PRODUCTION OF MONOCLONAL ANTIBODIES AGAINST *PENICILLIUM MARNEFFEI* ANTIGENS AND MOLECULAR EPIDEMIOLOGY OF *PENICILLIUM MARNEFFEI* INFECTION IN THAILAND

SOMPONG TREWATCHAREGON

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Penicilliosis marneffei, a disease caused by dimorphic fungus *Penicillium marneffei*, is currently the third most prevalent opportunistic infection in AIDS patients in northern Thailand. Incorrect diagnosis and delayed treatment contribute to its relatively high mortality rate. Clinical manifestations of penicilliosis marneffei closely resemble tuberculosis, histoplasmosis, cryptococcosis and other systemic fungal infections, therefore, specific and reliable diagnosis methods are needed.

Four MAbs specific for *P. marneffei*, 3C2, 8C3, 8B11 and 3B9, were produced from hybridomas raised from BALB/c mice immunized with crude culture filtrate (CCF) prepared from mycelial phase of growth. The hybridomas were screened and characterized using different fungal antigens by enzyme-linked immunosorbent assay (ELISA), immunoblotting and immunofluorescent staining. In the immunoblots, MAb 3C2 (IgG1 subclass) reacted specifically with a denatured form of 38-kDa antigen whereas MAbs 8B11 and 3B9 (IgM subclass) reacted most strongly with high molecular weight components (>200 kDa) produced during either mycelial or yeast phase of growth. The immunoreactive epitopes for these MAbs were most likely associated with carbohydrate moieties, judging from their susceptibility to periodate treatment and concanavalin A binding. This is in contrast to the immunoreactive epitopes for MAbs 8C3 (IgM subclass) and 3C2 which were resistant to periodate treatment. In immunofluorescent staining, the three IgM MAbs could react strongly with both mycelial and yeast phase of *P. marneffei*, but not with the yeast phase of *Histoplasma capsulatum* and *Cryptococcus neoformans* whose morphology are closely similar to *P. marneffei*. Thus, these MAbs showed diagnostic potential. They could be used to identify *P. marneffei* in culture and biopsy specimens by immunofluorescent staining.

For genomic epidemiology study of 67 *P. marneffei* isolates using PFGE, 2 macrorestriction patterns (MPs) and 9 MP subgroups were generated by *Not* I digestion of 67 *P. marneffei* isolates. Of the 64 human isolates, 42 isolates (65.6%) were of MPI and belonged to subgroups MPIa (8 isolates), MPIb (11 isolates), MPIc (10 isolates), MPId (3 isolates), MPIe (5 isolates) and MPIf (3 isolates). Whereas 22 isolates (34.4%) were of MPII and belonged to subgroups MPIIa (6 isolates), MPIIb (4 isolates) and MPIIc (7 isolates). Two bamboo rat isolates belonged to subgroup MPIa and one isolate was of subgroup MPIc. No significant correlation between the MP of *P. marneffei* isolates and geographical region nor specimen sources was observed. Notably, isolates obtained before 1995 were of MPI and we have seen increased incidence of infection with MPII isolates since then. However, further studies are necessary to confirm this finding.

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พ.ต. สมพงศ์ ตรีวัชรีกร: การผลิตโมโนโคลนอลแอนติบอดีต่อแอนติเจนของเชื้อเพ็นนิซิเลี่ยม มาร์เนฟฟิไอ และระบาดวิทยาระดับโมเลกุลของเชื้อเพ็นนิซิเลี่ยม มาร์เนฟฟิไอ ในประเทศไทย (PRODUCTION OF MONOCLONAL ANTIBODIES AGAINST *PENICILLIUM MARNEFFEI* ANTIGENS AND MOLECULAR EPIDEMIOLOGY OF *PENICILLIUM MARNEFFEI* INFECTION IN THAILAND) คณะกรรมการควบคุมวิทยานิพนธ์: สถิตย์ สิริสิงห (Ph.D.), ศันสนีย์ ไชยโรจน์ (Ph.D.), ชื่นจิตต์ บุญเฉิด (Ph.D.), อังคณา ฉายประเสริฐ (Dr.rer.nat), 167 หน้า. ISBN 974-664-591-9

โรคเพ็นนิซิลิโอซีส มาร์เนฟฟิไอ เป็นโรคราฉวยโอกาสที่พบมากเป็นอันดับที่ 3 ในผู้ป่วยโรคเอดส์ทางตอนเหนือของประเทศไทย ซึ่งมีสาเหตุมาจากการติดเชื้อราสองรูป *Penicillium marneffei* การให้การวินิจฉัยโรคที่ไม่ถูกต้องและการรักษาที่ล่าช้าทำให้ผู้ป่วยเสียชีวิตในอัตราที่สูง เนื่องจากลักษณะอาการแสดงของโรคเพ็นนิซิลิโอซีส มาร์เนฟฟิไอ คล้ายคลึงกับผู้ป่วยวัณโรค histoplasmosis, cryptococcosis รวมทั้งโรคติดเชื้อราทั้งระบบอื่นๆ ทำให้มีความต้องการวิธีการตรวจวินิจฉัยโรคที่มีความจำเพาะและเชื่อถือได้ ดังนั้น monoclonal antibody (MAb) ที่จำเพาะต่อเชื้อ *P. marneffei* คือ 3C2, 8C3, 8B11 และ 3B9 จึงได้ถูกผลิตขึ้นจากเซลล์ลูกผสมที่ได้จากหนู BALB/c ที่ถูกกระตุ้นด้วยแอนติเจน "crude culture filtrate" ที่เตรียมจากเชื้อในรูปราสาย จากนั้นทำการตรวจกรองและตรวจสอบเซลล์ลูกผสมที่ได้โดยใช้แอนติเจนที่เตรียมจากเชื้อราอื่นๆ โดยวิธี ELISA, immunoblotting และ immunofluorescent staining จากการตรวจสอบ MAbs ที่ได้ด้วยวิธี immunoblotting พบว่า 3C2 ซึ่งเป็น IgG_1 subclass ทำปฏิกริยาจำเพาะกับ denatured form ของ 38-kDa แอนติเจน ในขณะที่ 8B11 และ 3B9 ซึ่งเป็น IgM subclass ทำปฏิกริยากับสารที่มีน้ำหนักโมเลกุลสูง (มากกว่า 200 kDa) ที่ถูกผลิตจากเชื้อทั้งในรูปราสายและส่าได้ดี และพบว่า immunoreactive epitopes ของ 8B11 และ 3B9 น่าจะเป็นส่วนของ carbohydrate โดยพิจารณาจากการที่บริเวณดังกล่าวถูกย้อมได้ด้วย Concanavalin A และไวต่อการทำ ปฏิกริยากับ periodate ซึ่งตรงข้ามกับ immunoreactive epitopes ของ 8C3 ซึ่งเป็น IgM subclass และ 3C2 ที่ไม่มีการเปลี่ยนแปลงเมื่อทำปฏิกริยากับ periodate เมื่อตรวจสอบ MAbs ด้วยวิธี immunofluorescent staining พบว่า MAbs ชนิด IgM ทั้ง 3 ตัว ทำปฏิกริยากับเชื้อ *P. marneffei* ทั้งในรูปราสายและส่าได้ดี แต่ไม่ทำปฏิกริยากับเชื้อ *Histoplasma capsulatum* ในรูปส่าและเชื้อ *Cryptococcus neoformans* ซึ่งมีลักษณะคล้ายคลึงกับเชื้อ *P. marneffei* มาก ดังนั้น MAbs เหล่านี้จึงมีประโยชน์ที่จะนำมาใช้พัฒนาวิธีทดสอบเพื่อใช้วินิจฉัยโรคเพ็นนิซิลิโอซีส มาร์เนฟฟิไอ ดังเช่นการตรวจหาเชื้อ *P. marneffei* ในculture และ tissue biopsy specimens โดยวิธี immunofluorescent staining

สำหรับการศึกษาระบาดวิทยาจากพันธุกรรมของเชื้อ *P. marneffei* จำนวน 67 สายพันธุ์ โดยการใช้เทคนิค pulsed-field gel electrophoresis (PFGE) พบว่าวิธีดังกล่าวสามารถจำแนกเชื้อที่ตรวจสอบออกเป็น 2 กลุ่มใหญ่ (macrorestriction pattern, MP) และ 9 กลุ่มย่อย (MP subclass) หลังจากการย่อยสารพันธุกรรมของเชื้อด้วยเอ็นไซม์ *Not*I จากจำนวนเชื้อที่แยกได้จากผู้ป่วย 64 สายพันธุ์ พบว่า 42 สายพันธุ์ (65.6%) อยู่ในกลุ่ม MPI แบ่งออกเป็น MPIa 8 สายพันธุ์, MPIb 11 สายพันธุ์, MPIc 10 สายพันธุ์, MPId 3 สายพันธุ์, MPIe 5 สายพันธุ์ และ MPIf 3 สายพันธุ์ ในขณะที่ 22 สายพันธุ์ (34.4%) อยู่ในกลุ่ม MPII แบ่งออกเป็น MPIIa 6 สายพันธุ์, MPIIb 4 สายพันธุ์ และ MPIIc 7 สายพันธุ์ ส่วนเชื้อที่แยกได้จากตัวอ้นนั้น 2 สายพันธุ์อยู่ในกลุ่มย่อย MPIa และอีก 1 สายพันธุ์อยู่ในกลุ่มย่อย MPIc ผลการศึกษานี้ไม่พบความสัมพันธ์อย่างมีนัยสำคัญระหว่างพันธุกรรมของเชื้อที่ถูกจัดแบ่งเป็นกลุ่มต่างๆ โดยวิธี PFGE กับภูมิภาคหรือชนิดของตัวอย่างจากผู้ป่วย แต่มีข้อสังเกตคือพบว่าสายพันธุ์ที่แยกได้ก่อนปี พ.ศ. 2538 จะเป็น MPI ทั้งหมด ส่วนสายพันธุ์ในกลุ่ม MPII ค่อยๆ พบเพิ่มจำนวนมากขึ้นหลังจากเวลานั้นเป็นต้นมา อย่างไรก็ตามการนำข้อมูลทางระบาดวิทยาจากพันธุกรรมของเชื้อที่จะนำไปใช้นี้จะต้องมีการศึกษาสายพันธุ์ของเชื้อเพิ่มเติมต่อไป

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CHAPTER I

INTRODUCTION

Penicilliosis marneffei is a disseminated and progressive infection caused by a dimorphic fungus *Penicillium marneffei* which is endemic in southern part of China, Hong Kong and southeast Asian countries including Thailand. The organism was first isolated in bamboo rat in Vietnam in 1956 (1) and considered as a rare human pathogen in those residing or travelling in the endemic area. Since the massive epidemic of AIDS, the incidence of penicilliosis marneffei has increased markedly. In northern Thailand, penicilliosis marneffei has emerged as the third most prevalent opportunistic infection in AIDS patients after tuberculosis and cryptococcosis (2). The clinical manifestations of penicilliosis marneffei closely resemble tuberculosis and other systemic fungal infections (3-5). These usually include fever, anemia, weight loss, skin lesion, lymphadenopathy, hepatosplenomegaly and cough with abnormality on chest X-ray. If the diagnosis and proper treatment is delayed or misdiagnosed, the disease can be fatal with high mortality rate (6-9).

P. marneffei is rather unique fungus. It is the only dimorphic species in the genus *Penicillium*. It exhibits a mold form when grown on Sabouraud's dextrose agar (SDA) at 25°C. After 2 days of growth, the colony is grayish white and appears downy or wooly. A unique characteristic of *P. marneffei* is the production of soluble red pigment that diffuses into surrounding medium. This organism can be converted to yeast when incubated at 37°C on brain heart infusion agar (BHI). The yeast cells divide by binary fission and not by budding like *Histoplasma* and *Cryptococcus*.

A definitive diagnosis for penicilliosis marneffei is the culture positive for *P. marneffei*. However, this method often fails due to improper specimen collection, processing and coexistence with other opportunistic infections frequently found among immunocompromised hosts. A number of presumptive diagnostic methods based on serology and microscopic examination has been developed. Using polyclonal antibodies raised from rabbit or guinea pig, Sekhon *et al* (10) developed immunodiffusion test for specific and rapid identification of *P. marneffei*. Subsequently, an indirect immunofluorescent antibody technique, latex agglutination and ELISA method have been developed and evaluated for rapid presumptive diagnosis to fortify the conventional culture method (11-15).

With the exception of the immunological study aimed at developing sensitive and specific rapid diagnosis methods for penicilliosis marneffei, very little information is currently available at a molecular level of this fungus. Using nucleotide sequence of nuclear and mitochondrial ribosomal DNA regions of *P. marneffei*, LoBuglio and Taylor (16) established the phylogeny and developed identification method by PCR technique for this fungus. Vanittanakom and coworkers (17) studied molecular epidemiology of *P. marneffei* using RFLP technique and found two DNA types , type I and II, when the chromosomal DNA was digested with a restriction enzyme, *Hae* III.

The objectives of this study are as follows.

(1) Production of monoclonal antibodies (MAbs) specific for *P. marneffei* to be used for development of specific and reliable diagnostic method as well as valuable immunological reagent for the study on the biology of *P. marneffei*.

(2) Genomic epidemiologic study of *P. marneffei* using restriction endonuclease digestion and pulsed-field gel electrophoresis (PFGE), a method that should provide more information and understanding of molecular epidemiology of this organism.

CHAPTER II

BACKGROUND

1. Penicilliosis marneffei

Penicilliosis marneffei has emerged as an endemic systemic mycosis in southeast Asia especially among those infected with human immunodeficiency virus (3-9,18-37). Without early diagnosis and proper treatment, the disease is associated with high mortality rate, regardless of whether or not the HIV infection is involved (7,9,21,26). The causative agent of the disease is a dimorphic fungus *Penicillium marneffei*. The diagnosis of penicilliosis marneffei can be difficult because its clinical manifestations mimic those of tuberculosis and other mycotic infections (4,5,18,20,22) and microscopic examination of liver, bone marrow aspirate and of skin and lymph node biopsy specimen may be confused with *Cryptococcus* and *Histoplasma* (18,35,37,38). Although, a number of presumptive diagnostic methods have been developed (10-15,38-44), newer immunological and molecular-based methods are needed to improve the diagnosis, treatment and prevention of penicilliosis.

2. History

Penicilliosis marneffei is a disseminated and progressive disease caused by a dimorphic fungus *Penicillium marneffei*. The organism was first isolated from liver of a wild bamboo rat (*Rhizomys sinensis*) in Vietnam in 1956 by Capponi *et al* (1). Pathogenesis of *P. marneffei* infection in mice, hamsters and guinea pigs have been established. The organism was named after Dr. Marneffe who was once the Director of the Institute Pasteur in Indochina and Paris (45). In 1959, Segretain (46) reported the first

laboratory-acquired infection in humans. After he accidentally inoculated the fungi into his finger, lymphadenopathy developed and a nodule appeared at the inoculated site 9 days later. *P. marneffei* was subsequently isolated from the nodule. Fourteen years later, the first natural infection in humans was reported by Disalvo *et al* (45). The patient was a 61-year-old American minister working in Vietnam and travelling throughout southeast Asia. He had radiation therapy and splenectomy for Hodgkin's disease. *P. marneffei* was unexpectedly isolated from his enlarged and infarcted spleen. It was thought to be isolated accidentally until Jayanetra *et al* (6) reported on the presence of five human cases seen between 1974 to 1982 from Ramathibodi Hospital, Bangkok. Two of the patients had normal immunity and responded well with amphotericin B treatment. The others, had underlying conditions : tuberculosis, lymphoproliferative disorder or pregnancy complicated by SLE and died as a result of misdiagnosis. In 1985, Deng *et al* (18) claimed that they had identified the first case of patient with penicilliosis marneffei in Guangxi, China since 1964. Unfortunately, they were unable to publish their finding because of the suppression of academic endeavors during the Cultural Revolution (1966-1976).

In 1988, Piehl *et al* (23) reported the first case of penicilliosis marneffei associated with HIV infection. From that time, with the spread of AIDS epidemic, the incidence of penicilliosis marneffei associated with HIV infection cases has increased markedly. From 1991-1994, five hundreds and fifty cases of penicilliosis marneffei associated with HIV infection were diagnosed at Chiang Mai University Hospital. Now the disease is the third most prevalent opportunistic infection in AIDS patients from northern Thailand after tuberculosis and cryptococcosis (2).

3. Mycology

P. marneffei is the only species of *Penicillium* which is dimorphic. The colonies cultured at 25°C on SDA were moist at first but their surface soon become finely powdery, often umbonate and radially striated, and grayish pink. The reverse side are pink to red, resulting from the production of soluble red pigments that diffused into the media. Microscopically, the stipes are smooth and beared terminal verticils of 3 to 5 metulae. Each metulae produces several phialides whose basal part tapering to a pointed apex, bearing long chain of conidia, which are smooth, globose to sub-globose in shape (Fig. 1). When the isolates are grown on BHI agar at 37°C, their colonies become yeast-like and white to tan with a smooth to cerebriform surface. Microscopically, a tubular or oblong to oval shape of yeast-like cells are seen. The cells divide by binary fission.

For the taxonomy of *P. marneffei*, there appears to be two different lines of thoughts regarding the classification of this organism. The Raper and Thom system placed *P. marneffei* in the section *Asymmetrica*, subsection *Divaricata* (47) that was equivalent to Pitt's *Penicillium* subgenus *Furcatum* (48). However, the most recent taxonomic scheme for *Penicillium* species by Pitt assigned *P. marneffei* to subgenus *Biverticillium* rather than *Furcatum* because its conidiophores were most frequently biverticillate with poor growth on G25N medium (48).

The other classification, proposed by Ramirez (49), placed *P. marneffei* in the section *Asymmetrica,* subsection *fasiculata* owing to its strong synnematous character. However, he also noticed that this fungus was closer to the section *Biverticillata,*

subsection *symmetrica* and had questioned on the strong divaricate penicilli and the shape of phialides characteristics of the section *Asymmetric,* subsection *divaricata.*

Recently, the phylogenetic tree of *P. marneffei* has been established (16). The data showed *P. marneffei* to be closely related to species of *Penicillium* in the subgenus *Biverticillium* and sexual *Tararomyces* species with asexual biverticillate *Penicillium* states. Because the teleomorph of *P. marneffei* has never been reported, *P. marneffei* is therefore placed in Phylum *Deuteromycota.*

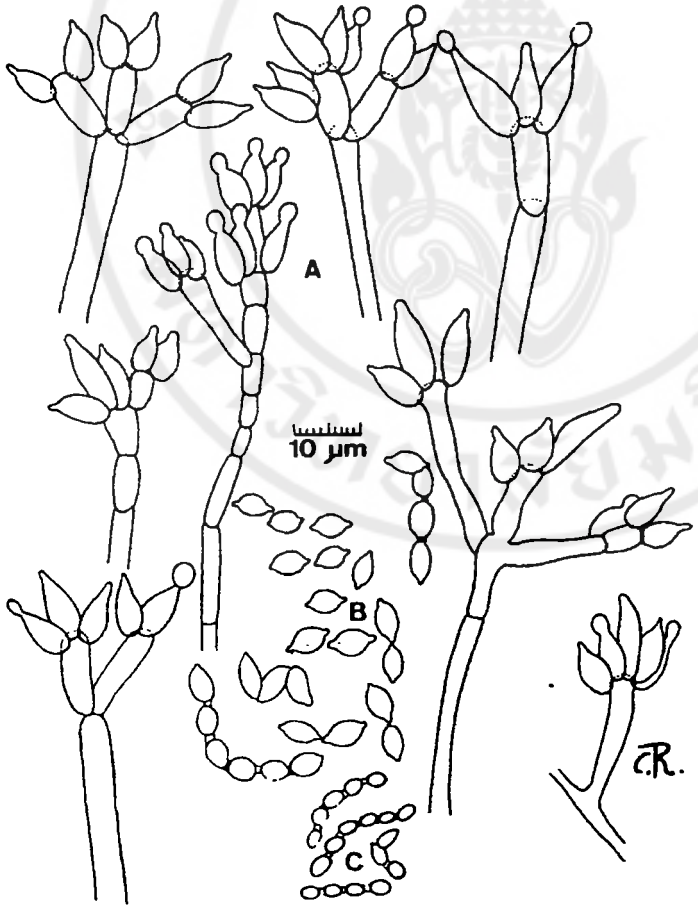

Fig. 182. *Camera lucida* drawings of *Penicillium marneffei* Segretain, Capponi *et* Sureau *ex* Ramírez CBS 324.59. A, Detailed drawings of the penicilli. B, Mature conidia (large ones). C, Mature small conidia.

Figure 1: Drawings of *Penicillium marneffei.* (Source: Carlos Ramirez. Manual and Atlas of the *Penicilia.* 1982. P.503)

4. Epidemiology and Ecology

Since the first isolate of *P. marneffei* was described in 1956 from a captive Chinese bamboo rat (*Rhizomys sinensis*) in Vietnam (1), a first survey of bamboo rat for natural infection by *P. marneffei* was carried out by Deng *et al* thirty years later (50). These investigators performed necropsies on apparently healthy 43 bamboo rats captured in Guangxi (*41 Rhizomys pruinosus* and 2 *R. sinensis*). *P. marneffei* was isolated from internal organs from 39 (91%) rats and from feces of 3 out of 4 bamboo rat examined. No gross pathological lesion was observed in any of the rats examined.

A survey of bamboo rat for this organism was also carried out in the central plains of Thailand during June-September 1987 by Ajello *et al* (51). Thirty-one small bamboo rats (C*annomys badius*) and eight hoary bamboo rats (*R. pruinosus*) were trapped. *P. marneffei* could be isolated from the internal organs of 6 *C. badius* (19%) and 6 *R. pruinosus* (75%). Altogether these findings suggested that the bamboo rats are a natural host and reservoir for *P. marneffei* and bamboo rats can serve as epidemiological markers for *P. marneffei* since the organism can be isolated from three species of bamboo rats in China, Thailand and Vietnam.

The source and route of *P. marneffei* infection are virtually unknown. In 1988, Deng *et al* (21) were able to isolate *P. marneffei* from soil collected from three burrows of the bamboo rats. Therefore, they believed that human and bamboo rats were infected by inhalation of conidia of *P. marneffei* from environmental source or ingestion of food contaminated with the organisms.

The seasonal variation of disseminated *P. marneffei* infection was compared among patients diagnosed between 1991 and 1994 at Chiang Mai University Hospital

(52). It was found that *P. marneffei* infection was more frequent in rainy than dry season of the year. The impressive and consistent seasonal increase of *P. marneffei* infections among HIV-seropositive patients in northern Thailand suggests that there might be an expansion of the environmental reservoirs with favorable conditions for growth of the fungus during the rainy season.

In 1996, Chariyalertsak *et al* (53) studied 75 captured bamboo rat in northern Thailand and found that *P. marneffei* could be isolated from the internal organs of 13 of 14 (92.8%) of large bamboo rat (*R. sumatrensis*) and 3 of 10 reddish-brown small bamboo rats (*C. badius*). All 51 grayish-black *C. badius* were negative on culture. The organism was also isolated from one soil sample collected from the burrow of bamboo rat (*R. sumatrensis*). This finding also supports the speculation that there are a wide range of natural hosts and environmental source of *P. marneffei* which may account for the natural infection of *P. marneffei*.

5. Clinical Manifestations

Doung (5) reviewed the clinical manifestations of 155 patients with penicilliosis marneffei. About 80% of the patients were immunocompromised. In the review, the infections usually manifested by fever, anemia and weight loss. Dissemination of the disease was characterized by skin lesion which appeared on the face, upper trunk, arm and mouth. Generalized lymphadenopathy and cough were commonly observed. Pericarditis, osteolytic lesions, arthritis and retropharyngeal purulent abscess causing upper-air-way obstruction were rare. The non-specific manifestations observed in penicilliosis marneffei cases closely resembled those of

tuberculosis, pneumocystis carrinii pneumonia and other systemic fungal infections. The latter makes diagnosis of *P. marneffei* infection more difficult.

6. Host Immune Response and Pathogenesis

The infection is presumed to originate in the lungs after inhalation of the airborn conidia of *P. marneffei*. It seems likely that attachment of the conidia to bronchioalveolar epithelium is a crucial step in the establishment of initial infection. Hamilton *et al* (54,55) showed that laminin that is an extracellular matrix glycoprotein present in the basement membrane of the lung tissue could bind to the surface of *P. marneffei* conidia. They believed that inflammatory process in the lungs may somehow expose the laminin to the attachment of conidia. Once the conidia colonize the lung tissue, disseminated penicilliosis marneffei can ensue. The histopathological consequence can be granulomatous reaction, suppurative reaction and focal necrosis. The granulomatous reaction occurs most often in bone marrow, liver, spleen and lymph nodes of immunocompetent host. Macrophages from these patients contain large number of yeast cells of *P. marneffei*. The round or oval shaped yeast cells are about 3-4 μm in diameter. The presence of septa and the lacking of budding distinguish the yeasts of *P. marneffei* from those of *H. capsulatum.* The suppurative reaction occurs in the lung, skin and subcutaneous tissue of immunocompetent host. A neutrophilic response is found in this pattern. Focal necrosis with surrounding macrophage containing yeast cells is also frequently seen in immunocompromised host.

Host defense mechanism against other fungal organisms such as *C. albicans, C. neoformans, H. capsulatum* have been investigated and elucidated to a great extent

(56-65). Resistance to these systemic fungal infections is due primarily to a cellular immune response mediated by T cells and macrophages. Moreover, interferon gamma is critical in activating macrophage which can kill the organisms. On the contrary, very little is known about the host defense mechanism against *P. marneffei*. Kudeken *et al* (66,67) established the experimental euthymic and athymic mouse model of chronic pulmonary and disseminated infection to examine the host immune response. They showed that cell-mediated immunity plays crucial role in defense against *P. marneffei* infection. The microorganisms inoculated intratracheally multiplied progressively in the lungs and disseminated to liver and spleen. In euthymic mice, the number of organisms decreased gradually with time. In contrast, congenitally athymic mice developed severe pulmonary and disseminated systemic mycosis. Transferring of spleen cells into athymic mice significantly reduced the number of yeasts in the organs of athymic mice. However, adverse effect of CMI also was found in their study. High dose of inoculation of live *P. marneffei* killed all immunocompetent mice. They demonstrated that CD4+ T cells were the predominant cells in the lungs, and played an important role in hyperinflammatory host reaction because neutralizing anti-CD4 MAbs increased the survival rate in infected mice despite the presence of a high number of live microorganisms in the lungs. Further study was done by Cogliati *et al* (68). They investigated the effect of nitric oxide (NO) and reactive nitrogen intermediate during the *in vitro* growth of *P. marneffei*, both in a cell free system and in a novel macrophage-culture system. Stimulation of interferon gamma production and the present of lipopolysaccharide (LPS) led to enhanced production of reactive nitrogen intermediates, thus resulting a significant reduction of *P. marneffei* viability in macrophages.

There has been no evaluation on the role of humoral immune response in eliminating *P. marneffei* infection. However, the role of humoral immune response in other fungal infections is a controversial subject (63-65). Nevertheless, the antibodies in sera from penicilliosis patients can be used for diagnosis and monitoring of the disease.

7. Laboratory Diagnosis

P. marneffei can be cultured from specimens of blood, bone marrow, skin lesion, liver tissue draining abscess and lymph node. Direct examination can be done microscopically for rapid presumptive diagnosis by staining with Wright's staining or Grocott's methenamine silver or H&E staining of clinical specimens (18,21,25,38). Intracellular and extracellular basophilic, elliptical yeast-like organisms could be seen. However, differentiation between *P. marneffei* and *H. capsulatum* is difficult because of the great resemblance between the two organisms. Hence, a number of presumptive diagnostic methods based on serology and exoantigens tests have been developed and evaluated for their reliability.

Immunodiffusion tests have been developed to diagnose infection caused by various pathogenic fungi. Sekhon *et al* (10) showed that only the extract of *P. marneffei* (6 isolates) reacted positively with rabbit anti-*P. marneffei* serum, giving at last 2 precipitin lines of identity with reference antigen by immunodiffusion test. None of the rabbit antisera made against five species of *Aspergillus*, four systemic fungi and three thermophilic actinomycetes reacted with *P. marneffei* antigens. Using micro-immunodiffusion method, Viviani *et al* (69) found that a patient with penicilliosis

marneffei had *P. marneffei* antibody approximately 2 months after the symptoms appeared and prior to the administration of antifungal therapy.

Immunohistochemical and indirect immunofluorescent antibody technique were also developed for identification of *P. marneffei* in clinical specimens. Estrada and coworker (41) used monoclonal antibody EB-A1, directed to *Aspergillus* galactomannan, to identify *P. marneffei* in tissue section by immunohistochemical technique. Kaufman *et al* (12) used polyclonal rabbit antibody, after appropriate adsorption, to develop indirect immunofluorescent antibody technique to identify *P. marneffei* in culture and clinical specimens.

Diagnosis by Western immunoblotting was also studied by Chongtrakool *et al* (43) who demonstrated that a 38-kDa antigen that is present in culture filtrate prepared from *P. marneffei* culture is specific to this organism. Anti-38-kDa antigen was detected in one-half of the HIV seropositive patients with confirmed penicilliosis marneffei. Vanittanakom *et al* (44) found that individual sera derived from 33 AIDS patients with penicilliosis marneffei reacted with 200, 88, 54 and 50 kDa antigens prepared from the yeast form of *P. marneffei* culture equal to 72.7, 93.9, 60.6 and 57.6% respectively. The 54 and 50 kDa antigens could be strongly detected two months before a definitive diagnosis by fungal culture was made. Recently, Jeavons *et al* (70) found that 86%, 71% and 48% of *P. marneffei* infected patients recognized 61, 54 and 50 kDa antigens respectively.

Recently, specific and sensitive rapid diagnosis based on enzyme-linked immunosorbent assay (ELISA) to detect specific antibodies and antigen in clinical specimens were developed by Cao *et al* (14,15) and Desakorn *et al* (71). In 1998, Cao

et al cloned mannoprotein 1 (MP1) gene from *P. marneffei*. The purified recombinant MP1 protein, found to be specific for *P. marneffei* by testing against guinea pig antisera to *P. marneffei* and other pathogenic fungi, was used to develop enzyme-linked immunosorbent assay (ELISA)-based antibody test. Clinical evaluation revealed that high levels of specific antibody were detected in two immunocompetent penicilliosis patients and approximately 80% (14 of 17) of penicilliosis patients with HIV infection. In their test, no false positive result was found for serum samples from 90 healthy blood donors, 20 patients with typhoid fever and 55 tuberculosis patients. In 1999, the detection of cell wall mannoprotein Mp1p in culture supernatants of *P. marneffei* and in sera of penicilliosis patients by enzyme-linked immunosorbent assay (ELISA) was developed (15) and 65% of penicilliosis patients (17 of 26) were Mp1p antigen test positive. Once the Mp1p antibody test was combined with Mp1p antigen test, the sensitivity of the tests reached 88% (23 of 26), with a positive predictive value of 100% and a negative predictive value of 96%.

A method for the quantitation of *P. marneffei* antigen in urine by fluorescein isothiocyanate-labelled purified rabbit immunoglobulin G in an enzyme-linked immunosorbent assay was developed by Desakorn *et al* (71). Urine samples from 33 culture-proven penicilliosis patients and 300 controls (52 healthy subjects, 248 hospitalized patients without penicilliosis) were tested. All 33 culture-proven penicilliosis patients were positive for antigen with a median titer of 1:20,480. With undiluted urine samples, 67 (27%) of 248 other hospitalized patients and 3 (6%) of 52 healthy controls were positive for urinary antigen. When using a cut-off titer of 1:40, the sensitivity and specificity of the test were 97% and 98%, respectively.

8. Treatment

P. marneffei responds well to currently available antifungal agents including miconazole, itraconazole, ketoconazole, 5-fluorocytosine and Amphotericin B (19,31,72,73,74). The successful treatment of patients depends on how early anti-fungal therapy is initiated. Successful rates to amphotericin B and itraconazole treatment were 77% and 75% respectively (73). However, amphotericin B given over the period of 6 weeks to 2 months gave some undesirable adverse side effects. Treatment with amphotericin B for 2 weeks, followed by a 400 mg/d dosage of itraconazole orally for 10 weeks on the other hand has been proven to be safe and effective and is now recommended as the treatment regimen of choice for disseminated *P. marneffei* infection (74).

9. Molecular Biology Study

The advent of molecular genetic technologies has made possible the comparison of microbial genome organization and has given rise to the science of molecular epidemiology. The availability of these technologies provides investigators with tools to determine genetic relatedness between strains of microbes within a given species rather than just on similarities in physiological and morphological characteristics. A variety of methods has been developed namely, restriction fragment length polymorphism (RFLP) with/without hybridization, random amplified polymorphic DNA (RAPD), electrophoretic karyotyping and DNA sequencing. However, not all genotyping methods are equally effective. Rather, each method has its own set of assets and limitations. For a method to be effective for epidemiological purposes, it must fulfill the following general requirements (75):

1. **Method must be resistance to environmental perturbations and to high-frequency genome organization.** The method must target chromosomal DNA of microbes and must resist to high frequency phenotypic switching. Hence, it can be employed effectively to identify microevolution within an infecting strain.

2. **Data generated should reflect genetic distance.** The method must provide a definite measure that reflects genetic distance between isolates or strains.

3. **Data generated must be relatively stable.** The method should assess primarily DNA sequences that have little recombination and are not highly reorganizational over many generations.

4. **Data generated should be amenable to automated computer-assisted analysis.** The method should generate data that could be stored and could be rapidly accessed by sophisticated computer-assisted systems.

Recently, genotyping methods are frequently applied to a variety of epidemiological problems of the infectious fungi (76-80). They can be used to decipher the complex relationship between commensalism and infection, identify the origin and time of infection or monitor the emergence of drug-resistant strains. In addition, some studies employed genotyping to determine the differences in virulence among isolates. For examples, virulence of *Aspergillus fumigatus* strains investigated by RAPD was performed by Mondon *et al* (76). The investigators used one hundred randomly designed oligonucleotide decamers to amplify the DNA of 34 isolates of *A. fumigatus*. One primer (OPQ6 : GAGCGCCTTG) generated RAPD products that enabled differentiation among *A. fumigatus* groups. Notably, the presence or absence

of a 0.95-kb fragment correlated with the nature of infection (non-invasive and invasive) and immune status of the patients.

As for *P. marneffei*, two molecular typing studies were reported. LoBuglio and Taylor (16) developed oligonucleotide primers from nucleotide sequence unique to this fungus and the phylogeny of *P. marneffei* was established using nucleotide sequences of the nuclear and mitochondrial ribosomal DNA regions. They believed that these sequences can serve as a specific primer for PCR technique for rapid diagnosis of *P. marneffei* in clinical specimens. Another study in Thailand by Vanittanakom and coworker (17) revealed that DNA of *P. marneffei* could be separated by RFLP without hybridization into two DNA types, type I and II, when digested with *Hae*III restriction enzyme.

9.1 Pulsed-field Gel Electrophoresis

The separation of a complex mixture of DNA into different sized fragments by electrophoresis is now a well-established technique. By treating with restriction enzyme, pieces of DNA molecule are small enough to be resolved by electrophoresis in agarose or acrylamide. The upper size limit of DNA in standard gel electrophoresis is not greater than 30-50 kb. In 1984, Schwartz and Cantor (81) described pulsed-field gel electrophoresis (PFGE), a technique which could raise the upper size limit of DNA to over 10 Mb. However, the original PFGE system used in homogeneous electric field, making interpretation of gel profile difficult. With the improvement of instrumentation and methods, a more reproducible and interpretable data could be achieved.

At present, the applications of PFGE are numerous and diverse. These include cloning of large DNA using yeast artificial chromosomes (YAC's) instead of cloning a large number of small fragments of DNA, identifying restriction fragment length polymorphics (RFLP's), construction of physical maps and determining the number and size of chromosome (electrophoretic karyotyping) from yeast, fungi and parasite such as *Leishmania*, *Plasmodium* and *Trypanosoma*.

9.2 Instrumentation

In standard gel electrophoresis (continuous field electrophoresis), DNA with the sizes above 30-50 kb migrate with the same electrophoretic mobility. This is seen as a single large diffuse band. If, however, the DNA is forced to change direction during electrophoresis, different sized fragments within this diffuse band can separate from each other. Therefore, the basis of PFGE is that with each reorientation of the field relative to the gel, smaller sized DNA begins moving in a new direction more quickly than the larger sized DNA because large DNA fragment needs more time to reorient according to the new electric field direction. Hence, the larger DNA fragments lag behind the smaller ones, giving a more clear-cut separation result.

