
# Final Report

## Evaluation of different avian embryonated eggs and cell cultures for efficient isolation and propagation of newly emerged duck Tembusu virus

**By Aunyaratana Thontiravong**

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# Evaluation of different avian embryonated eggs and cell cultures for efficient isolation and propagation of newly emerged duck Tembusu virus


| Researcher | Institute |
|---|---|
| 1. Aunyaratana Thontiravong | Chulalongkorn University |
| 2. Alongkorn Amonsin | Chulalongkorn University |


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## Abstract

**Project Code :** MRG6180017

**Project Title :** Evaluation of different avian embryonated eggs and cell cultures for efficient isolation and propagation of newly emerged duck Tembusu virus

**Investigator :** Aunyaratana Thontiravong
Faculty of Veterinary Science, Chulalongkorn University

**E-mail Address :** Aunyaratana.T@chula.ac.th

**Project Period :** 2 years

**Abstract :**

Duck Tembusu virus (DTMUV) has been identified as a causative agent of an emerging disease in ducks. Currently, DTMUV is widely distributed and becomes endemic in duck populations in Asia, causing significant economic losses to the duck producing industry. To early detect and control this emerging disease, the establishment of effective diagnostic methods, including host systems for virus isolation, is essential. In this study, various host systems, including different avian embryonated eggs (duck and chicken) and cell cultures (primary duck embryo fibroblast (DEF) cells, primary chicken fibroblast (CEF) cells, baby hamster kidney (BHK-21) cells, African green monkey kidney (Vero) cells and *Aedes albopictus* clone C6/36 (C6/36) cells), were evaluated and compared for their ability to support DTMUV isolation and propagation. Our results showed that all the host systems tested were susceptible to DTMUV infection; however, BHK-21 cells support more efficient replication of DTMUV compared to other host systems tested. In addition, BHK-21 cells had the highest DTMUV isolation rate when tested with experimental and field clinical samples. Notably, all circulating clusters of DTMUV, including DTMUV cluster 1, 2 and 3, were successfully isolated from duck clinical samples by using BHK-21 cells. In conclusion, our data support the use of BHK-21 cells as a host system for primary isolation of DTMUV from duck clinical samples. This study highlights the importance of selecting the most appropriate host system for efficient isolation and propagation of DTMUV from duck clinical samples.

**Keywords :** avian embryonated egg; cell culture; duck Tembusu virus; host system; virus isolation

**Executive summary**

**Introduction to Research**

Duck egg drop syndrome is a new emerging viral disease in ducks, causing huge economic losses in the duck producing industry. The 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 (90%-100%), while the mortality rate varied from 10%-30% depending on the farm management of affected flocks and the secondary bacterial infection (Yan et al., 2011b). A causative pathogen of this emerging disease was subsequently identified as a novel duck Tembusu virus (DTMUV). DTMUV was first detected in China in 2010 and then spread rapidly throughout the country (Su et al., 2011). Recently, DTMUV have been reported in several Asian countries, including Malaysia and Thailand (Chakritbudsabong et al., 2015; Homonnay et al., 2014; Thontiravong et al., 2015). In Thailand, a severe contagious disease in ducks caused by DTMUV has been detected since 2013, causing economic losses to both traditional and agro-industrial duck businesses in Thailand (Chakritbudsabong et al., 2015; Thontiravong et al., 2015). After its emergence, DTMUV has widely spread and become endemic in the duck population in several duck producing regions of China, Malaysia and Thailand. At present, a newly emerged DTMUV has become one of the most economically important pathogens of ducks in many Asian countries, including Thailand. To date, no commercial DTMUV vaccine is currently available for the prevention of DTMUV infection. Strict biosecurity and the culling of DTMUV infected ducks are the only effective measures to prevent and control DTMUV infection in ducks. Therefore, the establishment of effective diagnostic methods, including efficient host systems for virus isolation, is essential for effective control and prevention of this emerging disease.

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*. DTMUV is classified as a new genotype of Tembusu virus (TMUV) clustered within the group of mosquito-borne flaviviruses (O'Guinn et al., 2013; Platt et al., 1975). DTMUV was the first Flavivirus to cause disease in ducks (Su et al., 2011). DTMUV can infect a variety of avian and non-avian hosts, including several duck species, geese, chickens, pigeons, house sparrows and mice, indicating a wide host range of DTMUV (Chen et al., 2014; Han et al., 2013; Li et al., 2013; Liu et al., 2013; Tang et al., 2013a). As a member of the flavivirus genus, the public health concern regarding to the zoonotic potential of DTMUV has been raised. Although no human cases of DTMUV-related disease have been reported and a recent study showed that DTMUV is not pathogenic to non-human primates (Wang et al.,

2016), DTMUV-specific antibodies and DTMUV RNA were identified in duck farm workers in China (Tang et al., 2013b). Therefore, the establishment of effective diagnostic methods is essential for preventing economic losses in animal production as well as zoonotic potential in humans.

Currently, various methods have been used to detect DTMUV, including antigen detection methods, molecular-based assays, virus isolation in host system and serological assays (Cao et al., 2011; Tang et al., 2015; Wang et al., 2016; Yin et al., 2013). However, the virus isolation and/or reverse transcription polymerase chain reaction (RT-PCR) are the primary methods widely used to detect DTMUV from clinical samples (Homonnay et al., 2014; Su et al., 2011; Thontiravong et al., 2015). Although virus isolation is time-consuming method, this technique is a necessary step for confirming the virus infection, for investigating the antigenic and genetic characteristics of the epidemic strains and particularly for evaluating the viability of virus, which is important for determination of the risk of virus transmission among animal species. In addition, studies on viral pathogenesis, vaccine production and diagnostic test development rely on successful virus isolation and propagation (Li et al., 2014; Lu et al., 2016; Yin et al., 2013; Zhang et al., 2017b). Therefore, the establishment of an efficient host system for isolation and propagation of DTMUV is essential. Several host systems, including different avian embryonated eggs and cell cultures, have been used for DTMUV isolation and propagation (Chen et al., 2014; Lei et al., 2017; Su et al., 2011; Thontiravong et al., 2015). However, differences in their ability to support the replication of DTMUV have never been evaluated and the most efficient host system for DTMUV isolation is unknown. In this study, various host systems, including different avian embryonated eggs (chicken and duck) and cell cultures (primary duck embryo fibroblast (DEF) cells, primary chicken fibroblast (CEF) cells, African green monkey kidney (Vero) cells, baby hamster kidney (BHK-21) cells and *Aedes albopictus* clone C6/36 (C6-36) cells), were evaluated and compared for their ability to support DTMUV isolation and propagation. The infectivity and replication kinetic of DTMUV as well as isolation rate of DTMUV from duck experimental and clinical samples were examined in these host systems in order to identify the most suitable host system for DTMUV isolation and propagation.

