



**DETERMINATION OF MOSQUITOES SPECIES DIVERSITY AND
MOLECULAR DETECTION OF WEST NILE VIRUS IN WILD BIRD
HABITATS IN PERAK AND SELANGOR, MALAYSIA**

By

NATASHA BINTI JAFAR ALI

**Thesis Submitted to the School of Graduate Studies, Universiti Putra
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Science**

August 2022

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DEDICATIONS

Indeed, Allah is not timid to present an example – that of a mosquito or what is smaller than it. And those who have believed know that it is the truth from their Lord.

(Surah Al-Baqarah 2:26)



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Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirement for the degree of Master of Science

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West Nile virus (WNV) is a zoonotic and emerging arthropod-borne RNA virus from the *Flaviviridae* family. The virus is maintained in a sylvatic cycle between vectors and reservoirs host, the wild birds. Mosquitoes, especially *Culex* spp, acquired WNV from wild birds and transmitted it into the susceptible dead-end hosts such as humans and other mammals. Previous discovery of Kunjin virus, a subtype of WNV in mosquitoes from Malaysia, was more than half a century ago. Meanwhile, WNV antibodies and/or RNA have been demonstrated in wild birds, human, wildlife such as macaques and bats; and farm animals such as swine and horse from 2003 until now. Presence and transmission of WNV in these species certainly involved mosquito vectors, yet the study on the current status of WNV in mosquitoes is lacking, which further signifies the need of this study. The aim of this study was identifying the diversity of mosquito species found near to the wild bird habitats and determining the presence of WNV RNA in the mosquitoes in the selected areas of West Coast Malaysia. Mosquitoes were trapped by using CDC Light Trap baited with carbon dioxide followed by sex and species identification with morphological and taxonomic keys under stereomicroscope. The mosquitoes were pooled into 10 based on sex, species, and collection site. Subsequently, nested RT-PCR targeting conserved region between the capsid and the pre-membrane gene of WNV was conducted to detect WNV RNA. Positive amplicon from nested RT-PCR assay was further analysed using partial sequencing analysis. Overall, 2635 mosquitoes were collected from Kuala Gula Bird Sanctuary, Perak (1417 mosquitoes) at percentage of 53.78 and Kapar Energy Venture, Selangor (1218 mosquitoes) at percentage of 46.22. Nested RT-PCR revealed that WNV RNA was detected from 35 out of 285 (12.28%) mosquito pools, which belongs to six different species namely *Culex tritaeniorhynchus* (24 pools), *Culex vishnui* (6 pools), 2 pools of *Culex pseudovishnui*, 1 pool of *Culex gelidus*, 1 pool of *Armigeres*

subalbatus and 1 pool of *Coquillettidia* spp. Minimum infection rate (MIR) presenting the highest value on *Culex tritaeniorhynchus* which was 9.11. Partial sequencing analysis revealed that the isolates share 90.12- 97.01% similarity with the isolates found in Malaysia, Africa, Germany, Romania, Italy and Israel. Phylogenetic analysis signifies that isolates from this study is grouped in lineage 2. In conclusion, *Culex* species are the most significant species infected with WNV collected in this study suggesting that WNV is circulating among the mosquitoes in Malaysia. The MIR value on this species indicates high possible risk of WNV transmission in this study. Evidence of WNV in the mosquitoes further substantiate the need of enhancing vector control program and continuous surveillance of WNV in the reservoir, vector and susceptible host.

