Detection of a Coccidioidal Peptide in Plasma from Patients with Active Coccidioidomycosis

by

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ABSTRACT

Coccidioidomycosis, also known as Valley Fever, is a disease caused by the dimorphic soil-dwelling fungus, *Coccidioides* sp. Coccidioidomycosis is difficult to diagnose because symptoms are similar to community-acquired pneumonia. Current diagnostic tests rely on antibody responses, but immune responses can be delayed and aberrant, resulting in false negative diagnoses. Unlike serology, detection of coccidioidal proteins or other fungal components in blood could distinguish valley fever from other pulmonary infections and provide a definitive diagnosis. Using mass spectrometry (LC-MS/MS) we examined the plasma peptidome from patients with serologically confirmed coccidioidomycosis. Mass spectra were searched using the protein database from the Coccidioides species, generated and annotated by the Broad Institute. 15 of 20 patients with serologically confirmed coccidioidomycosis demonstrated the presence of a peptide in plasma, "PGLDSKSLACTFSQV" (PGLD). The peptide is derived from an open reading frame from a "conserved hypothetical protein" annotated with 2 exons, and to date, found only in the C. posadasii strain Silviera RMSCC 3488 genomic sequence. In this thesis work, cDNA sequence analysis from polyadenylated RNA confirms the peptide sequence and genomic location of the peptide, but does not indicate that the intron in the gene prediction of *C. posadasii* strain Silviera RMSCC 3488 is present. A monoclonal antibody generated against the peptide bound to a 16kDa protein in T27K coccidioidal lysate. Detecting components of the fungus plasma could be a useful diagnostic tool, especially when serology does not provide a definitive diagnosis.

DEDICATION

I dedicate this thesis to my family; my father, my mother, and my sisters who have continued to support my pursuit of knowledge of science and academia. Especially to my patient and understanding partner, Karin, for her constant encouragement and understanding along with her son, Nikolas, for his constant admiration for me and my love of science.

Finally, to my cat Marshall, who has been my constant encouragement to pursue all of my dreams and the opportunities that life has to offer.

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Special thanks to Wendi Simonson who, with her support and guidance, made the end of the road smoother with the obstacles before me. The SOLS Graduate College and students are blessed to have her.

Many thanks to the students and workers in Doug Lake's lab for assisting me throughout this study.

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CHAPTER 1

INTRODUCTION

1.1 Discovery of Coccidioidomycosis

In 1892, Wernecke and Posadas described an illness in an Argentinean soldier in South America with cutaneous manifestations relating to a fungal pathogen. A year after Posadas' initial report, a 40-year-old man entered a San Francisco hospital with skin lesions similar to those of Posadas' patient. He was a manual laborer who immigrated to the San Joaquin Valley and in 1895 and, upon his death, an autopsy revealed numerous nodules occupied the lungs, adrenals, lymph nodes, liver, peritoneum, prostate, spleen, and testes. It was believed that the pathogen was protozoan by nature but in 1899 William Ophüls and Herbert C. Moffitt discovered it to be a fungal pathogen. The pathogen, *Coccidioides immitis,* was not a protozoan but was a fungus that existed in 2 forms: mycelia and spherules containing endospores. In 1929, a 26-year-old, second-year medical student, Harold Chope, who was studying *C. immitis* opened an old, desiccated culture. He exhaled on the plate and a plume of spores arose. Chope endured severe pleuritic chest pain nine days later along with purulent sputum. He later developed erythema nodosum and his health recovered (Derensinski SC). In 1932, soil samples were taken from and around the barracks in Kern County as many of the men were becoming ill. Results indicated the soil contained *C. immitis.* Another most important case, a graduate student by the name of Charles E. Smith, developed pleuritic chest pain that he thought might be tuberculosis. He had, in fact, failed to diagnose his own case of

coccidioidomycosis. He further went on to study migratory farm laborers who had come to California in the 1930s after escaping from the "Dust Bowl" of the Midwest (Hirschmann, J.). During 1940–1941, Smith studied the outbreak of coccidioidomycosis amoung Army Air Forces pilots airmen whose training grounds were located in the San Joaquin Valley. Smith continued to study this endemic which revealed that 60% of infections were asymptomatic, and erythema nodosum occurred in ~5% of men (Lewis GG and J Mewha). Because of Pasadas work in the late 1800's the name *C. posadasii* was dedicated.

Coccidioidomycosis, or Valley Fever (VF), an understudied disease, is caused by a dimorphic fungus (Fig 1) endemic to the soils of Central and South America, Mexico, and the southwestern United States. *C. immitis,* one of two known species of the ascomycete fungus, is found in the central valley of California. *C. posadasii* is found in other areas such as Arizona, Texas, Mexico, and parts of Central and South America (Fisher MC, GL Koenig, TJ White, and JW Taylor). It has been noted that *C. immitis* has been discovered in parts of Baja California (Baptista-rosas, Raúl C, Jovani Catalán-dibene' Adriana L. Romero-olivares, Alejandro Hinojosa, Tereza Cavazos, and Meritxell Riquelme). *C. immitis* and *C. posadasii* are estimated to be 97% identical indicating they are highly syntenic (Fisher MC, GL Koenig, TJ White, and JW Taylor).

1.2 Clinical Symptoms

Coccidioidomycosis is typically a respiratory illness acquired through inhalation of airborne arthroconidia, or spores, found in soil. Coccidioidomycosis is not contagious between person-to-person as spore inhalation is the known route of transmission. In one

rare occasion, records indicate the transmission of coccidioidomycosis from a cat bite to a human leading to a cutaneous lesion (Gaidici, Adriana, and Michael Saubolle). Approximately 60% of humans who become infected with *C. immitis* or *C. posadasii* remain clinically asymptomatic (Blair, Janis, Anita Mayer, Jeremiah Curriera, Julia Files, Qing Wu). Those who become symptomatic may have symptoms anywhere from one to four weeks after infection resembling tuberculosis or histoplasmosis; including a dry cough, fever, lack of energy and appetite. Other species have been recorded to be susceptible to coccidioidomycosis include dogs, llamas, non-human primates, cats, horses, wild mammals, and snakes. Symptoms are generally similar to human mycosis with additional, yet less common, symptoms such as seizures or long bone pain and lesions (Centers for Disease Control and Prevention).

Disseminated Coccidioidomycosis occurs in approximately 5% of patients which initially presents with an acute infection (Drake, Kevin, and Richard Adam). Disseminated Coccidioidomycosis generally begins in the lungs with a granulomatous presentation (Fig 2). Although Valley Fever, also called Desert Fever, California Fever, and San Joaquin Valley Fever was named because of the high incidence of infection in Central California, the Phoenix and Tucson areas have been the epicenter for VF for many years. An estimated 150,000 human cases are diagnosed annually with 60% of those infections occurring in the people of the Maricopa, Pima, and Pinal counties of Arizona (Sunenshine RH, S Anderson, L Erhart, A Vossbrink, PC Kelly, D Engelthaler, and K Komatsu).

1.3 Current Diagnosis of Coccidioidomycosis

Upon inhalation the arthrospore settles in the lungs and develops into a spherule containing many endospores. Arthroconidia are phagocytized by host mononuclear cells followed by a respiratory burst. This respiratory burst may initiate the maturation of the arthroconidia into endosporulating spherules (Galgiani, J. N., R. Hayden, and C. M. Payne). This response would promote mononuclear cells to proliferate further. Diagnosis of VF is challenging due to the reliance of laboratory serology and the necessary presence of coccidioidal antibodies in the host. To avoid false negatives the serological test methods must be so fine tuned that a vast plethora of coccidioides immunogens are present. Current serological testing includes tube precipitation (TP) assay, a complement fixation (CF) assay, and immunodiffusion assays. All of these assays rely on the detection of an antigen-antibody complex. The TP assay detects IgM responses to Beta-glucosidase which are seen at approximately week 2 of primary infection (Pappagianis D) but drops dramatically around month 2 of infection. The CF assay detects IgG to chitinase which fixes complement. IgG is typically detected around month 2 post infection and fully around month 9 (Pappagianis D). Titers of antibodies, both IgM and IgG, can be obtained via an enzyme immunoassay (EIA) which is highly sensitive but not as specific as the TP and CF assays. Again, these serological assays depend on detecting antigen-antibody complexes with antibody titers being proportional to severity of disease. It is important to note that not all patients infected with coccidioides may have proper functioning humoral immune responses (Blair, Janis, Anita Mayer, Jeremiah Curriera, Julia Files, Qing Wu).

