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Experimental infection reveals transmission of tilapia lake virus (TiLV) from tilapia broodstock to their reproductive organs and fertilized eggs

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ABSTRACT

Early developmental stages of tilapia, including fertilized eggs were tested positive for TiLV in our previous study (Dong et al., 2017a). We, therefore, hypothesized that infected broodstock is able to pass the virus to their reproductive organs and then to the fertilized eggs. In order to prove this hypothesis, Nile tilapia (*Oreochromis niloticus*) broodstock were experimentally infected with TiLV by intramuscular injection and non-infected broodstock were used as control group. At day 6 post infection, eggs and semen from each breeding pair were aseptically collected for *in vitro* fertilization. Fertilized eggs at 3, 12 and 64 h post-fertilization were subjected to detection of TiLV by PCR, ISH, and cell culture. In parallel, blood, serum, liver and reproductive organs from each broodstock were subjected to TiLV analysis. The results revealed that all collected tissues (liver, blood, ovary and testis) from infected broodstock tested positive for TiLV by PCR, ISH, and cell culture. ISH revealed strong positive signals in hepatocytes surrounding blood vessels in the liver, connective tissue and membrane surrounding the oocytes in the ovary and the connective tissue close to blood vessels in the testis. These findings suggested that TiLV causes systemic infection in tilapia broodstock with the virus being able to spread into the reproductive organs, most likely through the blood circulatory system. Subsequently, the fertilized eggs produced by infected broodstock tested positive for TiLV by PCR and ISH revealed location of the virus inside the fertilized eggs. The results of this study suggested that TiLV can be transmitted vertically. We thus recommend for hatchery and multiplication center to use TiLV-tested negative broodstock for the production of TiLV-free tilapia seeds.

1. Introduction

Tilapia lake virus (TiLV) is a newly discovered virus of tilapines which was taxonomically updated as *Tilapia tilapinevirus* species, under *Tilapinevirus* genus, *Amnoonviridae* family (ICTV, 2018). The viral infections has been documented in either farmed or wild tilapines populations in 14 countries located in different parts of the world, i.e., Asia, Africa and South America (Jansen et al., 2018). Natural outbreaks of TiLV reportedly resulted in 20–90% mortality (Behera et al., 2018; Dong et al., 2017c; Eyngor et al., 2014; Ferguson et al., 2014; Jansen et al., 2018; Surachetpong et al., 2017). Experimental infection using cultivated virus caused 66–90% mortality in tilapia juveniles (Behera et al., 2018; Eyngor et al., 2014; Tattiyapong et al., 2017a). Current literature showed that TiLV seems to be most important in tilapia fry, fingerlings and juveniles although viral infections were also evidenced in sub-adult and adult fish (Jansen et al., 2018; Senapin et al., 2018). Additionally, subclinical infections with minor mortality impact were also reported (Senapin et al., 2018).

Horizontal transmission of the virus has been confirmed through cohabitation of infected fish with clinically healthy fish, which resulted in 80% mortality, confirming waterborne transmission (Eyngor et al., 2014). Experimental challenge of nine non-tilapia fish species with

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TiLV revealed that giant gourami is an additional susceptible species and cohabitation between tilapia and giant gourami caused cross-species transmission (Jaemwimol et al., 2018).

Our preliminary investigation indicated that there was a proportion of apparently healthy tilapia broodstock tested positive for TiLV (unpublished data). In tilapia hatcheries, detection of TiLV in fertilized eggs and very early developmental stages of tilapia, e.g., yolk-sac stage, fry and fingerlings suggest possible vertical transmission of this virus (Dong et al., 2017a). However, it is still unclear whether TiLV is able to transmit from infected broodstock to their reproductive organs and progeny. This study aims to investigate whether experimentally infected broodstock can pass the virus to their reproductive organs, eggs and sperm as well as *in vitro* fertilized eggs.

2. Materials and methods

2.1. Virus preparation

Tilapia lake virus strain NV18R from diseased red tilapia (*Oreochromis* sp.) were cultivated in E11 cell line. Viral isolation was performed as previously described (Eyngor et al., 2014). Purified viral stock was then confirmed by TiLV-specific PCR (Dong et al., 2017c). Viral titer of the stock was conducted by conventional assay for the 50% tissue culture infective dose ($TCID_{50}$) (Reed and Muench, 1938). Prior to challenge test, the original stock ($10^{7.5}$ $TCID_{50}$) preserved at −80 °C was thawed and diluted with saline solution (0.9% NaCl) to the designed injection dose of $10^{5.5}$ $TCID_{50}$ per fish.

