II. LITERATURE REVIEWS

2.1. History

Penicillium marneffei n. sp., Segretain, 1959 (Segretain, 1959a; 1959b), the only dimorphic pathogenic *Penicillium* among several hundred species of *Penicillium* was first isolated from the hepatic lesion of a bamboo rat (*Rhizomys sinensis*), rodents native to the highlands of central Vietnam (Capponi *et al.*, 1956). These rats had been maintained in captivity for experimental infections at the Pasteur Institute in Dalat, Vietnam. Capponi and collaborators (1956) first observed the spontaneous death of three bamboo rats due to reticuloendothelial mycosis. Subsequently, mice were experimentally infected with this fungus and sent to the Pasteur Institute in Paris for further study. The fungus was identified by Segretain as a new species, *Penicillium marneffei*, in honor of Dr. Hubert Marneffe, the director of the Pasteur Institute of Indochina (Segretain, 1959a; 1959b). Segretain pricked his finger accidentally during experimental studies, with needle used to inoculate hamsters (Segretain, 1959b). He developed a small nodule at the site of inoculation, followed by axillary lymphadenopathy. Antifungal sensitivity studied by Drouhet at that 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 pathogenic property of *P. marneffei* to cause the disease in humans.

The first naturally occurring human infection was described 17 years later by Di Salvo and collaborators; the patient was an American minister with Hodgkin's disease who had been living in Southeast Asia (Di Salvo *et al*., 1973). The second reported case was an American man who had traveled extensively in the Far East (Pautler *et al*., 1984). The patient had recurrent episodes of hemoptysis thought to be related to bronchitis and bronchiectasis. A pneumonectomy revealed granulomata, and tissue sections of the lung showed yeast-like cells of *P. marneffei* multiplying by fission while *P. marneffei* could be identified from the culture.

In the same year, five native cases were described in Bangkok, Thailand by Jayanetra *et al*. (1984). The first eight Chinese cases of *P. marneffei* were reported from southern China in 1985 (Deng *et al.*, 1985): these cases were observed during 1964 and 1983. A few years thereafter, 20 cases were reported from the Guangxi region, the most important Chinese endemic area of *P. marneffei* (Deng *et al*., 1988; Li *et al*., 1989). Six patients infected with *P. marneffei* were reported from Hong Kong (Chang and Woo, 1990; Chang *et al*., 1998; Yuen *et al*., 1986). From the year 1988, numerous cases of systemic penicilliosis due to *P. marneffei* were reported among AIDS patients who were traveling in the Southeast Asia, including patients from France (Grise *et al*., 1997; Hilmarsdottir *et al*., 1993; Valeyrie *et al*., 1999), United Kingdom (McShane *et al*., 1998; Vilar *et al*., 2000), Italy (Viviani *et al*., 1990), the Netherlands (Hulshof *et al*., 1990; Kok *et al*., 1994), USA (Nord *et al*., 1998) and Australia (Jones and See, 1992). Individuals presenting with penicilliosis marneffei in these countries had previous visits to the endemic regions. 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 endemic is possible. Among natives of the Southeast Asia, the first case of penicilliosis marneffei with human immunodefiency virus infection was reported in Bangkok, Thailand in 1989 (Satapatayavongs *et al*., 1989). In 1992, 21 cases were reported from Chiang Mai University Hospital (Supparatpinyo *et al*., 1992), from which the cutaneous lesions had been described in 5 cases (Chiewchanvit *et al*., 1991). Among, opportunistic infection affecting AIDS patients in Thailand, systemic penicilliosis marneffei is ranked as the fourth after tuberculosis, cryptococcal meningitis and *Pneumocystis carinii* pneumonia (Thongcharoen *et al*., 1992). The majority of infections by *P. marneffei* were diagnosed in AIDS patients in Thailand; however, infections were also observed in Cambodia (Bailloud *et al*., 2002), China (Liao *et al*., 2002), Hong Kong (Chang *et al*., 1998; Ko, 1994; Tsang *et al*., 1991; Tsui *et al*., 1992; Wong *et al*., 1998), India (Maniar *et al*., 2005; Ranjana *et al*., 2002; Sharma *et al*., 2007; Singh *et al*., 1999), Malaysia (Rokiah *et al*., 1995), Taiwan (Chang *et al*., 1995; Chiang *et al*., 1998; Hsueh *et al*., 2000; Hung *et al*., 1998; Liu *et al*., 1994) and Vietnam (Hien *et al*., 2001; Huynh *et al*., 2003). It is

indicating that the endemic areas of *P. marneffei* reach down to the tropical countries around Southeast Asia.

