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Research Article
Molecular Detection of Tilapia Lake Virus (TiLV) in Farmed Mono-sex Nile Tilapia (*Tilapia niloticus*) in Bangladesh

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Abstract

Background and Objectives: Tilapia lake virus (TiLV) has been marked as an emerging infectious agent that causes mass die-offs in farmed mono-sex Nile tilapia (*Tilapia niloticus*) in Bangladesh, indicates rapid diagnostic assay. This study was aimed to develop molecular detection method to confirm the TiLV in *Tilapia niloticus* and construct a genetic baseline to control this disease. **Materials and Methods:** The research aims to the detection of TiLV followed by complementary techniques of PCR based approaches such as reverse-transcription polymerase chain reaction (RT-PCR) and RT-quantitative (q) PCR using SYBR Green I dye. The RNA quantification, followed by a PCR protocol entailing, complementary deoxyribonucleic acid (cDNA) synthesis and detection of TiLV by either conventional PCR or quantitative identification via qPCR using SYBR Green I dye. **Results:** This research reported a novel RNA virus allowing its clinical signs lethargy, skin erosion, exophthalmia, detached scales and 15-82% morbidity rate. The RT-PCR amplified a 491 bp fragment from segment 3 in both cases. The PCR amplification efficiency of 98.5% over a wide linear range of 2.98×10^{1} to 2.98×10^{10} TiLV copies, while the NTC (no template control) produced no fluorescence and therefore no amplification. The sequence of amplified PCR products received 100, 98 and 97% identity. The phylogenetic relationship of 17 TiLV sequences was chosen to compare with GeneBank resulting a common ancestor while closely related with Columbia, India, Malaysia and Thailand. The highest pair-wise alignment score was received 90.20 for MH338228.1 (Columbia), 85.57 for MF582636.1 (India), 85.30 for MH213048.1 (Malaysia) and 86.93 for MH213039.1 (Thailand) using the sequence of TiLV segments of one TiLV-positive strain. **Conclusion:** The mono-sex Nile tilapia was infected with common fish pathogens, such as *Aeromonas* and *Streptococcus*. This newly developed SYBR Green-based RT-qPCR assay can be as an essential tool for TiLV diagnostics and should help to control the dissemination of this virus worldwide.

Key words: Tilapia lake virus (TiLV), Nile tilapia (*Tilapia niloticus*), PCR, RT-PCR, RT-qPCR

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Tilapia is a highly valuable source of fish protein in the world and are one of the most popular fish species for the aquaculture industry globally, including the Asia-Pacific[1]. The top producing countries include China, Egypt, Philippines, Thailand, Indonesia, Lao PDR, Costa Rica, Ecuador, Colombia, Honduras and Bangladesh[1]. Comparatively more than 100 species, tilapia is widely cultured and the second most farmed fish worldwide and essential to domestic and global food security[2] with the production of over 2.5 million tons annuall[3,4] and they serve as a primary protein source in the developing world. Tilapia production estimated globally 4.5 million metric tons valued more than $7.5 billion U.S. dollars and production is expected to increase[4] to 7.3 million metric tons by 2030. Currently estimated production 6.4 MMT, top 3 producers were the China, Indonesia, Egypt and other leading producers included Bangladesh, Vietnam and Philippines[5].

Tilapia aquaculture has been threatened by mass mortality since 2009 in Israel and Ecuador[2]. Tilapia is considered resistant to a wide range of parasites, bacteria, fungi, protozoa and viruses as challenged to tilapia aquaculture, recently virus implicated in large disease outbreaks with mass mortality[6-10]. These outbreaks featured betanodavirus and herpes-like viruses[11], reportedly mass die-offs have been identified as an orthomyxo-like (RNA) virus named as tilapia lake virus (TiLV)[2,9,12]. The TiLV has been reported an emerging virus that produces syncytial hepatitis of tilapia (SHT)[11].

The etiological agent of tilapia lake virus reported on Israel[9], Ecuador[10], Colombia[13], Egypt[14], Taiwan[15], Philipines[16], Thailand[17], Malaysia[18], India[19], Tanzania, Uganda[20], Peru[21] and Bangladesh[22,23]. Tilapia is one of the top one aquaculture species in Bangladesh, has shown susceptibility to the disease caused by TiLV, even co-farmed with Indian major carps[19,24], proving difficult to control[18,25] and due to the unavailability of TiLV vaccine or anti-viral drugs. Bacterial and fungal infection addressed with antibiotics or topical treatment, no specific treatment evolved for viral infections of tilapia[26].

In Bangladesh tilapia farms, disease outbreaks have recently occurred associated with high mortalities (60-80%), laboratory clinical test and histopathology confirmed non-specific causes, although[27] previously predicted that during imported tilapia and fingerlings and some may have unaware of risk that might be infected with TiLV and Peeler[23] reported tilapia mortality in Bangladesh might be the presence of TiLV. These findings indicate the lack of molecular diagnostic knowledge regarding virulence and toxicity of TiLV, might be necessary to minimize the risk of TiLV will depend upon a better understanding of epidemiology and biosecurity measures.

TiLV is caused by an envelope, single-stranded RNA of negative polarity with 10 genomic segments encoding 10 proteins[2,9,10,11,25] and identified to be an orthomyxo-like virus. Infected tilapia with clinical signs include lethargy, ocular alternations, anemia, loss of appetite, exophthalmia, abdominal swelling, skin erosion, scale protrusion, haemorrhagic patches, discoloration, fin rot and abnormal behavior[2,9,10,25,28]. Mortality levels of above 80% observed in affected farmed populations in Israel[9], 10-80% in Ecuador[10] and 20-90% in Thailand[22,25] within the 1st month after transfer to the grow-out pond.

To investigate and confirmation of the causative agent of TiLV histopathology[9], cell line culture[19,20], electron microscopy[29], RT-PCR[20,28], nested or semi-nested RT-PCR[13,27], RT-qPCR[13,29] have been used in different countries. With the ongoing investigations and characterization[12] of the TiLV among the tilapia producing countries, this study also conducted highly sensitive, specificity, scalability and rapid RT-qPCR[30] approaches to overcome the limitations of the other diagnostic tools. In this study, a SYBR Green 1 dye based RT-qPCR assay designed and developed to maximize the usability for the sensitive and specific detection TiLV. Genomic sequence of TiLV-positive samples was conducted for phylogenetic analysis and homology with reference TiLVs available in the GenBank database.

