

## Emergence of tilapia lake virus in Thailand and an alternative semi-nested RT-PCR for detection

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### ABSTRACT

The present study reports outbreaks of tilapia lake virus (TiLV), an emerging pathogen causing syncytial hepatitis of tilapia (SHT), in farmed tilapia in Thailand. Occurrence of the virus was confirmed by RT-PCR and nucleotide sequence homology to the TiLV from Israel. Diseased fish exhibited typical histopathological features of syncytial giant cells in the liver examined through H & E and semi-thin sections. Presence of intracytoplasmic viral particles was revealed by TEM. *In situ* hybridization using a specific DIG-labeled probe derived from a partial genome segment 3 of TiLV genome revealed multiple tissues tropism of the virus including liver, kidney, brain, spleen, gills and connective tissue of muscle. An alternative semi-nested RT-PCR protocol has been developed in this study for disease diagnosis. Additionally, comparative genetic analysis revealed genetic variations of Thailand-originated virus to the Israel TiLV strains, sharing 96.28 to 97.52% nucleotide identity and 97.35 to 98.84% amino acid identity. Outbreaks of TiLV in different continents might signal a serious threat to tilapia aquaculture globally.

### 1. Introduction

Tilapia (*Oreochromis* spp.) is ranked the second most important aquaculture species in Thailand after whiteleg shrimp (*Penaeus vannamei*) and currently accounts for over 45% of the national production of freshwater fish (Bhujel 2011; DOF 2014). Massive die-offs due to infectious diseases have brought huge economic losses not only to aquaculture producers but also to other related sectors. Among the identified fish killers of cultivated tilapia in Thailand, a significant number of emerging infectious pathogens have been reported during recent years, notably a novel genetic group of *Flavobacterium columnare* (Dong et al. 2015a), *Francisella noatunensis* subsp. *orientalis* (Dong et al. 2016; Jantrakajorn and Wongtavatchai 2016; Nguyen et al. 2016), *Hahella chejuensis* (Senapin et al. 2016), *Aeromonas jandaei* and *Aeromonas veronii* (Dong et al. 2015b, 2017), betanodavirus (Keawcharoen et al. 2015), and infectious spleen and kidney necrosis virus (ISKNV/Iridovirus) (Dong et al. 2015b; Suebsing et al. 2016).

Recently, a novel RNA virus, called tilapia lake virus (TiLV), has been discovered as the causative virus of high cumulative mortalities (80–90%) in farmed tilapia in Israel, Ecuador, and Colombia (Bacharach et al. 2016; Del-Pozo et al. 2017; Eyngor et al. 2014; Ferguson et al. 2014; Tsofack et al. 2017) and 5–15% mortality in Egypt (Fathi et al. 2017). Initially, Ferguson et al. (2014) described a novel disease with suspected viral etiology, namely syncytial hepatitis of tilapia (SHT) in fingerlings of Nile tilapia (*Oreochromis niloticus*) in Ecuador. In Israel, massive mortalities of both wild and farmed hybrid tilapia (*O. niloticus* × *O. aureus*) have been recorded from all over the country since 2009 and the etiological agent was subsequently identified as TiLV (Eyngor et al. 2014). Interestingly, later studies indicated that the virus causing SHT was genetically similar to TiLV and was classified as a novel Orthomyxovirus-like despite variations in histopathological features as described from the two countries (Bacharach et al. 2016).

In Thailand, severe die-offs have been observed in red tilapia

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**Table 1**  
Details of fish samples and RT-PCR result.

Year	Farm/province	Species	Fish stage	Mortality (%)	Number of positive/ number tested
2017	A/Pathumthani	Nile tilapia <i>Oreochromis niloticus</i>	Fingerling (2.5–3 cm)	~90	14/14
2016	CL/Phetchaburi	Red and Nile tilapia	Fingerling (3.5–4 cm)	~20	7/7
	CN/Chainat	Red tilapia <i>Oreochromis</i> sp.	Fingerling (8.5–9 cm)	~90	6/6
	Control	Nile tilapia	Juvenile (~15 cm)	–	0/2

(*Oreochromis* sp.) fingerlings during the first month after being transferred into floating cages. Abnormal mortality was also observed in tilapia hatcheries. Since the use of antibiotics did not show a significant reduction of mortality and the variety of sources of tilapia fingerlings have been stocked in the same culture system, tilapia producers in Thailand expressed their concern over TiLV infection in the country. Upon disease surveillance, this study 1) confirmed an emergence of TiLV associated with disease outbreaks in farmed tilapia in Thailand; 2) compared partial genome sequences of Thai TiLV to that of Israeli TiLV reported at GenBank; and 3) proposed an alternative semi-nested RT-PCR protocol and an *in situ* hybridization method for disease diagnosis.

## 2. Materials and methods

### 2.1. Fish samples

Naturally diseased fish used in this study were fingerlings of both red tilapia (*Oreochromis* sp.) and Nile tilapia (*O. niloticus*) collected from three affected farms located in three different provinces (Phetchaburi, Chainat, and Pathumthani) in Thailand in the years 2016 and 2017. Details of fish samples used in this study are summarized in Table 1. A mixture of the kidney, liver and spleen of each diseased fish (6–14 samples per farm) was collected for RNA extraction and molecular study. Internal organs of a separate set of 10 fish from an infected farm in Chainat province were preserved in formalin solution and, in parallel, small pieces of liver specimen from 5 fish were fixed in glutaraldehyde solution (see below).

