



**The Clinical and Environmental Epidemiology
of *Penicillium marneffe*i Infection in Vietnam**

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ABSTRACT

Infection due to *Penicillium marneffe* (renamed to *Talaromyces marneffe* in 2011) has emerged as an important public health problem over the past two decades due to the arrival of the HIV epidemic in Asia. Since 2004, *P. marneffe* has become the second most common pathogen isolated from routine blood culture, after *Cryptococcus neoformans*, at the Hospital for Tropical Diseases in Ho Chi Minh City, the largest referral centre for HIV care in southern Vietnam. The clinical epidemiology of *P. marneffe* infection has not been studied in Vietnam. The fundamental epidemiological questions regarding the pathogen reservoirs and risks of acquisition remain poorly understood. The diagnosis relies on isolation of the pathogen from clinical specimens and can take up to 14 days to identify, resulting in delayed initiation of therapy which is associated with worse treatment outcomes. This thesis aims to increase knowledge and understanding of the clinical and environmental epidemiology of *P. marneffe* infection and to improve the speed and accuracy of diagnosis of *P. marneffe* infection.

The Précis provides a brief background and rationale for the thesis.

Chapter 1 is an introductory chapter and provides an overview of the epidemiology, ecology, mycology, pathology, immunology, clinical features, diagnosis, and treatment of *P. marneffe* infection.

Chapter 2 summarizes the incidence and features of *P. marneffe* admissions at the Hospital for Tropical Diseases in Ho Chi Minh City over a 13 year period. During this period, 795 patients with *P. marneffe* infection were identified and hospital charts were obtainable for 513 (65%) patients. The data showed clear seasonality with an increase in incidence of approximately 30% during the rainy season compared to the dry season. The clinical and microbiological features and treatment outcomes of the patients were characterised. Poor outcome, defined as death or worsening disease at hospital discharge, occurred in 28% of patients. History of injection drug use, shorter duration of illness, absence of fever or skin lesions, higher respiratory rates, and lower platelet counts independently predicted poor outcome.

Chapter 3 describes an analysis of meteorological factors that determine penicilliosis incidence in Ho Chi Minh City. Humidity, rather than precipitation, was the most important factor that governs the seasonality of penicilliosis. Higher humidity was associated with increased odds of penicilliosis versus cryptococcosis admissions. The infection incubation period was estimated to be between one and three weeks.

Chapter 4 describes an analysis of exposure and behavioural risk factors for penicilliosis based on a matched case control study of 205 culture-confirmed HIV-infected penicilliosis cases and 405 HIV-infected controls recruited from two major HIV referral centres in Hanoi and Ho Chi Minh City. Penicilliosis was independently associated with proximity or exposure to tropical plants and

exposure to farmed animals. The geographical analysis showed that patients living in or traveling to the highland regions were at increased risk for penicilliosis in southern Vietnam.

Chapter 5 describes the development of a Taqman real-time PCR assay based on a novel *Mp1* gene target unique to *P. marneffeii* for rapid detection of *P. marneffeii* infection in patient plasma. The assay was tested in 70 plasma samples from HIV-infected patients (50 with culture-confirmed penicilliosis, 20 with other opportunistic infections) and showed a clinical specificity of 100% (20/20) and sensitivity of 70.4% (19/27) and 52.2% (12/23) prior to and within 24-48 hours of antifungal therapy administration, respectively.

Chapter 6 is an overview discussion interpreting the implications of the major findings and the future direction of *P. marneffeii* research.

The work of this thesis increases knowledge of the clinical epidemiology of *P. marneffeii* infection in Vietnam, providing essential data for the design of prospective studies to improve the diagnosis and treatment of *P. marneffeii* infection in Asia. The data suggest that multiple environmental factors including humidity, tropical plants, farmed animals, and highland location, are important drivers of *P. marneffeii* infection in southern Vietnam. The real-time PCR assay showed potential as a rapid 'rule-in' test for *P. marneffeii* in this pilot study and should be prospectively evaluated in a large cohort to determine if it can improve diagnostic speed and crucially, impact patient outcomes.

Prevention, diagnosis and elimination all require further research to reduce the high mortality following clinical disease caused by *P. marneffe* in Asia.

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For all studies described in this thesis, I am responsible for the design, obtaining funding, protocol writing, obtaining ethical approvals, collecting all clinical and microbiology data, enrolling patients (with assistance), performing laboratory work (with assistance), data management, statistical analyses (with assistance) and writing of the thesis.

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ABBREVIATIONS

AIDS	Acquired Immune Deficiency Syndrome
ALT	Alanine aminotransferase
ART	Antiretroviral therapy
ARV	Antiretrovirals
AST	Aspartate aminotransferase
BAL	Bronchial alveolar lavage
CI	Confidence interval
CNS	Central nervous system
Cp	Crossing point
CRF	Case Record Forms
CSF	Cerebrospinal Fluid
DNA	Deoxyribonucleic Acid
ELISA	Enzyme-Linked Immunosorbent Assay
FAM	6-Carboxyfluorescein
FSW	Female Sex Worker
GMS	Gomori-Grocott Methenamine
GFATM	Global Fund to fight AIDS, Tuberculosis and Malaria
HCMC	Ho Chi Minh City
HIV	Human Immunodeficiency Virus
HTD	Hospital for Tropical Diseases
ICD	International Classification of Disease
IDU	Injection drug use
IFAT	Indirect Fluorescent Antibody Test
IQR	Interquartile range
IRIS	Immune reconstitution inflammatory syndrome
IVDU	Intravenous drug user
LB	Luria-Bertani

MICs	Minimum inhibitory concentrations
MoH	Ministry of Health
MSM	Men who have sex with men
OIs	Opportunistic Infections
OR	Odds ratio
PEPFAR	President's Emergency Plan for AIDS Relief
PCR	Polymerase chain reaction
PJP	<i>Pneumocystis jiroveci</i> pneumonia
PMTCT	Prevention of Mother to Child Transmission
QGIS	Quantum Geographic Information System
SDA	Sabouraud dextrose agar
spp.	species
VAAC	Vietnam Administration for HIV/AIDS Control
VCT	Volunteer Counselling and Testing
WHO	World Health Organisation

LIST OF PUBLICATIONS

Original publications contributing to D.Phil. thesis

1. **Le T***, Hong Chau TT, Kim Cuc NT, Si Lam P, Manh Sieu TP, Shikuma CM, Day JN. AIDS-associated *Cryptococcus neoformans* and *Penicillium marneffe*i coinfection: a therapeutic dilemma in resource-limited settings. Clin Infect Dis 2010 Nov 1;51(9):e65-e68.
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2. Bulterys PL, **Le T**, Vo Minh Quang, Nelson KE, Lloyd-Smith JO. Determinants of Penicilliosis Seasonality in Ho Chi Minh City, Vietnam. 19th International AIDS Conference. 22-27 July 2012. Washington DC, USA. Oral presentation.
3. Hien HTA, Thanh TT, Thu NTM, Cuc NTK, Lan NPP, Hoa NT, Shikuma CM, Farrar J, Day J, **Le T**. Development of a Taqman Real-Time PCR Assay for Rapid Detection of *Penicillium marneffe* Infection. 19th Conference of Retroviruses and Opportunistic Infections. March 3-6, 2013. Atlanta, GA, USA.

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Invited lectures/presentations

1. *Penicillium marneffe*i case presentation, 13rd Bangkok International Symposium on HIV Medicine, Bangkok, Thailand, Jan 2010. Invited symposium speaker
2. Update on the epidemiology, diagnosis and treatment of penicilliosis, Academic Meeting, Hospital for Tropical Diseases, Ho Chi Minh City, Viet Nam, June 2013
3. Epidemiology, diagnosis and treatment of the Southeast Asian Tropical Fungus – *Penicillium marneffe*i. Harvard Medical School AIDS Initiative in Vietnam Continuing Medical Education Series, Ho Chi Minh City, Sept 2013. Invited speaker.
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PRÉCIS

Penicillium marneffe (renamed to *Talaromyces marneffe* in 2011) is the only fungus amongst a recently-accepted list of 354 saprophytic *Penicillium* species to exhibit temperature dependent dimorphism [1], and is capable of causing disease in both immunocompetent and immunocompromised individuals. In contrast to the worldwide and ubiquitous distribution of other *Penicillium* species, *P. marneffe* is endemic only in Southeast Asia, southern China, and northeastern India [2]. Infection due to *P. marneffe* has emerged as an important public health problem over the past two decades due to the arrival of the Human Immunodeficiency Virus (HIV) epidemic in Asia, where *P. marneffe* infection is an indicator of HIV infection [3,4] and ranks amongst the top three most common Acquired Immune Deficiency Syndrome (AIDS) defining illnesses in Southeast Asia and southern China [5,6]. Increasing numbers of cases are being diagnosed in the changing HIV landscape in Southeast Asia, mainland China, and India [7–9], and in immunocompromised individuals coming from Southeast Asian countries or those with remote or recent travel history to this area [10,11]. Since 2004 *P. marneffe* has emerged as the second most common pathogen (after *Cryptococcus neoformans*) isolated from routine blood culture at the Hospital for Tropical Diseases, the largest referral centre for HIV care in southern Vietnam. Despite the rapid emergence and importance of *P. marneffe* infection in clinical care in Vietnam, knowledge of the disease and its management have been based on clinician experience, small case series [7,12], and on studies conducted elsewhere in the region. There has not been a large case series that

evaluates the clinical epidemiology of *P. marneffe*i infection in relation to the HIV/AIDS epidemic and the antiretroviral therapy era in Asia. The impact of antiretroviral therapy on disease incidence and natural history has not been studied.

*P. marneffe*i causes a systemic mycosis called penicilliosis in immunocompromised patients. HIV infected persons are at greatly increased risk of developing penicilliosis when their CD4 counts are <100 cells/ μ L [3,5,7]. They typically present with disseminated disease with fever, weight loss, anemia, thrombocytopenia, hepatosplenomegaly, lymphadenopathy, and skin lesions. The infection is fatal if not treated, and mortality rates in patients treated with antifungal therapy are up to 20% [5,7,13,14]. The factors that govern penicilliosis severity and mortality have not been systematically studied but could provide insights to guide disease management and to improve treatment outcomes. The current diagnosis is based on conventional microscopic and cultural techniques, requiring isolation of the organism from clinical specimens and demonstration of temperature-dependent dimorphic growth which can take up to 14 days to identify [15]. Diagnostic delay, which delays antifungal therapy initiation, leads to significantly higher mortality in patients [3,5,8,15]. Several novel immunoassays and molecular assays have been developed and have the potential to accelerate diagnosis and allow antifungal therapy to be initiated earlier [16–21]; however none has been tested in clinical studies to evaluate their usefulness in detection of active disease in patients. There is a clear need for more rapid diagnostic tools to prevent early mortality in penicilliosis.

Critical features regarding the epidemiology including knowledge of pathogen reservoirs and modes of disease acquisition are poorly understood for *P. marneffe*, unlike the other phylogenetically-related dimorphic fungi that cause diseases in human, i.e. *Blastomyces dermatitidis*, *Histoplasma capsulatum*, *Coccidioides immitis*, *Paracoccidioides brasiliensis*, and *Sporothrix schenckii*. The bamboo rats are thus far the only known non-human vertebrate host of *P. marneffe*. The fungus has been isolated from the blood and internal organs of all 4 species of bamboo rats endemic in Asia with prevalence as high as 100% in some sampled populations [22–27]. The distribution of bamboo rats is similar to the distribution of human disease in the region [2,22,23], and it has been shown that isolates from bamboo rats have similar or identical genotypes to isolates from humans [23,25,28]. However evidence for zoonotic transmission is lacking. In a study in northern Thailand [29], exposure or consumption of bamboo rats was not associated with penicilliosis; whereas exposure to plants and animals, particularly during the rainy season, was associated with disease, suggesting a common reservoir in soil or soil related organic matter. In addition, penicilliosis incidence doubles during the rainy season compared to the dry season in northern Thailand [30], suggesting environmental disease reservoirs. However, despite considerable field sampling over the past two decades, the reservoirs for *P. marneffe* remain elusive. The major questions are: 1) Do humans develop infection from a single or from multiple sources of soil, plants, water, and invertebrates? 2) What is the geographical range of human penicilliosis? and 3) What is the role of bamboo rats, if any, in the maintenance, distribution, and

transmission of *P. marneffe* from environmental reservoirs to the human host? The answers to these questions might guide disease prevention strategies. Thus far most of the epidemiological studies that contribute to our understanding of the natural history and the environmental risk factors of penicilliosis have been exclusively conducted in northern Thailand [5,29,30], a region that bears the greatest case load to date. However most penicilliosis patients (75%) in northern Thailand live in rural settings [30], and more than 50% of patients have seen bamboo rats in the immediate proximity of their homes [29]. Penicilliosis is diagnosed in major urban settings across broad climate and geographical ranges from northern to southern Vietnam. The exposure and environmental risk factors might be very different in urban versus rural locations, highlighting the need to investigate these epidemiological questions in more diverse settings.

The main objectives of this DPhil thesis are to increase knowledge of the clinical epidemiology of *P. marneffe* in a changing HIV landscape in Vietnam and to investigate the environmental, behavioural, and geographic risk factors for penicilliosis in the diverse geographical regions of Vietnam. In addition, a novel real-time PCR assay is developed for the rapid diagnosis of *P. marneffe* infection. These objectives are organised into 4 data chapters in this thesis and are outlined in the following specific aims:

1. Aim 1: To describe the epidemiology, clinical and laboratory features, antifungal treatment options, and treatment outcomes of penicilliosis in a large retrospective case series of patients diagnosed over a 13 year

period at the largest referral centre for HIV care in southern Vietnam. To explore the association of clinical and laboratory variables present on admission with clinical outcomes and to assess the seasonality of disease incidence

2. Aim 2: To investigate the meteorological factors that determine the seasonality of penicilliosis in Ho Chi Minh City and to estimate a conditional incubation period of acute disease by modeling
3. Aim 3: To investigate the exposure, behaviour, and geographical risk factors for penicilliosis in HIV-infected patients in northern and southern Vietnam using a matched case control study design
4. Aim 4: To develop a Taqman real-time PCR assay for rapid detection of *P. marneffei* and to determine its accuracy in patients' plasma.

CHAPTER 1

Introduction

1.1 Epidemiology of *Penicillium marneffe* infection

1.1.1 Discovery of *Penicillium marneffe*

Penicillium marneffe was first discovered in captured bamboo rats (*Rhizomys sinensis*) used for an experimental infection model of *Rickettsia tsutsugamushi* at the Pasteur Institute in Da Lat, Vietnam in 1956 [31]. The organism was first described by Gabriel Segretain, a French mycologist working at the Pasteur Institute in Paris. He named the organism after Hubert Marneffe, the Director of the Pasteur Institute in Indochina at that time [32]. Segretain also described the first laboratory-acquired human infection when he accidentally pricked himself in the finger while transferring a needle containing *P. marneffe* yeast cells that were used to inoculate hamster in the laboratory. He developed a nodule at the site of infection followed by lymphangitis nine days after the incident [2,32]. The infection was reported to resolve after a 30 day course of oral nystatin, a polyene macrolide antibiotic. However, nystatin is now known to have poor oral bioavailability and likely poor activity against this fungus, and was therefore unlikely to have been responsible for the resolution of Segretain's infection.

1.1.2 The natural history of *Penicillium marneffe* infection

The first naturally occurring infection was reported in South Carolina in 1973, 17 years after the discovery of *P. marneffe*, in an American missionary with Hodgkin's lymphoma who had lived in Southeast Asia some years prior to the illness [33]. A splenectomy as part of the treatment for Hodgkin's disease revealed nodules in the spleen, from which *P. marneffe* was isolated. The

second case was reported in 1984 in an American who had traveled to Southeast Asia [34]. The patient had recurrent hemoptysis and underwent pneumonectomy. The pathology showed granuloma with yeast cells seen in tissue sections, which was confirmed by culture. From 1984 to 1988, cases began to appear in Asia, starting with 5 cases in Bangkok [35], 8 cases from China [36], followed by 20 cases in China and cases from Hong Kong [2,13]. *P. marneffei* infection was first diagnosed in patients with advanced HIV infection in 1989 and has since been increasingly diagnosed in Thailand, Cambodia, Malaysia, Vietnam, Hong Kong, Taiwan, China, and India, [2,15]. The infection became an indication of HIV infection and an AIDS-defining illness [3,4]. The greatest case burden documented to date has been in Chiang Mai province in northern Thailand, where a correlation between the temporal emergence of HIV and penicilliosis has been documented (Figure 1.1). Between 1984 to 2004, 6,709 cases of penicilliosis were diagnosed in Thailand [2]. Penicilliosis accounted for 20% of AIDS admissions, preceded only by tuberculosis and cryptococcosis in Chiang Mai [5]. In Hong Kong, penicilliosis accounted for 10% of AIDS admissions, preceded only by *Pneumocystis jiroveci* pneumonia (PJP) and tuberculosis [37]. Increasingly immunocompromised persons from outside of the endemic region, e.g. Belgium, France, Germany, Netherlands, Sweden, Switzerland, the United Kingdom, Australia, Japan, and the United States, are diagnosed with penicilliosis after traveling to Southeast Asia. The illness has been reported both in patients with recent or very remote travel history to the region [2,10,11,38].

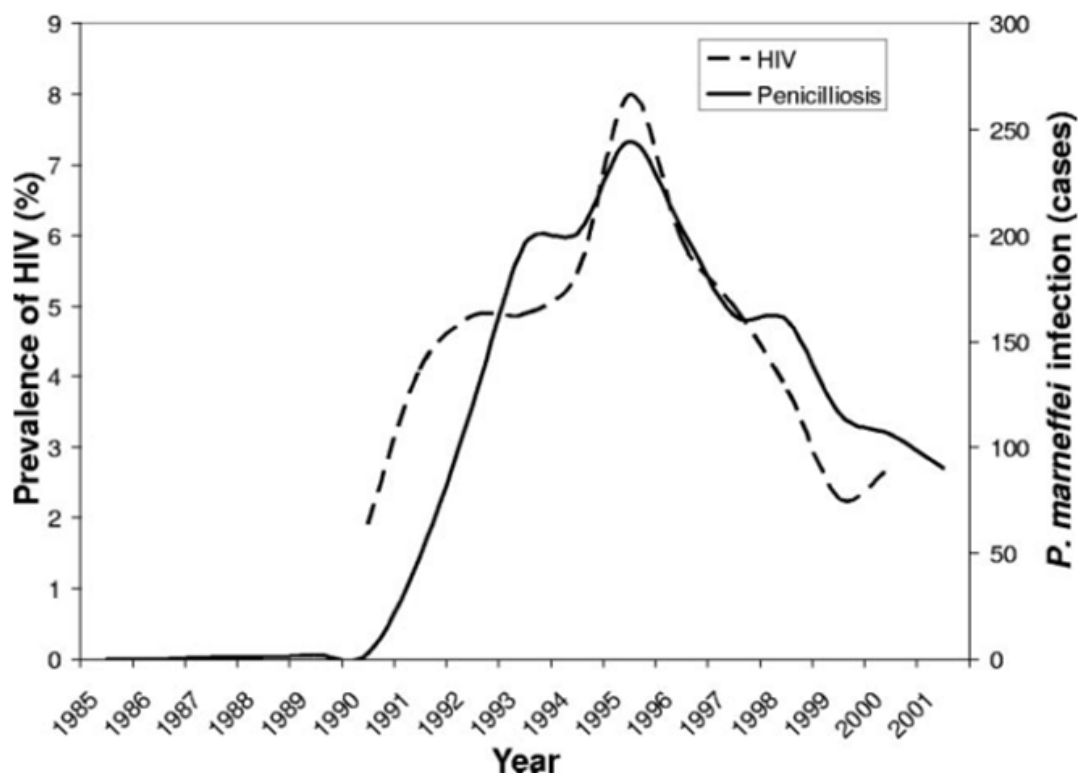


Figure 1.1 The temporal emergence of HIV and *Penicillium marneffeii* infections at Maharaj hospital in Chiang Mai, Thailand from 1985 to 2001

Source: Vanittanakom *et al.* Cllinical Microbiol. Rev. 2006; 19:95–110

1.1.3 *Penicillium marneffeii* infection and its relationship with the HIV epidemic in Vietnam

The first patient diagnosed with *P. marneffeii* infection was a 29 year old man who presented to the Hospital for Tropical Diseases (HTD) in Ho Chi Minh City (HCMC) with weight loss, oropharyngeal candidiasis, cervical lymphadenopathy, a maculopapular rash on his penis, and a hematocrit level of 12% in July 1996.

The second day he developed a generalised maculopapular rash with central necrotic umbilication. The family refused treatment and decided to take him home. He died 10 hours after leaving the hospital. His blood test was subsequently found to be HIV positive, and his blood culture eventually grew *P. marneffe*. Three more cases were diagnosed in the years following in 1997 and 1998 [12]. From May to September 2001, 12 of 273 (4.3%) HIV admissions were diagnosed with *P. marneffe* infection at the HTD [39]. This was followed by a case series of 94 of 874 (10.8%) HIV admissions at the Czech-Vietnam hospital in northern Vietnam, over a three year period from 2005 to 2008 [7]. A study investigated trends in bloodstream infections at the HTD from 1994 to 2008 showed that *Salmonella* Typhi was the predominant pathogen until 2002, when a significant shift occurred with *Cryptococcus neoformans* and *P. marneffe* becoming the predominant pathogens and increasing proportions of non-typhoidal *Salmonella* (Figure 1.2) [40]. In 2005 *C. neoformans* and *P. marneffe* became the two most commonly isolated bloodstream pathogens, in 113/425 (26.6%) and in 70/425 (16.5%) of all positive blood cultures, respectively. This dramatic shift correlated with an increase in HIV admissions over the same period. Figure 1.3 shows the temporal trend and relationship between proportions of HIV admissions, HIV related death, and the HIV-associated bloodstream pathogens *C. neoformans*, *P. marneffe*, and non-typhoidal *Salmonella* at the HTD over the 14 year period. HIV-associated fungal pathogens have now emerged as the leading cause of bloodstream infections and leading cause of mortality in hospitalised patients in Vietnam.

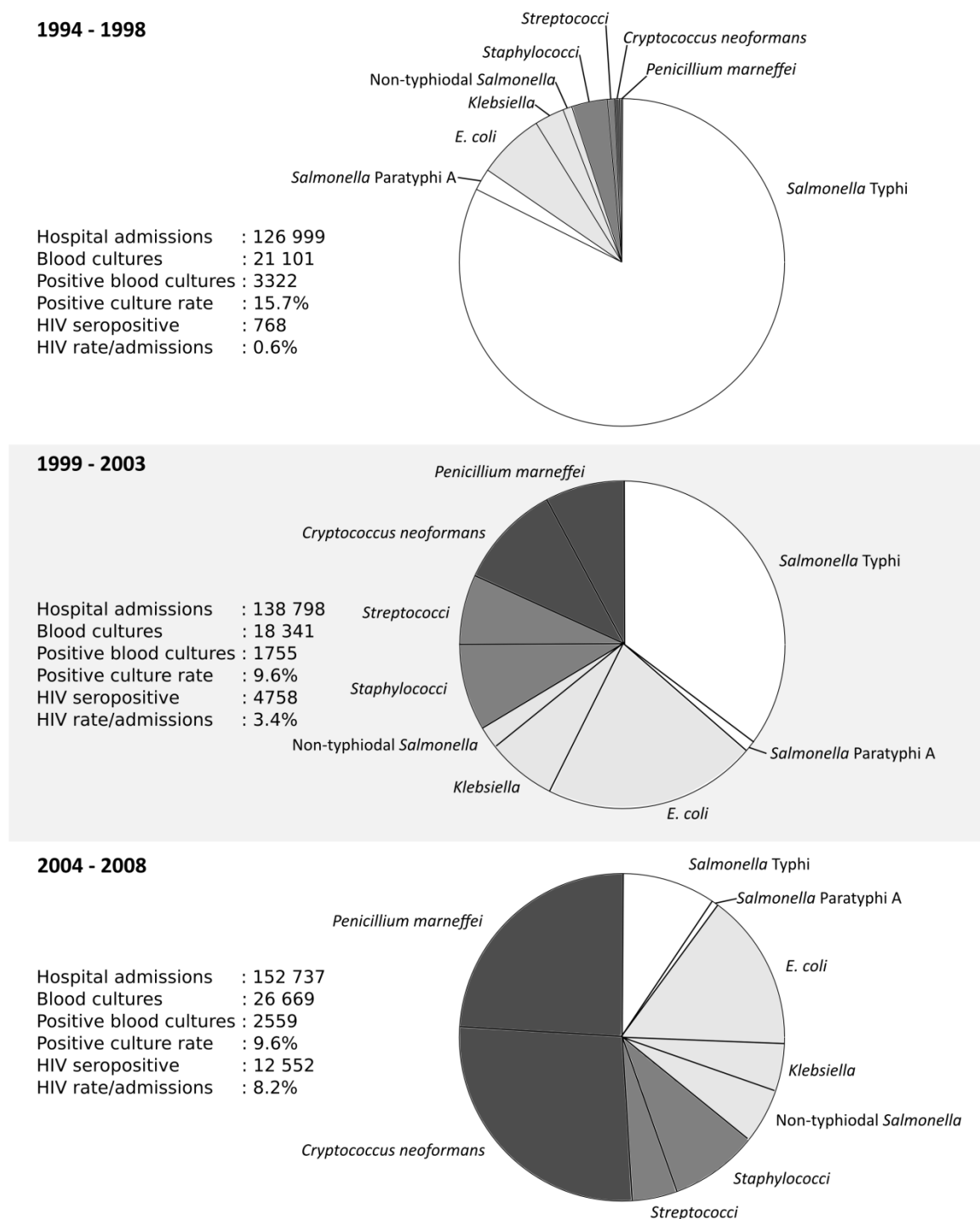


Figure 1.2: The distribution of blood stream pathogens over three successive five-year periods at the Hospital for Tropical Diseases in Ho Chi Minh City.

Pie charts showing combined data from 1004-1998, 1999-2003, and 2004-2008.

