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Antigen and Antibody Testing for the Diagnosis of Blastomycosis in Dogs

D. Spector, A.M. Legendre, J. Wheat, D. Bemis, B. Rohrbach, J. Taboada, and M. Durkin

Background: Early diagnosis and treatment are associated with an improved prognosis in blastomycosis. The diagnosis of blastomycosis may be missed by cytology, histopathology, culture, or serology. An enzyme immunoassay (EIA) for detection of *Blastomyces dermatitidis* galactomannan antigen in body fluids has been used for rapid diagnosis of blastomycosis in humans.

Hypothesis: Measurement of *Blastomyces* antigen in urine or serum by the MVista *Blastomyces* antigen EIA is more sensitive than measurement of anti-*Blastomyces* antibodies for diagnosis of blastomycosis in dogs.

Methods: Serum and urine samples from 46 dogs with confirmed blastomycosis were tested for *Blastomyces* antigen and serum was tested for anti-*Blastomyces* antibodies.

Results: The sensitivity for the detection of antigen in urine was 93.5% and it was 87.0% in serum. The sensitivity of antibody detection by agar gel immunodiffusion (AGID) was 17.4% and it was 76.1% by EIA. Antigen and antibody decreased during itraconazole treatment.

Conclusions and Clinical Importance: Antigen detection is a more sensitive test for diagnosis of blastomycosis than antibody testing by AGID, the only commercially available method. Antigen concentrations decreased with treatment.

Key words: Coccidioidomycosis; Histoplasmosis; Serology; Veterinary.

Blastomycosis is a fungal infection caused by the thermally dimorphic fungus *Blastomyces dermatitidis*. In the environment, *B. dermatitidis* grows as a saprophyte, generating an infectious asexual spore that can be aerosolized and inhaled. Once inside the terminal airways, the conidium transforms into a yeast, which multiplies and produces a primary lung infection. *B. dermatitidis* may disseminate throughout the body, localizing in sites such as the eyes, skin, bones, and lymph nodes in dogs. Infection induces an antibody response directed against antigens of the yeast. These antibodies are detectable in dogs and have been the target of the principal serologic test for the diagnosis of blastomycosis.[1]

Unfortunately, diagnosis of blastomycosis by serology is insensitive, in part because of the delay required to produce increased concentrations of antibodies against *B. dermatitidis*. For example, in 1 study, many of the dogs that tested positive for anti-*B. dermatitidis* antibodies at the referral hospital were seronegative when previously tested by the referring veterinarian.[2] Diagnosis of chronic blastomycosis by serology may also be hampered by insensitivity of the agar gel immunodiffusion (AGID) method. Thus, serologic testing has not been very helpful in the diagnosis of blastomycosis in dogs.

From the Department of Small Animal Clinical Sciences (Spector, Legendre), Department of Comparative Medicine, University of Tennessee College of Veterinary Medicine, Knoxville, TN (Bemis, Rohrbach); MiraVista Diagnostics, Indianapolis, IN (Wheat, Durkin); and the School of Veterinary Medicine, Louisiana State University, Baton Rouge, LA (Taboada). Work was done at University of Tennessee College of Veterinary Medicine and Mira Vista Diagnostics. Abstract was presented at the 2006 ACVIM Forum in Louisville, Kentucky.

Corresponding author: Alfred M. Legendre, 9037 Shallowford Road, Knoxville, TN 37923; e-mail: alegendr@utk.edu.

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The gold standard for the diagnosis of blastomycosis is a combination of clinical signs and cytologic or histopathologic identification or isolation of the organism from infected tissues. Although the diagnosis may be made easily by cytologic examination of skin lesions, in many cases the infected tissues may not be readily accessible or cytology may be falsely negative. Antigen testing may be useful in such cases.

