



## Short communication

## Evidence of TiLV infection in tilapia hatcheries from 2012 to 2017 reveals probable global spread of the disease

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## ABSTRACT

Recent outbreaks of tilapia lake virus (TiLV) in farmed tilapia in Thailand were the first indication of spread of the virus to the Southeast Asia region. Here we further investigate TiLV infection of archived and newly collected fish samples obtained from Thai hatcheries from 2012 to 2017. Fertilized eggs, yolk-sac larvae, fries, and fingerlings were tested for the TiLV using an established semi-nested RT-PCR assay. The results revealed that the majority of the tested samples were TiLV positive, including our earliest preserved samples collected in year 2012. DNA sequence analysis of representative amplified products also confirmed the presence of TiLV. Since the discovery of TiLV in 2012, over 40 countries worldwide have imported tilapia fry and fingerlings, and some may have been unaware of risk that they might be infected with TiLV. Thus, if they have not already done so, we recommend that countries that have imported tilapia for aquaculture carry out surveillance studies for its presence and also add TiLV to their import quarantine inspection list.

## 1. Introduction

Syncytial hepatitis of tilapia (SHT) or tilapia lake virus disease (TiLVD) is a newly emerging disease caused by a novel segmented RNA virus called tilapia lake virus (TiLV) that was named from a large freshwater lake in Israel (Kinneret Lake or the Sea of Galilee) where the disease was first recognized (Eyngor et al., 2014; OIE, 2017). The virus was then classified as an Orthomyxo-like virus based on its morphology of round to oval enveloped and filamentous/tubular viral particles with negative-sense, RNA genomes of 10-segments and 10,323 kb total length (Bacharach et al., 2016; Del-Pozo et al., 2017). TiLV-affected farms cultivating Nile tilapia (*Oreochromis niloticus*), red tilapia (*Oreochromis* sp.) and hybrid tilapia (*O. niloticus* × *O. aureus*) may experience up to 90% mortality (Dong et al., 2017; Eyngor et al., 2014; Ferguson et al., 2014; Surachetpong et al., 2017). To date, the disease has been reported from Ecuador (Ferguson et al., 2014), Israel (Eyngor et al., 2014), Colombia (Kembou Tsoufack et al., 2017), Egypt (Fathi et al., 2017; Nicholson et al., 2017) and Thailand (Dong et al., 2017; Surachetpong et al., 2017). Reported detection methods for TiLV

include nested RT-PCR, semi-nested RT-PCR, SYBR quantitative RT-PCR and in situ hybridization (Bacharach et al., 2016; Dong et al., 2017; Kembou Tsoufack et al., 2017).

Recent work has revealed the emergence of TiLV in Thailand, but the source of virus remains unknown (Dong et al., 2017; Surachetpong et al., 2017). However, these recent reports led us to hypothesize that some previous, unexplained mortality of tilapia fry in Thai hatcheries in the preceding years may have constituted unrecognized outbreaks of TiLVD. To test this hypothesis, we carried out a retrospective investigation to determine the presence of TiLV in archived samples from selected tilapia hatcheries that have records of both national sales and international export of tilapia fries/fingerlings since 2012. The results confirmed our hypothesis.

