Title: Development of a Highly Sensitive and Specific Blastomycosis Antibody Enzyme Immunoassay Using *Blastomyces dermatitidis* Surface Protein BAD-1

Running Title: Highly Sensitive and Specific Blastomycosis Antibody EIA

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Abstract.

Background: Serologic tests for antibodies to Blastomyces dermatitidis are not thought to be useful for diagnosis of blastomycosis in part due to the low sensitivity of immunodiffusion and complement fixation. Earlier studies have shown that enzyme immunoassay improves the sensitivity of antibody detection for diagnosis of blastomycosis.

Methods: Microplates coated with the B. dermatitidis surface protein BAD-1 were used for testing serum from patients with proven blastomycosis or histoplasmosis and controls. Semi-quantification was accomplished by using standards containing human anti-B. dermatitidis antibodies.

Results: Antibodies were detected in 87.8% of patients with blastomycosis by enzyme immunoassay compared to 15.0% by immunodiffusion. Specificity was 99.2% in patients with non-fungal infections and healthy subjects, and 94.0% in patients with histoplasmosis. Results were highly reproducible on repeat testing. When combined with antigen testing, antibody testing improved the sensitivity from 87.8% to 97.6%.

Conclusion: Enzyme immunoassay detection of antibodies against BAD-1 is highly specific, has greatly improved sensitivity over immunodiffusion, and may identify cases with negative results by antigen testing. This assay has the potential to aid in the diagnosis of blastomycosis.
Introduction

Blastomycosis is an endemic, systemic mycosis caused by the dimorphic fungus *Blastomyces dermatitidis*. The primary mode of infection is inhalation of the conidia and the subsequent conversion of these conidia into parasitic yeast (1). The endemic areas in the United States include the Ohio and Mississippi river valleys, the southeastern states, and the areas surrounding the Great Lakes. Diagnosis is often complicated by the similarity of symptoms to viral or bacterial respiratory infection and by the variety of manifestations that can range from asymptomatic to rapidly progressive dissemination, which is often fatal (2).

The diagnosis of blastomycosis is usually based upon direct visualization of broad-based budding yeast in a clinical specimen or culture of the organism (3-7). The methods can be time consuming or require invasive procedures. *B. dermatitidis* antigen detection (MiraVista Diagnostics, Indianapolis, IN) has high sensitivity and can be useful for diagnosis of fungal infection, but is limited by high cross reactivity with other dimorphic fungi including *Histoplasma capsulatum* (3). This can result in diagnostic uncertainty since the endemic areas of blastomycosis and histoplasmosis overlap (4). Further, around 10% of patients with blastomycosis have falsely negative results using the antigen detection test (5).

Serologic testing for *B. dermatitidis*-specific antibodies has not gained wide acceptance. According to a recent review, an agar gel immunodiffusion (AGID) test showed a sensitivity of only 32%, and in previous studies less than half of blastomycosis cases were seropositive (6-11). Complement fixation is less sensitive than AGID in patients with blastomycosis, more difficult to perform, and offers no advantage over AGID (7-9).
Enzyme immunoassay (EIA) is more sensitive than AGID, but previous tests were falsely positive in one quarter of patients with histoplasmosis (7-11). In one study using a commercially available EIA, the sensitivity was 100%, but false positives in non-fungal controls were detected in 20% of cases (10). In another study, sensitivity was 83%, but cross reactions occurred in one-third of patients with histoplasmosis (11). This assay is no longer commercially available.

However, a radioimmunoassay (RIA) for antibodies to the *B. dermatitidis* antigen, BAD-1 (*Blastomyces* adhesin-1) demonstrated positive results in 85% of patients with blastomycosis and only 3% of patients with other fungal diseases, which was superior to an EIA using the A antigen (58% seropositive) (12, 13). Subsequent reports validated the original findings (12, 14, 15), but this assay had never been made commercially available for clinical testing. An accurate serologic test could be useful for diagnosis of blastomycosis, has the potential to identify cases with negative results by antigen testing, and may assist in differentiating histoplasmosis and blastomycosis.

