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ANTIBODY AND ANTIGEN DETECTION IN *BLASTOMYCES DERMATITIDIS*

by

Jordan T. Hammon

A thesis

submitted in partial fulfillment

of the requirements for the degree of

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The members of the committee appointed to examine the thesis of Jordan T. Hammon find it satisfactory and recommend that it be accepted.

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Introduction

Blastomycosis, produced by the dimorphic fungal organism *Blastomyces dermatitidis,* is a systemic fungal infection of humans and other animals that is initiated by the inhalation of conidia (spores produced by the filamentous phase of the fungus). The organism exists in this stage in nature or in the laboratory at 25 C and has the ability to convert to the yeast phase at 37 C in the lungs of the infected host. The disease may be self-resolving or it may exist persist to an acute or chronic state in the pulmonary tissue, where it may be misdiagnosed as a form of pneumonia or even tuberculosis. If the disease is not diagnosed, or is left untreated while in the lungs, it may become invasive and disseminate to other regions of the body. In serious cases, the fungus has the ability to disseminate to the central nervous system where fatal meningitis may develop. Blastomycosis is cause for concern in individuals with AIDS or other deficiency diseases that compromise the immune system [1-4].

Traditionally, the geographic distribution of blastomycosis has been associated with southeastern and south-central states that border the Ohio and Mississippi Rivers and upper Midwestern states including areas in Wisconsin and Minnesota, which are highly endemic for the disease. Lower regions of Canada also contain the spores of *B. dermatitidis.* Recent studies have indicated sporadic cases being reported in Colorado, Texas, Kansas and Nebraska [6, 7].

Due to the increase in systemic fungal diseases, researchers have begun to devote more attention to developing ways of diagnosing, preventing and treating these mycoses. Blastomycosis has been a concern because problems have existed with regard to diagnosis of the disease or even the misdiagnosis as some other infectious disease. In some instances

culturing or histopathological examination may be beneficial, but in some patients these methods may not yield the desired results. This has led to more and more research being done to improve immunological assays which tend to provide a more rapid diagnosis, but problems still exist with regard to the sensitivity and specificity of immunoassays [4-5, 8-11].

For the past several years the thrust of research in our laboratory has been associated with studies on various strains of *B. dermatitidis* from human, animal or environmental specimens from many geographical locations in an effort to better understand how antigens prepared from these isolates might be useful as immunodiagnostic reagents. Our laboratory has developed novel methods for the preparation of yeast phase lysate antigens and utilized these in various comparative immunoassays for both antibody and antigen detection [12-25], but these studies have only opened up new avenues of approach with regard to how we might improve immunodiagnostic methods in the future.

Research objectives

The first objective of this study was to evaluate the ability of several lysate antigen preparations in detecting antibody produced in serum specimens from animals diagnosed with blastomycosis. The second objective of the study was to assess the ability of a variety of serum specimens' ability to detect antigen in urine specimens from dogs diagnosed with blastomycosis. The third and final objective was to compare antibody and antigen detection of common strains and better understand the differences of these two routes in diagnosing this evasive fungal infection. Completion of these objectives may assist clinicians in developing a more efficient diagnostic tool for blastomycosis.

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Reactivity of Diverse *Blastomyces dermatitidis* Lysate Antigens vs. Serum Specimens from Dogs with Blastomycosis

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Abstract

Blastomycosis is a disease caused by the parasitic, dimorphic fungus *Blastomyces dermatitidis*. This disease has been a diagnostic challenge due to problems with sensitivity and specificity of the assays. The present study assayed 8 lots of diverse *B. dermatitidis* yeast lysate antigens to determine the reactivity of the reagents. This was determined by comparing antibody detection (enzyme-linked immunosorbent assay; ELISA) with 15 serum specimens from dogs diagnosed with blastomycosis. Seven of the 8 reagents proved to be immunoreactive and were able to detect antibody in each of the 15 different serum specimens. Sensitivity mean absorbance values with the lysates ranged from 0.479 (48089; human isolate from Africa) to 1.464 (T-27; polar bear isolate from Tennessee) with a mean value for all 8 antigens equal to 1.144. This study generated data making it evident that

although the diverse antigenic lysates do display some differences in reactivity, each of them has ability to detect antibody in dog sera in an efficient manner.

Keywords: Blastomycosis, *Blastomyces dermatitidis*, ELISA, lysate antigens, antibody detection

Introduction

The systemic fungal disease blastomycosis, caused by the etiologic agent *Blastomyces dermatitidis*, is a disease of humans and other animals. Blastomycosis is an endemic disease found in the Southeastern, South-Central and upper Midwestern states of the United States, including areas of Wisconsin, Minnesota and regions of lower Canada. Evidence over the years has indicated that this fungus exists in areas with an abundance of moisture and decaying organic matter [1, 2]. *B. dermatitidis* is thermally dimorphic and acquired by inhalation of the infectious particle (mycelial phase spore) into the lung in which it then converts to a large yeast cell and produces a primary pulmonary acute infection. It may disseminate into other organs of the body including the central nervous system and, as the disease progresses, cutaneous lesions may develop. If a proper diagnosis is not made, or if the disease is misdiagnosed as a bacterial or viral infection, it may be fatal; especially in an immunosuppressed individual [4, 5].

Current laboratory diagnostic methods include culturing or histologic identification, but in many instances these methods may not provide a reliable diagnosis or they may take a considerable amount of time which can delay treatment. During the past several years investigators have made considerable progress with regard to the laboratory diagnosis by developing immunodiagnostic assays for the detection of antibodies or antigens present in patients with blastomycosis [6, 9].

In an effort to contribute to improved immunodiagnostic laboratory assays, our laboratory has been concerned with the preparation and comparative studies of *B. dermatitidis* yeast lysate antigens, prepared from various isolates of the fungus, for the detection of antibodies in sera from immunized and infected animals [10,12]. Encouraging results have been obtained with the *B. dermatitidis* lysate antigens, but other studies are needed to further evaluate the reagents with regard to sensitivity and specificity of the reagents prepared from diverse isolates of the fungus obtained from human, animal and environmental sources.

Materials and Methods

Lysate antigen preparation

Eight yeast lysate antigens were prepared from *B. dermatitidis* isolates (48089: human from Africa; 48938: bat lung from India; 56920: bat liver from India; T-27: polar bear from Tennessee; 449: polar bear from Illinois; 81: sea lion from Tennessee; 104: cat trachea from Tennessee; 103: cat skin from Tennessee). Each of the isolates was prepared by a method similar to one that was previously used for the production of yeast lysate antigen from *Histoplasma capsulatum* [13-15] and modified in our laboratory for *B. dermatitidis* lysate antigen production [10]. The yeast phase cells were grown for 7 days at 37°C in a chemically defined medium in an incubator shaker. They were then harvested by centrifugation (700 x g; 5 min), washed with distilled water, resuspended in distilled water and allowed to lyse for 7 days at 37°C in water with shaking. The preparations were centrifuged, filter sterilized, merthiolate added (1:10,000) and stored at 4°C for up to 22 years. Protein determinations were performed on the lysates using the BCA Protein Assay

Kit (Thermo-Fisher, Pierce Chemical Company, Rockford, IL) and dilutions of the antigenic reagents used in the ELISA assays were based on protein concentration.

Serum specimens

Fifteen different serum specimens from dogs with diagnosed blastomycosis were provided by Dr. A.M. Legendre (University of Tennessee College of Veterinary Medicine, Knoxville, TN). The dog sera in this study was used to evaluate each yeast lysate antigen's ability to detect antibody.

Enzyme-linked immunosorbent assay (ELISA)

The ability of each yeast lysate reagent to detect antibodies in the above serum specimens was determined using the indirect enzyme-linked immunosorbent assay (ELISA) as previously described [10-12]. Each lysate antigen was diluted (2000 ng/ml of protein) in a carbonate-bicarbonate coating buffer (pH 9.6) and then added to triplicate wells (100 ul) of a NUNC 96-well microplate (Fisher-Thermo). The plates were then incubated overnight at 4°C in a humid chamber followed by washing three times with phosphate buffered saline containing 0.15% Tween 20 (PBS-T). The serum specimens (1:2000 dilution; 100 ul) were added to the microplate wells in triplicate and incubated for 30 min at 37°C in a humid chamber. Following this incubation, the wells were washed as above and 100 ul of goat anti-dog IgG (H & L) peroxidase conjugate (Kirkegaard and Perry, Gaithersburg, MD, KPL) was added to each well and incubated for 30 min at 37°C. The plates were again washed as above and 100 ul of Sure Blue Reserve TMB peroxidase substrate (KPL) was added to each well and incubated for approximately 2 min at room

temperature. The reaction was stopped by the addition of Stop Solution (KPL) and the absorbance read at 450 nm using a BIO-RAD 2550 EIA reader.

