รายงานวิจัยฉบับสมบูรณ์

การศึกษายุงพาหะนำโรคมาลาเรียในคนชนิดที่ห้า *Plasmodium knowlesi*

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สัญญาเลขที่ MRG5580063

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มหาวิทยาลัยมหิดล

สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัย
และสำนักงานคณะกรรมการอุดมศึกษา
และมหาวิทยาลัยมหิดล

กิตติกรรมประกาศ

รายงานวิจัยฉบับสมบูรณ์ เรื่อง การศึกษายุงพาหะนำโรคมาลาเรียในคนชนิดที่ห้า เสร็จสมบูรณ์ได้โดย โครงการวิจัยนี้ได้รับทุนสนับสนุนโดยสำนักงานคณะกรรมการการอุดมศึกษา (สกอ.) และสำนักงานกองทุนสนับสนุนการวิจัย (สกว.) ระยะเวลาดำเนินโครงการ 2 ปี ตั้งแต่มีนาคม 2555 ถึง มีนาคม 2557 รายงานการวิจัยฉบับนี้จัดทำขึ้นเพื่อใช้เป็นข้อมูลทางวิชาการในการปรับกลยุทธในการควบคุมการแพร่เชื้อไข้มาลาเรียโดยยุงก้นปล่องได้ตรงจุดและทำได้อย่างมีประสิทธิภาพเพื่อตอบโจทย์ของ malaria elimination ที่กำลังดำเนินการอยู่ในปัจจุบัน

นอกจากนี้การศึกษาครั้งนี้ได้รับการสนับสนุนจาก ดร.เจตสุมน ประจำศรี นักวิจัยที่ปรึกษาในการให้คำปรึกษางานวิจัย ตลอดจนเจ้าหน้าที่ภาควิชากีฏวิทยาการแพทย์ทุกท่าน รวมทั้งเจ้าหน้าที่หน่วยวิจัยมหิดลไวแวกซ์ คณะเวชศาสตร์เขตร้อน มหาวิทยาลัยมหิดล ที่ช่วยสนับสนุนโอกาสในการทำงานวิจัยร่วมกันและคำแนะนำต่างๆ ขอขอบคุณ รอ.ดร. ปรินดา ภูคงเดือน ที่เอื้อข้อมูลทางภูมิศาสตร์และการแสดงผลของพื้นที่วิจัย ส่งผลให้โครงการวิจัยนี้สำเร็จได้ด้วยดี

ขอขอบพระคุณทุกท่าน

(ผศ.ดร.พัชรา ศรีวิชัย)
ผู้วิจัย

บทคัดย่อ

รหัสโครงการ: MRG5580063

ชื่อโครงการ : การศึกษายุงพาหะนำโรคมาลาเรียในคนชนิดที่ห้า *Plasmodium knowlesi*

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ระยะเวลาโครงการ : 2 ปี (กรกฎาคม 2555 - กรกฎาคม 2557)

จากการสำรวจกลุ่มยุงก้นปล่องในเขตบ้านผู้ป่วยมีรายงานโรคไข้มาลาเรียชนิด *Plasmodium knowlesi* ในรัศมีหนึ่งกิโลเมตร ณ อำเภอมะขาม จังหวัดจันทบุรีซึ่งอยู่ทางตะวันตกของประเทศไทย โดยทำการจับยุงใช้กับดักแสง CDC, การจับโดยใช้มนุษย์เป็นเหยื่อล่อและกับดัก BG ในช่วงฤดูแล้งและฤดูฝนในปี 2555-2556 สามารถจับยุงตัวเต็มวัยจำนวนทั้งหมด 1,125 ตัว จำนวน 9 จีนัส โดยพบยุงก้นปล่อง (Anopheles) จำนวน 4 ชนิดได้แก่ *An. barbirostirs* complex (96%) ตามด้วย *An. dirus* s.l. (3.3%), *An. asiaticus* (0.3%) และ *An. tessellatus* (0.3%) โดยยุงตัวเมียในกลุ่มยุงก้นปล่องนี้ทำการตรวจหาการติดเชื้อมาลาเรียในตัวยุง Anopheles ด้วยวิธี PCR เชิงปริมาณและ PCR เชิงซ้อน (Nested PCR) และหา Typing species ด้วยวิธี RFLP จากตัวอย่างทั้งหมด 243 ตัวพบการติดเชื้อ *Plasmodium vivax* (PV) ชนิด PV210ในยุง *An. barbirostirs* complex จำนวน 2 ตัวคิดเป็นอัตราการติดเชื้อร้อยละ 0.83 โดยหนึ่งในนั้นไม่สามารถจำแนก circumsporozoite typing ได้ ยุงทั้งสองตัวที่มีเชื้อถูกจับในช่วงฤดูแล้งของเดือนพฤษภาคมจากการใช้คนเป็นเหยื่อล่อในบ้านเวลา 21.00 น. และ 23.00 น. ซึ่งมีอัตราการกัด 7.40 และ 5.80 ครั้งต่อคนต่อชั่วโมงตามลำดับ ยุงกลุ่ม barbirostirs นี้มี พฤติกรรมที่เปลี่ยนแปลงไปโดยชอบกินเลือดคนในบ้าน (endophagic) ผลการศึกษานี้แม้จะไม่สามารถตรวจพบเชื้อ *P. knowlesi* แต่แสดงให้เห็นว่ายุงก้นปล่องกลุ่ม barbirostirs ในธรรมชาติเป็นพาหะของไข้มาลาเรียและมีศักยภาพและมีบทบาทสำคัญในการแพร่เชื้อมาลาเรียในบริเวณนี้ การศึกษาเพิ่มเติมนี้สามารถใช้เป็นข้อมูลสำคัญเพื่อทำความเข้าใจบทบาทของยุงพาหะในการแพร่เชื้อไข้มาลาเรียในพื้นที่นี้และช่วยในการปรับปรุงกลยุทธ์การควบคุมโรคมาลาเรียให้มีประสิทธิภาพและคุ้มค่าสุงสุด

คำหลัก : *Plasmodium vivax, Plasmodium falciparum, Plasmodium knowlesi,* ยุง *Anopheles barbirostris*, การติดเชื้อ, ไข้มาลาเรีย

Abstract

Project Code : MRG5580063

Project Title : *Plasmodium knowlesi* the fifth species of human malaria: investigation for mosquito vector in Thailand

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Project Period : 2 years (July 2012- July 2014)

Abstract

Wild caught *Anopheles* mosquitoes were collected in Chanthaburi province, western Thailand. One kilometer in diameter of malaria reported patient house by CDC light trap, human landing catch, and BG traps during specific wet and dry seasons in 2012-2013. Total 9 genus of 1,125 adult mosquitoes were collected, From 4 Anopheles species found, *An. barbirostirs group* was a dominant (96%) followed by *An. dirus group* (3.3%), *An. asiaticus* (0.3%), and *An. tessellatus* (0.3%). QMAL quantitative PCR and nested PCR were performed to detect malaria infection in Anopheles mosquitoes. Interestingly, there were two positive *Plasmodium vivax* (PV) infections, with PV210 and one of unclassified genotypic variants, from *An. barbirostirs* complex (0.83%, 2/243). They were captured from indoor human landing catch of the patient house at 21.00 and 23.00 for 7.40 and 5.80 bites person per hour, respectively, during May as a dry season. *An. barbirostirs* group was preference to endophagic behavior. The preliminary finding of this study indicated that the natural *barbirostirs* group can be a potential malaria vector and may play important role of malaria transmission in this area. Further study is required to better understand the potential role of secondary vectors in malaria transmission in this area and subsequently assist to improve malaria control strategies.

Keywords: *Plasmodium vivax, Plasmodium falciparum, Plasmodium knowlesi, Anopheles barbirostris* group, infection, malaria

Executive summary

This study investigated the malaria vector that was prevalent for the common human malaria, *Plasmodium falciparum* (PF), *P. vivax* (PV), and the new one, *P. knowlesi* (PK). The study area was focused at the patient data history appearance by PK infection. The specific malaria species and their susceptibility of mosquito vectors were determined by molecular techniques of real time and nested PCR as well as the species typing was also detected by RFLP technique. Vector biology and roles of transmission dynamics of Anopheles vector and malaria incidence need to be investigated in the study area. This can be imaged the transmission and more investigated vector biology to support human malaria including *P. knowlesi* transmission. It can be the guideline for the effective vector control program suitable for the local malaria vector as following the malaria elimination strategies of Thailand. The study represented the additionally local malaria vectors in Thai-Cambodia border that was *Anopheles barbirostrais* group to susceptible for *P. vivax* (PV210) with high infection rate up to 0.83%. It played the transmission role for malaria particularly PV as dominant species. The positives were compatible to peak of indoor man biting rates at 21.00 and 23.00 for 7.40 and 5.80 per person per hour, respectively. Entomological Inoculation rate (EIR) of indoor and outdoor presented for risk of malaria transmission in the area showed that indoor and outdoor household EIR of *An. barbirostirs* group were 17.29 and 6.16 infectious bite per night respectively. Moreover, this species had the changeable behaviour from exo to endophagic behaviour that contribute to human risk of malaria transmission in the household. Even it could not be detected *PK* in the mosquitoes, this study represented the natural malaria PV vectors in the household area with high endoghagic pattern to improve of more indoor transmission as the additional information not only *Anopheles dirus* as the main vector in this area. Vector control program should be adjusted to compatible with the correct time, place, and period of vector controls programs. More focus on self-protection strategy to decreasing the mosquito-human contact by of net use and outdoor repellent that should be analysed and evaluated for the practical actions particular during the agriculture activities both specific Thai and Foreigner with high risks of malaria exposure.

Potential *Plasmodium vivax* malaria vector of *Anopheles campestris* in focal endemic area along Thai-Cambodia border

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The most malaria incidence along Thai-Cambodia Border is *Plasmodium vivax* and there have been reported 3 cases with co-infection of *Plasmodium knowlesi* (PK) patient infected in Chantaburi province in 2011. Few investigations focusing on malaria vector with lacking of understanding in role of vector transmission. We have investigated the potential vectors that may help us to understand the malaria transmission in the endemic areas, such as in Chantaburi province. Wild caught mosquitoes were collected from 1 kilometer in diameter of *P. knowlesi* reported patient house by CDC light trap, human landing catch, and BG traps during specific wet and dry seasons in 2012-2013. Total 30 genus of 1,131 adult mosquitoes were collected, From 6 Anopheles species found, *An. campestris* was a dominant (95.4%) followed by *An. dirus* (2.0%), *An. baimai* (1.3%), *An. barbilostris* (0.7%), *An. asiaticus* (0.3%), and *An. tessellatus* (0.3%). qMAL quantitative PCR and nested PCR were performed to detect malaria infection in Anopheles mosquitoes. Interestingly, there was one positive PV210 infection from *An. campestris* (0.41%, 1/243) captured from indoor human landing catch of the patient house at 21.00 during May as a dry season. *An. campestris* represented the preference blood feed indoor than outdoor. The preliminary finding of this study indicated that *An. campestris* can be a potential malaria vector and may play important role of malaria transmission in this area. Further study is required to better understand the potential role of secondary vectors in malaria transmission in this area and subsequently assist to improve malaria control strategies.

