MOLECULAR DETECTION OF WEST NILE VIRUS IN HORSES IN KELANTAN, MALAYSIA

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BY

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CERTIFICATION

This is to certify that we have read this research paper entitled '**Molecular Detection of West Nile Virus in horses in Kelantan**' by Fatin Nadhirah Binti Hazarul Hisham, and in our opinion, it is satisfactory in terms of scope, quality, and presentation as partial fulfilment of the requirement for the course DVT55204 – Research Project.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

ABBREVIATIONS	DEFINITION
WNV	West Nile Virus
RT-PCR	Reverse Transcription Polymerase Chain Reaction
JE	Japanese Encephalitis
FCoV	Feline Corona Virus
RNA	Ribonucleic Acid
PCR	Polymerase Chain Reaction
IgM	Immunoglobulin M
ELISA	Enzyme Linked Immunosorbent Assay

MOLECULAR DETECTION OF WEST NILE VIRUS IN HORSES IN KELANTAN, MALAYSIA

ABSTRACT

West Nile Virus (WNV) is under-reported because it is usually asymptomatic in horses, only approximately 20% of infected horses will show signs. Furthermore, studies have shown that there were seropositive results for presence of West Nile Virus in migratory birds. This increases the chance for the virus to be transmitted to the horses in Kelantan with the *Culex* mosquito as the vector. Moreover, no research for molecular detection of West Nile Virus (WNV) in horses in Kelantan has been done before. The objectives of the study were: (1) to determine the molecular prevalence of West Nile Virus (WNV) in 30 horses in Kelantan and (2) to determine the risk factors associated with West Nile Virus (WNV) infection among horses in Kelantan. Thirty horses were randomly chosen for blood collection in this study. Molecular detection of West Nile Virus (WNV) was conducted using nested Reverse Transcription Polymerase Chain Reaction (RT-PCR). The prevalence of West Nile Virus in horses in this study was 0% through molecular detection. Risk factor analysis was unable to determine the correlation between the risk factors. The study's entirely negative outcome can be attributed to several factors. These encompass the sample size, the technique employed for West Nile Virus (WNV) detection, the absence of a specific positive control and the use of archived samples. The molecular detection method for West Nile Virus (WNV) used in this study has its own set of uncertainties and flaws. As a result, it can be that the negative findings from the archived samples in Kelantan does not definitively exclude the possibility of West Nile Virus (WNV) infection. Consequently, additional research is warranted.

Keywords: West Nile Fever, Horse, Molecular detection, Nested RT-PCR, Kelantan

PENGESANAN MOLEKUL VIRUS NIL BARAT PADA KUDA DI KELANTAN, MALAYSIA

ABSTRAK

Virus Nil Barat (WNV) sering dilaporkan kurang kerana biasanya tidak menunjukkan gejala pada kuda, hanya kira-kira 20% kuda yang dijangkiti akan menunjukkan tanda. Selain itu, kajian telah menunjukkan bahawa terdapat keputusan seropositif untuk kehadiran Virus Nil Barat dalam burung migrasi. Ini meningkatkan peluang untuk virus ini ditularkan kepada kuda di Kelantan dengan nyamuk *Culex* sebagai vektor. Selain itu, tiada kajian untuk pengesanan molekul Virus Nil Barat (WNV) pada kuda di Kelantan telah dilakukan sebelum ini. Objektif kajian ini adalah: (1) untuk menentukan prevalens molekul Virus Nil Barat (WNV) dalam 30 ekor kuda dari Kelantan dan (2) untuk menentukan faktor risiko yang berkaitan dengan jangkitan Virus Nil Barat (WNV) di kalangan kuda di Kelantan. Tiga puluh ekor kuda dipilih secara rawak untuk pengumpulan darah dalam kajian ini. Pengesanan molekul Virus Nil Barat (WNV) dilakukan dengan menggunakan Reverse Transcription Polymerase Chain Reaction (RT-PCR) berlapis. Prevalens Virus Nil Barat (WNV) dalam kuda dalam kajian ini adalah 0% melalui pengesanan molekul. Analisis faktor risiko tidak dapat menentukan korelasi antara faktor risiko. Hasil negatif sepenuhnya dalam kajian ini dapat dikaitkan dengan beberapa faktor. Ini merangkumi saiz sampel, teknik yang digunakan untuk pengesanan Virus Nil Barat (WNV), ketiadaan kontrol positif tertentu, dan penggunaan sampel arkib. Kaedah pengesanan molekul untuk Virus Nil Barat (WNV) yang digunakan dalam kajian ini mempunyai ketidakpastian dan kecacatan tersendiri. Oleh itu, adalah boleh dikatakan bahawa penemuan negatif dari sampel arkib di Kelantan tidak mengecualikan kemungkinan jangkitan Virus Nil Barat (WNV) dengan tegas. Oleh itu, penyelidikan tambahan diperlukan.

