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Diagnostic Antigenemia Tests for Penicilliosis Marneffei

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Disseminated penicilliosis marneffei is an emerging opportunistic mycosis seen in severely immunocompromised human immunodeficiency virus (HIV)-infected patients and is caused by the dimorphic fungus *Penicillium marneffei*. Early diagnosis and treatment improve clinical outcome. Proper diagnosis is complicated by nonspecific signs and symptoms and by difficulties in histologic recognition and species identification of the pathogen. Since no established immunodiagnostic methods for penicillosis marneffei are available, we attempted to develop separate immunodiffusion tests to detect *P. marneffei* antigens and antibodies in patient serum specimens and a latex agglutination test for antigenemia. Antigens consisted of 2-week-old fission arthroconidial filtrates produced in Pine's broth at 37°C. Rabbit antisera were prepared against the 10×-concentrated filtrate antigens. Studies were carried out with 17 serum specimens from HIV-seropositive adult Thai patients with penicilliosis marneffei and 15 control serum specimens from Thai persons free of HIV and *P. marneffei* infection. The immunodiffusion tests detected *P. marneffei* antigenemia in 10 (58.8%) of 17 patients, whereas the latex agglutination test detected antigenemia in 13 (76.5%) of the 17 patients. Antibody was demonstrated in only 2 of the 17 patient sera. All of the tests appeared to be highly specific, since none were positive with sera from 15 Thai control patients, six serum samples containing cryptococcal antigen, or six urine specimens positive for *Histoplasma* polysaccharide antigens.

Disseminated penicilliosis marneffei is an emerging mycosis seen in AIDS patients and is caused by the dimorphic fungus *Penicillium marneffei* (1, 7). Early diagnosis and treatment improve clinical outcome. However, proper diagnosis is complicated by nonspecific signs and symptoms and by difficulties in histologic recognition and species identification of the pathogen. Serologic tests appear to offer a means to achieve an early diagnosis of penicilliosis marneffei. In 1993, Viviani et al. (9), using immunodiffusion (ID) tests with mycelial culture filtrate antigens, detected precipitins in serial serum specimens from a patient with penicilliosis marneffei. Later, Yuen et al. (12), using indirect fluorescent antibody procedures with mycelial-form germinating conidia and yeast hyphae as antigens, showed that antibody detection permitted the early diagnosis of penicilliosis marneffei. In light of the successful application of these procedures, we investigated the value of ID tests to detect *P. marneffei* antigens and antibodies in sera from humans infected with *P. marneffei* and of a latex agglutination (LA) test for detecting antigenemia.

MATERIALS AND METHODS

Specimens. Seventeen serum specimens from 17 human immunodeficiency virus (HIV)-seropositive adult Thai patients with culture-confirmed penicilliosis marneffei at Chiang Rai Hospital in Northern Thailand were studied, as well as two urine specimens from two similarly infected patients. Negative controls consisted of 15 serum samples from Thai persons in Chaing Rai who were HIV seronegative and without evidence of penicilliosis marneffei infection and one urine sample from a normal individual. Specificity was further checked with *Histoplasma capsulatum* antibody-positive serum samples from 12 persons with culture-confirmed histoplasmosis and *H. capsulatum* polysaccharide antigen (HPA)-positive urine samples from 6 HIV-seropositive culture-positive histoplasmosis patients. In addition, cryptococcal antigen-positive serum samples from 6 culture-positive patients with cryptococcosis were studied. The cryptococcosis and histoplasmosis patients were from the United States. Lastly, specificity was also checked with culture filtrate antigens (4) of *Aspergillus flavus, Aspergillus fumigatus*, and the mycelial form of *H. capsulatum* and with a concanavalin A-purified galactomannan of *A. fumigatus*.

Fungal strain and antigen production. A stock culture of *P. marneffei* ATCC 18224 was maintained on laboratory-compounded Sabouraud glucose agar (pH 5.6) slants at 25°C and on Pine's agar slants (6) at 37°C. Fission-form arthroconidia of *P. marneffei* were grown in duplicate flasks containing 500 ml of Pine's broth (6) in accord with the method described by Kaufman et al. (3). The cultures were incubated for 2 weeks at 37°C while being rotated at 150 rpm in a New Brunswick Scientific gyratory shaker incubator (New Brunswick, N.J.). The cultures were killed by adding Merthiolate to a concentration of 0.02%. The supernatants obtained by centrifugation were concentrated 10 times by ultrafiltration through a PM-10 membrane (Amicon Corp., Lexington, Mass.). The retentate was sterilized by passage through a 0.45-μm-pore-size filter and was used as the antigen.