**Literature review**

Duck egg drop syndrome is a new emerging viral disease in ducks, causing huge economic losses in the duck producing industry. The 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 (90%-100%), while the mortality rate varied from 10%-30% depending on the farm management of affected flocks and the secondary bacterial infection (Yan et al., 2011b). A causative pathogen of this emerging disease was subsequently

identified as a novel duck Tembusu virus (DTMUV). DTMUV was first detected in China in 2010 and then spread rapidly throughout the country (Su et al., 2011). Recently, DTMUV have been reported in several Asian countries, including Malaysia and Thailand (Chakritbudsabong et al., 2015; Homonnay et al., 2014; Thontiravong et al., 2015). After its emergence, DTMUV has widely spread and become endemic in the duck population in several duck producing regions of China, Malaysia and Thailand. At present, a newly emerged DTMUV has become one of the most economically important pathogens of ducks in many Asian countries, including Thailand.

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*. The DTMUV genome is composed 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; Tang et al., 2011). The structural proteins are involved in cellular attachment, membrane fusion and virus assembly, while the non-structural proteins are associated with viral replication and the evasion of host immunity (Zhang et al., 2017b). Among these proteins, E protein is a major surface protein that plays an important role in virus receptor binding, entry and fusion (Yun et al., 2012). DTMUV is classified as a new genotype of Tembusu virus (TMUV) clustered within the group of mosquito-borne flaviviruses (O'Guinn et al., 2013; Platt et al., 1975). 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). TMUV was first isolated from *Culex tritaeniorhynchus* in Malaysia in 1957 and subsequently detected in Thailand with no report to cause illness in animals and humans (O'Guinn et al., 2013; Platt et al., 1975). In general, avian species usually serve as reservoirs for Flaviviruses (Liu et al., 2013). However, Sitiawan virus, a TMUV of chicken-origin, was reported to cause retarded growth and encephalitis in broiler chicks in Malaysia (Kono et al., 2000). Recently, DTMUV was the first Flavivirus to cause disease in ducks (Su et al., 2011). DTMUV can infect a variety of duck species, geese, chickens, pigeons and house sparrows, mostly resulting in neurological symptoms and severe egg drop (Chen et al., 2014; Han et al., 2013; Liu et al., 2013; Tang et al., 2013a). Moreover, previous studies reported that DTMUV can induce neurovirulence in mice (Li et al., 2013; Ti et al., 2016).

DTMUV can transmit through multiple routes, including Culex mosquito bites (O'Guinn et al., 2013), fecal-oral route (Ninvilai et al., 2020), airborne transmission (Li et al., 2015c) and vertical

transmission (Zhang et al., 2015). All duck ages are susceptible to DTMUV infection, especially young ducks which are most susceptible to this virus (Li et al., 2015a; Ninvilai et al., 2020; Sun et al., 2014). The signs of DTMUV infection include acute anorexia, retarded growth and a severe drop in egg production both in egg-laying and breeding ducks. The infected ducks also exhibit neurologic dysfunctions, including unable to walk, ataxia and paralysis (Su et al., 2011). The main pathological changes were ovaritis, severe ovarian hemorrhage and regression. In addition, encephalitis characterized by multifocal lymphoid infiltration with perivascular cuffing, is also detected in infected ducks. Pathological changes in other organs, including enlargement of spleen and liver, and hemorrhage of pancreas and liver, were also detected in some cases (Li et al., 2015a; Sun et al., 2014; Thontiravong et al., 2015). To date, no commercial DTMUV vaccine is currently available for preventing DTMUV infection. Strict biosecurity and the culling of DTMUV infected ducks are the only effective measures to prevent and control DTMUV infection in ducks. Thus, the efficient diagnostic methods for early detection of DTMUV are urgently needed for effective control and prevention of this emerging disease.

As a member of the flavivirus genus, which contains the viruses that cause serious diseases in humans and most of them are zoonotic pathogens, the public health concern regarding to the zoonotic potential of DTMUV has been raised. Although no human cases of DTMUV-related disease have been reported and a recent study showed that DTMUV is not pathogenic to non-human primates (Wang et al., 2016), DTMUV-specific antibodies and DTMUV RNA were identified in duck farm workers in China (Tang et al., 2013b). As described above, DTMUV can infect a variety of avian and non-avian species, indicating the expansion of its host range. Therefore, the establishment of the efficient diagnostic methods for DTMUV is essential for monitoring of DTMUV in animals and humans in order to prevent zoonotic potential in humans.

In Thailand, a severe, contagious disease in ducks, resembling to DTMUV infection, has been reported since 2013. The disease spread rapidly in the high density duck-producing areas of Thailand, causing economic losses in both traditional and agro-industrial duck businesses (Chakritbudsabong et al., 2015; Thontiravong et al., 2015). Our previous study demonstrated that this emerging disease is caused by DTMUV (Thontiravong et al., 2015). Genetic analysis revealed that Thai DTMUVs formed a cluster with mosquito-borne flaviviruses and were grouped with Chinese DTMUVs. Thai DTMUVs showed the higher nucleotide identity with DTMUVs reported from China (97.9%) than with those reported from Malaysia (90.3%) (Thontiravong et al., 2015). After the initial outbreak, DTUMV has been continuously caused outbreaks and has become widely spread in several duck producing areas of Thailand (Ninvilai et al., 2019). Our previous study revealed that

DTMUVs circulating in Thailand were divided into 3 distinct clusters, including cluster 1, subcluster 2.1 and cluster 3, indicating the high genetic diversity of DTMUVs in Thailand (Ninvilai et al., 2019). This finding increases the importance of DTMUV surveillance in ducks for early detection of the emergence of new genetic variant strains that may threaten animal and human health. Therefore, the efficient detection and isolation of DTMUV from both surveillance and clinical samples is essential.

