.9)	...	...
Overall	37/38	97.4 (86.2–99.9)	...	...
Control group	...	...	182/201	90.5 (85.6–94.2)
OD index ⩾2 × 0.5				
Proven IA	19/19	100 (85.4–100)	...	...
Probable IA	16/19	84.2 (60.4–96.6)	...	...
Overall	35/38	92.1 (78.6–98.3)	...	...
Control group	...	...	196/201	97.5 (94.3–99.2)

The authors also analyzed the effect of temporal proximity of sample collection to the day of conventional diagnosis (day 0). For this, all serum samples were collected between day -7 and day 7 of diagnosis. The results in Table II-7 show a better sensitivity at a cut-off of ≥ 0.5 between days 1 to 7 after diagnosis.

Table II -7: Changes in sensitivity of the Platelia Aspergillus EIA overtime for proven and probable IA patients, calculated according to test result at different OD cut-off values

Patient population, OD	Time to diagnosis	
	Week −1	Week 0
Leuven, Belgium		
No. of samples	105	131
OD index cutoff value		
⩾1.5	24.8 (16.9–34.1)	42.0 (33.4–50.9)
⩾1.0	44.8 (35.1–54.8)	58.0 (49.1–66.6)
⩾0.5	66.7 (56.8–75.6)	84.0 (76.6–89.8)
Nijmegen, The Netherlands[a]		
No. of samples	35	40
OD index cutoff value		
⩾1.5	34.4 (16.8–49.3)	47.5 (31.5–63.9)
⩾1.0	42.9 (26.3–60.6)	55.0 (38.5–70.7)
⩾0.5	60.0 (42.1–76.1)	65.0 (48.3–79.4)
Overall		
No. of samples	140	171
OD index cutoff value		
⩾1.5	26.4 (19.3–34.5)	43.3 (35.7–51.0)
⩾1.0	44.3 (35.9–52.9)	57.3 (49.5–64.8)
⩾0.5	65.0 (56.5–72.9)	79.5 (72.7–85.3)

NOTE. Data are percentage (95% CI) of PA-EIA–positive samples, unless otherwise indicated. Week −1 corresponds to day 7 through day 1 before diagnosis; week 0 corresponds to day of diagnosis through day 7 after diagnosis.

[a] Includes data from 15 episodes (5 proven cases and 10 probable cases of IA).

A comparison was made of the results originating at the two European centers in the study. Figure II-5 shows the differences in sensitivities and specificities of tests in the two centers. Although the specificities are similar there is a slight variation in the sensitivities with the sensitivities at center 1 being a little high than those at center 2 at all cut-off indices.

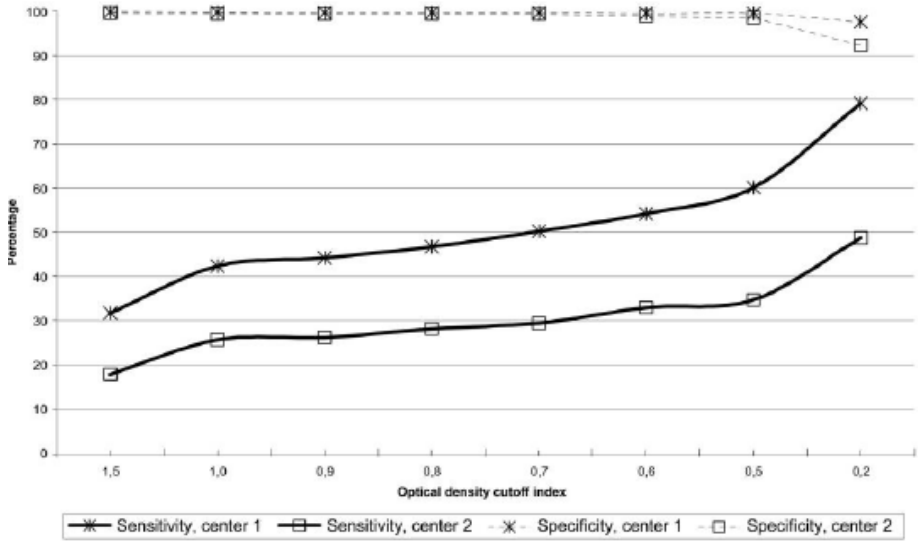


Figure II-5: Center specific sensitivity and specificity, from a per-test analysis, for proven and probable IA at variable static OD cut-offs for positivity (x-axis). Center1, Leuven, Belgium; Center2, Nijmen, The Netherlands.

(6) Becker *et al*., 2003: Study was conducted at the Erasmus Medical Center, Rotterdam, Netherlands. It was conducted in two parts. Part 1 consisted of a prospective blinded study between February 1999 and April 2000 and Part 2 a prospective non-blinded study between June 2000 and October 2001 focusing primarily on BAL fluid for diagnosing IPA. The population in both studies comprised of hematological oncological patients who were ≥18 years, neutropenic for at least 10 days with a neutrophil count of < 500 cells/μL. The criteria used for diagnosis of patients as proven, probable, or possible were similar to those recommended by EORTC/MSG, 2002. Patients received 500 mg of ciprofloxacin twice a day orally and either 200 mg of fluconazole once daily or 200 mg itraconazole twice daily for prophylaxis. During hospitalization, all patients were evaluated for fever and respiratory infection on a daily basis and underwent chest x-rays twice weekly except during febrile incidents (fever > 38.3^0 C) when chest x-rays were done every other day. During febrile incidents, CT scans were performed if the fever lasted for five days during antibiotic therapy or when chest x-rays were abnormal. Additionally, antifungal treatment was instituted under the following conditions: (a) if the chest x-rays showed abnormalities suggestive of IA during antibiotic treatment, (b) if molds were isolated from respiratory or blood cultures or (c) when fever lasted for 7 days while the patient underwent antibiotic therapy. One hundred and sixty patients ranging from 18 to 79 years of age were included in part 1 of the study. There were 249 neutropenic episodes in 160 patients; of these 2 were proven, 11 probable, 4 suspected, and 18 possible with respect to diagnosis of invasive pulmonary aspergillosis (IPA). There were 4 patients with other IFI and 121 with no IFI. The test for detection of serum galactomannan was the Platelia *Aspergillus* EIA (Sanofi Diagnostics Pasteur, Belgium) and the authors state that the manufacturer's instructions were followed. The reference standard supplied by the manufacturer was 1 ng/mL. An index of ≥1.0 was used as the cut-off limit. The GM positive sample was based on testing of two samples with the indices ≥1.0. Two serum samples were collected weekly from the start of neutropenia and terminated at its end. The samples were stored at -20^0C and tested for GM after patients classified as possible were discharged. The technician was unaware of the patients' identities and status and the clinicians were blinded to the results.

This was a prospective blinded study and a total of 1145 serum samples were collected during 249 neutropenic episodes and tested for GM. This resulted in a mean of 12.9 samples per patient. The authors state that of these 270 samples were drawn from patients with IPA and 26 samples were classified as positive with GM index range of 1.0 to 4.3. However, the number of patients from which 270 samples were collected was not specified. The authors state that of 17 patients with IPA, eight patients had two or more positive GM samples, with one patient having more than three positive samples. In seven patients the antigenemia was transient. Table II-8 shows the results of the GM test. Four (22%) of 18 patients with possible IPA tested positive. Five of the 44 patients without invasive fungal infection (IFI) tested positive. GM was detected more often in patients that died (4/6) than those that survived (4/11). The time of manifestation of fever and a positive serum GM test was 3.0 to 6.2 days, respectively. The other major indications of IPA such as CT scans (halo, crescent and cavitation), BAL fluid GM, and culture/histopathology were reported between 3.2 to 4.8, 5.8 to 8.0

and 9.8 to 19.8 days respectively. These results indicate that the serum GM was the earliest test to indicate a diagnosis of IPA.

Table II-8: GM detection in neutropenic patients between 1999 and 2000

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

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

The results were analyzed on the basis of different GM indices as cut-off for a positive result and testing of one versus two serum samples. Results in Table II.-9 show that under the conditions used in this study the chosen cut-off (2 specimens ≥1.0 index), the sensitivity was 47 ± 7%, the specificity 93 ± 5%, the PPV 73 ± 6% and the NPV 82 ± 5%. Increasing the cut-off to a GM index of 1.5 based on the testing of 2 serum samples decreased the sensitivity, PPV, and NPV; however the specificity was similar.

Table II-9: Sensitivity, specificity, positive and negative predictive values of GM detection in serum samples according to various levels of positivity

	Proven, probable and suspected IPA			
	Sensitivity ± SEM	Specificity ± SEM	PPV	NPV
Criterion positive				
1 × index > 0·7	59%	61%	36%	80%
2 × index > 0·7	53%	89%	64%	84%
1 × index > 1·0	59%	75%	48%	83%
2 × index > 1·0	47%	93%	73%	82%
1 × index > 1·5	18%	84%	30%	73%
2 × index > 1·5	12%	95%	50%	74%

Inter-laboratory reproducibility was tested using 200 randomly collected serum samples, 125 samples were taken from patients with IPA and 75 samples were taken from those without IPA. The samples were split and tested at another laboratory (Department of Medical Microbiology, University Medical Center Nijmegen, the Netherlands and, Erasmus Medical Centre Rotterdam, Rotterdam, the Netherlands). The criterion for positivity was the same in the comparator laboratory as it was in the primary laboratory. The results from both laboratories were the same for 188 (94%) serum samples. In the comparator laboratory, 2 samples showed discordant results (one patient who should have tested positive was positive and one who should have tested negative tested positive. Both tested negative in the original lab).

(7) Kawazu *et al*., 2004: A prospective study was performed in 96 hematological malignant patients with neutropenia (< 500 cells/µL for at least 10 days), from 149

treatment episodes between 2001 and 2002. The objective was to evaluate three non-invasive methodologies, Platelia *Aspergillus* EIA, a real time PCR for *Aspergillus* DNA, and the β-glucan test for the diagnosis of IA. Of the 96 patients, 9 were classified as having proven IA, 2 probable IA and 13 possible invasive fungal infections and the criteria used for diagnosis were as recommended by EORTC/MSG in 2002. The patients were monitored weekly. Positivity and negativity were calculated by treatment episodes. A treatment episode was defined as a period during which measurements were taken. Samples for laboratory tests were measured once weekly, approximately eight samples were analyzed per episode.

