GENETIC AND PATHOGENIC CHARACTERIZATIONS OF NEWLY EMERGED DUCK TEMBUSU VIRUS ISOLATED IN THAILAND

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การศึกษาลักษณะทางพันธุกรรมและพยาธิกำเนิดของไวรัสอุบัติใหม่เทมบูซูในเป็ดที่แยกได้ในประเทศไทย

น.ส.พัชรีภรณ์ นิลวิไล

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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พัชรีภรณ์ นิลวิไล : การศึกษาลักษณะทางพันธุกรรมและพยาธิกำเนิดของไวรัสอุบัติใหม่เทมบูซูในเป็ดที่แยกได้ในประเทศไทย. (GENETIC AND PATHOGENIC CHARACTERIZATIONS OF NEWLY EMERGED DUCK TEMBUSU VIRUS ISOLATED IN THAILAND) อ.ที่ปรึกษาหลัก : ผศ. สพ.ญ.ดร.อัญญรัตน์ ต้นธีรวงศ์, อ.ที่ปรึกษาร่วม : ศ. น.สพ.ดร.อลงกร อมรศิลป์,รศ. น.สพ. ดร.วิจิตร บรรลุนารา

ไวรัสเทมบูซูในเป็ด จัดอยู่ในกลุ่มฟลาวิไวรัสที่ถ่ายทอดโดยยุงเป็นพาหะ เป็นสาเหตุของโรคติดอุบัติใหม่ในเป็ดที่พบในเอเชียตั้งแต่ปีพ.ศ. 2553 ก่อให้เกิดความสูญเสียทางเศรษฐกิจในอุตสาหกรรมการเลี้ยงเป็ดเป็นอย่างมาก หลังจากรายงานการระบาดเมื่อปีพ.ศ. 2556 ยังคงพบการติดเชื้อไวรัสเทมบูซูในเป็ดอย่างต่อเนื่องในหลายฟาร์มในไทย ดังนั้นการตรวจเฝ้าระวังเชื้อและการศึกษาลักษณะทางพันธุกรรมของไวรัสเทมบูซูในเป็ดที่แยกได้ในไทยจึงมีความจำเป็นอย่างยิ่ง วัตถุประสงค์แรกของวิทยานิพนธ์นี้ คือ การศึกษาลักษณะทางพันธุกรรมของไวรัสเทมบูซูในเป็ดที่แยกได้ในประเทศไทย ผลการศึกษาย้อนหลังยืนยันการพบไวรัสเทมบูซูในเป็ดในประเทศไทยตั้งแต่ปีพ.ศ. 2550 โดยไวรัสที่แยกได้จัดอยู่ในคลัสเตอร์ 1 ซึ่งมีลักษณะทางพันธุกรรมแตกต่างจากไวรัสที่พบในไทยและจีนในปัจจุบัน นอกจากนี้ยังได้ทำการศึกษาลักษณะทางพันธุกรรมของไวรัสเทมบูซูในเป็ดที่พบในไทยในปัจจุบัน โดยทำการเก็บตัวอย่างจำนวน 288 ตัวอย่าง จากฟาร์มเป็ด 89 ฟาร์ม ระหว่างปีพ.ศ. 2558 ถึง 2560 พบตัวอย่างบวก 65 ตัวอย่าง (ร้อยละ 22.57%) จาก 34 ฟาร์ม (ร้อยละ 38.20) ผลการศึกษาบ่งชี้การกระจายของไวรัสเทมบูซูในเป็ดกระจายในพื้นที่เลี้ยงเป็ดในไทยเป็นวงกว้าง ผลการศึกษานี้พบว่าไวรัสเทมบูซูในเป็ดที่เป็นสาเหตุของการระบาดในเอเชียประกอบด้วยไวรัส 3 คลัสเตอร์ โดยมีการกระจายตัวสัมพันธ์กับลักษณะทางภูมิศาสตร์ การวิเคราะห์ความสัมพันธ์ทางพันธุกรรมพบว่าไวรัสเทมบูซูในเป็ดที่มีการระบาดในไทยจำแนกได้เป็น 3 คลัสเตอร์ (1, 2.1 และ 3) โดยคลัสเตอร์ย่อย 2.1 เป็นกลุ่มหลักที่พบระบาดในไทยระหว่างปี พ.ศ. 2558 ถึง 2560 และได้มีการพิสูจน์พบเชื้อในคลัสเตอร์ 3 เป็นครั้งแรกจากการศึกษานี้ ผลการศึกษาดังกล่าวบ่งชี้ถึงความหลากหลายทางพันธุกรรมของไวรัสเทมบูซูในเป็ด ผลการศึกษาข้างต้นชี้ให้เห็นถึงความจำเป็นในการพัฒนาวิธีตรวจวินิจฉัยที่มีประสิทธิภาพสามารถตรวจวินิจฉัยไวรัสเทมบูซูในเป็ดได้ทุกคลัสเตอร์ วัตถุประสงค์ที่สองของวิทยานิพนธ์นี้ คือ การพัฒนาและประเมินประสิทธิภาพของวิธีตรวจแบบ one-step RT-PCR ที่สามารถตรวจไวรัสเทมบูซูในเป็ดได้ทุกคลัสเตอร์ วิธีตรวจ one-step RT-PCR ที่พัฒนาขึ้นมีความจำเพาะต่อยีน NS5 ของไวรัสในบริเวณที่มีความแปรผันทางพันธุกรรมต่ำ วิธีตรวจนี้มีความจำเพาะสามารถตรวจไวรัสเทมบูซูในเป็ดได้ทุกคลัสเตอร์ โดยไม่มีปฏิกิริยาข้ามกับไวรัสที่พบในเป็ดและฟลาวิไวรัสชนิดอื่น และสามารถตรวจพบไวรัสเทมบูซูในเป็ดได้ปริมาณต่ำสุดที่ 0.001 ELD_{50}/มิลลิลิตร และพบว่าวิธีนี้มีประสิทธิภาพดีเมื่อทำการทดสอบกับตัวอย่างจากสัตว์ทดลอง การทดสอบนี้สามารถตรวจพบไวรัสเทมบูซูในเป็ดได้จากทุกตัวอย่างสัตว์ทดลอง และมีอัตราการตรวจพบไวรัสเทมบูซูในเป็ดจากตัวอย่างจากภาคสนาม (36%) มากกว่าผลการทดสอบด้วยวิธี RT-PCR ที่จำเพาะต่อยีน E (30%) ซึ่งมีการรายงานก่อนหน้านี้ นอกจากนี้วัตถุประสงค์ที่สามของวิทยานิพนธ์นี้ คือ การศึกษาพยาธิกำเนิดของการติดเชื้อไวรัสเทมบูซูที่ระบาดในประเทศไทยในเป็ดสามช่วงอายุ ผลการศึกษาพบว่าเป็ดทุกช่วงอายุไวต่อการติดไวรัสชนิดนี้ อย่างไรก็ตามพบว่าเป็ดอายุน้อยมีความไวรับต่อไวรัสเทมบูซูในเป็ดมากกว่าเป็ดอายุมาก บ่งถึงความสัมพันธ์ระหว่างอายุเป็ดและความไวต่อการโรค โดยสรุปวิทยานิพนธ์ฉบับนี้ให้ข้อมูลที่เป็นประโยชน์เกี่ยวกับลักษณะทางพันธุกรรมของไวรัสเทมบูซูในเป็ดที่พบในไทย การพัฒนาการตรวจด้วยวิธี one-step RT-PCR ที่สามารถตรวจไวรัสเทมบูซูในเป็ดได้ทุกคลัสเตอร์ และศึกษาพยาธิกำเนิดของการติดเชื้อไวรัสเทมบูซูในเป็ดที่ระบาดในประเทศไทยในเป็ดอายุต่างกัน โดยรวมความรู้ที่ได้จากการศึกษาในครั้งนี้ชี้ให้เห็นความสำคัญของการตรวจเฝ้าระวังไวรัสเทบูซูในเป็ดอย่างต่อเนื่อง โดยเฉพาะในเป็ดโตเต็มวัย และชี้ให้เห็นถึงความสำคัญของการประเมินประสิทธิภาพของวิธีตรวจวินิจฉัยอย่างต่อเนื่อง เพื่อสามารถตรวจวินิจฉัย ควบคุมและป้องกันโรคติดเชื้อไวรัสเทมบูซูในเป็ดได้อย่างรวดเร็วและมีประสิทธิภาพ

สาขาวิชา	พยาธิชีววิทยาทางสัตวแพทย์	ลายมือชื่อนิสิต .....
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Patchareeporn Ninvilai : GENETIC AND PATHOGENIC CHARACTERIZATIONS OF NEWLY EMERGED DUCK TEMBUSU VIRUS ISOLATED IN THAILAND. Advisor: Asst. Prof. AUNYARATANA THONTIRAVONG, D.V.M., M.Sc., Ph.D. Co-advisor: Prof. ALONGKORN AMONSIN, D.V.M., Ph.D., D.T.B.V.P.H.,Assoc. Prof. WIJIT BANLUNARA, D.V.M., Ph.D., D.T.B.V.P.

Duck Tembusu virus (DTMUV), a mosquito-borne Flavivirus, caused an emerging disease in ducks in Asia since 2010, resulting in significant economic loss in duck producing industry. After the initial outbreak in 2013, DTUMV cases have been continuously detected in several duck farms in Thailand. Thus, the disease surveillance and the genetic characterization of the Thai DTMUV are necessary. Therefore, the first objective of this dissertation was to investigate the genetic characteristic of DTMUVs circulating in Thailand. Our retrospective study demonstrated the presence of DTMUV in the Thai ducks since 2007. Phylogenetic analysis of the polyprotein gene sequence revealed that the 2007 Thai DTMUV was belonged within DTMUV cluster 1, which was genetically different from the currently circulating Thai and Chinese DTMUVs. In addition, the genetic characteristic of DTMUVs recently circulating in Thailand were investigated. Of the 288 clinical samples obtained from 89 ducks farms in Thailand during 2015-2017, 65 samples (22.57%) of 34 duck farms (38.20%) were DTMUV positive. Our results demonstrated the extensive distribution of DTMUV in duck raising areas of Thailand. Our finding indicated that three clusters of DTMUVs were associated with the current DTMUV outbreaks in Asia and suggested the correlation between virus cluster and geographic location. Phylogenetic analysis demonstrated that DTMUVs circulating in Thailand were divided into 3 clusters (1, 2.1 and 3), in which subcluster 2.1 was a predominant cluster of DTMUV circulating in ducks in Thailand during 2015-2017. A novel cluster of DTMUV (cluster 3) was first identified in this study. Our results highlight the high genetic diversity of DTMUVs. This highlighted the need for well-validated detection assay for broad detection of all DTMUV clusters. Therefore, the second objective of this dissertation was to develop and validate a universal one-step RT-PCR assay for broad detection of all DTMUV clusters. Our newly developed RT-PCR assay targeting conserved region of NS5 gene could specifically detect all clusters of DTMUV without cross-reaction with common duck viruses and other related flaviviruses. The assay was able to detect DTMUV as low as 0.001 ELD_{50}/ml. The performance of the assay was also evaluated by testing with experimental and field clinical samples. The assay could successfully detect DTMUV in all experimentally DTMUV infected samples and gave a higher DTMUV detection rate (36%) than the previously reported E-specific RT-PCR assay (30%) from field clinical samples. In addition, the third objective of this dissertation was to investigate the pathogenesis of a Thai DTMUV in three different ages of ducks. Our findings indicated that all duck ages were susceptible to Thai DTMUV infection. However, younger ducks were more susceptible to DTMUV infection than in older ducks, suggesting age-related susceptibility to Thai DTMUV in ducks. In summary, this dissertation provided useful information on genetic characteristic of Thai DTMUVs, developed the well-validated universal one-step RT-PCR assay for broad detection of all DTMUV clusters and demonstrated the pathogenesis of Thai DTMUV in different ages of ducks. Overall, this study highlights the necessity of the continued routine surveillance of DTMUV in ducks particularly in adult ducks as well as the importance of continuously validating the performance of currently used diagnostic assays against newly emerging strains for early and effective detection, control and prevention of DTMUV.

Field of Study:	Veterinary Pathobiology	Student's Signature .....
Academic Year:	2019	Advisor's Signature .....
		Co-advisor's Signature .....
		Co-advisor's Signature .....

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Patchareeporn Ninvilai

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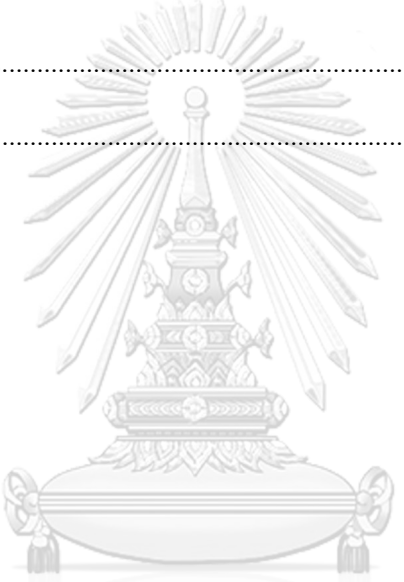

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LIST OF ABBREVIATIONS

ANOVA	Analysis of Variance
AIV	Avian influenza virus
AMV	Avian myeloblastosis virus
BHK-21	Baby Hamster Kidney 21
BAGV	Bagaza virus
BYDV	Baiyangdian virus
bp	Base pair
BMCMC	Bayesian Markov Chain Monte Carlo algorithm
BSA	Bovine serum albumin
C	Capsid protein
CMI	Cell-mediated immunity
CNS	Central nervous system
CEF	Chicken embryo fibroblast
CL swab	Cloacal swab
CPE	Cytopathic effect
dpi	Day post inoculation
DPC	Day post contact
DENV	Dengue virus
D	Domain
PKR	Double-stranded RNA-dependent *protein* kinase
DEDSV	Duck egg drop syndrome virus
DEF	Duck embryo fibroblast
DPV	Duck plague virus
DTMUV	Duck Tembusu virus
ELD_{50}	50% embryo lethal dose

ESS	Effective sample size
EDSV	Egg drop syndrome virus
ER	Endoplasmic reticulum
E	Envelope protein
ELISA	Enzyme-linked immunosorbent assay
GEF	Goose embryo fibroblast
HI	Hemagglutination inhibition
H&E	Hematoxylin and Eosin
HPD	Highest posterior density
HMI	Humoral immunity
H_2O_2	Hydrogen peroxide solution
ILHV	Ilheus virus
IHC	Immunohistochemical staining
IFA	Indirect immunofluorescence assay
IFN	Interferon
ISG	Interferon-stimulated gene
IL	Interleukin
ITV	Israel turkey meningoencephalomyelitis virus
JEV	Japanese encephalitis virus
MHC	Major histocompatibility complex
MCC tree	Maximum clade credibility tree
ML	Maximum-likelihood algorithm
MDA5	Melanoma differentiation-associated factor 5
MVEV	Murray Valley encephalitis virus
NJ	Neighbor-joining algorithm
NDV	Newcastle disease virus
NS	Non-structural protein

NTAV	Ntaya virus
NF-**K**B	nuclear factor kappa-light-chain-enhancer of activated B cell
OAS	2′,5′-oligoadenylate synthetase
OP swab	Oropharyngeal swab
PRR	Pattern recognition receptor
PNS	Peripheral nervous system
PBS	Phosphate buffered saline
PrM	Pre-membrane protein
qRT-PCR	Quantitative real time reverse transcription polymerase chain reaction
RIG-I	Retinoic acid-induced gene I
RT-LAMP	Reverse transcription loop-mediated isothermal amplification
RT-PCR	Reverse transcription polymerase chain reaction
ROCV	Rocio virus
SN test	Serum neutralization test
STWV	Sitiawan virus
SLEV	St. Louis encephalitis virus
SD	Standard deviation
TIM	T cell immunoglobulin mucin domain protein 1
TMUV	Tembusu virus
TLR	Toll-like receptor
TGN	Trans-golgi network
USUV	Usutu virus
WNV	West Nile virus

CHAPTER 1

Introduction

1.1 Importance and rationale

Tembusu virus (TMUV), a member of mosquito-borne flavivirus, was first isolated from *Culex* mosquitoes in Malaysia in 1955 (US-AMRU, 1957), and subsequently it was identified from *Culex* mosquitoes in Malaysia during 1968-1970 (Platt et al., 1975) and in Thailand in 1986 and 1992 (Leake et al., 1986; Pandey et al., 1999). However, disease associated with TMUV infection in both humans and animals had never been reported. The first evidence of TMUV causing disease was detected in broiler chicks in Malaysia in 2000. These infected chicks showed signs of encephalitis and growth retardation and later had been proven to be caused by Sitiawan virus, a chick-origin TMUV-related virus (Kono et al., 2000). Subsequently, TMUV was detected in *Culex* mosquitoes and serum of sentinel healthy ducks in Kanchanaburi province of Thailand in 2002 and 2015 without causing any clinical diseases (O'Guinn et al., 2013; Nitatpattana et al., 2017).

Since 2010, a newly contagious disease caused by Tembusu-related flavivirus has emerged in layer breeder and broiler duck farms in China. After the initial outbreak, the disease has quickly spread throughout the major duck-producing areas in the eastern and southern parts of China (Su et al., 2011; Yan et al., 2017). The affected ducks typically exhibited a severe reduction in egg production with severe neurological disorders including ataxia and paralysis (Su et al., 2011). The causative agent of the outbreaks was subsequently identified as the novel duck Tembusu virus (DTMUV) (Su et al., 2011). Besides China, this newly emerged DTMUV was also detected from several duck farms in the Southeast Asian countries, including

Malaysia and Thailand in 2012 and 2013, respectively (Homonnay et al., 2014; Chakritbudsabong et al., 2015; Thontiravong et al., 2015). The morbidity and mortality rates ranged from 20-50% and 10-30%, respectively (Thontiravong et al., 2015). After its first emergence, DTMUV has widely spread and become endemic in duck population in duck producing areas of China, Malaysia and Thailand, resulting in greater economic losses in poultry production sectors.

DTMUV is classified as a new genotype of Tembusu virus (TMUV), which was previously named as Baiyangdian (BYD) virus or duck egg drop syndrome virus (DEDSV). DTMUV is an enveloped, single-stranded, positive-sense RNA virus belonging to the Ntaya virus (NTAV) group in the genus *Flavivirus* of the Family *Flaviviridae*. Apart from several species of ducks, DTMUV infection was detected in a wide range of natural host species, including chickens, geese, pigeons and house sparrows (Yun et al., 2012; Han et al., 2013; Tang et al., 2013a; Chen et al., 2014b). In addition, previous studies demonstrated that BALB/C and Kunming mice were susceptible for DTMUV infection after intracerebral inoculation (Li et al., 2013; Ti et al., 2016). Furthermore, DTMUV-specific antibodies and DTMUV RNA were detected from serum samples and oral swabs from duck industry workers in China, highlighting the zoonotic potential of this virus (Tang et al., 2013b). However, DTMUV has not been reported to cause illness in humans and a recent study showed that it is not pathogenic to nonhuman primates (Wang et al., 2016).

At present, a newly emerged DTMUV has become one of the most economically important pathogens of ducks in many Asian countries, including Thailand. However, limited information is available on the genetic and pathogenic characteristics of DTMUV currently circulating in ducks in Thailand. Therefore, the genetic characterization of Thai DTMUVs currently circulating in Thailand and the pathogenesis study of Thai DTMUV in ducks are essential. In this study, the genetic

characteristic and diversity of DTMUVs isolated from ducks in Thailand in 2007 and during 2015-2017 were investigated. In addition, the pathogenicity of Thai DTMUV in different ages of Cherry Valley ducks were evaluated and compared. This study provided the important information on the geographic distribution, the genetic characteristic and diversity of DTMUVs circulating in ducks in Thailand in 2007 and during 2015-2017 as well as the pathogenic characteristic of Thai DTMUV. All of these data obtained from this study will be useful for effective disease diagnosis, control and prevention of DTMUV.

1.2 Objectives of study

1. To retrospectively investigate the DTMUV suspected cases in 2007
2. To determine the occurrence, genetic characteristic and diversity of DTMUV isolated from ducks in Thailand during 2015-2017
3. To develop and validate a universal one-step RT-PCR assay for broad detection of duck Tembusu virus
4. To evaluate the clinical signs and pathological features caused by Thai DTMUV in Cherry Valley ducks and determine the effect of duck ages on the pathogenesis of Thai DTMUV.

1.3 Hypothesis

1. DTMUV have been emerged in ducks earlier than 2010, prior to the first report of DTMUV in China in 2010.
2. Different clusters of DTMUVs can be detected in ducks in Thailand.
3. The detection assay targeting the highly conserved NS5 gene of DTMUV can increase the accuracy, specificity and sensitivity for broad DTMUV detection.

4. Thai DTMUV can cause different degrees of disease severity in various ages of ducks.

1.4 Keywords (English)

ducks, duck Tembusu virus, genetic characterization, pathogenesis, Thailand

1.5 Conceptual framework

To achieve the objectives of this study, this study was divided into 2 main parts as follows;

Part 1 Genetic characterization of duck Tembusu virus isolated from ducks in Thailand

1.1 The presence of duck Tembusu virus in Thailand since 2007: A retrospective study

1.2 Genetic characterization of duck Tembusu virus in Thailand, 2015-2017: Identification of a novel cluster

1.3 Development and validation of a universal one-step RT-PCR assay for broad detection of duck Tembusu virus

Part 2 Pathogenesis of Thai duck Tembusu virus in Cherry Valley ducks: the effect of age on susceptibility to infection

The conceptual framework of this study is shown in Figure 1.

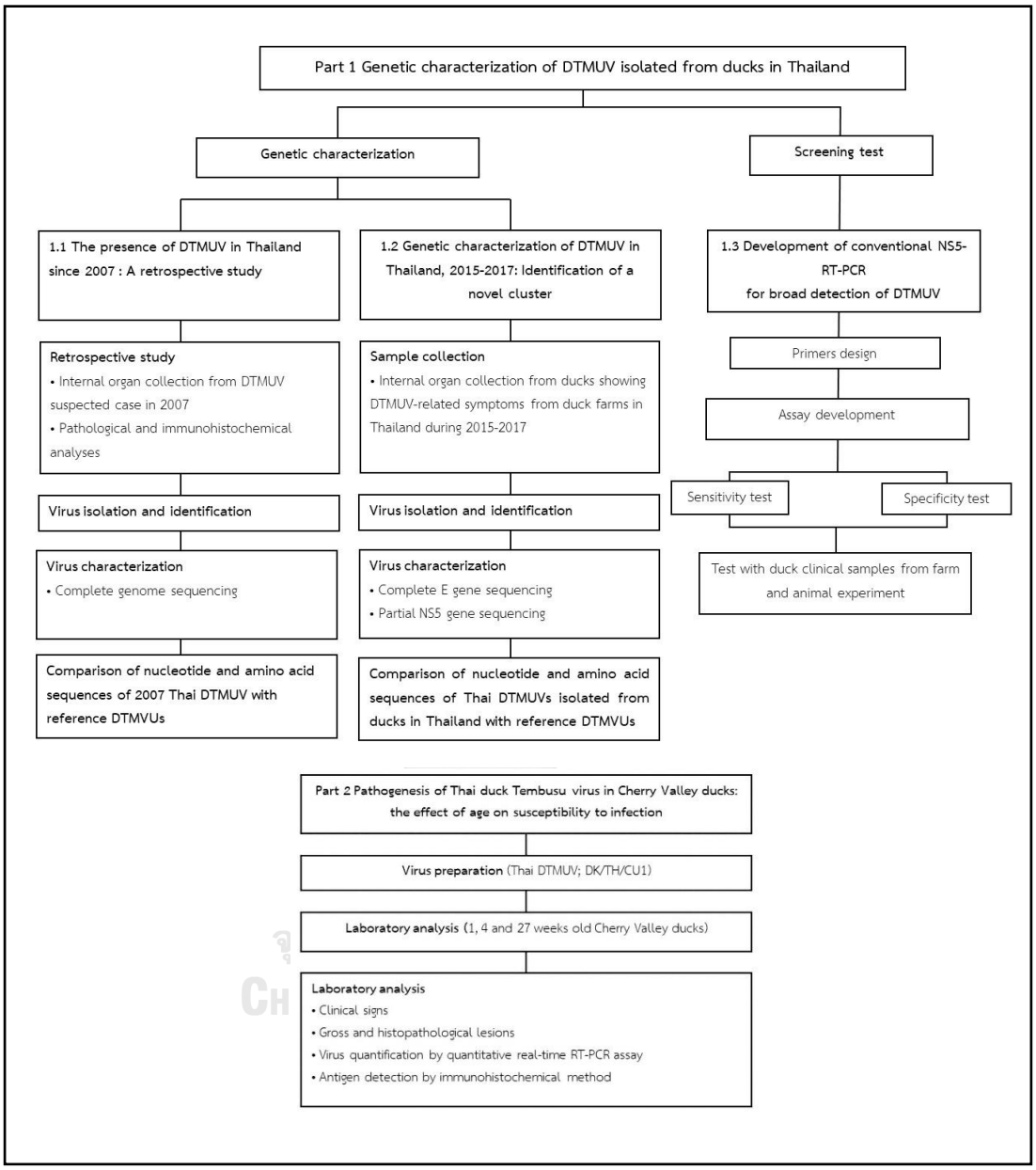

Figure 1 Conceptual framework of this study

1.6 Literature review

Tembusu virus (TMUV)

Tembusu virus (TMUV), a mosquito-borne flavivirus in Ntaya group, genus *Flavivirus*, family *Flavivirudae*, was first isolated from *Culex tritaeniorhynchus* mosquitoes in Malaysia in 1955(US-AMRU, 1957). Subsequently, TMUV was identified from *Culex tritaeniorhynchus* and *Culex gelidus* in Malaysia during 1968-1970 (Platt et al., 1975) and was detected from *Culex tritaeniorhynchus* in Thailand in 1986 and 1992 (Leake et al., 1986; Pandey et al., 1999). However, the association of TMUV with the animal infection had not been reported. In 2000, Sitiawan virus, a TMUV-related virus, was detected from affected chicken flocks that exhibited clinical signs of encephalitis and growth retardation in Malaysia in 2000 (Kono et al., 2000). Beside Malaysia, TMUV was also detected in *Culex* mosquitoes and serum samples of sentinel healthy ducks from the areas near Sangkhlaburi, Kanchanaburi province, Thailand in 2002 and 2015 without report of clinical illness in animals and humans (O'Guinn et al., 2013; Nitatpattana et al., 2017).

Duck Tembusu virus (DTMUV)

The outbreaks of a severe contagious disease caused by TMUV-related virus were first reported in layer, breeder and broiler duck farms in China in 2010 (Su et al., 2011). After the initial outbreak, this disease has spread throughout the duck-producing areas of China (Su et al., 2011; Yan et al., 2017). The affected ducks typically exhibited a severe reduction in egg production with severe neurological disorders, including ataxia and paralysis (Su et al., 2011). The morbidity rate of affected flocks was relatively high, while the mortality rate varied during 5-15% depending on the secondary infection and farm management (Su et al., 2011). The

causative agent of the outbreaks was subsequently identified as a Baiyangdian (BYD) virus. The genetic analysis of the partial envelope protein of BYDV showed that this virus was closely related to TMUV with 87–91% nucleotide identity and was classified as a novel duck Tembusu virus (DTMUV), a new genotype of Tembusu virus (TMUV) (Su et al., 2011). After the outbreaks in China, DTMUV was subsequently identified as a causative agent of a new emerging viral disease in ducks in several Southeast Asian countries, including Malaysia and Thailand in 2012 and 2013, respectively (Homonnay et al., 2014; Chakritbudsabong et al., 2015; Thontiravong et al., 2015). The clinical signs and lesions of affected ducks were similar to those of ducks infected with DTMUV in China. Since the initial outbreak in 2010, DTMUV has widely spread and become endemic in duck populations in duck producing areas of China, Malaysia and Thailand, resulting in massive economic losses in duck producing sectors.

DTMUV genome characteristics

DTMUV is classified as a new genotype of TMUV and grouped into the Ntaya virus (NTAV), genus *Flavivirus*, Family *Flaviviridae* (Cao et al., 2011; Su et al., 2011). DTMUV genome is a single stranded, positive-sense single stranded RNA, which is consists of single open reading frame encoding three structural proteins (capsid (C), pre-membrane (PrM), and envelope (E)) and seven non-structural (NS) proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5), flanked by the 5’ and 3’ untranslated regions (UTRs) (Liu et al., 2012). The size of DTMUV genome is approximately 11 kilobase in length. Genome organization of DTMUV is shown in Figure 2.

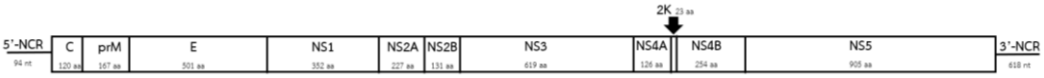

Figure 2 Genome organization of DTMUV

The structural proteins of DTMUV are involved in cellular attachment, membrane fusion and virus assembly. E protein is a large surface glycoprotein that is responsible for host cell receptor binding and virus entry. Like other flaviviruses, E protein, especially domain III, is highly variable as it is the major antigenic determinants involved in the induction of virus-neutralizing antibodies (Lindenbach et al., 2007). C protein is required for the viral nucleocapsid assembly (Oliveira et al., 2017). The function of PrM protein is to prevent E protein from acid-catalyzed rearrangement during transport through the secretory pathway (Lindenbach et al., 2007). In contrast to structural proteins, NS proteins are mostly responsible for viral genome replication and immune evasion (Lindenbach and Rice, 1999; Avirutnan et al., 2011; Youn et al., 2012; Akey et al., 2014). The detailed functions of DTMUV proteins are given in Table 1.

Table 1 The functions of the DTMUV proteins

Protein	Size (aa)	Predicted molecular weight (kDa) (Zhang et al., 2017)	Main functions	Reference
Structural proteins				
C	120	13	Nucleocapsid protein - requires for the viral nucleocapsid assembly - involves in endoplasmic reticulum (ER) translocation of prM - involves in nuclear localization	(Oliveira et al., 2017)
PrM	167	18	Precursor membrane glycoprotein - involves in the viral budding into the lumen of ER - heterodimeric interacts with E protein, which is involved in E proteins folding and the protection of immature virion during transport through acidic vesicles in the secretory pathway	(Lindenbach et al., 2013) (Heinz and Allison, 2000)
E	501	55	Envelope glycoprotein - involves in virus-host receptor binding and host cell entry - involves in the induction of virus-neutralizing antibodies	(Mukhopadhyay et al., 2005) (Lindenbach et al., 2007)
Non-structural proteins (NS)				
NS1	352	39	- Intracellular NS1 involves in early viral RNA replication. - Secreted NS1 activates the innate immune system.	(Guzman et al., 2016)
NS2A	227	25	- involves in virus replication and production of infectious particles	(Neufeldt et al., 2018)
NS2B	131	15	- Co-factor for serine protease NS3	(Guzman et al., 2016)
NS3	619	68	- involves in nucleoside triphosphatase and helicase functions during RNA synthesis	(Guzman et al., 2016)
NS4A	126	14	- requires for the formation of replication vesicles	(Guzman et al., 2016)

			- involves with membrane curvature-inducing activity	
2K peptide	23	23 amino acids	- A signal peptide involves in co-translational NS4B insertion into ER membrane	(Neufeldt et al., 2018)
NS4B	254	28	- inhibits the type I interferon response of host cells - modulates viral replication via its interaction with NS3 protein	(Guzman et al., 2016)
NS5	905	100	- involves in RNA synthesis: 5' RNA capping and methylation of the viral genome and a C-terminal domain with RNA-dependent RNA polymerase activity - involves in blockage of the IFN system	(Neufeldt et al., 2018)

Flavivirus replication cycle

Although the replication cycle of DTMUV has not been well characterized, as a member of flavivirus, its replication cycle seems to be similar to other flaviviruses. In brief, flavivirus particles bind to the specific receptors on the susceptible host cell surface and subsequently internalize into host cells through receptor-mediated endocytosis and a clathrin-dependent entry pathway. Although the specific receptors of DTMUV has yet been identified, many receptors of some flaviviruses are identified, including the mannose receptor (DENV receptor located on macrophages); the C-type lectin CD209 antigen (DC-SIGN) (DENV and WNV receptors located on dendritic cells); and members of the T cell immunoglobulin mucin domain protein 1 (TIM) and tyrosine protein kinase receptor 3 (TAM) family of phosphatidylserine receptors (DENV and ZIKV receptors located on epithelial cells, placenta cells, skin fibroblasts and glial cells) (Neufeldt et al., 2018). After entry into host cells, the viral particles are exposed to an acidic endosomal compartment, which facilitates the conformational rearrangements of E glycoproteins allowing fusion between viral and endosomal membranes leading to release the viral RNA into the cytosol.