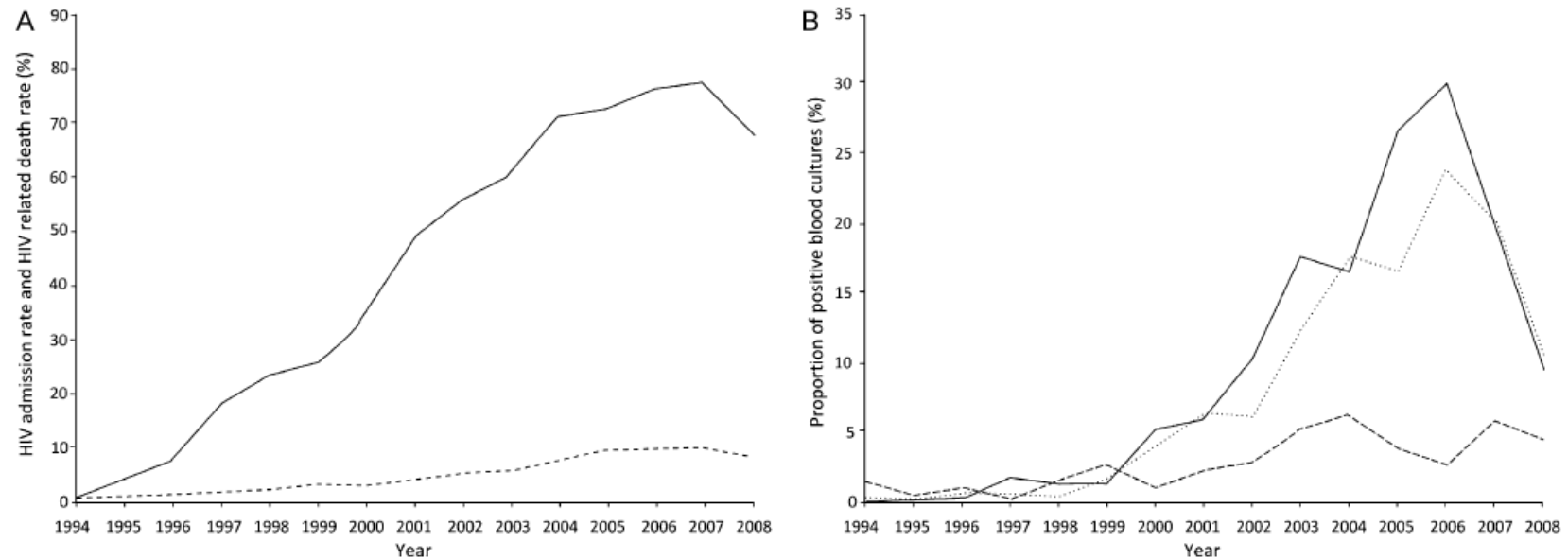


Figure 1.3: HIV prevalence and HIV related blood stream infections at the Hospital for Tropical Diseases in Ho Chi Minh City from 1994 to 2008

A: Graph showing the annual proportions of HIV admissions (broken line) and proportions of HIV-related death (solid line)

B: Graph demonstrating the proportion of positive blood culture from which *Cryptococcus neoformans* (solid line), *Penicillium marneffei* (dotted line), or Non-Typhoidal salmonella (broken line) was isolated

1.1.4 Brief overview of the HIV epidemic in Vietnam

The first case of HIV infection in Vietnam was diagnosed at the HTD in 1990. Since then the estimated number of people living with HIV has more than doubled from 120,000 to 260,000 between 2000 and 2005 [41]. The epidemic remains concentrated in at-risk populations of injection drug users (IDU), of female sex workers (FSW), and men who have sex with men (MSM). According to 2011 sentinel surveillance, HIV prevalence amongst IDU and FSW were 13.4% and 3%, respectively [42]. There is a rising HIV epidemic in MSM with prevalence as high as 16.3% in HCMC in 2011 [42]. HIV cases had been reported in all 63 provinces in the country. The number of HIV cases reported to the Ministry of Health held steady at 14,000 reports per year in 2010 and 2011. There has been a rise in reported cases of HIV-positive women who now represent 31% of newly reported cases, reflecting a steady transmission of HIV to women by men engaging in high-risk behaviours. The estimated number of people living with HIV in 2012 is 263,317 with the estimated prevalence in the general population (ages 15-49) of 0.45% [42].

Vietnam has made major advances in the response to HIV since 2004 launch of the National Strategy on HIV/AIDS Prevention and Control in Vietnam and the establishment of Vietnam Administration for HIV/AIDS Control (VAAC) under the Ministry of Health (MoH). There has been a significant increase in support from international donors, from US\$ 13 million in 2005 to US\$ 47.15 million in 2006. The major donors are the President's Emergency Plan for AIDS Relief

(PEPFAR), the Global Fund to fight AIDS, Tuberculosis and Malaria (GFATM), and the World Bank. Several HIV prevention programmes have been scaled up, including prevention of mother to child transmission (PMTCT), volunteer counselling and testing (VCT), and harm reduction interventions such as needle/syringe provision and methadone substitution. The latter are still in their infancy. The national antiretroviral therapy (ART) began in 2005. By 2014, close to 90,000 people living with HIV were on ART. However this is estimated to cover only 30 to 40% of those who meet the current criteria for treatment, i.e. CD4 count <350 cells/ μ L and/or HIV disease stage III or IV [43]. In a survey conducted in 2013 of close to 8,000 patients initiating ART in 30 randomly selected ART clinics amongst a total of 120 ART clinics in the country, the median CD4 count prior to ART initiation was 78 cells/ μ L, highlighting the need for increase screening for HIV infection in Vietnam. HIV-associated opportunistic infections will remain a major public health problem faced by Vietnam in the next decade.

1.2 Ecology

Despite two decades of field research efforts, the natural reservoirs and route of human acquisition of *P. marneffei* are poorly defined whereas they are somewhat more established for the other phylogenetically-related dimorphic fungi that cause diseases in human, i.e. *Blastomyces dermatitidis*, *Histoplasma capsulatum*, *Coccidioides immitis*, *Paracoccidioides brasiliensis*, and *Sporothrix schenckii*. The main questions are whether human infection ensues as a result of

transmission events from animal (zoonosis, the source is an infectious animal, interhuman transmission is uncommon), environmental (sapronosis, the source is an abiotic substrate, interhuman transmission is exceptional), or both (zoonosapronotic).

1.2.1 Bamboo rats and their association with *Penicillium marneffe*

P. marneffe was originally isolated from a captive *Rhizomys sinensis* bamboo rat used as an experimental infection model at Pasteur Institute in Da Lat, a city in the Central Highland of Vietnam [31]. Since then several studies have surveyed rodent species in the area and have established that all species of bamboo rats, *Rhizomys sinensis*, *R. pruinosis*, *R. sumatrensis*, and *Cannomys badius* carry *P. marneffe* [22,25–27,44,45]. A study captured 182 wild rodents from bamboo plantations in several regions in northeastern India and found that only the *Cannomys badius* bamboo rats endemic in the region carry *P. marneffe* (10/110, 9.1%), and none of the other 6 rat species including *Bendicota bengalensis*, *Rattus norvegicus*, *Rattus rattus*, *Rattus nitidus*, and *Mus musculus* [25]. Another survey from Chiang Mai, Thailand showed that 3/10 of the reddish-brown *C. badius* bamboo rats were positive for *P. marneffe* whilst all 51 bamboo rats from the greyish-black subspecies of *C. badius* were negative [27]. These rats share the same natural habitats and are presumably equally exposed to *P. marneffe* in the environment. However, only specific sub-species of rats are susceptible,

suggesting that there is a host factor governing the susceptibility to *P. marneffe* infection.

A recent study investigating domestic dogs as a zoonotic reservoir in Chiang Mai showed that 11/83 (13%) of nasal swab specimens from dogs were PCR test positive for *P. marneffe*. None of the 83 specimens were culture positive; however other environmental fungi were cultured from 68/83 (82%) of specimens, including *Cladosporium* sp., *Fusarium* sp., *Aspergillus* sp., *Penicillium* sp. (not *marneffe*), yeast and other fungus [46]. It appears that dogs may acquire *P. marneffe* from nasal contact with the pathogen environmental reservoir but are unlikely the carriers or zoonotic reservoir of *P. marneffe*. On the other hand, the oligonucleotide primers used in the nested PCR method in this study are based on a universal fungal ribosomal DNA sequence shared by many fungi. Therefore until other fungi from the soil are studied in greater detail it is unclear whether any may have identical sequences to the selected *P. marneffe* sequence used by this assay.

P. marneffe has been isolated from the blood and internal organs of all 4 species of bamboo rats endemic in Asia with prevalence ranging from 9 to 100% [22,23,26,27,45,47]. The distribution of bamboo rats is similar to the distribution of reported human penicilliosis cases in the region [3,22,26,27]. Using multilocus microsatellite typing, *P. marneffe* isolates from bamboo rats were shown to have similar, and sometimes identical, genotypes to those infecting humans in the same region [23,25,28,48]. One hypothesis is that bamboo rats are involved in

the transmission of *P. marneffe* to humans. Another hypothesis is that the habitats preferred by bamboo rats are shared with *P. marneffe*, and infections in both these rodents and humans are examples of sapronotic transmission. A case control study conducted in Chiang Mai, Thailand, an area endemic with bamboo rats, showed that exposure or consumption of bamboo rats did not pose an increased risk for penicilliosis; whereas an agriculture occupation with exposure to plants and animals, particularly during the rainy season, was an independent risk factor for disease [29]. Temporal analyses of penicilliosis incidence over a four year period in Chiang Mai showed that incidence increased 50% during the rainy months [30]. These studies suggest that bamboo rats are not the direct source of human acquisition. Rather agricultural exposure to soil or soil related organic matter, particularly during the rainy season, increases the risk of infection.

1.2.2 Soil and relation with *Penicillium marneffe*

Penicillium species are saprophytic organisms. They are well known as one of the most common fungi found in a diverse range of habitats, ranging from soil to vegetation to air, indoor environments and some food products [1]. Many *Penicillium* species are soil saprophytes. Soil is also a known environmental reservoir for the other known dimorphic pathogenic fungi, *B. dermatitidis*, *H. capsulatum*, *C. immitis*, *P. brasiliensis*, and *S. schenkii* [49–52]. However, field sampling efforts over the past two decades have not been very successful. Deng

et al. isolated *P. marneffe* from the stool of 3/4 *R. pruin* bamboo rats in Guangxi [13]. Chariyalertsak *et al.* isolated 1/28 soil samples collected from the *R. sumatrensis* bamboo rat burrows in Chiang Mai [27]. *P. marneffe* was not recovered from any of the 120 soil samples collected from *R. pruin* burrows in Guangdong [47], nor from any of the 215 soil samples collected from *C. badius* burrows in bamboo plantations in India and Nepal [53]. Since the discovery of *P. marneffe* in a captured bamboo rat in Vietnam in 1956, there are no published data on *P. marneffe* isolating from bamboo rats or from the environment in Vietnam.

To date attempts to recover *P. marneffe* from environments other than those that are in the immediate vicinity of bamboo rats have also been unsuccessful. Attempt to recover *P. marneffe* from the air surrounding the homes of penicilliosis patients has also been unsuccessful [15]. Using a flotation method to inoculate *P. marneffe* in soil, Vanittanakom *et al.* showed that 80 to 85% of *P. marneffe* colony forming units could be recovered from sterile soil after 3 days of incubation, whereas only 6% of *P. marneffe* colony forming units could be recovered from natural, nonsterile soil [54]. Another study demonstrated that *P. marneffe* can survive in sterile soil for several weeks but only survive a few days in nonsterile soil [55]. Despite epidemiological data pointing to soil as an environmental reservoir of *P. marneffe*, evidence of natural soil being a reservoir of *P. marneffe* is lacking and the natural reservoirs of *P. marneffe* remain elusive.

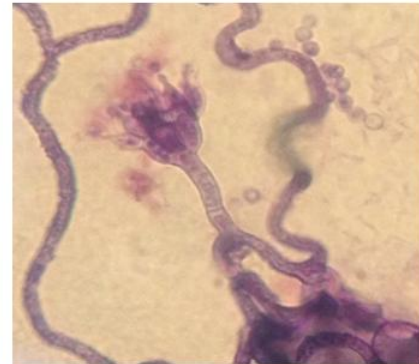
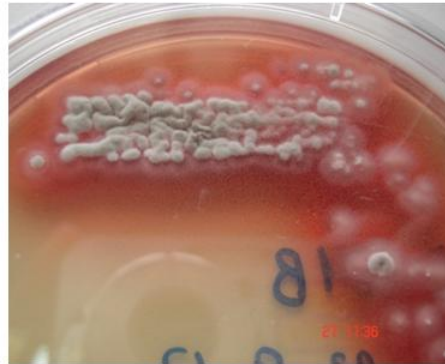
1.3 Mycology

Penicillium marneffei is the only fungus amongst a recently-accepted list of 354 saprophytic *Penicillium* species to exhibit the temperature dependent dimorphism [1]. Figure 1.4 shows the morphology of the colonies in Sabouraud agar medium and of the fungus under the microscope at 25°C and at 37°C. At 25°C the fungus grows as mycelia (a mold) with septate hyphae that bear conidiophores and conidia (similar to *Aspergillus* spp.). This feature is similar to other *Penicillium* species [2,15]. The colonies at 25°C produce a deep wine red, water-soluble pigment that diffuses into Sabouraud agar medium. Young colonies have whitish gray to yellow color; aged colonies are deep red. At 37°C *P. marneffei* converts to a yeast form that is sausage-shaped with prominent cross-walls (on artificial medium) or is spherical to oval (in human tissue). The intracellular yeast measure 2 to 3 µm; whereas the extracellular yeast can elongate up to 10 µm. They multiply by binary fission (like bacteria) and unlike other yeasts by budding. The presence of a midline septum (or cross-wall) in dividing yeast is a diagnostic feature of this fungus. The colonies of the yeast form have wrinkled or cerebriform surface and have light tan color. Figure 1.5 shows the fission yeast cells obtained from a Giemsa-stained skin touch smear of a patient seen under the microscope. The fission yeast cells represent the parasitic form of *P. marneffei*. This form is seen in the intracellular infection of macrophages. The temperature regulated mold to yeast conversion or phase transition is required

for the diagnosis of *P. marneffei* and is thought to be the key factor in its virulence.

P. marneffei has been classified under the *Penicillium* subgenus *Biverticillium* and sexual *Talaromyces* species [56]. In 2011 this classification was changed due to phylogenetic analyses of new sequencing data of a part of the RNA polymerase II largest subunit gene combined with phenotypic and extrolite data [57]. The *Penicillium* subgenus *Biverticillium* was found to form a monophyletic group with *Talaromyces* that is distinct from *Penicillium* at the genetic level. The species of *Penicillium* subgenus *Biverticillium* are now combined with the *Talaromyces* genera, and *P. marneffei* is now called *Talaromyces marneffei* [1,57]. This thesis uses the old name *Penicillium marneffei* to be consistent with names used in some of the thesis work that have been published.

25°C
SDA medium



37°C
SDA medium



Figure 1.4: The morphology of *Penicillium marneffe* colonies in Sabouraud agar medium and of the fungus under the microscope at 25°C and at 37°C

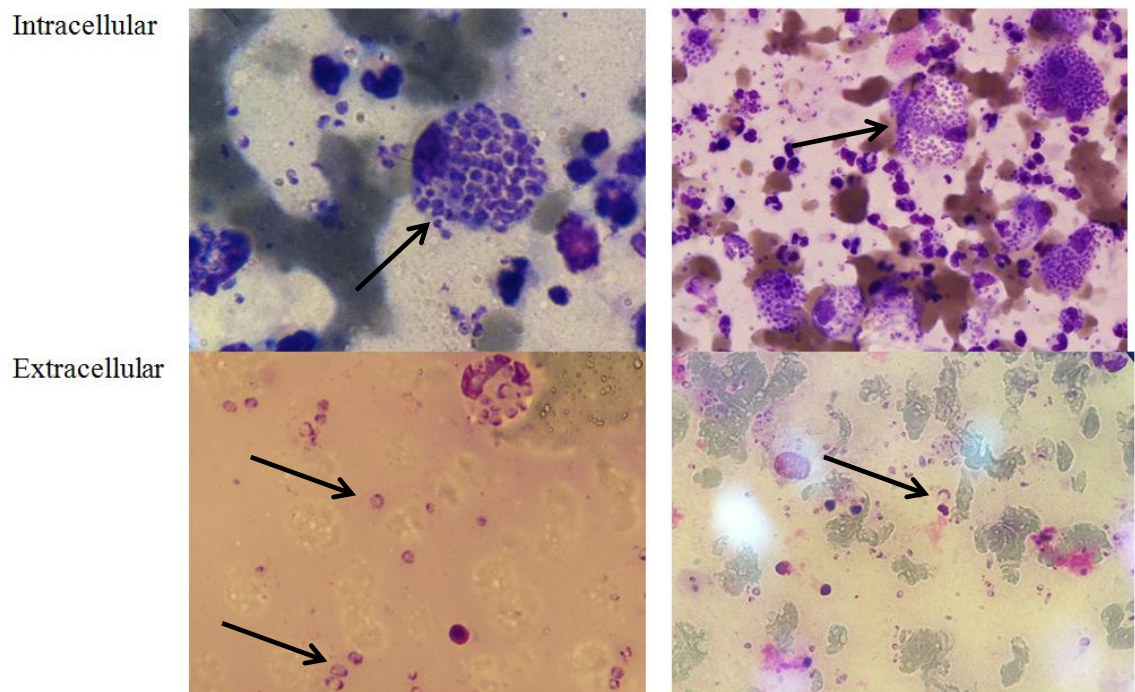


Figure 1.5: The *Penicillium marneffe* Giemsa-stained touch skin smear of HIV-infected patients showing *P. marneffe* yeast cells under the microscope at 100X magnification. The arrowheads show the intracellular yeast (upper photos) and extracellular yeasts (lower photos).

1.4 Pathology

Like other pathogenic dimorphic fungi, *Penicillium marneffei* is thought to be a primary pulmonary pathogen resulting from inhalation of infectious spores from the environment, although this has never been definitely shown. The first line of defense is thought to be the alveolar macrophages as necrotizing reactions with macrophage and histiocyte infiltrations is seen in lung biopsies of patients [13]. *P. marneffei* invades and multiplies in the histiocytes in the lung and the reticuloendothelial system, causing them to enlarge to accommodate the proliferating fungi. There are three distinct histopathological forms: granulomatous, suppurative, and anergic with necrosis. The granulomatous reaction, formed by histiocytes, lymphocytes, plasma cells, epithelioid and giant cells, tends to be seen in the reticuloendothelial organs in HIV-negative or immunocompetent patients. As the granulomas expand with release of organisms, the centres become necrotic with neutrophil infiltrations forming a central abscess. The suppurative reaction develops with the joining of multiple abscesses commonly seen in the lung and subcutaneous tissues of immunocompetent patients. The anergic and necrotising reaction is frequently seen in the lung, liver and skin of immunocompromised patients and is characterised by focal necrosis surrounded by distended histiocytes containing proliferating fungi. This reaction indicates progressive and disseminated infection [13].

1.5 Immunology

The mechanism of host and fungus interaction and host immune response to *P. marneffei* are not completely understood. Immune defects that lead to failure of macrophages to contain the infection result in dissemination of *P. marneffei* to other organs via the reticuloendothelial system [2]. Phagocytic cells are likely the primary line of host defense against this fungus. The ability of *P. marneffei* conidia to recognize fibronectin and to bind to laminin via a sialic acid-specific lectin may be important for conidia to attach to bronchoalveolar epithelia before being ingested by host mononuclear phagocytes [58]. Studies have shown that *P. marneffei* can be cleared within 2 to 3 weeks in healthy mice, whereas in nude mice or in T-cell-depleted mice, *P. marneffei* infection is fatal, demonstrating that T cells, and CD4⁺ T cells in particular, are necessary for clearing this fungal infection in mice [59]. It has been shown that rabbit pulmonary alveola macrophages and circulating human mononuclear phagocytes manifest an antifungal activity against *P. marneffei*. The circulating human monocytes have been shown to respond to *P. marneffei* conidia with an oxidative burst which was significantly enhanced by a macrophage colony-stimulating factor [60]. Macrophage colony stimulating factor enhances the antifungal activity of human neutrophils against the yeast form of *P. marneffei* but not the conidia. This activity is mediated by exocytosis of the granular cytolytic molecules from neutrophils rather than by oxygen radical-dependent mechanisms [61]. In vitro differential cytokine analysis of sub-lethal *P. marneffei* infection in wild type

BALB/c mice and IFN- γ knocked out mice indicates that protective immunity follows a Th1 response with involvement of T cells and macrophages, and IFN- γ is crucial for the activation of fungicidal macrophages and development of granulomas [62]. This finding is consistent with the general knowledge that a Th1 response plays a crucial role in host resistance to intracellular pathogens such as in mycobacteria infections and infections due to other pathogenic fungi.

1.6 Clinical manifestation

1.6.1 Typical clinical manifestation in patients with HIV infection

The most common manifestation in HIV-infected patients is disseminated infection. Common clinical features include fever, fatigue, weight loss, non-productive cough, diarrhea, lymphadenopathy, and hepatosplenomegaly, and characteristic skin lesions. Common laboratory findings include anaemia, thrombocytopenia, elevated serum aspartate aminotransferase and serum alanine aminotransferase [5,7,15,37]. With the exception of skin lesions, other clinical features are indistinguishable from other infections involving the reticuloendothelial system such as tuberculosis, systemic mycosis such as histoplasmosis or cryptococcosis, and infection due to intracellular pathogens such as *Salmonella* species. The CD4 count at presentation is generally < 100 cells/ μ L. Skin lesions are present in up to 70% of patients and tend to be papules with central necrosis, resembling molluscum contagiosum lesions presenting on the face, neck, chest, back, and upper more than lower extremities, in the order

of high to low frequency. The skin lesions are very similar to those seen in disseminated histoplasmosis and cryptococcosis. Figure 1.6 shows photos of skin lesions in HIV-infected patients with disseminated penicilliosis. After the skin, pulmonary system is involved in approximately 40% of patients [5,37]. Chest radiography findings are broad, ranging from diffuse reticulonodular, interstitial, or localised alveolar infiltrates to cavitation. The gastrointestinal system is frequently involved, with symptoms of diarrhea, abdominal distention, abdominal pain, and bloating.



Figure 1.6: Photos of skin lesions in HIV-infected patients with disseminated penicilliosis at the Hospital for Tropical Diseases in Ho Chi Minh City, Vietnam

Used with written informed consent of the patients

1.6.2 Less typical clinical manifestations

Less typical manifestations tend to occur in non-HIV infected patients with or without apparent underlying immune system impairment. Reported examples include chronic pulmonary cavitary lesion in a patient with chronic obstructive pulmonary disease on inhaled corticosteroid who had a distant travel history to Thailand and China [63], abdominal abscess [64], obstructive tracheal lesion in a non-immunocompromised patient from Guangxi province in China [65], genital chancroid-like lesion was described in an HIV-infected patient in China [66], chronic cutaneous ulcers in patients with lupus erythematosus [67], pericarditis with pericardial effusion in HIV-infected patients [35], primary central nervous system infections in patients with AIDS in Vietnam [68], and osteoarticular lesions of ribs, long bones, skull, lumbar vertebrae, scapula, and temporomandibular region in both HIV and non-HIV infected patients [69–71].

1.6.3 Concomitant opportunistic infections

Concurrent opportunistic infections are common, described in 20-60% of patients in case series. These include oral or esophageal candidiasis, tuberculosis, cerebral toxoplasmosis, Herpes simplex infections, cryptococcal meningitis, PJP, and Salmonella bacteremia [5,14,72].

1.6.4 Infection in non-HIV infected patients

1.6.4.1 Patients with underlying immune system dysfunction

*P. marneffe*i infection has been described in a wide range of hosts with underlying immune system dysfunction, including patients with primary immune defects [73,74], and patients with secondary immune system defects such as patients with lupus erythematosus on immunosuppressive therapy [67,75,76], patients with hematologic disorders who receive target therapy including monoclonal antibodies against CD20 and kinase inhibitors [77], a patient with idiopathic CD4+ lymphopenia [78], patients with solid organ transplants such as kidney transplant recipients with travel history to the endemic area [79,80], a lung transplant recipient from Australia who traveled to Vietnam [81], a liver transplant recipient in China [82], and patients with allogeneic bone marrow transplant patient in China [83].

1.6.4.2 Patients without immune system dysfunction

*P. marneffe*i infection has been described, albeit rarely, in HIV-negative patients without known underlying immune system disorders. Infection has been reported in 7 HIV-negative infants with a median age of 23.4 months from Guangxi, China [84]. Another study reported disseminated penicilliosis in 2 HIV-negative adult patients from Guangzhou, China who neither have history of infections nor apparent immune dysfunction [85]. Early case series showed that 6 of 92 penicilliosis patients from Chiang Mai and 3 of 47 penicilliosis patients from Hong

Kong were HIV-negative [5,86]; however underlying immune system dysfunction was not assessed in these patients. A recent study from Hong Kong reports detailed immunological investigation in 5 HIV-negative children with penicilliosis, and these children were all found to have some underlying immune abnormality [87]. Given the rapid emergence of *P. marneffei* infection in HIV-infected individuals and the opposing rarity of disease in immunocompetent individuals in the region, it is likely that the vast majority of HIV-negative penicilliosis patients have underlying immune defects yet to be characterised. The presence of *P. marneffei* infection in these patients should trigger investigations into the immune function of these patients, which can further our understanding of the immune variants increasing susceptibility to disease.

1.6.5 Immune Reconstitution Inflammatory Syndrome

Immune Reconstitution Inflammatory Syndrome (IRIS) is characterised by an excessive inflammatory response to an occult infection or an infection that is not clinically apparent (unmasking IRIS) or by a paradoxical worsening of a known infection (paradoxical IRIS) upon initiation of antiretroviral therapy [88]. The fast decline of HIV viral load and the opposing rise of CD4+ T cells lead to a paradigm shift of immune response from Th2 to Th1 type. Patients with very high HIV viral loads and very low CD4+ T cells are at increased risk of IRIS, particularly to intracellular pathogens [88]. Patients with penicilliosis IRIS present with swollen and tender joints [48]. Figure 1.7 shows photographs of an HIV-

infected patient from Vietnam presenting with skin lesions and multiple tender proximal interphalangeal joints in the hands associated with penicilliosis paradoxical IRIS. The skin lesions in IRIS are atypical including erythematous nodules, verrucous lesions, or erythematous plaques [74,89–91]. Figure 1.8 shows skin lesions in two HIV-infected patients with paradoxical IRIS. Figure 1.8A shows the difference in skin lesion appearance in a woman during the first penicilliosis illness and a subsequent illness 4 months later with paradoxical IRIS; the later showing skin nodules with inflammatory erythema. Figure 1.8B is a demonstration of erythematous plaques in a man 6 months after antiretroviral therapy initiation. The incidence of HIV-associated opportunistic infections in a cohort of nearly 8,000 patients initiating antiretroviral therapy in Nonthaburi, Thailand was 8% [92]. Of those, penicilliosis accounted for only 1.2%, compared to cryptococcosis at 6.2% and tuberculosis at 48.1%. However, prospective studies that define the incidence and risk factors of unmasking and paradoxical penicilliosis IRIS are lacking. Treatment with antifungal therapy has been the main approach to management, with effective use of non-steroidal anti-inflammatory medications reported in some patients, but use of corticosteroids has not yet been reported [89–91].



Figure 1.7: Photos of skin lesions and multiple swollen proximal interphalangeal joints on the hands of an HIV-infected patient at the Hospital for Tropical Diseases in Ho Chi Minh City with paradoxical penicilliosis immune reconstitution syndrome

Used with written informed consent of the patient



Figure 1.8: Photos of skin lesions in HIV-infected patients with paradoxical penicilliosis immune reconstitution syndrome showing in erythematous nodules (A) and inflammatory plaques (B).

