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Non-lethal sampling for Tilapia Lake Virus detection by RT-qPCR and cell culture

Pavarit Liamnimitr[a], Worrayanee Thammatorn[a], Sonicha U-thoomporn[a], Puntanat Tattiyapong[b], Win Surachetpong[a,b,*]

[a] Department of Veterinary Microbiology and Immunology, Faculty of Veterinary Medicine, Kasetsart University, Thailand
[b] Center for Advanced Studies for Agriculture and Food, Kasetsart University (CASAF, NRU-KU, Thailand), Institute for Advanced Studies, Kasetsart University, Bangkok 10900, Thailand

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ABSTRACT

Tilapia Lake Virus (TiLV) is an emerging virus of tilapia fish. Recently, outbreaks of TiLV associated mortality have been reported in many countries including Israel, Ecuador, Colombia, Egypt and Thailand. However, little is known about the route of transmission and how the virus is spread in fish populations. In this study, TiLV was detected in liver and mucus samples from moribund tilapia using reverse transcriptase quantitative polymerase chain reaction and virus isolation in the cell culture. Comparison of virus detection in the liver and mucus of field samples revealed that mucus could be applied for TiLV diagnosis and the virus in mucus was still viable and could cause a cytopathic effect in E-11 cells. The cohabitation of TiLV-infected fish with healthy fish resulted in 55.71% cumulative mortality of cohabitating fish suggesting that direct contact of infected fish is sufficient for disease transmission. Notably, the TiLV genomic RNA was identified in the mucus of cohabitation challenge fish as early as 1 day post infection (dpi) and the virus was isolated from mucus samples collected at 5 dpi. The presence of TiLV persisted up to 12–14 dpi in the mucus, liver and intestines of cohabiting fish. Taken together, the detection of TiLV in the mucus of field samples and cohabitating fish suggested that horizontal transmission is one of the important routes for the spread of TiLV. Importantly, this study revealed that mucus could be used for non-lethal sampling in TiLV detection.

1. Introduction

Tilapia Lake Virus (TiLV) is a recently discovered virus that affects wild and farm-raised tilapia fish. This emerging virus was first reported in Israel in 2014 (Eyngor et al., 2014) and subsequently found in South America, Africa and Asia (Bacharach et al., 2016; Fathi et al., 2017; Surachetpong et al., 2017). The clinical signs of TiLV infection include emaciation, swimming at the water surface, skin erosion and skin redness, body discoloration, exophthalmos and abdominal swelling (Surachetpong et al., 2017; Tattiyapong et al., 2017a). Experimental studies have shown that TiLV can cause disease in susceptible fish through cohabitation and intraperitoneal challenge of susceptible fish (Eyngor et al., 2014; Tattiyapong et al., 2017a). In severe infection, high mortality was observed within the two weeks that moribund fish were investigated (Surachetpong et al., 2017). Notably, a recent study has indicated that cohabitation of susceptible fish with TiLV-infected fish results in high mortality within 8–10 days, suggesting that horizontal transmission is one of the important routes of disease establishment (Eyngor et al., 2014).

The epidemic pattern of TiLV suggests that TiLV can be transmitted through direct contact with infected fish or possibly through contaminated water and equipment. Up to date, there is no specific prophylaxis or vaccine for TiLV prevention. Therefore, control strategies including strict biosecurity and screening carrier fish are essential to prevent TiLV transmission. However, appropriate control measures required a basic understanding of how the virus disseminates in the fish population. Such knowledge is still lacking for TiLV as the virus has been recently identified and no route of transmission has been reported.

Currently, diagnosis of TiLV relies on the detection of the virus in fish tissues such as the liver or brain using a highly sensitive molecular method such as reverse transcription polymerase chain reaction (RT-PCR) and reverse transcription quantitative polymerase chain reaction (RT-qPCR) or virus isolation in the cell culture (Tattiyapong et al., 2017b; Kembou Tsofack et al., 2017). However, non-lethal sampling techniques for TiLV detection have not been fully investigated. The objectives of this study were to determine the presence of TiLV in the

* Corresponding author at: Department of Veterinary Microbiology and Immunology, Faculty of Veterinary Medicine, Kasetsart University, 50 Ngam Wong Wan Road, Ladyao, Chatuchak, Bangkok 10900, Thailand.
E-mail address: fvetwsp@ku.ac.th (W. Surachetpong).

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mucus of tilapia and to evaluate whether mucus may be used for non-lethal sampling and the detection of TiLV.

