Quantitation of anti–*Pythium insidiosum* antibodies before and after administration of an immunotherapeutic product to healthy dogs

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OBJECTIVE
To evaluate the effect of an immunotherapeutic product on concentrations of anti–*Pythium insidiosum* antibodies in dogs.

ANIMALS
7 healthy hound-crossbreds.

PROCEDURES
Antibody concentrations were evaluated before (day 0) and after administration of the immunotherapeutic product. The immunotherapeutic product was administered on days 0, 7, and 21. Serum was obtained on days 0, 7, 14, 21, 28, 35, 42, 49, and 56. Anti–*P insidiosum* antibody concentrations were measured and reported as the percentage positivity relative to results for a strongly positive control serum.

RESULTS
Mean ± SD percentage positivity before administration of the immunotherapeutic product was 7.45 ± 3.02%. There was no significant change in anti–*P insidiosum* antibody concentrations after administration of the product, with percentage positivity values in all dogs remaining within the range expected for healthy dogs (3% to 15%).

CONCLUSIONS AND CLINICAL RELEVANCE
Administration of the immunotherapeutic product to healthy dogs in accordance with the manufacturer's suggested protocol did not induce a significant change in anti–*P insidiosum* antibody concentrations. These results suggested that administration of the immunotherapeutic product may not interfere with postadministration serologic monitoring. However, further investigations will be required to determine whether there is a similar effect in naturally infected dogs. (*Am J Vet Res* 2018;79:1160–1165)

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 historically been observed most often in tropical and subtropical climates, over the past 2 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 because 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 serologic testing[3] and microbial culture followed by molecular confirmation of isolate identity by use of species-specific PCR assay or ribosomal RNA gene sequencing.[4]

In addition to use as a tool for initial diagnosis, IgG antibody serologic testing has also been used to monitor response to treatment in dogs, with maintenance of high antibody concentrations after surgery suggesting incomplete excision or early relapse.[5] Conversely, decreasing anti–*P insidiosum* IgG concentrations have been detected in patients that are cured.[6]

ABBREVIATIONS

BSA	Bovine serum albumin
IL	Interleukin
PBST	PBS solution and 0.05% Tween
Th	T-helper

The most effective treatment for pythiosis is wide surgical excision, which is sometimes followed by antifungal chemotherapeutics. Unfortunately, complete surgical resection is often not possible because of lesion location, and the effectiveness of medical treatment alone is limited by the fact that ergosterol is not a major component of the oomycete cell membrane. As a result, alternative modes of treatment have been evaluated, including an immunotherapeutic product originally developed for use in horses that subsequently has been recommended for use in dogs and humans. Although there is evidence indicating some efficacy of that product 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 conducted to evaluate the effect

of the immunotherapeutic product on the immune response in any species.

In addition to a lack of information about the mechanism of action of the immunotherapeutic product, information regarding its potential effect on posttreatment monitoring of anti–*P insidiosum* IgG concentrations is limited. As a result, some clinicians avoid use of immunotherapeutics because of concerns that they may interfere with subsequent serologic monitoring. Although 4 dogs have been described in which anti–*P insidiosum* IgG concentrations were monitored after treatment that included immunotherapeutics, administration protocols (frequency of administration, number of administrations, and route of administration) and sampling intervals after product administration differed widely.[2,6,11,12]

Therefore, the objectives of the study reported here were to evaluate the effect of administration of an immuotherapeutic product on anti–*P insidiosum* IgG concentrations in healthy dogs to better characterize the effect of product administration on the canine humoral immune response and to provide initial information about the potential effect of product administration on posttreatment serologic monitoring. We hypothesized that administration of the immunotherapeutic product would result in significant increases in anti–*P insidiosum* IgG concentrations.

Materials and Methods

Animals

Seven adult sexually intact female purpose-bred hound-crossbreds were enrolled in the study. Median ± SD age of the dogs was 7 ± 2.73 years. Before the study began, all dogs were deemed healthy on the basis of results from a thorough physical examination, CBC, serum biochemical analysis, and urinalysis. Dogs were housed alone or in pairs in indoor kennels with controlled temperature and humidity for the duration of the study. All procedures were approved by the Institutional Animal Care and Use Committee at the Louisiana State University School of Veterinary Medicine.

