

Outbreaks of Tilapia Lake Virus Infection, Thailand, 2015–2016

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During 2015–2016, several outbreaks of tilapia lake virus infection occurred among tilapia in Thailand. Phylogenetic analysis showed that the virus from Thailand grouped with a tilapia virus (family Orthomyxoviridae) from Israel. This emerging virus is a threat to tilapia aquaculture in Asia and worldwide.

Viral diseases are common causes of illness and death in cultured fish; such viruses include infectious salmon anemia virus, infectious hematopoietic necrosis virus, and viral hemorrhagic septicemia virus (1). With regard to tilapia, some viral pathogens, including betanodavirus, iridovirus, and herpes-like virus (2,3), reportedly cause severe disease. In recent years, Thailand has experienced extensive losses of tilapia; most losses occurred 1 month after transfer of fish from hatchery to grow-out cages in public rivers or reservoirs (1-month mortality syndrome). During routine investigation of this syndrome, multiple bacterial and parasitic infections were identified. However, no association was established between the outbreaks and any primary causative agent(s). Most deaths occurred within 2 weeks after the first dead fish were found. Similar observations of extensive losses of raised tilapia and wild fish in Israel and Ecuador have been reported (4,5). These outbreaks led to identification of a virus affecting tilapia, called tilapia lake virus (TiLV). The epidemiologic pattern and clinical signs for infected fish in Thailand led to suspicion of an illness of unknown etiology that was similar to TiLV infection.

During 2015–2016, we investigated 32 outbreaks involving a large number of deaths of unknown cause among Nile tilapia (*Oreochromis niloticus*) and red hybrid tilapia (*Oreochromis* spp.). The outbreaks occurred at fish farms in central, western, eastern, and

northeastern Thailand (online Technical Appendix Figure 1, <https://wwwnc.cdc.gov/EID/article/23/6/16-1278-Techapp1.pdf>). Affected fish were commonly found within 1 month after transfer from the hatchery facility to grow-out ponds or cages. In general, clinical signs and high mortality rates were associated with fish weighing 1–50 g (online Technical Appendix Figure 2). Mortality rates among tilapia farms were 20%–90%; higher rates were associated with secondary bacterial and parasitic infections. Mortality rates peaked within 14 days after the first dead fish were found.

As part of the outbreak investigation, samples of brain tissue were taken from fish at each of the 32 outbreak locations (each with a mortality rate >1%/day for 3 consecutive days): 10–30 moribund fish and 5–10 apparently healthy fish from the same culture areas. In total, 325 samples were collected and tested for etiologic agent(s) (4,6). Samples from fish involved in 22 of the 32 outbreaks were positive for TiLV (online Technical Appendix Table 1).

For our study, we selected a field sample positive for TiLV (designated TiLV/Tilapia/Thai/TV1/2016) and processed it for whole-genome sequencing. Another 6 TiLVs were selected for sequencing of the putative polymerase basic 1 (PB1) gene (online Technical Appendix Table 2). TiLV genome sequencing was conducted by using newly designed primers based on reference TiLVs available in the GenBank database (7). Nucleotide sequences of 7 TiLVs from Thailand were submitted to GenBank (accession nos. KX631921–36).

Comparison of the TiLVs from Thailand with those from Israel showed high nucleotide and amino acid identities (95.18%–99.10%). Among TiLVs from Thailand, nucleotide and amino acid identities for segment 1 or the putative PB1 gene of the virus were high (99.61%–100%) (online Technical Appendix Table 3). Genetic analysis of the putative PB1 protein of TiLVs from Thailand and the viruses of the family *Orthomyxoviridae* showed that TiLVs from Thailand possessed motifs preA, A, B, C, D, and E similar to those of *Orthomyxoviridae* viruses, including influenza A, B, and C viruses; infectious salmon anemia virus; Dhori virus; and Thogoto virus (online Technical Appendix Table 4) (8–10). Phylogenetic analysis showed that TiLVs from Thailand were closely related to TiLVs from Israel and grouped with the viruses of the family *Orthomyxoviridae* but not *Arenaviridae* and *Bunyaviridae* (Figure). This result suggests that the genetic composition of this emerging virus was similar to that of orthomyxoviruses and homologous with previously published TiLV sequences.

