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Experimental infection of Tilapia Lake Virus (TiLV) in Nile tilapia (*Oreochromis niloticus*) and red tilapia (*Oreochromis* spp.)

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ABSTRACT

Since 2015, a novel orthomyxo-like virus, tilapia lake virus (TiLV) has been associated with outbreaks of disease and massive mortality of cultured Nile and red tilapia (*Oreochromis niloticus* and *Oreochromis* spp., respectively) in Thailand. In this study, TiLV was isolated from field samples and propagated in the permissive E-11 cell line, with cytopathic effect (CPE) development within 3–5 days post-inoculation. Electron micrographs of infected E-11 cells and fish tissues confirmed the rounded, enveloped virions of 60 to 80 nm with characteristics very similar to those of *Orthomyxoviridae*. *In vivo* challenge studies showed that high mortality in Nile (86%) and red tilapia (66%) occurred within 4–12 days post-infection. The virus was re-isolated from challenged fish tissues in the permissive cell line, and PCR analysis confirmed TiLV as a causative pathogen. The distinct histopathology of challenged fish included massive degeneration and inflammatory cell infiltration in the liver and brain as well as the presence of eosinophilic intracytoplasmic inclusions in hepatocytes and splenic cells. Our results fulfilled Koch's postulates and confirmed that TiLV is an etiologic agent of mass mortality of tilapia in Thailand. The emergence of this virus in many countries has helped increase awareness that it is a potential threat to tilapia aquacultured in Thailand, Asia, and worldwide.

1. Introduction

Tilapine cichilds comprise the second most important freshwater fish aquacultured worldwide, with annual production totaling more than 3.5 million tonnes (FAO, 2014a; FAO, 2014b). The main tilapia producers are China, Indonesia, Egypt, Brazil, the Philippines, and Thailand (FAO, 2014a). In Thailand, Nile tilapia (*Oreochromis niloticus*) and red tilapia (*Oreochromis* spp.) are commonly raised in cages in rivers, exposing them to multiple pathogens including bacteria, viruses, fungi, and parasites. During 2015–2017, multiple outbreaks of farm-raised Nile and red tilapia with an unknown etiology were observed in Thailand. The cumulative mortality rate were in the range 20–100%. The disease has been called "tilapia one month mortality syndrome" as it is frequently reported within one month after fry or juvenile tilapias have been moved from hatcheries to the grow-out cages. The clinical signs and pathology of infected fish include anorexia, poor body condition, abnormal swimming, severe anemia, bilateral exophthalmia, skin erosion and congestion, scale protrusion, and abdominal swelling. Recently, an emerging tilapia orthomyxo-like virus named tilapia lake virus (TiLV) was reported in many moribund fish in Thailand (Surachetpong et al., 2017). TiLV was first identified in farm-raised and wild tilapia in Israel (Eyngor et al., 2014). Subsequently, the virus was reported in Ecuador, Colombia, Egypt, and Thailand (Bacharach et al., 2016; Fathi et al., 2017; Kembou Tsofack et al., 2016; Surachetpong et al., 2017). TiLV is an enveloped single-stranded RNA virus consisting of ten genomic segments. Of the ten segments, only the first segment shares weak sequence similarity to viruses in the family *Orthomyxoviridae* (Bacharach et al., 2016). In addition, the 3′ and 5′ untranslated region repeatable sequences, which are characteristic of the influenza virus, have been identified in all segments of the TiLV (Bacharach et al., 2016). In 2014, the syncytial hepatitis virus (SHV) was reported in intensive farmed-raised tilapia in Ecuador (Ferguson et al., 2014). A nucleotide sequence analysis suggested that TiLV and SHV could be the same virus (Del-Pozo et al., 2016).

Although outbreaks of TiLV infection in tilapia have been reported in Thailand and other countries (Surachetpong et al., 2017), Koch's postulates have not been fulfilled for this pathogen. It is important to demonstrate that a pathogen from field outbreaks can cause clinical infection when used to challenge healthy animals and that the virus can be re-isolated from the experimentally infected animals. The aims of this study were to confirm that TiLV isolated from diseased tilapia could cause disease and mortality in healthy tilapia after experimental

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Table 1
Details of clinical isolates.

Sample no.	Location	Collection date	Clinical signs	PCR test
1	Ang Thong	02/06/16	+	+
2	Pathum Thani	22/06/16	+	+
3	Pathum Thani	28/06/16	+	+
4	Ang Thong	01/07/16	+	+
5	Pathum Thani	02/08/16	+	+
6	Pathum Thani	05/08/16	+	+
7	Ang Thong	16/08/16	+	+
8	Ang Thong	22/08/16	+	+
9	Kanchanaburi	24/08/16	+	+
10	Pathum Thani	16/09/16	+	+
11	Pathum Thani	02/10/16	+	+
12	Pathum Thani	05/10/16	+	+
13	Pathum Thani	16/10/16	+	+
14	Ang Thong	25/11/16	+	+
15	Pathum Thani	26/11/16	+	+

challenge and to develop laboratory-controlled challenge of TiLV infection. The identification and isolation of TiLV from clinically diseased fish, reproduction of clinical symptoms and mortality in healthy fish, and re-isolation of the same pathogen would fulfil Koch's postulates.