2. Materials and methods

2.1. Fish samples and virus detection

Moribund and normal red tilapia (*Oreochromis* spp.) were collected from different sampling locations in Thailand from August 2016 to January 2017. The moribund fish and healthy fish (three fish per outbreak) were randomly collected and brought to the laboratory as live fish or chilled carcass. Mucus samples (200 μL) were collected using a thick cover glass and skin scraping along the lateral line from the anterior to posterior direction. Feces were collected by gently pressing the ventral abdomen into a caudal direction. The pooled of livers (20 mg) and feces (5 mg) were placed into 1.5 mL tubes for RNA isolation. Of the 35 samples, TiLV was detected in 21 outbreaks using RT-qPCR assay (Tattiyapong et al., 2017b). The animal use protocol for this study was approved by the Kasetsart University Animal Ethics Committee (permit number OACKU00659). For humanized endpoint termination, severely moribund fish were euthanized using an overdose of eugenol solution at a concentration of 3 mL/L (Aquanes, Better Pharma, Thailand).

2.2. Challenge study

A sample of 220 red tilapia (*Oreochromis* spp.) with an average body weight of 10 g ± SE of 0.38 g were obtained from a local fish farm in Thailand. Fish were maintained and acclimated for two weeks at the Animal Research Facility, Faculty of Veterinary Medicine, Kasetsart University, Bangkok, Thailand. The dissolved oxygen, temperature, total nitrite and nitrate and total ammonia levels were monitored daily using commercial test kits. Prior to challenge, five fish were randomly selected for parasite examination using skin scraping, bacterial isolation on tryptic soy agar (TSA) and TiLV detection using RT-qPCR assay. For the cohabitation challenge, 60 red tilapia were sedated using eugenol solution at a concentration of 3 mL/L for 5 min and then were injected intraperitoneally (IP) with 50 μL TiLV strain VETKU-TV01 at 10^6 $TCID_{50}$/mL. The TiLV-IP challenge fish were clipped at the anal fin to differentiate from cohabitating fish. At 6 days post infection (dpi), 42 TiLV-IP challenge fish were equally separated and transferred into two 150 L/tank (tank A and tank B) each containing 70 normal red tilapia. Thus, the ratio of TiLV-IP challenge fish and cohabiting fish is approximate 1:3. The TiLV-IP challenge fish and normal red tilapia were allowed to cohabitat for 21 days. The clinical signs of TiLV infection and mortality rate were recorded daily from tank A. While tissue samples including liver, intestines, mucus and feces were randomly collected from challenged fish (three fish per day) in tank B at 0, 1, 2, 3, 4, 5, 6, 8, 10, 12, 14 and 21 dpi. The presence of TiLV in tissue samples was determined using RT-qPCR and virus isolation in the cell culture.

2.3. RNA isolation and cDNA synthesis

The pooled livers, feces, intestines or mucus were homogenized in a 1.5 mL centrifuge tube containing 1 mL Trizol® reagent according to the manufacturer's protocol (Invitrogen, USA). The RNA quantity and quality (A_{260}/A_{280}) were examined using a Nanodrop ND 2000 spectrophotometer (Thermo Scientific; Wilmington, DE, USA). A 1 μg of RNA template was used for cDNA synthesis using reverse transcription kit (ReverTra Ace qPCR RT kit, FSQ-101,Toyobo, Japan) following the manufacturer's instruction.

2.4. RT-qPCR

The amounts of TiLV in the liver, intestine and mucus samples were analyzed using a SYBR-based RT-qPCR assay (Tattiyapong et al., 2017b). Briefly, the reaction was performed in a 20 μL reaction containing 400 ng of cDNA, 10 μL of 2 × iTaq™ universal SYBR supermix (Bio-Rad, USA), 0.6 μL of each 10 μM forward and reverse primers and molecular water to adjust the final volume. The sequence of the forward primer, TiLV-112F, was 5′-CTGAGCTAAAGAGGCAATATGGATT-3′ and the sequence of the reverse primer, TiLV-112R, was 5′-CGTGCGTACT CGTTCAGTATAAGTTCT-3′. The reactions were carried out in a real-time PCR thermocycler CFX96 Touch™ (Bio-Rad, USA). At the end of the RT-qPCR reaction, samples were processed for melting curve analysis at 65 °C to 95 °C with 0.5 °C per 5 s increment. To extrapolate the viral copy numbers, the averaged C_t values were compared to the standard curve as previously described (Tattiyapong et al., 2017b).

