MOLECULAR DETECTION OF FLAVIVIRUSES IN BAT FLIES

OF BATS FROM KELANTAN AND TERENGGANU, MALAYSIA.

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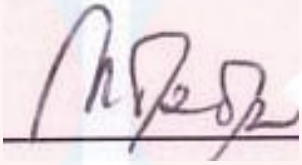
CERTIFICATION

It is hereby certified that I have read this proposal entitled "**Molecular Detection of Flaviviruses in Bat Flies of Bats from Kelantan and Terengganu, Malaysia.**" by Nur Fatin Akmal Binti Jenuwa and in our opinion it is satisfactory in terms of scope, quality, and presentation as partial fulfillment of the requirement for the course DVT 5436 – Research Project.



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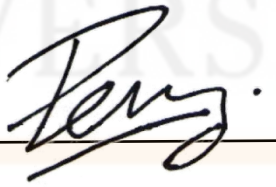

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DEDICATION

Everyone that loves and cares about me from morula until now.

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ABSTRACT

An abstract of the research paper presented to the Faculty of Veterinary Medicine in partial requirement on the course DVT 5436 - Research Project.

Flaviviruses are the most important pathogenic diseases in human and animal population that are transmitted by insect vectors to the hosts. Most Flaviviruses are host specific but also can infect a wide range of species including bats. Some of the flaviviruses isolated from bats are medically important vector-transmitted arboviruses such as St. Louis encephalitis virus (SLEV) and Japanese encephalitis virus (JEV). Currently there is no study conducted on the presence of Flavivirus in bat and its vectors in Malaysia. Therefore, this study was conducted to detect the presence of flaviviruses in bat flies in Kelantan and Terengganu, Malaysia. Thirty-five bat flies' samples from 106 bat flies were pooled and identified based on bat species in the same regions, the types of ectoparasites, and the morphological characteristics of the ectoparasites. A reverse transcriptase-polymerase chain reaction (RT-PCR) using published universal primers for *Flavivirus* were used to detect the presence of flaviviruses in the bat flies. The results were negative for all the 35 pooled samples of the bat flies. The findings of this research may serve some useful information regarding the prevalence of flaviviruses in bats'flies in Kelantan and Terengganu, Malaysia. Further investigation using more sample sizes, insect genus and locations are needed to increase the detection of flaviviruses in bats' ectoparasites in East Coast, Malaysia.

Keywords: Flaviviruses, Bat flies, RT-PCR, Kelantan and Terengganu

ABSTRAK

Abstrak daripada kertas penyelidikan dikemukakan kepada Fakulti Perubatan Veterinar untuk memenuhi sebahagian daripada keperluan kursus DVT 5436 – Projek Penyelidikan.

Flavivirus adalah penyakit patogen yang paling penting dalam populasi manusia dan haiwan yang disebarkan oleh vektor serangga kepada perumah. Kebanyakan flavivirus mempunyai perumah spesifik tetapi terdapat juga beberapa flavivirus yang boleh menjangkiti pelbagai spesies termasuk kelawar. Beberapa flavivirus kelawar yang disebarkan oleh serangga yang mempunyai kepentingan kesihatan seperti St. Louis encephalitis virus (SLEV) and Japanese encephalitis virus (JEV). Setakat ini, tidak terdapat kajian kehadiran flavivirus dalam ektoparasit kelawar yang dilaporkan di Malaysia. Oleh itu, kajian ini dijalankan untuk mengesan kehadiran flavivirus dalam lalat kelawar dari Kelantan dan Terengganu, Malaysia. Terdapat tiga puluh lima sampel lalat kelawar daripada 106 lalat kelawar telah dikumpulkan dan dikenal pasti berdasarkan spesies kelawar di kawasan yang sama, jenis ektoparasit, dan ciri -ciri morfologi ektoparasit. Sampel tersebut telah melalui reaksi '*Reverse Transcriptase Polymerase Chain Reaction* (RT-PCR)' menggunakan primer universal untuk mengesan flavivirus dalam lalat kelawar. Hasil kajian mendapati kesemua 35 sampel negatif terhadap virus tersebut. Walau bagaimanapun, penemuan penyelidikan ini boleh memberi maklumat berguna mengenai prevalens flavivirus didalam lalat kelawar di Kelantan dan Terengganu, Malaysia. Kajian lanjut menggunakan lebih banyak sampel, genus serangga dan lokasi adalah diperlukan untuk meningkatkan pengesanan flavivirus didalam serangga lalat di Pantai Timur, Malaysia.

Kata kunci: Flavivirus, Lalat kelawar, RT-PCR, Kelantan dan Terengganu

1.0 INTRODUCTION

Genus of *Flavivirus* has 70 recognized viruses that mainly affect humans and animals. Common Flaviviruses are Dengue virus, Zika virus, and West Nile virus which cause diseases in tropical and less developed countries, and it recently emerges worldwide (Vina *et al.*, 2017). *Flavivirus* is 50 nm in diameter, lipid enveloped with glycoprotein spikes, and surrounded with spherical nucleocapsid and icosahedral symmetry. The genome consists of a single molecule of liners and single-stranded RNA (Maclachlan *et al.,* 2011). Dengue virus, Japanese encephalitis virus, and Zika viruses are the most popular flaviviruses with high prevalence in Malaysia. Dengue fever and Japanese encephalitis are endemic in Malaysia due to the changes in the season or climate that cause the breeding of the mosquitoes (Kumar *et al*., 2018).

