2017

Quantitation of anti-Pythium insidiosum antibodies before and after immunotherapy in healthy dogs

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QUANTITATION OF ANTI-*PYTHIUM INSIDIOSUM* ANTIBODIES BEFORE AND AFTER IMMUNOTHERAPY IN HEALTHY DOGS

A Thesis

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
Requirements for the degree of
Masters of Science

in

The Interdepartmental Program in Veterinary Medical Sciences
through
The Department of Pathobiological Sciences

by
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B.S., University of Massachusetts Amherst, 2009
D.V.M., Louisiana State University School of Veterinary Medicine, 2014
August 2017

Acknowledgements

I would like to thank my family for all the love and support they have provided throughout my entire life. My grandparents planted the seed of curiosity in my life at a very young age and reminded me that the most important thing was your education as no one would be able to take that away. My grandfather, Frank, was the first to expose me to horses and thanks to him my life continued in the animal care and medicine field. Frank also provided me with the love of math and I owe him my ability to continue with biostatistics education. My mother, Carmen, was a pioneer in the study of HIV/AIDS in pregnant women and I owe my inquisitive nature to her. My father, Jose, taught me to take time to enjoy the little things, no matter how chaotic life is, to stop and be grateful for everything around you. My grandmother, Irma, always reminded me that no matter how much science you knew, there had to always be room for history, performing arts and literature. My grandfather, Lorenzo, taught me about patience and determination as no obstacle was too difficult to surpass. My grandmother, Maria Luisa, always reminded me of the value in science and development.

I would like to extend thanks to Michael for supporting me throughout this residency and graduate school endeavor. A special thanks to Nicole Arana for all her guidance and support while writing and the editing process.

I would also like to thank my best friend Filip for his effervescent personality and his love of life. Nothing could ever stop his will to live and to thrive. He reminds me every day that I need to fight in order to stay floating and there is never an option to succeed, that is the rule.

Many thanks and appreciation to all my committee members for their insight and guidance through my academic journey. Special thanks to my mentor Dr. Cherie Pucheu-Haston, for her dedication, support, patience, and humor. Her continual optimism and motivation allowed me to finish the thesis when others thought the road was too rough. Special thanks to my mentors Dr. Jon Fletcher and Dr. Rhett Stout for their guidance, encouragement, and compassion during my evolving projects in

graduate studies and residency. I appreciate that I had the liberty to make my own path but would offer me advice at any given time.

Special thanks to Michael Kearney for being my mentor in biostatistics and SAS programming. He is one of those professors that I aspire to be like in the future; kind, respectful, patient and passionate for his field. Additional thanks to Chin-Chi Liu for her technical expertise and assistance in ELISAs for this study.

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List of Abbreviations

PP - Percent positivity

IP - Immunotherapy product

CMI – Cell-mediated Immunity

DCs – Dendritic cells

ID- Immunodiffusion

ELISA - Enzyme-linked immunosorbent assay

Abstract

Pythium insidiosum is an aquatic oomycete that causes invasive, progressive granulomatous lesions of the skin in dogs, horses, and cats, and of the gastrointestinal tract in dogs. Quantitation of anti-*P. insidiosum* IgG antibodies can be used in dogs to both confirm a suspected diagnosis and to monitor response to therapy. Recently, an immunotherapeutic product (IP) has been marketed for the treatment of pythiosis in dogs, horses, and people. The aim of this study was to evaluate the effect of administration of this product on anti-*P. insidiosum* IgG concentrations in dogs. The IP was administered to seven, healthy hound mixes on days zero, seven and 21. Serum was collected on days zero, seven, 14, 21, 28, 35, 42, 49, and 56. Anti-*P. insidiosum* antibody concentrations were measured using a previously-described ELISA that utilizes a soluble mycelial-based antigen, with results reported as percent positivity (PP) in comparison to a strong positive control serum. Prior to immunotherapy administration, average PP was 7.45% +/- 3.02%. Following immunotherapy administration, there was no significant change in anti-*P. insidiosum* antibody concentrations, with PP values in all dogs remaining within the range expected for healthy dogs (3% - 15%) for the entire study period. In conclusion, the IP did not produce a significant change in anti-*P. insidiosum* IgG concentrations when administered to healthy dogs using the protocol suggested by the manufacturers. Further investigation will be required to determine whether a similar effect is observed in naturally infected dogs.

Chapter 1. Introduction and Literature Review

1.1 *Pythium insidiosum*

1.1.A Brief history

Pythium insidiosum is an aquatic oomycete of the kingdom Stramenopila, Class oomycetes, Order *Pythiales* and Family *Pythiaceae* [1]. It was historically called "bausette", from "bausett", the rainy season, by Veterinary Surgeon James Kerr in India in 1829; as a condition affecting horses "which appeared at the commencement of the rainy season and terminated as the dry season set in" [2]. The first cases were described as small tumors around the lips, face and scrotum [2]. If not treated, the tumors would "suppurate and become a *bausette* ulcer and from exposure to air become schirrous" [2]. It was thought that the smallest abrasion in susceptible horses was enough for the development and these would "rapidly degenerate into bausette, if exposed to air" [2]. At this time, the etiology for this condition was unknown. It wasn't until 1879 in the East Indies that Smith reported that "bursattee" originated in the subcutaneous tissue, referred to the tumors as "kunkur stones", and suggested the disease was caused by a mold-fungus [3]. In the United States in 1895, Dr. Pierre Fish wrote a historical account comparing "bursattee" of the East Indies and "leeches", a similar disease of horses and cattle in Florida, as identical in nature [2].

Around the early 1900's, de Haan and Hoogkamer described several cases of diseased horses in Indonesia and named the disease as "hyphomycosis destruens" [4, 5]. In 1961, Bridges and Emmons then described the etiologic agent as *Hyphomyces destruens*, a phycomycete (zygomycete) on the basis of its morphology in equine tissue as well as its broad, branched sparsely septate to coenocytic, non-sporulating mycelium in cultures [6]. At this time, the agent was unable to sporulate in the media used [6]. In 1974, Austwick and Copland reported the formation of biflagellate zoospores in isolates from horses with "swamp cancer" from Papua, New Guinea [7]. These were grown on Sabouraud dextrose agar and then transferred to an aqueous medium of rotten maize silage. From these results, they

concluded that *H. destruens* was a phycomycete belonging to the *Pythiaceae* in the *Peronosporales* and could be included in the genus *Pythium* Pringsheim [7].

