***** EXAMPLE of AAVD/ACVD GRANT *****

Intradermal immunotherapy with a heat-killed actinomycetales bacterin preparation as adjunctive treatment for canine pythiosis: a multicentric, randomized, controlled, clinical trial

Grant Proposal: August 01, 2021 – July 31, 2023

Intradermal immunotherapy with a heat-killed actinomycetales bacterin preparation as adjunctive treatment for canine pythiosis: a multicentric, randomized, controlled, clinical trial

Abstract

Pythium insidiosum, an aquatic fungal-like microorganism, causes disease in humans and animals. Infection results in a non-transmissible, severe, invasive, progressive gastrointestinal or cutaneous disease in dogs. Historically, pythiosis is a disease of tropical and subtropical climates. In the past two decades, its environmental niche has expanded to include more temperate (north) and more arid (southwest) regions of the United States of America. Infection is characterized by rapid clinical progression with a high mortality rate due to extensive, but typically localized, spread of the disease. Immunologically canine pythiosis is characterized by a highly inflammatory and potentially Th2-driven reaction pattern at the basis of its severe clinical features. There is currently no consensus on standard treatment recommendations, with complete surgical resection of affected tissues, where possible, and long-term adjunctive antifungal therapy commonly advised. Depending upon the location affected, surgical resection can result in up to 45% disease recurrence rendering treatment difficult, which often results in patients having a poor short to medium-term prognosis. The most common therapy instituted has been a combination of itraconazole/terbinafine, tetracycline antibiotics, glucocorticoids, and mefenoxam with variable success.

Very recently, the use of heat-killed actinomycetales as bacterial immunomodulators has shown potential beneficial effects on canine allergies (flea allergy and atopic dermatitis) associated with a lack of or very minimal (small self-resolving swelling at the site of injection) side effects. Actinomycetales have multiple immunological effects, including inhibition of Th2-mediated response and the ability to increase Th1 cells. They are also able to non-specifically decrease inflammation by down-regulating NF-κB, chemokines (e.g. CCL20), and pro-inflammatory cytokines. Because of the immunological similarities between canine

allergies and pythiosis, heat-killed actinomycetales may have a significant impact on the quality of life and control of clinical signs associated with of canine pythosis.

Specific Aims: **Thus, the aims of the present study are: 1) to evaluate the clinical benefits of the repeated administration of heat-killed actinomycetales in the treatment of canine pythiosis; 2) to evaluate the *in vitro* immunological and anti-inflammatory effects of heat-killed actinomycetales in canine pythiosis.** If successful, this double-blinded, randomized, controlled clinical trial has the potential to dramatically improve the quality of life and clinical signs of dogs affected by this disease.

Background and significance

Pythium insidiosum, an aquatic fungal-like microorganism, causes disease in humans and animals including horses, dogs, cats, and cattle.[1-4] Infection results in a non-transmissible, severe, invasive, progressive gastrointestinal or cutaneous disease in dogs, with one report of systemic dissemination.[1,4-9] Historically, pythiosis is a disease of tropical and subtropical climates.[3,5,10] In the past two decades, its environmental niche has expanded to include more temperate (north) and more arid (southwest) regions of the United States of America.[4,9,10] This geographic expansion is most likely due to environmental changes or landscape development but may also be a result of heightened awareness and increased accessibility to diagnostics such as serology.[6,7,10-12] *Pythium* organisms produce swimming zoospores that are responsible for the infection in animals.[6,11] Infective zoospores encyst, adhere, and germinate on damaged skin or gastrointestinal mucosa before converting to the hyphal form and invading gastrointestinal and/or cutaneous tissues.[5,13] Infection is characterized by rapid clinical progression with a high mortality rate due to extensive, but typically localized, spread of the disease.[2,14]

Immunologically, canine pythiosis is characterized by a humoral immunity (anti-*P. insidiosum* antibodies) as well as a cellular immunity (presence of macrophages, mast cells, eosinophils).[6] However, such immunity, believed to be related to a T helper 2 (Th2) immune response, is not protective against the organisms. Instead, it provides the basis for the severe local damage observed in affected dogs.[6] In addition, it has been theorized that *P. insidiosum* locks the immune response in a Th2 mode contributing to the worsening of disease.