Following the emergence of DTMUV, the efficient detection and isolation of DTMUV is needed for preventing economic losses in animal production as well as zoonotic potential in humans. Currently, various methods have been used to detect DTMUV, including antigen detection methods, molecular-based assays, virus isolation in host system and serological assays (Cao et al., 2011; Tang et al., 2015; Wang et al., 2016; Yin et al., 2013). However, the virus isolation and/or RT-PCR are the primary methods widely used to detect DTMUV from clinical samples (Homonnay et al., 2014; Su et al., 2011; Thontiravong et al., 2015). Although virus isolation is time-consuming method, this technique is a necessary step for confirming the virus infection, for producing enough virus to further detailed antigenic and genetic characterization of the epidemic strains and particularly for evaluating the viability of virus, which is important for determination of the risk of virus transmission among animal species. In addition, studies on viral pathogenesis, vaccine production and diagnostic test development rely on successful virus isolation and propagation (Li et al., 2014; Lu et al., 2016; Yin et al., 2013; Zhang et al., 2017a). Therefore, the establishment of an efficient host system for isolation and propagation of DTMUV is essential. Several host systems, including different avian embryonated eggs and cell cultures, have been used for DTMUV isolation and propagation (Chen et al., 2014; Lei et al., 2017; Su et al., 2011; Thontiravong et al., 2015). However, differences in their ability to support the replication of DTMUV have never been evaluated and the most efficient host system for DTMUV isolation is unknown. In this study, various host systems, including avian embryonated eggs (chicken and duck) and cell cultures (primary duck embryo fibroblast (DEF) cells, primary chicken fibroblast (CEF) cells, African green monkey kidney (Vero) cells, baby hamster kidney (BHK-21) cells and *Aedes albopictus* clone C6/36 (C6-36) cells), were evaluated and compared for their ability to support DTMUV isolation and propagation. The infectivity and replication kinetic of DTMUV as well as isolation rate of DTMUV from duck experimental and clinical samples were examined in these host systems in order to identify the most suitable host system for DTMUV isolation and propagation.

**Objectives**

1. To evaluate the infectivity and replication kinetic of newly emerged DTMUV in different host systems.
2. To examine the isolation rate of newly emerged DTMUV from duck experimental and clinical samples in different host systems.
3. To identify the most suitable host system for isolation and propagation of newly emerged DTMUV.

**Research methodology**

Methodology for achieving the objectives of this project is as follows (Fig. 1).

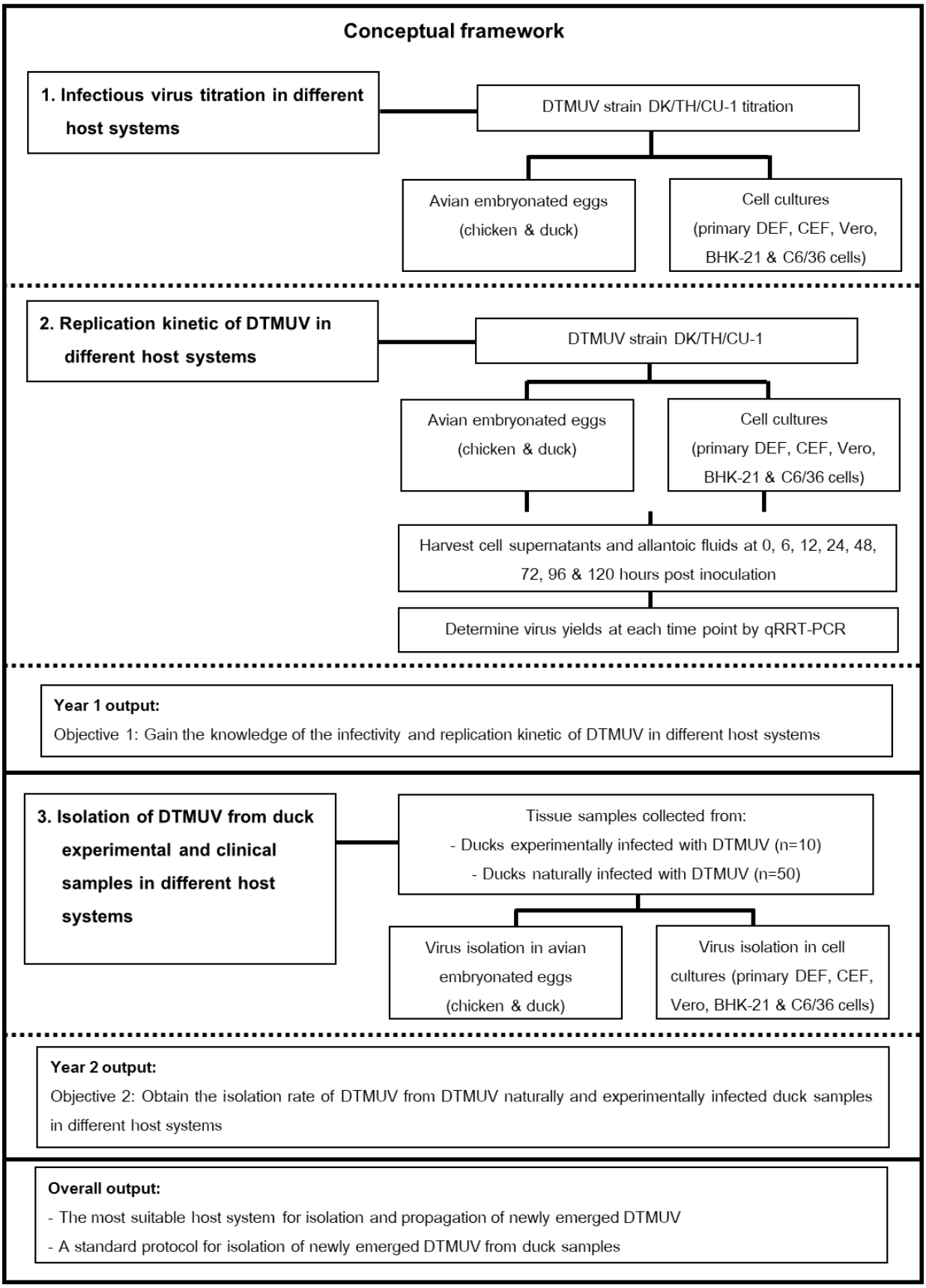


**Fig. 1.** Conceptual framework of this study.

**Objective 1:** To evaluate the infectivity and replication kinetic of newly emerged DTMUV in different host systems.

**Virus, cells and eggs**

DTMUV strain DK/TH/CU-1 (GenBank Accession No. KR061333) was used as a representative strain for evaluating the infectivity and replication kinetic of DTMUV in different host systems. This virus was isolated from DTMUV-infected ducks in Thailand and was genetically classified as DTMUV cluster 2, which is a predominant cluster of DTMUV circulating in duck populations in Asia (Ninvilai et al., 2019; Thontiravong et al., 2015). This virus was propagated in 9-day-old embryonated duck eggs as described previously (Thontiravong et al., 2015). Multiple aliquots of stock viruses were stored at 80°C until used for virus infectivity and virus replication kinetic studies. Virus propagation and handling were performed in a BSL-2 containment facility.

