

Comparison of *Blastomyces dermatitidis* Yeast Phase Lysate Antigens for Their Use as Diagnostic Reagents in the Competitive Inhibition ELISA for the Detection of Blastomycosis

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Abstract:

Competitive binding inhibition enzyme linked immunosorbent assays (ELISA) were used to detect *Blastomyces dermatitidis* antigens in urine specimens from dogs with blastomycosis. Sera from rabbits previously immunized with *B. dermatitidis* killed whole yeast cells were used as the primary antibody as well as for positive controls in the competitive ELISA. This study was performed to evaluate six different *B. dermatitidis* yeast lysates for their efficacy as reagents in the competitive ELISA. One hundred and seventy urine specimens were assayed from dogs residing within endemic regions of the United States. The level of antigen detection ranged from 92.86 to 46.47% and indicates that one isolate may be a good candidate as a reagent with the competitive ELISA.

Keywords: Antigen detection, *Blastomyces dermatitidis*, Competitive Inhibition ELISA, Urine

Introduction:

Blastomyces dermatitidis is a thermally dimorphic fungal organism with the mycelial phase being observed at 25°C. Inhalation of spores produced in the mycelial phase results in blastomycosis. Within the warm moist environment of the lungs or through culture at 37°C; the yeast form of this dimorphic fungus is observed. Conversion within the lungs leads to a primary pulmonary infection in the host, which may be self-resolving without the use of antifungal therapy. However, progressive pulmonary infection as well as dissemination to other body sites may result and without treatment blastomycosis may be fatal (2).

Various methods may be employed for the detection of blastomycosis: direct histological observation, lengthy culturing of the patient's specimen, and immunodiagnostic assays. Our laboratory has been involved in *B. dermatitidis* research for several years with a focus on utilizing enzyme linked immunosorbent assays (ELISA) to detect antibodies produced by humans and dogs (1, 3, 5, 8, 9) as well as the development of an antigen detection method utilizing a competitive inhibition ELISA (10).

In this study the competitive ELISA technique was used to compare different *B. dermatitidis* yeast phase lysates for their efficacy as reagents for the detection of blastomycosis antigens in urine specimens.

Materials and Methods:
Antigens

The *B. dermatitidis* isolates (Table 1) were prepared as yeast phase lysates. Mycelial phase cultures were converted to yeast cells in brain heart infusion agar and then grown in a chemically defined medium (glucose 0.056 M, KH₂PO₄ 0.011 M, CaCl 2H₂O 0.001M, MgSO₄ 7H₂O 0.002 M, (NH₄)₂SO₄ 0.015 M, L-asparagine 0.013 M, L-cysteine 0.001 M; pH 6.2) for 5 days at 37°C with shaking. The cells

Table 1. The *B. dermatitidis* yeast phase lysate antigens with the source and location. The * indicates an ATCC isolate.

Isolate	Source	Location
248	Soil	Wisconsin
592	Human	Wisconsin
48089*	Human	Africa
ER-3	Woodpile	Wisconsin
48938*	Bat	India
T-58	Dog	Tennessee

were harvested and washed 5 times by centrifugation (5 min at 700 x g) with sterile distilled water then lysed by incubation with shaking in sterile water for 7 days at 37°C to achieve maximal lysis of the yeast cells and optimal reactivity with the ELISA. The suspension was centrifuged (30 min at 700 x g) to remove debris then filter sterilized through a 0.2µm Nalgene filter (Nalge Company, Rochester, NY). Merthiolate (1:10,000 dilution) was added as a preservative (6). Protein determinations were made on the antigen preparations using bicinchoninic acid (BCA) method according to manufacturer directions (Pierce Chemical Company, Rockford, IL) and the solutions were stored at 4°C.