MATERIALS AND METHODS

Fish and tissue sample collection: During 2016-2018, 36 farms in central, southern and western part of Bangladesh was investigated with experiencing risk factors, co-infections and numerous mortalities of unknown causes in the Nile tilapia male mono-sex (*Oreochromis niloticus*) (Fig. 1a). The diseased 5-18 fish (weighing 30-85 g) were collected from grow-out ponds in the hatchery (Table 1), clinical signs and high mortality was observed (Fig. 2a). Morbidity rate (diseased fish/total fish sampled) and secondary infections with bacteria and parasites were also investigated (Table 1). Microscopically external and internally clinical examinations was conducted for pathological changes and for parasite identification (Fig. 2b). Tissue from the head kidney, spleen and liver were swabbed and subjected to bacterial isolation using tryptic soy agar (TSA), identification was performed using conventional biochemical test and AP120NE test (Table 1). The mucus samples were collected by cover glass or surgical blade[31] and the liver, spleen and head kidney were collected by removing the abdominal wall of the fish (Fig. 2c).

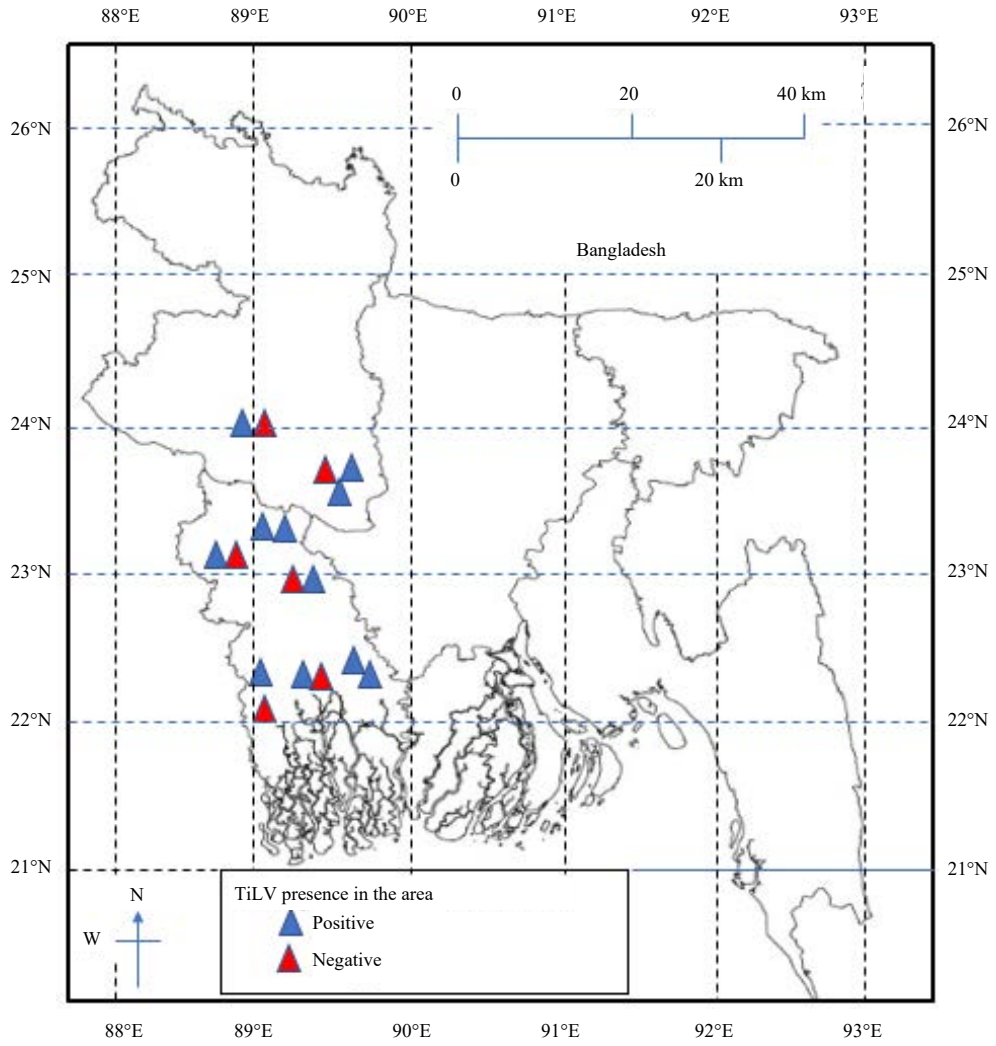

Fig. 1: Map of mono-sex Nile tilapia farming area highlighting the sampling sites (Rajbari, Faridpur, Magura, Norail, Jashore, Khulna and Bagerhat) and the presence of TiLV (Tilapia Lake Virus) in the study

Fig. 2(a-c): Tilapia necropsy study and clinical signs observations, (a) TiLV infected dead tilapia on the water surface of the culture pond, (b) Sample tilapia with the opaque eyeball, skin lesion, redness around the mouth and operculum and detached scale and (c) Liver, kidney and spleen tissue was collected from TiLV infected dead tilapia

Table 1: Details of TiLV outbreaks in Nile tilapia in Bangladesh* (in 2016-2018), to determine the morbidity rate, RT-PCR experiments to test for the presence of TiLV and conventional biochemical test and AP120NE test for the identification of fish pathogenic bacterial species in the specified Bangladeshi tilapia farms