### 2.2. RNA extraction and semi-nested RT-PCR detection of TiLV

Total RNA of pooled internal organs described above was extracted using Trizol reagent (Invitrogen) based on protocols from the manufacturer. The quality and quantity of the obtained RNA were measured

**Table 2**  
Primers used in this study.

Primer name	Sequence (5'-3')	Target gene	Product size (bp)	References
Nested ext-1	TATGCAGTACITTCCTGCC	Segment 3	415	Eyngor et al. (2014) Tsofack et al. (2017)
ME1	GTTGGGCACAAGGCATCCTA			
7450/150R/ME2	TATCACGTGCGTACTCGTTTCAGT	Segment 1	1614	This study
TiLV-Seg1-F	TCATTCGCTATATAGTTAC			
TiLV-Seg1-R	TTAATTACGCACTATTACTG	Segment 5	1073	This study
TiLV-Seg5-F	TTTTTCTCAGTTTACCACCTC			
TiLV-Seg5-R	TTATCTCAGACTCCAATAGC	Segment 9	522	This study
TiLV-Seg9-F	ACGTCCITAAAGTCATACTT			
TiLV-Seg9-R	ACAAGTCCGATTACTTTTTC			

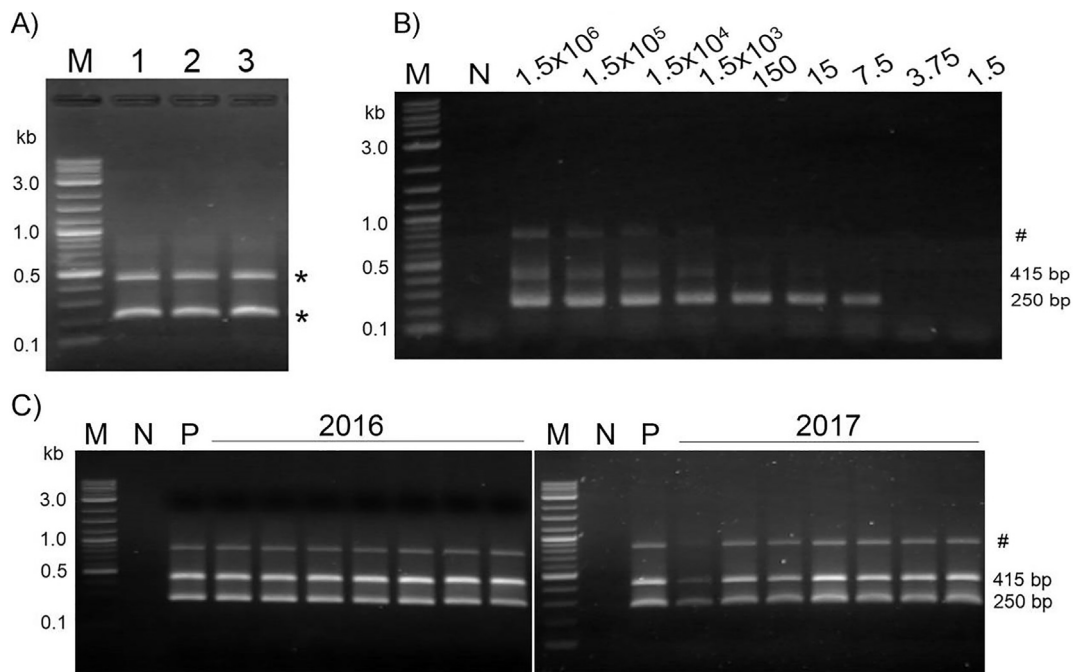
by spectrophotometry. An alternative detection procedure of TiLV was modified from Tsofack et al. (2017) that targeted TiLV genome segment 3 by omitting primer Nested ext-2. The first round RT-PCR reaction of 25 µl composed of 1.5 µl of RNA template (100–400 ng), 0.4 µM of each primer Nested ext-1 and ME1 (Table 2), 0.5 µl of SuperScript One-Step RT/Platinum Taq mix (Invitrogen), and 1 × reaction buffer. RT reactions performed at 50 °C for 30 min prior to heat inactivation at 94 °C for 2 min. PCR cycling was then carried out for 25 cycles at 94 °C for 30 s, 60 °C for 30 s, 72 °C for 30 s. Semi-nested PCR amplification was conducted in 20 µl reaction solution containing 1 µl of the first round RT-PCR reaction, 0.25 µM of each primer 7450/150R/ME2 and ME1 (Table 2), 1 unit of Taq DNA polymerase (RBC Bioscience), 0.2 mM dNTPs, and 1 × reaction buffer. PCR thermocycling was the same as the first round PCR. Expected amplicons of the first and second round amplification were 415 bp and 250 bp, respectively. Amplified amplicons were gel-purified, cloned into pGEM-T easy vector, and subjected to Sanger sequencing. The obtained sequences were blast to the GenBank database. Detection sensitivity was performed using serially diluted plasmid containing a 415-bp fragment (namely pGEM-415\_bp). The alternative semi-nested PCR was used to test for all clinical samples (Table 1). RNA extracted from internal organs of 2 apparently healthy Nile tilapia juveniles were served as control reactions. The plasmid pGEM-415\_bp was employed as a positive control while nuclease-free water was used as a negative control.

