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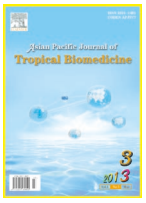

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Seasonal abundance and potential of Japanese encephalitis virus infection in mosquitoes at the nesting colony of ardeid birds, Thailand

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PEER REVIEW

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Comments

This is one of the most important studies on vector-borne zoonotic disease surveillance researches in view of increasing worldwide travelers and globally climate changes. The authors evaluated the seasonal abundance of mosquitoes vector transmitted JE and indicated that it is related with rice field status.

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ABSTRACT

Objective: To investigate the abundance and seasonal dynamics of mosquitoes, and to detect Japanese encephalitis virus (JEV) in these mosquitoes at the nesting colony of ardeid birds. **Methods:** Mosquitoes were collected bimonthly from July 2009 to May 2010 by Centers for Disease Control. Light traps and dry ice, as a source of CO_2, were employed to attract mosquitoes. Mosquitoes were first identified, pooled into groups of upto 50 mosquitoes by species, and tested for JEV infection by viral isolation and reverse transcriptase polymerase chain reaction. **Results:** A total of 20370 mosquitoes comprising 14 species in five genera were collected. The five most abundant mosquito species collected were *Culex tritaeniorhynchus* (95.46%), *Culex vishnui* (2.68%), *Culex gelidus* (0.72%), *Anopheles peditaeniatus* (0.58%) and *Culex quinquefasciatus* (0.22%). Mosquito peak densities were observed in July. All of 416 mosquito pools were negative for JEV. **Conclusions:** This study provides new information about mosquito species and status of JEV infection in mosquitoes in Thailand. Further study should be done to continue a close survey for the presence of this virus in the ardeid birds.

KEYWORDS

Mosquito, Japanese encephalitis virus, Vector, Abundance, Ardeid bird

1. Introduction

The Japanese encephalitis virus (JEV) is a mosquito-borne zoonotic infection recognized as the major causes of encephalitis in Eastern and Southern Asia[1]. It has been estimated an annual incidence of 45000 humans cases and 10000 deaths[2,3]. This virus is a member of the Japanese encephalitis (JE) serogroup of the genus *Flavivirus*, family Flaviviridae. Other important members in this serogroup are West Nile virus, St. Louis encephalitis virus, Kunjin virus and Murray Valley encephalitis virus[4]. JEV was first isolated in 1935 in Japan, and it has spread throughout Asia and Australia[3]. Since the first epidemic of JEV in 1969, this virus became endemic in Thailand with 1500 to 2500 reported cases of viral encephalitis each year during 1970-1985[2]. At present, this virus remains an important cause of encephalitis among hospitalized patients in Thailand[2].

JEV is maintained in enzootic transmission cycles among mosquitoes, wild birds and pigs[5]. The principal vector of JEV throughout Asia is *Culex tritaeniorhynchus* (*Cx. tritaeniorhynchus*) whereas other *Culex* mosquitoes also play a role as vectors. Pigs are one of the primary amplification hosts and probably the major determinant of human epidemic activity. Wild birds, particularly ardeid birds (*e.g.* egrets and herons) are also primary amplification hosts of JEV including black-crowned night herons (*Nycticorax nycticorax*), little egrets (*Egretta garzetta*), and intermediate or plumed egrets (*Egretta intermedia*)[1]. This virus can

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infect humans and horses, showing transient and low levels of viremia and are considered as dead-end hosts[1,5]. Several places in Thailand contain important colonies of resident and migratory birds. In particular, Ban Wang Pet (Bang Rakam, Phitsanulok) is one of the most important sites for ardeid birds, especially egrets. The objective of our study was to investigate the abundance, seasonal dynamics, and potential JEV infection in these mosquitoes at the nesting colony of ardeid birds in Ban Wang Pet. The present study was designed to investigate the abundance and seasonal dynamics of mosquitoes, and to detect JEV in these mosquitoes at the nesting colony of ardeid birds. The information obtained from this study will be useful for future research on epidemiological studies, detection and prevention of JEV in Thailand.

