

Research article

***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**

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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

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 more reactive when tested against *B. dermatitidis* serum specimens from immunized rabbits 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 lysate preparations 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 *Histoplasma capsulatum* [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 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 [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) 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 37° 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.

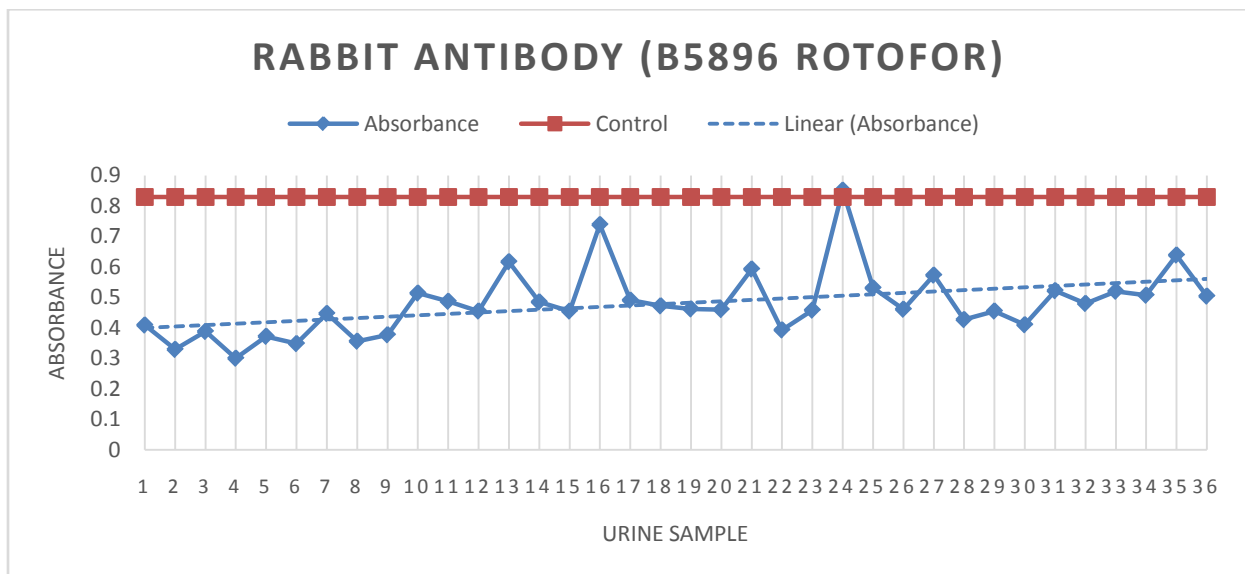


Figure 1. The absorbance values of the B5896 Rotofor(R) antibody against 36 different urine specimens from dogs with blastomycosis.