Keywords: *Anopheles campestris*, *Plasmodium vivax*, Thailand, qMAL real-time PCR, Nested PCR

เนื้อหางานวิจัย

Introduction

Malaria remains one of the most important infectious diseases in the world. The annual human malaria associated mortality approaches 1 million, with 2 children dying from the disease every minute worldwide causing by *Plasmodium falciparum, Plasmodium vivax, Plasmodium ovalae, and Plasmodium malariae* (WHO, 2017). The newcomer is *P. knowlesi*, which have known to cause malaria only in macaques. Zoonotic *P. knowlesi* infection is the normal mode of transmission by mosquito vectors that mainly feed in the forest (Cox-Singh and Singh, 2008). Usually, *P. knowlesi*, a malaria species that has the specific host with the long-tailed macaques, has found to be prevalent in humans in Southeast Asia (Kantele and Jokiranta, 2011). Most of the current researches are focused on characterization of parasite and the evidence within the natural human host (Jiang et al., 2010; Jongwutiwes et al., 2004; Kantele and Jokiranta, 2011; Vythilingam et al., 2008; Vythilingam et al., 2006a). There is still not a firm understanding of the potential vectors to support plasmodial infectivity to mosquitoes. It can be assumed that its vector behaves like the vectors of *Anopheles* species but few data on specific determination in the prevalent area. The extent to which the parasite can be transmitted from human back to mosquito vector in nature is also unclear. Thailand is the one of high case reports of *P. knowlesi* in human (Berry et al., 2011; Jongwutiwes et al., 2004; Putaporntip et al., 2009). This study will be investigated on the gap of the potential vector that is prevalent among of the common malaria, *P. falciparum*, *P. vivax*, and the new one, *P. knowlesi*. We select the study site in Chanthaburi province where the preliminary study has shown the positive *P. knowlesi* detection in human blood.

The obtained information of infectivity both in human and mosquito vectors will support the direction of control program for *P. knowlesi* malaria in Thailand. It is the initiate to understand the interactions of human host and *P. knowlesi* – vector.

Literature review

During current of malaria researches have developed the effective controls and eradicative programs by focusing on *P. falciparum* and *P. vivax*, *P. knowlesi* is the newcomer to appears as a natural parasite of macaques throughout the Southeast Asian region. It was not until 1965 that the first natural infection of *P. knowlesi* in humans was

reported in an American traveller returning from Peninsular Malaysia (Chin et al., 1965). Hundreds of human cases have been reported from Malaysia, several cases have been reported in other Southeast Asian neighboring countries, Thailand (Jongwutiwes et al., 2004; Putaporntip et al., 2009), Singapore (Ng et al., 2008), Brunei (Health protection agency 2011), Indonesia (Figtree et al., 2010), Myanmar (Jiang et al., 2010), Vietnam (Van den Eede et al., 2009), and the Philippines (Luchavez et al., 2008) (Figure 1). In 2004, *P. knowlesi* malaria was described in a patient who lived in a suburb of Bangkok, Thailand, and who had visited southern Thailand near the Myanmar border (Jongwutiwes et al., 2004). Subsequently*, P. knowlesi* has been identified in 10 patients from southern and southwestern Thailand (Putaporntip et al., 2009). A few cases have been reported in travellers visiting these areas (Thailand, Indonesia, Peninsular Malaysia, and Vietnam) ((Ta et al., 2010).

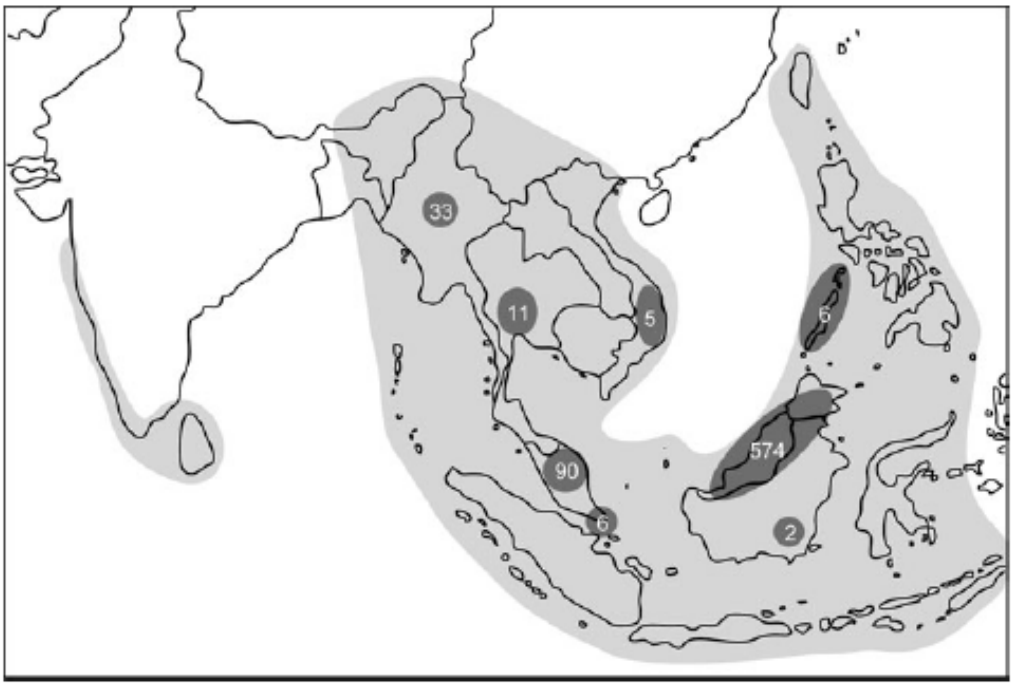


Figure 1: *Plasmodium knowlesi* infections reported in humans in Southeast Asia. *Anopheles leucosphyrus* is the main the main vectors for *P. knowlesi* that show in the grey area. The numbers represent the patients infected by *P. knowlesi* including both local patients and travellers returning from that area (Kantele and Jokiranta, 2011).

In environmental contexts, an overlapping of human and simian habitat becomes evident to a normally infested by mosquitoes of the genus *Anopheles* that are required to be the vehicle for malaria transmission to humans. Among 20 plasmodia species that are probably infect monkeys. Five of them have been documented as the potential infectious agents for humans: they are *Plasmodium simium, Plasmodium brasilianum*, *Plasmodium cynomolgi, Plasmodium inui,* and *P. knowlesi* (Garnham, 1966). These simian malaria infections were acquired by humans through blood passage or in laboratory settings through mosquito bites (Garnham, 1966). In the natural hosts, *P. knowlesi* causes

an asymptomatic low grade parasitism or mild disease (Cox-Singh et al., 2008; Singh et al., 2004). The possibility of zoonotic malaria in Southeast Asian forests, because of the transmission of *P. knowlesi* from monkeys to humans (Cox-Singh et al., 2008; Vythilingam et al., 2008), may form an additional complication (Marchand et al., 2011). It remains to be seen what kind of spreading has occurred in the past or will occur in the future, but to date, neither human-mosquito-human transmission nor spreading of *P. knowlesi* has been documented. *P. knowlesi* is not clear for the strict species-specific, because both experimental, and not natural, monkey-to-human and human-to human transmission have proved to be possible (Chin et al., 1968). It is not known whether human *P. knowlesi* infections are obtained only from mosquitoes fed on macaques or whether natural human-mosquito-human transmission occurs. Despite extensive studies in Malaysia in the 1960s, no additional reports appeared until 2004, when Singh *et al* described 120 cases of naturally acquired *P. knowlesi* infection in humans in Malaysian Borneo (Singh et al., 2004).

Other mechanisms of species specificity of plasmodia that are attributable to vector restriction, vector feeding preferences, and vector species specificity remain less information. *P. knowlesi* transmission is vector restricted in that the parasite can be transmitted only by certain *Anopheles* mosquitoes (Vythilingam et al., 2008). At least 2 main vectors in the *Anopheles leucosphyrus* group*, Anopheles latens* and *Anopheles cracens*, are known to be forest feeders, biting both humans and macaques at evening or during the night (Vythilingam et al., 2008). Taken together, numerous mechanisms restrict the nonhuman *Plasmodium* species from infecting humans, yet *P. knowlesi* has proved to make a significant exception to this rule.

P. knowlesi has also been detected in a patient who had spent time in a forested area on the Thai-Myanmar border (Jongwutiwes et al., 2004). Most cases have been described that the patients have a recent history of working or dwelling in a forest or forest fringe, which is the environment characteristic of the vector mosquitoes (Tan et al., 2008; Vythilingam et al., 2008). The situation might change if human-mosquito-human transmission occurred, because at least one widely distributed urban mosquito species (*Anopheles stephensi*) has experimentally been shown to be a possible vector for *P. knowlesi* (reviewed by Coatney *et al*., 2003). Thus, it is essential to identify the mosquito vectors responsible to determine the dynamics of *P. knowlesi* transmission to humans. The studies were initiated to determine the vectors of monkey malaria in different areas. However, few evidences show the specific species of vector to support their transmission. *An. latens* is in fact both of simian (*P. knowlesi*) and human malaria in Sarawak, Malaysia (Vythilingam et al., 2006b). Naturally, this species covers to bite both humans and monkeys that show the potential to transmit *P. knowlesi* to humans (Vythilingam et al., 2006b). If natural human mosquito- human transmission occurred, *P. knowlesi* could spread more widely in Asia. This is made possible by the wide distribution

of at least one vector species, *An. latens*, in Southeast Asia and in the southern parts of the Indian subcontinent, including the popular tourist areas in western India (Cox-Singh et al., 2008).

Anopheles hackeri (Wharton and Eyles, 1961) and *Anopheles introlatus* (as *An. balabacensis*) is the vector of *Plasmodium cynomolgi* (Eyles *et al*., 1963). However, it is known that *An. hackeri* is not attracted to humans and feeds mainly on monkeys in the natural environment (Reid and Weitz, 1961). In 2008, evidence was found for the co-infection of *P. knowlesi*, *P. falciparum,* and *P. vivax* in the salivary glands of 1 mosquito among 17 that had been diagnosis by PCR with malaria parasite species–specific primers (Nakazawa et al., 2009). Recently, the additional results both of 72 sporozoite–positive salivary glands of *An. dirus* mosquitoes and 211 blood samples from the local human population from Khanh Phu in Vietnam were detected by PCR (Marchand et al., 2011). There is the evidence both of single *P. knowlesi* infection and the combination of other Plamodia and *P. knowlesi* in both humans and mosquitoes (Marchand et al., 2011). A degree of interaction between these species may effect and precludes the establishment of co-infections. Such nonrandom association was, however, still not found yet in studies from Thailand, Malaysia, and Myanmar (Jiang et al., 2010; Jongwutiwes et al., 2004).
This study investigated the potential vector that was prevalent for the common malaria, *P. falciparum*, *P. vivax*, and the new one, *P. knowlesi*. We select the study site in Chanthaburi province where the preliminary study showed the positive *P. knowlesi* detection in human blood.

The information of infectivity both in human and mosquito vectors will support the direction of control program for *P. knowlesi* malaria in Thailand. This will initiate the understanding of interactions of human host and *P. knowlesi* – vector.

Objectives

The objective of this proposal is to investigate the specific species and their and susceptibility of mosquito vectors in the prevalence area of malaria and *P. knowlesi* infection in Chantaburi province, Thailand.