Competitive ELISA Method

Microdilution plates (96 well Immunomaxi modified flat bottom high binding, TTP, Switzerland) were coated with 100µl/well of the respective lysates (100ng/ml) diluted in carbonate-bicarbonate coating buffer (pH 9.6) and incubated in a humid chamber for 24hr at 4°C. The plates were rinsed 3 times with phosphate buffered saline with 0.15% TWEEN 20 (NaCl 0.1369M, KH₂PO₄ 0.0015 M, Na₂HPO₄ 0.0108 M, KCl 0.0027 M) (PBS-T, pH 7.4). Sera (1:5000 PBS-T) from rabbits immunized against *B. dermatitidis* killed yeast whole cells were used as the primary antibody, which was added to microcentrifuge tubes containing urine specimens from dogs with blastomycosis at various stages of anti-fungal therapy and incubated for 30 min at 37°C. Positive controls containing the rabbit sera and PBS-T were also used to determine maximal detection. After the incubation period, the urine/sera mix along with the positive controls were added to the microdilution plates (100µl/well) in triplicate and incubated in a humid chamber for 30 minutes at 37°C. The plates were washed as above followed by the addition of goat anti-rabbit antibody horseradish peroxidase conjugate (Kirkegaard and Perry Laboratories, KPL, Gaithersburg, MD) (1:2000, PBS-T; 100µl per well) and incubated as above. The plates were washed as indicated and enzyme substrate (1-step Ultra TMB, Pierce Chemical Company, Rockford, IL) was added (100µl per well) and incubated for 3 min (optimal time using the Ultra TMB) at room temperature. The reaction was stopped by the addition of 100µl of 2N H₂SO₄ to each well. The absorbance was read at 450nm using the BIO-RAD model 2550 EIA reader.

Statistical Analysis

A two way analysis of variance (ANOVA) was used to analyze the data with respect to the *B. dermatitidis* lysate antigens as well as the individual dog urine specimens. Means were obtained from a general linear model based on log-transformed absorbance values which were then back-transformed. Means were compared using a Tukey HSD multiple comparison to determine the efficacy of the lysates as reagents in the competitive ELISA.

Results:

Urine specimens were grouped together by date of sample then averaged

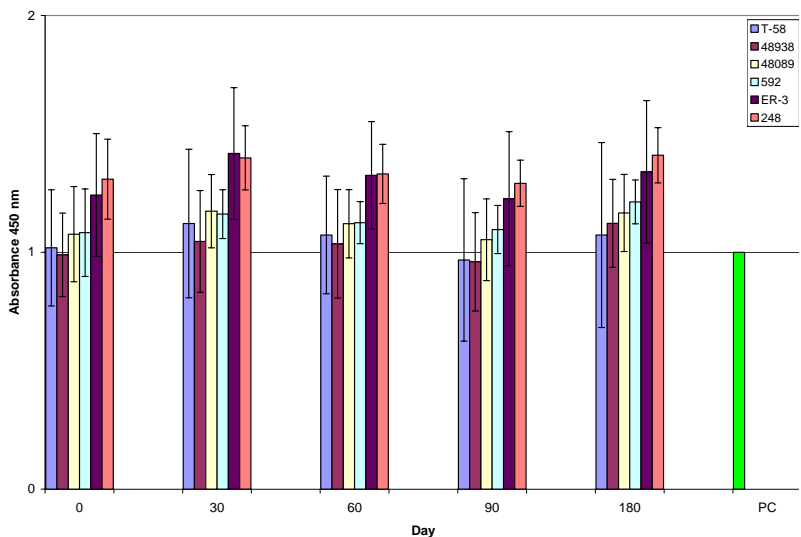


Figure 1. Standardized mean absorbance values for all dogs (N=170) averaged together at the respective date and lysate. Error bars indicate one standard deviation. The green bar indicates the positive control: PC used to standardize the data. No error bars are included with the positive control as this value was set to 1 and any samples with values greater than 1 indicate antigen detection.

together (Figure 1). The positive controls were used to standardize the data by taking the positive control absorbance value divided by the absorbance value of the specimens. Those values greater than 1 indicate antigen detection. The percent of antigen detection is shown in Table 2 and ranged from 46.47% for the India isolate to 92.86% for the 248 isolate.

Due to limited sample volumes and inconsistent sample collection dates, statistical analysis was only performed on the 90 day urine specimens (N=29) as this date encompassed the majority of the dogs in the study. A two way analysis of variance (ANOVA) was used to analyze the data with respect to the lysate antigens as well as the 29 individual dogs (Table 3). Means were obtained from a general linear model based on log-transformed absorbance values which were then back-transformed. Means were compared using a Tukey HSD multiple comparison (Figure 2). Isolates 248 and ER-3 had statistically significant differences from the T-58, Africa, and India isolates.