Kata Kunci: Virus Nil Barat, Kuda, Pengesanan Molekul, Nested RT-PCR, Kelantan

CHAPTER 1

1.0 INTRODUCTION

1.1 Introduction

Reports of West Nile Virus (WNV) disease was first published in 1963 (Pond,1963) and followed by detection of seropositive antibodies from captive birds in Selangor and from the Orang Asli Population from the second case in 2011 (Rais et al., 2011; Marlina et al., 2014). In 2018, there were seven out of 203 horses detected positive for West Nile Virus antigen in three selected areas in central part of Malaysia (Bande et al., 2023), indicating presence of West Nile Virus (WNV) in Malaysia with no clinical infections attributed to it in either humans or animals.

West Nile Virus (WNV) disease is not considered as a common disease in Malaysia, however this mosquito-borne virus that affects humans and other animals, particularly horses, is common in Africa, Europe, the Middle East, North America, and West Asia, in which horses represent 96.9% of reported non-human cases (Young, 2023). West Nile Virus (WNV) is an RNA virus, a spherical particle approximately 50 nm in diameter. This virus was first detected in West Nile Province in Uganda (World Health Organization: WHO, 2017). This virus has a zoonotic potential which can cause the spreading of the disease from infected horses and several species of birds to humans. Infected horses will show signs of ataxia, circling, hind limb weakness, limb paralysis, inability to stand, muscle fasciculation, blindness, fever, teeth grinding, and even acute death (Paré, 2018). Thus, equine West Nile Virus (WNV) is included in the World Organization for Animal Health-listed disease.

West Nile Virus can also replicate in birds, amphibians, reptiles, mammals, ticks, and mosquitoes which contribute to the spreading of the disease. Migratory birds from countries

endemic with West Nile Virus (WNV) that travel to Malaysia are one of the causes of the spreading of this disease. There are a few migratory birds that are endemic in Malaysia and are the potential sources of West Nile Virus (WNV) like Migratory birds Greater Sand Plover, Black-capped Kingfisher, Common Redshank, Terek Sandpiper and Lesser Sand Plover, and Common Sandpiper (Ain et al., 2020). Some of them migrate to countries that are endemic for West Nile Virus (WNV) like Africa, Middle east, West Asia, parts of Europe and also Australia according to the World Health Organization.

Mosquitoes are recognised as the main vector for West Nile Virus (WNV) transmission due to its abundance in Malaysia. According to Kumar et al. (2018), rice field plantations are common areas for mosquitoes to breed, the *Culex* primarily. *Culex* spp is the mosquito that acts as a vector for West Nile Virus (WNV) infection (Habarugira et al., 2020). Thus, horse stables located near the vicinity of migratory bird hotspots and with an environment that favours mosquito populations might be the risk factors for West Nile Virus (WNV) infection in horses.

Moreover, Kelantan is an area with abundance of rice field plantations and also active horse industry making it easily exposed to *Culex* spp. that could be the vector of West Nile Virus (WNV). The horse industry in Kelantan is very broad, there are horse breeding, equestrian sports, and others. Horse breeding is mostly conducted in Kelantan and Sabah (Hassan Nizam, 2019). Horse breeding was commonly conducted to improve the quality and breed of the horses. Equestrian sports like horse racing, show jumping and others are also frequently held in Kelantan.

1.2 Research Problem Statement

West Nile Virus (WNV) is under-reported because it is usually asymptomatic in horses, only approximately 20% of infected horses will show signs. Furthermore, studies have shown that there were seropositive results for presence of West Nile Virus (WNV) in migratory birds. This increases the chance for the virus to be transmitted to the horses in Kelantan with the *Culex* spp mosquito as the vector. Moreover, there is no research for molecular detection of West Nile Virus (WNV) in horses in Kelantan that has been conducted before, except in the central part of Malaysia.