Our PCR and whole-genome findings demonstrate genetic homology between TiLV from Thailand and the etiologic agent of a novel RNA virus infection of

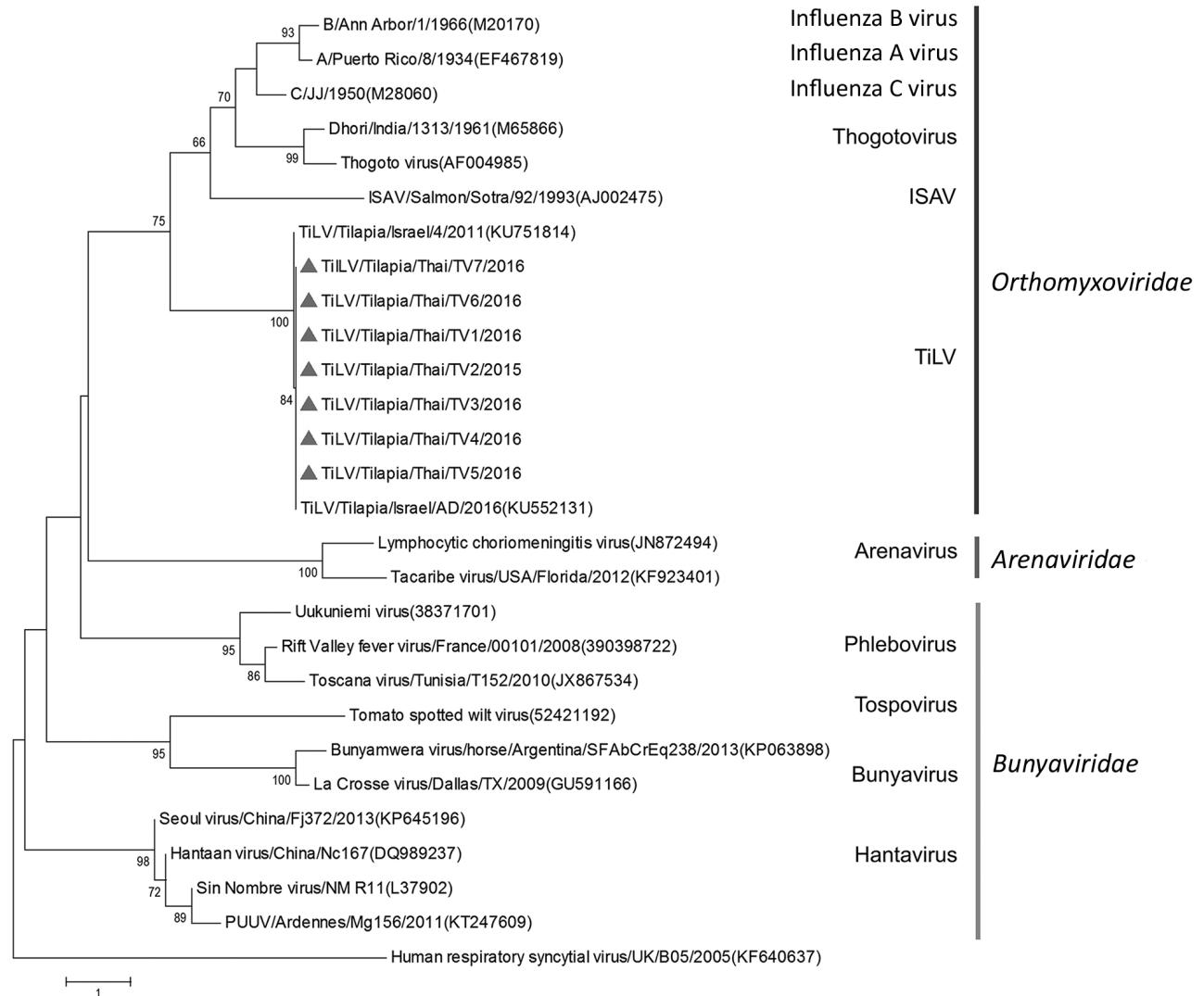


Figure. Phylogenetic analysis of the nucleotide sequences of RNA polymerase of TiLVs from Thailand (triangles) and reference viruses of the families Orthomyxoviridae, Arenaviridae, and Bunyaviridae. Genus and family groups are indicated; GenBank accession numbers are provided for reference viruses. The phylogenetic tree was constructed by using MEGA 6.0 (10) and applying a neighbor-joining bootstrap analysis (1,000 replications) with the Poisson model and gamma distribution. Human respiratory syncytial virus was used as an outgroup. ISAV, infectious salmon anemia virus; PUUV, Puumala virus; TiLV, tilapia lake virus. Scale bar indicates nucleotide substitutions per site.