## 2. Materials and methods

## 2.1. Fish samples and RNA preparation

Fish samples assayed in this study included fertilized eggs, yolk-sac

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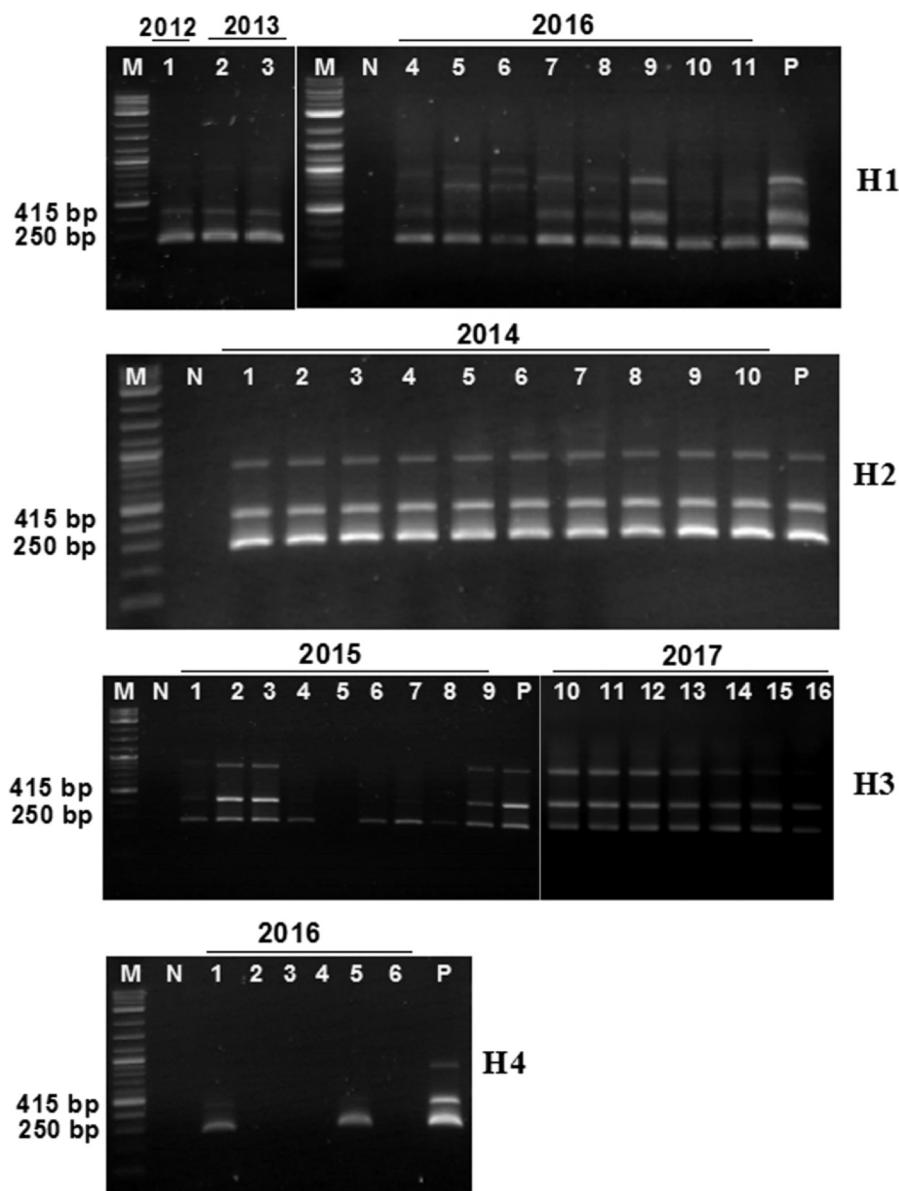
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**Table 1**  
Details of fish samples and TiLV detection results.

Hatchery code	Year collected	Species	Fish stage	Number of sample tested	Number of positive/number tested	Number of sample sequenced	% identity to prototype strain (KU751816)
H1	2012	Red tilapia	Yolk-sac larvae	1 pool	1/1	1 (250 bp)	97.2
	2013	Red tilapia	Fertilized eggs	1 pool	1/1	1 (250 bp)	97.6
		Nile tilapia	Fertilized eggs	1 pool	1/1	1 (250 bp)	97.2
	2016	Red tilapia	Fry	4	4/4	1 (250 bp)	96.0
		Nile tilapia	Fry	4	4/4	1 (250 bp)	97.6
H2	2014	Nile tilapia	Fry	10	10/10	1 (415 bp)	98.1
H3	2015	Nile tilapia	Fingerling	9	8/9	2 (415 bp)	96.9–97.8
	2017	Nile tilapia	Fry	7 pools	7/7	2 (415 bp)	97.3–97.6
H4	2016	Nile tilapia	Fingerling	6	2/6	0	n/a



**Fig. 1.** Agarose gels of amplicons from semi-nested RT-PCR tests for TiLV from tilapia samples from 4 Thai hatcheries (H1 to H4) collected in years 2012–2017. Product sizes of 415 bp and 250 bp represent amplicons from the first and semi-nested PCR steps, respectively. M = DNA marker (2-Log DNA Ladder, New England Biolabs); N = no template control; P = positive control reaction using pGEM-415\_bp plasmid.