1.3 Research Question

1.3.1 What is the molecular prevalence of West Nile Virus (WNV) infection among horses in Kelantan?

1.3.2 What are the risk factors associated with West Nile Virus (WNV) infection among horses in Kelantan?

1.4 Research Hypothesis

1.4.1 The molecular prevalence of West Nile Virus (WNV) is more than 5% among the horses in Kelantan.

1.4.2 The location of the stable is the risk factor associated with West Nile Virus (WNV) infection among the horses in Kelantan.

1.5 Research Objectives

1.5.1 To determine the molecular prevalence of West Nile Virus (WNV) in 30 horses in Kelantan.

1.5.2 To determine the risk factors associated with West Nile Virus (WNV) infection among horses in Kelantan.

CHAPTER 2

2.0 LITERATURE REVIEW

2.1 Horse industry in Kelantan

Kelantan's horse sector is diverse, with horse breeding, equestrian sports, and other activities. Horse breeding is mostly practised in Kelantan and Sabah (Hassan Nizam, 2019). Horse breeding was extensively used to improve the quality and breed of horses. Horse racing, show jumping, and other equestrian activities are very popular in Kelantan. Moreover, the horse endurance race is also one of the equestrian activities that can be found in Kelantan. A horse endurance race is a long-distance competition against the clock, it will test the speed and endurance of the horse. Although the rides are based on finishing time, they also emphasise the good condition of the horse upon finishing the race.

2.2 Common viral diseases in horse

Horses are susceptible to several common viral diseases, including Equine Influenza, Equine Infectious Anaemia and Equine Herpesvirus (Leith & Thometz, 2021). Equine influenza is a highly contagious respiratory disease that affects horses all over the world . It is caused by influenza viruses and spreads swiftly among nearby horses. Symptoms include fever, cough, nasal discharge, and a loss of appetite (Young, 2019). Vaccination is provided to help with illness prevention and control. Moreover, Equine herpesvirus is a virus that is spread through horses. It can produce nasal discharge, coughing, and fever as well as other respiratory symptoms (Lascola, 2023). It can cause neurological disorders, abortion in pregnant mares, and even death in extreme cases. Equine Infectious Anemia (EIA) is a viral illness that is spread by blood-sucking insects, infected needles, or blood transfusions. Fever, anaemia, weight loss, and oedema may occur in infected horses. The EIA is a notifiable sickness in

Malaysia, and strict control measures are in place, including mandatory testing and quarantining of infected horses.

Furthermore, West Nile Fever is not a widespread disease in Malaysia since it is more prevalent in the western area and temperate nations. The West Nile Virus (WNV) is a mosquito-borne virus that affects humans and other animals, with horses accounting for 96.9% of non-human cases reported (Young, 2023). The West Nile virus (WNV) is an RNA virus with a spherical particle diameter of around 50 nm. This virus was first identified in Uganda's West Nile Province (World Health Organization: WHO, 2017). This virus has zoonotic potential, which implies it may transmit disease to people from infected migratory bird species and mosquitoes. West Nile Virus may also replicate in birds, amphibians, reptiles, mammals, ticks, and mosquitos, aiding to spread the disease. Migratory birds from West Nile Fever-endemic areas that travel to Malaysia are a major source of disease spread. There are a few countries where West Nile Virus (WNV) is endemic with West Nile Virus (WNV) like Africa, Europe, the Middle East, North America, and West Asia (World Health Organization: WHO, 2017). Furthermore, Malaysia is residence to a number of migratory birds that are endemic to the country, including the Oriental Honey Buzzard, Black Baza, Japanese Sparrowhawk, Greater Spotted Eagle, Asian Openbill, Grey-faced Buzzard, Blue-tailed Bee-eater, Common Sandpiper, Pacific Golden Plover, Asian Brown Flycatcher, Arctic Warbler, and Brown Shrike. Some of them travel to places where West Nile Fever is prevalent. The vector for this disease is *Culex* mosquitoes also endemic in Malaysia with a prevalence of 9.6% in Pahang (Ali et al., 2011). Clinical signs of West Nile Fever in horses are ataxia, circling, hindlimb weakness, limb paralysis, difficulty standing, muscular fasciculation, blindness, fever, teeth grinding, and even early death can occur in infected horses (Paré, 2018). RT-PCR can also be used to detect this virus.

Prevalence is the proportion or percentage of a population that has a specific disease or condition at a specific moment in time or throughout a specified timeframe. It is a measure of the disease's impact on a certain demographic. Prevalence is calculated by dividing the number of infected individuals by the total population at risk.

