JOURNAL OF CLINICAL MICROBIOLOGY, July 1996, p. 1834–1836 Vol. 34, No. 7
0095-1137/96/$04.00+0

Restriction Endonuclease Analysis of *Penicillium marneffei*

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Received 31 January 1996/Returned for modification 4 March 1996/Accepted 12 April 1996

Forty-six isolates of *Penicillium marneffei* were differentiated into two DNA types on the basis of their restriction fragment length polymorphisms. Of the 22 human isolates of *P. marneffei*, 16 (72.7%) were type I and 6 (27.3%) were type II. Of the 23 bamboo rat isolates, 20 from *Rhizomys sumatrensis* were type I and 3 from *Cannomys badius* were type II. The soil isolate was type II. These data represent the first molecular epidemiological study of this important emerging fungal pathogen.

Penicillium marneffei is a causative agent of an emerging systemic mycosis which has been suggested to be an AIDS-defining illness (31). The natural reservoirs of this fungus are four species of bamboo rats, *Rhizomys sumatrensis*, *Rhizomys pruinosus*, *Rhizomys sinensis*, and *Cannomys badius* (1, 3, 4, 8, 10, 17), and soil samples of bamboo rat burrows (4, 10, 34). Before the AIDS era, *P. marneffei* infection occurred in both healthy and immunocompromised hosts (9, 10, 12, 16, 19, 25, 28). Since the beginning of the AIDS pandemic, however, a striking increase in the occurrence of this mycosis as an opportunistic infection in AIDS patients has been reported (5, 14, 15, 20, 22, 23, 27, 30–33, 36). A presumptive diagnosis can be made by microscopic examination of bone marrow aspirate stained with Wright's stain and/or touch smears of skin biopsy or lymph node biopsy specimens. The definite diagnosis must be confirmed by culture. An unusual feature of this fungus is its thermally dimorphic nature (25). The exoantigen test (26) can also be used to confirm the identification of the fungus. Other powerful methods that can identify or differentiate this organism should be developed for epidemiological distribution studies as well as for identification purposes. Molecular techniques were used to analyze the DNA fingerprints or the restriction fragment length polymorphisms of various human pathogenic fungi, such as *Candida albicans* (6, 24), *Histoplasma capsulatum* (29, 35), *Aspergillus fumigatus* (2, 11), *Blastomyces dermatitidis* (13), and *Sporothrix schenckii* (7). The method has become a useful tool for the epidemiologic study of these pathogens. The purpose of the study described here was to analyze the restriction profiles of DNA extracted from *P. marneffei* strains isolated from the environment and from AIDS patients with penicilliosis.

Twenty-two isolates of *P. marneffei* from human sources and 24 *P. marneffei* isolates from nature were used in the study (Table 1). The organisms were identified as *P. marneffei* on the basis of standard methods (25, 26). DNA from the yeast form of *P. marneffei* was extracted (7, 37). Briefly, *P. marneffei* was subcultured onto a brain heart infusion (BHI) agar slant, and the slants were incubated at 37°C for 5 days. This culture was used as the inoculum for 100 ml of BHI broth. The broth culture was incubated in a water bath shaker for 48 h at 37°C. The culture was centrifuged at 3,000 × *g* for 15 min, washed twice with phosphate-buffered saline, and washed once with 0.6 M $MgSO_4$. The cells were suspended in filter-sterilized osmotic medium (1.2 M $MgSO_4$, 10 mM sodium phosphate [pH 5.8]; 5 ml/g of yeast cells), and the suspension was placed on ice. A filter-sterilized solution of NovoZym 234 (20 mg/ml in osmotic medium; 2 ml/g of yeast cells) was added, and the cells were incubated on ice for 5 min. Next, a filter-sterilized solution of bovine serum albumin (12 mg/ml in osmotic medium; 0.5 ml/g of yeast cells) was added. The suspension was then incubated at 37°C for 2 h. The spheroplasts were pelleted by centrifugation, washed twice with ST buffer (0.6 M sorbitol, 100 mM Tris-HCl [pH 7.0]), and resuspended in 10 ml of lysis buffer (10 mM Tris, 1 mM EDTA, 1% sodium dodecyl sulfate [pH 8.0]). The suspension was mixed vigorously and was incubated at 65°C for 1 h. Three milliliters of 5 M potassium acetate was added. The suspension was mixed by inversion and was incubated on ice for 1 h. The supernatant was pipetted into a new tube after centrifugation at 12,000 × *g* and 4°C for 10 min. After an equal volume of 2-propanol was added, the solution was placed at −20°C for 1 h. The nucleic acid was collected by centrifugation at 12,000 × *g* and 4°C for 10 min and was resuspended in TE buffer (10 mM Tris, 1 mM EDTA [pH 8.0]). Following phenol, phenol-chloroform, and chloroform extractions of the nucleic acids, ethanol precipitation was performed. The pellet was resuspended in TE buffer containing RNase A (50 μg/ml), and the suspension was incubated at 37°C for 30 min. The DNA concentration was determined by measuring the A_{260} with a spectrophotometer (Spectronic Genesys 5; Milton Roy Company) or by gel electrophoresis. Different restriction enzymes were used to digest the DNA. Digestion reactions were performed in a total volume of 20 μl of digestion reaction mixture containing 3 μg of DNA and 10 U of restriction enzyme, and the mixture was incubated for 1 h at 37°C. The reaction was stopped by adding 4 μl of stop buffer (25% Ficoll 400, 50 mM EDTA, 0.25% bromphenol blue, 0.25% xylene cyanole). Electrophoresis was carried out for about 3 to 4 h at 3 to 4 V/cm in a horizontal gel containing 0.7% agarose.

