

Induction and Detection of Antibodies in Immunized Rabbits with *Blastomyces dermatitidis* Yeast Phase Lysate Antigens

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Abstract

The systemic fungal disease blastomycosis, caused by the dimorphic agent *Blastomyces dermatitidis*, has continually presented clinicians with major concerns with regard to prevention and laboratory diagnosis. Our laboratory has been involved in the production and evaluation of yeast lysate antigens from various isolates of the fungus for several years. This present study was performed to compare six lysate antigens (B5931, B5927, B5896, B5898, B5929, and B5926) for their ability to induce an antibody response in immunized rabbits and also for their ability to detect antibodies in sera from the animals. The enzyme-linked immunosorbent assay (ELISA) was used to evaluate the two parameters by determining antibody levels in the serum specimens. The results indicated that antibody induction (mean ELISA absorbance values) ranged from 0.259 (B5926) to 1.168 (B5931) and antibody detection ranged from 0.560 (B5929) to 0.821 (B5927). These data provide evidence that a great deal of variation exists among the lysate antigens produced from the different *B. dermatitidis* isolates when used to induce an antibody response, but only minimal variation was observed when the same reagents were used for the detection of antibodies in the immunized rabbits. Studies are continuing in an effort to explain these findings and to further evaluate these lysate antigens as immunizing agents and for their utility as immunodiagnostic antigens for blastomycosis.

Introduction

Blastomyces dermatitidis is a dimorphic fungal organism and the causative agent of blastomycosis in humans and animals. Pulmonary disease results from the inhalation of airborne spores which transform into yeast cells once in the lungs. The infection may exist as a primary acute or chronic infection in the lungs, or it may disseminate to other organs or the central nervous system with a potentially fatal outcome. In September of 1999, a cluster of blastomycosis cases was reported from the town of Mountain Iron, Minnesota (2). Eighteen human cases were diagnosed and there seemed to be a relationship of disease to soil excavation within the area. We were able to obtain several cultures from the Centers for Disease Control from this outbreak. For several years our laboratory has been interested in the preparation and evaluation of antigens prepared from *B. dermatitidis* isolates from various geographical regions endemic for blastomycosis (1,3,4,8,9). In the present study, assays were performed using yeast phase lysates prepared from *B. dermatitidis* isolates obtained from the outbreak to determine the antibody induction and antibody detection profile of each antigenic reagent.

Materials and Methods

Antigens: Killed *B. dermatitidis* yeast lysate antigens were prepared from six different strains (B5896: human, Minnesota; B5926: human, Minnesota; B5927 human, Minnesota; B5931: human, Minnesota; B5898: human, Minnesota; B5929: human, Minnesota) prior to the date of inoculation. Preparations were made by a method similar to one that was previously used for the production of antigens from strains of *Histoplasma capsulatum* (6,7) and modified in our laboratory for *B. dermatitidis* lysate

antigen production (5). The yeast phase cells were grown for seven days at 37°C in a chemically defined medium with shaking, harvested by centrifugation, followed by washing with distilled water and then allowed to lyse for seven days at 37°C in water with shaking. The lysed reagent was centrifuged, filter sterilized, merthiolate added (1:10,000) to prevent microbial proliferation, and stored at 4°C. Protein determinations and standardizations were performed on the lysates using the BCA Protein Assay Kit (Pierce Chemical Company, Rockford, IL) and dilutions of the antigenic reagents were based on protein concentration. All reagents were standardized to 500 µg of protein per milliliter of diluent (H₂O).

Immunization/serum specimens: Twelve New Zealand white rabbits were immunized with the above six yeast lysate antigens. They were inoculated intramuscularly (2ml) and subcutaneously (1ml) with an inoculum containing 500 µg/ml of protein. At day zero, approximately 5 ml of blood was obtained from the rabbits via bleeding from the median “ear” artery. Afterwards, rabbits were bled on days 7, 14, 21, 35, 42, and 49 for the purpose of measuring the humoral primary immune response of the animals. On day 49, the rabbits were boosted with the same killed yeast lysate reagent and subsequent bleedings were performed at days 7 post-inoculum and 14 post-inoculum in order to obtain serum for assaying the secondary response of the rabbit to *B. dermatitidis* via the ELISA.

