Effects of sample preservation and storage times on the detection of tilapia lake virus (TiLV) RNA in tilapia tissues

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Highlights

- Different preservation methods and storage conditions affect the amount of TiLV genomic RNA in fish samples.
- RNAlater® and deep-freezing at −20 °C offered the best storage conditions to maintain TiLV genomic RNA.
- The benefits of this study can be applied to preserve samples efficiently and to transport samples properly.

Abstract

Tilapia lake virus (TiLV) is a novel orthomyxo-like virus, and has genomic RNA material that is easily degradable. Hence, proper sample storage and preservation should be applied for accurate diagnostic results. In this study, we investigated various conditions for preserving tilapia tissues for the detection of TiLV. Our results revealed that RNA*later*® and deep-freezing at −20 °C are the best practices to maintain TiLV genomic RNA for subsequent diagnosis. Samples stored in these conditions could maintain TiLV genomic RNA for 365 days with minimal reduction. In contrast, TiLV genomic RNA is substantially degraded in ethanol and on Whatman® FTA® classic cards with a reduction of TiLV genomic material of 2 logs and up to 3 logs within 30 days of storage, respectively. Besides, all preservation methods showed a difference in the amount of TiLV genomic RNA between the initial day, during, and after 30 days of storage. The benefits of this study can be applied to preserve samples efficiently and to transport samples properly from remote areas to a laboratory that is suitably-equipped for disease examination.

Keywords: Tilapia lake virus; Preservation; Storage time; Tilapia

1. Introduction

Tilapia is an essential fish for aquaculture practice that provides an inexpensive protein source globally with a production of 6.4 million tonnes and a trade value worth of USD 9.8 billion in 2015 (FAO, 2017). Although tilapia is easy to raise and adaptable to different environments, recent disease outbreaks associated with the emergence of tilapia lake virus (TiLV) have been reported in many countries (Eyngor et al., 2014; Jansen et al., 2019; Surachetpong et al., 2017; Tattiyapong et al., 2018). The spread of TiLV may be caused by moving of live fish across countries as both horizontal and vertical transmission of TiLV to susceptible fish have been reported (Dong et al., 2020; Eyngor et al., 2014; Tattiyapong et al., 2017; Yamkasem et al., 2019), whereas frozen tilapia products showed minimal risk of TiLV transmission (Thammatorn et al., 2019). Hence, multiple strategies such as the implementation of proper control measures, and early and rapid detection of the pathogen are applied to limit the transmission of this virus and its economic impact on farmers. To date, various molecular methods including reverse transcription polymerase chain reaction (RT-PCR), RT-quantitative PCR (RT-qPCR) and reverse transcription loop-mediated isothermal amplification (RT-LAMP) assay have been developed for TiLV detection (Eyngor et al., 2014; Kembou Tsofack et al., 2017; Nicholson et al., 2018; Phusantisampan et al., 2019; Waiyamitra et al., 2018; Yin et al., 2019). However, sample collection and preservation remain one of the most critical steps to maintain the accuracy of the testing result.

Generally, fish tissues are processed and submitted to a laboratory within 24 to 48 h for disease screening. This situation is not achievable in many cases due to logistical and geographical limitations. For example, samples collected from local fish farms in African countries might take several days to reach a national laboratory or a few weeks for overseas delivery. Likewise, limited resources and capacity of a laboratory for fish diseases in many parts of the world could hamper early detection and surveillance of emerging and re-emerging diseases in aquaculture. Hence, there is a crucial need to be able to effectively preserve and deliver samples to a suitable laboratory for fish disease diagnosis. The choice of a suitable preservation method will provide confidence in the laboratory disease diagnosis (DiEuliis et al., 2016).

To date, few studies on sample preservation for subsequent disease investigation have been reported in aquaculture practice. It has been suggested that temperature-controlled storage at subzero temperatures provides an effective means to preserve aquaculture viruses in biological samples (Durand et al., 2000; Ørpetveit et al., 2010) however, it requires cold-chain logistics, which may be expensive and highly variable. Alternative approaches such as the use of RNA preserving buffers and paper-based techniques should, therefore, be considered. RNA*later*® is gaining popularity as a valid alternative to frozen storage (Foley et al., 2010). It has been shown to counteract the effects of temperature and endogenous nucleases present in the tissues (Gorokhova, 2005), thereby preserving nucleic acids without a need for freezing. Since commercial RNA*later*® is relatively expensive, a homemade version equivalent of RNA*later*® (Passow et al., 2019) and concentrated ethanol (Siah et al., 2014) can be applied to protect RNA degradation in tissue samples for short-term storage. Alternatively, Whatman® FTA® cards have been reported to sufficiently preserve RNA viruses for specific durations. A previous study on other RNA viruses in fish revealed that RNA persisted on Whatman® FTA® classic cards at 4 °C for up to 28 days (Krishnan et al., 2016). Despite some limitations being reported, Whatman® FTA® classic card is more convenient for the collection of samples in the field where chemicals and resources are limited.

