Concentration and quantification of *Tilapia tilapinevirus* from water using a simple iron flocculation coupled with probe-based RT-qPCR

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Background. *Tilapia tilapinevirus* (also known as tilapia lake virus, TiLV) is an important virus responsible for die-off of farmed tilapia globally. Detection and quantification of the virus from environmental RNA (eRNA) using pond water represents a potential, non-invasive routine approach for pathogen monitoring and early disease forecasting in aquaculture systems.

Methods. Here, we report a simple iron flocculation method for viral concentration from water combined with a newly developed hydrolysis probe quantitative RT-qPCR method for detection and quantification of TiLV.

Results. The RT-qPCR method targeting a conserved region of TiLV genome segment 9 has a detection limit of 10 viral copies per µL of template. The method had a 100% analytical specificity and sensitivity for TiLV. The optimized iron flocculation method was able to recover 16.11 ± 3.3% of virus from water samples spiked with viral cultures. During disease outbreak cases from one open-caged river farming system and two earthen fish farms, both tilapia and water samples were collected for detection and quantification of TiLV. The results revealed that TiLV was detected from both clinically sick and asymptomatic fish. Most importantly, the virus was successfully detected from water samples collected from different locations in the affected farms i.e., river water samples from affected cages (8.50×10^3 to 2.79×10^5 copies/L) and fish-rearing water samples, sewage, and reservoir (4.29×10^3 to 3.53×10^4 copies/L). By contrast, TiLV was not detected in fish or water samples collected from two farms that had previously experienced TiLV outbreaks and from one farm that never had a TiLV outbreak. In summary, this study suggests that the eRNA detection system using iron flocculation coupled with probe based-RT-qPCR is feasible for concentration and quantification of TiLV from water. This approach might be useful for non-invasive monitoring of TiLV in tilapia aquaculture systems and support evidence based decisions on biosecurity interventions needed.

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Abstract

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Introduction

Tilapia tilapinevirus (commonly called tilapia lake virus, TiLV) is a ~~novel and only virus of the genus *Tilapinevirus* under the family *Amnoonviridae*~~ (International Committee on Taxonomy of Viruses. 2019). TiLV is an RNA virus with a 10 segmented negative sense single stranded genome of approximately 10.323 kb in size (Bacharach et al. 2016). Since its first discovery in 2014, the virus had significant impacts on tilapia aquaculture worldwide (Eyngor et al. 2014; Ferguson et al. 2014; Jansen et al. 2019). Disease caused by TiLV usually results in cumulative mortality from 20 to 90% (Behera et al. 2018; Dong et al. 2017a; Eyngor et al. 2014; Ferguson et al. 2014; Surachetpong et al. 2017). So far, there are 16 countries that reportedly confirmed detection of TiLV (Jansen et al. 2019; Surachetpong et al. 2020), but a wider geographical spread has been hypothesized due to active movements of live tilapia among countries (Dong et al. 2017b). Waterborne spread of TiLV might also contribute to pathogen dissemination to new areas as well as transmission to other fish species (Chiamkunakorn et al. 2019; Eyngor et al.

2014; Jaemwimol et al. 2018; Piamsomboon & Wongtavatchai 2021). Experimental evidences have already demonstrated that TiLV is both horizontally and vertically transmitted (Dong et al. 2020; Eyngor et al. 2014; Jaemwimol et al. 2018; Yamkasem et al. 2019).

With respect to waterborne transmission of fish pathogens, several studies employed various viral concentration methods from water for pathogen detection (For example, Haramoto et al. (2007); Kawato et al. (2016); Minamoto et al. (2009); Nishi et al. (2016)). The concept is one of the applications of environmental DNA or RNA (eDNA/eRNA) which is nucleic acids extracted from environmental samples such as water, soil, and feces (Bass et al. 2015; Gomes et al. 2017). The eDNA/eRNA gives advantages in disease monitoring, control measure design, risk factor analysis and studies of viral survival nature (example review in Oidtmann et al. (2018)). The work described by Kawato et al. (2016) used an iron flocculation method to concentrate red sea bream iridovirus (RSIV) in a challenge model with Japanese amberjack (*Seriola quinqueradiata*). Results from that study showed that detection by qPCR of RSIV from fish-rearing water samples peaked more than five days before fish mortality occurred, suggesting potential benefit of using iron flocculation method for disease forecast. Others studies used a cation-coated filter method to detect DNAs of cyprinid herpesvirus 3 (CyHV-3) (also known as koi herpesvirus, KHV) from concentrated river water samples three to four months before mass mortalities events occurred in wild carp in Japan (Haramoto et al. 2007; Minamoto et al. 2009). Additionally, the virus was still detectable in river water for at least three months after the outbreaks (Minamoto et al. 2009). These findings helped local authorities and farmers to make rapid decisions for emergency harvest, biosecurity implementation, follow appropriate disinfection procedures and fallowing periods.

