Dynamics and diversity of mosquito vectors of Japanese encephalitis virus in Kandal province, Cambodia

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Title

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Highlights

- Vectors of Japanese Encephalitis Virus are abundant all the year in Cambodia
- One-year study and seasonality of JEV vectors
- Effect of host presence on JEV vector abundance in pig farms and cattle house
- The most abundant vectors are *Culex vishnui*, *Cx. gelidus* and *Cx. tritaeniorhynchus*

Abstract

The Japanese encephalitis virus (JEV) is one of the main causes of encephalitis in Asia, including Cambodia. An understanding of the interactions between JEV hosts and vectors (Diptera: Culicidae) remains rare in the context of expanding urbanization. The relative abundance, species diversity and population dynamics of potential JEV vectors were studied between August 2015 and July 2016 on a peri-urban and rural pig farm in Kandal province, Cambodia, where JEV is circulating. Five similar environments in the two farms were selected for mosquito trapping: pig farm, cattle house, river/canals, household/ponds and paddy fields. The main objective was to describe the distribution and the dynamics of the main JEV vector mosquito species. In total, 83,013 mosquitoes from 20 species were caught in rural and peri-urban areas, and 82.3% of the mosquitoes were potential JEV vector species. In peri-urban areas, *Culex (Cx.) gelidus* was the most abundant species, followed by *Cx. vishnui* subgroup and *Cx. tritaeniorhynchus*. In rural areas, the same species were dominant: *Cx. vishnui* subgroup, *Cx. gelidus* and *Cx. tritaeniorhynchus*. The vast majority of mosquitoes (95.9%) were collected in close proximity to pigs and cattle. In conclusion, JEV vectors were present at all study sites and throughout all months of the year, supporting a continuous circulation of JEV in Cambodia.

Keywords

Japanese encephalitis; Vectors; Mosquitoes; Culex vishnui; Culex gelidus; Culex tritaeniorhynchus; Cambodia

Introduction

Japanese encephalitis (JE), an arthropod-borne and zoonotic disease, is one of the main causes of encephalitis, especially in the Asia Pacific region (Campbell et al. 2011). JE has an estimated annual incidence rate of 67,900 cases among 24 JE endemic countries (van den Hurk et al. 2009; Campbell et al. 2011). This vector-borne disease caused by Japanese encephalitis virus (JEV) was first identified in Japan, in 1935 from an encephalitis patient's brain (Lewis et al. 1947) and has since been found throughout Australasian and Asian countries including Cambodia (van den Hurk et al. 2009; Rosen, 1986). JEV belongs to the viral family *Flaviviridae* and the genus *Flavivirus.* It is maintained in the complex transmission cycle between water birds, mosquitoes and domestic pigs. Humans and horses are incidental hosts and considered dead-end hosts for JEV transmission because of low viremia levels incapable of infecting mosquitoes (Scherer et al. 1959; Impoinvil et al., 2013). Water bird species (heron [*Ardeola grayii*, *Nycticorax nycticorax*], and egrets [*Bulbucus ibis*, *Egretta garzetta*, *Egretta intermedia*]) and wild ducks [*Anas platyrhynchos*] are reservoirs for the maintenance and dissemination of the virus (Buescher et al. 1959; Soman et al. 1977; Rodrigues et al. 1981). Pigs represent an important amplification host, and pig farming has been identified as a risk factor for JEV infection in humans (Cao et al. 2010; Liu et al. 2010). However, the role of pigs in JE epidemiology needs to be reassessed in order to identify and implement efficient control strategies for both human and animal health (Ladreyt et al. 2019).

JEV transmission occurs primarily in rural areas but there are reports of occasional human cases in urban areas (Gingrich et al. 1987). For example, in India, JEV occurs in peri-urban and rural settings in nearly half of its 29 states (Pattan et al. 2009). In Bangkok, Thailand (Thisyakorn and Nimmannitya, 1985; Gingrich et al. 1987), Vientiane, Lao PDR (Vallee et al. 2009), and Can Tho, Vietnam (Lindhal et al. 2012), JEV or its vectors were identified in sub-urban areas that are comparable to the outskirts of Phnom Penh, Cambodia.

To date, 34 mosquito species have been studied for their involvement in Japanese encephalitis transmission: *Armigeres* species (n=1), *Ochlerotatus* (n=2), *Mansonia* (n=2), *Aedes* (n=4), *Anopheles* (n=8) and mainly *Culex* (n=17). Of these 34 species, 14 species are considered potential JEV vectors and seven species as confirmed vectors (following the definitions of potential and confirmed vectors from Tantely et al. 2015). The seven confirmed species are *Culex tritaeniorhynchus, Cx. bitaeniorhynchus, Cx. fuscocephala, Cx. gelidus*, *Cx. quinquefasciatus*, *Cx. sitiens* group, and *Aedes vexans*. Notably, *Cx. tritaeniorhynchus* and *Cx. vishnui* are considered the main vectors of JEV (Le Flohic et al. 2013; Oliveira et al. 2018).

