Mycopathologia **119**: 89–95, 1992.

Evaluation of two vaccines for the treatment of pythiosis insidiosi in horses

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Received 19 June 1991; accepted in revised form 15 December 1991

Key words: Pythiosis insidiosi, horses, vaccine therapy, *Pythium insidiosum*

Abstract

Two vaccines to treat pythiosis insidiosi in horses were evaluated in 71 Costa Rican horses between 1982 to 1988. One vaccine used a cell-mass (CMV) as antigen and the other a soluble concentrated antigen (SCAV). Both vaccines cured horses infected with *Pythium insidiosum* (p value ~ 14%). The age of lesions prior to vaccination was important in the response of the horses to immunotherapy. All horses with lesions 0.5 months or less in duration were cured regardless of the vaccine used. Horses with lesions two or more months old did not respond to either vaccine. The age of the horses did not have any influence on their response to the vaccinations. The CMV produced a prominent inflammatory reaction at the side of injection, while the SCAV gave a low inflammatory reaction. In addition, the CMV lost its effectiveness two to three weeks after its preparation. By contrast, the SCAV maintained its ability to cure horses even after 18 months. Immunotherapy using SCAV can thus be used as the vaccine of choice in early cases of equine cutaneous pythiosis insidiosi.

Introduction

Pythiosis insidiosi is a skin and intestinal disease in horses [1–4], cattle [5], dogs [6, 7], cats [8], and humans [9] that occurs primarily in the tropical and subtropical areas of the world. The disease has been most frequently reported in equines [10–12]. The etiological agent *Pythium insidiosum* is a member of the Kingdom Protista, in the Class Oomycetes and Family Pythiaceae [13]. An oomycete isolated from horses in Australia was named *P. destruens* [14], but it was proven to be synonymous with *P. insidiosum* [15]. Moreover, Mendoza et al. [16] found that nine *Pythium* strains isolated from humans, horses and dogs with active pythiosis belonged to the same species, suggesting that *P. insidiosum* is the only specie of this genus pathogenic for humans and animals.

It has been postulated but not proven that *P. insidiosum* parasitizes aquatic plants (usually lilies) in stagnant water, as part of its life cycle producing biflagellate zoospores that may penetrate open lesions in the skin of horses submerged in such waters [17]. After infection, the invaded areas characteristically increase rapidly in size. There is no record in the literature of spontaneous recovery in horses afflicted with pythiosis insidiosi. Therefore, if this oomycotic disease is not treated in its early stages, the disease

commonly progresses to a chronic state that in 100% of the cases leads to the host's death [11, 18].

Due to the clinical similarities between equine pythiosis insidiosi, equine cutaneous habronemiasis and zygomycotic skin infections of horses caused by *Basidiobolus ranarum* and *Conidiobolus coronatus* [17, 19], pythiosis insidiosi, has often been misdiagnosed [18] and treated unsuccessfully. Radical surgery has been used to treat cutaneous granulomas caused by *P. insidiosum* in horses [18, 20, 21]. However, surgery on lesions located on limbs is often not possible since critical anatomical structures are involved in this area [4, 18]. Furthermore, lesions can reappear after surgical treatment [4, 22]. Drugs, like amphotericin B and iodine combined with surgery have been found to be effective but amphotericin B is nephrotoxic, expensive and time consuming to administer [17, 18]. Iodine therapy requires many hours of daily attention and also has toxic side effects. Recently, immunotherapy, involving vaccination with antigens derived from *P. insidiosum* cultures, has been recommended in Australia and Costa Rica [11, 18]. Immunotherapy has several drawbacks due mostly to the lack of trial controls, since experimental equine infection have not yet been possible [23], and to the development of severe inflammatory reactions at the vaccination site [18].

In this paper we describe evaluation trials of two vaccines used in the immunotherapy of 71 Costa Rican mixed breed horses afflicted with *P. insidiosum* between 1982 and 1988.

Materials and methods

Animals

From 1982 to 1988 seventy-one Costa Rican mixed breed horses, suffering from pythiosis in different parts of the body, were injected with two vaccine preparations. Horses were evaluated weekly for up to four months. Blood and biopsies were also collected for serological examination [24] and for histopathology and cultural study. The age of the lesions was determined either by their size or by the day when the lesions were first noticed or both.

