

was employed as CMV prophylaxis as reported previously (8). Beginning December 2002, all patients received valganciclovir 450 mg twice daily (with dosage adjusted for renal dysfunction) as CMV prophylaxis. Valganciclovir was continued for 6 months in CMV seronegative recipients of seropositive allografts, and for 3 months in all others.

Prior to July 2002, all patients received antifungal prophylaxis with fluconazole 200 mg orally daily for 3 months. In patients with pre- or posttransplant colonization with *Aspergillus* spp. (except *A. niger*), fluconazole was substituted with oral itraconazole 200 mg twice daily, with or without inhalational amphotericin B deoxycholate. Since July 2002, oral voriconazole 200 mg twice daily was employed for 4 months as antifungal prophylaxis.

Definitions

Patients were considered as having proven or probable IA as per criteria proposed by the European Organization for Research and Treatment of Cancer/Mycoses Study Group and as previously reported in lung transplant recipients (8, 11). Patients with *Aspergillus* spp. cultured from respiratory tract samples who did not fulfill the criteria for IA or tracheobronchitis were considered to have colonization (12).

BAL Sampling and Galactomannan Antigen Testing

A total of two 50-mL and one 25-mL aliquots of normal saline were instilled sequentially via a bronchoscope wedged into a segmental airway of the right middle lobe or the lingula, and aspirated into sterile collection traps. Approximately 6 mL of the BAL fluid was submitted to the virology and microbiology laboratories for bacterial, mycobacterial, and respiratory viral cultures. The remaining pooled BAL fluid was spun and the resultant supernatant was stored at -80°C for future analysis.

The galactomannan antigen testing was performed at the conclusion of the study by Mira Vista Diagnostics (Indianapolis, IN) using one-stage immunoenzymatic sandwich microplate assay (Bio-Rad Laboratories Platelia *Aspergillus* EIA, Bio-Rad Laboratories, Marnes, France) as per manufacturer's instructions. Briefly, 300 μL of the BAL was added to 100 μL of 4% ethylenediamine tetraacetic acid solution. After vigorous homogenization, the tubes were heated at 100°C in a water bath for 3 min, followed by centrifugation. Then 50 μL of the supernatant and the horseradish peroxidase-labeled monoclonal antibody were incubated for 90 min at 37°C . After five washes, the plates were incubated with 200 μL of substrate chromogen reaction solution for 30 min. The reaction was stopped with 1.5 N sulfuric acid solution. Optical density (OD) was read at 450 nm (reference 620 nm). Positive, negative, and cutoff controls were incorporated in each assay. An OD index of 0.5 was considered positive. All positive samples were retested and considered positive only if the repeat test was also positive.

Statistical Analysis

Sensitivity and specificity were calculated in reference to the diagnosis of IA by using the total number of patients in the study. Specificity was also determined based on the total number of tests in the patients without evidence of IA. Since all observations (galactomannan test) within a patient are correlated, we used the generalized estimating equations

(GEE) analysis to account for the correlation in multiple values from the same subject. The binary outcome is analyzed using a clustered logistic model with robust variance to assess the sensitivity and specificity (13, 14). Receiver operative curves (ROC) were also generated to determine the optimal cutoff point of the test. The number of tests performed per patient and the days posttransplant to onset of infection were compared using the Mann-Whitney test or Kruskal-Wallis test. Categorical data (use of antifungal agents, prior history of rejection, etc.) were compared using the chi-square or Fisher Exact test.

RESULTS

Demographic and clinical characteristics of the study patients are outlined in Table 1. IA was documented in 5%

TABLE 1. Demographic and clinical characteristics of lung transplant recipients (n=116)

Characteristic	Median (range) or percent (n)
Age, years	52.3 (18–65)
Sex	
Male	44.0% (51)
Female	56.0% (65)
Type of transplant	
Bilateral lung	55.2% (64)
Single lung	43.1% (50)
Heart/lung	1.7% (2)
Underlying lung disease	
Emphysema	27.6% (32)
Idiopathic pulmonary fibrosis	20.7% (24)
Cystic fibrosis	20.7% (24)
Alpha 1 antitrypsin deficiency	10.3% (12)
Sarcoidosis	5.2% (6)
Primary pulmonary hypertension	4.3% (5)
Scleroderma	2.6% (3)
Retransplant	1.7% (2)
Silicosis	1.7% (2)
Calcinosis, Raynauds, esophageal dysmotility, sclerodactyly, and telangiectasia	1.7% (2)
Eosinophilic granuloma	1.7% (2)
Other ^a	1.7% (2)
Cytomegalovirus status ^b	
Primary or mismatch (R-/D+)	19.8% (22)
Recipient positive	58.5% (65)
Recipient and donor negative	21.6% (24)
Diabetes mellitus	27.8% (32)
Induction regimen	
None	10.4% (12)
Daclizumab	27.6% (32)
Thymoglobulin	26.7% (31)
Alemtuzumab	35.3% (41)

^a Other includes one case of obliterative bronchiolitis and one case of pulmonary fibrosis.

