

Penicillium marneffei Infection and Recent Advances in the Epidemiology and Molecular Biology Aspects

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INTRODUCTION

Penicillium marneffei is an emerging pathogenic fungus that can cause a fatal systemic mycosis in patients infected with human immunodeficiency virus (HIV). *P. marneffei* infection is endemic in tropical Asia, especially Thailand, northeastern India, China, Hong Kong, Vietnam, and Taiwan (25, 33, 61, 125, 151, 185). The organism is a relatively recent addition to known southeast Asian mycoses, being discovered in 1956 as an infection of bamboo rats (16, 136). However, the importance of *P. marneffei* as a human disease was recognized only when the HIV pandemic arrived in Asia and prevalences of infection increased in local populations as well as in visitors from areas where the infection is not endemic (63, 119, 120, 147, 180). The disease is, after tuberculosis and cryptococcosis, the third most common opportunistic infection in patients with AIDS in northern Thailand (151). Common manifestations of disseminated *P. marneffei* infection in AIDS patients are fever, anemia, weight loss, lymphadenopathy, hepatosplenomegaly,

respiratory signs, and skin lesions. Patients who do not receive the appropriate antifungal treatment have a poor prognosis; however, primary treatment with amphotericin B and secondary prophylaxis with itraconazole are effective (153). *P. marneffei*-infected patients who are HIV positive need prolonged suppressive therapy to prevent relapse. Laboratory diagnosis of *P. marneffei* infection requires microscopic demonstration of intracellular *P. marneffei* yeast cells in the infected tissue and the culture of the fungus from clinical specimens. Under the microscope, *P. marneffei* appears as a unicellular organism with round to oval cells. These cells may divide by cross wall formation within macrophages, or, alternatively, to form extracellular elongated cells. The unique feature of *P. marneffei* relative to other penicillia is its thermal dimorphism (136). This ability to grow as a mycelium at 25°C and as a yeast at 37°C is the organism's principle virulence factor. General laboratory diagnosis is time-consuming despite the development of several tests for early diagnosis, including serodiagnostic and molecular assay-based diagnostic methods. There is a clear need for more rapid diagnostic tools to help the physician to make more rapid diagnoses and thus initiate an treatment early.

P. marneffei has been isolated from the internal organs of four species of rodents (*Rhizomys sinensis*, *Rhizomys pruinosus*, *Rhizomys sumatrensis*, and *Cannomys badius*) and from soil

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samples that have been collected from bamboo rat burrows (55, 177). Studies on the molecular epidemiology of this fungus have been reported (46, 64, 71, 95, 163, 171), and recently developed typing systems coupled with modern population genetic analyses have shown that humans and bamboo rats share genetically identical isolates (46, 47, 55). Expanding such surveys will further characterize *P. marneffei* and help in explaining the enigmatic natural history of this fungal infection. Recently, novel molecular tools have been developed to study the genetics of *P. marneffei*. Several genes of this fungus that are involved in asexual development and/or fungal morphogenesis have been cloned and characterized (7–9, 12, 13, 30, 160, 193, 194). Other genes that are involved in the host immune response have been found (15, 123). The determination of the *P. marneffei* genome, anticipated for 2005, will enable a study of the molecular networks underpinning these expressed genetic factors. Such analyses will clarify the molecular mechanisms of fungal morphogenesis, pathogenesis, and host-fungus interactions in future studies of this important and unique pathogen.

HISTORY AND CLASSIFICATION

Discovery of *P. marneffei* and *P. marneffei* Infection

Penicillium marneffei was first isolated from the hepatic lesions of a bamboo rat (*Rhizomys sinensis*) that had been maintained in captivity for experimental infections at the Pasteur Institute of Indochina, Dalat, South Vietnam, in 1956 (16). This bamboo rat died spontaneously from the reticuloendothelial mycosis. The fungus was named *Penicillium marneffei*, in honor of Hubert Marneffe, director of the Pasteur Institute of Indochina (136). Human penicilliosis marneffei was first described as a laboratory-acquired infection when researcher G. Segretain accidentally pricked his own finger with a needle filled with *P. marneffei* that was being used to inoculate hamsters (137). He developed a small nodule at the site of inoculation, followed by axillary lymphadenopathy; this accidental infection was cured by intensive treatment with oral nystatin for 30 days (40).

The mycology of *P. marneffei* was first described by Segretain in 1959 (136). *P. marneffei* was classified in the section *Asymetrica*, subsection *Divaricata*, in the classification of Raper and Thoms (126), which is equivalent to Pitt's subgenus *Furcatum*. Pitt (122) later assigned *P. marneffei* to the subgenus *Biverticillium*. This classification was confirmed by Frisvad and Filtenborg (51) on the basis of similar physiology and secondary metabolite profiles. A phylogenetic analysis of *P. marneffei*, as assessed by the nucleotide sequences of nuclear and mitochondrial rRNA gene regions, demonstrated that *P. marneffei* is closely related to the species of the *Penicillium* subgenus *Biverticillium* and sexual *Talaromyces* species with asexual biverticillate *Penicillium* states (105).

The first naturally occurring human case of penicilliosis marneffei was reported in 1973 by Di Salvo and collaborators (39); the patient was an American minister with Hodgkin's disease who had been living in southeast Asia. The second reported case, in 1984, was also in an American who had traveled in the Far East (118). Here, the patient had recurrent episodes of hemoptysis, which were thought to be caused by bronchiectasis. A pneumonectomy revealed granulomata, and

tissue sections showed yeast-like cells of *P. marneffei* that were confirmed by fungus culture. In the same year, five more cases were reported from Bangkok, Thailand (72). Eight cases of *P. marneffei* infection were reported from China in 1985 (32); these were observed between 1964 and 1983. A further 20 cases were subsequently reported from the Guangxi region in southern China (33, 99, 182), and six more were reported from Hong Kong (18, 19, 146, 190).

The rarity of human penicilliosis marneffei changed when the global HIV-AIDS pandemic arrived in southeast Asia. From 1988, cases of *P. marneffei* infection started being observed in patients with advanced HIV infection. The first cases were in foreign AIDS patients who had visited regions of endemicity (119, 120) and in HIV patients who were native to regions of endemicity within Thailand (135). The majority of infections by *P. marneffei* were diagnosed in AIDS patients in Thailand; however, infections were also observed in Cambodia (4), China (101), Hong Kong (20, 85, 164, 166, 185), India (125), Malaysia (132), Taiwan (21, 25, 64, 66, 103), and Vietnam (61, 67). Cases from outside the region of endemicity were observed in HIV-infected patients from Australia (60, 75), Belgium (35), France (54, 62, 63, 169), Germany (129, 147), Japan (113, 167), Sweden (77), Switzerland (10, 52, 88), The Netherlands (65, 87), the United Kingdom (6, 111, 119, 178), and the United States (114). Currently, the prevalence of human penicilliosis marneffei infection is increasing in areas where HIV infection is on the increase, for instance, Vietnam (67). However, where transmission of HIV has been reduced by control methods, concomitant decreases in the numbers of cases of penicilliosis marneffei have been seen (Fig. 1).

Mycology

Penicillium marneffei is the only known *Penicillium* species that exhibits temperature-dependent dimorphic growth. At temperatures below 37°C, the fungus grows as mycelia with the formation of septate hyphae, bearing conidiophores and conidia typical of the genus *Penicillium*. At 37°C on artificial medium or in human tissue, the fungus grows in a yeast-like form with the formation of fission arthroconidium cells. The fission yeast cells represent the parasitic form of *P. marneffei*. This form is seen in the intracellular infection of the macrophages. The mold-to-yeast conversion or phase transition, which is thermally regulated, is a diagnostic characteristic of *P. marneffei*. In contrast to *P. marneffei*, the other *Penicillium* species are not dimorphic and are more like *Aspergillus*, with hyphae in tissue.

