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Journal Articles

PRELIMINARY SURVEILLANCE OF JAPANESE ENCEPHALITIS VIRUS IN MOSQUITO CULEX SPECIES IN THE WEST PENINSULAR MALAYSIA

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SUMMARY

Japanese encephalitis virus (JEV), a mosquitoes-borne virus primarily spread by Culex mosquitoes, and the infection is capable of causing a significant public health concern particularly in Malaysia. By collecting mosquitoes from two distinct sites namely migratory bird areas and pig farms, this study aimed to employ molecular screening of JEV by using reverse transcription-polymerase chain reaction (RT-PCR). These localities were selected based on their association with JEV reservoirs and dynamics of transmission. Results indicated no evidence of JEV was detected in any of the collected mosquitoes. Absence of JEV infection in the area suggests low prevalence and necessity to prolong the study timeframe and extend the locality. This study emphasises the need for constant surveillance on the aspect of epidemiology and ecological factors influencing the dynamics of JEV transmission in Malaysia.

Keywords: Japanese encephalitis virus, Malaysia, migratory birds, mosquitoes, pig farms

INTRODUCTION

Japanese encephalitis (JE) is the clinical manifestation of a brain inflammation caused by the Japanese encephalitis virus (JEV). The virus belongs to the family Flaviviridae and the genus Flavivirus (Calisher et al., 2003; Thiel et al., 2005). It is enveloped, positive sense single-stranded RNA virus that measures around 50 nm in diameter and has spheroid cubical symmetry shape. This virus causes irreparable neurological damage, presenting a significant burden on public health and society. Although many flaviviruses may also lead to encephalitis, JEV has been linked to very severe neurological illness (Sharma et al., 2021). Approximately 1.15 billion people are at risk, and thousands suffer from permanent neurological damage, with 10,000 to 15,000 people dying each year due to JE (Yadav et al., 2022). To this day, there is no cure for the disease, meaning that no licenced anti-JEV medicine is available. Treatment only focuses on alleviating severe clinical indications and assisting the patient to overcome the infection. However, JE infection can be prevented with the usage of safe and effective vaccines (Srivastava et al., 2023).

Nowadays, JEV infection spreads across a large portion of Eastern and Southern Asia in the north, to South-East Asia and northern Australasia and, has a potential to spread to new geographical locations (Mackenzie et al., 2022). Globally, 75% of cases occur in children and adolescents, with an annual incidence of 5.4 per 100,000 population, making JE the major cause of viral paediatric encephalitis in Asia (Campbell et al., 2011). Previous records indicated that both adults and children were victims of JE in Malaysia. However, recent data shows that JE infection is only lethal to children in Malaysia. JE is considered endemic only in Sarawak (Kumar et al., 2018a). A seroprevalence study on livestock and companion animals in Malaysia revealed that JEV are circulating among the animals where pigs, dogs, and cattle are most susceptible to JEV infections. Evidently, JEV is highly prevalent across peninsular Malaysia with sporadic encephalitis cases being reported each year.

JE is a significant public health concern in Malaysia, where the virus is endemic. Studying JEV in Malaysian mosquitoes is vital for disease control, public health protection, epidemiological understanding, environmental management, vaccine development. Since the virus is amplified in the wild birds and pigs, the data on the status of JEV in the mosquitoes circulating among the reservoir host is limited. By considering this fact, this study aimed to screen JEV in mosquitoes in Peninsular Malaysia in high-risk area approximate to pig farming and migratory birds stopover.