It wasn't until 1980 that Chandler, Kaplan and Ajello proposed changing the to the more appropriate term "pythiosis" (also called: bursatii, Florida leeches, granular dermatitis, hyphomycosis destruens equi, phycomycosis, phycomycotic granuloma and swamp cancer) [8]. In 1980, Ichitani and Amemiya isolated a *Pythium* sp. from a Japanese case of equine pythiosis [9]. Based on the filamentous zoosporangia, smooth oogonia, and aplerotic (oospores not filling the oogonium), smooth oospores that it produced, it was considered part of the pre-existing species called *Pythium gracile* Schenk [9]. During a two-year study of pythiosis in Costa Rican horses, numerous isolates of a *Pythium* species were studied at the Centers for Disease and Control in Atlanta, Georgia [10]. A reliable and sensitive immunodiffusion test for diagnosing pythiosis was then developed by Mendoza, Kaufman, and Standard [11]. Using the immunodiffusion and fluorescent-antibody tests, Mendoza et al. demonstrated that isolates from the horses in Costa Rica; Papua, New Guinea; United States; and Japan; isolates from humans in Thailand; and isolates from dogs in the United States were identical [11]. Additional morphologic studies demonstrated the formation of zoosporangia in water, which led the researchers to conclude that this was a new species of *Pythium* [10]. De Cock, Mendoza, Padhye, Ajello and Kaufman finally described the etiologic agent for pythiosis as *Pythium insidiosum* [10].

1.1.B Organism

The organism's cell walls lack chitin and ergosterol but contain cellulose and β-glucan. In this manner, *P. insidiosum* is more closely related to algae than it is to fungi [1]. *P. insidiosum* incorporates sterols from the environment rather than producing them itself [1]. The sexual reproductive structures are oogonia and antheridia, and the asexual reproductive structures are zoospores [10]. It undergoes asexual reproduction, which is characterized by the production of "motile, reniform, biflagellate zoospores that develop by progressive cleavage within a vesicle that forms at the end of a discharge

tube produced by a zoosporangium" [10]. Its sexual reproductive structures are "characterized by intercalary, smooth, and subglobose oogonia; diclinous antheridia that produce a rigid fertilization tube from their tip; and oospores that are aplerotic and often pressed to one side of the oogonium" [10].

Due to the difficulties associated with induction of sporulation under laboratory conditions, the organism has been classified as a zygomycete or "phycomycete" [1]. *P. insidiosum* grows well at 37°C and produces motile biflagellate zoospores in water culture [1]. Since *P. insidiosum* isolates rarely produce sexual reproductive structures in the lab, presence and description of the hyphae, which are broad (mean of 4 µm; range 2-7 µm), rarely septate, and occasionally branching at right angles [1] help the clinician with diagnosis.

P. insidiosum is found in subtropical to tropical aquatic environments in Asia, Australia, and parts of Central and South America [12]. Most infections with *P. insidiosum* (pythiosis) in the United States are seen during the summer and fall along the Gulf of Mexico [12]. Areas with stagnant water (especially associated with flooding or hurricanes) are frequently associated with outbreaks of pythiosis.

1.1.C Pathogenesis

The infectious form of *P. insidiosum* is the motile biflagellate zoospore which usually infects by encysting in damaged skin or GI mucosa [1]. The zoospore has an anterior tinsel flagellum and a posterior whiplash flagellum [10]. These allow the zoospore to locate, move toward, and encyst on specific host tissues or other substrates [1]. Zoospores then use chemotaxis, electrotaxis, and auto-aggregation (i.e., dense accumulations of zoospores attract others, thereby increasing inoculum for infection) [1]. Once the zoospores contact an open wound, they "form a germ tube that mechanically penetrates tissues" [13]. Once into the tissues, *P. insidiosum*'s hyphae produce exoantigens that trigger an immune response with eosinophils, mast cells and sometimes IgE [13]. It has been proposed that the constant production of this exoantigen causes the host's immune response to develop a Th2 immunologic polarization [13]. However, no work to support this assertion has been performed. The

"overwhelming number of degranulated eosinophils (Splendore-Hoeppli phenomenon) and mast cells around the hyphae of *P. insidiosum* are primarily responsible for the extensive and rapid tissue damage encountered during pythiosis" [13]. Splendore-Hoeppli reaction is thought to be a localized immunological response to an antigen-antibody precipitate related to fungi, parasites, bacteria or inert materials [14]. The formation of the Splendore-Hoeppli reaction may inhibit phagocytosis and intracellular killing of the agent leading to persistence of infection [14]. This hypothesis is supported by the fact that viable hyphae of *P. insidiosum* have been found only inside the eosinophilic reaction or inside kunkers in equine infections, suggesting that *P. insidiosum* might use the Splendore-Hoeppli phenomenon and kunkers for survival [13].

1.1.D Species affected and clinical syndromes

Species commonly affected by *P. insidiosum* include dogs, humans and horses, although there have also been cases reported in other animals such as cattle, sheep, birds, as well as animals held in captivity [15]. Affected dogs and horses are generally immunocompetent and otherwise healthy overall [1, 13, 15]. In contrast, affected humans often suffer from some preexisting systemic illness, such as β-thalassemia [16].

Healthy dogs with access to water in the tropical and subtropical regions are commonly affected with *P. insidiosum* [12]. Usually infections appear to occur through damage of the skin, where the organism gains access while the animal is in flooded areas or when swimming [12]. The incidence is highest in young, male, large breed dogs, and lesions are most commonly on the limbs, perineum, tail, and head [17]. Pythiosis typically takes one of two clinical forms in the dog: cutaneous and gastrointestinal [1, 13, 15]. Cutaneous and gastrointestinal pythiosis seldom occur in the same patient [1].

Cutaneous lesions typically appear as non-healing wounds or invasive, progressively enlarging, pyogranulomatous masses that contain ulcerated nodules and draining tracts [17]. Regional

lymphadenopathy is often present and may reflect extension of infection rather than just reactive inflammation [1].

Gastrointestinal pythiosis is characterized by severe transmural thickening of the stomach, small intestine, colon, rectum, or, rarely, the esophagus [18]. Mesenteric lymphadenopathy is common, and the gastric outflow area and ileocecal junction are the most frequently infected portions [1]. It is not uncommon to find two or more segmental lesions in the same patient [1]. Most commonly, the submucosa is affected with variable mucosal ulceration and occasional extension through the serosal surfaces, which can result in adhesion formation and peritonitis [1]. Treatment of gastrointestinal pythiosis is challenging, as no treatment modality is 100% effective for all cases presented, and the prognosis is poor to grave [19]. It is best treated with wide (i.e., 3 to 4 cm margins) surgical excision, followed by prolonged courses (> 4 months) of antifungal chemotherapy [19]. Reports of survival have been documented in one case treated with partial gastrectomy, as well as another dog treated medically with a combination of itraconazole, terbinafine, and mefenoxam [19].

