Evaluation of the Incubation Parameters of an Enzyme Immunoassay for the Detection of *Blastomyces dermatitidis* Antibodies

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Abstract

The objective of this study was to evaluate incubation time parameters of the enzyme-linked immunosorbent assay (ELISA) in order to optimize the assay for detection of antibodies to *Blastomyces dermatitidis*. The indirect ELISA was used to compare the reactivity of yeast lysate antigens (598 and 248) for antibody detection in sera from rabbits immunized with killed yeast cells of *B. dermatitidis* or *Histoplasma capsulatum*. Absorbance values were determined following 37 C incubation periods of 10, 20 and 30 minutes after addition of primary antibody and anti-rabbit antibody-peroxidase conjugate. Mean absorbance values ranged from 0.506 (10 min), 0.960 (20 min) and 1.163 (30 min) when the *B. dermatitidis* sera were assayed with the 598 antigen. Values of 0.420 (10 min), 0.698 (20 min) and 0.900 (30 min) were obtained with the 248 antigen. Cross-reactivity mean values were considerably lower (0.194, 10 min;0.298, 20 min; 0.324, 30 min) and (0.298, 10 min; 0.416, 20 min; 0.532, 30 min) when the 598 and 248 antigens were used to detect antibodies in the *H. capsulatum* sera. The greatest reactivity (reactivity vs cross-reactivity) was observed at the 30 minute incubation times, but antibody was also detected after 10 and 20 minute incubation periods.

Introduction

Blastomyces dermatitidis, a thermally dimorphic fungal organism, is the causative agent of blastomycosis. Blastomycosis, a systemic and sometimes fatal disease of humans and animals, is often difficult to diagnose in the clinical laboratory (3). Various methods have been used including culturing, microscopic examination and serological assays. Immunological assays such as complement fixation and immunodiffusion have been of limited value due to problems with sensitivity and specificity, but the enzyme-linked immunosorbent assay (ELISA) has shown promise as a diagnostic tool. Thus our primary concern is to develop an ELISA with optimal sensitivity and specificity. Our laboratory has been utilizing an indirect ELISA for the detection of *B. dermatitidis* antibodies in serum specimens from humans and animals for the past several years (1,2,4,5,7,9). The objective of this present study was to determine if the incubation periods that we have been using in our ELISA are optimal or if other parameters might be more appropriate with regard to the development of a reliable clinical laboratory assay.

Materials & Methods

Antigens/Sera

Two *B. dermatitidis* yeast phase lysate antigens, prepared from different isolates of the organism, (598; human, Wisconsin and 248, soil, Wisconsin) were used in the ELISA for the detection of antibodies in serum specimens from rabbits that were previously immunized with *B. dermatitidis* or *Histoplasma capsulatum* killed whole yeast cells. The lysate antigens were prepared by a method previously described for *H. capsulatum* antigen production and modified in our laboratory for *B. dermatitidis* lysate antigen production (4,6,8).

ELISA

An indirect ELISA (peroxidase enzyme system) was utilized to determine the amount of antibody present in the serum specimens. All assays were performed in triplicate.

(a) Lysate antigen (100 ng/ml) was added to microdilution plates (Immunomaxi modified flat bottom high binding plates, TTP, Switzerland) and incubated overnight at 4˚C. The plate was rinsed 3 times with phosphate buffered saline containing 0.15% Tween-20 (PBS-T).

(b) Rabbit serum diluted 1:5000 (9) was added to the plates and incubated at 37˚C for periods of 10, 20 or 30 minutes. The plates were rinsed three times as above to remove any primary antibody.

(c) A secondary antibody (goat anti-rabbit H & L horseradish peroxidase conjugate, 1:2000 dilution, KPL) (9) was added and incubated at 37˚C for 10, 20 or 30 minutes followed by rinsing to remove any unbound secondary antibody.

(d) Finally, the substrate (1-Step Ultra TMB-ELISA, Pierce) was added and allowed to incubate at room temperature for 3 minutes +/- 30 seconds to detect antibody. The reaction was stopped with 2 N H2SO4 and the absorbance was read at 450 nm using a BIO-RAD EIA reader.

Results

A comparison of *B. dermatitidis* and *H. capsulatum* antibody detection (mean absorbance values) in the rabbit sera with 598 and 248 lysate antigens following incubation of the primary and secondary antibodies for 10, 20 or 30 minutes is shown in Figures 1, 2, 3 and 4 respectively. The 598 lysate antigen exhibited a greater degree of reactivity and less cross-reactivity than the 248 reagent when the two antigens were reacted against *B. dermatitidis* or *H. capsulatum* sera at the three incubation periods. Optimal reactivity

was evidenced at the 30 minute incubation times, with lesser reactivity at the 10 and 20 minute times.

