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Short communication

Inapparent infection cases of tilapia lake virus (TiLV) in farmed tilapia

Saengchan Senapin[a,b], K.U. Shyam[a], Watcharachai Meemetta[a], Triwit Rattanarojpong[c], Ha Thanh Dong[c,*]

[a] Fish Health Platform, Center of Excellence for Shrimp Molecular Biology and Biotechnology (Centex Shrimp), Faculty of Science, Mahidol University, 272 Rama VI Road, Bangkok 10400, Thailand
[b] National Center for Genetic Engineering and Biotechnology (BIOTEC), National Science and Technology Development Agency, Pathumthani 12120, Thailand
[c] Aquaculture Vaccine Platform, Department of Microbiology, Faculty of Science, King Mongkut's University of Technology Thonburi, Bangkok 10140, Thailand

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ABSTRACT

Tilapia farming has been affected by a newly discovered Orthomyxovirus-like, tilapia lake virus (TiLV), which has caused considerable economic loss to farmers. Currently, mortality-associated TiLV infections have been reported in tilapia farms in Israel, Ecuador, Colombia, Egypt, Thailand, Chinese Taipei, India and Malaysia. In this study, sets of samples collected from clinically healthy adult and fingerling tilapia with no signs of diseases or mortality were randomly diagnosed for TiLV. The tissue samples were examined by semi-nested RT-PCR, histopathology, and *in situ* hybridization (ISH). Unexpectedly, individual organs (liver, kidney, spleen, brain, and heart) of the tested adult fish (2/2) and liver of the fingerlings (9/19) exhibited positive results in the second step RT-PCR, indicating a low viral load of TiLV in the fish tissues. Sequencing analysis of 250-bp amplicons revealed 97.2% identity to the prototype strain from Israel. Histopathology was investigated in the adult fish specimens and pathological features resembling syncytial hepatitis were observed while ISH yielded no detectable signal. Unlike previous reports, this study revealed cases of inapparent or subclinical infections of TiLV in tilapia. Underlying factors and mechanisms between host and virus resulting in inapparent infection require further scientific investigation.

1. Introduction

Tilapia provides an inexpensive source of protein for the majority of developing countries and contributes significantly to global food security through its production of 4.5 million metric tons yearly (Bacharach et al., 2016; Cleasby et al., 2014; Gomna, 2011). However, fish farmers continue to identify diseases as one of the main problems in their intensive culture system. Recently, a newly discovered segmented RNA virus resembling Orthomyxovirus named TiLV that caused syncytial hepatitis of tilapia (SHT) has been considered ~~as~~ a potential threat to global tilapia production (Bacharach et al., 2016; CGIAR, 2017; Eyngor et al., 2014; FAO, 2017; OIE, 2017a). Besides SHT, the disease caused by TiLV has been documented as tilapia lake virus disease (TiLVD), summer mortality syndrome (SMS) or tilapia one month mortality syndrome (TOMMS) (Del-Pozo et al., 2017; Eyngor et al., 2014; Fathi et al., 2017; Ferguson et al., 2014; OIE, 2017a; Tattiyapong et al., 2017a). The disease occurs mainly in fingerling and juvenile stages of tilapia and can kill up to 90% of the fish population (Dong et al., 2017a; Eyngor et al., 2014; Ferguson et al., 2014; Surachetpong et al., 2017). TiLV was initially discovered in tilapia farms in Ecuador and Israel in 2014 (Eyngor et al., 2014; Ferguson et al., 2014). The virus may have been involved in massive mortalities in Israel since 2009 (Bacharach et al., 2016; Eyngor et al., 2014). Subsequently, occurrences of the disease have been confirmed in Colombia, Egypt (Fathi et al., 2017; Kembou Tsofack et al., 2017; Nicholson et al., 2017), Thailand (Dong et al., 2017a; Surachetpong et al., 2017), Chinese Taipei, India and Malaysia (Amal et al., 2018; Behera et al., 2018; OIE, 2017b, 2017c). DNA based-methods including SYBR Green-based PCR, nested RT-PCR, semi-nested RT-PCR and *in situ* hybridization have been established for TiLV diagnosis (Bacharach et al., 2016; Dong et al., 2017a; Kembou Tsofack et al., 2017; Tattiyapong et al., 2017b). Additionally, successful cultivation and isolation of TiLV using fish cell lines has been established (Behera et al., 2018; Eyngor et al., 2014; Kembou Tsofack et al., 2017; Tattiyapong et al., 2017a). Although all of the previous studies described TiLV association with tilapia mortality, the present work reports the detection of TiLV in clinically healthy tilapia that showed no symptoms of being infected.

