Roles of BALB/c mice and *Aedes* mosquitoes in the transmission of duck Tembusu virus

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A Dissertation Submitted in Partial Fulfillment of the Requirements
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บทบาทของหนูไมซ์สายพันธุ์ BALB/c และยุงลายในการถ่ายทอดเชื้อไวรัสเทมบูซูในเป็ด

น.ส.ณิชาภัทร ยุรญาติ

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ณิชาภัทร ยุรญาติ : บทบาทของหนูไมซ์สายพันธุ์ BALB/c และยุงลายในการถ่ายทอดเชื้อไวรัสเทมบูซูในเป็ด. (Roles of BALB/c mice and *Aedes* mosquitoes in the transmission of duck Tembusu virus) อ.ที่ปรึกษาหลัก : รศ. น.สพ. ดร.สนธยา เตียวศิริทรัพย์, อ.ที่ปรึกษาร่วม : รศ. สพ.ญ. ดร.อัญญรัตน์ ต้นธีรวงศ์,รศ. น.สพ. ดร.ธีระยุทธ แก้วอมตวงศ์

ไวรัสเทมบูซูในเป็ดเป็นเชื้ออุบัติใหม่ในกลุ่มฟลาวิไวรัสที่ก่อโรครุนแรงต่อระบบประสาทและระบบต่างๆ ในสัตว์ปีก ไวรัสนี้แบ่งเป็น 3 คลัสเตอร์ คลัสเตอร์ที่พบมากในประเทศไทยคือ 2.1 พยาธิกำเนิดของไวรัสมีการศึกษาอย่างกว้างขวางในสัตว์ปีก แต่สำหรับสัตว์เลี้ยงลูกด้วยนมนั้นข้อมูลยังมีจำกัด วัตถุประสงค์แรกของวิทยานิพนธ์นี้คือ ศึกษาพยาธิกำเนิดของเชื้อไวรัสเทมบูซูในเป็ดสายพันธุ์ไทยในสัตว์เลี้ยงลูกด้วยนม โดยฉีดเชื้อให้หนู BALB/c อายุ 6 อาทิตย์ ทางสมองและทางใต้ผิวหนัง เพื่อศึกษาอาการทางคลินิก การเปลี่ยนแปลงทางพยาธิวิทยา ปริมาณไวรัส และการกระจายตัวของไวรัส ผลการศึกษาพบว่าไวรัสก่อโรครุนแรงเฉียบพลันและเป็นสาเหตุการตายของหนูที่ได้รับเชื้อไวรัสเทมบูซูในเป็ดทางสมอง หนูแสดงอาการทั้งทางร่างกายและระบบประสาท การเปลี่ยนแปลงทางพยาธิวิทยา และการกระจายตัวของไวรัสพบได้ในทุกอวัยวะ ปริมาณเชื้อไวรัสในสมองสูงกว่าในอวัยวะอื่นๆ อย่างมีนัยสำคัญ ($p<0.05$) อย่างไรก็ตามไม่พบไวรัสในตัวอย่างน้ำลายและอุจจาระของหนู การศึกษานี้แสดงให้เห็นว่าเชื้อไวรัสเทมบูซูในเป็ดสายพันธุ์ไทยมีความสามารถก่อโรครุนแรงในสัตว์เลี้ยงลูกด้วยนม สำหรับการติดต่อของโรค ไวรัสติดต่อโดยมียุงเป็นพาหะนำเชื้อ แต่ขณะนี้ยังไม่มีข้อมูลความสัมพันธ์ระหว่างเชื้อไวรัสเทมบูซูในเป็ดและยุงลายซึ่งเป็นยุงที่ชอบดูดเลือดสัตว์เลี้ยงลูกด้วยนม ดังนั้น วัตถุประสงค์ที่สองคือ การศึกษาความสามารถของยุงลายบ้านและยุงลายสวนในการเป็นพาหะของเชื้อไวรัสเทมบูซูในเป็ดสายพันธุ์ไทย ผลการศึกษาพบว่ายุงลายทั้งสองชนิดสามารถนำเชื้อไวรัสได้ก็ต่อเมื่อได้รับเชื้อไวรัสในปริมาณอย่างน้อย 10^6 $TCID_{50}$/mL และเมื่อเพิ่มปริมาณเชื้อเป็น 10^7 $TCID_{50}$/mL พบว่าความสามารถในการเป็นพาหะของยุงลายทั้ง 2 ชนิดจะเพิ่มขึ้นอย่างมีนัยสำคัญ ($p<0.05$) ในทางตรงกันข้าม ยุงลายทั้ง 2 ชนิดไม่สามารถเอื้อให้เกิดการเพิ่มจำนวนของเชื้อไวรัสเทมบูซูในเป็ดที่แยกได้จากหนู BALB/c เนื่องจากเชื้อไวรัสจากหนูมีปริมาณน้อยกว่า 10^6 $TCID_{50}$/mL วัตถุประสงค์ที่สามคือการศึกษาสารพันธุกรรมของเชื้อไวรัสเทมบูซูในเป็ดที่แยกได้จากหนู BALB/c ยุงลายบ้าน และยุงลายสวน เปรียบเทียบกับสารพันธุกรรมของเชื้อไวรัสดั้งเดิม พบการกลายพันธุ์เฉพาะจุดทั้งในนิวคลีโอไทด์ และกรดอะมิโนของเชื้อไวรัสเทมบูซูในเป็ดที่แยกได้จากน้ำลายของยุงลายบ้าน ในขณะที่สารพันธุกรรมของเชื้อไวรัสเทมบูซูในเป็ดที่แยกได้หนู BALB/c และยุงลายสวนไม่มีการเปลี่ยนแปลง การศึกษานี้ให้ข้อมูลที่สำคัญของเชื้อเทมบูซูในเป็ดสายพันธุ์ไทย ในเรื่องของพยาธิกำเนิดของโรคในสัตว์เลี้ยงลูกด้วยนม ความสามารถของยุงลายในการเป็นพาหะของเชื้อ และการเปลี่ยนแปลงของสารพันธุกรรมของเชื้อที่พบในหนูและยุงลายซึ่งเป็นประโยชน์ต่อการป้องกันและควบคุมโรคทั้งในสัตว์ปีกและสัตว์เลี้ยงลูกด้วยนม

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Duck Tembusu virus (DTMUV) is an emerging flavivirus that causes severe nervous and systemic diseases in avian hosts. The virus has been classified into three clusters, and the predominant cluster in Thailand is cluster 2.1. The pathogenesis of the virus has been extensively studied in avian hosts but not in mammalian hosts. Therefore, the first objective of this dissertation was to investigate the viral pathogenesis in mammalian hosts. Six-week-old BALB/c mice were intracerebrally and subcutaneously inoculated with Thai DTMUV to examine clinical signs, pathological change, viral load, and virus distribution. Results demonstrated that Thai DTMUV caused an acute severe disease, and it was a cause of death in BALB/c mice inoculated by the intracerebral route. Infected mice showed both systemic and neurological symptoms. Pathological changes and virus distribution were observed in all tested organs. Viral load in the brain was significantly higher than in other organs ($p<0.05$). However, virus shedding was not recorded in saliva and feces. The findings suggested that Thai DTMUV has the potential to cause the threatening disease in mammalian hosts. In addition, one of the virus transmission routes is mosquito bites, but the interaction between Thai DTMUV and *Aedes* (*Ae.*) mosquito, which is a mammalian host preferred-mosquito, is lacking. Thus, the second objective was to examine the vector competence of *Ae. aegypti* and *Ae. albopictus* mosquitoes for Thai DTMUV. Results indicated that both *Aedes* mosquito species could serve as vectors for Thai DTMUV with minimum viral titer in a blood meal of 10^6 $TCID_{50}$/mL. When *Aedes* mosquitoes received more viral titer (10^7 $TCID_{50}$/mL), their competence significantly increased ($p<0.05$). In contrast, both *Aedes* species did not support the development of the isolated Thai DTMUV viruses from BALB/c mice because their titer was less than 10^6 $TCID_{50}$/mL. The third objective was to investigate the viral genomes that were isolated from BALB/c mice, *Ae. aegypti*, and *Ae. albopictus* mosquitoes compared with the original virus. A point mutation of nucleotide and the amino acid was found in all isolated DTMUV from *Ae. aegypti* saliva, while other viruses were similar to the positive virus. In summary, our results provide important information about Thai DTMUV in mammalian hosts, mosquito vectors, and virus genome that were useful for preventing and controlling the disease in both avian and mammalian hosts.

Field of Study:	Veterinary Pathobiology	Student's Signature .....
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Nichapat Yurayart

TABLE OF CONTENTS

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

LIST OF ABBREVIATIONS

Ae.	*Aedes*
ANOVA	Analysis of Variance
BBB	Blood-brain barrier
BHK-21	Baby hamster kidney-21
C	Capsid protein
CNS	Central nervous system
CSF	Cerebrospinal fluid
CULAC	Chulalongkorn University Laboratory Animal Center
CVV	Cache Valley virus
Cx.	*Culex*
DENV	Dengue virus
DPI	Day post-inoculation
DTMUV	Duck Tembusu virus
E	Envelop protein
EIP	Extrinsic incubation period
ER	Endoplasmic reticulum
H&E	Hematoxylin and eosin
HPF	High power field
IC	Intracerebral route
ID	Intradermal route
IP	Intraperitoneal route
IV	Intravenous route
JEV	Japanese encephalitis virus
M	Membrane protein
M-DTMUV	Mice-derived duck Tembusu virus
NS	non-structural protein
P-DTMUV	Positive duck Tembusu virus DK/TH/CU-1 strain
pbf	Post blood-feeding

pi	Post-inoculation
RdRp	RNA-dependent RNA polymerase
SAG	Spinal arachnoid granulation
SC	Subcutaneous route
SGE	Salivary gland extract
$TCID_{50}$	50% tissue cell culture infectious dose
TMUV	Tembusu virus
WNV	West Nile virus
YFV	Yellow fever virus
ZIKV	Zika virus

CHAPTER 1

Introduction

1.1 Importance and rationale

Duck Tembusu virus (DTMUV) is a newly emerging virus causing severe disease in the avian host, especially in ducks (Su et al., 2011). Clinical signs depend on their age. In duckling, symptoms are primarily found in nervous and systemic systems, including paralysis, rolling, depression, and retarded growth. Reproductive signs such as egg drop and ovaritis are mainly found in puberty, especially layer duck (Ninvilai et al., 2020). Morbidity was high up to 90%, and mortality was 5-30% (Chakritbudsabong et al., 2015). The disease has a negative impact on the economy, especially in agricultural countries such as Thailand. DTMUV which has been reported in Thailand consists of three clusters, but the predominant virus is classified as a member of cluster 2.1. Its identity is more closely related to Chinese DTMUV than the MM1775 strain, a prototype Tembusu virus (TMUV) found in Malaysia (Thontiravong et al., 2015; Ninvilai et al., 2019). Therefore, cluster 2.1 of DTMUV strain DK/TH/CU-1 was used as a model for our dissertation.

Also, DTMUV is a member of the *Flaviviridae* family, which is a large group of viral pathogens causing important public health diseases, including dengue virus (DENV), Japanese encephalitis virus (JEV), West Nile virus (WNV), yellow fever virus (YFV), and Zika virus (ZIKV) (Mackenzie et al., 2004). In general, flavivirus has a broad host range such as a human, non-human primate, mammal, and avian because it has the potential to adapt itself for a new host (Weaver and Barrett, 2004). A previous study showed that Chinese DTMUV could infect mice (Li et al., 2013; Ti et al., 2016). Moreover, the neutralizing antibody was found in farm workers during an outbreak (Tang et al., 2013). In contrast, Thai DTMUV infection in mammalian hosts lacks the information necessary to determine the disease's severity.

Originally, TMUV was firstly isolated from *Culex* (*Cx.*) *tritaeniorhynchus* mosquitoes collected from a pasture near cattle shed and human habitations in Kuala Lumpur, Malaysia. Subsequently, the virus was identified as a mosquito-borne flavivirus (Platt et al., 1975). Currently, the virus has been examined in several mosquito species to find out its vectors. Results showed that several *Culex* species were able to serve as virus vectors. Still, there was no report about vector competence of *Aedes* (*Ae.*) mosquitoes, particularly for Thai DTMUV, even though farmers and animal husbandry are close to each other (O'Guinn et al., 2013; Sanisuriwong et al., 2020).

DTMUV is a single-stranded RNA in the viral genome, and its replication process lacks a proofreading mechanism. Virus mutation or substitution can be found in their process (Holland and Domingo, 1998; Mukhopadhyay et al., 2005). Therefore, a viral genome study of the virus challenged into unnatural host and vector is fascinating because it has to survive or adapt to new environments.

This dissertation focused on Thai DTMUV in viral pathogenesis in the unnatural host, viral transmission in unnatural vectors, and viral genome. The findings will provide more information and understanding of Thai DTMUV that benefit disease prevention and control.

1.2 Objectives of study

1. To characterize the pathogenesis and distribution of DTMUV in BALB/c mice via intracerebral and subcutaneous routes

2. To evaluate the ability of *Aedes aegypti* and *Ae. albopictus* to support the replication and transmission of DTMUV

3. To investigate the viral characterization and genetic diversity of DTMUV in BALB/c mice and *Aedes* mosquitoes by using whole genome sequence analysis

1.3 Hypothesis

1. Duck Tembusu virus can cause different diseases between BALB/c mice inoculated by intracerebral and subcutaneous routes.

2. *Aedes aegypti* and *Ae. albopictus* can be the vectors for DTMUV.

3. The characterization of the DTMUV genome that was forced to change its host and vector may differ from the characterization of the original virus.

1.4 Keywords (English):

Aedes aegypti, *Aedes albopictus*, BALB/c mice, duck Tembusu virus, pathogenesis, vector competence, viral genome

1.5 Conceptual framework

This study was divided into three phases. A conceptual framework is shown in Figure 1.

Phase I: Pathogenesis of Thai duck Tembusu virus in BALB/c mice between intracerebral and subcutaneous routes

Thai DTMUV was challenged into BALB/c mice for a pathogenesis study. BALB/c mice were selected to serve as the new host and represent the small mammalian host. This mouse is a Th2 biased immune response strain suitable for a viral study (Mills et al., 2000).

Phase II: Vector competence study of *Ae. aegypti* and *Ae. albopictus* for Thai duck Tembusu virus

Thai DTMUV and mice isolated DTMUV were challenged in the unnatural vectors to study the interaction between viruses and vectors. *Aedes aegypti* and *Ae. albopictus* mosquitoes were selected for this study because both mosquito species were main vectors for human and mammalian hosts (Takken and Verhulst, 2013).

Phase III: Viral characterization and comparison of nucleotide and amino acid sequences

The whole genome sequence of isolated viruses from phase 1 and 2 was analyzed and compared with the Thai DTMUV DK/TH/CU-1 strain.

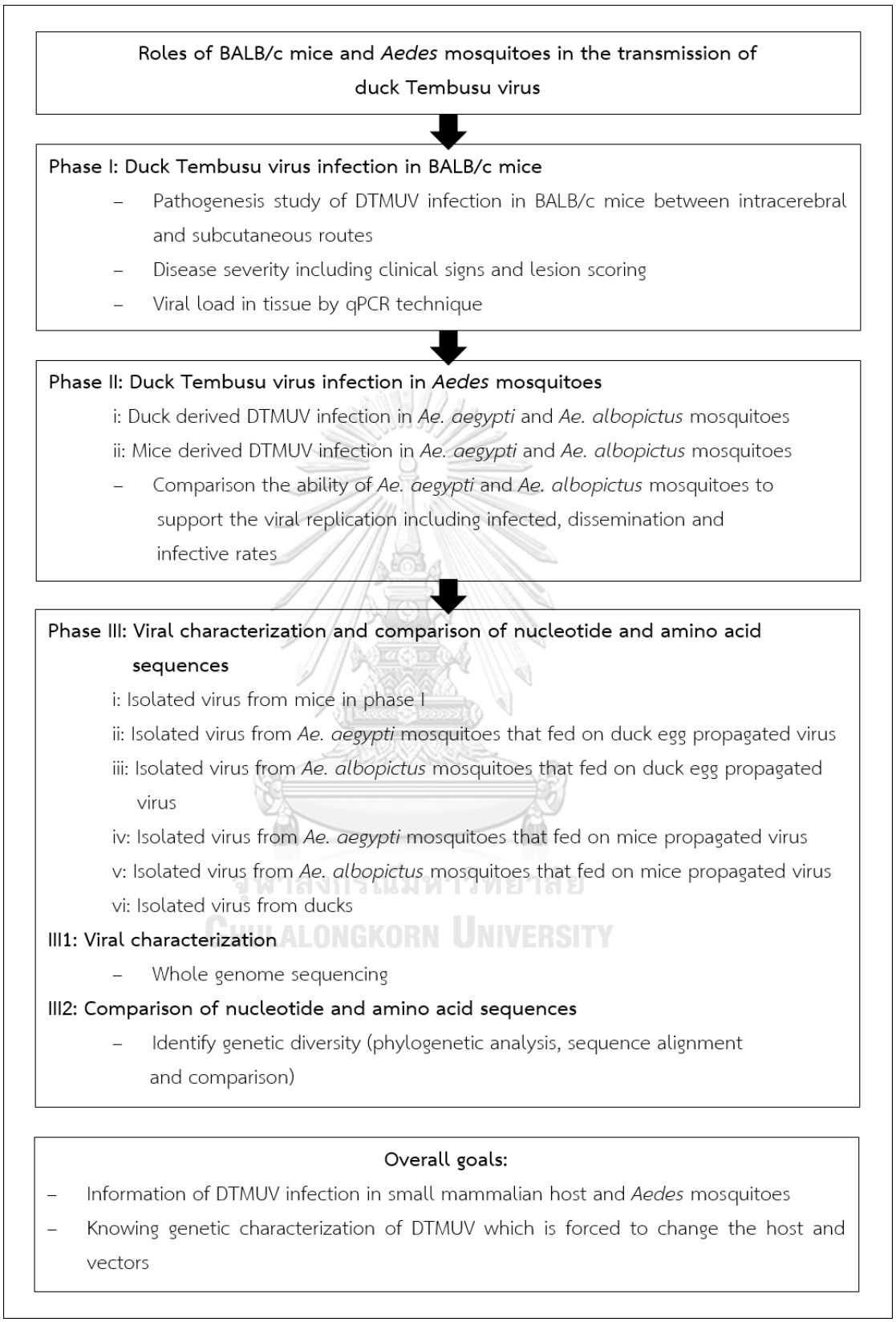

Figure 1 Conceptual framework of this dissertation

1.6 Literature review

Tembusu virus

Tembusu virus (TMUV) was firstly isolated from *Cx. tritaeniorhynchus* mosquitoes in Kuala Lumpur, Malaysia, in 1955. The virus was recorded as a prototype, MM1775 strain and identified as flavivirus (Platt et al., 1975). Then, TMUV was found in *Culex* mosquitoes such as *Cx. vishnui* and *Cx. tritaeniorhynchus* in several countries, including Indonesia and Thailand, between 1970-1997 (Leake et al., 1986; Pandey et al., 1999). Plus, neutralizing antibodies were detected in humans, domestic fowls, and Bornean orangutans. However, there was no disease report (Olson et al., 1983; Wolfe et al., 2001). In 2000, the disease was found in broiler chickens in Sitiawan, Malaysia, which infected chickens exhibited neurological signs. The confirmed causative agent was a Tembusu-related virus. Their genetic characteristics had 92% nucleotide sequences homologous with TMUV (Kono et al., 2000).

