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Isolation and Characterization of Immunoreactive Components of *Blastomyces dermatitidis* Yeast Lysate Antigens

By

Joshua Wright

A thesis

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Committee Approval

To the Graduate Faculty:

The members of the committee appointed to examine the thesis of JOSHUA WRIGHT find it satisfactory and recommend that it be accepted.

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Abstract

Over the past several years our lab has been working on novel ways to increase the sensitivity and specificity of tests for *Blastomyces dematitidis*. Our work has been done to help improve the diagnostics of current and future tests. Improving upon the current testing abilities will help to better diagnose the disease. This will be helpful because Blastomycosis, the disease caused by *B. dermatitidis,* mimics other diseases such as pneumonia and tuberculosis. The current endemic region is also under question. Improving these tests will also help to determine the true location and area that is susceptible to this fungal organism.

Introduction- Background information on the disease

Blastomycosis is a dimorphic fungal disease caused by the agent *Blastomyces dermatitidis*. This fungal organism is found in North America, specifically the eastern and mid-western parts of the country, South and Central America, Europe, Africa and parts of the Middle East [2,3]. The true endemic region is not fully known because of the lack of a specific and sensitive Assay. It is found in areas with acidic, moist and nitrogen-rich soils and most cases are found near bodies of fresh water such as rivers and lakes. The specific ecological factors that determine the absence or presence of the organism is not well understood due to the low recovery rates of the agent from soil samples. Overall the organism has only been recovered from a natural setting fewer than two dozen times[5]. Transmission of *B. dermatitidis* occurs when the agent is aerosolized when the soil that it is found in is disturbed and the mycelial cells are inhaled into the lungs of mammals. Once in the lungs it is then converted to the yeast form (at 37°C)[1, 2, 3]. The disease can spontaneously resolve or it can become progressive and disseminate to other organs of the body[1], including the meninges also cutaneous lesions may develop[2]. In endemic areas, such as Wisconsin, the prevalence rates can range from 10.4 to 41.9 per 100,000[5]. Specifically in dogs in highly endemic areas the rates of infection can be as high as 1,420 in 100,000[5].

Blastomycosis has not been found to have a distinct season of prevalence when rates of infection fluctuate, yet because of the varying incubation time it is difficult to determine the exact time of exposure and conversion into the pathogenic yeast form and onset of illness[2]. There is no apparent pattern of susceptibility based on age, sex or race, but those

at higher risk for the disease include persons working in the forestry or logging industry, where the soil is disturbed and the spores can become aerosolized, and those who spend large amounts of time outdoors in endemic areas[2].

Blastomycosis can be resolved and eliminated by the host's immune system in normal healthy individuals however it is specifically a concern in patients that are immunocompromised. Those with compromised immune systems have much higher mortality rates (68%) if the disease is misdiagnosed or left untreated[14]. Additionally, persons who are immunocompromised due to underlying medical conditions and live in endemic areas are also at higher risk for the disease[4]. In the medical community blastomycosis is known as the great mimicker of diseases and often presents with symptoms ranging from flu-like symptoms (headache, fever, chills, and productive cough) to bone and joint involvment[4]. It is often misdiagnosed as tuberculosis and the physician may prescribe antibiotics which will not affect the fungus and it will often disseminate non-specifically to other organs[6, 7]. It can also infect the central nervous system of the host as well as spread to bones and joints[2, 6]. If untreated this disease can potentially kill the host or compromise the individual significantly[4]. Blastomycosis mimics other diseases and as a result is difficult to diagnose. This leads to a need to develop diagnostic assays for the disease to detect and treat it early, however currently there is no quick and accurate way to diagnose the disease.

Diagnosis and treatment

Currently, the gold standard of diagnosis for blastomycosis is to add potassium hydroxide to a tissue or sputum sample and observe the broad-based budding yeast cells buds[4, 7]. This preliminary diagnosis is followed by culturing the cells in-vitro which can take 5-30 days to develop the mycelial cells and convert them into yeast cells[1]. Then the sample can either be identified using microscopic techniques, serology or DNA probes (Gen-Probe). The current serology methods use enzyme linked immunosorbent assays (ELISA) to detect antigens or antibodies made against the organism in the serum or urine. A complement fixation test can also be performed to identify any antibody's association with the *B. dermatitidis* antigen. Additionally a chest x-ray may also be done to observe the disease progression and dissemination in the host. In more developed cases brain MRIs and x-rays of bones may be taken to diagnose and evaluate the amount of dissemination of the fungal agent [1, 7, 14]. The ELISA has a sensitivity of approximately 90% and is currently the fastest and most successful test used to determine the presence of antigens or antibodies in the clinical lab[10]. In immunocompromised individuals, who are at the highest risk for fungal diseases, it is difficult to obtain a positive result, the presence of antibodies even with an active infection[10]. This is due to the antibody response not being as strong in these individuals and often their test results are negative 50% of the time[10]. As a result researchers are still working towards a skin test that may provide better specificity and sensitivity to give a more accurate diagnosis.

Many fungal diseases such as coccidioidomycosis and histoplasmosis have skin tests for clinical diagnosis by detect cell-mediated immunity against the fungus and serologic testing for IgM and IgG antibodies in serum or cerebral spinal fluid[8, 14]. This improves

the diagnosis process by making diagnosis faster and effective, however such a test for blastomycosis has not been reliably produced. In the past a skin test for *B. dermatitidis* antibodies was used to detect the presence of the A-antigen produced by *B. dermatitidis*, however because its accuracy was so low, 21%, the Food and Drug Administration took the test off the market in 1972[9]. *B. dermatitidis* false positive test results were the product of cross reactivity between *Histoplasma capsulatum*, the agent that causes histoplasmosis. Cross-reactivity is an issue in diagnosis because the diseases are endemic in the same areas and often have similar symptoms but, the treatment protocols are significantly different[10].

Research in DNA probe development and work to extract the DNA from clinical samples is still in exploration.

Currently, there are a few main groups of anti-fungal drugs used to treat patients with systemic fungal diseases, specifically blastomycosis. These drugs include ketoconazole, itraconazole, fluconazole, posaconazole, voriconazole and in severely immunosuppressed patients Amphotericin B may be prescribed as a more aggressive treatment option[11]. Many of these drugs may have mild to severe side effects ranging from flu-like symptoms, gastrointestinal upset, nausea, depression and anaphylaxsis to nephrotoxicity and liver failure [1, 2, 4, 11]. The side effects for antifungals are greater than in antibiotics because fungal cells are more similar to host cells because fungi are also eukaryotic so the anti fungals bind to similar cells[1, 12]. Antifungals interfere with the synthesis of ergosterol, a component of fungal cells, and can in turn bind cholesterol in host cells reducing its biosynthesis[12]. Specifically, the azole antifungals inhibit fungal cytochrome P450 (CYP) dependent enzyme lanosterol 14-α-demethylamylase, blocking ergosterol

formation[13]. Additionally, antifungals may cause drug to drug interactions between antibiotics, cholesterol, oral hypoglycemic, benzodiazepines, cardiac, and immunosuppressant medications[13]. These interactions must be anticipated by the healthcare providers and dosages should be adjusted accordingly to ensure the best treatment options with the fewest side effects.

Chapter 1

Introduction

This study evaluated the lysates of several strains of *B. dermatitidis*. The lysates were analyzed using a Rotofor, BCA, SDS PAGE, and ELISA. These lysates were assayed at to determine the presence of any reactive components that may be used in the future to design a specific and sensitive test for *B. dermatitidis*. In this study we analyzed 4 different strains of *B. dermatitidis*. The strains were chosen because of their wide range of infection. The strains used include two dog isolates T-58, ERC-2, and two human isolates B5931 and B5896.

The strains were first concentrated to a useful concentration to be used in further applications. The concentration allowed us to have enough useful amounts of the protein once fractionated to run on gels and perform concentration studies i.e. BCA.

Once they were all equilibrated via concentration they were run on the Rotofor® to fractionate the lysate into 20 separate fractions. The Rotofor® produces fractions that range from acidic to basic. This process is based on the isoelectricpoint (pI) of the proteins.

Once the proteins were fractionated the pH was then brought to a neutral level to be useful in further pH sensitive tests. The pH was adjusted using either HCl of NaCl to bring the fractions to a pH of 7 ± 0.2.

Once equilibrated a BCA protein assay was run to determine the concentration of the fractions. This was useful when doing initial strain comparisons and to determine the appropriate staining to use when performing gels.

The fractions were then run on SDS PAGE to determine the molecular weight of proteins that were involved in some of the fractions. These molecular weights were then analyzed to determine what fractions contain which reactive components.

Materials and methods

Rotofor®.

The ability of each yeast lysate reagent to detect antibodies in the serum specimens was determined using an indirect ELISA. Serum from infected dogs was used in this study. The early fractions from the B5931 lysate showed good reactivity with blastomycosis dog serum. This was in contrast to the poor reactivity with the histoplasmosis serum with the same fractions. However the *H. capsulatum* infected dogs show a higher reactivity to the later fractions than the dogs infected with *B. dermatitidis* [fig 1,2].

Fraction gels. (Silver stain)

To analyze these results the fractions were individually run on a 10% SDS page and (because of low protein concentration) a silver stain was performed to analyze the reactive proteins. In examining the first reactive set of proteins (fractions 1-6) we identified two proteins of interest. The first protein, a 120KD protein that has been identified as BAD1 was identified. BAD1 has been well characterized by Klein. and has

been used in an attempt to develop more sensitive and specific tests for *B. dermatitidis*. The second protein band that we are interested in is a 55KD protein that is present in some of the most reactive fractions [fig 3].

Preliminary data – Rotofor, SDS page/ Silver stain

Recent studies in our lab have produced results using the Rotofor® to fractionate proteins into their pI's (20 fractions in total). Using these fractionated proteins we have discovered that early fractions of strain B5896 show a greater reactivity with *B. dermatitidis* sera and low reactivity with *H. capsulatum* sera. Evaluating these fractions we were able to identify a 55kd protein that seems to be unique to this strain. A 120kd protein has previously been identified as BAD1. To determine if this could possibly be a portion of this protein we ran day one vs day seven lysate study on the several strains to determine the stability of BAD1. Our results showed that the 55kd protein was present in the day one and the day seven reagents.

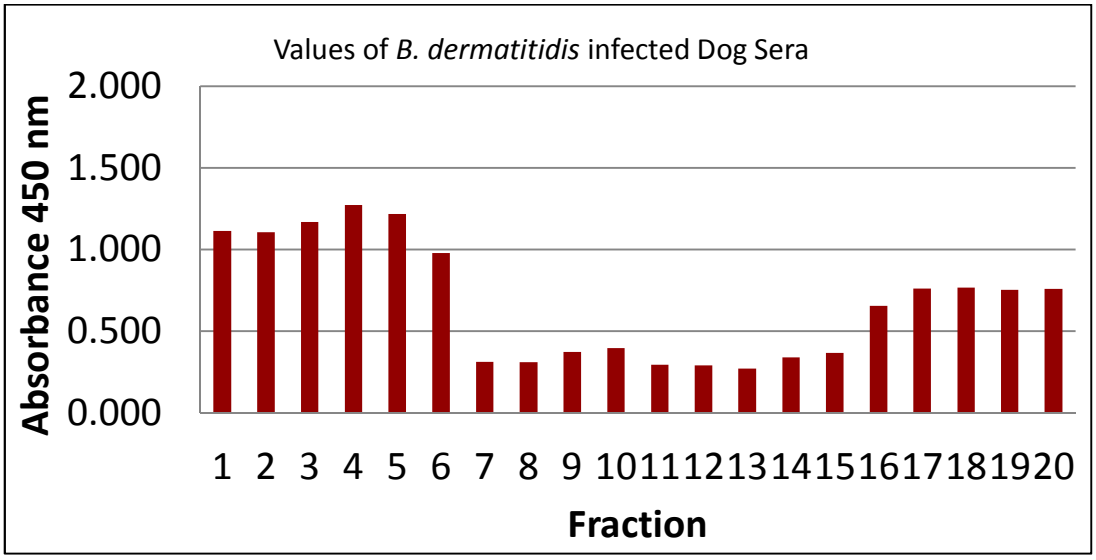

Figure 1.1. The mean absorbance values from the indirect ELISA using serum from *B. dermatitidis* infected dogs with the isoelectric fractions of the B5896 isolate (1-20). The early fractions show a greater reactivity with the dog serum than the *H. capsulatum* dog serum.

