

Comparison of colorimetric and chemiluminescent ELISAs for the detection of antibodies to *Blastomyces dermatitidis*

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Abstract

The infectious disease, blastomycosis, caused by *Blastomyces dermatitidis* can be present as an acute pulmonary infection that resolves on its own to full blown dissemination if not treated properly. Many immunodiagnostic assays have been developed to aid in the diagnosis of blastomycosis. Here, the enzyme-linked immunosorbent assay (ELISA) is discussed and a variation of the indirect procedure is evaluated that uses a chemiluminescent substrate in its ability to detect antibodies to *B. dermatitidis*. In conclusion, the chemiluminescent ELISA provides slight improvements in detection of antibodies to *B. dermatitidis* ($p=0.001$).

Introduction

Blastomycosis, caused by the potentially deadly pathogen *Blastomyces dermatitidis*, is a systemic fungal disease of humans and animals. The distribution in the United States is generally east of the Mississippi River in regions from Wisconsin to the Gulf of Mexico and in the eastern Canadian provinces including Ontario and Manitoba (3,5,9). It is also endemic in regions of Africa and India, and there have been sporadic cases of blastomycosis reported from the Middle East and Europe (5). *B. dermatitidis* is a thermally dimorphic fungus that exists in a mycelial mold-like form in nature at approximately 25°C, but when incubated at 37°C, it survives in a yeast form. As a mold it produces small ovoid conidia which can be inhaled into the alveoli of the lungs where they can replicate as broad based budding yeast cells as a result of the higher temperature (5). A primary infection in many cases, especially in healthy individuals, is asymptomatic; however, an acute pulmonary infection or dissemination may occur. The symptoms of an acute infection include: cough, chills, headaches, and malaise with some possibility of chest pain. Recovery can occur within 3 to 4 weeks. The more serious disseminated form of infection with *B. dermatitidis* is characterized by a productive cough and flu-like symptoms occurring initially, but if the infection is not treated, dissemination may occur by haematogenous or lymphatic routes, and almost any organ in the body can be infected. The common pathological symptoms include cutaneous lesions, osteomyelitis and arthritis due to lesions on the bones, and in the worst cases meningitis (4,5,15). Histopathology, culture techniques, radiography, and various immunodiagnostic assays such as complement fixation, immunodiffusion, radioimmunoassay, and more recently the enzyme-linked immunosorbent assay (ELISA) have been used in the detection and diagnosis of blastomycosis (8,14,17,18). Specifically, the colorimetric indirect ELISA technique has been a standard method used for the detection of antibodies produced as an immune response to infection with *B. dermatitidis* (2,7,16).

Recent studies have compared a colorimetric ELISA to a chemiluminescent ELISA in detecting various antibodies to many antigens, but none have been published that compare the two ELISA methods in dealing with *B. dermatitidis*. Some studies have shown the chemiluminescent procedure to be more rapid and convenient, yet some have also shown that the colorimetric and chemiluminescent ELISAs are comparable in sensitivity (13,19). The present study was designed to determine if the chemiluminescent ELISA is more sensitive in detecting antibodies against *B. dermatitidis*. Specifically, does the chemiluminescent ELISA obtain a lower minimum detection limit than the colorimetric ELISA in detecting antibodies against *B. dermatitidis*? If the minimum detection limit is statistically lower for the chemiluminescent ELISA, then it may be considered to replace the current colorimetric ELISA for detection of antibodies to *B. dermatitidis*.

Materials and Methods

Antigens: Three strains (T-58: dog, TN; B5931: human, MN; SOIL: Ontario, Canada (3)) of *B. dermatitidis* in the mycelial form, available as stock cultures in our laboratory, were converted to yeast cells by culturing on brain heart infusion agar containing cysteine at 37°C. Lysates from the yeast phase were prepared using a previously documented procedure for *Histoplasma capsulatum* (10,11) and modified by Johnson and Scalapone (7) for the preparation of *B. dermatitidis* yeast phase lysate reagents. Cell lysis was promoted by incubating the cells in sterile water with shaking for seven days at 37°C. The suspension was centrifuged (30 min at 700 x g) to remove any debris, and a 0.2 µm Nalgene filter (Nalge Company, Rochester, NY) was used to filter sterilize the yeast lysate. Merthiolate (1:10000) was then added to the yeast lysate to act as a preservative. The protein concentration of each

lysate preparation was determined using the BCA assay kit (Pierce Biotechnology, Inc. Rockford, IL). The yeast lysate preparations were stored at 4°C for further use.

