II. LITERATURE REVIEWS

2.1. History

Penicillium marneffei was first isolated from the hepatic lesion of a bamboo rat (*Rhizomys sinensis*) in the highlands of central Vietnam in 1956 (Capponi *et al.*, 1956). Bamboo rats with disseminated infection due to *P. marneffei* were kept at the Pasteur Institute in Dalat, Vietnam. The scientists first observed the spontaneous death of three bamboo rats due to disseminated *P. marneffei* infection involving the organs of the reticuloendothelial system. Subsequently, mice were experimentally infected with this fungus and sent to the Pasteur Institute in Paris for further study. A new species of the fungus was identified by Dr. Gabnella Segretain and named *Penicillium marneffei*, in honor of Dr. Hubert Marneffe, the director of the Pasteur Institute of Indochina (Segretain, 1959a). Segretain was the first record human victim of penicilliosis marneffei, which infected with this organism during his experimental studies (Segretain, 1959b). He accidentally stuck his finger with a needle used to inoculate the fungus in a hamster. The clinical manifestation of the infection was a subcutaneous small nodule at the site of the inoculation followed by lymphagitis and auxillary lymphnode hyperpathy. Seventeen years later, the first natural infection was reported in 1973 (Di Salvo *et al.*, 1973). The victim was a Caucasian 61 year-old man who suffered from Hodgkin's disease and had been living in Southeast Asia. The second reported case was in 1984 from United States (Pautler *et al.*, 1984). The patient was suffering from pulmonary tuberculosis and had a travel history to Southeast Asia. Additional reported cases were documented from endemic area. Five *P. marneffei*-infected patients were reported from Ramathibodi Hospital, Bangkok, Thailand (Jayanetra *et al.*, 1984). The first eight Chinese patients were reported from Guangxi Province of China (Deng *et al.*, 1985). In Hong Kong, the reported cases were documented in 1985 (So *et al.*, 1985), and in 1986 (Yeun *et al.*, 1986). In 1988, the first case of disseminated *P. marneffei* infection in an HIV-infected patient was reported (Piehl *et al.*, 1988). Since then, many HIV-infected patients with disseminated penicilliosis marneffei were reported in endemic areas. The majority of

reported cases occurred in AIDS patients from Maharaj Nakorn Chiang Mai Hospital (Supparatpinyo *et al.*, 1992; 1994). The other cases were reported in Taiwan, South China and Southeast Asia countries including Malaysia, Vietnam, Laos and Singapore (Supparatpinyo *et al.*, 1994). Moreover, cases of disseminated *P. marneffei* in HIV-infected patients from an eastern state of India, which shares borders with Myanmar (Singh *et al.*, 1999; Ranjana *et al.*, 2002), were reported. It is indicating that the endemic area of *P. marneffei* reaches down to the tropical countries around Southeast Asia. Cases of penicilliosis marneffei in HIV-infected individuals have also been reported in non-endemic countries such as Australia, France, Germany, Italy, Netherlands, Sweden, Switzerland, United Kingdom, and Japan (Sirisanthana, 1996; Mohri *et al.*, 2000). Individuals presenting with penicilliosis marneffei in these countries had previous visits to the endemic region. Additionally, there was a report of *P. marneffei* infection in an African AIDS patient who never visited Asia (Lo *et al.*, 2000). This case indicates that occurrence of *P. marneffei* infection outside the endemicity is possible.

2.2. Mycology

Since the discovery of *P. marneffei* in 1956 (Capponi *et al.*, 1956), the mycology of a new species of this fungus was first described by Segretain (1959a). *P. marneffei* was classified in the section Asymmetrica, subsection Divaricata by Raper and Thoms (1949). Later, *P. marneffei* was assigned to subgenus *Biverticillium* by Pitt (1979), which was confirmed by Frisvad and Filtenberg (1990) on the basis of similar physiology and secondary metabolite profiles. More recently, LoBuglio and Taylor (1995) classified this fungus by using phylogenetic analysis which was done by assessing the nucleotide sequences of nuclear and mitochondrial ribosomal DNA regions. The result revealed that *P. marneffei* is closely related to species of *Penicillium* subgenus *Biverticillium* and sexual *Talaromyces* species with asexual biverticillate *Penicillium* states.

Penicillium species are abundant in nature and frequently encountered in the laboratory as contaminants. *P. marneffei* is the only thermally dimorphic fungus of the genus *Penicillium*. The fungus grows rapidly as a mold at 25^{o}C on Sabauraud dextrose agar (Vanittanakom & Sirisanthana, 1997). Mycelial colonies appear within

1-2 days. Their growth begins as a white mycelial colony which then becomes downy to granular (Fig. 1B). As the colony ages, the center turns from white to yellow-green and finally to light green. Colony color varies amongst different fungal isolates based on the culture media and characteristics of each isolate. One of the most characteristic of mycelial phase is the production of a soluble red pigment that diffuses into the agar after growth for 2-3 days. However, red pigment production does not characterize solely this species and may be observed in several nonpathogenic *Penicillium* species. Conidia production of mold phase was induced by malt extract agar or potato dextrose agar. Microscopically, the mold phase of *P. marneffei* has typical penicillial features (Fig. 2B). The mold consists of septate-branched hyphae with lateral and terminal conidiophores which are smooth walled. Each conidiophore has basal tripes and terminal verticils of 3-4 metulae. The metulae bear 4-7 phialides, each of which produces long chain of ellipsoidal-shaped conidia. At 37^{o}C, *P. marneffei* grows rapidly as a yeast on brain heart infusion agar (Fig. 1A). The surface of yeast colonies have cerebriform wrinkle. There is a slight production of soluble brown pigment. The colony becomes light tan to brown in color. Microscopically, the yeast cells are globose to oval, measuring 2-3 x 2-6.5 µm, enmeshed by hyphae-like structures (Fig. 2A). As the culture ages, segments of varying length form, which appear to segment along septal planes. The ontogeny of these cells defines them as arthroconidia rather than yeast cells (Cooper and Haycocks, 2000). Uniquely, yeast cells divide by fission and not budding, and often have a central septum. The biochemical properties were assessed by Segretain (1959b). The studies demonstrated that *P. marneffei* has ability to assimilate glucose, lactose, xylose, levulose and manitol. The biochemical properties were confirmed by studies of 32 clinical isolates of *P. marneffei* in Hong Kong (Wong *et al.*, 2001a). All isolates were positive for urease and inhibited by 500 mg/L of cyclohexamide. For the assimilation, all isolates tested positive for glucose, maltose and cellobiose. The majority (65-85%) of the isolates assimilated trehalose, xylose and nitrate. However, none these biochemical properties are unique to the species, thus cannot be used for identification.

