II. LITERATURE REVIEWS

A. History

Penicillium marneffei is the only dimorphic member of the genus *Penicillium*. It can grow in living tissue or on culture media at 37 °C as a yeast like fungus, and on the culture media at temperature between 25-30 °C as a mold form. *P. marneffei* was discovered in 1956 from the bamboo rat, *Rhizomys sinensis*, in Vietnam (Capponi et al, 1956). Although bamboo rats are known to be asymptomatic carriers of the fungus, it is unclear whether they are an important reservoir for human infection or only a sentinel animal that is susceptible to infection from an environmental source. Up to now, the natural habitat of *P. marneffei* and an explanation for its geographic restriction, remain unknown. Among all *Penicillium* species, *P. marneffei* is regularly pathogenic to humans. Infection caused by *Penicillium* spp. due to species other than *P. marneffei* is rare. The ability of *P. marneffei* to cause disease in humans was first found as laboratory acquired infection. Segretain (1959) pricked his finger accidentally during experimental studies, with a needle used to inoculate the *P. marneffei* into the hamster. He developed a small nodule at the site of inoculation 9 days later, following with lymphangitis and auxillary lymphnode hyperthrophy. Antifungal sensitivity studied at the time demonstrated a high *in vitro* sensitivity of this fungus to nystatin. An intensive treatment with oral nystatin for 30 days cleared the infection. This accidental infection emphasized the possibility of human pathogenicity. The first identified natural human infection reported in the United States by DiSalvo (1973) was a 61-year-old man who had traveled in southeast Asia. Penicilliosis marneffei was diagnosed when the patient underwent a splenectomy for the Hodgkin's disease management. *P. marneffei* infection is one of the major opportunistic diseases in immunocompromised hosts, especially among AIDS patients. This fungal infection was reported in Thailand, Vietnam, Cambodia, Myanmar, Malaysia, northeastern India, Hong Kong, Taiwan and southern China (**Figure 1**) (Supparatpinyo et al, 1992). It was also reported among HIV-infected patients from the United States, the United Kingdom, the Netherlands, Italy,

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Germany, Switzerland, France, Sweden, Australia and Japan after they visited the *P. marneffei* endemic region (Sirisanthana, 1996). Though the fungus was initially isolated from the bamboo rats and has also been recovered from internal organs of bamboo rats, the rodents are unlikely to be important in the transmission of the disease in nature and to humans. Penicilliosis marneffei has been classified as a geoanthromycosis: the fungus probably exists as a saprophyte in the environment (e.g. in the soil), and humans, as well as bamboo rats, are infected through inhalation of the conidia. This postulation, however, has not been proven beyond doubt.

Figure 1. Endemic area of *P. marneffei.* Yellow area represents the endemic region of *P. marneffei* in tropical Asia. (This picture was taken from website:www.iapac.org)

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B. Mycology

Among all *Penicillium* species, *P. marneffei* is the only species that has thermal dimorphism. On Sabouraud's dextrose agar at 25 °C, *P. marneffei* grows as a mold form with velvety colony producing red to deep red, diffusable pigment (**Figure 2**). Microscopically, it consists of septate-branched hyphae with lateral and terminal conidiophores. The conidiophores have basal stripes and terminal verticils of 3-4 matulae. The matulae bear 4-7 phialides, each of which produces long chain of

conidia (**Figure 3**). At 37 °C *in vitro* or *in vivo,* it converts to a yeast form (**Figure 4**), displaying spherical or elliptical cells measuring 2-3 by 2-6.5 µm, which multiply by fission (**Figure 5**). The biochemical properties of *P. marneffei* were studied in 32 clinical isolates of *P. marneffei* in Hong Kong from 1996-1999 (Wong et al, 2001). The result showed that all isolates were positive for urease and inhibited by 500 mg/l of cyclohexamide. All assimilated glucose, maltose and cellobiose. 65.6%, 84.4%, and 71.9% of the isolates assiminated trehalose, xylose and nitrate, respectively. Fungal growth was inhibited by a low concentration (0.015-0.25%) of galactose. Overall, 17 different biotypes were identified (Wong et al, 2001). In addition, both mycelial and yeast forms expressed several interest enzymes, for examples: acid phosphatase, esterases and lipases, which might be contribute to the virulence of *P. marneffei* (Youngchim et al, 1999).

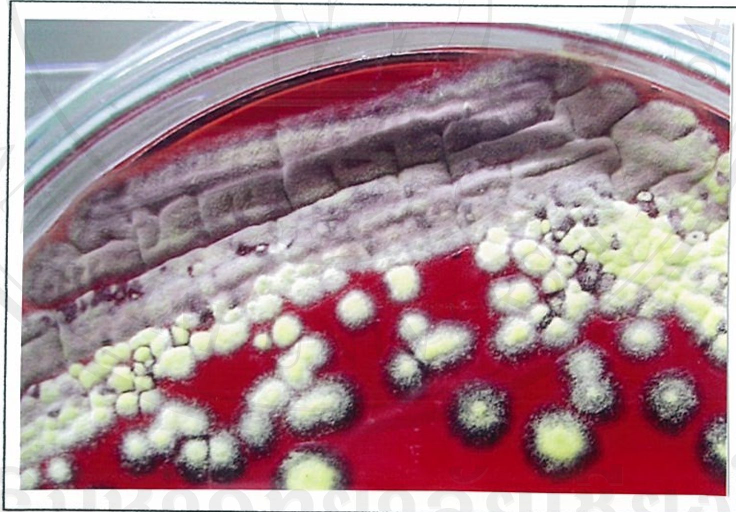

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Figure 2. Five-day-old of *P. marneffei* mold culture on Sabouraud dextrose agar at 25 °C