Anti-*P. marneffei* immunoglobulins. Large (weight, 4 kg), female New Zealand White rabbits were immunized with the concentrated culture filtrates. Antisera against the fission-form arthroconidial filtrate antigens were prepared by injecting four rabbits subcutaneously in the dorsal area with an emulsion consisting of 1.0 ml of 10×-concentrated filtrate and 1.0 ml of Freund's incomplete adjuvant. Three weeks after the initial injection, the rabbits were subcutaneously injected with another 1.0-ml dose of the concentrated filtrate-Freund's incomplete adjuvant emulsion. Two weeks after the second injection, the rabbits were injected intravenously with 0.5 ml of concentrated filtrate antigen.

One week after the final injection, each rabbit was bled and its serum was tested by ID for antibody against *P. marneffei*-concentrated filtrate antigen. Suitable antisera produced five immunoprecipitin bands. Anti-*P. marneffei* immunoglobulins were obtained by precipitation of antisera with 35% saturated ammonium sulfate.

ID tests. ID tests for antibodies and antigens were performed in plastic petri dishes (100 by 15 mm) into which 10 ml of 0.25% phenolized–1% purified agar (Difco) (2) was added. A seven-well pattern with 4-mm-diameter wells 4 mm apart was used. The reactants were added to the wells, and the wells were incubated for 24 h in a humid chamber at room temperature. Reagents consisted of rabbit anti-*P. marneffei* sera diluted 1:1.4 and 10×-concentrated culture filtrate antigen that was used undiluted. Optimal dilutions were determined in box titrations. Upon interaction, these reagents-, for which titers were determined and which were designated references, produced five precipitin bands. For antibody tests, reference antigen was placed in the central well and reference antiserum was placed in the upper and lower wells. Test sera were placed in the adjacent lateral wells. For antigen tests, reference antiserum was placed in the central well and reference antigen was placed in the upper and lower wells. Test sera were placed in the adjacent lateral wells. A serum sample was considered positive when it produced one or more bands of identity with any of the five reference immunoprecipitates.

LA test. Sera were inactivated at 56°C for 30 min before testing. A suspension

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TABLE 1. Immunologic reactions in ID and LA tests for *P. marneffei* antibodies and antigens in serum and urine specimens from 19 AIDS patients with penicilliosis marneffei

Specimen (*n*)	No. positive in ID test for		No. positive with LA test for antigen
	Antibody	Antigen	
Serum (17)	2	10	13
Urine (2)	0	1	2

of polystyrene beads prepared as described by Palmer et al. (4) was sensitized by being mixed with a 1:100 dilution of rabbit anti-*P. marneffei* globulin. The optimal quantity of immunoglobulin was the highest dilution that produced a clear 2+ agglutination with the highest reactive dilution of the 10×-concentrated culture filtrate in a box titration. The LA test was performed by adding 0.05 ml of the inactivated (56°C for 30 min) serum to 0.025 ml of latex-sensitized particles and by rotating the reactants at 150 rpm for 5 min. Sera were initially tested undiluted. Positive specimens were then diluted twofold to 1:8,192 with glycine-buffered saline (pH 8.4) containing 0.1% bovine serum albumin. The antigen titer was recorded as the highest dilution of each specimen showing an agglutination value of 2+, i.e., small but definite clumps with a slightly cloudy background.

RESULTS

Seventeen serum samples and two urine specimens from 19 patients with AIDS and penicilliosis marneffei were tested by ID for antigen and antibodies and by LA for antigenemia and antigenuria (Table 1). The ID test detected antigen in 10 of the 17 (58.8%) patient serum samples. Antibody was demonstrated in only 2 of the 17 (11.8%) patient serum samples, and 1 of these was antigen positive. ID tests for both serum antigens and antibodies detected 11 of the 17 cases (64.7%). The LA test detected antigens in 13 of the 17 (76.5%) serum samples. The ID test detected antigen in one of the two urine specimens tested, whereas the LA test was positive for antigen with both specimens.

Of the 19 cases, the ID tests for antigens and antibodies detected 12 (63.2%), whereas the LA test for antigens detected 15 (78.9%). Neither the ID nor the LA test reacted with any of the 16 negative-control specimens.

The numbers of *P. marneffei* precipitins and precipitinogens detected in the 17 serum samples and two urine specimens by ID tests, as well as the LA titers for *P. marneffei* antigens, are presented in Table 2. A single precipitin was detected in the two serum samples positive for antibody. In contrast, one to four precipitinogens were noted in the antigen-positive sera studied and one was noted in a single positive urine specimen. Serum LA titers ranged from 1:64 to 1:4,096. The LA test detected all of the ID antigen-positive sera plus three additional patient serum samples. One serum sample positive for antibody was negative in ID and LA tests for antigens. The LA test also reacted with both of the urine specimens, demonstrating titers of 1:128 and 1:512. The LA titers for four of the eight ID antigen-negative specimens ranged from 1:64 to 1:256; for those with one or two precipitin bands, they ranged from 1:128 to 1:2,048; and for those with three or four precipitin bands, they ranged from 1:4,096.