Bats are wild animals that carry a lot of diseases, and as reservoirs in zoonotic viruses such as Ebola, Sars, Rabies, Marburg, Nipah virus, and Hendra virus (Sauqi *et al.*, 2021). Furthermore, bats are host to many parasites. Bats can be divided based on the type of food they are eaten such as fruit eaters, insectivores, nectar eaters, and bloodsuckers (Sauqi *et al.*, 2021). Bats are a social creature that usually stay~~s~~ with their colonies and like to migrate from one place to another. This migration will increase the transmission of the viruses between the bats to other animals (Luis *et al.,* 2015*).*

Bats have varieties of ectoparasites such as bat flies (*Nycteribiidae* and *Streblidae*), bugs (*Cimicidae* and *Polyctenidae*), fleas (*Ischnopsyllidae*), mites (*Spinturnicidae* and *Macronyssidae*), and ticks (*Argas* spp., *Carios* spp., *Ixodes* spp., and *Ornithodoros* spp.) (Szentivanyi *et al.,* 2019). There was a study conducted previously that exhibited that ectoparasites are associated with pathogens that may

cause disease to humans and animals (wild and domestic animals) (Meis *et al.*, 2021). Therefore, it is very important to study the role of these ectoparasites as a potential vector in transmitting these diseases in Malaysia. In this study, we investigated the presence of flaviviruses in the bat flies in Kelantan and Terengganu using molecular method RT-PCR.

2.0 RESEARCH PROBLEM

Flaviviruses are the most important pathogenic diseases in human and animal population that are transmitted by insect vectors to the hosts. Most Flaviviruses are host specific but also can infect a range of the species. These are 11 species of the Flaviviruses that have been reported from different species in Malaysia. In mosquitoes, Japanese encephalitis virus has been isolated from *Culex* spp., Zika virus and dengue virus were isolated from *Aedes* spp. In tick, Langat virus was isolated from the *Ixodes* spp. (Kumar *et al.,* 2018). However, to date, there is no report on the presence of flaviviruses in the ectoparasites of the bats in Kelantan and Terengganu, Malaysia.

3.0 RESEARCH QUESTIONS

3.1 Are flaviviruses present in bat flies in Kelantan and Terengganu, Malaysia?

4.0 RESEARCH HYPOTHESIS

4.1 Flaviviruses are present in bat flies in Kelantan and Terengganu, Malaysia.

5.0 RESEARCH OBJECTIVES

5.1 To detect flaviviruses in bat flies in Kelantan and Terengganu, Malaysia.

6.0 LITERATURE REVIEW

6.1 *Flaviviridae*

Family *Flaviviridae* has four genera which are *Flavivirus, Pestivirus, Pegivirus,* and *Hepacivirus* (Maclachlan *et al*., 2011). These members have similar genomic organization, replication pattern, and physiochemical properties but the genetically and biologically different. Genus *Flavivirus* has over 90 viruses which are of the veterinary importance such as Japanese encephalitis, West Nile, louping ill, and others. There are also some members of the genus are arthropod-borne human pathogens that caused fever with rash to life-threatening hemorrhagic fever, encephalitis, and hepatic necrosis (Maclachlan *et al*., 2011). The diversity of the arthropod as a vector, disease characteristics, and geographical distribution of the *Flavivirus* makes them different from other viruses (Holbrook, 2017).

6.2 Structural characteristics of *Flavivirus*

Flavivirus is a positive-single stranded RNA genome, enveloped and spherical nucleocapsid which protected by lipid molecules on the external layer (Kumar *et al*., 2018). The genome has two separations which are coding and non-coding regions. The coding region is an area that involved with viral structure and replication. The viral structural proteins consist of the envelope (E), capsid (c), and membrane (M/prM) as well as seven nonstructural proteins that need for viral protein synthesis and replication that been shown at Figure 6.2.1. For the

noncoding region, it is involved in genome secondary structure for replication and translation (Kumar *et al.*, 2018).

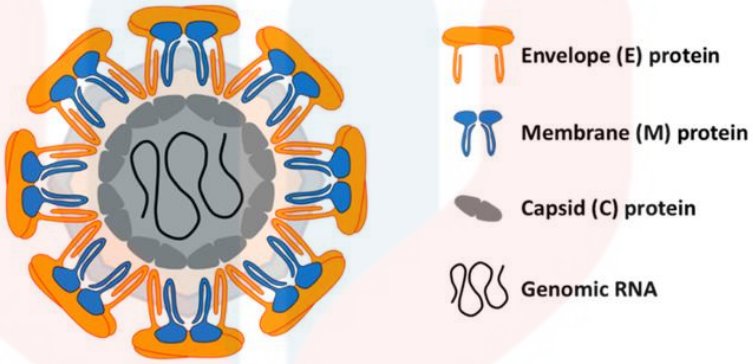


Figure 6.2.1: The structure of Flavivirus

The E protein content is 500 amino acids with six disulfide bonds that are used to hold the structure of the protein. These E proteins act as entry, fusion, cellular tropism, haemagglutination, the virulent factor of the virus, and for production of neutralizing antibodies of the immune system of the host (Kumar *et al.*, 2018).

