

A Proposed Vaccine for *Coccidioides immitis* in Dogs

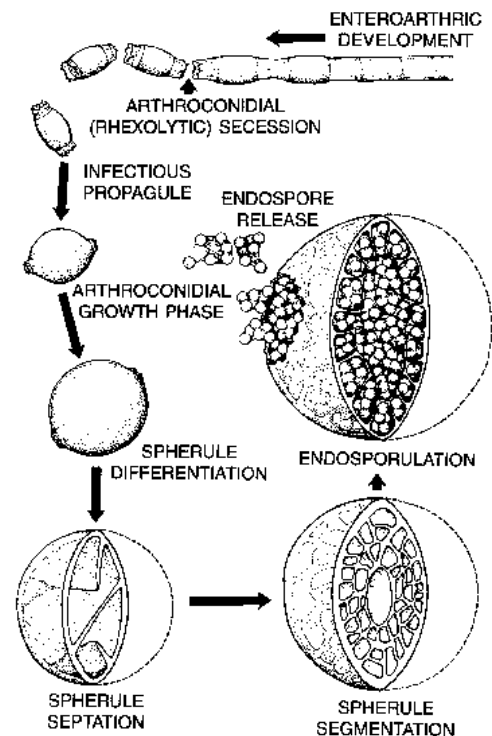
Literature Review:

Valley fever is a problem that continues to plague Southwestern United States. *Coccidioides immitis* is a fungus that lives in the soil. The fungus produces spores that, when the soil is disturbed, become airborne and are inhaled into the lungs. The resulting conditions are flu-like symptoms that can last for up to a month in humans. There is no difference between males and non-pregnant females as to incidence of infection, however pregnant women are more susceptible. Humans between the ages of 30 and 75 are the most often infected in Arizona (Saubolle, McKeller, and Sussland, 2007). According to the Arizona department of health services, the incidence of *Coccidioides immitis* has been steadily rising since it was first reported in the laboratory in 1997 (Arizona, 2007). In humans, the incidence of infection is most prevalent in the immunocompromised, women who are pregnant, and the African American as well as the Latino populations (Saubolle, McKeller, and Sussland, 2007).

Both humans and dogs are at risk for infection. When the soil is disturbed, spores are released into the air and inhaled into the lungs. Therefore, it naturally follows that those that reside in more dusty climates are more likely to become infected than those who do not (Saubolle, McKeller, and Sussland, 2007). Once the spores are inhaled, the fungus causes respiratory disease and can disseminate to other organs. Symptoms begin between 7-21 days past inhalation of the spores and can include fever, lethargy, cough and chest pain (Saubolle, McKeller, and Sussland, 2007).

In dogs, *C. immitis* is life threatening. As a result of their closer proximity to the ground and the constant sniffing in the dirt, dogs are even more susceptible to infection with *C. immitis* than humans. There are two main forms of *C. immitis* in dogs. In one form, the fungus resides in the lungs and in the second form, the infection begins in the lungs and disseminates to other organs. The form that does not disseminate causes fever, coughing, loss of appetite and loss of energy. The form that disseminates causes lameness, swollen joints, swollen lymph nodes and painful abscesses. According to a recent study, the disseminated form of the disease occurs in less than 5% of all symptomatic patients (Saubolle, McKeller, and Sussland, 2007). Furthermore, the occurrence of the disseminated form is much more prevalent in individuals who have the ability to mount an adequate Th2 immune response but have difficulty mounting a Th1 response to disease.

C. immitis is a dimorphic fungus that carries out a portion of its lifecycle in the soil. The phase of the lifecycle that is carried out in the soil is known as the saprobic phase, during which the fungus forms arthroconidia within a structure known as the hypha. These arthroconidia are released into the atmosphere when the soil is disrupted enough to rupture the hypha. The host, whether human or dog, becomes infected after inhaling the arthroconidia. The inhalation of the arthroconidia initiates the parasitic phase of the lifecycle,



during which the arthroconidia cluster in the lungs and develop into spherules (Kirkland and Fierer, 1996). The change from arthroconidium to spherule occurs because the pathogen recognizes the change in temperature (Saubolle, McKellar, and Sussland, 2007). The spherules undergo many internal divisions until they are filled with thousands of endospores, each of which has the ability to develop into a mature spherule upon the rupture of the spherule. This process of spherule development and maturation occurs over 48 to 72 hours (Kirkland and Fierer, 1996). The endospores can then be released into the bloodstream and move from the blood to other parts of the body, resulting in the disseminated form of the disease (Saubolle, McKellar, and Sussland, 2007).

