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Immunodiffusion Test for Diagnosing and Monitoring Pythiosis in Horses

LEONEL MENDOZA,[1] LEO KAUFMAN,[2]* AND PAUL G. STANDARD[2]

Escuela de Medicina Veterinaria, Universidad Nacional, Heredia, Costa Rica,[1] and Division of Mycotic Diseases, Center for Infectious Diseases, Centers for Disease Control, Atlanta, Georgia 30333[2]

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A practical, sensitive, and specific immunodiffusion test was developed for diagnosing and monitoring pythiosis in horses. Culture filtrates, a soluble cell mass, and trypsinized *Pythium* sp. antigens were evaluated against prepared rabbit anti-*Pythium* sp. serum and pythiosis horse case sera. The culture filtrate antigens demonstrated the greatest capacity for detecting precipitins and the greatest stability during storage. In contrast, the trypsinized antigens had the weakest capability for detecting multiple precipitins and the poorest stability. The 13 sera from horses with proven active pythiosis were positive in immunodiffusion tests with the culture filtrate antigens. Each serum contained from three to six precipitins. Treated horses lost precipitins, and some became antibody negative. No false-positive reactions were noted in tests with sera from normal horses and humans or with sera from a variety of heterologous horse and human infections.

Pythiosis is a disease of horses caused by an as yet unidentified species of *Pythium*. The *Pythium* species are members of the order Peronosporales of the phylum Oomycota, classified in the kingdom Protoctista (1, 2). (Since the *Pythium* species are identified on the basis of their sexual form, the etiologic agent of pythiosis cannot be identified. Accordingly, it will be simply referred to herein as *Pythium* sp. It should be noted that a Japanese horse isolate was identified as *Pythium* gracile [2], but that identification has not been confirmed.) The disease occurs primarily in tropical and subtropical areas of the world. It has been reported in Australia, Brasil, Burma, Colombia, Indonesia, Japan, New Guinea, the United States (6), and recently in Costa Rica (4). Equine lesions caused by *Pythium* sp. are clinically similar to those occurring in basidiobolomycosis, histoplasmosis farciminosi (epizootic lymphangitis), sporotrichosis, mycetomas, cutaneous habronemiasis, cutaneous glanders, and neoplasms (5).

Histopathologic studies do not always allow a definite diagnosis. Frequently, one observes abundant necrotic areas with broad, hyaline hyphae that are generally aseptate. Eosinophilic inflammatory reactions and the Splendore-Hoeppli reaction may also be observed around the hyphae. These reactions are not pathognomonic, since they may also occur in basidiobolomycosis, conidiobolomycosis, and cutaneous habronemiasis, particularly in the early stages of these diseases (4).

An experienced laboratorian can make an unequivocal diagnosis in 3 or more days by isolation of a *Pythium* sp. Unfortunately, few laboratory workers are acquainted with this organism and the methods used to identify it. The development of a simple serologic test with reliable reagents would permit a specific and rapid diagnosis. In 1982, Miller and Campbell (7) demonstrated that serologic tests could be used to diagnosis pythiosis. They prepared several antigens from a *Pythium* strain for use in immunodiffusion (ID) and complement fixation tests. The ID test was found to be entirely specific and sensitive. Horses with no history of pythiosis and animals which had recovered from pythiosis did not react in the ID test. In contrast, the complement fixation test detected only 82% of horses with pythiosis and yielded positive reactions with sera from recovered horses as well as with sera from horses with no history of pythiosis.

This study was undertaken to compare and evaluate an ID test with a culture filtrate antigen, as well as soluble cell mass antigen with the trypsinized antigen used by Miller and Campbell (7) for diagnosing equine pythiosis.

MATERIALS AND METHODS

Cultures. Two isolates of *Pythium* sp., designated H-6 and H-10, obtained from horses with pythiosis, were used for preparing antigens and antisera. The isolates were identified as *Pythium* sp. on the basis of the characteristics of their colonies and their ability to reproduce asexually with the production of motile zoospores (1, 6).

Sera. Thirteen sera from horses with proven pythiosis and five sera from horses with suspected pythiosis were examined. In addition, four sera from horses treated for pythiosis either by immunotherapy (6) and surgery or both were studied.

