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Development and validation of a reverse transcription quantitative polymerase chain reaction for tilapia lake virus detection in clinical samples and experimentally challenged fish

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Abstract

Tilapia lake virus (TiLV) is an emerging pathogen associated with high mortalities of wild and farm-raised tilapia in different countries. In this study, a SYBR green-based reverse transcription quantitative polymerase chain reaction (RT-qPCR) assay targeting segment three of the virus was developed to detect and quantify TiLV in clinical samples and experimentally challenged fish. All 30 field samples with clinical signs and history consistent with TiLV infection were positive for TiLV as detected by the developed RT-qPCR method. The RT-qPCR technique provided 100 and 10,000 times more sensitive for virus detection than those offered by the RT-PCR and virus isolation in cell culture methods, respectively. The detection limit of the RT-qPCR method was as low as two viral copies/μl. Moreover, the RT-qPCR technique could be applied for TiLV detection in various fish tissues including gills, liver, brain, heart, anterior kidney and spleen. Significantly, this study delivered an accurate and reliable method for rapid detection of TiLV viruses that facilitates active surveillance programme and disease containment.

KEYWORDS
diagnosis, RT-qPCR, tilapia, tilapia lake virus, TiLV

1 | INTRODUCTION

Since 2014, extensive losses of wild and farmed tilapia have been reported in different countries including Israel, Ecuador, Colombia, Egypt and Thailand (Bacharach et al., 2016; Eyngor et al., 2014; Fathi et al., 2017; Kembou Tsofack et al., 2017; Surachetpong et al., 2017). Subsequently, the disease investigation led to the identification of a novel, orthomyxo-like virus—tilapia lake virus (TiLV)—as a causative agent of these field outbreaks (Bacharach et al., 2016; Eyngor et al., 2014; Surachetpong et al., 2017). Recent study suggested that the isolated virus caused cytopathic effect in E-11 cells, and the disease could be reproduced in susceptible Nile and red hybrid tilapia (Tattiyapong et al., 2017). TiLV is a negative, single-stranded, RNA genome-enveloped virus comprising 10 genomic segments. Interestingly, only segment one of the virus shares weak homology to the PB1 subunit of the influenza C virus (Bacharach

et al., 2016). Other genomic segments of TiLV do not resemble any sequences of other organisms in the database.

For emerging diseases, the detection of the causative agent is based on the isolation of the pathogen or identification of the genetic components of a specific pathogen in clinical specimens using molecular techniques. To date, different diagnostic procedures have been developed for TiLV detection including viral isolation in cell cultures (Eyngor et al., 2014; Kembou Tsofack et al., 2017), in situ hybridization, and reverse transcription polymerase chain reaction (RT-PCR) (Eyngor et al., 2014). However, cell culture and in situ hybridization have certain limitations, including being time consuming, laborious and requiring well-trained technicians to perform the assays. Recently, a highly sensitive and specific nested RT-PCR protocol based on the detection of segment three of TiLV has been established (Kembou Tsofack et al., 2017). Although the nested RT-PCR method improves the specificity of the amplified products, it requires two-step amplification and is a semi-quantitative procedure which is difficult to apply for a comparison of amounts of pathogen among multiple samples.

The reverse transcription quantitative polymerase chain reaction (RT-qPCR) is a sensitive, rapid and specific assay for the detection of RNA viruses in aquatic animals (Hodneland et al., 2011; Kuo et al., 2011; Shi et al., 2017). A sensitive SYBR green-based RT-qPCR assay was developed to screen different strains of viral haemorrhagic septicaemia virus infection in Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*) (Matejusova et al., 2008). Moreover, the specific primers and RT-qPCR procedure have been established for the detection of infectious pancreatic necrosis virus in salmon (Vazquez et al., 2017). Comparison of the RT-qPCR and conventional RT-PCR methods revealed that the qPCR technique provides 100 times greater sensitivity for the detection of infectious salmon anaemia virus (Munir & Kibenge, 2004). In addition, the RT-qPCR assay could be applied for virus screening in asymptomatic carriers or valuable broodstock (Hodneland et al., 2011; Valverde et al., 2016).