Research on the biochemical properties of *P. marneffei* has been focused on its enzymatic activities (189). The secreted enzymatic activities of 10 *P. marneffei* isolates during the mid-log growth phase were analyzed. Both mycelia and yeast expressed alkaline phosphatase, acid phosphatase, and naphthol-AS-BI (bioMerieux UK, Basingstoke, United Kingdom)-phosphohydrolase activities, whereas a variety of other enzyme activities, including trypsin, chymotrypsin, and α -fucosidase, were absent. Active esterases, lipases, and galactosidases were found in some isolates. Several of these enzymes have been linked to virulence in pathogenic microorganisms, for example, acid phosphatase in *Coxiella burnetii* and phospholipase and esterase in *Candida albicans* (3, 69, 109, 165). In order to determine

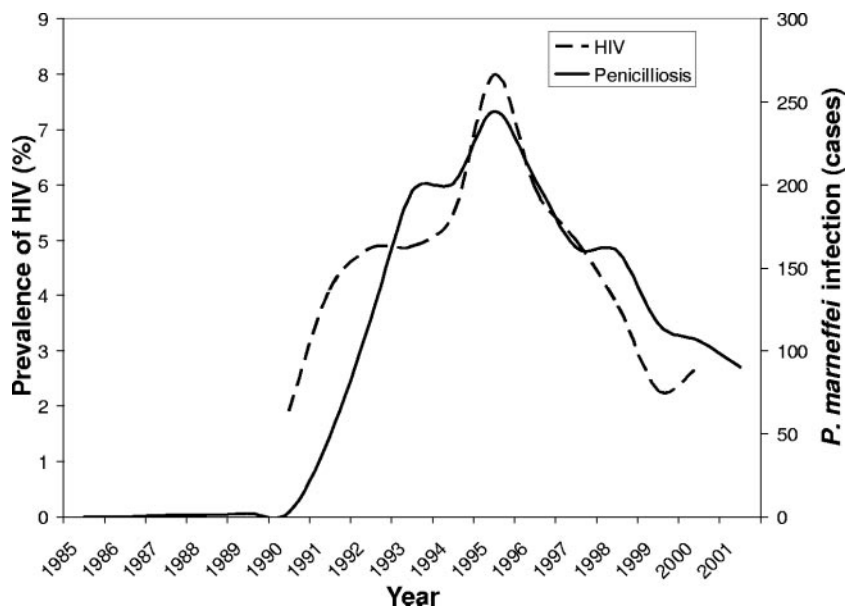


FIG. 1. Temporal emergence of HIV (antenatal data, 1990 to 2000; UNAIDS/WHO working group report, 2003) and *P. marneffeii*-associated penicilliosis (1985 to 2001; Maharaj Hospital, Chiang Mai) for the Chiang Mai region, northern Thailand.

whether secreted enzymes are linked to virulence in *P. marneffeii*, further work, focused on purifying such enzymes and characterizing their effects, is needed. A study by Wong et al. (186) on the biochemical properties of *P. marneffeii* secreted enzymes and their possible use in strain biotyping showed that all 32 isolates of *P. marneffeii* examined possessed the urease enzyme. All isolates assimilated glucose, maltose, and cellobiose. However, some heterogeneity between isolates was observed in their biochemical profiles. A total of 65, 84, and 72% of the isolates were able to assimilate trehalose, xylose, and nitrate, respectively, and when galactose (0.015 to 0.25%) was the sole carbon source in the medium, inhibition of growth occurred in all isolates. Several strains possessed the enzyme β -galactosidase. From these biochemical properties, 17 different biotypes were recognized.

PATHOGENESIS AND CLINICAL FEATURES

Clinical Manifestations

Between June 1990 and June 2004, 1,843 cases of disseminated *P. marneffeii* infection were seen in HIV-infected patients at Chiang Mai University Hospital. Approximately 6,709 cases of *P. marneffeii* infection were diagnosed in Thailand between September 1984 and October 2004 (HIV/AIDS epidemiology report, Division of Epidemiology, Ministry of Public Health, Thailand [<http://epid.moph.go.th>]). Patients infected with *P. marneffeii* had clinical histories that exhibited various degrees of severity. The symptoms and signs of 74 HIV-infected patients with disseminated *P. marneffeii* infection, observed between November 1993 and January 1996 at Chiang Mai University Hospital, have been summarized (177). Most cases presented with fever, weight loss, skin lesions, generalized lymphadenopathy, and hepatomegaly. Respiratory signs were also observed. Skin lesions were seen in 63 patients (85%). The observed lesions in 54 of these 63 patients were papules with central necrosis, and the rest were papules or maculopapules. All

patients acquired lesions on the face and neck. Other body sites where skin lesions were found included the upper extremities (33 patients [52%]), trunk (25 patients [39%]), and lower extremities (19 patients [30%]). Six patients also had papular lesions on the palate. The average number of CD4⁺ T lymphocytes in these patients was 64 cells/mm³ (standard deviation, 47 cells/mm³). Fifty-six patients (76%) were anemic, with a measured hemoglobin level of 10 g/dl or less. With the possible exception of the skin lesions, it was not possible to unequivocally attribute these abnormal clinical findings to *P. marneffeii* infection alone. This is because they may also be caused by other opportunistic infections associated with late-stage HIV infection, as well as the immunodeficiency virus itself.

Mucocutaneous, oral, and facial manifestations have been reported in patients with *P. marneffeii* infection (26, 79, 84, 151, 161, 181). Entire facial skin and oral manifestations have been described as papular and ulcerated lesions. Soft palate was also involved. Some patients having hepatic penicilliosis without any skin lesions have been described (78). Osteoarticular lesions were seen in multiple sites of patients with disseminated *P. marneffeii* infection; here the sites of bone infection were the ribs, long bones, skull, lumbar vertebrae, scapula, and temporomandibular region (72, 100, 107, 154). Arthritis involved both large peripheral joints and small joints of the fingers and multiple swollen joints. Multiple lytic bone lesions involving flat bones of the skull, long bones, and small bones of the fingers were also seen (19, 32, 107). The similarity of these lesions to those observed in other systemic infectious diseases, for example, cryptococcosis, blastomycosis, African histoplasmosis, and tuberculosis (40), makes differential diagnosis problematic.

Pathology

P. marneffeii 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. Clinical signs of *P. marneffei* infection in both HIV-positive and HIV-negative patients have been summarized (40). In the HIV-positive group, the rapid onset and severity of symptoms in the absence of early treatment were striking. Infected tissues can show different histopathological reactions. In immunocompromised patients, necrotizing reactions with macrophage and histiocyte infiltrations are seen (33, 40, 151). *P. marneffei* 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 to 3 μm in diameter, which multiply by binary fission. Elongated cells of up to 13 μm long can be observed extracellularly. In immunocompetent patients, granulomatous and suppurative reactions are frequently seen in the lung, skin, liver, and subcutaneous tissues (33, 72). 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 *Histoplasma capsulatum* exploit the macrophage as a host cell, and both organisms can cause acute or persistent pulmonary and disseminated infection and reactivation disease (31, 190).

Immunology

The mechanisms of host-fungus interaction and host immune response in *P. marneffei* infection are poorly understood. Infection is presumably via inhalation of conidia from the environment; however, aerosolization of infectious particles, and subsequent infection, has never been definitively shown. Phagocytic cells are likely to be the primary line of the host defense against this fungus. *P. marneffei* conidia are able to recognize fibronectin and bind to laminin via a sialic acid-specific lectin (56, 57). This reaction may play an important role in the attachment of conidia to bronchoalveolar epithelia before ingestion by host mononuclear phagocytes. A study of the interactions between human leukocytes and heat-killed yeast-phase *P. marneffei* revealed that monocyte-derived macrophages bind and phagocytose *P. marneffei*, even in the absence of opsonization. The entry of *P. marneffei* into macrophages is divalent cation independent, and the pathogen gains access through a process that is inhibitable by wheat germ agglutinin. For this reason, it is suggested that the major receptor(s) recognizing *P. marneffei* is a glycoprotein with exposed *N*-acetyl- β -glucosaminyl groups. In another intracellular fungus, *Histoplasma capsulatum*, the HSP60 protein has been identified as a specific ligand or adhesin on the surface of the fungus. This ligand is recognized by the CD11/CD18 receptors on the host macrophages (106). However, the surface ligand involved in the specific binding of *P. marneffei* to the host cell receptors has not thus far been identified.

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 (89, 90, 179). 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 (150). 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 (28, 91–93, 96, 155); it appears that this is also the case regarding infection by *P. marneffei*.

Both human and mouse macrophages are able to control *P. marneffei* growth and to kill intracellular yeast cells when activated in vitro by T-cell-derived cytokines, such as gamma interferon (IFN- γ). It was demonstrated that intracellular *P. marneffei* was damaged via the L-arginine-dependent nitric oxide (NO) pathway in murine macrophages stimulated with IFN- γ . *P. marneffei* could also stimulate a respiratory burst regardless of whether opsonins are present, and serum factors are required for *P. marneffei* to stimulate tumor necrosis factor alpha (TNF- α) release (134). The ability of unopsonized *P. marneffei* to parasitize mononuclear phagocytes without stimulating the production of TNF- α may enhance the virulence of this intracellular organism.