Antigen detection has been used for the diagnosis of histoplasmosis[3] and blastomycosis in humans.[4,5] The sensitivity of the diagnosis of blastomycosis was 93% and the reproducibility was 96% by the MVista *Blastomyces* antigen enzyme immunoassay (EIA). *Blastomyces* antigen concentrations decreased during treatment and increased with relapse, providing a method to monitor treatment.[5]

Cross-reactions in the MVista *Blastomyces* antigen EIA occurred in most human patients with other endemic mycoses.[4] Positive results were noted in 96% of patients with histoplasmosis. The antigen detected in the patients is a cell-wall galactomannan that is immunologically indistinguishable in histoplasmosis and blastomycosis.[6] Also, nearly complete cross-reactivity was observed in patients with penicilliosis marneffei and paracoccidioidomycosis,[4] endemic mycoses rarely encountered in the United States. The galactomannan detected in human patients with coccidioidomycosis was shown to cross-react in the MVista *Histoplasma* antigen EIA, and positive results were detected in 79% of patients with acute infection.[7] Cross-reactivity in coccidioidomycosis in humans was not observed in our earlier report by the MVista *Blastomyces* antigen EIA.[4] This may be explained by differences in the patients tested in the 2 studies or improvements to the original MVista *Blastomyces* antigen EIA that affected its sensitivity and specificity.[6] Cross-reactivity in coccidioidomycosis is expected in the MVista *Blastomyces* antigen EIA.

The purpose of this study was to evaluate the sensitivity of the MVista *Blastomyces* antigen and antibody EIAs for diagnosis of blastomycosis in dogs and

determine the effect of treatment on *Blastomyces* antigen and anti-*Blastomyces* antibody concentrations.

Materials and Methods

Dogs

Cases included 46 dogs with blastomycosis confirmed by cytology or histopathology. These dogs were previously reported in a multicenter study evaluating treatment with itraconazole.[8] The study sites from which the cases were obtained were Auburn University, University of Minnesota, Louisiana State University, and Mississippi State University. Serum and urine samples were obtained at diagnosis approximately 30, 60, 90, and 180 days after itraconazole was begun, but samples were not available for all dogs at all time points. Specimens were stored at −70 °C for 10 years before testing. Residual paired serum and urine (n = 44) specimens submitted for chemistry analysis and urinalysis from dogs at the University of Tennessee Veterinary Teaching Hospital were used as control samples. The samples were stored at −70 °C until testing. To exclude dogs with fungal infections from the control group, the medical records were reviewed by one of the investigators (DS). The diagnosis made by the veterinarians who evaluated the dogs was used to exclude dogs with fungal infections.

MVista Blastomyces Antigen EIA

The MVista *Blastomyces* antigen EIA development and characterization were evaluated in humans and reported elsewhere.[4] The performance characteristics of the test are summarized in the product information on the MiraVista Diagnostics website (miravistalabs.com). Briefly, the assay uses polyclonal immunoglobulin G (IgG) anti-*B. dermatitidis* antibodies produced in rabbits by immunization with a formalin-killed *B. dermatitidis* mold vaccine, emulsified with Freund's adjuvant.[a] The assay is a sandwich EIA that uses Immulon 2 HP microplates[b] coated with rabbit IgG anti-*Blastomyces* antibody.[c] The microplates are then blocked with 100 μL of Starting-Block[d] to reduce nonspecific binding of test specimens and detector antibodies.

One hundred microliters of the serum or the urine sample (single aliquot of each) was added to each well of the microplates and incubated at 37°C for 1 hour. Microplates were washed and 100 μL of biotinylated rabbit IgG anti-*B. dermatitidis* antibodies[c] was added. Next, the plates were incubated at 37°C for 1 hour, after which they were washed. Then 100 μL of streptavidin-horseradish peroxidase,[e] diluted to 1 : 75,000, was added to each well. Microplates were incubated at 37°C for 1 hour and then washed. Next, 100 μL of 3,3′,5,5′-tetramethylbenzidine[f] (TMB) substrate was added to each well and incubated at room temperature in the dark for 8 minutes. Sulfuric acid[g] (100 μL) was then added to each well and the wavelength was read at 450 λ in a microplate reader[h] to obtain the optical density (OD). Results were expressed as EIA units by dividing the OD for the test specimen by the cutoff OD, which was determined by receiver operator characteristic (ROC) analysis.