### 2.3. Comparative genetic characterization of TiLV originated from Thailand and Israel

Three primer sets targeting nearly complete genome segments 1, 5, and 9 of TiLV were designed (Table 2) based on available sequences of the Israel strain in GenBank (accession nos. KU751814, KU751818, and KU751822, respectively). RT-PCR amplifications were performed with pooled RNA samples from two infected farms (CL and CN; Table 1) using individual primer set. RT-PCR reactions were prepared in the same manner as mentioned above except that 35 cycles, 50 °C annealing step, and 1 kb/min at 72 °C extension step were used. Amplified products were purified, cloned into pGEM-T easy vector, and three recombinant plasmids of each segment were sequenced using T7 and SP6 primers. Consensus sequences were used for further analysis. Sequence homology was determined by nucleotide blast search (<https://blast.ncbi.nlm.nih.gov>). Nucleotide sequences were translated to deduced amino acid sequences using ExPASy program (<http://web.expasy.org/translate/>). Amino acid sequence alignments of the Thai and Israeli strains were performed using Clustal Omega (<https://www.ebi.ac.uk/Tools/msa/clustalo>).

### 2.4. Histopathology and *in situ* hybridization (ISH)

The clinically sick red tilapia (n = 10) collected from CN farm (Chainat province) were preserved in neutral buffer formalin (10%) for 24–36 h prior to long-term preservation in 70% ethanol. The samples



**Fig. 1.** Agarose gels of TiLV detection of clinical sick fish samples from Thailand. (A) Three nested RT-PCR reactions run at an annealing temperature 56 °C non-specifically amplified fish mRNA. \*Indicated bands whose sequences are shown in Supplemental Fig. 1. (B) Representative detection sensitivity assay from 3 replicates using our newly modified semi-nested RT-PCR protocol. Successive serial dilutions of positive control plasmid (pGEM-415 bp) are indicated. Expected band sizes of 415 bp and 250 bp represent amplicons from the first and semi-nested PCR, respectively. #Marks band probably derived from cross hybridizations of the amplified products. M, DNA marker (2-Log DNA Ladder, New England Biolabs); N, no template control. (C) Detection results using semi-nested RT-PCR assay with representative RNA template from the fish collected from farms in Thailand in years 2016 and 2017. All give positive results. P, positive control plasmid.

were processed for routine histology. Briefly, the specimens were dehydrated, embedded in paraffin, sectioned at 5  $\mu\text{m}$ , stained with haematoxylin and eosin (H & E) and examined under a light microscope. In order to investigate target tissues of TiLV, the tissues including liver, kidney, brain, gills and muscle collected from three infected fish (presumed from the presence of syncytial giant cells) were subjected to *in situ* hybridization (ISH). Digoxigenin (DIG)-labeled probes were prepared using DIG-labeling Mix (Roche, Germany). Plasmid pGEM-415 bp was used as a template in the labeling reaction while a 282-bp fragment derived from shrimp virus, infectious myonecrosis virus (IMNV) (Senapin et al. 2007), was employed as an unrelated negative probe. ISH was performed as previously described (Senapin et al. 2016) and the sections were examined and photographed under an Olympus BX51 digital microscope.

### 2.5. Semi-thin sections and TEM

The liver samples collected from five naturally diseased red tilapia were fixed in 2.5% glutaraldehyde solution prepared in 0.1 M phosphate buffer saline (PBS), for 2 h at 4 °C. The specimens were then washed with 0.1 M PBS and placed in 1% OsO<sub>4</sub> in 0.1 M PBS for 1 h at 4 °C. Subsequently, the specimens were dehydrated, embedded in Epon812 solution and allowed to polymerize at 70 °C. The samples were sectioned at 1  $\mu\text{m}$  thickness using an ultramicrotome (Leica UC-6), stained with toluidine blue and observed under an Olympus BX51 digital microscope. Selected areas of the embedded liver which contained giant cells (estimate based on the position observed in semi-thin sections) were sectioned by a diamond knife. Thin sections were placed onto mesh grids, stained with uranyl acetate and lead citrate prior to examination under a transmission electron microscope (TEM).

## 3. Results

### 3.1. Disease characterization

According to fish producers, abnormal cumulative mortalities (20–90%) due to unknown etiological agent(s) have been observed in fingerlings of both red tilapia and Nile tilapia farms in Thailand recently. In this study, the diseased fish samples were collected from three different sources in year 2016–2017 (Table 1). The red tilapia fingerlings collected from Chainat province (CN farm) were cultured in an open floating cage. Mortality occurred since the fish were stocked into the cages and reached up to ~90% within a month. The fish collected from Phetchaburi province (CL farm) were cultured in an earthen pond by mixing both red and Nile tilapia. Fish died gradually every day and accumulative mortality was ~20% on the day of sample collection. Nile tilapia fingerlings collected from a farm in Pathumthani province (A farm) showed massive mortality of 90%. In these cases, diseased fish exhibited loss of appetite, pale color, gathering in the bottom, slow movement, stopped schooling and eventually died.