As there is limited knowledge of the effect on storage time on different preservation methods of TiLV infected tissue, it is crucial to investigate the conditions that are most suitable for TiLV detection. In this study, different standard preservation methods and storage times have been investigated for the detection of TiLV in tilapia tissue.

2. Materials and methods

2.1. Fish samples and ethical statement

A total of 100 red hybrid tilapia (*Oreochromis* spp.) with 40 ± 3.0 g weight were purchased from a farm in Saraburi province, Thailand. Fish were allowed to acclimatize at the animal facility, Faculty of Veterinary Medicine, Kasetsart University, Bangkok, Thailand, and were quarantined in a 150-L aquarium tank with de-chlorinated water for 7 days. Water quality parameters were monitored, and water was changed at 50% daily. In three separate experiments, thirty red hybrid tilapia were randomly selected, and intraperitoneally (IP) injected with 50 µL of TiLV strain VETKU-TV01 at a titer of 10^5 $TCID_{50}$/mL to have at least 50% mortality. All laboratory protocols and animals used were approved by the Kasetsart Institutional Animal Care and Use Committee (IACUC) under the protocol number ACKU61-VET-009. Moribund fish with clinical signs indicative of TiLV infection were selected and euthanized using eugenol solution at a concentration of 5 mL/L for 10 min (Aquanes®; Better Pharma; Bangkok, Thailand) at 6 days post-infection (dpi). Subsequently, approximately 30 mg of the liver was collected and stored in different preservation conditions.

2.2. Preservation methods

2.2.1. Temperatures and chemicals

In the first experiment, liver tissues (weight 30 ± 1.0 mg) from six TiLV-infected fish were collected into 1.5 mL microcentrifuge tubes. The samples were stored under different preservation conditions including 1) 4 °C; 2) –20 °C; 3) 70% ethanol at room temperature; 4) 95% ethanol at room temperature; and 5) RNA*later*® at room temperature for 0, 3, 7, 14, and 30 days before further analysis. In the second experiment, long-term storages of TiLV-infected tissues were simulated during which three liver and two brain samples in RNA*later*® and two liver samples at −20 °C were kept for 365 days at room temperature.

2.2.2. Whatman® FTA® classic card

In the third experiment, the efficiency of a paper-based preservation technique was evaluated. Liver tissues (weight 30 ± 1.0 mg) from fish with clinical signs of TiLV infection (n = 8) were collected and applied onto Whatman® FTA® classic cards (Merck KGaA; Darmstadt, Germany). Tissue samples were smashed three times, each for 5 s onto the card paper, and left in an air-flow box for 2 h. After drying, all cards were stored in a zip-lock plastic bag containing desiccants, away from light, at room temperature for 0, 3, 7, 14, and 30 days. In the fourth experiment, livers of five TiLV-infected fish were stored on Whatman® FTA® classic cards for 120 days and processed for further analysis.

2.3. RNA extraction and complementary DNA (cDNA) synthesis

The tissue samples collected from different storage days and conditions were processed for RNA extraction using TRIzol®, an acid guanidinium thiocyanate-phenol-chloroform reagent (Thermo Fisher Science Inc.; Waltham, MA, USA) following the manufacturer's instruction. For RNA extraction, the Whatman® FTA® classic card was cut into small pieces (2 mm × 2 mm each), and all were placed into a 1.5 mL microcentrifuge tube. To determine the RNA integrity, samples preserved in different conditions were separated on 0.8% non-denaturing agarose gel. The total RNA concentration was determined using a micro-volume spectrophotometer (NanoDrop™2000; Thermo Fisher Scientific Inc.; Waltham, MA, USA). Subsequently, the concentration was adjusted to 200 ng/μL using nuclease-free water for cDNA synthesis. The cDNA was synthesized from the RNA, as mentioned above, using a reverse transcription kit (ReverTraAce®; Toyobo; Tokyo, Japan). The reaction was performed in a T100 PCR thermocycler (Bio-Rad Laboratories; Hercules, CA, USA) with the incubation at 42 °C for 60 min, followed by 98 °C for 5 min.