Methods

1. Study area

The study site was selected according the malaria reported in Makham district, Chanthaburi province where have represented 6:1 of *P. vivax*: *P. falciparum* cases in this area. The study was focused on the resident who reported for *P. knowlesi* in 2009 that was confirmed by Prapokklao hospital. The resident, female with 68 years old, has the rubber plantation and fruit garden as the occupation. She had to walk at 3.00-4.00AM for rubber trapping on her plant for 800-1,000 meters on the hill behind her house every

day. The house was surrounded by the foot hill (up to 1,700 above the mean sea level) along the West, North, and East side of the house with streaming lines (Figure1), and swamp nearby the house. *Anopheles* mosquitoes were collected by 1 kilometer in diameter of target house. The occupations in this area were fruit gardens (durian, rambutan, purple mangosteen, and longan), rubber plantation, and there are the movement across Thai-Cambodia as the labors in this area. These sites had the malaria seasonal incidence at major peak on dry season on December to March and minor peak on wet season of August to October (Average malaria cases from 3 years afterward). Malaria incidence in the study area was provided by Bureau of the Vector-Born Diseases at section number 3.5.2 Makham district.

2. Adult mosquito collection and identification

Traps were placed in the target house and 2 of neighbour houses by different methods of mosquito collections.

1) Light traps were set individually on each house as total of 3 indoor and 3 outdoor (more than 20 meters far from house), and 2 traps on the foot hill where was 500 meters far away from the target house. Three light traps were set at 0.5, 1.5, and 3 meters height hanging on the tree located on the foot hill in order to collect the mosquito diversity by height variation. CDC light trap were run from 18.00-6.00 and collected in the next morning.

2) Four human landing catches: 1 indoor, 1 outdoor in the positive house, and 2 along the foot hill were carried out. Inform consent for human landing catches were ethical approval (MUTM 2013-010-01) by ethical committee from Mahidol University (EC submission number TMEC 12-074). The volunteers were informed and described in enrolling study. Human landing catches were set during 18.00-06.00 by individually collection for every hour.

3) Two BG traps were placed either indoor or outdoor. The mosquitoes were captured for 5 consecutive nights on each season of wet (October) and dry (May).

All obtaining field–caught mosquitoes were preserved in dry ice and carried to Department of Medical Entomology, Faculty of Tropical Medicine, Mahidol University for malaria infectivity determination. Adult mosquitoes were morphological identified to species using the taxonomic keys (Rattanarithikul et al., 2006b) and trained Medical Entomology personal.

3. Malaria detection in the mosquitoes

Screening *Plasmodium* species detection by QMAL: real-time PCR. Primer set was PV18SRNA. Mosquito DNA was extracted DNA using the QIAamp® DNA mini kit (Qiagen,

Germany). Confirmed *Plasmodium* species *P. falciparum, P. vivax, P. ovalae, P. malariae*, and *P. knowlesi*) detection by Nested PCR (Primer set:18S rRNA) for the specific primers to each of species of *Plasmodium* parasite according (Kimura M, 1997; Van den Eede et al., 2009). The Fist strand and nested PCR primers were used according table1 and identified Genotypic species (PV210 and PV247) by RFLG *AluI and Hae*III restriction enzymes. The 5 µL of extracted DNA was resuspended in 45 µL of PCR buffer containing 0.4 µM of each primer with 2X GoTaqGreen mastermix and 200ng DNA template. The PCR amplification parameters were 94°C for 10 min, 40 cycles at 95°C for 30 s, 60°C for 90 sec, and 72°C for 1 min, followed by a final extension for 5 min at 72°C. For the Nested cycle, the reaction mixture for the second amplification round was done the same as for the first one, except for the reverse primers used instead of the P2R primers. First strand PCR product was performed 4 reactions for each type of reverse species-specific primers (FR, MR, OR and VR) and forward PF1 primer. The expected product is 110 bp.

For *P. knowlesi* detection, the primary reaction was carried out in a 50 µl reaction mixture PCR Buffer, 3 mM $MgCl_2$, 200 mM of each deoxynucleoside triphosphate, 250 nM of each primers rPLU1 and rPLU5, 0.1 µg/µL acetylated BSA (Promega, Madison USA) and 1 U HotStarTaq Plus DNA polymerase (Qiagen, Hilden Germany) and 2 µL of DNA template was used for each reaction. The nested PCR amplification was carried out in a 25 µL reaction mixture PCR buffer, 3 mM $MgCl_2$ (Qiagen, Hilden Germany), 200 mM of each deoxynucleoside triphosphate, 250 nM of each primers Pmk8, Pmk9r and 2 U HotStarTaq Plus DNA polymerase (Qiagen, Hilden Germany) and 2 µL of the primary PCR products were used as DNA templates. Five µL of the nested PCR products were loaded on a 2% agarose gel for 60 min at 5 V/cm using 0.5× TAE buffer. The gels were stained with ethidium bromide and visualized with UV.

Table 1: Specific primers for detection of *Plasmodium* parasites (Kimura *et al.* 1997 and Van den Eede *et al*., 2009).

Detection of *P. falciparum, P. vivax, P malariae,* and *P. ovalae* (PF,PV,PM, and PO)		
Specificity	Gene target	Sequence
A genus specific primers	18s rRNA	P1F: 5'-ACG ATC AGA TAC CGT CGT AT CTT-3' P2R:5'-GAA CCC AAA GAC TTT GAT TTC TCAT-3'
Species specific primers for PF, PV, PO, and PM		FR:5'-CAA TCT AAA AGT CAC CTC GAA AGA TG-3' MR: 5'-GGA AGC TAT CTA AAA GAA ACA CTC ATA T-3' OR:5'-ACT GAA GGA AGC AAT CTA AGA AAT TT-3' VR: 5'-CAA TCT AAG AAT AAA CTC CGA GAG GAA A-3'
Detection of *P. knowlesi*		
Specificity	Gene target	Sequence

Primary reaction	SSUrRNA	rPLU1: 5'-TCA AAG ATT AAG CCA TGC AAG TGA-3'; rPLU5: 5'-CCT GTT GTT GCC TTA AAC TCC3'),
Specific *P. knowlesi*	SSUrRNA	Pmk8: 5'-GTT AGC GAG AGC CAC AAA AAA GCG AAT-3'; Pmk9r: 5'-ACT CAA AGT AAC AAA ATC TTC CGT A-3')

ผลการวิจัย

1. Study site and Mosquito collection

Wild caught mosquitoes were collected from 1 kilometer in diameter of *P. knowlesi* reported patient house by 11 CDC light traps, 4 human landing catches, and 2 BG traps for 5 consecutive nights per season of wet (October) and dry (May) in 2012-2013 (Figure1). Total 9 genus of 1,125 adult mosquitoes were collected with the main genus of *Culex* (32.9%), *Anopheles* species/complex (26.8%), and *Armigeres* spp. (21.85%). Anopheline mosquito represented as the top two of the main captured species and showed *An. barbirostris complex* was a dominant species up to 96% within genus group followed by *An. dirus group* (3.3%), *An. asiaticus* (0.3%), and *An. tessellatus* (0.3%).

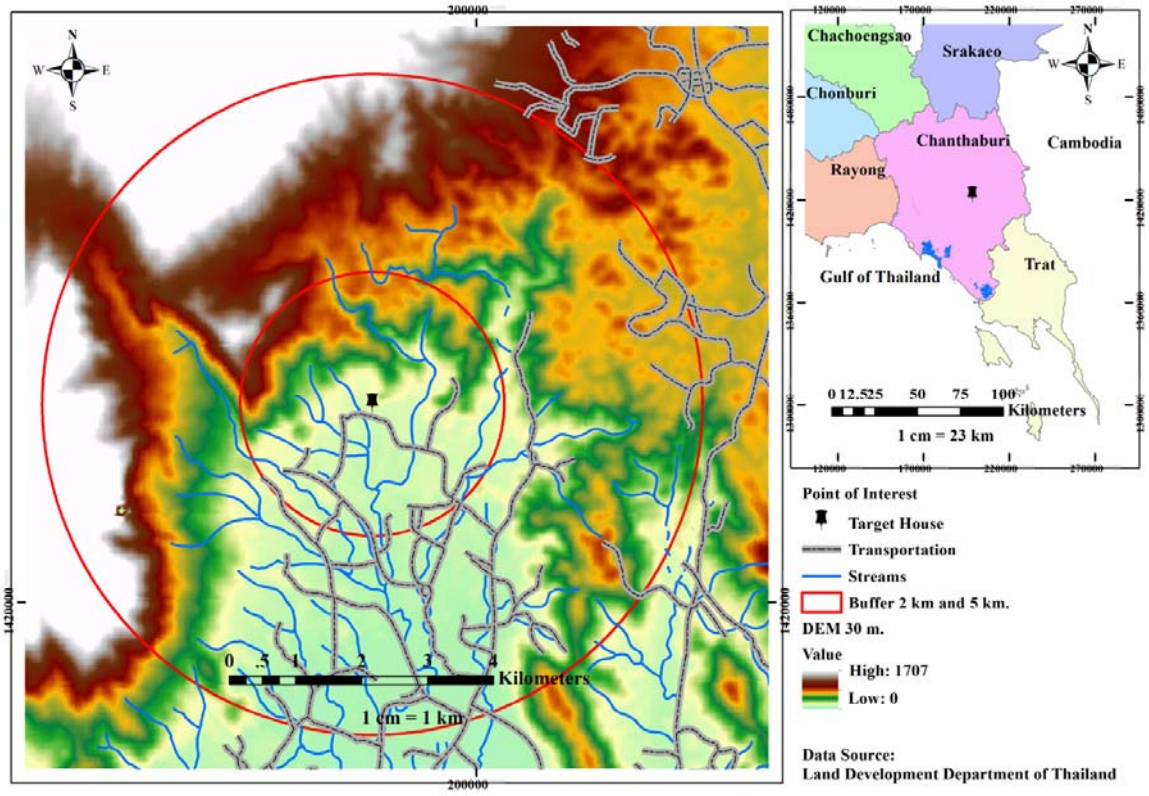


Figure1 Study site of mosquito collection in PK infected house in Makham district, Chanthaburi province nearby Cambodia. Map represented the buffer zone with transportation, streaming lines and surrounding by the mountain with hill height.

Table 2 *Anopheles* species collected by BG traps (BG), human landing catches (H) from indoor, outdoor, and mountain nearby target house, CDC light traps from indoor, outdoor houses (LH), from mountain sites (LM), and hanging on the trees with different height (LT) during dry (May) and wet (October) seasons in 2013. Blue high-light area is *Anopheles* species group that were used the thorax part for detection of malaria parasite.