tilapia in Israel and Ecuador (4,7). Furthermore, the clinical signs and pathological presentation of infection with TiLV from Thailand are similar to those of infection with TiLV from Israel (online Technical Appendix Figure 2). The clinical signs, gross lesions, and histopathologic lesions combined with virus identification and characterization highlight emerging TiLV in Thailand as the primary cause of the outbreaks. We also found that fish that survived massive die-offs rarely showed clinical signs, suggesting the development of specific immunity against the virus. It should be noted that the TiLVs from Thailand possessed 10 gene segments encoding 10

proteins, including segment 1 or putative PB1 protein. The pattern of protein motifs for this putative PB1 was similar to that for influenza viruses. To our knowledge, TiLV has infected tilapia only, no other aquatic or terrestrial animals.

Our results emphasize that the virus isolated from Thailand shares high sequence similarity with TiLV from Israel, suggesting that this virus spreads across continents. Given that tilapia are the main aquaculture species, control of TiLV will be improved by further efforts such as strict biosecurity, vaccine development, and selection of resistant tilapia breeds.

Acknowledgments

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Endemic Hantavirus in Field Voles, Northern England

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We report a PCR survey of hantavirus infection in an extensive field vole (*Microtus agrestis*) population present in the Kielder Forest, northern England. A Tatenale virus-like lineage was frequently detected ($\approx 17\%$ prevalence) in liver tissue. Lineages genetically similar to Tatenale virus are likely to be endemic in northern England.

Recently a new vole-associated hantavirus (Tatenale virus) was discovered in northern England (1), but only from an individual *Microtus agrestis* field vole. Previously only hantaviruses from murine-associated lineages (Seoul virus [SEOV] and SEOV-like viruses) had been reported in the United Kingdom, despite the abundance of potential vole hosts in the mainland United Kingdom and the endemicity of vole-associated hantavirus lineages (Puumala virus [PUUV] and Tula virus) in mainland Europe (2). Here we present data suggesting that the Tatenale virus lineage is endemic in northern England.

European hantaviruses are of public health significance because they are a causative agent of hemorrhagic fever with renal syndrome (HFRS). In the United Kingdom, HFRS cases have primarily been attributed to SEOV-like viruses on the basis of serologic tests. SEOV antibodies have been detected in both humans and Norway rats (*Rattus norvegicus*) in Northern Ireland and Yorkshire (3,4), and seropositivity in humans correlates with domestic or occupational exposure to rats (3,5). However, in the United Kingdom, HFRS cases with serologic cross-reactivity to PUUV (3), which might share antigenic determinants with Tatenale virus, have occurred.

To investigate the endemicity of hantavirus in field voles in the United Kingdom, we surveyed the extensive field vole population in the Kielder Forest, Northumberland (≈ 230 km distant from the locality where Tatenale virus was discovered). All sampled sites were grassy, clear-cut areas (adjacent to forest stands) where field voles were prevalent. Fieldwork was approved by the University of Liverpool Animal Welfare Committee and conducted subject to UK home office project license PPL 70_8210. Following the

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Technical Appendix

Materials and Methods

Clinical samples

Infected tilapia with clinical signs including loss of appetite, lethargy, swimming at the water surface, anemia, exophthalmia, abdominal swelling, and skin congestion and erosion were collected (Technical Appendix Figure 2). Internally, no specific gross pathological lesions were found. However, some fish developed brain congestion, pale gills and pale liver (Technical Appendix Figure 2). For external parasitic identification, the skin and gill samples were examined in wet mount under light microscope. For bacterial identification, anterior kidney samples were subjected to bacterial isolation using tryptic soy agar (TSA) or modified Shieh's agar. Bacterial species identification was performed using conventional biochemical test or API20NE test (BioMerieux, France). External parasitic identification findings were monogenean parasites (*Gyrodactylus* and *Dactylogyrus*) and ciliate protozoa (*Trichodina*). Bacterial findings were *Flavobacterium*, *Aeromonas* and *Streptococcus* (Technical Appendix Table 1).

