UNIVERSITI PUTRA MALAYSIA

SEROLOGICAL AND MOLECULAR DETECTION OF WEST NILE VIRUS IN RATTUS SP. IN KLANG VALLEY

THARSHAINI D/O MURUGASU

SEROLOGICAL AND MOLECULAR DETECTION OF WEST NILE VIRUS IN RATTUS SP. IN KLANG VALLEY

THARSHAINI D/O MURUGASU

A project paper submitted to the

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CERTIFICATION

It is hereby certified that we have read this project entitled "Serological and Molecular Detection of West Nile Virus in *Rattus* sp. in Klang Valley",by Tharshaini D/O Murugasu and in our opinion,it is satisfactory in terms of scope,quality,and presentation as part of fulfilment of the requirement for the course VPD 4999-Final Year Project.

DR NOR YASMIN BINTI ABD.RAHAMAN

DVM(UPM), PhD(UPM)

Senior Lecturer

Faculty of Veterinary Medicine

Universiti Putra Malaysia

(Supervisor)

PROFESSOR DR SITI SURI BINTI ARSHAD

DVM(UPM), M Sc.(UPM),PhD(England)

Senior Lecturer,

Department of Veterinary Pathology & Microbiology

Faculty of Veterinary Medicine,

Universiti Putra Malaysia

(Co-supervisor)

DR NUR FAZILA SAULOL HAMID

DVM(UPM),PhD(Liverpool)

Senior Lecturer,

Department of Veterinary Pathology & Microbiology

Faculty of Veterinary Medicine,

Universiti Putra Malaysia

(Co-supervisor)

DEDICATION

I would like to thank The Almighty God for the wisdom bestowed upon me, strength, peace of mind and good health in completing this project paper.

My humble effort I dedicate to my parents, Mr Murugase & Mrs Gunasundaree and my brothers,Vinoth and Linggeswaar for their love, motivation and support to reach this level.

To my dearest friends,Dr. Khanmani, Revathi,Kisyan and Manusha who provide me with emotional support and encouragement to complete this study.

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LIST OF ABBREVIATIONS

%	Percentage
× g	Relative Centrifuge force
°C	Degree Celcius
µL	Microliter
bp	Base pair
BSC	Biosafety cabinet
C	Capsid
CDC	Center of Disease Control
CNS	Central Nervous System
DC	Dendritic Cell
DDH20	Double Distilled water
DEET	Diethyltoluamide
DEV	Dengue Virus
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DPI	Day Post Infection
ELISA	Enzyme- Linked Immunosorbent Assay
C-ELISA	Competitive ELISA
CuOH	Conserved dinucleotide at 3'end
CCR5	C-C Chemokine receptor type 5
g	gram
IgA	Immunoglobulin A
IgG	Immunoglobulin G

IgM	Immunoglobulin M
IL-2	Interleukin 2
IFA	Immunofluorescent Assay
IFN- γ	Interferon gamma
IACUC	Institutional Animal Care and Use Committee
JEV	Japanese Encephalitis Virus
NS protein	Non-structural protein
NC	Negative Control
OD	Optical Density
PC	Positive Control
prE	Envelope protein
prM	pre-membrane protein
poly(A)	Polyadenylic acid
qRT-PCR	Real time PCR
RLR	retinoic acid-inducible gene 1 receptor (RIG-1)-like receptor
RNA	Ribonucleic acid
RT-PCR	Reverse Transcriptase – Polymerase Chain Reaction
TAE	Tri-Acetate-Ethylenediaminetetraacetic
TBEV	Tick borne Encephalitis Virus
UV	Ultraviolet
WNV	West Nile Virus

ABSTRAK

Abstrak daripada kertas projek ini akan dikemukakan kepada Fakulti Perubatan Veterinar,UPM untuk memenuhi sebahagian daripada keperluan kursus

VPD 4999-Projek Tahun Akhir

PENGESANAN VIRUS WEST NILE SECARA SEROLOGI DAN MOLEKULAR

DALAM SPECIES TIKUS DI LEMBAH KLANG

OLEH

THARSHAINI A/P MURUGASU

2020

Penyelia: Dr Nor Yasmin Binti Abd. Rahaman

Penyelia bersama: Prof. Dr Siti Suri Binti Arshad , Dr Nur Fazila Soulol Hamid

Virus West Nile (WNV) merupakan virus RNA bawaan nyamuk yang tergolong dalam genus Flavivirus. Ia disebarkan melalui kitaran enzootic yang melibatkan burung liar, nyamuk dan perumah terakhir seperti mamalia, reptilia dan amfibia. Perumah reservoir penguat adalah burung manakala vector primer adalah nyamuk sepsis Culex. Kajian yang dilakukan di

Malaysia menunjukkan antibodi dan/atau atigen WNV dijumpai dalam Orang Asli, kelawar, burung kurungan, burung hijrah liar, monyet, kuda dan lembu. Walaubagaimanapun, tiada bukti nyata prevalen WNV di dalam tikus liar meskipun ia dianggap sebagai perumah kebanyakan virus dari seluruh dunia. Kajian kami bertujuan untuk mengenalpasti WNV secara pendekatan serologi and molekular di dalam tikus liar. Dua puluh lima sampel arkib serum yang diperoleh daripada tangkapan tikus-tikus liar di kawasan pasar borong di Lembah Klang diuji dengan assay serologi dan molekular menggunakan masing-masing kit komersil IgG c-ELISA dan 'one –step RT-PCR' untuk mengenal pasti WNV antigen yang menyasar pada gen yang spesifik antara capsid (C) dengan pre-membran (PrM). Tiada sampel serum tikus liar yang positif kepada kedua-dua IgG c-ELISA dan RT-PCR. Secara konklusinya, tikus-tikus liar yang dijumpai pada kawasan itu mungkin tidak pernah terdedah kepada WNV atau mempunyai kadar viraemic yang sedikit sahaja virus dalam darah yg tidak dapat dikenalpasti.

Kata kunci:West Nile Virus,tikus-tikus liar,IgG c-ELISA dan RT-PCR

ABSTRACT

An abstract of the project paper is presented to Faculty of Veterinary Medicine,UPM in partial requirement of the course VPD 4999-Final Year Project

SEROLOGICAL AND MOLECULAR DETECTION OF WEST NILE VIRUS IN *RATTUS* SP. IN KLANG VALLEY

BY

THARSHAINI D/O MURUGASU

2020

Supervisor: Dr Nor Yasmin Binti Abd. Rahaman

Co-Supervisor: Prof. Dr Siti Suri Binti Arshad , Dr Nur Fazila Soulol Hamid

West Nile virus (WNV) is a mosquito borne RNA virus belonging to the genus of Flavivirus. It is transmitted via an enzootic cycle involving wild birds, mosquitoes and dead end hosts such as mammals, reptiles and amphibians. The amplifying reservoirs hosts are birds while the primary vector is Culex sp mosquitoes. Studies conducted in Malaysia revealed that WNV antibodies and/or antigen were detected in indigenous people, bats, captive birds, migratory wild birds, macaque, horses and cattle. However, there is no prominent evidence

on the WNV prevalence in wild rodents as they are considered reservoirs for most of the viruses worldwide. Our study was mainly aiming to detect WNV by both serological and molecular approach in *Rattus* sp. Twenty three archived blood serum samples collected from captured rats in Klang Valley wet market area, Malaysia were subjected to serological and molecular assay using commercial IgG c-ELISA kit and one-step RT-PCR, respectively targeting WNV pre-membrane and capsid protein . None of the wild rodent serum samples were positive for IgG c-ELISA and RT-PCR. In conclusion, the rats found in the area might be were not exposed to WNV or having a scarce viraemic level that cannot be detected.