1.4 Plasma as a diagnostic tool

Blood consists of approximately 22% solids and 78% water (Farley, Alistair, Charles Hendry, and Ella McLafferty). Blood consists of components such as red blood cells, white blood cells, platelets, with the plasma portion consisting of highly abundant proteins such as albumin, transferrin, macroblobulin, immunoglobulin, as well as other biologic molecules that are present down to femtomolar concentrations. Plasma is a rich vault of proteins and is an ideal source to detect biomarkers since it flows throughout every organ of the body (Zheng X, H Baker, and WS Hancock). Both host and pathogen proteins may be broken down by proteases leaving peptide fragments behind. Although these peptides can be as small as ~700 Da and may be in the femtomolar range chemists may use liquid chromatography–mass spectrometry (LC-MS/MS) as a useful tool in detecting these peptides (Cutillas, PR).

LC-MS is one of the most powerful tools to an analytical chemist. A multiple component mixture, such as plasma, is ionized after separation of the liquid into components, usually based on charge or hydrophobicity. These ions are electrostatically extracted onto a capillary tube which are analyzed by mass. Furthermore, an accelerated voltage is applied and once the acquired energy exceeds the ionization potential of the molecule the energy is dispersed though the molecule. When the dissociation energy from the molecule is reached fragmentation occurs. The fragmentation data can be analyzed for each amino acid allowing the amino acid sequence to be determined.

1.5 Peptide to Parent Protein

Peptide sequences are useful in that they can be traced back to the parent protein or transcript using such bioinformatic tools as the "Basic Local Alignment Search Tool" (BLAST) and other databases such as those provided by the Broad Institute. Bioinformatics allows a peptide to be aligned with a suspected transcript or parent protein. This information can be taken to the biology laboratory where wet lab experiments such as PCR and DNA gel electrophoresis can be performed to verify its presence in the genome.

The uses of a Western Blot along with monoclonal antibodies are paramount laboratory tools when searching for a parent protein. Compared to polyclonal antibodies, monoclonal antibodies are more specific as they are derived from a single B cell. A mammalian splenocyte is fused with a myeloma cell creating a hybridoma. This hybridoma produces one identical antibody of a single specificity. For this reason, the use of the MABs is a common tool to probe for a parent protein to verify translation.

1.6 Hypothesis

Diagnostic serology-detection of an antibody response is problematic for patients and their doctors, especially those who are immunocompromised. Patient plasma can be used to detect peptides secreted or released by the pathogen which can be traced back to either the host or pathogen genome. These peptides can be used as biomarkers to show the presence of an active infection in patients with Coccidioidomycosis.

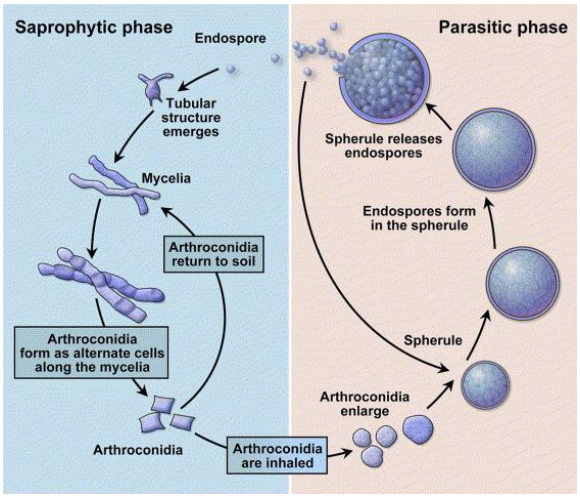

Figure 1 Life Cycle of Coccidioides: Life cycle of *C. posadasii* and *C. immitis (DiCaudo, 2006)*

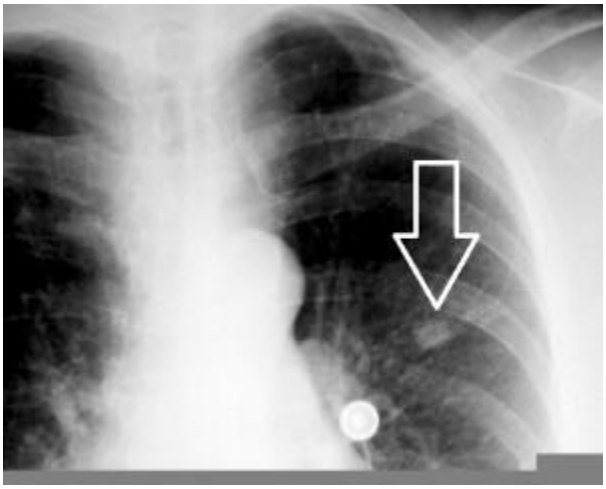

Figure 2 Patient with Lung Granuloma : Patient with Coccidioidomycosis lung granuloma (Burhan, 2011)

CHAPTER 2

EXPERIMENTAL: METHODS AND MATERIALS

2.1 ELISA, T27K, and Plasma

The antigen preparations T27K and Crushed Spherule Supernatant (CSS) were obtained through two different laboratories using two different techniques. To ensure antigenicity, an ELISA was performed to determine if they were immunogenic to patients with active coccidioidomycosis. The importance of knowing if there was antigenicity was because these antigen preparations would be paramount as they would be used in future experiments mentioned in this thesis.

Peripheral blood was obtained from both canine donors, graciously provided by Scottsdale Ranch Animal Hospital, and human donors within 8 weeks of diagnosis with coccidioidomycosis. Some of these patients were taking anti-fungal medication. Peripheral blood was also obtained from healthy donors, both canine and human, from the endemic area in Phoenix, Arizona, who had never been diagnosed with coccidioidomycosis. Plasma was harvested from separation of peripheral blood on a Ficoll gradient. One antigen preparation, T27K, is a thimerosal-inactivated spherule lysate that is prepared from mature endosporulating spherules of *C. posadasii* strain Silviera and centrifuged at 27,000 rpm. The supernatant is retrieved and thus called T27K. (Johnson, Susan M, KA Simmons, and Pappagianis). T27K was graciously provided by Dr. Susan Johnson, of UC Davis Medical School.

Preparing ELISA plate:

An Enzyme Linked Immuno Assay (ELISA) 96 well plate was coated with either 100uL of 10ug/mL of T27K or 100uL of 2ug/mL of Bovine Serum Albumin (- control) which was diluted in coating buffer (Na_2CO_3 +$NaHCO_3$). The 96 well plates were incubated for 1 hour at 37C. The plates were washed 3 times with 200uL of 0.05%tween in 1XPBS. The plates were dried by manual blotting against a paper towel. 200uL of blocking agent consisting of 1%BSA in 1XPBS was added to the wells in the washed plate and allowed to incubate for 2 hours at 37C. The plates were washed 3 times with 200uL of 0.05% tween in 1XPBS. The plates were dried by manual blotting against a paper towel.

Preparing plasma for introduction to the ELISA plate:

Canine Plasma – Canine plasma was diluted at 1:1000 with 1XPBS and introduced into each of the coated 96 well plates. 2-fold serial dilutions were made.

Human Plasma – Human plasma was diluted at 1:500 with 1XPBS and introduced into each of the coated 96 well plates. 2-fold serial dilutions were made.

The ELISA plate was incubated for 1 hour at 37C.

The ELISA plates were washed 3 times with 200uL of 0.05% tween-20 in 1XPBS. The plates were dried by manual blotting against a paper towel after each wash. 100uL of secondary antibody was added to every well in the plate at a dilution of 1:5000 for each

species. The secondary antibody for the ELISA plates with canine plasma consisted of goat-anti-canine horse radish peroxidase in 1%BSA in 1XPBS. The secondary antibody for the ELISA plates with canine plasma consisted of goat-anti-human horse radish peroxidase in 1%BSA in 1XPBS. This was incubated for 1 hour at 37C. The wells in the plates were washed 7 times with 200uL of 0.05%tween in 1XPBS. The plates were dried by manual blotting against a paper towel after each wash.

100uL of TMB was added to each well and allowed to develop at room temperature for 25 minutes. 50uL of 2N H_2SO_4 was used to stop the reaction. The ELISA plates were then read on a plate reader with Softmax Pro 5 software and optical density (OD) values were evaluated.