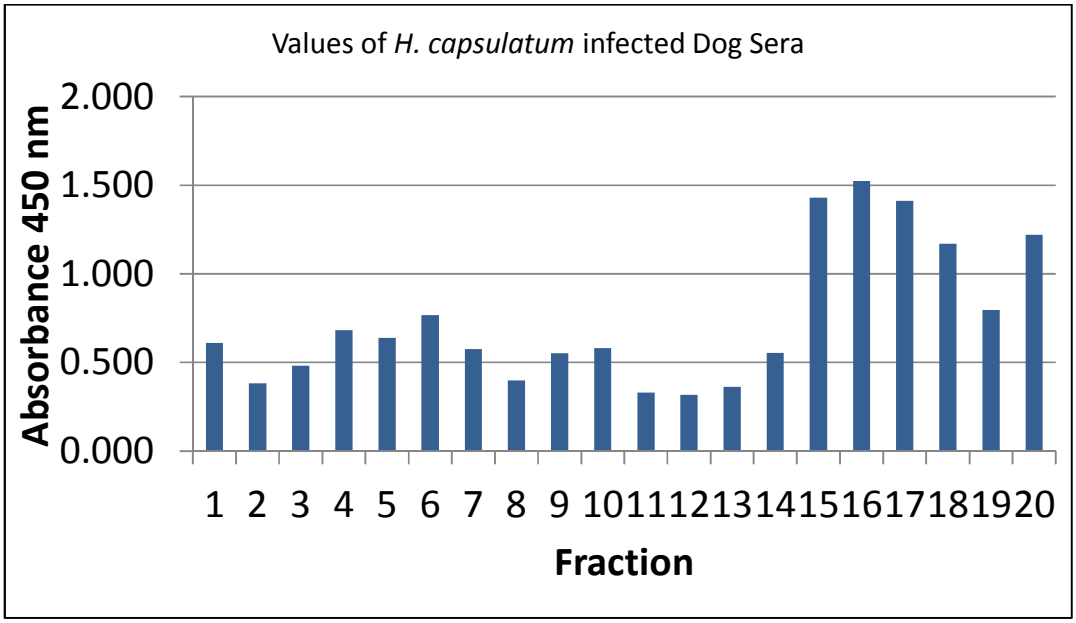

Figure 1.2. The mean absorbance values from the indirect ELISA using serum from *H. capsulatum* infected dogs with the B5986 isolate isoelectric fractions (1-20). The latter fractions here show a higher increase in reactivity.

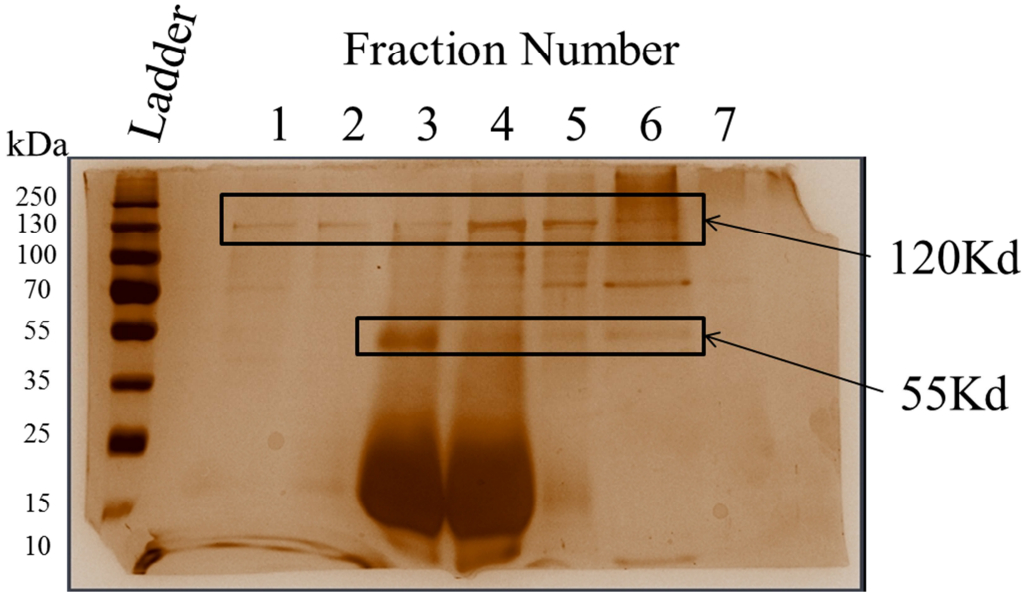

Figure 1.3. A SDS PAGE with a silver stain of the fractions of the B5896 isolate. The presence of the 120 kda (BAD 1) is in decent concentration across the fractions 1-6. The presence of a second protein of interest is present at the 55Kda size. These fractions were determined to be reactive with the *B. dermatitidis* dog serum and had low reactivity with the *H. capsulatum* dog serum. The large stained areas on the bottom of fractions 3 and four are presumed to be contamination.

Discussion

The use of antigens to create a sensitive and specific test for *B. dermatitidis* has shown promise in our lab and others. The initial analysis of the protein fractions from B5896 showed a reduction in cross reactivity and the presence of several proteins that we examined in greater detail to determine reactivity. If the reactivity and specificity shows promise we can then create protocols for the further purification and isolation of the proteins of interest. Tests now need to be performed on the other strains to determine the presence of any other reactive antigens. The presence of reactive antigens may allow us to use them to produce a highly sensitive and specific antibody to detect the presence of *B. dermatitidis* antigen in a patient. A skin test has been shown to be useful in other mycoses and would be useful if a sensitive and specific antigen could be produced for the detection of blastomycosis.

The preceding research was published

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Sensitivity and Specificity Determinations with Isoelectric Focusing Fractions of *Blastomyces dermatitidis* for Antibody Detection in Serum Specimens from Infected Dogs

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ABSTRACT

Blastomycosis and histoplasmosis manifest as lung and systemic fungal infections in mammals caused by *Histoplasma capsulatum*, and *Blastomyces dermatitidis.* These infections exhibit cross reactivity of antibodies which makes a correct diagnosis potentially elusive. The purpose of this study was to gain an understanding of which isoelectric focusing fractions (Rotofor™) of *B. dermatitidis* were reactive or cross reactive with serum specimens from dogs infected with *B. dermatitidis H. capsulatum*, and *Cryptococcus neoformans*. Three serum specimens from dogs that were infected with *B. dermatitidis*, two dogs infected with *H. capsulatum*, and one dog infected with *C. neoformans* were assayed against the 20 *B. dermatitidis* Rotofor™ fractions. Reactivity was determined using the indirect enzyme linked immunoassay (ELISA). Reactivity with B. dermatitidis was found predominantly in the protein fractions 1 - 6, and cross reactivity with *H. capsulatum*, and *C. neoformans* sera was found within the *B. dermatitidis* protein fractions 15 - 19.

1. Introduction

Blastomycosis is a systemic fungal infection of humans and animals that is initiated by the inhalation of conidia (spores produced by the filamentous phase of the fungus). The organism exists in this stage in nature or in the laboratory at 25°C and has the ability to convert to the yeast phase at 37°C in the lungs of the infected host. The disease may be self-resolving or it may exist as an acute or chronic state in the pulmonary tissue, where it may be misdiagnosed as tuberculosis. If the disease is not diagnosed or left untreated while in the lungs it may become invasive, and disseminate to other organs, and possibly to the central nervous system where fatal meningitis may develop [1-5]. Blastomycosis, as well as other systemic mycoses, are termed "emerging fungal threats" since they can not only infect persons with normal immune systems, but also they are a cause for concern in individuals with AIDS or other deficiency diseases that compromise the immune system [3,4,6].

Traditionally the geographic distribution of blastomycosis has been associated with southeastern and southcentral states that border the Ohio and Mississippi Rivers and upper Midwestern states including areas in Wisconsin and Minnesota, which are highly endemic for the disease. Recent studies have indicated that blastomycosis may be present in other regions with sporadic cases being reported in Colorado, Texas, Kansas and Nebraska [1,2,5].

Due to the increase in systemic fungal diseases researchers have begun to devote more attention to developing ways of diagnosing, preventing and treating these mycoses. The greatest problem that we face at present is that a considerable amount of research and development activities are needed if we are going to have a positive impact on this situation. For the past several years the thrust of research in our laboratory has been associated with studies on various strains of *B. dermatitidis* from human, animal or environmental specimens from many geographical locations in an effort to better understand the immunobiology of the organism. Diagnosis of the disease has presented major problems. In some instances culturing or histopathological examination may be beneficial, but in some patients these methods may not yield the desired results. This has led to more and more work being done to improve immunological assays which tend to provide a more rapid diagnosis, but problems still exist with regard to the sensitivity and specificity of immunoassays [2,5,7-10]. Our laboratory has

developed novel yeast phase lysate antigens and utilized these in various immunoassays for both antibody and antigen detection in blastomycosis [11-17], but these studies have only opened up new avenues of approach with regard to how we might improve immunodiagnostic methods in the future.

In recent years other investigators have been approaching the problem of immunodiagnosis and have produced encouraging results with antigen detection assays as second-generation assays have been developed [7,8,10]. Sensitivity values obtained by these researchers have generally been very good with antigen detection assays, but they evidenced less sensitivity when antibody detection assays were performed. The greatest problem that has become apparent in the majority of the investigations is the lack of specificity of the immunoassays. Our laboratory has performed a few studies on isoelectric focusing of *B. dermatitidis* yeast lysate antigens in an attempt to separate the immunoreactive and cross-reactive components of the preparations. The objective of this present study was to obtain isoelectric focusing fractions of yeast lysates and to evaluate these fractions with regard to ELISA antibody detection (reactivity vs. cross-reactivity) in serum specimens from dogs with blastomycosis, histoplasmosis and cryptococcosis.

2. Materials & Methods

2.1. Antigens

Lysate Antigen Preparation

Mycelial phase cultures of *B. dermatitidis* isolate (B5896), obtained from the Mountain Iron, Minnesota human outbreak of blastomycosis in 1999 (2), were converted to yeast cells by culturing at 37 C on brain heart infusion agar. Yeast phase lysate reagents were prepared by a method similar to one that was previously used for the production of antigen from *Histoplasma capsulatum* [18-20] and modified in our laboratory for *B. dermatitidis* lysate antigen production [11]. The yeast phase cells were grown for 7 days at 37 C in a chemically defined medium in an incubator shaker, harvested by centrifugation (700 × g; 5 min), followed by washing with distilled water, re-suspended in distilled water and then allowed to lyse for 7 days at 37 C in water with shaking. The preparations were centrifuged, filter sterilized, merthiolate added (1:10,000) and stored at 4 C for further use. Protein determinations were performed on the

lysates using the BCA Protein Assay Kit (Pierce Chemical Company, Rockford, IL) and dilutions of the antigenic reagents used in isoelectric focusing and ELISA assays were based on protein concentration.

