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RESEARCH ARTICLE

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Tilapia Lake Virus was not detected in non-tilapine species within tilapia polyculture systems of Bangladesh

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Abstract

Sixteen countries, including Bangladesh, have reported the presence of tilapia lake virus (TiLV), an emerging tilapia pathogen. Fish polyculture is a common farming practice in Bangladesh. Some unusual mortalities reported in species co-cultivated with TiLV-infected tilapia led us to investigate whether any of the co-cultivated species would also test positive for TiLV and whether they were susceptible to TiLV infection under controlled laboratory experiments. Using 183 samples obtained from 15 farms in six districts across Bangladesh, we determined that 20% of the farms tested positive for TiLV in tilapia, while 15 co-cultivated fish species and seven other invertebrates (e.g. insects and crustaceans) considered potential carriers all tested negative. Of the six representative fish species experimentally infected with TiLV, only Nile tilapia showed the typical clinical signs of the disease, with 70% mortality within 12 days. By contrast, four carp species and one catfish species challenged with TiLV showed no signs of TiLV infection. Challenged tilapia were confirmed as TiLV-positive by RT-qPCR, while challenged carp and walking catfish all tested negative. Overall, our field and laboratory findings indicate that species used in polycultures are not susceptible to TiLV. Although current evidence suggests that TiLV is likely host-specific to tilapia, targeted surveillance for TiLV in other fish species in polyculture systems should continue, in order to prepare for a possible future scenario where TiLV mutates and/or adapts to new host(s).

KEYWORDS

Bangladesh, carp species, nile tilapia, polyculture, susceptibility, TiLV, walking catfish

1 | INTRODUCTION

Tilapia lake virus (TiLV) is an enveloped, negative-sense, single-stranded RNA virus containing 10 genome segments ranging from 465 to 1641 bp, with a total genome size of 10,323 kb (Eyngor et al., 2014; Bacharach et al., 2016). The virus was first classified as a novel orthomyxo-like virus, but has now been classified as *Tilapia tilapinevirus*, the only species in the *Tilapinevirus* genus, and placed in the new *Amnoonviridae* family (Bacharach et al., 2019). TiLV is a highly contagious pathogen that could jeopardize the growth of the tilapia industry worldwide (Bacharach et al., 2016; Jansen et al., 2019). TiLV outbreaks purportedly cause mortality in the range of 20%–90% (Dong, Siriroob, et al., 2017; Jansen et al., 2019; Surachetpong et al., 2017). To date, TiLV has been detected and reported across Asia, Africa and North and South America in 16 tilapia-producing countries: Ecuador, Israel, Colombia, Thailand, Uganda, the United Republic of Tanzania, Egypt, India, Indonesia, Chinese Taipei, the Philippines, Malaysia, Peru, Mexico, the United States and Bangladesh (FAO, 2019; Jansen et al., 2019; Surachetpong et al., 2020).

In early 2017, in response to the rapid spread of TiLV, several international organizations issued and disseminated disease advisory alerts and information about the virus (CGIAR, 2017; FAO, 2017; NACA, 2017; OIE, 2017). At the time, it was expected that TiLV would have spread through the translocation of live tilapia for aquaculture in over 40 countries, including Bangladesh (Dong et al., 2017). As the fifth largest tilapia producer, since 1954, Bangladesh has been importing seeds and tilapia broodstock from various sources including Malaysia, Thailand and the Philippines (Rahman, 1985). In Bangladesh, TiLV was first detected from sick Nile tilapia in 2017, but the findings were only published recently (Chaput et al., 2020; Debnath et al., 2020; Hossain et al., 2020). The results from these three studies in Bangladesh (from 2017 to 2019) revealed the presence of TiLV in 13 of 64 districts. In the absence of adequate hatcheries and farms biosecurity and regular screening of live animals during production and before movement between production sites, TiLV may persist and continue to affect tilapia and, perhaps, new species in new locations across the country.(Debnath et al., 2020).

