รายงานการวิจัย

โครงการวิจัยเรื่อง

"โครงการวิจัยนำร่องโรคมาลาเรียอุบัติใหม่จากเชื้อพลาสโมเดียมโนวลิไซ: การเฝ้าระวังระดับอณูชีววิทยา การวิเคราะห์พันธุกรรมของเชื้อ และบทบาทของยุงก้นปล่องพาหะหลักของมาลาเรียในประเทศไทยในการนำโรค"

"*Plasmodium knowlesi*, an emerging human malaria: molecular surveillance, genetic characterization and role of anopheline malaria main vectors in Thailand in disease transmission"

หน่วยงานวิจัย

ภาควิชาปรสิตวิทยา คณะแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย

ศูนย์อบรมโรคติดต่อนำโดยแมลง (พระพุทธบาท)

กรมควบคุมโรค กระทรวงสาธารณสุข

คณะผู้วิจัย

ศ. นพ. ดร. สมชาย จงวุฒิเวศย์

รศ. ดร. จตุรงค์ พุทธพรทิพย์

น.พ. จีรพัฒน์ ศิริชัยสินธพ

สิงหาคม 2554

ได้รับทุนอุดหนุนการวิจัยจากกองทุนรัชดาภิเษกสมโภช

ปีงบประมาณ 2553

กิตติกรรมประกาศ

คณะผู้วิจัยขอขอบพระคุณ ผู้ป่วยทุกท่านที่อุทิศเวลาและให้ความร่วมมืออย่างดียิ่ง ผู้ร่วมวิจัยทุกท่าน เจ้าหน้าที่ สำนักโรคติดต่อนำโดยแมลง และหน่วยงานทุกแห่ง ที่ให้การสนับสนุนช่วยเหลือ จนทำให้งานวิจัยชิ้นนี้สำเร็จลุล่วงด้วยดี และการศึกษานี้ได้รับทุนอุดหนุนการวิจัยจากโครงการส่งเสริมการทำงานวิจัยเชิงลึกในสาขาวิชาที่มีศักยภาพสูง กองทุนรัชดาภิเษกสมโภช หรือ CU-CLUSTER-FUND

คณะผู้วิจัย

สิงหาคม 2554

บทคัดย่อภาษาไทย

ชื่อโครงการวิจัย

(ภาษาไทย) โครงการวิจัยนำร่องโรคมาลาเรียอุบัติใหม่จากเชื้อพลาสโมเดียมโนวลิไซ: การเฝ้าระวังระดับอณูชีววิทยา การวิเคราะห์พันธุกรรมของเชื้อ และบทบาทของยุงก้นปล่องพาหะหลักของมาลาเรียในประเทศไทยในการนำโรค

(ภาษาอังกฤษ) *Plasmodium knowlesi*, an emerging human malaria: molecular surveillance, genetic characterization and role of anopheline malaria main vectors in Thailand in disease transmission

บทคัดย่อ

การติดเชื้อมาลาเรียชนิดพลาสโมเดียม โนวลิไซของคนตามธรรมชาตินั้นพบอยู่ทางเขตเอเชียตะวันออกเฉียงใต้ ซึ่งจากการศึกษาของคณะผู้วิจัยในก่อนหน้านี้ระหว่างปี 2006-2007 พบการกระจายของเชื้อชนิดนี้ในประเทศไทยได้ทั่วไปแต่มีอัตราต่ำ การศึกษาในครั้งนี้เป็นการศึกษาต่อเนื่องโดยเก็บตัวอย่างจากพื้นที่การศึกษาเดียวกันในระหว่างปี 2008-2009 และผลการศึกษาพบว่าไม่มีการเปลี่ยนแปลงความชุกของเชื้อมาลาเรียชนิดพลาสโมเดียม โนวลิไซที่ตรวจพบในผู้ป่วยแต่ผลความชุกของเชื้อมาลาเรียอีก 4 ชนิดมีความแตกต่างกันอย่างมีนัยสำคัญ นอกจากนี้ยังตรวจพบเชื้อดังกล่าวจากการวิเคราะห์ตัวอย่างเลือดผู้ป่วยมาลาเรียย้อนหลังที่เก็บในปี 1996 จากหนึ่งพื้นที่การศึกษา จึงเป็นการยืนยันว่าเชื้อมาลาเรียชนิดพลาสโมเดียม โนวลิไซมีความชุกคงที่เมื่อเปรียบเทียบกับการวิเคราะห์ในปี 2006-2007 และ 2008-2009 ดังนั้นจึงแสดงให้เห็นว่ามาลาเรียที่ติดต่อจากลิงชนิดนี้ไม่ได้เป็นโรคมาลาเรียอุบัติใหม่ในประเทศไทย และยิ่งไปกว่านั้นผลการวิเคราะห์ลำดับเบสของยีนที่สร้างโปรตีนบนผิวเมอร์โรซอยต์ชนิดที่ 1 ที่มีความเหมือนกันระหว่างสายพันธุ์ที่พบในคนกับลิงกังที่เลี้ยงร่วมกับคน ซึ่งแสดงให้เห็นถึงความเป็นไปได้ที่จะมีการส่งถ่ายเชื้อชนิดนี้ไปมาระหว่างคนกับลิง