2.2. Fish infection, in vitro fertilization, and sample collection

The use of experimental fish in this study was approved by Mahidol University Animal Ethics Committee (No. MUSC62-017-481). Five pairs of fully mature Nile tilapia broodstocks (300–400 g) were obtained from a commercial tilapia hatchery which has no history of TiLV infection. Male individuals showing reddish color of protruded papilla and females releasing eggs after wiping their bodies were selected for artificial breeding. In order to obtain TiLV-infected broodstock, three pairs (families 1–3) of broodstock were anesthetized with clove oil (10 ppm) and intramuscularly injected with 0.1 mL TiLV inoculum at the dose of $10^{5.5}$ $TCID_{50}$ per fish while two pairs (families 4 and 5) of control group were injected with 0.1 mL of 0.9% saline solution without virus. Each pair of broodstock was then cultured in a 50-L fiberglass tank containing air stone and cotton filter. Fifty percent water and cotton filter in each tank were replaced every three days. The fish were fed twice daily with commercial tilapia feed pellets containing 28% crude protein (CP) at the rate of 5% body weight. At day 6 post infection, there was still no sign of any abnormal symptoms. Each fish was then injected with (Suprefact®), a gonadotropin releasing hormone analog at the dose of 10 μg/kg in combination of domperidone (motilium®) at dose of 10 mg/kg, and left for 6 h to induce fish spawning. Subsequently, both male and female fish from each family were anesthetized with clove oil (10 ppm), their body surface disinfected with 70% ethanol before being blotted with a soft towel. The ovulated eggs and semen from each broodstock were collected by gently pressing the fish abdomen with a thumb from the pectoral fin to the genital papilla. The stripped eggs were kept in a sterile glass beaker, gently washed three times with autoclaved distilled water before mixing with the semen collected from the male in the same family. The remaining unfertilized eggs were also kept for further PCR and viral culture experiments (described below). After 15 min, liquid in the beaker was discarded and the eggs were washed twice with distilled water and incubated in Petri dish with sterile water. The fertilized eggs at 3, 12 and 64 h post fertilization were randomly collected and preserved in Trizol reagent (Invitrogen) for PCR, in 10% neutral buffered formalin (NBF) for histology, and raw at −80 °C for virus culture. For each diagnostic test, pools of 10 unfertilized or fertilized eggs at each time course were used. Blood withdrawal of 1 mL/fish was performed from caudal vein of each broodstock using a 3 mL-syringe with a 23G-needle and then divided into two parts of 0.1 mL for PCR and 0.9 mL for serum collection, taking care of removing the needle before pushing blood out of the syringe into tubes. Blood was collected at room temperature, allowed to clot at 4 °C for 3 h, then centrifuged at 1000 g for 5 min to collect serum. Collected sera was kept at −80 °C until analysis. Fish were then euthanized by overdose of anesthetic and dissected carefully for liver and reproductive organ collection. These tissues were preserved for PCR, histology, and re-isolation onto cell cultures in the same manner as mentioned above.

2.3. Semi-nested RT-PCR detection of TiLV

Semi-nested RT-PCR protocol for TiLV detection in this study employed primers targeting genomic segment 1 of the virus (Senapin et al. unpublished). The first RT-PCR reaction of 25 μL composed of 200 ng of RNA template, 0.4 μM of primers TiLV/nSeg1F; 5′- TCT GAT CTA TAG TGT CTG GGC C-3′ and TiLV/nSeg1R; 5′- AGT CAT GCT CGC TTA CAT GGT-3′, 0.5 μL of SuperScript III RT/Platinum Taq Mix (Invitrogen), and 1X of supplied buffer. Amplification profile consisted of a reverse transcription step at 50 °C for 30 min; a denaturation step at 94 °C for 2 min; 30 cycles at 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s; and a final extension step at 72 °C for 2 min. Positive control was a reaction containing RNA extracted from a whole TiLV-infected red tilapia fry as template while no template (water) used as negative control. 5 μL of the first round product was used as template in the second round PCR reaction of 25 μL containing 0.5 μM of primer TiLV/nSeg1F, 0.6 μM of primer TiLV/nSeg1RN; 5′- CCA CTT GTG ACT CTG AAA CAG -3′, and 1X AccuStar II PCR SuperMix (Quantabio). Thermocycling conditions were the same as PCR steps described above. Expected amplicons from the first and nested reactions were 620 and 274 bp, respectively.

2.4. Histology and in situ hybridization

24 h formalin preserved samples of liver, ovary, testis, and fertilized eggs (6 and 12 h post fertilization) from each family were immersed in 70% ethanol before being processed for routine histology. Each embedded paraffin wax sample was sectioned at 5 μm thickness and four consecutive sections from each sample were subjected to H&E staining, ISH without probe, with unrelated probe and with TiLV-specific probe, respectively. DIG-labeled probe preparation was performed as previously described (Dong et al., 2017c). Briefly, a 415 bp probe specific for a partial TiLV genomic segment 3 and a 282 bp unrelated probe derived from a shrimp virus namely infectious myonecrosis virus (IMNV) (Senapin et al., 2007) were synthesized using a PCR Digoxygenin (DIG)-labelling mix (Roche, Germany). *In situ* hybridization was conducted following established protocols (Dong et al., 2017b, 2017c). Sections were incubated with a solution containing nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) (Roche, Germany), then counterstained with 0.5% Bismarck brown Y. Results from sections stained with H&E, ISH with/without probe were interpreted in parallel under a light microscope. ISH positive signals showed dark purple to black staining against yellow brown counterstain. Microphotographs were taken using an Olympus BX51 digital microscope equipped with a digital camera.

