P-ISSN 2586-9000
E-ISSN 2586-9027
Homepage : https://tci-thaijo.org/index.php/SciTechAsia
Science & Technology Asia
Vol.28 No.3 July – September 2023
Page: [239-246]

*Original research article*

# Efficiency of Tilapia Lake Virus Detection in Nile Tilapia by Nested RT-PCR and Semi-Nested RT-PCR

Umaporn Khimmakthong[1, 2,*], Sulaiman Madyod [1], Supaporn Noochu [1]

[1]*Faculty of Veterinary Science, Rajamangala University of Technology Srivijaya, Nakhon Si Thammarat 80240, Thailand*
[2]*Animal Infectious Diseases in South of Thailand Research Unit, Faculty Veterinary Science, Rajamangala University of Technology Srivijaya, Nakorn Si Thammarat 80240, Thailand*

Received 14 March 2023; Received in revised form 9 May 2023
Accepted 1 June 2023; Available online 26 September 2023

**ABSTRACT**

There are several methods for detecting Tilapia Lake virus (TiLV). The most popular methods for detecting RNA viruses are nested RT-PCR and semi-nested RT-PCR. The primers currently used are varied and are often designed to be specific for viruses found in each area. This study aimed to develop a detection method for TiLV in tilapia by designing two pairs of TiLV-specific primers and then detecting TiLV by nested RT-PCR and semi-nested RT-PCR, which examined suspected tilapia samples from fish cages in the Tapi River, Nakhon Si Thammarat Province. The results showed that nested RT-PCR (PCR product size of 307 bp) and semi-nested RT-PCR 2 (PCR product size of 496 bp) gave more accurate results than semi-nested RT-PCR 1 (PCR product size of 600 bp) while nested RT-PCR was more sensitive than semi-nested RT-PCR 2. The sensitivity and specificity of the nested RT-PCR method were each 100%.

**Keywords:** Detection; Nested RT-PCR; Semi-nested RT-PCR; Tilapia; TiLV

## 1. Introduction

Nile tilapia (*Oreochromis niloticus*) is an important economic animal in Thailand. It is the most productive freshwater fish and each year it generates income for many Thai farmers. At the same time, infectious diseases killed fish and reduced income greatly. Tilapia lake virus (TiLV) is an RNA virus in the family of Orthomyxovirus. The first TiLV outbreak in tilapia occurred in Israel, Ecuador, Colombia and Egypt in 2014 [1, 2]. In Thailand, the first TiLV outbreak began in 2015, found on farms in the central, western, eastern and northeastern regions of Thailand [3, 4]. Most infected fish were identified within one month of leaving the hatchery;

***Corresponding author:** tal_biot@hotmail.com

mortality rates were 20-90% at the tilapia farms and most of the affected fish were those that weighed 1-50g [4]. However, at present, infections are also found in large tilapia in the southern Thai province of Nakhon Si Thammarat.

Epidemics of TiLV are recent occurrences and there is still little research on developing methods of detection. In addition, detecting RNA viruses is more complicated than detecting DNA viruses. The detection methods currently in place involve checking the ability to induce CPE in the E11 [5] and SSN-1 cell lines [6], *in situ* hybridization [3] and histopathology [3]. All of which are techniques that require a specialist and take a long time. Therefore, molecular techniques with high accuracy and shorter waiting times are of interest. RT-PCR is the most basic technique but has a low sensitivity. Tsofack et al. developed a nested RT-PCR method which has higher sensitivity, but lacks specific primers [5]. Later, Dong et al. developed a semi-nested RT-PCR method but this method still has problems with unwanted DNA bands [3].

In this study, a new method was developed for detecting TiLV in tilapia by designing two pairs of primers that are specific to TiLV and then detecting TiLV using nested RT-PCR compared to semi-nested RT-PCR. The objective of this study was to address the problem of non-specificity seen in previously researched TiLV detection methods. This study uses samples of tilapia suspected to be infected from the Nile tilapia cage in the Tapee river, Nakhon Si Thammarat Province.