Duck Tembusu virus

In 2010, a significant disease outbreak was found in duck farms in several provinces of Southeast China. Clinical signs were egg drop, anorexia, and ataxia. Morbidity and mortality rates were 90% and 5-30%, respectively. The primary lesion was hemorrhagic ovaritis, so the disease was called duck egg-drop syndrome. The causative agent was tested by Koch's postulate technique and identified as a Baiyangdian virus (BYDV). Its genetics was closely related to TMUV and classified as a novel duck Tembusu virus (DTMUV). Moreover, *Culex* mosquitoes were examined as natural vectors (Cao et al., 2011; Su et al., 2011).

In 2013, the disease was found in layer and broiler duck farms in several Thailand provinces such as Nakhon Ratchasima, Prachinburi, Chonburi, and Suphanburi provinces. Clinical signs were severe neurological signs, and egg production decreased. Disease prevalence was 17.19%, and the morbidity and mortality rates were between

20-50% and 10-30%, respectively (Chakritbudsabong et al., 2015). Currently, DTMUV circulated in Thailand was characterized and compared with other TMUVs. The phylogenetic tree showed that Thai DTMUVs were divided into three clusters. Most of them were cluster 2, the same cluster with Chinese DTMUV (Ninvilai et al., 2019). Thai DTMUV, a DK/TH/CU-1 strain, was used in this study. Phylogenetic analysis showed that it was similar to Chinese DTMUV (97.3-98.3% identity) more than MM1775 (90.3% identity, mosquito-origin) and Sitiawan virus (89.4% identity, chicken-origin) (Thontiravong et al., 2015).

Duck Tembusu virus structure and virus characteristic

Duck Tembusu virus (DTMUV) is a member of the Ntaya virus (NTAV), which belongs to the *Flaviviridae* family. DTMUV is classified into a mosquito-borne cluster of *Flavivirus* genera. The viral genome is a positive sense single-stranded RNA, and its size is approximately 11 kilobases in length. Its genome has one open reading frame that encodes a single polyprotein, including three structural and seven non-structural proteins. Three structural proteins include capsid (C), membrane (M), which is expressed as the precursor to M (prM), and envelope (E) proteins. Seven non-structural proteins include NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5 proteins (Mukhopadhyay et al., 2005).

The capsid protein involves the viral genome packaging and forms the nucleocapsid (NC) core in terms of viral structural proteins. PrM protein plays a crucial role in the E protein's spatial structure and forms the viral envelope. E protein comprises three domains: envelop domain (ED) I, II, and III. The ED I is a structurally central domain related to viral production, pH sensitivity, and neuroinvasiveness. The ED II has a fusion loop and contributes to virus-mediated membrane fusion. The ED III interacts with host cellular receptors and plays a significant target of neutralization antibodies (Arora et al., 2013; Zhang et al., 2017).

For non-structural proteins, the NS1 protein consists of two forms, including secreted and membrane-associated forms. The secreted form found in the early phase of infection induces complement activity and forms the immune complex. The other involves the viral replication process, such as virus assembly and maturation at the endoplasmic reticulum (ER) lumen (Muller and Young, 2013). NS2A and NS2B proteins are cofactors and viral enzymes essential for viral replication and assembly (Leung et al., 2008). NS3 protein involves viral enzyme activities such as protease, helicase, and nucleotide triphosphatase (Yiang et al., 2013). NS4A protein induces membrane rearrangement for the viral replication complex. The 2K peptide is essential for proper co-translational membrane insertion and protein folding. NS4B protein facilitates viral replication, counteracts innate immune responses, and involves viral adaptation (Zou et al., 2014; Zmurko et al., 2015). NS5 protein is viral enzymes including RNA-dependent RNA-polymerase (RdRp) and methyltransferase (Klema et al., 2016) (Fig. 2)

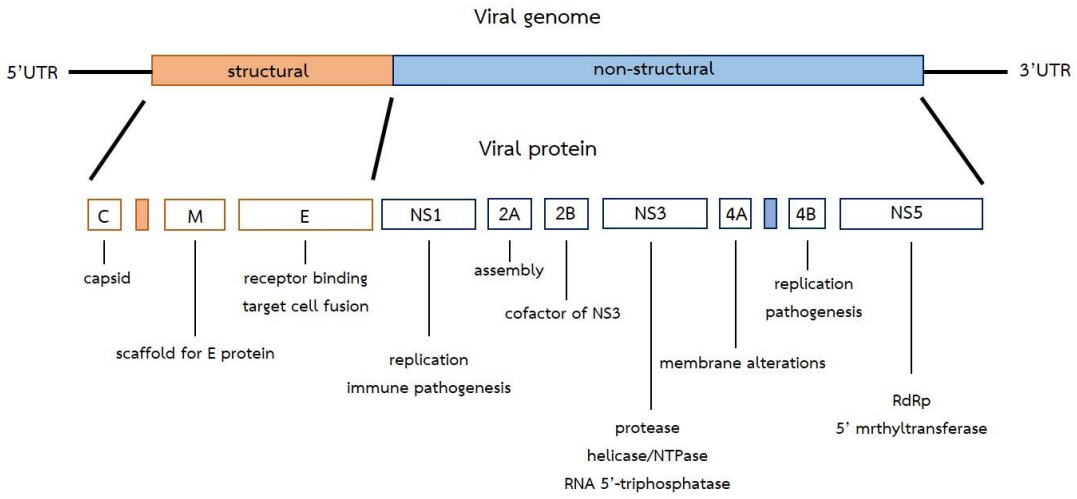

Figure 2 Summary of viral genome functions

DTMUV is a single-stranded RNA with an open reading frame consisting of three structural and seven non-structural proteins (modified from Zmurko et al., 2015).

Duck Tembusu virus in mammalian hosts

During the outbreak of DTMUV in China, the viral infection was investigated in the farmworkers because of public health concern. Results showed that viral RNA was found in saliva samples, and antibody was detected in blood samples, but clinical signs were not observed (Tang et al., 2013). In Malaysia, a virus surveillance project was also performed for humans and mammals around the disease outbreak area, but results were similar to the study in China (Wolfe et al., 2001).

The experiment of DTMUV infection in the mammalian host was first studied in BALB/c and Kunming mice using the outbreak strain in China. The virus caused disease in mice via the intracerebral (IC) inoculation route. In contrast, subcutaneous (SC) and intraperitoneal (IP) routes could not cause any disease. Clinical signs were similar to naturally infected ducks, in which the symptoms included hind limb paralysis, ataxia, blindness, loss of appetized, and weight loss. Morbidity and mortality were 100% and 20-80%, respectively, which the virus distributed to internal organs within two to three days post inoculation. In the early phase of infection, microscopic lesions were found in the brain, liver, and spleen suspected to be viral target organs, and late phase lesions were found in the kidney, heart, and intestine. Lesions included non-suppurative encephalitis, lymphoid depletion, and necrosis (Li et al., 2013; Ti et al., 2016).

Tembusu virus in mosquito vectors

Tembusu virus was firstly isolated from mosquitoes, and it was classified into the mosquito-borne cluster (Thontiravong et al., 2015). Therefore, the study of the interaction between TMUV and mosquitoes was evaluated, especially in *Culex* mosquito species. The result showed that each *Culex* mosquito species had a different ability to transmit the virus. For example, *Cx. vishnui* had highly susceptible to viral infection, but *Cx. fuscocephala* could not because it had a salivary gland barrier (O'Guinn et al., 2013). The interaction between pathogen and mosquito vector is

complicated. In general, a mosquito becomes a competent vector for pathogens depending on a mosquito's ability to acquire, maintain, and transmit the pathogens. On the other hand, some mosquito species are not competent vectors because they have barriers to protecting themselves from foreign bodies or pathogens. The most important tissue barriers are midgut and salivary gland barriers. The virus has to pass many steps to transmit to a new host including midgut infection and replication, dissemination from midgut to secondary tissues, amplification in secondary tissues, salivary gland infection and releasing to a proboscis (Franz et al., 2015).

Aedes aegypti and *Aedes albopictus* moquitoes

Aedes (*Stegomyia*) *aegypti* (L.) and *Aedes* (*Stegomyia*) *albopictus* (Skuse) mosquitoes are a member of the Culicidae family. They are highly competent for transmitting several flaviviruses such as DENV, YFV, and ZIKV (Black et al., 2002; Mousson et al., 2005). *Aedes aegypti* mosquitoes originated from Africa, and their habitats are urban areas or indoor houses. *Aedes albopictus* originated from Southeast Asia, and their habitats are forests, suburbs, and urban areas. Nowadays, both *Aedes* mosquitoes can adapt to a new environment to rapidly spread throughout the world (Paupy et al., 2009; Proestos et al., 2015).

About the blood-feeding behavior, only female mosquitoes take blood meals. Protein in the blood is necessary for egg production related to the host preference of the mosquitoes (Takken and Verhulst, 2013). Both mosquito species prefer to feed on human and mammalian hosts, but they can feed on other hosts, such as an avian host in a conditional area (Kek et al., 2014). Therefore, they are important vectors for zoonotic disease between humans and animals (Valderrama et al., 2017).

CHAPTER 2

Pathogenesis of Thai duck Tembusu virus in BALB/c mice: descending infection and neuroinvasive virulence

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Pathogenesis of Thai duck Tembusu virus in BALB/c mice: descending infection and neuroinvasive virulence

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2.1 Introduction

Duck Tembusu virus (DTMUV) is a newly emerging virus that causes disease in avian hosts, especially ducks and chickens. Infected animals manifest clinical symptoms, including loss of appetite, retarded growth, depression, and decreased egg production. DTMUV infected animals also demonstrate extraordinary clinical signs such as paralysis, walking in circles, and blindness signifying nervous system infection. Morbidity and mortality rates were recorded at 90% and 5-30%, respectively (Cao et al., 2011; Su et al., 2011). DTMUV belongs to an important flavivirus pathogen group that causes life-threatening diseases in both humans and animals, including dengue virus (DENV), Japanese encephalitis virus (JEV), West Nile virus (WNV), yellow fever virus (YFV), and Zika virus (ZIKV) (Mukhopadhyay et al., 2005). Most of these viruses cause zoonotic diseases transmitted by mosquitoes or tick bites (Dobler, 2010; Pandit et al., 2018). Global viral epidemics are considered a public health concern (Fernandez-Garcia et al., 2009).

In 2010, large outbreaks of DTMUV were reported in several duck farms in China and spread throughout Southeast Asia to Thailand and Malaysia. Koch's postulate technique confirmed the virus as a causative agent and transmitted via mosquito (Cao

et al., 2011; O'Guinn et al., 2013; Sanisuriwong et al., 2020). During the outbreak in China, sample collections were tested for DTMUV. Oral swabs and serum samples were positive for viral RNA and neutralizing antibodies, respectively (Tang et al., 2013). These indicated that DTMUV has the potential to transmit from ducks to humans or cause disease in other hosts. Pathogenesis study of Chinese DTMUV was investigated in mice (Li et al., 2013; Ti et al., 2016).

In Thailand, outbreaks of the virus have been recorded in both farms and free-grazing ducks since 2007. The disease is widely spread across several provinces and results in economic losses, especially in layer ducks (Chakritbudsabong et al., 2015; Ninvilai et al., 2018; Tunterak et al., 2018). Phylogenetic analysis showed the Thai DTMUV could be classified into three clusters, with the predominant Thai cluster as cluster 2.1 (Ninvilai et al., 2019). Thai DTMUV has the potential to transmit the disease in mammals; however, disease pathogenesis in a mammalian host remains unclear. Here, intracerebral and subcutaneous routes were used for DTMUV mammalian inoculation since DTMUV is known to cause neurological disease and is transmitted via a mosquito bite. BALB/c mice were inoculated with cluster 2.1 Thai DTMUV via intracerebral and subcutaneous routes to investigate the virus's pathogenesis, severity, and distribution.

2.2 Materials and Methods

2.2.1 Duck Tembusu virus

DK/TH/CU-1 strain of DTMUV was kindly provided by the Department of Veterinary Microbiology, Faculty of Veterinary Science, Chulalongkorn University. Initially, the virus was isolated from infected broiler ducks in Nakhon Ratchasima Province, Thailand (Thontiravong et al., 2015). This virus is classified as DTMUV cluster 2.1, a predominant cluster of DTMUV circulating in ducks in Thailand (Ninvilai et al., 2019). It was propagated in 9-day-old embryonated duck eggs, measured by virus titration assay in a baby hamster kidney-21 (BHK-21) cell line, and the infected cells

were proven by immunocytochemistry straining. Virus titer was expressed as a 50% tissue culture infectious dose per mL ($TCID_{50}$/mL).

2.2.2 Experimental animals and ethical considerations

Six-week-old specific pathogen-free BALB/c mice were selected as experimental animals representing a mammalian viral host because BALB/c mice are a Th2 biased immune response strain suitable for a viral study (Mills et al., 2000). All animal use was conducted in compliance with the Chulalongkorn University Laboratory Animal Care and Use Committee (Animal Use Protocol No. 1773002) at Chulalongkorn University Laboratory Animal Center (CULAC). Mice were reared in separated isolators in an ABSL-2 room under controlled conditions of 21±2 ºC, 50±5% relative humidity with 12 h light/dark cycle, and feed and water *ad libitum* to standard protocol. Before the experiment, mice were quarantined for two weeks and tested for DTMUV infection by reverse transcription-polymerase chain reaction (RT-PCR).

2.2.3 Experimental design and Thai DTMUV inoculation

One hundred and twenty mice (80 females and 40 males) were randomly divided into four groups (n=30), including two inoculated groups and two negative control groups. The inoculated groups received 30 µL of Thai DTMUV (DK/TH/CU-1 strain) (10^5 $TCID_{50}$/0.1 mL) via intracerebral (IC) and subcutaneous (SC) routes, respectively. Negative control groups were inoculated with allantoic fluid from specific pathogen-free duck eggs by the same routes. The experiment was performed for 14 days. Clinical signs were observed twice a day post-inoculation (dpi). The oral cavity was swabbed and kept in the transport medium every other day from the same five mice of each group. Three mice from each group were euthanized by isoflurane evaporation for blood collection and necropsy at 1, 3, 5, 7, 9, 12, and 14 dpi. Heparinized blood (1 mL) was collected from the heart. Organ samples (i.e., brain, heart, lung, liver, spleen, and kidney) were collected and divided into two parts. The first part was fixed in 10% neutral-buffered formalin for histopathological processing,

and the second part was frozen at -80 ºC until required for use. Gross lesions were observed and recorded during necropsy.

2.2.4 Histopathology and immunohistochemistry

For evaluating the pathological changes, fixed samples were stained with hematoxylin and eosin (H&E). In brief, fixed tissues were embedded in paraffin blocks, cut to 3 μm thick, and placed on a positively charged slide. Tissue sections were stained with H&E using standard methods. Lesions were scored according to the number of inflammatory areas using the scale 0: none, 1: 1-3 focal inflammatory areas per high power field (HPF), 2: lesions covering 25% of an HPF, and 3: lesions covering more than 75% of an HPF. Scoring was estimated from 10 HPFs over each tissue section. In the brain section, inflammatory lesions consisted of perivascular cuffing, neuronal necrosis, and gliosis. The spleen section criteria for lesions were lymphoid hyperplasia, lymphoid depletion, and splenic macrophage activation. The criterion for lesions in the liver, heart, lung, and kidney sections was inflammatory cell infiltration (Li et al., 2013). For detecting DTMUV infection in organs, tissue sections were stained with a specific antibody against the E protein of flavivirus. In brief, tissue sections were pretreated with citrate buffer pH 6.0 for 5 min, and then endogenous peroxidase was blocked by 3% H_2O_2 solution for 10 min. Next, the sections were stained with a mouse anti-flavivirus group antigen monoclonal antibody (1:400; Merck, Germany) at 4 ºC overnight, followed by incubation with a secondary antibody, a monoclonal rabbit anti-mouse immunoglobulin G/HRP (Envision Dako, Denmark) for 45 min. The positive reaction was labeled by a 3, 3' diamino-benzidine tetrahydrochloride (DAB) substrate. Finally, the sections were counterstained with hematoxylin for 1 min, dehydrated, mounted with permount, and observed under a light microscope. Each test consisted of positive and negative controls.

2.2.5 Viral load in tissue samples

The viral load in each tissue sample was measured using quantitative real-time PCR (qPCR) assay. Tissue samples including brain, heart, lung, liver, spleen, and kidney were homogenized with phosphate-buffered saline to make a 10% suspension. Plasma was separated from heparinized blood by centrifugation at 3,000g, 4 ºC for 5 min. RNA was extracted from tissue, plasma, red blood cells (RBCs), oral swab, and feces using a Viral Nucleic Acid Extraction Kit II (Geneaid, Taiwan). RNA concentration was adjusted to 250 ng RNA/sample for cDNA synthesis. Following the manufacturer's protocol, first-strand cDNA was synthesized using the ImProm-IITm Reverse Transcription System (Promega, USA). Real-time PCR was performed in a MicroAmp Fast Optical 96-well reaction plate using Applied Biosystems StepOnePlus™ Real-Time PCR System (Applied Biosystems, USA). Primers and TaqMan probes were specific for the E gene of DTMUV. The primer set was EF (5'-TGTCTTATGCAGTACCGATG-3') and ER (5'-CGTATGGGTTGACTGTTATCA-3'), with TaqMan probe sequences EP (FAM-AGTTCCCATATCCATGTC-MGB). Thermal cycling conditions were 95 ºC for 20 sec, 50 cycles at 95 ºC for 20 sec, and 54 ºC for 35 sec (Yan et al., 2011). The standard curve was obtained by serial dilutions of the recombinant plasmid (pUC-19) containing the E gene of DTMUV. The linear regression curve was obtained by plotting mean Ct values with copy number (log10) of each plasmid dilution. Absolute quantitation of DTMUV from samples was normalized per 250 ng of total RNA.