In addition to testing with the 15 control serum samples and one urine specimen from humans free of HIV and penicilliosis marneffei, the specificities of the tests were checked with cryptococcal antigen-positive serum samples from 6 patients with proven cryptococcosis, with antibody-positive serum samples from 12 patients with culture-positive histoplasmosis, and with HPA-positive urine specimens from 6 AIDS patients with histoplasmosis. All of the tests gave negative results with the heterologous case specimens.

TABLE 2. Number of *P. marneffei* ID precipitins and precipitinogens and LA antigen titers in 17 serum samples and two urine specimens from AIDS patients with penicilliosis marneffei

Specimen no.[a]	No. of ID antibody	No. of bands for antigen	LA titer for antigen[b]
S1	0	2	1,024
S2	0	0	0
S3	1	0	0
S4	0	0	0
S5	0	0	64
S6	0	1	128
S7	0	4	4,096
S8	0	3	4,096
S9	0	1	2,048
S10	1	1	1,024
S11	0	1	1,024
S12	0	2	1,024
S13	0	0	64
S14	0	1	1,024
S15	0	0	256
S16	0	2	256
S17	0	0	0
U1	0	0	128
U2	0	1	512

[a] S, serum specimen; U, urine specimen.
[b] Titers are expressed as reciprocals of each dilution factor.

In light of reports (3, 5, 8) indicating that *P. marneffei* shares antigens with *A. fumigatus* and *H. capsulatum*, we examined the reactivities of the ID and LA reagents with culture filtrate antigens of *A. flavus, A. fumigatus*, the mycelial form of *H. capsulatum*, and a concanavalin A-purified galactomannan of *A. fumigatus*. Our *P. marneffei* antibodies did not react with any of the aforementioned antigens.

DISCUSSION

Preliminary studies by Viviani et al. (9) and Yuen et al. (12) showed that penicilliosis marneffei can be specifically diagnosed serologically by using ID and IFA tests for *P. marneffei* antibodies. Yuen and coworkers noted diagnostic titers in all eight patients studied, one of whom was HIV positive. In contrast, our ID test with culture filtrate antigens produced by fission arthroconidia detected serum antibody in only 2 of 17 (11.8%) patients with penicilliosis marneffei (Table 1). One of these patients was also positive for antigen by the LA and ID tests. The insensitivity of our antibody test may be due to the fact that all of our patients were HIV positive and possibly had diminished humoral antibody responses.

Antigen detection tests may be more effective than antibody tests for diagnosing penicilliosis marneffei in immunocompromised patients. The newly developed ID and LA tests were capable of detecting antigens in both the serum and the urine specimens from the penicilliosis marneffei patients. The LA test detected antigenuria in both of the two available patient urine specimens, and the potential of the test is evident. However, the small number of urine specimens in this study did not permit a critical assessment of the usefulness of either the ID or the LA technique for detecting penicilliosis marneffei antigenuria. The LA test appeared superior in sensitivity (76.5%) to the ID test (58.8%) for immunodetection of *P. marneffei* antigenemia in HIV-infected patients (Table 1). Furthermore, it detected all of the cases positive by ID and was quantifiable; LA antigen titers ranged from 1:64 to 1:4,096 (Table 2). The presence of three to four serum precipitinogens appears to be associated with LA titers of greater than 1:2,048. Antigenemia

titrations may offer prognostic as well as diagnostic data; however, the prognostic significance of these titers could not be ascertained in this study, since clinical data on the extent and severity of *P. marneffei* infection in the HIV-positive patients studied were not documented.

The ID and LA tests appear to be quite specific; no positive reactions occurred with the 15 control serum samples and one urine specimen from persons without evidence of HIV or *P. marneffei* infection. Furthermore, no reactions occurred with human serum samples positive for cryptococcal antigen, serum samples positive for *H. capsulatum* antibody, and HPA-positive urine specimens from AIDS patients. Interestingly, although the HPA test (11) cross-reacts with urine specimens from HIV-infected patients with penicilliosis marneffei (10), the LA and ID tests for penicilliosis marneffei antigenuria do not cross-react with HPA-positive urine specimens.

