

Determination of Optimal Parameters for the Preparation of *Blastomyces dermatitidis* Yeast Phase Lysate Antigens

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Abstract

The objective of this present study was to determine the optimal period of lysis of *Blastomyces dermatitidis* yeast cells in order to produce a reagent with a high degree of sensitivity when used in antibody detection immunoassays. Antigens were prepared at three-day intervals (lysis period: 1 to 28 days) following the admixture of harvested yeast cells plus deionized water. The preparations were evaluated by the enzyme linked immunosorbent assay (ELISA) for the detection of antibodies in serum specimens from rabbits previously immunized with killed whole *B. dermatitidis* yeast cells. It was evidenced that the antibody detection activity gradually increased with time; reaching an optimal level of reactivity achieved between days 19 and 22. An additional evaluation using lysate antigens prepared on days 13 and 22 indicated that both reagents were able to detect antibody (various dilutions of the serum specimens), but optimal reactivity was obtained with the day 22 lysate. This antigen exhibited a high degree of sensitivity (antibody detected at an optimal serum dilution of 1:20,000). This *B. dermatitidis* preparation appears to show promise as an immunodiagnostic antigen for the clinical diagnosis of blastomycosis.

Introduction

The immunodiagnosis of the systemic fungal disease blastomycosis has presented problems to clinicians and laboratory personnel for many years (4). Various antigenic reagents have been used, but the reliability of the antigens for the definitive diagnosis of blastomycosis has been a major concern (3, 8). Our laboratory has been involved with

research on the development and evaluation of *Blastomyces dermatitidis* yeast phase lysate antigens for the past several years. In previous studies the lysate reagent was prepared at seven days of lysis (1,2,5,6,7). Our present study focuses on the optimal parameters for lysate preparation to be used for detection of antibodies to *B.*

dermatitidis. The first part of the study was to determine the optimal amount of days that the cells were allowed to lyse when admixed with distilled water. Based on the results from this first study, we then performed an evaluation using lysate antigens prepared on days 13 and 22 (the days in which we evidenced a high degree of reactivity) to determine dilution values that exhibit positive detection of antibodies to previous exposure to *B. dermatitidis*.

Materials and Methods

Antigens: Yeast lysate antigenic reagents were prepared from an isolate of *B. dermatitidis* (T-58) obtained from a dog infected with blastomycosis. We inoculated 20 flasks with *B. dermatitidis* yeast cells and they were allowed to grow for 7 days at 37 C with shaking. The cells were harvested and washed by centrifugation (5 min at 700 g) with sterile distilled water, repeated five times, and suspended in 350 ml of distilled water. This flask of cells was kept at 37 C and samples (25 ml aliquots) were collected every three days for a 28-day period of lysis. At each time interval the cells were centrifuged (30 min at 700 g) and the suspension sterilized by passage through a 0.2 µm Nalgene filter (Nalge, Rochester, NY). Merthiolate (1:10000 dilution) was added to the antigen preparations as a preservative and the solutions stored at 4 C (5). Protein determinations were made for each antigen preparation using the bicinchoninic acid (BCA, Pierce, Rockford, IL) method, according to manufacturer's specifications.

ELISA: An indirect enzyme linked immunosorbent assay (ELISA; 1,2,5-7) was utilized to determine the amount of antibody present in each serum specimen. Yeast lysate antigen (as above) was added to each plate (1000 ng/ml protein) and incubated overnight at 4°C. The plate was rinsed 3 times with PBS-T (phosphate buffered saline + 0.15% Tween 20). Rabbit serum diluted 1:5000 was added (100 ul) and the plate was incubated at 37°C for 30 minutes. Plates were rinsed three times to remove any unbound antibody. A secondary antibody (goat anti-rabbit IgG) conjugated to horseradish peroxidase was added (100 ul) and the plate was incubated at 37°C for 30 minutes followed by rinsing to remove any unbound secondary antibody. Finally, the substrate (Ultra TMB, Pierce) was added and allowed to incubate at room temperature for approximately 2 minutes to detect antibody. The reaction was stopped with 2 N H₂SO₄ and the absorbance was read at 450 nm using the BIO-RAD EIA reader (BIO-RAD, Hercules, CA). In the second evaluation, various dilutions of serum, ranging from 1:1250 to 1:80000, were assayed with the day 13 and 22 lysate antigens to determine the presence of antibody. These antigens were tested against two different samples of pooled sera, each collected from twelve rabbits that had been previously inoculated with different *B. dermatitidis* killed whole yeast cells. The normal value was established by determining the mean absorbance value obtained when sera from three non-immunized rabbits were assayed. This normal value was considered the baseline and absorbance values (serum dilutions) above the normal value were positive with respect to antibody titer.