2.2. Serum Specimens

Serum specimens from dogs with blastomycosis, histoplasmosis, and cryptococcosis were previously provided by Dr. A. M. Legendre (University of Tennessee College of Veterinary Medicine, Knoxville, TN).

2.3. Isoelectric Focusing

Isoelectric focusing was performed using the BIO-RAD Rotofor apparatus (BIO-RAD, Hercules, CA). Ampholytes (BIO-RAD) were added to the yeast phase lysate in a 2% to 98% ratio. These small charged molecules create a pH gradient in solution from a pH of 3 to 10 when an electrical current is applied so that proteins can be separated based on their isoelectric point. All proteins have a unique net charge that will force the proteins to move through the pH gradient until their net charge becomes zero (the isoelectric point). When proteins reach their unique isoelectric point in this pH gradient they are no longer able to migrate and forced to remain where their net charge is zero by the established pH gradient. Twenty protein fractions were collected after focusing (15 watts constant current) for approximately four hours at 4 C to ensure that no denaturing of the proteins occurred. The focusing was stopped when the voltage stopped fluctuating [15,16]. After collection of the fractions the pH was measured and adjusted to return the proteins to their physiologically active pH. This was accomplished by the addition of HCl or NaOH to either lower or raise the pH as required. Protein determinations were performed on the fractions using the Pierce BCA Protein Assay, as above.

2.4. 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 Costar 96-well microplate (Thermo/Fisher). The plates were then incubated overnight at 4 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 C in a humid chamber. Following this incubation the wells were washed as above and 100 ul of goat anti-dog IgG (H & L) peroxidase conjugate (Kirkegaard and Perry, Gaithersburg, MD) was added to each well and incubated for 30 min at 37 C. The plates were again washed as above and 100 ul of peroxidase substrate (Thermo/FisherPierce) was added to each well and incubated for approximately 2 min at room temperature. The reaction was stopped by the addition of sulfuric acid and the absorbance read at 450 nm using a BIO-RAD 2550 EIA reader.

3. Results

3.1. Reactivity of B5896 Protein Fractions with *B. dermatitidis* Dog Sera

Figures P1-P3 show that the reactivity of serum specimens from *B. dermatitidis* infected dogs against the Rotofor™ protein fractions of *B. dermatitidis* dog sera was found predominantly in the 1 - 6 range of protein fractions.

3.2. Reactivity of B5896 Protein Fractions with *H. capsulatum* Dog Sera

Figures P4 and P5 show that the cross reactivity of serum specimens from *H. capsulat*um infected dogs against the Rotofor™ protein fractions of *B. dermatitidis* was found within the 15 - 20 range of protein fractions.

3.3. Reactivity of B5896 Protein Fractions with *C. neoformans* Dog Serum

Figure P6 shows that the cross reactivity of serum specimens from dogs infected with *C. neoformans* against the Rotofor™ protein fractions of *B. dermatitidis* was found in the 15 - 19 range of protein fractions.

4. Discussion

B. dermatitidis, *H. capsulatum*, and *C. neoformans* are endemic in the United States mainly in the Ohio and Mississippi River drainages. The antigen and serum specimens used in the study were from different geographic regions

within the endemic area. The antigen used, (B5896), was from Minnesota. The dog serum specimens for B. dermatitidis were from Wisconsin, Louisiana, and Alabama. The dog serum specimens for *H. capsulatum* were from Tennessee and the *C. neoformans* dog serum specimen was from Tennessee. We believe that some of the variance in the results that we recorded are related to the serum specimen's different geographic origins.

Infections involving *B. dermatitidis*, *H. capsulatum*, and *C. neoformans* in mammals exhibit cross reactivity of antibodies which makes a correct diagnosis potentially elusive. In order to elicit an accurate diagnosis, the specificity of the antigen used in a diagnostic test would need to be improved in order to either reduce or eliminate any cross reactivity of antibodies.

We observed the potential to greatly reduce the cross reactivity of *B. dermatitidis* antigen with *H. capsulatum*, and *C. neoformans* antibodies via isoelectric focusing of antigen proteins. Rotofor™ separation of the antigenic proteins provides an accurate means to identify and isolate the most reactive antigen protein fractions in relation to cross reactive species antibodies.

This data may prove useful regarding future studies involving additional evaluations on the detection of antibodies, or the development of a skin testing antigen(s), [15-20] that may possibly eliminate the cross reactivity of B. dermatitidis to other fungal pathogens such as *C. neoformans* and *H. capsulatum.*

Figures:

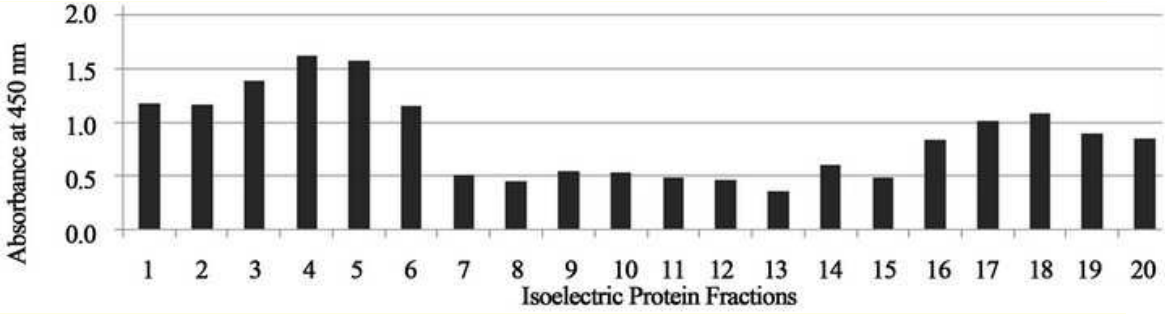

Figure P1. Absorbance values of protein fractions reacted with *B. dermatitidis* dog serum from Wisconsin.

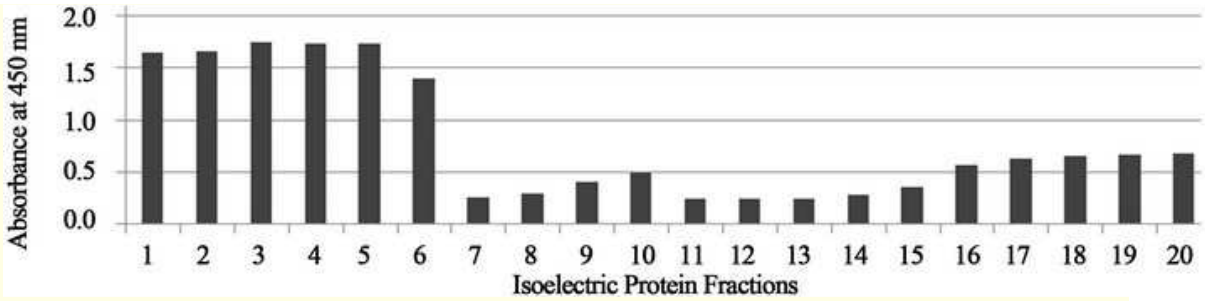

Figure P2. Absorbance values of protein fractions reacted with *B. dermatitidis* dog serum from Alabama.

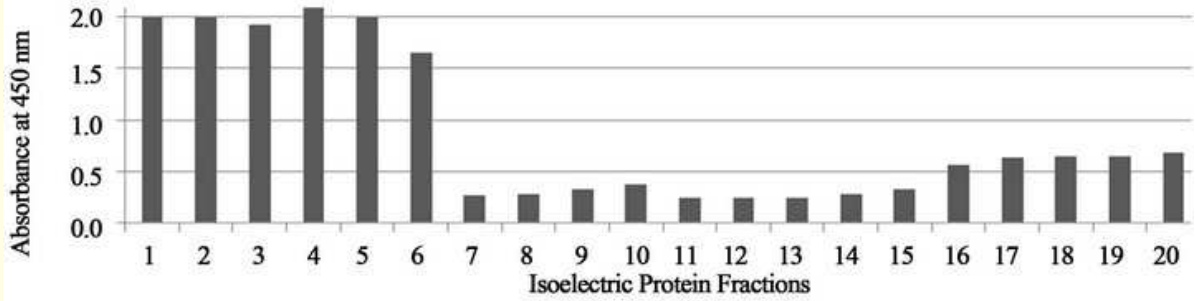

Figure P3. Absorbance values of protein fractions reacted with *B. dermatitidis* dog serum from Louisiana.

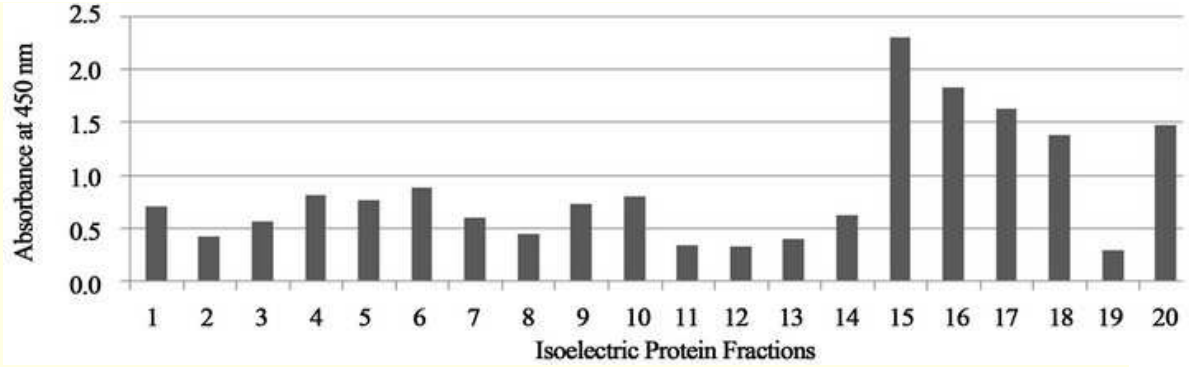

Figure P4. Absorbance values of protein fractions reacted with *H. capsulatum* dog serum (a), from Tennessee.

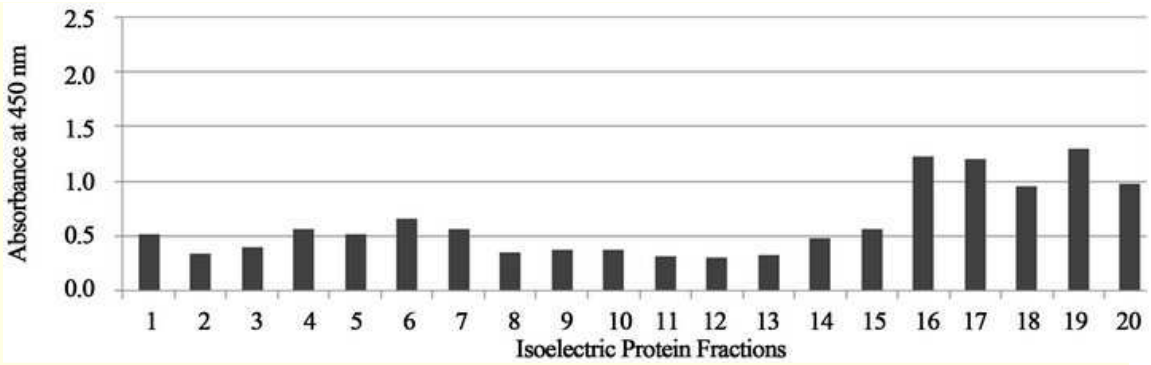

Figure P5. Absorbance values of protein fractions reacted with *H. capsulatum* dog serum (b), from Tennessee.