6.3 *Flavivirus* cases in Malaysia

Dengue fever, Japanese encephalitis, and Zika fever are the highest prevalent in Malaysia in where Dengue fever and Japanese encephalitis are endemic in Malaysia. Most of the dengue fever outbreaks were reported during the rainy and dry seasons because of the changes in climate that favor the breeding of the mosquitoes that act as a vector to transmit the dengue virus. Recently, Zika fever was re-emerging in Johor and Sabah, Malaysia. Furthermore, Japanese

encephalitis cases have also been reported in East Malaysia because of a residential area near with to wild boar population (Kumar *et al*., 2018).

6.4 Life cycle of *Flavivirus*

The monocytes, macrophages, and dendritic cells are the host cells that Flaviviruses can infect. The virus will attach at the host cell's surface (Wurtz *et al.*, 2010), but some studies said that cellular receptors that make the *Flavivirus* specific for different cell types and different host species. The virus enters the cell by receptor-mediated endocytosis and replicates in the cytoplasm of the host cell. After that, the virus replication is involved in the synthesis of the negative-sense RNA that acts as a template for positive-sense RNA. The virus mRNA is translated to single polyproteins to form structural and nonstructural virus proteins. Lipid envelope occurs on the membrane of the endoplasmic reticulum and the plasma membrane of the host cell, but capsids and budding formation are not seen. After this formation, the virions were formed and found at the cisternae of the endoplasmic reticulum. These virions were transported by using cytoplasmic vesicles of the host cell then been release by exocytosis or cell lysis (Maclachlan *et al*., 2011).

6.5 Transmission cycles of *Flavivirus*

Although, flaviviruses have similar genomic organization but they are different based on their host range and transmissibilities. Most flaviviruses are transmitted through horizontal transmission which is between hematophagous

arthropods and vertebrate hosts that are divided into mosquito-vertebrate (dengue virus, yellow fever virus, Japanese encephalitis virus, and West Nile virus) and tick-vertebrate viruses (tick-borne encephalitis virus, Langat virus, and Powassan virus). Some of the flaviviruses have a vertebrate-specific host range (No Known Vector flaviviruses) and insect-specific (Nounane virus, Barkedji virus, and Lammi virus) (Blitvich *et al.*, 2015).

6.6 Common bats in Peninsular Malaysia

Chiroptera is an order of bats that are 50% important to the mammal species in tropical forests and 20% of mammal species worldwide. A study has exhibited there were 106 bat species in Peninsular Malaysia, but the numbers keep on increasing because of the discoveries of the species. *Hipposideros bicolor* sensu lato is one of the most widespread species in Southeast Asia. The morphology identification is hard to do because they have similar morphology characteristics, but we can detect the differences by using molecular techniques such as DNA barcoding (Lim *et al.*, 2017).

Most of the diseases that emerged are caused by bats and rodents. Bats are also ideal reservoir hosts for viruses, and it is only mammals that can fly as well as can host viruses without causing the disease to the bats. This ability of the bats will lead to transmission of the viruses such as alphaviruses, flaviviruses, and bunyaviruses that may infect the bats through bitting via arthropods (Calisher *et al.*, 2006). Thus, investigating the presence of arbovirus such as flaviviruses in bat's ectoparasites may help to identify species that are reservoir sources of

these viruses and to understand factors influencing this richness, particularly those related to life or ecological traits.

6.7 Common viruses in bats

Bats are known as the species that capability to fly and disseminated viruses that lead to increase the chance of transmission from intraspecies or interspecies (Woo *et al.,* 2019). Most bats are reservoirs for many viruses such as Rabies virus, Coronavirus, and Nipah virus. The *Rhabdoviridae* and *Flaviviridae* are the viral families that are usually found in bat order which is *Chiroptera* (Abundes *et al*., 2017). There is a study has been done in China to detect Japanese Encephalitis Virus (JEV) in the bats and it shows positive JEV in all the bats' species. This show that the bats is a potential role in the cycle of JEV (Kading *et al.,* 2016).

6.8 Common ectoparasites in bats

Most flaviviruses are transmitted through horizontal transmission between arthropods and vertebrate hosts (Blitvich *et al.*, 2015). Various ectoparasitic groups are commonly discovered in the bats including bat flies (superfamily: Hippoboscoidea, Diptera: Nycteribiidae and Streblidae), fleas (Siphonaptera: Ischnopsyllidae), bugs (Hemiptera: Cimicidae and Polyctenidae), mites (Mesostigmata: Spinturnicidae and Macronyssidae) and ticks (*Argas* spp., *Ixodes* spp., *Carios* spp., and *Ornithodoros* spp.) (Szentivanyi *et al.,* 2019).