2.2. Mycology

*P. marneffe*i, the only dimorphic fungus of the genus *Penicillium*, grows as mycelial phase at 25^{o}C on Sabouraud's dextrose agar. In its mycelial form, the colony is greyish white, or yellowish-green to bluish gray-green and downy. During differentiation, the color of the colony turns to greyish pink. The reverse side becomes brownish red, and soluble red pigment diffuses into the agar medium (Chiewchanvit *et al.*, 1991; Cooper and McGinnis, 1997; Vanittanakom *et al*., 2006) (Figure 2-B1). However, not all penicillia producing a red pigment are *P. marneffei*. Recently, the pigment of *P. marneffei* was characterized, the pigment was found to have some structural resemblance with the copper-colored pigment (herquinone) produced by *Penicillium herquei* as both pigments contain the phenalene carbon framework (Bhardwaj *et al*., 2007). Nevertheless, the function of the pigment produced by *P. marneffei* is still not known and the presence of this pigment during infection has not been studied. Microscopically, hyaline septate branching hyphae with lateral and terminal conidiophores can be seen. The conidiophores consist of basal stripes with terminal verticals of 3 to 5 metulae, each metulae bearing 3 to 7 phialides. The conidia are oval, smooth-walled, measuring approximately 2 μm by 3 μm. They are formed basipetally in chains from each phialide. At 37^{o}C on brain-heart infusion agar, rough, glabrous, tan-colored colonies of yeast are seen within a few days (Andrianopoulos, 2002; Cooper and Haycocks, 2000; Cooper and McGinnis, 1997) (Figure 2-C1). Microscopic examination of this growth reveals unicellular yeast cells are ovoid or elongated measuring 2-3 μm x 2-6.5 μm. Similar forms are also observed in tissue samples obtained from patients, which may be seen within macrophages or extracellularly. In contrast to other yeasts, the yeast cells of *P. marneffei* divide not by budding, but by fission, with the result that a transverse septum is often seen in the dividing cell. This helps to differentiate *P. marneffei* from other dimorphic fungi in histological sections, especially *Histoplasma capsulatum*.

The biochemical properties of *P. marneffei* and their possible use in strain biotyping have been studied (Wong *et al*., 2001). They showed that all 32 isolates of

P. marneffei examined possessed the urease enzyme. All isolates assimilated glucose, maltose, and cellobiose. However, some heterogeneity between isolates was observed in their biochemical profiles. From these biochemical properties, 17 different biotypes were recognized. There was, however, no correlation between the biotype and other clinical parameters, including the HIV status of the patients. In addition, 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* revealed the presence of melanin in yeast cells inside skin tissue from penicilliosis marneffei patients. Interestingly, sera from *P. marneffei*–infected mice developed a significant antibody response 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*. More recently, Moon *et al*. (2006) have purified and characterized two acid proteinases (PMAP-1 and PMAP-2) and serine protease (PMNP) produced in culture supernatant by mold and yeast form of *P. marneffei*, respectively. The degradation of elastin, fibronectin and fibrinogen by PMNP *in vitro* suggests that this enzyme may play a significant role in the virulence of *P. marneffei*, especially considering its proposed route of infection. However, the role of the putative enzymes, PMAP-1 and PMAP-2, remains to be established.

2.3. Ecology and epidemiology

The ecology and possible environmental reservoirs of *P. marneffei* was first investigated in 1986 by Deng *et al*. In the Guangxi Province of region of the China, it was found that *P. marneffei* can be isolated in the internal organs of 18 out of 19 bamboo rats belonging to the species *Rhizomys pruinosus*. This finding was confirmed by subsequently study of Li and collaborators in 1989. *P. marneffei* was isolated from the internal organs of *Rhizomys pruinosus senex* bamboo rats (93%). The fungus was most commonly isolated from the lungs (87.5%), followed by liver (56.3%), spleen (56.3%) and mesentery lymph node (50%). The association between *P. marneffei* and bamboo rats had also been noted in Thailand, another endemic country of this infection. In several studies, *P. marneffei* was recovered from various

species of bamboo rats, including *Cannomys badius*, *Rhizomys pruinosus* and *Rhizomys sumatrensis* (Ajello *et al*., 1995; Chariyalertsak *et al*., 1996b; Gugnani *et al*., 2004). The distribution of the fungus in the internal organs was similar to the previous studies. In the study of Chariyalertsak *et al*. (1996b), *P. marneffei* was recovered from one soil sample collected from a burrow of *R. sumatrensis*. Infection is presumably via inhalation of conidia from the environment. However, aerosolization of infectious particles, and subsequent infection, has never been definitively demonstrated. It has been suggested that bamboo rats, like human victims, probably acquired the infection from a common environment source. The occurrence of the fungus in the liver could be a result of the propensity of the fungus to invade the recticuloendothelial system. The possible link to environment factors is demonstrated by two studies from northern Thailand which showed a significant clustering of cases of penicilliosis marneffei during the rainy seasons (Chariyalertsak *et al*., 1996a; 1997). A recent history of occupational or other forms of exposure to soil is also a significant risk factor (Joshi *et al*., 2003; Vanittanakom *et al*., 1995). The exact mode of transmission of the fungus from its natural habitats is still unsettled at the moment.

2.4.Pathogenesis and clinical features

2.4.1) Clinical Manifestations

Penicilliosis marneffei manifests clinically as a progressive systemic febrile illness as a results of infiltration and inflammation of the recticuloendothelial system by the yeast stage of *P. marneffei.* Common clinical features include systemic symptoms of fever, weight loss, anemia and those due to local organ involvement such as pulmonary syndrome, chest radiographic infiltrate, lymphadenopathy, hepatosplenomegaly, molluscum-contagiosum like skin lesions with central umbilication, osteolytic bone lesions, arthritis, subcutaneous abscesses and even endophthalmitis. Almost all organs could be involved in severe disseminated disease (Supparatpinyo *et al*., 1994; Vanittanakom and Sirisanthana, 1997). The incubation periods of the disseminated disease vary from a few weeks to many years of exposure to the organism (Jones and See, 1992; Peto *et al*., 1988), resulting in three possible processes: primary infection, reinfection, or reactivation of the latent disease.