The fungi have the ability to develop both virulence and morphologic forms which aid in its ability to multiply in the host. Some of the virulence factors include the ability of the capsule of the spherule to survive in heated or acidic conditions. Morphologic conditions include the ability to change into yeasts, hyphae and spherules. Since the lifecycle of the fungus includes these different morphological stages, the immune system of the host can have a difficult time fighting the disease (Kobayashi, 2002). Furthermore, the fungus has the ability to defend itself against the host's immune system by preventing phagosome-lysosome fusion and survive in the peritoneal macrophages while avoiding phagocytosis (Saubolle, McKellar, and Sussland, 2007). Even with all of the abilities of the fungus to survive in the host, dissemination is usually due to a lapse in the host defense system. The normal defense system has the ability to prevent dissemination. The immune system could lapse due to factors that inhibit the body's ability to fight diseases. These could include anything from immune compromising diseases to external stresses

In order to survive in the environment, *C. immitis* requires an area to be arid, light, slightly alkaline, a hot summer and a mild winter. The soil should be uncultivated with sparse vegetation, and only get 5-20 inches of rainfall per year (Kobayashi, 2002). These conditions are often found in the southwestern United States and Mexico.

Some previous treatments or vaccines include the FKS vaccine, the 27K vaccine that removed fluid from the spherule, and the 27K plus alum adjuvant vaccine that stimulated Th2 cells. The production of IL-10 was linked to the susceptibility of the individual to *C. immitis* (Fierer, Waters, and Walls, 2006). The production of IL-10 was found to increase in mice after infection, and the amount of IL-10 produced was related to how susceptible the mouse was to infection. Furthermore, the role of CD4 and CD8 T-cells were found to be an important component to vaccine induced immunity (Fierer, Waters, and Walls, 2006). Mice were administered with a mutant form of *C. immitis* which was temperature dependent, and four weeks later were given a virulent form of the disease. Spleen cells from vaccinated mice were then transferred to unprotected mice, and those who received CD4 or CD8 depleted cells had less TNF- α than those who received cells with CD4 and CD8 indicating that both CD4 and CD8 T cells provide protective immunity against coccidioidomycosis.

Description of Vaccine:

Coccidioidomycosis is an excellent target for a vaccine. First, *Coccidioides immitis* can elicit immunity. Dogs who recover from a benign or asymptomatic infection are resistant to a reinfection due to immunity induced by first infection. Second, the areas of potential infection are known. *C. immitis* lives in desert soil therefore it is geographically restricted. Finally, the target population is predictable. This population includes dogs that are genetically predisposed to

developing coccidioidomycosis, dogs that have a high probability of exposure due to their lifestyle (ex: outdoor versus indoor dog, etc.) or dogs that travel to these areas of potential infection (Cox and Magee, 2004).

In making a vaccine to *C. immitis*, it is important to consider which life cycle to target. *C. immitis* has two morphological cycles: the saprophytic cycle and the parasitic cycle. The vaccine in this proposal will contain elements from the parasitic cycle because parasitic forms induce better immunity than saprobic forms of *C. immitis* (Cox and Magee, 2004). The parasitic forms are also the structures that develop within the host. This vaccine will contain spherule antigens. In the parasitic cycle, arthroconidia or arthrospores grow into spherules which then continue to enlarge until they burst, releasing hundreds of endospores. Each endospore matures into a new spherule (Hung, Yu, Seshan, Reichard, and Cole, 2002). Spherules are the target for the vaccine because it is the last step before hundreds of potential infecting endospores are produced. If dogs inhale arthroconidia, the arthroconidia will become spherules, thus halting any further growth of an inhaled arthroconidia.

In developing a vaccine against the spherule form of *C. immitis*, it will be important to concentrate on increasing the ability of macrophages to phagocytize and destroy spherules. Spherules possess an extracellular fibrillar matrix that impedes physical contact with polymorphonuclear leukocytes (PMNL) and macrophages, thus allowing them to evade phagocytosis and subsequent death. In addition, there has been evidence that the fungus inhibits phagosome-lysosome fusion when phagocytized by macrophages. Therefore, it will be important to induce a good cell-mediated immune response, in order for the macrophages to be able to degrade the spherules, in addition to increasing the ability of macrophages to phagocytize spherules.