2.2 Liquid Chromatography-Mass Spectrometry (LC-MS)

Separation and Analysis of Peptides

Plasma samples from patients with serologically confirmed coccidioidomycosis and control subjects were filtered through Centricon (Millipore) centrifugal filters with MWCO of 3kDa. The filtrates were either analyzed immediately or transferred into siliconized tubes and stored at -20 °C until analysis. Each sample was analyzed by adding 0.1 mL of 3kDa filtrate to 0.9 mL of 0.1% TFA solution with pH adjusted to 2.5. Diluted samples were then desalted on Bond Elut-C18 EWP solid phase extraction (SPE) columns (Varian, Inc.) as follows. Columns were wet with 1 mL of 100% acetonitrile, washed with 1 mL of 0.1% TFA solution before loading diluted samples. Columns were

washed with 3% acetonitrile in 0.1% TFA. Peptides that were trapped on the column were eluted with 1 mL of 50% acetonitrile in 0.1% TFA solution and dried on Speedvac. The dried samples were reconstituted in 15 µl of 3% acetonitrile in 0.1% FA of which 12 µl were injected on C18 PicoFrit column (New Objective) on a Thermo Finnigan Surveyor HPLC system (Thermo Scientific). Peptides were resolved on the PicoFrit column with elution gradient running from 5 to 50% in 30 min and 50-100% in 10 min with mobile phase B (90% ACN, 0.1% formic acid in LCMS grade water) versus mobile phase A (3% ACN, 0.1% formic acid in LC-MS water) at a flow rate of 0.6 µL/min. The eluted gradient was analyzed on-line with nanoelectrospray ionization (nano-ESI) linear ion trap mass spectrometer (LTQ, Thermo Scientific) in the positive ion mode. The high voltage capillary was set at 2.60 kV. Mass spectrometer (MS) full scans were acquired from 350 to 1500 m/z in data-dependent mode. Ten most abundant ion peaks above the background in each mass spectrum were selected as precursor ions for tandem mass spectrometry (MS/MS) using collision-activated dissociation (CAD). MS/MS scan of the same ion was not allowed in more than two MS spectra that were obtained within a period of 1 min.

Synthetic Peptides

Because of the relatively large precursor mass tolerance (1.5 Da) used in the database search, PGLDSKSLACTFSQV peptide was chemically synthesized and run on the LC-MS/MS under the same conditions as described above for the natural peptide to make sure the retention time and spectra matched. Peptides were synthesized at the proteomics core facility at Arizona State University on a Milligen 9050 peptide synthesizer

(Millipore, Bedford, MA). Stable heavy isotope labeled peptide PGL*DSKSLACTFSQV, which had all six carbon-12 and nitrogen-14 in Leu12 (C6H13NO2) substituted with carbon-13 and nitrogen-15, respectively, was synthesized by Anaspec, Inc. (San Jose, CA). Mass of the peptide was determined by amino acid analysis. After HPLC purification, the purity of all synthetic peptides was estimated to be greater than 95%. Mass spectrometric analysis was used to confirm amino acid composition of peptides.

3.3 Transcriptomics

cDNA Sequence Analysis

cDNA sequence analysis was performed using total mRNA from *C. posadasii* RMSCC 3488. SuperScript® III First-Strand Synthesis System for RT-PCR (Invitrogen) was used to reverse-transcribe mRNA to cDNA. Genomic DNA from C. posadasii was used as a positive control to determine if genomic DNA contamination was present in the total RNA preparation. Primers (Forward: GTCTATGCGTGTCCCCACTT ; Reverse: CGTCGAAGATGCAAGAGTGA) for the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) specific for *C. posadasii* were developed and used to confirm the absence of genomic DNA. PCR primers within annotated exons flanking the PGLD peptide (Forward: GGTAATCCGGAAGCTCAACC; Reverse: GTCGATGTCCTGGACGAAGT) were synthesized based on the annotation of the predicted protein *C. posadasii* RMSCC 3488: CPAG_04641 (Broad Institute). cDNA was amplified by PCR using 5’ and 3’ primers flanking the PGLD peptide and

electrophoresed on a 1% agarose gel. DNA bands were excised and gel purified with GeneJET® Gel Extraction Kit (Thermo Scientific).

The extracted DNA was ligated and cloned into T-vector using Life Sciences TA Cloning Kit. Plasmid DNAs were purified using GeneJet plasmid miniprep kit (Thermo Scientific). Inserts were sequenced by the ASU DNA sequencing facility and analyzed with DNA Baser. The DNA sequence was then translated and aligned with the Broad Institute sequence using ClustalW2.

In addition to comparing the genomic amplified sequence with the cDNA-amplified sequence a "primer walk" was performed to evaluate the possibility that exon 1 (5' end) may actually be annotated incorrectly. To perform the primer walk cDNA was obtained from total mRNA from *C. posadasii* RMSCC 3488. SuperScript® III First-Strand Synthesis System for RT-PCR (Invitrogen) was used to reverse-transcribe mRNA to cDNA. The predicted protein *C. posadasii* RMSCC 3488: CPAG_04641 (Broad Institute) annotation of the genomic sequence was used to develop primers spanning the 1KB flanking genomic region of 5' and 3' ends. The primer walk on the 3' end lead to no cDNA amplification therefore primers are not listed. The primers for the 5' end of the 1KB genomic flanking region are as follows: 1) "889" - GGT GCA AAG GTG TAA CGA TTC, 2) "810" - AGA TCT CGT GGG ATC TCC TA, 3) "640" - CAG ATG ATC GAG AGG TGG AG, 4) "460" - ACA TCG CCA GCT GAT TCT AT, 5) "280" - CAA GTC TCC CTT TCC CGG AT, and 6) "192" - TAA ACC GTG AAA TAG GGT TGA AGA GG. The PCR primer within annotated exons flanking the PGLD peptide was used for the reverse (Reverse: GTCGATGTCCTGGACGAAGT) and were synthesized based on the annotation of the predicted protein *C. posadasii* RMSCC 3488:

CPAG_04641 (Broad Institute). cDNA was amplified by PCR using 5' and 3' primers flanking the PGLD peptide and electrophoresed on a 1% agarose gel. DNA bands were excised and gel purified with GeneJET® Gel Extraction Kit (Thermo Scientific). Extracted DNA was sequenced by the ASU DNA sequencing facility and analyzed with DNA Baser. The DNA sequence was then translated and aligned with the Broad Institute sequence using ClustalW2.

2.4 Peptide to Parent Protein

Monoclonal Antibody Generation

The PGLDSKSLACTFSQV peptide was synthesized by the proteomics facility at ASU and coupled to KLH (Thermo/Pierce, Rockford, IL). 50ug of peptide-KLH was used to immunize mice in Freund's complete adjuvant (Thermo/Pierce, Rockford, IL), followed by boosting with the peptide-KLH conjugate in Freund's incomplete adjuvant. When titers were greater than 12,800 against the peptide, mice were euthanized and splenocytes harvested. Splenocytes were then fused with P3 murine myeloma cells, plated into 96-well flat-bottom plates and incubated in 10HY media containing hypoxanthine aminopterin and thymidine for 2 weeks. Hybridoma supernatants were tested in a peptide-based ELISA for the ability to secrete antibodies specific for PGLD peptide against PGLDSKSLACTFSQV (PGLD) peptide and a control peptide. Positive hybridomas from the peptide screening were graciously cultured by the laboratory of David Azorza at TD2, Scottsdale, AZ, and subcloned by limited dilution (one cell per

well) at least three times to ensure monoclonality. Anti-PGLD hybridomas were mass cultured and purified on Protein A/G (Thermo/Pierce, Rockford, IL).

Western blot Analysis

Crushed Spherule Supernatant (CSS) is a supernatant derived from *C. posadasii* strain Silviera spherules were obtained from culture after 96 hours of incubation. The spherules are introduced to glass beads and vortexed at 3000rpm/3min. This is then centrifuged to pellet the cracked spherules and the supernatant is retrieved. T27K and CSS, generously provided by Dr. John Galgiani at the University of Arizona, were subjected to SDS-Page at 30ug per lane on a 12% Tris-tricine gel. Proteins were transferred to a PVDF membrane and probed with anti-PGLD monoclonal antibody (Mab). Goat anti-mouse IgG-alkaline phosphatase (Thermo Scientific) was used to detect anti-PGLD Mab. Blots were developed with NBT/BCIP (Thermo Scientific) for 45 minutes. The molecular weight marker used was Invitrogen See Blue® Pre-Stained Standard.

To confirm specificity of the anti-PGLD Mab, an inhibition assay was performed by pre-incubating 70uM of PGLD peptide with anti-PGLD Mab prior to adding the primary antibody Mab-peptide mixture to the PVDF membrane. Goat anti-mouse IgG-alkaline phosphatase (Thermo Scientific) was used to detect anti-PGLD Mab. Blots were developed with NBT/BCIP (Thermo Scientific) for 45 minutes. The molecular weight marker used was Invitrogen See Blue® Pre-Stained Standard.

N-linked Deglycosylation of T27K

T27K was subjected to N-linked deglycosylation using the New England Biolabs®Inc. PNGase-F Kit™. 20ug of T27K was combined with 1ul of 10X Glycoprotein Denaturing Buffer. The T27K was then heated at 100°C for 10 minutes in a thermocycler. 2uL of 10X G7 Reaction Buffer, 2 µl of 10% NP-40, H_20 and 2 µl of PNGase F were added to the T27K mixture and then incubated at 37°C for 1 hour.