2. Materials and methods

2.1. Study site

Ban Wang Pet (Bang Rakam, Phitsanulok) is one of the most important sites for ardeid birds, especially egrets. This place is actually classified as the largest colony of egrets in Thailand. The bird species found in this area include cattle egret (*Bubulcus ibis*), little egret (*Egretta garzetta*), intermediate egret (*Egretta intermedia*), great egret (*Egretta alba*), Chinese egret (*Egretta eulophotes*), black-crowned night heron (*Nycticorax nycticorax*) and little cormorant (*Phalacrocorax niger*). Therefore, the site might potentially be an important source vector borne diseases.

2.2. Mosquito collection

Mosquitoes were collected bimonthly by using CO_2-baited CDC light traps (John W. Hock Company, Gainesville, USA) with dry ice used as a source of CO_2 to attract mosquitoes from July 2009 to May 2010. Five traps were operated from 6 p.m. until 6 a.m. on each study day. Mosquitoes were transported alive to laboratory for species identification by using description and illustrated keys[6]. They were pooled by species ranged from 1 to 50 and stored at −80 °C until tested for virus.

2.3. Viral isolation

A pool of mosquitoes was homogenized in 1 000 μL of minimum essential medium (MEM 10×, Penicillin G, Streptomycin and Fungizone) in 1.5 mL Eppendorf tube by using plastic pestle. The homogenated mosquito was centrifuged at 4 000 rpm for 10 min and supernatant was passed 0.45 μm syring filter. The 200 μL of samples were inoculated into baby hamster kidney (BHK-21) cells and incubated at 37 °C in a 5% CO_2 incubator for 2 h. About 150 μL of the samples of BHK-21 cells were discarded and added 500 μL of maintenance medium (2% fetal bovine serum in MEM) and then incubated at 37 °C in 5% CO_2. The presence of cytopathic effect (CPE) was checked daily for 3 d. Positive CPE was confirmed by reverse transcription polymerase chain reaction (RT-PCR).

2.4. Viral ribonucleic extraction and reverse transcription polymerase chain reaction

Viral ribonucleic acid (RNA) was extracted from mosquitoes by using a viral nucleic acid extraction kit (Geneaid Biotech Ltd., Taiwan). The RT-PCR was performed using a one-step RT-PCR kit (QIAGEN Ltd., Germany) for detection of JEV infection. Briefly, the reaction mixture contained 0.125 μL of forward primer JE/WN-OF 5′-GRA ARM GDG ARG ACA TYT GGT GTG G-3′, 0.125 μL of reverse primer JE/WN-OR 5′-CGG GGT CTC CTC TAA CCT CTA GTC C-3′, 2 μL of template DNA, 5 μL of 5× one-step RT-PCR buffer (QIAGEN), 1 μL of 10 mmol/L dNTP mix (QIAGEN), 1 μL of one-step RT-PCR enzyme (QIAGEN) and RNase-free water was added to a total volume of 25 μL. PCR cycling conditions were as follows: 1 cycle at 50 °C for 30 min; 1 cycle at 95 °C for 15 min; 35 cycles at 94 °C for 45 seconds followed by 70 °C for 45 seconds, 72 °C for 90 seconds and final extension at 72 C° for 10 min. Positive control was obtained from JE vaccine strain Beijing-1. Negative control consisted of master mix minus RNA templates. PCR products were separated by gel electrophoresis and visualized under UV light. The specific size of PCR product for JEV was 591 base pairs. The RT-PCR positive specimens will be confirmed for JEV by using specific method, SYBR I-based real-time RT-PCR with primer JE-multi-forward 5′-AGA ACG GAA GAY AAC CAT GAC TAA-3′ and JE-multi-reverse 5′-CCG CGT TTC AGC ATA TTG AT-3′ as described by Shirato *et al*[7].