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MATERIALS AND METHODS

Sampling of field mosquitoes

A simple random sampling was carried out to screen the presence of JEV RNA in field-collected mosquitoes in selected high-risk areas of peninsular Malaysia, which include 3 localities in the states of Penang, Perak and Selangor from July until September 2018 (Figure 1). Institutional Animal Care and Use Committee (IACUC) approval was obtained from Universiti Putra Malaysia with the reference code UPM/IACUC/AUP NO: R043/17. Approval for the collection of mosquitoes from wild was received from the Department of Wildlife and National Parks (DWNP), Peninsular Malaysia under the research permit code JPHL & TN(IP):100-6/1/14. Pig farms and migratory bird stopover were selected and considered high risk since those animals are classified as JEV amplifying host and the fact that JEV is endemic in Malaysia (Kumar et al., 2018a). The mosquitoes were collected in Penang (pig farm) and in Perak and Selangor (wild birds). The bird is ardeid bird's species which renowned to serve as the common reservoir species JEV, as a source of blood meal and the location of having a vast freshwater body such as a small stream, pond and shallow stagnant water making it as an ideal breeding spot for mosquitoes.

Figure 1. Areas of field mosquitoes sampling for JEV studies in selected locations in the states of Penang, Perak and Selangor. Adapted from Google maps.

a. Pig farm; Sungai Bakap, Penang (5.2074 N, 100.491°E).

b. Seabird conservation centre; Kuala Gula, Perak (4.9375 N, 100.468°E).

c. Migratory bird rest stop areas; Kapar, Selangor (3.1373 N, 100.374°E).

Mosquitoes trapping

Mosquitoes from the field were collected using the battery-operated CDC miniature light traps (John W. Hock Company, USA). The CO2 gas was supplied as a bait to lure mosquitoes into the trap, by placing 1 kg dry ice slab (Linde, Malaysia) in an insulated container that was suspended beside the trap. The fan that pushes air downward into the collecting container (Becker et al., 2010) remains running to prevent live mosquitoes from escaping through the top of the trap until the battery is disconnected (Figure 2). The traps were installed at several strategic locations at the sampling site by late evening starting from 6.00 pm and retrieved back the next day at 8.00 a.m. in the morning.

Figure 2. A CDC miniature light trap used in the study to trap field mosquitoes. The blue insulated container was filled with dry ice during the operation of the trap (arrow A), and small holes (arrow B) at the top allowed the released of CO2 gas to be dispersed by the fan of the trap.

Identification of mosquito species

The mosquitoes collected from sampling sites were maintained alive and freeze immediately at -80 °C (SANYO, Japan) upon arrival at Virology Laboratory, Faculty of Veterinary Medicine, UPM in order to preserve any possible viral agent inside the mosquito's bodies. The species identification was performed by observing the morphological features of the mosquito (Reuben et al., 1994) under a stereomicroscope (Carl Zeiss Stemi DV4, Germany) with low surrounding temperature setup (23-25 °C). A group of 25 female mosquitoes were pooled together (Gu et al., 2008) in 1.5 mL tube (Eppendorf, Germany) according to their species and stored again at -80 °C until further use.

Extraction of total RNA from mosquito

The total RNA from mosquito samples was extracted using TRIsure reagent (Meridian Bioscience, USA) according to manufacturer instructions. Briefly, 500 µL of PBS was added into the pooled mosquito samples, grinded with tissue homogenizer (OMNI, USA) and centrifuged at 3,000 x g for 5 minutes at 4 °C (Heraeus Primo R Refrigerated Centrifuge, Thermo Fisher, USA). The supernatant was collected and transferred into a new tube. Next, 100 µL of TRIsure reagent was added into the supernatant and incubated for 5 minutes at room temperature. 200 µL Chloroform (Thermo Fisher, USA) was added to the suspension, vortexed and centrifuged at 12,000 x g for 15 minutes at 4 °C. The colourless upper phase layer containing RNA was collected and transferred into a new tube and precipitated by adding 500 µL isopropanol (Thermo Fisher, USA). The mixture was incubated for 10 minutes at room temperature and centrifuged at 12,000 x g for 10 minutes at 4 °C. The RNA

pellet was washed with 75 % ethanol (Thermo Fisher, USA) and centrifuged at 7,500 x g for 5 minutes at 4 °C. The step was repeated once again and the RNA pellet was air-dried for 5 minutes inside the biosafety cabinet (ESCO AC2-4E1, Singapore). The RNA pellet was resuspended in 30 µL DEPC water (Thermo Fisher, USA) and incubated in a 60 °C water bath (HOTECH 830-S1, Taiwan) for 10 minutes. Finally, the RNA samples were stored at -80 °C until further use.