Used with written informed consent of the patients

1.7 Laboratory diagnosis

1.7.1 Microscopy & Culture

Microscopically *P. marneffe* can be seen as oval or round intracellular and extracellular yeasts in biopsies of cutaneous lesions, fine needle aspirates of lymph nodes, or bone marrow aspirate using Wright, Giemsa, or Gomori-Grocott methenamine (GMS) stains. More rarely, the infection has been diagnosed from sputum, pleural fluid, peritoneal fluid, stool, urine, cerebrospinal fluid, pericardium fluid, and from blood smear of patients with fulminant infection [2,5,15]. *P. marneffe* has characteristic central septate or cross-wall formation that is essentially a diagnostic feature of the fungus. The differential diagnosis of such intracellular yeasts include histoplasmosis (which also has similar clinical presentation) [93], cryptococcosis (which is associated with more neurological symptoms and less respiratory involvement, lymphadenopathy or hepatosplenomegaly), and *Candida glabrata*. Unlike other endemic dimorphic fungi, *P. marneffe* grows readily in standard media and Sabouraud dextrose agar. The classical culture characteristics of thermal dimorphism and the production of red pigment can easily be demonstrated. Bone marrow, blood, and biopsies of skin lesions all have high culture yield (100%, 76%, and 90% respectively) [3,5]. The identification of *P. marneffe* is based on the morphology of colonies (see mycology section), on the demonstration of mold-to-yeast conversion (or vice versa), and on the microscopic morphology of the organisms. This process can take 4-14 days. Diagnostic delay results in delayed antifungal

therapy initiation which leads to significantly higher mortality in patients [3,5,8,15,94]. A presumptive diagnosis can be made rapidly based on Giemsa or Gram's staining of skin lesion touch smear showing round to oval intracellular and extracellular yeast approximately 3-8 μm in diameter with central septation (Figure 1.9). This allows empirical antifungal therapy to be initiated while awaiting culture confirmation; however skin lesions can be absent in 50% of patients with penicilliosis [11,86].

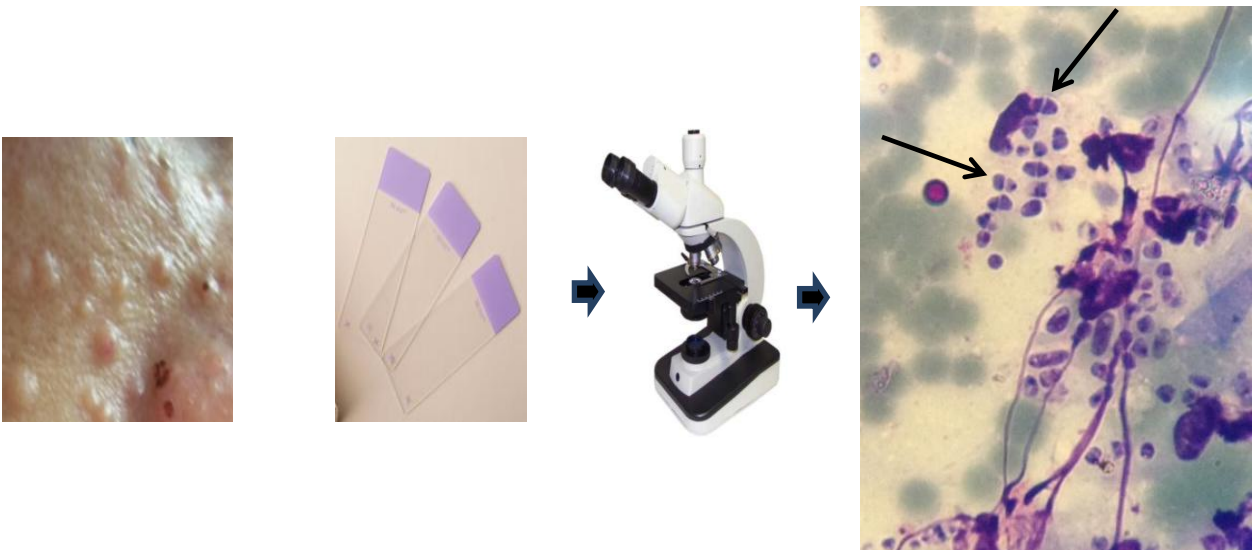


Figure 1.9: A simple bed-side procedure that can make a presumptive diagnosis of *Penicillium marneffeii* infection; giemsa staining of skin lesion touch smear showing 1-2 μm round to oval extracellular yeast cells under the microscope at 100X magnification. The arrowheads show a dividing yeast cell with an unstained midline septum.

1.7.2 Immunodiagnostics

Various methods have been developed assessing of *P. marneffei* antigen production, including immunoblot, indirect fluorescent antibody test (IFAT), latex agglutination, and enzyme-linked immunosorbent assay (ELISA). However these have so far been studied in only small numbers of patients or there have been issues with sensitivity and specificity, when compared to the gold standard method of culturing *P. marneffei* from clinical specimens [18,95,96]. There has been recent interest in detecting circulating galactomannan. The *Aspergillus* galactomannan has considerable homology to that of *Penicillium*, and commercial assays for the detection of galactomannan have recently been investigated in *P. marneffei* infection [94,97]. The sensitivity and specificity of the serum galactomannan for detection of active penicilliosis was 23/24 (95.8%) and 30/33 (90.9%), respectively (cutoff index=1.0). This test has the potential for use as a rapid diagnostic assay for *P. marneffei* infection.

Recently an ELISA test for detection of *P. marneffei* antigen in urine has been developed and prospectively evaluated in 33 HIV-positive Thai patients with culture-confirmed *P. marneffei* and 248 patients with other diagnoses [98]. This ELISA detected *P. marneffei* antigen in the urine samples of all 33 (100%) patients with penicilliosis with a median titer of 1:20,480. *P. marneffei* was not detected in 94% of samples from healthy volunteers; however it was detected in 27% of 248 urine samples from inpatients with diagnoses other than penicilliosis (including cryptococcosis, melioidosis, and other bacterial septicemia). Sensitivity

and specificity for this assay to detect penicilliosis at a cut off titer of 1:40 was 97% and 98% with a positive predictive value of 84.2% and negative predictive value of 99.7%.

The same polyclonal hyperimmune IgG was used to develop a simplified dot blot ELISA and a latex agglutination test for detecting *P. marneffei* antigenuria and prospectively evaluated in urine specimens from 37 patients with culture proven penicilliosis and 300 controls (52 healthy and 248 hospitalized patients without penicilliosis) [19]. The sensitivities for ELISA, dot blot ELISA, and agglutination test were 97.3%, 94.6%, and 100% respectively; specificities were 98%, 97.3%, and 99.3%, respectively. Of these 3 promising tests, the agglutination test seems to be the simplest, most rapid and robust and needs to be evaluated in larger prospective cohort studies for both diagnosis and use as a surrogate marker of treatment response and relapse.

Recently a novel diagnostic biomarker based on the *MP1* gene, which encodes the abundant cell wall manoprotein Mp1p specific for *P. marneffei*, was discovered [99]. Monoclonal antibody against the recombinant Mp1p protein and polyclonal antibodies against whole yeast cells have been developed for use in immunoassays for simultaneous detection of *P. marneffei* Mp1p antigen and antibody from patient sera with sensitivity of 15/20 (75%) and specificity of 537/540 (99.4%) when used in combination [100]. A recent serological surveillance of more than 8,000 archived HIV-infected sera samples from Guangzhou for Mp1p antigen showed an overall prevalence of 9.4%, which

increased to 28% in patients with CD4 count <50 cells/ μ L [101]. This assay has the potential to allow early detection or screening for infection before symptoms develop and potentially prevent penicilliosis and the associated morbidity and mortality with prophylactic treatment. The assay should be validated and evaluated in prospective clinical studies.

1.7.3 Molecular Diagnosis

Polymerase chain reaction (PCR) technology is a highly sensitive and specific molecular tool with wide applications in medical diagnostics and research. PCR assays have been developed for detection of several invasive fungal pathogens [102–106]. Molecular diagnostic development of *P. marneffei* has been based on specific oligonucleotide primers designed from the internally transcribed spacer regions and the 5.8S rRNA and the 18S rRNA gene of *P. marneffei* [107]. A PCR-hybridisation assay was developed based on the 18S rRNA gene and was shown to be specific when tested against 7 other fungi with sensitivities of 0.5 pg/ μ L and 0.1 pg/ μ L of DNA by PCR and southern hybridization, respectively [108]. However the hybridization technique is labor-intensive and requires high level of competency in the laboratory.

To address this, a nested PCR assay with primers designed based on 18S rRNA was subsequently developed by the same group which was able to detect *P. marneffei* from 2-day old culture supernatant [109]. This nested PCR assay was later tested in sera of 35 *P. marneffei*-infected patients, 15 non- *P. marneffei*-

infected patients, and 300 sera from healthy people. The sensitivity was 24/35 (68.6%), and specificity was 100% [20].

Compared to real-time PCR technology, conventional PCR methods are still relatively more labor-intensive and prone to post PCR contamination. A TaqMan real-time PCR assay was recently developed to detect *P. marneffei* in peripheral blood samples of 20 patients with penicilliosis and showed a sensitivity of 80% but clinical specificity was not assessed [21]. These assays have the potential to make a rapid diagnosis of *P. marneffei* within a few hours of patient presentation. However the accuracy and reliability of these assays need to be assessed in larger numbers of clinical samples and to be prospectively evaluated to determine their usefulness in clinical management of penicilliosis.

1.8 Antifungal susceptibility testing

In vitro antifungal susceptibility testing of *P. marneffei* clinical isolates has been performed using either broth dilution methods or E- test; however clinical outcome data are lacking [99–103]. The only study that relates *in vitro* antifungal susceptibility testing results to clinical outcome of patients was the very first case series of 80 patients from Chiang Mai Thailand [112]. Antifungal susceptibility was tested in 30 isolates using macro-dilution methods. They found that *P. marneffei* was highly susceptible to itraconazole, ketoconazole, miconazole, and 5-fluorocytosine. The minimum inhibitory concentrations (MICs) were ≤ 0.002 $\mu\text{g/ml}$ for itraconazole, ketoconazole, and miconazole and ≤ 0.015 $\mu\text{g/ml}$ for 5-

florocytosine. The MICs were higher for amphotericin B (0.25 µg/ml) and fluconazole (≤ 0.313 µg/ml). Some strains were resistant to fluconazole. The antifungal susceptibility results appear to have good clinical correlation in patients treated with itraconazole or fluconazole; however the clinical correlation in patients treated with amphotericin B is difficult to assess.

The other studies simply reported the MICs of clinical isolates without *in vivo* or clinical correlation; however the results of MICs for azole drugs and amphotericin B are in general in agreement [110,111,113]. A recent study reported the MIC results of 25 isolates of patients from Guangxi, China against various antifungal drugs, including the newer generation azole drug voriconazole. The voriconazole MICs ranged from 0.004 to 0.25 mg/l, and it had the lowest MICs compared to other antifungal agents. The MICs for other antifungal drugs, in the order of lowest to highest, were itraconazole (0.031-0.5 mg/l), terbinafine (0.031-2.0 mg/l), amphotericin B (0.125-2.0 mg/l), and fluconazole (1.0-16 mg/l) [111]. As there are significant variations in absorption of oral itraconazole, and there are significant side effects with amphotericin B treatment, voriconazole and terbinafine should be further evaluated as treatment options of penicilliosis.

1.9 Treatment and outcomes

Disseminated penicilliosis has a high mortality if untreated. All 12 patients who did not receive antifungal therapy either died or left the hospital without follow up in an early series [112]. In-hospital case fatality rates range from 10 to 20%.

Delay in diagnosis and treatment is associated with significantly higher mortality rates [3,5,8,15,94]. There have been no comparative clinical trials on the treatment of penicilliosis, and thus treatment choices and guidelines for this infection are still based on data from case series, *in vitro* antifungal susceptibility data, and expert opinions.

1.9.1 Induction and consolidation therapy

The most objective data came from an early case series in 1993 of 80 consecutive HIV positive Thai patients with penicilliosis describing responses to treatment with amphotericin B (0.3 to 0.6 mg/kg/day given intravenously for 6 to 8 weeks), itraconazole (400mg/day given orally twice daily for 8 to 12 weeks), or fluconazole (400mg/day orally twice daily for 8 weeks). At the time antifungal treatment choices were based on the attending clinician's preference without prior knowledge of *in vitro* sensitivities or antifungal drug efficacies against *P. marneffei* infection. The treatment success rates (defined as resolution of fever, skin lesions, and other signs/symptoms of disease with absence of fungaemia on follow up blood culture) were 27/35 (77.2%) for amphotericin B, 9/12 (75%) for itraconazole, 4/11 (36.4%) for fluconazole, and 0/9 (100%) for no treatment. Death occurred while on treatment in 6, 5, and 5 patients treated with amphotericin B, itraconazole, or fluconazole, respectively. Disease relapse was observed (1 to 6 months after therapy) in 4, 5, and 3 patients treated with amphotericin B, itraconazole, or fluconazole, respectively [112].

1.9.1.1 Amphotericin B induction therapy followed by itraconazole consolidation therapy

A subsequent series also from Chiang Mai in 1998 described 74 HIV-patients with disseminated penicilliosis treated with amphotericin B 0.6 mg/kg/day for 2 weeks followed by itraconazole 400 mg/day for 10 weeks. All patients received cotrimoxazole as primary prophylaxis for PJP. Remarkably there were no deaths. The treatment success rate (defined by negative blood culture and resolution of fever and skin lesions at the end of 12 weeks therapy) was 97%. It is not clear from the report which patients were selected for this treatment; however the high treatment success rate is very different from the early series by the same group. Amphotericin B intravenous therapy has significant side effects and is not widely available in many parts of Asia. Nevertheless, intravenous amphotericin B at 0.7 to 1.0 mg per kg per day for 14 days, followed by oral itraconazole 400mg per day for 10 weeks has been recommended for treatment of penicilliosis in major international and national guidelines [115,116].

1.9.1.2 Itraconazole induction therapy

In a small case series of 10 HIV-infected Thai patients with penicilliosis who were treated with itraconazole 400 mg/day monotherapy for 2 months, two patients died while on therapy; the other 8 achieved clinical improvement, but the mean duration to culture negative was long at 57 days [117]. A study from India described successful treatment with itraconazole 400 mg/day for 3-4 weeks with

a treatment response rate of 97% (N=40 patients) [9]. In a case series of 94 HIV-infected patients with disseminated penicilliosis from northern Vietnam, the mortality rates were not different in the 53 patients who were treated with itraconazole compared to the 41 patients who were treated with amphotericin B. The overall mortality was 18% [7]. In another case series of 47 patients from Hong Kong, the mortality was 9% and was not different in the 33/43 (77%) of those who received amphotericin B and the 10/43 (23%) who received itraconazole [86]. A multi-centre randomised clinical trial comparing itraconazole and amphotericin B as induction therapy for HIV-associated penicilliosis is being conducted in Vietnam, of which I am the Principal Investigator, with mortality and disease relapse as outcomes and should provide a definitive answer regarding the comparative efficacies of these two drugs for induction therapy of penicilliosis. The trial completed recruitment of 440 patients in December 2015 and the last patient will complete the last follow up visit in June 2016 (clinical trial registration number ISRCTN97524945).

1.9.2 Secondary prophylaxis

Before the widespread introduction of antiretroviral therapy it was recognized that disease relapse rate after initial treatment success can exceed 50% with the median relapse time of 24 weeks [118]. A subsequent randomized, double-blind, placebo-controlled study of itraconazole secondary prophylaxis (200 mg once/day) was discontinued early as all relapses were within the placebo arm

[119]. Long term maintenance therapy with itraconazole has since been widely adopted.

1.9.3 Discontinuation of secondary prophylaxis

There are no clinical trials which studied how long secondary prophylaxis therapy should be continued after immune reconstitution from antiretroviral therapy. One retrospective study reported no relapses after itraconazole discontinuation in 33 patients with a CD4 lymphocyte count $>100/\mu\text{L}$ for >6 months who were followed for a median time of 18 months [120]. Another retrospective study found no disease relapse in patients after immune reconstitution on antiretroviral therapy; however CD4 counts were not provided [121]. One relapse was described in a series of 19 patients who discontinued prophylaxis at a median CD4 lymphocyte count of $95/\mu\text{L}$ (18 patients had a CD4 count $<200/\mu\text{L}$ and ten $<100/\mu\text{L}$) equating to a relapse rate of 1.72/100 patient-years [122]. It therefore is reasonable to discontinue secondary prophylaxis after significant immune restoration from antiretrovirals, though exact criteria need to be established in prospective randomised studies. International guidelines recommend stopping secondary prophylaxis when CD4 >100 cells/ μL for >6 months [116].

1.9.4 Primary prophylaxis

The potential for primary prophylaxis for fungal opportunistic infection in advanced HIV infection has been explored with a randomized placebo-controlled double-blinded study conducted in Chiang Mai, Thailand, of itraconazole (200 mg/day) in those with CD4 lymphocyte counts $<200/\mu\text{L}$ [123]. There was a significant decrease in the incidence of both cryptococcosis and penicilliosis in the intervention group (primarily in those with CD4 count $<100/\mu\text{L}$). However, there was no survival advantage to being on itraconazole (though the study was not powered for this end-point). In a retrospective study (also conducted in Chiang Mai) the incidence of systemic fungal infection was not significantly different in HIV-infected patients receiving fluconazole 400mg weekly dosing or itraconazole 200mg daily dosing during a 12-month follow up period with reported incidences of 7/276 (2.5%) and 2/32 (6.3%), respectively [124]. Based on the results of these studies, international guidelines recommend primary prophylaxis with either itraconazole 200mg daily or fluconazole 400mg weekly in patients with CD4 count <100 cells/ μL living in or traveling to Thailand, Vietnam, and southern China, especially those in rural areas [125]. However this primary prophylaxis strategy has not been adopted widely in the region due to costs, drug-drug interactions, and concerns of potentially inducing drug resistance.

CHAPTER 2

The Clinical Epidemiology and Seasonality of HIV-Associated *Penicillium marneffe* Infection in Ho Chi Minh City, Vietnam

2.1 Abstract

Background

Penicillium marneffe is a common HIV-associated opportunistic fungal pathogen in Southeast-Asia. The clinical epidemiology, clinical outcomes, and the predictors of outcomes have not been systematically studied in Vietnam.

Methods

We performed a large retrospective study of culture-confirmed incident penicilliosis cases from 1996-2009 at the Hospital for Tropical Diseases in Ho Chi Minh City. Seasonality of penicilliosis was assessed using cosinor models. Logistic regression was used to assess the predictors of poor outcome, defined as death or worsening disease, based on predefined covariates. Cox regression was performed to model time-to-antifungal-initiation.

Results

795 patients were identified; hospital charts were obtainable for 513 (65%) patients. Cases increased exponentially and peaked in 2007 (156 cases), mirroring the trends in AIDS admissions during the study period. A highly significant seasonality for penicilliosis ($p<0.001$) but not for cryptococcosis ($p=0.63$) or AIDS admissions ($p=0.83$) was observed, with 27% (95% CI:14%-41%) increase in incidence during rainy months. All patients were HIV-positive; the median CD4 count (N=62 patients) was 7 (IQR:4-24) cells/ μ L. Hospital outcome was an improvement in 347 (68%), death in 101 (20%), worsening in 42

(8%), and non-assessable in 23 (5%) cases. Injection-drug use, shorter history, absence of fever or skin-lesions, elevated respiratory rates, higher lymphocyte and lower platelet counts independently predicted poor outcomes in both complete-case and multiple-imputation analyses. Time-to-treatment-initiation was significantly shorter for patients with skin lesions, HR=3.78 (95% CI: 2.96-4.84), $p<0.001$.

Conclusions

Penicilliosis incidence correlates with the HIV/AIDS epidemic in Vietnam. Injection-drug use, shorter history, absence of fever or skin-lesions, respiratory difficulty, higher lymphocyte, and lower platelet counts predict poor in-hospital outcome.

2.2 Introduction

The systemic mycosis caused by *Penicillium marneffe* is endemic in South and Southeast Asia and is a common Human Immunodeficiency Virus (HIV)-associated opportunistic infection, ranking third after tuberculosis and cryptococcal meningitis in Thailand and after *Pneumocystis jiroveci* pneumonia (PJP) and tuberculosis in Hong Kong [5,37,126]. It is increasingly diagnosed in immunocompromised individuals from non-endemic regions who have traveled to Southeast Asia [10,126]. HIV-infected individuals with CD4 count <100 cells/ μ L are particularly at risk and make up the majority of infections in endemic regions [5,126]. Untreated disseminated infection is fatal. Induction therapy with amphotericin B followed by itraconazole has been shown to be highly effective in one prospective non-comparative study of 74 patients in Chiang Mai, Thailand [118]. Itraconazole has been shown to be equally effective compared to amphotericin B in retrospective case series [7,86,112] and is recommended for treatment of mild to moderate disease [9,112]. However these treatment strategies have never been studied in randomized controlled trials. Despite appropriate antifungal therapy in-hospital fatality rates are up to 20% [3,5,86,112], and the determinants of poor outcome have not been identified.

The first reported human infection in Vietnam was a 29 year-old HIV-infected man who died before the identification of *P. marneffe* from his blood culture at the Hospital for Tropical Diseases (HTD) in Ho Chi Minh City (HCMC) in July 1996 [12]. *P. marneffe* has rapidly emerged as the second most common

pathogen isolated from all routine blood cultures after *Cryptococcus neoformans* at the HTD [40]. The epidemiology of penicilliosis in Vietnam is unknown, and apart from relatively small case-series in Southeast Asia [3,5,9,37,86,112,126], there has not been a large case-review of penicilliosis that spans the antiretroviral therapy (ART) era. In the setting of a growing HIV epidemic in Vietnam [127], we conducted a large retrospective chart-review to determine the clinical features, treatment outcomes, and outcome predictors of penicilliosis.

2.3 Methods

2.3.1 Study setting and population

The HTD is the largest primary, secondary and tertiary referral hospital for infectious diseases in Vietnam, with over 5,000 HIV-infected patients seen annually. The study reported here included all patients admitted with penicilliosis from July 1996 to December 2009. Penicilliosis was defined as any illness in which *P. marneffe* was isolated from blood, skin scrapings, bone marrow, lymph node, and/or other body fluids. Isolation of *P. marneffe* was according to standard culture techniques described elsewhere [128]. Cases were identified from the microbiology records, which has recorded all blood culture data (whether positive or negative) consistently since 1992. To ensure that cases with *P. marneffe* isolated from referral hospitals were included, we additionally searched for hospital charts with penicilliosis as a primary discharge-diagnosis. All cases were cross-checked for duplication in the database by name, date of

birth, and address. Multiple culture-positive specimens and/or multiple admissions from one patient were merged into one case. Only data from the first penicilliosis admission were extracted. The study was approved by the Scientific and Ethical Committee of the HTD.

2.3.2 Data collection and definition of hospital outcome

Clinical and laboratory information were entered into individual case record forms (CRF). Hospital outcome was evaluated by a physician investigator (in consultation with a second physician when there was uncertainty) and was categorized as follows: 1) death, 2) worsening disease at discharge (i.e. patients who were moribund or deteriorated clinically and were taken from hospital to die at home as per local custom), 3) an improvement (negative repeat culture, resolution of fever, skin lesions, and/or symptoms), or 4) non-assessable (unchanged or left hospital against medical advice). For analysis, 'poor outcome' was defined as death or worsening disease at discharge; 'good outcome' was an improvement, and non-assessable outcome was treated as missing data. Data from CRFs were independently double-entered into a Microsoft Access 2003 database to ensure data integrity.

2.3.3 Statistical analysis

2.3.3.1 Assessment of seasonality

Seasonality of penicilliosis, cryptococcosis, and Acquired Immunodeficiency Syndrome (AIDS) admissions were assessed using Poisson regression models, which modeled monthly admissions depending on a global trend (modeled with a natural cubic spline with five degrees of freedom) and a sinusoidal shape for seasonality effect (modeled with a sine- and a cosine-term, i.e. a cosinor model) [129]. The log-number of days per month was used as an offset to correct for unequal lengths of months. To detect seasonality we tested whether the sine- and cosine-term could jointly be omitted from the model using a likelihood ratio test. In a second step, we modeled the proportion of penicilliosis amongst all AIDS admissions using the same strategy based on a logistic regression model. Non-duplicate cryptococcosis cases were simultaneously identified from blood and cerebrospinal fluid (CSF) cultures from the microbiology records. AIDS admission numbers were identified from the hospital records based on the International Classification of Disease (ICD-10) coding B20.

2.3.3.2 Assessment of admission predictors of hospital outcomes

We performed univariate and multiple logistic regressions based on ten predefined admission-covariates: days of illness, history of fever, cough, injection-drug use (IVDU), presence of skin lesions, dyspnea, respiratory rate, absolute lymphocyte, platelet, and hemoglobin. These covariates were chosen

because they either are established HIV/AIDS prognostic factors (IDU, absolute lymphocyte as a CD4 cell surrogate), have been associated with death in AIDS-associated cryptococcosis or histoplasmosis (longer illness, respiratory difficulty, skin lesions, and hemoglobin) [130–132], or known outcome predictors in other infectious diseases (fever, platelet count) [133–135]. Multiple imputation was used to deal with missing covariates and non-assessable outcome. We performed a sensitivity analysis, which excluded patients with missing covariates and non-assessable outcome. In a second sensitivity analysis, we additionally excluded patients who left the hospital with worsening disease, i.e. only patients with documented death remained in the ‘poor outcome’ category. We described time-to-treatment-initiation using Kaplan-Meier estimates and modeled it with a Cox regression analysis depending on history of fever or presence of skin lesions. Patients who did not initiate treatment were treated as censored on the day of discharge or death. A multivariate analysis based on multiple imputed data and including all ten admission-covariates was also conducted.

All analyses were performed with the statistical software R version 2.9.1 [136] and the companion R package MICE for multiple imputation [137].

2.4 Results

2.4.1 Penicilliosis incidence

From 1996-2009 the search of microbiology records resulted in 728 cases, and the search by primary discharge diagnosis resulted in an additional 67 cases,

giving a total of 795 non-duplicate cases. Penicilliosis cases increased exponentially, from two in 1996 to a peak of 156 in 2007 then decreased subsequently to 90 cases per year (Figure 2.1). Penicilliosis incidence largely mirrored the trends in AIDS admissions at HTD during the same study period (Figure 2.1). From 2004-2009, penicilliosis accounted for on average 4.4% (range: 3.4-5.4%) of yearly AIDS admissions and accounted for on average 6.3% (range: 5-8.3%) of AIDS-associated in-hospital deaths at the HTD.

