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Research article

Antibody detection in dogs with blastomycosis and immunized rabbits: comparison of blastomyces antigens prepared from isolates from northern and southern regions of the United States

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Abstract

The objective of this study was to compare eight Blastomyces yeast phase lysate antigens, prepared from isolates from northern: N (Minnesota; Wisconsin) and southern: S (Tennessee, Alabama, Mississippi) regions of the United States, for antibody detection in serum specimens from dogs from these same regions with blastomycosis using the indirect enzyme-linked immunosorbent assay (ELISA). In Trial 1, the lysates were evaluated for antibody detection in sera from 36 dogs (N:18; S:18) with the mean absorbance values ranging from 0.672 (N antigen 643) to 1.504 (S antigen T-27). In addition when the N and S sera were evaluated separately, the mean absorbance values ranged from 0.564 (N) to 0.780 (S) with the 643 antigen and from 1.332 (N) to 1.677 (S) with the T-27 antigen. With all eight lysates greater reactivity was evidenced with the S sera than with the N sera. In addition, four combinations of both N and S antigens were compared with regard to antibody detection in the same dog sera as above. Mean absorbance values ranged from 0.967 (N antigen ERC-2 plus S antigen T-27) to 1.355 (N antigen plus S antigen T-27).The greatest reactivity with these combination antigens was less than was determined with the individual S antigen T-27 in Trial 1. In Trial 2 the same 8 lysates were evaluated for antibody detection in sera from 10 immunized rabbits with the mean absorbance values ranging from 0.978 (N antigen 643) to 2.603 (S antigen T-58). Trial 2 also had a total reactivity absorbance value range of 1.503 (N antigens) to 1.870 (S antigens). A higher level of antibody was detected in the serum specimens from infected dogs from S regions and in sera from rabbits immunized with the S isolates than with N dog sera or with N sera from immunized rabbits.

Additional studies may provide more evidence with regard to the utilization of N and S lysate antigens for the definitive diagnosis of blastomycosis in animals and humans.

Keywords: Blastomyces; ELISA; immunodiagnosis; lysate antigens; blastomycosis

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Introduction

Blastomycosis, a systemic mycosis of humans and animals, is endemic in Southeastern and North Central regions of the United States and in areas of Canada bordering the states of Minnesota and Wisconsin.

The disease is caused by two thermally dimorphic fungal agents; *Blastomyces dermatitidis* and another

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recently described cryptic species, *B.gilchristii* (DiSalvo, 1998; Pfaller MA and Diekema, 2010; Brown et al., 2013; McTaggart et al., 2016).

In nature, at approximately 25 C, Blastomyces is prevalent in moist environments that are rich in decaying matter as a mycelial form. Once the mycelial spore becomes airborne and is inhaled into the lungs, at 37 C, the filamentous form converts to a broad based budding yeast cell. In healthy patients, blastomycosis often presents as a potentially self-resolving primary pulmonary infection or may evolve into a chronic form; however, in immunocompromised individuals the disease may disseminate to other organs of the body, including the skin and even to the central nervous system, causing meningitis (DiSalvo, 1998; Bradsher et al., 2003; Bariola and Vyas, 2011).

The problem that physicians have faced for many years is that blastomycosis is difficult to diagnose. Culture of the organism or histopathological methods have been effective in some instances, but may be costly and time-consuming thus leading to delayed diagnosis and treatment (Bradsher et al., 2003; Bariola and Vyas, 2011).

In addition blastomycosis symptoms vary widely and misdiagnosis is common since it can be mistaken for various viral or bacterial diseases, including tuberculosis. Blastomycosis can be treated with systemic anti-fungal drugs, but only when a timely diagnosis is made. Therefore in recent years researchers have focused on attempts to develop improved and efficient immunodiagnostic assays for the detection antibody and antigen in human and animal blastomycosis (Spector et al., 2008; Vyas et al., 2008; McKinnell and Pappas, 2009; Saccente and Woods, 2010).

