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Research article

ELISA Comparative Evaluations of *Blastomyces dermatitidis* Antigenic Reagents for Antibody Detection in Serum Specimens from Immunized Rabbits

ChaoFang Tan, Enkhmaa Luvsannyam, Gamalyn Svancara, Jesse L. Atwood, Meghna Rathi, and Gene M. Scalarone

Department of Biological Sciences, Idaho State University, Pocatello, ID 83209, USA

Phone: (208) 282-3374, Fax: (208) 282-4570

E-mail: tanchao@isu.edu

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Abstract

The systemic fungal infection, blastomycosis, caused by *Blastomyces dermatitidis* infects both humans and animals. Blastomycosis has presented a diagnostic challenge and many investigators, including our laboratory, have continued attempting to develop improved immunodiagnostic methods for this disease. The purpose of this study was two-fold: (a) the primary aim was to evaluate *B. dermatitidis* yeast phase lysate antigens with respect to antibody detection in 48 sera from immunized rabbits and (b) to compare the sensitivity of three peroxidase substrates (A: Ultra TMB; B: Sure Blue TMB and C: Sure Blue Reserve TMB), using the enzyme immuoassay (ELISA) in two separate trials. Lysate antigens were prepared from *B. dermatitidis* isolates B5931 and B5896 (humans; Minnesota) and ERC-2 and T-58 (dogs; Wisconsin and Tennessee respectively).

In Trial 1, the mean absorbance antibody detection values in 24 serum specimens with B5931 ranged from 0.727 (B) to 1.171(C) and values for ERC-2 from 0.543 (B) to 0.770 (C). In Trial 2, the mean absorbance values obtained with the 24 sera with B5896 ranged from 0.575 (B) to 0.780 (C) and from 0.721 (B) to 1.143 (C) with T-58. Comparative studies are continuing to further evaluate various lysate antigens for antibody detection in blastomycosis.

Keywords: Blastomycosis, Antibody Detection, Lysate Antigen, ELISA, *Blastomyces dermatitidis*

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Introduction

Blastomyces dermatitidis is the causative agent of the systemic fungal disease blastomycosis. *B. dermatitidis* is a dimorphic fungal organism that exists in the mycelial phase in nature in the soil environment and converts to the yeast phase once spore inhalation occurs and the organism is exposed to the body temperature of the host [1]. This disease can present as an acute or chronic infection and closely resembles tuberculosis or other respiratory infections. If the disease is not diagnosed or untreated while in the lungs the fungus may also disseminate to other organs including skin, bones, and to the central nervous system which may result in meningitis.

B. dermatitidis can not only infect individuals with normal immune systems, but also this organism is extremely important as a disease agent in persons with AIDS or other immunodeficiency diseases that compromise the immune system [2-6].

The importance of a prompt and reliable diagnosis of the disease is of prime importance. Delayed diagnosis can result in a poor prognosis if dissemination has occurred prior to diagnosis. Several of the immunodiagnostic assays that have been used lack sensitivity and specificity, and many other culturing and histologic methods fail to produce desired results [1,7,8]. In recent years investigators have been devoting a considerable amount of effort in attempts to develop improved and reliable immunodiagnostic procedures for blastomycosis and other fungal diseases [6-10]. For several years our laboratory has been involved in the production and evaluation of novel yeast phase lysate antigens prepared from human, animal and environmental isolates of *B. dermatitidis* [11-18]. Encouraging results have been obtained with some of the lysate reagents in various immunoassays, but studies have continued with respect to evaluating new lysate preparations for antibody detection in serum specimens from infected and immunized animals in different ELISA formats [11,12,15,16,18].

Our current study was designed to determine the reactivity of four *B. dermatitidis* lysate antigens when three peroxidase substrates were used in the indirect ELISA to detect antibodies in serum specimens from immunized rabbits.

Materials and Methods

Antigens.