In horses, pythiosis can also be referred to as swamp cancer, leeches or summer sores [20]. The two most common presentations are cutaneous and subcutaneous, although gastrointestinal involvement has been described [20]. No predisposing factors (age, sex, and breed) have been described; however, immunocompetent horses exposed to warm, fresh water in swampy areas are at an increased risk of contracting the infection [20]. Transmission is through colonization of traumatic lesions by zoospore and/or hyphae of *P. insidiosum* [21] or by ingestion of *P. insidiosum*-contaminated water [22]. Lesions typically occur on the limbs and ventral abdomen and are often intensely pruritic. Patients may demonstrate mild to marked lymphadenopathy [20]. Lesions may demonstrate gritty, often branching yellow concretions (kunkers). Kunkers in horses are formed by degranulation of eosinophils over the invading hyphae of *P. insidiosum* [21]. In chronic infections (> 4 weeks), the infection may spread to the underlying bone and cause lameness [21]. The only place where the hyphae can be found in these

chronic infections is within the kunkers [20]. Though most cases of pythiosis in horses are cutaneous and subcutaneous, intestinal infections have also been reported [21, 22]. Intestinal infections consist of stenotic fibrous and disseminated gastrointestinal lesions [21]. Cases with intestinal pythiosis are associated with clinical signs of colic that can be associated with partial luminal obstruction [22].

The development of pythiosis in humans is rare in the United States, but is commonly seen in Southeast Asia. There are 4 types of pythiosis in humans: vascular (59%), ocular (33%), cutaneous/subcutaneous (5%) and miscellaneous forms (3%) [23]. In contrast to horses and dogs (which are typically otherwise healthy), humans with pythiosis are often immunologically compromised [23]. These patients suffer from diseases such as aplastic anemia, β-thalassemia, α-thalassemia, paroxsysmal nocturnal hemoglobinuria and Hemoglobin E disease, all caused by mutations in the *HBB* gene [16, 23, 24]. Patients typically have histories of exposures to rice fields or access to flooded or swampy areas for swimming. Clinical signs depend on the type of infection, but most vascular pythiosis patients present with chronic arterial insufficiency syndrome of the lower extremities, which varies from intermittent claudication to gangrenous ulceration [23].

1.1.E Methods of diagnosis

1.1.E.1 Histology and immunohistochemistry

The diagnosis of infection with *P. insidiosum* can be challenging. Histologic examination of biopsy material may demonstrate the presence of broad, irregularly branching hyphae. Although hyphae are difficult to visualize with H&E-stained sections, they may appear as hyphal-shaped "clear spaces" surrounded by a narrow band of eosinophilic material [1]. Hyphae are visualized easily in sections stained with Gomori's methenamine silver (GMS), but not with periodic acid-Schiff (PAS) [1]. Unfortunately, histologic examination alone cannot reliably distinguish the hyphae associated with *P. insidiosum* from those of some species of true fungi (e.g., *Basidiobolus*) [25].

Immunohistochemical techniques based on polyclonal antibodies have been developed for P. *insidiosum* by Brown [26] and Newton [27] and had been used as confirmatory tests [1]. However, these tests have been found to be cross-reactive with other species such as *Conidiobolus, Basiodiobolus* and *Lagenidium* hyphae [1]. Therefore, specificity of this antibody for the immunohistochemical diagnosis of pythiosis is questionable [1].

An additional factor complicating the diagnosis of gastrointestinal pythiosis is the depth of the infection. In these cases, granulomatous inflammation is often centered on the submucosal and muscular layers rather than the mucosa and lamina propria [1]. For this reason, the diagnosis can be missed on endoscopic biopsies that fail to reach deeper tissues [1]. Pythiosis should be considered a differential diagnosis when endoscopic biopsies reveal eosinophilic or pyogranulomatous inflammation without identification of an etiologic agent [1].

1.1.E.2 Culture

Pythium may be cultured from tissue, but the organism is somewhat susceptible to temperature extremes and may be killed by prolonged refrigeration, making transport inconvenient [25]. In addition, the use of inappropriate media and technique during culture can also lead to false negative results [25]. In addition to this, induction of sporulation, which can facilitate proper identification, can be difficult in a laboratory setting [25].

1.1.E.3 PCR

Although molecular diagnostics can identify *P. insidiosum*, these methods are not readily available to most practitioners in a clinical setting [25]. Nested polymerase chain reaction (PCR) based assays for identification of *P. insidiosum* can be applied to DNA extracted from cultured isolates or from appropriately preserved infected tissue samples [28]. When using these tissues, Grooters found that freezing samples at -70° C, or storing them at ambient temperature in 95% ethanol adequately

preserved DNA for subsequent amplification [1]. Due to the sensitivity of this assay, archival samples may be also tested [1].

1.1.E.4 ELISA

Perhaps the most practical method of diagnosis of *P. insidiosum*, especially for the dog, is by the demonstration of anti-*P. insidiosum* IgG antibodies using an enzyme-linked immunosorbent assay (ELISA) [25]. An ELISA utilizes an enzyme conjugated to an anti-canine IgG antibody [29]. This conjugate reacts with a colorless substrate to generate a colored reaction product, with the amount of color indicating quantity of the product [29].

This method also has the advantage of permitting evaluation of the course of treatment in dogs, as antibody levels generally rise and fall in conjunction with the host's pathogen burden [18, 30]. In general, concentrations increase with the progression or worsening of infection [19, 30].

The IgG ELISA used in this study has been previously described and evaluated in dogs and has a 100% specificity and sensitivity for pythiosis [25]. Results of the assay are expressed as percent positivity (PP) in comparison with a strong positive control sample, with values > 40% positivity having been shown to be 100% sensitive and specific for canine pythiosis and values in healthy dogs ranging from 3-15% [25].

1.1.E.5 Other assays

Agar gel immunodiffusion (AGID) detects precipitating antibodies in the serum of most equine and human patients with active pythiosis, but often is negative in affected dogs [1]. Western immunoblot analysis has been used successfully to demonstrate the ability of sera from Pythium-infected horses and dogs to recognize antigens of *P. insidiosum*, and it has the added advantage of high specificity and sensitivity [1]. However, this immunoblot technique is time and labor intensive compared to other techniques (e.g., ELISA) for the detection of antibodies in the serum [1].

