

Canine antibody response to *Blastomyces dermatitidis* WI-1 antigen

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Objective—To assess whether dogs with blastomycosis produce antibodies against the WI-1 and A-antigens of *Blastomyces dermatitidis* and whether the antibodies are useful in serodiagnosis.

Sample Population—359 serum samples obtained from 245 dogs.

Procedure—233 samples from 122 dogs with blastomycosis, and 1 sample each from 24 dogs with suspected blastomycosis, 51 control dogs without infection, and 48 healthy dogs from an enzootic region were obtained. Antibodies against WI-1 antigen were detected by radioimmunoassay (RIA). Serum samples were tested in parallel for antibodies against the A-antigen of *B dermatitidis* by commercial agar-gel immunodiffusion (AGID) in a reference laboratory.

Results—Antibodies were detected in 92% of infected dogs by RIA and in 41% by AGID. For 29 serum samples that were obtained 11 to 1,545 days after diagnosis, antibodies were detected in 92% of samples by RIA and 7% by AGID. For 93 serial serum samples from 29 dogs with blastomycosis, the mean anti-WI-1 titer was 1:18,761 at the time of diagnosis, and decreased to a mean of 1:1,338 by 210 days after treatment was initiated. Of 24 dogs with suspected infection, antibodies were detected in 67% by RIA and 33% by AGID. Control dogs without blastomycosis had no detectable antibodies in either assay. Thus, sensitivity was 92% for RIA and 41% for AGID, and specificity was 100% for both tests.

Conclusions and Clinical Relevance—Anti-WI-1 antibodies are readily detected by RIA in dogs with blastomycosis. Titers become high, decline during treatment, and persist for months. Anti-A antibodies are sometimes detected with AGID, but these decrease quickly. (*Am J Vet Res* 2000;61:554–558)

Blastomycosis is one of the principal systemic mycoses of dogs and humans in North America.^{1,2} It has also been reported in a variety of other mammalian species, including cats,³ horses,⁴ a Stellar's sea lion,⁵ an African lion,⁶ and an Atlantic bottlenose dolphin.⁷ The disease has been diagnosed in humans in Europe, South America, Asia, and Africa, but it is most

common in North America, particularly in the Mississippi and Ohio River basins.

The incidence of clinical blastomycosis in dogs has been estimated to be approximately 10 times higher than in humans.⁸ Although veterinarians working in enzootic areas are familiar with the disease, a diagnosis may be difficult to confirm. Cytologic or histologic detection of 8- to 30- μ m broad-based budding yeasts in tissue or exudate is usually sufficient for diagnosis in patients with compatible clinical signs. However, these methods are insensitive, commonly yielding false-negative results in infected patients. Likewise, commercially available serologic tests based on detection of *B dermatitidis* antigen A are eschewed by many veterinarians who are experienced in dealing with blastomycosis in dogs because of perceived problems with sensitivity. Finally, culture of the organism from tissue or exudate is time consuming, leads to diagnostic delay, and poses some health risk to laboratory personnel. Thus, a rapid, safe, noninvasive diagnostic test of high sensitivity and specificity would be a valuable addition to the diagnostic armamentarium of veterinarians working in enzootic areas.

It has been reported in recent studies of human patients that the WI-1 antigen of *B dermatitidis* is a chief target of antibodies that are formed during infection, and that a radioimmunoassay (RIA) can detect blastomycosis, with nearly 90% sensitivity and 100% specificity.^{9,10} To the authors' knowledge, no studies have addressed the formation of antibodies against the WI-1 antigen in dogs. The purpose of the study reported here was to investigate the antibody response against the WI-1 antigen in dogs and to evaluate the use of RIA for detection of these antibodies. In addition, because commercial tests for detection of anti-A-antigen antibodies are widely used to make a diagnosis of blastomycosis, the commercial agar-gel immunodiffusion (AGID) test for detection of anti-A antibodies was compared with the RIA for detection of anti-WI-1 antibodies.