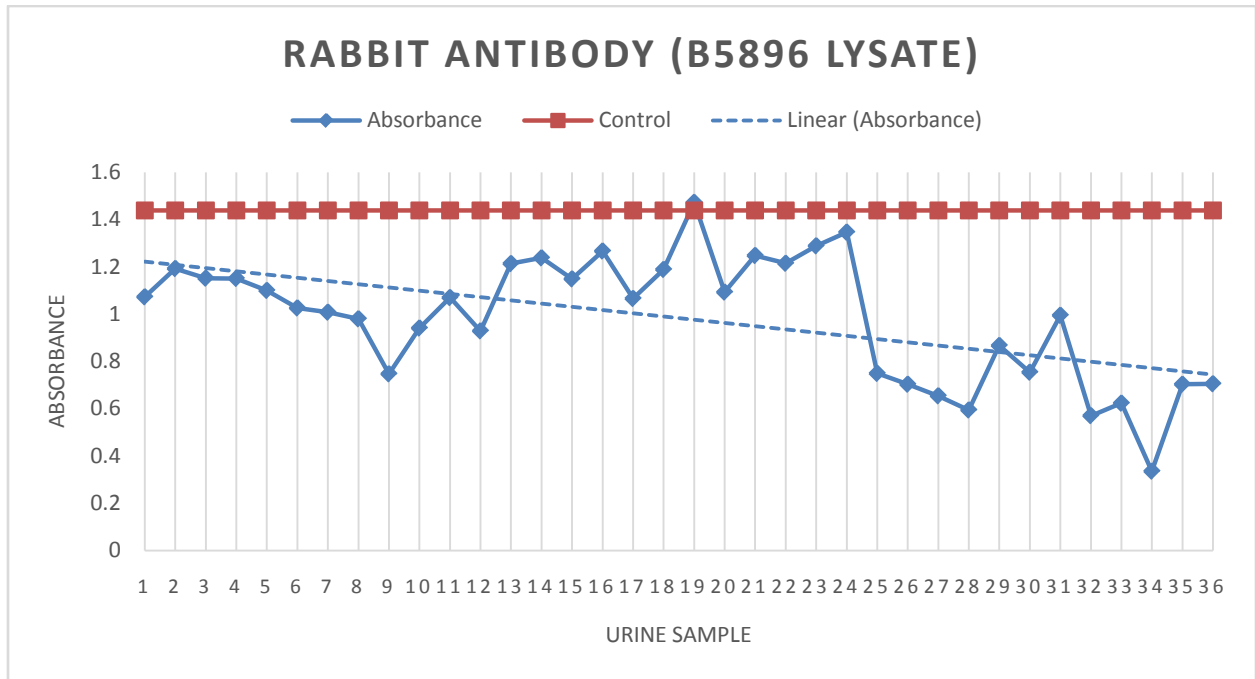


Figure 2. The absorbance values of the B5896 lysate (L) antibody against 36 different urine specimens from dogs with blastomycosis.