The West Nile Virus (WNV) prevalence in Central Malaysia was 3.5%, with Seven out of 203 horses testing positive for West Nile Virus (WNV) antigen (Bande et al., 2023). However, the incidence is limited in the central part of Malaysia, hence additional research in the other regions is required.

2.3 Characteristics of West Nile Virus

The West Nile Virus (WNV) is an RNA virus in the genus of Flavivirus and the family of Flaviviridae. West Nile Virus (WNV) is also categorised as arbovirus which means an arthropod-borne virus. West Nile Virus (WNV) virions are spherical in shape with a diameter of 50 nm and composed of enveloped positive single-stranded viruses where the size of the genome is between 11 000 and 12 000 nucleotides long with icosahedral capsid (Bande et al., 2023). The West Nile Virus (WNV) is also an enveloped virus with positive-stranded ribonucleic acid (RNA) virus.

2.4 Transmission of West Nile Virus

West Nile Virus (WNV) can be transmitted to horses through the bite of infected *Culex* mosquitoes. The mosquitoes act as vectors by transmitting the virus from the reservoir host, for example from birds to the host. The virus can only be transmitted through mosquitoes, not from humans to horses and vice versa. This is because humans and horses are the 'end-stage' hosts thus the virus cannot spread from humans and horses (Venter et al., 2010). Birds are the amplifier hosts, which indicate that the virus will replicate in the bird before the birds

are bitten by mosquitoes and spread the disease to humans and horses. Moreover, birds that commonly act as the reservoir are crows, jays, and sparrows. The mosquito transmits the virus through its saliva when biting the 'end-hosts'. After biting the infected bird, the virus will move from the midgut to the salivary glands after the incubation period. Thus, that is why the virus is present in the saliva of the mosquitoes (Styer et al., 2011).

Moreover, vaccination for West Nile Virus (WNV) is only available for horses and not for humans. This is because horses are more susceptible to this disease and have a higher risk of severe illness and death compared to humans (Saiz, 2020). It is important to vaccinate horses against West Nile Virus (WNV) as a preventive measure, especially if the horse travels frequently to countries that are endemic to this disease.

2.5 Risk factors of West Nile Virus infection

Risk factors for West Nile Virus (WNV) infection include geographic location, mosquito contact, absence of immunisation, age, exposure to infected birds, and environmental variables (Montgomery & Murray, 2015). In Kelantan, horses are at great risk of contracting West Nile Virus (WNV). This might be owing to the number of horses of all ages in Kelantan, which is recognised for horse breeding. First and foremost, geographic location is one of the risk factors because horses that are in the endemic region have a higher risk for West Nile Virus (WNV) infection. Furthermore, mosquito exposure is also one of the risk factors. This is because *Culex* mosquitoes are vectors for the virus, thus exposure to the mosquitoes will increase the risk as mosquitoes can act as a vector and spread the disease (Yuseri et al., 2019). Moreover, unvaccinated horses are also one of the factors. Unvaccinated horses also have a higher risk (Gardner et al., 2007). After all, they are more susceptible to West Nile Virus (WNV) compared to vaccinated horses because they have antibodies from the vaccine. Furthermore, age is also one of the risk factors. Younger and

older animals are more susceptible because they have weaker immune systems (Long, 2023). Last but not least, the environmental factor is also one of the risk factors. This is because environments like standing water or stagnant pools might enhance the likelihood of mosquito populations and subsequent West Nile Virus (WNV) transmission. Besides, the quantity of paddy fields in Kelantan acts as a danger factor because the mosquitoes can breed in the paddy fields increasing the mosquito population.

2.6 Molecular detection of West Nile Virus

In order to detect West Nile Virus (WNV) by doing RT-PCR it is important to extract the RNA first from the collected sample by using a commercial RNA extraction kit or other methods. This step is important in RT-PCR to isolate the RNA that contains the virus's genetic material. After that, reverse transcription will be done to convert the virus RNA into complementary DNA (cDNA) (Lanciotti et al., 2000). Reverse transcriptase enzymes and specific primers can be used to synthesise the cDNA. After that, for PCR setup, it is important to prepare the PCR reaction mix that contains cDNA, West Nile Virus (WNV) -specific primers, nucleotides, and thermostable DNA polymerase enzymes. The primers should be tailored to amplify specific areas of the West Nile Virus (WNV) genome. Furthermore, for the PCR amplification, a thermal cycler and a specialised PCR programme will be used. A series of heating and cooling stages are typically utilised to denature the DNA, anneal the primers to the target regions, and extend the DNA strands. If the West Nile Virus (WNV) -specific DNA fragment is discovered in the sample, it is amplified (Shukla et al., 2012). After that, gel electrophoresis will be performed by running the PCR products on an agarose gel, this will allow the amplified DNA fragments to be visualised. Lastly, to properly visualise the amplified DNA bands, they can be stained with DNA-specific fluorescent dye (Farkas & Holland, 2009).

