



Anti-*Pythium insidiosum* intradermal immunotherapy in horses: diagnosis and therapy¹

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ABSTRACT. Santos C.E.P., Loreto E.S., Zanette R.A., Bortolini J., Santurio J.M. & Marques L.C. 2024. Anti-*Pythium insidiosum* intradermal immunotherapy in horses: diagnosis and therapy. *Pesquisa Veterinária Brasileira* 44:e07370, 2024. Núcleo de Clínica e Cirurgia Veterinária, Faculdade de Medicina Veterinária, Universidade Federal do Mato Grosso, Av. Fernando Corrêa da Costa 2367, Boa Esperança, Cuiabá, MT 78069-900, Brazil. E-mail: carlos.favet@gmail.com

Pythiosis is a life-threatening disease that affects various species and is prevalent in regions with high humidity. The ailment is increasingly reported globally, and in Brazil, it is an important cause of profound economic and health losses in equines. This study aimed to explore the potential of intradermal immunotherapy as both a diagnostic and therapeutic approach for pythiosis in horses from the Pantanal region of Mato Grosso, Brazil. The horses were divided into three groups: those never diagnosed with pythiosis or with lesions that could be mistaken for pythiosis (Group 1); previously infected but successfully treated horses (Group 2); and horses with a positive diagnosis for pythiosis that were under treatment (Group 3). An immunotherapeutic product (PitiumVac®) was administered intradermally. Injection site reaction and response to immunotherapy were assessed. The results indicated that intradermal immunotherapy could be a viable diagnostic and therapeutic tool, particularly in remote areas where traditional laboratory diagnosis methods are not readily accessible. The efficacy of intradermal administration was comparable to that of subcutaneous administration in treating pythiosis in horses, and the combined use of immunotherapy and triamcinolone acetonide yielded promising results for treating pythiosis in horses. However, further research is required to validate these findings. This study contributes to understanding and managing pythiosis more efficiently by providing a simple, cost-effective, and potentially efficient alternative approach to diagnosis and treatment.

INDEX TERMS: Pythiosis, intradermal immunotherapy, equines, diagnosis, treatment.

RESUMO.- [Anti-*Pythium insidiosum* imunoterapia intradérmica em equinos: diagnóstico e terapia.] A pitiose é uma doença potencialmente fatal que afeta várias espécies e é prevalente em regiões com alta umidade. A doença é cada vez mais relatada globalmente, e no Brasil é uma causa importante de profundas perdas econômicas e de saúde em equinos. Este estudo teve como objetivo explorar o potencial da imunoterapia intradérmica como

uma abordagem diagnóstica e terapêutica para a pitiose em cavalos na região do Pantanal de Mato Grosso, Brasil. Os cavalos foram divididos em três grupos: aqueles que nunca foram diagnosticados com pitiose ou com lesões que poderiam ser confundidas com pitiose (Grupo 1); cavalos previamente infectados, mas tratados com sucesso (Grupo 2); e cavalos com diagnóstico positivo de pitiose em tratamento (Grupo 3). Um produto imunoterapêutico (PitiumVac®) foi administrado

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intradermicamente. A reação no local da injeção e a resposta à imunoterapia foram avaliadas. Os resultados indicaram que a imunoterapia intradérmica pode ser uma ferramenta diagnóstica e terapêutica viável, particularmente em áreas remotas onde os métodos tradicionais de diagnóstico laboratorial não estão prontamente acessíveis. A eficácia da administração intradérmica foi comparável à da administração subcutânea no tratamento de pitiose em cavalos, e o uso combinado de imunoterapia e triacinaclona acetônica produziu resultados promissores para o tratamento da pitiose em cavalos. No entanto, são necessárias mais pesquisas para validar esses achados. Este estudo contribui para o entendimento e o gerenciamento da pitiose de forma mais eficiente, fornecendo uma abordagem alternativa simples, econômica e potencialmente eficaz para diagnóstico e tratamento.