Histopathology and Electron microscopy

For histopathological examination, brain, liver, spleen, heart, and kidney were collected from 3 fish (per outbreak) and kept in 10% buffered formalin. The samples were cut at 4 μm thick and processed for standard H&E staining. Histopathological findings were aggregation of lymphocytes and perivascular cuffing in brain tissue. For electron microscopic examination, the infected fish brains were filtered at 0.22 μm and prepared for EM transmission. Electron micrographs of negatively stained revealed enveloped virus particles with diameter between 50 to 80 nm (online Technical Appendix Figure 2).

Polymerase chain reaction

For molecular identification, PCR with specific primers for TiLV were performed (1). In addition, samples were tested for other viral infections including Betanodavirus and Iridovirus by specific PCR primers (2,3). In brief, RNA was extracted from brains (pooled sample of 3–5 brains) of infected and normal fish from the same culture area. RNA was subjected to PCR with TiLV specific primers.

Phylogenetic analysis

Phylogenetic analysis of TiLVs was conducted by comparing segment 1 (putative PB1 gene) of Thai TiLVs, Israel TiLV and reference viruses of Orthomyxoviridae, Arenaviridae and Bunyaviridae using program MEGA 6.0 (4).

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Technical Appendix Table 1. Description of TiLV outbreaks in Thailand*

Outbreak	Date	Location	Species	Laboratory diagnosis		
				Ectoparasite†	Bacteria identification‡	TiLV Identification§
1	15/10/2015	Ang Thong	RT	ND	ND	+
2	30/10/2015	Ang Thong	RT	ND	ND	+
3	11/11/2015	Ang Thong	RT	ND	ND	+
4	29/12/2015	Kanchanaburi	RT	ND	No growth	–
5	29/12/2015	Chai Nat	RT	ND	<i>Flavobacterium</i>	+
6	29/12/2015	Kanchanaburi	RT	ND	<i>Flavobacterium, Aeromonas</i>	+ (TV2)
7	29/12/2015	Chai Nat	RT	ND	<i>Flavobacterium</i>	–
8	05/01/2016	Nakhon Ratchasima	RT	1+	<i>Flavobacterium</i>	+ (TV3)
9	05/01/2016	Pathum Thani	RT	ND	No growth	+
10	15/01/2016	Pathum Thani	RT	2+	<i>Aeromonas</i>	+
11	15/01/2016	Chachoengsao	T	3+	<i>Aeromonas</i>	+ (TV4)
12	15/01/2016	Pathum Thani	RT	ND	ND	–
13	19/01/2016	Ratchaburi	RT	1+	<i>Aeromonas</i>	+ (TV5)
14	04/02/2016	Pathum Thani	RT	0	<i>Aeromonas</i>	+
15	05/02/2016	Kanchanaburi	RT	ND	<i>Aeromonas</i>	+
16	09/02/2016	Kanchanaburi	RT	1+	<i>Aeromonas</i>	+
17	16/02/2016	Samut Songkhram	RT	2+	ND	–
18	16/02/2016	Samut Songkhram	RT	3+	<i>Aeromonas</i>	+
19	18/02/2016	Pathum Thani	RT	3+	<i>Aeromonas</i>	–
20	26/02/2016	Pathum Thani	RT	2+	<i>Flavobacterium, Aeromonas</i>	+ (TV1)¶
21	27/02/2016	Samut Songkhram	RT	1+	No growth	+
22	30/03/2016	Pathum Thani	RT	ND	<i>Aeromonas</i>	+
23	28/04/2016	Nakhon Ratchasima	RT	ND	ND	+
24	28/04/2016	Pathum Thani	RT	ND	ND	+
25	06/05/2016	Pathum Thani	RT	2+	<i>Aeromonas</i>	+
26	06/05/2016	Prachin buri	T	0	<i>Streptococcus</i>	–
27	10/05/2016	Pathum Thani	T	1+	ND	–
28	13/05/2016	Nong Khai	T	3+	ND	–
29	20/05/2016	Phitsanulok	RT	0	<i>Aeromonas</i>	+ (TV6)
30	20/05/2016	Phitsanulok	T	0	<i>Streptococcus, Aeromonas</i>	–
31	23/05/2016	Chai Nat	RT	0	<i>Aeromonas</i>	–
32	24/05/2016	Khon Kaen	T	2+	<i>Aeromonas</i>	+ (TV7)

*Outbreaks of massive tilapia death were investigated in 9 provinces during Oct 2015 to May 2016. Epidemiologic information and laboratory findings were shown.

†Ectoparasite: External parasites were examined from skin and gills under light microscope. The majority of external parasites were monogenean parasites (*Gyrodactylus* and *Dactylogyrus*) and ciliate protozoa (*Trichodina*).