Five cell cultures, including primary duck embryo fibroblast (DEF) cells, primary chicken fibroblast (CEF) cells, African green monkey kidney (Vero) cells, baby hamster kidney (BHK-21) cells and *Aedes albopictus* clone C6/36 (C6-36) cells, were used in this study. These cell cultures were selected for evaluation in this study because they are often used for isolation and propagation of many flaviviruses, including DTMUV (Chen et al., 2014; Lei et al., 2017; Su et al., 2011; Thontiravong et al., 2015). Primary CEF cells and primary DEF cells were prepared from 10-day-old chicken and duck embryos, respectively. Primary DEF cells, primary CEF cells and Vero cells were maintained in minimal essential medium (MEM) (Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA). BHK-21 cells and C6-36 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS (Invitrogen, Carlsbad, CA). All cell cultures were grown and maintained at 37°C with 5% $CO_2$ using standard culture procedure. Nine-day-old embryonated chicken and duck eggs were used in this study for evaluation of the infectivity and replication kinetic of DTMUV as well as the isolation rate of DTMUV from duck clinical samples.

**Infectious virus titration in different host systems**

The infectivity of DTMUV in different host systems was evaluated by virus titration in avian embryonated eggs (chicken and duck) and cell cultures (primary DEF cells, primary CEF cells, Vero cells, BHK-21 cells and C6/36 cells). To determine the infectious titer of DTMUV in different cell cultures, 100 µl of 10-fold serial dilution of DTMUV strain DK/TH/CU-1 in MEM or DMEM supplemented with 10% FBS was inoculated onto 96-well plates containing each cell culture. Plates were incubated at 37ºC with 5% $CO_2$ for 5 days and examined microscopically for the presence of a

cytopathic effect (CPE) (Wang et al., 2016). Virus presence was confirmed by immunocytochemistry staining using an anti-flavivirus group antigen antibody (clone D1-4G2-4-15, Merck Millipore, Billerica, MA) and by RT-PCR using DTMUV E gene specific primers as described previously (Thontiravong et al., 2015). The virus titers were expressed as $\log_{10}$ 50% tissue culture infectious dose ($TCID_{50}$)/ml calculated by the Reed and Muench method (Reed and Muench, 1938). To determine the infectious titer of DTMUV in embryonated eggs, six 9-day-old embryonated chicken or duck eggs were inoculated via the allantoic cavity with 100 µl of the same 10-fold serial dilution of DTMUV strain DK/TH/CU-1 that was inoculated into the cell cultures. Embryos were examined twice a day for 5 days and allantoic fluids were harvested at 5 days post inoculation or upon embryo death. DTMUV E gene specific RT-PCR was performed to confirm the presence of DTMUV in embryonated eggs as described previously (Thontiravong et al., 2015). The virus titers were expressed as $\log_{10}$ 50% embryo infectious dose (EID50)/ml calculated by the Reed and Muench method (Reed, 1938). The infectious doses ($ID_{50}$)/ml of DTMUV in different host systems were compared for evaluation of the ability of these host systems to support DTMUV infection and replication (Jahangir et al., 2010; Moresco et al., 2010).

**Replication kinetic of DTMUV in different host systems**

The replication kinetic of DTMUV in different host systems was evaluated and compared by virus replication kinetic study in avian embryonated eggs (chicken and duck) and cell cultures (primary DEF cells, primary CEF cells, Vero cells, BHK-21 cells and C6/36 cells). To determine virus replication kinetics in cell cultures, monolayers of each cell culture were inoculated in triplicate with DTMUV strain DK/TH/CU-1 at a multiplicity of infection (MOI) of 0.01 at 37°C. After 1 hour of inoculation, cell monolayers were washed and overlaid with MEM or DMEM supplemented with 10% FBS and then placed at 37°C. To determine virus replication kinetics in embryonated eggs, 9-day-old embryonated chicken or duck eggs were inoculated in triplicate with the same titer of DTMUV strain DK/TH/CU-1 that was used in cell cultures ($10^3$ $TCID_{50}$/ml). The eggs were incubated at 37°C. Cell supernatants and allantoic fluids were harvested at 0, 6, 12, 24, 48, 72, 96 and 120 hours post inoculation. Virus yields at each time point in different host systems were determined by quantitative real-time RT-PCR assay (qRRT-PCR) using DTMUV E gene specific primers and probe and expressed as $\log_{10}$ DTMUV RNA copies/ml as described previously (Yan et al., 2011a).

**Objective 2:** To examine the isolation rate of newly emerged DTMUV from duck experimental and clinical samples in different host systems.

**Isolation of DTMUV from duck experimental samples in different host systems**

To examine the isolation rate of DTMUV from duck experimental samples in different host systems, virus isolation was performed on tissue samples from ducks experimentally infected with DTMUV obtained from our previous study (Ninvilai et al., 2020). Briefly, target tissue samples collected from ducks experimentally infected with DTMUV (brain and spleen; n=10) were homogenized and then filtered through 0.45 µm filters. The tissue suspensions were used for virus isolation in avian embryonated eggs (chicken and duck) and cell cultures (primary DEF cells, primary CEF cells, Vero cells, BHK-21 cells and C6/36 cells). For virus isolation in cell cultures, tissue suspensions were inoculated onto each cell culture and incubated at 37ºC with 5% $CO_2$. Each sample was inoculated in three wells of 24-well plates containing each cell culture. After 1 hour of incubation, the cell culture medium was added, and the inoculated cells were incubated at 37ºC with 5% $CO_2$ for 5 days. Inoculated cells were examined daily for the presence of CPE. The presence of DTMUV in cell cultures was confirmed by RT-PCR using DTMUV E gene specific primers as described previously (Thontiravong et al., 2015). For virus isolation in embryonated eggs, the tissue suspensions were inoculated into the allantoic cavity of embryonated chicken or duck eggs. Each sample was inoculated in three eggs. Embryos were examined twice a day for 5 days and allantoic fluids were harvested at 5 days post inoculation or upon embryo death. DTMUV E gene specific RT-PCR was performed to confirm the presence of DTMUV in embryonated eggs as described previously (Thontiravong et al., 2015). The DTMUV isolation rates from duck experimental samples in different host systems were compared for evaluation of the ability of these host systems to support DTMUV isolation.