larvae, fry and fingerlings of both red (*Oreochromis* sp.) and Nile tilapia (*Oreochromis niloticus*) (Table 1). The samples included those collected recently (2016–2017) and those collected from 2012 to 2015 (preserved at  $-20\text{ }^{\circ}\text{C}$ ) from 4 tilapia hatcheries located in 4 different provinces of Thailand. Details of sample information are presented in Table 1. Most of the samples were collected during occurrence of

abnormal, unexplained mortalities. Total RNA was extracted from 2 to 3 pooled specimens of whole fertilized eggs, larvae, fry or pooled internal organs (liver, kidney and spleen) of individual fingerlings using Trizol reagent (Invitrogen) according to protocol recommended by the manufacturer. Extracted RNA was quantified by spectrophotometry at 260 and 280 nm and adjusted to desirable template concentration.

## 2.2. Detection of TiLV by semi-nested RT-PCR

Semi-nested RT-PCR detection of TiLV was carried out as previously described (Dong et al., 2017). Briefly, primers used for the semi-nested RT-PCR assay were Nested ext.-1 (5'-TAT GCA GTA CTT TCC CTG CC-3'), ME1 (5'-GTT GGG CAC AAG GCA TCC TA-3') and 7450/150R/ME2 (5'-TAT CAC GTG CGT ACT CGT TCA GT-3') targeting TiLV genome segment 3 (Eyngor et al., 2014; Kembou Tsofack et al., 2017). For PCR assays, a plasmid containing a 415-bp fragment of the partial TiLV genome segment 3 (pGEM-415\_bp) (Dong et al., 2017) was used as the positive control while nuclease-free water served the negative control. PCR products were stained with RedSafe nucleic acid staining solution (iNtRON Biotechnology), electrophoresed using 1% agarose gel in 1 × TBE buffer prior to visualization under UV light of the gel documentation system. Product sizes of expected amplicons from respective first and second step reactions were 415 bp and 250 bp (Dong et al., 2017).

## 2.3. DNA cloning and sequencing

Representative 415 bp amplicons from RT-PCR-positive samples collected from hatcheries H2 and H3 in the years 2014, 2015 and 2017 as well as 250 bp amplicons from hatchery H1 in the years 2012, 2013 and 2016 were selected for DNA sequencing (Table 1). PCR products were gel-purified and ligated into pGEM-T-easy vector. Successful cloning was verified by colony PCR using vector primers (T7 and Sp6 promoter primers) and recombinant plasmids were subsequently extracted and subjected to DNA sequencing (Macrogen, South Korea). Nucleotide sequence homology was determined using BLASTN in the GenBank database (NCBI). Multiple alignments of 250-bp consensus sequences of 10 sequences obtained from this study and that of the TiLV genome segment 3 of the prototype strain from Israel (GenBank accession no. KU751816) were conducted using Clustal Omega (<http://www.ebi.ac.uk/Tools/msa/clustalo/>).

## 3. Results and discussion

Fish specimens collected from Thailand in 2012–2017 were obtained from 4 different hatcheries located in 4 provinces as listed in Table 1. By TiLV-semi-nested RT-PCR, all samples collected from hatcheries H1 (years 2012, 2013 and 2016), H2 (year 2014) and H3 (year 2017) gave positive results for TiLV while only 8 out of 9 samples from H3 (year 2015) and 2/6 samples from H4 (year 2016) gave positive results for TiLV (Table 1 and Fig. 1). Most of the samples generated 2 expected amplicons (415 and 250 bp) indicating heavy TiLV infection, while a few samples generated only the 250 bp product indicating light infections (Fig. 1). Also present in the agarose gels of heavily-infected samples and in the plasmid positive control was a non-specific band at approximately ~800 bp. As previously described (Dong et al., 2017), this appeared to result from cross-hybridization between the 415 and 250 bp amplicons.