The aim of this study was to develop a sensitive and reliable assay for TiLV detection in clinically moribund and asymptomatic fish. The RT-qPCR protocol was evaluated on materials prepared from positive and negative field samples and from experimentally infected fish. The RT-qPCR assay could be applied as a sensitive and specific method to determine the infection intensity and examine the viral load in fish tissues.

2 | MATERIALS AND METHODS

2.1 | Clinical samples and virus isolation

Red hybrid tilapia (*Oreochromis* spp.) and Nile tilapia (*Oreochromis niloticus* L.) with clinical signs of TiLV infection were obtained from multiple locations throughout Thailand during October 2015 to February 2017. A total of 30 field samples with 10 fish per sampling

[Correction added on 22 November 2017, after first online publication: Primer design for qPCR was updated in this version.]

event were included in the study. The TiLV was isolated from a pool of livers (*n* = 3) of clinically sick fish from each sampling event. Briefly, 100 mg of TiLV-infected liver was homogenized in 900 μl Hank's balanced salt solution (Sigma). Samples were centrifuged at 3,000 × *g* for 10 min at 4°C. The suspension was filtered using a 0.22 μm pore size filter and then inoculated into confluent E-11 cells. The E-11 cell lines were purchased from the European Collection of Authenticated Cell Cultures (ECACC), UK (catalogue number 01110916) and were maintained in L-15 Leibovitz medium (Sigma) supplemented with 2% foetal bovine serum and 2 mM L-glutamine. After the cytopathic effect (CPE) was observed, culture medium was harvested using centrifugation at 3,000 × *g* for 10 min at 4°C. An aliquot of viral suspension was stored at −80°C for further use.

2.2 | Plasmid construction for standard quantification

A 491 bp cDNA fragment from the segment three of the TiLV genome (GenBank accession number KX631923) was amplified using specific primers (Eyngor et al., 2014) (Nested ext-2: TTGCTCTGAG-CAAGAGTACC and Nested ext-1: TATGCAGTACTTTCCCTGCC) and cloned into the pTG19-T vector (Vivantis). The RT-PCR protocol was performed as previously described (Tattiyapong et al., 2017). The recombinant plasmid later called pTiLV was transformed into the *E. coli* strain DH5 alpha. Plasmid DNA was isolated from the transformed *E. coli* using a plasmid extraction kit (Geneaid) and subjected to DNA sequencing (Macrogen). The pTiLV concentrations were measured using a Nanodrop 2,000 spectrophotometer (Thermo Scientific) and used as a standard for the determination of the sensitivity of RT-qPCR assay. The concentration of stock plasmid DNA was 1 ng/μl, which is equivalent to 2.7×10^8 copies/μl.

2.3 | Primer design for qPCR

The RT-qPCR primers were designed based on the sequence of segment three of TiLV isolated from Israel (Genbank accession no. KJ605629 using primer three online software [http://frodo.wi.mit.edu/primer3/]. The forward and reverse primers are TiLV-112F (5′-CTGAGCTAAAGAGGCAATATGGATT-3′) and TiLV-112R (5′-CGTGCGTACTCGTTCAGTATAAGTTCT-3′), respectively. The predicted size of RT-qPCR product is 112 bp.

2.4 | RNA extraction and cDNA synthesis

For RNA extraction, livers and brains (*n* = 3) from each field sample were collected and homogenized in 1 ml Trizol reagent (Invitrogen). The samples were extracted according to the manufacturer's protocol. The concentration of RNA was measured using Nanodrop 2,000 spectrophotometer (Thermo Scientific). The amount of RNA was adjusted to 200 ng/μl in molecular-grade water. The first strand cDNA was synthesized using the RNA reverse transcription kit (Vivantis). Briefly, one microgram of total RNA was added into 20 μl reaction mixture containing 2 μM Oligo d(T), 0.5 mM dNTPs mix,

100 U of M-MuLV reverse transcriptase and $1 \times$ M-MuLV buffer. The reactions were incubated under the following conditions: 65°C for 5 min, 42°C for 60 min and 85°C for 5 min in the T100 thermal cycler (Bio-Rad).

