



Massive Surveillance of Tilapia Lake Virus (TiLV) in Tilapia Farms and Feral from Various Districts of Tamil Nadu, India

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ABSTRACT

Background: Tilapia is one of the most widely consumed farmed fish due to high protein content and rapid growth. It is the second-most important group of farmed fish globally. Although tilapia is generally resistant to many diseases, Tilapia Lake virus (TiLV) has emerged as a devastating viral pathogen, affecting both wild and farmed tilapia. Early detection and surveillance of TiLV are crucial for effective disease management and prevention of mass mortalities. Despite its importance to assess the prevalence of disease in Tamil Nadu, a surveillance program was undertaken, focusing on tilapia grow-out farms and feral ("wild") populations across multiple districts.

Methods: *Oreochromis niloticus* (Nile tilapia) samples (102 Nos.) were collected from 34 sites across 11 districts in Tamil Nadu, India between February 2020 to May 2022. These sites include disease outbreak farms and feral area. Semi-nested reverse transcription PCR (RT-PCR), targeting segment 3, was used to screen and confirm TiLV infection. Positive samples were further sequenced using commercial sequencing services and phylogenetic analysis was performed using the Maximum-Likelihood method.

Result: 13 sites experienced higher than usual mortalities, of which 10 were tested positive for TiLV. Asymptomatic samples were collected from 21 sites, with 13 tested positive. Infected fish exhibited clinical signs and symptoms typical of TiLV, including open wounds, ulcers, lethargy, gasping at the water surface, discoloration, exophthalmia and mortalities ranging from 40% to 90%. Tissue samples confirmed for TiLV by semi-nested RT-PCR and displayed syncytial cells in liver, a characteristic of TiLV infection as seen in the histopathological sections. The overall prevalence of TiLV recorded in the samples was 67.64% (69/102). Phylogenetic analysis of TiLV segment 3 revealed that Tamil Nadu sequences were closely related to each other along with Israel, indicating a possible transmission link. These findings underscore the importance of understanding phylogenetic relationships in developing effective control measures to prevent further spread of TiLV.

Key words: Mass mortality, Nile tilapia, *Tilapia tilapinevirus*, TiLV surveillance.

INTRODUCTION

Tilapia is one of the fastest-growing fish species in the global aquaculture, with an estimated production of 7 million tonnes in 2020 (FAO, 2020). By 2032, India aims to produce 2.155 million metric tons of tilapia, (USD 4.398 billion) and expected tilapia export earnings might be USD 3.92 billion (WorldFish and CII, 2022). Its demand is driven by fast growth, hardiness, adaptability and higher consumer demand leads to global food security and economic development (Wasso *et al.*, 2024; Debnath *et al.*, 2023). Although tilapia is considered as hardy and relatively disease-resistant (Ferguson *et al.*, 2014), Disease outbreaks now pose a major challenge in tilapia farming, affecting both production and trade, tilapia is susceptible to several bacterial, viral and parasite infections (Uma *et al.*, 2024; Philominal *et al.*, 2024; Bigarre *et al.*, 2009; Kaviarasu *et al.*, 2022; Ponsrinivasan *et al.*, 2023). No viral diseases were reported in tilapia until 2009 (Aich *et al.*, 2022), but the emergence of Tilapia Lake virus (TiLV) has since become a major threat.

TiLV a negative sense RNA virus, with 10 genome segments (10,323 kb) encoding 14 functional genes with high potential for genetic reassortment (Bacharach *et al.*, 2016; Ferguson *et al.*, 2014; Acharya *et al.*, 2019; Chatput

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et al., 2020), belongs to the family Amnoonviridae, genus *Tilapinevirus* and species *Tilapia tilapinevirus* (Adams *et al.*, 2017; Koonin *et al.*, 2023). Mass tilapia disease outbreaks

in many countries across four continents in the farmed as well as feral populations of Tilapia (He *et al.*, 2023), first reported in 2014 in Ecuador and Israel (Eyngor *et al.*, 2014; Ferguson *et al.*, 2014). Although studies suggest it may have originated 5 to 10 years earlier (Thawornwattana *et al.*, 2021). Asymptomatic carriers of TiLV, causing vertical transfer of the virus to their offspring and horizontally transmitted from one fish to another through water and co-habitation (Eyngor *et al.*, 2014; Mugimba *et al.*, 2018; Senapin *et al.*, 2018; Dong *et al.*, 2020). In India, TiLV outbreaks were first reported in 2018 in polyculture farms in Ernakulam district of Kerala and northsouth 24 parganas district, West Bengal (Behera *et al.*, 2018). Subsequent outbreaks in wild *O. mossambicus* populations were seen in Mysuru and Mandya districts, Karnataka (Suresh *et al.*, 2023), Nile tilapia in cage culture, Pune district, Maharashtra (Rao *et al.*, 2024) and *O. niloticus* mortality were also documented in Katpadi lake, Vellore district, Tamil Nadu (Saranya and Sudhakaran, 2020). TiLV causes mortality of wild outlets, *Sarotherodon* (Tilapia) *galilaeus* in the Sea of Galilee, Israel (Eyngor *et al.*, 2014), *O. niloticus* in Malaysia (Abdullah *et al.*, 2018), wild Nile tilapia from Tanzanian, Ugandan parts of lake victoria (Mugimba *et al.*, 2018) and Peru (Hardaker and Chudleigh, 2021). Stress due to high stocking densities, bacteria and parasitic infections have often been documented during TiLV outbreaks, particularly in poorly managed aquaculture systems (Tatiyapong *et al.*, 2017; Amal *et al.*, 2018; Rao *et al.*, 2021; Munangandu *et al.*, 2016; Mugimba *et al.*, 2018). Targeted surveillance for TiLV has been initiated in various countries to prevent its spread