* Corresponding author.
E-mail address: hadongntu@gmail.com (H.T. Dong).

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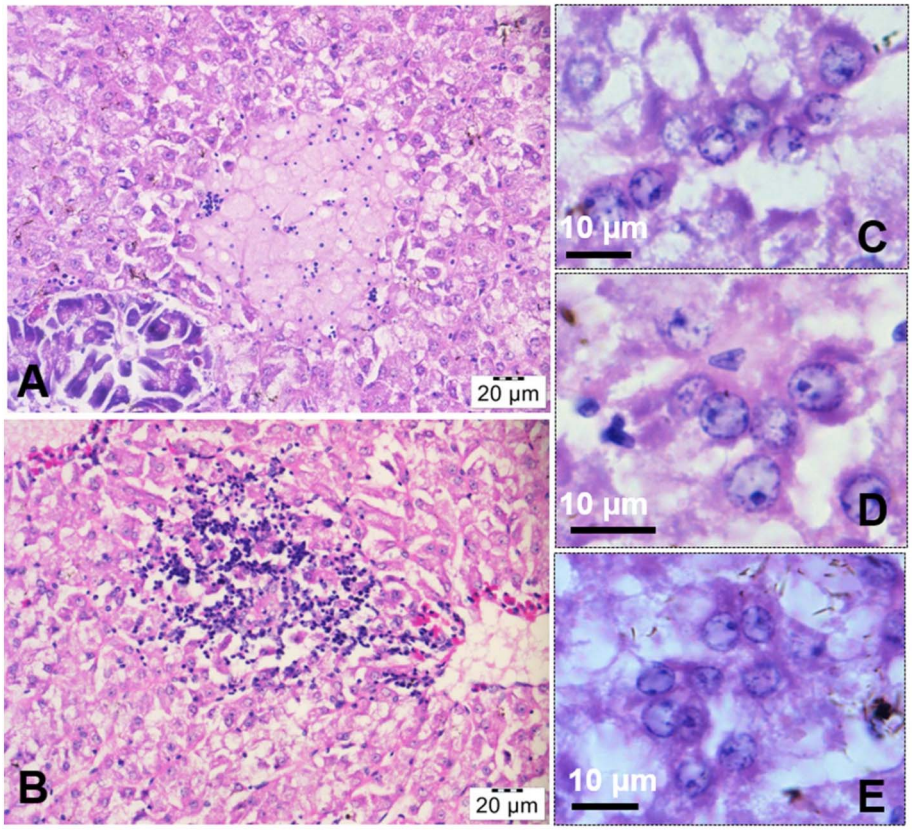

Fig. 1. Representative photomicrographs of H&E stained sections of the fish liver showing focal necrosis of hepatocytes and infiltration of lymphocytic inflammatory cells (A, B). Presence of hepatocytes resembling giant cells which contained multiple nuclei (C–E) was also observed, H&E staining.

2. Materials and methods

2.1. Fish samples and RNA preparation

The samples used in this study were obtained from clinically healthy fish. This included 1 red tilapia (*Oreochromis* sp.) and 1 Nile tilapia (*Oreochromis niloticus*) adults (350–380 g) and a batch of 19 Nile tilapia fingerlings (1–2 g). The adult fish were collected from a grow-out pond in a commercial tilapia farm with no previous record of TiLV while fingerling fish were sampled from a nursery pond in another farm which experienced occurrences of TiLV in 2015 and 2017. It is noted that the fingerlings were produced within a closed system. Prior to and post sample collection, the fish from two farms exhibited no abnormal clinical signs or unusual behaviors. The internal organs (spleen, liver, heart, kidney, and the brain) from each of adult fish were individually collected and preserved in 95% ethanol for TiLV diagnosis by RT-PCR assay while only the liver tissue was fixed in 10% buffered formalin for 24 h before being transferred into 70% ethanol solution for histopathological examination. For the fingerlings, only liver tissues were processed for RT-PCR detection and histology was not performed. Total RNA was extracted using Trizol reagent (Invitrogen), adjusted to desirable concentration and stored at −20 °C until used.

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

TiLV detection assay was performed using a semi-nested RT-PCR protocol as described in the previous works (Dong et al., 2017a, 2017b). Oligo-sequences of primers targeting TiLV genome segment 3 were followed from Eyngor et al. (2014). RNA extracted from internal organs of tested red and Nile tilapia was used as a template. A recombinant plasmid containing a 415-bp fragment of TiLV segment 3 (pGEM-415_bp) (Dong et al., 2017a) was employed in a positive control reaction while nuclease-free water served as the negative control. Amplified products were electrophoresed and visualized under UV light. The expected amplicons in the first and second round PCR were 415 and 250 bp, respectively.