Keywords: West Nile virus, mosquitoes, nested RT-PCR, sequencing, West Coast Malaysia.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia
sebagai memenuhi keperluan untuk ijazah Master Sains

**PENENTUAN KEPELBAGAIAN SPESIES DAN PENGESANAN
MOLEKULAR VIRUS WEST NILE DALAM NYAMUK DI HABITAT BURUNG
LIAR DI PERAK DAN SELANGOR, MALAYSIA**

Oleh

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Virus West Nile (WNV) ialah virus RNA muncul bawaan arthropod dan zoonotik daripada keluarga *Flaviviridae*. Virus ini kekal dalam kitaran silvatik antara vektor dan perumah reservoir, iaitu burung liar. Nyamuk terutamanya *Culex* spp. memperoleh WNV daripada burung liar dan menyebarkan ia kepada perumah terakhir yang mudah terdedah seperti manusia dan mamalia lain. Penemuan virus Kunjin sebelum ini, subjenis WNV dalam nyamuk dari Malaysia, adalah lebih daripada setengah abad yang lalu. Sementara itu, antibodi dan/atau RNA WNV ditemui dalam burung liar, manusia, hidupan liar seperti kera dan kelawar dan haiwan ladang seperti babi dan kuda dari tahun 2003 hingga sekarang. Kehadiran dan penularan WNV dalam spesies ini pastinya melibatkan vektor nyamuk, namun kajian tentang status semasa WNV dalam nyamuk masih kurang, di mana menandakan kepentingan keperluan kajian ini. Matlamat kajian ini adalah untuk mengenal pasti kepelbagaian spesies nyamuk yang terdapat berhampiran dengan habitat burung liar dan untuk menentukan kehadiran RNA WNV dalam nyamuk di kawasan terpilih di Pantai Barat Malaysia. Nyamuk diperangkap dengan menggunakan trap lampu CDC yang diumpan dengan karbon dioksida dan diikuti dengan pengenalan jantina dan spesies dengan kunci morfologi dan taksonomi di bawah stereomikroskop. Nyamuk tersebut dikumpulkan kepada bilangan 10 berdasarkan jantina, spesies dan tapak kajian. Seterusnya, nested RT-PCR yang menyasarkan kawasan terpelihara di antara gen kapsid dan pra-membran WNV telah dijalankan untuk mengesan RNA WNV. Amplikon positif daripada ujian nested RT-PCR dianalisis selanjutnya dengan menggunakan analisis penjujukan separa. Secara keseluruhan, 2635 nyamuk telah dikumpul dari Santuari Burung Kuala Gula, Perak (1417 nyamuk) pada peratusan 53.78 dan Kapar Energy Venture, Selangor (1218 nyamuk) pada peratusan 46.22. Nested RT-PCR mendedahkan bahawa RNA WNV telah dikesan daripada 35 daripada 285 (12.28%) himpunan nyamuk, yang tergolong

kepada enam spesies berbeza iaitu *Culex tritaeniorhynchus* (24 himpunan), *Culex vishnui* (6 himpunan), 2 himpunan *Culex pseudovishnui*, 1 himpunan *Culex gelidus*, 1 himpunan *Armigeres subalbatus* dan 1 himpunan *Coquillettidia* spp. Kadar jangkitan minimum (MIR) menunjukkan nilai tertinggi pada *Culex tritaeniorhynchus* iaitu 9.11. Analisis penjujukan separa mendedahkan bahawa isolat daripada kajian ini berkongsi 90.12- 97.01% persamaan dengan strain yang dijumpai di Malaysia, Afrika, Jerman, Romania dan Israel. Analisis filogenetik menandakan bahawa pencilan daripada kajian ini dikelompokkan dalam susur galur 2. Kesimpulannya, spesies *Culex* adalah spesies paling ketara yang dijangkiti WNV yang dikumpul dalam kajian ini menunjukkan bahawa WNV sedang beredar di kalangan nyamuk di Malaysia. Nilai MIR pada spesies ini menunjukkan risiko transmisi WNV yang tinggi dalam kajian ini. Bukti WNV dalam nyamuk menekankan lagi keperluan untuk meningkatkan program kawalan vektor dan pengawasan berterusan WNV di dalam reservoir, vektor dan perumah yang mudah terdedah.

Kata kunci: Virus West Nile, nyamuk, nested RT-PCR, penjujukan, Pantai Barat Malaysia.