The first part of the vaccine will utilize an altered form of the 27K vaccine. The 27K vaccine is composed of the supernatant of whole killed spherules, of strain Silveira, after lysis and centrifugation combined with 3.5mg/ml concentrated saline. Both protein and carbohydrates are present in this vaccine, but the soluble vaccine is colorless, opalescent and devoid of intact visible fragments (Zimmermann, 1998). The previous 27K vaccine used alum as the adjuvant. This adjuvant triggers a Th2 response characterized by the secretion of the cytokines IL-4 and IL-5 and the generation of IgG1 and IgE. The Th2 response involves Th2 cells which are helper CD4 T-cells that stimulate B cells to produce antibodies. Given that alum triggers a Th2 response, it will enhance antibody production. The previous 27K vaccine was relatively successful in inducing protection against infection, even though the adjuvant alum induces a humoral immune response rather than a good cell-mediated immune response. However, the 27K vaccine without adjuvant has also shown limited success in inducing protection against the pathogen.

Since coccidioidomycosis is dependent on cell-mediated immunity for host defense, it requires a Th1 immune response to eliminate *C. immitis* (Cox and Magee, 2004). Therefore alum is not a good choice to induce a Th1 response. In order to improve the previous 27K vaccine, a new adjuvant that enhances a Th1 immune response should replace alum. With this 27K vaccine, QS-21 should be used as the adjuvant. QS-21 is a natural saponin taken from the bark of the Chilean tree *Quillaja saponaria*. It is a water-soluble triterpene glycoside that can be mixed with a soluble antigen to produce a fully soluble vaccine. QS-21 induces a Th1 response characterized by secretion of IL-2 and IFN- γ , the generation of IgG2a, and increased cytotoxic T-lymphocyte activity. The Th1 response involves Th1 cells which are inflammatory CD4 T-cells that activate macrophages. Using QS-21 will increase IFN- γ production, which will activate macrophages to

increase phagosome-lysosome fusion. The phagocytized spherule can not keep up with inhibiting this fusion, therefore it will be killed. Saponin adjuvants also work by intercalating into cell membranes and releasing antigens into the cytoplasm. Now that the antigen is in an “infected” cell, it can be degraded and presented on class I MHC. Cytotoxic T-cells will recognize the MHC class I and antigen peptide and then kill the infected cell. (Cox and Magee, 2004).

The 27K vaccine plus the adjuvant QS-21, will be injected intramuscularly in the left leg. This injection will induce immunity to the disseminated form of coccidioidomycosis, by inducing a systemic immune response. Now that the body is able to mount a sufficient cellular immune response to many possible antigens of the pathogen (since the initial vaccine was comprised of a cell extract of spherules) that can be presented on cells and APCs, a second vaccine will follow up as a booster. This booster will be given three months later to allow the first vaccine some time to induce immunity. This second vaccine will be a subunit vaccine comprised of recombinant spherule outer wall glycoproteins (SOWgp) from *Coccidioides* strains C634 and C735. SOWgp is a major parasitic cell antigen that has been strongly suggested to function as an adhesin, allowing the pathogen to colonize lung tissue. The antigen has been found to be expressed at detectable levels only during the parasitic cycle, with peak expressions occurring during the early stages of isotropic growth of the presegmented spherules (Cole, Hung and Delgado, 2002). It was shown that SOWgp was able to elicit protection from the pathogen in mice when challenged with fifty arthroconidia (Cox and Magee, 2004). Thus, even though the antigen is not present in detectable levels in the arthroconidia, it is still able to elicit an immune response against the spherule that the arthroconidia will become. The gene that encodes for the SOWgp antigen is highly conserved among *Coccidioides* isolates, with the exception of the number of tandem repeats found within the hydrophilic domain of the protein, and thus SOWgp isolates from two different strains are being used in the vaccine (Cole, Hung and Delgado, 2002).