There is currently no consensus on standard treatment recommendations, with complete surgical resection of affected tissues, where possible, and long-term adjunctive antifungal therapy commonly advised.[1,3,4] Surgical resection alone can result in up to 45% disease recurrence, which often results in patients having a poor short to medium-term prognosis.[6,8] The most common therapy instituted has been a combination of itraconazole

and terbinafine, which appears to have variable success.[4,5,15] Other proposed therapies, as either sole or in combination with the above-mentioned antifungals, include β-glucan synthesis inhibitors (caspofungin), macrolide antibiotics (azithromycin), tetracycline antibiotics (minocycline), glucocorticoids, and agricultural pesticides (mefenoxam).[1,8,16-20] Variable response to treatment has prompted the investigation of other compounds and/or combination treatment protocols as alternative options for the management of *P. insidiosum* infections in animals. However, the prognosis of pythiosis remains grave with the majority of dogs succumbing to the disease.

The use of heat-killed actinomycetales bacteria (*Mycobacterium vaccae*, *Gordonia bronchialis*, *Rhodococcus coprophilus*, and *Tsukamurella inchonensis*) has shown potential rapid beneficial effects in dogs affected by allergies (flea allergy and atopic dermatitis).[21-23] Actinomycetales have multiple immunological effects, the most relevant of which includes the inhibition of Th2-mediated inflammation, favoring a Th1-mediated response.[24] They are also able to non-specifically decrease inflammation, down-regulating the expression of NF-κB, CCL20, and the pro-inflammatory cytokine Interleukin(IL)-1β.[25] Because canine pythiosis is thought to be characterized by a Th2 immune response which is not protective, but rather destructive and highly inflammatory, switching to a more protective Th1 type immune response would be highly beneficial in the treatment of canine pythiosis.

To date, no studies have been published on the use of such therapy for canine pythiosis. **Thus, the purpose of this multicentric, double-blinded, placebo-controlled, randomized clinical trial is to evaluate the clinical and immunological beneficial effects of heat-killed intradermal injections of *G. bronchialis* in canine pythiosis. It is hypothesized that the use of heat-killed bacteria will significantly improve the clinical signs and life expectancy in dogs.**

Preliminary data

In the past two years, one of the PIs has conducted and completed a preliminary clinical trial in atopic dogs (n=22) using repeated intradermal injections of heat-killed *G. Bronchialis* and *R. coprophilus* alone or in combination. Bacteria were grown in Sauton's liquid medium at 32°C until the end of the logarithmic phase. Harvested bacterial cell mass was suspended in physiological saline and autoclaved at 15 lbs./square inch at 121°C for 15 min and then tested for endotoxin concentration. The suspensions were diluted at $1x10^{10}$ bacteria/ml with phenol buffered saline and doses of 0.1 ml (containing $1x10^{9}$ bacilli, equivalent to 1 mg wet weight) were administered intradermally on the shoulder. A total of four injections were administered over one-year period (days 0, 20, 40, and 180). During the study time, no side effects were reported. Clinically, the treated dogs had a significant improvement of the clinical signs after only 90 days (three injections only) (Figure 1).