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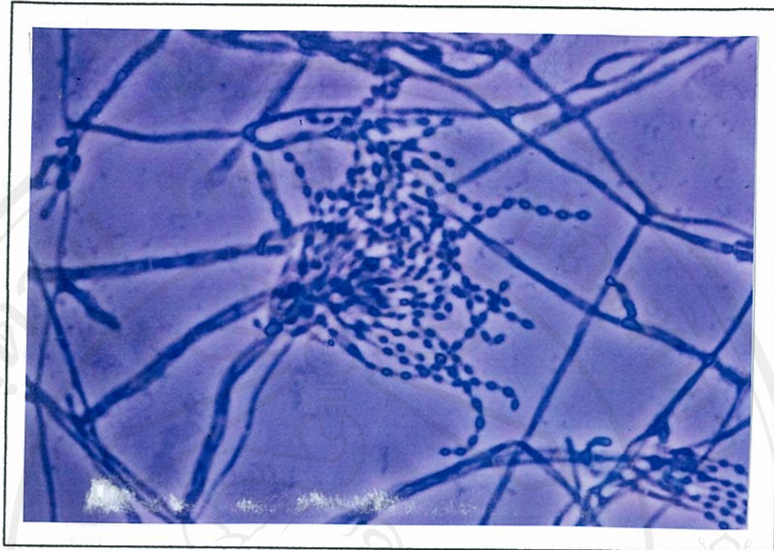

Figure 3. Photomicrograph of 5-day-old mold-form of *P. marneffei* on slide culture; *phase contrast, x400.*

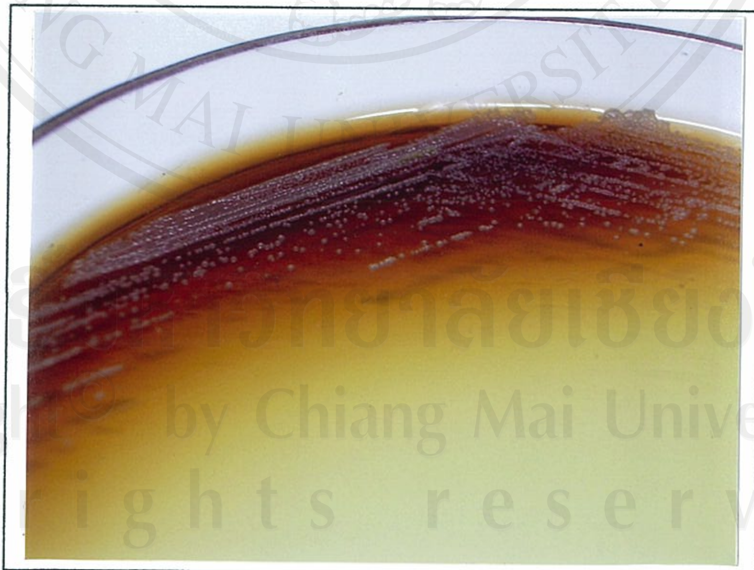

Figure 4. Five-day-old of *P. marneffei* yeast culture on brain heart infusion agar at 37 °C

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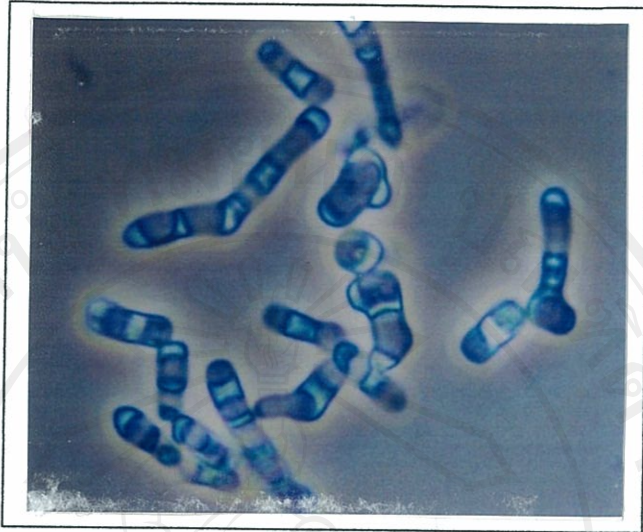

Figure 5. Photomicrograph of 5-day-old culture of *P. marneffei* at 37 °C; *phase contrast, x400.*

C. Ecology and epidemiology

The ecology and environmental reservoirs of *P. marneffei* were first investigated in 1986 by Deng and colleagues in China. It was found that *P. marneffei* was isolated from internal organs of 18 out of 19 bamboo rats belonging to the species *Rhizomys pruinosus* (*R. pruinosus*). The association between *P. marneffei* and bamboo rats had also been noted in Thailand and another endemic countries. Several investigators reported that *P. marneffei* was isolated from various species of bamboo rats, including *Cannomys badius* (*C. badius*), *Rhizomys sumatrensis* (*R. sumatrensis)* and *R. pruinosus* (Ajello et al, 1995; Chariyalertsak et al, 1996B). Chariyalertsak and collegues also reported that *P. marneffei* was isolated from one of the soil samples collected from a burrow of *R. sumatrensis* (Chariyalertsak et al, 1996B). The mycological characteristics of *P. marneffei* isolated from bamboo rats and humans were very similar. The results indicated that *R. sumatrensis* and *C. badius* may be important animal hosts of *P. marneffei* in northern Thailand.