1.1.F Prognosis and treatment

1.1.F.1 Traditional treatment

Treatment of pythiosis is challenging and consists of a multi-modal approach by wide-margin surgical excision (when possible) and antifungal chemotherapy for prolonged periods of time [25, 30, 31]. Although *P. insidiosum* resembles fungi in culture and in tissue, it is not a true fungus and contains neither ergosterol nor chitin in its cell walls [10]. As most antifungal drugs primarily target these molecules, medical management alone generally has limited efficacy against *P. insidiosum*. In addition, infections with the organism are typically very locally invasive, and often extend microscopically far further than their clinical appearance would suggest. For this reason, radical excision or amputation of the affected area is usually recommended. Unfortunately, diagnosis of pythiosis is often delayed as the lesions typically seen with *P. insidiosum* infections may easily be mistaken for other conditions, such as acral lick granulomas in dogs or "proud flesh" in horses. Thus, lesions may become very large and/or extensive by the time a definitive diagnosis is made.

A dramatic decrease in IgG concentrations has been seen in dogs and cats following successful surgical resection [1]. In contrast, antibody levels remain high in animals that develop recurrence following surgical treatment [1]. In this case, it is recommended to continue medical therapy with re-evaluation of the ELISA every 2-3 months [1]. Efficacy of treatment depends on location of the lesion (thoracic vs limb), type of infection (vascular, cutaneous, or visceral), chronicity of the infection and the individual patient's immune response (immune-compromised or immune-competent). It has been reported that long-term infections of more than 4 weeks have a worse prognosis than early detected and treated infections [32].

1.1.F.2 Immunotherapeutic Product development as newer form of treatment

Recently, an immunotherapeutic product (IP) has been developed and marketed for use in the treatment of pythiosis in horses, dogs and humans. The first IP for *P. insidiosum* was designed for

horses. This product used a whole cell, killed, sonicated hyphal preparation of *P. insidiosum* and was developed by Miller in the 1980's [33]. A positive response was observed within seven to 14 days after the first injection. However, swelling and sterile abscesses were frequently observed at the injection site [34]. A subsequent improved formulation was designed to have enhanced immunotherapeutic properties due to the addition of both secreted exoantigens as well as cytoplasmic antigens of *P. insidiosum* [13]. In studies conducted by Mendoza et al. the new IP formulation, containing an equal mixture of exoantigens plus cytoplasmic immunogens, has been reported to produce clinical cure of 72% of the 26 injected horses, including chronic cases [13]. These findings suggested that the addition of cytoplasmic antigens dramatically increased the IP's curative properties [13].

There have been several reports in which the IP has been administered to dogs. While some of these cases appear to have been successfully treated, the efficacy in canines seems to be less than that reported in horses [12, 19]. The majority of these cases have received both traditional therapy (medical or surgical) and the IP. Oldenhoff reported administration of the IP to a 4-year old German shepherd; however, new lesions formed despite the initiation of the IP. Traditional medical management (itraconazole and terbinafine) was added but the patient was later lost to follow-up [18]. Pereira reported a 2.5-year old Beagle in which intestinal pythiosis caused luminal obstruction, requiring exploratory laparotomy and biopsy. After surgery, the beagle was administered the IP in addition to terbinafine and itraconazole. The remaining affected area was completely resolved by 60 days [15]. Hensel reported a 4-year old Labrador retriever with a lesion on the carpus. After partial surgical excision, the retriever was administered the IP twice. The lesion had resolved by one year post treatment [17]. Dykstra published a report of 2 dogs which were treated using a combination of immunotherapy and medical management [35]. Neither animal responded to treatment and both were euthanized shortly after presentation. Schmiedt reported a 1.5-year mixed-breed dog with 1-month history of vomiting and anorexia, later diagnosed as intestinal *P. insidiosum* [19]. This patient was

treated by a subtotal colectomy, followed by administration of the IP. The patient achieved significant improvement by two months post-surgery [19]. In contrast, Mendoza reported two dogs which achieved a cure following administration of the IP alone, after prior medical management had failed [13].

1.1.F.3 Prognosis

Long-term prognosis in dogs infected with *P. insidiosum* depends on chronicity of infection, location of lesion and treatment modalities used. In general, the prognosis of chronic infections (> 2 months) is poor [13]. Medical management alone is generally thought to have limited efficacy, as *P. insidiosum* does not contain most of the structures targeted by traditional antifungal medications. For this reason, surgical excision is currently considered the mainstay of effective therapy, especially in the dog. It is suspected that only an amputation or radical surgical excision with wide-surgical margins can truly stop the spreading of the infection through the subcutaneous planes. Unfortunately, radical excision is difficult in cases where the lesions include the stomach and small intestine or in areas that are not surgically resectable, such as the hip or thorax.

Because of the limited efficacy of these traditional therapies, there has been an interest in the development of a product to direct and amplify the patient's own immunologic response to the organism. One attempt to achieve this has resulted in the development of the previously described IP. Unfortunately, valid assessment of this product is complicated due to the paucity of knowledge regarding the immune response against *P. insidiosum*, either with or without administration of the IP.

While serum concentrations of anti-*P. insidiosum* IgG have been measured after administration of this product in naturally-infected dogs, these measurements have not been performed at any consistent time (or in any consistent manner) after administration of the product. For this reason, it is unknown whether administration of this immunotherapy product (IP) can be expected to interfere with serologic-based monitoring techniques. Adoption of this technology has been avoided by many veterinary

clinicians because of concerns that it might interfere with serologic monitoring using IgG concentrations with an ELISA.

1.1.G Objectives

The specific objectives of this study are to characterize the IgG antibody responses of healthy dogs treated with a purified protein mixture made from *Pythium insidiosum,* using the manufacturer's suggested protocol, and evaluate IP site reactions. An additional goal was to obtain serum samples for future analysis of cytokine and other antibody isotype levels in serum. We hypothesize that repeated administration of this commercially available immunotherapeutic product will increase IgG concentrations over time following each administration of the IP in a population of healthy dogs. We do not expect significant injection site reactions in the dogs in this study.

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Chapter 2. Quantitation of Anti-*Pythium insidiosum* Antibodies before and after Immunotherapy in Healthy Dogs

2.1 Introduction

Pythium insidiosum is an aquatic oomycete that causes invasive, progressive granulomatous lesions of the skin in dogs, horses, and cats, and of the gastrointestinal tract in dogs. Although pythiosis has traditionally been observed most often in tropical and subtropical climates, over the past two decades it has been recognized in a broader area, including California [1] and Wisconsin [2] in the United States. Obtaining a definitive diagnosis may be challenging, as histologic findings are insufficiently unique to differentiate pythiosis from lagenidiosis, paralagenidiosis, and zygomycosis. Methods that have been used to confirm a diagnosis include IgG antibody serology [3] and culture followed by molecular confirmation of isolate identity by species-specific PCR or ribosomal RNA gene sequencing [4]. In addition to being used as a tool for initial diagnosis, IgG antibody serology has also been used to monitor response to treatment in dogs, with maintenance of high antibody concentrations post-surgery suggesting incomplete excision or early relapse [5]. Conversely, decreasing anti-*P. insidiosum* IgG concentrations have been observed in patients that are cured [6].