**Isolation of DTMUV from duck clinical samples in different host systems**

To examine the isolation rate of DTMUV from duck clinical samples in different host systems, virus isolation will be performed on DTMUV-positive samples. A total of 50 RT-PCR-positive tissue samples (1 DTMUV cluster 1, 48 DTMUV cluster 2 and 1 DTMUV cluster 3) obtained from DTMUV suspected cases in Thailand during 2007-2017 were used in this study (Ninvilai et al., 2018; Ninvilai et al., 2019; Thontiravong et al., 2015). Briefly, tissue samples were homogenized and then filtered through 0.45 µm filters. The tissue suspensions were used for virus isolation in avian embryonated eggs (chicken and duck) and cell cultures (primary DEF cells, primary CEF cells, Vero cells, BHK-21 cells and C6/36 cells). The tissue suspensions were inoculated into each of three 10-day old embryonated chicken or duck eggs and three wells of 24-well plates containing each cell

culture as mentioned above. The inoculated cells and embryos were incubated at 37ºC for 5 days. The presence of DTMUV was confirmed by RT-PCR using DTMUV E gene specific primers as described previously (Thontiravong et al., 2015). The virus isolation rates from duck clinical samples in different host systems were compared for evaluation of the ability of these host systems to support DTMUV isolation.

**Statistical analysis**

Data obtained from virus replication kinetic and virus isolation studies were expressed as the mean ± standard deviation and DTMUV isolation rate (%), respectively. Differences in virus infectious titers, virus yields and DTMUV isolation rates among different host systems were evaluated by analysis of variance (ANOVA) or Chi-Square test, respectively, using the GraphPad Prism 5.0 software (GraphPad Software Inc. La Jolla, CA). All *P* values < 0.05 were considered statistically significant.

**Results**

**Objective 1:** To evaluate the infectivity and replication kinetic of newly emerged DTMUV in different host systems.

**Infectious virus titration in different host systems**

To assess which host system could be used as the most suitable host system for DTMUV isolation and propagation, the infectivity of DTMUV in different host systems was first determined. In this study, DK/TH/CU-1 was titrated in various avian embryonated eggs and cell cultures. The $EID_{50}$ results from avian embryonated eggs inoculated with DK/TH/CU-1 were compared to TCID50 from cell cultures inoculated with the same virus. The results showed that DK/TH/CU-1 could infect all host systems tested in this study, indicating the susceptibility of these host systems to DTMUV infection (Table 1; Fig. 2). However, the highest infectious titer of DTMUV was obtained from primary DEF (7 $\log_{10}$ $ID_{50}$/ml) and BHK-21 (7 $\log_{10}$ $ID_{50}$/ml) cells, followed by embryonated duck eggs (6 $\log_{10}$ $ID_{50}$/ml), C6/36 (5 $\log_{10}$ $ID_{50}$/ml) cells, embryonated chicken eggs (4.5 $\log_{10}$ $ID_{50}$/ml), primary CEF (4 $\log_{10}$ $ID_{50}$/ml) and Vero (2.67 $\log_{10}$ $ID_{50}$/ml) cells (Table 1; Fig. 2). It is noted that the DTMUV infectious titers in primary DEF and BHK-21 were significantly higher than those in Vero cells (Table 1). In cell cultures, the typical CPE for DTMUV infection was characterized by cell rounding and focal detachment (Fig. 2). CPE was observed in primary DEF, CEF and BHK-21 cells (48 hpi) earlier than in Vero and C6/36 cells (72 hpi) (Table 1). It is noted that CPE observed in primary DEF and BHK-21 cells was found to be more prominent than that observed in other cell

cultures tested (Fig. 2). DTMUV infection in different cell cultures and avian embryonated eggs was confirmed by ICC staining and DTMUV E gene specific- RT-PCR. All these host systems were found to be positive for DTMUV by ICC staining (for cell cultures) and DTMUV E gene specific- RT-PCR (for cell cultures and avian embryonated eggs) (Fig. 2). In addition, our result showed that duck embryos infected with DK/TH/CU-1 died earlier (72 hpi) when compared to chicken embryos infected with the same virus (96 hpi) (Table 1). Taken together, these results indicated that all the host systems tested in this study, particularly primary DEF, BHK-21 cells and embryonated duck eggs, were susceptible to DTMUV infection.

**Table 1** Comparison of the infectivity of DTMUV in different avian embryonated eggs and cell cultures.

| **Host** | **CPE/embryo dead (hpi)**[†] | **DTMUV infectious titer ($log_{10}$ $ID_{50}$/ml)**[‡] |
|---|---|---|
| Duck eggs | 72 | 6 |
| Chicken eggs | 96 | 4.5 |
| Primary DEF cells | 48 | 7* |
| Primary CEF cells | 48 | 4 |
| BHK-21 cells | 48 | 7* |
| Vero cells | 72 | 2.67 |
| C6/36 cells | 72 | 5 |

[†]hpi indicates hours post inoculation when CPE or embryo dead was observed.

[‡]$ID_{50}$/ml indicates $TCID_{50}$/ml for cell cultures or $EID_{50}$/ml for avian embryonated eggs.

*$P$<0.05, one-way ANOVA for DTMUV infectious titers compared between primary DEF, BHK-21 and Vero cells.