Results and Discussion

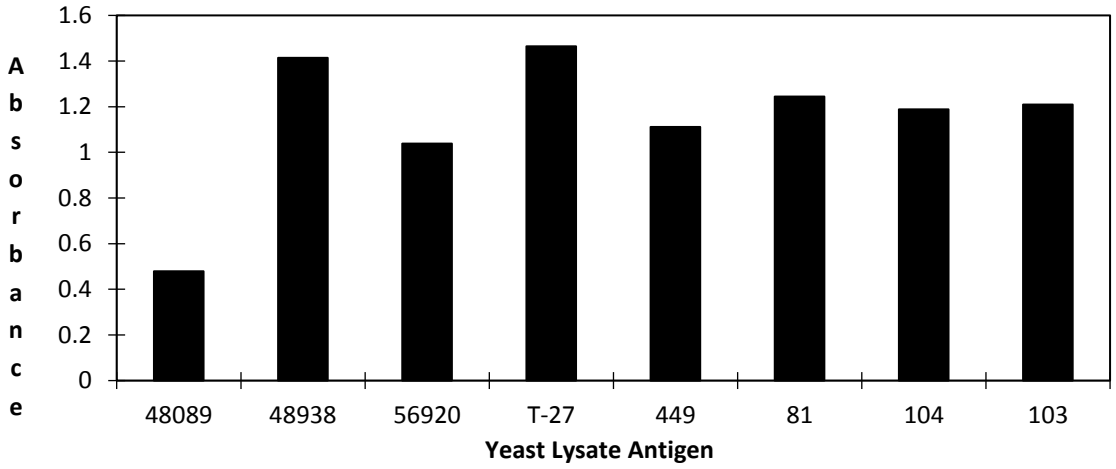

Figure 1. Mean reactivity of 8 diverse Blastomyces dermatitidis yeast lysate antigens (48089: human from Africa; 48938: bat from India; 56920: bat from India; T-27: polar bear from Tennessee; 449: polar bear from Illinois; 81: sea lion from Tennessee; 104: cat from Tennessee; 103: cat from Tennessee) reacted with 15 different dog serum specimens.

Figure 1 illustrates that all of *B. dermatitidis* yeast lysate antigens from these diverse environments except 48089 (human from Africa) were reactive with dog serum specimens. Mean absorbance values ranged from 0.479 (human from Africa) to 1.464 (polar bear from Tennessee) with a mean value for all 8 antigens equal to 1.144. The mean absorbance value difference between the lysate showing the greatest value and the lysate with the lowest value was 0.985.

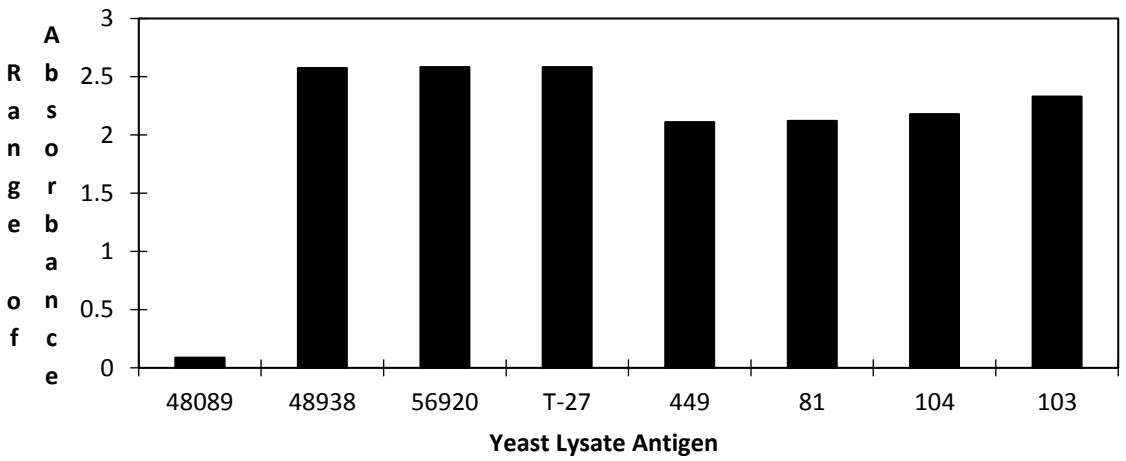

Figure 2. Range of reactivity between 8 diverse Blastomyces dermatitidis yeast lysate antigens (48089: human from Africa; 48938: bat from India; 56920: bat from India; T-27: polar bear from Tennessee; 449: polar bear from Illinois; 81: sea lion from Tennessee; 104: cat from Tennessee; 103: cat from Tennessee) reacted with 15 different dog serum specimens.

Figure 2 shows the range of these exotic yeast phase lysate antigens in their reactivity with a total of 15 different dog sera. The T-27 (polar bear from Tennessee) lysate antigen showed the greatest range of reactivity among the dog sera with a range of 0.395-2.504 while the 48089 (human from Africa) lysate antigen showed the lowest range of reactivity between dog serum specimens with a range of 0.323-0.410. The average range between yeast lysate antigens with respect to their reactivity with 15 different dog sera was 2.071. The aim of this current research was to compare the antibody detection capability of diverse yeast lysate antigens of *B. dermatitidis* from a variety of environments. This was achieved by reacting those lysate antigens with a variety of dog sera acquired from dogs infected with *B. dermatitidis*. The lysates were prepared and then stored at 4°C. Seven of the 8 yeast lysate antigens exhibited a great deal of reactivity and were able to detect antibody in each of the 15 *B. dermatitidis* dog sera. The variations in reactivity that were

observed with the 8 different lysates were likely associated with antigenic differences and variations in the amount of antibody present in the sera from the dogs. The ability of some yeast lysate antigens to detect antibody better than others provides evidence that needs to be considered when using such antigens as immunodiagnostic tools in clinical situations. This is certainly an important consideration in the production and use of such preparations for the laboratory diagnosis of fungal diseases. This present study demonstrates that the yeast lysate antigens from diverse environments had the ability to detect *B. dermatitidis* antibodies in a sensitive manner and to a similar degree. The continuation of studies in this respect will further examine yeast lysate antigens for their ability to detect antibody in specimens from humans and other animals with blastomycosis.

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Antibody Detection in Dogs with Blastomycosis: Comparison of *Blastomyces dermatitidis* Lysate Antigens Prepared from Dog and Soil Isolates

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Abstract

Blastomyces dermatitidis, the causative agent of blastomycosis, has presented diagnostic dilemmas to clinicians for many years due to problems with sensitivity and specificity. This study compared six *B. dermatitidis* yeast lysate antigens, three prepared from soil isolates and three from dog isolates to assess antibody detection using the enzyme-linked immunosorbent assay [ELISA] in 20 serum specimens from dogs with diagnoses blastomycosis. All six reagents proved to be immunoreactive and were able to detect antibody in each of the sera with only slight variations in the mean absorbance values evidenced. Sensitivity mean absorbance values ranged from 0.658 (47; dog from Minnesota) to 0.733 (98 and 42913; dog from Minnesota and dog from Tennessee

respectively) with a mean value for all six antigens equal to 0.710. This study generated data making it apparent that although the antigenic lysates do display some differences in reactivity, each of them has ability to detect antibody in dog sera in an efficient manner. This study is part of ongoing evaluations in which we have been performing comparative studies on a large number of *B. dermatitidis* yeast phase lysate antigens prepared from human, animal and environmental isolates of this dimorphic fungus.

Keywords: *Blastomyces dermatitidis*, lysate antigens, antibody detection, ELISA, blastomycosis

Introduction

Blastomycosis, a pulmonary and potentially systemic fungal disease caused by *Blastomyces dermatitidis*, is an infection of humans and other animals. This disease has been associated with regions of the United States where there is an abundance of water and decaying vegetation including states that border the Mississippi and Ohio Rivers and also states like Minnesota, Wisconsin, areas of lower Canada, and even in certain regions of Africa and India [1,2].

The disease state originates when an individual inhales mycelial spores into the lung which then have the ability to convert to broad-based budding yeast cells. This primary acute infection may progress to a chronic state or even disseminate to other organs including the production of cutaneous lesions or infection of the central nervous system which may be fatal depending on the immunological status of the patient. Often times the disease is not diagnosed in the desired time frame or a misdiagnosis is made as a bacterial or viral infection which may certainly lead to problems regarding antimicrobial treatment [3,4].

Various techniques have been used in the clinical laboratory for the diagnosis of blastomycosis including microscopy, culturing and histopathologic methods. In some instances these methods have provided a reliable diagnosis, but in other instances a diagnosis may not be achieved or the time interval required for the diagnosis of the infection may be quite lengthy. Therefore investigators have spent much effort during the past several years in attempting to develop improved immunodiagnostic assays for antibody and antigen detection in blastomycosis [5-9]. For many years our laboratory has developed and evaluated yeast phase lysate antigens prepared from a variety of *B. dermatitidis* isolates and the utilization of such reagents for the detection of antibodies in serum specimens from immunized and infected animals [10-14]. In many instances we have shown the utility of some of these novel lysate antigens with regard to antibody detection, but these comparative studies have also indicated that additional evaluations with some of the more recently prepared lysates are necessary. This study compares six new lysate preparations (three each from dog isolates and soil isolates) for antibody detection in serum specimens from dogs with diagnosed blastomycosis.

Materials and Methods

Lysate antigen preparation

Six yeast lysate antigens were prepared from *B. dermatitidis* isolates (397: soil from Georgia; 98: dog from Minnesota; 47: dog from Tennessee; 248: soil from Eagle River Minnesota; 42913: dog from Tennessee; A2: soil from Canada). Each of the isolates was prepared by a method similar to one that was previously used for the production of yeast lysate antigen from *Histoplasma capsulatum* [15-17] and modified in our laboratory for *B. dermatitidis* lysate antigen production [10]. The yeast phase

cells were grown for 7 days at 37°C in a chemically defined medium in an incubator shaker. They were then harvested by centrifugation (700 x g; 5 min), washed with distilled water, resuspended in distilled water and allowed to lyse for 7 days at 37°C in water with shaking. The preparations were centrifuged, filter sterilized, merthiolate added (1:10,000) and stored at 4°C. Protein determinations were performed on the lysates using the BCA Protein Assay Kit (Thermo-Fisher, Pierce Chemical Company, Rockford, IL) and dilutions of the antigenic reagents used in the ELISA assays were based on protein concentration.

Serum specimens

Twenty different serum specimens from dogs with diagnosed blastomycosis were provided by Dr. A.M. Legendre (University of Tennessee College of Veterinary Medicine, Knoxville, TN). The dog sera in this study were used to assess each yeast lysate antigen's ability to detect antibody.

