# *SEROLOGICAL AND MOLECULAR PREVALENCE OF WEST NILE VIRUS INFECTION IN WILD BIRDS, MACAQUES AND BATS IN SELECTED AREAS IN PENINSULAR MALAYSIA*

**NUR AIN NAJWA BINTI MOHD YUSERI**

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By

**NUR AIN NAJWA BINTI MOHD YUSERI**

**Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Master of Science**

**August 2019**

**DEDICATION**

I dedicate this thesis to my beloved parents, Mohd Yuseri Isa and Faridah Akma Mohd Zin, for supporting and nurturing my dreams with love, guidance and personal sacrifice. You have always been here during my hardship time in completing this research. This thesis also dedicated to my siblings (Amir Syafiq, Isamuddin, Ain Nadia, Naqib Naufal and Qistina Balqis) and also my grandmother, Gayah.

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the Degree of Master of Science

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West Nile virus (WNV) is the aetiological agent for mosquito-borne zoonotic virus which originated from a genus of Flavivirus and belongs to the family of Flaviviridae. WNV transmission cycles involve birds as a reservoir host, mosquitoes as the vector while susceptible animals mainly mammals are the incidental hosts. In Malaysia, seropositivity and nucleic acid detection of WNV have been demonstrated in captive birds, Orang Asli and horses. Previous studies have very limited information on the status of WNV in wildlife. On the other hand, other wildlife such as macaques and bats are renowned as a reservoir for deadly zoonotic viruses such as Ebola, Marburg, Severe Acute Respiratory Syndrome (SARS) Coronavirus and Henipavirus, and they also have been demonstrated to shed WNV in other countries. By considering all these facts and WNV has become a global threat to the public health, this study aimed to determine the seroprevalence (antibody) and molecular prevalence (nucleic acid) status of WNV in wildlife particularly wild birds, macaques and bats from selected areas in Peninsular Malaysia. In addition to that, risk factors associated with WNV seropositivity and infection were identified. The serum was collected from 236 (n=236) wild birds and macaques in selective states of Perak, Pahang, Selangor and Johor and followed by screened for WNV antibodies by using commercial competitive ELISA (c-ELISA) kit (ID Screen® West Nile Competition Multi-species ELISA, ID VET, Montpellier, France). Due to the cross-reaction with another genus of Flavivirus, the samples tested seropositive to WNV were further analysed by using Japanese encephalitis virus (JEV) double-antibody sandwich ELISA (DAS-ELISA) kit (Sunred, China). In addition, a total of 240 oropharyngeal and rectal swabs from wild birds, macaques and bats were collected in selective states of Perlis, Perak, Pahang, Selangor and Johor. The swabs were subjected to one-step RT-PCR assay to detect WNV nucleic acids by targeting the conserved region of WNV between capsid and pre-membrane regions. The positive band from RT-PCR assay were subjected to DNA sequencing and phylogenetic tree analyses. The risk factors associated with WNV exposure from ELISA result and infection from RT-PCR result in wildlife were analysed by using Chi-square ($X^2$), Fisher's exact test, multiple logistic regression and student t-test. The prevalence was calculated as a

percentage for the positive samples. The seroprevalence of WNV in this study are 18.71% (29/155) at 95% CI (0.131 to 0.260) in wild birds and 29.63% (24/81) at 95% CI (0.203 to 0.410) in macaques. In wild birds, significant risk factors associated with WNV seroprevalence are a category of wild bird, family, species, age, locality, presence of paddy field and migratory period. In macaques, all risk factors include age and sex were not significantly associated with WNV seroprevalence. Molecular analysis by using RT-PCR revealed that 15.2% (16/105) at 95% CI (0.092 to 0.239) of wild birds, 8.3% (6/72) at 95% CI (0.034 to 0.179) of bats and none of the macaque samples were positive. Sequencing analysis by using DNA Sanger sequencing method showed that the positive samples in this study resembled 98-99% similarity and closely related to the strain from South Africa in lineage 2 of WNV. Overall, this is the first study to investigate WNV status in wildlife in Peninsular Malaysia as well as the risk factors associated with WNV exposure and infection. Although there is no WNV outbreak in Malaysia and no clinical reports of WNV infections have been made yet, precaution and preventive measures should be taken as WNV could possibly become pathogenic to animals and humans.