Regarding Cambodia, JEV was first detected in 1965 from *Cx. tritaeniorhynchus* (Chastel and Rageau, 1966) and again in 1967 from *Cx. tritaeniorhynchus* and *Cx. gelidus* (Chen et al. 1990). Several studies between 1996 and 2008, and in 2017, demonstrated serological evidence of JEV infection in humans (Srey et al. 2002; Chhour et al. 2002; Touch et al. 2009; Horwood et al. 2017). Furthermore, more than 60% of the 505 swine sera sampled from eight provinces in Cambodia were positive to JEV antibodies (Duong et al. 2011). Recently, evidence of infections in pigs, mosquitoes and humans demonstrated an intensive circulation of JEV in peri-urban areas of Cambodia (Cappelle et al. 2016; Duong et al. 2017). Importantly, Cappelle et al. (2016) showed that all female vector species have a low minimum infection rate (MIR) of 0.091 / 1,000. These previous studies have established the presence of JEV in Cambodia but there is limited data regarding the diversity and population dynamics of JEV vectors driving the transmission. The main objectives of this study were to (i) describe the diversity of potential JEV vectors in the vicinity of pig farms in a peri-urban and a rural area of Cambodia and to (ii) compare their relative abundance and describe their dynamics.

Materials and methods

Study sites

Between August 2015 and July 2016, mosquitoes were collected from two different geographical areas classified as peri-urban and rural (Figure 1).

The peri-urban area was approximately 11 km south of Phnom Penh in a village of Ta Krapeu Ha, Preaek Ruessei commune, Ta Khmau city (11.4739 °N, 104.9376 °E). This area is located between a densely populated urban area and a rural landscape dominated by cultivated areas (Figure 1). The peri-urban study site was located on an experimental pig farm where 15 pigs were kept in a garden with no other domestic animals (Di Francesco et al. 2018).

The rural area was approximately 45 km south of Phnom Penh and adjacent to the Bassac River (Figure 1), in a village of Kbal Chhroy, Porti Ban commune, Koh Thom district (11.219846 °N, 105.039502 °E). Most inhabitants of the rural area are farmers and land usage is predominantly agricultural and livestock, rearing mainly pigs, cattle, chickens and ducks (Figure 1). Within this area, the selected dwelling had a backyard farm with a constant presence of pigs, and 2 cows in the farm.

The number of pigs at each pig farm varied over the study period. In the peri-urban area, 15 pigs were present at the experimental pig farm between August and October 2015. No pigs were present after the monitoring study of JEV circulation in pigs ended in October 2015. In the rural area, more than 100 pigs were present between August 2015 and January 2016. From February to July 2016, the number of pigs was reduced to 15 after the owner relocated pigs to another pigsty.

Five different locations were defined in both peri-urban (PU) and rural (R) areas for mosquito trapping: the pig farm (PU-P & R-P), a cattle house (PU-C & R-C) and the household to estimate the mosquito abundance in proximity to humans (PU-H & R-H). Two aquatic sites per study area were sampled: a canal and paddy field in the peri-urban area (PU-Ca and PU-PF respectively); and a riverbank and paddy field in the rural area (R-R and R-PF respectively (Figure 1).

Mosquito trapping and identification

Mosquitoes were captured over a 12-month period during the second or the third week of each month. Each month a CDC light-trap was in operation at each location between 5-7 pm until 6-7 am for three consecutive nights. In the pig farms, the suspended light-trap was outside and close to the pig garden. In the human and cattle houses, the light-trap was suspended outside from the ceiling under the stilt house. In the river/canals, ponds and paddy fields, the traps were attached to a tree branch.

The morphological identification of adult mosquitoes was performed according to the identification key to mosquitoes of Southeast Asia countries (Stojanovich and Scott 1966; Reuben et al. 1994; Rattanarithikul et al. 2005). To avoid bias, any mosquitoes captured in malfunctioning light-traps (i.e. light, fan or battery issues) were excluded from analysis.

Statistical analysis

Statistical analysis used RStudio software (Core Team (2017). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/). Differences between species abundance within the different areas and locations were assessed using ANOVA tests followed by a post-hoc analysis, Tukey-HSD for multiple comparisons, to indicate differences between groups. The ANOVA also tested the interactions between the location of the traps and the sampling area, and between the trapping month and the sampling area.

Results

Diversity and relative abundance of mosquitoes in peri-urban and rural areas

In total, 360 collections were performed (10 traps were used) across both areas (PU and R) during the 36 nights of capture. 340 (94.4%) traps operated correctly and 20 (5.6%) failed (12 times in rural and 8 in peri-urban). From correctly working traps, mosquitoes belonging to 10 genera and 20 species were collected from both areas, with 14 of these species known to be JEV vectors: *Aedes* (n=2), *Anopheles* (n=2), *Armigeres* (n=1), *Culex* (n=7) and *Mansonia* (n=2; Table 1).