## 2. Materials and Methods

### 2.1 Materials

The main materials used in this experiment were GENEzol$^{TM}$ reagent (Geneaid, Taiwan), QIAGEN® OneStep RT-PCR (QIAGEN, Germany), TopTaq master mix kit (QIAGEN, Germany), primers purchased from Eurofins Genomics (Germany), and 100bp marker purchased from GeneDireX (China). Maestro-Gen NanoDrop spectrophotometer (Taiwan) was used to measure the concentration and purity of extracted RNA. PCR was run in FlexCycler2, Biometra GmbH (Germany). Electrophoresis was run in Wide Mini-Sub Cell GT (Bio-Rad, USA).

### 2.2 Fish samples

This research was granted permission to use animals for scientific purposes under the ANIMALS FOR SCIENTIFIC PURPOSES ACT, B.E. 2558 with the approval of the Supervisory Committee on Raising and Use of Animals, Rajamangala University of Technology Srivijaya (RMUTSV-IAC No. IAC 03-11-61).

The samples from tilapia suspected to be infected with TiLV were collected from a tilapia cage in the Tapee river, Nakhon Si Thammarat. Samples were taken from a total of 27 fish, of which 5 were used for the preliminary test while the other 22 were used for the examination of suspected infected samples. Tilapia suspected to be infected with TiLV virus were observed for external symptoms as follows: scratched skin, scattered wounds on the body that become enlarged, turbid eye lens, sunken eyes, loss of balance, lethargy, and pale body color. The target organs of the virus, the liver and brain, were separated using aseptic technique. The separated organs were immersed in GENEzol™ reagent and kept at -80 °C until RNA extraction was performed.

### 2.3 Primer design

The primers for nested PCR were designed using sequences from the GenBank database (http://www.ncbi.nlm.nih.gov/genbank/). The segment 3 of the TiLV virus (Accession no. KU751816.1) was used to design 2 pairs of primers, TiLV outer-F, TiLV outer-R, TiLV inner-F and TiLV inner-R. Primers were designed using Primer3 Input (version 0.4.0) (Source: http://bioinfo.ut.ee/primer3-0.4.0/). Analysis of primers was done using Oligo Calc: Oligonucleotide Properties Calculator (Source: http://biotools.nubic.north

western.edu//OligoCalc.html) to find $T_m$ and probability of self-complementarity.

### 2.4 Experimental design

For the preliminary experiment, the liver and brain of 5 fish (3 infected fish and 2 healthy fish) were used. The tilapia brain tissue samples were divided into two parts, the first part for RNA extraction and the second part for viral isolation. Infection was confirmed by observing the occurrence of CPE in the SSN-1 cell line. After that, the brains of 22 fish (unknow infection status) were used for testing this technique. The tissue samples were separated using aseptic technique. The total RNA was extracted and tested using the method described below.

### 2.5 Confirmation of infection in SSN-1 cells

SSN-1 cells were cultured in 75 ml cell culture flasks with L15 cell culture medium. One hundred ml of medium contained 94 ml of L15 media, 1 ml of 10,000 µg/ml Pen-strep, and 5 ml of FBS. Then these flasks were incubated at 25ºC to observe cell density of 80-90%. The cells were then seeded into a 24 well plate (containing 2.5 x $10^5$ cells/well).

TiLV in the brain was isolated in cell culture medium. Briefly, fish brains were ground in L15 cell culture medium, then centrifuged at 12,000 rpm for 10 minutes at 4 °C twice to remove tissue debris. They were then filtered through a 0.45 µM membrane. Then, virus suspension was added to the SSN-1 cells (in the 24 well plate) and incubated at 25 °C. The suspension from healthy fish brain samples was added to the control group. CPE formation was observed at 7 days post-inoculation.

### 2.6 One-step RT-PCR

Total RNA from the liver and brain were extracted using GENEzol™ reagent according to the manufacturer's instructions. Quantity and purity of RNA was measured by NanoDrop spectrophotometer. The RNA was taken to run one-step RT-PCR with the first primer pair. The total reaction mixture of 12.5 µL contained 2.5 µL of 5x QIAGEN One-Step RT-PCR Buffer (containing 12.5 mM $MgCl_2$), 400 µM of each dNTP, 0.6 µM of TiLV outer-F, 0.6 µM of TiLV outer-R, 2.0 µL of QIAGEN One-Step RT-PCR Enzyme Mix, and 0.5 µg of RNA. Thermal cycler conditions including reverse transcription were set as follows: 50°C for 30 min, initial PCR activation step: 95°C for 15 min, 3-steps cycling: 40 cycles at 94°C for 45 s, 55°C for 45 s, 72 °C for 45 s, and final extension: 72 °C for 10 min. The 18S rRNA gene was amplified at the same time to be used as control.