The foremost aquaculture production systems in Bangladesh are extensive, semi-intensive and small-scale pond-based polyculture systems (Belton & Azad, 2012). Pond polyculture systems in Bangladesh are typically optimized to produce multiple fish species together, generally tilapia, carps and catfish (Castine et al., 2017). While tilapia production is high in Bangladesh, carp species are the primary culture crop, with tilapia serving as a surplus crop. Carp species accounted for 33.5% of entire aquaculture production (fiscal year 2018–19), with a total production volume of 1.47 million metric tonnes (DoF, 2019). The total value of carp produced is estimated to be USD 2.94 billion using an average market price of USD 2/kg for 1–1.5 kg/fish. Prominent carp species farmed in Bangladesh include rohu (*Labeo rohita*), catla (*Catla catla*), mrigal (*Cirrhinus cirrhosis*), silver carp (*Hypophthalmichthys molitrix*), grass carp (*Ctenopharyngodon idella*), common carp (*Cyprinus carpio*), bighead carp (*Hypophthalmichthys nobilis*), mohashol (*Tor putitora*) and black carp (*Mylopharyngodon piceus*). Prominent catfish species include striped catfish, locally called pangas. Without further evidences to prove the contrary, TiLV must be considered as a potential threat to Bangladesh polyculture systems, with the virus's possible ability to adapt and spread from tilapia to other non-tilapine species. There are several examples of fish viruses that have jumped from one fish species to another. Infectious pancreatic necrosis virus (IPNV) and nervous necrosis virus (NNV) are notable examples of RNA viruses in fish, whereas the infectious spleen and kidney necrosis virus (ISKNV) is a good example of a DNA virus. IPNV was first isolated from a diseased rainbow trout (*Oncorhynchus mykiss*) fingerling and later discovered worldwide in a wide host range of diseased and non-diseased salmonid/non-salmonid fish species and invertebrates (Hill & Way, 1995; Reno, 1999). Viral nervous necrosis (VNN) caused by *Betanodavirus* was first reported in Australian farmed barramundi (*Lates calcarifer*), and a year later, in turbot (*Scophthalmus maximus*), European sea bass (*Dicentrarchus labrax*), red-spotted grouper (*Epinephelus akaara*) and striped jack (*Pseudocaranx dentex*) (Glazebrook et. al., 1990; Breuil et al., 1991; Yoshikoshi & Inoue, 1990; Bloch et al., 1991; Mori et al., 1992; Munday et al., 2002;). ISKNV has been detected from both freshwater and euryhaline fish species, including tilapia (*O. niloticus*) and farmed barramundi (*L. calcarifer*) (Suebsing et al., 2016; Dong, Jitrakorn, et al., 2017). To date, there are a number of tilapia species known to be susceptible to TiLV, including hybrid tilapia (*O. niloticus* ×*O. aureus* hybrids), Nile tilapia (*O. niloticus*), grey tilapia (*O. niloticus* ×*O. aureus*), red tilapia (*Oreochromis* sp.), Mozambique tilapia (*O. mossambicus*), mango tilapia (*Sarotherodon galilaeus*), redbelly tilapia (*Tilapia zillii*), blue tilapia(*O. aureus*) and wild tilapia (*Tristamellasimonis intermedia*) (Eyngor et al., 2014; Ferguson et al., 2014; Fathi et al., 2017; Surachetpong et al., 2017; Mugimba et al., 2018; Waiyamitra et al., 2021). In addition to tilapia, giant gourami (*Osphronemus goramy*) naturally infected with TiLV have been found (Chiamkunakorn et al., 2019) and also shown to be susceptible to TiLV in an experimental challenge study (Jaemwimol et al., 2018). TiLV has also been identified in wild tinfoil barb (*Barbonymus schwanenfeldii)* in Malaysia (Abdullah et al., 2018) as well as in farmed barramundi (*L. calcarifer*) in Thailand (Piamsomboon & Wongtavatchai, 2021). In Israel, Egypt and India, there have been no reports of TiLV detected in co-cultivated species during TiLV outbreaks in tilapia (Eyngor et al., 2014; Fathi et al., 2017; Behera et al., 2018). There is still a scarcity of information about the host range of TiLV.

Bangladesh is one of the very few countries where tilapia, carp and catfish species are produced together by small-scale farmers in semi-intensive, homestead and backyard ponds. During unusual and unexplained disease outbreaks in species produced in polyculture systems, there are often suspicions among farmers that TiLV might be the cause of the mortalities in species other than tilapia. Here, we investigated whether co-cultivated species were TiLV-positive in tilapia polyculture farms experiencing abnormal mortalities and also conducted controlled laboratory TiLV experiments with various carp species and walking catfish to assess their susceptibility to the virus. This research was set to investigate the TiLV status and

TiLV susceptibility of non-tilapine species co-cultured with tilapia. Monoculture of tilapia is very rare in Bangladesh. Majority of freshwater farming systems undertake polyculture, and it is very common to see tilapia raised with carps, catfish, among other species. Our preliminary results show that—in a limited number of tilapia polyculture farms experiencing abnormal mortalities and under experimental conditions—non-tilapine species were negative and not susceptible to TiLV. If future evidences point towards susceptibility of carps or catfish species to TiLV, this will have major implications to small-holder Bangladeshi farmers, management and biosecurity risk mitigation for the industry including legislating against polyculture of tilapia. Therefore, pursuing this line of research is very important for the country and also for the National Competent Authorities.

2 | MATERIALS AND METHODS

2.1 | Field sample collection and preservation

The utilization of fish in this investigation was approved by the Animal Care and Use Committee of the National University of Malaysia (approval no. UKM.PPI.AEC.800-4/3/1). Field samples were collected from 15 polyculture farms from 2017 to 2020, where mortalities for tilapia and other co-cultivated species were documented. A total of 183 samples belonging to 23 species of fish, crustaceans and insects were collected from 15 polyculture farms (Table S1). Samples of the affected stock included moribund fish, along with crustaceans and insects from the same ponds, while samples of the non-affected stock were clinically healthy fish. The 15 affected farms were located in six districts of Bangladesh, including Cumilla, Chandpur, Chittagong, Jashore, Satkhira and Gazipur (Table 1). For each fish, we collected and pooled a small piece (approximately, 5 × 5 × 5 mm) of liver, kidney, spleen and brain. For crabs, snails and bivalves, a small piece of muscle was collected. For small shrimp, copepod and insects, whole specimens were taken. All of these tissues were preserved in RNAlater (Qiagen) for reverse transcription–polymerase chain reaction (RT-PCR) analyses.