7.0 MATERIALS AND METHODOLOGY

7.1 Sample collection

Samples were obtained from FPV, UMK virology laboratory archived samples bank of Faculty of Veterinary Medicine, University Malaysia Kelantan which were collected at Gunung Reng, Kelantan, and Sekayu, Terengganu. The study has obtained animal ethical approval from the Animal Ethics Committee, FPV, UMK (UMK/FPV/ACUE/PG/6/2021). The samples were kept in Eppendorf tubes that contained 80% diluted ethanol and were stored in a -80ºC freezer.

7.2 Sample identification

The samples were divided based on species of ectoparasites using a dissecting microscope (Olympus Corporation, Japan). The species were identified based on the morphological identification of the parasites. Then, each species was placed in different Eppendorf tubes that contain 80% diluted ethanol and the ID was recorded.

7.3 Samples processing

First, these samples were pooled based on bats species in the same regions, the types of ectoparasites, and morphological characteristics of the ectoparasites and were placed in different Eppendorf tubes that contain 80% ethanol. Then, the tube needs to centrifuge at 1500 x g for three minutes at 4°C using a centrifuge machine (KUBOTA, Japan). After centrifuged, the ethanol was

discarded and 500 µL 1x Phosphate Buffered Saline (PBS) (1st BASE, Singapore) was added into the tube followed with centrifuged again at 1500 x g for three minutes at 4°C. The 1x Phosphate Buffered Saline (PBS) (1st BASE, Singapore) was discarded, and the centrifuged samples were homogenized using micro pestle until completely crashed within the Eppendorf tube. Then, 400 µL 1x Phosphate Buffered Saline (PBS) (1st BASE, Singapore) was added to the tube and the homogenized samples were stored at -80°C until use.

7.4 RNA extraction

The RNA extraction was done in a biosafety cabinet (BCS) class II. One ml GENEzol ™ Reagent (Geneaid Biotech Ltd, Taiwan) was added to the sample in the Eppendorf tube. A 200 µL of chloroform was added and the tube was shaken vigorously for 10 seconds. Then, the samples were centrifuged at 14,000 x g for 5 minutes at 4°C using a centrifuge machine (KUBOTA, Japan) to separate the phases. Only 600 µL of the aqueous (clear) phase containing the RNA was transferred to a new Eppendorf tube by slowly pipetting out the solution and not disturbing the interphase layer (white) or the organic phase layer (red).

Next, the RNA was precipitated using the following steps, one volume of isopropanol (600 µL) was added to the aqueous phase, and it is mixing by inverting the tube several times to completely combine. Then, the sample mixture was left for incubation for 10 minutes at room temperature and centrifuged at 14,000 x g for 10 minutes at 4°C to allow the formation of a dense

RNA pellet. The supernatant was removed carefully by pouring it into a special waste tube for isopropanol waste.

The pellet was resuspended with the addition of one ml of 70% cold ethanol to wash the RNA pellet and was vortexed for five seconds. The sample mixture was centrifuged at 14,000 x g for five minutes at 4°C. The supernatant was discarded with a micropipette. The RNA pellet was air dried for five minutes at room temperature and 30 µL of RNAse-free water was added to the pellet for RNA resuspension. The RNA pellet was then incubated for 15 minutes at 60°C to completely dissolve the pellet and was stored at -80°C until use.

7.5 RT-PCR

The primers used in this study for RT-PCR were shown in table 7.5.1 below:

Table 7.5.1: Primers used for RT-PCR reaction.

Primers	**Sequence (5'-3')**	**Gene**	**Expected size (Base pair)**	**Reference**
Flavi forward	TGYRBTTAYAACATGATGGG	*NS5*	270	PREDICT (EcoHealth Alliance, USA)
Flavi reverse	GTGTCCCAICCNGCNGTRTC			

The master mix was prepared as in table 7.5.2 using AccessQuick RT-PCR reagent (Promega, USA).

Table 7.5.2: Components of the RT-PCR reaction.

Solution	Volume (µL)
2X buffer	12.5
AMV-RT enzyme	0.5
Primer 1 (10 uM)	1
Primer 2 (10 uM)	1
RNAse inhibitor (RNAsin)	0.2
Taq Polymerase (8 units)	0.5
dH_2O	4.3
Total volume	25

Five µL of the extracted RNA extraction was preheated for five minutes at 95^{o}C. After that, the master mix was added 20 µL and top up with nuclease free water to the total volume of 25 µL reaction. Then, RT-PCR was carried out using a T100 thermocycler (Bio-Rad, USA) and the RT-PCR was conducted based on table 7.5.3 below:

Table 7.5.3: Condition of RT-PCR cycle

Cycle step	Temperature (^{o}C)	Time	Cycles
Initial denaturation	95	5 minutes	1
Denaturation	94	30 seconds	40
Annealing	50	1 minute	
Extension	72	45 seconds	
Final extension	77	5 minutes	1

Killed vaccine Japanese encephalitis (Nisseiken, Japan) and previously tested negative Japanese encephalitis sample were used as a positive and negative control respectively.