2.5. Virus isolation from fish mucus and feces

Collected mucus samples (200 μL) and feces (5 mg) were diluted 1:4 in Hank's balanced salt solution (HBSS), homogenized and then centrifuged at 3000 × *g* for 10 min at 4 °C. The supernatant was filtered through a 0.22 μM pore-size syringe filter (Costar, USA). An aliquot of 200 μL mucus and fecal samples was inoculated to a confluent E-11 cell line in a 24-well cell culture plate. The E-11 cell line was purchased from the European Collection of Authenticated Cell Cultures (ECACC) catalog number 01110916, UK. Cells were maintained in L-15 Leibovitz medium (Sigma, USA) supplemented with 2% fetal bovine serum. The cytopathic effect (CPE) was monitored for two weeks.

2.6. Detection of TiLV using RT-PCR

RNA samples were isolated from E-11 cells inoculated with non-infected mucus and TiLV-infected mucus. The E-11 cells were centrifuged for 15 min at 3000 × *g* at 4 °C. The supernatant was either kept at − 80 °C or directly subjected to RNA extraction using a GF-1 Nucleic Acid Extraction Kit (Vivantis, Malaysia). Total RNA was finally re-suspended in 30 μL RNase-free water. A 1 μg sample of total RNA was processed for cDNA synthesis as described earlier. The PCR reaction was performed in a 20 μL reaction containing 1 × of Tag buffer with KCl (ThermoScientific, USA), 2 mM of $MgCl_2$, 0.2 mM of dNTP mix, 0.125 μM of each primer, 0.25 μL of 5 U/μL Taq DNA polymerase and 200 ng of cDNA. The sequence of forward and reverse primers were TiLV-112F (Tattiyapong et al., 2017b) 5′-CTGAGCTAAAGAGGCAATA TGGATT-3′ and Nested ext-1 (Eyngor et al., 2014) 5′-TATGCAGTACT TTCCCTGCC-3′, respectively, which amplify the segment 3 of TiLV isolated from Thailand (GenBank accession no. KX631923). The PCR reaction was carried out in a T100 thermal cycler (Bio-Rad, USA) with cycle conditions of initial denaturation at 95 °C for 30 s, 40 cycles of denaturation at 95 °C for 10 s, annealing at 60 °C for 30 s and extension at 72 °C for 30 s, with a final extension at 72 °C for 5 min. The PCR products were separated on 3% NuSieve™ 3:1 agarose gel and stained with ethidium bromide.

3. Results

3.1. Detection of TiLV in fish mucus

Of the 35 field samples tested, 21 samples were confirmed positive for TiLV using the RT-qPCR method. TiLV was present in the livers with mean Ct values ranging from 8.4 (2.8×10^8 copies/μg of total RNA) to 32.9 (2.0×10^1 copies/μg of total RNA) as shown in Table 1. Similarly, the virus was detected in the mucus of all 21 field samples with Ct values ranging from 14.7 (3.8×10^6 copies/μg of total RNA) to 33.4 (1.5×10^1 copies/μg of total RNA). Overall, the comparison of mean Ct values for virus detection between the liver and mucus samples revealed that the virus levels were relatively close (Table 1).

Table 1
Detection of TiLV from mucus and liver of field samples.