2.2 | Experimental challenge

For the challenge experiment, we used four carp species and one catfish species commonly stocked with tilapia by polyculture farmers in Bangladesh. These were rohu (*L. rohita*), silver carp (*H. molitrix*), mrigal (*Cirrhinus cirrhosus*), mohashol or Putitor mahseer (*Tor khudree*) and walking catfish (*Clarias batrachus*). Nile tilapia (*O. niloticus*) was used as our positive control (Table 2). The number of fish used for each species was 20 for challenge and 20 for control groups, with the exception of mrigal and walking catfish, which had a lesser number of fish utilized due to a shortage of the required number of fish at the time of the experiment (Table 2). All of the fish utilized in this experiment were of approximately similar size (5 ± 1 cm). All non-tilapia species were sourced from a commercial Thai hatchery that was not linked with any past tilapia seed production. Tilapia were sourced from a known TiLV-negative population. All fish species were shipped to the laboratory in temperature-controlled boxes supplied with oxygen. Upon arrival to the laboratory, all fish were disinfected using 5 parts per thousand (ppt) salt water for 30 min and then left to acclimatize for 2 h in 500 L freshwater holding tanks within a quarantine room. Following the period of disinfection and acclimatization, individual species were stocked in separate 200 L fibreglass tanks with air stone and biological cotton filter units. Cotton filters were exchanged once every three days, while water was replaced with new tap water disinfected with 60 parts per million (ppm) chlorination at the rate of 50%. Prior to the infection trial, the fish were conditioned within their respective tanks for an additional seven days and fed twice daily with a commercial feed containing 28% protein at a rate of 5% body weight. Water quality parameters for the period of the experiment were recorded as were kept as follows: temperature (28,128 ± 1)°C, pH 7.6–8.4, dissolved Oxygen 8 mg/L, NH3 <3 mg/L and NO2 <1 mg/L. The original TiLV stock, NV18R, was prepared as previously described (Dong et al., 2020). All fishes were divided into two groups—control and experimental groups—with one replicate tank per species (Table 2). All fish were anaesthetized using 100 ppm clove oil before being injected with 0.1 ml TiLV inoculum intraperitoneally at a dose of 10^{-6} $TCID_{50}$ per fish. Control fish were injected with 0.1 ml 1× phosphate-buffered saline (PBS), pH 7.4, and stocked separately. All fish were returned to their original tank and monitored four times per day for the typical clinical signs of TiLV disease. Any moribund fish was immediately killed by overdose with 250 ppm clove oil and small pieces (approximately, 5 × 5 × 5 mm) of liver, kidney, spleen and brain were collected and pooled for RT-qPCR, as previously described (Debnath et al., 2020). After 21 days post-infection, all remaining surviving fish from both groups were humanely killed and subjected to sampling, as described above, for RT-qPCR test.

2.3 | Total RNA isolation and PCR amplification for detection of TiLV

2.3.1 | Field samples tested by semi-nested RT-PCR

Following manufacturer's protocol, TRIzol reagent (Invitrogen) was used for total RNA extraction of pooled samples of liver, kidney, spleen and brain for each individual fish, crustacean, copepod and insect species. All field samples were subjected to semi-nested RT-PCR using TiLV genome segment 1 primers (Taengphu et al., 2020). The primers used were TiLV/nSeg1F: 5'-TCT GAT CTA TAG TGT CTG GGC C-3'; TiLV/nSeg1R: 5'-AGT CAT GCT CGC TTA CAT GGT-3'; and TiLV/nSeg1RN: 5'-CCA CTT GTG ACT CTG AAA CAG −3'. PCR master mix composition and thermocycling conditions were the same as described by Taengphu et al. (2020). A plasmid with a 620-bp fragment of the partial TiLV genome segment 1 (pGEM-620 bp) (Taengphu et al., 2020) was used as the positive control, and nuclease-free water served as the negative control. Expected

TABLE 1 Samples collected from fish farms experiencing abnormal mortalities in six districts of Bangladesh