CHAPTER 3

3.0 MATERIALS AND METHODS

3.1 Study area and horse sampling

In this study, a total of 30 blood samples which had been previously collected and archived were used. These samples were taken from horses that were housed in private stables located in three different districts: Kota Bharu, Machang, and Bachok, all of which are in Kelantan. Before the blood samples were collected, each horse was thoroughly examined for any signs of abnormalities to ensure the accuracy and reliability of the study. Despite the thorough examination, none of the 30 horses showed any clinical signs or abnormalities. The study also took into account various risk factors that could potentially influence the results. One of the factors is the presence of paddy fields near the stables. It is generally understood that rural areas have a higher abundance of paddy fields compared to urban areas. Therefore, the proximity of the stables to these fields was considered a significant factor.

In this context, the horse stables located in Machang and Bachok were considered to be near paddy fields due to their rural location. This is in contrast to the city of Kota Bharu, which is more urban and therefore has fewer paddy fields. Out of the 30 horses included in the study, 25 were from stables located in rural areas that were near to paddy fields.

3.2 Blood sample collection

All the procedures conducted on animals were reviewed by the animal ethic committee of FPV, UMK to ensure that the procedures were conducted appropriately and humanely, with animal ethic approval code of UMK/FPV/ACUE/PG/001/2023 (Appendix A). Blood samples were collected by using an 18G needle with vacutainer and transferred to plain tubes and kept in an ice box during transportation. Upon arrival at the UMK laboratory, the samples were centrifuged and serum was taken. The serum was stored in a -80 °C freezer.

3.3 Molecular analysis

RNA was extracted from 250 µl of blood serum by using Acid guanidinium thiocyanate-phenol-chloroform (AGPC) extraction method, it is a liquid to liquid extraction technique. The RNA was isolated from DNA by using an acidic solution containing guanidinium thiocyanate, sodium acetate, phenol and chloroform accompanied with centrifugation (Chomczyński & Sacchi, 2006). Total RNA remains in the upper aqueous phase in acidic circumstances, but most DNA and proteins remain in the interphase or the lower organic phase. Then, total RNA is then recovered via isopropanol precipitation and can be implemented for a variety of purposes (Chomczyński & Sacchi, 2006). The RNA pellets were dissolved in 20 µl of nuclease free water and kept at -80 °C for nested RT-PCR and gel electrophoresis. Primer used to detect West Nile Virus (WNV) and the conditions are stated in Table 1. Positive samples of Feline Coronavirus are used as positive control due to absence of specific positive control for West Nile Virus (WNV). The primer used for detection of West Nile Virus (WNV) and the conditions are stated in Table 1. Nuclease-free water was used as negative control for both West Nile Virus (WNV) and Feline Coronavirus. The resulting PCR products were compared with positive control to determine if the PCR is done correctly with proper reagent.

Table 1: Primer Sequence for West Nile Virus and nested RT-PCR cycling conditions

Primer Name	Primer Sequence	Amplification size (bp)	PCR Cycling Conditions
1WNV_F	CCAATACGTTTCGTGTTGG	437	1. Reverse transcription: 45°C/20min 2. Polymerase activation: 95°C/1min 3. Denaturation: 95°C/30s; 40 cycles 4. Annealing: 52°C/30s 5. Extension: 72°C/30s 6. Final extension: 72°C/5min
1WNV_R	GGAAATGACCCTGAAGACAT		
2WNV_F	GCTGGATCGATGGAGAGGTG	114	1. Initial denaturation: 95°C/1min 2. Denaturation: 95°C/30s; 30 cycles 3. Annealing: 52°C/30s 4. Extension: 72°C/30s 5. Final extension: 72°C/1min
2WNV_R	CGGCGGAGCTCAAAACAAAA		
Primer set was designed Apical Scientific Sdn Bhd by using the NCBI primer designing tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast/)			

3.5 Data Analysis

The results obtained through gel electrophoresis in serum specimens were statistically assessed. Data collected was compiled in Google spreadsheet and statistical analysis was performed using SPSS statistics. The data were planned to be analysed comparatively, comparing different groups of horses. SPSS and Pearson Chi-Square were also planned to be used to analyse the data. The prevalence of West Nile Fever in horses in Kelantan was also calculated, with the formula:

Prevalence (%) = (number of horses with West Nile Virus (WNV) / total study population) x 100%

CHAPTER 4

4.0 RESULTS

4.1 Samples' Demographic

A total of 30 horse blood serum samples collected from the private stables in Kelantan.