ELISA: In order to detect (how well the individual strains of *B. dermatitidis* elicited antibody production) antibodies within the individual sera, we utilized the enzyme linked immunosorbent assay (ELISA). Each lysate antigen was diluted (1000ng of protein per milliliter) in a carbonate-bicarbonate coating buffer (pH 9.6) and then added in triplicate

to wells (100µl) of an Immunomaxi 96-well microplate (TTP, Switzerland). The plates were then incubated overnight at 4°C in a humid chamber followed by three consecutive washings with phosphate buffered saline containing 0.15% Tween 20 (PBS-T).

Afterwards, the serum specimens were diluted (1: 5000) in PBS-T and then added (100µl) to the microplate wells and incubated for 30 minutes at 37°C in a humid chamber. Following this incubation the wells were washed as above. Next, 100µl of goat anti-rabbit IgG (H&L) peroxidase conjugate (Kirkegaard and Perry, Gaithersburg, MD) at a 1:2000 dilution was added to each well and incubated for 30 minutes at 37°C. The plates were washed again as above and 100µl of peroxidase substrate (Pierce) was added to each well. The substrate was allowed to incubate for approximately 2 minutes 30 seconds at room temperature. The reaction was stopped by the addition of 2N sulfuric acid and the absorbance read at 450 nm using a BIO-RAD 2550 EIA reader.

Results

The ability to induce an immune response varied greatly among the lysate antigens produced from the individual strains of *B. dermatitidis*. The absorbance values indicate that the yeast lysate antigen B5931 is greatest at inducing an immune response within the animal model followed by strains B5927 and B5896 (Figure 1). Strains B5898 and B5929 were able to produce a minimal immune response (Table 1). Strain B5926 did not produce an immune response (Figure 1 and Table 1). These findings were consistent even when the serum was placed with the different strains of *B. dermatitidis* utilized in this experiment. The ability to detect antibodies within the ELISA was comparable among all strains (Figure 2 and Table 2).

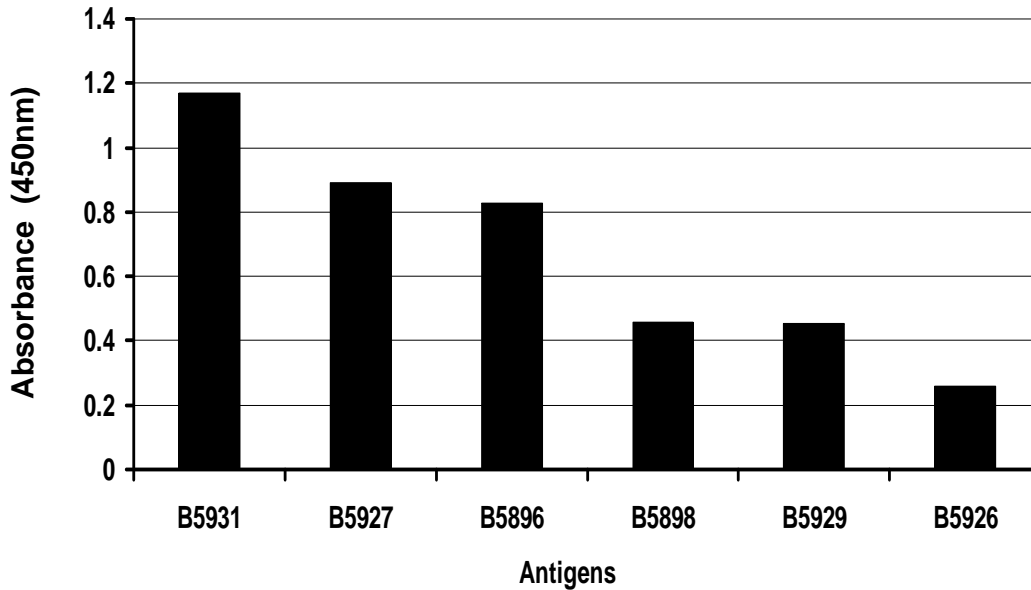


Figure 1. Comparison of antibody induction utilizing the six yeast lysate antigens

Table 1. Induction: Summary of the mean absorbance value ranges with the six lysate antigens.