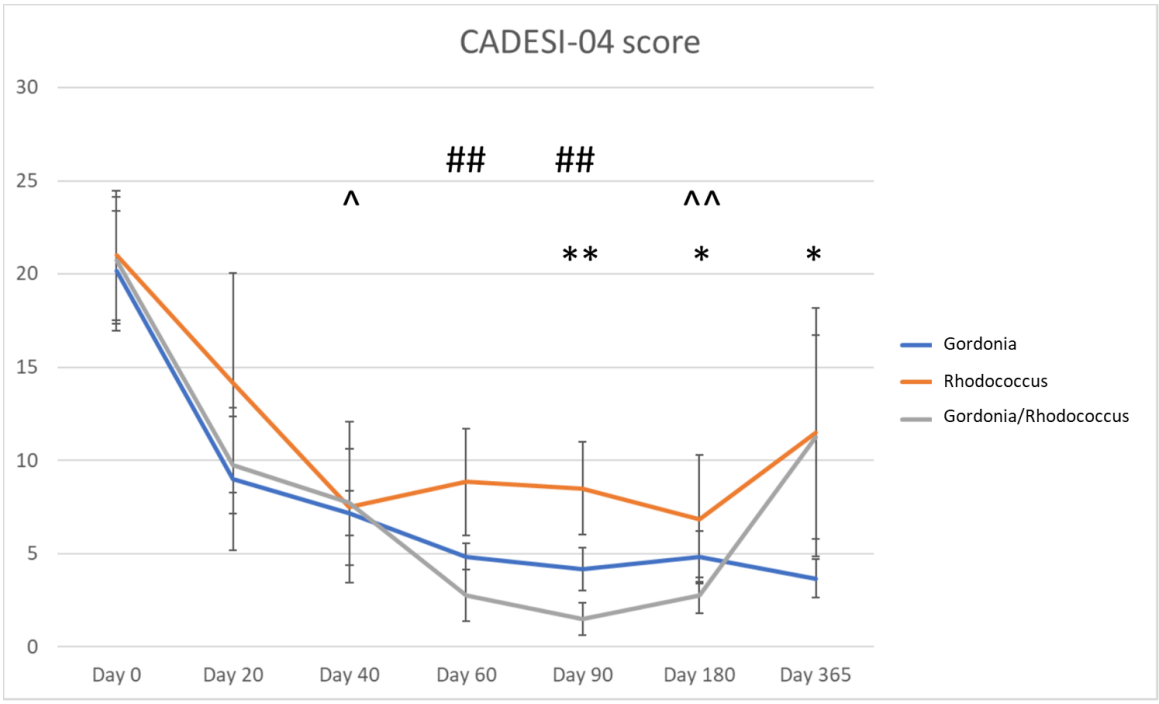

Figure 1: Clinical severity score (CADESI-04) of the dogs (n=22) enrolled in the different groups. Bars: standard errors of the mean; *: a significantly different over-time expression in the *Gordonia* group (compared to day 0); ^: a significantly different over-time expression in the *Rhodococcus* group (compared to day 0); #: a significantly different over-time expression in the *Gordonia/Rhodococcus* group (compared to day 0); *,^,#: $p<0.05$; **, ^^, ##: $p<0.01$

Experimental methods and design

Aim #1: To evaluate the clinical benefits of the repeated administration of heat-killed actinomycetales in canine pythiosis.

Aim #2: To evaluate the *in vitro* immunological and anti-inflammatory effects of heat-killed actinomycetales in canine pythiosis.

Animals

Dogs with cutaneous and gastrointestinal pythiosis (n=40) will be enrolled in this study by three academic institutions. Dogs with pythiosis will be included in the study if they have compatible clinical signs of cutaneous and/or gastrointestinal pythiosis. Other co-morbidities will be evaluated based on physical examination and history. The clinical diagnosis will be confirmed by histopathological (immunohistochemistry), microbiological (culture) and immunological/molecular analysis (ELISA and/or PCR). In addition, in order to be enrolled, dogs will have undergone a previous surgical resection with disease recurrence, surgical resection is not recommended because of location, or owners will have declined recommendations of surgical resection for naturally occurring untreated disease.