Asymptomatic infections occur in healthy individuals. One study reported serologic evidence of asymptomatic infection in 2 laboratory personnel (Vanittanakom *et al.*, 1997). The likely route of infection in most cases is inhalation of *P. marneffei* conidia. Patients with localized bronchopulmonary disease have also been reported (Chan *et al*., 1989). Brochopneumonia with or without adenopathy and cavity lung disease also occur. Chronic cervical lymphadenitis resembling tuberculosis has been described (Yuen *et al*., 1986). Although the disease is more common in immunocompromised persons, disseminated infections can be found in normal hosts as well. The tissue damage is mainly associated with granulomatous inflammation with multinucleated giant cells, lymphocytes and neutrophils. A suppurative inflammation dominated by neutrophils resulting in abscess formation can be present. Immunosuppressed hosts, an anergic and necrotizing reaction is found with diffused infiltration of macrophage engorged with yeast cells. Osteolytic bone lesions may be feature of disseminated disease, especially in infants and children (Chan and Woo, 1990). Disseminated *P. marneffei* infections occur in patients with AIDS or in those whose immunocompromised state is caused by other underlying illnesses. Infections in non-HIV infected patients have also been described (Wong *et al.*, 2001), primarily among immunocompromised patients and less frequently in patients without any known underlying diseases. 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.

2.4.2) Pathology

P. marneffei appears to be a primary pulmonary pathogen that disseminates to other internal organs by hematogenous spread. Severity of the disease depends upon the immunological status of the host. In the HIV-positive group, the rapid onset and severity of symptoms were striking in the absence of early treatment. Infected tissues can show different histopathological reactions (Vanittanakom *et al.,* 2006). In immunocompromised patients, necrotizing reaction with macrophage and histiocyte infiltrations are seen. *P. marneffei*'s yeast-like cells are observed to be associated with both the intracellular and extracellular environments of both macrophages and histiocytes. The intracellular yeasts are oval or spherical cells of 2-3 μm in diameter,

which multiply by binary fission. Elongated cells of up to 13 μm long can be observed extracellulary. 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 (Cooper and McGinnis 1997; Vanittanakom *et al.,* 2006). In immunocompetent patients, granulomatous and suppurative reactions are frequently seen in the lung, skin, liver, and subcutaneous tissues. The formation of central necrosis and multiple abscesses could be seen in the reactions. *P. marneffei* infection may be considered the homolog of histoplasmosis, since both *P. marneffei* and *H. capsulatum* exploit the macrophage as a host cell, and both organisms can cause acute or persistent pulmonary and disseminated infection, and reactivation disease (Vanittanakom *et al.,* 2006*)*.

2.5. Immunology

The route of transmission and infection of *P. marneffei* is unknown at the moment. However, it is generally believed that inhalation of the conidia is a likely route, in line with the mode of infection for other moulds. The attachment of *P. marneffei* conidia to host cells and tissues is the first step in the establishment of an infection. The conidia-host interaction may occur via adhesion to the extracellular matrix proteins, laminin and fibronectin via a sialic acid-dependent process. Using immunofluorescence microscopy, Hamilton *et al*. 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; 1999). The interaction between human leukocytes and heat-killed yeast-phase of *P. marneffei* has been studied by Rongrungruang and Levitz (1999). The data suggested that monocyte-derived macrophages phagocytosed *P. marneffei* even in the absence of opsonization and the major receptor(s) recognizing *P. marneffei* could be a glycoprotein with *N*-acetyl-β-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. In healthy hosts, *P. marneffei* can be cleared within 2 to 3 weeks, depending on the size of the inoculum, whereas in nude mice or in T-cell-depleted mice, *P. marneffei* infection is fatal (Kudeken *et al*., 1996; 1997; Viviani *et al*., 1993). These results demonstrated that T cells, and in particular $CD4^+$ T cells, are necessary for clearing this fungal infection in mice. For humans, it was also shown that the deficiency of a $CD4^+$ T-cell-dependent immunity contributes to the development of fatal disseminated penicilliosis marneffei in AIDS patients (Supparatpinyo *et al*., 1992). A commonality in the host immunological response to intracellular pathogens is that activation of macrophages by T-cell-derived cytokines is necessary for defense against such infections (Cogliati *et al*., 1997; Kudeken *et al*., 1998; 1999a; 1999b; Taramelli *et al*., 2000) it appears that this is also the case regarding infection by *P. marneffei*.

Although *P. marneffei* is capable of infecting and replicating inside mononuclear macrophages, it is also evident that macrophages do possess antifungal activities. The fungicidal activity of macrophages is likely to involve the generation of reactive nitrogen intermediates, as described by Kudeken *et al*. (1999b). Moreover, the responses of rabbit pulmonary alveolar macrophages and circulating human mononuclear phagocytes to *P. marneffei* conidia have been reported (Roilides *et al*., 2003). These cells manifested antifungal activity against *P. marneffei*; the circulating monocytes responded to conidia with an oxidative burst which was significantly enhanced by a macrophage colony-stimulating factor (M-CSF), suggesting a potential role of this cytokine in the host defense mechanism against *P. marneffei* infection. In addition, Kogushi *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. As described above, several studies have proved that T cells and macrophages are important for protection against the *P. marneffei* infection. However, little is known about the role of T-cell cytokines in the immune response against this fungus. The *in vitro* analysis of a sublethal *P. marneffei* infection in BALB/c mice indicated that protective immunity

follows a Th1 response that involves T cells and macrophages and that IFN-γ is an essential mediator (Sisto *et al*., 2003). The data suggested that the polarization of a 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 the development of granulomas.