The digestion of DNA with 10 restriction enzymes (*Bgl*II, *Cfo*I, *Eco*RI, *Hae*III, *Hin*dIII, *Kpn*I, *Msp*I, *Sal*I, *Xba*I, and *Xho*I) was first performed to select the most appropriate enzymes for further study. The results indicated that the enzymes *Cfo*I and *Hae*III could digest the DNA of *P. marneffei* 391H (ATCC 200050). *Cfo*I generated DNA fragments with esti-

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TABLE 1. Clinical and natural *P. marneffei* isolates used in the study

Sources of isolates	Designation of isolates[a]
Humans[b]	231H, 233H, 235H, 299S, 299BM, 302BM (ATCC 200051), 332H, 389H, 391H (ATCC 200050), 397S, 423H, 429H, 444H, 475H, 495H, 496H, 497H, 500H, 502H, 515H, 517H, 518H
Bamboo rats[c]	
R. sumatrensis	LuB0301, SpB0401, LuB0401, LuB0501, LuB0601, SpB0601, LiB0601, SpB0701, LuB3701, LuB3801, SpB4001, LuB4001, LuB4901, SpB4901, LiB4901, LuB5001, SpB5001, LuB5101, LuB7401, LiB7401
C. badius	LiB6201, SpB6203, LuB8001
Soil isolate[d]	SB0501

[a] H, hemoculture; S, skin lesion; BM, bone marrow aspirate; LuB, lung tissue of bamboo rat; SpB, spleen tissue of bamboo rat; SB, soil from bamboo rat's burrow.
[b] *P. marneffei* organisms were isolated from AIDS patients with penicilliosis.
[c] *P. marneffei* organisms were isolated from the internal organs of *R. sumatrensis* and *C. badius* bamboo rats.
[d] The *P. marneffei* organism was isolated from a soil sample from the burrow of an *R. sumatrensis* bamboo rat.

mated sizes of 19.0, 4.9, 3.7, 3.1, 2.0, and 1.9 kbp, and *Hae*III generated DNA fragments of about 18.0, 6.5, 3.3, 2.9, 2.7, 2.2, and 2.0 kbp. Use of DNA from the mold and yeast forms resulted in no difference in the results of restriction endonuclease analysis. For further study, the DNA restriction digestion patterns of 22 human isolates and 24 natural isolates of the yeast form of *P. marneffei* were analyzed with both enzymes. All 46 isolates of *P. marneffei* had the same DNA restriction pattern with the enzyme *Cfo*I. In contrast, digestion with *Hae*III exhibited two DNA patterns. These two distinct DNA fingerprints were designated *P. marneffei* DNA types I and II. DNA type I consisted of six distinctly staining DNA bands with estimated sizes of 18.0, 6.5, 4.6, 3.3, 2.9, and 2.5 kbp. DNA type II consisted of seven bands of about 18.0, 6.5, 3.3, 2.9, 2.7, 2.2, and 2.0 kbp. The bands of DNA which were smaller than 2.7 kbp could not be clearly seen. Of the 22 human isolates, 16 (72.7%) were type I and 6 (27.3%) were type II. Twenty isolates from the internal organs of large bamboo rats (*R. sumatrensis*) were DNA type I, and three isolates from the reddish brown bay bamboo rats (*C. badius*) were type II. The soil isolate recovered from a burrow of *R. sumatrensis* was classified as DNA type II (Table 2).