Vaccine production

Two different vaccine preparations were processed. The first one, a cell-mass vaccine (CMV), was prepared by modification of the technique described by Miller [18]. Essentially, this involved the use of 1 mm blocks of a culture of *P. insidiosum* grown on Sabouraud dextrose agar (SDA) (ATCC 58643 = CBS 574.85) and incubated at 37 °C for five days. Each block was transferred to 1.0-liter flasks containing 500 ml of nutrient broth. The flasks were incubated statically at 37 °C for 10 days. The growth was then filtered through a Watman No 40 filter paper and fungal mass was washed three times with 200 ml of 0.75% of NaCl solution. In the last wash, the cell-mass was resuspended in 15 ml of sterile saline solution and then broken in a Braun MSK cell homogenizer (Bronwill Scientific, Inc., Rochester, NY) until microscopically 80% of the hyphae were observed to have been fragmented. The hyphae were transferred to a previously measured container and then desiccated overnight at 55 °C. The final dose was adjusted to 5 mg dry weight/ml with 5% phenolized, sterile saline solution. Sterility was checked by plating out 0.1 ml of each batch on SDA and rabbit blood agar (RBA) and incubating at 37 °C for 1 week. The CMV was stored at 4 °C for no more than one week.

The second vaccine (SCAV) was prepared according to the procedure described by Mendoza and Alfaro [11]. Briefly, this involved the use of filtrate concentrated antigens. Small portions of SDA containing hyphae of *P. insidiosum* (ATCC 58643) were inoculated in 1.0-liter flasks containing 500 ml of Sabouraud dextrose broth (Difco) and incubated at 37 °C on a shaker rotating at 100 rpm. After five days of growth, the cultures were killed with phenol (0.5% final concentra-

tion), and then concentrated 20-fold in a stir cell (Amicon Corp., Lexington, MA). The 25 ml of concentrated soluble antigen obtained from each 1.0-liter flask were precipitated with 50 ml of chilled acetone twice and centrifuged at 10,000 Xg. The supernatant was drained and the precipitated antigen was resuspended in 25 ml of 0.75% NaCl sterile solution. The antigen's protein concentration was determined by the Lowry method [25] and then adjusted to 2.0 mg/ml. Its sterility was checked by inoculating SDA and RBA plates and incubating them at 37 °C for 1 week. The SCAV vaccine was stored at 4 °C until used.

Immunotherapy

The CMV (freshly prepared vaccine) was injected subcutaneously in 30 horses over their superficial pectoral muscles using 2 ml of the antigen. The vaccine was given once and the horses were observed weekly. The SCAV was also applied subcutaneously to a total of 41 horses by injecting 0.1 ml of the vaccine in the middle of their necks. The vaccine was applied once. Vaccination was repeated seven days later if the horses did not show improvement with either vaccine. The horses were recorded as cured if all traces of infection had been eliminated; this included: lesion epithelilization, cessation of discharge and closure of sinus tracts.

Results

Reactions at the site of injection (using CMV or SCAV) were evident 24 h after vaccination and in some cases sooner. In those cases in which the lesions's age was 1.5 or less months before vaccination, the inflammatory reaction due to the vaccine was between 250 to 330 mm in diameter. The zone was edematous, painful, and hot. The reaction however, decreased in size between two and five days after injection. Half of the horses vaccinated with CMV developed violent reactions with sterile abscesses. Horses with lesions between 1.5 or less months in age always developed a marked inflammatory reaction to both vaccines, whereas horses with lesions two or more months in age showed weaker or negative responses.

Of the 30 horses vaccinated with the CMV, 18 were cured (60%) and 12 did not respond (40%). In the 41 horses vaccinated with the SCAV, 29 responded satisfactorily (70.7%), while 12 showed no improvement (29.3%). When both vaccines were evaluated statistically, no differences in their capacity to cure the horses were found (p value $\sim$ 14%).

According to the age of lesions, the horses reacted to both vaccines as follows: 100% of the horses with lesions aged 15 days or less reacted adequately to both vaccines (CMV showed abscesses on the site of the injection in this group of animals); 75.0% and 86.6% of the horses with lesions aged from 0.5 to 1 month were cured using CMV and SCAV respectively; 71.4% (CMV) and 70.0% (SCAV) of the vaccinated horses with lesions of 1 to 1.5 months duration were cured upon vaccination; only 20.0% (CMV) and 40.0% (SCAV) of horses with lesions with a duration between 1.5 to 2 months were cured and finally, no horses with lesions of two or more months duration responded to either of the vaccines (Table 1). These data could not be evaluated statistically, since the population involved was affected by the owners decision as to when to call the veterinary practitioner for treatment of their horses.