TERMOS DE INDEXAÇÃO: Pitiose, imunoterapia intradérmica, equinos, diagnóstico, tratamento.

INTRODUCTION

Pythiosis is a globally distributed disease commonly occurring in tropical, subtropical, and temperate regions with high moisture levels. This potentially fatal disease affects many species and is increasingly reported worldwide. Antimicrobial drugs are often ineffective, and radical surgery is frequently necessary, with recurrences and reinfections being common. Immunotherapy with *Pythium insidiosum* antigens has emerged as an alternative treatment, usually used with surgery to increase effectiveness (Santos et al. 2011, Loreto et al. 2014, Loreto et al. 2020, Yolanda & Krajaejun 2021).

Numerous laboratory techniques exist to validate clinical diagnoses, given the potential for misdiagnosing the disease with other conditions, especially when manifested in cutaneous form in horses. Serological and molecular methods serve as potent instruments for prompt and precise diagnoses (Grooters et al. 2002, Santurio et al. 2006, Gaastra et al. 2010, Azevedo et al. 2012, Htun et al. 2020). Additional methodologies, including histopathological staining, immunodiffusion, immunohistochemistry, and mycological culture, are also accessible and have been meticulously examined (Martins et al. 2012, Chareonsirisuthigul et al. 2013, Tartor et al. 2020). These studies elucidate the typical clinical, pathological, and hematological manifestations of *P. insidiosum* infection, culture identification, direct sample PCR techniques, and immunohistochemical investigations. Nevertheless, the accessibility of these diagnostic resources is constrained in field settings, notably in isolated areas like the Brazilian Pantanal and Amazon biomes (Santos et al. 2011, 2014, Barbosa et al. 2023).

This study aims to describe the use of intradermal immunotherapy for diagnostic and therapeutic purposes in horses in the Pantanal region of Mato Grosso, Brazil.

MATERIALS AND METHODS

Animal Ethics. All animal procedures were approved by the Ethical Committee on Animal Use (CEUA) of "Universidade Federal do Mato Grosso" (UFMT), number 23108.040953/12-1.

This study prospectively analyzed pythiosis in horses from the Pantanal region of Mato Grosso, Brazil, from 2007 to 2022. Animal data were obtained on-site based on requests from local

veterinarians or animal owners. Technical visits were made to previous epidemiological surveys to raise awareness of the importance of early identification and treatment of animals affected with pythiosis or other misdiagnosed skin diseases.

To test the intradermal immunotherapy, a 1/10 fraction of the immunotherapeutic PitiumVac® (0.2mL) (Santurio et al. 2003) was used, which was inoculated intradermally (ID) in the neck of the experimental groups. The characteristics of the local lesion were verified between 24 and 72 h. The animals were divided into three groups: Group 1 (control; n=8), horses that never developed pythiosis or developed lesions that could be misdiagnosed; Group 2 (historical; n=6), healthy horses that previously had clinical pythiosis and were successfully treated; and Group 3 (cases; n=8), horses with skin lesions, with a positive diagnosis for pythiosis, and that have been treated with 2mL of the immunotherapeutic product. The animals in Group 3 were subdivided based on lesion diameter. They were subjected to a new therapeutic protocol, which included the application of PitiumVac® (0.2mL, ID) and triamcinolone acetone (50mg, IM) every 15 days until complete recovery.

Serum samples from all animals were obtained by jugular venipuncture and evaluated by indirect ELISA for pythiosis diagnosis (Santurio et al. 2006). Biopsies of more advanced lesions were collected for histopathological examination stained with hematoxylin-eosin (HE) and Grocott's methenamine silver (GMS) staining. In addition, fragments of cutaneous tissues were subjected to the immunohistochemistry technique (Martins et al. 2012) and molecular characterization (PCR) in the affected tissues using specific primers for *Pythium insidiosum*, according to Azevedo et al. (2012).

