Journal de Mycologie Médicale (2008) **18**, 129—133

Disponible en ligne sur www.sciencedirect.com

ScienceDirect

journal homepage: www.elsevier.com/locate/mycmed

ORIGINAL ARTICLE/ARTICLE ORIGINAL

Comparison between immunotherapy and caspofungin as agents to treat experimental pythiosis in rabbits

Essai comparatif entre l'immunothérapie et le traitement par la caspofungine dans la pythiose expérimentale du lapin

D.I.B. Pereira [a,d], J.M. Santurio [a,*], S.H. Alves [a], M.I. de Azevedo [a], F. Silveira [a], F.F. da Costa [a], E.S.V. Sallis [b], L. Pötter [c], L. Ferreiro [d,e]

[a] *Mycology Research Laboratory — Lapemi, Department of Microbiology, Universidade Federal de Santa Maria (UFSM), Microbiologia, Prédio 20, sala 4139, 97105-900 Santa Maria, RS, Brazil*
[b] *Department of Preventive Veterinary Medicine, Universidade Federal de Pelotas (UFPel), Pelotas, RS, Brazil*
[c] *Department of Animal Science, Universidade Federal de Santa Maria (UFSM), Santa Maria, RS, Brazil*
[d] *Program of Pos-graduate in Veterinary Science, Universidade Federal do Rio Grande do Sul (UFRGS), Porto Alegre, RS, Brazil*
[e] *Mycology Laboratory, Department of Veterinary Clinical Pathology, Faculdade de Medicina Veterinária (UFRGS), Porto Alegre, RS, Brazil*

Received 5 December 2007; received in revised form 15 May 2008; accepted 16 May 2008
Available online 21 July 2008

KEYWORDS
Pythium insidiosum;
Pythiosis;
Caspofungin;
Immunotherapy;
Pitium Vac®;
Rabbits

Summary Pythiosis is an emerging disease that is difficult to treat and is caused by the oomycete *Pythium insidiosum*. This study evaluated the efficacy of two different treatments for pythiosis using rabbits as an experimental model. Fifteen rabbits subcutaneously inoculated with *P. insidiosum* zoospores were divided into three groups of five animals (group 1, control; group 2, treated with Pitium Vac® immunotherapic and group 3, treated with caspofungin). The treatments were started 25 days after the inoculation, and consisted of: (1) eight doses of the immunotherapic administered at 14-day intervals and (2) 1 mg/kg per day of caspofungin during 20 consecutive days. The animals were necropsied 18 weeks after the experiment onset, and lesion fragments were collected for histopathologic and morphometric analysis. The animals in the control group displayed larger lesion sizes compared to the animals treated with Pitium Vac® or caspofungin ($P < 0.05$). The histological aspect of the lesions was similar among the groups

* Corresponding author.
E-mail address: janio.santurio@gmail.com (J.M. Santurio).

1156-5233/$ — see front matter
doi:10.1016/j.mycmed.2008.05.001

under study, and the morphometric evaluation showed that the animals in groups 2 and 3 had lower amounts of hyphae in necrotic areas ($P < 0.05$). The results of the present study indicate that, even though the treatments did not differ significantly, the immunotherapic treatment is still the best alternative to treat pythiosis due to its lower cost. Caspofungin fungistatic effect on *P. insidiosum* in addition to its high cost makes its use for the treatment of animal pythiosis impractical.

MOTS CLÉS
Pythium insidiosum ;
Pythiose ;
Caspofungine ;
Immunothérapie ;
Pitium Vac® ;
Lapins