Although many types of PFGE instrumentation are available (Fig. 2), they generally fall into two categories. For the first category, the polarity of the electrodes during electrophoresis is periodically inverted. Field inversion gel electrophoresis (FIGE) (82,83) subjects the DNA to a 180° reorientation, the DNA spends a certain amount of time moving backwards. FIGE has mobility inversions in which larger DNA can move ahead of smaller DNA during electrophoresis.

The other category consists of an instrument that reorients the direction of DNA at small oblique angle, generally between 96° and 120°. This causes DNA to always move forward in a zigzag pattern down the gel. Compared with FIGE, this type of separation is faster and resolves a wider size range. Contour-clamped homogeneous electric field (CHEF) (83,84), transverse alternating field electrophoresis (TAFE) (84), Rotating field gel electrophoresis (RFGE) are examples of other commonly used transverse angle reorientation techniques.

In contrast with FIGE, these systems require both a special gel box with a specific electrode and gel geometry, and the associated electronic control for studying and programming the electrophoresis run. Increasing both the separation range and the resolution of large DNA requires smaller reorientation angles (96°-140°). Smaller angles increase the mobility of the DNA. The lower limit is approximately 96°. TAFE uses a more complicated geometry between the electrodes and vertically placed gel to gel straight lanes, while CHEF and RFGE maintain a homogeneous electric field in combination with horizontal gel. CHEF, moreover, electronically changes the direction of the electric field to reorient the DNA. These changes are made by alternating the polarity of an electrode array. With RFGE the electric field is fixed and the gel is rotated to move the DNA in a new direction.

During PFGE running, several parameters must act in concert. Separation parameters include applied voltage (e.g., 6V/cm); pulse length (e.g., 5/50 sec); reorientation angle (e.g.,120°); buffer (0.5X TBE); type and concentration of agarose (e.g., SeaKem Gold 1.1%); the buffer chamber temperature (e.g., 12°C) and quantity of DNA loaded.

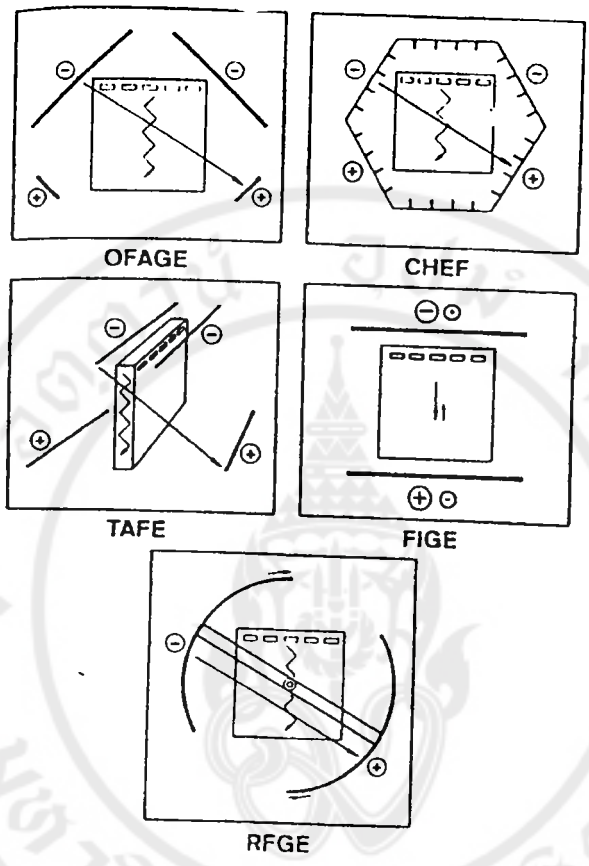

Figure 2 : Schematic diagram of different PFGE systems. The most common PFGE apparatus for Orthogonal Field Alternation Gel Electrophoresis (OFAGE). Contour-clamped Homogeneous Electric Field (CHEF), Transverse Alternating Field Electrophoresis (TAFE), Field-Inversion Gel Electrophoresis (FIGE), and Rotating Field Gel Electrophoresis (RFGE) are shown. The zigzags represents the migration of chromosomal DNA. Electrode geometrics are indicated by anodes (-) and cathodes (+). The long arrows indicate the resulting field vector corresponding to one pulse. (Waltz M.,1995)

Along with the ability to separate large DNA came the need for new sample preparation and handling procedures. Large DNA (e.g., yeast chromosomes) is easily sheared and also difficult to pipet due to its high viscosity. The solution to this problem is to first embed the bacteria or yeast in agarose plugs and then treat the plugs with enzymes to digest away the cell wall and proteins, thus leaving the naked DNA undamaged in the agarose. The plugs then are cut to size, treated with restriction enzymes if necessary, loaded in the sample well, and sealed into place with agarose.

The field strength has a profound effect on pulsed-field separation and is a compromise between separation time and resolution of a particular size class. Four to six V/cm is generally required for resolving DNA up to 2000 kb in a reasonable period of time. However, these field strengths trap and immobilize even bigger DNA in the agarose plug, and DNA greater than 3000 kb requires 2 V/cm or less for separation.

Pulse time primarily changes the size range of separation. Longer pulse time leads to separation of larger DNA. For the reorientation angle, any angle between 96° and 165° produces roughly equivalent separation. The smaller the angle is, however, the faster is the DNA mobility. As for separation of extremely large DNA, 96° to 105° is almost a requirement to get a good separation in the shortest possible time.

The common buffers used in PFGE are TAE (1X TAE : 40 mM Tris acetate, 1 mM EDTA, pH 8.0) and TBE (1X TBE : 89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH8.0). Both are used at a relatively low ionic strength to prevent heating and carry the designation of either 0.25 X or 0.5 X to indicate the dilution relative to

the standard concentration. An added benefit to low ionic strength buffer is an increase in DNA mobility.

For the type of agarose, the fastest mobility and best resolution achieved in gels made of low electroendosmosis (EEO) agarose. Although most standard electrophoresis grades of agarose are suitable for PFGE, agarose with minimal EEO will provide a faster separation. The concentration of agarose affects both the resolution and mobility of DNA. Higher concentrations of agarose yield sharper, but slower moving bands. The typical concentrations used (0.8%-1.2%) represent a tradeoff between speed and resolution. High percentages of low EEO agarose may improve resolution without sacrificing the speed of separation.

Temperature is a factor that affects the DNA mobility. The temperature must be low and constant during the runs. Although higher temperature increase DNA mobility, it does so at the expense of resolution.

9.3 Electrophoretic Karyotyping and Restriction Endonuclease Analysis by PFGE

Nowadays, a large number of yeast and mycelial fungi have been subjected to PFGE for electrophoretic karyotyping (Fig. 3). Using CHEF-DRII (Bio-Rad) with a pulse time of 60 seconds for 15 hours and then 90 seconds for 9 hours at a constant voltage of 200 V at 14°C, at least 11 bands of intact chromosomes from *Saccharomyces cerevisiae* could be visualized (85). Separation of *Paracoccidiodes brasilliensis* chromosomes was archived with 1.4 V/cm, switching at 60 minutes for 68 hours, with the circulating buffer and temperature maintain at 10-12 °C (83). The

isolated chromosomes showed molecular sizes ranging from 3.2 to 10 Mb according to *Schizosaccharomyces pombe* molecular size standard. Three different patterns observed had bands of 3.2, 3.8, 4.1, 5.2, 6.7, 7.2, 8.8, and 10 Mb. Electrophoretic karyotyping of *Histoplasma capsulatum* was successfully analysed using CHEF and FIGE technique (82). Six chromosomal DNA bands of *H. capsulatum* (Downs strain) were resolved by CHEF gel electrophoresis whereas at least five chromosomal DNA bands of this isolate could be resolved by FIGE gel.

In order to give various degrees of discrimination, restriction endonuclease analysis (REA) using rare cutting enzyme was also used in combination with PFGE. *Candida albicans* and *C. parapsilosis* were successfully typing by Riederer *et al* (77). Among three restriction enzymes tested (*Sfi* I, *Sma* I and *BssH* II), digestion by *Sfi* I (running on the CHEF DR-III in 0.8% agarose gel, 0.5X TBE, pulsed time of 120 seconds at 150 V for 16 hours, 300 seconds at 150 V for 3 hours and 300 seconds at 115 V for 16 hours at a 120° angle, constant temperature of 14 °C), yielded the better results for *C. albicans* whereas digestion by *BssH* II (pulsed at 5-35 seconds ramped at 200 V for 24 hours at 120° in 1% agarose and 0.5X TBE), yielded the better results for *C. parapsilosis*. From 14 isolates of *C. albicans*, 7 banding patterns were obtained whereas 6 banding patterns were obtained from 15 isolates of *C. parapsilosis*. Although these investigators found that the results of karyotyping of *C. albicans* and *C. parapsilosis* was better than REA but it falls short of the desired discrimination.

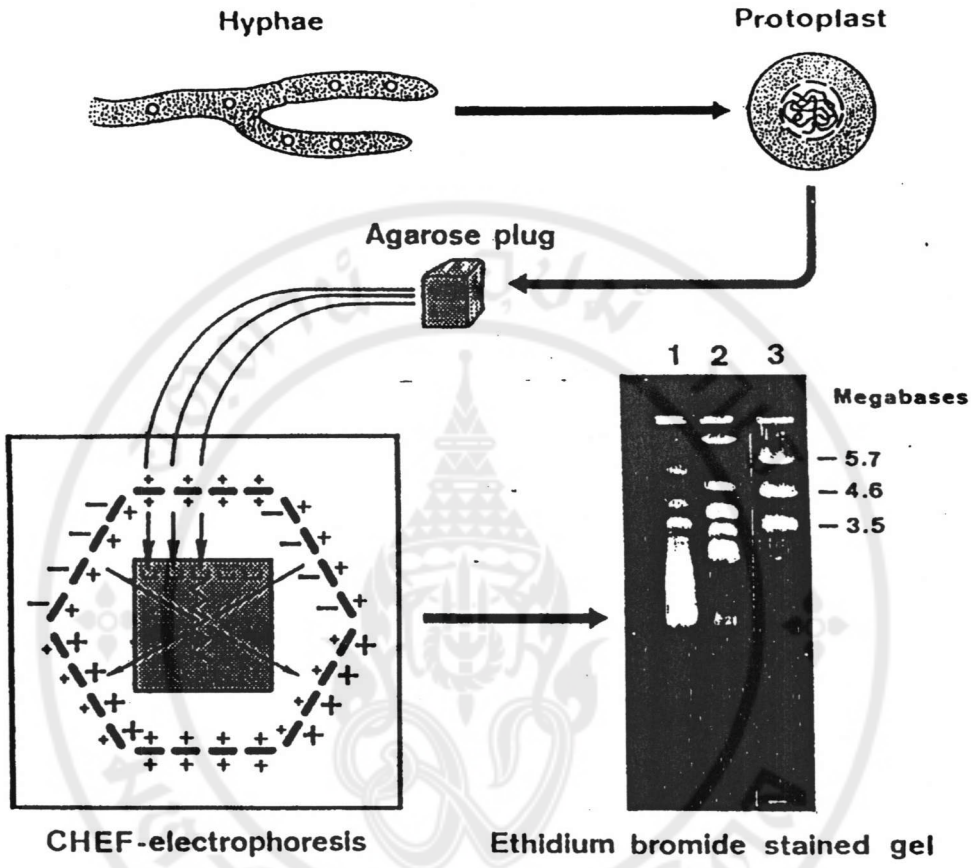

Figure 3 : Preparation of chromosome plugs and PFGE. Sample plugs are embedding protoplasts isolated from fungal hyphae in agarose. After lysis of protoplasts, samples are subjected to PFGE. As an example, separation of chromosomes from *Aspergillus niger* (1), *Acremonium chrysogenum* (2) and *Schizosaccharomyces pombe* (3) by CHEF electrophoresis is shown. Samples were electrophoresed in 0.8% agarose for 96 hours with a pulse of 60 minutes and 72 hours with a pulse of 45 minutes 0.5X TBE (chilled to 14°C) and a voltage of 45 V were used. (Walz M.,1995)

CHAPTER III

MATERIALS AND METHODS

A. Experimental Infection in Mice with *P. marneffei*

Six-week old, female BALB/c mice (National Institute of Health, Ministry of Public Health, Nonthaburi, Thailand) were used in this experiment. One million conidia of *P. marneffei* ATCC 64102 were injected into 2 groups of mice (eight mice per group) via an intraperitoneal (IP) or subcutaneous (SC) route. At different time intervals, i.e. 4, 6, 8, 12 and 16 weeks, one mouse was sacrificed. Internal organs were removed and macroscopically examined for pathological changes. Sera from all mice were collected from each animal and subjected to indirect ELISA and Western immunoblotting assay.

B. Production and Characterization of Monoclonal Antibodies.

1. Fungal Isolates used for immunological study

Four reference strains (ATCC 64102, ATCC 64101, ATCC 18224 and ATCC 24100) strains, 11 local strains of *P. marneffei* and other fungi including *P. chrysogenum* (2 strains) *P. siamensis, P. pinophilum,* one unidentified *Penicillium sp., Aspergillus fumigatus* (3 strains), *A. flavus, A. nidulans, A. niger* (3 strains), *A. terreus*, *Histoplasma capsulatum* (3 strains), *Cryptococcus neoformans* (11 strains) and *Candida albicans* (3 strains) were used in this study. The ATCC reference strains were purchased from the American Type Culture Collection (ATCC-12301 Parklawn Drive, Rockville, MD). *Penicillium sp., Aspergillus sp.*, *H. capsulatum*, *C. albicans*

and *C. neoformans* were obtained from stock culture collection kept at Microbiology Laboratory, Department of Pathology, Faculty of Medicine, Ramathibodi Hospital, Bangkok. *P. siamensis, P. pinophilum* were obtained from Bangkok MIRCEN, Thailand Institute of Science and Technological Research, Bangkok, Thailand.

2. Culture and Maintenance Conditions

All mycelial fungi were grown on Sabouraud dextrose agar (SDA) (Difco, Detroit, MI) under aerobic condition at 25°C. Lyophilized *P. marneffei* ATCC 64102, ATCC 64101, ATCC 18224 and ATCC 24100 were first resuspended in sterile distilled water and inoculated on Emmon 's modification of Sabouraud's dextrose agar (Em-SDA) at 25°C following the ATCC protocol. Subsequent subcultures were done on SDA. All monomorphic yeasts were grown and maintained on SDA under aerobic condition at 37°C. Spore or yeast suspensions in sterile distilled water were kept as stock culture at 4°C , -20°C , and -70°C .

3. Growth Conditions for *P. marneffei*

The organisms were grown and maintained in the mycelial phase on SDA under aerobic condition at 25-30°C. To convert to the yeast phase, the mycelial cultures were transferred to brain heart infusion (BHI) agar (Difco) whose surface was kept moist with a few drops of sterile distilled water. After 5-7 days of incubation at 37°C, the fungal colonies were similarly subcultured onto the moist BHI agar and again incubated at 37°C for another 5-7 days. This procedure was repeated several times. Within 1-2 months of this periodical subculturing, yeast-like colonies could be observed. Under microscopic examination, a conversion to sausage-shaped cells

reproduced by binary fission could be observed. The organisms were thereafter maintained in the yeast phase by subculturing them at 37°C onto the BHI agar supplemented with 10% fetal calf serum (FCS).

4. Preparation of Antigens

4.1 Preparation of Crude Culture Filtrate Antigen (CCF)

4.1.1 Preparation of Inocula

All mycelial fungi were cultured on SDA and incubated at 25°C for 5-7 days. For the slow growers such as *H. capsulatum*, the incubation period was extended to 21 days. Culture filtrate antigen of *P. marneffei* was prepared from the reference *P. marneffei* ATCC 64102. Monomorphic yeasts were grown on SDA while *P. marneffei* yeasts were cultured on BHI. All yeasts were incubated at 37°C for 3 days. To harvest the cells, 3 ml of 0.01% Tween 80 (Sigma) were added to each fully grown culture. Spore (conidia) or yeast suspensions were thoroughly mixed in this solution. A suspension of 10^6 cells/ml was counted using hemacytometer (American Optical, Buffalo, NY). Then 2 ml of 10^6 cells/ml suspension were inoculated to a 500 ml flat bottom-bottle containing 200 ml of SDB. The suspension was finally checked for purity by inoculating on SDA and incubated at 25°C. The cultures were placed in a temperature controlled gyratory shaker (New Brunswick Scientific Co., Inc. Edison, NJ) set at 150 rpm for 6 weeks and incubated at 25-30°C for mycelial fungi or at 37°C for yeasts.

4.1.2 Preparation of Antigens

At the end of the incubation period , the culture was checked for purity by subculturing the inocula on SDA before merthiolate (Sigma) was added to the final concentration of 0.01% and the incubation was continued for another 24 hours before harvesting. The culture fluid collected after filtration through several layers of sterile gauze, Whatman filter paper No.2 and finally 0.45 μm Whatman membrane. The filtrate was precipitated by adding slowly chilled acetone (1:3 v/v, Merck). The suspension was left standing overnight at 4°C. Then, they were centrifuged at 2000 rpm at 4°C for 60 minutes (International Portable Refrigerated Centrifuge model PR-2, IEC, Needham, HTS., MA). The precipitates were collected and left dry at room temperature for a few hours and then resuspended in sterile 0.15 M PBS pH 7.2 (see Appendix) to 1:20 of the original volume. After dialysis against PBS at 4°C and sterilization by 0.45 μm membrane filtration, phenylmethylsulfonylfluoride (PMSF, Sigma) and L-1-tosylamide-2-phenylethyl chloromethyl ketone (TPCK, Sigma) were added to the final concentration of 0.1 mM. The protein concentration of the culture filtrate antigen was determined according to the method of Lowry (86) and kept in small aliquots at –70°C.

4.2 Preparation of Exoantigen (Exo-Ag)

Exoantigen was prepared from the yeast phase of growth. Briefly, yeast-like colonies were incubated at 37°C for 72 hours on BHI agar. The agar surface was then flooded with 50 ml culture medium containing of 10 g glucose, 1 g asparagine, 1.7 g YNB (yeast nitrogen base, Difco) and 20 ml RPMI in 980 ml distilled water and the culture was incubated further at 37°C for another 72-96 hours. The fluid layer was

collected, Millipore filtered, dialyzed and concentrated 10 times using Amicon PM-10 concentrator (Amicon Inc., 72 Cherry Hill Drive, Beverly, MA). Then PMSF and TPCK were added to the final concentration of 0.1 mM. The protein concentration of the exoantigen was determined according to the method of Lowry (86) and kept in small aliquots at –70°C.

5. Protein Determination

Protein concentration was determined by a Folin-Ciocalteau tyrosine method (Lowry) with minor modification (86). In brief, 1 ml of working solution (see Appendix) was added to 0.2 ml of each sample. After 10 minutes of incubation at room temperature, 0.1 ml of 1 N Folin reagent (Sigma) was added and the tube was vigorously shaken. The absorbance at 750 nm was determined for each reaction after 30 minutes incubation at room temperature (Spectronic 1201, Milton Roy Company, Rochester, NY). Distilled water was used as a reagent blank whereas bovine serum albumin (BSA, Sigma) was used as a standard.

6. Production of Monoclonal Antibodies (MAbs)

6.1. Immunization Schedule

Six-week old, female BALB/c mice were used for MAbs production. The immunogen used was a crude culture filtrate of *P. marneffei* ATCC 64102. Two immunization protocols were used in this study.

Protocol I :

Four female BALB/c mice, 6-8 weeks old, were injected subcutaneously with 100 μg of cell culture filtrate antigen from *P. marneffei* ATCC 64102 emulsified in complete Freund's adjuvant (CFA) and followed by 2 doses of 100 μg of the same antigen in incomplete Freund's adjuvant (IFA) at 21-day intervals. Trial bleedings were made at intervals to monitor the antibody levels and the animal that gave satisfactory antibody titer by ELISA was given a prefusion booster by an intravenous route with 50 μg of antigen in PBS and the fusion was performed 4 days later.

Protocol II:

Four BALB/c mice , 6-8 weeks old, were injected subcutaneously with 50 μg of the cell culture filtrate antigen from *P. marneffei* ATCC 64102 in TiterMax (Vaxel Inc., Norcross, GA) and followed by 1 dose of 50 μg of antigen in TiterMax on day 14. On day 35, the mice were injected intraperitoneally with 50 μg of antigen in PBS. On day 36, 50 μg of antigen in PBS was injected intraveneously. Trial bleedings were made at intervals to monitor the antibody levels and the animal that gave satisfactory antibody titer by ELISA was given a prefusion booster by an intravenous route with 50 μg of antigen in PBS and the fusion was performed 4 days later.

6.2. Myeloma Cell Line

Myeloma partners used for fusion were P3.653 (P3X63-Ag8.653) and PAI myeloma cell lines. The P3.653 myeloma cell line was obtained from Laboratory of Immunology, Chulabhorn Research Institute, Bangkok, Thailand. The PAI

myeloma cell line was obtained from Dr. Tadashi Tai (Department of Tumor Immunology, The Tokyo Metropolitan Institute of Medical Science, Japan). Prior to being used, these myelomas were cultured in complete medium containing 8-Azaquanine (see Appendix) and then maintained in complete medium. Culture was incubated in a humidified incubator in the presence of 5% CO_2 at 37°C. Subculturing was performed every 2 or 3 days.

TABLE 1 Immunization protocols for monoclonal antibody production.

Fusion no.	Days of injection	Route	Immunogen † (μg)	Adjuvant	Fusion partner
1	0 21 42 84	SC SC SC IV	100 100 100 50	CFA IFA IFA -	P3.653
2	0 14 35 36	SC SC IP IV	50 50 50 50	TiterMax®* TiterMax® - -	P3.653
3	0 14 35 36	SC SC IP IV	50 50 50 50	TiterMax® TiterMax® - -	PAI

SC = subcutaneous, IP = intraperitoneal, IV = intravenous,

CFA = complete Freund's adjuvant, IFA = incomplete Freund's adjuvant

† Acetone-precipitated culture filtrate

* Vaxcel Inc. (Norcross, GA, USA)

6.3 Fusion Procedure

Hybridomas were prepared by fusing the myelomas and spleen cells from immunized animal using polyethylene glycol (PEG 300-3000, Sigma) as fusogen according to the method of Heddy (87). Before being euthanized by cervical dislocation, the hyperimmune animal was bled from the eye to obtain prefusion immune serum. The animal was then soaked in 70% ethanol and the abdomen was surgically opened. Spleen was aseptically removed and placed in a sterile petri-dish containing cold RPMI 1640 (Gibco BRL, Life Technologies, Inc., Grand Island, MO). Fat was trimmed away as much as possible from the surface of spleen. A single cell suspension was prepared by teasing and chopping the spleen with surgical blades. The spleen cells were washed twice with cold RPMI 1640 and the viable cells were determined by the exclusion of 0.1% trypan blue in PBS.

The fusion was done by mixing approximately 1×10^8 spleen cells with 1×10^7 myeloma cells in a total volume of 20 ml cold RPMI in a 50-ml conical polypropylene tube. These cells were collected by light centrifugation at 200X g for 7 minutes at room temperature. Supernatant was removed by gentle aspiration and residual RPMI was carefully sucked out as much as possible before the pellet was tapped very gently. One milliliter of warm PEG was added dropwise to the pellet over a period of 1 minute. The mixture was then left in a 37°C humidified incubator with 5% CO_2 for one more minutes. One milliliter of warm RPMI 1640 was slowly added over a period of one minute while the tube was kept under light rotation. During the next 4 minutes, 20 ml of warm RPMI were added at ever increasing rate. The cell suspension was then immediately centrifuged at 200X g for 7 minutes at room temperature. The supernatant was discarded and the cells were suspended in 100 ml of

HAT medium (see Appendix). About 0.1 ml aliquots of the cell suspension were plated in eight 96-well tissue culture plates (NUNC, Denmark). Four wells each containing 2 x 10^5 myelomas in HAT medium per plate were left for control. All plates were incubated in 5% CO_2 humidified incubator at 37°C.

6.4 Maintenance and Monitoring of Hybridoma Growth.

Four to five days after fusion, the control wells with $2x10^5$ myelomas were observed for growth. Unfused myeloma cells died within 4-5 days of incubation in the presence of HAT medium while the unfused spleen cells normally died within 7 to 10 days because they are not immortalized. Therefore, only the hybrids could survive. Clones of hybridoma could be detected 4-7 days after the fusion was initiated. After 7-10 days, one half of a total volume of culture supernatant in each well was removed and replaced with an equal volume of fresh HT medium (see Appendix). When the medium turned yellow, the culture supernatants were collected and kept for antibody screening. The HT medium finally replaced the HAT medium in the wells. The hybrids were kept for a few days in this condition before being cultured in a complete medium.

6.5 Screening of Hybridomas for MAbs Production.

Hybridomas producing MAbs reactive with *P. marneffei* antigens were screened by indirect ELISA using native and heat-denatured (in the presence of 1% SDS and 5% 2-mercaptoethanol) crude culture filtrates to coat the microtiter plates (88,89). In brief, 50 µl of the culture filtrates diluted to a final concentration of 25 µg protein/ml with 0.15M PBS pH 7.2 were added to the microtiter wells and incubated at 4°C overnight. The plates were washed three times with PBS-T (0.15M PBS

containing 0.05% Tween20). Then 200 µl of 5% skim milk PBS-T were added to each well. After 1 hour of incubation at 37°C, the plates were washed three time with PBS-T. Then 50 µl of each hybridoma supernatant were added to each well and incubated at 37°C for 2 hours. After washing three times with PBS-T, 50 µl of 1:2000 diluted horseradish peroxidase conjugated goat anti-mouse immunoglobulins (Dako A/S, Copenhagen, Denmark) were added to the wells and incubated for 1 hour at 37°C. After washing three times with PBS-T, 100 µl of freshly prepared substrate solution [10 mg /ml O-phenylenediamine (Sigma) with 0.003% H_2O_2 in 0.1M citrate phosphate buffer pH 5.0] were added to the wells and the plates were incubated in the dark at room temperature for 40 minutes. Enzyme activity was stopped by adding 25 µl of 5N H_2SO_4 to the wells and the color reaction was determined spectrophotometrically at 492 nm by a Titertek Multiskan (Flow laboratories, Bonn, West Germany).

The clones secreting antibodies that cross reacted with other fungal antigens were screened out by indirect ELISA and immunoblotting assays using a panel of fungal antigens prepared from *A. niger, A. flavus, A. fumigatus, A. terreus, A. nidulans, C. neoformans, H. capsulatum, C. albicans* and four other species of *Penicillium* (*P. chrysogenum, P. pinophilum, P. siamensis* and one other *unidentified Penicillium sp.*). Only the clones that reacted specifically with the *Penicillium* antigens were propagated and isotyped, using Mouse Monoclonal-ID Kit (Zymed Laboratories, Inc., San Francisco, CA).

6.6 Single-Cell Cloning by Limiting-dilution Method

The selected hybridomas were transferred to 24-well plates and cloned as soon as possible by a limiting dilution method in order to prevent the loss of desirable hybridomas by the overgrowth of non-secretors. The procedure for single-cell cloning by limiting dilution method consisted of dilution the cell suspension from a selected well until a single cell per well (100 µl) was obtained and the cell was allowed to proliferate in the well containing feeder cells to support the growth of a single hybridoma cell. Briefly, a small volume of hybridoma cell suspension was taken from each positive well and viable cells were counted by staining with 0.1% trypan blue. The cell suspension was serially diluted with complete medium until the proportions of 1 cell/100 µl and 0.5 cell/100 µl were obtained. Each proportion was plated, 100 µl per well, to 60 wells (one plate) containing 100 µl of feeder cell suspension (Section 6.7). The plates were incubated in a 37°C incubator with 5% CO_2. Subsequently, the plates were examined for a single colony growth under microscope 4-5 days later. After 7 to 10 days, only the supernatant from each well containing a single clone was assayed for antibody secretion. The positive well was subjected to a second limiting-dilution to ensure that they were really monoclones. Desirable hybridomas were transferred to a new 24-well plate, rechecked for antibody secretion, expanded and kept by cryopreservation as stock (Section 6.8).

6.7 Feeder Cell Preparation.

Feeder cell culture was normally prepared from peritoneal exudate cells of BALB/c mice. The exudate cells, composing of fibroblasts and macrophages, were obtained by injecting 5 ml of complete medium into peritoneal cavity of the animal

that was euthanized by cervical dislocation and then withdrawing the medium aseptically. The peritoneal macrophages were then suspended in HAT medium to the final volume of 50 ml and were plated in 96-well plates, 100 µl/well. The plates were incubated overnight in a 37°C humidified incubator with 5% CO_2.

6.8 Cryopreservation

The mid-log phase proliferating hybridomas were centrifuged at 200X g for 10 minutes at 4°C. Hybridomas in the supernatant were collected and approximately 1-2 x 10^6 cells were added to 1 ml of cold RPMI medium containing 10% dimethyl sulfoxide (DMSO) and 50% fetal bovine serum (Flow Laboratories). The cells were transferred to cryopreservation vials (NUNC) and slowly frozen to –70°C over a 24-hour period. The cryovials could be kept at –70°C in the freezer for several months. For longer term storage, the cryovials kept at –70°C were transferred to liquid nitrogen tank (-196°C). To thaw the hybridomas, the vial was placed in a 37° C water bath, taking care to avoid contact of the water with the seal area of the vial. The vial was swabbed with 70% ethanol, dried and opened. The cell were washed immediately with RPMI medium and resuspended at approximately 2 x 10^5 cells per ml in complete medium, plated in a 24-well plate and incubated at 37°C humidified incubator with 5%CO_2. Growth could be observed within 2 days later. Those developed to a cell line could be maintained indefinitely. The cryopreservation was done periodically.

6.9 Isotype Analysis

The heavy chain classes and light chain types of MAbs were determined using Mouse Monoclonal-ID Kit (Zymed Laboratories, Inc., San Francisco,

CA). The assay is based on an indirect ELISA principle. Rabbit anti-mouse class and subclass specific antibodies were employed in isotyping the mouse immunoglobulins secreted into the culture supernatant. Peroxidase-labelled goat anti-mouse IgG serves as the signal generating reagent. The procedure was performed as recommended by the instruction. In brief, 50 µl of goat anti-mouse IgGAM were added to the microtiter wells. After incubation the plate at 4°C overnight, the solution were decanted and slapped the plate onto paper towel until dry. Then 200 µl of diluted blocking solutions were added to all wells. After incubation the plate at 37°C for 1 hour, the solutions were decanted and slapped the plate onto paper towel until dry. Next, 50 µl of each MAb were added into the wells of each row and incubated at 37°C for 30 minutes. After the plate was washed 4 times with PBS-T, 50 µl of buffer were added into the wells of first column (blank column) and 50 µl of normal rabbit serum were added into the wells of second column (negative control). Next, 50 µl of subclass specific rabbit anti-mouse immunoglobulins (IgG_1, IgG_{2a}, IgG_{2b}, IgG_3, IgA, IgM, Kappa and Lamda) were added to wells of each column and incubated at 37°C for 30 minutes. After the plate was washed 4 times with PBS-T, 50 µl of diluted HRP-goat anti-rabbit IgG were added into all wells. After 30 minutes of incubation at 37°C, the plate was washed 4 time with PBS-T. Then 100 µl of working substrate solution were added into all wells and incubated at room temperature for 30 minutes. The color reaction was determined spectrophotometrically at 405 nm by a Titertek Multiskan.

6.10 Production and Storage of Monoclonal Antibodies

6.10.1 Cell Culture Production

Production of MAbs by cell culture is a simple method (89). The concentration of the MAbs present in the cell culture supernatant is usually high enough for most assay. In order to produce high-titered supernatant, each culture was allowed to grow for several days and the cells were removed by centrifugation. The supernatant fluid could be kept at 4°C for several months with only a slight reduction of the antibody titer (89). Concentration and partially purification of MAbs was done by precipitation in half-saturated ammonium sulfate solution and subsequently dialyzed against 1X PBS pH 7.2 before storage at –20°C. In brief, equal volume of ice-cold saturated ammonium sulfate solution was added dropwise to the culture supernatant fluid to the final concentration of 50% saturation with continuous gentle agitation overnight at 4°C. The solution was then centrifuged at 2000X g for 30 minutes at 4°C. The precipitate was collected, reconstituted with PBS to a required volume and dialyzed against 6 changes of PBS. MAbs concentration was determined by measuring the absorbance at 280 nm (A_{280}) and calculating using the extinction coefficients for protein concentration. The A_{280} of 1.43 and 1.18 are equal to 1 mg/ml solution of IgG and 1 mg/ml solution of IgM, respectively.

6.10.2 Minifermentor Production

Minifermentor system (miniPERM HDC 5 module) by standard procedures in Heraeus BB6220 incubator (Heraeus Sepatech GmbH, 37520 Osterode/Harz, Germany) equipped with CO_2/O_2 regulator systems and a roller apparatus were used for the *in vitro* production of high concentration of monoclonal

antibodies. The hybridomas were harvested from customary stationary cultures in tissue culture flasks, centrifuged and adjusted to a cell density of 2 x 10^6 cells per ml with complete medium. Five milliliters of the cell suspension was injected into HDC5 production module. The minifermentor was operated on a roller system with a rolling speed in the range of 10 rpm. Since gas exchange is passively achieved through the silicone membrane, high-density culture of hybridoma and enriched monoclonal antibodies were obtained within one to four weeks in a HDC 5 production module. The supernatant was harvested by centrifugation at 600X g for 10 minutes at 4°C and kept at –20°C.

7. Characterization of Monoclonal Antibodies

7.1 Immunofluorescence

Indirect immunofluorescent antibody (IFA) technique was used to reconfirm antibody specificity and to further characterize the MAbs (12). The mycelial and yeast phases of *P. marneffei* and other fungi were smeared and heat-fixed onto poly-L-lysine coated glass slides before adding the MAbs and the reaction was allowed to take place at room temperature for 1 hour. After gently rinsing with 0.15M PBS, 20 µl of 1:50 diluted FITC-conjugated rabbit anti-mouse immunoglobulins (Dako A/S) was added and the slides were incubated in the dark for 45 minutes at room temperature. After washing with PBS, the fluorescent staining was observed under epifluorescent microscope (Nikon LOBOPHOT-2) equipped with a mercury lamp and B-2A filter. Pooled sera from uninfected and *P. marneffei* infected (intraperitoneally) mice used at a dilution of 1:100 served as negative and

positive controls. Additional control slides using irrelevant MAb of the same isotype were made to rule out non-specific binding and autofluorescence.

In some experiments, 7-10 day-old slide cultures of *P. marneffei* and other fungi (e.g., *P. chrysogenum*, *P. siamensis*, *P. pinophilum*, *Penicillium sp.*, *A. fumigatus*, *A. flavus*, *A. nidulans*, *A. niger*, *A. terreus*, and *H. capsulatum*) were employed to study the topological relationship of hyphae, conidiophores and conidia. After incubation, the coverslips were removed, heat-fixed and washed thoroughly before staining.

In order to evaluate immunohistological potential of these MAbs for differential diagnosis of penicilliosis marneffei, particularly in distinguishing it from histoplasmosis and other common fungal infections, simulated infected tissues were prepared. These were done by mixing finely minced human tissue pellets with the tissue-forms of *P.marneffei*, *H. capsulatum*, *C. albicans*, *C. neoformans* or *A. fumigatus* and the mixtures were centrifuged and the pellets were smeared onto poly-L-lysine coated slides. After air-drying, the slides were fixed in 10% formalin, washed in running water and then subjected to IFA.

Paraffin-embedded formalin-fixed tissue sections, kindly provided from Pathology Section, Army Institute of Pathology, Pramongkutklao Medical center, Army Medical Department, Bangkok, were stained to evaluate immunohistological potential of these MAbs for differential diagnosis of penicilliosis marneffei. The tissue sections from patients with other systemic fungal diseases were deparaffinized by incubating the slides overnight at 37°C, after which the slides were placed in 60°C for 1 hour, dipped in xylene (changing 3 times, 5 minutes each) and

followed by 2 changes of absolute ethanol (2 minutes each), 95% ethanol (10 dips), 70% ethanol (10 dips) and rinsed with running water for 5 minutes. Before staining, the slides were washed with 2 changes of Tris-borate saline (TBS) (5 minutes each) and non-specific binding sites were blocked with 3% BSA in TBS for 30 minutes.

