

Immunodiffusion Test for Diagnosis and Monitoring of Human Pythiosis Insidiosus

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To facilitate the laboratory diagnosis of human cases of pythiosis insidiosus, an immunological test was evaluated. A soluble antigen was prepared from a human isolate of *Pythium insidiosum*, an aquatic, thermotolerant oomycete that causes infections in cattle, dogs, horses, and humans. Sera from seven proven cases of disseminated human pythiosis insidiosus were tested in an immunodiffusion test along with appropriate control sera from patients with a variety of actinomycotic, bacterial, and mycotic diseases as well as sera from uninfected individuals. Titers ranged from 1:1 to 1:32 in the seven serum samples from the disseminated cases of pythiosis insidiosus of varying severity. The heterologous sera gave negative reactions. The rapidity and specificity of the immunodiffusion test makes it a useful diagnostic tool for the serodiagnosis of *P. insidiosum* infections.

Human cases of pythiosis insidiosus were first described as a new disease entity in 1989 in patients from Thailand (1, 5, 6). The disease is caused by *Pythium insidiosum*, an aquatic oomycete that produces biflagellate zoospores. The clinical features and signs of pythiosis insidiosus in humans are characterized by chronicity and progressive arterial insufficiency followed by the development of gangrene, aneurysms, and at times, fatal arterial leakage. The subcutaneous form is slowly progressive and nonfatal. The disease syndrome was found among farmers who had thalassemia hemoglobinopathy syndrome as a possible risk factor. Isolation and identification of *P. insidiosum* is difficult. Serodiagnosis by means of a serological test would be a useful tool for diagnosis, as described by Mendoza et al. (3), who used a soluble antigen for diagnosing and monitoring pythiosis insidiosus in horses. The objective of this study was to determine whether this test could be similarly used for the diagnosis of human cases of this disease and whether decreasing titers could be used to monitor the disease.

Sera from seven farmers with clinical features of leg gangrene were collected during the active phase of their infection, prior to surgical treatment, and were collected serially thereafter. These seven patients had histological proof of mycotic arteritis at the site of their arterial occlusions. The morphology of the hyphae in their tissues was consistent with that of *P. insidiosum*, i.e., broad, rarely septate hyphae with right-angle branching. Only three of the patients were culture positive. Although the morphology of the hyphae was not unique for *P. insidiosum*, the unique clinical features and pathology make this diagnosis highly probable, so we included them in our series. In addition, two serum samples from farmers with culture-proven pythiosis insidiosus of the eye were also studied. Control sera were collected from 20 patients with other culture-proven infec-

tions, i.e., invasive aspergillosis (*Aspergillus fumigatus*; $n = 2$), cerebral cryptococcosis (*Cryptococcus neoformans*; $n = 2$), pulmonary cryptococcosis (*Cryptococcus neoformans*; $n = 2$), actinomycotic mycetoma (*Nocardia asteroides* [$n = 2$] and *Actinomyces madural* [$n = 1$]), penicilliosis marneffei (*Penicillium marneffei*; $n = 1$), phaeohyphomycosis (*Phialophora parasitica*; $n = 1$), zygomycosis (*Mucor* sp. [$n = 1$], *Basidiobolus ranarum* [$n = 1$], and *Conidiobolus coronatus* [$n = 1$]), and melioidosis (*Pseudomonas pseudomallei*; $n = 6$). Serum samples from 15 uninfected thalassemic farmers and 14 healthy blood donors were also included as controls. All sera were frozen at -20°C until they were tested.

For antigen production, a culture filtrate antigen was prepared by the method described by Mendoza et al. (4). *P. insidiosum* F-RTCC 1472 obtained from an aneurysm in a human case of pythiosis insidiosus, which was confirmed at the Centers for Disease Control, Atlanta, Ga., was used as a reference strain for preparing our antigen. The isolate was subcultured on Sabouraud dextrose agar (Difco Laboratories, Detroit, Mich.) and was incubated at 25°C for 3 days. A small portion of the growth was transferred to each of three flasks containing 500 ml of brain heart infusion broth, and the flasks were incubated at 37°C for 1 month. The mycelium was killed by storage at 4°C for 1 week. The supernatant from the broth cultures was separated and concentrated 20-fold with polyvinyl pyrrolidone (BDH, Poole, England). This soluble antigen was used in the immunodiffusion (ID) tests.