2.3. Epidemiology and natural reservoir

Penicillium marneffei was considered rarely infection until the spread of HIV through Southeast Asia. The opportunistic fungal infection occurs among HIV-infected and other immunocompromised patients in several regions of Southeast Asia. The majority of cases were reported in AIDS patients from Northern Thailand (Supparatpinyo *et al.*, 1992; 1994). Areas where *P. marneffei* infection is known to be endemic include Thailand, Southern China, Taiwan, Hong Kong, Malaysia, Indonesia, Vietnam, Myanmar and India (Deng *et al.*, 1988; Supparatpinyo *et al.*, 1994; Duong, 1996; Singh *et al.*, 1999; Ranjana *et al.*, 2002). However, there was a single case of disease in an African from Ghana, who had no history of travel to Asia (Lo *et al.*, 2000). Non-endemic area have also been documented the cases of penicilliosis marneffei in HIV-infected individuals such as, Australia, France, Germany, Italy, Netherlands, Sweden, Switzerland, United Kingdom, and Japan (Drouhet, 1993; Sirisanthana, 1996; Mohri *et al*., 2000). All the patients had traveled to the endemic areas.

After the first isolation of *P. marneffei* from bamboo rat *(Rhizomys sinensis*) captured in Vietnam (Capponi *et al.*, 1956), many investigators attempted to describe the relationship between the two organisms. Two genera of bamboo rats are found in endemic areas (Cooper, 1998). One genus of bamboo rats, *Rhizomys*, contains at least three species including *R. sinensis, R. pruinosus* and *R. sumatrensis.* The second genus, *Cannomys*, contains only one species, *C. badius*, which includes two distinct group: reddish-brown and grayish-black rats. From several reports (Table 1 and Table 2), *P. marneffei* is a naturally occurring infection in a high proportion of bamboo rat species in endemic areas. The data showed that the bamboo rat is often a carrier of this fungus. The other rodents (*Bandicota bengalensis, Rattus norvegicus, Rattus rattus, Rattus nitidus,* and *Mus musculus*) were investigated in India (Gugnani *et al.*, 2004). None of the 72 rodents of the five species trapped on bamboo plantations from northeast India were found to harbor *P. marneffei*. In a case control study, Chariyalertsak *et al.* (1996a) could not establish exposure to bamboo rats as a risk factor for acquiring penicilliosis marneffei, even though some people live in proximity to rat habitats and consume them as food. They proposed that exposure to soil, especially during the rainy season in the tropical climes of Thailand, is the

critical risk factor associated with acquisition of *P. marneffei* infection. However, when soil samples and rat burrow samples were cultured, only one of 95 samples proved to be culture positive (Chariyalertsak, 1996b). In a current study in India, Gugnani *et al.* (2004) could not isolate *P. marneffei* from any the 25 samples of soil from the burrows of *C. badius* or from 10 samples (each) of bamboo shoots and leaves from the surrounding areas. They also did not find *P. marneffei* in 120 soil samples collected from burrows of other species of rats or from sites other than rodent burrows in bamboo plantations in different parts of northeast India. Additionally, investigators attempted to establish an environmental reservoir for *P. marneffei*. Vanittanakom *et al.* (1995) demonstrated 80 to 85.1 % CFU recovery after 3 days of incubation from sterilized soil seeded with *P. marneffei*, but the recovery from nonsterile soil seeded with the fungus was only 6%. The evidence of survival *of P. marneffei* in soil was confirmed by Joshi *et al.* (2003). They demonstrated that *P. marneffei* can survive in sterile soil for several weeks but only a few days in nonsterile soil. Therefore, exactly how and where both humans and bamboo rats acquire *P. marneffei* and natural occurrence of this fungus in soil remain unclear.

2.4. Mode of transmission, pathogenesis and clinical characteristics

The type of exposure and the route of entry of *P. marneffei* leading to infection in humans are still unclear. However, microbiologists presumed that the infection was acquired from the inhalation of an aerosol containing *P. marneffei* conidia suggesting a respiratory route of infection. There was evidence that proved this presumption is possible. One patient, an HIV-positive Congolase physician, was infected with *P. marneffei* at the Pasteur Institute after attending a course in tropical microbiology (Peto *et al.*, 1988). The organism was not handled directly by the patient, but by other students and laboratory workers in the same building. This case assumed that the patient acquired the infection from an aerosol containing *P. marneffei* spores. Additionally, the study by Deng *et al.* (1988) revealed that there might be a common environmental source of infection and the spores of the organism may be inhaled or ingested, thereby infecting both rats and humans. Since bamboo rats live in remote, mountainous areas and have limited contact with people, direct transmission is not expected to be a significant cause (Deng *et al.*, 1986).

Similar to *Histoplasma capsulatum*, *P. marneffei* is likely acquired via respiratory route and then disseminates through the body by hematogenous means (Cooper and McGinnis, 1997). The first step of pathogenesis is the attachment of *P. marneffei* conidia to host cells and tissues. The conidia-host interaction may occur via adhesion to the extracellular matrix protein laminin and fibronectin via a sialic-dependent process. Hamilton *et al.* (Hamilton *et al.*, 1998; 1999) demonstrated that fibronectin binds to the conidia surface and to phialides, but not to hyphae. In histiocytes, the fungus can survive and proliferate in the phagosome. Cooper and McGinnis (1997) demonstrated that histiocytes of tissues infected with *P. marneffei* contain a few to many globose-to-oval yeastlike cells of *P. marneffei* that measure 2-3 x 2-7 μm. As the lesion progresses, the intracellular fungal cells were released following cellular necrosis and subsequent abscess formation. Free fungal cells or phagocytes containing fungal cells can disseminate throughout the body.

There were many reported cases and clinical characteristics of penicilliosis marneffei. Supparatpinyo *et al.* (1994) demonstrated that the most common presenting symptoms and signs were fever, anemia, weight loss, and cutaneous lesions. Cough, generalized lymphadenopathy, and hepatomegaly also occurred frequently. The most common skin lesion was a papular rash with central umbilication, resembling molluscum contagiosum. These lesions were found predominantly on the face, scalp, and upper extremities, but spared on surface including the palate. Chest radiographs were frequently abnormal; diffuse reticulonodular or localized alveolar infiltrates being the most common findings. The incubation periods of the disseminated disease vary from a few weeks to many years of exposure to the organism (Peto *et al.*, 1988; Jones and See, 1992), resulting in three possible processes: primary infection, reinfection, or reactivation of the latent disease. Moreover, there was a publication that compared of the clinical manifestations of penicilliosis in HIV-positive and HIV-negative patients (Wong *et al.*, 2001b). The HIV-infected patients were more likely to have a higher incidence of fungaemia than non-HIV-infected patients. There was a significant delay in establishing the diagnosis in non-HIV-infected patients when compared with HIV-infected patients.

