Penicilliosis, caused by the dimorphic fungus *Penicillium marneffei*—a facultative intracellular pathogen for humans—is a disseminated and progressive infection which is endemic to certain parts of southeast Asia and China (4, 6, 7, 21). The first natural infection in humans was described in 1973 in a man suffering from Hodgkin’s disease who had travelled to southeast Asia (5). Only 21 further cases were reported during the next 15 years (4, 9), most of which had no evidence of underlying impaired immunity. With the increasing prevalence of human immunodeficiency virus (HIV) infections in Thailand, *Penicillium marneffei* has emerged as a major opportunistic infection in AIDS patients (8, 19, 21). The common clinical manifestations of penicilliosis include fever, anemia, leukopenia, weight loss, diarrhea, hepatosplenomegaly, generalized lymphadenopathy, cough, and characteristic molluscum contagiosum-like lesions, predominantly on the face and trunk (4, 8, 19, 20, 21). The presentation may mimic tuberculosis, melioidosis, leishmaniasis, and other AIDS-related opportunistic infections, such as histoplasmosis and cryptococcosis (6, 8, 14, 21, 26). A presumptive diagnosis of penicilliosis can be made by finding characteristic yeast cells in smears of skin lesions, blood, bone marrow, or lymph nodes, but these may be confused easily with those of *Histoplasma capsulatum* and, occasionally, *Cryptococcus neoformans* or *Leishmania sp*. Definitive diagnosis relies upon the identification or isolation of *P. marneffei* in clinical specimens. However, conventional culture usually takes ≥3 days. Histological recognition of the pathogen (12, 15) is relatively straightforward, although the septa which distinguish *P. marneffei* from *H. capsulatum* may not always be seen. Identification of by means of an immunohistochemical approach (2) and exoantigen tests (7, 11, 17) is specific; however, these tests are also time-consuming and are not generally available. Rapid diagnosis is important, because disseminated *P. marneffei* infection has a high mortality, and effective antifungal treatments are available (7, 8, 18, 21).

A number of serological diagnostic tests have been developed for the detection of antibody to *P. marneffei*. These include an immunodiffusion test (12, 13, 23) and use of an indirect fluorescent antibody technique (25). We report the development and prospective evaluation of an enzyme-linked immunosorbent assay (ELISA) for the detection of *P. marneffei* antigen in urine, using a fluorescein isothiocyanate (FITC)-anti-FITC amplification system, in an area of northeastern Thailand in which penicilliosis is endemic.

**MATERIALS AND METHODS**

**Patients.** The study was conducted between June 1995 and November 1997. Adult patients with suspected or confirmed penicilliosis admitted to Sappasitprasong Hospital, Ubon Ratchathani, northeastern Thailand, were included in the study. All patients were seen by one of the study team. Full clinical details were recorded on a standard form. Routine hematological and biochemical tests were performed, and HIV antibody was measured when indicated with the ELISA. As part of the routine diagnostic workup, blood (15 ml), urine, and where appropriate, lymph node biopsy or liver aspirate specimens were collected for bacterial and fungal culture. Smears were made from any suspicious skin lesions or aspirates, and were then fixed on a glass slide and stained with Gram and Wright’s stains. Throat and skin lesion swabs were also collected. All specimens were plated on Sabouraud’s medium (with added chloramphenicol) and cultured at 30°C in air for up to 3 weeks.

Urine samples were obtained on admission and, in cases of confirmed penicilliosis, at weekly intervals thereafter. All urine samples were stored at −30°C and thawed only at the time of testing.

**Antigen preparation.** A stock culture of *P. marneffei* (a clinical isolate from a patient in Ubon Ratchathani) was maintained on horse blood agar (HBA) at 37°C for several days. Fission-form arthroconidia (yeast-like cells) of *P. marneffei* were grown in 50 ml of brain heart infusion broth (BHB) at 37°C for 2 weeks while being rotated on a rotary shaker at 150 rpm. The culture was checked weekly for the degree of turbidity and microscopic morphology. After 2 weeks of incubation, the culture was centrifuged at 2,900 × g for 10 min. The cell pellet was collected for bacterial and fungal culture. Smears were made from any suspicious skin lesions or aspirates, and were then fixed on a glass slide and stained with Gram and Wright’s stains. Throat and skin lesion swabs were also collected. All specimens were plated on Sabouraud’s medium (with added chloramphenicol) and cultured at 30°C in air for up to 3 weeks.