7.6 Agarose Gel Electrophoresis

RT-PCR product was analyzed using agarose gel electrophoresis. First, agarose powder (Transgen Biotech, China) was put into the beaker and 1.5% Tris-borate EDTA (TBE) (Base Asia, Singapore) was poured into the beaker. Next, the solution was heated using a microwave for two minutes and warmed it to 55^{o}C. Then, one µL of Midori Green DNA stain (Nippon Genetics Europe, Germany) was added and stirred gently. The solution was poured into the mould with the wells comb and left for 30 minutes under room temperature until it is solidify. Then, the 100bp DNA Ladder (Vivan Technologies, USA) was loaded into the first well followed by positive, negative control, and 35 samples to the corresponding wells. The electrophoresis is conducted for 40 minutes at 100 V and 400 mA. The result was observed and analyzed using a UV transilluminator (Bio-Rad, USA).

8.0 RESULT

There were 19 bats from Sekayu, Terengganu and 52 bats from Gunung Reng, Kelantan were collected. These samples were pooled based on bat species in the same regions, the types of ectoparasites, and the morphological characteristics of the ectoparasites. The samples were all identified as bat flies (superfamily: Hippoboscoidea; families: Streblidae and Nycteribiidae) were shown in figure 8.1 and were placed in different Eppendorf tubes based on each of the species of the bats in the same regions. There were 15 samples from Sekayu, Terengganu, and 20 samples from Gunung Reng, Kelantan.

Figure 8.1: The bat fly's families: Streblidae (wing fly) on the left and Nycteribiidae (wingless fly) on the right.

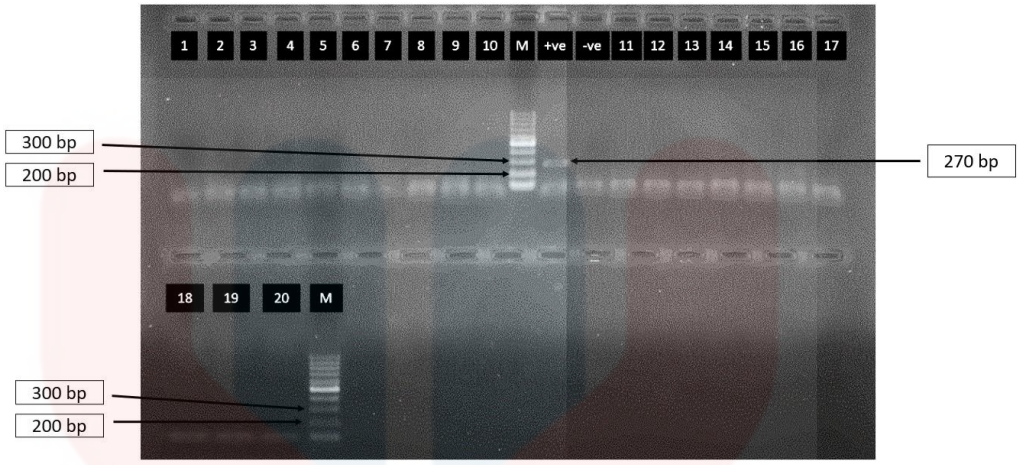


Figure 8.2A: Detection of Flavivirus in ectoparasites of bats using agarose gel electrophoresis where M represents ladder, +ve positive control, -ve negative control, and well 1 to 20 represent samples.

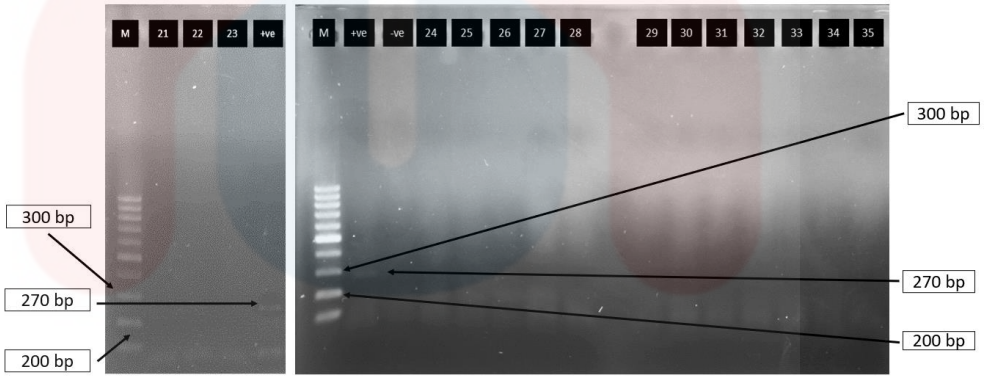


Figure 8.2B: Detection of Flavivirus in ectoparasites of bats using agarose gel electrophoresis where M represents ladder, +ve positive control, -ve negative control, and well 21 to 35 represent samples.

The results were as shown in Figure 8.2A and Figure 8.2B with an expected band result of 270 bp as shown in the positive (+ve) control wells and there was no band observed in the negative (-ve) control wells. The results from Figure 8.2A and Figure 8.2B showed that all the 35 samples were negative for the presence of *Flavivirus*.

9.0 DISCUSSION

All the 35 samples were negative for the presence of *Flavivirus*. These results concluded that bat flies taken from the different species of bats found in Gunung Reng, Kelantan, and Sekayu, Terengganu did not carry the *Flavivirus*. The fact that the results were negative could be attributed to several different things, such as the fact that the samples were delayed in the sampling process which resulted in a lower concentration of the virus within the samples. This is supported by the evidence that RNA detection will be high in the acute phase of the illness, and late processing of the samples can cause degradation of RNA which reduces the amount of RNA detection in the samples (Gibbs and Smith., 2016). Therefore, processing the samples as soon as possible can increase the likelihood of getting a positive result from the RT-PCR in comparison to delaying the processing of the samples.