and prevent the negative consequences (Debnath *et al.*, 2020; Abdullah *et al.*, 2022; Contreras *et al.*, 2021). This study aims to investigate the prevalence of TiLV in *Oreochromis* spp. in Tamil Nadu, India, focusing on key farming districts by conducting purposive surveillance.

MATERIALS AND METHODS

Sample collection and processing

Oreochromis niloticus samples (102 Nos) were collected from 11 districts (Ariyalur, Chengalpattu, Chennai, Krishnagiri, Ranipet, Tiruvarur, Tiruvallur, Tenkasi, Thoothukudi, Thirunelveli and Vellore) in Tamil Nadu, India. A total of 34 sites (21 commercial farm sites (5 districts-63 samples)/13 feral sites (8 districts-39 samples)) were selected due to their higher than usual mortality events observed from February 2020 to May 2022 (Table 1 and 2). The fish were transported live in aerated plastic bags to the State Referral Laboratory for Aquatic Animal Health at Tamil Nadu Dr. J. Jayalithaa Fisheries University, Chennai, Tamil Nadu. Clinical signs in the fish were recorded onsite and examined microscopically for the presence of parasites. Fish were euthanized using MS-222 (250 mg/L) (Sigma-Aldrich) and dissected. Organs, including the gills, brain, liver, intestine and kidney, were transferred aseptically into RNA later reagent for RT-PCR detection of TiLV. For pathological investigation, liver samples were dissected and fixed in 10% neutral buffered formalin. The liver tissue samples were sectioned at 4-5 µm, stained with Hematoxylin and Eosin (H and E) and examined microscopically (EVOS,

Table 1: Details of *O. niloticus* samples collected from tilapia farms.

Farm	District	Period	Outbreak or surveillance	% Mortality level	Number of TiLV positive /tested samples
A1	Ariyalur	Jan-2020	Surveillance	-	0/3
A2		Feb-2022	Surveillance	-	0/3
D1		Feb-2020	Outbreak	50	3/3
D2	Tiruvarur	Mar-2020	Outbreak	60	3/3
F1		Feb-2021	Surveillance	-	0/3
F2			Surveillance	-	3/3
F3			Surveillance	-	3/3
F4			Surveillance	-	3/3
F5			Surveillance	-	3/3
F6			Surveillance	-	3/3
F7			Surveillance	-	3/3
F8			Surveillance	-	3/3
F9			Surveillance	-	3/3
F10			Surveillance	-	3/3
F11			Surveillance	-	3/3
F12	Tiruvallur	Feb-2022	Surveillance	-	3/3
G1		Jun-2020	Surveillance	-	0/3
G2		Feb-2021	Surveillance	-	3/3
G3	Tenkasi	Aug-2021	Surveillance	-	3/3
H1		Jan-2021	Outbreak	50	0/3
H2		Jan-2021	Outbreak	50	0/3

Thermo Scientific) to assess histopathological changes specific to TiLV. Statistical analysis Fisher Exact Test was performed using RStudio to determine whether the mortality was significantly associated with TiLV infection (R Core Team, 2016).

RT-PCR diagnosis for TiLV

Total RNA was extracted from gill, brain, liver, intestine and kidney tissues using RNA Isoprep reagent (Takara Bio Inc., Japan). The purity and concentration of the extracted RNA were determined using a NanoPhotometer (Thermo Fisher Scientific Inc., USA). For cDNA synthesis, reverse transcription (RT) was performed using a cDNA synthesis kit (Thermo Fisher Scientific Inc., USA) according to the manufacturer's instructions. Semi-nested reverse transcription PCR (RT-PCR) targeting segment 3 was followed to screen and confirm TiLV infection (Dong *et al.*, 2017a). The RT-PCR amplicons were resolved by horizontal gel electrophoresis on a 1.5% agarose gel containing ethidium bromide (0.8 mg/ml) at 100 V and visualized under UV illumination using a gel documentation system (Bio-Rad, USA).