Enzyme-linked immunosorbent assay (ELISA)

The ability of each yeast lysate reagent to detect antibodies in the above serum specimens was determined using the indirect enzyme-linked immunosorbent assay (ELISA) as previously described [11-14]. Each lysate antigen was diluted (2000 ng/ml of protein) in a carbonate-bicarbonate coating buffer (pH 9.6) and then added to triplicate wells (100 ul) of a NUNC 96-well microplate (Fisher-Thermo). The plates were then incubated overnight at 4°C in a humid chamber followed by washing three times with phosphate buffered saline containing 0.15% Tween 20 (PBS- T). The serum specimens (1:2000 dilution; 100 ul) were added to the microplate wells in triplicate and incubated

for 30 min at 37°C in a humid chamber. Following this incubation, the wells were washed as above and 100 ul of goat anti-dog IgG (H & L) peroxidase conjugate (Kirkegaard and Perry, Gaithersburg, MD, KPL) was added to each well and incubated for 30 min at 37°C. The plates were again washed as above and 100 ul of Sure Blue Reserve TMB peroxidase substrate (KPL) was added to each well and incubated for approximately 2 min at room temperature. The reaction was stopped by the addition of Stop Solution (KPL) and the absorbance read at 450 nm using a BIO-RAD 2550 EIA reader.

Results

Figure 1 demonstrates that each of the six *B. dermatitidis* yeast lysate antigens from these isolates were reactive with the dog serum specimens. Mean absorbance values ranged from 0.658 (dog; Tennessee) to 0.733 (dog; Minnesota and dog; Tennessee) with a mean value for all six antigens equal to 0.710. The mean absorbance value difference between the lysate showing the greatest value and the lysate with the lowest value was 0.075. Figure 2 shows the range of these yeast phase lysate antigens based on their reactivity with a total of 20 different dog sera. Lysate 98 (dog; Minnesota) showed the greatest range of reactivity among the dog sera with a range of 0.440-1.468 while lysate 47 (dog; Tennessee) presented the lowest range of reactivity between dog serum specimens with a range of 0.412-1.003. The average range between yeast lysate antigens with respect to their reactivity with 20 different dog sera was 0.812.

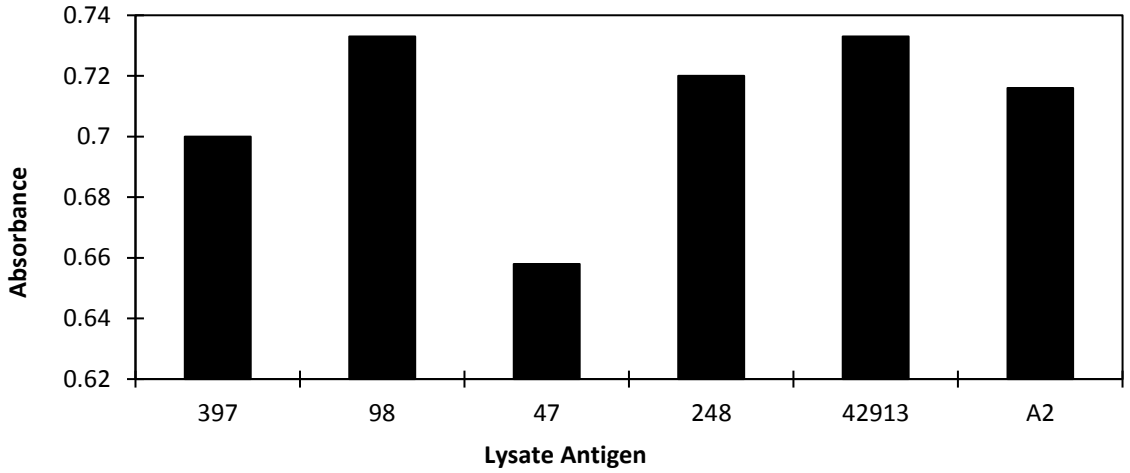

Figure 1. Mean reactivity of 6 Blastomyces dermatitidis yeast lysate antigens (397: soil from Georgia; 98: dog from Minnesota; 47: dog from Tennessee; 248: soil from Eagle River Minnesota; 42913: dog from Tennessee; A2: soil from Canada); reacted with 20 different dog serum specimens.

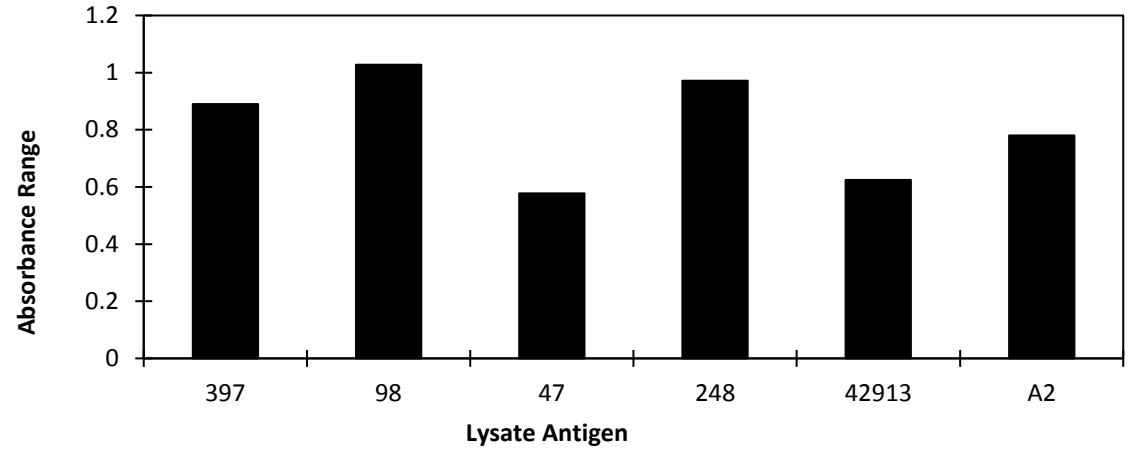

Figure 2. Range of absorbance values at 450nm between 6 Blastomyces dermatitidis yeast lysate antigens (397: soil from Georgia; 98: dog from Minnesota; 47: dog from Tennessee; 248: soil from Eagle River Minnesota; 42913: dog from Tennessee); A2: soil from Canada; reacted with 20 different dog serum specimens.

Discussion/Conclusion

The focus of this study was to compare the antibody detection capability of six

different yeast lysate antigens of *B. dermatitidis* from various locations. This was accomplished by reacting the lysate antigens with an assortment of dog sera acquired from dogs that had been infected with *B. dermatitidis*. Five of the six lysate antigens proved to have similar ability to detect antibody in the dog sera. As illustrated in Figure 1, lysate antigens 98 (dog; Minnesota) and 42913 (dog; Tennessee) had the highest absorbance values of 0.733 and were thus slightly more efficient at detecting antibody in the dog serum specimens. Lysate antigen 47 (dog; Tennessee) indicated it was the least reactive at detecting antibody by displaying a mean absorbance value slightly lower than the others, a value equal to 0.658. The slight differences in reactivity of the lysates were likely due to antigenic differences and variations in the amount of antibody present in the sera from the dogs. Because some yeast lysate antigens detect antibody better than others, this fact needs to be considered when using such antigens as immunodiagnostic tools in clinical situations. This is certainly an important consideration in the production and use of such preparations for the laboratory diagnosis of fungal diseases. This study makes evident that the yeast lysate antigens from these environments had the ability to detect *B. dermatitidis* antibodies to a similar degree. The continuation of studies in this respect will further examine yeast lysate antigens for their ability to detect antibody in serum specimens from humans and other animals. The ultimate aim of this experiment and ones to follow is to decrease the number of misdiagnosed cases of blastomycosis.

Acknowledgements

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Blastomyces dermatitidis Antigen Detection: A Comparative Study with Rabbit Antibodies Produced from Isoelectric Focusing Fractions and Yeast Lysates from Two Human Isolates of the Organism

US Open Animal Health and Veterinary Medicine Journal, Vol. 2, No. 2, August 2015, pp. 1-8.

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Abstract

The laboratory diagnosis of blastomycosis has been an enigma to medical personnel for many years. Recently investigators have concentrated on the development of antigen detection immunoassays for this disease. This present study was designed to evaluate rabbit antibodies produced from *Blastomyces dermatitidis* isoelectric focusing fractions (Rotofor:R) of yeast lysate preparations and lysate (L) reagents from two human isolates for the detection of antigen in 36 urine specimens from dogs with diagnosed blastomycosis. A competitive inhibition enzyme-linked immunosorbent assay (ELISA) was used to compare the antibodies. All four antibodies were able to detect antigen in the urine specimens with sensitivity values ranging from 97% (B5896: R and L) to 100% with the B5931 (R and L) antibody. When the positive control value of each antibody was compared to the mean inhibition value (the lower the value=more antigen detected) of all 36

specimens, the absorbance value differences were 0.191 (B5931:R), 0.350 (B5896:R), 0.495 (B5896:L) and 0.510 (B5931:L). The degree of inhibition (antigen detection) was greater with antibodies from both yeast lysate reagents as compared to antibodies produced from the isoelectric focusing fractions. Therefore this study indicated the potential of using any of the four antibody preparations for the detection of antigen in urine specimens from dogs with blastomycosis.

Keywords: *Blastomyces dermatitidis*, antigen detection, competitive ELISA, lysate antigens, isoelectric focusing fractions

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Introduction

Blastomycosis, caused by the thermally dimorphic systemic fungal organism *Blastomyces dermatitidis*, is a disease of humans and animals. Blastomycosis is endemic in southeastern regions of the United States as well as in upper midwestern states including Minnesota and Wisconsin and regions of lower Canada. The fungus grows in the mycelial form in areas where there is an abundance of moisture and rich organic matter present [1-3]. Individuals become infected by inhaling the infectious mycelial spore into the lung where the spore may convert into a broad-based budding yeast cell. The disease may present as an acute or chronic infection in the lung or it may disseminate to other internal organs or even to the central nervous system where a fatal meningitis may develop [4-6].