‡Bacterial Identification: Bacteria were isolated from anterior kidney and identified by conventional biochemical tests and API20NE test.

§TiLV Identification: Tilapia Lake Virus (TiLV) identification was performed by PCR with specific primers.

¶TV1 was subjected to whole genome sequencing.

Technical Appendix Table 2. List of Thai Tilapia lake viruses (TiLVs) characterized in this study*

Virus	Host species	Province	Date collection	Gene sequenced	GenBank accession no.
TiLV/Tilapia/Thai/TV1/2016	Red tilapia	Pathum Thani	Feb-2016	Whole genome	KX631921 – KX631930
TiLV/Tilapia/Thai/TV2/2015	Red tilapia	Kanchanaburi	Dec-2015	Complete Seg No. 1	KX631931
TiLV/Tilapia/Thai/TV3/2016	Red tilapia	Nakhon Ratchasima	Jan-2016	Complete Seg No. 1	KX631932
TiLV/Tilapia/Thai/TV4/2016	Nile tilapia	Chachoengsao	Jan-2016	Complete Seg No. 1	KX631933
TiLV/Tilapia/Thai/TV5/2016	Red tilapia	Ratchaburi	Jan-2016	Complete Seg No. 1	KX631934
TiLV/Tilapia/Thai/TV6/2016	Red tilapia	Phitsanulok	May-2016	Complete Seg No. 1	KX631935
TiLV/Tilapia/Thai/TV7/2016	Nile tilapia	Khonkean	May-2016	Complete Seg No. 1	KX631936

*Seg No. 1: putative PB1.

Technical Appendix Table 3. Nucleotide and amino acid identities of Thai Tilapia Lake Virus (TiLV) against reference TiLV available in the GenBank database*

Viruses	Segment No., nucleotide (amino acid) identities, %									
	1 (1560 bp)	2 (1368 bp)	3 (1260 bp)	4 (1065 bp)	5 (1023 bp)	6 (954 bp)	7 (588 bp)	8 (525 bp)	9 (351 bp)	10 (342 bp)
Israel/4/2011	95.85 (99.22)	96.33 (98.73)	95.88 (99.52)	97.12 (99.15)	95.65 (97.90)	95.56 (96.74)	95.59 (97.37)	98.62 (99.42)	98.18 (97.35)	99.10 (99.11)
Israel/AD/2016	96.52 (99.42)	96.80 (99.49)	95.69 (99.27)	97.12 (99.43)	95.18 (97.90)	95.56 (97.74)	95.78 (96.83)	98.41 (100)	97.86 (96.43)	98.19 (98.20)
Thai/TV1/2016	100 (100)	100 (100)	100 (100)	100 (100)	100 (100)	100 (100)	100 (100)	100 (100)	100 (100)	100 (100)
Thai/TV2/2015	95.35 (99.81)	N/A								
Thai/TV3/2016	95.42 (99.81)	N/A								
Thai/TV4/2016	99.42 (100)	N/A								
Thai/TV5/2016	95.70 (99.81)	N/A								
Thai/TV6/2016	96.13 (99.61)	N/A								
Thai/TV7/2016	95.84 (99.61)	N/A								

*The number without blanket indicated % nucleotide identity and the number within blanket indicated % amino acid identity.
 Note: Common amino acid variation of each segment. The alphabet before number indicates amino acid in Israel TiLV. The number indicates a position of amino acid variation. The alphabet post number indicates amino acid in Thai TiLV. Putative PB1: K446R, Segment No.2: K6R and A231T, Segment No.3: V275M, Segment No.4: S24G and A33V, Segment No.5: I17M, D109E, R155K, T163A, V291I and L294F, Segment No.6: L13F, K55I, M95R, R159H, K274R, N276D and I301M, Segment No.7: S22I, E72R, H113R, C177S and K189E, Segment No.8: V45I, S66G and R114K, Segment No.9: V45I, S66G and R114K, Segment No.10: R89I.