Serum Specimens: Serum samples, from rabbits that had previously been immunized with *B. dermatitidis* were used for this study. Serum samples stored at -20°C were taken prior to immunization and at 7 days following a booster immunization to be used as controls and test samples, respectively. Each test serum sample and negative control serum were diluted with 0.15% Tween 20 in phosphate buffered saline (PBS-T, pH 7.4 without adjustment) two-fold beginning with a 1:5000 dilution and up to 1:320000.

ELISA Procedure: Yeast phase lysate antigens were diluted to obtain a protein concentration of 1000 ng/mL using a carbonate-bicarbonate coating buffer (pH 9.6 without adjustment). The diluted antigens (100 µL) were added to each of the wells of a 96 well microtiter plate. Clear microtiter plates (Corning Incorporated, Corning, NY) were used for the colorimetric ELISA whereas opaque white microtiter plates (Pierce Biotechnology, Inc. Rockford, IL) were used for the chemiluminescent assay. The plates were placed in humid containers and incubated (20-24 hrs at 4°C). Following incubation, the microtiter plates were washed three times using PBS-T. The diluted serum samples, as above, were added in triplicate to the wells of both clear and opaque plates. Negative control sera were added in triplicate in the same manner as the test samples. Each test sample and respective dilution had a negative control sample with the same dilution. After addition of serum samples, the plates were placed in humid containers and incubated at 37°C for 30 min after which the plates were washed three times with PBS-T. A commercially available antibody conjugate (peroxidase labeled goat anti-rabbit IgG (H+L) liquid conjugate (Kirkegaard & Perry Laboratories, Inc. Gaithersburg, MD)), was diluted 1:2000 and added (100 µL) to each well of the plates. The plates were again placed in humid containers and incubated at 37°C for 30 min before being washed three times with PBS-T. Enzyme substrate (1-step Ultra TMB, Pierce Biotechnology, Inc. Rockford, IL) (100 µL) was added to each well of the clear microtiter plates and allowed to incubate at room temperature for approximately 2 to 3 minutes. The reaction was stopped by addition of 100 µL of 2N H₂SO₄ to each well. For the chemiluminescent assay, 100 µL of a SuperSignal R ELISA Pico Chemiluminescent Substrate working solution (Pierce Biotechnology, Inc. Rockford, IL) was added to each well. These plates were mixed gently for 1 minute before measuring luminescence. All plates were analyzed using a BioTek Synergy H-T Microplate Reader (Bio Tek Instruments Inc., Winooski, VT) controlled by KD4 programming. The plates used for the colorimetric assay were analyzed by measuring absorbance at 450 nm, whereas the plates used for the chemiluminescent assay were analyzed by measuring luminescence with the Lum/E setting and the sensitivity setting at 150. A total of 18 serum samples were tested against the 3 different antigens (total of 54 results), and each of these was repeated giving 108 comparable results for both ELISA methods.

Data Analysis: A negative cutoff value was calculated for each serum dilution tested. These negative cutoff values were calculated by multiplying the standard deviation for each negative serum sample by three and adding this number to the mean result of each negative sample. Test samples at each dilution were considered positive if the individual well's reading was greater than the negative cutoff value for the serum at the same dilution. Minimum detection limits were determined to be the highest dilution at which all three replicate test samples were considered positive. The minimum detection limits for the colorimetric assay and the chemiluminescent assay were compared statistically using the Wilcoxon test (SPSS 15.0, Chicago, USA). The results of the Wilcoxon test were used to compare the performance of the chemiluminescent assay to the colorimetric assay in detecting antibodies against *B. dermatitidis*. This method provided a way to compare the results of each test directly since it was a number with no values attached. It also provided a way of detecting the end point at which the assay detected only antibodies to *B. dermatitidis*.

Reliability of the data was analyzed by repeating each colorimetric and chemiluminescent ELISA test. A Pearson correlation coefficient was calculated (Minitab, State College, PA, USA) for the colorimetric ELISA and the chemiluminescent ELISA results to determine if one method is more reliable than the other.