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. Recently, by the use of an in vitro analysis of a sublethal *P. marneffei* infection in BALB/c mice (3×10^5 conidia/mouse), it was demonstrated that protective immunity follows a Th1 response, with high levels of interleukin-12 (IL-12), IFN- γ , and TNF- α being developed (144). This finding is consistent with the general knowledge that a Th1 response plays a crucial role in host resistance to intracellular pathogens (133), as in mycobacterial infections (45) and infections with other fungi, such as *Histoplasma capsulatum*, *Coccidioides immitis*, and *Candida albicans* (59). The data also 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 (144).

The role of the phosphoprotein osteopontin (OPN) in IL-12 production from peripheral blood mononuclear cells stimulated with *P. marneffei* was investigated (86). The results of that work demonstrated that OPN, secreted from monocytes, is involved in the production of IL-12 from peripheral blood mononuclear cells after stimulation with *P. marneffei* and that the OPN production is regulated by granulocyte-macrophage colony-stimulating factor. These results also indicated the possible involvement of the mannose receptor as a signal-transducing receptor for triggering the secretion of OPN by *P. marneffei*-stimulated peripheral blood mononuclear cells. These findings suggested that OPN may polarize the Th1 cytokine response and may also contribute to the host's defense against *P. marneffei* infection.

The responses of rabbit pulmonary alveolar macrophages and circulating human mononuclear phagocytes to *P. marneffei* conidia have been reported (131). 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. In addition, studies utilizing electron microscopy demonstrated that intact conidia were enclosed in phagosomes at 7 h. Subsequently, the conidia then became intraphagosomal, and they exhibited damage, by 12 h. The role of human neutrophils in

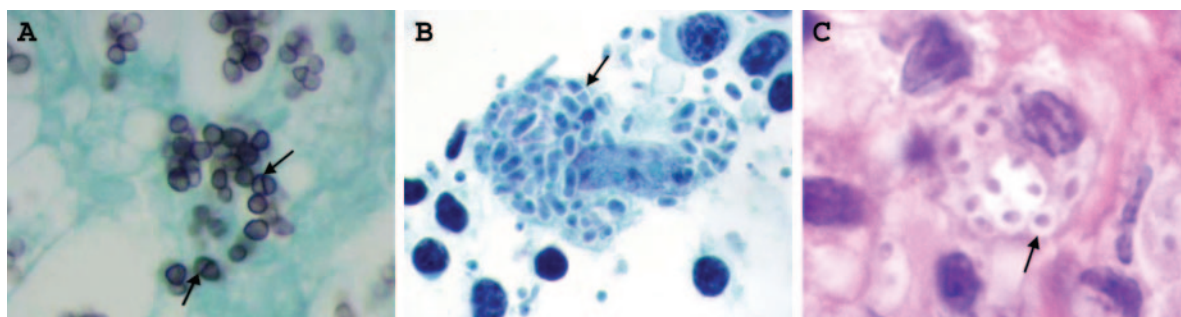


FIG. 2. (A and B) Photomicrographs of yeast cells of *Penicillium marneffeii* (courtesy of Benjaporn Chaiwun) in the fine-needle-aspirated material obtained from deep-seated lymph nodes from a *P. marneffeii* infected patient. The characteristic transverse septum (arrows) within the yeast cell can be seen by Grocott methenamine silver staining (A) and Papanicolaou staining (B). (C) Histopathologic picture of *Histoplasma capsulatum*. Yeast cells without transverse septa are seen by hematoxylin-eosin staining. Magnification, $\times 1,000$.

the host defense against *P. marneffeii* infection has been studied (93, 94). These results indicated that granulocyte-macrophage colony-stimulating factor enhanced the antifungal activity of human neutrophils against the yeast form of *P. marneffeii* but not the conidia. This activity was mediated by exocytosis of the granular cytolytic molecules from neutrophils rather than by oxygen radical-dependent mechanisms.

As for other intracellular pathogens, surviving within the phagocytes is the primary key to a successful invasion by *P. marneffeii*. However, the mechanism of survival of *P. marneffeii* under oxidative stress within the macrophage remains unknown. Many fungi have been shown to survive within the phagocytic environment (97). 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. (189) found an expression of acid phosphatase activity by *P. marneffeii*. Production of acid phosphatase is considered to be one of the virulence factors for intracellular pathogens, such as *Coxiella burnetii* (3), and *Francisella tularensis* (127). 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 has found some support from studies of the antimicrobial activity of chloroquine against *P. marneffeii* by using human THP1 and mouse J774 macrophages. These results revealed that the drug's antifungal activity was due to an increase in the intravacuolar pH and a disruption of pH-dependent metabolic processes (156); 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. In addition, iron overload was found to significantly reduce the antifungal activity of gamma interferon-lipopolysaccharide-activated human THP1 and mouse J774 macrophages (155). That work suggested that iron availability critically affects immunity and the pathogenicity of *P. marneffeii*. In *H. capsulatum*, the expression of at least three catalases which detoxify hydrogen peroxide has been identified (188). The expression of these enzymes could contribute to survival of *H. capsulatum* inside the host cell. In *P. marneffeii*, an antigenic catalase-peroxidase protein-encoding gene (*cpeA*) was recently isolated by antibody screening of a cDNA yeast-phase library of this fungus (123). The

high expression of this *cpeA* gene at 37°C may contribute to the survival of this fungus within the host cells.

LABORATORY DIAGNOSIS

Diagnosis by Staining Methods and Cultures

Diagnosis of infection by *Penicillium marneffeii* is commonly made by identifying the fungus in clinical specimens by microscopy and culture. Clinical specimens that are commonly used include bone marrow aspirate, blood, lymph node biopsies, skin biopsies, skin scrapings, sputum, bronchoalveolar lavage pellet, pleural fluid, liver biopsies, cerebrospinal fluid, pharyngeal ulcer scrapings, palatal papule scrapings, urine, stool samples, and kidney, pericardium, stomach, or intestine specimens (40, 151). Rapid presumptive diagnosis can be made by microscopic examination of Wright-stained bone marrow aspirates and/or touch smear of skin biopsy or lymph node biopsy specimens (150). In patients with fulminant infection, *P. marneffeii* could be observed in peripheral blood smears (112, 151). *P. marneffeii* can be seen in histopathological sections stained with hematoxylin and eosin, Grocott methenamine silver, or periodic acid-Schiff stain. The organisms appear as fission arthroconidia or unicellular round to oval cells, which may divide by cross wall formation in macrophages or histiocytes (26, 63, 65, 112, 119, 120, 141, 142, 150, 164, 168, 180). The cross wall formation can differentiate yeast cells of *P. marneffeii* from those of *Histoplasma capsulatum*, which also appear as intracellular yeasts (Fig. 2). Extracellular elongated or sausage-shaped cells of *P. marneffeii*, with one or two septa, may also be seen (40, 151).

P. marneffeii in paraffin-embedded, formalin-fixed tissue from infected guinea pigs could be identified by the use of a monoclonal antibody directed to *Aspergillus* galactomannan (44). Here, it seems that the monoclonal antibody used detected a specific galactomannan that appeared to have at least one identical epitope in *P. marneffeii* and *Aspergillus* spp. (115). These two fungi have different morphologies as seen in tissue sections. *Aspergillus* appears as mycelial mats composed of radiating hyphae with regular septation and dichotomous branchings. Conversely, the tissue form of *P. marneffeii* develops as a unique yeast-like structure of 2 to 3 μm in diameter,

which multiplies by binary fission or schizogony. Immunoperoxidase staining for the tissue form of *P. marneffei* was developed by using rabbit antisera raised against the 3-day-old yeast culture filtrate antigens of *P. marneffei* (175). *P. marneffei* in deparaffinized tissue sections of skin biopsies from patients with *P. marneffei* infection could be clearly demonstrated as intracellular yeasts.