2.2.6 Statistical analysis

Descriptive statistics were used to describe clinical signs, morbidity and mortality rates, and immunohistochemistry. Macroscopic and microscopic lesions were scored and compared between inoculated and negative control groups using a non-parametric test. Viral loads in different tissue samples were compared using analysis of variance (ANOVA). Statistical analysis was performed using GraphPad Prism 5.0 software (GraphPad Software Inc. La Jolla, CA) with $p<0.05$ considered statistically significant.

2.3 Results

2.3.1 Clinical signs

To investigate the pathogenesis of Thai DTMUV, BALB/c mice were inoculated with the virus through intracerebral (IC) and subcutaneous (SC) routes. Clinical signs were observed in the inoculated IC group but not in inoculated SC and negative control groups. Ten out of 27 mice showed clinical signs at three days post-inoculation (dpi), including depression, ruffled hair, and loss of appetite (Fig. 3a). All mice exhibited clinical signs at five dpi. Nine out of 21 mice, including seven females and two males, died at six dpi. At nine dpi, two mice exhibited left side eyelid inflammation (blepharitis), right side hind limb paralysis, and right circling walk (Fig. 3b-c). General clinical signs were observed until the end of the experiment, and mice did not recover. No clinical signs were shown in the negative control inoculated IC group (Fig. 3d).

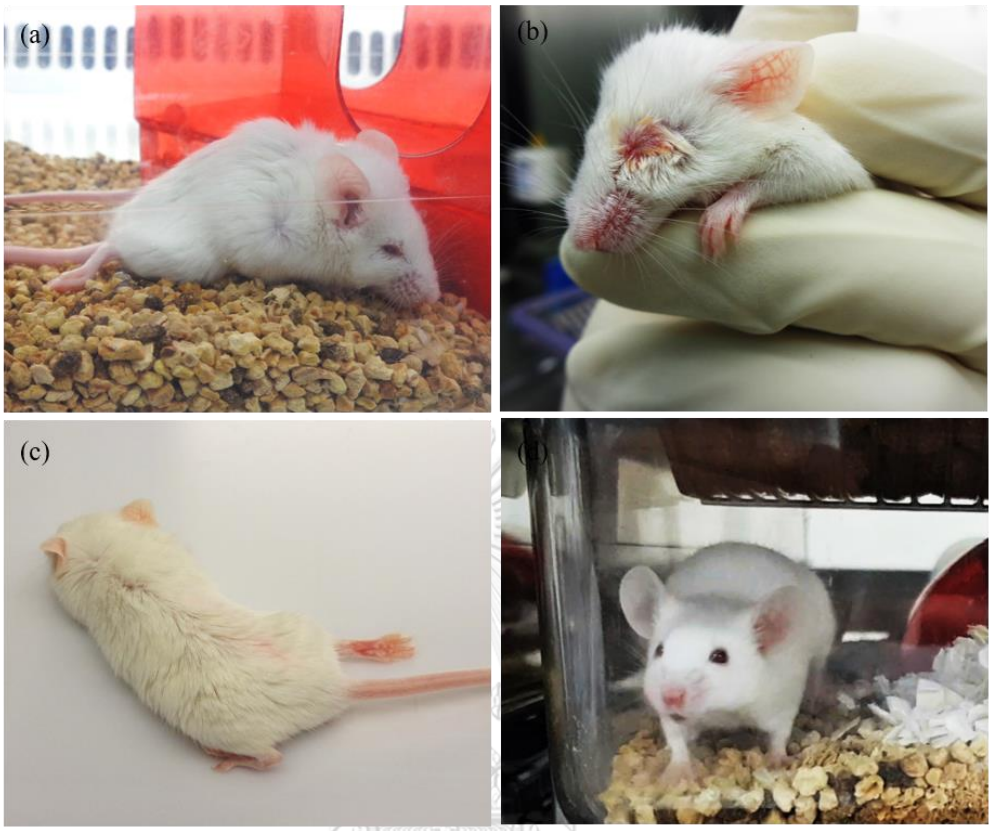


Figure 3 Clinical signs exhibited by BALB/c mice inoculated with Thai DTMUV
(a-c) Mice inoculated with Thai DTMUV via intracerebral (IC) route showed severe clinical signs including depression, ruffled hair, blepharitis, and hind limb paralysis; **(d)** Negative control IC inoculation group.

2.3.2 Macroscopic and microscopic findings

Macroscopic lesions were observed only in inoculated IC group. No lesion was observed in inoculated SC and negative control groups. In inoculated IC group, lesions were found in both brain and visceral organs. In the brain, a lesion was found at 3 dpi. The severity was mild degree and increased to a moderate degree at 5 dpi until 9 dpi. At the end of the experiment, no lesion was observed in the brain. Brain's lesions were edema, meningeal congestion, and soft consistency (Fig. 4a-c). In the spleen, mild lesions were found at 3 dpi, increased the severity at 7 dpi, and decreased between day 9 and 12 pi. The lesions were splenomegaly, edema, and rounded shape compared with the negative control group (Fig. 4m-o). In the heart, lung, liver, and kidney, macroscopic lesions were mild and found at 5 dpi. The severity increased to moderate between day 7 to 9 pi and then decreased between day 12 to 14 pi. Heart's lesions were white streak, hemorrhage, and firm consistency (Fig. 4d-f). Lung's lesion was congestion with pleural effusion (Fig. 4g-i). The liver was hepatomegaly with pale and soft consistency (Fig. 4j-l). Kidney lesions were edema, and the renal cortex was pale color (Fig. 4p-r). Moreover, peritoneal fluid and gallbladder distention were observed at 5 and 7 dpi, respectively. Mice with hind limb paralysis had complex lesions, including severe brain congestion, a white streak in the heart, and a pale spleen. Macroscopic lesions in dead mice were not different from necropsied mice. The severity of lesions and the number of mice with lesions are shown in Table 1.

Microscopic lesions were observed in the inoculated IC group's organs. The severity is shown in Table 2. In contrast, no microscopic lesion was observed in the inoculated SC and both negative control groups. In the inoculated IC group, most lesions were found in the brain. In the brain, lesions were reported in two parts, including the cerebrum and cerebellum. In the cerebrum, lesions were non-suppurative encephalitis with lymphocytes and plasma cells infiltration. Congestion in blood vessels and two to three layers of mononuclear cell infiltration cuffing around the vessel were observed (Fig. 5a-c). Lesions were found at 3 dpi, and the severity increased from mild to moderate at 7 dpi. Then, the lesion degree decreased to mild

between 9 and 14 dpi. Neuronal necrosis and gliosis were shown in the cerebellum at 3 dpi, and lesion scores were more severe at 5 to 7 dpi and decreased to a mild degree at 12 dpi (Fig. 5d-f). In the heart, a small number of cardiomyocytes were lost and replaced by a loosely arranged band of edematous collagen, fibrin, edema, and occasionally plasma cells and lymphocyte infiltration (Fig. 5g-i). Heart lesions were found at 3 dpi, and the severity was mild until the end of the experiment.

In the lung, alveolar septa were expanded five to seven times average size, infiltrated by numerous macrophages and lymphocytes, and mixed with degenerated epithelium and mild hemorrhage. Consolidation and atelectasis also affected approximately 60-70% of the lung (Fig. 5j-l). The severity of lung lesions was mild from day 5 to day 14 pi. In the liver, mild lesions were found at 5 dpi until 14 dpi. Hepatocytes were ballooning, pale granular cytoplasm with mild sinusoidal congestion (Fig. 5m-o). The white pulp of the spleen showed multifocal lymphoid depletion, characterized by lymphocyte necrosis with loss of lymphocytes replaced by small amounts of cellular debris and fibrin. The red pulp of the spleen showed increased numbers of erythrophagocytic macrophages and histiocytes infiltrations (Fig. 5p-r). The severity of lesions was mild to moderate between 3 and 5 dpi. After that, the severity decreased to mild at 14 dpi. In the kidney, lesions were moderate congestion with increased cellular components, macrophages, and lymphocytes in glomeruli and interstitium (Fig. 5s-u).

Immunohistochemical staining detected DTMUV antigen in the brain and spleen of mice in the inoculated IC group. Virus antigens in the brain at 5 dpi were detected in neurons' cytoplasm in the cerebral cortex and cerebellum (Fig. 6a, c). These findings related to the brain's microscopic lesions on this day; the brain had neuron necrosis. In the spleen, the DTMUV antigen was detected in the cytoplasm of macrophages in the white pulp, where lymphoid depletion was observed in the microscopic lesion (Fig. 6e). By contrast, the DTMUV antigen was not detected in other organs in the SC inoculated and negative control groups (Fig. 6).

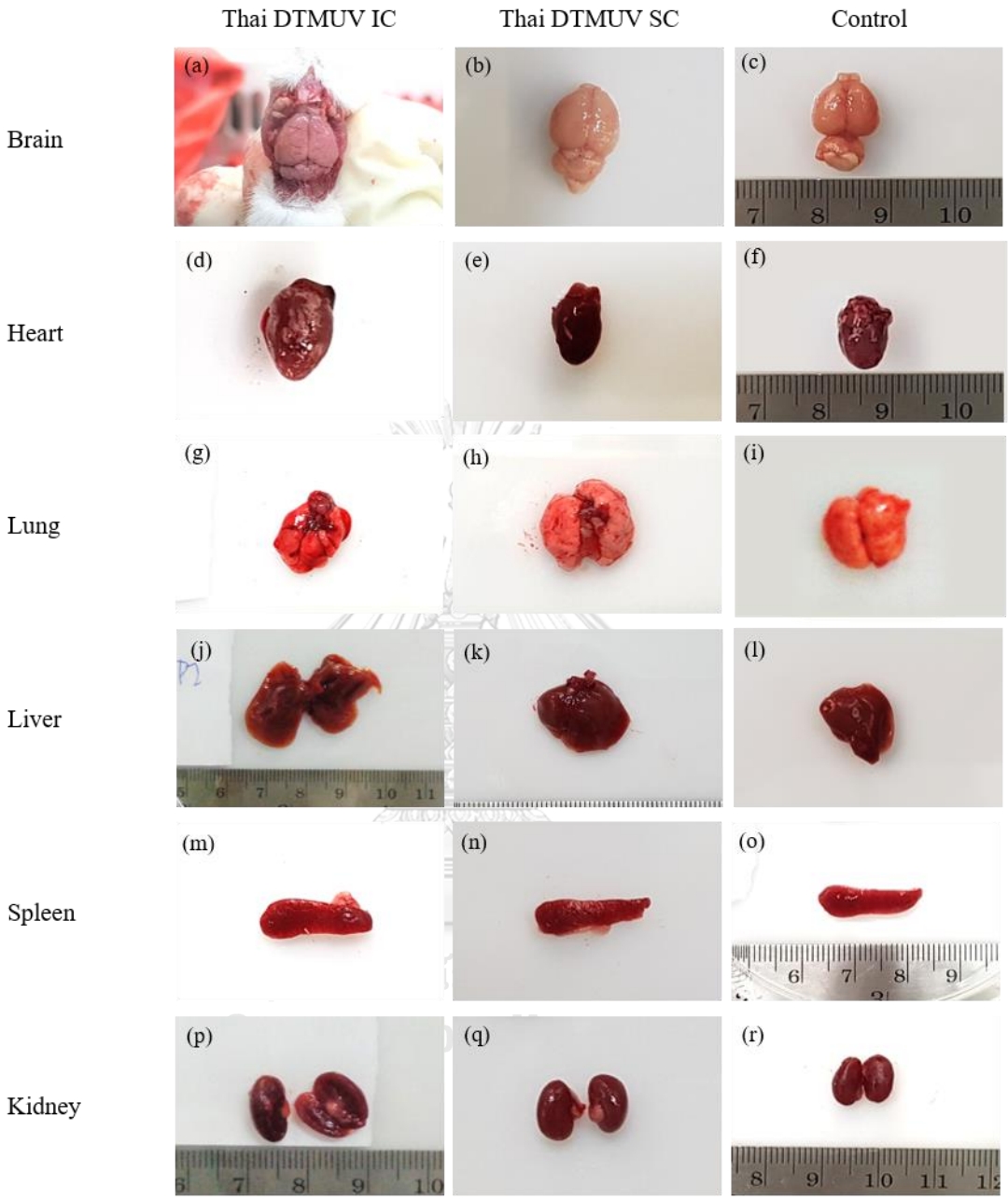
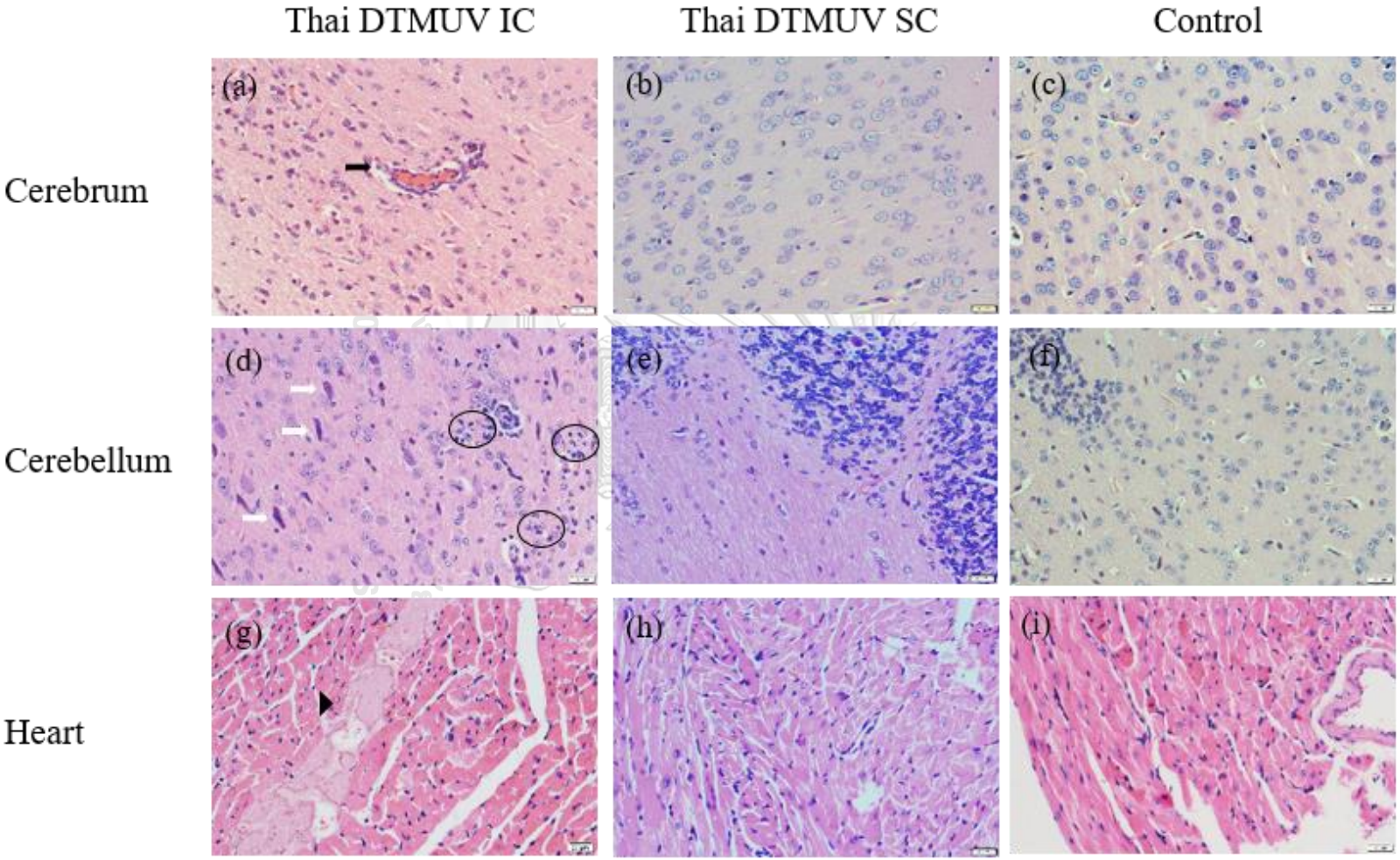


Figure 4 Macroscopic lesions of BALB/c mice inoculated with Thai DTMUV

In the first, second, and third rows, figures exhibit internal organs of BALB/c mice in IC and SC inoculation and negative control IC inoculation groups, respectively. **(a)** Brain: congestion, 5 dpi; **(d)** Heart: white streak and edema, 7 dpi; **(g)** Lung: edema and congestion, 9 dpi; **(j)** Liver: edema and yellowish, 7 dpi; (m) Spleen: rounded shape and edema, 5 dpi; **(p)** Kidney: edema and pale, 9 dpi.