The laboratory diagnosis of blastomycosis has presented a challenge to physicians for many years. Routine microbiological or histological may be performed, but in many instances these methods may not yield an accurate diagnosis [3,7,8]. Therefore, in recent

years, researchers have devoted a considerable amount of effort in the development of immunodiagnostic assays for the detection of *B. dermatitidis* antibodies or antigens in serum or urine specimens from humans or dogs with blastomycosis or other fungal diseases [7-16].

For several years our laboratory has been involved in the development of *B. dermatitidis* yeast phase lysate antigens from various isolates of the fungus and the evaluation of such lysates for the detection of antibodies in animals and humans. The lysates have also been used to induce antibodies in rabbits and the utilization of such antibodies in antigen detection assays [17-22]. These studies have produced data/results that has been encouraging for continued studies on these reagents as immunodiagnostic tools. We have also performed initial studies on the use of isoelectric focusing (Rotofor) to separate the yeast lysate preparations in various fractions in order to determine the immunoreactive components associated with the lysates. Data has indicated that the initial fractions (1-5) of the total of 20 seem to be morereactive when tested against *B. dermatitidis* serum specimens from immunizedrabbits or infected dogs [23].

The objective of this present study was to evaluate isoelectric focusing (Rotofor) antibodies produced from 4 “early” fractions and to compare these to standard yeast lysate antibody preparations from human isolates (B5896,B5931) for the detection of antigen in urine specimens from dogs with diagnosed blastomycosis. The competitive inhibition ELISA was used for the comparative assays.

Materials and Methods

Antibodies/Urine Specimens

The four antibodies were obtained from rabbits immunized with either *B. dermatitidis* yeast lysate (L) antigens or isoelectric focusing (Rotofor) fractions (R) from lysatepreparations from human isolates B5896 and B5931. Urine specimens (36) were provided by Dr. A.M. Legendre (University of Tennessee College of Veterinary Medicine, Knoxville, TN)

Yeast Lysate Antigens

Two *B. dermatitidis* yeast phase lysate reagents, from a human outbreak of blastomycosis in Mountain Iron, Minnesota, (B5898 and B5931) were prepared by a method similar to one that was previously used for the production of yeast lysate antigen from *Histoplasmacapsulatum* [24-26] and modified in our laboratory for *B. dermatitidis* lysate antigen production [17]. The yeast phase cells were grown for 7 days at 37°C in a chemically defined medium in an incubator shaker. They were then harvested by centrifugation (700 x g; 5 min), washed with distilled water, resuspended in distilled water and allowed to lyse for 7 days at 37°C in water with shaking. The preparations were centrifuged, filter sterilized, merthiolate added (1:10,000) and stored at 4 C. Protein determinations were performed on the lysates using the BCA Protein Assay Kit (Thermo-Fisher, Pierce Chemical Company, Rockford, IL) and dilutions of the antigenic reagents used as immunizing agents and ELISA assays were based on protein concentration.

Isoelectric Focusing (Rotofor)

Isoelectric focusing was performed using the BIO-RAD Rotofor apparatus (BIO-RAD, Hercules, CA). Ampholytes (BIO-RAD) were added to the yeast phase lysate in a 2% to 98% ratio. These small charged molecules create a pH gradient in solution from a pH of 3 to 10 when an electrical current is applied so that proteins can be separated based on their isoelectric point. All proteins have a unique net charge that will force the proteins to move through the pH gradient until their net charge be-comes zero (the isoelectric point). When proteins reach their unique isoelectric point in this pH gradient they are no longer able to migrate and forced to remain where their net charge is zero by the established pH gradient. Twenty protein fractions were collected after focusing (15 watts constant current) for approximately four hours at 4 C to ensure that no denaturing of the proteins occurred. The focusing was stopped when the voltage stopped fluctuating [23]. After collection of the fractions the pH was measured and adjusted to return the proteins to their physiologically active pH. This was accomplished by the addition of HCl or NaOH to either lower or raise the pH as required. Protein determinations were performed on the fractions using the Pierce BCA Protein Assay, as above.

Competitive ELISA

The horseradish peroxidase competitive binding inhibition ELISA was used for the detection of *B. dermatitidis* antigens in the urine specimens. Microdilution plates (96 well NUNC, Thermo-Fisher) were coated with 100 µl of B5896 (Minnesota human isolate) lysate antigen that was diluted (2000 ng ml-1) in a carbonate-bicarbonate coating buffer (pH 9.6). The plates were incubated overnight at 4° C in a humid chamber followed by washing three times with phosphate buffered saline containing 0.15% Tween 20 (PBS-T).

Dog urine and 1:100 or 1:1250 antibody obtained from rabbits immunized with either B5931 (Minnesota human) or B5896 (Minnesota human) isoelectric focusing fractions (R) or whole lysate (L) preparationswere added to microcentrifuge tubes (200 µl plus 200 µl of each urine specimen) and incubated for 30 min at 37° C. Following this incubation step 100 µl of the antibody-urine mixture from the microdilution tubes was added to the above plates containing the B5896 antigen and incubated for 30 min at 37º C. The plates were again washed as above and 100 µl of goat anti-rabbit IgG horseradish peroxidase conjugate (Kirkegaard and Perry Laboratories, KPL) was added to each well and incubated for 30 min at 37° C and were washed as above. Then 100 µl of Sure Blue Reserve TMB peroxidase substrate (KPL) was added to each well and incubated for approximately 6 min at room temperature. Stop Solution (KPL) was added to each well and the absorbance was read using a BIO-RAD 2550 EIA reader at 450 nm. Positive controls containing known B5896 antigen coated on the plate and the above sera from the immunized rabbits were used to determine the baseline value to which all of the urine specimens were compared.

Results and Conclusion

The results depicted in the figures below show that each of the four antibodies had the ability to detect antigen in a sensitive manner. The absorbance values for the B5896 Rotofor (R) antibody ranged from 0.300-0.852 with a mean absorbance value of 0.480 while the absorbance values for the B5896 lysate antibody (L) fell in the range of 0.335-1.473 with a mean absorbance value of 0.944. The B5931 Rotofor (R) antibody showed a range of absorbance of 0.335-0.678 and a mean absorbance of 0.507 and the B5931 lysate antibody (L) had an absorbance range of 0.265-1.323 with a mean absorbance value of 0.880.

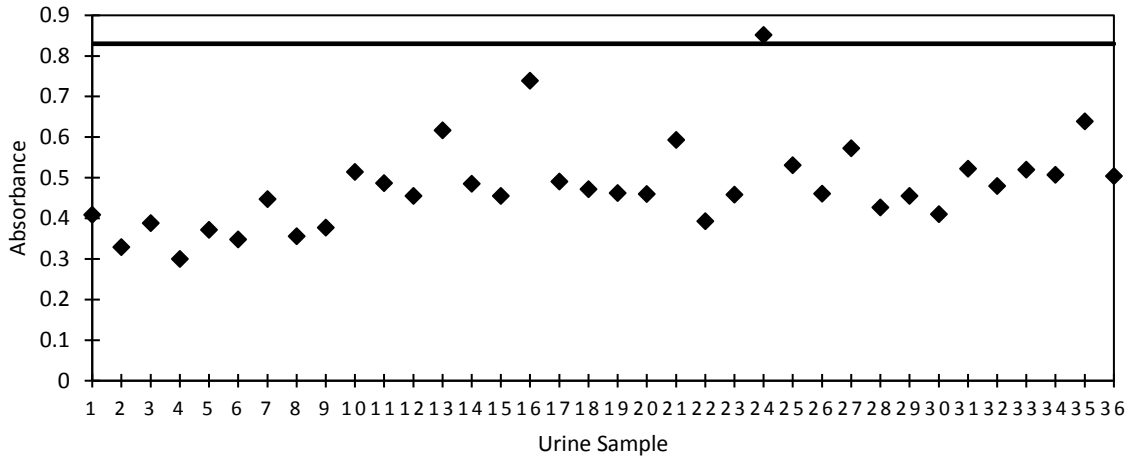

Figure 1. The absorbance values of the B5896 Rotofor (R) antibody against 36 different urine specimens from dogs with blastomycosis.

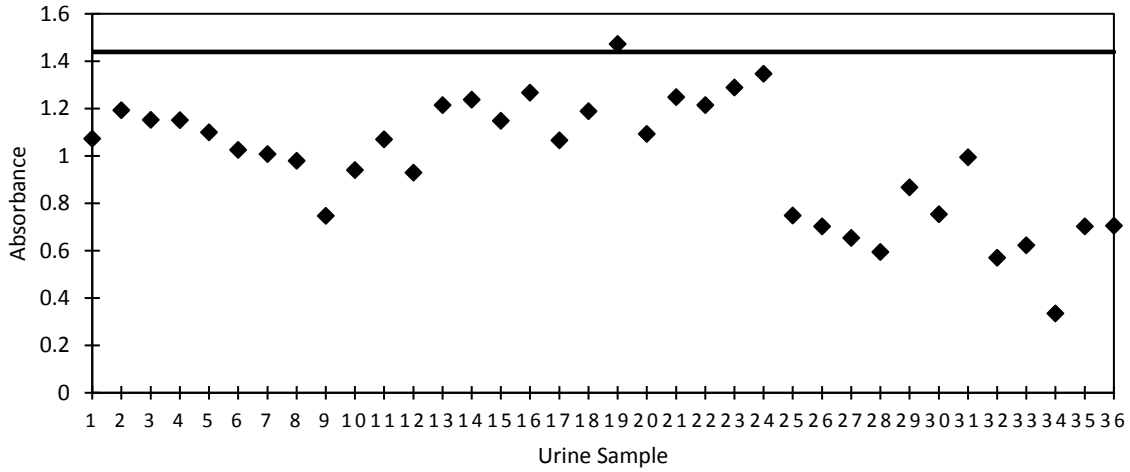

Figure 2. The absorbance values of the B5896 lysate (L) antibody against 36 different urine specimens from dogs with blastomycosis.