2.5. Viral re-isolation using fish cell line

Viral isolation was performed using E11 cell line as originally described by Eyngor et al. (2014) and later by Tattiyapong et al. (2017a) with some minor modifications. Prior to cell cultivation of the virus, liver, ovary, testis, unfertilized eggs, and fertilized eggs (at 3, 12 and 64 h post fertilization) that were collected from each family and preserved at −80 °C were homogenized in four volumes of cell culture medium Leibovitz's L-15 (Gibco). The suspension was centrifuged at

9000 × *g* for 5 min and the respective supernatant was filtered through 0.22 μm filter. The filtrates were then used for inoculation of E11 cells. In case of serum, samples were directly filtered as mentioned above before inoculation of cell cultures. The E11 cell lines were cultured in 24 well-plates using Leibovitz's L-15 medium supplemented with 5% fetal bovine serum (FBS) (Sigma) and incubated at 26 °C. Infection was performed when the cells reach ~90% confluence (monolayer). Each well was infected with 200 μL of the tissue filtrate or 30 μL of serum filtrate and incubated for 1 h before medium was removed and replaced with fresh L-15 (5% FBS) for further incubation at 26 °C. Cytopathic effect (CPE) was confirmed daily by observation under the microscope and compared between cells that received inoculum prepared from infected- and non-infected fish specimens. After 7 days post inoculation, 200 μL of the supernatant obtained from each well was passaged to a new E11 cell plate and incubated under the same conditions for 10 more days. CPEs observed from this passage were considered as positive.

3. Results

3.1. Investigation of TiLV infection in broodstock and in vitro fertilized eggs by PCR

In this study, tissues from 3 families of the experimental TiLV-infected broodstock as well as their *in vitro* fertilized eggs were subjected to TiLV detection by PCR. Liver, blood, ovary, and unfertilized eggs were collected from female broodstock and liver, blood, and testis from male fish were used. Artificially fertilized eggs were collected at 3, 12 and 64 h post fertilization. Corresponding specimens from the 2 control families were processed in the same manner. The results summarized in Table 1 demonstrated a systemic infection with TiLV in the experimentally challenged fish. All tested samples from the challenged families including the liver, blood, reproductive organs of both male and female broodstock, and the unfertilized eggs were tested positive for TiLV by PCR. The fertilized eggs at 3, 12 and 64 h post fertilization collected from three infected families (1–3) were also positive, except for samples collected at 3 and 64 h from family 1. Samples from two control families (4–5) were all negative for TiLV (Table 1). Representative PCR testing results from an infected and non-infected control family are shown in Fig. 1.

3.2. Localization of TiLV in fish tissues revealed by in situ hybridization

In situ hybridization (ISH) was employed to further investigate the presence of TiLV in the liver, reproductive organs and artificially fertilized eggs of experimentally infected fish with TiLV and control non-infected families. The results are summarized for each specimen as follows:

Liver: Using the TiLV-specific probe, liver samples collected from 6 infected broodstock (3 males and 3 females) showed strong reactive ISH signals (Fig. 2, Table 1). Representative microphotographs of ISH results from an infected fish are shown in Fig. 2. When compared to H&E stained sections, the strong ISH positive signals were localized in areas surrounding blood vessels in the liver (arrows, Fig. 2C). At higher magnification, positive signals seem to be found in both nucleus and cytoplasm of infected hepatocytes (Fig. 2F). These hepatocytes also showed signs of degeneration with accumulation of lipofuscin (brown color) in their cytoplasm (Fig. 2D) while sign of syncytial hepatitis was not clearly observed. No positive signal was detected from the sections of infected fish without probe (data not shown) or with unrelated probe (IMNV-specific probe) (Fig. 2B, E) or from the sections prepared from control fish incubated with TiLV, without probe and with IMNV unrelated probes (Table 1; Fig. S1).

Ovary: By H&E staining, different developmental stages of oocytes were observed from the female ovary samples (Fig. 3, Table 1). Infiltration of inflammatory lymphocytes, macrophages containing melanin were notably observed in the connective tissue of infected ovaries (Fig. 3A, D). Representative ISH results of fish 2 and 3 are shown in Fig. 3. In agreement with the PCR results, ovaries from all three infected females (1–3) showed strong TiLV positive signals in the connective tissue surrounding different developmental stages of the oocytes (Fig. 3C, F). Degenerated oocytes also called atretic oocytes appeared to have stronger reactivity located on their membrane and inside the oocytes (Fig. 3F). Some areas of the oocyte membrane were also found to be TiLV positive (arrows, Fig. 3C, F). No signal was detected in those respective tissue positions when assayed with unrelated probe (Fig. 3B, E) or without probe (data not shown). Non-infected control fish sections gave no reactivity to either TiLV or IMNV probes (Table 1; Fig. S1).

Testis: Similar to the ovary, ISH using TiLV-specific probe gave positive signals in all testes samples collected from the infected male broodstock (Fig. 4, Table 1). No signal was found in the same samples assayed with unrelated probe (Fig. 4B), no probe (data not shown), as well as non-infected control sections (Table 1; Fig. S1). Location of positive signals appeared mainly in the connective tissue and some cells resembling lymphocytes, especially in tissue areas surrounded by blood vessel (arrows, Fig. 4C, E and G). No positive signal was detected at the areas of spermatocytes, spermatids or spermatozoa.