Several molecular methods have been developed for detection of TiLV including RT-PCR (Eyngor et al. 2014), nested and semi-nested PCR (Dong et al. 2017a; Kembou Tsofack et al. 2017; Taengphu et al. 2020), RT-qPCR (Tattiyapong et al. 2018; Waiyamitra et al. 2018), loop-mediated isothermal amplification (LAMP) (Kampeera et al. 2021; Phusantisampan et al. 2019; Yin et al. 2019) and Nanopore-based PCR amplicon approach (Delamare-Debouteville et al. 2021). However, all of these methods target fish tissue specimens for diagnosis, none of which reported any application for TiLV detection from environmental water samples. Previous probe-based RT-qPCR methods developed to detect TiLV from tilapia clinical samples with detection limits of 2.7×10^4 or ~70,000 copies (Kembou Tsofack et al. 2017; Waiyamitra et al. 2018) might

not be sensitive enough to detect low viral loads of TiLV in environmental water samples. Moreover, at the time of earlier primer and probe design, there were a limited number of TiLV genome sequences in the NCBI database. As a result, sequence variation among viral isolates and within the genome segments may not be accounted for in the design of those previous methods. The objective of this study was to develop a new RT-qPCR assay (based on updated publicly available TiLV genomic sequences data) to detect and quantify TiLV in fish tissues and in environmental RNA (eRNA) concentrated from fish-rearing water samples using an iron flocculation method.

Materials & Methods

Development of a probe-based quantitative RT-qPCR method for TiLV

Primer & probe design and establishment of PCR conditions

A hydrolysis probe-based RT-qPCR method was developed and optimized for detection and quantification of TiLV following the MIQE guidelines (Bustin et al. 2009). Out of the 10 segments of the TiLV genome, segment 9 was reported to have relatively high identity (97.44 - 99.15%) among various TiLV isolates (Pulido et al. 2019). Primers and probe were manually designed based on conserved regions of TiLV genome segment 9 following multiple sequence alignments of all complete coding sequences (n=25) retrieved from the GenBank database at NCBI as of November 2021 (Fig. S1). Primer Seg9-TaqMan-F (5'-CTA GAC AAT GTT TTC GAT CCA G-3') had a 100% perfect match with all retrieved sequences while primer Seg9-TaqMan-R (5'-TTC TGT GTC AGT AAT CTT GAC AG-3') and probe (5'-6-FAM-TGC CGC CGC AGC ACA AGC TCC A-BHQ-1-3') had one mismatch nucleotide from the compared sequences (Fig. S1). Size of the amplified product is expected at 137 bp. Primers and probe were synthesized by Bio Basic Inc (Canada). Specificity of the primers and probe was assessed *in silico* using Primer-BLAST program (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). Gradient PCR reactions with annealing temperatures ranging from 55 to 65 ºC were used to determine the optimal temperature of the designed qPCR primers. The final composition of the optimized TiLV RT-qPCR 20 µL reaction consists of 1X master mix (qScript XLT 1-Step RT-qPCR ToughMix Low ROX buffer) (Quanta Bio, Beverly, MA, cat no. 95134-500), 1.5-2 µL (≤300 ng) of RNA template, 450 nM of each forward and reverse primers, and 150 nM of Seg9-TaqMan-Probe. Cycling conditions include a reverse transcription step at 50 °C for 10 min, then

an initial denaturation step at 95 °C for 1 min followed by 40 cycles of 95 °C for 10 s and 58 °C for 30 s. RT-qPCR amplification was carried out using Bio-Rad CFX Connect Real-Time PCR machine.

Construction of a positive control plasmid

Positive control plasmid (pSeg9-351) was constructed in ~~our~~ previous study (Thawornwattana et al. 2021). In brief, a 351 bp-TiLV segment 9 open reading frame (ORF) product was obtained from an RT-PCR amplification using TiLV-S9-F (5'-ATG TCA CGA TGG ATA GAA-3') and TiLV-S9-R (5'-TCA TAA AGT TTT ATC GCC AG-3') primers (Pulido et al., 2019) and RNA extracted from TiLV infected tilapia as template. The amplicon was purified before being cloned into the pGEM T-easy vector (Promega, Madison, WI). The sequence of the recombinant clone was verified using the Sanger technique (Macrogen, South Korea). The obtained pSeg9-351 plasmid was used as positive control and used in RT-qPCR analytical sensitivity assays (see below). TiLV copy numbers in the stock vials were determined using an online calculator (http://www.scienceprimer.com/copy-number-calculator-for-realtime-pcr) based on the pSeg9-351 concentration (ng) and length (bp), then adjusted with sterile water to 10^6 copies/µL working concentration.

