

Isoelectric Focusing Comparative Studies on Yeast Phase Lysate Antigens

Produced from Two Animal Isolates of *Blastomyces dermatitidis*

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Abstract:

The purpose of this study was to continue our comparative investigations on the purification and evaluation of yeast lysate reagents prepared from two unique *Blastomyces dermatitidis* isolates (T-27; polar bear and 449; sea lion). The non-purified lysate preparations were subjected to isoelectric focusing (IEF) utilizing the BIO-RAD Rotofor apparatus in order to separate the reactive antigenic components. An indirect enzyme-linked immunosorbent assay (ELISA) was performed to test the twenty fractions for sensitivity and specificity. Fractions 3 and 4 of the 449 isolate exhibited the greatest reactivity. Fractions 1-3 proved to be the most reactive in the T-27 isolate when used to detect antibodies in a pooled serum specimen from rabbits immunized with *B. dermatitidis* killed whole yeast cells. In contrast, only a low level of reactivity was evidenced when the fractions were tested against a *Histoplasma capsulatum* serum specimen. This study indicated that IEF may be a valuable technique for the purification of *B. dermatitidis* antigens. Studies are continuing in an effort to further evaluate such preparations as reliable reagents for the immunodiagnosis of blastomycosis.

Introduction:

Blastomyces dermatitidis is a systemic dimorphic fungus that causes blastomycosis in humans and animals. Infection is produced by the inhalation of the saprophytic airborne mycelial spores (conidia) into the lungs where a morphologic transition to the parasitic form (yeast cells) takes place. Infection is

only acquired by the inhalation of spores and cannot be transmitted from person to person. Sixty to 90% of *B. dermatitidis* infections are either asymptomatic or are of very short duration, but in some instances the organism may disseminate from the lungs to other organs including the central nervous system. In such instances the disease process may result in death of the patient. Since blastomycosis is acquired by the inhaling the airborne spores, persons working construction, excavating and wood clearing are especially at risk of infection with this fungal organism.

The endemic areas of the fungus *B. dermatitidis* are the eastern United States, extending northward to the Great Lakes region and adjacent areas in Canada, and also the disease has been reported in Africa and India (3).

The laboratory diagnosis and specifically the immunodiagnosis of blastomycosis remains problematic. A major concern in this area has been the lack of a reliable laboratory diagnostic test method due to problems with sensitivity and specificity of the standard assays, including the immunodiffusion and complement fixation tests, that have been used for many years (4).

Our laboratory has been involved in the production, evaluation and utilization of *B. dermatitidis* yeast phase lysate antigens produced from a variety of isolates of the fungus. We have performed numerous comparative studies on the detection of *B. dermatitidis* antibody in sera from infected humans and animals (1,2,5) and also assays for antigen detection in urine specimens (6). Most of the previous studies have utilized crude (non-purified) preparations of the yeast lysate

antigens, but improved immunodiagnostic techniques will probably have to rely on purified antigens in order to be able to detect the disease in a reliable manner. The purpose of this study was to fractionate two *B. dermatitidis* yeast lysate preparations and evaluate the immunoreactivity of each fraction in order to determine if more specific diagnostic reagents could be developed. Previous studies in our laboratory (7) indicated that isoelectric focusing could be used to separate specific and non-specific antigenic components. The BIO-RAD Rotoform apparatus was used in the present study to isolate antigenic fractions obtained following isoelectric focusing of two yeast lysate preparations from animal isolates of *B. dermatitidis*. These fractions were then evaluated by ELISA to determine optimal reactivity.

Materials and Methods:

Antigens

Mycelial phase cultures of *B. dermatitidis* isolates 449 and T-27 were transformed to yeast cells, and were grown in a chemically defined medium for 7 days at 37 °C with shaking. The yeast cells were then harvested and washed five times with distilled water by centrifugation (5 min at 700g) then were resuspended in distilled water 10x original concentration), and allowed to lyse for 7 days at 37 C with shaking. The preparation was then centrifuged and filter sterilized using a 0.2-µm porosity Nalgene filter (Nalge Company, Rochester, NY) to eliminate debris. Merthiolate (1:10,000) was added as a preservative and

stored at 4 °C (4). Protein determinations were made on the antigen preparations using the bicinchoninic (BCA) method according to manufacturer's directions (Pierce Chemical Company, Rockford, IL).