Subsequently, the ribosomes recognize the positive-sense RNA (+RNA) and initiate the translation on rough endoplasmic reticulum (ER) membrane to produce a single polyprotein. Then, the proteases catalyze the co-translational and post-translational cleavage of the polyprotein into major structural and non-structural proteins (Figure 3) (Guzman et al., 2016; Neufeldt et al., 2018).

Three structural proteins are the major components of virion, while NS proteins are involved in ER membrane invaginations. ER membranes are the genome amplification site, where the negative-sense RNA (–RNA) is generated. The +RNA molecules that are produced by viral replicase complex are incorporated into viral particles and processed to RNA encapsidation and budding into the ER lumen as an immature particle. This immature particle containing prM is transported via the secretory pathway. The low pH of trans-golgi network (TGN) is involved in the rearrangement of prM-E heterodimers that permits the cleavage of prM by furin protease to form the mature virion. The mature flavivirus particles is released from the host cell through the conventional secretory pathway (Figure 3 and 4) (Guzman et al., 2016; Neufeldt et al., 2018).

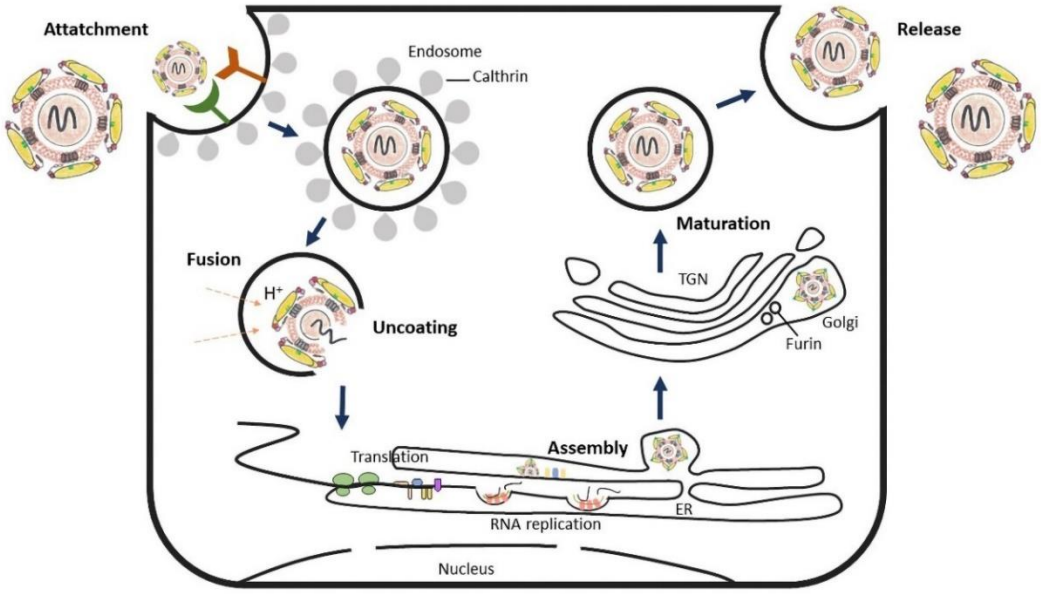

Figure 3 Schematic diagram of flavivirus replication cycle.

(adapted from Neufeldt et al., 2018)

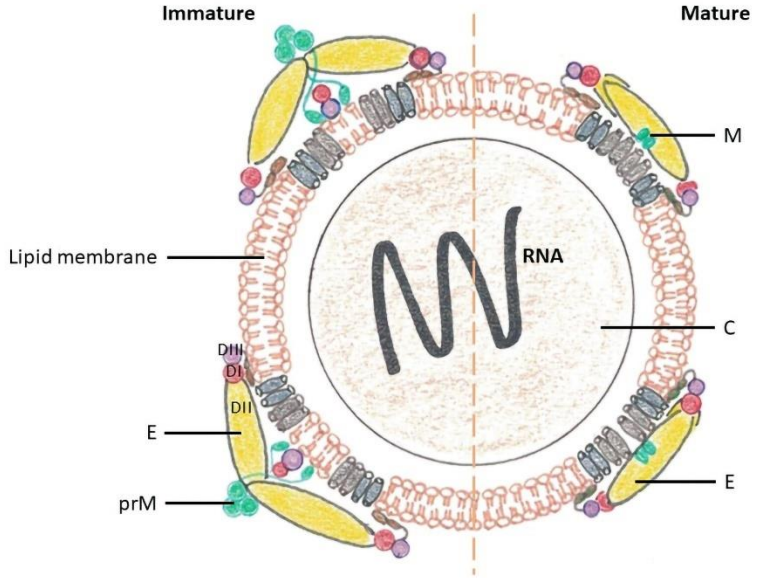

Figure 4 Schematic diagram of immature and mature flavivirus virion structures and proteins

(C: capsid, E: envelope, M: membrane protein and prM: precursor membrane protein)

Envelope glycoprotein of DTMUV

E protein is the major surface protein of flavivirus with approximately 53 kD and comprises of 1,503 nucleotides. E protein involves in virus replication cycle by mediating receptor binding, membrane fusion, host cell entry (Mukhopadhyay et al., 2005; Lindenbach et al., 2007). Each E protein subunit of flavivirus consisted of three domains; domain I, which forms a β-barrel; domain II, which projects along the virus surface between the homodimer subunits and the transmembrane regions, and domain III that maintains an immunoglobulin-like fold. The putative fusion peptide, which locates at the tip of domain II, appears to be involved in the mediation of insertion into the target cell membrane (Rey et al., 1995). Domain III involves in receptor binding and is a major target of neutralizing antibodies. Neutralizing antibodies against the domain III of flavivirus E protein can inhibit a post attachment step of viral entry (Nybakken et al., 2005). Immature flavivirus virions after budding in ER are covered by spiky complexes of PrM-E hetrodimers (Figure 4). E protein dimers dissociate into the monomeric subunits and form trimers when exposure to low pH and the proteolytic cleavage of PrM, which involves in the rearrangement of E proteins in TGN (Figure 4). The trimeric E ectodomains (post fusion form) interact with their fusion peptides, which extend back toward the fused membrane. Domain III rotates and folds back in relation to domain I for its conformational rearrangement into flat position against the surface of virions. Finally, PrM proteins are cleaved by furin protease. The disassociation of Pr portions of PrM occurs when the flavivirus particles release from the host cell to form the mature virions (Figure 3) (Pierson and Diamond, 2012; Rey et al., 2017).

Among all of the DTMUV proteins, E protein is the most important immunogenic component of the virus as it contains epitopes inducing neutralizing antibodies and plays a major role in virus-host cell recognition and host cell receptor

binding (Mukhopadhyay et al., 2005; Lindenbach et al., 2007). As it contains several neutralizing epitopes, E protein shows a very high degree of genetic variation. Therefore, the clustering of DTMUVs are usually classified based on the genetic variation of the E proteins (Yu et al., 2013).

Natural and experimental hosts of DTMUV

DTMUV infects a wide range of avian species, including ducks, chickens, geese, pigeons and house sparrows (Yun et al., 2012; Han et al., 2013; Tang et al., 2013a; Chen et al., 2014b; Liang et al., 2019). A variety of duck breeds, including Beijing ducks, Pekin ducks, Muscovy ducks, shelduck and Khaki Campbell ducks, can be infected with DTMUV (Yun et al., 2012; Zhu et al., 2012). Moreover, DTMUV has been reported to induce the cytopathic effect in many cell lines, including several mammalian cell lines (baby hamster kidney fibroblasts (BHK-21), cancerous cells (Hela), kidney epithelial cells extracted from African green monkey (Vero), human liver cancer cell lines (HepG2) and neuroblastoma cell lines (SH-SY5Y), avian cells (duck embryo fibroblasts (DEF), chicken embryo fibroblasts (CEF), goose embryo fibroblasts (GEF) and chicken fibroblast cell lines (DF-1)) and mosquito cells (C6/36 and *Aedes albopictus*) (Su et al., 2011; Tang et al., 2015a; Wang et al., 2016; Lei et al., 2017; Zhang et al., 2017). In addition, previous studies demonstrated that DTMUV is neurovirulent in BALB/C mice and Kunming mice after inoculation via intracerebral inoculations (Li et al., 2013; Ti et al., 2016). Furthermore, DTMUV-specific antibodies and DTMUV RNA were detected in serum samples (>70%) and oral swabs (approximately 50%) from duck industry workers in China (Tang et al., 2013b). However, DTMUV has not been reported to cause illness in humans and a recent study showed that DTMUV was not pathogenic in nonhuman primates (Wang et al., 2016).

Transmission of DTMUV

Like other flaviviruses, DTMUV can transmit by arthropod vector, which are Culex mosquitoes. A previous study demonstrated that *Culex vishnui* can develop a high TMUV titer after experimental feeding on TMUV-infected chickens and can transmit virus to other naïve chickens (O'Guinn et al., 2013). In addition, TMUV was also detected in other *Culex* species, including *Culex tarsalis* and *Culex quinquefasciatus* (Petz et al., 2014; Nitatpattana et al., 2017). Besides transmission via mosquito bite, DTMUV can transmit through non-vector routes, including fecal-oral route (Tang et al., 2013a), airborne transmission (Li et al., 2015d) and vertical transmission (Zhang et al., 2015). In fecal-oral route, DTMUV can transmit through both direct and indirect contacts via virus-contaminated fomites (Yan et al., 2011a). In addition, a previous study showed that DTMUV can transmit from infected ducks to naïve ducks housed in different separate isolators connected with air pipe, indicating the possibility of DTMUV to transmit via airborne route (Li et al., 2015d). Interestingly, vertical transmission of DTMUV in duck seems to be possible. A previous study demonstrated that TMUV-SDDE strain was isolated from dead duck embryos in China, providing an evidence of vertical transmission of DTMUV (Li et al., 2015d). Moreover, several studies showed that DTMUV could be detected in organs from reproductive system of infected ducks, which strongly supports the possible vertical transmission of DTMUV (Cao et al., 2011; Su et al., 2011).

Pathogenesis study of Chinese DTMUV

The pathogenesis study of several Chinese DTMUV strains in different ages of ducks were reported (Sun et al., 2014; Li et al., 2015b; Lu et al., 2016; Shen et al., 2016). The incubation period of Chinese DTMUV infection ranges from 2-5 days. A previous study showed that clinical signs of DTMUV infection, including depression, inappetence, tilted head and neurological dysfunction, were observed as early as 2 days post inoculation (dpi) in 1 week-old Cherry Valley ducks after experimentally infection with Chinese DTMUV and some of them died during 5-7 dpi (Li et al., 2015b). In contrast to 1-week-old ducks, all 3 week-old ducks infected with Chinese DTMUV survived and showed milder clinical symptoms than those observed in 1-week-old ducks. No significant clinical symptoms and death were observed in 7-week-old ducks experimentally infected with Chinese DTMUV (Li et al., 2015b). Therefore, a previous study revealed that 1- week-old group is the most susceptible duck age to Chinese DTMUV infection when compared to other age groups. Another previous study demonstrated that the severity of DTMUV infection in ducks was also related to age of ducks, in which 7-10 and 18-21 week-old were more susceptible to DTMUV infection than 14-16 week-old ducks (Lu et al., 2016).

The major gross lesions observed in Chinese DTMUV infected ducks were hyperemia and hemorrhage of ovarian follicles, ovarian atrophy, meningeal congestion and myocardial hemorrhage. Amyloid degeneration and splenomegaly were also observed in spleens of some infected ducks (Su et al., 2011; Lu et al., 2016; Shen et al., 2016). Interestingly, gross pathology lesions gradually reduced in severity as ducks matured (Li et al., 2015b). The major histopathology demonstrated that all DTMUV infected ducks had viral encephalitis lesions, including lymphocytic infiltration with perivascular cuffing, gliosis and central chromatolysis of neurons (Li et al., 2015b). Lymphoid depletion was observed in spleen. In addition, mononuclear

cells infiltration, hyperemia, degeneration and necrosis of different cells were observed in kidney, heart and ovary (Lu et al., 2016).

A high copy number of DTMUV RNA was mostly detected in several vital organs including spleen, kidney, heart, pancreas, lung, brain and liver, during 1-9 dpi. A previous study revealed that DTMUV could be detected in brains of 1-week-old ducks as early as 1 dpi. Moreover, DTMUV RNA detected from vital organs of 1-week-old ducks were significantly higher than that of the older ducks at 3-5 dpi. In addition, the virus clearance in 1 week-old ducks was slower comparing with the older ducks (Li et al., 2015b). Serum neutralizating (SN) antibodies could be detected in serum of 7-week-old ducks as at 3 dpi, which was earlier than the younger ducks. DTMUV-SN titers were continuously detected in serum samples of 1, 3 and 7 weeks-old ducks and reached peak titers at 17, 19 and 21 dpi, respectively (Li et al., 2015b). It is noted that pathogenesis study of DTMUV in ducks have been restricted to only Chinese DTMUVs. No information is available on the pathogenesis of DTMUV circulating in Thailand.

Immune response to DTMUV infection

As a member of flavivirus genus, immune responses to DTMUV infection are most likely to those to flavivirus infection. Recently, a previous study demonstrated that DTMUV could replicate rapidly in various organs and could activate innate immune responses of ducks in the early phase of infection (Li et al., 2015c). A previous study revealed that DTMUV could invade lymphocytes and macrophages in spleen (Sun et al., 2019), and could effectively activate host innate immune response mainly through pattern recognition receptors (PRR) including melanoma differentiation-associated factor 5 (MDA5), retinoic acid-induced gene I (RIG-I) and

Toll-like receptors (TLR) 3-dependent signaling pathways (Li et al., 2015c; Chen et al., 2016). The upregulation of MDA5, RIG-I and TLR3 are detected in brain and spleen of DTMUV infected ducks (Li et al., 2015c). Moreover, nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) is activated in both chicken and human cells during the TMUV infection (Chen et al., 2016).

In general, type I interferons (IFN) (IFN-α or IFN-β) involved in blocking of flavivirus infection by preventing translation and replication of infectious viral RNA. Several studies demonstrated that TMUV infection significantly up-regulates the expression of IFN (type I and III) and proinflammatory cytokines, such as interleukin (IL)-1β, IL−2, IL−6 and C-X-C motif chemokine ligand 8 (Cxcl8) (Li et al., 2015c; Chen et al., 2016). Subsequently, the binding of type I IFN to IFN-α/β receptor activates the JAK/STAT pathway, leading to the induction of interferon-stimulated gene (ISG) expression, including Mx proteins, 2′,5′-oligoadenylate synthetase (OAS) and double-stranded RNA-dependent *protein* kinase *(*PKR*)* (Li et al., 2015c). These antiviral proteins, especially Mx and OAS, were significantly increased in brain and spleen of DTMUV infected ducks (Li et al., 2015c). Moreover, antigen presentation by major histocompatibility complex (MHC) molecules is essential for the activation of adaptive immune response. The previous study demonstrated that the expression MHC-I is upregulated in brain and spleen, but the production of MHC-II is slightly upregulated in brain and downregulated in spleen (Li et al., 2015c).

Apart from innate immune response, adaptive immune response has been shown to be more effectively control flavivirus infection (Arjona et al., 2011). The adaptive immune system consists of humoral immunity (HMI) and cell-mediated immunity (CMI) (Chaplin, 2010). The HMI response mediated by neutralizing antibodies plays an important role in the control of flavivirus infection and

dissemination (Ngono and Shresta, 2018). Neutralizing antibodies, that recognize neutralizing epitopes located in the envelope glycoprotein, can inhibit attachment and replication of flaviviruses in host cells (King et al., 2007) and anti-NS antibodies can elicit protective humoral immunity and prevent lethal infection in West Nile virus (WNV) infected mice (Chung et al., 2006). Several DTMUV challenge studies demonstrated that the presence of DTMUV neutralizing antibodies correlates directly with the decrease of viral loads in the visceral organs (Sun et al., 2014; Li et al., 2015b). In addition to HMI, the CMI response also plays an important role to control and eliminate the infection of many flaviviruses, such as WNV (Suthar et al., 2013) and dengue virus (DENV) (Slon Campos et al., 2018). A previous study revealed that DTMUV can promote $CD8^+$ T cells and induce proinflammatory cytokines (IL-1β and IL-6) and IFN-γ in tissues of DTMUV infected ducks (Zhou et al., 2016). However, the information on the immunological mechanisms that involved in the control of DTMUV infection are remain largely unknown.

Diagnosis

The presumptive diagnosis of DTMUV infection is mainly based on the observation of clinical signs and pathological lesions, including appetite loss, a severe reduction in egg production with hyperemia and hemorrhage of ovarian follicles, and severe neurological disorders (ataxia and paralysis) (Su et al., 2011). However, due to the similar clinical signs and lesions of DTMUV infection in ducks with other avian viruses, the laboratory diagnosis is needed for providing the definitive diagnosis of DTMUV infection. Currently, various methods have been used in the detection of DTMUV, including virus detection methods, virus isolation in host system and serological assays (Su et al., 2011; Sun et al., 2014; Li et al., 2015b; Thontiravong et al., 2015; Lu et al., 2016; Shen et al., 2016). DTMUV isolation can be performed by

the inoculation of 10% tissue suspension of target organs into allantoic cavity of embryonated duck or chicken eggs (Thontiravong et al., 2015; Shen et al., 2016). In addition to embryonated eggs, DTMUV can also be isolated by using various types of cell cultures, including mammalian cell lines, avian cells and mosquito cells (Su et al., 2011; Tang et al., 2015a; Wang et al., 2016; Lei et al., 2017; Zhang et al., 2017). Several techniques of DTMUV detection were reported, including antigen detection (e.g. immunohistochemistry and immunofluorescence tests) and DTMUV nucleic acid detection (e.g. reverse transcription polymerase chain reaction (RT-PCR), quantitative real time reverse transcription polymerase chain reaction (qRT-PCR) and reverse transcription loop-mediated isothermal amplification (RT-LAMP) assays) (Su et al., 2011; Yan et al., 2011b; Tang et al., 2012; Yan et al., 2012; Tang et al., 2015b; Ti et al., 2016). Currently, most of the currently used RT-PCR assays for detection of DTMUV in ducks are focusing on E gene, which is considered to be the most highly variable gene among all genes of flaviviruses (Lindenbach et al., 2007; Su et al., 2011; Yu et al., 2013). To increase the accuracy, specificity and sensitivity of RT-PCR assay for DTMUV detection, NS5 gene, which is known as the most conserved gene of flaviviruses (Lim et al., 2015; Duan et al., 2017; Wang et al., 2017), should be used as a target gene for the development of RT-PCR assay for DTMUV detection. In this study, specific primers for NS5 gene of DTMUV will be developed and used for a one-step RT-PCR assay for more effective detection of DTMUV.

Besides virus detection, serological assays have also been used to evaluate the DTMUV infection by determining the levels of DTMUV specific antibodies that can be detected for a longer period after infection than antigen detection. Several techniques of serological assay have been used for DTMUV specific antibody detection, including serum neutralization (SN) test, hemagglutination inhibition (HI) test, enzyme-linked immunosorbent assay (ELISA) and indirect immunofluorescence

assay (IFA). Among all of the serological assays, ELISA and SN tests have been commonly used for evaluating DTMUV specific antibodies (Kono et al., 2000; Li et al., 2012; Ma et al., 2013). It is well known that SN test is considered to be a gold standard method for detection of the flavivirus specific antibodies (Timiryasova et al., 2013).

CHAPTER 2

The presence of duck Tembusu virus in Thailand since 2007: A retrospective study

This part has been published in the topic of

The presence of duck Tembusu virus in Thailand since 2007: A retrospective study

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2.1 Introduction

Duck Tembusu virus (DTMUV) is considered to be a causative agent of a new emerging viral disease in ducks. This newly emerging disease is characterized by a significant drop in egg production and severe neurological dysfunctions, including ataxia and paralysis (Su et al., 2011). The morbidity rate was relatively high, while the mortality rate varied from 10-30% depending on the farm management of affected flocks (Thontiravong et al., 2015). Apart from ducks, DTMUV was also detected in several avian species, including chickens, geese, pigeons and sparrows, indicating a wide host range of DTMUV (Yun et al., 2012; Han et al., 2013; Tang et al., 2013a; Chen et al., 2014b).

DTMUV is classified as a new genotype of Tembusu virus (TMUV) grouped into the Ntaya virus (NTAV) group within the genus *Flavivirus* of the Family *Flaviviridae* (Cao et al., 2011; Su et al., 2011). Its genome is composed of a positive-sense single

stranded RNA, which encodes three structural proteins (capsid (C), pre-membrane protein (prM) and envelope (E)) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) (Liu et al., 2012). DTMUV was first reported in China in 2010 (Su et al., 2011), and subsequently it was identified in many Southeast Asian countries, including Malaysia and Thailand (Homonnay et al., 2014; Chakritbudsabong et al., 2015; Thontiravong et al., 2015). Since then, DTMUV has widely spread and become endemic in duck populations in several duck producing areas of China, Malaysia and Thailand, resulting in massive economic losses in duck producing industries. Although, DTMUV infection had never been reported in ducks prior to 2010, an unknown disease associated with severe neurological signs and losses in egg production in ducks was found in Thailand since 2007. This indicated that DTMUV might have been emerged in ducks earlier than 2010, the first report in China. To determine the presence of DTMUV in 2007, the clinical samples from affected ducks presenting DTMUV like symptoms collected from the suspected cases in 2007 were retrospectively tested for DTMUV using pathological and virological analyses.

2.2 Materials and Methods

Retrospective clinical samples

In July 2007, an unknown severe contagious disease characterized by severe neurological dysfunctions and significant decreases in egg production was observed in several layer and broiler duck farms located in the northeastern (Nakhon Ratchasima) and central (Lopburi and Saraburi) provinces of Thailand. During the outbreak in 2007, 4 duck carcasses *(Anas platyrhynchos)* from affected flocks were submitted to the Pathology Unit, Faculty of Veterinary Science, Chulalongkorn University, Thailand for pathological and virological examinations of an unknown disease. These ducks were necropsied for gross examination and sample collection.

Clinical samples from affected ducks, including brain, spinal cord, spleen, lung, heart, kidney, liver, intestine and pancreas, were collected. Fresh tissue samples and formalin-fixed paraffin embedded tissues were stored at -80ºC and room temperature, respectively, from the time of collection in 2007 until retrospective testing for DTMUV.

Pathological and immunohistochemical analyses

For retrospective histopathological examination, the formalin-fixed paraffin embedded tissues were selected and subjected to hematoxylin and eosin (H&E) staining. In addition, the presence of flavivirus-specific antigens in tissues, including brain, spinal cord and spleen, was evaluated by the immunohistochemical (IHC) staining, as previously described with some modifications (Palmieri et al., 2011). In brief, the paraffin blocks were recut in 4 μm thickness, then deparaffinized and rehydrated with xylene and graded alcohol and put in phosphate buffered saline (PBS) solution. The sections were pretreated in citrate buffer pH 6.0 with microwave oven. For inactivation of endogenous peroxidase, the sections were immersed in 3% hydrogen peroxide solution (H_2O_2) at room temperature for 10 min. Sections were blocked with 5% skimmed milk in PBS for 15 min at 37ºC. Subsequently, the primary antibody, mouse monoclonal antibody against Flavivirus antigen group, clone D1-4G2-4-15 (EMD Millipore Corporation, CA, USA) diluted at 1:400, was added and incubated overnight at 4ºC. After 3 washes in PBS, the sections were incubated with the secondary antibody of polymer system (Dako REAL™ Envision™/HRP, Rabbit/Mouse, Dako, Denmark) at room temperature for 45 min, followed by addition of the substrate, 3, 3’ diamino-benzidine tetrahydrochloride (DAB). Sections were counterstained with hematoxylin, dehydrated, mounted with permount and then observed under the light microscope (10x and 40x). Each test included positive and negative controls.

Virus isolation and identification

Frozen tissue samples, including brain, spinal cord and spleen, were homogenized in sterile phosphate-buffered saline (PBS) at a 10% suspension (w/v), centrifuged at 3000 g for 15 min, filtered through 0.2 μm filters. The filtered suspensions were inoculated into the allantoic cavities of 9-day-old embryonated duck eggs. All embryonated duck eggs used in this study were obtained from healthy breeder duck flocks that were routinely tested and confirmed negative for common duck viruses, including DTMUV, avian influenza virus (AIV), Newcastle disease virus (NDV) and duck plague virus (DPV), by PCR and standard serological tests (Li et al., 2006; Liu et al., 2007; Suarez et al., 2007). Viral RNAs were extracted from tissue suspensions and allantoic fluids using NucleoSpin Extract Viral RNA Kit (Macherey-Nagel, Düren, Germany) following the manufacturer's recommendation. The samples were initially tested for common duck viruses, including AIV, NDV and DEV (Li et al., 2006; Liu et al., 2007; Suarez et al., 2007). The samples were also examined for the presence of DTMUV by RT-PCR using E gene specific primers (Su et al., 2011).

Whole genome sequencing and phylogenetic analysis

Viral RNA of DTMUV isolated from diseased ducks in 2007 (DK/TH/CU-DTMUV2007) (2007 Thai DTMUV) was subjected to whole genome sequencing using the primer set for amplification of the polyprotein gene sequence of DTMUV as described previously (Thontiravong et al, 2015). The nucleotide sequences were assembled using SeqMan software v.5.03 (DNASTAR Inc.,Wisconsin, USA). A whole polyprotein gene sequence of the 2007 Thai DMTUV was submitted to GenBank database under the accession number MF621927.

Phylogenetic analysis was conducted by comparing the polyprotein gene sequence of the 2007 Thai DTMUV with the previously reported Chinese, Malaysian

and Thai DTMUVs, identified during 2010-2015. The phylogenetic trees based on the polyprotein gene sequences of DTMUV were constructed by neighbor-joining (NJ) and maximum-likelihood (ML) algorithms with 1000 bootstraps using MEGA v.6.0 program (Tamura et al., 2011). To evaluate the nucleotide and amino acid identities among DTMUV strains, the polyprotein gene sequence of the 2007 Thai DTMUV was aligned and compared with the previously reported Chinese, Malaysian, Thai DTMUVs identified during 2010-2015 and other selected reference flaviviruses in MegAlign software v.5.03 (DNASTAR Inc., Wisconsin, USA). Additionally, the polyprotein sequence of the 2007 Thai DTMUV was aligned and compared with those of Chinese, Malaysian and 2013 Thai DTMUVs using MEGA v.6.0 program to determine the signature amino acid mutations.

To determine the evolution of DTMUV, the time-scaled phylogenetic analysis was performed as previously described (Yu et al., 2013; Dai et al., 2015; Moureau et al., 2015). The polyprotein gene sequences of the 2007 Thai DTMUV and the previously reported Chinese, Malaysian and Thai DTMUVs, identified during 2010-2015, were aligned using Muscle v.3.6 (Edgar, 2004). Maximum clade credibility (MCC) tree of the polyprotein gene sequence was generated by BEAST 1.8 with Bayesian Markov Chain Monte Carlo (BMCMC) algorithm. Strict clock model with coalescent exponential growth population and GTR with gamma 4 substitution was used as model parameters to obtain mean substitution rate (Drummond et al., 2002; Drummond et al., 2012). The Bayesian MCMC chain length was 50,000,000 generations with sampling every 5,000 generations for the polyprotein sequence analysis. The effective sample size (ESS) value was assessed by using Tracer (v1.6.0) (Molecular evolution, phylogenetics and epidemiology, Edinburgh, Scotland, UK) (Rambaut et al., 2014). The polyprotein sequence analysis had ESS value greater than 200 suggesting minimal standard error. The resulting tree of each iteration was

summarized for a representative clustering pattern by using a tree annotator with 10% discarding of the chains as burn-in and the resulting maximum clade credibility tree was visualized with FigTree software (v1.4.2) (Molecular evolution, phylogenetics and epidemiology, Edinburgh, Scotland, UK).

2.3 Results

Clinical and pathological findings

In July 2007, an unknown severe contagious disease associated with severe neurological dysfunctions and egg production losses was observed in several layer and broiler duck farms located in the northeastern (Nakhon Ratchasima) and central (Lopburi and Saraburi) provinces of Thailand. Clinical signs were usually observed in 3-4-weeks-old broiler ducks and in 40-48-weeks-old layer ducks during their production period. The affected ducks showed the clinical signs of severe neurological disorders, including ataxia, imbalance movement, head tremor and reluctance to walk (Figure 5a, b). A significant drop in egg production (40%) was also detected. Overall, the infected ducks exhibited the clinical signs resembling to ducks infected with DTMUV previously reported in 2013 (Thontiravong et al, 2015). The morbidity and mortality rates of affected duck flocks were estimated to be 30% and 15%, respectively.

Retrospective macroscopic and microscopic examinations showed that the most significant macroscopic changes were severe ovarian hemorrhage and atrophy (Figure 5c). No remarkable lesions were observed in other visceral organs. The prominent microscopic changes were observed in the cerebrum and spinal cord, which showed non-suppurative meningoencephalitis characterized by perivascular cuffing with 2-3 layers of mononuclear cells, multifocal gliosis and generalized

demyelination (Figure 5d). In addition, lymphoid follicular necrosis of spleen, multifocal mononuclear cell infiltration and perivascular cuffing with fibrinoid degeneration in myocardium were also observed. Flavivirus-specific antigens were detected by IHC method in the cytoplasm of affected neurons and glial cells in cerebellum, brain stem and spinal cord, and in the cytoplasm of affected lymphocytes and macrophages in spleen (Figure 5e).

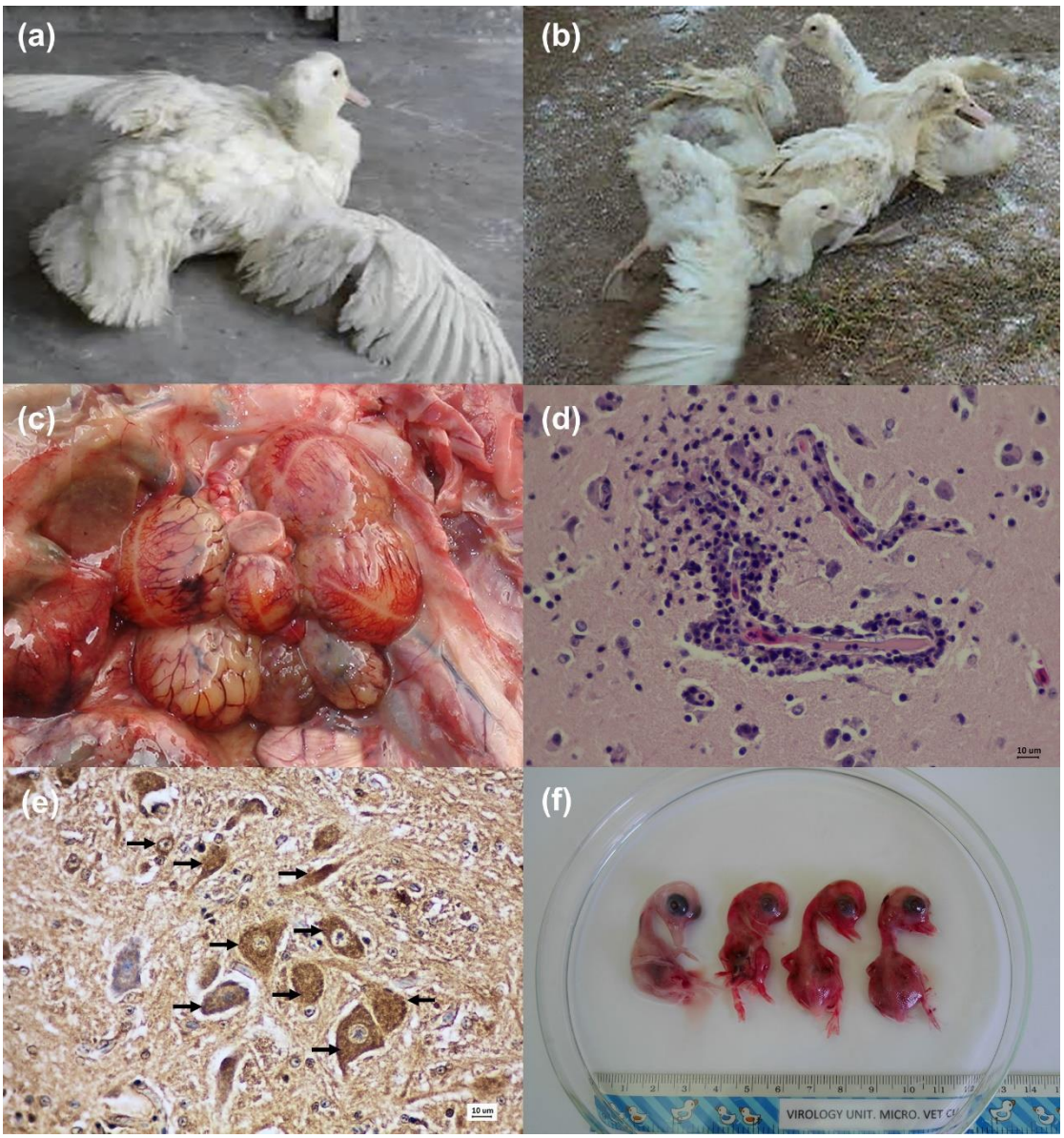

Figure 5 Clinical signs and pathological lesions of ducks infected with the 2007 Thai DTMUV (Ninvilai et al., 2018).