2. Materials and methods

2.1. Clinical samples

Nile tilapia (*Oreochromis niloticus*) and red tilapia (*Oreochromis* spp.) were collected from the provinces of Ang Thong, Kanchanaburi, and Pathum Thani which are located in central and western Thailand (Table 1). These three separate locations (80 to 180 km apart) suffered from multiple outbreaks with an unidentified etiology and a mortality rate above 3% per day for 3–5 consecutive days. Twenty Nile and red tilapia were collected from each outbreak (a total of 15 outbreaks) to investigate the disease. The experimental protocols and animals used were approved by the Institutional Animal Care and Use Committee of Kasetsart University under permit number OACKU00659. The permit allowed the collection of clinical samples of Nile and red tilapia from natural outbreaks. In addition, the permit allowed the use of laboratory animals for virus isolation and experimental challenge.

2.2. RNA isolation and polymerase chain reaction

Total RNA was extracted from the fish brains or inoculated E-11 cells using TRIzol™ reagent (Invitrogen, USA) according to the manufacturer's protocol. For cDNA synthesis, reverse transcription (RT) was carried out using a Viva 2-steps RT-PCR kit (Vivantis, Malaysia) in 20 µl reaction containing 2× mastermix, 4 µM oligo(dT) primer, and 1 mM dNTP. The RT reaction was performed at 42 °C for 60 min and inactivated at 85 °C for 5 min. For PCR reaction, cDNA templates were amplified with specific primers for TiLV and other viruses (Table 2). Each PCR mastermix contained 2 µl cDNA template, 0.2 µM of forward and reverse primer, 2 mM $MgCl_2$, 0.2 mM dNTP, and 0.05 U/µl Taq DNA polymerase (Invitrogen, USA). The PCR cycling conditions were denaturation at 95 °C for 5 min, followed by 40 cycles at 95 °C for 30 s, 58 °C for 30 s, and 72 °C for 30 s, with final elongation at 72 °C for 5 min using a T100 PCR thermocycler (Bio-Rad, USA). The PCR products were separated on 2% agarose gel and stained with ethidium bromide. The β-actin gene was used as an internal control gene to show the quality of the genomic RNA and DNA.

2.3. Virus isolation

The virus was isolated in E-11 cells, a continuous cell line from snakehead fish (*Ophicephalus striatus*). The E-11 cells were purchased from the European Collection of Authenticated Cell Cultures (ECACC), England (catalogue number 01110916) and were propagated in Liebovitz L-15 supplemented with 5% (vol/vol) fetal bovine serum (Sigma, USA) and 2 mM L-glutamine, and maintained at 25 °C without CO_2. Briefly, brain tissues were homogenized in 10% Hank's balanced salt solution (HBSS) and then centrifuged at 3,000 × *g* for 10 min. The supernatant was collected and filtered through a 0.22 µm filter membrane. Five hundred microliters of the filtered sample were inoculated into confluent E-11 cells in a 25 cm^2 flask and maintained at 25 °C for 14 days. The cells were observed daily. The supernatant was collected and stored at −80 °C for animal challenge studies.

2.4. Challenge study

A sample of 35 Nile tilapia and red tilapia weighing 30 g ± 0.52 g each were housed in the animal facility, Faculty of Veterinary Medicine, Kasetsart University. The water quality parameters of temperature, dissolved oxygen, pH, ammonia, and nitrite were monitored daily. The animals were monitored three times per day with all efforts made to minimize animal distress. Prior to the experimental challenge study, five fish were randomly selected to undergo screening for bacterial and viral infections, consisting of *Streptococcus agalactiae*, *Aeromonas* spp., betanodavirus, iridovirus, and TiLV, using a bacterial culture on tryptic soy agar (TSA) and PCR analysis. To prepare for the viral challenge, fish were sedated in 40 mg/L eugenol solution (Aquanes®, Better Pharma, Thailand). Fish in the control group were injected intraperitoneally with supernatant from normal E-11 cells, while fish in the infected group were injected with supernatant from E-11 cells infected with TiLV at a dose of 1×10^6 $TCID_{50}$/fish. The $TCID_{50}$ was calculated following the method of Reed and Muench (Muench, 1938). Clinical signs after virus inoculation were monitored daily for 14 days and the gross pathology and mortality rate were recorded daily. The decision criteria to euthanize animals included two or more clinical signs (poor body condition, severe skin erosion and hemorrhage, loss of balance, extensive abdominal swelling, scale protrusion, and exophthalmia). At 14 dpi, all surviving fish were euthanized using an overdose of eugenol solution. Brains from the control and virus challenge groups were taken for PCR examination. Cumulative mortality curves from the challenge study were generated using the GraphPad Prism software (version 5.01).

Table 2
Primers used in this study.