Fertilized eggs: 10 artificially fertilized eggs from each experimental fish family were investigated by H&E staining and ISH assays in the same manner as fish tissues. Among the three TiLV-infected families, only families 2 and 3 but not family 1 gave TiLV positive results (Fig. 5, Table 1). There were 40–50% of the fertilized eggs exhibited

Table 1
Summary of TiLV detection results from broodstock specimens and fertilized eggs.

Samples		TiLV-infected families									Control families		
		Family 1			Family 2			Family 3			Family 4 & 5		
		PCR	ISH	CPE	PCR	ISH	CPE	PCR	ISH	CPE	PCR	ISH	CPE
F	Liver	+	+	+	+	+	+	+	+	+	–	–	–
	Blood	+	ND	+	+	ND	+	+	ND	+	–	ND	–
	Ovary	+	+	+	+	+	+	+	+	+	–	–	–
	Eggs	+	ND	+	+	ND	+	+	ND	+	–	ND	–
M	Liver	+	+	+	+	+	+	+	+	+	–	–	–
	Blood	+	ND	+	+	ND	+	+	ND	+	–	ND	–
	Testis	+	+	+	+	+	+	+	+	+	–	–	–
FE	3 h	–	–	–	+	+	–	+	+	ND	–	–	–
	12 h	+	–	–	+	+	–	+	+	ND	–	–	–
	64 h	–	ND	–	+	ND	–	+	ND	ND	–	ND	–

F, female broodstock; M, male broodstock; FE, fertilized eggs; Eggs, unfertilized eggs; PCR, polymerase chain reaction; ISH, *in situ* hybridization, CPE, cytopathic effect; +, positive; -, negative; ND, not determined.

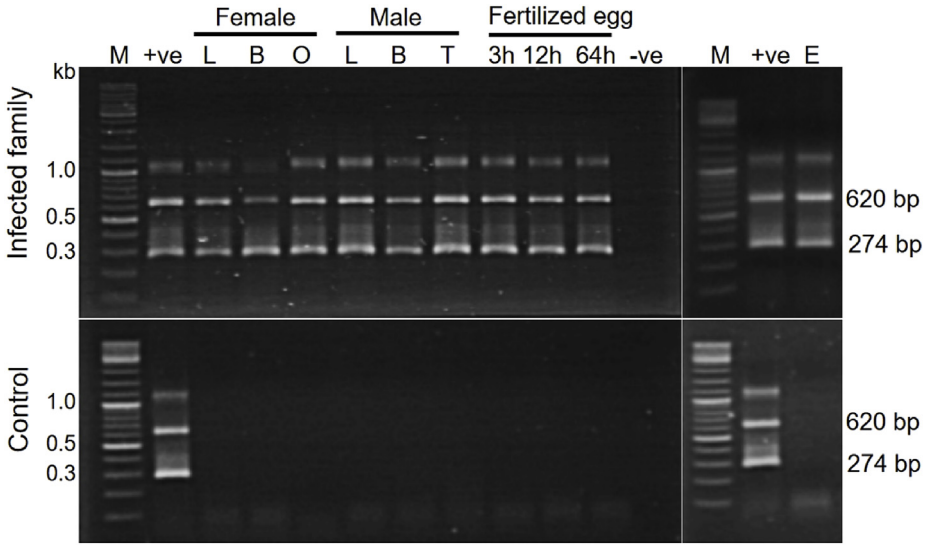

Fig. 1. Representative PCR testing results of samples obtained from infected and control (non-infected) families. M, DNA marker (New England Biolabs); +ve, positive control; -ve, negative control; L, liver; B, blood; O, ovary; T, testis; E, unfertilized eggs.

