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Random amplified polymorphic DNA (RAPD) analysis of *Penicillium marneffei* strains isolated from AIDS patients in Thailand

Nanthawan Mekha[1], Natteewan Poonwan[1], Yuzuru Mikami[2],*, Katsukiyo Yazawa[2], Tohru Gonoi[2], Shuji Hasegawa[3] and Kazuko Nishimura[2]

[1] National Institute of Health, Department of Medical Sciences, Nonthaburi, Thailand
[2] Research Center for Pathogenic Fungi and Microbial Toxicoses, Chiba University, Chuo-ku, Chiba-city, Chiba 260, Japan
[3] Chiba City Institute of Health, Mihama-ku, Chiba-city, Chiba 261, Japan

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Results of random amplified polymorphic DNA (RAPD) analysis using three different primers showed that 16 strains of *Penicillium marneffei* isolated from AIDS patients in Thailand belonged to a genetically homogenous group, but differed slightly from an isolate from bamboo rat in China. Six PCR fragments (from about 200 to 600 bp) that were commonly observed in the RAPD fingerprint of all strains were extracted and sequenced. Usefulness of this sequence information for identification of *P. marneffei* is discussed.

Key Words——AIDS; genome typing; PCR; *Penicillium marneffei*; RAPD method.

The first report of the isolation of *Penicillium marneffei* Segretain, Capponi et Sureau ex Ramirez from a bamboo rat in Vietnam was made by Capponi et al. in 1956 (Capponi et al., 1956). Since then, epidemics of penicilliosis marneffei have been reported in Southern China, Indonesia, Vietnam and Thailand as a systemic mycosis among native healthy as well as immunocompromised patients (Supparatpinyo et al., 1992; Drouhet, 1993; Sirisanthana, 1996). Coupled with the increase in the number of AIDS patients, numerous fungal infections due to *P. marneffei* have recently been reported in Southeast Asia among these patients or those who are HIV-positive (Drouhet, 1993; Sirisanthana, 1996). The association between HIV infection and disseminated *P. marneffei* infection has thus been confirmed (Drouhet, 1993; Sirisanthana, 1996). Laboratory diagnosis of *P. marneffei* infection was reported to be difficult in many cases because symptoms of this mycosis are very similar to those of infections by other fungal pathogens such as *Histoplasma capsulatum* Darling in epidemic areas (Drouhet, 1993). In addition, firm identification of etiological agents by current culture-based diagnostic methods takes a long time, and detection of the organism in a patient is often delayed until infection has reached an advanced state. The development of a rapid and accurate identification system of *P. marneffei* has therefore been needed from the point of view of treatment, since delay in the diagnosis and initiation of therapy of this disease has led to a high mortality rate (Drouhet, 1993; Boon-Long et al., 1996). We isolated and identified 16 strains of *P. marneffei* from AIDS patients in Thailand in 1995, and confirmed that the PCR method using the primers reported by LoBuglio and Taylor (1995) was the most useful for rapid identification of this fungus.

A PCR-based random amplified polymorphic DNA (RAPD) fingerprint analysis method has been applied to various microorganisms to confirm similarity at the strain level (Goodwin and Annis, 1991; Bostock et al., 1993; Poonwan et al., 1996). We were, therefore, interested in determining the genetic homogeneity of *P. marneffei* in the Thai AIDS patients by this method. Here, we report the results of the RAPD analysis of the present isolates and development of PCR primers based on RAPD patterns.

Materials and Methods

Isolation of *P. marneffei* *Penicillium marneffei* was isolated from the clinical specimens shown in Table 1 by using potato dextrose (PDA, Difco) and brain heart infusion (BHI, Difco) agars. *Penicillium marneffei* was characterized as a dimorphic fungus that showed mold growth on PDA at 25°C and yeast growth on BHI agar at 37°C (Drouhet, 1993). *Penicillium marneffei* IFM 41708, an isolate from bamboo rat in China, was used as reference

*Correspondence: Dr. Y. Mikami, Division of Experimental Chemotherapy, Research Center for Pathogenic Fungi and Microbial Toxicoses, Chiba University, 1–8–1, Inohana, Chuo-ku, Chiba-city, Chiba 260, Japan.
FAX: 043-226-2486, TEL. 043–222–7171, EX. 5923.
e-mail: mikami@myco.pf.chiba-u.ac.jp.

strain. Sixteen strains of *P. marneffei* were isolated from AIDS patients in 1995: 13 strains from blood samples, 1 from pus, and 2 from lymph nodes (Table 1). Each strain was identified by the following typical features of thermal dimorphism: unicellular oval or elongated cells which multiplied by scission in vitro at 37°C on BHI agar, and mold-like growth on PDA medium at 27°C. Final species identification was done by microscopic observation of morphology on PDA at 27°C. The production of diffusible red pigments in PDA, which is another key characteristic of *P. marneffei*, was seen in all strains.