Thai DTMUV IC
Thai DTMUV SC
Control
Cerebrum
Cerebellum
Heart
(a)
(b)
(c)
(d)
(e)
(f)
(g)
(h)
(i)

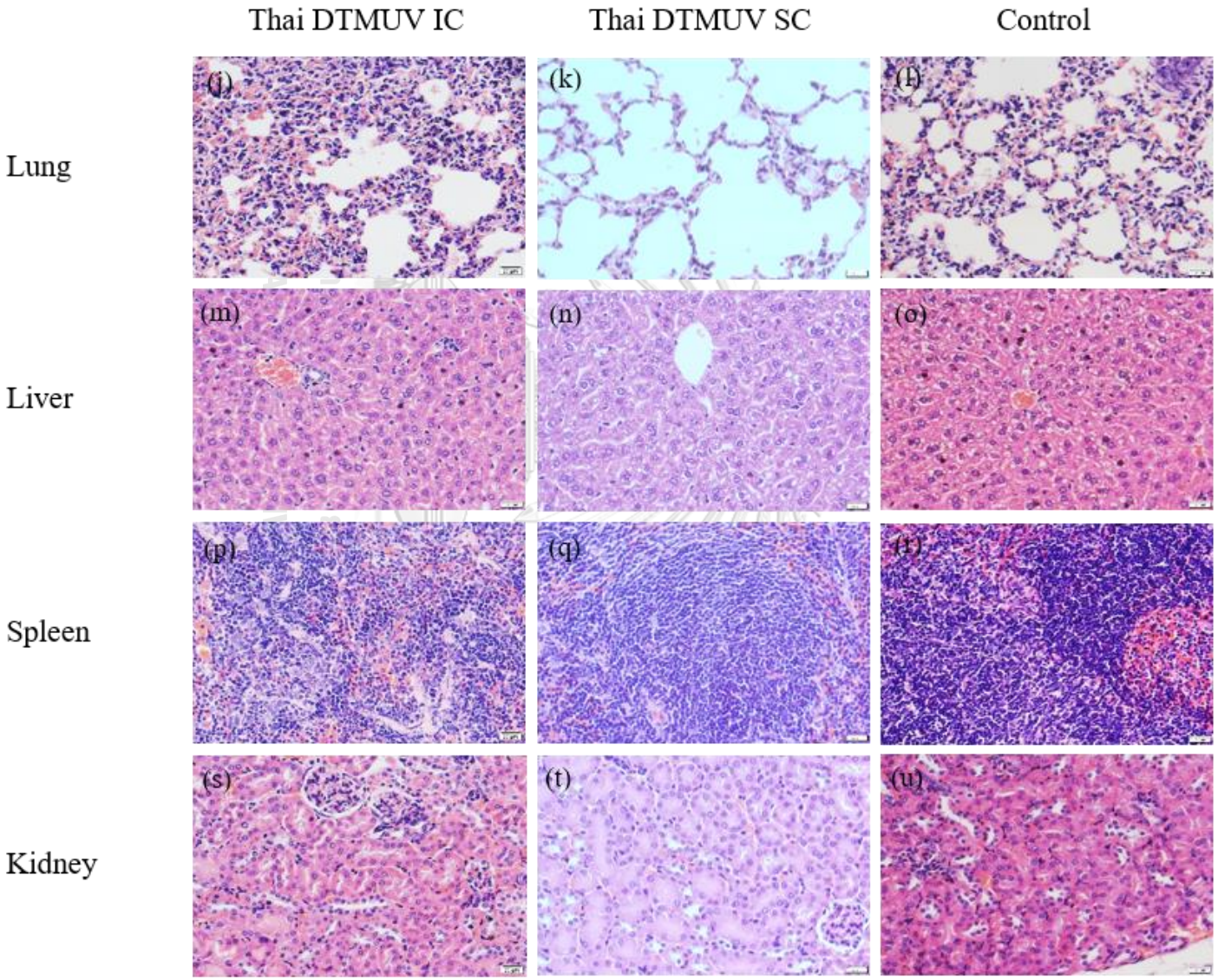

Thai DTMUV IC
Thai DTMUV SC
Control
Lung
Liver
Spleen
Kidney
(j)
(k)
(l)
(m)
(n)
(o)
(p)
(q)
(r)
(s)
(t)
(u)

Figure 5 Microscopic lesions of BALB/c mice inoculated with Thai DTMUV

Tissue sections were stained with H&E and observed under a light microscope. Scale bars = 20 μm. Figures in the first, second and third lanes exhibit histopathology of BALB/c mice's organs in IC and SC inoculation and negative control IC inoculation groups. **(a)** Cerebrum: multifocal non-suppurative meningoencephalitis with mononuclear cell perivascular cuffing (black arrow), 5 dpi; **(d)** Cerebellum: moderate acute multifocal non-suppurative meningoencephalitis with perivascular cuffing, neuronal necrosis (white arrows), and gliosis (circle), 5 dpi; **(g)** Heart: multifocal lymphoplasmacytic myocarditis with loosely arranged bands (arrowhead), 5 dpi; **(j)** Lung: severe lymphohistiocytic interstitial pneumonia, 7 dpi; **(m)** Liver: mild panlobular fatty degeneration and hepatic congestion, 5 dpi; **(p)** Spleen: splenic lymphoid necrosis, 5 dpi; **(s)** Kidney: moderate interstitial nephritis, 7 dpi. No lesion was observed in tissue sections of BALB/c mice' organs in SC group (the second lane) compared with the negative control group (the third lane).

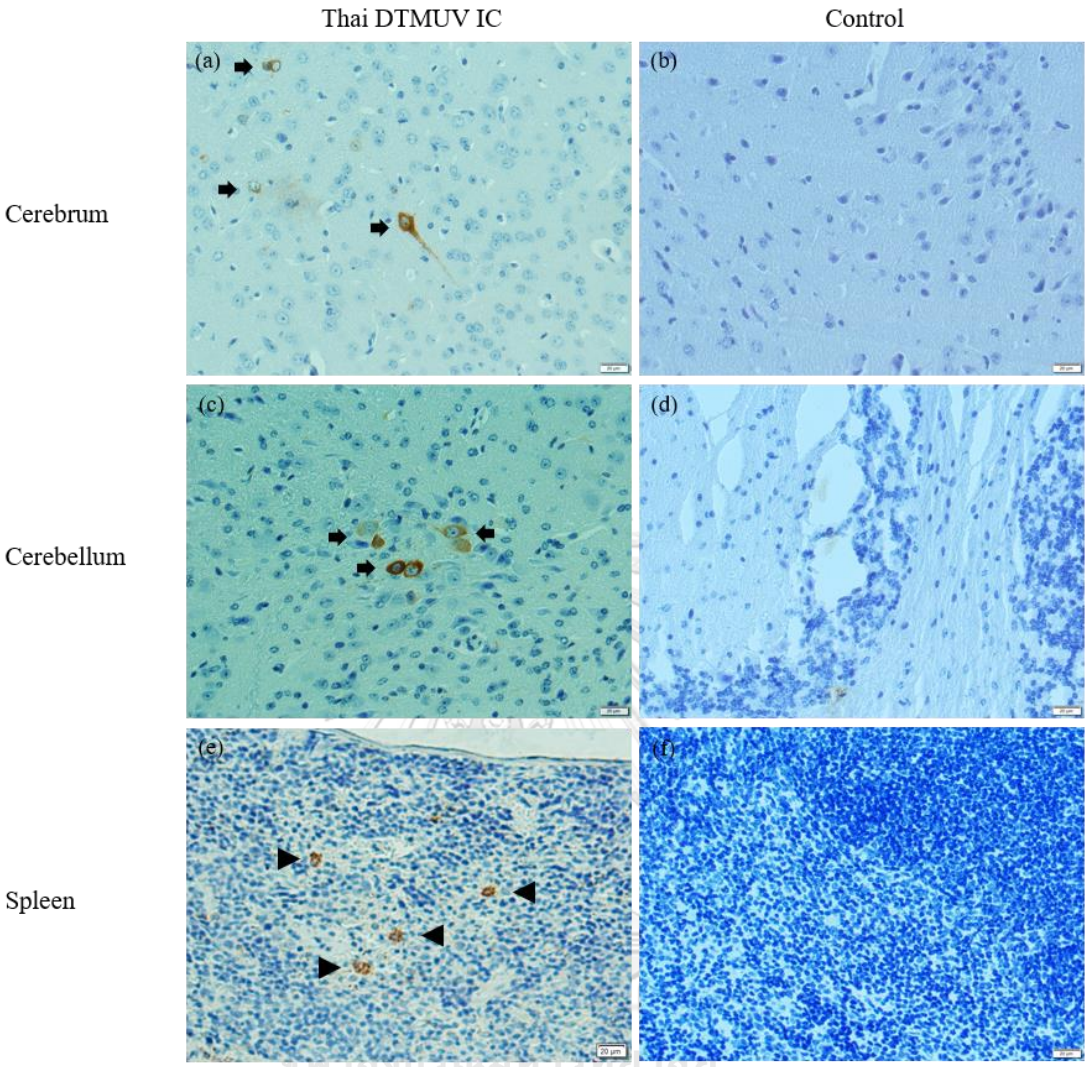


Figure 6 Immunohistochemistry staining of BALB/c mice inoculated with Thai DTMUV in IC group

Tissue sections were stained with a specific antibody against the E gene of flavivirus and observed under a light microscope. Scale bars = 20 μm. Figures in the first and second rows exhibit section organs of BALB/c mice in IC inoculation and negative control IC inoculation groups, respectively. **(a, c)** Cerebrum and cerebellum: positive Thai DTMUV immunostaining in neurons (black arrow), 5 dpi; **(e)** Spleen: positive Thai DTMUV immunostaining in macrophages (arrowhead), 5 dpi; **(b, d, f)** negative control IC inoculated group.

Table 1 Macroscopic lesion scoring of BALB/c mice inoculated with Thai DTMUV via IC route

Organ	Lesion	Day post inoculation							
		1	3	5	7	9	12	14	Dead mice
Brain	congestion	- [a](3/3)[b]	+ (1/3)	++ (1/3)	++ (3/3)	++ (1/3)	++ (1/3)	- (3/3)	++ (6/9)
			- (2/3)	+ (2/3)		+ (2/3)	+ (1/3)		+ (3/9)
							- (1/3)		
Heart	white streak/hemorrhage	- (3/3)	- (3/3)	+ (1/3)	+ (2/3)	+ (1/3)	+ (1/3)	- (3/3)	+ (3/9)
				- (2/3)	- (1/3)	- (2/3)	- (2/3)		- (6/9)
Lung	congestion	- (3/3)	- (3/3)	- (3/3)	+ (1/3)	+ (1/3)	+ (1/3)	- (3/3)	+ (5/9)
					- (2/3)	- (2/3)	- (2/3)		- (4/9)
Liver	hepatomegaly	- (3/3)	- (3/3)	- (3/3)	+ (1/3)	+ (1/3)	+ (1/3)	- (3/3)	+ (4/9)
					- (2/3)	- (2/3)	- (2/3)		- (5/9)
Spleen	splenomegaly	- (3/3)	+ (2/3)	+ (3/3)	++ (1/3)	+ (2/3)	+ (1/3)	- (3/3)	++ (2/9)
			- (1/3)		+ (1/3)	- (1/3)	- (2/3)		+ (5/9)
					- (1/3)				- (2/9)
Kidney	edema	- (3/3)	- (3/3)	+ (1/3)	+ (1/3)	+ (1/3)	- (3/3)	- (3/3)	+ (3/9)
				- (2/3)	- (2/3)	- (2/3)			- (6/9)

[a] The degree of lesions are shown as **-** no lesion, + mild, ++ moderate, and +++severe.

[b] Number of the lesion showing mice /total number of necropsied mice

Table 2 Microscopic lesion scoring of BALB/c mice inoculated with Thai DTMUV via IC route

Organ	Day post inoculation							
	1	3	5	7	9	12	14	Dead mice
Brain								
Cerebrum	0.0±0.0[a]	1.0±0.2	1.7±0.5	2.1±0.8	1.6±0.2	1.1±0.3	0.4±0.3	1.8±0.4
Cerebellum	0.0±0.0	0.6±0.2	1.7±0.4	1.7±0.7	1.4±0.0	0.9±0.1	0.4±0.2	1.7±0.4
Heart	0.0±0.0	0.2±0.3	0.5±0.2	0.8±0.3	0.6±0.2	0.3±0.2	0.1±0.1	0.8±0.3
Lung	0.0±0.0	0.0±0.0	0.9±0.6	0.6±0.5	0.5±0.2	0.3±0.1	0.1±0.1	1.1±0.4
Liver	0.0±0.0	0.0±0.0	0.5±0.3	0.4±0.2	0.2±0.2	0.2±0.2	0.2±0.2	0.6±0.3
Spleen	0.0±0.0	1.1±0.2	1.6±0.3	1.0±0.6	0.4±0.3	0.6±0.3	0.2±0.1	1.1±0.5
Kidney	0.0±0.0	0.0±0.0	0.9±0.1	0.5±0.3	0.4±0.4	0.3±0.2	0.0±0.1	1.1±0.4

[a] The average of microscopic lesion score ± standard deviation (SD). The severity of lesions is shown as 0: no lesion, 1: mild, 2: moderate, and 3: severe.

2.3.3 Virus load and virus distribution in mice

To measure the viral load and viral distribution in the tissue of BALB/c mice inoculated with Thai DTMUV, organs (i.e., brain, heart, lung, liver, spleen, and kidney) were tested using the DTMUV specific qPCR method. RNA of Thai DTMUV was only detected in the inoculated IC group, where the viral load was also early detected at one dpi in all organs. Viral load increased and peaked at seven dpi and then gradually decreased until 14 dpi. Viral load patterns were similar in all organs (Fig. 7a). Viral load in the brain was significantly higher than in other organs at all tested days, and the highest level was $10^{6.42\pm1.62}$ DTMUV genome copies per 250 ng RNA ($p<0.05$). Viral load in dead mice was similar to euthanized mice (Fig. 7b). Viral load in the brain was significantly different from all other organs ($p<0.05$). No viremia and oral and fecal sheddings were detected in the inoculated and negative control groups. Viral load in each mouse was shown in appendix 1.

These findings suggested that firstly, Thai DTMUV could infect and replicate in BALB/c mice through the IC route while, secondly, the virus rapidly disseminated from the brain to systemic within 24 hr. Thirdly, the brain was a tissue tropism of DTMUV, and fourthly, cause of death of nine mice at six dpi was due to the high level of viral load in the brain. Finally, the virus was not shed via saliva and feces.

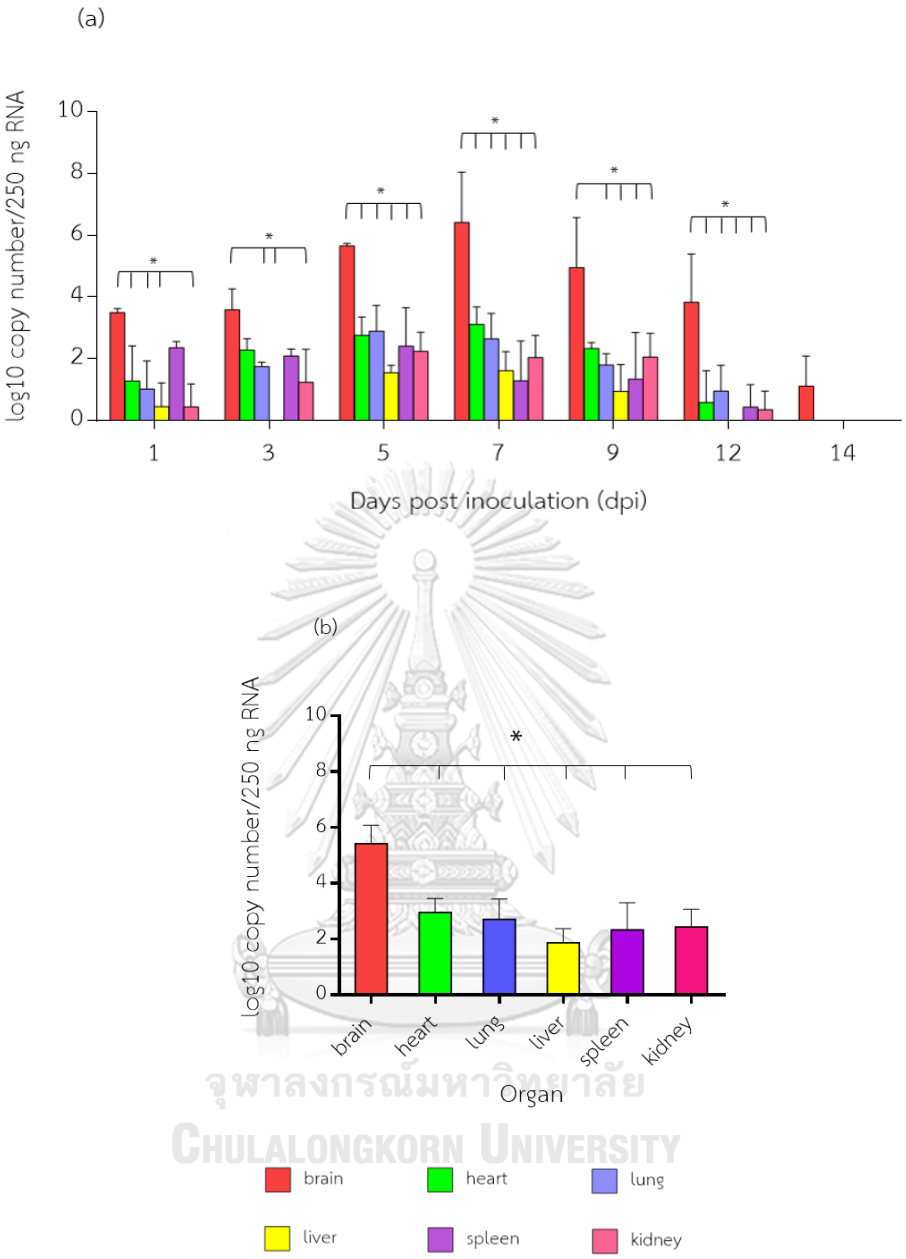


Figure 7 Viral load and tissue distribution of BALB/c mice inoculated with Thai DTMUV via IC route

Viral load was determined using the qPCR technique and showed as $\log_{10}$ DTMUV genome copies number per 250 ng RNA. **(a)** Viral load in organs of euthanized mice at 1 dpi to 14 dpi. **(b)** Viral load in organs of dead mice at six dpi. (*) indicates statistically significant differences among organs at that time point ($p<0.05$, one-way ANOVA).

2.4 Discussion

DTMUV is a newly emerging flavivirus in mainland China and Southeast Asia. The virus causes severe disease in avian hosts, especially in ducks and chickens (Kono et al., 2000; Su et al., 2011; Chakritbudsabong et al., 2015). Currently, DTMUVs are classified into three distinct clusters based on the nucleotide sequences of polyprotein genes (Ninvilai et al., 2019). A previous study showed that the pathogenesis of DTMUV in both avian and mammalian hosts mostly aligned with cluster 2.2 Chinese DTMUV. By contrast, information lacks concerning the pathogenesis of cluster 2.1 Thai DTMUV, the predominant cluster in Thailand, especially in mammalian hosts (Thontiravong et al., 2015). Here, cluster 2.1 Thai DTMUV infection pathogenesis in BALB/c mice was investigated for disease severity and virus distribution. Findings indicated that Thai DTMUV infected and caused severe disease in BALB/c mice by the intracerebral inoculation route. Mice exhibited neurological and systemic signs with acute death. Macro- and microscopic lesions were observed in all organs, especially the cerebral cortex and cerebellum. Virus antigen was detected in the cytoplasm of neurons and macrophages by immunohistochemistry (IHC). Virus distribution was detected in every organ but not recorded in oral swabs and blood. The virus did not manifest in mice through the subcutaneous inoculation route. These findings provided important information to understand disease mechanisms in the mammalian host.