2.4. Reverse transcription quantitative polymerase chain reaction (RT-qPCR)

TiLV was quantified using SYBR® green-based RT-qPCR assay, according to Tattiyapong et al. (2018). Briefly, the 10 μL reaction was comprised of 4 μL of cDNA, 5 μL of 2× iTaq™ universal SYBR® green-based supermix (Bio-Rad Laboratories; Hercules, CA, USA), 0.3 μL each of 10 μM forward and reverse primers (accession no. KJ605629), and then the mixture was adjusted to a volume of 10 μL using DNase free water. All samples were run in triplicate. Later, the reaction was performed in a real-time PCR thermocycler (CFX96 Touch™; Bio-Rad Laboratories; Hercules, CA, USA) with 2-step cycle conditions including a cycle of 95 °C for 3 min, and 40 cycles of 95 °C for 10 s and 60 °C for 30 s. To determine the specificity of qPCR reactions, the products were further verified by melting curve analysis with the step of 65–95 °C with 5 °C per 5 s increment, and products were separated on low melting temperature agarose gel (NuSieve®, Lonza, Japan) and visualized under UV light. The amount of virus was then extrapolated from the Ct value of each sample by comparing it to the standard curve as previously described (Nicholson et al., 2018).

2.5. Statistical analysis

The viral load in each fish sample was tested for normality using Shapiro-Wilk test. For non-parametric data, data were compared using the Wilcoxon signed-rank test. Samples that follow a normal distribution were tested by the paired *t*-test. The calculated probability (*p*-value) lesser than 0.05 was considered significant. The R Stats Package was used to perform all statistical analyses.

3. Results and discussion

3.1. Effects of different preservation methods on TiLV loads

Liver of six fish with TiLV copy number ranging from 3.67×10^1 to 3.82×10^7 copies/μg of total RNA, preserved under five conditions: 4 °C, −20 °C, 70% ethanol, 95% ethanol, and RNA*later*®, showed a gradual decline in the amount of TiLV genomic RNA from 0 to 30 days (Table 1). Samples (fish no. 1, 2, and 4) stored at 4 °C presented a two-log decline of TiLV concentration at days 30. In contrast to 4 °C, −20 °C showed only one log reduction in those fish samples having a low viral load at day 3 (fish no. 4, 5, and 6) and two fish samples

having a high viral load (fish no.1 and 2) on day 30. This result indicated that temperature is an important parameter influencing viral reduction. Although a standard method for preserving TiLV-infected tissues has yet to be established, the OIE recommends a shipping time of 48–72 h (≤10 °C) for subsequent analysis of infectious hematopoietic necrosis virus (IHNV) and viral hemorrhagic septicemia virus (VHSV) (OIE, 2019).

Table 1. Effect of preservation methods and storage times on TiLV viral load. Livers were collected from TiLV-challenged red hybrid tilapia and stored at the following conditions: (1) 4 °C, (2) –20 °C, (3) 70% ethanol at room temperature, (4) 95% ethanol at room temperature, and (5) RNA*later*®. Samples were preserved for 0, 3, 7, 14, 30 days and analyzed using RT-qPCR assay.

