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Diagnosis of Invasive Aspergillosis by Galactomannan Antigenemia Detection using an enzyme immunoassay

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Abstract

Invasive aspergillosis is a serious and often fatal infection in patients who are neutropenic or have undergone solid organ or stem cell transplantation. Delayed diagnosis and therapy may lead to poor outcomes. Diagnosis may be facilitated by a test for galactomannan antigen detection using an enzyme immunoassay. Other rapid methods for diagnosis include (1→3)- β-D-glucan determination and polymerase chain reaction. The sensitivity and specificity of galactomannan antigenemia testing in serum and bronchoalveolar lavage specimens are high in patients with hematological malignancy, neutropenia, and receipt of stem-cell transplants. False-positivity can be seen with concomitant administration of some antibiotics and infection by fungi other than Aspergillus.

Introduction

Invasive aspergillosis occurs in about 10% of stem cell transplant patients and 5% of solid organ transplant patients. Diagnosis often is difficult (1) and antifungal therapy is usually initiated empirically, based upon fever and abnormal findings on chest CT. Typically the diagnosis is not proven by histopathology or culture. If treatment is targeted for invasive aspergillosis, and the patient has zygomycosis or another mold infection, it may not be effective. Thus, tests to assist in the accurate diagnosis of invasive aspergillosis may improve outcome. Also, a test that assists in early diagnosis may improve outcome by permitting treatment before the infection becomes irreversible (2). Furthermore, a test that indicates that invasive aspergillosis is unlikely may alert the physician to modify therapy or to pursue additional diagnostic procedures. An enzyme immunoassay (EIA) which detects an *Aspergillus* cell wall galactomannan, may assist in these tasks.

The test is primarily used for monitoring immunosuppressed patients for development of galactomannan antigenemia, as an early marker for invasive aspergillosis. The test also is used for diagnosis of suspected cases of invasive aspergillosis. The sensitivity for diagnosis on pulmonary invasive aspergillosis may be improved by testing bronchoalveolar lavage (BAL) specimens. Although the assay has its limitations, it is useful and complements other diagnostic methods.

Other tests used for diagnosis of invasive aspergillosis include (1→3)- β-D-glucan (BG) and polymerase chain reaction (PCR), but those methods have not been adequately evaluated to recommend them in the workup of invasive aspergillosis. This review will focus on the effective use of the galactomannan antigenemia EIA for diagnosis of invasive aspergillosis, but will not attempt to thoroughly summarize the vast literature, which is available in several reviews (3-7).

EIA for detection of *Aspergillus* galactomannan antigen.

The test is an enzyme immunoassay that uses rat monoclonal antibodies, which recognize ß (1→5)-linked galactofuranose (Platelia® *Aspergillus,* Bio-Rad Laboratories, Hercules, CA) (8). The assay is performed in sequential steps (Figure 1), and can be completed in about 4 hours (6). Results are expressed as "galactomannan index" (GMI), by comparison to the "cutoff" control. GMIs of 0.5 or higher are regarded as positive in the US and 1 to1.5 or higher elsewhere. All positive results should be verified by retesting a new aliquot of the specimen that was originally positive, and demonstration of reproducibility in a new specimen. Furthermore, the laboratory must perform the test at least daily Monday to Friday to provide the physician prompt results to be used in patient management. If testing is performed less frequently, several days may elapse before results are available, during which the infection may have progressed.

Aspergillus is common in the hospital and laboratory environment, thus contamination of the serum specimen at collection, processing or transport, or testing may be a cause for false positivity. During testing a splash from a positive kit control or another positive patient is also a cause for a false-positive test. Because of complexity and cost, the test is usually performed in reference laboratories or hospitals that specialize in bone marrow transplantation and treatment of hematological malignancies.

Monitoring for invasive aspergillosis during immunosuppression

The approved indication for the test is monitoring for *Aspergillus* galactomannan antigenemia in high-risk patients (Table 1). Using the test for this purpose, Maertens et al., requiring consecutive results with a GMI of 0.5 or higher as criteria for positivity, reported that sensitivity was 100% and specificity 97.5% (9). Others have reported lower sensitivity and specificity (6). A meta-analysis noted a sensitivity of 79% and specificity of 86%, with an overall accuracy of 89% (10).