Comparing of Thai DTMUV pathogenesis between BALB/c mice and Cherry Valley duck, a natural host. Our results showed that Thai DTMUV caused severe disease in BALB/c mice similar to duck, but the pathogenesis differed. In terms of clinical signs and lesions, BALB/c mice exhibited neurological and systemic signs similar to clinical signs found in young ducks. Infected ducks showed severe clinical signs, including weight loss, ataxia, and paralysis. The onset of disease in both mice and ducks was rapid. In terms of viral load and distribution, the virus was early detected in mice's organs at 1 dpi, peaked at 7 dpi, and decreased after that. In contrast, duck viral load, especially in brain and spleen, showed that the virus was early detected at 1 dpi and increased until the end (21 days) (Ninvilai et al., 2020). Previous research indicated that

duck is a natural host of DTMUV; therefore, it is more susceptible to the virus than mice. Moreover, inoculation routes affected the pathogenesis of the virus. Li et al. (2015) and Sun et al. (2019) showed that the spleen was the first replication site when duck was inoculated by intranasal or intramuscular routes. In addition, lymphocytes, macrophages, and neurons were targeted cells of the virus. Our results suggested that BALB/c mice could serve as a viral host, but its performance was less than the duck.

Comparing Thai and Chinese DTMUV pathogenesis in mice showed that both viruses caused the disease by the IC route with similar clinical signs. Still, the severity and distribution of the viruses were different. Thai and Chinese DTMUVs were characterized into different clusters, the Thai strain was cluster 2.1, but the Chinese strain was cluster 2.2. The nucleotide identity was about 98%. Between Thai and Chinese strains, nucleotide and amino acid were different in several genes. The most mutation sites located NS5 gene, followed by NS1, E, NS2, NS4A, C, PrM, NS4B, and NS3 genes (Ninvilai et al., 2018). All of these genes were important genes for viral pathogenesis. Therefore, pathogenesis between Thai and Chinese DTMUVs was different. Moreover, replication sites of Chinese DTMUV were restrictive, with viral RNA only found in some organs, including the brain, spleen, and kidney, while the viral antigen was only detected in neurons (Li et al., 2013; Ti et al., 2016). By contrast, the distribution of Thai DTMUV was more widespread. Viral RNA and lesions were found in all organs. The viral antigen was observed inside and outside the central nervous system (CNS), including neurons in the cerebral cortex and cerebellum and macrophage in the spleen. Therefore, our findings suggested that neurons and macrophages were tissue tropisms of DTMUV. Likewise, tissue tropism of JEV and WNV, a neuroinvasive flavivirus, occurs in neuron cells but differs in the CNS. For instance, JEV was found at the thalamus and brain stem, while WNV was found at the brain stem and anterior horns of the spinal cord (Guarner et al., 2004; German et al., 2006; Brown et al., 2007; Sips et al., 2012). Viral shedding was not found in the oral swab of mice in Thai and Chinese DTMUV, whereas viral load was found in the lung. Our results showed that viral load in the lung was very low, with viral antigens not found. This

study suggested that the lung was not a target organ of Thai DTMUV, and the virus was not shed from the respiratory system.

This study determined virus distribution as a descending infection pathway that started from the brain as an inoculation site and disseminated to peripheral organs without viremia. This result contrasted with the theory of flavivirus pathogenesis as an especially mosquito-borne group with an ascending infection pathway from the bitten site to the CNS. After infected mosquitoes take a blood meal, the virus replicates in local tissues and regional lymph nodes. Subsequently, primary viremia leads to extraneural tissue such as the reticuloendothelial system, followed by secondary viremia to the nervous system. The virus uses three mechanisms to cross the blood-brain barrier (BBB): direct infection of brain microvascular endothelial cells, transcellular transportation, and a Trojan horse mechanism by carrying an infected monocyte. Dissemination of the virus relies on viremia to target organs (Mukhopadhyay et al., 2005; Brown et al., 2007; Kimura et al., 2010). Previous studies of cerebrospinal fluid (CSF) outflow in animals can be used to explain how the virus spreads out from the inoculation site. Brain waste and CSF are physically removed from the CNS to the systemic circulation by the lymphatic drainage system. They are absorbed through spinal arachnoid granulations (SAG) of cranial and spinal nerve roots that directly connect with lymphatic vessels (Brierley and Field, 1948; Boulton et al., 1999; Chen et al., 2015). Moreover, CSF is also found in connective tissues surrounding the nerve roots, including optic and nasal cavities (Brinker et al., 1997; Lüdemann et al., 2005; Killer et al., 2007). Clinical signs of eyelid inflammation might be caused by a virus infection around the optic nerve. Unfortunately, infected mice were not tested for the smell. Therefore, our results suspected that Thai DTMUV disseminated from the brain to visceral organs via lymphatic vessels.

Surprisingly, Thai and Chinese DTMUVs do not transmit infection in mice by SC, intradermal (ID), and intraperitoneal (IP) inoculation routes. O'Guinn et al. (2013) reported that DTMUV infected mosquitoes transmitted the virus to naïve chicken and infected chicken also showed clinical signs. This finding confirmed that DTMUV is a

mosquito-borne virus, with mosquito inoculation causing the disease in animals. When a mosquito takes a blood meal, the proboscis extends into the skin's dermis layer; therefore, most inoculation routes used for studying mosquito-borne pathogens are SC and ID (Hopp and Sinnis, 2015). However, pathogenesis in infected animals between needle and mosquito inoculations is different. Cox et al. (2012) and Pingen et al. (2016) showed that mosquitoes' saliva and probing mechanisms modulated skin response and supported virus infection. Disease outcomes of other flaviviruses, including DENV and WNV, were more severe when mice were inoculated with viruses mixed with mosquito saliva and salivary gland extract (SGE) compared with methods including SC, IP, and intravenous (IV) inoculation routes. Virus infection was enhanced and more progressive, resulting in rapid viremia, higher virus load in tissue, and accelerated neuroinvasion (Schneider et al., 2007; Styer et al., 2011; Schmid et al., 2016). Moreover, Edwards et al. (1998) reported that mice inoculated with Cache Valley virus (CVV) by SC inoculation did not contract the disease. Still, mice immediately inoculated with the virus in the mosquito spot feeding area became infected. While a mosquito is probing for blood vessels, its stylet causes tissue trauma, hemorrhage, and induces local inflammation (Fontaine et al., 2011). Also, mosquito saliva components activate local cells in the dermis, including endothelial cells, dermal mast cells, and dendritic cells that induce faster immune processes and drainage to lymph nodes (Depinay et al., 2006; Schneider et al., 2007). Thus, the pathogenesis of DTMUV using mosquito saliva or a mosquito model in a mammalian host is interesting and requires further investigation, especially since infection through mosquitoes cause viremia and increase disease spreading opportunities.

This is the first study of Thai DTMUV pathogenesis in mice. Our data demonstrated that Thai DTMUV has the potential to cause disease in a mammalian host, but the inoculation route limits this. The occurrence of the disease in other mammalian hosts should be investigated.

CHAPTER 3

Vector competence of *Aedes aegypti* and *Aedes albopictus* mosquitoes for duck Tembusu virus

This part has been published on the topic of

Interactions of duck Tembusu virus with *Aedes aegypti* and *Aedes albopictus* mosquitoes: vector competence and viral mutation

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3.1 Introduction

Duck Tembusu virus (DTMUV) is an emerging pathogen that causes severe neurological and reproductive diseases in avian hosts, especially in ducks and chickens. The disease impacts agriculture and economic systems with high morbidity and mortality rates (Su et al., 2011). Since 2010, virus outbreaks have been reported in several Asian countries, including Thailand, China, and Malaysia (Thontiravong et al., 2015). DTMUV is a member of the *Flavivirus* genus and is classified as a mosquito-borne cluster similar to dengue virus (DENV), Japanese encephalitis virus (JEV), West Nile virus (WNV), Zika virus (ZIKV), and yellow fever virus (YFV) (Mukhopadhyay et al., 2005). The viral genome is a positive sense, single-stranded RNA that encodes three structural proteins (capsid (C), pre-membrane (PrM), and envelope (E)) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) (Bollati et al., 2010). Transmission routes consist of mosquito biting and direct contact (Li et al., 2015; Ninvilai et al., 2020).

In general, Tembusu (TMUV) was firstly found in *Culex tritaeniorhynchus* mosquitoes in Malaysia. Subsequently, the virus has been reported in several *Culex* species in other countries such as Thailand and Indonesia (Platt et al., 1975). Since the

disease outbreak, vector competence for DTMUV has been examined in several species of *Culex* mosquitoes. Results showed that, *Cx. vishnui* and *Cx. tritaeniorhynchus* had the potential to be a vector, but their abilities were different in particular for infectious dose and extrinsic incubation period (EIP). However, other *Culex* species, including *Cx. quinquefasciatus* and *Cx. fuscocephala* mosquitoes could not because they had midgut and salivary gland barriers, respectively (O'Guinn et al., 2013; Guo et al., 2020; Sanisuriwong et al., 2020). In contrast, vector competence study has not been investigated in other mosquito species, including *Aedes* mosquitoes, although several types of research showed that DTMUV could infect mammalian hosts. For instance, both Chinese and Thai DTMUVs caused a contagious disease in mice (Ti et al., 2016; Yurayart et al., 2020). Plus, the virus was found in the saliva of duck farm workers (Tang et al., 2013).

Thai DTMUV DK/TH/CU-1 strain used in our work was tested that it caused the neurological disease in BALB/c mice (results were shown in chapter 2). Therefore, an investigation of the ability of *Aedes* mosquitoes to support DTMUV is interesting. Moreover, our work will provide important information for viral prevention and control. Here, two types of DTMUV, including duck-derived and mice-derived viruses, were challenged into *Ae. aegypti* and *Ae. albopictus* mosquitoes to examine their competence to be viral vectors.

3.2 Materials and methods

3.2.1 Viruses

3.2.1.1 Positive DTMUV

Positive DTMUV (P-DTMUV) was sourced as a duck-derived Tembusu virus isolated from sick ducks in the Thai disease outbreak area. The virus strain used was DK/TH/CU-1 cluster 2.1, a predominant cluster in Southeast Asia, with virus accession number in GenBank as KR061333 (Thontiravong et al., 2015; Ninvilai et al., 2019). P-

DTMUV was provided by the Department of Veterinary Microbiology, Faculty of Veterinary Science, Chulalongkorn University, Thailand. The virus was propagated in nine-day-old embryonated duck eggs, and the allantoic fluid was harvested on day 5 post-inoculation (pi). Viral titer was measured using a virus serial titration assay in a baby hamster kidney-21 (BHK-21) cell line. The viral cytopathic effect was confirmed by a flavivirus-specific immunocytochemistry staining assay (Tunterak et al., 2020). Viral titer was expressed and calculated as a 50% tissue culture infectious dose per mL ($TCID_{50}$/mL), and P-DTUMV titer was 10^7 $TCID_{50}$/mL (Reed and Muench, 1938). All experiments were performed in the BSL-2 containment facility, and viruses were preserved at -80 ℃ until required for use.

3.2.1.2 Mouse DTMUV

Mouse DTMUV (M-DTMUV) was a mice-derived virus isolated from BALB/c mice in chapter 2. Briefly, six-week-old BALB/c mice were inoculated with P-DTMUV via the intracerebral route and euthanized on day 7 pi. The virus was isolated from the brain with the highest viral load measured by qPCR assay (10^8 DTMUV genome copies). The brain sample was mixed with a 4% FBS opti-mem medium to prepare M-DTMUV suspension. It was prepared in the fit volume for the artificial membrane feeding method. After blood-feeding, the virus was measured for the alive virus by serial titration assay in the BHK-21 cell line, and the virus titer was 10^4 $TCID_{50}$/mL.

3.2.2 Mosquitoes and mosquito rearing

3.2.2.1 *Aedes* (*Ae.*) *aegypti* and *Ae. albopictus* mosquitoes

Aedes aegypti mosquitoes were collected from Bangkok Province, Thailand, while *Ae. albopictus* mosquitoes were provided by Dr. Usawadee Thavara, Department of Medical Sciences, Ministry of Public Health, Thailand. The mosquito species were identified using physical morphology following a pictorial key presented by Rueda (2004) and molecular techniques. Unique characteristics of *Ae. aegypti* mosquitoes

include silver-white lyre markings on their thoraxes, and white bands on their legs and bodies, while *Ae. albopictus* mosquitoes have a white stripe running along the middle of their heads and thoraxes. Molecular identification was conducted using PCR and DNA sequencing techniques with universal primers LCO 1490 and HCO 2198 for invertebrate phyla, which amplified a fragment of the mitochondrial cytochrome c oxidase subunit I gene (COI) (Folmer et al., 1994).

3.2.2.2 Mosquito rearing

Mosquito rearing and experimental methods were approved and conducted according to the Chulalongkorn University Laboratory Animal Care and Use Committee (Animal Use Protocol No. 1831048). All experiments were performed at the Entomology Laboratory of the Veterinary Parasitology Unit, Faculty of Veterinary Science, Chulalongkorn University. Adult mosquitoes were reared in an incubator under controlled conditions including 25 °C and 80% relative humidity with a 12 h light/dark cycle, and fed on 10% sucrose solution and distilled water (DW) *ad libitum*. The mosquitoes were starved for 24 h and then allowed to feed on sheep blood using an artificial membrane feeding technique. A filter paper was lined inside a cup with DW for mosquito oviposition. Mosquito larvae were hatched by immersing the eggs in deoxygenated DW and reared under controlled conditions, including 25 °C and 12 h light/dark cycle, with daily fish feed (Clemons et al., 2010).

3.2.3 Infection of DTMUV in *Aedes* mosquitoes

Virus infection in mosquitoes was studied in three topics: extrinsic incubation period (EIP), survival rate, and vector competence. For EIP and survival rate experiments, P-DTMUV was tested. For the vector competence experiment, P-DTMUV and M-DTMUV were tested.

3.2.3.1 Mosquito infection

A large number of five-day-old *Ae. aegypti* and *Ae. albopictus* mosquitoes were sucrose-starved for 24 h and then allowed to feed on virus-infected blood meal using an artificial membrane feeder for 30 min. Post blood-feeding (pbf), the engorged mosquitoes were transferred to a new container and reared under the controlled conditions described above for subsequent studies. The virus-infected blood meal comprised 12.5% sheep blood, 7.5% fetal bovine serum (FBS), 1% sucrose, 900 µM adenosine triphosphate, and P-DTMUV in 4% FBS opti-MEM medium and 100U/mL gentamycin (Roundy et al., 2017). The final concentration of virus-infected blood meal varied from 10^0-10^7 $TCID_{50}$/mL, while viral titers were the same before and after mosquito feeding. P-DTMUV was replaced by M-DTMUV suspension for other study groups, and viral-free allantoic fluid for the negative control group.

3.2.3.2 Extrinsic incubation period experiment

Ten engorged mosquitoes from all P-DTMUV study groups were selected daily to detect the virus in each mosquito for 14 days (n=150 per group). The virus was detected by the RT-PCR assay described below. On day 0-7 pbf, the virus was detected in the whole body. On day 8-14 pbf, the saliva was also collected saliva from each mosquito. If the body sample was positive for the virus, the saliva sample was also tested.

3.2.3.3 Survival rate experiment

Thirty engorged mosquitoes from the P-DTMUV study and negative control groups were separated into new containers (n=30 per group). Mosquito death was daily recorded for 14 days

3.2.3.4 Vector competence experiment

Thirty engorged mosquitoes from all P-DTMUV and M-DTMUV study groups were individually dissected to collect three samples on day 14 pbf. The dissected day was selected from the EIP result. These samples consisted of two wings and six legs, saliva, and whole body included head, thorax, and abdomen. In brief, mosquitoes were starved for sugar and water at least 24 hr before the sample collation time. First, the mosquito was sedated by freezing in -20 ℃ incubator for 1.30 min and placed on a sterile petri dish. Second, wings and legs were cut under a light stereo microscope. Third, proboscis was inserted into a capillary tube filled with 10 µl of saliva media for 20 min, and the petri dish was placed on ice all the time. Saliva medium was 50% sucrose and FBS in ratio 1:1. Finally, the mosquito body was collected. Samples were collected and preserved in a 4% FBS opti-MEM medium. The virus was examined by using a RT-PCR assay.

3.2.3.5 Viral detection

DTMUV in all the samples was examined using the RT-PCR technique and partial NS 5 gene-specific primers. Samples were manually homogenized in a sterilized medium. Viral RNA was extracted using Viral Nucleic Acid Extraction Kit II (Geneaid, Taiwan), following the manufacturer's protocols. RT-PCR was performed using SuperScript® III One-Step RT-PCR system with Platinum® Taq DNA Polymerase (Invitrogen, USA) with thermal cycling conditions as follows; 48 ℃ for 45 min, 94 ℃ for 3 min, 40 cycles at 94 ℃ for 30 sec, 55 ℃ for 30 sec and 72 ℃ for 1 min, with a final extension at 72 ℃ for 10 min (Thontiravong et al., 2015).

3.2.4 Statistical analysis

Survival rates of mosquitoes were compared among infected blood meal levels and the negative control group using the Chi-square test. Vector competence was evaluated using three criteria of infected, disseminated, and infective rates. The viral

infected rate was calculated from the presence of the virus in the body of the mosquito. In contrast, the disseminated rate as viral distribution throughout the mosquito was calculated from the presence of the virus in the wings and legs. The infective rate as a virus that was ready to transmit to a new host was calculated from the presence of the virus in mosquito saliva. Vector competence between both *Aedes* mosquito species was compared using the Chi-square test. All statistical analyses were performed using GraphPad Prism 5.0 software (GraphPad Software Inc. La Jolla, CA). Statistical significance was considered at $p < 0.05$.