Date–Month–Year[b]	Farm	Districts	Fish Species (Common Name)[c]	(%) Mortality	# Sample(s) Collected *	# TiLV-Positive/# Sample Tested (%)
3 September 2017	Farm 1	Satkhira	Corsula mullet	~5	3	0/3 (0)
			Tilapia	~30	8	2/8 (25)
3 September 2017	Farm 2	Satkhira	Corsula mullet	~10	4	0/4 (0)
			Tilapia	~50	5	1/5 (20)
10 January 2019	Farm 3	Jashore	Gonia	~10	4	0/4 (0)
			Rohu	~10	1	0/1 (0)
			Silver carp	~10	1	0/1 (0)
			Tilapia	~10	4	0/4 (0)
28 January 2019	Farm 4	Satkhira	Rohu	~5	3	0/7 (0)
			Tilapia	5–10	7	0/7 (0)
9 November 2019	Farm 5	Gazipur	Stinging catfish	~25	5	0/5 (0)
			Gulsha	~25	4	0/4 (0)
			Tilapia	~40	10	3/10 (30)
29 September 2020	Farm 6	Cumilla	Common carp	~5	1	0/1 (0)
			Snail[a]	No mortality	2	0/2 (0)
			Tilapia	~50	5	0/5 (0)
30 September 2020	Farm 7	Cumilla	Common carp	~5	2	0/2 (0)
			Rohu	~10	2	0/2 (0)
			Bighead carp[a]	No mortality	2	0/2 (0)
			Silver hatchet chela[a]	No mortality	1	0/1 (0)
			Climbing perch[a]	No mortality	1	0/1 (0)
			Small shrimp[a]	No mortality	1	0/1 (0)
			Crab[a]	No mortality	2	0/2 (0)
			Copepod[a]	No mortality	1	0/1 (0)
			Tilapia	~80	5	0/5 (0)
1 October 2020	Farm 8	Cumilla	Rohu[a]	No mortality	2	0/2 (0)
			Pangasius	~10	2	0/2 (0)
			Silver hatchet chela[a]	No mortality	1	0/1 (0)
			Flying barb[a]	No mortality	1	0/1 (0)
			Bivalve[a]	No mortality	2	0/2 (0)
			Damselfly larvae[a]	No mortality	2	0/2 (0)
			Water striders[a]	No mortality	2	0/2 (0)
			Tilapia	~80	5	0/5 (0)
2 October 2020	farm 9	Cumilla	Rohu	~10	1	0/1 (0)
			Pangasius	~5	2	0/2 (0)
			Flying barb[a]	No mortality	3	0/3 (0)
			Damselfly larvae[a]	No mortality	2	0/2 (0)
			Water striders[a]	No mortality	1	0/1 (0)
			Tilapia	~40	5	0/5 (0)
3 October 2020	Farm 10	Cumilla	Silver carp[a]	No mortality	1	0/1 (0)
			Rohu[a]	No mortality	1	0/1 (0)
			Pangasius	~5	2	0/2 (0)
			Damselfly larvae[a]	No mortality	2	0/2 (0)
			Water spider[a]	No mortality	2	0/2 (0)
			Tilapia	~70	5	0/5 (0)
14 October 20	Farm 11	Chandpur	Mrigal[a]	No mortality	1	0/1 (0)
			Silver barb[a]	No mortality	2	0/2 (0)
			Tilapia	~30	5	0/5 (0)

(Continues)

TABLE 1 (Continued)

Date–Month–Year[b]	Farm	Districts	Fish Species (Common Name)[c]	(%) Mortality	# Sample(s) Collected *	# TiLV-Positive/# Sample Tested (%)
16 October 2020	Farm 12	Cumilla	Rohu	~10	4	0/4 (0)
			Climbing perch[a]	No mortality	2	0/2 (0)
			Tilapia	~60	5	0/5 (0)
17 October 2020	Farm 13	Cumilla	Silver barb[a]	No mortality	2	0/2 (0)
			Bata labeo[a]	No mortality	2	0/2 (0)
			Rohu[a]	No mortality	2	0/2 (0)
			Common carp	~40	1	0/1 (0)
			Climbing perch[a]	No mortality	1	0/1 (0)
			Tilapia	~50	5	0/5 (0)
18 October 2020	Farm 14	Cumilla	Silver carp[a]	No mortality	2	0/2 (0)
			Silver barb[a]	No mortality	2	0/2 (0)
			Rohu	~5	2	0/2 (0)
			Common carp	~20	2	0/2 (0)
			Pangasius[a]	No mortality	1	0/1 (0)
			Bata labeo[a]	No mortality	1	0/1 (0)
			Tilapia	~80	5	0/5 (0)
19 October 2020	Farm 15	Chittagong	Rohu	~5	2	0/2 (0)
			Common carp	~10	1	0/1 (0)
			Tilapia	~40	5	0/5 (0)
					183	6/183 (3.3)

*DIFFERENT number of samples collected per fish and per farm, due to a limited number of moribund fish available at time of sampling.
[a]Sample found to be clinically healthy.
[b]In 2018, no sampling was carried out.
[c]Scientific name for all of the species mentioned in Table S1.

amplicon sizes from the first and nested reactions were 620 and 274 bp, respectively. The amplified products were electrophoresed in 1.5% agarose gel and stained with ethidium bromide.