Technical Appendix Table 4. Summary of Motifs preA, A, B, C, D and E of Orthomyxoviridae PB 1 aa alignment (5, 6)*

Viruses	Premotif A	Motif A	Motif B	Motif C	Motif D	Motif E
IAV	KDAER G KLKRRRAIATPGM QIRGFVYFVET	TELSFTITGDNT KWNENQN	ASLSPGMMMGMF NMLSTVLGVS	TYWWDGLQ SSDDFAL	GINMSK K K S-YINR	TGTFEFTSF FYR
IBV	KDAER G KLKRRRAIATAGIQI RGFVLVVEN	GGISMTVTGDN TKWNECLN	ASLSPGMMMGMF NMLSTVLGVA	EYLWDGLQ SSDDFAL	GINMSK K K S-YCNE	TGMFEFTSM FYR
ICV	KDGER G KLQRRRAIATPGMI VRPFSKIVET	DQFAVNITGDN SKWNECQQ	CFLPGGMLMGFMF NMLSTVLGVS	GCFWTGLQ SSDDFVL	GINMSLEKS -YGSL	PELFEFTSM FFD
ISAV	KNSERTKLEPRAVFTAGVP WRAFIFVLEQ	GQTLVTLTGDN SKYNESMC	IRVRRGMLMGMA NNAFTTASTI	PEAVYTLQS SDDFVT	GLNVSQKK SFYVEG	TT- FEFNSMFVR
Dho	KHLER G RLLNRRTIATPSML ARGFVKIVED	SEVTGELSGDQ EKFNECLD	IRCTLGMFMGMFN LSSTLLALI	EITGDHVES SDDFIH	GINMSPSK CILISP	AGIGEFNSK YHH
Tho	KHLER G RLLNRRTIATPSML IRGFVKIVED	TAVTGELSGDQ EKFNECLD	ISCR L GMFMGMYN LTSTLLALI	ELTGSHVES SDDFIH	GINMSPSK CILISP	AGIGEFNSK FHH
TiLV4-11	RDQER G KPKSRAIFLSHPF FRLSSVVET	ESRK H VLNGDC TKYNEAID	— GGMLMGMFNATA TLA—	— GTTDRFLSF SDDFIT	—NLSLK K S- YISV	AS- LEIN S CTLT
TiLVAD-16	RDQER G KPKSRAIFLSHPF FRLSSVVET	ESRK H VLNGDC TKYNEAID	— GGMLMGMFNATA TLA—	— GTTDRFLSF SDDFIT	—NLSLK K S- YISV	AS- LEIN S CTLT
Thai TiLV	RDQER G KPKSRAIFLSHPF FRLSSVVET	ESRK H VLNGDC TKYNEAID	— GGMLMGMFNATA TLA—	— GTTDRFLSF SDDFIT	—NLSLK K S- YISV	AS- LEIN S CTLT

*Gap represented by -. Residues that are invariant for all RNA polymerases are shown in Bold. Conserved residues among negative-stranded RNA are shown in bold and underline. Description of viruses are as follows: IAV: influenza A/Puerto Rico/8/1934 (EF467819), IBV: influenza B/Ann Arbor/1/1966 (M20170), ICV: influenza C/JJ/1950(M28060), ISAV: Infectious salmon anemia virus strain Sotra 92/93(AJ002475), Dho: Dhori virus (M65866), Tho: Thogoto virus (AF004985).