Table 2

Samples' Demographic from the gender and the stables' location

Variables		Number	Percentage (%)
Stable Location			
	Machang	11	36.67
	Kota Bharu	5	16.67
	Bachok	14	46.67
Gender			
	Mare	23	76.67
	Stallion	5	16.67
	Gelding	1	3.33

Note: Total number of samples n=30

As stated in Table 3, most of the samples were taken from private stables that are located in Bachok (n=14, 46.67%) and Machang (n=11, 36.67%). Majority of the samples were taken from mare (n=23, 76.67%).

4.2 Molecular detection of West Nile Virus

There was no positive result of West Nile Virus infection in private stables in Kelantan out of 30 serum samples taken. Figure 1 shows no presence of band at the samples except from the positive control and DNA ladder. Based on the nested RT-PCR results, the prevalence of West Nile Virus infection in 30 horses in Kelantan is 0%.

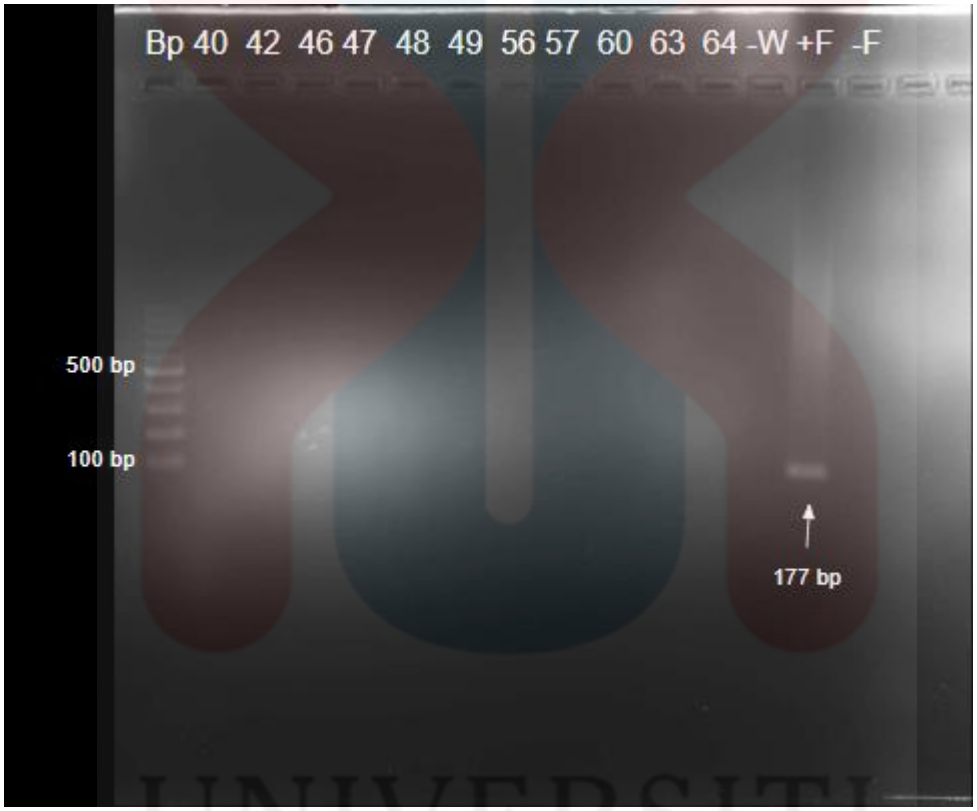

Figure 1: Gel electrophoresis of PCR products, using primers for West Nile Virus and Feline Coronavirus: Lane Bp: molecular marker; -W: negative control for West Nile Virus (WNV) ; +F: positive control for FCoV with 177 bp; -F: negative control for FCoV; negative lanes: 40-64. 40 - 64 are the sample number identification as this is an archived sample. Gel electrophoresis of PCR products for other 19 samples also appeared the same as shown in Figure 1.