The horses with lesions of one month or less before vaccination showed a quick response (in one week the lesions were dry and no secretions were observed) regardless of the vaccine used. Contamination of lesions with bacteria was absent or minimal in this group of horses. Horses with lesions of two or more months duration did not respond adequately to either vaccine and finally died. Secondary bacterial contamination of lesions in this group of equines was always observed. These horses were not cured even after five injections and were considered to be anergic. In these particular cases, the diagnosis of pythiosis insidiosi was corroborated by the isolation

Table 1. Response of horses to two vaccines (CMV and SCAV) according to the evolution of lesions before vaccination

Evolution of lesions prior to vaccination	CMV				SCAV			
	N	c	nc	%	N	c	nc	%
15 days or less	6	6	–	100	7	7	–	100
15 to 30 days	8	6	2	75.0	15	13	2	86.6
1 to 1.5 months	7	5	2	71.4	10	7	3	70.0
1.5 to 2 months	5	1	4	20.0	5	2	3	40.0
2 months and up	4	–	4	0.0	4	–	4	0.0

N = Number of horses injected; % = Percentage of cured horses; c = Cured cases; nc = Non cured cases; CMV = Cell mass vaccine; SCAV = Soluble concentrated antigen vaccine.

Table 2. Number of horses cured and non-cured after treatment with CMV and SCAV vaccines according to their ages

Ages of horses (year)	CMV			SCAV		
	N	c	nc	N	c	nc
1 to 3	9	5	4	9	7	2
3 to 6	6	5	1	11	8	3
6 to 9	5	3	2	9	7	2
9 to 12	4	3	1	7	4	3
12 and up	6	2	4	5	3	2

N = Number of treated horses; c = Cured cases; nc = Non-cured cases.

of *P. insidiosum* from their lesions. The anergic horses showed negative results in the ID tests before and after vaccination.

Sixty-two horses showed three to six precipitin bands in the ID test before vaccination. After vaccination, the same horses did not show an increase in the number of bands but a decrease was recorded in cured cases ($n = 47$). After seven months of successful treatment, no precipitin bands were observed in the sera tested with the ID procedure.

The age of the treated horses did not have a decisive influence on the response to the two vaccines (Table 2). Twelve younger horses (1 to 3 years old) were cured with both vaccines, and six were not. Five older horses (10 years old and older) were also cured, while six were not.

The relationship between the age of the treated horses and the age of their lesions before vaccination showed that the horses responded to the vaccination according to the age of their lesions rather than their own age. All horses between one and 14 years of age with lesions less than 0.5 month duration were cured. In contrast, all horses between one and 14 years old with lesions with a duration of two months did not respond to the immunological treatment (Tables 3 and 4). Twenty-four non-cured horses (12 vaccinated with CMV and 12 with SCAV) in which the age of their lesions was between 1.0 to two months or more (Table 1) responded clinically at first, but later their lesions became infected with other organisms and the horses finally died.

Batches of the CMV two to three weeks old did not cure horses in any stage of their disease. By contrast, the potency of the SCAV was observed to be adequate in the cure of horses even 18 months after preparation.

Discussion

The use of CMV as well as of SCAV resulted in the cure of horses according to the age of their lesions. The inflammatory reaction at the site of injection was an expected side effect that had been reported previously [11, 18, 26]. However, the reaction was less severe when we used the SCAV. This was apparently a function of the amount of the vaccine inoculated. We inoculated 2 ml of CMV (10 mg/ml) and 0.1 ml (200 g/ml) of SCAV, obtaining a suitable response in the horses without the development of severe inflammatory reactions. Furthermore, we worked with a relatively purified antigen acquired through the precipitation of liquid cultures with acetone. Further

Table 3. Relationship between the age and evolution of lesions before vaccination, and the response of horses to the CMV vaccine

Age of lesions prior to vaccination	Age of horses (years)									
	1–3		3–6		6–9		9–12		12 and up	
	c	nc	c	nc	c	nc	c	nc	c	nc
0.5 or less month	2	*	1	*	2	*	1	*	*	*
0.5 to 1 month	3	*	2	1	*	1	*	*	1	*
1 to 1.5 month	*	1	1	*	1	*	2	*	1	1
1.5 to 2 months	*	2	1	*	*	1	*	*	*	1
2 and up months	*	1	*	*	*	*	*	1	*	2

c = Cured cases; nc = Non-cured cases; * = No. cases.

Table 4. Relationship between the age and evolution of lesions before vaccination, and the response of horses to the SCAV vaccine

Age of lesions prior to vaccination	Age of horses (years)									
	1–3		3–6		6–9		9–12		12 and up	
	c	nc	c	nc	c	nc	c	nc	c	nc
0.5 or less month	2	*	3	*	*	*	1	*	1	*
0.5 to 1 month	3	*	4	1	3	*	2	1	1	*
1 to 1.5 month	1	*	*	1	4	1	1	1	1	*
1.5 to 2 months	1	1	1	*	*	*	*	1	*	1
2 and up months	*	1	*	1	*	1	*	*	*	1

c = Cured cases; nc = Non-cured cases; * = No. cases.

investigation is needed to elucidate which components of SCAV are responsible for the immunity that they elicit in horses. Although different batches of SCAV and CMV were used through the six years of study, even new batch of both vaccines cured the horses according to the age of their lesions as described above.