2.3.2 | Experimentally TiLV-challenged animals tested by RT-qPCR

In addition to TiLV detection from field samples by semi-nested RT-PCR, TiLV in tissue samples from experimentally TiLV-challenged animals was detected using a newly developed quantitative one-step RT-qPCR protocol targeting TiLV genome segment 9 (Taengphu et al. 2021). This method was used as it offers quantifiable results which can describe the potential multiplication of the virus inside fish cells. TaqMan primer sequences for TiLV segment 9 were as follows: forward primer, Seg9-TaqMan-F, 5'-CTA GAC AAT GTT TTC GAT CCA G-3'; reverse primer, Seg9-TaqMan-R, 5'-TTC TGT GTC AGT AAT CTT GAC AG-3'; and probe primer, Seg9-TaqMan-Probe, 5'-6-FAM-TGC CGC CGC AGC ACA AGC TCC A-BHQ-1–3', with a product size of 137 bp. The one-step RT-qPCR was carried out in a 20 μl volume, which included 10 μl of 2X qScript™ XLT 1-Step RT-qPCR ToughMix Low ROX (QuantaBio, Beverly), 0.9 μl each of 10 μM forward primer (450 nM) and 10 μM reverse primer (450 nM), 0.3 μl of 10 μM TaqMan probe (150 nM), 2 μl of RNA template (100 ng/μl) and 5.9 μl of RNase-free water. Amplification was performed at 50°C for 10 min, followed by 95°C for 1 min and 40 cycles at 95°C for 10 s and then 58°C for 30 s.

3 | RESULTS

3.1 | Field samples

All investigated polyculture farms (n = 15), from the six considered districts, experienced abnormal mortality in tilapia first, that is before other co-cultivated species were affected. Out of 15 co-cultivated fish species and seven other aquatic organisms, only seven species (i.e. corsula mullet, gonia, rohu, silver carp, Asian stinging catfish, gulsha and common carp) were found to experience abnormal mortalities along with tilapia. Clinical signs observed in moribund fish from affected polyculture farms are shown in Figure 1. Swollen eyes, lesions on body surface, ascitic fluid, scale protrusion, haemorrhagic skin and loss of appetite are the major clinical signs in tilapia (Figure 1). Mortality in tilapia from all 15 affected farms ranged from 10% to 80%, with mortality in farms testing positive for TiLV (n = 3 farms) ranging from 30% to 50% (Table 1). Clinical signs found in affected co-cultivated species included lesions on the opercula, jaw and body surface, haemorrhagic skin, and fin rot and tail rot, with mortality ranging from 5% to 40%, depending on the farm and species (Table 1 and Figure 1).

TABLE 2 Experimental challenge test results of Nile tilapia, rohu, mohashol, silver carp, mrigal and catfish injected with TiLV NV18R isolate at a dose of 10^{-6} $TCID_{50}$ per fish in the peritoneal cavity

Fish Species	Photograph and Body Length (cm)	Group	Number of Fish Used	Mortality	RT-qPCR Test Result (+ve/ Tested Samples)	Viral Loads (Copies per Reaction)
Nile tilapia (*Oreochromis niloticus*)		PBS	20	1	0/5	0
		TiLV	20	14	9/10	6.12×10^5–2.35×10^8
Rohu (*Labeo rohita*)		PBS	20	0	0/5	0
		TiLV	20	0	0/10	0
mohashol or Putitor mahseer (*Tor khudree*)		PBS	20	0	0/5	0
		TiLV	20	0	0/10	0
Silver carp (*Hypophthalmichthys molitrix*)		PBS	20	0	0/5	0
		TiLV	30	0	0/10	0
Mrigal (*Cirrhinus cirrhosus*)		PBS	15	0	0/5	0
		TiLV	20	0	0/10	0
Walking catfish (*Clarias batrachus*)		PBS	7	0	0/5	0
		TiLV	8	3	0/8	0

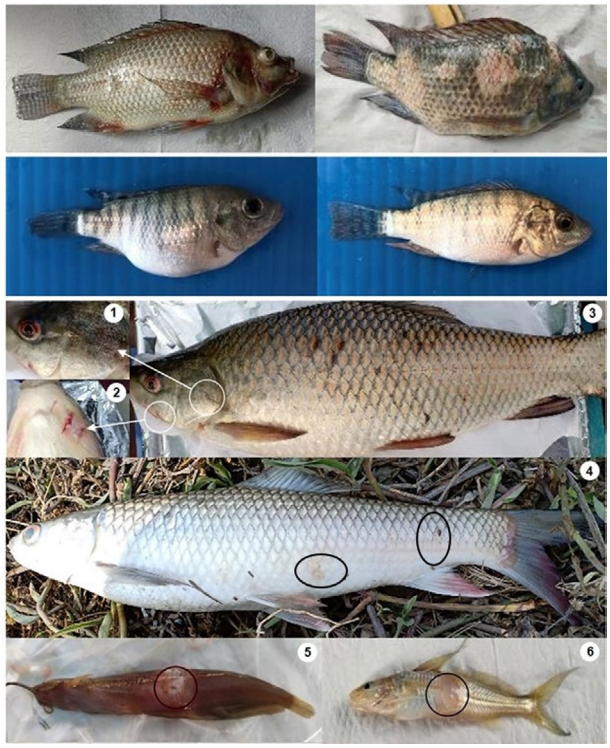

FIGURE 1 Pictures of the major clinical signs observed in moribund fish from affected polyculture farms and those experimentally challenged with TiLV: (a) field-collected Nile tilapia displaying swollen eyes, body lesions and haemorrhagic skin; (b) laboratory TiLV-injected tilapia with scale protrusion, swollen eyes and swollen abdomen; and (c) field-collected co-cultivated species (C1–3: carp, C4: mullet, C5–6: catfish), showing lesions on opercula, jaw, head region and body surface, as well as fin rot and tails with petechial haemorrhage