Untreated T27K along with PNGase-F treated T27K were subjected to SDS-Page at 30ug per lane on a 12% Tris-tricine gel. Proteins were transferred to a PVDF membrane and probed with anti-PGLD monoclonal antibody (Mab). Goat anti-mouse IgG-alkaline phosphatase (Thermo Scientific) was used to detect anti-PGLD Mab. Blots were developed with NBT/BCIP (Thermo Scientific) for 45 minutes. The molecular weight marker used was Invitrogen See Blue® Pre-Stained Standard.

To confirm specificity of the anti-PGLD Mab, an inhibition assay was performed by pre-incubating 70uM of PGLD peptide with anti-PGLD Mab prior to adding the primary antibody Mab-peptide mixture to the PVDF membrane. Goat anti-mouse IgG-alkaline phosphatase (Thermo Scientific) was used to detect anti-PGLD Mab.

An additional inhibition assay was performed to also confirm specificity of the anti-PGLD Mab and to also discount an unknown band at 35kD by pre-incubating 2ug/mL of PNGase-F with goat anti-mouse IgG-alkaline phosphatase (Thermo Scientific). This was done by adding the goat-anti-mouse/PNGase-F mixture to the PVDF membrane. All

blots were developed with NBT/BCIP (Thermo Scientific) for 45 minutes. The molecular weight marker used was Invitrogen See Blue® Pre-Stained Standard.

CHAPTER 3

RESULTS AND INTERPRETATION

3.1 ELISA, T27K, CSS, and Plasma

Canine Serology

Canine serology commercial lab results were graciously provided by Scottsdale Ranch Animal Hospital (SRAH) as IgM and IgG titers. These results were compared to those obtained from the ELISA assay performed using T27K. (See Table 1) As seen in the comparison between the positive canine laboratory results versus the T27K ELISA results, results would suggest that there are immunogenic and antigenic properties to the antigen prep T27K.

Patient	Plasma Date Drawn	Commercial Lab results	T27K ELISA Results
Kowboy (- control)	9-Oct	(-)	(-)
Bandit Roberts	10-Jun	(-)	(-)
Bart Brown	10-Mar	(+)	(+)
Bo Czajka	10-Jun	(-)	(-)
Bo Czajka	10-Sep	(-)	(-)
Brutis Brodt	10-May	(+)	(+)
Chance Benwell	9-Dec	(-)	(+)
Chance Benwell	10-Sep	(-)	(-)
Chippers Reagan	9-Oct	(+)	(+)
Chippers Reagan	10-Mar	(+)	(+)
Chippers Reagan	10-Jul	(+)	(+)
Cooper Pasque	9-Dec	(-)	(+)
Corky Hice	10-Feb	(-)	(-)
Daisy Coakley	10-Jun	(-)	(-)
Daisy Coakley	10-Sep	(-)	(-)
Gretta Sinski	10-Feb	(-)	(-)
Jazz Karpinski	10-May	(-)	(-)
Jazz Leonow	10-Jan	(-)	(-)
Lexie Gross	10-May	(+)	(+)
Lexie Gross	10-Aug	(+)	(+)
Masie Martinson	10-Jan	(-)	(+)
Maya Callahan	10-Feb	(+)	(+)
Motion Rostan	10-Sep	(-)	(-)
Moxie Goodman	10-Mar	(-)	(-)
Reggie Bartley	9-Dec	(-)	(-)
Reggie Bartley	10-Sep	(-)	(-)
Sophie Greer	9-Oct	(-)	(-)
Wynni Marchetta	9-Dec	(-)	(+)
Wynni Marchetta	10-Oct	(+)	(+)

Table 1 Canine Coccidioidomycosis ELISA Results: Commercial laboratory tests compared to T27K ELISA results. 9 canines tested (+) via the commercial veterinary diagnostic lab. 13 canines tested (+) via the ELISA using T27K as the antigen prep. (For purposes of consistency, positive results were based off of 3 known VF healthy non-immune canines with OD readings lower than 0.250.)

Interestingly, canine patient, Roxie Rowe, was serologically tested for Coccidioidomycosis with a commercially available diagnostic test when she presented with a skin lesion in August 2009. As seen in Table 2, Roxie was serologically negative for Coccidioidomycosis yet a skin scrape culture proved a positive result for a coccidioidal skin lesion. In October 2010 Roxie tested positive via a commercial serological test for Coccidioidomycosis. No plasma was available from either of these dates to test the plasma for antibody reactivity with T27K antigen prep. In March and August of 2010 Roxie was again serologically tested via a commercial diagnostics lab which resulted in negative responses. Whole blood was given to ASU and processed to obtain the plasma from these dates. These plasma samples did show antibody reactivity to T27K when used in an ELISA assay. Lastly, in December of 2010 Roxie continued to show negative results to the antigen preparation used in the commercially available diagnostic assay.

Report Date	Commercial Lab Results	Commercial Lab Skin Scrape Results	T27K ELISA Results
8/26/2009	-	+	No plasma
10/15/2009	+	ND	No plasma
3/21/2010	-	ND	+
8/27/2010	-	ND	+
12/02/2010	-	ND	No plasma

Table 2 Roxie Rowe Coccidioidomycosis ELISA Results: "Roxie" showing clinical symptoms of Valley fever in August 2009 with a positive Coccidioidomycosis skin scrape test yet commercial test show negative serological results. Two months later commercial serology is positive with 2 negative results following. According to T27K ELISA results "Roxie" continued to show an immunological response to T27K prep. (For purposes of consistency, positive results were based off of 3 known VF healthy non-immune canines with OD readings lower than 0.250.

Human Results:

Human serological assays were performed by a commercial laboratory and results were graciously provided by Janis Blair, MD, of Mayo Clinic along with whole blood from each patient or donor through IRB-approved protocols at Mayo Clinic and Arizona State University. Human patients were grouped into 3 categories: 1 = actively infected with coccidioidomycosis; 2 = never diagnosed with coccidioidomycosis; and 3 = healthy immune to coccidioidomycosis. These results were compared to those obtained from the ELISA using CSS at ASU. (See table 3) As seen in the comparison between the positive laboratory results versus the CSS ELISA results, evidence would suggest that there are immunogenic and antigenic properties to the antigen prep CSS.

Sample	Lab Results	CSS ELISA Results	Sample	Lab Results	CSS ELISA Results
ND 82	N/A	-	1-0027	+	+
ND 90	N/A	-	1-0028	Negative	+
1-0001	+	+	1-0029	Negative	+
1-0002	Negative	+	1-0030	Negative	+
1-0003	+	+	1-0031	+	+
1-0004	+	+	2-0001	N/A	+
1-0005	+	+	2-0002	N/A	-
1-0006	+	+	2-0003	N/A	-
1-0007	N/A	+	2-0004	N/A	-
1-0008	+	+	2-0005	N/A	-
1-0009	+	+	2-0006	N/A	-
1-0010	N/A	+	2-0007	N/A	-
1-0011	+	+	2-0008	N/A	-
1-0012	+	+	2-0009	N/A	-
1-0013	+	+	2-0010	N/A	+
1-0014	+	+	3-0001	N/A	+
1-0015	+	+	3-0002	N/A	+
1-0016	N/A	+	3-0003	N/A	+
1-0017	+	+	3-0004	N/A	+
1-0018	N/A	+	3-0005	N/A	-
1-0020	N/A	+	3-0006	N/A	+
1-0022	Negative	+	3-0007	N/A	+
1-0023	Suspect	+	3-0008	N/A	N/A
1-0024	Suspect	+	3-0009	N/A	-
1-0026	+	+	3-0010	N/A	+

Table 3 Human Coccidioidomycosis ELISA Results: Human Valley Fever patient results from a commercially available diagnostic assay were compared to results from an ELISA using Crushed Spherule Supernatant (CSS). Group 1 patients were diagnosed with active Coccidioidomycosis, Group 2 donors were "healthy non-immune" donors, and Group 3 were "healthy immune" donors. (For purposes of consistency positive results were based off of known VF healthy non-immune normal donors with OD readings lower than 0.340.)

All 26 Group 1 patients showed a positive result to the CSS based ELISA.

In addition, 7 of the 10 patients in Group 3, those who reported being previously diagnosed with Valley Fever and were not considered as an active case, had a significant antibody response to the CSS antigen prep.

Comparing the number of patients with active Coccidioidomycosis, whose plasma was evaluated using both the commercially available assay and the CSS ELISA, suggests that there are antigenic and immunogenic properties to the antigen preparation CSS.

3.2 Liquid Chromatography-Mass Spectrometry (LC-MS)

LC-MS/MS analysis was performed on the plasma peptidome from 20 patients with active coccidioidomycosis from the Phoenix area revealed over 100 peptides from *C. posadasii* translated database (data not shown). A spectrum corresponding to one peptide, PGLDSKSLACTFSQV, was common to 15 of 20 patients with active disease, but was not present in any healthy control donors from the Phoenix endemic area (Table 4).