Résumé La pythiose est une maladie émergente très difficile à traiter due au développement de l'oomycète *Pythium insidiosum*. Le but de cette étude est d'évaluer l'efficacité de deux traitements différents de la pythiose expérimentale chez le lapin. Trois groupes de cinq lapins sont inoculés par la voie sous-cutanée avec une suspension de zoospores de *P. insidiosum*. Les animaux du premier groupe représentent les témoins. Le vingt-cinquième jour après l'inoculation, le second groupe reçoit huit doses de l'immunothérapie qui sont administrées à 14 jours d'intervalle et les lapins du troisième lot sont traités avec de la caspofungine: 1 mg/kg par jour pendant 20 jours consécutifs. Les animaux sont sacrifiés 18 semaines après le début de l'expérience et des biopsies sont réalisées au niveau des lésions pour des études morphométriques et histopathologiques. Des lésions plus importantes en dimension sont constatées chez le groupe témoin par rapport aux animaux traités avec le Pitium Vac® et la caspofungine ($p < 0,05$). Pour chaque groupe, l'aspect histologique des lésions est similaire, en revanche, l'évaluation morphométrique des lapins des groupes 2 et 3 révèle des quantités d'hyphes inférieures au niveau des zones nécrosées ($p < 0,05$). Les résultats de cette étude montrent que, même si les traitements ne diffèrent pas significativement, le traitement au moyen de l'immunothérapie, de par son prix abordable, s'avère le meilleur choix pour traiter la pythiose. Malgré l'effet fongistatique de la caspofungine sur *P. insidiosum*, son utilisation est rendue impraticable pour le traitement des animaux atteints de pythiose en raison de son coût élevé.

Introduction

Pythiosis is an emerging disease of animals and humans in the tropical, subtropical and temperate regions of the world [23]. Most published reports describe this disease in horses and dogs. In horses, the disease manifests itself as an ulcerative, proliferative, pyogranulomatous lesion, most often involving skin and subcutaneous tissues of the legs and ventral body wall [14]. In dogs, the infection includes cutaneous/subcutaneous and gastrointestinal lesions [6]. In humans, the disease occurs in a localized form as well as in systemic or vascular forms [23]. Pythiosis is caused by *Pythium insidiosum*, a fungus-like microorganism of the Stramenopila kingdom. Pythiosis can be a life-threatening infection in all species, if not treated [10].

Successful resolution of pythiosis with antifungal treatment alone is difficult due to *Pythium* spp. cell wall and cytoplasmic membrane compositions. While true fungi contain chitin and β-glucan in their cell wall, stramenopilan microorganisms are devoid of ergosterol, the target of most antifungal drugs [5,6]. Various attempts to treat animals as well as humans with antifungal drugs such as amphotericin B, ketoconazole, miconazole, fluconazole, itraconazole and terbinafine have yielded variable, and sometimes contradictory results [1–4,6,7,9,17,18,20,22]. The lack of therapeutic choices for pythiosis has encouraged the use of immunotherapy [10]. Initially proposed by Miller [12], immunotherapy constitutes an important alternative for the treatment of equine pythiosis, with cure rates that vary around 70–80% [10,15]. Wanachiwanawin et al. [23] achieved the cure in 50% of the cases when using immunotherapy to treat arterial pythiosis in humans. However, immunotherapy did not show satisfactory results in dogs and cats [11]. Although the cure rates obtained using immunotherapy for the treatment of pythiosis are considered good and inexpensive, there are cases that do not respond to this therapy and demand new approaches.

Hope for the treatment of pythiosis with antifungal agents appeared when a new group of antimycotics derived from echinocandins was discovered. Caspofungin (L-743, 872, MK-0991) is a semisynthetic derivative of pneumocandin Bo, whose mechanism of action consists of blocking the synthesis of β(1,3)-D-glucan of the fungal cell wall through the non-competitive inhibition of the enzyme β(1,3)-D-glucan synthase [8]. This drug can be efficient against *P. insidiosum* since this oomycete contains great amounts of β-glucan in its cell wall [6].

In this work, rabbits were experimentally infected with *P. insidiosum* zoospores, in order to evaluate the efficacy of two different treatments for pythiosis: immunotherapy and caspofungin administration.

Materials and methods

P. insidiosum

A *P. insidiosum* strain (CBS 101555), isolated from a case of equine pythiosis in Brazil, was employed. This strain was initially cultivated in Corn Meal Agar at 27 °C and subsequently

subjected to zoosporogenesis. The resulting zoospores were inoculated into the rabbits.

Zoosporogenesis

Previously autoclaved pieces of *Paspalum notatum* grass were distributed over *P. insidiosum* cultures and incubated at 37 °C for five days. Infected grass pieces were transferred to an induction medium containing solution 1 (K_2HPO_4 [1.0 mol/L], KH_2PO_4 [1.0 mol/L], $(NH_4)_2HPO_4$ [3.66 mol/L], 500 mL of distilled water) and solution 2 ($MgCl_2{\cdot}6H_2O$ [0.5 mol/L]), $CaCl_2{\cdot}2H_2O$ [0.5 mol/L], 250 mL of distilled water). The induction medium final composition was 0.5 mL of solution 1, 0.1 mL of solution 2 and 1000 mL of sterile distilled water. Induction medium containing infected grass pieces was incubated at 37 °C for 8 h. The grass pieces were observed under a microscope and the zoospores were counted using a Neubauer chamber.