2.5. Dimorphism and putative virulence factors

The growth of *P. marneffei* can be divided into two phases based on natural growth and during infection: saprobic phase at 25°C and parasitic phase at 37°C. The saprobic phase *(in vitro* model) at 25°C under suitable conditions (Fig. 3), spore rapidly grows within the first 6 h and then polarises to produce germ tube by 12 h. The germ tube grows apically to a hypha, and cellular compartments are established behind the growing tip by septation. Unlike apical cells, sub-apical cells are capable of repolarising to produce branched cells with a new apical growth point. When hyphal growth was fully established and the presence of an air interface, such as growth on a solid medium in Petri dish, was provided then reproductive asexual (conidia) were produced. Additionally, conidia production was induced by using malt extract agar (MEA) as well as potato dextrose agar (PDA). However, Sabouraud dextrose agar (SDA) induced filamentous growth and less spore production in late-phase cultures.

When conidia are germinated in parasitic phase (*in vitro* model, Fig. 3) at 37°C, they do not direct morphogenesis to produce yeast cells. Initially, germination of conidia at 25°C and 37°C is indistinguishable with conidia swelling and polarization that lead to germ tube formation to produce germ tube, eventually a hyphal network. However, the cellular differentiation stage at 37°C is slower than at 25°C as follow spores grow within the first 12 h and then polarise to produce germ tube by 24. Germinated conidia produce highly branched hyphal cells. Cellular differentiation to yeast-like cells begins between 24 to 48 h. By 48 h post germination, most branched hyphae go through arthroconidiation and segmentation. Completed yeast cells are observed at 96 h after germination. Conversely, *P. marneffei*–infected macrophages only contain yeast cells. The establishment of a hyphal network by *P. marneffei* conidia after they have been phagocytosed by macrophages has not been described (Chan and Chow, 1990; Garrison and Boyd, 1973). In addition, experimental *P. marneffei* infection in mice was investigated by Cui *et al*. (1997). They demonstrated that conidia were phagocytosed by Kuffer cells soon after inoculation with viable conidia of *P. marneffei* through the tail vein, and proliferated by fission in the cytoplasm. At 1-day stage, conidia phagocytosed by Kuffer cells became yeast-like cells. The phagocytosed cells elongated and curved. Two and 3 days after

inoculation, yeast cells proliferated in phagosomes with some cells multiplying in phagocytic vacuoles. The yeast cells in phagosomes were globose, ovoid or elongated in shape. Some of the yeast cells had one or two septa which were electron lucent. The other observation, when yeast cells are shifted from 37°C to 25°C, yeast cell rapidly turned to germ tube (mycelial phase) within 24 h. Therefore, dimorphic switching in *P. marneffei* occurs in both the mycelial (or spore) to yeast-like and the yeast-like to mycelial directions and is triggered by simple temperature cue (Andrianopoulos, 2002). Other factors such as nutritional conditions can also influence the growth state of *P. marneffei*, but these factors cannot effect dimorphic switching in the absence of the temperature signal. Thermal dimorphism of *P. marneffei* plays an important role for survival in host cells. It is responsible for the temperature change of host body. Thus, the dimorphic switching is one of the virulence factors of *P. marneffei*.

Virulence factors of *P. marneffei* are still unclear. There are a few reported observations that contribute to putative virulence factors. Analysis of the enzymatic activity of mycelial and yeast phases of *P. marneffei* was reported by Youngchim *et al.* (1999). They demonstrated that both mycelia and yeast expressed alkaline phosphatase, acid phosphatase and naphthol-AS-BI-phosphohydrolase activity, whereas a variety of other enzyme activities, including trypsin, chymotrypsin and α–fucosidase were absent. The authors discussed that production of acid phosphatase is considered to be one of the virulence factors for survival of *P. marneffei* in phagocytes. Recently, Pongpom *et al.* (2005) isolated an antigenic catalase-peroxidase protein-encoding gene (*cpeA*) by antibody screening of a cDNA yeast phase library of this fungus. The *cpeA* transcript was accumulated in the yeast phase, which is the pathogenic form of *P. marneffei*. Enzymatic assays also revealed that the yeast phase of *P. marneffei* exhibited high levels of catalase-peroxidase activity. The authors suggested that catalase-peroxidase protein may contribute to the survival or interaction of this fungus to the host cells. More recently, melanization of *P. marneffei in vitro* and during infection was investigated by Youngchim *et al.* (2005). They found melanin in both conidia and yeast cells *in vitro*. Further investigation *in vivo*, they found melanin in yeast cells inside skin tissue from penicilliosis marneffei patients. In addition, sera from *P. marneffei* – infected mice developed a significant antibody

response (both IgG and IgM) against melanin. Phenoloxidase activity capable of synthesizing melanin from L-DOPA was detected in cytoplasmic yeast cell extracts. This data indicated that *P. marneffei* conidia and yeast cells can produce melanin or melanin-like compounds *in vitro* and that the yeast cells can synthesize pigment *in vivo*. The authors speculated that pigments may play some role in the virulence of *P. marneffei*.

2.6. Immunity to *Penicillium marneffei.*

Penicillium marneffei is an intracellular opportunistic fungus causing invasive mycosis in immunocompromised patients, especially AIDS patients who have a CD4+ count of less than 100 cells/cu.mm.(Supparatpinyo *et al.*, 1994; Sirisanthana *et al.*, 1998a). Disseminated infection with *P. marneffei* is considered to be the indicator disease for AIDS (Li *et al.*, 1992). The immune response against *P. marneffei* infection is mediated mainly by T-cells and macrophages (Kudeken *et al.*, 1996; 1997). The role of T-cell cytokines in the immune response against *P. marneffei* was studied by Sisto *et al.* (2003). They demonstrated that splenic and hepatic granulomas were present in wild-type (wt) mice, whereas disorganized masses of macrophages and yeast cells were detected in IFN- γ knockout (GKO) mice. The infection resolved faster in the spleen than in the liver of wt mice and associated with the local expression of Type 1 cytokines (high levels of IL-12 and IFN-γ) but not Type 2 cytokines (low levels of IL-4 and IL-10). Conversely, both Type 1 and Type 2 cytokines were detected in the livers of wt mice. The inducible nitric oxide synthase mRNA level was low and the TNF-α was high in both spleen and liver in GKO mice compared to wt mice. These data suggested that the polarization of protective Type 1 immune response against *P. marneffei* is regulated at the level of individual organs and that the absence of IFN- γ is crucial for the activation of fungicidal macrophages and for the development of granulomas.

