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# Antigenic Relationship between the Animal and Human Pathogen *Pythium insidiosum* and Nonpathogenic *Pythium* Species

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**Identification of the newly named pathogenic oomycete *Pythium insidiosum* and its differentiation from other *Pythium* species by morphologic criteria alone can be difficult and time-consuming. Antigenic analysis by fluorescent-antibody and immunodiffusion precipitin techniques demonstrated that the *P. insidiosum* isolates that cause pythiosis in dogs, horses, and humans are identical and that they were distinguishable from other *Pythium* species by these means. The immunologic data agreed with the morphologic data. This indicated that the animal and human isolates belonged to a single species, *P. insidiosum*. Fluorescent-antibody and immunodiffusion reagents were developed for the specific identification of *P. insidiosum*.**

Pythiosis is a cutaneous-subcutaneous and intestinal disease of animals that is caused by *Pythium insidiosum* (synonym, *P. destruens* [15]), a newly described species (4) found in tropical and subtropical areas of the world. *Pythium* spp. are classified in the kingdom *Protoctista*, class *Oomycetes*, order *Peronosporales*, and family *Pythiaceae*. The first isolations of this oomycete from animals were done by de Haan (5) in 1902. In 1961, Bridges and Emmons (3) isolated the organism from eight horses with subcutaneous lesions and named it *Hyphomyces destruens*. They considered it to be a zygomycete, perhaps a species of *Mortierella*, but no asexual or sexual spores were found to support this contention. Unfortunately, their cultures were lost before a definitive identification could be made. A similar organism, isolated from a case of granular dermatitis in a horse in Japan (1), was also claimed to be related to *Mortierella* spp. This organism was identified as *H. destruens*. Later Austwick and Copland (2) found that the etiological agent of subcutaneous granulomas in horses produces biflagellate zoospores and belongs to the genus *Pythium* rather than to *Mortierella*.

The identification of *Pythium* species is based in part on their production of motile biflagellate zoospores and oospores, which are not easy to induce in some species of the genus. For example, Ichitani and Amemiya (6) reported that an isolate from a Japanese horse produced oogonia resembling those of *P. gracile*. That identification, however, could not be confirmed by other investigators because of their inability to obtain oogonia and oospores in their *Pythium* isolates.

In recent years, other animal isolates obtained from Australia (16), Costa Rica (10), Japan (6), New Guinea (2), and the United States (13, 14) developed sufficient morphologic characteristics that they could be classified in the genus *Pythium*; however, species identification of these pathogenic isolates was never carried out.

Recent collaborative morphologic studies by de Cock et al. (4) showed that the isolates causing pythiosis in animals and humans belong to an unnamed species of *Pythium*, for which they proposed the binomial *P. insidiosum*. Our present study was carried out simultaneously to elucidate the antigenic relationship existing among animal, plant, and soil isolates of *Pythium* spp. by using immunodiffusion (ID) and fluorescent-antibody (FA) techniques.

## MATERIALS AND METHODS

**Cultures.** Five isolates of *P. insidiosum* that caused pythiosis in horses and in a dog, plus five *Pythium* spp. isolated from plants and soil, were studied. The sources of these isolates and their reference numbers are listed in Table 1. In addition, two isolates obtained from cases of pythiosis in humans in Thailand were examined. Since these two isolates (CDC B-4307 and CDC B-4309 [CBS 673.85]) were initially suspected to be *Lagenidium* spp. (A. W. A. M. de Cock, personal communication), *L. giganteum* CBS 580.84 was included as a control.