7.2 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) was used for analysis of protein components present in fungal antigens according to a modified method of Laemmli (90). The molecular weight (MW) of individual components were also determined using prestained standard molecular weight markers (High range) (Gibco BRL, Life Technologies, Inc., Grand Island, MO).

7.2.1 Reagents and Buffers

a. Polyacrylamide solution : A stock solution of 30% (W/V) acrylamide (Sigma) and 0.8% (W/V) of N-N'-methylene-bis-acrylamide (Sigma) was prepared in distilled water by allowing them to dissolve at room temperature for approximately 2 hours. The solution was then filtered through a Whatman filter paper No.1 and stored at 4 °C in a dark bottle.

b. Sodium dodecyl sulfate (SDS, Sigma) was prepared as a 10% (W/V) stock solution in distilled water and stored at room temperature.

c. Gel buffer for preparing separation gel was a 3.0 M Tris-HCl buffer, pH 8.9 and stored at 4 °C.

d. Gel buffer for preparing stacking gel was a 0.5 M Tris-HCl buffer, pH 6.8 and stored at 4 °C.

e. N,N,N',N'-tetramethylethylenediamine (TEMED, Sigma)

f. Ammonium persulfate (Gibco, BRL) was prepared as a 10% (W/V) solution in distilled water and stored at 4 °C for 1 week.

g. Electrophoresis buffer was composed of 25 mM Trisma-base (Sigma), 192 mM glycine (Sigma) and 0.1% (W/V) SDS with pH adjusted to 8.3 by using 1 N HCl.

7.2.2 Preparation of separation gel and stacking gel

All reagents were prepared as described in the Appendix 2. The separation gel was cast between 2 glass plates using a 10% acrylamide solution in separation gel buffer containing 0.1% (W/V) SDS, 0.05% (V/V) TEMED and 0.05% (W/V) ammonium persulfate. The solution was allowed to polymerize between 2 glass plates at room temperature for at least 1 hour. The stacking gel was prepared with 4% acrylamide solution in stacking gel buffer containing 0.1% (W/V) SDS, 0.025% (V/V) TEMED and 0.05% (W/V) ammonium persulfate and was layered over the separation gel. Comb was gently inserted on top of the stacking gel to make slots for sample application.

7.2.3 Preparation of samples

Samples were reduced and denatured in sample buffer (see Appendix 2) composed of 0.0625 M Tris-HCl pH 6.8, 1% (W/V) SDS, 10% (V/V) glycerol (Sigma), 5% (V/V) 2-mercaptoethanol (Sigma) and 0.001% (W/V)

bromphenol blue (Sigma) (as an indicator dye). Then, the mixture was heated in a boiling water-bath for 3 minutes before loading.

7.2.4 Electrophoresis

Electrophoreses were carried out on a Mini-Protean apparatus (Bio-Rad, Richmond, CA) on 10% polyacrylamide containing 0.1% SDS slab gel (0.75 mm thick) according to the methods of Laemmli (90) and Weber and Osborn (91). Protein markers with a molecular mass ranging from 14 to 200 kDa (Gibco BRL) were used as standard. The samples were electrophoresed at a constant voltage of 100 V for 1 hour 15 minutes. After terminating electrophoresis the distance of dye migration and gel length was measured. Thereafter, the gel was carefully taken out for staining or Western blotting.

7.2.5 Coomassie brilliant blue staining

To visualize the protein bands, the gel was stained and fixed in a 0.2% (W/V) Coomassie brilliant blue R (Sigma) solution containing 7% acetic acid (Merck) and 46.5% methanol (Merck) (see Appendix) for 2 hours. Excess staining was removed by soaking the gel in several changes of destaining solution (see Appendix 2) until the background was clear and specific protein bands could be easily visualized.

7.2.6 Molecular weight determination

A procedure used for molecular weight determination was modified from the methods described by Weber and Osborn (88) and by Davies and Stark (89). The molecular weight of any unknown components could be determined by comparing their electrophoretic mobilities with those of standard protein markers

of known molecular weights. A linear relationship was obtained when the relative mobility of the standard markers were plotted against the logarithmic values of their molecular weight (Fig. 4). The prestained molecular weight markers used in the present study were myosin (200 kDa), phosphorylase b (97.4 kDa), bovine serum albumin (68 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa), β-lactoglobulin (18.4 kDa) and lysozyme (14.3 kDa). The relative mobility of the unknown was calculated according to the following formula :

$$\text{Relative mobility} = \frac{\text{Distance of protein migration}}{\text{Gel length after destaining}} \times \frac{\text{Gel length before staining}}{\text{Distance of dye migration}}$$

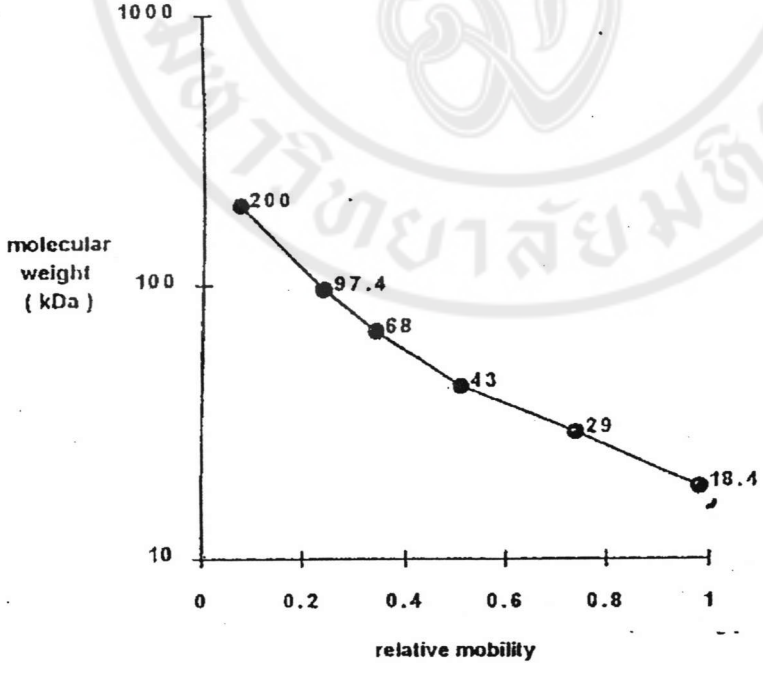

Figure 4: A typical standard curve used for molecular weight estimation of the protein components separated by SDS-PAGE.

7.3 Western Blotting.

The protein components, separated by electrophoresis as described above, were electro-transferred onto a nitrocellulose membrane of 0.45 μm pore size (Scheilder and Schuell BA 85, West Germany) using a transfer electroblotting unit (Bio-Rad Laboratories, Richmond, CA) according to the method of Towbin *et al* (93). The transfer was performed in Towbin's buffer containing 25 mM Trisma base, 192 mM glycine and 20% methanol at constant voltage of 100 V for 1 hour at 4°C. The electrophoretic blots were incubated overnight at 4°C in 5% skim milk in PBS-T (0.15M PBS containing 1% Tween 20) to saturate additional protein binding sites. Then, the membranes were washed three times with PBS-T prior to reacting with MAbs for 3 hours at room temperature with continuous agitation. After incubation, the membranes were washed as above and alkaline phosphatase conjugated goat anti-mouse immunoglobulins diluted 1:1000 in 5% skim milk PBS-T was added and incubated at room temperature for 1 hour. The membranes were washed two times with PBS-T and followed by two additional washing with alkaline phosphate buffer pH 9.5 before the freshly prepared substrate solution [BCIP/NBT Combo (Gibco BRL)] was added. The enzymatic reaction was stopped by washing with distilled water.

7.4 Lectin Binding Assay.

Lectins used in binding assay to determine the carbohydrate nature of antigens in this study included concanavalin A (con A, specific for mannose and glucose) (94), wheat germ agglutinin (WGA, specific for N-acetyl glucosamine and sialic acid) (95) and *Vicia villosa* (VV, specfic for N-acetyl galactosamine) (96). For

the con A staining, the membrane was washed with PBS-T and then incubated overnight at 4°C with 3% BSA in PBS-T to saturate additional lectin binding sites. After incubation, the membrane was washed 3 times with 10 mM Trisma-base buffer pH 8.0 before reacting with 200 µg /ml con A (Sigma) in Trisma-base buffer for 1 hour at room temperature. Then, the membrane was washed 6 times with Trisma-base buffer (15 minutes intervals) and reacted with 100 µg /ml horseradish peroxidase (Sigma) diluted in 3% BSA PBS-T for 2 hours at room temperature. The membrane was washed again 6 times as described above before a substrate solution was added and the enzymatic reaction was stopped by washing with distilled water. For WGA staining, the membrane was washed and additional lectin binding sites were saturated as described above. After incubation, the membrane was washed 3 times with PBS, pH 7.2 before reacting with 1 µg/ml biotin-labelled WGA (Sigma) in PBS for 2 hours at room temperature. The membrane was washed again 6 times with PBS (15 minutes intervals) and reacted with 250 µg/ml of streptavidin-conjugated peroxidase (Sigma) at the dilution 1:1000 in 3%BSA PBS-T. The membrane was washed again 6 times as described above before a substrate solution was added and the enzymatic reaction was stopped by washing with distilled water. For VV staining, the staining procedure was the same as for WGA staining with the exception that a 5 µg/ml biotinylated VV (Sigma) in PBS was used instead of biotinylated WGA solution.

7.5 Periodate Oxidation.

Periodate oxidation was used to determine the carbohydrate nature of the immunoreactive epitope according to the method of Woodward *et al* (97). The nitrocellulose membrane containing transferred proteins was immersed in PBS-T and

incubated overnight at 4°C with 3% BSA PBS-T to saturate additional protein binding sites. After incubation, the membrane was washed 3 times with PBS-T and rinsed with 50 mM sodium acetate buffer pH 4.5. The blot was soaked in 50 mM sodium-m-periodate in sodium acetate buffer for 1 hour in the dark at room temperature. Finally, the membrane was rinsed briefly with sodium acetate buffer and incubated with 1% glycine in PBS pH 7.2 for 30 minutes at room temperature before subjected to immunostaining and lectin binding assay.

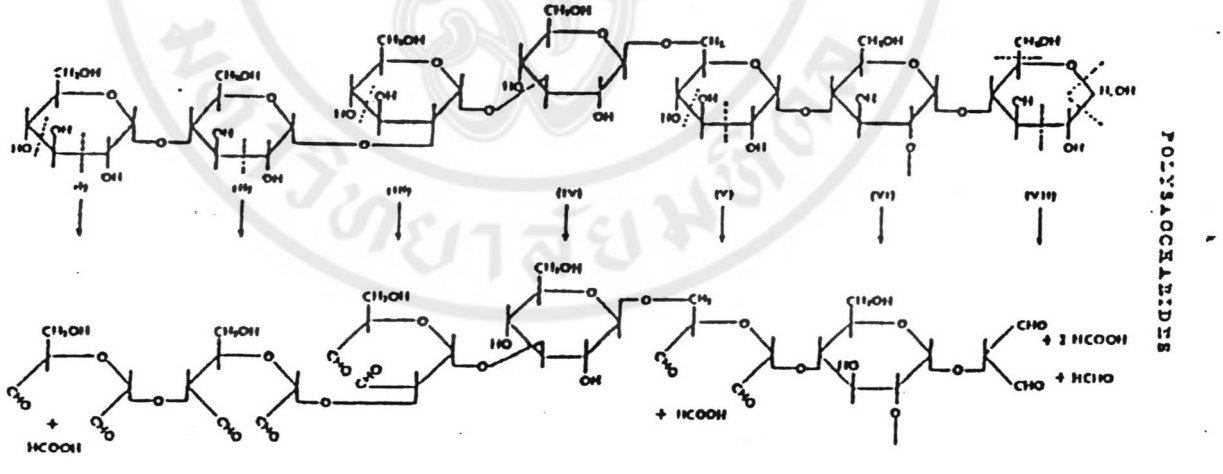

Figure 5: Action of periodate on different types of polysaccharide linkage (98).

C. Genome Fingerprinting by Restriction Endonuclease Digestion and PFGE.

1. Fungal Isolates.

Sixty-two local patient isolates of *P. marneffei,* one Thai bamboo rat isolate and 4 reference strains used for genotypic study are summarized in Table 2. The details of each isolate obtained from different geographical regions and bamboo rats are shown in Tables 3 – 6. All strains of *P. marneffei* were cultured on SDA and incubated at 25°C for 1 to 2 weeks. For DNA preparation, 2 ml of 0.01% Tween 80 (Sigma) in sterile distilled water was added to each fully grown culture. Spores (conidia) were thoroughly mixed in this solution. Approximately 10^6 cells/ml were inoculated into a 500-ml flat bottom-bottle containing 50 ml of SDB. The cultures were incubated at 25°C in a gyratory shaker (New Brunswick Scientific Co., Inc.) set at 160 rpm for 2 days.

Table 2 *P. marneffei* isolates used in this study.

Origin of isolates	No of isolates
Clinical isolates	
-Northern Thailand	14
-Southern Thailand	23
-Central region of Thailand	25
Thai bamboo rat isolate	1
ATCC isolates	4
Total	**67**

Table 3 List of *P. marneffei* isolates from patients in northern Thailand.

No.	Isolates	Hospital	HIV Status	Source	Collection date
1	PM-VV-2	Lanna Medical Laboratory, Chiang Mai	Unknown	Unknown	25/08/95
2	PM-AR-3	Lanna Medical Laboratory, Chiang Mai	Unknown	Blood	18/10/95
3	PM-AR-4	Lanna Medical Laboratory, Chiang Mai	Unknown	Blood	18/10/95
4	PM-AR-5	Lanna Medical Laboratory, Chiang Mai	Unknown	Blood	18/10/95
5	PM-AR-6	Lanna Medical Laboratory, Chiang Mai	Unknown	Blood	18/10/95
6	PM-AR-7	Lanna Medical Laboratory, Chiang Mai	Positive	Blood	16/11/95
7	PM-AR-8	Lanna Medical Laboratory, Chiang Mai	Unknown	Unknown	16/11/95
8.	PM-AR-14	Lanna Medical Laboratory, Chiang Mai	Positive	Blood	unknown
9.	PM-LN-46	Lanna Medical Laboratory, Chiang Mai	Unknown	Unknown	20/05/98
10.	PM-AR-70	Lanna Medical Laboratory, Chiang Mai	Unknown	Unknown	21/10/98
11.	PM-AR-71	Lanna Medical Laboratory, Chiang Mai	Unknown	Unknown	21/10/98
12.	PM-AR-73	Lanna Medical Laboratory, Chiang Mai	Unknown	Bone marrow	08/06/99
13.	PM-AR-74	Lanna Medical Laboratory, Chiang Mai	Unknown	CSF	08/06/99
14.	PM-AR-75	Lanna Medical Laboratory, Chiang Mai	Unknown	CSF	05/09/99

Table 4 List of *P. marneffei* isolates from patients in southern Thailand.

No.	Isolates	Hospital	HIV Status	Source	Collection date
1.	PM-RT-12-1	Prince of Songkla University	Unknown	Skin	Unknown
2.	PM-RT-12-2	Prince of Songkla University	Unknown	Blood	Unknown
3.	PM-RT-12-3	Prince of Songkla University	Unknown	Liver	Unknown
4.	PM-RT-13	Prince of Songkla University	Unknown	Unknown	Unknown
5.	PM-RT-18	Prince of Songkla University	Unknown	Skin	Unknown
6.	PM-RT-25	Prince of Songkla University	Unknown	Unknown	17/05/98
7.	PM-RT-26	Prince of Songkla University	Unknown	Unknown	17/05/98
8.	PM-RT-27	Prince of Songkla University	Unknown	Unknown	17/05/98
9.	PM-RT-28	Prince of Songkla University	Unknown	Unknown	17/05/98
10.	PM-RT-33	Prince of Songkla University	Unknown	Unknown	21/01/98
11.	PM-RT-34	Prince of Songkla University	Unknown	Unknown	23/05/98
12.	PM-RT-45	Prince of Songkla University	Unknown	Unknown	-/08/98
13.	PM-RT-54	Prince of Songkla University	Unknown	Bone marrow	15/06/98
14.	PM-RT-55	Prince of Songkla University	Unknown	Blood	15/06/98
15.	PM-RT-56	Prince of Songkla University	Unknown	Sputum	15/06/98
16.	PM-RT-57	Prince of Songkla University	Unknown	Bone marrow	15/06/98
17.	PM-RT-58	Prince of Songkla University	Unknown	Oral cavity	15/06/98
18.	PM-RT-64	Prince of Songkla University	Unknown	Oral cavity	22/09/98

Table 4 List of *P. marneffei* isolates from patients in southern Thailand (cont).

No.	Isolates	Hospital	HIV Status	Source	Collection date
19.	PM-RT-65	Prince of Songkla University	Unknown	Blood	22/09/98
20.	PM-RT-66	Prince of Songkla University	Unknown	Blood	22/09/98
21.	PM-RT-67	Prince of Songkla University	Unknown	Blood	22/09/98
22.	PM-RT-68	Prince of Songkla University	Unknown	Blood	22/09/98
23.	PM-RT-72	Prince of Songkla University	Unknown	Oral cavity	03/10/98

Table 5 List of *P. marneffei* isolates from patients in central Thailand.

No.	Isolates	Hospital	HIV Status	Source	Collection date
1.	PM-RP-15	Ramathibodi Hospital, Bangkok	Unknown	Unknown	Unknown
2.	PM-RP-16	Ramathibodi Hospital, Bangkok	Unknown	Unknown	Unknown
3.	PM-AC-23	Siriraj Hospital, Bangkok	Positive	Blood	Unknown
4.	PM-AC-24	Siriraj Hospital, Bangkok	Positive	Blood	Unknown
5.	PM-RP-30	Ramathibodi Hospital, Bangkok	Unknown	Unknown	Unknown
6.	PM-SR-35	Siriraj Hospital, Bangkok	Unknown	Unknown	Unknown
7.	PM-N-36	Ramathibodi Hospital, Bangkok	Unknown	Unknown	Unknown
8.	PM-N-37	Ramathibodi Hospital, Bangkok	Unknown	Unknown	Unknown
9.	PM-BE-38	Bamrasnaradura Hospital, Nonthaburi	Unknown	Blood	23/04/98
10.	PM-BE-39	Bamrasnaradura Hospital, Nonthaburi	Unknown	Blood	23/04/98
11.	PM-BE-41	Bamrasnaradura Hospital, Nonthaburi	Unknown	Blood	28/05/98
12.	PM-BE-42	Bamrasnaradura Hospital, Nonthaburi	Unknown	Blood	22/05/98
13.	PM-BE-43	Bamrasnaradura Hospital, Nonthaburi	Unknown	Unknown	12/06/98
14.	PM-BE-44	Bamrasnaradura Hospital, Nonthaburi	Unknown	Unknown	12/06/98
15.	PM-N-47	Ramathibodi Hospital, Bangkok	Unknown	Unknown	Unknown
16.	PM-N-48	Ramathibodi Hospital, Bangkok	Unknown	Unknown	Unknown
17.	PM-N-50	Ramathibodi Hospital, Bangkok	Unknown	Unknown	Unknown
18.	PM-N-51	Ramathibodi Hospital, Bangkok	Unknown	Unknown	Unknown
19.	PM-N-52	Ramathibodi Hospital, Bangkok	Unknown	Unknown	Unknown
20.	PM-RP-53	Ramathibodi Hospital, Bangkok	Unknown	Unknown	15/06/98

Table 5 List of *P. marneffei* isolates from patients in central Thailand. (Cont.)

No.	Isolates	Hospital	HIV Status	Source	Collection date
21.	PM-N-59	Ramathibodi Hospital, Bangkok	Unknown	Unknown	Unknown
22.	PM-BE-60	Bamrasnaradura Hospital, Nonthaburi	Unknown	Blood	14/08/98
23.	PM-BE-61	Bamrasnaradura Hospital, Nonthaburi	Unknown	Lymph node	14/08/98
24.	PM-BE-62	Bamrasnaradura Hospital, Nonthaburi	Unknown	Blood	14/08/98
25.	PM-BE-63	Bamrasnaradura Hospital, Nonthaburi	Unknown	Blood	14/08/98
26.	PM-BE-69	Bamrasnaradura Hospital, Nonthaburi	Unknown	Blood	16/10/98
27.	PM-MK-76	Pramongkutklao Hospital, Bangkok	Unknown	Blood	31/12/99

Table 6 List of *P. marneffei* isolates from bamboo rat and ATCC.

No.	Isolate	Country	Source	Year of collection
1.	PM-BR-49	Thailand	Bamboo rat lung	1987
2.	ATCC 18224	Vietnam	Bamboo rat liver (*R. sinensis*)	1956
3.	ATCC 64102	China	Bamboo rat lung (*R. pruinosus*)	1986
4.	ATCC 24100	U.S.A.	Human spleen (having history of working in SEA)	1973
5.	ATCC 64101	China	Human	1985

2. Preparation of DNA.

Forty milligrams of fungal mycelia were washed three times with 50 mM EDTA and suspended in 1 ml of cell wall-lytic buffer containing 125 mM EDTA, 50 mM sodium citrate, 25 µg/ml chitinase (Sigma) and 200 units/ml lyticase (Sigma). The mycelial suspension was mixed with low-melting point agarose (SEA PLAQUE, FMC Bioproducts, Rockland, ME) to the final concentration of 1.0 % and the mixture was immediately pipetted into mold chamber to make agarose plugs. The plugs were incubated twice in a buffer containing 0.4 M EDTA, 50 mM sodium citrate supplemented with 1% 2-mercaptoethanol for 24 hours at 37°C. The plugs were washed three times with 50 mM EDTA before the cells and protoplasts in the plugs were lyzed and proteins were degraded with a buffer containing 0.5 M EDTA, 10 mM Tris-HCl, 1% N-sodium lauroylsarcosine and 2 mg/ml proteinase K for 24 hours at 50°C. The plugs were then washed three times with 50 mM EDTA and stored in EDTA solution at 4°C until use.

3. Restriction Endonuclease Digestion.

Prior to restriction digestion, the plugs were treated twice with 1 ml of TE buffer (10 mM Tris-HCl, 0.1 mM EDTA) containing 1 mM PMSF (Sigma) for 1 hour at room temperature with gentle agitation. The plugs were treated with TE buffer for 1 hour at room temperature and then incubated with 300 µl of appropriate digestion buffer for 2 hours. Digestions were performed by incubating the plugs with genome-grade restriction enzymes (10-20 units/1 µg of DNA) suspended in 100 µl digestion buffer. Incubation was done overnight at the optimum temperature for digestions. The following restriction

endonucleases were used: *Ase* I, *Bfa* I, *BamH* I, *Spe* I, *Sac* II, *Xba* I, *BssH* II (Biolabs, Beverly, MA), *Xho* I, *Nhe* I, *Sma* I, *Sal* I, *Bgl* I, *Sfi* I, *Sgf* I, *Csp* I, I-*Ppo* I and *Not* I (Promega, Madison, WI).

4. General Electrophoretic Condition and Image Analysis

Gel consisting of 1.0 % SeaKem Gold agarose (SeaKem Gold, FMC Bioproducts, Rockland, ME), 1 cm thick, was cast in 14 cm x 13 cm casting stand with the wells formed by a plastic comb in 0.5X TBE buffer (10.3 g. Trisma base, 5.5 g. boric acid and 0.93 g. disodium EDTA per litre). DNA markers and fungal plugs were inserted in the wells and sealed with 1% low-melting-point agarose (SEA PLAQUE). Pulsed-field gel electrophoresis (PFGE) of the digested DNA were performed by the contour-clamped homogeneous electric field method on a CHEF-DRIII system (Bio-Rad Laboratories) under predetermined condition (22-40 hours at a temperature of 12°C, 120° constant angle, 6 V/cm and with ramped pulsed time of 10 to 50 seconds). The DNA size marker used was a bacteriophage λ ladder consisting of concatemers starting at 48.5 kbp (Bio-Rad Laboratories). The gel was stained with 0.5 μg/ml ethidium bromide in 0.5X TBE and photographed using a UV transilluminator (Vilber Lourmat, 77202 Marne-Lavallee, France, 280 nm).

5. Macrorestriction Analysis

DNA banding patterns of digested DNA from each isolate were compared for similarity by Dice coefficient (99) and cluster analysis was performed by the unweighted pair group matching band average (UPGMA) and displayed as a dendogram with the aid

of BIO-PROFIL (Vilber Lourmat, Marne-Lavallee, France). In brief, the location of the band is considered with ± around its value in Kb where was a percentage directly read on the drawn curve of the marker (Fig. 6).

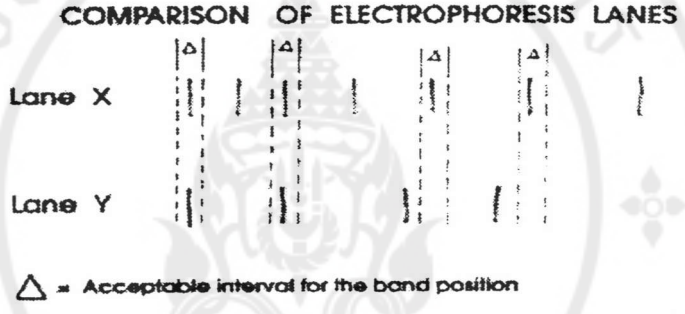

Figure 6: Drawing of the comparison of electrophoresis lanes by Dice coefficient calculation.

The equation of Dice coefficient was indicated below:

$$\text{Coeff} : b = nxy/(nx + ny - nxy)$$

Where nx and ny are the number of bands in lane "x" and in lane "y" respectively, and nxy the number of shared bands between the 2 lanes.

After the comparison, the dendrogram was calculated from the similarity value in the matrix and using the UPGMA algorithm (Unweighted Paired-Group Method) (Fig.

7). The bigger the coefficient was the higher the number of matching bands was found, and conversely.

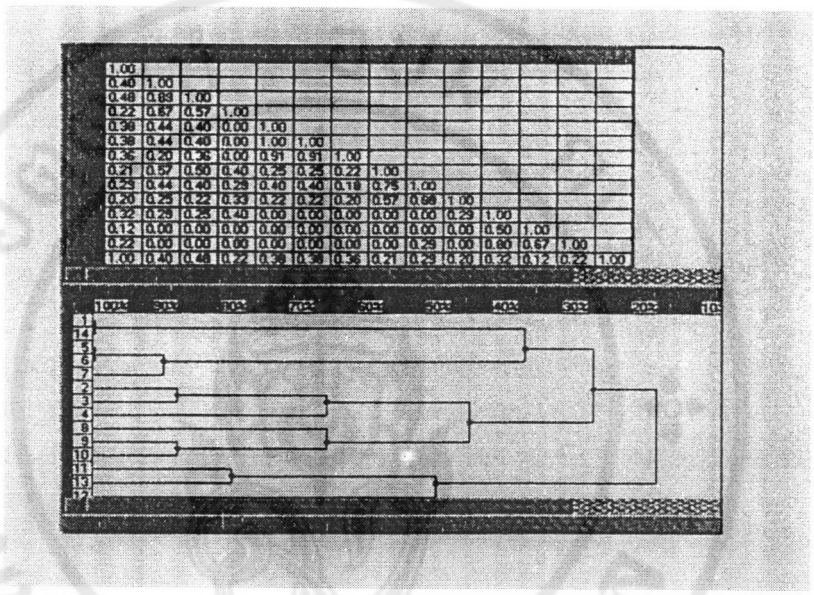

Figure 7: Drawing of comparison matrix between the lanes (above) and the corresponding dendrogram.

6. Restriction Endonuclease Analysis Using *Hae*III Restriction Enzyme.

Restriction fragment length polymorphism (RFLP) of *P. marneffei* using *Hae*III restriction enzyme was performed by the method previously described by Vanittanakom *et al* with some modification (17). In brief, 40 mg of fungal mycelia were washed three times with 50 mM EDTA and suspended in 1 ml of cell wall-lytic buffer containing 0.6M KCl, 25 μg/ml chitinase (Sigma) and 200 units/ml lyticase (Sigma) and incubated the mixture at room temperature for 2 hours. After centrifugation

at 1000X g for 2 minutes, the protoplasts were collected and resuspended in 1 ml of lysis buffer containing 50 mM Tris-HCl, 100 mM EDTA, 0.5% SDS and 0.3M sodium acetate. After incubation at 65°C for 30 minutes, an equal volume of phenol/chloroform/isoamyl alcohol was added and centrifuged at 1600X g for 10 minutes. The upper aqueous phase was transferred to a new microfuge and an equal volume of isopropanol was added with gentle mixing. After centrifugation at 2000X g for 5 minutes, the pellet of DNA was washed with 70% ethanol, centrifuged and left air dry. Finally, the pellet was resuspended with TE buffer containing RNaseA (50 μg/ml).

Digestion reactions were performed in a total volume of 20 μl of digestion reaction mixture containing 3 μg of DNA and 10 units of *Hae*III restriction enzyme (1 μl), 0.2 μl of 100X BSA, 2 μl of 20X restriction buffer and 16.8 μl of distilled water. After incubation at for 1 hour, electrophoresis was carried out for 3 hours at 3V/cm in a horizontal gel containing 1% agarose.

CHAPTER IV

RESULTS

A. Experimental Infection in Mice with *P. marneffei.*

1. Immune Response in Mice Experimentally Infected with *P. marneffei*

Immune response in mice experimentally infected with *P. marneffei* was studied as described in the Materials and Methods (Section 6). After injection of 1 x 10^6 conidia of *P. marneffei* ATCC 64102 into 2 groups of mice via intraperitoneal (IP) or subcutaneous (SC) routes, each mouse developed an abscess at the injection site within one month (Fig. 8). Each month, one animal was sacrificed and the internal organs of both groups of mice were also investigated for pathological lesions one month after the initial injection. In the mice infected via the intraperitoneum, splenomegaly to be relatively common was found (Fig. 9). There were also a large number of abscesses found on the intestine, liver (Fig. 9, IC) and spleen (Fig. 9, IIC). Samples taken from these organs were all culture positive for *P. marneffei*. In every mouse infected subcutaneously, only a slight splenomegaly, with no abscess was found in other internal organs (Fig. 9, IIB). Only in the pus from the abscess formed at the injection site gave positive culture results for *P. marneffei*.

The levels of antibody production against *P. marneffei* were determined by indirect ELISA and Western blotting using CCF. Marked elevation of antibody titers was detected within 4 weeks after intraperitoneal challenge comparing to 2 months for

the SC group (Fig. 10) Antibody titer in the group receiving IP infection (1:625 – 1:3,125) was higher than that of the SC group (1:125 – 1:625). The immunoblot pattern looked similar in both groups (Fig. 11). The dominant bands were 38,40, and 97 kDa position.

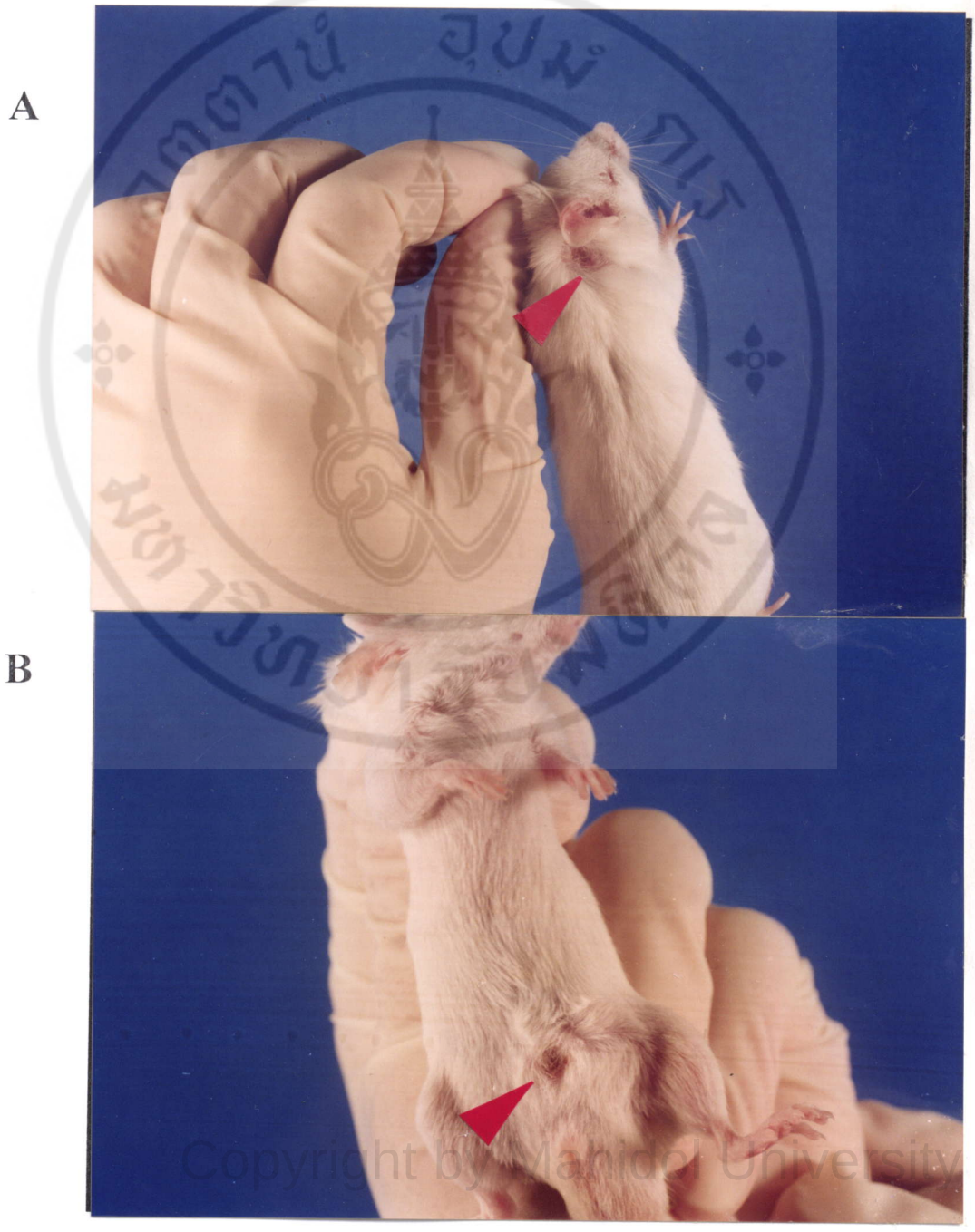

Figure 8: Lesions developed at the injection site of SC (A) and IP (B) groups of mice.