For control purposes, the following equine sera were evaluated: five from horses with high agglutination titers for sporotrichosis, three from horses with proven cutaneous habronemiasis, five from horses with proven conidiobolomycosis, three from horses with pneumonia caused by *Streptococcus* sp., and five from horses without evidence of any disease.

The following sera from humans were also tested: one from a case of proven conidiobolomycosis, two from cases of basidiobolomycosis, five from cases of aspergillosis, four from patients with candidiasis, and five from apparently healthy humans.

Four sera from rabbits inoculated with *Pythium* sp. strains H-6 and H-10 were also studied.

Antigen production. Cultures of *Pythium* sp. strains H-6 and H-10 were transferred to brain heart infusion (Difco Laboratories, Detroit, Mich.) and Sabouraud dextrose agar slants and incubated at 37°C for 3 and 10 days. Small portions of growth from the brain heart infusion slant cultures were transferred to each of six 1.0-liter flasks containing 500 ml of brain heart infusion broth. The flasks were incubated at 37°C on a shaker rotating at 150 rpm. Two

* Corresponding author.

flasks, one for each strain, were removed after 5, 10, and 15 days of incubation.

The cultures were killed with merthiolate (0.02%), filtered, and concentrated 20-fold in an Amicon stir cell (Amicon Corp., Lexington, Mass.). This preparation was designated the culture filtrate antigen (CFA). The cell mass obtained by filtration was broken with a Braun MSK cell homogenizer (Bronwill Scientific, Inc., Rochester, N.Y.) and centrifuged at 3,000 × *g* for 20 min. The supernatant so obtained was designated the soluble cell mass antigen (SCMA).

Trypsinized antigen was prepared as previously described by Miller and Campbell (7). Essentially, this involved using 10-day-old cultures of the H-6 strain that were removed from the surface of the Sabouraud dextrose agar slants and dried at 37°C. The fungal mass was then ground to a powder, and 1.0 g was added to 0.5 g of trypsin in 8 ml of 0.05 M phosphate buffer (pH 8.0). This was incubated at 37°C for 24 h in a mechanical shaker rotating at 150 rpm. After centrifugation at 3,000 × *g* for 20 min, the supernatant antigen was collected.

Antiserum production. The CFA preparations obtained from strains H-6 and H-10 were inoculated into four albino rabbits. Each rabbit was injected intramuscularly with 0.5 ml of CFA and 0.5 ml of Freund incomplete adjuvant (Difco) on days 1, 2, and 3. CFA (0.5 ml) was also injected intravenously on days 7 and 14. On day 21 the rabbits were bled, and their sera were tested for precipitins by the ID test against the CFA, SCMA, and trypsinized antigens. Sera demonstrating four to six strong precipitins were used as positive reference sera.

ID test. Agar-gel double diffusion was carried out in 100- by 15-mm plastic petri dishes into which 7.5 ml of purified 1% phenolized agar was added. A pattern with 4-mm-diameter wells 4 mm apart was used. The reactants were added to the wells and incubated for 24 h in a humid chamber at room temperature.

TABLE 1. Immunodiffusion reactions of sera from two rabbits immunized with *Pythium* sp. H-6 CFA with *Pythium* sp. H-6 and H-10 culture filtrate, soluble cell mass, and trypsin-treated antigens

Strain	Antigen derivation		No. of precipitin bands produced	
	Type of antigen	Age (days)	Rabbit A	Rabbit B
H-6	CFA	5	6	5
		10	5	5
		15	4	4
H-6	SCMA	5	(2)[a]	(2)
		10	(2)	(2)
		15	(2)	(2)
H-6	Trypsinized, nonconcentrated	10	1	1
	Trypsinized, 2X concentrated	10	2	2
H-10	CFA	5	5	4
		10	4	3
		15	4	3
H-10	SCMA	5	(2)	(2)
		10	(2)	(2)
		15	(4)	(3)

[a] Parentheses indicate weak bands.