We have developed an EIA assay using BAD-1 to detect antibodies to *B. dermatitidis*. Herein, we describe the preparation of this protein and determine the sensitivity and specificity of our assay.
Methods.

BAD-1 preparation. The *B. dermatitidis* antigen, BAD-1, was isolated from a clinical isolate and prepared according to Klein, *et al.* (16, 17) with the following modifications. Native BAD-1 was purified using a low stringency nickel purification where the buffers contain 300 mM NaCl and no imidazole was included in the wash buffer. An additional Concanavalin A purification step was also added to this protocol. Briefly, agarose bound Concanavalin A resin (Vector Labs; Burlington, CA) was added to the nickel column elution fraction and the sample was incubated for 30 minutes at 4°C. The supernatant was then isolated and prepared as described. Sample concentration was quantified by Optical Density (OD) at 280 nm and purity and antigen activity were confirmed by SDS-PAGE, Western blotting, and EIA. GelCode Blue Stain Reagent (Thermo Scientific; Rockford, IL) was used for sensitive SDS-PAGE detection with bands visible down to 8 ng.

Patient Samples. Active cases of blastomycosis from nine U.S. endemic states were evaluated; 39 are proven and two are probable cases. Serum was available from 36 cases of culture proven blastomycosis. Of the remaining five cases, three were diagnosed by pathology and classified as proven blastomycosis, one by *Blastomyces* antigenuria and antibody (A precipitin by AGID, probable), and one based on *Blastomyces* antigenuria and clinical information from the ordering physician (probable). Clinical information was available for 14 of the samples that were previously reported (3, 6) and reviewed with the approval of the Clarian Health – now Indiana University Health - institutional review committee. Limited clinical and laboratory information for the remaining 27 cases were provided by the ordering physician who managed those cases.
Controls included 50 individuals with histoplasmosis who had elevated titers of complement fixing antibodies and/or positive AGID Histoplasma precipitins, including specimens obtained during an outbreak investigation by the CDC (18) or from clinical testing at the Clarian Health—now Indiana University Health - Medical Center pathology laboratory. Additional controls included 25 non-fungal clinical specimen, and 100 healthy subjects from an endemic area for blastomycosis and histoplasmosis (Memphis, Tennessee, 50 subjects) and a non-endemic area (Miami, Florida, 50 subjects). Specimens had been stored at -20\(^\circ\) C for up to six years prior to testing.

**BAD-1 EIA Calibrators.** BAD-1 calibrators were prepared from serum pooled from 5 patients with confirmed blastomycosis. These samples were positive in the BAD-1 EIA and dilutions of this pool in StartingBlock blocking buffer (Thermo Scientific; Rockford, IL) were prepared in order to obtain a standard curve. Each point of the curve was assigned an EIA unit value ranging from 1 to 128 units to allow for semi-quantification. SigmaPlot statistical analysis software (Systate Software, Inc.) was used for transformation of OD values from individual serum samples into EIA unit values based on the standard curve.

**Antibody immunoassay.** Immulon 2 HP microplates were coated with 100 \(\mu\)L of B. dermatitidis BAD-1 antigen at 50 ng/mL, and then blocked with StartingBlock blocking buffer (Thermo Scientific; Rockford, IL). Between each step the plates were washed with PBS-Tween (Bioreba; Reinach BL 1, Switzerland). 100 \(\mu\)L of the test serum diluted to 1:1000 was added to each well and incubated at 37\(^\circ\) C for 1 hour, after which bound antibody was detected with biotinylated goat anti-human IgG antibody (Vector Laboratories; Burlingame, CA) by incubation at 37\(^\circ\) C for 1
hour. Plates were then incubated with 100μl of streptavidin-horseradish peroxidase at 37°C for 1 hour, followed by 3,3′,5,5′-tetramethylbenzidine (TMB; SurModics; Eden Prairie, MN) for 8 minutes at room temperature in the dark. 2N Sulfuric acid was then added to each well to stop the reaction, after which plates were read in a microplate reader at 450 nm with a 620 nm reference filter. Results were expressed as EIA units by comparison to calibrators. Reproducibility for each sample was investigated (n = 3).

