CLINICAL AND DIAGNOSTIC LABORATORY IMMUNOLOGY, Nov. 1997, p. 715–718 Vol. 4, No. 6
1071-412X/97/$04.00+0

Serodiagnosis of Human and Animal Pythiosis Using an Enzyme-Linked Immunosorbent Assay

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Received 14 May 1997/Returned for modification 31 July 1997/Accepted 15 August 1997

Conventional serodiagnosis of *Pythium insidiosum* infections involves the use of the immunodiffusion (ID) test. This test specifically diagnoses human and animal pythiosis. The test, however, has limited sensitivity and does not detect some culturally proven cases of the disease. Because of the increased recognition of pythiosis among humans and animals, we developed and evaluated an enzyme-linked immunosorbent assay (ELISA) using a soluble antigen from broken hyphae of *P. insidiosum*. Studies were carried out with sera from five humans and eight animals with culturally and/or histologically proven pythiosis. Some of these sera were negative in the ID test for pythiosis. Heterologous case sera from thirteen humans and two horses, plus 5 sera from healthy humans and 17 from healthy animals, were tested. Of the pythiosis case sera tested, the ID test detected only 8 of 13 (61.5%), whereas the ELISA detected all of them (100%). The ID and ELISA tests were entirely specific and gave negative results or low titers respectively, with sera from humans and animals with heterologous fungal infections or with no apparent illness. No correlation was found between the height of the ELISA titers and negative or positive sera in the ID test. Our results indicate that the ELISA is a reliable serodiagnostic test for pythiosis. It is as specific as the ID test but more sensitive.

Infections caused by *Pythium insidiosum*, a fungus-like organism of the kingdom *Chromista* and phylum *Pseudofungi* (6, 7), are characterized by the development of cutaneous and subcutaneous granulomatous lesions (4, 24, 27). Lymph and blood vessel invasion (5, 27, 28) as well as intestinal (2, 22), lung (9), and bone (1, 8) tissue invasion have been well documented in the last two decades. In addition, devastating orbital-facial (24, 27) and corneal infections caused by *P. insidiosum* in humans (11, 28) are being seen increasingly in tropical and subtropical areas of the world. Pythiosis was reported early in the 20th century in equines afflicted with cutaneous granulomas (4). However, it was only after 1971 that this disease was recognized as being of significance in other species, including humans (7, 10, 24–29). Recent reviews on pythiosis have covered the past and current aspects of the disease in humans and animals (4, 19, 26).

Conventional drugs for treating pathogenic fungi are usually ineffective against infections caused by this organism. This may be due to the fact that *Pythium* spp. do not synthesize sterols in the cytoplasmic membrane as do members of the kingdom *Fungi*. Consequently, drugs such as amphotericin B and imidazoles may be useless on *P. insidiosum*. Surgery and iodides have been frequently used, but reports of failure or relapses with these procedures are common. Because pythiosis is difficult to treat, it is life threatening in more than 70% of diagnosed patients. Recently, an immunotherapeutic vaccine for pythiosis was described (14, 20). The vaccine works well in horses with acute disease but failed when tested in horses with chronic pythiosis (18). The efficacy of the vaccine in humans has yet to be determined.

Since chronic pythiosis responds poorly to the available treatments, early diagnosis is essential to assure timely and appropriate management. The availability of a sensitive and specific serologic method could enable rapid and successful management of the disease by either surgery, iodides, chemotherapy, or immunotherapy. An immunodiffusion (ID) test using *P. insidiosum* culture filtrate antigens containing six precipitinogens (15) has proven useful for diagnosing human and animal pythiosis (12, 16, 23). The ID test, however, did not demonstrate the sensitivity necessary to detect all cases of pythiosis, especially those in dogs and humans with subcutaneous, intestinal, and/or blood vessel infection (5, 12, 29). The present study describes our efforts to improve the serodiagnosis of pythiosis through the use of an enzyme-linked immunosorbent assay (ELISA) with a soluble antigen from broken hyphae (SABH) of *P. insidiosum.*

MATERIALS AND METHODS

Sera. Fifty sera from humans, dogs, equines, and a cat were tested in an ELISA. Five pythiosis case sera were from humans and eight were from animals. Three of the human sera were from patients with arteritis (94-Thai 03, 95-Thai 02, and 96-Thai 01 [see Table 1]). Two sera were from culturally proven cases of subcutaneous human pythiosis (96-010728 and 95-Thai 04 [see Table 1]). One pythiosis case cat serum was studied. The cat had subcutaneous pythiosis from which *P. insidiosum* was isolated in pure culture (97-Flo 001 [see Table 1]). Two proven canine pythiosis case sera were also studied, one of which was from a dog with gastrointestinal lesions (95-001355 [see Table 1]). Pythiosis in this case was confirmed by the detection of *P. insidiosum* hyphae in biopsied tissue with fluorescent antibodies (16). The second canine case (96-010735 [see Table 1]) presented with a thoracic mass from which *P. insidiosum* was isolated. Five of the animal sera tested were from horses with cutaneous and subcutaneous pythiosis proven by culture.

