*Original Article*

# Mosquito Distribution and Japanese Encephalitis Virus Infection in a Bat Cave and Its Surrounding Area in Lopburi Province, Central Thailand

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***Abstract***

Mosquito distribution and Japanese encephalitis virus (JEV) infection were studied in a bat cave and its surrounding area in Lopburi province, central Thailand. Mosquitoes were collected from May 2009 to April 2010 by using $CO_2$-baited CDC-light traps, and dry ice as a source of $CO_2$ to attract mosquitoes. Mosquitoes were identified and tested for JEV infection by using reverse transcription polymerase chain reaction (RT-PCR). Five genera of mosquitoes collected from the bat cave include *Aedes, Anopheles*, *Armigeres, Culex* and *Uranotaenia*, and eight genera of mosquitoes collected from the area close to the bat cave include *Aedes, Aedeomyia*, *Anopheles, Armigeres, Coquillettidia, Culex, Mansonia* and *Uranotaenia*. The dominant species of the collected mosquitoes from these two areas were *Cx. quinquefasciatus, Cx. tritaeniorhynchus* and *Ar. subalbatus*. There was only one species of blood-fed mosquitoes collected from the bat cave in this study, which was *Cx. quinquefasciatus*. Blood meal identification based on cytochrome b sequences in this study indicated that all mosquito blood meals were blood from the fruit bat, *Rousettus leschenaulti*. A total of 61 pools of mosquitoes were collected from the bat cave: six pools of *Ar. subalbatus*, one pool of *Cx. gelidus*, 39 pools of *Cx. quinquefasciatus* and 15 pools of *Cx. tritaeniorhynchus*. A total of 110 pools of mosquitoes were collected from the area close to the bat cave: six pools of *Ae. albopictus*, three pools of *An. stephensi*, 23 pools of *Ar. subalbatus*, two pools of *Cx. gelidus*, 36 pools of *Cx. quinquefasciatus* and 40 pools of *Cx. tritaeniorhynchus*. Each of these pools in this study, which contains 10 mosquitoes was tested for JEV infection by using RT-PCR, and all of them were negative.

