PREVALENCE OF WEST NILE VIRUS IN DOMESTICATED MAMMALS (CATTLE, GOAT, HORSE AND PIG) IN SELECTED AREAS OF PENINSULAR MALAYSIA

By

MOHAMMED MOHAMMED NMA

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

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Chairman : Nor Yasmin binti Abd Rahaman, PhD
Faculty : Veterinary Medicine

West Nile virus (WNV) is the most widespread cause of arboviral encephalitis in the world. It is a re-emerging zoonotic virus that has the potential of significantly impacting public health and animal welfare. In nature, the virus is maintained in a cycle between wild birds that served as amplifiers and Culex mosquitos which are the vectors. Non-avian species are dead-end hosts that can become infected with WNV. The reservoirs and vectors of WNV are abundant in Malaysia and are often found in close to livestock farms, yet limited information is available on WNV status in domesticated mammals in Malaysia. This study was carried out to determine the WNV seroprevalence in domesticated mammals, including cattle, goats, horses and pigs in selected areas of Peninsular Malaysia and to determine the association between WNV infection and host factors (objective 1); to detect WNV infection through reverse transcriptase- Polymerase chain reaction (RT-PCR) in cattle, goats, horses and pigs and the association between WNV infection and host factors (objective 2); and to carry out phylogenetic analysis on the WNV isolates from this study to determine their genetic relatedness to other strains (objective 3). A total of 283 animals (n=283; of which were 80 pigs, 91 horses, 29 goats and 83 cattle) were sampled in this study, which included 283 serum samples and 203 nasopharyngeal swab samples. The serum samples were screened for WNV IgG using a competitive ELISA (c-ELISA) kit (ID VET, France), and for Japanese encephalitis virus (JEV) IgG using double-antibody sandwich ELISA kit (Sunred, China). Total RNA extracted from nasopharyngeal swabs were tested using reverse-transcriptase polymerase chain reaction (RT-PCR) test targeting conserved gene of WNV capsid and pre-membrane. The samples that produced bands following RT-PCR test were purified and subjected to partial DNA sequencing. Using MEGA 7, the sequences were aligned with the MUSCLE method. Phylogenetic analysis was done with BEAST2 with the Bayesian MCMC method, and statistical analyses were done using Chi-square (Fisher's exact test) and logistic regression in IBM

SPSS 28. In total, 140 samples were positive for WNV IgG. Among the different species, the highest seroprevalence was in pigs with 62.5% [(50/80); 95% CI (0.5155 – 0.7231)], followed by 53.85% [(49/91); 95% CI (0.4366 – 0.6373)] in horse, 48.3% [(14/29); 95% CI (0.3139 – 0.4828)] in goats and 32.53% [(27/83); 95% CI (0.2339 – 0.4322)] in cattle. WNV seroprevalence was associated with the species, age of the animal and location of sampling. Among the species, pigs were more likely to be WNV seropositive. In the cattle, location of sample collection was associated with WNV seropositivity, whereas in the goat, age was associated with WNV seropositivity. Location was the only factor in the horse that was associated with WNV seropositivity. Meanwhile, both age and location were associated with WNV seropositivity in the pigs. Serological results indicate past exposure to WNV in all the species in this study. For the molecular analysis, 7.7% [(7/91) at 95% CI (0.0353 to 0.1528)] of the horses are positive for WNV RNA, comprising of four males and three females. The horses were from Cheras (n=4) and Putrajaya (n=3). There were no significant differences between sex and location with the WNV molecular positivity ($p > 0.05$). RT-PCR test positivity indicating a recent and ongoing infection in the horse at the time of sample collection. From the phylogenetic analysis of the partial sequence of the isolates, they grouped closely with lineage 2 WNV isolates, with close similarity (greater that 98%) with South African strain, and with recently reported Malaysian isolates of WNV. These findings show that domesticated mammals in Malaysia were exposed to WNV infection of which highlights the need for increased vigilance and surveillance for WNV to prevent future outbreak.

Keywords: Prevalence, West Nile virus, cattle, goat, horse, pig, mosquito, c-ELISA, RT-PCR.

