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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% morality 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 fingerings 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 *Oreochromis niloticus*	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 *Oreochromis* 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 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

Table 2
Primers used in this study.

Primer name	Sequence (5′-3′)	Target gene	Product size (bp)	References
Nested ext-1	TATGCAGTACTTTCCCTGCC	Segment 3		Eyngor et al. (2014)
ME1	GTTGGGCACAAGGCATCCTA		415	Tsofack et al. (2017)
7450/150R/ME2	TATCACGTGCGTACTCGTTCAGT		250	
TiLV-Seg1-F	TCATTCGCCTATATAGTTAC	Segment 1	1614	This study
TiLV-Seg1-R	TTAATTACGCACTATTACTG			
TiLV-Seg5-F	TTTTTCTCAGTTTACCACTC	Segment 5	1073	This study
TiLV-Seg5-R	TTATCTCAGACTCCAATAGC			
TiLV-Seg9-F	ACGTCCTTAAAGTCATACTT	Segment 9	522	This study
TiLV-Seg9-R	ACAAGTCCGATTACTTTTTC			