Species	Traps					Total	Percentage
	BG	H	LH	LM	LT		
Aedeomyia catustica			2			2	0.2%
Aedes aegypti	21	13				34	3.0%
Aedes albopictus	10	36	12	21	38	117	10.4%
Aedes khazani		1				1	0.1%
Aedes lineatopenne			1			1	0.1%
Aedes poicilia				1		1	0.1%
Aedes vexans		1	3	1		5	0.4%
Anopheles asiaticus				1		1	0.1%
Anopheles barbirostris group	2	280	7			289	25.7%
Anopheles dirus group		9	1			10	0.9%
Anopheles tessellatus		1				1	0.1%
Armigeres annulitarsis		1				1	0.1%
Armigeres malayi		6	1	2	8	17	1.5%
Armigeres subalbatus	1	160	12	12	42	227	20.2%
Coquinletidae crassipes			4	1	8	13	1.2%
Culex bitaeniorhnychus					2	2	0.2%
Culex fascocephala	1	1	1			3	0.3%
Culex pseudovishnui	1	3	1	1	1	7	0.6%
Culex quinquefasciatus	86	76	6			168	14.9%
Culex sitiens		6	18	3	3	30	2.7%
Culex tritaeniorhynchus			2			2	0.2%
Culex vishnui	1	24	93	7	27	152	13.5%
Culex whitmorei			6			6	0.5%
Mansonia annulifera		1				1	0.1%
Mansonia indiana		13	3			16	1.4%
Mansonia uniformis		12	2			14	1.2%
Mimomyia spp.		2	1			3	0.3%
Uranotenia spp.					1	1	0.1%
Grand Total	**123**	**646**	**176**	**50**	**130**	**1125**	100%

For the hypothesis of the species variation of mosquito flight height might be impact to the mosquito-animal or monkey contact. There was no *Anopheles* spp. found in this experiment but *Armigeres* group was the most captured genus and showed that *Armigeres* and *Aedes albopictus* preferred to flight at the low level (0.5 meters) but different season available. *Aedes albopictus* was the most collection in Wet season while *Armigeres* was

the most preference in dry season. *Culex vishnui* flight most at the highest range at 5 meters and more numbers in the wet season but some other *Culex* species was zero captured numbers in dry season (Table 3).

Table 3 Mosquitoes captured from the different of 0.5, 3, and 5 meters height hanging CDC Light traps on the trees (LT) located on the high hill site behind the reported malaria infected house during dry (May) and wet (October) seasons in 2013.

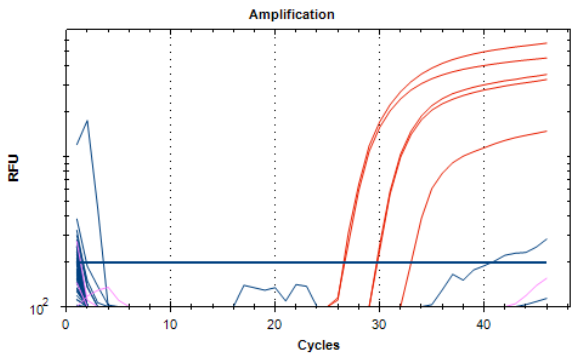
Species	Light traps								Grand Total	Percentage
	Dry season			Total	Wet season			Total		
	0.5m	3m	5m		0.5m	3m	5m			
Aedes albopictus	7	1		8	11	14	5	30	38	29.23%
Armigeres malayi	1			1	3	3	1	7	8	6.15%
Armigeres subalbatus	29	2	2	33	4	2	3	9	42	32.31%
Coquinletidae crassipes		3	3	6			2	2	8	6.15%
Culex bitaeniorhnychus						2		2	2	1.54%
Culex pseudovishnui							1	1	1	0.77%
Culex sitiens					2		1	3	3	2.31%
Culex vishnui	7	1	12	20		5	2	7	27	20.77%
Uranotenia spp.							1	1	1	0.77%
Grand Total	**44**	**7**	**17**	**68**	**20**	**26**	**16**	**62**	**130**	**100%**

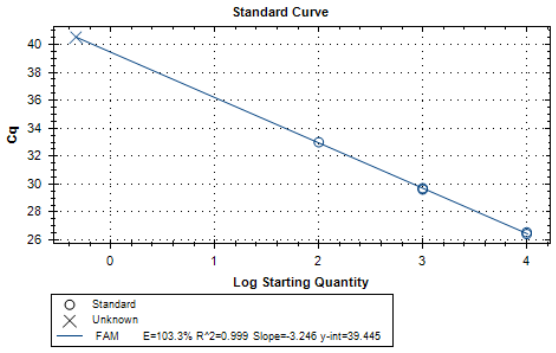
2. Malaria detection in adults of female *Anopheles* mosquitoes

2.1 Screening *Plasmodium* species detection by qMAL-real-time PCR

Total 243 samples of Anopheles female mosquitoes were dissected to use thorax part for detection of actively infected stage of malaria in the mosquitoes. The screening method showed 2 malaria positive samples of *An. barbirostris* group (HI1.6/5Anc-23 and HI1.4/9Anc-161) collecting from indoor patient house (Figure 1).

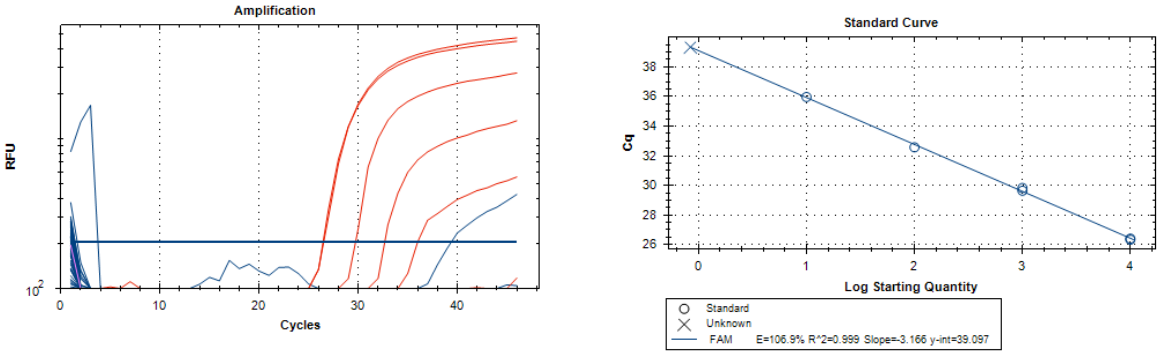
Sample HI1.6/5Anc-23





Sample HI1.4/9-161

Figure2 Positive qMAL PCR detection in two *An. barbirostirs* complex. Red lines are STD



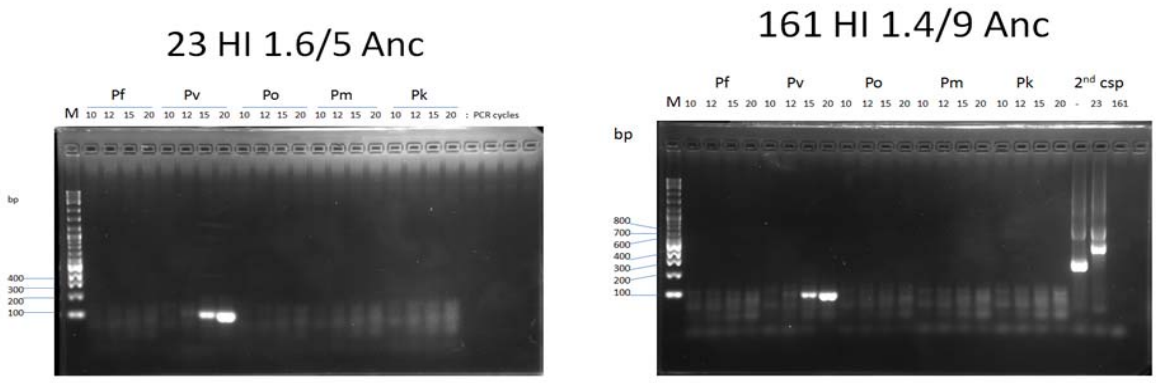
10000 copy, 1000 copy, 100 copy, 10 copy. Blue curve lines are positive unknown samples with the over cut off range (bold horizontal blue line). Right graph pictures are standard curve that used for calculate of the copy numbers of PCR products.

Regression equations are HI1.6/5Anc-23: Cq value = -3.246 × 10 log DNA/PCR + 39.445, r^2 = 0.999, $p < 0.001$; HI1.4/9Anc-161: Cq value = -3.166 × 10 log DNA/PCR + 39.097, r^2 = 0.999, $p < 0.001$.

2.2 Confirmed *Plasmodium* species detection by Nested PCR

Malaria species identification for *P. falciparum* (F), *P. vivax* (V), *P. ovalae* (O), *P. malariae* (M), and *P. knowlesi* (K) were detected in two qMAL screening positive reactions by using Nested PCR with 18S rRNA primers (Figure 3.1) and recheck in Figure3.2.

Figure3.1 PCR detection to identify *Plasmodium* species; *P. falciparum* (F), *P. vivax* (V),



P. ovalae (O), *P. malariae* (M), and *P. knowlesi* (K) by using 0.5 uL of DNA of sample 23 and 2 uL of sample HI 1.6/5 Anc-23. Nested PCR were used 5 uL of primary PCR product as the template. One hundred bp band represented for the positive PV malaria species and 2nd csp was detected 700 bp to confirm positive PV in HI 1.6/5 Anc-23 but could not identify in sample HI 1.4/9 Anc-161.

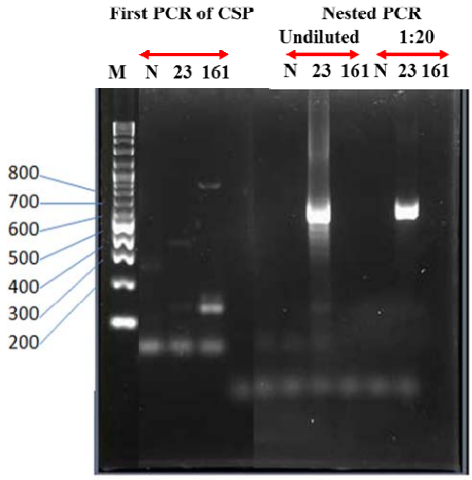


Figure 3.2 Recheck of Nested PCR for detection of Circumsporozoite typing in sample HI 1.6/5 Anc-23 and HI 1.4/9 Anc-161. N is negative control, M is DNA ladder, Positive Circumsporozoite (csp) product located at 600-700 bp.

Both positive samples from Nested PCR were detected malaria strain (PV210 and PV247) by RFLP (AluI and HaeII). It represented the positive PV210 in sample 161 HI 1.6/5 Anc but could not identify in sample 23 HI 1.4/9 Anc (Figure 4).

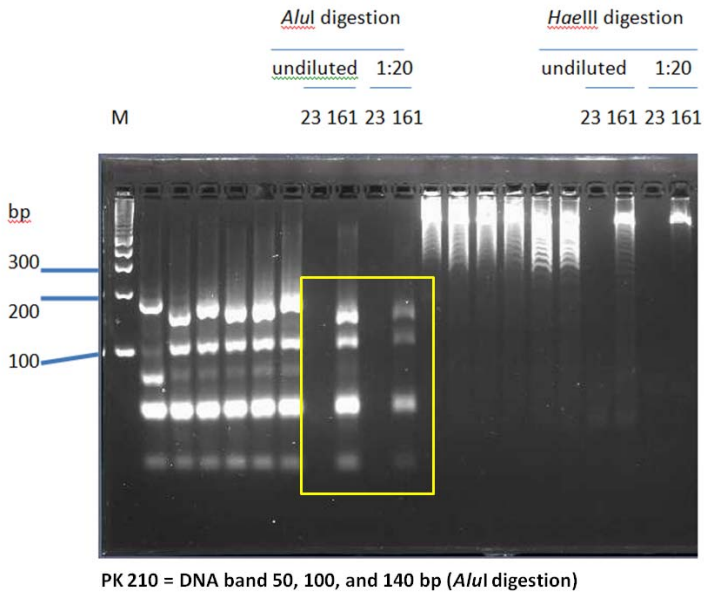


Figure 4 RFLP to detect PV210 and PV247 in 2 positive nested PCR sample number 23 and 161.