**Keywords:** bat cave, Japanese encephalitis virus, mosquito, Thailand

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# บทคัดย่อ

## ชนิดของยุงและการติดเชื้อไวรัสสมองอักเสบเจอีในบริเวณถ้ำค้างคาวในจังหวัดลพบุรี ประเทศไทย

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ทำการศึกษาชนิดของยุงและการติดเชื้อไวรัสสมองอักเสบเจอีในบริเวณถ้ำค้างคาวในพื้นที่จังหวัดลพบุรี ภาคกลางของประเทศไทย โดยจับยุงตั้งแต่เดือนพฤษภาคม 2552 ถึงเดือนเมษายน 2553 โดยใช้กับดักยุงชนิด CDC-light trap ร่วมกับการใช้น้ำแข็งแห้งเป็นแหล่งของคาร์บอนไดออกไซด์ในการดึงดูดยุง ยุงที่จับได้จะถูกนำมาจำแนกชนิดและตรวจหาเชื้อไวรัสสมองอักเสบเจอีโดยใช้เทคนิค reverse transcription polymerase chain reaction (RT-PCR) ผลการศึกษาพบ ในถ้ำค้างคาวมียุงจำนวน 5 สกุล ได้แก่ *Aedes, Anopheles, Armigeres, Culex* และ *Uranotaenia* ส่วนบริเวณภายนอกถ้ำค้างคาวนั้นพบยุงจำนวน 8 สกุล ได้แก่ *Aedes, Aedeomyia, Anopheles, Armigeres, Coquillettidia, Culex, Mansonia* และ *Uranotaenia* สำหรับชนิดของยุงที่พบได้มากจากทั้งสองบริเวณคือ *Cx. quinquefasciatus*, *Cx . tritaeniorhynchus* และ *Ar. subalbatus* และพบยุงเพียงชนิดเดียวคือ *Cx. quinquefasciatus* ซึ่งจับได้จากภายในถ้ำค้างคาวที่พบว่ามีก้อนเลือดอยู่ภายในส่วนท้องของยุง และเมื่อได้ทำการศึกษาถึงชนิดของเลือดดังกล่าวโดยอาศัยการศึกษาลำดับเบสของ cytochrome b ทำให้ทราบว่าเลือดที่พบในยุงนั้นเป็นเลือดของค้างคาวบัวฟันรี ที่มีชื่อทางวิทยาศาสตร์ว่า *Rousettus leschenaulti* ซึ่งเป็นค้างคาวกินผลไม้ขนาดกลางชนิดหนึ่ง สำหรับการตรวจหาเชื้อไวรัสสมองอักเสบเจอี ได้ทำการทดสอบยุงที่จับมาจากภายในถ้ำค้างคาวจำนวน 61 กลุ่มตัวอย่าง ซึ่งประกอบไปด้วย *Ar. subalbatus* จำนวน 6 กลุ่มตัวอย่าง *Cx. gelidus* จำนวน 1 กลุ่มตัวอย่าง *Cx. quinquefasciatus* จำนวน 39 กลุ่มตัวอย่าง และ *Cx. tritaeniorhynchus* จำนวน 15 กลุ่มตัวอย่าง และได้ทำการทดสอบยุงที่จับมาจากภายนอกถ้ำค้างคาวจำนวน 110 กลุ่มตัวอย่าง ซึ่งประกอบไปด้วย *Ae. albopictus* จำนวน 6 กลุ่มตัวอย่าง *An. stephensi* จำนวน 3 กลุ่มตัวอย่าง *Ar. subalbatus* จำนวน 23 กลุ่มตัวอย่าง *Cx. gelidus* จำนวน 2 กลุ่มตัวอย่าง *Cx. quinquefasciatus* จำนวน 36 กลุ่มตัวอย่าง และ *Cx. tritaeniorhynchus* จำนวน 40 กลุ่มตัวอย่าง โดยที่แต่ละกลุ่มตัวอย่างประกอบไปด้วยยุงจำนวน 10 ตัว อย่างไรก็ตามตรวจไม่พบเชื้อในทุกกลุ่มตัวอย่างที่ทำการทดสอบ

**คำสำคัญ:** ถ้ำค้างคาว ไวรัสสมองอักเสบเจอี ยุง ประเทศไทย

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## *Introduction*

Japanese encephalitis virus (JEV) is a zoonotic mosquito-borne virus. The outbreak of this virus can be found in several countries, particularly in Asia. This virus was first isolated in 1935 in Japan and has spread throughout Asia and Australia. JEV belongs to the Japanese encephalitis (JE) serogroup of the genus *flavivirus*, family flaviviridae. Other important viruses in this serogroup are West Nile virus, St. Louis encephalitis virus, Rocio virus, and Kunjin virus. There are five genotypes of JEV, including genotype I, II, III, IV and V. The genotype that was recently found in Thailand is genotype I (Nitatpattana et al., 2008). Transmission cycle of JEV in nature involves amplifying in mosquito vectors, particularly *Culex* species and in reservoir hosts which allow the virus to propagate into the optimal level at which the transmission can occur (Mitchell and Chen, 1973; Maeda et al., 1978; Ali et al., 1995). Avian and swine are animals that play a major role in the JEV transmission cycle (Kedarnath et al., 1987; Ting et al., 2004).

Bat is another creature that might be involved in the transmission cycle of several infectious diseases, particularly viral ones. Bat is a mammal species in the order Chiroptera. There are 18 families with 203 genera, and 1,100 species were discovered; however, there are 10 genera with 120 species of bats which can be found in Thailand. Most of them are insectivores while others are frugivores and a few are carnivores. Bat can serve as a reservoir host for several viruses: rabies, Lyssavirus, Marburg virus, Tioman virus, Nipah virus and JEV (Butler 2004; Wacharapluesadee et al., 2005; Towner et al., 2007; Yaiw et al., 2008; Preuss et al., 2009).