(a and b) Clinical signs; 48 weeks old ducks showed severe neurological signs, including ataxia, imbalance movement and reluctance to walk. (c) Gross lesion; severe hemorrhage and atrophy of ovarian follicles. (d) Histopathological lesions; non-suppurative encephalitis with mononuclear cells, perivascular cuffing and gliosis in cerebrum, Hematoxylin and eosin (H&E) stain. (e) Immunohistochemical findings; positive flavivirus-specific antigen was detected in the cytoplasm of affected neurons and glial cells in thalamus (black arrows), Envision-DAB method. (f) Three duck

embryos infected with DK/TH/CU-DTMUV2007 died within 3-5 days with subcutaneous hemorrhage (right) and a normal embryo is shown at left. The suspensions were inoculated into the allantoic cavities of 9-day-old embryonated duck eggs.

Isolation and identification of the 2007 Thai DTMUV

Following inoculation with tissue suspensions prepared from brain, spinal cord and spleen of affected ducks in 2007, all duck embryos died with subcutaneous hemorrhage within three to five days post inoculation, resembling to the observation from DTMUV infection in the previous report (Thontiravong et al., 2015) (Figure 5f). Initially, the samples were tested for common duck viral pathogens and were shown to be negative for NDV, AIV and DPV. Interestingly, DTMUV was clearly detected in all tissue suspensions of tissue samples collected from the outbreaks in 2007 and all of the inoculated allantoic fluids by RT-PCR followed by confirmation with DNA sequencing (100% DTMUV positive rate).

Phylogenetic and evolutionary analyses of the 2007 Thai DTMUV

To further characterize the 2007 Thai DTMUV, the virus designated as DK/TH/CU-DTMUV2007 was subjected to whole genome sequencing and analyses. The whole genome length of DK/TH/CU-DTMUV2007 is 10,278 nucleotides, encoding 3,426 amino acids, which is identical to that of the previously reported DTMUV isolates (Liu et al., 2012; Thontiravong et al., 2015). Phylogenetic analysis of the polyprotein gene sequence showed that the 2007 Thai DTMUV was grouped together with the previously reported DTMUVs and with the most closely related to Malaysian DTMUVs, D192/1/3/MY and D1977/1/MY, isolated in 2012 (97.5%-97.7% nucleotide identity) (Figure 6, Table 2). However, the polyprotein gene sequence of the 2007 Thai DTMUV shared only 91.9%-92% and 91.4%-92.2% nucleotide identity with the previously reported Thai DTMUVs isolated in 2013 and Chinese DTMUVs isolated

during 2010-2015, respectively (Table 2). It is noted that the 2007 Thai DTMUV showed only 88.7% and 87.7% nucleotide identity with MM1775 and Sitiawan viruses, respectively (Table 2). Overall, our findings based on the nucleotide identity of the polyprotein gene sequence indicated that the 2007 Thai DTMUV was different from the other recently reported DTMUVs, although it was closely related to Malaysian DTMUVs.

Analyses of NJ, ML and MCC trees consistently revealed that DTMUVs could be divided into two distinct clusters, 1 and 2, of which cluster 2 was further divided into 2 subclusters (2.1 and 2.2) (Figure 6, 8). The cluster 1 was predominantly circulated in Malaysia, while subcluster 2.1 and 2.2 were the dominant lineages commonly circulating in Thailand and China, respectively. Interestingly, the 2007 Thai DTMUV was grouped into a unique position within cluster 1 located on a separate branch with the Malaysian DTMUVs. In contrast, the 2007 Thai DTMUV was distinct from any of the 2013 Thai DTMUVs and the Chinese DTMUVs (Figure 6, 8). This finding indicated the presence of DTMUV, which was genetically unrelated to the recently reported DTMUV, in Thailand since 2007. From our analysis, it could be suggested that in Thailand, DTMUVs were classified into 2 groups, cluster 1 (2007 Thai DTMUV) and subcluster 2.1 (2013 Thai DTMUV). These two DTMUV clusters were associated with the outbreak of DTMUV in Thailand.

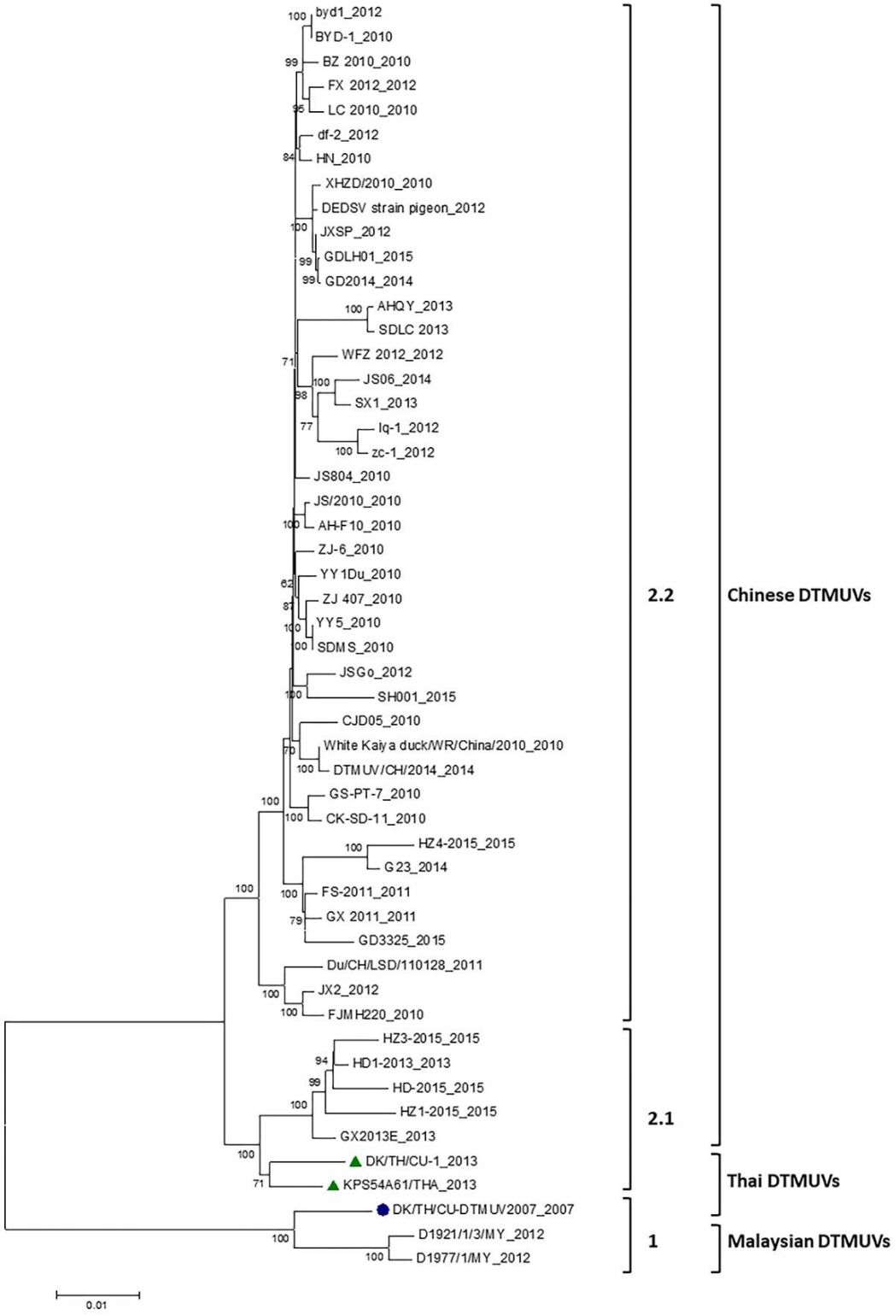
byd1_2012
BYD-1_2010
BZ 2010_2010
FX 2012_2012
LC 2010_2010
df-2_2012
HN_2010
XHZD/2010_2010
DEDSV strain pigeon_2012
JXSP_2012
GDLH01_2015
GD2014_2014
AHQY_2013
SDLC 2013
WFZ 2012_2012
JS06_2014
SX1_2013
lq-1_2012
zc-1_2012
JS804_2010
JS/2010_2010
AH-F10_2010
ZJ-6_2010
YY1Du_2010
ZJ 407_2010
YY5_2010
SDMS_2010
JSGo_2012
SH001_2015
CJD05_2010
White Kaiya duck/WR/China/2010_2010
DTMUV/CH/2014_2014
GS-PT-7_2010
CK-SD-11_2010
HZ4-2015_2015
G23_2014
FS-2011_2011
GX 2011_2011
GD3325_2015
Du/CH/LSD/110128_2011
JX2_2012
FJMH220_2010
HZ3-2015_2015
HD1-2013_2013
HD-2015_2015
HZ1-2015_2015
GX2013E_2013
DK/TH/CU-1_2013
KPS54A61/THA_2013
DK/TH/CU-DTMUV2007_2007
D1921/1/3/MY_2012
D1977/1/MY_2012
0.01
2.2
2.1
1
Chinese DTMUVs
Thai DTMUVs
Malaysian DTMUVs

Figure 6 Phylogenetic analysis of the nucleotide sequences of polyprotein gene (10,278 bp) of a 2007 Thai DTMUV (DK/TH/CU-DTMUV2007) and selected reference strains of DTMUV isolated from Thailand, Malaysia and China (Ninvilai et al., 2018). The nucleotide sequences were aligned using Muscle version 3.6 (Edgar, 2004). The phylogenetic trees were constructed in MEGA version 6.0 by using the neighbor-joining algorithm with the Kimura-2 parameter model applied to 1,000 replications of bootstrap (Tamura et al., 2011). Blue circle indicates the DK/TH/CU-DTMUV2007 isolated from this study, while green triangles indicate the previously reported Thai DTMUVs isolated in 2013. Similar results were observed when applying the maximum-likelihood algorithm. Three distinct clusters/subclusters, including 1, 2.1 and 2.2, are shown.

To identify the signature amino acid mutations of the 2007 Thai DTMUV, the polyprotein sequence of the 2007 Thai DTMUV was aligned and compared with those of the Chinese, Malaysian and 2013 Thai DTMUVs using MEGA v.6.0 program. Amino acid mutation analysis showed that the 2007 Thai DTMUV have 76 signature amino acid differences across the polyprotein sequence compared to the Chinese, Malaysian and 2013 Thai DTMUVs, most of which were located in NS5 protein, followed by NS1, E, NS2, NS4A, C, PrM, NS4B and NS3 proteins (Figure 7). In addition, our result also showed that 53 amino acid mutations unique to each cluster (cluster 1 and 2) were observed across the polyprotein sequence, indicating that cluster 1 and 2 had their specific distinctive amino acid characteristics (Figure 7). This finding also supported the phylogenetic analysis result, in which DTMUV were classified into two distinct clusters.

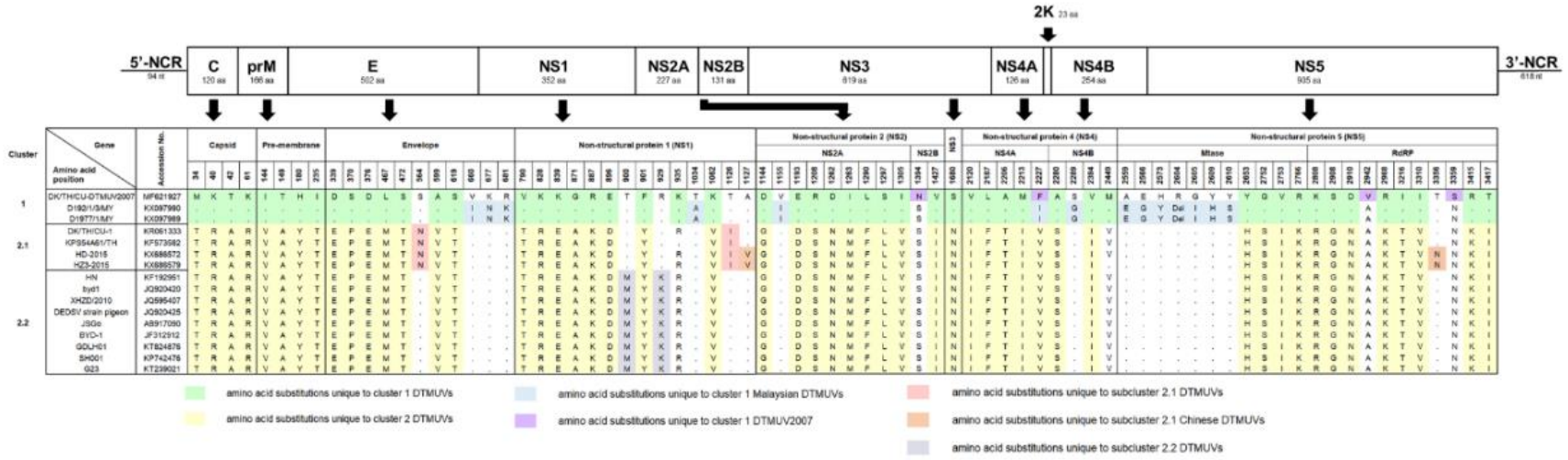

Figure 7 Signature amino acid changes across the polyprotein sequence between the 2007 Thai DTMUV and other DTMUV reference strains (Ninvilai et al., 2018).

Table 2 Comparison of the nucleotide and amino acid identities of the polyprotein sequences of a 2007 Thai DTMUV (DK/TH/CU-DTMUV2007) with reference DTMUVs and TMUVs isolated from mosquitos (MM1775) and chickens (Sitiawan virus) (Ninvilai et al., 2018).

Reference DTMUVs	Accession No.	Host	Location	Year of collection	% Nucleotide identity	% Amino acid identity
Malaysian DTMUVs						
D1921/1/3/MY	KX097990	Duck	Malaysia	2012	97.7	99
D1977/1/MY	KX097989	Duck	Malaysia	2012	97.5	99
Thai DTMUVs						
DK/TH/CU-1	KR061333	Duck	Thailand	2013	91.9	97.8
KPS54A61	KF573582	Duck	Thailand	2013	92	97.7
Chinese DTMUVs						
JS804	JF895923	Goose	China	2010	92.2	97.6
HN	KF192951	Duck	China	2010	92.1	97.7
BYD-1	JF312912	Duck	China	2010	92.1	97.8
XHZD/2010	JQ595407	Duck	China	2010	92	97.8
LC_2010	KC990543	Duck	China	2010	91.9	97.6
GX_2011	KC990542	Duck	China	2011	92	97.7
JX2	JQ920426	Duck	China	2012	92.2	97.7
df-2	KJ489355	Duck	China	2012	92.2	97.8
byd1	JQ920420	Duck	China	2012	92.1	97.8
JSGo	AB917090	Goose	China	2012	92	97.8
DEDSV strain pigeon	JQ920425	Pigeon	China	2012	92	97.8
GX2013E	KM275940	Duck	China	2013	91.9	97.7
SX1	KM066945	Chicken	China	2013	91.7	97.5
G23	KT239021	Goose	China	2014	91.7	97.7
JS06	KR869106	Chicken	China	2014	91.6	96.9
GDLH01	KT824876	Duck	China	2015	92	97.8
SH001	KP742476	Duck	China	2015	91.7	97.6
HD-2015	KX686572	Duck	China	2015	91.5	97.4
HZ1-2015	KX686570	Duck	China	2015	91.4	97.4
HZ4-2015	KX686571	Duck	China	2015	91.4	97.1
MM1775	JX477685	*Culex tritaeniorhynchus*	Malaysia	1955	88.7	96.7
Sitiawan virus	JX477686	Chicken	Malaysia	2000	87.7	96.3

To evaluate the evolution characteristic of DTMUV, the time-scaled phylogenetic analysis based on the polyprotein gene sequence of DTMUV identified during 2007-2015 was conducted. A strict clock model with coalescent exponential growth population and GTR with gamma 4 substitution was used as model parameters to obtain mean substitution rate. The mean nucleotide substitution rate of the polyprotein gene sequence of DTMUV isolated during 2007-2015 was estimated to be 1.113×10^{-3} substitution/site/year. In addition, a time-scaled Bayesian MCC tree based on the polyprotein gene sequence revealed that DTMUVs emerged in Asia around 1960 (with 95% highest posterior density (HPD) = 1951.2-1967.4), when cluster 2 diverged from cluster 1. Subsequently, cluster 2 is further divided into two major subclusters, 2.1 and 2.2, around 1998 (95% HPD = 1996.1-2000.7) (Figure 8).

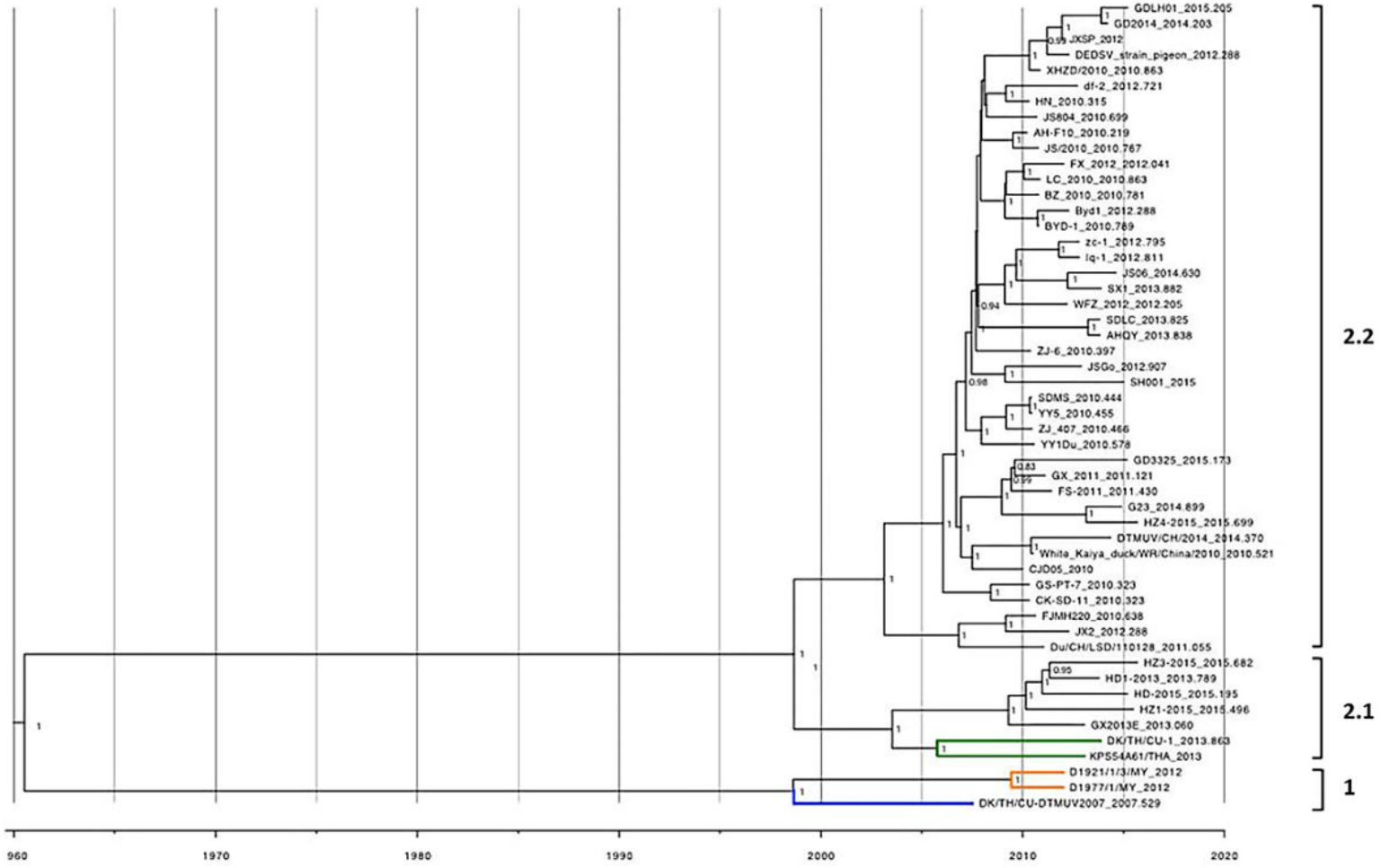
GDLH01_2015.205
GD2014_2014.203
JXSP_2012
DEDSV_strain_pigeon_2012.288
XHZD/2010_2010.863
df-2_2012.721
HN_2010.315
JS804_2010.699
AH-F10_2010.219
JS/2010_2010.767
FX_2012_2012.041
LC_2010_2010.863
BZ_2010_2010.781
Byd1_2012.288
BYD-1_2010.789
zc-1_2012.795
lq-1_2012.811
JS06_2014.630
SX1_2013.882
WFZ_2012_2012.205
SDLC_2013.825
AHQY_2013.838
ZJ-6_2010.397
JSGo_2012.907
SH001_2015
SDMS_2010.444
YY5_2010.455
ZJ_407_2010.466
YY1Du_2010.578
GD3325_2015.173
GX_2011_2011.121
FS-2011_2011.430
G23_2014.899
HZ4-2015_2015.699
DTMUV/CH/2014_2014.370
White_Kaiya_duck/WR/China/2010_2010.521
CJO05_2010
GS-PT-7_2010.323
CK-SD-11_2010.323
FJMH220_2010.638
JX2_2012.288
Du/CH/LSD/110128_2011.055
HZ3-2015_2015.682
HD1-2013_2013.789
HD-2015_2015.195
HZ1-2015_2015.496
GX2013E_2013.060
DK/TH/CU-1_2013.863
KPS54A61/THA_2013
D1921/1/3/MY_2012
D1977/1/MY_2012
DK/TH/CU-DTMUV2007_2007.529
2.2
2.1
1
960
1970
1980
1990
2000
2010
2020

Figure 8 A time-scaled Bayesian maximum clade credibility tree of the polyprotein gene sequences of DTMUV. Maximum clade credibility (MCC) tree was generated by BEAST 1.8 with Bayesian Markov Chain Monte Carlo (BMCMC) algorithm (Ninvilai et al., 2018).

Strict clock model with coalescent exponential growth population and GTR with gamma 4 substitution was used as model parameters to obtain mean substitution rate. The MCC tree representing the estimated timing of DTMUV divergence from their ancestor is provided after the sequence name. Posterior probability above 0.7 is shown at each node. DK/TH/CU-DTMUV2007, previously reported Thai and Malaysian DTMUVs isolated during 2012-2013 are labeled by blue, green and yellow, respectively. All non-labeled sequence names are DTMUVs identified from China. Three distinct clusters/subclusters, including 1, 2.1 and 2.2, are shown.

2.4 Discussion

In 2007, an unknown severe contagious disease characterized by severe neurological dysfunctions and significant decreases in egg production was observed in several layer and broiler duck farms located in the northeastern and central provinces of Thailand. The objective of this study was to investigate the etiological agent of this unknown disease in 2007. Our findings based on retrospective laboratory analysis revealed that the pathogen responsible for the unknown disease of 2007 was duck Tembusu virus (DTMUV), suggesting the presence of DTMUV in Thailand since 2007, prior to the first report of DTMUV in China in 2010 (Su et al., 2011).

The clinical signs of ducks infected with the 2007 Thai DTMUV observed in this study include severe neurological disorders and egg production losses. Clinical signs correlated with the pathological changes and immunohistochemical findings, which were mainly affected in the nervous system and the ovary. Despite lack of the experimental pathogenicity testing of the 2007 Thai DTMUV, clinical features and pathological changes of DTMUV cases in 2007 are in agreement with the DTMUV infections reported previously in China, Malaysia and Thailand (Cao et al., 2011; Su et al., 2011). This indicates the possible involvement of DTMUV in the outbreak in ducks in 2007. Like the DTMUV outbreak in 2013 (Thontiravong et al., 2015), our observation demonstrated that the 2007 DTMUV outbreak also occurred in the rainy month, suggesting that rainy might be a season of high prevalence of DTMUV infection, possibly associated with the highest numbers of mosquitos in Thailand. However, the transmission routes and pathogenicity of the 2007 Thai DTMUV could not be evaluated in this study. Further studies on the transmission routes and pathogenicity of the 2007 Thai DTMUV should be conducted.

Genetic analyses demonstrated that the 2007 Thai DTMUV appeared to be most closely related to the Malaysian DTMUVs, while it was genetically distinctive to the Thai and Chinese DTMUVs identified during 2010-2015. In addition, phylogenetic analysis showed that the 2007 Thai DTMUV was grouped within cluster 1 located on a separate branch with the Malaysian DTMUVs, while it formed a distinct cluster with any of the currently circulating Thai and Chinese DTMUVs, which belonged to cluster 2. Overall, these findings based on phylogenetic and MCC tree analyses indicated that the 2007 Thai DTMUV was genetically different from any of the currently circulating DTMUVs; although, it appeared to share a common ancestor with the recently reported DTMUVs. However, the origin of the 2007 Thai DTMUV remains unknown and requires further investigation.

Our time-scaled phylogenetic analysis based on the largest number of the polyprotein gene sequences of DTMUV identified during 2007-2015 showed that DTMUV might have emerged since 1960. In contrast with a previous report using a different set of DTMUV data collected only from China between 2010 and 2013 to establish the clusters of DTMUVs into four clusters (I, II-a, II-b and II-c) (Dai et al., 2015), three clusters of DTMUVs (1, 2.1 and 2.2) were established in this study by using sequences of DTMUV identified in China and Southeast Asia during 2007-2015. It is noted that all of the DTMUV sequences in cluster I, II-a, II-b and II-c described in Dai et al. (2015) were grouped into cluster 2.2 described in this study. Our finding indicated that three clusters of DTMUVs (1, 2.1 and 2.2) established in this study, were associated with the current DTMUV outbreaks in China and Southeast Asia in which the correlation between virus cluster and geographic location was observed. In Thailand, two DTMUV clusters, including cluster 1 (2007 Thai DTMUV) and subcluster 2.1 (2013 Thai DTMUV), were associated with the outbreaks of DTMUV.

Amino acid mutation analysis revealed that most of the signature amino acid mutations of the 2007 Thai DTMUV were located in NS5, NS1 and E proteins. It is known that NS5 and NS1 proteins are responsible for viral genome replication and viral immune evasion, respectively (Lindenbach and Rice, 1999; Avirutnan et al., 2011; Youn et al., 2012; Akey et al., 2014). Therefore, whether these amino acid changes in NS5 and NS1 protein affect the functions of these proteins needs to be further investigated. Besides NS1 and NS5 proteins, amino acid changes of the 2007 Thai DTMUV were also mainly detected in E protein, particularly in the domain III (DIII). Since E protein, especially DIII, is involved in the induction of virus-neutralizing antibodies (Lindenbach et al., 2007), the amino acid mutations in DIII may affect the cross protection reactivity between the 2007 Thai DTMUV and other DTMUV strains, particularly cluster 2 DTMUV. Thus, the cross-reactivity between the 2007 Thai DTMUV (cluster 1) and the 2013 Thai DTMUV (cluster 2), was additionally determined in this study by serum neutralization (SN) test as previously described (Chen et al., 2014a). From our preliminary serological study, our result showed that the 2013 Thai DTMUV (cluster 2)-positive duck serum exhibited cross-reactivity with the 2007 Thai DTMUV (cluster 1) (mean SN titer 5), although the level of cross-reactivity was relatively lower than those tested with their homologous virus (mean SN titer 12.73) (data not shown). This finding suggested the amino acid mutations found in E protein had no effect on the cross protection reactivity between these two different DTMUVs. However, the difference of mean SN titer between the 2007 and 2013 Thai DTMUVs may be related to the high number of amino acid mutations in the domain III, where most of the neutralizing epitopes were located (Lindenbach et al., 2007). However, the factor of the difference of mean SN titer between these 2 viruses requires further investigation. In addition, it is known that E protein has important functions in virus replication cycle, including virus receptor binding and host cell entry (Mukhopadhyay et al., 2005). Therefore, whether the amino acid mutations in E

protein would be effected on the other functions of E protein remains to be investigated. In addition, our result showed that cluster 1 and 2 had their specific distinctive amino acid characteristics. This finding provides the information that could be possibly used to differentiate DTMUV strains between two different clusters, which will be useful for DTMUV diagnostic and vaccine development.

The mean evolutionary rate of the DTMUVs detected in this study was estimated at 1.113×10^{-3} substitution/site/year, which is higher than previous reports observed in DTMUVs (Yu et al., 2013; Dai et al., 2015) and some other Flaviviruses, including Japanese encephalitis virus (JEV) and West Nile virus (WNV) (Pan et al., 2011; Añez et al., 2013). This finding indicated the continued evolving of DTMUVs with relatively high evolutionary rate. Therefore, continued DTMUV surveillance in ducks is essential to monitor the emergence of the new distinct DTMUV variants with possible enhanced virulence.

In conclusion, our data demonstrated the presence of DTMUV in diseased ducks since 2007 and the co-circulation of different DTMUV clusters in Asia. These findings highlight high genetic diversity and rapid evolution of DTMUVs in Asia. Our study emphasized the need for the continuous DTMUV surveillance to monitor the emergence of the novel DTMUV variants that may cause new epidemics in ducks.

CHAPTER 3

Genetic characterization of duck Tembusu virus in Thailand, 2015-2017: Identification of a novel cluster

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Genetic characterization of duck Tembusu virus in Thailand, 2015-2017: Identification of a novel cluster

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3.1 Introduction

Duck Tembusu virus (DTMUV) is an emerging flavivirus that causes severe neurological disorder and acute egg drop syndrome in ducks and some other avian species, including geese and chickens (Su et al., 2011; Yun et al., 2012; Han et al., 2013; Tang et al., 2013a; Chen et al., 2014b). DTMUV is classified as a new genotype of Tembusu virus (TMUV), which is a member of the Ntaya virus (NTAV) group in the genus *Flavivirus* of the Family *Flaviviridae* (Cao et al., 2011; Su et al., 2011). Like other flaviviruses, the DTMUV genome consists of a positive-sense single-strand RNA encoding three structural proteins (capsid (C), pre-membrane (PrM), and envelope (E)) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) (Liu et al., 2012). Among these proteins, E protein has high variability since it is associated with host cell entry and contains virus-neutralizing epitopes (Mukhopadhyay et al., 2005; Lindenbach et al., 2007). Therefore, besides the polyprotein gene, the clusters of DTMUV are usually classified based on the genetic variation of the E gene (Dai et al., 2015; Lei et al., 2017). Recently, DTMUV has been genetically described into two

different clusters, 1 and 2, of which cluster 2 has been further divided into 2 subclusters (2.1 and 2.2) (Ninvilai et al., 2018).

DTMUV was first detected in China in 2010 (Su et al., 2011) and it was later spread rapidly to other Asian countries, including Malaysia and Thailand (Homonnay et al., 2014; Chakritbudsabong et al., 2015; Thontiravong et al., 2015). Currently, DTMUV is widely distributed and becomes endemic in duck populations in Asia, causing significant economic losses to the duck producing industry. In Thailand, we previously reported the emergence of DTMUV infection in domestic ducks in 2013 (Thontiravong et al., 2015). After this initial report, an increasing number of DTMUV infected cases have continuously been observed in several duck farms in Thailand. However, the genetic characteristic of DTMUVs recently circulating in ducks in Thailand remains unknown. In this study, we investigated the geographic distribution and genetic characteristic of DTMUVs recently circulating in ducks in Thailand during 2015-2017.

3.2 Materials and Methods

Clinical samples

A total of 288 clinical samples (brain, spinal cord, spleen and ovary) were collected from individual broiler and layer ducks showing clinical signs of DTMUV infection, including neurological dysfunctions and significant decreases in egg production. The samples were obtained from 89 duck farms from 16 provinces located in duck raising areas of Thailand, including the central (Ang Thong, Lopburi, Nakhon Pathom, Phetchabun, Saraburi, Sing Buri and Suphan Buri), eastern (Chachoengsao, Chonburi, Rayong, Prachinburi and Sa Kaeo), northern (Uttaradit), north-eastern (Nakhon Ratchasima), western (Kanchanaburi) and southern

(Chumphon) regions of Thailand, from January 2015 to December 2017. The samples were stored at -80ºC until tested.