To achieve at least 90% accuracy, monitoring should be performed at least twice weekly and consecutive samples with positive results should be required as criteria for positivity (Table 2). Additionally, monitoring should be restricted to patients who are at high risk for invasive aspergillosis (10). This includes patients with hematological malignancy, neutropenia, and allogeneic SCT. Monitoring is not recommended in solid organ transplant patients.

Several factors affect the accuracy of the test. First, antibiotics may cause false-positive results. The greatest problem has been in patients receiving piperacillin-tazobactam, amoxicillin or amoxacillin-clavulate, all fermentation products of *Penicillium* spp (11). More recently intravenous administration of the electrolyte solution, Plasmalyte®, has been reported to cause false-positive antigenemia (12,13). False-positivity should be excluded in a patient receiving one of these antibiotics or Plasmalyte. However, the positive galactomannan antigenemia result in a patient receiving these drugs may in fact be caused by invasive aspergillosis, and should not be disregarded without careful evaluation to exclude the diagnosis.

Other causes for false-positive results have been reviewed but are rare (Table 2) (3,6). Among these are contamination of the blood specimen with cotton, contamination of micro centrifuge tubes with cardboard (14), enteral feeding with soybean protein (15), and possibly gastrointestinal colonization with *Bifidobacterium* (16), several of which are thought to explain the higher false-positive rate in very young children (17).

Second, a positive result may occur in patients infected with fungi containing a cross reactive galactomannan. The monoclonal antibody used reacts with β-1-5-linked galactofuranose in the galactomannan of *Penicillium, Paecilomyces, Alternaria, Trychophyton, Botrytis, Wallemia, Cladosporium* (8,18), all which could give a positive result in the assay. False positivity has been observed in about half of specimens from patients with histoplasmosis who had positive results in the MVista™ *Histoplasma* antigen assay (19). At high concentrations (~1μg/ml) the *Histoplasma* galactomannan is detected in the galactomannan antigenemia. Others reported false-positive results in a patient with cryptococcosis (20), which was not confirmed by others (21,22). False-positive results also were reported in patients with ***P. marneffei*** (22), *Geotrichum* (23) and *Neosartoria* infections (24).

Third, treatment with antifungal drugs with activity against *Aspergillus* reduces the sensitivity of test. In one report sensitivity was 52% in patients receiving antifungal therapy versus 89% in those who were not (25). False-negativity should be considered in patients who are receiving antifungal agents that are active against *Aspergillus* if clinical, roentgenographic,

laboratory findings suggest the diagnosis. Although antifungal therapy reduced sensitivity, it did not eliminate the utility of the assay for diagnosis of aspergillosis, however.

Given the difficulty of establishing the diagnosis of invasive aspergillosis rapidly, and the importance of early therapy, the benefits outweigh the limitations, if the test is used properly. Nevertheless, a negative result does not exclude the diagnosis and a positive result does not establish the diagnosis of invasive aspergillosis. As with all tests, the antigenemia results must be considered in the context of the clinical, radiographically, and laboratory findings.

Evaluation for suspected pulmonary invasive aspergillosis.

Diagnosis usually requires bronchoscopy but the sensitivity of the routine mycological tests is low, and the imaging characteristics are neither sensitive nor specific. While a halo-sign is suggestive of invasive aspergillosis, its sensitivity was only 25%, and the air-crescent sign, cavities, and nodules did not differentiate invasive aspergillosis from bacterial infection (26). Furthermore, cytology or culture of bronchoalveolar lavage (BAL) fluid were positive in less than one-quarter of cases who underwent bronchoscopy (27). Sensitivity may be improved by testing BAL in the antigenemia EIA. Becker reported positive results in BAL in 100% compared to serum in 47% of cases of invasive aspergillosis in hematological patients (28) (Table 3).