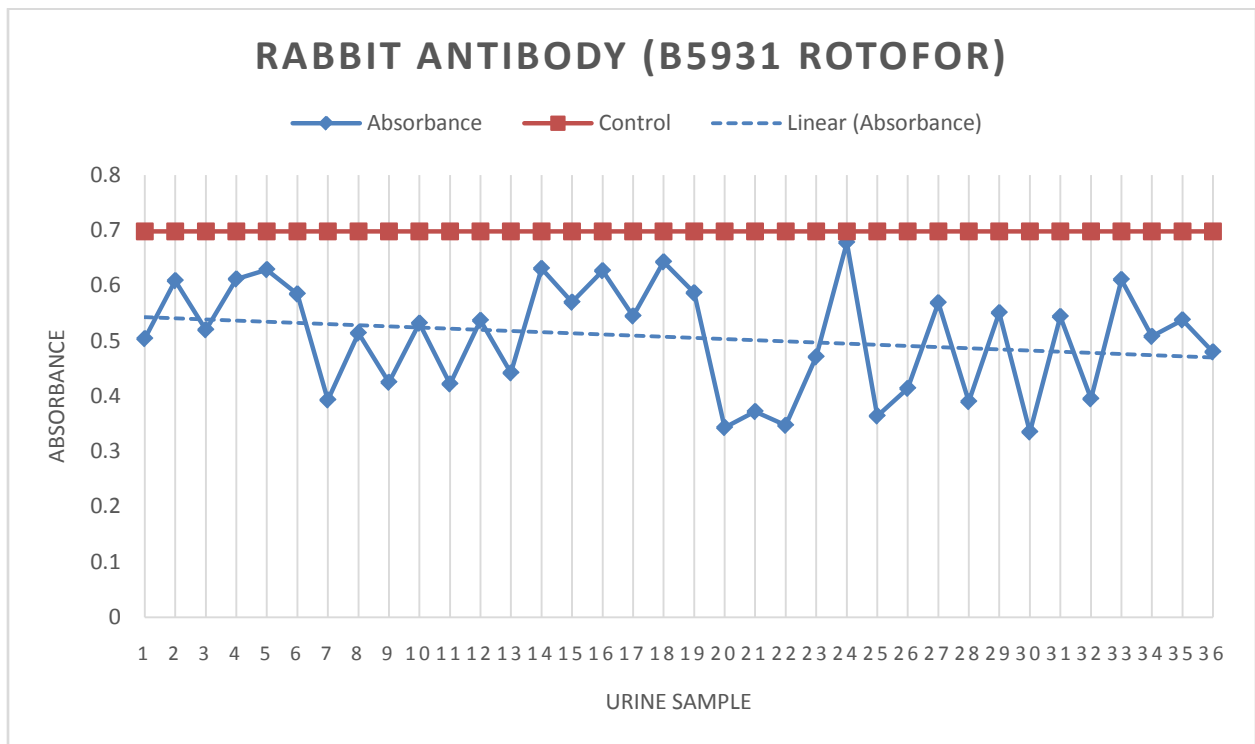


Figure 3. The absorbance values of the B5931 Rotofor(R) antibody against 36 different urine specimens from dogs with blastomycosis

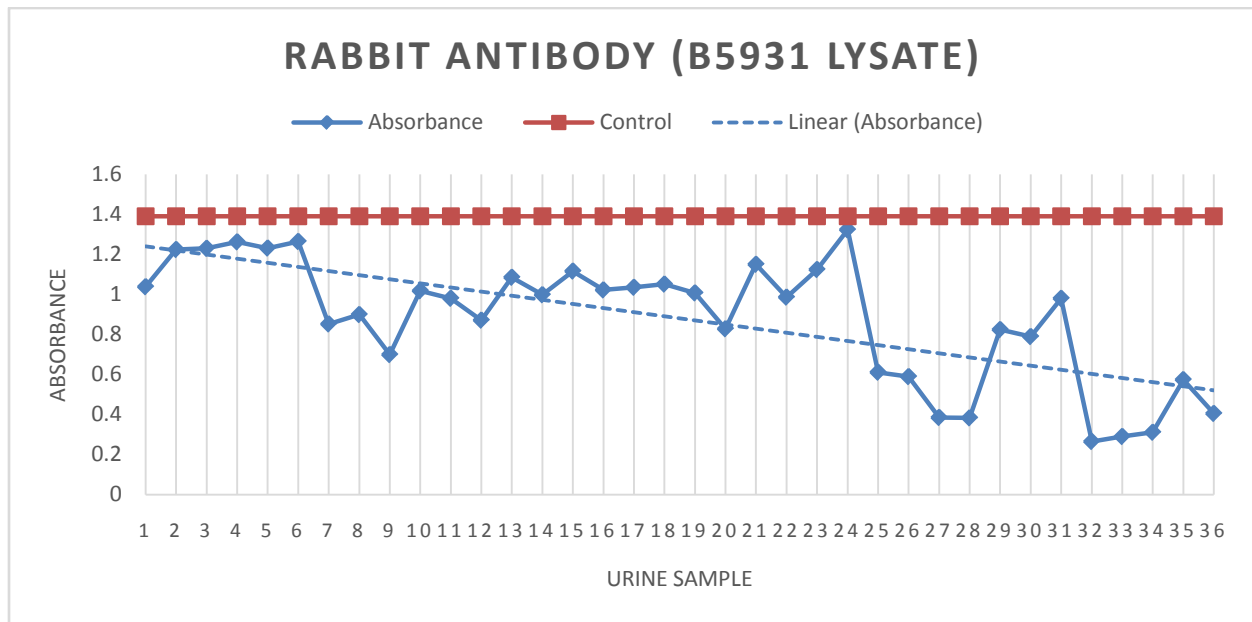


Figure 4. The absorbance values of the B5931 lysate (L) antibody against 36 different urine specimens from dogs with blastomycosis.

The B5896 Rotoforand 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.

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