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D. The Mode of transmission

The mode of transmission of *P. marneffei* to man is still unclear. However, Chariyalertsak et al. demonstrated the significant increase of the incidence of penicilliosis marneffei during the rainy season in the northern Thailand (Chariyalertsak et al, 1996A). This result suggested that the transmission of *P. marneffei* may be by inhalation of conidia and may relate with the meterological factors such as the wind speed. The occurrence of the fungus in the liver could be a result of the propensity of the fungus to invade the reticuloendothelial system.

E. Clinical manifestations and treatment

P. marneffei infection in humans may either be disseminated or localized. In immunocompromised hosts, *P. marneffei* infection is usually disseminated at diagnosis. The clinical signs of penicilliosis marneffei vary in degree of severity such as fever, sometimes accompanied by chills; respiratory signs including persistent cough, pneumonia, pulmonary infiltration, pleural effusion or dyspnea; hepatomegaly, splenomegaly or hepatosplenomegaly; lymphadenopathy; osteoarticular lesions including diarrhea, tonsil ulceration or nasopharyngitis; cutaneous and subcutaneous lesions, as well as weight loss, anorexia and asthenia. The skin lesions are papules, rushes, necrotic papules, acne-like pustules and nodules which are usually located on face, trunk and extremities (**Figure 6**). The X-ray findings respiratory field varied from a normal chest picture to abnormal including pulmonary infiltration, diffused or localized inflammation of the bronchial mucosa or the alveolar spaces. Fungaemia is present in the majority of cases in organ systems including kidney, bones and joints. The pericardium may also be involved.

For the clinical and microbiological responses, in patients who are seriously ill, treatment could be started with intravenous amphotericin B and followed by oral medication with itraconazole or ketoconazole until cultures were negative and clinical findings had been resolved. In the treatment of mild to moderate *P. marneffei* infection, itraconazole or ketoconazole should be the drug of choice. The current recommended treatment regimen is to give 0.6 mg/kg/day of amphotericin B for 2 weeks, followed by 400 mg/day of itraconazole orally in two divided doses for the next 10 weeks. After initial treatment, the patient should be given 200 mg/day of

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itraconazole as secondary prophylaxis (Sirisanthana et al, 1998). One recent interesting observation is that several 4-aminoquinoline agents including chloroquine (CQ) were found to be able to inhibit the growth of *P. marneffei* inside macrophages. The activity of CQ on *P. marneffei* is postulated to be due to an increase in the intravacuolar pH and a disruption of pH-dependent metabolic processes. This finding could be of value in the chemotherapy or chemoprophylaxis of penicilliosis marneffei (Taramelli et al, 2001). The antimicrobial activities of CQ and several 4-aminoquinoline drugs were tested against *P. marneffei.* CQ is a widely available and inexpensive drug belonging to the family of 7-chloro-4-aminoquinoline compounds (O'Neill et al, 1998). It is currently employed for malaria prophylaxis and therapy; its hydroxy derivative is also used as an anti-inflammatory agent in the treatment of rheumatoid arthritis and systemic lupus erythematosus. These antimicrobial properties prompted to investigate the potential inhibitory effects of CQ and several quinoline derivatives on *P. marneffei.* An in vitro macrophage model that allows evaluation of the extent of intracellular *P. marneffei* growth was developed. The human THP1 and mouse J774 macrophages were infected in vitro with *P. marneffei* conidia and treated with different doses of drugs for 24 to 48 h followed by cell lysis and the counting of *P. marneffei* CFU. The results revealed that CQ and amodiaquine exerted a dose-dependent inhibition of fungal growth. CQ's antifungal activity is due to an increase in the intravacuolar pH and a disruption of pH-dependent metabolic processes. These results suggest that CQ has a potential for use in prophylaxis of *P. marneffei* infections in human immunodeficiency virus-infected patients in countries where *P. marneffei* is endemic.

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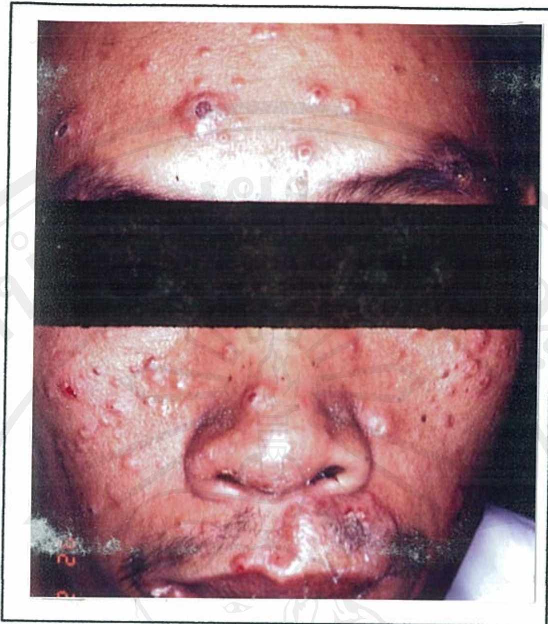

Figure 6. Skin lesions of a Thai AIDS patient with *P. marneffei* infection (This picture was taken from Associate Professor Siri Chiewchanvit, Faculty of Medicine, Chiang Mai University)