In addition to macrophages, the neutrophils also exhibit antifungal properties. The role of a neutrophil in the host defense mechanism against *P. marneffei* infection was observed by Supparatpinyo and Sirisanthana (1994). The yeast cells could be seen inside neutrophil in a blood smear taken from an AIDS patient. The fungicidal activity of neutrophils is significantly increased in the presence of proinflammatory cytokines, especially GM-CSF, G-CSF and IFN-γ. In addition to GM-CSF, G-CSF and IFN- γ, other cytokines such as TNF-α and IL-8 are capable of enhancing the inhibitory effects of neutrophil on germination of *P. marneffei* conidia. The strongest effect was observed with GM-CSF (Kudeken *et al*., 1999a). Conidia are, however, generally not susceptible to being killed 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).

As for other intracellular pathogens, surviving within the phagocytes is the primary key to a successful invasion by *P. marneffei*. However, the mechanism of survival of *P. marneffei* under oxidative stress within the macrophage remains unknown. Many fungi have been shown to survive within the phagocytic environment (Levitz and Diamond, 1985). The mechanism of resistance for these organisms may function by inhibiting the production of reactive oxygen metabolites or by neutralizing inhibitory host metabolites. Youngchim *et al*. (1999) found an expression of acid phosphatase activity by *P. marneffei*. When a pathogen produces acid phosphatase, the concomitant decrease in intracellular pH may improve the survival of the organism by inhibiting the phagocyte respiratory burst. This hypothesis could be supported by the study of antimicrobial activity of chloroquine against *P. marneffei* by using human THP1 and mouse J774 macrophages. The results from this study revealed that the drug's antifungal activity was due to an increase in the intravascular pH and a disruption of pH-dependent metabolic processes (Taramelli *et al*., 2001), the increase in pH within the phagocytic vacuole may directly reduce fungal growth, or it

may inhibit pH-dependent yeast virulence factors, such as acid phosphatase activity. Like most other pathogens, the availability of iron is crucial to the survival of *P. marneffei* in the human host. Studies by Taramelli *et al*. (2000) have shown that the antifungal activity of macrophages is markedly suppressed in the presence of iron overload and that iron chelators could inhibit the extracellular growth of *P. marneffei*.

2.6. Laboratory diagnosis

2.6.1) Diagnosis by Staining Methods and Cultures

A presumptive diagnosis of *P. marneffei* can be made by examining the organism microscopically from clinical specimens such as bone marrow aspirates, lymph node biopsies, skin biopsies, sputum, pleural fluid, liver biopsies, cerebrospinal fluid, pharyngeal ulcer scrapings, urine, kidney, pericardium, stomach or intestine and stool samples (Drouhet, 1992; Supparatpinyo *et al.*, 1994) 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). 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 intracellular fungal cells (Supparatpinyo *et al*., 1994). *P. marneffei* can be seen in histopathological sections stained with hematoxylin and eosin, Grocott methenamine silver, or periodic acid-schiff stain. The organisms appear as arthroconidia-like cells or unicellular round to oval cells, which may divide by cross wall formation in macrophages or histiocytes (Chiewchanvit *et al*., 1991; Hilmarsdottir *et al*., 1993; Hulshof *et al*., 1990; Sirisanthana *et al*., 1993; 1995; Supparatpinyo *et al*., 1992; Ukarapol *et al*., 1998; Viviani *et al*., 1993). The cross wall formation can differentiate yeast cells of *P. marneffei* from those of *H. capsulatum*, which also appear as intracellular yeasts. Kaufman *et al*. (1995) using rabbit *P. marneffei* anti-globulins adsorbed with yeast form antigens of *H. capsulatum*, successfully developed an immunofluorescent antibody test that specifically identified *P. marneffei* in formalin-fixed, paraffin embedded tissue sections. Conventional fungal culture remains the diagnostic of choice in most settings. The fungus may be cultivated from appropriate clinical specimens in most cases, such as blood cultures, skin lesions, and respiratory tract specimens. *P. marneffei* grows in a mycelial phase at 25°C on Sabouraud glucose

agar. Mold-to-yeast conversion is achieved by subculturing onto brain heart infusion agar and incubating at 37°C (Segretain, 1959a). Identification of *P. marneffei* is based upon the morphology of the colony, its mold-to-yeast conversion, and the organism's microscopic morphology.

2.6.2) Serologic Diagnosis

Although the gold standard of diagnosis of *P. marneffei* infection is a positive culture from appropriate tissues, invasive biopsies often delayed and patients are often treated empirically for other diseases. Thus, a serologic testing helps to establish an early diagnosis. Viviani *et al*. (1993) could detect *P. marneffei* antibodies in serum specimens from HIV-infected and immunocompetent patients, using immunodiffusion (ID) methods with a mycelial phase culture filtrate antigens. In each case, the test demonstrated both diagnosis and prognosis value. Later, Kaufman and colleagues (1996) used ID with specific fission arthroconidial filtrate antigens, however, this test showed low sensitivity. In a study of Yuen *et al*. (1994), an indirect immunofluorescent antibody test was developed which provided rapid, presumptive diagnosis and supplement to conventional culture. However, cross-reactivity with other potential fungal pathogens remains to be tested. 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 individual sera derived from 33 AIDS patients with penicilliosis marneffei (Vanittanakom *et al*., 1997). The results indicate that there are at least two yeast-phase immunoreactive proteins (54 and 50 kDa), which are relatively specific for *P. marneffei*. The use of these yeast-phase immunoreactive proteins for diagnosis should be studied further with more serum samples. A study by Chongtrakool *et al*. (1997) revealed 38-kDa antigen of *P. marneffei* appeared to be specific to this fungus. In another study, the 61-kDa, 54-kDa and 50-kDa antigens were purified and found to be specific for *P. marneffei*, with no-cross reactivity with sera from patients with other mycoses (Jeavons *et al.*, 1998). The study revealed that the 61-kDa antigen was an additional candidate for the development of diagnostic tests for disseminated *P. marneffei* infection.