B. dermatitidis yeast phase lysate reagents (B5931, human Minnesota; ERC-2, dog Wisconsin; B5896, human Minnesota; T-58, dog Tennessee) were prepared following a protocol similar to the one used for the production of lysate antigen from *Histoplasma capsulatum* [18-20] and modified in our laboratory for *B. dermatitidis* lysate antigen production 16]. The yeast cells were grown for 7 days at 37°C in an incubator shaker in a chemically defined medium (glucose, 10.0 g; potassium phosphate monobasic, 1.5 g; calcium chloride dehydrate 0.15 g; magnesium sulfate, 0.5 g; ammonium sulfate, 2.0 g; L-asparagine, 2.0 g; L-cysteine, 0.2g; and pH adjusted to 6.2 with 5 N sodium hydroxide) in an incubator shaker, harvested by centrifugation (700 xg; 5 min) followed by washing with distilled water, re-suspended in distilled water, and then allowed to lyse for 1 day 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 (Pierce Chemical Company, Rockford, IL, USA), and dilutions of the antigenic reagents used in the ELISA assays were based on protein concentration.

Serum Specimens.

Forty eight serum specimens from rabbits immunized with various *B. dermatitidis* yeast lysate antigens were previously produced and available in our laboratory.

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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 of protein/mL) in a carbonate-bicarbonate coating buffer (pH 9.6) and then added to triplicate wells (100 uL) of a NUNC 96-well microplate (Fisher-Thermo). The plates were then incubated over night at 4^{o}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^{o}C in a humid chamber. Following this incubation the wells were washed as above and 100 uL of goat anti-rabbit IgG (H &L) peroxidase conjugate (Kirkegaard and Perry, Gaithersburg, MD, USA) was added to each well and incubated for 30 min at 37^{o}C. The plates were again washed as above and 100 uL of each of the TMB peroxidase substrates [A:1-Step^{TM} Ultra TMB-ELISA; Thermo Scientific (Rockford, IL, USA), B: SureBlue^{TM} TMB Microwell Peroxidase 1-Component; KPL (Gaithersburg, MD, USA) and C: SureBlue Reserve^{TM} TMB Microwell Peroxidase 1-Component; (KPL) was added to each well and incubated for approximately 3 min at room temperature. The A reaction was stopped by the addition of sulfuric acid and the B and C reactions were stopped by TMB Stop Solution. Finally, the absorbance was read at 450 nm using a BIO-RAD 2550 EIA reader.

Results and Discussion

The mean absorbance values of the 4 different antigens (B5931, human Minnesota; ERC-2, dog Wisconsin; B5896, human Minnesota; T-58, dog Tennessee) when used in the indirect ELISA to detect antibodies in 48 rabbit sera are shown in Figure 1 and Figure 2. The antigen, B5931 exhibited the highest absorbance values of all the antigens tested, ranging from 0.727-1.171 depending on the substrate used, followed closely by the second most reactive antigen, T-58, with mean absorbance values from 0.72-1.143. ERC-2 and B5896 had similar absorbance ranges (0.543-0.77 and 0.575-0.78 respectively) and are the least reactive of the four. Among the three substrates, Sure Blue TMB Reserve peroxidase substrate (C) produced the greatest reactivity to the antigens tested, second was substrate Ultra TMB (A) and lastly, Sure Blue TMB microwell peroxidase substrate (B).