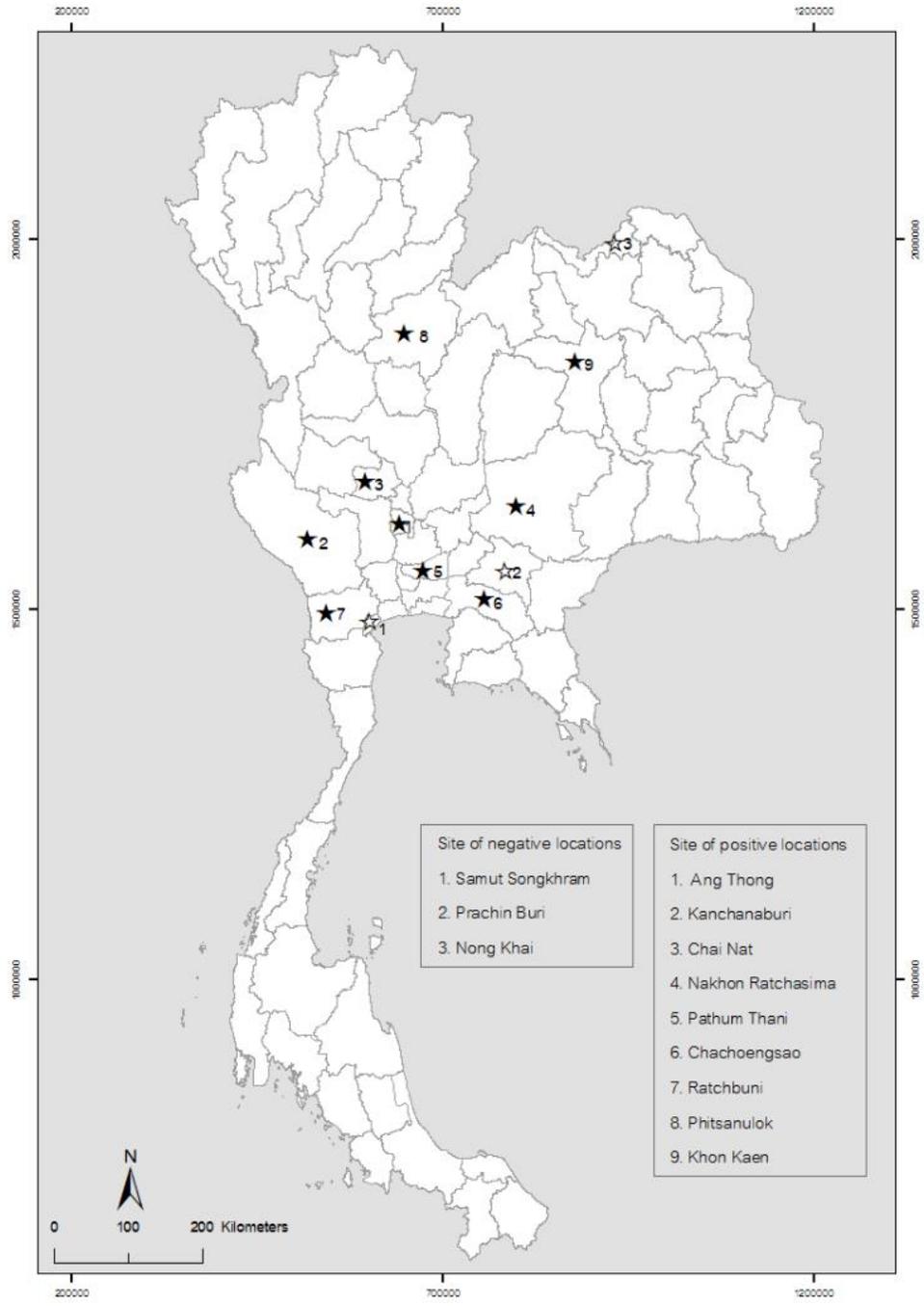
Technical Appendix Table 5. List of Oligonucleotide primers for Thai TiLV sequencing*

Segment	Primer Name	Primer Sequence (5' – 3')	Bp	Product size (Bp)
1	TiLV1F	CCAAACGTTATCTCTTAATTACGCAC	26	1641
	TiLV1R	GCAAATATTTCTCTCATTGCGCT	23	
2	TiLV2F	ACTCTCTATTACCAAATACATTTACT	26	1445
	TiLV2R	TTACCATATATAGTGAAGGC	22	
3	TiLV3F	ACCCCTTAATCCTTAATAGACCGTTA	26	1352
	TiLV3R	CCCATAATCCTCTATTAGAACGTCGT	26	
4	TiLV4F	CCAAAGTTTACTCCTATTACCCAGA	25	1250
	TiLV4R	GCAAATCTTTCTCCAATTACCGTCT	25	
5	TiLV5F	CCAAATGTTTCTTATCTCAGACTC	26	1087
	TiLV5R	CTTTTTCTCAGTTTACCACCTTTATG	25	
6	TiLV6F	CCAAATTTTACCTCTCGCAT	20	1027
	TiLV6R	TCAAGCACTTAAAAGTGTACC	21	
7	TiLV7F	CTCTCTTTGCATTGCATACCGT	22	704
	TiLV7R	GACCAATTATCCCTGCTTTCA	21	
8	TiLV8F	ACCTCATCTACACTAACATTTCCA	24	637
	TiLV8R	TCATCATTACACAAATGGAGTAGCT	25	
9	TiLV9F	ACAAGTCCGATTACTTTTTCCGC	23	530
	TiLV9R	TCTTTCTCAGTCCTTAAAGTCA	23	
10	TiLV10F	AACCCTACTAACACCAAATATAGCT	25	450
	TiLV10R	CTTCCCTCTGACACCCTGT	20	