Results

The results obtained in the initial experiment indicate that the antibody detection absorbance values remained relatively constant for the first 10 days, and began to increase on day 13. The absorbance values for both serum samples increased until day 22 where they began to level off (Figure 1). For the antibody dilution detection assays, the normal absorbance value for the first serum was 0.203. Both lysate antigens had absorbance values higher than 0.203 up to a dilution of 1:20000 (Figures 2 and 3). The normal value for the second serum was 0.253 with absorbance values higher than the normal up to a 1:20000 dilution (Figures 4 and 5).

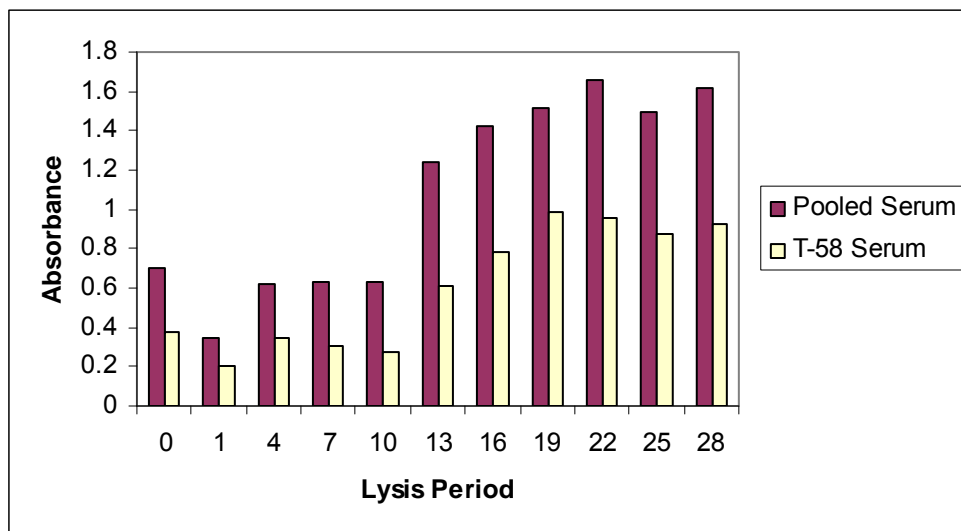


Figure 1: Absorbance value readings of the different number of days that the yeast phase *B. dermatitidis* antigens were allowed to lyse.

