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Sensitivity and Specificity Determinations with Isoelectric Focusing Fractions of *Blastomyces dermatitidis* for Antibody Detection in Serum Specimens from Infected Dogs

Joshua C. Wright, Terrick E. Harrild, Gene M. Scalarone
Department of Biological Sciences, Idaho State University, Pocatello, USA
Email: wrigjos2@isu.edu

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ABSTRACT

Blastomycosis and histoplasmosis manifest as lung and systemic fungal infections in mammals caused by *Histoplasma capsulatum*, and *Blastomyces dermatitidis.* These infections exhibit cross reactivity of antibodies which makes a correct diagnosis potentially elusive. The purpose of this study was to gain an understanding of which isoelectric focusing fractions (Rotofor™) of *B. dermatitidis* were reactive or cross reactive with serum specimens from dogs infected with *B. dermatitidis H. capsulatum*, and *Cryptococcus neoformans.* Three serum specimens from dogs that were infected with *B. dermatitidis*, two dogs infected with *H. capsulatum*, and one dog infected with *C. neoformans* were assayed against the 20 *B. dermatitidis* Rotofor™ fractions. Reactivity was determined using the indirect enzyme linked immunoassay (ELISA). Reactivity with *B. dermatitidis* was found predominantly in the protein fractions 1 - 6, and cross reactivity with *H. capsulatum*, and *C. neoformans* sera was found within the *B. dermatitidis* protein fractions 15 - 19.

Keywords: Isoelectric Focusing; ELISA; Blastomycosis; Lysate Antigen; Antibody Detection

1. Introduction

Blastomycosis is a systemic fungal infection of humans and 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 as an acute or chronic state in the pulmonary tissue, where it may be misdiagnosed as tuberculosis. If the disease is not diagnosed or left untreated while in the lungs it may become invasive, and disseminate to other organs, and possibly to the central nervous system where fatal meningitis may develop [1-5]. Blastomycosis, as well as other systemic mycoses, are termed “emerging fungal threats” since they can not only infect persons with normal immune systems, but also they are a cause for concern in individuals with AIDS or other deficiency diseases that compromise the immune system [3,4,6].

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. Recent studies have indicated that blastomycosis may be present in other regions with sporadic cases being reported in Colorado, Texas, Kansas and Nebraska [1,2,5].

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. The greatest problem that we face at present is that a considerable amount of research and development activities are needed if we are going to have a positive impact on this situation. 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 the immunobiology of the organism. Diagnosis of the disease has presented major problems. 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 work 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 [2,5,7-10]. Our laboratory has developed novel

yeast phase lysate antigens and utilized these in various immunoassays for both antibody and antigen detection in blastomycosis [11-17], but these studies have only opened up new avenues of approach with regard to how we might improve immunodiagnostic methods in the future.

In recent years other investigators have been approaching the problem of immunodiagnosis and have produced encouraging results with antigen detection assays as second-generation assays have been developed [7,8,10]. Sensitivity values obtained by these researchers have generally been very good with antigen detection assays, but they evidenced less sensitivity when antibody detection assays were performed. The greatest problem that has become apparent in the majority of the investigations is the lack of specificity of the immunoassays. Our laboratory has performed a few studies on isoelectric focusing of *B. dermatitidis* yeast lysate antigens in an attempt to separate the immunoreactive and cross-reactive components of the preparations. The objective of this present study was to obtain isoelectric focusing fractions of yeast lysates and to evaluate these fractions with regard to ELISA antibody detection (reactivity vs. cross-reactivity) in serum specimens from dogs with blastomycosis, histoplasmosis and cryptococcosis.

2. Materials & Methods

2.1. Antigens

Lysate Antigen Preparation

Mycelial phase cultures of *B. dermatitidis* isolate (B5896), obtained from the Mountain Iron, Minnesota human outbreak of blastomycosis in 1999 (2), were converted to yeast cells by culturing at 37 C on brain heart infusion agar. Yeast phase lysate reagents were prepared by a method similar to one that was previously used for the production of antigen from *Histoplasma capsulatum* [18-20] and modified in our laboratory for *B. dermatitidis* lysate antigen production [11]. The yeast phase cells were grown for 7 days at 37 C in a chemically defined medium in an incubator shaker, harvested by centrifugation (700 × g; 5 min), followed by washing with distilled water, re-suspended in distilled water and then 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 further use. Protein determinations were performed on the lysates using the BCA Protein Assay Kit (Pierce Chemical Company, Rockford, IL) and dilutions of the antigenic reagents used in isoelectric focusing and ELISAsassays were based on protein concentration.

2.2. Serum Specimens

Serum specimens from dogs with blastomycosis, histoplasmosis, and cryptococcosiswere previously provided by Dr. A. M. Legendre (University of Tennessee College of Veterinary Medicine, Knoxville, TN).