Molecular detection of JEV in mosquitoes by One Step RT-PCR

Reference JEV Nakayama Strain

The whole genome of JEV Nakayama strain genotype III MY 2009 P578662 (GenBank accession: HE861351) in the form of RNA samples was used as a positive control in one step RT-PCR in an attempt to screen for JEV RNA in mosquitoes' samples. A total of 2 µL of viral RNA added into the PCR master mix to serve as positive control in every PCR reaction.

Oligonucleotide primers to amplify JEV NS3 gene

The forward (NS3F) and reverse (NS3R) primers were used to screen the JEV antigen in this study (Kumar et al., 2018b). The NS3F/NS3R primer pairs were designed to flank at specific sites in NS3 regions of the JEV genome to produce 602 bp PCR amplicons (Table 1).

Table 1: List of oligomer DNA primers used for PCR amplification of JEV NS3 gene.

Primer	Primer sequence	Product size (bp)	Nucleotide location (5'-3')
NS3F	5' AAG ACG CGG GAA GCT TTA GAC 3'	602	6519-6540
NS3R	5' TGA GCC AGC TTG TGA GTT AAT TGA G 3'		7145-7122

One Step RT-PCR assay

The one step reverse transcription-polymerase chain reaction (RT-PCR) was performed to detect and amplify JEV NS3 genes in the total RNA extracted from the mosquito homogenate samples, by using NS3F/NS3R primer pair (Kumar et al., 2018b). The AccessQuick RT-PCR System (Promega, USA) master mix was prepared to a final volume of 25 µL per reaction which comprised of 5 µL of 5X AMV/Tfl reaction buffer, 1 µL of 25 mM of Magnesium sulphate (MgSO4), 0.5 µL of 10 mM dNTP mixture, 0.5 µL of Tfl DNA polymerase, 0.5 µL of ribonuclease inhibitor, 0.5 µL of reverse transcriptase, 0.5 µL of 10 µM forward and reverse primers (NS3F/ NS3R), 14 µL of nuclease free water and finally 2 µL of RNA template. The PCR mixture was mixed well by pulse vortexing (FINE VORTEX, Korea) and spun down by centrifugation (VWR Mini Centrifuge, USA).

The detection and amplification reaction of the JEV NS3 gene was carried out in a programmed thermocycler (Eppendorf, Germany). The PCR cycling parameters for amplification of JEV NS3 gene were set as follows: 1 cycle of initial heating temperature to activate reverse transcriptase at 45°C for 45 minutes, 1 cycle of initial denaturation at 94°C for 2 minutes, followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing of NS3F/NS3R primers at 54°C for 30 seconds and extension at 72°C for 30 seconds. The 1 cycle of the final extension step was set at 72°C for 10 minutes. The amplified PCR products were examined by gel electrophoresis using 1.5 % (w/v) agarose gel (Vivantis, Malaysia). As the electrophoresis process completed, the DNA band was visualised and imaged under UV transilluminator (Gel Doc 2000 Gel, Bio-Rad, USA).

RESULTS

Sampling and identification of mosquitoes' species

A total of 1,700 female mosquitoes were captured and subjected to JEV RNA screening using one-step RT-PCR assay. Only the Culex female mosquitoes were selected based on their morphological features which include the thorax, wing, proboscis and abdomen. In total, there were 68 pooled mosquito samples which comprises of 25 mosquitoes per pool. There were 18 pool samples from pig farms (Sungai Bakap, Penang), 30 pool samples from seabird sanctuary park (Kuala Gula, Perak), and 20 pool samples from migratory bird stopover area (Kapar, Selangor). Upon examination, there were a total of 6 species identified namely (A) Cx. tritaeniorhynchus, (B) Cx. gelidus, (C) Cx. vishnui, (D) Cx. quinquefasciatus, (E) Cx. sitiens, and (F) Cx. whitei. Of the six species identified, Cx. tritaeniorhynchus was the most common species sampled at Perak and Selangor sampling sites, whereas Cx. gelidus was the most common species sampled in the pig farm (Figure 3).