Urine samples were obtained on admission and, in cases of confirmed penicilliosis, at weekly intervals thereafter. All urine samples were stored at −30°C and thawed only at the time of testing.

**Antigen preparation.** A stock culture of *P. marneffei* (a clinical isolate from a patient in Ubon Ratchathani) was maintained on horse blood agar (HBA) at 37°C for several days. Fission-form arthroconidia (yeast-like cells) of *P. marneffei* were grown in 50 ml of brain heart infusion broth (BHB) at 37°C for 2 weeks while being rotated on a rotary shaker at 150 rpm. The culture was checked weekly for the degree of turbidity and microscopic morphology. After 2 weeks of incubation, the culture was centrifuged at 2,900 × g for 10 min. The cell pellet was
were prepared by injecting three female New Zealand White rabbits subcutaneously in the dorsal area with an emulsion consisting of 1 ml of 2 × 10^8 cells/ml of killed-whole fission arthroconidia of *P. marneffei* and 2 ml of Freund’s incomplete adjuvant, at weekly intervals for 3 weeks. One week after the third injection, the rabbits were injected intravenously with 0.5 ml of 5 × 10^5 cells of killed-whole fission arthroconidia of *P. marneffei* per ml every 3 to 4 days for a further 2 weeks.

Three days after the final injection, each rabbit was bled, and the serum was collected. This serum was tested for antibody against killed whole-fission arthroconidia of *P. marneffei* by using an immunodiffusion (ID) test similar to that described by Sekhon et al. (17). A single broad band was produced both before and after pooling the serum samples. The purified IgG fraction of the pooled immune serum was obtained by precipitation with 35% saturated ammonium sulfate and followed by fractionation by protein A-Sepharose CL-4B chromatography (Pharmacia, Uppsala, Sweden) by standard methods. The concentration of protein in the purified preparation was estimated by measuring the optical density at 280 nm.

**Anti-*P. marneffei* IgG preparation.** Hypermimmune sera against *P. marneffei* were prepared by injecting three female New Zealand White rabbits subcutaneously in the dorsal area with an emulsion consisting of 1 ml of 2 × 10^8 cells/ml of killed-whole fission arthroconidia of *P. marneffei* and 2 ml of Freund’s incomplete adjuvant, at weekly intervals for 3 weeks. One week after the third injection, the rabbits were injected intravenously with 0.5 ml of 5 × 10^5 cells of killed-whole fission arthroconidia of *P. marneffei* per ml every 3 to 4 days for a further 2 weeks.

Three days after the final injection, each rabbit was bled, and the serum was collected. This serum was tested for antibody against killed whole-fission arthroconidia of *P. marneffei* by using an immunodiffusion (ID) test similar to that described by Sekhon et al. (17). A single broad band was produced both before and after pooling the serum samples. The purified IgG fraction of the pooled immune serum was obtained by precipitation with 35% saturated ammonium sulfate and followed by fractionation by protein A-Sepharose CL-4B chromatography (Pharmacia, Uppsala, Sweden) by standard methods. The concentration of protein in the purified preparation was estimated by measuring the optical density at 280 nm.

**Anti-*P. marneffei* IgG-FTTC conjugate.** FITC-labeled antibody was prepared by the method of Samual and others (16). Briefly, FITC (5 mg/ml in dry absolute ethanol) was added at a molar ratio of 20:1 to a stirred preparation of purified anti-*P. marneffei* IgG, diluted in 0.1 M carbonate buffer, containing 0.5 M NaCl (pH 9.2) in the dark at room temperature. The reaction was allowed to proceed for a further 60 min, and then the anti-*P. marneffei* IgG-FTTC was separated from free FITC by gel filtration through a Sephadex G-25 M PD-10 column (Pharmacia) preblocked with 1% bovine serum albumin, and equilibrated with phos- phate-buffered saline (PBS). Fractions containing conjugate were pooled and stored at 4°C in PBS (33% vol/vol) containing 0.02% merthiolate.