Treatment allocation

This is a multicentric, double-blinded, placebo-controlled, randomized, clinical trial. Dogs will be randomly assigned to one of the following two groups:

1. Treatment (heat-killed *Gordonia bronchialis*) (n=20)
2. Placebo (sterile phenolated saline) (n=20)

Bacterial preparation

The preparations to test will be heat-killed cultures of the bacterial species *G. bronchialis* (ATCC-25592) (ATCC, Manassas, VA, USA). The organisms will be grown on Sauton's liquid medium (Teknova, Hollister, CA, USA), which is free from other antigens and compliant with all relevant transmissible spongiform encephalitis regulations. Culture will be performed at 32°C and continued until the end of the logarithmic phase. Once the logarithmic phase is reached, the bacterial suspension will be centrifuged and resuspended in sterile water. Multiple washes of the pellet will be performed to eliminate any residual medium. After the last wash (5 total), the bacterial suspension will be autoclaved, at 15 lbs/in^2 at 121°C for 15 min, and aliquoted. After autoclave and before the administration of the intradermal injections, 100µl of bacterial suspensions will be plated on agar plates and cultured at 32°C for 48 hours to verify that the organisms tested will be dead. In addition, the autoclaved bacterial suspension will be tested for endotoxin using the ToxinSensor

Endotoxin Detection System by GenScript. The suspensions will then be diluted $1x10^{10}$ bacteria/ml with phenolated saline and doses of 0.1 ml (containing $1x10^{9}$ bacilli, equivalent to 1 mg wet weight) will be administered by intradermal injection into the shoulder area in each dog, rotating sites at each visit.

Study design

Dogs will be examined every three weeks for three times and monthly afterwards, up to 1 year. At each visit a clinical evaluation, blood draw and an intradermal injection of the treatment or placebo will be performed as described below:

1. Pets enrolled in group one will receive intradermal injections of heat-killed *G. bronchialis* every three weeks (20±3 days interval) for three times and monthly afterwards. Dogs will be allowed to receive prednisone orally (0.5-1mg/kg/day) for the first 3 weeks and "as needed" basis after that. Topical therapy with antiseptic and emollient shampoos will be allowed. In addition, minocycline (10mg/kg/day), terbinafine (20-30mg/kg/day), and mefenoxam (8mg/kg/day) will be administered orally as standard treatment.

2. Pets enrolled in group two will receive intradermal injections of vehicle (placebo) every three weeks (20±3 days interval) for three times and monthly afterwards. Dogs will be allowed to receive prednisone orally (0.5-1mg/kg/day) for the first 3 weeks and "as needed" basis after that. Topical therapy with antiseptic and emollient shampoos will be allowed. In addition, minocycline (10mg/kg/day), terbinafine (20-30mg/kg/day), and mefenoxam (8mg/kg/day) will be administered orally as standard treatment.

Pets will be randomly assigned to one of the two groups using the Research Randomizer Program® (www.randomizer.com). Investigators performing the clinical assessments will be blinded to the group allocation. At each visit, the needed administration of prednisone will be recorded; the decrease in use of prednisone will be used as indirect evaluation of the clinical efficacy of the intradermal therapy.

Aim #1: To evaluate the clinical benefits of the repeated administration of heat-killed actinomycetales in canine pythiosis.

Clinical evaluation

During the visit, the dogs will be evaluated for clinical signs as follows.

1. For cutaneous pythiosis, skin lesions will be assessed for extent and severity (e.g. distribution and size of the lesions). Pictures of the lesions will be taken and the surface area affected calculated using the image J software.

2. For the gastrointestinal pythiosis, gastrointestinal lesions will be assessed for extent and severity (e.g. distribution and size of the lesions) via abdominal ultrasound. For each dog, a physical examination assessing the general condition of each dog (e.g. canine chronic enteropathy clinical activity index (CCECAI), body condition score, and quality of life) will be assessed.

Blood draws will be performed monthly to assess CBC and chemistry. A total of 2mL of whole blood will be collected and apportioned into an EDTA-containing tube and a serum tube for chemistry.