The most effective treatment for pythiosis is wide surgical excision, which is sometimes followed by antifungal chemotherapy. Unfortunately, complete surgical resection is often not possible because of lesion location, and the effectiveness of medical therapy alone is limited by the fact that ergosterol is not a major component of the oomycete cell membrane. As a result, alternative modes of therapy have been explored, including an immunotherapy product (IP) that was originally developed for and evaluated in horses, and which has subsequently been recommended for use in dogs and people. Although there is published evidence showing some efficacy in horses, [7, 8] efficacy in dogs has not been well evaluated and anecdotally appears to be poor [9]. In addition, although a mechanism of action for the product has been proposed, [10] there have been no studies designed to evaluate the effect of the IP on the immune response in any species.

In addition to a lack of information about the mechanism of action of the IP, information regarding its potential effect on post-treatment monitoring of anti-P. *insidiosum* IgG concentrations is limited. As a result, some clinicians avoid use of immunotherapy because of concerns that it may interfere with subsequent serologic monitoring. Although four individual canine cases have been described in which anti-*P. insidiosum* IgG concentrations were monitored following treatment that included immunotherapy, administration protocols (frequency, number, route) and post-IP administration sampling intervals varied widely [2, 6, 11, 12].

Therefore, the goals of this study were to evaluate the effect of IP administration on anti-*P. insidiosum* IgG concentrations in healthy dogs in order to better characterize the effect of IP administration on the canine humoral immune response and to provide initial information about the potential effect of IP administration on post-treatment serologic monitoring.

2.2 Materials and methods

Seven adult, female, purpose bred, intact hound-mixes from the Division of Laboratory Animal Medicine (DLAM) colony were enrolled in this study with a median of 7 +/- 2.73 years. Dogs were housed in either single or paired indoor AALAC-International-approved kennels, with controlled temperature and climate for the duration of the study. Temperature and relative humidity (RH) were measured using Onset Computer Corporation HOBO (Bourne, MA). Prior to the beginning of the study, all dogs were screened for preexisting health conditions by a thorough physical examination, complete blood count (CBC), biochemistry profile and urinalysis. All procedures described in this experiment were approved by the Institutional Animal Care and Use Committee of Louisiana State University School of Veterinary Medicine.

The IP was obtained from Pan American Veterinary Laboratories (Hutto, TX) and was kept refrigerated at 4° C until use. On days zero, seven and 21, the IP was administered as per manufacturer's instructions (1 mL subcutaneously; Figure one). Injection sites were rotated so as to not inject in the same site more than once (cranial left thorax, cranial right thorax and caudal left thorax). All administration sites were shaved and photographed pre- and post-injection to facilitate examination. Injection sites were monitored for pruritus, swelling, induration, erythema, erosion, ulceration, necrosis and discomfort. Body temperature was measured twice daily for seven days after every injection. A temperature of 103.5° F or above was considered to be elevated [13]. Full physical examinations were performed weekly for the duration of the study.

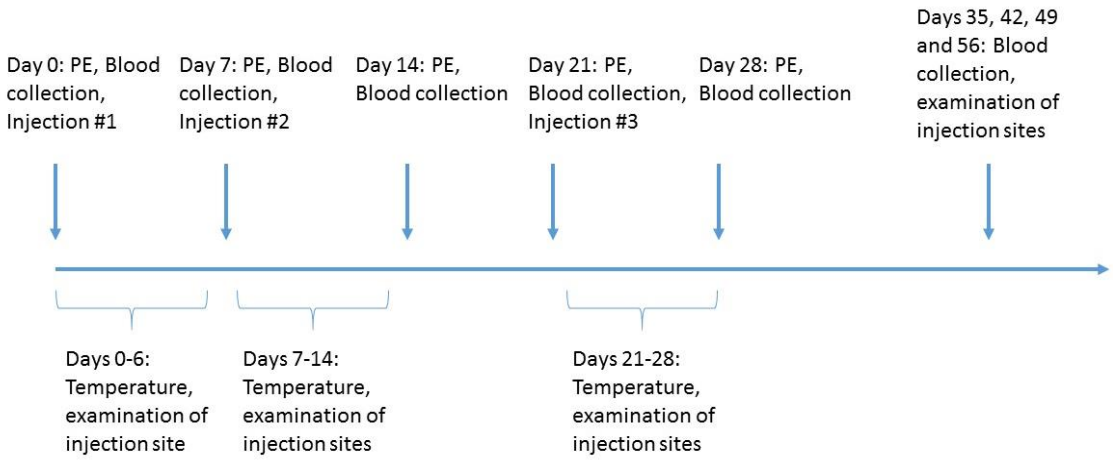

Figure 2.1. Timeline for the quantitation of anti-*Pythium insidiosum* IgG concentrations before and after immunotherapy in healthy dogs. Serum was collected every seven days until Day 56 and three injections of the immunotherapy product (IP) were administered on Day zero, seven and 21. Injection sites were evaluated after each IP and temperature was collected twice daily for seven days post-injection.

Blood collection (20 mL) via jugular or saphenous venipuncture was performed weekly (D0 until D56; Figure one). Serum was harvested and stored in 200 µL aliquots at -80° C until analyzed. An ELISA previously described for the serodiagnosis of pythiosis in dogs was used to measure anti-*P. insidiosum* IgG concentrations [3]. Briefly, 96-well microtiter plates (Immulon 2HB, Thermo Scientific, Rochester, NY) were coated overnight with a soluble mycelial antigen solution prepared from vortexed *P. insidiosum* cultures. Wells were washed with phosphate-buffered saline-0.05% Tween (PBST), and then blocked with bovine serum albumin in PBST (BSA-PBST). Sera were diluted in PBST (1:2000) and plated in quadruplicate wells. Bound anti-*P. insidiosum* IgG was detected using a horseradish peroxidase-conjugated anti-canine IgG (Rockland Antibodies and Assays, Limerick, PA) in BSA-PBST, followed by the addition of TMB two-component substrate (SeraCare, Milford, MA). Absorbance was measured at 450 nm using a BioTek Plate Reader (Epoch Microplate Spectrophotometer, Winooski, VT). Results were recorded as percent positivity (PP) relative to a strong positive control serum run in quadruplicate on each plate, calculated as: $\frac{Median\ optical\ density\ sample\ serum}{Median\ optical\ density\ reference\ serum}\ x\ 100\%$. A negative control of BSA-PBST was included on each plate.