Sample IDs	Location	Cycle threshold value (copies/μg of total RNA)	
		Liver	Mucus
VETKU-01	Ang Thong	8.4 (2.8×10^8)	18.8 (2.6×10^5)
VETKU-02	Uthai Thani	13.2 (1.1×10^7)	15.3 (2.7×10^6)
VETKU-03	Ratchaburi	13.3 (1.0×10^7)	21.7 (3.7×10^4)
VETKU-04	Kanchanaburi	14.9 (3.5×10^6)	30.3 (1.2×10^2)
VETKU-05	Kanchanaburi	16.5 (1.2×10^6)	29.0 (2.6×10^2)
VETKU-06	Prachinburi	17.1 (7.7×10^5)	29.5 (2.0×10^2)
VETKU-07	Ratchaburi	17.5 (6.1×10^5)	21.6 (3.9×10^4)
VETKU-08	Ratchaburi	19.3 (1.8×10^5)	23.3 (1.2×10^4)
VETKU-09	Prachinburi	19.7 (1.4×10^5)	24.5 (5.5×10^3)
VETKU-10	Nakhon Si Thammarat	19.9 (1.2×10^5)	19.4 (1.7×10^5)
VETKU-11	Ratchaburi	20.2 (1.0×10^5)	23.1 (1.4×10^4)
VETKU-12	Phitsanulok	20.2 (1.0×10^5)	30.0 (1.3×10^2)
VETKU-13	Phitsanulok	22.0 (2.9×10^4)	25.7 (2.5×10^3)
VETKU-14	Phra Nakhon Si Ayutthaya	22.7 (1.9×10^4)	14.7 (3.8×10^6)
VETKU-15	Pathum Thani	24.5 (5.7×10^3)	28.7 (3.2×10^2)
VETKU-16	Pathum Thani	26.6 (1.3×10^3)	32.8 (2.2×10^1)
VETKU-17	Phra Nakhon Si Ayutthaya	28.6 (3.5×10^2)	20.0 (1.1×10^5)
VETKU-18	Kanchanaburi	28.8 (3.1×10^2)	29.0 (2.8×10^2)
VETKU-19	Nakhonnayok	32.2 (3.2×10^1)	32.3 (3.0×10^1)
VETKU-20	Samut Sakhon	32.8 (2.2×10^1)	18.1 (4.0×10^5)
VETKU-21	Uttaradit	32.9 (2.0×10^1)	33.4 (1.5×10^1)
VETKU-22	Saraburi	ND	ND
VETKU-23	Saraburi	ND	ND
VETKU-24	Ratchaburi	ND	ND
VETKU-25	Kanchanaburi	ND	ND
VETKU-26	Phitsanulok	ND	ND
VETKU-27	Ang Thong	ND	ND
VETKU-28	Kanchanaburi	ND	ND
VETKU-29	Ang Thong	ND	ND
VETKU-30	Samut Songkhram	ND	ND
VETKU-31	Chai Nat	ND	ND
VETKU-32	Phitsanulok	ND	ND
VETKU-33	Chai Nat	ND	ND
VETKU-34	Phatthalung	ND	ND
VETKU-35	Nakhon Pathom	ND	ND

ND = No fluorescence detection.

3.2. Virus isolation from fish mucus

To further confirm that the presence of TiLV in the fish mucus represented the infective virus, the mucus samples from normal and TiLV positive fish were infected with E-11 cells. A total of nine independent field samples confirming positive TiLV using RT-qPCR and two negative samples were selected for virus isolation in E-11 cells (Table 2). At 3–7 dpi, the characteristics of CPE formation including syncytial formation and round and swollen cell structure were observed in the E-11 cells inoculated with TiLV-positive mucus (Fig. 1D). Extensive cell detachment, cell aggregation and vacuolation were clearly detected at 4–9 dpi. No CPE formation appeared in the cells inoculated with the mucus from the RT-PCR negative fish (Fig. 1C). Additionally, the TiLV genomic RNA was present in the E-11 cells inoculated with the TiLV positive mucus (Fig. 2). None of the E-11 cells inoculated with TiLV-negative samples showed a specific PCR product (Fig. 2). In particular, the amount of CPE correlated with virus titer in the mucus, with samples with a low C_t value including VETKU-01 and VETKU-10 with C_t values 18.8 (2.6×10^5 copies/μg of total RNA) and 19.4 (1.7×10^5 copies/μg of total RNA), respectively, showed CPE development within 3–5 dpi. In contrast, samples inoculated with a high C_t value (30 − 32) such as VETKU12 and VETKU-19 showed CPE at 6–7 dpi.

Table 2
Comparison of RT-qPCR and viral isolation from positive mucus samples.

Sample IDs[a]	Location	Cycle threshold value (copies/μg of total RNA)	CPE first observed (dpi)
VETKU-01	Ang Thong	18.8 (2.6×10^5)	3
VETKU-10	Nakhon Si Thammarat	19.4 (1.7×10^5)	5
VETKU-17	Phra Nakhon Si Ayutthaya	20.0 (1.1×10^5)	6
VETKU-11	Ratchaburi	23.1 (1.4×10^4)	6
VETKU-09	Prachinburi	24.5 (5.5×10^3)	5
VETKU-13	Phitsanulok	25.7 (2.5×10^3)	5
VETKU-06	Prachinburi	29.5 (2.0×10^2)	5
VETKU-12	Phitsanulok	30.0 (1.3×10^2)	6
VETKU-19	Nakhonnayok	32.3 (3.0×10^1)	7
VETKU-23	Saraburi	ND	ND
VETKU-24	Ratchaburi	ND	ND

dpi = days post infection ND = No detection.
[a] Samples were arranged by the Ct-value.