Keywords: West Nile virus, arbovirus, wild bird, macaque, bat, c-ELISA, RT-PCR.

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

**SEROPREVALENSI DAN MOLEKUL PREVALENS INFEKSI VIRUS NIL BARAT DALAM BURUNG LIAR, KERA DAN KELAWAR DI KAWASAN TERPILIH DI SEMENANJUNG MALAYSIA**

Oleh

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**Ogos 2019**

**Pengerusi : Nor Yasmin binti Abd. Rahaman, PhD**
**Fakulti : Perubatan Veterinar**

Virus Nil barat (WNV) adalah agen etiologi untuk virus zoonosis bawaan nyamuk yang berasal daripada genus Flavivirus dan tergolong dalam keluarga Flaviviridae. Kitaran penjalaran WNV melibatkan burung sebagai perumah reservoir, nyamuk sebagai vektor manakala haiwan yang mudah terdedah terutamanya mamalia adalah perumah insidental. Di Malaysia, seropositiviti dan pengesanan asid nukliek WNV telah ditunjukkan dalam burung teman, Orang Asli dan kuda. Kajian terdahulu mempunyai maklumat yang sangat terhad mengenai status WNV dalam hidupan liar. Sebaliknya, hidupan liar lain seperti kera dan kelawar terkenal sebagai reservoir untuk virus zoonosis yang membawa maut seperti Ebola, Marburg, sindrom pernafasan akut Coronavirus yang teruk (SARS) dan Henipavirus, dan mereka juga telah menunjukkan untuk menurunkan WNV di daerah lain. Dengan mempertimbangkan semua fakta ini dan WNV menjadi ancaman global kepada kesihatan awam, kajian ini bertujuan untuk menentukan status seroprevalensi (antibodi) dan molekul prevalens (asid nukliek) WNV dalam hidupan liar terutamanya burung liar, kera dan kelawar dari kawasan terpilih dalam Semenanjung Malaysia. Tambahan pula, faktor risiko yang dikaitkan dengan seropositiviti dan infeksi WNV telah dikenalpasti. Serum itu dikumpulkan daripada 236 (n = 236) burung liar dan kera di negeri-negeri terpilih Perak, Pahang, Selangor dan Johor dan diikuti dengan menyaring antibodi WNV dengan menggunakan komersial kit ELISA bersiang (c-ELISA) (ID Screen® West Nile Competition Multi-species ELISA, ID VET, Montpellier, Perancis). Oleh kerana tindak balas silang dengan genus Flavivirus yang lain, sampel yang diuji seropositif kepada WNV telah dianalisis lagi dengan menggunakan virus ensefalitis Jepun (JEV) ELISA sandwic antibodi-berganda (DAS-ELISA) (Sunred, China). Tambahan, sejumlah 240 sampel orofaring dan rektum daripada burung liar, kera dan kelawar dikumpulkan di negeri-negeri terpilih Perlis, Perak, Pahang, Selangor dan Johor. Swab itu telah menjalani asai satu-langkah RT-PCR untuk mengesan asid nukliek WNV dengan menyasarkan kawasan terabadi WNV di antara kawasan kapsid dan pra-membran. Jalur positif daripada asai RT-PCR telah menjalani pengurutan DNA dan analisis pokok filogenetik. Faktor-faktor risiko yang berkaitan dengan pendedahan WNV daripada hasil ELISA dan infeksi daripada hasil RT-