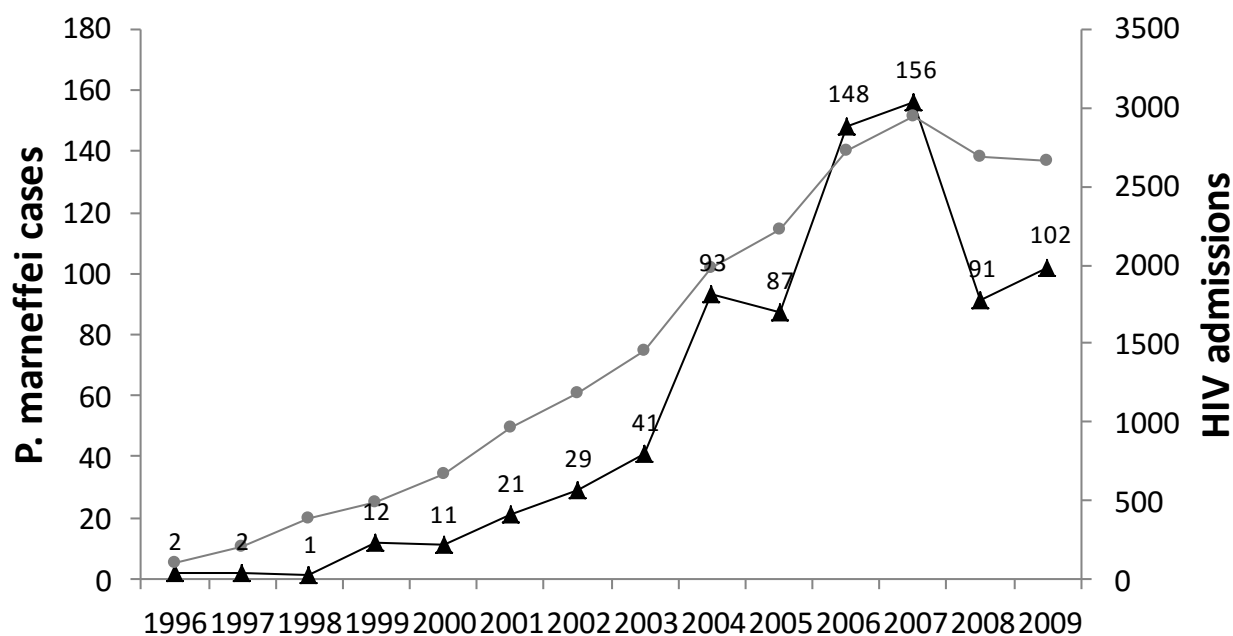


Figure 2.1: *P. marneffei* cases (black line) and AIDS admissions (grey line) at HTD from 1996-2009

2.4.2 Season association

2.4.2.1 Absolute number of admissions

Monthly admission numbers of penicilliosis, cryptococcosis, and AIDS from 2004-2009 are displayed in Figure 2.2 (left panel). The Poisson regression model revealed a highly significant seasonality for penicilliosis ($p < 0.001$) but not for cryptococcosis ($p = 0.63$) or AIDS admissions ($p = 0.83$). The seasonality term for penicilliosis has an estimated peak in August, which is in the middle of rainy months lasting from May to November in HCMC, and an amplitude corresponding to an estimated increase in penicilliosis cases by 27% [95% confidence interval (CI):14%-41%] during the peak month as compared to the average. Regression diagnostics did not indicate any problems with the model assumptions or evidence of any unexplained seasonality in the residuals.

2.4.2.2 Proportion of penicilliosis cases amongst all AIDS admissions

Monthly proportion of penicilliosis cases amongst all AIDS admissions are shown in Figure 2.2 (right panel). The logistic regression model revealed a highly significant seasonality for the proportion of penicilliosis ($p < 0.001$). Again, the peak month was estimated to be August, and the amplitude corresponded to a 32% (95% CI:17%-48%) higher odds for a penicilliosis admission during the peak month as compared to the average.

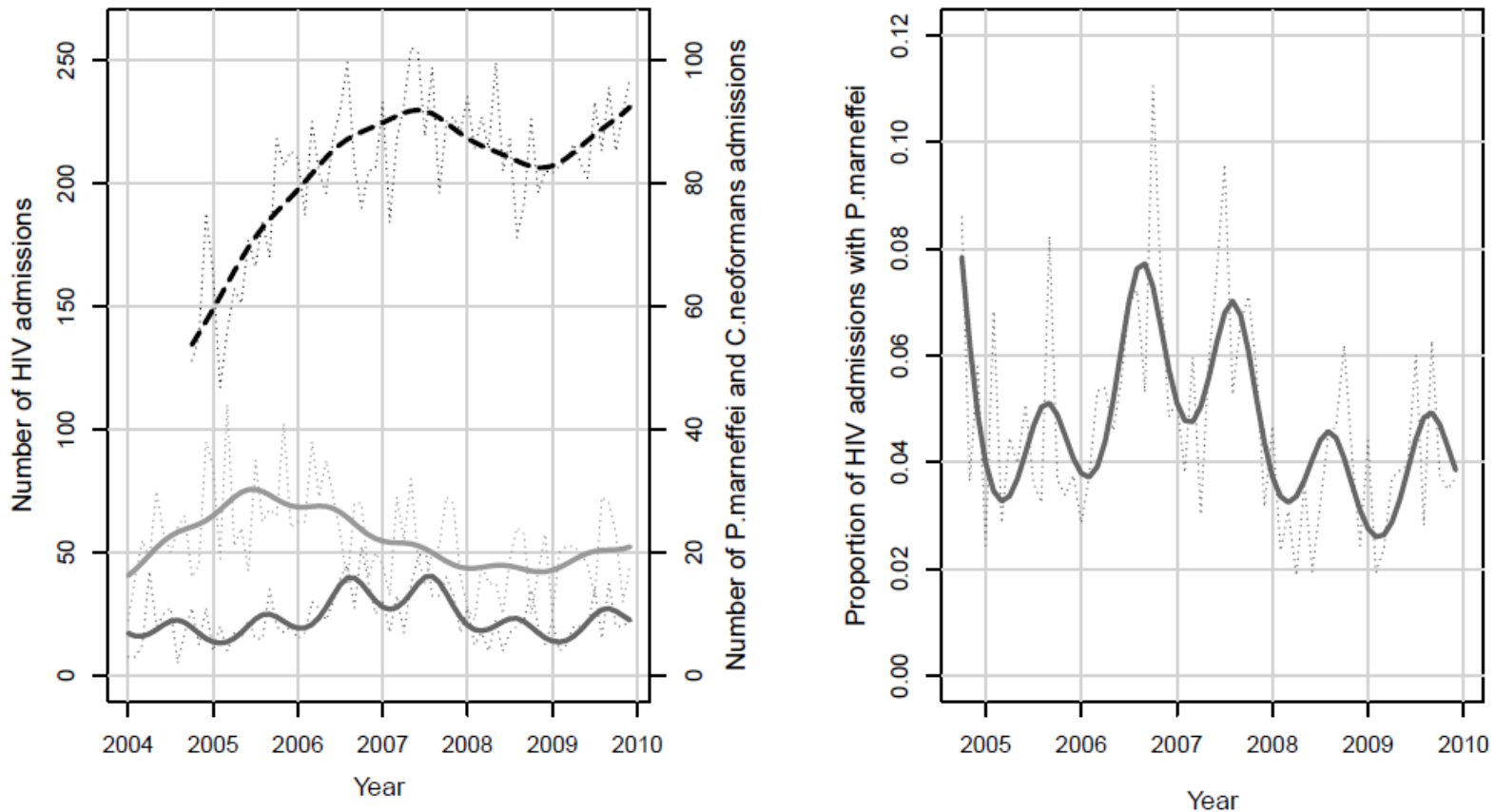


Figure 2.2: Seasonality of penicilliosis at the Hospital for Tropical Diseases from 2004 to 2009

Left panel: Monthly admission numbers of AIDS (black, dashed), *P. marneffei* (dark gray, solid), and *C. neoformans* (light gray, solid) patients from 2004-2009. Thin dotted lines correspond to actual counts, thick lines to smoothed values based on a Poisson regression model with terms for a global trend and seasonality. **Right panel:** Monthly admission numbers of *P. marneffei* as a proportion of all AIDS admissions. The dotted line corresponds to observed proportions, the solid line to smoothed values based on a logistic regression model with terms for a global trend and seasonality.

2.4.3 Clinical and laboratory features

Hospital charts were obtainable for 513/795 (65%) patients who were admitted at the HTD from 2004 to 2009. Charts prior to 2004 had been destroyed to make room for new charts. All patients were HIV-infected. The median CD4 count (available for 62 patients) was 7 (IQR: 4-24) cells/ μ L. Penicilliosis was the HIV presenting illness in 126/513 (25%) patients. 103/513 (20%) patients had been on ART for a mean duration of 30 (IQR: 15-120) days. The median age was 28 (IQR: 25-32) years. 421/513 (82%) patients were men. History of injection drug use was present in 332/485 (68%) patients. 431/513 (84%) patients were from HCMC; the remaining 16% were from 21/32 southern provinces of Vietnam.

The clinical and laboratory characteristics of the 513 patients are summarised in Table 2.1. Symptoms were non-specific including fever, anorexia, fatigue, cough, and gastrointestinal complains. Common signs included cachexia, paleness, skin lesions, hepatomegaly and/or splenomegaly. The most common laboratory abnormalities were anaemia, thrombocytopenia, and elevated transaminases. Isolation of *P. marneffe*i from clinical specimens obtained for diagnostic evaluation is reported in Table 2.2, with the highest sensitivities for skin scraping, blood, and lymph node culture. The cultural yields for peritoneal fluid, CSF, bronchial alveolar lavage (BAL) and bone marrow were high, but the denominators were small. The median time to identify *P. marneffe*i from skin and blood cultures were 4 (IQR:3-5) days and 5 (IQR:4-6) days, respectively. Skin microscopy by Giemsa stain revealed intra and extracellular yeasts in 123/153

(80%) specimens examined; however, the detailed description of the yeasts, i.e. whether or not a diagnostic midline septum was observed, was lacking.

Clinical pneumonia was diagnosed in 148/513 (29%) patients. Of those a pathogen was identified in 74/148 (50%) patients: sputum/BAL smear-positive *M. tuberculosis* in 36, PJP in 13 (not all specimens were tested for PJP), and *P. marneffei* in 12. Central nervous system (CNS) infection was suspected in 59/513 (12%) patients and was definitively diagnosed by CSF culture and/or microscopy in 29/59 (50%) patients: *P. marneffei* was isolated in twenty; *C. neoformans* was microscopically observed and isolated in seven; *M. tuberculosis* was seen on Ziehl-Neelsen stain in one, and concurrent *P. marneffei* and *C. neoformans* was isolated in one. Concurrent opportunistic infections were diagnosed in 285/513 (56%) patients, including oral candidiasis (49%), microscopically-confirmed tuberculosis (22%), PJP (4.6%), cutaneous herpes (4.6%), cryptococcal meningitis (2.5%), salmonellosis (3.5%), and other bacterial infections (3.2%). The clinical features of the patients with *P. marneffei* infection of the CNS and penicilliosis patients with concurrent *C. neoformans* infection will be described in chapter 3.

Table 2.1: Clinical features of 513 patients with penicilliosis in Ho Chi Minh City, Vietnam

Patient Characteristics	
Demographics	Value (% or IQR)
Male	421 (82%)
Age in years	28 (25-32)
IVDU	332/485 (68%)
On ARV	103 (20%)
New HIV diagnosis	126 (25%)
Symptoms	
Days of illness	15 (7-30)
Fever	419 (82%)
Anorexia	318 (62%)
Fatigue/malaise	239 (47%)
Cough	205 (40%)
Abdominal pain	160 (32%)
Diarrhea	150 (30%)
Weight loss	92 (18%)
Signs	
Admission temperature (°C)	38 (37-38.5)
Highest temperature (°C)	39.5 (38.5-40)
Respiratory rates (breaths/min)	24 (22-28)
Emaciated/cachexia	369/467 (79%)
Pale	286/397 (72%)
Thrush	139/496 (28%)
Skin lesions	346/487 (71%)
Hepatosplenomegaly	286 (56%)
Lymphadenopathy	131 (26%)
Dyspnoea	117/468 (25%)
Decreased mentation	24 (4.7%)
Meningismus	2 (0.4%)
Laboratory Findings	
Absolute lymphocyte count (cells/ μ L)	395 (232-696)
Hemoglobin (g/dL)	8 (6-9.8)
Hematocrit (%)	24.9 (19.3-30.3)
Platelets ($\times 10^3$ cells/ μ L)	82 (42-150)
AST (U/L) ^a (N=410)	122 (63-230)
ALT (U/L) ^b (N=410)	60 (34-119)
AST/ALT ratio	2 (1.85-1.93)

CD4 cell count (cells/ μ L) (N=62)	7 (4-24)
Imaging Findings	
Abnormal chest x-ray	357 (70%)
Reticulonodular	177/357 (50%)
Interstitial	144/357 (39%)
Alveolar consolidation	71/357 (20%)
Pleural effusion	25/357 (7%)
Abnormal ultrasound	312 (60%)
Hepatomegaly, size in cm	197/312 (63%), 15.8 (14.3-17.6)
Splenomegaly, size in cm	176/312 (56%), 14.5 (13.2-16)
Hepatosplenomegaly	159/312 (51%)
Abdominal lymphadenopathy	159/312 (33%)
Ascites	73/312 (23%)
Spleen abscess	8/312 (2.6%)
Concurrent Diagnoses	
Clinical pneumonia	148 (29%)
<i>M. tuberculosis</i>	36/148 (24%)
PJP	13/148 (8.8%)
<i>P. marneffei</i>	12/148 (8.1%)
Undiagnosed	87/148 (59%)
Clinical CNS infection	59 (11.5%)
<i>P. marneffei</i>	20/59 (34%)
<i>C. neoformans</i>	7/59 (11.9%)
<i>P. marneffei</i> and <i>C. neoformans</i> co-infection	1
<i>M. tuberculosis</i>	1
No pathogen	30/59 (50.8%)
Concurrent OIs	285 (56%)
Oral candidiasis	139/285 (49%)
Tuberculosis	62 (22%)
PJP	13/285 (4.6%)
Herpes	13/285 (4.6%)
<i>C. neoformans</i>	7/285 (2.5%)
<i>Salmonella</i> spp.	10/285 (3.5%)
Other bacteria	9/285 (3.2%)
Data are in absolute count (%) for categorical variables and median (interquartile range, IQR) for continuous data. The denominator N=513 unless otherwise stated. Normal ranges: ^a ≤ 37 U/L; ^b ≤ 40 U/L. IV DU, intravenous drug user; ARV, antiretrovirals; PJP, <i>Pneumocystis jiroveci</i> pneumonia; CNS, central nervous system; OIs, opportunistic infections	

Table 2.2: *P. marneffei* culture from clinical specimens of 513 patients with penicilliosis

Clinical Specimens	Number (%)
Blood	395/472 (84%)
Skin biopsy/scrapings	186/195 (95%)
Lymph node	17/20 (85%)
Peritoneal fluid	9/23 (39%)
CSF	21/59 (36%)
Bronchial alveolar lavage	7/9
Bone marrow	2/2
Pleural fluid	0/2
Oral pharyngeal lesions	8/13
Stool	6/10

Data refer to the number of *P. marneffei*-positive specimens (%) amongst all specimens sampled for diagnostic evaluation within 48 hours of admission.

2.4.4 Treatment and outcomes

Appropriate antifungal treatment was initiated in 410/513 (80%) patients with an estimated median time-to-treatment-initiation of 2 (IQR:0-5) days. 254/410 (62%) patients received itraconazole 400mg/day. 147/410 (36%) patients received amphotericin B at dosages 0.6 to 1mg/kg/day. 4/410 (1%) patients received fluconazole 400mg/day. The remaining 5/410 (1.2%) patients received fluconazole followed by itraconazole. Blood transfusion was given for 144/513 (28%) patients with symptomatic anaemia.

Outcome at discharge was an improvement in 347/513 (68%), death in 101/513 (20%), worsening in 42/513 (8%), and non-assessable in 23/513 patients (5%), 4 left the hospital against medical advice, 19 were discharged with unchanged clinical conditions. The median duration of hospitalization was 9 (IQR:4-17) days and was strongly dependent on the outcome group: patients with documented death died a median of 2 (IQR:1-5) days after admission, and those with worsening disease stayed in hospital for 2.5 (IQR:1-4) days. In contrast, patients who showed an improvement at discharge had a median hospitalization duration of 14 (IQR: 8-19) days. Amongst 254 patients receiving itraconazole, 208 (82%) had an improvement; 36 (14%) died or had worsening disease. By comparison, 111/147 (76%) patients receiving amphotericin B had an improvement; 33/147 (22%) died or had worsening disease. The fatality rate amongst patients receiving amphotericin B tended to be higher but the difference did not reach statistical significance ($p=0.054$, Fisher's exact test).

2.4.5 Predictors of poor outcome

The ten admission covariates used to assess the prediction of outcome are listed in Table 2.3. The most frequently missing covariates were IDU and presence of skin lesions (both missing in 5%); the other covariates were missing in <2%. All covariates were jointly non-missing and outcome assessable in 411 (80%) patients. The results of the logistic regression are displayed in Table 2.3. All pre-defined covariates except for cough, dyspnoea (highly significant in univariate

analysis, borderline significant in multiple logistic regression), and hemoglobin were significant predictors of outcome in both univariate and multiple logistic regression analyses. Multiple logistic regression was based on multiple imputation of missing data but a complete case analysis gave highly consistent results. The only difference was that dyspnoea also reached statistical significance ($p=0.043$) in the multiple regression analysis. If patients with worsening disease were also regarded as non-assessable, i.e. only patients with death remained in the 'poor outcome' group, results were also consistent, except that the presence of skin lesions was no longer significant in the adjusted analysis, $OR=0.70$ (95% $CI:0.33-1.48$), $p=0.35$.

Table 2.3: Covariates at hospital admission and association with poor outcome in 513 patients with penicilliosis

Characteristics	All patients (N=513)	Good outcome ^a (N=347)	Poor outcome ^b (N=143)	Univariate effect ^c : OR (95% CI)	Adjusted effect from multiple logistic regression ^d : OR (95% CI)
IVDU	332/485 (68%)	208/331 (63%)	108/134 (81%)	2.46 (1.52 to 3.98), p <0.001	2.97 (1.67 to 5.29), p <0.001
Days of illness ^e	n=502 15(7,30)	n=341 15(10,30)	n=138 14(7,30)	0.80 (0.69 to 0.92), p=0.003	0.77 (0.65 to 0.92), p=0.004
History of fever	436/502 (87%)	315/342 (92%)	106/138 (77%)	0.28 (0.16 to 0.50), p <0.001	0.28 (0.14 to 0.55), p <0.001
Respiratory rate ^f (breaths/minute)	n=504 24(22,28)	n=338 24(22,28)	n=143 28(24,38)	3.47 (2.52 to 4.76), p <0.001	3.02 (2.08 to 4.40), p <0.001
Cough	205/513 (40%)	143/347 (41%)	51/143 (36%)	0.79 (0.53 to 1.18), p=0.25	0.66 (0.40 to 1.10), p=0.11
Dyspnoea	61/513 (12%)	22/347 (6%)	36/143 (25%)	4.97 (2.80 to 8.82), p <0.001	2.07 (0.99 to 4.35), p=0.05
Skin lesions	346/487 (71%)	252/337 (75%)	81/128 (63%)	0.58 (0.38 to 0.90), p=0.01	0.54 (0.31 to 0.93), p=0.03
Absolute Lymphocyte count ^e (cells/ μ L)	n=513 399.0(232.3,699.6)	n=347 343.1(221.6,595.4)	n=143 601.8(313.7,1231.3)	1.36 (1.18 to 1.58), p <0.001	1.19 (1.01 to 1.40), p=0.04
Platelet count ^e ($\times 10^3$ cells/ μ L)	n=509 82(42,150)	n=344 93.1(46.9,165.3)	n=142 62.5(33.8,102.3)	0.73 (0.63 to 0.85), p <0.001	0.72 (0.61 to 0.86), p <0.001
Hemoglobin ^g (g/dL)	n=508 8.0(6.0,9.8)	n=345 8.0(6.0,9.7)	n=140 8.0(5.9,10.3)	1.03 (0.97 to 1.08), p=0.31	1.02 (0.95 to 1.09), p=0.59

Summary statistic is absolute count (%) for categorical variables and median (IQR) for continuous data. IVDU, intravenous drug user; OR, odds ratios. ^aDefined as an improvement at hospital discharge with negative repeat culture, resolution of fever, skin lesions, and/or symptoms; ^bDefined as death or worsening disease at discharge; ^cBased on patients with non-missing outcome and covariate; ^dBased on multiple imputation; ^eOR correspond to the effect of a 2-fold higher value; ^fOR correspond to the effect of a +10 breaths/minute higher respiratory rate; ^gOR correspond to the effect of a +1 g/dL higher hemoglobin value

2.4.6 Time to treatment initiation

The time-to-treatment-initiation was significantly shorter for patients with skin lesions than those without, HR=3.78 (95%CI: 2.96-4.84), $p<0.001$, and the estimated median time-to-treatment-initiation was 1 (IQR:0-2) day after admission for patients with skin lesions compared to 5 (IQR:3-8) days for those without skin lesions. There was no evidence that fever affected time-to-treatment-initiation, HR=0.91 (95%CI: 0.66-1.24), $p=0.54$. When all covariates were jointly included in a multiple Cox regression model, only presence of skin lesions was significantly associated with a more rapid treatment initiation, HR=3.27 (95%CI: 2.51-4.27), $p<0.001$.

2.5 Discussion

This is a large penicilliosis case-review that spans the ART era in Vietnam. New epidemiological and clinical data is reported on an emerging opportunistic mycosis from an endemic region that faces one of the fastest growing HIV epidemics in the world. The seasonality of penicilliosis reported from Thailand [30] is also demonstrated for Vietnam. Important and easily measurable predictors of outcome in penicilliosis are identified for the first time. Albeit penicilliosis cases in this study do not represent true incidence of disease, they provide estimates of penicilliosis burden (4.4% amongst hospitalized patients with AIDS) and mirrored the trends in AIDS admissions at the HTD over more than a decade. Both penicilliosis and AIDS trends reflect the HIV/AIDS epidemic

in Vietnam with an estimated number of people living with HIV increased from 120,000 to 310,000 since 2000 [127]. The fall in penicilliosis and AIDS incidence at the HTD after 2007 may be a reflection of the successful roll-out of the ART programme in Vietnam. In partnership with international agencies, Vietnam has made considerable progress in the national response to HIV, including launching of the National Strategy on HIV/AIDS Prevention and Control in 2005 and decentralizing its HIV management approach by expanding provincial AIDS centers to 58 of 64 provinces in Vietnam [138]. This may have reduced the numbers of patients referred to the HTD. Alongside this, with increased awareness and experience amongst physicians, penicilliosis is often being treated in outpatient settings, further reducing the numbers of admissions.

Penicilliosis has been associated with agricultural exposure to plants and animals in the rainy season [53]. The number of cases doubled during the wet months in Chiang Mai [30], suggesting that precipitation facilitates an expansion of environmental reservoirs of *P. marneffei*. In this study there was an approximately 30% increase in penicilliosis cases during the rainy months of May to November in HCMC, whereas cryptococcosis and AIDS admissions showed no seasonal pattern. This study demonstrated the seasonality of penicilliosis and should re-energize research interest in the ecological niche of *P. marneffei*.

The in-hospital case fatality rate of penicilliosis at the HTD was 20%, which increased to 28% if patients with worsening disease at discharge were included. This fatality rate was observed despite initiation of appropriate antifungal therapy

in 50% of patients who subsequently died. More patients treated with amphotericin B than those treated with itraconazole had a poor outcome, but the difference did not reach statistical significance, and amphotericin B may have been chosen for patients with more severe disease. In both complete-case and multiple-imputation analyses, IDU, shorter history, absence of fever or skin lesions, higher respiratory rates, higher absolute lymphocyte and lower platelet counts independently predicted poor outcome. IDU is an established independent risk factor for death and/or development of AIDS. The association of shorter illness and poor outcome suggests differences in disease courses and host pathogen factors are contributing to outcome, rather than delay in seeking care. Absence of fever in patients with systemic mycosis may be a sign of profound immune-suppression, which is itself a poor prognosis. However, the presence of fever may be advantageous in that it stimulates investigation and admission, and subsequently earlier diagnosis and treatment. Similarly presence of skin lesions is protective since it results in more rapid initiation of empirical antifungal treatment. The analysis of time-to-antifungal-initiation in association with all admission-covariates showed that only presence of skin lesions (not fever) was significantly associated with a more rapid time-to-antifungal-initiation. Respiratory rate, an easily measureable and objective physical sign, was an independent predictor of outcome, unlike cough and dyspnoea. The finding that higher rather than lower lymphocyte counts predicted poor outcome is unexpected and should be further investigated. It is possible that lymphocyte count is not a good outcome predictor at such low numbers and over a relatively

narrow range. As in other infectious diseases, thrombocytopenia was not a predictor of poor outcome [134,135]; however, admission hemoglobin level perhaps did not predict outcome because patients with symptomatic anaemia received blood transfusion. It was not possible to ascertain follow up information beyond hospital discharge on these patients due to a limitation in the current medical record system at the HTD, which generates a different hospital number for every inpatient visit from the same patient and does not link with the outpatient care.

Notwithstanding the inherent limitations of a retrospective study design including confounding and selection bias, this study sheds new light on the epidemiology and clinical prognosis of penicilliosis. The previously reported seasonality of penicilliosis has been confirmed in this large dataset. Better understanding of the ecological niche of *P. marneffei* may shed light on the modes of disease transmission and may bring about new strategies for disease prevention. The in-hospital mortality remains high and is associated with IDU, shorter illness duration, absence of fever or skin lesions, respiratory difficulty, and the degree of thrombocytopenia. These and other prognostic factors should be validated in prospective studies to guide treatment strategies for this emerging opportunistic infection, particularly in disease-endemic and resource-poor settings.

CHAPTER 3

Environmental Predictors and Incubation Period of HIV-Associated *Penicillium marneffe* Infection in Ho Chi Minh City, Vietnam

3.1 Abstract

Background

The factors determining the seasonality of *P. marneffe* infection are unknown and may yield critical insights into possible reservoirs or modes of acquisition. The incubation period is also unknown due to lack of knowledge on the exposure source in humans.

Methods

This study investigated the association between temperature, humidity, wind, precipitation and *P. marneffe* and *Cryptococcus neoformans* admissions to the Hospital for Tropical Diseases in Ho Chi Minh City, Vietnam from 2004-2010. Multivariate regression modeling was used to identify factors associated with *P. marneffe* and *C. neoformans* admissions. The *P. marneffe* incubation period was estimated by considering profile likelihoods for different exposure-to-admission delays.