4.2 Data on Risk factors

However, it is important to note that all the samples tested negative. This indicates that no statistical analysis could be performed on the data. Despite this, the data collected was still valuable and can be presented in a descriptive manner. This allows for a comprehensive understanding of the conditions and factors surrounding the horses and their environment. There were no horses with clinical signs with 83.33% of the horses from stables located near to paddy plantations. Since there was no West Nile Virus (WNV) detected from these animals, it indicates that there was no correlation acquired from the Pearson Chi Square for the risk factors as all the samples were negative for West Nile Virus (WNV) .

Table 3: Percentage of horses located in rural area and showing clinical signs

	Variables	**Number**	**Percentage (%)**
Stable Location			
	Rural	25	83.33
	Urban	5	16.67
Clinical Signs			
	Presence	0	0
	Absence	30	100

Note: Total number of samples n=30

CHAPTER 5

5.0 DISCUSSION

The study revealed that, despite the abundance of rice plantations in Kelantan, none of the 30 horses tested there were found to be infected with the West Nile Virus (WNV). This contrasts with Central Malaysia, where 3.5% of the 203 horses tested were found to be infected through RT-PCR. The same study also found that 24.14% of the 203 horses had been exposed to West Nile Virus (WNV) , as indicated by the seroprevalence rate (Bande et al., 2023). This dissimilarity can be explained by the fact that West Nile Virus (WNV) infection in horses can often be asymptomatic, with only about 20% of infected horses developing clinical signs (Paré, 2018). Therefore, it is likely that many of the horses were infected but did not show clinical signs, making diagnosis difficult. Alternatively, the horses may have already recovered from the infection by the time of testing. This would explain why the West Nile Virus (WNV) antigen was not detected in the RT-PCR tests.

Several factors contributed to the all-negative result in this study. These include the size of the sample, the method used to detect the West Nile Virus (WNV), the lack of a specific positive control for West Nile Virus (WNV) , and the use of archived samples.

The primary reason for the lack of West Nile Virus (WNV) detection in all samples in this study was the small sample size. It is generally accepted that a minimum sample size of 30 is required for research, as this number of data points should provide sufficient information to draw a statistically valid conclusion about a population (Pannell, 2023). This principle is also known as the Law of Large Numbers, which states that the accuracy of results improves as the sample size increases. In this study, a total of 30 horse serum samples were collected. Ideally,

the sample size should be at least 10% of the population, but this was not feasible due to time constraints (Bullen, 2022). Hypothetically, increasing the sample size would improve the results. Therefore, despite the presence of risk factors such as an abundance of rice plantations in rural areas of Kelantan, the prevalence of West Nile Virus (WNV) was still found to be 0% in this study.

Moreover, the detection method for West Nile Virus (WNV) used in this case is nested RT-PCR. According to the Centers for Disease Control and Prevention (CDC), it is said that it is fairly low likelihood to detect West Nile Virus (WNV) infection through molecular detection, negative results from the tests does not rule out West Nile Virus (WNV) infection. Immunoglobulin M antibody for West Nile Virus (WNV) can be detected by using enzyme-linked immunosorbent assay (ELISA) by using serum or cerebrospinal fluid from the horse that has not been vaccinated for West Nile Virus (WNV) (Paré, 2018). This will confirm the diagnosis alongside the presence of clinical signs. Detection of IgM by using ELISA is the diagnostic workup of choice because IgM antibodies are short-lived and it also indicates recent infection (Hou et al., 2020). In this case, Reverse Transcription Polymerase Chain Reaction (RT-PCR) was used instead to detect the antigen itself instead of antibodies. IgM can be detected as early as three days after infection because it acts as a first line humoral immunity defence compared to Immunoglobulin G that plays a key role in long term immune memory (Hou et al., 2020). Thus, using nested RT-PCR may contribute to negative results of this study.

Other than that, in this study there was no positive control for West Nile Virus (WNV) available to be used. That is why another virus was used as the positive control. Feline Coronavirus was used as the positive control, the RT-PCR for West Nile Virus (WNV) and FCoV was done concurrently following the PCR protocol from West Nile Virus (WNV) . In this study, FCoV

was chosen because it also has the primer for nested RT-PCR so it can be done concurrently. Theoretically, a positive sample of West Nile Virus (WNV) or synthetic RNA transcript or plasmid should be used as positive control. Other viruses that are closely related to West Nile Virus (WNV) like Japanese Encephalitis can also be used as they are from the same genus which is Flavivirus. However, in this study all of the options mentioned were unavailable that is why FCoV was chosen.