3. Results

3.1. Seasonal abundance of mosquitoes

There were 20 370 mosquitoes collected in this study. Five genera in 14 species of mosquitoes were collected including *Aedes*, *Anopheles*, *Armigeres*, *Culex* and *Mansonia*. The five most abundant mosquito species collected were *Cx. tritaeniorhynchus* (95.46%), *Culex vishnui* (*Cx. vishnui*) (2.68%), *Culex gelidus* (*Cx. gelidus*) (0.72%), *Anopheles peditaeniatus* (*An. peditaeniatus*) (0.58%) and *Culex quinquefasciatus* (*Cx. quinquefasciatus*) (0.22%) (Table 1). The number of mosquitoes in July 2009, September 2009, November 2009, January 2010, March 2010 and May 2010 were 10 070, 3 035, 187, 5 691, 1 052 and 335 respectively (Figure 1).

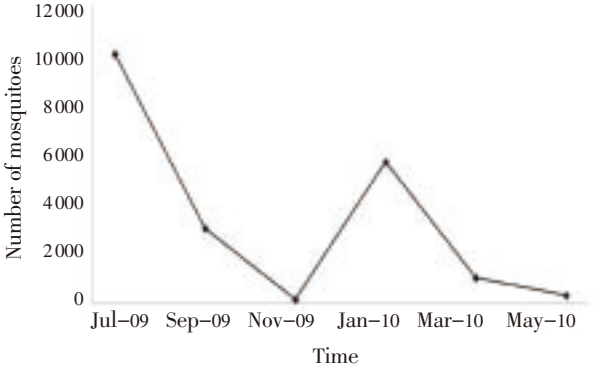

Figure 1. Mosquito seasonal dynamics at the nesting colony of ardeid birds in Phitsanulok Province, Thailand.

3.2. JEV infection in mosquitoes

We tested a total of 416 mosquito pools, and all of them were negative for JEV isolation. These results were confirmed by using RT-PCR, and all of them were negative (Table 1).

Table 1
Total number of each mosquito species collected and their test outcome for JEV at the nesting colony of ardeid birds in Phitsanulok Province, Thailand.

Mosquito species	Collection No.	Percent (%)	Pools No.	Positive pools
Ae. aegypti	1	0.00	1	0
An. argyropus	2	0.01	1	0
An. barbirostris	2	0.01	1	0
An. peditaeniatus	118	0.58	2	0
Ar. subalbatus	3	0.01	1	0
Cx. bitaeniorhynchus	2	0.01	1	0
Cx. fascocephala	4	0.02	1	0
Cx. gelidus	146	0.72	3	0
Cx. pseudovishnui	36	0.18	1	0
Cx. quinquefasciatus	44	0.22	1	0
Cx. tritaeniorhynchus	19445	95.46	390	0
Cx. vishnui	545	2.68	11	0
Mansonia indiana	18	0.09	1	0
Mansonia uniformis	4	0.02	1	0
Total	20370	100.00	416	0

4. Discussion

This study reports the mosquito abundance at the nesting colony of ardeid birds at Ban Wang Pet site. The site is located near the Yom River and surrounded by rice fields which are suitable breeding place for *Culex* sp. and Hyrcanus group of *Anopheles* sp.[8,9], especially *Cx. tritaeniorhynchus, Cx. vishnui, Cx. gelidus* and *An. peditaeniatus*. However, other mosquito genera in this study such as *Aedes, Armigeres* and *Mansonia* were also found in this area but in smaller numbers. *Culex* mosquitoes were the genus most frequently collected in this study. These mosquitoes are known to have a nocturnal feeding pattern and many of them have been collected in large numbers at night by using light traps[8]. *Ae. aegypti* was the mosquito species least collected in this study, probably because this species has a predominant diurnal activity[6].

Cx. tritaeniorhynchus, the most abundant mosquito species collected in this study, is the principal vector of JEV[1]. This species was identified as a JEV vector species in Thailand since the stains of the disease, when it was isolated from this species in Chiangmai Valley during 1970[10]. Other species of *Culex* mosquito frequently collected in our study *i.e., Cx. gelidus, Cx. fuscocephala* and *Cx. vishnui* were also reported as JEV vectors in Thailand[10,11]. *Cx. pseudovishnui* and *Cx. bitaeniorhynchus*, also found in our study, were reported to transmit JEV in India and Malaysia[12,13]. It is also important to remark that *Cx. quinquefasciatus* has recently became another potential mosquito vector for JEV in Thailand[14]. In addition, JEV has been isolated from other genera of mosquitoes such as *Aedes* sp., *Anopheles* sp. and *Mansonia* sp.[15,16]. *An. peditaeniatus*, the 4th most abundant mosquito species in this study, has been found to be the secondary vector of JEV in India[16]. This mosquito species has been found abundantly and widely distributed in Thailand[17], but the role as vector of JEV in Thailand remains unclear.