Results

A total of 719 HIV infected patients with *P. marneffe* infections and 1,598 HIV infected patients with *C. neoformans* infections were hospitalised during the study period. *P. marneffe* admissions were strongly associated with humidity ($p < 0.001$), and not with precipitation, temperature, or wind speed. *C. neoformans* admissions were not seasonal and were associated with maximum wind speed. *P. marneffe* admissions were more common relative to *C. neoformans*

admissions during months of high ($\geq 85\%$) humidity, odds ratio = 1.49 (95% confidence interval: 1.10 - 2.01). Maximum likelihood estimation suggested an incubation period of 1 week (95%CI: 0 - 3 weeks) for acute disseminated penicilliosis.

Conclusions

Humidity, rather than precipitation, was found to be the most important environmental predictor of *P. marneffei* admissions. Humidity may drive exposure by facilitating fungal growth or spore release in the environment. Modeling data suggest an incubation period of 1 to 3 weeks for acute disseminated disease in susceptible patients.

3.2 Introduction

Critical aspects of the epidemiology of *P. marneffei* infection have yet to be elucidated, including its environmental reservoirs, mode of acquisition, and incubation period. The incidence of *P. marneffei* infections is closely correlated with the HIV prevalence in Chiang Mai, Thailand [3,15] and with HIV/AIDS admissions in Ho Chi Minh City, Vietnam [Chapter 2]. In addition, *P. marneffei* infections were shown to vary seasonally with more infections occurring during the rainy months in Chiang Mai and in Ho Chi Minh City (HCMC), whereas *C. neoformans* infections were non-seasonal [30]. However, specific seasonal drivers of *P. marneffei* infections such as temperature, humidity, precipitation, and wind speed have not been studied.

One case-control study identified agricultural exposure to plants and animals during the rainy season as an important risk factor for *P. marneffei* infection, but not exposure to the soil-burrowing bamboo rat, the only known non-human natural host of *P. marneffei* [29], suggesting that humans and bamboo rats may acquire the infection from a common soil reservoir. However, as *P. marneffei* cases occur both in rural and urban settings, it is unclear whether infection ensues from exposure to an immediate soil reservoir, wind-blown spores, construction related activities (especially in urban settings), or a combination of these factors. Analysis of specific seasonal drivers that influence such factors can provide clues to these questions. The objective of this study was therefore to examine the association between *P. marneffei* hospital admissions and a set of

environmental variables, including precipitation, temperature, humidity, and wind speed. Hospital admissions data for *P. marneffe* and *C. neoformans* at the Hospital for Tropical Diseases (HTD) in HCMC in Vietnam from 2004 to 2010 was examined in relation to high resolution meteorological and HIV admissions data from HCMC for the corresponding period. The aim was to identify factors that could account for the observed seasonality of *P. marneffe* infection. A Conditional estimate of the *P. marneffe* incubation period was also generated, which has been inaccessible to direct study due to the lack of serological data and the lack of knowledge of the source of exposure, by incorporating different exposure-to-admission delays in our models and comparing the goodness-of-fit of these models.

3.3 Methods

3.3.1 Study population and data collection

The study included all patients admitted with *P. marneffe* infection, *C. neoformans* infection, and HIV/AIDS-related illness to the HTD from January 2004 to June 2010. The HTD is the largest infectious disease referral hospital in southern Vietnam, caring for greater than 5,000 HIV infected patients annually. *P. marneffe* and *C. neoformans* cases were identified from hospital microbiology records and were defined as a compatible illness in which *P. marneffe* or *C. neoformans* was isolated from blood, skin scrapings, cerebrospinal fluid, bone marrow, lymph node, and/or other bodily fluids. Standard culture techniques were

used and have been described elsewhere [128], as were data collection details [Chapter 2].

Daily weather data from the HCMC weather station (latitude = 10.81, longitude = 106.66) for the 2004 to 2010 period were extracted from the website TuTiempo.net, which compiles global climactic data that have been used in epidemiological studies [139,140]. Weather variables included in this analysis were: precipitation (mm); mean humidity (%); visibility (km); minimum, maximum, and mean temperature (°C); wind speed (km/h); and maximum sustained wind speed (km/h). All data were double entered into Microsoft Excel 2008. The study was approved by the Scientific and Ethical Committee of the HTD.

3.3.2 Statistical analysis

P. marneffe and *C. neoformans* admissions were aggregated by week and by month. Units of aggregation were selected to allow sufficient resolution to generate a conditional estimate of the incubation period (in weeks), as well as sufficient sample sizes to detect an annual trend (in months). Weather variables were averaged (means of temperature, humidity, visibility, and wind speed) or were summed (total precipitation) over the corresponding units of time. HIV admission numbers were aggregated by month. Univariate and multivariate negative binomial regressions (to account for over-dispersion of count data) were performed, with meteorological factors as the independent variables, by week and by month to identify factors associated with *P. marneffe* and *C. neoformans*

admissions. Factors found to be significantly associated with *P. marneffe* admissions in univariate analyses were included in multivariate regression models. *P. marneffe* and *C. neoformans* cases were also stratified by low (< 70%), intermediate (70-84%), and high ($\geq$ 85%) monthly humidity to evaluate the odds of *P. marneffe* relative to *C. neoformans* admissions at different levels of humidity. The cutoffs were chosen to allow approximately equal case counts in the low and high categories.

The dates of penicilliosis disease onset were estimated by subtracting the patient reported duration of symptomatic illness from the date of admission. The dates of exposure were estimated by subtracting a range of hypothetical incubation periods (0 - 7 weeks) from the dates of symptom onset, and from the dates of hospital admission. Negative binomial regression models were fitted to examine hypothetical dates of *P. marneffe* exposure. By examining the likelihood scores corresponding to models fitted with different assumed incubation periods, a conditional estimate of the *P. marneffe* incubation period was generated (conditional on the assumption that pathogen exposure is directly linked to climate variables). A 95% confidence interval (CI) was calculated by standard methods of likelihood profiling, and included all values that yielded log-likelihood scores within 1.92 units of the maximum score [141]. All statistical analyses were conducted using the statistical software R, version 2.12.1 [142]. Regressions were performed using the glm.nb function in the MASS package, with a log link function [143]. We performed a likelihood ratio test to determine that the negative

binomial regression model was required instead of a standard Poisson model, due to over-dispersion in the count data. We examined residuals and found that errors were not skewed across seasons.

3.4 Results

This study included 719 HIV infected patients admitted with *P. marneffe* infections, and 1,598 HIV infected patients admitted with *C. neoformans* infections between 2004 and 2010. The clinical features of the patients with *P. marneffe* infection have been described in detail in chapter 2. *P. marneffe* admissions peaked annually during the rainy season (May to November) and decreased during the dry season (December to April). *C. neoformans* admissions did not show a seasonal trend. The associations between *P. marneffe* and *C. neoformans* admissions and environmental variables are reported in Table 3.1. Amongst all environmental variables examined, *P. marneffe* admissions were associated with humidity ($p=0.0004$) and precipitation ($p=0.001$) in univariate regression analyses. The association between *P. marneffe* admissions and humidity was significant when examined at the weekly ($p=0.0004$) and monthly levels ($p=0.002$). The association between *P. marneffe* admissions and precipitation was also significant at the weekly ($p=0.001$) and monthly levels ($p=0.004$). Other weather variables, including minimum, maximum, and mean temperature, visibility, wind speed, and maximum sustained wind speed were not significantly associated with *P. marneffe* admissions. *C. neoformans* admissions

were significantly associated with low maximum wind speeds ($p=0.026$), but not with other environmental variables.

Table 3.1: Univariate and multivariate associations between monthly *Penicillium marneffe* admissions, *Cryptococcus neoformans* admissions, HIV/AIDS-related admissions, total admissions, and environmental variables, Ho Chi Minh City, Vietnam, 2004-2010

Variable	<i>P. marneffe</i>				<i>C. neoformans</i>
	Univariate <i>P</i>	β (SE)	Multivariate <i>P</i>	β (SE)	Univariate <i>P</i>
Mean Humidity (%)	0.0015	0.023 (0.0072)	0.00082*	0.023 (0.0069)	0.87
Precipitation (mm)	0.0040	0.0012 (0.00041)	0.30**	0.00055 (0.00053)	0.46
Min temp (°C)	0.054	0.077 (0.040)			0.081
Max temp (°C)	0.18				0.43
Mean temp (°C)	0.86				0.28
Mean visibility (km)	0.25				0.92
Mean wind speed (km/h)	0.73				0.19
Max wind speed (km/h)	0.57				0.026
HIV admissions	0.0023	0.0015 (0.00040)	0.00014**	0.0017 (0.00052)	0.071
Total admissions	0.011	0.00021 (0.000063)	0.42**	0.000084 (0.00010)	0.27

Statistically significant associations are in **bold**.

* *P* value generated by simultaneous examination of humidity and HIV admissions with regard to *P. marneffe* admission.

** *P* values generated by simultaneous examination of the variable in question and humidity with regard to *P. marneffe* admission.

Upon simultaneous examination of humidity and precipitation in a multivariate regression model, only humidity remained significantly associated with *P. marneffe* admissions ($p=0.01$). When humidity and HIV admissions were examined simultaneously, both remained significantly associated with *P. marneffe* admissions ($p=0.0008$ and $p=0.0001$, respectively) [Table 3.1]. When humidity and total admissions to the HTD were examined simultaneously, only humidity was significantly associated with *P. marneffe* admissions ($p=0.036$). The distribution of total *P. marneffe* and *C. neoformans* cases and mean humidity by month, from 2004 to 2010, is illustrated in Figure 3.1. When *P. marneffe* and *C. neoformans* admissions were stratified by monthly humidity (Table 3.2), *P. marneffe* admissions were more common relative to *C. neoformans* admissions during months of intermediate (70-84%) humidity, odds ratio = 1.22 (95% CI: 0.96 - 1.55) and high ($\geq 85\%$) humidity, OR = 1.49 (95%CI: 1.10 -2.01).

The median duration of illness in patients with penicilliosis was 15 days (interquartile range: 7 - 30 days) and was not correlated with humidity ($r^2 = 0.09$). Severity of disease, as measured by the risk of death upon hospital admission (mean = 20%), was also not correlated with humidity ($r^2 = 0.16$). The CD4 cell counts at admission for patients presenting during the rainy season (May to November) and dry season (December to April) were similar (median of 6 and 7 cells/ μ L, respectively; two-tailed T-test $p=0.18$). The date of penicilliosis disease onset was estimated by subtracting the patient reported days of illness from the

date of hospital admission for each patient. The association between the estimated dates of penicilliosis onset and monthly humidity was statistically significant ($p=0.01$); however, the correlation between the calculated dates of penicilliosis onset and humidity was lower ($r=0.28$) than that between the dates of admission and humidity ($r=0.39$), leading us to hypothesize that the patient reported duration of illness may have included illness time attributable to other HIV related infections.

Table 3.2: Odds of *P. marneffei* admission relative to *C. neoformans* admission by low (<70%), intermediate (70-84%), and high ($\geq 85\%$) monthly humidity.

Monthly Humidity (%)	Number of Patients (2004-2009)		Odds Ratio (95% Confidence Interval)
	<i>P. marneffei</i>	<i>C. neoformans</i>	
<70	115	330	Reference (1.0)
70-84	436	1025	1.22 (0.96-1.55)
≥ 85	126	243	1.49 (1.10-2.01)

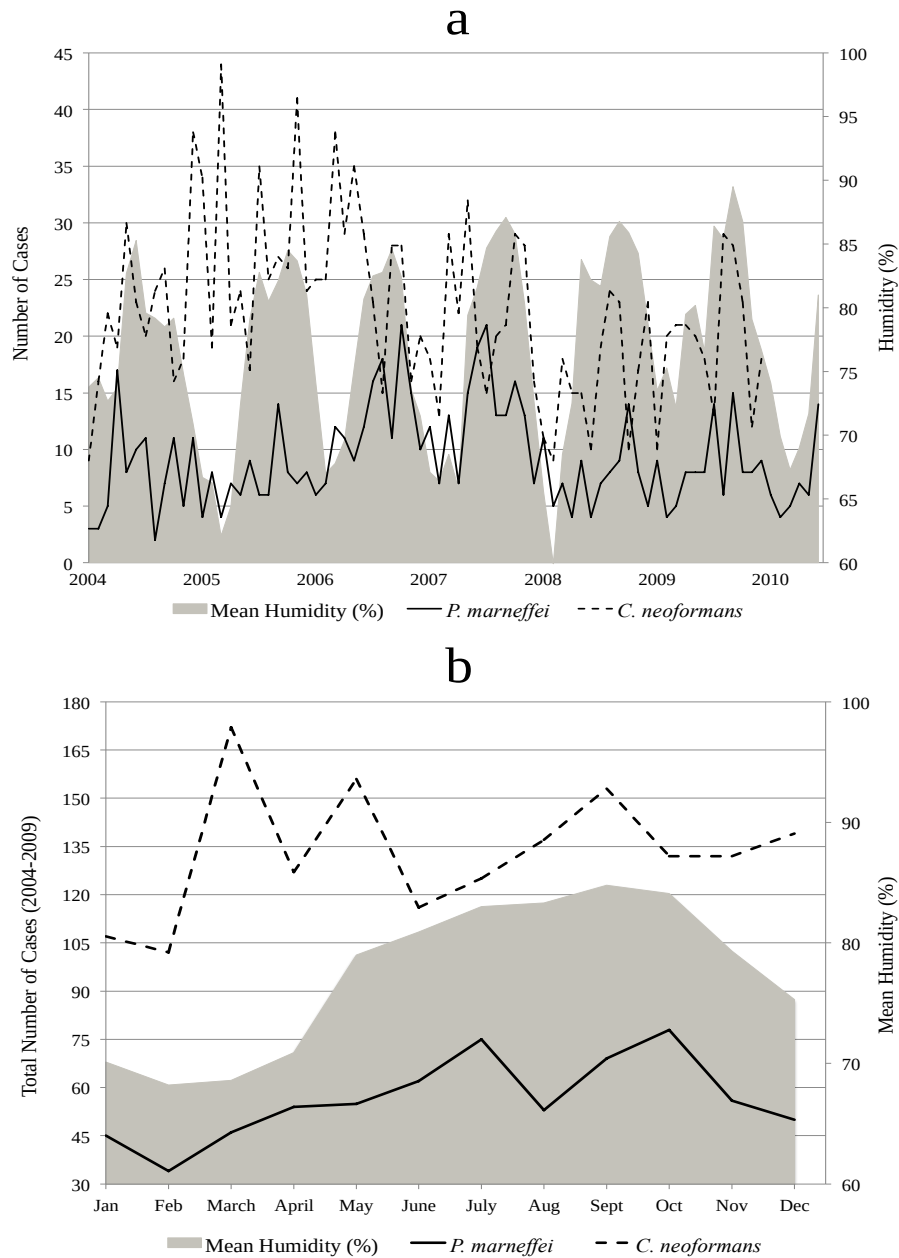


Figure 3.1: Distribution of *P. marneffei* and *C. neoformans* cases in relation to humidity in Ho Chi Minh City

(a) Monthly *P. marneffei* and *C. neoformans* hospital admissions and mean humidity (%), Ho Chi Minh City, Vietnam, 2004-2010. (b) Distribution of total *P. marneffei* and *C. neoformans* cases and mean humidity by month, Ho Chi Minh City, Vietnam, 2004-2009.

Subtraction of hypothetical incubation periods from the dates of admission was a more successful approach to estimating the dates of exposure. We incorporated hypothetical exposure-to-admission delays in our regression model linking *P. marneffe* cases to humidity, and compared the goodness-of-fit of these models for delays from 0 to 7 weeks. In this way we obtained an estimate of the *P. marneffe* incubation period, conditional on the validity of the association with humidity. The likelihood scores for the model with different values of the incubation period are shown in Figure 3.2, and reveal a maximum likelihood estimate of the *P. marneffe* incubation period of 1 week (95%CI: 0 - 3 weeks). The association between *P. marneffe* admissions and precipitation was not significant at any incubation period.

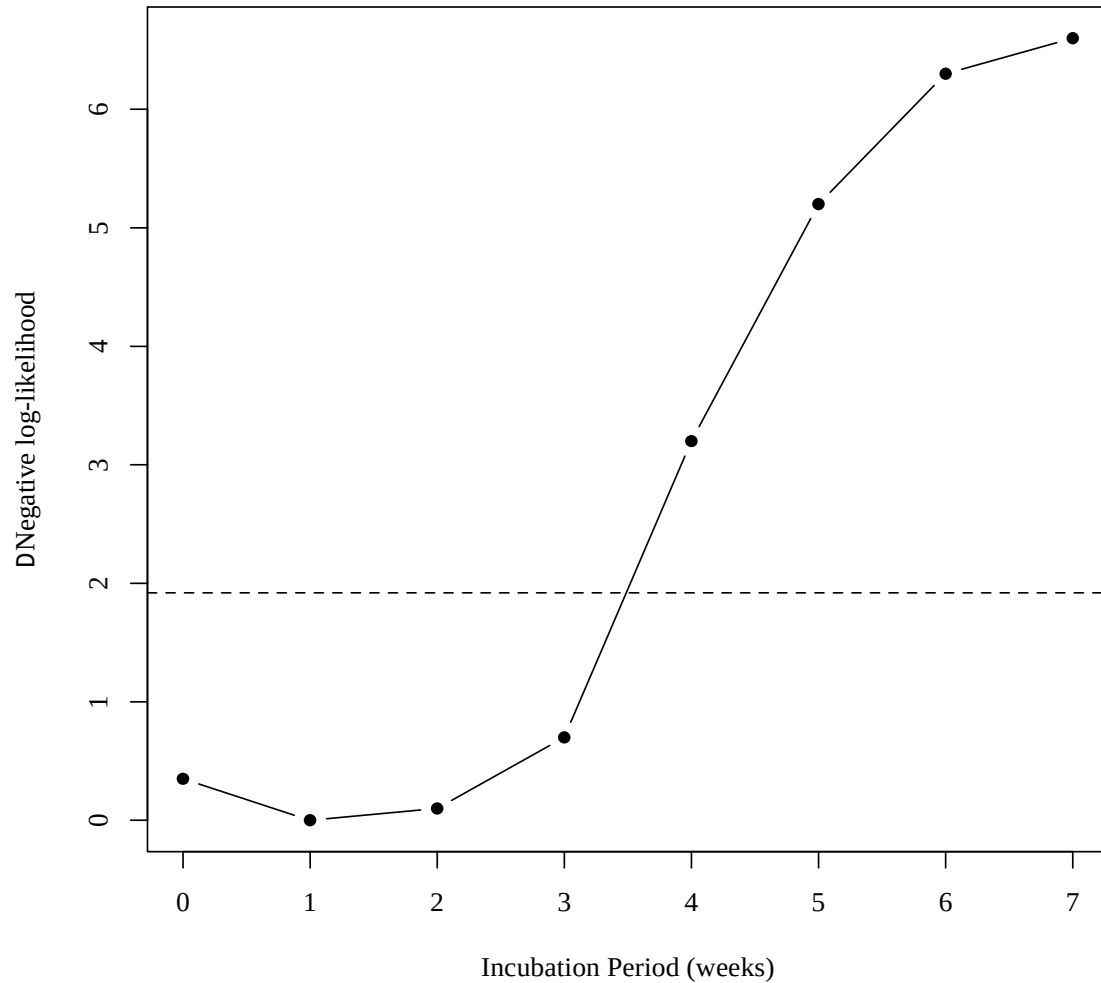


Figure 3.2: Maximum likelihood estimation of the *Penicillium marneffe* incubation period

The plot shows negative log-likelihood values obtained from negative binomial regression of weekly admission data and mean humidity, incorporating different exposure-to-admission delays (corresponding to incubation periods of 0 to 7 weeks). The maximum likelihood value (minimum negative log-likelihood) is 1-week incubation (95% confidence interval: 0–3 weeks), suggesting an incubation period of ≤ 3 weeks. The dashed line corresponds to a difference of 1.92 log-likelihood units from the optimum value; points beneath this line fall within the 95% confidence interval.

3.5 Discussion

The data presented in chapter 2 and a previous study from Chiang Mai have demonstrated the seasonality of penicilliosis infection, but it is unclear what factors are involved in maintaining this seasonal pattern [29]. In this study, a set of environmental variables was considered to determine which could best account for the observed admissions data. Humidity, rather than rainfall, was the strongest predictor of *P. marneffe* hospital admissions. This study is the first to perform a formal statistical analysis of *P. marneffe* seasonality with unaggregated data across seasons and years, and to report associations between infection and environmental factors at high resolution. These findings suggest that humidity may drive *P. marneffe* incidence, perhaps by promoting expansion of the environmental reservoir by facilitating fungal growth or by enhancing spore release or spore dispersal in the environment. These findings are consistent with proposed mechanisms of *P. marneffe* spillover from the environment into susceptible human populations via inhalation of infectious spores or hyphal fragments from a soil reservoir [2,144], and with established risk factors for infection [29,30]. As a dimorphic fungus, *P. marneffe* exists as a mold in the as-yet unidentified environmental reservoir. If humidity does in fact facilitate fungal growth, this may indicate that the mold is growing on air-exposed plant and soil surfaces, whereas rainfall would be expected to influence fungal growth in deeper soil and animal burrows. Therefore, these findings suggest that humidity-mediated fungal growth and/or spore release from air-exposed plant

and soil surfaces may serve as a crucial step in the exposure and infection of immunocompromised human populations in endemic areas.

Expert opinions assert that dimorphic yeasts, including *Histoplasma capsulatum*, *Blastomyces dermatiditis*, and *Coccidioides immitis*, present clinically as either primary pulmonary infections or as disseminated infections arising from reactivation of latent infection upon significant impairment of cellular immunity [145–147]. The strong association of disseminated *P. marneffe*i infections with humidity in this study indicates that disseminated disease can be a manifestation of primary infections in immunocompromised individuals shortly after exposure to the fungus in the environment. This hypothesis is more plausible, as the alternative hypothesis linking immunosuppression and season is unfounded. No difference was observed in CD4 counts at admission during the rainy and dry seasons, and there is currently no evidence to suggest that immunosuppression is seasonal. Longitudinal CD4 data of patients prior to *P. marneffe*i infections were not available as the HIV diagnosis had only been recently made in 80% of patients, and *P. marneffe*i infections were the HIV presenting illness in 25% of patients [Chapter 2]. Such data could inform whether infection was driven primarily by environmental exposure or by a decline in host immune function or both, and can guide prevention strategies. The strong seasonal signal observed here suggests that primary disseminated infection can occur soon after exposure. However, a high background level of disseminated infection was consistently observed in non-humid months, indicating that exposure continues

to occur during non-humid months (but to a lesser degree), and/or that these cases are the result of reactivation of latent infection upon profound HIV related immunosuppression. The relative impact of these different processes to the observed levels of disseminated infection at different times of the year should be studied further. The high level of primary disseminated infection, as suggested by the strong seasonal signal, may indicate that *P. marneffe*i pathophysiology differs somewhat from that of other dimorphic fungi in pathogen, transmission, or host response. For instance, *P. marneffe*i may exhibit an enhanced ability to cause primary disseminated infection, relative to other dimorphic fungi, due to increased virulence upon initial infection. Alternatively, the high levels of primary disseminated infection observed may simply reflect some unique characteristics of the HIV epidemic in Vietnam with regards to the immune status of the population at risk, demographics, geography, setting, and environmental factors. Certainly, the fact that *C. neoformans*, a pathogen with an overlapping target population to *P. marneffe*i, shows no seasonal pattern suggests that *P. marneffe*i may exhibit a unique pathophysiology or route of acquisition or reservoir.

This is the first study to my knowledge to estimate the incubation period of *P. marneffe*i infection. It has thus far been impossible to generate an estimate given the lack of serological data. This study used a modeling approach conditioning on the association with humidity to generate the first estimate of the incubation period. The maximum likelihood estimates suggest an incubation period between 1 and 3 weeks. If we consider the possibility of delay in patient presentation to

the hospital following symptom onset, as well as a possible lag for fungal growth or spore release from the reservoir, we can reasonably presume that the true incubation period is shorter. The finding that the estimated dates of patient-reported illness onset were less associated with humidity than the dates of admission was surprising, as the onset of symptoms should more closely approximate the time of exposure than the date of admission. One likely explanation is that this finding was biased by the inclusion of illness time not directly attributable to *P. marneffe*i infection. With the exception of umbilicated skin lesions that are present in up to 70% of patients, most symptoms of penicilliosis are non-specific. In addition, other opportunistic infections were diagnosed in 56% of the patients and may have confounded the reported days of illness.

An alternative, but perhaps less plausible, explanation for the association between humidity and *P. marneffe*i admissions is that, rather than contributing to exposure to *P. marneffe*i, humidity may stimulate the development of clinical manifestations such as skin lesions in latently-infected individuals that would trigger patients to seek care. This seems less probable however, as skin lesions in disseminated penicilliosis typically develop on relatively open and dry surfaces of the body such as face, neck, chest, back, and extremities with sparing of palms and soles. Moreover, skin lesions in penicilliosis are often preceded by other manifestations of systemic infection that humidity would be unlikely to affect. Further investigation is planned to examine possible associations of skin

lesion development in patients. Assuming that humidity is indeed an indicator of pathogen exposure rather than disease onset, it can be inferred that the approach of subtracting hypothetical incubation periods from the date of hospital admission was more accurate than subtracting the reported duration of illness in approximating the time of exposure, and that the estimated incubation period, given the limitations of this method, is ≤ 3 weeks. The finding that *C. neoformans* admissions were not associated with most environmental variables was expected. The association with low maximum wind speeds may be an artifactual result driven by multiple comparisons in the univariate analyses, as the p value is above the Bonferroni-corrected threshold for significance of 0.005 for 10 comparisons.

This study is limited in that it did not include a metric of agricultural activity or construction work in Ho Chi Minh City, both of which could hypothetically contribute to exposure to *P. marneffe* and confound the results. The study is strengthened by the inclusion of other potential confounders, such as HIV admissions, total admissions, and other weather variables. In addition, the large sample size and detailed admission and weather data over a six year period enabled precise quantification of associations between weather variables and *P. marneffe* admissions, as well as maximum likelihood estimation of the incubation period. These findings should help guide efforts to understand the environmental reservoir and transmission of *P. marneffe*, which may inform the design and timing of prevention strategies for susceptible individuals living in or traveling to

endemic regions of Asia. Earlier HIV diagnosis and placement on antiretroviral therapy, and seasonal antifungal drug prophylaxis for patients with low CD4 counts in the early stages of antiretroviral therapy may prevent penicilliosis in patients presenting very late. Further research to investigate the causal and temporal relationships among humidity, exposure, immunosuppression, symptom onset, and ultimate presentation to the hospital will broaden our understanding of the transmission and pathophysiology of this emerging dimorphic yeast.

CHAPTER 4

Penicillium marneffe Infection – a Putative Zoonosapronotic Disease in Regional Hotspots: Results of a Case Control Study in Vietnam

4.1 Abstract

Background

The disease reservoirs and transmission risks of *P. marneffe* remain unknown although bamboo rats (zoonotic) and/or soil exposure (sapronotic) are both leading suspects.