To evaluate test specificity, sera from 13 humans and two horses with proven heterologous fungal infections (see Table 1) and control sera from five humans, 10 horses, two dogs, and five cats without apparent illness were studied. The heterologous human sera were from three patients with aspergillosis, two with candidiasis, three with coccidioidomycosis, three with histoplasmosis, and two with proven subcutaneous zygomycosis caused by *Basidiobolus ranarum*. Two sera from proven cases of equine subcutaneous zygomycosis caused by *Conidiobolus coronatus* were also tested (see Table 1).

Antigen production. *P. insidiosum* ATCC 58643 was subcultured at 37°C on Sabouraud dextrose agar every 20 days and then transferred to slants of cornmeal agar. One 3-mm^2 block with hyphal elements of *P. insidiosum* from a 24-h cornmeal agar slant culture incubated at 37°C was used to inoculate a 1.0-liter

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TABLE 1. *P. insidiosum* ELISA titers and ID reactions with sera from humans and animals with pythiosis and various heterologous fungal diseases

Host	Serum sample	ELISA titer (ID reaction)[a] with serum from human or animal with:						
		Aspergillosis	Basidiobolomycosis	Candidiasis	Coccidioidomycosis	Conidiobolomycosis	Histoplasmosis	Pythiosis insidiosi
Human	93-026515	<1:100 (−)						
	93-020462	1:100 (−)						
	95-032405	1:200 (−)						
	95-019522		1:100 (−)					
	95-030669		1:200 (−)					
	93-022162			1:100 (−)				
	93-033227			No titer (−)				
	92-021053				<1:100 (−)			
	95-019512				1:200 (−)			
	92-033243				<1:100 (−)			
	92-033351						<1:100 (−)	
	95-01612						No titer (−)	
	94-036785						No titer (−)	
	96-Thai 01							1:6,400 (−)
	95-Thai 02							1:3,200 (−)
	94-Thai 03							1:6,400 (+)
	95-Thai 04							1:3,200 (−)
	96-010728							1:3,200 (+)
Dog	96-001355							1:25,600 (−)
	96-010735							1:6,400 (−)
Cat	97-Flo 001							1:12,800 (+)
Equine	95-009341					<1:100 (−)		
	95-030669					<1:100 (−)		
	Five 94-Tex sera							1:3,200–1:25,600 (+)[b]

[a] Titers are expressed as the reciprocal of the dilution factor. ID results are expressed as positive (+) (band[s] of identity to *P. insidiosum* antigens) or negative (−).
[b] All five sera were positive by ID.

flask containing 500 ml of Sabouraud dextrose broth. The broth culture was incubated at 37°C while being rotated at 150 rpm for 5 days. After incubation, the culture was killed with merthiolate (0.02%, wt/vol) and then filtered. The cell mass was then transferred to a mortar and ground in the presence of liquid nitrogen. The resulting powder was resuspended in 5 ml of sterile distilled water and incubated at 4°C for 24 h. After incubation, the mixture was centrifuged at 5,000 × *g* for 10 min, and the supernatant was collected. The SABH was dialyzed with a membrane molecular weight cutoff point of 10,000. The protein concentration was estimated by the Bio-Rad microassay procedure (Bio-Rad Laboratories, Hercules, Calif.) and adjusted to ~3.0 μg of protein per ml. The SABH was stored at 4°C until used.

The antigen for the ID test was prepared according to Mendoza et al. (15). Briefly, broth cultures of *P. insidiosum* (ATCC 58643) were prepared and incubated as above. The filtrate was concentrated by ultrafiltration under positive pressure in a stirred cell fitted with a PM-10 membrane (Amicon Corp., Lexington, Mass.). The culture filtrate antigen (CFA) was stored at 4°C until used.