บทคัดย่อภาษาอังกฤษ

ชื่อโครงการวิจัย

(ภาษาไทย) โครงการวิจัยนำร่องโรคมาลาเรียอุบัติใหม่จากเชื้อพลาสโมเดียมโนวลิไซ: การเฝ้าระวังระดับอณูชีววิทยา การวิเคราะห์พันธุกรรมของเชื้อ และบทบาทของยุงก้นปล่องพาหะหลักของมาลาเรียในประเทศไทยในการนำโรค

(ภาษาอังกฤษ) *Plasmodium knowlesi*, an emerging human malaria: molecular surveillance, genetic characterization and role of anopheline malaria main vectors in Thailand in disease transmission

Abstract

Naturally acquired human infections with *Plasmodium knowlesi* are endemic in Southeast Asia. Our previous survey in 2006-2007 has shown a wide-spread and low prevalence of this simian malaria in Thai patients. This follow-up study in the same endemic areas in 2008-2009 has revealed a stable prevalence of *P. knowlesi* among malaria patients whereas a significance difference in the prevalence of 4 human malaria species occurred. Retrospective analysis of blood samples from malaria patients collected in 1996 in one of these endemic areas has reaffirmed a stable prevalence of *P. knowlesi* when compared to those in 2006-2007 and 2008-2009, indicating that this simian malaria is not newly emergent human malaria in Thailand. Importantly, identical merozoite surface protein-1 sequences were observed between isolates from a patient and a pig-tailed macaque living in vicinity, suggesting potential cross-transmission of *P. knowlesi* from naturally infected macaques to humans.

สารบัญเรื่อง

สารบัญตาราง

สารบัญภาพ

คำอธิบายสัญลักษณ์และคำย่อที่ใช้ในการวิจัย

DNA	=	deoxyribonucleic acid
RNA	=	ribonucleic acid
SSU rRNA	=	small subunit ribosomal RNA
A	=	adenine
T	=	thymine
G	=	guanine
C	=	cytosine
dNTP	=	deoxyribonucleotide triphosphate
dATP	=	deoxyadeninosine triphosphate
dTTP	=	deoxythymine triphosphate
dCTP	=	deoxycytosine triphosphate
dGTP	=	deoxyguanosine triphosphate
PCR	=	polymerase chain reaction
Tm	=	melting temperature
Taq	=	*Thermus aquaticus*
ddH_2O	=	double distilled water
bp	=	base pair
Kb	=	kilobases
ml	=	millilitre
mm	=	millimetre
mM	=	millimolar
ng	=	nanogram
µg	=	microgram
µl	=	microlitre
µM	=	micromolar
UV	=	ultraviolet
^{o}C	=	degree celsius
%	=	percent
λ	=	lambda
EDTA	=	ethylene diamine tetra acetate
KCl	=	potassium chloride
$MgCl_2$	=	magensium chloride
PBS	=	phospate buffer saline

Introduction

Plasmodium knowlesi has been known to circulate mainly among long-tailed macaques (*Macaca fascicularis*) and pig-tailed macaques (*M. nemestrina*) inhabiting a wide geographic range of Southeast Asia (*1*). Although the first naturally acquired human infection with *P. knowlesi* was reported in 1965, it was not until 2004 that naturally acquired infections in humans were rediscovered in Malaysian Borneo and southern Thailand (*2-4*). Failure in microscopy-based detection of *P. knowlesi* stemmed from the morphological resemblance of young trophozoites of *P. knowlesi* with those of *P. falciparum* and the characteristic band-shaped growing trophozoites mimicking those of *P. malariae* (*2-4*). Although the presence of characteristic 'Sinton and Mulligan's stippling in *P. knowlesi*-infected erythrocytes could be a diagnostic clue, it may neither be well-discernible nor unequivocally identified (*1,3*). To date, the effective tools for diagnosing *P. knowlesi* is PCR targeting multicopy gene targets such as small subunit ribosomal RNA (SSU rRNA) and mitochondrial cytochrome *b* (*3-5*).

Human infections with *P. knowlesi* exhibit geographic variation with the highest prevalence in Malaysian Borneo whereas individual cases have been increasingly identified in Southeast Asian countries (*6*). Meanwhile, annual malaria cases in Thailand during the past 4 decades have an overall decline due to several control measures through early case detection, timely change of nation-wide anti-malarial drug policy and active implementation of vector control measures (*7*). Importantly, the data on annual malaria cases in Thailand based on microscopic examination of Giemsa-stained blood smears undoubtedly underdiagnosed mixed species infections, misdiagnosed *Plasmodium* species possessing morphologically similar parasites and underestimated those with parasite density below microscopic detection threshold (*8*). Our large-scale molecular-based survey of malaria in Thailand during 2006-2007 has shown that *P. knowlesi* was widely distributed

in low prevalence in several endemic areas bordering Myanmar, Cambodia and Malaysia (*8*). Correct diagnosis of malaria has a significant impact on malaria control in terms of treatment outcomes, disease transmission and interpretation of efficiency of a given malaria control measure.