Neutralizing antibodies against JEV were examined and identified in the old world fruit bat, *Rousettus leschenaulti*, which is the bat in the suborder Megachiroptera. Virus particle, however, cannot be detected from both brain and liver of the infected bats (Cui et al., 2008).

This study investigated the distribution of mosquito species and JEV infection in the bat cave and its surrounding area in Lopburi province, central Thailand. Since the major sources of the swine production in Thailand are located in the central of Thailand, including Lopburi province, these areas are of public health concern for the outbreak of JEV. The initial data from our study would be useful for future research on the relationship among JEV, mosquitoes and bats in Thailand.

## *Materials and Methods*

***Mosquito collection:*** Mosquitoes were collected by using $CO_2$-baited CDC light traps, and dry ice as a source of $CO_2$ to attract mosquitoes from May 2009 to April 2010. Eight traps were operated, from 5 pm to 9 am of the next day, in the bat cave and surrounding area in Lopburi province, central Thailand. Collected mosquitoes were identified according to Rattanarithikul and Panthusiri (1994) and kept at -80$^0$C until tested.

***Mosquito blood meal identification***

***Mosquito blood meal DNA extraction:*** The DNA was extracted from the homogenized blood-fed mosquito using DNAzol BD® (Molecular Research Center, Cincinnati, OH) according to the manufacturer's instructions. Briefly, whole mosquito was homogenized with 200 µl of DNAzol BD® (Molecular Research Center, Cincinnati, OH) in microcentrifuge tube using a plastic pestle. The homogenized mosquito was then kept at room temperature for 10 min, centrifuged at 10,000 rpm for 10 min, and the supernatant was removed into a new tube. Three µl of polyacryl carrier (Molecular Research Center, Cincinnati, OH) was added and kept at room temperature for 3 min. A total of 80 µl of isopropanol was added, kept at room temperature for 10 min, and centrifuged at 6,000 rpm for 6 min. The supernatant was removed into waste and 100 µl of DNAzol BD® (Molecular Research Center, Cincinnati, OH) was added, mixed with the DNA pellet, and centrifuged at 6,000 rpm for 5 min. The supernatant was removed into waste, and 1 ml of 95% ethanol was added and mixed with the DNA pellet, and centrifuged at 2,000 rpm for 5 min. DNA pellet was dried at room temperature for 5 min after ethanol was removed. DNA pellet was then resuspended with 50 µl of TE buffer (pH 8) and kept at -20$^0$C.

***PCR amplification and sequencing of mosquito blood meal:*** Isolated DNA from the mosquito blood meals served as DNA templates in subsequent PCR reactions. PCR primers were based on a multiple alignment of cytochrome b sequences of mammalian and avian species obtained from GenBank (National Center for Biotechnology Information). All DNA templates were initially screened with mammalian- and avian-specific primer pairs, and the sequences were analyzed. Mammalian-specific PCR primers were Mammal-570F (5'-CCC TAG TCC TAG TCC ACC TCC T-3') and Mammal-960R (5'- GGC TAA GTG GTC GRA ATA TTA TGC -3') with amplified product size of 390 bp. The PCR reactions were performed in a 25 µl reaction containing PCR buffer (10 mM Tris-HCl, pH 9.0, 1.5 mM $MgCl_2$, 50 mM KCl) (Amersham Phamacia, Freiburg, Germany), 200 µM each of dATP, dCTP, dGTP, and dTTP (Promega, Madison, WI), 0.625 units Taq DNA polymerase (Amersham Phamacia, Freiburg, Germany), 5 pmol of each primer and DNA template. After incubation at 94$^0$C for 5 min, amplification was carried out for 35 cycles with the following temperature cycling parameters: 94$^0$C for 30 sec of denaturation, 57$^0$C for 30 sec of annealing, and 72$^0$C for 30 sec of extension. The final amplification cycle included an addition of 10 min extension at 72$^0$C.