Aim #2: To evaluate the *in vitro* immunological and anti-inflammatory effects of heat-killed actinomycetales in canine pythiosis.

Immunological evaluation

In all enrolled dogs, modifications in the systemic (blood) and local (skin) inflammatory response will be tested before (day 0), every three months after that, and at the end of the study. Selected biomarkers of inflammation (IL-4, -10, -17, -22, and IFN-γ) will be tested in the serum and in the skin using the reagents in Table 1, using the manufacturer's recommendations.

Target	**Species**	**Manufacturer**	**Substrate**
Interleukin 1 beta	Canine	R&D Systems	Serum and tissue extract
Interleukin 4	Canine	R&D Systems	Serum and tissue extract
Interleukin 10	Canine	R&D Systems	Serum and tissue extract
Interleukin 17	Canine	R&D Systems	Serum and tissue extract
Interferon gamma	Canine	R&D Systems	Serum and tissue extract

Table 1: ELISA kits information

Blood sample collection

Five milliliters of blood will be collected by jugular venipuncture after at least 12 hours fasting and placed into serum tubes without anticoagulant agents. After clotting for 30 minutes at room temperature, the blood will be centrifuged at 327g for 15 minutes and the serum obtained will be stored at -80 C° until analysis. Serum samples will be used to detect IL-1β, -4, -10, -17, and IFN-γ by ELISA following the manufacturers' protocols.

Skin sample collection

One 8 mm punch biopsy will be taken from the affected skin and placed in 1.5 ml microfuge tubes, and flash frozen in liquid nitrogen. These samples will be stored at – 80 ^{0}C

until processed for protein extraction and quantification. Briefly, one half of the 8 mm skin biopsy specimen will be homogenized using a PowerGen 125 (Fisher Scientific) and then the proteins will be extracted using the T-PER™ Tissue Protein Extraction Reagent (Fisher Scientific), according to the manufacturer's protocol. The amount of total protein in each sample (skin and serum) will be measured using a commercially available BSA protein assay kit (Pierce™ BCA Protein Assay Kit, Thermo Scientific, Rockford, Illinois, USA).

Statistical analysis

The number of 20 dogs per group was based on a power analysis expecting positive changes of the analyzed parameters (reduced inflammation markers) in 10% of the placebo group and a 60% positive changes in the treatment group (α=0.5 and β=0.2). The three institutions participating see an average of 10 cases per year (data collected over the past 5 years). This caseload is without any advertising. We do expect to collect more than 10 case per institution with proper advertising. In fact, in order to reach the proposed number of 40 cases, each institution needs to enroll about 14 dogs. The power analysis was performed using MedCalc 12.0 statistical software (MedCalc Software, Mariakerke, Belgium). Data will be analyzed using the intention-to-treat analysis with the last value carried forward if at least two data points will be present. Descriptive analysis will be performed. Once collected, the data will first be tested for normal distribution using the Kolmogorov-Smirnov test (alpha = 0.05). Unpaired Student's t test (or Mann-Whitney test if not normally distributed) will be performed to evaluate the behavior of each data variable between the two groups (placebo and treatment) at each time point. Then repeated measures ANOVA (Friedman's test if not normally distributed) will be performed to evaluate the behavior of each data variable over time in either group. If statistically significant (P values of ≤ 0.05), a Dunnett's (Dunn's if not normally distributed) Multiple Comparison Test will be performed as post-hoc analysis. Finally, a survival time analysis will be performed to compare the difference in survival time between the two group. All statistical analyses will perform using GraphPad Prism 6.09 statistical software.

Expected outcomes: We expect that repeated intradermal injections of *G. bronchialis* will be associated with a significant amelioration of the clinical signs and a prolongation of the life expectancy when compared with the standard of care treatments. In addition, we expect a switch in the systemic and local immune response from a predominantly Th2 to a predominantly Th1 immune environment.