For determination of precision, 4 aliquots of a single urine specimen from a dog with blastomycosis were tested on a single occasion. Reproducibility was assessed by testing 2 serum and 2 urine specimens from dogs with blastomycosis and 1 serum and 1 urine sample from negative control dogs on 2 microplates in assays performed on 2 different days.

MVista B. dermatitidis Antibody EIA

This is an indirect IgG antibody detection EIA that uses Immulon 2 HP microplates[b] coated with a 1 : 10,000 dilution of commercially available crude mold-phase *B. dermatitidis* antigen[i] and blocked with 100 μL of Starting-Block.[d] One hundred microliter of the 1 : 1,000 diluted test serum was added to each well and incubated at 37°C for 1 hour. Plates were then washed and 100 μL of biotinylated goat anti-dog IgG antibody[j] was added to each well and incubated at 37°C for 1 hour. Plates were again washed and 100 μL of streptavidin-horseradish peroxidase[e] diluted to 1 : 75,000 was added to each well and incubated at 37°C for 1 hour. Plates were washed and 100 μL TMB[f] substrate was added to each well and incubated for 5 minutes at room temperature in the dark. Sulfuric acid[g] (100 μL) was then added to each well to stop the reaction. Microplates were read in a microplate reader at a wavelength of 405 λ to obtain the OD. Results were expressed as EIA units by dividing the OD for the test specimen by the cutoff OD, which was determined by ROC analysis.

For determination of precision of the antibody EIA, 4 aliquots of a single serum specimen from a dog with blastomycosis were tested on a single occasion. Sera from 4 dogs with blastomycosis and 2 negative control dogs were tested on 2 microplates in assays performed on 2 different days for determination of reproducibility.

Blastomyces Antibody AGID

AGID was performed with a commercial kit, according to the manufacturer's recommendations.[k] A single pretreatment aliquot of serum from each dog was tested undiluted, and those showing a line of identity with the positive kit control were regarded as positive.

Statistical Analysis

Statistical analysis was performed by MedCalc for Windows, version 9.3.0.0.[l] The cutoff that defines positivity was determined by ROC analysis. The Fisher exact test was used to compare categoric variables in the analysis of sensitivity, and $P < .05$ was considered to be statistically significant. Clearance of antigen and antibody was evaluated in a subset of 23 cases that met the following criteria: pretreatment urine antigen positive and at least 3 urine and 3 serum specimens available between baseline and day 180 after starting itraconazole treatment, including the baseline specimen.

Results

ROC analysis of MVista *Blastomyces* antigen EIA results in the urine of 46 dogs with blastomycosis and 44 controls showed that an OD of 0.078 best discriminated dogs with blastomycosis and controls (Fig 1). At this cutoff, the sensitivity for detection of antigen was 93.5% (95% confidence interval [95% CI]: 82.5%, 97.7%) in urine and 87% (95% CI, 74.3%, 93.9%) in serum (Fig 2). The sensitivity in urine was not statistically different from that of serum ($P = .465$). At this cutoff, results were negative in the serum in 100% (95% CI, 92.07%, 100%) and in urine in 97.7% (95% CI, 88.2%, 99.6%) of the control dogs.

The precision of the MVista *Blastomyces* antigen EIA was determined by testing 4 aliquots of urine from a dog with blastomycosis. The mean antigen concentration was 26.5 U, the standard deviation was 2.5 U, and the coefficient of variation (CV) was 9.6%. Reproducibility was determined by testing single aliquots from 4 dogs and 2 negative controls on 2 microplates on 2 different days. The means, standard deviations, and coefficients of variation are shown in Table 1. Except for the negative control and the dog with the lowest antigen concentration (1.89 U), the coefficients of variation were <10%. ROC analysis of the MVista *Blastomyces* antibody EIA

