Mycopathologia (2006) 162: 33–37
DOI 10.1007/s11046-006-0028-7
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Detection of IgG and IgM in sera from canines with blastomycosis using eight *Blastomyces dermatitidis* yeast phase lysate antigens

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Received 5 August 2005; accepted in revised form 5 April 2006

Abstract

The objective of this study was to compare the efficacy of eight *Blastomyces dermatitidis* yeast phase lysate antigens (T-58: dog, Tennessee; T-27: polar bear, Tennessee; ERC-2: dog, Wisconsin; B5894: human, Minnesota; SOIL: soil, Canada; B5896: human, Minnesota; 48089: human, Zaire; 48938: bat, India) in the detection of the immunoglobulins IgG and IgM in serum specimens from canines with blastomycosis. An indirect enzyme-linked immunosorbent assay (ELISA, peroxidase system) was used to analyze sera collected during four different intervals post-infection. The yeast lysate antigen 48938 was a reactive antigen for the detection of both IgG (mean absorbance value range: 1.198–2.934) and IgM (mean absorbance value range: 0.505–0.845). For the same sera, antigen T-27 was also effective in the detection of IgG (mean absorbance value range: 0.904–3.356) and antigen 48089 was useful for the detection of IgM (mean absorbance value range: 0.377–0.554). The yeast lysate antigen B5894 proved to be a poor antigen for the detection of both IgG and IgM (mean absorbance value ranges: 0.310–0.744 for IgG, 0.025–0.069 for IgM). Inherent variations in yeast lysate antigens such as these may be utilized to develop improved immunoassay procedures for the specific detection of IgG or IgM in cases of blastomycosis.

Key words: *Blastomyces dermatitidis*, ELISA, IgG/IgM, immunodiagnosis, pathogenic fungi

Introduction

Blastomycosis is a potentially serious disease that is caused by the pathogenic fungus *Blastomyces dermatitidis*, a thermally dimorphic organism that exists in soil and rotting wood in the mycelial form and transforms into large, budding yeast cells in the host [1, 2]. Cases of blastomycosis have been reported in both humans and canines and the disease typically presents itself as a mild, chronic respiratory infection [3, 4]. Individuals can become infected with *B. dermatitidis* by inhaling the conidia produced by the organism. If left untreated, the infection can disseminate from the lungs to other parts of the body, resulting in a number of symptoms that may include skin lesions, bone and joint infections, meningitis, and death [3].

Diagnosis of blastomycosis can be quite challenging, but the enzyme-linked immunosorbent assay (ELISA) has proven to be one of the most sensitive tools for detecting *B. dermatitidis* infections [4–10]. An indirect ELISA procedure can be used to identify different classes of immunoglobulins that are directed against *B. dermatitidis* antigens in the serum of an infected individual. A yeast phase *B. dermatitidis* lysate antigen may be utilized in the ELISA procedure to detect these antibodies. This procedure is generally more sensitive than complement fixation or immunodiffusion procedures, with reported sensitivity ranging from 77% to 100% [6, 11–14]. Although cross-reactivity has been reported with ELISA procedures designed to diagnose blastomycosis [15], improved specificity has been achieved with

different preparations of *B. dermatitidis* yeast lysate antigens [16]. Two classes of antibodies that may be detected with these procedures are IgG and IgM [17]. Since IgM is the first antibody produced in a primary antibody response, the ability to detect this immunoglobulin would aid in the early diagnosis of blastomycosis. Thus, treatment could be initiated before the onset of any invasive symptoms.

The objective of this study was to compare eight *B. dermatitidis* yeast phase lysate antigens in the detection of the immunoglobulins IgG and IgM in serial serum samples from canines with blastomycosis. This comparison provides guidelines for improving the detection of IgG and establishes new methods for detecting IgM. Each antigen's interaction with antibodies directed against *B. dermatitidis* was monitored by measuring absorbance to identify the antigens that were efficient and inefficient at detecting IgG or IgM in sera.