Molecular detection of JEV in mosquitoes by one-step RT-PCR

One-step RT-PCR assay using NS3F/NS3R primer pairs targeting the NS3 region of the pooled mosquito samples revealed no amplification of JEV RNA was detected in all of the 68 pooled mosquito samples (Figure 4).

DISCUSSIONS

This study approach involved an attempt to detect and isolate JEV RNA from mosquito's pools in high risk area of mosquitoes breeding sites. Field sampling of mosquitoes was performed in a pig farm and migratory bird rest stop areas by considering the fact that pig and ardeid water bird species develop high viremia following JEV infection, hence demonstrates their role as a natural reservoir and amplifying host for JEV (Gresser et al., 1958). Currently, research shows that pig's involvement in JEV transmission is less due to wide application of JEV vaccines at pig farms worldwide. As a consequence, due to

A) *Cx. tritaeniorhynchus* **B) *Cx. gelidus*** **C) *Cx. vishnui***

Figure 3. Identification of Culex mosquitoes' species. The species was classified by observing their morphological features including thorax, wing, proboscis and abdomen (Reuben et al., 1994). Stereomicroscope. (Mag. x 20).

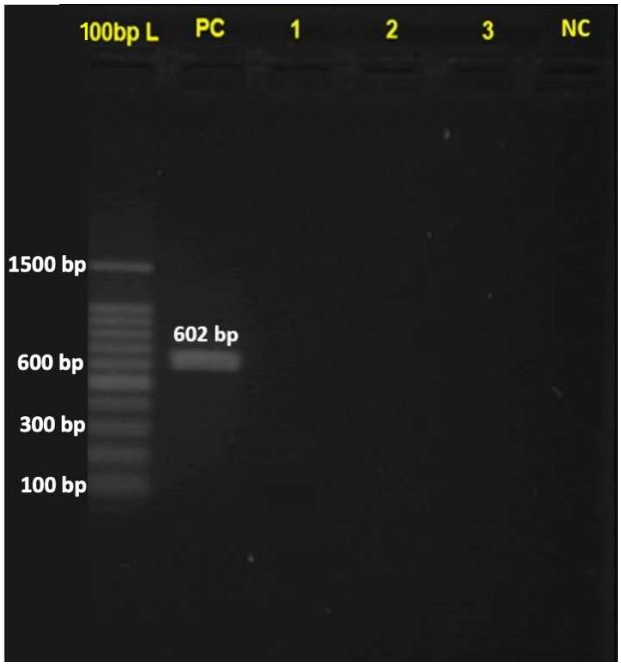

Figure 4. Representative of One-step RT-PCR assay to detect JEV antigen in 68 pooled mosquito homogenates samples by using NS3F/NS3R primer pair targeting the NS3 region of JEV producing 602 bp. Electrophoresis was carried out on 1.5% (w/v) agarose gel. Lanes 1-3: mosquito homogenates samples show no amplification. Lane PC: Positive control with 602 bp. Lane NC: No template control. Lane M: 100 bp DNA marker (Promega, USA).

lack of JEV vaccination in wild birds, this species might play an important role in JEV transmission compared to pigs (Hameed et al., 2021).

In this study, the sampling of field mosquitoes was carried out for three months consecutively starting from July until September 2018 which coincided with the peak season of migratory birds' occurrence in Malaysia. Most migratory birds fly across Malaysia to escape the cold winter or to reach breeding grounds at the end of July. Malaysia is located on the path of the East Asian-Australasian Flyway that provides strategic and important resting sites for migratory birds, that fly between wintering sites in Australia and Southeast Asia and the breeding sites in Japan, China, Korea, Mongolia and Siberia (Bakewell et al., 2017). Perhaps during the sampling period none of the migratory bird species developed high viremia to enable the transmission of JEV in mosquito vectors.

