A warning and an improved PCR detection method for tilapia lake virus (TiLV) disease in Thai tilapia farms

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Background

Tilapia lake virus (TiLV) is a novel RNA virus resembling Orthomyxovirus. It has been reported as a newly emerging virus that causes syncytial hepatitis of tilapia (SHT) in Israel, Ecuador, Colombia, and Egypt (Ferguson *et al*. 2014; Eyngor *et al*. 2014; Bacharach *et al*. 2016; Del-Pozo *et al*. 2017, Fathi *et al.* 2017). The disease caused massive mortality up to 90% in farmed tilapia and is considered to be a potential threat to global tilapia farming (Eyngor *et al*. 2014). In Thai tilapia farms, disease outbreaks have recently occurred associated with high cumulative mortalities (20-90%), and we have found that the diseased fish show typical histopathological features of SHT. Infection has been confirmed by transmission electron microscopy (TEM), *in situ* hybridization and high nucleotide sequence identity to TiLV from Israel (Dong *et al*. 2017). Our research progress was delayed by use of nested RT-PCR primer sequences previously published by Eyngor et al. (2014) together with detailed RT-PCR protocols later released by Tsofack *et al*. (2016). Our preliminary detection assays performed using the earlier report (Eyngor et al. 2014) with assumed PCR conditions (not specified in the publication) resulted in amplification of nonspecific fish genes. This probably resulted from the fact that 13-18 out of 20 nucleotides for the primer "Nested ext-2" matched fish genes. We, therefore, modified the nested RT-PCR protocols into a semi-nested RT-PCR by omitting the primer "Nested ext-2" to avoid false positive results.

The semi-nested RT-PCR protocol given below may be used freely for non-commercial applications to detect TiLV. Heavily-infected samples will generate 2 amplicon bands of 415 bp and 250 bp while lightly-infected samples will generate a single 250-bp amplicon band (Fig. 1). Since this is the first report of TiLV in Asia, the authors urge fish health laboratories in Asia to test for TiLV when abnormal mortality of tilapia occurs. Please contact Centex Shrimp (saengchan@biotec.or.th) to obtain a free positive control plasmid (pGEM-415_bp).

Detailed PCR protocol

- **Primer sequences**

Primer name	Sequence (5' to 3')	Amplicon size (bp)	Reference
First RT-PCR			
Nested ext-1	TATGCAGTACTTTCCCTGCC	415	Eyngor et al. 2014, Tsofack et al. 2016
ME1	GTTGGGCACAAGGCATCCTA		
Semi-nested PCR			
7450/150R/ME2	TATCACGTGCGTACTCGTTCAGT	250	Eyngor et al. 2014, Tsofack et al. 2016
ME1	GTTGGGCACAAGGCATCCTA		

- **Reaction conditions**

Step	Reagents	Volume (µl)	Thermocycler parameters
First RT-PCR	100-400 ng of total RNA	1.5	Reverse transcription 50°C, 30 min Denaturation 94°C, 2 min 25 cycles of ▪ Denaturation 94°C, 30 s ▪ Annealing 60°C, 30 s ▪ Extension 72°C, 30 s Final 72°C, 5 min
	2X buffer	12.5	
	10 µM primer Nested ext-1	1	
	10 µM primer ME1	1	
	SuperScript One-Step RT/Platinum *Taq* mix (Invitrogen)	0.5	
	Sterile water	8.5	
	Total reaction	25	
Semi-nested PCR	The first PCR product	1	Denaturation 94°C, 2 min 25 cycles of ▪ Denaturation 94°C, 30 s ▪ Annealing 60°C, 30 s ▪ Extension 72°C, 30 s Final 72°C, 5 min
	10X buffer	2	
	10 mM dNTP	0.4	
	10 µM primer 7450/150R/ME2	0.5	
	10 µM primer ME1	0.5	
	Taq DNA polymerase (5 units/µl)	0.2	
	Sterile water	15.4	
	Total reaction	20	

