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Diagnosis of pulmonary histoplasmosis and blastomycosis by detection of antigen in bronchoalveolar lavage fluid using an improved second-generation enzyme-linked immunoassay

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Summary Antigen detection is a useful adjunct for the diagnosis of histoplasmosis. The purpose of this study was to evaluate antigen detection in bronchoalveolar lavage (BAL) fluid using an improved second-generation *Histoplasma* antigen assay. Antigen was detected in 16 of 19 (84%) cases of histoplasmosis and 5 of 6 (83.3%) blastomycosis cases using the second-generation assay vs. 13 of 19 (68%) and 4 of 6 (66.7%), respectively, in the original assay. Ten-fold concentration permitted detection of antigen in an additional case of histoplasmosis and another with blastomycosis, for an overall sensitivity of 23 of 25 (92.0%). Specificity was 98.2% in both assays in controls with other pulmonary infections. These findings support the diagnostic utility of the second-generation assay in patients with pulmonary histoplasmosis and blastomycosis.
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Introduction

Antigen detection has proven useful in the diagnosis of histoplasmosis.[1] In disseminated disease, antigen was detected in the urine in 92% and the serum in 85% of patients.[2] *Histoplasma* antigen can be detected in the urine in 80% of patients with

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acute, diffuse pulmonary involvement, 34% with subacute pulmonary involvement, and 14% with chronic pulmonary involvement.[3] Those studies used the originally described radioimmunoassay method.[1] Subsequently detection of antigen in bronchoalveolar lavage (BAL) fluid in 70% of specimens from patients with AIDS and disseminated histoplasmosis was reported.[4] The Histoplasma antigen test was adapted to an enzyme immunoassay format in 1997,[5] and recently modified to improve sensitivity and specificity. In the new second-generation *Histoplasma* antigen assay, antigen was detected in the urine in 24 of 33 (73%) cases with false negative results using the original method (Wheat, *Transpl Inf Dis* 2006, in press).

Cytology or histopathology on BAL or transbronchial lung biopsy specimens has limited sensitivity for diagnosis of histoplasmosis.[2] While histopathology using Gomori methenamine silver and Giemsa stain was positive in 70% of BAL specimens in patients with AIDS and disseminated histoplasmosis with pulmonary infiltrates, the sensitivity of these methods in patients with the other histoplasmosis pulmonary syndromes is expected to be lower. For example, in the self-limited, subacute pulmonary histoplasmosis cultures of bronchial washings were positive in none of 35 patients, sputum in 7 of 72 (10%) and lung biopsy in 3 of 14 (21%).[6] Since culture is more sensitive than histopathology in histoplasmosis,[2] diagnosis by cytology or histopathology is likely to be even lower than 10%. Although culture is more sensitive in chronic pulmonary histoplasmosis, positive in half to three-quarters of cases,[2,6,7] organisms were seen by fungal stain of respiratory specimens in only one of six (17%) patients in one review.[2] Fungal stains of respiratory specimens are positive in less than 20% of cases of the "epidemic" form of acute pulmonary histoplasmosis following high inoculum exposure (Wheat, Expert Opin Biol Ther, 2006, in press). Furthermore, fungal stains may be falsely positive due to infection or colonization with yeast morphologically similar to *Histoplasma capsulatum*. These limitations of antigen testing of urine and serum, and cytology and histopathology of respiratory specimens for rapid diagnosis of the pulmonary histoplasmosis syndromes, and the improvement in sensitivity of the second generation *Histoplasma* antigen assay forms the basis for evaluation of BAL specimens in the new assay.

The following is an illustrative case where *Histoplasma* antigen detection and analysis of the BAL provided a rapid diagnosis when the urine antigen was borderline positive and the serum antigen was negative:

A 61-year-old woman had undergone an orthotopic liver transplant in 1993 for primary biliary cirrhosis. Six years later she developed pulmonary histoplasmosis, was successfully treated, and placed on maintenance fluconazole therapy until February 2001. She developed lung infiltrates associated with a chronic pulmonary illness from November 2001 until March 2002. The *Histoplasma* urine antigen was weakly positive at 2.4 units and the serum antigen was negative. A bronchoscopy with BAL was performed. The *Histoplasma* antigen was strongly positive when performed on the BAL fluid (11.15 units using the old assay and 52.2 units when retested using the new assay). The fungal stains of the BAL were interpreted as small budding yeast, not specifically noted as resembling *H. capsulatum*. Three weeks later the BAL cultures grew *H. capsulatum*, confirming the diagnosis of pulmonary histoplasmosis.

Antigen detection in the BAL fluid assisted in the rapid diagnosis of histoplasmosis in this complicated patient.