An ELISA-based antibody test was developed by using purified recombinant Mp1p antigen (Cao *et al*., 1998a; 1998b; 1999). Evaluation of the test with guinea pig

sera against *P. marneffei* and other pathogenic fungi indicated that this assay was specific for *P. marneffei*. This ELISA-based test for the detection of anti-Mp1p antibody can be of significant value as a diagnostic test for penicilliosis. Desakorn *et al.* (1999) later used purified hyperimmune IgG, from rabbits immunized with yeast cells, in an enzyme-linked immunosorbent assay (ELISA) to quantitate *P. marneffei* yeast antigens in urine samples. This polyclonal antibody was used further in a dot blot ELISA and a latex agglutination test for the detection of *P. marneffei* urinary antigen (Desakorn *et al.*, 2002). All tests were highly sensitive and specific. A monoclonal antibody-based sandwich ELISA was developed for the detection of *P. marneffei* antigen in clinical specimens from patients with *P. marneffei* infection (Panichakul *et al*., 2002; Trewatcharegon *et al*., 2000). The test was also useful for the detection of secreted antigen in urine samples. In addition, Chaiyaroj *et al.* (2003) developed an antigen capture ELISA by using the mixture of two monoclonal antibodies for the detection of *P. marneffei* antigens in sera of humans in areas where the organism is endemic with high sensitivity and specificity of the test. Several additional methods for detecting circulating *P. marneffei* antigens have been developed. Pastorex *Aspergillus* is a latex agglutination test kit using a monoclonal antibody to detect *Aspergillus fumigatus* galactomannan in serum specimens from patients with aspergillosis. This monoclonal antibody was found to cross-react with *P. marneffei* antigen (van Cutsem *et al*., 1990; Pierard *et al*., 1991). The reagent was used to detect galactomannan in an experimental infection with *P. marneffei*. Recently, a novel immunogenic protein in *P. marneffei* was identified by using ΔAFMP1ΔAFMP2 deletion mutant of *A. fumigatus*. The recombinant 55-kDa protein of *P. marneffei* reacted strongly with both sera from guinea-pig and patients infected with *P. marneffei* (Woo *et al*., 2006).

2.6.3) Molecular Diagnosis

P. marneffei infection could be diagnosed by using molecular-based methods. A number of studies reported on the detection of *P. marneffei* genomic DNA in clinical specimens. LoBuglio and Taylor (1995) used primers PM2 and PM4 to amplify a 347 bp fragment of the internal transcribed spacer (ITS) region between 18S rDNA and 5.8S rDNA. A pair of these primers was used successfully to identify *P. marneffei* from a skin biopsy (Tsunemi *et al*., 2003). On the other hand,

Vanittanakom *et al*. (1998) 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. In 2002, Vanittanakom *et al*. described a nested PCR assay which might prove to be useful in the detection of *P. marneffei* and identification of young fungal cultures. Additionally, Prariyachatigul *et al.* (2003) developed a one-tube seminested PCR assay to identify *P. marneffei* DNA based on the 18S rRNA sequences. This assay was sensitive and could identify *P. marneffei* DNA both from pure cultures and two clinical samples. The utility of these PCR methods for the early diagnosis of the disease needs to be studied further.

2.7. Therapy and prophylaxis

Antifungal therapy should be initiated promptly in patients with disseminated *P. marneffei* infection. Amphotericin B or itraconazole has been successful in approximately 75% of patients; fluconazole has been less so (Supparatpinyo *et al*., 1993). In a nonrandomized study, 63.3% of patients failed in the treatment with flucozazole. The therapeutic recommendation is amphotericin B 0.6 mg/kg/day or oral itraconazole 400 mg/day. Total duration of therapy with either drug is 8 to 10 weeks. A different regimen using both of these antifungal agents consists of IV amphotericin B 0.6mg/kg/day for 2 weeks followed by 10 weeks of oral itraconazole in 2 divided does of 400 mg/day (Sar *et al*., 2006; Sirisanthana *et al*., 1998; Supparatpinyo *et al.*, 1998). Because disseminated penicilliosis marneffei occurs generally in patients with very low $CD4^+$ cell counts and markedly impaired immunologic function, continued antifungal therapy is important to prevent relapse. In at least 50% cases reported by Supparatpinyo and colleagues (1993), the absence of continued prophylaxis resulted in relapse or death within a year. The only prophylaxis drug that has been studied to date is itraconazole; second prophylaxis with itraconazole at a dosage of 200 mg/day has been effective in preventing relapses in a controlled clinical trial (Supparatpinyo *et al*., 1998).