**Urinary antigen ELISA.** Flat-bottom microtiter plates (Falcon 3912 Microtest III; Becton Dickinson, Oxnard, Calif.) were coated with capture antibody by adding 100 µl of purified rabbit anti-*P. marneffei* IgG (10 mg/ml) in PBS (pH 7.2) containing 0.02% (wt/vol) merthiolate to each well. The plates were incubated at 4°C for 48 h, and the wells were then washed three times with PBS containing 0.1% (vol/vol) Tween 20 (PBS-Tween).

Urine samples were heated in a boiling waterbath for 6 min, cooled, and centrifuged at 5,000 × g for 6 min to remove any precipitate. Urine (100 µl), either undiluted or diluted 1:10 and then serially diluted twofold in PBS-Tween, was added to each well. The plate was incubated at 37°C for 60 min and then washed three times in PBS-Tween. Anti-*P. marneffei* IgG-FTTC (100 µl) diluted 1:2,000 in blocking buffer (PBS-Tween, 0.5% bovine serum albumin) was then added to each well and the plate incubated as above. After further washing, 100 µl of the rabbit anti-FTTC horseradish peroxidase conjugate (Dakopatts, Glostrup, Denmark), diluted 1:2,000 in blocking buffer, was added to each well followed by an incubation and washing step as described above. Finally, 100 µl of tetramethyl benzidine substrate was added, and this mixture was then incubated for 30 min at room temperature. The reaction was stopped by adding 100 µl of 2 M sulfuric acid. The results were read spectrophotometrically at 450 nm in a TiterTek Multilink MCC plate reader (Flow Laboratories, Ltd., Ayerst, Scotland). Blank wells were treated as described above, but the test specimen was omitted. A positive reading was defined as a value greater than the mean plus 3 standard deviations of the optical density of the blank wells.

**Patient group** (no. of patients) | **Median ELISA titer** (range)**a**
---|---
**Penicilliosis** (33) | 20,480 (1–327,680)
Other fungal infection (34) | 0 (0–5,120)
Melioidosis (168) | 0 (0–20)
Other septicemia (12) | 0 (0–1,024)
Other bacterial infection (7) | 0 (0–160)
Culture negative (27) | 0 (0–10)
Normal healthy controls (52) | 0 (0–1)

**RESULTS**

**Preliminary evaluation.** Broth cultures of *C. albicans*, *C. kefyr*, *C. neoformans var. neoformans*, *C. neoformans var. gattii*, *P. grisoeofulum*, *P. chrysogenum*, *P. notatum*, *A. terreus*, *A. fumigatus*, *A. flavus*, *H. capsulatum var. capsulatum*, and *T. beigelli* were nonreactive in the ELISA, even at cell concentrations of 10^7 cells/ml. All of the clinical strains of *P. marneffei* (both fission and hyphal forms) were reactive down to concentrations of 100 yeast cells/ml. Bacterial strains were all nonreactive, except for *Staphylococcus aureus* (10^6 CFU/liter), which was reactive only when undiluted.

**Diagnostic sensitivity and specificity.** Urine samples from 33 HIV-seropositive adult Thai patients with culture-confirmed *P. marneffei* infection were collected on admission. Urine samples from 248 patients with other diagnoses were also tested. These included 34 urine samples from HIV-seropositive patients with fungal infections other than *P. marneffei*. Of these, 31 were from patients with culture-confirmed cryptococcosis, 1 was from a patient with culture-confirmed histoplasmosis, and 2 were from patients with culture-confirmed candidiasis.