Analytical sensitivity and specificity tests

Analytical sensitivity of the Seg9-targeted RT-qPCR was investigated using 10-fold serial dilutions of pSeg9-351 plasmid template from 10^6 to 1 copies/µL template. The assays were performed in duplicate. Standard curves were prepared by plotting the $\log_{10}$ of serial plasmid dilutions versus quantification cycle (Cq) values. Viral copy numbers in each tested sample were calculated by extrapolating the Cq values to the generated standard curve using the equation and Cq values:

$$\text{Viral copy number} = 10^{(\text{Cq - Intercept})/\text{Slope}}$$

$$\text{i.e., } 10^{(\text{Cq} - (-42.295))/-3.476}$$

Specificity of the method was tested with RNA extracted (150 ng/reaction) from clinically healthy tilapia, 15 common fish bacterial pathogens, and fish tissues infected with nervous necrosis virus (NNV), infectious spleen and kidney necrosis virus (ISKNV), or scale drop disease virus (SDDV) (as listed in Table S1).

Validation of the RT-qPCR assay

We assessed the Seg9 RT-qPCR assay against RNA extracted from 65 samples held in our laboratory. Forty-four samples originated from known TiLV outbreaks and 21 from known non-diseased samples (healthy tilapia). Diagnostic test results were obtained using semi-nested RT-PCR methods as described before (Dong et al. 2017a; Taengphu et al. 2020). Diagnostic specificity and sensitivity of the assay were calculated according to formulas described by Martin (1984) as:

- Sensitivity % = [number of true positive samples / (number of true positive samples + number of false negative samples)] × 100
- Specificity % = [number of true negative samples / (number of true negative samples + number of false positive samples)] × 100

Optimization for viral concentration protocol

Virus preparation

Viral stock used in this study was isolated from TiLV-infected Nile tilapia using E-11 cell line, a clone of the cell line SSN-1 derived from whole fry tissue of snakehead fish (Sigma-Aldrich cat no. 01110916-1VL). The virus was propagated as described in Dong et al. (2020). Briefly, 200 µL of TiLV stock (~10^8 copies/mL) was added into a 75 mL cell culture flask containing a monolayer of E-11 cell and 5 mL of L15 medium (Leibovitz), incubated at 25 °C for 5 days. The culture supernatant containing viral particles was collected after centrifugation at 15,000 x g for 10 min at 4 ˚C. The viral stock was kept in aliquots of 1 mL at -80 ˚C until used.

Iron flocculation

Viral concentration using iron flocculation method was performed using the protocol previously described by Kawato et al. (2016) with some modifications. Workflow of this method is illustrated in Fig. 1. Briefly, 100 µL of TiLV viral stock containing ~10^7-10^8 viral copies was added into 500 mL of sterile water that contained 1% marine salt and 36 µM ferric chloride. The viral copy numbers were quantified by RT-qPCR using RNA extracted from viral stock vials. The suspension was stirred at room temperature for 1 h before being mechanically filtered through a 0.4-µm pore size polycarbonate filter (Advantec) with a vacuum pump connected to a filter holder KG-47 (Advantec) under < 15 psi pressure. The flocculate-trapped filters were then subjected to nucleic acid extraction using Patho Gene-spin DNA/RNA extraction kit (iNtRON Biotechnology). In comparison studies, the flocculate-trapped filters were soaked in oxalate-EDTA buffer to re-suspend the trapped particles (John et al. 2011) prior to nucleic acid

extraction. Experiments were carried out in two to four replicates. Viral concentration and percentage (%) recovery of the virus copies were calculated from Cq values after flocculation compared to that of the starting viral stock.

Tilapia and water samples used in this study

~~The aforementioned optimized methods for viral concentration and RT-qPCR for TiLV detection and quantification were then used in both fish and water samples collected from six tilapia farming sites between 2020 and 2021.~~ Out of the three TiLV outbreak cases, one occurred in a river's floating cages from a farm producing hybrid red tilapia, *Oreochromis* sp. (Table 3, Farm 1) and two in earthen ponds culturing Nile tilapia, *O. niloticus* (Table 4, Farms 2 and 3). Three other fish farms had no abnormal mortality reported at the time of sample collection~~, of which~~ Farms 4 and 5 had previously experienced TiLV outbreaks and Farm 6 never had a TiLV outbreak (Table 4).

~~From the TiLV outbreak in river open-cages (Farm 1), we received specimens from diseased fish (n=2-3 fish per cage) and water samples (two bottles of 500 mL per cage) collected from four cages namely A, B, C and D in close proximity sharing the same river water body (Table 3). From the TiLV outbreak that occurred on Farm/Hatchery 2, internal organs from both diseased and healthy looking tilapia (fingerlings and broodstock) as well as snails and sludge were collected from different ponds (Table 4, Farm 2).~~ Water samples (500 mL per sample per location) were collected from fish ponds, reservoir, and sewage (outgoing waste water from ponds) (Table 4, Farm 2). ~~From the TiLV outbreak on Farm 3, survivor tilapia and water samples were collected when the disease severity decreased. From the two farms with history of TiLV outbreaks, we obtained specimens from normal looking fish and water samples from Farm 4 and only water samples from Farm 5. From Farm 6 with no history of TiLV outbreak, only water samples were collected (Table 4).~~ All fish samples were preserved in Trizol reagent (Invitrogen) and kept on ice with water samples during transportation and shipped to our laboratory within 24 h.