Plasma Sample from:	Frequency of PGLD peptide in plasma
Active Disease	15/20
Non-immune healthy donor	0/13

Table 4 Frequencies of PGLD Peptide in Human Plasma

Sample	Commercial Lab Results	LC/MS-MS PGLD Detection
1-0001	+	-
1-0002	-	+
1-0003	+	-
1-0004	+	+
1-0005	+	+
1-0006	+	-
1-0007	Not Available	+
1-0008	+	+
1-0009	+	-
1-0010	Not Available	+
1-0011	+	+
1-0012	+	-
1-0013	+	+
1-0014	+	+
1-0015	+	+
1-0016	Not Available	+
1-0017	+	+
1-0018	Not Available	+
1-0020	Not Available	+
1-0023	Suspected Cocci	+

Table 5 Frequencies of PGLD peptide in plasma with Commercial lab results

The identity of the PGLD peptide was validated using LC-MS/MS methods. A PGLD peptide was chemically synthesized with a "heavy leucine" in the third position such that the mass of the synthetic peptide is 7 Daltons heavier than the natural plasma-derived peptide. This peptide was then spiked into plasma from patients with active disease followed by LC-MS/MS. Selective Reaction Monitoring (SRM) of the peptide ion fragmentation is shown in figure 3. The top spectra show b and y ion fragmentation of heavy PGLD such that selected SRM peaks are 7 Daltons heavier than the identical b and y ion peaks in the natural peptide in the bottom spectra.

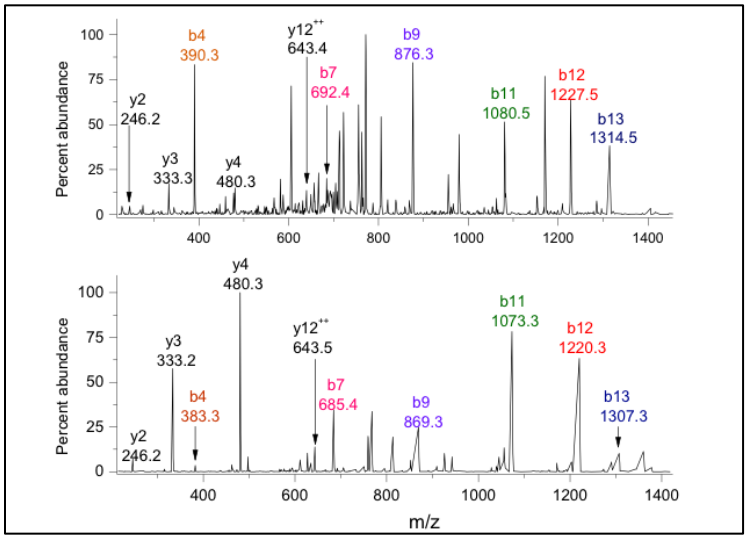

Figure 3 Mass Spec Data of PGLD peptide: Mass spectra of synthetic heavy PGLD peptide (top) compared to natural plasma-derived peptide from a human donor with active disease (bottom). B-ions are colored and represent peptide fragmentation mass from the C-terminal end of the peptide at each peptide bond. For each labeled b-ion the top spectra shows a 7-Dalton difference in mass for each fragmentation.

3.3 PGLD RNA Analysis

PGLDSKSLACTFSQV mapped back to the open reading frame of CPAG_04641.1-predicted protein in the *C. posadasii* strain RMSCC 3488 genome by Broad Institute (See Figure 4). This ORF is conserved throughout all *C. posadasii* strains, but is not annotated by Broad Institute as an ORF in *C. immitis*.

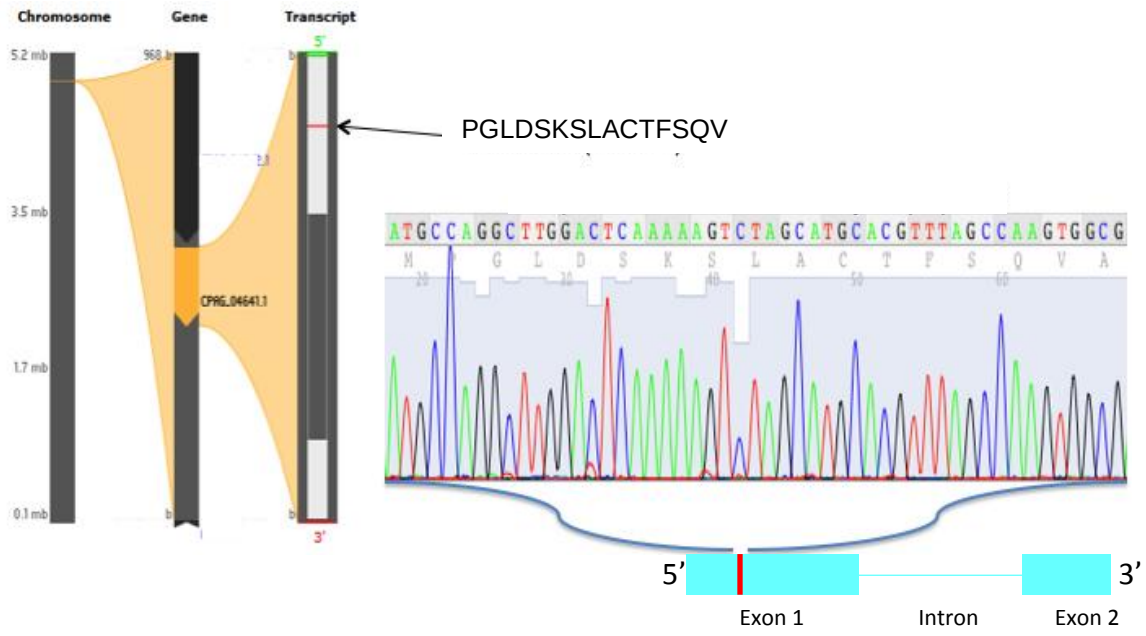

Figure 4 PGLD Peptide Identified on Predicted Protein: PGLD peptide maps to "CPAG_04641.1-predicted protein" on chromosome 2. Arrow pointing to red line indicates location of peptide in CPAG_04641.1. DNA sequence chromatogram from PCR amplification of *C. posadasii* mRNA/cDNA shows the nucleotide and amino acid sequence of the region flanking and including PGLD peptide.

Sequencing of a cDNA amplified from mRNA, with oligonucleotides flanking the predicted gene CPAG_04641.1 indicated that the intron predicted between exons 1 and 2, does not exist. Because it was a possibility that the polyA mRNA was contaminated with genomic DNA, the presence of genomic DNA was tested for by amplifying the

Coccidioides GAPDH gene with primers on adjacent exons using either coccidioidal cDNA or genomic DNA as template (figure 2). The GAPDH genomic DNA PCR product is 272 bp, while the predicted cDNA product is 197 bp, lacking a 75 nt intron. As shown in figure 2, there was no evidence of amplification of the genomic DNA product in the cDNA lane for GAPDH indicating the mRNA used for cDNA preparation was not contaminated with genomic DNA. Using the PGLD primers the same size amplicon is present in the genomic DNA and cDNA template lanes Fig 2, lanes 3 and 4), indicating that there is no intron in the CPAG_04641.1 transcript as annotated by Broad Institute (figure 5). Sequence analysis confirms the identity of both genomic and cDNA PCR products. This strongly suggests that the predicted intron does not exist in this gene. Removal of the intron suggests that the ORF containing the PGLD peptide is 55 residues in length (figure 6).

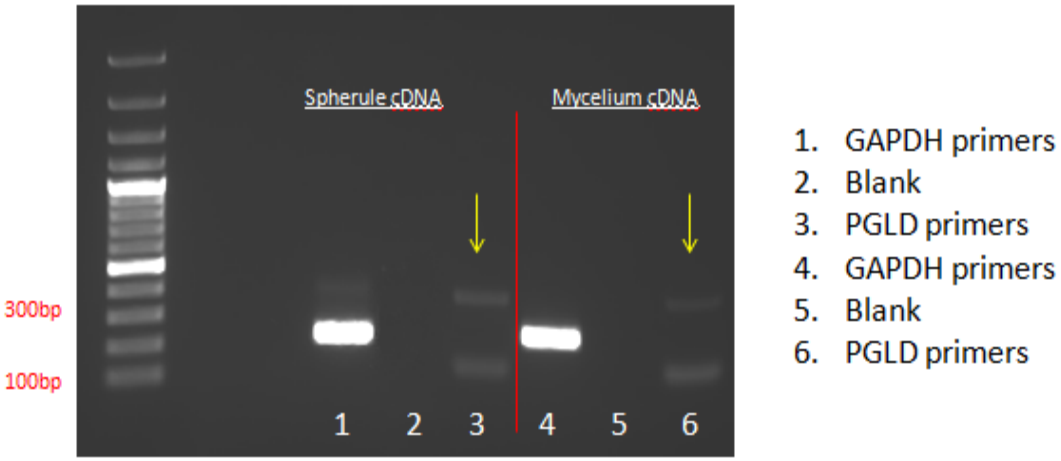

Figure 5 PCR Amplification of PGLD Transcript: Amplification of PGLD in both Spherule cDNA and Mycelium cDNA

Primer Walking

A primer walk ~1KB upstream of PGLD genomic region of the *C. posadasii* is shown in Figure 6. Sequencing of the amplified cDNA with oligonucleotides flanking 1KB of the Broad Institute annotated genomic region of the 5' end of the predicted gene CPAG_04641.1 indicated that there are 10 stop codons in this region and does not reflect the presence of start codons (Figure 7). This further indicates that the actual size of the transcript is not due to upstream transcription of the 5' end but instead that there is no intron in the CPAG_04641.1 transcript as annotated (Broad Institute). Sequence analysis confirms the identity of all cDNA PCR products in the primer walk to contain PGLD. This strongly suggests that the intron predicted by the Broad institute does not exist in this gene.