Table 5 Summary malaria detection in *Anopheles barbirostirs* complex with positive malaria detection of genus (using qMAL), species (using Nested PCR) and circumsporozoite typing (using RFLP).

In summary, there was 2 positives malaria infection in *An. barbirostirs group* with 0.82% infection rate (2/243). The positive samples were captured from indoor human landing catch during wet season (table 5). One was positive for PV210 but unclear for species typing in sample HI 1.4/9 Anc-161. The man biting rate of *An. barbirostirs* complex in indoor, outdoor of house hold and outdoor at the hill side were 21.08, 8.01 and 7.52 bites per person per night. The positives were compatible to peak of indoor man biting rates at 21.00 and 23.00 for 7.40 and 5.80 bites per person per hour, respectively (Figure5). This species is endophagic pattern while the outdoor of the household when compared to the outdoor hill-side were the same biting rate in range 0-2 bite per person per hour.

Theory, risk of transmission in term of the entomological surveillance can be represented basically by using the estimate of Entomological inoculation rate (EIR) in each period by standard method based on infective bite per person per time period.
Data indicated that Indoor and outdoor household EIR of *An. barbirostirs* group were 17.29 and 6.16 infectious bite per night respectively while the outdoor EIR at the hill side was 6.57 infectious bite per night. Most of high EIR was at the beginning of Rain season in May of this study. These estimates showed the large variations over short distances in time and space. They were all markedly higher than those reported from the previous study.

Sample numbers	QMAL screening (Cq)	Starting Quantity (SQ)	Species typing	CSP typing
23 HI 1.6/5 Anc (at 23.00)	36.44	7.17592	PV	not identify
161 HI 1.4/9 Anc (at 21.00)	35.9	10.26102	PV	210

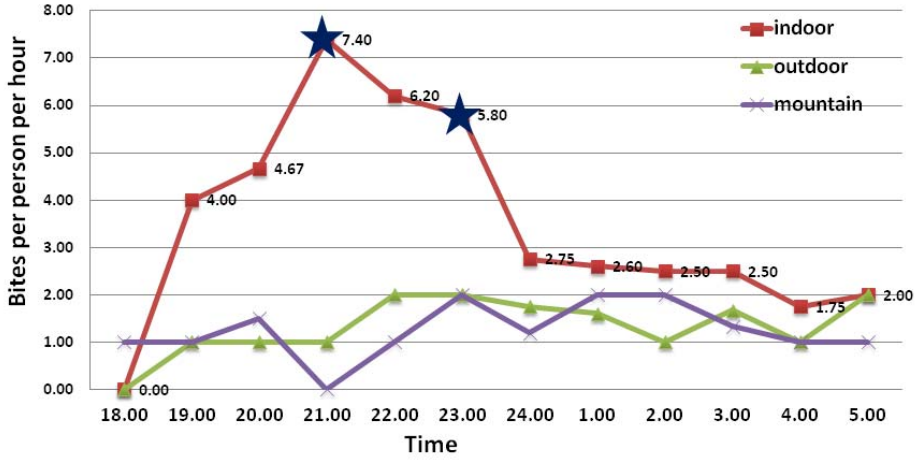


Figure 5 Biting activities of *An. barbirostirs* group among locations of indoor, outdoor, and the hill nearby the target house. ★ Represented the positive malaria (PV210 and unidentified species typing) infection in *An. barbirostirs* group detected by real time and nested PCR.

สรุปและวิจารณ์ผลการทดลอง

The update finding of natural potential PV malaria vector, *An. barbirostirs* group, may play important role of malaria transmission in this area. Very few information of vector biology and vector capacity for this species complex extremely requested to investigate in order to manage properly of vector control program in the specific local vector in this area. Meanwhile, current data showed the higher risks of the potential vectors by represented of the endophagic pattern that was changed from the previous report in the role of *An. barbirostris s.l.* as an outdoor biting vector in maintaining malaria transmission along Thai-Cambodia border, Sae Kaeo province and in Thai-Myanmar border (Apiwathnasor et al., 2002; Limrat et al., 2001), Kanchanburi province (Green et al., 1991). Biting time was at the early night faster than the previous report (Apiwathnasor et al., 2002; Limrat et al., 2001).

The Barbirostris subgroup of this group includes 5 species (J. Overgaard et al., 2015; Taai and Harbach, 2015) : *An. barbirostris* van der Wulp s.l., *An. campestris* Reid, An. donaldi Reid, An. hodgkini Reid, and *An. pollicaris* Reid (Rattanarithikul et al., 2006a; Saeung et al., 2008; Saeung et al., 2012; Saeung et al., 2007). *An. barbirostris* and *An. campestris* are the most common species of this group, and they are closely associated with humans. We also found PV infection in natural *An. campestris* in Sae Kaeo province (unpublished data) as the main vector in the study area. However, the capture of an oocyst-positive specimen of *An. barbirostris/campestris* in the prolonged absence of the dominant vector species (Sinka et al., 2011) highlighted *An. campestris* and *An. hodgkini* were naturally infected with sporozoites (Coleman et al., 2002), suggesting their potential roles of secondary vectors in habitats experiencing land use and environmental changes in Thailand. The wild strain (F0-3) can provide 33% PV susceptibility until infective stage

and ready for transmission. Unclear of mechanism and vector competent for susceptible of PF needs to be investigated. In term of PV and PF mix infection, if impact to the level of malaria susceptibility or vector-malaria selective susceptible species is needed to further study as well. This study showed 2 positive PV infection that would not detect for their species complex. However, we can purpose that two samples has the potential to be *Anopheles campestris* based on morphological identification in the more white scales on the dorsal thorax segments. It will be further to collect *An. barbirostris* s.l. for confirmation of species complex by detection of pupa skin and molecular techniques.

In term of Malaria evidence in the area as shown since 2009-2013 in table 6, PV was the high ratio up to 90% infection rate. Interestingly, most of the patients were Thai people and could be detected more in dry season that contrasted to foreign patients with representing more in wet season. This might be impact from the labor movement in the agriculture season as the basic pattern along Thai borders. This area was also found relapse of PV infection during most in dry season up to 3 time of PV reinfection.

Table6 Malaria case incidence in Makham district, Chanthaburi province from 2009-2013. Under line numbers were PF patients.

				----------Dry----------					----------Wet----------					---- Dry ---	
	Thai			Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec
Year	PV	PF	mix												
2009	12	2	1	1	3	1, 1, PK	1	1	mix ,4		1		1		1, 1
2010	12	0	0					2			1	1	1	2	5
2011	4	0	0	2		1				1					
2012	2	3	0		1		1	1		2					
2013	3	1	0	1		1, 1	1								
	Foreigners			Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec
Year	PV	PF	mix												
2009	2	0	0								1	1			
2010	0	1	0												1
2011	4	0	0					1	1			2			
2012	2	0	0					1	1						
2013	2	0	0							2					

ข้อเสนอแนะสำหรับงานวิจัยในอนาคต

This study represented the better understand of the potential malaria transmission in focal area of Makham district. It could subsequently assist to improve malaria control strategies. This study provides the spot survey of seasonal malaria vector transmission and can be summarized accordingly;

1. *Anopheles barbirostirs* s.l. was the dominant malaria vector captured in this area to susceptible for PV infection and mainly detected in late of dry season. *Anopheles dirus* was not the main household malaria vector in this area.
2. Main malaria patients' incidence were come from Thai local residences particularly on dry season (November to May). The foreign patients for fruit garden labors were represented and had the additionally potential causing of malaria transmission during wet season (June to October) to compatible with their staying in the fruit garden during cultivation season.
3. Even data could not detect the potential vector for PK but this data participate on the natural malaria vector as the additional concern for vector surveillance and control where the main of the study site is barbirostirs group. Even integrated vector control program for malaria vector have ever done in 1995 by Integration of trapping system, application of IGR, use of both residual spraying and impregnated bed-net methods with etofenprox successfully interrupted malaria infection (Kanda et al., 1995), they need to be concerned of our additional vector species as the main population in focal sites where should be re-analysed of vector control programs with determination of insecticide resistant status that could be found for *An. campestris* s.l. as in Sa-Keaw province with the herbicide induction from the agriculture system (publication data).
4. Vector control programs should correspond to the period of vector exist;
    a. If there is malaria case detection, IRS should be treated in the correct time by focusing the high risk of local Thai household in dry season.
    b. Self-protection for decreasing of mosquito contact should be more focus in the foreigner who involved in labor and cultivation season.
    c. In-depth follow up the cases and blocking transmission blocking of indoor contact by promote of net usage either of household or in the fruit garden.
    d. Monitor of net usage and determine the net efficiency by priority of the transmission risk level.
    e. Education and training of entomological surveillance system within the limited budget by setting the priority area to compatible with man power of local staffs.
    f. People education to improve self-protection of mosquito bite and malaria fever should be focused for the translation knowledge from children in

school to family as the practical life for mosquito biology and biting protection.

Output จากโครงการวิจัยที่ได้รับทุนจาก สกว.

1. ผลงานตีพิมพ์ในวารสารวิชาการนานาชาติ (ระบุชื่อผู้แต่ง ชื่อเรื่อง ชื่อวารสาร ปี เล่มที่ เลขที่ และหน้า) หรือผลงานตามที่คาดไว้ในสัญญาโครงการ

ได้ Manuscript แล้วเรื่อง "Natural susceptibility of malaria vector (*Anopheles barbirostris* group) to *Plasmodium vivax* in Thai-Cambodia border" และคาดว่าจะ submit in Parasitology Research ได้ในเดือน ตุลาคม 2561

2. การนำผลงานวิจัยไปใช้ประโยชน์

- เชิงพาณิชย์ (มีการนำไปผลิต/ขาย/ก่อให้เกิดรายได้ หรือมีการนำไปประยุกต์ใช้โดยภาคธุรกิจ/บุคคลทั่วไป)
- **เชิงนโยบาย (มีการกำหนดนโยบายอิงงานวิจัย/เกิดมาตรการใหม่/เปลี่ยนแปลงระเบียบข้อบังคับหรือวิธีทำงาน)**

เป็นข้อมูลสำคัญทางระบาดวิทยาที่แสดงให้เห็นถึงพาหะนำโรคไข้มาลาเรียไม่ใช่เพียงแค่ยุง *An. dirus, An. minimus, An. maculatus* ที่นักกีฏวิทยาการแพทย์ให้ความสนใจและเฝ้าระวังและควบคุมการแพร่เชื้อในพื้นที่ระบาดเท่านั้น และยังแสดงให้เห็นถึงความเป็นพาหะนำเชื้อที่เป็นยุงพาหะหลักในพื้นที่ซึ่งควรทำการควบคุมจำนวนยุงในแหล่งเพาะพันธุ์ที่เป็นลำน้ำเล็กๆบริเวณตีนเขาและเฝ้าระวังยุงชนิดนี้ให้มากขึ้นเพราะเป็นยุงพาหะหลักไข้มาลาเรียในธรรมชาติของพื้นที่นี้ซึ่งเคยรายงานงานว่ายุง *An. dirus* คือพาหะหลัก

- เชิงสาธารณะ (มีเครือข่ายความร่วมมือ/สร้างกระแสความสนใจในวงกว้าง)
- เชิงวิชาการ (มีการพัฒนาการเรียนการสอน/สร้างนักวิจัยใหม่)