2.5 | Quantitative PCR assay

The RT-qPCR assay was performed in a CFX96 Touch thermal cycler (Bio-Rad). The assay was carried out in a 20 μl reaction mixture containing 10 μl of $2 \times$ iTaq™ universal SYBR green supermix (Bio-Rad) 0.3 μM of forward or reverse primer, 4 μl of cDNA template and molecular-grade water to adjust the final volume. The cycling conditions consisted of denaturation at 95°C for 3 min followed by 40 cycles of 95°C for 10 s and 60°C for 30 s. At the end of the qPCR cycle, melting curve analysis was performed at a temperature ranging from 65°C to 95°C with 0.5°C per 5 s increments. All samples, including the no template control were run in triplicate, and each qPCR reaction was repeated twice. The specificity of RT-qPCR products was confirmed on low melting temperature agarose NuSieve 3:1 agarose (Lonza) to verify the size of the PCR products.

2.6 | Standard curve and reproducibility of the assay

The standard curve was generated using 10-fold serial dilutions of the purified plasmid (pTiLV). The plasmid dilution ranged from 10^{-1} to 10^{-8} representing 2.7×10^7 copies to 2.7 copies/μl and was tested in triplicate as described above. The plasmid copy number was calculated as described by Guan M. Ke (Ke et al., 2006). The standard curve was plotted between the standard plasmid pTiLV concentration (log copy number) and cycle threshold (Ct). In addition, the standard curve was generated from 10-fold serial dilutions of cDNA prepared from TiLV-infected tissue samples. To determine the reproducibility of the RT-qPCR protocol, the intra-assay and interassay were evaluated using 10-fold serial dilutions of plasmid pTiLV ranging from 10^{-1} to 10^{-8}. The assay was performed in triplicate in a single run [intra-assay] or with three independent times [interassay]. The measured Ct values from each assay were calculated to determine the standard deviation (SD) and coefficient of variation (%CV), which were used to assess the reliability of the RT-qPCR protocol.

2.7 | Challenge experiment

A group of 20 red hybrid tilapia (Oreochromis spp.) were equally divided into two groups: TiLV challenge and non-challenge fish. Fish were intraperitoneally injected with 50 μl of 10^6 TCID$_{50}$ ml^{-1} TiLV strain KU-TV01 or an equal volume of L-15 medium (non-challenge fish). The virus titer and TCID$_{50}$ were calculated according to the Reed and Muench method (Reed & Muench, 1938). Clinical signs of virus infection were observed daily. Severely moribund fish were killed using an overdose of eugenol solution (Aquanes). Liver tissues were collected from individual fish and processed for RNA isolation

or virus isolation in E-11 cells. The animal use protocol followed the standard guidelines of the Institutional Animal Use Committee, Kasetsart University, Bangkok, Thailand.

2.8 | Detection limit of TiLV virus in clinical samples

To compare the sensitivity of virus isolation in cell culture, conventional RT-PCR and RT-qPCR assay, an equal amount of infected liver (100 mg) was used for RNA extraction and viral isolation. Total RNA samples were isolated using Trizol reagent (Invitrogen) as previously described. The RNA sample was 10-fold serially diluted and processed for RT-PCR or RT-qPCR analysis. The 10-fold serial dilutions of the tissue suspension were prepared as described, and 50 μl of each dilution was inoculated into confluent E-11 cells. Each dilution was performed in triplicate. Each plate was incubated at 25°C for 1 hr. The development of a cytopathic effect (CPE) was monitored daily until 10 day post-inoculation.