Target name	Primer name	Sequence (5′-3′)	Size (bp)	References
Iridovirus	1-F	5′-CTC-AAA-CAC-TCT-GGC-TCA-TC-3′	570 bp	(Kurita et al., 1998)
	1-R	5′-GCA-CCA-ACA-CAT-CTC−CTA-TC-3′		
Betanodavirus	OPVP75	5′-CGT-GTC-GGT-GCT-GTG-TCG-CT-3′	421 bp	(Bigarre et al., 2009)
	R3	5′-CGA-GTC-AAC-ACG-GGT-GAA-GA-3′		
TiLV	Nested ext-2	5′-TTG-CTC-TGA-GCA-AGA-GTA-CC-3′	491 bp	(Eyngor et al., 2014)
	Nested ext-1	5′-TAT-GCA-GTA-CTT-TCC−CTG-CC-3′		
β-actin	β-actin _F	5′-TCC-AAT-TTA-TTG-GCC-TTC-GTT-GC-3′	114 bp	(Wang et al., 2014)
	β-actin _R	5′-CTT-CCA-TTT-TCT-GTG-TGA-GGG-AGG-3′		

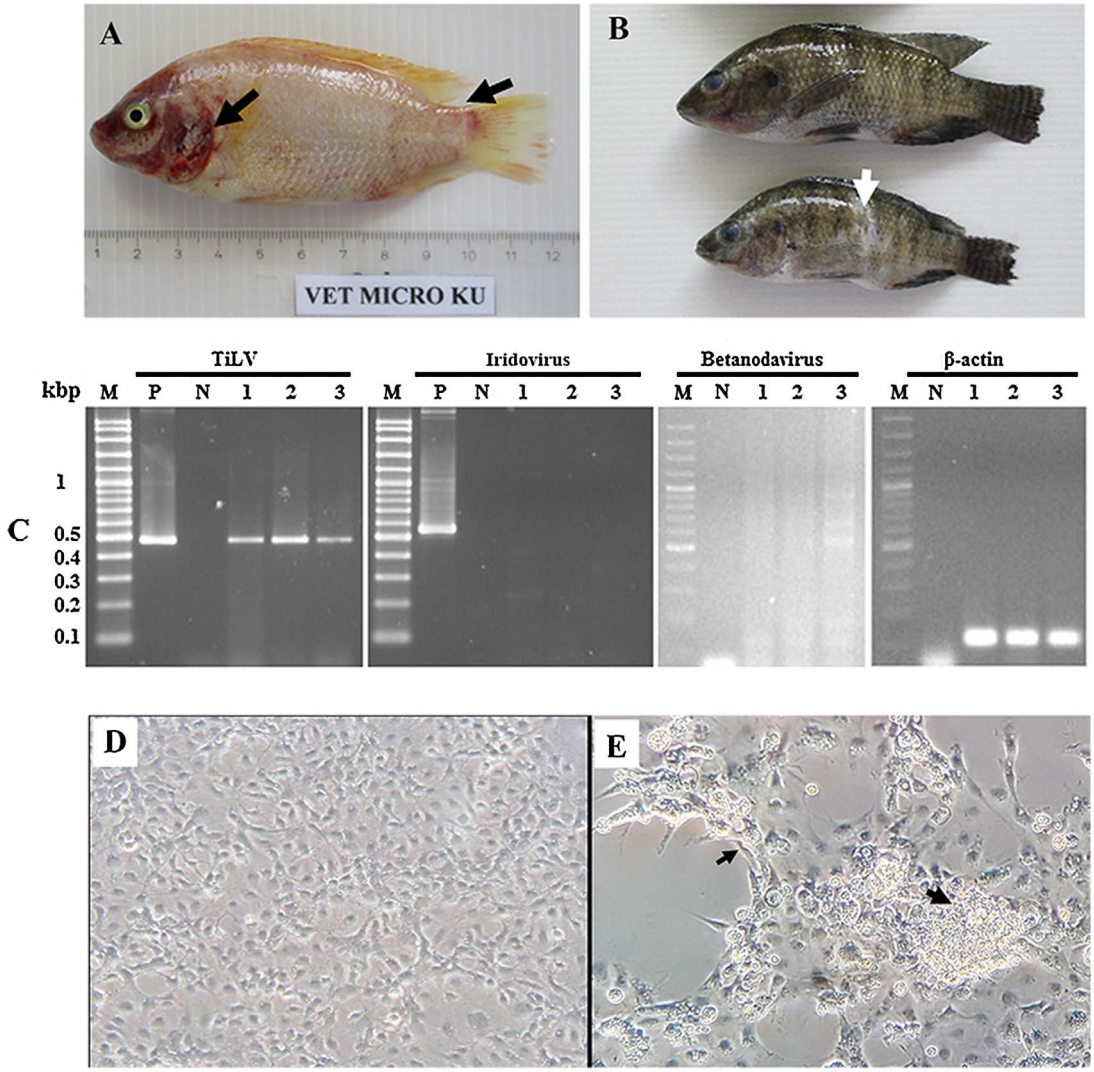

Fig. 1. Detection and isolation of TiLV in clinical samples. (A) Gross signs of infected red tilapia; distinct skin redness (black arrow) and skin erosion. (B) Nile tilapia; skin erosion (white arrow). (C) Infected fish were screened for virus infections using specific primers to TiLV, iridovirus, and betanodavirus. The β-actin was used as an internal control. Viruses were isolated in E-11 cells. (D) E-11 cells inoculated with normal tilapia brain. (E) E-11 cells inoculated with TiLV-positive brain, CPE with cell shrinkage and syncytial formation (black arrow), 4 dpi at a magnification of 20×.