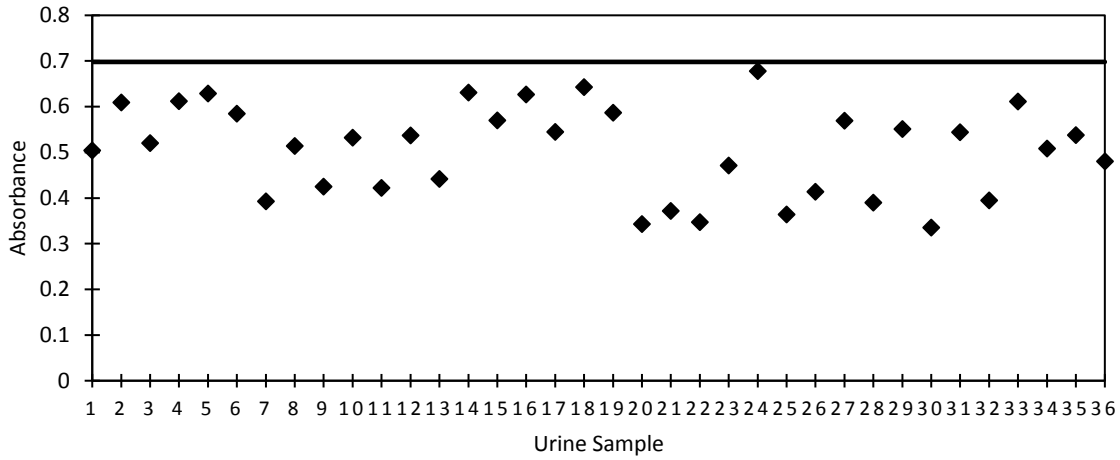

Figure 3. The absorbance values of the B5931 Rotofor (R) antibody against 36 different urine specimens from dogs with blastomycosis.

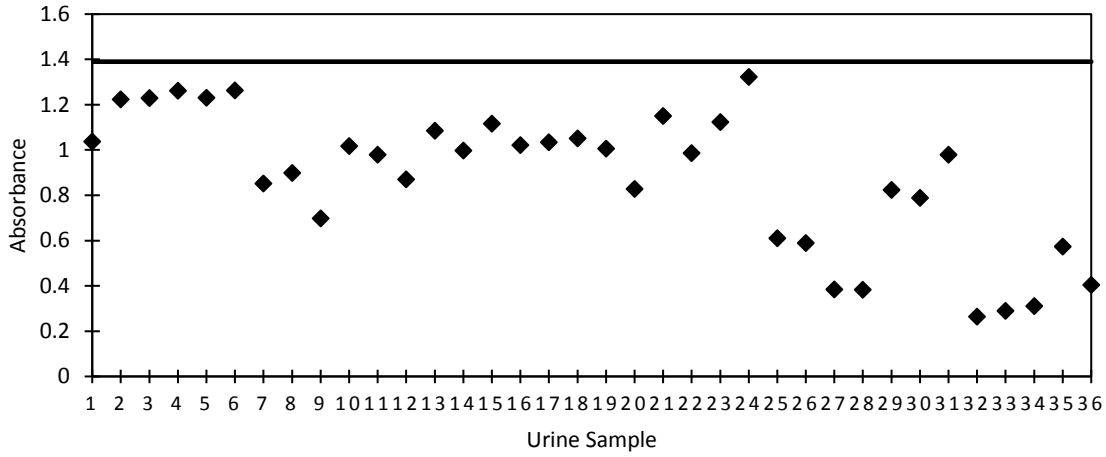

Figure 4. The absorbance values of the B5931 lysate (L) antibody against 36 different urine specimens from dogs with blastomycosis.

The B5896 Rotofor and B5896 lysate antibodies exhibited a 97% antigen detection rate while the B5931 Rotofor and lysate antibodies had a detection rate of 100%. These results demonstrate the high capacity of each of these four antibodies to detect antigen in urine specimens from dogs with diagnosed blastomycosis.

Acknowledgement

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Blastomyces dermatitidis Antigen Detection: Comparison of Antibodies Prepared from Lysates and Killed Yeast Cells from a Human and Dog Isolate of the Fungus

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Abstract

Blastomycosis is a difficult disease to diagnose in animals. Recently investigators have concentrated on the development of antigen detection immunoassays for this disease. The current study was designed to evaluate rabbit antibodies produced from *Blastomyces dermatitidis* lysates and killed whole cells from a human and dog isolate of the fungus. The antibodies were used for the detection of antigen in 30 urine specimens from dogs that were known to be infected with blastomycosis. A competitive inhibition enzyme-linked immunosorbent assay (ELISA) was used to compare the antibodies. All four antibodies were able to detect antigen in the urine specimens at different rates with sensitivity values ranging from 46.7% (ERC2 whole cell antibody) to 93.3% (B5931 lysate antibody). When the positive control value of each antibody was compared to the mean inhibition value (the lower the value=more antigen detected) of all 30 specimens, the absorbance value differences between the controls and the urines of each antibody were -0.405 (B5931 lysate), -0.137 (ERC2 lysate), -0.214 (B5931 whole cell) and 0.043 (ERC2 whole cell).

The degree of inhibition (antigen detection) was greater with antibodies from both yeast lysate reagents as compared to antibodies produced from killed whole cells.

Keywords: *Blastomyces dermatitidis*, antigen detection, competitive ELISA, lysate antigens, whole cell antigens

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Introduction

Blastomycosis, caused by the thermally dimorphic systemic fungal organism *Blastomyces dermatitidis*, is a disease of humans and animals. Blastomycosis is endemic in southeastern regions of the United States as well as in upper midwestern states including Minnesota and Wisconsin and regions of lower Canada. The fungus grows in the mycelial form in areas where there is an abundance of moisture and rich organic matter present [1-3]. Individuals become infected by inhaling the infectious mycelial spore into the lung where the spore may convert into a broad-based budding yeast cell. The disease may present as an acute or chronic infection in the lung or it may disseminate to other internal organs or even to the central nervous system where fatal meningitis may develop [4-6].

The laboratory diagnosis of blastomycosis has presented a challenge to physicians for many years. Routine microbiological or histological assays may be performed, but in many instances these methods may not yield an accurate diagnosis [3,7,8]. Therefore, in recent years, researchers have devoted a considerable amount of effort in the development of immunodiagnostic assays for the detection of *B. dermatitidis* antibodies or antigens in serum or urine specimens from humans or dogs with blastomycosis or other fungal diseases [7-14].

For several years our laboratory has been involved in the development of *B. dermatitidis* yeast phase lysate antigens from various isolates of the fungus and the evaluation of such lysates for the detection of antibodies in animals. The lysates have also been used to induce antibodies in rabbits and the utilization of such antibodies in antigen detection assays [15-18]. These studies have produced data/results that has been encouraging for continued studies on these reagents as immunodiagnostic tools.

The purpose of this study was to evaluate four separate antibody preparations produced from both lysates and whole cells and to compare them for their ability to detect antigen in urine specimens from dogs that were diagnosed with the disease blastomycosis. The competitive inhibition ELISA was used for the comparative assays.

Materials and Methods

Antigens

Two *B. dermatitidis* yeast phase lysate reagents, from a human outbreak of blastomycosis in Mountain Iron, Minnesota, (B5896 and B5931) and ERC2 (dog isolate, Wisconsin) were prepared by a method similar to one that was previously used for the production of yeast lysate antigen from *Histoplasma capsulatum* [19-21] and modified in our laboratory for *B. dermatitidis* lysate antigen production [15]. The yeast phase cells were grown for 7 days at 37°C in a chemically defined medium in an incubator shaker. They were then harvested by centrifugation (700 x g; 5 min), washed with distilled water, resuspended in distilled water and allowed to lyse for 7 days at 37°C in water with shaking. The preparations were centrifuged, filter sterilized, merthiolate added (1:10,000) and stored at 4 C. Protein determinations were performed on the lysates using the BCA Protein Assay Kit (Thermo-Fisher, Pierce Chemical Company, Rockford, IL) and dilutions of the

antigenic reagents used as immunizing agents and ELISA assays were based on protein concentration.

Antibodies/Urine Specimens

The four antibodies were obtained from rabbits immunized with either *B. dermatitidis* yeast lysate antigens or with formalin killed whole yeast cell preparations from human isolates B5931 and dog isolate ERC2. Urine specimens (30) from dogs diagnosed with blastomycosis were provided by Dr. A.M. Legendre (University of Tennessee College of Veterinary Medicine, Knoxville, TN)

Competitive ELISA

The horseradish peroxidase competitive binding inhibition ELISA was used for the detection of *B. dermatitidis* antigens in the urine specimens. Microdilution plates (96 well NUNC, Thermo-Fisher) were coated with 100 µl of B5896 (Minnesota human isolate) lysate antigen that was diluted (2000 ng ml-1) in a carbonate-bicarbonate coating buffer (pH 9.6). The plates were incubated overnight at 4° C in a humid chamber followed by washing three times with phosphate buffered saline containing 0.15% Tween 20 (PBS-T). Dog urine and 1:1000 (whole cell antibodies) or 1:1250 lysate antibodies obtained from rabbits immunized with either B5931 or ERC2 preparations were added to microcentrifuge tubes (200 µl plus 200 µl of each urine specimen) and incubated for 30 min at 37° C. Following this incubation step 100 µl of the antibody-urine mixture from the microdilution tubes was added to the above plates containing the B5896 antigen and incubated for 30 min at 37º C. The plates were again washed as above and 100 µl of goat anti-rabbit IgG horseradish peroxidase conjugate (Kirkegaard and Perry Laboratories, KPL) was added to

each well and incubated for 30 min at 37° C and were washed as above. Then 100 µl of Sure Blue Reserve TMB peroxidase substrate (KPL) was added to each well and incubated for approximately 6 min at room temperature. Stop Solution (KPL) was added to each well and the absorbance was read using a BIO-RAD 2550 EIA reader at 450 nm. Positive controls containing known B5896 antigen coated on the plate and the above sera from the immunized rabbits were used to determine the baseline value to which all of the urine specimens were compared.