Budget

Category	Year 1	Total
Personnel: Technician Salary (.6 calendar months) Fringe Benefits (39.1%) Total Salaries & Wages	 $2,380 $931	 $3,311
Materials and Supplies: Expenses encountered by the primary institution (e.g., admission fees, abdominal ultrasound, skin biopsy, etc.) Bacterial preparation Protein extraction kit Elisas (IL1β, IL4, IL10, IL17,and IFNγ) Miscellaneous Lab materials and supplies	 $6,100 $1,000 $400 $4,850 $4,000	 $16,350
Other Costs: Clinical Trial Support Samples Shipping Fee	 $5,600 $300	 $5,900
Subcontract(s) – expenses encountered by the other institutions (e.g., admission fees, abdominal ultrasound, skin biopsy, etc.) Subcontract 1 Subcontract 2	 $5,700 $5,700	 $5,700 $5,700
Subtotal of All Categories:		$39,961
Indirect Costs: 8%		$2,957
Total Funds Requested:		$39,918

Budget justification

The salary for the investigators is not requested since they are all Tenure and Clinical track faculty. Salaries for the scientific bio scientist helping with the project is required. The $16,350 include expensed encountered by the primary institution, bacterial preparation, extraction and ELISA kits, and laboratory material (reagents and materials necessary to perform the study). The clinical trial support includes the fee for the clinical trial team to organize enrollment and data (requested by the institution). Finally, the subcontracts include expensed encountered by the other institutions (e.g., admission fees, abdominal ultrasound, skin biopsy, etc.).

Animal and human Use

Privately owned dogs will be enrolled in this study. All the procedures have been submitted and approved (IACUC# 202011241) by the PI's Institutional Animal Care and Use Committee (IACUC) and the College of Veterinary Medicine's Veterinary Hospital Research Review Committee (VHRRC) (VHRRC# 2020-24). Signed consent will be obtained from the owners in accordance with the VHRRCC's policies and procedures.

Facilities

The PI's comparative laboratory is equipped with a forced-air incubator and –70^{o}C freezer, liquid nitrogen, ELISA readers, and flow cytometers, as well as all the other general laboratory equipment necessary to perform this study. Level II Biological safety hoods and a laboratory fume hood are available for this study. In addition, two senior bio scientists with extensive experience in cell culturing and immunological methodologies will help in performing the study.