Isoelectric focusing

The BIO-RAD Rotofor apparatus (BIO-RAD, Hercules, CA) was pre-cleaned by running distilled H₂O through until readings of 2 mA were acquired. Lysate (19ml) was added to Bio-Lyte ampholyte (2%, pH 3-10, BIO-RAD), focused at 4 °C until the voltage remained stable for one hour and the current remained constant, according to the manufacturer's instructions. BIO-RAD DuoFlow low-pressure liquid chromatography with HiTrap 5 ml desalting columns (Amersham Bio-sciences, Piscataway, NJ) were used to eliminate the ampholyte from each of the protein fractions. The protein concentrations of each of the twenty fractions were determined as described above (7).

ELISA Method

The antigenicity of each fraction (449 and T-27) was determined by indirect ELISAs employing sera from rabbits immunized with either *B. dermatitidis* (reactivity) or *H. capsulatum* (cross-reactivity) killed whole yeast cells. Lysate fractions, diluted to 1000 ng/ml in carbonate-bicarbonate coating buffer (Na₂CO₃ 0.0015 M, NaHCO₃ 0.0035 M, pH 9.6), were added to triplicate wells of Immunomaxi modified flat bottom high binding plates (TTP, Switzerland) and incubated overnight at 4 °C in a humid chamber. The plates were washed three times with phosphate buffered saline (NaCl 0.1369 M, KH₂PO₄ 0.0015 M, NaHPO₄ 0.0108 M, KCl 0.0027 M, pH 7.4) with 0.15% TWEEN 20 (PBS-T).

Sera from rabbits immunized against either *B. dermatitidis* or *H. capsulatum* were used as the primary antibody. The serum was diluted 1:5,000 in PBS-T, added to the plates and then incubated in a humid chamber for 30 minutes at 37 °C. The plates were washed as above. Goat anti-rabbit IgG horseradish peroxidase conjugate (Kirkegaard and Perry Laboratories, Gaithersburg, MD), diluted 1:2,000 in PBS-T, was added (100 µl per well) and incubated as above. The plates were washed and enzyme substrate (1-step Ultra TMB, Pierce) was added (100 µl per well) and incubated for approximately 3 minutes at room temperature. The reaction was stopped by the addition of 100 µl of 2N H₂SO₄ to each well. The absorbance was read at 450 nm using the BIO-RAD model 2550 EIA reader. (1,2,5-7).

Results:

Certain fractions of the two yeast phase lysate antigens demonstrated greater reactivity than others with respect to ELISA antibody detection in the *B. dermatitidis* serum specimens following isoelectric focusing. Fractions 3 and 4 from the isolate 449 (sea lion) proved to be the most reactive, with absorbance values of 1.568 and 1.564, when compared to the other twenty fractions, which demonstrated less reactivity (Figure 1). When fractions from the T-27 (polar bear) isolate were evaluated evidence indicated that fractions 1 through 6 were the most reactive with absorbance values ranging from 0.933 to 1.769 (Figure 3). In contrast, when each of the 20 fractions obtained from the two isolates were

used for the detection of *H. capsulatum* antibodies, only a low level of cross-reactivity was evidenced with no absorbance values above 0.456 (Figures 2 and 4).