Fish no.	**Day(s)**	**TiLV copies/µg of total RNA**				
		4 °C	**–20 °C**	**70% ethanol**	**95% ethanol**	**RNA*later*®**
1	0	3.82×10^7	3.82×10^7	3.82×10^7	3.82×10^7	3.82×10^7
	3	1.95×10^7	3.11×10^7	1.26×10^5	3.39×10^4	1.16×10^7
	7	1.36×10^7	2.58×10^7	4.14×10^5	1.06×10^6	3.95×10^7
	14	1.13×10^7	4.66×10^7	3.11×10^5	6.21×10^5	2.68×10^7
	30	3.34×10^5	1.23×10^6	1.85×10^5	6.38×10^4	1.30×10^7
2	0	1.31×10^6	1.31×10^6	1.31×10^6	1.31×10^6	1.31×10^6
	3	1.84×10^6	1.18×10^6	2.54×10^5	1.25×10^5	3.41×10^6
	7	5.77×10^5	2.65×10^6	1.20×10^5	2.46×10^5	3.46×10^6
	14	3.53×10^5	1.32×10^6	2.23×10^5	7.38×10^4	8.66×10^5
	30	4.28×10^4	2.11×10^5	4.22×10^4	4.79×10^5	1.42×10^6
3	0	1.94×10^6	1.94×10^6	1.94×10^6	1.94×10^6	1.94×10^6
	3	9.01×10^6	5.69×10^6	4.51×10^4	9.83×10^5	1.53×10^7
	7	1.60×10^6	1.49×10^6	3.01×10^5	9.07×10^4	7.19×10^6
	14	5.77×10^5	1.23×10^6	1.37×10^4	9.57×10^4	2.17×10^6
	30	1.70×10^6	6.59×10^6	2.20×10^5	4.60×10^5	1.04×10^7
4	0	9.77×10^2	9.77×10^2	9.77×10^2	9.77×10^2	9.77×10^2
	3	2.78×10^2	6.13×10^1	1.21×10^2	4.76×10^1	2.48×10^3
	7	1.64×10^1	6.01×10^1	ND	2.06×10^1	4.95×10^1
	14	1.93×10^1	3.90×10^1	1.86×10^1	9.45×10^1	3.37×10^2
	30	ND	6.82×10^2	ND	1.06×10^2	6.30×10^1
5	0	3.67×10^1	3.67×10^1	3.67×10^1	3.67×10^1	3.67×10^1
	3	7.95×10^1	ND	1.06×10^2	6.01×10^1	5.12×10^1
	7	1.50×10^1	1.36×10^1	1.11×10^1	5.89×10^1	5.23×10^1
	14	ND	5.77×10^1	2.79×10^2	7.48×10^2	2.58×10^1
	30	7.95×10^1	3.75×10^1	ND	1.88×10^1	4.22×10^1
6	0	6.50×10^2	6.50×10^2	6.50×10^2	6.50×10^2	6.50×10^2
	3	5.53×10^3	3.13×10^3	8.01×10^2	2.28×10^3	1.02×10^3
	7	3.34×10^3	8.01×10^2	6.58×10^2	1.24×10^3	1.24×10^3
	14	2.19×10^3	1.52×10^2	2.65×10^3	4.69×10^2	1.10×10^4
	30	3.62×10^2	3.61×10^2	3.28×10^3	2.42×10^2	5.06×10^2

ND = not detected.

No difference of TiLV viral load among treatments/storage times ($p > 0.05$).

In cases where a cold chain may not be achieved during transportation, common laboratory chemicals such as ethanol can be applied. Concentrated ethanol is the most frequently utilized medium for preserving specimens due to ease of use, ready availability, and low cost. Our study found that 70% ethanol caused a rapid 1 to 2 log reduction in high viral load samples (fish no. 1, 2, and 3) on day 3, whereafter the amount of viral RNA remained the same until day 30. A one to three log reduction was recorded in samples preserved in 95% ethanol at day 3, and most samples lost at least 1 to 3 log of viral genomic RNA by 30 days of preservation. However, 95% ethanol appeared to retain a higher TiLV load in subclinically infected tissues than 70% ethanol. Besides ethanol, samples preserved in RNA*later*®, a common reagent used for RNA storage were analyzed. In particular, our study revealed that the viral concentration in all samples kept in RNA*later*® at 30 days of storage was close to the beginning of the experiment (Table 1).

In all preservation methods, the virus experienced a certain degree of degradation, albeit no statistical difference. They appeared to offset the natural deterioration of TiLV in the starting material. This finding suggests that despite the cold chain being the best practice procedure, preservatives such as ethanol and RNA*later*® may be used in a resource-limited scenario. Additional experiments to determine the RNA concentration and integrity of samples preserved in different conditions were examined. As shown in Fig. 1A and B, RNA degradation was found in samples stored in 70% ethanol, 95% ethanol, and FTA card, while specific bands at 18S and 28S, suggesting of RNA intact, were still observed in samples kept at 4 °C, −20 °C, and RNA*later*® (Fig. 1A). Although the RNA degraded in some storage conditions with the reduction of RNA concentration, 260/280 ranged between 1.80 and 2.02 (Supplementary Table 1), it had little impact on the detection of the virus.

Table 2. TiLV viral load in liver and brain tissues preserved in RNA*later*® and − 20 °C for 365 days.