Materials and Methods

Study population—More than 600 veterinarians in Wisconsin were contacted by mail and telephone to solicit suspected and confirmed cases of blastomycosis. Nearly 50 veterinarians in Tennessee submitted cases to one of the authors (AML) for enrollment into a previously reported study of itraconazole treatment of blastomycosis at the University of Tennessee College of Veterinary Medicine.¹¹ Blood samples were obtained from 245 dogs: 122 dogs from Wisconsin and Tennessee with confirmed blastomycosis; 24 dogs from Wisconsin with suspected blastomycosis; 51 control dogs from California with no history of travel to geographic areas in which blastomycosis is enzootic;

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and 48 healthy dogs from a northern Wisconsin county where blastomycosis is enzootic (these dogs constituted a survey population judged to be at risk for blastomycosis).

Diagnostic and clinical information—The diagnosis of blastomycosis was confirmed by standard histologic or cytologic evaluation or by isolation of *B dermatitidis* by fungal culture. These diagnostic methods were used as the gold standard against which serologic test results were compared. Clinical information was solicited regarding dogs from Wisconsin about diagnosis and treatment of blastomycosis and response to treatment. For dogs from Tennessee, relevant clinical and laboratory information was recorded during enrollment in the itraconazole study and on follow-up after treatment as described.¹¹

Blood samples—Serum or heparinized plasma samples were obtained from dogs, shipped frozen on dry ice, and stored at -20 C until assayed. Serum samples were aliquoted so that samples were frozen and thawed no more than 3 times. All samples were coded and assayed, with investigators blinded to the case or control status of the sample. After results were obtained, samples were uncoded and results analyzed.

Detection of antibody to WI-1 antigen—The WI-1 antigen was isolated from *B dermatitidis* yeasts of ATCC strain 60636 and radiolabeled with ¹²⁵I as described.⁹ Specific radioactivities between 2,000 and 10,000 cpm/ng of antigen protein were obtained. Radioimmunoassays were performed in plastic microfuge tubes. Radiolabeled WI-1 antigen (2.5 ng) in 100 µl of phosphate-buffered saline (PBS; 0.04 M NaPO₄ and 0.15 M NaCl; pH 7.6) with 1% bovine serum albumin (BSA) was added to each tube as a target. Test serum or plasma was diluted with PBS/BSA (1:20); 100 µl was added to each tube, to yield a final dilution in the reaction mixture of 1:40. Samples were incubated at 37 C for 1 to 2 hours, then at 4 C overnight. The next morning, 200 µl of fresh staphylococcal protein A^a (5 mg/ml in PBS) was added to each reaction tube. After incubation at room temperature for 10 minutes, precipitates were centrifuged and pelleted at 2,000 × g for 10 minutes. A 200-µl sample of supernatant was transferred to a 12- × 75-mm plastic tube and counted in a gamma counter. Coprecipitation controls were performed to test for nonspecific binding to radiolabeled WI-1 antigen. Controls contained 100 µl PBS/BSA in place of diluted serum or plasma. Coprecipitation control values were typically < 5%. Each experimental and control sample was analyzed in duplicate and the mean calculated. Values for nonspecific binding were subtracted from values for total binding to derive values for specific binding. Samples that specifically bound ≥ 20% of the labeled antigen were designated positive. The endpoint titer of positive samples was defined as that dilution of serum that specifically bound 20% of radiolabeled WI-1 antigen.

Detection of antibody to A-antigen—Samples were tested for antibodies directed against the A-antigen of *B dermatitidis* by use of conventional AGID assay. Tests were performed by experienced personnel at a certified veterinary laboratory^b by use of commercial test and reagent kits.^c Manufacturers' recommendations were followed precisely for the use of test plates,^d A-antigen,^e and antibody.^f

Statistical analyses—Sensitivity, specificity, and positive and negative predictive values were calculated for each test.¹² Data were analyzed by use of a Fisher exact test. A *P* value of < 0.05 was considered significant.

Results

Study population and samples—The study population consisted of 4 groups, including 245 dogs from which 359 serum samples were available for serologic

testing. In the first group, blastomycosis was confirmed by histologic or cytologic evaluation in 122 dogs. Of these, 70 dogs from Wisconsin each had 1 serum sample available either at the time of diagnosis (*n* = 41 samples) or from 11 to 1545 days after diagnosis (29 samples). The 52 dogs from Tennessee each had a sample available when treatment was begun (52 samples) and often serially from 30 to 210 days after receiving treatment with itraconazole (114 samples). Thus, of the 233 sera from confirmed cases of blastomycosis, 93 were obtained from dogs at the time blastomycosis was first diagnosed. The remainder were single serum samples obtained from dogs from Wisconsin after the diagnosis was made, or serial samples obtained from dogs from Tennessee after receiving treatment.