3.3. Detection of TiLV in mucus of cohabitation fish

At 3 dpi, the clinical signs of TiLV infection were clearly seen in the cohabitating fish including loss of feed intake, skin erosion, skin redness, scale protrusion and abdominal swelling. The mortality of cohabitating fish lasted from 3 dpi until 15 dpi with cumulative mortality at 55.71% (Fig. 3). Noteworthy, TiLV RNA was identified in the livers of cohabitating fish at 2–14 dpi, in mucus at 1–12 dpi and in intestines at 5–12 dpi with the viral loads ranging from 1.1×10^1 copies/μg of total RNA (1 dpi) to 1.7×10^2 copies/μg of total RNA (14 dpi) (Fig. 4). Notably, no amplification of TiLV genomic RNA was detected in any feces sample, and no virus was isolated from feces. However, the infective virus was isolated from mucus collected at 5–12 dpi (Table 3).

4. Discussion

In the present study, mucus was examined as a form of non-lethal sampling for TiLV detection. Comparatively, the virus was detected in the mucus and liver samples collected from the field and experimentally challenged fish. The presence of TiLV genomic RNA in the liver and mucus suggested that these samples are suitable for TiLV diagnosis. Although the storage condition and viability of the virus has not been fully investigated in this study, TiLV RNA could be detected from mucus samples kept at − 20 °C for 30 days (data not shown). Prior to this study, the standard lethal sampling technique using pooled brains or livers was routinely performed for TiLV diagnosis using the RT-qPCR method and virus isolation in the permissive cell line (Eyngor et al., 2014; Del-Pozo et al., 2017; Surachetpong et al., 2017). Notably, the present results showed that pooled mucus samples collected from 2 to 5 fish is sufficient for TiLV detection. Beneficially, mucus collection through skin scraping using cover glass or surgical blade is much easier to perform in the field and in the laboratory without causing much damage to the fish. In addition, the non-lethal sampling techniques could be applied for TiLV detection in valuable bloodstocks or large fish that are close to market size. Non-lethal sampling methods using the mucus, feces (Graham et al., 2012; Griffiths and Melville, 2000) and blood (Lopez-Jimena et al., 2010) have been reported for viral detection in salmon. Giray et al. (2005) described the detection of infectious salmon anemia virus (ISAV) in the blood and mucus of moribund salmon using RT-PCR and virus isolation in susceptible cells. Similarly, blood samples may be used as a non-lethal source for ISAV detection using RT-PCR and virus culture from clinically sick and asymptomatic carrier fish (Snow et al., 2003). Moreover, Salmonid alphavirus (SAV) RNA could be amplified from mucus and feces samples from cohabiting challenged Atlantic salmon using RT-PCR (Graham et al., 2011). Comparison of non-lethal sampling methods including kidney