In the ID test, agar gel diffusion was carried out on a slide which contained 7 ml of 1% purified agar in Veronal buffer (pH 7.8). The antigen and antibody were added to 4-mm-diameter wells which were 4 mm apart. The slides were then incubated in a humid chamber at room temperature for 24 h. Positive sera were subjected to serial twofold dilutions and were retested for antibody titers in a similar way.

Seven serum samples, which were collected at the time of active infection before surgical treatment, were positive in the ID test, with titers ranging from 1:1 to 1:32. Serum

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TABLE 1. Reciprocal of antibody titers with a *P. insidiosum* soluble antigen before and after surgical treatment

| Case no. | Culture | Reciprocal antibody titer at the following times (mo) after surgery ^a : | | | | | | |
|----------|---------|--|---|---|---|---|---|----|
| | | 0 | 1 | 2 | 3 | 4 | 5 | 12 |
| 1 | + | 8 | 1 | | | | 1 | - |
| 2 | - | 4 | | - | - | | | |
| 3 | - | 32 | 8 | 1 | | | | |
| 4 | - | 2 | | - | - | | | |
| 5 | + | 8 | | 4 | | 2 | - | |
| 6 | + | 1 | | 4 | | 2 | | |
| 7 | - | 8 | | 4 | | | | |

^a -, negative antibody titer.

samples from the two patients with ocular pythiosis insidiosum and from 20 patients with bacterial and fungal infections other than pythiosis insidiosum and 29 control serum samples were all negative. The antibody titers of the sera from the seven cases of pythiosis insidiosum, which were originally antibody positive, were serially determined, as shown in Table 1. A significant decrease in the antibody titer (greater than fourfold) was observed in cases 1 to 5. This appeared to correlate well with clinical improvement after surgical treatment. The follow-up of these five cases over a 2- to 12-month period revealed no signs or symptoms of relapse. One patient (case 6), who had rising and persistent antibody titers, subsequently died as a result of progressive infection. The last case (number 7) had an initial failure following surgical removal and further resection of the infected arteritis. This patient had no significant decrease in antibody titer, but his followup was lost. Retesting of these antisera with another soluble antigen, which was kindly provided by P. Imwidthaya from the Siriraj Hospital in Bangkok, showed the reproducibility of the test; the antibody titers varied only within one dilution.

Pythiosis insidiosum arteritis in humans has rarely been reported in the literature (5). The diagnosis of the disease on the basis of the isolation of *P. insidiosum* presents problems because of laboratory limitations in the ability to isolate and identify this aquatic oomycete. A provisional diagnosis can be made by histopathological means. However, the broad aseptate or rarely septate hyphae of *P. insidiosum* cannot be differentiated from those of the pathogenic zygomycetes. Thus, a serodiagnostic method as simple as the ID test would be an important tool in diagnosing this disease. An ID test with soluble antigen was developed and was shown to be

quite specific and sensitive for the diagnosis of equine pythiosis insidiosum (3). Imwidthaya and Srimuang reported similar findings in human cases of subcutaneous and systemic infection (2). Our results confirmed that this test is sensitive and specific for diagnosing human cases of arteritis. In addition, we also demonstrated that the antibody titer determined by the ID test correlated well with the activity of the infection. Clinical cure is associated with the disappearance of antibody. Mendoza et al. reported similar findings in their cases of equine pythiosis insidiosum (3). Considering that the clinical course of oomycotic arteritis is gradually progressive and mainly silent (5), this test not only will be useful for the early diagnosis of *P. insidiosum* infection but may very well serve as an indicator of treatment failure, which would alert physicians of the need to reevaluate their treatment procedure.

No antibody was detected in sera from two patients who had ocular pythiosis insidiosum. The corneal infections may have been inaccessible to their immunological defense mechanisms. This may explain the negative serology in these patients.

In conclusion, we demonstrated that the ID test for antibody to *P. insidiosum* is a sensitive and specific test to help in diagnosing *Pythium* arteritis and in monitoring the activity of the disease.

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