Although malaria caused by *P. knowlesi* was generally benign and well responsive to chloroquine treatment, severe and fatal cases akin to complicated falciparum malaria patients have been documented (*6,9*). Therefore, it is crucial to determine the public health burden of this simian malaria. To date, it is unknown whether the occurrence of human infections with *P. knowlesi* in Thailand was a result of newly emerging malaria species or the parasite has been circulating cryptically with other human malaria parasites since a long time ago. Furthermore, it is intriguing to explore spatio-temporal distribution of malaria species in humans and analyze genetic characteristics of *P. knowlesi* circulating among naturally infected macaques and human cases. These data could lead to a better understanding of malaria transmission and provide information for a more effective malaria control policy at a nation-wide level.

Materials and Methods

Prospective study and sample collection

In Thailand, the majority of malaria infections occurred along country borders and malaria transmission exhibited a bimodal pattern, peaking in May-July and October-November (*7*). Venous blood or finger pricked blood samples were collected from 3,770 febrile individuals (2577 males, 1193 females; mean age=27.4 years, age range=1-87 years) who attended malaria clinics at northwestern (Tak Province, n=1,354), eastern (Chantaburi Province, n=401) and southern (Yala Province, n=1,552 and Narathiwat Province, n=463) areas of Thailand from October 2008 to September 2009 (Figure 1). Of these, 470 blood

samples were negative for malaria parasites under microscopy 153 from Tak, 179 from Yala and 138 from Narathiwat).

Retrospective study

In 1996, a total of 210 blood samples from microscopy-positive symptomatic malaria patients (139 males, 71 females; mean age=25.1 years, age range=12-72 years) living in Tak Province were included for comparative analysis. Of these, 143 blood samples were collected from May to July and the remaining 70 samples from October to December. Blood samples were preserved in EDTA as an anticoagulant and kept at -40°C until use.

Microscopic diagnosis

Both thin and thick blood smears were prepared from each blood sample, stained with Giemsa solution and examined by experienced microscopists who were blinded to clinical information and the results of PCR detection. At least 200 leucocytes for thick blood film and at least 200 microscopic fields with the 100x objective were examined before a slide was declared negative.

PCR-based diagnosis

DNA was extracted from the 200 µl of blood by using the QIAGEN DNA minikit (Hilden, Germany) following the manufacturer's instruction. Malaria species was identified by nested PCR using *Plasmodium* genus-specific outer primers (M18SF0 and M18SR0) derived from *SSU rRNA* for primary PCR. Nested PCR was carried out in separate reaction tubes for each pair of the species-specific primers (*P. falciparum*, PF18SF and PF18SR; *P. vivax*, PV18SF and PV18SR; *P. malariae*, PM18SF and PM18SR; *P. ovale*, PO18SF and PO18SR; and *P. knowlesi*, PK18SF and PK18SR)(*8*). The thermal cycling profiles for the primary and nested PCR contained 35 and 25 cycles (94°C for 40 s, 60°C for 30 s and 72°C for 1 min), respectively. The PCR products were analyzed by agarose gel electrophoresis.

Sequencing of the complete merozoite surface protein-1 gene of *P. knowlesi* from humans and macaques

During December 2008-June 2009, a prospective survey of malaria in monkeys inhabiting Yala Province (n=70) and Narathiwat Province (n=566) was performed as reported (*10*). Analysis of the mitochondrial cytochrome oxidase *b* locus has revealed a number of malaria and *Hepatocystis* spp. in these monkey populations whereas *P. knowlesi* was detected in 5 *M. nemestrina* and one each in *M. fascicularis* and *Semnopithecus obscurus* in Narathiwat Province. The complete nucleotide sequences of the merozoite surface protein-1 gene of *P. knowlesi* (*Pkmsp-1*) from these monkeys and humans in the current survey were performed by direct sequencing of the PCR-amplified products as described previously (*11*). This study has been approved by the Institutional Review Board of Faculty of Medicine, Chulalongkorn University.

Data analysis

Sequences were aligned by the CLUSTAL X program with minor manual adjustment made by visual inspection (*12*). Phylogenetic construction was inferred from the maximum likelihood methods using the HKY model with 1,000 bootstrap iterations as implemented in the MEGA 5.01 program (*13*). Results of malaria species distribution in 2006–2007 from our previous analysis were included for comparison (*8*). Difference between the numbers of malaria cases was computed by using the chi-square or the Fisher's exact test. A 2-tailed p value of <0.05 denoted statistical significance.

Results

Malaria species distribution

Microscopic examinations of blood samples from 3,770 febrile patients collected from October 2008 to September 2009 revealed that 3,260 individuals had malaria parasites

in their circulation whereas 470 patients were negative. Most malaria cases diagnosed by microscopy were either *P. falciparum* (48.75%) or *P. vivax* (45.62%) whereas co-infections with both species contributed to 0.17%. Conversely, 186 of 470 microscopy-negative samples turned to be positive by nested PCR. In total, 3446 blood samples contained malarial DNA with a remarkable difference in species distribution and mixed species infections compared to microscopy (Table 1). Mixed species infections accounted for 13.26% of all PCR positive cases and were most pronounced in Tak Province (Table 2). Microscopy failed to diagnose *P. knowlesi* in these samples. By contrast, PCR identified 23 patients having *P. knowlesi* infections (8 monoinfections and 15 co-infections with other *Plasmodium* species) (Table 1).