Preservation conditions	**Samples**	**TiLV copies/µg of total RNA**	
		Day 0	**Day 365**
RNA*later*®	Liver	7.90×10^6	7.03×10^6
	Liver	5.46×10^6	5.80×10^6
	Liver	5.82×10^6	6.34×10^6
	Brain	8.13×10^6	7.37×10^6
	Brain	6.51×10^6	5.79×10^6
–20 °C	Liver	7.53×10^6	4.42×10^6
	Liver	8.45×10^6	6.08×10^6

No difference of TiLV viral load among treatments ($p > 0.05$).

We further tested infected samples (brain and liver) under these conditions for a more extended storage period of 365 days. All liver samples kept in RNA*later*® showed comparable TiLV load at day 0 ($5.46–7.90 \times 10^6$ copies/µg RNA) vs. day 365 ($5.80–7.03 \times 10^6$ copies/µg RNA). Slight reductions were observed in brain samples at day 0 ($6.51–8.13 \times 10^6$ copies/µg RNA) vs. day 365 ($5.79–7.37 \times 10^6$ copies/µg RNA). Although the results suggested that RNA*later*® provides the most suitable preservation for TiLV, the reagent is expensive and may not be available in some locations where tilapia are cultured. Accordingly, we tested the effect of storage condition by keeping samples at −20 °C for up to 365 days. There was a minimal reduction of TiLV RNA in liver tissues preserved at −20 °C at day 0 ($7.53–8.45 \times 10^6$ copies/µg RNA) vs. day 365 ($4.42–6.08 \times 10^6$ copies/µg RNA)