**Antigen production.** All of the cultures were transferred to cornmeal agar slants (Difco Laboratories, Detroit, Mich.). The equine and canine isolates were incubated at 37°C, while those from plants or soil were incubated at 25°C, for 5 to 15 days. Small portions of growth from the cornmeal agar slant cultures of all of the *Pythium* isolates except *P. monospermum* were transferred to 1.0-liter flasks containing 500 ml of brain heart infusion broth (Difco). We attempted to grow all of the *Pythium* spp. statically in brain heart infusion broth. Those species which grew poorly under the aforementioned growth conditions were shaken or grown in Sabouraud dextrose broth (Difco). *P. monospermum* produced sparse growth in brain heart infusion broth and therefore was grown in 500 ml of Sabouraud dextrose broth. The flasks containing the animal pathogens were statically incubated at 37°C, whereas those containing isolates from the nonanimal sources, other than *P. monospermum*, were incubated at 25°C on a shaker rotating at 150 rpm. *P. monospermum* was statically incubated at 25°C. After 5 days of growth, all of the cultures except *P. monospermum* were killed with Merthiolate (0.02%), filtered, and concentrated 20× in a stir cell (Amicon Corp., Lexington, Mass.). The *P. monospermum* cultures grew slowly and were similarly treated after 30 days of growth. The concentrated soluble antigens were used to inoculate animals and in ID tests. The cell masses obtained by filtration were used for adsorptions in FA studies. The cell masses were stored in 0.02% aqueous Merthiolate at 4°C until used.

**Antiserum production.** The soluble antigens harvested from each culture were injected into albino rabbits. Each

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TABLE 1. Isolation data for the *Pythium* and *Lagenidium* species studied

| Isolate identity | Culture collection no.[a] | Source |
|---|---|---|
| *P. insidiosum* | CDC B-4295 = ATCC 58642 | Horse, Costa Rica |
| *P. insidiosum* | CDC B-4298 = ATCC 28251 = *Pythium* sp. sensu Austwick & Copeland | Horse, New Guinea |
| *P. insidiosum* | CDC B-4299 = ATCC 46947 = *P. gracile* sensu Ichitani & Amemiya | Horse, Japan |
| *P. insidiosum* | CDC B-4300 = LSU P-25156 (R. Miller) | Canine gastrointestinal infection, United States |
| *P. insidiosum* | CDC B-4301 = LSU P-609325 (R. Miller) | Horse, United States |
| *P. insidiosum* | CDC B-4302 = LSU P-609224 (R. Miller) | Horse, United States |
| *P. debaryanum* | ATCC 10393 | Unknown |
| *P. diclinum* | CBS 664.79 | Plant |
| *P. graminicola* | ATCC 28458 | Plant |
| *P. inflatum* | ATCC 38894 | Soil |
| *P. monospermum* | CBS 158.73 | Soil |
| *L. giganteum* | CBS 580.84 | Mosquito larvae |

[a] CDC, Centers for Disease Control; ATCC, American Type Culture Collection; LSU, Louisiana State University; CBS, Centraalbureau voor Schimmelcultures.

rabbit was injected intramuscularly with 0.5 ml of soluble antigen and 0.5 ml of Freund incomplete adjuvant (Difco) on days 1, 2, and 3. A 0.5-ml portion of the antigen was also injected intravenously on days 7 and 14. On day 21 the rabbits were bled, and their sera were tested for precipitins in ID tests against homologous antigens (11).

**Conjugation procedures.** FA reagents were prepared as described by Jones et al. (7). Accordingly, rabbit antiserum to *P. insidiosum* B-4295 was precipitated with an equal volume of cold, saturated ammonium sulfate solution. The precipitated antiglobulin was recovered by centrifugation and, after being dissolved in distilled water, was dialyzed against 0.85% saline solution. A biuret protein determination was made, and the antiglobulin was labeled with fluorescein isothiocyanate with 0.05 mg of dye per mg of antiglobulin.

Unreacted fluorescent dye was removed by gel filtration with Sephadex G-25 (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.). The conjugate was then concentrated by pervaporation to a volume equal to that of the original serum.

**ID test.** Agar-gel double diffusion was carried out in plastic petri dishes (100 by 15 mm) to which 7.5 ml of 0.25% phenolized, 1% purified agar (Difco) was added. A pattern consisting for 4-mm-diameter wells 4 mm apart was used. The reactants, consisting of 20×-concentrated antigens and undiluted antisera, were added to the wells and incubated for 24 h in a humid chamber at room temperature.

**Staining procedures and visualization of stained preparations.** The procedure used to stain the cell preparations was that described by Kaplan and Ivens (8). The cells were examined with a Leitz Ortholux II indirect-light fluorescence microscope. Based on the intensity of staining, the results were recorded as −, ±, 1+, 2+, 3+, and 4+; −, ±, and 1+ were considered negative, and 2+, 3+, and 4+ were considered positive.