Procedures

The immunotherapeutic product[a] was refrigerated at 4°C until use. Antibody concentrations were evaluated before (day 0) and after dogs received the immunotherapeutic product. On days 0, 7, and 21, the product was administered as per manufacturer's instructions (1 mL, SC). Injection sites were changed (cranial portion of the left thorax, cranial portion of the right thorax, and caudal portion of the left thorax) so that the product was injected into the same site only once. Hair over each administration site was shaved prior to the injections, and photographs were obtained before and after injection to facilitate examination. Injection sites were monitored for pruritus, swelling, induration, erythema, erosion, ulceration, and necrosis and evidence that the site caused the dog discomfort daily for 7 days after each injection. Rectal temperature was measured twice daily for 7 days after each injection; temperatures ≥ 39.7°C were considered to be elevated.[13] Thorough physical examinations were performed weekly for the duration of the study. All procedures were performed at the Louisiana State University School of Veterinary Medicine vivarium.

Blood collection (20 mL) via jugular or saphenous venipuncture was performed weekly (days 0, 7, 14, 21, 28, 35, 42, 49, and 56). Serum was harvested and stored at –80°C until analyzed. An ELISA previously described for the serodiagnosis of pythiosis in samples obtained from dogs was used to measure anti–*P insidiosum* IgG concentrations.[3] Briefly, 96-well microtiter plates[b] were incubated overnight (18 hours) with a soluble mycelial antigen solution prepared from vortexed *P insidiosum* cultures. Wells then were washed with PBST and blocked with BSA-PBST. Sera were diluted in PBST (1:2,000) and plated in quadruplicate wells. Bound anti–*P insidiosum* IgG was detected by use of horseradish peroxidase–conjugated anti-canine IgG[c] in BSA-PBST, followed by the addition of a 2-component substrate.[d] Absorbance was measured at 450 nm.[e] Results were recorded as the percentage positivity relative to a strongly positive control serum sample assayed in quadruplicate on each plate. Percentage positivity was calculated as (median optical density of a serum sample/median optical density of the strongly positive control serum sample) × 100. A negative control sample that consisted of BSA-PBST was included on each plate.

Statistical analysis

Anti–*P insidiosum* IgG concentrations were evaluated over time and compared with values for day 0. Data were evaluated for normality by use of the Kolmogorov-Smirnov test; data were analyzed by use of a repeated-measures ANOVA[f] as a randomized block design on the plates. Animal was included as a random effect. Significance was set at $P \leq 0.05$.

Results

Clinically important adverse effects attributable to injection of the immunotherapeutic product (including induration, swelling, and pruritus at the injection site) were not observed. Rectal temperature remained within reference limits in all dogs at all time points. Six dogs developed transient erythema at the injection site; 5 of these 6 dogs developed erythema only once (1 after the first injection, 2 after the second injection, and 2 after the third injection), whereas 1 dog developed erythema after 2 injections (both the first and second injections). There appeared to be no obvious relationship between the injection number and whether erythema developed. Erythema developed 8 hours to 4 days after the injection; erythema was considered mild to moderate, with a duration of 4 to 8 days. Necrosis or ulceration was not detected at any injection site. A small crust was noticed at the site of the first injection in one dog