2.5. Histopathology

Tissue samples (brain, anterior kidney, liver, and spleen) from the control and infected groups (three per group) were collected for histological study at 7 dpi. These samples were preserved in 10% (vol/vol) neutral buffered formalin. After 24 h, the formalin was replaced with fresh 10% formalin solution. Tissue sections were embedded with paraffin and stained with hematoxylin and eosin (H & E) using standard histological procedures.

2.6. Electron microscopy

For electron microscopy studies, infected cells with CPE were harvested at 5 dpi. Cells were scraped from culture flasks and centrifuged (3,000 × *g* for 10 min) to separate the supernatant from the cell pellets. The cell pellets were fixed in 2.5% cacodylate buffered glutaraldehyde and post-fixed in 1% cacodylate buffered osmium tetroxide with a pH of 7.2 for 2 h, and then dehydrated in ethanol and embedded in resin. A drop of the sample was placed on a thin copper grid for 15 min and stained with 2% uranyl acetate. To analyze infected brain tissue, samples were collected from infected fish and fixed in 2.5% cacodylate buffered glutaraldehyde and post-fixed in 1% cacodylate buffered osmium tetroxide for 45 min, and then washed three times with sodium cacodylate buffer, dehydrated in acetone and embedded in resin. Tissue sections were cut 1 μm thick and stained with toluidine blue for observation under a light microscope. The ultrathin sections were trimmed and stained with 2% uranyl acetate. All micrographs were taken at 80 kV using a Hitachi HT7700 transmission electron microscope (Hitachi, Germany) at the Scientific Equipment and Research Division, Kasetsart University, Bangkok, Thailand.

3. Results

3.1. A novel, orthomyxo-like virus, TiLV was identified and isolated from field samples

Our previous study indicated that a novel, orthomyxo-like virus, TiLV, could be isolated from field samples associated with massive mortality of both Nile and red tilapia in Thailand. In this study, we investigated additional field outbreaks to further confirm TiLV as the primary cause of tilapia deaths in Thailand (Table 1). From the 15 field outbreaks sampled, TiLV could be detected in all samples with clinical signs of infection including distinct skin hemorrhage and erosion, head congestion and severe anemia (Fig. 1A and B). It was likely that TiLV