This vaccine will be comprised of the lipid-rich, membraneous outer wall that spherules shed. This outer wall may be similar to the extracellular matrix that prevents phagocytosis of spherules by macrophages and neutrophils (Cox and Magee, 2004). This vaccine will be administered intranasally to induce immunity especially in the lungs where Valley Fever initiates. This vaccine only contains outer spherule proteins because the spherule surface is what is recognized by the lungs before phagocytosis and antigen presentation. With this vaccine, antibody production will increase because SOWgp induces a strong Th2 immune response. Th2 cells are helper CD4 T-cells that stimulate B cells to produce antibodies. Since the infection is found initially in the lungs, antibodies of isotype IgA would be present in the lungs, because they are heavily involved in mucosal immunity. A strong Th2 immune response alone could be detrimental to host immunity. Since the pathogen is able to inhibit the fusion of the phagosome with the lysosome when engulfed by a macrophage, any opsonization done by the antibodies produced from the Th2 response would be ineffective. Even if the IgA antibodies help macrophages take up the pathogen better, the macrophages cannot degrade the pathogen (Cox and Magee, 2004). However, since an effective Th1 response was already established with the initial vaccine, macrophages are activated and able to effectively mediate phagosome-lysozyme fusion. The purpose of this booster is to increase IgA antibody production: to help opsonize spherules, to prevent the pathogen from colonizing lung tissues by preventing adhesion, or to help activate complement. Overall, the action of these antibodies is to help assist macrophages in phagocytizing spherules.

The problem with the 27K vaccine with the QS-21 is that QS-21 has not been used in studies of *C. immitis* vaccines yet. Therefore, it may not be useful against *C. immitis*. QS-21

strongly induces a potent cytotoxic T-cell response, whereas cytotoxic T-lymphocyte activity has not been demonstrated in coccidioidomycosis. Our proposed vaccine may be triggering a CTL response where it is not necessarily needed. However, previous research supports the need of a Th1 immune response against *C. immitis*. QS-21 is a new proposed adjuvant that is trying to be FDA approved. It has not been put with the 27K vaccine yet. The main purpose of the QS-21 adjuvant is its ability to elicit a Th1 immune response despite its unnecessary CTL response. Also another disadvantage of QS-21 is that in large doses of 200 µg, it causes side effects, such as severe pain. To minimize the side effects, smaller doses less than 200 µg will have to be used (Cox and Magee, 2004).

The problem with the booster of SOWgp is that it is parasite-phase specific. If any saprobic forms are ingested, such as an arthroconidium, the immune system may not be able to mount as effective an immune response as it would against a spherule. In addition, the arthroconidia sometimes grow into hyphae (another saprobic form) inside the macrophages. This hyphae form will not be recognized by the immunity the booster has helped induce. Since both parts of the vaccine mostly concentrate on the spherule form of *C. immitis*, it could be problematic in having immunity against endospores and arthroconidia. Another problem that exists with both parts of the vaccine is that the vaccines are strain specific. It is unknown how many strains of *C. immitis* exist or how closely related the spherule forms of each are. These vaccines may not induce immunity against all strains of *C. immitis* (Cox and Magee, 2004).

Description of Immunity Assessment:

Vaccine administration will include an intramuscular injection of the 27K vaccine with the QS-21 adjuvant into the left quadriceps of the canine. This injection will establish an appropriate and fast-acting, systemic dissemination of the killed vaccine. Three months following the initial injection, an intranasal version of the vaccine will be administered. This intranasal booster will allow for the most direct route for immune responses (considering the infection starts by the inhalation of the spores of the fungus). However, this booster will not establish strong immune responses; it is merely a follow-up to a strong systemic infection produced by the initial vaccine injection, producing a strong immune response. After the two administrations of the vaccine, the immune system of the animal will then be able to provide a suitable defense to the infection.

In order to determine if the vaccine has produced the appropriate immune responses, an array of assays and diagnostic procedures will be performed. Latex agglutination (LA) assays can be done. This test will be able to detect antibodies of the IgM class mostly. Antibodies in the serum of patients infected with *C. immitis* will agglutinate the antigen coated latex particles contained in this system. Samples usually show positive results early in the course of primary infection and become negative within the few months of a chronic infection. Immunodiffusion (ID) assays can also be performed to examine the progression of the vaccine. Immunodiffusion assays can be used to detect antibodies of the IgG and IgA classes that have formed in response to antigens of *C. immitis*. This test will detect recent and active infections mostly. One such ID test is the Agarose Gel Immunodiffusion (AGID) assay. In this test, an antigen of interest (in this case the proteins of the spherule wall or its supernatant) and a serum sample from one of the test canines is added to adjoining wells of an agar plate. Antigen/antibody complexes form solid precipitates in the agar to indicate positive test results. However, AGID tests are not very

specific and its sensitivity of this test is questionable because it is based on one's ability to visually detect the results. This test is easy and inexpensive to perform and can be used on a great number of samples but, however, detects only the presence of antibodies for the antigen instead of providing more specific information. Positive AGID tests need to be followed by more in depth analysis.