Antigen	Absorbance Range	Mean
B5931	0.917 – 1.555	1.168
B5927	0.683 – 1.145	0.891
B5896	0.647 – 1.036	0.827
B5898	0.407 – 0.580	0.459
B5929	0.279 – 0.571	0.454
B5926	0.198 – 0.299	0.259

Discussion

The goal of this study was to examine the humoral immune response of six *B. dermatitidis* lysate (B5931, B5927, B5896, B5898, B5929, and B5926) preparations from an outbreak at Mountain Iron, Minnesota. Due to the strains being located in the same area our laboratory hypothesized that the induction and detection of antibodies within the animal system would be approximately the same. However, there were considerable differences when these strains were compared to one another. Currently,

there are no reliable reagents that can detect antibodies for the purpose of blastomycosis diagnosis.

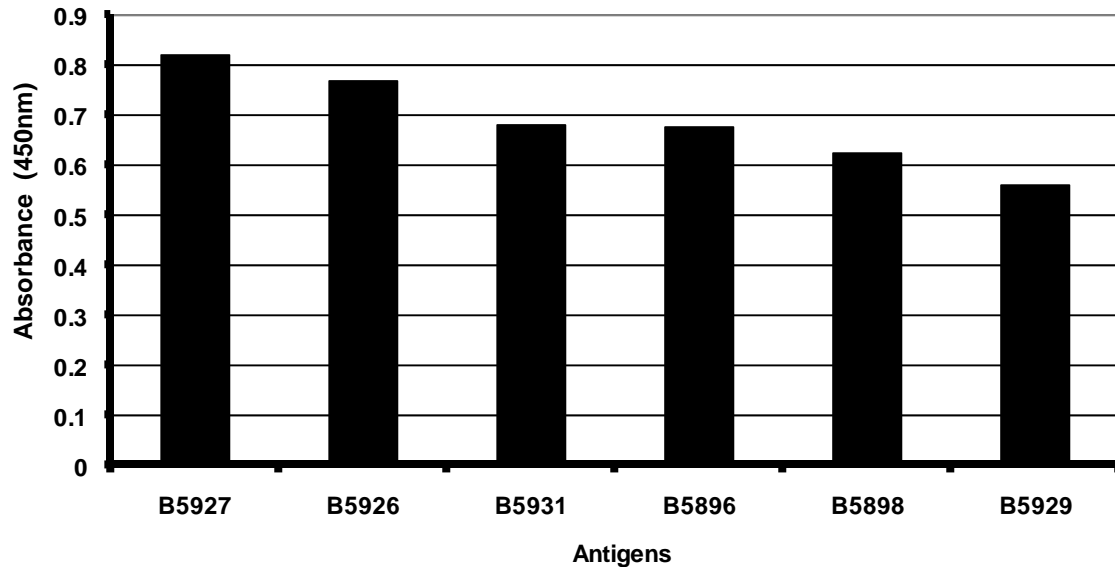


Figure 2. Comparison of antibody detection utilizing the six yeast lysate antigens

Table 2. Detection: Summary of the mean absorbance value ranges with the six lysate antigens

Antigens	Absorbance Range	Mean
B5927	0.299 – 1.372	0.821
B5926	0.285 – 1.110	0.770
B5931	0.240 – 1.555	0.680
B5896	0.284 – 1.036	0.623
B5929	0.247 – 0.917	0.560

The lack of diagnostic tools and techniques leaves clinicians with an inability to detect this pathogen in a timely manner which ultimately may cost the livelihood of the patient or even their life. Based on the results of this experiment, it appears that the B5931 strain would be an optimal candidate for the purpose of creating an immunizing and diagnostic reagent. However, when strain B5926 was considered as an inductive antigen, no strains within this study were able to detect levels of antibody within the sera following immunization with this antigen. B5926 is puzzling due to its inability to induce

an immune response. This might indicate that the B5926 *B. dermatitidis* strain is unable to be detected/processed by the immune system of the host. Thus, the humoral response would be latent throughout the entire infection in the case of blastomycosis or through the entire research project as is our case.

The differences in the inductive capabilities of B5931 and B5926 are being studied further through gel electrophoresis, Western blot analysis, immunohistochemistry, and isoelectric focusing. These efforts are being made for the purpose of determining if there are any differences in cellular composition of antigenic and virulent components of the various *B. dermatitidis* isolates. With this information we may be able to tell further why the strains were different and if any reagent for diagnosis could be created for clinical use. Comparative studies, such as this, are continuously needed as mycologists strive to find a potential *B. dermatitidis* strain that would act as an optimal immunoassay antigen. This would give physicians greater ability to diagnose and treat both human and animal blastomycosis.

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