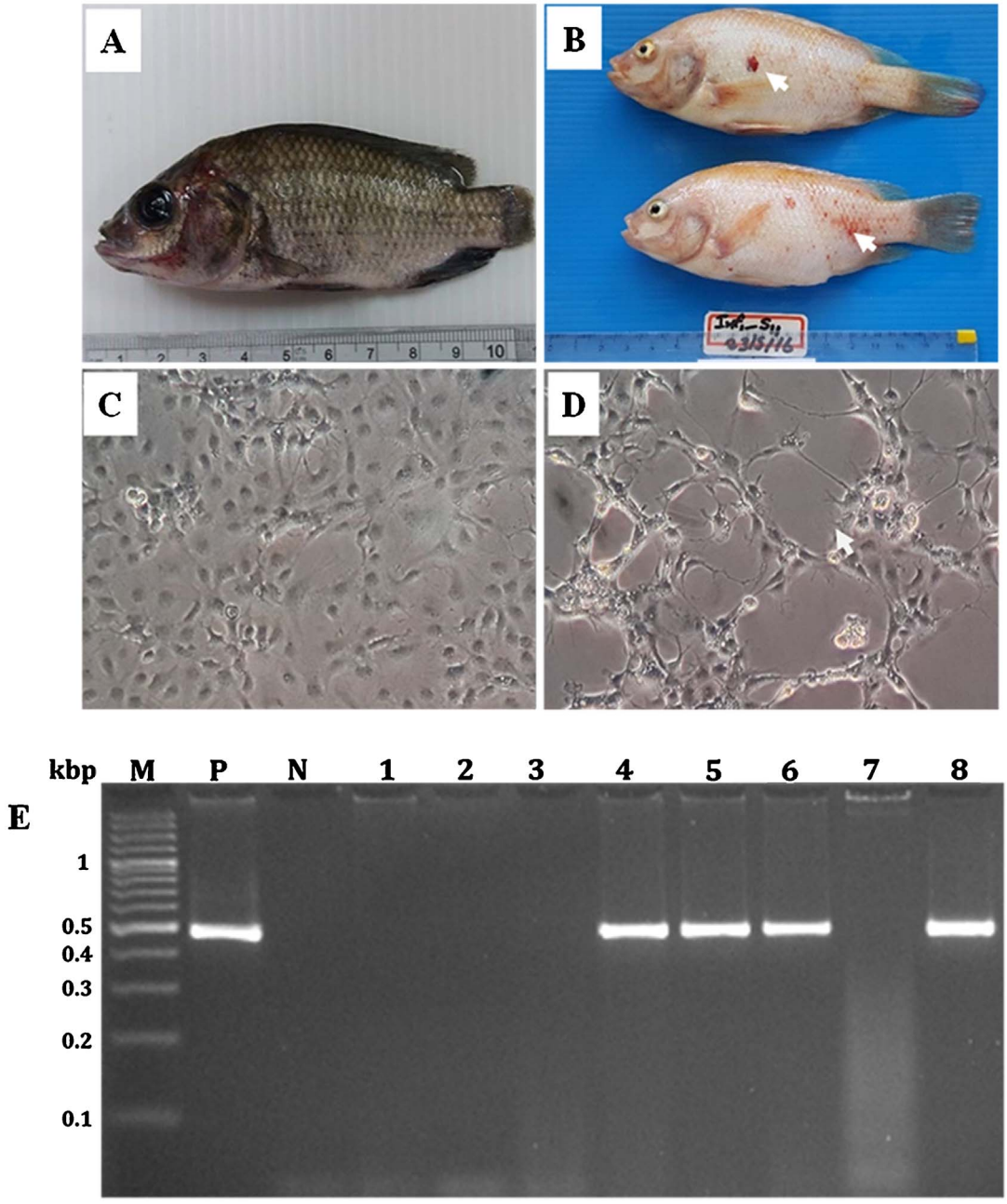

Fig. 2. Experimental challenge of TiLV in Nile and red tilapia. Nile and red tilapia were injected intraperitoneally with TiLV grown in E-11 cells. At 5 to 7 dpi, distinct clinical signs of infected fish include (A) Nile tilapia; skin erosion and hemorrhage (B) Red tilapia; skin hemorrhage (arrow), mild exophthalmos and abdominal swelling. Brains of challenged fish were collected and subjected to virus isolation in E-11 cells. (C) E-11 cells inoculated with brain from PBS control fish. (D) E-11 cells inoculated with brain from TiLV-challenged fish, with appearance of CPE at 5 dpi (arrow). (E) A specific PCR product was observed in TiLV-challenged fish. M = 100 bp marker; P = plasmid containing TiLV fragment; N = no template control; lane 1–3 = brain from PBS injected fish; lane 4–6 = Brain from TiLV-challenged fish; lane 7 = uninfected E-11 cells; lane 8 = infected E-11 cells.

was the primary cause of such massive mortality as no other viruses including iridovirus and betanodavirus could be detected in the field samples (Fig. 1C). Notably, comparison of the nucleotide sequence of the PCR products revealed 98% nucleotide sequence identity to TiLV (GenBank accession no. KY381578).

Brain tissues of infected fish were inoculated into confluent E-11 cells and at 4 dpi, CPE was clearly observed in E-11 cells inoculated with materials prepared from TiLV-positive fish, but not from normal fish (Fig. 1D). The characteristics of infected cells included an increase in cell aggregation, cellular vacuolation, cell shrinkage, and formation of foci of detached, rounded cells (Fig. 1E). More pronounced cell detachment and syncytial formation were observed at 5 to 7 dpi.

3.2. TiLV produces clinical infection in experimental, challenged fish and re-isolation of virus from challenged fish

To identify if TiLV isolated from diseased fish can cause disease in naïve susceptible fish, both Nile and red tilapia were injected with cell culture suspension prepared from TiLV-infected E-11 cells. At 3 to 5 dpi, the clinical signs, including skin erosion, hemorrhage, and abdominal distension, were observed in challenged fish (Fig. 2A and B). Notably, these clinical signs in the experimental, challenged fish were similar to the clinical observations under field conditions. Virus was re-isolated in E-11 cells from liver and brain of experimentally infected fish. Of note, distinct CPE was observed in inoculated E-11 cells within 5 dpi (Fig. 2D). The characteristics of infected cells included a round shape,

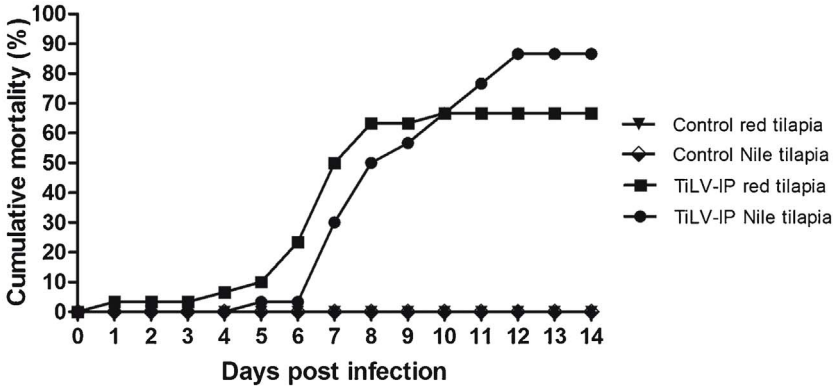

Fig. 3. Cumulative mortality in TiLV-challenged red tilapia. Nile and red tilapia (n = 30) were injected intraperitoneally with TiLV at 1×10^6 $TCID_{50}$/fish. The control Nile and red tilapia were injected with cell culture medium. Survival rate was recorded daily until 14 dpi.