Sequencing results for 5 clones containing the 250-bp amplicon obtained from hatchery H1 and 5 clones harboring the 415-bp amplicon from hatcheries H2 and H3 (Table 1) revealed 96.0–98.1% nucleotide identity to the sequence of the TiLV prototype strain from Israel (GenBank accession no. KU751816) (Table 1). Note that there was 96.0–100% nucleotide sequence identity among the TiLV amplified fragments from infected samples from Thailand. Alignment of the 250-bp consensus sequences obtained from 10 samples in this study and the corresponding sequence of the prototype strains revealed variation in 21 nucleotide positions throughout the fragments (Fig. 2). Analysis of partial genome sequences of TiLV isolates from different countries revealed genetic variation (Bacharach et al., 2016; Dong et al., 2017; Nicholson et al., 2017; Surachetpong et al., 2017). However, the possibility of correlation between genetic variation and virulence remains unexplored. In this regard, it is notable that TiLV infections result in

highly variable mortality ranging from 9.2 to 90% (Dong et al., 2017; Eyngor et al., 2014; Fathi et al., 2017; Surachetpong et al., 2017) that may be the result of TiLV genetic variation or host status as a result of age or size, overall health, environmental stress, etc. All these variables need to be examined in further research.

TiLV was first documented as a novel viral pathogen in farmed tilapia in Israel in 2014 (Eyngor et al., 2014) after a report of outbreak of syncytial hepatitis (SHT) associated with an uncharacterized virus in Ecuador in 2013 (Ferguson et al., 2014). A few years later, further evidence revealed that the viruses from the two countries were genetically homologous, suggesting the same or similar causative viral agents in the two countries (Bacharach et al., 2016; Del-Pozo et al., 2017). Subsequently, occurrences of TiLV in the Colombia (Ecuador's neighbor) and Egypt (Israel's neighbor) were revealed (Fathi et al., 2017; Kembou Tsofack et al., 2017; Nicholson et al., 2017), suggesting serious transboundary spread of infectious disease in the tilapia aquaculture industry. The present study and recent publications (Dong et al., 2017; Surachetpong et al., 2017) have included Thailand in the geographical distribution of TiLV. Thus, the list of countries with published evidence of TiLV has been increased to include Ecuador, Israel, Colombia, Egypt, and Thailand (Fig. 3).

Four tested tilapia hatcheries in Thailand were confirmed to be infected with TiLV by both RT-PCR and DNA sequence analysis, including archived samples collected from 2012 to 2015. This proves the virus was in circulation in Thailand as early as 2012, even before TiLV became known to science, and it is probable that it initially arrived in the kingdom in 2008 when very significant, unexplained disease outbreaks occurred nationwide and continued thereafter. Nucleic acid sequences of TiLV found in early developmental stages of tilapia (fertilized eggs and yolk-sac larvae) in this study suggests possible vertical transmission or nonspecific absorption of viral particles on these life stages, an issue that requires further investigation. Evidence of vertical transmission of both bacterial and viral pathogens including *Streptococcus* spp., *Francisella noatunensis* subsp. *orientalis* (*Fno*) and tilapia larvae encephalitis virus (TLEV) have already been reported in tilapia (Pradeep et al., 2017; Pradeep et al., 2016; Sinyakov et al., 2011). To limit impact of infectious diseases globally, research programs should be promoted towards development of tilapia stocks that are specific pathogen free (SPF) for TiLV and other important infectious pathogens of farmed tilapia such as *Iridovirus*, TLEV, *Streptococcus agalactiae* and *Fno* (Dong et al., 2015; Kayansamruaj et al., 2014; Nguyen et al., 2016; Sinyakov et al., 2011).

It is of concern, that three of the commercial Thai hatcheries (H1, H2 and H3) have provided tilapia fry/fingerlings nationwide and worldwide. Therefore, dispersal of the virus to at least some importing countries is highly probable. Based on the list of recorded destination countries for tilapia fry/fingerlings from those hatcheries since 2012 (the first year of TiLV positive results in our archived samples), we propose the following list of 40 countries that may have been at high risk of TiLV spread: Algeria, Bahrain, Bangladesh, Belgium, Burundi, Canada, China, Congo, Germany, Guatemala, India, Indonesia, Japan, Jordan, Laos, Malaysia, Mozambique, Myanmar, Nigeria, Pakistan, Philippines, Romania, Rwanda, Saudi Arabia, Singapore, South Africa, Sri Lanka, Switzerland, Tanzania, Togo, Tunisia, Turkey, Turkmenistan, Uganda, Ukraine, United Arab Emirate, United Kingdom, United States, Vietnam and Zambia. There are also 3 lower risk countries (El-Salvador, Mexico, and Nepal) that imported fish from these hatcheries before year 2012 (Fig. 3). Thus, we recommend the countries which have been translocating tilapia fry/fingerlings from the five countries with confirmed reports of TiLV infection should rapidly initiate disease surveillance and biosecurity, especially for translocated fish, to prevent wider spread of the virus and reduce the impact of the disease. In addition, retrospective diagnosis using archived samples in aquatic animal health laboratories (especially samples from unexplained tilapia disease outbreaks) is recommended to gain a better understanding on epidemiology of TiLV.