PCR dalam hidupan liar dianalisis dengan menggunakan Chi-square ($X^2$), ujian tepat Fisher, regresi logistik berganda dan ujian-t pelajar. Prevalens dikira sebagai peratusan bagi sampel positif. Seroprevalensi WNV dalam kajian ini adalah 18.71% (29/155) pada 95% CI (0.131 hingga 0.260) dalam burung liar dan 29.63% (24/81) pada 95% CI (0.203 hingga 0.410) dalam kera. Dalam burung liar, faktor risiko keertian yang dikaitkan dengan seroprevalensi WNV adalah kategori burung liar, keluarga, spesies, umur, Kawasan, kehadiran sawah padi dan tempoh penghijrahan. Dalam kera, semua faktor risiko termasuk umur dan jantina tidak dikaitkan dengan seroprevalensi WNV. Analisis molekul dengan menggunakan RT-PCR pula menunjukkan bahawa 15.2% (16/105) pada 95% CI (0.092 hingga 0.239) burung liar, 8.3% (6/72) pada 95% CI (0.034 hingga 0.179) kelawar dan tidak ada sampel kera yang positif. Analisis urutan dengan menggunakan kaedah pengurutan DNA Sanger menunjukkan bahawa positif sampel dalam kajian ini menyerupai 98-99% kesamaan dan berkait rapat dengan strain dari Afrika Selatan dalam nasabah 2 WNV. Keseluruhannya, ini adalah kajian pertama untuk mengkaji status WNV dalam hidupan liar di Semenanjung Malaysia serta faktor risiko yang berkaitan dengan pendedahan dan infeksi WNV. Walaupun wabak WNV di Malaysia belum ada dan tiada laporan klinikal infeksi WNV telah dibuat, langkah berjaga-jaga dan pencegahan perlu diambil kerana WNV mungkin boleh menjadi patogenik kepada haiwan dan manusia.

Kata kunci: virus Nil barat, arbovirus, burung liar, kera, kelawar, c-ELISA, RT-PCR.

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Master of Science. The members of the Supervisory Committee were as follows:

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## LIST OF ABBREVIATIONS

| | |
|---|---|
| A | Ampere |
| AUP | Animal use protocol |
| BLAST | Basic local alignment search tool |
| bp | Base pair |
| BSL | Biosafety laboratory level |
| C | Capsid |
| c-ELISA | Competitive ELISA |
| CDC | Centres for Disease Control and Prevention |
| CFT | Complement fixation test |
| CI | Confidence interval |
| CNS | Central nervous system |
| CPE | Cytopathic effect |
| CSF | Cerebrospinal fluid |
| DAS-ELISA | Double-antibody sandwich ELISA |
| $ddH_2O$ | Double-distilled water |
| DENV | Dengue virus |
| DEPC water | Diethylpyrocarbonate water |
| DNA | Deoxyribonucleic acid |
| DWNP | Department of Wildlife and National Parks |
| E | Envelope |
| ELISA | Enzyme-linked immunosorbent assay |
| ER | Endoplasmic reticulum |
| $g$ | Gravity |
| g | Gram |
| G | Gauge |
| $H_A$ | Alternate hypothesis |
| $H_O$ | Null hypothesis |
| HI | Hemagglutination inhibition |
| HRP | Horseradish peroxidase |
| IACUC | Institutional Animal Care and Use Committee |
| IBM | International business machines corporation |
| ID | Identity document |
| IgG | Immunoglobulin G |
| IgM | Immunoglobulin M |
| IFAT | Indirect fluorescent antibody assay |
| IFN | Interferon |
| IHC | Immunohistochemistry |
| JEV | Japanese encephalitis virus |
| km | Kilometre |
| KUNV | Kunjin virus |
| LCs | Langerhans cells |
| m | Metre |
| M | Membrane |
| MAC-ELISA | IgM antibody capture enzyme-linked immunosorbent assay |
| MEGA | Molecular Evolutionary Genetics Analysis |
| MHC | Major histocompatibility |
| mL | Millilitre |
| mm | Millimetre |