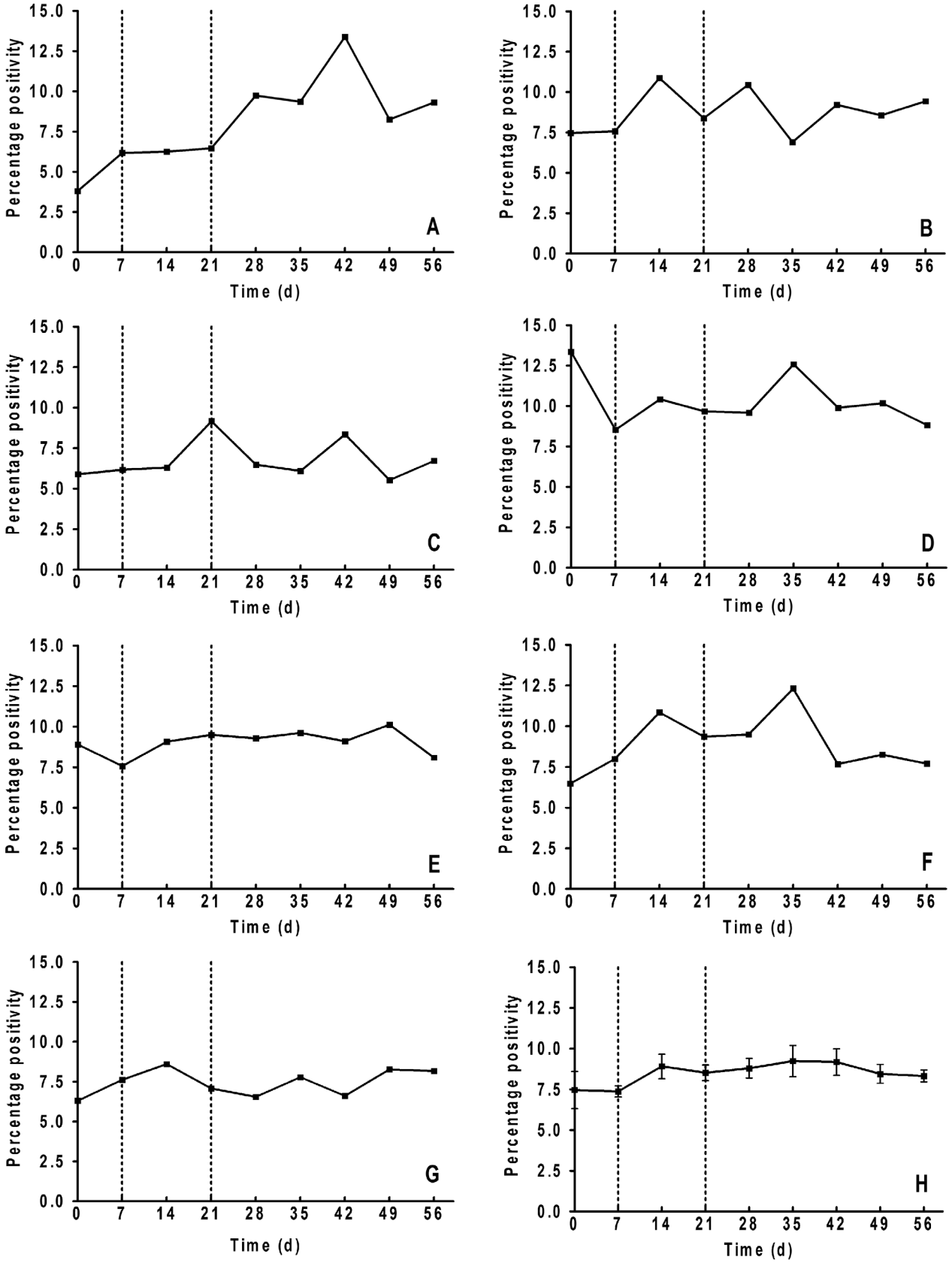

Figure 1—Serum anti–*Pythium insidiosum* antibody concentrations for each of 7 dogs (A through G) and mean ± SD antibody concentration for all 7 dogs (H) before (day 0) and after they received an anti–*P insidiosum* immunotherapeutic product on days 0, 7, and 21 (vertical dotted lines). Results represent percentage positivity relative to that of a strongly positive canine serum.

(day 42), at the sites of the first and second injections in another dog (day 35 for both), and at the site of the second injection in a third dog (day 35).

Anti–*P insidiosum* IgG concentrations remained within a previously described reference interval[3] (percentage positivity, 3% to 15%) in all dogs at all time points of the study **(Figure 1)**. No significant change in percentage positivity was detected over time.

Overall, percentage positivity for the dogs was highly variable, and significant differences were observed among the dogs at all time-points (including on day 0; Figure 1). The anti–*P insidiosum* IgG concentration of 1 dog was considerably higher on day 0 (percentage positivity, 13.35%), relative to that of the other 6 dogs, and remained at that concentration throughout the study. Removal of data for that dog from the statistical analysis did not result in a significant overall increase in the percentage positivity for the remaining 6 dogs.