2.3. Cloning and sequences analysis

In order to confirm specific amplification of RT-PCR assay, 2 representative amplicons of 250-bp yielded from one red and one Nile tilapia samples were gel-purified, cloned into pGEM-T easy vector, and sequenced (Macrogen, South Korea). Three recombinant clones derived from each sample were randomly picked for DNA sequence analysis. Nucleotide sequence identity of the obtained consensus sequences was determined using BLASTN (https://blast. ncbi.nlm. nih.gov). Translation of nucleotide sequences to deduced protein sequences was conducted using ExPASy (http://web.expasy.org/translate/). Multiple nucleotide and amino acid sequence alignments of the TiLV in this study and the previously reported isolates from Israel (GenBank accession no. KU751816), Egypt (KY817384), and Thailand (Dong et al., 2017a) were performed using Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo).

2.4. Histopathological investigation and in situ hybridization (ISH)

Formalin-preserved liver specimens were processed for histology, and subjected to *in situ* hybridization (ISH). TiLV specific digoxigenin (DIG)-labelled DNA probe was prepared from a 415-bp fragment of TiLV genome segment 3 (Dong et al., 2017a) while an unrelated control DIG probe was made from shrimp virus IMNV genome and used as a negative control (Senapin et al., 2007). *In situ* hybridization assay was carried out following previously described protocols (Dong et al., 2017a; Senapin et al., 2016) and a liver of natural TiLV-infected fish collected in a previous study (Dong et al., 2017a) was used as a positive control for ISH assay.

3. Results and discussion

3.1. Adult fish samples exhibited histopathological signs resembling syncytial hepatitis

Liver specimens of adult, clinically healthy red and Nile tilapia were histologically investigated for signs of syncytial hepatitis of tilapia (SHT). The results from H&E stained sections revealed that the fish hepatocytes in some areas of the liver were abnormally enlarged and had lost their usual polyhedral shape (Rocha et al., 1997) (Fig. 1). Other notable lesions observed in the liver were focal necrosis of hepatocytes and severe infiltration of lymphocytic inflammatory cells (Fig. 1, A–B). Frequently, swollen hepatocytes contained multiple nuclei (syncytial cells), in some areas of the liver sections were visualized (Fig. 1, C–E). The syncytial hepatitis was previously described in not only TiLV infected tilapia (Del-Pozo et al., 2017; Dong et al., 2017a; Ferguson et al., 2014) but also in herpesviruses infected turbot (*Scophthalmus maximus*) and rainbow trout, (*Oncorhynchus mykiss*) (Wolf, 1988), and reovirus-like infected halibut (*Hippoglossus hippoglossus*) (Cusack et al., 2001; Ferguson et al., 2003). Therefore, further confirmation of TiLV infection in the clinically healthy tilapia was conducted using molecular techniques (see below).

3.2. TiLV infection were confirmed by RT-PCR and sequence analysis

Semi-nested RT-PCR assay for TiLV detection was performed using total RNA extracted from the fish internal organs (liver from fingerlings while liver, kidney, brain, spleen, and heart from adult) as templates. The results showed that all tested organs from adult tilapia and 9 out of 19 livers from Nile tilapia fingerlings were positive for TiLV. A representative RT-PCR detection result obtained from the adult fish samples is shown in Fig. 2. All positive samples gave a single amplified product of 250 bp indicating a low viral load in the tested tissues interpreted based on the detection protocol described previously (Dong et al., 2017a). Low viral load in the fish tissues might explain no detectable signals in the subsequent ISH results (not shown).

Subsequent DNA analysis of the partial TiLV genome segment 3 was performed from a representative 250-bp product from each of the red and Nile adult tilapia samples. The consensus sequences of TiLV segments derived from 3 recombinant clones obtained from each of both fish revealed 100% identity (Fig. 3). The fragments exhibited 97.2% homology (7 differences) to the prototype TiLV from Israel (GenBank accession no. KU751816). Further comparison of the TiLV partial genome segment 3 among isolates in this study and from Israel, Thailand, and Egypt revealed 92.4–97.8% nucleotide sequence identity with 21 variable positions (Fig. 3A). Putative amino acid sequence alignment revealed 1–3 residue differences (Fig. 3B).