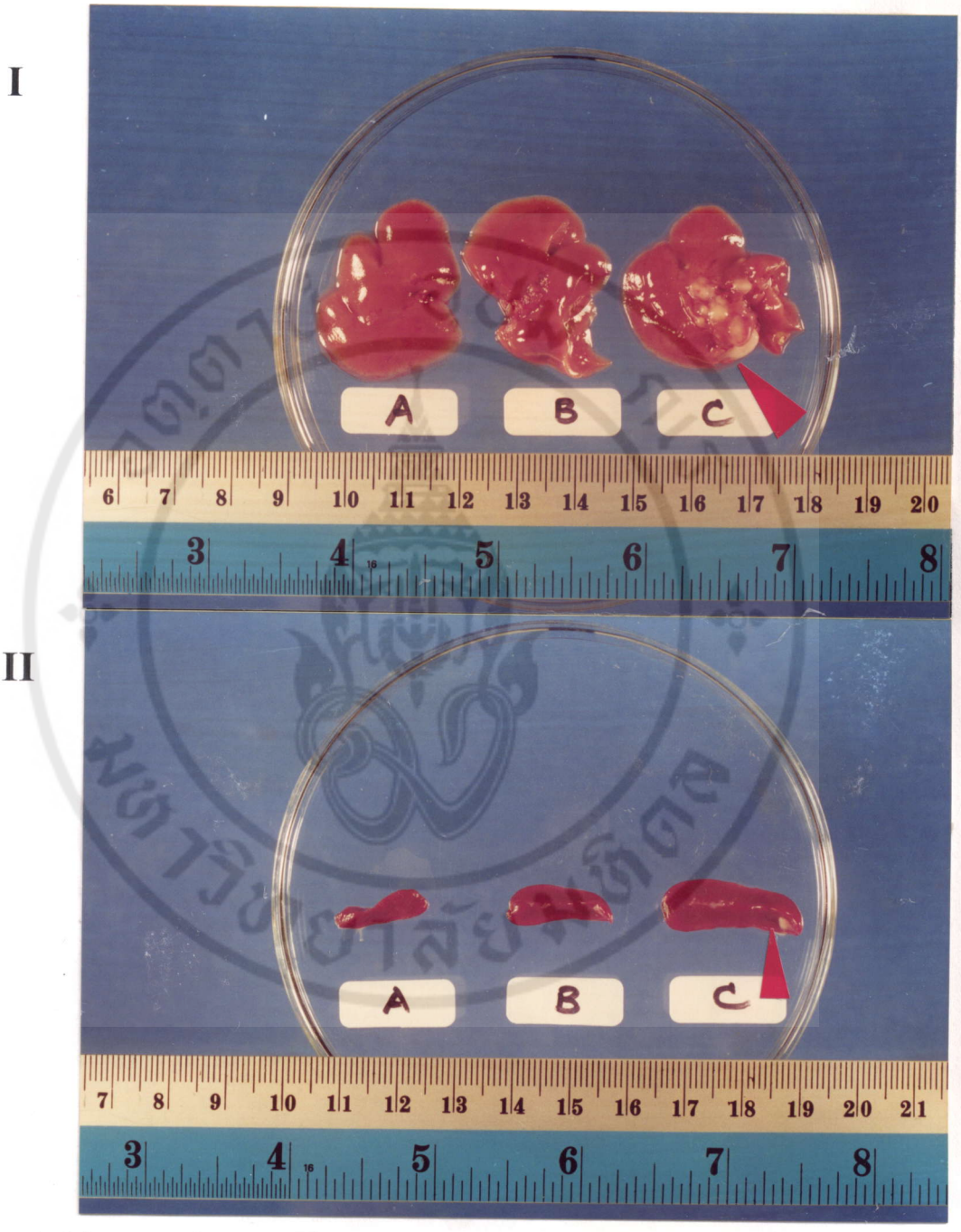

Figure 9: Macroscopic appearances of livers (I) and spleens (II) of BALB/c mice.

I: Several abscesses can be seen on the liver of an IP mouse (C) compared to the livers of a normal mouse (A) and a SC mouse (B).

II: The spleen obtained from the same IP mouse was markedly enlarged with an abscess (C) compared to the spleens of the normal mouse (A) and the SC mouse (B).

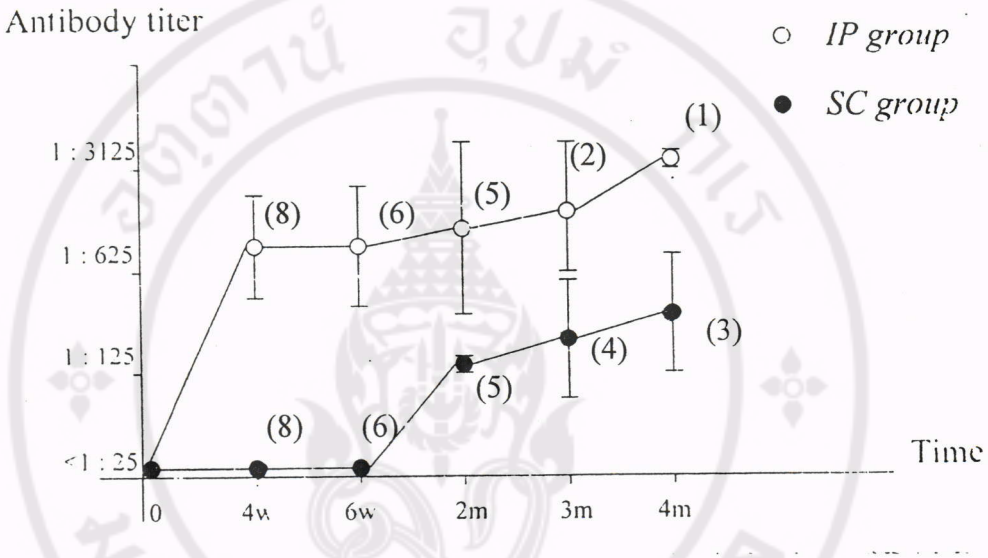

Figure 10: Immune response of BALB/c mice infected with *P. marneffei.* The levels of antibody against *P. marneffei* were determined by ELISA using CCF as antigen. The results shown represent mean and SD of the antibody titer at different time intervals. The number of blood specimens at each point is shown in parenthesis.

Figure 11: Immunoblot patterns of CCF reacting with sera from infected mice.

A-H represent the reactions of fungal antigens with sera from the mice infected by intraperitoneal route, collected at 4 weeks after infections (left lanes), compared with corresponding sera collected from the same animals before the injection (right lanes).

I-M represent the reactions of fungal antigens with sera from the mice infected by subcutaneous route, collected at 2 months after infections (left lanes), compared with corresponding sera collected from the same animals before the injection (right lanes).

+ and – represent the positive and negative controls, respectively

B. Production and Characterization of Monoclonal Antibodies.

1. *Penicillium marneffei* Antigens

P. marneffei antigens used in the study were *P. marneffei* CCF prepared from the mycelial phase of *P. marneffei* and Exo-Ag prepared from the yeast phase of this organism. Both antigen preparations were characterized by SDS-PAGE and the protein bands were stained with Coomassie brilliant blue as well as lectins.

Figure 12 depicted many different protein components present in CCF and Exo-Ag after staining with Coomassie brilliant blue. Many faint bands in both antigenic preparations were observed. The distinct bands observed in CCF (Fig. 12A) and Exo-Ag (Fig. 12B) ranged between 29-97 kDa. Proteints with molecular weight less than 29 kDa and those with higher molecular weight greater than 97 kDa stained weakly with Coomassie brilliant blue. The most prominent band at 68 kDa position was observed in the Exo-Ag. As most fungal antigens are highly glycosylated, the carbohydrate parts or glycoprotein properties of the components present in both antigen preparations were demonstrated by staining with Con A, WGA and VV. Staining of CCF and Exo-Ag with Con A (Fig. 13, A&B) yielded the strongest staining. High molecular weight molecules (between 29-200 kDa) bound more strongly than those low molecular weight glycoproteins (lower than 29 kDa). For CCF antigens, smeary pattern was observed around 200 kDa position. Staining of CCF with WGA (Fig. 13C) yielded faint staining whereas staining of Exo-Ag with WGA (Fig.

13D) yielded very poor staining. In contrast, staining of CCF and Exo-Ag with VV (Fig. 13, E&F) yielded very poor staining in both antigens.

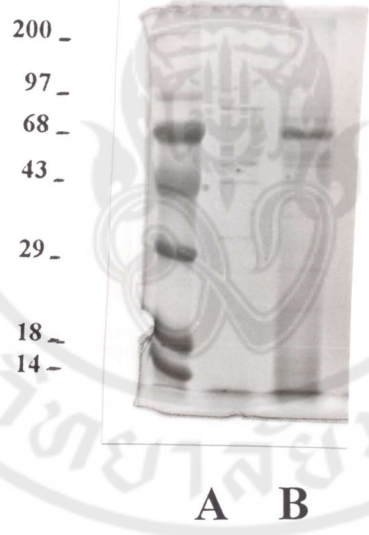

Figure 12: SDS-PAGE analysis of protein components present in CCF (A) and Exo-Ag (B) after staining with Coomassie brillient blue. The numbers on the left are the size of protein markers (in kilodalton).

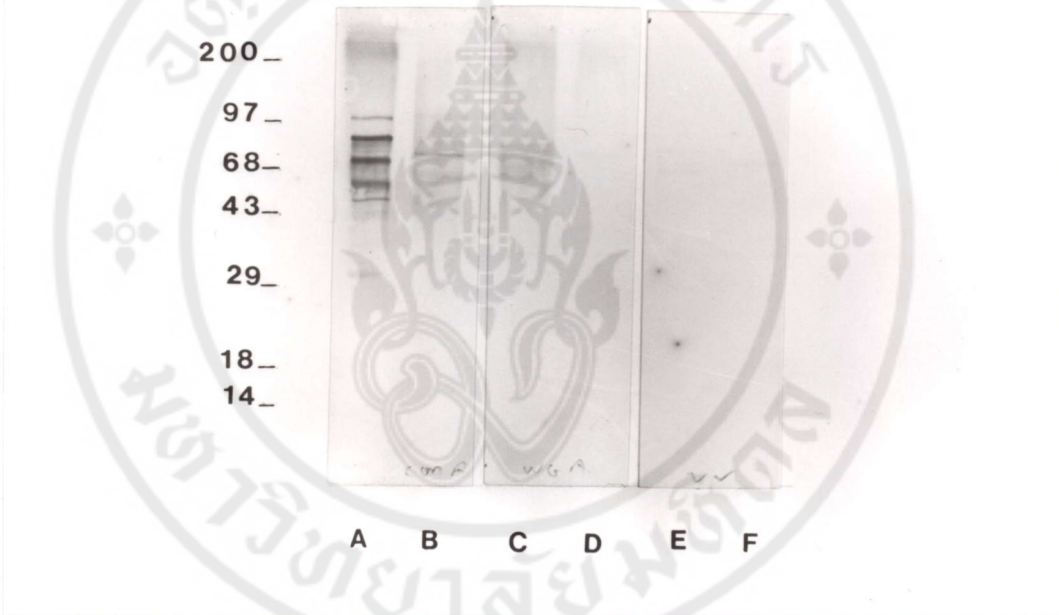

Figure 13: Lectin binding assay for the determination of the nature of carbohydrate parts in the components present in CCF (A, C, E) and Exo-Ag (B, D, F). After SDS-PAGE and Western blotting, nitrocellulose membrane were stained with ConA (A&B), with WGA (C&D) and with VV (E&F). The numbers on the left are the size of protein markers (in kilodalton).

2. Production of Monoclonal Antibodies

Screening and identification of hybridoma secreting specific antibodies against *P. marneffei* were done by using indirect ELISA. Both native and denatured CCF at a concentration of 25 μg/ml were used to coat the wells of microtiter plates. Supernatant from 465 wells with growing hybridomas obtained from the first fusion (showing 96.88% fusion efficiency) were tested. The positive hybridomas were cloned immediately and the supernatant from each clone was further characterized. Only one hybridoma clone, namely 3C2, was obtained. It reacted strongly with denatured CCF and Exo-Ag by ELISA. The optical density (O.D.) value was higher than 0.8. By immunoblotting analysis, MAb produced from hybridoma clone 3C2 reacted strongly with protein components presented in both CCF and Exo-Ag at a 38-40 kDa position (Fig. 14, G&H). MAb 3C2 did not react with either mycelial or yeast phase of *P. marneffei* when it was tested by immunofluorescent staining.

In the second fusion, the fusion efficiency was 91.88%. Supernatant from 441 wells with hybridomas were tested. Four positive hybridomas, namely 8C3, 9B9, 8E8 and 9C11, were obtained. However, only one hybridoma clones, namely 8C3, was chosen for further characterization. The supernatant from this clone reacted poorly with native CCF by ELISA. The O.D. value was closed to 0.2. MAbs produced from hybridoma clone 8C3 did not react with denatured form of CCF. The O.D. value was lower than 0.2. Testing with Exo-Ag by ELISA, MAb 8C3 showed moderate reaction with both native and denatured form. The O.D. values were 0.6 and 0.4, respectively. For immunoblotting analysis, MAb 8C3 reacted with protein components in CCF at the 68, 74, 79, (97) kDa position (Fig. 14E) and those in Exo-Ag at the 51, 60, 62, 76,

108 kDa position. However, the components, ranging in sizes from 45 – 200 kDa, exhibited smearing pattern (Fig. 14F). For immunofluorescent staining, MAb 8C3 showed strong reaction with yeast phase and the tip of newly germinating hyphae of *P. marneffei* (Fig. 15). Fluorescent staining of the older hyphae or the hyphae and conidia prepared from slide culture showed negative staining with the MAb 8C3 (Fig. 16) but positive staining of few hyphae could be observed. In contrast, fluorescent staining of *P. marneffei* yeast cells from the culture that were kept over 2-week period was still positive with MAb 8C3.

In the third fusion, the fusion efficiency was only 40.80%. Supernatant from 196 wells with growing hybridomas were tested. Nine hybridoma clones, namely 8B11, 3B9, 8B6, 1C7, 6E4, 1F3, 3C9, 8F4 and 9F3, were obtained. However, two hybridoma clones, namely 3B9 and 8B11, were chosen. The supernatants from hybridoma clone 8B11 reacted with both native and denatured form of CCF by ELISA. The O.D. values were >0.8 and 0.6, respectively. The MAbs produced from hybridoma clone 3B9 also reacted with both native and denatured form of CCF by ELISA. The O.D. values were 0.6 and 0.4, respectively. Testing with Exo-Ag by ELISA, the supernatant from both clones showed poor reactivity with both native and denatured form of antigens. For immunoblotting analysis, MAb 8B11 reacted with protein components in CCF at 79 kDa and greater than 200 kDa positions in smearing pattern (Fig. 14A) and reacted with those in Exo-Ag at the sizes greater than 79 kDa and also in smearing pattern (Fig. 14B). MAb 3B9 reacted with protein components in CCF at 68, 79 (97) and greater than 200 (smear) kDa position (Fig. 14C) and reacted

with protein components in Exo-Ag with the sizes greater than 77 kDa in smearing pattern (Fig. 14D). Both clones showed strong reaction with yeast phase (Fig. 17), newly germinating hyphae and hyphae of *P. marneffei* (Fig. 18). Conidia were negatively stained with both MAbs 8B11 and 3B9.

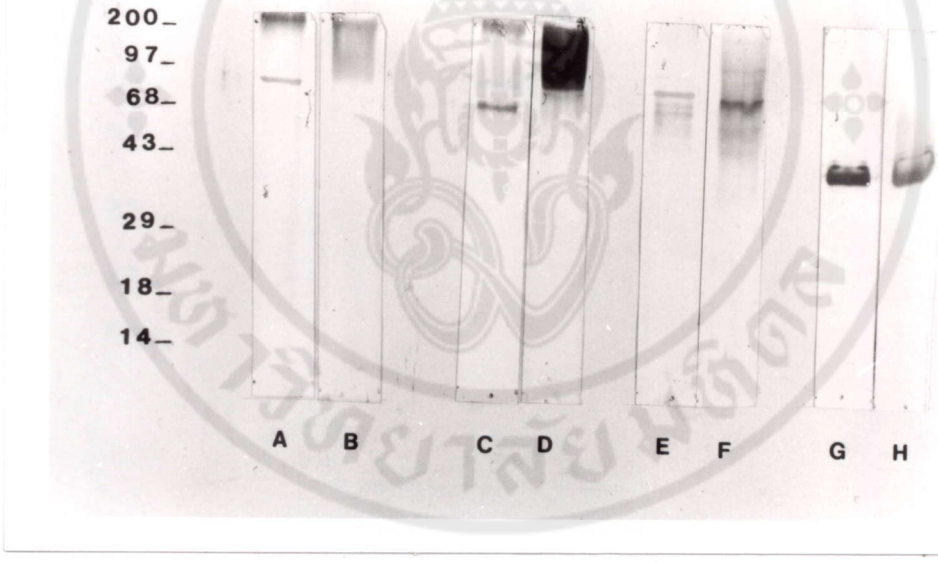

Figure 14: Immunoblotting analysis of four MAbs reacted with CCF (lane A, C, E&G) and Exo-Ag (lane B, D, E&H). The reaction was probed with 8B11 (lane A&B), 3B9 (lane C&D), 8C3 (lane E&F) and 3C2 (lane G&H). The numbers on the left are the size of protein markers (in kilodalton).

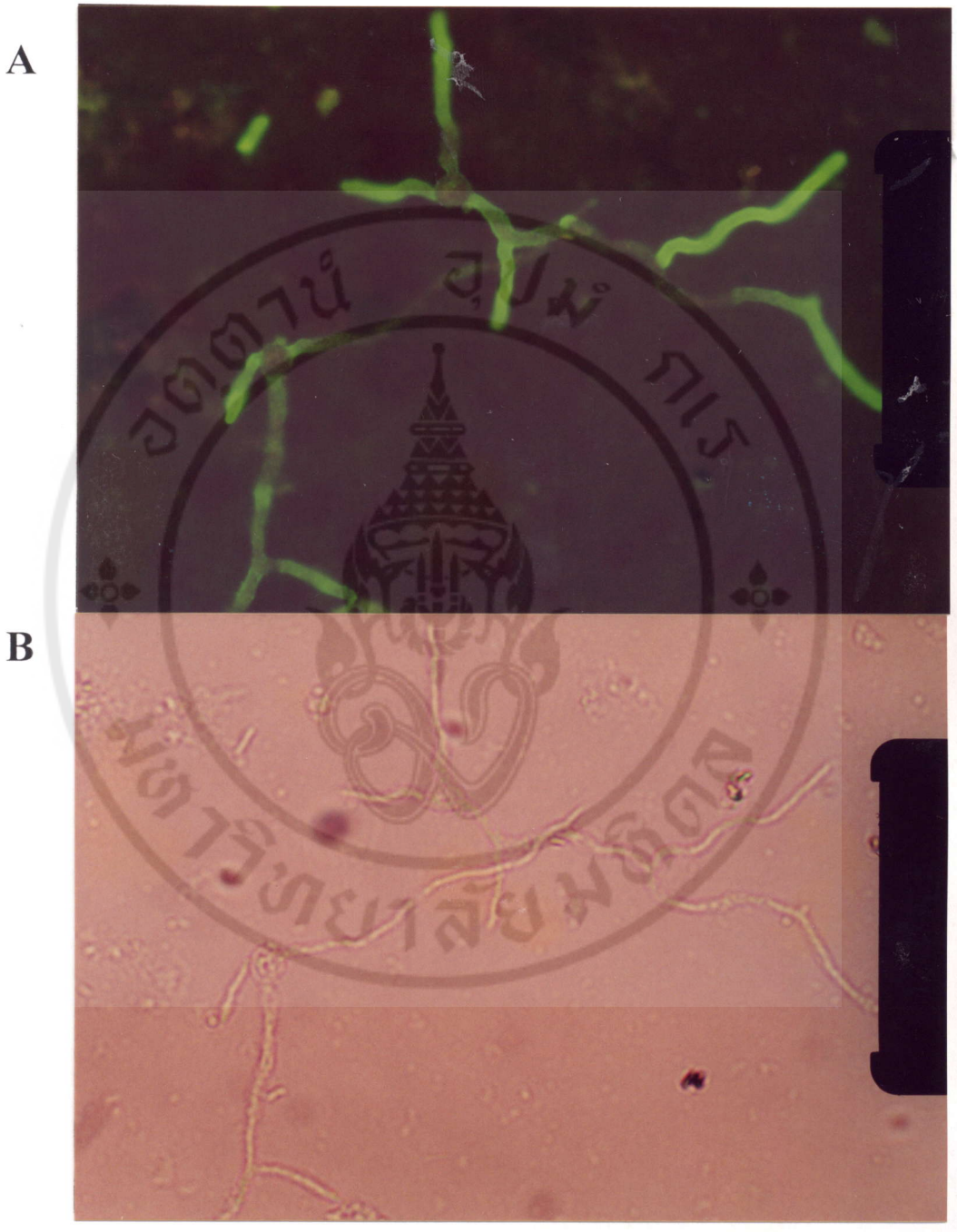

Figure 15: Immunofluorescent staining of *P. marneffei* hyphae (1-day old) with MAb 8C3 (A). The tips of newly germinating hyphae fluoresced strongly. Light microscopic image of the corresponding field is shown for comparison (B). (400X)

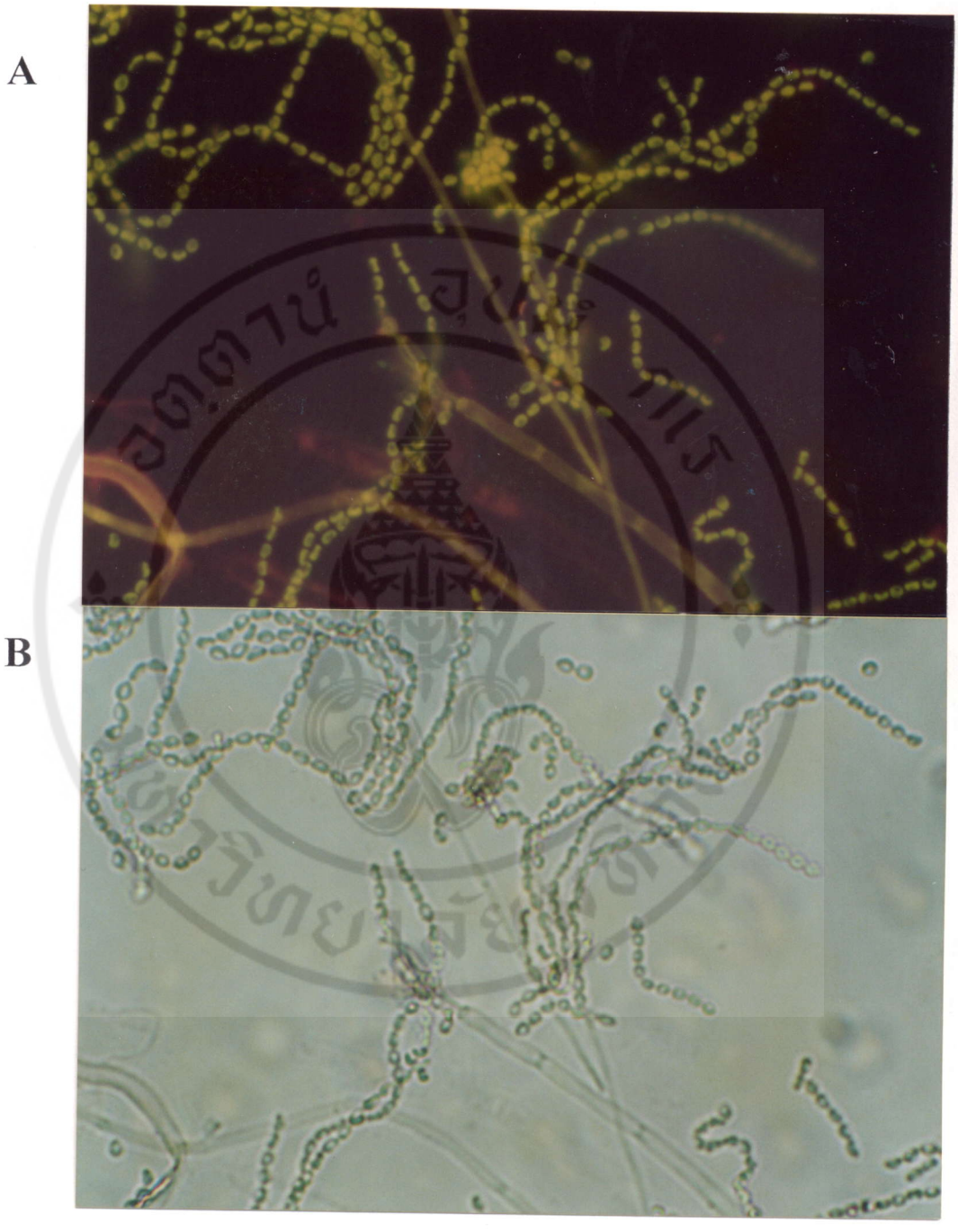

Figure 16: Immunofluorescent staining of the hyphae and conidia of *P. marneffei* prepared from slide culture with MAb 8C3 (A). The yellow staining due to autofluorescence of the fungus can be readily observed. Light microscopic image of the corresponding field is shown for comparison (B). (400X)

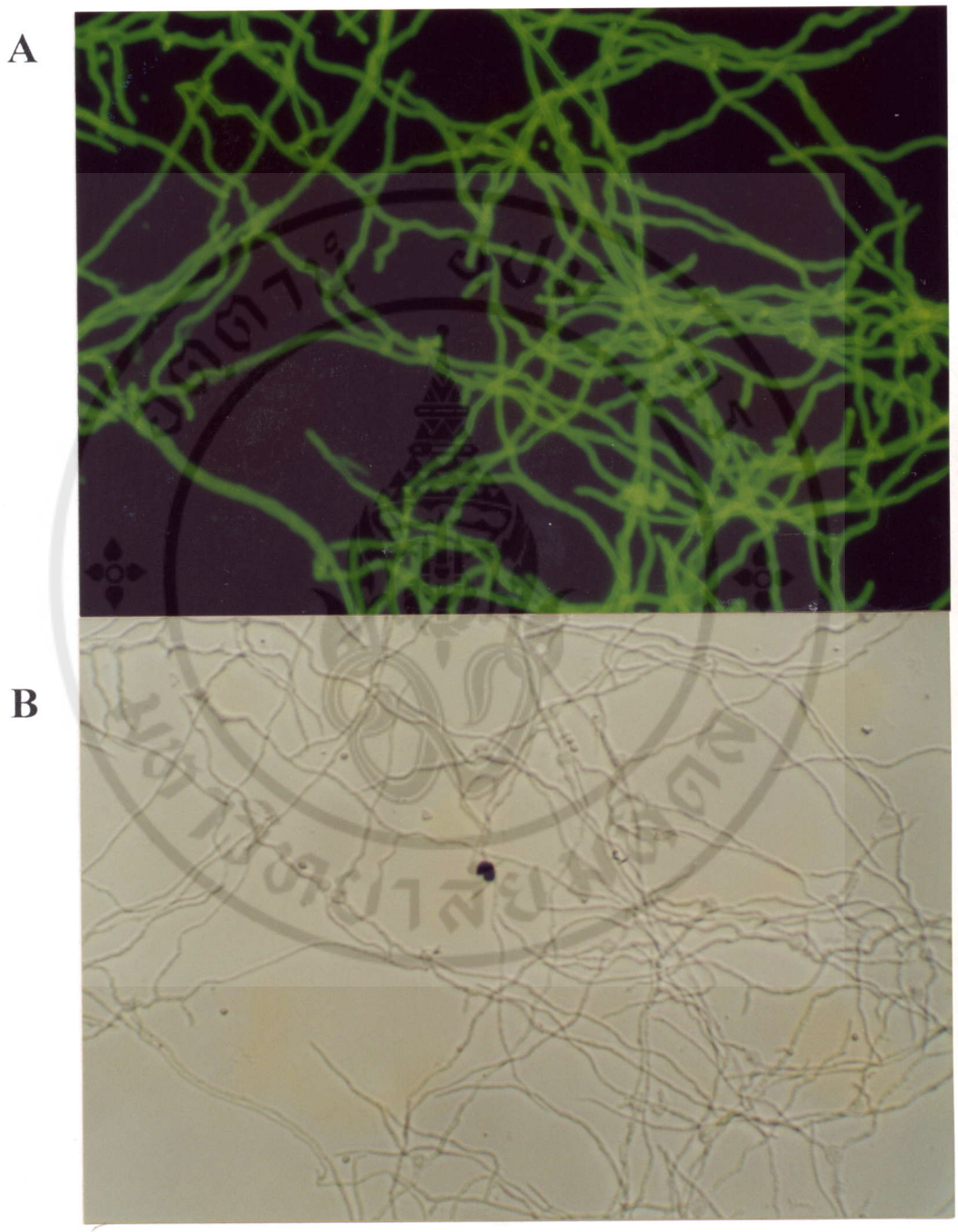

Figure 17: Immunofluorescent staining of *P. marneffei* hyphae with MAb 8B11 (A). Light microscopic image of the corresponding field is shown for comparison (B). (200X)

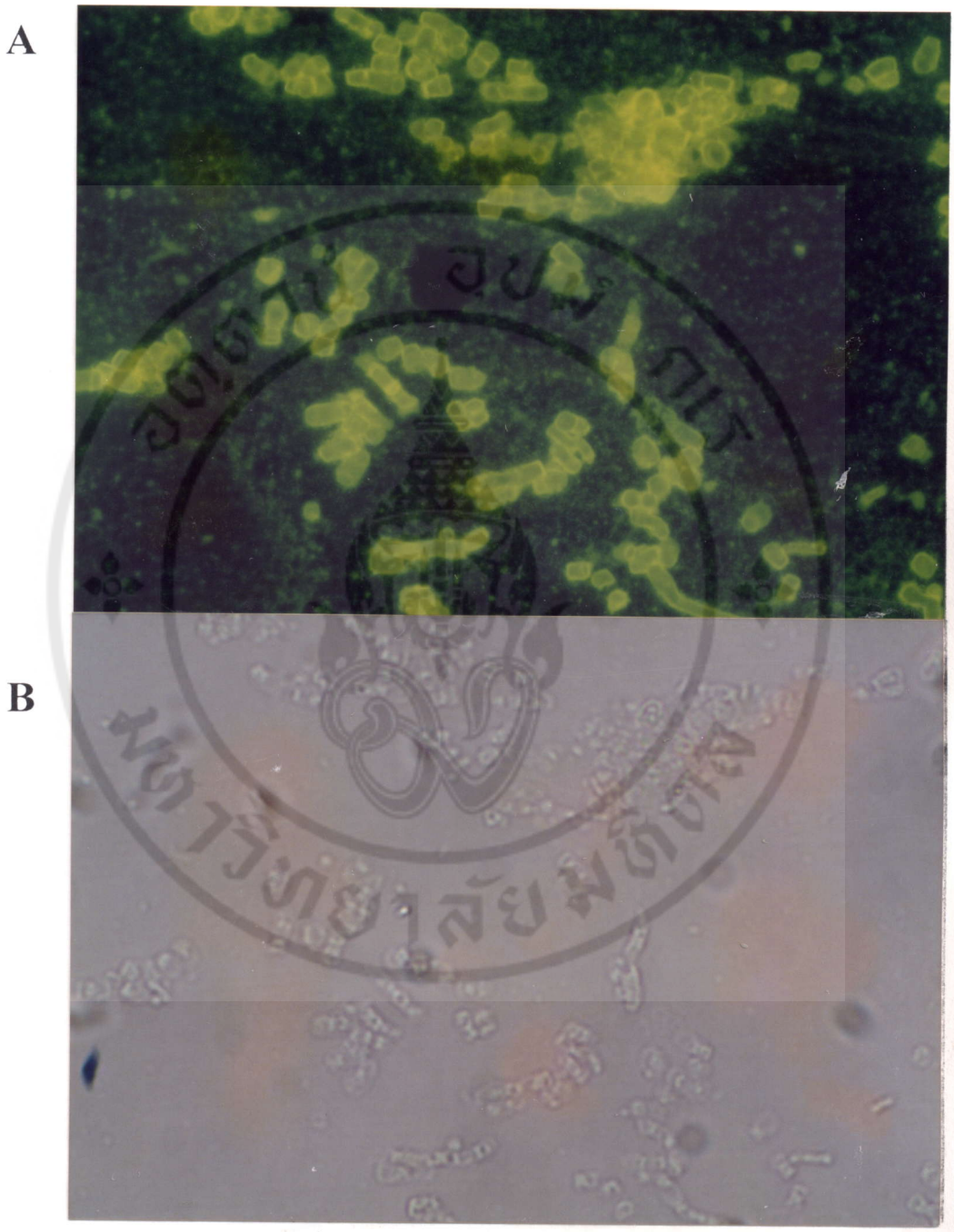

Figure 18: Immunofluorescent staining of the yeast cells of *P. marneffei* with MAb 8B11 (A). Light microscopic image of the corresponding field is shown for comparison (B). (400X)

Table 7 Immunoreactivity of MAbs.

Fusion No.	Clones	ELISA (O.D. value)				Immunoblot (kDa)		Immunofluorescence	
		Native CCF	Denatured CCF	Native Exo-Ag	Denatured Exo-Ag	CCF	Exo-Ag	Yeast	Hyphae
1	3C2	-	>0.8	–	>0.8	38, 40	38, 40	–	–
2	8C3	0.2	-	0.6	0.4	68, 74, 79, (97)	51,60,62,76,108 45-200 (smear)	+	±[1]
3	3B9	0.6	0.4	-	-	68, >200 (smear)	>77 (smear)	+	+
	8B11	>0.8	0.6	-	-	79, >200 (smear)	>79 (smear)	+	+

[1] Positive with the tip of newly germinating hyphae and young hyphae of *P. marneffei*

3. Characterization of Monoclonal Antibodies

3.1 Specificity of MAbs

Different fungi including *H. capsulatum* (both yeast phase and mycelial phase) *Penicillium spp., P. chrysogenum, P. siamensis, P. pinophilum, A. fumigatus, A. flavus, A. niger, A. terrius, A. nidulans, C. neoformans, C. albicans*, were used to evaluate the specificity of these four MAbs by indirect ELISA, IFA and immunoblotting assay.

Cross reactivity by indirect ELISA of the MAbs to the native form of CCF from the fungi mentioned above was summarized as shown in Table 8. MAbs 8B11 and 3B9 showed cross reactivity to the native form of CCF prepared from *P. siamensis* and *P. pinophilum*. MAb 8C3 showed cross reactivity to the native form of CCF prepared from *A. nidulans* whereas MAb 3C2 did not react with the native CCF of *P. marneffei* and also failed to react with all native CCF of the above fungi by indirect ELISA.

Cross reactivity of MAbs to denatured form of the CCF by indirect ELISA was summarized as shown in Table 9. MAb 8B11 showed cross reactivity to denatured form of the CCF prepared from *P. pinophilum* whereas MAb 3B9, MAb 8C3 and MAb 3C2 did not show cross reactivity to all denatured form of CCF.

Cross reactivity of MAbs to the above fungi by IFA was summarized as shown in Table 10. MAb 8B11 showed cross reactivity to hyphae of *H. capsulatum* and *P. chrysogenum*. MAb 3B9 showed cross reactivity only to hyphae of *H.*

capsulatum. MAbs produced from hybridoma clone 8C3 gave poor reactivity to hyphae and macroconidia of *H. capsulatum* whereas MAb 3C2 did not react with any of the fungi tested. Negative fluorescent staining with the yeast phase of *H. capsulatum, P. siamensis, P. pinophilum, A. fumigatus, C. neoformans* and *C. albicans* by MAb 8B11 were shown in Fig. 19, 20, 21, 22, 23 and 24, respectively.

Figure 25 demonstrated reactivity of four MAbs to *P. marneffei* against a panel of fungal antigens including *A. flavus, A. fumigatus, A. niger, P. pinophilum, P. chrysogenum, H. capsulatum, C. neoformans* and *C. albicans* by immunoblotting assay. MAbs 8B11 and 3B9 showed cross reactivity to *P. pinophilum* (Fig. 25(a), E and Fig. 25(b), E). MAb 8C3 showed cross reactivity to *P. pinophilum* (Fig. 25(c), E) and *P. chrysogenum* (Fig. 25(c), F). In contrast, MAb 3C2 did not show cross reactivity to these fungal antigens (Fig. 25(d)).

Table 8 Cross reactivity of monoclonal antibodies to native CCF prepared from different fungi by ELISA (O.D. value).

	3C2	8C3	3B9	8B11	Immune mouse sera	Relevant MAbs
P.marneffei -yeast	-	0.6	-	-	0.3	-
P.marneffei -mycelium	-	0.2	0.6	0.9	0.6	-
H.capsulatum -mycelium	-	-	-	-	-	-
Penicillium spp.	-	-	-	-	-	-
P. chrysogenum	-	-	-	-	-	-
P. pinophilum	-	-	0.4	0.8	0.2	-
P. siamensis	-	-	0.6	0.7	-	-
A. fumigatus	-	-	-	-	-	-
A. flavus	-	-	-	-	-	-
A. niger	-	-	-	-	-	-
A. terreus	-	-	-	-	-	-
A. nidulans	-	0.2	-	-	-	-
C. albicans	-	-	-	-	-	-
C. neoformans	-	-	-	-	-	-

Note: Microtiter wells were coated with 50 µl of native CCF (25 µg/ml) prepared from the above fungi and 50 µl of hybridoma supernatant were used to determine the cross reactivity by ELISA. The O.D. values were calculated from duplicate experiments.

Table 9 Cross reactivity of monoclonal antibodies to denatured CCF prepared from various organism by ELISA (O.D. value).

	3C2	8C3	3B9	8B11	Immune mouse sera	Relevant MAbs
P.marneffei -yeast	0.8	0.4	-	-	0.5	-
P.marneffei -mycelium	0.9	-	0.4	0.6	0.7	-
H.capsulatum -mycelium	-	-	-	-	-	-
Penicillium spp.	-	-	-	-	-	-
P. chrysogenum	-	-	-	-	-	-
P. pinophilum	-	-	-	0.3	-	-
P. siamensis	-	-	-	-	-	-
A. fumigatus	-	-	-	-	-	-
A. flavus	-	-	-	-	-	-
A. niger	-	-	-	-	-	-
A. terreus	-	-	-	-	-	-
A. nidulans	-	-	-	-	-	-
C. albicans	-	-	-	-	-	-
C. neoformans	-	-	-	-	-	-

Note: Microtiter wells were coated with 50 µl of denatured CCF (25 µg/ml) prepared from the above fungi and 50 µl of hybridoma supernatant were used to determine the cross reactivity by ELISA. The O.D. values were calculated from duplicate experiments.