^b Not available for five patients.

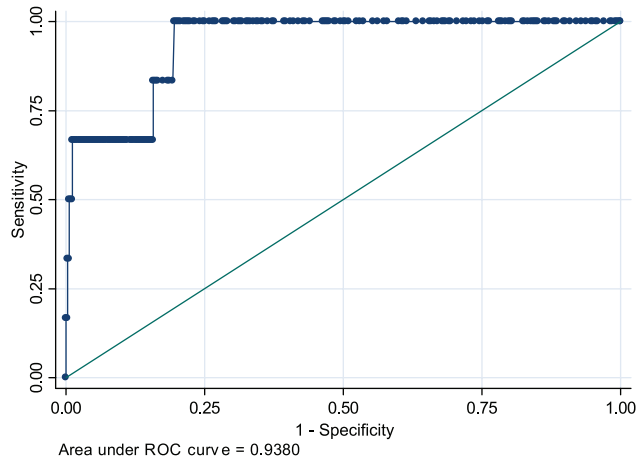


FIGURE 2. Receiver operative characteristics (ROC) curve in the generalized estimation equation (GEE) model for the galactomannan test. The ROC is plotted between the true-positive rate (sensitivity) on the y-axis, and the false-positive rate (1-specificity) on the x-axis. Area under curve (AUC) represents the accuracy of the galactomannan test and was 0.938 (standard error: 0.03, 95% CI: 0.86–1.0).

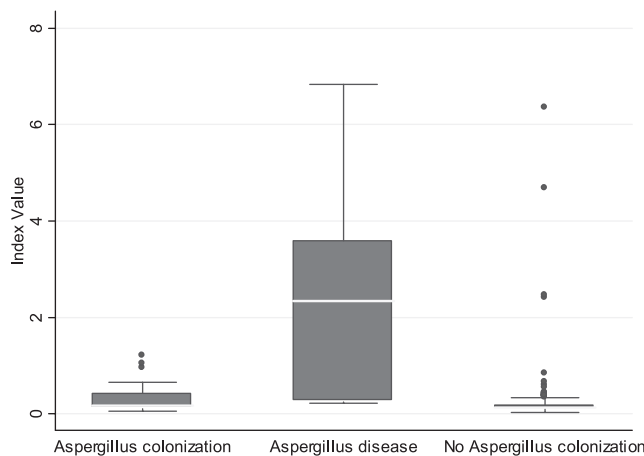


FIGURE 3. Box plots of the median Galactomannan index values between BAL samples of patients with *Aspergillus* colonization, *Aspergillus* disease, and no *Aspergillus* colonization ($P=0.0001$).

Overall, 13 patients had 15 false-positive tests (≥ 0.5). Four of the 13 patients subsequently developed IA. In two patients (patients 2 and 4, Table 3), galactomannan in the BAL was positive at least 60 days prior to the diagnosis of IA. In patient 2, the index increased from an earlier value of 0.54 to 1.21 at the time of the diagnosis of IA, and in patient 4 the galactomannan index increased to 6.83 from an initial value of 0.63. Two other patients (patients 5 and 10) had positive indices >1 year prior to IA. Patient 5 received antifungal prophylaxis with voriconazole for 6 months; the index value of 1.20 declined to negative indices later. Patient 10 underwent endobronchial stent placement and was subsequently placed on long-term prophylaxis with itraconazole for bronchomalacia in the transplanted lung.

False-positive tests with index values >2.0 were documented in four patients (Table 3). Of these, two patients (pa-

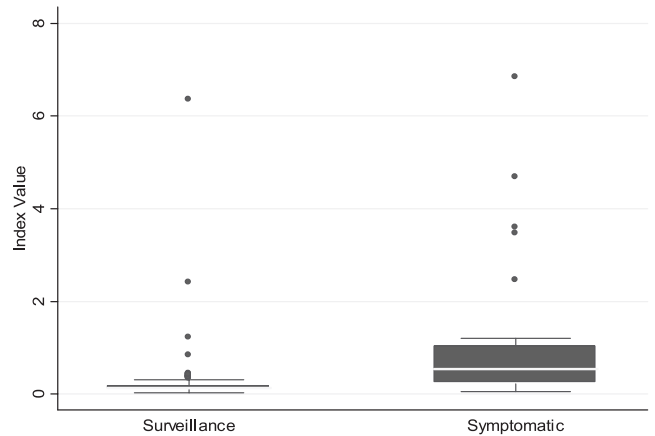


FIGURE 4. Box plots of the median Galactomannan index values between BAL samples of patients who underwent bronchoscopy due to the development of respiratory symptoms vs. those who underwent surveillance bronchoscopy ($P<0.0001$).