Overall, 83,013 individual mosquitoes were captured from both areas: 59.4% (n=49,305) from rural areas and 40.6% (n=33,708) from peri-urban areas. Of the total collection, 17.1% (n=14,192) were not identified at the species level due to poor physical condition of the specimen: 86.8% (n=12,317) of these mosquitoes belonged to the *Culex* genus. In total, *Culex* mosquitoes represented 97.4% (n=32,843) and 96.2% (n=47,428) of the total mosquito collection in peri-urban and rural areas respectively. (Table 1).

Furthermore, 99.3% (n=80,271) of these JEV vectors belong to the *Culex* genus, with 83.0% (n=66,604) from three main species, namely *Cx. gelidus*, *Cx. tritaeniorhynchus,* and *Cx. vishnui* subgroup. In the rural sites, *Cx. vishnui* subgroup accounted for 51.7% (n=25,488) and were the most abundant, followed by *Cx. gelidus* (14.9%; n=7,322) and *Cx. tritaeniorhynchus* (10.8%; n=5,327) (Table 1). In the peri-urban sites, the more abundant species were *Cx. gelidus* (36.9%; n=12,421), *Cx. vishnui* subgroup (28.0%; n=9,453) and *Cx. tritaeniorhynchus* (19.6%; n=6,593) (Table 1).

<u>Main JEV vector species population dynamics</u>

The three main mosquito species, *Cx. vishnui* subgroup, *Cx. gelidus* and *Cx. tritaeniorhynchus*, were present throughout the year but with a variation in their abundance depending on the month for the two areas (p=0.009 for rural area; p=0.049 for peri-urban area). In the peri-urban area, the three species displayed a similar trend with an abundant peak in October 2015 (rainy season), especially *Cx. gelidus* and *Cx. vishnui* with a maximum relative density of approximately 300 mosquitoes per night. The population of those species rapidly declined from November 2015 to April 2016, and then *Cx. vishnui* gradually started to increase again with the beginning of the rainy season in May, and *Cx. gelidus* later in July 2016 (Figure 2). In the rural area, the three species were present throughout the year with two peaks of abundance in December 2015 and July 2016 (Figure 2). Particularly, *Cx. vishnui* had two peaks of abundance at the beginning and at the end of the rainy season, while *Cx. gelidus* peaked at the beginning of the rainy season and *Cx. tritaeniorhynchus* to a lesser extent at the end of the rainy season concomitantly *Cx. vishnui* (Figure 2).

Relative abundance of mosquitoes in different trap locations

More mosquitoes were trapped in pig farms and cattle houses compared to the household, river/canals, ponds and paddy fields ($p<10^{-4}$). In total, 95.9% of mosquitoes were caught in pig farms (71.0%; n=58,971) and in cattle houses (24.8%; n=20,606), while only 4.1% of trapped mosquitoes were from the other locations. In the peri-urban area, we captured a similar average number of mosquitoes per night in the pig farm (n=459) compared to the cattle house (n=463) (p=1.00). In the rural area, the average nightly capture rate was more than 8 times higher in the pig farm (n=1212) compared to the cattle house (n=146) ($p<10^{-4}$).

There was no significant effect of the location of the traps and the sampling area on the number of caught mosquitoes for the three main species (p=0.33) (Figure 3). However, in the rural area, there were more *Cx. vishnui* subgroup (640 vs. 68, p=0.0004) and *Cx. tritaeniorhynchus* (127 vs. 20, p=0.033) mosquitoes in the pig farm (n=640) than in the cattle house (n=68) (p=0.043; Figure 4). Even though the difference was not significant with *Cx. gelidus* (p=0.053), there were more mosquitoes caught per night in pig farm (n=191) than in the cattle house (n=15). The captured mosquito numbers were lower in the river/canal, household/pond and rice fields compared to the pig farms and cattle house, and there was an overall difference between the number of *Cx. vishnui* subgroup between rural and peri-urban areas (p=0.008; Figure 5). In the rural areas*,* *Cx. vishnui* was more abundant in the river and canal than near households and paddy fields.

Discussion

Japanese encephalitis is mainly considered a rural disease, but there is growing evidence of a peri-urban and urban transmission in several countries, including Cambodia (Di Francesco et al. 2018). Moreover, JE represents one of the most important cause of human viral encephalitis in South East Asia and particularly in Cambodia (Horwood et al. 2017). Our study focused on the dynamics of JEV mosquito vectors and was implemented to monitor mosquito populations in peri-urban and rural areas in Cambodia. JE studies in Cambodia mainly focused on JEV isolation from humans, pigs or mosquitoes, but few studies have focused on JEV vectors. A previous study showed predominance of *Cx. quinquefasciatus* in Phnom Penh during the rainy season (Kohn, 1990). Beyond the difference of habitats (urban area vs peri-urban and rural areas in our study), this discrepancy could also be explained by the different method of trappings and/or the locations of the study. Kohn (1990) used an aspirator and netting in households that are likely to capture highly anthropophilic mosquitoes (Gowda and Vijayan, 1992), while our study used light traps near pigs and cattle, and likely captured mosquitoes that are more zoophilic. A more recent study demonstrated similar findings in terms of species and vector diversity to our peri-urban area in Ta Khmau city (Cappelle et al. 2016). They also found *Cx. tritaeniorhynchus* (69%), *Cx. gelidus* (17%) and Cx. vishnui (12%) as the predominant species with a higher density between April and July for the two first ones. The same abundance peaks are observed at the beginning of the rainy season (June and July), and the beginning of the dry season (December).