TABLE 2. Immunodiffusion reactions of sera from horses with pythiosis and from horse and human control sera with *Pythium* sp. strain H-6 antigens

Serum source	Disease state	No. of animals		No. of precipitins identical to those in rabbit reference H-6 antiserum
		Tested	Positive	
Horse	Proven active pythiosis	13	13	3-6
Horse	Suspected active pythiosis	5	3	4-6
Horse	Treated pythiosis	4	2	1
Horse	Streptococcal pneumonia	3	0	0
Horse	Conidiobolomycosis	5	0	0
Horse	Granulation tissue	3	0	0
Horse	Habronemiasis	3	0	0
Horse	Suspected sporotrichosis	4	0	0
Horse	Normal	5	0	0
Human	Aspergillosis	5	0	0
Human	Basidiobolomycosis	1	0	0
Human	Candidiasis	5	0	0
Human	Conidiobolomycosis	1	0	0
Human	Normal	5	0	0

RESULTS

Studies were performed to determine the reactivity of *Pythium* sp. (H-6 and H-10) CFA, SCMA, and trypsinized antigens undiluted (Table 1) and diluted 1:2, and 1:4 with phosphate-buffered saline (pH 7.2). The ID test antigens prepared with the H-6 and H-10 *Pythium* sp. strains gave the following results upon reaction with the rabbit anti-*Pythium* sp. H-6 CFA serum. The CFA produced two to six strong precipitin bands in the ID test. The maximum number of bands was observed with the undiluted antisera that reacted with 5-day-old CFA; 10- and 15-day-old CFA showed diminished reactivity with the rabbit antisera. One to four weak precipitin bands were produced with the SCMA preparations. Negative reactions occurred with some 1:2 to 1:4 antigen dilutions. Twice-concentrated H-6 and H-10 SCMA antigens did not demonstrate enhanced reactivity over the unconcentrated preparations. The trypsinized antigen prepared from strain H-6, unconcentrated and concentrated two times elicited one and two bands, respectively. Patterns of antibody reactivity similar to those obtained with the rabbit antisera were noted with the three antigens and horse sera from pythiosis cases.

During this study, we found that the reactivity of the trypsinized antigen decreased rapidly. Trace reactivity was noted 1 month after preparation, and no reactivity was observed after 2 months. In contrast, the CFA preparations remained stable for at least 1 year after their preparation. In these studies, the trypsin antigen was always used within 1 day of preparation.

The ID studies were carried out with the 5-day-old H-6 CFA and rabbit reference H-6 antiserum to seek antibodies in undiluted sera from horses with proven pythiosis, suspected pythiosis and *Pythium* sp.-infected horses cured either by immunotherapy or surgery or both (Table 2). The 13 horses with proven active pythiosis contained between three and six precipitins in their sera that were identical to those of the reference serum. Some of the horses had one precipitin that was unrelated to any of those in the reference specimen. Of five horses with suspected pythiosis, the sera of three had four to six precipitins of identity, whereas two

TABLE 3. Number of *Pythium* sp. precipitins in sera from horses with pythiosis before and after immunotherapy, surgery, or both

Horse case no.	No. of precipitins[a]	Time interval for serum collection
1	4	8 months after infection
	1	4 months after immunotherapy
2	4	2 months after infection
	1	7 months after immunotherapy and surgery
3	4	5 months after infection
	0	10 months after immunotherapy
4	–[b]	
	0	5 years after surgery

[a] Number of horse anti-*Pythium* sp. precipitins detected upon reaction with *Pythium* sp. H-6 CFA that were identical to those in rabbit reference serum.
[b] Pretreatment serum not available.

were negative. Sera obtained from horses with heterologous diseases such as cutaneous habronemiasis, conidiobolomycosis, suspected sporotrichosis, and streptococcal pneumonia and sera from five normal horses were completely negative in the ID test with the CFA. Sera from humans with a variety of heterologous fungal infections including conidiobolomycosis, basidiobolomycosis, aspergillosis, and candidiasis as well as sera from healthy humans were also studied. All were negative for diagnostic precipitins (Table 2).

Sera from four horses with pythiosis treated either by immunotherapy, surgery, or both were studied. Two were positive with only a single precipitin, and two were negative. In three of the cases, where pretreatment sera were available, it was evident that a significant decline in precipitins had occurred with immunotherapy or surgery (Table 3).