A single serum sample was available from each of the dogs in the remaining 3 groups. The second group of 24 dogs from Wisconsin had been evaluated for suspected blastomycosis, on the basis of clinical findings selected from history, physical examination, thoracic radiographs, or prior serologic tests, but the diagnosis was not firmly established for these dogs. A third group of 51 dogs from California had no history of travel to areas known to be enzootic for blastomycosis and were used as negative controls. The last group consisted of 48 healthy dogs that resided in a highly enzootic area of northern Wisconsin. Their serum samples were evaluated to assess the prevalence of antibodies against *B dermatitidis*, as a serologic marker of the prevalence of recent or remote infection.

Detection of anti-WI-1 antibody by use of RIA—Of 122 dogs with confirmed blastomycosis, 112 (92%) had detectable antibodies against WI-1 antigen by RIA (Table 1). Results were comparable in the 2 confirmed case groups: 91% of dogs from Wisconsin were seropositive (titer > 1:40), and 92% of dogs from Tennessee were seropositive. These results reflected RIA performed on all 70 serum samples obtained from dogs from Wisconsin, and the 52 samples obtained from dogs from Tennessee when treatment was begun, so that each proven case was represented once in the analysis. To emulate the situation that clinicians confront with a clinically ill dog, however, we analyzed the data further, including only samples obtained from dogs at or around the time of diagnosis. In this instance, 29 dogs from Wisconsin that were evaluated from 11 to 1545 days after a diagnosis was made were excluded from analysis. Thirty-seven (90%) of the 41 samples

Table 1—Results of radioimmunoassay (RIA) and a commercial agar-gel immunodiffusion (AGID) test used to detect serum antibodies against WI-1 and A-antigens, respectively, in dogs with blastomycosis and control dogs

Group	No. positive (%)	
	RIA	AGID
Confirmed blastomycosis		
Wisconsin (n = 70)	64 (91)	29 (41)
Tennessee (n = 52)	48 (92)	NT*
Suspected blastomycosis (n = 24)	16 (67)	8 (33)
Controls from non-enzootic area (n = 51)	0 (0)	0 (0)
Healthy dogs in enzootic area (n = 48)	2 (4)	0 (0)

*NT = Not tested

obtained from dogs from Wisconsin within 10 days of diagnosis were seropositive by RIA. Thus, of the 93 serum samples obtained from dogs from Wisconsin and Tennessee around the time that blastomycosis was diagnosed, 85 (92%) tested seropositive by RIA.

Anti-WI-1 antibodies also were detected in serum samples obtained > 10 days after a diagnosis was made. Of the 29 dogs from Wisconsin that serum was obtained > 10 days after the diagnosis was made, 27 (93%) were seropositive to *B dermatitidis*. Similarly, 95 (83%) of the 114 samples obtained 30 to 210 days after treatment was begun in dogs from Tennessee were seropositive by RIA.

Clearance of anti-WI-1 antibodies—Clearance of anti-WI-1 antibodies was analyzed in dogs from Tennessee that had samples taken serially. A total of 103 samples were available from 29 dogs; 23 dogs were treated successfully, 3 relapsed, and 3 died from blastomycosis. Pretreatment serum samples were obtained at the time a diagnosis was confirmed, and samples were subsequently obtained at 30-day intervals after treatment was initiated. Two to 5 serum samples (median, 3 samples) were available following treatment, extending as far as 210 days. To assess the decrease in antibody titer, the anti-WI-1 endpoint titer was measured. At the time of diagnosis, the serum sample obtained from each dog was seropositive for antibodies against WI-1 antigen. The geometric mean titer of anti-WI-1 in these samples was 1:18,761 (Fig 1), with a range of titers from 1:2,201 to 1:32,140,700. During treatment with itraconazole, the mean anti-WI-1 titer for the group decreased substantially during the first 2 months, although 7 dogs had a small increase in titer between the pretreatment and 30-day posttreatment samples. Geometric mean titers for the group diminished by about 50% at day 30 and by a further 75% by day 60. Subsequent titers remained stable with little decay from 90 to 210 days. During that interval, titers