<u>Diversity and relative abundance of JEV vectors in peri-urban and rural areas</u>

This entomological survey provides direct evidence for the abundance of JEV vectors in close proximity to humans and in areas with known JEV circulation (Cappelle et al. 2016). Among all mosquito species caught in either peri-urban or rural areas, *Cx. gelidus* and *Cx. vishnui* subgroup were the predominant species in peri-urban areas and in rural areas, respectively. In India, *Cx*. *gelidus* and *Cx*. *tritaeniorhynchus* were also the predominant species in peri-urban and rural areas, respectively (Arunachalam et al. 2009; Murty et al. 2010). In both countries, the presence and importance of these two species could explain the circulation of JEV.

In India, JE cases occurred with a seasonality shortly after the mosquito density reached its peak (Mishra et al. 1984; Kanojia et al. 2003). A study in Thailand showed that *Cx*. *tritaeniorhynchus* and *Cx*. *gelidus* populations increased during the rainy season (Gingrich et al. 1992) with a peak in July when the farmers plough their rice fields (Somboon et al. 1989; Changbunjong et al. 2013). Even if the JEV transmission is described as seasonal in Thailand and Vietnam, the transmission was presumed to occur from May to October in Cambodia and Laos (Pattan et al. 2009). However, the serological monitoring of pigs during the rainy and dry seasons (Cappelle et al. 2016), and the high abundance of JEV vectors during the rainy and dry season in the present study, strongly suggest that JEV transmission occurs all year round. The abundance of *Culex* species can be explained by several factors such as the irrigation of rice paddies two or three times per year, and the importance of several different mosquito-breeding sites such as streams, rivers, seepages, marshy areas, water containers, ponds and other water reservoirs during the dry season. Specifically, *Cx. gelidus*, *Cx. tritaeniorhynchus* and *Cx. vishnui* subgroup breeding sites in Asia are rice fields and general ground pools such as stagnant water with plants, ditches, ponds (Sirivanakarn, 1976; Abu Hassan et al. 2010; Kumar et al. 2012) that are present in our areas. Further studies to define the specific breeding sites for each species and to determine the impact of climate variability on the dynamics of the species should be conducted.

JEV vector abundance and hosts

The vast majority of mosquitoes (95.8%) were captured near hosts, particularly pigs. It has to be noted, notably in the rural area, that the number of hosts, especially the number of pigs, certainly play a role as a better attractant than the 2 cows present in the cattle house. The three main species *Cx. vishnui* subgroup, *Cx. tritaeniorhynchus* and *Cx. gelidus* are zoophilic species mainly attracted to animals for blood feeding (Lord et al. 2016; Tuno et al. 2016). As our results show, particularly in peri-urban areas, the mosquito number dramatically decreased after the pigs were removed from the pigsty; pigs likely attract JEV vector species. In Vietnam, the abundance of *Cx. tritaeniorhynchus* in an urban area was related to the presence of pigs (Nitatpattana et al. 2005). Studies in Indonesia and China also found that JEV cases in human are associated to the proximity of the residence to rice fields and pig ownership, respectively (Liu et al. 2010, Ren et al. 2017). Considering the role of pigs in JEV amplification and transmission to humans, the attraction of JEV vectors to pigs is of importance, especially pigs that are kept in close proximity to human residences.

Conclusion: importance for Public Health

In conclusion, this study has shown that JEV vectors are present throughout the year in both peri-urban and rural areas, with a high abundance in both rainy and dry seasons in Cambodia. More mosquitoes were collected near pigs and cattle. Based on these findings, we can conclude that the JEV transmission can occur throughout the year in peri-urban and rural areas where pigs are present. It is recommended for people to avoid the presence of pigs near the house in order to limit the abundance of JEV vectors. In addition, it would be effective to protect these animals with insecticide-treated nets even when breeding sites are unknown (Dutta et al. 2011).

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Figure Legends

Table 1. Number and percentage of each species captured in peri-urban and rural areas. Number of mosquito species captured between August 2015 and July 2016 in peri-urban and rural areas. Highlighted species with * are the potential know JEV vectors.

Figure 1. Geographic locations of traps in the peri-urban and rural areas. Peri-urban pig farm (PU-P), Peri-urban household (PU-H), Peri-urban canal (PU-Ca), Peri-urban cattle house (PU-C), Peri-urban paddy fields (PU-PF). Rural pig farm (R-P), Rural cattle house (R-C), Rural river (R-R), Rural ponds (R-Po), Rural paddy fields (R-PF).

Figure 2. Population dynamic of the three main JEV vectors in rural and peri-urban areas.