Materials and methods

Antigens

Yeast phase lysate antigens were prepared from eight different strains of *B. dermatitidis* available through our laboratory (T-58: dog, Tennessee; T-27: polar bear, Tennessee; ERC-2: dog, Wisconsin; B5894: human, Minnesota; SOIL: soil, Canada; B5896: human, Minnesota; 48089: human, Zaire; 48938: bat, India). Filamentous phase cultures were first converted to yeast cells by culturing at 37 °C on brain heart infusion agar containing cysteine. The yeast phase lysates were prepared by a method that was previously used for the preparation of yeast lysate reagents from *Histoplasma capsulatum* [18–20] and modified by Johnson and Scalarone [5] in our laboratory. The yeast cells were grown at 37 °C for five days in a chemically defined medium with shaking and then harvested by centrifugation (5 min at $700 \times g$). The harvested cells were then washed five times with sterile distilled water by centrifugation and incubated in distilled water for 7 days at 37 °C with shaking in order to promote lysis. Following this incubation period, the preparation was centrifuged (30 min at $700 \times g$) to remove cellular debris and filter sterilized through a Nalgene filter (Nalge Company, Rochester, NY). Merthiolate (1:10000) was added to each lysate preparation as a preservative and the reagents were stored at 4 °C for future use.

Protein concentrations of the preparations were determined using the BCA assay kit (Sigma Chemical Company, St. Louis, MO) and appropriate antigen dilutions were prepared based on these concentrations. Western blotting was performed using a procedure modified from Hurst and Kaufman to check the antigen preparations prior to this project [21].

Serum specimens

The serum specimens used in this study were collected from canines diagnosed with blastomycosis. All of these cases were the result of natural infections reported to veterinarians and occurred in endemic areas in Alabama, Tennessee, and Mississippi. Diagnosis of blastomycosis was confirmed via histopathological examination and direct culture of the clinical specimens. Serum samples were collected on the day of diagnosis and the initiation of treatment (Day 0), 30 days after diagnosis (Day 30), 60 days after diagnosis (Day 60), and 90 days after diagnosis (Day 90). For each case, Day 0 only represents the day of diagnosis and not the day of infection. Serial serum samples from eight different dogs with blastomycosis were used. Normal sera from eight dogs inhabiting the endemic areas without blastomycosis were also used as controls for the indirect ELISA procedure. Prior to use, all serum samples were stored at −20 °C.

Indirect ELISA procedure

The efficacy of each yeast phase lysate reagent in detecting either IgG or IgM in serum specimens was determined by the indirect enzyme-linked immunosorbent assay (ELISA). This assay was duplicated for each antigen and serum sample.

Yeast phase lysate antigen was diluted to 100 ng/ml in a carbonate–bicarbonate buffer (pH 9.6) and placed into the wells of an Immunomaxi 96-well modified flat bottom high binding microdilution plate (100 μl/well; TPP, Switzerland). The plate was incubated in a humid chamber at 4 °C for 24 h and then washed three times with 0.15% Tween 20 in phosphate buffered saline (PBS-T; pH 7.4). Serum specimens diluted 1:1000 in PBS-T

were added to the wells (100 μl/well). Each sample was run in duplicate and a total of 32 serum samples were used. The microdilution plate was incubated in a humid chamber for 30 min at 37 °C. The plate was then washed three times with 0.15% Tween 20 in phosphate buffered saline (PBS-T; pH 7.4).

To detect IgG in sera, a 1:2000 dilution of goat-anti-dog IgG conjugated antibody (peroxidase; Kirkegaard and Perry Laboratories, Gaithersburg, MD) was added to each well (100 μl/well). To detect IgM in sera, a 1:500 dilution of goat anti-dog IgM conjugated antibody (peroxidase; KPL, Gaithersburg, MD) was added to each well (100 μl/well). The microdilution plate was incubated in a humid chamber for 30 min at 37 °C. The plate was washed three times as previously described. Peroxidase substrate (1-Step Ultra TMB-ELISA, Pierce Chemical Company, Rockford, IL) was added to each well (100 μl/well). Reactions were allowed to proceed at room temperature for 2 min. A solution of 2 N H_2SO_4 was added to each well to stop the reactions. The absorbance value for each well at 450 nm was obtained using the BIO-RAD model 2550 EIA reader.