DNA preparation from fungal cells Chromosomal DNA of *P. marneffei* was extracted by the modified method of Zhu et al. (1993) and Fujimori and Okuda (1995). The strains were grown on BHI agar at 37°C for 1 wk. Three or four loopfuls of yeast cells from BHI agar slants were suspended in 500 μl of TE buffer (100 mM Tris-HCl, pH 8.0, 1 mM EDTA) in an Eppendorf tube (1.5 ml), and after agitation for 1 min, the suspension was centrifuged at 12,000 rpm and the sediment was mixed with 0.5 ml of extraction buffer (100 mM Tris-HCl, pH 9.0 with 40 mM EDTA), 0.1 ml of 10% SDS and 0.3 ml of benzyl chloride. After shaking with a vortex mixer, the fungal cells were incubated at 65°C for 30 min to kill them, then broken with a beadbeater (Wakenyaku, Japan) for 20 s and centrifuged at 12,000 rpm at 4°C for 5 min. After transfer of the supernatant into a new Eppendorf tube, 3 M sodium acetate was added in 1/10 volume of the supernatant and the mixture was cooled at 0°C for 10 min. DNA was precipitated with 0.5 ml of 2-propanol (−70°C, overnight), and the precipitate was washed with 70% ethanol, dried and resuspended in 100 ml of TE buffer.

Identification by PCR The species of the fungus was determined by PCR method using two primers: MAE-1=5′-GATGGACTGTCTGAGTACC-3′ and MAE-2=5′-ATGGTGGTGACCAACCCCCGCA-3′. The primer set used in this experiment was prepared based on the information of LoBuglio and Taylor (1995). Amplification reactions were performed in a volume of 50 μl containing 5 μl of 10 X buffer, 3 μl of $MgCl_2$ (25 mM), 4 μl of dNTP mixture (2.5 mM each), 2.5 μl of each primer (20 pM), 2.5 μl of genomic DNA (1 μg/ml) and 1 μl of *Taq* polymerase (5 U/ml, Biotech International). Distilled water was added to a total volume of 50 μl. The PCR was performed by initially heating the samples at 94°C for 10 min; this was followed by 25 cycles of denaturation (94°C, 1 min), annealing (50°C, 1 min) and extension (72°C, 2 min), then final extension at 72°C for 10 min.

RAPD analysis The three primers used, R-1=(5′-ATTGCGTCCA-3′), R-2=(5′-TCACGATGCA-3′) and R-3=(5′-ATGGATC(GC)(GC)C-3′), were prepared based on the report of Goodwin and Annis (1991) or on information from our preliminary experiments. A 5-μl portion of a diluted DNA preparation containing 20 ng of DNA was subjected to RAPD amplification in 50 μl (final aqueous volume). The reaction mixture was the same as described in the identification by PCR except that a single primer was used instead of the two primer sets. The PCR was performed by initially heating the samples at 94°C for 4 min; this was followed by 35 cycles of denaturation at 94°C for 2 min, annealing at 32°C for 2 min, extension at 72°C for 2 min, and the final extension at 72°C for 10 min in a thermoreactor. All reaction products were characterized by electrophoresis on 1.5% agarose gels, then stained in 0.5 μg/ml of ethidium bromide solution.

DNA sequence determination The PCR reaction pro-

Table 1. *Penicillium marneffei* strains tested.