In stem cell transplant patients, a sensitivity of 61% and specificity 98% was reported (29). In solid organ transplants, sensitivity ranged from 67%-100%, while specificity ranged from 91% to 98% (30,31). Specificity was 98-100% in those studies using a cutoff for positivity of 1.0 GMI. Positive results between 0.5 and 1.0 GMI may be seen in patients with airway colonization without evidence for invasive aspergillosis. However, airway colonization is concerning in transplant patients, and a few such patients later developed invasive aspergillosis in the Husain study (31).

In a study of immunocompetent patients, the galactomannan EIA test for BAL specimens was inferior to the combination of microscopy and culture (32). BAL specimens were also evaluated in intensive care unit patients (33). Culture or direct examination was positive for Aspergillus in 60% of cases, while serum antigen was positive in 42%. However, antigen was detected in BAL in 88%. Specificity for antigen detection in BAL (87%) was higher than for culture or direct examination (70%). The galactomannan EIA test has been validated for testing BAL specimens at MiraVista Diagnostics (see www.miravistalabs.com). In patients with a BAL and serum specimen obtained within one week of each other, and in which at least one was positive, the serum was positive in only 27% of cases in which the BAL was positive (Figure 2).

Although the Platelia® *Aspergillus* EIA has not been FDA cleared for testing BAL, these reports support a recommendation to test BAL if bronchoscopy is performed in immunocompromised patients at risk for invasive aspergillosis (Table 4). Detection of *Aspergillus* antigen in a patient with a compatible clinical syndrome would provide a compelling basis for treatment. However, careful assessment using traditional laboratory methods also is indicated, and clinical judgment is essential, considering the many potential causes for inaccurate results. Furthermore, if a positive result in serum is the sole laboratory basis for diagnosis, a follow-up specimen should be tested before mold-active antifungal therapy is initiated. It may be

appropriate, however, to initiate therapy for invasive aspergillosis before results of the second test are available.

Monitoring therapy for invasive aspergillosis.

Antigenemia declines during therapy (34-36). Woods and colleagues reported that failure of antigenemia to decline during therapy was associated with a poor outcome (37), supporting earlier reports (34-36). Survival was better in patients whose antigenemia cleared than in those with persistent antigenemia. Furthermore, in a subset of patients whose imaging studies worsened following neutrophil recovery, who otherwise did well without a change in antifungal therapy, antigenemia levels declined (37,38). These cases were felt to represent an immune constitution inflammatory syndrome (38). Renal failure may reduce antigen clearance (39).

Woods et al. recommended frequent antigenemia testing during therapy for invasive aspergillosis, and concluded that persistent antigenemia implied unresolved infection, requiring treatment modification (37). While these studies support a hypothesis that monitoring antigenemia during therapy may be useful, prospective studies are needed.

β-D-glucan detection.

(1→3)- β-D-glucan (BG), a cell wall component of many fungi, activates factor G of the horseshoe crab coagulation cascade causing activation of a chromogenic substrate(40). BG is found in most fungi, including *Aspergillus* and *Candida*. When compared to the galactomannan antigenemia test, the sensitivity was 55% in the BG test versus 100% for the EIA (41). Others reported a similar sensitivity for the two methods (42).

However, the BG test is not specific for invasive aspergillosis. Candidiasis is common in patients at risk for invasive aspergillosis, and the BG test does not differentiate between the two mycoses. The BG test is frequently falsely-positive for a variety of other reasons: bacteremia, hemolysis; intravenous administration of plasma proteins or coagulation factors, treatment with certain medications; hemodialysis using cellulose membranes; exposure to cotton bandages; heat stroke; and unknown causes (43,44). Several antibiotics, at high concentrations, may cause positive results in the BG test (45). For these and logistical reasons, the galactomannan antigenemia test is preferred for diagnosis of invasive aspergillosis.

Polymerase chain reaction.