Positive GM results were based on two cut-off values of ≥0.6 and ≥1.0. Two methods were used to define GM positivity. For method 1, an episode was considered positive if at least one sample for the patient was positive. Criteria for method 2 required that any two consecutive samples be positive. When the cut-off value was decreased from ≥1.0 to ≥ 0.6 the sensitivity of the Platelia *Aspergillus* EIA increased for Method II but were similar under Method I. Results showed that when method 1 was changed to method 2, the area under the curve (AUC) in the Platelia *Aspergillus* assay was further increased thereby suggesting increased sensitivity. On the other hand, even though the decrease in cut-off increased the sensitivity of the PCR and β–Glucan test the specificity was negatively affected. Additionally, the AUC of the latter two tests decreased in a change from Method 1 to 2. The effect on the AUC indicated that the Platelia *Aspergillus* EIA has greater reproducibility than that of the other two tests. The statistical parameters of the Platelia with cut-offs of ≥ 0.6 resulted in sensitivities, specificities, PPV and NPV of 100%, 55%, 16%, and 100%, under method I and 100%, 93% 55% and 100% using method II. Figure II-6 shows that the AUC increased in *Aspergillus* EIA while that of PCR and β-glucan decreased using methods 1 (one sample) and II (two consecutive samples). It was not clear whether each positive sample was retested or whether each sample was run in duplicate.

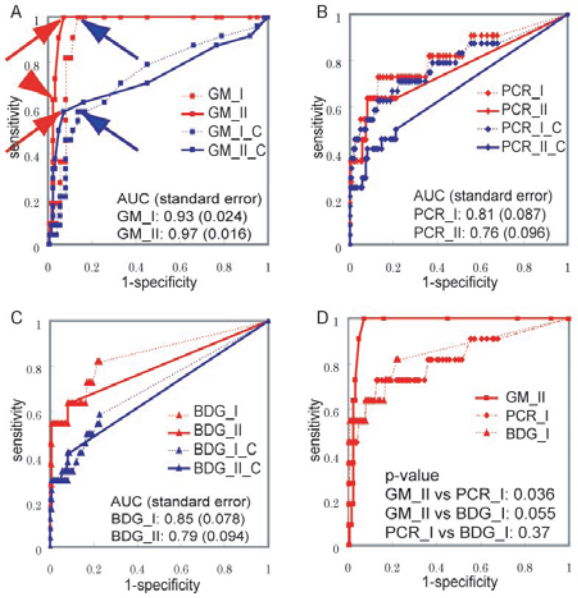


Figure II-6: Curves for Platelia aspergillus EIA, PCR and β-glucan tests when the number of tests for positivity was increased from one to two consecutive specimens

(8) Yoo *et al*. 2005: The results of the Platelia™ *Aspergillus* EIA were compared with nucleic acid sequence-based amplification (NASBA) test for diagnosis of IA in neutropenic patients. The criterion used for diagnosis of IA was based on EORTC/MSG criteria of 2002 in patients with HSCT. The patient population comprised of 128 febrile neutropenic patients two of whom had proven IA and 12 were classified as probable (Table II-10). The determination of positivity of the EIA was a cut-off index of ≥ 0.5 in at least two consecutive samples for patients with proven or probable IA. The results in Table 11-11 show that the results of the galactomannan EIA that resulted in a sensitivity of 86% and specificity of 78%. The PPV and NPV were 33% and 98% respectively. The NASBA resulted in a sensitivity of 86% and specificity of 72%.

Table II-10: Characteristics of patients with proven and probable IA

Patient	Age, years	Sex	Primary disease or risk factor	Treatment	IA classification	GM-EIA result	Duration of hospitalization, days	Duration of antibiotic therapy, days	Duration of AmB therapy, days	Total dose of AmB, mg	Duration of neutropenia, days	Recovery from neutropenia	Clinical course and outcome
1	49	F	ALL	ChemoTx	Probable	Positive	44	25	22	1209	25	Yes	Condition manifested as presumptive fungal pneumonia; patient survived
2	55	F	AML	HSCT	Probable	Positive	50	20	20	1274	15	Yes	Condition manifested as presumptive fungal pneumonia; patient survived
3	63	F	AML	ChemoTx	Probable	Positive	51	16	12	534	14	Yes	Halo sign on chest CT; patient survived
4	21	F	ALL	HSCT	Probable	Positive	29	16	24	1151	17	No	Multiple fungal balls on chest CT; grade III acute GVHD; patient died
5	45	M	ALL	ChemoTx	Proven	Negative	34	20	10	264	18	Yes	Confirmed septate hyphae on tissue biopsy of fungal ball in lung; patient survived
6	24	F	AML	ChemoTx	Probable	Positive	56	13	19	534	20	Yes	Multiple fungal balls on chest CT; patient survived
7	58	F	AML	ChemoTx	Proven	Positive	67	31	30	2259	17	Yes	Confirmed septate hyphae on tissue biopsy of fungal ball in lung; patient survived
8	28	F	ALL	HSCT	Probable	Negative	54	15	12	354	13	Yes	Halo sign on chest CT; patient survived
9	61	M	AML	ChemoTx	Probable	Positive	53	30	24	1134	27	No	Condition manifested as presumptive fungal pneumonia; patient died
10	65	F	AML	ChemoTx	Probable	Positive	35	12	19	927	19	Yes	Condition manifested as presumptive fungal pneumonia; air crescent on chest CT; patient survived
11	49	M	MDS	HSCT	Probable	Positive	91	37	30	1024	24	Yes	Condition manifested as presumptive fungal pneumonia; patient died
12	40	F	ALL	HSCT	Probable	Positive	39	21	21	940	29	No	Presumptive rhinocerebral aspergillosis invading frontal lobe, orbit, and ethmoid sinus; patient died
13	51	M	AML	ChemoTx	Probable	Positive	40	19	15	474	23	Yes	Fungal balls on chest CT; patient survived
14	32	M	AML	ChemoTx	Probable	Positive	35	12	19	927	19	Yes	Condition manifested as presumptive fungal pneumonia; patient survived

NOTE. ALL, acute lymphocytic leukemia; AmB, amphotericin B; AML, acute myelogenous leukemia; ChemoTx, cytotoxic chemotherapy; GM-EIA, galactomannan EIA; GVHD, graft versus host disease; HSCT, hematopoietic stem cell transplantation; MDS, myelodysplastic syndrome.

Table 11 -11 Results of GM –EIA testing using a cut-off of ≥ 0.5

Diagnosis	GM positive	GM negative	Total assays
Proven and probable IA	12	2	14
No IA	25	89	114
Total	37	91	128

(9) Yoo *et al*., 2007: Study was performed in 22 febrile, neutropenic (HSCT) patients with IA using the Platelia *Aspergillus* EIA (BioRad Laboratories) and a real-time nucleic acid sequence-based amplification (Rti-NASBA), conventional NASBA in patients with HSCT patients in Korea. Seven of the patients were classified as probable and 15 as possible according to EORTC/MSG criteria 2002. There were 56 patients without fungal infections. The EIA cut-off positivity index was ≥ 0.5 based on testing of at least two consecutive serum samples. The EIA showed good specificity (98%) but poor sensitivity (45%). The PPV was 91% and NPV 82% (Table II-12). Five of 7 probable and 5/15 possible patients were identified by EIA with false positive results of

1/56. The correlation between the two nucleic acid tests was kappa 0.8 suggesting good concurrence.

Table II -12: Specificity, sensitivity, PPV and NPV by 3 different assays

	Proba-ble	Possi-ble	NFI	sn	sp	ppv	npv
NASBA							
<3.5	0	1	25	0.95	0.45	0.40	0.96
≥3.5	7	14	31				
RTi-NASBA							
Negative	1	0	24	0.96	0.43	0.40	0.96
Positive	6	15	32				
GM-EIA							
<0.5	2	10	55	0.45	0.98	0.91	0.82
≥0.5	5	5	1				

NASBA, nucleic acid sequence-based amplification; NFI, non-fungal infection.

(10) Lai *et al*. (2007): The Platelia™ *Aspergillus* EIA (BioRad Laboratories) was evaluated in a prospective blinded study of 189 patients with hematological disorders, in Taiwan. The study comprised five proven, nine probable, 26 possible and 149 no IA. The IA diagnosis was based on the basis of EORTC/MSG criteria of 2002. The median duration of neutropenia was 25.5 days, however, the actual neutrophil cell count for characterizing neutropenia was not specified. The authors have stated that two consecutive patient serum samples with cut-off ≥1.5 were required to classify a patient as galactomannan positive. However, some of the positive results are based on testing of less than 2 samples being positive and for some patients 3 samples. For example, of the 5 patients with proven IA, 4 were GM positive (Table II- 13). Of these 4 patients, 2 consecutive serum samples with positive GM results were reported in 1 patient and for the remaining 3 patients positive result was based on testing of one serum sample. Similarly, 15 serum samples were tested from the 9 probable patients and 7 were GM positive (6 patients were positive based on testing of 1 sample and 1 based on 3 samples). Of the 26 possible patients 5 were GM positive (4 patients based on 1 sample and 1 patient based on testing of 3 samples). Nine of the remaining 149 subjects without any radiological and clinical evidence of IA were GM positive (based on testing of 20 samples- 3 patients with 2 consecutive positives and 6 patients with a single positive result). The statistical parameters were sensitivity 78.6 %, specificity 93.9 %, PPV 55.0 % and NPV 97.9 %. The authors stated that when the cut-off was lowered to ≥0.5 the sensitivity was 100% and the specificity decreased to 65.7%.

Table II -13 Platelia Aspergillus EIA test results of patients with proven, probable and possible IA or without retained diagnosis of IA

Category	Platelia results (No. of patients)		
	Positive	Negative	Total
Proven IA	4	1	5
Probable IA	7	2	9
Possible IA	5	21	26
IA not retained	9	140	149
Total	25	164	189

The GM index at different time intervals was available for 3 patients with proven or probable IA who survived and 5 patients who died (Figure II-7). The authors state that GM index decreased after antifungal treatment. However, the number is too small to evaluate the effect of antifungal treatment.