Figure 3. Average number of the three main species collected per night in the pig farm and cattle house and compared between peri-urban and rural areas. Average comparison of the number of mosquitoes / night was tested with a Tukey HSD test (NS meaning non-significant with $p > 0.05$; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

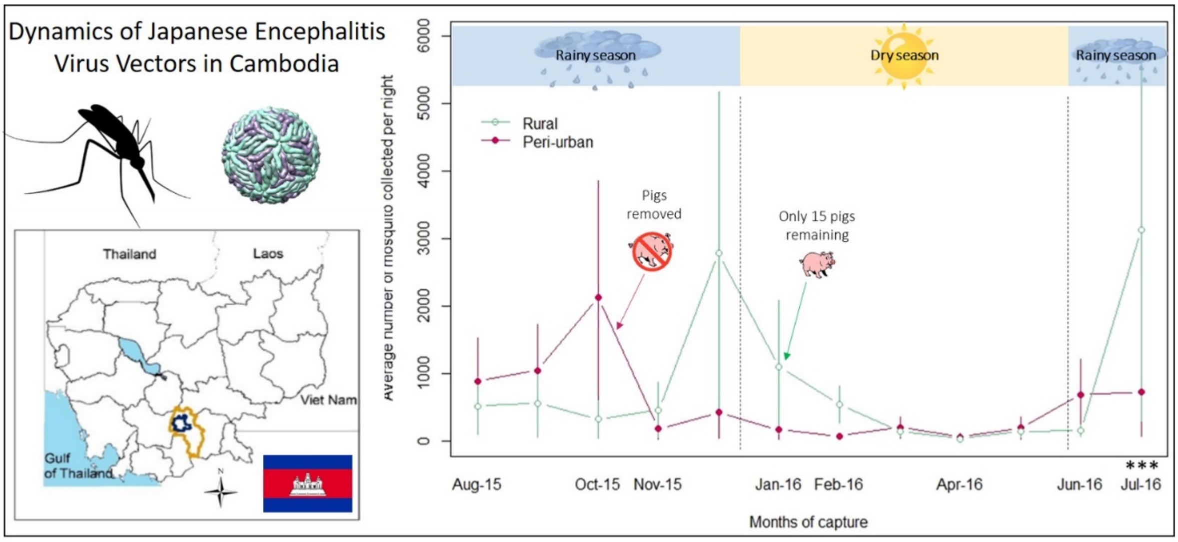
Figure 4. Average number of the three main species collected per night in peri-urban and rural areas and compared between the pig farm and cattle house. Average comparison of the number of mosquitoes / night was tested with a Tukey HSD test (NS meaning non-significant with $p > 0.05$; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

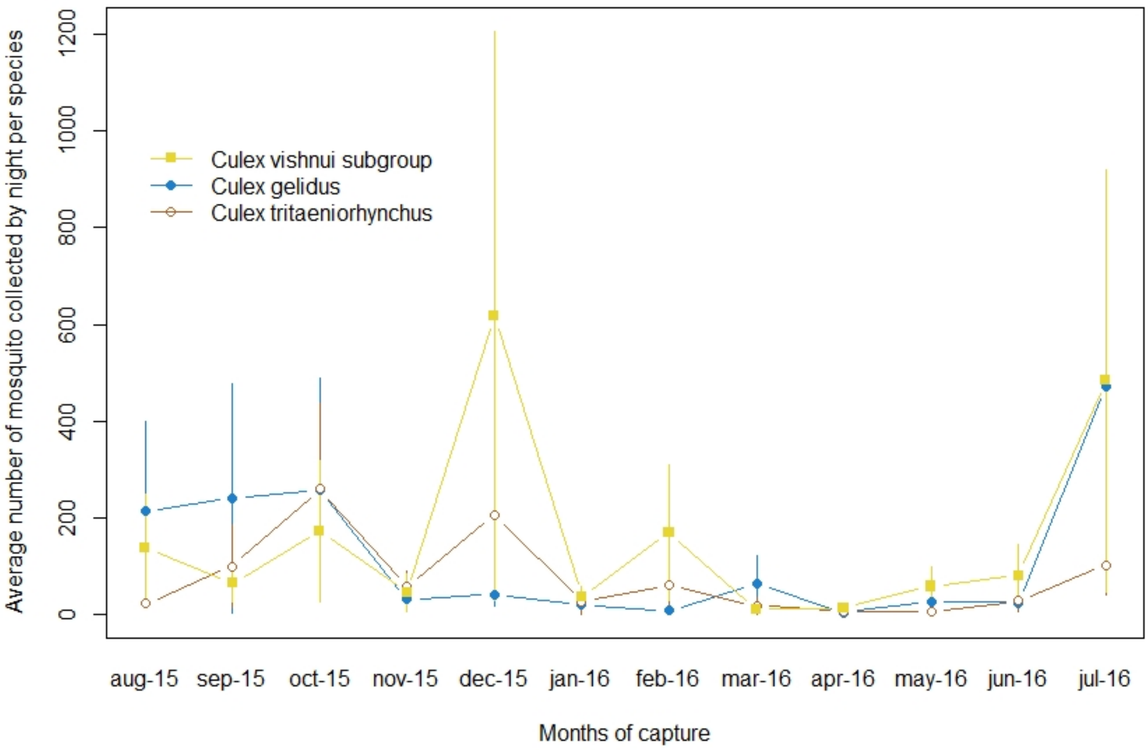
Figure 5. Average number of the three main species collected per night in paddy fields, river/canals and household/ponds trap locations in peri-urban and rural areas. Average comparison of the number of mosquitoes / night was tested with a Tukey HSD test (NS meaning non-significant with $p > 0.05$; for *Cx vishnui*, a and b means there is a statistical difference with $p <0.05$).