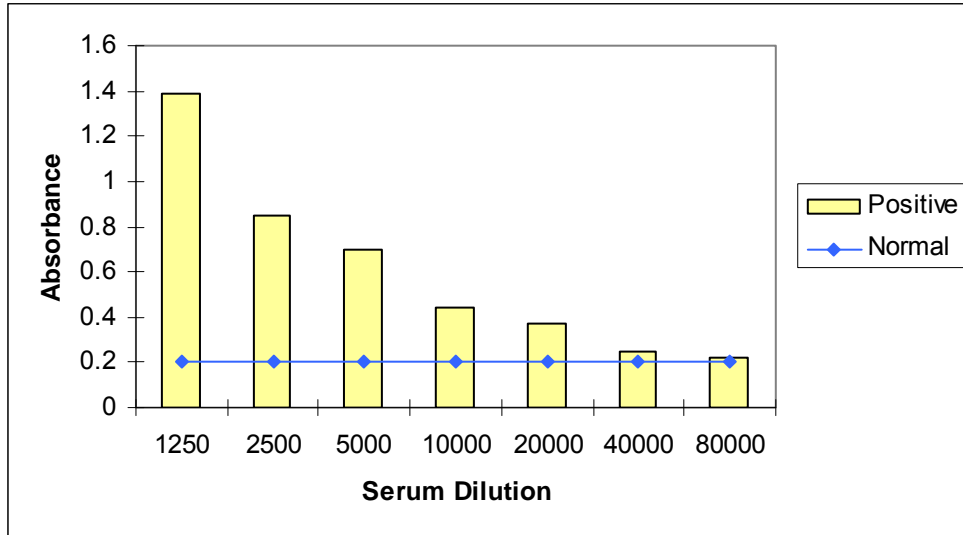


Figure 2: Absorbance values of the day 13 lysate antigen tested against a pooled serum.

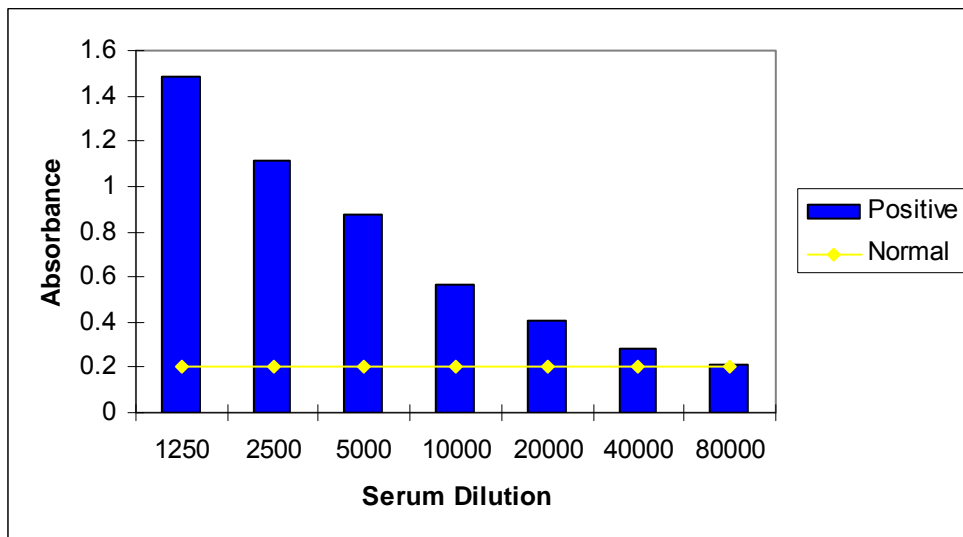


Figure 3: Absorbance values of the day 22 lysate antigen tested against a pooled serum.

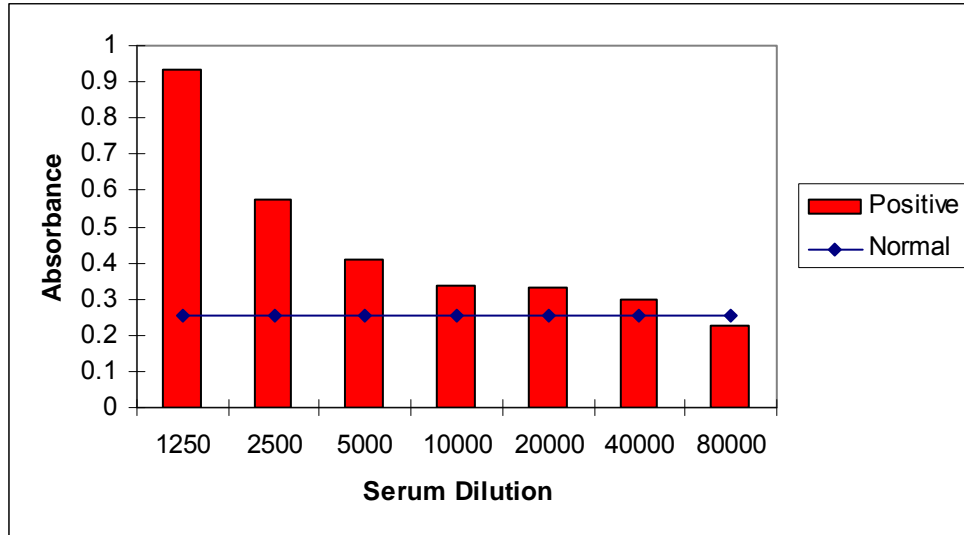


Figure 4: Absorbance values of the day 13 lysate antigen tested against second pooled serum.