Studies showing the feasibility of PCR for diagnosis of invasive aspergillosis by detection of *Aspergillus* DNA in the blood have been reviewed elsewhere (4,46). Sensitivity has ranged from 36% to 98% and specificity from 72% to 100% (41,43,47,48). However, when compared, PCR is no more sensitive than the galactomannan antigenemia test. In one report PCR was less sensitive than antigen detection, 45% versus 93%, respectively (41).

Several unanswered questions must be addressed before the role of PCR is known. Some of these include the optimal specimen type, DNA extraction method, primer specificity, and PCR

format. An additional factor in choosing between PCR and antigen detection is cost, as PCR is likely to be more labor intensive and expensive than antigen testing. For general use, a commercially-available, validated procedure is required. To date these issues discourage use of PCR for diagnosis of invasive aspergillosis.

Table 1. Use of the galactomannan antigenemia test for monitoring at risk patients

Population: Prolonged neutropenia, allogeneic SCT
Frequency: Two or three times weekly during high level immunosuppression
Criteria for positivity:

- Two consecutive serum specimens with GMI $\geq$ 0.5
- Always repeat the test before implementing therapy for invasive aspergillosis

Considerations:

- The galactomannan antigenemia EIA does not replace other tests in the workup of invasive aspergillosis
- Antibiotics produced by *Penicillium* spp. may cause false-positivity
- Medications/IV additives containing materials produced by *Aspergillus* (sodium gluconate) or *Penicillium* (certain antibiotics) may cause false-positivity
- Histoplasmosis (and other endemic mycoses) may cause false-positivity
- Mold-active antifungal drugs may cause false-negativity: repeat the test before implementing therapy for invasive aspergillosis
- Falsely-positivity or falsely-negativity may occur for other reasons: clinical correlation is imperative

Table 2. Causes for false-positivity or cross reactivity in the galactomannan antigenemia test

False-positivity caused by galactomannan contamination	Cross-reactivity caused by similar cell wall galactomannan
Piperacillin-tazobactam	*Penicillum* spp. including *P. marneffei*
Amoxacillin-clavulanate	*Histoplasma capsulatum*
Other beta-lactam antibiotics	*Geotrichum*
Plasmalyte (sodium gluconate)	*Neosartoria*
Other intravenous hydration or nutrition fluids containing sodium gluconate	Possibly *Paecilomyces, Alternaria, Trychophyton, Botrytis, Wallemia, Cladosporium, Bifidobacterium*
Possibly cotton, cardboard, soybean protein	

Table 3. Diagnosis of invasive pulmonary aspergillosis using the galactomannan antigenemia EIA on BAL specimens

Population	Cutoff 0.5		Cutoff 1.0		Reference
	Sensitivity-%	Specificity-%	Sensitivity-%	Specificity-%	
Hematology	Not stated	Not stated	100	100	(28)
Bone marrow transplant	76	94	61	98	(29)
Solid organ transplant	100	84	100	91	(30)
Solid organ transplant	67	95	67	98	(31)
Intensive Care Unit	88	87	Not stated	Not stated	(33)
Nonimmunocompromised	100	78	100	88	(32)

Table 4. Use of the galactomannan antigenemia test for diagnosis of invasive aspergillosis

BAL and serum for evaluation of suspected pulmonary invasive aspergillosis
Validate positive result by repeat testing before starting empiric therapy
Search for other evidence of invasive aspergillosis
- CT scan of lungs and sinuses and
- Histopathology and culture

Evaluate for causes for inaccurate results
- Galactomannan contamination
- Cross-reactive mycosis
- Mold-active antifungal therapy

Figure 1. Galactomannan antigenemia detection in the Platelia® Aspergillus EIA. The test specimen in heated at 100° C. for 3 minutes in the presence of 4% ethylenediaminetetraacetic acid (EDTA), after which the supernatant is removed and mixed with the enzyme labeled detector antibody. This mixture is incubated in the microplate wells precoated with the capture antibody. Then the plates are incubated with a chromogenic substrate, tetramethylbenzidine (TMB). If antigen is present color develops, which is recorded using a microplate reader.