F. Pathogenesis and immunology of *P. marneffei*

It is generally believed that inhalation of the conidia is a likely route, in line with the mode of infection for other molds. The attachment of *P. marneffei* conidia to host cells and tissues is the first step in the establishment of an infection. Infection with *P. marneffei* is presumed to originate in the lung following the inhalation of airborne conidia. The recognition of laminin by *P. marneffei* conidia may facilitate attachment to the bronchoalveolar epithelium (Hamilton et al, 1998). The conidia-host interaction may occur via adhesion to the extracellular matrix protein laminin and fibronectin via a sialic acid-dependent process. Using immunofluorescence microscopy, Hamilton and colleagues demonstrated that fibronectin binds to the conidia surface and to phialides, but not to hyphae. The investigators suggested that there could be a common receptor for the binding of fibronectin and laminin on the surface of *P. marneffei* (Hamilton et al, 1998; Hamilton et al, 1999). In susceptible hosts, the fungus undergoes phase transition and reproduces as yeast cells. The

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histopathology of penicilliosis marneffei is characterized by the intracellular infection of macrophages (Cooper et al, 1997). Yeast cells of *P. marneffei* measured approximately 2-3 by 2-7 μm reproduce inside the macrophage by schizogony. In AIDS patients, the fungal burden is usually high, and intracellular *P. marneffei* organisms are readily seen in infected tissue specimens. In murine models of penicilliosis marneffei, T cells are critical for protection. Athymic mice are hypersusceptible to infection, and partial protection can be adoptively transferred via nylon wool nonadherent splenocytes (Kudeken et al, 1996). In immunocompetent mice inoculated intranasally with *P. marneffei*, an exuberant $CD4^+$ T-cell infiltration into the lungs is observed (Kudeken et al,1997). Presumably then, analogous to many other intracellular infections (Levitz, 1992), the activation of macrophages by T cell-derived cytokines is necessary to control penicilliosis marneffei. In 1997, Cogliati and colleagues investigated the effect of nitric oxide (NO) and reactive nitrogen intermediates on the *in vitro* growth of *P. marneffei* both in a cell-free system and in a novel macrophage culture system. In the cell-free system, NO that was chemically generated from $NaNO_2$ in acid media (pH 4 and 5) markedly inhibited the growth of *P. marneffei.* On the contrary, inhibition of growth did not occur in neutral medium (pH 7.4) in which NO was not produced. The inverse correlation between intramacrophage growth and the amount of nitrite detected in culture supernatants supports the hypothesis that the L-arginine-dependent NO pathway plays an important role in the murine macrophage immune response against *P. marneffei* (Cogliati et.al, 1997). The interaction between human leukocytes and heat-killed yeast-phase *P. marneffei* has been studied by Rongrungruang and colleagues (1999). Their data suggested that monocyte-derived macrophages phagocytose *P. marneffei* even in the absence of opsonization and the major receptor(s) recognizing *P. marneffei* could be a glycoprotein with N-acetyl-beta-D-glucosaminyl groups. *P. marneffei* stimulates the respiratory burst of macrophages regardless of whether opsonins are present, but tumor necrosis factor-α (TNF-α) secretion is stimulated only in the presence of opsonins. The authors thus speculated that the ability of unopsonized fungal cells to infect mononuclear phagocytes in the absence of TNF-α production is a possible virulence mechanism. Although *P. marneffei* is capable of infecting and replicating inside macrophages, it is also evident that macrophages do possess antifungal

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activities. The fungicidal activities of macrophages is likely to involve the generation of reactive nitrogen intermediates, as described by Kudeken and colleagues (1999A). In addition to macrophages, the neutrophils also exhibit antifungal properties. The fungicidal activity of neutrophils is significantly increased in the presence of proinflammatory cytokines, especially granulocyte macrophage-colony stimulating factor (GM-CSF), granulocyte-colony stimulating factor (G-CSF) and interferon-gamma (IFN-γ). In addition to GM-CSF, G-CSF and IFN-γ, other cytokines such as TNF-α and interleukin-8 (IL-8) are capable of enhancing the neutrophil's inhibitory effects on germination of *P. marneffei* conidia. The strongest effect was observed with GM-CSF (Kudeken et al, 1999B). Conidia are, however, generally not susceptible to killing by phagocytes. The fungicidal activity exhibited by neutrophils is believed to be independent of superoxide anion, but through exocytosis of granular enzymes (Kudeken et al, 2000). Recently, Koguchi and colleagues demonstrated that osteopontin (secreted by monocytes) could be involved in IL-12 production by peripheral blood mononuclear cells during infection by *P. marneffei,* and the production of osteopontin is also regulated by GM-CSF (Koguchi et al, 2002). It is also likely that the mannose receptor is involved as a signal-transducing receptor for triggering the secretion of osteopontin by *P. marneffei*-stimulated peripheral blood mononuclear cells.