Table 10 Cross reactivity of monoclonal antibodies to different fungi by indirect immunofluoresent test.

	8B11	3B9	8C3	3C2	Immune mouse sera	Relevant MAbs
P.marneffei -yeast	+	+	+	-	+	-
P.marneffei -mycelium	+	+	+	-	+	-
H.capsulatum -yeast	-	-	-	-	+	-
H.capsulatum -mycelium	+/-[1]	+	+[2]	-	+	-
Penicillium spp.	-	-	-	-	+	-
P. chrysogenum	+/-[1]	-	-	-	+	-
P. pinophilum	-[3]	-[3]	-	-	+	-
P. siamensis	-[3]	-[3]	-	-	+	-
A. fumigatus	-	-	-	-	+	-
A. flavus	-	-	-	-	-	-
A. niger	-	-	-	-	-	-
A. terreus	-	-	-	-	-	-
A. nidulans	-	-	-	-	-	-
C. albicans	-	-	-	-	-	-
C. neoformans	-[4]	-	-	-	-	-

[1] Faint staining with some hyphae
[2] Positive staining with macroconidia and some hyphae
[3] Yellow-green satining of hypae and conidia represents the autofluorescent of the fungi
[4] Yellow-green staining of yeast cells represent the autofluorescent of the fungi.

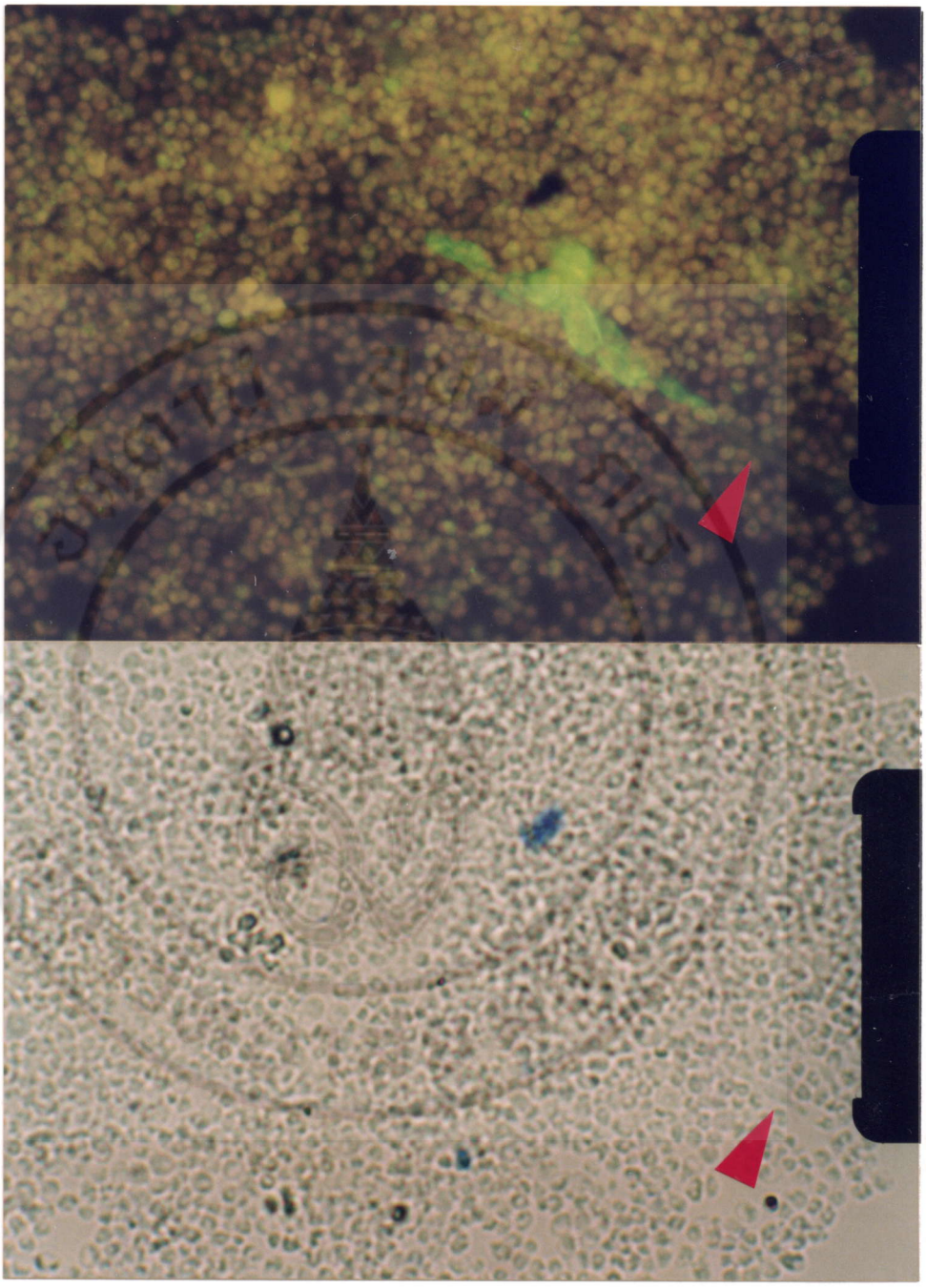

Figure 19: Immunofluorescent staining of the yeast phase of *H. capsulatum* with MAb 8B11 (A). Light microscopic image of the corresponding field is shown for comparison (B). Autofluorescence of the fungi (yellow color) can be observed. The MAb stained positively with some unidentified component which may represent some technical artifact during the preparation of the slide. It should be noticed that the nearby hyphae-like filament (arrowhead) gave negative staining. (200X)

Figure 20: Immunofluorescent staining patterns of *P. siamensis* (2-week old slide culture) stained with MAb 8B11 (A). Light microscopic image of the corresponding field is shown for comparison (B). Autofluorescence of the fungi (yellow color) is shown. (200X)

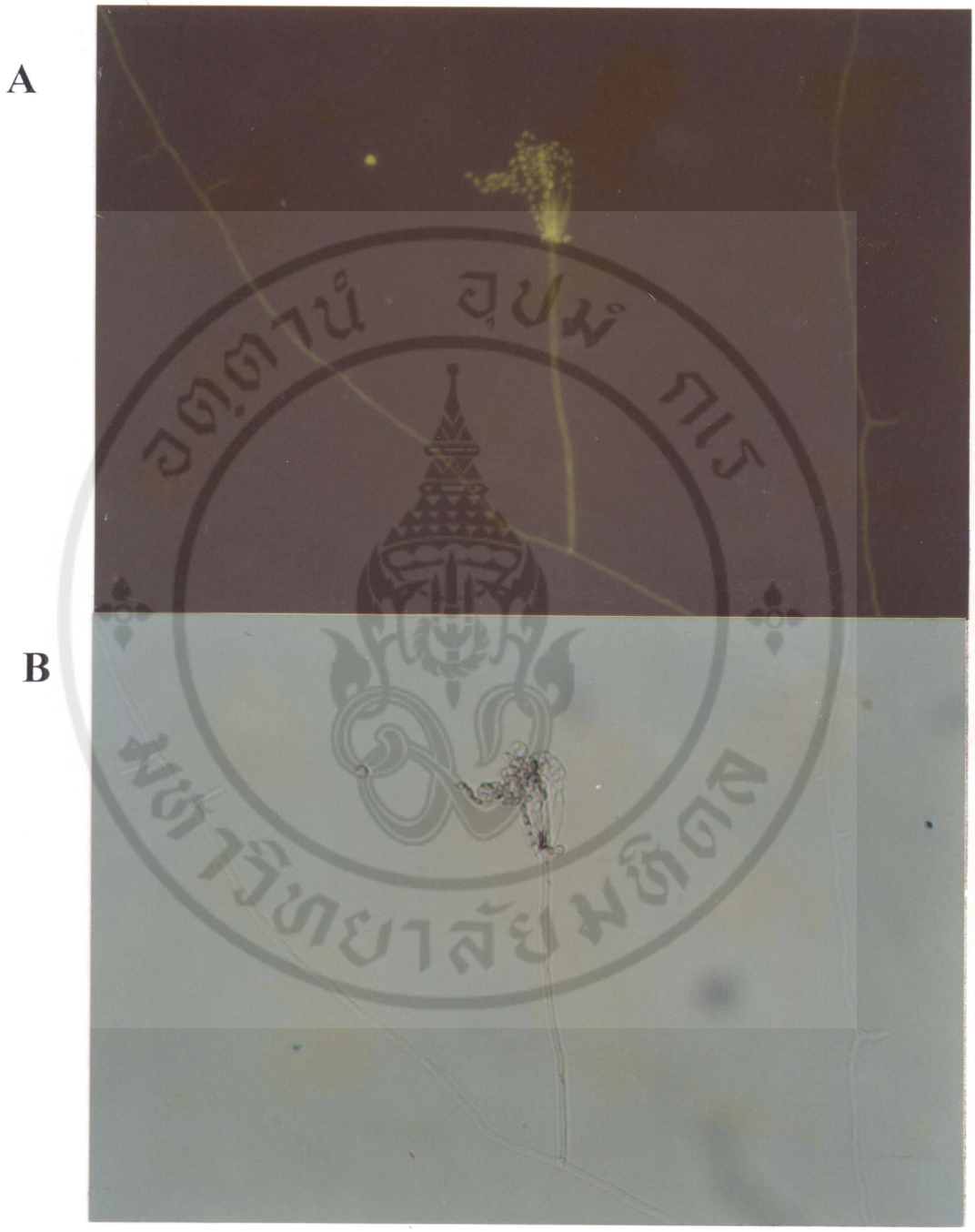

Figure 21: Immunofluorescent staining patterns of *P. pinophilum* (2-week old slide culture) stained with MAb 8B11 (A). Light microscopic image of the corresponding field is shown for comparison (B). A weak autofluorescence of the fungi (yellow color) could be observed. (200X)

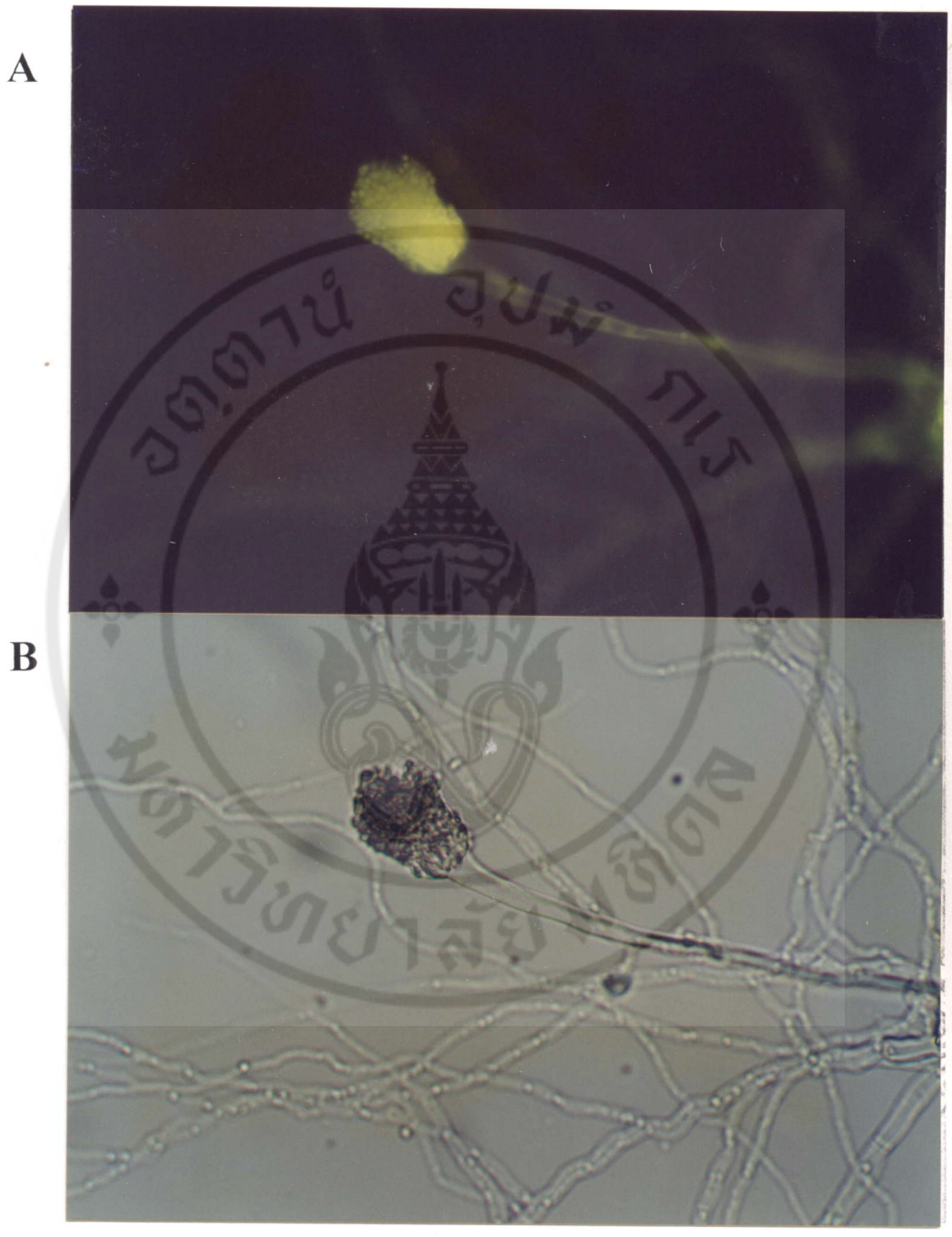

Figure 22: Immunofluorescent staining patterns of *A. fumigatus* (2-week old slide culture) stained with MAb 8B11 (A). Light microscopic image of the corresponding field is shown for comparison (B). Autofluorescence of the fungi (yellow color) could be observed. (200X)

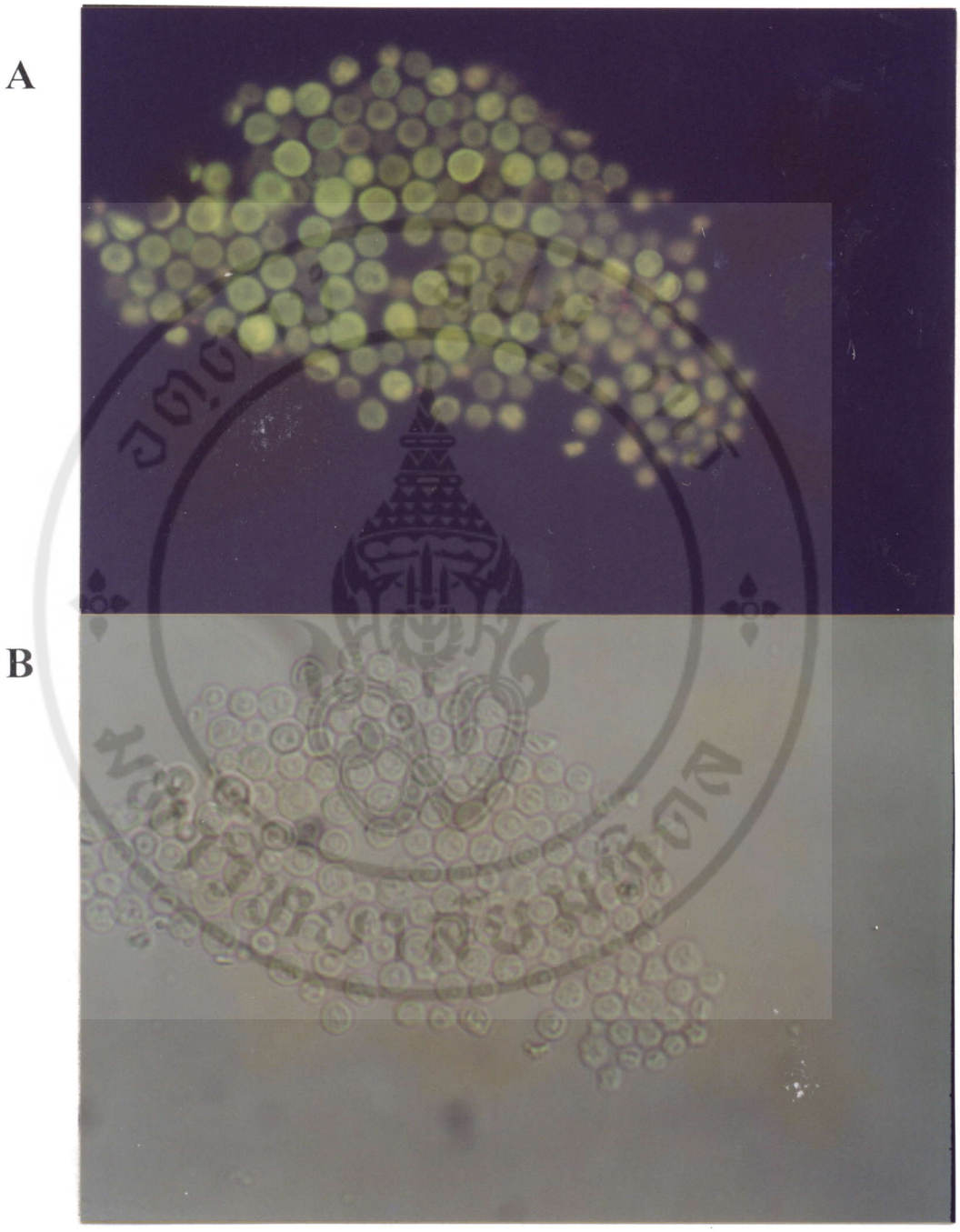

Figure 23: Immunofluorescent staining patterns of *C. neoformans* stained with MAb 8B11 (A). Light microscopic image of the corresponding field is shown for comparison (B). Autofluorescence of the fungi (yellow-green color) could be observed. (200X)

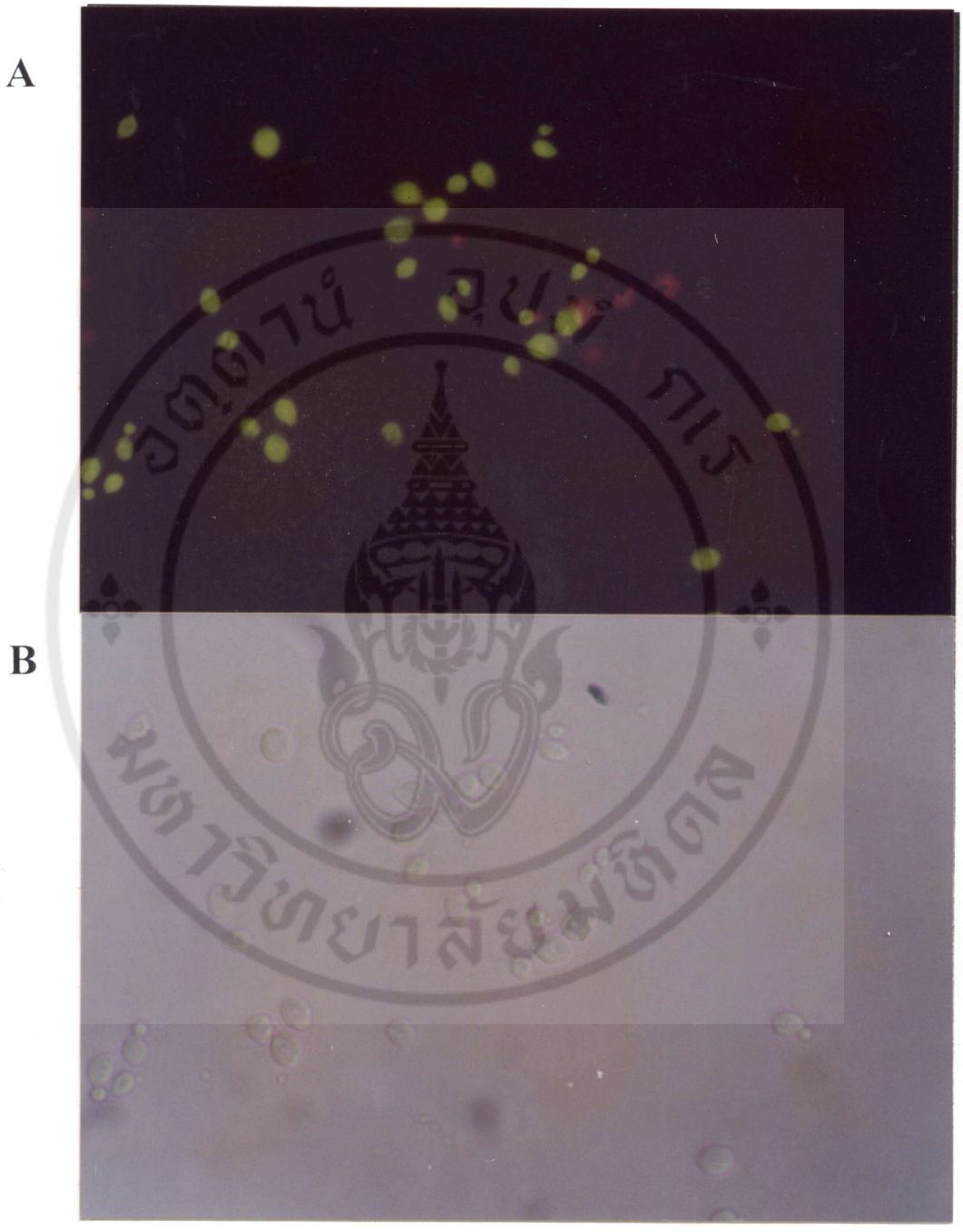

Figure 24: Immunofluorescent staining patterns of *C. albicans* stained with MAb 8B11 (A). Light microscopic image of the corresponding field is shown for comparison (B). Autofluorescence of the fungi (yellow color) could be observed. (200X)

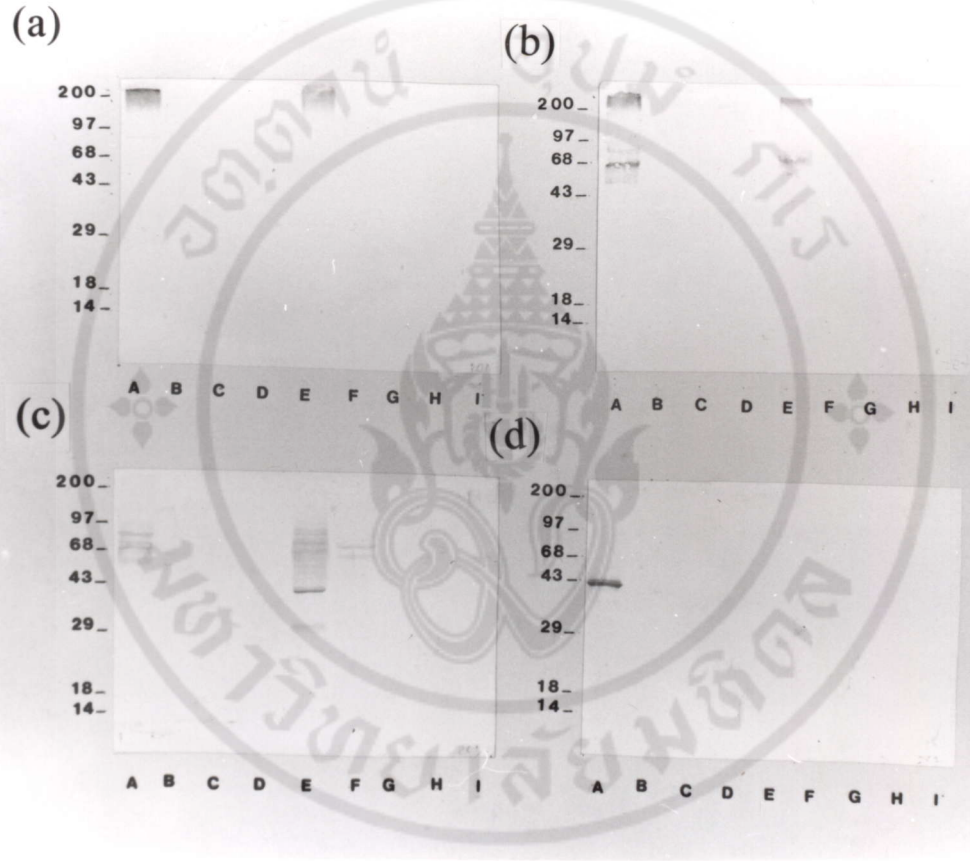

Figure 25: Reactivity of four MAbs to *P. marneffei* against a panel of fungal antigens including *P. marneffei* (A), *A. flavus* (B), *A. fumigatus* (C), *A. niger* (D), *P. pinophilum* (E), *P. chrysogenum* (F), *H. capsulatum* (G), *C. neoformans* (H) and *C. albicans* (I). The antigens were subjected to SDS-PAGE, blotted onto nitrocellulose membrane and then probed with MAbs 8B11 (a), 3B9 (b), 8C3 (c) and 3C2 (d). The numbers on the left are relative molecular weights of protein markers (in kilodalton)

3.2 The Nature of Immunoreactive Epitopes of Monoclonal Antibodies

P. marneffei culture filtrate prepared form both yeast phase and mycelial phase of this fungus were highly glycosylated. This was demonstrated by staining the antigens with lectins (Fig.13) and treating the antigens with periodate before reacting with MAbs. Periodate treatment was performed as described in Materials and Methods (Section 7.5). The epitope(s) reactive with MAbs 8B11 and 3B9 were most likely associated with high molecular weight glycoprotein or polysaccharide components of *P. marneffei* as they were susceptible to periodate treatment. In contrast, the immunoreactive epitopes that reacted with MAb 8C3 and MAb3C2 were resistant to periodate treatment (Fig. 26).

3.3 Isotyping of Monoclonal Antibodies

Isotyping of MAbs were performed using commercial reagent kit as described in Materials and Methods (Section 6.9). The heavy chain type of MAbs 8B11, 3B9 and 8C3 was μ while that of MAb 3C2 was γ1. The type of light chain of all MAbs was κ (Table 11).

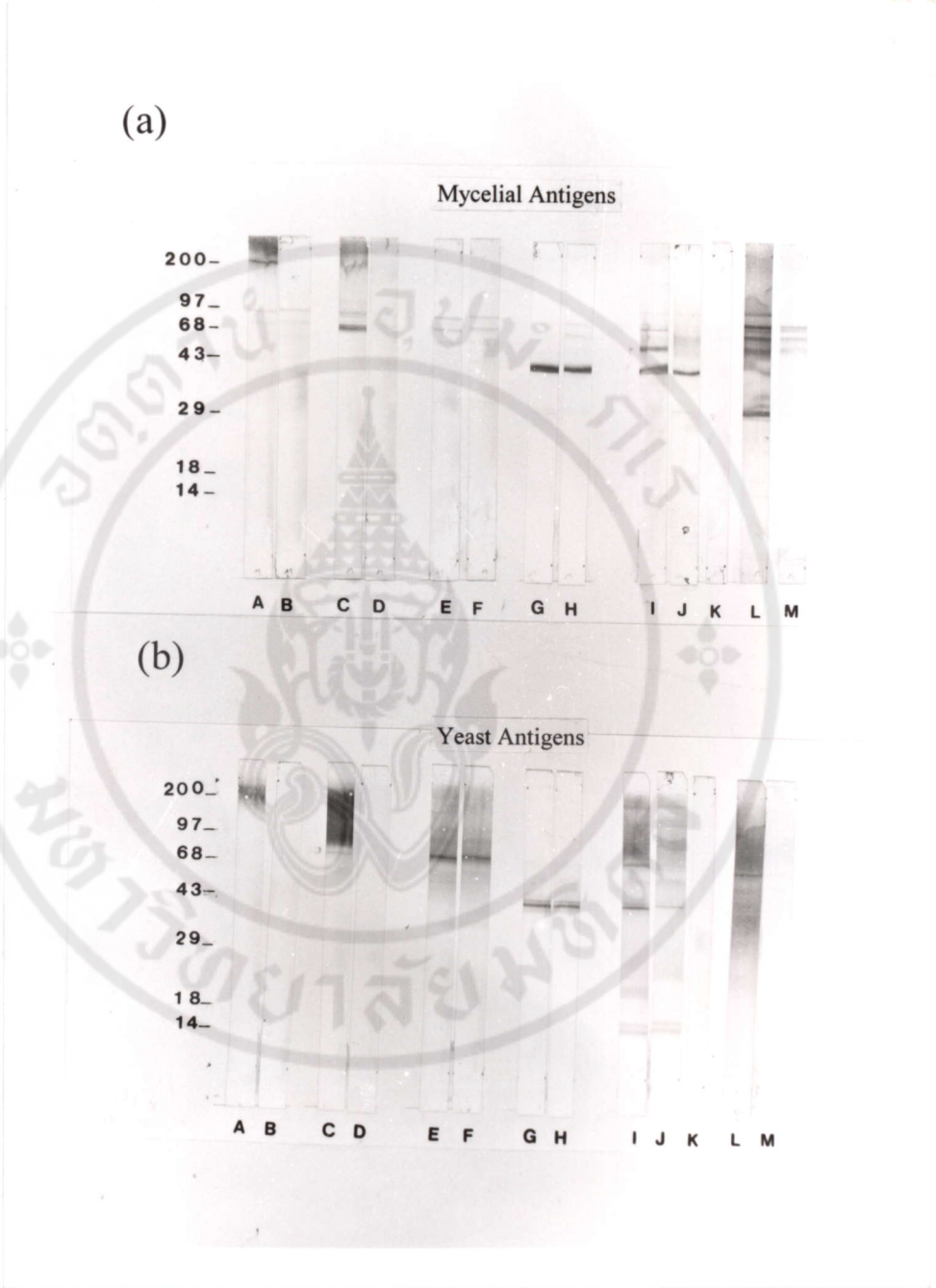

Figure 26: Immunoblot patterns of mycelial (a) and yeast (b) phase antigens before (lane A, C, E, G, I and L) and after (lanes B, D, F, H, J and M) periodate oxidation. The reaction was subsequently probed with MAb 8B11 (lane A&B), MAb 3B9(lane C&D), MAb 8C3 (lane E&F) and MAb3C2 (lane G&H), immune mouse serum (lane I&J) and ConA (lane L&M). Lane K was the antigen developed with normal mouse serum which served as a control. The numbers on the left are the size of protein markers (in kilodalton).

Table 11 Isotyping of monoclonal antibodies.

Fusion	Hybridoma clone	Heavy chain subclass	Light chain types
First	3C2	Gamma 1	Kappa
Second	8C3	Mu	Kappa
Third	8B11 3B9	Mu Mu	Kappa Kappa

4. Potential of MAbs for the Detection of *P. marneffei* in Culture and Tissue Specimens

4.1 Fungal Culture

A time-course analysis to demonstrate the appearance and disappearence of immunoreactive components distributed on the cell wall of *P. marneffei* was performed as described in Materials and Methods. MAbs from hybridoma clone 8B11 and 3B9 gave positive immunofluorecent reactivity to swelling conidia, newly germinating hyphae or germ tubes and hyphae of *P. marneffei.* For the 15-day old slide culture, MAbs 8B11 and 3B9 gave positive reactivity to hyphae whereas conidia were negatively stained. MAb 8C3 gave positive immunofluorecent staining with swelling conidia, newly germinating hyphae or germ tubes and hyphae of *P. marneffei.* For the 15-day old slide culture, MAb 8C3 gave negative reactivity to hyphae, conidiophore and conidia but positive staining to the tip of a few hyphae could be observed. MAb 3C2 showed negative fluorescent staining with all stages of *P. marneffei*. The potential of MAbs for the detection or identification of *P. marneffei* by IFA depended on the age of fungal culture (Table 12). However, there was no problem in distinguishing the yeast phase of *P. marneffei* from the yeast phase of *H. capsulatum, C. neoformans* and *C. albicans* when MAbs 8B11, 3B9 and 8C3 were employed.

Table 12 A time course analysis for the appearance and disappearance of immunoreactive components on fungal cell wall by IFA.

MAbs	1-day old culture				2-day old culture		3-day old culture		10-day old culture		15-day old slide culture		
	Hyphae	Tip	Germtube	Swelling conidia	Hyphae	Tip	Hyphae	Tip	Hyphae	Tip	Hyphae	Tip	conidia
8B11	+	+	+	+	+	+	+	+	+	+	+	+	-
3B9	+	+	+	+	+	+	+	+	+	+	+	+	-
8C3	+	+	+	+	+w	+	+w	+	+w	+	-	-	-
3C2	-	-	-	-	-	-	-	-	-	-	-	-	-

+, represent for positive staining; -, represent for negative staining; +w, represents weak reaction.

4.2 Simulated Tissues

Simulated tissues prepared as described in Materials and Methods were used to represent the presence of fungus in tissue when tissue specimens were not available. In addition, there is a possibility that normal tissue may interfere with a positive reaction by giving non-specific reaction, particularly with the available MAbs were largely of the IgM isotype. In the experiment, irrelavant MAbs with the same isotype were included as isotype controls. Moreover, keratin may give autofluorescent reactivity. With the exception of *P. marneffei*, none of the artificial tissue-fungal mixture gave positive immunofluorescent staining with any of the MAbs tested (Table 13).

Table 13 Fluorescent-antibody staining reactions of simulated tissue prepared by mixing human tissues with different fungi.

	3C2	8C3	3B9	8B11	Positive mouse serum	Negative mouse serum	Human histoplasmosis serum
P. marneffei (yeast form)	-	+	+	+	+	-	+
P. spp	-	-	-	-	+/-	-	-
H. capsulatum (yeast form)	-	-	-	-	+	-	+
A. Fumigatus	-	-	-	-	+/-	-	-
C. neoformans	-	-	-	-	-	-	-
C. albicans	-	-	-	-	-	-	-

4.3 Indirect Immunofluorescent Staining of Clinical Tissue Biopsies

Histological tissue sections from the patients suspected of having fungal infections, without culture confirmation, were tested. The presumptive diagnosis was based on microscopic examinations of tissue sections stained with H&E. By light microscopic examination, differentiation between the yeast cells of *P. marneffei* and *H. capsulatum* was difficult except by experienced personnel who could distinguish central septation (Fig. 27). Tissue sections of all 3 patients diagnosed of penicilliosis marneffei and two out of four patients diagnosed of histoplasmosis gave positive immunofluorecent staining with MAbs 8B11, 3B9 and 8C3. The strongly positive fluorescent staining of round to oval shape of yeast cells present in the tissue section could be easily seen (Figs. 29 - 31) compared to those with negative fluorescent staining (Figs. 32 - 34). Staining of oral tissue lesion from a patient diagnosed of penicilliosis marneffei with immune mouse serum was used as positive control (Fig. 28). Tissue sections from patients diagnosed of aspergillosis (3 cases), candidiasis (4 cases), cryptococcosis (2 cases), chromomycosis (1 case), phycomycosis (2 cases) and actinomycosis (1 case) gave negative immunofluorescent staining with all MAbs tested (Table 14).