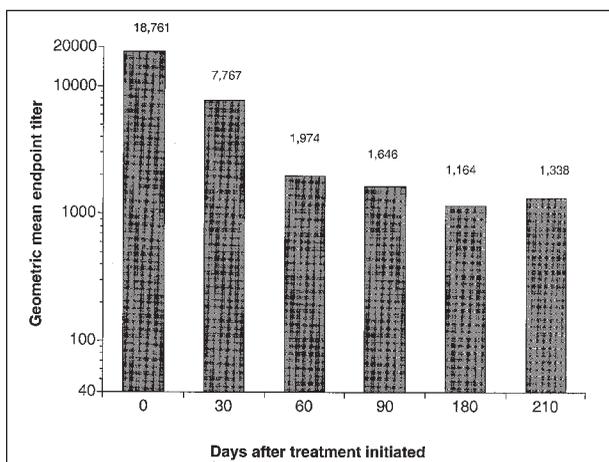


Figure 1—Clearance of anti-WI-1 antibodies, as measured by radioimmunoassay, in dogs from Tennessee with blastomycosis. Endpoint titer is the serum dilution that yields 20% specific binding of radiolabeled WI-1. Endpoint titer was converted to natural log to calculate geometric mean titer (GMT) for the group. The reciprocal of the GMT is illustrated. Number of samples tested at each time was: pretreatment, n = 27 samples; 30 days, 28 samples; 60 days, 25 samples; 90 days, 14 samples; 180 days, 6 samples; and 210 days, 3 samples.

in the 22 serum samples ranged from 1:60 to 1:20,480, with a mean titer of < 1:2,000.

To evaluate the association between serologic response and clinical outcome, anti-WI-1 titer was analyzed in the 6 dogs from Tennessee that relapsed or died from blastomycosis. Serum samples were available at the time of relapse or death for only 2 of these dogs. One dog relapsed several months after a 60-day course of treatment. At the time of initial evaluation, the dog had a pretreatment anti-WI-1 titer of 1:277,151. The titer decreased to 1:31,084 at 60 days after treatment commenced and to 1:9,671 at 90 days. After a 3-month interval without disease, the dog relapsed, and its anti-WI-1 titer increased to 1:20,480. One dog died 30 days after blastomycosis was diagnosed. The titer in this dog had decreased from 1:5,915 before receiving treatment to 1:3,428 30 days after treatment. One dog had a strikingly high anti-WI-1 titer of 1:32,140,700 at the time of initial evaluation. However, this dog was treated with itraconazole for 90 days and was healthy 510 days after treatment was initiated. In this dog and the other 28 dogs, antibody titers also did not appear to correlate with severity of blastomycosis, as judged by extent of lung involvement radiographically.

Detection of anti-A antigen antibody by AGID—Dogs from Wisconsin with blastomycosis also were evaluated by use of the commercial AGID test. Of the 70 serum samples tested, only 29 (41%) had detectable antibodies against A-antigen in the AGID test. However, these samples had been collected not only at the time of infection but also at various times afterward. To evaluate whether the sensitivity of the commercial AGID test may be influenced by this variable, results of the assay were analyzed in those dogs that were tested within 10 days of disease confirmation. In this analysis, serologic results from dogs from Wisconsin that were tested in both assays also were compared. Indeed, the sensitivity of AGID was much higher during the interval shortly after diagnosis, especially between days -2 and 10, when 66% of samples yielded positive results (Table 2). In contrast, at all subsequent times, < 10% of samples tested yielded positive results. Nevertheless, RIA remained much more sensitive than AGID at every interval analyzed, including just before diagnosis and up to 10 days afterward. One remarkable feature of this comparison was the seropositivity rate in 29 dogs that were evaluated more than 10 days after a diagnosis was established. More than 90% of dogs remained seropositive by RIA for anti-WI-1 at 11 to 200 days after diagnosis and at 201

Table 2—Serologic results using radioimmunoassay (RIA) or the commercial agar-gel immunodiffusion (AGID) test, according to the time interval between blood sampling and diagnosis in 70 dogs from Wisconsin with blastomycosis that were evaluated with both tests in parallel

Days from diagnosis	No. of dogs	No. positive (%)		P value
		RIA	AGID	
-2 to 10	41	37 (90)	27 (66)	< 0.015
11 to 200	13	12 (92)	1 (8.0)	< 0.001
201 to 1545	16	15 (94)	1 (6.0)	< 0.001
Total	70	64 (92)	29 (41)	< 0.001

to 1,545 days after diagnosis, whereas < 10% of dogs were seropositive by AGID at these times.