The potential reactivity (*B. dermatitidis* reactivity minus *H. capsulatum* cross-reactivity; mean absorbance values) of the two lysate antigens following incubation at the three time intervals is shown in Figure 5.

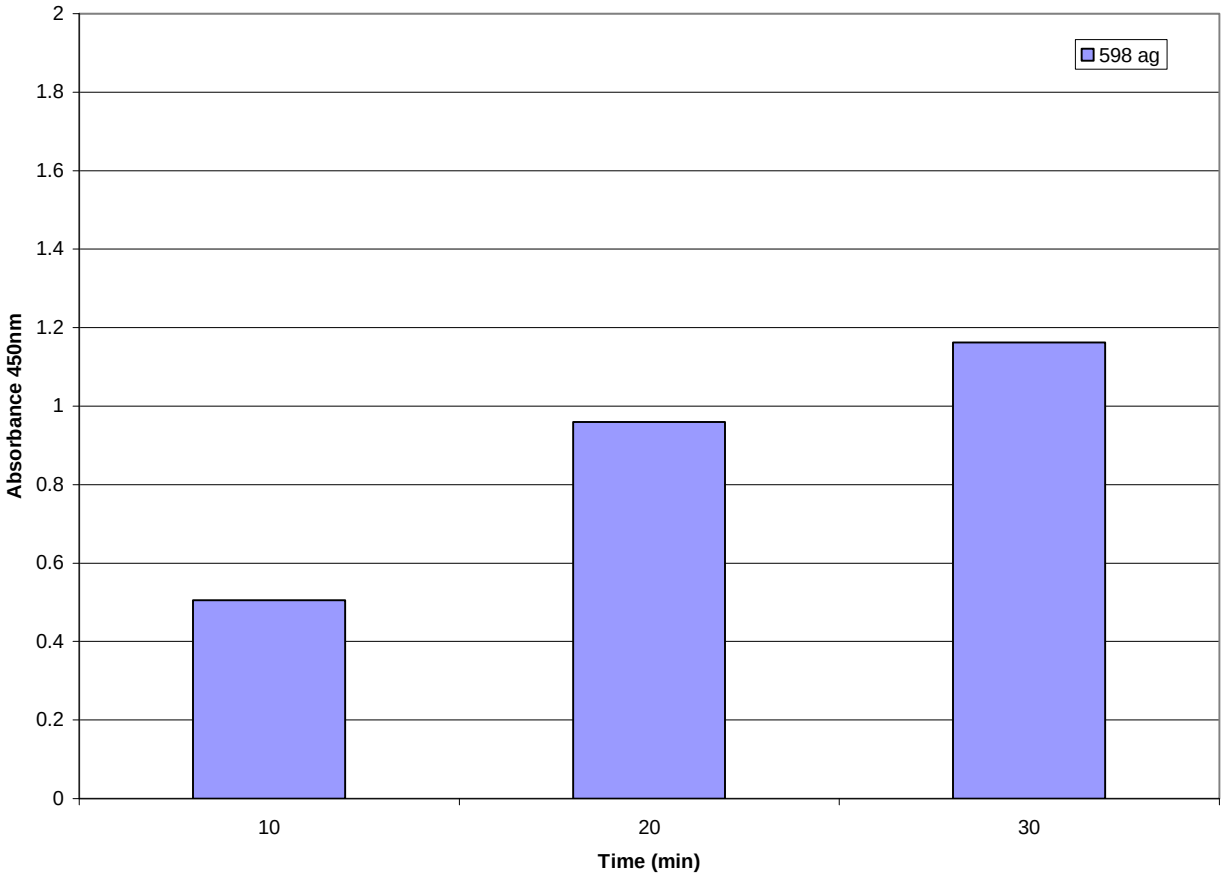

Figure1: The ELISA detection of antibody with *B. dermatitidis* 598 lysate antigen in serum specimens from rabbits immunized with *B. dermatitidis* killed yeast cells. Evaluations performed following incubation of primary and secondary antibody for 10,20 or 30 minutes.