2.9 | Analysis of viral loads in different infected tissues

Gills, liver, brain, heart, anterior kidney and spleen were collected from two TiLV-challenged fish at 9 days post-infection. Total RNA isolation and cDNA synthesis were processed for RT-qPCR analysis. Viral copy numbers were calculated by extrapolating the averaged Ct values to the standard curve.

3 | RESULTS

3.1 | Standardization of RT-qPCR assay

The standard curve of the RT-qPCR assay was determined using 10-fold serial dilutions of pTiLV (10^{-1}-10^{-8} dilutions) which is equivalent to 2.7×10^7 to 2.7 copies/μl. The mean Ct values of each dilution were reproducibly obtained from three replicates. A linear regression relationship between the mean Ct values and log plasmid DNA concentration was observed with the coefficient of determination ($R^2 = 0.9994$) and a slope of -3.4312 demonstrating 95.64% efficiency of the qPCR reaction (Figure 1a). Similarly, serial dilutions from cDNA of TiLV-infected fish tissue produced a standard curve with a slope and amplification efficiency close to pTiLV (Figure 1b). In addition, amplification of pTiLV at different concentrations yielded consistent results over a range of eight dilutions (Fig S1a).

The melting curve analysis revealed a temperature that verified the size of the PCR amplicons. In particular, the melting temperatures (Tm) of TiLV amplicons were in the range 79.50-80.0°C (± 0.18 standard deviation: SD). No melt peak appeared in the no template control. Analysis of the PCR product on 5% low melting agarose gel showed a specific band product of 112 bp (Fig S1b). Of note, the band intensity decreased gradually which was correlated with the amount of template from 2.7×10^7 to 2.7 copies/μl (Fig S1c).
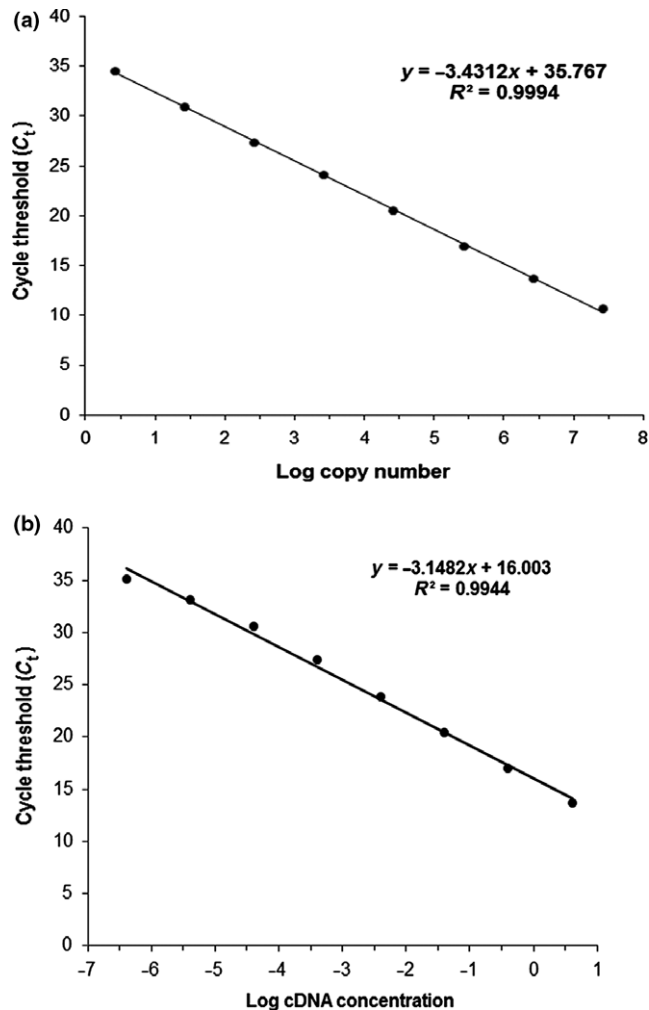
FIGURE 1 Standard curve of SYBR green-based RT-qPCR amplification of plasmid pTiLV containing segment three of TiLV and infected tissue. (a) Standard curve was plotted between mean Ct values obtained from each dilution of standard plasmid pTiLV against calculated log copy number (slope = −3.4312, R^2 = 0.9994). (b) Standard curve of cDNA prepared from TiLV-infected fish tissue showed slopes = −3.1482, R^2 = 0.9944