Results

In comparing all results for each ELISA method, the minimum detection limit for the chemiluminescent ELISA was significantly higher than that of the colorimetric ELISA ($p = 0.001$). This was consistent for Ag T-58 ($p = 0.017$) and Ag B5931 ($p = 0.047$), but not for Ag SOIL ($p = 0.138$). A direct comparison of the difference between the amounts of dilutions to reach the minimum detection limit for each assay is shown in Figures 1-3. The average difference of the minimum detection limit between the two assays was less than one two-fold dilution.

The analysis using Pearson's correlation coefficient indicated a statistically significant linear relationship between the two replicate colorimetric ELISA tests ($r = 0.686$, $p = 0.000$, $n = 54$) and also for the replicate chemiluminescent tests ($r = 0.613$, $p = 0.000$, $n = 54$).

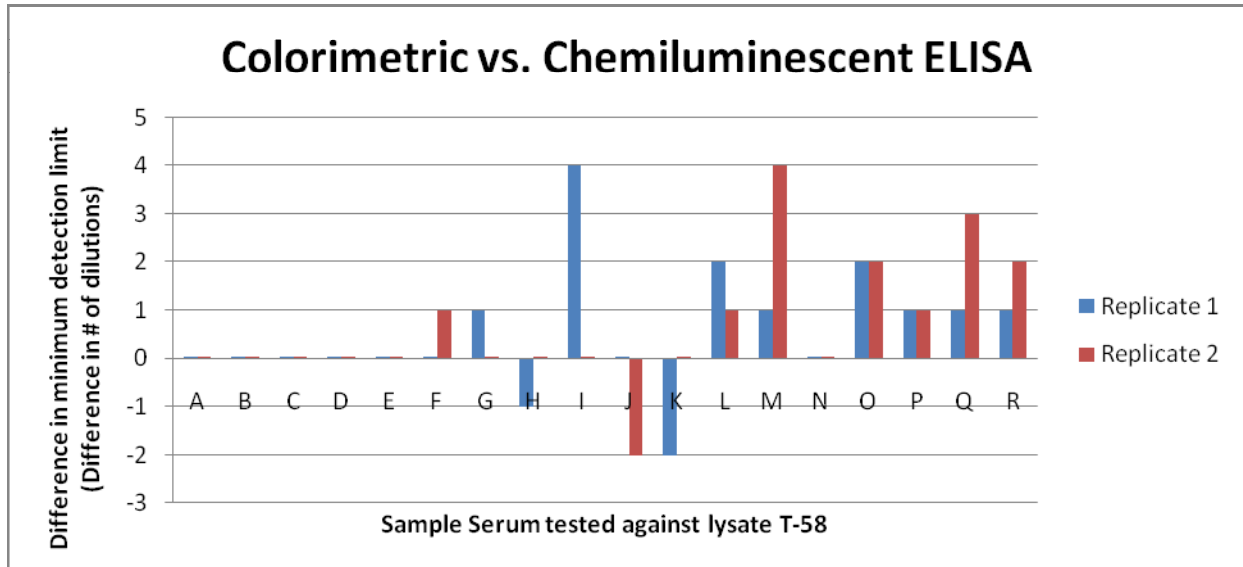


Figure 1. Comparison of the minimum detection limits for colorimetric and chemiluminescent ELISAs using sera A-R against yeast phase lysate T-58. Positive values are the result of the chemiluminescent ELISA having a lower minimum detection limit, whereas negative values are shown when the colorimetric ELISA had a lower minimum detection limit. The absolute value is the number of dilutions between the minimum detection limits of the two assays for the same serum sample. A zero value indicates that the minimum detection limit was the same for the two assays for that sample.