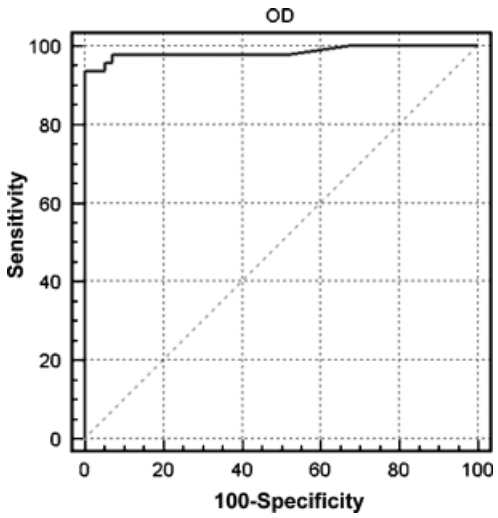

Fig 1. Receiver operator characteristics analysis of urine antigen results in the 46 cases and 44 control dogs.

showed that an OD of 0.042 best discriminated the dogs from the controls. Antibody was detected in 76.1% (95% CI, 62.1%, 86.1%) of the cases by EIA (Fig 2) and 17.4% (95% CI: 9.1%, 30.7%) by AGID. The sensitivity for detection of antibodies was lower than that for detection of antigen in urine ($P = .039$), but not significantly different from the sensitivity for detection of antigen in serum ($P = .282$). The sensitivity for detection of antibody by EIA was higher than that for detection by AGID ($P < .001$).

The precision of the MVista *Blastomyces* antibody EIA was determined by testing 4 aliquots of serum from a dog with blastomycosis. Reproducibility was determined by testing single aliquots from 4 dogs with blastomycosis and 2 negative controls on 2 microplates on 2 different days. The means, standard deviations, and coefficients of variation are shown in Table 1. CVs were $< 10\%$, except for a single specimen from a dog with blastomycosis.

Sequential specimens were tested for 23 dogs with blastomycosis that had specimens available at baseline and at least 2 follow-up specimens. Antigen concentrations at baseline were lower and appeared to decrease more rapidly in serum than in urine (Fig 3). The median antigen concentrations in both serum and urine decreased below a concentration considered to be positive by day 180; however, the median antibody concentration at day 180 was 3.57 EIA units (positive ≥ 1.0 U).

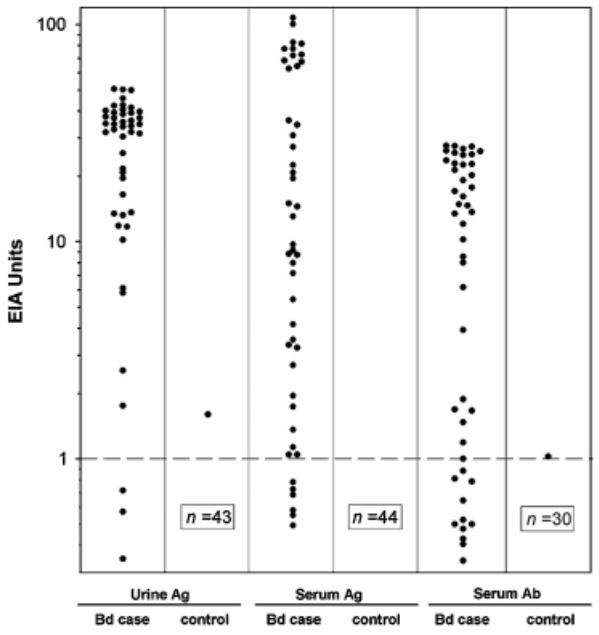

Fig 2. Antigen (Ag) concentration expressed as EIA units, as measured in the MVista *Blastomyces* antigen EIA, and antibody response expressed as EIA units, as measured in the MVista *Blastomyces* antibody EIA. The broken line drawn at 1 EIA unit indicates the cut-off value separating positive from negative results. The number within the box below the line represents the number of dogs with negative results.