Upon gross species identification, there are a total of three species of mosquito namely Culex, Aedes, and Anopheles have been identified. Of all the species, Culex is the most common species sampled at Penang, Perak and Selangor sampling sites compared to other species of mosquitoes. The use of CDC-Light Trap baited with CO2 in this study may have contributed to the attraction of more Culex species compared to others, especially when the trap is placed in outdoors location (Sriwichai et al., 2015). Since JEV is primarily vectored by Culex mosquito, therefore the method to collect mosquitoes used in this study is the most appropriate. However, the CDC-CO2 trap was reported as a less efficient method for capturing blood-fed females' mosquitoes. The BG-Sentinel trap with attractants and CO2 is significantly better at capturing mosquitoes that had fed on mammals than the CDC-CO2 traps (Roiz et al., 2012).

The RT-PCR is extensively used for screening of flavivirus in susceptible animals including mosquitoes. The assay is highly sensitive, rapid and capable to detect broad-range flavivirus-specific, making it a valuable tool for epidemiological studies and rapid detection of flaviviruses in livestock for clinical diagnosis (Patel et al., 2013). No JEV RNA was detected from the field collected mosquitoes in this study after several attempts using one-step RT-PCR assay. The unsuccessful detection of JEV antigen could be caused by several factors. There could be insufficient amount of mosquito samples as limited sampling area were selected and only one trap was place for each sampling site. The duration of the study should be extended of which more mosquitoes can be collected and screened for the presence of JEV RNA (Yap et al., 2020; Lim et al., 2022). For instance, a study conducted in Singapore managed to collect 10 to 50 mosquitoes in a pool as the light trap was placed for 16 hours over two nights at four sites with five traps each (Yap et al., 2020).

Apart from that, the cross-neutralisation among flaviviruses could be the factors for inability to detect JEV RNA from the field collected mosquitoes. Following JEV infection in pigs and birds, the virus could be cross neutralised by existing flavivirus antibodies (Li et al., 2016). Since the outbreaks of JEV cases in human in peninsular Malaysia is sporadic, the mosquitoes collected in the field at that time might not carrying JEV nor the amplifying host. When this study was conducted in 2018, there was absence of JEV outbreak. However, it was only in year 2020 that the outbreak happened. Two cases of JEV infection in humans involving eight years old and two years old children were confirmed in the area of mosquitoes sampling sites at Penang, indicates that JEV cases are seasonal in that exact location where sampling was performed for this study. For future detection of JEV in mosquitoes, the sampling of mosquitoes should cover more regions of Malaysia including Sabah and Sarawak. The latter state is the only state in Malaysia where JEV cases are still endemic (Kumar et al., 2018a).

In addition, the vector-free transmission and persistence of JEV in pigs are reported to be possible. It has been discovered that JEV can be transmitted between pigs in the absence of arthropod vectors. Pigs shed virus in oronasal secretions and transmitted to other susceptible pigs via oronasal infection (Lyons et al., 2018). In pigs, tonsils being the important site of JEV replication in which the virus is found to persist for at least 25 days despite the presence of high levels of neutralizing antibodies (Ricklin et al., 2016). For future study in an attempt to isolate JEV from pig, a schedule of oronasal swabs collection should be performed and tested (Redant et al., 2020). Once the samples are positive, the animal should be sacrificed by harvesting their tonsils and subjected to virus isolation and propagation in susceptible cell lines.

CONCLUSIONS

In this study, absence of JEV was observed from collected mosquitoes in the area approximate to amplifying host such as pigs and wild birds. The finding may reflect that low risk of JEV transmission in the area at that particular period, possibly due to timing and limited area of sampling.

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CONFLICT OF INTEREST

The authors declare there is no conflict of interest upon completion of this study.

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REFERENCES

Bakewell, D., Wong, A., Kong, D. & Au, R. (2017). Waterbird Surveys of the Sarawak Coast (2010-2012). [A Report by the Malaysian Nature Society-Bird Conservation Council (MNS-BCC) Waterbirds Group in partnership with the Sarawak Forestry Corporation]. Kuala Lumpur: Malaysian Nature Society. (MNS Conservation Publication No. 13).