ELISA. Flat-bottom polystyrene microtiter plates (96-well Immulon 2; Dynatech Laboratories Inc., McLean, Va.) were coated overnight at 4°C with 100 μl of 5 μg of SABH per ml. The wells were then washed three times with phosphate-buffered saline with 1% (vol/vol) Tween 20 (PBS-T) and blocked for 1 h at 37°C with 5% gelatin. Test sera were diluted twofold from 1:100 to 1:102,400, and 50 μl of each dilution was added to each well and incubated with the antigen for 1 h. Four wells each containing positive and negative control sera, as well as wells for buffer and substrate controls, were included and washed three times, as described above. One hundred microliters of a horseradish peroxidase-conjugated rabbit anti-human immunoglobulin G, or equivalent anti-canine, -feline, or -equine (heavy and light chain) antibody, was diluted in PBS-T, and 100 μl was added to each well and then incubated at 30°C for 1 h. The wells were washed three times, and 100 μl of freshly prepared chromogen buffer (0.1 mg of *O*-phenylenediamine dihydrochloride and 3.0 μl of 30% H_2O_2/1 ml of citrate borate buffer [pH 5.6]) was added to each well and allowed to react at room temperature for up to 20 min. Color development was recorded in a Dynatech MR 5000 ELISA plate reader at 490 nm. The reaction was stopped with 100 μl of 4 N H_2SO_4. Sera were recorded as being positive if their optical density readings were higher than the average value of the negative control sera plus 2 standard deviations. The titer was recorded as the highest serum dilution giving a reading double that of the saline control.

RESULTS

All of the five proven human pythiosis case sera, regardless of their ID reactivity, showed very high titers in the ELISA (Table 1; Fig. 1). Titers of 1:3,200 to 1:6,400 were observed with these sera. All of the 13 proven human and animal pythiosis case sera reacted positively in the ELISA (100%), whereas only 8 reacted in the *P. insidiosum* ID test (61.5%). Two sera from dogs with proven pythiosis were ID negative but showed 1:6,400 and 1:25,600 titers in the ELISA (Table 1; Fig. 1). Similar titers were obtained with the sera from horses with proven pythiosis and positive ID reactions. ELISA titers were monitored in serum from a successfully treated human

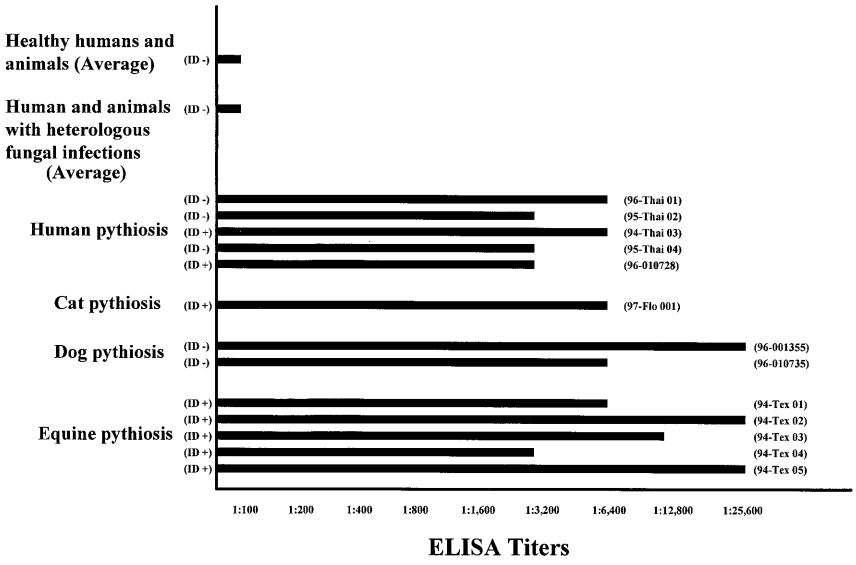

FIG. 1. Distribution of *P. insidiosum* ELISA titers obtained for 63 sera categorized according to their hosts. The result obtained for each serum in the ID test for pythiosis is shown on the left side of the figure.

with pythiosis arteritis. The disease in this case was confirmed by histopathologic and cultural studies (96-Thai 01 [Table 1]). In this case, the initial ID test was negative while the ELISA test gave a titer of 1:6,400. After therapy, serial serum specimens taken after 2, 4, and 6 months showed diminishing titers of 1:3,200, 1:1,600, and 1:1,600, respectively. All of these sera were negative in the ID test (data not shown).