A specific indirect fluorescent-antibody reagent for the rapid identification of *P. marneffei* in histologic sections was developed by using rabbit antiglobulins against yeast-like culture filtrate antigens of *P. marneffei* (80). The antiglobulins, adsorbed with *Histoplasma capsulatum*, specifically stained the yeast-like cells of *P. marneffei* in tissue sections from six patients with *P. marneffei* infection. None of the tissue sections from 10 humans with histoplasmosis stained with this specific antiglobulin. Murine immunoglobulin (IgM) monoclonal antibodies raised against *P. marneffei* mycelial culture filtrate antigens were also proved to react strongly in immunofluorescent staining with the yeast cells in the tissue biopsies of patients (162). Rapid diagnosis of *P. marneffei* infection, particularly in patients with lymphadenopathy, could be made by the use of fine-needle aspiration cytology (Fig. 2A and B) (17, 108). The use of this method of diagnosis is potentially beneficial to patients in whom lymphadenopathy is confined to deep intra-abdominal nodes.

Definite diagnosis of disseminated *P. marneffei* infection is based on mycological culture, and studies have demonstrated high sensitivity from bone marrow (100%), blood (76%), and skin biopsy (90%) (151). The fungus grows in a mycelial phase at 25°C on Sabouraud glucose agar without cycloheximide. Mold-to-yeast conversion is achieved by subculturing onto brain heart infusion agar and incubating at 37°C (136). Identification of *P. marneffei* is based upon the morphology of the colony, its mold-to-yeast conversion, and the organism's microscopic morphology.

A specific exoantigen test using the immunodiffusion (ID) technique was developed to identify *P. marneffei* culture (139). The concentrated culture filtrate from 6-week-old, 25°C shake cultures of a standard strain of *P. marneffei* was employed as a reference antigen. Rabbit antiglobulin raised against this antigen was used in an ID test to identify *P. marneffei* and proved especially useful for cultures that were difficult to convert or that had an atypical colony morphology (82). Two to four specific precipitin lines are seen in a positive reaction, and the exoantigen test can be used to differentiate *P. marneffei* from other *Penicillium* species, such as *P. citrinum* and *P. commune* (138). The anti-*P. marneffei* antiglobulin adsorbed with *P. primulinum* antigens can also be used in an ID test to differentiate between these two closely related species.

Serologic Diagnosis

Several methods have been developed for detecting specific antibodies against antigens of *P. marneffei* in clinical specimens. These tests have the potential to provide a rapid diagnostic method for identifying *P. marneffei* infection, thus enabling initial therapeutic management. The micro-ID test using *P. marneffei* (mycelial phase) exoantigens was applied to detect precipitin antibodies in a patient's serum specimen. Thirteen serum specimens which were collected serially from

the same HIV-positive patient infected with *P. marneffei* gave positive results 2 months after the initial treatment (180). However, another application of the ID test to detect *P. marneffei* antibodies in individual sera from 17 patients with *P. marneffei* infection revealed positive results for only 2 of the 17 serum specimens, showing low sensitivity of the test (81).

An indirect fluorescent-antibody test for detecting IgG antibodies in patients infected with *P. marneffei* was developed using germinating conidia and yeast forms as antigens (192). The test was evaluated with serum samples from 103 patients with persistent fever and 78 normal subjects. Eight cases with persistent fever and documented *P. marneffei* infection had an IgG titer of 160 or more. The remaining 95 patients without *P. marneffei* infection and 78 healthy controls had a titer of 40 or below. This test has the potential to provide rapid presumptive diagnosis and might supplement conventional culture.

Immunoblot assay using crude antigens of *P. marneffei* and serum specimens from patients were analyzed. The protein antigens produced during the growth phase of the yeast form were found to be more immunoreactive than the antigens obtained from the mycelial form (172). These findings corresponded to the infective stage of this organism, which was the intra- and extracellular yeast phase in the patient's lesions. Four immunogenic yeast proteins of 200, 88, 54, and 50 kDa were produced in a large quantity during the deceleration and early stationary phases of growth. When these four proteins were tested with serum specimens from 33 *P. marneffei*-infected AIDS patients, reactivities to them were detected in 73, 94, 61, and 58%, respectively. The 200- and 88-kDa proteins may be common antigens that occur in other environmental fungi. These proteins elicited weak reactivity in high proportions of serum specimens from HIV-infected patients without *P. marneffei* infection and normal persons. However, the 54- and 50-kDa proteins proved more promising and were strongly reactive with serum specimens from *P. marneffei*-infected patients (172). Interestingly, in one serum specimen from an HIV-infected patient, strong reactivities to the 54- and 50-kDa proteins could be detected 2 months before the definite diagnosis was made by fungal culture. These 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. Another diagnostic antigen of 38 kDa, which was prepared from acetone-precipitated culture filtrate of mycelial-phase cells, has also been reported (27).

A study of whole cytoplasmic antigens from yeast-form *P. marneffei* confirmed that *P. marneffei* infection could trigger a humoral response (73). Antigens of 88, 61, 54, and 50 kDa, identified by Western blotting, were purified by a combination of liquid isoelectric focusing (Rotofor system) and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Prep-cell system). These antigens were screened separately with pooled sera from patients infected with *P. marneffei*, pooled normal sera, and pooled sera from patients with candidiasis, aspergillosis, histoplasmosis, and cryptococcosis. The 61-, 54-, and 50-kDa antigens were specific for *P. marneffei*, with no cross-reactivity with sera from patients with other mycoses. The 88-kDa antigen was slightly cross-reactive with sera from patients with cryptococcosis. The 61-kDa antigen was an addi-

tional candidate for the development of specific antibody tests for disseminated *P. marneffe* infection.

An enzyme-linked immunosorbent assay (ELISA)-based antibody test was developed with a recombinant *P. marneffe* mannoprotein (Mp1p) for the serodiagnosis of *P. marneffe* infection (15). Evaluation of the test revealed high specificity (100%) and approximately 80% sensitivity (14 of 17) in HIV-seropositive patients infected with *P. marneffe*. The anti-Mp1p antibody was also used in an ELISA-based method for the detection of the mannoprotein Mp1p in the sera of patients (14). The antigen test had an overall sensitivity of 65% (17 of 26). The combined antibody and antigen tests for the diagnosis of *P. marneffe* infection had a sensitivity of 88% (23 of 26), with a positive predictive value of 100% and a negative predictive value of 96%.

Several additional methods for detecting circulating *P. marneffe* 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. marneffe* antigen (121, 170). The reagent was used to detect galactomannan in an experimental infection with *P. marneffe*. However, the titer of antigen detected was lower than that in infection with *Aspergillus* (170). A specific latex agglutination (LA) for the detection of *P. marneffe* antigens was developed (81). The LA test detected antigens in 13 of 17 serum samples (76%) and in 2 urine specimens. The LA titers ranged from 1:64 to 1:4,096. The use of ID antibody and LA antigenemia tests concurrently increased the sensitivity to 82%. These tests appeared to be highly specific (100%), since none were positive with sera from 15 Thai control patients, 6 serum samples containing cryptococcal antigen, or 6 urine specimens positive for *Histoplasma* polysaccharide antigens.

An enzyme immunoassay for the quantitation of *P. marneffe* urinary antigen was developed by using fluorescein isothiocyanate-labeled purified rabbit hyperimmune IgG (37). The hyperimmune serum was prepared by injecting New Zealand White rabbits subcutaneously with killed whole yeast-like cells of *P. marneffe*. The urine antigen detection assay had a diagnostic sensitivity of 97% and specificity of 98% at a cutoff titer of 1:40. The same polyclonal anti-*P. marneffe* antibody was used further in a dot blot ELISA and a latex agglutination test for the detection of *P. marneffe* urinary antigen (36). The overall sensitivities of the tests were as follows: dot blot ELISA, 94%; ELISA, 97%; and LA test, 100%. The specificities were 97, 98, and 99%, respectively. All tests were highly sensitive and specific. These tests have the potential to be used to assess responses to antifungal therapy in serial urinary antigen testing during treatment and subsequent follow-up.

A monoclonal antibody-based sandwich ELISA was developed for the detection of *P. marneffe* antigen in clinical specimens from patients with *P. marneffe* infection (116, 162). When used at a 1:2 dilution, 13 of 18 culture-positive serum samples proved to be antigen positive. When samples were used undiluted, sensitivity was improved, with three additional specimens proving positive. The test was also found to be useful for the detection of secreted antigen in urine samples. However, to determine the actual sensitivity and specificity of

the test, a larger number of specimens from patients with *P. marneffe* and other diseases need to be tested.