3. อื่นๆ

Poster presentation เรื่อง "Potential *Plasmodium vivax* malaria vector of *Anopheles campestris* in focal endemic area along Thai-Cambodian border"ในงานนักวิจัยรุ่นใหม่พบเมธีวิจัยอาวุโส สกว. ครั้งที่ 13 วันที่ 16-18 ตุลาคม 2556 ณ โรงแรมเดอะรีเจ้นท์ชะอำบีช รีวอร์ท หัวหิน ชะอำ จังหวัดเพชรบุรี

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ภาคผนวก

1. **การเสนอผลงานในที่ประชุมวิชาการ**
   Poster presentation เรื่อง “Potential *Plasmodium vivax* malaria vector of *Anopheles campestris* in focal endemic area along Thai-Cambodian border”ในงาน นักวิจัยรุ่นใหม่พบเมธีวิจัยอาวุโส สกว. ครั้งที่ 13 วันที่ 16-18 ตุลาคม 2556 ณ โรงแรมเดอะรีเจ้นท์ ชะอำบีช รีวอร์ท หัวหิน ชะอำ จังหวัดเพชรบุรี

2. Manuscript เรื่อง “Microscale of Potential *Plasmodium vivax* malaria vector of *Anopheles campestris* in focal endemic area along Thai-Cambodian border”

Natural susceptibility of malaria vector (*Anopheles barbirostris* group) to *Plasmodium vivax* in Thai-Cambodia border

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Wild caught *Anopheles* mosquitoes were collected in Chanthaburi province, western Thailand. One kilometer in diameter of malaria reported patient house by CDC light trap, human landing catch, and BG traps during specific wet and dry seasons in 2012-2013. Total 30 genus of 1,131 adult mosquitoes were collected, From 6 Anopheles species found, *An. campestris* was a dominant (95.4%) followed by *An. dirus* (3.3%), *An. barbirostris* (0.7%), *An. asiaticus* (0.3%), and *An. tessellatus* (0.3%). QMAL quantitative PCR and nested PCR were performed to detect malaria infection in Anopheles mosquitoes. Interestingly, there were two positive *Plasmodium vivax* (PV) infections, with PK210 and one of unclassified genotypic variants, from *An. campestris* (0.83%, 2/243). They were captured from indoor human landing catch of the patient house at 21.00 and 23.00 for 7.40 and 5.80 per person per hour, respectively, during May as a dry season. *An. campestris* was preference to endophagic behavior. The preliminary finding of this study indicated that *An. campestris* can be a potential malaria vector and may play important role of malaria transmission in this area. Further study is required to better understand the potential role of secondary vectors in malaria transmission in this area and subsequently assist to improve malaria control strategies.

Keywords: *Anopheles campestris*, *Plasmodium vivax*, Thailand, qMAL real-time PCR, Nested PCR

Introduction

Malaria remains one of the most important infectious diseases in the world. The annual human malaria associated mortality approaches 1 million, with 2 children dying from the disease every minute worldwide causing by *Plasmodium falciparum, Plasmodium vivax, Plasmodium ovalae, and Plasmodium malariae* (WHO, 2017). The newcomer is *P. knowlesi*, which have known to cause malaria only in macaques. Zoonotic *P. knowlesi* infection is the normal mode of transmission by mosquito vectors that mainly feed in the forest (Cox-Singh and Singh, 2008). Usually, *P. knowlesi*, a malaria species that has the specific host with the long-tailed macaques, has found to be prevalent in humans in Southeast Asia (Kantele and Jokiranta, 2011). Most of the current researches are focused on characterization of parasite and the evidence within the natural human host (Jiang et al., 2010; Jongwutiwes et al., 2004; Kantele and Jokiranta, 2011; Vythilingam et al., 2008; Vythilingam et al., 2006a). There is still not a firm understanding of the potential vectors to support plasmodial infectivity to mosquitoes. It can be assumed that its vector behaves like the vectors of *Anopheles* species but few data on specific determination in the prevalent area. The extent to which the parasite can be transmitted from human back to mosquito vector in nature is also unclear. Thailand is the one of high case reports of *P. knowlesi* in human (Berry et al., 2011; Jongwutiwes et al., 2004; Putaporntip et al., 2009). This study will investigate the potential vector that is prevalent for the common malaria, *P. falciparum*, *P. vivax*, and the new one, *P. knowlesi*. We select the study site in Ranong province where the preliminary study has shown the positive *P. knowlesi* detection in human blood. The information of infectivity both in human and mosquito vectors will support the direction of control program for *P. knowlesi* malaria in Thailand. This will initiate the understanding of interactions of human host and *P. knowlesi* – vector.

Methods

1. Study area

The study site was selected according the malaria reported in Makham district, Chanthaburi province where have represented 6:1 of *P. vivax*: *P. falciparum* cases in this area. The study was focused on the resident who reported for *P. knowlesi* in 2009 that was confirmed by Prapokklao hospital. The resident, female with 68 years old, has the rubber plantation and fruit garden as the occupation. She had to walk at 3.00-4.00AM for rubber trapping on her plant for 800-1,000 meters on the hill behind her house every day. The house was surrounded by the foot hill (up to 1,700 above the mean sea level) along the West, North, and East side of the house with streaming lines (Figure1), and swamp nearby the house. *Anopheles* mosquitoes were collected by 1 kilometer in diameter of target house. The occupations in this area were fruit gardens (durian, rambutan, purple mangosteen, and longan), rubber plantation, and there are the movement across Thai-Cambodia as the labors in this area. These sites had the malaria seasonal incidence at major peak on dry season on December to March and minor peak on wet season of August to October (Average malaria cases from 3 years afterward). Malaria incidence in the study area was provided by Bureau of the Vector-Born Diseases at section number 3.5.2 Makham district.

2. Adult mosquito collection and identification

Traps were placed in the target house and 2 of neighbour houses by different methods of mosquito collections.

1) Light traps were set individually on each house as total of 3 indoor and 3 outdoor (more than 20 meters far from house), and 2 traps on the foot hill where was 500 meters far away from the target house. Three light traps were set at 0.5, 1.5, and 3 meters height hanging on the tree located on the foot hill in order to collect the mosquito diversity by height variation. CDC light trap were run from 18.00-6.00 and collected in the next morning.

2) Four human landing catches: 1 indoor, 1 outdoor in the positive house, and 2 along the foot hill were carried out. Inform consent for human landing catches were ethical approval (MUTM 2013-010-01) by ethical committee from Mahidol University (EC submission number TMEC 12-074). The volunteers were informed and described in enrolling study. Human landing catches were set during 18.00-06.00 by individually collection for every hour.

3) Two BG traps were placed either indoor or outdoor. The mosquitoes were captured for 5 consecutive nights on each season of wet (October) and dry (May).

All obtaining field–caught mosquitoes were preserved in dry ice and carried to Department of Medical Entomology, Faculty of Tropical Medicine, Mahidol University for malaria infectivity determination. Adult mosquitoes were morphological identified to species using the taxonomic keys (Rattanarithikul et al., 2006b) and trained Medical Entomology personal.

3. Malaria detection in the mosquitoes

Screening *Plasmodium* species detection by QMAL: real-time PCR. Primer set was PV18SRNA. Mosquito DNA was extracted DNA using the QIAamp® DNA mini kit (Qiagen, Germany). Confirmed *Plasmodium* species *P. falciparum*, *P. vivax*, *P. ovalae*, *P. malariae*, and *P. knowlesi*) detection by Nested PCR (Primer set:18S rRNA) for the specific primers to each of species of *Plasmodium* parasite according (Kimura M, 1997; Van den Eede et al., 2009). The Fist strand and nested PCR primers were used according table1 and identified Genotypic species (PV210 and PV247) by RFLG *AluI and Hae*III restriction enzymes. The 5 µL of extracted DNA was resuspended in 45 µL of PCR buffer containing 0.4 µM of each primer with 2X GoTaqGreen mastermix and 200ng DNA template. The PCR amplification parameters were 94°C for 10 min, 40 cycles at 95°C for 30 s, 60°C for 90 sec, and 72°C for 1 min, followed by a final extension for 5 min at 72°C. For the Nested cycle, the reaction mixture for the second amplification round was done the same as for the first one, except for the reverse primers used instead of the P2R primers. First strand PCR product was performed 4 reactions for each type of reverse species-specific primers (FR, MR, OR and VR) and forward PF1 primer. The expected product is 110 bp.

For *P. knowlesi* detection, the primary reaction was carried out in a 50 µl reaction mixture PCR Buffer, 3 mM $MgCl_2$, 200 mM of each deoxynucleoside triphosphate, 250 nM of each primers rPLU1 and rPLU5, 0.1 µg/µL acetylated BSA (Promega, Madison USA) and 1 U HotStarTaq Plus DNA polymerase (Qiagen, Hilden Germany) and 2 µL of DNA template was used for each reaction. The nested PCR amplification was carried out in a 25 µL reaction mixture PCR buffer, 3 mM $MgCl_2$ (Qiagen, Hilden Germany), 200 mM of each deoxynucleoside triphosphate, 250 nM of each primers Pmk8, Pmk9r and 2 U HotStarTaq Plus DNA polymerase (Qiagen, Hilden Germany) and 2 µL of the primary PCR products were used as DNA templates. Five µL of the nested PCR products were loaded on a 2% agarose gel for 60 min at 5 V/cm using 0.5× TAE buffer. The gels were stained with ethidium bromide and visualized with UV.

1. Study site and Mosquito collection

Wild caught mosquitoes were collected from 1 kilometer in diameter of *P. knowlesi* reported patient house by 11 CDC light traps, 4 human landing catches, and 2 BG traps for 5 consecutive nights per season of wet (October) and dry (May) in 2012-2013 (Figure1). Total 9 genus of 1,125 adult mosquitoes were collected with the main genus of *Culex* (32.9%), *Anopheles* species/complex (26.8%), and *Armigeres* spp. (21.85%). Anopheline mosquito represented as the top two of the main captured species and showed *An. barbirostris complex* was a dominant species up to 96% within genus group followed by *An. dirus group* (3.3%), *An. asiaticus* (0.3%), and *An. tessellatus* (0.3%).

For the hypothesis of the species variation of mosquito flight height might be impact to the mosquito-animal or monkey contact. There was no *Anopheles* spp. found in this experiment but *Armigeres* group was the most captured genus and showed that *Armigeres* and *Aedes albopictus* preferred to flight at the low level (0.5 meters) but different season available. *Aedes albopictus* was the most collection in Wet season while *Armigeres* was the most preference in dry season. *Culex vishnui* flight most at the highest range at 5 meters and more numbers in the wet season but some other *Culex* species was zero captured numbers in dry season (Table 3).

2. Malaria detection in adults of female *Anopheles* mosquitoes

2.1 Screening *Plasmodium* species detection by qMAL-real-time PCR

Total 243 samples of Anopheles female mosquitoes were dissected to use thorax part for detection of actively infected stage of malaria in the mosquitoes. The screening method showed 2 malaria positive samples of *An. barbirostris* group (HI1.6/5Anc-23 and HI1.4/9Anc-161) collecting from indoor patient house (Figure 2).

2.2 Confirmed *Plasmodium* species detection by Nested PCR

Malaria species identification for *P. falciparum* (F), *P. vivax* (V), *P. ovalae* (O), *P. malariae* (M), and *P. knowlesi* (K) were detected in two qMAL screening positive reactions by using Nested PCR with 18S rRNA primers (Figure 3) and recheck in Figure4.
Both positive samples from Nested PCR were detected malaria strain (PV210 and PV247) by RFLP (AluI and HaeII). It represented the positive PV210 in sample 161 HI 1.6/5 Anc but could not identify in sample 23 HI 1.4/9 Anc (Figure 5).