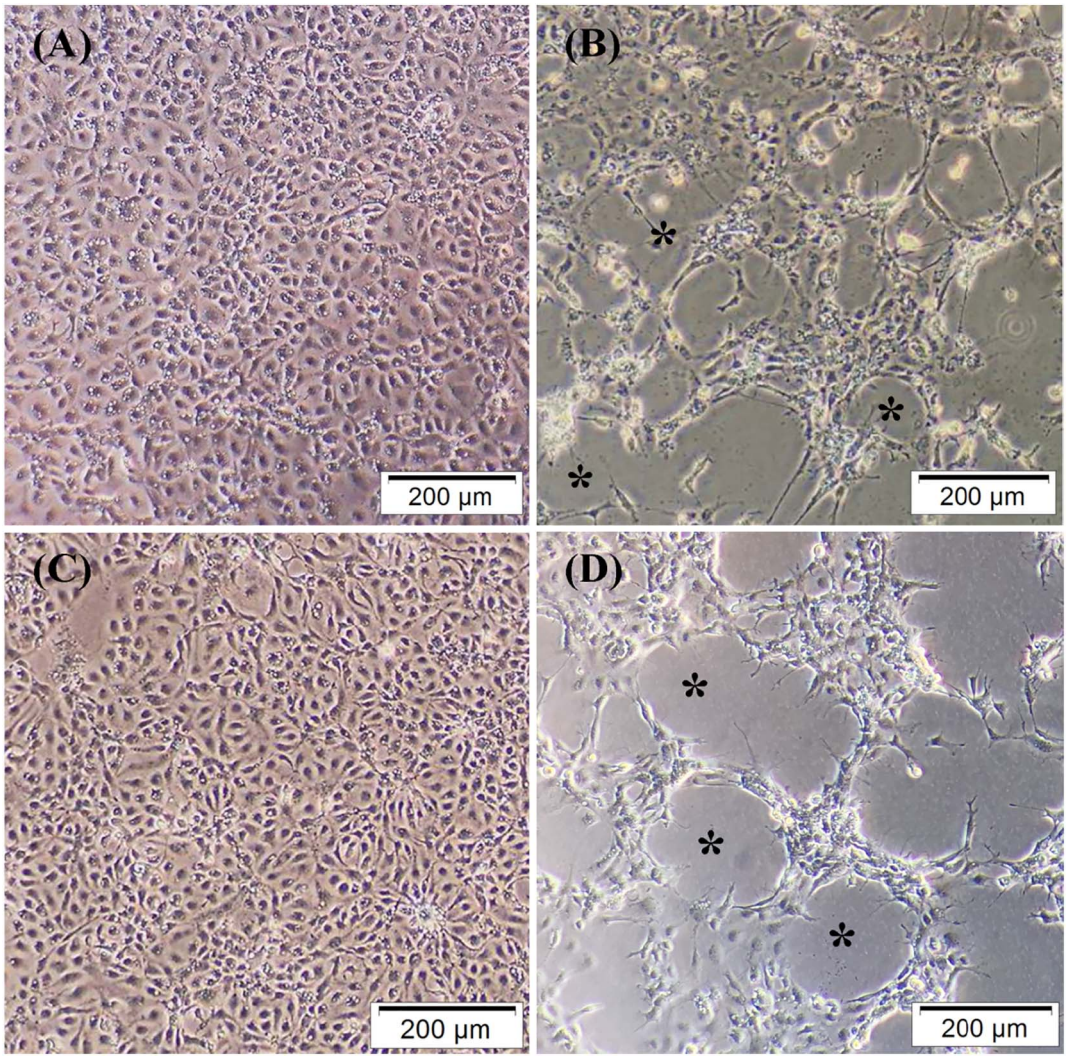

Fig. 1. Cytopathic effects (marked with asterisk) in E11 cells after inoculation with TiLV-infected mucus: (A) Uninfected E-11 cells; (B) Positive control, E-11 cells inoculated with TiLV; (C) E-11 cells inoculated with TiLV-uninfected mucus; (D) E-11 cells inoculated with TiLV-infected mucus.

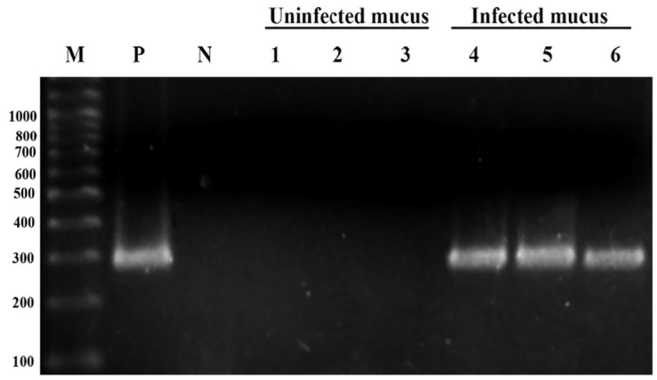

Fig. 2. Amplification of PCR products from E-11 cells inoculated with uninfected or TiLV-infected mucus. (M) 100 bp marker; P = Positive control (pTiLV), N = No template control, Lanes 1–3; E-11 inoculated with mucus from normal fish (uninfected mucus), Lanes 4–6; E-11 inoculated with mucus from TiLV-positive fish (infected mucus). The uninfected and infected mucus analysis was based on three clinical samples representing different geographic locations.

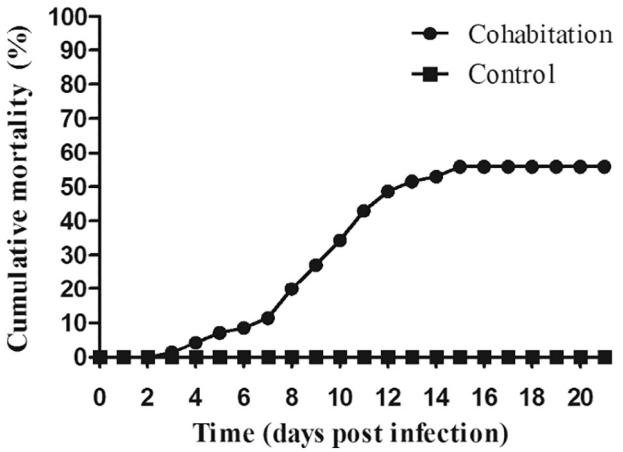

Fig. 3. Transmission of TiLV through cohabitation. Normal red tilapia (n = 70) were cohabited with TiLV infected fish (n = 21). The cumulative mortality was recorded daily until 21 days.