In 1999, Rongrungruang and Stuart (1999) examined the interactions of human peripheral blood mononuclear cells (PBMC) and monocyte-derived macrophages (MDM) with heat-killed yeast-phase of *P. marneffei.* Monocyte-derived macrophages bound and internalized *P. marneffei* in the presence of complement-sufficient pooled human serum (PHS). Binding and phagocytosis were still seen if PHS was heat inactivated or omitted altogether. The binding of unopsonized *P. marneffei* to MDM occurred in the absence of divalent cations and was not affected by inhibitors of mannose and β–glucan receptors or monoclonal antibodies directed against CD14 and CD11/CD18. Binding was profoundly inhibited by wheat germ agglutinin. A vigorous respiratory burst was seen in PBMC stimulated with *P. marneffei*, regardless of whether the fungi were opsonized. However, TNF-α released from PBMC stimulated with *P. marneffei* occurred only if serum was present. These data demonstrate that (i) MDM bind and phagocytose *P. marneffei* even in the absence of opsonization, (ii)

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binding is divalent cation independent but is inhibited by wheat germ agglutinin, suggesting that the major receptor(s) recognizing *P. marneffei* is a glycoprotein with exposed *N*-acetyl-D-glucosaminyl groups, (iii) *P. marneffei* stimulates the respiratory burst regardless of whether opsonins are present, and (iv) serum factors are required for *P. marneffei* to stimulate TNF-α release. The ability of unopsonized *P. marneffei* to parasitize mononuclear phagocytes without stimulating the production of TNF-α may be critical for the virulence of this intracellular parasite.

G. Laboratory diagnosis

Penicillium marneffei infection is relatively amenable to antifungal therapy and a cure is potentially possible. Early recognition of the infection is therefore essential for timely initiation of effective therapy. Identification of *P. marneffei* infection by routine laboratory tests includes microscopic examination and isolation of the organisms from clinical specimens such as blood, skin biopsy, bone marrow aspirate, sputum, lymphnode biopsy, liver biopsy, skin scraping, cerebrospinal fluid, pleural effusion and pharyngeal ulcer scraping. Bone marrow is the most sensitive specimen (100%) and more invasive than blood culture (76%) (Supparatpinyo et al, 1994).

In the AIDS patients with high levels of fungaemia, it has been occasionally reported that a direct smear of the peripheral blood may reveal the fungus. In HIV-positive patients, fungaemia could be detected in at least 55% of the patients Unfortunately, fungal culture suffers from the drawback of a long turnaround time and that sometimes invasive tissue biopsies are necessary for obtaining a satisfactory specimen. In tissue, histopathologic examination is available for identification of *P. marneffei*. The samples are usually stained with hematoxylin and eosin, Grocott methenamine silver, periodic acid schiff, and Ziehl-Neelsen stain. *P. marneffei* are seen as intracellular yeast cells of 3 μm in diameter dividing by binary fission. However, elongated shaped cells with septa, up to 8 μm long, may be present extracellularly. (Supparatpinyo et al, 1994). In a series of HIV-infected patients from Hong Kong, 50% of them had documented fungaemia (Wong et al, 2001). The HIV-negative patients pose a more difficult situation: 28.6% of cases did not have fungaemia at the time of presentation, more invasive procedures of obtaining a deep tissue sample for culture were therefore frequently necessary. Piérard and his

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coleagues reported that the monocloncal antibody EB-A1 against the galactomannan of *Aspergillus* species may also be used to detect *P. marneffei* in formalin-fixed, paraffin-embedded tissues (Pierard et al, 1991).

A number of presumptive diagnostic methods have been developed including serodiagnostic techniques, a number of studies aimed at detecting fungal antibodies and/or antigens in the serum and body fluids of infected patients. In earlier studies, culture filtrates or whole cell extracts were being used as antigens. *P. marneffei* was cultured in liquid media, and the culture filtrate was concentrated and used to immunize rabbits. The culture filtrate and the rabbit anti-*P. marneffei* sera were incorporated in an immunodiffusion test to detect antibodies or antigens respectively (Sekhon et al, 1982; Viviani et al, 1993; Imwidthaya et al, 1997). An indirect immunofluorescent antibody test (IFAT) for serodiagnosis of *P. marneffei* infection was reported, using the yeast-hyphae (representing tissue multiplication phase) or the germinating conidia (representing initial tissue invasion phase) as antigens (Yuen et al, 1994). None of the eight sera from culture-documented patients tested at 1:10 dilution gave a positive result for IgM. High IgG titers (of the respective phases, geometric mean 1:905 and 1:1280) were found in all eight penicilliosis marneffei patients, in contrast to that obtained from 78 healthy controls (with a respective geometric mean of 1:1.34 and 1:2.14). Sera from patients with cryptococcosis (n=2) or candidaemia (n=2) did not show cross-reactivity (IgG titer <1:40, which is similar to that of the healthy controls). Overall, the IgG titer was higher than IgA titer but there was little difference in using the germinating conidia or the yeast-hyphae form as the testing antigen. Moreover, IgA could not be detected in two out of eight positive cases. Three HIV patients with culture-documented penicilliosis marneffei were tested positive (IgG titers 1:80 - 1:160). An IgG titer >1:80 is suggestive of penicilliosis marneffei. Jeavons and colleagues characterized purified three cytoplasmic yeast antigens of 50-, 54- and 61-kDa, which were found respectively in 48, 71 and 85% of serum samples from 21 *P. marneffei* culture-positive patients (Jeavons et al, 1998). Chongtrakool and colleagues isolated a 38-kDa antigen partially-purified from yeast culture filtrate, where 45% of *P. marneffei* culture-positive HIV patients (n=51), 17% of HIV positive asymptomatic patients (n=262)

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and 25% of other fungal culture-positive HIV patients (n=67) have developed antibodies against this antigen (Chongtrakool et al, 1997).