For FA studies, the cellular elements of each *Pythium* isolate were suspended in distilled water and washed three times. During the last wash, the tubes were shaken vigorously until the mycelium had been broken into small fragments as visualized by direct microscopic examination; these fragments usually were found in the upper part of the suspension. A few drops of each sample were taken from the surface of the suspension and smeared directly onto ringed slides. Since the hyphal fragments of the *Pythium* spp. autofluoresced, unstained controls were always run in parallel with the stained preparations.

**Adsorption procedures and testing of adsorbed conjugates.** One volume of conjugate was mixed with one volume of the packed cell mass and incubated for 2 h in a 37°C water bath. The mixture was centrifuged at 3,000 × *g* for 10 min. Two adsorptions were carried out with cellular elements of *P. diclinum*, and one was carried out with cellular elements of *P. graminicola*. After each adsorption, the conjugate titers were determined against homologous and heterologous organisms. The conjugates were stored at 4°C.

Rabbit antiserum for ID studies was adsorbed by mixing 1 volume of packed cells of *P. diclinum* with an equal volume of rabbit antiserum to *P. insidiosum* B-4295. The serum was adsorbed for 2 h at 37°C, and after separation it was evaluated for specificity. ID antiserum was stored at 4°C.

## RESULTS

ID and FA studies were performed to determine the antigenic relationships between the *P. insidiosum* isolates and nonpathogenic *Pythium* spp. isolated from plants and soil. The ID test carried out with the six antigens prepared from the isolates that were pathogenic for animals produced at least six intense precipitin bands upon reaction with the rabbit anti-*P. insidiosum* B-4295 serum. Using the antiserum and antigen from the B-4295 isolate as a reference system for studying heterologous antigens, we noted that all of the horse isolates had six antigens in common (Table 2). These six bands are identical to those described and shown in a recent report (11). One isolate, B-4299, produced an additional unrelated antigen. Antiserum prepared against all of the horse isolates contained the six precipitins noted in the reference rabbit antiserum. Adsorption of the B-4295 antiserum to cell mass antigens from the other *Pythium* isolates that were pathogenic for animals eliminated all antibody reactivity. Tests with the rabbit anti-*P. insidiosum* B-4295 serum and antigens derived from the five plant and soil isolates revealed that the antiserum contained antibodies that were cross-reactive with one or two antigens among the heterologous *Pythium* spp.

Adsorption of the B-4295 antiserum with *P. diclinum* antigens or antigens from any of the other *Pythium* spp. removed the cross-reacting antibodies and rendered the antiserum specific for the animal pathogens. The adsorbed antiserum contained six or seven precipitins that were demonstrable with all of the equine antigens (Table 2). Similar results were obtained with rabbit antisera to the other horse isolates before and after adsorptions with antigens of the other *Pythium* spp. Five or six bands of identity were observed with the *P. insidiosum* reference reagents when the unadsorbed or adsorbed *P. insidiosum* antiserum reacted with antigens derived from the two human isolates.

Rabbit antiserum was produced against each of the five *Pythium* spp. Except for the antiserum to *P. monospermum*, which produced one precipitin with its homologous antigen, each of the antisera tested produced two precipitins upon

TABLE 2. Immunodiffusion reactions of rabbit anti-*Pythium insidiosum* B-4295 serum and antiserum to five *Pythium* plant and soil isolates with homologous and heterologous soluble antigens