Statistical analysis

The absorbance values obtained for the detection of either IgG or IgM with each antigen and within each time period were analyzed using standard within-subjects analysis of variance (ANOVA). Each time period (Day 0, Day 30, Day 60, and Day 90) was analyzed individually. All sample groups exhibited normality, with the exception of the absorbance values for IgG detection on Day 60 and the absorbance values for IgM detection on Day 90. For these sample groups, Friedman's test was substituted for the within-subjects ANOVA. Values of $P < 0.05$ were considered significant.

Results

The range of reactivity for the eight *B. dermatitidis* yeast phase lysate antigens in the detection of either IgG or IgM in canine sera varied (Figures 1 and 2). Absorbance values higher than the mean absorbance values obtained with each antigen combined with normal sera were considered positive for the detection for both IgG and IgM. The absorbance data indicates that the yeast lysate antigens 48938, T-27, and T-58 were the most effective at detecting IgG. Although these antigens were not statistically different from one another ($P > 0.05$), they were statistically different from the five remaining antigens, with 48938 exhibiting the highest absorbance values for all days except Day 0 (mean absorbance value ranges: 1.198–2.934 for 48938, 0.904–3.356 for T-27, and 0.742–2.605 for T-58, $P < 0.05$). The yeast lysate antigen 48938 was statistically superior to all other antigens in detecting IgM (mean absorbance value range: 0.505–0.845, $P < 0.05$).

The yeast lysate antigen B5894 was shown to be the least effective antigen for the detection of both IgG and IgM, with a statistical difference indicated in the detection of IgM (mean absorbance value ranges: 0.310–0.744 for IgG, 0.025–0.069 for IgM, $P < 0.05$ for IgM detection).

Discussion

All eight yeast phase lysate antigens exhibited varying degrees of reactivity when used in an indirect ELISA for the detection of both IgG and IgM in sera from canines with blastomycosis. Figures 1 and 2 illustrate that certain antigens were more efficient than others with respect to immunoglobulin detection, an important factor that must be considered when selecting an antigen to use in an immunodiagnostic procedure such as the indirect ELISA. Based on mean absorbance values, the yeast phase lysate antigen 48938 was quite effective for detecting both IgG and IgM. Of the eight antigens tested, this antigen is the best candidate to utilize for procedures designed to effectively detect antibodies present in canine serum as a result of blastomycosis. Abuodeh et al. [16] previously demonstrated that the 48938 antigen exhibited high relative sensitivity and specificity in the detection of anti-*B. dermatitidis* IgG in human sera when compared to ten other antigens, further supporting its use for diagnostic procedures. The antigen B5894 exhibited low absorbance values for the detection of both IgG and IgM, indicating that it would be a poor choice for use in the same procedures. Interestingly, there is no apparent correlation between antibody detection ability and geographic origin for any of the *B. dermatitidis* antigens used in this study. Despite