The remaining patient samples were from 168 patients with culture-confirmed melioidosis, 12 patients with septicemia due to other bacterial species, 7 patients from patients with other bacterial infections, and 27 from culture-negative patients (who were being screened for melioidosis as part of another study). All patients in the control groups mentioned above were admitted to Sapphaprakiat Hospital during the same period as the *P. marneffei* patients. They had demographic characteristics similar to those of the *P. marneffei* patients. Fifty-two urine samples from healthy volunteers living in Ubon Ratchathani also served as negative controls, and 3 were used for the calculation of the ELISA standard deviations.

The results of *P. marneffei* antigen detection in urine from patients with *P. marneffei* infection and the control groups are given in Table 1 and Fig. 1. The ELISA detected antigen in the urine samples of all 33 (100%) patients with *P. marneffei* infection, with a median titer of 1:20,480 (range, neat to 1:327,680). Of the 52 samples from healthy volunteers, 49 (94%) were negative for antigen, whereas 3 (6%) reacted in the assay when tested undiluted. A higher proportion, 67 (27%) of 248 urine samples from inpatients with diagnoses other than *P. marneffei* were reactive in the assay, with a median titer of neat (range, neat to 1:5120) (Table 2). Of the 34 urine samples from patients with other fungal infections, 9 (26.5%) gave positive results (all had cryptococcosis). Of these, four (11.8%) were reactive in the assay only when tested undiluted, one (2.9%) was reactive at a titer of 1:10, 2 (5.9%) were reactive at a titer of 1:80, one was reactive at a titer of 1:160, and one was reactive at a titer of 1:5120. False-positive results were also found in 39 (23.2%) of 168 urine samples from patients with...
melioidosis; 32 (19.0%) of these were reactive undiluted, 6 (3.6%) were reactive at a titer of 1:10, and 1 (0.6%) was reactive at a titer of 1:20. Ten (83.3%) of 12 urine samples from patients with other bacterial septicemias and 1 (14.3%) of 7 from patients with other bacterial infections gave positive results. In the group of patients with other septicemias, nine (75%) were reactive only when tested undiluted, and one (8.3%) was reactive at a titer of 1:5,120, whereas one (14.3%) of the patients with other bacterial infections was reactive at a titer of 1:160. False-positive results also occurred in 8 (29.6%) of 27 patients who were culture negative: 1 patient at a titer of 1:10, and the remainder in neat urine only.

The median ELISA titer for \( P. marneffei \) urinary antigen in the group of patients with penicilliosis (1:20,480; range, neat to 1:3,276,800) was significantly higher than those of the controls (Table 1), including the other fungal infections (median, negative; range, negative to 1:5,120; \( P < 0.0001 \)), melioidosis (median, negative; range, negative to 1:20; \( P < 0.0001 \)), other septicemia (median, negative; range, negative to 1:5,120 \( P < 0.0001 \)), other bacterial infections (median, negative; range, negative to 1:160; \( P < 0.0001 \)), those whose cultures were negative (median, negative; range, negative to 1:10; \( P < 0.0001 \)), and normal healthy volunteers (median, negative; range, negative to neat; \( P < 0.0001 \)).

**Optimum cutoff titer.** The sensitivity and specificity of the ELISA in the diagnosis of penicilliosis are summarized in Table 2. Progressive dilution of urine samples reduced the sensitivity of the test, but the specificity increased to 100%. In order to achieve the best cutoff titer, a receiver-operating curve was used to assess the ability of the test to correctly identify patients with penicilliosis. This suggested that the best cutoff titer (i.e., the point that maximized the sum of the sensitivity and specificity) was at 1:40. At this cutoff titer, the ELISA was 97% sensitive and 98% specific (positive predictive value, 84.2%; negative predictive value, 99.7%). Repeated testing of a single sample with this initial titer gave values of 1:40 and 1:80. At a cutoff titer of 1:320, the specificity rose to 99%, but the sensitivity fell to 90% (positive predictive value, 91.3%; negative predictive value, 90.5%).