Israel-2011	TATCAGTGGCTACTCGTTTCAGTATAAGTCTCTTGCCCTCTGGTCAAGACCACACTCCT	60
H1-2012 Y-Red	TATCAGTGGCTACTCGTTTCAGTATAAGTCTCTTGCCCTCTGGTCAAGACCACACTCCT	60
H1-2013 F-Nile	TATCAGTGGCTACTCGTTTCAGTATAAGTCTCTTGCCCTCTGGTCAAGACCACACTCCT	60
H1-2013 F-Red	TATCAGTGGCTACTCGTTTCAGTATAAGTCTCTTGCCCTCTGGTCAAGACCACACTCCT	60
H1-2016 Fr-Red	TATCAGTGGCTACTCGTTTCAGTATAAGTCTCTTGCCCTCTGGTCAAGACCACACTCCT	60
H1-2016 Fr-Nile	TATCAGTGGCTACTCGTTTCAGTATAAGTCTCTTGCCCTCTGGTCAAGACCACACTCCT	60
H2-2014 Fr-Nile	TATCAGTGGCTACTCGTTTCAGTATAAGTCTCTTGCCCTCTGGTCAAGACCACACTCCT	60
H3-2015 Fr-Nile	TATCAGTGGCTACTCGTTTCAGTATAAGTCTCTTGCCCTCTGGTCAAGACCACACTCCT	60
H3-2015 Fr-Nile	TATCAGTGGCTACTCGTTTCAGTATAAGTCTCTTGCCCTCTGGTCAAGACCACACTCCT	60
H3-2017 Fi-Nile	TATCAGTGGCTACTCGTTTCAGTATAAGTCTCTTGCCCTCTGGTCAAGACCACACTCCT	60
H3-2017 Fi-Nile	TATCAGTGGCTACTCGTTTCAGTATAAGTCTCTTGCCCTCTGGTCAAGACCACACTCCT	60
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Israel-2011	CACCGCAGGCGAGGAACCTTTGAGCACTCGAAGAATCCATATGCTCTTTAGCTCAGCTG	120
H1-2012 Y-Red	CACCGCAGGCGAGGAACCTTTGAGCACTCGAAGAATCCATATGCTCTTTAGCTCAGCTG	120
H1-2013 F-Nile	CACCGCAGGCGAGGAACCTTTGAGCACTCGAAGAATCCATATGCTCTTTAGCTCAGCTG	120
H1-2013 F-Red	CACCGCAGGCGAGGAACCTTTGAGCACTCGAAGAATCCATATGCTCTTTAGCTCAGCTG	120
H1-2016 Fr-Red	CACCGCAGGCGAGGAACCTTTGAGCACTCGAAGAATCCATATGCTCTTTAGCTCAGCTG	120
H1-2016 Fr-Nile	CACCGCAGGCGAGGAACCTTTGAGCACTCGAAGAATCCATATGCTCTTTAGCTCAGCTG	120
H2-2014 Fr-Nile	CACCGCAGGCGAGGAACCTTTGAGCACTCGAAGAATCCATATGCTCTTTAGCTCAGCTG	120
H3-2015 Fr-Nile	CACCGCAGGCGAGGAACCTTTGAGCACTCGAAGAATCCATATGCTCTTTAGCTCAGCTG	120
H3-2015 Fr-Nile	CACCGCAGGCGAGGAACCTTTGAGCACTCGAAGAATCCATATGCTCTTTAGCTCAGCTG	120
H3-2017 Fi-Nile	CACCGCAGGCGAGGAACCTTTGAGCACTCGAAGAATCCATATGCTCTTTAGCTCAGCTG	120
H3-2017 Fi-Nile	CACCGCAGGCGAGGAACCTTTGAGCACTCGAAGAATCCATATGCTCTTTAGCTCAGCTG	120
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Israel-2011	TTTCCTTGGATATGTCGCGAAGTCTGGGTGGTGCCACCCACTCAATACGAGGCTTCGGGC	180
H1-2012 Y-Red	TTTCCTTGGATATGTCGCGAAGTCTGGGTGGTGCCACCCACTCAATACGAGGCTTCGGGC	180
H1-2013 F-Nile	TTTCCTTGGATATGTCGCGAAGTCTGGGTGGTGCCACCCACTCAATACGAGGCTTCGGGC	180
H1-2013 F-Red	TTTCCTTGGATATGTCGCGAAGTCTGGGTGGTGCCACCCACTCAATACGAGGCTTCGGGC	180
H1-2016 Fr-Red	TTTCCTTGGATATGTCGCGAAGTCTGGGTGGTGCCACCCACTCAATACGAGGCTTCGGGC	180
H1-2016 Fr-Nile	TTTCCTTGGATATGTCGCGAAGTCTGGGTGGTGCCACCCACTCAATACGAGGCTTCGGGC	180
H2-2014 Fr-Nile	TTTCCTTGGATATGTCGCGAAGTCTGGGTGGTGCCACCCACTCAATACGAGGCTTCGGGC	180
H3-2015 Fr-Nile	TTTCCTTGGATATGTCGCGAAGTCTGGGTGGTGCCACCCACTCAATACGAGGCTTCGGGC	180
H3-2015 Fr-Nile	TTTCCTTGGATATGTCGCGAAGTCTGGGTGGTGCCACCCACTCAATACGAGGCTTCGGGC	180
H3-2017 Fi-Nile	TTTCCTTGGATATGTCGCGAAGTCTGGGTGGTGCCACCCACTCAATACGAGGCTTCGGGC	180
H3-2017 Fi-Nile	TTTCCTTGGATATGTCGCGAAGTCTGGGTGGTGCCACCCACTCAATACGAGGCTTCGGGC	180
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Israel-2011	CACTCTTTGGATGTGGTAGTTCCAATAGCCGTTCCCTTAGCTCAGCATCGTAGGATGCCT	240
H1-2012 Y-Red	CACTCTTTGGATGTGGTAGTTCCAATAGCCGTTCCCTTAGCTCAGCATCGTAGGATGCCT	240