Discussion

Blastomyces antigen was detected in the serum in 87.0% and urine in 93.5% of cases of blastomycosis in dogs, similar to earlier studies in humans.[4] Also, as seen in humans,[5] antigen concentrations in dogs decreased during treatment. *Blastomyces* antigen detection in urine was more sensitive than antibody detection determined either by AGID (17.4%) or by EIA (76.1%). Of note is that the *Blastomyces* antibody EIA is a research procedure, and that AGID is the method used for serologic testing in clinical practice. We were unable to compare antigen detection with histopathology or culture, because those tests were required for inclusion in the treatment study from which the specimens were obtained.[8] Studies are in progress to compare the sensitivity of antigen detection with histopathology and culture.

We were unable to assess specificity because too few specimens were available from dogs with other mycoses. Specificity of the MVista *Blastomyces* antigen EIA has been established in humans.[4] Extensive cross-reactivity occurred in humans with other endemic mycoses such as histoplasmosis, and similar cross-reactivity is expected in dogs. Cross-reactivity was rare in other fungal infections,[4] but positive results by the MVista *Blastomyces* antigen EIA were noted in 1 of 88 (1.1%) humans with aspergillosis and 2 of 66 (2.9%) with cryptococcosis. Subsequent research, however, shows that the positive results in aspergillosis and cryptococcosis probably represent dual infections rather than cross-reactivity.[9] False antigenuria occurred in 2% dogs with nonfungal diseases in this study, similar to earlier findings in humans. Studies are in progress to define cross-reactivity in the MVista *Blastomyces* antigen EIA in dogs with histoplasmosis or coccidioidomycosis, other mycoses, and nonfungal infections.

Cross-reactivity does not substantially limit the usefulness of the antigen test for the diagnosis of blastomycosis.

Table 1. Reproducibility in the MVista *Blastomyces* antigen and antibody EIAs.

	MVista *Blastomyces* Antigen EIA			MVista *Blastomyces* Antibody EIA		
Dog[a]	Mean[b]	SD	CV (%)	Mean[b]	SD	CV(%)
1	27.53	0.66	2.39	28.16	2.38	8.46
2	2.02	0.19	9.43	4.30	0.75	17.54
3	49.67	3.12	6.28	2.09	0.20	9.59
4	1.89	0.41	21.82	13.37	0.82	6.12
5	0.44	0.09	20.56	0.53	0.05	8.73
6	0.44	0.10	22.35	0.47	0.05	9.75

[a]Dogs 1–4 represent cases of blastomycosis and dogs 5 and 6 are negative controls. In the antigen EIA, results for dogs 1 and 2 are for urine specimens and dogs 3 and 4 are for serum specimens. The specimen type for the antigen EIA for control dog 5 is urine and control 6 is serum. In the antibody EIA, the results for all 6 dogs are for serum.

[b]The mean was determined in single aliquots tested in 2 microplates on 2 different occasions, and thus 4 data points for each mean.

EIA, enzyme immunoassay; CV, coefficient of variation; SD, standard deviation.

The drugs used for treatment of histoplasmosis and blastomycosis are the same, and early diagnosis based on a positive antigen test should benefit patients with either mycosis. Amphotericin B is the treatment of choice for dogs with brain involvement with blastomycosis. Severe cases of either mycosis may respond better to amphotericin B than itraconazole, but no significant difference was noted in the survival rates of dogs with blastomycosis treated with amphotericin B or itraconazole.[8] Itraconazole is preferred for less severe infection. Cross-reactions in coccidioidomycosis are less important because of the different geographic patterns of endemnicity.

Antigen concentrations declined during treatment. By day 90, antigenemia had cleared in 75% of dogs with blastomycosis, whereas antigenuria had cleared in only 12%. By day 180, antigenuria was still present in one third of the patients and antigenemia in none. Antibody persisted for >180 days in 75% of the cases. Approximately one quarter of the dogs experience a recurrence of blastomycosis after treatment is stopped.[8] A study is in progress to determine whether persistent antigenuria or antigenemia at the completion of treatment correlates with the risk for recurrence of blastomycosis.

Another study to detect *Blastomyces* antigen in dogs was carried out at Idaho State University with polyclonal rabbit anti-*B. dermatitidis* antibodies in a competitive EIA.[10] Antigenuria was detected in all 12 dogs with blastomycosis, one of the 2 dogs with histoplasmosis, and one of the 4 uninfected dogs.[10] The findings of the study would have been strengthened by testing a larger number of dogs. Demonstration of false-positive results in one of the 4 uninfected control dogs suggests that specificity of the assay may not be sufficient for clinical application.