Avian-specific PCR primers were Avian-475F (5'-CAC ACC CTA GTA GAG TGA GC-3') and Avian-1120R (5'- GTG TTC CGR TTR TGG GGA AG -3') with amplified product size of 645 bp. The PCR reactions were performed in a 25 µl reaction containing PCR buffer (Amersham Phamacia, Freiburg, Germany), 200 µM each of dNTP (Promega, Madison, WI), 0.625 units Taq DNA polymerase (Amersham Phamacia, Freiburg, Germany), 5 pmol each of primer and DNA template. After incubation at 94$^0$C for 5 min, amplification was carried out for 35 cycles with the following temperature cycling parameters: 94$^0$C for 45 sec of denaturation, 55$^0$C for 45 sec of annealing, and 72$^0$C for 45 sec of extension. The final amplification cycle included an addition of 10 min extension at 72$^0$C. PCR reactions were performed with the GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA). Analysis of PCR product was performed by electrophoresis on ethidium bromide-stained 2% agarose gel.

***Sequencing of blood meal PCR products:*** PCR-amplified products were purified by using QIAquick PCR Purification Kit (QIAGEN, Valencia, CA) and then subjected to direct sequencing with the Big Dye Terminator kit according to the manufacturer's protocol (Applied Biosystems, Foster City, CA) on an ABI PRISM3100 DNA Sequencer (Applied Biosystems, Foster City, CA). Sequences were annotated by using ChromasPro version 1.22 (Technelysium Pty Ltd, Tewantin, Australia) and identified by comparing to the GenBank DNA sequence database (National Center for Biotechnology Information).

***Virus infection in collected mosquitoes***

***Viral RNA extraction:*** Viral RNA was extracted from each mosquito pool collected from the bat cave and the surrounding area. Each mosquito pool had 10 mosquitoes. Viral RNA was subsequently tested for JEV infection by using reverse transcription polymerase chain reaction (RT-PCR). Viral RNA was extracted by using Tri Reagent LS® (Molecular Research Center, Cincinnati, OH) according to the manufacturer's recommendation with slight modification. Briefly, a pool of 10 mosquitoes was homogenized in 500 µl of Minimum Essential

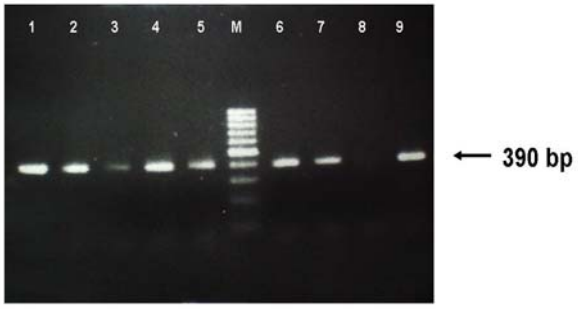


**Figure 1** Polymerase chain reaction (PCR) amplification of mammalian cytochrome b sequences. Template DNAs for each lane are as follows: lane 1-7: Mosquito (*Culex quinquefasciatus*) from field, lane 8: Chicken (*Gallus gallus*) (PCR negative control for mammalian specific primer), lane 9: Bat (*Rousettus leschenaulti*) (PCR positive control), M: 100 DNA ladder.

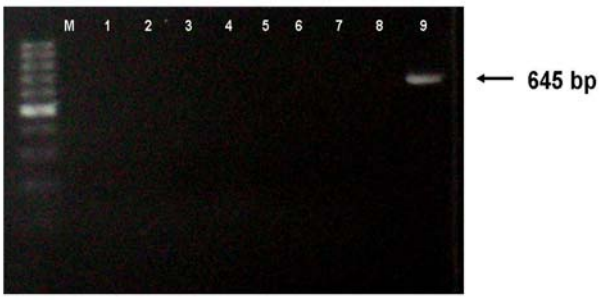


**Figure 2** Polymerase chain reaction (PCR) amplification of avian cytochrome b sequences. Template DNAs for each lane are as follows: lane 1-7: Mosquito (*Culex quinquefasciatus*) from field, lane 8: Bat (*Rousettus leschenaulti*) (PCR negative control for avian specific primer), lane 9: Chicken (*Gallus gallus*) (PCR positive control), M: 100 DNA ladder.