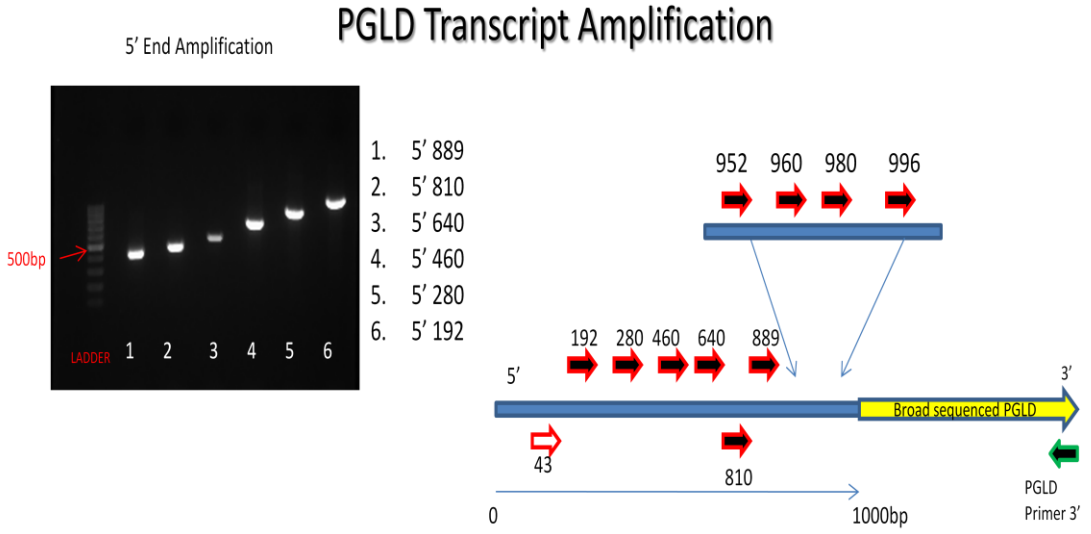

Figure 6 Primer Walking of Genomic PGLD Region: Primer Walking via PCR of 1kb genomic sequence on the 5' end.

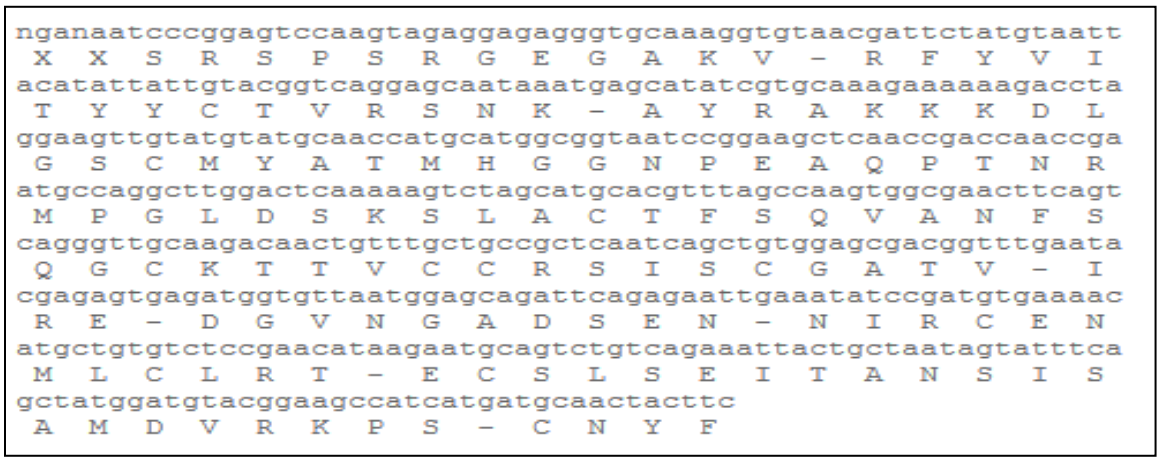

Figure7 Amino Acid Sequence from Primer Walk: DNA and Amino Acid Sequence results of "Primer Walk" to determine possible alternative start codons. Translation performed by ExPASy.

3.3 Peptide to Parent Protein

Characterization of anti-PGLD Monoclonal Antibodies (MAbs):

Hybridoma supernatants from mice immunized with PGLD peptide that tested positive by peptide ELISA were re-tested in Western blotting against CSS and T27K. A hybridoma was identified that secretes anti-PGLD MAb and also recognizes a ~16kDa protein in the T27K lysate, but does not bind to antigen in the CSS (Figure 8). As proof that the antigen-PGLD Mab recognizes a coccidioidal protein containing the PGLD peptide sequence, an inhibition was performed using PGLD peptide of the MAb binding to the parent protein in the T27K by PGLD peptide, while a control peptide with a different sequence does not inhibit binding of anti-PGLD Mab (Figure 9).

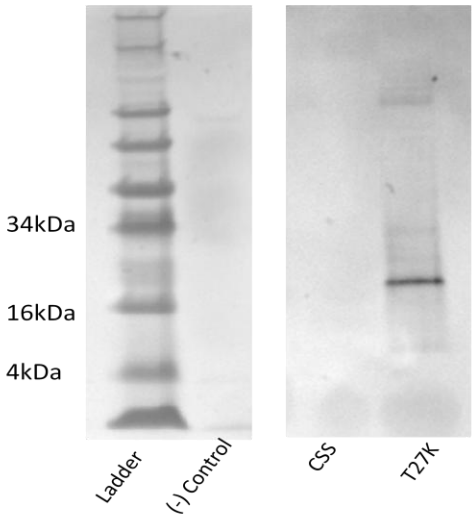

Figure 8 Reactivity of anti-PGLD MAb with T27K. Left blot: (-) Control = No PGLD Mab with T27K antigen preparations (secondary = antibody only). Right blot: Anti-PGLD Mab 5ug/mL with CSS and T27K antigen preparations.

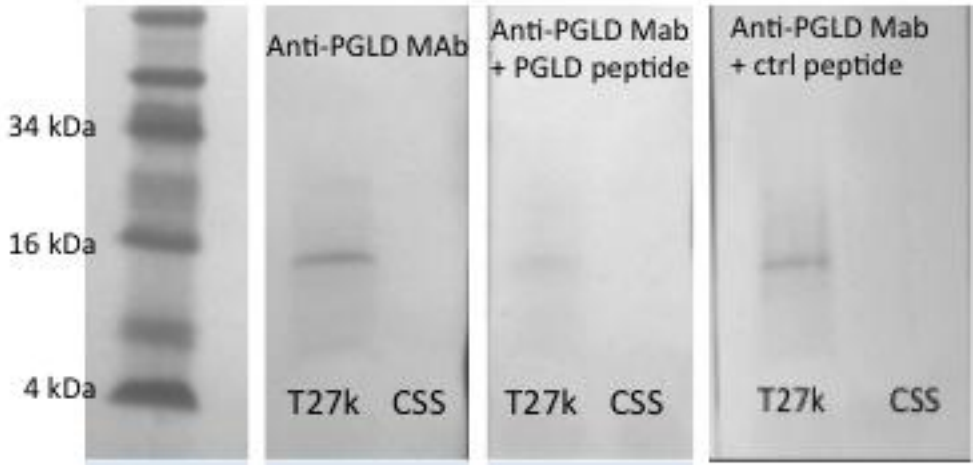

Figure 9 Reactivity of anti-PGLD MAb with T27K and Crushed Spherule Supernatant (CSS). Left blot: Anti-PGLD Mab (5ug/mL) was incubated with each blot containing lanes T27K and CSS antigen preparations. Middle blot: Anti-PGLD Mab (5ug/mL) was pre-incubated with 70□M PGLD peptide for 30 minutes, then the mixture was incubated with the blot. Right blot: Same as middle blot, except with 70 □M control peptide (VVAGLGRAVTRL).