Keywords: West Nile virus, *Rattus* sp. ,IgG c-ELISA,one-step RT-PCR

1.0 INTRODUCTION

West Nile virus(WNV) is an enveloped, single stranded RNA virus belong to the genus of Flavivirus.It is a zoonotic arthropod borne virus and was originally isolated from the blood of a Ugandan woman (Smithburn et al.,1940).Since then,the WNV outbreak were reported in Africa, America, Europe and India(Sule et al.,2018). WNV is sustained in an enzootic cycle that involves Culex mosquito species as the principal vector and wild birds as the amplifying hosts that are known as major vertebrate reservoirs (Molaei et al.,2006).The transmission of WNV virus usually occur when the culex mosquitoes feed on the wild birds and transmit the virus to other mammals or humans that are incidental dead end host (Ain et al.,2020). In addition to birds, at least 30 other vertebrate species including reptiles, amphibians, and mammals, are susceptible to WNV infection.

It has been reported in several countries that *Rattus* sp. is seropositive to WNV with a low viraemia. A study carried out in Slidell ,Louisiana showed that WNV seroprevalence in *Rattus* sp. is very low around 5.6% (Dietrich et al.,2005). A recent study in two peri-domestic species of wild rats in Merida, Mexico suggest that many black rats (R. rattus) and house mice (M. musculus) had antibodies reactive to flaviviruses but none of the individuals were positive for WNV antibodies by plaque reduction neutralization tests (PRNT) (Cigarroa-Toledo et al.,2016).

In Malaysia, there were very few information found on WNV prevalence from several studies conducted on certain species. A study performed on indigeneous people in peninsular Malaysia, seropositivity towards WNV were detected in 1.21% of the

population(Marlina et al.,2014). There were around 4.41% seroprevalence in captive birds from four states in Selangor(Rais et al.,2011).Besides, further studies for molecular detection of WNV were conducted on wild birds and horses where 0.25% of the sample shows positive result respectively(Sifa et.al,2018;Ain Najwa et al.,2020a). Apart from that, WNV were also detected in primates, pigs, cattles and bats by either serological and molecular method(Ain Najwa et al.,2019). Furthermore, Culex mosquitoes can be abundantly found in Malaysia (Low et al.,2012) .However, there is no study conducted to determine WNV prevalence in *Rattus* sp. in Malaysia.

Therefore,the objective of the study is:

1) To detect the presence of West Nile Virus in *Rattus* sp. in Malaysia using serological and molecular method.

2.0 LITERATURE REVIEW

2.1 Geographical Distribution of West Nile Virus Worldwide

2.1.1 History And Worldwide Distribution of West Nile Virus

West Nile virus (WNV) was first isolated in 1937 from the blood of an infected woman in Uganda (Smithburn et al., 1940)). Further serological investigations in Egypt had been identified the virus in birds and animals too (Smithburn et al., 1954; Ciota, 2017). The first outbreak of neuroinvasive disease caused by WNV was reported among the elderly in Israel in 1957(Chancey.C et al., 2014). Subsequent outbreaks of WNV infection reported from Israel and Africa were mild illnesses. In the mid-1990s, more frequent and more severe outbreaks were reported in Romania, Russia, and Israel. After a few years, WNV was first identified in the United States in August 1999 and since then has spread nationwide. In the 2000s, WNV was found to cause severe neuroinvasive signs such as meningioencephalitis in horses in Africa and Europe countries such as Italy and Hungary (Samantha et al., 2014). Almost 80% human with WNV infections are asymptomatic and symptomatic infections may vary from flu-like malaise to serious neuroinvasive diseases in less than 1% people, where there is no specific treatment observed (Lindsey et al.,2010). In 2002, more than 4000 WNV cases were reported to the CDC while in 2003 outbreak, at least 31 cases of WNV encephalitis and 79 cases of WNV meningitis have been occurred (Hayes & O'Leary, 2004).

Besides, WNV was isolated from mosquitoes in Portugal and the Czech Republic in 1972; migrating birds in Slovakia in 1977, and western Ukraine; and Ixodes sp. ticks in Hungary and the Moldavia region in 1967. Recent seroprevalance of WNV in Kenya has also been

detected in ticks collected from 2010–2012 and mosquitoes from 2007–2011(Lwande et al., 2013;Ochieng et al., 2013). In Asian countries like South Korea, 5/1531 wild bird specimens were seropositive to WNV in a 2009 study, but none of the positives are resident birds (Yeh et al., 2011). In India, sporadic cases were documented during 1970s–2000s with the first paediatric fatalities due to WNV encephalitis (George et al., 1984). Most sequenced isolates from India are WNV lineage 5 and lineage 1C with lineage 5 being more pathogenic to mice (Bondre et al., 2007; Chowdhury et al., 2014). In China, WNV seropositivity was first reported in birds from Yunnan province in 1988(Yang et al., 1988). Seropositivity for WNV in Shanghai was reported in 14.9% of cats and 4.9% of dogs tested in 2010, as well as in captive resident birds from 2009-2010 (Lanet et al., 2011).Figure 1 shows the distribution of WNV worldwide.

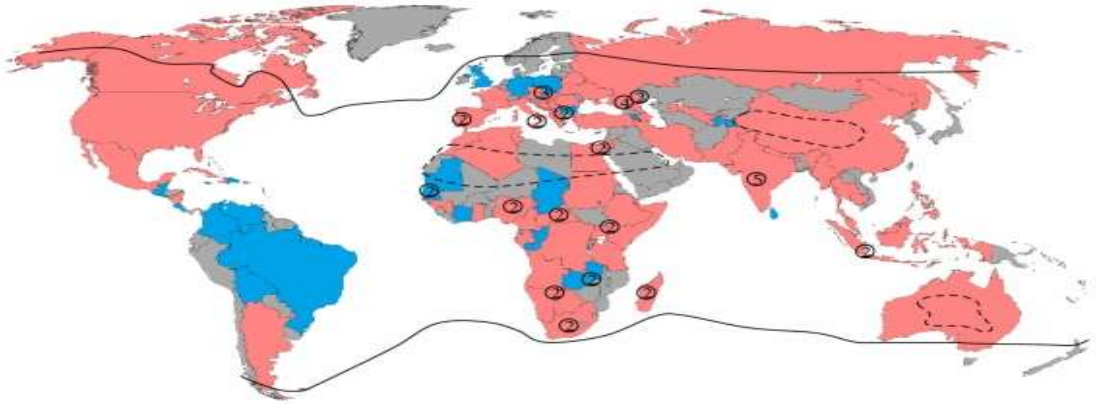

Figure 1: Global distribution of West Nile Virus **.**Note: Red zone-seropositivity in human cases, Blue-Other species/mosquito cases seropositivity, Grey-no data or no seropositivity reported Black lines represent worldwide distribution of WNV mosquito vectors and do not include the areas of extreme climate shown by dashed lines. Circled regions indicate the presence of WNV lineages other than lineage 1 in that specific area(Chancey et al.,2015)

2.1.2 West Nile Virus in Malaysia

Thus far, there is no WNV outbreak reported in Malaysia. WNV was first isolated as Kunjin virus, a genetically distinct subtype of WNV lineage 1 in Sarawak, Malaysia (Karabotsos, 1985; Bowen et al., 1970). The strains were acquired from the pool of *Culex pseudovishnui* that includes some *C.alienus* and *C.annulus* in the inland area of Sarawak (Hills et al.,1969;Bowen et al.,1970).