Affected areas	Sample ID	Outbreaks	Ectoparasites†	Bacterial identification¶	TiLV identification	Morbidity (%)
Rajbari	R1	1	1+	*Aeromonas*	-	3/11 (27)
Rajbari	R2	2	2+	*Streptococcus*	-	7/10 (70)
Rajbari	R3	3	ND	ND	-	2/8 (25)
Rajbari	R4	4	ND	*Streptococcus*	-	3/5 (60)
Rajbari	R5	5	ND	*Streptococcus*	-	1/6 (17)
Rajbari	R6	6	3+	*Aeromonas*	+	6/8 (75)
Faridpur	F1	7	ND	*Streptococcus*	-	4/7 (57)
Faridpur	F2	8	ND	*Streptococcus*	+	11/14 (77)
Faridpur	F3	9	2+	ND	-	3/15(20)
Faridpur	F4	10	ND	*Aeromonas*	-	2/12 (17)
Faridpur	F5	11	1+	*Streptococcus*	-	5/10 (50)
Magura	M1	12	ND	*Streptococcus*	-	2/10 (20)
Magura	M2	13	3+	*Streptococcus*	-	3/11 (27)
Magura	M3	14	ND	*Aeromonas*	-	1/7 (14)
Norail	N1	15	2+	*Streptococcus*	+	14/18 (78)
Norail	N2	16	1+	*Streptococcus*	-	10/13 (77)
Norail	N3	17	ND	*Streptococcus*	+	4/17 (23)
Norail	N4	18	3+	*Streptococcus*	+	10/18 (56)
Norail	N5	19	ND	ND	-	7/11 (64)
Norail	N6	20	1+	*Aeromonas*	-	4/8 (50)
Jashore	J1	21	ND	ND	-	2/12 (17)
Jashore	J2	22	ND	*Streptococcus*	-	5/14 (36)
Jashore	J3	23	2+	*Streptococcus*	-	4/17 (24)
Jashore	J4	24	ND	*Aeromonas*	-	2/13 (15)
Jashore	J5	25	2+	*Aeromonas*	+	7/18 (39)
Jashore	J6	26	1+	ND	-	5/15 (33)
Khulna	K1	27	ND	ND	-	3/7 (42)
Khulna	K2	28	3+	*Streptococcus*	+	2/10 (20)
Khulna	K3	29	ND	*Streptococcus*	-	4/7 (57)
Bagerhat	B1	30	ND	*Streptococcus*	-	4/10 (40)
Bagerhat	B2	31	1+	*Streptococcus*	-	9/11 (82)
Satkhira	S1	32	ND	ND	+	9/12 (75)
Satkhira	S2	33	ND	*Streptococcus*	+	14/18 (78)
Satkhira	S3	34	2+	*Streptococcus*	-	9/15 (60)
Satkhira	S4	35	2+	ND	-	3/10 (30)
Satkhira	S5	36	ND	*Aeromonas*	-	7/10 (70)

*Massive deaths investigated 8 districts, †Ectoparasites: Light microscope revealed external monogenean parasites from skin and gills, ¶Bacterial Identification: Conventional biochemical test and AP120NE test confirmed bacteria found in head kidney, liver and spleen tissues of tilapia, TiLV identification: PCR amplification with specific primers confirmed TiLV, morbidities were determined at the throughout the year of 2016-2018, the sampling of fish might have favoured diseased tilapia

Twenty to hundred miligram tissues from the liver of the tilapia placed into a 1.5 mL micro-centrifuge tube. The total RNA extracted from tissue cells, processed immediately store in -80°C kept them undamaged or stabilized for further use and isolated carefully to prevent RNase contamination.

Extraction of RNA and quantify RNA concentration by spectrophotometer: A tube containing tissue sample (20-100 mg) added with 1 mL of monophasic solution (phenol and guanidinium isothiocyanate), homogenized and store at -80°C until further use. Two hundred microliter of chloroform was added for phase separation, mixed well by inversion for 15 sec, incubate for 3 min at room temperature (RT) and centrifuge for 15 min at 12,000×g at 4°C. A white interphase has a light pink appearance and an upper phase contained RNA, transferred the upper aqueous layer (approximately 500 µL) to a fresh micro-centrifuge tube. To precipitate the RNA 1 µL isopropanol was added, mixed well by inversion several times, store for overnight at -20°C and centrifuged for 15 min at 12,000×g at 4°C. Discarded the supernatant, the RNA pellet at the bottom of micro-centrifuge tube mixed with 1 mL of 75% ethanol (v/v) by inverting the tube several times, centrifuge for 15 min at 10,000×g at 4°C and store at -20°C until further use. Drew remaining ethanol using a pipette and the RNA was air-dried at RT for 5-10 min. To solubilize the RNA pellet, added 30-60 µL of RNase-free water and pre-warmed at 55-60°C. The total RNA placed on ice for immediate use or stored at -80°C for later use. To quantify the RNA concentration, 1-2 µL of RNase-free water was used as a black. To assess, the RNA quantity 1-2 µL of each RNA sample was used, recorded the reading at 230, 260 and 280 nm for each sample and 200 ng μL^{-1} RNase-free water was used to dilute the RNA.