Molecular Diagnosis

The PCR technique is extremely sensitive and has been used effectively for the specific detection of many fungi. Molecular diagnosis of *P. marneffe* is based on specific oligonucleotide primers designed from the internally transcribed spacer and 5.8S rRNA gene (ITS1-5.8S-ITS2) of *P. marneffe* (105). The specificity of these *P. marneffe* primers was tested in a nested PCR; fungal DNA was first amplified with the primer pair ITS5 and ITS4 (184) and nested PCR subsequently performed with primer pair PM1 and PM4 or PM2 and PM4. The primer pair PM2 and PM4 was 100% successful in amplifying *P. marneffe* DNA, yielding a 347-bp PCR product, and this method was used successfully to identify *P. marneffe* from a skin biopsy (167). To identify medically important yeast-like fungi, specific oligonucleotide probes for several pathogens, including *P. marneffe*, have been developed and tested in a PCR-enzyme immunoassay method (102). In those studies, minor cross-reactivity has been observed for a *Blastomyces dermatitidis* probe used against *C. immitis* DNA and for an *H. capsulatum* probe used against *C. albicans*. However, no cross-reactivity to *P. marneffe* was seen. The method was able to rapidly identify DNA obtained from fungi in a pure culture, and the application of this test in a clinical laboratory setting needs to be evaluated further.

An oligonucleotide probe based on the 18S rRNA gene of *P. marneffe* has been designed and has proved specific for *P. marneffe* in a PCR-hybridization reaction, regardless of whether the fungus was isolated from humans or from natural habitats. The sensitivity of the technique was about 0.1 pg/ μ l of DNA. In addition, this PCR-hybridization technique was used to detect *P. marneffe* DNA in EDTA-blood samples collected from AIDS patients with *P. marneffe* infection (174). Although the method was shown to be highly sensitive and specific, the hybridization technique as described is labor-intensive and requires a high level of competence in the laboratory. To address these concerns, single and nested PCR methods for the rapid identification of *P. marneffe* were then developed using newly designed specific primers, also based on the 18S rRNA gene sequence of *P. marneffe* (176). The sensitivities of single and nested PCR were 1.0 pg/ μ l and 1.8 fg/ μ l, respectively, and successful discrimination of a very young culture of *P. marneffe* (2-day-old filamentous colony, 2 mm in diameter) could be performed by the use of this assay. Further diagnostic PCR methods have also been described; a one-tube seminested PCR assay was developed to identify *P. marneffe* DNA based on the 18S rRNA sequences (124). This assay was sensitive and could identify *P. marneffe* DNA both from pure cultures and two clinical samples. The utility of both of these PCR methods for the early diagnosis of the disease needs to be studied further.

TREATMENT

The mortality rate of patients with *P. marneffe* infection has been very high, especially when physicians do not make an early diagnosis and commence treatment early. By using an in

vitro susceptibility test, the fungus was demonstrated to be highly susceptible to miconazole, itraconazole, ketoconazole, and flucytosine (152). Amphotericin B showed intermediate antifungal activity, while fluconazole was the least active. Some strains of *P. marneffeii* were resistant to fluconazole. The clinical and microbiological responses correlated with the overall patterns of in vitro susceptibility to the azoles, whereas results with amphotericin B were more difficult to assess. In this study of 80 patients with disseminated *P. marneffeii* infection, no maintenance therapy was used. The study found that 12 out of 40 patients who initially responded to treatment relapsed within 6 months (152). These results suggested that a long-term secondary prophylaxis might be needed to prevent recurrence of this infection.

A double-blind trial was conducted to evaluate itraconazole as a secondary prophylaxis against *P. marneffeii* infection in 71 HIV-infected patients, who were enrolled in a maintenance study (153). The initial standard antifungal therapy comprised 2 weeks of parenteral amphotericin B at a dose of 0.6 mg/kg/day, followed by 400 mg of itraconazole per day orally in two divided doses for the next 10 weeks. The patients were then randomly assigned to receive either oral itraconazole (200 mg daily) or a placebo as maintenance therapy. None of the 36 patients assigned to itraconazole had a relapse within 1 year, whereas 20 of the 35 patients assigned to a placebo had relapses. The results suggest that in patients infected with HIV who successfully complete primary treatment of *P. marneffeii* infection, a secondary prophylaxis with oral itraconazole (200 mg once daily) is well tolerated and prevents relapses of *P. marneffeii* infection.

ECOLOGY AND EPIDEMIOLOGY

Despite a decade of research on the epidemiology and ecology of *Penicillium marneffeii*, the basic ecology of this unique pathogen remains enigmatic. A core issue is whether the human disease, penicilliosis marneffeii, occurs as a consequence of zoonotic (animal) or sapronotic (environmental) transmission. In other words, the ecological reservoir(s) of human penicilliosis marneffeii remains unknown to this date.

Penicillium marneffeii was originally isolated from a bamboo rat, *Rhizomys sinensis*, by Capponi et al. in 1956 (16). Since then, a number of studies have surveyed rodent species and firmly established four species of bamboo rats as enzootic reservoirs of infection: *Rhizomys sinensis*, *R. pruinosus*, *R. sumatrensis*, and the reddish-brown subspecies of *Cannomys badius* (1, 24, 34, 55, 98, 183). These studies have shown that, within these susceptible species, the prevalence of infection varies widely across southeast Asia. This finding suggests either that there are regional variations in the endemicity of infection or that there are geographical variations in the predisposition to infection within different species of bamboo rats. A recent study by Gugnani et al. (55) comprehensively surveyed six species of sympatric rodents (*Bandicota bengalensis*, *Rattus norvegicus*, *Rattus rattus*, *Rattus niditus*, *Mus musculus*, and indigenous reddish-brown *C. badius* [$n = 182$]) from bamboo plantations in Manipur State, northeastern India, and found that only *C. badius* harbored infection. As all the rodents in this area presumably have approximately similar exposure rates, this study provides strong evidence that there exist host-

specific factors that govern infection. Surveillance of the rodent reservoirs of the ascomycete pathogens *Coccidioides immitis* and *Coccidioides posadasii* in the United States have shown similar interspecific variation in the prevalence of infection, suggesting that this may be a general feature of pathogenic fungi that infect rodents (42, 43). Intraspecific variation in the prevalence of infection has also been found between subspecies of bamboo rat, where a study by Chariyalertsak et al. (24) in the Chiang Mai region, Thailand, showed that all 51 animals of the greyish-black subspecies of *C. badius* were negative for *P. marneffeii* while 3 of the 10 rats (30%) in the reddish-brown group were positive. Taken together, these studies indicate that the distribution of prevalences of *P. marneffeii* in rodents has (i) a host component and (ii) a geographical component.

A correct description of the factors governing the distribution of *P. marneffeii* infection in bamboo rats is necessary to describe the epidemiology of this disease in human populations. The reporting of autochthonous infections in humans has so far provided the best method of describing the geographic range of *P. marneffeii*, and such reports have defined the endemic range to include Thailand, southern China, Taiwan, Hong Kong, Laos, Cambodia, Malaysia, Vietnam, Myanmar (Burma), and northeastern India (4, 33, 41, 125, 140, 143). While a single case of the disease has also been observed in an African from Ghana who had no history of travel to southeast Asia (104), no further cases have been reported, suggesting that *P. marneffeii* is in fact endemic to southeast Asia. The geographic ranges of the lesser and greater bamboo rats (*Cannomys* spp. and *Rhizomys* spp.) broadly follow the known distribution of *P. marneffeii*. While this may be considered circumstantial evidence that bamboo rats are an obligate stage in the life cycle of the fungus, it may also be that the ecotype favored by these rodents is simply shared with *P. marneffeii* and that infections within these rodents are examples of sylvatic sapronotic infections. Early attempts to epidemiologically link bamboo rats and human infection showed no association; a case-control study of HIV-positive patients, performed in northern Thailand by Chariyalertsak et al. (23), did not implicate bamboo rats as a reservoir of infection for humans. Rather, age (16 to 30 years) and an agricultural occupation were found as factors that were independently associated with an increased risk of infection. Temporal analyses of the incidence within northern Thailand over a 4-year period (22) showed that there was extensive seasonal variation in infection rates and that increased disease was associated with the rainy season. These studies suggest that soil exposure, especially during the rainy season, is a critical risk factor associated with infection by *P. marneffeii*.