According to some research that the majority of flaviviruses have the potential to undergo global distribution as a result of the biting behavior of arthropods like mosquitoes and ticks (Kuhn., 2011; Sonja M., 2016). Flaviviruses were commonly isolated in mosquitoes and ticks but very rare in flies. The samples of this project were all hippoboscid flies because we could not trap the mosquitoes due to the unavailability of mosquitoes traps during the sampling period and the absence of ticks on our bat samples. However, there was a study reported the presence of flaviviruses in bat flies (Diptera: Streblidae) in Mexico (Abundes *et al*., 2017). They have detected *Flavivirus* sequences in 38 pools of bat flies of vampire bats where six sequences were clustered together with the dengue virus (Abundes *et al*., 2017).

In addition, different sequences of *Flavivirus* strains, especially those belonging to the classical insect-specific (IS) flaviviruses group, are more likely contributed to the difficulty in detecting flaviviruses using universal primers specific non-structural (NS) proteins due to its high variations (Daidoji *et al*., 2021). In this study, the universal primers that we used also flanked the non-structural region (*NS5*). There is a possibility that the virus strains present in the samples cannot be detected by the primers used in this study due to differences in the sequences. Thus, it is recommended to use many sets of primers based on more conserved regions such as using precursor membrane (preM) or Capsid (C) proteins.

Degradation of the samples can occur if it is handled incorrectly, which is a possibility. This is because flaviviruses are RNA viruses, which, in comparison to DNA viruses, are much more likely to be degraded because of hydrolysis caused by enzyme or chemical-based compounds known as RNases, which can typically be found on human hands as well as in microorganisms found in the environment. This RNase is the one responsible for the degradation of RNA (Relova *et al*., 2018).

In addition to this, RNA is extremely susceptible to oxidation when exposed for an extended time to an environment at a particular temperature. Therefore, if the sample is allowed to thaw in its environment for an extended time, it may become degraded. Most RNA viruses are extremely sensitive to temperatures of 35^{o}C because this temperature is the optimal temperature for RNase activity, which can ultimately result in the virus being degraded (Relova *et al*., 2018). This can be one of the factors that contribute to negative results.

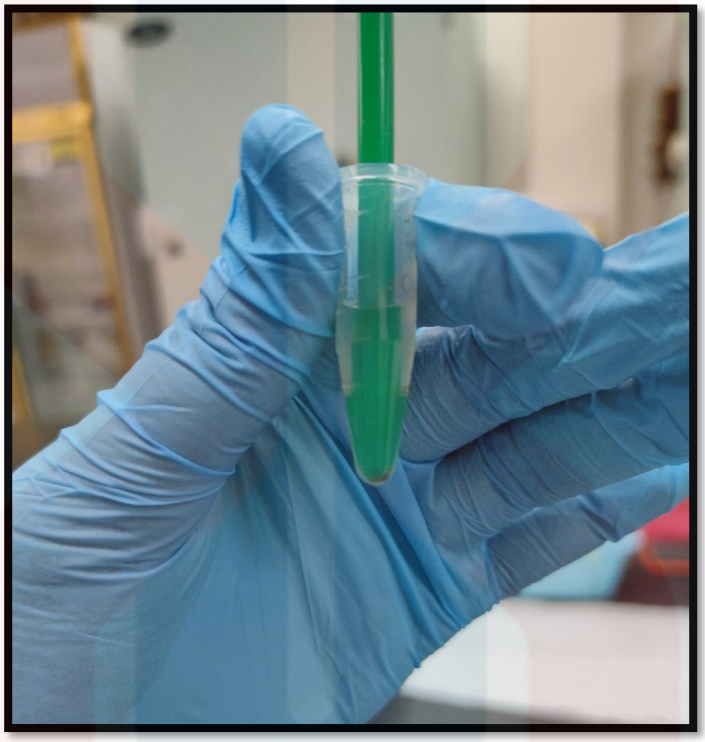
10.0 CONCLUSION

In conclusion, based on the reverse transcriptase-polymerase chain reaction (RT-PCR) that was used in this study, no flaviviruses were detected in all 35 tested bat flies' samples. This indicates that the bat flies from Gunung Reng, Kelantan, and Sekayu, Terengganu, was free of flaviviruses.