Results and Conclusion

The four figures below represent the data from this experiment. Each of the four antibodies had the ability detect antigen; however, some preparations were found to be more sensitive than others. The absorbance values for the B5931 lysate antibody presented a range of 0.689-2.159 with a mean absorbance value of 1.276 while the absorbance values for the ERC2 lysate antibody fell in the range of 0.327-1.669 with a mean absorbance value of 0.609. The B5931 whole cell antibody showed a range of absorbance of 1.183-2.436 and a mean absorbance of 1.687 and the ERC2 whole cell antibody had an absorbance range of 0.522-1.963 with a mean absorbance value of 1.213. The B5931 lysate antibody effectively detected antigen in 28 of the 30 urine samples while the ERC2 lysate antibody did this in 25 of the 30 urine specimens. The B5931 whole cell antibody was efficient at detecting antigen in 24 of the 30 urine samples and the ERC2 whole cell antibody was able to detect antigen in only 14 of the 30 urine specimens.

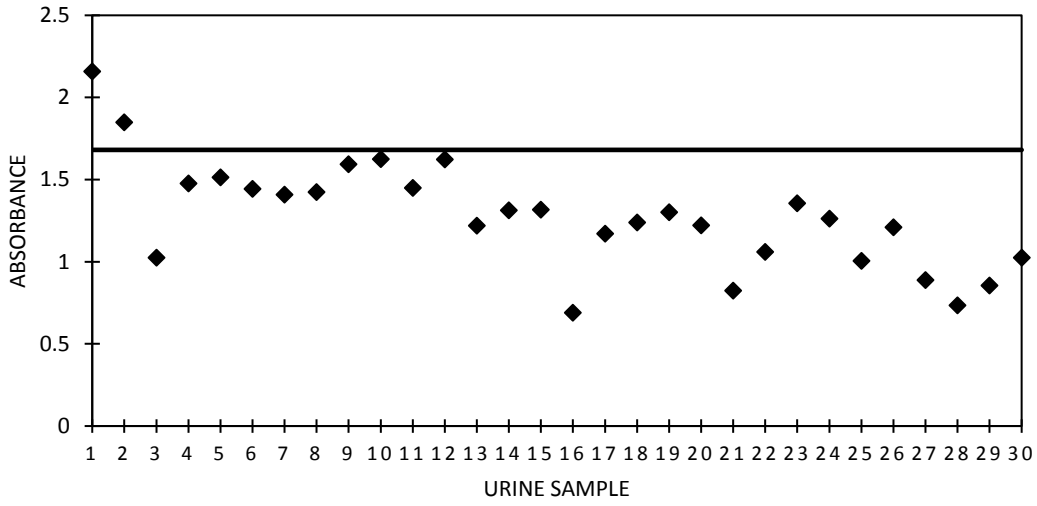

Figure 1. Absorbance values of the B5931 lysate antibody against 30 different urine specimens from dogs with blastomycosis.

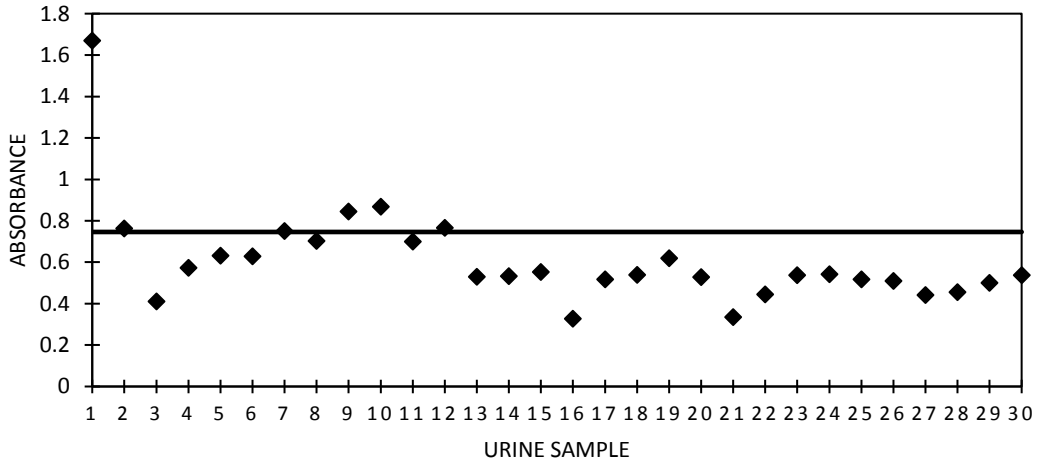

Figure 2. Absorbance values of the ERC2 lysate antibody against 30 different urine specimens from dogs with blastomycosis.

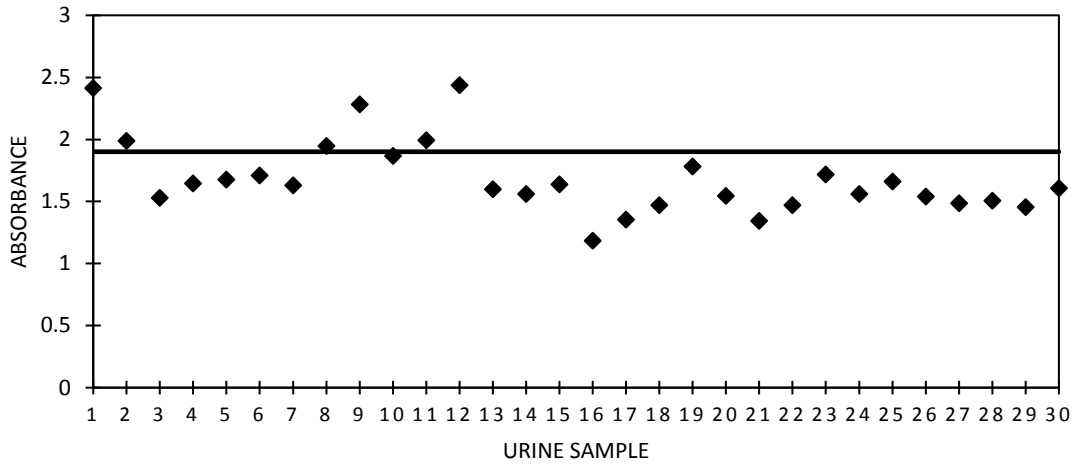

Figure 3. Absorbance values of the B5931 whole cell antibody against 30 different urine specimens from dogs with blastomycosis.

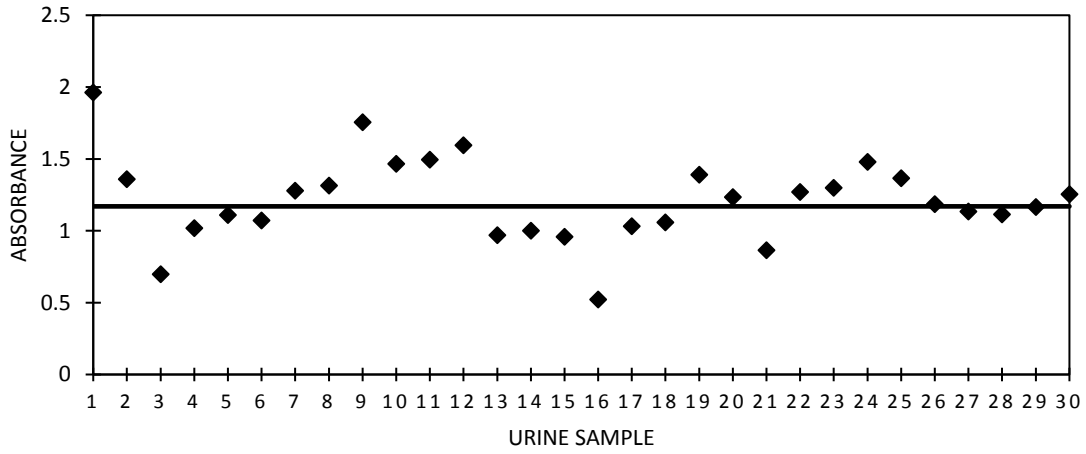

Figure 4. Absorbance values of the ERC2 whole cell antibody against 30 different urine specimens from dogs with blastomycosis.

These results show the differences between the two lysate antibodies and the two whole cell antibodies with regard to antigen detection. The capacity of each of these four antibodies to detect antigen in urine specimens from dogs diagnosed with blastomycosis varied. The B5931 lysate exhibited a 93.3% antigen detection rate while the ERC2 lysate showed an 83.3% detection rate. The study showed that the B5931 lysate antibody was indeed the more effective lysate of the two examined. The B5931 whole cell displayed an antigen detection rate of 80.0% while the ERC2 whole cell fell to only a 46.7% antigen

detection rate. The data indicates that the B5931 whole cell antibody is the better one of the two whole cells at detecting antigen in a sensitive fashion. Ultimately, with regard to the four antibodies examined in this study, the results of the experiment suggest that the most efficient, most dependable antibody to use when detecting antigen is the B5931 lysate antibody while the least effective antibody at detecting antigen is the ERC2 whole cell antibody. The use of the B5931 lysate antibody would therefore be the optimal reagent when presented with a clinical situation where antigen detection could play a role in discovering an accurate diagnosis.