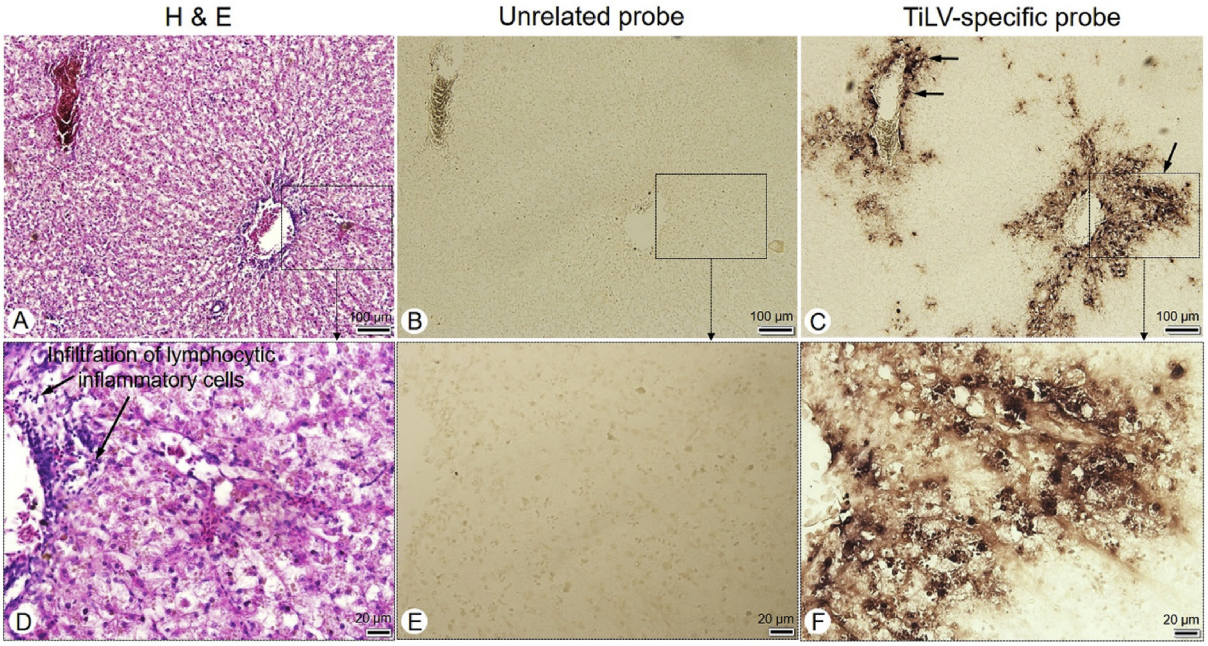

Fig. 2. Representative microphotographs of liver sections of a TiLV-infected tilapia female broodstock taken at low (A–C) and high (D–F) magnifications. H&E stained section (A and D), ISH with unrelated probe (B and E) and ISH with TiLV-specific probe (C and F). Dark brown color (arrows in C) indicated strong positive signals in the area around blood vessel. Arrows in D indicate infiltration of lymphocytic inflammatory cells. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

positive reactivity that was observed mainly within the eggs. No signals were observed in sections from same samples assayed with IMNV unrelated probe (Fig. 5) or without probe (data not shown) as well as egg sections from non-infected control families (Table 1; Fig. S1).

3.3. Cell culture revealed viability of the virus

The viability and infectivity status of TiLV strain NV18R used for the experimental challenge and collected from fish specimens were evaluated by cell culture using E11 cell lines for two consecutive passages. Filtrates were prepared from serum, liver, ovary, testis, fertilized and unfertilized eggs collected from both experimentally TiLV-infected families and control families. Typical cytopathic effects (CPE) of plaque formation and cell shrinkage previously described for TiLV infection in the E11 cells (Eyngor et al., 2014) was clearly seen at 2–5 days post inoculation with preparations from serum, liver, ovary, testis and unfertilized eggs from the infected fish (Table 1). A representative micrograph shows CPEs in E11 cells inoculated with the supernatant prepared from ovary of a female broodstock IM infected with TiLV are shown in Fig. 6B. No CPE were observed when using inoculum from non-infected control female ovary (Fig. 6A). With respect to the artificially fertilized eggs, filtrates were prepared from only families 1 and 2. Interestingly, the viral inoculum derived from these fertilized eggs gave no CPE (Table 1). Preparation from the control fish specimens showed no detectable changes in E11 cells (Table 1).