Some co-cultivated species in the affected farms were found to be clinically healthy, with no mortality or clinical signs observed (Table 1). The RT-PCR test results showed that samples from three out of 15 farms tested positive for TiLV, with 20%–30% of tilapia samples from these farms testing positive (Table 1 and Figure 2). By contrast, 99 non-tilapia samples collected from 15 co-cultivated species and seven other aquatic organisms on those same 15 farms were all negative for TiLV (Table 1). Representative test results are shown in Figure 2. TiLV-affected farms were identified in 2017 ($n = 2/2$) and 2019 ($n = 1/3$), but all farms sampled in 2020 ($n = 10$) were TiLV-negative (Table 1). Within the samples obtained from 2017 to 2020, 7% of tilapia samples (6 out of 84 tilapia samples) were positive for TiLV (Table S2).

3.2 | Carp and catfish species are not susceptible to TiLV under experimental challenge

None of the individual fish from the four carp species in both infected and control groups showed any clinical signs of TiLV disease manifestation, with no mortality observed until 21 days post-infection (DPI), when the experiment was terminated (Table 2). Of the walking catfish that were TiLV-challenged, three out of eight individuals (37.5%) died at 13 DPI (Figure 3), with no major clinical signs but observed some injured area in head region (Table 2). None of the control (PBS injected) walking catfish died (Table 2).

However, after 5 DPI, positive control Nile tilapia individuals injected with TiLV started to exhibit clinical signs, including anorexia, lethargy, bilateral exophthalmia, scale protrusion and abdominal swelling (Figure 1b). Final mortality (70%) in the infected tilapia group started at 6 DPI and continued until 12 DPI (Figure 3). No clinical signs were observed in any of the control tilapia individuals injected with PBS, with only one fish dying at 9 DPI. The RT-qPCR test results from 40 individual samples taken from 40 challenged fish of the four carp species were all TiLV-negative (40 out of 40), and similarly, all 20 individual samples collected from 20 fish of the same four carp species from the control group were also TiLV-negative (20 out of 20) (Figure 4, Table 2). Similarly, all eight walking catfish individuals (including the three dead individuals) from the challenged group and five from the control group were found to be TiLV-negative (Figure 4, Table 2). Ninety per cent (9 out of 10) of the tilapia individuals from the challenged group, where 8 samples were obtained from dead fish and one from surviving fish, were confirmed to be TiLV-positive by RT-qPCR, whereas none (0 out of 5) in the control group (Table 2, Figure 4) tested positive. For each TiLV-positive tilapia sample, the RT-qPCR result revealed a TiLV load of 6.12×10^5–2.35×10^8 copies per reaction containing 200 ng RNA template (Table 2).

4 | DISCUSSION

The use of polycultures consisting of multiple species (e.g. tilapia, carp, catfish, shrimp, prawn and others) is the most common production strategy in Bangladesh, as it is perceived by many farmers to be a strong resilient strategy to reduce production risks (DoF, 2019). Both extensive and semi-intensive homestead to entrepreneur commercial farming practices in Bangladesh follow polyculture farming. The choice of species for polyculture farming depends on the geographical location, water type, seasonality and market demand. Tilapia has always been considered as a hardy fish, capable of surviving and thriving in sub-optimal conditions but, with the intensification of its production globally, there have been an increasing number of pathogens shown to infect tilapia, with TiLV being one of them. In Bangladesh, TiLV was first identified in 2017 in tilapia farmed in the district of Mymensingh (Chaput et al., 2020) then in tilapia farmed in six districts (Hossain et al., 2020). Additional cases have been recently reported in five more districts (Bagerhat, Barguna, Cumilla, Cox's Bazar and Gazipur), in 2017 and 2019 (Debnath et al., 2020).

Among fish-farming communities and the competent authorities of Bangladesh, there is great concern that TiLV could spread to new geographies, not only affecting tilapia but potentially other major economically important co-cultivated species. Tilapia remains the major fish group susceptible to TiLV, while only rare