The highest and lowest numbers of mosquitoes collected in this study occurred in July and November respectively. Such peaks in mosquito abundance concords with the study carried out by Somboon *et al.* in Northern Thailand[18]. They found a peak of rice field mosquitoes occurred during rainy season and showed a sharp rise in the population in July when most of the rice fields were ploughed, and a marked decline in mosquito population densities occurred after transplanting in August when the fields were flooded. In addition, they also showed that the average number of larvae plus pupae per square meter in rice fields was the highest in July when the fields were ploughed, but in the period from transplanting to harvesting (August to November), the densities were very low. Furthermore, the study of Takagi *et al.* also supported rice cultural practices that have the effect on the abundance of mosquitoes in Northern Thailand[19]. At any rate, the number of mosquitoes may also vary depending on several factors such as rice field density, insect predators and artificial control of the water supply to the rice field[20,21].

Our study shows no evidence of JEV for the collected mosquitoes. A recent study of mosquito surveillance for JEV also found JEV negative in the mosquitoes collected at the Asian open-billed stork nested area and bat cave in Thailand[22,23]. Although these are promising results on the status of JEV in Thailand, mosquito active surveillance should be continuously conducted because 1) we can not definitively rule out that other sites are affected by JEV, 2) mosquitoes at important bird areas will continue being a potential disease vector, 3) JEV remains an important cause of encephalitis in Thailand, and 4) to prevent any potential JEV epidemics. In a further study JEV antigens and antibodies should be studied in order to help conclude whether ardeid birds are a natural important reservoir for JEV in Thailand.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgements

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Comments

Background

JE has a wide geographical distribution in Asia. JEV is a mosquito-borne member of the family Flaviviridae, genus *Flavivirus*, and mainly affects humans. Infection can occur in a number of animal species including horses and pigs. The virus is transmitted by mosquitoes and is maintained by a mosquito-aquatic bird cycle. The primary mosquito vector of JEV is *Cx. tritaeniorhynchus*, although species such as *Cx. gelidus, Cx. fuscocephala*, and *Cx. annulirostris* are important secondary or regional vectors.

Research frontiers

This work is performed to investigate the abundance and seasonal dynamics of mosquitoes collected from Ban

Wang Pet sites classified as the largest colony of egrets in Thailand. The mosquitoes were used as materials to detect JEV infection using reverse transcription polymerase chain reaction.

Related reports

Results in this study correspond to previous studies reported by Somboon *et al.* (1989) and Tiawsirisup (2010). The former found a peak of rice field mosquitoes occurred in July and markedly declined in August in Northern Thailand, and the latter reported that they could not detect any JEV in mosquitoes collected from nested area in central Thailand.

Innovations and breakthroughs

This study has shown that the highest and the lowest numbers of mosquitoes were collected in July and November, respectively, and *Cx. tritaeniorhynchus* is the most common encountered mosquito species among five genera in 14 species collected. However, there are no any mosquito vectors harboring JEV agents.

Applications

It is significant to know the distribution of vector-borne diseases and related viruses in migrating birds. The present study suggests that mosquito active surveillance should be continuously conducted due to several reasons including they cannot definitively rule out that other sites are affected by JEV, and mosquitoes at important bird areas will continue being a potential disease vector. Therefore, it is important to monitor globally the distribution of mosquitoes associated with JEV, including other Flavivirus.

Peer review

This is one of the most important studies on vector-borne zoonotic disease surveillance researches in view of increasing worldwide travelers and globally climate changes. The authors evaluated the seasonal abundance of mosquitoes vector transmitted JE and indicated that it is related with rice field status.

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