### 2.7 Nested RT-PCR

One µL of one-step RT-PCR product was taken to run nested-PCR. The total reaction mixture of 12.5 µL contained 6.25 µL of 2x TopTaq Master Mix (containing 1.25 units of TopTaq DNA Polymerase, 1x PCR Buffer (containing 1.5 mM $MgCl_2$), 200 µM of each dNTP), 0.2 µM of TiLV inner-F, 0.2 µM of TiLV inner-R, 1x CoralLoad Concentrate, and 1 µL of one-step RT-PCR product. Three-step PCR cycling conditions were set as follows: 40 cycles at 94°C for 45 s, 55°C for 45 s, 72°C for 45 s, and final extension: 72°C for 10 min. Cycling was initiated at 94°C for 5 min and terminated at 72°C for 10 min.

### 2.8 Semi-nested RT-PCR

#### *2.8.1 Semi-nested RT-PCR 1*

One µL of one-step RT-PCR product was taken to run semi-nested RT-PCR 1. The total reaction mixture of 12.5 µL contained 6.25 µL of 2x TopTaq Master Mix (containing 1.25 units of TopTaq DNA Polymerase, 1x PCR Buffer (containing 1.5 mM $MgCl_2$), 200 µM of each dNTP), 0.2 µM of TiLV outer-F, 0.2 µM of TiLV inner-R, 1x CoralLoad Concentrate, and 1 µL of one-step RT-PCR product. 3-step PCR cycling conditions were set as follows: 40 cycles at 94°C for 45 s, 55°C for 45 s, 72°C for 45 s, and final extension: 72°C for 10 min. Cycling was initiated at 94°C for 5 min and terminated at 72°C for 10 min.

### *2.8.2 Semi-nested RT-PCR 2*

One µL of one-step RT-PCR product was taken to run semi-nested RT-PCR 2. The total reaction mixture of 12.5 µL contained 6.25 µL of 2x TopTaq Master Mix (containing 1.25 units of TopTaq DNA Polymerase, 1x PCR Buffer (containing 1.5 mM $MgCl_2$), 200 µM of each dNTP), 0.2 µM of TiLV inner-F, 0.2 µM of TiLV outer-R, 1x CoralLoad Concentrate, and 1 µL of one-step RT-PCR product. 3-step PCR cycling conditions were set as follows: 40 cycles at 94°C for 45 s, 55°C for 45 s, 72°C for 45 s, and final extension: 72°C for 10 min. Cycling was initiated at 94°C for 5 min and terminated at 72°C for 10 min.

## 2.9 The sensitivity of nested RT-PCR

The sensitivity and specificity of the nested RT-PCR method was calculated using the formula [8]:

$$\text{Method sensitivity} = \frac{\text{Number of true positive(TP)}}{\text{(Number of true positive(TP) +Number of false negative(FN))}},$$

$$\text{Method specificity} = \frac{\text{Number of true negative (TN)}}{\text{(Number of true negative (TN) +Number of false positive (FP))}}.$$

The TP, FN, TN, and FP values were obtained by comparing the results with the gold standard method (the observation of CPE in SSN-1).

## 2.10 Data Analysis

The results of one-step RT-PCR, nested-PCR, and semi-nested RT-PCR are shown in the form of the PCR product compared with 100 bp markers. The number of positive PCR products are presented as a proportion of the total sample number.

# 3. Results

## 3.1 Primer design

The segment 3 of TiLV has been reported in previous research as the popular selective segment for primer design [9]. The nucleotide sequence of the designed primers used in the experiment are shown in Table 1. The primer for 18S rRNA gene was synthesized according to a previous trial [7].