Strain No. NIH[a]	Date of isolation	Place of hospital[b]	HIV condition	Source of specimen
1965-102 (1)[c]	1995-6-13	Chiang Rai hosp. (N)	+	blood
1963-100	1995-6-13	Chiang Rai hosp. (N)	+	blood
1845-137	1995-5-29	Chiang Rai hosp. (N)	+	blood
1803-95 (2)	1995-5-18	Hadyai hosp. (S)	+	blood
2000-137 (3)	1995-6-16	NIH[a] (C)	+	blood
2437-83 (4)	1995-8-11	Chiang Rai hosp. (N)	+	pus
2515-161	1995-8-23	Chiang Rai hosp. (N)	+	blood
2344-256	1995-6-31	Bamrasnaradura hosp. (C)	+	blood
2436-82	1995-8-11	Chiang Rai hosp. (N)	+	blood
2221-133	1995-6-14	Chiang Rai hosp. (N)	+	blood
2223-135	1995-6-14	Chiang Rai hosp. (N)	+	blood
2224-136	1995-6-14	Chiang Rai hosp. (N)	+	blood
2267-179 (5)	1995-6-20	Bamrasnaradura hosp. (C)	+	lymph nodes
2597-243 (6)	1995-8-30	Chiang Rai hosp. (N)	+	blood
2596-242	1995-8-30	Chiang Rai hosp. (N)	+	blood
2452-98	1995-8-14	Bamrasnaradura hosp. (C)	+	lymph nodes

a) NIH: National Institute of Health, Thailand.
b) Regional distribution in Thailand shown by (N), northern; (C), central; and (S), southern.
c) The numbers in parentheses show the strain No. tested in the RAPD experiments of Figs. 1a–c.

ducts were subjected to phenol-chloroform extraction and precipitated with ethanol. The amplified DNA fragments were then sequenced using a dye terminator reagent kit including *Taq* polymerase, and the protocol recommended by the manufacturer (a model 373 automated DNA sequencer, Applied Biosystems, Japan).

Results

Identification by PCR was done to confirm the species, and the results showed that the PCR primer combination of MAE-1 and MAE-2 was 100% successful in the identification of all tested Thai strains of *P. marneffei*.

RAPD analysis of the 16 strains of *P. marneffei* isolated from AIDS patients showed a certain characteristic pattern. We used three PCR primers, R-1, R-2, and R-3, and each one produced a characteristic fingerprint pattern (Figs. 1a–c). Interestingly, the PCR patterns of tested Thai strains were very similar with each primer. Although the reference strain IFM 41708 and the tested Thai strains were related in most features, there were some differences. Namely, one or two additional PCR bands were seen in the reference strain from China, such bands being missing in the Thai strains. Figure 1 shows representative PCR patterns using the six strains, and patterns of other strains with each PCR primer were almost the same as these.

Since the R-3 primer showed ladder-like PCR band patterns, we were interested in the base sequences of each fragment, and we reasoned that these ladder band might be advantageous in characterizing this fungus. These PCR bands, which were believed to be common to all *P. marneffei*, were then extracted and the DNA sequence of each band (from 200 to 600 bp) was determined. We were able to determine base sequences of six fragments: 208, 246, 347, 430, 516 and 545 bp (data not shown). No homology was observed among them by alignment (data not shown). We then prepared new PCR primers based on this base sequence information and compared the PCR band patterns. Interestingly, the amount of amplified PCR bands in agarose gel differed depending on the primer used. Comparison of PCR amplified bands by various primer sets tested (347, 430, 516 and 545 bp) showed that the largest amount of products was given by the set: Pm-545 sen, 5′-ATGGATCGGCCATAAGCAGA-3′; Pm-545-antisen, 5′-ATGGATCGCCAATAGCCGAG-3′. The amplified fragment by PCR is shown in Fig. 2.

Discussion

The mortality rate of patients with disseminated *P. marneffei* infection has been reported to be very high (Drouhet, 1993; Sirisanthana, 1996). However, clinically it is accepted that penicillosis marneffei is a treatable disease, although delay in treatment can be fatal. Therefore, early diagnosis and subsequent therapy are very important. In most laboratories, morphological and physiological characteristics are still used to identify this fungus, and these tests take one to two weeks for the final identification. Recently, LoBuglio and Taylor (1995) proposed a primer set for identification of *P. marneffei*; we tested the usability of the primer set using 16 strains of *P. marneffei* and found that it was 100% successful. We then prepared a new primer set based on the RAPD patterns and found that it was also 100% successful for the identification of *P. marneffei*. The most recent new PCR primer set (Pm-545) is also expected to be a candidate for the rapid identification of this fungus, although further detailed specificity studies of this primer are necessary.