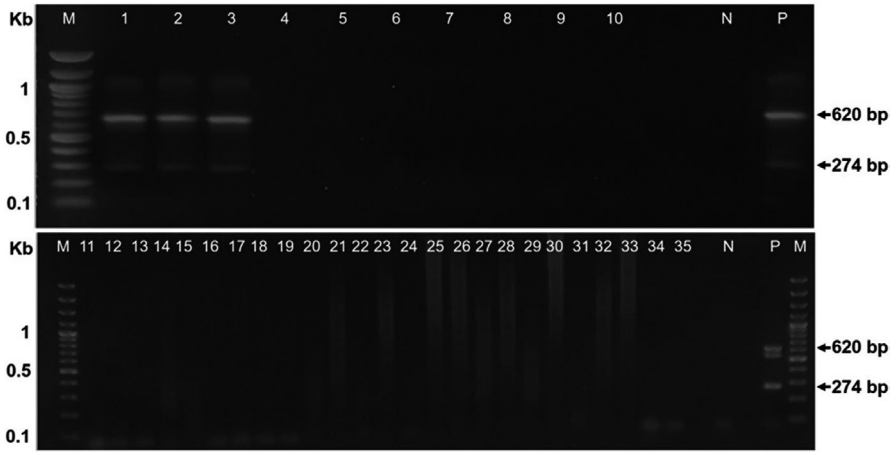

FIGURE 2 Analysis of 35 RT-PCR products acquired using TiLV semi-nested PCR primers electrophoresed on a 1.5% agarose gel. Lanes 1–10: field samples of tilapia; lanes 11–16: field samples of rohu; lanes 16–20: field samples of *Cyprinus carpio*; lanes 21–26: field samples of silver barb, lanes 27–32: field samples of Pangasius; lanes 32–35: field samples of damselfly larvae. M, DNA marker (New England BioLabs, Hitchin, United Kingdom); P, positive control using RNA extracted from TiLV-infected tilapia as template (note the presence of two bands at 620 and 274 bp); N, no RNA negative control, using nuclease-free water as template

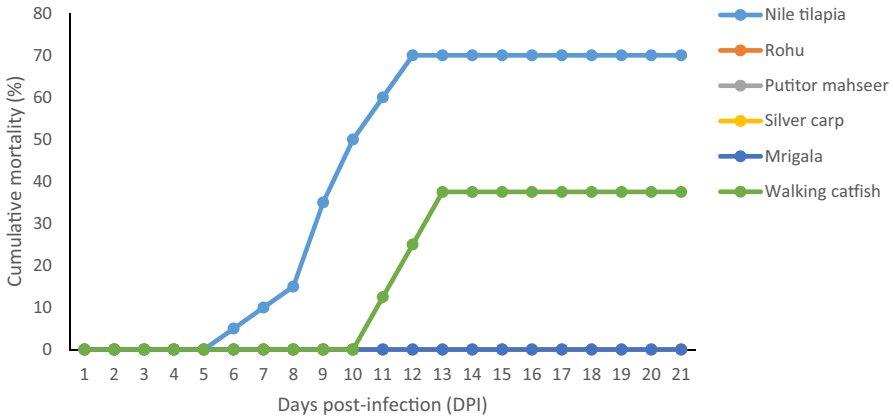

FIGURE 3 Cumulative mortality rate of all challenged fish species in TiLV challenge experiment. Number of fish used is summarized in Table 2

cases have been found in non-tilapia species, such as tinfoil barb (*Puntius schwanenfeldii*) farmed in Malaysia (Abdullah et al., 2018) and giant gourami (*Osphronemus goramy*) and barramundi (*Lates calcarifer*) farmed in Thailand (Chiamkunakorn et al., 2019; Piamsomboon & Wongtavatchai, 2021). Experimental evidence has also been provided that giant gourami (*Osphronemus goramy*) is susceptible to TiLV in response to challenge in a laboratory setting (Jaemwimol et al., 2018). With increased awareness and fear among producers regarding the potential impact of TiLV, increased numbers of abnormal mortality events have been reported by farmers for tilapia, carp and catfish, but in the absence of proper disease investigation with sample collection for diagnostic purposes, those mortalities tend to be incorrectly attributed to TiLV by farmers, often leading producers to use inadequate chemical and drug treatments.

Both our field and challenge findings revealed no evidence of TiLV infection in co-cultured fish species or other aquatic organisms such as crustaceans and insects. During the challenge experiment, no mortality was recorded in carp, while 37.5% mortality was observed in walking catfish; however, the TiLV test results revealed that these samples were TiLV-negative. This unexpected mortality in walking catfish might be attributed to the fact that these species were aggressive and fought each other, leading to the death of some individuals. In Israel, during TiLV outbreaks in tilapia, other co-cultivated species, such as grey mullet (*Mugil cephalus*) and carp (*Cyprinus carpio*), did not show the clinical signs of TiLV with no mortalities recorded (Eyngor et al., 2014). Similar observations were made in Egypt with co-cultivated grey mullet (*M. cephalus*) and thin-lipped mullet (*Liza ramada*) (Fathi et al., 2017), and in India with co-cultivated Indian major carps, including rohu (*Labeo rohita*), catla (*Catla catla*), mrigal (*Cirrhinus cirrhosus*), milk fish (*Chanos chanos*) and pearl spot (*Etroplus suratensis*) (Behera et al., 2018). Supporting confirmations were found in Indian major carp (rohu), which were shown not to be susceptible to TiLV infection (Pradhan et al., 2020). Additionally, a TiLV experimental challenge in 10 warm-water fish species, including giant gourami (*Osphronemus goramy*), snakeskin gourami (*Trichogaster pectoralis*), iridescent shark (*Pangasianodon hypophthalmus*), walking catfish (*Clarias macrocephalus*), striped snakehead fish (*Channa striata*), climbing perch (*Anabas testudineus*), common carp (*Cyprinus carpio*), silver barb (*Barbodes gonionotus*), Asian sea bass (*Lates calcarifer*) and red hybrid tilapia (*Oreochromis* spp.), showed that all species, apart from giant gourami, were not