### 3.2. Development of an alternative semi-nested RT-PCR detection

Before the recent publication by Tsoufack et al. (2017), an initial work on TiLV detection has already published PCR primer sequences but did not describe the amplification reaction conditions (Eyngor et al. 2014). We roughly estimated the melting temperatures of the established primers and 56 °C annealing temperature were then used in our preliminary nested RT-PCR assays with TiLV suspected fish samples from Thailand. The results showed that the nested RT-PCR test generated two bands with similar sizes to expected products (491 bp and 250 bp) (Fig. 1A). After cloning the amplified amplicons, 3 recombinant clones with inserted size of ~400–500 bp and 1 clone

with ~200-bp fragment were sent for sequencing. Sequence analysis revealed that all of the PCR products were amplified by outer primers (Nested ext-1 and Nested ext-2) but the sequences matched (99–100% nucleotide identity) to 4 different mRNA sequences of *O. niloticus* (Supplemental Fig. 1). 13–18 out of 20 nucleotides of primer Nested ext-2 bound upstream to the fish sequences while 6–10 nucleotides of primer Nested ext-1 bound downstream of the amplified products. Since the error was likely to be from primer Nested ext-2, we then developed an alternative semi-nested RT-PCR detection by omitting this primer. Nested ext-1 and ME1 were employed in the first round amplification and 7450/150R/ME2 and ME1 used in the second round PCR. Optimal annealing temperatures and number of thermal cycling cycles were selected from gradient PCR and cycling test conditions (not shown). After obtaining TiLV positive samples (see below), a recombinant plasmid (pGEM-415\_bp) containing a 415-bp fragment derived from TiLV genome segment 3 (Supplemental Fig. 2) was constructed and used in detection sensitivity assay. Amplification results from 3 replicates using serial dilutions of pGEM-415\_bp indicated that detection sensitivity of the newly modified detection protocol was found to be 7.5 viral copies/reaction. The result of one replicate is shown in Fig. 1B. A band of approximately 750 bp might be derived from cross hybridizations of the amplified products (Fig. 1B).

### 3.3. Clinical samples from Thailand were TiLV positive

A total of 27 representative clinical sick samples from 3 different farms in Thailand (Table 1) were subjected to disease diagnosis by an alternative semi-nested RT-PCR protocol. The results showed that all tested samples exhibited positive results (Fig. 1C, Table 1) except for two apparently healthy tilapia juveniles (Table 1). The 415-bp and 250-bp amplicons from an RT-PCR positive sample of CN farm was cloned and sequenced (Supplemental Fig. 2). Blast result showed 97.35% and 98.80% nucleotides identity to the genome segment 3 of the Israel strain (Til-4-2011) (GenBank accession no. KU751816).

### 3.4. Genetic variation of TiLV originated from Thailand and Israel

Sequence comparison of three genome segments (segment 1, 5, and 9) of TiLV was investigated between Thailand and Israel-originated strains. RT-PCR using primers targeting the selected segments of TiLV yielded expected amplicons of 1614-bp (segment 1), 1073-bp (segment 5) and 522-bp (segment 9) from a pooled RNA sample extracted from fish specimens from CL farm while only a 522-bp amplicon was obtained from a pooled sample of CN farm. After cloning and sequence analysis, the consensus genome sequences (from three clones of each sample) have been submitted to the GenBank under accession numbers KY615742-KY615745. The Thailand-originated TiLV shared 96.28 to 97.52% nucleotide identity and 97.35 to 98.84% amino acid identity to the Israeli TiLV strains (Table 3). Several variations of amino acid residues in the segments 1, 5, and 9 between Thai and Israeli strains are

presented in Table 3 and Supplemental Fig. 3.

### 3.5. Histopathological changes and TEM

Histopathological examination of infected fish noticeably revealed hepatocytes that were swollen and dissociated. Presence of syncytial giant cells containing 2–11 nuclei (Fig. 2) was observed in some liver samples. Appearance of syncytial cells was more clearly seen in semi-thin sections stained with toluidine blue (Fig. 3), including more numbers of liver samples whose giant cells were not previously visualized by the H & E technique. From semi-thin sections, cytoplasm of syncytial cells stained light blue compared to normal cells that stained dark blue. Swollen mitochondria (Mt) with round or oval shape were also notably observed in the syncytial cells (Fig. 3). Ultrastructurally, presence of multinuclear cells was clearly revealed by TEM (Fig. 4A). With higher magnification, the swollen mitochondria were clearly observed in TEM sections (Fig. 4B). Interestingly, only a few viral particles were seen within the syncytial cells. Instead, infected cells containing numerous intracytoplasmic viral particles (~100 nm) with a round shape were observed at the area resembling capillary in the liver (Fig. 4C, D).