Medium Alpha (Invitrogen, Carlsbad, CA) by using a plastic pestle. The mosquito homogenate was then centrifuged at 3,000 rpm for 10 min. Two hundred and fifty µl of the homogenate was mixed with 750 µl of Tri Reagent LS®. Two hundred µl of chloroform was added, and RNA was precipitated in 500 µl of isopropanol. RNA pellet was then washed with 75% ethanol and dissolved in 20 µl of ultrapure water (Invitrogen, Carlsbad, CA). RNA was kept at -80$^0$C until tested.

***Reverse transcription polymerase chain reaction (RT-PCR)***: RNA samples extracted from mosquito pools were tested for Japanese Encephalitis virus (JEV) by using RT-PCR. The primers for C/Pr M gene of JEV were forward 5′ GAC TAA AAA ACC AGG AGG GC and reverse 5′ CTC CCC ATG TGT TTG GAC CG. After the reverse transcription step at 48$^0$C for 45 min and the initial PCR activation step at 94$^0$C for 3 min, the amplification was carried out for 35 cycles with the following temperature cycling parameters: 94$^0$C for 30 sec of denaturation, 58$^0$C for 30 sec of annealing, and 72$^0$C for 30 sec of extension. The final amplification cycle included an addition of 7 min extension at 72$^0$C.

RT-PCRs were performed in 25 µl-reaction. One and a half µl of RNA was mixed with 12.5 µl of master mix (AccessQuick®, Promega, Madison, WI), 0.2 µM of forward and reverse primer, 2.5 U of AMV reverse transcriptase (AccessQuick®, Promega, Madison, WI), and 8.5 µl of ultrapure water (Invitrogen, Carlsbad, CA). RNA was amplified by using thermocycler (Perkin Elmer Cetus 9600, Perkin Elmer, Waltham, MA). The PCR product was analyzed in 1.2% agarose gel (UltraPure™, Invitrogen, Carlsbad, CA) with expected 681 base pair band.

## *Results*

***Mosquito distribution:*** There were 2,901 mosquitoes collected in this study, from May 2009 to April 2010, by using eight mosquito traps. There were five genera of the mosquitoes collected from the bat cave: *Aedes, Anopheles, Armigeres, Culex* and *Uranotaenia*, and there were eight species of the mosquitoes: *Ae. albopictus, An. barbirostris, An. stephensi, Ar. subalbatus, Cx. gelidus, Cx. quinquefasciatus, Cx. tritaeniorhynchus* and *Ur. lateralis* (Table 1). There were eight genera of the mosquitoes collected from the area close to the bat cave: *Aedes, Aedeomyia, Anopheles, Armigeres, Coquillettidia, Culex, Mansonia* and *Uranotaenia* and there were 16 species of the mosquitoes: *Ae. aegypti, Ae. albopictus, Ae. lineatopennis, Ae. vigilax, Ae. Niveus subgroup, Ad. catasticta, An. barbirostris, An. stephensi, Ar. subalbatus, Cq. crassipes, Cx. gelidus, Cx. quinquefasciatus, Cx. tritaeniorhynchus, Ma. annulata, Ma. uniformis and Ur. lateralis* (Table 2). The dominant species of the collected mosquitoes from the bat cave and the surrounding area were *Cx. quinquefasciatus, Cx. tritaeniorhynchus and Ar. subalbatus.*