**Table 1.** All primers used in this study.

| The study | Primer sequence (5'→3') | $T_m$ (°C) | PCR product size (bp) | Ref. |
|---|---|---|---|---|
| One step RT-PCR | **Outer F:** ACCCTTGTAGAGTCGAGGCA<br>**Outer R:** TTTGGAGATCGACGGGGTTG | 60.25<br>60.04 | 789 | This study |
| Nested PCR | **Inner F:** ACTGTGCTTTCCAGAGTCGC<br>**Inner R:** GTGCTCAAAGTTCCTCGCCT | 60.60<br>60.60 | 307 | This study |
| Semi-nested PCR 1 | **Outer F:** ACCCTTGTAGAGTCGAGGCA<br>**Inner R:** GTGCTCAAAGTTCCTCGCCT | 60.25<br>60.60 | 600 | This study |
| Semi-nested PCR 2 | **Inner F:** ACTGTGCTTTCCAGAGTCGC<br>**Outer R:** TTTGGAGATCGACGGGGTTG | 60.60<br>60.04 | 496 | This study |
| 18S rRNA | **18S rRNA F:** GGACACGGAAAGGATTGACAG<br>**18S rRNA R:** GTTCGTTATCGGAATTAACCAGAC | 58.92<br>57.97 | 111 | [7] |

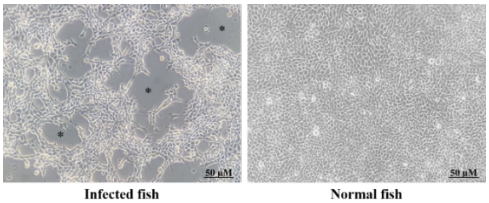


**Fig. 1.** Cytopathic effects (marked with asterisk) in SSN-1 cells, after inoculation with TiLV-infected fish brain samples, compared to healthy fish brain.

## 3.2 Confirmation of infection in SSN-1 cells

Virus suspensions obtained from the brains of healthy and infected fish were added to the cells. CPE was observed at 7 days post inoculation. Extensive cell detachment, cell aggregation and vacuolation were clearly detected at 7 days post-inoculation. No CPE formation appeared in the cells inoculated with the brain samples from healthy fish.

### 3.3 One-step RT-PCR

RNA was extracted from the livers and brains of 5 tilapia fish. Then, it was examined for TiLV infection by the one-step RT-PCR method using the outer F and outer R primers, and 18S rRNA as the control gene. The results were examined by 1.5% agarose gel electrophoresis. The results showed no target DNA bands for TiLV in any of the samples (for both liver and brain). However, 18S rRNA was positive in all samples, as shown in Fig. 2.

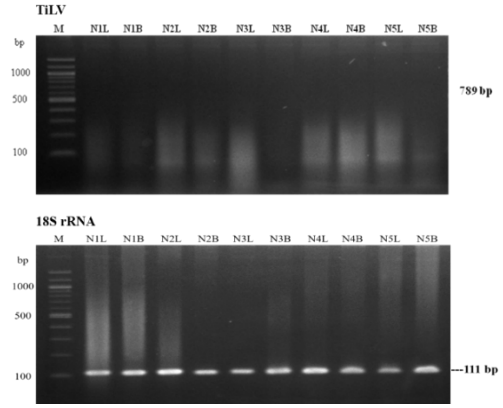


**Fig. 2.** One-step RT-PCR product using the outer F and outer R primers and 18S rRNA of liver and brain samples.

### 3.4 Nested RT-PCR

The one-step RT-PCR products were further analyzed with nested RT-PCR using the inner F and inner R primers at 60ºC. The product had a size of 307 bp, exactly as designed. A non-specific DNA band was also observed, shown in Fig. 3. The number of positive PCR products from brain tissue as a proportion of the total sample number was 3/5. This was consistent with the results seen in SSN-1 cells that also had a proportion of 3/5.