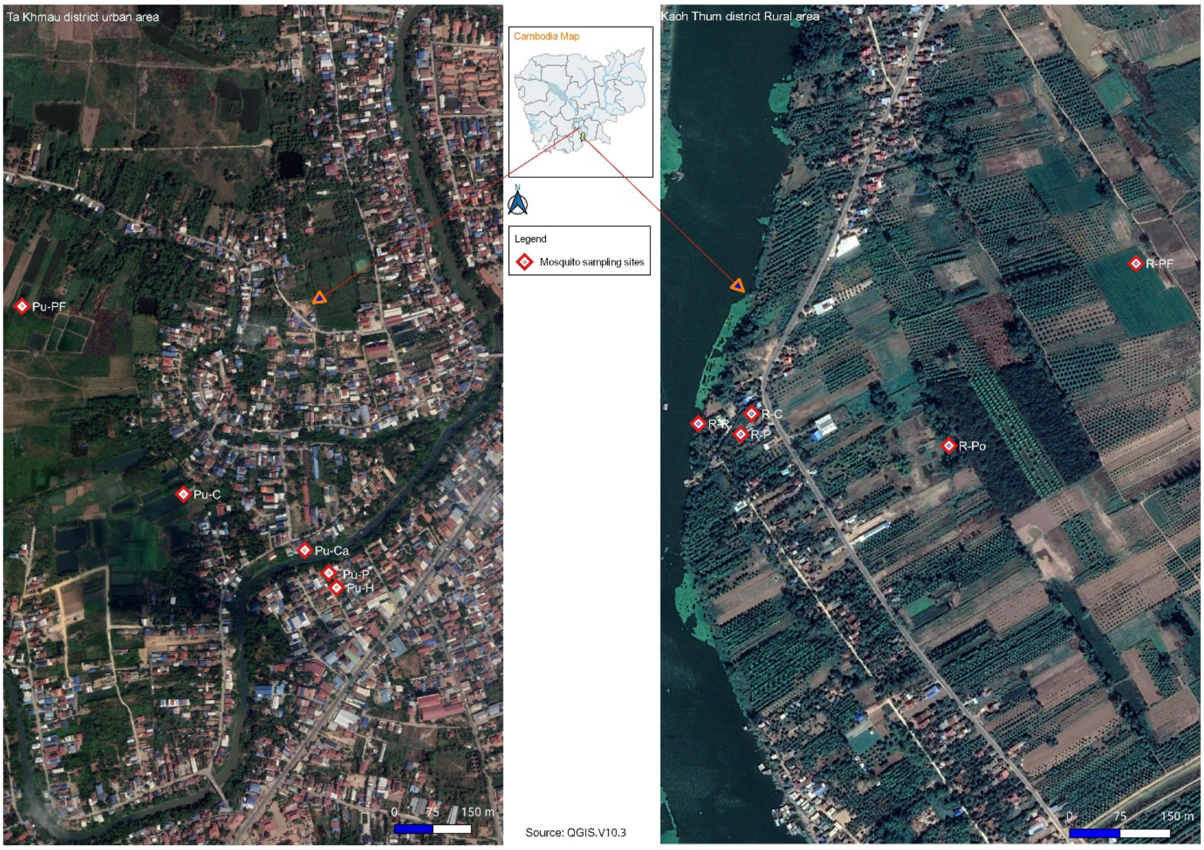
Highlights.