H1-2013 F-Nile	CACTCTTTGGATGTGGTAGTTCCAATAGCCGTTCCCTTAGCTCAGCATCGTAGGATGCCT	240
H1-2013 F-Red	CACTCTTTGGATGTGGTAGTTCCAATAGCCGTTCCCTTAGCTCAGCATCGTAGGATGCCT	240
H1-2016 Fr-Red	CACTCTTTGGATGTGGTAGTTCCAATAGCCGTTCCCTTAGCTCAGCATCGTAGGATGCCT	240
H1-2016 Fr-Nile	CACTCTTTGGATGTGGTAGTTCCAATAGCCGTTCCCTTAGCTCAGCATCGTAGGATGCCT	240
H2-2014 Fr-Nile	CACTCTTTGGATGTGGTAGTTCCAATAGCCGTTCCCTTAGCTCAGCATCGTAGGATGCCT	240
H3-2015 Fr-Nile	CACTCTTTGGATGTGGTAGTTCCAATAGCCGTTCCCTTAGCTCAGCATCGTAGGATGCCT	240
H3-2015 Fr-Nile	CACTCTTTGGATGTGGTAGTTCCAATAGCCGTTCCCTTAGCTCAGCATCGTAGGATGCCT	240
H3-2017 Fi-Nile	CACTCTTTGGATGTGGTAGTTCCAATAGCCGTTCCCTTAGCTCAGCATCGTAGGATGCCT	240
H3-2017 Fi-Nile	CACTCTTTGGATGTGGTAGTTCCAATAGCCGTTCCCTTAGCTCAGCATCGTAGGATGCCT	240
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Israel-2011	TGTGCCAAC	250
H1-2012 Y-Red	TGTGCCAAC	250
H1-2013 F-Nile	TGTGCCAAC	250
H1-2013 F-Red	TGTGCCAAC	250
H1-2016 Fr-Red	TGTGCCAAC	250
H1-2016 Fr-Nile	TGTGCCAAC	250
H2-2014 Fr-Nile	TGTGCCAAC	250
H3-2015 Fr-Nile	TGTGCCAAC	250
H3-2015 Fr-Nile	TGTGCCAAC	250
H3-2017 Fi-Nile	TGTGCCAAC	250
H3-2017 Fi-Nile	TGTGCCAAC	250
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**Fig. 2.** Multiple alignment of 250-bp consensus sequences of TiLV isolates obtained from infected tilapia hatcheries (H1 to H3) from 2012 to 2017 in Thailand and the sequence of the prototype strain from Israel (GenBank accession no. KU751816). There was variation in 21 nucleotide positions (grey-shaded nucleotides) throughout the fragment. Red and Nile indicate red tilapia (*Oreochromis* sp.) and Nile tilapia (*Oreochromis niloticus*) samples. Stages of fish were Y (yolk-sac larvae), F (fertilized eggs), Fr (fry), and Fi (Fingerling). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Despite the fact that TiLV was discovered recently, the occurrence of the disease has been suspected in Ecuador and Israel since 2009 (Eyngor et al., 2014; Ferguson et al., 2014). Therefore, the countries that imported tilapia from Ecuador and Israel during this period might also have a potential risk of viral transmission. In Thailand, the earliest occurrence of TiLV was confirmed in 2012 but the source of viral transmission remains unclear. It may be that the disease spread from Ecuador to Vietnam with import of red tilapia stocks for a selective breeding program and that the disease then spread to Thailand via import of tilapia or other species in 2008. The disease is particularly harmful to red tilapia which is cage reared in natural water bodies in Thailand. In fact, a cage farmer raising tilapia in the Kwai River in Kanchanaburi province was the first to report mass mortality in his fish in April 2008 and within a few months the disease was being reported nationwide. Pond-based hatcheries and grow-out farms subsequently quickly became infected as the disease was already in all the waterways and irrigation systems.