Virus isolation and identification

The tissue samples, including brain, spinal cord, spleen and ovary, were homogenized in sterile phosphate-buffered saline (PBS) at a 10% suspension (w/v), centrifuged at 3000 x g for 10 minutes and filtered through 0.2 µm filters. The filtered suspensions were then inoculated into the allantoic cavities of 9-day-old specific pathogen-free (SPF) embryonated duck eggs. Following inoculation with tissue suspensions, most of the duck embryos died within 3-5 days post inoculation and the allantoic fluids were harvested upon embryo death or at 7 days post inoculation. Viral RNAs were extracted from tissue suspensions and allantoic fluids using QIAamp Viral RNA Mini Kit (Qiagen®, Hilden, Germany) following the manufacturer's instructions. The RNA samples were examined for the presence of DTMUV by RT-PCR using E and NS5 gene specific primers (Su et al., 2011; Thontiravong et al., 2015). The samples were also tested and confirmed to be negative for other common duck viruses, including avian influenza virus (AIV), Newcastle disease virus (NDV) and duck plague virus (DPV) (Li et al., 2006; Liu et al., 2007; Suarez et al., 2007).

DNA sequencing and genetic analysis

In this study, 61 DTMUV positive samples were selected based on location, type of duck raising and year of collection, and subjected to complete E gene sequencing. Primers for complete E gene sequencing were designed based on the E gene sequences of DTMUV available in the GenBank database. The primer sequences are available upon request. In addition, 53 DTMUV positive samples were selected based on location, type of duck raising and year of collection, and subjected for partial NS5 gene sequencing as described previously (Thontiravong et al., 2015). Due

to the largest gene of flaviviruses, partial NS5 gene is commonly used for phylogenetic analysis of flaviviruses, including DTMUV (Regato et al., 2008; Cao et al., 2011; Adiga, 2016; Monastiri et al., 2018). The nucleotide sequences were validated and assembled using SeqMan software v.5.03 (DNASTAR Inc.,Wisconsin, USA). The nucleotide sequences of the Thai DTMUVs characterized in this study were submitted to the GenBank database under the accession numbers MK276414 - MK276474 and MK276475 - MK276527 for E and NS5 genes, respectively.

To investigate the genetic characteristic of the recently circulating Thai DTMUVs, phylogenetic analysis was performed by comparing the complete E and partial NS5 gene sequences of the 2015-2017 Thai DTMUVs with those of the previously reported Chinese, Malaysian and Thai DTMUVs available in the GenBank database. The phylogenetic trees were generated by MEGA v.6.0 using neighbor-joining (NJ) and maximum-likelihood (ML) algorithms with 1000 replications of bootstrap (Tamura et al., 2013). To assess the nucleotide and amino acid identities, the nucleotide and amino acid sequences of the 2015-2017 Thai DTMUVs were aligned and compared with the previously reported Chinese, Malaysian, Thai DTMUVs and TMUV isolated from mosquitoes (MM1775) and chickens (Sitiawan virus) using MegAlign software v.5.03 (DNASTAR Inc., Wisconsin, USA). In addition, the complete amino acid sequence of the E protein of a novel cluster 3 DTMUV was aligned and compared with those of the previously reported cluster 1 and 2 DTMUVs using MEGA v.6.0 program.

To determine the evolution of DTMUVs circulating in Asia, the time-scaled phylogenetic analysis was conducted as previously described (Yu et al., 2013; Dai et al., 2015; Moureau et al., 2015; Ninvilai et al., 2018). The complete E gene sequences

of the 2015-2017 Thai DTMUVs and the previously reported Chinese DTMUVs, Malaysian DTMUVs, Thai DTMUVs, chicken and mosquito-derived TMUVs were aligned using Muscle v.3.6 (Edgar, 2004). Maximum clade credibility (MCC) tree of the complete E gene sequence was generated by BEAST 1.10.4 with Bayesian Markov Chain Monte Carlo (BMCMC) algorithm. Mean substitution rate of DTMUV was analyzed by using the strict clock model with coalescent exponential growth population and GTR with gamma 4 substitution model parameters (Drummond et al., 2002; Drummond et al., 2012). The Bayesian MCMC chain length was 30,000,000 generations with sampling every 3,000 generations. The effective sample size (ESS) value was assessed by using Tracer (v1.7.1) (Molecular evolution, phylogenetics and epidemiology, Edinburgh, Scotland, UK) (Rambaut et al., 2018). The complete E gene sequence analysis had ESS value greater than 200 suggesting minimal standard error. The MCC tree of each iteration was generated by TreeAnnotator software v.1.10.4 with 10% discarding of the chains as burn-in. The resulting MCC tree was visualized with FigTree software (v1.4.4) (Molecular evolution, phylogenetics and epidemiology, Edinburgh, Scotland, UK).

3.3 Results

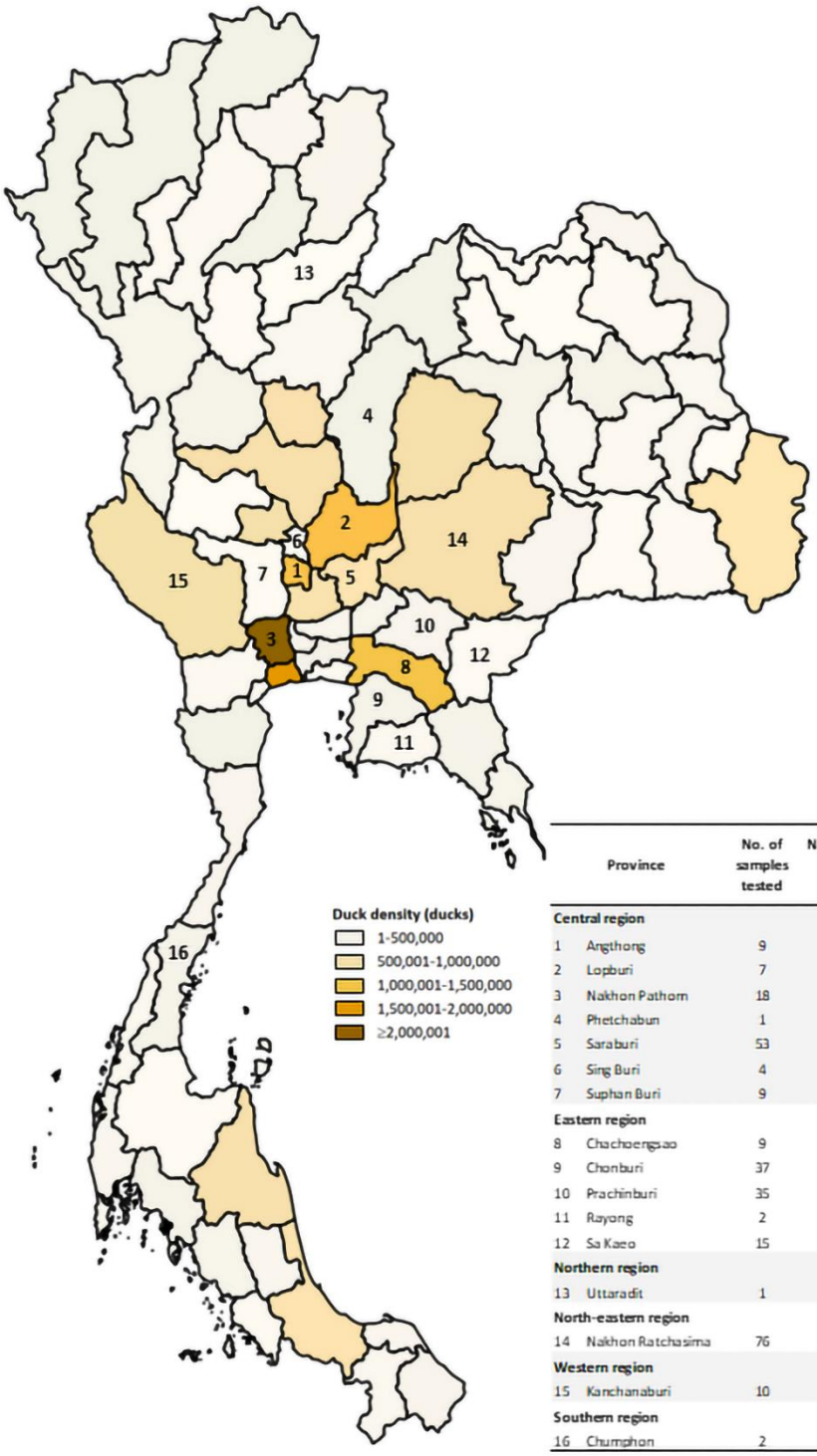
DTMUV isolation and identification

From 2015 to 2017, 288 clinical samples with suspected DTMUV infection were collected from 89 duck farms in 16 provinces located in duck raising areas of Thailand (Figure 9a). The DTMUV suspected ducks exhibited the clinical signs associated with severe neurological disorders and egg production losses. Clinical signs of neurological dysfunctions, including ataxia, imbalance movement and reluctance to walk, were usually observed in 3-5 weeks old broiler ducks. Meanwhile, a significant drop in egg production occurred in 35-45 weeks old layer ducks during

their production period. The morbidity and mortality rates of affected duck flocks ranged from 10%-20% and 5%-10%, respectively, correlating with secondary bacterial infection and farm management.

Of the 288 clinical samples from 89 duck farms, 65 samples (22.57%) and 34 duck farms (38.20%) were DTMUV positive. All of the DTMUV positive samples were negative for other common duck viruses, including NDV, AIV and DEV. Among 16 provinces tested, DTMUV positive samples were observed in 10 provinces located in the central (Lopburi (57.14%), Nakhon Pathom (22.22%), Saraburi (11.32%), Sing Buri (50.00%) and Suphan Buri (33.33%)), eastern (Chachoengsao (66.67%), Chonburi (18.92%) and Prachinburi (54.29%)), north-eastern (Nakhon Ratchasima (17.11%)) and southern (Chumphon (50.00%)) regions of Thailand (Figure 9a). In addition, DTMUV positive samples were found in every year tested, including 2015 (15.84%), 2016 (26.79%) and 2017 (25.33%). Although DTMUV positive samples were generally observed throughout the year, they were detected more frequently during rainy and winter seasons of Thailand (May-December) (Figure 9b).

(a)



	Province	No. of samples tested	No. of DTMUV positive samples	DTMUV positive rate (%)
Central region				
1	Angthong	9	0	0.00
2	Lopburi	7	4	57.14
3	Nakhon Pathom	18	4	22.22
4	Phetchabun	1	0	0.00
5	Saraburi	53	6	11.32
6	Sing Buri	4	2	50.00
7	Suphan Buri	9	3	33.33
Eastern region				
8	Chachoengsao	9	6	66.67
9	Chonburi	37	7	18.92
10	Prachinburi	35	19	54.29
11	Rayong	2	0	0.00
12	Sa Kaeo	15	0	0.00
Northern region				
13	Uttaradit	1	0	0.00
North-eastern region				
14	Nakhon Ratchasima	76	13	17.11
Western region				
15	Kanchanaburi	10	0	0.00
Southern region				
16	Chumphon	2	1	50.00

(b)

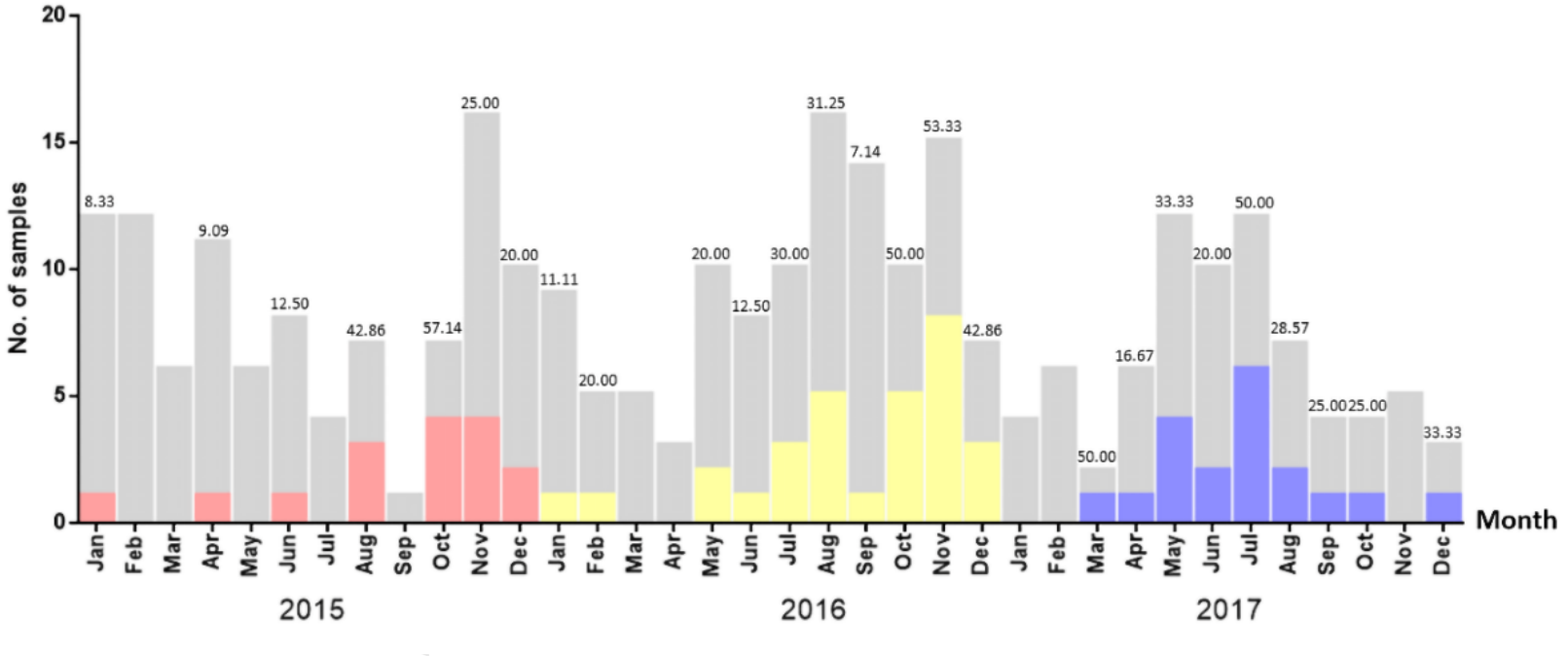


Figure 9 Details of DTMUV distribution in Thailand during 2015-2017 (Ninvilai et al., 2019).

(a) Geographic distribution of DTMUV in Thailand.

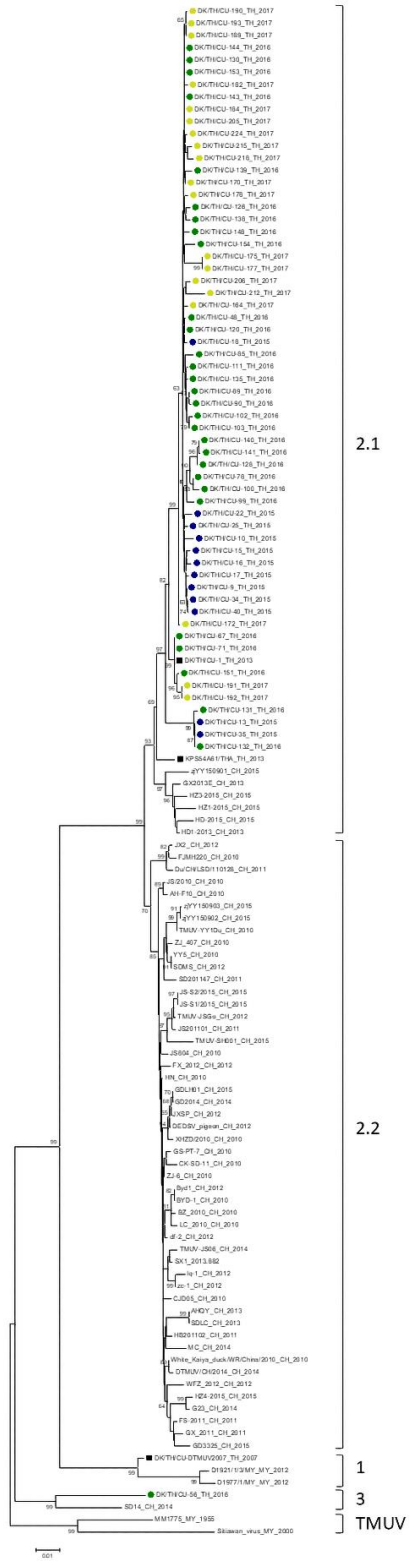
(b) Monthly distribution of DTMUV positive samples in Thailand during 2015-2017. Grey bars indicate the number of samples tested for DTMUV. Pink, yellow and purple bars indicate the number of DTMUV positive samples in 2015, 2016 and 2017, respectively. The numbers above each column show the percentage of DTMUV positive samples.

Phylogenetic and genetic analyses of DTMUV

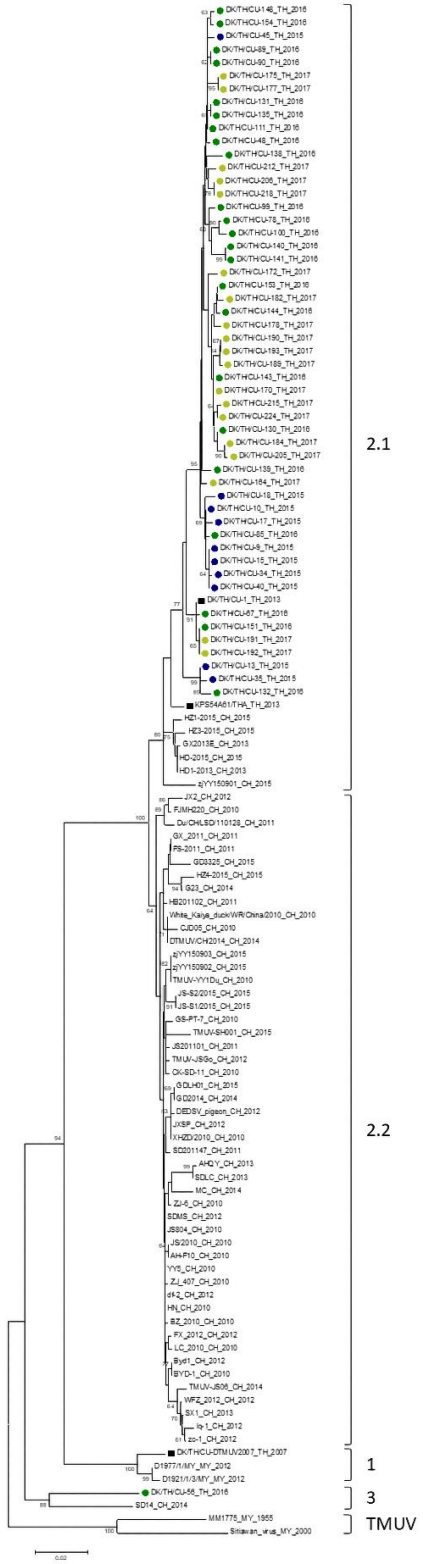
To genetically characterize the recently circulating Thai DTMUVs, 61 Thai DTMUVs isolated during 2015-2017 were subjected for complete E gene sequencing (Appendix 3). Phylogenetic analysis of the complete E gene using NJ, ML and BMCMC algorithms showed that most of the 2015-2017 Thai DTMUVs (n=60, 98.36%) were grouped within cluster 2 (subcluster 2.1) and most closely related to the 2013 Thai DTMUVs (97.3%-100% nucleotide identity) (Figure 10a, Table 3). The 2015-2017 Thai DTMUVs shared 97.1%-100% nucleotide identity to each other, while they shared only 95.7%-98.1% and 89%-90.2% nucleotide identity with Chinese and Malaysian DTMUVs, respectively (Table 3). It should be noted that the subcluster 2.1 contained two separated branches of the recent Thai DTMUVs identified during 2013-2017 and other DTMUVs isolated from China (Figure 10a). Interestingly, phylogenetic tree of the E genes also showed that one Thai DTMUV isolated in 2016 (DK/TH/CU-56) and a Chinese DTMUV strain SD14 was grouped together and formed a novel cluster, proposed namely the DTMUV cluster 3, which was distinctly separated from any of the previously reported DTMUV clusters (Figure 10a). This virus (DK/TH/CU-56) shared only 88.3%-89.4%, 88.6%-89.7% and 89.6%-89.9% nucleotide identity with other 2013-2017 Thai DTMUVs, Chinese DTMUVs and Malaysian DTMUVs, respectively (Table 3). It should be noted that the cluster 1 reported previously in 2007 were not detected in ducks in this study during 2015-2017. Analyses of NJ, ML and MCC trees of the partial NS5 gene of the 2015-2017 Thai DTMUVs displayed similar findings with those of E genes (Figure 10b; Appendix 4). Overall, our findings based on the phylogenetic analysis of the E and NS5 genes suggested that DTMUVs were classified into three distinct clusters, cluster 1, cluster 2 (2.1 and 2.2) and a novel cluster, cluster 3. The cluster 1 was predominantly circulated in Malaysia, while subcluster 2.2 was the predominant cluster commonly circulating in China (Figure 11). In Thailand, 3 clusters of DTMUV were circulated in ducks during 2007-2017, including

cluster 1 (2007 Thai DTMUV), subcluster 2.1 (2013-2017 Thai DTMUV) and a novel cluster 3 (a 2016 Thai DTMUV). Among these clusters, subcluster 2.1 was the predominant cluster circulating in duck populations in Thailand during 2015-2017 (Figure 11). It is interesting to note that although different clinical signs were observed in broiler and layer ducks in Thailand, the genetic characteristics of DTMUVs isolated from these different breeds of ducks were similar and mostly grouped within subcluster 2.1 (Figure 10; Appendix 3).

(a)



(b)



(c)

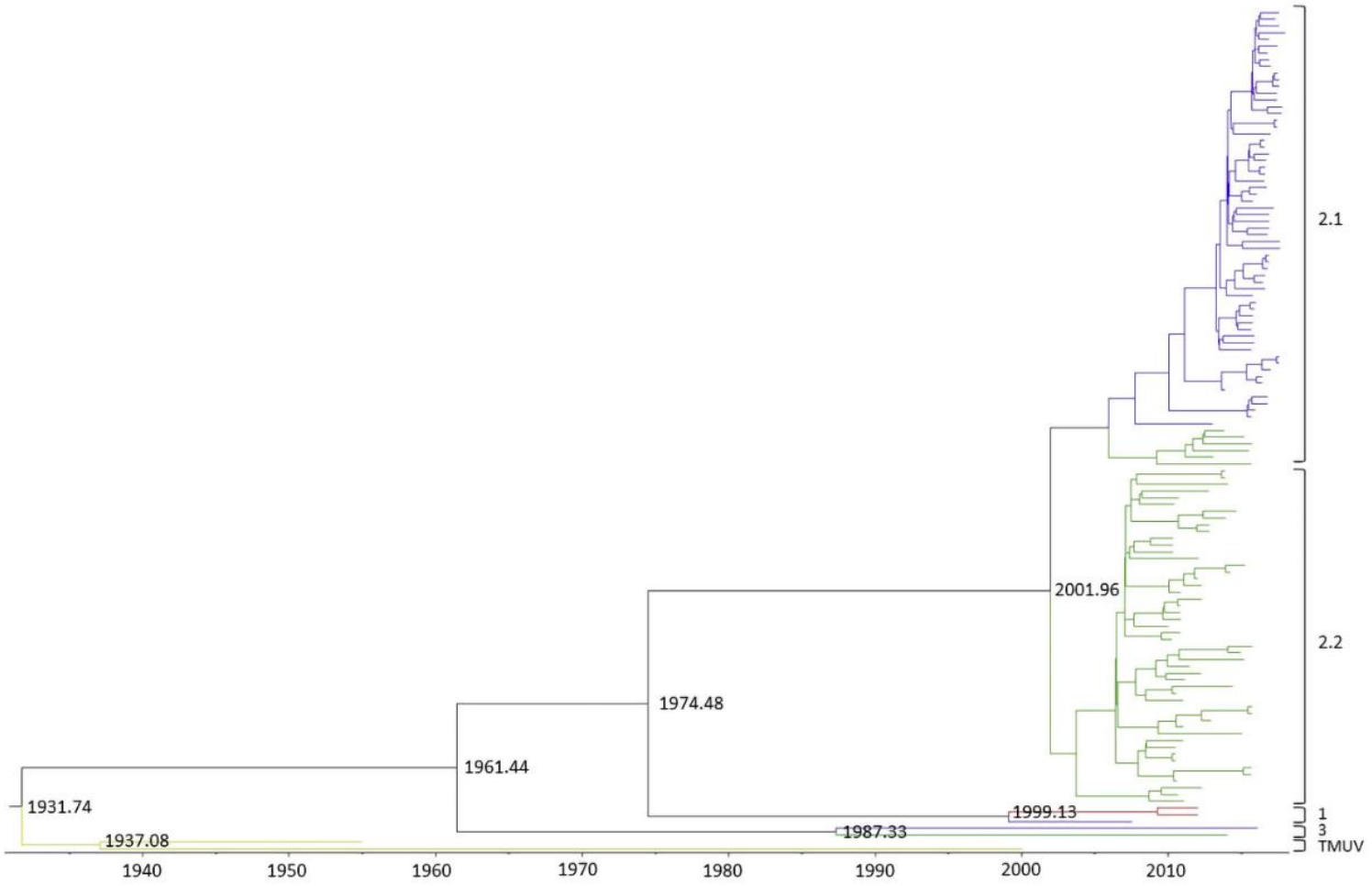

2.1
2.2
1
3
TMUV
2001.96
1974.48
1961.44
1931.74
1937.08
1999.13
1987.33
1940
1950
1960
1970
1980
1990
2000
2010

Figure 10 Phylogenetic and evolutionary analyses of DTMUV. The phylogenetic trees were constructed using neighbor-joining (NJ) algorithm based on the nucleotide sequences of the complete E (1,503 bp) (Ninvilai et al., 2019)

(a) and partial NS5 (750 bp) (b) genes of the 2015-2017 Thai DTMUVs and selected reference strains of DTMUV and TMUV isolated from China, Malaysia and Thailand. The countries and year of detection of DTMUVs are labeled behind the strain names. Dark blue, green and yellow circles indicate the 2015, 2016 and 2017 Thai DTMUVs, respectively, while black squares indicate the 2007 and 2013 Thai DTMUVs. Similar results were observed when applying the maximum-likehood (ML) and Bayesian Markov Chain Monte Carlo (BMCMC) algorithms. (c) The time-scaled Bayesian maximum clade credibility (MCC) tree of the complete E gene sequences of DTMUV was generated by BEAST 1.8 with BMCMC algorithm. The MCC tree representing the estimated timing of DTMUV divergence from their ancestor is provided at each node. Four distinct clusters/subclusters of DTMUV, including 1, 2.1, 2.2 and 3, and TMUV strains are shown. Thai, Chinese and Malaysian DTMUVs are labeled by blue, green and red, respectively, while TMUV strains are labeled by yellow.

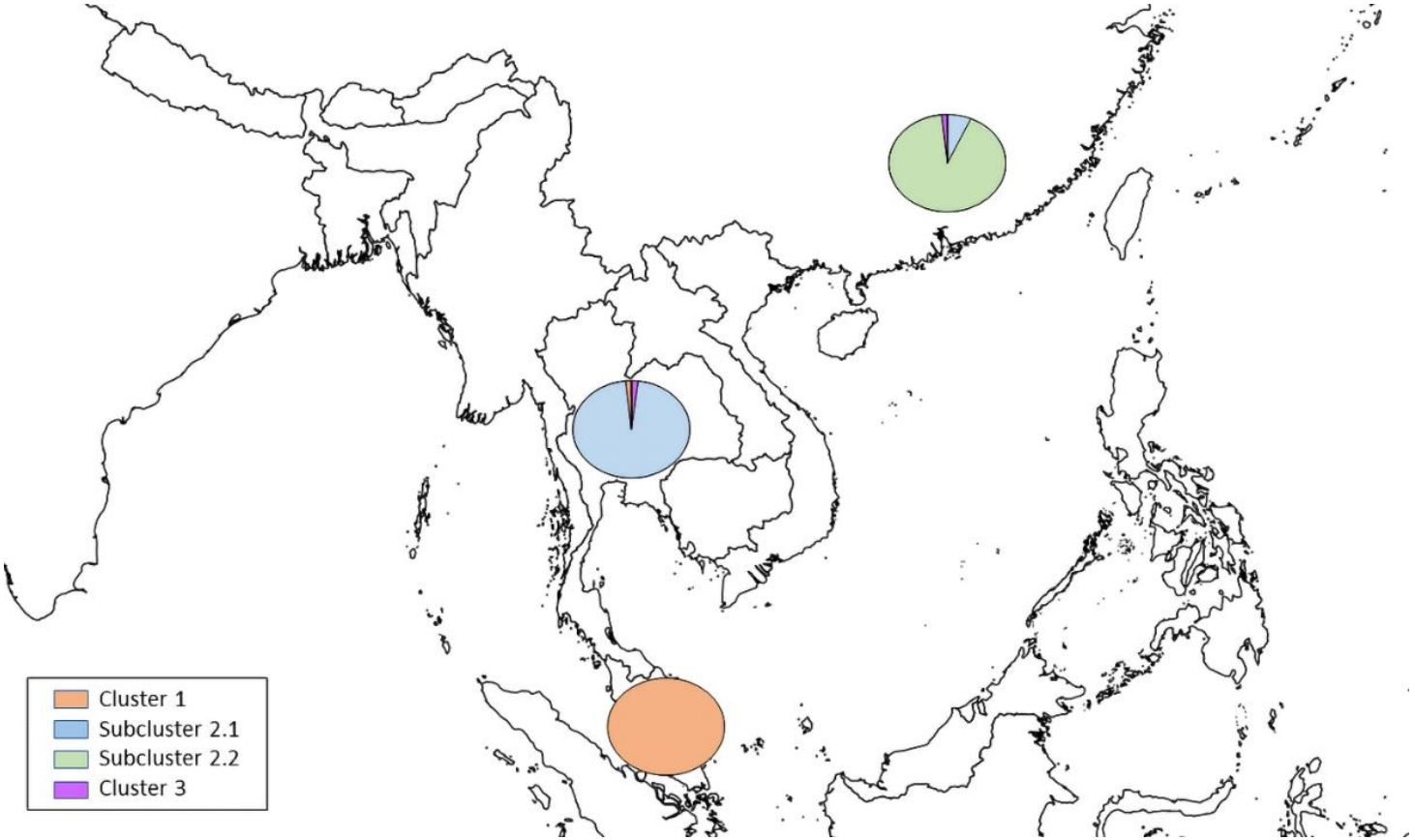


Figure 11 Geographic distribution of DTMUV clusters circulating in Asia during 2007-2017. The DTMUV cluster distribution was summarized using country-specific pie-chart whose slide size is proportional to the frequency of cluster (Ninvilai et al., 2019)

Table 3 Comparison of the nucleotide and amino acid identities of the complete E sequences of the 2015-2017 Thai DTMUVs with reference DTMUVs and TMUVs isolated from mosquitoes (MM1775) and chickens (Sitiawan virus) (Ninvilai et al., 2019).