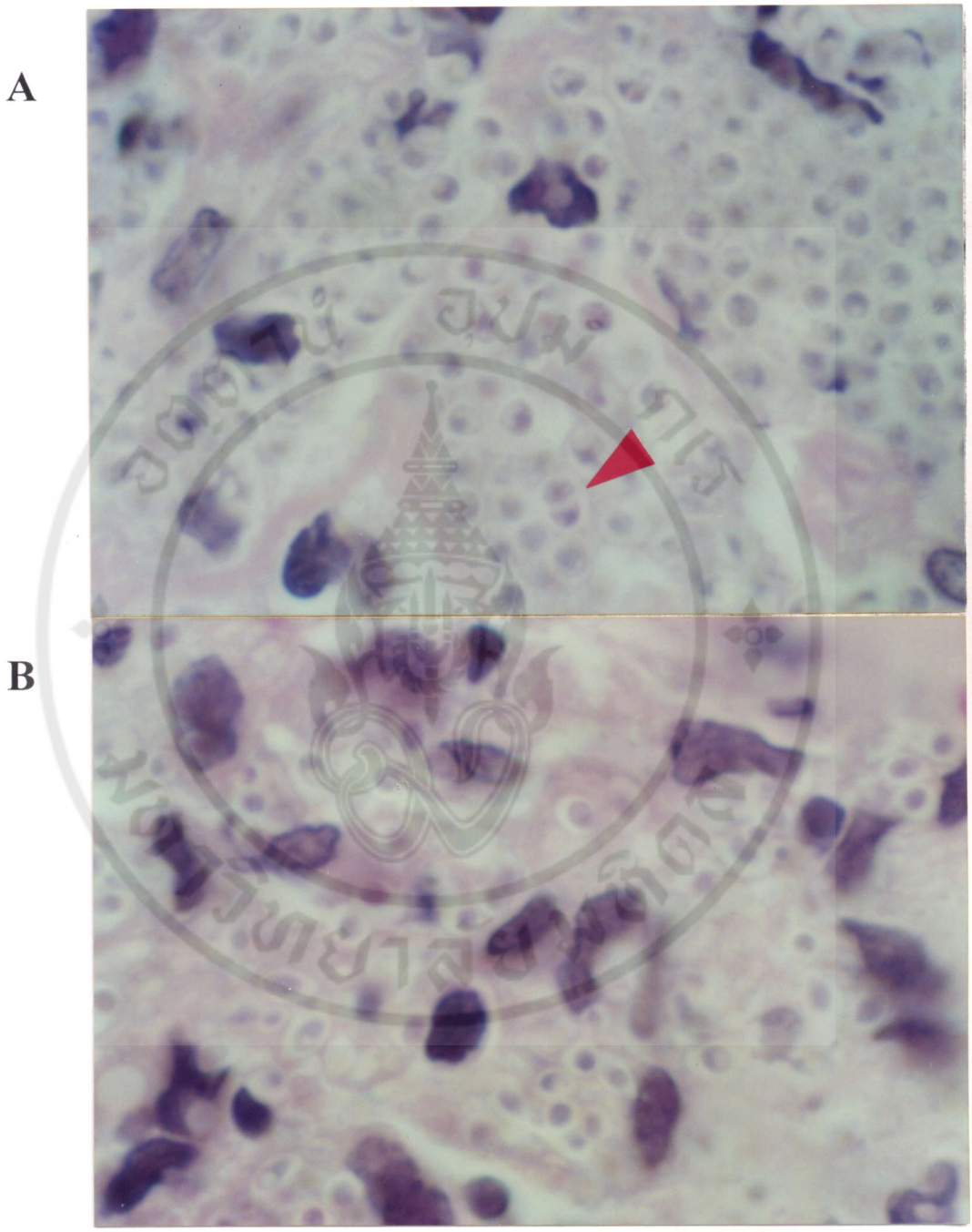

Figure 27: H&E staining of liver sections from a patient with penicilliosis marneffei (A) and a patient with histoplasmosis (B). There was no difference in morphology (tissue form) of both fungi. The only way to differentiate between the tissue form of *P. marneffei* and *H.capsulatum* is the central septation of the yeast cells that could be observed in *P. marneffei* (arrow).(1000X).

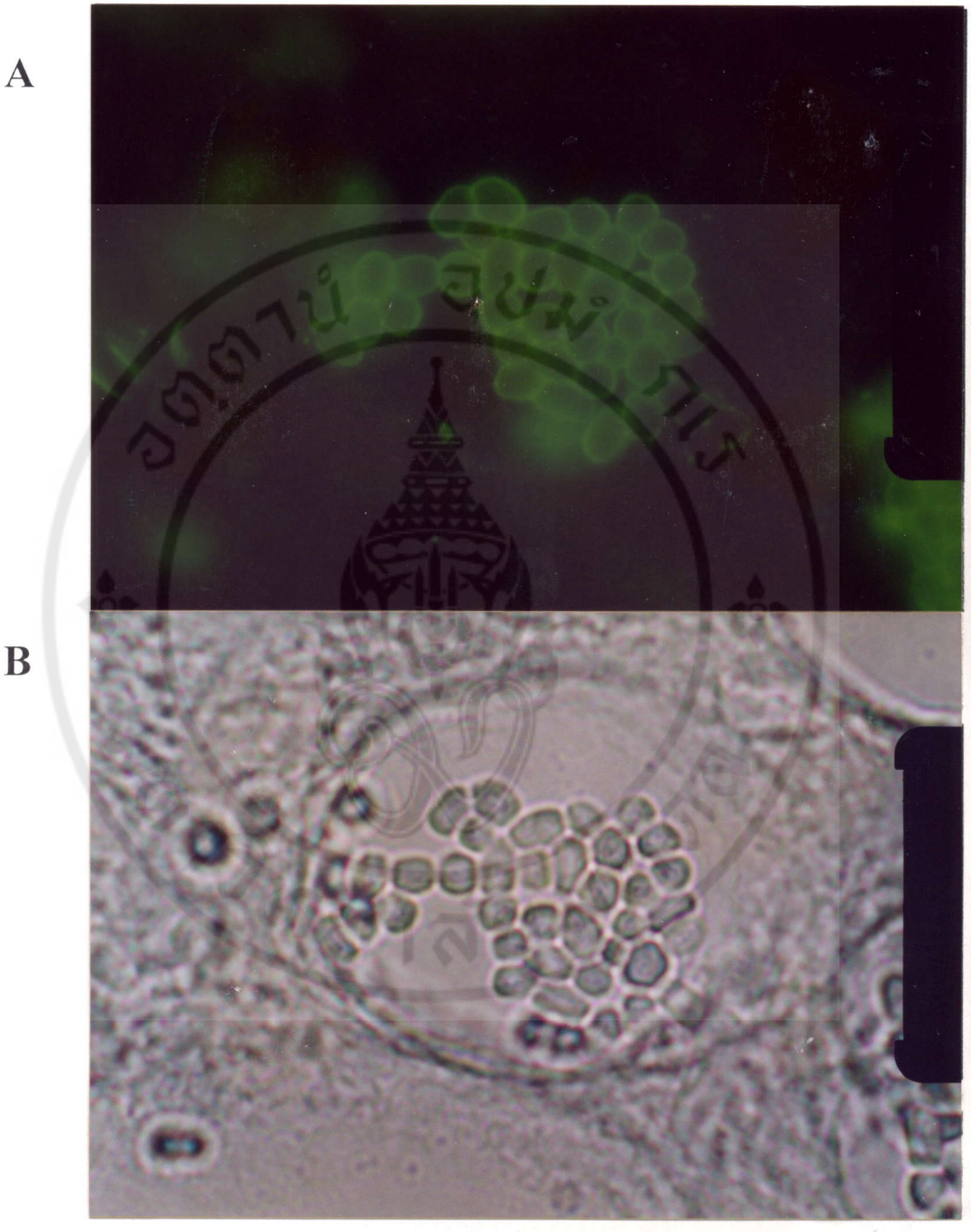

Figure 28: Immunofluorescent staining of an oral tissue section from a patient with penicilliosis marneffei with immune mouse serum (A). Light microscopic image of the corresponding field is shown for comparison (B). Positive staining of the yeast cells of *P. marneffei* could be observed. (1000X)

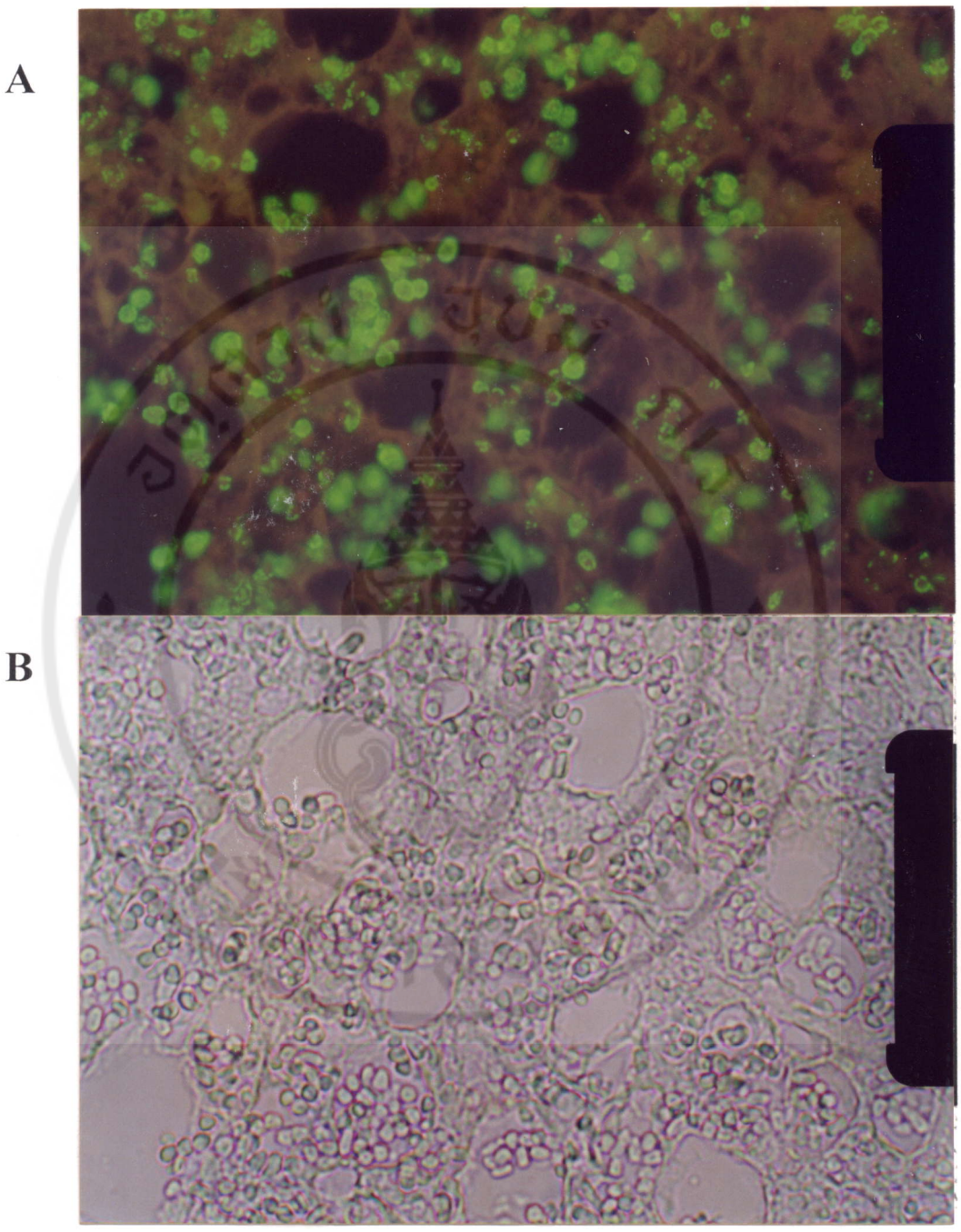

Figure 29: Immunofluorescent staining of an oral tissue section from a patient with penicilliosis marneffei with MAb 8B11 (A). Light microscopic image of the corresponding field is shown for comparison (B). Positive staining of the yeast cells of *P. marneffei* could be observed. (400X)

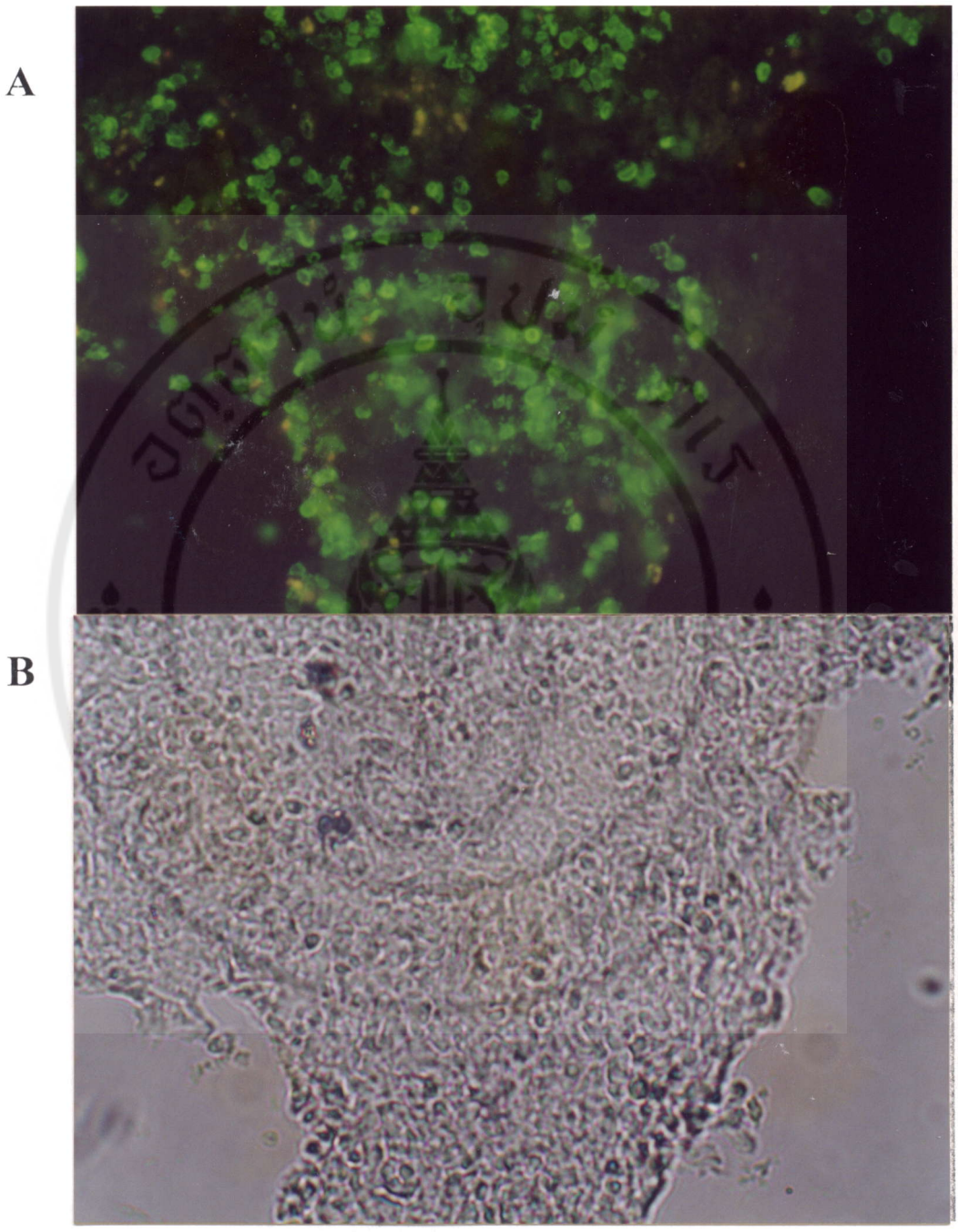

Figure 30: Immunofluorescent staining of a liver section from a patient with penicilliosis marneffei with MAb 8B11 (A). Light microscopic image of the corresponding field is shown for comparison (B). Positive staining of the yeast cells of *P. marneffei* could be observed. (400X)

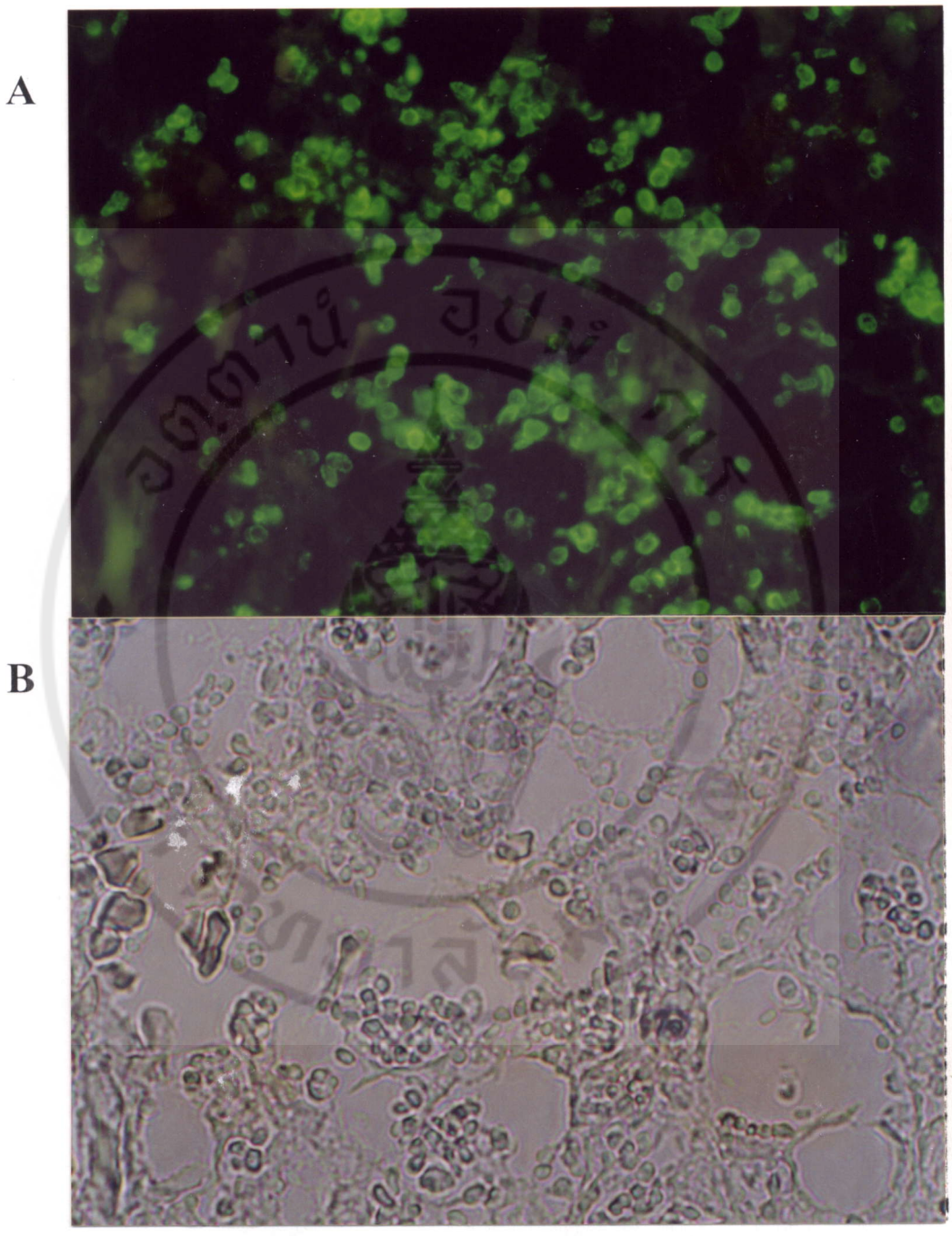

Figure 31: Immunofluorescent staining of a skin section from a patient with penicilliosis marneffei with MAb 8B11 (A). Light microscopic image of the corresponding field is shown for comparison (B). Positive staining of the yeast cells of *P. marneffei* could be observed. (400X)

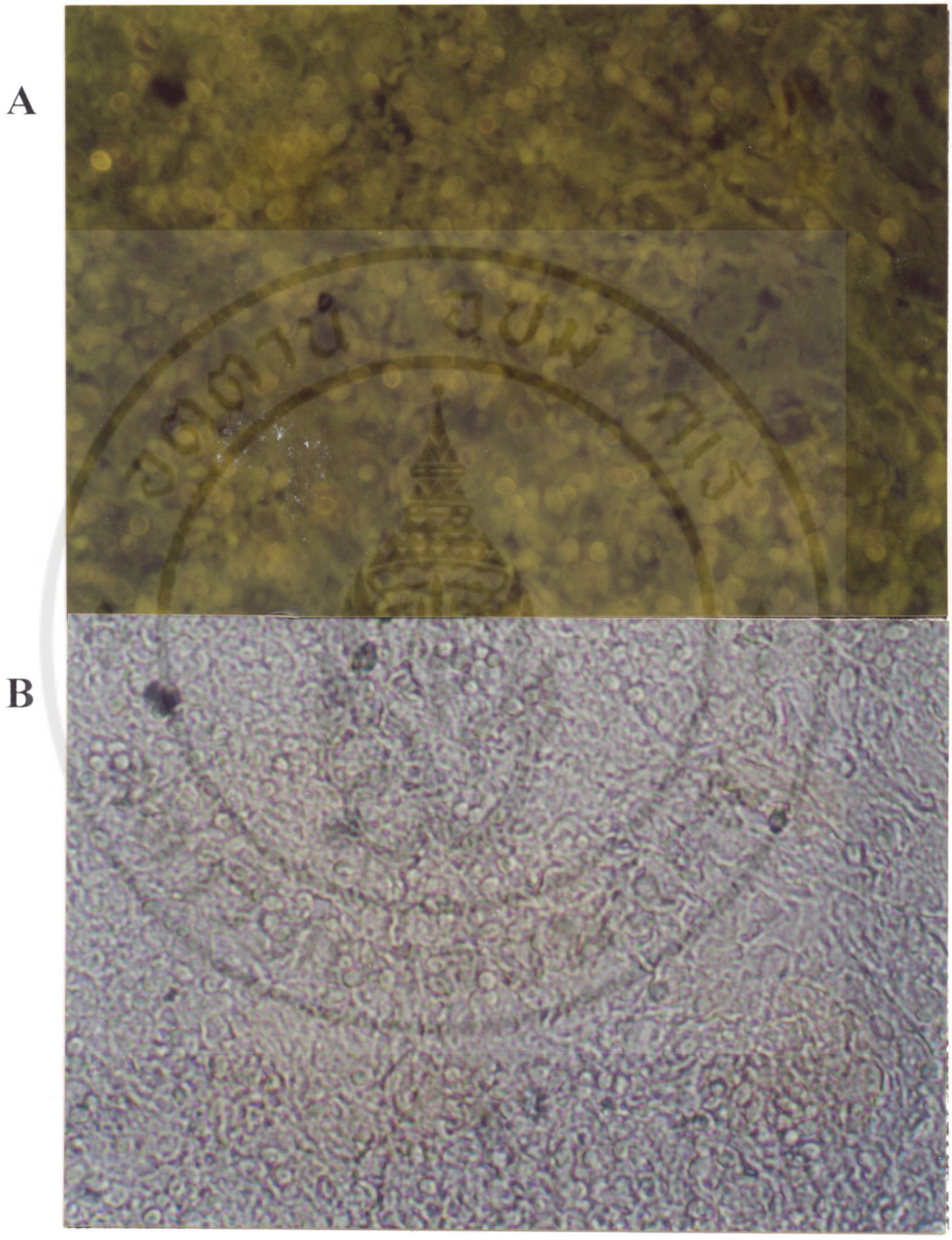

Figure 32: Immunofluorescent staining of a nasal tissue section from a patient with histoplasmosis with MAb 8B11 (A). Light microscopic image of the corresponding field is shown for comparison (B). No staining of the yeast cells of *H.capsulatum* could be observed. (400X)

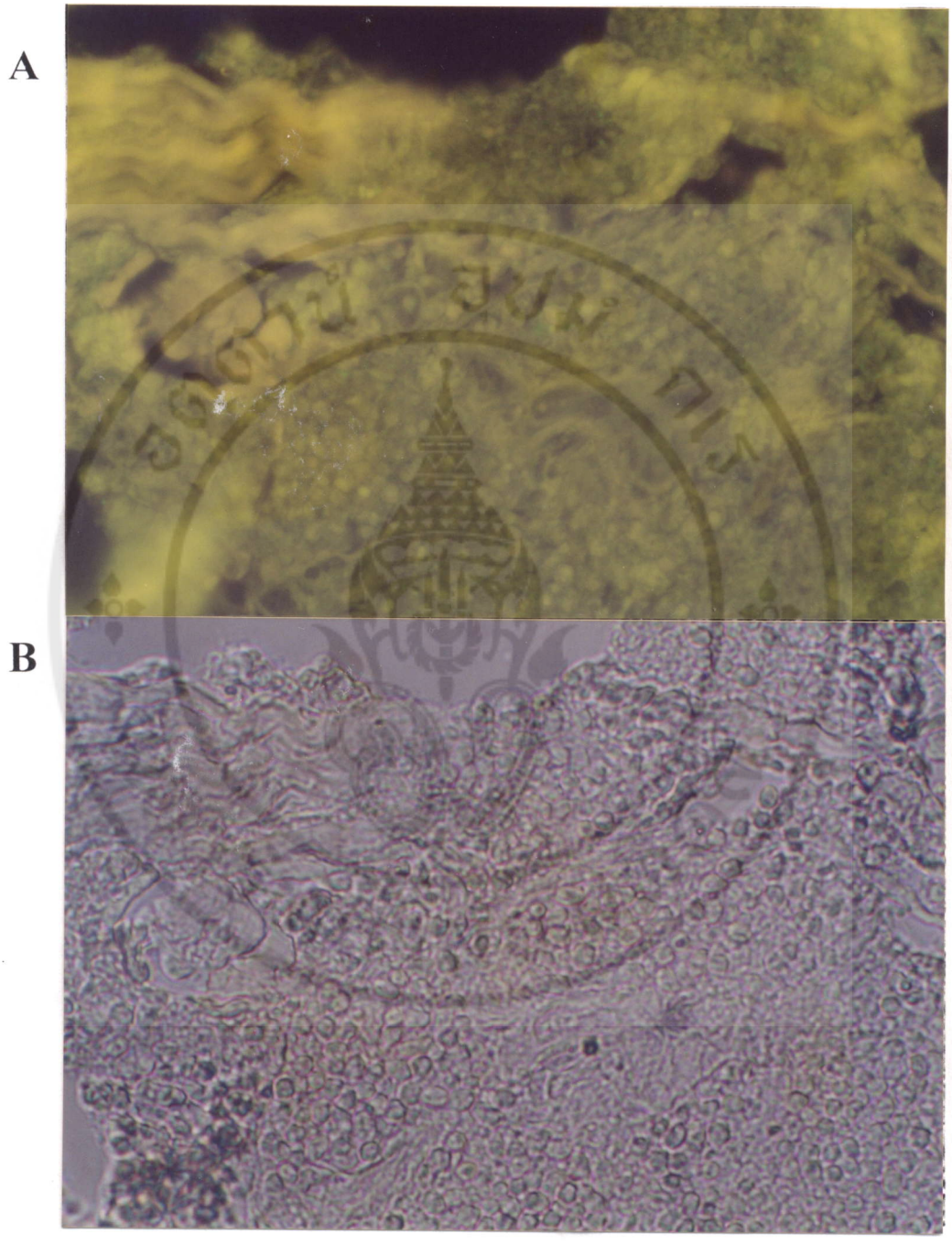

Figure 33: Immunofluorescent staining of a skin section from a patient with penicilliosis marneffei with MAb 3C2 (A). Light microscopic image of the corresponding field is shown for comparison (B). No staining of the yeast cells of *P. marneffei* could be observed. (400X)

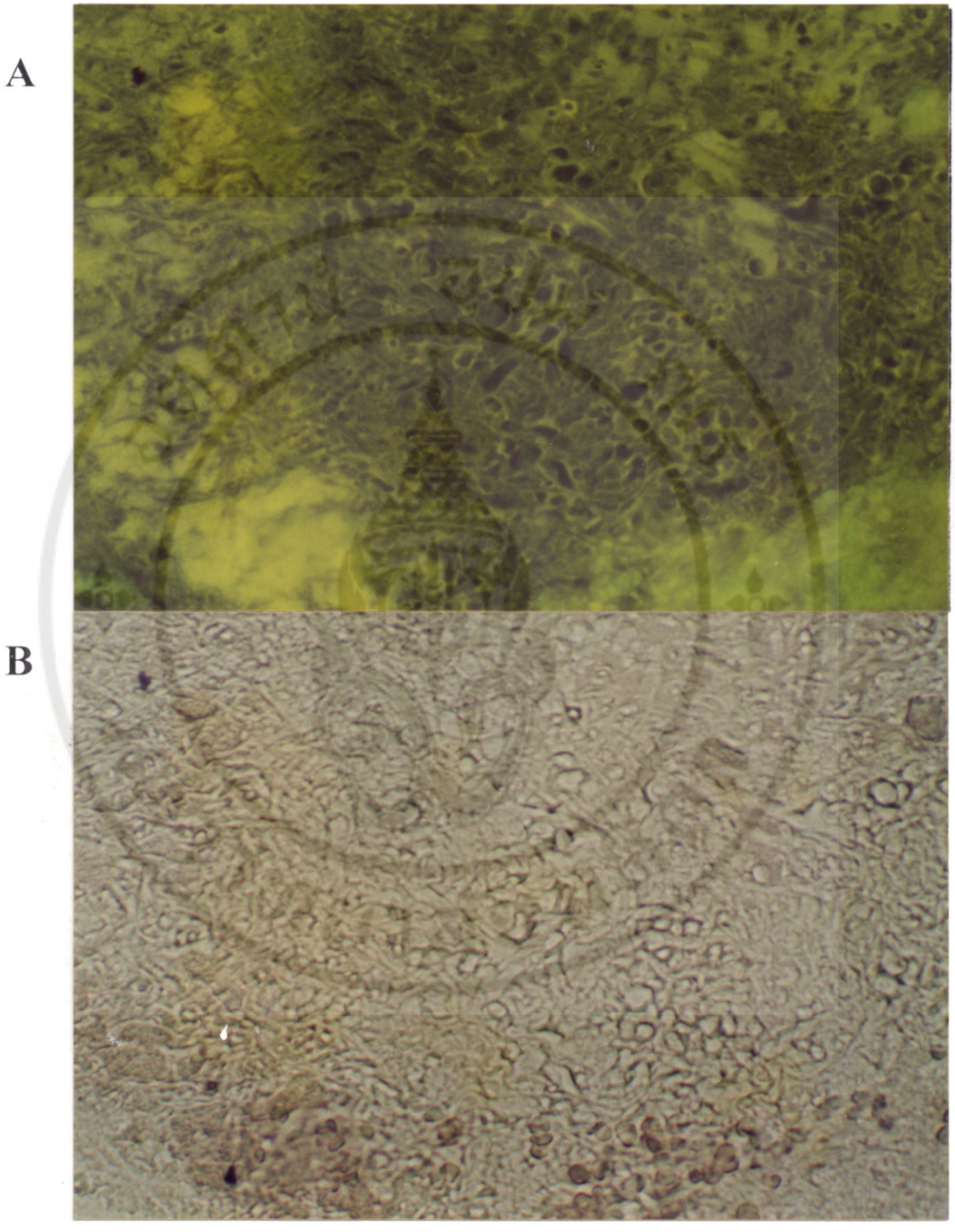

Figure 34: Immunofluorescent staining of a maxillary sinus tissue section from a patient with aspergillosis with MAb 8B11 (A). Light microscopic image of the corresponding field is shown for comparison (B). No staining of the fungal mycelium could be observed. (400X)

TABLE 14 Fluorescent-antibody staining reactions of histological sections of biopsy specimens from patients with different fungal infections

Biopsy taken from	Microscopic examination compatible with[a]	Fluorescence reaction[c]
Oral cavity	Peniciliosis marneffei	+
Liver	Peniciliosis marneffei[b]	+
Skin	Peniciliosis marneffei	+
Skin	Histoplasmosis[b]	+
Bone marrow	Histoplasmosis	-
Nasal cavity	Histoplasmosis[b]	-
Skin	Histoplasmosis[b]	+
Maxillary sinus	Aspergillosis	-
Maxillary sinus	Aspergillosis	-
Lung	Aspergillosis	-
Skin	Candidiasis	-
Skin	Candidiasis	-
Stomach	Candidiasis	-
Vagina	Candidiasis	-
Skin	Cryptococcosis	-
Scalp	Cryptococcosis	-
Skin	Chromomycosis	-
Joint	Phycomycosis	-
Skin	Phycomycosis	-
Neck	Actinomycosis	-

[a] Presumptive diagnosis based on microscopic examination of tissue sections stained with H & E.

[b] HIV positive

[c] Similar fluorescence reaction observed with all 3 MAbs (8B11, 3B9 and 8C3).

C. Genome Fingerprinting by Restriction Endonuclease Digestion and PFGE

1. Fungal Isolates.

Sixty-two clinical isolates of *P. marneffei* used in genome analysis were from penicilliosis marneffei patients in Thailand. One isolate from a bamboo rat and 4 isolates from the American Type Culture Collection were also included. Of 62 clinical isolates, 14 isolates were obtained from Lanna Medical Laboratory, Chiang Mai, northern Thailand. Twenty-three isolates were kindly provided by Dr. Rawee Teanpaisan, Microbiology Laboratory, Prince of Songkla Hospital, Songkla, southern Thailand. Twenty-seven isolates were from several hospitals located in the central region of the country (Table 2).

Among 14 isolates from the northern region of the country, 6 isolates were cultured from patients' blood, two isolates were from CSF, 1 isolate was from bone marrow and 5 isolates were of unknown origin. Only 2 of the 14 isolates were from patients having history of HIV infection. Regarding the period of specimen collection, half of the isolates were received in 1995. Three isolates were obtained in 1998 while three isolates were collected in 1999. Only one isolate had no specimen collection record (Table 3).

With 23 isolates from the southern Thailand, *P. marneffei* were obtained from skin lesions (2 isolates), blood samples (6 isolates), liver (1 isolate), bone marrow (2 isolates), sputum (1 isolate) and oral tissue (3 isolates). The rest were of unknown

origins. All patients had no record of HIV infection. Among these 23 isolates, 18 isolates were obtained in 1998 while 5 isolates had no record on the period of specimen collection (Table 4).

For 25 isolates from central Thailand, 12 isolates were received from Ramathibodi Hospital and 3 isolates were from Siriraj Hospital. One isolate was obtained from a patient admitted to Pramongkutklao Hospital, Bangkok. Nine isolates were received from Bamrasnaradura Hospital, Nonthaburi. Details of specimen sources were as follows: 11 isolates from blood specimens; 1 isolate from lymph node; 13 isolates from unknown sources. Eleven isolates were from the patients having history of HIV infection. Regarding the period of specimen collection, 10 isolates were obtained in 1998, one isolate was obtained in 1999 and fourteen isolates had no record on the collection period (Table 5).

The bamboo rat isolate was a gift from Dr. Samaniya Sukroongreung, Department of Clinical Microbiology, Faculty of Medical Technology, Mahidol University. This isolate was collected in 1987.

Four isolates were purchased from the ATCC. Two (ATCC 64101 and 24100) were clinical isolates and the others (ATCC 64102 and ATCC 18224) were bamboo rat isolates. The ATCC 18224 isolate was obtained from the liver of a bamboo rat (*R. sinensis*) captured in Vietnam in 1956 whereas ATCC 64102 was obtained from the lung of a bamboo rat (*R. pruinosus*) captured in China in 1986. The ATCC 24100 isolate was cultured from spleen of an American patient in 1973. This patient had a history of working in Vietnam and travelling throughout Southeast Asia

(SEA). The ATCC 64101 isolate was obtained from a Chinese patient in 1985 (Table 6).

2. Restriction Endonuclease Digestions and PFGE

Accessment on the type of restriction enzymes used to generate a suitable number of well-separated fragments was performed. Among all 17 enzymes tested (*Ase* I, *Bfa* I, *BamH* I, *Spe* I, *Sac* II, *Xba* I, *BssH* II, *Xho* I, *Nhe* I, *Sma* I, *Sal* I, *Bgl* I, *Sfi* I, *Sgf* I, *Csp* I, I-*Ppo* I and *Not* I), *Not* I (5' GCGGCCGC 3') and *Sfi* I (5' GGCCNNNNGGCC 3') provided discrete fragments of DNA ranging in size from 50-550 kb (Fig. 35). However, *Not* I gave the best optimum number of fragments and the clearest restriction endonuclease analysis patterns. Using *Not* I restriction endonuclease, 31 different DNA banding patterns were generated among 67 strains of *P. marneffei* examined. Among 67 isolates tested, two very different patterns are distinctly different from one another and can be clearly seen. Forty-five isolates (67.16%) were classified as MPI and 22 isolates (32.84%) were classified as MPII (Table 15). The differences between the 2 MPs are clearly noted. There is one or no DNA fragment ranging in size between 400 – 500 kb presented in MPI whereas a few DNA fragments between 400 – 500 kb are clearly noticeable in MPII. DNA bands with the size less than 250 kb could not be well-separated. We therefore chose to concentrate on fragments in the size range of 250 – 550 kb. Using Bio-profil[@] software at 95% confidential, the isolates belong to the MPI could be separated into 6 subgroups (MPIa – MPIf) whereas those of the MPII could be separated into 3 subgroups (MPIIa – MPIIc) (Fig. 36-41). There were 7 isolates that can

not be placed in any subgroup. PFGE patterns of isolates within each subgroup was identical by DNA fragment sizes as shown in Figure 42. A filled regular line represents a common DNA fragments found in all *P. marneffei* member of each subgroup while a dotted line represents a DNA fragments in some member of each subgroups. For instance, among 10 isolates belonging to MPIa subgroup, DNA fragments of 500, 410, 320, 300 kb were found in most isolates of this subgroup whereas DNA fragments in the sizes of 460, 350, 290 and 280 kb were presented some isolates.