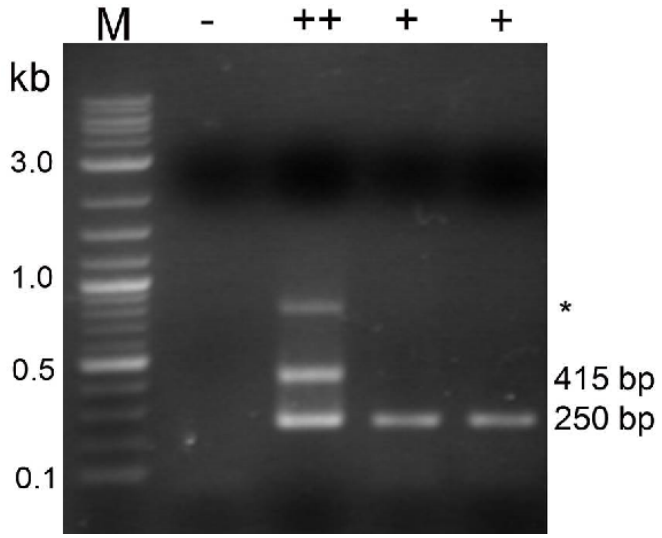

Fig. 1 A sample agarose gel of TiLV detection from fish RNA extracts. Expected band sizes of 415 bp and 250 bp represent amplicons from the first and semi-nested PCR, respectively, with lanes marked ++ for a heavy infection, + for a light infection and - for the negative control. The band marked with an asterisk (*) on the right side of the gel probably arose from cross hybridization of the amplified products. M = DNA marker (2-Log DNA Ladder, New England Biolabs).

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References

1. Bacharach, E., Mishra, N., Briese, T., Zody, M.C., Kembou Tsofack, J.E., Zamostiano, R., Berkowitz, A., Ng, J., Nitido, A., Corvelo, A., Toussaint, N.C., Abel Nielsen, S.C., Hornig, M., Del Pozo, J., Bloom, T., Ferguson, H., Eldar, A., Lipkin, W.I., 2016. Characterization of a novel Orthomyxo-like virus causing mass die-offs of tilapia. MBio. 7, e00431-00416.
2. Del-Pozo, J., Mishra, N., Kabuusu, R., Cheetham, S., Eldar, A., Bacharach, E., Lipkin, W.I., Ferguson, H.W., 2017. Syncytial hepatitis of tilapia (*Oreochromis niloticus* L.) is associated with Orthomyxovirus-like virions in hepatocytes. Vet. Pathol. 54, 164-170.
3. Dong HT, S Siriroob, W Meemetta, W Santimanawong, W Gangnonngiw, N Pirarat, P Khunrae, T Rattanarojpong, R Vanichviriyakit, S Senapin (2017). Emergence of tilapia lake virus in Thailand and an alternative semi-nested RT-PCR for detection. Aquaculture [Under revision].
4. Eyngor, M., Zamostiano, R., Kembou Tsofack, J.E., Berkowitz, A., Bercovier, H., Tinman, S., Lev, M., Hurvitz, A., Galeotti, M., Bacharach, E., Eldar, A., 2014. Identification of a novel RNA virus lethal to tilapia. J. Clin. Microbiol. 52, 4137-4146.
5. Fathi, M., Dickson, C., Dickson, M., Leschen, W., Baily, J., Muir, F., Ulrich, K., Weidmann, M., 2017. Identification of Tilapia Lake Virus in Egypt in Nile tilapia affected by 'summer mortality' syndrome. Aquaculture. 473, 430–432.
6. Ferguson, H.W., Kabuusu, R., Beltran, S., Reyes, E., Lince, J.A., del Pozo, J., 2014. Syncytial hepatitis of farmed tilapia, *Oreochromis niloticus* (L.): a case report. J. Fish Dis. 37, 583-589.
7. Tsofack, J.E.K., Zamostiano, R., Watted, S., Berkowitz, A., Rosenbluth, E., Mishra, N., Briese, T., Lipkin, W.I., Kabuusu, R.M., Ferguson, H., Del Pozo, J., Eldar, A., Bacharach, E., 2016. Detection of tilapia lake virus (TiLV) in clinical samples by culturing and nested RT-PCR. J. Clin. Microbiol. doi:10.1128/JCM.01808-16.