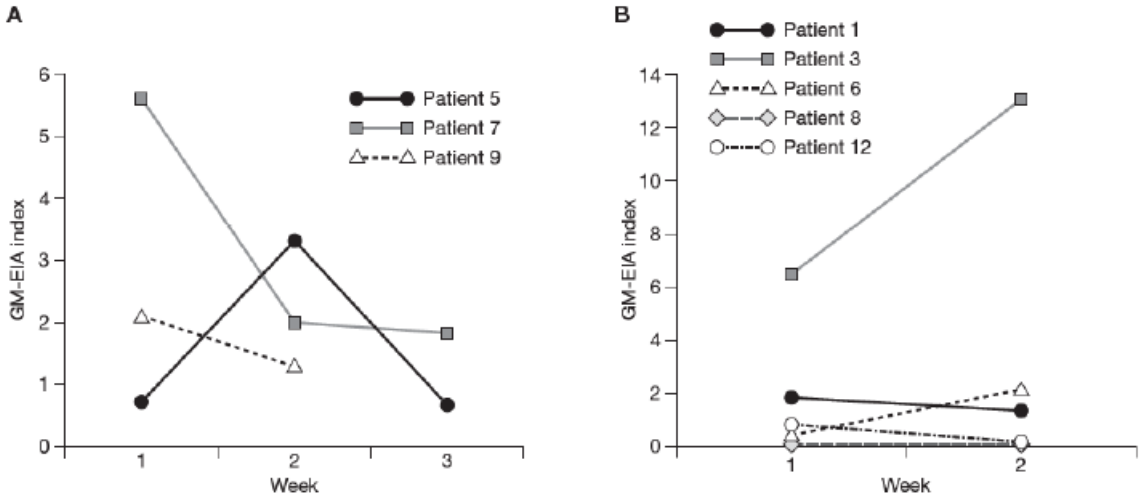


Figure II-7: Kinetics of galactomannan EIA index in 3 patients who survived (A) and in 5 patients who died (B)

(11) Buchheidt *et al*., 2004: Study was done between 1999 to 2002 and compared the Platelia™ *Aspergillus* EIA with the nested–PCR in patients with hematologic malignancies or recipients of HSCT. The 177 patient episodes and 1228 samples from 165 patients with hematological malignancies were classified according to the 2002 EORTC/MSG criteria. The cut-off index was ≥ 1.5 using two or more consecutive positive samples to define positivity. The sensitivity and specificity were 33% and 98.9% respectively for the Platelia™ *Aspergillus* EIA as compared to results for the PCR of 63.6% and 63.5% for the same parameters. The above sensitivity of the *Aspergillus* EIA was obtained when two patients with probable IA based on GM serology were included in the calculation, but when they were excluded the sensitivity dropped to 14.3%. When the cut-off of 0.7 for the EIA was applied to the same results the authors state that the results did not change. No actual numbers were provided for the 0.7 cut-off in the paper.

(12) Ulusakarya *et al.* (2000): Study was conducted in France in 135 patients with hematological malignancies, undergoing chemotherapy, and neutropenia (<500 neutrophils/µL for more than 7 days; mean duration of neutropenic episode was 36 days (range 9-95 days). Serum samples (n= 507) were collected on a weekly basis from 193 neutropenic episodes and tested by Platelia *Aspergillus* EIA (Sanofi Diagnostics Pasteur, Marnes-La-Coquette, France). An index of ≥ 1.5 was considered positive and between 1.0 and 1.5 undetermined. Positive result was based on testing of 2 serum samples. Criteria for diagnosis were similar to that recommended by EORTC/MSG in 2002. Ten patients were diagnosed as proven IA, 6 as probable IA, and 2 as possible IA (Table II-14). At a cut-off of >1.5 the sensitivity and specificity were 69% and 96%, respectively. However at a cut-off of ≥ 1.0 the sensitivity, specificity PPV, and NPV were 100%, 92%, 64%, and 100% respectively. The authors recommended lowering the cutoff to 1.

Table II-14: Patients with proven, probable or possible IA.

Patient	*Sex/Age*	*Underlying disease*	*Allogeneic HSCT*	*Duration of neutropenia (days)*	*Type of IA*	*Organ involvement by IA*	*ELISA results*	*Days from diagnosis of IA to first P or U ELISA result*	*Treatment for IA*	*Outcome*
1	M/51	AML	No	22	Probable	Lung	1 U	5	Yes	Survived
2	F/66	AML	No	20	Proven	Lung	1 U	9	Yes	Died
3	M/44	AML	No	30	Proven	Lung	4 P, 1 U	5	Yes	Survived
4	F/8	ALL	Yes	31	Proven	Lung	3 P, 1 U	−8	Yes	Survived
5	F/46	AA	No	25	Possible	Lung	1 U	10	Yes	Died
6	M/63	ALL	No	27	Proven	Lung	2 P, 3 U	7	Yes	Died
7	F/37	ALL	No	38	Probable	Lung	3 P, 1 U	11	Yes	Died
8	M/51	CLL	No	94	Probable	Lung	1 U	6	Yes	Died
9	M/51	MM	No	23	Proven	Lung	2 U	4	Yes	Died
10	M/35	MM	Yes	52	Proven	Lung	1 P	11	Yes	Died
11	F/47	NHL	No	95	Probable	Lung	1 U	−6	Yes	Survived
12	F/47	NHL	No	9	Proven	Lung	1 P	7	No	Died
13	M/43	AML	No	31	Probable	Lung	4 P, 2 U	10	Yes	Survived
14	F/40	ALL	Yes	35	Proven	Lung, brain, myocardium	4 P	12	Yes	Died
15	F/38	ALL	No	28	Probable	Lung, MS	1 P	0	Yes	Died
16	F/33	CML	Yes	10	Proven	Lung	1 P	0	Yes	Died
17	F/29	ALL	Yes	43	Possible	Lung	2 P	14	Yes	Died
18	M/46	AML	No	28	Proven	Lung	6 P, 1 U	12	Yes	Survived

P = positive; U = undetermined; MS = maxillary sinus.

(13) Herbrecht *et al.* (2002): The authors summarized a randomized unblinded, non-inferiority, multicountry study of voriconazole versus amphotericin B for primary therapy of invasive aspergillosis.

The major study was subdivided into two smaller studies 150-307 and 150-602 using similar protocols and conducted in a total of 19 countries. The countries included Europe, Israel, Australia, U. S. A., Canada, Mexico, Brazil and India. To be included in the study the subjects must have been diagnosed with definitive or probable invasive – aspergillosis, 12 years of age or older and be immuno-compromised. The cause of the immuno-competency included a wide range of disorders such as allogeneic or autologous hematopoietic-cell transplantation, hematologic cancer, aplastic anemia, myelodysplastic syndrome; or other immuno-compromising conditions, including the acquired immunodeficiency syndrome (AIDS), receipt of corticosteroid therapy, and solid-organ transplantation. The study enrolled a total of 391 patients between July 1997 and October 2000 from 95 centers.

The study specific definitions were as follows:

- Definite invasive aspergillosis was defined as clinically relevant symptoms plus evidence of the presence of *Aspergillus spp.* by culture or histology, radiologic evidence of pulmonary lesions that were not attributable to other factors and a culture of bronchoalveolar-lavage fluid that was positive for *Aspergillus spp*. in patients who had undergone allogeneic hematopoietic-cell transplantation or who had a neutropenic hematologic condition or tracheobronchial lesions confirmed by bronchoscopy with a positive culture for *Aspergillus spp.*
- Neutropenia was defined by a neutrophil count of < 500 per cubic millimeter at some time during the two weeks prior to study selection.
- Probable invasive aspergillosis included the same specific clinical symptoms in conjunction with one or more of the following hyphae consistent with the presence of *Aspergillus spp.* but without culture or the presence of a halo or an air-crescent sign on a chest X-ray in subjects who had undergone allogeneic HSCT or who had a neutropenic hematologic condition; radiologic evidence of new pulmonary lesions that were not attributable to other factors with either hyphae consistent with *Aspergillus* in BAL fluid or sputum or a sputum culture that was positive for *Aspergillus*; clinical evidence of sinusitis, opacification of a sinus on CT or MRI, and positive histopathological examination or culture of *Aspergillus* from a lesion in the nose or paranasal sinus .
- A successful response was classified as a complete or partial response all other responses were deemed failures. The following criteria were used to classify the effect of treatment on the aspergillosis patient.
- Complete response was described as the disappearance of > 90% of the lesions caused by invasive aspergillosis along with the resolution of all of the clinical signs and symptoms.
- Partial response was described as improvement of > 50% of the lesions caused by invasive aspergillosis in conjunction with improvement in the clinical signs and symptoms.
- Stable response was described as no change from baseline or change in clinical signs and symptoms or <50% improvement from baseline.
- Failure of therapy was defined as worsening disease.

Patients were excluded if they had already been diagnosed with any stage of aspergillosis or had received therapy for more than 96 hours with either 0.5 mg of amphotericin B / kg of body weight /day or > 200 mg itraconazole /day ≤14 days preceeding entry to the trial
. The most common causes for exclusion were the inability to confirm the presence of a halo or air crescent in patients sign at baseline with supporting mycological or pathological evidence. The number of patients excluded because of this criterion was 35 in the voriconazole arm and 25 in the amphotericin B arm. It should be noted that halo sign is found in a number of hematological diseases and is not specific for the diagnosis of IA. The characteristics of subjects in the two groups are listed in table **II-15.** The data show that 37% % of the patients in both groups had positive microscopy for the presence of fungal elements indicative of *Aspergillus. spp.* while 54% of the patients had positive cultures for *Aspergillus spp.* The most prevalent species isolated was *A. fumigatus* in 85 patients. *A. niger* was isolated from 9 patients, *A. flavus* from 7

patients, *A. terreus* from 6 patients, and *A. glaucus, A. nidulans* and *A. sydowii* from one patient each. Histological examination was positive at baseline in only 20% of patients and the halo or air-crescent sign was present in 34% of patients. From this study microbiological culture appears to be a better diagnostic tool than histological examination. Hussain et. al., 2000 stated that regarding the use of histological, and microbiological results as a means for diagnosis of invasive aspergillosis that culture fails to detect between 30 to 50% of invasive aspergillosis cases, the baseline data in these two studies are consistent with this observation. Histological examination from these studies appears to be of much less value than the culture for the diagnosis of IA.