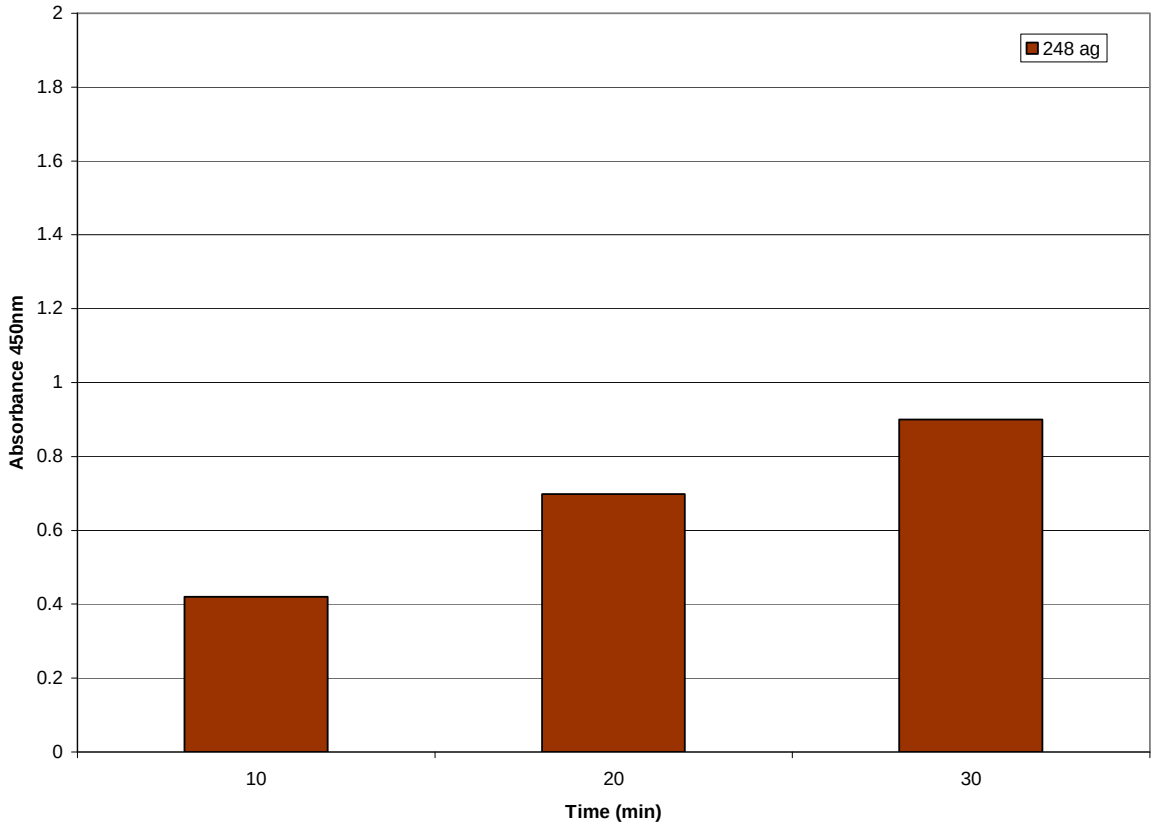

Figure 2: The ELISA detection of antibody with *B. dermatitidis* 248 lysate antigen in serum specimens from rabbits immunized with *B. dermatitidis* killed yeast cells. Evaluations performed following incubation of primary and secondary antibody for 10,20 or 30 minutes.