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PREVALENS VIRUS NIL BARAT DALAM MAMALIA DOMESTIK (LEMBU, KAMBING, KUDA DAN KHINZIR) DI KAWASAN TERPILIH DI SEMENANJUNG MALAYSIA

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Virus Nil barat (WNV) adalah penyebab ensefalitis arboviral yang paling meluas di dunia. Ia adalah virus zoonotik bangkit semula yang berpotensi secara signifikan dalam mempengaruhi kesihatan awam dan kebajikan haiwan. Secara semula jadi, virus ini dikekalkan di dalam kitaran di antara burung liar yang berfungsi sebagai amplifier dan nyamuk Culex sebagai vektor. Spesis bukan unggas adalah perumah terakhir yang dijangkiti WNV. Reservoir dan vektor WNV terdapat begitu banyak di Malaysia dan sering dilihat berhampiran ladang ternakan, namun maklumat yang ada mengenai status WNV pada mamalia domestik di Malaysia adalah terhad. Kajian ini dilakukan untuk menentukan seroprevalensi WNV pada mamalia domestik, termasuk lembu, kambing, kuda dan khinzir di kawasan terpilih di Semenanjung Malaysia dan untuk menentukan hubungan antara jangkitan WNV dan faktor-faktor perumah (objektif 1); untuk mengesan jangkitan WNV melalui tindak balas rantai polymerase transkripsi balikan (RT-PCR) pada lembu, kambing, kuda dan khinzir dan hubungan antara jangkitan WNV dan faktor-faktor perumah (objektif 2); dan untuk melakukan analisis filogenetik pada pencilan WNV dari kajian ini bagi menentukan hubungan genetik mereka dengan strain lain (objektif 3). Sebanyak 283 haiwan (n = 283, di mana khinzir sebanyak 80 ekor, kuda sebanyak 91 ekor, kambing sebanyak 29 ekor dan lembu sebanyak 83 ekor) diambil sampel dalam kajian ini, yang merangkumi 283 sampel serum dan 203 sampel nasofaring. Sampel serum diperiksa untuk menentukan WNV IgG menggunakan kit ELISA bersaing (c-ELISA) (ID VET, Perancis), dan virus ensefalitis Jepun (JEV) IgG menggunakan kit ELISA berapit antibodi-berganda (Sunred, China). Jumlah RNA yang diekstrak dari kesatan nasofaring diuji menggunakan ujian RT-PCR yang menyasarkan gen terpelihara kapsid dan pra-membran WNV. Sampel yang menghasilkan jalur dari ujian RT-PCR ditulenkan dan menjalani penjujukan DNA separa. Menggunakan MEGA 7, jujukan-jujukan disekutukan dengan kaedah MUSCLE. Analisis filogenetik dilakukan dengan BEAST2 dengan

kaedah Bayesian MCMC dan analisis statistik dilakukan dengan menggunakan khi-kuasa dua (ujian tepat Fisher) dan regresi logistik di IBM SPSS 28. Secara keseluruhan, 140 sampel adalah positif WNV IgG. Di segi spesis berlainan, seroprevalensi tertinggi adalah pada khinzir dengan 62.5% [(50/80); 95% CI (0.5155 - 0.7231)], diikuti oleh 53.85% [(49/91); 95% CI (0.4366 – 0.6373)] dalam kuda, 48.3% [(14/29); 95% CI (0.3139 – 0.4828)] dalam kambing dan 32.53% [(27/83); 95% CI (0.2339 – 0.4322)] dalam lembu. Seroprevalensi WNV dikaitkan dengan spesis, usia haiwan dan lokasi pengambilan sampel. Di antara spesis tersebut, khinzir lebih cenderung untuk seropositif WNV. Dalam lembu, lokasi pengumpulan sampel dikaitkan dengan seropositif WNV, sedangkan pada kambing, usia dikaitkan dengan seropositif WNV. Lokasi adalah satu-satunya faktor dalam kuda yang dikaitkan dengan seropositif WNV. Sementara itu, usia dan lokasi dikaitkan dengan seropositif WNV pada khinzir. Hasil serologi menunjukkan pendedahan masa lalu terhadap WNV pada semua spesis dalam kajian ini. Untuk analisis molekul, 7.7% [(7/91) pada 95% CI (0.0353 to 0.1528)] kuda positif untuk WNV RNA, di mana terdiri daripada empat ekor jantan dan tiga ekor betina. Kuda itu berasal dari Cheras (n = 4) dan Putrajaya (n = 3). Tidak terdapat perbezaan yang signifikan antara jantina dan lokasi dengan positif molekul WNV (p> 0.05). Uji positif RT-PCR menunjukkan jangkitan terkini dan sedang berlaku pada kuda pada masa kutipan sampel. Daripada analisis filogenetik jujukan separa pencilan-pencilan, mereka berkelompok rapat dengan pencilan WNV susur galur 2, yang hampir bersamaan (lebih besar daripada 98%) dengan strain Afrika Selatan, dan juga dengan pencilan WNV Malaysia yang baru dilaporkan. Penemuan ini menunjukkan bahawa mamalia domestik di Malaysia telah terdedah kepada jangkitan WNV, di mana ia menyerlahkan kepada keperluan untuk penambahan kewaspadaan dan pengawasan terhadap WNV untuk mencegah wabak di masa depan.