Reference DTMUVs	Accession No.	Host	Location	Year of collection	% Nucleotide identity		% Amino acid identity	
					2015-2017 Thai DTMUVs (cluster 2.1)	2016 Thai DTMUV (DK/TH/CU-56) (cluster 3)	2015-2017 Thai DTMUVs (cluster 2.1)	2016 Thai DTMUV (DK/TH/CU-56) (cluster 3)
Cluster 1								
DK/TH/CU-DTMUV2007	MF621927	Duck	Thailand	2007	91.4-92.3	90.2	97.2-98.2	96.2
D1977/1/MY	KX097989	Duck	Malaysia	2012	89-90	89.9	96.4-97.2	95.6
D1921/1/3/MY	KX097990	Duck	Malaysia	2012	89.2-90.2	89.6	96.2-97	95.6
Cluster 2.1								
DK/TH/CU-1	KR061333	Duck	Thailand	2013	98.3-100	89.4	99.2-100	95.6
KPS54A61	KF573582	Duck	Thailand	2013	97.3-98.5	89.1	98.8-99.8	95.6
GX2013E	KM275940	Duck	China	2013	96.8-98.1	89.1	98.6-99.6	95.4
HD-2015	KX686572	Duck	China	2015	96.2-97.5	88.8	98.2-99.2	95
HZ1-2015	KX686570	Duck	China	2015	96.1-97.5	88.7	98.2-99.2	95
HZ3-2015	KX686579	Duck	China	2015	96.5-97.7	88.8	98.2-99.2	95
zjYY150901	MF522174	Duck	China	2015	96.5-97.7	88.6	98.2-99.2	95

Cluster 2.2

JS804	JF895923	Goose	China	2010	96.6-97.7	89.4	98.2-99	95.4
BYD-1	JF312912	Duck	China	2010	96.3-97.4	89.7	98.6-99.4	95.8
YY5	JF270480	Duck	China	2010	96.4-97.5	89.5	98.4-99.2	95.6
CK-SD-11	JQ627862	Chicken	China	2010	96.3-97.3	89.7	98.2-99	95.8
GX_2011	KC990542	Duck	China	2011	96-97	89.3	98.2-99	95.8
FS-2011	KX686578	Duck	China	2011	96.2-97.3	89.4	98.4-99.2	95.6
df-2	KJ489355	Duck	China	2012	96.6-97.7	89.7	98.6-99.4	95.6
byd1	JQ920420	Duck	China	2012	96.3-97.4	89.7	98.6-99.4	95.8
JSGo	AB917090	Goose	China	2012	96.3-97.5	89.7	98.6-99.4	96
DEDSV strain pigeon	JQ920425	Pigeon	China	2012	96.5-97.5	89.4	98.6-99.4	95.8
AHQY	KJ740748	Duck	China	2013	95.8-96.8	89.1	98.6-99.4	95.8
SX1	KM066945	Chicken	China	2013	96.4-97.5	89.4	98-98.8	95.2
G23	KT239021	Goose	China	2014	95.7-96.9	89.2	98.6-99.4	96.2
JS06	KR869106	Chicken	China	2014	96.2-97.3	89.4	97.8-98.6	95
GD2014	KU323595	Duck	China	2014	96.4-97.5	89.5	98.4-99.2	95.6
DTMUV/CH/2014	KP096415	Duck	China	2014	96.3-97.4	89.6	98.6-99.4	95.8
GDLH01	KT824876	Duck	China	2015	96.4-97.5	89.5	98.4-99.2	95.6
SH001	KP742476	Duck	China	2015	95.7-96.7	89.2	98.2-99	95.8
HZ4-2015	KX686571	Duck	China	2015	95.7-96.9	89	98.6-99.4	96.2

Cluster 3								
DK/TH/CU-56	MK276427	Duck	Thailand	2016	89.4-88.3	100	94.8-95.6	100
SD14	MH748542	Duck	China	2014	89-90	93.9	94.6-95.4	95.2
MM1775	JX477685	*Culex tritaeniorhynchus*	Malaysia	1955	88-88.6	89.6	96-96.8	97.4
Sitiawan virus	JX477686	Chicken	Malaysia	2000	86.2-86.9	87.8	96-96.8	97.2

To further investigate the characteristic of a novel cluster 3 virus (DK/TH/CU-56), amino acid sequence of the E protein of DK/TH/CU-56 was aligned and compared with those of the DTMUV cluster 1 and 2 using MEGA v.6.0 program. Amino acid mutation analysis showed that a novel cluster 3 virus, DK/TH/CU-56, carried 9 amino acid substitutions in the E protein that differed from the previously reported cluster 1 and 2, most of which located in the domain I (DI), followed by DIII and DII (Figure 12). It is noted that this novel virus contained a serine at position 156 in the E protein, which was reported to be critical for efficient replication and transmissibility of DTMUV in ducks (Yan et al., 2018). In addition, our findings also revealed that 19 amino acid mutations in the E protein were unique to each cluster, indicating that cluster 1, 2 and 3 had their specific different amino acid characteristics (Figure 12). This finding also supported the phylogenetic analysis result, in which DTMUVs were divided into three different clusters.

To determine the evolution of DTMUVs circulating in Asia, the time-scaled phylogenetic analysis based on the complete E gene sequence of DTMUVs and TMUVs identified during 1955-2017 was conducted. The mean nucleotide substitution rate of the E protein sequence of DTMUVs and TMUVs isolated during 1955-2017 was estimated to be 1.507×10^{-3} substitution/site/year with a 95% highest posterior density (HPD) interval of 1.183×10^{-3} - 1.887×10^{-3} substitution/site/year. In addition, a time-scaled Bayesian MCC tree based on the E gene sequence revealed that TMUV emerged in Asia around 1931 (95% HPD = 1915.4-1946.4), when DTMUV diverged from TMUV. Subsequently, DTMUV cluster 3 diverged from DTMUV cluster 1 and 2 around 1961 (95% HPD =1946.8-1976.8) and DTMUV cluster 2 diverged from DTMUV cluster 1 around 1974 (95% HPD = 1963.2-1985.4). DTMUV cluster 2 was further divided into 2 subclusters, 2.1 and 2.2, in 2002 (95% HPD = 1998.5-2005) and became the predominant cluster circulating in ducks in Asia (Figure 10c)

	DI	DII	DI	DII	DI	DIII
	1-50	51-132	133-197	197-278	279-300	301-415

Cluster	Strain name	Accession No.	Envelope																		
			2	52	72	83	89	149	150	157	180	185	236	277	312	332	373	390	391	394	487
1	DK/TH/CU-DTMUV2007_TH_2007	MF621927	S	D	S	S	D	A	S	A	L	S	K	S	A	S	V	K	G	R	V
	D192/1/3/MY_MY_2012	KX097990	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	N	.	K	.
	D1977/1/MY_MY_2012	KX097989	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	N	.	K	.
2.1	DK/TH/CU-1_TH_2013	KR061333	.	E	.	P	E	.	.	.	M	T	.	N	V	T	.	.	.	.	A
	KPS54A61/TH_TH_2013	KF573582	.	E	.	P	E	.	.	.	M	T	.	N	V	T	.	.	.	.	.
	HD-2015_CH_2015	KX686572	.	E	.	P	E	.	.	.	M	T	.	N	V	T	.	.	.	.	.
	HZ1-2015_CH_2015	KX686570	.	E	.	P	E	.	.	.	M	T	.	N	V	T	.	.	.	.	.
	DK/TH/CU-18_TH_2015	MK276420	.	E	.	P	E	.	.	.	M	T	.	N	V	T	.	.	.	.	.
	DK/TH/CU-130_TH_2016	MK276442	.	E	.	P	E	.	.	.	M	T	.	N	V	T	.	.	.	.	.
	DK/TH/CU-175_TH_2017	MK276459	.	E	.	P	E	.	.	.	M	T	.	N	V	T	.	.	.	.	.
2.2	BYD-1_CH_2010	JF312912	.	E	.	P	E	.	.	.	M	T	.	.	V	T	.	.	.	.	A
	ZJ-6_CH_2010	JF459991	.	E	.	P	E	.	.	.	M	T	.	.	V	T	.	.	.	.	A
	FS-2011_CH_2011	KX686578	.	E	.	P	E	.	.	.	M	T	.	.	V	T	.	.	.	.	A
	JSGo_CH_2012	AB917090	.	E	.	P	E	.	.	.	M	T	.	.	V	T	.	.	.	.	A
	AHQY_CH_2013	KJ740748	.	E	.	P	E	.	.	.	M	T	.	.	V	T	.	.	.	.	A
	GD2014_CH_2014	KU323595	.	E	.	P	E	.	.	.	M	T	.	.	V	T	.	.	.	.	A
	GDLH01_CH_2015	KT824876	.	E	.	P	E	.	.	.	M	T	.	.	V	T	.	.	.	.	A
3	DH/TH/CU-56_TH_2016	MK276427	N	E	P	P	.	T	N	V	.	A	R	.	.	.	.	.	E	K	A

amino acid substitutions unique to cluster 1 DTMUVs
amino acid substitutions unique to cluster 1 Malaysian DTMUVs
amino acid substitutions unique to cluster 2 DTMUVs
amino acid substitution unique to subcluster 2.1 DTMUVs
amino acid substitutions unique to cluster 2 and cluster 3 DTMUVs
amino acid substitution unique to subcluster 2.2 and cluster 3 DTMUVs
amino acid substitutions unique to cluster 3 DTMUV

Figure 12 Signature amino acid substitutions across the E protein sequences between a novel DTMUV cluster 3 (DK/TH/CU-56) and other reference strains of DTMUV cluster 1 and 2 (Ninvilai et al., 2019).

3.4 Discussion

Since the first report in 2013, DTMUV infected cases have been increasingly detected in several duck farms in Thailand. However, no information is available on the genetic characteristic of DTMUVs recently circulating in ducks in Thailand. The objective of this study was to investigate the geographic distribution and genetic characteristic of DTMUVs circulating in Thailand during 2015-2017. Our results demonstrated that DTMUV was extensively distributed in duck raising areas of Thailand. Our findings also revealed that 3 clusters of DTMUV, including cluster 1, subcluster 2.1 and a novel cluster 3, were found to be circulated in ducks in Thailand during 2007-2017, indicating the high genetic diversity of DTMUV in Thailand. Among these 3 clusters, subcluster 2.1 was the predominant cluster associated with recent DTMUV outbreaks in Thailand. In addition, we also identified a novel distinct cluster of DTMUV, the DTMUV cluster 3, in ducks in Thailand. To the best of our knowledge, this is the first study reporting a novel DTMUV cluster 3 in ducks.

Our epidemiological data of DTMUV in Thailand during 2015-2017 revealed that DTMUV had wider distribution in Thailand than previously reported since the virus could be detected in some provinces, including Chachoengsao, Chumphon and Nakhon Pathom, where the DTMUV outbreaks have never been reported (Thontiravong et al., 2015; Ninvilai et al., 2018) This indicates the wide distribution and endemicity of DTMUV in ducks in Thailand. This finding is consistent to our recent study, which reported the widespread DTMUV infection in free-grazing ducks in Thailand (Tunterak et al., 2018). In addition, our results also showed that DTMUV were detected throughout the year during 2015-2017, indicating the continued circulation of DTMUV in ducks in Thailand. Interestingly, although a high rate of DTMUV detection was observed in the rainy season as reported previously, this virus

could be frequently detected in the winter season, when mosquito activity in Thailand is relatively low. Corresponding to this finding, a high seropositive rate of DTMUV was observed during the winter season in China (Li et al., 2015d). This suggests that, in addition to vector transmission route, non-vector transmission routes may also play an important role in the spread of DTMUV in Thailand. However, a low rate of DTMUV detection were observed in summer season, when the number of *Culex* mosquitoes in Thailand is lowest (our unpublished data). Since *Culex* mosquito is known to be a vector of DTMUV (O'Guinn et al., 2013), the lowest number of *Culex* mosquitoes in summer season might affect the rate of DTMUV detection. However, the factor that contributes to a low rate of DTMUV detection in summer season requires further investigation.

Genetic analysis demonstrated that DTMUVs circulating in Thailand could be divided into 3 clusters, including cluster 1 (2007 Thai DTMUV), subcluster 2.1 (2013-2017 Thai DTMUV) and a novel cluster 3 (a 2016 Thai DTMUV). Meanwhile, only 1 and 2 clusters of DTMUV were reported in Malaysia (cluster 1) and China (subcluster 2.1 and 2.2) (Dai et al., 2015; Ninvilai et al., 2018). Our findings suggest that DTMUVs circulating in Thailand showed a high level of genetic diversity when compared to those circulating in other two countries. Although 3 clusters of DTMUV were found to be circulated in Thailand, only 2 clusters (subcluster 2.1 and novel cluster 3) had been recently detected in ducks in Thailand during 2015-2017. Notably, subcluster 2.1 was the predominant cluster recently circulating in duck populations in Thailand, while cluster 1 and subcluster 2.2 were predominantly circulated in Malaysia and China, respectively (Dai et al., 2015; Ninvilai et al., 2018). This finding suggests the association between virus cluster and geographic location. Interestingly, a novel cluster of DTMUV, cluster 3, was first identified in this study. This novel cluster 3 was genetically different from any of the previously reported DTMUV clusters. Although

this novel virus was isolated from sick ducks, the pathogenicity and transmissibility of this virus remain unknown and require further investigation. In addition, genetic analysis revealed that this novel virus contained various amino acid changes in the E protein, particularly in DI and DIII, compared to the previously reported cluster 1 and 2. As reported previously, DI is responsible for the replication and transmissibility of DTMUV in ducks (Yan et al., 2018), while DIII is involved in host cell entry and the induction of virus-neutralizing antibodies (Mukhopadhyay et al., 2005; Lindenbach et al., 2007). Therefore, whether these amino acid changes contribute to virus pathogenicity, antigenic cross-reactivity among different clusters of DTMUV and other biological characteristics need to be further investigated.

The estimated evolutionary rate for the E gene of DTMUVs and TMUVs isolated during 1955-2017 was 1.507×10^{-3} substitution/site/year, which is the highest among previously observed in DTMUV (Yu et al., 2013; Dai et al., 2015; Ninvilai et al., 2018). This finding indicates that DTMUV had continually and rapidly evolved, possibly leading to the emergence of the novel virus variants with genotypic and phenotypic changes. Therefore, the monitoring of DTMUV in ducks and other species should be routinely conducted. In addtion, time-scaled Bayesian MCC tree revealed that TMUV first appeared in Asia in 1931, which is consistent to a previous report showing that the recent common ancestor of TMUV might have appeared before 1934 (Lei et al., 2017). This finding is supported by the first report of mosquito-derived TMUV in 1955 (Kono et al., 2000). Our result also showed that although DTMUV cluster 3 was recently identified in this study, it might have emerged earlier than DTMUV cluster 1 and 2.

In conclusion, our data demonstrated the circulation of different clusters of DTMUV and the presence of a novel DTMUV cluster in ducks in Thailand. Our findings indicate the high genetic diversity and continued evolution of DTMUV in ducks in Thailand as well as in Asia. This study provides useful information for DTMUV diagnostic and vaccine development and highlights the necessity of the routine DTMUV surveillance for early detection of emergence of novel DTMUV strains.

CHAPTER 4

Development and validation of a universal one-step RT-PCR assay for broad detection of duck Tembusu virus

Manuscript in preparation

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4.1 Introduction

Duck Tembusu virus (DTMUV) is an emerging flavivirus that causes severe neurological disorder and significant egg drop syndrome in ducks and some other avian species, including geese and chickens (Su et al., 2011; Yan et al., 2017) . DTMUV was first reported in China in 2010 (Su et al., 2011), and subsequently it was identified in Malaysia and Thailand (Homonnay et al., 2014; Chakritbudsabong et al., 2015; Thontiravong et al., 2015). At present, DTMUV is widely distributed and becomes one of the highly contagious viral disease in ducks in Asia, causing significant economic losses to the duck producing industry. However, no commercial vaccine is currently available for DTMUV infection. Strict biosecurity and the culling of DTMUV infected ducks are the only effective measures to prevent and control DTMUV infection. Therefore, accurate, early and rapid diagnosis of DTMUV infection is crucial for effective control and prevention of DTMUV.

DTMUV is classified as a new genotype of Tembusu virus (TMUV) belonging to the genus *Flavivirus* of the Family *Flaviviridae* (Cao et al., 2011; Su et al., 2011). Its genome consists of a positive-sense single stranded RNA, which encodes three structural proteins (capsid (C), pre-membrane protein (prM) and envelope (E)) and

seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) (Liu et al., 2012). Among these proteins, NS5 protein is highly conserved and is frequently used as a target for pan flavivirus diagnostic assays (Chao et al., 2007; Regato et al., 2008; Johnson et al., 2010; Patel et al., 2013; Adiga, 2016; Monastiri et al., 2018). Like most single-stranded RNA viruses, DTMUV has a high nucleotide substitution rate, resulting in a high level of DTMUV genetic diversity (Dai et al., 2015; Ninvilai et al., 2018; Ninvilai et al., 2019). Since its first emergence in 2010, several clusters of DTMUV, including cluster 1, cluster 2 and a novel cluster 3, have increasingly been identified (Ninvilai et al., 2019). These highlight the need for improved and novel pan detection assays in order to keep up-to-date with the circulating clusters of DTMUV.

Besides the "gold standard" virus isolation, molecular diagnostic assays, including conventional reverse transcription polymerase chain reaction (RT-PCR) and real-time RT-PCR, are widely used to detect DTMUV due to rapid, high sensitivity and specificity (Yan et al., 2011a; Liu et al., 2013; Tang et al., 2015b). Several RT-PCR and real-time RT-PCR assays have previously been developed for the detection of DTMUV; however, none of them have been validated against all clusters of DTMUV (Yan et al., 2011a; Tang et al., 2012; Liu et al., 2013; Tang et al., 2015b). In addition, the assay suitable for broad detection of all clusters of DTMUV has not yet been established. Therefore, this study aims to develop and validate a universal one-step RT-PCR assay targeting highly conserved region of NS5 gene for broad detection of all DTMUV clusters. A one-step RT-PCR was chosen since it is simple, cost saving and practical for field sample screening in local diagnostic laboratories in remote areas.

4.2 Materials and methods

Viruses and samples

Reference strains of all three DTMUV clusters, including DK/TH/CU-DTMUV2007 (DTMUV cluster 1), DK/TH/CU-1 (DTMUV cluster 2) and DK/TH/CU-56 (DTMUV cluster 3), were used for RT-PCR assay validation. All three isolates were propagated in 9-day-old embryonated duck eggs (Ninvilai et al., 2019). The viruses were harvested, clarified by centrifugation, and the 50% embryo lethal doses (ELD_{50}) of viruses were calculated by the Reed and Muench method (Reed and Muench, 1938). A panel of common duck viruses and other related flaviviruses, including avian influenza virus (AIV) subtype H5N1, Newcastle disease virus (NDV), egg drop syndrome virus (EDSV), duck plague virus (DPV), Japanese encephalitis virus (JEV), dengue virus (DENV) and West Nile virus (WNV), were used to determine the specificity of the assay. NDV and AIV were obtained from the Center of Excellence for Emerging and Re-emerging Infectious Diseases in Animals (CUEIDAs), Chulalongkorn University, Thailand. EDSV and DEV were kindly provided by Animal Health Diagnostic Center, CPF (Thailand) Public Company Limited. JEV and WNV were received from the Animal Vector-Borne Disease Research Group, Department of Pathology, Faculty of Veterinary Science, Chulalongkorn University. DENV was kindly provided by Assoc. Prof. Padet Siriyasatien, Department of Parasitology, Faculty of Medicine, Chulalongkorn University.

To validate the newly developed RT-PCR assay, known DTMUV positive (n=60) and negative control (n=60) tissues (brain and spleen), oropharyngeal and cloacal swab samples obtained from experimentally DTMUV infected and negative control ducks from our previous study were tested by this assay (Ninvilai et al, unpublished data). In addition, a total of 100 clinical samples, including brain, spinal

cord, spleen and ovary, obtained from DTMUV suspected cases in Thailand during 2015- 2017 were also tested. Viral RNAs were extracted from each virus isolate and samples using QIAamp Viral RNA Mini Kit (Qiagen®, Hilden, Germany) following the manufacturer's instructions and were stored at -80ºC until used.

Primers design

In order to design an RT-PCR assay capable of detecting all clusters of DTMUV, all complete genome and NS5 sequences of DTMUV available on the GenBank database (3 DTMUV cluster 1, 101 DTMUV cluster 2 and 2 DTMUV cluster 3) were used. A multiple sequence alignment was carried out using the ClustalW algorithm implemented in the BioEdit software package version 7.2. Primers were selected from conserved regions in the NS5 gene of all known DTMUV sequences available on the GenBank database. Primers were chosen and analyzed using the OLIGO primer design software (version 9.1). To avoid non-specific cross-reactions with other related flaviviruses, primers were analyzed *in silico* by BLAST analysis on NCBI and by multiple sequence alignment using the ClustalW program with sequences of other related flaviviruses in Japanese encephalitis virus (JEV) group (Usutu virus (USUV), Murray Valley encephalitis virus (MVEV), JEV, West Nile virus 1 (WNV-1), West Nile virus 2 (WNV-2) and St. Louis encephalitis virus (SLEV)) and Ntaya group (Rocio virus (ROCV), Israel turkey meningoencephalomyelitis virus (ITV), Bagaza virus (BAGV), Ilheus virus (ILHV), Ntaya virus, Tembusu virus (TMUV) and Sitiawan virus (STWV)).

Universal one-step RT-PCR assay

Universal RT-PCR assay was carried out in a single-step reaction using the AccessQuick RT-PCR System (Promega, Madison, WI) on SimpliAmp Thermal Cycler (Applied Biosystems, Foster City, CA). Primers were used at a final concentration of 0.5 μM each. A combination of 3.0 μL of RNA sample with a reaction mixture containing 12.5 μL of AccessQuick Master Mix, 5 U of avian myeloblastosis virus (AMV) reverse transcriptase and DEPC-treated water was used in a final volume of 25 μL. The amplification condition consisted of a reverse transcription at 48°C for 45 min, an initial denaturation at 94°C for 3 min, 40 cycles of amplification (94°C for 30 sec, 58°C for 30 sec and 72°C for 30 sec) and a final extension at 72°C for 10 min. A total of 10 μL of PCR product was analyzed on a 1.5% agarose gel stained with SYBR® Safe DNA gel stain (Invitrogen, Thermo Fisher Scientific, Waltham, MA) and visualized by ultraviolet transilluminator.

Sensitivity and specificity analyses

The analytical sensitivity of the newly developed universal RT-PCR assay was determined by using RNA extracted from ten-fold serial dilutions of stock virus (DTMUV, DK/TH/CU-1) ranging from 10^5 to 10^{-5} ELD_{50}/ml in triplicate. In addition, the specificity of this assay was evaluated using RNA or DNA extracted from all three clusters of DTMUV (DTMUV cluster 1, 2 and 3), a panel of common duck viruses (AIV subtype H5N1, NDV, EDSV and DPV) and other related flaviviruses (JEV, DENV and WNV).

Validation of universal RT-PCR assay with experimental and field clinical samples

The performance of the newly developed universal RT-PCR assay was initially evaluated with 60 known DTMUV positive and 60 known DTMUV negative control tissue and swab samples obtained from experimentally DTMUV infected and negative control ducks from our previous study (Ninvilai et al, unpublished data). In addition, the newly developed universal RT-PCR assay was further validated with 100 clinical samples obtained from DTMUV suspected cases in Thailand during 2015-2017. All samples were also tested by the previously reported E-specific RT-PCR assay (Su et al., 2011). All DTMUV positive samples were subjected to DNA sequencing to confirm the presence of DTMUV in the samples as described previously (Thontiravong et al., 2015).

4.3 Results

Primer analysis

To broadly detect all clusters of DTMUV, a newly developed universal RT-PCR assay was designed by targeting the conserved region in NS5 gene of DTMUV. BLAST analysis of designed primers showed high homology with target regions of all DTMUV sequences available on the GenBank database (data not shown). *In silico* analysis also demonstrated that designed primers were specific to all DTMUV strains originating from different regions of Asia (100% coverage) without cross-reactivity with other related flaviviruses in JEV group and Ntaya group (Table 4). These *in silico* findings indicated that the newly developed universal RT-PCR assay was potentially highly specific to all clusters of DTMUV. Sequences and genome positions of designed primers are shown in Table 4.

Table 4 Primers used in the universal one-step RT-PCR assay

Primer	Sequence (5'-3')	Position	Tm (°C)	Amplicon (bp)	% Coverage[a] (matched/total[b])
NS5_F8911	ACCTGCATTTACAACATGATGG	8911-8932	62	249	100% (104/104)
NS5_R9140	GCGTACATAAGTCCTCCTTCTT	9149-9160	64		100% (104/104)

[a] Percentage of DTMUV strain coverage

[b] Number of matched DTMUV sequences/total DTMUV sequences used in the analysis

Specificity and sensitivity evaluation

To evaluate the specificity of the newly developed universal RT-PCR assay, all three clusters of DTMUV (DTMUV cluster 1, 2 and 3), a panel of common duck viruses (AIV subtype H5N1, NDV, EDSV and DPV) and other related flaviviruses (JEV, DENV and WNV) were tested. Corresponding to the findings from *in silico* analysis, the newly developed universal RT-PCR assay could specifically detect all clusters of DTMUV without cross-reactions with common duck viruses and other related flaviviruses (Figure 13a). The nucleotide identity of all amplicons was confirmed by DNA sequencing (data not shown). These results indicated the broad range detection of DTMUV and the high specificity of the newly developed universal RT-PCR assay.

To determine the sensitivity of the newly developed universal RT-PCR assay, RNAs from ten-fold serial dilutions of stock virus (DTMUV, DK/TH/CU-1) ranging from 10^{5} to 10^{-5} ELD_{50}/ml were tested in triplicate. The newly developed universal RT-PCR assay was able to detect DTMUV as low as 10^{-3} or 0.001 ELD_{50}/ml (Figure 13b).

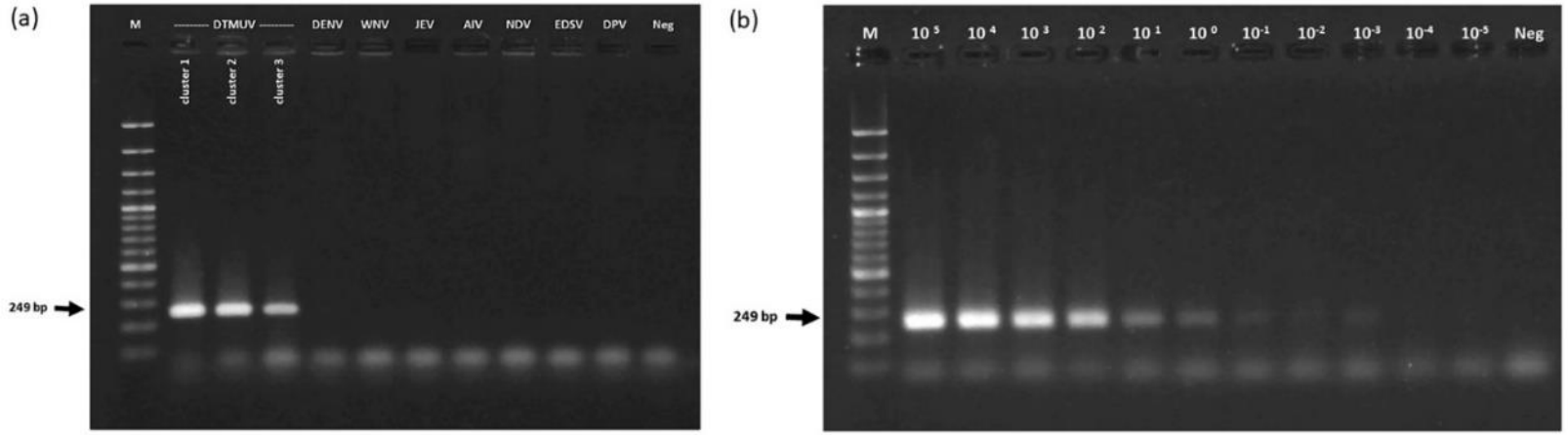

Figure 13 Specificity and sensitivity of the universal one-step RT-PCR assay for DTMUV detection.

(a) Specificity of the universal one-step RT-PCR assay for DTMUV detection. The PCR amplicons could be specifically observed when three clusters of DTMUV were tested. No amplification products were detected when common duck viruses (avian influenza virus (AIV), Newcastle disease virus (NDV), egg drop syndrome virus (EDSV) and duck plague virus (DPV)) and other related flaviviruses (Japanese encephalitis virus (JEV), dengue viruses (DENV) and West Nile virus (WNV) were tested.

(b) Sensitivity of the universal one-step RT-PCR assay for DTMUV detection. Ten-fold serial dilutions of stock virus were indicated on the top of the lanes (10^{5}-10^{-5} embryo lethal dose (ELD_{50})/ml). The DNA bands were visualized at viral RNA dilutions as low as 10^{-5} or 0.001 ELD_{50}/ml. Lane M: 100-bp plus ladder; lane Neg: negative control; arrow: the expected amplification product of 249 bp.

Validation of universal RT-PCR assay with experimental and field clinical samples

To validate the newly developed universal RT-PCR assay, the assay was initially tested with 60 known DTMUV positive and 60 known DTMUV negative control tissue and swab samples obtained from experimentally DTMUV infected and negative control ducks from our previous study (Ninvilai et al, unpublished data). The results showed that the newly developed universal RT-PCR assay could successfully detect DTMUV in all samples from ducks experimentally infected with DTMUV, whereas no DTMUV was detected in samples from negative control ducks (Table 5). In addition, the newly developed universal RT-PCR assay was further validated by comparing with the previously reported E-specific RT-PCR assay on the detection of 100 clinical samples obtained from DTMUV suspected cases in Thailand during 2015-2017 (Su et al., 2011; Ninvilai et al., 2019). The results showed that the newly developed universal RT-PCR assay gave a higher DTMUV positive rate (36%) than the previously reported RT-PCR targeting E gene (30%) when tested with field clinical samples (Table 5). It should be noted that the samples that tested positive by E-specific RT-PCR assay were also positive by universal RT-PCR assay. The presence of DTMUV in all DTMUV positive samples were confirmed by DNA sequencing (data not shown). Overall, these findings indicated the newly developed universal RT-PCR assay could be used to screen DTMUV in clinical samples.

Table 5 Detection of DTMUV in duck experimental and clinical samples using the newly developed universal RT-PCR assay and previously reported E-specific RT-PCR assay (Su et al., 2011).

Sample	Type of sample	No. of positive/No. of tested samples (%)	
		Universal RT-PCR	E-specific RT-PCR
Experimental samples			
- DTMUV infected ducks	Tissue	30/30 (100)	30/30 (100)
	Swab	30/30 (100)	30/30 (100)
	Total	**60/60 (100)**	**60/60 (100)**
- Negative control ducks	Tissue	0/30 (0)	0/30 (0)
	Swab	0/30 (0)	0/30 (0)
	Total	**0/60 (0)**	**0/60 (0)**
Clinical samples	Tissue	36/100 (36)	30/100 (30)
	Total	**36/100 (36)**	**30/100 (30)**

4.4 Discussion

Since its emergence in 2010, several clusters of DTMUV have increasingly been identified, indicating a high level of DTMUV genetic diversity (Ninvilai et al., 2019). This highlights the need for novel broad detection assays in order to keep up-to-date with the circulating clusters of DTMUV. Rapid diagnostic assays which can broadly detect a wide range of DTMUV may facilitate accurate and early detection of infection, allowing the prompt implementation of control measures and thus preventing further outbreaks. Although rapid and sensitive DTMUV assays have been developed previously, none of them have been validated for detecting all clusters of DTMUV (Yan et al., 2011a; Tang et al., 2012; Liu et al., 2013; Tang et al., 2015b). In this study, a universal one-step RT-PCR assay was developed and validated for broad detection of all DTMUV clusters. Our results demonstrated that the newly developed universal RT-PCR assay was able to detect all clusters of DTMUV with high accuracy,

sensitivity and specificity. In addition, this assay is simple and utilizes a conventional PCR machine; thus, it is cost saving, requires minimal expertise, and can be easily conducted in local laboratories in remote areas.

In this study, the newly developed universal RT-PCR assay was designed based on the conserved region in the NS5 gene of DTMUV, which is known as the highly conserved gene of flaviviruses (Lim et al., 2015; Duan et al., 2017; Wang et al., 2017). As a result, this assay not only can detect all the currently known clusters of DTMUV, but also has a good potential of detecting novel emerging DTMUV strains. Accordingly, our results revealed that the newly developed universal RT-PCR assay was able to detect all three circulating clusters of DTMUV, including the recently identified cluster 3. The newly developed universal RT-PCR assay showed high specificity and no cross-reactions with common duck viruses and other related flaviviruses, suggesting that this assay could be used as DTMUV screening in avian species infected with those viruses without false-positive results. In addition, the newly developed universal RT-PCR assay could detect a low amount of DTMUV RNA with limit of detection of 0.001 ELD_{50}/ml, which was more sensitive than other previous reported RT-PCR assays (Yan et al., 2012). Although sensitivity of this assay was lower than the real-time RT-PCR assays (Tang et al., 2015b), the real-time RT-PCR assays required probes and an expensive real-time PCR instrument, which increase the cost of the method and limit their use in local diagnostic laboratories in remote areas. The performance of the newly developed universal RT-PCR assay was further evaluated by using experimental and field clinical samples. This assay could successfully detect DTMUV in all samples from ducks experimentally infected with DTMUV. In addition, our assay provided a higher DTMUV detection rate from field clinical samples when compared to the previously reported RT-PCR assay targeting E gene (Su et al., 2011). This finding may be caused by the high variability of E gene

(Lindenbach et al., 2007; Yu et al., 2013), which might affect the sensitivity of detection. This supported the fact that the assays targeting highly variable gene of viruses is not suitable for detecting a broad range of viruses, while the highly conserved NS5 gene is predominantly used for flavivirus screening (Johnson et al., 2010; Patel et al., 2013; Vina-Rodriguez et al., 2017). Overall, these findings indicated the effectiveness of the newly developed universal RT-PCR assay to detect DTMUV in clinical samples with high sensitivity, and its suitability for using as diagnostic tool of DTMUV infection in ducks

In conclusion, the newly developed universal RT-PCR assay provides a simple, accurate, specific and sensitive tool for broad detection of DTMUV clusters, which improve the efficiency of DTMUV screening method for routine detection and epidemiology surveillance of DTMUV. This assay may also be useful in finding and identifying novel DTMUV clusters in different avian species. This study highlights the importance of continuously validating the performance of currently used diagnostic assays against newly emerging strains to ensure the broad detection of the assay.