Furthermore, the use of archived serum samples may have contributed to the study's negative results for West Nile Virus (WNV) detection. This can be due to Ribonucleic Acid (RNA) degradation in archived samples. Time, temperature, nuclease enzymes and chemicals can cause RNA degradation (Wang & Liu, 2022). The temperature at which the archived samples are kept is also important to prevent RNA degradation, it is crucial to store the archived samples at low temperatures, such as -20 °C or -80 °C, or under liquid nitrogen (Fabre et al., 2013). Furthermore, repeated freeze-thaw cycles might cause RNA degradation in archival samples. As a result, one of the reasons for the unfavourable outcomes in this study is the use of archived serum samples.

Last but not least, the study did not find any correlation between the risk factors, such as whether the horses were from urban or rural areas, and the negative results. Rural areas are often considered risk factors due to the prevalence of rice plantations, which are more common in these areas compared to urban ones. Rice plantations are seen as a risk factor because they contain stagnant water, which is a breeding ground for the *Culex* species. As previously mentioned, the *Culex* species can carry the West Nile Virus (WNV) and transmit it to horses. To better understand this risk factor, further research is necessary.

CHAPTER 6

6.0 CONCLUSION & RECOMMENDATION

In summary, this study indicates a low prevalence of West Nile Virus (WNV), specifically 0%, in horses in Kota Bharu, Machang and Bachok, Kelantan, Malaysia, as detected by nested Reverse Transcription-Polymerase Chain Reaction (RT-PCR). However, given the uncertainties and imperfections associated with the molecular detection method of West Nile Virus (WNV) used in this study, it can be concluded that the negative results from the archived samples in Kelantan do not conclusively rule out West Nile Virus (WNV) infection. Therefore, further research is needed. This study has also conducted screenings for the virus during the study period.

This study, being the initial attempt at molecular detection of West Nile Virus (WNV) in Kelantan, encountered several limitations. These included the lack of a positive control for West Nile Virus (WNV) and a limited sample size. To enhance the accuracy of prevalence results, it is advisable to expand the study population. Furthermore, the inclusion of a positive control for West Nile Virus (WNV) could significantly improve the study's precision. Additional research could also be conducted in Kelantan to determine the seroprevalence of West Nile Virus (WNV) using ELISA for antibody detection. Lastly, for future West Nile Virus (WNV) detection, it would be beneficial to use fresh samples that have been stored properly.

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APPENDIX

Appendix A

UNIVERSITI MALAYSIA KELANTAN

Fakulti Perubatan Veterinar

RUJ. KAMI *(Our Ref.)* : UMK/FPV/ACUE/PG/001/2023
TARIKH *(Date)* : 20 MARCH 2023

ASSOC. PROF. DR. RUMAIZI BIN SHAARI
Main Supervisor
Faculty of Veterinary Medicine
Universiti Malaysia Kelantan

Dear Dr,

APPROVAL OF INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE (IACUC) TO CONDUCT RESEARCH INVOLVING ANIMALS

We are pleased to inform you that your application for approval to conduct research from Institutional Animal Care and Use Committee (IACUC), Faculty of Veterinary Medicine, Universiti Malaysia Kelantan has been approved. Please refer the table below for approval code:

APPROVAL CODE	UMK/FPV/ACUE/PG/001/2023
TITLE	EPIDEMIOLOGY OF EQUINE HEMOPARASITE IN MALAYSIA
NAME OF STUDENT	NUR ZUL IZZATI BINTI MOHD RAJDI (D21E006F)

2. Please be noted for the Final Year Project, you are responsible to supervise your student to conduct all animal-related procedures as stated during ethic application. The co-supervisor(s) for the project are encouraged to help with the procedures as well.

3. You are advised to always follow "3R" (REDUCE, REFINE, & REPLACE) and all animal ethics and animal welfare principles to reduce suffering in animal.

Thank you.

"ALLAH DITAATI, RAJA DISANJUNGI, RAKYAT DIKASIHI"
"MALAYSIA MADANI"
"BERKHIDMAT UNTUK NEGARA"

Yours sincerely,

(DR. NOR FADHILAH BINTI KAMARUZZAMAN)
Chairman
Institutional Animal Care and Use Committee
Faculty of Veterinary Medicine
Universiti Malaysia Kelantan