Agar gel immunodiffusion (AGID). The blastomycosis AGID assay was performed according to the manufacturer's instructions using commercially available reagents (Immuno-Mycologics; Norman, OK).

Antigen enzyme immunoassay. *B. dermatitidis* antigen in urine and in serum was determined by comparison to calibrators containing known amounts of *B. dermatitidis* galactomannan at MiraVista Diagnostics, as described (3, 4).

Statistics. Receiver operating characteristic (ROC) curve analysis was performed to determine the cut-off for positivity that would give the optimal sensitivity and specificity. Linear Regression analysis was used to analyze reproducibility and precision according to the Passing and Bablock method (MedCalc).
Results.

Patients. Of the 41 patients with active blastomycosis, 39 were classified as proven cases based on positive cultures and/or pathology. The remaining two patients were classified as probable cases based on Blastomyces antigenuria and clinical information from the attending physician.

ROC determination of cutoff for positivity. ROC analysis determined the optimal cutoff for Blastomyces antibody detection to be an OD of 0.042, at which point the sensitivity was 95.1% and specificity was 93.6% (Figure 1). However, an OD of 0.085 (corresponding to 1.5 units) was chosen for further analysis to increase specificity. At this cut off sensitivity was 87.8% and specificity 99.2%, area under the curve was 0.980 (95% confidence interval [CI] 0.946 to 0.995), and the standard error was 0.0122, p < 0.0001.

Clinical cases and controls. Antibody levels in the blastomycosis cases ranged from undetectable to greater than 128 units, had an average of 45 units, and were positive at ≥1.5 units in 36 of 41 samples (87.8%) (Figure 2). It should be noted that our blastomycosis sample set did not allow for correlations between time of onset and antibody levels. Precipitins to the Blastomyces A antigen were detected in 6 of 40 patients (15.0%). Antibody levels were ≥1.5 units in 22 of 24 (91.7%) patients with positive Blastomyces antigenemia compared to 9 of 12 (75.0%) without antigenemia, and mean antibody levels were 55.2 units and 24.1 units respectively. Antibody levels were ≥1.5 units in 32 of 36 (88.9%) of patients with positive Blastomyces antigenuria compared to 4 of 5 (80.0%) without antigenuria, and mean antibody levels were 45.7 units and 5.7 units respectively (3). Results from histoplasmosis patients were positive in three out of 50 cases (6.0%), at 2.18, 2.59, and 2.98 units. Results in clinical controls (25 specimen) and healthy
subjects from non-endemic areas (50 specimen) were all undetectable, and one of 50 subjects
from endemic areas exhibited a low positive result at 3.1 units.

*B. dermatitidis* antigenuria was detected in 34 of 39 cases (87.2%) and antigenemia in 24 of 36
cases (66.7%) in which testing was performed (3). Of all *Blastomyces* cases, patients were
antigen and antibody positive in 32 of 41 cases (78.0%) and antigen or antibody positive in 40 or
41 cases (97.6%)

Precision and reproducibility. Of the blastomycosis, histoplasmosis, non-fungal, and healthy
subject samples, results were reproducibly positive or negative in 205 of 209 instances (98.1%).
Comparison of initial and repeat antibody results (n = 3) in blastomycosis cases by linear
regression showed strong correlation with a coefficient of determination (R²) of 0.9880, residual
standard deviation of 5.069, 95% slope CI of 0.970 to 1.050, and slope p<0.0001 (Passing and
Bablock Method, Figure 3).
Discussion.