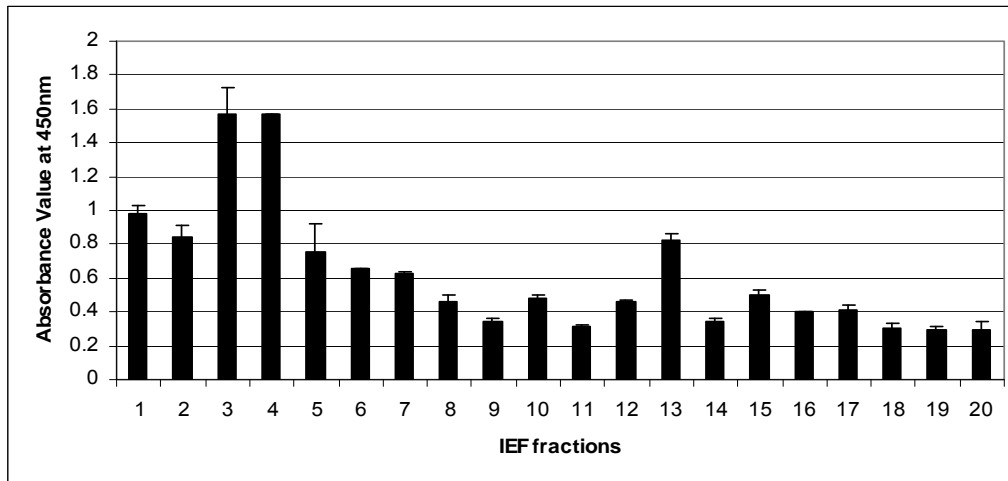


Figure 1. Reactivity of IEF fractions 1-20 from *B. dermatitidis* isolate 449 using sera from rabbits immunized against *B. dermatitidis*.

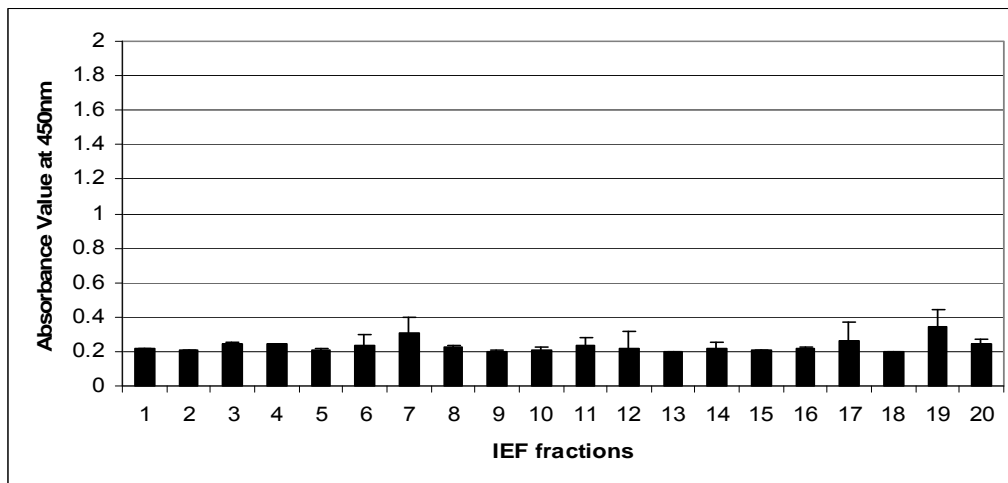


Figure 2. Cross reactivity of IEF fractions 1-20 from *B. dermatitidis* isolate 449 against sera from rabbits immunized against *H. capsulatum*.

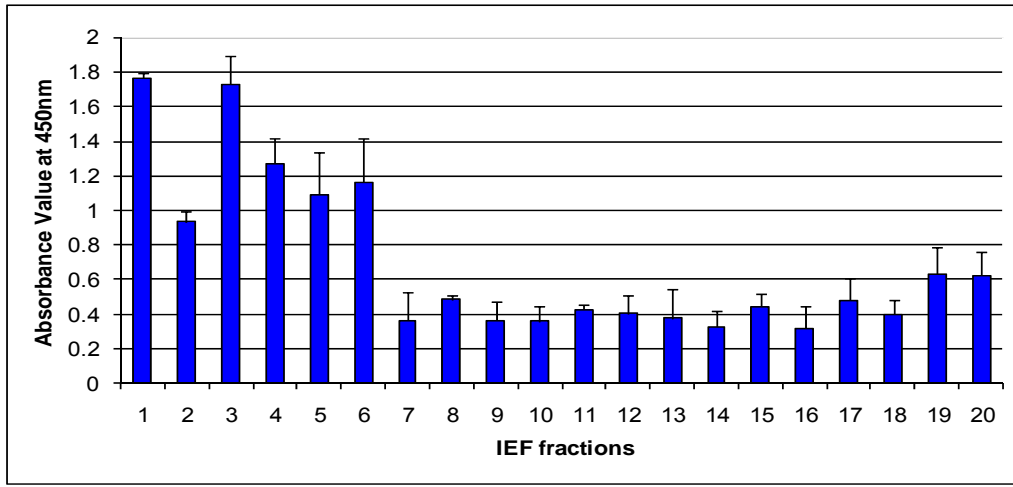


Figure 3. Reactivity of IEF fractions 1-20 from *B. dermatitidis* isolate T-27 using sera from rabbits immunized against *B. dermatitidis*.