Acknowledgement

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Blastomyces dermatitidis Antibody and Antigen Detection: the Use of Four Lysate Antigens Prepared from a Human Outbreak of Blastomycosis

Abstract

Blastomycosis is a fungal disease in humans and other animals. The newer studies have focused on the capabilities of antibody and antigen detection in the development of immunoassays for diagnosis of blastomycosis. This study was designed to evaluate four *B. dermatitidis* antigenic preparations (591,592,597,598) prepared from human isolates of an outbreak of blastomycosis in Eagle River, Wisconsin. By utilizing the enzyme-linked immunosorbent assay [ELISA], these antigens were used to detect antibodies in serum specimens from dogs with blastomycosis. Antibodies prepared from the same strains were used to detect antigen in urine specimens from the same dogs. This study compared those four *B. dermatitidis* serum preparations (591,592,597,598) in their ability to detect antigen by using the competitive enzyme-linked immunosorbent assay [Competitive ELISA] in 18 urine specimens from dogs with blastomycosis. All four reagents proved to be immunoreactive and were able to detect antigen in each of the urine samples with only slight variations in the mean absorbance values evidenced. Regarding antibody detection, sensitivity mean absorbance values ranged from 1.522 (592 lysate antigen) to 2.039 (597 lysate antigen). Regarding antigen detection, sensitivity mean absorbance values ranged from 89% effective (598 serum specimen) to 100% (591 and 592 serum specimens).

Introduction

Blastomycosis, a potentially fatal systemic fungal disease of humans and animals, is caused by the dimorphic organism *Blastomyces dermatitidis*. Blastomycosis is endemic in states that border the Ohio and Mississippi Rivers in the southeast and upper Midwest and it is highly endemic in regions of Minnesota, Wisconsin and lower Canada [1-3].

The fungus exists in the mycelial phase in nature and an infection is initiated by inhalation of the conidia into the lungs. The disease may be self-resolving or it may develop into an acute primary pulmonary infection. It may then progress into a chronic state in which the symptoms may present diagnostic problems to physicians because the symptoms/pathology of blastomycosis mimics various bacterial or viral pulmonary infections. If not diagnosed properly and treated while in the lungs, the organism may become invasive and disseminate to other organs and possible to the central nervous system where fatal meningitis may result [4-6].

The clinical diagnosis of blastomycosis has been difficult because in many instances culturing or histological determinations have not yielded the desired results, therefore investigators have concentrated on the development and use of immunodiagnostic assays for antibody or antigen detection in serum specimens or urine specimens from humans or other animals with blastomycosis [3,7-14].

The focus of research in our laboratory has been on comparative studies to improve the immunodiagnosis of blastomycosis. We have studied a number of different isolates of the fungus and prepared yeast phase lysate antigens for the detection of antibodies in immunized or infected animals. These lysate preparations have also been use to immunize

rabbits and the antibodies produced have been utilized in immunodiagnostic assays for antigen detection in urine specimens from dogs with blastomycosis [15-18]. These previous results have been encouraging with regard to continuing these comparative immunoassays.

The aim of this study was to evaluate four *B. dermatitidis* antigenic preparations (591,592,597,598) prepared from human isolates of an outbreak of blastomycosis in Eagle River, Wisconsin. These antigens were used to detect antibodies in serum specimens from dogs with blastomycosis and antibodies prepared from the same antigens were used to detect antigen in urine specimens from the same dogs.

Materials and Methods

Antigens

Four *B. dermatitidis* yeast phase lysate reagents, from a human outbreak of blastomycosis in Eagle River, Wisconsin (591,592,597,598) were prepared were prepared by a method similar to one that was previously used for the production of yeast lysate antigen from *Histoplasma capsulatum* [19-21] and modified in our laboratory for *B. dermatitidis* lysate antigen production [15]. The yeast phase cells were grown for 7 days at 37°C in a chemically defined medium in an incubator shaker. They were then harvested by centrifugation (700 x g; 5 min), washed with distilled water, resuspended in distilled water and allowed to lyse for 7 days at 37°C in water with shaking. The preparations were centrifuged, filter sterilized, merthiolate added (1:10,000) and stored at 4 C. Protein determinations were performed on the lysates using the BCA Protein Assay Kit (Thermo-

Fisher, Pierce Chemical Company, Rockford, IL) and dilutions of the antigenic reagents used as immunizing agents and ELISA assays were based on protein concentration.

Antibodies/Urine Specimens

The four antibodies were obtained from rabbits immunized with *B. dermatitidis* yeast lysate antigens (591,592,597,598). Urine specimens (18) from dogs diagnosed with blastomycosis were provide by Dr. A.M. Legendre (University of Tennessee College of Veterinary Medicine, Knoxville, TN). In addition 28 serum specimens from immunized rabbits were assayed from antibody with the four lysate antigens.

Enzyme-linked immunosorbent assay (ELISA)

The ability of each yeast lysate reagent to detect antibodies in the above serum specimens was determined using the indirect enzyme-linked immunosorbent assay (ELISA) as previously described [10-12]. Each lysate antigen was diluted (2000 ng/ml of protein) in a carbonate-bicarbonate coating buffer (pH 9.6) and then added to triplicate wells (100 ul) of a NUNC 96-well microplate (Fisher-Thermo). The plates were then incubated overnight at 4°C in a humid chamber followed by washing three times with phosphate buffered saline containing 0.15% Tween 20 (PBS-T). The serum specimens (1:2000 dilution; 100 ul) were added to the microplate wells in triplicate and incubated for 30 min at 37°C in a humid chamber. Following this incubation, the wells were washed as above and 100 ul of goat anti-dog IgG (H & L) peroxidase conjugate (Kirkegaard and Perry, Gaithersburg, MD, KPL) was added to each well and incubated for 30 min at 37°C. The plates were again washed as above and 100 ul of Sure Blue Reserve TMB peroxidase substrate (KPL) was added to each well and incubated for approximately 2 min at room

temperature. The reaction was stopped by the addition of Stop Solution (KPL) and the absorbance read at 450 nm using a BIO-RAD 2550 EIA reader.

Competitive ELISA

The horseradish peroxidase competitive binding inhibition ELISA was used for the detection of *B. dermatitidis* antigens in the urine specimens. Microdilution plates (96 well NUNC, Thermo-Fisher) were coated with 100 µl of 591, 592, 597, and 598 lysate antigens that was diluted (2000 ng ml-1) in a carbonate-bicarbonate coating buffer (pH 9.6). The plates were incubated overnight at 4° C in a humid chamber followed by washing three times with phosphate buffered saline containing 0.15% Tween 20 (PBS-T). Dog urine and 1:1000 lysate antibodies obtained from rabbits immunized with 591, 592, 597, and 598 preparations were added to microcentrifuge tubes (200 µl plus 200 µl of each urine specimen) and incubated for 30 min at 37° C. Following this incubation step 100 µl of the antibody-urine mixture from the microdilution tubes was added to the above plates containing the antigen and incubated for 30 min at 37º C. The plates were again washed as above and 100 µl of goat anti-rabbit IgG horseradish peroxidase conjugate (Kirkegaard and Perry Laboratories, KPL) was added to each well and incubated for 30 min at 37° C and were washed as above. Then 100 µl of Sure Blue Reserve TMB peroxidase substrate (KPL) was added to each well and incubated for approximately 6 min at room temperature. Stop Solution (KPL) was added to each well and the absorbance was read using a BIO-RAD 2550 EIA reader at 450 nm. Positive controls containing known antigen coated on the plate and the above sera from the immunized rabbits were used to determine the baseline value to which all of the urine specimens were compared.

Results and Conclusion

The six figures below represent the data gathered from this experiment. All four lysates had the capacity to detect antibody. In addition, each of the four antibody preparations had the ability detect antigen; however, some preparations were found to be more sensitive than others. When considering antibody detection in the 28 rabbit sera, the mean absorbance for the 591 lysate antigen presented with a value of 1.563 while the mean absorbance value for the 592 lysate antigen landed at 1.522. The mean absorbance for the 597 lysate antigen was 2.039 and the mean absorbance value for the 598 lysate antigen came in at 1.904. Regarding the 18 dog sera, the mean absorbance for the 591 lysate antigen presented with a value of 1.507 while the mean absorbance value for the 592 lysate antigen landed at 1.638. The mean absorbance for the 597 lysate antigen was 1.881 and the mean absorbance value for the 598 lysate antigen came in at 1.712. Both the 591 and the 592 serum preparations displayed an antigen detection rate of 100% while the 597 and 598 serum preparations showed an antigen detection rate of 94% and 89%, respectively.

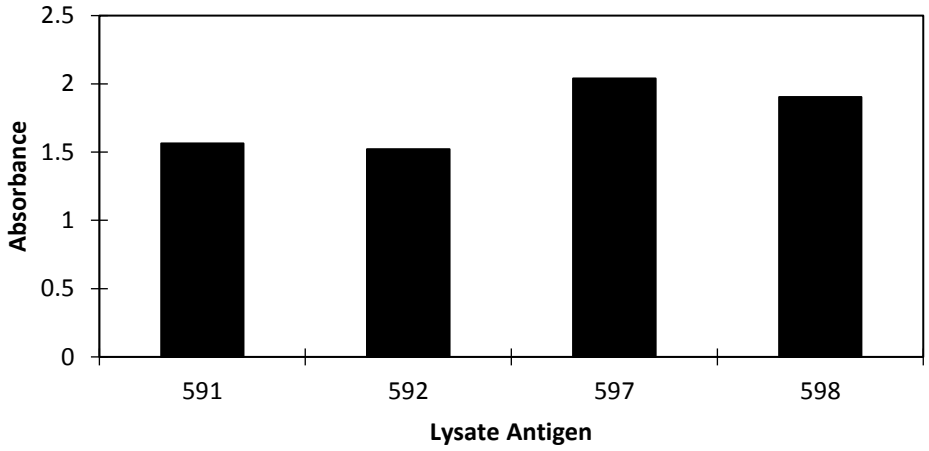

Figure 1. Mean absorbance values of the four lysate antigens reacted with 28 rabbit sera.