Table 2. The percentage of antigen detection observed for each of the isolates, N=170 urine specimens. Note sample 248 had an N=168.

Isolate	% detection
248	92.86
592	78.24
48089	70.59
ER-3	85.29
48938	46.47
T-58	61.18

Table 3. Analysis of variance for log transformed standardized mean, using Adjusted SS for Tests.

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Lysate	5	0.442154	0.442154	0.088431	10.40	<0.001
Dog	29	1.016860	1.016860	0.035064	4.12	<0.001
Error	145	1.232963	1.232963	0.008503		
Total	179	2.691977				

There was a significant difference in the absorbance values of the lysates ($F_{5, 145} = 10.40, P < 0.001$). There were significant differences among the urine specimens of the dogs that were tested ($F_{29, 145} = 4.12, P < 0.001$).

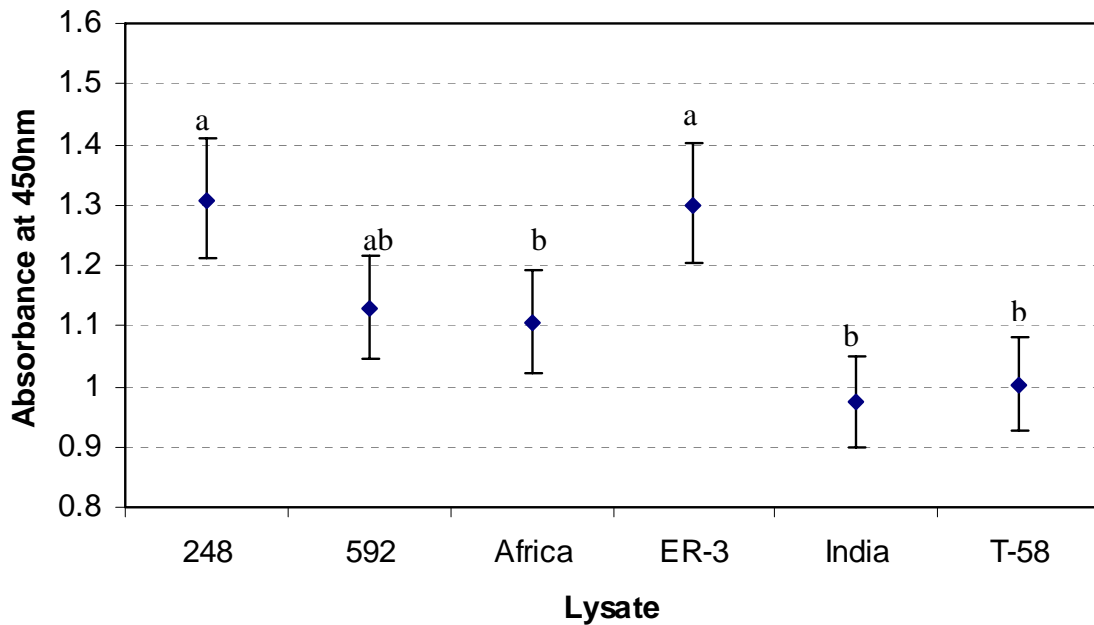


Figure 2. Mean absorbance at 450 nm (\pm 95% CI) for 6 lysates. Means were obtained from a general linear model based on log-transformed absorbance values which were then back

transformed. Means with different letters were significantly different using a Tukey HSD multiple comparison ($P < 0.05$).

Discussion:

Each lysate showed the ability to detect blastomycosis antigen in the dog urine specimens. The urine specimens were collected from endemic areas within the United States, with the majority of the specimens 155/170 being Southern isolates (Tennessee, Louisiana, and Mississippi) and 15 were from Minnesota. Differences were observed between the lysates, which may be due to strain variation (4, 7). However, it is interesting to note that the greatest amount of detection was observed with two Northern isolates (248: 92.86 and ER-3: 85.29% detection) whereas the Southern isolate, T-58, only showed 61.18% detection. Levels of detection may vary due to the stage of infection in the dogs as well as the initial amount of inoculum. Further work is in progress to examine the differences between the lysates to determine if a more sensitive and specific reagent can be produced for the detection of blastomycosis.

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