In conclusion, the findings in this study have raised awareness for tilapia producers on potential global dispersal of an emerging virus; a hidden pathogen that was discovered recently. Thus, the countries with potential risk should increase the measures to control and prevent further spread of the disease.

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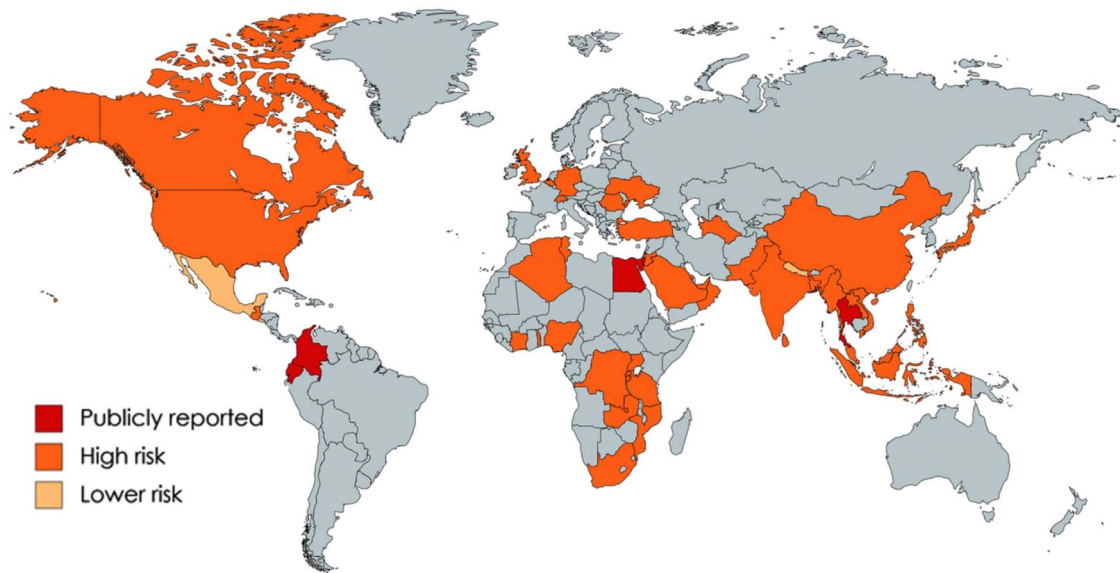


Fig. 3. The geographical distribution map of tilapia lake virus (TiLV). Red colour indicated 5 countries with firm evidence of the presence of TiLV. Orange and light orange colors represent 40 and 3 countries with respective high risk and lower risk of TiLV spread through translocation of tilapia fry/fingerlings that may have been infected with TiLV. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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