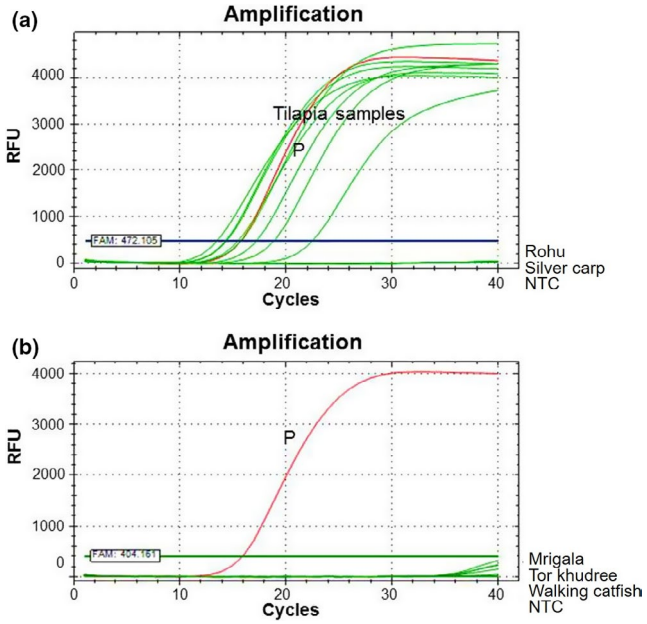

FIGURE 4 TiLV RT-qPCR results from TiLV experimental challenge samples. (a) Detection for TiLV in Nile tilapia, rohu and silver carp samples of both challenged and control groups. B) Detection in mrigal, *Tor khudree* and walking catfish samples. Tested specimen numbers are indicated in Table 2. P, positive control using RNA template extracted from TiLV-infected tilapia; NTC, no template control

susceptible to TiLV infection (Jaemwimol et al., 2018). On the other hand, adult zebrafish (*Danio rerio*) and Mozambique Tilapia (*Oreochromis mossambicus*) were found to be TiLV susceptible through experimental challenge (Rakus et. al., 2020; Waiyamitra et. al., 2021). Those studies are coherent and in support of our findings of our study: that all the co-cultivated species, along with other aquatic organisms (crustaceans and insects), were unlikely to benot susceptible to TiLV infection. In summary, both our field samples collected from outbreaks on farms in Bangladesh, along with our susceptibility experimental challenge findings, confirmed that those co-cultivated species, together with other aquatic organisms, presumably were not susceptible to TiLV. The lack of viral receptors or factors that allow the virus to enter and proliferate in these fish, crustacean and insect species may be one of the reasons for them being refractory to TiLV—a hypothesis proposed by Surachetpong et al. (2020). As a result, these species may have a limited probability of becoming TiLV carriers.

While 22 species from tilapia polyculture farms were examined in this study, we acknowledge the limitation in terms of number of samples collected per species and number of TiLV-affected farms. We also targeted only TiLV, while other infectious agents and/or possible environmental factors that may have been associated with the observed mortality were not explored. Most of the farms included in this study came from districts with previous reports of TiLV. Future disease investigations from affected polyculture farms should first confirm TiLV in tilapia and then test other species present on the farm; this with a sufficient number of samples from each species. Similarly, for future TiLV tests, relevant polyculture species can be challenged with new TiLV isolate retrieved from affected farms.

A cautionary approach should always prevail, as the nature of RNA viruses such as IPNV, avian influenza and SARS-CoV-2 can evolve rapidly and adapt to new host(s) (Hill & Way, 1995; Reno, 1999; Stallknecht & Shane, 1988; Wu et al., 2020). While in this initial investigation, co-cultivated species and other aquatic organisms were found to be apparently not susceptible to TiLV; further research and regular disease investigations are required to validate our observations. Until now, there is very little knowledge available regarding TiLV host range, evolution, transmission route and disease pattern, so it is very important that farmers and health experts continue to report and investigate the origins of those mortalities occurring in tilapia and co-cultivated species. TiLV needs to be kept in the priority list of potential pathogens as part of the national disease surveillance programme of Bangladesh and other countries where it has been reported. If implemented in the long term, this will minimize further spread of TiLV as well as limiting its potential transmission to other co-cultivated species in tilapia polyculture systems. Due our limitations in knowledge regarding the TiLV host range, evolution, transmission route and disease pattern, it is important that farmers and health experts continue to report and investigate abnormal mortalities occurring in tilapia and co-cultivated species, maintaining TiLV screening as part of the national disease surveillance both in Bangladesh and in other countries.

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CONFLICTS OF INTEREST

There are no conflicts of interest declared by the authors. The funders had no involvement in the study design, data collection, analysis, or interpretation, manuscript writing or the decision to publish the findings.

AUTHOR CONTRIBUTIONS

P. P. D., C. R. and H. T. D. conceptualized the study; P. P. D., J. D. D. and H. T. D. contributed to methodology; P. P. D. and H. T. D. validated the study; P. P. D., N. D. H., V. V. N. and S. T. contributed to investigation and formal analysis; C. V. M., C. R. and S. S. contributed to resources; P. P. D contributed to writing—original draft preparation; and all authors contributed to writing—review and editing; all authors have read and agreed to the published version of the manuscript.

DATA AVAILABILITY STATEMENT

The data used in this investigation are available in the article.

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SUPPORTING INFORMATION

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