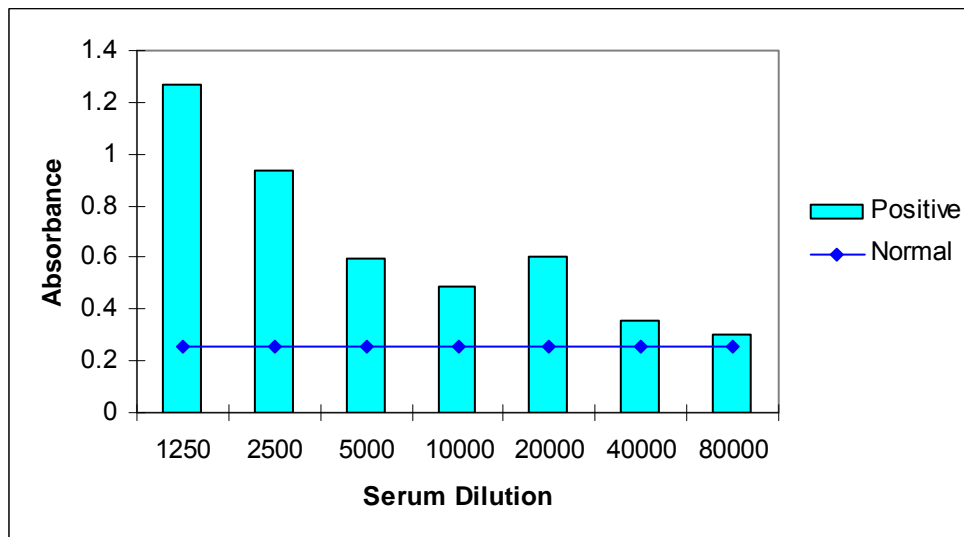


Figure 5: Absorbance values of the day 22 lysate antigen tested against second pooled serum.

Discussion

The purpose of the research was to determine if we might be able to enhance the efficacy of a *B. dermatitidis* yeast phase lysate antigenic preparation by allowing the

yeast cells to lyse in distilled water for an extended period of time. Prior to this, cells were typically allowed to lyse for 5 to 7 days (1,2,5,6,7). In this study we evidenced a substantial increase in the sensitivity of the antigens when the yeast cells were allowed to lyse for 13 plus days as compared to lysates prepared at earlier times in the antigen production process. The activity of the antigens increased following day 13, reaching an optimal level of sensitivity between days 19 and 22. Therefore the first phase of this study provided evidence that a new method of antigen production, which yielded a reagent with a high degree of sensitivity, may be a more appropriate laboratory procedure for lysate antigen preparation. Additional studies on not only sensitivity, but also specificity determinations will be necessary to further investigate this process. Another phase of the study involved diluting the serum specimens in order to determine the optimal level of antibody detection with the day 13 and day 22 lysate antigen preparations. With lysates prepared on day 13 and day 22 we were able to detect antibodies in serum specimens from immunized rabbits up to a dilution of 1:80000 with the optimal dilutions being between 1:1250 and 1:20000. This indicates that the lysate antigens have the ability to detect antibodies to *B. dermatitidis* in an efficient manner at very high dilutions of the serum specimens.

In summary, this present investigation of lysate antigen production provided additional information on the preparation and utility of *B. dermatitidis* yeast lysate antigens with respect to the clinical laboratory detection of antibodies to *B. dermatitidis*. This investigation concludes that in order to enhance the sensitivity of this study, longer cell lysis is essential. Studies will continue to determine if a similar pattern of reactivity will be evidenced with lysates prepared from different isolates of the fungus.

References

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Acknowledgement

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