The Shapiro-Wilk test was conducted to assess the adherence of the response variable "difference" data to the normal distribution in the control (n=8), historical (n=6), and case (n=7) animal groups. A non-normal distribution of data was observed in the historical group. Therefore, to compare the means of the response variable "difference" among the groups, the t-test with bootstrap was adopted, considering the presence of heterogeneous variances among the mentioned groups. Additionally, 95% confidence intervals were constructed using the percentile bootstrap method for the group means (Dwivedi et al. 2017). The analyses were performed using R software, with a significance level set at 5% and employing 1,000 bootstrap resamples to obtain the confidence intervals.

RESULTS

The results of the ID application of the immunotherapeutic product were consistent and coherent across all stratified groups. Group 1, consisting of animals that had never been diagnosed with pythiosis, showed discreet increases in volume (mild edema) as a reaction to intradermal inoculation, along with slight scaling (Fig.1). Among these animals, five had no skin lesions, two had trauma-related non-specific granulomas, and one was diagnosed with sarcoidosis on the face. A significant cutaneous reaction was observed in Group 2, composed of animals that had developed cutaneous pythiosis in previous years and recovered after using immunotherapy or its combination with surgery (Fig.1). All animals showed a substantial increase in local volume and moderate to severe edema around the application site. Moreover, partial local necrosis was observed in four animals. Abscess formation that fistulated and drained blood and pus was observed in two (Fig.2). In Group 3, consisting of animals with clinical pythiosis, the reactions were similar to those in Group 2, with edema ranging from moderate to severe (Fig.3). Two

animals in this group, with initial circumscribed pythiosis lesions that were smaller than 5cm in diameter, recovered with only two intradermal applications of immunotherapy. However, one horse with a chronic multifocal pythiosis wound greater than 10cm in diameter did not elicit a reaction (Fig.4). In this severe pythiosis case, after unresponsive treatments with potassium iodide, amphotericin, and other therapies, we opted for the intradermal application of PitiumVac® along with

triamcinolone treatment (50mg/animal/IM, every 15 days) due to the animal's depleted state that did not allow surgery. After three applications, the animal left the anergic state, expressed local edema after ID applications, and recovered in about 90 days (Fig.5 and 6). The success of this protocol was repeated in more advanced cases, in single ($n=3$) or multiple ($n=3$) lesions, with complete recovery of all the animals. Epidemiological data are shown in Table 1.



Fig.1-6. Intradermal reaction sites of horses intradermally inoculated with PitiumVac®. (1) Mild edema was predominantly observed in animals from Group 1 (without pythiosis). (2) Moderate to severe edema was observed in animals from Group 2 (animals that had pythiosis in the past and that were successfully cured). (3) Severe edema was the main reaction observed in animals from Group 3 (with clinical pythiosis). (4-6) Animal with chronic, unresponsive pythiosis that did not elicit a reaction to the intradermal application of the immunotherapeutic product. However, it was cured after receiving a treatment combining intradermal immunotherapy and triamcinolone acetonide every 15 days for three months.

The results of the Shapiro-Wilk test suggested that the distribution of the control and case groups was normal (p -value = 0.097 and p -value = 0.396). In contrast, the historical group had a non-normal distribution (p -value = 0.036). The analysis of means and standard deviations showed that the case, historical, and control groups had means of 99.429mm, 71mm, and 5.5mm, respectively, with standard deviations of 65.23mm, 27.698mm, and 2.268mm. By comparing the means through the t-test with bootstrap, significant differences were found between the control and historical groups (t = -5.778 and p -value = 0.003) and between the control and case groups (t = -3.808 and p -value = 0.006). However, there were no significant differences between the historical and case groups (t = -1.048 and p -value = 0.304). Based on the percentile bootstrap method with 1,000 resamples, the 95% confidence intervals for the means were as follows: for the case group, the interval ranged from 56.571 to 144.571mm; for the historical group, the interval was from 53.333 to 92.667mm; and for

the control group, the interval was from 4.125 to 7.125mm, reflecting the homogeneity of measurements in this group. These confidence intervals reinforce the estimates of the means and the data interpretation within the research context.