If these studies are correct and *P. marneffeii* infection is in fact a sapronosis (rather than a zoonosis), then the fungus must have a reservoir in the environment. Many species of penicillia are soil saprophytes. However, despite extensive efforts, attempts to recover soil isolates of *P. marneffeii* have met with only limited success. Vanittanakom et al. (173) demonstrated 80 to 85% recovery of CFU after 3 days of incubation from sterilized soil seeded with *P. marneffeii*; however, the recovery from nonsterile soil seeded with the fungus was only 6%. A recent laboratory study has demonstrated that *P. marneffeii* can survive in sterile soil for several weeks but can survive for only

a few days in nonsterile soil (76); these studies suggest that survivability in the soil when faced with natural fungal competitors may be limited. Deng et al. isolated *P. marneffeii* from three soil samples collected from the burrows of *Rhizomys pruinosus* (33) and Chariyalertsak et al. (24) were able to recover *P. marneffeii* from one out of 28 soil samples collected from the burrows of *Rhizomys sumatrensis*. However, to date no attempts to recover *P. marneffeii* from environments other than those that are intimately associated with bamboo rats have been successful, and definitive proof of an environmental reservoir for *P. marneffeii* within the soil, or other substrates, is still lacking.

Molecular Epidemiology

Modern molecular methods provide the best opportunity for dissecting the epidemiology of *P. marneffeii* and revealing its fundamental life history traits (158). This is because multilocus genotypes can be generated that (i) identify isolates of a similar or identical genetic background that are derived from a common infective population, (ii) when applied to systematically sampled isolates can describe the hierarchical organization of population structure (i.e., whether there is a single large population or a number of genetically isolated subpopulations), (iii) can identify the reproductive mode (i.e., whether the fungus is asexual and clonal, sexual and recombining, or a combination of both), and (iv) can provide information on the deeper phylogenetic and evolutionary history of the pathogen.

Randomly sampling genetic variation within the *P. marneffeii* genome. Until recently, molecular approaches to typing *P. marneffeii* have relied on surveying the genome by using methods that randomly sample for genetic variation. Vanittanakom et al. (171) used HaeIII digests of genomic DNA to search for restriction fragment length polymorphisms (RFLPs) in order to differentiate *P. marneffeii* isolates from the Chiang Mai region, northern Thailand. The 22 human isolates in their study were classified in two DNA types, with type I representing 16 (73%) and type II representing 6 (27%) isolates; of the 23 bamboo rat isolates, all 20 from *R. sumatrensis* were type I and all 3 from *C. badius* were type II. In a separate study of 20 *P. marneffeii* isolates from Taiwanese patients, Hsueh et al. (64) used the same restriction digestion assay of genomic DNA to uncover the same two HaeIII RFLP patterns that had been found in Thailand and at approximately the same frequencies (type I, 75%; type II, 25%). However, the use of randomly amplified polymorphic DNA (RAPD) assays yielded eight different RAPD patterns, suggested that there was greater genetic diversity in the Taiwanese population than had been uncovered by the RFLP assay (64). Imwidthaya et al. (71) developed a fingerprinting assay for *P. marneffeii* by using the tetranucleotide repeat primer (GACA)₄ and the phage M13 core sequence. This assay identified four genotypes that varied in frequency between northern and southern Thailand. A separate study used pulsed-field gel electrophoresis of 69 *P. marneffeii* isolates from several regions of Thailand, using the restriction enzyme NotI (163). This study revealed two macrorestriction patterns (MPI and MPII) that could be grouped into nine subprofiles (MPIa to MPIf and MPIIa to MPIIc) by using HaeIII digestion, yielding 54 genotypes in total. How-

ever, no correlation between the restriction patterns from various *P. marneffeii* isolates and geographic region or specimen source was observed in these studies.

Substantial drawbacks of all these typing systems are that (i) discriminatory power is low due to the small numbers of alleles, (ii) the reproducibility of RAPD and macrorestriction profiles between laboratories can be low (5), and (iii) alleles may not be homologous despite being of similar sizes (128, 158). In order to address these concerns, recent efforts have focused on developing typing schemes that focus on specific, sequence-characterized regions of the genome that have been isolated by data mining of the DNA sequence that has been generated by *P. marneffeii* genomics programs (191).

Sequence-specific assays of genetic variation in the *P. marneffeii* genome. There are two main technologies that target specific regions of the fungal genome in order to discriminate between isolates: multilocus sequence typing (MLST) (46, 110) and multilocus microsatellite typing (MLMT) (46, 49, 50). MLST is becoming the technique of choice for bacterial species and the fungus *Candida albicans* (11, 130) and works by characterizing isolates by sequencing housekeeping genes (usually seven). The alleles present at each locus are combined into a multilocus sequence type, which is then deposited in a species-specific online database held at <http://www.mlst.net/>. However, the use of MLST is inappropriate when the species being typed contain insufficient genetic variation to differentiate isolates, as is the case for a number of bacterial and eukaryotic species (48, 148). At this stage, it is unclear whether levels of genetic diversity found in *P. marneffeii* housekeeping loci are high enough to allow MLST to be used to discriminate between isolates.

To circumvent the problem of low levels of genetic variation, MLMT targets loci that contain di-, tri-, or tetranucleotide repeats. These repeats ("microsatellites") (157) are more highly variable than housekeeping loci due to the accumulation of length polymorphisms as a consequence of slippage by DNA polymerase during genome replication. The alleles at each locus are scored by electrophoresing PCR-amplified loci through an automated sequencer, typing the length polymorphisms, and then combining the alleles from each locus into a multilocus microsatellite type (MT). These MTs can then be used to query online databases held at <http://www.multilocus.net/>. The resulting outputs from these queries can be used to analyze the population genetic structure of the organism or to test epidemiological hypotheses.

Fisher et al. (47) screened 1.7 Mb of *P. marneffeii* genome sequence for microsatellite motifs, using all possible permutations of di-, tri-, and tetranucleotide motifs with a minimum repeat number of six; this search resulted in 30 dinucleotide, 14 trinucleotide, and 5 tetranucleotide repeats being discovered. A similar study on the same genome sequence by Lasker and Ran (95) uncovered only three microsatellites; why this discrepancy between the two studies exists is unclear, although the software used in the latter study excluded tri- and tetranucleotide repeats. Of the 49 loci identified by Fisher et al. (47), 24 were chosen and amplified as multiplex PCRs in four groups of six loci and used to type a panel of 29 clinical and bamboo rat isolates chosen from across the endemic range of *P. marneffeii* (46, 47, 55). Of the 24 loci, 23 were amplifiable and 21 were polymorphic with between 2 and 14 alleles present at

each locus, comprising 19 unique MTs in total. Clustering of isolates based on the microsatellite genetic distance D_1 (53) showed that isolates occur within one of two geographically separated clades that account for 26% of the total observed genetic diversity (46). The “eastern” clade contained isolates from mainland China, Hong Kong, Indonesia, and Vietnam, while the “western” clade contained isolates from Thailand and India, showing that *P. marneffei* has an extensive geographic component to its population genetic structure. Within the eastern and western clades, extensive linkage disequilibria existed between loci (measured by the index of association) (145), suggesting that there were either genetically differentiated subpopulations or extensive clonal reproduction occurring within the existing populations. A study over a smaller geographical scale in Manipur, India, showed that while the MTs of isolates were identical within bamboo plantations, they were dissimilar between plantations (55). This finding suggests that the population genetic structure of *P. marneffei* may in fact be partitioned over local, as well as large, geographical scales, although further studies are necessary to confirm the generality of this finding.

Fisher et al. (46) genotyped the type isolate *P. marneffei* CBS 388.87 (136) and showed that it was characterized by a unique MT and was therefore dissimilar to any human isolates (55); a similar result was found by comparing 2 bamboo rat isolates with 32 clinical isolates in another study (95). However, a comparison of 10 bamboo rat isolates collected in Manipur, India, showed that a single bamboo rat isolate was identical at all 21 microsatellite loci to the human isolate CBS 101038, isolated in 1998 (125). This is the first direct molecular epidemiological evidence that humans and bamboo rats share identical strains of *P. marneffei* and that host-to-host transmission may occur. However, it is equally possible that, in this case, coinfection had occurred from a common environmental source, and this possibility needs to be investigated. To address these issues, ongoing studies in northern Thailand are using highly sensitive real-time PCR assays to screen rigorously sampled cohorts of (i) bamboo rat populations, (ii) environmental isolate samples, and (iii) human clinical populations. The application of highly discriminatory MLMT techniques to characterize these isolates will then provide a unique means to definitively identify the natural cycles of infection by *P. marneffei* in nature.