2.8. Molecular genetic studies

2.8.1) Morphogenesis in *P. marneffei*

The mechanism of thermal dimorphism and morphogenesis in *P. marneffei* is not fully understood. Many studies mainly characterized genes having homologous function in *Aspergillus* as well as other fungi (Andrianopoulos, 2002; Borneman *et al*., 2000; 2001; 2002; Boyce *et al*., 2001; 2003; 2005; Pongsunk *et al*., 2005; Todd *et al*., 2003; Zuber *et al*., 2002; 2003). Several genes identified from *P. marneffei* that are involved in intracellular signaling, transcription regulation and cellular developments were summarized (Cooper and Vanittanakom, 2008; Vanittanakom *et al*., 2006). However, no genes have been identified that specifically induced dimorphism of *P. marneffei.*

A genome project of *P. marneffei* has been conducted (Yuen *et al*., 2003). Investigating of the genome size and chromosome number by pulse-field gel electrophoresis and telomeric fingerprinting revealed that *P. marneffei* contains 6 chromosomes. Its total genome size was estimated from analysis of 11 strains to be about 17.8-26.2 Mb. Random exploration of the genome of *P. marneffei* yield 2,303 random sequence tags (RSTs) representing about 9% of the whole genome (with a genome size of 20 Mb). Phylogenetic analysis of the 18S and 28S rRNA genes revealed close relationship to the *Talaromyces* species. This result confirmed the first report by LoBuglio and Taylor (1995). Considering that the *P. marneffei* genome size is twice as large as that of *Talaromyces* and that it is uncommon to isolate the organism from natural sources, a hypothesis was made that *P. marneffei* is likely to be an anamorph of *Talaromyces.* Analysis of the RSTs revealed genes for information transfer (ribosomal protein genes, tRNA synthetase subunit genes, translation initiation genes, and elongation factor genes), metabolism, and compartmentalization, including several multi-drug resistance protein genes and homolog of fluconazole resistance genes. In addition, the RSTs uncovered the presence of genes encoding pheromone homolog and ankyrin repeat-containing proteins of other fungi and algae. These findings indicate that *P. marneffei* may have a sexual component to its life cycle; however, further work is necessary to determine whether these genes are still functionally intact and capable of undergoing transcription to form functional gene products. Later, sequencing of *P. marneffei* mitochondrial genome was reported.

From this *P. marneffei* sequencing project, a contig that contains the complete sequence (35 kb) of the mitochondrial genome was assembled (Yuen *et al*., 2003). According to the phylogenetic analysis, the sequences of mitochondrial genes, of *P. marneffei* are more closely related to those of molds than the mitochondria of yeasts (Woo *et al*., 2003). Subsequently, due to the possibility of a sexual cycle in *P. marneffei* and its major clinical and biological significance, genomic and other evidence of the potential and occurrence of a sexual cycle in *P. marneffei* was explored (Wong *et al*., 2006). In this study, all the genes in the putative sexual cycle of *P. marneffei* are found to be most closely related to their orthologues in *Aspergillus* species. This shows that the sexual cycles in *Penicillium* and *Aspergillus* were probably present in their common ancestor, possibly a homothallic fungus, and then evolved independently after the divergence into the two genera and the individual species, with some of the species becoming heterothallic fungi.

2.8.2) Molecular determinants of virulence

In the face of this vast array of pulmonary defenses, dimorphic fungal pathogens are remarkably effective at establishing infection and causing disease, highlighting the need to better understand the molecular mechanisms of pathogenesis. Underlying the success of fungal pathogens compared with related but nonpathogenic fungi must be the expression of so-called virulence factors. For the dimorphic fungi, a virulence factor can be functionally defined as having an effect on the survival and growth of the organism in its mammalian host but is not essential for growth of the parasitic phase *in vitro*. Virulence of dimorphic fungi is also affected by factors that are responsible for the transition from the saprophytic mold phase to the parasitic phase. Distinct from the definition of virulence factor above, these developmentally important factors are still important as prerequisites for pathogenesis mediated by the parasitic form. Research on the characteristic morphologies and dimorphic switching of *P. marneffei* has identified isocitrate lyase gene (*icl1* or *acuD*), a gene which encodes a relatively conserved protein that critically controls the glyoxylate bypass, a metabolic pathway supplementary to the tricarboxylic acid cycle when the micro-organism needs to survive in mammalian hosts (Lorenz and Fink, 2002). The *P. marneffei icl1* was identified in a differential display expression screen as a gene highly expressed in yeast cells at 37^{o}C (Cooper and Haycock, 2000). In addition, it

was found that the expression of isocitrate lyase protein increased significantly in the yeast phase by using two-dimensional difference gel electrophoresis (Xi *et al*., 2007). Recently, Cánovas and Andrianopoulos (2007) reported that the *P. marneffei acuD* gene which is required for growth on gluconeogenic carbon sources such as acetate and fatty acids is strongly induced by acetate and is dependent on the FacB transcriptional activator for acetate induction. Interestingly, *P. marneffei acuD* is also independently regulated by the dimorphic switching developmental program and part of this control is through the AbaA transcriptional activator. The developmental regulation of *P. marneffei acuD* has both cis- and trans-acting elements which are not present in the *A. nidulans acuD* gene or in *A. nidulans*, showing a unique evolutionary path for acetate and fatty acid regulation in this dimorphic pathogen.

An antigenic catalase-peroxidase protein-encoding gene (*cpeA*) was isolated by antibody screening of a cDNA yeast phase library of *P. marneffei* (Pongpom *et al.*, 2005). The *cpeA* transcript was accumulated in the yeast phase, which is the pathogenic form of *P. marneffei*. The authors suggested that catalase peroxidase protein may contribute to the survival or interaction of this fungus to the host cells. In addition, the study of proteins differentially expressed in the yeast and mycelial phases demonstrated an increase in the protein level of catalase peroxidase in the yeast phase of more than 14-fold compared with that in the mycelial phase. As an intracellular pathogen, *P. marneffei* survives as yeast inside phagocytes, protecting itself from the host defense machinery. It will be of great interest to investigate whether catalase peroxidase serves as a virulence factor of *P. marneffei* that counteracts the oxidative defense reactions of the host phagocytes (Xi *et al*., 2007).