The role of nitric oxide (NO) and reactive nitrogen intermediates in the *in vitro* growth of *P. marneffei* both in a cell-free system and in a novel macrophage culture system was studied by Cogliati *et al.* (1997). In the cell-free system, pH-dependent NO markedly inhibited the growth of *P. marneffei*. The inverse correlation between

intramacrophage growth and the amount of nitrite detected in culture supernatants support the hypothesis that the L-arginine-dependent NO pathway plays an important role in the murine macrophage immune response against *P. marneffei.*

The interaction between human leukocytes and heat-killed yeast-phase *P. marneffei* has been studied by Rongrungruang *et al.* (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. The fungicidal activity of macrophages is likely to involve the generation of reactive nitrogen intermediates, as described by Kudeken *et al.* (1999b). The addition of superoxide dismutase enhanced the overall fungicidal activity of the macrophages. Recently, effect of elutriated human monocytes (EHMs) on the elimination of the *P. marneffei* conidia was reported (Roilides *et al*., 2003).

The role of human neutrophils was observed by Kudeken *et al.* (1999a). They demonstrated that 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 gamma interferon (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. Conidia are, however, generally not susceptible to killing by phagocytes. The fungicidal activity exhibited by neutrophils is believed to be independent of the superoxide anion, acting through exocytosis of granular enzymes instead (Kudeken *et al.*, 2000). Recently, Koguchi *et al.* (2002) 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. 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.

2.7. Molecular studies of *Penicillium marneffei*

There are a few studies of genes encoding immunogenic proteins by using a cloning approach. Cao *et al*. (1998a) reported a *MP1* gene that encoded Mp1p protein, which is an abundant antigenic cell wall mannoprotein. Recently, a cDNA library from the yeast form of *P. marneffei* was constructed by Pongpom (2004). Immunogenic clones were isolated by Ab screening approach using pooled sera of *P. marneffei*-infected patients. The author demonstrated that immunogenic genes were the genes encoded catalase-peroxidase, heat shock protein 30, fructose-1,6-bisphosphatase, 60S ribosomal protein, cytochrome C oxidase, NADH-ubiquinone oxidoreductase, Mp1p-like protein, glutathione peroxidase, thymine synthase, stearic acid desaturase and 7 unknown proteins.

Studies of molecular morphogenesis are importance to understand the mechanism of morphogenesis control in this fungus. Several genes involving in this mechanism were reported since 2000. A homologue of *Aspergillus nidulans abaA* gene encoding ATTS/TEA DNA-binding domain transcriptional regulator was the first gene described by Borneman *et al.* (2000). They demonstrated that deletion of *abaA* blocked asexual development at 25^{o}C before spore production, resulting in aberrant conidiophores with reiterated terminal cells. They also found the mutant strain failed to switch correctly from multinucleate filamentous to uninucleate yeast cells. The *abaA* gene of the filamentous monomorphic fungus *A. nidulans* could complement both conidiation and dimorphic switching defects in the *P. marneffei abaA* mutant. Then, Borneman *et al.* (2001) described a *stlA* gene*, a STE12* homologue which was predicted to encode a protein containing two potential DNA-binding domains: a homeobox domain and a $C_2H_2Zn^{2+}$ finger motif. Typically, the STE protein is a protein in family of transcriptional regulators that control mating and dimorphic transition. However, deletion of *stlA* didn't appear to have any phenotypic effect in *P. marneffei*, but the gene appeared fully functional as it is capable of complementing the sexual cycle defects of a *steA* mutant in *A. nidulans*. A *CDC42* homologue (*cflA* gene) of *Saccharomyces cerevisiae*, a member of the Rho family of

small GTPase-encoding gene, was described by Boyce *et al.* (2001). They demonstrated that CflA is required for polarized growth of vegetative mycelium at 25°C and correct morphogenesis of yeast cells. However, CflA is not required for dimorphic switching and did not appear to play a role during the generation of specialized structures during a sexual development. In 2002, a basic helix-loop-helix protein-encoding a homologue of *A. nidulans*, *stuA* gene, was reported by Borneman *et al.* (2002). Deletion of *stuA* in *P. marneffei* showed that the gene was required for metula and phialide formation during conidiation but was not required for dimorphic growth. The *gasA* gene encoding a Gα subunit of a heterotrimeric G protein, an ortholog of *A. nidulans FadA*, was studied by Zuber *et al.* (2002). A dominant activating $gasA^{G42R}$ mutant did not express the conidiation-specific regulator gene (*brlA*) and was locked in vegetative growth, while a dominant interfering $gasA^{G203R}$ mutant showed inappropriate *brlA* expression and conidiation. GasA was also a regulator of secondary metabolites, but was not involved in dimorphic switching or yeast growth at 37°C. Later, Zuber *et al.* (2003) described *gasC* gene encoding a G-protein α -subunit that showed high homology to members of the class III fungal G α–subunits. GasC was a regulator of secondary metabolites and conidiation similarly to GasA. A Δ*gasC* mutant severely delayed in germination, whereas strains carrying a dominant-activating $gasC^{G45R}$ allele showed a significantly accelerated germination rate. This was the first report showing that a Gα-subunit was involved in germination in filamentous fungi. A study by Todd *et al.* (2003) showed that deletion of *tupA*, the *TUP1* homologue, confered reduced-filamentation and inappropriated yeast morphogenesis at 25°C. Deletion of *tupA* also conferred premature *brlA*-dependent asexual development, unlike reduced asexual development in the corresponding *A. nidulans rco* deletion mutant. Therefore *tupA* represented a focal point for regulation of the switches between undifferentiated filamentous growth and development. Moreover, the *cflB* gene, which encoded a RAC homologue of family of GTPase proteins, was described by Boyce *et al.* (2003). Their data showed that the overlapping of CflB and CflA function controlled a vegetative hyphal growth. CflB also affected cellular polarization during asexual development, but not during yeast growth.

2.8. Diagnosis

2.8.1.) Direct staining. The primary diagnosis of *Penicillium marneffei* infection is usually made by identifying the fungus in clinical specimens by microscopy. The clinical specimens include bone marrow aspirates, blood, lymph node biopsies, skin biopsies, skin scrapings, sputum, bronchoalveolar lavage pellets, pleural fluid, liver biopsies, cerebrospinal fluid, pharyngeal ulcer scrapings, palatal papule scrapings, urine, kidney, pericardium, stomach or intestine and stool samples (Supparatpinyo *et al.*, 1994; Drouhet, 1993). Staining with Wright's (or Giemsa) stains of bone marrow aspirates and/or touch smear of skin biopsy or lymph node biopsy is a rapid and sensitive diagnostic technique (Supparatpinyo *et al.*, 1992). It is low-cost and convenient method, suitable for local laboratories in endemic areas. The fungus can be seen in histopathological sections stained with Grocott methenamine silver or periodic-acid-Schiff, whereas when stained with hematoxylin-eosin, *P. marneffei* cells may result in the false impression that a capsule similar to *Histoplasma capsulatum* (Nelson *et al.*, 1999). Within histiocytes, the fungi are ovoid or round and tend to be uniform in size at about 3 mm in diameter (Deng and Conner, 1985). Many of the extracellular *P. marneffei* cells are elongated, occasionally curved and measure 8 to 13 μm in length (Cooper and McGinnis, 1997). Rarely, short hyphae no longer than 20 μm are observed. Because *P. marneffei* reproduces by schizogony (fission), the dividing cells of *P. marneffei* characteristically contain a single, centrally located-transverse septum, although cells with two septa can also be observed.