Detection of antibody in suspected cases by use of RIA—Among the 24 dogs from Wisconsin in which blastomycosis was suspected but unconfirmed, 16 (67%) had detectable antibodies against WI-1 antigen by RIA, whereas 8 (33%) were seropositive by the A-antigen AGID.

Sensitivity, specificity, and predictive values of tests—Overall sensitivity of the tests was 92% for the WI-1 RIA and 41% for the A-antigen AGID when serologic results from all confirmed cases were analyzed. When only results from serum samples that were obtained shortly after diagnosis were included, however, sensitivity of RIA remained high at 91% (85/93 seropositive) and improved to 67% (27/41 seropositive) for the AGID test.

The 51 control dogs that had never traveled to an area enzootic for blastomycosis were tested for antibody production in both assays; none were seropositive. On the basis of results from this group of control dogs, both assays were judged to be 100% specific for antibodies against *B dermatitidis*. Predictive values for the 2 tests were determined initially, using serologic results in all confirmed cases. Here, the predictive value of positive test results was 100% for RIA and AGID. The predictive value of negative test results was 84% for RIA and 55% for AGID. When predictive values were determined using only the serologic results from samples obtained shortly after blastomycosis was diagnosed (within 10 days), the predictive value of negative test results improved to 86% for RIA and 79% for AGID.

Serologic survey of dogs residing in the enzootic area—Forty-eight serum samples were tested from healthy dogs residing in Spooner, Wisconsin, which is part of the enzootic area for blastomycosis. Two (4%) dogs were seropositive for anti-WI-1 antibody by RIA and both dogs had a titer of only 1:80; neither dog was seropositive by AGID. Neither of the seropositive dogs had a history of blastomycosis, nor were they ill with signs of infection at the time blood samples were collected.

Discussion

Dogs in southeastern, southcentral, and upper midwestern regions of the United States are at risk for contracting blastomycosis after exposure to infected soil. In states in enzootic regions, such as Wisconsin, as many as 1 to 2% of dogs acquire blastomycosis annually in hyperenzootic areas.¹³ The fact that nearly a quarter of infections are fatal makes blastomycosis a major health problem for dogs in the United States.^{14,15} Despite the clinical importance of blastomycosis, veterinarians may have difficulty establishing a timely diagnosis necessary for early treatment, because they lack a sensitive test to detect antibodies.

The primary problem with the development of reliable serologic tests is that well-defined *B dermatitidis* antigens have not been available. The major surface protein antigen, termed WI-1, was recently discovered in *B dermatitidis* yeasts.⁹ The molecule is an

adhesin^{16,17} and key virulence factor of *B dermatitidis*¹⁸ that induces strong immune responses during human infection.^{9,19,20} Studies have recorded that WI-1 is an immunodominant antigen that induces circulating antibodies^{9,19} and sensitized T-cells in humans with blastomycosis.²⁰ These findings have led to the development of more reliable serologic tests for diagnosis of human infection, using WI-1 for detection of antibody by RIA. When compared with commercially available assays that incorporate the A-antigen, such as ELISA, immunodiffusion, and complement fixation tests, the WI-1-based RIA has been more reliable for diagnosis of blastomycosis in humans.^{9,21} Radioimmunoassay is more sensitive and more specific than other assays, and yields greater predictive values.^{9,10}