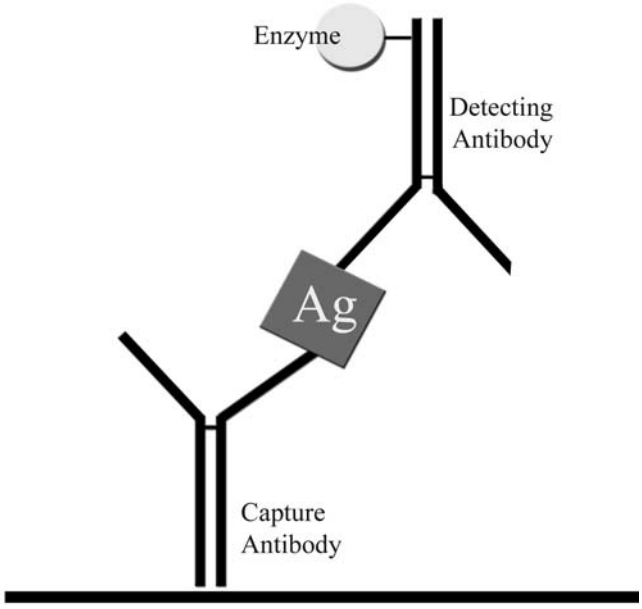
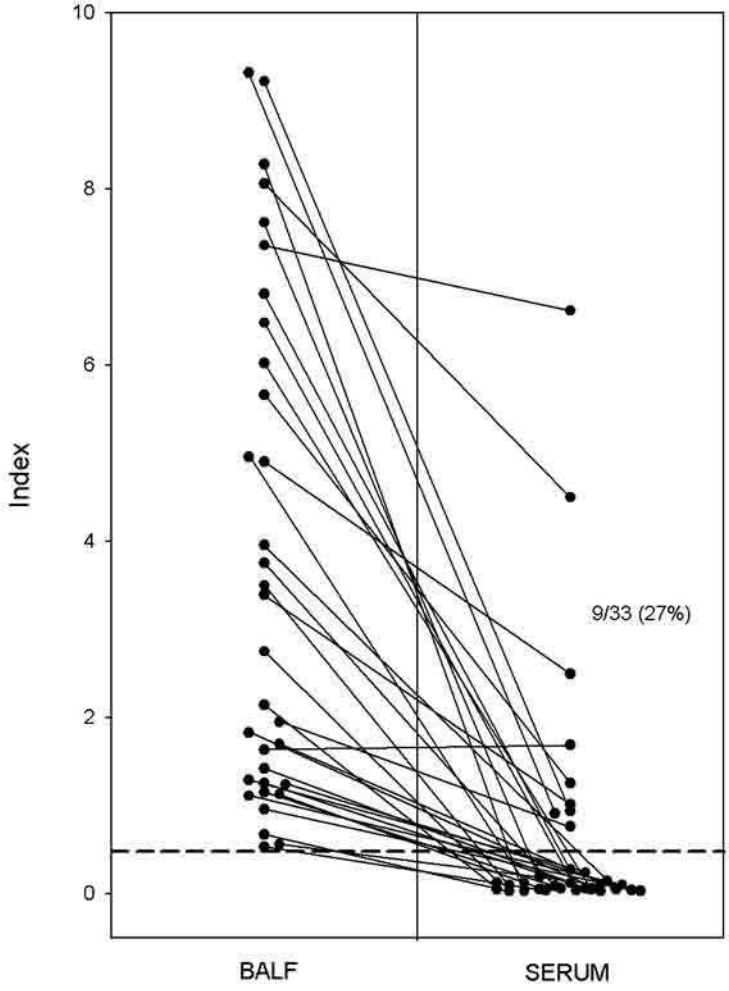


Figure 2. Galactomannan antigenemia EIA on BAL fluid (BALF) versus serum. These represent data for 33 cases in which either BAL or serum, obtained within one week of one another, were positive. All 33 BAL specimens but only 9 of 33 serums specimens (27%) were positive. In 29 of the 33 (88%) BAL specimens the result was >1.0.



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