2.8.2.) Culture. Culture is the gold standard of *P. marneffei* infection diagnosis. Bone marrow culture is the most sensitive (100%), as followed by culture of the specimen obtained from skin biopsy (90%) and blood culture (76%) (Supparatpinyo *et al.*, 1994). It is also possible to isolate the organism from cerebrospinal fluid, sputum, pleural fluid and liver biopsy (Chiewchanwit *et al.*, 1991). However, most of fungal isolates in routine laboratory screening are usually obtained from the blood culture of HIV-infected patients. *P. marneffei* is identified by macroscopic and microscopic examination. The fungus grows in saprophytic mycelial phase at 25^{o}C on SDA. Its morphological characteristics are similar to the genus *Penicillium* (Fig. 2). A typical red soluble pigment secretion is common at the

saprophytic mycelial phase. However, the temperature-dependent conversion from saprophytic mycelial form to parasitic yeast-like form is the confirmative test to identify this fungus. The fungus grows in parasitic yeast-like phase on BHI at 37°C. The yeast-like phase cells of *P. marneffei* are usually elongated with one or more septa found as the result of division by fission (Fig. 2). Arthroconidia-like cells and short septate hyphae are also found.

2.8.3.) Serodiagnosis. Several serodiagnostic tests were developed to provide rapid diagnosis of *P. marneffei* infection. In a study by Viviani *et al.* (1993), antigens derived from a mycelial culture were used in an immunodiffusion test to detect precipitins in the serum samples derived from an AIDS patient infected with *P. marneffei*. Positive antibody reactions were noted early in the course of the disease, but sera taken up to 5 months following therapy were negative. From a study by Yuen *et al.* (1994), an indirect immunofluorescent antibody test was developed in which germinating conidia and yeast-like cells were employed as antigens. The test could provide a rapid presumptive diagnosis and it does supplement conventional cultures. Both an immunodiffusion and latex agglutination test have been used to diagnose antigenemia in patients during *P. marneffei* infection (Kaufman *et al.*, 1996). The results showed that immunodiffusion tests detected *P. marneffei* antigenemia in 10 (58.8%) of 17 patients, whereas the latex agglutination test detected *P. marneffei* antigenemia in 13 (76.5%) of the 17 patients. These highly specific tests do not cross-react with antigens from *Aspergillus* species or *Histoplasma capsulatum* and appear much more sensitive than antibody detection techniques. A study by Chongtrakool *et al.* (1997) revealed that *P. marneffei* produces and secretes a 38-kDa antigen that appeares to be specific to this dimorphic fungus. Antibody reaction with this antigen was found in a large proportion of HIV-patients indicating a presumptive diagnosis of *P. marneffei* infection. A Western blot analysis of protein antigens of *P. marneffei*, which prepared during the yeast and mould phase of *in vitro* growth, was studied by Vanittanakom *et al.* (1997). The results showed that four immunogenic proteins of 200, 88, 54 and 50 kDa, which were produced in large quantity during the deceleration and early stationary yeast phase of growth, could react with individuals sera derived from 33 AIDS patients with penicilliosis marneffei. The reactivities to these proteins were high for about 60-94 %, with the strong reactions of the bands of

88, 54 and 50 kDa. Interestingly, the strong reactivities to the 54 and 50 kDa were observed in one serum derived from an AIDS patient who was definited diagnosis by fungal culture two months later. They suggested that there were at least two yeast-phase immunoreactive proteins (54 and 50 kDa) that would be useful for clinical application to diagnosis. Identification and purification of specific *P. marneffei* antigens of 50, 54 and 61 kDa and their recognition by human immune sera have been studied (Jeavons *et al.*,1998). The three antigens were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotting and to immunoenzyme development with individual patient sera. The study revealed that sera from 86% of the *P. marneffei*-infected patients recognized the 61-kDa antigen, sera from 71% recognized the 54-kDa antigen and sera from 48% recognized the 50-kDa antigen.

An ELISA-based antibody test was developed with a recombinant *P. marneffei* mannoprotein (Mp1p) for the serodiagnosis of *P. marneffei* infection (Cao *et al.*, 1998b). Evaluation of the test revealed high specificity: approximately 80 % (14 of 17) sensitivity in HIV seropositive patients infected with *P. marneffei.* The anti-Mp1p antibody was also used in an ELISA-based method for the detection of the mannoprotein, Mp1p, in the sera of patients (Cao *et al.*, 1999). The antigen test had an overall sensitivity of 65 % (17 of 26). The combined antibody and antigen tests for the diagnosis of *P. marneffei* infection had a sensitivity of 88 % (23 of 26), with a positive predictive value of 100% and a negative predictive value of 96%. An ELISA for the quantitation of *P. marneffei* antigen in urine was developed using fluorescein isothiocyanate-labelled purified rabbit hyperimmune Ig that was prepared from killed whole-fission-form arthroconidia of *P. marneffei* (Desakorn *et al.*, 1999). Urinary antigen was found in all 33 patients with penicilliosis, with a median titer of 1: 20,480. The urine antigen detection assay had a diagnostic sensitivity of 97 % and specificity of 98 % at a cutoff titer of 1 : 40. Later, Desakorn *et al.* (2002) developed a dot blot ELISA and a latex agglutination (LA) test by using the same polyclonal antibody and compared with the ELISA for detection of *P. marneffei* urinary antigen. Sensitivities and specificities of the diagnostic tests were as follows: sensitivities; dot blot ELISA (94.6%), ELISA (97.3%) and LA test (100%): specificities; 97.3, 98 and 99.3%, respectively. Recently, Chaiyaroj *et al.* (2003) developed an antigen capture

ELISA by using the mixture of two monoclonal antibodies (8B11 and 8C3) for the detection of *P. marneffei* antigens in sera of humans in areas where the organism is endemic. The diagnostic sensitivity, specificity, and accuracy of the ELISA were 92.45, 97.5 and 96.59%, respectively.