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LIST OF ABBREVIATIONS'

%	percentage
°C	degree Celcius
µL	microlitre
µM	micromolar
A	absorbance
aa	amino acid
<i>Ad.</i>	<i>Aedeomyia</i>
<i>Ady.</i>	<i>Aedeomyia</i> subgenus
<i>Ae.</i>	<i>Aedes</i>
<i>Aed.</i>	<i>Aedes</i> subgenus
AMCA	American Mosquito Control Association
<i>An.</i>	<i>Anopheles</i>
<i>Ar.</i>	<i>Armigeres</i>
<i>Arm.</i>	<i>Armigeres</i> subgenus
BLAST	Basic Local Alignment Search Tool
bp	basepair
C	capsid
CDC	Centers for Disease Control and Prevention
<i>Cel.</i>	<i>Cellia</i> subgenus
cm	centimetre
<i>Coq.</i>	<i>Coquillettidia</i> subgenus
<i>Cq.</i>	<i>Coquillettidia</i>
CRISPR/Cas9	Clustered Regularly Interspaced Short Palindromic Repeats -associated protein 9
<i>Cux.</i>	<i>Culex</i> subgenus
<i>Cx</i>	<i>Culex</i>

DEET	<i>N, N</i> -diethyl- <i>m</i> -toluamide
DWNP	Department of Wildlife and National Parks
E	envelope
g	gram
<i>g</i>	gravity
GIS	Geographic Information System
H _A	Alternate hypothesis
<i>Hez.</i>	<i>Heizmannia</i> subgenus
H ₀	Null hypothesis
<i>Hz.</i>	<i>Heizmannia</i>
IU/mL	international unit per millilitre
JEV	Japanese encephalitis virus
kb	kilobases
kDa	kilodalton
kg	kilogram
<i>Lop.</i>	<i>Lophoceraomyia</i> subgenus
mA	milliAmpere
<i>Ma.</i>	<i>Mansonia</i>
MIR	Minimum Infection Rate
mL	millilitre
<i>Mnd.</i>	<i>Mansonioides</i> subgenus
MOH	Ministry of Health
MW	molecular weight
NCBI	National Center for Biotechnology Information
ng / μL	nanogram per microlitre
nm	nanometre
NS	non-structural

PAST	Palaeontological Statistics Software
PBS	phosphate buffer saline
PCR	polymerase chain reaction
PFU/mL	plaque-forming units per millilitre
PPE	personal protective equipment
prM	premembrane
qRT-PCR	quantitative reverse transcription- polymerase chain reaction
RNA	ribonucleic acid
RT-PCR	reverse transcription- polymerase chain reaction
spp.	species
<i>To.</i>	<i>Topomyia</i>
<i>Ur.</i>	<i>Uranotaenia</i>
UTR	untranslated region
UV	ultraviolet
<i>Ve.</i>	<i>Verralina</i>
<i>Ver.</i>	<i>Verralina</i> subgenus
WHO	World Health Organization
WNV	West Nile virus

CHAPTER 1

INTRODUCTION

Mosquitoes are extremely important vectors that play a role in transmitting zoonotic viruses and parasites worldwide. Arboviruses such as dengue, chikungunya, West Nile, Japanese encephalitis viruses, and parasites like *Plasmodium* spp., *Wuchereria bancrofti* and *Brugia* spp. are broadly transmitted through mosquito bites (WHO, 2020). West Nile virus (WNV) is a zoonotic emerging disease transmitted by the bite of mosquitoes primarily *Culex* species because of the ornithophilic properties (Petersen et al., 2013). Infection caused by WNV are distributed globally resulting in significant outbreaks in birds, humans, and other mammals like horses (Asnis et al., 2006). Abundance of mosquito vectors combined with the migratory flying behaviour of wild birds across the globe have exacerbated the dynamics of WNV infection (Maquart et al., 2016).