tients 7 and 12, Table 3) colonized with *Penicillium* spp. had index values of 2.40 and 6.34 each, one patient (patient 11) colonized with *Paecilomyces* had an index value of 4.67, and one patient receiving piperacillin-tazobactam had galactomannan index value of 2.40. However, the rate of colonization with *Penicillium* or *Paecilomyces*, and the receipt of piperacillin-tazobactam did not differ significantly for patients with false positive and true negative tests. Three of 18 patients colonized with *Aspergillus* spp. had a false-positive test (patients 1, 3, 9; Table 3). Two of these (patients 3 and 9) were concomitantly receiving itraconazole; both had index values of <1.0 , while the third patient without receipt of antifungal prophylaxis (patient 1) had index value of >1.0 .

We also calculated the sensitivity, specificity, and the likelihood ratios using the GEE regression analysis. Based on this model, at a cutoff of ≥ 0.5 , the sensitivity was 60%; specificity was 95%, with positive and negative likelihood ratios of 14 and 0.14, respectively. Increasing the cutoff value to ≥ 1.0 retained the sensitivity of 60%, while the specificity increased to 98% with positive and negative likelihood ratios of 28 and 0.44, respectively (Fig. 2).

DISCUSSION

We have previously reported that serum galactomannan had a sensitivity of 30% for the diagnosis of IA in lung transplant recipients. Since a vast majority of the *Aspergillus* infections after lung transplantation comprise tracheobronchial or pulmonary infections, we evaluated the performance characteristics of galactomannan assay in the BAL for the diagnosis of IA in these patients. Using the standard cutoff index value of 0.5 for the serum, galactomannan detection in the BAL had a sensitivity of 60% and a specificity of 95%. The specificity increased to 98% when the index cutoff value of 1.0 was used, while the sensitivity remained the same.

The higher diagnostic yield of galactomannan detection in the BAL in our study may be a function of greater hyphal burden in the BAL. Galactomannan is released predominantly from the hyphae and to a lesser extent from the conidia. Furthermore, the antigenic determinants released by

TABLE 3. False-positive galactomannan tests in 13 lung transplant recipients

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

the conidia are only weakly reactive in immunologic assays in contrast to those released by the hyphae (15). The presence of galactomannan in the BAL fluid therefore is likely to be a better diagnostic indicator for hyphal growth than routine mycological culture (15, 16).

Studies in animal models and in other immunocompromised hosts have evaluated galactomannan in the BAL for the diagnosis of IA. In a rabbit model of invasive pulmonary aspergillosis, the galactomannan at a cutoff index value of 0.75 had a sensitivity and specificity of 100% (17). In another animal model study, the sensitivity of galactomannan assay in the BAL increased from 25% to 100% by day 5 (16). However, in two studies comprising neutropenic patients, serum as compared to BAL galactomannan was more sensitive in diagnosing IA (18, 19). To our knowledge, ours is the first report that has utilized galactomannan in the BAL for establishing the diagnosis of IA in lung transplant recipients. Our data show that the sensitivity and specificity of the galactomannan test in BAL was higher than the serum as previously reported in lung transplant recipients (8).

As per the FDA-approval criteria, an index cutoff value of ≥ 0.5 in serum is considered positive. However, an optimal cutoff index value for galactomannan in the BAL has not been determined. Previous studies have employed index cutoff values ranging from ≥ 1.0 –1.5 with sensitivity of 85–100% (18–21). However, none of these studies performed the ROC analysis which is widely used in medical data analysis to study the effect of varying the threshold on the numerical outcome of a diagnostic test. One study which performed the ROC analysis in hematopoietic stem cell transplant recipients reported a sensitivity of 76% at index cutoff of ≥ 0.5 ; the sensitivity decreased to 61% with index cutoff value of ≥ 1.0 (22). Using a GEE regression analysis; we have documented a sensitivity of 60% at both cutoff index values of ≥ 0.5 and ≥ 1.0 .