P. knowlesi in blood samples collected in 1996

Analysis of 210 blood samples from microscopy-positive patients in Tak Province collected in 1996 revealed that PCR identified 55 and 96 patients having single infections of *P. falciparum* (26.19%) and *P. vivax* (45.71%), respectively. The remaining 59 patients (28.10%) had mixed species infections comprising co-infection with *P. falciparum* and *P. vivax* (n=50), co-infection with *P. vivax* and *P. malariae* (n=7), co-infection with *P. vivax* and *P. knowlesi* (n=1) and triple infection with *P. falciparum*, *P. vivax* and *P. malariae* (n=1). Therefore, *P. knowlesi* has circulated among malaria patients in Thailand since 1996, about 15 years ago.

Spatial variation

The distribution of *P. falciparum* and *P. vivax* displayed remarkable spatial variation in Thailand. PCR assays revealed that *P. falciparum* and *P. vivax* almost equally contributed to malaria cases in Tak Province in 2008-2009, being 50.55% and 48.68%, respectively, whereas *P. vivax* was remarkably more prevalent than *P. falciparum* in Chantaburi Province and the reverse was true for Yala and Narathiwat Provinces. Despite a

low overall prevalence of the remaining malaria species (<2%), *P. knowlesi* could be detected more often than *P. malariae* and *P. ovale* in these endemic areas (Table 3).

Temporal variation

The distribution of *P. falciparum*, *P. vivax*, *P. malariae* and *P. ovale* in Thailand among samples collected in 2006-2007 and in 2008-2009 exhibited significant temporal variation (χ^2= 99.9, 92.2, 24.6 and 20.5 respectively; all *p* values <0.0001). On the other hand, the occurrence of *P. knowlesi* remained stable over these periods (χ^2= 0.17; p=0.68). Considering only Tak Province where samples were available for comparison in 3 different periods (1999, 2006-2007 and 2008-2009), *P. knowlesi* contributed to malaria cases not significantly different in all pair-wise comparison (p>0.05), suggesting a stable prevalence of this simian malaria over a decade in this endemic area. By contrast, there was a significant difference in the distribution of *P. falciparum*, *P. vivax* and *P. malariae* collected from these three periods in each endemic area (p<0.05 in all pair-wise comparisons) while *P. ovale* was not detected in samples collected in 1996 but contributed to 1.41% and 0.21% of samples collected during 2006-2007 and 2008-2009, respectively (Table 3). Meanwhile, the prevalence of mixed species infections exhibited both regional and seasonal variations characterized by a significantly higher prevalence in dry season than in raining season in isolates from Chantaburi Provinces (χ^2=5.94, p=0.015; OR, 2.59 [95% CI, 1.20-5.60]) and the reverse was true for isolates from Yala Province (χ^2=101.7, p<0.001; OR, 7.2 [95% CI, 4.65-10.90]). No significant seasonal difference in the prevalence of mixed species infections occurred in isolates from Tak (χ^2=1.09, p=0.60; OR, 1.09 [95% CI, 0.81-1.46]) and Narathiwat Provinces (χ^2=0.10, p=0.76; OR, 1.09 [95% CI, 0.64-1.87]).

Characteristics of *P. knowlesi*-infected patients

Of 5,407 PCR-positive samples collected in 1996, 2006-2007 and 2008-2009, *P. knowlesi* was detected in 34 patients (0.63%) (Tables 2 and 3)(*8*). All patients infected with *P. knowlesi* including the first case identified in 2000 (n=35) had uncomplicated malaria symptoms (*3,8*). The age range was from 4 to 59 years (mean, 30 years; mode, 19 years; median, 33.5 years) with the majority of cases (73.5%) occurring between ages 16-45 years. Knowlesi malaria was diagnosed in males about twice more often than in females (male:female=2.18:1) akin to gender distribution of total malaria cases (male:female=2.16:1); thereby no sex preference was observed (p=0.97). Almost half of knowlesi malaria patients acquired the infections in Southern Thailand bordering Malaysia. Single infections with *P. knowlesi* were observed in 10 patients whose parasite density ranged from 0 to 145,000 parasites/μl. The remaining 24 cases had co-infection with *P. falciparum* (n=16), co-infection with *P. vivax* (n=9) and triple infection with *P. falciparum* and *P. vivax* (n=5). Although the geometric mean of parasite density of *P. knowlesi* moninfection seems to be more than those of mixed species infections, no significant difference was observed (p>0.05, Mann-Whitney U-test) (Table 4). Initial microscopy diagnosis was *P. malariae* for those having single infections with *P. knowlesi* whereas either *P. falciparum* or *P. vivax* was determined in patients with mixed species infections. About two-third (23/35) of knowlesi malaria occurred in rainy season. However, the ratio of *P. knowlesi*-infected patients to total malaria cases identified in rainy and dry seasons was not significantly different (χ^2=0.25; p=0.62; OR=1.20; 95%CI=0.60-2.38). The majority of human infection with *P. knowlesi* (73.5% of total cases) occurred in areas with macaques living in vicinity.