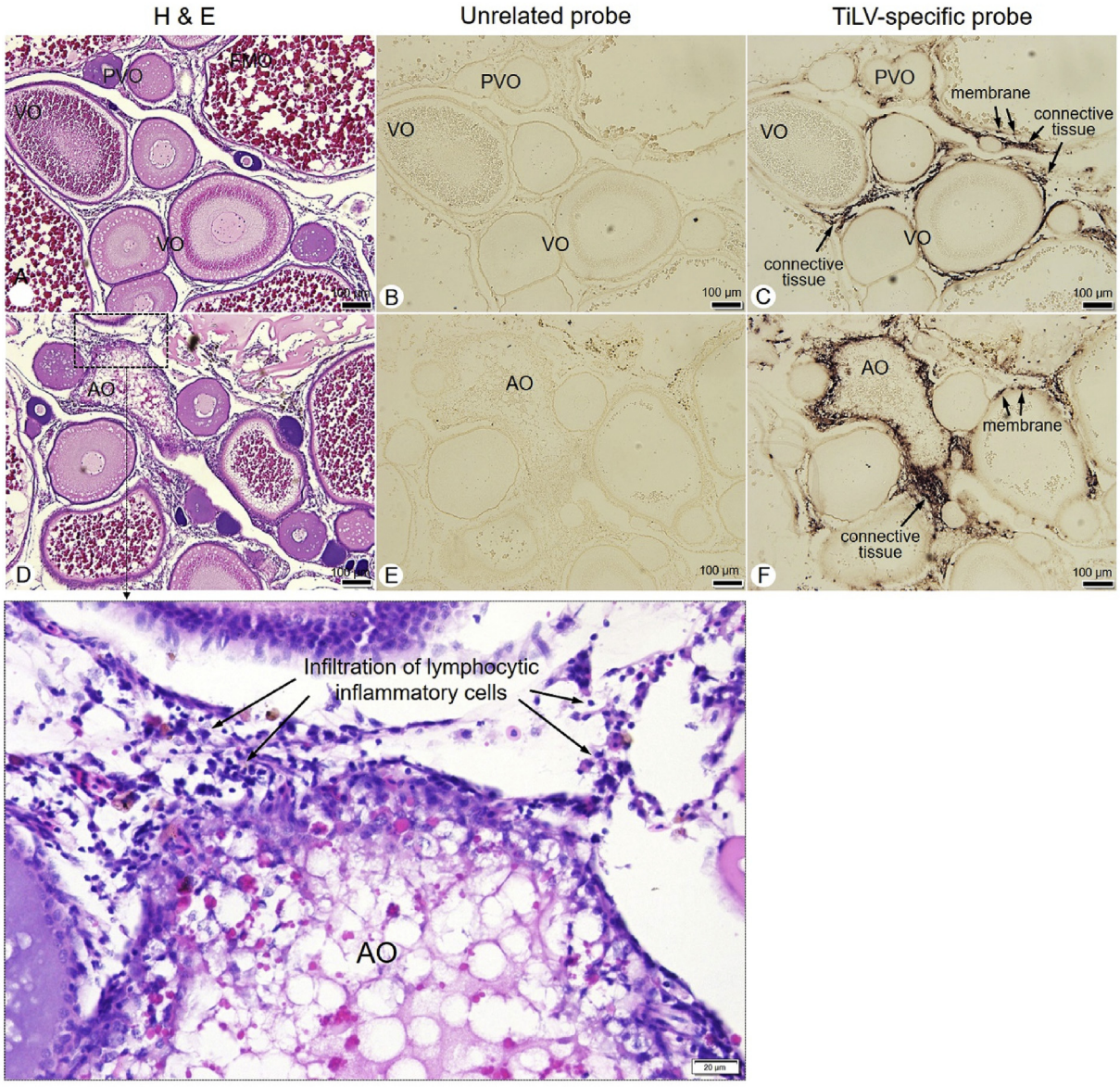

Fig. 3. Representative microphotographs of ovary sections of a TiLV-infected female broodstock. H&E stained sections (A and D), ISH sections with unrelated probe (B and E) and ISH with TiLV-specific probe (C and F). Strong TiLV positive ISH signals were found mainly in the connective tissue surrounding all developmental stages of oocytes in the ovary (C and F) and the atretic (degenerated) oocytes (F). Some positive signals were detected on the membrane of the oocytes (C and F). PVO, pre-vitellogenic oocytes; VO, vitellogenic oocytes; AO, atretic oocytes. Higher magnification of H&E stained ovary section (dotted box) reveals infiltration of lymphocytic inflammatory cells.

4. Discussion

Understanding disease pathogenesis and transmission route of TiLV are important scientific bases for further studies on effective prevention approaches. In this experiment, despite the fact that the virus was injected intramuscularly into broodstock, the virus was later detected in the blood, liver and reproductive organs, which was confirmed by PCR, ISH and cell culture. Large areas surrounding blood vessels of the tissues assayed (liver and testis) strongly reacted with TiLV-specific probe by ISH. To our best knowledge, this tropism has not yet been reported for TiLV infection. All these findings suggest that TiLV is systemic in nature and spreads to other organs most probably through the circulatory system. Tissue areas close to blood vessels are thus most likely the initial targets of infection and viral spread to neighboring target cells. If this was the case, TiLV would be detected from all fish organs where blood circulates to. This is consistent with previous observations that various tissues (e.g. liver, spleen, kidney, brain, gills, heart and connective tissues of muscle) from infected fish harbor the virus (Bacharach et al., 2016; Dong et al., 2017c; Tattiyapong et al., 2017b). In this study, an under lethal dose that allows broodstock to survive from infection and produce no clinical signs was used. This might explain the absence of typical syncytial hepatitis pathology in the infected broodstock that were apparently healthy at day 6 post infection.

To the best of our knowledge, this is the first laboratory controlled study to prove that TiLV-experimentally infected broodstock can pass the virus to their reproductive organs. It was observed that based on the comparative frequencies of strong, widespread of ISH reactive signals in the reproductive organs, viral infection in the ovary seems to be more severe than in the testis. Additionally, many infected areas in the liver, ovary and testis were areas with infiltration of cells resembling lymphocytes. It was suspected that lymphocytes might become infected during the course of the immune response to TiLV infection in these organs. Previously, viral infection in lymphocytes has been described for various viruses including influenza virus, a member of