A latex agglutination test was developed to detect antigenaemia, where polystyrene beads were coated with rabbit anti-*P. marneffei* globulin, obtained from rabbits immunized with yeast culture filtrate (Kaufman et al, 1996). 77% of the 17 *P. marneffei* culture-positive HIV patients were positive. Purified hyperimmune IgG, from rabbits immunized with yeast cells was used in an enzyme-linked immunosorbent assay (ELISA) to quantitate *P. marneffei* yeast antigens in urine samples (Desakorn et al, 1999). All urine samples from 33 *P. marneffei* culture-positive HIV patients were positive with a median titer of 1:20.

The detection of the *P. marneffei* genomic DNA in clinical specimens have also been reported. A polymerase chain reaction (PCR)-hybridization assay and PCR method have been developed for the rapid identification of *P. marneffei.* LoBuglio and Taylor used primers PM2 and PM4 to amplify a 347 bp fragment of the internal transcribed spacer region between 18S ribosomal deoxyribonucleic acid (rDNA) and 5.8S rDNA (LoBuglio et al,1995). On the other hand, Vanittanakom and colleagues used a PCR-Southern hybridization format, where primers RRF1 and RRH1 were used to amplify a 631 bp fragment of the 18S rDNA, followed by hybridization with a *P. marneffei*-specific 15-oligonucleotide probe (Vanittanakom et al, 1998). Recently, Vanittanakom and colleagues described a nested PCR assay which might prove useful in the detection of *P. marneffei* and identification of young fungal cultures (Vanittanakom et al, 2002).

Molecular diagnosis revealed the first gene cloned from *P. marneffei* was the *MP1* gene (Cao et al, 1998A). Serum from guinea pigs immunized with *P. marneffei* yeast cells was used to screen the cDNA library of *P. marneffei*. The *MP1* gene which encodes an abundant antigenic cell wall mannoprotein in *P. marneffei* was subsequently cloned. *MP1* is a unique gene without homologues in sequence databases. It codes for a protein, Mp1p, of 462 amino acid residues, with a few sequence features that are present in several cell wall proteins of *Saccharomyces cerevisiae* and *Candida albicans*. Specific anti-Mp1p antibody was generated with recombinant Mp1p protein purified from *E. coli* to allow further characterization of Mp1p. Western blot analysis with anti-Mp1p antibody revealed that Mp1p has

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predominant bands with molecular masses of 58 and 90 kDa and that it belongs to a group of cell wall proteins. It was observed that infected patients develop a specific antibody response against Mp1p, suggesting that this protein represents a good cell surface target for host humoral immunity. The combined antibody and antigen tests for *P. marneffei* carry a sensitive of 88% (23 of 26), with a positive predictive value of 100% and a negative predictive value of 96%. The value of antigen (Mp1p) and antibody (anti-Mp1p) detection in the diagnosis of penicilliosis marneffei is best evaluated by comparing the results in patients with or without underlying HIV infection. Thus, this ELISA-based test for the detection of anti-Mp1p antibody can be of significant value as a diagnostic for penicilliosis.

H. Researches on proteins and molecular biology of *Penicillium marneffei*

H.1 Molecular biology

The mechanism of thermal dimorphism and morphogenesis in *P. marneffei* is not fully understood. However, studies by Borneman and Andrianopoulos started to provide important information on this area. It was shown that a homologue gene of the *Aspergillus nidulans (A. nidulans) abaA* gene is involved in the regulation of cell cycle and morphogenesis in *P. marneffei* (Borneman et al, 2000). An *STE12* homologue of *P. marneffei* (*stlA* gene) was subsequently shown to be able to complement the sexual defect of an *A. nidulans steA* mutant (Borneman et al, 2001). Until now sexual stage of *P. marneffei* is still unknown, therefore postulated to be present. This is further supported by recent genomic analysis of *P. marneffei*. Other genes which are involved in the growth and development of *P. marneffei* have been described recently. A CDC42 homologue (*cflA* gene) is shown to be required for polarization and determination of correct cell shape during yeast-like growth, and for the separation of yeast cells (Boyce et al, 2001). Deletion of the homologue of *A. nidulans stuA* gene in *P. marneffei* showed that the gene is required for metula and phialide formation during conidiation but is not required for dimorphic growth (Borneman et al, 2002).

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H.2 Vaccination

Some recent studies showed that the vaccine development is potentially feasible. The *P. marneffei* mannoprotein Mp1p (encoded by the *MP1* gene) has been tested in a mouse model as a potential vaccine candidate (Wong et al, 2002). The relative efficacy of intramuscular *MP1* DNA vaccine, oral mucosal *MP1* DNA vaccine using live-attenuated *Salmonella typhimurium* carrier, and intraperitoneal recombinant Mp1p protein vaccine were compared. Intramuscular *MP1* DNA vaccine appears to give the best protection against *P. marneffei.*

H.3 Researches on proteins and molecular biology

Vanittanakom and colleagues identified four major immunogenic proteins of *P. marneffei* with molecular masses of 50, 54, 88 and 200 kDa using gel electrophoresis and immunoblot assay (Vanittanakom et al,1997A). These proteins were prepared from culture filtrate of *P. marneffei* during the deceleration and early stationary yeast phase. The reactivities to the 88-kDa and 200-kDa proteins were detected in 93.9% and 72.7% of penicilliosis pateints, respectively. These two proteins may be common antigens that occur in other environmental fungi because they elicited weak reactivity in high proportions of both AIDS patients without penicilliosis and normals. However, the 88-kDa band was strongly recognized by a half of the serum samples derived from *P. marneffei*-infected AIDS patients (Vanittanakom et al, 1997A). The reactivities of the 50-kDa and 54-kDa proteins were detected in 57.6% and 60.6% in AIDS patients with penicilliosis and approximately 10% or less in AIDS patients without penicilliosis and in normals. In one serum sample, the 50-kDa and 54-kDa proteins were detected by Western blot assay two months before the definite diagnosis of fungal culture. These results suggested that at least two yeast phase immunoreactive proteins (50 and 54 kDa) may be specific to the *P. marneffei* infection.