Penicilliosis marneffei has emerged as an endemic systemic mycosis in Southeast Asia among humans and wild bamboo rats. Ajello et al. (1995) reported that most bamboo rats (*Cannomys badius* and *Rhizomys pruinosus*) are carriers of the fungus and suggested a close association between these animals and humans in its infection. Epidemiological studies by other researchers (Drouhet, 1993; Vanittanakom et al., 1995) have also suggested that bamboo rats in Thailand are hosts of *P. marneffei*. However, it is still controversial whether

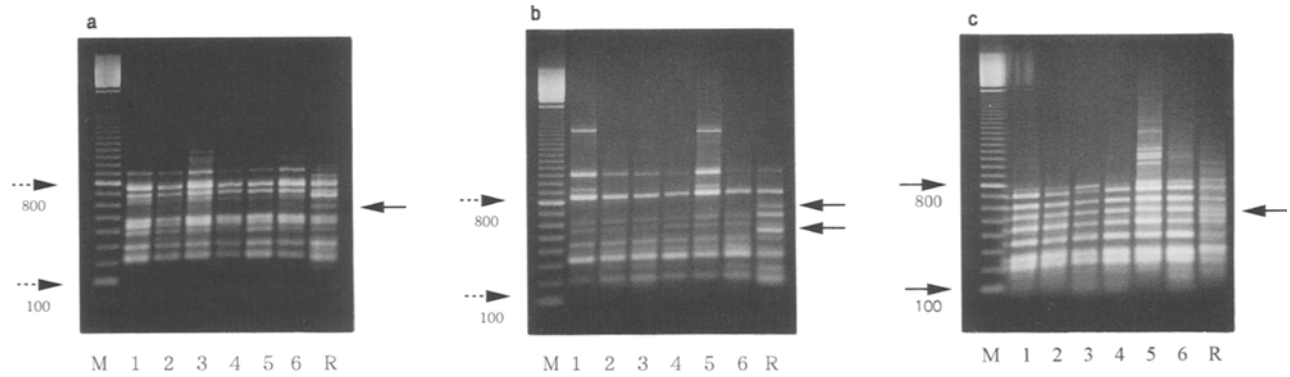

Fig. 1. RAPD fingerprint band patterns of six representative strains of *Penicillium marneffei* isolated from AIDS patients after PCR with 10-mer primers.
DNA ladder was used as the molecular size standard (M). R shows reference strain (*P. marneffei* IFM 40718). a, R-1 primer; b, R-2 primer; c, R-3 primer. Arrows at right show the specific PCR bands of the reference strain that were not observed in Thai isolates (see Table 1 for the strain No. used).

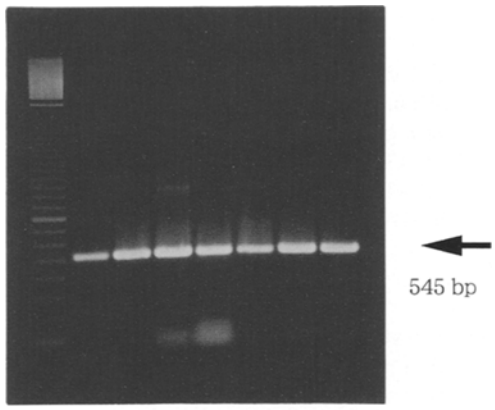

Fig. 2. PCR patterns of *Penicillium marneffei* isolates in Thailand by using newly prepared PCR primers based on the information of RAPD analysis.
PCR bands of seven isolates are shown.

bamboo rats are the actual carrier of this fungus.

During the present studies we demonstrated that the isolates of *P. marneffei* in Bangkok were genetically homogenous, although they were slightly different from the reference strain of *P. marneffei* from China. We isolated the fungus from various hospitals in Thailand including the Northern, Central and Southern regions. Although we tested only 16 strains, all *P. marneffei* strains in Thailand may be genetically homogenous, and further studies on their genetic relatedness using many strains from various sources including bamboo rats in Thailand are in progress. The present studies also showed the RAPD method to be very useful in the determination of strain variability. In fact, our experiment demonstrated that even minor differences between the reference strain and the Thai strains could be identified by this method. Application of the RAPD method is thus expected to provide useful information on the epidemiological survey of penicilliosis marneffei in AIDS patients.

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