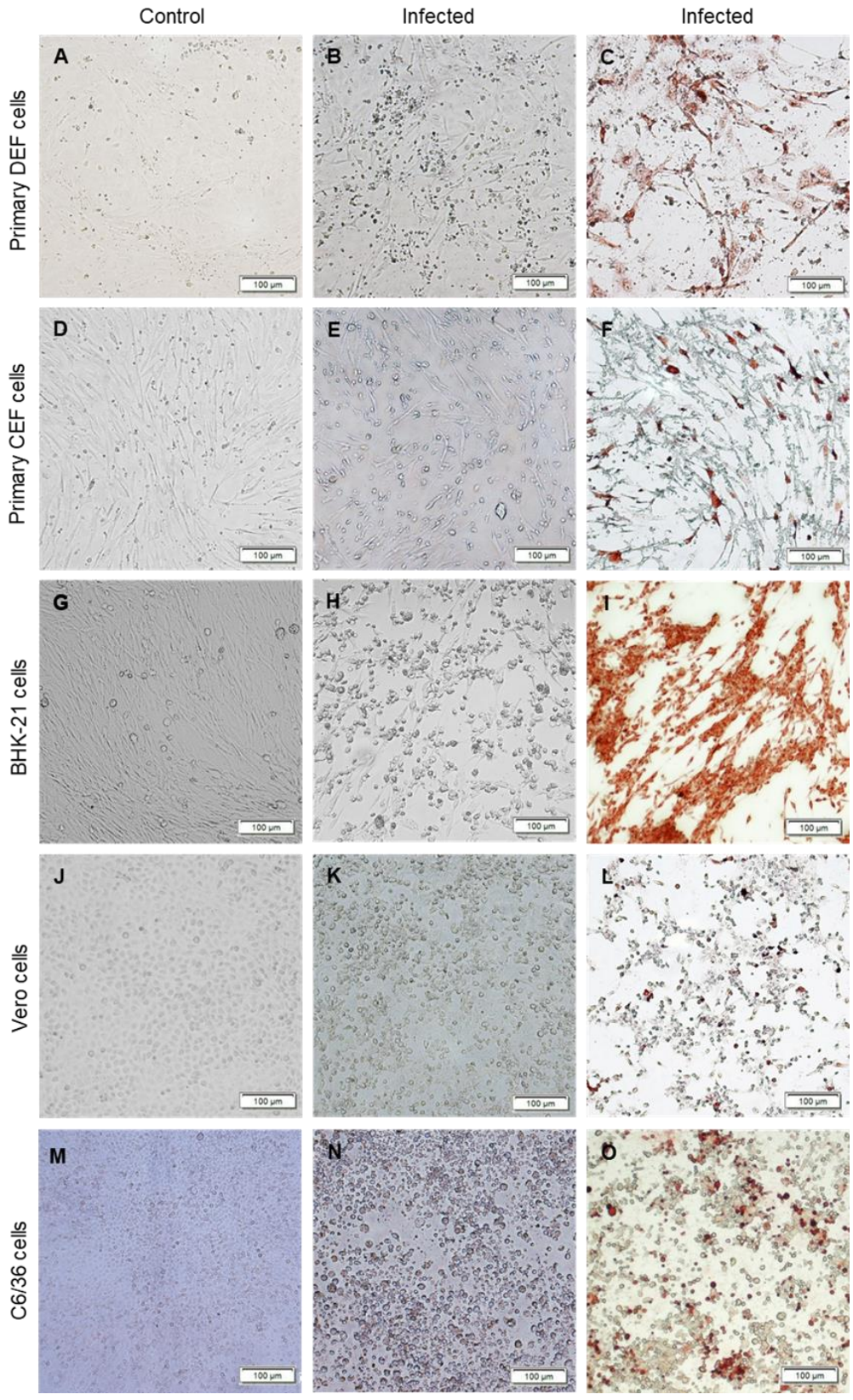


**Fig. 2.** Susceptibility of different cell cultures to DTMUV infection. The cells were infected with DTMUV at MOI of 0.01 and observed for the development of cytopathic effect (CPE) for 5 days. CPE with cell rounding and focal detachment (B, E, H, K and N) and flavivirus antigen stained red

brown (C, F, I, L and O) were observed in DTMUV-infected cells at 72-96 hours post inoculation (hpi). Scale bar, 100 μm.

**Replication kinetics of DTMUV in different avian embryonated eggs and cell cultures**

To evaluate the replication kinetics of DTMUV in different host systems, various avian embryonated eggs and cell cultures were infected with DK/TH/CU-1, after which the allantoic fluids or cell supernatants were harvested at different time points. In general, DK/TH/CU-1 replicated efficiently in all host systems tested with peak viral RNA levels ranging from 5.16 to 9.89 $\log_{10}$ copies/μl within 72-120 hpi (Table 2; Fig. 3). However, BHK-21 cells had the highest ability to support the replication of DTMUV among host systems tested, as observed by the highest maximum viral RNA level in BHK-21 cells (Table 2; Fig. 3). It is noted that the maximum viral RNA level in BHK-21 cells (9.89 $\log_{10}$ copies/μl) was significantly higher than that in embryonated chicken eggs (8.72 $\log_{10}$ copies/μl), primary CEF (7.14 $\log_{10}$ copies/μl) and C6/36 (5.16 $\log_{10}$ copies/μl) cells ($P$<0.05) but did not differ significantly from that in embryonated duck eggs (9.12 $\log_{10}$ copies/μl), primary DEF (9.53 $\log_{10}$ copies/μl) and Vero (9.83 $\log_{10}$ copies/μl) cells (Table 2; Fig. 3). However, our result showed that DK/TH/CU-1 reached maximum viral RNA level in embryonated duck eggs and primary DEF cells within 72 hpi, which was earlier than in other host systems (Table 2; Fig. 3). Collectively, these findings demonstrated that DTMUV replicated more efficiently in BHK-21 cells, albeit with slightly slower than in embryonated duck eggs and primary DEF cells.

**Table 2** Replication kinetics of DTMUV in different avian embryonated eggs and cell cultures. Cells and eggs were infected with each virus at MOI of 0.01 or $10^3$ $TCID_{50}$/ml, respectively. The level of viral RNA was determined by qRT-PCR at the indicated time points. Each data point represents the mean ± standard deviation (SD) of three independent experiments.

| Hours post inoculation | Mean DTMUV RNA copy number ± SD ($log_{10}$ copies/ml) | | | | | | |
|---|---|---|---|---|---|---|---|
| | **Duck eggs** | **Chicken eggs** | **DEF** | **CEF** | **BHK-21** | **Vero** | **C6/36** |
| 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 6 | 4.80 ± 0.21 | 5.56 ± 0.23 | 4.62 ± 0.46 | 3.91 ± 0.23 | 4.34 ± 0.20 | 4.85 ± 0.27 | 3.60 ± 0.21 |
| 12 | 6.07 ± 0.22 | 5.57 ± 0.29 | 5.57 ± 0.21 | 3.24 ± 0.30 | 4.82 ± 0.16 | 4.81 ± 0.10 | 3.69 ± 0.28 |
| 24 | 6.36 ± 1.63 | 5.58 ± 0.25 | 7.75 ± 0.28 | 3.99 ± 0.35 | 5.17 ± 0.27 | 3.81 ± 0.21 | 4.73 ± 0.04 |
| 48 | 8.27 ± 0.30 | 6.76 ± 0.96 | 9.26 ± 0.17 | 6.86 ± 0.53 | 6.88 ± 0.42 | 6.78 ± 0.52 | 5.22 ± 0.00 |
| 72 | 9.12 ± 0.57* | 6.00 ± 0.49 | 9.26 ± 0.56 | 7.14 ± 0.25 | 8.49 ± 0.04 | 7.87 ± 0.15 | 5.16 ± 0.14 |
| 96 | 8.92 ± 0.65 | 8.72 ± 0.36 | 8.76 ± 0.53 | 6.33 ± 0.14 | 9.17 ± 0.27 | 8.26 ± 0.30 | 4.87 ± 0.27 |
| 120 | 7.40 ± 0.66 | 4.70 ± 0.27 | 9.53 ± 0.34* | 6.91 ± 0.21 | 9.89 ± 0.29* | 9.83 ± 0.09* | 5.00 ± 0.47 |

* $P<0.05$ (one-way ANOVA) when compared the maximum viral RNA levels among different host systems tested.