DNA-based typing methods have successfully been used for the delineation of strains within fungal species as well as for epidemiological studies (18, 21). Similar analyses have previously been used with other fungi such as *S. schenckii* (7) and *A. fumigatus* (2). Our results indicated that the DNA patterns of *P. marneffei* isolates from humans in Chiang Mai province were defined as DNA types I and II, with the majority being type I. Similarly, the isolates obtained from Chiang Rai, Mae Hong Son, and Phayao were type I. This does not necessarily help in the study of the epidemic in provinces outside Chiang Mai because only a few isolates from those areas were analyzed. It is interesting, however, that two human isolates from Amphoe Hang Dong of Chiang Mai and one soil isolate from the same district had the same DNA type (type II). These two human isolates were cultured from a bone marrow aspirate and a skin lesion from the same patient (Fig. 1, lane 6 and 7). *P. marneffei* type I was found in 8 isolates from *R. sumatrensis* rats in this area. The true distribution of *P. marneffei* in this area, however, needs more study. The different DNA type (type II) found in

TABLE 2. DNA types of 46 clinical and natural *P. marneffei* isolates generated by digestion of whole-cell DNA with restriction enzyme *Hae*III

Source of isolates	Isolate designation[a]	Total no. of isolates	DNA type
Human isolates			
Province Chiang Mai			
Amphoe Muang[b]	231H, 302BM, 332H, 389H, 391H, 397S, 423H, 429H, 475H, 495H, 496H, 497H, 502H, 515H, 517H	15	I, II
Amphoe Mae Rim	233H	1	I
Amphoe Samoeng	235H	1	I
Amphoe Hang Dong	299S, 299BM	2[c]	II
Province Chiang Rai	518H	1	I
Province Mae Hong Son	500H	1	I
Province Phayao	444H	1	I
Bamboo rat isolates			
Province Chiang Mai			
Amphoe Hang Dong	LuB0301, SpB0401, LuB0401, LuB0501, LuB0601, SpB0601, LiB0601, SpB0701	8	I
Amphoe Mae Taeng	LuB3701, LuB3801, LuB4001, SpB4001, LuB4901, LiB4901, SpB4901, LuB5001, SpB5001, LuB5101	10	I
Amphoe Mae Rim	LuB7401, LiB7401	2	I
Province Lampang	LiB6201, SpB6203, LuB8001	3	II
Soil isolate, Amphoe Hang Dong	SB0501	1	II

[a] See footnote *a* of Table 1 for descriptions of isolate designations.
[b] Amphoe indicates a district within a province.
[c] Two isolates from the same patient.
[d] Three isolates of *P. marneffei* from two reddish brown *C. badius* bamboo rats.

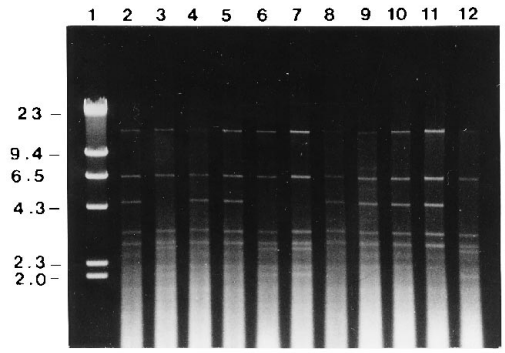

FIG. 1. *Hae*III-generated DNA types of *P. marneffei* human isolates from four provinces in northern Thailand and *P. marneffei* natural isolates. Lane 1, molecular size markers of standard *Hin*dIII-digested bacteriophage lambda DNA, with approximate sizes given in kilobases; lanes 2 and 3, human isolates from Amphoe Muang, Chiang Mai (isolate 496H, DNA type I, and isolate ATCC 200050, DNA type II, respectively); lane 4, human isolate 233H from Amphoe Mae Rim, Chiang Mai, DNA type I; lane 5, human isolate 235H from Amphoe Samoeng, Chiang Mai, DNA type I; lanes 6 and 7, human isolates 299BM and 299S, respectively, from Amphoe Hang Dong, Chiang Mai, DNA type II; lanes 8 to 10, human isolates 518H from Chiang Rai, 500H from Mae Hong Son, and 444H from Phayao, respectively; DNA type I; lane 11, bamboo rat isolate LuB0501 from Amphoe Hang-Dong, Chiang Mai, DNA type I; lane 12, soil isolate SB0501 from Amphoe Hang Dong, Chiang Mai, DNA type II.

the three isolates of *P. marneffei* recovered from *C. badius* may reflect the different sensitivities of bamboo rats to a particular strain. In summary, DNA restriction analysis may be useful for identifying different the genotypes of *P. marneffei* isolates. However, further studies are necessary to analyze the existence of multiple genotypes of *P. marneffei* and their geographic variations.

This work was supported in part by grants from the Fogarty International Center, National Institutes of Health (grant D43TW00010), the National Research Council of Thailand (1994–1995), and the Faculty of Medicine, Chiang Mai University, Chiang Mai, Thailand.

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