Methods

We conducted a case-control study to evaluate the exposure, behavioral, and geographical risk factors of penicilliosis in HIV-infected patients in northern and southern Vietnam. For each penicilliosis case, two controls were matched by age (± 5 years), sex, and CD4 count (± 50 cells/ μ L) or disease staging. Analyses were based on conditional logistic regression.

Results

205 cases and 405 controls were recruited between January 2011 and July 2013. In the multivariable analysis, proximity or exposure to tropical plants and exposure to farm animals were independent risk factors of penicilliosis, ORs (95% CI): 1.84 (1.17-2.90), $p=0.008$ and 2.03 (1.18-3.49), $p=0.010$, respectively. In southern Vietnam, case-to-control ratios were the highest in the mountainous regions (Central Highlands, Southeast, and South Central Coast), and the risk of penicilliosis was significantly higher in these regions compared to the Mekong Delta and Ho Chi Minh City (adjusted p -values for all pair-wise comparisons

<0.05). The risk of penicilliosis in those with prior residence or travel history to these regions was also higher, OR (95%CI): 3.15(1.49 – 6.64), $p=0.003$.

Conclusions

This study provides the first epidemiological link between human penicilliosis and geospatial proximity or travel to highland regions, thus informing prophylaxis recommendations for travelers. Based on this work and the literature, we hypothesize that bamboo rats, which are prevalent in the highlands, are a zoonotic reservoir and animal- and tropical-plant-associated soil a saprophytic reservoir of human penicilliosis.

4.2 Introduction

The HIV epidemic has transformed penicilliosis from a rare infection to one of the most common AIDS-defining diagnoses in Asia [3,4,14,40,86,148]. Incidence continues to increase in the changing HIV landscape in mainland China and India [8,9,13,149], with evidence to suggest that the region of endemicity within these countries is expanding [64,150–152]. Disease prevention is hampered by the lack of understanding of disease reservoirs and exposure risk factors. The four species of bamboo rats *Rhizomys sinensis*, *R. pruinosus*, *R. sumatrensis*, and *Cannomys badius* (Figure 4.1) are the only known non-human hosts, with asymptomatic infection prevalence approaching 100% [22–27]. Their geographical distribution generally follows the distribution of human penicilliosis [2,22,23,25]. Using multilocus microsatellite typing, *P. marneffei* isolates from bamboo rats were shown to have similar, and sometimes identical, genotypes to those infecting humans in China, Thailand, and India [23,25,28]. However, a case-control study conducted in a bamboo-rat-abundant region in Chiang Mai, Thailand did not find an association between penicilliosis and exposure or consumption of bamboo rats [29]. The zoonotic link between bamboo rats and human penicilliosis is unclear. If bamboo rats are merely susceptible and not the source of human infection, then *P. marneffei* must have a reservoir in the environment; however, the source remains elusive. The Thai case-control study identified agricultural exposure to animals or plants as a risk factor particularly during the rainy season [29], suggesting a reservoir in soil. However, *P.*

marneffe could not be isolated from soil samples in the residential areas of penicilliosis patients, except occasionally in bamboo rat burrows [22,24,27,47]. *P. marneffe* does not survive beyond several days in natural soil [55,153] and cannot be cultured from the air surrounding the homes of infected patients [15]. Definitive proof that soil is the environmental reservoir for *P. marneffe* is still lacking.

Geographic clustering has been seen in mountainous regions in Chiang Mai, Thailand, in Guangxi and Guangdong, China, and in the Manipur state, India [2,8,9]. This suggests there might be geographical 'hotspots' within the endemic region; however penicilliosis is diagnosed in urban settings from northern to southern Vietnam across remarkably diverse climate and geographical ranges [7,12,14]. The climate in Vietnam varies from humid subtropical in the north to tropical savanna in the south. Three quarters of Vietnam's territory is comprised of hills and mountains spreading from the Northwest to the Central Highland regions; the remaining quarter is comprised of lowlands in the deltas and the coastline [154]. This makes Vietnam an appealing setting to elucidate the geographical risk of human penicilliosis. The exposure risk factors for infection might be very different in more urban environment than in Chiang Mai. In the context of limited understanding of environmental risk factors of penicilliosis, a prospective epidemiological study exploring the exposure risk factors for penicilliosis in Vietnam was performed.



Rhizomys sinensis

Photo by Pham Quang Tung
Source: <http://dongvathoangda.com.vn>



Rhizomys pruinosus

Photo by Phung My Trung
Source: <http://www.vncreatures.net>



Rhizomys sumatrensis

Photo by Omar Ariff
Source: <http://omarariff.photoshelter.com/>



Cannomys badius

Photo by Klaus Rudloff
Source: <http://www.biolib.cz/>

Permission was obtained from all photographers
FOUR SPECIES OF BAMBOO RATS IN ASIA

Figure 4.1: Four species of bamboo rats in Southeast Asia

4.3 Methods

4.3.1 Study design and setting

This is a case-control study designed to investigate the exposure, behavioral, and geographical risk factors for penicilliosis in susceptible patients. Because penicilliosis primarily affects HIV-infected patients with CD4 cell counts of <100 cells/ μ L [5,7,86], we recruited HIV-infected patients from the inpatient and outpatient departments of the National Hospital for Tropical Diseases in Hanoi and the Hospital for Tropical Diseases in Ho Chi Minh City (HCMC). The hospitals are the largest primary, secondary, and tertiary referral centres for infectious diseases and HIV in Vietnam. Together they share a catchment population of 72 of the 91 million people in Vietnam [155] and receive at least 40% of all HIV inpatient referrals in Vietnam [156].

4.3.2 Definition and selection of case patients and sample size

Cases were HIV-infected patients aged ≥ 18 years with incident penicilliosis. Penicilliosis was defined as an illness in which *P. marneffe*i was isolated from blood, skin scrapings, bone marrow, lymph node, and/or other body fluids. HIV diagnosis was made according to national and international guidelines, requiring serum to be reactive to three different HIV antibody tests [157,158]. Patients with recurrent penicilliosis and those with memory impairment who were unable to respond to the study questionnaire (e.g. patients with mental disorders, AIDS dementia, or acute/chronic neurological conditions) were excluded. All patients

with penicilliosis and control patients were evaluated for eligibility by the site investigators, and consenting patients were enrolled. The target sample size of 200 penicilliosis cases was chosen to allow for a robust assessment of 13 candidate risk factors (15 cases per risk factor).

4.3.3 Definition and selection of control patients

Controls were randomly recruited amongst HIV-infected individuals seen at the study centres for routine or acute care. Controls had the same eligibility and exclusion criteria as cases but did not have penicilliosis, i.e. they could not be suspected by clinicians to have penicilliosis based on characteristic skin lesions and had blood culture performed to exclude *P. marneffe*i fungaemia. For each case, two matched controls were recruited within one week to account for known disease variation with seasons and humidity [30,159]. Controls were matched to the corresponding case based on age (± 5 years), sex, and CD4 cell count (± 50 cells/ μ L) or absolute lymphocyte count (± 330 cells/ μ L) or WHO disease stage when CD4 count was not available. The matching scheme was designed to ensure that controls were similar to cases in immune susceptibility and host characteristics. Age and sex may be related to the risk factors being investigated in that younger males may be more likely to hold a job with exposure to soil, e.g. construction work, or to engage in risky behavior, e.g. injection drug use and practices.

4.3.4 Assessment of exposure variables

A standardized questionnaire was used by study nurses who were trained to elicit exposure questions consistently across study participants. The questionnaire covered 13 host and exposure candidate risk factors of interest. These variables (listed in Table 4.1) were selected based on data linking penicilliosis with agricultural exposure to plants and animals [29], to humidity [Chapter 3], and data linking histoplasmosis, another HIV-associated mycosis that infects rodents, with exposure to soil contaminated with bird or bat droppings [49]. To minimize assessment bias, the study nurses were blinded to the assignment of cases and controls and the study hypotheses. Photographs of bamboo rats (Figure 4.1) were shown to all subjects to ensure that history of exposure was correctly elicited.

Table 4.1: The 13 risk variables included in the study questionnaire

Risk variables
1. Antiretroviral therapy
2. Antifungal prophylaxis
3. Cigarette smoking
4. Injection drug use
5. Outdoor occupations (Current or previous job in construction, agriculture, gardening, or long-distant truck driving...)
6. Soil exposure (Living within 100-metre from agricultural/industrial soil-excavation sites or direct occupational exposure to construction work, farming, soil digging, gardening, rubbish collection...)
7. Natural water (Living within 100-metre from or direct occupational exposure to river, lake, pond, canal, ditch)
8. Tropical plants (Living within 100-metre from or direct occupational exposure to any type of bamboo, sugarcane, or rice)
9. Highland plants (Living within 100-metre from or direct occupational exposure to rubber, cashew, coffee or tea)
10. Bamboo rats (Any direct contact or consumption)
11. Farming animals (Direct occupational exposure to pigs, cows, chickens, or ducks)
12. Domestic animals (Frequent contact or caring of dogs, cats, birds, or fishes)
13. Raw animal products (Any consumption of uncooked meats, eggs, milk, blood...)

4.3.5 Mapping of case and control patients

Vietnam is divided into 58 provinces and 5 city municipalities treated as provinces. These provinces are grouped into 8 municipal regions as shown in Figure 4.2A. Patients' residential addresses during the preceding 3 months were collected at the interview. Patients were categorized into municipal regions based on their provinces. Data from HCMC was categorized as a separate region due to the high number of patients recruited within the city limits. Maps of the number of cases, controls, and case-to-control ratios in different geographic regions were visualized using the Quantum Geographic Information System (QGIS) version 1.5 [160].

4.3.6 Analysis of exposure and geographic risks

Univariate and multivariable conditional logistic regression was used to evaluate the candidate risk factors for penicilliosis while accounting for the matching. All 13 pre-defined exposure variables in Table 4.1 were entered into the regression model. If a composite variable (i.e. exposure to tropical plants) was found to independently predict disease, then data of all individual sub-variables (i.e. exposure to bamboo, sugarcane, or rice) would be entered into the multivariate regression model and reanalyzed to identify specific sub-variables that predict disease. To test the regional risk for penicilliosis, conditional logistic regression with region as the only covariate was used. Pair-wise comparisons between regions were adjusted for multiple comparisons using a parametric single-step

method [161]. Patients recruited in northern Vietnam were excluded from this analysis due to the small number of patients recruited in these regions. The number of recruited penicilliosis cases per total HIV population in the southern regions was also summarized. The latest HIV prevalence data (2007) were obtained from the Vietnam Ministry of Health Administration of HIV/AIDS Control [43]. All analyses are complete-case analyses, i.e. no imputation of missing data was performed, and were performed with the statistical software R version 3.2.1[162].

4.3.7 Ethics

The study was approved by the ethical and scientific committees of the two study sites in Vietnam, by the University of Hawaii, USA, and by the University of Oxford Tropical Research Ethics Committee, UK.

4.4 Results

4.4.1 Patient population and characteristics

Recruitment at the two hospitals started in January 2011 in HCMC and in November 2012 in Hanoi (due to later ethical approval). Until July 2013, of 236 penicilliosis cases diagnosed at the two hospitals, 205 cases were recruited (29 of them in Hanoi) along with 405 matched controls. For five cases, it was not possible to identify a second matched control. Two control patients were

recategorised as cases because their blood culture subsequently grew *P. marneffe*. Of 31 cases that failed screening, 25 (80.6%) patients died or were discharged before the penicilliosis diagnosis was made; the remaining 6 patients declined participation. The characteristics of cases and controls are shown in Table 4.2. By design, cases and controls were similar in age, sex, and had similar CD4 and absolute lymphocyte counts. The WHO disease staging was more diverse in controls. All cases and 91% of controls were hospitalized patients; 9% of controls were recruited from outpatient clinics. 84% of controls had an acute opportunistic infection; the list of opportunistic infections is shown in Table 4.2.

Table 4.2: The characteristics of case and control patients

Characteristics	All patients (N=610)	Cases (N=205)	Controls (N=405)
Age (years)	34 (31 – 38)	33 (30 – 38)	34 (31 – 39)
Sex (male)	456 (74.8%)	154 (75.1%)	302 (74.6%)
CD4 (cells/ μ L)	n=194 16.5 (7.0 – 36.0)	n=66 9.0 (5.0 – 18.8)	n=128 25.5 (9.0 – 54.3)
Absolute Lymphocyte (cells/ μ L)	n=585 520 (300 – 750)	n=197 410 (230 – 600)	n=388 570 (380 – 810)
WHO stage	n=606	n=204	n=402
1	3 (0.5%)	0 (0%)	3 (0.7%)*
2	16 (2.6%)	0 (0%)	16 (4.0%)*
3	146 (24.1%)	0 (0%)	146 (36.3%)*
4	441 (72.8%)	204 (100%)	237 (59.0%)
Inpatient	574 (94.1%)	205 (100%)	369 (91.1%)
Outpatient	37 (6.1%)	0 (0%)	37 (9.1%)
Concomitant OIs			
◦ Penicilliosis	205 (33.6%)	205 (100%)	0 (0%)
◦ Non-tuberculosis pneumonia, including PJP	130 (21.3%)	24 (11.7%)	106 (26.2%)
◦ Oral/esophageal candidiasis	90 (14.8%)	14 (6.8%)	76 (18.8%)
◦ Tuberculosis	84 (13.8%)	23 (11.2%)	61 (15.1%)
◦ Cryptococcosis	45 (7.4%)	0 (0%)	45 (11.1%)
◦ Toxoplasmosis	40 (6.6%)	1 (0.5%)	39 (9.6%)
◦ Herpes simplex	12 (2.0%)	3 (1.5%)	9 (2.2%)
◦ AIDS-associated wasting syndrome	23 (3.8%)	2 (1.0%)	21 (5.2%)
◦ Other OIs	73 (12.0%)	22 (10.7%)	51 (12.6%)
◦ No OIs	66 (10.9%)	0 (0%)	66 (16.3%)

Note: Data are in absolute count (%) for categorical variables and median (interquartile range [IQR]) for continuous data; *These control patients had CD4 counts or absolute lymphocyte counts in the same ranges as their matched cases; OIs, opportunistic infections

4.4.2 Results of the exposure risk analysis

The 13 covariates used to assess the risk of penicilliosis and the results of the conditional logistic regression analyses are shown in Table 4.3. Data on fluconazole prophylaxis were missing in 2% of patients; the other 12 co-variables were not missing. In the univariate analysis, cases were significantly less likely to be on antiretroviral therapy, more likely to work in outdoor settings, more likely to live within 100-metre of or have direct occupational contact with tropical plants, and more likely to have direct occupational contact with highland plants and farm animals. However, in the multivariable analysis, only proximity or exposure to tropical plants and exposure to farm animals were independent risk factors of penicilliosis, ORs [95% confidence interval (CI)]: 1.84(1.17-2.90), $p=0.008$ and 2.03(1.18-3.49), $p=0.010$, respectively. The results of the repeat analysis treating exposure to bamboo, sugar cane, and rice as separate variables are shown in Table 4.4. None of these sub-variables were shown to predict penicilliosis in both univariate and multivariable analyses.

Table 4.3: The association of exposure covariates with penicilliosis

Exposure covariates	All patients (N=610)	Cases (N=205)	Controls (N=405)	Univariate effect OR (95% CI), p-value	Multivariate effect OR (95% CI), p-value
Antiretroviral therapy	250/610(41.0%)	72/205(35.1%)	178/405(44.0%)	0.68 (0.47 to 0.97), p=0.04	0.75 (0.50 to 1.13), p=0.17
Fluconazole prophylaxis	61/596 (10.2%)	15/198 (7.6%)	46/398 (11.6%)	0.59 (0.31 to 1.11), p =0.10	0.68 (0.35 to 1.34), p =0.27
Cigarette smoking	413/610(67.7%)	130/205 (63.4%)	283/405 (69.9%)	0.65 (0.42 to 1.01), p =0.06	0.71 (0.43 to 1.18), p=0.19
Injection drug use	232/610 (38.0%)	71/205 (34.6%)	161/405 (39.8%)	0.79 (0.54 to 1.15), P=0.21	0.85 (0.54 to 1.35), p =0.50
Outdoor occupation	263/610 (43.1%)	100/205 (48.8%)	163/405 (40.2%)	1.47 (1.03 to 2.09), p=0.04	1.23 (0.81 to 1.87), p=0.34
Soil exposure	409/610 (67.0%)	143/205 (69.8%)	266/405 (65.7%)	1.22 (0.85 to 1.75), p=0.29	1.06 (0.69 to 1.63), p=0.80
Natural water exposure	285/610 (46.7%)	90/205 (43.9%)	195/405 (48.1%)	0.83 (0.58 to 1.19), p=0.31	0.76 (0.51 to 1.13), p=0.18
Tropical plant exposure	218/610 (35.7%)	90/205 (43.9%)	128/405 (31.6%)	1.75 (1.22 to 2.56), p=0.002	1.84 (1.17 to 2.90), p=0.008
Highland plant exposure	49/610 (8.0%)	25/205 (12.2%)	24/405 (5.9%)	2.25 (1.24 to 4.01), p=0.008	1.71 (0.86 to 3.41), p=0.13
Bamboo rat exposure or consumption	6/610 (1.0%)	3/205 (1.5%)	3/405 (0.7%)	2.00 (0.40 to 9.91, p=0.40	1.71 (0.33 to 8.87), p=0.53
Farming animal exposure	93/610 (15.2%)	40/205 (19.5%)	53/405 (13.1%)	1.60 (1.02 to 2.51) p=0.04	2.03 (1.18 to 3.49) p=0.010

Exposure covariates	All patients (N=610)	Cases (N=205)	Controls (N=405)	Univariate effect OR (95% CI), p-value	Multivariate effect OR (95% CI), p-value
Domestic animal Exposure	170/610 (27.9%)	57/205 (27.8%)	113/405 (27.9%)	1.01 (0.67 to 1.51) p=0.97	1.39 (0.87 to 2.22) p=0.17
Raw animal product consumption	411/610 (67.4%)	132/205 (64.4%)	279/405 (68.9%)	0.82 (0.57 to 1.18) p=0.28	0.91 (0.60 to 1.37) p=0.64

Note: Summary statistic is absolute count (%); OR, odds ratio based on conditional logistic regression. Multivariate analysis is a complete case analysis excluding 7 cases and 7 controls with missing fluconazole prophylaxis data. For definition of exposure covariates, refer to Table 4.1.

Table 4.4: Association of exposure to bamboo, sugar cane, and rice as separate variables on penicilliosis

Exposure covariates	All patients (N=610)	Cases (N=205)	Controls (N=405)	Matched univariate effect OR (95% CI)	Matched multivariate effect OR (95% CI)
Antiretroviral therapy	250/610(41.0%)	72/205(35.1%)	178/405(44.0%)	0.68 (0.47 to 0.97), p=0.035	0.74 (0.49 to 1.11), p=0.144
Fluconazole prophylaxis	61/596 (10.2%)	15/198 (7.6%)	46/398 (11.6%)	0.59 (0.31 to 1.11), p =0.099	0.70 (0.35 to 1.37), p =0.294
Cigarette smoking	413/610(67.7%)	130/205 (63.4%)	283/405 (69.9%)	0.65 (0.42 to 1.01), p =0.056	0.70 (0.42 to 1.16), p=0.162
Injection drug use	232/610 (38.0%)	71/205 (34.6%)	161/405 (39.8%)	0.79 (0.54 to 1.15), P=0.213	0.86 (0.54 to 1.35), p =0.502
Outdoor occupation	263/610 (43.1%)	100/205 (48.8%)	163/405 (40.2%)	1.47 (1.03 to 2.09), p=0.035	1.28 (0.83 to 1.96), p=0.263
Soil exposure	409/610 (67.0%)	143/205 (69.8%)	266/405 (65.7%)	1.22 (0.85 to 1.75), p=0.288	1.13 (0.74 to 1.73), p=0.580
Natural water exposure	285/610 (46.7%)	90/205 (43.9%)	195/405 (48.1%)	0.83 (0.58 to 1.19), p=0.311	0.77 (0.52 to 1.16), p=0.214
Bamboo exposure	218/610 (35.7%)	90/205 (43.9%)	128/405 (31.6%)	1.23 (0.80 to 1.88), p=0.342	1.04(0.64 to 1.70), p=0.873
Rice exposure	218/610 (35.7%)	90/205 (43.9%)	128/405 (31.6%)	1.39 (0.89 to 2.16), p=0.148	1.26 (0.74 to 2.14), p=0.398
Sugar cane exposure	218/610 (35.7%)	90/205 (43.9%)	128/405 (31.6%)	1.65 (0.88 to 3.13), p=0.121	1.82 (0.89 to 3.75), p=0.103

Exposure covariates	All patients (N=610)	Cases (N=205)	Controls (N=405)	Matched univariate effect OR (95% CI)	Matched multivariate effect OR (95% CI)
Highland plant exposure	53/610 (8.0%)	28/205 (13.7%)	25/405 (6.2%)	2.43 (1.37 to 4.31), p=0.00238	1.69 (0.86 to 3.41), p=0.13
Bamboo rat exposure or consumption	6/610 (1.0%)	3/205 (1.5%)	3/405 (0.7%)	2.00 (0.40 to 9.91, p=0.396	1.86 (0.36 to 9.63), p=0.460
Farming animal exposure	93/610 (15.2%)	40/205 (19.5%)	53/405 (13.1%)	1.60 (1.02 to 2.51), p=0.042	1.94 (1.13 to 3.32), p=0.016
Domestic animal Exposure	170/610 (27.9%)	57/205 (27.8%)	113/405 (27.9%)	1.01 (0.67 to 1.51), p=0.973	1.32 (0.83 to 2.1), p=0.245
Raw animal product consumption	411/610 (67.4%)	132/205 (64.4%)	279/405 (68.9%)	0.82 (0.57 to 1.18), p=0.283	0.91 (0.60 to 1.36), p=0.641

Note: Summary statistic is absolute count (%); OR, odds ratio based on conditional logistic regression. Multivariate analysis is a complete case analysis excluding 7 cases and 7 controls with missing fluconazole prophylaxis data. For definition of exposure covariates, refer to Table 4.1.

4.4.3 Mapping of cases and controls and geographical risk analysis

Geographical data was available for 204 cases and 403 controls. Figure 4.2 (B) and (C) present the regional distribution of cases and controls in Vietnam's 8 municipal regions, with data from HCMC represented as a separate region. Due to the earlier start of recruitment in southern Vietnam, 175 (86%) cases and 348 (86%) controls were recruited in southern Vietnam, with 59 (29%) cases and 186 (46%) controls recruited in HCMC. Figure 4.2 (D) presents the regional case-to-control ratios in southern Vietnam. Case-to-control ratio demonstrated regions with a higher or lower-than expected number of cases relative to controls, with 0.5 as the expected case-to-control ratio given that 2 controls were recruited for every case. Ratios were highest in the Central Highlands (2.18), South Central Coast (1.20), and Southeast region (0.87). Ratios were lowest in HCMC (0.32) and the Mekong Delta (0.25). Conditional logistic regression demonstrated that patients in the Central Highlands, Southeast, and South Central Coast regions were significantly more likely to develop penicilliosis than those residing in the Mekong Delta or HCMC (multiplicity-corrected $p < 0.05$ for all pair-wise comparisons) (Table 4.4). Other pair-wise regional comparisons did not reach conventional statistical significance though there was some evidence that the risk was also higher in the Central Highlands compared to the Southeast region ($p = 0.06$). Table 4.5 shows the number of recruited penicilliosis cases relative to

the HIV populations in the southern regions with the highest relative case numbers being in the Central Highlands.

Latent infection can last for years, and there is extensive urbanization and migration into HCMC, therefore, an *ad hoc* follow-up phone interview of participants residing in HCMC and the Mekong Delta was carried out, asking whether they had previously lived in or had ever traveled (at minimum several days) to any of the three identified risk regions. The interviewing nurses were blinded to the patients' status as case or control. A total of 69/77 (90%) cases and 82/254 (32%) controls responded to the phone interview, and patients who had lived or traveled to these risk regions had significantly higher risk of penicilliosis [55/69 (81%) cases and 47/82 (57%) controls, OR (95%CI): 2.90 (1.33–6.59), Fisher's exact test: $p=0.005$].

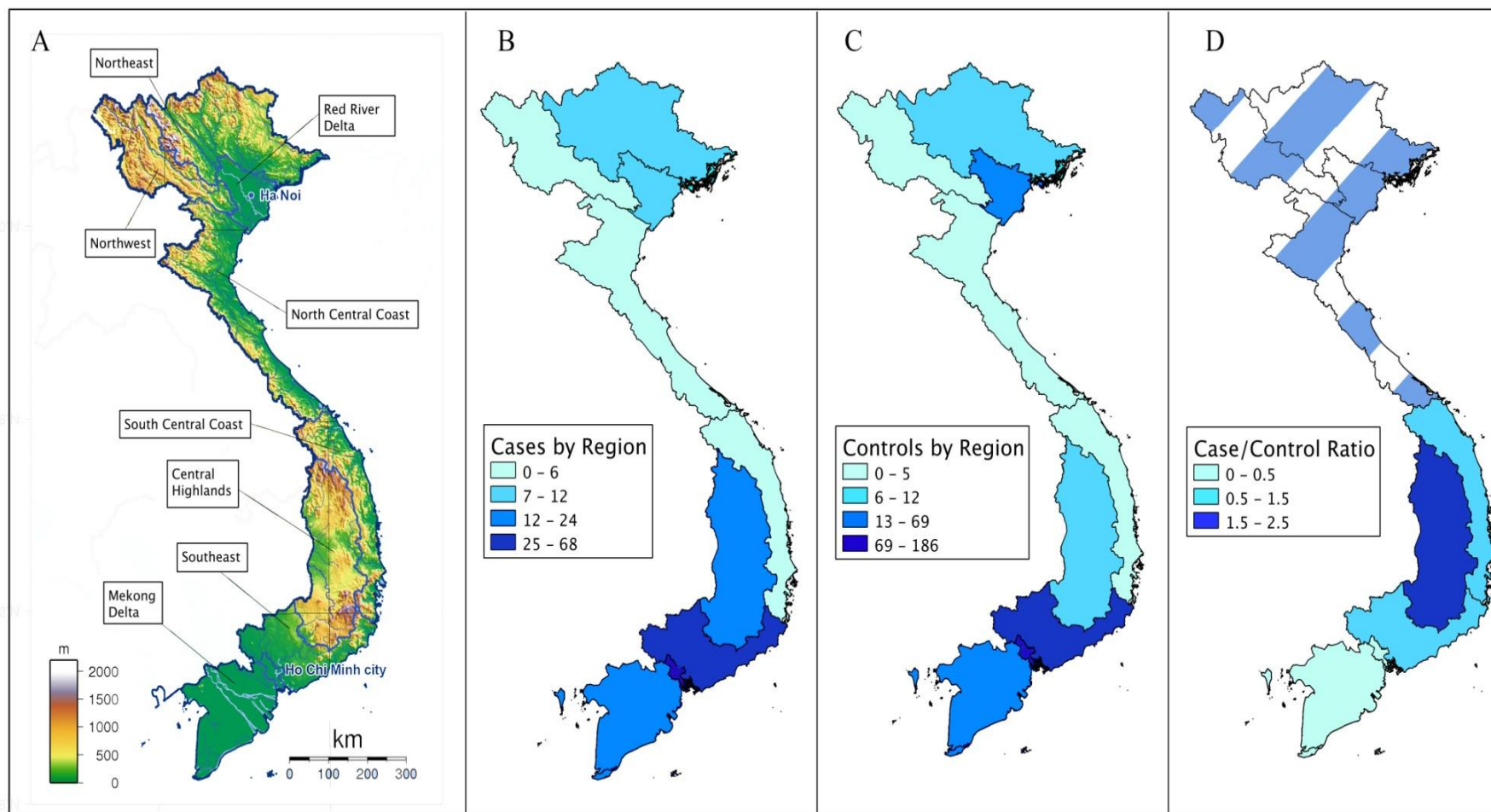


Figure 4.2: Regional distribution of penicilliosis cases and controls in Vietnam

Figure 4.2. Regional maps of Vietnam and distribution of recruited cases and controls (from left to right) (A) Municipal regions of Vietnam with locations of recruitment centers in Hanoi and Ho Chi Minh City. (B) Number of cases by region and (C) Number of controls by region. Regions in maps B and C are shown in one of four color categories based on quartiles, with darker color indicating a higher number of cases or controls in that region. (D) Ratio of cases to controls by region. Regions in map D are placed in one of three color categories, with darker color representing a higher number of cases relative to controls for that region. A case-to-control ratio of 0.5 is expected as 2 controls were recruited for every case. A ratio lower than 0.5 indicates a lower-than-expected number of cases relative to controls. A ratio higher than 0.5 indicates a greater-than-expected number of cases relative to controls. Data for northern regions of Vietnam is excluded from map D, as study subject enrollment from these regions was insufficient to draw meaningful conclusions from analysis. Both cases and controls are concentrated in Southern Vietnam where most subjects were recruited. The ratios of cases to controls are higher in the Central Highlands and the adjacent Southeast and South Central Coast regions than in Ho Chi Minh City and the Mekong Delta, with the highest ratio in the Central Highlands region.