References

1. Hummel J, Grooters A, Davidson G, et al. Successful management of gastrointestinal pythiosis in a dog using itraconazole, terbinafine, and mefenoxam. *Med Mycol* 2011;49:539-542.
2. Grooters A, Gee M. Development of a nested polymerase chain reaction assay for the detection and identification of Pythium insidiosum. *J Vet Intern Med* 2002;16:147-152.
3. Grooters A, Leise B, Lopez M, et al. Development and evaluation of an enzyme-linked immunosorbent assay for the serodiagnosis of pythiosis in dogs. *J Vet Intern Med* 2002;16:142-146.
4. Grooters AM, Foil CS. Miscellaneous fungal infections. In: Greene CE ed. *Infectious diseases of the Dog and Cat.* 4th ed. Athens: Elsevier, 2012; 677-681.
5. Grooters A. Pythiosis, lagenidiosis, and zygomycosis in small animals. *Vet Clin North Am Small Anim Pract* 2003;33:695-720.
6. Gaastra W, Lipman L, Cock AD, et al. Pythium insidiosum: an overview. *Vet Microbiol* 2010;146:1-16.
7. Reagan K, Marks S, Pesavento P, et al. Successful management of 3 dogs with colonic pythiosis using itraconzaole, terbinafine, and prednisone. *J Vet Intern Med* 2019;33:1434-1439.
8. Pereira D, Botton S, Azevedo M, et al. Canine gastrointestinal pythiosis treatment by combined antifungal and immunotherapy and review of published studies. *Mycopathologia* 2013;176:309-315.
9. Arsuaga-Zorrilla C, Grooters A, Pucheu-Haston C. Quantitation of anti-Pythium insidiosum antibodies before and after administration of an immunotherapeutic product to healthy dogs. *Am J Vet Res* 2018;79:1160-1165
10. Oldenhoff W, Grooters A, Pinkerton M, et al. Cutaneous pythiosis in two dogs from Wisconsin, USA. *Vet Dermatol* 2014;25:52-e21.
11. Presser J, Goss E. Environmental sampling reveals that Pythium insidiosum is ubiquitous and genetically diverse in North Central Florida. *Med Mycol* 2015;53:674-683.
12. Berryessa N, Marks S, Pesavento P, et al. Gastrointestinal pythiosis in 10 dogs from California. *J Vet Intern Med* 2008;22:1065-1069.
13. Mendoza L, Hernandez F, Ajello L. Life Cycle of the Human and Animal oomycete Pathogen *Pythium insidiosum*. *J Clin Microbiol* 1993;31:2967-2973.
14. Schmiedt C, Stratton-Phelps M, Torres B, et al. Treatment of intestinal pythiosis in a dog with a combination of marginal excision, chemotherapy, and immunotherapy. *J Am Vet Med Assoc* 2012;241:358-363
15. Grooters AM. Pythiosis, lagenidiosis, and zygomycosis. In: Sykes JE, ed. *Canine and feline infectious diseases*. St. Louis: Saunders, 2014;668-678.
16. Brown TS, Grooters AM, Hosgood GL. In vitro susceptibility of Pythium insidiosum and Lagenidium sp. to itraconazole, posaconazole, voriconazole, terbinafine, caspofungin, and mefenoxam. *Am J Vet Res* 2008;69:1463-1468.
17. Fonseca A, Pereira D, Filho FM, et al. In vitro susceptibility of zoospores and hyphae of Pythium insidiosum to antifungals. *J Antimicrob Chemother* 2014;69:1564-1567.
18. Jesus F, Ferreiro L, Loreto E, et al. In vitro synergism observed with azithromycin, clarithromycin, minocycline, or tigecycline in association with antifungal agents against *Pythium insidiosum*. *Antimicrob Agents Chemother* 2014;58:5621-5625.
19. Jesus F, Loreto E, Ferreiro L, et al. In vitro and in vivo antimicrobial activities of minocycline in combination with azithromycin, clarithromycin, or tigecycline against Pythium insidiosum. *Antimicrob Agents Chemother* 2015;60:87-91.
20. White AG, Smart K, Hathcock T *et al*. Successful management of cutaneous paralagenidiosis in a dog treated with mefenoxam, minocycline, prednisone, and hyperbaric oxygen therapy. *Med Mycol Case Rep* 2020;29:38-42.
21. Ricklin Gutzwiller ME, Reist M, Peel JE *et al*. Intradermal injection of heat-killed Mycobacterium vaccae in dogs with atopic dermatitis: a multicenter pilot study. *Vet Dermatol* 2007;18:87-93.
22. Marro A, Pirles M, Schiaffino L *et al*. Successful immunotherapy of canine flea allergy with injected Actinomycetales preparations. *Immunotherapy* 2011;3:971-978.
23. Tartara G, Gonzalez Beltran S, Bottai H *et al*. Beneficial effects of immunotherapy with Gordonia bronchialis on canine flea allergic dermatitis. *Vet Dermatol* 2013;24:395.
24. Stanford JL, Stanford CA, Grange JM. Environmental echoes. *Sci Prog* 2001;84(Pt 2):105-124
25. Smaldini PL, Stanford J, Romanin DE *et al*. Down-regulation of NF-κB signaling by Gordonia bronchialis prevents the activation of gut epithelial cells. *Innate Immun* 2014; 20: 626-638.