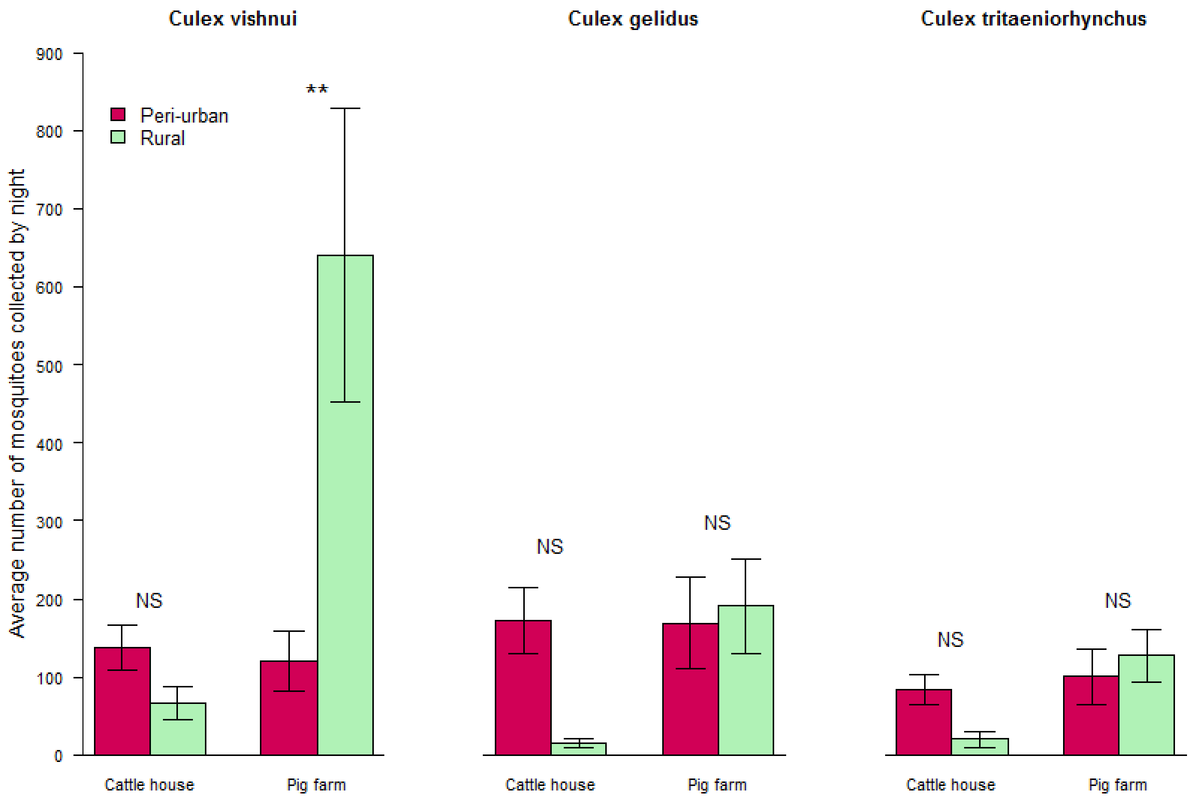
- Vectors of Japanese Encephalitis Virus are abundant all the year in Cambodia

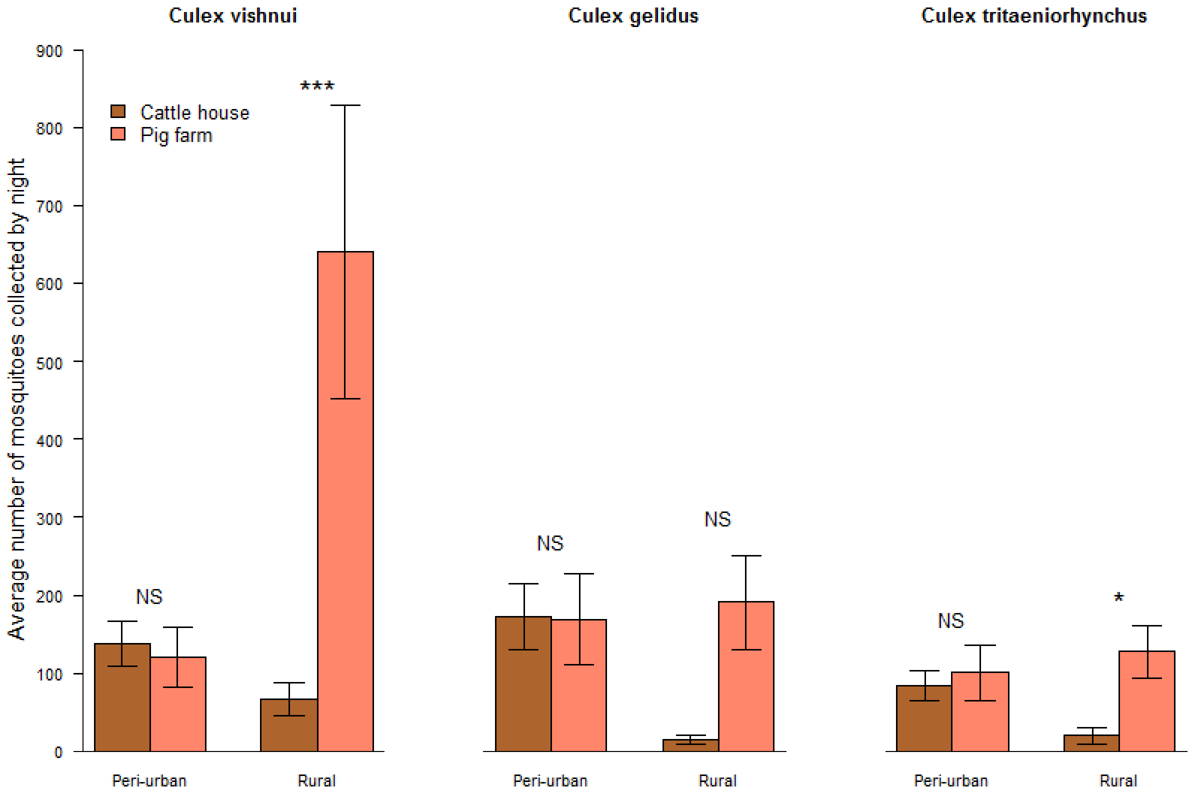
- One-year study and seasonality of JEV vectors
- Effect of host presence on JEV vector abundance in pig farms and cattle house
- The most abundant vectors are *Culex vishnui*, *Cx. gelidus* and *Cx. tritaeniorhynchus*