2.8.4.) Molecular diagnosis. The polymerase chain reaction (PCR) technique for detection of the *P. marneffei* genomic DNA was first reported by LoBuglio and Taylor (1995). Specific oligonucleotide primers were designed from the internal transcribed spacer and 5.8S rRNA gene (ITS1-5.8S-ITS2) of *P. marneffei*. A pair of PM2 and PM4 primers was 100% successful in amplifying *P. marneffei* DNA yielding a 347 bp PCR product and selecting against the amplification of the other tested species, which included *Penicillium* Subgenus *Biverticillium* and *Talaromyces* species and several well-known fungal pathogens (*Aspergillus fumigatus*, *Coccidioides immitis*, *Histoplasma capsulatum* and *Pneumocystis carinii*). On the other hand, Vanittanakom *et al.* (1998) developed a PCR-Southern hybridization method by using a pair of RRF1 and RRH1 primers to amplify a 631 bp fragment of the 18S rDNA and a *P. marneffei*-specific oligonucleotide probe (Pm3) which was designed from the internal region of 18S rDNA of *P. marneffei*. Their data demonstrated that the Pm3 probe could hybridize with only the 631-bp PCR products from *P. marneffei*, but not from six other fungi including *Penicillium* spp., *A. fumigatus*, *A. flavus*, *H. capsulatum*, *Cryptococcus neoformans*, *Candida albicans* and *C. krusei*. The sensitivity of the assay was approximately 0.5 and 0.1 pg/µl of DNA by PCR and Southern hybridization, respectively. Later, Vanittanakom *et al.* (2002) developed a nested PCR assay by using the outer primers (RRF1 and RRH1) and the *P. marneffei* specific inner primers (Pm1 and Pm2) which were derived from the sequence of 18S rRNA gene of *P. marneffei*. The primers Pm1 and Pm2 were specific to amplify the fragment of approximately 400-bp from both mold and yeast forms of *P. marneffei*, but not from other fungi, bacteria and human DNA. The nested PCR (1.8 fg/µl) was more sensitive than the single PCR (1.0 pg/µl, only Pm1 and Pm2 primers). Additionally, Prariyachatigul *et al.* (2003) developed a one-tube seminested PCR assay by using F3, CLP1 and PM primers which designed from 18S rDNA region. They demonstrated that this assay detected 100% specifically amplified products of 251 and 331 bp from all *P. marneffei* DNA (47 strains) and

from two blood samples of AIDS patients suspected to suffer from penicilliosis marneffei. The assay was sensitive to detect as little as 10 pg purified DNA, which is equivalent to 250 cells. The author suggested that this PCR assay might be useful as an alternative test, if a rapid diagnosis of penicilliosis marneffei is needed.

2.9. Therapy and prophylaxis

Penicilliosis marneffei patients have poor prognosis without treatment (Supparatpinyo *et al.*, 1994). Even with treatment, mortality is approximately 20%. The fungus was highly susceptible to amphotericin B, itraconazole, and ketoconazole *in vitro* (Supparatpinyo *et al.*, 1993). However, fluconazole appears to be less active *in vitro*. Clinical failure is more common with fluconazole (63.8%) compared to treatment with amphotericin B (22.8%) or itraconazole (25%). The current recommended treatment regimen is to give amphotericin B 0.6 mg/kg/day intravenously for 2 weeks followed by a 400 mg/day dosage of oral itraconazole for 10 weeks (Sirisanthana *et al.*, 1998b). This regimen was effective in 97% of the treated 74 HIV-infected patients. All patients had cleared fungaemia by the end of their second week of treatment. Patients tolerated the regimen without any major adverse reactions. After completing initial treatment, patients with *P. marneffei* infection should receive secondary prophylaxis with itraconazole 200 mg daily to prevent relapse of infection (Supparatpinyo *et al.*, 1998). None of the patients receiving itraconazole had a relapse while 57% of those in the placebo group had a relapse within one year after completing initial treatment. Recently, itraconazole (200 mg per day) was proved to be an effective prophylatic agent against *P. marneffei* and *Cryptococcus neoformans* infection among advanced AIDS-patients in Thailand (Chariyalertsak *et al.*, 2002). It is reasonable to consider primary prophylaxis with itraconazole in HIV-infected patients with CD4 + lymphocyte counts of less than 100 cells/cu.mm who live in developing countries, especially if the patients cannot be treated with highly active combination antiretroviral therapy.

2.10. Knowledge involving heat shock proteins (Hsps) and heat shock protein 70

2.10.1.) Heat shock proteins. The heat shock response was discovered by Ritossa (1962) who observed a pattern of *Drosophila* salivary gland chromosome puffs that were induced in response to transient exposures to elevated temperatures. Since then, efforts from a large number of investigators have shown that the heat shock response is ubiquitous and highly conserved, in all organisms from bacteria to plants and animals, as an essential defense mechanism for protection of cells from harmful conditions including heat shock, alcohols, inhibitors of energy metabolism, heavy metals, oxidative stress, fever or inflammation (Lindquist, 1986; Morimoto, 1993). Heat shock proteins can be classified into groups based primarily on size which vary from 10 – 170 kDa. The six major size classes recognized were Hsp100, Hsp90, Hsp70, Hsp60, Hsp40 and the small heat shock proteins which can range from10-30 kDa (Fink and Goto, 1998; Gething, 1997). Within each gene family are members that are constitutively expressed, inducibly regulated, and/or targeted to different compartments. The induction of heat shock proteins upon exposure to heat stress was coined the heat shock response although it was subsequently observed that diverse stressors such as heavy metals, ischemia, radiation, anoxia, glucose deprivation and a variety of chemical stressors also elicited the induction of heat shock proteins (Linquist, 1986). The heat chock proteins function as molecular chaperones which bind to and stabilize proteins that are in a non-native conformation. Interactions with these unstable protein conformations prevent the formation of large protein aggregates and facilitate normal protein folding, membrane translocation and the degradation and removal of damaged proteins (Feder and Hofmann, 1999). The cellular response to stress has been an invaluable tool for investigating the mechanisms and dynamics of inducible gene expression in eukaryotes (Lindquist, 1986; Morimoto, 1993). The molecular analysis of Hsp genes identified the heat shock element (HSE), a stress-responsive promoter element essential for heat shock inducibility, which comprises multiple adjacent inverted arrays of the bindings site (5'-nGAAn-3') (Wu, 1995; Morimoto, 1998). HSEs are positioned at various distances upstream of the site of transcription initiation; in vertebrates, inducible transcription requires the *de novo* binding of heat shock transcription factors (HSFs) transiently to the HSEs. Whereas vertebrates and plants have at least four members of

the HSF gene family, only a single HSF is expressed in *Saccharomyces cerevisiae, Caenorhabditis elegans* and *Drosophila* (Wu, 1995; Morimoto, 1998). Activation triggers vary within the HSF family. For instance HSF is activated by heat and other stressors, HSF2 activation occurs during cell development and differentiation, while the exact triggers of HSF3 and HSF4 are unclear (Nakai *et al.*, 1993; Nakai, 1997). A model of the heat shock response was presented in review by Morimoto (1993). The response is controlled primarily at the transcription level by a heat shock factor (HSF). In unstressed cells, HSF is present in the cytoplasm and the nucleus in a monomeric form that has no DNA binding activity through its interactions with Hsp70. In response to stress, the monomeric forms combine into trimers and accumulate within the nucleus. The response is very rapid, starting within minutes of the temperature rise. In the nucleus, the trimers bind to the heat shock elements (HSE). When attached to DNA, HSF becomes phosphorylated. Finally, the trimeric forms of HSF dissociate from the DNA and are converted back into non-active monomers.