Several studies have investigated the seroprevalance of WNV in various species namely Orang Asli, captive birds, wild birds, horses, bats, primates and pigs. The seropositivity in the Orang Asli population from Perak and Pahang aged under 16 years old is around 1.21% (9/ 742) (Marlina et al.,2014). In captive birds, WNV was detected serologically which accounts for 4.41% (3/68) in Selangor (Rais et al., 2011). From a study conducted on the horses in central part of Selangor, there are 7/20 horses were molecularly detected positive for WNV antigen and 19/20 horses are seropositive to WNV (Sifa et al., 2018) .Other than that, 5/41 bats from Hutan Simpang Wang Mu were detected positive WNV RNA using RT-PCR (Ain-Najwa et al., 2020b). Wild bird from Perak and Selangor were studied and around 18.75%(29/155) of the population were seropositive towards WNV while molecular detection using RT-PCR revealed that 15.2% (16/105) of wild birds were tested positive for WNV antigen(Ain-Najwa et al., 2020a). Furthermore, a study performed in macaques from Pahang, Perak and Johor have the seroprevalance of 29.6% (24/81) (Ain Najwa et al., 2018; Ain-Najwa et al., 2020b). Ruminants also proven to have the antibody against WNV which is around 17 out of 30 cattle from a study done in Ladang Angkat UPM (Fatihah et al.,2019).

2.2 STRUCTURE AND GENOME OF WEST NILE VIRUS

West Nile Virus (WNV) is a mosquito borne virus which is one out of 70 viruses that belong to the genus Flavivirus, family Flaviviridae (Habarugira et al.,2020; Linda et al.,2011). WNV is a member of Flavivirus sero-complex that includes Japanese encephalitis virus, Murray Valley Encephalitis Virus and St Louis encephalitis virus (Petersen et al., 2013; Mansfield et al., 2011;. Van der Meulen et al., 2005).

The structure of WNV virion appear spherical, enveloped with icosahedral nucleocapsid and approximately 50 nm in diameter. The genome is single-stranded, positive sense consist of 11kb RNA (Habarugira et al., 2020). The genome is transcribed as a single polyprotein which is cleaved by host and viral proteases producing three structural proteins and seven non-structural proteins. The three structural proteins are capsid(C), pre-membrane (prM) and envelope (E) proteins as shown in Figure 2. The C protein involve in protecting viral RNA through encapsidation and uncoating during virus replication. The pr-M facilitates blocking of premature viral fusion while E protein mediates viral attachment, membrane fusion, and viral assembly as well as elicit neutralizing antibodies. The viral non-structural (NS) proteins are NS1, NS2A, NS2B, NS3, NS4A NS4B, and NS5 which is shown in Figure 3 involve in virus assembly, regulate viral transcription, replication and reduce host antiviral responses by evading the immune system (Chambers et al.,1990;Mansfield et al.,2011;Habarugira et al.,2020) .The non-coding region of WNV are the type 1 cap structure (m7GpppAmp) that is present at the 5’ end with no internal base of methylated adenine residues while the 3’ end terminates with CuOH that represent the lack of terminal po1y(A) tract(Chambers et al.,1990;Brinton et al.,2002).

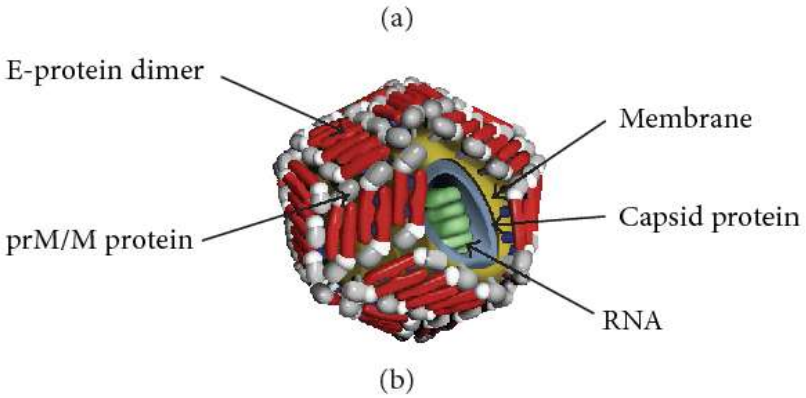

Figure 2: Structural virion of West Nile Virus**.** Structured protein are blue in colour while non-structural protein represented by the green colour. The structured protein consist of capsid(C), pre-membrane (PrM/M) and E protein. (Chancey et al.,2015)

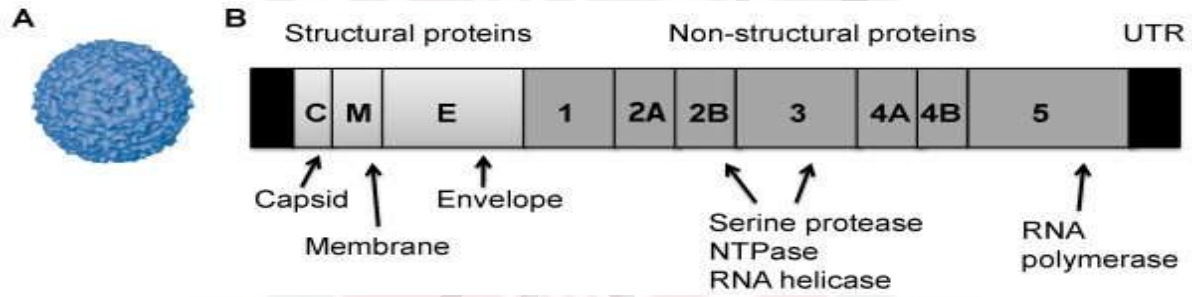

Figure 3: 11kb RNA genome of West Nile Virus **.** Note: WNV viral genome constitute of structural and non-structural protein (Marina et.al,2012).