Rabbits and treatments

Fifteen, three-month-old New Zealand rabbits, including males and females, were used in this work. All animals received 1 mL of induction medium containing approximately 20,000 viable *P. insidiosum* zoospores applied subcutaneously in the right costal region, as described by Santurio et al. [19]. Inoculated rabbits were checked every three days by measuring the subcutaneous nodular area (centimeter cube) using a sliding caliper. Rabbits that developed pythiosis lesions were divided into three groups of five rabbits each:

- group 1 (control, without treatment);
- group 2 (treated with the immunotherapic Pitium Vac® — Lapemi/Embrapa, developed by Santurio et al. [19]);
- group 3 (treated with caspofungin — Cancidas® — MSD Sharp and Dohme GmbH, Haar, Germany).

The treatments were started 25 days after zoospore inoculations. Rabbits in group 2 received 2 mL of the immunotherapic by the subcutaneous route in the left costal region. This procedure was repeated every 14 days, at eight time points. Caspofungin in the form of 50 mg was commercially acquired. The drug was dissolved in sterile distilled water according to the manufacturer's instructions, and applied at a dosage of 1 mg/kg per day by the intraperitoneal route during 20 days.

The procedure was approved by the Animal Welfare Committee of Federal University of Santa-Maria.

Necropsy and histopathologic and morphometric analyses

All rabbits were necropsied after 18 weeks of observation. Representative fragments of the subcutaneous lesions were fixed in 10% buffered formalin, routinely processed for histopathologic evaluation, and stained with Hematoxylin and Eosin (HE) or Grocott's stain. An Olympus Oly-200 color video camera coupled to a BX51/BX52 Olympus binocular microscope was used for image capturing. Images were acquired at 400 × magnification. Hyphae were quantified using Alpha Ease FC software (Alpha Innotech Corporation version 4.0). Seven microscopic fields were used for each slide. The images were converted into 255 grayscale intensities. Among different intensity ranges, a dark range (values 0 to 175) corresponded to hyphae stained by silver in Grocott's stain, and was selected using a selection tool. The data obtained were submitted to statistical analysis.

Statistics

The areas of the lesions (centimeter cube) were transformed into percentage and submitted to analysis of variance and *F*-test using a significance level of 5%. The Tukey test was carried out when differences between the treatments were detected. Regression analysis of the dates of the measurements was also performed, with adjustments in the polynomial model made up to the third order.

Results

All rabbits developed granulomatous lesions with areas ranging from 4 to 31.3 cm^2, 25 days after subcutaneous inoculation with *P. insidiosum* zoospores. One animal from each group died between the 8th and 12th weeks after inoculation; necropsies indicated that their death was caused by pythiosis metastases in the lungs and kidneys. The remaining animals were kept in their groups until the end of the experiment.

When the mean percent lesion areas were compared using the Tukey test, the animals in the control group showed larger lesion sizes as compared to the animals treated with Pitium Vac® or caspofungin ($P < 0.05$). The evolution of the lesions in the course of the experimental period showed a quadratic behavior for all treatments ($P < 0.0001$). Subcutaneous lesion areas exhibited a reduction in their increase during the treatment in the rabbits that received caspofungin; however, lesions resumed growth after the end of the drug administration (Fig. 1).

The subcutaneous lesions cut surfaces were multilobulated, hard, white-pinkish and vascularized. The histopathology

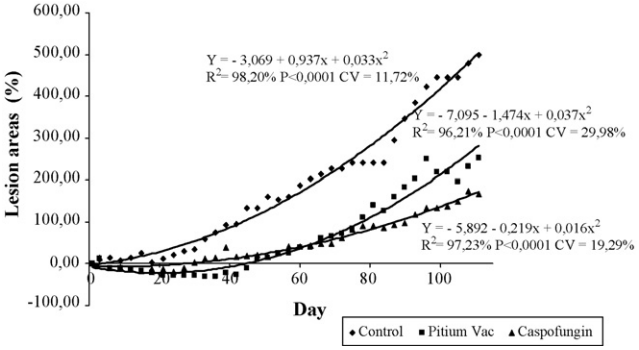

Figure 1 Percent variation of subcutaneous lesion areas in rabbits experimentally inoculated with *P. insidiosum* zoospores and treated with caspofungin or the immunotherapic Pitium Vac®.