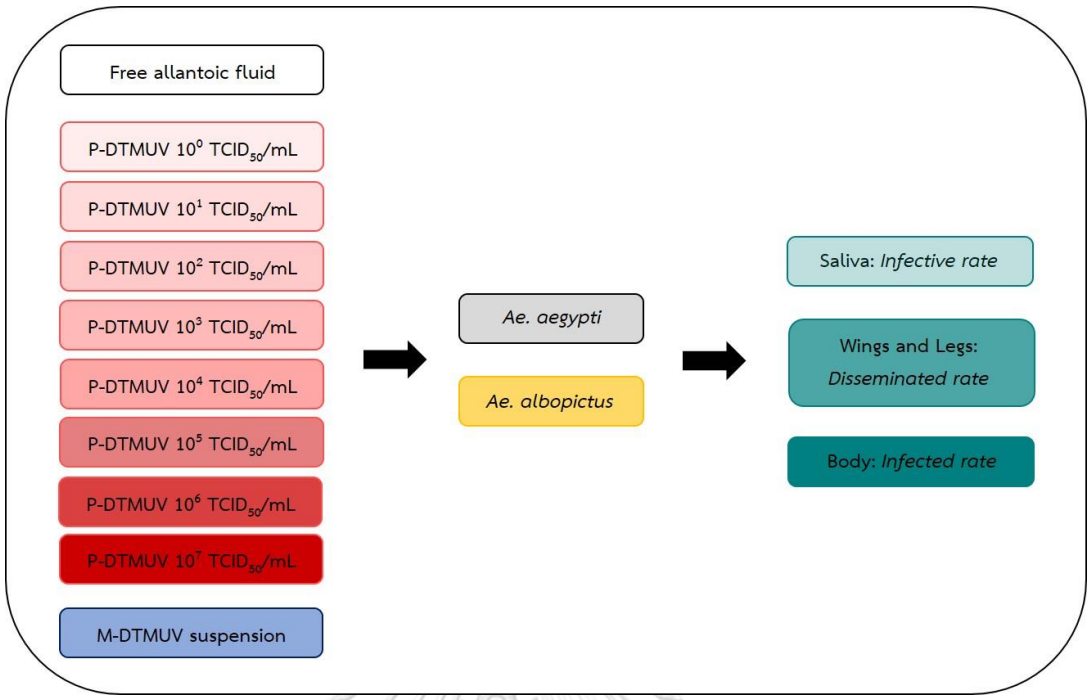

Figure 8 Vector competence experiment

Aedes aegypti and *Ae. albopictus* mosquitoes were allowed to feed on three types of blood meal, including free allantoic fluid, serial P-DTMUV dilutions, and M-DTMUV suspension. Vector competence was estimated by the virus detection in each part of mosquito body.

Table 3 Description of the terminology used for vector competence experiment

Terminology	Viral detection site		
	Whole body	Wing and leg	Saliva
Infected	✓	-	-
Disseminated	✓	✓	-
Infective	✓	✓	✓

3.3 Results

3.3.1 Extrinsic incubation period

To evaluate the extrinsic incubation period (EIP) of DTMUV in *Ae aegypti* and *Ae. albopictus* mosquitoes, serial dilution of virus (10^0-10^7 $TCID_{50}$/mL) was challenged into mosquitoes and examined daily from day 0 to 14 pbf using RT-PCR assay. On day 0 pbf, the virus was 100% detected in both *Aedes* species to confirm that all of the mosquitoes received the virus. For *Ae. aegypti* species, the virus was 100% detected in mosquitoes in 10^0-10^5 $TCID_{50}$/mL groups from day 0-4 pbf after that virus was not detected. For 10^6 and 10^7 $TCID_{50}$/mL groups, results were similar with other groups but the virus was detected again on day 13 pbf. According to the method, after day 7 pbf if the virus was detected in the mosquito body, the virus was also tested in saliva. The result showed that the virus was also detected in saliva on day 13 pbf. Infected rates were 60-70%, and infective rates were 10-50%. However, infective rates between day 13 and 14 pbf were not significantly different (Table 4a).

For *Ae. albopictus* species, results were similar with *Ae. aegypti* species. The virus was detected in saliva samples on day 13 pbf in 10^6 and 10^7 $TCID_{50}$/mL groups. The other groups showed negative results. Infected rates were 60-80%, and infective rates were 10-40%. However, infective rates between day 13 and 14 pbf were not significantly different (Table 4b). Results indicated that EIP of DTMUV in *Ae. aegypti* and *Ae. albopictus* were 13 days pbf.

3.3.2 Survival rate

To estimate the effect of virus infection in the mosquito, mosquito death was daily observed in challenged *Ae. aegypti* and *Ae. albopictus mosquitoes* for 14 days pbf. In *Ae. aegypti* mosquitoes, the negative control and study groups' survival rates were 93.33% and 93.33-100%, respectively, while *Ae. albopictus* mosquitoes recorded the negative control and study groups' survival rates at 96.67% and 90-100%,

respectively. Mosquito death was found throughout the extrinsic incubation period, including early and late phases. However, no difference was apparent between the negative control and study groups in both mosquito species. Therefore, DTMUV infection was not a cause of death in mosquitoes (Table 5). All the mosquitoes laid eggs; however, the transovarial transmission was not examined in this study.

3.3.3 Vector competence

Both *Aedes* mosquito species' vector competence was assessed using two types of virus, P-DTMUV and M-DTMUV, as described above. In P-DTMUV study groups, a viral titer equal to or higher than 10^6 $TCID_{50}$/mL infected, replicated, and migrated to the salivary glands of both mosquito species. Significantly different disseminated and infective rates were shown between *Ae. aegypti* mosquitoes after feeding on 10^6 and 10^7 $TCID_{50}$/mL of the virus ($p < 0.05$). Significantly different infective rates were recorded between *Ae. albopictus* mosquitoes after feeding on 10^6 and 10^7 $TCID_{50}$/mL of the virus ($p < 0.05$) but no difference in infected and disseminated rates. Data indicated that the vector competence of both *Aedes* mosquito species depended on viral titers. Moreover, vector competence between *Ae. aegypti* and *Ae. albopictus* was not different (Table 6). In M-DTMUV study groups, the viral titer of infected blood meal was 10^4 $TCID_{50}$/mL, and the virus was not detected in all mosquito parts in both *Aedes* species.

These findings suggested that, firstly, *Ae. aegypti* and *Ae. albopictus* mosquitoes were able to act as vectors for DTMUV, but their competence depended on viral concentration. The viral infection did not affect mosquito physical fitness, including egg production, appetite, and death. The extrinsic incubation period of the virus was 13 days post oral infection.

Table 4 The presence of duck Tembusu virus in mosquitoes: extrinsic incubation period experiment

(a) *Aedes aegypti*

Blood meal levels	Infected rates[a]							
($TCID_{50}$/mL)	0	1	2	3	4	5	6	7
10^7	100% (10/10)	100% (10/10)	100% (10/10)	100% (10/10)	100% (10/10)	0% (0/10)	0% (0/10)	0% (0/10)
10^6	100% (10/10)	100% (10/10)	100% (10/10)	100% (10/10)	100% (10/10)	0% (0/10)	0% (0/10)	0% (0/10)
10^5	100% (10/10)	100% (10/10)	100% (10/10)	100% (10/10)	100% (10/10)	0% (0/10)	0% (0/10)	0% (0/10)
10^4	100% (10/10)	100% (10/10)	100% (10/10)	100% (10/10)	100% (10/10)	0% (0/10)	0% (0/10)	0% (0/10)
10^3	100% (10/10)	100% (10/10)	100% (10/10)	100% (10/10)	100% (10/10)	0% (0/10)	0% (0/10)	0% (0/10)
10^2	100% (10/10)	100% (10/10)	100% (10/10)	100% (10/10)	100% (10/10)	0% (0/10)	0% (0/10)	0% (0/10)
10	100% (10/10)	100% (10/10)	100% (10/10)	100% (10/10)	100% (10/10)	0% (0/10)	0% (0/10)	0% (0/10)
1	100% (10/10)	100% (10/10)	100% (10/10)	100% (10/10)	100% (10/10)	0% (0/10)	0% (0/10)	0% (0/10)

Blood meal levels	Infected rates							Infective rates[b]	
($TCID_{50}$/mL)	8	9	10	11	12	13	14	13[c]	14[c]
10^7	0% (0/10)	0% (0/10)	0% (0/10)	0% (0/10)	0% (0/10)	60% (6/10)	70% (7/10)	40% (4/10)	50% (5/10)
10^6	0% (0/10)	0% (0/10)	0% (0/10)	0% (0/10)	0% (0/10)	60% (6/10)	60% (6/10)	20% (2/10)	10% (1/10)
10^5	0% (0/10)	0% (0/10)	0% (0/10)	0% (0/10)	0% (0/10)	0% (0/10)	0% (0/10)	ND[d]	ND
10^4	0% (0/10)	0% (0/10)	0% (0/10)	0% (0/10)	0% (0/10)	0% (0/10)	0% (0/10)	ND	ND
10^3	0% (0/10)	0% (0/10)	0% (0/10)	0% (0/10)	0% (0/10)	0% (0/10)	0% (0/10)	ND	ND
10^2	0% (0/10)	0% (0/10)	0% (0/10)	0% (0/10)	0% (0/10)	0% (0/10)	0% (0/10)	ND	ND
10	0% (0/10)	0% (0/10)	0% (0/10)	0% (0/10)	0% (0/10)	0% (0/10)	0% (0/10)	ND	ND
1	0% (0/10)	0% (0/10)	0% (0/10)	0% (0/10)	0% (0/10)	0% (0/10)	0% (0/10)	ND	ND

(b) *Aedes albopictus*

Blood meal levels	Infected rates[a]							
($TCID_{50}$/mL)	0	1	2	3	4	5	6	7
10^7	100% (10/10)	100% (10/10)	100% (10/10)	100% (10/10)	100% (10/10)	0% (0/10)	0% (0/10)	0% (0/10)
10^6	100% (10/10)	100% (10/10)	100% (10/10)	100% (10/10)	100% (10/10)	0% (0/10)	0% (0/10)	0% (0/10)
10^5	100% (10/10)	100% (10/10)	100% (10/10)	100% (10/10)	100% (10/10)	0% (0/10)	0% (0/10)	0% (0/10)
10^4	100% (10/10)	100% (10/10)	100% (10/10)	100% (10/10)	100% (10/10)	0% (0/10)	0% (0/10)	0% (0/10)
10^3	100% (10/10)	100% (10/10)	100% (10/10)	100% (10/10)	100% (10/10)	0% (0/10)	0% (0/10)	0% (0/10)
10^2	100% (10/10)	100% (10/10)	100% (10/10)	100% (10/10)	100% (10/10)	0% (0/10)	0% (0/10)	0% (0/10)
10	100% (10/10)	100% (10/10)	100% (10/10)	100% (10/10)	100% (10/10)	0% (0/10)	0% (0/10)	0% (0/10)
1	100% (10/10)	100% (10/10)	100% (10/10)	100% (10/10)	100% (10/10)	0% (0/10)	0% (0/10)	0% (0/10)

Blood meal levels	Infected rates							Infective rates[b]	
($TCID_{50}$/mL)	8	9	10	11	12	13	14	13[c]	14[c]
10^7	0% (0/10)	0% (0/10)	0% (0/10)	0% (0/10)	0% (0/10)	80% (8/10)	70% (7/10)	40% (4/10)	40% (4/10)
10^6	0% (0/10)	0% (0/10)	0% (0/10)	0% (0/10)	0% (0/10)	70% (7/10)	60% (6/10)	10% (1/10)	20% (2/10)
10^5	0% (0/10)	0% (0/10)	0% (0/10)	0% (0/10)	0% (0/10)	0% (0/10)	0% (0/10)	ND[d]	ND
10^4	0% (0/10)	0% (0/10)	0% (0/10)	0% (0/10)	0% (0/10)	0% (0/10)	0% (0/10)	ND	ND
10^3	0% (0/10)	0% (0/10)	0% (0/10)	0% (0/10)	0% (0/10)	0% (0/10)	0% (0/10)	ND	ND
10^2	0% (0/10)	0% (0/10)	0% (0/10)	0% (0/10)	0% (0/10)	0% (0/10)	0% (0/10)	ND	ND
10	0% (0/10)	0% (0/10)	0% (0/10)	0% (0/10)	0% (0/10)	0% (0/10)	0% (0/10)	ND	ND
1	0% (0/10)	0% (0/10)	0% (0/10)	0% (0/10)	0% (0/10)	0% (0/10)	0% (0/10)	ND	ND

[a] Percentage of DTMUV presented in mosquito body on day 0-14 pbf; the numbers in brackets are number of DTMUV positive samples/number of tested samples

[b] Percentage of DTMUV presented in mosquito saliva on day 13-14 pbf

[c] Infective rates between day 13 and 14 pbf in both *Aedes* mosquitoes were not different

[d] Not determined (ND) due to virus was not detected in the body

Table 5 Number of alive mosquitoes post blood feeding

(a) *Aedes aegypti*

Blood meal levels	Number of alive mosquitoes															Survival
($TCID_{50}$/mL)	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	rate[a]
Negative group	30	30	29	29	29	29	29	28	28	28	28	28	28	28	28	93.33%
10^7	30	30	30	30	30	30	29	29	29	29	29	29	29	29	29	96.67%
10^6	30	30	30	30	30	30	30	30	30	30	29	29	28	28	28	93.33%
10^5	30	30	30	30	30	30	30	30	30	30	30	30	30	30	30	100%
10^4	30	30	30	30	30	30	30	30	30	30	30	30	30	30	30	100%
10^3	30	30	30	30	30	30	30	30	30	30	30	30	30	30	30	100%
10^2	30	30	30	30	30	30	30	30	30	30	30	30	30	30	30	100%
10	30	30	30	30	30	30	30	30	30	30	30	30	30	30	30	100%
1	30	30	30	30	30	30	30	30	30	30	30	30	30	30	30	100%

[a] Survival rates of both mosquitoes were not different from negative groups.

Dead dates were marked by gray color.

(b) *Aedes albopictus*

Blood meal levels	Number of alive mosquitoes															Survival
($TCID_{50}$/mL)	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	rate[a]
Negative group	30	30	30	29	29	29	29	29	29	29	29	29	29	29	29	96.67%
10^7	30	30	30	30	30	30	30	30	29	29	29	29	29	29	29	96.67%
10^6	30	30	30	30	30	30	30	30	30	30	30	30	30	30	30	100%
10^5	30	29	29	29	29	29	29	29	29	29	29	28	28	27	27	90%
10^4	30	30	30	30	30	29	29	29	29	29	29	29	29	29	29	96.67%
10^3	30	29	29	29	29	28	28	28	28	28	28	28	28	28	28	93.33%
10^2	30	30	30	30	30	30	30	30	28	28	28	28	28	28	28	93.33%
10	30	30	30	30	29	28	27	27	27	27	27	27	27	27	27	90%
1	30	30	30	30	30	30	30	30	30	30	30	30	30	30	30	100%

[a] Survival rates of both mosquitoes were not different from negative groups.

Dead dates were marked by gray color.

Table 6 Vector competence of mosquitoes for duck Tembusu virus

Virus types	Blood meal levels ($TCID_{50}$/mL)	*Aedes aegypti*			*Aedes albopictus*		
		Infected rate	Disseminated rate	Infective rate[d]	Infected rate	Disseminated rate	Infective rate[d]
P-DTMUV	10^7	90% (27/30)	83.33% (25/30)[a]	63.33% (19/30)[b]	83.33% (25/30)	80% (24/30)	43.33% (13/30)[c]
	10^6	70% (21/30)	56.67% (17/30)[a]	16.67% (5/30)[b]	90% (27/30)	90% (27/30)	13.33% (4/30)[c]
	10^5	0% (0/30)	0% (0/30)	0% (0/30)	0% (0/30)	0% (0/30)	0% (0/30)
	10^4	0% (0/30)	0% (0/30)	0% (0/30)	0% (0/30)	0% (0/30)	0% (0/30)
	10^3	0% (0/30)	0% (0/30)	0% (0/30)	0% (0/30)	0% (0/30)	0% (0/30)
	10^2	0% (0/30)	0% (0/30)	0% (0/30)	0% (0/30)	0% (0/30)	0% (0/30)
	10	0% (0/30)	0% (0/30)	0% (0/30)	0% (0/30)	0% (0/30)	0% (0/30)
	1	0% (0/30)	0% (0/30)	0% (0/30)	0% (0/30)	0% (0/30)	0% (0/30)
M-DTMUV	10^4	0% (0/30)	0% (0/30)	0% (0/30)	0% (0/30)	0% (0/30)	0% (0/30)
Negative group	-	0% (0/30)	0% (0/30)	0% (0/30)	0% (0/30)	0% (0/30)	0% (0/30)

[a, b, c] Viral detection rates were significantly difference ($P<0.05$).

[d] Infective rates between *Ae. aegypti* and *Ae. albopictus* were no different.

3.4 Discussion

DTMUV was proven to cause severe disease in mammalian hosts, including mice, similar to duck as the natural host (Ti et al., 2016; Yurayart et al., 2020). The virus is transmitted by mosquito vectors (Tang et al., 2015). However, no research has been conducted to assess the interaction between DTMUV and the *Aedes* mosquito as a mammalian host-preferred mosquito species. This study investigated the vector competence of *Ae. aegypti* and *Ae. albopictus* mosquitoes for DTMUV. Results indicated that both *Aedes* mosquito species supported viral replication and were ready to transmit the virus to a vertebrate host. Significantly, the viral infection did not affect mosquito physiology and death. Our findings suggested a relationship between virus and mosquito that may affect the pathogenesis and variety of vectors.

Aedes aegypti and *Ae. albopictus* mosquitoes were orally infected with DTMUV to investigate the probability of mosquitoes serving as a vector between avian to mammalian hosts or mammalian to mammalian hosts. Findings suggested that both *Aedes* species were found to be competent vectors. First, the virus was detected in the saliva of both mosquitoes when the oral infection dose was 10^6 $TCID_{50}$/mL. Viremia is naturally found in the bloodstreams of both ducks and chickens, but levels vary due to several factors, including host age, duration of infection, and infectious dose. For example, viremia levels in Cherry Valley ducks inoculated with DTMUV strain DK/TH/CU-1 ranged 10^4-10^6 DTMUV genome copies per 50 ng RNA and in white leghorn chickens inoculated with TMUV strain Thai-MLO305 ranged 10^4-10^7 PFU/mL (O'Guinn et al., 2013; Ninvilai et al., 2020). In humans and non-human primates (orangutans), viral RNA and antibodies were detected in oral swabs and blood, respectively. However, the quantity of virus was not reported (Tang et al., 2013). Detection of the virus in oral swab and saliva related to viremia levels and viral pathogenesis in the natural host. Viremia was present in both humans and orangutan bloodstreams. Second, the survival rate of infected mosquitoes was no different from non-infected mosquitoes. This indicated that viral infection did not affect mosquito physiology. Third, the extrinsic incubation period was 13 days and less than the life span of the

Aedes mosquito. Fourth, the *Aedes* mosquito prefers to feed on humans but can also feed on animals.