Methods

Patient specimens

Specimens were obtained from the following sources: (1) specimens submitted to the Indiana University Medical Center for microbiological testing, (2) specimens submitted to the Clinical Bronchoalveolar Lavage Laboratory at the Indiana University Medical Center for analysis of immunologic lung disease, or (3) specimens submitted to MiraVista Diagnostics for antigen testing. As the specimens were obtained from three different laboratories, procedures for processing the specimen storage differed. Specimens were stored refrigerated or frozen for variable time periods prior to testing. In order to evaluate the utility of a concentration step, 16 specimens were concentrated ten-fold using 5000 MWCO Amicon Ultra Centrifugal Filter Devices (Millipore).

Medical records were reviewed to confirm the final diagnosis of fungal infection for the specimens obtained at Indiana University Medical Center. For specimens from patients with histoplasmosis and blastomycosis submitted to MiraVista Diagnostics, minimal clinical information was available, namely the basis for diagnosis (positive culture, serology, histopathology or antigen). Cases were classified as histoplasmosis or blastomycosis if confirmed by microbiological or histopathology. Control specimens included 16 with other fungal infections and 38 with non-fungal infections. The other fungal infections included cases with positive histopathology or culture for *Cryptococcus neoformans* and

Pneumocystis carinii, and specimens containing elevated levels of *Aspergillus galactomannan* in the Platelia *A. galactomannan* assay.[8] Non-fungal infection controls included specimens from patients with positive culture for mycobacteria, bacteria, and fungal organisms that clinically represented colonization (*Aspergillus, Candida*) rather than true infection.

Histoplasma antigen detection assay

The "original" assay was a sandwich enzyme immunoassay (EIA).[1] Briefly, the assay uses microplates coated with rabbit IgG antibodies to *H. capsulatum.* Antigen present in body fluids attaches to the solid phase capture antibody, and is detected with an enzyme labeled detector antibody, creating a color reaction that is measured using a microplate reader. The new second-generation assay employs a biotinylated detector antibody that has been chemically modified to improve sensitivity and specificity (Wheat, Transpl Inf Dis, 2006, in press). In both assays, results were classified as positive or negative by comparison to a negative control specimen[5]; and result greater than twice the optical density of the negative control cutoff were considered positive. The optical density of the patient specimen was divided by the cutoff optical density, and results were reported as assay units, with results above one unit being positive.

Statistical analysis

Wilcoxon Rank Sum Test was used to compare the results of the antigen test using the two assays described. Chi-square test was used to compare the proportions between the old and the second-generation assay.

Results

To compare the second-generation assay with the original enzyme immunoassay, BAL specimens from 19 patients with histoplasmosis, 6 cases of blastomycosis and 54 controls were tested in both assays simultaneously. Among the histoplasmosis cases, antigen was detected in 16 out of 19 (84%) in the second-generation assay vs. 13 of 19 (68%) in the original assay. The second-generation assay was positive in 5 of the 6 (83%) patients with blastomycosis compared to 4 of the 6 (66%) in the original assay (Fig. 1). Thus, overall BAL from 21 of 25 (84%) histoplasmosis or blastomycosis patients were
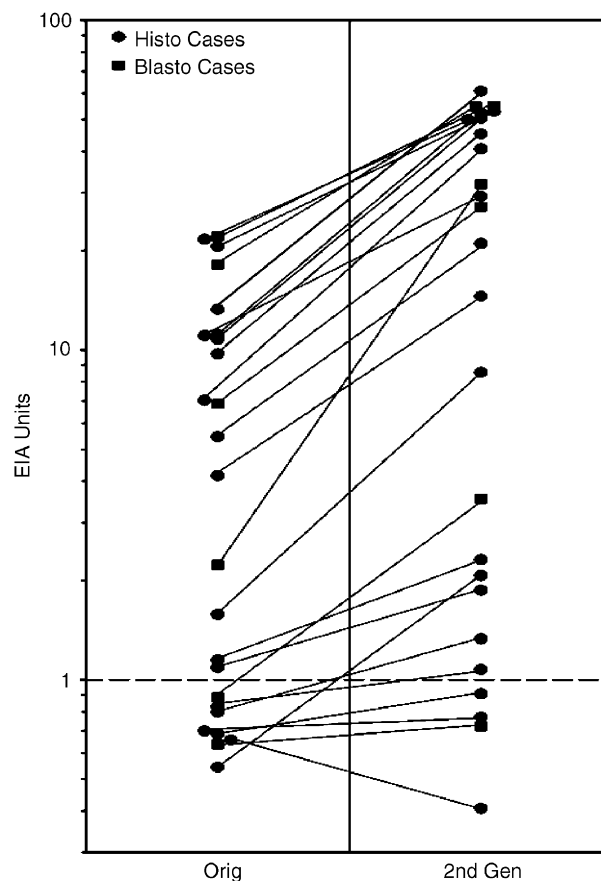

Figure 1 BAL fluid specimens ($n = 25$) were tested using the original and the second-generation *Histoplasma* antigen test simultaneously. Nineteen samples were from cases of confirmed histoplasmosis (circles). The remaining 6 were from cases of blastomycosis (squares). The second-generation test yielded a higher sensitivity than the original assay ($P = 0.125$).