MOLECULAR GENETICS

Morphogenesis in *P. marneffei*

The generalized life cycle of *Penicillium marneffei* can be divided into three distinct phases: (i) a multicellular, filamentous (mold) vegetative form; (ii) an asexual reproductive stage (conidiogenesis); and (iii) a unicellular yeast-like/arthroconidial phenotype. Temperature and nutrition play a significant role in determining which particular phase is exhibited. Current evidence suggests that these two environmental conditions actually influence the expression, or the absence of expression, of particular genes that regulate cellular differentiation in *P. marneffei* (2, 30, 31).

Mold phase of *P. marneffei*. Laboratory cultures of *P. marneffei* incubated at 25°C to 30°C resemble all other species of

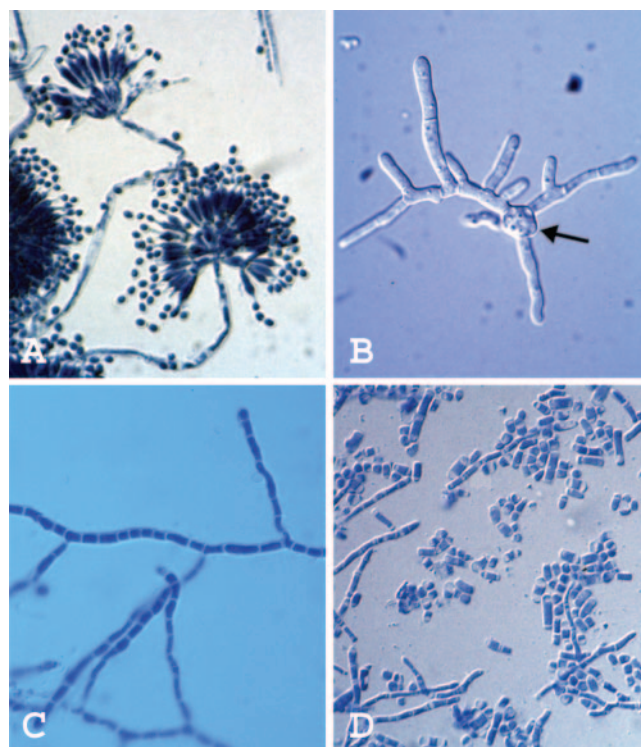


FIG. 3. Microscopic morphologies of *Penicillium marneffei* observed using differential interference contrast optics. (A) Conidiophores of *P. marneffei* bearing phialides and chains of conidia; (B) early stage of hyphal formation at 37°C, showing branched, septated filaments originating from a single conidium (arrow); (C) Intact hyphae on a solid medium that has undergone arthroconidiogenesis after incubation at 37°C; (D) arthroconidia and some remaining segmented hyphae in a liquid culture incubated at 37°C.

Penicillium in that growth occurs as a mold bearing the asexual reproductive structures (conidia) typical of the genus (Fig. 3A). In particular, young colonies grown on Sabouraud glucose agar are relatively thin and powdery to velvet in texture. Cultures may appear bluish gray-green initially, but as the *P. marneffei* colony matures, it becomes reddish yellow in color and produces a pink or red-rose pigment that readily diffuses into the surrounding medium (31). However, not all penicillia producing a red pigment are *P. marneffei*.

Microscopically, the conidiophores of *P. marneffei* (70 to 150 by 2.5 to 3.0 μm) usually possess bivaricated penicilli, but monoverticilliate structures may also be present (Fig. 3A). Each penicillus is composed of three to five metulae (7 to 11 μm long) bearing four to seven phialides (6 to 10 by 2.5 to 3.0 μm), ampulliform to acerose in shape. Attached to the phialides in short chains are single-celled, smooth-walled to echinulated conidia (3 to 4 μm in diameter). Conspicuous connectors between each conidium in the chain are readily observed. Spiral hyphae are occasionally present (39). This unusual structure of hyphae has also been observed in several isolates of *P. marneffei* which were isolated from HIV-AIDS patients (175).

Typically, the conidia are uninucleate, and when incubated at 25°C under the appropriate conditions, they swell isotropically for up to 6 to 12 h before undergoing polarized growth to produce a germ tube (2, 30, 31). Continued incubation at 25°C

results in the formation of true hyphae by apical extension of the germ tubes. The establishment of cross walls subapically produces individual cells. The subapical cells may undergo a repolarization to form branches of filaments that also grow by apical extension to form a mycelial network (Fig. 3B). Within this thallus, older cells tend to be uninucleate, whereas actively growing cellular compartments are usually multinucleate. The latter observation suggests that nuclear and cellular division become uncoupled during periods of rapid cellular growth.

Molecular studies have identified a gene intimately involved in germination (194). This gene, designated *gasC*, encodes a G α protein subunit belonging to the G-protein signaling pathway that regulates normal germination. Deletion of this gene results in a significant delay of the germination process. However, *gasC* apparently has no role in vegetative growth, yeast development, or the dimorphic switch. Curiously, it does seem to be involved in a secondary metabolic pathway related to the red pigment production by *P. marneffeii*.

Other studies have identified a transcription factor encoded by the gene *tupA* (160). This gene represses yeast development at 25°C while maintaining hyphal growth. However, *tupA* is not responsible for the initiation of hyphal formation. Instead, it appears to be required for the correct polarity of growth by yeast cells (see below). Interestingly, the *P. marneffeii* gene *stlA* also has no apparent role in the initiation of vegetative hyphal development, although it is a homolog of the *STE12* gene in *Saccharomyces cerevisiae* and *Candida albicans*, which regulates the yeast-hyphal transition (8).

Hyphal development is also affected by the normal deposition of actin. The *P. marneffeii* gene *cfIB*, which encodes a RAC homolog of a family of GTPase proteins, plays a crucial role in activity (13). The absence of this gene affects polarized growth and cell division in both hyphal development and conidiogenesis.

Conidiogenesis in *P. marneffeii*. The formation of conidia by *P. marneffeii* results from a well-programmed series of cellular changes that produce the complex, multicellular conidiophores described above. At 25°C, conidiogenesis begins following the establishment of aerial hyphae on a solid medium. Liquid shake cultures of *P. marneffeii* do not usually permit the formation of conidia (2, 30, 31). Hence, an air interface appears to be essential for conidiogenesis.

Conidiophore development begins as multicellular stalk cells arise from specialized “foot cells” of the hyphae lying at the culture/air interface. Secondary stalks, termed rama, may be formed by septation of the primary stalk cells. Eventually, the tips of the stalk cells differentiate into the uninucleate cells comprising the sterigmata (metulae and phialides). The latter divide by budding rather than apical extension and septation. The metulae are formed first and then gives rise to the various phialides. In turn, the phialides generate uninucleate conidia in a similar budding fashion. Interestingly, the conidia are produced in a basipetal mode; i.e., older conidia are displaced by newer ones. Hence, the first conidium in a chain represents the oldest cell, whereas the youngest is closest to the phialide from which it was born. Such cellular production is opposite of the acropetal mode of development exhibited by the remaining cells of the fungal thallus; i.e., the younger cell is the first one in the hypha or conidiophore stalk.

The molecular signals that induce conidiogenesis are regulated by a transcription factor encoded by the *abaA* gene (7).

This gene is expressed only during conidiogenesis, and its transcript is not found during normal vegetative hyphal development. Deletion of *abaA* results in defective phialides and the absence of conidia. Other evidence suggests that this transcription factor also helps control cell cycle events during conidiogenesis.

Other genes involved in the formation of conidia include the transcription factor gene *stuA* and the *gasA* gene, encoding the G α protein subunit (9, 193). Like *abaA*, *stuA* is expressed only during conidiogenesis. Loss of *stuA* results in the absence of metulae and phialides. Curiously, conidia are still formed, but they are located at the tips of conidiophores. *gasA* plays a role in the normal timing of conidiogenesis and appears to be involved in the molecular decision between vegetative growth or formation of conidia. Interestingly, like *gasC*, this gene appears to also have a role in the secondary metabolic pathway leading to red pigment synthesis by the hyphal form of *P. marneffeii*.

Dimorphism in *P. marneffeii*: arthroconidiogenesis and the yeast phase. Among the penicillia, few species exhibit a sexual means of reproduction. Most species of *Penicillium* grow only as molds, reproducing solely via the asexual production of conidia. None are known to be dimorphic except *P. marneffeii* (29, 30). The means by which *P. marneffeii* undergoes dimorphism, a process termed phase transition, has been well studied at the morphological level and has triggered great interest in the molecular mechanisms governing this unique type of cellular differentiation.