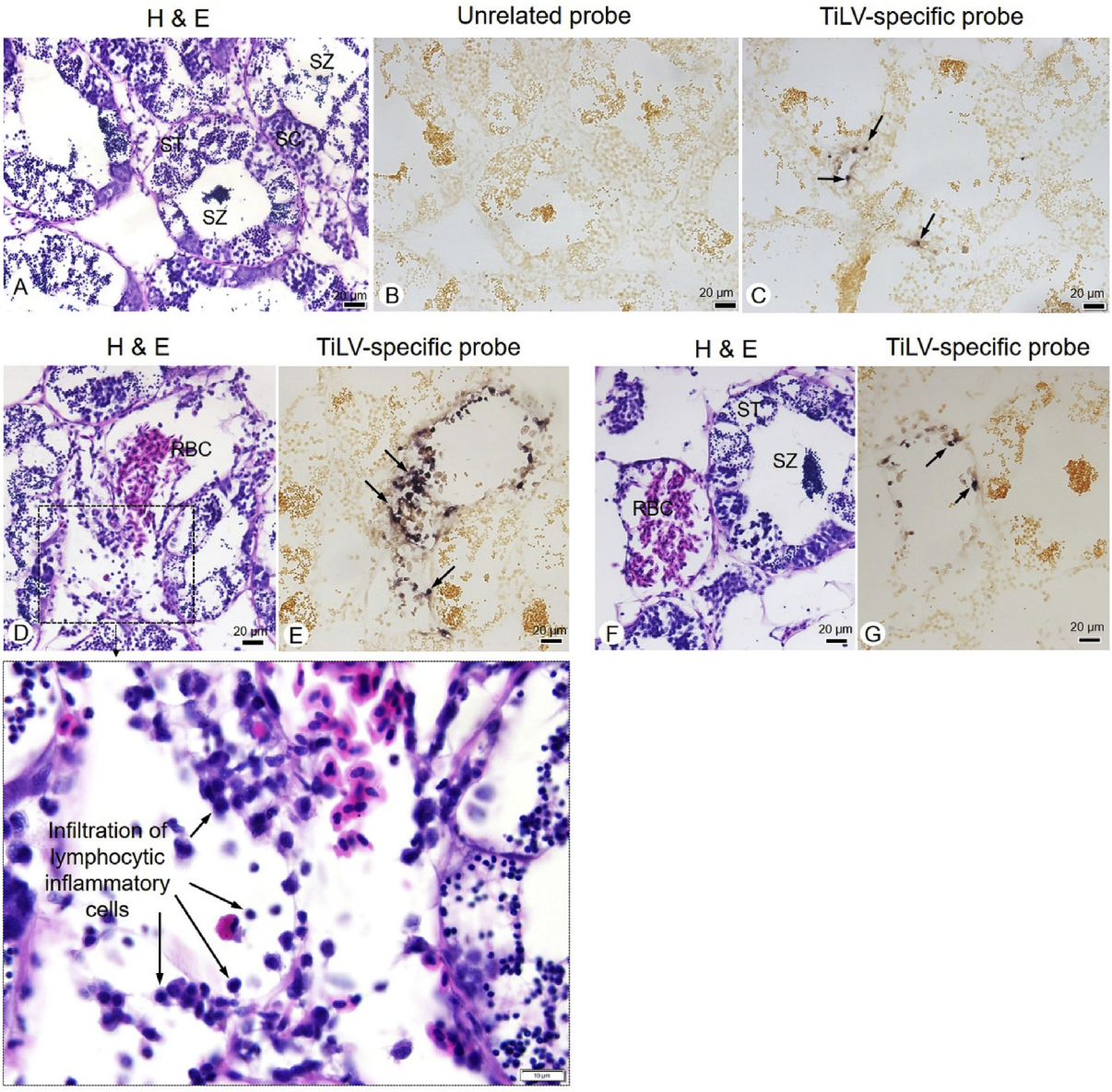

Fig. 4. Representative microphotographs of testis sections of TiLV-infected male broodstock subjected to H&E staining and ISH assay using unrelated probe or TiLV-specific probe. TiLV positive signals (arrows) were detected in the connective tissue (C), especially connective tissue near blood vessels (E, G). SC, spermatocytes; ST, spermatids; SZ, spermatozoa; RBC, red blood cells. Higher magnification of H&E stained testis section (dotted box) reveals infiltration of lymphocytic inflammatory cells. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Orthomyxoviridae (Mock et al., 2018). Observation of ISH reactive signals being detected in both nucleus and cytoplasm of hepatocytes (see Fig. 2) suggested that the replication cycle of TiLV involved a transcription step in the nucleus similar to that of influenza virus (Dou et al., 2018; Mikulásová et al., 2000).

With respect to ISH results from the oocytes, TiLV was found mainly in the connective tissue surrounding all stages of oocytes and the oocytes membrane but not inside the oocytes. Interestingly, the ISH signals were more clearly seen in both membrane and inside the cells of the atretic (degenerated) oocytes. It is not known whether viral infection resulted in oocyte degeneration or degrading oocytes are preferred sites for TiLV. The stripped unfertilized oocytes were consistently tested positive for TiLV by different methods. Although the fish testis tissues were tested positive for TiLV by PCR, no TiLV ISH reactive signals found in spermatozoa from male reproductive organ. This might imply that TiLV infection in fertilized eggs likely came from infected unfertilized eggs. Unexpectedly, the fertilized eggs that were tested positive for TiLV by both PCR and ISH yielded no formation of CPE by the cell culture method. The underlying reason for this is still unknown. It is possible that the viral preparation process for the fertilized eggs was not successful probably due to the presence of the sticky vitellogenin (yolk). Alternatively, the virus might be inactivated by various factors such as the increased enzyme activity post-fertilization, maternal immunoglobulin transferred from female broodstock or antiviral activity of vitellogenin derived proteins (Garcia et al., 2010; Wang et al., 2012; Zhang et al., 2013, 2015). Another limitation of this study was that, in our laboratory conditions, we were not able to obtain further developmental stages of the fish beyond fertilized eggs. Therefore, despite the fact that in our experimental infection challenge, TiLV was able to transmit vertically to fertilized eggs, it remains unknown whether those infected fertilized eggs would be able to develop to fry and fingerlings and whether the virus would still be infectious through their development stages. These questions are worthy of further investigation in future research.