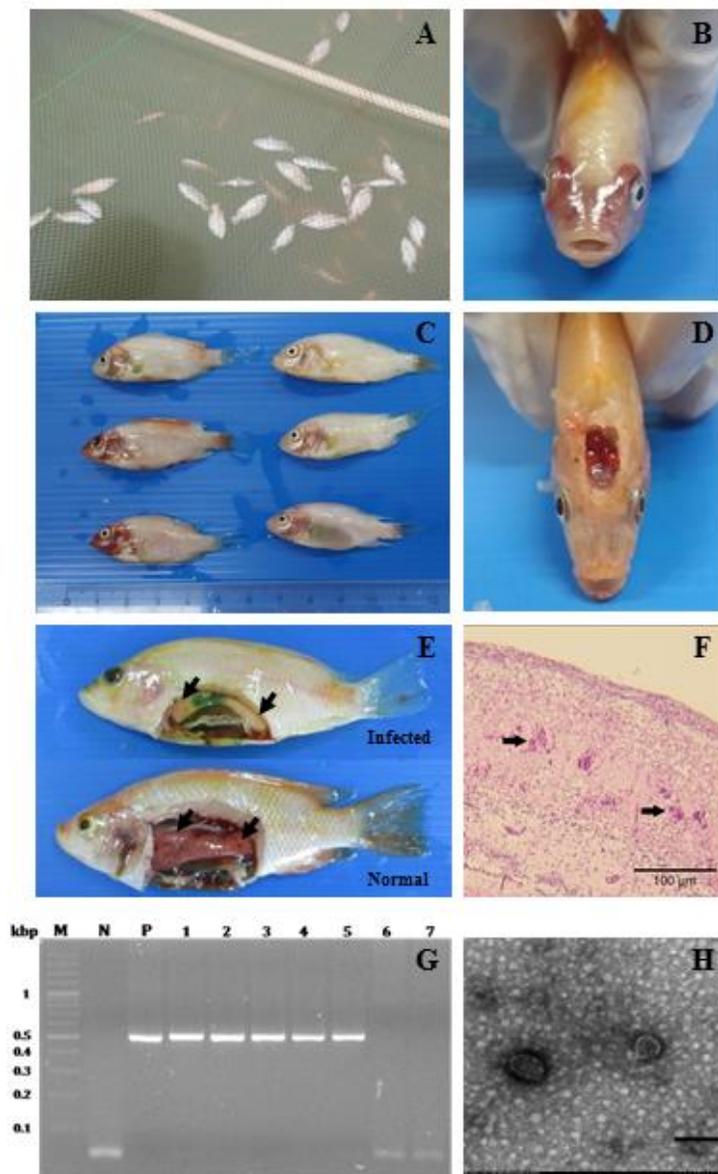
*Primers were designed based on available TiLV reference (7).

Technical Appendix Table 6. Detail description of genome composition of Thai TiLV (TV1)

Contig	Israel TiLV, TiLV4/2011*		Thai TiLV, THA1/2015	
	Segment length (Bp)	Predicted protein (AA)	Segment length (Bp)	Predicted protein (AA)
1	1,641	519	1562 (1560)	519
2	1,471	457	1368 (1368)	455
3	1,371	419	1301 (1260)	419
4	1,250	356	1170 (1065)	354
5	1,099	343	1024 (1023)	340
6	1,044	317	988 (954)	317
7	777	195	685 (588)	195
8	657	174	588 (525)	174
9	548	118	484 (351)	116
10	465	113	405 (342)	113



Technical Appendix Figure 1. Locations of sample collection covering central, eastern, northeastern and western of Thailand. Stars (solid) represent site of virus positive location.



Technical Appendix Figure 2. Gross and histopathological lesions of infected tilapia, Thailand. (A) Massive losses of fish due to mortality at 2 weeks post transfer into cages. (B and C) Gross appearance of infected tilapia included skin congestion and mild exophthalmia, (D and E) brain congestion and pale liver. (F) Histological findings showed influx of mononuclear lymphocytes in the brain consistent with non suppurative meningoencephalitis with multifocal hemorrhage. (G) PCR identification of TiLV from infected fish. M = marker, N = negative, p = positive control (plasmid containing TiLV fragment), Lane 1–5: brain from moribund fish, Lane 6–7: brain from normal fish. Samples were pools of 3 brains. (H) Morphology of virus prepared from infected brain. The virion size is 50–80 nm with electron dense aggregate surface; bar size = 50 nm.