We sought to evaluate the response to WI-1 during infection with *B dermatitidis* in dogs, and the usefulness of the WI-1-based RIA for serologic diagnosis. We demonstrated that WI-1 is also an immunodominant antigen for infected dogs. Most of the 122 dogs with blastomycosis had evidence of an immune response to WI-1 by RIA, based on detection of antibodies in sera collected at or after the time infection was confirmed. This observation held true for dogs in different regions of the country (Tennessee and Wisconsin) and, thus, was seemingly not influenced by geographic variation of antigen between fungal strains. Overall, 92% of dogs with blastomycosis had anti-WI-1 antibodies and positive serologic test results at the time of active disease. This result suggests that RIA is a sensitive method for detecting infection in dogs, as it is in humans.^{9,10} Among an additional 24 dogs, for which blastomycosis was suspected yet undiagnosed at the time of sample collection, 18 (67%) were also seropositive. Whereas some of these 24 dogs may not have had the infection, the seropositive dogs were most likely infected. Serologic test results in 51 control dogs from California were negative for anti-WI-1 by RIA, suggesting that this test is specific. Testing in humans also has revealed a high degree of specificity of RIA.^{9,10,21}

On the basis of results of our study, the predictive value of a positive RIA result is 100%. The seroprevalence rate of 4% seropositive for anti-WI-1 in healthy dogs from a hyperenzootic county in Wisconsin suggests that preexisting antibodies against the WI-1 antigen in dogs should not confound interpretation of a positive RIA result. In dogs with clinical features suggestive of blastomycosis, detection of antibodies against WI-1 should be interpreted to represent an active infection rather than a previous infection. Conversely, the predictive value of a negative RIA result is 84%. Thus, there is a reasonable degree of assurance that a negative RIA result reflects the absence of disease in a dog with compatible clinical signs. However, a negative serologic AGID result offers clinicians little assurance that a dog is not infected.

The study reported here confirms findings regarding the insensitivity of commercial assays formatted with the A-antigen.^{9,22,23} Limitations of the AGID test are underscored by the low predictive value of a negative result, which was about 50%. Consequently, when

a clinician receives a report of a negative AGID test result, there is a 50% chance that the result is a false-negative one. However, a positive AGID test result is highly accurate, according to results in this study based on 51 control dogs.

Surprisingly, anti-WI-1 antibodies persisted for an extended period after a diagnosis of blastomycosis was confirmed. Antibodies in dogs from Wisconsin persisted for up to 1,545 days after an initial diagnosis was made. Although antibodies in dogs from Tennessee decreased gradually with time, they were still detectable 210 days after initial diagnosis, when the mean titer was > 1:1000. Thus, seroconversion seems unlikely using RIA, at least within the time frame analyzed. This finding contrasts with results from the AGID test, in which seropositive results were found primarily at the time of illness, but much less often afterward, implying that antibody concentrations detected by AGID decrease quickly. Serial serum samples from dogs from Tennessee were tested only by RIA; therefore, the kinetics of seroconversion by AGID with time could not be characterized precisely.

It has been reported that WI-1 and A-antigens are similar in several ways. A key feature is that both contain a 25 amino acid tandem repeat.²¹ This sequence is evident in WI-1 in up to 34 copies,¹⁷ and is the chief site of recognition by antibodies in human sera directed against WI-1 or A-antigen.¹⁹ A distinguishing feature, however, is the large amount of carbohydrate on A-antigen (which is lacking on WI-1) that is responsible for antibody cross-reactivity with sera of patients having other fungal diseases and false-positive test results for blastomycosis.²¹ Although WI-1 is well-defined and more reliable in serologic diagnosis, an obvious difference between the 2 tests studied here was not just the target antigen but also the assay format. The greater sensitivity of the WI-1 RIA may have been the result of the assay format, the antigen, or both (it is most likely that both features contributed).

The WI-1 antigen and the tandem repeat have been cloned and expressed as recombinants in *Escherichia coli*.^{17,19,21} Both recombinants are recognized by monoclonal and patient antibodies,²¹ suggesting that the antigens, which lack carbohydrate, could be incorporated and functional in serologic assays. Such material is worth investigating in future studies of serologic diagnosis of blastomycosis and should be considered in commercial development of better tests for detection of blastomycosis in dogs.

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^bMarshfield Laboratories, Marshfield, Wis.

^cImmunomycologics, Norman, Okla.

^d#CA1019, Immunomycologics, Norman, Okla.

^e#B30110, Immunomycologics, Norman, Okla.

^f#B30110, Immunomycologics, Norman, Okla.

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