aspiration, nasal wash, gill swab, and fecal and blood collection revealed that all of these methods are appropriate for *Streptococcus agalactiae* isolation in tilapia (Tavares et al., 2016). Of the three non-lethal samples (mucus, gills and fin tissue), gill biopsy provided the best non-lethal sampling method for the detection of active viral hemorrhagic septicemia virus (VHSV) and infectious hematopoietic necrosis virus (IHNV) infection (Monaghan et al., 2015; Burbank et al., 2017). However, gill biopsy or blood samples required trained staff to better obtain

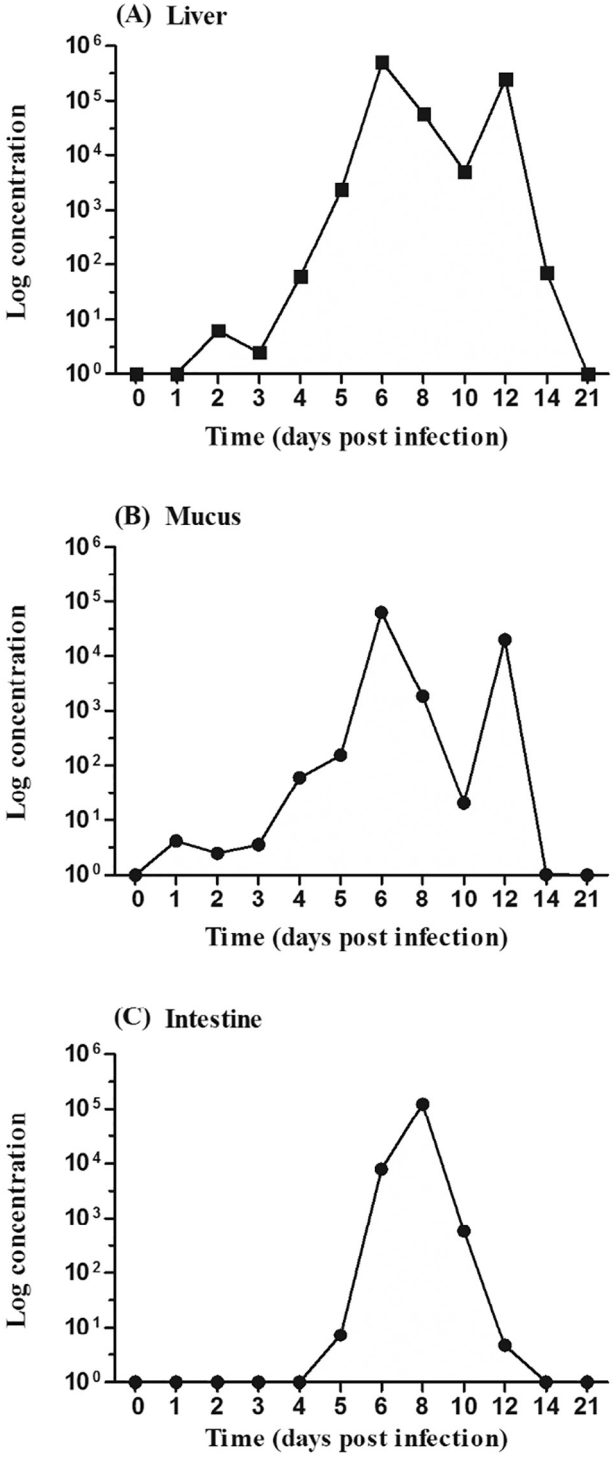

Fig. 4. Detection of TiLV in different tissues of cohabiting challenged tilapia. Tissues consisting of liver (A), mucus (B) and intestine (C) were collected from infected fish at 0–21 dpi. The amounts of TiLV (log_{10} (copy number/μL of infected tissue)) were determined using RT-qPCR.

the samples and the collection procedures may cause extensive damage in small fish. In the current study, TiLV could not be identified or isolated from the feces of field samples and experimentally challenged fish. However, the genomic RNA of virus could be detected in the intestines of challenged fish at 5–12 dpi. Nevertheless, it remains inconclusive whether feces could spread the virus under naturally occurring conditions.