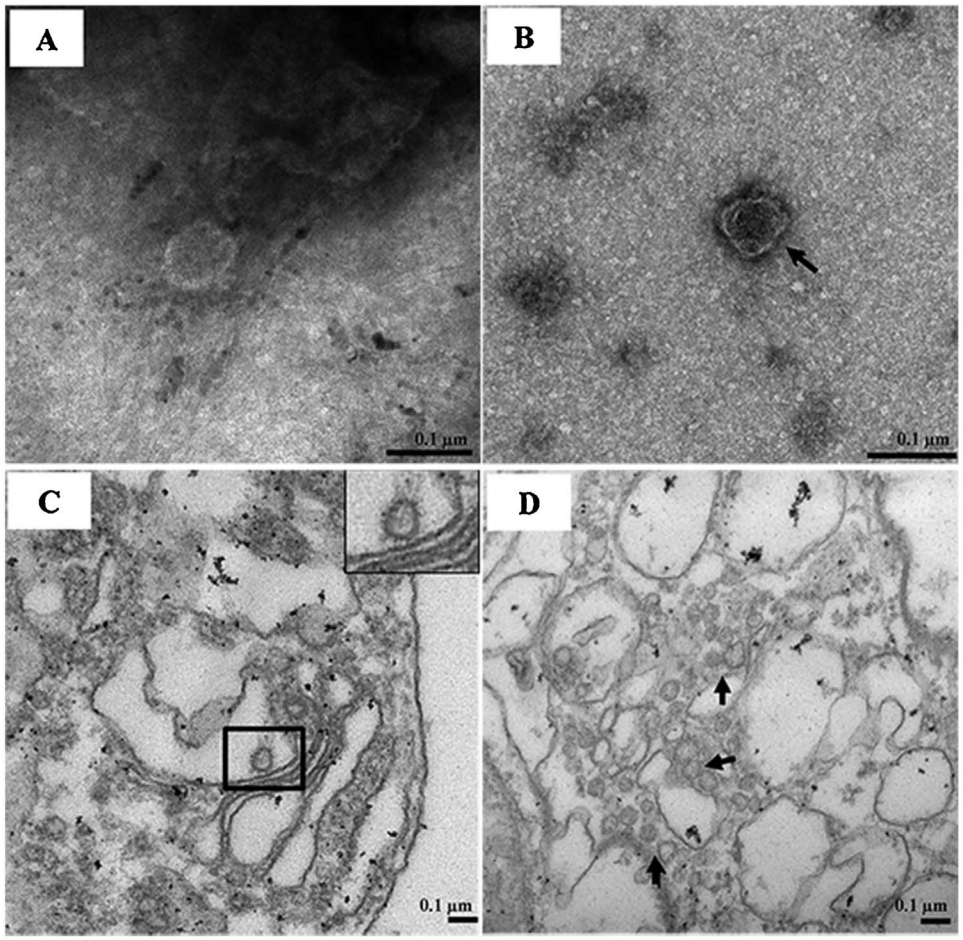

Fig. 4. Transmission electron micrograph of infected E-11 cells and ultrathin sections of infected tilapia brain tissue. (A and B) High magnification of a free virion showing a round enveloped viral particle with 60 to 80 nm diameter. (C and D) Ultrathin section of infected tilapia brain showed multiple viral particles in the cytoplasm of infected cells (C) marked with square box or (D) marked with black arrows. Scale bars: 0.1 μm.

syncytial formation, and cell detachment, while E-11 cells inoculated with brain from the control group did not show any CPE (Fig. 2C). Three fish each from the control group and the infected group were subjected to PCR analysis to confirm the presence of TiLV. All infected fish showed a positive PCR product on agarose gel (Fig. 2E). In challenged fish, mortality started at 4 dpi and lasted until 10 dpi with accumulative mortality at 86.67% and 66.67% for Nile and red tilapia, respectively. The control fish did not show any clinical signs of infection or mortality (Fig. 3)

3.3. The viral particles were present in infected E-11 cells and brains of challenged fish

At 5 dpi, the supernatant of infected E-11 cells and brain tissue from TiLV-challenged fish was processed for transmission electron microscopy. The electron micrographs of infected E-11 cell lines revealed single, round-shaped, enveloped viral particles (Fig. 4A and B). In the tissue sections, the infected brain tissues contained various enveloped viral particles located in the cytoplasm of the brain cells (Figs. 4C and 4D). The viral particles were consistently round or oval, 60–80 nm in diameter with a central variable electron-dense core. These virus-like particles usually appeared as a single particle or aggregate in small groups in the brain cells.