Table II-15 : A Evidence supporting Base-line Diagnosis in the treatment population

VARIABLE	VORICONAZOLE GROUP (N=144)	AMPHOTERICIN B GROUP (N=133)
	no. (%)	
Site of the infection		
Lung only	123 (85.4)	117 (88.0)
Sinus	8 (5.6)	7 (5.3)
Cerebral*	5 (3.5)	5 (3.8)
Disseminated†	4 (2.8)	1 (0.8)
Other	4 (2.8)	3 (2.3)
Level of certainty of the diagnosis of aspergillosis		
Definite‡	67 (46.5)	41 (30.8)
Probable	77 (53.5)	92 (69.2)
Initial evidence of aspergillosis§		
Positive finding on microscopy	56 (38.9)	46 (34.6)
Positive culture	84 (58.3)	65 (48.9)
Positive histologic examination	35 (24.3)	22 (16.5)
Halo or air-crescent sign only	46 (31.9)	49 (36.8)

*Category includes those with other organ involvement.

†Category excludes those with cerebral involvement.

‡There were significantly more definite cases in the voriconazole group (P=0.01).

§Some patients had more than one type of biologic evidence.

After exclusion the voriconazole arm studied 144 subjects while the amphotericin B arm had 133 subjects. The duration of the trial was 12 weeks.
On Day 1 patients were first treated either twice daily with 6 mg / kilogram voriconazole intravenously followed by 4 mg / kilogram intravenously twice daily for at least 7 days switching to either 200 mg oral voriconazole, twice daily or once daily intravenous amphotericin B deoxycholate (1.0 to 1.5 mg/kilogram once daily). Patients who did not tolerate or did not respond to the initial therapy could be switched to another antifungal agent. Radiologists reviewed the digital images but the computerized planimetry was used for visual evaluation of the invasive aspergillosis lesions. The study indicated that treatment with voriconazole was superior to treatment with amphotericin B. This study was done prior to the development of the EORTC criteria and the use of *Aspergillus* galactomannan assay and gives an overview of diagnosis of IA without the use of the EIA.

(14) Herbrecht *et al.* (2002): Study was conducted in France in adult and pediatric neutropenic (< 500 cells/µL) patients. Four patient groups were studied (1) fever of unknown origin (FUO) during neutropenia, (2) suspected pulmonary infection (PI), (3) nonpulmonary aspergillosis (NPA) and (4) hematopoietic stem-cell transplant (HSCT). Patients could be included in the study more than one time if they were not diagnosed with IA. A total of 3,297 serum samples were collected during 797 episodes (Table II-16). Serum was tested for GM detection by Platelia *Aspergillus* assay (BioRad Laboratories). Diagnosis of IA was based on EORTC/MSG criteria of 2002.

FUO patients (cohort 1): These patients had no documented evidence of an infectious disease. Specimens were collected up to 3 times when no cause of fever could be determined in patients despite antibiotic therapy. There were 261 episodes in this cohort and only one patient (a child) was diagnosed as possible IA. The sensitivity was 100%, specificity was 95%, PPV 7% and NPV 100%. There were 44% (11/25) false positive results in children and 0.9% (2/235) in adults. There were no false negative results and no mycological evidence was obtained.

Table II -16: Demographics of patients included in the study

	Reason for Antigenemia Determination			
	Fever of Unknown Origin	Suspected Pulmonary Infection	Extrapulmonary Signs	Surveillance in Transplant Recipients
No. of episodes	261	297	28	211
No. of different patients	220	274	28	206
Age, years				
Median	50	55	54	41
Range	1-85	1-88	22-80	4 months-68 years
Male/female	147/114	202/95	15/13	110/101
No. of antigenemia assays	540	1481	122	1151
No. of antigenemia assays per episode				
Mean	2.1	5.0	4.4	5.5
Range	1-11	1-28	1-19	1-32

Suspected pulmonary infection (cohort 2): A serum sample was collected as soon as infection was suspected and up to three consecutive days thereafter. Sputum samples were routinely collected for fungal and bacterial culture, CT scans were obtained when feasible and BAL was collected when no microbiological evidence was found in the blood or sputum. There were 297 episodes in this cohort (53 possible, 67 probable, 25 proven and 152 were controls). The sensitivity was 28% specificity was 99 % PPV 95 % and NPV 58 %. One hundred and five antigenemia tests were false negative, eight of which were proven IA, 56 were probable and 41 were possible.

Suspected extra pulmonary aspergillosis (cohort 3): Serum samples were collected from all patients with non-pulmonary or radiologic signs consistent with IA for three consecutive days and then every seven days until the resolutions of signs, non *Aspergillus* cause of infection or death. Of twenty-eight episodes in this cohort, five of were diagnosed as proven IA. The sensitivity was 40%, specificity was 100%, PPV 100%, and NPV 88 %.

HSCT recipients (cohort 4): This cohort comprised of patients undergoing both autologous and allogenic HSCT. Samples were taken on a weekly basis. There were

211 episodes in this cohort and one patient was diagnosed as possible IA and another as proven IA. The sensitivity was 100%, specificity was 91%, PPV 10%, and NPV 100%.

Overall, specificity was 94.8%; specificity was lower in children than in adults and lower in adult allo-HSCT patients compared to adults auto-HSCT recipients or non-transplant patients. GM testing was not useful in patients with febrile neutropenia with no clinical or radiological signs suggestive of pulmonary disease. Based on analysis of adults auto HSCT the authors have recommended a cut-off of 0.7 compared to 1.5. The authors have stated that this increases the sensitivity by 24% and decreases the specificity by 5.5%; the PPV decreases from 92% to 70%. Table II-17 shows the diagnosis of the episodes and results of antigen determination in each cohort. Results in Table II-18 show that, in adult HSCT patients, as the cut-off is increased from 0.6 to 1.5 the sensitivity decreased and specificity increased.

Table II-17: Demographics and number of antigenemic tests according to reason for determination

	Reason for Antigenemia Determination			
	Fever of Unknown Origin	Suspected Pulmonary Infection	Extrapulmonary Signs	Surveillance in Transplant Recipients
No. of episodes	261	297	28	211
No. of different patients	220	274	28	206
Age, years				
Median	50	55	54	41
Range	1-85	1-88	22-80	4 months-68 years
Male/female	147/114	202/95	15/13	110/101
No. of antigenemia assays	540	1481	122	1151
No. of antigenemia assays per episode				
Mean	2.1	5.0	4.4	5.5
Range	1-11	1-28	1-19	1-32

Table II.-18: Impact of different cut-offs on performance of the assay in adult nonallogeneic HSCT patients

	Cutoff					
	1.500 (%)	1.000 (%)	0.900 (%)	0.800 (%)	0.700 (%)	0.600 (%)
Sensitivity						
Definite IA (n = 26)	57.7	61.5	61.5	65.4	73.1	76.9
Probable IA (n = 61)	16.4	26.2	31.1	36.1	44.3	49.2
Possible IA (n = 47)	21.3	31.9	34.0	36.2	44.7	53.2
All cases (n = 134)	26.1	35.1	38.1	41.8	50.0	56.0
Specificity	99.4	98.5	97.5	95.4	93.9	88.7
PPV	92.1	87.0	81.0	71.8	69.8	58.1
NPV	82.7	84.4	84.8	85.3	87.0	87.7
Clinical efficiency*	83.3	83.8	84.4	83.6	84.3	81.5

*Clinical efficiency: (true-positive + true-negative)/total number of tests.

(15) Pinel *et al*., 2003: Study was conducted in France and 3237 serum samples from 807 patients (proven 3, probable 31, possible, 22 and 751 non IA) at high risk for IA, from Hematologic Departments and Intensive Care Units, were collected. One to two serum samples were collected each week. Diagnosis was made according to EORTC/MSG criteria of 2002. GM testing was done by Platelia *Aspergillus* EIA (BioRad Laboratories, France), and an O.D. index of ≥1.0 based on testing of 2 consecutive serum samples was considered a positive result. The results in Table II-19 show poor sensitivity (50%) of the assay for the detection of GM in patients with proven and probable IA. Specificity among non IA patients was 99.6%. The PPV and NPV were 85% and 98%, respectively.

Table II-19: GM antigenemia in patients with proven, probable, or possible IA or without IA.

Patient case category	No. of patients with:		Total no. of patients
	Positive Platelia result[a]	Negative Platelia result	
Proven IA	0	3	3
Probable IA	17	14	31
Possible IA	14	8	22
IA not retained	3	748	751
Total	34	773	807

[a] Patients with at least two positive antigenemia results were included.

(16) Steinbach *et al.*, 2007: The study was conducted in the U.S.A. and comprised of 64 pediatric subjects at high risk of developing IA infection who were undergoing hematopoietic stem cell transplant or with acute graft vs host disease. The serum samples were collected before initiation of prophylaxis and then 2 times a week during the period of neutropenia (< 500 cells/µL). Of the 64 patients, one patient was diagnosed as probable IA and 63 patients as non IA according to the EORTC/MSG criteria of 2002. GM detection was by Platelia *Aspergillus* EIA (BioRad laboratories). Positivity was based on a cut-off index of ≥0.5 as per manufacturer's instructions. The specificity was 87% overall and 92%, when patients on piperacillin/tazobactam therapy were excluded from testing. The researchers concluded that the test could be used in the early diagnosis of IA in high risk children. The level of false positive results was low and when false positive results were obtained the cause of the false result was explainable in terms of the physical status of the patient.