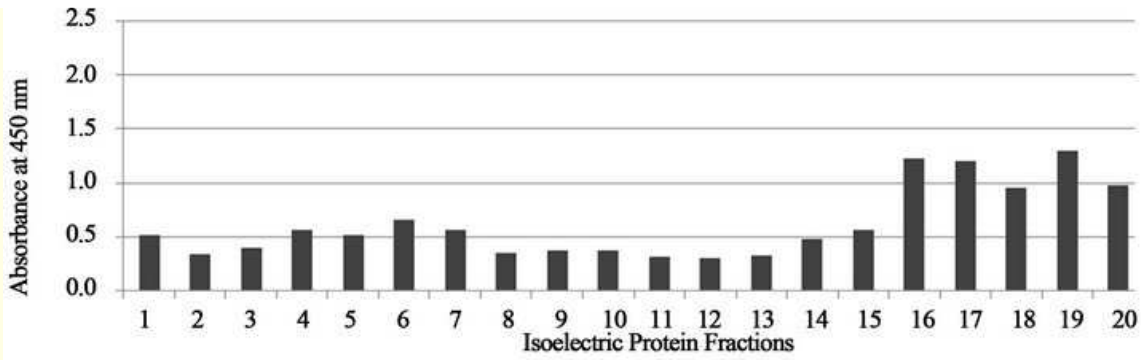

Figure P6. Absorbance values of protein fractions reacted with *C. neoformans* dog serum, from Tennessee.

5. Acknowledgements

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Chapter 2.

Further Analysis and Data Collection.

Introduction

The previous studies showed that there may be a difference between the early fractions and reactivity with dog sera and late fractions with the same dog sera. This led us to determine if other strains of *B. dematitidis* follow a similar pattern. To do this we chose four (4) other strains, these strains were chosen because of their range of diversity (human and nonhuman isolates) (see supplemental S1).

Materials and Methods

Culturing and Lysis

The strains were first cultured and lysed to collect the cellular components. The strains were grown in a chemically defined medium broth in a shaker at 37°C for a period of seven days. The cells were then collected by centrifugation (5 minutes at 2500 RPM). After collection the cells are then lysed by placing in sterile DI water in a shaker at 37°C for a period of 1 or 7 days. The lysates were then collected and centrifuged at 700 X g for 30 minutes to remove debris. They were then filtered through a Corning 0.22μm low protein binding filter and then merthiolate (1: 10,000 dilution) was added as a preservative and stored at 4°C. Before use, the lysate solution protein concentration was determined using a BCA method according to the manufacturer's directions (Pierce Chemical Company Rockford, IL).

Concentration of proteins

The lysates were then concentrated to a useful level using the Thermo Scientific Pierce® concentrator (20mL with a 20K molecular weight cutoff). The whole cell lysates were placed in the upper chamber of the concentrator. We used a swinging bucket rotor so we could use the full 20mL of sample per the literature/ instructions. The samples were run for 30 minutes at 2500xg. Once the run was finished we then removed 5mL of concentrated sample and added it to another concentrator tube and then mixed with 15mL of un-concentrated sample in the tube. This was run again for 30 minutes at 2500xg. The remaining concentrated samples were run again at the same parameters and an average of 2mL was recovered resulting in an average increase in concentration. This protocol was created experimentally from the initial protocol provided with the Pierce® Concentrator kit. According to the literature we should have seen an increase of greater than 100 fold increase, however, this was not the case. We also saw some precipitate form in the bottom of the tubes; these were aspirated using the pipette to dissolve them back into solution. The concentration allowed us to have enough useful amounts of the protein once it is fractionated to run gels and preform further tests.

Rotofor®

To fractionate the lysates we used the Bio Rad Rotofor® (Bio-Rad, Hercules, CA) to give us 20 fractions of the cellular lysates. The Rotofor® separates proteins into fractions that range from acidic to basic; this fractionating is based on a protein's isoelectric point (pI). To standardize the Rotofor® controls were run to determine that the machine was operating within specified parameters. We used three proteins with known pI's

purchased from BIO-RAD. The proteins used were Phycocyanin 2mg/ml with a pI range of 4.5 – 5.5 and a blue color. The second was Hemoglobin 2mg/ml with a pI range of 6.0 – 7.5 and a red color. The third protein was Cytochrome c 2mg/ml with a pI range of 8.0 -9.0 and an orange color [Fig. 1]. This method has been used before in our lab to evaluate other lysates [1, 2].

Figure 2.1. The Rotofor® with the test proteins showing the separation of the known proteins to their prospective pI range.

Protein Concentration determination and neutralization

The use of the Rotofor® gave us a range of proteins that varied from acidic to basic. The proteins were fractionated and the pH was then brought to a neutral level to be useful in further pH sensitive tests. The pH was adjusted using either HCl or NaCl added drop wise to the glass collection vials to bring the fractions to a pH to 7, ± 0.2.

Once equilibrated a BCA assay was run, in accordance with the manufacturer's instructions, on the fractions to determine the concentration. This was useful when preforming initial strain comparisons and to determine the appropriate staining to use when preforming gels.

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 Costar 96-well microplate (Thermo/Fisher). The plates were then incubated overnight at 4° 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° C in a humid chamber. Following this incubation the wells were washed as above and 100 ul of anti-rabbit IgG (H & L) peroxidase conjugate (Kirkegaard and Perry, Gaithersburg, MD) was added to each well and incubated for 30 min at 37° C. The plates were again washed as above and 100 ul of peroxidase substrate (Thermo/Fisher Pierce) was added to each well and incubated for approximately 2 min at room temperature. The reaction was stopped by the addition of sulfuric acid and the absorbance read at 450 nm using a BIO-RAD 2550 EIA reader.

ELISA Procedure

Coating buffer:

Na_2CO_3 0.159g

$NaHCO_3$ 0.293g

D.I. H_20 100mL

pH= 9.6 without adjusting

PBS-T (phosphate buffered saline + Tween 20):

NaCl 16g

KH_2PO_4 0.4g

$Na_2HPO_{4\text{-}7}H_2O$ 5.8g

KCl 0.4g

Tween 20 3.0 mL

D.I. H_2O

pH= 7.4 (without adjusting)

[Indirect ELISA for Ab detection]

1. Antigen diluted (2000ng/mL) in coating buffer (pH 9.6) (see above) and added (100µL) to each well of micro-dilution plate – Incubated overnight at 4°C in a humid chamber.
2. After incubation the wells are washed 3 times with PBS-T (see above) to remove unbound antigen.
3. Serum specimen (antibodies) diluted in PBS-T (1:2500 or other dilutions) and added to each well (100 µL) – Incubated for 30 min at 37°C.
4. Wells washed as above and anti-species Ab + enzyme (horseradish peroxidase or others) conjugate (1:2000 or other dilutions) is added to each well (100 µL)- Incubated for 30min at 37°C.
5. Wells washed as above and the enzyme substrate added (100 µL) and incubated for a few minutes until a blue color forms. (peroxidase + TMB substrate). The reaction is stopped by adding 2N sulfuric acid (100 µL) = yellow color (peroxidase system).
6. Absorbance is then read with a BIO-RAD EIA reader at 450nm.

SDS PAGE

Due to the low protein concentrations in our fractions we determined that the best course of action to perform an adequate SDS PAGE would be to use the silver stain technique. We used a 5% stacking and 10 % running gel to preform our test.

10% SDS PAGE

			Stacking
		Gel percentage	5
	1	H2O (mL)	3.33
	2	0.5M Tris(pH6.8)(mL)	1.5
	3	30% Polyacrylamide (mL)	1.02
	4	10% SDS (**uL**)	60
	5	TMED (**uL**)	6
	6	10% Ammonium persulfate (**uL**)	60
		Bromophenol blue 1% (uL)	20

			Separating
		Gel percentage	10
	1	H2O (mL)	3.96
	2	1.5M Tris(pH8.8)(mL)	2.5
	3	30% Polyacrylamide (mL)	3.33
	4	10% SDS (**uL**)	100
	5	TMED (**uL**)	4
	6	10% Ammonium persulfate (**uL**)	100

Results

ELISA

Analyzing the results using an Enzyme Linked Immunosorbent Assay (ELISA) as stated above we were able to determine the reactivity between the "Early" and "Late" fractions that were acquired using the Rotofor®. See Table 1 for the data code. A full list or results can be found on table S1.

Antigen B5896 was a human strain isolated from an outbreak in Minnesota [2, 3]. The twenty fractions were divided into three sections; an early, middle and late. These consist of fractions 1-6, 7-14 and 15-20 respectively. After the initial results were analyzed it appeared that there was indeed a difference between the early and late fractions as we had seen in previous tests [Fig. 1, 2]. The numbers were then analyzed using a standard T-test to determine if there was any statistical difference between the fractions. What we found was between the first 6 and late 6 of the day 1 lysates there was no real significant difference between the fractions. However if we focus the test to the first 3 and last 3 we see a difference (Table 3). This also leads us to believe that there is something important with the 55kDa protein that we have located. (Fig 2). By looking at the day 7 lysate results we showed that there was a significant difference between the early and late fractions for both the 6 early and the 6 late and the 3 early and the 3 late. However we do show that there is slightly better result for the early and late 3 fractions (Table 4).

Antigen B5931 was a human strain, it was tested the same as the above strain and the twenty fractions were separated into an early, middle and late group. While there is a difference between the fractions, only at a statistical significance of 90% do we find that

there is a significant difference between the early and late fractions. Here however there is a reversal of the groups. In the day one lysates we showed that there was a difference in only the 3 early and 3 late fractions and when we group them together as 1-6 and 15-20 we see no significant difference. However, in the day seven lysates we noticed that there was a significant difference between the 1-6 and the 15-20 groups and no difference between the 1-3 and 15-20 [Table 5, 6].

Antigen T-58 was isolated from an infected dog (Tennessee). It was tested the same as the above strains and the twenty fractions were separated into an early, middle and late group. We found that the 1-6 and the 15-20 day one lysates and the 1-3 and 18-20 day one lysates both showed no significant differences in reactivity. The same is true for the day seven lysates where the 1-6 and 15-20 and the 1-3 and 18-20 groups showed no significant difference. Only at a significance of 90% do we see a difference between the two groups but only in the day seven lysates [Table 7, 8].

ERC-2 was a dog isolate from Wisconsin, it was tested the same as the above strains and the twenty fractions were separated into an early, middle and late group. Here we find that there was a significant difference between the 1-3 and the 18-20 groups of the day one lysates. While there was a difference between the 1-6 and 15-20 fractions, only at a statistical significance of 90% do we find that there was a significant difference between them. In the day seven lysates however, we showed that there is a significant difference between both groups, the 1-6 and 15-20 group and the 1-3 and 18-20 group [Table 9, 10].

Determining what proteins were in our samples

After we analyzed the fractions using the ELISA (above), and from the results of our previous experiments we used a SDS PAGE with a silver stain to observe what size proteins were involved in the reactions. A silver stain was used because of the relatively small amounts of protein that was left after fractionating the cells in the Rotofor®.