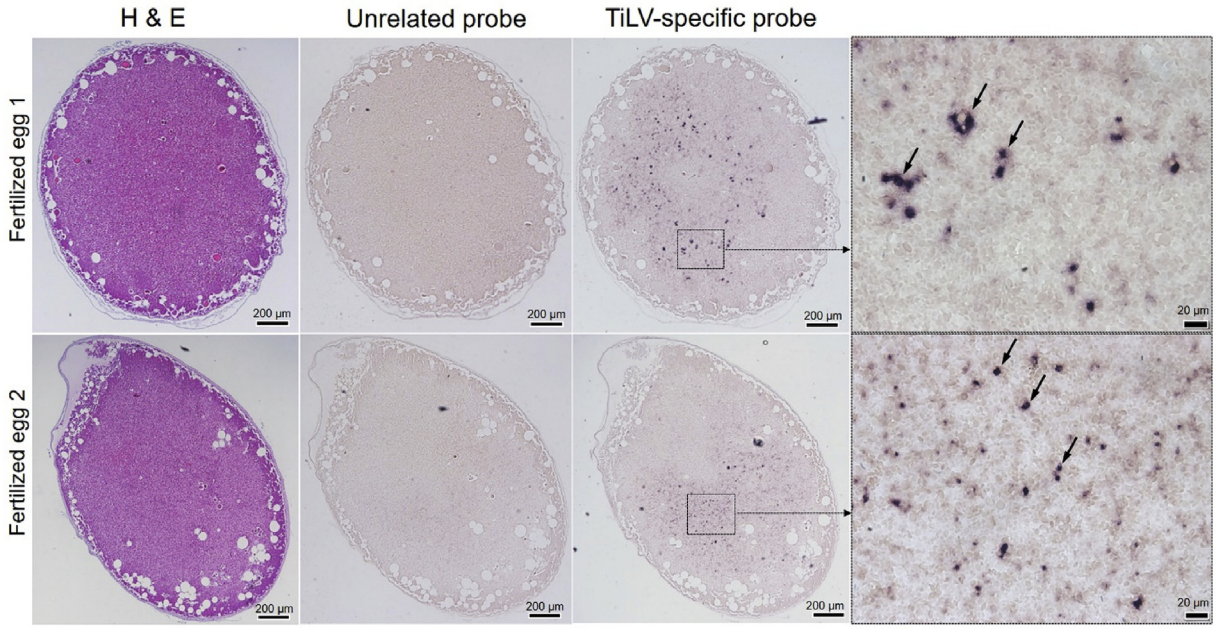

Fig. 5. Representative microphotographs of sections of 3 h-post fertilized eggs obtained from two separate TiLV-infected families. Three consecutive sections from each egg specimen were subjected to H&E staining, ISH with unrelated probe and ISH with TiLV-specific probe, respectively. TiLV positive signals were detected inside the fertilized eggs. High magnification of the area marked by dotted boxes reveal multifocal ISH positive signals (arrows).

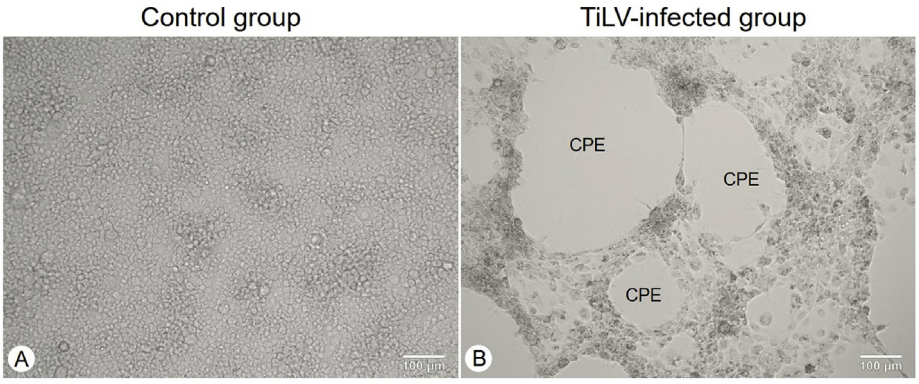

Fig. 6. Representative microphotographs of the E11 cell line at 5 days-post inoculation with the extracted supernatant prepared from ovary of the non-infected (A) and TiLV infected broodstock female (B). CPEs, cytopathic effects.

In summary, this study suggests that TiLV caused systemic infection in tilapia broodstock and that the virus is capable to circulate into the reproductive organs of both male and female via the blood circulatory system. The infected female fish produced infected eggs, when fertilized with sperms from infected male broodstock resulted in infected fertilized eggs. Therefore, to produce TiLV-free seeds, we highly recommend using TiLV-free broodstock for seed production.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aquaculture.2019.734541.

Disclaimer

The opinions expressed here belong to the authors, and do not necessarily reflect those of FISH, WorldFish or CGIAR.

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