<u>The msp-1 sequence of *P. knowlesi*</u>

The complete Pkmsp-1 gene was analyzed using 8 isolates from patients collected during the current survey in Narathiwat (n=3), Chantaburi (n=3) and Yala Provinces (n=2),

3 isolates from patients in Prachuab Khirikhan Province collected in 2000 and 2006-2007 (*3,8*) and 5 isolates from naturally infected pig-tailed macaques in Narathiwat Province collected in 2008-2009 (*10*). All of these isolates had single Pkmsp-1 sequences because no superimposed signals occurred in electropherograms. Size variation was observed amongst *Pkmsp-1* of these isolates, ranging from 5430 to 5613 bp. Phylogenetic analysis revealed that both human and macaque isolates displayed genetic diversity at the Pkmsp-1 locus that could be placed into 2 clusters with 100% bootstrap support. One cluster contained 6 human isolates (BMC151, CT273, MC128, NR234, YL975 and YL978) and a monkey isolate from Narathiwat Province (isolate HB3) whereas the remaining isolates belonged to the other cluster (Figure 2). Interestingly, identical *Pkmsp-1* sequences were found in an isolate from a patient in Narathiwat Province (NR280) and an isolate from a pig-tailed macaque (HB149) dwelling around, in the neighborhood. Furthermore, isolates from 2 patients living in Yala Province (YL975 and YL978), who concurrently got *P. knowlesi* infection in the same endemic areas, shared completely identical *Pkmsp-1* sequences. Likewise, the other identical sequences were found in isolates from 2 patients in Chantaburi Province (CT157 and CT190) who had onset of febrile illness a few days apart (Figure 2).

Discussions

Despite a dramatic decline in the annual incidence of malaria cases in Thailand since the nation-wide implementation of malaria control program in the 1950s, disease transmission remains persistent in several areas mostly along international borders of the country and in forest or forest-fringes (*14,15*). Our PCR analysis on a large number of samples representing 12.4% (3770/30425) of total malaria cases in the study areas (*16*) has revealed a remarkable spatio-temporal heterogeneity in the prevalence of both *P.*

falciparum and *P. vivax* in line with previous analyses based on microscopy-based detection (15,17). Interestingly, the prevalence of *P. falciparum* and *P. vivax* in Yala and Narathiwat Provinces located adjacent to each other also exhibited significant spatial difference in species distribution in 2006-2007 whereas a non-significant difference was observed in 2008-2009, supporting a spatio-temporal pattern on a micro-geographic level as noted in other endemic areas in Thailand (*15*). Furthermore, spatio-temporal variation in the prevalence of both *P. malariae* and *P. ovale* was found. Cross-border migration of infected individuals, predominantly along Thailand-Myanmar and Thailand-Cambodia borders, could plausibly contribute to both spatial and temporal variation in the prevalence of these malaria species (*14,15*). However, migration *per se* could not explain similar findings in Yala and Narathiwat Provinces because almost all malaria cases in these areas were autochthonous cases (*16*). Meanwhile, a dramatic increase in *P. vivax* prevalence in endemic areas bordering Cambodia during the past decade has reportedly been due to relative changes in species distribution of local vectors with difference in vectorial capacity (*18-20*). Furthermore, intrinsic difference in parasite biology such as the presence of hypnozoites in *P. vivax* and *P. ovale* could contribute to additional episodes of infections if not radically treated. Therefore, it is likely that spatio-temporal variation in these human malarias in Thailand could stem from multiple variable factors.

Our previous survey in 2006-2007 has shown that co-infections of different malaria species displayed spatial variation, characterized by a high prevalence (~23-24%) along Thailand-Myanmar border, contrasting with a low prevalence of 3% and 5% occurring in endemic areas bordering Cambodia and Malaysia, respectively (*8*). Our current survey has detected changing prevalence of mixed species infections compared to the survey in 2006-2007 in these same endemic areas that could be due to the changing prevalence of each malaria species. Microscopy failed to detect either *P. falciparum* or *P. vivax* in isolates

containing co-infections of both species at a comparable rate (p = 0.35). When one of these two species coexisted with other malaria species (including *P. knowlesi*), only *P. falciparum* or *P. vivax* was diagnosed by microscopy. Failure to diagnose mixed species infections could lead to requirement for repeated diagnosis and treatment, economic loss, misinterpretation of drug or vaccine efficacies and inadequate control policy (*21,22*).

Co-infections of *P. falciparum* and *P. vivax* reportedly could be advantageous to the hosts in terms of reduced disease severity, lower chance for gametocyte carriage and decrease in parasite density whereas detrimental outcomes have been observed in other studies (*21-23*). Although the parasite densities of *P. knowlesi* monoinfections and co-infections with other species were not significantly different, the geometric mean of the former was more than twice the latter (Table 4). Therefore, interference by a high prevalence of mixed species infection (~14% in total for the current survey) and probably cross-species immunity could partly contribute to the low prevalence of *P. knowlesi* in Thailand. By contrast, a lower prevalence of mixed species infections was observed in Sarawak where the majority of malaria patients had *P. knowlesi* monoinfections (~91%) (*4,6*).