2.3 TRANSMISSION OF WEST NILE VIRUS

WNV transmission analogue the concept of enzootic cycle within wild bird-mosquitoes-birds and other mammals as shown in Figure 4 .Birds are the primary reservoir vertebrate host while mosquitoes are predominate arthropod vector (Mohammed et.al, 2019). The virus usually persist in circulation between mosquito vectors and avian species. Mammals, reptilians and amphibians are the dead end host. A competent mosquito vector has specific receptors for WNV on endothelial cells lining of the mosquito's midgut that allow WNV to invade and replicate in the cell. From the midgut, the virus travels to the mosquito salivary

glands via hemolymph. The virus then penetrate and replicate in the salivary glands and transmit the virus to definitive hosts through salivary secretions during blood meals (Girard et al., 2004; Molaei et al., 2006; Mendelin et al., 2016). The competent *Culex (C) sp.* mosquitoes are *C.pipiens, C. vishnui* and *C. Quinquefasciatus* (Molaei et al, 2006; Sule et al., 2018).

Birds are the amplifying reservoir host in which the replication of the WNV occur rapidly at very high titre and able to infect the mosquitoes that bite them. Crows, magpies , house sparrows (Passer domesticus), house finches and other passerines appear to develop the highest concentrations of virus in the blood and have the longest duration of viraemia. The virus persist in the infected birds at a very high viraemic load that causes mosquito vector to get the infection through blood meals. The infected bird also could spread the virus directly to other birds or mammals through oral- fecal route .The shedding of the virus can occur through the faeces of infected birds too (Kilpatrick et al.,2007;Colpitts et al.,2012).

Mammals such as humans, horses and rodents are incidental dead end host that do not produce viraemic load to be uptaken by the mosquitoes (Crook et al.,2002). While, experimental intraperitoneal inoculation or mosquito bites in rodents such as mice or hamster showed development of high viraemic loads which affect the brain tissues causing encephalitis (Linda et al.,2011).

For humans, spending more time outdoor or travelling to endemic regions pose a higher risk to be exposed to mosquito bites (Hayes et al., 2005;Esser et al.,2019). Healthcare, laboratory workers, veterinarians, animal handlers, animal slaughterers and butchers are

also at higher risk of being in contact with WNV via needle sticks injury, accidental cuts or contamination of open wound (Vonesch et al., 2019).

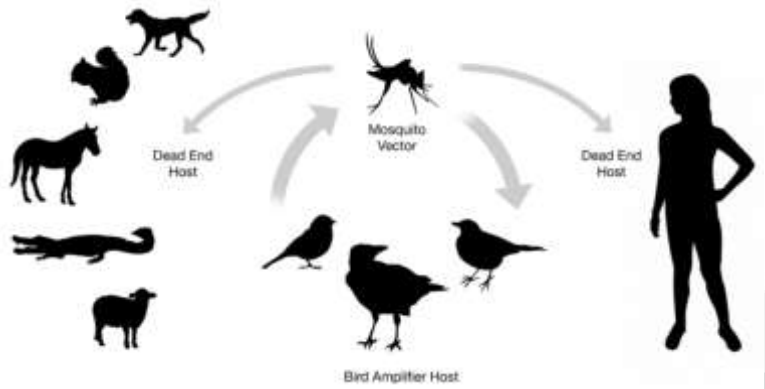

Figure 4: West Nile Virus transmission cycle. West Nile virus circulates between Culex species mosquitoes and avian amplifying hosts. Infected humans and other vertebrate species can be affected as incidental dead-end hosts (Byas & Ebel,2020).

2.4 CLINICAL SIGNS OF WEST NILE VIRUS

West Nile virus infection in humans are usually asymptomatic and to know if the person really being infected, it would take around five to fifteen days of incubation period (Chowers et al.,2001). The person would show mild febrile illness such as fever with chills, headache; muscle, bone and joint pains; nausea and vomiting, accompanied by rashes and it is typically self-limiting which would usually lasts for few days to weeks (Chowers et al.,2001;Petersen &Marfin ,2002). Less than 1% of infected patients would be susceptible to a neuroinvasive form of the disease such as meningitis, encephalitis, acute flaccid paralysis with poliomyelitis, which would lead to death especially in the elderly people (Petersen et al.,2003).

Birds are amplifying reservoir host of WNV. If disease occurs in birds, it is characterized by neurological signs that include ataxia, paralysis, paddling, torticollis, opisthotonus, and incoordination and the non-neurological signs are depression, lethargy, ruffled feathers, weight loss and myocarditis. Horses are amplifying dead end hosts of WNV. Disease in horses is mainly characterized by fever, anorexia, weakness, ataxia and recumbency or paralysis, abnormal behaviour, cranial nerve deficits, head pressing and teeth grinding (Van der meulen et al.,2005)

Other species that affected by WNV are primates, bats, pigs, ruminants and reptiles. Affected bats are mostly weak and succumb to paralysis (Davis et al., 2006). In reptilians like snakes ,the main clinical signs are weakness and immobility (Dahlin et al. ,2016) .Pigs develop low viremias and it is unlikely that they are amplifying hosts, but they can be useful as sentinels due to their serological reaction (Teehe et al. ,2005). Macaque prone to scrotal edema, anorexia, weight loss, and weakness (Ratteree et al., 2004).Ruminants such as sheeps and white tailed deer diagnosed with WNV had clinical signs of anorexia, fever, tremors and ataxia (Miller et al.,2005;Rimoldi et al.,2016)

Rodents especially wild rats are not naturally infected with WNV unlike other virus namely Hantavirus and Tick borne Encephalitis where they are the reservoir hosts. However, symptomatic neurological signs such as ataxia, tremor, depression and severe viraemia were showed by experimental rodents inoculated with WNV (Kiupel et al., 2003; Lim et al.,2011). There is a study done on squirrels in which they elicited uncoordinated movement, paralysis, circling, lethargy and death (Padgett et al. ,2007)

2.5 PATHOGENESIS OF WEST NILE VIRUS

As shown in Figure 5,following an infectious mosquito bite, initial WNV replication occur locally in the keratinocytes and Langerhans dendritic cells of the skin epidermis. The local virus replication is enhanced due to the immune modulation of the host response by the mosquito saliva through two mechanisms, including alteration of leukocyte proliferation and recruitment to the site of bite, and cytokine signalling by suppressing the production of interleukin (IL) 2 and IFN γ (Lim et al.,2011). The infected Langerhans cells migrate to the draining lymph nodes. Infected cells and free virus particles are picked up by macrophages and cleared either directly through phagocytosis or indirectly enhanced antigen presentation, cytokine, and chemokine secretion (Habarugira et al., 2020). While macrophages clear the infection, virus replication continues in dendritic cells in the lymph nodes resulting in primary viraemia and subsequent infection of peripheral organs such as kidney and spleen via hematogenous route. The primary clinical signs usually present at the end of the incubation period, within the range of 2 to 14 days post infection. WNV invades the CNS via hematogenous spread due to enhanced viral burden in serum correlates with earlier viral entry into the brain . In mice, WNV crosses the BBB and infects the CNS after the peak of viremia at day 3 (Samuel et al.,2007).The mechanisms that may contribute to WNV CNS infection, including the infection or passive transport through the endothelium or choroid plexus epithelial cells due to increased cytokine mediated vascular permeability followed by infection of olfactory neurons and spread to the olfactory bulb as well as the "Trojan horse" mechanism in which the virus is transported by infected immune cells that traffic to the CNS and lastly ,the direct axonal retrograde transport from infected peripheral neurons(Petersen et al.,2013).