**DISCUSSION**

Penicilliosis is now recognized as one of the most important opportunistic infections in AIDS patients in Thailand. It is the fourth or fifth most common infection in this group of patients. Although in many cases, the diagnosis is readily made from microscopic examination of skin lesion smears, some patients do not have dermatological manifestations of the infection. In others, there may be confusion with histoplasmosis or disseminated cryptococcosis. Thus a rapid diagnostic or confirmatory test would be of clinical value and would direct appropriate antifungal therapy.

Yuen and colleagues (25) have developed an indirect immunofluorescence test to detect antibody in serum from patients infected with \( P. marneffei \). All 78 healthy controls and 95 patients with fever from other causes had IgG titers of <1:40. Although there were only eight patients with \( P. marneffei \) infection in the series, all had IgG titers \( \geq 160 \), but none had demonstrable IgM. Thus there was 100% sensitivity for this antibody test in this small series, although the study was performed in an area of low endemicity (Hong Kong) and included only two patients with other fungal infections (cryptococcosis). In an area of high endemicity, such as northern Thailand, background IgG seroprevalence could affect the specificity of the indirect fluorescent antibody IFA test.

Recently a specific 38-kilodalton antigen has been used to detect antibody to \( P. marneffei \) in HIV-seropositive patients in Thailand (3). Antibody was detected by immunoblotting in 30 (46%) of 65 patients with penicilliosis. In patients from an area in which \( P. marneffei \) is endemic, antibody was also found in 17 (25%) of 67 patients with cryptococcosis or candidiasis and in 45 (17%) of 262 asymptomatic HIV-positive individuals, but only one of 60 healthy controls. Thus, the specificity and usefulness of antibody tests for the diagnosis of penicilliosis in endemic areas remain uncertain. Other antigens from \( P. marneffei \) have been purified (10); one of these, a 61-kDa antigen, was recognized by sera from 18 of 21 (86%) \( P. marneffei \)-infected patients, but further evaluation of this in diagnostic tests is required.

Detection of \( P. marneffei \) antigen indicates active infection. However, it is known that there is some cross-reactivity be-

**TABLE 2. Sensitivity and specificity of \( P. marneffei \) urinary antigen ELISA at different cutoff values**

<table>
<thead>
<tr>
<th>ELISA cutoff titer</th>
<th>% Sensitivity</th>
<th>% Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>All (n = 300)</td>
<td>Other inpatients (n = 248)</td>
</tr>
<tr>
<td>Neat</td>
<td>100.0</td>
<td>76.7</td>
</tr>
<tr>
<td>10</td>
<td>97.0</td>
<td>95.0</td>
</tr>
<tr>
<td>20</td>
<td>97.0</td>
<td>97.7</td>
</tr>
<tr>
<td>40</td>
<td>97.0</td>
<td>98.0</td>
</tr>
<tr>
<td>80</td>
<td>90.9</td>
<td>98.0</td>
</tr>
<tr>
<td>160</td>
<td>90.9</td>
<td>98.7</td>
</tr>
<tr>
<td>320</td>
<td>90.9</td>
<td>99.0</td>
</tr>
<tr>
<td>640</td>
<td>87.9</td>
<td>99.0</td>
</tr>
<tr>
<td>1,280</td>
<td>84.9</td>
<td>99.3</td>
</tr>
<tr>
<td>2,560</td>
<td>84.9</td>
<td>99.3</td>
</tr>
<tr>
<td>5,120</td>
<td>78.8</td>
<td>99.3</td>
</tr>
<tr>
<td>10,240</td>
<td>69.7</td>
<td>100.0</td>
</tr>
<tr>
<td>20,480</td>
<td>60.6</td>
<td>100.0</td>
</tr>
<tr>
<td>40,960</td>
<td>33.3</td>
<td>100.0</td>
</tr>
<tr>
<td>81,920</td>
<td>24.2</td>
<td>100.0</td>
</tr>
<tr>
<td>163,840</td>
<td>12.1</td>
<td>100.0</td>
</tr>
<tr>
<td>327,680</td>
<td>3.0</td>
<td>100.0</td>
</tr>
</tbody>
</table>
between A. fumigatus and P. marneffei antigens, because antibody to A. fumigatus galactomannan reacts with P. marneffei in a latex agglutination test (22) and on immunohistochemical staining (2). A urinary antigen detection test developed for diagnosis of H. capsulatum var. capsulatum infection, using a rabbit IgG, has been reported to give positive results in 17 of 18 confirmed penicilliosis patients (24). Also, during the development of fluorescent-antibody tests for the tissue form of P. marneffei, Kaufman et al. (12) reported that the rabbit antiserum reacted with the yeast form of H. capsulatum but not with the hyphal form or the hyphal forms of A. flavus, A. fumigatus, and H. capsulatum or with Candida albicans, C. glabrata, or C. neoformans. Prior adsorption of the antiserum with yeast-form H. capsulatum could overcome this cross-reaction. These findings are consistent with those of Sekhon et al. (17), who reported no reactivity when rabbit antisa to mycelial elements of P. marneffei were tested with the exoantigens of eight mono- morphic species of Penicillium or with hyphal antigens of As- pergillus spp. in the immunodiffusion test. Furthermore, Kauf- man et al. (13) have developed an immunodiffusion test and a latex agglutination test to detect serum antigen. Both tests used rabbit antiserum raised against a culture filtrate of fission arthroconidia of P. marneffei which had the same antigens as those reported by Kaufman et al. (12). The tests were evaluated by using a small number of human sera. Serum antigen was detected by latex agglutination in 13 (76%) of 17 infected patients and by immunodiffusion in 10 (60%) patients. Antigen was not detected in serum samples from 15 normal Thai controls and six patients with cryptococcosis. P. marneffei antigen was also found in the urine from two infected patients, but not in six urine specimens containing Histoplasma antigen (13).