Table 4.5: Case and control numbers and risk of penicilliosis per region (Northern regions excluded)

Region	Number of cases	Number of control	Case-control ratio#	OR (95%CI), p-value *
Mekong	17	69	0.25	- Reference category
HCMC	60	185	0.32	1.31 (0.56-3.03); p=0.91
Southeast	68	78	0.87	3.42 (1.44-8.10); p=0.001
South Central Coast	6	5	1.20	8.76 (1.25-61.56); p=0.02
Central Highlands	24	11	2.18	11.36 (2.92-44.24); p<0.0001

#Total number of cases: 175, controls: 348, ratio: 0.50.

*OR, odds ratio based on conditional logistic regression. 95%CI and p-values are multiplicity-adjusted for pair-wise comparisons between all regions. Comparisons to the Mekong Delta are shown above.

The remaining pair-wise comparisons are [OR (95%CI), p-value]:

- Comparisons to HCMC: Southeast OR=2.62(1.41-4.85), p=0.0003; South Central Coast OR= 6.71 (1.04-43.45), p=0.04; Central Highlands OR=8.70 (2.61-28.97), p<0.0001.
- Comparisons to Southeast: South Central Coast OR= 2.57 (0.40-16.52), p=0.63; Central Highlands OR=3.32 (0.98-11.26), p=0.06.
- Comparison to Central Highlands: South Central Coast OR= 0.77 (0.12-5.06), p=1.00.

Table 4.6: Recruited penicilliosis cases per regional HIV population in southern Vietnam

Region	Penicilliosis cases	Estimated HIV Population in 2007	Cases/100,000 HIV population
Mekong Delta	17	103,615	16
Ho Chi Minh City	60	72,566	83
Southeast	68	52,132	130
South Central Coast	6	11,878	51
Central Highlands	24	12,123	200

4.5 Discussion

Penicilliosis is an emerging opportunistic mycosis of growing regional and international importance. The source of infection remains ambiguous despite considerable research efforts. Because penicilliosis almost always requires medical attention and can readily be diagnosed, a case-control design with cases diagnosed in the hospital settings and concurrent recruitment of matched controls was chosen to assess risk factors for the development of penicilliosis. In this study, the factors associated with increased risk of penicilliosis included: not being on antiretroviral therapy, outdoor occupations, proximity or exposure to tropical plants, proximity or exposure to highland plants, and exposure to farm animals. However, in the multivariate analysis, only proximity or exposure to tropical plants (i.e. bamboo, sugarcane, or rice) and exposure to farm animals

were independent risk factors of penicilliosis. Our findings are consistent with results of the Thai case-control study, which found a two-fold risk with recent exposure to animals or plants [29]. The strengths of the association are similar in the two studies, despite the significantly larger sample size and the more urban settings in the Vietnamese study, supporting the validity of the findings. Exposure to soil in itself was not a risk factor in this study. However it is possible that a specific type of soil, presumably soil associated with animal excreta and/or with tropical plant substrates, is preferred by *P. marneffe*. This hypothesis is plausible because soil is a known reservoir for major pathogenic (and non-pathogenic) fungal species, including the dimorphic fungi *Coccidioides spp.*, *Paracoccidioides spp.*, *Histoplasma capsulatum*, and *Sporothrix schenckii* [49–52]. The reservoirs for these dimorphic fungi are usually soil contaminated with animal droppings (bird or bat) in the case of histoplasmosis [49] and soil mixed with dead plant materials in the case of sporotrichosis [51], yet efforts to isolate *P.marneffe* from bamboo rat burrows have yielded limited success in the past (1/28 samples by Chariyalertsak et al., 0/120 samples by Li et al., and 0/215 by Gugani et al.) [27,47,53]. In 2015 Chinese investigators surveyed soil samples from bamboo rat burrows in Guangdong and showed a higher prevalence of *P.marneffe* in bamboo rat burrows than at non-rat-associated sites (15/184 versus 1/50), and *P.marneffe* could be isolated from bamboo roots deep in the burrow and from bamboo rat stool [24]. *P.marneffe* DNA has also been detected in animal-associated soil in Thailand [153]. Collectively, these recent studies provide evidence for bamboo-rat- and other animal-associated soil being an ecological

niche of *P.marneffe*, and these data suggest that farm animals and tropical plants might be important for *P.marneffe* growth and survival in natural soil.

The mapping data and the geographical analyses showed that, in southern Vietnam, the Central Highlands, the Southeast, and the South Central Coast are penicilliosis high-risk regions. The odds of penicilliosis in susceptible patients living in these regions were significantly higher than in HCMC and the Mekong Delta. Assuming the referral patterns of HIV-infected patients were similar across regions, cases per 100,000 HIV populations were 8 and 10 times higher in the Southeast and Central Highlands compared to the Mekong Delta and were 2 and 3 times higher compared to HCMC. Further, patients with previous residence or travels to these regions had almost 3-fold increased odds to develop penicilliosis than those without such history. One possible explanation for these findings is that the identified high-risk regions are comprised of mountainous terrain which forms the natural habitat of bamboo rats. Bamboo rats are known to forage amongst dense bamboo thickets in the highlands in Southeast Asia [163,164]. Their geographical distribution is similar to the distribution of human penicilliosis in Thailand, India, and China [2,22,23,25]. *R. pruinosis* is the most prevalent bamboo rat species in Vietnam [165]. Their distribution, shown in Figure 4.3 , is consistent with the data from this study on regional risk in southern Vietnam [165,166]. However, like the Chiang Mai study [29], there was no association between exposure or consumption of bamboo rats and penicilliosis, hence there are no evidence to suggest direct animal-to-human transmission. Because of the

very high prevalence of asymptomatic infection [22–27], one hypothesis is that bamboo rats may be a zoonotic reservoir, rather than a vector, of human penicilliosis. The soil excavation and foraging behaviors of bamboo rats may have been exploited by *P. marneffei* to expand its biomass and biogeography. From an evolutionary standpoint, the ability of *P. marneffei* to invade warm-blooded hosts must have engendered a survival advantage. Comparative genome analysis of *Paracoccidioides* reveals the evolution of genes that allow dimorphic fungi to degrade not only cellulosic plant materials in soil but also animal substrates [167]. The fact that *P. marneffei* is the only one of 354 plant and environmental *Penicillium* species to acquire the ability to interchange from a saprophytic to a zoonotic lifestyle further supports the hypothesis that an interaction with the bamboo rat is an integral part of its lifecycle.

Case-control studies employ an efficient design to investigate diseases with long latency periods; however they tend to be susceptible to biases that can undermine the validity of the findings [168]. There are several potential sources of bias in the study described here. First, the 31/236 (13%) penicilliosis patients who did not enter the study may be different in terms of exposure risk from those in the study. 80% of these patients died or were discharged before the diagnosis was made. Second, there was potential for misclassification of control patients with concurrent bacteremia, as bacteria can outgrow *P. marneffei* leading to false-negative blood culture results. However this occurred in only 2 of 407 control patients recruited. Third, CD4 count was only performed for 30% of

patients. Absolute lymphocyte counts and HIV disease staging are not ideal ways to measure immune susceptibility. Fourth, there was potential for interviewer assessment bias as up to 70% of penicilliosis patients had the characteristic umbilicated skin lesions [3,15]. Fifth, the differential referral pattern between cases and controls in different regions could bias the geographical results; however, the laboratory capacity to differentiate between AIDS-associated infections outside of major referral centres is still very limited in Vietnam. Finally, the low recruitment number from northern Vietnam restricted the geographical analyses only to southern Vietnam.

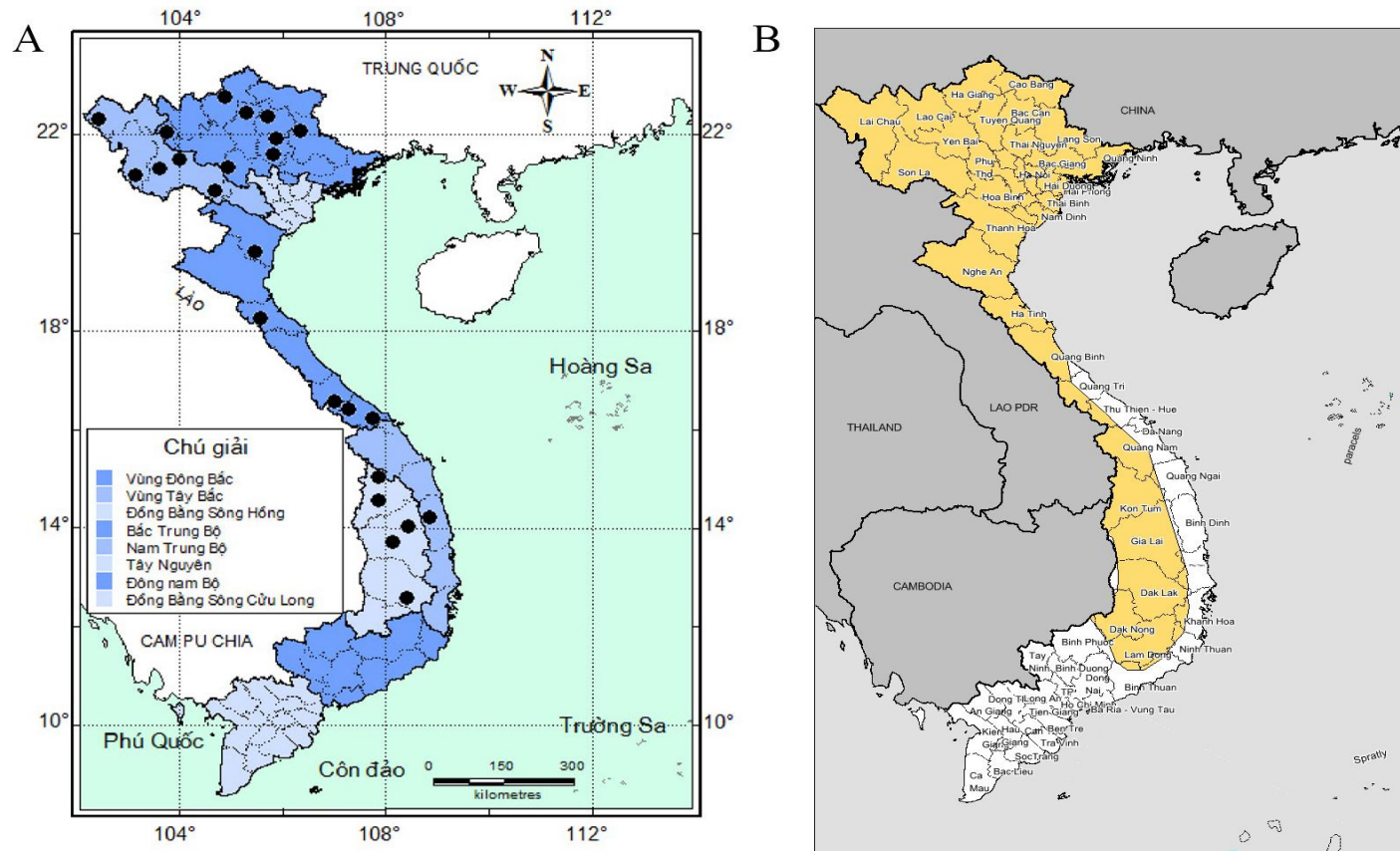


Figure 4.3: Distribution of Hoary Bamboo Rat *Rhizomys pruinosus* in Vietnam

Maps of distribution of *Rhizomys pruinosus* in Vietnam. (A) Map created by Dang Ngoc Can et al. in the Checklist of Wild Mammal Species of Vietnam published in 2008 by the Institute of Ecology and Biological Resources, Hanoi, Vietnam and the Primate Research Institute, Inuyama, Japan (B) Map compiled in 2015 by Hoang Minh Duc on the International Union for Conservation of Nature Red List, webpage: <http://www.iucnredlist.org/mammals>. Both maps were used with permissions from the original authors.

4.6 Conclusions

This study provides the first epidemiological link between human penicilliosis and geospatial proximity or travel to highland regions, thus informing prophylaxis recommendations for susceptible travelers to and within the endemic region. The previous findings of risk associated with exposure to plants and animals are confirmed in this large dataset. Based on this work and the current body of literature, I hypothesize that the bamboo rat, which is prevalent in the highlands, is a zoonotic reservoir, rather than a vector, and animal- and tropical-plant-associated soil is a plausible saprophytic reservoir of human penicilliosis. Comparative genome studies of *P. marneffe* isolated from bamboo rats and human and hypothesis-driven soil sampling studies are needed to elucidate the ecology of human penicilliosis.

CHAPTER 5

Development of a TaqMan Real-Time PCR Assay Based on *MP1* gene for Rapid Diagnosis of *Penicillium marneffe* Infection in Human Plasma

5.1 Abstract

Background

The systemic mycosis caused by *Penicillium marneffe* is amongst the most common AIDS-defining illnesses in South and Southeast Asia. Diagnosis is often delayed as culture can take 7 to 14 days for identification. Treatment delay is associated with increased mortality.

Methods

We developed a TaqMan real-time PCR assay with primers and probe designed to be highly specific for the *MP1* gene encoding an abundant cell wall protein specific to *P. marneffe*. Sensitivity and specificity were evaluated using *MP1*-containing plasmids, in clinical isolates, and in patients' plasma samples.

Results

The assay consistently detected 10 copies of *MP1*-containing plasmids per reaction and 100 *P. marneffe* yeast cells per milliliter of plasma. There was no amplification with 7 other *Penicillium* species and 6 other HIV-associated fungal pathogens tested. The assay was evaluated in plasma specimens of 70 HIV-infected patients: 50 patients with microbiological-confirmed penicilliosis and 20 patients with other opportunistic infections. The clinical sensitivities for detecting penicilliosis were 70.4% (19/27, 95% CI: 51.5%-84.1%) and 52.2% (12/23, 95% CI: 33.0%-70.8%) prior to and within 24-48 hours of antifungal therapy

administration, respectively. The clinical specificity was 100% (20/20, 95% CI: 83.9%-100%).

Conclusions

The assay takes 5-6 hours to perform and should be evaluated further to determine its role in improving the management and outcomes of patients with *P. marneffei* infection.

5.2 Introduction

The diagnosis of *P. marneffei* infection is often delayed as it can take up to 14 days to isolate and identify the pathogen from clinical specimens. The signs and symptoms in patients without skin lesions are indistinguishable from other common causes of AIDS-defining illnesses [5,15,118]. Typical skin lesions develop in up to 70% of patients [3,5,118] which enable a presumptive diagnosis to be made based on Gram's or Giemsa staining of skin smear, showing intra- and extracellular-yeast-like organisms of approximately 3-8µm in diameter. Some of these yeast cells have a clear midline septum, which is characteristic of *P. marneffei* [15]. Patients without skin lesions experience a significant delay in antifungal therapy initiation compared to those with skin lesions [median 5 (IQR: 3-8) days compared to 1 (IQR: 0-2) day] [Chapter 2]. These patients have significantly higher mortality, and they frequently die before the diagnosis is made [3,5,15]. Development of non-culture-based assays for rapid detection of *P. marneffei* infection has the potential to improve treatment outcomes particularly in patients without skin lesions.

Several serological assays detecting antigen and antibody to *P. marneffei* have been developed [19,98,100,169,170]; however none has been evaluated in prospective clinical studies to show their usefulness in the diagnosis of active *P. marneffei* infections. Polymerase chain reaction (PCR) techniques offer a sensitive and specific tool to detect several invasive fungal pathogens [102–104,171]. Conventional PCR-based assays have been developed to detect *P.*

marneffe in clinical isolates and in small number of clinical specimens [20,102,108,109]. A TaqMan real-time PCR assay was developed to detect *P. marneffe* in peripheral blood samples of 20 patients with penicilliosis and a sensitivity of 80% was reported but clinical specificity was not assessed [21]. To date, the molecular targets of these PCR assays are based on a highly conserved region of the universal fungal ribosomal DNA. When tested on the BLAST database, some of these primers were non-specific and were also predicted to amplify from common HIV-associated fungal pathogens *Cryptococcus neoformans*, *Candida albicans* and *Histoplasma capsulatum*. A novel *MP1* gene encoding the *P. marneffe* specific antigenic cell wall manoprotein has been cloned [99]. Immunoassays based on *MP1* have been developed to detect *P. marneffe* antigen and antibodies from cultured and clinical specimens with high sensitivity and specificity [16,17,100,101]. *MP1* is a unique gene without homologs in sequence databases, thus cross-reaction to related fungal pathogens is minimised. A Taqman real-time PCR was designed to detect *P. marneffe* based on *MP1* gene in the sera of patients with penicilliosis.

5.3 Methods

5.3.1 Fungal species and strains

Fungal strains used in this study included clinical isolates obtained from HIV-infected patients at the Hospital for Tropical Diseases (HTD) in Ho Chi Minh City,

including 7 isolates of *P. marneffe*, one isolate each of *Candida albicans*, *Candida tropicalis*, *Cryptococcus neoformans*, *Aspergillus fumigatus*, *Aspergillus terreus*, and *Aspergillus flavus* and 7 American Type Culture Collection (ATCC) laboratory *Penicillium* strains, including *P. chrysogenum* (ATCC 10106), *P. variable* (ATCC 32333), *P. aurantiogriseum* (ATCC 16025), *P. citrinium* (ATCC 10499), *P. crustosum* (ATCC 10122), *P. expansum* (ATCC 1117), and *P. glabrum* (ATCC 10444). The fungal isolates were sub-cultured using Sabouraud Dextrose Agar (SDA) at 37⁰C for the yeast isolates (*P. marneffe*, *Candida spp.*, and *Cryptococcus neoformans*) and at 25⁰C for the mold isolates (*Aspergillus spp.* and non-*marneffe* *Penicillium spp.*) for 3-7 days. The fungal strains were harvested re-suspended in PBS, and were stored at -20⁰C until used.

5.3.2 Clinical samples

Seventy random plasma samples collected between January 2011 and July 2013 from HIV-infected patients with culture-confirmed penicilliosis and patients with other HIV-associated opportunistic infections were used to evaluate the diagnostic accuracy of the assay. Plasma samples were stored at -80⁰C and were thawed one-time before use. Samples came from patients who participated the case-control study described in chapter 4, to investigate the risk factors of penicilliosis in patients with AIDS at the HTD. Penicilliosis was defined as an illness in which *P. marneffe* was isolated from blood, skin scrapings, bone marrow, lymph node, and/or other body fluids. Blood culture was performed

using the automated Bactec™ 9240 culture system (Becton Dickinson Microbiology Systems, Sparks, MD, USA). Patients with other opportunistic infections had blood culture performed to rule out *P. marneffei* fungaemia. This diagnostic study was approved as a laboratory sub-study of the case-control study by the Scientific and Ethical committee of the HTD.

5.3.3 Template DNA preparation

Fungal DNA extraction was performed according to a method described previously with minor modifications [21]. Plasma was used in place of whole blood. Briefly, the fungal cell wall was removed to form spheroplasts by adding 200 µL of re-suspended cultured fungal isolates or of clinical plasma specimens to 600 µL of sorbitol buffer (1 mol/L sorbitol, 100 mmol/L EDTA and 14 mmol/L β mercaptoethanol) supplemented with 200 U of lyticase enzyme (Sigma, Singapore). The mixture was incubated at 30°C for 30 minutes. Spheroplast DNA was extracted using QIAamp DNA blood mini kit (QIAGEN, Hilden, Germany) and was eluted in 30µL of elution buffer (provided with the kit). DNA concentration was determined using UV-spectrophotometer (NanoDrop, Thermo Scientific, Wilmington, DE, USA) and DNA at concentrations of 10 to 110 ng/µL was used in real-time PCR reactions.

5.3.4 Oligonucleotide primers and hybridization probe

All 23 nucleotide sequences of *P. marneffei* MP1 (accession number DQ08822 to DQ08845) were retrieved from Genbank. The sequences were aligned using BioEdit software (v7.0.1, Ibis Biosciences, Carlsbad, CA, USA). The primers and TaqMan probes were designed to be highly specific to a conserved region of MP1 using Primer Express software (v2.0, Applied Biosystem, Foster City, CA, USA). The specificity of primers and probes were tested by performing the BLAST searches. Table 5.1 shows the primers and probe sequences selected for this assay. They were synthesized and provided by Sigma-Proligo, Singapore.

Table 5.1: Primers and probe for the real-time PCR assay

Primers and probe	Nucleotide sequences ¹	Nucleotide position	T _m (°C)
Forward primer	5'-TCTGGACGGYGTTCAGTC-3'	222 - 239	57
Reverse primer	3'-TGATTGCTTAAATCCTGAACA-5'	294 – 314	52
Probe	5'-FAM-AAAATGAGCCTCCGCTTA GCTCCATGG-BHQ1-3'	249 - 271	52

¹ Y = T or C; 5'-FAM = 5' carboxyfluorescein; BHQ = Black Hole Quencher

5.3.5 TaqMan real-time PCR assay and product detection

Real-time PCR was performed using the LightCycler® 480 (Roche Applied Science, Penzberg, Germany). The reaction was conducted in a total volume of 25µL containing 2.5µL of buffer 2.5X (QIAGEN), 500nM MgCl₂ (QIAGEN),

400nM dNTP (Invitrogen), 400nM of each primer, 200nM of TaqMan probe, 1 unit Hotstart Taq polymerase (QIAGEN), and 5µL of template DNA. The real-time PCR conditions were optimized as follows: one cycle at 95°C for 15 minutes, 45 cycles at 95°C for 15 seconds, at 56°C for 30 seconds, and at 72°C for 15 seconds. The fluorescent signal of 6-carboxyfluorescein (FAM) was detected at 72°C in every reaction cycle. All real-time PCR reactions were performed in triplicate. A crossing point (Cp) threshold is defined as the PCR cycle at which an increase in fluorescent above the baseline signal is detected in at least 2 of the 3 PCR reactions. Mean Cp values and standard deviations were calculated.

5.3.6 Preparation of *P. marneffei* plasmid controls

The real-time PCR products from amplification of *P. marneffei* *MP1* gene were purified using the QIAquick® PCR purification kit (QIAGEN, Hidden, Germany), were cloned into pCR2.1-TOPO plasmid, and electro-transformed into *E. Coli* DH5α™-T1^R (TOPO TA cloning kit, Invitrogen, Carlsbad, CA, USA). Plasmids derived from a single colony were sequenced to confirm the presence of the *P. marneffei* *MP1* gene by using ABI BigDye Terminate v3.1 Cycle Sequencing kit (Applied Biosystem, USA). *E. Coli* clone carrying plasmids with the *MP1* insert were cultured overnight in liquid Luria-Bertani (LB) medium supplemented with ampicillin 100mg/mL at 37°C to select for successful transformation. Plasmids were purified using QIAprep® Miniprep Kit (QIAGEN), and plasmid concentration

was determined using UV-spectrophotometer. The quantity of plasmids was calculated using the following formula [172]:

$$DNA(copies) = \frac{6.23 \times 10^{23} \left(\frac{copies}{mol} \right) \times DNA\ amount(g)}{DNA\ length(bp) / 660 \left(\frac{g}{mol} \right)}$$

5.3.7 Preparation of *P. marneffei* cell controls

P. marneffei yeast cells were cultured on SDA at 37°C for 5 to 7 days until single cells were created. The cells were homogenised by a vortex mixer for 2 minutes in 5ml PBS (pH 7.2) containing Tween 20 (5%) (Sigma, Singapore). Cell pellets were collected by centrifugation at 8000xg for 5 minutes, the condition we found the most optimal for cell recovery. The pellets were thoroughly washed 2 times with PBS and were re-suspended in 5ml PBS. *P. marneffei* yeasts were counted on a cell counting chamber under a light microscope.

5.3.8 Sensitivity of the TaqMan real-time PCR assay for detection of *P. marneffei* MP1 gene

To assess the analytical sensitivity of the TaqMan-PCR assay, 10-fold serial dilutions of purified plasmid DNA (10^6 to 1 copies/ μ L) in sterile distilled water were tested in triplicate. The remaining plasmids were stored at -80°C for use as the positive control for real-time PCR. The smallest number of *P. marneffei* yeast cells detected by the TaqMan-PCR assay (limit of detection) was also

determined. Ten-fold serial dilutions of *P. marneffe* yeast cells (10^6 to 10^1 cells/mL) mixed with human plasma collected from healthy volunteers were tested in triplicate. Two hundred microliters of plasma of each dilution was used for DNA extraction.