3.3. Inapparent infection of TiLV

Natural outbreaks of infection with TiLV associated with high mortalities (20–90%) were reported in fingerling and juvenile stages of tilapia (Behera et al., 2018; Dong et al., 2017a; Eyngor et al., 2014; Ferguson et al., 2014; Surachetpong et al., 2017). However, lower mortalities of TiLV infected farms were found in Egypt (9.2%), Chinese Taipei (6.4%), and Malaysia (0.71 and 15%). The former case was observed from medium to large fish greater than 100 g (Fathi et al., 2017) while no description on fish sizes were mentioned in the 2 latter reports (OIE, 2017b, c). It is not known if the TiLVD is life stage specific or not. However, in this study, both young and adult tilapia were found to be TiLV positive without showing abnormal symptoms or unusual mortality observed one month before and after the confirmation of TiLV. Such an inapparent infection and a wide range of mortality rates might theoretically suggest the existence of a genetic variability of TiLV as also described in a fish orthomyxovirus called infectious salmon anemia virus (ISAV) having more than 20 variants with differences in pathogenicity (Christiansen et al., 2011; Cottet et al., 2011). If it is also the case for TiLV, investigation on virus genotypes and their correlation with virulence should be a priority. Molecular diagnosis and control measures could then emphasize on highly pathogenic variant(s).

On the other hand, a detectable but low viral load could be indicative of TiLV being present in tilapia in a carrier state or chronic state that can be a potential reservoir source for viral transmission. In case of koi herpesvirus (KHV) in carp, latent infection of the virus in fish

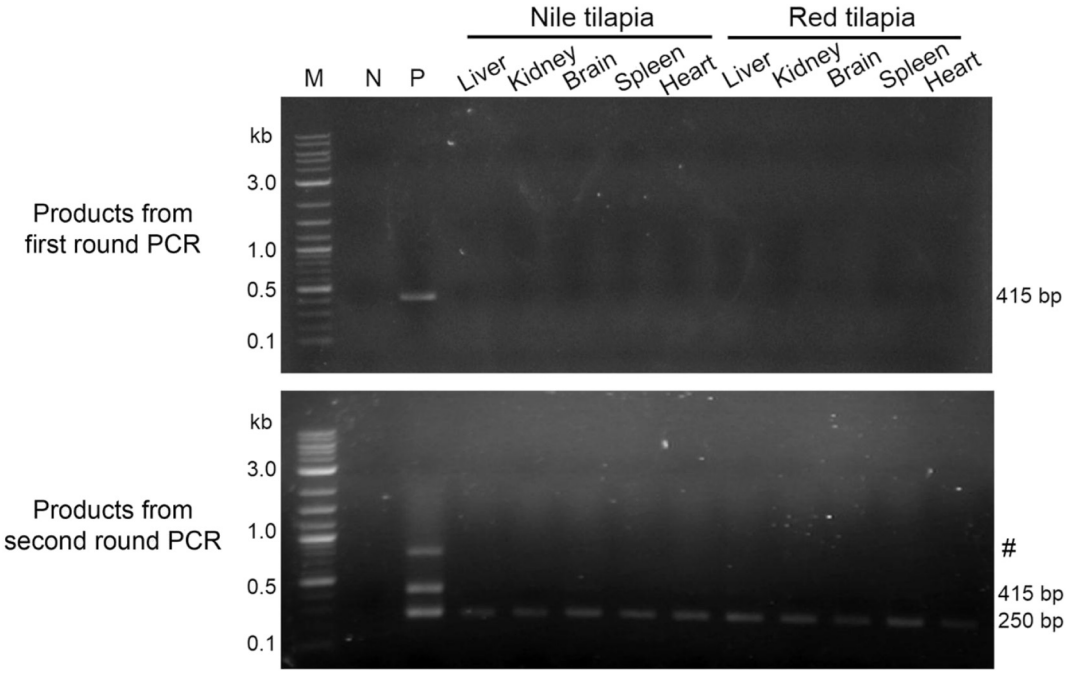

Fig. 2. Agarose gels of TiLV detection using semi-nested RT-PCR assay with RNA template from clinically healthy red and Nile tilapia. All tested organs yielded a 250-bp amplicon. M-DNA ladder (New England Biolabs); N-negative control; P-positive control. Bands of 415 bp and 250 bp representing amplicons from respective first and nested PCR are indicated. # marks band probably derived from cross hybridizations of the amplified products.