As the parasitic phase is closely linked with the higher temperature of the mammalian host, the developmental class factors will likely include heat shock proteins (HSPs) and other temperature-related stress responses. It is assumed that heat shock genes are upregulated upon infection in order to prevent mis-folding and aggregation of damaged proteins (Lindquist and Craig, 1988). During the mycelia-to-yeast transition upon temperature shift, *P. marneffei hsp70* becomes significantly elevated and also in yeast or mycelial cells encountering heat shock condition at 39^{o}C (Kummasook *et al*., 2007). These imply that Hsp70 may be required for the

adaptation to higher-temperature growth in *P. marneffei* enabling parasitic growth in the mammalian host.

The number of defined virulence factors for *P. marneffei* is relatively small due to the infancy of molecular genetic tools typically required to demonstrate that a gene is essential for virulence. Investigation of differential gene expression during transition from the mycelial to the yeast form of *P. marneffei* may lead to the discovery of candidate genes for pathogenicity (Liu *et al*., 2007). In this study, 43 differential expression genes were identified by suppression subtractive hybridization combined with real-time semi-quantitative RT-PCR. These genes were sorted into broad functional categories including cell wall synthesis, signal transduction, cell cycle, substance transport, general metabolism and stress response, etc. This suggests that a very complex series of molecular mechanisms are involved in the switching process of *P. marneffei*.

2.9. The putative virulence factors of *P. marneffei*

Additional genes and gene products with putative roles in dimorphic fungal pathogenicity have been identified by a variety of biological, biochemical, and molecular biological techniques. With the development and adaptation of new molecular genetic tools, the contributions of such candidate genes to fungal virulence should now be assessed. Many of these candidates were identified by close examination of the characteristics of infection or by expression studies to catalog parasitic phase-specific genes. Expression analysis can be valuable as a first step in virulence gene discovery, but we focus on candidate genes for which additional experimental data support their involvement in dimorphic fungal pathogenesis and raise their priority for functional tests.

Oxidative stress response

2.9.1) Superoxide dismutase

Cellular components of innate defense of the host include monocytes, macrophages and polymorphonuclear cells. These immune effecter cells play an essential role in the killing of pathogenic fungi by producing reactive oxygen species (ROS), including superoxide radical anion (O_2^-), hydrogen peroxide (H_2O_2) and

hydrogen radicals (OH^-). Superoxide dismutase (SOD), one of essential elements of the antioxidant defense system, mainly removes O_2^-. SODs are metalloenzymes that detoxify oxygen radicals through the conversion of superoxide to hydrogen peroxide and oxygen. The primary role of SODs is to protect cells from endogenously generated superoxide anion, which is by-product of normal aerobic respiration. SODs can be complexed with iron, manganese, and copper plus zinc. Eukaryotic cells generally contain Mn SOD in the mitochondrial matrix and a Cu, Zn SOD, which is located predominantly in the cytoplasm and to a lesser extent in peroxisomes. In addition to superoxide resulting from endogenous production, human-pathogenic organisms are exposed to ROS generated by phagocytic cells. After phagocytosis by polymophonuclear cells or macrophages, pathogens in the phagolysosomes are exposed to various toxins, including superoxide. For some bacteria, SOD has been shown to play a role in virulence when the organisms have been tested in animal models, and it has been thought that decreased virulence of SOD mutant strain was due to increases susceptibility to host phagocytic cells (Lynch, 2000; Piddington, 2001). The role of SOD in the pathogenesis of fungal infections is not clear. In *C. albicans*, the activity of Cu, Zn SOD increased 100 times when these organisms were incubated at the 37°C (Romandini *et al*., 1994). *C. albicans* mutated in the Cu, Zn SOD gene showed a delay of five days in hyphal growth when compared to wild-type cells. These mutants also were sensitive to menadione and H_2O_2 (Hwang *et al*., 2002). The expression of Cu, Zn SOD increased three times in *Cryptococcus neoformans* grown at 37°C, when compared with growth at 25°C (Cox *et al*., 2003). In *C. albicans* and *C. neoformans*, Cu, Zn SOD took part in virulence. This was concluded because Cu, Zn SOD mutants of these organisms were less virulent to BALB/c mice (Hwang *et al*., 2002). Exposure of *C. albican*s to blood caused increased expression of the genes coding ROS detoxification enzymes, including Cu, Zn SOD (*Sod1*), a catalase (*Cat1*), a supposed thiol-specific antioxidant protein, and a thioredoxin reductase (Trr1) (Fradin *et al*., 2003). These data showed that *C. albicans* responded to signals present in the blood, most probably leukocytes. Infection by *P. marneffei* appears to begin following phagocytosis of inhaled conidia by host alveolar macrophages. As a facultative intracellular pathogen, *P. marneffei* survives and replicates as a yeast inside the phagosome (Chan and Chow, 1990).

Within the phagosome, *P. marneffei* must protect itself from host defense mechanisms, such as the ROS and reactive nitrogen intermediate. Although the mechanisms by which this fungus survives within macrophages are not fully understood, the role of Cu, Zn SOD in the response of *P. marneffei* to the host cell is thought to be important.