B5931 SDS PAGE

Observing the gel in Fig 2.1, we noticed that there was the 120kD protein that we expected to find. There was also a great difference in concentration between the day 1 lysates and the day 7 lysates. There were other proteins present as well and the concentration in the day 7 lysates appeared to be much greater than the day one preparations. We showed that the majority of the proteins were isolated in the early fractions. This was the case for the day one and the day seven lysates. The day one reagent showed that there is the appearance of the 120 kDa protein in both the early and late fractions.

B5986 SDS PAGE

Observing the gel in Fig 2.2, we once again noticed that there was the presence of the 120 kDa protein, however, there was also what appeared to be a large band of 55 kDa proteins in greater concentration than the B5931 yeast lysate for both the day one and the day seven reagents.

T-58 SDS PAGE

Observing the gel in Fig 2.3, we observed a large concentration in the early fractions compared to the late fractions. We also observed, as in the previous results, that the 120 and 55 kDa proteins, along with the majority of the proteins, were located in the early fractions 1-7. The day seven once again showed a greater concentration of proteins than the day one lysate.

ERC-2 SDS PAGE

Finally we analyzed the results from the ERC-2 SDS PAGE Fig 2.4. There was a large concentration of proteins that spanned the entire gel. There appears to be a large concentration of the 120 kDa protein in every lane. We also noticed some over staining in the empty wells. While the gel was not as clear we saw a concentration of the 55 kDa protein.

Discussion

Fractionating the yeast lysates allowed us to be able to break the overall reactions down into 20 separate reactions. Using this we were able to better determine what proteins were reacting. When we ran the ELISA for the fractionated B5986 strain we saw that the day one lysates showed a significant difference between the early and late fractions only when we reduced the comparison to the first 3 and last 3 fractions (Table. 3). These results were then visualized using SDS PAGE. The results from the gel showed us that there was a large amount of the 120 kDa and the 55 kDa proteins (Fig. 2). The results from these evaluations showed that there was a greater reactivity when the 120 kDa and the 55 kDa protein were present. While it is inconclusive which protein, the 55 kDa or

the 120 kDa proteins are responsible for the spike in reactivity it was clear that there was a definite difference between the pooled early and late fractions for the B5986 *B. dermatitidis* strain.

Tables and Figures

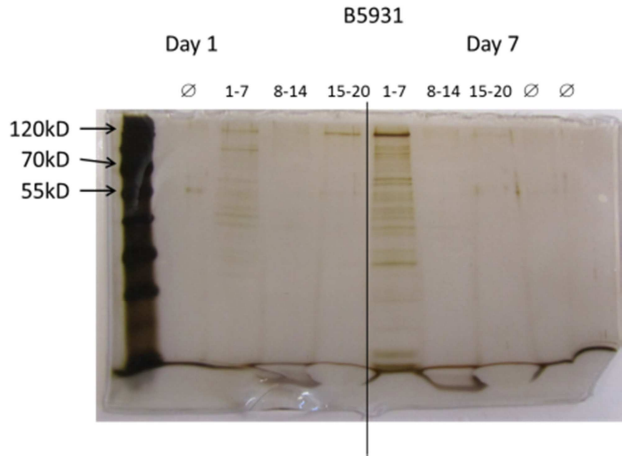

FIGURE 2.2. SDS PAGE WITH SILVER STAIN. THE GEL SHOWS THE PROTEINS THAT HAVE BEEN SEPARATED BY USING THE ROTOFOR®, THE PROTEINS WERE POOLED TO SAVE LANES AND EXPEDITE RESULTS. LANE ONE CONSISTS OF PROTEIN LADDER IT IS LABELED TO SHOW THE RELATIVE WEIGHTS OF THE PROTEINS. LANE 2 IS EMPTY. LANE 3 CONTAINS THE FRACTIONS 1-7 FROM THE DAY ONE LYSIS. LANE 4 CONTAINS THE FRACTIONS FROM THE 8-14 DAY ONE LYSIS. LANE 5 CONTAINS THE FRACTIONS FROM THE 15-20 DAY ONE LYSIS. LANE 6 CONTAINS THE FRACTIONS FROM THE 1-6 DAY 7 LYSIS. LANE 7 CONTAINS THE POOLED FRACTIONS FROM 8-14 DAY 7 LYSIS. LANE 8 CONTAINS THE POOLED FRACTIONS FROM THE 15-20 DAY 7 LYSIS. LANES 9 AND 10 WERE LEFT EMPTY.

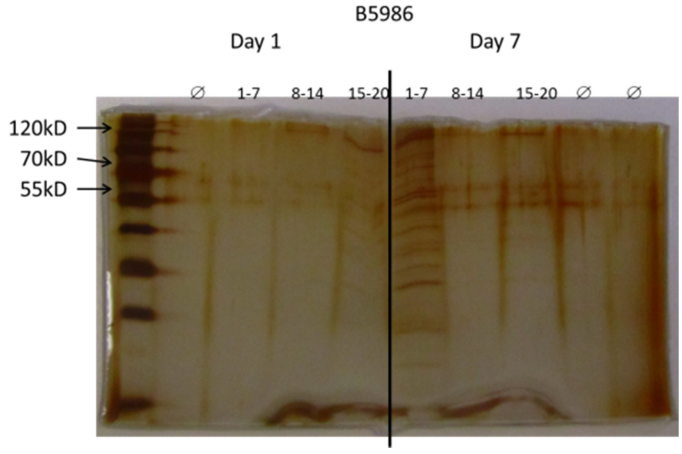

FIGURE 2.3. SDS PAGE WITH SILVER STAIN. THE GEL SHOWS THE PROTEINS THAT HAVE BEEN SEPARATED BY USING THE ROTOFOR®, THE PROTEINS WERE POOLED TO SAVE LANES AND EXPEDITE RESULTS. LANE ONE CONSISTS OF PROTEIN LADDER IT IS LABELED TO SHOW THE RELATIVE WEIGHTS OF THE PROTEINS. LANE 2 IS EMPTY. LANE 3 CONTAINS THE FRACTIONS 1-7 FROM THE DAY ONE LYSIS. LANE 4 CONTAINS THE FRACTIONS FROM THE 8-14 DAY ONE LYSIS. LANE 5 CONTAINS THE FRACTIONS FROM THE 15-20 DAY ONE LYSIS. LANE 6 CONTAINS THE FRACTIONS FROM THE 1-6 DAY 7 LYSIS. LANE 7 CONTAINS THE POOLED

FRACTIONS FROM 8-14 DAY 7 LYSIS. LANE 8 CONTAINS THE POOLED FRACTIONS FROM THE 15-20 DAY 7 LYSIS. LANES 9 AND 10 WERE LEFT EMPTY.

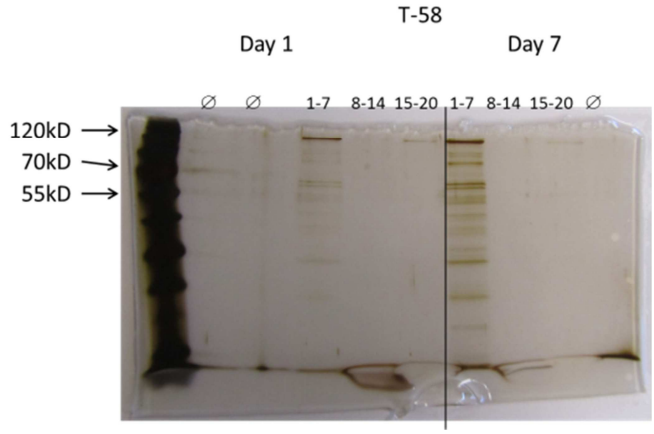

FIGURE 2.4. SDS PAGE WITH SILVER STAIN. THE GEL SHOWS THE PROTEINS THAT HAVE BEEN SEPARATED BY USING THE ROTOFOR®, THE PROTEINS WERE POOLED TO SAVE LANES AND EXPEDITE RESULTS. LANE ONE CONSISTS OF PROTEIN LADDER IT IS LABELED TO SHOW THE RELATIVE WEIGHTS OF THE PROTEINS. LANE 2 AND 3 WERE LEFT EMPTY. LANE 4 CONTAINS THE FRACTIONS 1-7 FROM THE DAY ONE LYSIS. LANE 5 CONTAINS THE FRACTIONS FROM THE 8-14 DAY ONE LYSIS. LANE 6 CONTAINS THE FRACTIONS FROM THE 15-20 DAY ONE LYSIS. LANE 7 CONTAINS THE FRACTIONS FROM THE 1-6 DAY 7 LYSIS. LANE 8 CONTAINS THE POOLED FRACTIONS FROM 8-14 DAY 7 LYSIS. LANE 9 CONTAINS THE POOLED FRACTIONS FROM THE 15-20 DAY 7 LYSIS. LANE 10 WAS LEFT EMPTY.

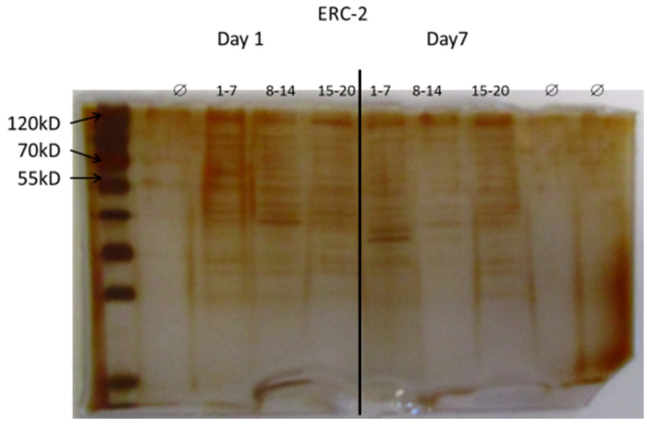

Figure 2.5. SDS PAGE with silver stain. The gel shows the proteins that have been separated by using the Rotofor®, the proteins were pooled to save lanes and expedite results. Lane one consists of protein ladder it is labeled to show the relative weights of the proteins. Lane 2 is empty. Lane 3 contains the fractions 1-7 from the Day one lysis. Lane 4 contains the fractions from the 8-14 day one lysis. Lane 5 contains the fractions from the 15-20 day one lysis. Lane 6 contains the fractions from the 1-6 day 7 lysis. Lane 7 contains the pooled fractions from 8-14 day 7 lysis. Lane 8 contains the pooled fractions from the 15-20 day 7 lysis. Lanes 9 and 10 were left empty.