Discussion

Various *P insidiosum* antigen extracts have been used for the treatment of horses with pythiosis for almost 40 years.[14] More recently, a commercially available product has been marketed for the treatment of horses and dogs with *P insidiosum* infections. This product is a combination of hyphal and secreted antigens of *P insidiosum*.[8,15] There is evidence to suggest that this immunotherapeutic product has some efficacy against *P insidiosum* infections in horses and humans.[7]

The response to immunotherapy has been studied most thoroughly in horses. In a 2003 study,[8] pythiosis resolved in 13 of 18 horses that received the immunotherapeutic product evaluated in the present study. The horses of that study[8] had previously failed to respond to topical medications or treatment via surgical excision. In another study,[10] investigators claimed that approximately 360 of 600 (60%) horses were successfully treated by use of immunotherapy, but specific details were not provided.

Immunotherapy has also been used with some success in humans. Administration of immunotherapeutics was associated with clinical cure in a 14-year-old boy with vascular pythiosis who had previously failed to respond to antifungal or surgical treatment.[16] In a subsequent case series of affected humans,[17] 4 of 8 patients treated with the immunotherapeutic product were without clinical or radiographic signs of disease (ie, arterial occlusion) for approximately 24 to 30 months after treatment, and 2 other patients had a partial response. All of these patients had previously failed to respond to medical or surgical treatment.[17] In another case series,[18] authors reported a treatment success rate of 55.5% and 44.4% for vascular and ocular pythiosis, respectively. However, these patients also received systemically administered antifungal treatments (itraconazole and terbinafine, with or without voriconazole, ketoconazole, or posaconazole). Furthermore, all the patients with vascular pythiosis had also been treated via radical surgical excision, with clinical cure observed only in patients in which clean surgical margins had been obtained.[18]

In contrast, although there have been a small number of reports of a good clinical outcome in canine patients receiving the immunotherapeutic product (typically in conjunction with other treatments), the clinical efficacy of this product in dogs appears anecdotally to be poor.[2,6,8,11,12,19] Of 12 reports in which immunotherapeutics were used for the treatment of pythiosis in dogs, a favorable outcome was evident in only 5 dogs.[6,8,11,12] In a case series,[8] an immunotherapeutic product was used alone to treat 6 dogs that had previously failed to respond to surgery or antimicrobial treatment. Two of the 6 dogs (1 with intestinal involvement and 1 with cutaneous involvement) had clinical resolution of the disease. In other reports, clinical resolution was also detected in 1 mixed-breed dog with intestinal pythiosis (which had also been treated by means of subtotal colectomy as well as with a combination of itraconazole and terbinafine),[6] 1 Beagle with intestinal pythiosis (which had also received a combination of itraconazole and terbinafine),[11] and 1 dog with cutaneous pythiosis.[12] Other dogs (all with cutaneous disease) failed to have evidence of clinical improvement after administration of immunotherapeutics.[2,19]

Development of an effective immunotherapeutic product should ideally be based on a thorough understanding of the immunologic response to the organism. It was beyond the scope of the present report to provide a detailed summary of the current knowledge about antifungal immunity; however, some of the key features should be mentioned. Effective antifungal immunity is dependent on a complex interaction between cells and elements of the innate and adaptive immune systems. For most fungi, development of a Th1 immune response appears to be the most critical factor for effective elimination of a pathogen.[20–22] These responses are characterized by a robust cell-mediated response in which phagocytes (especially macrophages) become strongly activated, which increases their rate of phagocytosis and their ability to kill phagocytized organisms.[20,22] The cell-mediated response may involve other cells (including natural killer cells), which may induce apoptosis and elimination of infected cells and may possibly be able to directly damage extracellular fungi.[23] The Th17 immune responses may also play a role in antifungal defense. These responses are characterized by the recruitment and activation of neutrophils, which can phagocytize and kill small fungal elements directly as well as damage or inhibit larger fungal elements via the elaboration of neutrophil extracellular traps.[22,23] Whereas the critical role of Th1 and Th17 effector elements is clearly established for most fungal infections, the relevance of Th2-mediated antifungal antibody responses appears to be more variable and less certain. Although antibodies appear to play an

important role in the defense against certain organisms (namely *Aspergillus* spp), their role in effective defense against other organisms (eg, *Candida* spp) is less clear, and they may even be counterproductive.[20]

In contrast to information for many true fungal pathogens (eg, *Candida* spp and *Aspergillus* spp), there is a paucity of information regarding the immunologic processes associated with 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.[3,24,25] However, the role (if any) that these antibodies have in clearance of infection is unknown.