In immunocompetent animals, WNV is largely cleared from the serum and peripheral organs within the end of first week and infection of the central nervous system (CNS) is observed. Rodents that succumb to infection develop a CNS pathology similar to that observed in human WNV cases.WNV infection is not significantly detected in non-neuronal CNS cell populations in humans or animals. In surviving wild-type rodents, WNV is cleared from all tissue compartments within 2 to 3 weeks after infection. However, persistent viral infection in the brains of CD8 T-cell negative or perforin-deficient mice and in the brains and kidneys of infected hamsters has been reported. Mice that lacks IFN-α/β/ receptors or IFN- γ or the IFN- γ receptor showed a greater peripheral viral burden, entry into the CNS, and increased fatality (Lazear et al., 2011).

In rodent species, the golden hamster develops persistent shedding of WNV in the urine for up to 8 months. As for rat species which is experimentally inoculated, the viraemic load is higher in the brain sample similar to the human at severe WNV infection stage. Some fox squirrels apparently shed WNV orally for long periods of time, at relatively low levels as fox squirrels are highly peri-domestic and often forage at bird feeders (Dietrich et al., 2005).

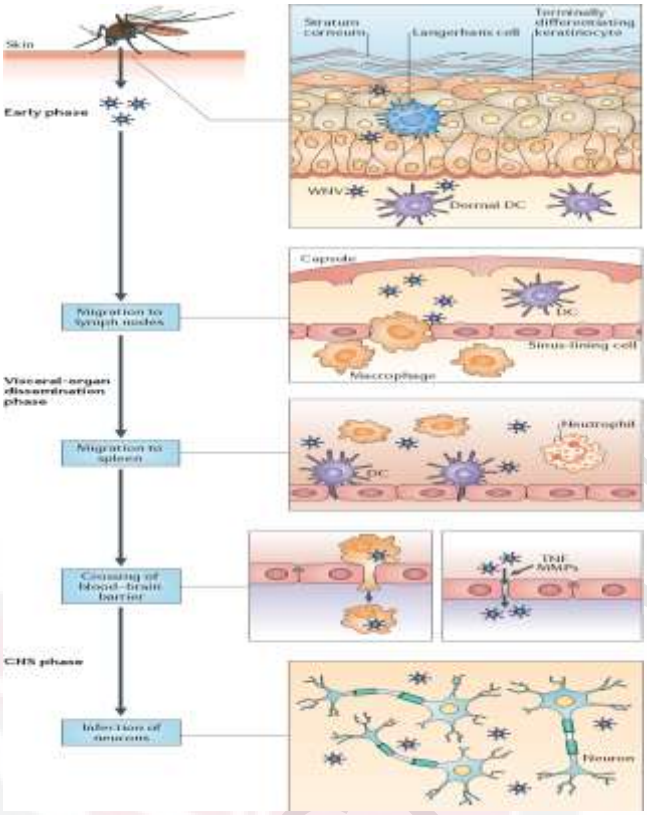

Figure 5: Schematic Diagram of West Nile Virus infection (Suthar et al.,2013). Following a subcutaneous bite of mosquito, WNV replicates in keratinocytes, dendritic cells (DCs) and Langerhans cells. Infected DCs migrate to the regional lymph node and the virus seed into the node . Replication within the draining lymph node leads to viraemia and subsequent infection of peripheral organs such as spleen, kidney and liver. By day 4, viral replication peaks in the spleen and serum. Between day 6 and day 8 after infection, WNV is cleared from peripheral organs. Then, in the severe infection, the virus cross the blood brain barrier and enter brain and spinal cord via increased permeability of the endothelial cell by cytokines, infection of olfactory neurons or through'Trojan Horse' mechanism, Lastly, the WNV is transported to the central nervous system (CNS) which would eventually infects and causes injury to neurons within the brain stem, hippocampus, cortex, cerebellum and spinal cord (Eldadah et al., 1967).

2.6 DIAGNOSIS OF WEST NILE VIRUS

Diagnosis of WNV is based on the history of exposure, clinical signs and the result of diagnostic tests. The presumption of the WNV disease in susceptible hosts is confirmed by laboratory tests (Ledermann et al., 2011). The preferred rapid and efficient serological assays to detect the WNV antibodies are competitive enzyme-linked immunosorbent assay (ELISA), plaque reduction neutralization test and immunofluorescence assay (IFA) (Malan et al., 2003; Weingartl et al., 2003). For the molecular detection of specific WNV antigen, the process would be nucleic acid amplification by reverse transcriptase polymerized chain reaction (RT-PCR).

2.6.1 Competitive Enzyme-linked Immunosorbent Assay (c-ELISA)

IgM and IgA are the first antibodies that usually detected 3 days post infection. The duration of IgM present depends on various species. In horses, IgM would be secreted for 7-14 days post infection (DPI),4-5 DPI in chickens and around 500 days DPI in humans(Bunning et al.,2002;Roehrig et al.,2003). IgG antibodies appear by the seventh day of illness and can be detected by 3rd week of infection.

Evidence of WNV exposure was determined by using serological assays namely WNV IgG based competitive ELISA(c-ELISA). This assay is a reliable marker in screening presence of WNV neutralizing antibodies in animal which is neither vaccinated nor infected in the past (Ain Najwa et al., 2020). However, WNV IgG c-ELISA has the tendency to cross-react with other Flavivirus particularly Dengue virus(DEV), Japanese Encephalitis Virus(JEV) and Tick-borne virus (TBEV)(Petersen et al.,2003) .Therefore, WNV IgG c-ELISA is less specific compared to IgM based capture ELISA which can detect only WNV during acute infection and prevent cross reactivity with other flavivirus.

2.6.2 Plaque Reduction Neutralisation Test

The plaque reduction neutralization test (PRNT) is the gold standard serological assay for WNV which is acquired in measuring protective and neutralizing antibodies in serum (Kuno, 2003). PRNT is usually used to identify the specific serum antibodies for WNV because of its high sensitivity and specificity to avoid the cross reactivity of the antibodies with other flavivirus (Johnson et al., 2010). PRNT usually require the serum samples which are tested with WNV together with other flavivirus.The specific virus is determined when the virus exceed the 4 folds neutralizing titre compared to other viruses (Di Gennaro et al., 2014).However, the test would take several days to complete, requires an environment with a high level of biosafety for manipulating infectious WNV and it is not suitable for large-scale screening of susceptible animals.

2.6.3 Nucleic acid amplification by Reverse Transcriptase Polymerized Chain Reaction

The molecular detection of WNV RNA can be performed using quantitative real time PCR(qRT-PCR), reverse transcriptase PCR (rt-PCR) and nested PCR (Shi&Wong,2003).The standard RT-PCR has equal sensitivity to real-time RT-PCR which is useful for the testing of small numbers of specimens or as a confirmatory assay. A nested PCR assay is utilized to further improve the detection limit by approximately 10-fold. Nucleic acid-based techniques, especially reverse transcription (RT)-PCR, have the advantages of speed, specificity, and sensitivity for detection of viral RNA.