| | |
|---|---|
| n | Sub-total population |
| NA | Not applicable |
| NCBI | National Centre for Biotechnology Information |
| NCR | Non-coding regions |
| ng/µL | Nanogram per microliter |
| nm | Nanometre |
| No. | Number |
| NS | Non-structural |
| NY99 | New York 1999 |
| $OD_{NC}$ | Optical density for negative control |
| $OD_{PC}$ | Optical density for positive Control |
| OR | Odds ratio |
| PBS | Phosphate buffer saline |
| PCR | Polymerase chain reaction |
| PERHILITAN | Jabatan Perlindungan Hidupan Liar dan Taman Negara |
| PPE | Personal protective equipment |
| prE | Envelope protein |
| prM | Pre-membrane |
| PRNT | Plaque reduction neutralization test |
| Ref | Reference |
| RNA | Ribonucleic acid |
| RNase | Ribonuclease |
| ROC | Receiver operating characteristics |
| RT-PCR | Reverse transcriptase PCR |
| S/N% | Percentage of sample over negative control |
| SARS | Severe Acute Respiratory Syndrome |
| SE | Standard error |
| SLEV | Saint Louis encephalitis virus |
| Spp. | Species |
| SPSS | Statistical Package for the Social Sciences |
| ssRNA | Single stranded RNA |
| $T_m$ | Melting temperature |
| TAE | Tris-acetate Ethylenediaminetetraacetic acid |
| TBEV | Tick-borne encephalitis virus |
| UK | United Kingdom |
| UPM | Universiti Putra Malaysia |
| USA | United States of America |
| UV | Ultraviolet |
| V | Volt |
| VNT | Virus neutralization test |
| WEE | West Equine encephalitis |
| WNF | West Nile fever |
| WNV | West Nile virus |
| w/v | Weight per volume |
| X | Times |
| YFV | Yellow fever virus |
| α | Alpha |
| β | Beta |
| °C | Degree celsius |
| % | Percentage |
| µL | Microliter |

# CHAPTER 1

## INTRODUCTION

Over the past decade, number of zoonotic and non-zoonotic viruses have emerged and re-emerged in Malaysia namely Nipah virus, Japanese encephalitis virus (JEV), Rabies virus and Avian Influenza virus (Ganesan & Sinniah, 1993; Looi & Chua, 2007; Nur Adibah *et al*., 2017; Kumar *et al*., 2018a). Several of these viruses' outbreaks lead to significant morbidity and mortality to humans and animals which had imposed immense public health and economic burden to the country. West Nile virus (WNV) is one of the significantly zoonotic viruses that could affect a large number of animals including wildlife as well as humans worldwide and it is a causative agent for febrile illness and meningitis. It was first reported in Uganda in 1937 with febrile illness symptoms from a woman (Smithburn *et al*., 1940; Sambri *et al*., 2013).

West Nile virus (WNV) is a zoonotic enveloped RNA virus derived from the genus of Flavivirus under the family of Flaviviridae in which Japanese encephalitis virus (JEV), St. Louis encephalitis virus (SLEV), Zika, Dengue virus and Yellow fever virus are grouped in. All of the viruses under this family are categorised as an arthropod-borne virus or known as arbovirus. These viruses spread by mosquitoes namely *Culex* and *Aedes* spp. that have been shown to cause infection in human and animal (Antipa *et al*., 1984; Davis *et al*., 2006). The virus replicates in mosquitoes' salivary glands and transmits to other hosts through secretions during a blood meal (Chancey *et al*., 2015). West Nile virus (WNV) is maintained in zoonotic cycles among the wild birds as amplifying host and mosquitoes as vector (Petersen *et al*., 2003). West Nile virus (WNV) also can infect human and many species of mammals, reptiles and amphibians. Therefore, all of these factors attributed to why WNV has been successfully spread over a large geographical area.

Besides wild birds, other wildlife such as macaques and bats might play a crucial role in spreading of WNV. In bird species, the virus builds up in the blood in higher concentrations than in other mammals. Most of the infected birds will not show symptoms, but increased levels of WNV in birds make them as amplifier host (Petersen, 2013). Macaques species could be infected with WNV, but they often developed a low level of viraemia, making them unlikely to perpetuate the virus (Olberg *et al*., 2004). Some medically important Flaviviruses that have been isolated from bats are mosquito-transmitted arboviruses such as SLEV, WNV and JEV (Pilipski *et al*., 2004; Bunde *et al*., 2006). These findings reported that bats may act as competent amplifying hosts for arthropod-borne Flaviviruses (Kading & Schountz, 2016).