There was no significant difference in temporal and spatial distribution of *P. knowlesi* in the study areas, suggesting that the transmission pattern could be different from other human malaria. It is noteworthy that natural vectors for *P. knowlesi* in Malay Peninsular were *Anopheles cracens* and *An. latens,* members of the Leucosphyrus group (*24,25*), whereas the main vectors for human malaria in Thailand comprised *An. minimus*, *An. maculatus* and *An. dirus* (*26*). Although *An. dirus* also belongs to the Leucosphyrus group and has been incriminated as a potential vector for *P. knowlesi* in Vietnam (*27*), this vector species exhibited a drastic decline in abundance in all major malaria endemic areas of this country during the past decade (Putaporntip et al, unpublished) whereas their

feeding behaviors were zoonotic rather than anthropophilic in certain areas (*28*). Therefore, transmission of *P. knowlesi* to human could be limited and distinct from other human malaria in Thailand. On the other hand, identification of *P. knowlesi* cryptically circulated among malaria patients in Tak Province collected in 1996 has implied its past occurrence at least 15 years ago in Thailand with relatively stable prevalence. Likewise, recent analysis of archival blood samples from Sarawak patients collected over a decade ago has supported that knowlesi malaria in humans was not newly emergent zoonotic malaria species (*29*).

Our recent survey on simian malaria in macaque populations in western and southern Thailand (n=754) has revealed a prevalence of 26% (196/754) (*10*). Although *P. inui* was the most frequently identified malaria species, *P. knowlesi* was found in naturally infected long-tailed macaques and pig-tailed macaques accounting for 5.6% and 2.3%, respectively (*10*). Sequence analysis of the complete Pkmsp-1 sequences from 5 *P. knowlesi*-infected macaques living in vicinity to infected human cases has shown that all displayed unique sequences, reaffirming genetic heterogeneity of *P. knowlesi* in its natural hosts in Thailand (*8*). Importantly, *P. knowlesi* from a patient in Narathiwat Province shared an identical Pkmsp-1 sequence with that from a pig-tailed macaque living in vicinity, suggesting that *P. knowlesi* could be transmitted from macaques to humans and vise versa through anopheline vectors. However, the higher prevalence of *P. knowlesi* in macaques than in humans and the chronic course of parasitemia in asymptomatic macaque natural hosts could provide a higher chance for transmission from macaques to humans. Furthermore, the acute clinical course of malaria infection in humans would be rapidly eliminated by antimalarial treatment resulting in a lower chance for further transmission of *P. knowesi* gametocytes to the vectors (*1,8*). Meanwhile, an identical *PkMsp-1* sequence observed in 2 patients in Chantaburi Province who lived within neighborhood and had symptomatic malaria within the same week could suggest that both acquired the infections

from a common source. Alternatively, parasites harboring this identical *PkMsp-1* allele could predominate among infected macaques in the region. Unfortunately, no extensive survey of malaria in wild macaques around Chantaburi Province could be performed during the period.

A spectrum of clinical manifestations has been observed in individuals infected with *P. knowlesi* (*6,30*). However, the majority of patients with knowlesi malaria presented with febrile illness and associated symptoms were indistinguishable from those caused by other malaria species (*2,3*). It is noteworthy that *P. knowlesi* spends 24 hours to complete its asexual erythrocytic cycle resulting in a unique quotidian type of fever pattern contrasting with other 4 human malaria species (*1*). However, such paroxysms could not be of practical value for presumptive diagnosis because characteristic fever patterns would not be observed during early phase of infections while mixed species infections could further complicate the febrile symptoms (*31*). Furthermore, *P. knowlesi* malaria seems to be well-responsive to chloroquine treatment and would be probably responsive to other antimalarial drugs (*4*). Therefore, reappearance of *P. knowlesi* among patients with mixed infections after initial malaria treatment of another malaria species in Thailand seems to be less likely. Importantly, *P. knowlesi* infections in humans reportedly caused severe symptoms and fatal outcome accounting for 6.5% and 1.8%, respectively, in Kapit population in Sarawak where it contributed to 70% of all malaria cases (*4*). Provided that knowlesi malaria could cause similar prevalence of complicated symptoms, the probability to observe such severe and fatal consequences from *P. knowlesi* infections among patients in Thailand would be low.

In conclusion, human malaria caused by *P. knowlesi* occurred in Thailand over a decade ago. Despite significance variation in the prevalence of all 4 human malaria species, *P. knowlesi* remained persistence at almost stable prevalence rates, suggesting different

transmission cycles. Human infections with *P. knowlesi* in Thailand could be from macaque natural hosts as evidenced by the shared *PkMsp-1* sequences of human and macaque origins.

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Figure captions

Figure 1. Map of Thailand showing provinces where blood samples were collected: Tak (blue), Prachuab Khirikhan (orange), Yala (purple), Narathiwat (yellow) and Chantaburi (red).

Figure 2. Maximum-likelihood tree inferred from the complete msp-1 sequences of *P. knowlesi* from human (red circle) and macaque (blue circle) origins. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Bootstrap values >50% are shown. Human isolates are from the following provinces: Narathiwat (NR280, NR234 and NR522), Yala (YL975 and YL978), Chantaburi (CT157, CT190 and CT273) and Prachuab Khirikhan (BMC151, MC128 and DQ220743). Isolates HB3, HB92, HB126, HB132 and HB149 are from macaques in Narathiwat Province. Sequence of *P. knowlesi* strain H is from GenBank accession number XM_002258546.