| Antigen | No. of precipitin bands with rabbit antiserum to: | | | | | | |
|---|---|---|---|---|---|---|---|
| | *P. insidiosum* B-4295 serum | Plant and soil isolates | | | | | *P. insidiosum* B-4295 adsorbed with *P. diclinum* |
| | | *P. debaryanum* | *P. diclinum* | *P. graminicola* | *P. inflatum* | *P. monospermum* | |
| *P. insidiosum* | | | | | | | |
| B-4295[a] | 6 | 1 | 1 | 1 | 1 | 1 | 6 |
| B-4299 | 7[b] | 1 | 1 | 1 | 1 | 1 | 7[b] |
| B-4298 | 6 | 1 | 1 | 1 | 1 | 1 | 6 |
| B-4302 | 6 | 1 | 1 | 1 | 1 | 1 | 6 |
| B-4301 | 6 | 1 | 1 | 1 | 1 | 1 | 6 |
| B-4300 | 6 | 1 | 1 | 1 | 1 | 1 | 6 |
| Plant and soil isolates | | | | | | | |
| *P. debaryanum* | 1[c] | 2 | 1 | 1 | 0 | 1 | 0 |
| *P. diclinum* | 1[c] | 2 | 2 | 1 | 1 | 2 | 0 |
| *P. graminocola* | 2[c] | 2 | 1 | 2 | 2 | 1 | 0 |
| *P. inflatum* | 1[c] | 2 | 1 | 1 | 2 | 1 | 0 |
| *P. monospermum* | 1[c] | 1 | 1 | 1 | 2 | 1 | 0 |

[a] Reference antigen.
[b] One nonidentity band.
[c] Band(s) of nonidentity with *P. insidiosum* B-4295 reference precipitates.

reaction with their homologous antigens (Table 2). Although all of the *P. insidiosum* isolates produced antigens that were reactive with antiserum to the plant pathogens, none of the antigens were identical to the reference *Pythium* antigens.

Antigens prepared for each of the horse and other *Pythium* spp. were tested against serum samples obtained from three horses with active pythiosis (Table 3). The three horse serum samples produced similar reactions against the test antigens. Although six to eight precipitin bands were observed in ID tests with the different animal isolate antigens, six of the bands were identical. Precipitin bands were not noted after the three horse serum samples were adsorbed with cells prepared from any of the animal isolates. In addition, no precipitin bands were observed in the ID tests when the antigens from the other *Pythium* spp. were reacted against the unadsorbed horse sera.

Fluorescein isothiocyanate-conjugated rabbit antiglobulin to *P. insidiosum* B-4295 was used to stain *P. insidiosum* and the other *Pythium* spp. (Table 4). The unadsorbed conjugate diluted 1:8 to 1:16 stained the cellular elements of all of the *Pythium* cultures studied with a 4+ intensity. The cell walls and cytoplasm stained uniformly. When the 1:8-diluted conjugate was adsorbed with hyphal elements of *P. diclinum*, it stained the animal isolates with 4+ intensity, the *P. graminicola*, *P. diclinum*, and *P. inflatum* isolates with 2+ to 3+ intensity, and the *P. debaryanum* and *P. monospermum* isolates with 1+ to 2+ intensity. A second adsorption was made with *P. diclinum* cells. The twice-adsorbed conjugate stained the animal isolates with a 3+ to 4+ intensity, whereas significant reductions in staining were noted with the other *Pythium* spp., notably *P. inflatum* and

TABLE 3. ID tests of sera from horses with pythiosis after reaction with antigens from animal, plant, and soil *Pythium* isolates

| Antigen | No. of precipitin bands with unadsorbed sera from infected horses[a] |
|---|---|
| *P. insidiosum* | |
| B-4295 | 7 |
| B-4299 | 8 |
| B-4298 | 6 |
| B-4302 | 6 |
| B-4301 | 6 |
| B-4300 | 7 |
| *P. debaryanum* | 0 |
| *P. diclinum* | 0 |
| *P. graminicola* | 0 |
| *P. inflatum* | 0 |
| *P. monospermum* | 0 |

[a] No precipitin bands were observed when the horse sera were adsorbed with any of the animal isolate antigens.