**Table 1** Species and percentage of mosquitoes collected from the bat cave, Lopburi province, Thailand from May 2009 to April 2010

| Mosquito species | Month | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | May 09 | Jun 09 | Jul 09 | Aug 09 | Sep 09 | Oct 09 | Nov 09 | Dec 09 | Jan 10 | Feb 10 | Mar 10 | Apr 10 |
| *Aedes albopictus* | 0.7 | 0 | 0 | 0.9 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 16.7 |
| *Anopheles barbirostris* | 0 | 0 | 0 | 0 | 0 | 0 | 9.1 | 4.6 | 0 | 0 | 0 | 0 |
| *Anopheles stephensi* | 0 | 0 | 0 | 0.9 | 3.1 | 3.1 | 9.1 | 1.5 | 1.5 | 0 | 0 | 0 |
| *Armigeres subalbatus* | 5.4 | 9.2 | 19.1 | 42.2 | 9.4 | 11.0 | 0 | 1.5 | 4.5 | 7.1 | 20.0 | 0 |
| *Culex gelidus* | 0 | 1.1 | 0 | 0 | 15.6 | 6.3 | 0 | 6.2 | 1.5 | 0 | 0 | 16.7 |
| *Culex quinquefasciatus* | 90.3 | 88.5 | 80.9 | 35.8 | 31.3 | 18.9 | 45.5 | 44.6 | 92.5 | 92.9 | 80.0 | 66.6 |
| *Culex tritaeniorhynchus* | 3.6 | 1.1 | 0 | 20.2 | 40.6 | 60.6 | 27.3 | 41.5 | 0 | 0 | 0 | 0 |
| *Uranotaenia lateralis* | 0 | 0 | 0 | 0 | 0 | 0 | 9.1 | 0 | 0 | 0 | 0 | 0 |

**Table 2** Species and percentage of mosquitoes collected from the area close to the bat cave, Lopburi province, Thailand from May 2009 to April 2010

| Mosquito species | Month | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | May 09 | Jun 09 | Jul 09 | Aug 09 | Sep 09 | Oct 09 | Nov 09 | Dec 09 | Jan 10 | Feb 10 | Mar 10 | Apr 10 |
| *Aedes aegypti* | 1.1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 15.4 | 0 |
| *Aedes albopictus* | 1.6 | 4.7 | 4.7 | 14.9 | 1.7 | 4.7 | 1.0 | 0 | 13.7 | 28.6 | 15.4 | 57.1 |
| *Aedes lineatopennis* | 0.5 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *Aedes vigilax* | 0 | 0 | 0 | 2.4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *Aedes* Niveus subgroup | 0 | 0 | 0 | 0.8 | 0 | 2.2 | 0 | 0 | 0 | 0 | 0 | 0 |
| *Aedeomyia catasticta* | 0 | 2.7 | 0.6 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *Anopheles barbirostris* | 0 | 0 | 0 | 0 | 0 | 1.2 | 6.7 | 0 | 2.0 | 0 | 0 | 0 |
| *Anopheles stephensi* | 0 | 0 | 1.1 | 3.6 | 3.3 | 4.0 | 13.3 | 3.8 | 11.8 | 0 | 0 | 0 |
| *Armigeres subalbatus* | 10.8 | 25.1 | 8.0 | 30.5 | 12.5 | 7.2 | 6.7 | 5.8 | 11.8 | 14.3 | 7.7 | 0 |
| *Coquilletidia crassipes* | 0 | 1.2 | 0.6 | 0 | 0.3 | 0.9 | 1.0 | 0 | 5.9 | 0 | 0 | 0 |
| *Culex gelidus* | 0.5 | 1.2 | 0 | 0.4 | 5.8 | 0 | 1.0 | 0 | 0 | 0 | 0 | 0 |
| *Culex quinquefasciatus* | 83.2 | 60.4 | 82.5 | 18.9 | 2.8 | 1.2 | 1.0 | 3.8 | 25.5 | 57.1 | 61.5 | 42.9 |
| *Culex tritaeniorhynchus* | 2.2 | 4.7 | 2.2 | 28.5 | 73.5 | 76.6 | 69.5 | 78.8 | 23.5 | 0 | 0 | 0 |
| *Mansonia annulata* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 7.7 | 0 | 0 | 0 | 0 |
| *Mansonia uniformis* | 0 | 0 | 0.3 | 0 | 0 | 0 | 0 | 0 | 5.9 | 0 | 0 | 0 |
| *Uranotaenia lateralis* | 0 | 0 | 0 | 0 | 0 | 1.9 | 0 | 0 | 0 | 0 | 0 | 0 |