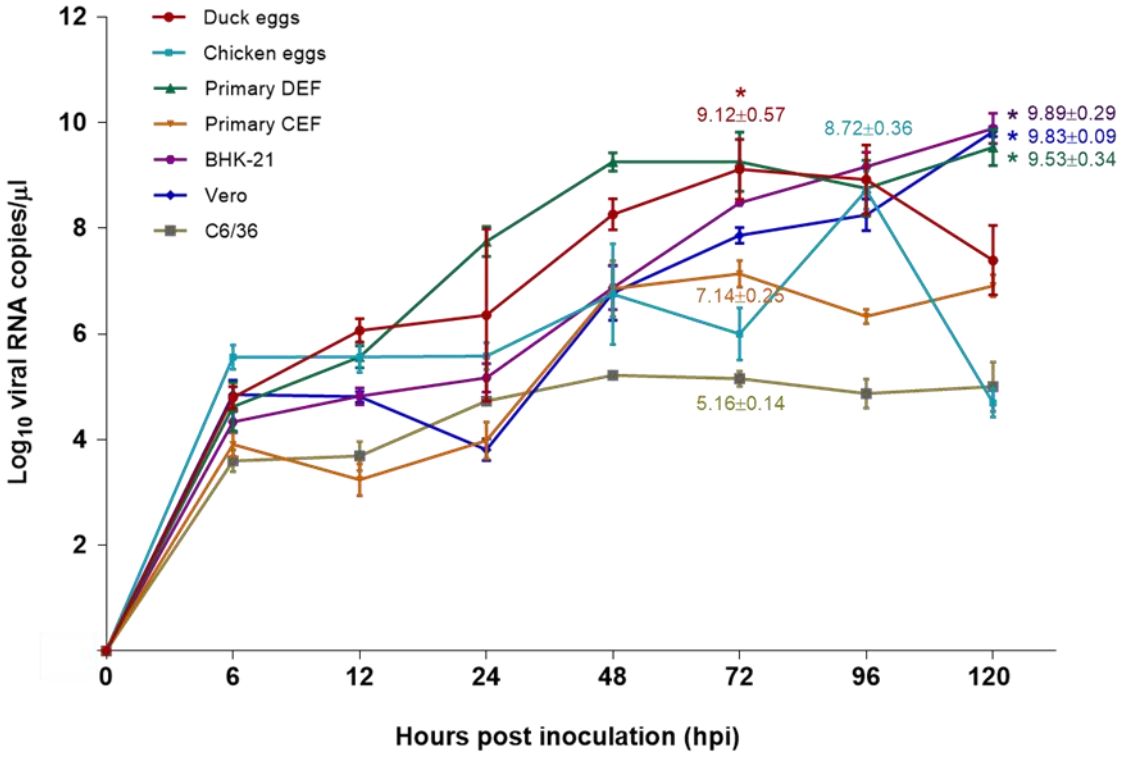


**Fig. 3.** Replication kinetics of DTMUV in different avian embryonated eggs and cell cultures. Cells and eggs were infected with each virus at MOI of 0.01 or $10^3$ $TCID_{50}$/ml, respectively. The level of viral RNA was determined by qRT-PCR at the indicated time points. Each data point represents the mean ± standard deviation (SD) of three independent experiments. Asterisks indicate the comparison of maximum viral RNA levels among different host systems tested ($P$<0.05, one-way ANOVA). The numbers at data points show the maximum viral RNA level ± SD of each host system.

**Objective 2:** To examine the isolation rate of newly emerged DTMUV from duck experimental and clinical samples in different host systems.

**Isolation of DTMUV from duck experimental and field clinical samples in different avian embryonated eggs and cell cultures**

To evaluate the efficiency of DTMUV isolation in different host systems, known DTMUV positive tissue samples (n=10) of experimentally DTMUV-infected ducks obtained from our previous study were first inoculated into various avian embryonated eggs and cell cultures (Ninvilai et al., 2020). The results showed that embryonated duck eggs, primary DEF, BHK-21, Vero and C6/36 cells gave a higher DTMUV isolation rate (100%) than embryonated chicken eggs and primary CEF cells (70%) when tested with known DTMUV positive tissue samples (Table 3).

In addition, a total of 50 RT-PCR-positive clinical samples obtained from DTMUV suspected cases in Thailand during 2007-2017 were tested by virus isolation in different host systems. Of the 50 RT-PCR-positive clinical samples tested, 23/50 (46%), 18/50 (36%), 25/50 (50%), 22/50 (44%), 32/50 (64%), 24/50 (48%) and 22/50 (44%) were positive for DTMUV by virus isolation in embryonated duck and chicken eggs, primary DEF, primary CEF, BHK-21, Vero and C6/36 cells, respectively (Table 4). All DTMUV positive samples by virus isolation were confirmed to be DTMUV positive by RT-PCR and DNA sequencing (data not shown). Among the host systems tested, BHK-21 cells had the highest DTMUV isolation rate even though the differences were not statistically significant (Table 4). Notably, although DTMUV cluster 1 and 2 were successfully recovered from all host systems with different isolation efficiencies, DTMUV cluster 3 was isolated only from BHK-21 and C6/36 cells (Table 4).

Taken together, these findings indicated that BHK-21 cells were the most efficient host system for isolation of all circulating DTMUV clusters from duck clinical samples.

**Table 3** DTMUV virus isolation rates from duck experimental samples in different avian embryonated eggs and cell cultures.

| **Host** | **No. of virus isolates recovered/No. of samples tested** | **DTMUV isolation rate (%)** |
|---|---|---|
| Duck eggs | 10/10 | 100.00 |
| Chicken eggs | 7/10 | 70.00 |
| DEF cells | 10/10 | 100.00 |
| CEF cells | 7/10 | 70.00 |
| BHK-21 cells | 10/10 | 100.00 |
| Vero cells | 10/10 | 100.00 |
| C6/36 cells | 10/10 | 100.00 |

**Table 4** DTMUV virus isolation rates from duck clinical samples in different avian embryonated eggs and cell cultures.

| Host | DTMUV isolation rate (No. of virus isolates recovered/No. of samples tested) | | | |
|---|---|---|---|---|
| | **Total samples** | **DTMUV cluster 1** | **DTMUV cluster 2** | **DTMUV cluster 3** |
| Duck eggs | 46% (23/50) | 100% (1/1) | 45.83% (22/48) | 0% (0/1) |
| Chicken eggs | 36% (18/50) | 100% (1/1) | 35.42% (17/48) | 0% (0/1) |
| Primary DEF cells | 50% (25/50) | 100% (1/1) | 50% (24/48) | 0% (0/1) |
| Primary CEF cells | 44% (22/50) | 100% (1/1) | 43.75% (21/48) | 0% (0/1) |
| BHK-21 cells | 64% (32/50) | 100% (1/1) | 62.50% (30/48) | 100% (1/1) |
| Vero cells | 48% (24/50) | 100% (1/1) | 47.92% (23/48) | 0% (0/1) |
| C6/36 cells | 44% (22/50) | 100% (1/1) | 41.67% (20/48) | 100% (1/1) |