The *B. dermatitidis* EIA antibody assay using BAD-1 offers several advantages over the current methods. First, the sensitivity and specificity are high, at 87.8% and 99.2%, respectively, at the cutoff chosen for this analysis. This corresponds to a nearly 6-fold increase in sensitivity of this antibody assay over conventional blastomycosis AGID analysis in this study. Second, cross-reactivity in patients with histoplasmosis was low, at only 6%, allowing for differentiation between these two similar mycoses. Of note is that the positive results in histoplasmosis cases were all low positive, between 1.5 and 3.0 units. Higher results were more likely to occur in blastomycosis than histoplasmosis. Finally, sensitivity is improved when antibody and antigen testing are combined. In this study, combined antigen and antibody detection improved sensitivity from 87.8% to 97.6%. Further, studies in histoplasmosis infection have shown antibody detection to be considerably more sensitive than antigen detection for syndromes including acute, subacute and chronic pulmonary infection (19, 20). It is possible a similar benefit may be seen in blastomycosis.

Not unexpectedly, antibody results were negative in 12% of cases. Several factors may be responsible for these false negative results. IgG antibodies, detected by this EIA assay, may require more than one month to reach detectible levels following acute blastomycosis. Among cases with acute pulmonary blastomycosis identified during an outbreak, only 45% were positive during the first month after the onset of illness (8). Whether testing for IgM antibodies would improve the sensitivity for diagnosis of early cases remains to be determined. Investigation into the relationship between antibody levels and time from onset of infection and the development of an blastomycosis IgM assay will be investigated as specimen become available. Second, the
BAD-1 antigen may not contain epitopes recognized by the antibodies produced in some patient samples. This could be associated with the genetic variability of *B. dermatitidis* that has recently been described (21-23). Third, anti-BAD-1 antibodies may be complexed with antigens in the specimen and therefore not free for the detection in our assay. Finally, some patients may not be able to mount an antibody response.

In conclusion, detection of antibodies to *B. dermatitidis* BAD-1 antigen has the potential to aid in the diagnosis of blastomycosis, identifying cases that are falsely negative by antigen testing or microscopy and differentiating histoplasmosis from blastomycosis in cases diagnosis based on antigen detection. Combining antibody with antigen testing seems to provide the highest diagnostic yield for blastomycosis.
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Potential conflicts of interest. L.J.W. is an owner of MiraVista Diagnostics. S.M.R., M.L.S., M.M.D., P.A.C, and L.J.W. are employees of MiraVista Diagnostics and MiraBella Technology and intend to offer the described test commercially. T.T.B. and B.S.K. are the inventors of the antigen purification scheme, have submitted the patent application, and the Wisconsin Alumni Research Foundation (WARF) will be the recipient of a portion of royalties from the license. All other authors have no conflict of interest.
References.


Figure 1.

ROC curve for determination of Blastomyces antibody cutoff. The ROC recommended cutoff 0.042 with a sensitivity of 95.1% and a specificity of 93.6%. However, an OD of 0.085 (corresponding to 1.5 ELA units) was chosen for further analysis to increase specificity. At this cut off sensitivity was 87.8% and specificity 99.2%, area under the curve was 0.980 (95% confidence interval [CI] 0.946 to 0.995), and the standard error was 0.0122, p<0.0001.
Figure 2.

Antibody response with BAD-1. Antibody levels in patients with proven blastomycosis (39), probable blastomycosis (2), histoplasmosis (50) and healthy patients or non-fungal clinical controls (125). Antibody levels as antibody units are shown on the vertical axis. The cutoff for positivity (1.5 units) is indicated by the broken horizontal line and the numbers below the broken line represent the number of patients with negative results. Abbreviations: Bd, blastomycosis; Hc, histoplasmosis.
Reproducibility by linear regression. Interassay agreement of unit values obtained from repeat 3 testing of Blastomyces patient samples showed strong correlation with a coefficient of determination (R²) of 0.988, residual standard deviation of 5.069, 95% slope CI of 0.970 to 1.050, and slope p<0.0001 (Passing and Bablock Method).