Upon arrival at our laboratory, fish specimens were processed for RNA extraction and water samples were centrifuged (5,000 x g for 5 min) to remove suspended matters before being subjected to iron flocculation and subsequent nucleic acid extraction by Patho Gen-spin DNA/RNA extraction kit (iNtRON Biotechnology). Viral detection and quantification were then performed to investigate the presence of TiLV by the established Seg 9 RT-qPCR assay described above. Plasmid template pSeg9-351 was used in a positive control reaction while nuclease-free water was used for negative control.

Results

A new probe-based RT-qPCR method for detection and quantification of TiLV

The Seg9 RT-qPCR method developed in this study had a detection limit (sensitivity) of 10 copies/µL template with mean Cq ± SD values of the detection limit at 38.24 ± 0.09 (Fig. 2a). Hence, samples with a Cq value ≥ 38.15 were considered TiLV negative or under the limit of this detection method. Based on the standard curve analysis, the established RT-qPCR was found to be highly efficient with Slope = -3.476, $R^2 = 0.998$, and E (amplification efficiency) = 94.0% (Fig. 2b). The formula, copy number = $10^{(Cq - Intercept)/Slope}$ i.e., $10^{(Cq - (-42.295))/-3.476}$ can be used to calculate TiLV copy numbers present in the assayed samples. Analytical specificity test revealed that the method was highly specific to TiLV only since no amplifications were found when the method was assayed with RNA templates extracted from three other viruses, 15 bacterial species, and healthy tilapia (Fig. 2c, Table S1). The method had 100% diagnostic specificity and 100% diagnostic sensitivity when assayed with previously diagnosed TiLV infected and non-infected fish samples (n =65 with Cq value ranges 13.02 – 34.85) (Table 1).

Conditions for viral concentration and percentage recovery

Percentage recovery of TiLV after iron flocculation without suspension of the membrane filter in oxalate-EDTA buffer was only 2.04 ± 0.5% (n=2) compared to the original viral stock (Table 2). This was significantly improved with an additional suspension step of the flocculate-trapped filters into oxalate-EDTA buffer prior to RNA extraction. The percentage recovery of TiLV increased to 16.11 ± 3.3% (n=4) in viral concentration after iron flocculation (Table 2). Figure 2d showed representative results of viral quantification using Seg 9 RT-qPCR assays of TiLV from water after iron flocculation with the resuspension step.

TiLV detection and quantification from tilapia and water samples

The results of TiLV detection and quantification from tilapia tissues and water samples from different farms and water sources are shown in Tables 3 and 4. In the first disease outbreak (Farm 1; river open- cages), TiLV was detected from both fish and water samples from all four cages (A-D) (Table 3). Fish samples had Cq values ranging from 12.40 to 36.22, equivalent to 3.98×10^8 to 5.6×10^1 viral copies/150 ng RNA template, respectively (Table 3, Fig. 2e). Eight water samples collected from four cages in close proximity ~~sharing the same river~~ water body

had Cq values ranging from 31.19 to 36.76, equivalent to a viral load ranging from 3.40×10^5 to 8.50×10^3 viral copies/L, respectively (Table 3, Fig. 2f).

In the second disease event (Table 4, Farm 2), samples were collected from eight ponds; one had unusually mortality (C1), five showed no sign of disease (C2-C3, B1-B3), one was a sewage pond and one a reservoir pond. In the affected fingerling pond C1, TiLV was detected from five diseased fish (9.53×10^7 to 1.17×10^9 copies/150 ng RNA template), one asymptomatic fish (3.80×10^3 copies/150 ng RNA template), and water sample from one location (8.41×10^4 copies/L) (Table 4, Farm 2). TiLV was undetectable from snail and sludge samples originating from pond C1. TiLV investigation from the remaining seven other ponds of Farm 2 revealed that TiLV was also detectable— but in relatively low viral loads from some asymptomatic fish (both fingerling and brood fish) and water from culture ponds C2 and B1 as well as water from the reservoir and sewage ponds that were collected during the disease event (Table 4, Farm 2). In case of TiLV outbreak on Farm 3, both survivor tilapia were positive for TiLV (Cq 36.45-37.22), and two out of the three water samples contained TiLV at 1.50 $x10^4$ to 2.59 $x10^4$ viral copies/L (Table 4). Despite the fact that Farms 4 and 5 had experienced a TiLV outbreak a few years earlier, TiLV was not detected in samples taken from these farms or from Farm 6 with no history of TiLV infection (Table 4).