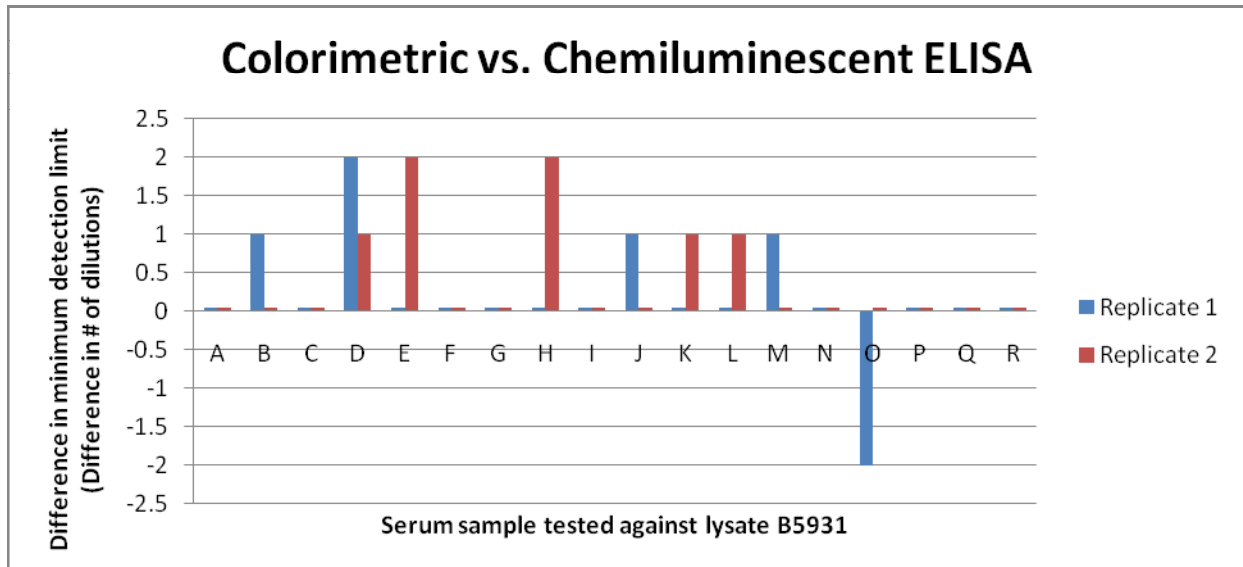


Figure 2. Comparison of the minimum detection limits for colorimetric and chemiluminescent ELISAs using sera A-R against yeast phase lysate B5931. Positive values are the result of the chemiluminescent ELISA having a lower minimum detection limit, whereas negative values are shown when the colorimetric ELISA had a lower minimum detection limit. The absolute value is the number of dilutions between the minimum detection limits of the two assays for the same serum sample. A zero value indicates that the minimum detection limit was the same for the two assays for that sample.