Anti-*P. insidiosum* IgG concentrations were evaluated over time in comparison to baseline. Data was checked for normality using a Kolmogorov-Smirnoff Test, and was analyzed using a Repeated Measures Analysis of Variance (ANOVA) as a Randomized Block Design (RBD) on the plates. Animal ID was a random effect using Proc Mixed Procedure. Significance was set at $P \leq 0.05$. SAS Version 9.4 (SAS Institute Inc) was used for data analysis.

2.3 Results

Clinically significant adverse effects of the IP (including injection site induration, swelling and pruritus) were not observed. Body temperature remained normal in all dogs at all time points. Erythema was noted at 7/21 injection sites, but resolved within seven days of IP administration in all instances. No dog developed erythema at all three injection sites. Mild crusting was noted in three

dogs at either the first or the second injection site on Days 35 to 42, but these lesions resolved by Day 56 without treatment. These animals also had mild erythema associated with either site one (cranial left thorax) or site two (cranial right thorax) during the study. By day 56, injection sites were normal in all dogs.

Anti-*P. insidiosum* IgG concentrations remained within the previously-described reference interval during all time points of this study (Table 1, Figure 2), and no significant change in PP was observed over time ($p = 0.2814$; Figure 3, Table 1).

Overall, the PP demonstrated by the dogs was highly variable, and significant differences were observed between all of the dogs at all time-points (including at baseline; Figure 2). One of the seven dogs started with considerably higher baseline anti-*P. insidiosum* concentrations relative to the other six dogs (Dog D, Figure 2, Baseline PP = 13.35%) and remained at this level throughout the study. When this individual was removed from the model, the overall increase in PP was still not significant ($p = 0.1157$, Table 1).

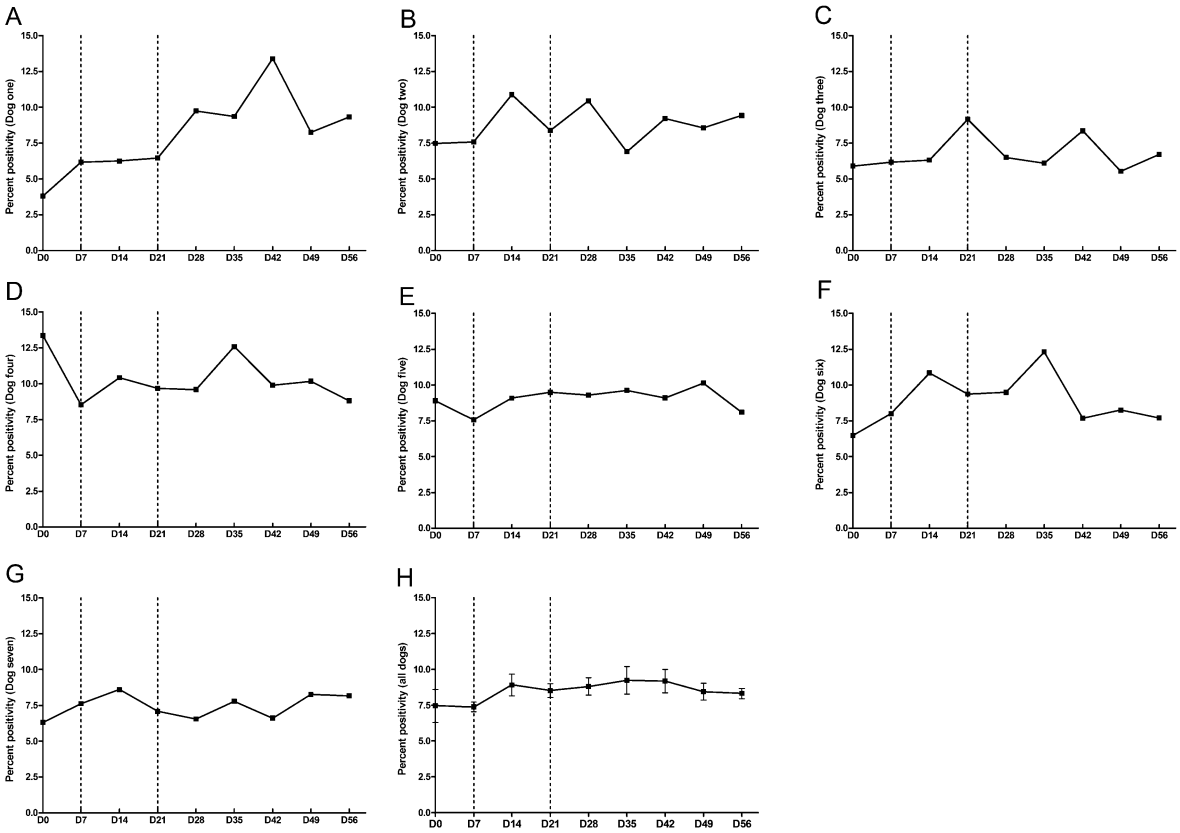

Figure 2.2 Anti-*P. insidiosum* IgG concentrations reported as percent positivity (PP) in individual dogs before and after immunotherapy.