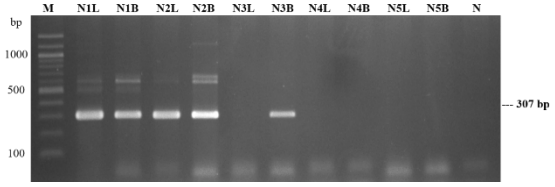


**Fig. 3.** Nested RT-PCR results of liver and brain samples using inner F and inner R primers at 60 ºC.

### 3.5 Semi-nested RT-PCR

#### *3.5.1* Semi-nested RT-PCR 1

The one-step RT-PCR method was performed with semi-nested RT-PCR using the outer F and inner R primers (semi-nested RT-PCR 1) at 60ºC. The results revealed that the target DNA bands in samples 1 and 2 matched the designed size, 600 bp, and no non-specific DNA bands occurred, as is shown in Fig. 4. The number of positive PCR products in brain tissue as proportion of the total sample number was 2/5.

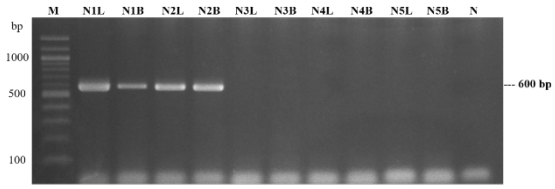


**Fig. 4.** Semi-nested RT-PCR 1 results of liver and brain samples using outer F and inner R primers at 60ºC.

#### *3.5.2* Semi-nested RT-PCR 2

The one-step RT-PCR method was performed with semi-nested RT-PCR using the inner F and outer R primers (semi-nested RT-PCR 2) at 60ºC. The results were similar to those of nested RT-PCR, but the DNA bands from semi-nested RT-PCR 2 were fainter, with the target DNA bands found in samples 1, 2, and 3 that were 496 bp in size, exactly as designed. A non-specific DNA band was also found. Additionally, in sample 3, the target DNA band was found in the brain but not the liver, as shown in Fig. 5. The number of positive PCR products in brain tissue as proportion of the total sample number was 3/5.

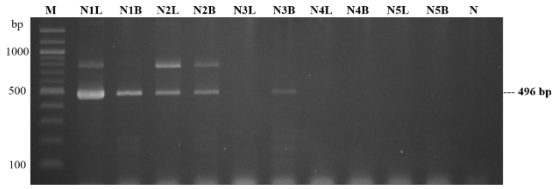


**Fig. 5.** Semi-nested RT-PCR 2 results of liver and brain samples using inner F and outer R primers at 60ºC.

### 3.6 Comparison of nested RT-PCR and semi-nested RT-PCR 2

The results from the nested RT-PCR, semi-nested RT-PCR 1, and semi-nested RT-PCR 2 methods for TiLV detection showed that nested RT-PCR and semi-nested RT-PCR 2 gave the correct results. However, the DNA bands obtained from the nested RT-PCR and semi-nested RT-PCR 2 still had non-specific DNA bands. So, this experiment was chosen to compare nested RT-PCR and semi-nested RT-PCR 2; the temperature was raised to 61ºC to reduce non-specific DNA bands.

Twenty-two tilapia brain samples were taken for RNA extraction. Then, they were examined for TiLV infection by one-step RT-PCR using the outer F and outer R primers at 60 ºC. The resulting RT-PCR products were then investigated by nested RT-PCR and semi-nested RT-PCR 2 at 61 ºC, with 18S rRNA used as the control gene. The results were examined by 1.5% agarose gel electrophoresis.

Nested RT-PCR showed positive results for 9 out of 22 samples, i.e., samples 3, 4, 6, 10, 11, 12, 13, 14, and 15. Non-specific DNA bands were present, as shown in Fig. 6(A). The number of positive PCR products as proportion of the total sample number was 9/22. This was consistent with the results in SSN-1 cells that also gave a positive proportion of 9/22.

Semi-nested RT-PCR 2 showed positive results in 7 out of 22 samples, i.e., samples 6, 10, 11, 12, 13, 14, and 15. Non-specific DNA bands were not found, as shown in Fig. 6(B). The results of the 18S rRNA primer test were positive in all samples (data not shown). The number of positive PCR products as proportion of the total sample number was 7/22.