3.4. Distinct histopathological findings in multiple organs of challenged fish

The histological appearance of multiple organs (brain, liver, spleen, and anterior kidney) from the control fish showed normal structures (Fig. 5A, C, E, and G). In TiLV-challenged fish, the pathological findings included multifocal hemorrhages with severe blood congestion and

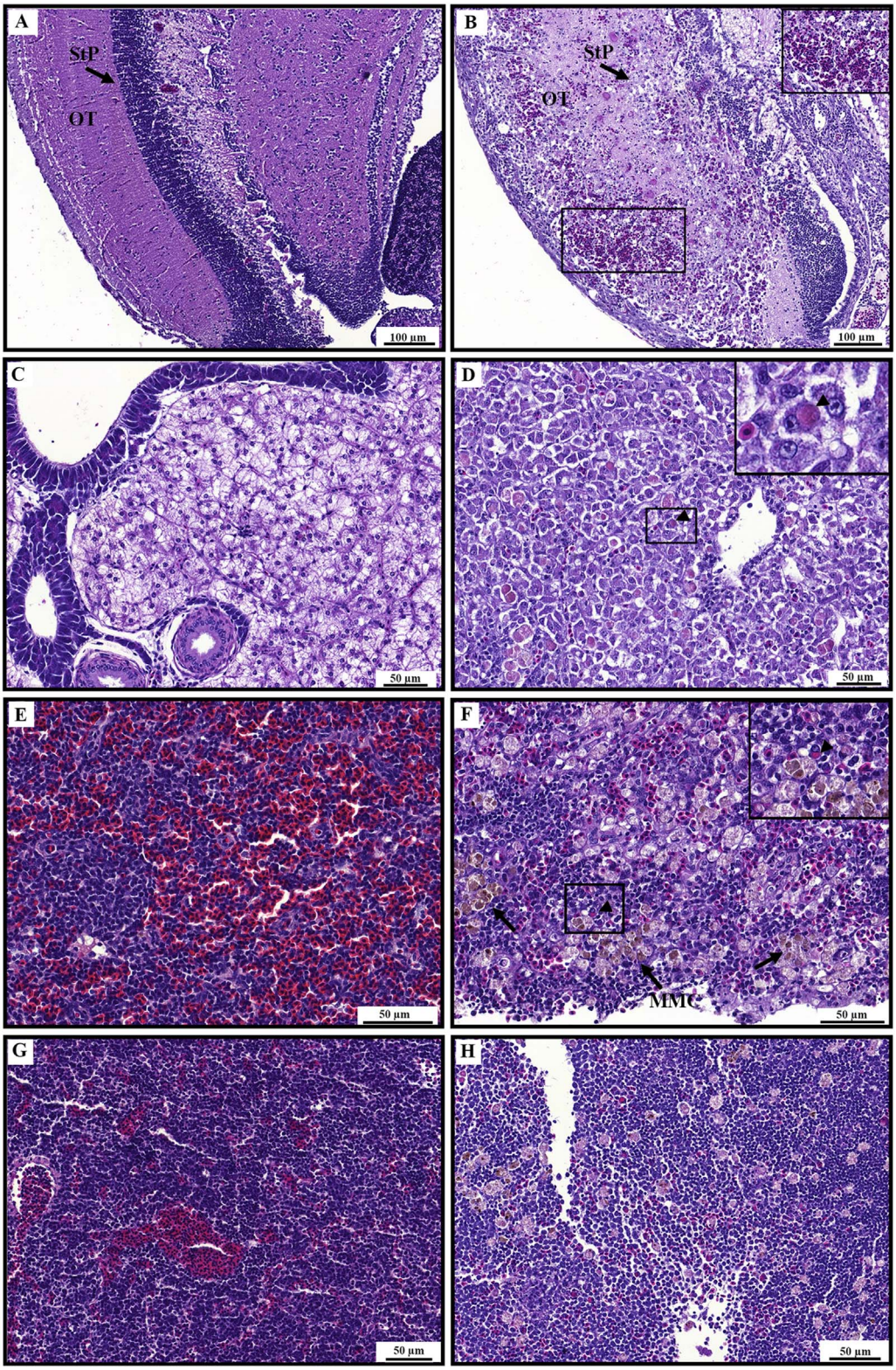

Fig. 5. Histopathology in TiLV-challenged tilapia. Section of normal fish tissues: (A) Brain, (C) Liver, (E) Spleen, (G) Anterior kidney. In infected fish, the pathological findings include (B) Multifocal hemorrhages with severe blood congestion in the brain, optic tectum (OT), stratum periventriculare (StP). (D) Extensive hepatocellular necrosis with eosinophilic intracytoplasmic inclusion body in liver cells (higher magnification). (F) Increased melanomacrophage center (MMC) in the spleen (arrows) with eosinophilic intracytoplasmic inclusion body (arrow head, higher magnification). (H) Multifocal area of necrosis and inflammatory cells infiltration in the anterior kidney.

proliferation of glial cells in the brain (Fig. 5B). Notably, syncytial cell formation and massive hepatocellular necrosis with pyknotic and karyolytic nuclei were observed in the liver cells of infected fish (Fig. 5D). Moreover, eosinophilic intracytoplasmic inclusion bodies were present in the liver cells and splenic cells of infected fish (Fig. 5D and F). Multiple necrotic foci were observed in the anterior kidney (Fig. 5H) and increased melanomacrophage centers and dispersion of melanin granules were observed in the spleens of infected fish (Fig. 5F).