Pourcentage de variation au niveau de la taille des lésions souscutanées après inoculation expérimentale des lapins avec des zoospores de P. insidiosum et traités avec de la caspofungine ou avec l'immunothérapie Pitium Vac®.

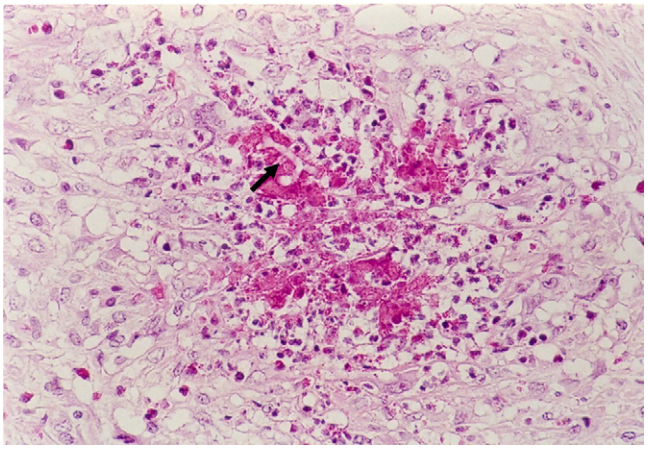

Figure 2 Subcutaneous tissue of a rabbit inoculated with *P. insidiosum* and necropsied after 18 weeks. Necrotic area with cell debris and hyphae-like structures surrounded by Splendore-Hoeppli reactive areas (arrow). HE 10 × objective.
Tissu sous-cutané d'un lapin inoculé avec des zoospores de P. insidiosum et sacrifié après 18 semaines. L'aspect histologique montre un envahissement ainsi qu'une nécrose tissulaire avec présence de débris tissulaires et d'ébauches d'hyphes accompagnés d'une importante réaction éosinophilique à leur périphérie. HE 10 × objectif.

of the lesions stained with HE was characterized by the presence of multifocal to coalescent necrotic areas delimited by inflammatory infiltrates predominantly constituted by PMN (eosinophils). Hyphae-like structures surrounded by irregular, eosinophilic material could be observed in the necrotic areas and corresponded to Splendore-Hoeppli reactions (Fig. 2). Some of these reactive areas were delimited by Langhans' giant cells; some of these cells had hyphae in their interior. There was also intense proliferation of fibrous connective tissue cells, and presence of eosinophils, plasma cells, lymphocytes, macrophages, some giant cells and epithelioid cells. Irregularly ramified, scarcely septate hyphae with thick brown walls were visualized with Grocott staining; these were located especially in the periphery of the necrotic areas.

The morphometric analysis demonstrated that the lesions in the animals of the control group had a greater number of hyphae as compared to those in the rabbits belonging to the groups treated with Pitium Vac® or caspofungin ($P < 0.05$) (Fig. 3).

Discussion

Rabbits are used to study serology and efficacy of immunotherapy for the treatment of pythiosis nowadays because this disease can be experimentally reproduced in this species, but not in others [11,19]. In the present study, the development of subcutaneous nodules 25 days after inoculation with viable *P. insidiosum* zoospores and the histological characteristics of the lesions were similar to those described in other studies [13,19]. These findings indicate that the experimental reproduction of pythiosis in the rabbits was successful.

Even though none of the animals in the group treated with Pitium Vac® showed signs of clinical cure, their lesion areas showed less growth when compared to those of the animals in the control group ($P < 0.05$). These results differ from other study [19], that reported the cure of two animals using the same number of doses and the same immunotherapic formulation. We believe that the absence of complete lesion regression may have happened due to the animals' immunological status, since the efficacy of the immunotherapic used in the present work has been previously demonstrated in rabbits with experimental pythiosis [19] and in horses naturally infected by *P. insidiosum* in Brazil [15].

Some authors affirmed that the immunotherapic's mechanism of cure involves a change from a Th2 lymphocyte-mediated response to a Th1 lymphocyte-mediated reaction, as evidenced: by changes in the interleukin and histopathological lesion patterns [10,21,23]. In this study, we did not observe differences in the pattern of the histological findings between the groups studied. On the other hand, the lower number of hyphae observed in the necrotic areas of the animals treated with Pitium Vac® suggest that this immunotherapic was able to stimulate an immune response that resulted in destruction of *P. insidiosum* hyphae.