In summary, there was 2 positives malaria infection in *An. barbirostirs group* with 0.82% infection rate (2/243). The positive samples were captured from indoor human landing catch during wet season (Table 5). One was positive for PV210 but unclear for species typing in

sample HI 1.4/9 Anc-161. The man biting rate of *An. barbirostirs* complex in indoor, outdoor of house hold and outdoor at the hill side were 21.08, 8.01 and 7.52 bites per person per night. The positives were compatible to peak of indoor man biting rates at 21.00 and 23.00 for 7.40 and 5.80 bites per person per hour, respectively (Figure6). This species is endophagic pattern while the outdoor of the household when compared to the outdoor hill-side were the same biting rate in range 0-2 bite per person per hour.

Theory, risk of transmission in term of the entomological surveillance can be represented basically by using the estimate of Entomological inoculation rate (EIR) in each period by standard method based on infective bite per person per time period.

Data indicated that Indoor and outdoor household EIR of *An. barbirostirs* group were 17.29 and 6.16 infectious bite per night respectively while the outdoor EIR at the hill side was 6.57 infectious bite per night. Most of high EIR was at the beginning of Rain season in May of this study. These estimates showed the large variations over short distances in time and space. They were all markedly higher than those reported from the previous study.

Discussion

The update finding of natural potential PV malaria vector, *An. barbirostirs* group, may play important role of malaria transmission in this area. Very few information of vector biology and vector capacity for this species complex extremely requested to investigate in order to manage properly of vector control program in the specific local vector in this area. Meanwhile, current data showed the higher risks of the potential vectors by represented of the endophagic pattern that was changed from the previous report in the role of *An. barbirostris s.l.* as an outdoor biting vector in maintaining malaria transmission along Thai-Cambodia border, Sae Kaeo province and in Thai-Myanmar border (Apiwathnasor et al., 2002; Limrat et al., 2001), Kanchanburi province (Green et al., 1991). Biting time was at the early night faster than the previous report (Apiwathnasor et al., 2002; Limrat et al., 2001).

The Barbirostris subgroup of this group includes 5 species (J. Overgaard et al., 2015; Taai and Harbach, 2015) : *An. barbirostris* van der Wulp s.l., *An. campestris* Reid, An. donaldi Reid, An. hodgkini Reid, and *An. pollicaris* Reid (Rattanarithikul et al., 2006a; Saeung et al., 2008; Saeung et al., 2012; Saeung et al., 2007). *An. barbirostris* and *An. campestris* are the most common species of this group, and they are closely associated with humans. We also found PV infection in natural *An. campestris* in Sae Kaeo province (unpublished data) as the main vector in the study area. However, the capture of an oocyst-positive specimen of *An. barbirostris/campestris* in the prolonged absence of the dominant vector species (Sinka et al.,

2011) highlighted *An. campestris* and *An. hodgkini* were naturally infected with sporozoites (Coleman et al., 2002), suggesting their potential roles of secondary vectors in habitats experiencing land use and environmental changes in Thailand. The wild strain (F0-3) can provide 33% PV susceptibility until infective stage and ready for transmission. Unclear of mechanism and vector competent for susceptible of PF needs to be investigated. In term of PV and PF mix infection, if impact to the level of malaria susceptibility or vector-malaria selective susceptible species is needed to further study as well. This study showed 2 positive PV infection that would not detect for their species complex. However, we can purpose that two samples has the potential to be *Anopheles campestris* based on morphological identification in the more white scales on the dorsal thorax segments. It will be further to collect *An. barbirostris* s.l. for confirmation of species complex by detection of pupa skin and molecular techniques.

In term of Malaria evidence in the area as shown since 2009-2013 in table 6, PV was the high ratio up to 90% infection rate. Interestingly, most of the patients were Thai people and could be detected more in dry season that contrasted to foreign patients with representing more in wet season. This might be impact from the labor movement in the agriculture season as the basic pattern along Thai borders. This area was also found relapse of PV infection during most in dry season up to 3 time of PV reinfection.

Figures and tables

Table 1: Specific primers for detection of *Plasmodium* parasites (Kimura *et al.* 1997 and Van den Eede *et al*., 2009).

Detection of *P. falciparum, P. vivax, P malariae,* and *P. ovalae* (PF,PV,PM, and PO)		
Specificity	**Gene target**	**Sequence**
A genus specific primers	18s rRNA	P1F: 5'-ACG ATC AGA TAC CGT CGT AT CTT-3' P2R:5'-GAA CCC AAA GAC TTT GAT TTC TCAT-3'
Species specific primers for PF, PV, PO, and PM		FR:5'-CAA TCT AAA AGT CAC CTC GAA AGA TG-3' MR: 5'-GGA AGC TAT CTA AAA GAA ACA CTC ATA T-3' OR:5'-ACT GAA GGA AGC AAT CTA AGA AAT TT-3' VR: 5'-CAA TCT AAG AAT AAA CTC CGA GAG GAA A-3'
Detection of *P. knowlesi*		
Specificity	**Gene target**	**Sequence**
Primary reaction	SSUrRNA	rPLU1: 5'-TCA AAG ATT AAG CCA TGC AAG TGA-3'; rPLU5: 5'-CCT GTT GTT GCC TTA AAC TCC3'),
Specific *P. knowlesi*	SSUrRNA	Pmk8: 5'-GTT AGC GAG AGC CAC AAA AAA GCG AAT-3'; Pmk9r: 5'-ACT CAA AGT AAC AAA ATC TTC CGT A-3')

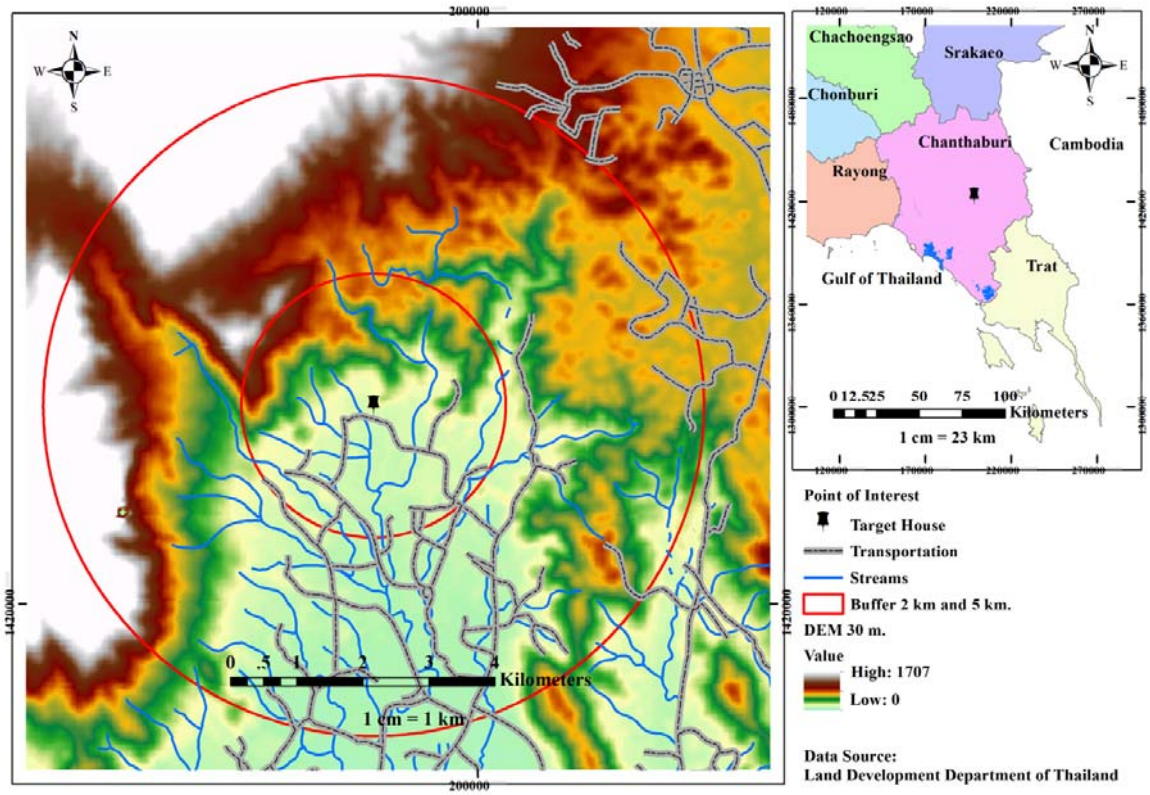


Figure1 Study site of mosquito collection in PK infected house in Makham district, Chanthaburi province nearby Cambodia. Map represented the buffer zone with transportation, streaming lines and surrounding by the mountain with hill height.

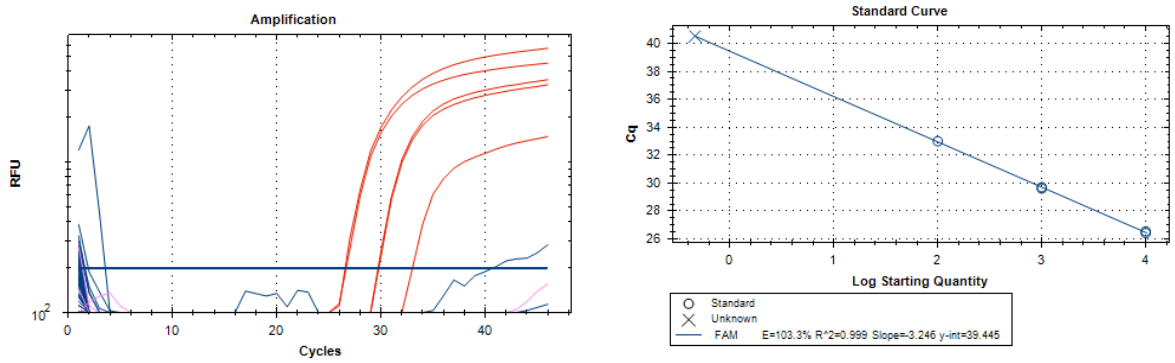
Table 2 *Anopheles* species collected by BG traps (BG), human landing catches (H) from indoor, outdoor, and mountain nearby target house, CDC light traps from indoor, outdoor houses (LH), from mountain sites (LM), and hanging on the trees with different height (LT) during dry (May) and wet (October) seasons in 2013. Blue high-light area is *Anopheles* species group that were used the thorax part for detection of malaria parasite.