(17) Bretagne *et al.*, 1997: This was a prospective study in 50 patients with hematological malignancy or aplastic anemia. Patients were either neutropenic (< 500 cells/µL), on steroid therapy for > 10 days, or re-hospitalized with graft vs. host disease. Diagnosis of IA was similar to the criteria recommended by the EORTC/MSG in 2002. Serum samples were prospectively collected once a week for each patient until the risk factors ceased. A positive GM result by the EIA (Sanofi Pasteur, Paris) was based on a cut-off of ≥1 ng/mL (OD ≥ 0.8; it is not known to what extent the cut-off using a concentration of fungus can be equated with the cut-off index used in other assays) and a mean of testing of duplicate samples. Of the 50 patients, 3 were diagnosed as proven IA (*A. flavus*, *A. fumigatus*, and *A. ustus*, respectively), 3 probable IA (2 *A. fumigatus* and 1 *A. niger*), 9 suspected IA, 5 proven other fungal infections, 9 suspected of other fungal infections and 21 no fungal infections (Table II-20). About 6 samples (range 2 to7) per patient (a total of 310 samples) were tested. The authors state that 75 serum samples from 18 patients were GM positive based on a cut-off of ≥ 1 ng/mL but 16 were patients were positive based on testing of 2 consecutive samples. The 6 patients with proven or probable IA were all GM positive (Table II- 21). Of the 9 patients with suspected IA, 6 were GM positive when tested consecutively and died with high GM titers 3 patients were GM negative (2 were diagnosed with other infections and died). The specificity, sensitivity, PPV and NPV were 76%, 100%, 60%, and 100%, respectively.

Table II-20: Patient characteristics and mycological findings in 50 patients at risk of IA.

	Proven and probable IA	Suspected IA	Other proven fungal infections	Suspected other fungal infection	No fungal infection
Number of patients	6	9	5	9	21
Sex M/F	4/2	9/0	3/2	4/5	13/8
Mean age, years (range)	29 (9–47)	38 (22–53)	44 (21–62)	34 (7–51)	35 (6–68)
Underlying diseases					
Acute leukaemia	5	6	4	5	13
Other	1	3	1	4	8
Allogeneic bone marrow transplant	3	4	2	5	7
Autologous bone marrow transplant				2	3
Average of GM tests per patient (range)	11 (4–14)	6 (2–17)	7 (3–16)	6 (3–9)	5 (3–8)
GM positive patients*	6	6	1	2	1
Identified fungus	*Aspergillus fumigatus*: 3 *A. flavus*: 1 *A. ustus*: 1 *A. niger*: 1		*Candida tropicalis*: 1 *C. krusei*: 1 *Trichosporon capitatum*: 1 *Phialophora americana*: 1 *Fusarium solani*: 1		

* Patients with more than two consecutive GM titres ≥ 1 ng/ml.

Table II -21: Patient characteristics and mycological findings in 6 patients with proven or probable IA.

Patient	1	2	3	4	5	6
Sex/age	F/44	M/11	M/9	F/47	M/44	M/21
Underlying disease	AML	ALL	AML	AML	AML	AML
Allogeneic bone marrow transplant	No	Yes	Yes	No	No	Yes
Isolated *Aspergillus*	*A. flavus*	*A. fumigatus*	*A. ustus*	*A. fumigatus*	*A. fumigatus*	*A. niger*
Mean of isolation	Retro-orbital biopsy	Sinus biopsy	Pulmonar biopsy	Bronchoalveolar lavage	Protected tracheal catheter	Bronchoalveol
Positive GM (tests*/number of tests)	9/13	2/9	2/14	6/14	6/13	2/4
Outcome	Deceased	Deceased	Deceased	Survived	Survived	Deceased

* Positive GM titres were all consecutively positive.

(18) Rohrlich *et al.*, 1996: The study was performed in France and included 37 pediatric hematology patients. The patients were neutropenic (200 cells / µL for more than five days) caused by either chemotherapy, aplastic anemia or allogenic or autologous BMT procedure. Blood samples were collected upon entry to the study, before neutropenia or before BMT and then twice a week. The Platelia *Aspergillus* EIA (Sanofi Diagnostics Pasteur) with a cut-off of 0.9 ng/mL using two consecutive positive samples to define patient positivity. Twelve patients obtained positive GM results of these 10 presented with signs of IA. Six of the 12 positive patients had accompanying radiologic evidence. Other patients including 13 of the patients had cystic fibrosis were negative. The authors stated the PPV to be 83%. Sensitivity, specificity, and NPV were not specified but can be calculated to be 100%, 93%, and 100%, respectively.

(19) Penack *et al*., 2008: A prospective study was undertaken to establish the accuracy of the Platelia *Aspergillus* EIA (BioRad Laboratories) and to determine the optimal frequency of serum sampling using a cut-off index of ≥0.5 and testing only one serum sample per patient. Different groups (proven IA 6%, probable IA 5.5%, and possible IA 13%) of hematological malignant patients (n=200) with neutropenia (<500 cells/µL for >10 days) were tested at three time periods after the start of a febrile episode. Diagnosis of IA was according to EORTC/MSG criteria of 2002. The results in Table II-22 show the performance of the Platelia assay for diagnosis according to EORTC criteria (with GM positive results) and by exclusion of GM results.

Table II-22: PPV, NPV, sensitivity and specificity to predict proven or probable IA of the maximum positive GM test result of each patient.

	According to EORTC–MSG criteria	Exclusion of GEI for definition of IA
Incidence of proven/probable IA	23 patients (11.5%)	17 patients (8.5%)
PPV	82.1%	60.7%
NPV	100%	100%
Sensitivity	100%	100%
Specificity	97.2%	93.8%

Separate analyses were carried out using the EORTC–MSG criteria to define IA and excluding the GEI results for definition of IA.
PPV, positive predictive value; NPV, negative predictive value; IA, invasive aspergillosis; GEI, galactomannan enzyme immuno assay; EORTC, European Organization for Research and Treatment of Cancer; MSG, Mycosis Study Group.

Of the 200 patients, 126 patients were tested at the time of onset of fever and 118 were GM negative and eight were positive; all eight of the positive patients were eventually classified as either proven or probable IA (Table II-23). Seventy-six patients were tested three days after the onset of fever and treatment with a broad spectrum antibiotic, 58 were GM negative; all of the 18 positive patients were eventually classified as either proven or probable. Fifty-five patients were tested six days after the onset of fever and treatment with a broad spectrum antibiotic, 34 were negative and the remaining 21 patients that tested positive were eventually diagnosed as having proven or probable IA. The study did not indicate if the subjects in each group were the same. This study suggests that accurate results could be obtained by the use of one serum sample and that within a single patient the GM indices correlated with the clinical course of IA. The specificity and PPV of the tests on different days were 100% for all groups and sensitivity after 6 days of fever was 94.7%.

Table II-23: Diagnostic accuracy of the Platelia GM EIA for predicting a diagnosis of proven or probable IA

Clinical situation	Number of patients	PPV (%)	NPV (%)	Sensitivity (%)	Specificity (%)
Onset of fever	126	100	89.8	40	100
After 3 days of fever	76	100	95	84.2	100
After 6 days of fever	55	100	97.3	94.7	100
At diagnosis of pneumonia	48	100	81.8	71.4	100

Further, in this study the median indices for patients who recovered and who died were 1.7 and 9.5, respectively. This study used 76 patients who had been febrile for three days and did not improve with the treatment of broad spectrum antibiotics. Eighteen of those patients received positive GM results and were classified as either proven or probable IA. Three of the 58 negative patients subsequently developed IA. Another set of 55 patients was tested after six days of persistent fever and non responsiveness to broad spectrum antibiotics. Of these 21 were positive and were classified as proven or probable IA. Two of the 34 negative patients subsequently developed IA.

All of the patients who died had a GM index > 2.5 and only 3/18 GM positive patients who survived had an index > 2.5. In most patients the serum GM level tended to rise at the onset of fever and the increase in GM tended to rise faster in the patients who died than in those who survived. Figure II-8 shows a comparison between the GM levels of patients who recovered and those who died. The GM level of the recovered patients ranged from 0.6 to 3.9 whereas the level in those who died ranged from 2.9 to 22.8. As the patient responded to therapy the GM level decreased. Figure II-9 shows the difference in the increase of GM in patients who died indicated by a steep incline followed by a decrease as compared to the more gradual increase in the patients who recovered. The study showed that in febrile patients with no response to broad spectrum antibiotics for ≥ 6 days, the Platelia *Aspergillus* EIA was highly accurate in detecting GM. The additional time allowed the fungus more time to grow and therefore the concentration of GM in the blood was high.

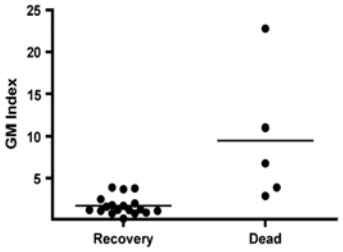


Figure II-8: Comparison of Serum GM levels in patients who recovered as compared to those who died

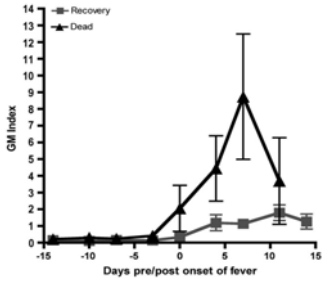


Figure II-9. The GM concentrations increase in patients who died ▲ vs those who recovered ■

The study also showed that the GM levels appeared earlier in bronchoalveolar lavage fluid as compared to serum (for details see microbiology review by Dr Shurland).

(20) Busca *et al*., 2006: In one prospective study, conducted between 2002 and 2004, 74 patients with allogenic HSCT (n=71) and solid tumors (n=3) were tested by Platelia *Aspergillus* EIA (BioRad Laboratories). Invasive pulmonary aspergillosis (IPA) was diagnosed according to EORTC/MSG criteria of 2002. Of these 7 were diagnosed as possible IA and 2 as proven IA. All patients received prophylaxis with fluconazole until day 100 post-transplant. Serum samples were collected twice per week for hospitalized patients and once a week for outpatients. GM positive results were based on a cut-off of ≥ 1.0. The time intervals between abnormal CT scans and those of a positive GM test were analyzed. The CT scans were taken after a patient showed any sign of pulmonary infection or after fever lasted for more than three days during treatment with a broad spectrum antibiotic. Of the nine patients who tested GM positive an abnormal CT scan was obtained in five patients, seven days (range 1 to 12 days) prior to the positive GM test result was reported. In four cases the GM became positive approximately four days (range 0 to 9 days) after the CT abnormality (Table II-24). The median time of CT abnormality was three days after the febrile episode. By the Platelia *Aspergillus* assay, five patients showed false positive results and there were no false negative results. Based on testing of 2 serum samples and a cut-off of ≥1.0, the sensitivity, specificity, PPV and NPV were 100%, 93%, 64% and 100%, respectively.