Previous research showed that both *Aedes* species were generalist feeders. Their host blood meals were a wide range of hosts depending on the host species in their areas. Data collected in Thailand revealed that *Aedes'*s blood-feeding habits were both single and multiple-host bloodmeals, including humans, bovine, swine, chicken, dog, cat, and monkey (Ponlawat and Harrington, 2005; Khaklang and Kittayapong, 2014). Habitats of *Ae. aegypti* and *Ae. albopictus* mosquitoes are endophilic and exophilic, respectively. Thus, they have the opportunity to contact both humans and animals (Takken and Verhulst, 2013). These factors support the ability of *Aedes* mosquitos to act as potential vectors for DTMUV.

Vector competence of both *Aedes* mosquito species was determined from the presence of the virus in parts of the mosquito, including body, wing and leg, and saliva. Results showed that competence differed between low and high viral titers in the blood meal. For high viral titers (10^6-10^7 $TCID_{50}$/mL), the virus was detected in every part of the mosquito, and infective rates between the two titer levels were significantly different. This suggested that the virus replicated in mosquito cells and escaped from both midgut and salivary gland barriers and mosquito immunity. By contrast, viral infection was limited within the midgut barrier for low viral titers (10^0-10^5 $TCID_{50}$/mL). Moreover, M-DTMUV (an isolated DTMUV from mice) infection in mosquitoes was similar to low viral titer groups because its titer (10^4 $TCID_{50}$/mL) was not enough to cause infection in the mosquitoes. These findings indicated that vector competence of *Ae. aegypti* and *Ae. albopictus* for DTMUV followed a dose-dependent pattern. The midgut infection barrier was also found in a natural vector of the virus, i.e., *Culex vishnui* and *Cx. tritaeniorhynchus*, and low viral titers were unable to infect and replicate in epithelial cells. Viral titers in blood meal at more than $10^{5.5}$ PFU/mL and 10^5 $TCID_{50}$/mL were required for successful viral transmission by *Cx. vishnui* and *Cx. tritaeniorhynchus*, respectively (O'Guinn et al., 2013; Sanisuriwong et al., 2020). This midgut mechanism was mostly found in flavivirus infection including DENV, YFV, and

ZIKV (Franz et al., 2015), with DENV low viral titer affecting affinity between receptors of midgut epithelial cells and domain III of the flavivirus E protein. Thus, the virus could not enter into the epithelial cells (Smith, 2012). Another function that prevents viral infection is the RNA interference pathway (RNAi) as a primary inhibitor for viral replication (Khoo et al., 2010; Sim et al., 2014). Therefore, the vector competence of both *Aedes* mosquito species depended on viral infection dose, while the midgut barrier played a crucial role in preventing viral infection for lower viral titers.

DK/TH/CU-1 virus used in this study was classified as cluster 2.1, and its partial E gene shared 96.7-98.9% nucleotide identity with Chinese DTMUV. Currently, Thai DTMUV strain DK/TH/CU-1 was already examined for the vector competence in *Ae. aegypti*, *Ae. albopictus*, *Cx. tritaeniorhynchus*, and *Cx. quinquefasciatus* mosquitoes. Data showed that their competencies depended on viral dose for both *Aedes* species and Cx. *tritaeniorhynchus,* but *Culex* mosquito required a viral amount less than *Aedes* mosquitoes. Oral infectious doses for *Aedes* and *Culex* mosquitoes were 10^6 and 10^5 $TCID_{50}$/mL, respectively. In contrast, *Cx. quinquefasciatus* mosquito did not support the DTMUV propagation. The virus was only limited to the midgut site (Sanisuriwong et al., 2020). Also, different strains of TMUV-related virus could develop in mosquitoes differently. For instance, Chinese DTMUV, including BYD-1 virus classified into cluster 2.2 could be transmitted by *Cx. pipiens* (*p.*) *pallens*, *Cx. p. quinquefasciatus*, and *Cx. tritaeniorhynchus* mosquitoes and EIP was 7 days pbf. The viral dose requirement was 10^6 PFU/mL. Plus, the virus could well develop in *Ae. albopictus* but there was no transmission data (Guo et al., 2020). Table 7 showed the summary of potential mosquitoes for the duck Tembusu virus.

This is the first study of duck-origin flavivirus in human-preferring mosquitoes. Vector competencies of *Ae. aegypti* and *Ae. albopictus* mosquitoes for DTMUV were investigated. Results demonstrated that both *Aedes* mosquito species served as vectors under optimal conditions, with complicated interactions between the virus and mosquitoes. Vector competence depended on viral infection dose, while the midgut was shown to be a major barrier against viral infection.

Further studies are required to investigate the viral genome of unnatural host and vector and compare with the natural host. Our findings increase the current awareness of viral crossing between avian and mammalian hosts.

Table 7 Summary of potential mosquito vectors for duck Tembusu virus

Virus strain	Cluster	Mosquito species	Transmission	Viral dose	EIP (day)	Reference
DK/TH/CU-1	2.1	*Ae. aegypti*	✓	10^6 $TCID_{50}$/mL	13	This study
		Ae. albopictus	✓	10^6 $TCID_{50}$/mL	13	This study
		Cx. tritaeniorhynchus	✓	10^5 $TCID_{50}$/mL	14	Sanisuriwong et al., 2020
		Cx. quinquefasciatus	✖	10^4- 10^5 $TCID_{50}$/mL	-	Sanisuriwong et al., 2020
Thai-ML0305	ND	*Cx. vishnui*	✓	$10^{5.5}$ PFU/mL	15-18	O'Guinn et al., 2013
		Cx. fuscocephala	✖	$10^{5.5}$ PFU/mL	-	O'Guinn et al., 2013
BYD-1	2.2	*Cx. p. pallens*	✓	10^6 PFU/mL	7	Guo et al., 2020
		Cx. p. quinquefasciatus	✓	10^6 PFU/mL	ND	Guo et al., 2020
		Cx. tritaeniorhynchus	✓	10^6 PFU/mL	ND	Guo et al., 2020
		Ae. albopictus	ND	10^6 PFU/mL	ND	Guo et al., 2020

ND: no reported data

CHAPTER 4

Viral characterization and comparison of nucleotide and amino acid sequences of Thai DTMUV in BALB/c mice and *Aedes* mosquitoes

This part has been published on the topic of

Interactions of duck Tembusu virus with *Aedes aegypti* and *Aedes albopictus* mosquitoes: vector competence and viral mutation

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4.1 Introduction

Duck Tembusu virus (DTMUV) belongs to the Flaviviridae family, which is a large group of viral pathogens such as yellow fever virus (YFV) and dengue virus (DENV) (Su et al., 2011). The viral genome is a positive sense single-stranded RNA. Its genome is approximately 10-11 kb in length and consists of one open reading frame encodes a single polyprotein (Mousson et al., 2005). The polyprotein contains three structural proteins (capsid (C), pre-membrane (PrM), and envelop (E)) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5), which are crucially for viral replication and immune evasion (Bollati et al., 2010).

The first outbreak of DTMUV was found in a duck that served as a natural host. The clinical sign was mainly shown in the nervous system. Plus, the virus was transmitted between hosts by *Culex* mosquitoes (Tang et al., 2015). The virus's features were similar to flaviviruses in the Japanese encephalitis serocomplex group, including West Nile virus (WNV), St Louis encephalitis virus (SLEV), and Murray Valley encephalitis virus (MVEV) (Turtle et al., 2012). Under laboratory conditions, DTMUV had the potential to cause disease in the mammalian host and replicate in *Aedes* mosquitoes which

were not a natural host and vector, respectively (Yurayart et al., 2020; Yurayart et al., 2021).

Previous research showed that changing host or vector by the flavivirus related to viral genome mutation, especially in genes that play a crucial role in viral entry, replication, and immune evasion, including E, NS1, and NS5 genes (Weaver and Barrett, 2004; Lei et al., 2017). Currently, the genetic of DTMUV forced to change host and vector is lack of information. This study aimed to study the genetic of the virus that was isolated from mice, *Ae. aegypti*, and *Ae. albopictus* and compare with duck-origin DTMUV. The concept of this study is shown in figure 9.

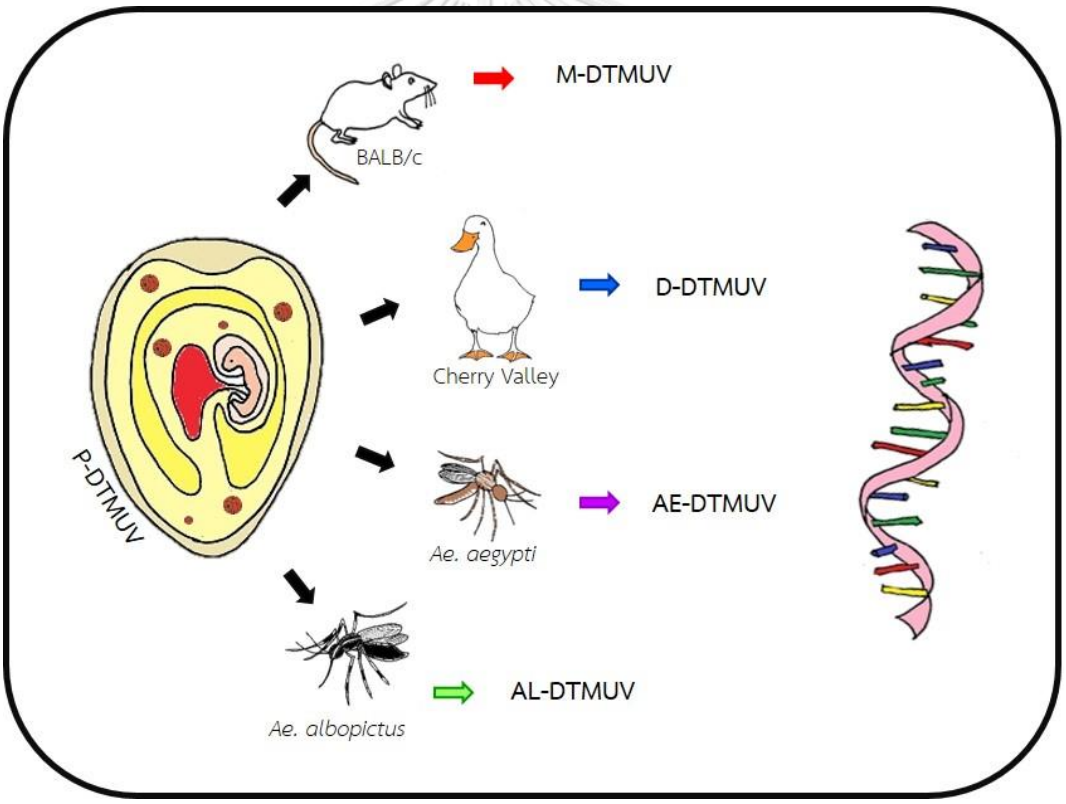


Figure 9 Concept of phase III experiment

P-DTMUV or virus propagated in duck embryonated egg was challenged into BALB/c mice, duck, *Ae. aegypti*, and *Ae. albopictus*. Then, the virus was harvested. Their genetics were characterized and compared with the original virus.

4.2 Materials and methods

4.2.1.1 Positive DTMUV

Positive DTMUV (P-DTMUV) was the same DTMUV used in viral pathogenesis (phase I) and vector competence (phase II) studies. In brief, the virus was originally isolated from a sick duck in a disease outbreak farm in Thailand. It was reported as DK/TH/CU-1 strain and characterized in cluster 2.1, a predominant cluster in Southeast Asia, and the virus accession number in GenBank was KR061333 (Thontiravong et al., 2015; Ninvilai et al., 2019). The virus was propagated in nine-day-old embryonated duck eggs, harvested on day 5 post-inoculation (pi), and preserved at -80 ºC until required for use. All used viruses are summarized in Table 8.

4.2.1.2 Mouse DTMUV

Mouse DTMUV (M-DTMUV) was an isolated virus from infected BALB/c mice in phase I. In brief, BALB/c mice were inoculated with P-DTMUV by an intracerebral route and euthanized every other day to collect samples. Tissue samples were measured viral load by qRT-PCR assay. M-DTMUV was isolated from mice's brain which was a viral target organ and had a high viral load (n=3). Selected brains belonged to mice euthanized on day 7 pi. Their viral loads were at the peak of infection. The average viral load of M-DTMUV was $10^{6.75}$ DTMUV genome copies.

4.2.1.3 Duck DTMUV

Duck DTMUV (D-DTMUV) was an isolated virus from an infected duck with P-DTMUV. The virus was provided by the Department of Veterinary Microbiology, Faculty of Veterinary Science, Chulalongkorn University (Ninvilai et al., 2020). In brief, four-week-old Cherry Valley ducks were inoculated with P-DTMUV and euthanized every other day. D-DTMUV was selected similarly with M-DTMUV (n=3). The virus reached

the high viral load in the duck's brain on day 7 pi. The average viral load was $10^{5.5}$ DTMUV genome copies.

4.2.1.4 *Aedes aegypti* DTMUV

Aedes aegypti DTMUV (AE-DTMUV) was an isolated virus from *Ae. aegypti*'s saliva in phase II. Briefly, *Ae. aegypti* mosquitoes were allowed to feed on P-DTMUV viremic blood meal. On day 14 post blood feeding (pbf), mosquitoes were sedated to collect saliva. Samples were tested for virus using the RT-PCR technique, which was specific to the NS5 gene. Then, the PCR product was measured DNA concentration by Nano drop spectrophotometer. AE-DTMUV was selected from a saliva sample that had a high DNA concentration (n=3).

4.2.1.5 *Aedes albopictus* DTMUV

Aedes albopictus DTMUV (AL-DTMUV) was an isolated virus from *Ae. albopictus*'s saliva in phase II. AL-DTMUV was processed similarly to AE-DTMUV.

Table 8 Summary of used virus for chapter 4

Virus	Abbreviation	Host	Sample site	Source
Positive DTMUV (n=1)	P-DTMUV	Embryonated duck egg	Allantoic fluid	Vet. Micro. CU
Mouse DTMUV (n=3)	M-DTMUV	BALB/c mouse	Brain	This study (Phase I)
Duck DTMUV (n=3)	D-DTMUV	Cherry Valley duck	Brain	Ninvilai et al., 2020
Aedes aegypti DTMUV (n=3)	AE-DTMUV	*Aedes aegypti* mosquito	Saliva	This study (Phase II)
Aedes albopictus DTMUV (n=3)	AL-DTMUV	*Aedes albopictus* mosquito	Saliva	This study (Phase II)

4.2.2 Viral genome analysis

The viral genome of all viruses (Table 8) was sequenced using primer sets specific to the viral whole genome from start to stop codons (Thontiravong et al., 2015). Primers and thermal cycling conditions are shown in appendix 3. Then, nucleotide and amino acid sequences were analyzed and compared with P-DTMUV. In brief, viral RNA was extracted using Viral Nucleic Acid Extraction Kit II (Geneaid, Taiwan) following the manufacturer's protocols. RT-PCR was performed using SuperScript® III One-Step RT-PCR system with Platinum® Taq DNA Polymerase (Invitrogen, USA) following the manufacturer's instructions. Subsequently, the amplicons were subjected to DNA sequencing (1st Base, Malaysia). Simultaneously, viral nucleotide sequences were assembled by SeqMan software (DNASTAR Inc., Wisconsin, USA) and translated to an amino acid by EditSeq software (DNASTAR Inc.).

4.3 Results

Viral genomes of DTMUV isolated from mice, duck, *Ae. aegypti* and *Ae. albopictus* saliva were analyzed and compared with original DTMUV from the sick duck. Results revealed that viral genomes of all viruses from mice, duck, and Ae. albopictus were not different from P-DTMUV. Surprisingly, all viruses from *Ae. aegypti* (n=3) showed a point mutation or substitution in their genomes but at different positions (Table 9). A stop codon was not found and viral genomes could normally translate to amino acids.

When translating to an amino acid, AE-DTMUV 1 found a silent mutation from cysteine (Cys) to Cys, an unchanged amino acid, located at residue 92 of envelope structural domain II (EDII). AE-DTMUV 2 showed a conservative missense mutation from phenylalanine (Phe) to leucine (Leu), a mutation of amino acid in the same group, located at residue 133 of the NS1 gene. Phenylalanine and leucine are grouped in a non-polar hydrophobic amino acid group. In contrast, AE-DTMUV 3 showed a non-conservative missense mutation from isoleucine (Ile) to threonine (Thr), which was a mutation of amino acid in a different group, located at residue 167 of the NS4B gene (Fig. 10). Isoleucine is a non-polar hydrophobic amino acid, but threonine is a polar hydrophilic amino acid. The location of genes was referenced from DTMUV DK/TH/CU-1, complete genome in GenBank.

Table 9 Nucleotide bases and amino acids between P-DTMUV and AE-DTMUV

Virus type	Nucleotide base position			Amino acid position		
	1137	2763	7298	E-92	NS1-133	NS4B-167
P-DTMUV	C	T	T	Cys (TG**C**)[b]	Phe (TT**T**)	Ile (A**T**T)
AE-DTMUV 1	T	-[a]	-	Cys (TG**t**)	-	-
AE-DTMUV 2	-	A	-	-	Leu (TT**a**)	-
AE-DTMUV 3	-	-	C	-	-	Thr (A**c**T)

[a] At this position was not changed.

[b] Codon of amino acid was shown in the bracket.