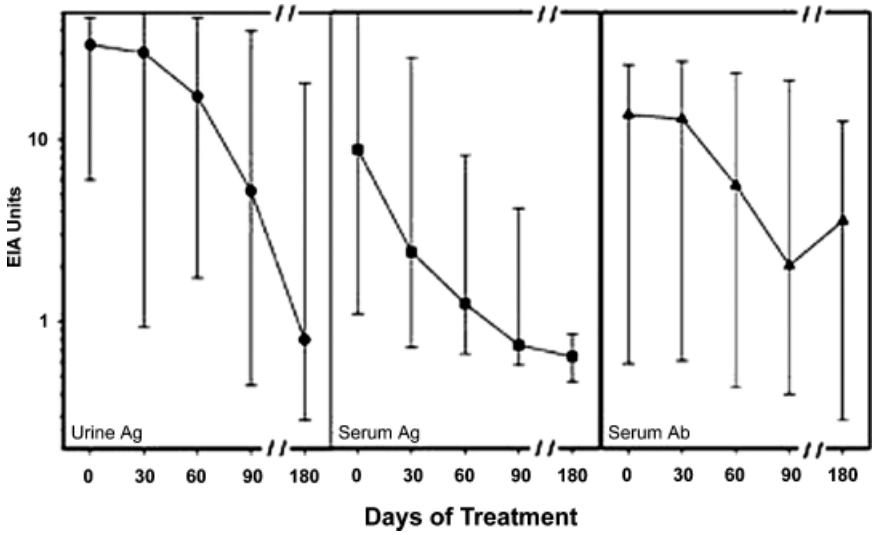

Fig 3. Antibody and antigen clearance after initiation of treatment with itraconazole. Ag, antigen; Ab, antibody. Data are expressed as antigen or antibody units, as determined by EIA, on the vertical axis. The number of days after initiation of itraconazole treatment is depicted on the horizontal axis, and the break in the axis indicates that the scale was truncated between days 90 and 180. The median for specimens tested at each time point is represented by the symbol, and the 10th and 90th percentiles are indicated by the whisker bars. The total number of animals tested at each time point for each sample type is as follows: urine antigen: day 0, n = 23; day 30, n = 18; day 60, n = 20; day 90, n = 16; day 180, n = 9; serum antigen: day 0, n = 23; day 30, n = 21; day 60, n = 23; day 90, n = 16; day 180, n = 11; and serum antibody: day 0, n = 23; day 30, n = 20; day 60, n = 23; day 90, n = 17; day 180, n = 12.

In summary, detection of *Blastomyces* antigen in urine or serum may aid in the early diagnosis of blastomycosis in dogs. In such cases, detection of antigen in urine or serum may allow prompt initiation of treatment. Cross-reactions in histoplasmosis must be recognized but do not detract from the usefulness of this test because treatment is the same for both mycoses. Furthermore, the correct diagnosis usually can be suspected based on different clinical and epidemiologic characteristics of the 2 diseases. Monitoring for changes in antigen concentration during treatment may facilitate decisions to stop treatment or resume treatment in patients with a relapse, hypotheses requiring investigation.

Footnotes

[a] Sigma-Aldrich, St Louis, MO
[b] Therm Electron Corp, Milford, MA
[c] MiraBella Technologies, Indianapolis, IN
[d] Pierce, Rockford, IL
[e] Roche Diagnostics, Indianapolis, IN
[f] BioFX Laboratories, Owings Mills, MD
[g] LabChem Inc, Pittsburgh, PA
[h] Tecan, Research Triangle Park, Raleigh Durham, NC
[i] Immuno-Mycologics, Norman, OK
[j] Vector Laboratories, Burlingame, CA
[k] Meridian Bioscience, Cincinnati, OH
[l] MedCalc Software, Mariakerke, Belgium

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