Discussion

Methods to concentrate and recover viral particles from environmental water samples have been ~~long~~ applied in human health studies especially with waterborne diseases caused by enteric viruses (example review in Cashdollar & Wymer (2013); Haramoto et al. (2018)). It has ~~later~~ become an essential process for aquatic environment research (Jacquet et al. 2010). Several techniques have been used for viral concentration from aquatic environment, including coagulation/flocculation, filtration/ultrafiltration, and centrifugation/ultracentrifugation (Cashdollar & Wymer 2013; Ikner et al. 2012). Our present study employed an iron flocculation method which was initially described for virus removal from freshwater (Chang et al. 1958) and virus concentration from marine water (John et al. 2011). It was later adapted to detect and quantify two fish viruses: nervous necrosis virus (NNV) (an RNA virus) and red sea bream iridovirus (RSIV) (a DNA virus) that were experimentally spiked in fish-rearing water (Kawato et al. 2016; Nishi et al. 2016). The recovery rate was estimated by qPCR and yielded >50 and

>80% for NNV and RSIV, respectively. In this study, while the recovery rate of TiLV (an RNA virus) from spiked-water was considerably lower (16.11 ± 3.3%), it is in a similar range of practical methods used for concentrating and detecting human viruses from water environments (Haramoto et al. 2018). For example, murine norovirus-1 (MNV-1) used as a viral model in viral concentration assay of human enteric viruses was recovered from spiked-water at 5.8–21.9% using the electronegative hydroxyapatite (HA)-filtration combined with polyethylene glycol (PEG) concentration method. The protocol was then used for detection of human noroviruses (NoV) and hepatitis A virus (HAV) in all water types (De Keuckelaere et al. 2013). More recently, researchers used porcine coronavirus (porcine epidemic diarrhea virus, PEDV) and mengovirus (MgV) as model viruses to concentrate severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) from water samples (Randazzo et al. 2020). By using an aluminum hydroxide adsorption-precipitation concentration method, PEDV and MgV spiked in water were recovered at 3.3-11.0%. The method can then be applied to detect SARS-CoV-2 RNA in untreated wastewater samples of ~$10^{5.4}$ genomic copies/L (Randazzo et al. 2020).

Despite a low recovery rate from water samples in this study, we confirmed the usefulness of the iron flocculation and RT-qPCR approach to concentrate and determine the concentration of TiLV from fish-rearing water and other water sources from two aquaculture production systems during disease outbreaks. The inherent nature of DNA and RNA viruses and their ability to survive outside their hosts may also contribute to those differences observed in recovery rates (Cashdollar & Wymer 2013; Pinon & Vialette 2018). Other viral concentration techniques using different coagulant/flocculant chemicals as well as more efficient RNA extraction methods should be tested for further improvement of TiLV recovery from water.

Following the viral concentration and recovery processes, viral detection is generally performed using PCR-based assays, cell culture methods, or viral metagenomics analysis (example review in Haramoto et al. (2018)). Here, we employed RT-qPCR technique for detection and quantification of TiLV, although the detected amounts did not represent the viral viability. Using all TiLV genomic sequences publicly available, we designed a new set of conserved primers and probe targeting the viral genomic segment 9. The newly established RT-qPCR protocol was highly specific to TiLV and did not cross-amplify RNA extracted from other common bacterial

and viral aquatic pathogens. The method is very sensitive as it can detect as low as 10 viral copies per µL of template, >2,700 times more sensitive than previous probe-based RT-qPCR methods (Kembou Tsofack et al. 2017; Waiyamitra et al. 2018), reflecting high specificity of the newly designed primers and probe. Our RT-qPCR method has 100% diagnostic specificity and sensitivity in agreement with previous results (n=65) obtained using semi-nested RT-PCR protocols (Dong et al. 2017a; Taengphu et al. 2020). Increased number of sample sizes with diverse geographical sources may be required for further investigation. Most importantly, this new Seg 9 RT-qPCR assay was able to detect and quantify TiLV load from various types of field samples, including clinically sick fish, asymptomatic fish, and water samples, as opposed to other molecular diagnostic methods optimized solely for fish specimens.

The viral loads from water samples collected during the two disease events were approximately ~ 10^3 viral copies/L (earthen ponds) and ~10^4 viral copies/L (open-cages), but in reality, these concentrations might be significantly higher due to substantial losses during the concentration and recovery process. Higher viral loads observed in some of the water samples collected during the disease outbreak were probably due to active shedding of the virus from diseased fish into the environment, and might be additional evidence of the waterborne transmission nature of TiLV reported previously (Eyngor et al. 2014; Yamkasem et al. 2019). Potential application for TiLV outbreak forecasting should be further investigated by experimental infection to monitor viral loads in water in relation to fish morbidity and mortality as previously described for other fish pathogens (Haramoto et al. 2007; Kawato et al. 2016; Minamoto et al. 2009; Nishi et al. 2016).

Conclusions

In summary, the viral concentration method by iron flocculation used in concert with a newly developed probe-based RT-qPCR was not only successful for detection and quantification of TiLV from water in diseased pond/cages, but also from unaffected ponds, reservoir, and sewage water. This method, apart from its potential practical use for future monitoring programs of TiLV viral load in water samples from various culturing units, our approach could become useful to detect possible TiLV contamination from incoming and outgoing waste water as well as to test the systems after disinfection treatments. Such application will support health professionals and

farmers to design appropriate biosecurity interventions to reduce the loss caused by TiLV in tilapia farms and hatcheries.