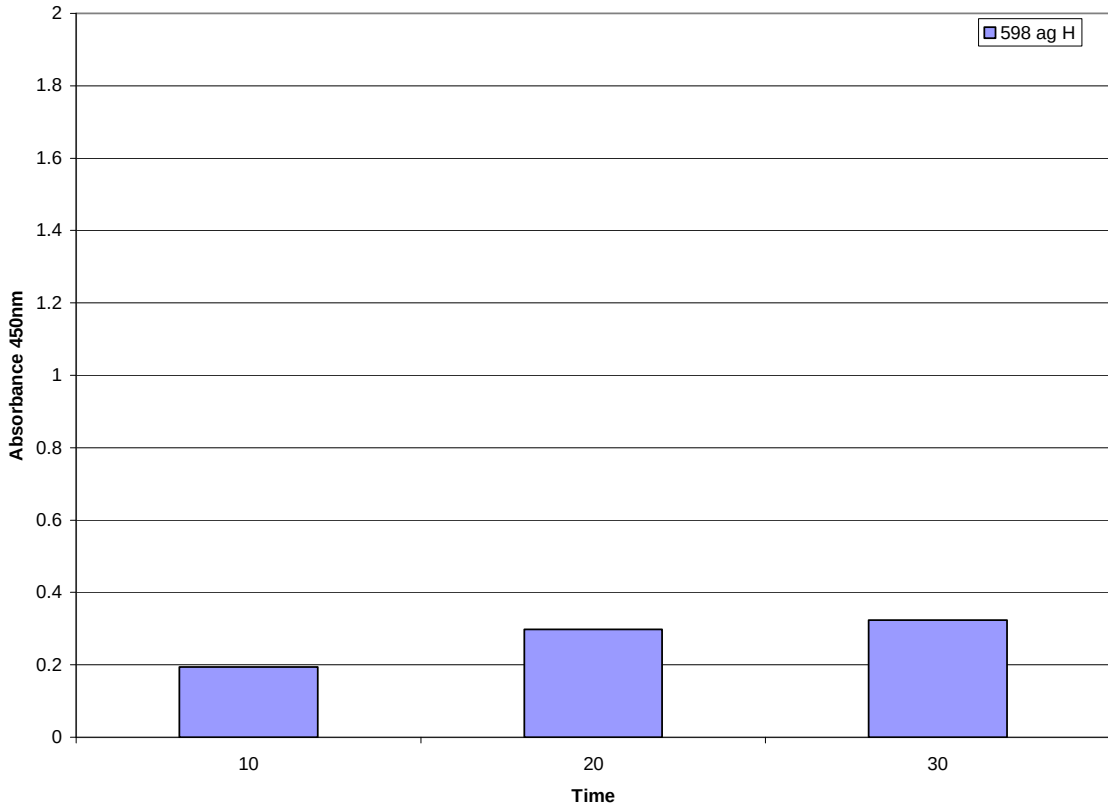

Figure 3: The ELISA detection of antibody with *B. dermatitidis* 598 lysate antigen in serum specimens from rabbits immunized with *H. capsulatum* killed yeast cells. Evaluations performed following incubation of primary and secondary antibody for 10,20 or 30 minutes.

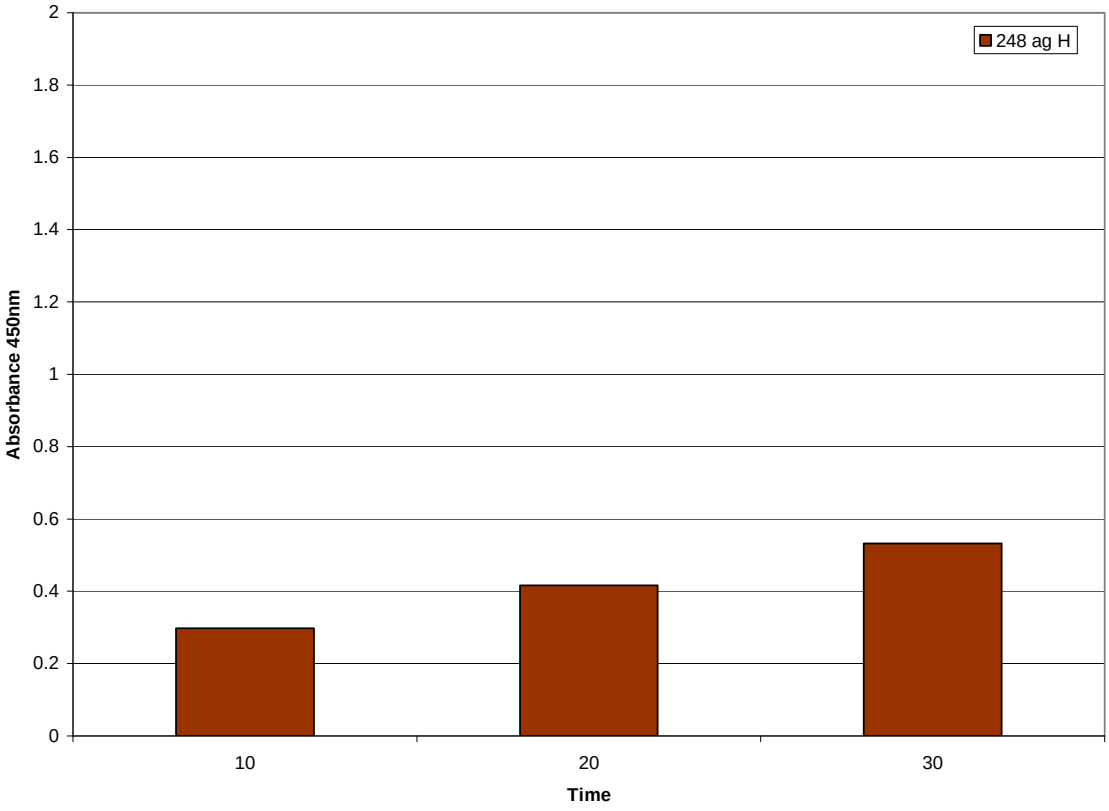

Figure 4: The ELISA detection of antibody with *B. dermatitidis* 248 lysate antigen in serum specimens from rabbits immunized with *H. capsulatum* killed yeast cells. Evaluations performed following incubation of primary and secondary antibody for 10,20 or 30 minutes.