For several years research work in our laboratory has focused on determining the efficacy of immunodiagnostic assays with regard to the laboratory diagnosis of blastomycosis. We have developed methods for the preparation of Blastomyces yeast lysate antigens and the utilization of such reagents for the detection of antibody in various serum specimens from infected and immunized animals (Johnson and Scalarone,1989; Chester et al., 2003; Sestero and Scalarone, 2006; Shurley and Scalarone, 2007; Hatch and Scalarone, 2013; Kennell and Scalarone, 2015; Kennell and Scalarone, 2016; Kennell et al., 2017). Such studies have provided considerable data and the potential for improved and reliable laboratory assays, but additional comparative investigations will certainly contribute to the definitive diagnosis of blastomycosis in humans and animals.

In the present study, various Blastomyces antigens prepared from both southern (Tennessee, Georgia, Kentucky) and northern (Minnesota, Wisconsin) regions of the United States were tested against serum specimens from dogs with diagnosed blastomycosis from both regions. In addition, various combinations of northern and southern lysates were also evaluated for their ability to detect antibody in the same serum specimens (Trial 1). The same lysates were further tested against sera from rabbits immunized with the Blastomyces lysates (Trial 2).

Materials and Methods

Lysate antigens

Eight yeast lysate antigens were prepared from northern (ERC-2, dog,Wisconsin, ER-3, soil, Wisconsin, 643, human, Wisconsin, 248, soil, Wisconsin) and southern (T-58, dog, Tennessee, 85, soil, Georgia, KyC, human, Kentucky, T-27, polar bear, Tennessee) isolates of *Blastomyces.* Strain ERC-2 (ATCC-2585) has recently been classified as *B. gilchristii,* while the other isolates are assumed to be *B.dermatitidis.* 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 (Levine et al., 1977; Scalarone et al., 1978) and modified in our laboratory for *B. dermatitidis* lysate antigen production (Johnson and Scalarone. 1989). 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, re-suspended 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

Thirty-six serum specimens from dogs with diagnosed blastomycosis were provided by Dr. A.M. Legendre (University of Tennessee College of Veterinary Medicine, Knoxville, Tennessee).

Twelve serum specimens came from rabbits immunized with the same eight lysate antigens in our laboratory. The rabbits were housed in accordance to the NIH guide for Care and Use of Laboratory Animals with approval from the Idaho State University IACUC.

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). 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 µl) 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 µl) 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 µl 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 µl of Sure Blue Reserve TMB peroxidase substrate (KPL) was added to each well and incubated for approximately 3 min at room temperature. The reaction was stopped by the addition of Stop Solution (KPL) and the absorbance was read at 450 nm using s Bio-Tek Synergy HT reader.

Results

In Trial 1, eight lysate antigens from northern (Minnesota and Wisconsin) and southern (Tennessee, Alabama, and Mississippi) regions were tested against 36 sera from dogs with blastomycosis from both northern and southern regions. As shown in Figure 1, the mean absorbance values ranged from 0.672 (Antigen 643) to 1.504 (AntigenT-27).

In Figure 2, the reactivity of same eight lysate antigens is contrasted between the northern and southern dog sera. All eight antigens detected higher levels of antibody in the southern specimens than the northern specimens. For the northern sera the mean absorbance values ranged from 0.564 (Antigen 643) to 1.332 (Antigen T-27) and for the southern sera the mean absorbance values ranged from 0.780 (Antigen 643) and 1.677 (Antigen T-27).

The four combinations of antigens (A: ERC-2+T-27; B: ERC-2; 85; C: 643+T-27; D: 643+85) prepared from Blastomyces isolates from both northern and southern regions of the United States were tested for antibody detection in the same 36 serum specimens from dogs diagnosed with blastomycosis from these same regions as above. Figure 3 shows that the absorbance values ranged from 0.967 (Antigen Combination A: ERC-2+T-27) to 1.355 (Antigen Combination C: 643+T-27).