ประวัติและผลงานนักวิจัย

1. ชื่อ-นามสกุล (ภาษาไทย) นายแพทย์ ดร. สมชาย จงวุฒิเวศย์
   ชื่อ-นามสกุล (ภาษาอังกฤษ) Dr. Somchai Jongwutiwes
2. เลขหมายประจำตัวประชาชน 3 1005 0309 5200
3. ตำแหน่งปัจจุบัน ศาสตราจารย์
4. หน่วยงานที่อยู่ที่สามารถติดต่อได้ ภาควิชาปรสิตวิทยา คณะแพทยศาสตร์
   จุฬาลงกรณ์มหาวิทยาลัย กรุงเทพฯ 10330
   โทรศัพท์ 02-2528181 ต่อ 3685
   โทรสาร 02-2524963
   e-mail address: fmedsjw@md2.md.chula.ac.th;
   malaria1823@gmail.com

5. ประวัติการศึกษา

มหาวิทยาลัย	ปริญญา	สาขา	ปีพุทธศักราชที่จบ
จุฬาลงกรณ์มหาวิทยาลัย	วิทยาศาสตร์บัณฑิต (เกียรตินิยม)	วิทยาศาสตร์การแพทย์	2525
จุฬาลงกรณ์มหาวิทยาลัย	แพทยศาสตร์บัณฑิต	แพทยศาสตร์	2527
Nagasaki University	Doctor of Philosophy	Molecular Protozoology	2536

6. สาขาที่มีความชำนาญพิเศษ Medical Parasitology, Molecular Parasitology, Molecular Population Genetics

7. ประสบการณ์ที่เกี่ยวข้องกับการบริหารงานวิจัยทั้งภายในและภายนอกประเทศ

7.1 หัวหน้าโครงการวิจัย: ชื่อโครงการวิจัย

1. Molecular evolution of *Plasmodium vivax*
2. Molecular analysis of the cpg40 gene of *Cryptosporidium* isolated from HIV-infected patients

7.2 งานวิจัยที่ทำเสร็จแล้ว : ชื่อผลงานวิจัย ปีที่พิมพ์ การเผยแพร่

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Table 1 Distribution of *Plasmodium* species in 3446 malaria patients in Thailand from October 2008 to September 2009

Nested PCR	Microscopy						Total
	P.falciparum	*P.vivax*	*P.malariae*	*P.ovale*	*P.falciparum* + *P.vivax*	Negative	
P.falciparum	1397	78	0	0	2	79	1556
P.vivax	52	1301	0	0	1	68	1422
P.malariae	1	1	1	0	0	0	3
P.ovale	0	0	0	0	0	0	0
P.knowlesi	3	3	1	0	0	1	8
P.falciparum+P.vivax	211	181	0	0	3	37	432
P.falciparum+P.malariae	1	0	0	0	0	1	2
P.falciparum+P.ovale	3	0	0	0	0	0	3
P.falciparum+P.knowlesi	6	0	0	0	0	0	6
P.vivax+P.malariae	0	3	0	0	0	0	3
P.vivax+P.ovale	1	1	0	0	0	0	2
P.vivax+P.knowlesi	3	1	0	0	0	0	4
P.falciparum+P.vivax+P.knowlesi	2	3	0	0	0	0	5
Total	1680	1572	2	0	6	186	3446

a

Table 2 Distribution of *Plasmodium* species by endemic areas of Thailand from October 2008 to September 2009

Nested PCR	Region				Total
	Northwestern (Tak)	Eastern (Chantaburi)	Southern (Yala)	Southern (Narathiwat)	
P.falciparum	730	76	919	279	2004 (51.28%)
Single infection	507	27	810	212	1556
Mixed infection	223	49	109	67	448
P.vivax	703	370	592	203	1868 (47.80%)
Single infection	481	320	485	136	1422
Mixed infection	222	50	107	67	446
P.malariae	3	1	2	2	8 (0.20%)
Single infection	1	1	0	1	3
Mixed infection	2	0	2	1	5
P.ovale	3	0	1	1	5 (0.13%)
Single infection	0	0	0	0	0
Mixed infection	3	0	1	1	5
P.knowlesi	5	7	5	6	23 (0.59%)
Single infection	0	3	2	3	8
Mixed infection	5	4	3	3	15
Total	1216	401	1408	421	3446
Single infection	989 (81.33%)	351 (87.53%)	1297 (92.12%)	352 (83.61%)	2989 (86.74%)
Mixed infection	227 (18.67%)	50 (12.47%)	111 (7.88%)	69 (16.39%)	457 (13.26%)

b

Table 3 Temporal variation in distribution of *Plasmodium* species by endemic regions of Thailand

Nested PCR	% Distribution								
	Northwestern (Tak)			Eastern (Chantaburi)		Southern (Yala)		Southern (Narathiwat)	
	1996 (n=210)	2006-7 (n=681)	2008-9 (n=1216)	2006-7 (n=261)	2008-9 (n=401)	2006-7 (n=286)	2008-9 (n=1408)	2006-7 (n=370)	2008-9 (n=421)
P.falciparum	39.26	44.54	50.55	8.42	16.74	57.14	60.50	23.31	56.82
P.vivax	57.41	52.63	48.68	91.03	81.50	41.86	38.97	76.17	41.34
P.malariae	2.96	1.16	0.21	0	0.22	0	0.13	0	0.41
P.ovale	0	1.41	0.21	0.27	0	0	0.07	0	0.20
P.knowlesi	0.37	0.26	0.35	0.27	1.54	1.00	0.33	0.52	1.22