2.3. Isoelectric Focusing

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 becomes 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 [15,16]. 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.

2.4. 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 of protein/ml) in a carbonate-bicarbonate coating buffer (pH 9.6) and then added to triplicate wells (100 ul) of a Costar 96-well microplate (Thermo/Fisher). 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 (1:2500 dilution; 100 ul) were added to the microplate wells 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) was added to each well and incubated for 30 min at 37 C. The plates were again washed as above and 100 ul of peroxidase substrate (Thermo/Fisher- Pierce) was added to each well and incubated for approximately 2 min at room temperature. The reaction was stopped by the addition of sulfuric acid and the absorbance read at 450 nm using a BIO-RAD 2550 EIA reader.

3. Results

3.1. Reactivity of B5896 Protein Fractions with *B. dermatitidis* Dog Sera

Figures 1-3 show that the reactivity of serum specimens from *B. dermatitidis* infected dogs against the Rotofor™ protein fractions of *B. dermatitidis* dog sera was found predominantly in the 1 - 6 range of protein fractions.

3.2. Reactivity of B5896 Protein Fractions with *H. capsulatum* Dog Sera

Figures 4 and **5** show that the cross reactivity of serum specimens from *H. capsulatum*infected dogs against the Rotofor™ protein fractions of *B. dermatitidis* was found within the 15 - 20 range of protein fractions.

3.3. Reactivity of B5896 Protein Fractions with *C. neoformans* Dog Serum

Figure 6 shows that the cross reactivity of serum specimens from dogs infected with *C. neoformans* against the Rotofor™ protein fractions of *B. dermatitidis* was found in the 15 - 19 range of protein fractions.

4. Discussion

B. dermatitidis, *H. capsulatum*, and *C. neoformans* are

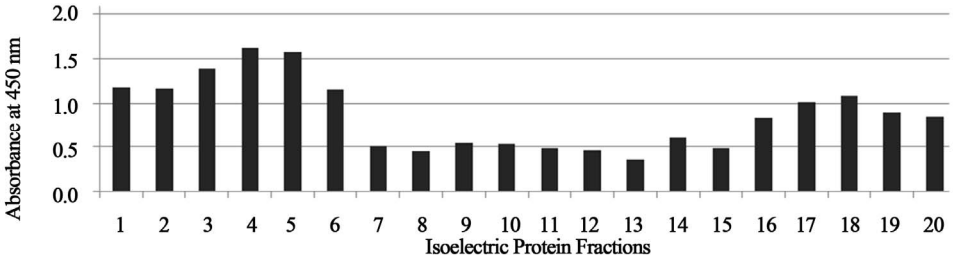

Figure 1. Absorbance values of protein fractions reacted with *B. dermatitidis* dog serum from Wisconsin.

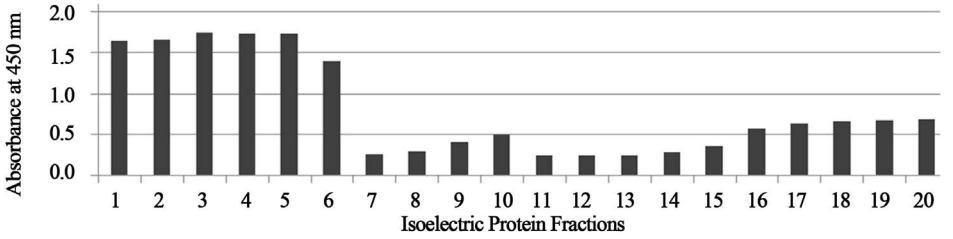

Figure 2. Absorbance values of protein fractions reacted with *B. dermatitidis* dog serum from Alabama.

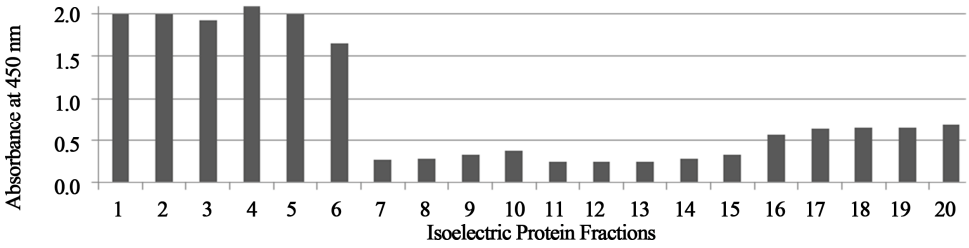

Figure 3. Absorbance values of protein fractions reacted with *B. dermatitidis* dog serum from Louisiana.