2.10.2.) The HSP 70 family. The Hsp70 are ancient, highly conserved among all species from bacteria to humans (Craig and Linquist, 1988). The DnaK chaperones are the prokaryotic homologues of Hsp70s, having about 50% identity to all eukaryotic Hsp70s (Rensing and Maier, 1994). The eukaryotic proteins are between 50 and 98% identical. A comparison of all the known Hsp70 sequences revealed that the N-terminal two-third of the protein is more highly conserved than the C-terminal one-third (Boorstein *et al.*, 1994; Craig *et al.*, 1993).

Structurally, Hsp70 typically ranges from 613 to 650 amino acids in length; it shares a high degree of similarity amongst widely divergent groups (Boorstein *et al.*, 1994; Ha *et al.*, 1998). The N-terminal region of the protein contains an ATP binding site of approximately 385 amino acid residues which has a weak ATPase activity. The portion of the Hsp70 responsible for binding to peptides lies approximately 160 residues from the ATPase domain and consists of a channel formed by two β–sheets and an α–helical subdomains (Morshauser *et al.*, 1995). The two β–sheets structures confirmed by nuclear magnetic resonance are the most hydrophobic parts of Heat shock cognate (Hsc70) and thus likely to be involved in the binding of hydrophobic peptides (Leung and Hightower, 1997; Kiang and Tsokos, 1998). The approximately

100 residues of C-terminal region of the protein varies among the different Hsp70 subfamilies (Ha *et al.*, 1998). The highly conserved EEVD terminal sequences are present in all eukaryotic HSP70 and HSP90 (Kiang and Tsokos, 1998).

Functionally, the Hsp70 comes in two flavors constitutively expressed and inducible. The constitutively expressed proteins or cognate proteins assist in the day to day cell functions of protein folding and unfolding, prevention of polypeptide aggregation, disassembly of large protein complexes and aid in the translocation proteins between cellular compartments (Gething, 1997). The inducible form of Hsp70 was initially recognized for its rapid synthesis upon exposure to thermal stress. Despite this early label as a "heat shock" protein it has subsequently been recognized that exposure to a variety of proteotoxic stressors results in the rapid induction of Hsp70. These stressors either damage proteins directly or result in the abnormal synthesis of proteins leading to the expression of Hsp70 (Kozutsumi *et al.*, 1988; Edington *et al.*, 1989; Mifflin and Cohen, 1994). Transcription of the *hsp70* gene is due to the activation of heat shock transcription factor (HSF) which binds to the heat shock elements (HSE) that are located upstream of the coding region (Morimoto, 1998).

2.10.3.) Expression of fungal Hsp70s. The *Saccharomyces cerevisiae* genome contains at least ten genes that are related to the Hsp70 encoding genes of higher eukaryotes (Craig, 1992, Mukai *et al.*, 1993). These genes were named on the basis of structural and functional similarities including *SSA1-4*, *SSB1-2*, *SSC1*, *SSD1* (*KAR2*) and *SSE1-2* and then were the model for studying in fungal Hsp70s. *SSA1-4* and *SS1-2* are the genes that encode the cytoplasmic-localized proteins, whereas *KAR2* and *SSC1* are essential genes that encode the protein localized in the endoplasmic reticulum and the matrix of mitochondria, respectively. The expression of the family members is modulated differently in response to changes in temperature. The transcripts of *SSA1*, *SSC1* and *KAR2* were abundance during steady-state growth and increased three to tenfold upon an upshift in temperature (Craig, 1992). Genes *SSA3* and *SSA4* were expressed at very low levels during steady-state growth at 23^{o}C, but their expression was greatly enhanced upon upshift to 39^{o}C (Werner-Washburne *et al.*, 1987; Craig, 1992). The *SSE1* mRNA was moderately abundant during steady-state growth at 23^{o}C, whereas *SSE2* was present low level (Mukai *et al.*, 1993). The

expression of *SSA2* and *SSE1* changed little upon shift to a higher or lower temperature (Ellwood and Craig, 1984, Mukai *et al.*, 1993). The expression of *SSE2* greatly increased upon upshift to 37°C (Mukai *et al.*, 1993). However, *SSB1* and *SSB2* transcripts, which were abundant during steady-state growth, rapidly decreased in amount upon an upshift in temperature (Craig and Jacobsen, 1985).

A study of Caruso *et al.*, (1987) in dimorphic *Histoplasma capsulatum*, the *hsp70* gene was isolated using a Drosophila *hsp70* gene to screen a cosmid library of the DNA from the temperature-sensitive Downs strain (low level of thermotolerance for mice). The gene was constitutively transcribed at low levels, both in the yeast and the mycelial stages. Synthesis of *hsp70* mRNA was transiently increased 1 to 3 h after the temperature shifts. By northern analysis, peak levels of transcription were shown to occur at 34°C in the Downs strain and at 37°C in the more pathogenic G222B strain. They speculated that the low levels of transcription of the *hsp70* gene in the Downs strain at 37°C correlated with its greater temperature sensitivity and low level of virulence.

In dimorphic *Paracoccidioides brasilensis*, the first *hsp70* gene was isolated by da Silva *et al.* (1999). The *hsp70* gene was induced during both heat shock of yeast cells at 42°C and the mycelial to yeast transition. A differential expression of this gene could be observed between mycelial and yeast forms, with a much higher level of expression in the yeast. Then, Florez *et al.* (2003) described two *hsp70* homologue genes from *P. brasiliensis*. The highest level of *hsp70* transcripts occurred between 30 min to 6 h after temperature shift during mycelial transition to yeast phase. The *hsp70* transcripts reduced significantly after 36 to 48 h. However, after 72 h, the level of the transcription increased until yeast phase was reached. This data suggested that as a response to temperature increase, *hsp70* genes are expressed during the transition phases and possibly play a role in the differentiation process.