Clinical pythiosis is frequently referred to as a Th2-polarized immune response, whereas successful treatment of *P insidiosum* infections is commonly attributed to a Th1 immune response.[8] Although these statements are frequently repeated in the literature, the authors are currently not aware of any published data regarding the response of immune cells or secreted factors to infection with *P insidiosum*, and it may not be valid to extend assumptions about immune responses to this nonfungal organism on the basis of knowledge about antifungal immune responses. Histologic lesions induced by *P insidiosum* are generally characterized by eosinophilic inflammation, whereas resolving lesions typically contain few eosinophils and large numbers of macrophages and lymphocytes. There is 1 report[17] of a human patient that had a relative decrease in serum *P insidiosum*–specific IgE concentrations as well as concentrations of IL-4 and IL-5 and a relative increase in the concentration of IL-2 after successful treatment. However, similar studies have not been performed for nonhuman species. Although these observations might be consistent with a switch from a Th2-polarized to a Th1-polarized immune response, the evidence is far from definitive.

For the study reported here, administration of the immunotherapeutic product to healthy dogs was not associated with a significant change in anti–*P insidiosum* IgG concentrations. One potential explanation would be that the product may simply not be able to induce an effective immune response in this species. This idea would be supported by clinical observations that the immunotherapeutic product is fairly ineffective for the treatment of pythiosis in dogs. Given the relatively higher clinical response rate after administration of the immunotherapeutic product in horses and humans, compared with the response rate in dogs, it would be interesting to determine whether there are substantial IgG responses to administration of the product in these species. Another explanation, which is perhaps more likely, would be that the immunotherapeutic product stimulated a predominantly cell-mediated response rather than a humoral response.

The present study had some limitations. The first was the relatively small sample size. In general, there was significant variability in anti–*P insidiosum* IgG concentrations (both before and after administration of the immunotherapeutic product) among dogs, although the percentage positivity remained within the reference interval for healthy dogs.

Perhaps a more important limitation was that the study was performed on healthy dogs with no known exposure to *P insidiosum*. Although selection of such dogs was necessary to determine the expected antibody response to the immunotherapeutic product for controlled conditions, the results might not necessarily be reflective of results for naturally infected dogs. It is possible that natural infection would cause sufficient immunologic priming that subsequent challenge exposure with the immunotherapeutic product would be associated with a significant increase in anti–*P insidiosum* IgG concentrations. Further investigation would require administration of the immunotherapeutic product to infected dogs. However, it may be difficult to determine the relative impact of the immunotherapeutic product versus that of the infection. In addition, multiple forms of treatment (eg, surgery or antifungal treatments) are often provided concurrent with the immunotherapeutic product to infected dogs, which might be expected to further complicate analysis.

For the study reported here, administration of a commercially available *P insidiosum* immunotherapeutic product in accordance with the manufacturer's recommendations to healthy dogs did not induce a significant increase in anti–*P insidiosum* IgG concentrations. This outcome did not support our original hypothesis. These results may suggest a failure of the product to induce a productive immune response in this species, or it might be indicative that factors other than IgG are responsible for the resolution of *P insidiosum* infections in dogs. Regardless, the lack of impact on serum anti–*P insidiosum* IgG concentrations suggested that administration of the immunotherapeutic product would not be expected to interfere with subsequent serologic monitoring of affected dogs. However, further evaluation of the antibody responses to this immunotherapeutic product in naturally affected dogs will be required before firm conclusions can be prudently drawn.

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Footnotes

a. Pan American Veterinary Laboratories, Hutto, Tex.
b. Immulon 2HB, Thermo Scientific, Rochester, NY.
c. Peroxidase-conjugated affinity-purified anti-dog IgG, Rockland Antibodies and Assays, Limerick, Pa.

d. TMB 2-component microwell peroxidase substrate kit, SeraCare, Milford, Mass.
e. Epoch Microplate spectrophotometer, BioTek instruments Inc, Winooski, Vt.
f. PROC MIX, SAS, version 9.4, SAS Institute Inc, Cary, NC.

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