The CFA, SCMA, and trypsin antigens were compared in ID tests with sera from a horse infected with *Pythium* sp. It was apparent that all of the antigens present in the SCMA and trypsin preparations were also contained in the CFA. The CFA contained at least six antigens, and the unconcentrated trypsin antigen contained only one antigen (Fig. 1), whereas the SCMA had two weak antigens.

DISCUSSION

The serodiagnosis of pythiosis can circumvent the need for extensive, time-consuming, costly cultural and histopathological studies in horses with suspected pythiosis. Miller and Campbell (7) prepared a trypsinized antigen from mycelial elements of a *Pythium* sp. which they found to be sensitive and specific in ID tests for pythiosis. Our comparative studies with CFA and SCMA preparations revealed that nontrypsinized antigens derived from culture filtrates provide the greatest sensitivity with a specificity equivalent to that of the antigen recommended by Miller and Campbell (7). The CFA detected between three and six precipitins in infected horses, whereas the trypsinized antigen consistently detected only one precipitin. The SCMA contained two weak antigens. It proved to be inadequately sensitive. Our data also indicated that the trypsin antigen was highly unstable. It lost considerable activity within 2 weeks and in many cases all reactivity in 1 month. Use of such an antigen after storage for 1 month could result in false-negative diagnoses. The CFA preparations proved to be stable for at least 1 year, thus eliminating the continuous need for fresh antigens.

Using undiluted CFA from a 5-day-old culture of *Pythium* sp. H-6, we developed a practical ID test using rabbit reference anti-*Pythium* sp. serum for detecting pythiosis antibodies. The reference system produced five to six precipitin bands. A horse serum reacting with CFA and producing one or more lines of identity with the reference precipitate was considered positive for pythiosis. Three to six such precipitins were observed in sera from 13 horses with culturally proven pythiosis. Miller and Campbell (7) observed no such precipitins in sera from normal horses. We also did not find precipitins in sera from normal horses or humans. In addition, none was found in sera from horses and humans with heterologous diseases. The horse antisera usually contained more precipitins than the sera from immunized rabbits. A band of identity, however, with any of the rabbit precipitins was always diagnostic. Our experience to date suggests that any reaction with CFA is diagnostic for pythiosis. Thus, the presence of precipitins is a reliable indicator of pythiosis. It is interesting to note that three of the five horses with suspected pythiosis were serologically positive. These three horses also had hyphae in biopsied tissue, whereas tissue from the serologically negative horses was free of hyphal elements.

As noted by Miller and Campbell (7), horses with pythiosis, regardless of the length of illness, were always seropositive. Although our test detected multiple precipitins, the number of precipitins detected did not appear to relate to the duration of the illness or its severity. Our test appears to have prognostic value. Horses treated surgically or by immunotherapy or both not only demonstrated clinical cures but also showed definite declines in the number of precipitins, with either one precipitin or none being detectable (Tables 2 and 3). Our data suggest that treated horses with one or no serum precipitins can be considered cured. Miller and Campbell (7), using the trypsinized antigen, found that 6 weeks after successful treatment the diagnostic band disappeared. Our study with the CFA indicates that successfully treated horses may show a single precipitin 4 to 7 months after treatment and may become seronegative 9 to 10 months later.

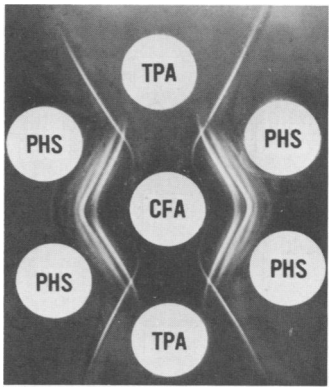

FIG. 1. Reactivity of *Pythium* sp. culture filtrate and trypsinized antigens with serum from a horse with pythiosis. Abbreviations: TPA, freshly prepared trypsinized antigen (<24 h old); PHS, pythiosis case horse serum. The horse serum produced the maximal number of precipitins, six with the CFA. Only one precipitin, also detectable with the CFA, was evident with the TPA.

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