5.4 Results

5.4.1 Amplification of *MP1* gene in the clinical and laboratory fungal isolates

The primers and probe successfully detected all 7 *P. marneffe* clinical isolates. No significant elevated signal was detected in all 13 non-*P. marneffe* isolates, including *P. chrysogenum*, *P. variable*, *P. aurantiogriseum*, *P. citrinium*, *P. custosum*, *P. expansum*, *P. glabrum*, *Candida albicans*, *Candida tropicalis*, *Cryptococcus neoformans*, *Aspergillus fumigatus*, *Aspergillus terreus*, and *Aspergillus flavus*. The absence of DNA was confirmed by agarose gel electrophoresis of TaqMan-PCR products derived from these non-*P.marneffe* isolates, indicating that the assay is highly specific.

5.4.2 The analytical sensitivity of the TaqMan-PCR assay

The TaqMan-PCR assay was able to detect *MP1*-containing plasmids at 1 copy per reaction, but detection was consistent at 10 copies per reaction. Figure 5.1 shows the quantification of *MP1*-containing plasmids that were serially diluted to concentrations from 10^6 to 10^1 and the linear regression of the C_p values versus

the $\log_{10}$ concentrations of *MP1* plasmids. Linearity was achieved within a plasmid DNA range from 10^6 to 10^1 copies per reaction, correlation coefficient (R^2 value) = 0.996.

The TaqMan-PCR assay was able to detect *P. marneffe* yeast cells at a concentration of 100 cells per milliliter of plasma. Linearity was achieved within a range of yeast concentrations from 10^6 to 10^2 cells per milliliter plasma, R^2 value = 0.997.

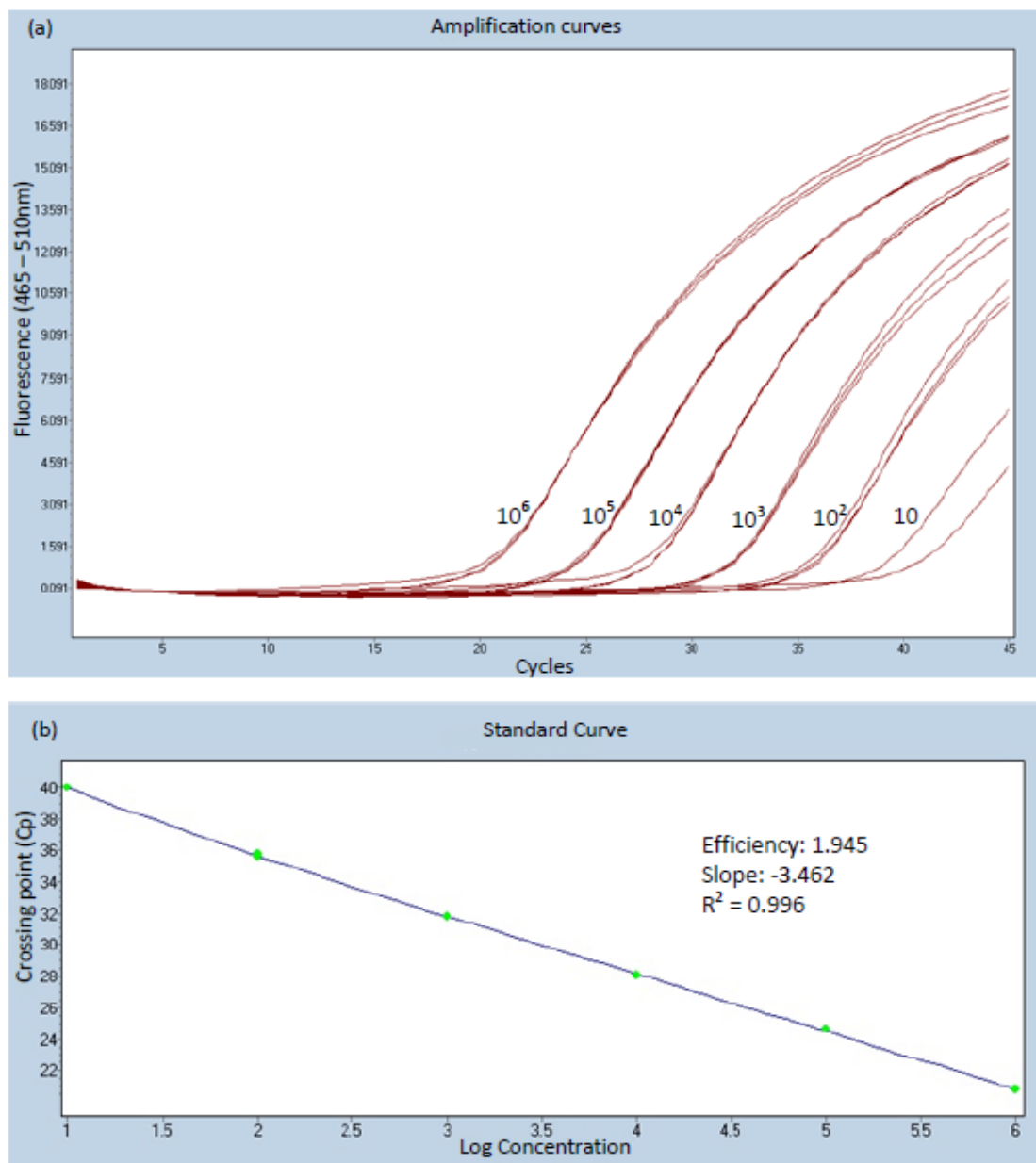


Figure 5.1: Amplification plots and standard curve of real-time PCR assay

(a) Amplification plots of PM1 plasmids with dilutions from 10^6 to 10 copies per reaction

(b) Standard curve showing crossing point (Cp) versus the log concentration of PM1 plasmids.

All the samples were performed in triplicate.

5.4.3 The clinical sensitivity and specificity of the TaqMan-PCR assay

The clinical sensitivity and specificity of the assay were evaluated in randomly selected plasma samples of 50 HIV-infected patients with culture-confirmed penicilliosis and in 20 patients with other HIV-associated opportunistic infections. Figure 5.2 shows the clinical features and the PCR results of these patients. The assay was able to detect *P. marneffei* MP1 gene in 19 of 27 (70.4%, 95% CI: 51.5%-84.1%) patients in whom samples were collected prior to antifungal therapy and in 12 of 23 (52.2%, 95% CI: 33.0%-70.8%) patients in whom samples were collected 24 to 48 hours on antifungal therapy. This compares to the Bactec system which detected *P. marneffei* growth in 44 of 50 (88.0%, 95% CI: 76.2% to 94.3%) patients, with all samples collected prior to antifungal therapy. A statistical comparison between the sensitivity of the new TaqMan PCR assay and the Bactec system in patients where both samples were collected prior to antifungal therapy did not reach significance, 19/27 (70.4%) vs. 24/27 (88.9%), p-value (McNemar test) = 0.29. No visible signal was detected in all 20 plasma specimens belonging to patients infected with other common HIV-associated infections, giving an observed specificity of 100%.

Table 5.2 shows the Cp values in the 50 plasma samples of the patients with culture-confirmed penicilliosis with respect to timing of blood collection for the TaqMan-PCR assay and the duration of the Bactec culture system to detect fungal growth from the hospital microbiology laboratory.

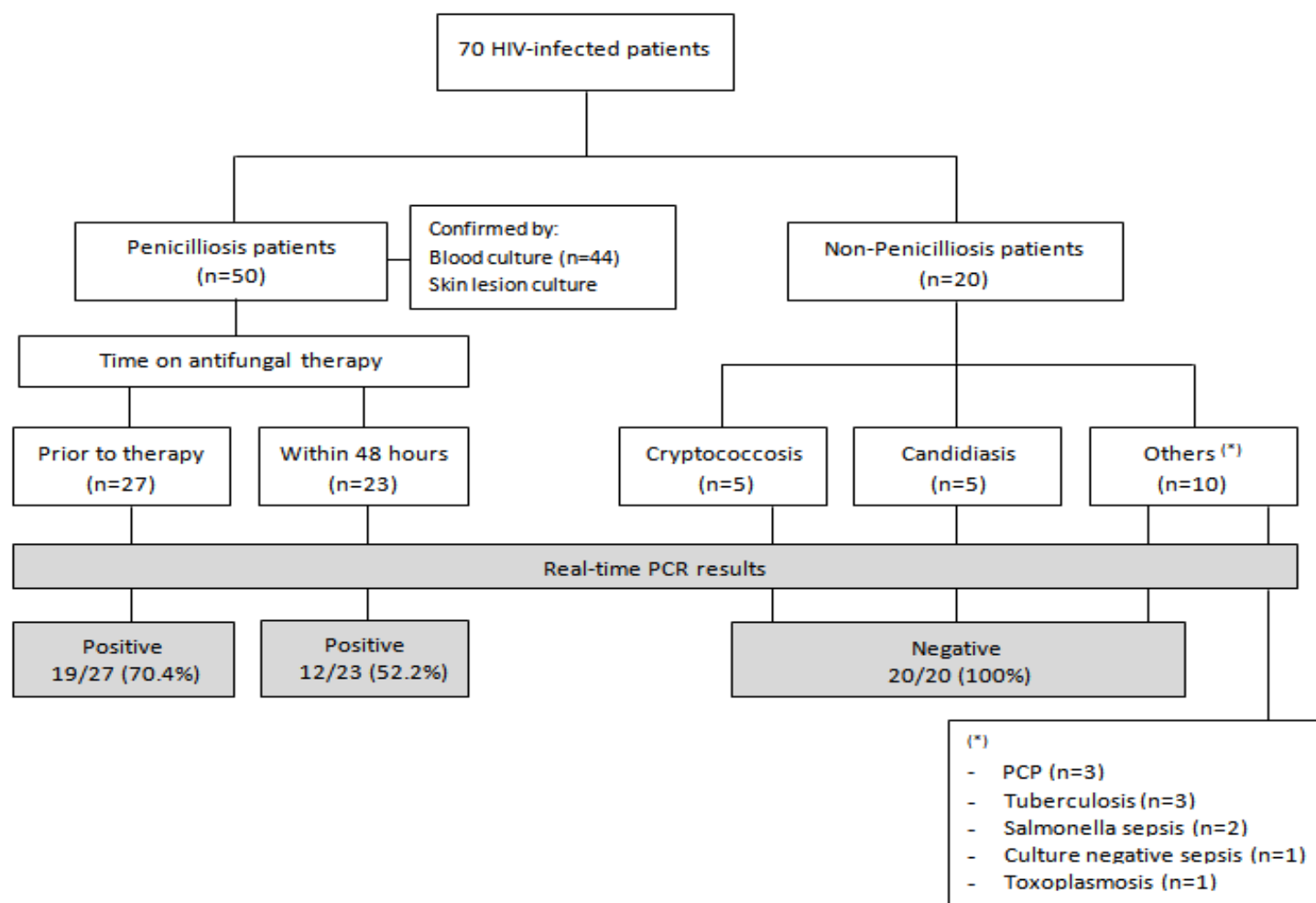


Figure 5.2: The characteristics of the study population and real-time PCR results

Table 5.2: TaqMan real-time PCR detection of *P. marneffeii* MP1 gene in 50 plasma samples of patients with microbiological-confirmed penicilliosis

Patients	Time on antifungal therapy (hours) ¹	Mean C _p values (standard deviation)	Time to Bactec blood culture identification (days) ²	Specimens diagnosed with <i>P. marneffeii</i>
1	0	39.5 (±0.95)	9	blood and skin
2	0	40.0 (±0.00)	data not available	skin
3	0	34.4 (±0.05)	data not available	blood and skin
4	0	40.0 (±0.00)	data not available	skin
5	0	35.7 (±0.97)	3	blood
6	0	not detected	data not available	skin
7	0	40.0 (0.00)	4	blood and skin
8	0	not detected	6	blood
9	0	not detected	7	blood and skin
10	0	39.0 (±1.74)	6	blood and skin
11	0	38.6 (±0.67)	3	blood and skin
12	0	35.0 (±0.24)	4	blood and skin
13	0	38.1 (±1.25)	5	blood and skin
14	0	not detected	data not available	blood
15	0	39.3 (±0.63)	4	blood and skin
16	0	34.0 (±0.34)	data not available	blood
17	0	not detected	5	blood and skin
18	0	39.6 (±0.62)	6	blood
19	0	35.2 (±0.11)	5	blood and skin
20	0	not detected	data not available	blood and skin
21	0	40.0 (0.00)	7	blood and skin
22	0	not detected	3	blood and skin
23	0	not detected	7	blood and skin
24	0	40.0 (0.00)	5	blood and skin
25	0	40.0 (0.00)	7	blood and skin
26	0	40.0 (0.00)	5	blood and skin
27	0	33.0 (±1.25)	6	blood
28	24	not detected	data not available	skin
29	24	not detected	7	blood and skin
30	24	38.2 (±0.23)	5	blood and skin
31	24	37.2 (±0.10)	3	blood and skin
32	24	not detected	Contaminated with (<i>Burkholderia cepacia</i>)	skin

Patients	Time on antifungal therapy (hours) ¹	Mean C _p values (standard deviation)	Time to Bactec blood culture identification (days) ²	Specimens diagnosed with <i>P. marneffe</i>
33	24	not detected	6	blood
34	24	40.0 (0.00)	4	blood and skin
35	24	40.0 (0.00)	6	blood and skin
36	24	not detected	5	blood
37	24	40.0 (0.00)	9	blood and skin
38	24	40.0 (0.00)	5	blood
39	24	not detected	7	blood
40	24	not detected	5	blood
41	24	31.0 (± 1.20)	3	blood and skin
42	24	33.0 (± 0.47)	5	blood and skin
43	24	not detected	7	blood and skin
44	48	37.8 (± 0.33)	data not available	skin
45	48	not detected	data not available	blood
46	48	36.9 (± 0.29)	7	blood
47	48	not detected	6	blood
48	48	not detected	7	blood
49	48	39.9 (± 0.24)	5	blood
50	48	39.3 (± 0.63)	7	blood and skin

¹ Time on antifungal therapy prior to blood collection for real-time PCR assay, time 0 = prior to antifungal therapy; ² Time to detect a growth signal in blood culture bottles using the automated Bactec culture system; median: 5 days (interquartile range: 5-7). All reactions were performed in triplicates

5.5 Discussion

The fluorogenic real-time PCR technology has revolutionised the way clinical microbiology laboratories diagnose pathogens. The closed system for amplification and integrated detection minimizes the risk of cross-contamination with amplicons from previous reactions. Compared to conventional PCR and traditional culture-based or immunoassay-based methods, the real-time PCR

method is faster, more efficient, and can be more accurate in the detection of many infectious disease pathogens [173]. It is useful in detecting slow and/or difficult growing pathogens. The current diagnosis of *P. marneffe* infection is based on conventional microbiological techniques, relying on the isolation of *P. marneffe* from clinical specimens which can take 5-14 days [5,15]. Presumptive diagnosis can be made early based on microscopic examination of skin lesions; however up to 50% of patients do not present with skin lesions [11,68]. These patients experience significant treatment delay and higher mortality rates [3,5,15].

In this study, a new TaqMan real-time PCR assay was developed with primers and probe designed to be highly specific for the *P. marneffe* *MP1* gene. The assay was evaluated for accuracy in clinical and laboratory isolates, in *MP1*-containing plasmids, and in plasma samples of patients. The assay was shown to be highly specific for *P. marneffe* with no amplification from 13 different fungal isolates within the *Cryptococcal*, *Aspergillus*, *Candida*, and *Penicillium* genera, and no amplification was observed in plasma specimens of 20 patients infected with other common HIV-associated pathogens.

The assay consistently detected 10 copies of *P. marneffe* *MP1* per reaction and 100 *P. marneffe* yeast cells spiked into one milliliter of plasma. It could detect *P. marneffe* in patients' plasma, with higher sensitivity in samples collected prior to antifungal therapy (70.4%) than in patients in whom samples were collected 24 to 48 hours after antifungal therapy initiation (52.2%). The assay takes 5 to 6 hours

to perform. Although this is not ideal and would probably result in a next day diagnosis in most routine laboratories, this compares to a median time of 5 days (interquartile range: 5-7) to detect a growth signal in the blood culture of these patients using the highly efficient Bactec system (Table 5.2). Additionally identification of *P. marneffei* requires a demonstration of temperature-dependent dimorphic growth, and subculture at 25°C generally takes another 2 days. This TaqMan-PCR assay therefore can make a significant difference in patient management by allowing antifungal therapy to begin sooner and has the potential to improve the outcomes of *P. marneffei*-infected patients.

To my knowledge this is the first use of the *MP1* gene as the target for PCR-based diagnostics of *P. marneffei*. Compared to the previous two PCR-based assays that were tested in clinical samples [20,21], this assay had equivalent specificity (100%) but the highest sensitivity (70.4%) (tested in patients prior to antifungal therapy). The nested PCR assay developed by Pongpom et al. yielded a similar sensitivity (24 of 35 sera from *P. marneffei*-infected patients, 68.6%) [20]. However, the real-time PCR method is faster, less labor-intensive, and less prone to post-PCR contamination [173]. The TaqMan-PCR assay developed by Pornprasert et al. had relatively lower clinical sensitivity (12 of 20 whole blood specimens from *P. marneffei*-infected patients, 60%) [21]. The difference in sensitivity of the two real-time PCR assays could be due to patient sample selection, DNA extraction methodology, molecular targets chosen, or types of specimen used (i.e. the Thailand group use whole blood; I use plasma); however

the difference is small. The sensitivity estimate generated in the study reported here may be more reliable as it was tested in a substantially larger number of patients.

Compared to the Bactec culturing method, this assay was less sensitive (70% vs. 88%) but the difference did not reach statistical significance. A major advantage of culturing is that a large volume of sample is used, i.e. approximately 5 milliliter of blood compared to 200 μ L of plasma used in this assay. Real-time PCR assays will not replace the need for conventional microbiology methods in modern microbiology. Rather, this real-time PCR assay can be used in combination with culture to improve the rapid diagnosis of patients without skin lesions in whom *P. marneffe*i is in the differential diagnoses. In addition to detection, the assay gives a quantitative result which can be used to measure fungal load in clinical samples and thus may have the potential to be used for disease prognosis and for monitoring treatment response. Further work to improve the sensitivity of the assay, to determine prognostic value of fungal burden and to ensure quality control of the results is needed.

In summary, a sensitive and highly specific TaqMan-PCR assay was developed based on the *MP1* gene that can detect *P. marneffe*i in patient plasma in 5-6 hours. The assay should be further refined to improve the sensitivity and has the potential to improve the management and outcomes of patients infected with *P. marneffe*i.

CHAPTER 6

General Discussion and concluding remarks

The research presented in this thesis aims to increase knowledge and understanding of the clinical epidemiology, environmental epidemiology, exposure and/or behavioural risk factors, and geographical risk of *Penicillium marneffe* infection. Moreover, the development of a novel real-time PCR diagnostic assay addresses the diagnostic delay in up to 50% of patients who do not present with skin lesions and experience worse outcome. The main findings are specific to the questions raised by each study; thus syntheses of these findings are presented by chapters and, where applicable, integrated implications are discussed.

Chapter 2 documented the incidence and prevalence of penicilliosis admissions amongst all HIV-related admissions at the Hospital for Tropical Diseases over a 13 year period. Penicilliosis accounted for 4.4% of HIV admissions from 1996 to 2009. The emergence of penicilliosis was in close correlation with the HIV/AIDS epidemic in southern Vietnam, which had an exponential peak in 2007 and has gradually decreased, corresponding to the timing of the national antiretroviral therapy rollout. This study is the largest reported penicilliosis case series and includes detailed characterisation of the clinical and microbiological features, treatment, and outcomes of more than 500 patients. Clinical factors routinely collected on hospital admission that predict poor treatment outcome were identified for the first time, including history of injection drug use, shorter duration of illness, absence of fever or skin lesions, higher respiratory rates, and lower platelet counts. These data will be used to inform a prognostic scoring system

based on combined outcome data from this retrospective cohort and the case control study cohort. The resulting scoring system will be validated (and refined if necessary) in an ongoing multi-centre clinical trial of penicilliosis of which I am the Principal Investigator. This may provide clinicians with a useful tool to assess disease severity at the bed side, which may dictate the level of care (i.e. whether to hospitalise a patient, whether to admit a patient to an intensive care unit) and may guide treatment options for patients in the future.

Chapter 3 was informed by the findings from chapter 2 and previous data from Chiang Mai that penicilliosis cases increase in the rainy months compared to the dry months [30]. The subsequent analysis of environmental drivers of seasonality identified humidity as the key driver. Cryptococcal meningitis admissions were not seasonal, and the odds of penicilliosis admission were increased relative to cryptococcal meningitis as humidity increased. These findings suggest that humidity facilitates *P. marneffe* growth, maintenance, and/or dispersal in the environment. This is not surprising as humidity is well known to promote growth of environmental molds in indoor settings. Higher level of humidity increase the odds of elevated levels of *Penicillium* and *Aspergillus* species four fold in urban homes of people living in Syracuse, New York [174]. This finding is consistent with the proposed mechanism that human penicilliosis occurs as a result of *P. marneffe* spillover from the environment to susceptible human hosts by inhalation of infectious spores or hyphal fragments from a soil reservoir. This leads to the hypothesis that humidity-mediated *P. marneffe* growth from certain

air-exposed plants and soil surfaces may be an important step in the exposure and infection in immunocompromised human populations living in or traveling to the endemic areas.

Contrary to the prevailing opinions that dimorphic mycosis, including histoplasmosis, blastomycosis, and coccidioidomycosis, manifest clinically as either primary pulmonary infection or as disseminated disease arising from reactivation of latent infections upon the onset of profound immunosuppression [145–147], the strong association of penicilliosis incidence with humidity suggests that disseminated disease can occur in primary infection shortly after exposure to the fungus in the environment. Whether this means that *P. marneffe* may exhibit increased virulence relative to the other dimorphic pathogens or whether this reflects some unique host factors and/or unique features of the HIV epidemic in Vietnam, with patients being diagnosed late and being more prone to disseminated disease, is unclear and require further research. The impact of humidity on the clinical manifestation (e.g. whether there are skin lesions), on penicilliosis severity and treatment outcomes should be evaluated in patients presenting with penicilliosis at different times of the year.

Our estimate of the incubation period is conditional on the assumption that humidity drives the exposure to *P. marneffe* rather than by stimulating some clinical manifestations that would prompt patients to go to the hospital to seek care. The latter seems less plausible as skin lesions in penicilliosis develop on relatively drier surfaces of the body, and symptoms preceding the onset of skin

lesions are very non-specific. Humidity, as discussed, has been shown to promote growth of environmental fungi and, as shown in Chapter 3, to drive penicilliosis incidence which has a clear seasonal component [30]. The seasonality of penicilliosis, if not driven by host factors or behavioural changes, must indicate environmental changes that impact pathogen behaviours or characteristics, resulting in increased exposure and acute primary infections in susceptible hosts. Thus the hypothetical incubation periods were modeled based on plausible assumptions and grounded disease parameters. The estimated incubation period of between 1 and 3 weeks is similar to the short time window from birth to clinical infection in a report of a 2-month old infant born to an asymptomatic HIV-infected mother who presented with disseminated penicilliosis in Chiang Mai [175]. Data of *P. marneffei*-infected travelers from outside of the endemic region documented a range of times from estimated exposure to clinical infection of 4 weeks to more than two decades [10,11,38,63,176]. The wide variations in these 'incubation periods' are most likely driven by the immune status of these individuals, but clearly indicating that *P. marneffei* is able to remain latent for many years in as yet unknown cellular compartments within the human host.

Chapter 4 aimed to answer the following questions: 1) Do human infections develop from a single or multiple sources of soil, plants, water, and invertebrates? 2) What are the geographical risks of penicilliosis? First, it was shown that infection was associated with proximity or exposure to tropical plants

and with exposure to farm animals. Despite being conducted in very urban settings, this is the second epidemiological study that points to an agricultural reservoir associated with plants and farm animals. Although there is no evidence of disease risk with direct soil exposure, it is possible that a soil in presence of animal excreta and/or decayed tropical plant materials is favored by *P. marneffei*. This assertion comes from the knowledge of soil, usually a mixed source of soil, plants, and animals, being the reservoir for the phylogenetically-related dimorphic fungi *Histoplasma capsulatum* and *Sporothrix schenckii* [49,51]. A recently published field study conducted in 2012 in Guangdong showed that *P. marneffei* was isolated in 15/184 soil samples from *R. prinosus* burrows and only in 1/50 soil samples collected from non-rat-associated sites, suggesting a close ecological connection between *P. marneffei*, bamboo rats, and bamboo-rat-associated soil.

The second major finding came from the geographical analysis. Although penicilliosis tends to cluster in specific geographical areas in the region, this study provided the first epidemiological evidence of infection risk in susceptible individuals living in or traveling to highland regions within an endemic country. These risk regions correspond to the distribution of the most prevalent *R. prinosus* bamboo rats in Vietnam (according to survey data from wild-life conservation groups). Although this is only circumstantial evidence that bamboo rats may be important in the acquisition of *P. marneffei* in humans, these data taken collectively with the findings of the high prevalence of asymptomatic

disease in bamboo rats and higher prevalence of *P. marneffei* isolated from bamboo-rat-associated soil than from non-rat-associated soil lead to the hypothesis that the bamboo rat may be a zoonotic reservoir, rather than a vector, of human penicilliosis. They may play an important role in the maintenance and distribution, rather than transmission, of *P. marneffei*. Comparative genome studies to evaluate the relationship between human, bamboo rat, and environmental isolates will advance knowledge in the ecology of this emerging mycosis.

Chapter 5 described the development of a Taqman real-time PCR assay based on the novel *MP1* gene for rapid detection of *P. marneffei* in patient plasma. The assay had an analytical and clinical specificity of 100% in the relatively small sample assessed. The clinical sensitivity is estimated to be 70.4% (95% CI: 51.5-84.2%) in patients prior to antifungal therapy initiation. The assay takes 5-6 hours to perform and has the potential to make a significant difference in the management of patients. Further work to improve the assay sensitivity and quality are currently underway. Prospective studies are needed to evaluate the positive and negative predictive values of this test in patients clinically suspected to have *P. marneffei* infection. If the assay proves to be accurate, the effect on time to treatment initiation and resulting impact on clinical outcome should be assessed.

In conclusion, this thesis provides several new insights into the clinical epidemiology of *P. marneffei* infection in Vietnam and furthers knowledge and

understanding of the ecology of *P. marneffe*. This thesis provides fundamental data for the design of prospective studies to improve the diagnosis and management of penicilliosis not only in Vietnam. The newly identified environmental and geographical risks for penicilliosis should re-energise research interests, combining hypothesis-driven field work with tools in pathogen genetics, to decipher the ecology and life cycle of this enigmatic pathogen. Such knowledge is important not only to understand the nature and impact of the pathogen on the environment and human health but also to inform the design of the most cost effective prevention strategies in susceptible populations. Importantly, screening and early treatment of HIV should remain the corner stone of public health approaches against HIV and HIV-associated infections. Alongside this, evaluation of current immunoassay candidates to detect *P. marneffe* antigen for further development into a point-of-care test (such as the Cryptococcal Antigen Lateral Flow assay) could lead to early detection and preemptive treatment before *P. marneffe* infection and its complications develop.

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APPENDICES