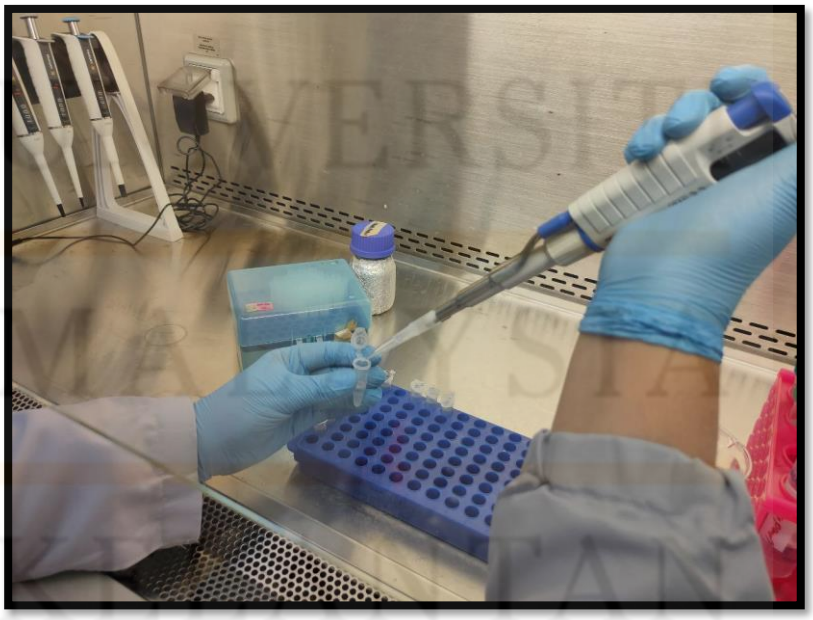
11.0 RECOMMENDATION

Firstly, more samples collected from various districts and regions to increase the possibility of detecting flaviviruses in the bats. In addition, samples such as ticks and mosquitoes need to be collected to increase the probability of obtaining flaviviruses because flaviviruses are most found in mosquitoes and ticks. A mosquito trap (which we do not have in our study) can be used to collect the mosquitoes, while a forcep can be used to remove ticks from bats. Secondly, the utilization of other few sets of universal primers for flaviviruses are something that could be done in the future to increase the chances of detecting flaviviruses within ectoparasites due to high sequence variations in flavivirus genes. Lastly, the utilization of various methods to detect the flavivirus that is more sensitive increases the probability of obtaining a positive result. It is possible to achieve a better result by using different methods such as TaqMan real-time reverse RT-PCR, which is more sensitive than conventional RT-PCR. In addition to that, RT-LAMP is also a method that can be to detect flaviviruses because of their high levels of specificity and sensitivity due to the use of many primers in the reaction.

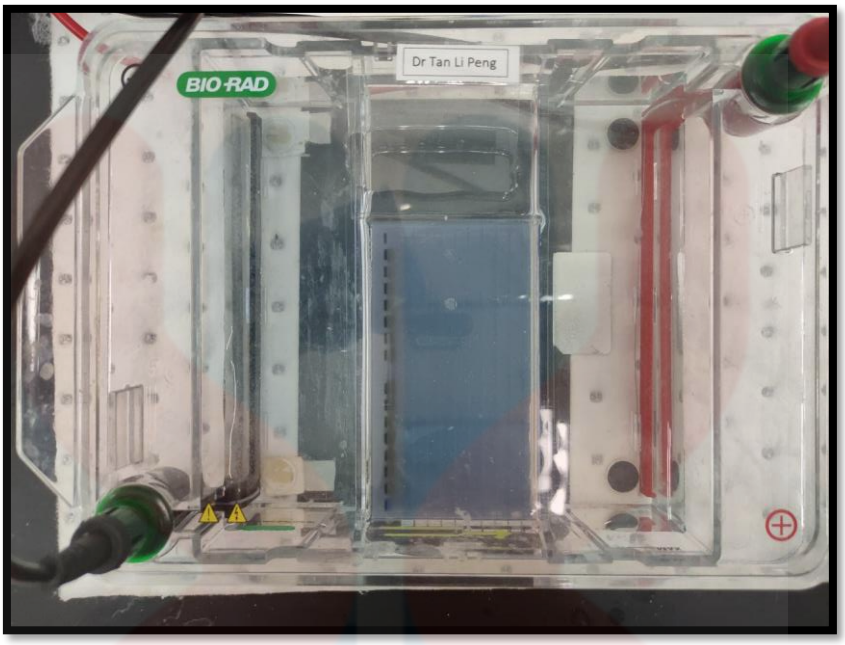
APPENDIX A



Appendix A.1: RNA extraction processing



Appendix A.2: RNA extraction in biosafety cabinet (BSC) class II



Appendix A.3: Agarose gel electrophoresis

REFERENCES

Abundes-Gallegos J; Salas-Rojas M; Galvez-Romero G; Perea-Martínez L; Obregón-Morales CY; Morales-Malacara JB; Chomel BB; Stuckey MJ; Moreno-Sandoval H; García-Baltazar A; Nogueda-Torres B; Zuñiga G; Aguilar-Setién A; (2017). Detection of dengue virus in bat flies (Diptera: Streblidae) of common vampire bats, Desmodus Rotundus, in Progreso, Hidalgo, Mexico. Retrieved May 23, 2022, from https://pubmed.ncbi.nlm.nih.gov/29232534/

Blitvich, B. J., & Firth, A. E. (2015, April 10). *Insect-specific flaviviruses: A systematic review of their discovery, host range, mode of transmission, superinfection exclusion potential and genomic organization*. Viruses. Retrieved January 1, 2022, from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4411683/