The lower sensitivity in our study may be due to two reasons (18, 19). First, our study population included non-neutropenic patients in whom serum galactomannan gener-

ally has lower sensitivity than neutropenic hosts. Neutropenic patients may have greater fungal burden due to impaired clearance of fungal mannan from the bloodstream by granulocytes (4, 23–27). It is plausible that galactomannan in the BAL may have correspondingly lower sensitivity in nonneutropenic patients. Second, we used GEE regression model for the calculation of sensitivity and specificity. This model is well suited for analyzing correlated and repeated measures such as multiple galactomannan values in the same patient, but is more conservative compared to the ROC or sensitivity/specificity calculations reported in the literature (13, 14).

Antifungal agents can potentially lower the sensitivity of the serum and BAL galactomannan for the detection of IA by lowering the residual fungal burden (16, 17, 20, 22, 28, 29). In our study, false-negative tests were documented in two patients with IA; both were receiving antifungal agents. Additionally, 13 patients had false-positive results. Of these, four patients subsequently developed IA, including two in whom the positive results preceded the diagnosis by 60 days. In patients with hematologic malignancy and hematopoietic stem cell transplant recipients, positive serum galactomannan indices have been shown to precede the diagnosis of IA by 12–30 days (4, 30, 31). We believe that the positive galactomannan in two patients in whom the test preceded the clinical diagnosis of IA represented an early indicator of IA. The other two patients with false positive tests received voriconazole and itraconazole after the bronchoscopy resulting in negative galactomannan tests. In these patients, antifungal therapy may have aborted the progression of subclinical infection to IA.

False-positive galactomannan tests have also been reported in patients receiving piperacillin-tazobactam (32–34). Two of the four patients who received piperacillin-tazobactam in our study had positive galactomannan index values. We have previously shown that the timing of the collection of serum samples from patients may influence the test results, with reactivity being less likely in samples collected at trough levels or prior to

the administration of the antibiotic (35). Moreover, the epithelial lining fluid concentration of piperacillin is 56% of the serum steady state concentrations (36). Thus galactomannan in BAL may be potentially less likely to yield a false-positive test as compared to the serum. Whether the two patients with false positive tests received piperacillin-tazobactam just prior to the bronchoscopy and achieved peaked concentration in the epithelial lining fluid at the time of procedure is a possibility that we are unable to confirm.

Galactomannan may have cross reactivity with other fungal antigens, such as *Penicillium*, *Paecilomyces*, and *Alternaria* species (37, 38). In our study, only 10% (2 of 20) of the patients with *Penicillium* colonization had a positive test. This suggests that despite colonization of the respiratory tract with *Penicillium*, galactomannan in the BAL can be used to diagnose IA with a reasonable accuracy. Only 17% (3/18) of patients with *Aspergillus* colonization had true false-positive tests. This is comparable to our previous study in which the false-positive rate of serum galactomannan was 14% (8). We note that we used sterile saline and not Plasmalyte (Baxter Healthcare Corporation Deerfield, IL) for BAL sampling, which may cause false-positive galactomannan tests in the BAL (39). Plasmalyte is an intravenous solution that contains sodium gluconate and is produced by fermentation in mold cultures, including *Aspergillus flavus* and *Penicillium* (39).

Our study represents the largest cohort of lung transplant recipients in whom consecutive BAL samples were prospectively collected. However, there are limitations of our study that deserve to be acknowledged. First, the number of cases of IA was small. We, however, note that the clinical investigators were blinded to BAL results and the criteria employed for determining the type of IA, and fungal colonization were defined a priori. Likewise, the laboratory personnel were blinded to the clinical diagnosis of the patient. Furthermore, data were prospectively collected thereby obviating any subjective bias that may confound a retrospective study. Second, the optimal processing of BAL fluid for the performance of the galactomannan assay is not known and the quantity or type of specimen (cell pellet vs. supernatant) or the preparation method may alter the performance characteristics of the test. Finally, we did not collect concurrent serum samples. We have previously reported the results of the galactomannan testing in sera collected prospectively in lung transplant recipients and believe that concomitant blood collection would have added little to the robustness of the data (8).

In summary, the findings have important clinical implications for the management of lung transplant recipients. We show that a galactomannan index value of ≥ 1.0 may be used as a decision facilitating tool in discerning the treatment threshold for IA in these patients. Assuming 6% prevalence of IA, index value of ≥ 1.0 as a positive test will increase the posttest probability of IA to 62%, whereas an index value of < 1.0 will decrease the posttest probability to 2%. This is in sharp contrast to our earlier study of serum galactomannan in lung transplant recipients where neither a positive nor a negative galactomannan test altered the treatment threshold (8). Thus, although future studies validating our findings are warranted, the test may be used in all lung transplant recipients undergoing bronchoscopy as an aid in the diagnosis of IA.

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