Table II- 24 Characteristics of patients with IPA

Patient	Underlying disease	Type of HSCT	Conditioning regimen	Neutropenia during diagnosis	Steroid >14 days	Type of IA	CT scan result	Time elapsed between +AGA and CT scan	Max OD index	Sites of aspergillus isolation	Time of diagnosis of IA post-HSCT	Treatment	Outcome
1	MM	MUD	RIC	Yes	No	Possible	Pulmonary infiltrate	−6	4.2	Nonisolation	+21	L-Amb	Dead, progressive disease
2	ALL	MUD	myeloablative	Yes	No	Possible	Pulmonary infiltrate	9	2.5	Nonisolation	+14	L-Amb	Dead, progressive disease
3	NHL	MSD	RIC	Yes	Yes	Proven	Pulmonary infiltrate Plus nodules	−1	1.2	Autopsy specimen	+175	Caspo	Dead, progressive disease and IPA
4	NHL	PMRD	RIC	Yes	Yes	Possible	Bilateral infiltrate	7	6.3	Nonisolation	0	L-Amb	Suspected IPA
5	MM	MUD	myeloablative	No	Yes	Possible	Pulmonary infiltrate cavitation	0	2.4	Nonisolation	+116	Caspo/L-Amb	Dead, progressive disease
6	CML	MSD	myeloablative	No	Yes	Proven	Infiltrate plus nodules	−8	7.3	Autopsy specimen	+343	ABLC	IPA and PCP
7	MM	MUD	myeloablative	Yes	No	Possible	Pulmonary infiltrate Plus nodules	−12	1.5	Nonisolation	+8	Caspo	Alive and well
8	AML	MSD	RIC	Yes	No	Possible	Pulmonary nodules cavitation	−8	1.3	Nonisolation	0	L-Amb	Alive and well
9	AML	MUD	myeloablative	Yes	Yes	Possible	Pulmonary infiltrate cavitation	2	1.5	Nonisolation	+16	Caspo	Alive and well

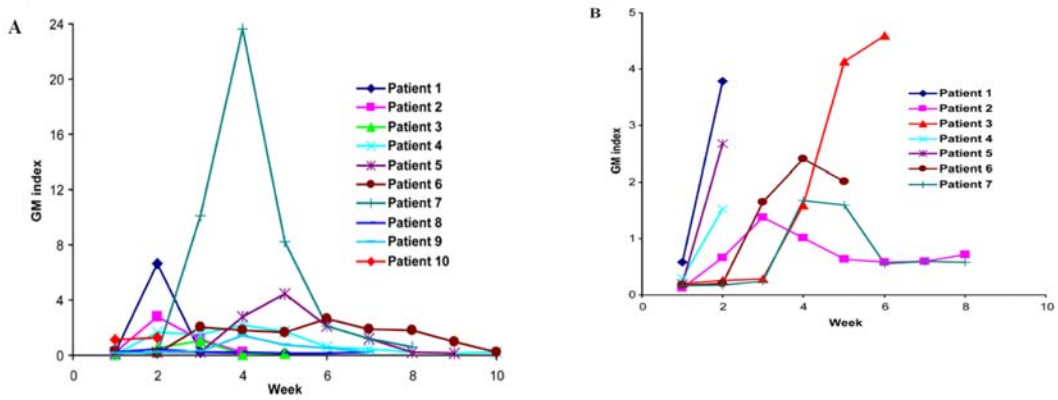
Abbreviations: MM, multiple myeloma; ALL, acute lymphoblastic leukemia; NHL, non-Hodgkin lymphoma; CML, chronic myelogenous leukemia; AML, acute myeloid leukemia; MUD, matched unrelated donor; MSD, matched sibling donor; PMRD, partially matched related donor; RIC, reduced intensity conditioning; IA, invasive aspergillosis; AGA, *Aspergillus galactomannan* antigen; HSCT, hematopoietic stem cell transplantation; L-Amb, liposomial amphotericin B; Caspo, caspofungin; IPA, invasive pulmonary aspergillosis; PCP, *Pneumocystis carinii* pneumonia.

(21) Suankratay *et al*., 2006: This prospective study took place in Thailand. The purpose was to evaluate the performance of The Platelia *Aspergillus* assay in the diagnosis of IA. The study took place between 2002 and 2004 in patients with hematologic disorders who were at risk of developing IA. Inclusion criteria were that the patients were older than 16 years of age, on chemotherapy and were expected to have neutropenia of < 500 cells/μL for at least seven days. Also, patients were undergoing allogenic bone marrow or peripheral stem cell transplantation. Patients who were undergoing autologous bone marrow transplant were excluded. The study patients were chosen consecutively. Patients were treated with broad spectrum antibiotics as soon as they became febrile and with amphotericin B if fever persisted more than 5 to 7 days, until the fever was resolved. Additionally, they were treated with 200- 400 mg/day of oral itraconazole solution twice a day throughout the period of neutropenia. Blood samples were drawn once or twice weekly and serum samples were stored at -70^{0}C until ready to be tested. Samples were tested according to Platelia *Aspergillus* EIA (Sanofi Diagnostics, Pasteur) with a cut off of ≥1 using two consecutive samples to determine positivity. Diagnosis was classified according to EORTC/MDG 2002 criteria.

The performance parameters based on five cut-off indices are shown in Table II-25. At the ≥ 1.0 cut off the sensitivity, specificity, PPV and NPV were 88%, 97%, 94% and 94% percent, respectively. The authors also studied the kinetics of GM indices in order to correlate indices with the clinical outcome. Figure II-10 (A and B) shows that patients whose GM indices returned to the baseline level survived whereas those who had persistently high GM indices eventually died. The study does not identify the number of possible or no IA episodes separately but classifies then collectively as 33 episodes.

Table II -25: Performance Parameters based on five GM cut-off indices for proven and probable IA patients in Thailand using Platelia *Aspergillus* EIA (Sanofi) using 2 consecutive samples.

GM index	Sensitivity	Specificity	PPV	NPV
0.50	94	67	59	96
0.75	94	79	70	96
1.00	88	97	94	94
1.25	89	100	100	94
1.50	77	100	100	89



FigureII-10: Kinetics of GM antigenemia from patients with proven and probable IA. (A) patients who survived and (B) patients who died.

(22) Pazos *et al.*, 2005: Forty neutropenic patients at high risk for IA were studied retrospectively to assess the utility of the (1→3) β-D-glucan test (Glucatell) for diagnosis of patients with IA. The study took place in 2001 and 2002 in Spain. The patients had previously been prospectively studied using the Platelia *Aspergillus* assay (BioRad) and the results had been used to classify the 40 patients as five proven, three probable, three possible IA and 29 patients without IA. The samples had been collected twice weekly and were stored after testing at -70^{0}C until they were used to perform the (1→3) β-D-glucan test. True positive results were defined as consecutive positive serum samples at an index of ≥1.5 with the retesting of the first positive sample. The criteria for classification of patient outcomes were according to EORTC/MSG, 2002. Fluconazole was given prophylactically to 9/40 of the patients; one patient received amphotericin B and another itraconazole. Treatment of fever included β-lactam and aminoglycoside antibiotics with the addition of vancomycin if the fever persisted more than two days after the initiation of treatment. Fungal blood cultures, with incubation up to 15 days were done as required using the BACTEC 9240. The study showed that the sensitivity, specificity, PPV and NPV for both the Platelia *Aspergillus* assay and the (1→3) β-D-glucan test were the same 88%, 90%, 70%, and 96%, respectively. All of the five patients with proven IA by Platelia *Aspergillus* EIA grew *Aspergillus* spp, four with *A. fumigatus* and one with *A. flavus*. All three of the probable patients grew *A. fumigatus* whereas none of the possible patients grew *Aspergillus spp.* None of the 29

non IA patients by the Platelia *Aspergillus* EIA grew *Aspergillus* spp. but there were 10 % false positive results in both tests.

(23) Marr *et al*., 2005: The purpose of this study was to investigate *in vivo* the impact of time and a clinical variable, treatment of patients with a mold-active agent on the sensitivity and specificity of the Platelia *Aspergillus* assay utilizing 46 patients with hematologic disorders and IA and 269 control patients. One arm compared treatment with itraconazole (92.5 mg/kg t.i.d.) versus fluconazole (400 mg q.d.) for antifungal prophylaxis. Secondly, febrile patients enrolled in the study received amphotericin B (1.0 mg/kg). A third cohort of hematologic malignant patients received cytotoxic therapy during neutropenic periods and antifungal therapy was administered at the discretion of the physician. Blood samples were collected twice weekly until Day 75 after stem cell transplant and the serum was stored at -70^{0}C. The Platelia *Aspergillus* assay was performed according to the manufacturer's specification. Results were based on three cut-off indices of 1.5, 1.0 and 0.5. Sensitivity and specificity were calculated on both a per-patient and per-test outcome. The data were restricted to those patients who had positive samples within 14 days prior or subsequent to diagnosis of IA. The day of diagnosis was defined as the day in which confirmatory tests were obtained. Patients were categorized as positive if they received a positive result during the 14 day diagnostic period. No patient classified as possible was included in the study. The results show that when the cut-off index was decreased from 1.5 to 0.5, the sensitivity for a proven or probable patient test increased from 43% to 70% and the specificity decreased from 93% to 70%.

To determine the effect of antifungal therapy and the closeness of sample collection to the diagnosis, the length of time between specimen collection date and diagnosis date was calculated. These data took into consideration whether or not the patient was receiving antifungal therapy at the time. Figure II-11 shows the results of the sensitivity of the assay from specimen collection to the time of patient diagnosis with probable and proven IA. Figure II-12 shows two receiver operator characteristic curves the upper curve drawn from results of the week before diagnosis and the lower curve the week of diagnosis (Week 0). The sensitivity of the test was highest during the week of diagnosis. The specificity was more accurate during the week of diagnosis and also when the patient was not being treated with an anti-fungal agent.