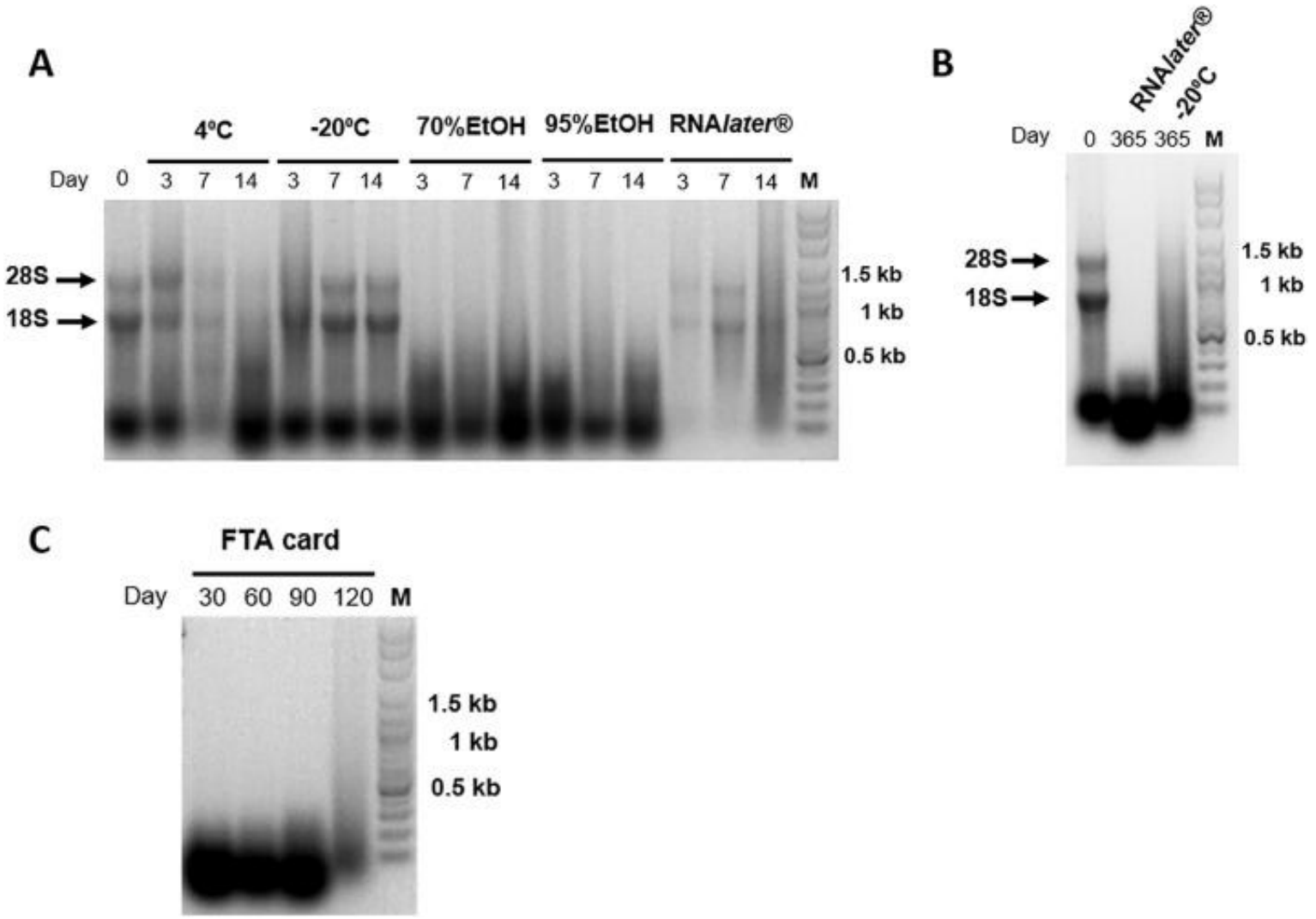

Fig. 1. RNA integrity under different storage conditions. RNA samples were separated on a 0.8% non-denaturing agarose gel (A) Liver tissues stored at 4 °C, −20 °C, 70% ethanol (EtOH), 95% EtOH, and RNA*later*® for 0, 3, 7 and 14 days. (B) Liver tissues preserved in Whatman® FTA® classic cards at room temperature for 30, 60, 90 and 120 day. M is 100 bp DNA marker. 18S and 28S are ribosomal RNA subunits.

(Table 2). The results revealed comparable amounts of viral genomic load could be detected at 30 and 365 days of storage. These results are in accordance with a previous report showing that TiLV could be detected after tissue samples had been archived at −20 ℃ (Dong et al., 2017; Nicholson et al., 2018; Rakus et al., 2020).

Table 3. TiLV viral load on Whatman® FTA® classic cards stored at room temperature for 30 days.

Fish no.	Preserved day(s)	TiLV copies/μg of total RNA on Whatman® FTA® classic cards[a]
1	0	2.03×10^7
	3	1.71×10^6
	7	6.21×10^5
	14	3.16×10^5
	30	6.46×10^4
2	0	4.90×10^6
	3	5.77×10^5
	7	6.40×10^5
	14	4.33×10^5
	30	1.32×10^5
3	0	1.58×10^7
	3	1.42×10^6
	7	1.22×10^6
	14	2.44×10^5
	30	2.32×10^5
4	0	4.63×10^6
	3	2.54×10^5
	7	3.03×10^5
	14	2.66×10^5
	30	1.63×10^5
5	0	9.38×10^6
	3	1.40×10^6
	7	6.64×10^5
	14	1.84×10^5
	30	7.53×10^4

[a]Difference of TiLV viral load of fish ($n = 5$) at 0 to 30 days ($p < 0.05$).

3.2. Preservation of samples on Whatman® FTA® classic card

Recently, a paper-based technique has become an alternative to reagents and temperature-controlled atmosphere for archiving nucleic acids. Whatman® FTA® classic cards provide a more convenient option for field-based sample collection using a pre-treated paper. Despite being extensively used for preserving pathogens of humans, animals, and plants, few studies have reported using FTA® cards in the diagnosis of viral diseases in aquaculture. To further explore the application of Whatman® FTA® classic cards for TiLV preservation, we placed liver tissues of five fish onto Whatman® FTA® classic cards. Within seven days, all liver

samples showed one log reduction, while at least 1 to 3 log reductions were found in samples preserved on Whatman® FTA® classic cards at 30 days ($p < 0.05$) (Table 3). As the transportation of samples from the remote areas could take more than 30 days, we also tested the stability of TiLV genomic RNA from livers of five TiLV-infected fish on Whatman® FTA® classic cards kept for 120 days at 0, 30, 60, 90, and 120 days. Similar to the previous experiment (Table 3), the amount of the viral RNA on Whatman® FTA® classic cards decreased within 30 days (1 to 3 log reduction; $p < 0.05$) (Fig. 2). The remaining viral RNA was retained at the same level from day 30 to day 120 days of storage in all fish (Fig. 2). It should be noted that FTA® cards may allow the preservation of infected tissues with very low TiLV load for no longer than 60 days due to the reduction to an undetectable level after 90 days. Despite a certain degree of loss of TiLV, in our study, the remaining genomic RNA was sufficient for subsequent TiLV detection. Limitation on storage duration on FTA® card was previously described in studies on betanodavirus at 4 °C (Kirti et al., 2019; Krishnan et al., 2016).