2.9.2) Catalase

Catalases are widespread enzymes in aerobic organisms. They catalyze the reaction $2H_2O_2 \rightarrow O2 + 2H_2O$. Catalase plays an important role in the pathogenicity of *Histoplasma capsulatum* (Johnson *et al*., 2002). There are three catalase genes in *H. capsulatum*, *CATA*, *CATB* (encoding the M antigen) (Zancopé-Oliveira *et al*., 1999), and *CATP*. All three catalase enzymes are synthesized by yeast cells during exposure to the respiratory burst of neutrophils and macrophages (Johnson *et al*., 2002). *Aspergillus fumigatus* produces two mycelial catalases, one that is monofunctional and one that is bifunctional catalase-peroxidase (Hearn *et al*., 1992). Paris *et al*. (2003) examined the role of all of the conidial and mycelial catalases of *A. fumigatus* in the pathogenicity. These included three active catalases, one expressed in conidia and another two in mycelium. The conidial catalase did not protect conidia against the oxidative burst of macrophages, but it protected against H_2O_2 *in vitro*. The ROS produced by alveolar macrophages played an essential role in killing *A. fumigatus* conidia (Philippe *et al*., 2003). However, the mycelial catalases are needed to scavenge harmful peroxide *in vitro* and in the rat model of infection, but they provide only partial resistance to polymorphonuclear leukocytes. A lack of catalase (CAT1) activity could make *A. fumigatus* vulnerable to rapid killing by the H_2O_2-generating phagocytes, and thus, *A. fumigatus* CAT1 has been supposed to be a virulence factor (Calera *et al*., 1997; Hamilton and Holdom, 1999). In *P. marneffei*, a diagnostically useful 61-kDa antigen has been found to have N-terminal amino acid sequence, which has high homology with other catalases (Jeavons *et al*., 1998). The study of genome of *P. marneffei* by Yuen and his colleagues (2003), found one of random sequence tag that has high similarity to catalase gene of *A. fumigatus* (*CatA*) and *H. capsulatum* (*CATA*). The role of catalase in pathogenesis and phase transition of *P. marneffei* needs further investigations.

Adhesion and adaptation

2.9.3) Glyceraldehyde-3-phosphate dehydrogenase

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) catalyzes the oxidative phosphorylation of glyceraldehyde-3-phosphate into 1, 3-biphosphoglycerate in the presence of nicotinamide adenine nucleotide (NAD^+) and inorganic phosphate, in the glycolysis pathway. It is a tetramer composed of four subunits with a molecular mass of 36 kDa. GAPDH appears to be a multifunctional protein found in several subcellular locations in eukaryotes, displaying functions unrelated to glycolysis, such as membrane transport and fusion, nuclear RNA transport, among others (McDonald and Moss, 1993; Singh and Green, 1993; Sirover, 1997; 1999). Also, in some pathogens such as *C. albicans*, *Staphylococcus aureus*, and *Staphylococcus epidermidis*, GAPDH has been found on the cell wall, where it may have differing roles in host–pathogen interactions (Gil-Navarro *et al*., 1997; Gozalbo *et al*., 1998; Modun and Williams, 1999; Modun *et al*., 2000; Taylor and Heinrichs, 2002). The GAPDH has long been thought to be the product of a housekeeping gene whose transcript level does not vary. However, several studies have shown that the expression of *P. brasiliensis gapdh* gene and the cognate protein were developmentally regulated in the different growth phases of *P. brasiliensis*, with a higher expression in the yeast parasitic phase, thereby reinforcing possible new functions attributed to the enzyme (Barbosa *et al*., 2006). In addition, the GAPDH has been found to be upregulated mainly in *P. brasiliensis* recovered from infected mice (Bailão *et al.*, 2006). The *P. brasiliensis* GAPDH is a molecule located at the fungal cell wall, which binds components of the extracellular matrix and is capable of mediating the adherence and internalization of *P. brasiliensis* to *in vitro* cultured cells, suggesting its involvement in fungal pathogenesis (Barbosa *et al*., 2006).

2.9.4) Calmodulin

Calmodulin (CaM) belongs to a large family of calcium binding proteins that participate in cellular processes involving protein kinases, phosphokinase, phosphodiesterases and cytoskeletal proteins. Calmodulin is one of the most conserved proteins known to date, and is usually genetically represented by a single gene that varies from 1 to 15 kb. The main feature of the members of this protein family is the presence of the structural EF-hand motif composed of a helix-loop-helix

arrangement. Generally, calmodulin is composed of 149 amino acid residues organized in four EF-hand motifs that contain the calcium binding loops. Each calmodulin molecule binds to four calcium ions becoming able to act as a signal transduction component. These motifs are in the N and C terminus of the protein, two in each region, connected by a long α-helix. Calmodulin is intracellular calcium signaling modulator and acts on several metabolic pathways and gene expression regulation in many eukaryotic organisms including human fungal pathogens, such as *H. capsulatum* (el-Rady and Shearer, 1996). In *H. capsulatum*, CaM was found to be expressed as a single major transcript in both the yeast and mycelial form. Interestingly, CaM transcript was approximately two-fold more abundant in the yeast form than the mycelial form, indicating that differential expression of CaM plays a role in dimorphism. The actual role of CaM in *H. capsulatum* dimorphism, however, remains to be tested. In *C. albicans*, the transition to the filamentous growth form is important for its virulence and mutants that are unable to grow in the filamentous form are avirulent (Lo *et al*., 1997). Calcium signaling via calmodulin is important for this transition to filamentous growth because compounds that inhibit calmodulin function could prevent this transition (Sabie and Gadd, 1989), but a recent report suggests that calmodulin contributes to *C. albicans* filamentous growth via an avenue other than the activation of calcineurin (Blankenship *et al*., 2003). De Carvalho *et al*. (2003) have cloned and characterized the calmodulin gene from *P. brasiliensis* using PCR approach with degenerated primers designed from conserved sequence regions. They showed that calmodulin is probably involved in the transition of *P. brasiliensis* from the mycelial to yeast phase.