Dimorphism in *P. marneffeii* is thermally controlled. At 37°C, germinated conidia produce hyphae that are generally shorter in length and wider in diameter than those produced at 25°C (2, 30, 31). Moreover, the 37°C-derived hyphae are much more highly branched than their 25°C-derived counterparts. Within 48 h upon a suitable growth substrate, each compartment within a hyphal strand produced at 37°C becomes uninucleate as cell and nuclear division are coupled. These individual cells, containing a single nucleus, are divided from each other by a double-layered septum. These cells, termed prearthroconidia (Fig. 3C), are shorter (approximately 15 to 20 μ m) along their axis than vegetative hyphal cells formed at 25°C. Left undisturbed, the prearthroconidia tend to remain attached to one another, yet even slight disturbances cause the prearthroconidial cells to break apart from one another along the midline of the double septum separating them. By definition, these cells are now termed arthroconidia, and they function as independent asexual propagules. In liquid cultures incubated at 37°C, the action of aeration by shaking leads to a population consisting mostly of single, unattached arthroconidia (Fig. 3D).

Arthroconidial formation, however, is merely a transitory stage to production of a yeast phase. Continued incubation of arthroconidia at 37°C initiates polarized growth and subsequent elongation of the cell shape. Eventually, following nuclear division, the cell divides by fission to form two uninucleate cells, much in the same way that cell division occurs in *Schizosaccharomyces pombe*. These newly formed cells can then again propagate by fission. Ultimately, the culture will consist entirely of yeast cells. In a liquid culture, the cells appear elliptical and generally detached from one another. On many rich solid media, a smooth cream-to-beige colony that

consists almost entirely of yeast cells is formed. Notably, cultures of *P. marneffei* grown at 37°C do not produce the diffusible red pigment that is so readily secreted by the mold phase.

A number of genetic studies have focused upon genes involved in the dimorphic switch in *P. marneffei*. Several of those previously mentioned, i.e., *stuA*, *stlA*, *gasA*, *gasC*, and *cflB*, have no role in yeast cell development or the dimorphic switch. However, others do have some role to play in the regulation of dimorphism or yeast growth. For example, the *abaA* gene appears to control the coupling of nuclear and cellular division in prearthroconidial cells (7). However, once arthroconidia begin to develop as yeasts, a second series of genes appears to take over the coupling of cell cycle events.

With regard to yeast growth, two genes seem to be intimately involved in cell polarity. One gene in *P. marneffei*, designated *cflA*, is a homolog of *CDC42* of *S. cerevisiae* (12). *CDC42* encodes a Rho-related GTPase that functions in cell signaling and polarity. In *P. marneffei*, *cflA* controls the correct morphogenesis of yeast cells. Mutants with mutations in the *cflA* gene form aberrant yeast cells, although they are still capable of switching to filamentous growth when the incubation temperature is shifted to 25°C. However, the hyphae formed by these mutants exhibit defects in the hyphal tips where apical growth occurs. Curiously, the *cflA* mutations have no effect upon conidiogenesis. Similarly, *tupA* regulates the correct polarity in yeast cells that form at 37°C (160).

Two additional points regarding dimorphism in *P. marneffei* are significant to note. First, arthroconidial development and subsequent yeast cell formation can also occur by shifting mold cultures from 25°C to 37°C. Initially, nuclear and cellular division must become coupled in the apical cells of the hyphal filaments. Once this event is established, then arthroconidiogenesis occurs, followed by yeast cell formation. Conversely, yeast cells and arthroconidia formed at 37°C will cease their unique mode of development when shifted to 25°C and initiate apical growth with concomitant uncoupling of nuclear and cellular division typical of the mold phase. These observations clearly indicate that phase transition in *P. marneffei* is a readily controllable and reversible process that is regulated solely by temperature. While nutritional factors may influence the extent to which phase transition may proceed, the event of the dimorphic switch rests entirely upon a temperature-induced signal that presently remains unknown.

A final important observation regarding the dimorphic nature of *P. marneffei* pertains to its *in vivo* state. Conidia are regarded as the infectious propagule of *P. marneffei*. Upon inhalation, conidia are believed to be phagocytized by alveolar macrophages, where they reside as intracellular parasites growing in the yeast phase. From there, the fungus can spread to other parts of the body. Interestingly, the yeast phase appears to develop directly from the phagocytized conidia without first forming hyphae, as is typical of conidia incubated at 37°C *in vitro*. Hyphae have never been reported in patients infected with *P. marneffei*. Until now, no studies have reported data on the yeast-hyphal transition in animal models. It is therefore not known whether mycelia occur in the initial stages of infection. This is in contrast to the case for non-*P. marneffei* species, which grow filamentously *in vivo* on those rare occasions when they cause disease. Why such a distinct type of phase transition occurs remains unclear and will require further investigation.

The focus of such investigations must be twofold and include the response of the invading fungus to the host as well as that of the host to the engulfed fungus.

GENOMICS OF *P. marneffei*

To understand the fundamental cellular machinery that underpins thermal dimorphism, disease pathogenesis, and the interaction between the fungus and the immune system, a comprehensive knowledge of the *P. marneffei* genome is required. The field of human fungal pathogen genomics is currently progressing at great speed; complete genome sequences have currently been completed for *Candida albicans*, *Candida glabrata*, *Aspergillus fumigatus*, and *Coccidioides immitis*, and four genomes are currently available for *Cryptococcus neoformans*. However, there is currently no publicly available genome sequence for *P. marneffei*.

Pulsed-field gel electrophoresis of genomic DNA of *P. marneffei* has revealed three chromosomes (5.0, 4.0, and 2.2 Mb), although this is likely an underestimate of the true number (149), as genomes in the genus *Aspergillus* tend to have eight or more chromosomes (<http://www.broad.mit.edu>). Prospective genome sequencing of *P. marneffei* has been performed by Yuen and collaborators (191). The genome size of *P. marneffei* has been estimated to be in the range of 17.8 to 26.2 Mb (191), compared to an estimated 22 to 35 Mb in *H. capsulatum* (170) and 31 Mb in *Aspergillus nidulans* (<http://www.broad.mit.edu>). Sequencing a shotgun library of the *P. marneffei* genome yielded 2,303 random sequence tags (RSTs), corresponding to a 9% genome coverage, with 11.7, 6.3, and 17.4% of the RSTs having sequence similarity to yeast-specific sequences, nonyeast fungus sequences, and both (common sequences), respectively (191). 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 multidrug resistance protein genes and homologs of fluconazole resistance genes. In addition, the RSTs uncovered the presence of genes encoding pheromone homologs 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.

From this *P. marneffei* sequencing project, a contig that contains the complete sequence (35 kb) of the mitochondrial genome was assembled (191). The gene contents, gene orders, and gene sequences were analyzed and compared to those of yeasts and molds (187). Interestingly, the results of comparative analyses revealed that the mitochondrial genome of *P. marneffei* is more closely related to those of molds, especially to that of *Aspergillus nidulans*, than to those of yeasts.

Many investigators have focused on phase-specific genes in the pathogenic dimorphic fungi, for example, in *H. capsulatum*. Several genes that are expressed predominantly in the yeast phase of *H. capsulatum* have been identified: *yps-3* (83), *cdc2* (38), and *CBP1* (117). Some genes specific to the mold phase have been identified: alpha- and beta-tubulin genes (58), *MS8* (159), and *CATA* (74). In addition, several genes that were

highly expressed in the mold or yeast phase of *H. capsulatum* have been identified using a genomic shotgun microarray (68). In *P. marneffeii*, the phase-specific genes have been studied by utilizing differential display techniques (30). This work has shown that many of the genes whose expression is upregulated during the mold-to-yeast transition are related to those genes involved in energy metabolism. The malate synthase-encoding gene was identified as being upregulated in the yeast phase (30). Recently, an antigenic catalase-peroxidase protein-encoding gene (*cpeA*) was isolated by antibody screening of a cDNA library (123). Interestingly, high-level expression of this gene was induced when the temperature was shifted to 37°C, whereby the pathogenic yeast phase of *P. marneffeii* is formed. The role of *cpeA* in the pathogenesis of *P. marneffeii* will require further investigations.

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