DISCUSSION

The findings of this study emphasize the effectiveness of the intradermal immunotherapy test in diagnosing and treating pythiosis in horses, especially in the challenging field conditions of the Pantanal region. Based on delayed-type hypersensitivity reaction to *Pythium insidiosum* antigens, the test has proven to be an efficient diagnostic tool for pythiosis, allowing for an immediate therapeutic response. This is particularly significant as pythiosis progresses rapidly, and advanced laboratory diagnostics are often inaccessible in remote areas (Hagebock et al. 1993, Silva et al. 2021).

Table 1. Epidemiology data of horses diagnosed and treated using an intradermal injection containing 0.2ml of the immunotherapeutic product PitiumVac®

Animal profile				Induration size (mm)			Induration characteristics			Results				Lesion characteristics (location, evolution, and size) and diagnosis	
Group	Sex	Age	Breed	T0	T1	Dif	Size	Pain	Nec	Fis	ID**	Elisa*	IHC*	PCR*	
Control animals															
G11	M	4	Pant	15	21	6	-	-	-	-	-	-	ND	ND	Left hind limb; unspecific granuloma
G12	M	5	Pant	16	20	4	-	-	-	-	-	-	ND	ND	Right forelimb; unspecific granuloma
G13	M	16	Grade	17	23	6	-	-	-	-	-	-	ND	ND	-
G14	M	10	Grade	17	20	3	-	-	-	-	-	-	ND	ND	-
G15	F	11	Grade	16	26	10	-	-	-	-	-	-	ND	ND	-
G16	F	3	Pant	18	24	6	-	-	-	-	-	-	ND	ND	Face; sarcoid
G17	M	8	Pant	15	21	6	-	-	-	-	-	-	ND	ND	-
G18	F	3	Pant	16	19	3	-	-	-	-	-	-	ND	ND	-
Successfully treated animals†															
G21	M	12	Pant	18	70	52	++	-	++	++	+	+	ND	ND	Right hind limb; 40 days; Ø 10cm
G22	F	10	Pant	12	130	118	+++	+	+	-	+	+	ND	+	Abdomen; 40 days; Ø 30cm
G23	F	9	Pant	17	75	58	++	++	++	++	+	+	ND	+	Abdomen; 60 days; Ø 25cm
G24	M	18	Pant	18	110	92	+++	+	+	-	+	+	+	+	Left forelimb; 30 days; Ø 15cm
G25	M	16	Pant	19	70	51	++	-	+	-	+	+	ND	+	Flank; 20 days; Ø 10cm
G26	F	6	Pant	17	72	55	++	-	-	-	+	+	ND	+	Right forelimb; 20 days; Ø 10cm
Pythiosis active animals															
G31	F	4	Pant	18	19	1	-	-	-	-	-	+	+	ND	Ribs and flank; 70 days; Ø 35cm
G32	F	3	Pant	16	40	24	+	-	+	-	+	+	+	ND	Right rump, 30 days, Ø 10cm
G33	F	4	Pant	19	80	61	+++	+	+	++	+	+	+	ND	Right forelimb; 30 days; Ø 10cm
G34	F	3	Pant	18	190	172	+++	+	-	-	+	+	+	ND	Right hind limb; 10 days; Ø 5cm
G35	F	6	Mang	19	210	191	+++	-	+	-	+	+	+	ND	Abdomen; 40 days; Ø 10cm
G36	F	6	Mang	18	90	72	+++	-	+	-	+	+	+	ND	Abdomen; 40 days; Ø 10cm
G37	M	12	Mang	20	65	45	++	-	++	+	+	+	+	ND	Abdomen; 30 days; Ø 10cm
G38	F	3	Pant	19	150	131	++	-	++	-	+	+	ND	+	Right forelimb; 15 days; Ø 5cm