In Trial 2, the same eight lysates were tested against 10 sera from rabbits immunized with lysates from northern and southern regions. The amount of antibody detected in sera induced by the lysates is shown in Figure 4. Whether the lysate antigen was from a northern or southern isolate, it was determined that the reactivity was always greater in the sera

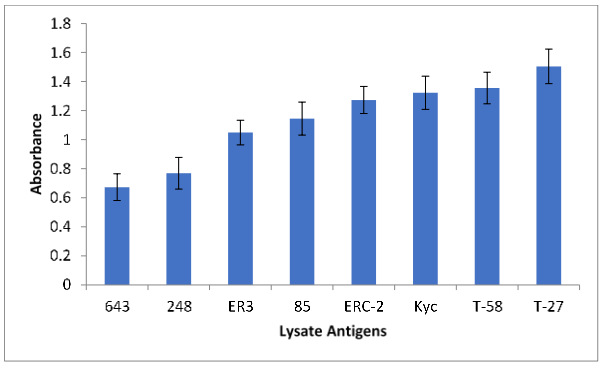

Fig. 1: Comparison of eight lysate antigens prepared from Blastomyces isolates from both northern and southern regions of the United States for antibody detection in 36 dog serum specimens from these same regions.

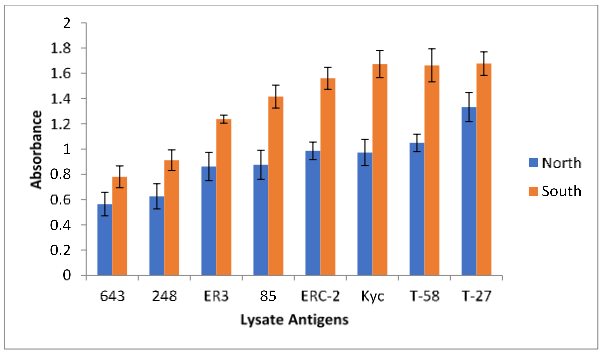

Fig. 2: Comparison of eight lysate antigens prepared from Blastomyces isolates from both northern and southern regions of the United States for antibody detection in 18 dog serum specimens from the north (blue) and 18 dog serum specimens from the south (red).

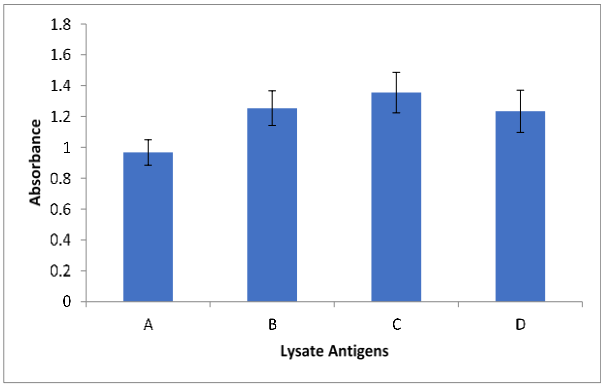

Fig. 3: Comparison of four lysate antigens combinations (A: ERC-2+T-27; B: ERC-2; 85; C: 643+T-27; D: 643+85) prepared from Blastomyces isolates from both northern and southern regions of the United States for antibody detection in 36 dog serum specimens from these same regions with blastomycosis.

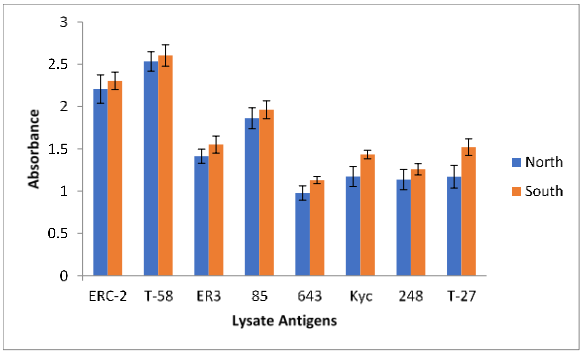

Fig. 4: Comparison of eight lysate antigens prepared from Blastomyces isolates from both northern and southern regions of the United States for antibody detection in serum specimens from rabbits immunized with lysates from the North (blue) and serum specimens from the south (red).