TABLE 4. Staining reactions of adsorbed and unadsorbed fluorescein isothiocyanate-labeled *P. insidiosum* B-4295 antiglobulin with antigens of animal, human, plant, and soil *Pythium* isolates

| Isolate | Reaction[a] of antiglobulin | | |
|---|---|---|---|
| | Unadsorbed | Adsorbed twice with *P. diclinum* | Adsorbed twice with *P. diclinum* and once with *P. graminicola* |
| *P. insidiosum* | | | |
| B-4295 | 4+ | 3+–4+ | 2+–3+ |
| B-4299 | 4+ | 4+ | 2+–3+ |
| B-4298 | 4+ | 3+–4+ | 2+–3+ |
| B-4302 | 4+ | 3+ | 2+–3+ |
| B-4301 | 4+ | 3+ | 2+–3+ |
| B-4300 | 4+ | 3+–4+ | 2+–3+ |
| B-4307[b] | ND | ND | 2+–3+ |
| B-4309[b] | ND | ND | 2+–3+ |
| *P. debaryanum* | 4+ | 1+ | 0 |
| *P. diclinum* | 4+ | ±–1+ | 0 |
| *P. graminicola* | 4+ | 1+–2+ | 0 |
| *P. inflatum* | 4+ | 0 | 0 |
| *P. monospermum* | 4+ | 0 | 0 |

[a] 2+ to 4+, Positive staining; 0, ±, or 1+, negative staining; ND, not done.
[b] Human isolate from Thailand.

*P. monospermum*, which were negative. *P. graminicola* demonstrated 1+ to 2+ staining. Accordingly, a third adsorption was made with *P. graminicola* as the antigen. The third adsorption eliminated the staining of the other *Pythium* spp. and rendered the conjugate specific for the animal *Pythium* isolates (Table 4). This conjugate stained the human isolates B-4307 and B-4309 with a 2+ to 3+ intensity and was nonreactive with *L. giganteum*.

## DISCUSSION

Our immunologic analyses revealed that all of the *Pythium* species studied, regardless of source, shared antigens with *P. insidiosum*. The antigenic relationships were sufficiently different, however, to allow distinction between *P. insidiosum* and the other *Pythium* spp. The ID studies indicated that the animal isolates produced and shared six antigens, which were identical to those of the reference culture of *P. insidiosum* B-4295. These data suggest that the animal isolates are antigenically identical to *P. insidiosum*. This contention is further supported by the fact that adsorptions of the rabbit reference anti-*P. insidiosum* serum or sera from horses and a dog with proven pythiosis with antigens derived from heterologous animal isolates completely eliminated antibody reactivity with the animal isolates.

The plant and soil isolates share at least one antigen with the animal isolates, as revealed by cross-precipitin and cross-staining reactions in the ID and FA studies with rabbit *P. insidiosum* antiserum and conjugated antiglobulin. None of the *P. insidiosum* precipitins that were reactive with the precipitinogens of other *Pythium* spp. were identical to any of the six reference precipitins that were reactive with the animal isolate antigens. These differences permitted the production of serological reagents capable of distinguishing the animal isolates from the plant and soil isolates. Adsorption of rabbit *P. insidiosum* antiserum with cellular elements of *P. diclinum* eliminated all precipitin activity with the antigens of the *Pythium* spp.

White (18) attempted to produce a specific FA reagent for *P. graminicola*. His studies revealed common antigens between *P. graminicola* and other plant *Pythium* spp. Our FA studies confirm his findings and indicate that the spread of common antigens occurs also among the animal *Pythium* isolates. A conjugated *P. insidiosum* antiglobulin, specific for the animal isolates, was produced only after adsorptions with *P. diclinum* and *P. graminicola*. Unadsorbed sera from horses with pythiosis demonstrated variability in cross-precipitin reactivity with the plant and soil isolate antigens. Some sera showed no reactivity, whereas others showed reactions of nonidentity. This was not the case, however, with conjugated horse antiglobulins, which always demonstrated cross-staining of the plant and soil isolates. These cross-reactions were easily removed by adsorptions with *P. diclinum* alone or in combination with *P. graminicola*.

Our studies revealed that the Japanese horse isolate ATCC 46947, identified as *P. gracile* by Ichitani and Amemiya (6), was antigenically identical to *P. insidiosum* and that it could not be equated with *P. diclinum* or *P. monospermum*, as suggested by Middleton (12) and Van der Plaats-Niterink (17), respectively. In addition, none of the antigens derived from the animal and human isolates produced lines of identity with the reference bands of *P. diclinum*, *P. monospermum*, or any of the other plant or soil isolate antigen-antibody precipitates.