Table 1. Code to the following data tables

Absorbance =	Reactivity

Code	
Strain	1 = B5968
	2 = T-58
	3 = B5931
	4 = ERC-2
Serum: Histo/ Blasto	1 = Blasto B5896
	2 = Histo G217 A
Day	1 = one day lysis
	7 = seven day lysis

Histo serum	
1 thru 7 =	1
8 thru 14 =	2
15 thru 20 =	3

No data

Table 2 B5896 Day 1 Lysis

B5896 Day 1

Fraction	pH	Day	Histo/Blasto serum	Strain	Absorbance
1	2.37	1	1	1	0.468
2	3.92	1	1	1	0.525
3	3.99	1	1	1	0.580
4	4.51	1	1	1	0.562
5	5.06	1	1	1	0.545
6	5.6	1	1	1	0.613
15	8.17	1	1	1	0.387
16	8.55	1	1	1	0.418
17	8.75	1	1	1	0.459
18	9.16	1	1	1	0.597
19	9.87	1	1	1	0.703
20	10.1	1	1	1	0.820

p=	0.421755	1-6 and 15-20	Shows no significant difference
p=	0.032324	1-3 and 18-20	Shows a significant difference between the late 3 and early 3

Table 3 B5896 Day 7 Lysis

B5896 Day 7

Fraction	pH	Day	Histo/Blasto serum	Strain	Absorbance
1	2.91	7	1	1	0.898
2	3.18	7	1	1	0.774
3	4.17	7	1	1	0.918
4	4.87	7	1	1	0.956
5	5.35	7	1	1	1.151
6	5.81	7	1	1	1.041
15	8.19	7	1	1	0.472
16	No Data	7	1	1	0.476
17	8.78	7	1	1	0.593
18	9.08	7	1	1	0.668
19	9.4	7	1	1	0.719
20	10.02	7	1	1	0.766

p=	0.000444	1-6 and 15-20	Shows a significant difference between the late 6 and early 6
p=	0.002271	1-3 and 18-20	Shows a significant difference between the late 3 and early 3

Table 4 B5931 Day 1 Lysis

B5931 Day 1

Fraction	pH	Day	Histo/Blasto serum	Strain	Absorbance
1	2.96	1	1	3	0.423
2	3.59	1	1	3	0.394
3	4.39	1	1	3	0.690
4	5.09	1	1	3	0.665
5	5.59	1	1	3	0.535
6	5.88	1	1	3	0.620
15	8.07	1	1	3	0.439
16	8.24	1	1	3	0.580
17	8.51	1	1	3	0.539
18	8.81	1	1	3	0.627
19	9.44	1	1	3	0.761
20	9.72	1	1	3	0.872

1-6	15-20		
p=		0.171092	Shows no significant difference
1-3	18-20		
p=		0.052781	Shows no significant difference

Table 5 B5931 Day 7 Lysis

B5931 Day 7

Fraction	pH	Day	Histo/Blasto serum	Strain	Absorbance
1	2.93	7	1	3	2.552
2	3.57	7	1	3	0.881
3	4.26	7	1	3	1.231
4	5.02	7	1	3	0.887
5	5.53	7	1	3	0.872
6	5.74	7	1	3	0.746
15	8.13	7	1	3	0.544
16	8.4	7	1	3	0.614
17	8.62	7	1	3	0.549
18	9.03	7	1	3	0.576
19	9.69	7	1	3	0.782
20	10.34	7	1	3	0.833

1-6 15-20 p=	0.05471	Shows no significant difference
1-3 18-20 p=	0.122449	Shows no significant difference

Table 6 B5931 Day 7 Lysis

B5931 Day 7

Fraction	pH	Day	Histo/Blasto serum	Strain	Absorbance
1	2.93	7	1	3	2.552
2	3.57	7	1	3	0.881
3	4.26	7	1	3	1.231
4	5.02	7	1	3	0.887
5	5.53	7	1	3	0.872
6	5.74	7	1	3	0.746
15	8.13	7	1	3	0.544
16	8.4	7	1	3	0.614
17	8.62	7	1	3	0.549
18	9.03	7	1	3	0.576
19	9.69	7	1	3	0.782
20	10.34	7	1	3	0.833

1-6 15-20 p=	0.05471	Shows no significant difference
1-3 18-20 p=	0.122449	Shows no significant difference

Table 7 T-58 Day 7 Lysis

T-58 Day 7

Fraction	pH	Day	Histo/Blasto serum	Strain	Absorbance
1	2.92	7	1	2	0.604
2	3.46	7	1	2	0.706
3	4.28	7	1	2	1.601
4	5.02	7	1	2	1.950
5	5.54	7	1	2	0.734
6	5.8	7	1	2	0.736
15	8.17	7	1	2	0.448
16	8.38	7	1	2	0.635
17	8.68	7	1	2	0.623
18	9.16	7	1	2	0.550
19	9.69	7	1	2	0.642
20	10.24	7	1	2	0.817

1-6 15-20 p= 0.060962 Shows no significant difference

1-3 18-20 p= 0.222402 Shows no significant difference

Table 8 ERC-2 Day 1 Lysis

ERC-2 Day 1

Fraction	pH	Day	Histo/Blasto serum	Strain	Absorbance
1	3.01	1	1	4	0.375
2	3.71	1	1	4	0.414
3	4.1	1	1	4	0.422
4	4.91	1	1	4	0.559
5	5.48	1	1	4	0.380
6	5.74	1	1	4	0.410
15	8.04	1	1	4	0.403
16	8.26	1	1	4	0.416
17	8.39	1	1	4	0.518
18	8.68	1	1	4	0.460
19	9.56	1	1	4	0.644
20	10.15	1	1	4	0.719

1-6 15-20 p= 0.066316 Shows no significant difference

1-3 18-20 p= 0.056493 Shows no significant difference

Table 9 ERC-2 Day 7 Lysis

ERC-2 Day 7

Fraction	pH	Day	Histo/Blasto serum	Strain	Absorbance
1	2.78	7	1	4	1.612
2	3.56	7	1	4	1.468
3	4.2	7	1	4	2.275
4	4.77	7	1	4	2.771
5	5.22	7	1	4	3.000
6	5.51	7	1	4	2.275
15	7.82	7	1	4	1.081
16	8.08	7	1	4	0.375
17	8.3	7	1	4	0.310
18	8.55	7	1	4	0.341
19	9.44	7	1	4	0.435
20	10.97	7	1	4	0.444

1-6 15-20 p= 0.000183 Shows significant difference

1-3 18-20 p= 0.014596 Shows significant difference

Chapter 3.

Histoplasma capsulatum reactivity comparison

Introduction

To determine if our fractions produced any cross reactivity we tested them against *H. capsulatum*. The fractions were pooled in this test to save space.

Materials and Methods

Culturing and Lysis

The strains were first cultured and lysed to collect the cellar components. The strains were grown in a chemically defined medium broth in a shaker at 37°C for a period of seven days. The cells were then collected by centrifugation (5 minutes at 2500 RPM). After collection the cells are then lysed by placing in sterile DI water in a shaker at 37°C for a period of 1 or 7 days. The Lysates were then collected and centrifuged at 700 X g for 30 minutes to remove debris. They were then filtered through a Corning 0.22μm low protein binding filter and then merthiolate (a 1:10,000 dilution) was added as a preservative and stored at 4°C. Before use, the lysate solution concentration was determined using a BCA method according to the manufacturer's directions (Pierce Chemical Company Rockford, IL).

Concentration of proteins

The lysates were then concentrated to a useful level using the Thermo Scientific Pierce® concentrator 20mL with a 20K molecular weight cutoff. The whole cell lysates were

placed in the upper chamber of the concentrator. We used a swinging bucket rotor so we could use the full 20mL of sample per the literatures instructions. The samples were run for 30 minutes at 2500xg. Once the run was finished we then removed 5mL of concentrated sample and added it to another concentrator tube and then mixed with 15mL of un-concentrated sample in the tube. This was run again for 30 minutes at 2500xg. The remaining concentrated samples were run again at the same parameters and an average of 2mL was recovered resulting in an average increase in concentration. This protocol was created experimentally from the initial protocol provided with the Pierce® Concentrator kit. According to the literature we should have seen an increase of greater than 100 fold increase however, this was not the case. We also saw some precipitate form in the bottom of the tubes; these were aspirated using the pipette to dissolve them back into solution. The concentration allowed us to have enough useful amounts of the protein once it was fractionated to run gels and perform further tests.

Rotofor®

To fractionate the lysates we used the Bio Rad Rotofor® (Bio-Rad, Hercules, CA) to give us 20 fractions of the cellular lysates as above. The Rotofor® separates proteins into fractions that range from acidic to basic; this fractionating is based on a protein's isoelectric point (pI). To standardize the Rotofor® controls were run to determine that the machine was operating within specified parameters. We used three proteins with known pI's purchased from BIO-RAD. The proteins used were Phycocyanin 2mg/ml with a pI range of 4.5 – 5.5 and a blue color. The second was Hemoglobin 2mg/ml with a pI range of 6.0 – 7.5 and a red color. The third protein was Cytochrome c 2mg/ml with a

pI range of 8.0 -9.0 and an orange color. This method has been used before in our lab to evaluate other lysates [19].

Protein Concentration determination and neutralization

The use of the Rotofor® gave us a range of proteins that varied from acidic to basic. The proteins were fractionated and the pH was then brought to a neutral level to be useful in further pH sensitive tests. The pH was adjusted using either HCl or NaCl added drop wise to the glass collection vials to bring the fractions to a pH to 7, ± 0.2.

Once equilibrated a BCA assay was run in accordance with the manufacturer's instructions, on the fractions to determine the concentration. This was useful when preforming initial strain comparisons and in determining the appropriate staining to use when preforming gels.

ELISA

The ability of each yeast lysate reagent to detect antibodies in the above serum specimens was determined using the indirect enzyme-linked immunosorbant 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 Costar 96-well microplate (Thermo/Fisher). The plates were then incubated overnight at 4 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 C in a humid chamber. Following this incubation the wells were washed as above and 100 ul of anti-rabbit IgG (H & L) peroxidase conjugate (Kirkegaard and Perry, Gaithersburg, MD) was added to each well and

incubated for 30 min at 37 C. The plates were again washed as above and 100 ul of peroxidase substrate (Thermo/Fisher Pierce) was added to each well and incubated for approximately 2 min at room temperature. The reaction was stopped by the addition of sulfuric acid and the absorbance read at 450 nm using a BIO-RAD 2550 EIA reader.

RESULTS

B5896 Pooled Fractions with *Histoplasma capsulatum* G217A Serum.

To determine the reactivity compared to a *H. capsulatum* strain we used pooled fractions due to the previous results showing that the early, mid and late fractions showed the greatest variance. We ran the group of pooled fractions against the G217A serum to determine if reactivity was evident with the *B. dermatitidis* antigens. With the B5896 fractions we found that we didn't achieve any variation between the day one and day seven lysates Fig 3.1.

T-58 Pooled Fractions with *Histoplasma capsulatum* G217A Serum.

Once again we noticed very little reactivity between the day one and the day seven lysates. We also noticed very little difference between the early and late fractions3.2.

ERC-2 Pooled Fractions with *Histoplasma capsulatum* G217A Serum Fig.

Once again we noticed very little reactivity between the day one and the day seven lysates. We also noticed very little difference between the early and late fractions3.3.

B5931 Pooled Fractions with *Histoplasma capsulatum* G217A Serum.

Here we were able to observe a difference between the early and late fractions Fig 3.4. This is similar to the results that we obtained in the early preliminary results Fig 1.2. We didn't, however, notice any significant difference between the day one lysates and the day seven lysates.