In humans, the viral load is higher in whole blood compared to plasma at time of infection. Besides, the WNV persist for several months in kidney cells which is found in shedding of urine especially in dogs, humans and experimental animal like rat (Root et al.,2005).

2.7 TREATMENT , PREVENTION AND CONTROL OF WEST NILE VIRUS

The treatment for WNV infection is mainly supportive. Several therapeutic approaches include immune γ-globulin, West Nile virus–specific neutralizing monoclonal antibodies, corticosteroids, ribavirin and interferon α-2b but the efficacy is not proven due to less people for clinical trials (Gyure K.A. et al.,2009). For animals like birds and horses, the treatment is supportive followed by anti-inflammatory medications such as flumixine meglumine, dexamethasone, and DMSO . Mannitol aids in reducing cerebral oedema and vitamin E facilitate healing by providing antioxidant effect (Habarugira et al.,2020)

The prevention and control measure for WNV infection would be vaccination. As for now, there are no vaccines for WNV in humans due to under development and viral amplification cannot be controlled in nature with human as incidental host. There are vaccines for horses as a measure to control the spread of WNV. The ultimate choice to prevent WNV would be through community based mosquito control programs using integrated pest management principles. Mosquito repellent that contains diethyltoluamide or permethrin can be sprayed on exposed skin and clothing and eliminate standing water in vicinity if possible to prevent mosquitoes from breeding (Ain najwa et al., 2019). Insect repellents containing DEET, IR3535, oil of lemoneucalyptus, and picaridin are safe and proven high efficacy in preventing mosquito biting (Petersen et al.,2013).

3.0 MATERIALS AND METHOD

3.1 Study Design

The application for Institutional Animal Care and Use Committee (IACUC) ethical clearance has been approved for this project (RO43/2017). Twenty three (23) wild rats archived serum samples were obtained from Laboratory of Virology UPM in August 2019 which were sampled in Pasar Borong Seri Kembangan, Selangor. Data of the collected wild rats such as species, age and sex were recorded as shown in Table 1.

Table 1: Number of rats captured in relative to the species, sex and age

Rat species	**Sex**		**Age**	
	Male	Female	Adult	Juvenile
Rattus rattus	8	9	9	8
Rattus norwegicus	4	2	3	3
Total	23		23	

3.2 Sample collection, processing and storage

Blood were collected from the wild rats to obtain serum samples for serological and molecular detection of WNV using ELISA and RT-PCR, respectively. The blood collection sites were either saphenous vein, tail blood vessels, retro-orbital plexus or heart to obtain maximum blood for serum extraction. The blood collection site were wiped with alcohol swab to be aseptically prepared and the blood were taken using 23 gauge needle (B.Braun Sterican, USA) with 5mL syringe(Terumo®Syringe, Japan) from each wild rats and placed

in plain tube (BD Vacutainer®, New Jersey, USA). The samples were processed in the Laboratory of Veterinary Virology UPM .The serum were centrifuged at 4000*g* for 10 minutes and transferred into 1.5 mL tube (Eppendorf, USA) and were stored at -80°C freezer (SANYO Ultra Low, Osaka, Japan) until further use.

3.3 Enzyme-linked Immunosorbent Assay (ELISA)

Competitive ELISA kit (ID Screen® West Nile Competition Multi-species, France) was used to detect the anti-envelope protein (prE) antibody of WNV. The test procedure were carried out based on the manufacturer's instructions. The microwells were coated with WNV envelope protein antigen. Firstly, 50 µL of dilution buffer together with 50 µL of samples were added in each microwells. 50 µL of Positive Control (PC) was added to well A1 and B1 followed 50 µL Negative Control (NC) were added in well E1 and F1 while C1 and D1 were the blanks. The microwell was let to be incubated for 90 minutes. Then, washing was done 3 times using 300 µL of Wash Solution.100 µL of Conjugate 1X were added to each well and incubated for 30 minutes. After that, washing process continued. Next,100 µL of the substrate solution were added to all the wells and let to be incubated for 15 minutes.Lastly,100 µL of stop solution was added to each well to stop the reaction.

The absorbance value is used to measure the result quantitatively for high specificity. The absorbance value was read at 450nm by using ELISA reader (Tecan M200 Infinite Pro Microplate, Switzerland) to get average optical density (OD). The result were obtained by using the calculation of Sample(S)/Negative Control (NC) (%) = OD sample/OD (Negative control) ×100 as shown in Table 2. The changes in colour of the samples is the qualitative measure of c-ELISA reaction. If more antibody-conjugate with WNV antigen were formed, then there is no antigen-antibody complex. The colour will appear yellow in negative

results. If more sample anti pr-E antibody reacts with WNV pr-E, more antigen-antibody complex formed. Thus, the colour intensity reduced to colourless in positive results.

Table 2: Interpretation of WNV status using ELISA ID Screen® West Nile Competition Multi-species kit

Result	Status
S/N(%)≤40%	Positive
40%≤ S/N(%)≤50%	Doubtful
S/N(%)≥50%	Negative

Note: S: sample, N: Negative control

3.4 Reverse Transcriptase Polymerase Chain Reaction(RT-PCR)

3.4.1 RNA Extraction

RNA extraction was performed on serum sample using TRIsure™ (Bioline, London, UK). RNA extraction procedure was divided into several processes which were homogenization/lysis phase separation, RNA precipitation, washing, dissolving RNA and determination of RNA concentration and purity. In homogenization step, serum samples were thawed and vortex for about 10 seconds. Then, 150 µL of serum samples were transferred into 2 mL tubes and 900 µL of TRIsure™ was added to each tubes. The mixture were vortexes and been incubated for 5 minutes at room temperature. For the phase separation, 200 µL of chloroform were added to each tubes and vortex for 30 seconds followed by incubation for 5 minutes. After that, the tubes were centrifuged at -4 °C 12000 *g* speed for 15 minutes in a refrigerated centrifuge (Eppendorf, USA). Three layers of

separation were formed namely the aqueous layer (upper part), interphase (middle) and pale green (bottom) which contain RNA, protein and DNA, respectively. The aqueous layer were chose and transferred into the 1.5 mL microcentrifuge tubes without interrupting the interphase layer. RNA precipitation step were performed by adding 500 µL of isopropyl alcohol to each tube. The tubes were inverted gently to mix well and then incubated for 10 minutes. The tubes were centrifuged at -4 °C at 12000 *g* speed for about 10 minutes. Later, the RNA precipitates were seen viable at the bottom of the tubes. Supernatants were removed gently. The next step would be the washing step which was carried out twice to remove the contaminants. After that, 1000 µL of 75% ethanol were added to each tubes and the tubes were vortex for 15 minutes followed by centrifuge at -4 °C, 7500 *g* for 5 minutes and the supernatant were discarded. The tubes were air dried for about one and half hour or longer until no ethanol droplet could be seen. Then, precede to RNA re-dissolving in which 15µL of ddH2O were added to each tubes to dissolve the RNA pellet. The RNA pellet was pipetted up and down to mix properly. The tubes with the samples were then placed in water bath set (830-S1, Taiwan) at 60 °C for 10 minutes. Finally, RNA purity and concentration were determined using spectrophotometer at absorbance value of 260/280 (Eppendorf, Germany). The range within 1.8-2.0 value indicates RNA with good purity.