Kata kunci: Prevalens, Virus Nil barat, lembu, kambing, kuda, khinzir, nyamuk, 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

α	Alpha
β	Beta
μL	Micro litre
μM	Micro molar
%	Percentage
A	Ampere
Ab	Antibody
Ae.	Aedes
Ag	Antigen
AUP	Animal use protocol
BEAST	Bayesian Evolutionary Analysis Tool
BLAST	Basic Local Alignment Search Tool
Bp	Base pair
BSL	Biosafety level
C	Capsid
°C	Degree Celsius
C-	Negative control
c-ELISA	Competitive enzyme-linked immunosorbent assay
C.	Culex
C+	Positive control
cDNA	Complementary deoxyribonucleic acid
CI	Confidence interval
CNS	Central nervous system
CRISPR	Clustered regularly interspaced short palindromic repeat

Cryo-EM	Cryogenic electron microscopy
CSF	Cerebrospinal fluid
DAS-ELISA	Double antibody sandwich enzyme-linked immunosorbent assay
DNA	Deoxyribonucleic acid
dsRNA	Double-stranded ribonucleic acid
E	Envelope
ELISA	Enzyme-linked immunosorbent assay
EM	Electron microscopy
ER	Endoplasmic reticulum
F	Forward
G	Gauge
g	Gravity
HI	Haemagglutination inhibition
HIV	Human immunodeficiency virus
IACUC	Institutional Animal Care and Use Committee
ID	Identification
IFN	Interferon
IgG	Immunoglobulin class G
IgM	Immunoglobulin class M
JEV	Japanese encephalitis virus
Kb	Kilo base
M	Membrane
MCMC	Markov Chain-Monte Carlo
MEGA	Molecular Evolutionary Genetics Analysis
mL	Milli litre

MUSCLE	Multiple Sequence Comparison by Log- Expectation
N	Number
NC	Nucleocapsid
NGS	Next generation sequencing
Nm	Nanometre
NY99	New York 99
OAS	oligoadenylate synthetase
OD	Optical density
ODNC	Optical density negative control
ODPC	Optical density positive control
OR	Odds ratio
ORF	Open reading frame
P	Prevalence
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PFU	Plaque forming units
prM	Pre-membrane
PRNT	Plaque reduction neutralisation test
R	Reverse
RBC	Red blood cells
Ref	Reference
RNA	Ribonucleic acid
RNAi	Ribonucleic acid interference
RNase	Ribonuclease
RT-PCR	Reverse transcriptase-polymerase chain reaction

S/N%	Percentage of sample over negative control
Spp.	Species
TAE	Tris-acetate ethylenediaminetetraacetic acid
UK	United Kingdom
UPM	Universiti Putra Malaysia
USA	United States of America
UTR	Untranslated region
UV	Ultraviolet
V	Volt
WN	West Nile
WNF	West Nile fever
WNV	West Nile virus

CHAPTER 1

INTRODUCTION

Most emerging infectious agents infecting humans have an animal origin (Woolhouse & Gaunt, 2007). The domestication of livestock for food and companionship, and expansion of human settlements into wildlife habitats, has led to the spill over of animal pathogens into human populations. Out of the more than 1400 human pathogens globally identified presently, more than half are transmitted to humans from animal reservoirs (van Doorn, 2014). A substantial number of these pathogens are viruses, many of which can remain dormant in their natural habitats but are capable of causing widespread outbreaks once introduced into naïve population of humans and animals, as was witnessed in the dramatic outbreak and subsequent spread of West Nile virus (WNV), WN fever (WNF) and WN encephalitis in the United States, beginning in 1999 (Nash et al., 2001; Trock et al., 2001).

West Nile virus (WNV) is an emerging zoonotic virus that has had a significant impact on global public health in the past decades due to its ability to cause neurological disease and occasionally death in people and several animal species (Bode et al., 2006; Hubálek et al., 2018; Kleiboeker et al., 2004; McLean, 2006). The pathogen has a potential to emerge in new areas and cause large epidemics as was witnessed in the United States following its introduction in 1999 (Gubler, 2007). The identification of WNV as the cause of encephalitis outbreaks affecting horses, birds and humans in the United States of America (USA) beginning in 1999 marked its first appearance in the Western Hemisphere (Nash et al., 2001; Trock et al., 2001). It quickly spread throughout the USA, into Canada to the north and southwards through the Caribbean to South America (Gubler, 2007). This alarming spread, partly due to the virus's ability to mutate rapidly and evolve new traits, including increased pathogenicity and better fit in vectors and hosts allowing for a more rapid replication and greater transmission efficiency (Grubaugh et al., 2017; Rückert and Ebel, 2018), has allowed the virus to become established around the globe over a short period of time. Experience from previous outbreaks have shown that outbreaks due to WNV are difficult to predict especially with limited data (DeFelice et al., 2018).