We are currently developing studies on the *in vitro* susceptibility of *P. insidiosum* to several antifungal drugs, including caspofungin. The *in vitro* evaluation of 26 Brazilian *P. insidiosum* isolates showed that this oomycete is poorly susceptible to caspofungin, and the *in vivo* observations revealed that this drug displays fungistatic activity in rabbits

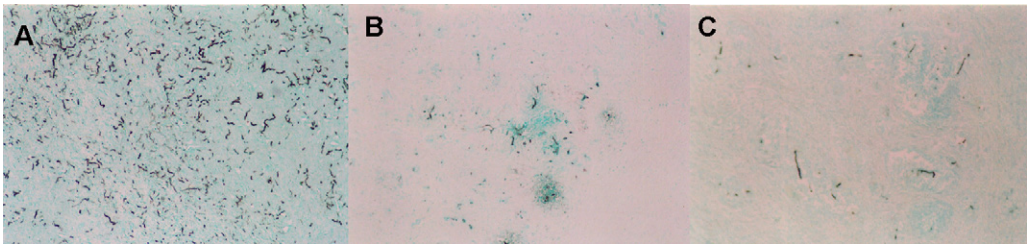

Figure 3 Subcutaneous tissue of rabbits inoculated with *P. insidiosum* and necropsied after 18 weeks. The structures stained black correspond to *P. insidiosum* hyphae. Panels A (control); B (Pitium Vac®) and C (caspofungin) show the differences in the amount of hyphae between the treatments. Grocott. 10 × objective.
Tissu sous-cutané d'un lapin inoculé avec des zoospores de P. insidiosum et sacrifié après 18 semaines. Les structures pigmentées en noir correspondent à des hyphes de P. insidiosum. On peut observer les différences dans la quantité d'hyphes par rapport aux trois groupes : témoin (A), Pitium Vac® (B) et caspofungine (C). Grocott. Objectif 10 ×.

with experimental pythiosis [16]. In the present study, the area of the lesions in animals treated with caspofungin showed less growth as compared to the control group ($P < 0.05$). This finding reinforces that caspofungin effect on *P. insidiosum* is fungistatic. This drug was more effective in containing the lesions than Pitium Vac®, as evidenced by:

- slow growth of the lesions even 12 weeks after the end of the treatment;
- quantification of hyphae in Grocott's staining;
- no significant difference as compared to the Pitium Vac® group.

Notwithstanding, cure was not achieved using caspofungin, whereas it has been reported with the use of Pitium Vac® [19]. Prolonged therapies with caspofungin are very expensive, and this justifies the treatment for only 20 days. The application of caspofungin for a time extent similar to that used for Pitium Vac® would require approximately 115 bottles of the drug. This is impracticable, since the cost of this antifungal drug is something around US$ 1000 per bottle in Brazil. Our observations, add to other studies [2–4,7,9,18], report the difficulty in treating pythiosis with antifungal drugs.

In conclusion, the comparison of two therapies based on different mechanisms to treat pythiosis provided evidence that, even though there was no difference between the treatments evaluated, immunotherapy is still the best alternative to treat pythiosis due to its lower cost and possibility of cure, in spite, of the longer treatment time required. Caspofungin's lack of fungicidal effect on *P. insidiosum*, in addition, to its high cost and inability to cure, makes its use for the treatment of pythiosis in animals impracticable, especially in the horse, the species, in which this disease is more frequently observed.

Acknowledgments

This work was supported by National Council for Scientific and Technological Development of Brazil (CNPq). This manuscript is based on the thesis submitted by the first author in partial fulfillment of requirements for the obtainment of the degree of Doctor in Veterinary Science, PPGC/FAVET, UFRGS, RS, Brazil.

We thank Cristiane Danesi and Adriano Tony Ramos for the morphometric analysis.

References

[1] Bissonnette KW, Sharp NJH, Dykstra MH, Robertson IR, Davis B, Padhye AA, et al. Nasal and retrobulbar mass in a cat caused by *Pythium insidiosum*. J Med Vet Mycol 1991;29:39–44.