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Table 1(on next page)

Diagnostic specificity and sensitivity of the Seg9 probe-based RT-qPCR method

1 **Table 1**: Diagnostic specificity and sensitivity of the Seg9 probe-based RT-qPCR method

Test results	Diseased samples (n=44)	Non-diseased samples (n=21)
Positive (+)	True positive 44	False positive 0
Negative (-)	False negative 0	True negative 21
Diagnostic sensitivity (%)	100	
Diagnostic specificity (%)	100	

2

Table 2(on next page)

Percentage (%) recovery of TiLV from water using iron flocculation method with or without a resuspension step of flocculate-trapped filters soaked in oxalate-EDTA buffer

*Representative RT-qPCR results are depicted in Fig. 2d.

Table 2: Percentage (%) recovery of viruses from water using different conditions

Sample type	Before and after flocculation	Suspension step	Total viral copy number	% recovery	Fold reduction
Water spiked with TiLV culture	Before (viral stock)		3.92×10^8		
	After (Rep.1)	No	9.34×10^6	2.38	41.93
	After (Rep.2)	No	6.62×10^6	1.69	59.18
	Mean ± SD			**2.04 ± 0.5**	**50.55± 12.2**
	Before (viral stock 1)		1.27×10^8		
	After (Rep.1)	Yes	2.67×10^7	21.08	4.74
	Before (viral stock 2)		3.21×10^7		
	After (Rep.2)	Yes	4.67×10^6	14.55	6.87
	Before (viral stock 3)*		4.16×10^7		
	After (Rep.3)*	Yes	5.85×10^6	14.07	7.10
	Before (viral stock 4)*		3.07×10^7		
	After (Rep.4)*	Yes	4.52×10^6	14.74	6.78
	Mean ± SD			**16.11 ± 3.3**	**6.38± 1.1**

Rep, replicate; * denotes experiments where qPCR results were shown in Fig. 2d.

Table 2: Percentage (%) recovery of TiLV from water using iron flocculation method with or without a resuspension step of flocculate-trapped filters soaked in oxalate-EDTA buffer

Conditions	Number of replications	% Recovery
Without resuspension step	2	2.04 ± 0.5
With resuspension step	4	16.11 ± 3.3*

*Representative RT-qPCR results are depicted in Fig. 2d.

Table 3(on next page)

Quantification of TiLV from fish and water samples during an active outbreak in river open-cages

Gray highlights water samples; *viral copy (per reaction for 150 ng fish extracted RNA & per L of water sample); +, detected.

Table 3: Quantification of TiLV from fish and water samples during an active outbreak in river open-cages

Cage in Farm 1 ~~Cage~~	Samples	Cq	TiLV load*	Interpretation
A	Diseased fish A1-1 (liver + spleen)	13.02	2.64×10^{8}	+
	Diseased fish A1-2 (liver + spleen)	30.69	2.18×10^{3}	+
	Diseased fish A1-3 (liver + spleen)	13.11	2.49×10^{8}	+
	Water sample A1	36.76	8.50×10^{32}	+
	Water sample A2	31.95	2.06×10^{54}	+
B	Diseased fish B1-1 (liver + spleen)	14.35	1.10×10^{8}	+
	Diseased fish B1-2 (liver + spleen)	17.49	1.37×10^{7}	+
	Diseased fish B1-3 (liver + spleen)	13.13	2.46×10^{8}	+
	Water sample B1	32.54	1.39×10^{54}	+
	Water sample B2	31.60	2.59×10^{54}	+
C	Diseased fish C1-1 (liver + spleen)	14.76	8.34×10^{7}	+
	Diseased fish C1-2 (liver + spleen)	13.87	1.50×10^{8}	+
	Water sample C1	32.71	1.24×10^{54}	+
	Water sample C2	31.49	2.79×10^{54}	+
D	Diseased fish D1-1 (liver + spleen)	36.22	5.6×10^{1}	+
	Diseased fish D1-2 (liver + spleen)	12.40	3.98×10^{8}	+
	Diseased fish D1-3 (liver + spleen)	18.67	6.26×10^{6}	+
	Water sample D1	35.90	1.50×10^{54}	+
	Water sample D2	31.19	3.40×10^{54}	+

Gray highlights water samples; *viral copy (per reaction for 150 ng fish extracted RNA & per L of water sample); +, detected.

Table 4(on next page)

Quantification of TiLV from fish and pond water samples from earthen ponds

Gray highlights water samples; *viral copy (per reaction for 150 ng fish extracted RNA & per L of water sample); #, liver, kidney, spleen, gill, gonad; -, not detected; +, detected.