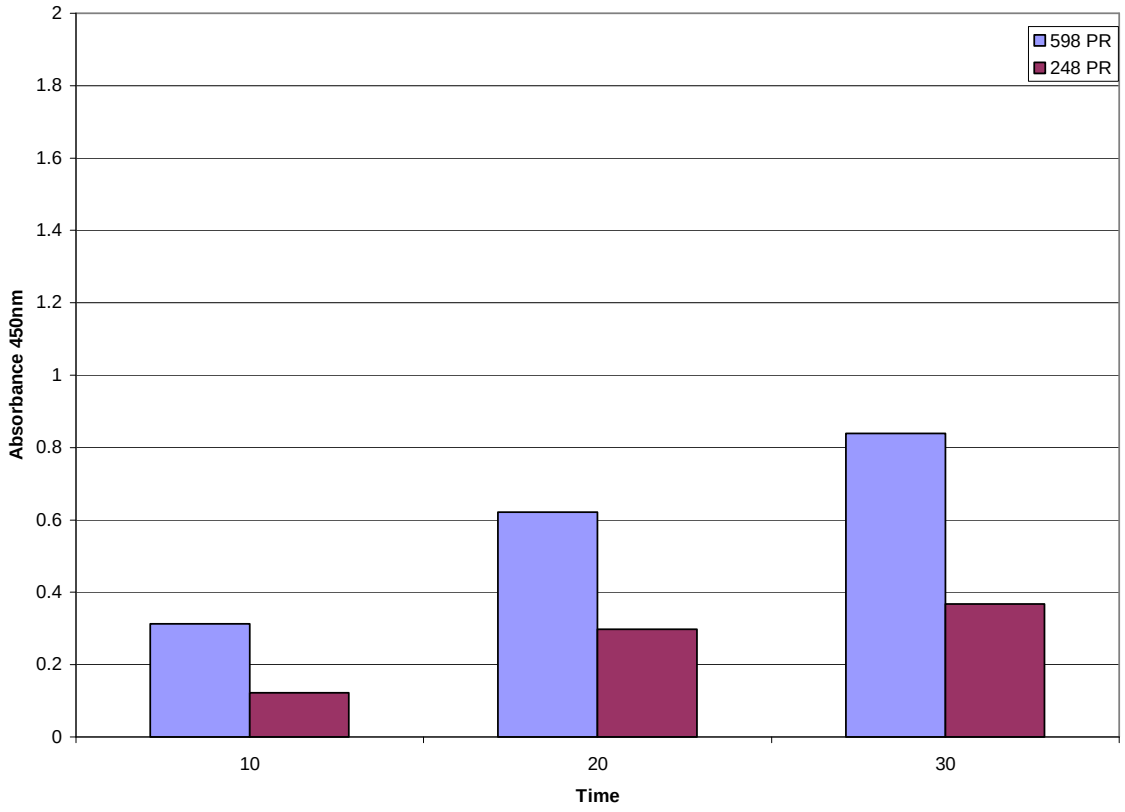

Figure 5: The ELISA potential reactivity (mean absorbance reactivity values minus mean cross-reactivity absorbance values) of *B. dermatitidis* 598 and 248 lysate antigens in serum specimens from rabbits immunized with *B. dermatitidis* or *H. capsulatum* killed yeast cells. Evaluations performed following incubation of primary and secondary antibody for 10,20 or 30 minutes.

Discussion and Conclusion

Antibody in the serum specimens was detected following incubation at 10, 20 or 30 minutes after addition of primary and secondary antibodies to the plates. The greatest sensitivity and specificity was achieved after incubation of the two antibodies for 30 minutes (598 yeast lysate antigen preparation). The data indicate that the 30-minute incubation periods that our laboratory has been using for a number of years is optimal for

greatest sensitivity, but even the 10-minute incubation periods may be used if time is a major factor with regard to clinical immunodiagnostic applications.

Therefore the study provides evidence with regard to incubation parameters, but other aspects of the assay require optimization including additional comparative studies with different antigenic reagents, as above, and also a number of studies using different enzyme-substrate systems. Research is continuing in an effort to develop a reliable immunodiagnostic assay for the clinical detection of blastomycosis in humans and animals.

Acknowledgement

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