### 3.6. Tissue tropisms revealed by in situ hybridization

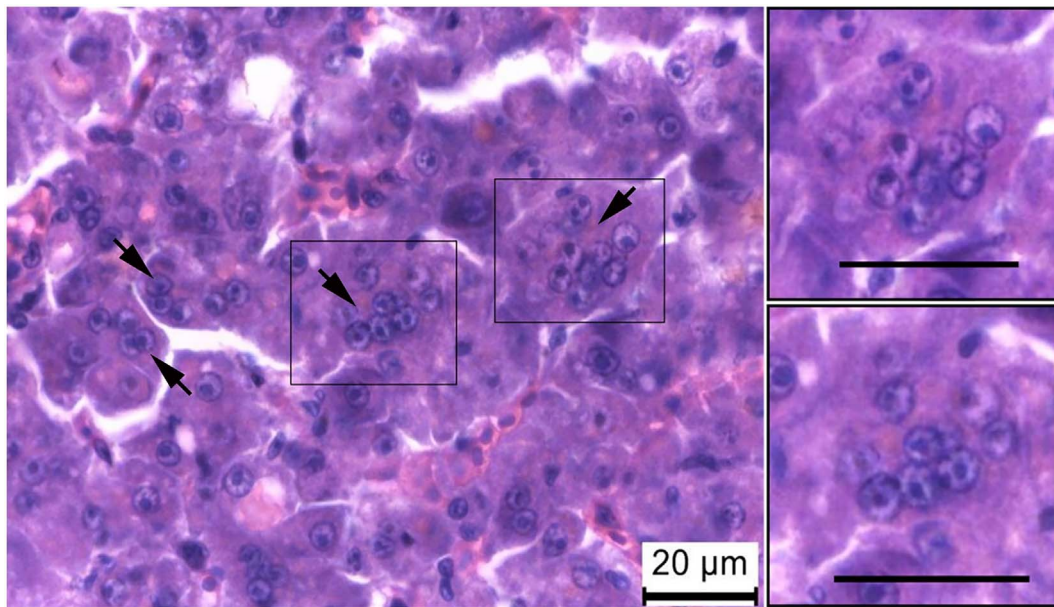
ISH revealed positive hybridization signals in multiple organs of the naturally diseased fish including liver, kidney, brain, gills (Fig. 5), spleen, and connective tissue in the muscle (not shown). Strongest positive signals were observed in the liver, both peripheral membrane and hepatocytes (Fig. 5B). Numerous areas in the kidney showed positive signals in both epithelial cells of tubules and hematopoietic tissue (Fig. 5D). Fewer positive signals were found in the brain, notably at an outer part (Fig. 5F). Both primary and secondary lamellae of the gills also exhibited strong positive signals (Fig. 5H). ISH assay using an unrelated probe derived from the shrimp virus, IMNV, performed with consecutive sections of the same samples, revealed no positive signals (Fig. 5A, C, E, and G).

## 4. Discussion

Up-to-date, TiLV has been publicly reported in Israel, Ecuador, Colombia, and Egypt (Eyngor et al. 2014; Ferguson et al. 2014; Tsofack et al. 2017; Fathi et al. 2017). This study has confirmed the presence of TiLV associated with high mortalities (20–90%) in tilapia farms in Thailand, one of the major tilapia producers in Asia. Occurrences of TiLV infections contributing to high mortalities have been reported in Nile tilapia (*O. niloticus*) fingerlings in Ecuador (Ferguson et al. 2014), in *O. niloticus* × *O. aureus* hybrid in Israel (Eyngor et al. 2014), and in unspecified species and stage of tilapia in Colombia (Tsofack et al. 2017) while an average of 9.2% mortality was found in medium to large sized *O. Niloticus* in Egyptian farms (Fathi et al. 2017). This study also

**Table 3**  
Comparison of nucleotide and amino acid sequences between Thai and Israeli TiLV strains.

Segment	GenBank accession no.	% Identity with TiLV from Israel		Residue differences between Israeli and Thai strains	
		GenBank accession no.	nt		aa
1	KY615742 (CL)	KU751814 (Til-4-2011)	96.28	98.84	L31 → I, G98 → S, R104 → K, V258 → A, A310 → V, D406 → G
		KU552131 (AD-2016)	96.96	–	–
5	KY615743 (CL)	KU751818 (Til-4-2011)	96.74	97.35	A13 → V, I17 → T, N92 → D, R111 → K, I117 → V, S135 → T, D152 → E, V219 → I, Y321 → F, S330 → G
		KU552136 (AD-2016)	96.84	–	–
9	KY615744 (CL)	KU751822 (Til-4-2011)	97.52	98.27	S68 → G, R116 → K
		KU552140 (AD-2016)	96.37	–	–
	KY615745 (CN)	KU751822 (Til-4-2011)	97.13	97.41	T26 → I, S68 → G, R116 → K
		KU552140 (AD-2016)	96.35	–	–