The relatedness between subgroups was as follows; the relatedness between MPIa and MPIb was 92%; MPIc and MPIa&b was 90%; MPIe&d and MPIa&b&c was 76%. For those isolates of MPII, the similarity between MPIIa and MPIIb was 92% while MPIIc and MPIIa&b displayed 90% similarity.

Based on the geographical distribution of *P. marneffei* isolates, 7 isolates (50%) from northern Thailand were of MPI and the other 7 isolates from the same geographical region were of MPII. Fourteen isolates (59.1%) from the outbreak in southern Thailand were of MPI whereas 9 isolates (40.9%) belonged to MPII. Among 25 isolates obtained from central region of the country, 19 isolates (76%) were of MPI and 6 isolates (24%) belonged to MPII. The MPs of three bamboo rat isolates appears to have similar patterns to those of human isolates and belonged to MPI (Table 15).

To determine the patterns of strains obtained in 1956, 1973, 1985, 1986 and 1987, all isolates were of MPI and belonged to subgroups MPIc, MPIa, MPIb, MPId and MPIa, respectively. Four of seven isolates (57.1%) obtained in 1995 were classified as MPI and belong to subgroups MPIc (2 isolates), MPId (1 isolate) and MPIa (1 isolate).

The others were of MPII and belonged to subgroups MPIIa (1 isolates) and MPIIb (2 isolates). In 1998, thirty-one isolates were obtained. Fifteen isolates (48.4%) were of MPI and belonged to MPIa (4 isolates), MPIb (4 isolates), MPIc (6 isolates), MPIe (2 isolates) and MPIf (2 isolates). Sixteen isolates (51.6%) were of MPII and belonged to MPIIa (4 isolates), MPIIb (2 isolates) and MPIIc (6 isolates). There were five isolates that could not be classified into any subgroup. Four isolates obtained in 1999 possessed MPIb (2 isolates) and MPIIc (2 isolates) (Table 16).

There was no particular MP specific for specimen of any source (Table 17). Among 23 isolates obtained from blood specimens, 17 isolates were of MPI and belonged to subgroups MPIa (3 isolates), MPIb (1 isolates), MPIc (4 isolates), MPId (1 isolate), MPIe (1 isolates) and MPIf (2 isolates). Six isolates were of MPII and belonged to subgroups MPIIa (2 isolates), MPIIb (1 isolates), MPIIc (1 isolate) and two isolates can not be placed in any subgroup. Two isolates cultured from CSF belonged to subgroups MPIb and MPIIc (Fig. 43,A). Three isolates obtained from bone marrow specimens belonged to MPIc, MPIIa and MPIIc (Fig. 43,B). Three isolates obtained from oral tissue belonged to subgroups MPIa and MPIIa (Fig. 43,C). There were two isolates from skin abscesses classified as subgroups MPIc and MPIIa, respectively (Fig. 43,D).

In addition, we also performed RFLP using *Hae* III digestion as previously described by Vanitanakom *et al* (17). The purpose of this study was to verify whether there was any correlation between patterns generated by the two methodologies (Fig. 44, I&II). We observed that isolates classified as DNA type I by *Hae* III RFLP fell into

MPIIa, MPIa, MPIb, MPIc and MPIe subgroup (Fig. 44, IIA) whereas those of DNA type II were classified as MPIIb, MPIIc, MPIb, MPIc, MPId subgroup (Fig. 44, IIB).

Finally, three bamboo rat isolates were of MPI and belonged to different subgroups namely, MPIa, MPIb and MPId.

Figure 35: Pulsed-field gel electrophoresis of genomic DNA of *P. marneffei* after digestion with different restriction endonuclease enzymes: *Not* I (A), *Sfi* I (B), *Sma* I (C), *Bgl* I (D), *ApaI* (E), *BssH* I (F), and *Sac* I (G). In this figure, *Not* I gave the best optimum number of fragments and the clearest restriction endonuclease analysis patterns. Size markers (bacteriophage lambda concatemer ladder) are indicated to the left of the panel.

Table 15 The macrorestriction patterns (MPs) of all *P. marneffei* isolates tested.

Origin of isolates tested	**Total**	**MPI**	**MPII**
Northern Thailand	14	7 (50%)	7(50%)
Southern Thailand	23	14 (59.1%)	9 (40.9%)
Central Thailand	25	19 (76%)	6 (24%)
Bamboo rat isolate	1	1 (100%)	-
ATCC isolates	4	4 (100%)	-
Total	**67**	**45 (67.16%)**	**22 (32.84%)**

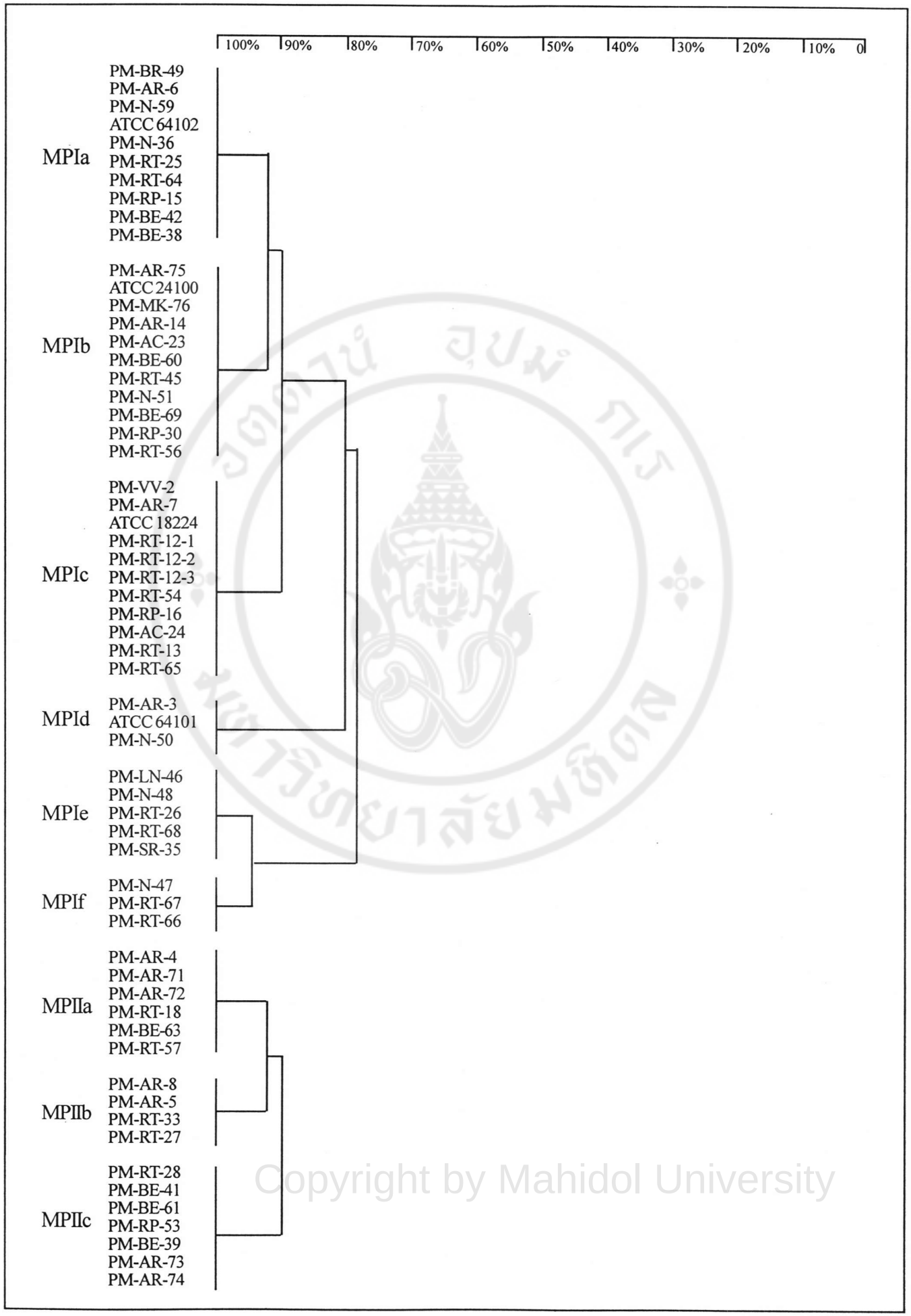

Figure 36: Dendogram of *P. marneffei* isolates.

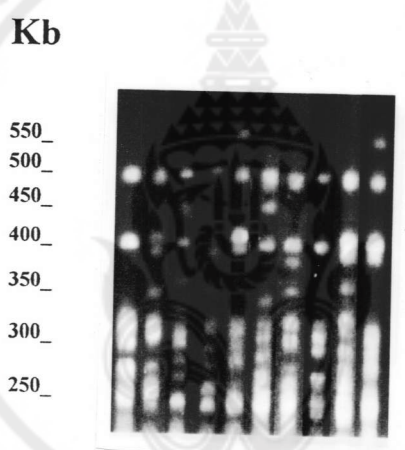

Figure 37: PFGE pattern of whole genome of *P. marneffei* isolates classified as MPIa subgroup. DNA fragments of 500, 410, 320, 300 kb were found in most isolates of this subgroup whereas DNA fragments of 460, 350, 290 and 280 kb were presented in some isolates. Size markers (bacteriophage lambda concatemer ladder) are indicated to the left of the panel.

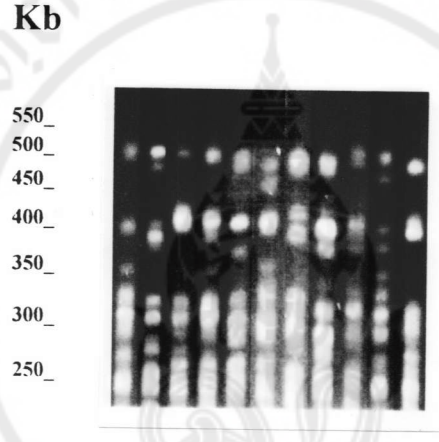

Figure 38: PFGE pattern of whole genome of *P. marneffei* isolates classified as MPIb subgroup. DNA fragments of 500, 410, 320, 300 kb were found in most isolates of this subgroup whereas those of 380, 350, 290 were found in some isolates. Size markers (bacteriophage lambda concatemer ladder) are indicated to the left of the panel.

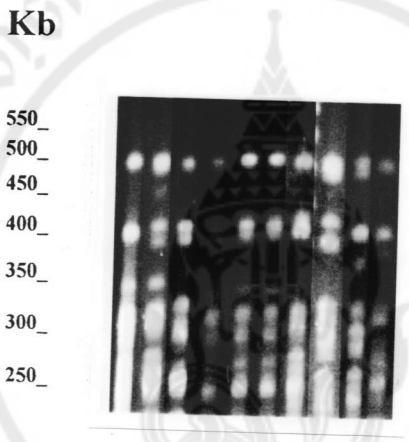

Figure 39: PFGE pattern of whole genome of *P. marneffei* isolates classified as MPIc subgroup. DNA fragments of 500, 400, 300 kb were found in most isolates of this subgroup whereas those of 420 and 280 kb were found in some isolates. The sizes of the marker are indicated to the left of the panel.

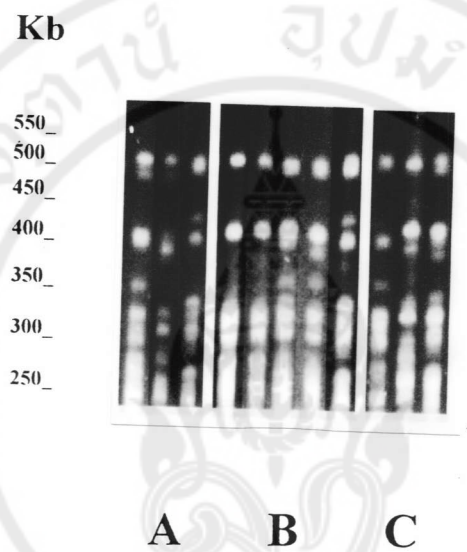

Figure 40: PFGE pattern of whole genome of *P. marneffei* isolates classified as MPId (A), MPIe (B) and MPIf (C) subgroup. DNA fragments of 500, 410, 330, 310 and 290 kb were found in most isolates of MPId subgroup whereas those of 430, and 340 kb were found in some isolates. DNA fragments of 500, 410, 320, 310 and 290 kb were found in most isolates of MPIe subgroup whereas those of 390 and 350 kb were found in some isolates. Only DNA fragments in the sizes of 500, 400 and 290 kb were found in most isolates of MPIf subgroup. Size markers (bacteriophage lambda concatemer ladder) are indicated to the left of the panel.

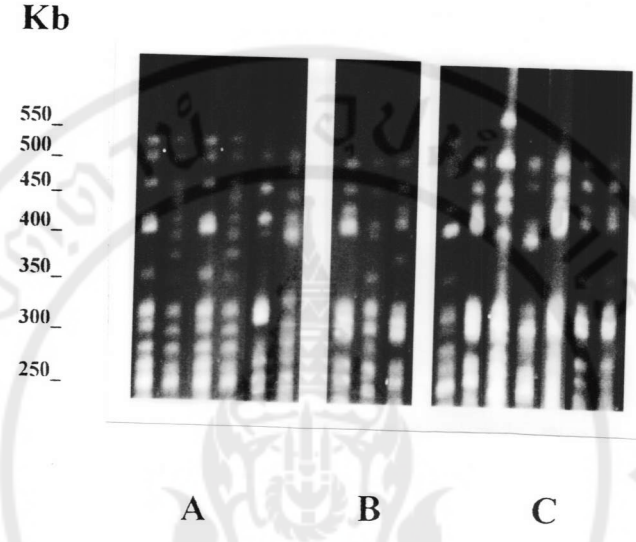

Figure 41: PFGE pattern of whole genome of *P. marneffei* isolates classified as MPIIa (A), MPIIb (B) and MPIIc (C) subgroup. DNA fragments in the sizes of 508, 490, 450, 400, 306 and 290 kb were found in most isolates of MPIIa subgroup whereas those of 430 and 340 kb were found in some isolates. DNA fragments of 480, 400, 305 and 290 kb were found in most isolates of MPIIb subgroup whereas those of 500, 440, 420, and 360 kb were found in some isolates. DNA fragments of 490, 450, 400, 320 and 290 kb were found in most isolates of MPIIc subgroup whereas those of 520, 420 and 350 kb were found in some isolates. Size markers (bacteriophage lambda concatemer ladder) are indicated to the left of the panel.

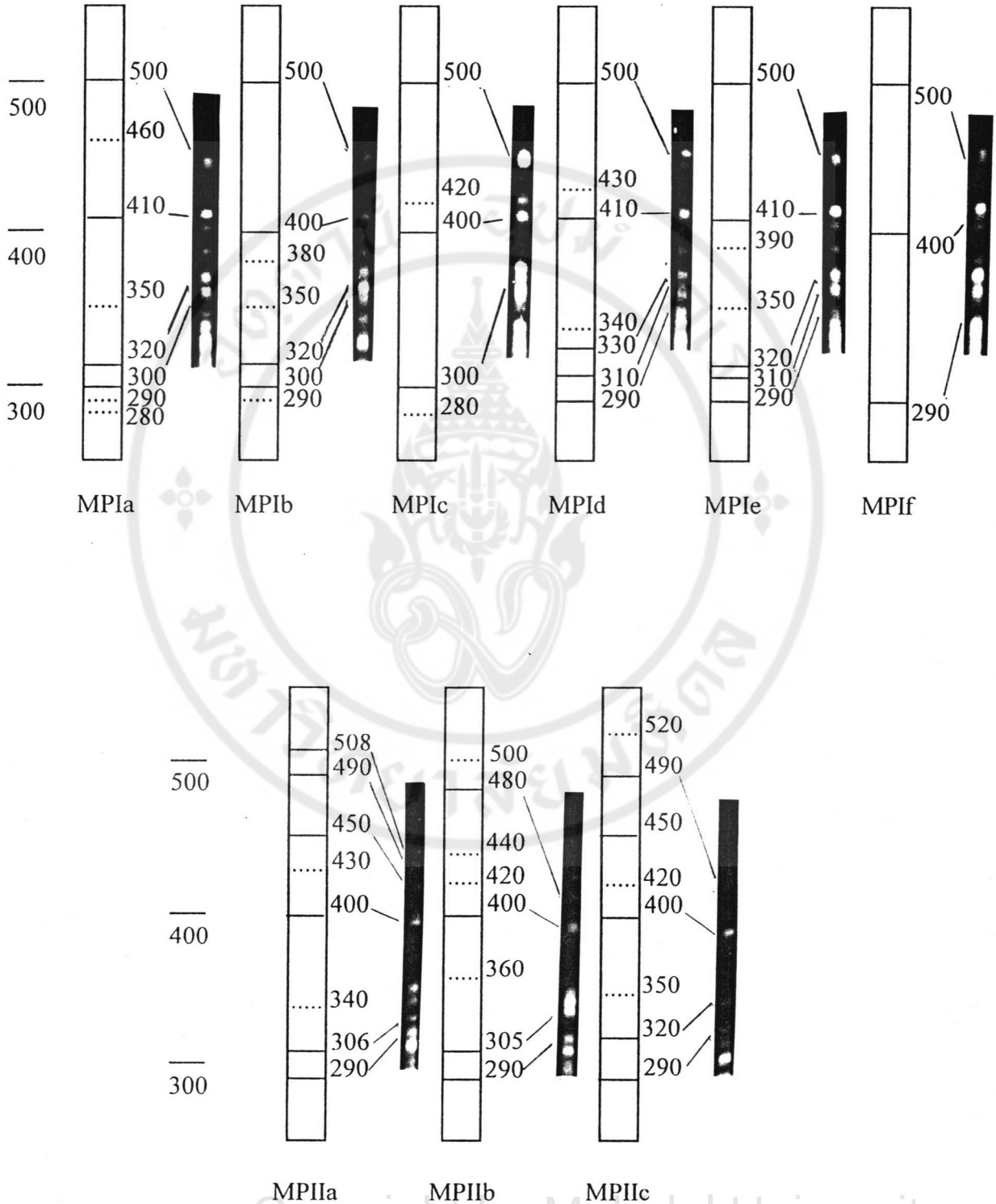

Figure 42: Drawing of the identical DNA fragment sizes within the subgroups.
—— represent the DNA fragment found in most isolates.
..... represent the DNA fragment found in some isolates.

Table 16 The macrorestriction patterns (MPs) of *P. marneffei* isolates collected at different periods.

Year of collection	Total	MPI	MPII
1956	1	1 (100%)	-
1973	1	1 (100%)	-
1985	1	1 (100%)	-
1986	1	1 (100%)	-
1987	1	1 (100%)	-
1995	7	5 (71.41%)	2 (28.57%)
1998	31	15 (48.39%)	16 (51.61%)
1999	4	2 (50%)	2 (50%)

Table 17 The macrorestriction patterns (MPs) of *P. marneffei* isolates cultured from clinical specimen.

Clinical specimen	**Total**	**MPI**	**MPII**
Blood	23	17	6
CSF	2	1	1
Oral cavity	3	1	2
Bone marrow	3	1	2
Skin	2	1	1
Liver	1	1	-
Spleen	1	1	-
Sputum	1	1	-
Total	**36**	**24**	**12**

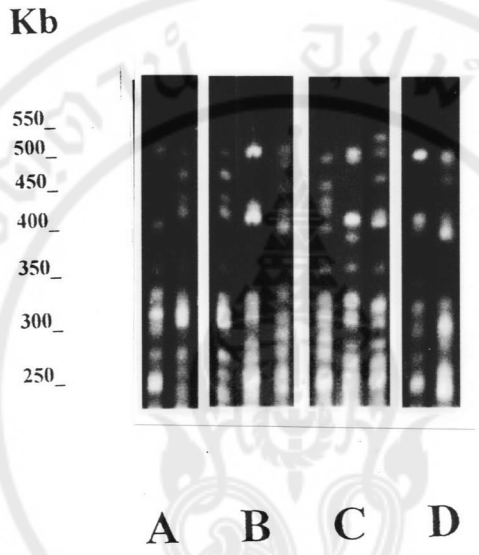

Figure 43: PFGE patterns of *P. marneffei* isolates obtained from different clinical specimens: CSF (A), bone marrow (B), oral cavity (C) and skin (D). There was no particular MP specific for specimen of any source. Both DNA banding patterns, MPI and MPII, of *P. marneffei* could be observed in all specimens. Size markers (bacteriophage lambda concatemer ladder) are indicated to the left of the panel.

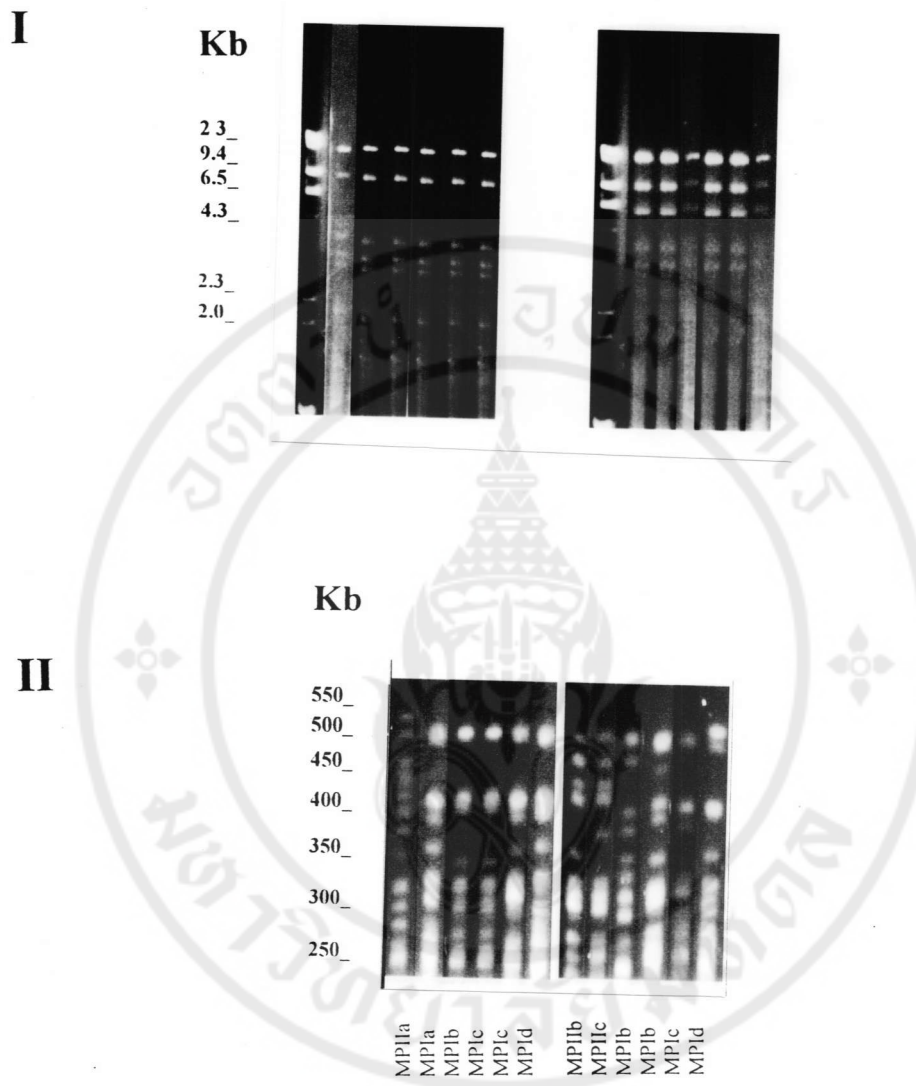

Figure 44: Comparison of RFLP patterns generated by *Hae*III restriction endonuclease digestion of genomic DNA of *P. marneffei* isolates (I) and PFGE pattern generated by *Not*I restriction endonuclease digestion of whole genome of *P. marneffei* isolates (II) were shown. No specific correlation of RFLP patterns to any macrorestriction patterns generated by PFGE. Size markers, bacteriophage lamda DNA *Hind* III digested and bacteriophage lambda concatemer ladder, are depicted on to the left of each panel, respectively.

CHAPTER V

DISCUSSION

At the beginning of this study, very little information was available on the pathogenesis of penicilliosis marneffei, and the immunology and molecular biology of the *P. marneffei* itself. Hence, the immune response in animal model was first performed in order to gain insights into some basic knowledge need for MAbs production and other biological studies. Then, the genomic fingerprinting of *P. marneffei* isolates obtained from different geographical regions and sources using a restriction endonuclease digestion and PFGE was performed for molecular epidemiology study of this organism.

1. Immune Response in Mice Experimentally Infected with *P. marneffei*

The initial step of establishing *P. marneffei* infection in susceptible hosts is virtually unknown at present time. The fact that *P. marneffei* could be cultured from soil samples (50,53) suggests that both humans and bamboo rats are infected by inhalation of fungal spores (conidia) into the lungs and followed by multiplication and dissemination. From surveys of the bamboo rats for natural infection by *P. marneffei* both Ajello *et al* (51) and Chariyalertsak *et al* (53) found that among the internal organs of culture-positive rats, the lungs were the most common organ harboring *P. marneffei*. Later, Hamilton *et al* (54,55) demonstrated that lung tissue could bind to the surface of *P. marneffei* conidia. The study of *in vitro* growth of *P. marneffei* both in a cell-free system and in a macrophage system by Cogliati *et al* (68) demonstrated that *P. marneffei* conidia were phagocytized by non-stimulated murine macrophages

J774 after 2 hours of incubation and thereafter the *P. marneffei* grew as yeast-like cells during the next 24 hours. In penicilliosis marneffei cases, the histopathology of the infected tissues is characterized the intracellular localization in macrophages and the three distinct reactions, namely granulomatous, supperative and anergic with necrosis, could be observed (3,7,22,31,35,38,45). The granulomatous reaction is seen especially in organs of the reticuloendothelial system in immunocompetent patients. The granulomas form by accumulation of histiocytes, epithelioid cells and lymphocytes. The supperative reaction with multiple abscesses develops in a variety of organs but the lung, skin and subcutaneous tissue of patients with normal immunity are the most common organs involved. The anergic and necrotizing reaction usually occurs in immunocompromised patients. The reaction is characterized by focal necrosis surrounded by histiocytes.

In general, principal mechanism of innate immunity against intracellular microbes is phagocytosis. However, this immunity is quite ineffective in controlling the colonization and spread of some of these microorganisms. Cell-mediated immunity (CMI) is generally considered to be the major protective immune response against intracellular microbes. However, there is still a debate on the possible involvement of humoral-mediated immunity (HMI) in protection against these intracellular microbes. The protective role of CMI in intracellular infection has been clearly demonstrated in the *Listeria monocytogenes* infection model. After the uptake of *L. monocytogenes*, the macrophages induced NK cells to release interferon gamma, and the interferon gamma in turn induced nitric oxide (NO) production by macrophages. NO is a direct listericidal molecule. It also can induce vascular dilation

that induces the migration of leukocytes to the site of infection (100). Similarly, the yeast cells of *H. capsulatum* which resided within macrophage were also stimulated very effective CMI response both under *in vitro* and *in vivo* situations (64). Production of interferon gamma and TNF-α by macrophages and NK cells allow the macrophages to have increased intracellular killing activity against *H. capsulatum* by increasing NO production (57,58,64).

In animal model, Kudeken *et al* (66,67) demonstrated that CMI played crucial role in defense against *P. marneffei* infection. The enhanced production of reactive nitrogen intermediates by interferon gamma-activated macrophages led to a significant reduction of *P. marneffei* viability. However, the role of humoral immune response in this infection has not been investigated in details. In our experiment, we put emphasis on the pathology of infected mice and on the kinetics of antibody production to determine immune response in these experimentally infected animals. The pathology of mice experimentally infected with *P. marneffei* by injection of the fungal conidia via SC or IP route was similar to those reported by Deng *et al* (50) and DiSalvo *et al* (45). These investigators also found the formation of an abscess at the injection site as well as at several other abscesses in remoted internal organs of the infected animals. However, these investigators did not investigate the humoral immune response of these infected animals. We found that the antibody levels (1:125 – 1:625) in the infected animals could be detected within 4-8 weeks after the infection was initiated (Fig. 10). The immunoblot patterns obtained from the reaction of CCF with immune mouse sera (Fig. 11) looked similar to those obtained from the reaction of CCF with sera from HIV patients with culture confirmed penicilliosis marneffei reported earlier by Chongtrakool *et al* (43). In that study, the 38-kDa antigen showed

very strong reaction by Western blotting. However, only 23 out of 51 penicilliosis marneffei patients were antibody positive for this 38-kDa antigen. This may be explained based on the defect immune response in HIV infected patients (101,102) or the possible occurrence of circulating immune complex (103,104). In contrast, Vanitanakom *et al* (44) found that the immunogenic proteins of molecular weight around 54 and 50 kDa in the culture filtrate of the yeast phase of *P. marneffei* growth gave strong reaction with sera from AIDS patients with penicilliosis marneffei by Western blotting. The reactivity of 54 and 50 kDa proteins were detected in 60.0 and 57.6% from 33 sera of this group of patients.

However, the role of these antibodies in host defense is still virtually unknown. Although, adoptive transfer of spleen cells from mice immunized with *H. capsulatum* results in immunity to challenge with *H. capsulatum* in the recipients (64), the experiment did not rule out the involvement of cell-mediated response. So, additional experiments should be performed to demonstrate a role of antibody in host defense against *P .marneffei* infection.

2. Identification and Characterization of *P. marneffei* Components in CCF and Exo-Ag.

In general, a large number of fungi produce many different antigens having a large varieties of biological activity. Some of them are cell associated, e.g. cell wall and cell membrane components. Some are secreted extracellularly into the medium. Fungal cell walls are made mostly of polysaccharides that comprise typically about 80% of their dry weight. Chitin, glucan, mannan, chitosan and galactomannan are the

most predominant polysaccharides or carbohydrate moiety of the glycoproteins of fungal cell wall. Proteins are present in lower proportion, typically representing only between 3 and 20% of the dry weight. Lipids are also present but in much smaller proportion (105). Most proteins present in the fungal cell walls are in the form of glycoproteins and these components are extremely important since they are one of the most predominant antigenic determinants of fungal cells (105-110). Some of these glycoproteins are enzymes responsible for structural modification of the wall or degradation of large molecules to release small products prior to entering the cell such as invertase, melibiase and β-glucosidase (105). In the present study, two different preparations of *P. marneffei* antigens were used, i.e., CCF and Exo-Ag. The CCF was prepared from the mycelial phase of growth of *P. marneffei* whereas the Exo-Ag was prepared from the yeast phase of growth. Both antigen preparations contained various fungal products and fungal cell wall components that were cleaved off during development or aging. There were more than 20 components ranging in size from 29 kDa to 200 kDa. Most of these were glycoproteins or polysaccharides, judging from their Coommassie blue staining (Fig. 12) and lectin binding assay (Fig. 13). No significant difference between the components present in both antigen preparations was observed. However, judging from intensity of the bands, the CCF appeared to contain higher content of proteins. This finding was similar to the study reported by Vanittanakom *et al* (44). The later group also found a similarity of the proteins profile of the mycelial phase antigen and yeast phase antigen but their mycelial phase antigen contained lower amount of protein content and showing more diffusible bands compared to those found in yeast phase antigen. Both of their antigen preparations also contained many other components with molecular weight ranging in size from 29

kDa to 200 kDa. They also found that the protein profiles of the culture filtrate antigen prepared from different *P. marneffei* isolates were similar to each other, although the relative proportion of various components varied from strain to strain. However, after probing with sera from 33 AIDS patients having penicilliosis marneffei, no 38-kDa antigen could be demonstrated. Such a discrepancy should be investigated. It is unlikely that the difference is caused by the difference of fungal strain used. The most logical reason may be related to difference in the culture conditions or the methods of antigen preparation used by these investigators.

The components present in both antigen preparations were proven to be highly antigenic when the blots were probed with immune mouse sera (Fig. 11) or sera of culture-proven penicilliosis marneffei cases (43,44). Many fungi are able to excrete a large variety of proteins and polysaccharides. The cell wall is made mostly of polysaccharides (105). Several studies have been carried out to elucidate the structure of these extracellular polysaccharides (EPS) which are highly immunogenic (106-108). It is clear from these studies that EPS produced from many fungi appeared to have similar structure, and that mannose, galactose, glucose are the dominant polysaccharides (109-110). Hence, the cross reactivity commonly found in serodiagnosis for fungal infection especially between closely related genera such as between *Penicillium* and *Aspergillus* is common. Both genera contain and produce galactomannan with very similar immunogenic galactofuranosyl side chain (106,108,110). The main chain of the polysaccharide of *A. fumigatus*, *A. niger*, *P. chorlesii* and *Paecilomyces variotii* (a filamentous fungus closely related to *Penicillium*) is composed of either 1→2 or 1→6 linked α-D mannopyranosyl residues.

The galactose is present as side chain of 1→5 linked β-D-galactofuranosyl residues (106,108,111). Brouwer (109) demonstrated that IgG anti-*Penicillium* reacted with several *A. fumigatus* antigens with molecular weights between 28 and 130 kDa and *vice versa*. With *P. marneffei,* the cross reactivity of this organism with *A. fumgatus*, *H. capsulatum* and *P. primulinum* could be readily observed (10,12,41). However, some components in CCF and Exo-Ag have been proven to be the candidate antigens with diagnostic potential such as the 38-kDa antigen, 50-kDa antigen and 54-kDa antigen (43,44). Recently, *MP1* gene of *P. marneffei* had been cloned by Cao and associates (112). This gene encoded an abundant and highly antigenic cell wall mannoprotein designated as Mp1p. It was a secreted product found in the cell culture supernatant (15). Western blotting with polyclonal rabbit anti-Mp1p antibody revealed that Mp1p has predominant band with molecular weight of 90 kDa and proven to be specific for *P. marneffei* since anti-Mp1p antibody did not cross react with any other proteins from lysate of *C. albicans*, *H. capsulatum* or *C. neoformans.*

3. MAb Production, Characterization and Application.

The data presented in the present study showed that MAbs reactive against the mycelial and yeast antigens of *P. marneffei* could be readily produced in BALB/c mice immunized only with the *P. marneffei* mycelial crude culture filtrate antigen. For example, components recognized by MAbs 8B11 and 3B9 are most likely distributed on the *P. marneffei* cell walls and the immunoreactive epitopes appear to be common between the two forms of this fungus demonstrated by IFA. The immunoblot patterns, particularly those showing broad smearing bands with the Exo-Ag (Fig. 14 B&D) suggested that these components are high molecular weight polysaccharides or

glycoproteins. *Penicillium* and *Aspergillus* seem to share many common antigenic epitopes. Nigeran, a hot water-soluble polysaccharide made of D-glucopyranose with alternating α-1,3 and α-1,4 linked, and D-galactofuranoside with β-1,4 linked are immunodominant in EPS of both fungi (105). Moreover, Estrada *et al* (41) showed that their MAb EB-A1 directing against *A. fumigatus* galactomannan could react with *P. marneffei* presented in tissue specimens using immunohistochemistry staining. However, the components recognized by both of these MAbs (8B11 & 3B9) are unlikely to be the same components reported previously for *Aspergillus* and *Penicillium* by immunohistochemistry staining (41) because these MAbs did not react with any of the 4 *Aspergillus* species tested. Although, the data presented in our study indicated that these high molecular weight glycoprotein or polysaccharide components recognized by both MAbs (8B11 & 3B9) contained an abundant of mannose, glucose or N-acetyl glucosamine in their carbohydrate moiety, they were unlikely to be the same as present in Mp1p because they have molecular weights greater than this mannoprotein.

It should be mentioned, however, that both Mabs may not be entirely specific for *P. marneffei* because they also reacted by ELISA with the CCF prepared from *P. siamensis* and *P. pinophilum.* The seemingly positive immunofluorescent staining noted with *H. capsulatum* preparation (Fig. 19) may represent some unknown technical artifact as it did not resemble any known structure associated with the fungus. On the other hand, a nearby structure with morphological appearance of typical mycelium (in the same slide) was negatively stained by immunofluorescent test.