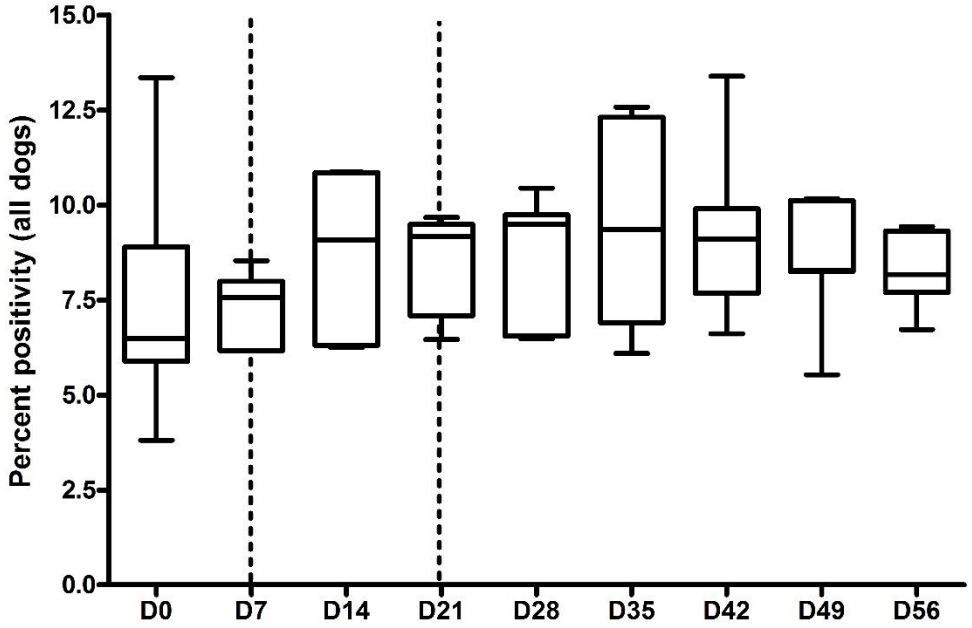

Figure 2.3: Median, quartiles and ranges of IgG PP (percent positivity) across time for anti-*Pythium insidiosum* antibodies before and after immunotherapy product (IP).

Table 1: Averages, standard deviation and standard error for IgG PP (percent positivity) across time for anti-*P. insidiosum* antibodies before and after immunotherapy product.

	Anti-*P. insidiosum* PP Data p > 0.2814			PP Data Excluding Dog 4 p > 0.1157		
Days	Mean	Standard Deviation	Standard Error	Mean	Standard Deviation	Standard Error
0	7.457	3.023	1.142	3.874	3.241	1.871
7	7.374	0.889	0.336	2.866	3.913	2.259
14	8.911	1.995	0.754	3.887	4.395	2.537
21	8.514	1.272	0.480	3.422	4.427	2.556
28	8.797	1.601	0.605	3.667	4.469	2.580
35	9.234	2.525	0.954	4.238	4.397	2.538
42	9.177	2.152	0.813	4.047	4.492	2.593
49	8.448	1.552	0.586	3.529	4.287	2.475
56	8.32	0.955	0.361	3.212	4.433	2.559

2.4 Discussion

Immunotherapy in the form of various *P. insidiosum* antigen extracts has been used for almost 40 years for the treatment of horses with pythiosis [14]. More recently, a commercially-available product has been marketed for the treatment of *P. insidiosum* infections in horses and dogs. This product is a combination of *P. insidiosum* hyphal and secreted antigens. There are several manuscripts suggesting that this immunotherapy has some efficacy against *P. insidiosum* infections in horses and humans [7].

The response to immunotherapy has been studied most thoroughly in the horse. In 2003, Mendoza reported resolution of pythiosis in 13 out of 18 horses using the IP evaluated in the current study [8]. These horses had previously failed to respond to topical medications or by surgical excision. A second manuscript by Mendoza claimed that approximately 60% of 600 horses had been successfully treated by immunotherapy, but specific details were not provided [10].

Immunotherapy has also been used with some success in humans. Administration of immunotherapy was associated with clinical cure in one 14-year old boy with vascular pythiosis who had previously failed to respond to antifungal or surgical therapy [15]. A subsequent case series demonstrated that four out of eight humans treated with the IP were "without clinical or radiographic signs of disease, i.e. arterial occlusion for about 24-30 months of follow-up" [16], while two patients showed a partial response. All of these patients had previously failed to respond to medical and/or surgical therapy. A later case series report described treatment success rates of 55.5% and 44.4% for vascular and ocular pythiosis, respectively [17]. However, these patients also received systemic (itraconazole and terbinafine, +/- voriconazole, ketoconazole or posaconazole) antifungal therapy. Furthermore, all of the vascular pythiosis cases had also been treated by radical surgical excision, with clinical cure observed only in patients in which clean surgical margins had been obtained.

In contrast, while there have been a small number of manuscripts demonstrating a good clinical outcome in canine patients receiving the IP (in conjunction with other therapy), anecdotally the clinical

efficacy of this product in this species appears to be poor [2, 6, 8, 11, 12, 18]. Of the 12 published reports in which immunotherapy was used for the treatment of canine pythiosis, only five had a favorable outcome. In one case series by Mendoza, immunotherapy alone was used to treat six dogs which had previously failed to respond to surgery or antibiotic or antifungal therapy [8]. Two of these dogs (one with intestinal involvement and one with cutaneous involvement) demonstrated clinical resolution of the disease. Clinical resolution was also reported in one mixed breed dog with intestinal pythiosis (which had also been treated by subtotal colectomy as well as a combination of itraconazole and terbinafine); in one beagle with intestinal pythiosis (which had also received a combination of itraconazole and terbinafine) and one dog with cutaneous pythiosis [6, 11, 12]. The remaining cases (all with cutaneous disease) failed to demonstrate clinical improvement following immunotherapy administration [2, 18].

The development of an effective immunotherapeutic product should ideally be based upon a thorough understanding of the immunologic response to the organism in question. Although it is beyond the scope of this manuscript to provide a detailed summary of the current knowledge of antifungal immunity, it is possible to include a brief discussion of some of the key features. Effective antifungal immunity is dependent upon a complex interaction between cells and elements of the innate and adaptive immune systems. For most fungi, the development of a Th1 immune response appears to be the most critical for effective elimination of the pathogen [19-21]. These responses are characterized by a robust cell-mediated response, in which phagocytes (especially macrophages) become strongly activated, increasing their rate of phagocytosis and their ability to kill phagocytized organisms [19, 21]. Other cells may also participate in the cell mediated response, including natural killer cells, which may induce apoptosis and elimination of infected cells, and which may possibly be able to directly damage extracellular fungi [22]. Th17 immune responses may also play a role in antifungal defense. These are characterized by the recruitment and activation of neutrophils, which can both phagocytize and kill

small fungal elements directly as well as damage or inhibit larger fungal elements via the elaboration of neutrophil extracellular traps [21, 22]. While the critical role of Th1 and Th17 effector elements is well established for most fungal infections, the relevance of Th2-mediated anti-fungal antibody responses appears to be more variable and less certain. Although antibodies do appear to play a significant role in the defense against certain organisms (namely *Aspergillus*), their role in effective defense against other organisms (such as *Candida*) is less certain and may even be counterproductive [19].

In contrast to many true fungal pathogens (such as *Candida and Aspergillus*), there is a paucity of knowledge regarding the immunologic processes associated with either the development of pythiosis or with its resolution. In dogs and horses, infection with *P. insidiosum* has been associated with development of anti-*P. insidiosum* IgG antibodies, and resolution of infection is typically associated with a decrease in antibody concentrations [3, 23, 24]. However, it is unknown what role (if any) these antibodies play in clearance of infection.