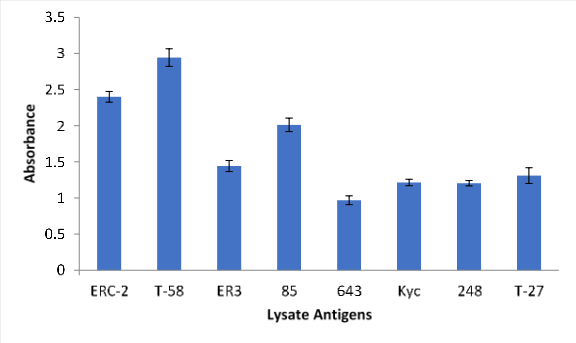

Fig. 5: Comparison of eight lysate antigens prepared from Blastomyces isolates from both northern and southern regions of the United States for antibody detection in 10 serum specimens from rabbits immunized with lysates from these same regions.

induced by the southern isolate antigen. For the northern sera, the mean absorbance ranged from 0.978 (Antigen 643) to 2.533 (Antigen T-58). In the southern sera, the reactivity ranged from 1.130 (Antigen 643) to 2.603 (Antigen T-58).

Figure 5 shows the total reactivity of the eight lysates with the 10 rabbit sera. Mean absorbance values ranged from 0.969 (Antigen 643) to 2.400 (Antigen ERC-2). The mean absorbance for all eight lysates in all the northern sera was 1.503 as compared to a mean absorbance value of 1.870 with the southern sera.

Discussion

The focus of our research has been on the production and comparative evaluations of Blastomyces dermatitidis yeast phase lysate antigens prepared from human, animal and environmental isolates of the fungus for the detection of antibodies to blastomycosis in animals and humans. Our initial studies on the development and preparation of these novel yeast phase Blastomyces lysate reagents was based on previous research work that we were associated with on the development of lysate antigens as skin-testing reagents for the clinical diagnosis of cocccidioidomycosis and histoplasmosis (Scalarone et al., 1978).

In all of our previous studies with the various Blastomyces isolates only one species, *B. dermatitidis*, was known as the causative agent of blastomycosis. However one of the isolates that we having been working with, ERC-2, has been recently identified as *B. gilchristii* (Brown et al, 2013; McTaggart et al, 2016). Based on this research this species seems to be found in the Northern United States and regions of Canada.

Therefore, based on these findings that there appears to be a species of Blastomyces other than dermatitidis, it was decided to evaluate various Blastomyces lysate antigens produced from isolates from both northern and southern regions of the United States for their ability to detect antibodies in serum specimens from dogs with blastomycosis from both northern and southern regions. In addition rabbits immunized with the same lysate antigens and antibody induced by these antigens was evaluated for comparison.

The objective of this study was to attempt to determine if Blastomyces antigens from isolates from northern (Minnesota and Wisconsin), as compared to southern (Tennessee, Alabama and Mississippi), exhibited differences with regard to their ability to detect antibody in the infected dogs and immunized rabbits. Overall, all eight lysate antigens were able to detect antibody in the 36 dog sera and 10 rabbit sera; however, as shown above, efficacy varied. All eight antigens detected higher levels of antibody in the southern sera than in the northern specimens. The southern Blastomyces strains appear to be inducing more antibody with comparable detection whether a northern or southern antigen was used in the assay. These results lead one to question if the differences seen in the comparative studies between the northern and southern isolates are related to species differences. Are all of the northern isolates, in addition to ERC-2, actually *B. gilchristii*? It would be interesting to speculate that if the northern isolates are *B. gilchristii* is this species different with regard to virulence, as compared to the southern isolates, and possibly better at evading the immune system of the host. It seems like the host is possibly able to mount a better defense against the southern isolates which leads to a greater production of antibody as compared to the northern strains. This leads to the question of are there actual differences in chemical and molecular characteristics between organisms from the different geographical regions that allow for the host to react different immunologically when infected or immunized with the

various antigens since it was determined that the antigens from the southern isolates that infect dogs or those southern isolate antigens used for immunizing rabbits induce a greater antibody response in the hosts than with the northern isolate antigens? Research is continuing in an attempt to further elucidate these characteristics.

Acknowledgements

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