**Table 3** Pools of mosquitoes collected from the bat cave and tested for Japanese encephalitis virus by using reverse transcription polymerase chain reaction (RT-PCR)

| Mosquito species | Month | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | May 09 | Jun 09 | Jul 09 | Aug 09 | Sep 09 | Oct 09 | Nov 09 | Dec 09 | Jan 10 | Feb 10 | Mar 10 | Apr 10 |
| *Aedes albopictus* | - | - | - | - | - | - | - | - | - | - | - | - |
| *Anopheles barbirostris* | - | - | - | - | - | - | - | - | - | - | - | - |
| *Anopheles stephensi* | - | - | - | - | - | - | - | - | - | - | - | - |
| *Armigeres subalbatus* | 1 | - | - | 4 | - | 1 | - | - | - | - | - | - |
| *Culex gelidus* | - | - | - | - | 1 | - | - | - | - | - | - | - |
| *Culex quinquefasciatus* | 10 | 7 | 3 | 3 | 3 | 2 | - | 2 | 6 | 1 | 2 | - |
| *Culex tritaeniorhynchus* | 1 | - | - | 2 | 3 | 7 | - | 2 | - | - | - | - |
| *Uranotaenia lateralis* | - | - | - | - | - | - | - | - | - | - | - | - |
| **Total** | **12** | **7** | **3** | **9** | **7** | **10** | **0** | **4** | **6** | **1** | **2** | **0** |

**Table 4** Pools of mosquitoes collected from the area close to the bat cave and tested for Japanese encephalitis virus by using reverse transcription polymerase chain reaction (RT-PCR)

| Mosquito species | Month | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | May 09 | Jun 09 | Jul 09 | Aug 09 | Sep 09 | Oct 09 | Nov 09 | Dec 09 | Jan 10 | Feb 10 | Mar 10 | Apr 10 |
| *Aedes aegypti* | - | - | - | - | - | - | - | - | - | - | - | - |
| *Aedes albopictus* | - | 1 | 1 | 3 | - | 1 | - | - | - | - | - | - |
| *Aedes lineatopennis* | - | - | - | - | - | - | - | - | - | - | - | - |
| *Aedes vigilax* | - | - | - | - | - | - | - | - | - | - | - | - |
| *Aedes* Niveus subgroup | - | - | - | - | - | - | - | - | - | - | - | - |
| *Aedeomyia catasticta* | - | - | - | - | - | - | - | - | - | - | - | - |
| *Anopheles barbirostris* | - | - | - | - | - | - | - | - | - | - | - | - |
| *Anopheles stephensi* | - | - | - | - | 1 | 1 | 1 | - | - | - | - | - |
| *Armigeres subalbatus* | 2 | 6 | 2 | 7 | 4 | 2 | - | - | - | - | - | - |
| *Coquilletidia crassipes* | - | - | - | - | - | - | - | - | - | - | - | - |
| *Culex gelidus* | - | - | - | - | 2 | - | - | - | - | - | - | - |
| *Culex quinquefasciatus* | 10 | 10 | 10 | 4 | 1 | - | - | - | 1 | - | - | - |
| *Culex tritaeniorhynchus* | - | 1 | - | 7 | 10 | 10 | 7 | 4 | 1 | - | - | - |
| *Mansonia annulata* | - | - | - | - | - | - | - | - | - | - | - | - |
| *Mansonia uniformis* | - | - | - | - | - | - | - | - | - | - | - | - |
| *Uranotaenia lateralis* | - | - | - | - | - | - | - | - | - | - | - | - |
| **Total** | **12** | **18** | **13** | **21** | **18** | **14** | **8** | **4** | **2** | **0** | **0** | **0** |

Mosquito blood meal identification: A total of 40 blood-fed *Cx. quinquefasciatus* collected from the bat cave were tested by using PCR, and all the blood meals were mammal blood. Twenty one samples of the mosquito blood meals were randomized, and the PCR products were sequenced. All of them were *Rousettus leschenaulti* (Figs 1 and 2).