The components distributed on the cell wall of both mycelial and yeast phases were also reactive with MAb 8C3. These components were unlikely to be the same components recognized by MAbs 8B11 and 3B9. The smearing in the immunoblotting patterns suggested that the components recognized by MAb 8C3 were most likely glycoproteins, thus unlikely to be the common component found on *Penicillium* and *Aspergillus* or the Mp1p. The reactive epitope recognized by MAb 8C3 was not destroyed by periodate treatment, suggesting that it is present on the protein moiety of the glycoprotein. Like MAbs 8B11 and 3B9, MAb 8C3 reacted with the components present in CCF prepared from *A. nidulans* by ELISA and it also reacted with some components distributed on mycelial cell wall of *H. capsulatum* by indirect immunofluorescent antibody test, suggested that this protein is not unique for *P. marneffei.* This finding, as well as those noted with 8B11 and 3B9, favors the conclusion that the positive immunofluorescent staining of the component present in the 2-week old slide culture with these *P. marneffei* MAbs was due to a true cross reactivity between these two fungi.

The 38-kDa antigen recognized by MAb 3C2 and appeared to be a specific antigen of *P. marneffei* was also most likely to be glycoprotein, although, at this position, the Coomassie blue staining was invisible and the Con A staining was very weak. Like MAb 8C3, the immunoreactive epitope of MAb 3C2 was not destroyed by periodate treatment, suggesting that it is the protein moiety of the glycoprotein. But the amount of 38-kDa antigen present in both CCF and Exo-Ag was very low. Due to the fact that the MAb 3C2 recognized only the denatured form of the 38-kDa antigen, it was impossible to use MAb 3C2 to demonstrate the intracellular location of 38-kDa antigen in the cell. In *Paracocidioides brasiliensis*, a 43-kDa glycoprotein antigen of

unknown function was shown to be a paracoccidioidomycosis-specific antigen and was secreted extracellularly (113). *In A. fumigatus*, a 23-kDa antigen was proven to be a serine protease (105). Thus, further studies are required to elucidate the nature and function of this low molecular weight 38-kDa antigen.

Due to the fact that *P. marneffei* become a new emerging pathogenic fungus, very little information or specific reagents are currently available. Once the MAbs against *P. marneffei* are available, they can be used as probes to study the differential distribution of specific cell wall components within different fungal structures, or to monitor local changes of wall architecture and biochemistry during hyphal and conidia morphogenesis. For dimorphic fungi, these MAbs could be used as probes to study the differential distribution of specific wall components during the phase conversion between the mold form and yeast form. For examples, both 8B11 and 3B9 reacted with all components of various developmental stages except the conidia, while 8C3 reacted only with the swelling conidia, germ tube and mature mycelium. These findings are similar to the study reported by Marshall *et al* (114). Their MAbs, raised from mice immunized with hyphal walls of *Neurospora crassa* or *Paxillus involutus,* also recognized only some developmental stages of respective fungi used for immunization. These MAbs can be used in the affinity purification of these components, thus making it possible to have purified components for elucidating the structure. The latter could be of value in searching of target for new antifungal agents and so on. *Collectotrichum largenarium* conidia, for instance, contain xylose and large amount of rhamnans which are absent in the cell walls of mycelium, whereas the content of mannose decreases after spore germination (105). *Fonsecaea pedrosoi*

conidia also contain large amount of rhamnose, whereas large amount of galactose is prominent in mycelium (115). The chemical changes in the cell walls of *P. notatum* during germination were also investigated (116). The resting stage of conidia cell wall contained glucose, galactose, mannose and rhamnose, but the rhamnose was absent during germination (105). The change in the cell wall occurring during aging could also be observed, although it must be remembered that this change is mainly quantitative. The mycelial cell wall of 24-hour-old culture of *Trichoderma voridae* contained less β-1,6-linkage glucan, but more chitin and protein than cell wall obtained from the same fungus grown for 18 hours only (105). In our study, we found that several mycelial cell wall components of *P. marneffei* recognized by 8C3 were absent in aging mycelium (2-week-old culture). The differences in the cell wall composition of dimorphic fungi have been observed previously (117-119). Cell wall from the mycelial form of *H. capsulatum* contained more mannose than its yeast counterpart. On the other hand, the wall of some dimorphic fungi has higher content of α-1,3-glucan in the yeast form (105,119). Hence, it is not surprising why our MAbs stained only weakly with the mycelial phase of *H. capsulatum* but not at all with its respective yeast form.

In addition to being valuable for study on fungal cell development or morphogenesis, these MAbs also have diagnostic potential as well. Although in many cases of penicilliosis marneffei, the diagnosis can be readily made from microscopic examination of smear taken from skin lesion, the sensitivity is very low and it may be confused with histoplasmosis or dissemminated cryptococcosis. A number of serological diagnosis tests have been developed for the detection of antibody to *P.*

marneffei. These include an immunodiffusion test (10), indirect immunofluorescent antibody test (11,12), Western blotting (43,44) and ELISA (14,15). Although, these tests are somewhat specific, higher specificity and sensitivity are needed.

Yuen and colleagues (11) have developed an indirect immunofluorescence test to detect antibody in the serum from patients with penicilliosis marneffei using germinating conidia and yeast-hyphal forms of *P. marneffei* as antigens. All 8 documented *P. marneffei* cases had an IgG titer of 1:160 or more while 95 patients with others infections and all 78 healthy controls had an IgG titer of 1:40 or below. Detection of antibody specific *to P. marneffei* antigens by Western blotting has been developed (43,44). However, antibody specific to 38-kDa antigen was detected in only 30 (46%) of 65 patients with penicilliosis marneffei but it was in 45 (17%) of 262 asymptomatic HIV-positive individuals. The titer was also found in 17 (25%) of 67 patients with cryptococcosis or candidiasis (43).

Detection of antibodies specific to 54 and 50 kDa protein antigens of *P. marneffei* was evaluated by Vanittanakom *et al* (44). Although these antibodies could be detected two months before a definite diagnosis by fungal culture was made, these antibodies could be detected only in 60.0 and 57.6%, respectively. Recently, antibody specific to a 61-kDa antigen was reported to be present in 18 (86%) of 21 penicilliosis marneffei patients (70). It is clear that these results are at present not sufficiently useful for diagnosis purpose and requires further investigation if one is to have a more reliable test for this fungal infection.

Detection of *P. marneffei* organisms or *P. marneffei* antigens in clinical specimens indicates active infection and proven to be more useful than detection of antibody to *P. marneffei* for the diagnosis of *P. marneffei* infection. However, one of the major problems often encountered with the tests is the widespread occurrence of cross reactivity between related or even unrelated fungi. Antibody to *A. fumigatus* galactomannan reacted with *P. marneffei* on immunohistochemical staining (41). A urinary antigen detection test developed for diagnosis of *H. capsulatum* infection, using rabbit IgG antibody has been reported to give positive results in 17 of 18 culture-confirmed penicilliosis patients (120). In contrary, Kaufman *et al* (12) reported that rabbit antiserum raised against a culture filtrate of the yeast phase of growth of *P. marneffei* reacted with the yeast form of *H. capsulatum* but not with the hyphal form of *A. flavus*, *A. fumigatus*, *H. capsulatum*, *C. albicans* and *C. neoformans*.

In the present study, we showed that our MAbs could be readily used as specific immunofuorescent reagent for the identification of *P. marneffei* in culture and in the tissue. The tissue phase of *P. marneffei* and *H. capsulatum* are difficult to distinguish from one another by microscopic examination (Fig. 27). This problem is of paramount importance because both fungal infections are important opportunistic infections in patients with HIV infection, particularly in those from Southeast Asian countries (5,36). Moreover, the managements of these two conditions are also different. Using these MAbs, three cases of penicilliosis marneffei and two out of four cases of histoplasmosis gave positive immunofluorecent staining. Other fungal infection including aspergillosis (3 cases), candidiasis (4 cases), cryptococcosis (2 cases), chromomycosis (1 case), phycomycosis (2 cases) and actinomycosis (1 case) were negative for immunofluorecent staining with all MAbs (Table 14). We believe

that the two cases of histoplasmosis that stained positively with our MAbs were originally misdiagnosed, because (i) the presumptive diagnosis of these patients was based on microscopic examination of the tissue sections stained only with H&E and it is difficult to be certain except by experienced personnel who could detect the central septum of *P. marneffei* (Fig. 27) and (ii) the yeast cells of *H. capsulatum* from culture could not be stained by our MAbs. Moreover, our MAbs gave positive immunofluorescent staining only with simulated tissue prepared from the mixing of human tissue with *P. marneffei* but not with other fungi. For more certain, additional clinical specimens with culture proven cases should be available for analysis.

Although, these MAbs have been proven to be valuable immunological reagent for immunofluorescent staining of tissue biopsy especially for the diferentiation between *P. marneffei* and *H. capsulatum*, more rapid and simply diagnostic method with reliable sensitivity and specificity still required.

Kaufman *et al* (53) have developed an immunodiffusion test and a latex agglutination test to detect antigen in the serum of patients. Both tests used polyclonal rabbit antiserum raised against a culture filtrate of fission arthroconidia of *P. marneffei*. Antigen in serum was detected in 13 (76%) of 17 infected patients by latex agglutination and in 10 (60%) patients by immunodiffusion. Cao *et al* (112) have cloned *MP1* gene and developed an ELISA method to detect mannoprotein Mp1p that might be present in the serum of these patients. It was found that Mp1p could be detected in 17 (65%) of 26 penicilliosis marneffei patients whereas none of the 40 blood donors and 29 patients with tuberculosis was positive in the Mp1p detection assay. Recently, using polyclonal rabbit antiserum, Desakorn *et al* (71) were able to

detect immunoreactive component(s) in the heated urine of most patients with *P. marneffei* infection. All 33 culture-proven penicilliosis patients were positive for this test with a median titer of 1:20,480. However, with undiluted samples, 67 (27%) of 248 other hospitalized patients and 3 (6%) of 52 healthy controls were also positive. The sensitivity and specificity of the test were 97% and 98%, respectively, when using a cut-off titer of 1:40. The availbility of MAbs will certainly help to make these tests more reproducible as the quality of the reagents can be more readily controlled.

4. Genomic Fingerprinting by Restriction Endonuclease Digestion and PFGE

Epidemiology studies of fungal infections are essential if we are to understand the biology of the fungi and so as to obtain improved treatments for infections. Serotyping was used as an epidemiological tool for many years (121-124). However, due to the increase in the power of discrimination of strains, the DNA typing methods become more popular among epidemiologist. The success of DNA typing is dependent on variation in the DNA sequence, degree of discrimination with restriction endonuclease as well as discriminatory supplementation of Southern hybridization. Burnie *et al* (78) has developed a genome based DNA fingerprinting system for *A. fumigatus*. From 21 isolates of *A. fumigatus* obtained from 8 aspergilloma patients, all isolates were identical when their DNA's were cut by *Eco*RI whereas *Xba*I delineated six DNA types by REA. Cooper *et al* (79) also used this technique to type *Sporothrix schenckii.* From 36 clinical isolates and environmental isolates, 8 different DNA types were obtained after digestion with *Hae*III. The DNA fingerprinting pattern of *H. capsulatum* using PCR-based RAPD was reported by Poonwan *et al* (80). The investigators found that the DNA patterns of all 13 Thai

isolates of *H. capsulatum* were similar. However, the DNA patterns of the Thai isolates were clearly different from that of the U.S. reference strain (*H. capsulatum* IFM 46159).

Recently, PFGE is widely used in epidemiology study of many bacterial and fungal pathogens. These include the typing of *Staphylococcus aureus* (125), *Salmonella typhi* (126), *Yersinia enterocolitis* (127), *Klebsiella pneumoniae* (128), *Burkhoderia pseudomallei* (129), *C. albicans* (77), *H. capsulatum* (82) and *P. brasilliensis* (76). PFGE have been demonstrated as a useful and reliable typing method for investigating the source, transmission, or spread of nosocomial infections (125,126,128). PFGE was superior to ribotyping in strain differentiation of several microbial pathogens. For instance, ribotyping of 26 methicillin resistant *Staphylococcus aureus* isolates using *Eco* RI, *Hin*d III or *Cla* I and then probing with *Escherichia coli* 16S and 23 S rRNAs generated 6, 4, and 4 ribotyping patterns, respectively. In contrary, 24 different fingerprints were generated by PFGE after digestion of genomic DNA of the above 26 bacterial isolates with *Sma* I (125). The REA patterns produced by PFGE are stable and reproducible and are relatively easier to interpret when compared to the patterns produced by separation of the digested DNA by conventional agarose gel electrophoresis (125-130).

In this study, we used restriction endonuclease digestion of genomic DNA of *P. marneffei* and PFGE to type the fungal isolates obtained from the penicilliosis marneffei patients and bamboo rats. Clinical isolates included the isolates obtained from Thai penicilliosis marneffei patients admitted in hospitals located in different geographical regions of the country and two ATCC isolates collected from Chinese

and American patients. Three bamboo rat isolates were obtained from bamboo rats trapped from Thailand, Vietnam and China. Among all enzymes tested, high frequency cutting enzymes and enzymes that recognize AT-rich regions failed to generate the discrimination of DNA banding patterns, suggesting that the GC content in genomic DNA of *P. marneffei* is not too high. In general, the GC content of genomic DNA is variable. The quantitative difference of base composition could be reflected phylogenetically between each organism. For examples, the GC content of *Staphylococcus*, *Micrococcus*, *Enterobacteriaceae*, *Yersinia*, *Vibrio*, *Pseudomonas, H. capulatum* and *B. dermatitidis* are 30-35%, 66-75%, 39-58%, 46-47%, 40-50%, 57-70%, 47.3% and 48.2% respectively (131-135). Therefore, restriction enzymes that recognize GC-rich regions such as *Not* I (5' GCGGCCGC 3') and *Sfi* I (5' GGCCNNNNGGCC 3') can be predicted to give a discriminatory pattern.

Although *Not* I and *Sfi* I provided discrete fragments of DNA ranging in size from 50-550 kb (Fig. 35), *Not* I reproducibly gave the best optimum number of fragments and the clearest restriction endonuclease analysis patterns. In our study, among 67 isolates tested, 2 MP and 9 MP subgroups were obtained by *Not* I digestion. Our results are in contrast to those published by LoBuglio and Taylor (16) and Vanittanakom *et al* (17) in that LoBuglio and Taylor performed restriction enzyme digestion of ITS1-5.8S-ITS2 rDNA PCR products obtained from six isolates of *P. marneffei*. The restriction fragment length polymorphism patterns generated by *Ava*I, *Ava*II, *Hha*I, *Mbo*II and *Tag*I were identical among the six isolates. Although, the technique could not discriminate the difference of each isolate because the length of ITS1-5.8S-ITS2 rDNA region is too short and this region is a highly conserved region, the investigators claimed that their primers have the potential to be incorporated in a PCR identification of this

pathogenic fungus from clinical specimens (16). Subsequently, RFLP of genomic DNA of *P. marneffei* using *Hae*III restriction endonuclease digestion was reported by Vanittanakom *et al* (17). Only two DNA profiles (DNA type I and II) were obtained in their study. The DNA type I consisted of 18.0, 6.5, 4.6, 3.3, 2.9 and 2.5 kb whereas DNA type II consisted of 18.0, 6.5, 3.3, 2.9, 2.7, 2.2 and 2.0 kb. We also employed this method to type our isolates. The same findings were obtained. Half of *P. marneffei* isolates tested were of DNA type I and the other half was of DNA type II. Moreover, we found that the MPs of our *P. marneffei* isolates classified as DNA type I were of MPIb, MPIc, MPId, MPIIb and MPIIc and the MPs of isolates classified as DNA type II were of MPIa, MPIb, MPIc, MPIe and MPIIa. Therefore, the epidemiological application by this technique was limited when compared to the PFGE developed by our group.

With the limit on clinical data of each isolates obtained from different sources and sporadic cases, we can not make any definitive conclusion on the correlation between MPs and all known clinical parameters. For examples, 7 *P. marneffei* isolates obtained from the northern region of Thailand in 1995 were of MPIa (1 isolate), MPIc (2 isolates), MPId (1 isolate), MPIIa (1 isolate) and MPIIb (2 isolates). Fifteen isolates of *P. marneffei* isolates obtained from the southern region of Thailand in 1998 were of MPIa (2 isolates), MPIb (2 isolates), MPIc (2 isolates), MPIe (2 isolates), MPIf (2 isolates), MPIIa (2 isolates), MPIIb (2 isolates) and MPIIc (1 isolates). Moreover, we found that all three bamboo rat isolates were of MPI. One Thai bamboo rat isolate and the isolate obtained from bamboo rat captured in China (ATCC 64102) belonged to MPIa. Another bamboo rat isolate obtained from Vietnam (ATCC 18224) belonged to MPIc (Fig. 36).

Notably, the DNA banding pattern of Thai bamboo rat isolate was similar to PM-LN-46 and PM-N-48 (MPIe) whereas those of the other two bamboo rats isolates (China and Vietnam) were different from to any DNA banding pattern of Thai clinical isolates. More studies are required with *P. marneffei* isolates obtained from bamboo rats to verify the true susceptibility.

We also interested in using PFGE as a tool for discrimination of the virulent strains from avirulent stains of *P. marneffei*. In *H. capsulatum*, α-1,3 glucan-containing cell walls may be a virulence factor. The avirulent mutants that may be caused by the genetically defect strains that have 1,000-fold less α-1,3 glucan in their cell wall (105,136). In *Blastomyces dermatitidis*, avirulent strain has lost all α-1,3 glucan (105,136). We tried to relate the virulent of *P. marneffei* isolate or ability of fungal strain with particular MPs that can cause more severity of the disease by comparison between the source of specimens and MPs. Unfortunately, in this study, no correlation was observed (Fig. 43).

Notably, one interesting finding is that the isolates belonged to MPII did not exist among the *P. marneffei* obtained before 1995. In 1995, we found 71.4% of *P. marneffei* isolates belonged to MPI whereas 28.6% were of MPII. In 1998 and 1999, the incidence had increased to 50%.

Recently, PFGE have been used as a tool to type *C. albicans* for nosocomial fungal infection by Riederer *et al* (77). From 11 isolates of *C. albicans* that were collected from individual infants in neonatal intensive care unit (NICU) between January 1994 and December 1995, 7 DNA banding patterns were obtained using PFGE after

BssH II digestion. There were two pairs of identical DNA banding patterns. Both identical NICU pairs were recovered from infants who were in NICU at the same time.

In our case, however, we are currently collecting more isolates to verify an increase in MPII infected cases over a period of time.

CHAPTER VI

CONCLUSION

Four monoclonal antibodies (MAbs) highly reactive with the mycelial and yeast antigens of *P. marneffei* could be produced in BALB/c mice immunized with *P. marneffei* mycelial crude culture filtrate. By Western blot analysis, one IgG_1 MAbs (3C2) reacted specifically with 38-kDa antigen whereas the MAbs 8B11 and 3B9 (IgM subclass) mostly reacted with high molecular weight components (>200 kDa) produced during either mycelial or yeast phase of growth. The immunoreactive epitopes for these MAbs are most likely associated with carbohydrate moieties, judging from their susceptibility to periodate treatment and concanavalin A binding. This is in contrast to the immunoreactive epitopes for MAbs 8C3 (IgM) and 3C2 (IgG_1) which were resistant to destruction by periodate treatment. By immunofluorescent staining, MAbs 8C3 stained strongly with the yeast cells, swelling conidia, germ tube and mycelium, but not with aging mycelium and conidia, whereas MAbs 8B11 and 3B9 stained strongly with yeast cells, swelling conidia, germ tube and mycelium, but not with conidia. The yeast phase of *Histoplasma capsulatum* and *Cryptococcus neoformans* whose morphology is are closely similar to *P. marneffei* stained negatively with these MAbs. Hence, these MAbs exhibited diagnostic potential for *P. marneffei* in culture and tissue biopsy specimens by immunofluorescent staining. Using these MAbs, development of specific and reliable diagnosis tools can be archieved.

For genomic study of *P. marneffei*, PFGE was used to compare and analyze 67 isolates of *P. marneffei* obtained from penicilliosis marneffei patients admitted in

different hospitals located at different geographical regions (64 isolates) as well as from 3 bamboo rats. Digestion of genomic DNAs from these *P. marneffei* isolates with restriction endonuclease *Not* I and analyzed through PFGE, discrete DNA bands ranging in size from 50 – 550 kb could be observed. Using Dice coefficient analysis by the aid of Bioprofil software, 2 macrorestriction patterns (MPI and MPII) with 9 subgroups were obtained. Of the 64 human isolates, 42 isolates (65.6%) were of MPI and belonged to subgroups MPIa (8 isolates), MPIb (11 isolates), MPIc (10 isolates), MPId (3 isolates), MPIe (5 isolates) and MPIf (3 isolates). Whereas 22 isolates (34.4%) were of MPII and belonged to subgroups MPIIa (6 isolates), MPIIb (4 isolates) and MPIIc (7 isolates). Two bamboo rat isolates belonged to subgroup MPIa and one isolate was of subgroup MPIc. No significant correlation between the MPs and geographical regions or source of specimens was found. Instead it was found that all three bamboo rat isolates belonged only to the MPI group. This finding may reflect preferential sensitivities of bamboo rats to a particular fungal strain. Hence, the true susceptibility of bamboo rat infected by *P. marneffei* strains belonged to MPI need more study. Moreover, we also found that isolates obtained before 1995 were of the MPI group and increasing number of MPII isolates was observed since then. In this study, we demonstrated that PFGE of *P. marneffei* genomic DNA digested with *Not* I is a useful and reproducible method for comparing and analyzing different *P. marneffei* isolates for epidemiology purpose.

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APPENDIX 1

CULTURE MEDIA FOR FUNGAL CULTURE

1. SABOURAUD DEXTROSE AGAR (SDA)

Glucose	40.00	gm
Peptone	10.00	gm
Agar	20.00	gm
Distilled water	1000.00	ml

Sterilized by autoclaving at 121°C for 15 minutes and adjusted to a final pH of 5.5 to 5.6. This medium is commercially available.

2. BRAIN HEART INFUSION AGAR

Brain-heart infusion	37.00	gm
Glucose	20.00	gm
L-cysteine hydrochloride	1.00	gm
Agar	20.00	gm
Distilled water to	1000.00	ml

Adding 1 ml of 4N HCl and autoclaved at 121°C for 15 minutes.

APPENDIX 2

SODIUM DODECYL SULFATE-POLYACRYLAMIDE GEL ELECTRPHORESIS (SDS-PAGE)

1. Stock Acrylamide (30%)		
Acrylamide (Sigma)	30.00	gm
N,N-bis-methylene acrylamide (Sigma)	0.80	gm
Distilled water to	100.00	ml
2. Gel Buffer pH 8.9 (3.0M)		
Trisma base (Sigma)	9.075	gm
1N HCl	12.00	ml
Distilled water to	25.00	ml
3. Gel Buffer pH 6.8 (0.5M)		
Trisma base (Sigma)	3.00	gm
Adjusted pH with concentrated HCl		
Distilled water to	50.00	ml
4. Electrophoresis Buffer pH 8.3		
Trisma base (Sigma)	3.00	gm
Glycine	14.40	gm
SDS	1.00	gm
Distilled water to	1000.00	ml
5. Sample Buffer 5X pH 6.8		
Trisma base (Sigma)	0.3784	gm
SDS	0.50	gm
Glycerol	5.00	ml
2-mercaptoethanol	2.50	ml
Bromphenol blue	0.005	gm
Distilled water to	10.00	ml
6. Coomassie Blue Staining		
Coomassie brilliant blue R	0.20	gm
Glacial acetic acid	7.00	ml
Methanol	46.50	ml
Distilled water	46.50	ml
7. Destaining Solution		
Methanol	50.00	ml
Glacial acetic acid	70.00	ml
Distilled water to	1000.00	ml

APPENDIX 3

WESTERN BLOTING AND IMMUNOENZYMATIC STAINING

1. Towbin's Buffer

Trisma base (Sigma)	3.035	gm
Glycine	14.413	gm
Methanol	200.00	ml
Distilled water to	1000.00	ml

2. 0.15M PBS pH 7.2

NaCl	8.90	gm
Na_2HPO_4	1.28	gm
$NaH_2PO_4.H_2O$	0.156	gm
Distilled water to	1000.00	ml

3. Substrate Buffer for Horseradish Peroxidase (50mM Tris-HCl) pH 7.4

Trisma base (Sigma)	6.06	gm
Adjust pH with 1N HCl		
Distilled water to	1000.00	ml

4. Substrate Working Solution for Horseradish Peroxidase

Diaminobenzidine (DAB) (Sigma)	12.00	mg
Substrate buffer	20.00	ml
35% H_2O_2	30.00	µl

5. Substrate Buffer for Alkaline Phosphatase pH 9.5

Trisma-base	12.10	gm
NaCl	5.84	gm
$MgCl_2.6H_2O$	10.74	gm
Distilled water to	1000.00	ml
Adjusted pH to 9.5 with 1N NaOH		

6. Substrate Working Solution for Alkaline Phosphatase

Substrate buffer	10.00	ml
NBT (Sigma)	44	µl
BCip (Sigma)	33	µl

APPENDIX 4

HYBRIDOMA PRODUCTION

1. Complete Medium

Stock RPMI 1640	100.00	ml
Fetal bovine serum	25.00	ml
L-glutamine (200 mM)	1.25	ml
2-mercaptoethanol (0.01M)	0.625	ml

2. Stock RPMI 1640

RPMI 1640 powder (Gibco, BRL)	1	package
$NaHCO_3$	2.00	gm
HEPES	3.57	gm
Penicillin (10^5 unit/ml)	1.00	ml
Streptomycin (10^5 µg/ml)	1.00	ml

Sterilization by 0.2 µm millipore membrane filtration

3. L-glutamine (200mM)

L-glutamine (Sigma)	2.922	gm
Steriled distilled water to	100.00	ml

Sterilization by 0.2 µm millipore membrane filtration

4. 2-mercaptoethanol (0.01M)

14.34 M 2-mercaptoethanol (Sigma)	0.07	ml
Sterile PBS	100.00	ml

Sterilization by 0.2 µm millipore membrane filtration
Stored at 4°C for 2 weeks

5. PBS pH 7.2

NaCl	8.50	gm
$Na_2HPO_4.2H_2O$	1.34	gm
$NaH_2PO_4.2H_2O$	0.39	gm
Distilled water to	1000.00	ml

Sterilized by autoclaving at 121°C for 15 minutes.

6. 0.1% Trypan Blue

Trypan Blue	0.10	gm
NaCl	0.82	gm
NaN_3	0.04	gm
6% acetic acid	100.00	ml

7. Hypoxanthine and Thymidine solution (HT solution)

HT (50X) (Sigma)	1	vial
RPMI medium	10.00	ml

8. **HT medium**

HT solution	2.00	ml
Complete medium	100.00	ml

9. **Hypoxanthine Aminopterine and Thymidine solution (HAT solution)**

HAT (50X) (Sigma)	1.00	vial
RPMI medium	10.00	ml

10. **HAT medium**

HAT solution	2.00	ml
Complete medium	100.00	ml

11. **Polyethylene Glycol 4000 (MW. 3000-3700)**

PEG (ATCC)	2.00	gm
RPMI medium	1.80	ml
DMSO	0.20	ml

Warming the solution at 60°C until completely dissolved and adjusting the volume to 4.0 ml with RPMI medium.

12. **8-Azaguanine**

Complete medium	100.00	ml
8-Azaguanine (100X) (Sigma)	1.00	ml

APPENDIX 5

ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

1. Coating Buffer (0.15M PBS pH 7.2)

NaCl	8.900	gm
Na_2HPO_4	1.280	gm
$NaH_2PO_4.H_2O$	0.156	gm
Distilled water to	1000.00	ml

2. Washing Buffer

NaCl	8.900	gm
Na_2HPO_4	1.280	gm
$NaH_2PO_4.H_2O$	0.156	gm
Tween 20	0.50	ml
Distilled water to	1000.00	ml

3. Substrate Buffer (Phosphate citrate buffer) for OPD, pH 5.0

Na_2HPO_4	7.19	gm
Citric acid	5.19	gm
Distilled water to	1000.00	ml

4. Substrate Stock

Ortho-phenylenediamine (Sigma)	100.00	mg
Mthanol	10.00	ml

5. Substrate Working Solution

Substrate buffer	19.80	ml
Substrate stock	200	µl
3% H_2O_2	20	µl

PROTEIN DETERMINATION

A. 1 N Folin reagent (Sigma)
B. 0.5% $CuSO_4.5H_2O$ in 0.1% Na or K Tartrate
C. 2% Na_2CO_3 in 0.1 N NaOH

Working soluion: 1 ml of reagent B was added into 50 ml of reagent C.

APPENDIX 6

PULSED-FIELD GEL ELECTROPHORESIS

1. 0.5 M EDTA (ethylenediamine tetraacetic acid)

$Na_2EDTA.\ H_2O$	186.10	gm
Distilled water	700.00	ml
Adjust pH to 8.0 with 10 M NaOH (~50 ml)		
Add distilled water to	1000.00	ml
Sterilized by autoclaving at 121°C for 15 minutes.		

2. 1M Tris.HCl [tris(hydroxymethyl)aminomethane]

Tris base	121.00	gm
Distilled water	700.00	ml
Adjust to pH 8.0 with concentrated HCl		
Add distilled water to	1000.00	ml
Sterilized by autoclaving at 121°C for 15 minutes.		

3. TBE electrophoresis buffer (10X stock solution)

Tris base	108.00	gm
Boric acid	55.00	gm
0.5 M EDTA		
Sterilized by autoclaving at 121°C for 15 minutes		

4. TE buffer pH 8.0

1M Tris.HCl	5.00	ml
0.5M EDTA	1.00	ml

5. Ethidium bromide

Ethidium bromide	0.2	gm
Distilled water	20.0	ml
Mix well and store at 4°C in dark.		

BIOGRAPHY

NAME	Major Sompong Trewatcharegon
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LIST OF PUBLICATIONS AND PRESENTATIONS:

1. Chongtrakul P, Chaiyaroj SC, Vithayasai V, Trawatcharegon S, Teanpaisan R, Kalnawakul S, Sirisinha S. Immunoreactivity of a 38-kilodalton *Penicillium marneffei* antigen with human immunodeficiency virus-positive sera. J Clin Microbiol 1997; 35: 2220-3.
2. Trewatcharegon S, Chaiyaroj SC, Chongtrakul P, Sirisinha S. Production and characterization of monoclonal antibodies reactive with the mycelial and yeast phases of *Penicillium marneffei.* Med Mycol 2000; 38: 91-6.

3. Sirisinha S, Trewatcharegon S, Chaiyaroj SC. Monoclonal antibodies reactive with the mycelial and yeast phases of a dimorphic *Penicillium* and their potential for diagnosis of penicilliosis marneffei. 10th International Congress of Immunology. NewDelhi, India. November 1-6, 1998.
4. Trewatcharegon S, Sirisinha S, Romsai A, Teanpaisan R, Eampokalap B, Chaisavaneeyakorn S, Chaiyaroj SC. Genome fingerprinting of *Penicillium marneffei* isolates by restriction endonuclease digestion and pulsed-field gel electrophoresis. 100th General Meeting of American Society for Microbiology. Los Angeles Convention Center. Los Angeles, California. May 21-25, 2000.
5. Trewatcharegon S, Chongtrakul P, Chaiyaroj SC, Sirisinha S. Immune response in mice experimentally infected with Penicillium marneffei. 22nd Congress on Science and Technology of Thailand. Bangkok Convention Center. Bangkok, Thailand. October 16-18, 1996.

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Immunoreactivity of a 38-kilodalton *Penicillium marneffei* antigen with human immunodeficiency virus-positive sera

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Abstract:

Penicillium marneffei produced and secreted 38-kDa antigen that appeared to be specific for this dimorphic fungus. This component could not be detected in antigenic extracts of *Histoplasma capsulatum, Cryptococcus neoformans, Aspergillus niger, Aspergillus fumigatus, Aspergillus flavus, Aspergillus terreus, Candida albicans*, and two other species of *Penicillium* by immunoblot analysis against the sera from patients with culture-confirmed penicilliosis marneffei. Antibodies reactive with this antigen was found in a large proportion of human immunodeficiency virus (HIV)-positive patients, indicating a presumptive diagnosis of *P. marneffei* infection. A small

number of asymptomatic HIV-seropositive patients and HIV-seropositive patients with other fungal infections were also found to be positive by this analysis, suggesting that subclinical or mixed fungal infections involving *P. marneffei* are not uncommon.

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Production and characterization of monoclonal antibodies reactive with the mycelial and yeast phases of *Penicillium marneffei.*

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Abstract:

A definitive diagnosis of *Penicillium marneffei* is hampered by a microscopic similarity to the yeast form of *Histoplasma capsulatum* and *Cryptococcus neoformans*. In order to obtain a better discrimination for accurate diagnosis, monoclonal antibodies (MAbs) were produced from hybridomas raised from Balb/c mice immunized with mycelial culture filtrate. By indirect immunofluorescent or immunoblot analyses, one immunoglobulin (Ig) G_1 (3C2) MAb and three IgM (8B11, 3B9 and 8C3) MAbs were found to react strongly with *P. marneffei* antigens. In the immunoblots, the MAbs 8B11 and 3B9 reacted most strongly with a high molecular weight component (>200 kDa) produced during either the mycelial or yeast phase of growth. The immunoreactive epitopes for these two IgM MAbs were most likely associated with carbohydrate moieties, judging from their susceptibility to periodate oxidation and concanavalin A binding. This is in contrast to immunoreactive epitopes for MAbs 8C3 and 3C2, which were resistant to destruction by periodate treatment and did not bind to the lectin. Judging from immunofluorescent intensity, the three IgM MAbs could react strongly with the yeast cells present in the tissue biopsies of patients with *P. marneffei* infection.

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Monoclonal antibodies reactive with mycelial and yeast phases of a pathogenic dimorphic *Penicillium* and their potential for diagnosis of penicilliosis marneffei

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Summary:

Penicillium marneffei is a dimorphic fungus that caused an important emerging opportunistic infection in people with HIV infection, particularly in the Southeast Asian region. Reliable and rapid diagnosis of penicilliosis marneffei is currently not available. In this study, monoclonal antibodies (MAbs) were produced in mice immunized with the mycelial culture filtrate. Three clones producing IgM antibodies specifically reacting with both the mycelial and yeast phase antigens in ELISA, immunoblot and immunofluorescence assays. IFA of biopsy specimens using these MAbs made it possible to distinguish the infection caused by *P. marneffei* from other opportunistic fungal infections common in AIDS patients.

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Genome Fingerprinting of *Penicillium marneffei* Isolates by Restriction Endonuclease Digestion and Pulsed-Field Gel Electrophoresis

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Summary:

1. Among 67 *P. marneffei* isolates, 2 macrorestriction patterns (MPs) and 9 MP subgroups were generated by *Not* I digestion.
2. Forty-two isolates (65.6%) were of MPI and belonged to subgroups MPIa (8 isolates), MPIb (11 isolates), MPIc (10 isolates), MPId (3 isolates), MPIe (5 isolates) and MPIf (3 isolates). Whereas 22 isolates (34.4%) were of MPII and belonged to subgroups MPIIa (6 isolates), MPIIb (4 isolates) and MPIIc (7 isolates).
3. Two bamboo rat isolates belonged to subgroup MPIa and one isolate was of subgroup MPIc.
4. No significant correlation between the MP of *P. marneffei* isolates and geographical region or specimen sources.
5. Isolates obtained before 1995 were of MPI and we have seen an increase in isolates with MPII since then. We are now conducting an investigation to comfirm this finding.

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IMMUNE RESPONSE IN MICE EXPERIMENTALLY INFECTED WITH *PENICILLIUM MARNEFFEI*

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Summary :

1. The pathology caused by *P. marneffei* were included skin abcess, liver enlargement and generalized distributed of abcesses of abdominal organs in the mice injected intraperitoneally with the spore of *P. marneffei.* The pathology of the mice injected subcutaneously only exhibit a skin abcess at the injection site.
2. The antibodies to P. marneffei can be detected within 4 weeks in the mice infected intraperitoneally and within 2 months in the mice infected subcutaneously.
3. Antibodies produced by all infected mice reacted with antigen of molecular weight 43, 43-68, and 97 kDa.

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