West Nile virus is an emerging zoonotic arbovirus that is maintained in nature between mosquitos of the Culex genera and multiple species of birds. It is one of the members of the Flaviviridae family of viruses and is classified in the genus Flavivirus. First discovered in Uganda in 1937 (Smithburn et al., 1940), the virus caused sporadic outbreaks across Africa, the Middle East, Mediterranean and Europe throughout the 20th century, and has since become established on every continent except Antarctica (Chancey et al., 2015). Its genome is composed of a single-stranded RNA approximately 11kb with a single open reading frame (ORF), enclosed in an icosahedral virion (Barrows et al., 2018). Based on similarities in nucleotide sequence, WNV strains are divided into distinct lineages

that are somewhat region-specific (Bondre et al., 2007; Chancey et al., 2015) and have varying pathogenicity in birds, horses and humans.

Many species of animals including birds, reptiles and mammals can become infected with WNV with varying degree of severity (Rimoldi et al., 2017). Natural infection occurs following a blood meal from vector mosquitoes harbouring the virus in their saliva. Although WNV has been detected from several insects, mosquitoes of the Culex genus are the primary vectors for the virus (Ciota, 2017). Malaysia has a tropical climate that supports a rich biodiversity, and is home to several species of mosquitoes, including Culex species, as well as many avian species (Jambari et al., 2012; Morris, 2013). Malaysia is also important to several migratory avian species, as many of such birds are known to travel across parts of its territories (Medway, 1970, 1973; Nisbet and Medway, 1972). Ideal conditions for the establishment of WNV and perhaps, for an outbreak, are thus present are present. WNV has had a long history in Malaysia, having been detected for the first time over 5 decades ago (Ching et al., 1970), with current reports indicating that the virus is still circulating in the country (Ain-Najwa, Yasmin, Arshad, et al., 2020; Ain-Najwa, Yasmin, Omar, et al., 2020).

Elsewhere, WNV outbreaks are typically first noticed in animals before human infections are observed (Nash et al., 2001; Trock et al., 2001). Effective outbreak prevention and control measures depend on rapid and accurate identification of illnesses due to the virus in sentinel animals and in patients. The abundance of both the vectors and reservoir host for WNV in Malaysia is a challenge especially for preventing animal infections. Although the virus has been reported to be present in mosquitos, humans, wild birds and bats in Malaysia, the role of livestock in the epidemiology of this virus has not been well studied. As such, this study was carried out to determine the exposure of livestock animals to WNV and infection with the virus in Malaysia using ELISA and RT-PCR respectively, as well as to determine risk factors for WNV infection in these species.

1.1 Hypothesis

1.1.1 Scientific hypothesis

1 WNV antibody is detectable in serum of horse, ruminants and pigs from selected areas in Peninsular Malaysia.

2 West Nile virus RNA is present in horses, cattle, goats and pigs in selected areas in Peninsular Malaysia.

3 There is an association between WNV infection and host factors including age, sex and location of the animals.

1.1.2 Statistical hypothesis

1 HO: WNV antibodies are not detectable in horses, cattle, goats and pigs in selected areas in Peninsular Malaysia.

2 HA: WNV antibodies are detectable in horses, cattle, goats and pigs in selected areas in Peninsular Malaysia.

1 HO: WNV RNA is not present in horses, cattle, goats and pigs in selected areas in Peninsular Malaysia.

2 HA: WNV RNA is present horses, cattle, goats and pigs in selected areas in Peninsular Malaysia.

1 HO: There is no association between WNV infection and host factors (age, sex and location) in this study.

2 HA: There is an association between WNV infection and host factors (age, sex and location) in this study.

1.2 Research objective

1 To determine the association between WNV seropositivity and host factors of horse, cattle, goats and pigs in Malaysia using competitive ELISA (c-ELISA).

2 To detect the presence of WNV in cattle, goats, horses and pigs using reverse transcriptase polymerase chain reaction (RT-PCR).

3 To determine the genetic relatedness of the positive WNV isolates using phylogenetic analysis via MEGA-7 and BEAST analysis.

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