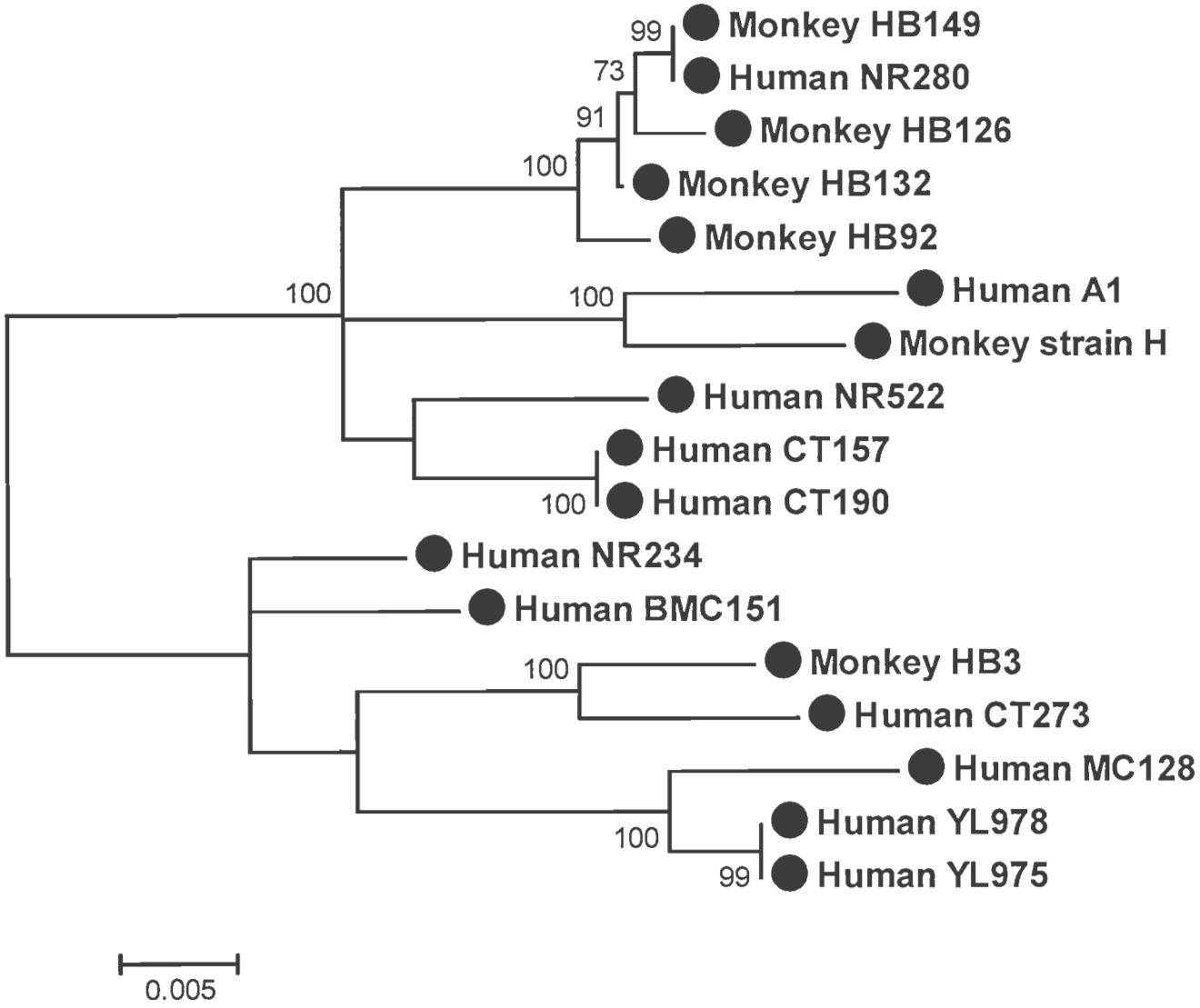
Table 4 Parasite densities of patients with *P. knowlesi* monoinfection and co-infection with other malaria species

Category	Parasite density (parasites/μl)*		
	Geometric mean	Ratio of geometric means**	Range
P. knowlesi monoinfection (n=10)	4,165	-	0 – 145,000
P. knowlesi mixed with			
P. falciparum (n=11)	1,632	2.55	440 – 6,560
P. vivax (n=9)	1,686	2.47	320 – 13,120
P. falciparum and *P. vivax* (n=5)	487	8.55	320 – 1,520
All mixed infection (n=25)	1,271	3.28	320 – 13,120

* Parasite densities between categories were not significantly different ($p>0.05$, Mann-Whitney U-test).

** Ratio of parasite densities of monoinfection to mixed infection.

(Fig. 1 Jongwutiwes et al)



B

Fig. 2 Jongwutiwes et al.