Malaysia has an abundance of *Culex* and *Aedes* spp. of mosquitoes and as WNV is arbovirus that needs a vector to transmit the virus, the virus may spread widely and easily. Wildlife is constantly exposed to mosquitoes bite due to the nature of their habitat and WNV might potentially being transmitted during the blood meal. Furthermore, the possibility of the spill over of the viruses from wildlife to human could occur due to

many reasons such as deforestation and urbanisation that lead to loss of wildlife habitat (Plowright *et al*., 2017).

Thus far, there is no WNV outbreak reported in Malaysia. However, several studies have shown evidence of WNV exposure among animals and human. A study by Marlina *et al*., (2014) reported that 1.21% (9/742) WNV seroprevalence in Orang Asli from several states in Peninsular Malaysia. Besides that, research by Rais *et al*., (2011) reported that the seroprevalence in captive bird populations is 4.41% (3/68) in Selangor while Sifa *et al*. (2018) demonstrated nucleic acid detection of WNV in horses in the central part of Peninsular Malaysia. Kunjin virus (KUNV) which is sub-type of WNV that was endemic in Australia was detected from mosquitoes of the *Culex pseudovishnui* group in Sarawak in 1970 (Ching *et al*., 1970). Kunjin virus (KUNV) was closely related to the strain of the Usutu virus that was isolated from mosquitoes in Spain in 2006 (Vazquez *et al*., 2010).

Previous studies have insufficient information on the status of WNV in susceptible animals, especially in wildlife. As wildlife in most cases served as the reservoir of the deadly emerging and re-emerging zoonotic viruses including WNV, a study on the status of exposure and infection of WNV in wildlife by using ELISA and PCR, respectively is extremely important in order to prevent future outbreaks among human and animal population. The risk factors that associated with the WNV infection were also not documented in Malaysia. Therefore, the risk factors of WNV will be determined in this study by using Chi-square analysis and Fisher's Exact test. As the seroprevalence and molecular prevalence of WNV among wild birds, macaques and bats population are still not documented yet in Malaysia and by considering the potential effect of WNV become pathogenic to humans and wildlife, thus the necessity to conduct this study is extremely important. Therefore, this study proposed the following hypotheses and objectives:

## 1.1 Hypothesis

### 1.1.1 Scientific Hypotheses

1. WNV antibody is presence in wild birds and macaques in selected areas in Peninsular Malaysia.
2. The nucleic acid of WNV is presence in wild birds, macaques and bats in selected areas in Peninsular Malaysia.
3. There is an association between WNV infection and the risk factors in wild birds, macaques and bats in selected areas in Peninsular Malaysia.

### 1.1.2 Statistical Hypotheses

1) **$H_O$:** WNV antibodies are absence in wild birds and macaques in selected areas in Peninsular Malaysia.
**$H_A$:** WNV antibodies are presence in wild birds and macaques in selected areas in Peninsular Malaysia.

2) **$H_O$:** Nucleic acid of WNV are absence in wild birds, macaques and bats in selected areas in Peninsular Malaysia.
**$H_A$:** Nucleic acid of WNV are presence in wild birds, macaques, and bats in selected areas in Peninsular Malaysia.

3) **$H_O$:** There is no association between WNV infection and the risk factors in wild birds, macaques and bats in selected areas in Peninsular Malaysia.
**$H_A$:** There is an association between WNV infection and the risk factors in wild birds, macaques and bats in selected areas in Peninsular Malaysia.

## 1.2 Research Objectives

There are four objectives in this study which are:

1. To determine the exposure to WNV by detecting the antibodies against WNV in wild birds and macaques by using competitive ELISA (c-ELISA) in selected areas in Peninsular Malaysia.
2. To determine the status of infection by detecting the nucleic acids of WNV from oropharyngeal and rectal swabs of wild birds, macaques and bats by using reverse transcriptase PCR (RT-PCR) in selected areas in Peninsular Malaysia and further characterised by using phylogenetic tree analysis.
3. To identify the risk factors associated with WNV seropositivity from ELISA analysis in wild birds and macaques in selected areas in Peninsular Malaysia.
4. To identify the risk factors associated with WNV infection from RT-PCR analysis in wild birds, macaques and bats in selected areas in Peninsular Malaysia.

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