Table 2: PCR amplification of TiLV cDNA, the established primer pairs

Target TiLV genome segment	Forward primer 5'-3'	Reverse primer 5'-3'	PCR product size (bp)	Temperature (°C)	References
1	CCAAACGTTATCTCTTAATTACGCAC	GCAAATATTTCTCTCATTCGCCT	1641	50	Surachetpong *et al.*[25]
1	CCTCATTCCTCGTTGTGTAAGT	AGGAGTTGCTGTTGGGTTATAG	1000	62	Mugimba *et al.*[20]
2	ACTCTCTATTACCAAATACATTTACT	TTACCATATATATAGTGAAGGC	1445	45	Surachetpong *et al.*[25]
2	GTCCAGGGCGGTATGTATTG	CTTACGGCTGACAAGTCTCTAAG	834	62	Mugimba *et al.*[20]
3	GTTGGGCACAAGGCATCCTA	TATCACGTGCGTACTCGTTCAGT	250	56	Eyngor *et al.*[9]
3	TATGCAGTACTTTCCCTGCC	TTGCTCTGAGCAAGAGTACC	491	57	Eyngor *et al.*[9]
3	ACCCCTTAATCCTTAATAGACCGTTA	CCCATAATCCTCTATTAGAACGTCGT	1352	50	Surachetpong *et al.*[25]
3	GTCGAGGCATTCCAGAAGTAAG	GAGCTAAGGGAACGGCTATTG	834	62	Mugimba *et al.*[20]
4	AGCAGCAGCAGGAGAAAGAG	ACCGTCCTGTTTCTGAATGG	358	60	Nicholson *et al.*[24]
4	CCAAAGTTTACTCCTATTACCCAGA	GCAAATCTTTCTCCAATTACCGTCT	1250	50	Surachetpong *et al.*[25]
4	GCCCAATGGTTCCCATATCT	GCCCAATGGTTCCCATATCT	524	62	Mugimba *et al.*[20]
5	CCAAATGTTTCTCTTATCTCAGACTC	CTTTTTCTCAGTTTACCACTTTATG	1087	57	Surachetpong *et al.*[25]
5	CAACTCTTAGCCTCCGGAATAC	CGTTCTGCACTGGGTTACA	696	62	Mugimba *et al.*[20]
6	CCAAATTTTACCTCTCGCAT	TCAAGCACTTAAAACTGTACC	1027	45	Surachetpong *et al.*[25]
6	CCCACACGACAGGACATATAG	GAGTTGGCTTAGGGTGATAAGA	948	62	Mugimba *et al.*[20]
7	CTCTCTTTGCATTGCATACCGT	GACCAATTATCCCTGCTTTCA	704	57	Surachetpong *et al.*[25]
7	TCCTTTAGGGATTGGCACTAAC	TTCCATCGACTGCTCCTAGA	486	62	Mugimba *et al.*[20]
8	ACCTCATCTACACTAACATTTCCA	TCATCATTACACAAATGGAGTAGCT	637	50	Surachetpong *et al.*[25]
8	CTTAAGGGCCATCCTGTCATC	TGGCTCAAATCCCAACACTAA	476	62	Mugimba *et al.*[20]
9	TTGGTGATGTCACGATGGATA	AGTTCTATCGCCAGCCATGT	351	60	Nicholson *et al.*[24]
9	ACAAGTCCGATTACTTTTTCCGC	TCTTTCTCACGTCCTTAAAGTCA	530	50	Surachetpong *et al.*[25]
9	GATATCCTCCACATGACCCTTC	GTACGTCACTTTGTGCCATTAC	261	62	Mugimba *et al.*[20]
10	AACCCTACTAACACCAAATATAGCT	CTTTCCCTCTGACACCCTGT	450	50	Surachetpong *et al.*[25]
10	TCCTCTCTGTCCCTTCTGTT	CAGGATGAGTGTGGCAGATTAT	276	62	Mugimba *et al.*[20]

Underline primer were used to generate the representative result revealed in Fig. 3 and 4

cDNA synthesis using total RNA: To bring the final volume of 10 µL with nuclease-free water, 1 µg of total RNA was mixed with 2 µM oligo (dT), 0.5 dNTPs mixture and mixed well followed by brief centrifugation, incubated at 65°C for 5 min followed by a 2 min incubation on ice and brief centrifuge to collect all the bottom liquid of the tubes. To bring the final volume of each sample to 20 µL using nuclease-free water added 100 U reverse transcriptase (1×reverse transcriptase buffer) mixed well followed by brief centrifugation, incubate at 42°C for 60 min followed by 85°C for 5 min. To desired concentration, the synthesized cDNA was diluted by adding nuclease-free water and place the cDNA on ice for immediate use and store it at -20°C for further use.

Conventional PCR: The cDNA used as templates for PCR reaction using primer pairs[28] detailed in Table 2 (NTC, non-template control included nuclease-free water instead of cDNA, a positive control included previously TiLV cDNA fragment cloned into a plasmid). PCR master-mix included the primer pair (Forward+Reverse), dNTPs, $MgCl_2$, selected DNA polymerase, buffer, suggested the amount of cDNA samples and control samples and PCR cycling followed annealing temperature in Table 2, initial denaturation at 95°C for 2-5 min, followed by 30-40 cycles of denaturation at 95°C for 30 sec, annealing for 30 sec and elongation at 72°C for 30 sec, followed by a final elongation 72°C for 5-10 min. Loaded 10 µL of each PCR reaction and DNA ladder into wells of a 1% agarose gel, stained the gel by ethidium bromide (EthBr), visualized the expected size of DNA bands in a gel documentation machine using UV light.

Quantitative polymerase chain reaction (qPCR): A plasmid containing the appropriate TiLV genomic segment 3 cDNA (pTiLV)[29], prepared triplicated 10-fold serial dilution. Ten-fold serial dilution of the p-TiLV (Malaysia) containing a 491 bp cDNA fragment from TiLV segment 3 were prepared to cover the range 2.98×10^{1} to 2.98×10^{10} copies of TiLV. The plotting log TiLV copy number versus cycle threshold (Ct) was shown in a standard curve constructed by running RT-qPCR reactions in triplicate. The PCR amplification efficiency was calculated[32] as:

$$100\times(10^{1/m}-1)$$

A qPCR master-mix per reaction prepared to utilize 0.4 µL of nuclease-free water, 0.3 µL of primer (Forward+Reverse) and 5 µL of 2×SYBR Green DNA polymerase (comprise: SYBER Green I dye, hot-start tag DNA polymerase, dNTPs, $MgCl_2$ and passive reference dyes). The primers pairs for p-TiLV as: forward primer: p-TilV-F (5'-CTGAGCTAAAGAGGCAATA TGGATT-3'), reverse primer: p-TiLv-R (5'-CGTGCGTACTCGTT CAGTATAAGTTCT-3') at a concentration of 10 µM.

Six microliter of qPCR master-mix dispensed in a 96 well plate compatible with qPCR machine 4 µL of cDNA template was added, serially diluted, covered and mixed the using a centrifuge and collected liquid in the bottom, placed the 96-well plates. The qPCR thermocycler performed an initial denaturation at 95°C for 3 min, followed by 40 cycles, annealing 95°C for 10 sec, elongation 60°C for 30 sec. The amplification curves of the samples and standards were evaluated and set the threshold in a region were the amplification rate of the cDNA is the same in all samples. The number of TiLV copies was calculated using the standard curve.