Table 1. Prevalence of *P. marneffei* in bamboo rats in endemic area.*

Country (province)	Species (no. of rats examined)	No. (%) of rats positive	Reference
China (Guangxi)	*R. pruinosus* (19)	18 (94.7)	Deng *et al*, 1986
China (Guangxi)	*R. pruinosus* (179)	114 (63.7)	Wei *et al*, 1987
China (Guangxi)	*R. pruinosus* (16)	15 (93.8)	Li *et al*, 1989
China (Guangxi)	*R. pruinosus* (22)	19 (86.4)	Deng *et al*, 1988
	R. sinensis (2)	2 (100)	
Thailand (Kanchanaburi)	*C. badius* (31)	6 (19.4)	Ajel1o *et al*, 1995
Thailand (Lopburi, Prachuap Khri Khan)	*R. pruinosus* (8)	6 (75.0)	
Thailand (Chiang Mai)	*C. badius* (10)	3 (33.3)	Chariyalertsak, 1996b
	R. sumatrensis (14)	13 (92.8)	
Vietnam (central Vietnam)	*R. sinensis* (1)	1 (100)	Capponi *et al*, 1956
India (Manipur)	*C. badius* (110)	10 (9.1)	Gugnani *et al*, 2004

* This table was adapted from Vanittanakom & Sirisanthana (1997) and Gugnani *et al*. (2004)

Table 2. Frequency of *P. marneffei* among captured bamboo rats.*

Bamboo rat species	*P. marneffei* isolation frequency
Rhizomys sinensis	100 % (3 / 3)
Rhizomys sumatrensis	92.86 % (13 / 14)
Rhizomys pruinosus	70.49 % (172 / 244)
Cannomys badius	12.58 % (19 / 151)

* This table was adapted from Cooper (1998) and table 1.

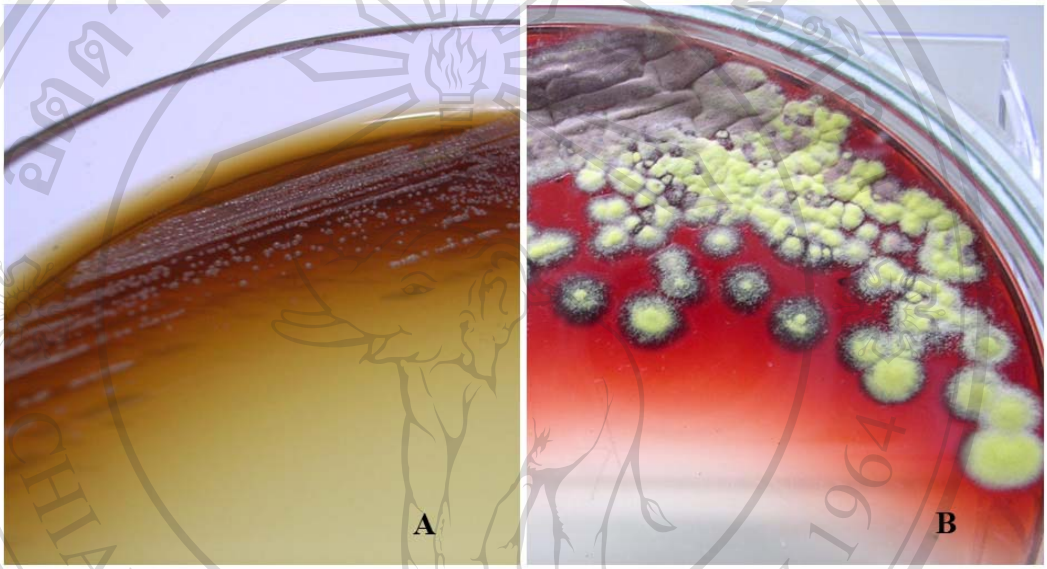

Figure 1. Characteristics of *P. marneffei* colony. Five-day-old of *P. marneffei* yeast culture on brain heart infusion agar at 37°C (A) and mold culture on Sabouraud dextrose agar at 25°C (B).

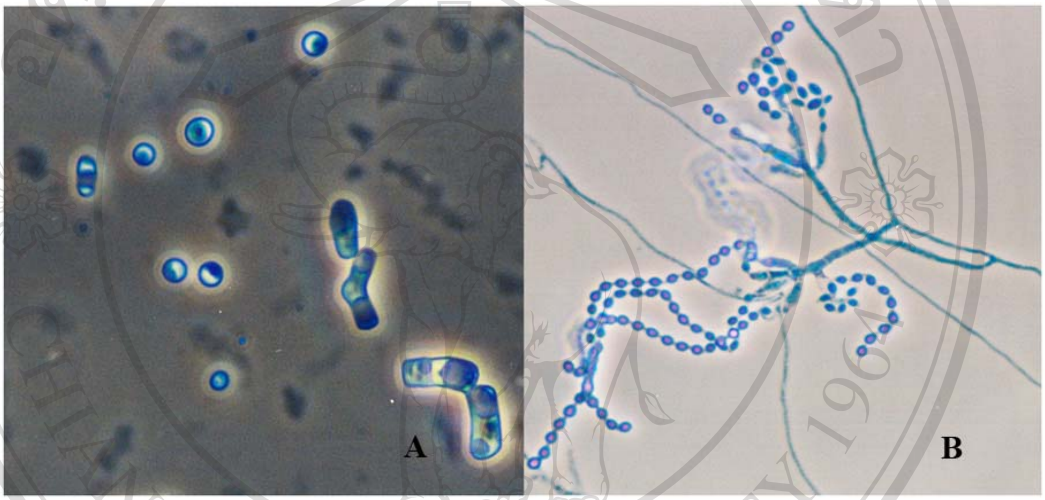

Figure 2. Microscopic morphology of *P. marneffei*. Photomicrograph of 4-day-old yeast-form of *P. marneffei* in brain heat infusion agar at 37°C (A) and 5-day-old mold-form on slide culture at 25°C (B); phase contrast, x400.

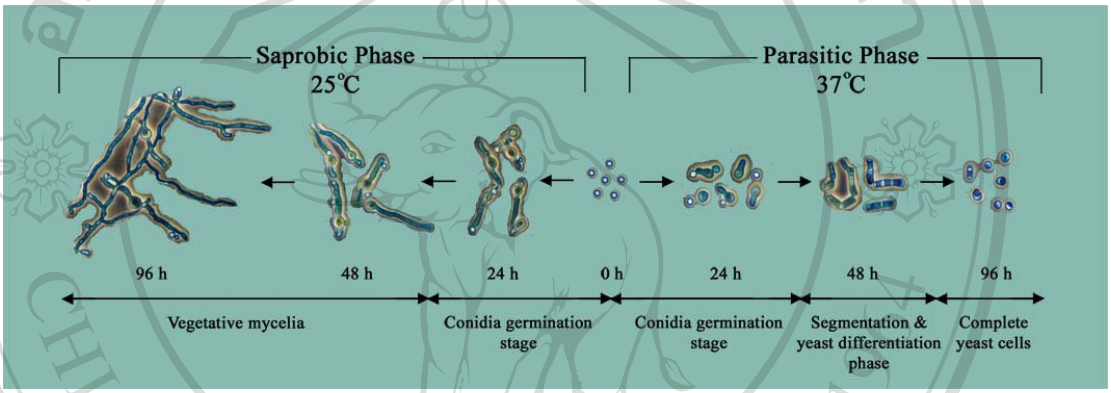

Figure 3. Diagrammatic representation of the generation of saprobic mycelial and parasitic yeast forms of *P. marneffei* (*in vitro* model).