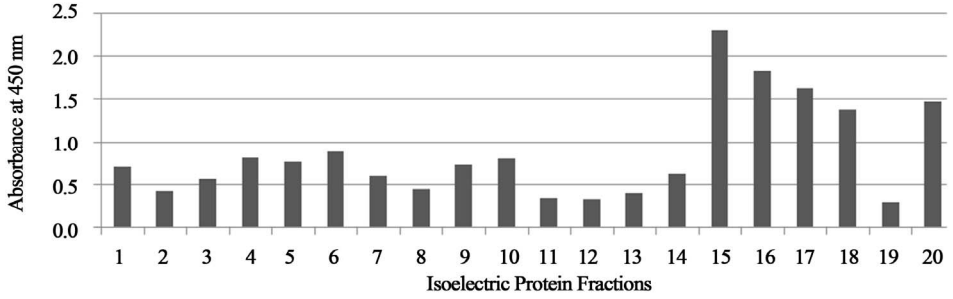

Figure 4. Absorbance values of protein fractions reacted with *H. capsulatum* dog serum (a), from Tennessee.

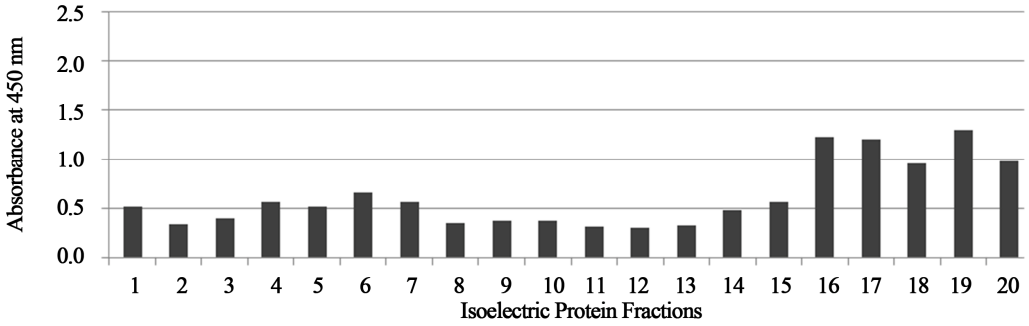

Figure 5. Absorbance values of protein fractions reacted with *H. capsulatum* dog serum (b), from Tennessee.

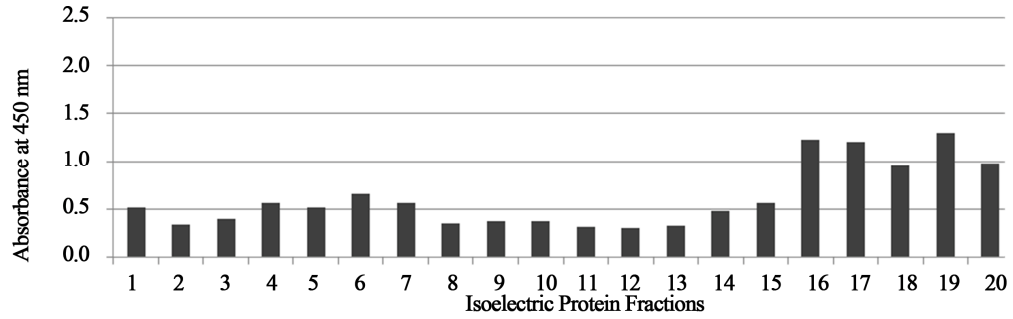

Figure 6. Absorbance values of protein fractions reacted with *C. neoformans* dog serum, from Tennessee.

endemic in the United States mainly in the Ohio and Mississippi River drainages. The antigen and serum specimens used in the study were from different geographic regions within the endemic area.The antigen used, (B5896), was from Minnesota. The dog serum specimens for *B. dermatitidis* were from Wisconsin, Louisiana, and Alabama. The dog serum specimens for *H. capsulatum* were from Tennessee and the *C. neoformans*dog serum specimen wasfrom Tennessee. We believethat some of the variance in the results that we recorded are related to the serum specimen's different geographic origins.

Infections involving *B. dermatitidis*, *H. capsulatum*, and *C. neoformans* in mammals exhibit cross reactivity of antibodies which makes a correct diagnosis potentially elusive. In order to elicit an accurate diagnosis, the specificity of the antigen used in a diagnostic test would need to be improved in order to either reduce or eliminate any cross reactivity of antibodies.

We observed the potential to greatly reducethe cross reactivity of *B. dermatitidis* antigen with *H. capsulatum*, and *C. neoformans* antibodiesvia isoelectric focusing of antigen proteins. Rotofor™ separation of the antigenic proteins provides an accurate means to identify and isolate the most reactive antigen protein fractions in relation to cross reactive species antibodies.

This data may prove useful regarding future studies involving additional evaluations on the detection of antibodies, or the development of a skin testing antigen(s), [15-20] that may possibly eliminate the cross reactivity of *B. dermatitidis* to other fungal pathogens such as *C. neoformans* and *H. capsulatum*.

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