Clinical pythiosis is frequently referred to as a Th2 "polarized" immune response, while successful treatment of *P. insidiosum* infections is commonly attributed to a Th1 immune response. Although these statements are frequently repeated in the literature, there are no published data regarding the response of immune cells or secreted factors to infection with *P. insidiosum*, and it may or may not be valid to extend assumptions based upon knowledge of antifungal immune responses to this non-fungal organism. Evidence cited in support of this assertion includes the observation that histologic lesions induced by *P. insidiosum* are generally characterized by eosinophilic inflammation, while resolving lesions typically contain fewer eosinophils and larger numbers of macrophages and lymphocytes. There is a single published report of a human patient demonstrating a relative decrease in serum *P. insidiosum*-specific IgE as well as interleukins 4 (IL-4) and IL-5, and a relative increase in IL-2 after successful therapy [16]. However, no similar studies have been performed in non-human species.

While it is true that these observations might be consistent with a switch from a Th2 to a Th1-polarized immune response, this evidence is far from definitive.

In the current study, administration of the IP to healthy dogs was not associated with a significant change in anti-*P. insidiosum* IgG concentrations. One potential explanation is that the IP may simply fails to induce a productive, effective immune response in this species. This idea would be supported by clinical observations that the IP is fairly ineffective for the treatment of pythiosis in dogs. Given the relatively higher clinical response rate to the IP in horses and humans in comparison to dogs it would be interesting to determine whether there are significant IgG responses to IP administration in these species. Another, perhaps more likely, explanation would be that the IP generates a predominantly cell-mediated rather than humoral response.

The current work does have some limitations. The first involves the relatively small sample size. In general, anti-*P. insidiosum* IgG concentrations (both pre- and post-IP administration) demonstrated significant variability between individuals, although still remaining within the reference interval for healthy dogs.

Perhaps a more significant limitation was that this study was performed on healthy dogs with no known exposure to *P. insidiosum*. While this selection was necessary to determine the expected antibody response to the IP under controlled conditions, our results might not necessarily reflect the results seen in naturally infected dogs. It is possible that natural infection would produce enough immunologic "priming" that subsequent challenge with the IP would be associated with a significant rise in anti-*P. insidiosum* IgG concentrations. Further investigation would require administration of the IP to infected animals. However, under these circumstances, it may be difficult to determine the relative impact of the IP versus that of the infection itself.

In conclusion, this study demonstrated that administration of a commercially-available *P. insidiosum* immunotherapeutic product does not produce a significant increase in anti-*P. insidiosum* IgG

concentrations when administered to healthy dogs using the protocol suggested by the manufacturers. These results may suggest a failure of the product to induce a productive immune response in this species, or might indicate that factors other than IgG are responsible for the resolution of *P. insidiosum* infections in the dog. Regardless, the lack of impact on serum anti-*P. insidiosum* IgG levels suggests that administration of the IP would not be expected to interfere with subsequent serologic monitoring in affected dogs. However, further evaluation of the antibody responses to this product in naturally affected dogs will be required before a firm conclusion can be prudently drawn.

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Chapter 3. Conclusions

Pythium insidiosum is an aquatic oomycete found in tropical, subtropical and some temperate climates worldwide, especially in the Gulf Coast States [1]. Pythiosis is characterized by severe, progressive cutaneous or gastrointestinal disease [1] in dogs, horses and sometimes humans. Treatment for this infection typically consists of surgical intervention combined with prolonged antifungal treatment. Unfortunately, traditional antifungal medications have limited efficacy due to the lack of ergosterol and chitin in *P. insidiosum's* cellular wall, as the organism is more closely related to algae than to fungi.

Recently, and immunotherapeutic product (IP) has been developed for use in horses, dogs and humans. The IP evaluated in this study is a modification of Miller's immunotherapy [2] and contains both cytoplasmic antigens and secreted exoantigens from *P. insidiosum* [3]. Although this product appears to be efficacious in horses and in some humans, anecdotally the response in dogs has been poor. Evaluation of the efficacy of this product has been limited by several factors, including a lack of information regarding its mechanism of action. In addition, many veterinarians have been hesitant to use this product, as it is unknown whether administration of the IP affects anti-*P. insidiosum* IgG antibody concentrations, which are commonly used to monitor the efficacy of treatment [4].

In this study, administration of the IP did not induce significant changes in anti-*P. insidiosum* IgG antibody concentrations, nor were significant injection site reactions observed in the dogs used in this study. The lack of a response in IgG antibody concentrations might suggest that administration of this product does not induce the development of a productive immune response in the dog. Alternately, the product might be more effective at producing a cell-mediated response rather than a humoral response. Investigation of this hypothesis might include the determination of whether an IgG response is generated after administration of this product to horses, in which the clinical response to this product is better documented.

Future studies include the characterization of the IgG antibody responses in healthy horses as well as determination of the responses of other antibody isotypes such as IgE. Another area of future research would include determination of serum cytokine profiles after administration of this product, as well as in naturally infected animals at different stages of infection. There is a single report of a human patient demonstrating a relative decrease in anti-*P. insidiosum* serum IgE concentrations as well as IL-4 and IL-5, and a relative increase in IL-2 after successful therapy [5].

In conclusion, this study demonstrated that administration of a commercially-available *P. insidiosum* immunotherapy product does not produce a significant increase in anti-*P. insidiosum* IgG concentrations when administered to healthy dogs using the protocol suggested by the manufacturers. These results suggest that administration of the IP would not interfere with subsequent serological monitoring in affected dogs. However, further evaluation of the antibody responses to this product in naturally infected dogs will be required before a firm conclusion can be prudently drawn.

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Vita

Carmen Beatriz Arsuaga-Zorrilla was born in San Juan, Puerto Rico. She graduated from Cupeyville School in 2005. After graduation, she attended University of Massachusetts Amherst where she graduated in 2009 with a Bachelor of Science in Veterinary and Animal Sciences. After undergraduate school, she moved back to Puerto Rico for a year and worked as a Laboratory Assistant in the Laboratory of Parasite Immunology and Pathology in University of Puerto Rico Medical Sciences Campus in San Juan. She continued her studies at Louisiana State University School of Veterinary Medicine where she obtained her Doctorate in Veterinary Medicine in 2014. Immediately following graduation, Carmen began a residency in Laboratory Animal Medicine within the Division of Laboratory Animal Medicine (DLAM) and Master's in Biomedical and Veterinary Sciences at Louisiana State University School of Veterinary Medicine. She completed her programs in 2017.