Table 4: Quantification of TiLV from fish and pond water ~~during an outbreak in~~samples from earthen ~~closed~~ ponds

Farm	Pond	Samples		Cq	TiLV load*	Interpretation
Farm/Hatchery 2 (Active TiLV outbreak)	Fingerling pond C1 (TiLV affected pond)	Fish	Diseased F1 (liver + spleen)	12.42	3.93×10^{8}	+
			Diseased F2 (liver + spleen)	14.56	9.53×10^{7}	+
			Diseased F3 (liver + spleen)	12.11	4.83×10^{8}	+
			Diseased F4 (liver + spleen)	10.77	1.17×10^{9}	+
			Diseased F5 (liver)	13.46	4.17×10^{8}	+
			~~Normal~~ Healthy looking F1 (whole fish)	29.85	3.80×10^{3}	+
		Water	Location 1	39.73	-	-
			Location 2	33.30	8.41×10^{43}	+
		Snail	Pooled sample	-	-	-
		Sludge	Pooled sample 1	-	-	-
			Pooled sample 2	-	-	-
	Fingerling pond C2 (No signs of TiLV)	Fish	~~Normal~~ Healthy looking F1 (whole fish)	-	-	-
			~~Normal~~ Heathy looking F2 (whole fish)	32.88	5.11×10^{2}	+
		Water	Location 1	34.66	3.42×10^{43}	+
			Location 2	39.76	-	-
	Fingerling pond C3 (No signs of TiLV)	Fish	~~Normal~~ Healthy looking F1 (whole fish)	37.34	2.6×10^{1}	
			~~Normal~~ Healthy looking F2 (whole fish)	-	-	-
		Water	Location 1	-	-	-
			Location 2	-	-	-
	Broodstock pond B1 (No signs of TiLV)	Fish	Female brood 1, ~~normal~~ Healthy looking#	37.08	3.10×10^{1}	
			Female brood 2, ~~normal~~ Healthy looking#	35.42	9.50×10^{1}	
			Male brood 1, ~~normal~~ Healthy looking#	38.28	-	-
			Male brood 2, ~~normal~~ Healthy looking#	36.18	5.70×10^{1}	
		Water	Location 1	37.79	4.29×10^{32}	+
	Broodstock pond B2 (No signs of TiLV)	Water	Location 1	-	-	-
			Location 2	-	-	-

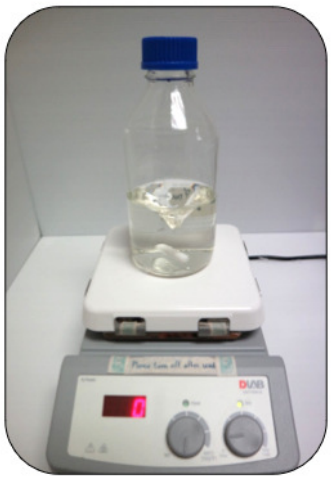
	Broodstock pond B3 (No signs of TiLV)	Water	Location 1	-	-	-
			Location 2	-	-	-
	Sewage	Water	Location 1	34.61	3.53×10^{43}	+
			Location 2	-	-	-
	Reservoir	Water	Location 1	-	-	-
			Location 2	37.78	4.32×10^{32}	+
Farm 3 (Active TiLV outbreak)		Fish	Survivor F1 (spleen)	36.45	4.80×10^{1}	+
			Survivor F2 (spleen)	37.22	2.88×10^{1}	+
		Water	Location 1	35.08	2.59×10^{4}	+
			Location 2	39.03	-	-
			Location 3	35.90	1.50×10^{4}	+
Farm 4 (With history of TiLV outbreak)		Fish	Healthy F1 (whole fish)	-	-	-
			Healthy F2 (whole fish)	-	-	-
		Water	Location 1	39.27	-	-
			Location 2	-	-	-
Farm 5 (With history of TiLV outbreak)		Water	Location 1	39.18	-	-
			Location 2	38.24	-	-
			Location 3	-	-	-
Farm 6 (No history of TiLV outbreak)		Water	Location 1	-	-	-
			Location 2	-	-	-

Gray highlights water samples; *viral copy (per reaction for 150 ng fish extracted RNA & per L of water sample); #, liver, kidney, spleen, gill, gonad; -, not detected; +, detected~~; C2, C3, B1-B3 apparently healthy ponds with no signs of disease~~ .

Figure 1

Workflow of TiLV flocculation, concentration and quantification used in this study

An iron flocculation method was used to concentrate viruses from water (a). The water suspension containing the virus was filtered through a 0.4-µm pore size polycarbonate membrane filter with a vacuum pressure pump (b-c). The flocculate-trapped filter (d) was then resuspended in oxalate-EDTA buffer (e) prior to nucleic acid extraction (f) and TiLV quantification (g).