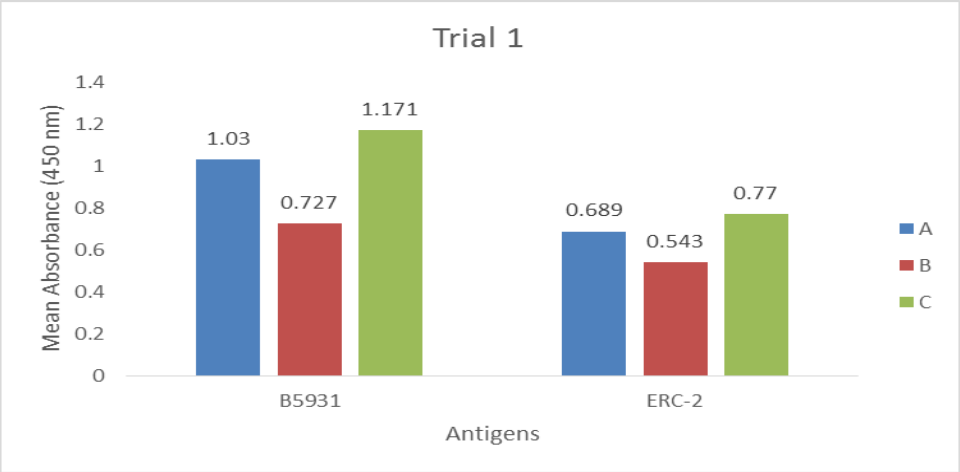

Figure 1: Comparison of the reactivity (Mean absorbance values) of two *B. dermatitidis* yeast lysate antigens: B5931, human isolate from Minnesota and ERC-2, dog isolate from Wisconsin, with respect to antibody detection in 24 rabbit serum specimens. Reactivity was detected using three substrates, A: Ultra TMB, B: Sure Blue TMB peroxidase substrate, and C: Sure Blue TMB Reserve peroxidase substrate.

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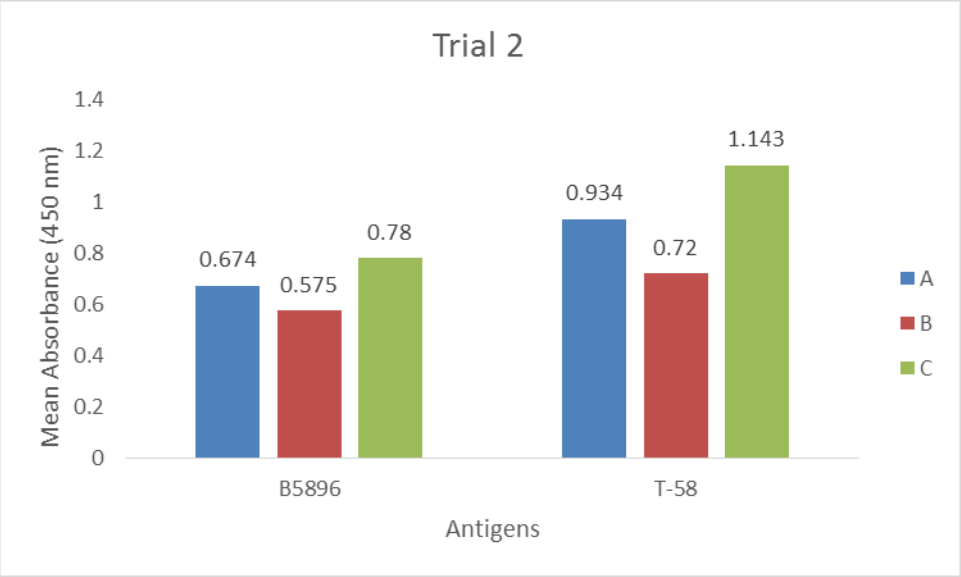

Figure 2: Comparison of the reactivity (Mean absorbance values) of two *B. dermatitidis* yeast lysate antigens: B5896, human isolate from Minnesota and T-58, dog isolate from Tennessee, with respect to antibody detection in 24 different rabbit serum specimens. Reactivity was detected using three substrates, A: Ultra TMB, B: Sure Blue TMB microwell peroxidase substrate, and C: Sure Blue TMB Reserve peroxidase substrate.

Conclusion

The selection of antigens for the diagnosis of blastomycosis is key for the sensitivity of the ELISA test, as is the selection of the peroxidase substrate.

The results of Trial 1 and Trial 2 indicate that antigens prepared from *B. dermatitidis* isolates from both the north (Minnesota) and south (Tennessee) are efficient as for antibody detection in sera from rabbits. This study showed that B5931 antigen used in Trial 1 provided the highest absorbance values and thus is the most reactive antigen. T-58 antigen used in Trial 2 also showed a high degree of reactivity as well and showed absorbance values only slightly lower than B5931. The results also indicated that Sure Blue TMB Reserve peroxidase substrate was the most reactive substrate used in all trials with all of the antigens tested. Based on these results, future testing for blastomycosis would be greatly benefited by the use of either B5931 or T-58 lysate antigens in the assay in combination with Sure Blue TMB Reserve peroxidase substrate to detect *B. dermatitidis* antibodies.

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