positive in the second-generation assay compared to 17 of 25 (64%) in the original assay ($P = 0.125$, Chi-square). Additionally, antigen levels were higher in the second generation EIA for both histoplasmosis ($P < 0.02$) and blastomycosis ($P < 0.032$). Among the 54 controls, results were positive in a single specimen (1.8%) in both assays, and it was included because of elevated levels of *A. galactomannan.* The *Histoplasma* antigen test was negative on four other specimens containing elevated levels of *A. galactomannan.* To further assess potential cross reactivity with *A. galactomannan,* 24 additional BAL specimens that were positive in the Platelia assay were tested in the *Histoplasma* antigen assay, of which none were positive.

Ten-fold concentration was performed on 16 BAL fluid samples (histoplasmosis, $n = 5$; blastomycosis, $n = 2$; and non-fungal controls, $n = 9$). The addition of a concentration step yielded positive results in two false-negative specimens, one with

histoplasmosis (unconcentrated, 0.91 units; concentrated, 1.85 units) and another with blastomycosis (unconcentrated, 0.72; concentrated, 4.96 units). These two specimens were not tested in the original assay. Antigen levels significantly increased with concentration in two other cases in which the unconcentrated specimens were positive (3.32 vs. 19.7 units, and 36.85 vs. 53.87 units). Concentration did not increase antigen levels in 9 antigen-negative control specimens. With concentration, 23 of 25 (92%) specimens were positive in the second-generation assay.

Discussion

In this report, we have demonstrated the diagnostic utility of *Histoplasma* antigen testing in BAL fluid for histoplasmosis and blastomycosis. The second-generation *Histoplasma* antigen assay showed a trend toward increased sensitivity in the BAL when compared to the original EIA (84% vs. 68%), for both histoplasmosis and blastomycosis. Although the difference in sensitivity was not statistically significant, the second-generation test yielded higher antigen levels in all but one of the 25 specimens tested, suggesting that the lack of statistical significance is due to the small sample size. Sensitivity was further improved to 92% by concentration of BAL specimens before testing. We estimate that a sample size of at least 30 of each disease will be necessary to reach statistical significance, and a prospective study is in progress to expand the sample size and determine the sensitivity in the different pulmonary syndromes.

We have previously reported the utility of antigen detection for diagnosis of blastomycosis.[9] The antigen detected in histoplasmosis,[5] and blastomycosis[9] EIA is a cross-reactive polysaccharide.[10] Although the current assays are unable to distinguish the two mycoses, a specific diagnosis can usually be made by histopathology or culture, or on clinical and epidemiological differences. Even if the two mycoses cannot be differentiated, antigen detection provides a basis for early treatment, which is the same in blastomycosis and histoplasmosis. The importance of rapid diagnosis is emphasized by the often fatal outcome in patients with pulmonary blastomycosis, highlighted in a recent report of death in four children with blastomycosis.[11] Testing BAL may improve the sensitivity of antigen detection for diagnosis of pulmonary blastomycosis.

False-positive results were observed in 1.8% of BAL specimens from controls, a specimen containing high levels of *A. galactomannan*. Additional testing found no false-positive results in specimens containing *A. galactomannan*. Cross-reactions are to be expected in specimens from patients with paracoccidioidomycosis and penicillosis marneffei.[10] More recently, we have detected a cross-reactive antigen in the urine from patients with severe coccidioidomycosis (Wheat, unpublished observation, 2006). These findings provide a basis for the hypothesis that antigen testing in BAL can improve the sensitivity for diagnosis of pulmonary histoplasmosis and blastomycosis.

Prospective studies are in progress to answer several important questions not addressed adequately in this preliminary investigation. First, what is the relative sensitivity of antigen detection compared to cytology and histopathology? In an earlier report, the sensitivity was 70% for antigen detection and histopathology.[4] In the current study, histopathology was not available for specimens submitted for clinical testing. Second, what is the relative sensitivity of antigen detection in BAL compared to urine and serum? The number of cases with concurrent specimens was inadequate for that analysis. Third, what is the sensitivity for diagnosis of the different pulmonary syndromes? In the earlier study, all patients had AIDS complicated by disseminated histoplasmosis. In the current study, the type of pulmonary infection was unknown for the cases that were included based upon results of antigen testing. Fourth, the specificity of the antigen test requires further evaluation, since only 16 patients with other fungal diseases were studied. Finally, the utility of concentration of the BAL before testing requires further evaluation, as there were too few false-negative specimens with sufficient volume to study following concentration.

In summary, the second-generation *Histoplasma* antigen test has improved sensitivity for the diagnosis of histoplasmosis and blastomycosis. Although important questions remain to be answered through a prospective study, these preliminary findings support the utility of antigen testing as an adjunct to histopathology and cytology for evaluation of BAL in patients suspected to have pulmonary histoplasmosis or blastomycosis.

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