Sequencing of TiLV segment 3 and phylogenetic relationship: RT-PCR assays were performed to observed multiple sequence alignment with cDNA from representative one infected fish (F2 strain) from Bangladesh, using Nested ext-2 (5'-TTGCTCTGAGCAAGAGTACC-3) and nested ext-1 (5'-TATGCAGTACTTTCCCTGCC-3) primers amplifying a 491 bp fragment of TilV genomic segment 311. Purified the PCR products and Sanger sequenced was used as previously outlined[29]. The neighbor-joining method[33] was used to derive the evolutionary history of representative isolates. The maximum composite likelihood method[34] figured out the evolutionary distance and analyzed by MEGA-X. The NCBI-BLAST and Smart BLAST was used for sequence analysis and multiple sequence alignment was monitored using Geneious Prime, ClustalW and DNAMAN software.

RESULTS

Risk factors for disease outbreaks and co-infections: Clinical outbreaks have been found during the hot season, namely April to August at water temperatures of 25-31°C in Bangladesh. In Bangladesh, large farm size, intensive aquaculture systems, high stocking densities, tilapia-carp poly culture, live tilapia entering in the country, microbial agents interacting with the host, pathogens reduced fitness of the host, vertical transmission, nucleus breeding centre, multiple hatcheries and production systems had identified as risk factors for TiLV outbreaks. Variations in mortality have been found ranging from around 15% in farms with a mixed stocking to approximately 82% in farms with mono-sex Nile tilapia monoculture in earthen ponds. Affected fingerlings were commonly detected within 10-15 days post transfer to grow-out ponds, with mortality ranging from a low level of 15-20% to a high level of 82% (Table 1). This study revealed the co-infections in TiLV-positive fish from Bangladesh were *Aeromonas* and *Streptococcus* (Table 1) and some external and internal parasites.

Clinical signs of TiLV infected tilapia: The massive mortality was observed and found TiLV infected moribund tilapia on the water surface of the aquaculture farm (Fig. 2a). The TiLV infected tilapia displayed clinical signs (Fig. 2b) were euthanized by bathing in a high concentration of MS222 as an anaesthetics. The clinical symptoms were variable and appeared lethargy, skin erosion and discolouration, exophthalmia, detached scales, open wounds/lesion and abnormal behaviour[10,25,27-29] clearly seen in Fig. 2b with 60-80% mortality (Fig. 2c).

Spectrometric quantification of the total RNA: The RNA quantification was performed to assess sample purity and examination of special profiles (Table 3). The successful total RNA extraction procedure was shown in Table 3, while poor RNA preparation represents in this table, bold marks indicates possible protein or phenol contamination. The purity of each sample was measured at 260 nm for nucleic acids and at 280 nm for proteins indicates pure RNA for the sample and ratios of 1.8-2.2. The sample contamination for pure RNA preparations was calculated at 230 nm as showed by a value of 2.01 for the sample in while ratio should be in the range of 2.0-2.2. The low ratio A260/A230 ratio of 1.60 indicates residual guanidine or phenol in the sample (Table 3). To improve the purity of the sample, the RNA was reprecipitated to remove the contamination. The absorbance A260/A280 ration ranged from 1.88-2.03, which indicates little or no protein contamination (Table 3).

Detection of TiLV by RT-PCR: The pure RNA samples by RT-PCR represented in Table 3 were reverse transcribed into cDNA and used for PCR amplification as a template shown in

Table 3: Spectrophotometric quantification of total RNA extracted from the TiLV infected tilapia

	Absorbance ratios		
Treatment ID	A260/A280	A260/A230	Total RNA (ng µL^{-1})
R6	1.97	2.32	357
F2	1.93	2.05	478
N1	2.01	2.02	396
N3	1.98	1.60	416
N4	1.88	2.27	345
J5	1.94	2.08	422
K2	2.03	2.25	418
S1	2.00	2.36	435
S2	1.92	2.47	332

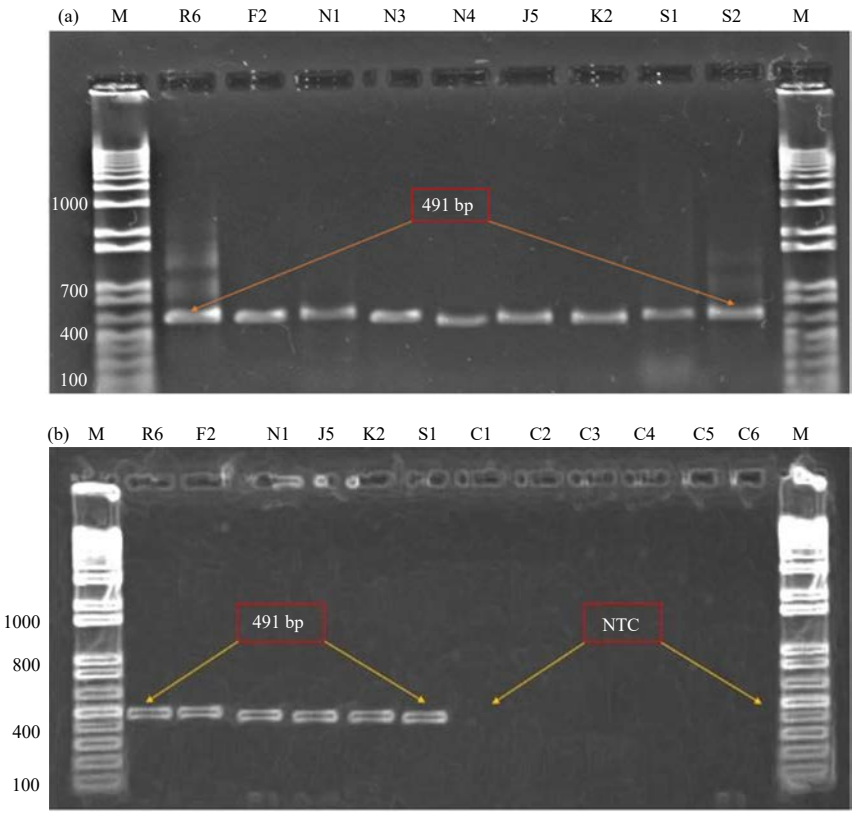

Fig. 3(a-b): TiLV detection by RT-PCR, (a) A 2-step RT-PCR assay using cDNA samples from liver tissue of diseased tilapia were tested for TiLV infection using specific primers for segment 3 and (b) Pure RNA samples of representative 6 areas produced from head kidney tissues of diseased tilapia were the screen for TiLV using the same primers as in 3A using a one-step RT-PCR assay