CHAPTER 5

Pathogenesis of Thai duck Tembusu virus in Cherry Valley ducks: the effect of age on susceptibility to infection

Manuscript in preparation

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5.1 Introduction

Duck Tembusu virus (DTMUV) is a causative agent of a new emerging viral disease in ducks and several avian species, including chickens and geese. This disease is primarily characterized by a significant decrease in egg production and severe neurological disorders, including ataxia and paralysis (Su et al., 2011). The morbidity rate was relatively high, while the mortality rate varied from 10%-30% depending on the farm management of affected flocks (Thontiravong et al., 2015). Currently, DTMUV has widely spread and become endemic in duck populations in duck producing areas of China, Malaysia and Thailand, resulting in significant economic losses in the duck producing industry. To establish the effective control and prevention strategies, a better understanding of the pathogenesis and host-pathogen interaction of this newly emerging virus is needed.

DTMUV is an enveloped, single-stranded, positive-sense RNA virus belonging to the Ntaya virus (NTAV) group in the genus *Flavivirus* of the Family *Flaviviridae* (Su et al., 2011; Liu et al., 2012; Homonnay et al., 2014). Its genome is composed of an open reading frame, which encodes three structural proteins (capsid (C), pre-membrane

(PrM), and envelope (E)) and seven non-structural (NS) proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5), flanked by the 5' and 3' untranslated regions (UTRs) (Liu et al., 2012). At present, DTMUV is genetically classified into three distinct clusters, including cluster 1, cluster 2 (2.1 and 2.2) and cluster 3 (Ninvilai et al., 2019). Cluster 1 and subcluster 2.2 were predominantly circulated in Malaysia and China, respectively, while subcluster 2.1 was the predominant cluster commonly circulating in Thailand (Ninvilai et al., 2019). This indicates a relatively high level of DTMUV genetic diversity, and the correlation between virus cluster and geographic location. However, whether different DTMUV clusters from different regions affected the pathogenicity in ducks remains unknown.

Recently, the pathogenicity of DTMUV have been evaluated in different ages of ducks (Sun et al., 2014; Li et al., 2015b; Lu et al., 2016). These studies demonstrated that although all duck ages were susceptible to DTMUV, DTMUV infection induces greater disease severity in young ducks than in adult ducks, indicating age-related susceptibility to DTMUV in ducks (Sun et al., 2014; Li et al., 2015b). However, only cluster 2.2 Chinese DTMUV strains were evaluated in these studies. Limited information is available on the pathogenesis of other DTMUV clusters circulating in other countries, particularly in Thailand. In the present study, the pathogenesis of a Thai DTMUV was investigated in three different ages of Cherry Valley ducks. This duck breed was chosen for pathogenesis evaluation in this study due to the extensive raising in Asia.

5.2 Materials and methods

Virus

The DTMUV strain DK/TH/CU-1 isolated from DTMUV infected ducks in Thailand in 2013 was used in this study (Thontiravong et al., 2015). This virus is known to circulate endemically among the duck populations in Thailand (Thontiravong et al., 2015; Ninvilai et al., 2019). The polyprotein gene sequence of DK/TH/CU-1 revealed that this virus belongs to DTMUV cluster 2.1, which is a predominant cluster of DTMUV circulating in duck populations in Thailand (Thontiravong et al., 2015; Ninvilai et al., 2018; Ninvilai et al., 2019). The DK/TH/CU-1 was propagated in the allantoic cavity of 9-day-old embryonated duck eggs as described previously (Thontiravong et al., 2015). The virus was harvested and the 50% embryo lethal dose (ELD_{50}) of virus was calculated by the following serial titration in embryonated duck eggs (Reed and Muench, 1938) . Virus propagation and handling were performed in a BSL-2 containment facility.

Animals

Animal experiment was approved and conducted in accordance with the ethical guidelines of the Chulalongkorn University Animal Care and Use Committee (approval number 1673012). Animal experiment was performed in the ABSL-2 containment facility at Chulalongkorn University Laboratory Animal Center (CULAC). Two hundred and twenty-five female Cherry Valley ducks (*Anas platyrhynchos*) were all obtained from a commercial hatchery and private research farm operated with high biosecurity standard and transferred to CULAC. All ducks were confirmed negative for DTMUV by virus-specific RT-PCR or PCR on oropharyngeal (OP) and cloacal (CL) swabs and negative for DTMUV antibody by serum neutralization (SN)

test (Su et al., 2011; Tunterak et al., 2018). All ducks were housed in the ABSL-2 containment facility at CULAC. Food and water were provided *ad libitum*.

Pathogenesis study in ducks

The pathogenesis of Thai DTMUV was evaluated in 1-, 4- and 27-week-old Cherry Valley ducks. In each age, 70 DTMUV-negative ducks were randomly allocated into two groups: DTMUV inoculated group (n=35), and negative control group (n=35). Ducks from DTMUV inoculated group were inoculated with 10^5 ELD_{50}/ml of Thai DTMUV virus (DK/TH/CU-1 strain) in a total volume of 1 ml via the intranasal (0.5 ml) and intramuscular (0.5 ml) routes. Ducks from negative control group were inoculated with allantoic fluid from specific pathogen free duck eggs in the same fashion. To evaluate virus transmission, 5 naïve ducks were introduced to each DTMUV infection group 1-day post inoculation (dpi) to allow direct contact. Clinical signs and body weight were monitored and recorded daily for 21 dpi. OP and CL swabs were collected from each duck on 1-7, 9, 11, 13, 15, 17, 19 and 21 dpi for virus shedding determination. Swabs were collected in viral transport medium (MEM containing 2% bovine serum albumin (BSA) and antibiotics) and stored at -80°C until tested. On 1, 3, 5, 7, 9, 14 and 21 dpi, 5 ducks from each group were bled for viremia detection and SN test, and subsequently humanly euthanized for gross examination. Tissue samples, including brain, spleen, bursa, thymus, heart, pancreas, liver, kidney, uterus and ovary were collected and stored at -80°C for viral load determination or fixed in 10% neutral buffered formalin for histopathological and immunohistochemical (IHC) examinations.

Histopathological and immunohistochemical examinations

Tissue samples were fixed in 10% neutral buffered formalin solution, embedded in paraffin, cut into 4 µm thickness and stained with hematoxylin and eosin (H&E) according to standard histopathological procedures. For the histological examination, the stained sections of lymphoid organs were evaluated and scored with the following criteria: 0 = no lesion; 1 = depletion <30%; 2 = depletion >30-60% and 3 = depletion >60% as previously described with some modifications (Salle et al., 2014). The sections of organs in nervous system were scored with the following criteria: 0 = no lesion, 1 = mild lesions/ mild perivascular cuffing with 2-3 layers of mononuclear cells and focal gliosis, 2 = moderate lesions/ moderate perivascular cuffing with 4-6 layers of mononuclear cells and multifocal gliosis) and 3 = severe lesions/severe perivascular cuffing with >6 layers of mononuclear cells and diffuse gliosis) as previously described with minor modification (Ecco et al., 2011). In addition, the histopathological score of other tissues were classified with the following criteria; 0 = no lesion, 1 = mild mononuclear cells infiltration, 2 = moderate congestion with moderate mononuclear cells infiltration and 3 = severe congestion and necrosis with severe mononuclear cells infiltration as previously described (Pantin-Jackwood et al., 2017).

In addition, the presence of flavivirus-specific antigens in the target tissues, including brain, spleen, thymus and bursa and heart, were evaluated by the immunohistochemical staining as previously described with some modifications (Palmieri et al., 2011; Ninvilai et al., 2018). Briefly, the paraffin sections were deparaffinized, rehydrated and pretreated in citrate buffer pH 6.0 with microwave oven, then immersed in 3% hydrogen peroxide solution (H_2O_2) at room temperature for 10 min for inactivation of endogenous peroxidase. Sections were blocked with 2% BSA for 30 min at 37ºC. Subsequently, virus staining was performed with a mouse

monoclonal antibody against Flavivirus antigen group, clone D1-4G2-4-15 (EMD Millipore Corporation, CA, USA) as the primary antibody diluted at 1:400, incubated overnight at 4ºC. The antibody of polymer system (Dako REAL™ Envision™/HRP, Rabbit/Mouse, Dako, Denmark) was used as the secondary antibody at dilution 1:50 and followed by addition of the substrate chromogen, 3, 3' diamino-benzidine tetrahydrochloride (DAB). Finally, the sections were counterstained with hematoxylin, dehydrated, mounted with permount and then observed under the light microscope. Each test included positive and negative controls.

RNA extraction and quantitative real-time RT-PCR (qRT-PCR)

DTMUV loads in OP and CL swabs, serum and tissue samples were determined by qRT-PCR targeting the conserved region in E gene of DTMUV as previously described with minor modifications (Yan et al., 2011a; Sun et al., 2014; Li et al., 2015b). In brief, viral RNA was extracted from OP and CL swabs, serum and tissue samples using QIAamp Viral RNA Mini Kit (Qiagen®, Hilden, Germany) and RNeasy Mini Kit (Qiagen®, Hilden, Germany) following the manufacturer's instructions. One µg of total RNA was subsequently converted to cDNA using random hexamers and an Improm-II reverse transcription system (Promega, Wisconsin, USA) following the manufacturer's instructions. The cDNA was used as a template for qPCR using a TaqMan™ Fast Advanced Master Mix (Applied Biosystems, TX, USA). The standard curve was generated from serial 10-fold dilutions of the recombinant plasmid containing DTMUV E gene. DTMUV loads of samples were calculated using the linear regression equation from the standard curve (Log_{10} vs Ct value) from the recombinant plasmid. The absolute quantitation of DTMUV from samples were normalized per 1 µg total RNA. Standards and samples were assayed in triplicate.

Serum neutralization (SN) test

The presence of DTMUV specific antibodies in serum samples was determined by SN test using BHK-21 cells and DK/TH/CU-1 as previously described with minor modifications (Tunterak et al., 2018). In brief, triplicate serial two-fold dilutions of heat inactivated sera were incubated with 100 $TCID_{50}$ of DK/TH/CU-1 for 1 h at 37°C. The virus-serum mixture was then added into 96-well plates containing BHK-21 cells. The cells were further incubated at 37°C and were checked for the presence of cytopathic effects (CPE) daily for 5 days. Reference DTMUV antibody positive and negative sera, uninfected BHK-21 cells and back titration of used virus served as controls. SN antibody titers were expressed as the reciprocal of the highest serum dilution capable of inhibiting CPE.

Statistical analysis

Data were reported as the mean ± standard deviation (SD). Differences in body weights between DTMUV inoculated group and negative control group in each age were determined with two-tailed Student's unpaired t test. Differences in virus shedding, viral loads in tissues and serum, SN antibody titers and histopathological lesion scores among three age groups were evaluated by analysis of variance (ANOVA) or a non-parametric Kruskal-Wallis test, respectively. All statistical analyses were performed using the GraphPad Prism 5.0 software (GraphPad Software Inc. La Jolla, CA). All $P < 0.05$ was considered statistically significant.

5.3 Results

Clinical assessment

All Thai DTMUV inoculated ducks in three age groups showed depression, loss of appetite, conjunctivitis and mild diarrhea as early as 2-3 dpi. Most ducks in 1-week (12/35) and 4 weeks (18/35) of age groups exhibited severe neurological signs, including ataxia, reluctance to walk, and progressive paralysis, starting at 4-5 dpi and continuing through 14-21 dpi, while mild neurological signs were observed in some ducks in 27 weeks of age group (7/25) from 6 dpi to 14 dpi (Figure 14). In 27 weeks of age group, decrease in egg production was observed during 2-21 dpi. From 3 to 21 dpi, DTMUV inoculated ducks in 1-week and 4 weeks of age groups had significantly lower mean body weights than negative control ducks within each age ($P<0.05$), while no significant difference in mean body weights was observed in 27 weeks of age group (Figure 15). From 5 to 14 dpi, 8 out of 35 ducks in both 1-week and 4 weeks of age groups died or were early euthanized as showing severe clinical signs. However, only 3 ducks in 27 weeks of age group found dead at 5 and 6 dpi. Overall, the morbidity rates of 1, 4 and 27 weeks of age groups were 34.29%, 51.42% and 17.14%, while the mortality rates were 22.86%, 22.86% and 8.57%, respectively. In addition, severe neurological signs were also observed in most contact ducks of three age groups at 6-7 days post contact (dpc). The morbidity rates of 1-, 4- and 27-week-old contact ducks were 60%, 100% and 20%, while the mortality rates were 40%, 60% and 20%, respectively. No clinical signs or mortality were observed in any of the negative control ducks during the 21 days of observation. Taken together, 1-week-old and 4-week-old ducks developed more severe and earlier neurological signs and had higher morbidity and mortality rates compared to 27-week-old ducks.

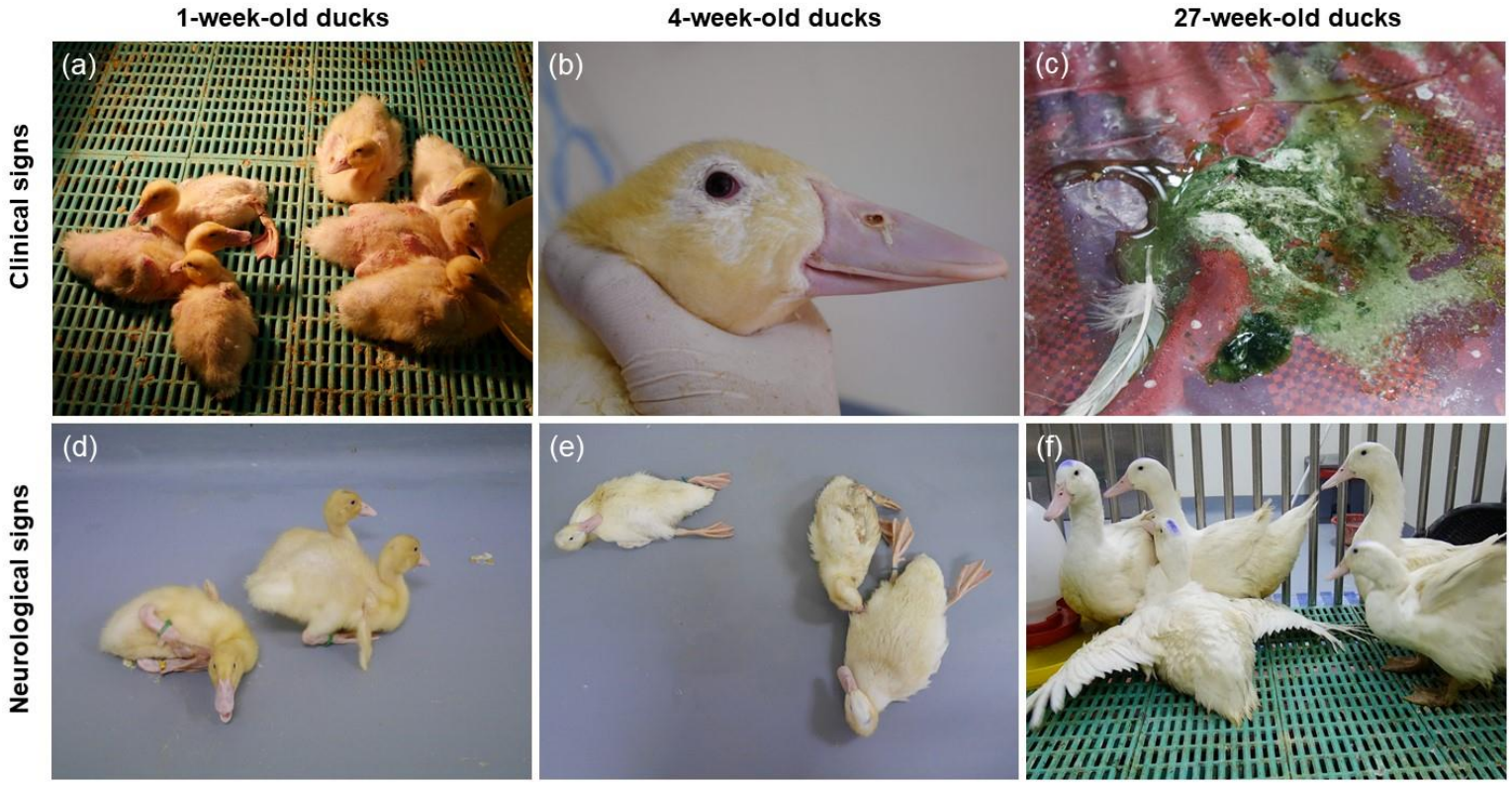

Figure 14 Clinical assessment of 1-, 4- and 27-week-old ducks inoculated with Thai DTMUV.

1- (a), 4- (b) and 27- (c) week-old ducks displayed clinical signs, including depression and conjunctivitis and diarrhea. Neurological signs, including ataxia, reluctance to walk, and progressive paralysis were observed in 1- (d), 4- (e) and 27- (f) week-old ducks.

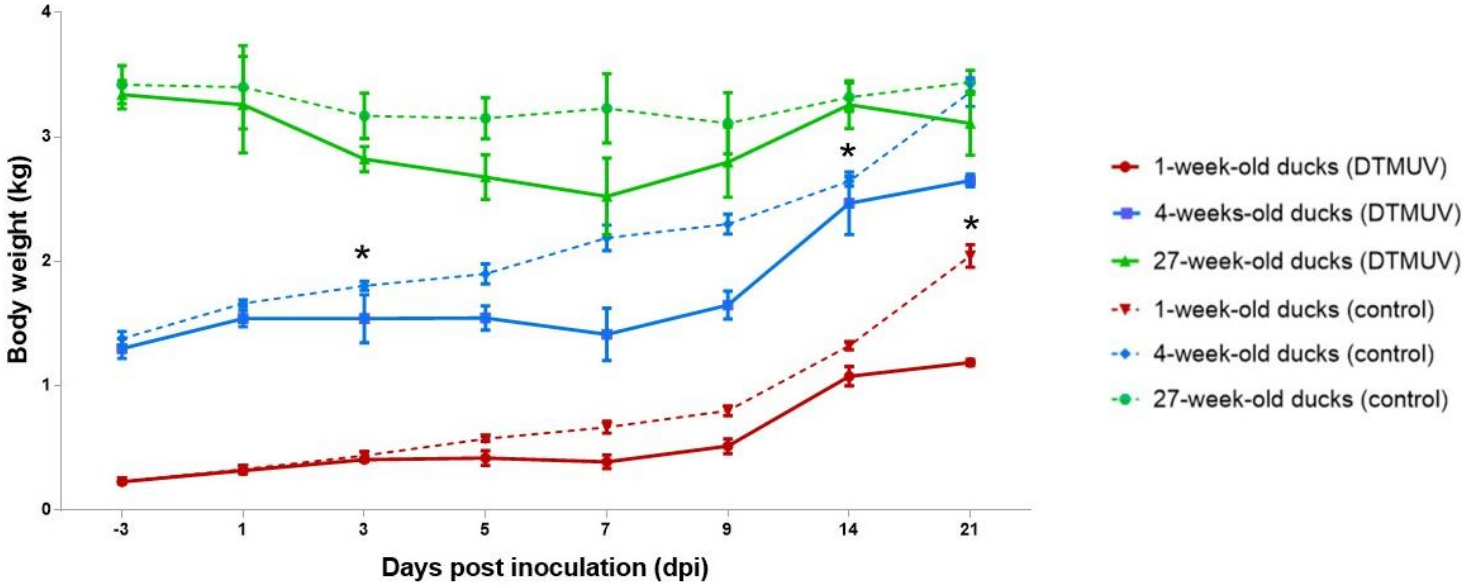

Figure 15 Mean body weights of DTMUV inoculated ducks in 1, 4 and 27 weeks of age groups compared to negative control ducks within each age.

Each data point represents the mean ± standard deviation (SD) of 5 ducks. Asterisks (*) indicate statistically significant difference between DTMUV inoculated and negative control groups within each age ($P < 0.05$, two-tailed Student's unpair t-test).

Gross and histopathological lesions

No gross lesions were observed in tissues from any of the negative control ducks. The most significant gross lesions in 1- and 4-week-old ducks were cerebral edema and meningeal congestion, and hemorrhages in myocardium, proventiculus and pancreas (Figure 16a, b, c and g). Major gross lesions of immune organs in these 2 age groups were swelling with petechial hemorrhage in thymus, marble spleen with splenomegaly and bursal atrophy (Figure 16d, e and f). In contrast, the prominent gross lesions in 27-week-old ducks were severe ovarian atrophy and ovarian hemorrhage (Figure 16h and i). In addition, mild meningeal congestion, splenomegaly, diffuse hemorrhage in pancreas and proventiculus were also detected. Only mild or no remarkable gross lesions were observed in other organs of 27 weeks old ducks. In general, the number of DTMUV inoculated ducks with gross lesions gradually increased until 7-9 dpi (Table 6). Overall, extent and degree of severity of the gross lesions in 1- and 4-week-old ducks appeared to be higher when compared to 27-week-old ducks (Table 6).

The major histopathological findings in DTMUV inoculated ducks in all groups were congestion, perivascular cuffing with mononuclear cells and necrosis of lymphoid cells in various organs. In central and peripheral nervous systems (CNS and PNS), the prominent histopathology were observed in cerebrum, hippocampus, thalamus, cerebellum, brainstem and nerve, which showed multifocal gliosis, dark neurons, neuronophagia and perivascular cuffing with mononuclear cells (Figure 17a). It should be noted that 1-week-old ducks showed histopathological lesions in brains as early as 3 dpi, which was earlier than other groups (Table 7). Overall, 1- and 4-weeks old ducks exhibited more severe histopathological lesions in brains than 27 weeks old ducks. In lymphoid tissues, severe lymphoid depletion, lymphocytic death and hemorrhage were observed in spleen, thymus and bursa as early as 1 dpi (Figure

17d, e and f). In addition, non-suppurative perivascular cuffing and mononuclear cells infiltration were mostly observed at perivascular area of various organs in all age groups, including myocardium, pancreas, liver and kidney. An increase in number of acinar cell and islet cell deaths in pancreas, single hepatic cell death and myocardial necrosis was also detected. For 27-week-old ducks, mononuclear cells infiltration in the interstitial tissues of oviduct and uterus, and parenchymatous zone of ovaries could be observed (Figure 17i). Overall, one-week old ducks inoculated with Thai DTMUV had significantly higher mean histopathological scores for cerebellum, spleen and heart than ducks in 4 and 27 weeks of age groups ($P<0.05$) (Table 7). No histopathological lesions were observed in any of the tested organs from negative control ducks. In addition, flavivirus antigens could be detected in all examined organs of DTMUV inoculated ducks from all groups, indicating the systemic infection of Thai DTMUV in three different ages of ducks (Figure 18). It is interesting to noted that flavivirus antigens were mainly observed in monocytes/macrophages in various organs of all age groups. This corresponded to a recent report demonstrating monocytes/macrophages as the key targets of DTMUV infection (Ma et al., 2019). Taken together, similar to the result of gross lesions, histopathological findings demonstrated that 1- and 4-weeks old ducks generally showed more severe lesions than 27 weeks old ducks.

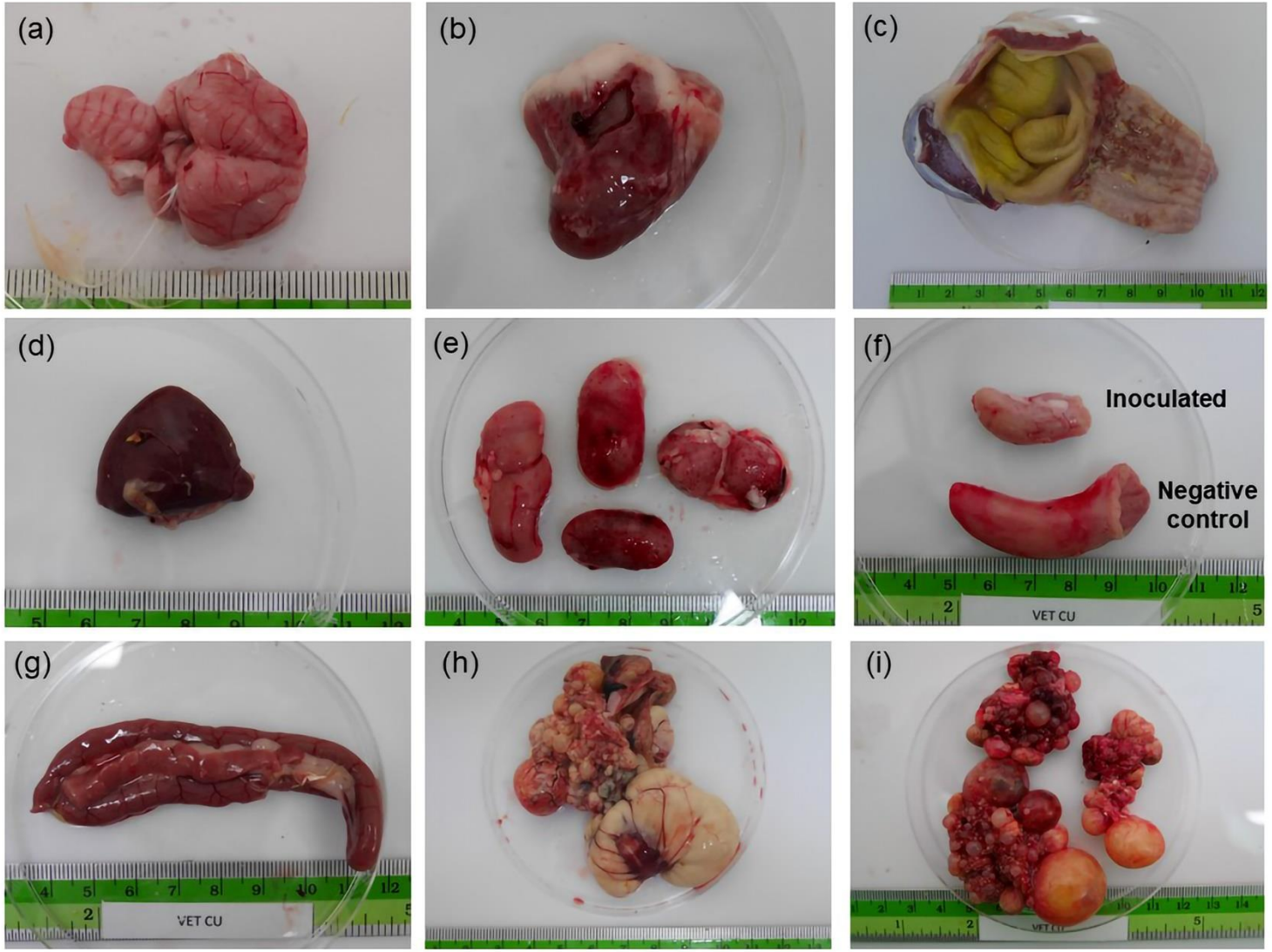
(a)
(b)
(c)
(d)
(e)
(f)
Inoculated
Negative control
VET CU
(g)
VET CU
(h)
(i)

Figure 16 Gross lesions of Thai DTMUV inoculated ducks in 1, 4 and 27 weeks of age groups.

(a) Brain: congestion and cerebral edema (4-week-old ducks, 14 dpi) **(b) Heart:** petechial hemorrhage in coronary fat, myocardial white streak and edema (4-week-old ducks, 7 dpi) **(c) Proventiculus:** patchy hemorrhage (4-week-old ducks, 7 dpi) **(d) Spleen:** splenomegaly (4 weeks old ducks, 14 dpi) **(e) Thymus:** diffuse pin point hemorrhage and edema (4-week-old ducks, 21 dpi) (**f) Bursa:** bursa atrophy compared with normal bursa (4-week-old ducks, 14 dpi) **(g) Pancreas:** hemorrhage and necrotic white foci (1-week-old ducks, 14 dpi) **(h)-(i) Ovaries:** ovarian atrophy and hemorrhage (27-week-old ducks, 5 and 21 dpi)

Table 6 Gross lesions of Thai DTMUV inoculated ducks in 1, 4 and 27 weeks of age groups.

Organ	Lesion	1-week-old ducks							4-week-old ducks							27-week-old ducks						
		1 dpi	3 dpi	5 dpi	7 dpi	9 dpi	14 dpi	21 dpi	1 dpi	3 dpi	5 dpi	7 dpi	9 dpi	14 dpi	21 dpi	1 dpi	3 dpi	5 dpi	7 dpi	9 dpi	14 dpi	21 dpi
Brain	Congestion	-[a] (0/5)[b]	+ (1/5)	++ (3/5)	++ (4/5)	+ (3/5)	+ (2/5)	+ (2/4)	- (0/5)	+ (2/5)	+ (3/5)	++ (4/5)	+ (2/5)	+ (2/3)	+ (1/3)	- (0/5)	- (0/5)	+ (2/5)	+ (2/5)	- (0/5)	- (0/4)	- (0/4)
Spleen	Splenomegaly	- (0/5)	++ (5/5)	++ (5/5)	++ (5/5)	++ (4/5)	+ (3/5)	+ (2/4)	- (0/5)	++ (4/5)	++ (5/5)	+ (5/5)	+ (4/5)	+ (3/3)	+ (2/3)	- (0/5)	++ (4/5)	++ (5/5)	+ (3/5)	++ (3/5)	+ (1/4)	+ (1/4)
Thymus	Hemorrhage	- (0/5)	++ (4/5)	++ (4/5)	++ (5/5)	+ (5/5)	+ (3/5)	+ (2/4)	- (0/5)	++ (4/5)	++ (4/5)	++ (5/5)	+ (3/5)	+ (2/3)	+ (2/3)	- (0/5)	ND[c]	ND	ND	ND	ND	ND
Bursa	Atrophy/hemorrhage	- (0/5)	+ (3/5)	+ (3/5)	+ (5/5)	+ (5/5)	+ (3/5)	+ (4/4)	- (0/5)	+ (2/5)	++ (4/5)	++ (5/5)	+ (3/5)	+ (3/3)	+ (3/3)	- (0/5)	ND	ND	ND	ND	ND	ND
Heart	Hemorrhage	- (0/5)	+ (3/5)	++ (3/5)	++ (4/5)	++ (4/5)	+ (2/5)	+ (1/4)	- (0/5)	+ (2/5)	+ (4/5)	++ (4/5)	++ (4/5)	++ (2/3)	+ (1/3)	- (0/5)	+ (2/5)	+ (3/5)	+ (2/5)	+ (2/5)	+ (1/4)	- (0/4)
Pancreas	Hemorrhage	- (0/5)	+ (2/5)	++ (3/5)	++ (4/5)	+ (3/5)	+ (1/5)	- (0/4)	- (0/5)	+ (1/5)	+ (2/5)	++ (3/5)	+ (3/5)	+ (1/3)	- (0/3)	- (0/5)	+ (1/5)	++ (2/5)	+ (2/5)	+ (1/5)	- (0/4)	- (0/4)
Liver	Hepatomegaly	- (0/5)	- (0/5)	+ (1/5)	+ (2/5)	- (0/5)	- (0/5)	- (0/4)	- (0/5)	+ (1/5)	+ (2/5)	+ (1/5)	- (0/5)	- (0/3)	- (0/3)	- (0/5)	- (0/5)	- (0/5)	- (0/5)	- (0/5)	- (0/4)	- (0/4)
Ovary	Atrophy/hemorrhage	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	- (0/5)	+ (5/5)	++ (5/5)	++ (5/5)	++ (4/5)	++ (4/4)	++ (4/4)

[a] The severity of gross lesions are shown as – (no lesion), + (mild), ++ (moderate) and +++ (severe).

[b] Number of ducks showing gross lesions / Number of DTMUV inoculated ducks

[c] Not determined (ND) since no organs exist in that age.