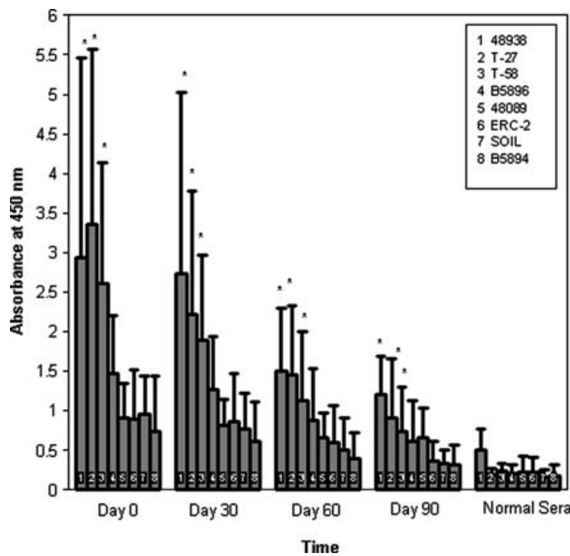

Figure 1. Comparison of indirect ELISA absorbance values for the eight *Blastomyces dermatitidis* lysate antigens used for the detection of IgG in serial samples of canine sera. Normal serum samples from dogs without blastomycosis are also presented for comparison. Error bars represent one standard deviation. An asterisk is used to indicate that 48938, T-27, and T-58 form a group of lysate antigens that is statistically superior to the remaining antigens in the detection of IgG in this study, although these antigens do not differ statistically from one another in detection ability.

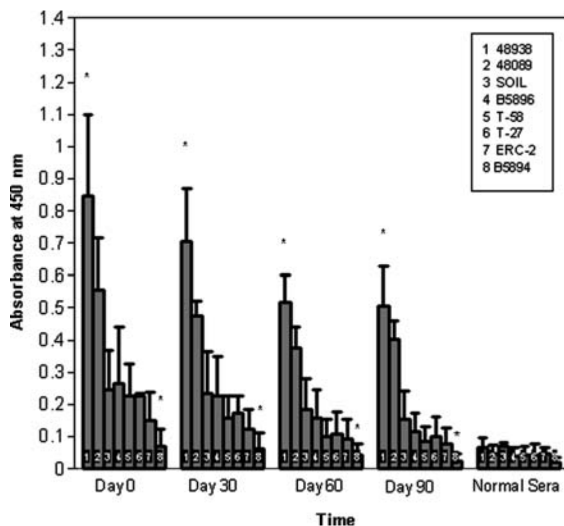

Figure 2. Comparison of indirect ELISA absorbance values for the eight *Blastomyces dermatitidis* lysate antigens used for the detection of IgM in serial samples of canine sera. Normal serum samples from dogs without blastomycosis are also presented for comparison. Error bars represent one standard deviation. An asterisk is used to indicate that 48938 is statistically superior and B5894 is statistically inferior to the other antigens in the detection of IgM in this study.

the fact that all sera were from dogs inhabiting the southern part of the United States, the best overall immunoglobulin detection was obtained using the 48938 antigen from an Indian *B. dermatitidis* strain.

It is important to note that antigens effective for detecting IgG are not necessarily ideal for detecting IgM, and vice versa. The antigen 48089 exhibited high absorbance values for the detection of IgM, indicating that it may be effective to use in immunodiagnostic procedures for diagnosing blastomycosis. However, the absorbance data for IgG detection using the antigen 48089 does not support this same conclusion. An antigen that would be a better second choice for detecting IgG would be T-27, although this antigen was not effective for detecting IgM. Other variations in the abilities of each antigen to detect IgG and IgM can be observed by comparing relative absorbance values. Another option for this type of ELISA procedure is to use a pooled antigen comprised of various strains of *B. dermatitidis*. Such an antigen may increase sensitivity, but only if the antigens selected are genuinely effective at detecting the immunoglobulins of interest. For this reason, different pooled antigen preparations may be required for detecting either IgG or IgM. Ongoing investigations include utilizing the antigens judged in this study to be the optimal detectors of IgG and IgM in both individual and pooled preparations with larger numbers of canine serum samples in order to better define detection efficiencies. The data suggest that choosing antigens to use in an indirect ELISA or other immunodiagnostic procedure merits consideration of the immunoglobulin class to be detected.

Acknowledgements

This research was funded by a grant from the Idaho State University Graduate Student Research and Scholarship Committee (F02-21) and the Department of Biological Sciences at Idaho State University. A.M. Legendre, D.V.M. (University of Tennessee School of Veterinary Medicine, Knoxville, TN) provided the canine serum samples used for this project. Teri Peterson provided guidance on statistical analysis. Bret Birrer and Pahresah Roomiany are acknowledged for their assistance with this project.

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