T0 = day of intradermal application of 0.2 ml of immunotherapy, T1 = 24 or 72h after application, Dif = difference between T0 and T1 measurements, Nec = necrosis, Fis = fistulae, ID** = intradermal test obtained for diagnosis purpose, (*) laboratory tests obtained at the time pythiosis lesions appeared, IHC = immunohistochemistry, PCR = polymerase chain reaction, M = male, F = female, ND = not done, Pant = Pantaneira, Mang = Mangalarga; Ø circumference, (-) absent, (+) mild, (++) moderate, (+++) strong; † Animals from this group were diagnosed one or more times with pythiosis, were successfully treated with immunotherapy, and were ELISA negative and without lesions when the study was conducted; Years of the diagnosis: G21 = 2008 and 2012, G22 = 2019, G23 = 2021, G24 = 2007, 2011 and 2018, G25 = 2020, G26 = 2020.

In the Pantanal region, it is only sometimes possible to use traditional laboratory methods such as mycological culture, PCR, and immunohistochemistry (Ubiali et al. 2013). In these cases, the intradermal test is a practical and reliable alternative. Although laboratory methods are the gold standard for confirming pythiosis diagnosis, they require specialized equipment and expertise, making them less accessible in field settings. On the other hand, as demonstrated in our study, the intradermal test is easy to administer. It provides prompt results, which are crucial for early intervention and improved outcomes, especially under field conditions.

Notably, the advanced stage of the disease - chronic pythiosis - can lead to an anergic condition (Denotta & Mcfarlane 2023), which may affect the test results. Nevertheless, the intradermal test using a 0.2mL dosage via ID was as effective as the subcutaneous route for treating pythiosis in horses. These findings are consistent with experiments conducted by Weiblen et al. (2019), which demonstrated that treatment effectiveness and cure rates did not differ significantly between intradermal and subcutaneous routes in treating experimentally induced pythiosis in rabbits. In addition, combining immunotherapy via ID with triamcinolone acetonide showed promising results in treating infected animals. However, further studies are necessary to investigate this therapeutic approach. Cardona-Álvarez et al. (2016) proposed that the effectiveness of triamcinolone acetonide may be due to the immunomodulatory mechanism of glucocorticoids, which inhibit the synthesis, release, and action of cytokines and other mediators that promote the immune or inflammatory response. The main mechanism involves blocking the synthesis of the IL-5 cytokine and granulocyte-macrophage colony-stimulating factors, which induces apoptosis, thereby reducing the half-life and functions of eosinophils. This is because IL-5 is crucial for eosinophilopoiesis and enhances mature eosinophils' functions (such as degranulation, adhesion, and cytotoxicity), prolonging cell survival.

Conversely, Yolanda & Krajaejun (2021) reported that in natural infection, Th2 activation produces IL-4 and IL-5 to attract and activate eosinophils and mast cells, thus blocking the host's immune response. The immunomodulatory mechanism of triamcinolone may contribute to the effectiveness of the combined therapy. Immunotherapy modifies the host's immune response, promoting cellular differentiation to Th-1 and inducing the release of cytokines such as IFN-γ and IL-2, which attract macrophages and cytotoxic T lymphocytes to the site of infection, helping to eliminate the pathogen.

CONCLUSIONS

Overall, the intradermal application of PitiumVac® as an immunodiagnostic agent shows promise in simplicity, low cost, and ease of use. It may also reduce the cost of treatment, as only 10% of the therapeutic dose is needed for initial pythiosis cases. The combined use of immunotherapy with triamcinolone acetonide may have additive results, improving the clinical response of affected hosts. However, further studies are necessary to validate this approach.

The main limitation is the dependence on natural cases, as it is impossible to reproduce the disease in the equine host.

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Conflict of interest statement.- The authors declare that there are no conflicts of interest.

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