Cys=Cysteine, Phe=Phenylalanine, Leu=Leucine, Ile=Isoleucine, Thr=Threonine

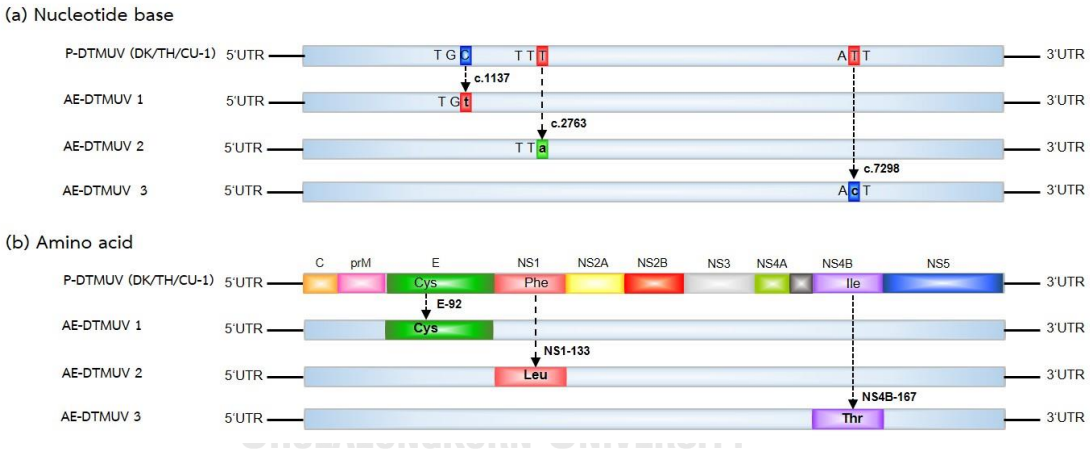


Figure 10 Positions of nucleotide base and amino acid mutations of AE-DTMUVs

4.4 Discussion

Originally, the Tembusu virus (TMUV) was firstly found in Malaysia. The virus was isolated from *Culex* mosquitoes, and it did not cause disease. In 2010, DTMUV occurred in duck farms in China. The virus was identified as a new genotype of TMUV belonging to the *Flavivirus* genus (Su et al., 2011). Currently, DTMUVs are classified into three clusters by phylogenetic analysis of the polyprotein gene. The predominant virus was reported in Thailand as Thai DTMUV, which was different from Chinese and Malaysian DTMUVs (Ninvilai et al., 2019). Thai DTMUV strain DK/TH/CU-1 used in this study was already tested its virulence in both vertebrate and non-vertebrate hosts. The virus had the ability to cause disease in both avian and mammalian hosts, including duck and BALB/c mouse, respectively (Ninvilai et al., 2020; Yurayart et al., 2020). Plus, the virus could develop and release into the saliva of *Culex* and *Aedes* mosquitoes (Sanisuriwong et al., 2020; Yurayart et al., 2021). However, we did not know the change occurring at the gene level of DTMUV found in unnatural conditions. Here, the genetic of Thai DTMUV collected from the unnatural host and vector was analyzed and compared with the original Thai DTMUV. Results indicated that a point mutation was shown in DTMUV collected from *Ae. aegypti* mosquito's saliva while any change was not found in viruses collected from duck, BALB/c mouse, and *Ae. albopictus*. Our finding revealed that viral infection in unnatural environments had an opportunity to be a predisposing cause for the viral mutation. These were the next steps in the related study of host, vector, and virus.

In this study, DTMUVs collected from several hosts and vector types were investigated in their genetics. Results showed that all AE-DTMUVs were found a point mutation in their nucleotide sequences. First of all, the accuracy of our results was discussed. Sanger sequencing method was selected to analyze DNA sequences because this method was suitable to determine a point mutation, small deletion, and duplication. Plus, this method reported the major population at each nucleotide position (Gomes and Korf, 2018). Moreover, this method was operated by specific primers covering all specific DNA regions and by both forward and reverse primers to

check each other. In addition, the RT-PCR method was performed by Platinum® *Taq* DNA Polymerase, high fidelity. This enzyme mixture comprised a 3'⟶5' exonuclease activity which served as a proofreading activity or a defense mechanism to correct DNA polymerase errors (Khare and Eckert, 2002). The performance of Platinum® Taq DNA polymerase was higher than other enzymes 6 times. All these methods resulted in high accuracy.

Duck Tembusu virus was challenged into an unnatural host and vector to investigate what happened with viral genetics. Point mutations were found in the virus collected from *Ae. aegypti* saliva. There were revealed in three positions, including EDII, NS1, and NS4B genes, and mutation's type had both silent and missense types. In general, EDII is a dimerization domain in the viral replication cycle. It contributes to viral-mediated membrane fusion (Arora et al., 2013; Zhang et al., 2017). NS1 protein is an essential cofactor for RNA replication and involves in virus assembly and maturation. Post-translational modification of NS1 protein has two secreted and membrane-associated forms related to pathogenesis (Muller and Young, 2013). Functions of NS4B facilitate viral replication at the endoplasmic reticulum membrane and counteract innate immune responses (Zou et al., 2014; Zmurko et al., 2015). However, the results just reported the genetic study which did not cover the phenotype of the virus. The point mutation is a change of a single nucleotide of DNA or RNA sequences and is mostly found in flavivirus because its genomic structure is a single strand. Another mutation type, including reassortment, frequently occurs in the virus with a segmented genome (Pérez-Losada et al., 2015). In addition, the function of the viral enzyme also affects the viral mutation. RNA-dependent RNA polymerase is a low-fidelity viral enzyme. It lacks proofreading and mismatch-repair mechanisms leading to the viral mutation. Mutation rates up to 10^{-4} substitutions per nucleotide (Holland et al., 1982; Huang et al., 2014). The viral mutation is the beginning of the evolution of populations because it may affect protein dynamics and conformation that cause a change in the original phenotypes of the virus, including pathogenesis and host adaptability (Aharoni et al., 2005). For instance, point mutation of WNV-T198F at domain I-II hinge caused disconfirmation dynamics that reduced viral stability and attenuated its pathogenesis

in mice (Goo et al., 2017). Point mutation of ZIKV and DENV-2 at NS1-A188V and NS1-T164S, respectively, showed that the virus was able to induce secreted forms and inhibit host interferon, resulting in increased viral infection virulence in mice. Moreover, the viral infection rate in *Ae. aegypti* mosquitoes increased and facilitated viral transmission (Liu et al., 2017; Xia et al., 2018; Chan et al., 2019). Point mutation of WNV at NS4B-P38G enhanced strong innate and adaptive immune responses and reduced viremia and severity of wild-type NY-99 in mice. The effect of the viral mutation was increasing and decreasing the potential of the virus (Welte et al., 2011; Xie et al., 2015). However, we could not conclude that viral mutations found in this study were caused by *Ae. aegypti* mosquitoes.

The interactions among virus, host, and vector are complicated and need further study. Firstly, the sample size should be increased to determine whether the viral mutation is significantly related to the host and vector. Second, the serial passage should be conducted. For instance, the study of DENV mutation in cell lines showed that DENV mutated in vertebrate host cell lines faster than non-vertebrate host cell lines. Plus, a viral mutation was found when the passage was more than 10 times (Vasilakis et al., 2009). Third, next-generation sequencing will be added to our method because this technique can provide data of subpopulations in DNA fragments (Gupta and Gupta, 2014). Thus, results will be more informative. Moreover, phenotypes or functions of observed amino acid mutations should be determined for their viral abilities.

In conclusion, our works demonstrated that mutation was found in Thai DTMUV genomes when it was challenged into *Ae. aegypti*. However, the data were not enough to suggest that this mutation event was specified to the vector. Our findings indicate an increase in the current awareness of viral diversity in the transmission cycle that may increase the severity of the disease.

CHAPTER 5

Conclusions

Duck Tembusu virus (DTMUV) has emerged in China and Southeast Asia since 2010. The virus causes a threatening disease in ducks and chickens. However, Chinese DTMUV was reported that it had the potential to infect mice. Clinical signs were moderate to severe, including acute death, paralysis, disorientation, and depression which were similar to avian hosts. The virus is classified as a mosquito-borne flavivirus, the same group as the Japanese encephalitis virus and West Nile virus, and its natural vector is *Culex* mosquito (Su et al., 2011; Li et al., 2013; O'Guinn et al., 2013; Ti et al., 2016). Because of viral diversity, DTMUVs were currently separated into three clusters. In Thailand, the predominant cluster was cluster 2.1, which was distinctly divided from the Chinese strain (cluster 2.2) (Ninvilai et al., 2019). Interestingly, no information of Thai DTMUV has been reported about viral pathogenesis in another host especially a mammalian host, although the virus has a wide range of hosts. Therefore, the pathogenesis of Thai DTMUV in the mammalian host was investigated in this dissertation. In addition, a vector competence of *Aedes* mosquitoes for the virus was also studied because this was important information for disease prevention if the virus could cause the disease in a mammalian host. Plus, the viral genome in unnatural host and vector were characterized for the study of viral evolution. Understanding the nature of pathogens is necessary for disease prevention and control.

Thai DTMUV has been proved its pathogenesis in an avian host. However, the potential of the virus in a mammalian host is limited. The first objective of this study was to investigate the pathogenesis of Thai DTMUV in BALB/c mice. Our findings suggested that the virus causes severe disease in mice via the intracerebral route. Both female and male mice got the infection. Morbidity and mortality rates were 100% and 20%, respectively. Clinical signs were found in nervous and systemic systems. Viral pathogenesis was a descending infection from the brain to internal organs. Clinical signs and lesions supported our suggestion. Thai DTMUV infection had more virulent than

Chinese DTMUV. Viral loads were found in all essential organs. Notably, severe lesions were found in the brain and spleen, and tissue tropisms of the virus were neuron cells and macrophages. By contrast, the pathogenesis of Chinese DTMUV was mainly in the nervous system, and other organs showed mild infection (Ti et al., 2016). Unfortunately, viremia was not detected, and the virus did not infect mice by subcutaneous route. In conclusion, our results demonstrated that Thai DTMUV had an ability to cause the disease in a mammalian host. These findings raised awareness of the virulence of Thai DTMUV.

Previous research indicated that mammals, including mice, were susceptible to Thai DTMUV, and mice showed heavy infection (Yurayart et al., 2020). Subsequently, vector competence of *Ae. aegypti* and *Ae. albopictus* mosquitoes for Thai DTMUV were also investigated by using virus propagated from duck embryonated eggs and mice. Our results revealed that both Aedes mosquitoes had a high potential to serve as a viral vector. Their abilities depended on viral infectious dose, which was at least 10^6 $TCID_{50}$/ml. Moreover, viral infection was not affected the normal physiology of mosquitoes, including egg production and laying, and the virus did not make the mosquitoes die. However, Thai DTMUV propagated from infected mice could not develop in both mosquito species because viral titer was lower than the threshold. In conclusion, *Aedes* mosquitoes had a chance to transmit the virus from avian to mammalian hosts, especially humans. However, the transmission cycle needs to be further investigated. These studies raised awareness of the opportunity of zoonotic disease.

Changing host and vector of flavivirus associate with the viral mutation that is a starting point of evolution. In this study, Thai DTMUV was proved to develop in mice and *Aedes* mosquitoes which were unnatural host systems for the virus. Therefore, the viral genome in unnatural hosts was also compared with the original virus. Results showed that nucleotide and amino acid mutations were detected in Thai DTMUV that was collected from *Ae. aegypti* saliva. The mutations were found in the E, NS1, and NS4B genes. These genes are necessary for host cell entry, replication, and immune

evasion (Mukhopadhyay et al., 2005; Muller and Young, 2013; Zmurko et al., 2015). However, we could not conclude whether mutations related to vectors or not. Further study will be required.

In summary, our dissertation provided important information on the interaction among mammalian hosts, mosquito vectors, and Thai DTMUV in terms of pathogenesis, vector competence, and viral genome. The benefit of our information is to know the virus's potential to be a zoonotic pathogen that is useful for disease prevention and control.

APPENDIX

Appendix 1

Table 10 Viral load in organs of BALB/c mice inoculated with Thai DTMUV via IC route

Day post inoculation	Mice No.	Viral load (log10 copy number/RNA250ng)					
		Brain	Heart	Lung	Liver	Spleen	Kidney
1	A1	3.37	0.26	1.32	0.41	2.37	0
	A2	3.48	1.71	0	1.34	2.55	0.64
	A3	3.64	2.14	1.74	0.52	2.14	1.30
	Mean±SD	3.50±0.13	1.37±0.99	1.02±0.91	0.76±0.51	2.36±0.21	0.65±0.65
3	A4	3.37	2.04	1.90	0.62	2.35	1.88
	A5	3.06	2.71	1.62	0.00	1.97	1.83
	A6	4.34	2.08	1.73	0.00	1.95	0.63
	Mean±SD	3.59±0.67	2.28±0.38	1.75±0.14	0.21±0.36	2.09±0.23	1.45±0.70
5	A7	5.59	2.50	3.08	1.34	3.50	2.15
	A8	5.64	2.34	1.98	1.49	1.06	1.66
	A9	5.74	3.44	3.62	1.81	2.66	2.90
	Mean±SD	5.65±0.08	2.76±0.59	2.89±0.84	1.55±0.24	2.41±1.24	2.24±0.62
7	A10	4.76	2.66	1.87	1.19	0.93	1.69
	A24	8.00	2.93	2.54	1.34	1.30	1.56
	A12	6.51	3.75	3.52	2.32	2.57	2.87
	Mean±SD	6.42±1.62	3.11±0.57	2.64±0.83	1.62±0.61	1.60±0.86	2.04±0.72
9	A13	3.10	2.22	2.07	0.31	1.01	1.26
	A28	6.13	2.22	1.38	1.12	0.86	2.08
	A14	5.63	2.56	1.93	1.71	2.99	2.82
	Mean±SD	4.95±1.63	2.33±0.20	1.79±0.37	1.05±0.70	1.62±1.19	2.05±0.78
12	A16	2.55	0.51	0	0	0	0.78
	A15	5.58	1.77	1.50	0.75	0	1.05
	A25	3.35	0.91	1.37	0	1.28	0
	Mean±SD	3.83±1.57	1.06±0.64	0.96±0.83	0.25±0.43	0.43±0.74	0.61±0.55
14	A11	1.87	0	0	0	0	0
	A23	0	0	0	0	0	0
	A29	1.46	0.52	0.80	0	0	0
	Mean±SD	1.11±0.98	0.17±0.30	0.27±0.46	0	0	0

Appendix 2

Table 11 Viral load in organs of dead BALB/c mice at 6 dpi

Mice No.	Viral load (log10 copy number/RNA250ng)					
	Brain	Heart	Lung	Liver	Spleen	Kidney
A17	5.86	2.99	2.04	1.39	1.19	2.03
A18	5.51	3.36	3.20	2.43	3.36	3.16
A19	5.98	2.95	2.90	1.86	2.65	2.93
A20	5.37	2.47	3.41	2.68	3.14	3.02
A21	5.43	2.84	3.40	1.64	2.19	2.92
A26	3.71	3.65	3.28	2.41	2.99	1.60
A27	5.47	2.59	2.72	1.32	1.23	1.61
A22	5.72	2.15	1.94	1.38	0.91	1.94
A30	5.62	3.55	1.43	1.61	3.25	2.68
Mean±SD	5.41±0.67	2.95±0.50	2.70±0.73	1.86±0.52	2.32±0.98	2.43±0.63

Appendix 3

Table 12 Primers used for whole-genome sequencing

No.	Primer name	Sequence (5'-3')
1	P1f	AGAAGTTCRYCTGTGTGA
	DF_R638	CAGCAGTCTATGTCTTCAGG
2	DF_F441	CGATAGTTGCTGGGCTGAAGC
	DF_R1115	GCAGTAAGATCTCACAACCGC
3	DF_F954	GCTTCAGCTGTCTGGGGATGC
	DF_R1650	CAATGACTCTTTGTTTTGCCACG
4	DF_F1480	CTACACYGCTGAGATGGAGG
	DF_R2463	GCCAAGTCGATTGAGCACCCC
5	DF_F2353	GGCACTGCTATTGTGGATGGG
	DF_R3339	GGTGGGGTGGTGCAAGACC
6	DF_F3302	GGAACAACTGTCACAGTAACG
	DF_R4694	GCATGACTCCCACTCCAGCC
7	DF_F4406	GCATCACAGAGATTTGATGTGG
	DF_R5162	CCTGAACCTGGATGTAGGTCC
8	DF_F4874	GCAAGTCATCGTCGTGCAACC
	DF_R5582	GCTCTTCAATGTCTGTTATTGGC
9	DF_F5399	GCTCACACCTCAGCGAGTGC
	DF_R6249	GGTCATTGTAACTTATCCCAGC
10	DF_F5928	CCAGTTCCTATAACATCAGCC
	DF_R6678	GCTCCAAGACCTGTCTTCCC
11	DF_F6494	CGCTCACAGAATGACAGAATCC
	DF_R7348	GGAACATCTGTAGCCACTATGC
12	DF_F6807	GAACCAGAGAGACAGAGATCGC
	DF_R8158	CCCTAGCTAGCCATTCCTCGG
13	DF_F7940	GCAGGTTCAGGAAGTGAGAGG
	DF_R8536	GGATTGTCTTGGTCATAATGCC
14	DF_F8383	GGATGCACAAAACCAACCGC
	DF_R9215	GGCCGAGATGTCACGCAGC
15	DF_F8084	GCTGTGTGACATAGGTGAAGC
	DF_R9449	CCACTTCCCCTCTGGTCTTCC
16	DF_F9274	GGGACACTAGAATAACCAAGGC
	DF_R10485	CCAACATCCGGTGGCAGGG

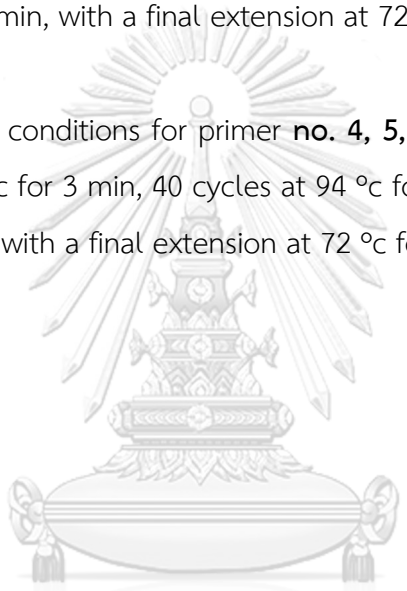
(Ref. Technical Appendix Table 1, Thontiravong et al., 2015)

Thermal cycling conditions

- Thermal cycling conditions for primer **no. 1** were 48 °c for 45 min, 94 °c for 3 min, 40 cycles at 94 °c for 30 sec, 47 °c for 30 sec, and 72 °c for 1 min, with a final extension at 72 °c for 10 min.

- Thermal cycling conditions for primer **no. 2, 3, 7, 8, 9, 10, 11, 13, and 14** were 48 °c for 45 min, 94 °c for 3 min, 40 cycles at 94 °c for 30 sec, 55 °c for 30 sec, and 72 °c for 1 min, with a final extension at 72 °c for 10 min.

- Thermal cycling conditions for primer **no. 4, 5, 6, 12, 15, and 16** were 48 °c for 45 min, 94 °c for 3 min, 40 cycles at 94 °c for 30 sec, 47 °c for 30 sec, and 72 °c for 2 min, with a final extension at 72 °c for 10 min.



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AWARD RECEIVED

1) The 90th Anniversary of Chulalongkorn University Scholarship

2) “Best Poster Research Award”, CUVC 2018, Bangkok, Thailand, 2018