The skin and mucus of fish are parts of the mucosal barrier that is important for the prevention of pathogen entry into the host (Benhamed et al., 2014; Rombout et al., 2014). Although the TiLV genomic RNA could be identified in infected mucus, it is important to determine the viability of the virus in fish mucus. The development of CPE in E-11 cells revealed that the virus was still viable and capable of infecting the permissive cells. Furthermore, the detection of TiLV in infected E-11 cells was confirmed using the RT-PCR method. The transmission of the virus via contaminated water and cohabitation with clinically sick fish and normal fish has been reported for many aquatic viruses including rhabdoviruses (Schonherz et al., 2013), iridovirus (Jeong et al., 2008), betanodaviruses (Korsnes et al., 2012), aquabirnaviruses (Munang'andu et al., 2016) and orthomyxoviruses (Aamelfot et al., 2015; Jones and Groman, 2001). SAV has been found to spread from infected to susceptible fish through direct contact with contaminated water and infected fish (Graham et al., 2011; McLoughlin et al., 1996). A previous study suggested that cohabitation of diseased fish with susceptible tilapia is sufficient to cause TiLV infection indicating horizontal transmission through direct contact of infected fish or contaminated water (Eyngor et al., 2014). Likewise, the cohabitation in the present study confirmed that direct contact of infected fish allows TiLV transmission. The transmission could occur through cannibalism of an infected carcass or mucus from moribund fish. Cannibalism has been described as the source of dwarf gourami iridovirus infection in Murray cod (Rimmer et al., 2017). In tilapia, a high stocking density and the increased age of fish promote cannibalism (Fessehaye et al., 2006). Moreover, high mortality due to Streptococcus infection was reported in Nile tilapia rearing with a fish density over 11.2 g/L (Shoemaker et al., 2000). As such, active biosecurity such as the removal of moribund or dead fish should be implemented to limit TiLV shedding in the infected pond or cage.

From the cohabitation study, TiLV could be identified in the mucus of challenged fish as early as 1 dpi while the virus was detected in the liver and intestines at 2–5 dpi. As the disease progressed, the virus was present in the liver and intestines until 14 and 12 dpi, respectively. Consistently, the virus was isolated from fish mucus collected at 5–12 dpi in E-11 cells in which the virus reached high amounts with fish showing severe clinical signs of infection. Interestingly, the cohabitation study indicated that healthy tilapia may acquire TiLV infection within 1–2 days after exposure to infected fish. During early infection, the highly sensitive RT-qPCR may amplify a low amount of TiLV in infected tissue, whereas the virus isolation requires a high viral titer in the samples. It has been shown that virus isolation in cell culture is less sensitive than the RT-qPCR method (Tattiyapong et al., 2017b). All this information could be applied to set up the quarantine period and sample collection during epidemiological studies. Previously, the reproduction of disease depended on intraperitoneal injection; therefore,

Table 3
Isolation of TiLV from mucus and feces samples of cohabitating fish.

Sample IDs	Days post infection	Days first observed CPE (number of positive fish/number of fish tested)	
		Mucus	Feces
Co-hab0	0	ND	ND
Co-hab1	1	ND	ND
Co-hab2	2	ND	ND
Co-hab3	3	ND	ND
Co-hab4	4	ND	ND
Co-hab5	5	4(2/3)	ND
Co-hab6	6	3(3/3)	ND
Co-hab8	8	4(3/3)	ND
Co-hab10	10	4(1/3)	ND
Co-hab12	12	5(2/3)	ND
Co-hab14	14	ND	ND
Co-hab21	21	ND	ND

ND = No detection (0/3).

the cohabitation challenge model could better support future challenge studies that resembled the natural route of infection. While the cumulative mortality in this study was 55.7%, Eyngor et al. (2014) reported high mortality at 80% in cohabitation challenged tilapia. This difference could be accounted for by the different genetic background of fish, the challenge procedure or the genetic diversity of the virus, any of which may influence the outcome of challenge studies. Collectively, the present study and previous reporting (Eyngor et al., 2014) confirmed that horizontal transmission via infected fish is sufficient for TiLV transmission, possibly through the direct contact of infected fish materials including skin and mucus.

In summary, the present study demonstrated that mucus is a potential route of horizontal transmission of TiLV. Moreover, the detection and isolation of live virus from mucus could be applied as a non-invasive sampling technique for TiLV detection. To the authors' best knowledge, this is the first report of detection of TiLV using a non-lethal sampling method. Hence, this finding suggests that strict biosecurity and farm management such as the removal of moribund or dead fish, and proper disinfection of water and equipment could limit the spread of TiLV and reduce the economic impact of this emerging viral disease.

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