Enzyme-Linked Immunosorbent Assay, or the ELISA test, elicits the use of two different antibodies. One antibody will detect the specific antigen present (the proteins used as antigens in the 27K vaccine) and the other is attached to an enzyme that catalyzes fluorogenic changes to indicate an antigen/antibody complex. This test would provide more specific results than an AGID test would. Complement fixation assays can also be done to detect if antibodies have been made in the patient's blood serum to the antigen added. If the test shows positive and antibody is present in the patient's serum it binds to the antigen, and the complement reagent is consumed.

A Fluorescent Immunoassay can be done not as a qualitative analytical test but to quantify the presence of antibodies. This will show the efficacy of protection of the vaccine. These tests can also be modified to show T-cell production. Flow Cytometry is a fluorescence assay that will easily perform these tasks. Dilutions of serum can be reacted with the same concentration of the *C. immitis* antigen attached to a fluorochrome.

Latex agglutination tests and AGID assays will be done 3 weeks after the booster is administered to monitor the presence of antibodies. Once antibodies are detected, ELISA tests and fluorescence assays will be done to provide more specific results of how well the vaccine is working. The ELISA, AGID, LA, and flow cytometry assays will be done every month afterward to follow the immune progress.

At the 3 month marker Hematologic changes will be recorded. With infections of *C. immitis* it is not uncommon to see cases of anemia, hyperglobulinemia, or hypoalbuminemia. An entire blood profile is not necessarily needed to evaluate status of infection, only a complete blood count should be necessary which will include hematocrit, total protein, white blood cell, differential and platelet readings. At 4 months after the booster is administered, cytology analysis will be performed on different areas of the respiratory system. Bronchial and tracheal washings will be conducted. These procedures include anesthetizing the animal, inserting an endotracheal tube and flushing out the lungs with a urinary catheter tube and immediately collecting the flushed sample. The animal will only be slightly anesthetized so as to still provide a cough reflex to recover more of the fluid. A diuretic will most likely need to be administered after the collection to dry up any excess fluid. Smears of the samples will be made and read immediately after collection to possibly discover any signs of the fungus. This method will only be used every six months due to the strain that will occur on the animal. Animals over 5 years of age will not be used for this experiment due to the danger of placing older animals under anesthesia.

Smears will also be made at the 4 month marker and every six months after that of alveolar and lymph node aspirates. Spherules are often found in lymph nodes. Skin, bone, and lung lesions should also be sampled and smeared if there is any presence of them. If they are present, at least 2 samples must be taken during the duration of the study. Any smears made must be stained with Papamicolaou's (PAP) stain. With this stain the spherules will display a purple/black capsule and a yellow cytoplasm. Endospores will produce a red/brown color. Neutrophils will often be found surrounding the spherules so careful scrutiny must be used when studying the smears.

If there should be any presence of abscesses, samples should be taken and a biopsy should be conducted as these abscesses are excellent places to find spherules. Bone lesion

biopsies are not worth evaluating as the lesion commonly fuses with the bone. The most common place to find a lesion, not surprisingly, is the lung. Careful physical examination of the animals should be done with every other analytical technique performed done to make sure that any abscesses present are found and studied.

Radiographs can sometimes be helpful in diagnosis of Valley Fever. However, bone and thoracic abnormalities take longer to detect by x-ray photography. To be on the safe side and to conduct thorough analysis, radiographs will be taken every 6 months after the booster is administered. Valley Fever can take up to 2 years to show bone abnormalities which is the reason that this study will be in the works for at least 2 years. Cultures of samples can also be grown, however results will not be seen until the third day of incubation.

For the vaccine to be considered successful, by the end of the study the animals should have high titers showing that antibodies have been made and are fighting off the fungal infection. Titers of 1:2 show infection and even titers of 1:4, 1:8, and 1:16 can show asymptomatic infection of *C. immitis*. The final immunological assays performed at the 2 year marker should show ample ability of the immune system to control the fungal infection. Smears of aspirates and lesions should not show any spherule infection. Biopsies should not display spherule infection. Complete blood counts should show no presence of anemia or any associated abnormalities and white blood cell counts should be in “healthy dog” range. Radiographs should show no bone abnormalities, thoracic lesions or respiratory infections. General behavior of the animal should be normal without any painful signs, limping, cough (or any other respiratory infection symptoms), skin abnormalities, seizures, etcetera. The animal’s appetite should be healthy and adequate.

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