Cytoplasmic yeast antigens of *P. marneffei* were partially purified by using the isoelectric focusing technique followed by preparative gel electrophoresis (Jeavons et al, 1998). From the Western blotting with sera derived from *P. marneffei*-infected patients, the proteins of 50, 54 and 61 kDa were recognized by sera from 48%, 71% and 86% respectively. This result also demonstrated that a 39-kDa protein was a

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processed product of the 61-kDa protein. The 39-kDa protein was reactive with 48% of the sera from *P. marneffei*-infected AIDS patients (Jeavons et al, 1998). The 61-kDa protein was purified to homogeneity whereas the 50-kDa and 54-kDa proteins could be partially purified. The 50-kDa and 88-kDa proteins were partially purified from crude culture filtrate using preparative polyacrylamide gel electrophoresis (Poolsri, 1999). The result revealed that the 50-kDa and 88-kDa proteins had isoelectric point values of approximately = 4.5 to 5.1 and = 4.5 to 5.6, respectively. Both proteins are glycoproteins and characterized as mannoproteins. From these data, the antigens of 39, 50, 54, 61 and 88 kDa or various combinations of these antigens are potential candidates of the effective diagnostic markers on identification of *P. marneffei* infection.

Cao and colleagues cloned the *MP1* gene which encoded an abundant antigenic cell wall mannoprotein with 462 amino acid residues (Cao et al, 1998A). Western blot analysis with anti-Mp1p antibody revealed that Mp1p has predominant bands with molecular masses of 58 and 90 kDa and that it belongs to a group of cell wall proteins. In addition, Mp1p is an abundant yeast glycoprotein and has high affinity for concanavalin A, a characteristic indicative of a mannoprotein. *In vitro*, Mp1p is found to be secreted into the cell culture supernatant detected by Western blotting. In addition, this Mp1p antigen-based ELISA is also specific for *P. marneffei* since the cell culture supernatants of the other three fungi gave negative results. The specific anti-Mp1p antibody was produced by immunization of Mp1p recombinant protein into a rabbit. The anti-Mp1p antibody is specific since it fails to react with any protein lysates of *C. albicans*, *H. capsulatum*, or *C. neoformans* by Western blotting. An ELISA based antibody test with purified Mp1p was produced (Cao et al, 1998B; Cao et al, 1999). An Mp1p antibody test was performed with sera from penicilliosis patients. An ELISA-based antigen test was performed with these serum specimens from penicilliosis patients. The result indicated that 17 of 26 (65%) patients are Mp1p antigen test positive. The combined antibody and antigen tests for *P. marneffei* carry a sensitive of 88% (23 of 26), with a positive predictive value of 100% and a negative predictive value of 96%. The specificity of the tests are high since none of the 85 control sera was positive by either test. The value of antigen (Mp1p) and antibody (anti-Mp1p) detection in the diagnosis of penicilliosis marneffei is best evaluated by

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comparing the results in patients with or without underlying HIV infection. Concomitant testing of the serum antigen and antibody levels could therefore improve the diagnostic yield of serology in immunocompromised patients. When serial serum samples were available for the HIV-positive patients, it was found that the serum antigen and antibody titers against *P. marneffei* were elevated as early as 30 days before the day of positive cultures. The titers of both serum antigen and antibody dropped with the initiation of amphotericin B therapy and itraconazole prophylaxis. Upon subsequent follow up, there was no clinical and mycological evidence of relapse and this was associated with a persistently negative serum antigen and antibody ELISA. Evaluation of the test with guinea pig sera against *P. marneffei* and other pathogenic fungi indicated that this assay was specific for *P. marneffei.* Clinical evaluation revealed that high levels of specific antibody were detected in two immunocompetent penicilliosis patients. Furthermore, approximately 80% (14 of 17) of the documented penicilliosis patients with human immunodeficiency virus tested positive for the specific antibody. No false-positive results were found for serum samples from 90 healthy blood donors, 20 patients with typhoid fever, and 55 patients with tuberculosis, indicating a high specificity of the test.

Recently, Pongpom constructed and characterized the cDNA library from the yeast form of *P. marneffei* (Pongpom, 2004). The constructed cDNA library containing the genes of the yeast phase was used for the screening of genes encoding protein antigens by using pooled patients' sera. The clones of interest are ongoing characterized. The synthesis of protein is more complex than the synthesis of RNA because a greater number of factors have to be taken into account when attempting to maximize the amount of product that is obtained (Brown, 2000). High level protein synthesis requires that the vector carries a variety of sequence signals that are individually efficient, compatible with one another and ideally, easily regulated. Protein synthesis occurs inside the bacterium, so steps must be taken to avoid immediate degradation of the protein by cellular activities and there must be a means of obtaining the protein in pure form from the culture. These general considerations will be covered before specific vectors for protein expression are described.