Our FA and ID studies indicate that the animal and human isolates are antigenically identical and that specific fluorescent antibodies and precipitins could be produced against *P. insidiosum*. These specific reagents reacted with the two human isolates and thus identified them as *P. insidiosum*. It is thus apparent that pythiosis is not a disease limited to cattle, dogs, and horses, but that it also occurs in humans.

The ability to identify *P. insidiosum* and to distinguish it serologically from the *Pythium* spp. that are not pathogenic to animals is an important accomplishment because the morphologic characteristics needed for species identification are frequently difficult to induce and because many of their specific characteristics show variability (9).

## LITERATURE CITED


1. **Amemiya, J., and S. Nishiyama.** 1968. Isolation of a fungus of Mortierellaceae from an equine granular dermatitis. Bull. Fac. Agric. Kagoshima Univ. **18**:185–191.
2. **Austwick, P. K. C., and J. W. Copland.** 1974. Swamp cancer. Nature (London) **250**:84.
3. **Bridges, C. H., and C. W. Emmons.** 1961. A phycomycosis of horses caused by *Hyphomyces destruens*. J. Am. Vet. Assoc. **138**:579–589.
4. **de Cock, A. W. A. M., L. Mendoza, A. A. Padhye, L. Ajello, and L. Kaufman.** 1987. *Pythium insidiosum* sp. nov., the etiologic agent of pythiosis. J. Clin. Microbiol. **25**:344–349.
5. **de Haan, J.** 1902. Bosartige Schimmelkrankheit des Pferdes (*Hyphomycosis destruens equi*). Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg. Abt. 1 Orig. **31**:758–763.
6. **Ichitani, T., and J. Amemiya.** 1980. *Pythium gracile* isolated from the foci of granular dermatitis in the horse (*Equus caballus*). Trans. Mycol. Soc. Jpn. **21**:263–265.
7. **Jones, G. L., G. A. Hebert, and W. B. Cherry.** 1978. Fluorescent antibody techniques and bacterial applications. Center for Disease Control, Atlanta.
8. **Kaplan, W., and M. S. Ivens.** 1969. Fluorescent antibody staining of *Sporotrichum schenckii* in cultures and clinical materials. J. Invest. Dermatol. **35**:154–159.
9. **Krywienczyk, J., and C. E. Dorworth.** 1980. Serological relationships of some fungi of the genus *Pythium*. Can J. Bot. **58**:1412–1417.
10. **Mendoza, L., and A. A. Alfaro.** 1986. Equine pythiosis in Costa Rica: report of 39 cases. Mycopathologia **94**:123–129.
11. **Mendoza, L., L. Kaufman, and P. G. Standard.** 1986. Microimmunodiffusion test for diagnosing and monitoring pythiosis in horses. J. Clin. Microbiol. **23**:813–816.
12. **Middleton, J. T.** 1943. The taxonomy, host range and geographic distribution of the genus *Pythium*. Mem. Torrey Bot. Club **20**:1–171.
13. **Miller, R. I., C. W. Qualls, and G. H. Turnwald.** 1983. Gastrointestinal phycomycosis in a dog. J. Am. Vet. Med. Assoc. **183**:1245–1246.
14. **Miller, R. I., D. Wold, W. A. Lindsay, R. E. Beadle, J. J. McClure, J. R. McClure, and D. J. McCoy.** 1983. Complications associated with immunotherapy of equine phycomycosis. J. Am. Vet. Med. Assoc. **182**:1227–1229.
15. **Shipton, W. A.** 1987. *Pythium destruens* sp. nov., an agent of equine pythiosis. J. Med. Vet. Mycol. **25**:137–151.
16. **Shipton, W. A., R. I. Miller, and I. R. Lea.** 1982. Cell wall, zoospore and morphological characteristics of Australian isolates of a *Pythium* causing equine phycomycosis. Trans. Br. Mycol. Soc. **79**:15–23.
17. **Van der Plaats-Niterink, A. J.** 1981. Monograph on the genus *Pythium*. Stud. Mycol. **21**:1–142.
18. **White, D. G.** 1976. The preparation and use of a fluorescent antibody reagent for the detection of *Pythium graminicola*. Phytopathology **66**:523–525.