Deglycosylation of T27K

PGLD Mabs were used to probe for PGLD in Western blotting against T27K, both untreated and N-linked deglycosylation with PNGase-F. A doublet band was seen around 16kDa and 13kDa in the T27K deglycosylated with PNGase-F (Figure 10). As proof that the antigen-PGLD Mab recognizes a coccidioidal protein containing the PGLD peptide sequence in T27K, both untreated and deglycosylated, an inhibition was performed using PGLD peptide (Figure 11).

An additional protein band was seen around 35kDa with deglycosylation of T27K. The molecular weight of PNGase-F is 36kDa. As proof that this band was not related to any coccidioidal protein an inhibition was performed to determine nonspecific binding of the secondary antibody vs. the anti-PGLD Mab to PNGase-F (Figure 12).

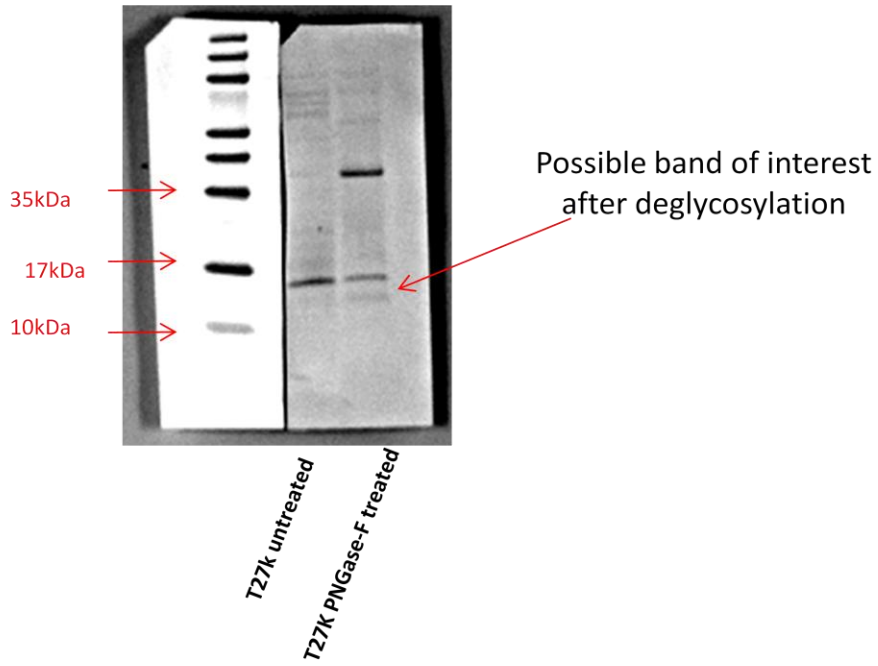

Figure 10 Reactivity of anti-PGLD Mab with T27K Deglycosylated: Reactivity of anti-PGLD MAb with T27K and T27K deglycosylated with PNGase-F. Anti-PGLD Mab (5ug/mL) was incubated with blot containing lanes T27K and T27K deglycosylated preparations.

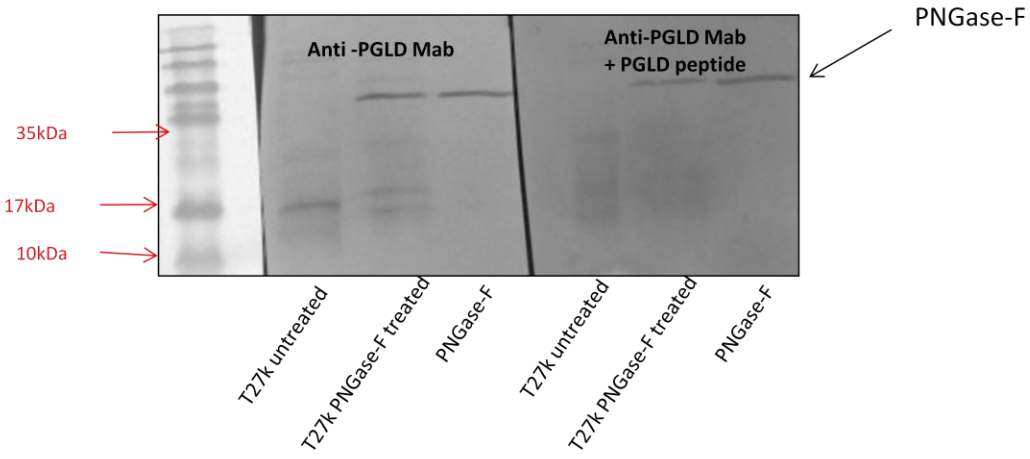

Figure 11 Reactivity of anti-PGLD Mab with T27K and PNGase-F: Reactivity of anti-PGLD MAb with T27K, T27K after being deglycosylated with PNGase-F, and PNGase-F alone. Left blot: Anti-PGLD Mab (5ug/mL) was incubated with each blot containing lanes T27K, T27K after being deglycosylated with PNGase-F, and PNGase-F alone. Right blot: Anti-PGLD Mab (5ug/mL) was pre-incubated with 70□M PGLD peptide for 30 minutes, then the mixture was incubated with the blot.

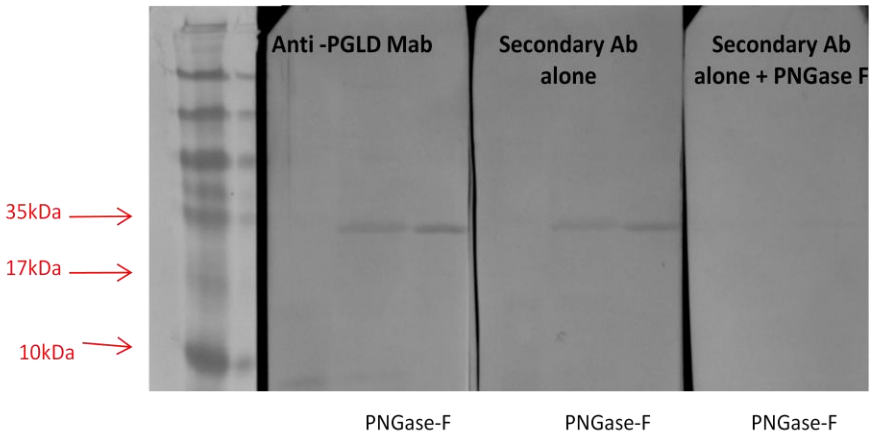

Figure 12 Reactivity of Secondary Ab and PNGase-F : Reactivity of anti-PGLD MAb and Goat-anti-mouse Ab with PGNase-F. Left blot: Anti-PGLD Mab (5ug/mL) was incubated with each blot containing 2 lanes of PNGase-F. Middle blot: Negative control = secondary antibody was incubated with each blot containing 2 lanes of PNGase-F. Right blot: Secondary antibody (1ug/mL) was pre-incubated with 2ug/mL of PGNase-F for 30 minutes, then the mixture was incubated with the blot.

CHAPTER 4

SUMMARY AND CONCLUSIONS

4.1 Summary and Discussion

Coccidioidomycosis is caused by the soil dwelling dimorphic fungus *C. immitis* and *C. posadasii*. Endemic areas in the United States include California, Arizona, and Texas. The route of exposure is through inhalation of the arthroconidia, or spores, which are found in soil. Approximately 40% of those who become infected Coccidioides will become symptomatic (Sunenshine RH, S Anderson, L Erhart, A Vossbrink, PC Kelly, D Engelthaler, and K Komatsu). Symptoms include those similar tuberculosis including dry cough, fever, lack of energy, and appetite (Smith, 1955). Disseminated Coccidioidomycosis occurs in approximately 5% of those infected (Sunenshine RH, S Anderson, L Erhart, A Vossbrink, PC Kelly, D Engelthaler, and K Komatsu). Current diagnostic tests include serological tests, cultures, and sputum smears. For those with a compromised immune system these diagnostic tests may show false negative results since serological testing is dependent on antigen-antibody reactivity. Even in otherwise healthy individuals with no known immune suppression, immune responses to the fungus may be delayed or aberrant.

Plasma is an excellent tool in diagnostics as it contains valuable biomarkers both from the host and the pathogen. Plasma has been used to detect biomarkers in multiple different diseases which has enabled development of new and innovative ways to detect disease (Zheng X, H Baker, and WS Hancock). Disadvantages of using plasma to detect biomarkers include the dynamic qualitative and quantitative range of proteins. Great care must be taken in sample preparation due to the interference of intrinsic plasma proteins

like albumin and immunoglobulins (Heide, K., H Haupt, and H.G. Schwick). Bioinformatics must be utilized to eliminate host peptides prior to determining useful biomarkers. This thesis represents very early attempts to detect and validate coccidioidal antigens as biomarkers in plasma for definitive diagnosis of valley fever.

