Aquaculture 486 (2018) 75–80

Contents lists available at ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aquaculture

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

ARTICLE INFO

Keywords:
Mucus
Tilapia
Tilapia Lake Virus
Horizontal transmission
Detection

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).

https://doi.org/10.1016/j.aquaculture.2017.12.015
Received 10 October 2017; Received in revised form 14 November 2017; Accepted 10 December 2017
Available online 12 December 2017

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).