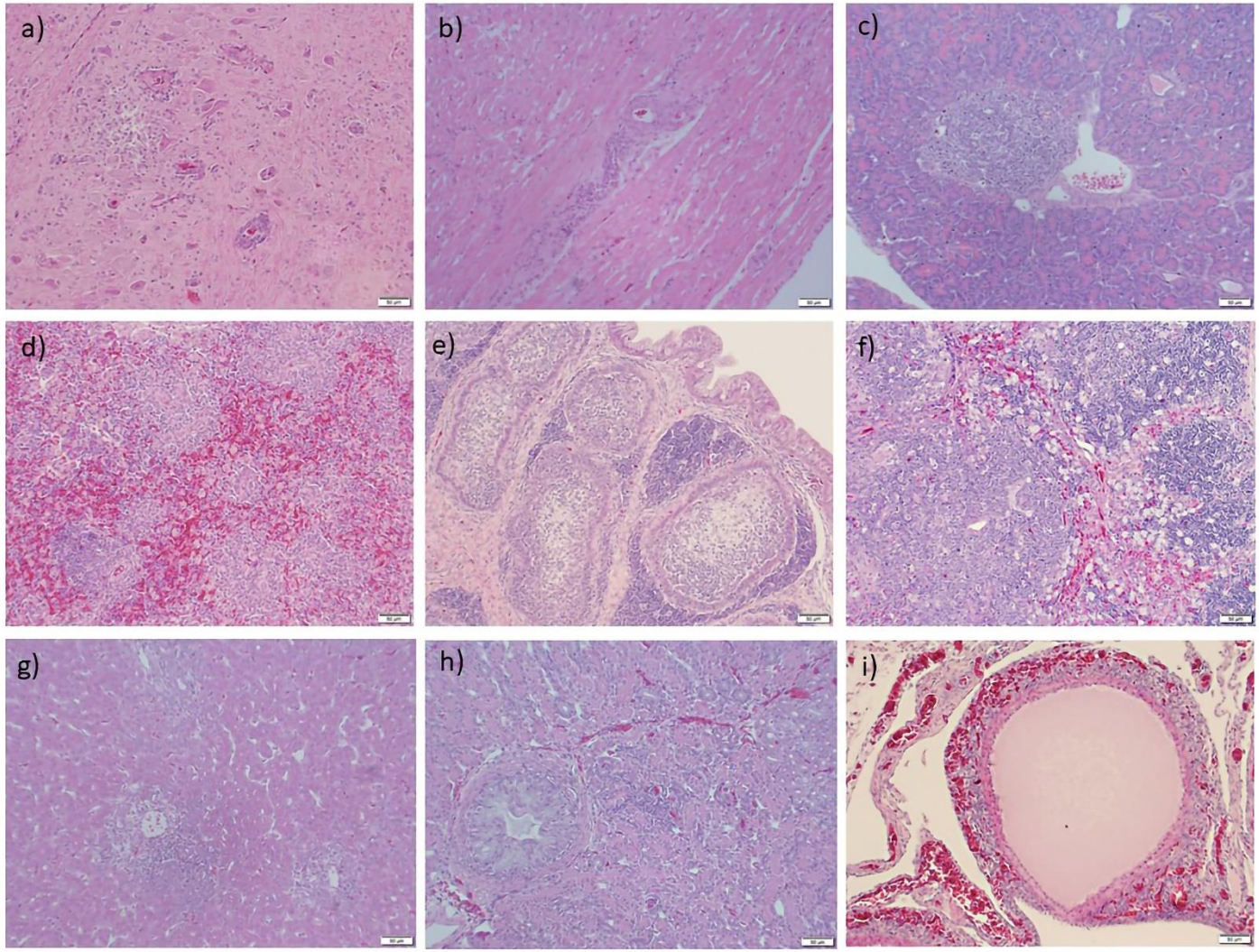
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Figure 17 Histopathological lesions of Thai DTMUV inoculated ducks in 1, 4 and 27 weeks of age groups. H&E stained tissue sections of various organs from different age groups.

(a) Brain: multifocal gliosis, dark neurons, neuronophagia and non-suppurative perivascular cuffing (4-week-old ducks, 7 dpi) **(b) Heart:** perivascular cuffing and infiltration by mononuclear cells observed mostly at perivascular area in myocardium (1-week-old ducks,7 dpi) **(c) Pancreas***:* mononuclear cells infiltration and newly lymphoid follicular formation (1-week-old ducks, 7 dpi) **(d) Spleen:** severe lymphoid depletion, lymphocytic death and marked congestion (1-week old-ducks, 7 dpi) **(e) Bursa:** severe lymphoid depletion and lymphocytic death (4-week-old ducks, 7 dpi) **(f) Thymus:** severe lymphoid depletion, lymphocytic death and hemorrhage (1-week-old ducks, 7 dpi) **(g) Liver:** mononuclear cells infiltration observed mostly at perivascular area (1-week-old ducks, 7 dpi) **(h) Kidney:** non-suppurative perivascular cuffing and mononuclear cells infiltration in interstitial tissues around renal tubules (1-week-old ducks, 7 dpi) **(i) Ovary:** mononuclear cells infiltration and hemorrhage in interstitial tissue of parenchymatous zone (27-week-old ducks, 7 dpi). Scale bars = 50 μm.

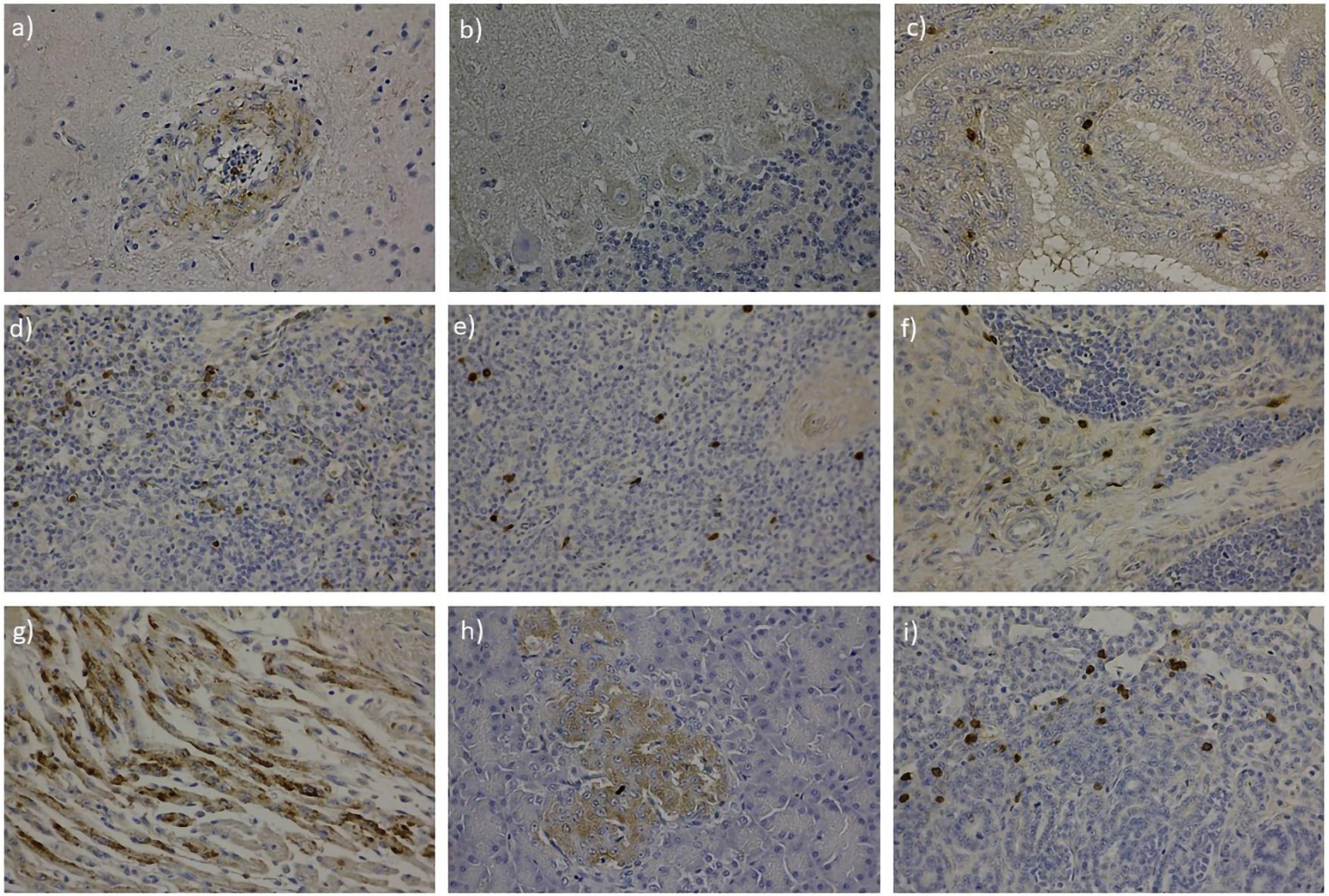
a)
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Figure 18 Immunohistochemical (IHC) staining of Thai DTMUV inoculated ducks in 1, 4 and 27 weeks of age groups.

(a) Positive flavivirus antigens in endothelial cells and mononuclear cells in blood vessels in brain (4-week-old ducks, 7 dpi). **(b)** Positive flavivirus antigens in Purkinje cells in cerebellum of 4-week-old duck, 21 dpi. **(c)** Positive flavivirus antigens in mononuclear cells in choroid plexus of 27-week-old duck, 14 dpi. **(d)-(f)** Positive flavivirus antigens in mononuclear cells in spleen, thymus and bursa of 4 week-old-duck, 14 dpi. **(g)** Positive flavivirus antigens in myocytes of 4 week-old-duck, 7 dpi. **(h)** Positive flavivirus antigens in acinar cells of pancreas of 4 week-old-duck, 21 dpi. **(i)** Positive flavivirus antigens in mononuclear cells in interstitial tissue of kidney of 1 week-old-duck, 7 dpi. Scale bars = 10 μm.

Table 7 Histopathological lesions of Thai DTMUV inoculated ducks in 1, 4 and 27 weeks of age groups. Mean histopathological lesion scores ± SD for 1-, 4- and 27-week-old ducks inoculated with Thai DTMUV.

Organ	1-week-old ducks							4-week-old ducks							27-week-old ducks						
	1 dpi	3 dpi	5 dpi	7 dpi	9 dpi	14 dpi	21 dpi	1 dpi	3 dpi	5 dpi	7 dpi	9 dpi	14 dpi	21 dpi	1 dpi	3 dpi	5 dpi	7 dpi	9 dpi	14 dpi	21 dpi
Brain																					
Cerebrum	0.0±0.0[a] (0/5)[b]	0.6±0.5 (3/5)	1.0±0.0 (5/5)	1.6±0.5 (5/5)	2.4±0.9 (5/5)	1.5±1.0 (4/5)	1.0±0.0 (4/4)	0.0±0.0 (0/5)	0.0±0.0 (0/5)	1.2±0.4 (5/5)	1.8±0.4 (5/5)	1.8±0.8 (5/5)	1.7±0.6 (3/3)	1.0±1.0 (2/3)	0.0±0.0 (0/5)	0.2±0.4 (1/5)	1.0±0.0 (5/5)	1.0±0.7 (4/5)	2.0±0.0 (5/5)	1.0±0.0 (4/4)	1.0±0.8 (4/4)
Hippocampus	0.0±0.0 (0/5)	0.2±0.4 (1/5)	0.6±0.5 (3/5)	1.4±0.5 (5/5)	2.0±1.2 (4/5)	1.0±1.2 (3/5)	0.3±0.5 (1/4)	0.0±0.0 (0/5)	0.0±0.0 (0/5)	0.2±0.4 (1/5)	1.2±1.0 (3/5)	2.0±0.7 (5/5)	1.7±0.6 (3/3)	0.3±0.6 (1/3)	0.0±0.0 (0/5)	0.0±0.0 (0/5)	0.8±0.4 (5/5)	1.0±0.7 (4/5)	1.8±0.4 (5/5)	1.3±0.5 (4/4)	1.0±0.8 (2/4)
Thalamus	0.0±0.0 (0/5)	0.8±0.8 (3/5)	0.6±0.5 (3/5)	1.4±0.5 (5/5)	2.2±1.3 (4/5)	1.4±1.1 (3/5)	0.0±0.0 (0/4)	0.0±0.0 (0/5)	0.0±0.0 (0/5)	1.0±0.7 (4/5)	2.2±0.4 (5/5)	2.0±0.7 (5/5)	1.7±0.6 (3/3)	1.0±1.0 (2/3)	0.0±0.0 (0/5)	0.0±0.0 (0/5)	1.6±0.9 (5/5)	1.4±0.5 (5/5)	1.6±0.5 (5/5)	1.5±0.6 (4/4)	1.3±0.5 (3/4)
Brainstem	0.0±0.0 (0/5)	1.0±0.0* (5/5)	1.0±0.0 (5/5)	1.6±0.6 (5/5)	2.6±0.9 (5/5)	1.4±0.9 (5/5)	1.0±0.0 (4/4)	0.0±0.0 (0/5)	0.0±0.0 (0/5)	1.2±0.8 (4/5)	2.2±0.8 (5/5)	2.2±0.4 (5/5)	1.8±0.5 (3/3)	1.3±0.6 (2/3)	0.0±0.0 (0/5)	0.0±0.0 (0/5)	1.8±0.4 (5/5)	1.4±0.5 (5/5)	1.6±0.5 (5/5)	1.8±1.0 (4/4)	1.5±1.0 (4/4)
Cerebellum	0.0±0.0 (0/5)	0.6±0.5 (3/5)	0.6±0.5 (3/5)	1.6±0.5 (5/5)	2.8±0.4* (5/5)	1.4±0.9 (5/5)	1.0±0.8 (4/4)	0.0±0.0 (0/5)	0.4±0.5 (2/5)	1.6±0.5 (5/5)	2.2±0.8 (5/5)	2.2±0.4 (5/5)	1.7±0.6 (3/3)	1.3±0.6 (3/3)	0.0±0.0 (0/5)	0.0±0.0 (0/5)	1.0±0.7 (5/5)	1.4±0.5 (5/5)	1.6±0.5 (5/5)	2.3±0.5 (4/4)	1.8±0.5 (4/4)
Spleen	1.0±0.0 (5/5)	1.6±0.5 (5/5)	2.8±0.4* (5/5)	2.6±0.5* (5/5)	1.7±0.3 (5/5)	1.2±0.3 (5/5)	1.3±0.5 (4/4)	1.0±0.0 (5/5)	1.3±0.4 (5/5)	1.0±0.0 (5/5)	1.5±0.5 (5/5)	1.2±0.3 (5/5)	1.5±0.5 (3/3)	1.2±0.3 (3/3)	1.0±0.0 (5/5)	2.0±0.0 (5/5)	1.5±0.5 (5/5)	1.3±0.4 (5/5)	1.4±0.5 (5/5)	1.8±-/3 (4/4)	1.1±0.3 (4/4)
Thymus	1.0±0.0 (5/5)	2.0±0.0 (5/5)	2.6±0.5 (5/5)	2.8±0.4 (5/5)	1.6±0.5 (5/5)	1.4±0.5 (5/5)	1.5±0.6 (4/4)	1.6±0.5 (5/5)	2.0±0.0 (5/5)	2.4±0.5 (5/5)	1.8±0.8 (5/5)	1.2±0.4 (5/5)	1.7±0.6 (3/3)	2.0±0.0 (3/3)	ND[c]	ND	ND	ND	ND	ND	ND
Bursa	1.4±0.5 (5/5)	2.0±0.0 (5/5)	2.2±0.4 (5/5)	2.4±0.5 (5/5)	1.8±0.4 (5/5)	1.6±0.5 (5/5)	1.8±0.5 (4/4)	1.8±0.4 (5/5)	2.0±0.0 (5/5)	2.4±0.5 (5/5)	1.8±0.8 (5/5)	1.2±0.4 (5/5)	1.7±0.6 (3/3)	2.0±0.0 (3/3)	ND	ND	ND	ND	ND	ND	ND
Heart	0.0±0.0 (0/5)	0.4±0.4 (3/5)	1.2±0.4 (5/5)	1.6±0.5* (5/5)	2.1±0.7 (5/5)	1.2±0.4 (5/5)	1.0±0.0 (4/4)	0.0±0.0 (0/5)	0.2±0.4 (1/5)	1.2±0.4 (5/5)	1.0±0.0 (5/5)	1.8±0.4 (5/5)	1.7±1.2 (3/3)	0.8±0.3 (3/3)	0.0±0.0 (0/5)	0.8±0.4 (4/5)	0.8±0.4 (5/5)	0.8±0.4 (5/5)	1.0±0.0 (5/5)	0.9±0.3 (4/4)	1.1±0.3 (4/4)
Pancreas	0.0±0.0 (0/5)	0.9±0.7 (4/5)	2.8±0.4* (5/5)	1.2±0.4 (5/5)	1.4±0.5 (5/5)	1.4±0.5 (5/5)	1.75±0.5 (4/4)	0.0±0.0 (0/5)	0.9±0.5 (4/5)	1.6±0.5 (5/5)	0.8±0.4 (5/5)	1.4±0.5 (5/5)	1.6±0.6 (3/3)	2.0±1.0 (3/3)	0.0±0.0 (0/5)	2.0±1.0 (5/5)	1.8±0.8 (5/5)	2.8±0.4* (5/5)	2.1±0.7 (5/5)	1.8±0.5 (4/4)	2.0±1.2 (4/4)
Liver	0.0±0.0 (0/5)	0.5±0.4 (4/5)	1.2±0.4 (5/5)	1.6±1.1 (5/5)	1.6±0.5 (5/5)	1.2±0.4 (5/5)	1.0±0.8 (4/4)	0.2±0.4 (1/5)	0.6±0.4 (3/5)	1.0±0.0 (5/5)	1.6±1.1 (5/5)	1.6±0.5 (5/5)	1.7±06 (3/3)	1.2±0.3 (3/3)	0.0±0.0 (0/5)	2.2±0.8* (5/5)	2.8±0.4* (5/5)	1.6±0.5 (5/5)	1.8±0.8 (5/5)	1.8±1.0 (4/4)	1.3±0.5 (4/4)
Ovary	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	1.0±0.0 (5/5)	1.4±0.5 (5/5)	2.0±0.7 (5/5)	1.8±0.8 (5/5)	1.6±0.5 (5/5)	1.5±0.6 (4/4)	1.8±0.5 (4/4)

[a] Mean histopathological lesion scores ± SD obtained by combining individual scores from the same organ type at each time point.

Scoring was based on the degree of severity in the lesions as described in the materials and methods: 0 (no lesion), 1 (mild), 2

(moderate), and 3 (severe). Asterisks (*) indicate statistically significant difference among three age groups on the same organ type at the indicated time point ($P < 0.05$, non-parametric Kruskal-Wallis test).

[b] Number of ducks showing histopathological lesions / Number of DTMUV inoculated ducks

[c] Not determined (ND) since no organs exist in that age.

Virus distribution and shedding

To assess the level of viremia in 1-, 4- and 27-week-old ducks inoculated with Thai DTMUV, serum samples collected from each group on 1, 3, 5, 7, 9, 14 and 21 dpi were evaluated for the presence of DTMUV using qRT-PCR. The results showed that all ducks in three age groups developed viremia as early as 1 dpi, in which the levels of viremia detected in 1-week old ducks were significantly higher than those detected in other groups ($P<0.05$) (Figure 19e). At 3 dpi, viremia in all ducks reached the maximum level as high as $10^{5.20\pm1.02}$ (1-week-old ducks), $10^{4.73\pm0.63}$ (4-week-old ducks) and $10^{6.06\pm0.88}$ (27-week-old ducks) DTMUV genome copies per 50 ng RNA and then gradually decreased (Figure 19e). The period of viremia in 1-week-old ducks lasted for 9 days, which was longer than that in 4- and 27-week-old ducks with no DTMUV RNA being detected after 7 dpi (Figure 19e). These results indicated that DTMUV induced a shorter-term viremia in 4- and 27-week-old ducks compared to 1-week-old ducks, which correlated with a lower degree of disease severity in older ducks. No DTMUV RNA was detected in serum samples from any of the negative control ducks.

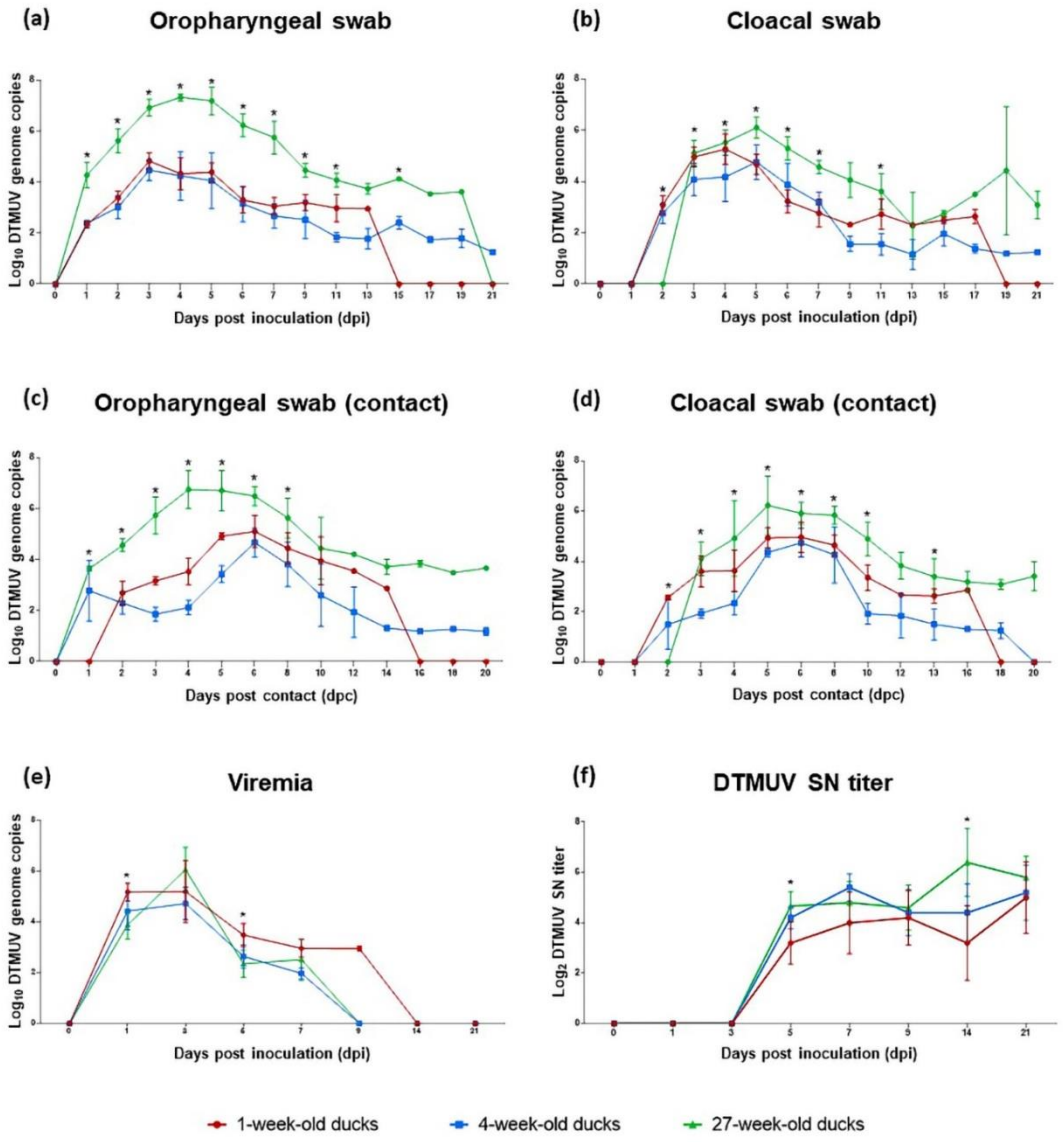


Figure 19 Viral shedding, viremia and neutralizing antibody response to DTMUV in 1-, 4- and 27-week-old ducks inoculated with Thai DTMUV. Viral shedding and viremia levels were determined by qRT-PCR. Neutralizing antibody titers against DTMUV was determined by SN test.

(a)-(d) Viral shedding in oropharyngeal (a), (c) and cloacal (b), (d) swabs from DTMUV inoculated and contact ducks, respectively, were expressed as log10 DTMUV genome copy number per 250 ng total RNA. (e) Viremia levels in serum were expressed as log10 DTMUV genome copy number per 50 ng total RNA. Each data point represents

the mean ± standard deviation of 5 ducks. (f) Neutralizing antibody titers in serum samples from DTMUV inoculated ducks. Asterisks (*) indicate statistically significant difference among three age groups at the indicated time point ($P < 0.05$, one-way ANOVA).

To determine the viral load and tissue distribution of Thai DTMUV in 1-, 4- and 27-week-old ducks, tissue samples, including brain, spleen, thymus, heart, pancreas, liver, uterus and ovary, collected on 1, 3, 5, 7, 9, 14 and 21 dpi were tested using DTMUV-specific qRT-PCR. Corresponding to viremia detection, DTMUV RNA could be detected in all examined organs of all ducks as early as 1 dpi, indicating a rapid systemic dissemination of Thai DTMUV (Figure 20). Viral loads in all examined organs reached the maximum levels during 3-5 dpi and gradually decreased and mostly disappeared from 7 to 14 dpi (Figure 20). However, the virus remained detectable in brains and spleens of ducks in all groups until 21 dpi (Figure 20). These findings indicated that high levels of viral loads in visceral organs may be the main cause of death in some infected ducks. Notably, the highest viral loads were consistently detected in spleen of ducks in all groups, indicating that spleen might be the target organ and primary replication site of Thai DTMUV. No DTMUV RNA was detected in any of the negative control ducks. Overall, 1-week-old ducks had significantly higher levels of viral loads in most examined organs compared to 4- and 27-week-old ducks (Figure 20). Taken together, these results along with the level of viremia and the severity of clinical signs and lesions indicated that Thai DTMUV induced more severe disease in younger ducks than older ducks.

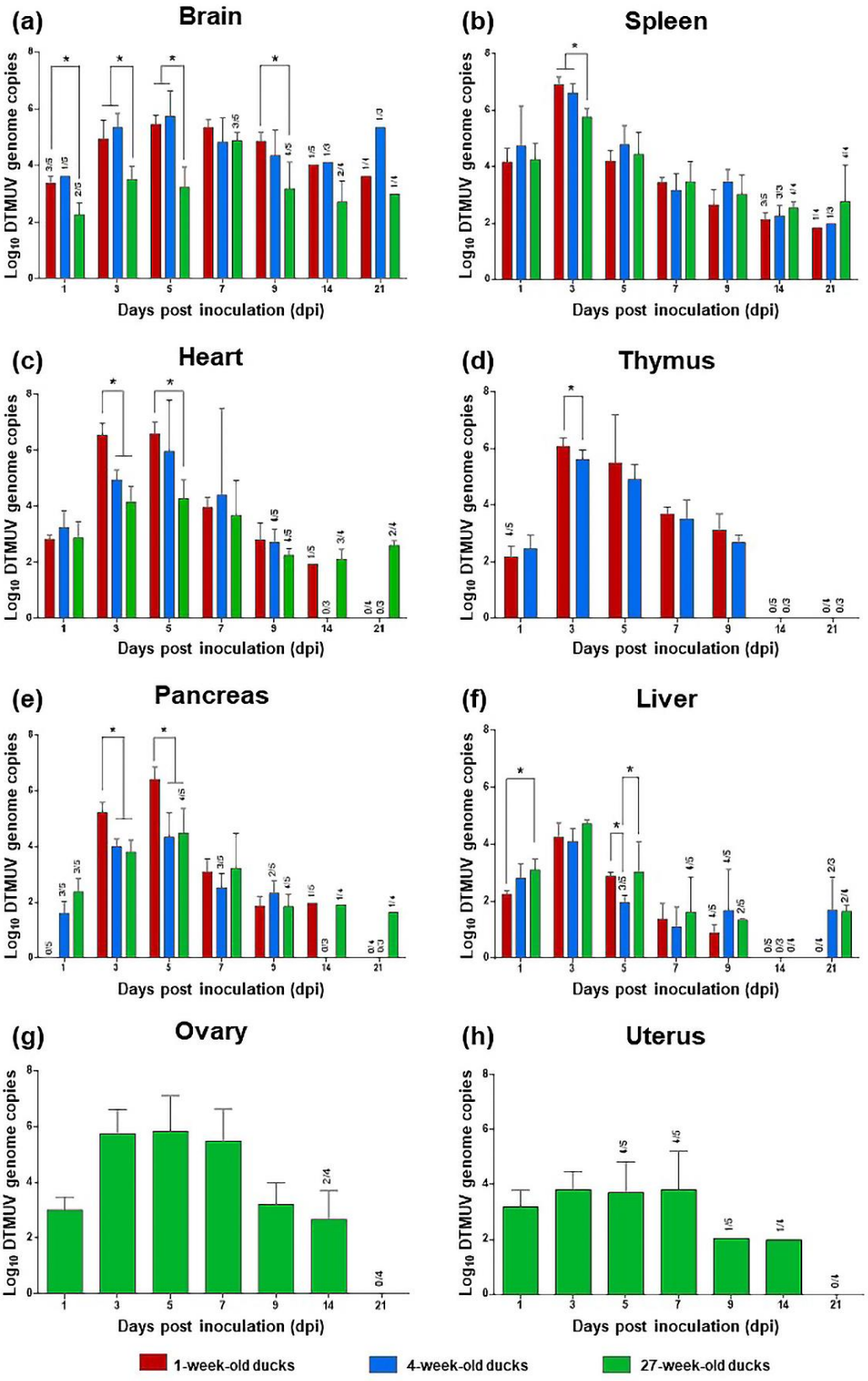

(a) Brain
(b) Spleen
(c) Heart
(d) Thymus
(e) Pancreas
(f) Liver
(g) Ovary
(h) Uterus
Log10 DTMUV genome copies
Days post inoculation (dpi)
1-week-old ducks
4-week-old ducks
27-week-old ducks

Figure 20 Viral loads and tissue distribution of Thai DTMUV in 1-, 4- and 27-week-old ducks.

Levels of DTMUV RNA in brain (a), spleen (b), heart (c), thymus (d), pancreas (e), liver (f), ovary (g) and uterus (h) were determined by qRT-PCR. Each data point represents the mean log10 DTMUV genome copy number per 250 ng total RNA ± standard deviation. Asterisks (*) indicate statistically significant difference among three age groups at the indicated time point ($P < 0.05$, one-way ANOVA).

To evaluate the shedding pattern and dynamic of Thai DTMUV in 1-, 4- and 27-week-old ducks, OP and CL swabs collected on 1-7, 9, 11, 13, 15, 17, 19 and 21 dpi were tested using DTMUV-specific qRT-PCR. No DTMUV RNA was detected from any of the negative control ducks. All ducks inoculated with Thai DTMUV shed virus in OP and CL swabs from 1 dpi and up to 13-21 dpi (Figure 19a, b). Peak viral shedding on OP and CL swabs were detected on 3-4 dpi and 4-5 dpi, respectively (Figure 19a, b). Overall, ducks in all groups generally shed virus in OP swabs earlier and at higher levels than those in CL swabs. It is interesting to note that 27-week-old ducks generally shed virus in both OP and CL swabs at significantly higher levels and for longer durations than 1- and 4-week-ducks ($P<0.05$) (Figure 19a, b). In addition, DTMUV RNA remained detectable in both swabs and tissues following recovery from DTMUV infection in some 27-week-old ducks. These findings together with less disease severity observed in 27-week-old ducks suggested that 27-week-old ducks may serve as the potential carriers of DTMUV infection. Corresponding to the pattern of virus shedding in inoculated ducks, contact ducks also shed virus in OP and CL swabs from 1-2 dpc and up to 15-21 dpc (Figure 19c, d). Peak viral shedding on OP and CL swabs of contact ducks were detected on 4-6 dpc (Figure 19c, d). Overall, these findings together with clinical findings in contact ducks revealed that Thai DTMUV transmitted efficiently among ducks in all age groups. Like virus shedding in

inoculated ducks, contact ducks in all groups also generally shed virus in OP swabs earlier and at higher levels than those in CL swabs (Figure 19c, d). Interestingly, similar to the findings from inoculated ducks, contact ducks in 27 weeks of age group generally shed virus in both OP and CL swabs at significantly higher levels and for longer periods than other groups ($P<0.05$) (Figure 19c, d). This supported the potential role of the older ducks as the carriers for transmitting DTMUV to other hosts.

Neutralizing antibody response against Thai DTMUV

All DTMUV inoculated ducks seroconverted to DTMUV and developed neutralizing antibodies as early as 5 dpi (Figure 19f). Neutralizing antibody titers reached the maximum levels at 7-14 dpi and were still detected in all surviving ducks until the end of experiment on 21 dpi (Figure 19f). Overall, 27-week-old ducks had significantly higher neutralizing antibody titers in serum samples than 1- and 4-week-ducks ($P<0.05$), which correlated directly with less disease severity in this age group (Figure 19f). It is noted that the increasing neutralizing antibody titers in infected ducks in all groups occurred simultaneously with decreasing disease severity, viremia, viral load in tissues and virus shedding, suggesting the important role of neutralizing antibodies in viral clearance during DTMUV infection. Taken together, the results demonstrated an early and potent neutralizing antibody response following Thai DTMUV infection in ducks.