Commercial lab diagnostic tests results were used to evaluate immunogenicity of T27k and CSS via ELISA. This was done to provide evidence of the antigenicity and immunogenicity of the antigen preparations as they would be used in future experiments. All of the human plasma samples with an active case of coccidoidomycosis indicated a positive signal to CSS. In the canine ELISA using T27K antigen preparation 15 out of the 20 samples reflected a positive signal. Without delving deeper into antibody cross reactivity, these results would indicate that T27K and CSS are antigenic to those with active cases of coccidoidomycosis and these preparations would be adequate for further investigations. Current commercially available diagnostic assays may present false negative results. Since the objective was not to prove this statement it cannot be confirmed by the results presented. In fact, the reason for the uncertainty lies in the unknown composition of the antigen preparations T27K and CSS. A more thorough examination of the immunogenic factors in T27K and CSS would need to be performed. The fact that CSS and T27k have shown to be immunogenic by use of the ELISA makes them an ideal candidate for use for searching for parent proteins.

An alternate way to detect infection is to detect components (peptides, proteins, nucleic acid, carbohydrates, etc…) of the infectious agent itself. Screening of plasma was done by filtering with a 3KDa filter and analyzed with LC-MS/MS a 15-mer peptide (PGLDSKSLACTFSQV) was found in 15 of 20 patients with active

Coccidioidomycosis. The fact that PGLD was not found in 5 of the 20 patients cannot adequately be explained since the plasma samples were subjected only once LC-MS/MS and no validation of the results were performed due to lack of project funding. Additional validation, running the samples again using LC-MS/MS, would give a better level of confidence of the absence of PGLD. As mentioned, PGLDSKSLACTFSQV mapped back to the CPAG_04641.1-predicted protein in the *C. posadasii* strain Silviera RMSCC 3488. The fact that PGLD was only amplified in *C. posadasii* cannot be explained nor can it definitively be stated that it only exists in *C. posadasii* in this thesis. As mentioned previously, the two species are said to be 97% genetically identical so it should not be assumed that *C. immitis* contains the gene containing the PGLD ORF. In fact, The Broad Institute database indicates that *C. posadasii* is the only one of the two species containing the ORF. Due to lack of funding, RNA from *C. immitis* was not able to be obtained nor purchased for any of these experiments and, therefore, should be further investigated. The absence of PGLD in *C. immitis* relies solely on bioinformatics and remains defined as absent by The Broad Institute database. On the other hand, the presence of the gene containing the PGLD ORF has been verified in *C. posadasii* by experimentation as noted in this thesis.

PCR amplification indicated that the intron predicted between exons 1 and 2 does not exist since the predicted size is 161 bp and the amplified size was 340bp. Before making the assumption that the Broad Institute annotation was incorrect, and to ensure this was not due to genomic contamination, GAPDH was used as a housekeeping gene which validated the cDNA did not contain any genomic DNA. To further evaluate the discrepancy in the size of the nucleotide sequence a primer walk was performed. The

primer walk for the 3' end yielded no amplification. The lack of amplification indicates that the transcript of CPAG_04641.1-predicted protein ends at the annotated region and that no adjacent gene exists within 1KB downstream. The primer walk for the 5' end yielded amplification in the 1KB annotated genomic region flanking the 5' end. Although it does not provide an explanation for the increase in size for the product amplified it does indicate that there is an adjacent transcript upstream within 1KB of the PGLD ORF. After further investigation using Broad Institute BLAST this region is actually amplification of the 3' end of an upstream gene CPAG_04640. Although highly unlikely, the upstream gene CPAG_04640 may be fused to the gene containing the PGLD ORF. On the other hand, due to the presence of multiple stop codons in this region it was hypothesized that this sequence was not associated with the predicted protein CPAG_04641.1.

A monoclonal antibody to the 15-mer peptide PGLDSKSLACTFSQV was generated from splenocytes from Balb/C mice inoculated with the peptide. Hybridomas were generated from the monoclonal antibodies and used to probe in Western blotting. The results of the Western blotting show the PGLD Mab reacted with a protein approximately 16KDa in the T27K antigen preparation but not in the CSS. To verify for antibody specificity an inhibition assay was performed using the PGLD peptide as the inhibitor and VVAG peptide as the control peptide. Results indicate that the PGLD Mabs were inhibited by the PGLD peptide but not the VVAG peptide. Unless antibody cross reactivity is unknowingly involved, the parent protein for the PGLD peptide is ~16kDa.

Interestingly, use of the CSS did not yield any antibody-antigen reactivity when probing with the PGLD Mab. Although the Silviera strain from the two antigen preps T27K and

CSS is the same, the culture method is different. Possible reasons for PGLD Mab reactivity with T27K and not CSS could be that the fungus used in T27K is more closely related to that which infects humans. Another possible reason could be due to the method and time at which the spherules are retrieved. The fact that the T27K antigen preparation is prepared from endosporulating spherules may play a role. In addition, although the strains are identical, genetic variation between the two preparations could be possible. None of these possible explanations could be validated at the time and deserve future investigation.

A primary question raised by this study is the fact that the PGLD Mabs reacted with a protein approximately 16KDa. According to the results of the sequence analysis performed using the RNA from the PGLD regions the protein should be approximately 6KDa. Since transcripts are all subject to post transcriptional modifications (PTM) the PTM glycosylation was evaluated. Glycosylation can be N-linked (linked to an asparagine) or O-linked (linked to serine or threonine). The gene which was amplified has the possibility of (11) O-linked glycosylation sites and (3) N-linked glycosylation sites. To test this hypothesis the use of an N-linked deglycosylation technique using PNGase-F was used in attempt to remove any N-linked sugars. Deglycosylation of O-linked sugars could not be performed at the time of this study due to lack of funding. After N-linked deglycosylation of T27K the Western blotting shows PGLD Mab reactivity with both a 16KDa protein and a 13KDa protein. Since it is well known that fungi heavily glycosylate their proteins, the increase in the expected size of the protein could be due to glycosylation. The fact that there was only a slight decrease in mass could be due to only performing N-linked deglycosylation. An experiment using an O-

link deglycosidase would have been much more beneficial as the protein has many more possible O-linked sites. Since O-linked deglycosylation was not performed to confirm if the higher molecular weight was due to heavy glycosylation of the protein, it cannot be confidently concluded that the size difference is due completely to glycosylation.

Plasma has been shown to be a valuable vault for biomarkers. A 15-mer peptide mapped back to a predicted protein in the coccidioidal proteome. The transcript containing the peptide was amplified and showed that the annotated intron does not exist. This in itself shows that bioinformatic modeling of proteins may be erroneous and should appropriately be verified by experimentation.

This discovery along with the bench work confirmation indicates that a coccidioidal peptide has been detected by LC-MS/MS in patients with an active case of disseminated coccidioidomycosis. Since the Broad Institute BLAST indicates that the peptide maps back to a parent protein LC-MS/MS may be used as a valuable clinical tool in diagnosing pathogen presence long before an immune system response.

A more specific and selective diagnostic tool must be discovered for patients, especially those who are immunocompromised. Patient plasma can be used to detect peptides present in the host which can be mapped back to either the host or pathogen peptidome. These peptides, such as the 15-mer peptide P G L D S K S L A C T F S Q V reviewed in this thesis, can be used as biomarkers to show the presence of an active infection in patients with Coccidioidomycosis.

4. 2 Future Studies

To help define the PGLD parent protein and its function further studies should be performed. Since the fungal pathogen is known to be highly glycosylated it would be beneficial to perform O-linked deglycosylation and continue with a Western Blot. This would affirm or negate the hypothesis that the higher molecular weight is primarily due to glycosylation. In addition, further investigation should be done to verify the absence of CPAG_04641.1-predicted protein in *C. immitis* to determine if it is specific for *C. posadasii.*

Knowing functionality of the protein is essential in future investigations. Purification should be done by passing T27K through a PGLD Mab immunoprecipitation column. Purification and isolation would allow for LC-MS/MS on the parent protein giving a better annotation the amino acid sequence. Once purified the parent protein may be expressed to investigate functionality. Cloning into expression vectorss would allow us to determine the protein function. Cloning in itself would not necessarily assist in learning the function but would allow for further 'knock down' studies. Knocking down a gene and observing phenotypical differences can lead to understanding the function. Knowing the function can help in determining if the gene contributes to pathogenicity or if it can be manipulated to assist in treatment.

Lastly, genetic identification should be performed on the strains from both antigen preps T27K and CSS along with the techniques used to generate each. Knowing if and what the genetic difference is between the two would allow an investigator to choose the one more closely resembling that of the human pathogen activity. This would allow for

future in vitro studies which more closely resemble in vivo pathogen activity without harming the patient.

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