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I. Glutathione S-transferase (GST)

The glutathione S-transferase, a group of multigene isoenzymes represents an integral part of the detoxication machanism. They are widely distributed in nature, being found in bacteria, yeast, molds, fungi, mollusks, crustacean, worm parasites, frogs, insects, plants, fish, birds and mammals (Daniel, 1993). These intracellular enzymes protect cells against both xenobiotic and endogenous compound (Salinas et al, 1990), and stress by catalyzing the nucleophillic addition of the thiol of reduced glutathione (γ-glutamyl-cysteinyl-glycine) to electrophillic centers in organic compounds. The resultant glutathione conjugates, more water-soluble, can be exported from animal cells by putative membrane ATP-dependent pump systems (Hayes et al, 1990; Ishikawa, 1992) after which they are metabolized via the mercapturic acid pathway and eventually eliminated (Habig et al, 1974). Essentially all eukaryotic species appear to possess multiple isoenzymes. Multiple forms of GSTs have been discovered in virtually every organism in which GST activity has been found. The cytosolic GSTs exists as either homo- or hetero- dimeric forms due to multiple genes and monomer hybridization (Manervik et al, 1988). Heterodimers have been identified between molecules of different classes (Kuzmich et al, 1992). Members within any class exhibit similar monomer sizes, about 20 to 34-kDa, share high amino acid sequence identity, typically 60 to 80%, and have distinctive but overlapping substrate specificities (Board et al, 1990). The membrane bound or microsomal GSTs are quite distinct from their soluble counterparts. Molecular weights of GSTs have been found in general to fall within the range 36 to 50 kDa. Almost invariably, they consist of two subunits of reported molecular weight between 20-27 kDa. Molecular weight cited for the native enzymes cluster around a mean of 43.0 ± 0.8 kDa, and for a subunit, a mean of 24.9 ± 3.1 kDa (Clark, 1989). Isoelectric points (pI) have been determined for enzymes from a variety of organisms, generally by isoelectrofocussing or by chromatofocussing. In the case of housefly GSTs, it appears that those enzymes with a pI higher to pH 6.5 are catalytically more versatile than those of low pI, but it does not appear possible generally to establish any relationship between pI and function (Clark et al, 1984; Clark et al, 1986). Analysis of data presented by Dierickx shows that the K_m with respect to 1-Chloro-2,4-dinitrobenzene (CDNB) appears to vary systemically with isoelectric point for these

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enzymes (Dierickx, 1985). The multifunctional nature of GSTs allowed a variety of affinity gels to be designed that can be used to isolate GSTs. These include agarose containing immobilized bromosulfothalein (BSP), choric acids, glutathione, S-hexylglutathione, S-octylglutathione, thyroxine and triazine dye (Hayes et al, 1990). Among these affinity gels two matrices in particular, glutathione-agarose (Simon et al, 1977) and S-hexylglutathione-agarose (Gluthenberg et al, 1979) have been widely used to purify GSTs as they display both excellent specificity and yield of these enzymes.

The GST gene fusion system is a versatile system for the expression, purification, and detection of fusion proteins produced in *E. coli.* The system is based on inducible, high-level expression of genes or gene fragments as fusions with *Schistosoma japonicum (S. japonicum)* GST (Smith et al, 1988). Expression in *E. coli* yields fusion proteins with the GST moiety at the amino terminus and the protein of interest at the carboxyl terminus. GST fusion proteins are constructed by inserting a gene or gene fragment into the multiple cloning site of the pGEX vectors. Most transformation procedures are relatively inefficient under optimal conditions only a few percent of all bacteria are transformed. It is essential that one can discriminate transformed and non-transformed bacteria. With most vectors this is accomplished by making use of dominant selection markers, such as genes carrying an antibiotic resistance marker which, if present on the plasmid, provide the bacterium with resistance to that antibiotic. The most commonly used marker is that for ampicillin resistance (*amp*) originating from transposon Tn3. This marker is similar in that direct selection for plasmids containing one of the genes is easy and efficient. For example, *E. coli* bacteria are sensitive to very low concentrations of ampicillin (a few μg/ml), but become resistant to very high levels (up to 1 mg/ml) if the *amp* gene is expressed. Expression is under the control of the *tac* promoter, which is induced by the lactose analog isopropyl β-D thiogalactoside (IPTG). The protein accumulates within the cell cytoplasm. GST occurs dimer molecular weight naturally as a M_r 26,000 protein that can be expressed in *E. coli* with full enzymatic activity. Fusion proteins that possess the complete amino acid sequence of GST also demonstrate GST enzymatic activity and can undergo dimerization similar to that observed in nature (Parker et al, 1990; Ji et al, 1992; Maru et al, 1996). The crystal structure of recombinant *S. japonicum*

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GST from pGEX vectors has been determined (McTique et al, 1995) and matches that of the native protein. GST fusion proteins are purified from bacterial lysates by affinity chromatography using immobilized glutathione. GST fusion proteins are captured by the affinity medium, and impurities are removed by washing. Fusion proteins are eluted under mild, non-denaturing conditions using reduced glutathione. The purification process preserves protein antigenicity and function. If desired, cleavage of the protein from GST can be achieved using a site-specific protease whose recognition sequence is located immediately upstream from the multiple cloning site on the pGEX plasmids. Fusion proteins can be detected using colorimetric or immunological methods.

The aim of this study is to construct the plasmid containing the desired DNA fragment from the cDNA encoding *P. marneffei* immunogenic proteins, express and purify their recombinant proteins by using GST fusion system. Then, the recombinant *P. marneffei* proteins were used to detect specific antibody in sera of *P. marneffei*-infected AIDS patients by Western blot analysis.

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