3.2 | Reproducibility of RT-qPCR assay

The reproducibility of the method was characterized by analysis of inter and intra-assay variations (Table 1). The interassay was analysed by measuring the mean Ct values of independent runs on three consecutive days using 10-fold serial dilutions of standard plasmid pTiLV (10^{-1}-10^{-8}). The coefficient of variation (%CV) calculated from measured mean Ct values ranged from 0.27% to 2.31% with SD values from 0.05 to 0.75. Moreover, the %CV of mean Ct values of the intra-assay was 0.10%-1.60% and SD 0.03-0.56. Similar results were obtained from the interassay and the intra-assay using TiLV-infected fish tissues (data not shown).

To further determine the specificity of RT-qPCR primers for non-specific amplification of other pathogens and host nucleic acids, samples were prepared from fish infected with *Iridovirus*, *Aeromonas*

TABLE 1 Reproducibility of intra-assay and interassay with a 10-fold serial dilution of standard plasmid pTiLV

Plasmid concentrations (copy no/µl)	Intra-assay			Interassay		
	Mean Ct	SD^a	CV (%)	Mean Ct	SD^a	CV (%)
2.7×10^7	10.71	0.10	0.95	11.16	0.07	0.66
2.7×10^6	13.56	0.05	0.37	13.80	0.08	0.59
2.7×10^5	16.50	0.03	0.19	17.47	0.05	0.27
2.7×10^4	20.13	0.16	0.90	21.05	0.10	0.48
2.7×10^3	23.87	0.16	0.68	24.66	0.15	0.59
2.7×10^2	27.32	0.03	0.10	28.09	0.13	0.48
2.7×10^1	30.72	0.23	0.74	31.54	0.40	1.26
2.7×10^0	35.05	0.56	1.60	35.37	0.75	2.31

[a]Standard deviation was calculated from three independent experiments.

hydrophila and *Streptococcus agalactiae*. None of these samples yielded amplification or melting curve signals at the end of the RT-qPCR reaction. Moreover, the nucleotide sequences of a 112 bp RT-qPCR product matched with those of TiLV previously reported in Thailand (GenBank accession no. KX631923).

3.3 | Detection of TiLV in fish tissues using RT-qPCR protocol

In total, 30 field samples with clinical signs of TiLV infection were tested for TiLV using the newly developed RT-qPCR method. The clinical specimens were collected from different geographic locations in Thailand (Table S1). All 30 field samples showed positive detection of TiLV with the mean Ct values ranging from 12.83 to 32.89 representing 1.37×10^7 – 1.97×10^1 viral copies/µg of total RNA and Tm of the RT-qPCR product at 80.0°C. The RT-qPCR assay was further tested for detection of TiLV in experimentally challenged fish (Table 2). All challenged fish developed clinical signs of TiLV infection within 5 day post-inoculation. Livers were individually collected from 10 TiLV-challenged fish and 10 non-challenged fish for RT-qPCR analysis. The mean Ct values of experimentally challenged fish ranged from 20.08 to 27.28 which is equivalent to 1.00×10^5 – 8.50×10^2 viral copies/µg of total RNA. No amplification curve and melting curve were detected in non-challenged fish (Table 2). Analysis of viral loads in different infected tissues including gills, liver, brain, heart, anterior kidney and spleen using the RT-qPCR protocol revealed that TiLV was found in all tissues of experimentally challenged fish with the viral loads ranging from 6.3×10^3 to 6.3×10^5 viral copies/µg of total RNA (Table 3).