This study showed that

- GM indices in patients receiving mold-active agents are not as high as those from patients who are not being treated with mold-active agents. This might signify that fungal growth is inhibited or decreased in the presence of the agent. It is also suggested that different anti-fungal agents might affect the GM levels to varying degrees.
- GM sensitivity is greatest when a low cut-off is used and this is particularly important when the patient is receiving mold active agents.
- Indices from serum samples are highest closest to the time of diagnosis.

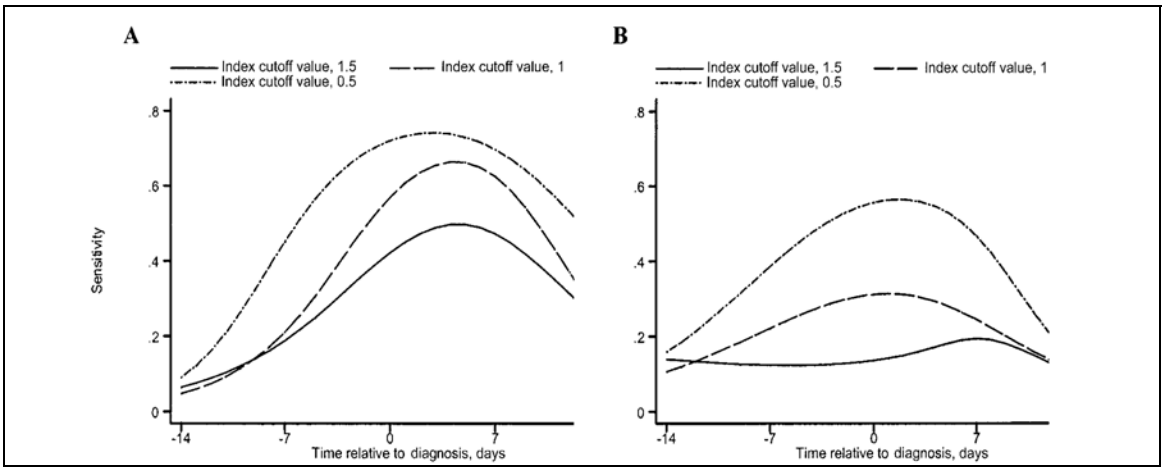


Figure II-11: Sensitivity of the Platelia *Aspergillus* assay in proven and probable patients as a function of time relative to diagnosis using cut-off indices of 1.5, 1.0 and 0.5. (A) shows patients not receiving antifungal treatments (B) patients receiving antifungal treatments

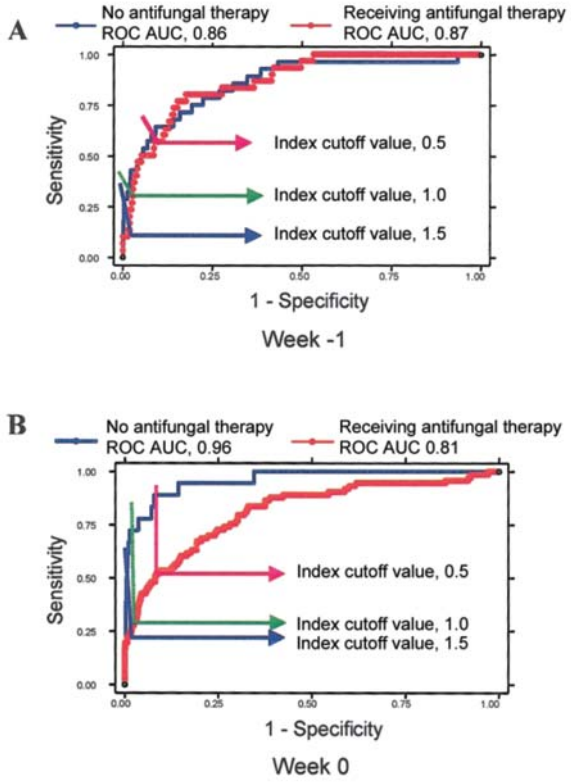


Figure II-12: Receiver operator characteristic curve graphing sensitivity versus specificity using multiple cut-off values to define positivity

(24) Marr *et al*., 2004: Sixty seven patients who received BMT for GVHD were evaluated. The patients received prophylaxis with fluconazole and amphotericin B if fever persisted inspite of antibiotic treatment, until the resolution of fever. Itraconazole was added after the diagnosis of IA was made. The diagnosis was made by X-ray, CT scan and culture of sputum and bronchoalveolar lavage (BAL) and biopsy if indicated There were13 proven cases, 11 probable, 8 possible and 35 control patients. The performance of the Platelia *Aspergillus* assay was evaluated on the basis of whether or

not the participants received antifungal treatment. Table II-26 shows the sensitivity of the samples if patients were treated with either itraconazole or amphotericin B within two weeks prior to the diagnosis of IA or whether they were not treated with those agents. The table shows that at a cut off of ≥1.0 treatment with mold-active agents decreased the sensitivity from 88 % to 20% for proven and 80% to 17 % for probable. In order to determine the optimal cut-off index the sensitivity and specificity of the test was measured at various cut-off values. The results in Figure II-13 show that the overall specificity did not vary significantly between the cut off indices of 1.5 and 0.8. The study indicated that the Platelia *Aspergillus* assay is useful for the screening of immunosuppressed patients especially in BMT with neutropenia and that the performance of the Platelia *Aspergillus* assay can be optimized by decreasing the cut-off index to define positivity. Additionally, factors such as antifungal therapy that reduce the fungal burden affects the results of the assay.

Table II -26: Performance of GM EIA at cut-off of 1.0 in patients being treated with antifungal agents compared to the untreated patients.

Diagnosis, by patient group	No. of patients testing positive	Sensitivity, %
Overall cohort		
Proven or probable IA (*n* = 24)	13	54.2
Proven IA (*n* = 13)	8	61.5
Probable IA (*n* = 11)	5	45.5
Receiving antifungal compounds		
Proven IA (*n* = 5)	1	20
Probable IA (*n* = 6)	1	16.7
Not receiving antifungal compounds		
Proven IA (*n* = 8)	7	87.5
Probable IA (*n* = 5)	4	80

NOTE. IA, invasive aspergillosis.

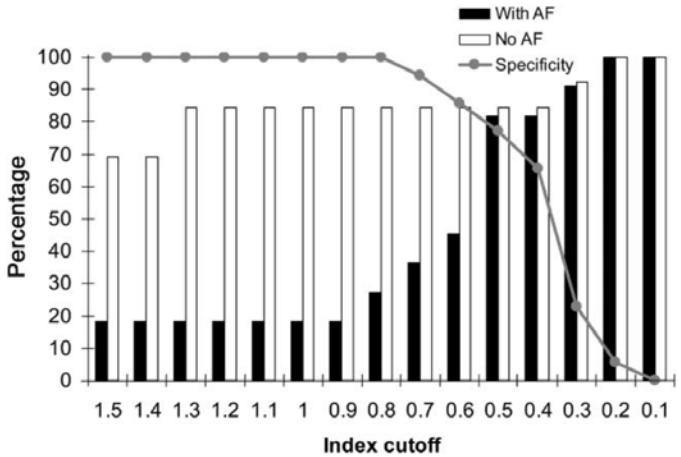


Figure II-13: Sensitivity and specificity of Platelia GM assay for proven and probable aspergillosis at various cut-off indices on the basis of whether the patient was receiving antifungal treatment or not.

(25) Rovira *et al.,* 2004. This prospective study took place between January, 1999 and January, 2001 in Spain and included 74 consecutive adult patients undergoing allogenic hematologic stem cell transplant. During hospitalization the patients were treated prophylactically with fluconazole for 60 days or itraconazole if the patient suffered from graft-versus-host disease. Febrile patients received meropenem (β-lactam) and vancomycin or an aminoglycoside was added if the fever persisted longer than 48 hours. If the fever persisted after 5 days of adequate intravenous antibiotic therapy or if pulmonary infiltrates had developed while receiving antibacterial therapy or if molds from upper or lower respiratory tract were isolated or if there was a relapse of fever after an afebrile interval of at least 48 hours when the patient was still on broad-spectrum antibiotic therapy, amphotericin B was added to the regimen. The patients were categorized as having proven, probable or possible IA according to EORTC/MSG 2002 criteria. There were one proven, five probable and two possible patients in the group.

Blood samples were collected twice weekly from the time of admission until discharge or death. If outpatient immunosuppressive treatment was continued samples were collected once weekly. Positivity was determined on the basis of a single sample at a cut-off index of >1.5, values between 1.0 and 1. 5 were categorized as indeterminate and < 1.0 was negative. If indeterminate, a second sample was immediately collected and tested Specimens collected from the lung (bronchoalveolar lavage samples) were cultured for bacteria and fungus. The fungi isolated from six patients were *A. fumigatus, A. flavus* and *A. terreus.* Two of the patients that grew no fungi were classified as possible.

The Platelia *Aspergillus* assay (Sanofi Diagnostics Pasteur) identified six of the IA patients (1 proven, 3 probable, 2 possible) but failed to identify two of the probable patients. There were no false positive results. Of the eight patients classified as either proven, probable or possible IA, six had been receiving amphotericin B when the EIA became positive. Two of the positive GM results preceded the development of pulmonary infiltrates and one coincided with pulmonary isolates. Overall, the study showed that the impact of patient treatment on the GM result was not significant. The sensitivity of the test was 75% and specificity was 100%; the PPV 100% and NPV 97%.