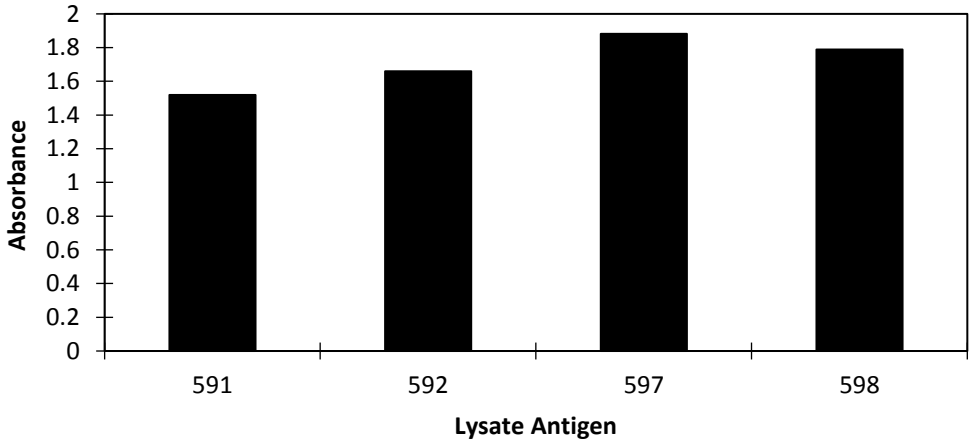

Figure 2. Mean absorbance values of the four lysate antigens reacted with 18 dog sera.

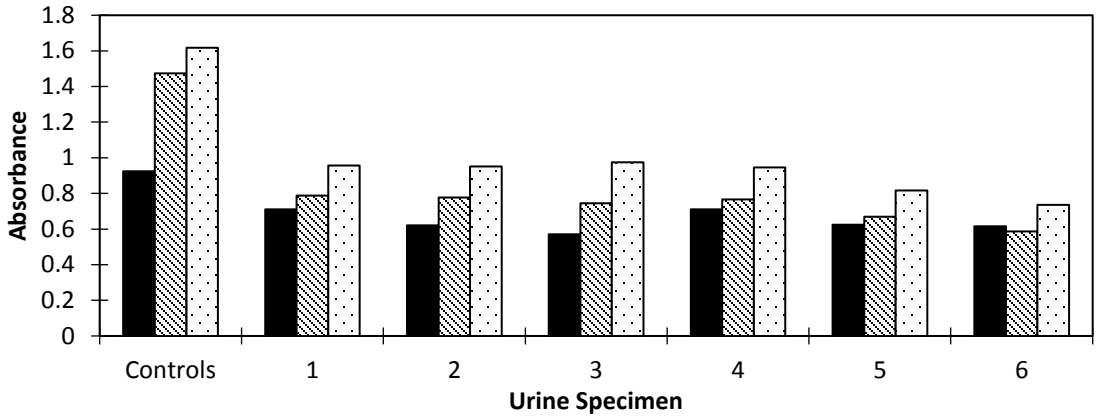

Figure 3. Mean absorbance values of lysate antigen 591 reacted with 18 urine specimens. The solid black bar represents the first trial of 1-6 urine specimens. The hatched bar represents the second trial of 7-12 urine specimens. The dotted bar represents the third trial of 13-18 urine specimens.

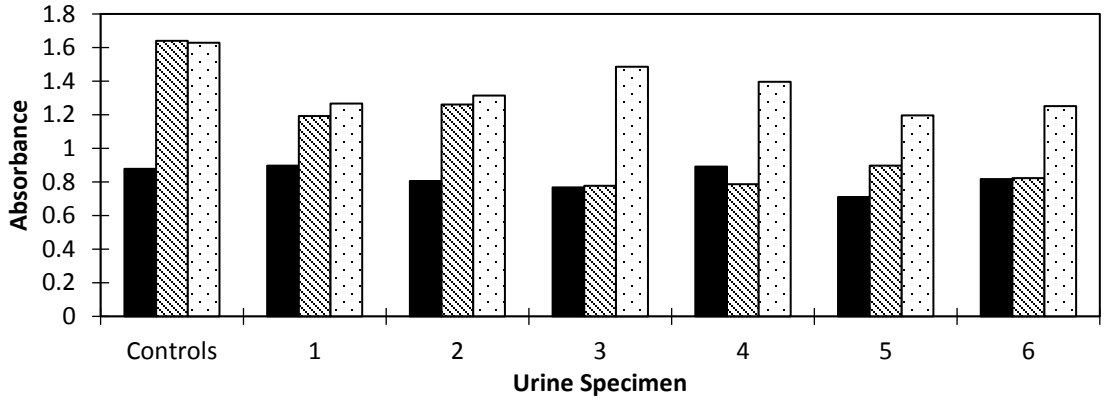

Figure 4. Mean absorbance values of lysate antigen 592 reacted with 18 urine specimens. The solid black bar represents the first trial of 1-6 urine specimens. The hatched bar represents the second trial of 7-12 urine specimens. The dotted bar represents the third trial of 13-18 urine specimens.

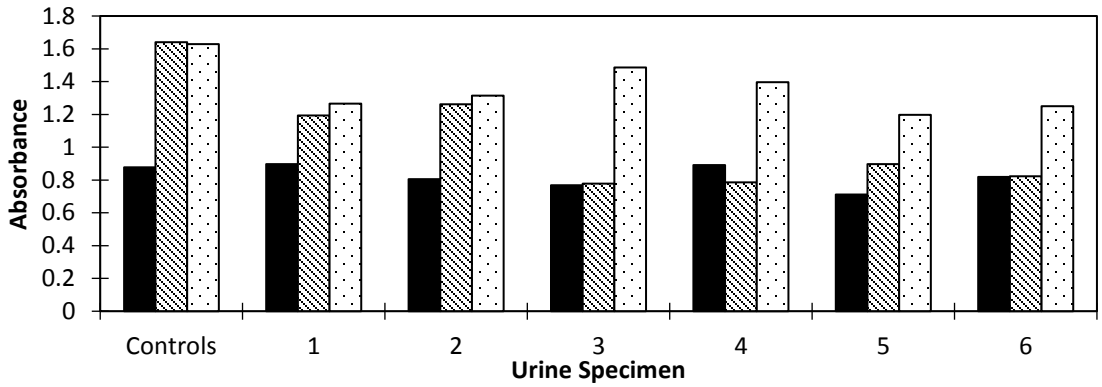

Figure 5. Mean absorbance values of lysate antigen 597 reacted with 18 urine specimens. The solid black bar represents the first trial of 1-6 urine specimens. The hatched bar represents the second trial of 7-12 urine specimens. The dotted bar represents the third trial of 13-18 urine specimens.

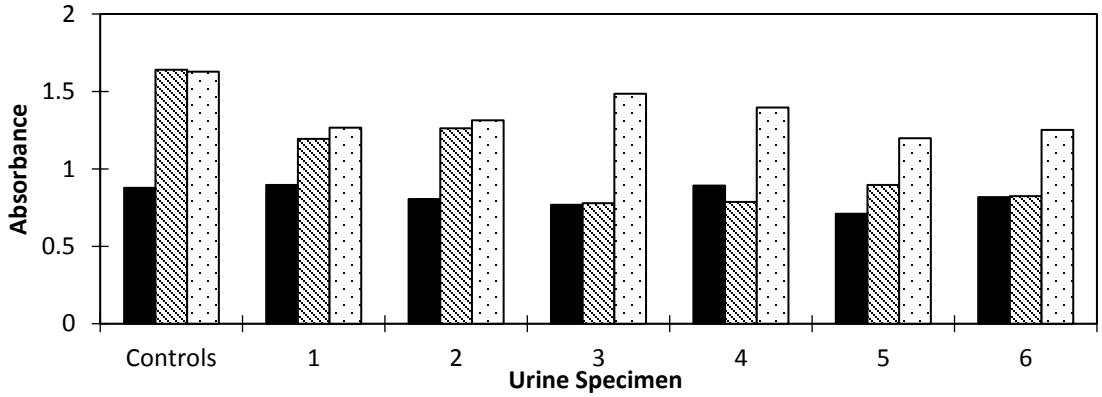

Figure 6. Mean absorbance values of lysate antigen 598 reacted with 18 urine specimens. The solid black bar represents the first trial of 1-6 urine specimens. The hatched bar represents the second trial of 7-12 urine specimens. The dotted bar represents the third trial of 13-18 urine specimens.

These results show the differences between the four lysate antigens in detecting antibody and the difference between the four serum preparations in detecting antigen. Each of the four lysate antigens had the ability to detect antibody in a sensitive manner. The 597 lysate antigen was the most sensitive as it had the highest ability to detect antibody. The capacity of each of these four serum specimens to detect antigen in urine specimens from dogs diagnosed with blastomycosis varied. The 591 and 592 antibodies were the most

effective with an antigen detection rating of 100%. The results of this experiment suggest that the most efficient, most dependable lysate antigen to use when detecting antibody is the 597 lysate and the most useful and most dependable serum preparation to use when detecting antigen is either the 591 or 592 serum specimen. As demonstrated by the data gathered in this study, the use of multiple strains to develop lysates and serum preparations will be optimal in creating the most efficient skin test for the diagnosis of blastomycosis in humans and other animals. Further research could be pursued to discover even more efficient strains for use in antibody and antigen detection; however, the results of this study certainly begin to shed light on more efficient skin testing and advancement of the clinical diagnosis of the evasive fungal infection blastomycosis.

Acknowledgement

This study was supported by the Department of Biological Sciences at Idaho State University

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