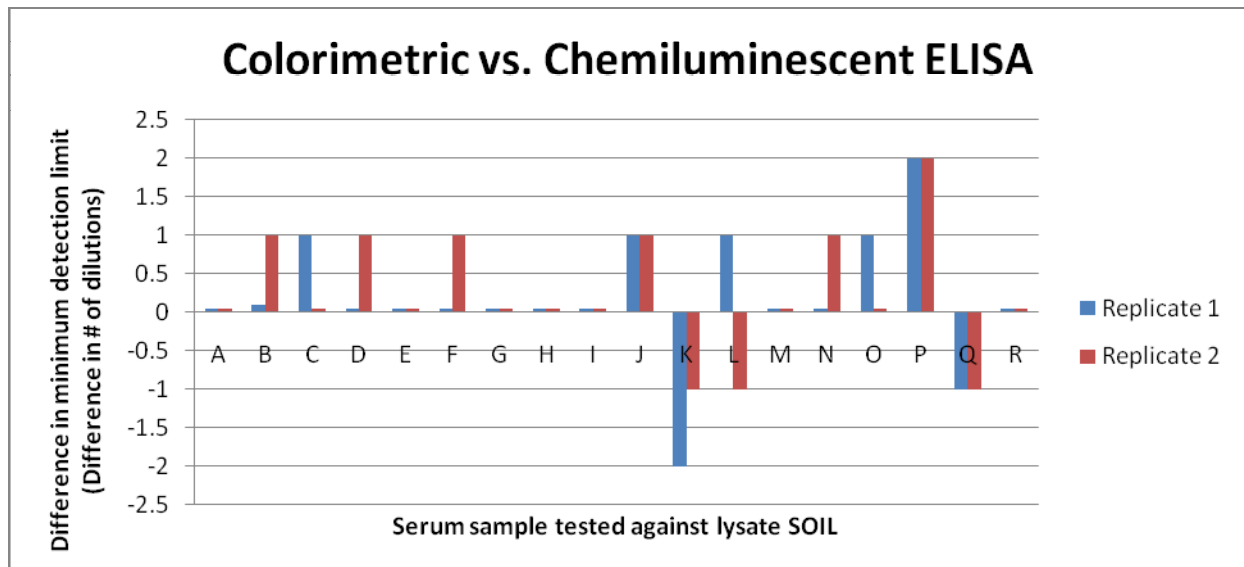


Figure 3. Comparison of the minimum detection limits for colorimetric and chemiluminescent ELISAs using sera A-R against yeast phase lysate SOIL. Positive values are the result of the chemiluminescent ELISA having a lower minimum detection limit, whereas negative values are shown when the colorimetric ELISA had a lower minimum detection limit. The absolute value is the number of dilutions between the minimum detection limits of the two assays for the same serum sample. A zero value indicates that the minimum detection limit was the same for the two assays for that sample.

Discussion

The results of this study suggest that there is a significant difference between the chemiluminescent ELISA and the colorimetric ELISA in their minimum detection limit in detecting antibodies to *B. dermatitidis* in an infected host. For the most part though as Figures 1-3 describe, the chemiluminescent ELISA only duplicated what the colorimetric ELISA detected, but the chemiluminescent ELISA may at times give a better result when detecting low amounts of antibody to *B. dermatitidis*. These results are comparable with other research done in comparing the two ELISA methods, as others have seen higher end-titers and greater sensitivity with the use of the chemiluminescent ELISA over the colorimetric ELISA (12,13,19). This work now provides support that the chemiluminescent ELISA can also provide greater sensitivity in detecting antibodies against *B. dermatitidis*.

It is interesting to note that for Ag T-58 and B5931 there is a significant difference, but this was not true for Ag SOIL. This may be due to antigenic variation between strains of the organism used in this study. Each lysate was made from a *B. dermatitidis* isolate obtained from various places, in particular the SOIL lysate was from an environmental sample while the other two lysates were obtained from infected individuals.

The results of replicated tests indicate some variability in the standard ELISA procedure itself, but results of the Pearson correlation show that there was a significant relationship between the replicate tests of each ELISA method individually. The Pearson correlation coefficient was between 0.60 and 0.70 for both the chemiluminescent and the colorimetric ELISA methods which is much lower than a perfect correlation for duplicate tests, but it was still significant ($p < 0.001$ for both). This variability may be due in part to our use of crude *B. dermatitidis* lysates. The lysate was obtained from a whole yeast cell which likely has many proteins acting as antigens. We do not know specifically what antigenic components of the lysates our immunized rabbits produced antibodies against; therefore, future work may benefit to focus on more purified antigens. The rabbit sera may also contain general antibodies, including polyclonal antibodies, not directed against a particular *B. dermatitidis* antigen that may react with the lysate. The evidence of variability was seen in the results of the negative controls. The negative controls should have had consistently low numerical results since there were not any antibodies to *B. dermatitidis* present in the sera, but a range from 0.06 to 1.70 in absorbance values for the colorimetric ELISA and a range of 10 to 8000 in relative light units (RLUs) for the chemiluminescent ELISA were recorded. This is a great variation in negative results giving evidence that reliability was an issue in this test and should not be overlooked in future research. Previous studies have shown consistent results using the ELISA to detect antibodies with whole cell lysates. For example, Goodell et al recently achieved reproducible results with correlation of variation values of less than 15% in antibody detection

(6). On the other hand, variation among serum samples used and lysate antigens has been seen consistently among previous ELISA results for detection of antibodies to *B. dermatitidis* (1,16).

Further testing in comparing these assays would benefit from the use of purified antigens of *B. dermatitidis* which should limit variability and give greater consistency in results. Use of sera from dogs and/or humans that have been infected with *B. dermatitidis* should be tested in addition to rabbit sera. Specificity tests would help to show how well the two ELISA methods compare when detecting positive and negative infections with *B. dermatitidis*. Suggesting that the chemiluminescent ELISA can detect antibodies produced against *B. dermatitidis* in smaller amounts than the colorimetric ELISA could have an impact on earlier diagnosing of blastomycosis in the clinical setting, but is it worth a higher cost for supplies and no real saving of time or effort to get similar results with the chemiluminescent ELISA that may only detect slightly smaller concentrations of antibody than the colorimetric ELISA.

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