5.4 Discussion

Since the first emergence, several clusters of DTMUV have been identified and the predominant cluster causing the outbreak in different countries has recently been described, including cluster 1 (Malaysia), cluster 2.1 (Thailand) and cluster 2.2 (China). However, the pathogenesis evaluation of DTMUV has been restricted to Chinese isolates that belong to cluster 2.2 (Sun et al., 2014; Li et al., 2015b; Ti et al., 2015; Lv et al., 2019). Limited information is available on the pathogenesis of other DTMUV clusters circulating in other countries, particularly in Thailand. Therefore, the objective of this study was to investigate the pathogenesis of a cluster 2.1 Thai DTMUV in three different ages of Cherry Valley ducks. Our results demonstrated that Thai DTMUV induced more severe disease in younger ducks (1- and 4-week-old) than older ducks (27-week-old), indicating age-dependent susceptibility to infection with Thai DTMUV in ducks. Corresponding to the degrees of disease severity, longer-term viremia, higher levels of viral loads in tissues and lower neutralizing antibody titers were also detected in younger ducks compared to those in older ducks. However, it is interesting to note that a prolonged shedding period of high viral load was observed in older ducks even in the convalescence phase, suggesting the potential role of the older ducks as the carriers of Thai DTMUV infection. To the best of our knowledge, this is the first study reporting the pathogenesis of Thai DTMUV in ducks.

The clinical and pathological findings revealed that all duck ages were susceptible to cluster 2.1 Thai DTMUV; however, differences in the disease severity were observed among ages. Notably, most younger ducks inoculated with Thai DTMUV developed severe and early clinical signs and lesions, and had high mortality rate, while some inoculated older ducks showed only mild clinical symptoms and low mortality. These observations indicated that Thai DTMUV is more pathogenic to

younger ducks than older ducks, corresponding to the findings of previous studies conducted with cluster 2.2 Chinese DTMUV (Sun et al., 2014; Li et al., 2015b). As reported in the natural infection (Cao et al., 2011; Su et al., 2011; Thontiravong et al., 2015), pathological lesions caused by Thai DTMUV were mainly observed in the nervous system and ovary of younger and older ducks, respectively. These findings correlated with the typical clinical signs presenting in those duck ages, including severe neurological signs in younger ducks and egg production losses in older ducks (Su et al., 2011; Sun et al., 2014; Lv et al., 2019). However, Thai DTMUV could also cause pathological changes with positive flavivirus staining in various organs of inoculated ducks in all groups. This indicates the systemic infection of Thai DTMUV, which possibly leads to multiple organ failure in the infected ducks. It should be noted that flavivirus positive mononuclear cell infiltration was widely observed at perivascular areas in various vital organs of infected ducks. This finding supports the essential role of monocytes/macrophages as the key targets of DTMUV infection, replication and dissemination (Garcia-Nicolas et al., 2019; Ma et al., 2019). In addition, flavivirus antigen was also detected in mononuclear cells and vascular endothelium in the brains of all duck ages. This observation suggests that DTMUV is likely to enter the CNS via infected infiltrating leukocytes and infection of vascular endothelial cells through hematogenous route as previously reported in other neurotropic flaviviruses (Neal, 2014; Li et al., 2015a; Mustafa et al., 2019).

In this study, DTMUV RNA was detected in serum samples of all duck ages as early as 1 dpi, indicating that viremia could occur in the acute stage of infection with Thai DTMUV. Consistent with early detection of viremia, DTMUV load in multiple organs could also be detected in all duck ages as early as 1 dpi. These suggest the rapid systemic dissemination and broad tissue tropism of Thai DTMUV in all three duck ages regardless of the clinical outcomes. However, the differences in level and

duration of viremia as well as viral load in tissues were observed among duck ages, in which younger ducks generally had longer-term viremia and higher levels of viral loads in tissues than older ducks. These findings correlated directly with higher degree of disease severity in younger ducks, in which high mortality observed in these ducks might be mainly caused by high levels of viral loads in visceral organs. Overall, these findings were consistent with the previous observations conducted with cluster 2.2 Chinese DTMUVs (Sun et al., 2014; Li et al., 2015b). Interestingly, the highest viral loads with prolonged duration were consistently detected in spleen of all duck ages. This finding along with histopathological findings showing severe lymphoid depletion in spleen support the notion that spleen is likely to be the target organ and primary replication site of DTMUV and several flaviviruses (Prestwood et al., 2012; Bryan et al., 2018; Sun et al., 2019). Apart from spleen, high DTMUV loads could also be detected in brains of all duck ages throughout the experiment, suggesting that, as with other related flaviviruses (Ricklin et al., 2016; Mustafa et al., 2019), DTMUV is a neurotropic flavivirus that can infect and causes disease in the nervous system. However, we also found that older ducks generally had lower levels of viral loads in brains compared to younger ducks. Therefore, less neurovirulence of Thai DTMUV observed in older ducks may be influenced by low ability of virus to invade the nervous system of older ducks. Notably, levels of viremia and viral load decreased after the appearance of neutralizing antibodies correlating with the reduction of clinical signs and lesions. These observations indicate that, similar to other flaviviruses (Sitati and Diamond, 2006; Pestka et al., 2007; Slon Campos et al., 2018), neutralizing antibodies may play an important role in virus clearance during DTMUV infection.

The patterns and dynamics of virus shedding following Thai DTMUV infection were monitored in OP and CL swabs of ducks from all age groups. Our result

demonstrated that all ducks inoculated with Thai DTMUV shed virus in OP and CL swabs with relatively high viral loads for up to approximately 21 dpi. Compared to a previous study conducted with Chinese DTMUVs (Lu et al., 2016), viral shedding in ducks inoculated with Thai DTMUV could be detected longer and at higher levels in both OP and CL swabs. The reason of this difference is unknown but may be related to virus strains and duck ages. Notably, OP shedding was generally higher and earlier than CL shedding for all Thai DTMUV inoculated ducks, suggesting that OP swabs might be another appropriate specimen for DTMUV detection. Our results also demonstrated that DTMUV RNA could be consistently detected in both swabs from contact ducks showing typical signs of DTMUV infection, indicating that Thai DTMUV transmitted efficiently among ducks in all age groups. Collectively, these data suggest that secretions/excretions from oropharynx and cloaca may serve as the sources for DTMUV transmission, supporting the notion that DTMUV might transmit through fecal-oral route (Cao et al., 2011). This finding might thus explain the rapid spread of the disease during winter season, when the mosquito activity is low (Li et al., 2015d; Ninvilai et al., 2019). Interestingly, prolonged shedding of DTMUV with high viral loads could be observed in older ducks even in the absence of clinical signs, suggesting the potential role of the older ducks as the carriers of Thai DTMUV. Therefore, awareness of DTMUV transmission and continued monitoring of DTMUV in adult ducks is crucial for early control and prevention of DTMUV.

Although all duck ages were susceptible to Thai DTMUV infection, the susceptibility and the pathogenicity of Thai DTMUV in younger ducks were much higher than those in older ducks. This finding agreed with previous studies demonstrating that young birds are highly susceptible to infection with various mosquito-borne flaviviruses, including Chinese DTMUVs, Tembusu virus, West Nile virus and Israel turkey meningoencephalitis virus (Benzarti et al., 2019). The difference

in disease severity among duck ages may be related to the maturity of immune system, in which older ducks developed more fully effective immune response as evidenced by significantly higher neutralizing antibody titers compared to younger ducks. This indicates that the induction of high neutralizing antibody titers early in the course of DTMUV infection in older ducks could limit viremia and virus dissemination into the nervous system and other visceral organs, resulting in protection against a severe and lethal outcome of DTMUV infection in older ducks.

In conclusion, our data collectively indicated that all duck ages were susceptible to Thai DTMUV; however, Thai DTMUV infection induced greater disease severity in younger ducks than in older ducks, suggesting age-related susceptibility to Thai DTMUV in ducks. Our finding also raises the awareness of adult ducks as the potential carriers of DTMUV infection. This highlights the importance of monitoring DTMUV in adult ducks as well as preventing the transmission of DTMUV among duck ages. Overall, this study demonstrated the pathogenesis and infection dynamics of Thai DTMUV in ducks. This information will help in development of improved diagnostic methods, effective prevention and control strategies of DTMUV.

CHAPTER 6
Conclusions

Since 2010, a newly contagious disease caused by Tembusu-related Flavivirus has emerged in duck farms in China. The causative agent of the outbreaks was subsequently identified as the novel duck Tembusu virus (DTMUV) (Su et al., 2011). After the initial outbreak, the disease has quickly spread throughout the major duck-producing areas in China and Asian countries, including Malaysia and Thailand, resulting in greater economic losses in poultry production sectors (Su et al., 2011; Homonnay et al., 2014; Chakritbudsabong et al., 2015; Thontiravong et al., 2015; Yan et al., 2017). The affected ducks typically exhibited a severe reduction in egg production with severe neurological disorders including ataxia and paralysis (Su et al., 2011). The morbidity and mortality rates ranged from 20-50% and 10-30%, respectively (Thontiravong et al., 2015). At present, a newly emerged DTMUV has become one of the most economically important pathogens of ducks in many Asian countries, including Thailand. However, the information on the genetic and pathogenic characteristics of DTMUV currently circulating in ducks in Thailand is limited. To establish the effective control and prevention strategies, a better understanding of the genetic characteristic and pathogenesis of DTMUV circulating in Thailand is essential.

The first objective of this dissertation was to investigate the genetic characteristic and diversity of DTMUVs isolated from ducks in Thailand in 2007 and during 2015-2017. DTMUV infection had never been reported in ducks prior to 2010; however, an unknown disease associated with severe neurological signs and losses in egg production in ducks was found in Thailand since 2007. This indicated that DTMUV might have been emerged in ducks earlier than 2010, the first report in China. To

determine the presence of DTMUV in 2007, the clinical samples from affected ducks presenting DTMUV like symptoms collected from the suspected cases in 2007 were retrospectively tested for DTMUV using pathological and virological analyses. Overall, our results showed the presence of DTMUV in clinical samples collected from affected ducks in 2007. Gross and histopathological lesions of affected ducks were mostly observed in ovary, brain and spinal cord, and correlated with the presence of flavivirus antigen in these organs. Subsequently, DTMUV was isolated, identified by RT-PCR and nucleotide sequencing. Genetic analyses of the polyprotein gene sequence revealed that the 2007 Thai DTMUV was closely related to the Malaysian DTMUVs, while it was genetically distinctive to the currently circulating Thai and Chinese DTMUVs. Interestingly, the 2007 Thai DTMUV was a unique virus, belonged within DTMUV cluster 1, but distinctively separated from the Malaysian DTMUV. Moreover, the 2007 Thai DTMUV was genetically different from the currently circulating Thai and Chinese DTMUVs, which belonged to cluster 2. Our findings indicated that this 2007 Thai DTMUV emerged earlier from a common ancestor with the recently reported DTMUVs; however, it was genetically distinctive to any of the currently circulating DTMUVs. In summary, our retrospective study demonstrated the presence of DTMUV in the Thai ducks since 2007, prior to the first report of DTMUV in China in 2010. These findings also indicated that three clusters of DTMUVs (1, 2.1 and 2.2) established in this study, were associated with the current DTMUV outbreaks in China and Southeast Asia in which the correlation between virus cluster and geographic location was observed. Our data indicated the continued evolving of DTMUVs with relatively high evolutionary rate.

In this study, the geographic distribution and genetic characteristic of recently circulating DTMUVs in ducks in Thailand during 2015-2017 were also investigated. Of the 288 clinical samples obtained from 89 ducks farms located in duck raising areas

of Thailand, 65 samples (22.57%) of 34 duck farms (38.20%) were DTMUV positive. Our results demonstrated that DTMUV was extensively distributed in duck raising areas of Thailand. Phylogenetic analysis revealed that DTMUVs circulating in Thailand were divided into 3 distinct clusters, including cluster 1, subcluster 2.1 and a novel cluster 3. Among these 3 clusters, subcluster 2.1 was a predominant cluster of DTMUV circulating in duck populations in Thailand during 2015-2017. Interestingly, a novel cluster of DTMUV (cluster 3) was first identified in this study. The genetic analysis revealed that this novel virus contained various amino acid changes in the E protein compared to the previously reported cluster 1 and 2. As reported previously, E protein is responsible for host cell entry, replication, transmissibility of DTMUV in ducks and involved in the induction of virus-neutralizing antibodies (Mukhopadhyay et al., 2005; Lindenbach et al., 2007; Yan et al., 2018). Therefore, whether these amino acid changes contribute to virus pathogenicity, antigenic cross-reactivity among different clusters of DTMUV and other biological characteristics need to be further investigated. In conclusion, our data demonstrated the circulation of different clusters of DTMUV and the presence of a novel DTMUV cluster in ducks in Thailand. Our findings indicate the high genetic diversity and continued evolution of DTMUV in ducks in Thailand as well as in Asia. This study highlights the necessity of routine surveillance of DTMUV for early detection and monitoring the emergence of the new distinct DTMUV variants with possible enhanced virulence.

Our previous study demonstrated that several clusters of DTMUV have increasingly been identified since its first emergence, indicating a high level of DTMUV genetic diversity (Ninvilai et al., 2019). Therefore, this highlights the need for improved and novel broad detection assays in order to keep up-to-date with the circulating clusters of DTMUV. The second objective of this study was to develope and validate a universal one-step RT-PCR assay targeting highly conserved region of

NS5 gene for broad detection of all DTMUV clusters. The newly developed universal RT-PCR assay showed high specificity and no cross-reactions with common duck viruses and other related flaviviruses. Our newly developed universal RT-PCR assay could detect a low amount of DTMUV RNA with limit of detection of 0.001 50% ELD_{50}/ml. In addition, the performance of the assay was further evaluated using experimental and field clinical samples. The assay could successfully detect DTMUV in all experimentally DTMUV infected samples and gave a higher DTMUV detection rate (36%) than the previously reported E-specific RT-PCR assay (30%) from field clinical samples. This finding may be caused by the high variability of E gene (Lindenbach et al., 2007; Yu et al., 2013), which might affect the sensitivity of detection. In conclusion, our newly developed universal RT-PCR assay exhibited high accuracy, specificity and sensitivity in broad DTMUV detection, thus providing an improved screening assay for routine detection and epidemiology surveillance of DTMUV. This study highlights the importance of continuously validating the performance of currently used diagnostic assays against newly emerging strains to ensure the broad detection of the assay.

Although the pathogenesis of DTMUV have recently been evaluated in different ages of ducks (Sun et al., 2014; Li et al., 2015b; Ti et al., 2015; Lv et al., 2019), this evaluation has been restricted to Chinese isolates that belong to cluster 2.2 (Sun et al., 2014; Li et al., 2015b; Ti et al., 2015; Lv et al., 2019). Limited information is available on the pathogenesis of other DTMUV clusters circulating in other countries, particularly in Thailand. Therefore, the third objective of this study was to investigate the pathogenesis of a cluster 2.1 Thai DTMUV in three different ages of Cherry Valley ducks. Our findings revealed that all duck ages were susceptible to Thai DTMUV infection; however Thai DTMUV induced more severe disease in younger ducks (1- and 4-week-old) than older ducks (27-week-old),

indicating age-dependent susceptibility to infection with Thai DTMUV in ducks. Corresponding to the degrees of disease severity, longer-term viremia, higher levels of viral loads in tissues and lower neutralizing antibody titers were also detected in younger ducks compared to those in older ducks. The difference in disease severity among duck ages may be related to the maturity of immune system, in which older ducks developed more fully effective immune response as evidenced by significantly higher neutralizing antibody titers compared to younger ducks. Our study demonstrated the rapid systemic dissemination and broad tissue tropism of Thai DTMUV in all three duck ages regardless of the clinical outcomes. Notably, levels of viremia and viral load decreased after the appearance of neutralizing antibodies correlating with the reduction of clinical signs and lesions. In addition, the patterns and dynamics of virus shedding following Thai DTMUV infection were monitored in OP and CL swabs of ducks from all age groups. Our result demonstrated that all ducks inoculated with Thai DTMUV shed virus in OP and CL swabs with relatively high viral loads for up to approximately 21 dpi. Our results also showed that DTMUV RNA could be consistently detected in both swabs from contact ducks, indicating that Thai DTMUV transmitted efficiently among ducks in all age groups. These data suggest that the secretions/excretions from oropharynx and cloaca may serve as the sources for DTMUV transmission, supporting the notion that DTMUV might be transmit through fecal-oral route (Cao et al., 2011). Interestingly, a prolonged shedding period of high viral load was observed in older ducks even in the convalescence phase, suggesting the potential role of the older ducks as the carriers of Thai DTMUV infection. In conclusion, our pathogenic study indicated that all duck ages were susceptible to Thai DTMUV; however, Thai DTMUV infection induced greater disease severity in younger ducks than in older ducks, suggesting age-related susceptibility to Thai DTMUV in ducks. Our finding also raises the awareness of adult ducks as the potential carriers of DTMUV infection. This highlights the importance of

monitoring DTMUV in adult ducks as well as preventing the transmission of DTMUV among duck ages.

In summary, this dissertation provided information on the genetic characteristic and pathogenesis of DTMUVs circulating in Thailand as well as established the well-validated universal RT-PCR assay for broad detection of DTMUV clusters in ducks. This information is useful for improving the development of the effective control and prevention strategies of DTMUV. Overall, this study highlights the necessity of the continued surveillance of DTMUV in ducks particularly in adult ducks as well as the importance of continuously validating the performance of currently used diagnostic assays against newly emerging strains for early and effective detection, control and prevention of DTMUV.

CHAPTER 7

Application, suggestion and recommendation

Application

Genetic & pathogenesis characterization

1. The genetic classification system of DTMUV using DTMUV sequences identified in China and Southeast Asia during 2007-2015 was established in this study. Based on this classification, three clusters of DTMUV, including cluster 1, subcluster 2.1, subcluster 2.2 and cluster 3, were associated with the DTMUV outbreaks in China and Southeast Asia.

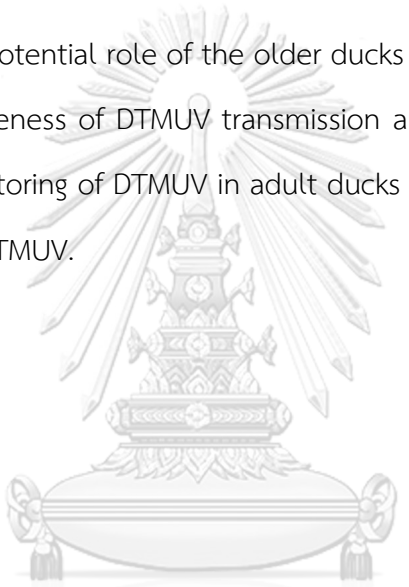
2. Several diagnostic methods for DTMUV detection were established in this study, including a universal RT-PCR assay for broad detection of all DTMUV clusters and a modified immunohistochemical staining assay for detecting flavivirus-specific antigens in tissue samples. All these techniques could be used as effective methods for detection and epidemiology surveillance of DTMUV.

3. This study revealed the systemic infection of Thai DTMUV in three different ages of ducks; however, difference in level of viral load was observed among sample types. Our study showed that higher viral loads with prolonged duration were generally detected in spleen, brain and OP swabs than those from other tissue and swab samples. This indicates that spleen, brain and OP swabs might be the most appropriate specimens for DTMUV detection.

4. Our data suggest that secretions/excretions from oropharynx and cloaca may serve as the sources for DTMUV transmission, supporting the notion that DTMUV might transmit through fecal-oral route (Cao et al., 2011). Therefore,

the biosecurity control among duck flocks within/between farms was necessary for disease prevention. Effective measures for DTMUV prevention included controlling the movement of suspected duck flocks, avoiding contact with DTMUV suspected flocks, DTMUV positive ducks, and contaminated environments.

5. Our results revealed that the prolonged DTMUV shedding with high viral loads could be observed in older ducks even in the absence of clinical signs, suggesting the potential role of the older ducks as the carriers of Thai DTMUV. Therefore, awareness of DTMUV transmission among different age flocks and continued monitoring of DTMUV in adult ducks is crucial for early control and prevention of DTMUV.



Suggestion and recommendation

Farm level

1. Our study highlights the importance of preventing transmission of DTMUV among duck ages. Therefore, we suggest that implementation of the “one age flocks” and “one site management” with “all in all out” production programs are essential for preventing and reducing the risks of DTMUV transmission among duck flocks.

2. Strict biosecurity, sanitary and hygiene control of duck farms, staffs, farm workers and suppliers are necessary for preventing DTMUV spread into duck farms, including avoiding close contact between farm ducks or farm workers and wild birds , treating water used in farms with approved disinfection with appropriated concentration and contact time, strict controlling the entry of visitors and vehicles into the farm and eliminating the mosquitoes during the rainy season.

3. Ducklings and/or pullet ducks must be obtained from healthy parent stock flocks and must be tested and confirmed negative for DTMUV and other common duck diseases before transportation and receiving into the rearing farms.

4. Our information highlights the importance of routine surveillance and monitoring DTMUV in ducks, especially in adult ducks, for early detection, control and prevention of DTMUV.

5. During DTMUV outbreaks, several measures must be implemented, including culling the affected ducks, restricting the movement of suspected flocks and carcasses, disinfecting DTMUV contaminated equipment, vehicles and poultry

house with appropriated disinfection and providing an appropriated period of downtime before replacement with new flocks.

6. Veterinarians, farm owners and farm workers need to be educated in order to improve the awareness and understanding about DTMUV infection, transmission control and prevention through the effective communication and training.

National level

1. To monitor and prevent the emergence and spread of novel DTMUV strains, the awareness and understanding about DTMUV infection, transmission, diagnosis, control and prevention must be improved through the effective communication, education and training among related parties, including veterinarians, staffs from government sector (department of livestock development) and private sectors, farm owners, farm workers and related laboratories.

2. This study highlights the need for the continuous DTMUV surveillance to monitor the emergence of new DTMUV outbreaks in ducks in Thailand. Genetic characterization of DTMUV isolates should be routinely conducted for early detection of novel DTMUV variants, effective control and prevention of DTMUV.

3. The effective measures for controlling DTMUV outbreaks must be issued and implemented, including culling the affected ducks, restricting the movement of suspected flocks and carcasses, disinfecting DTMUV contaminated equipment, vehicles and poultry house with appropriated disinfection and

providing an appropriated period of downtime before replacement with new flocks.

4. This study highlights the importance of continuously validating the performance of currently used diagnostic assays against newly emerging strains to ensure the broad detection of the assay.

APPENDIX

Appendix 1

Table A1 Detail description of Thai DTMUVs characterized in this study.

Virus name	Duck type	Time of collection	Location	Accession No.
DK/TH/CU-DTMUV2007	Layer	Jul 2007	Nakhon Ratchasima	MF621927
DK/TH/CU-9	Broiler	Aug 2015	Nakhon Ratchasima	MK276414
DK/TH/CU-10	Broiler	Aug 2015	Prachinburi	MK276415
DK/TH/CU-13	Layer	Aug 2015	Nakhon Pathom	MK276416
DK/TH/CU-15	Broiler	Oct 2015	Nakhon Ratchasima	MK276417
DK/TH/CU-16	Layer	Oct 2015	Nakhon Pathom	MK276418
DK/TH/CU-17	Layer	Oct 2015	Chumphon	MK276419
DK/TH/CU-18	Broiler	Oct 2015	Lopburi	MK276420
DK/TH/CU-22	Broiler	Nov 2015	Prachinburi	MK276421
DK/TH/CU-25	Broiler	Nov 2015	Prachinburi	MK276422
DK/TH/CU-34	Broiler	Nov 2015	Nakhon Ratchasima	MK276423
DK/TH/CU-35	Broiler	Nov 2015	Chachoengsao	MK276424
DK/TH/CU-40	Broiler	Dec 2015	Nakhon Ratchasima	MK276425
DK/TH/CU-48	Broiler	Jan 2016	Nakhon Ratchasima	MK276426
DK/TH/CU-56	Pullet	Feb 2016	Saraburi	MK276427
DK/TH/CU-67	Layer	May 2016	Nakhon Ratchasima	MK276428
DK/TH/CU-71	Pullet	May 2016	Saraburi	MK276429
DK/TH/CU-78	Layer	Jun 2016	Nakhon Ratchasima	MK276430
DK/TH/CU-85	Layer	Jul 2016	Lopburi	MK276431
DK/TH/CU-89	Layer	Jul 2016	Sing Buri	MK276432
DK/TH/CU-90	Broiler	Jul 2016	Nakhon Ratchasima	MK276433
DK/TH/CU-99	Layer	Aug 2016	Nakhon Ratchasima	MK276434
DK/TH/CU-100	Layer	Aug 2016	Nakhon Ratchasima	MK276435
DK/TH/CU-102	Layer	Aug 2016	Saraburi	MK276436
DK/TH/CU-103	Layer	Aug 2016	Saraburi	MK276437
DK/TH/CU-111	Broiler	Aug 2016	Lopburi	MK276438
DK/TH/CU-120	Broiler	Sep 2016	Chachoengsao	MK276439
DK/TH/CU-126	Layer	Oct 2016	Chonburi	MK276440
DK/TH/CU-128	Layer	Oct 2016	Chonburi	MK276441
DK/TH/CU-130	Broiler	Oct 2016	Prachinburi	MK276442
DK/TH/CU-131	Broiler	Oct 2016	Prachinburi	MK276443
DK/TH/CU-132	Broiler	Oct 2016	Prachinburi	MK276444
DK/TH/CU-135	Broiler	Nov 2016	Chachoengsao	MK276445
DK/TH/CU-138	Broiler	Nov 2016	Prachinburi	MK276446
DK/TH/CU-139	Broiler	Nov 2016	Chachoengsao	MK276447

DK/TH/CU-140	Broiler	Nov 2016	Chonburi	MK276448
DK/TH/CU-141	Broiler	Nov 2016	Chonburi	MK276449
DK/TH/CU-143	Broiler	Nov 2016	Chachoengsao	MK276450
DK/TH/CU-144	Broiler	Nov 2016	Chachoengsao	MK276451
DK/TH/CU-148	Broiler	Nov 2016	Chonburi	MK276452
DK/TH/CU-151	Broiler	Dec 2016	Nakhon Ratchasima	MK276453
DK/TH/CU-153	Broiler	Dec 2016	Prachinburi	MK276454
DK/TH/CU-154	Layer	Dec 2016	Chachoengsao	MK276455
DK/TH/CU-164	Layer	Mar 2017	Sing Buri	MK276456
DK/TH/CU-170	Broiler	Apr 2017	Prachinburi	MK276457
DK/TH/CU-172	Broiler	May 2017	Chonburi	MK276458
DK/TH/CU-175	Layer	May 2017	Nakhon Ratchasima	MK276459
DK/TH/CU-177	Layer	May 2017	Nakhon Ratchasima	MK276460
DK/TH/CU-178	Broiler	May 2017	Prachinburi	MK276461
DK/TH/CU-182	Broiler	Jun 2017	Prachinburi	MK276462
DK/TH/CU-184	Broiler	Jul 2017	Prachinburi	MK276463
DK/TH/CU-189	Broiler	Jul 2017	Nakhon Pathom	MK276464
DK/TH/CU-190	Broiler	Jul 2017	Nakhon Pathom	MK276465
DK/TH/CU-191	Broiler	Jul 2017	Prachinburi	MK276466
DK/TH/CU-192	Broiler	Jul 2017	Prachinburi	MK276467
DK/TH/CU-193	Broiler	Jun 2017	Nakhon Ratchasima	MK276468
DK/TH/CU-205	Broiler	Jul 2017	Prachinburi	MK276469
DK/TH/CU-206	Broiler	Aug 2017	Prachinburi	MK276470
DK/TH/CU-212	Layer	Aug 2017	Suphan Buri	MK276471
DK/TH/CU-215	Broiler	Sep 2017	Chonburi	MK276472
DK/TH/CU-218	Layer	Oct 2017	Chachoengsao	MK276473
DK/TH/CU-224	Broiler	Dec 2017	Prachinburi	MK276474

Appendix 2

Table A2 Comparison of the nucleotide and amino acid identities of the partial NS5 sequences of all clusters of DTMUVs and TMUVs isolated from mosquitoes (MM1775) and chickens (Sitiawan virus).

Reference DTMUVs	Accession No.	Host	Location	Year of collection	% Nucleotide identity		% Amino acid identity	
					2015-2017 Thai DTMUVs	2016 Thai DTMUV (DK/TH/CU-56)	2015-2017 Thai DTMUVs	2016 Thai DTMUV (DK/TH/CU-56)
Cluster 1								
D1977/1/MY	KX097989	Duck	Malaysia	2012	91.5-92.7	91.2	98-98.4	98
D1921/1/3/MY	KX097990	Duck	Malaysia	2012	91.5-92.4	91.2	98-98.4	98
DK/TH/CU-DTMUV2007	MF621927	Duck	Thailand	2007	91.2-92.7	90.7	97.6-98	97.6
Cluster 2.1								
DK/TH/CU-1	KR061333	Duck	Thailand	2013	98-99.9	90.4	99.6-100	98.8
KPS54A61	KF573582	Duck	Thailand	2013	97.5-98.4	91.1	99.6-100	98.8
GX2013E	KM275940	Duck	China	2013	97.3-98.4	90.9	99.6-100	98.8
HD-2015	KX686572	Duck	China	2015	97.3-98.4	90.8	99.6-100	98.8
HZ1-2015	KX686570	Duck	China	2015	96.9-98	90.4	99.6-100	98.8
HZ3-2015	KX686579	Duck	China	2015	96.8-97.9	90.3	99.6-100	98.8
zjYY150901	MF522174	Duck	China	2015	96.5-97.6	90.7	99.6-100	98.8
Cluster 2.2								
JS804	JF895923	Goose	China	2010	96.4-97.5	91.3	99.6-100	98.8
BYD-1	JF312912	Duck	China	2010	96.1-97.2	91.5	99.6-100	98.8
YY5	JF270480	Duck	China	2010	96.4-97.5	91.3	99.6-100	98.8
ZJ-6	JF459991	Duck	China	2010	96.1-97.2	91.3	99.6-100	98.8
XHZD/2010	JQ595407	Duck	China	2010	96.4-97.5	91.2	99.6-100	98.8
LC_2010	KC990543	Duck	China	2010	96.1-97.2	91.1	99.2-99.6	98.4

CK-SD-11	JQ627862	Chicken	China	2010	96.1-97.2	91.1	99.2-99.6	98.4
GX_2011	KC990542	Duck	China	2011	96.4-97.2	91.1	99.6-100	98.8
FS-2011	KX686578	Duck	China	2011	96.4-97.2	91.1	99.6-100	98.8
JX2	JQ920426	Duck	China	2012	95.6-96.9	90.5	98.8-99.2	98.4
df-2	KJ489355	Duck	China	2012	96.4-97.5	91.3	99.6-100	98.8
byd1	JQ920420	Duck	China	2012	96.1-97.2	91.5	99.6-100	98.8
zc-1	KF557894	Duck	China	2012	95.7-96.7	90.0	99.2-99.6	98.4
JSGo	AB917090	Goose	China	2012	96.1-97.2	91.1	99.6-100	98.8
DEDSV strain pigeon	JQ920425	Pigeon	China	2012	96.3-97.3	91.1	99.6-100	98.8
AHQY	KJ740748	Duck	China	2013	95.5-96.5	90.8	99.2-99.6	98.4
SX1	KM066945	Chicken	China	2013	95.7-96.8	91.1	99.2-99.6	98.4
G23	KT239021	Goose	China	2014	96.1-96.9	90.7	99.6-100	98.8
JS06	KR869106	Chicken	China	2014	95.6-96.7	91.2	98.8-99.2	98
GD2014	KU323595	Duck	China	2014	96.3-97.3	91.1	99.6-100	98.8
DTMUV/CH/2014	KP096415	Duck	China	2014	96.3-97.3	90.9	99.6-100	98.8
GDLH01	KT824876	Duck	China	2015	96.1-97.2	90.9	98.4-99.2	97.6
SH001	KP742476	Duck	China	2015	95.9-96.9	90.8	99.6-100	98.8
HZ4-2015	KX686571	Duck	China	2015	95.6-96.7	90.7	99.6-100	98.8
Cluster 3								
DK/TH/CU-56	MK276427	Duck	Thailand	2016	89.9-90.4	100	98.4-98.8	100
SD14	MH748542	Duck	China	2014	90-90.9	93.7	98.4-98.8	99.2
MM1775	JX477685	*Culex tritaeniorhynchus*	Malaysia	1955	86.9-88	89.5	97.2-97.6	97.2
Sitiawan virus	JX477686	Chicken	Malaysia	2000	86.9-88	88.1	98.4-98.8	98.4

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AWARD RECEIVED

The 90th Anniversary of Chulalongkorn University Scholarship