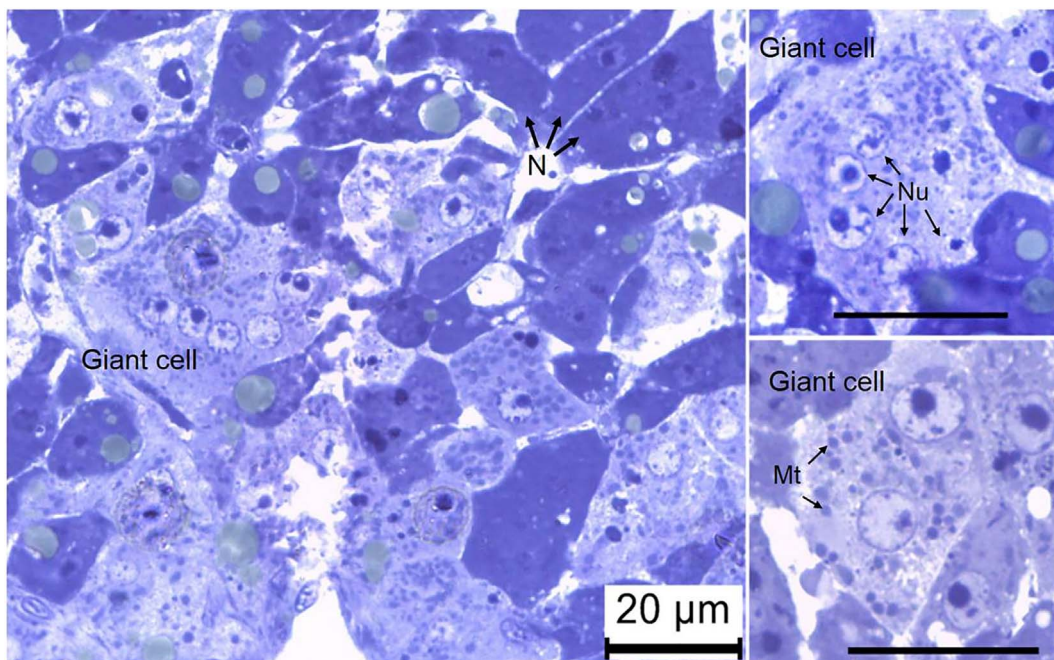


**Fig. 2.** The liver of naturally diseased fish exhibited dissociation, basophilic stained hepatocytes. Syncytial giant cells contained multiple nuclei (arrows). Higher magnification of syncytial giant cells (squared boxes), (H & E staining section, 1000 ×; scale bars, 20 μm).

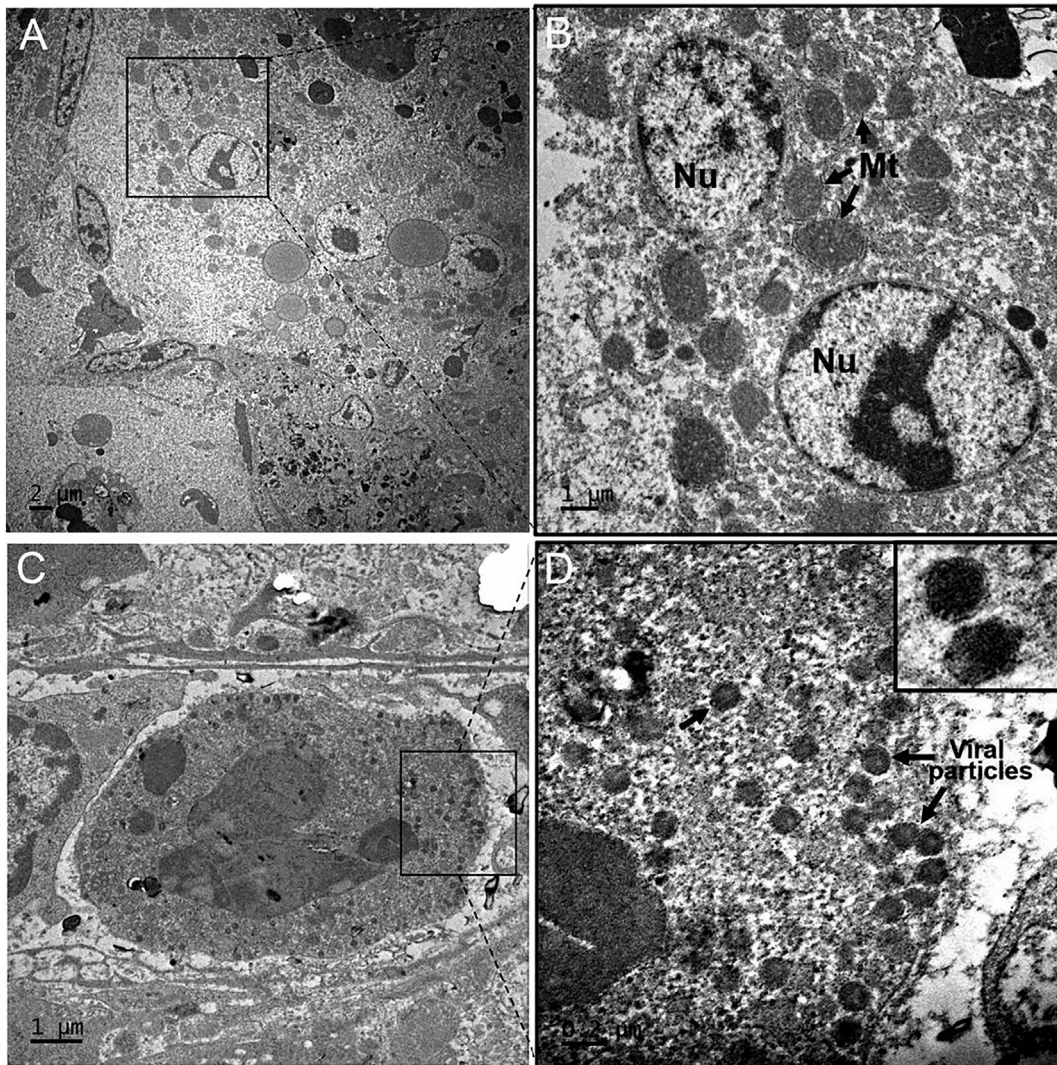
found natural infections of TiLV in fingerlings of red and Nile tilapia reared in both earthen pond and open cage system in three different provinces in Thailand. [Eyingor et al. \(2014\)](#) suggested that waterborne is a route of disease transmission. Thus, the disease is likely wide-spread across the whole aquaculture system if one farm was infected with the virus. Since it is unclear whether the deadly virus has already affected other Asian countries, the tilapia aquaculture industry should therefore be aware that the incidence of TiLV could pose a threat to regional and global aquaculture production. Prior to having established effective control and prevention measures, biosecurity strategies and rapid diagnosis should be applied immediately to stop the disease spreading. This study also provided an alternative semi-nested RT-PCR protocol for