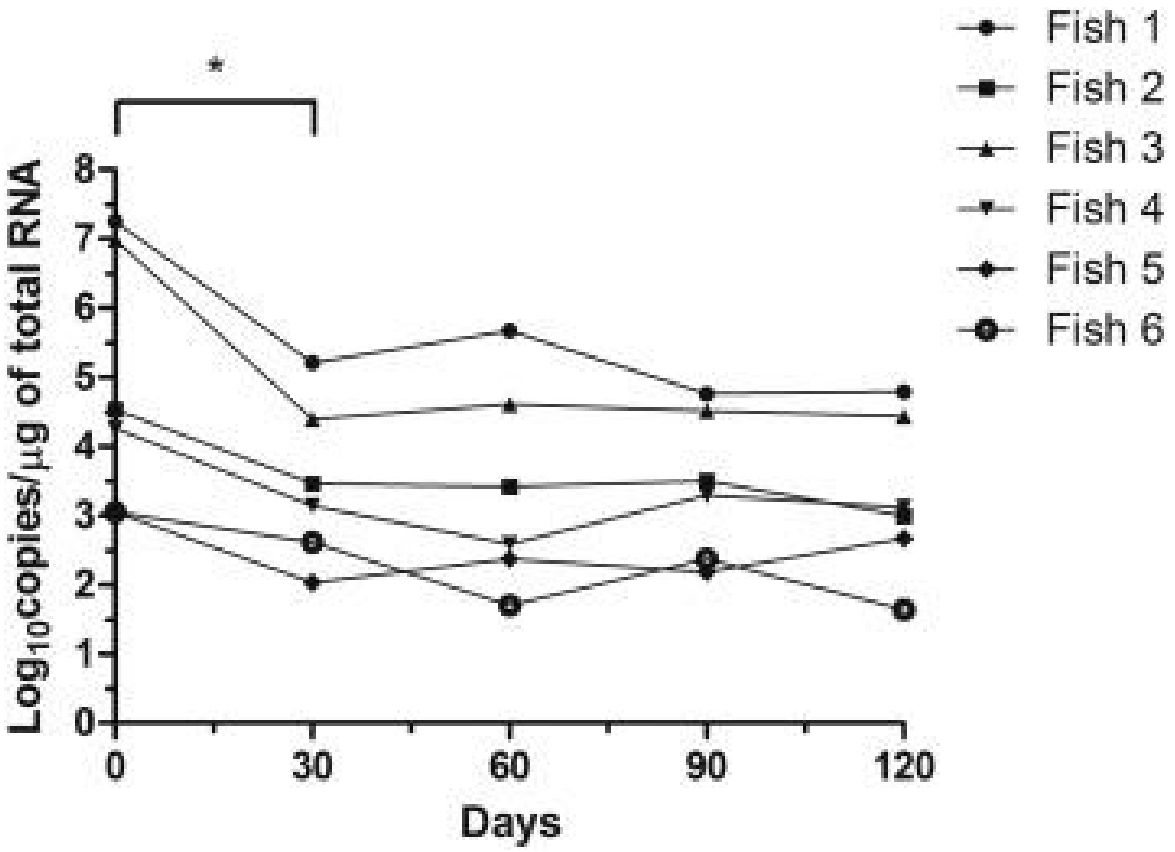

Fig. 2. TiLV viral load in liver ($n = 6$) stored on Whatman® FTA® classic cards at room temperature for 120 days. (*$p < 0.05$).

4. Conclusions

In summary, the results suggested that preservation methods and storage time contribute greatly to the reduction of TiLV genomic load. Based on the comparison, RNA*later*® and deep-freezing at −20 °C showed higher efficiency in maintaining TiLV genomic RNA than other techniques tested. Despite the superior preservation of the virus, the high cost of these methods may limit access for many users. Ethanol, therefore, could be recommended as an alternative due to its low cost and availability. Alternatively, Whatman® FTA® classic cards

may be of benefit in field applications for ease of use and transportation. Taken together, the data suggest that storage conditions and transportation can dramatically affect viral integrity. Therefore, the selection of appropriate preservation methods is an essential prerequisite for accurate TiLV diagnosis and disease management.

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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