3.4 | Comparison of RT-qPCR assay and virus titration in cell culture

The sensitivity of the RT-qPCR assay was compared with the conventional RT-PCR and virus isolation in permissive E-11 cells. The detection limit of both assays was determined using 10-fold serial

TABLE 2 TiLV detection in clinical samples using RT-qPCR procedure

Fish samples	Number of samples	TiLV positive (%)	Mean Ct values (range)	Estimated viral loads (Copy numbers)[c]
Clinical samples[a]	30	30/30 (100)	22.86 (12.83 - 32.89)	1.65×10^4 (1.37×10^7-1.97×10^1)
TiLV-challenged fish	10	10/10 (100)	23.65 (20.08 - 27.28)	9.72×10^3 (1.00×10^5-8.50×10^2)
Non-challenged fish	10	0/10 (0)	ND[b]	ND[b]

[a]Clinical samples were collected from 30 field outbreaks with history of massive mortality.
[b]ND = No detection.
[c]Copy numbers of TiLV template per μg of total RNA.

TABLE 3 Analysis of viral loads in different tissues

	Viral loads (copies/μg of total RNA)					
Sample No.	Gills	Liver	Brain	Heart	Anterior kidney	Spleen
1	2.2×10^5	1.7×10^5	3.4×10^5	6.3×10^5	1.6×10^5	2.3×10^4
2	3.1×10^5	6.3×10^3	1.3×10^6	3.9×10^5	3.1×10^5	2.8×10^4

dilutions of viral suspension prepared from TiLV-infected fish tissue. Notably, the sensitivity of RT-qPCR assay was at 10^{-7} dilution, while the sensitivity of RT-PCR and virus isolation in cell culture was at 10^{-5} and 10^{-3} dilution, respectively (Table 4 and Fig S2).

4 | DISCUSSION

The data in this study present the development and validation of a RT-qPCR assay for the detection of TiLV in tilapia. The RT-qPCR procedure was accurate and reliable for quantification of TiLV in the field samples and experimentally challenged fish. Specifically, the correlation coefficient (R^2 value) of the standard curve from the plasmid pTiLV and infected fish tissues showed an efficiency of higher than 95% indicating the high precision of the assay. The intra- and interassay showed high reproducibility of the RT-qPCR method with coefficients of variation ranging from 0.10% to 1.6% and 0.27% to 2.31%, respectively. These data were in a comparable range with previous qPCR protocols in other fish viruses (Panzarin et al., 2010; Yue et al., 2008). Comprehensively, the assay can differentiate

normal fish and infected fish collected from field samples and experimentally challenged fish, suggesting that this method could be applied for routine diagnosis. Indeed, the viral load in field samples and laboratory challenged fish using RT-qPCR method demonstrated that the assay could identify TiLV ranging from 10^1 to 10^7 copies/μg of total RNA. Although it is difficult to compare the levels of virus between the field samples and laboratory challenged fish as we do not know when the moribund fish acquire the infection, many factors such as the dates of sample collection, the level of virus in an individual fish, the immune response of each fish, as well as different farm management, may contribute to the level of TiLV in field samples.

The limitation of a SYBR-based qPCR is the non-specific amplification of the double strand DNA templates in the samples or primer-dimer formation (Vandesompele et al., 2002). In this study, no non-specific peaks were observed in either amplification curve or melting curve from non-TiLV-infected samples, suggesting that the developed primers used in this study did not cross-react with other pathogens or host RNA. It has been shown that genetic variation among target viruses could affect the primer binding and PCR assay, causing false negative results (Osman et al., 2007). Sequence

TABLE 4 Comparison of RT-qPCR, conventional RT-PCR and virus isolation in cell culture

	Template dilution							
Detection method	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}
RT-qPCR[a]	+	+	+	+	+	+	+	−
Conventional RT-PCR[b]	+	+	+	+	+	−	−	−
Virus isolation in cell culture[c]	+	+	+	−	−	−	−	−