TiLV detection whose detection sensitivity (7.5 viral copies/reaction) was similar to an established nested RT-PCR (7 copies) but higher than qRT-PCR (70 copies) ([Tsofack et al. 2017](#)).

Histological changes have been described mainly in the liver (syncytial cells) ([Ferguson et al. 2014](#)) and central nervous system ([Eyingor et al. 2014](#)) of the infected fish. Both liver and brain have been suggested for use as target tissues for molecular diagnosis of TiLV ([Tsofack et al. 2017](#)). In this study, ISH assay using a TiLV-specific probe revealed additional tissue tropisms of the virus including the kidney, spleen, and connective tissue of muscle. Strong positive signals were visualized predominantly in the liver, kidney, and gills of the clinically sick fish while fewer signals were viewed in the brain,



**Fig. 3.** Representative micrographs of semi-thin sections through the liver of the fish infected with TiLV revealed syncytial cells containing 2–5 nuclei (Nu), stained lighter than normal cells (N). Cytoplasm of giant cells in the bottom right figure revealed numerous round and oval shaped, well-stained particles of swollen mitochondria (Mt) (toluidine blue staining, 1000 ×; scale bars, 20 μm).



**Fig. 4.** Ultrastructure of liver from diseased tilapia showing multinuclear hepatocytes (A) with notable presence of numerous swollen mitochondria within cytoplasm (B). Infected cell contained typical viral particles in cytoplasm (C). Intracytoplasmic virions observed at higher magnification showing round-shaped particles with diameter around 100 nm (D). Nu, nuclei; Mt, mitochondria.