### 3.7 The sensitivity of nested RT-PCR

TiLV infections were investigated by nested RT-PCR compared to the gold standard method (the observation of CPE in SSN-1) and calculate the value out as method sensitivity and method specificity. It was found that the sensitivity and specificity of the nested RT-PCR method were each 100%.

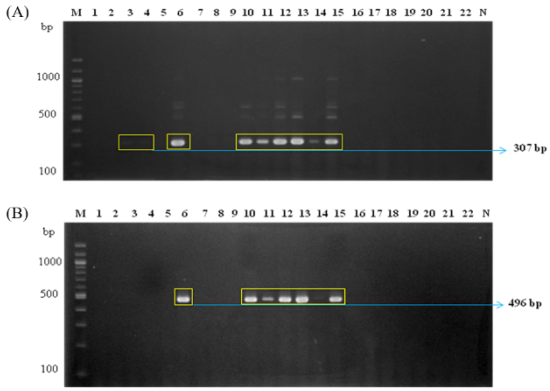


**Fig. 6.** Nested RT-PCR (A) and semi-nested RT-PCR 2 (B) results of 22 brain samples at 61ºC.

## 4. Discussion

Snakehead-fish cell line (SSN-1 cells) are derived from the striped snakehead fish (*Ophicephalus striatus*). The characteristics of infected cells includes an increase in cell aggregation, cellular vacuolation, cell shrinkage, and formation of foci of detached, rounded cells. More pronounced cell detachment and syncytial formation were observed at 5 to 7 dpi [10]. In this study, extensive cell detachment, cell aggregation, and vacuolation were clearly detected at 7 days post-inoculation.

The target tissues of TiLV are the gills, liver, brain, heart, kidney, and spleen [11]. The use of mucus membranes has also been reported in the detection of TiLV, but the results were only demonstrated in live fish [12]. According to a study by Dong et al., the liver is the most effective tissue for TiLV detection by histopathological and in situ hybridization [3], while the liver and brain are the tissues that are most suitable for the detection of viruses by molecular methods [5]. The results of semi-nested RT-PCR 2 confirmed the results of the nested RT-PCR experiment, which showed that using brain tissue for detecting TiLV was more accurate than liver tissue. This is consistent with the results reported by Tattiyapong et al. that

measured the viral loads in various tissues of infected fish including gills, liver, brain, heart, kidney, and spleen. The highest amount of virus was detected in the brain [11].

Nested PCR is a modification of PCR that was designed to improve sensitivity and specificity [13]. There are two forms of nested PCR assays, fully nested and semi- nested. Fully nested PCR assays involve the initial use of a pair of external primers that amplify the entirety of the target sequence to produce a primary amplicon. Then in a second PCR amplification, an internal pair of primers is used that amplifies a target sequence internal to both ends of the initial amplicon. With the semi- nested PCR assay, an initial pair of external primers is used to produce a primary amplicon. The secondary pair of primers consists of either the initial forward or the initial reverse primer used in conjunction with an opposing internal primer so that the resulting secondary amplicon has only one end internal to the initial amplicon [14]. In this study, the results showed that detection of TiLV by nested RT- PCR was more accurate than semi-nested RT-PCR 1, but that the DNA band obtained by semi- nested RT-PCR 1 was more specific than nested RT-PCR method. The experimental results (Fig. 6.) show that increasing the temperature prevented the non-specific DNA band of semi- nested RT- PCR 2 from forming, while the non- specific DNA band of nested RT-PCR remained. The resulting non-specific DNA band did not influence the diagnosis of the experimental results and increasing the temperature above 61ºC may also decrease the target DNA band. Therefore, the optimum temperature for detection is 60ºC. It has been shown that nested RT-PCR is more sensitive than semi-nested RT-PCR 2.

## 5. Conclusion

From these results, it can be concluded that using brain tissue to detect TiLV is more accurate than using liver tissue. The optimum temperature for PCR was 60°C. Nested RT-PCR and semi- nested RT-PCR 2 gave more accurate results than semi- nested RT- PCR 1. Meanwhile, nested RT-PCR was more sensitive than semi-nested RT-PCR 2. The sensitivity and specificity of nested RT- PCR method were each 100%.

## Acknowledgements

The authors would like to thank the Rajamangala University of Technology Srivijaya for research funding and the RMUTSV Faculty of Veterinary Science for kindly providing research facilities.