M: Marker shown in base-pairs (bp), R6, F2, N1, N3, N4, J5, K2, S1, S2: Samples, C1-C6: No template control (NTC) collected from the same areas

Fig. 3a. To amplify a 491 bp fragment of TiLV genomic segment 311 primer was used in bold in Table 1. The 1% gel electrophoresis was separated the PCR products and stained with ETBr for visualization. A two-step RT-PCR using 9 cDNA sample (R6, F2, N1, N3, N4, J5, K2, S1 and S2), derived from the liver of diseased tilapia isolated in Bangladesh and resulting in each sample a clean single band 491 bp (Fig. 3a), indicates 9 samples are TiLV positive. A one-step RT-PCR using representative 6 RNA samples (R6, F2, N1, J5, K2 and S1) derived from the head kidney tissues of tilapia and 6 no template control (NTC) from the same areas with the same primers as in Fig. 3a as shown in Fig. 3b. This detection assay was determined that samples R6, F2, N1, J5, K2 and S1 are TiLV positive while 6 NTCs did not generate any PCR products at the correct size (Fig. 3b).

RT-qPCR amplification efficiency (E) and sensitivity: The amplification curve was consistent over the range of the 10 p-TiLV dilutions indicating that SYBR Green I dye was optimized in RT-qPCR assay has a wide linear range from 2.98×10^{1} to 2.98×10^{10} of TiLV per reaction, while the NTC produced no fluorescence and thus no amplification. The SYBR Green I dye qPCR confirmed the amplification of TiLV genome in ten-fold serial dilutions and expressed the linear ground phase, early exponential phase, late exponential phase and the plateau phase. The cDNA quantities produced an insufficient signal with no duplication identified in the linear ground phase; after that, target cDNA starts to double in concentration with inducing detectable signal and increase exponentially. The amplification efficiency was very high (near 100%) in the beginning reactions and remain stabled during the early exponential phase. A standard curve was generated by plotting log copy number versus cycle threshold for each dilution (Fig. 4), resulting -3.3576 slope, which was extrapolated to give efficiency of 98.5%. The coefficient of determination R^2 (0.9767) indicating that there was a high correlation between cycle number and dilution factor for each dilution series.

Phylogenetic tree and sequence alignment of target genes of TiLV: The amplified PCR products of the target genes with TiLV-positive F2 strain was sequenced received 100, 98 and 97% identity (Sequence ID: MK425012.1, MK392374.1, KU751816.1, NCBI, BLAST). To construct phylogenetic relationship, 17 TiLV sequences (including TiLV 10-segments sequences) were chosen from GeneBank to compare with target genes sequence, derived from a common ancestor while closely related with MH338228.1 (Columbia), MF582636.1 (India), MH213048.1 (Malaysia) and MH213039.1 (Thailand) in Fig. 5. The highest pair-wise alignment score for the sequence of TiLV segments of TiLV-positive strain F2 was 90.20 for MH338228.1 (Columbia), 85.57 for MF582636.1 (India), 85.30 for MH213048.1 (Malaysia) and 86.93 for MH213039.1 (Thailand) aligned with seventeen reference sequences from GeneBank (Fig. 6). Variations included a missing nucleotide position in the sequence of all target genes in this study analyzed by ORF finder, RCSB PDB, using Smart BLAST (CDD), NCB[35-37]. All the sequences in the 28th position of nt "a" showed the highly conserved and 1st position of the nt for all sequences showed low conserved regions (Fig. 6).

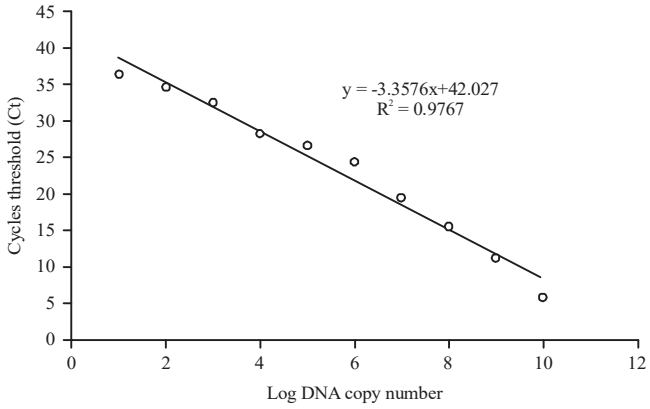

Fig. 4: SYBR Green I dye based RT-qPCR showed standard curves. Amplification curves and ten-fold serial dilutions (dilution labelled 2.98×10^{1} to 2.98×10^{10} copies respectively) of TiLV-positive samples, RT-qPCR standard curves, ten serially diluted p-TiLV samples were tested, all of the known concentration and correlated to the number of TiLV copies/reaction, standard curve was generated by plotting log copy number vs. cycle threshold (Ct), Slope (m): -3.3576, R^2: 0.9767 and the PCR efficiency is 98.5%