Becker, N., Petric, D., Zgomba, M., Boase, C., Madon, M., Dahl, C. & Kaiser, A. (2010). Mosquito research techniques. Mosquitoes and their Control, 43-61.

Calisher, C. H., & Gould, E. A. (2003). Taxonomy of the virus family Flaviviridae. Advances in virus research, 59, 1–19.

Campbell, G. L., Hills, S. L., Fischer, M., Jacobson, J. A., Hoke, C. H., Hombach, J. M., Marfin, A. A., Solomon, T., Tsai, T. F., Tsu, V. D., & Ginsburg, A. S. (2011). Estimated global incidence of Japanese encephalitis: a systematic review. Bulletin of the World Health Organization, 89(10), 766–774.

Gresser, I., Hardy, J. L., Hu, S. M., & Scherer, W. F. (1958). Factors influencing transmission of Japanese B encephalitis virus by a colonized strain of Culex tritaeniorhynchus Giles, from infected pigs and chicks to susceptible pigs and birds. The American Journal of Tropical Medicine and Hygiene, 7(4), 365-373.

Gu, W., Unnasch, T. R., Katholi, C. R., Lampman, R., & Novak, R. J. (2008). Fundamental issues in mosquito surveillance for arboviral transmission. Transactions of the Royal Society of Tropical Medicine and Hygiene, 102(8), 817–822.

Hameed, M., Wahaab, A., Nawaz, M., Khan, S., Nazir, J., Liu, K., Wei, J., & Ma, Z. (2021). Potential role of birds in Japanese encephalitis virus zoonotic transmission and genotype shift. Viruses, 13(3), 1–15.

Kim, H. C., Takhampunya, R., Tippayachai, B., Chong, S. T., Park, J. Y., Kim, M. S., Seo, H. J., Yeh, J. Y., Lee, W. J., Lee, D. K., & Klein, T. A. (2015). Japanese encephalitis virus in Culicine Mosquitoes (Diptera: Culicidae) of the Republic of Korea, 2008–2010. Military Medicine, 180(2), 158–167.

Kumar, K., Arshad, S. S., Selvarajah, G. T., Abu, J., Toung, O. P., Abba, Y., Yasmin, A. R., Bande, F., Sharma, R., & Ong, B. L. (2018a). Japanese encephalitis in Malaysia: An overview and timeline. Acta Tropica, 185(May), 219–229.

Kumar, K., Arshad, S. S., Selvarajah, G. T., Abu, J., Toung, O. P., Abba, Y., Bande, F., Yasmin, A. R., Sharma, R., Ong, B. L., Rasid, A. A., Hashim, N., Peli, A., Heshini, E. P., & Shah, A. K. M. K. (2018b). Prevalence and risk factors of Japanese encephalitis virus (JEV) in livestock and companion animal in high-risk areas in Malaysia. Tropical Animal Health and Production, 50(4), 741–752.

Li, J., Gao, N., Fan, D., Chen, H., Sheng, Z., Fu, S., Liang, G., & An, J. (2016). Cross-protection induced by Japanese encephalitis vaccines against different genotypes of Dengue viruses in mice. Scientific Reports, 6, 1–9.

Lim, M. J., Loh, Z. Y., Yeo, H. L., Yenamandra, S. P., Kong, M., Pang, H. Y., Lee, M. H., Humaidi, M., Chua, C., Griffiths, J., Ng, L. C., Hapuarachchi, H. C., & Mailepessov, D. (2022). Isolation and Genetic Characterization of Japanese Encephalitis Virus Two Decades after Its Elimination in Singapore. Viruses, 14(12), 1–18.

Lyons, A. C., Huang, Y. J. S., Park, S. L., Ayers, V. B., Hettenbach, S. M., Higgs, S., McVey, D. S., Noronha, L., Hsu, W. W., & Vanlandingham, D. L. (2018). Shedding of Japanese Encephalitis Virus in Oral Fluid of Infected Swine. Vector-Borne and Zoonotic Diseases, 18(9), 469–474.