Figures:

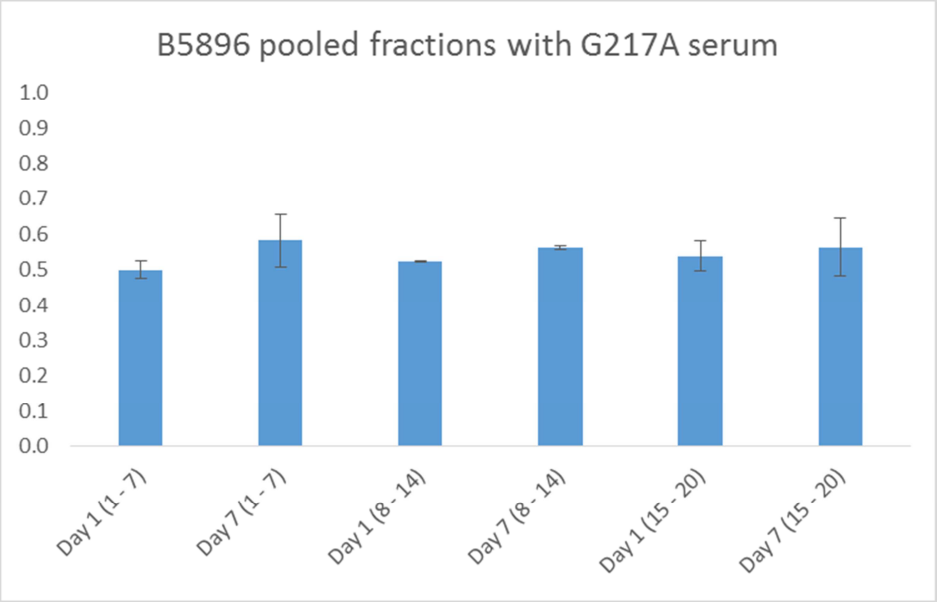

Figure 3.1 shows the comparison between day one and day seven pooled fractions. Pooling fractions from the B5896 strain and testing their reactivity in an ELISA. Of the 20 total fractions we divided and pooled them each into three separate solutions. The solutions are as follows; fractions 1-7 1mL was taken from each fraction and pooled with the rest of the fractions to create a 7mL solution containing fractions 1-7. The same procedure was used to combine fractions 8-14 and 15-20. This was done for the Day 1 samples and the Day 7 samples. The results of the B5896 pooled fractions and their reactivity with G217A serum shows that there was a small difference with the day 7 being slightly more reactive but no real statistical significance between the two days.

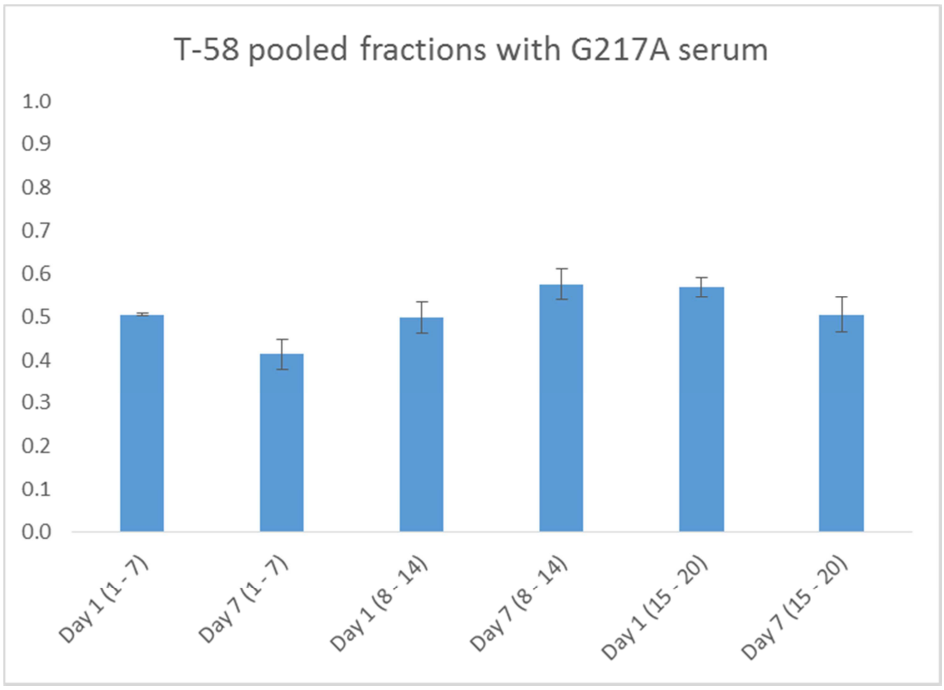

Figure 3.2 Once again using the G217a serum we were able to observe the reaction between the Day 1 and the Day 7 lysed *B. dermatitidis* cells. Once again we obserevd little to no statistical significant difference between the two days or the separate pooled fractions.

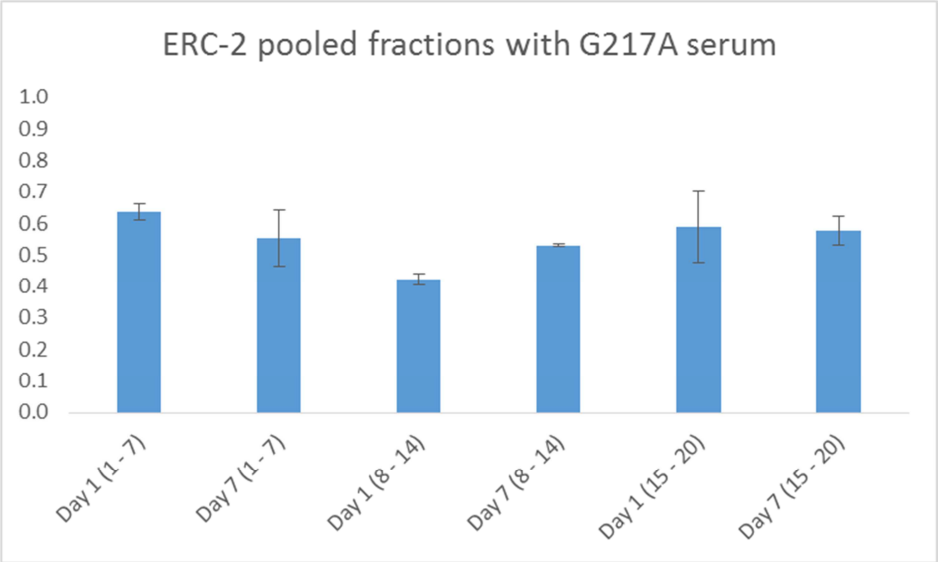

Figure 3.3 Once again using the G217a serum we were able to observe the reaction between the Day 1 and the Day 7 lysed *B. dermatitidis* cells. Once again we obserevd little to no statistical significant difference between the two days or the separate pooled fractions.

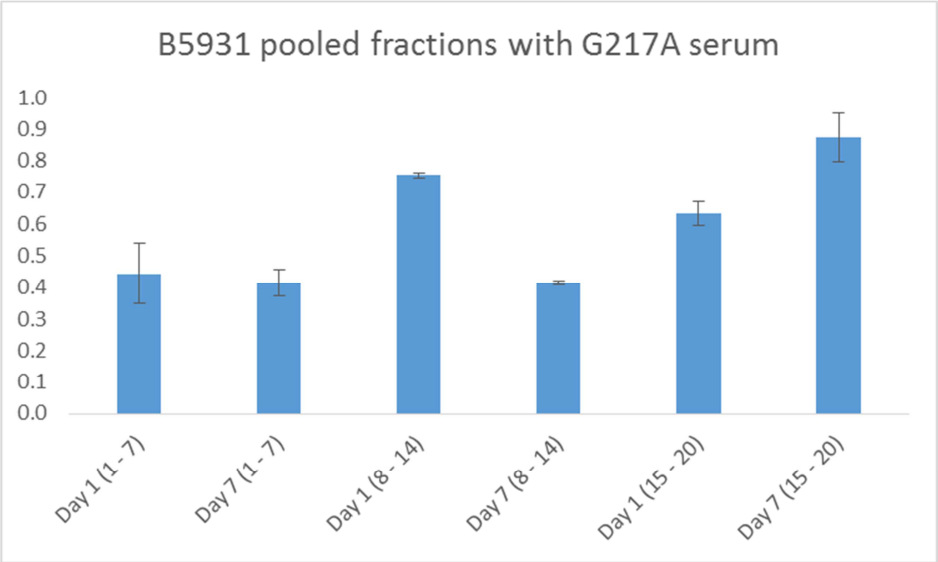

Figure 3.4 Once again using the G217a serum we were able to observe the reaction between the Day 1 and the Day 7 lysed *B. dermatitidis* cells. We obsereved a difference between the early and late fractions. This is simmular to the preliminary results seen in chapter 1. However we once again we obserevd little to no statistical significant difference between the two days.

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Table S1. The following table shows the full data set from the ELISA results. Lane one is the fraction number that was extracted from the yeast lysate via the Rotofor®. Lane two is the pH of that fraction before it was adjusted to 7 ± 0.2. Lane 3 indicates the date of growth as to when the cells were lysed, (1 or 7 days). Lane 4 indicates whether the serum used was from the *Histoplasma capsulatum* or the *Blastomyces dermatitidis* organisms, a 1 indicates *B. dermatitidis* and a 2 indicates *H. capsulatum*. Lane 5 indicates the strain of the organism that was used 1-4. Lane 6 gives the results from the ELISA as read in the mean absorbance from three wells. Lane 7 gives the standard deviation among the three well values.

Fraction	pH	Day	Histo/Blasto serum	Strain	Absorbance	Stdev
1	2.37	1	1	1	0.468	0.034
2	3.92	1	1	1	0.525	0.023
3	3.99	1	1	1	0.580	0.076
4	4.51	1	1	1	0.562	0.009
5	5.06	1	1	1	0.545	0.035
6	5.6	1	1	1	0.613	0.028
7	5.79	1	1	1	0.438	0.047
8	6.26	1	1	1	0.416	0.02
9	6.57	1	1	1	0.439	0.033

10	6.81	1	1	1	0.406	0.051
11	6.71	1	1	1	0.710	0.057
12	7.38	1	1	1	0.380	0.007
13	7.81	1	1	1	0.486	0.015
14	8.03	1	1	1	0.403	0.016
15	8.17	1	1	1	0.387	0.003
16	8.55	1	1	1	0.418	0.023
17	8.75	1	1	1	0.459	0.006
18	9.16	1	1	1	0.597	0.009
19	9.87	1	1	1	0.703	0.016
20	10.1	1	1	1	0.820	0.035
1	2.91	7	1	1	0.898	0.044
2	3.18	7	1	1	0.774	0.055
3	4.17	7	1	1	0.918	0.033
4	4.87	7	1	1	0.956	0.109
5	5.35	7	1	1	1.151	0.098
6	5.81	7	1	1	1.041	0.017
7	6.08	7	1	1	0.840	0.023
8		7	1	1		
9	6.71	7	1	1	0.559	0.018
10	6.98	7	1	1	0.541	0.086
11	7.02	7	1	1	0.784	0.005
12	7.37	7	1	1	0.655	0.017
13	7.81	7	1	1	0.531	0.047

14	7.91	7	1	1	0.506	0.020
15	8.19	7	1	1	0.472	0.019
16		7	1	1	0.476	0.038
17	8.78	7	1	1	0.593	0.026
18	9.08	7	1	1	0.668	0.013
19	9.4	7	1	1	0.719	0.009
20	10.02	7	1	1	0.766	0.045
1	2.97	1	1	2	0.654	0.006
2	3.73	1	1	2	0.695	0.046
3	4.31	1	1	2	0.659	0.0135
4	5.02	1	1	2	0.591	0.244
5	5.46	1	1	2	0.494	0.049
6	5.77	1	1	2	0.372	0.034
7	6.12	1	1	2	0.702	0.0595
8	6.56	1	1	2	0.458	0.05
9	6.73	1	1	2	0.474	0.0465
10	6.96	1	1	2	0.323	0.0035
11	7.27	1	1	2	0.363	0.031
12	7.42	1	1	2	0.345	0.006
13	7.63	1	1	2	0.633	0.025
14	7.89	1	1	2	0.403	0.0035
15	8.1	1	1	2	0.386	0.027
16	8.29	1	1	2	0.429	0.0245
17	8.51	1	1	2	0.625	0.01