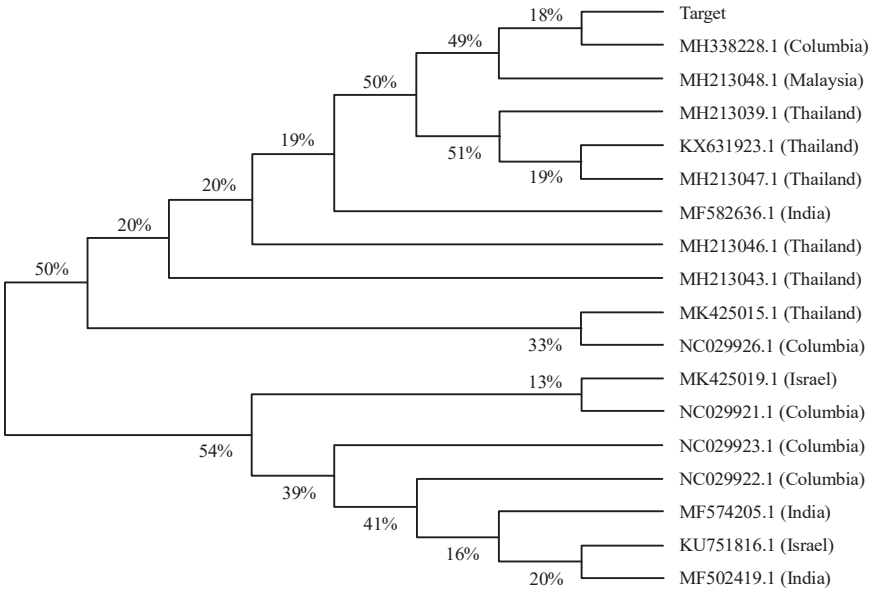

Fig. 5: Phylogenetic tree of TiLV-positive F2 strain as a representative strain and the reference strains. The Maximum Likelihood method and Kimura (Kimura, 1980) 2-parameter model shows the phylogenetic relationships among the sequence of TilV genomic segment 3 isolated from TiLV-positive strain and closely related sequences downloaded from GenBank. All clades are derived from different traits with low genetic distance. The number at each branch points represents the percentage supported by bootstrap. Bar: 0.05% sequence divergence. The scale bar represents 0.5 nucleotide change/position

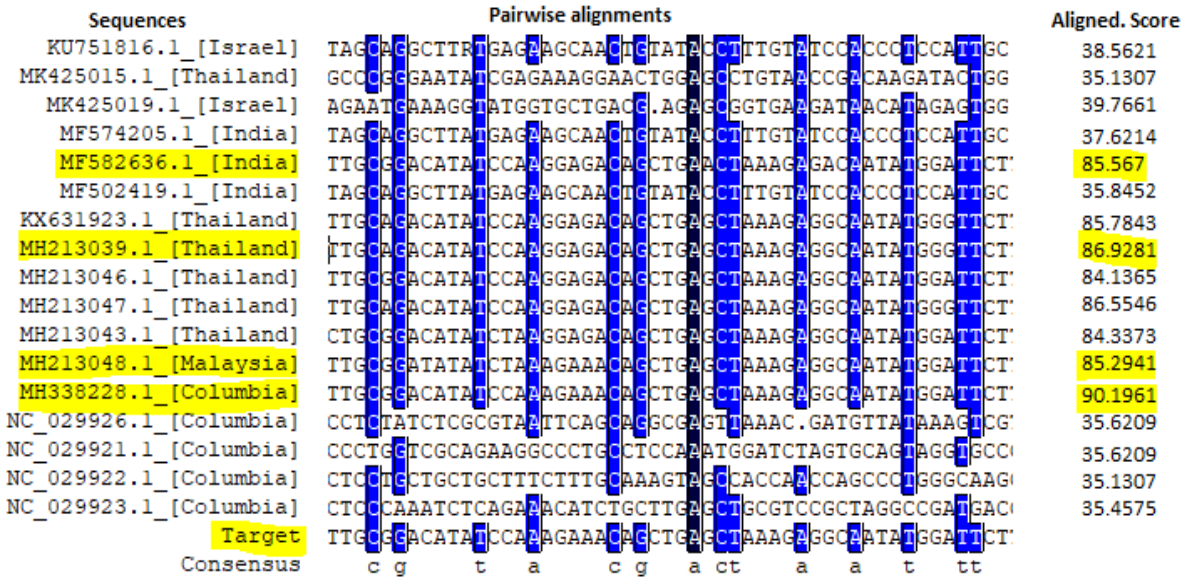

Fig. 6: Multiple sequence alignment and pairwise alignment score (right) of TilV genomic segment 3 sequence with the reference sequences of TiLV

DISCUSSION

In this study, the RNA and cDNA synthesis of TiLV infected tilapia isolated to detect followed the methods of complementary techniques of PCR based approaches such as reverse-transcription polymerase chain reaction (RT-PCR) and RT-quantitative (q) PCR using SYBR Green I dye. The software analysis and graphical presentations of all genotypic data revealed that the primer pairs detected the etiological agent of this disease, a novel RNA virus, was reported and procedures allowing its clinical signs lethargy, skin erosion, exophthalmia, detached scales with 15-82% morbidity rate. The cDNA sample derived from the liver of diseased tilapia using a diagnostic RT-PCR amplified a 491-bp fragment of segment 3. The 6 RNA samples obtained from the head kidney of diseased tilapia amplified a 491 bp fragment from segment 3. The standard curve and ten-fold serial dilutions showed PCR amplification efficiency of 98.5% over a wide linear range of 2.98×10^{1} to 2.98×10^{10} TiLV copies, while the NTC (no template control) produced no fluorescence and therefore no amplification. The amplified PCR products of the target genes of one TiLV-positive strain was sequenced received 100, 98 and 97% identity and resulting a common ancestor while closely related with Columbia, India, Malaysia and Thailand in phylogenetic relationship. The highest pair-wise alignment score was received 90.20 and diagnosed the mono-sex Nile tilapia was not only infected with common fish pathogens, such as *Aeromonas* and *Streptococcus* but also highly infected TiLV.