Species	Traps					Total	Percentage
	BG	H	LH	LM	LT		
Aedeomyia catustica			2			2	0.2%
Aedes aegypti	21	13				34	3.0%
Aedes albopictus	10	36	12	21	38	117	10.4%
Aedes khazani		1				1	0.1%
Aedes lineatopenne			1			1	0.1%
Aedes poicilia				1		1	0.1%
Aedes vexans		1	3	1		5	0.4%
Anopheles asiaticus				1		1	0.1%
Anopheles barbirostris group	2	280	7			289	25.7%
Anopheles dirus group		9	1			10	0.9%
Anopheles tessellatus		1				1	0.1%
Armigeres annulitarsis		1				1	0.1%
Armigeres malayi		6	1	2	8	17	1.5%
Armigeres subalbatus	1	160	12	12	42	227	20.2%
Coquinletidae crassipes			4	1	8	13	1.2%
Culex bitaeniorhnychus					2	2	0.2%
Culex fascocephala	1	1	1			3	0.3%
Culex pseudovishnui	1	3	1	1	1	7	0.6%
Culex quinquefasciatus	86	76	6			168	14.9%
Culex sitiens		6	18	3	3	30	2.7%
Culex tritaeniorhynchus			2			2	0.2%
Culex vishnui	1	24	93	7	27	152	13.5%
Culex whitmorei			6			6	0.5%
Mansonia annulifera		1				1	0.1%
Mansonia indiana		13	3			16	1.4%
Mansonia uniformis		12	2			14	1.2%
Mimomyia spp.		2	1			3	0.3%
Uranotenia spp.					1	1	0.1%
Grand Total	**123**	**646**	**176**	**50**	**130**	**1125**	100%

Table 3 Mosquitoes captured from the different of 0.5, 3, and 5 meters height hanging CDC Light traps on the trees (LT) located on the high hill site behind the reported malaria infected house during dry (May) and wet (October) seasons in 2013.

Species	Light traps								Grand Total	Percentage
	Dry season			Total	Wet season			Total		
	0.5m	3m	5m		0.5m	3m	5m			
Aedes albopictus	7	1		8	11	14	5	30	38	29.23%
Armigeres malayi	1			1	3	3	1	7	8	6.15%
Armigeres subalbatus	29	2	2	33	4	2	3	9	42	32.31%
Coquinletidae crassipes		3	3	6			2	2	8	6.15%
Culex bitaeniorhnychus						2		2	2	1.54%
Culex pseudovishnui							1	1	1	0.77%
Culex sitiens					2		1	3	3	2.31%
Culex vishnui	7	1	12	20		5	2	7	27	20.77%
***Uranotenia* spp.**							1	1	1	0.77%
Grand Total	44	7	17	68	20	26	16	62	130	100%

Sample HI1.6/5Anc-23



Sample HI1.4/9-161

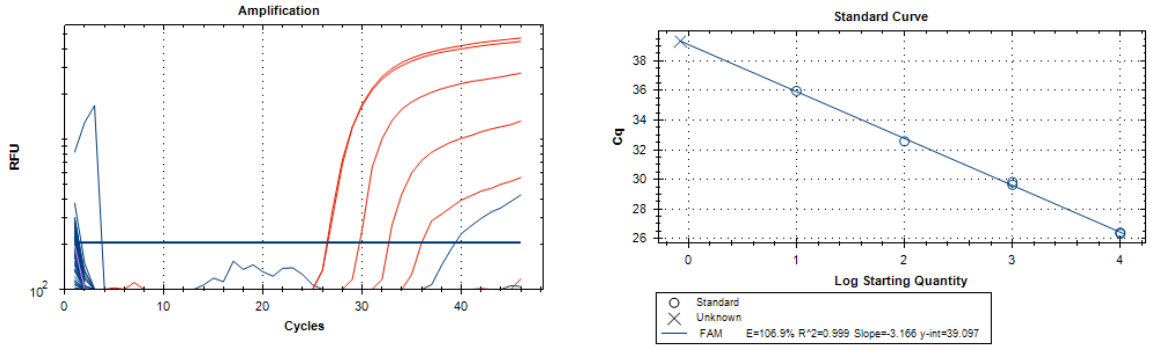


Figure2 Positive qMAL PCR detection in two *An. barbirostirs* complex. Red lines are STD 10000 copy, 1000 copy, 100 copy, 10 copy. Blue curve lines are positive unknown samples with the over cut off range (bold horizontal blue line). Right graph pictures are standard curve that used for calculate of the copy numbers of PCR products.

Regression equations are HI1.6/5Anc-23: Cq value = -3.246 × 10 log DNA/PCR + 39.445, $r^2 = 0.999$, $p < 0.001$; HI1.4/9Anc-161: Cq value = -3.166 × 10 log DNA/PCR + 39.097, $r^2 = 0.999$, $p < 0.001$.

Figure3 PCR detection to identify *Plasmodium* species; *P. falciparum* (F), *P. vivax* (V), *P. ovalae* (O), *P. malariae* (M), and *P. knowlesi* (K) by using 0.5 uL of DNA of sample 23 and 2 uL of sample HI 1.6/5 Anc-23. Nested PCR were used 5 uL of primary PCR product as the template. One hundred bp band represented for the positive PV malaria species and 2nd csp was detected 700 bp to confirm positive PV in HI 1.6/5 Anc-23 but could not identify in sample HI 1.4/9 Anc-161.

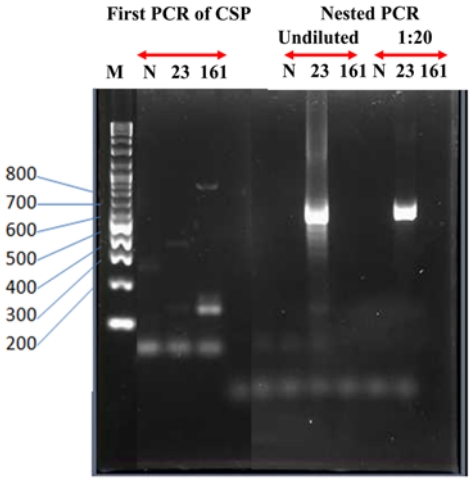
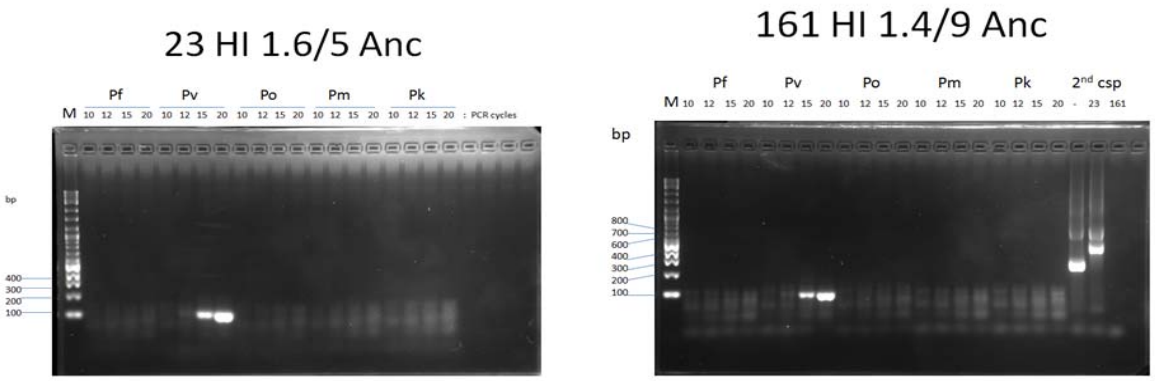


Figure 4 Recheck of Nested PCR for detection of Circumsporozoite typing in sample HI 1.6/5 Anc-23 and HI 1.4/9 Anc-161. N is negative control, M is DNA ladder, Positive Circumsporozoite (csp) product located at 600-700 bp.



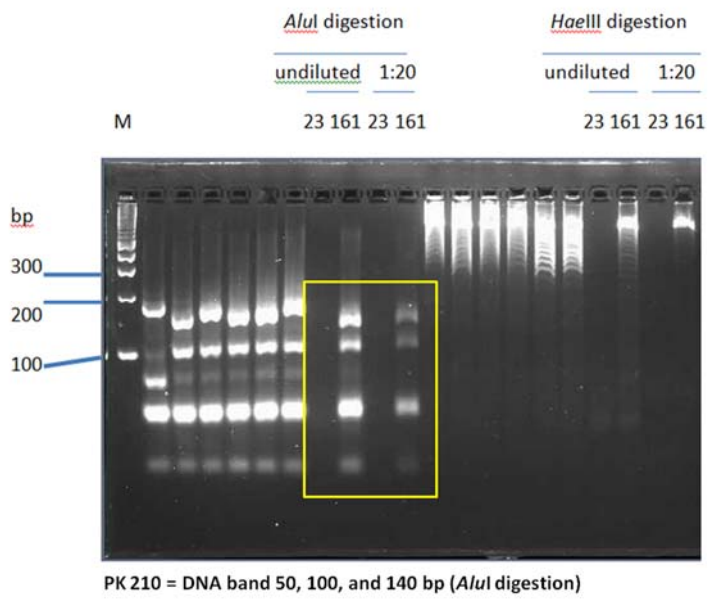


Figure 5 RFLP to detect PV210 and PV247 in 2 positive nested PCR sample number 23 and 161.

Table 5 Summary malaria detection in *Anopheles barbirostirs* complex with positive malaria detection of genus (using qMAL), species (using Nested PCR) and circumsporozoite typing (using RFLP).

Sample numbers	QMAL screening (Cq)	Starting Quantity (SQ)	Species typing	CSP typing
23 HI 1.6/5 Anc (at 23.00)	36.44	7.17592	PV	not identify
161 HI 1.4/9 Anc (at 21.00)	35.9	10.26102	PV	210

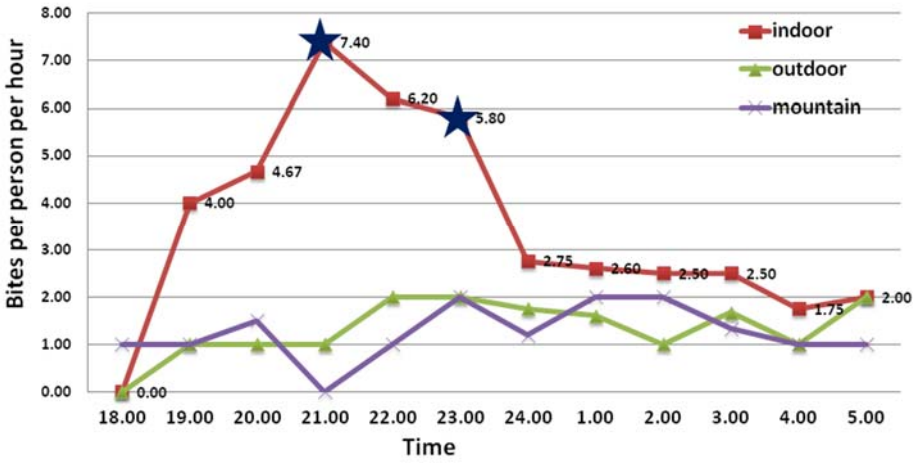


Figure 6 Biting activities of *An. barbirostirs* group among locations of indoor, outdoor, and the hill nearby the target house. ★ Represented the positive malaria (PV210 and unidentified species typing) infection in *An. barbirostirs* group detected by real time and nested PCR.

Table 6 Malaria case incidence in Makham district, Chanthaburi province from 2009-2013. Under line numbers were PF patients.

				----------Dry----------					----------Wet----------					---- Dry ---	
Year	Thai PV	PF	mix	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec
2009	12	2	1	1	3	1, 1, PK	1	1	mix ,4		1		1		1, 1
2010	12	0	0					2			1	1	1	2	5
2011	4	0	0	2		1				1					
2012	2	3	0		1		1	1		2					
2013	3	1	0	1		1, 1	1								
Year	Foreigners PV	PF	mix	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec
2009	2	0	0								1	1			
2010	0	1	0												1
2011	4	0	0					1	1			2			
2012	2	0	0					1	1						
2013	2	0	0							2					

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