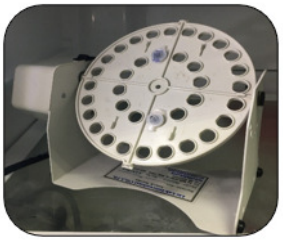


(a) Viral flocculation

(b) Vacuum pressure pump setting

(c) Membrane filtration

(d) Viral concentration



(e) Resuspension

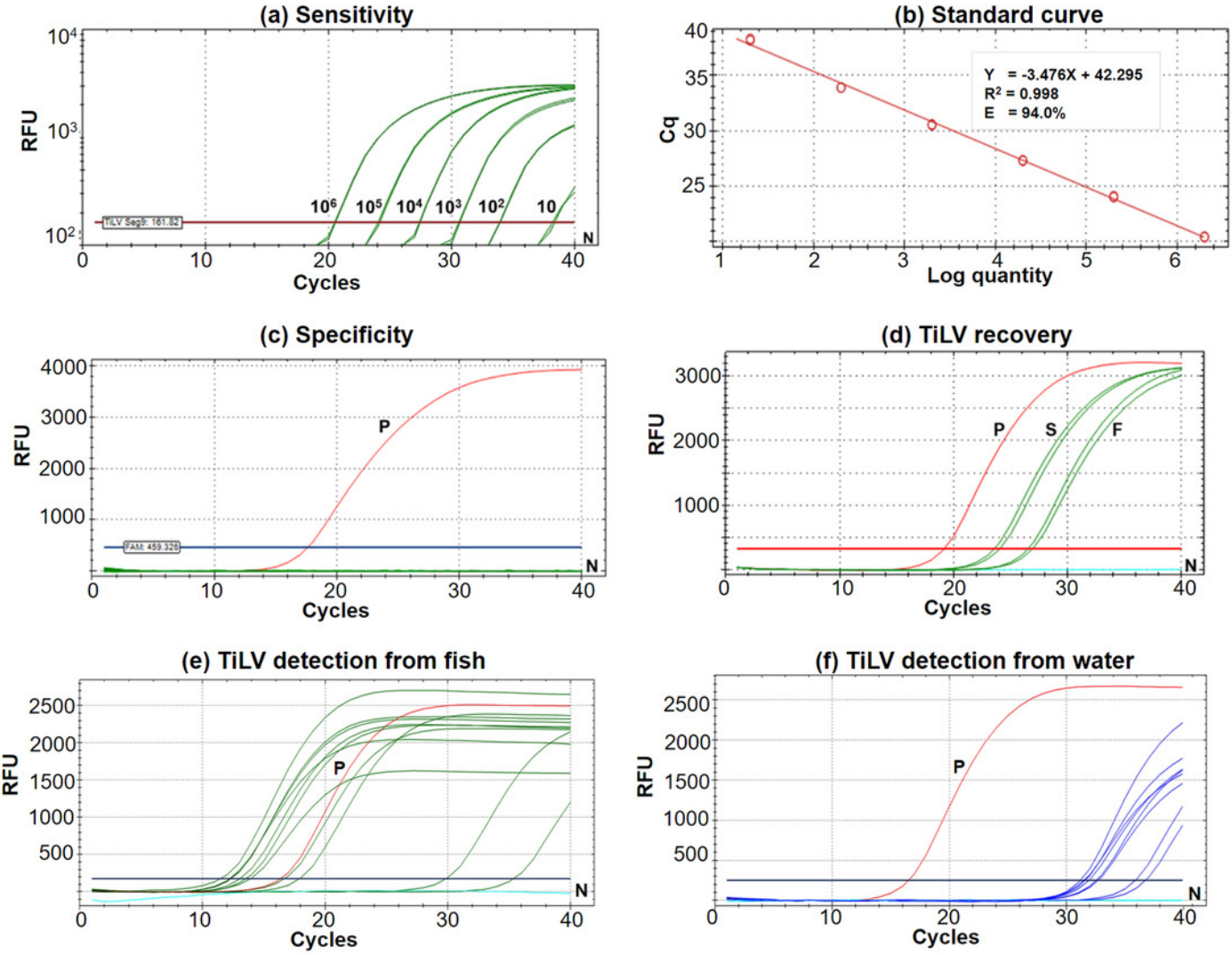
(f) RNA extraction

(g) Viral quantification

Figure 2

Performance of the newly established probe-based RT-qPCR detection of TiLV genomic segment 9

a) Analytical sensitivity assay determined using serial dilutions of plasmid DNA containing a 351-bp TiLV segment 9 insert. Amplification results were from two technical replicate tests. b) A standard curve was derived from the assays in (a) showing an amplification efficiency (E) of 94.0%. c) Analytical specificity test of the RT-qPCR protocol against RNAs extracted from common pathogens of fish and healthy looking tilapia as listed in Table S1. d) TiLV quantification from template extracted from stock virus (S) and flocculate-trapped filters (F) with resuspension step using two replicates. e) TiLV quantification from fish samples collected from an outbreak open cage. f) TiLV quantification from water samples collected from an outbreak open cage. P, positive control; N, no template control; RFU, relative fluorescence units.


(a) Sensitivity
RFU
Cycles
10^6 10^5 10^4 10^3 10^2 10
N
(b) Standard curve
Cq
Log quantity
Y = -3.476X + 42.295
R² = 0.998
E = 94.0%
(c) Specificity
RFU
Cycles
P
N
(d) TiLV recovery
RFU
Cycles
P S F
N
(e) TiLV detection from fish
RFU
Cycles
P
N
(f) TiLV detection from water
RFU
Cycles
P
N