In previously, TiLV was first reported in Israel[9], it has been identified in multiple countries including Israel[9], Ecuador[10], Colombia[13], Egypt[24], Taiwan[15], Philipines[16], Thailand[22], Malaysia[18,38], India[19], Tanzania, Uganda[20], Peru[21] and Bangladesh[23,26]. To prevent and to control of TiLV in tilapia producing countries a detailed protocol for TiLV detection in tilapia tissue, covering sample collection, RNA isolation, cDNA synthesis, PCR and qPCR assays has been explained. TiLV has been identified in fish spanning a variety sizes[9,14,20,21,28] and species of tilapia including farmed hybrid tilapia (*O. niloticus*×*O. aureus*)[9,16], Nile tilapia (*O. niloticus*)[9,10,19,20,22,24,25,28], red tilapia[22,25], wild Nile tilapia[20,21], black tilapia[16] and very recently TiLV was identified in wild carp (*Barbonymus schwanenfeldii*)[38].

The outbreaks of TiLV had been reported during the hot season, namely May to October (at water temperatures of 22-32°C) in Israel[9], June to October (over 25°C) in Egypt[14] and May to November (25-27°C) in Ecuador[10]. In the present study, clinical outbreaks have been reported during the hot season, namely April to August at water temperatures of 25-31°C in Bangladesh. Some samples yielding positive TiLV detection in Thailand were collected in the months between October and May[25]. In Egypt, large farm size, high stocking densities, tilapia-mullet poly culture have been identified as risk factors for TiLV outbreaks[15]. The results of the research indicated that the large farm size, intensive aquaculture systems, high stocking densities, tilapia-carp poly culture, live tilapia entering in the country, microbial agents interacting with the host, pathogens reduced fitness of the host, vertical transmission, nucleus

breeding centre, multiple hatcheries and total production systems have been reported as risk factors for TiLV outbreaks in Bangladesh. The mortality was reported in previously ranging a low level of 10-20% and high level of 80% at 4-7 days in affected grow out ponds in Ecuador[10]. In this study, detected TiLV in fingerlings within 10-15 days post transfer to grow-out ponds, with mortality ranging from a low level of 15-20% to a high level of 82%. Variations in mortality have been have been found ranging from around 15% in farms with mixed stocking to around 82% in farms with mono-sex Nile tilapia monoculture in Bangladesh. Similarly report was obtained from Thailand, the mortality around 20% was observed in mixed culture farms with red tilapia and Nile tilapia in earthen pond in Phetchaburi province and 90% in farms with Nile tilapia in Pathum Thani province and farms with red tilapia in Chai Nat province[22].

In this study, it is clear that the co-infections in TiLV-positive fish from Bangladesh were *Aeromonas* and *Streptococcus* and some external and internal parasites. In Thailand, reported that co-infections with bacteria (*Flavobacterium*, *Aeromonas* and *Streptococcus*) and parasites (*Gyrodactylus*, *Dactylogyrus* and *Trichodina*) in TiLV-positive fish showed mortality level up to 80% in experimental populations[9]. TiLVD is an emerging and highly contagious disease caused by a novel orthomyxo-like virus in tilapia[30]. This study aimed to develop a SYBR Green-based RT-qPCR assay for the specific and fast detection of TiLV. The genomic segments-3 of TiLV as its stands was focused, this region of TiLV has the highest amount of genomic information publicly available[9,19,29]. Comparison of SYBR[28] and TaqMan qPCR[30] protocols revealed that both qPCR methods quantified cDNA prepared from infected materials with unbiased quantification of viral copy number (paired Student's t-test; $p>0.05$). The TiLV TaqMan and SYBR green RT-qPCR assays generally considered more specific and performed equally well, as reported by other comparing TaqMan and SYBR Green assays[30,39].

The disease distribution pattern of TiLV in some regions of Bangladesh is not known clearly, this may be due to international trade involving tilapia as TiLV hosts. Although the TiLV variants showed clear geographical boundaries based on molecular markers, the distribution pattern of this disease remain unclear. Ongoing detection and monitoring is urgently needed to determine the current condition associated with the presence of TiLV in the tilapia and the possibility of TiLV to infect other freshwater fish outside of the specific host, as we know that the evolutionary of TiLV to infect the other fish is not known yet. Implementation of strict biosecurity and quarantine measures is also indispensable especially to prevent the spread of TiLV to other district of Bangladesh. As a recommendation, the conventional RT-PCR was commonly used for TiLV diagnosis[13,18,19,20,25,27,28,40], while RT-qPCR was detected and quantification of small amounts of TiLV in tissue or mucus[31,40] for generating precise results[41] and with high sensitivity, specificity, good reproducibility, dynamic range and speed[42] as well as in epidemiological studies of TiLV.

CONCLUSION

This study will help to broaden our understanding of the genetic structure, pathology and pathogenicity, RNA fragment amplifications and their efficiency analysis, phylogenetic relationship and detection of pair-wise alignment score of TiLV in tilapia. In this study, it was clearly delivered a sensitive, rapid and specific SYBR Green-based Rt-qPCR assay for the detection of TiLV in farm samples. The development of high-performance diagnostic tools is of utmost importance for the implementation of screening and containment measures. It can be concluded that this information's on molecular detection of SYBR RT-qPCR method might be of interest as a starting point for diagnostic purposes aiming to better understand of TiLV to control or eradicate the TiLV along with common pathogenic bacteria in farmed tilapia aquaculture system of Bangladesh.

SIGNIFICANCE STATEMENT

This study discovers a novel RNA virus allowing its clinical signs lethargy, skin erosion, exophthalmia, detached scales with 15-82% morbidity, detection of TiLV was followed by complementary techniques of PCR, RT-PCR and RT-qPCR using SYBR Green I dye. This study also revealed the a 491 bp fragment of segment 3 by RT-PCR amplification, 100, 98 and 97% identity with the sequence of TiLV-positive strain and resulting a common ancestor while closely related with Columbia, India, Malaysia and Thailand in the phylogenetic relationship of seventeen TiLV sequences. This newly developed SYBR Green-based RT-qPCR assay might be an essential tool for TiLV diagnostics, that can be beneficial for fisheries community (hatchery, wild and pond aquaculture) in Bangladesh to control this diseases. This study will help the researcher to uncover the critical areas of molecular detection and genetic analysis that many researchers were not able to explore in Bangladesh. This new approach can be applied to data in several science fields and which can help reduce the drug use by farmers to control TiLV and also complete eradication using all genetic information's may have arrived.

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