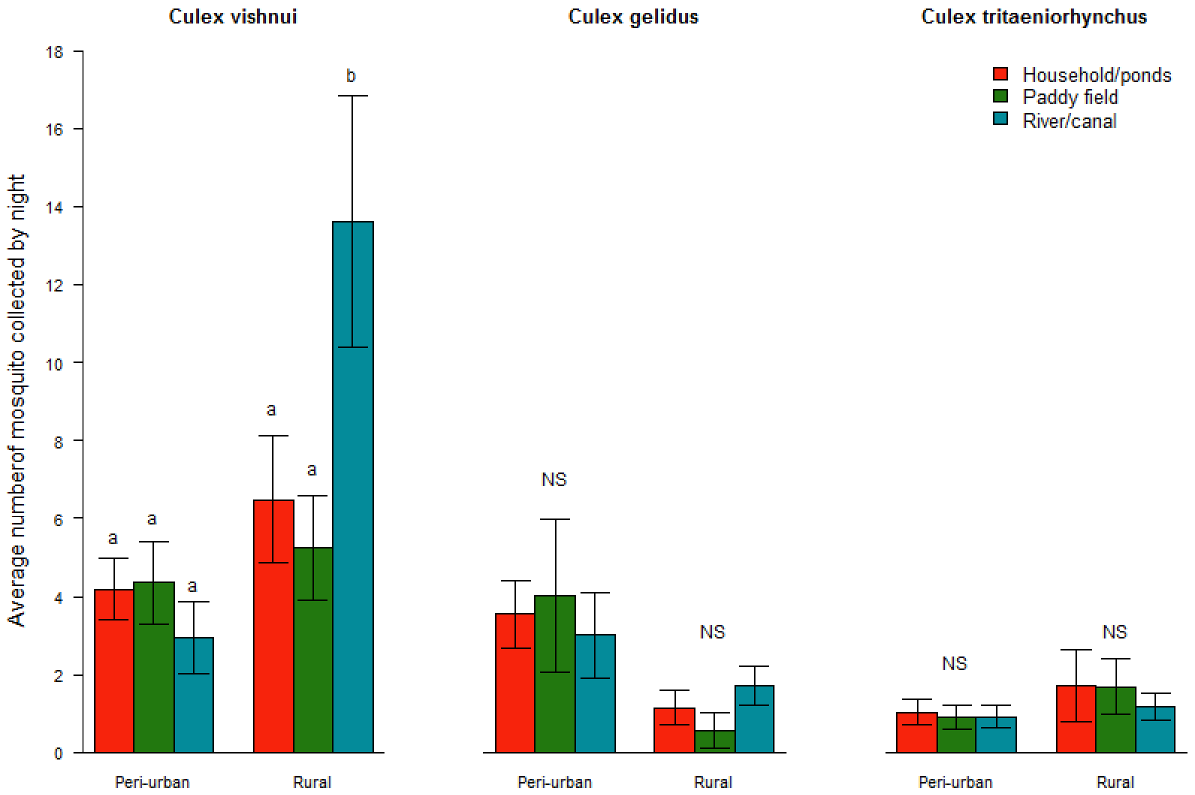



Ta Khmau district urban area
Pu-PF
Pu-C
Pu-Ca
Pu-P
Pu-H
0 75 150 m
Cambodia Map
Legend
Mosquito sampling sites
Source: QGIS.V10.3
Kaoh Thum district Rural area
R-PF
R-C
R-P
R-Po
0 75 150 m


Culex vishnui
Culex gelidus
Culex tritaeniorhynchus
Average number of mosquitoes collected by night
0
100
200
300
400
500
600
700
800
900
Peri-urban
Rural
**
NS
Cattle house
Pig farm



Culex vishnui
Culex gelidus
Culex tritaeniorhynchus
Average number of mosquitoes collected by night
Cattle house
Pig farm
NS
***
NS
NS
NS
*
Peri-urban
Rural
Peri-urban
Rural
Peri-urban
Rural



Culex vishnui
Culex gelidus
Culex tritaeniorhynchus
Average numberof mosquito collected by night
Household/ponds
Paddy field
River/canal
a
a
a
a
a
b
NS
NS
NS
NS
Peri-urban
Rural
Peri-urban
Rural
Peri-urban
Rural