4. Discussion

Our attempts to identify the causative agent of massive losses of tilapia at multiple sites throughout Thailand led to the identification of TiLV infection in tilapia (Surachetpong et al., 2017). Although TiLV has been detected from many field outbreak samples, the reproduction of disease and re-isolation of the causative pathogen from challenged fish is an important step to confirm the etiology of disease. In this study, we

fulfilled Koch's postulates for TiLV using molecular techniques, histopathology, electron microscopy, and a challenge experiment: (i) the virus was isolated from clinically infected fish but not from normal fish; (ii) inoculation of materials prepared from cell cultures obtained from isolated, diseased fish resulted in CPE formation; (iii) electron micrographs of virus particles from the brains of infected fish revealed round, enveloped virions; (iv) injection of cultured supernatant from infected E-11 cells into naïve fish reproduced clinical symptoms similar to field specimens; and (v) the virus was re-isolated and detected in challenged fish. Our data indicated that TiLV is sufficient to produce clinical disease and mortality in Nile and red tilapia. In addition to TiLV, other emerging viruses and bacteria including iridovirus and betanodavirus have been previously identified in diseased tilapia in Thailand (Keawcharoen et al., 2015; Suebsing et al., 2016). However, neither of these viruses were detected in any of our clinical specimens. In addition, we did not find other emerging bacterial pathogens such as *Francisella* spp. in field samples using PCR analysis (Jantrakajorn and Wongtavatchai, 2016; Soto et al., 2009) (data not shown). TiLV is a single stranded, negative-sense RNA virus containing 10 genomic segments (Bacharach et al., 2016). In addition to TiLV, infectious salmon anemia virus (ISAV) and rainbow trout orthomyxovirus (RbtOV) are viruses in the *Orthomyxoviridae* family that cause disease in fish (Batts et al., 2017; Merour et al., 2011).

To our knowledge, this newly identified virus is very contagious in Nile and red tilapia, making it a great threat to tilapia aquacultured worldwide. Global awareness of this problem must be raised. Previous reported indicated that only tilapia cichlids are highly susceptible to TiLV infection (Eyngor et al., 2014). Thus, it is worthwhile to investigate the infection in other piscine species or to identify the natural potential sources of this virus. Our results also suggest that red hybrid tilapia (*Oreochromis* spp.) is susceptible to TiLV infection. Although the mortality rates in both Nile and red tilapia were relatively high under laboratory challenge, our observations from the field outbreaks suggested that the mortality rate in red tilapia is 30–100%, while the mortality rate of Nile tilapia is below 30%. Other factors in the natural environment may impact clinical outcomes of infection and the challenge route through intraperitoneal injection, which is not the natural means of infection by this virus which may impact on the outcome. Indeed, cohabitation of susceptible fish with infected fish may provide an alternative strategy for a TiLV challenge study (Eyngor et al., 2014). It is probable that different genetic backgrounds of Nile and red tilapia may also influence susceptibility to TiLV as well as concomitant infection with other bacteria and viruses. For example, cross-breeding disease-resistant lines of Nile tilapia with those that are susceptible produced more hybrids that were disease-resistant against *A. hydrophila* (Sarder et al., 2001). Moreover, red tilapia may express different patterns of preferred receptors for TiLV. In Atlantic salmon, the tissue distribution of 4-*O*-acetylated sialic acid is important for virus entry and cell tropism of ISAV (Aamelfot et al., 2012). All of these research questions await further investigation.

The viral morphology of TiLV isolated in Thailand has a close resemblance to that of viruses in the *Orthomyxoviridae* family (Fontana and Steven, 2015; Weli et al., 2013). In our infected tilapia, free and aggregate virions with or without envelopes were observed in the brains of diseased fish. Previous observation revealed that similar viral particles of SHV were found in the liver cells of diseased tilapia in Ecuador (Ferguson et al., 2014). It has been emphasized that TiLV isolated from tilapia in Israel produces histopathology in the brain (Eyngor et al., 2014), while the closely related SHV isolated from Ecuador mainly causes lesions in the liver (Del-Pozo et al., 2016; Ferguson et al., 2014). For other piscine viruses, distinct genotypes may result in different pathological outcomes of infection and infection tissues, such as in the case of infectious salmon anemia virus (ISAV) (Aamelfot et al., 2014; Godoy et al., 2014). Nevertheless, the different genotypes and tissue tropisms of this emerging virus require additional study. In our experiment, extensive histopathological changes were observed in both liver and brain tissues of fish after experimental challenge including severe syncytial hepatitis and meningoencephalitis. Our current study and previous reports indicated that both liver and brain samples could be used for TiLV detection using RT-PCR or virus isolation in the cell cultures (Kembou Tsofack et al., 2016; Surachetpong et al., 2017).

In summary, the results clearly showed that TiLV isolated in Thailand can cause disease in challenged Nile and red tilapia. Our study provided evidence that fulfills Koch's postulates regarding TiLV infection in tilapia. The importance of tilapia culture worldwide highlights that effective control measures, biosecurity programs, and the development of vaccines should be implemented to reduce the impact of this emerging viral disease.

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