3.4.2 Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

The RT-PCR procedure begin by preparing the master mix. The preparation of Master Mix took place in the BSC to avoid contamination. Table 3 shows sequence of primers used which targeted the highly conserved region between capsid (C) and pre-membrane (PrM) of WNV. Synthetic gene which is 5 µL plasmid used as positive control and 5µL of ddH20 is used as negative control with the addition of 20 µL Master Mix respectively (Table 4). The other reactions involve 5µL RNA template from sera samples with 20µl Master mix added to other 1.5 mL tubes used as templates. The tubes with the mixtures were vortex and placed into thermal cycler (Eppendorf, Germany) and was set to run with optimized RT-PCR protocol for WNV as shown in Table 5.

Table 3: Forward and Reverse Primer used in RT-PCR for West Nile Virus detection

Primer	Primer sequence	Product size (bp)
Forward	5’ CCA ATA CGT TTC GTG TTG G 3’	470
Reverse	5’ ATG TCT TCA GGG TCA TTT CC 3’	

Table 4: List of reagents used to prepare Master Mix (1X reaction)

Reagent	Volume(µL)/tube
ddH2O	10
2x MyTaq One step	8
WNV Forward primer	0.5
WNV Reverse Primer	0.5
Reverse Transcriptase	0.5
Ribosafe RNase	0.5
RNA Template	5
Total volume	**25µL/tube**

Table 5: Optimized Thermal Cycling Protocols using RT-PCR for molecular detection of West Nile Virus

Protocol	Temperature(°C)	Time	Cycle
Reverse Transcriptase	45	20 minutes	1
Polymerase Activation	95	1 minutes	1
Denaturation	95	10 seconds	40
Annealing	52	10 seconds	40
Extension	72	30 seconds	40
Final Extension	72	5 minutes	1
Hold	10	Infinity	1

3.4.3 Gel Electrophoresis

Gel electrophoresis was conducted to separate DNA fragment according to sizes. First of all, 1.5% gel was prepared by mixing 1.5 g of agarose gel powder (Biotechnology 1st Base, Singapore) in 100 mL x TAE buffer solution (Trisacetate-EDTA). The gel solution then microwaved at medium high temperature for 2 minutes until the solution boils to dissolve the gel. Five µL of Red Safe™ (Red Safe™, South Korea) was added to the mixture and the bottle flask was swirled gently to mix well. The mixture was poured into a cast tray with well combs had been placed on it. The gel was allowed to solidify for 30 minutes .The gel was transferred to the gel tank once it had fully solidified. The tank was filled with 1xTAE buffer and DNA of the samples, positive control and negative control were loaded in respective wells on the solidified gel. Then, 3µL of molecular marker (Bioline, UK) was added into the second lane without loading dye. Depending on the number of reactions, 1 µL of blue loading dye was pipette on the paraffin film. From the third lane, 5 µL PCR products of the samples were mixed with 1 µL loading dye and loaded carefully without puncturing the gel followed by positive control were in the last lane and negative control in the first lane. After loading, the electrodes on the gel tank were connected to the power supply (Bio-Rad, California, USA).The gel was run at 90V for 30-35 minutes to reach the last third line and finally the gel was viewed under UV trans illuminator (Syngene, United Kingdom

4.0 RESULTS

4.1 Enzyme Linked Immunosorbent Assay(ELISA)

Sera samples of 23 wild rats subjected to c-ELISA showed none of the rats were positive to WNV antibody. The colour change into colourless solution using the c-ELISA kit shows qualitative measure of positive WNV IgG antibody as shown in Figure 6 while the absorbance values with Sample(S)/Negative control(NC) (%) ≤ 40 in ELISA reader indicates the positive quantitative measure of WNV-IgG antibody as shown in Figure 7. All the wild rat samples are negative for c-ELISA result. as observed from Figure 6 and Figure 7.

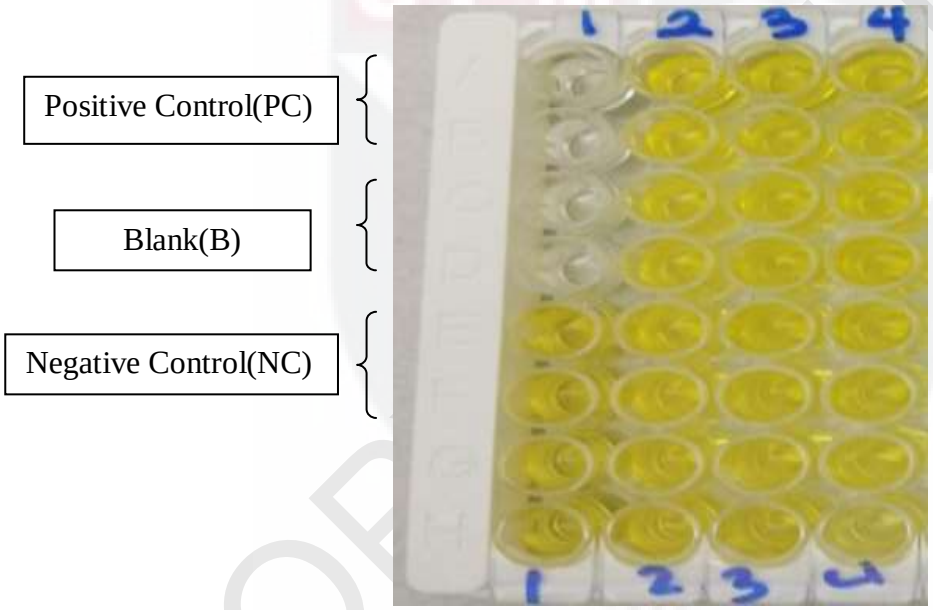

Figure 6:Qualitative WNV ELISA result . All 23 samples are yellow in colour excluding,2 positive control(PC) in well A1 and B1,2 blanks in well C1 and D1 and 2 negative control(NC) in well E1 and F1 shows negative results

S/N (%)=OD Sample/ OD Negative control × 100

<>	1	2	3	4
A(PC)	-1%	94%	92%	91%
B(PC)	-2%	95%	86%	84%
C(BLANK)	1%	84%	88%	89%
D(BLANK)	-1%	87%	88%	94%
E(NC)	94%	84%	87%	80%
F(NC)	89%	84%	83%	73%
G	78%	78%	72%	63%
H	74%	78%	71%	

Figure 7: Quantitative WNV ELISA result. All 23 samples excluding blanks, PC - and NC shows the value of S/N(%)≥50 indicates negative WNV antibody in the samples.

4.2 Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

Based on RT-PCR analysis of the wild rats sera, all 23 wild rats were negative for WNV RNA as shown in gel electrophoresis in Figure 8.There are no single band formed at 470 base pair (bp) of positive control in all the samples.