Mackenzie, J. S., Williams, D. T., van den Hurk, A. F., Smith, D. W., & Currie, B. J. (2022). Japanese Encephalitis Virus: The Emergence

of Genotype IV in Australia and Its Potential Endemicity. Viruses, 14(11), 1–26.

Patel, P., Landt, O., Kaiser, M., Faye, O., Koppe, T., Lass, U., Sall, A. A., & Niedrig, M. (2013). Development of one-step quantitative reverse transcription PCR for the rapid detection of flaviviruses. Virology Journal, 10(1), 1.

Redant, V., Favoreel, H. W., Dallmeier, K., Van Campe, W., & De Regge, N. (2020). Efficient control of Japanese encephalitis virus in the central nervous system of infected pigs occurs in the absence of a pronounced inflammatory immune response. Journal of Neuroinflammation, 17(1), 1–17.

Reuben, R., Tewari, S. C., Hiriyan, J., & Akiyama, J. (1994). Illustrated keys to species of Culex (Culex) associated with Japanese Encephalitis in Southeast Asia (Diptera: Culicidae). Mosquito Systematics, 26(2), 75–96.

Ricklin, M. E., García-Nicolás, O., Brechbühl, D., Python, S., Zumkehr, B., Nougairede, A., Charrel, R. N., Posthaus, H., Oevermann, A., & Summerfield, A. (2016). Vector-free transmission and persistence of Japanese encephalitis virus in pigs. Nature Communications, 7.

Roiz, D., Roussel, M., Munõz, J., Ruiz, S., Soriguer, R., & Figuerola, J. (2012). Efficacy of mosquito traps for collecting potential West Nile mosquito vectors in a natural mediterranean wetland. American Journal of Tropical Medicine and Hygiene, 86(4), 642–648.

Sharma, K. B., Vrati, S., & Kalia, M. (2021). Pathobiology of Japanese encephalitis virus infection. Molecular Aspects of Medicine, 81(July), 100994.

Srivastava, K. S., Jeswani, V., Pal, N., Bohra, B., Vishwakarma, V., Bapat, A. A., Patnaik, Y. P., Khanna, N., & Shukla, R. (2023). Japanese Encephalitis Virus: An Update on the Potential Antivirals and Vaccines. Vaccines, 11(4), 1–25.

Sriwichai, P., Karl, S., Samung, Y., Sumruayphol, S., Kiattibutr, K., Payakkapol, A., Mueller, I., Yan, G., Cui, L., & Sattabongkot, J. (2015). Evaluation of CDC light traps for mosquito surveillance in a malaria endemic area on the Thai-Myanmar border. Parasites and Vectors, 8(1), 1–10.

Thiel, H. J., Collett, M. S., Gould, E. A., Heinz, F. X., Houghton, M., & Meyers, G. (2005). Family Flaviviridae In: Fauquet CM, Mayo MA, Maniloff J, Desselberger U, Ball LA, editors. Virus taxonomy Eighth report of the International Committee on Taxonomy of Viruses. San Diego: Elsevier Academic Press; 2005. pp. 981–998.

Yadav, P., Chakraborty, P., Jha, N. K., Dewanjee, S., Jha, A. K., Panda, S. P., Mishra, P. C., Dey, A., & Jha, S. K. (2022). Molecular Mechanism and Role of Japanese Encephalitis Virus Infection in Central Nervous System-Mediated Diseases. Viruses, 14(12), 1–25.

Yap, G., Mailepessov, D., Lim, X. F., Chan, S., How, C. B., Humaidi, M., Yeo, G., Chong, C. S., Lam-Phua, S. G., Lee, R., Okumura, C., Vythilingam, I., & Ng, L. C. (2020). Detection of Japanese Encephalitis virus in Culex mosquitoes in Singapore. American Journal of Tropical Medicine and Hygiene, 103(3), 1234–1240.