[a]Detection of RT-qPCR product (+) amplification curve, (−) no amplification curve.
[b]Detection of RT-PCR product (+) positive band, (−) no band.
[c]Virus isolation (+) CPE, (−) no CPE.

variations of Spring viremia of carp isolated from different geographic locations have been reported (Maj-Paluch et al., 2016). D. Vazquez (Vazquez et al., 2017) developed an RT-qPCR method that is specific for the detection of seven strains and 23 field viral isolates of infectious pancreatic necrosis virus. Currently, only the complete genome sequences of TiLV isolated from Israel and Thailand are available on the public database (Bacharach et al., 2016; Surachetpong et al., 2017). Although only TiLV isolated from Thailand have been tested for the developed RT-qPCR method, alignment of genetic sequences at primer binding sites of TiLV isolated from Israel and Thailand demonstrated only one nucleotide mismatch at the forward and reverse primers, suggesting that primers used in this study may amplify other TiLV isolates including the Israel or Ecuador viral isolates (Bacharach et al., 2016; Del-Pozo et al., 2017; Eyngor et al., 2014). Nevertheless, more validation is required to test the application of this pair of primers and the RT-qPCR protocol for detection of other TiLV isolates from different geographic locations.

Currently, molecular techniques based on the amplification of genetic material in samples such as qPCR have been developed and validated for detection of other fish viruses (Ciulli et al., 2015; Dalla Valle et al., 2005; Starkey et al., 2006). A one step, real-time RT-PCR was developed to detect the RNA1 genomic sequence of the betanodavirus infection in various fish species including tilapia (Baud et al., 2015). The qPCR method offers multiple advantages over other diagnostic methods such as a rapid result within a short period, allowing direct quantification of pathogens in tissue samples, being highly specific to the target viral pathogen. For many viral diseases, the RT-qPCR could be used as a screening tool to establish viral-free broodstock, to examine viral kinetics and to determine viral genome copies in asymptomatic carrier fish. For instance, asymptomatic carrier gilthead seabream containing lymphocystis disease virus were identified using a qPCR procedure (Valverde et al., 2016). Tissue distribution and target organs of red spotted grouper nervous necrosis virus were examined in juvenile European seabass (*Dicentrarchus labrax*) using RT-qPCR, allowing the detection of viral replication in nervous and non-nervous tissues (Lopez-Jimena et al., 2011). In the present study, analysis of TiLV in six tissues: gills, liver, brain, heart, anterior kidney and spleen, using the developed RT-qPCR revealed that the virus distributed in different fish tissues. Previously, brain and liver were the main targets for TiLV PCR analysis (Del-Pozo et al., 2017; Eyngor et al., 2014; Surachetpong et al., 2017; Tattiyapong et al., 2017). Thus, additional kinetic studies to examine the viral replication in different fish tissues could benefit from the developed RT-qPCR method.

The data in this study also suggested that the RT-qPCR method is highly sensitive and specific for the detection of TiLV in clinical samples and challenged fish. Previous reports showed that the standard RT-PCR method has a detection limit of 70,000 viral copies, while the more sensitive nested RT-PCR could detect as low as seven copies in the fish tissue (Kembou Tsofack et al., 2017). In this study, the amplification curve of standard plasmid pTiLV and infected tissues showed that the newly developed RT-qPCR assay could detect as low as 2 copies/μl. Furthermore, the RT-qPCR method is 100 and 10,000 more sensitive for virus detection compared with the conventional RT-PCR and viral isolation in cell culture.

In summary, the RT-qPCR method described in this study provides a rapid, sensitive and accurate molecular method for the detection of TiLV in field samples and experimentally challenged fish. The method could be applied as a diagnostic tool for sample screening in epidemiological studies and to evaluate the virus load in challenged animals.

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SUPPORTING INFORMATION

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