Japanese Encephalitis virus infection in mosquitoes: A total of 61 pools of mosquitoes collected from the bat cave: six pools of *Ar. subalbatus*, one pool of *Cx. gelidus*, 39 pools of *Cx. quinquefasciatus*, and 15 pools of *Cx. tritaeniorhynchus*, each of which had 10 mosquitoes, were tested for JEV infection by using RT-PCR, and all of them were negative (Table 3). A total of 110 pools of mosquitoes collected from the area close to the bat cave: six pools of *Ae. albopictus*, three pools of *An. stephensi*, 23 pools of *Ar. subalbatus*, two pools of *Cx. gelidus*, 36 pools of *Cx. quinquefasciatus* and 40 pools of *Cx. tritaeniorhynchus*, each of which had 10 mosquitoes, were tested for JEV infection by using RT-PCR, and all of them were negative (Table 4).

## Discussion

This study was the initial exploration of the relationship among Japanese encephalitis virus (JEV), mosquitoes and bat population in nature in Thailand. The dominant species of the collected mosquitoes from the bat cave and its surrounding area in this study were *Cx. quinquefasciatus*, *Cx. tritaeniorhynchus* and *Ar. subalbatus*. The bat cave is located on top of the mountain, surrounded by some trees and no water reservoir in this area. Large numbers of *Cx. quinquefasciatus* were collected from the bat cave and also its surrounding area. The result was different from our previous study (Tiawsirisup et al., 2008; Tiawsirisup and Nuchprayoon, 2010), which investigated the mosquito distribution in the migratory bird nested area surrounded by water reservoirs, and two most collected species were *Cx. tritaeniorhynchus* and *Cx. gelidus*.

There was only one species of blood-fed mosquitoes collected from the bat cave in this study, which was *Cx. quinquefasciatus.* Agarose gel diffusion technique, molecular technique (e.g. method based on cytochrome b gene), antibody sandwich enzyme-linked immunosorbent assay, and Microdot ELISA have been used to study mosquito blood meal identification (Chow et al., 1993; Thapar et al., 1998; Molaei et al., 2006; Philip Samuel et al., 2008). Amplified mammal and avian cytochrome b genes from this study had the product sizes of 390 and 645 bp, respectively. Blood meal identification based on cytochrome b sequences in this study indicated that all mosquito blood meals were blood from the fruit bat, *Rousettus leschenaulti*. Mosquito blood meal or host preference identification is an important tool for the epidemiological study of mosquito-borne diseases since it can be used to indicate the role and relationship between mosquito vectors and reservoir hosts in nature.

Our data revealed that *Cx. quinquefasciatus* might play an important role as the vectors for any pathogen found in the blood of this bat and could replicate in this species of mosquitoes. Identification of blood meal from blood-fed insects as demonstrated in the current study also helps researchers to gain insights about the bat species without taking the blood samples directly from them. Even though *Cx. quinquefasciatus* may not be an important vector for JEV, the study by Nitatpattana et al. (2005) indicated the isolation of JEV from *Cx. quinquefasciatus* collected from Thailand. Therefore, this mosquito species cannot be excluded from the epidemiological study of the JEV ecology in Thailand. *Cx. tritaeniorhynchus*, one of the majorities of the mosquitoes collected from this study, is an important species as the vector for JEV (Gingrich et al., 1987; Arunachalam et al., 2009). However, no JEV from the collected mosquitoes could be detected.

Viral nucleic acid or antibodies against Nipah virus and lyssavirus have been identified from bats collected from Thailand; however, no study has been undertaken to examine the correlation between bat and JEV in Thailand (Lumlertdacha et al., 2005[a]; Lumlertdacha et al., 2005[b]; Wacharapluesadee et al., 2005). More studies are needed to understand this type of correlation in nature in Thailand.

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