Miller in 1981 [18] suggested that success in the treatment of pythiosis insidiosi 'appears dependent on a number of actors such as type of treatment, species of fungus, size, site and duration of lesions and possibly age and physiological status of the horse'. Our data (Table 1) indicate that the age of the lesions prior to vaccination is a critical feature in the curing of horses. The age of the horses were not related to the response to both vaccines as shown in Tables 2, 3 and 4. Miller [18] reported an 82.5% success rate using immunotherapy in Australia (30% vaccination + surgery). Our data showed similar results. Nonetheless, we believe that the results cannot be evaluated as cured and non-cured, without taking into account the age of the lesions before vaccination. In addition, CMV and SCAV did not cure cases of zygomycosis due to *C. coronatus* [19] in horses in Costa Rica (unpublished data).

The complications of lesions attributed to the immunotherapy [26] are due to secondary bacterial contamination rather than the immunotherapy per se. We do not agree with Miller et al. [26] that after vaccination degenerated 'kunkers' (necrotic masses found in horses with active pythiosis insidiosi), which contained dead hyphae of *P. insidiosum*, acted as foreign bodies and caused tissue necrosis. Our observations are that, in cured cases of early pythiosis, the 'kunkers' are digested without any secondary complications. In chronic pythiosis, however, these 'kunkers' are heavily coontaminated by bacteria which involve all of the affected tissue. For this reason, in cases

with lesions 1.5 months old or more, the horses showed an improvement after immunotherapy (when the hyphae were killed in the kunkers), but later the bacteria present in the affected tissue exacerbated the original lesions. Miller et al. [26] found that one of five treated horses responded to the vaccine while the remainder were complicated by the presence of bacteria. Two horses developed a septic arthritis, osteitis and tenosynovitis. The evolution of lesions in these horses was between 1.5 to 3 months, which supports our findings shown in Table 1.

These data show that the number of precipitin bands did not increase after vaccination but decreased over the following months in the cured cases, corroborating previous observations [24]. This might suggest that cellular immunity rather than humoral immunity is involved in the elimination of the infection. This is supported by the fact that when immunotherapy was used in Australia [18] and Costa Rica (unpublished data), the initial eosinophilic inflammatory reaction present in the affected tissue changed to an infiltrate with numerous neutrophils, plasma cells, giant cells, and mononuclear macrophases. Later, the infiltrate, which surrounded the 'kunkers', digested them killing the hyphae of *P. insidiosum*. These observations and the data obtained in this study suggest that cellular immunity may be responsible for the cure of horses after vaccination. Further studies are necessary to investigate the meaning of the cellular changes after vaccination.

Lack of trial controls is the major drawback in the evaluation of immunotherapy in the treatment of horses with pythiosis insidiosi. Experimental equine infections, using zoospores or hyphae of *P. insidiosum*, so far, have been unsuccessful [23]. Thus, the evaluation of immunotherapy is based mainly on horses with naturally acquired disease. During this study, two or more equines, afflicted with pythiosis insidiosi, were sporadically observed in some farms, but the disease was more frequently observed as single cases rather than in outbreaks. Therefore, horses with natural infections to be used as controls were difficult to include in each farm. Interestingly, farms in which vaccination of horses with active pythiosis insidiosi diagnosed by ID test was not possible, and the animals did not receive any type of treatment, all died later (data not shown), confirming that if this disease is not treated early, the rate of mortality is 100%. Horses, which had not responded to immunotherapy ($n = 24$), all died later of toxic shock due to contamination, by gram-negative bacteria, systemic parasitic infections, or due to other complications not fully investigated during this study.

The propyhlactic properties of these vaccines has yet to be evaluated. During this study, however, we found that some horses cured by immunotherapy were reinfected in the following summer, suggesting that if vaccination confers immunity, it is of short duration.

In summary, SCAV proved to be more practical than CMV since the SCAV maintained its effectiveness even up to one year after preparation. The inflammatory reaction of SCAV at the site of injection was less violent than that with the CMA. Hence, immunotherapy using SCAV could be used to treat equine pythiosis insidiosi in its early stages. In cases of lesions 1.5 or more months old, we recommend the use of antibiotics and a diet rich in vitamins and minerals before starting immunotherapy to improve the response to vaccination.

Acknowledgment

We gratefully acknowledge the constructive criticism of Dr. J.F. Prescott. This investigation was supported in part by grants of the Ganadera La Emilia S.A., Heredia, Costa Rica, and by the Foundation Hospital Veterinario, Escuela de Medicina Veterinaria, Universidad Nacional.

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