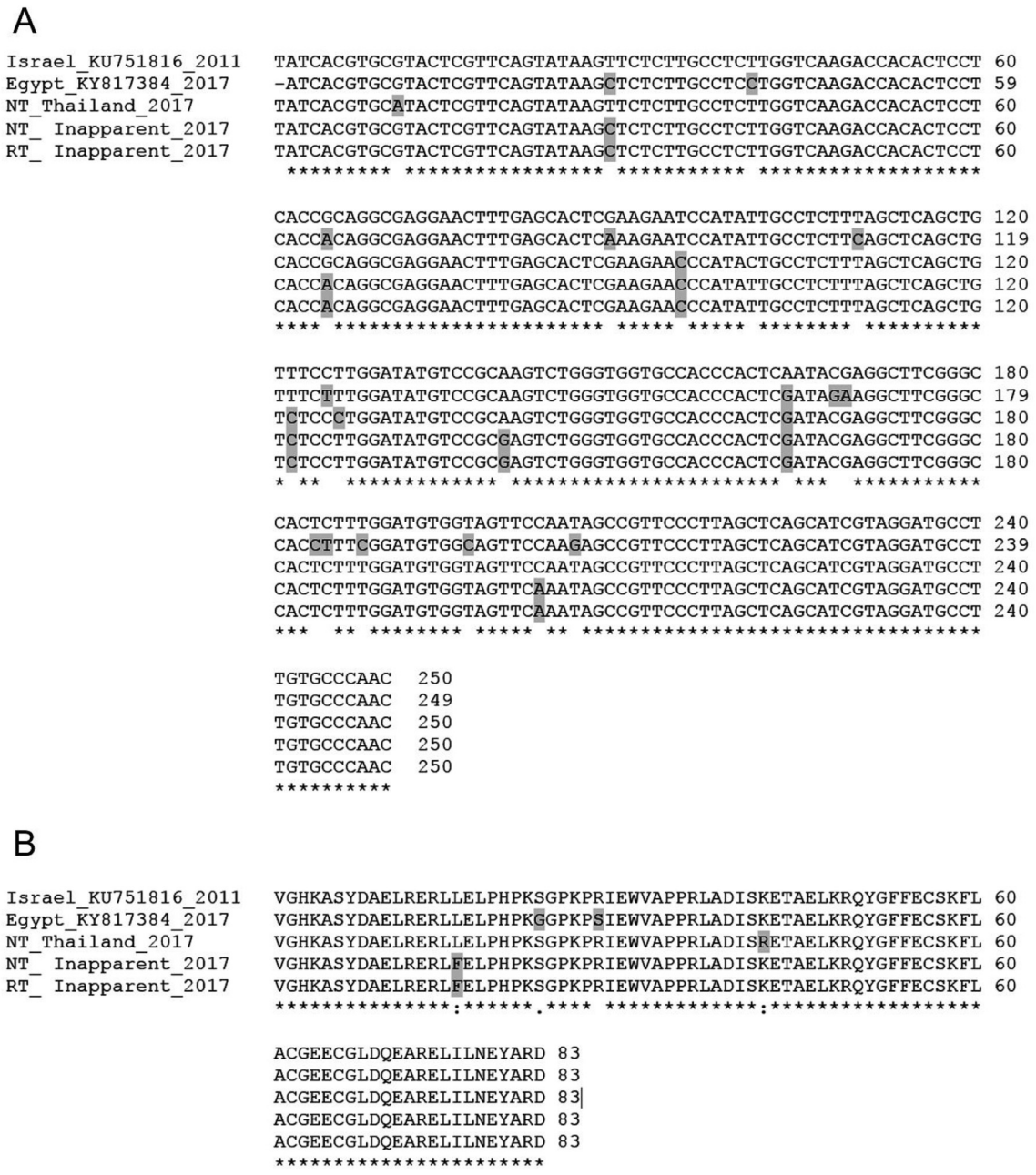

Fig. 3. Nucleotide (A) and amino acid (B) sequence alignments of the partial genome segment 3 of TiLV originated from different reported countries. Grey highlights indicated variations compared to the prototype TiLV from Israel. NT-Nile tilapia; RT-red tilapia.

survivors is generally common and the virus can be reactivated and widespread in fish populations following stress induction (Eide et al., 2011; Zheng et al., 2017). Important knowledge gaps such as susceptibility of fish strains, culture methods, co-infections, and risk factors for TiLVD manifestation have been previously addressed (Jansen and Mohan, 2017). Due to potential carrier status, we recommend that investigation of inapparent infection should be included in TiLV surveillance programs and comparative genomic analysis of TiLV strains associated with clinical and subclinical infections might shed light on pathogenic diversity of the virus.

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