## References

[1] Ferguson HW, Kabuusu R, Beltran S, Reyes E, Lince JA and del Pozo J. Syncytial hepatitis of farmed tilapia, *Oreochromis niloticus* (L.): a case report. J Fish Dis. 2014;37:583-89.

[2] Eyngor M, Zamostiano R, Tsofack JEK, Berkowitz A, Bercovier H, Tinman S, Lev M, Hurvitz A, Galeotti M, Bacharach E and Eldar A. Identification of novel RNA virus lethal to tilapia. J Clin Microbiol. 2014;52:4137-46.

[3] Dong HT, Siriroob S, Meemetta W, Santimana-wong W, Gangnonngiw W, Pirarat N, Khunrae P, Rattanarojpong T, Vanichviri-yakit R and Senapin S. Emergence of tilapia lake virus in Thailand and an alternative semi-Nested RT-PCR for detection. Aquaculture. 2017;476:111-8.

[4] Surachetpong W, Janetanakit T, Nonthaben-jawan N, Tattiyapong P, Sirikanchana K and Amonsin A. Outbreaks of tilapia lake virus infection, Thailand, 2015- 2016. Emerg Infect Dis. 2017;23(6):1031-3.

[5] Tsofack JEK, Zamostiano R, Watted S, Berkowitz A, Rosenbluth E, Mishra N, Briese T, Lipkin WI, Kabuusu RM, Ferguson H, del Pozo J, Eldar A, and Bacharach E. Detection of tilapia lake virus in clinical samples by culturing and nested reverse transcription-PCR. J Clin Microbiol. 2017;55(3):759-67.

[6] Al-Hussinee L, Subramaniam K, Surachetpong W, Popov V, Hartman K, Starzel K, Yanong R, Watson C, Ferguson H, Frasca Jr S and Waltzek T. Tilapia lake virus (TiLV): a globally emerging threat to tilapia aquaculture. UF/IFAS. 2019;2019(2):1-7.

[7] Wang E, Wang K, Chen D, Wang J, He Y, Long B, Yang L, Yang Q, Geng Y, Huang X, Ouyang P and Lai W. Evaluation and selection of appropriate reference genes for real-time quantitative PCR analysis of gene expression in nile tilapia (*Oreochromis niloticus*) during vaccination and infection. Int J Mol Sci. 2015;16(5):9998-10015.

[8] Jacobson RH. Principles of validation of diagnostic assays for infectious disease. In: Manual of diagnostic tests and vaccines for terrestrial animals. Paris, France: World Organisation for Animal Health (OIE); 2006. Chapter 1.1.4.

[9] Waiyamitra P, Tattiyapong P, Sirikanchana K, Mongkolsuk S, Nicholson P and Surachetpong W. A TaqMan RT-qPCR assay for tilapia lake virus (TiLV) detection in tilapia. Aquaculture. 2018;497:184-8.

[10] Tattiyapong P, Dachavichitlead W and Surachetpong W. Experimental infection of tilapia lake virus (TiLV) in Nile tilapia (*Oreochromis niloticus*) and red tilapia (*Oreochromis* spp.). Vet Microbiol. 2017;207:170-7.

[11] Tattiyapong P, Sirikanchana K and Surachetpong W. Development and validation of a reverse transcription quantitative polymerase chain reaction for tilapia lake virus detection in clinical samples and experimentally challenged fish. J Fish Dis. 2018;41(2):255-61.

[12] Liamnimitr P, Thammatorn W, Uthoomporn S, Tattiyapong P and Surachetpong W. Non-lethal sampling for Tilapia Lake Virus detection by RT-qPCR and cell culture. Aquaculture. 2018;486:75-80.

[13] Carr J, Williams DG and Hayden RT. Molecular detection of multiple respiratory viruses. Mol Diagn. 2010;289-300.

[14] Levin RE, Ekezie FGC. and Sun DW. DNA-based technique: polymerase chain reaction (PCR). In: Sun DW, editors. Modern techniques for food authentication. 2nd ed. London, UK: Academic Press; 2018. p. 527-616.