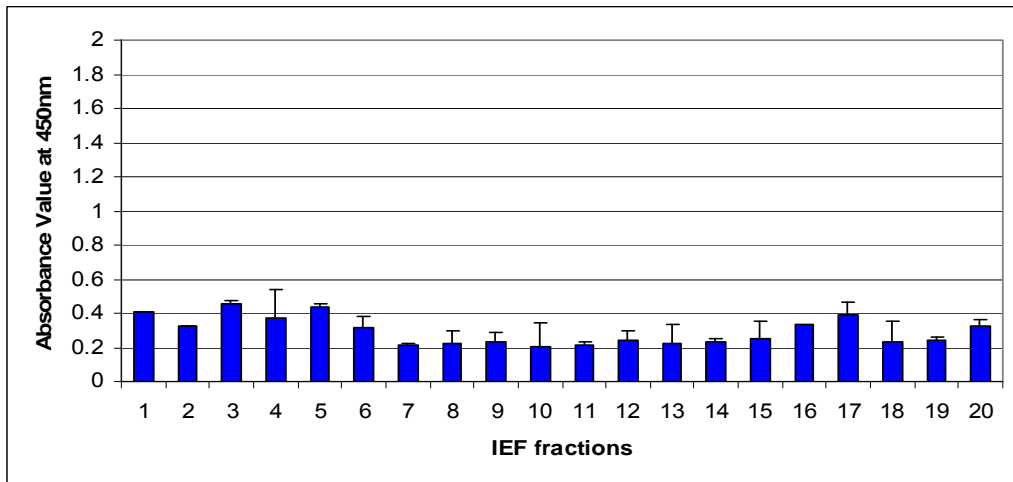


Figure 4. Cross reactivity of IEF fractions 1-20 from *B. dermatitidis* isolate T-27 against sera from rabbits immunized against *H. capsulatum*.

Discussion:

The diagnosis of blastomycosis has presented problems to physicians and laboratory personnel for many years. Microscopic evaluation and culturing methods have been useful, but in certain instances these techniques have failed to provide the necessary evidence for a reliable diagnosis. Immunodiagnostic methods have been employed, including immunodiffusion, agglutination and complement fixation assays, but in many instances investigators have experienced cross-reactivity problems with other fungal organisms (1,2,5). The thrust of our research has been to subject crude antigenic preparations to various purification procedures in an attempt to separate the reactive components from the cross-reactive ones. With the use of isoelectric focusing (7) it appears possible to further purify the protein fractions that possess a high level of reactivity.

In this present study the Rotofor isoelectric focusing apparatus was used to fractionate yeast lysate reagents produced from two *B. dermatitidis* animal isolates. As described above it was possible to isolate the highly reactive fractions from the yeast lysates. The two isolated differed slightly with regard to which fractions exhibited the optimal reactivity, but generally the immunoreactive (ability to detect *B. dermatitidis* antibodies) components resided in the first few protein fractions (Figures 1 and 3). In contrast only minimal cross-reactivity was evidenced when the fractions were used in the ELISA for the detection of anti-*H. capsulatum* antibodies (Figures 2 and 4).

Therefore isoelectric focusing offers another option for investigators attempting to produce antigenic reagents of high sensitivity and specificity. These highly reactive proteins may possibly be considered as an immunodiagnostic tool for future clinical laboratory applications for the diagnosis for blastomycosis. Studies are in progress to not only produce these immunoreactive antigenic components, but also to perform comparative evaluations on the presence or absence of these antigens in various isolates of *B. dermatitidis*. The ultimate aim of our studies is to produce an immunodiagnostic reagent for the definitive diagnosis of human and animal blastomycosis.

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