The heterologous case sera from humans and animals were either negative or gave titers of 1:100 and 1:200 by the ELISA (Table 1; Fig. 1). Sera from apparently healthy humans and animals exhibited similar negative reactions or low titers in the ELISA. Similarly, the ID test was negative with the sera from the heterologous case infections and from the apparently healthy humans and animals (Fig. 1). In addition, new batches of SABH showed the same strength and sensitivity as the first batch when tested in the ELISA.

DISCUSSION

The ID test has proven to be valuable for detecting equine pythiosis (4, 15, 21). However, it may be negative when applied to some sera from dogs and humans with pythiosis (5, 12, 29). In an attempt to achieve a higher degree of sensitivity, we ascertained the value of SABH in the ELISA. This ELISA detected not only all of the pythiosis case sera positive by ID but also those proven pythiosis case sera which were negative by the ID test.

Although ELISA readings of up to 1:25,600 were recorded with some of the proven pythiosis case sera, no correlation was observed between titers of sera positive by ID and those negative by ID. For instance, sera from three human pythiosis cases negative by ID showed titers similar to those of two positive by ID. Similarly, the two canine sera negative by ID showed ELISA titers of 1:25,600 and 1:6,400, respectively. The ELISA may be of value in monitoring treatment for pythiosis. Sera from a successfully treated human pythiosis arteritis case showed a decline in titer from 1:6,400 to 1:1,600 after 6 months of therapy.

Cross-reaction with antibodies to the agents of entomophthoramycosis does not appear to be a problem. ELISA readings similar to those obtained with the negative control sera were recorded when the new *P. insidiosum* antigen was used with sera of humans infected with *B. ranarum* and equines infected with *C. coronatus*. Cross-reactivity was previously noted in an ID test with *P. insidiosum* CFA and sera from rabbits inoculated with *B. ranarum* and *C. coronatus* antigens (13). No cross-reaction, however, was observed when sera from humans with subcutaneous zygomycosis caused by *B. ranarum* and *C. coronatus* were tested against the *P. insidiosum* CFA (16). Likewise, rabbit polyclonal antibody to *P. insidiosum* hyphae, used in an indirect immunoperoxidase technique applied to tissue sections containing *P. insidiosum*, *B. ranarum*, and *C. coronatus*, reacted only with *P. insidiosum* hyphae (3, 27).

Clinically and histologically, *P. insidiosum* infections mimic infections caused by members of the class *Zygomycetes* (4, 13, 14); thus, it is important to serologically differentiate these diseases. Fluorescent antibody (17) and immunoperoxidase (3) studies as well as the ELISA-SABH (this study) have indicated that antibodies to the hyphae of *P. insidiosum* are useful for diagnosing pythiosis in humans and animals. Previous studies (3, 16) and the results obtained in our study indicate that *P. insidiosum* hyphal antigens possess unique epitopes not encountered in members of the kingdom *Fungi*. The fact that organisms in the class *Oomycete* were recently placed in the kingdom *Chromista* (6) further corroborates the antigenic and taxonomic divergence of *P. insidiosum* from members of the kingdom *Fungi*. Interestingly, SABH appears to be of little value when used in the ID test (data not shown). In contrast, strong reactions demonstrating four to six bands occurred in ID tests using the CFA and pythiosis case sera (15). This suggests that CFA is more efficient in detecting precipitin antibodies than the SABH used in the ELISA.

The first human pythiosis cases were diagnosed in 1986 (7, 25, 26). Since then, more than 28 cases have been reported in areas where *P. insidiosum* was previously known only as an animal pathogen (26). The upsurge of cases of pythiosis in areas where the disease is endemic is due in part to the increasing awareness of its presence in those areas, the dissemination of knowledge of its clinical and epidemiolog-

ical features, and the development of new serological tests for its diagnosis (4, 15, 19, 21, 26). The widespread use of the ELISA with SABH should provide a sensitive and accurate assay for detection of pythiosis cases in areas where it is endemic, regardless of the type of host. In addition, this test may be useful for detecting pythiosis in humans and animals in cases in which the current ID test has failed. Due to its sensitivity, the ELISA test may also be valuable for diagnosing early and chronic stages of the disease and for epidemiological studies.

ACKNOWLEDGMENTS

We thank Ploenchan Chetchotsakd, Arunee Thitithanyanont, and Randall Thomas for some of the sera used in this study.

This research was supported in part by Bio-Medical International.

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