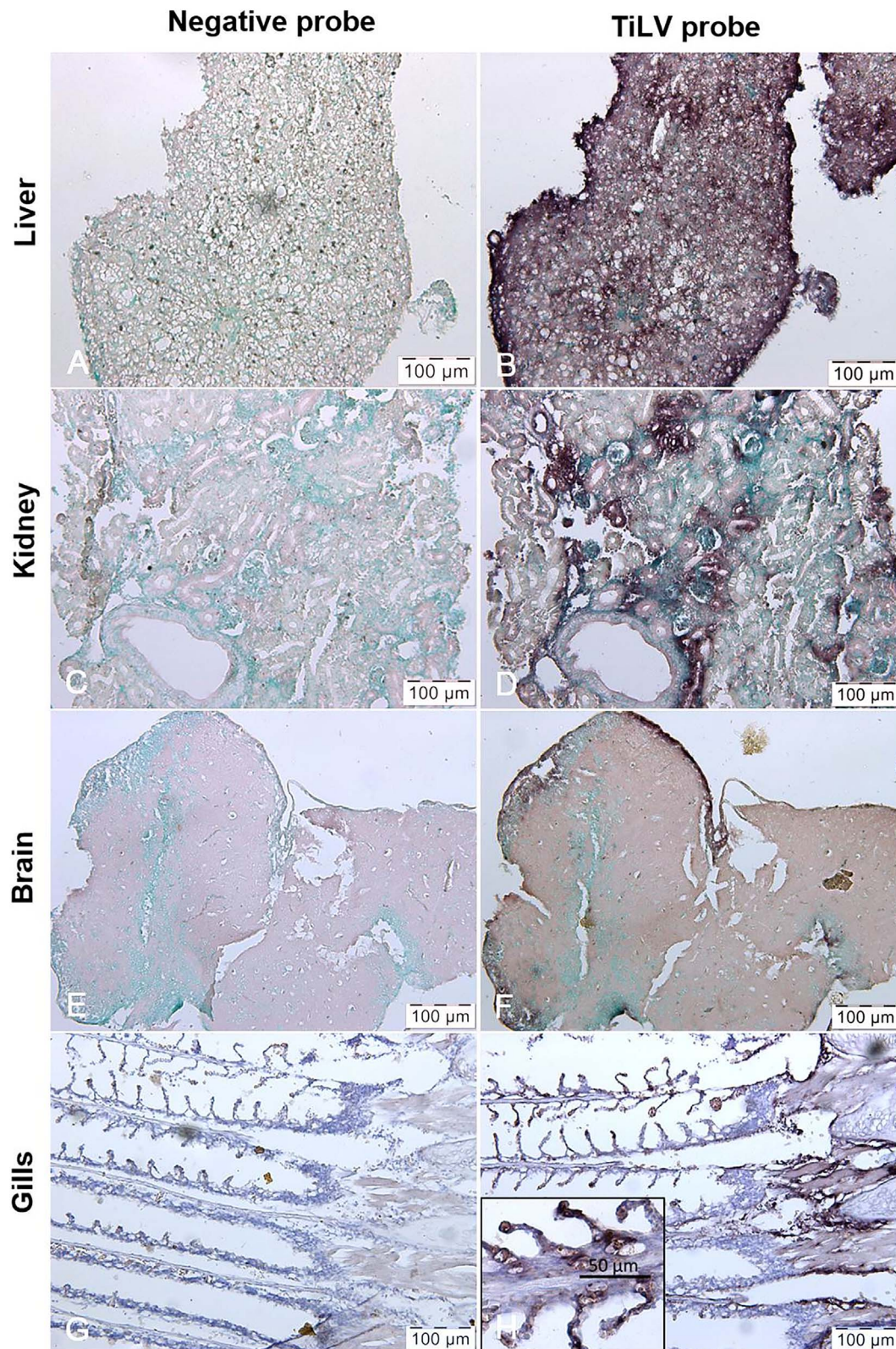
spleen and connective tissue of muscle. This suggests that liver, kidney and gills could also be used as target tissues for molecular diagnosis. The use of a newly modified detection protocol and ISH assay described in this study might be useful to retrospectively investigate archived samples to better understand history and epidemiology of the disease. It was also noted that semi-thin sections (1  $\mu\text{m}$  thickness) could help visualization of syncytial cells better than H & E sections (5  $\mu\text{m}$  thickness) and swollen mitochondria could also be observed.

Bacharach et al. (2016) discovered that TiLV is a negative sense RNA virus which has 10 genome segments. Segments size range from 456 to 1641 nucleotides and genome total size is 10.323 kb. All segments have conserved, complementary sequences at 5' and 3' termini. The genome organization and ultrastructural morphology of TiLV resemble other Orthomyxoviruses (Del-Pozo et al. 2017; Eyngor et al. 2014). However, the genome segments showed no homology to known viruses except for the segment 1 exhibiting weak homology to PB1 subunit of the influenza virus C, a member of Orthomyxoviridae. Comparative genome analysis of TiLV originated from Israel and Ecuador revealed 97.20 to 99.00% nucleotide identity which resulted in 98.7 to 100% amino acid identity among the genome segments, indicating geographically genetic variation (Bacharach et al. 2016).

In this study, a genetic comparison partially investigated 3 genome segments (segment 1, 5, and 9) between Thailand-originated TiLV to

the Israeli virus. It was revealed that genetic variation was relatively higher (96.28–97.52% nucleotide identity and 97.35–98.84% amino acid identity) among the corresponding sequences from these two strains (Table 3). Most of the amino acid changes in selected TiLV segments between Thailand-Israel strains are located in different positions from the differences between Ecuador-Israel strains. Note that the sequences of the Ecuador, Colombia, and Egypt TiLV strains are not yet available in the GenBank database for comparison in this study. Interestingly, the 3 selected segments could be amplified from fish RNA samples from the CL farm but only 1 segment was obtained from another fish farm (CN farm) that was previously confirmed as TiLV positive by semi-nested RT-PCR and sequence analysis. This might suggest variations among isolates in Thailand too. Whether these variations affect evolution and the virulence of the virus needs further investigation.

In summary, the present study reported emergence of TiLV in both Nile tilapia and red tilapia, which have been cultured in either earthen ponds or floating cages in Thailand. An alternative semi-nested RT-PCR protocol and an *in situ* hybridization method have been developed for detection of this virus. Genetically, a partial genome obtained from Thailand-originated TiLV exhibited genetic variations with those from Israel.



**Fig. 5.** *In situ* hybridization using a DIG-labeled probe derived from TiLV genome segment 3 revealed strongest positive signals in the liver capsule and hepatocytes (B) of the naturally diseased fish. Positive signals were visualized in both epithelial cells of the kidney tubules and hematopoietic tissue (D). Fewer positive signals found in the brain which located mainly in outer part (F). Both primary and secondary lamellae of gill filaments showed strong positive (H). ISH assay using an unrelated probe as a negative control revealed no signal with the same tested samples (A, C, E and G).

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.aquaculture.2017.04.019>.

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