[2] Bosco SMG, Bagagli E, Araújo Jr JP, Candeias JMG, Franco MF, Marques MEA, et al. Human pythiosis, Brasil. Emerg Infect Dis 2005;11:715–8.

[3] Dykstra MJ, Sharp NJH, Olivry T, Hillier A, Murphy KM, Kaufman. et al. A description of cutaneous-subcutaneous pythiosis in fifteen dogs. Med Mycol 1999;37:427–33.

[4] English PB, Frost AJ. Phycomycosis in a dog. Aust Vet J 1984;61:291–2.

[5] Foil CS. Update on pythiosis (oomycosis). The North American Veterinary Conference 1996; 57–63.

[6] Grooters AM. Pythiosis, lagenidiosis, and zygomycosis in small animals. Vet Clin North Am Small Anim Pract 2003;33:695–720.

[7] Jaeger GH, Rotstein DS, Law JM. Prostatic pythiosis in a dog. J Vet Intern Med 2002;16:598–602.

[8] Letscher-Bru V, Herbrecht R. Caspofungin: the first representative of a new antifungal class. J Antimicrob Chemother 2003;51:513–21.

[9] McMullan WC, Joyce JR, Hanselka BV, Heitmann JM. Amphotericin B for the treatment of localized subcutaneous phycomycosis in the horse. J Am Vet Med Assoc 1977;170:1293–7.

[10] Mendoza L, Mandy W, Glass R. An improved *Pythium insidiosum*-vaccine formulation with enhanced immunotherapeutic properties in horses and dogs with pythiosis. Vaccine 2003;21: 2797–804.

[11] Mendoza L, Newton JC. Immunology and immunotherapy of the infections caused by *Pythium insidiosum*. Med Mycol 2005;43: 477–86.

[12] Miller RI. Treatment of equine phycomycosis by immunotherapy and surgery. Aust Vet J 1981;57:377–82.

[13] Miller RI, Campbell RSF. Experimental pythiosis in rabbits. Sabouraudia 1983;21:331–41.

[14] Miller RI, Campbell RSF. The comparative pathology of eqüine cutaneous phycomycosis. Vet Pathol 1984;21:325–32.

[15] Monteiro AB. MS. Dissertation. Immunotherapy of equine pythiosis: testing the efficacy of a biological and evaluation of the leukocytic response to the treatment in horses naturally infected with *Pythium insidiosum*. Federal University of Santa Maria, Brazil 1999; p.54.

[16] Pereira DI, Santurio JM, Alves SH, Argenta JS, Pötter L, Spanamberg A, et al. Caspofungin *in vitro* and *in vivo* activity against Brazilian *Pythium insidiosum* strains isolated from animals. J Antimicrob Chemoter 2007;60:1168–71.

[17] Rakish PM, Grooters AM, Tang K. Pythiosis in two cats. J Vet Diagn Invest 2005;17:262–9.

[18] Rivierre C, Laprie C, Guiard-Marigny O, Bergeaud P, Berthelemy M, Guillot J. Pythiosis in Africa. Emerg Infect Dis 2005;11: 479–81.

[19] Santurio JM, Leal AT, Leal ABM, Festugatto R, Lubeck I, Sallis ESV, et al. Three types of immunotherapics against *Pythiosis insidiosi* developed and evaluated. Vaccine 2003;21:2535–40.

[20] Shenep JL, English BK, Kaufman L, Pearson TA, Thompson JW, Kaufman RA, et al. Successful medical therapy for deeply invasive facial infection due to *Pythium insidiosum* in a child. Clin Infect Dis 1998;27:1388–93.

[21] Thitithanyanont A, Mendoza L, Chuansumrit A, Pracharktam R, Laothamatas J, Sathapatayavongs B, et al. Use of an immunotherapeutic vaccine to treat a life-threatening human arteritic infection caused by *Pythium insidiosum*. Clin Infect Dis 1998;27:1394–400.

[22] Triscott JA, Weedon D, Cabana E. Human subcutaneous pythiosis. J Cutan Pathol 1993;20:267–71.

[23] Wanachiwanawin W, Mendoza L, Visuthisakchai S, Mutsikapan P, Sathapatayavongs B, Suwanagool P, et al. Efficacy of immunotherapy using antigens of *Pythium insidiosum* in the treatment of vascular pythiosis in humans. Vaccine 2004;22:3613–21.