18	9.01	1	1	2	0.578	0.017
19	9.63	1	1	2	0.78	0.019
20	10.55	1	1	2	0.845	0.08
1	2.92	7	1	2	0.604	0.0135
2	3.46	7	1	2	0.706	0.028
3	4.28	7	1	2	1.601	0.043
4	5.02	7	1	2	1.950	0.116
5	5.54	7	1	2	0.734	0.0245
6	5.8	7	1	2	0.736	0.052
7	6.17	7	1	2	0.600	0.009
8	6.62	7	1	2	0.815	0.0185
9	6.88	7	1	2	0.851	0.027
10	7.02	7	1	2	0.341	0.0075
11	7.35	7	1	2	0.402	0.0115
12	7.61	7	1	2	0.614	0.047
13	7.75	7	1	2	0.749	0.0705
14	7.95	7	1	2	0.384	0.01
15	8.17	7	1	2	0.448	0.0535
16	8.38	7	1	2	0.635	0.12
17	8.68	7	1	2	0.623	0.0675
18	9.16	7	1	2	0.550	0.0235
19	9.69	7	1	2	0.642	0.0095
20	10.24	7	1	2	0.817	0.0575
1	2.96	1	1	3	0.423	0.010

2	3.59	1	1	3	0.394	0.003
3	4.39	1	1	3	0.690	0.003
4	5.09	1	1	3	0.665	0.052
5	5.59	1	1	3	0.535	0.064
6	5.88	1	1	3	0.620	0.059
7	6.22	1	1	3	0.496	0.040
8	6.67	1	1	3	0.550	0.094
9	6.89	1	1	3	0.413	0.045
10	6.98	1	1	3	0.589	0.016
11	7.29	1	1	3	0.610	0.014
12	7.53	1	1	3	0.655	0.074
13	7.72	1	1	3	0.438	0.059
14	7.90	1	1	3	0.423	0.024
15	8.07	1	1	3	0.439	0.063
16	8.24	1	1	3	0.580	0.043
17	8.51	1	1	3	0.539	0.039
18	8.81	1	1	3	0.627	0.018
19	9.44	1	1	3	0.761	0.042
20	9.72	1	1	3	0.872	0.037
1	2.93	7	1	3	2.552	0.130
2	3.57	7	1	3	0.881	0.015
3	4.26	7	1	3	1.231	0.101
4	5.02	7	1	3	0.887	0.074
5	5.53	7	1	3	0.872	0.002

6	5.74	7	1	3	0.746	0.003
7	6.17	7	1	3	0.908	0.081
8	6.52	7	1	3	0.561	0.043
9	6.71	7	1	3	0.528	0.048
10	6.98	7	1	3	0.45	0.012
11	7.27	7	1	3	0.386	0.045
12	7.47	7	1	3	0.496	0.062
13	7.66	7	1	3	0.495	0.033
14	7.95	7	1	3	0.465	0.018
15	8.13	7	1	3	0.544	0.030
16	8.4	7	1	3	0.614	0.033
17	8.62	7	1	3	0.549	0.032
18	9.03	7	1	3	0.576	0.005
19	9.69	7	1	3	0.782	0.016
20	10.34	7	1	3	0.833	0.041
1	3.01	1	1	4	0.375	0.008
2	3.71	1	1	4	0.414	0.014
3	4.1	1	1	4	0.422	0.010
4	4.91	1	1	4	0.559	0.026
5	5.48	1	1	4	0.380	0.022
6	5.74	1	1	4	0.410	0.004
7	6.11	1	1	4	0.419	0.006
8	6.42	1	1	4	0.481	0.012
9	6.82	1	1	4	0.452	0.030

10	7.03	1	1	4	0.350	0.008
11	7.25	1	1	4	0.357	0.015
12	7.33	1	1	4	0.355	0.020
13	7.54	1	1	4	0.443	0.012
14	7.73	1	1	4	0.370	0.003
15	8.04	1	1	4	0.403	0.037
16	8.26	1	1	4	0.416	0.007
17	8.39	1	1	4	0.518	0.004
18	8.68	1	1	4	0.460	0.019
19	9.56	1	1	4	0.644	0.008
20	10.15	1	1	4	0.719	0.043
1	2.78	7	1	4	1.612	0.084
2	3.56	7	1	4	1.468	0.069
3	4.2	7	1	4	2.275	0.104
4	4.77	7	1	4	2.771	0.206
5	5.22	7	1	4	3.000	0.001
6	5.51	7	1	4	2.275	0.221
7	6.01	7	1	4	1.322	0.118
8	6.11	7	1	4	0.940	0.075
9	6.34	7	1	4	0.777	0.074
10	6.63	7	1	4	0.784	0.084
11	6.75	7	1	4	0.307	0.008
12	7.11	7	1	4	0.621	0.049
13	7.37	7	1	4	0.867	0.125

14	7.63	7	1	4	1.119	0.109
15	7.82	7	1	4	1.081	0.034
16	8.08	7	1	4	0.375	0.014
17	8.3	7	1	4	0.310	0.006
18	8.55	7	1	4	0.341	0.008
19	9.44	7	1	4	0.435	0.021
20	10.97	7	1	4	0.444	0.047
1		1	2	1	0.498	0.024
1		1	2	1	0.498	0.024
1		1	2	1	0.498	0.024
1		1	2	1	0.498	0.024
1		1	2	1	0.498	0.024
1		1	2	1	0.498	0.024
1		1	2	1	0.498	0.024
2		1	2	1	0.521	0.0015
2		1	2	1	0.521	0.0015
2		1	2	1	0.521	0.0015
2		1	2	1	0.521	0.0015
2		1	2	1	0.521	0.0015
2		1	2	1	0.521	0.0015
2		1	2	1	0.521	0.0015
3		1	2	1	0.537	0.0425
3		1	2	1	0.537	0.0425
3		1	2	1	0.537	0.0425

3		1	2	1	0.537	0.0425
3		1	2	1	0.537	0.0425
3		1	2	1	0.537	0.0425
1		7	2	1	0.58	0.0745
1		7	2	1	0.58	0.0745
1		7	2	1	0.58	0.0745
1		7	2	1	0.58	0.0745
1		7	2	1	0.58	0.0745
1		7	2	1	0.58	0.0745
1		7	2	1	0.58	0.0745
2		7	2	1	0.561	0.0055
2		7	2	1	0.561	0.0055
2		7	2	1	0.561	0.0055
2		7	2	1	0.561	0.0055
2		7	2	1	0.561	0.0055
2		7	2	1	0.561	0.0055
2		7	2	1	0.561	0.0055
3		7	2	1	0.562	0.08
3		7	2	1	0.562	0.08
3		7	2	1	0.562	0.08
3		7	2	1	0.562	0.08
3		7	2	1	0.562	0.08
3		7	2	1	0.562	0.08
1		1	2	2	0.506	0.004

1		1	2	2	0.506	0.004
1		1	2	2	0.506	0.004
1		1	2	2	0.506	0.004
1		1	2	2	0.506	0.004
1		1	2	2	0.506	0.004
1		1	2	2	0.506	0.004
2		1	2	2	0.500	0.036
2		1	2	2	0.500	0.036
2		1	2	2	0.500	0.036
2		1	2	2	0.500	0.036
2		1	2	2	0.500	0.036
2		1	2	2	0.500	0.036
2		1	2	2	0.500	0.036
2		1	2	2	0.500	0.036
3		1	2	2	0.570	0.023
3		1	2	2	0.570	0.023
3		1	2	2	0.570	0.023
3		1	2	2	0.570	0.023
3		1	2	2	0.570	0.023
3		1	2	2	0.570	0.023
3		1	2	2	0.570	0.023
1		7	2	2	0.414	0.035
1		7	2	2	0.414	0.035
1		7	2	2	0.414	0.035
1		7	2	2	0.414	0.035
1		7	2	2	0.414	0.035

1		7	2	2	0.414	0.035
1		7	2	2	0.414	0.035
2		7	2	2	0.577	0.035
2		7	2	2	0.577	0.035
2		7	2	2	0.577	0.035
2		7	2	2	0.577	0.035
2		7	2	2	0.577	0.035
2		7	2	2	0.577	0.035
2		7	2	2	0.577	0.035
3		7	2	2	0.506	0.041
3		7	2	2	0.506	0.041
3		7	2	2	0.506	0.041
3		7	2	2	0.506	0.041
3		7	2	2	0.506	0.041
3		7	2	2	0.506	0.041
1		1	2	3	0.445	0.095
1		1	2	3	0.445	0.095
1		1	2	3	0.445	0.095
1		1	2	3	0.445	0.095
1		1	2	3	0.445	0.095
1		1	2	3	0.445	0.095
1		1	2	3	0.445	0.095
2		1	2	3	0.754	0.009
2		1	2	3	0.754	0.009

2		1	2	3	0.754	0.009
2		1	2	3	0.754	0.009
2		1	2	3	0.754	0.009
2		1	2	3	0.754	0.009
2		1	2	3	0.754	0.009
3		1	2	3	0.634	0.037
3		1	2	3	0.634	0.037
3		1	2	3	0.634	0.037
3		1	2	3	0.634	0.037
3		1	2	3	0.634	0.037
3		1	2	3	0.634	0.037
1		7	2	3	0.416	0.04
1		7	2	3	0.416	0.04
1		7	2	3	0.416	0.04
1		7	2	3	0.416	0.04
1		7	2	3	0.416	0.04
1		7	2	3	0.416	0.04
1		7	2	3	0.416	0.04
2		7	2	3	0.417	0.006
2		7	2	3	0.417	0.006
2		7	2	3	0.417	0.006
2		7	2	3	0.417	0.006
2		7	2	3	0.417	0.006
2		7	2	3	0.417	0.006

2		7	2	3	0.417	0.006
3		7	2	3	0.875	0.076
3		7	2	3	0.875	0.076
3		7	2	3	0.875	0.076
3		7	2	3	0.875	0.076
3		7	2	3	0.875	0.076
3		7	2	3	0.875	0.076
1		1	2	4	0.637	0.025
1		1	2	4	0.637	0.025
1		1	2	4	0.637	0.025
1		1	2	4	0.637	0.025
1		1	2	4	0.637	0.025
1		1	2	4	0.637	0.025
1		1	2	4	0.637	0.025
2		1	2	4	0.424	0.018
2		1	2	4	0.424	0.018
2		1	2	4	0.424	0.018
2		1	2	4	0.424	0.018
2		1	2	4	0.424	0.018
2		1	2	4	0.424	0.018
2		1	2	4	0.424	0.018
3		1	2	4	0.590	0.111
3		1	2	4	0.590	0.111
3		1	2	4	0.590	0.111

3		1	2	4	0.590	0.111
3		1	2	4	0.590	0.111
3		1	2	4	0.590	0.111
1		7	2	4	0.555	0.089
1		7	2	4	0.555	0.089
1		7	2	4	0.555	0.089
1		7	2	4	0.555	0.089
1		7	2	4	0.555	0.089
1		7	2	4	0.555	0.089
1		7	2	4	0.555	0.089
2		7	2	4	0.532	0.005
2		7	2	4	0.532	0.005
2		7	2	4	0.532	0.005
2		7	2	4	0.532	0.005
2		7	2	4	0.532	0.005
2		7	2	4	0.532	0.005
2		7	2	4	0.532	0.005
2		7	2	4	0.532	0.005
3		7	2	4	0.577	0.045
3		7	2	4	0.577	0.045
3		7	2	4	0.577	0.045
3		7	2	4	0.577	0.045
3		7	2	4	0.577	0.045
3		7	2	4	0.577	0.045