**Conclusion and Discussion**

Since its emergence in 2010, DTMUV has widely spread and become endemic in duck populations in Asia, causing significant economic losses to duck producing industry (Su et al., 2011). To early detection and control of this disease, the establishment of effective diagnostic methods, including host systems for virus isolation, is essential. Several host systems have been used for isolation of DTMUV (Chen et al., 2014; Lei et al., 2017; Su et al., 2011; Thontiravong et al., 2015), but the most suitable host system for DTMUV isolation has never been reported. In this study, various host systems, including different avian embryonated eggs and cell cultures, were evaluated and compared for their ability to support the DTMUV isolation. Our results showed that all the host systems tested were susceptible to DTMUV infection, particularly primary DEF, BHK-21 cells and embryonated duck eggs. However, BHK-21 cells allowed more efficient replication of DTMUV and had the highest DTMUV isolation rate compared to other host systems tested. Collectively, our findings revealed that BHK-21 cells were the most efficient host system for isolation of DTMUV from duck clinical samples. To the best of our knowledge, this study is the first report on the evaluation of the host systems for efficient isolation and propagation of DTMUV.

In this study, all the host systems tested were found to be susceptible to DTMUV infection, which is consistent with previous studies showing successful isolation of DTMUV by using these host systems (Chen et al., 2014; Lei et al., 2017; O'Guinn et al., 2013; Su et al., 2011; Thontiravong et al., 2015). However, it is noted that DTMUV yielded higher titers in primary DEF, BHK-21 cells and embryonated duck eggs than in other host systems tested. This corresponds with recent studies reporting the presence of DTMUV specific receptors in primary DEF and BHK-21 cells (Wu et al., 2019; Zhao et al., 2018). In addition, this finding indicates that host systems derived from the same host species where virus were isolated displayed a high susceptibility to virus infection (Li et al., 2015b). Our result demonstrated that CPE was observed in primary DEF, CEF and BHK-21 cells within 48 hpi, which was earlier than that from the previous studies showing the presence of CPE of Chinese TMUV in BHK-21 cells after 72 hpi (Cao et al., 2011; Lei et al., 2017). In virus replication kinetic study, DK/TH/CU-1 replicated efficiently in all host systems tested, which corresponded to previous studies reporting the ability of mosquito-borne flaviviruses, including DTMUV, to infect and replicate in a variety of cell lines (Blitvich and Firth, 2015; Wang et al., 2016). However, among the host systems tested, DK/TH/CU-1 reached the highest maximum viral RNA level in BHK-21 cells, indicating the preference of DTMUV to grow in BHK-21 cells. This finding is consistent with a previous study reporting that Chinese DTMUV reached the highest peak titer in BHK-21 cells compared to other cell cultures tested, including Vero cells and chicken

embryo fibroblast cells (Wang et al., 2016). This suggests that DTMUV replicated more efficiently in BHK-21 cells, even though it grew slightly slower than in embryonated duck eggs and primary DEF cells. In addition, CPE observed in BHK-21 and primary DEF cells could easily be detected when compared to that observed in other cell cultures, indicating the advantage of using these cell cultures for DTMUV isolation. Taken together, these findings suggest that primary DEF, BHK-21 cells and embryonated duck eggs were highly susceptible to DTMUV infection and allowed DTMUV replication at high efficiency.

Corresponding to the findings from virus infectivity and replication kinetic studies, the isolation rate of DTMUV from duck clinical samples in BHK-21 cells was higher than that in other host systems. In addition, all circulating clusters of DTMUV were successfully recovered from BHK-21 cells. These findings suggest that BHK-21 cells appear to be the most efficient host system for isolation of all DTMUV clusters from duck clinical samples. BHK-21 is a widely used cell line, which provides several advantages over embryonated eggs and primary cell culture for virus isolation, including convenient, less time-consuming, high-volume sample processing and cost-effective (Leland and Ginocchio, 2007; Zhang et al., 2017a). It should be noted that low isolation rates of DTMUV from both experimental and clinical samples were observed in primary CEF cells and embryonated chicken eggs, indicating that these chicken-derived host systems had relatively low sensitivity for isolation of DTMUV from duck clinical samples. However, these findings might not be true for all strains of DTMUV circulating in other host species. Additional studies will be required to evaluate the host systems for efficient DTMUV isolation with other strains of DTMUV. Furthermore, although BHK-21 cells showed better results for isolation of DTMUV than other host systems, the use of other host systems, especially primary DEF cells, should be considered when BHK-21 cells fail to isolate DTMUV from RT-PCR-positive samples.

In conclusion, our data collectively support the use of BHK-21 cells as a host system for primary isolation of DTMUV from duck clinical samples. Our findings will also be useful for DTMUV research and possibly for vaccine production. This study highlights the importance of selecting the most appropriate host system for efficient isolation and propagation of DTMUV from duck clinical samples. However, regarding to the rapid evolution of DTMUV (Dai et al., 2015; Ninvilai et al., 2019), the continued validation of the virus isolation protocol with recent DTMUV strains will be required to ensure the highest sensitivity and accurate results.

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**Output** (Acknowledge the Thailand Research Fund)

**1. International journal publication**

1. Tunterak, W, Ninvilai, P, Tuanudom, R, Prakairungnamthip, D, Oraveerakul, K, Amonsin, A, **Thontiravong, A**. Evaluation of various host systems for efficient isolation and propagation of duck Tembusu virus (under review, Veterinary Microbiology; T1/Q1; Impact factor 2.791)
2. Ninvilai, P, Tunterak, W, Oraveerakul, K, Amonsin, A, **Thontiravong, A**. 2019. Genetic characterization of duck Tembusu virus in Thailand, 2015-2017: Identification of a novel cluster. Transboundary and Emerging Diseases 66(5):1982-1992. (T1/Q1; Impact factor 3.554)
3. Ninvilai, P, Limcharoen, B, Tunterak, W, Prakairungnamthip, D, Oraveerakul, K, Banlunara, W, **Thontiravong, A**. 2020. Pathogenesis of Thai duck Tembusu virus in Cherry Valley ducks: The effect of age on susceptibility to infection. Veterinary Microbiology 243: 108636. (T1/Q1; Impact factor 2.791)
4. Ninvilai, P, Tunterak, W, Prakairungnamthip, D, Oraveerakul, K, **Thontiravong, A**. 2020. Development and validation of a universal one-step RT-PCR assay for broad detection of duck Tembusu virus. Avian Diseases. Article in Press. (Q2; Impact factor 1.306)