The polyclonal rabbit antiserum used in this study provides a highly sensitive and specific method for the diagnosis of penicilliosis in this area of endemicity. Of the 33 penicilliosis patients, 1 had detectable antigen only in neat urine (this patient had coinfection with C. neoformans) and a further 2 had titers of 1:40, while of the remaining 30 patients, 28 had titers of 1:2,560 or greater. Both of the patients with titers of 1:40 had very scanty skin lesions. One of these patients and the patient whose urine was positive only when undiluted had presented with disseminated cryptococcal infections. Because four of the six patients with false-positive antigen titers of >1:40 had cryptococcal infections, use of this cutoff titer alone for diagnosis would have lead to incorrect prescription of antifungal drugs in only two cases. Furthermore, these four patients were all HIV positive, and we cannot exclude the possi- bility that they were also infected with P. marneffei, although we were unable to culture it from suitable specimens. Of the other patients with false-positive results, one was also HIV positive and had a Salmonella septicemia (titer, 1:5,120). This patient died shortly after blood cultures were collected and before other samples could be collected, so we cannot rule out coexisting penicilliosis. The other patient (HV test not done) was culture negative from blood and cerebrospinal fluid, but a coliform was grown from urine (titer, 1:160). This is not an area where leishmaniasis is prevalent, and so we cannot comment on test results for patients with this infection.

This antigen detection test should prove useful in diagnosis of P. marneffei infection and may be modifiable to a simple dot blot or latex agglutination form. The antigens recognized by the polyclonal antisera should now be characterized. The applicability of the test to serodiagnosis and the value of serial antigen detection in monitoring the response to treatment are currently being evaluated.

ACKNOWLEDGMENTS
We thank Wipada Chaowagul and Yupin Supputamongkol, Boong- gong Pimsa-ard, and Sayan Langla for assistance and Sornchai Lou- reesuwon (Faculty of Tropical Medicine, Mahidol University) and Sirivan Vanijanond (Department of Clinical Tropical Medicine) for support and encouragement.

This study was part of the Wellcome-Mahidol University, Oxford Tropical Medicine Research Programme, funded by the Wellcome Trust of Great Britain.

REFERENCES