Van Cutsem et al. (8), using LA, and Pierard et al. (5), using immunostaining, found that monoclonal antibody to *A. fumigatus* galactomannan cross-reacted with *P. marneffei* antigen. More recently, Kaufman et al. (3) reported that in indirect fluorescent antibody tests, antibody to soluble antigens of the tissue form of *P. marneffei* stained the yeast-form cells of *H. capsulatum* but not the hyphal forms of *A. flavus, A. fumigatus*, or *H. capsulatum*. Our present ID and LA studies indicated that the anti-*P. marneffei* globulins to the fission arthroconidial culture filtrates, like those used in the indirect fluorescent antibody test, did not react with the soluble culture filtrate antigens of *A. flavus* and *A. fumigatus* or of the mycelial form of *H. capsulatum*. Our studies indicate that the aforementioned antiglobulins lack antibody to the common *Aspergillus* sp. antigen and that histoplasmin, like the mycelium of *H. capsulatum*, is devoid of a shared epitope with the tissue form of *P. marneffei*. A common epitope may be on the *Aspergillus* spp. antigens, but it is not demonstrable with our *P. marneffei* antibody.

It is evident from our studies that a reliable reagent for detecting penicilliosis marneffei antigenemia can readily be produced by sensitizing latex particles with antiglobulin to concentrated culture filtrate antigens of fission arthroconidia. In our preliminary evaluations, the test demonstrated a sensitivity of 76.5%. Use of ID antibody and LA antigenemia tests concurrently increased the sensitivity to 82.4%. Further increases in diagnostic sensitivity and predictive value might be achieved with development of more-improved assays and/or through the serial testing of clinical specimens obtained over time.

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REFERENCES

1. **Drouhet, E.** 1993. Penicilliosis due to *Penicillium marneffei*: a new emerging systemic mycosis in AIDS patients travelling or living in Southeast Asia. J. Mycol. Med. **4:**195–224.
2. **Kaufman, L., and E. Reiss.** 1992. Serodiagnosis of fungal diseases, p. 506–528. *In* N. R. Rose, E. C. De Macario, J. L. Fahey, H. Friedman, and G. M. Penn (ed.), Manual of clinical laboratory immunology, 4th ed. American Society for Microbiology, Washington, D.C.
3. **Kaufman, L., P. G. Standard, S. A. Anderson, M. Jalbert, and B. L. Swisher.** 1995. Development of specific fluorescent-antibody test for tissue form of *Penicillium marneffei*. J. Clin. Microbiol. **33:**2136–2138.
4. **Palmer, D. F., L. Kaufman, W. Kaplan, and J. J. Cavallaro.** 1977. Serodiagnosis of mycotic diseases, p. 7, 96, and 118–120. Charles C. Thomas, Publisher, Springfield, Ill.
5. **Pierard, G. E., J. A. Estrada, C. Pierard-Franchimont, A. Thiry, and D. Stynen.** 1991. Immunohistochemical expression of galactomannan in the cytoplasm of phagocytic cells during invasive aspergillosis. Am. J. Clin. Pathol. **96:**373–376.
6. **Pine, L.** 1977. *Histoplasma* antigens: their production, purification and uses. Contrib. Microbiol. Immunol. **3:**138–168.
7. **Supparatpinyo, K., C. Khamwen, V. Baosoung, K. E. Nelson, and T. Sirisanthana.** 1994. Disseminated *Penicillium marneffei* infection in Southeast Asia. Lancet **344:**110–113.
8. **Van Cutsem, J., L. Meulemans, F. Van Gerven, and D. Stynen.** 1990. Detection of circulating galactomannan by Pastorex *Aspergillus* in experimental invasive aspergillosis. Mycoses **33:**61–69.
9. **Viviani, M. A., A. M. Tortorano, G. Rizzardini, T. Quirino, L. Kaufman, A. A. Padhye, and L. Ajello.** 1993. Treatment and serological studies of an Italian case of penicillosis marneffei contracted in Thailand by a drug addict infected with the human immunodeficiency virus. Eur. J. Epidemiol. **9:**79–85.
10. **Wheat, H., J. Wheat, P. Connolly, M. Goldman, K. Nelson, K. Supparatpinyo, R. Bradsher, and A. Restrepo.** 1996. Causes for cross-reactivity in the *Histoplasma* antigen immunoassay, abstr. F-38, p. 80. *In* Abstracts of the 96th General Meeting of the American Society for Microbiology, New Orleans, La., 1996. American Society for Microbiology, Washington, D.C.
11. **Wheat, L. J., P. Connolly-Stringfield, R. B. Kohler, P. T. Frame, and M. R. Gupta.** 1989. *Histoplasma capsulatum* polysaccharide antigen detection in diagnosis and management of disseminated histoplasmosis in patients with acquired immunodeficiency syndrome. Am. J. Med. **87:**396–400.
12. **Yuen, K., S. S. Wong, D. N. Tsang, and P. Chau.** 1994. Serodiagnosis of *Penicillium marneffei* infection. Lancet **344:**444–445.