Calisher, C. H., Childs, J. E., Field, H. E., Holmes, K. V., & Schountz, T. (2006, July). *Bats: Important reservoir hosts of emerging viruses*. Clinical microbiology reviews. Retrieved January 3, 2022, from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC1539106/

Daidoji, T., Morales Vargas, R., Hagiwara, K., Arai, Y., Watanabe, Y., Nishioka, K., . . . Nakaya, T. (2021, September 15). Development of genus-specific universal primers for the detection of flaviviruses. Retrieved May 23, 2022, from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC8442469/

Holbrook, M. R. (2017, April 30). *Historical Perspectives on Flavivirus Research*. Viruses. Retrieved January 3, 2022, from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5454410

Gibbs, T., & Smith, D. W. (2016, December 16). Flavivirus laboratory case definition. Retrieved May 23, 2022, from https://www1.health.gov.au/internet/main/publishing.nsf/Content/cda-phlncd-flavivirus.htm

Kading, R., & Schountz, T. (2016, November 2). Flavivirus infections of bats: Potential role in zika virus ecology. Retrieved May 28, 2022, from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5094249/#:~:text=Isolates%20of%20RBV%2C%20DBV%2C%20and,glands%2C%20suggesting%20transmission%20by%20bite.&text=However%2C%20some%20flaviviruses%20that%20have,Japanese%20encephalitis%20virus%20(JEV).

Kuhn, R. (2011). Flaviviruses. In 1942376441 1355458143 N. H. Acheson (Author), Fundamentals of Molecular Virology (2nd ed., pp. 137-147). S.l., United State: Wiley-Blackwell.

Kumar K; Arshad SS; Toung OP; Abba Y; Selvarajah GT; Abu J; A R Y; Ong BL; Bande F; (2018, September 17.). *The distribution of important sero-complexes of flaviviruses in Malaysia*. Tropical animal health and production. Retrieved January 3, 2022, from https://pubmed.ncbi.nlm.nih.gov/30604332/

Lim, V.-C., Ramli, R., Bhassu, S., & Wilson, J.-J. (2017, July 25). *A checklist of the Bats of Peninsular Malaysia and progress towards a DNA Barcode Reference Library*. PloS one. Retrieved January 3, 2022, from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5526618/

Luis, A. D., Hayman, D. T., O'Shea, T. J., Cryan, P. M., Gilbert, A. T., Pulliam, J. R., and Webb, C. T. 2015. *A comparison of bats and rodents as reservoirs of zoonotic*

viruses: Are bats special? Proceedings. Biological sciences. Retrieved January 3, 2022, from https://pubmed.ncbi.nlm.nih.gov/23378666/

Maclachlan, N. J., & Fenner, F. (2011). *Fenner's Veterinary Virology*. Elsevier Academic Press.

Meis, N., Tiltje, R., Handry, L., Roni, K., & Alice, L. (2021, June 6). *Diversity of fruit bats (Pteropodidae) and and their ectoparasites in Batuputih Nature Tourism Park, Sulawesi, Indonesia.* Retrieved January 3, 2022, from https://smujo.id/biodiv/article/download/8127/4853/42166

Pastorino, B., Nougairède, A., Wurtz, N., Gould, E., & Lamballerie, X. de. (2010, May 7). *Role of host cell factors in flavivirus infection: Implications for pathogenesis and development of Antiviral Drugs*. Antiviral Research. Retrieved January 3, 2022, from https://www.sciencedirect.com/science/article/pii/S0166354210006030

Relova, D., Rios, L., Acevedo, A., Coronado, L., Perera, C., & Pérez, L. (2020, March 18). Impact of RNA degradation on viral diagnosis: An understated but essential step for the successful establishment of a diagnosis network. Retrieved May 23, 2022, from https://experts.illinois.edu/en/publications/impact-of-rna-degradation-on-viral-diagnosis-an-understated-but-e

Sauqi, M. S., Restiadi, T. I., Koesdarto, S., Hastutiek, P., Setiawan, B., & Wijaya, A. (2021, September). *Identification of ectoparasites and endoparasites on fruit bats (Cynopterus Brachyotis) in Ketapang Timur village, Ketapang Sub-district, Sampang District*. Journal of Parasite Science (JoPS). Retrieved January 3, 2022, from https://e-journal.unair.ac.id/JoPS/article/view/30365/15739

Sonja, M. B. (2016, December 19). Current Biology Magazine - cell. Retrieved May 23, 2022, from https://www.cell.com/current-biology/pdf/S0960-9822(16)31080-6.pdf

Szentiványi, T., Christe, P., & Glaizot, O. (2019, April 24). *Bat Flies and their microparasites: Current knowledge and distribution*. Frontiers in veterinary science. Retrieved January 3, 2022, from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6492627/

Vina-Rodriguez, A., Sachse, K., Ziegler, U., Chaintoutis, S. C., Keller, M., Groschup, M. H., & Eiden, M. (2017, May 24). *A novel pan-flavivirus detection and identification assay based on RT-qPCR and Microarray*. BioMed Research International. Retrieved January 3, 2022, from https://www.hindawi.com/journals/bmri/2017/4248756/

Woo, P., & Lau, S. (2019, September 21). Viruses and bats. Retrieved May 28, 2022, from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6832948/