A study by Bisanzio et al. (2011) documented that WNV transmission were influenced by the emergence of migratory birds, the reservoir host and mosquito species primarily *Aedes* spp. and *Culex* spp. as the vector of WNV. The ornithophilic properties on *Culex* species mosquitoes influenced the transmission of WNV to be maintained in a bird-mosquito cycle (Becker et al., 2010). Migratory birds with WNV infections are competent to transmit WNV and able to maintain the viraemic phase which lasting two to seven days even after long flying distance which classifying them as reservoir hosts (Reisen et al., 2005). A study showed the avian host experienced WNV clinical manifestations differ from each species. The Great Gray Owl, raptors were suffered from compound fracture on shoulder girdle, internal hemorrhage and ocular and beak trauma, corneal ulcer and poor feather condition meanwhile the barred owl affected to neurologically leading to depression, recumbency which prone to euthanasia (Lopes et al., 2007).

According to Zhao and Alto (2016), Africa, Asia, the Middle East, Europe, and Australia are now considered as endemic countries of WNV pathogen. Due to these outbreaks, the study of virus transmission and vector-virus relationships are taking into consideration globally (Valiakos et al., 2013). In Malaysia, a first study on WNV detection in mosquitoes was reported in 1967 in which Kunjin virus, a WNV subtype was detected from *Culex pseudovishnui* in Sarawak, a state in West Malaysia (Bowen et al., 1970). Up to now, WNV RNA and/or antibodies were discovered in multi species in companion birds, migratory birds, swine, bats and macaques (Ain-Najwa et al., 2020a; Ain-Najwa et al., 2020b; Mohammed et al., 2021).

Malaysia is located within tropical climate which is an ideal environment for mosquito population to breed and survive but a study on the competent vector of the WNV are yet to be conducted. Apart from that, there are no continuous or

follow up study were carried out around Peninsular Malaysia on mosquito species following the presence of the Kunjin virus from the *Culex* mosquitoes in Sarawak. Identification of possible vector species among mosquitoes followed by the susceptible sites that have higher risk of WNV transmission are essential prevent future unprecedented WNV outbreaks.

The clinical representation of WNV infections is quite similar with other arboviral diseases such as dengue and Japanese encephalitis leading to WNV as one of the negligible culprits. Most of the people usually were prone to left undiagnosed or misdiagnosed. Hence, detection of WNV in mosquito populations are essential for monitoring viral activity and provide an insightful information in the field of medical about adding WNV as one of the possible suspected with other arboviruses.

1.1 Problem Statement

The problem statements for this study are as follows:

- 1) Although wild bird is known as the reservoir for WNV, the mosquito diversity in wild bird's habitat or landing area are poorly known.
- 2) Previously, the presence of WNV RNA and antibodies was found in the migratory and resident birds in Kuala Gula Bird Sanctuary, Perak and Kapar Energy Venture, Selangor. However, there is no current data that could confirm the presence of WNV on mosquitoes, the vector.

1.2 Research Hypothesis

1.2.1 Scientific Hypothesis

The scientific hypotheses for this study are as follows:

- 1) *Culex* species are abundantly distributed near to bird habitats around West Coast Malaysia.
- 2) WNV RNA are detected among mosquito species collected around bird habitats in West Coast Malaysia.

1.2.2 Statistical Hypothesis

The statistical hypotheses for this study are as follows:

- 1) **a. H_0 :** *Culex* spp. is not abundant mosquito population near to wild bird habitats in Perak and Selangor, Malaysia.
b. H_A : *Culex* spp. is abundant mosquito population near to wild bird habitats in Perak and Selangor, Malaysia.
- 2) **a. H_0 :** WNV RNA is not present among mosquito population near to wild bird habitats in Perak and Selangor, Malaysia.
b. H_A : WNV RNA is present among mosquito population near to wild bird habitats in Perak and Selangor, Malaysia.

1.3 Research Objectives

The objectives of this study are as follows:

- 1) To identify the species diversity among mosquito species in wild bird habitats in Perak and Selangor, Malaysia by using the morphological characteristics.
- 2) To detect the presence of WNV RNA among mosquito species in wild bird habitats in Perak and Selangor, Malaysia by using molecular method.

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