(26) Sulahian *et al.*, 1996: The purpose of this study was to compare the *Aspergillus* EIA (Pastorex *Aspergillus*, Sanofi Diagnostics) with a latex agglutination test for the diagnosis of IA. The study was conducted in France from August 1992 to October 1994. The 211 allogenic BMT recipients with previous hematological malignancies were chosen consecutively. There is a discrepancy between the stated (215) and actual (211) total of subjects listed in the table. The patients were housed in a laminar flow room and received fluconazole and ketoconazole for prevention of fungal infection and acyclovir for protection against herpes viral infection. There were 25 patients with confirmed IA, 15 probable cases, eight intermediate and 163 control patients with no IFI. Serum samples were obtained 15 or 7 days before BMT, weekly for the first

month after transplant, and then monthly for a mean of 90 days post- transplant (range 1 to 150 days), and daily when aspergillosis was suspected. The sera were stored at 4^0C if tested within 48 hours and -20^0C for longer periods. The specificity of the test was determined by adding sera from patients with other diseases e.g. candidiasis, cryptococcosis, protozoal and bacterial, and dysproteinemia and anti-lymphocytic sera either alone or with cyclosporin A. At a cut-off was O.D. 0.7, there were no false positive or false negative results for the controls. The sensitivity was 83%, specificity 81%, PPV 54% and NPV 95%. This study was conducted in the very early stages of the *Aspergillus* EIA. The authors state that the development of the EIA was an improvement over the other serologic tests being used at that time for the diagnosis and follow-up of patients with aspergillosis. The low threshold (<1 ng/mL) would allow aspergillosis to be suspected prior to or at the same time of the onset of clinical symptoms. The threshold was lower than that of the latex agglutination test with which it was being compared and which did not detect concentrations of GM below15 ng/mL.

(27) Sulahian *et al.*, 2001: The prospective study evaluated the sensitivity and specificity of the Platelia *Aspergillus* assay in detecting GM. The study conducted over a 4-year period (1995 to1998) involved 347 children with hematologic malignancies and 450 patients from the Bone Marrow Transplantation (BMT) Unit in France. Five of the pediatric patients were diagnosed as proven and four probable; of the BMT patients 22 were proven and 22 probable IA. The Platelia *Aspergillus* EIA (BioRad) was used as recommended by the manufacturer. A true positive result was defined as positive results at a cut-off index of ≥ 1.5 in two consecutive samples. The results in the BMT samples confirmed that there were 27 proven cases and 26 probable cases. Among the patients with proven or probable IA the EIA was positive in 48 patients that includes 39/44 BMT patients and 9/9 pediatric patients. In five patients positive GM results were obtained at the same time as the positive culture or positive CT scan. While in 19/48 patients positive GM results were obtained before the clinical symptoms became manifested with a mean delay of -6.9 days (range -21 to -1 day). Five patients with proven aspergillosis were consistently negative although at least three (3 - 32) samples were tested. Among 744 patients that did not present clinical symptoms 10/406 BMT and 34/338 presented with transient antigenemia. The study suggested that two possible causes of 'false positive' results in children were mucositis and early antifungal therapy with either amphotericin B or itraconazole or both. The sensitivity and specificity of the BMT patients were 89% and 98%, respectively and for pediatric patients 100% and 90%, respectively resulting in an overall sensitivity and specificity were 91% and 94 % respectively.

(28) Machetti *et al.*, 1998. This study was performed to compare the Platelia *Aspergillus* assay (Sanofi Diagnostic Pasteur) with the Latex Agglutination Pastorex *Aspergillus* test. The subjects comprised of 22 bone marrow transplant recipients. Both the EIA and the latex agglutination tests utilized the same monoclonal antibody EB-A2. EIA detects GM at 1ng/ml whereas the latex agglutination test detects GM at a minimum concentration of 1.5 nag/ml. The duration of this study was six months and tested patients from the day of transplant, Day 0 to Day 90 post transplant. No routine microbiological cultures were performed but were done during febrile episodes. The patients were treated prophylactically with pefloxacin and fluconazole during the

neutropenic period. When fever developed the antibacterial regimen was changed to ceftazidime and amikacin with the addition of vancomycin after 24 to 48 hours. After 5 days of treatment prophylactic fluconazole was discontinued amphotericin B started. Based on EORTC/MSG criteria patients were classified as proven, probable, possible, and no IA. Serum samples were collected three times per week in the first month after BMT and once / week in the second and third months. Positivity was defined as ≥ 2 positive consecutive samples at a cut-off index of > 1.5. Sample with indices between 1 and 1.5 were retested and were designated as positive only if the same index or greater was obtained. Of the 22 patients one was diagnosed as proven, three were probable and one was possible, the EIA identified three of the five positive IA patients with a sensitivity of 60% and a specificity of 82%. The latex agglutination test identified two of the positive patients and had a sensitivity and specificity of 40% and 94%, respectively. Therefore the Platelia *Aspergillus* assay was more sensitive but less specific than the latex agglutination tests

(29) Ja¨rv *et al*., 2004: In the case study of a 17 year old boy with stage IV Hodgkin's disease serum was tested for GM using the Platelia™ *Aspergillus* EIA (BioRad Laboratories) and the resulting GM indices were 0.7, 1.63, 0.8 and 0.5. The researchers in lowering the cut-off from 1.5 and 1.0 to 0.7 detected the presence of *Neosartorya pseudofischeri.* Had the cut-off been 1.5 or 1.0 and no fungus would have been isolated from the blood culture, the patient might not have been diagnosed with fungemia and successfully treated. The single index of > 1.0 might have been attributed to a contaminant and so too would the blood culture isolate. Therefore in this case cut-off indices of 1.5 and 1.0 were not sensitive enough for patient diagnosis.

B. Liver Transplant:

(1) Fortun *et al*., 2009: This was a prospective study and tested GM antigenemia in serum samples from 88 liver transplant patients. The patients were treated prophylactically with ampicillin and cefotaxime in the first two to five days of transplant. Patients who were allergic to β–lactam antibiotics, donors or recipients who were previously colonized or infected with microorganisms that were resistant to β-lactam antibiotics, patients with retransplants, or other special conditions were given other appropriate antibiotics. Antifungal prophylaxis was administered if patients met established criteria set out by the study. The test used was the Platelia *Aspergillus* assay and a true positive was defined as two consecutive positive serum samples at a cut-off of ≥ 0.5. During the study two patients were diagnosed as proven IA and one as probable and 85 as negative for IA. The sensitivity of the test was 67% and the specificity was 67%. There were 28 patients that were reported as false positives. Multivariate analysis showed that the only factor associated with false positive results was treatment with ampicillin. Authors tested aliquots from vials of ampicillin, piperacillin tazobactam and cefotaxime and negative controls of 0.9% saline solution in a retrospective study. None of the negative controls gave an index of ≥ 0.5, none of the cefotaxime were positive, 67% of the ampicillin samples were positive and so were 50% of the piperacillin-tazobactam. The study showed that administration of ampicillin or piperacillin –tazobactam soon after transplantation could give a false positive result.

(2) Kwak *et al.*, 2004: The purpose of this blinded study was the evaluation of the utility of the Platelia *Aspergillus* assay (BioRad) in the diagnosis of IA in liver transplant recipients. The study was conducted between September 2001 and October 2002 and in 154 liver transplant recipients. EORTC/MSG criteria of 2002 was used to categorize the patients. The patients were immuno-suppressed with tacrolimus, with / without mycophenolate mofetil, and low-dose prednisone. Routine anti-fungal prophylaxis was not administered. Blood samples were collected twice a week post transplant Positivity was determined by the positive results of ≥ 2 consecutive samples the first of which was retested. The cut-off index was ≥ 0.5. One patient was diagnosed as IA probable and 153 patients were negative. There were 20 false positive patients and the results showed that patients undergoing transplantation for autoimmune liver disease were more likely than others to have false positive results, possibly due to auto-reactive antibodies or paraproteins. Of the false positive patients seven received treatment with piperacillin-tazobactam. Additionally, it was shown that patients receiving dialysis were also more likely to have false positive results than those who were not. Twenty-six patients received mold-active anti-fungal agent and a false positive results was reported in 40% of the patients that died and 5% who survived. Among the 128 patients who did not receive mold-active anti-fungal agents false positive results were reported in 31 % of the patients died and 10 % of the survivors. Patients who died had a high number of false positives than those who survived and false positive test preceded death by approximately three weeks. The specificity of the test was 87% however, because only one positive patient was found an accurate determination of the sensitivity of the test could not be made. The sample agreement was 98.5%.

C Lung Transplant:

(1) Husain *et al.*, 2004. The purpose of this prospective, blinded study was the evaluation of the utility of the Platelia *Aspergillus* assay (BioRad) in the diagnosis of IA in lung transplant recipients. The study was conducted between June, 2001 and December 2002 in the U.S.A. and involved 70 consecutive lung transplant recipients, who were not neutropenic. The patients were followed for 18 months post transplant. EORTC/MSG criteria of 2002 were used to categorize the patients. The patients were immuno-suppressed with tacrolimus, cyclosporine A, mycophenolate mofetil, sirolimus and/or azathioprine, received ganciclovir for cytomegalovirus, and antifungal prophylaxis with fluconazole, itraconazole, amphotericin B or voriconazole. Samples were collected and the serum stored at -80^0C within 24 hours of collection. Positivity was based on positive samples with a retest of the first positive sample at a cut-off ≥ 0.5.

The study included nine patients with proven IA and three with probable IA. The Platelia *Aspergillus* assay was positive in three of the 12 patients with IA The positive GM test result from a patient with pulmonary aspergillosis preceded the histological diagnosis by three days; in a patient with systemic aspergillosis the positive result followed the histological diagnosis by four days after diagnosis of proven aspergillosis and seven days for the third patient. The assay sensitivity was 25% and the specificity

was 76%. Fourteen patients received false positive results. Patients who had not received antifungal prophylaxis had multiple positive tests but one patient who had received itraconazole prophylaxis for 14 days prior to diagnosis received only one positive test. Nine of the 12 patients with IA did not have a positive test within one week of the diagnosis. Five of the 14 false positive patients had an median of three (range 2 to 9) consecutive positive tests. Three of these patients had cystic fibrosis, had been treated with either voriconazole, amphotericin B, or itraconazole as prophylaxis and were colonized with *Pseudomonas aeruginosa.* Another patient was not treated with antifungal prophylaxis but was infected with methicillin-resistant *Staphylococcus aureus*. The fifth patient suffered from bronchiectasis as a result of graft-versus-host disease because of a sibling matched BMT. Ten of the 70 patients died within one year of transplantation, three of the patients had IA, two with pulmonary and one with systemic IA. Thus the Platelia *Aspergillus* assay in this population showed high specificity (76%) but poor sensitivity (25%) with PPV and NPV of 18 and 83%, respectively.