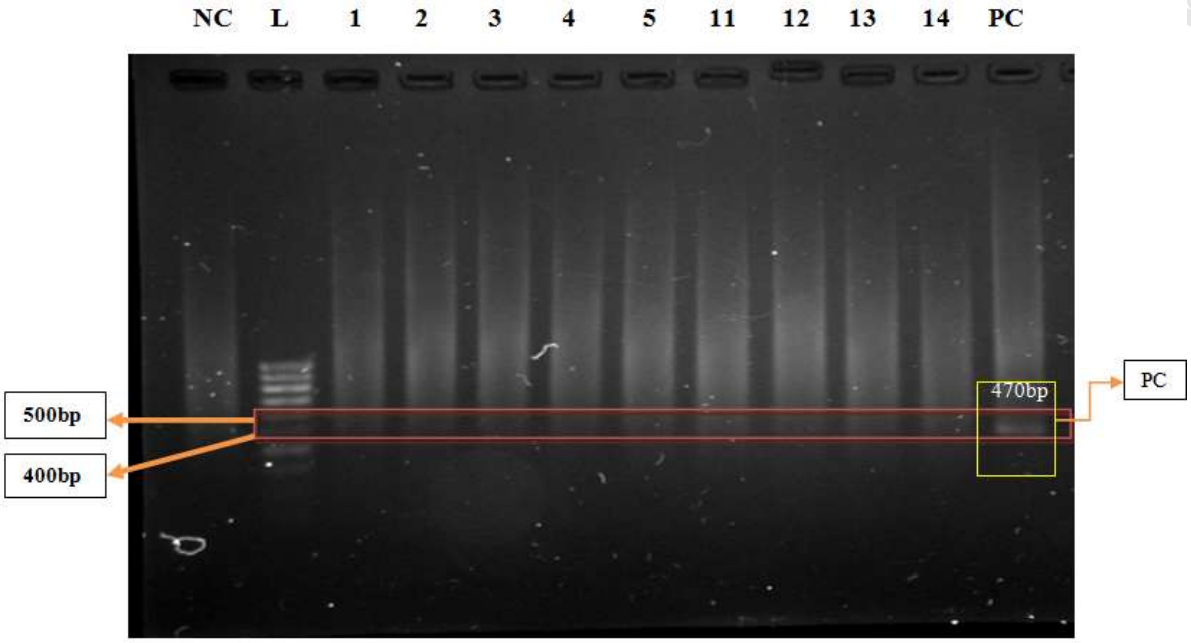

Figure 8: Nucleic acid amplification by RT-PCR in sample 1-5 and11-14 samples. There was no band formation at 470bp of positive control. Note: NC=Negative control, L= Ladder, PC=positive control

5.0 DISCUSSION

Studies shows that West Nile Virus (WNV) was detected in captive birds,wild birds, bats, horses, macaques, pig and Orang Asli (Rais et al.,2011; Marlina et al.,2014; Ain et al.,2018; Sifa et al.,2018;Mohammed et al.,2019; Ain et al.,2020a;Ain et al.,2020b). However in Malaysia, there are no reported cases in either human or animals with clinical infection of West Nile Virus (Mohammed and Yasmin, 2019). Rattus sp. are pests known to be seropositive to Yellow Fever Virus, West Nile Virus, Japanese Encephalitis Virus and Tick borne Encephalitis Virus (*Flaviviridae)* ,Hantavirus (*Bunyaviridae*) and Chikungunya virus (*Togaviridae)* (Cigarroa-Toledo et al.,2016;. Rattus sp. have similar vector-feeding patterns as other mammal species such as monkeys, bats and humans and their free roaming and scavenging lifestyles would expose them to multiple pathogens including pathogen present in wider populations at a low prevalence or in populations that are difficult to sample (Heinz-Taheny et al., 2004). Besides, Culex mosquitoes which plays important role as a primary vector of WNV can be widely found in tropical climate country like Malaysia that enable the survivability of the mosquitoes (Ain Najwa et al.,2019). Although rats had been recognized as principle reservoir of multiple pathogens, the status of WNV infection in wild rodents particularly in Malaysia remain poorly understood. Therefore, our study mainly focused on the seroprevalance and molecular detection of WNV) in Rattus sp.

Serological analysis of WNV using competitive ELISA showed that all wild rats sera were negative for WNV. This is might be due to no previous exposure to WNV that causes no IgG antibodies being produced towards WNV (Lim et al., 2013). Apart from that, WNV antibody prevalence in Rattus sp. is very low around 5.6% (2/36) in a study carried out in Slidell, Louisiana (Dietrich et al., 2005). Besides, a recent study conducted in Merida, Mexico suggest that many black rats (*R. rattus*) had antibodies reactive to flaviviruses but none of them were positive for WNV antibodies serologically (Cigarroa-Toledo et al.,

2016). However, studies conducted in Pakistan, Israel, Austria, Tunisia, central Africa and Madagascar showed that WNV at a low seroprevalence were detected in brown rats (*R. norvegicus*) and roof rats (*R.rattus*) (Akov & Goldwasser, 1966; Abdel-Wahab and Imam, 1970; Darwish et al.,1983; Fontenille et al.,1989; Sixl et al.,1989 ; Dietrich et al.,2005; Blitvich BJ ,2009; Root, J.J., 2013).

The negative result of all samples for WNV RNA indicate absence or low transient viraemic load in Rattus sp.(Hirota et al.,2013).Besides, blood serum sample used in this experiment is suitable enough to detect WNV antigen in a sufficient viraemic levels . However, in a scarce low viraemic load, WNV may be persist in the site where viral shedding occur especially in faeces, urine and oropharyngeal swabs (Root,J.J., 2013).

Moreover, there are some receptors and controlled tissue tropism generally acquired by these rattus sp. that resist WNV infection during innate and adaptive immune response. The actions of the RIG-I like receptor (RLR) and type I interferon (IFN) signalling pathways are responsible to enhance innate immune response against WNV and restrict tissue permissiveness to WNV (Loo YMet al., 2008; Suthar MS et al.,2013). Rattus species with chemokine receptor like CCR5, was found to be a strong host defence factor against WNV at early clinical infection (Lim J.K. et al., 2010). Furthermore, these wild rodents too might acquire CD8+ T cells that are important in controlling viral load in rodents towards WNV infection with the concurrent involvement perforin role (Wang et al., 2003; Shreshta et al., 2006). These rodents might have WNV-specific murine CD4 T cell which produces IFN- γ and IL-2 That lead to direct antiviral activity (Colpitts et al., 2012).

6.0 CONCLUSION AND RECOMMENDATION

Based on the study conducted, wild rats were found to be negative to WNV antibody and nucleic acid through serological and molecular methods, respectively. The rats in this study might possibly were not exposed to WNV, not in viraemic state or immunologically may have host receptors that hinder the infection.

Further study however, should be conducted in larger sample size in various different geographical locations to have a comprehensive and accurate overview of the disease in wild rodents found in Malaysia. Besides, as detection of WNV antigen at low viraemic level is challenging, it is recommended to complement the study by taking samples such as faeces, urine or oropharyngeal swab to determine WNV shedding status.

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