Phylogenetic analysis

The PCR amplified products of TiLV segment 3 were purified and sequenced using commercial sequencing

services (Eurofins, Bengaluru, India). Consensus sequences were generated using DNA Sequence Assembler v4.31.0 and submitted to GenBank (NCBI). This study analyzed segment 3 sequences from 20 TiLV isolates, including four from this study and 16 from GenBank (Indonesia, Bangladesh, Ecuador, Peru, Thailand, Colombia, Egypt, Uganda, Israel, Vietnam and Malaysia). Phylogenetic analysis was performed using the Maximum Likelihood method, based on the General Time-Reversible model with gamma-distributed rate heterogeneity (GTR+G). Node confidence was assessed with 100 bootstrap replicates using MEGA11 (Tamura *et al.*, 2021).

RESULTS AND DISCUSSION

Prevalence of TiLV

Tilapia samples collected from disease outbreak sites, exhibited symptoms *viz.*, weakness, lethargy, gasping at the water surface, skin discoloration/skin darkening, exophthalmia/endophthalmia, abdominal dropsy, fin/tail erosion, open wounds, ulcers, haemorrhages on the body surface and mortalities ranging from 40 to 90% (Fig 1). Fisher Exact Test revealed significant difference ($p = 7.42 \times 10^{-7}$) in mortality rates associated with the TiLV infection. Microscopic observations of the wet mount squashes of

Table 2: Details of *O. niloticus* samples collected from feral outlets.

Feral	District	Period	Outbreak or surveillance	% Mortality level	Number of TiLV positive /tested samples
B1	Chengalpatu	Dec-2020	Surveillance	-	0/3
C1	Chennai	Feb-2022	Outbreak	70	3/3
C2		Mar-2022	Outbreak	70	3/3
D3	Krishnagiri	Feb-2021	Outbreak	70	3/3
D4		Mar-2021	Outbreak	70	3/3
D5		May-2021	Outbreak	70	3/3
E1	Ranipet	Mar-2021	Outbreak	60	3/3
G4	Tiruvallur	May-2022	Outbreak	40	3/3
G5		May-2022	Outbreak	60	3/3
I1	Thoothukudi	Jan-2021	Surveillance	-	0/3
J1	Thirunelveli	Jan-2021	Surveillance	-	0/3
J2		Jan-2021	Surveillance	-	0/3
K1	Vellore	Oct-2021	Outbreak	70	0/3



Fig 1: TiLV infected tilapia showing clinical signs.

the gills and body swab (40X) showed no parasitic infestation. The observations recorded in the infected tilapia in this study align with earlier reports which documented symptoms such as lethargy, abnormal dropsy, loss of appetite, skin lesions or erosions, discoloration, loss of scales, scale protrusion, exophthalmia and abdominal distension (Jaemwimol *et al.*, 2018; Surachetpong *et al.*, 2017; Kembou-Tsofack *et al.*, 2017). Variations in clinical signs of TiLV infections have been reported in different geographical location (Aich *et al.*, 2022).

A total of 102 samples from 34 sampling sites were screened for TiLV using semi-nested PCR targeting segment 3 of TiLV produced specific amplicons in all tissues (gills, brain, liver, intestine and kidney) tested. The overall prevalence of TiLV in the samples was 67.64% (69/102). Specifically, 20.58% (21/102) of samples showed amplification of 415 bp in the first-step PCR and 50.0% (51/102) showed amplification at 250 bp in the second-step PCR (Fig 2). Samples from disease outbreak sites showed a 100% prevalence of TiLV in Chennai (C1 and C2), Krishnagiri (D1 to D5) and Ranipet (E1) districts. Samples from non-outbreak areas showed a TiLV prevalence of 80% in Tiruvallur. PCR amplification of TiLV and histopathological changes in liver tissue were confirmed with syncytial multinuclei hepatocytes (Fig 3). Since the liver and brain is considered the primary target organ for TiLV (Kembou-Tsofack *et al.*, 2017). TiLV demonstrates broad tissue tropism, inducing systemic infections and being detected in multiple organs, including the brain, liver, kidney, muscles, gills, fins, spleen, intestine, eyes, heart, ovaries and testis, making it a highly lethal pathogen (Kembou-Ringert *et al.*, 2023). Mortality rates associated with TiLV vary widely in wild and farmed tilapia,

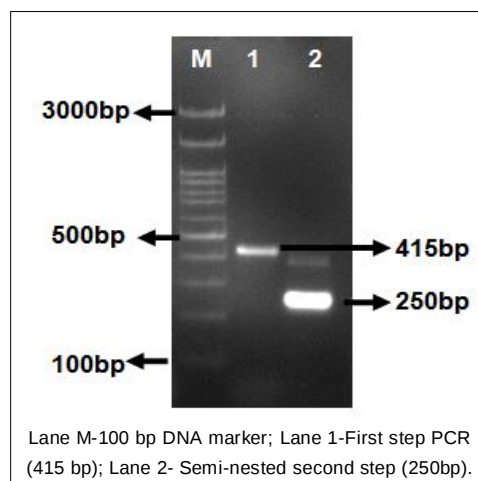


Fig 2: Semi-nested PCR amplification of TiLV.

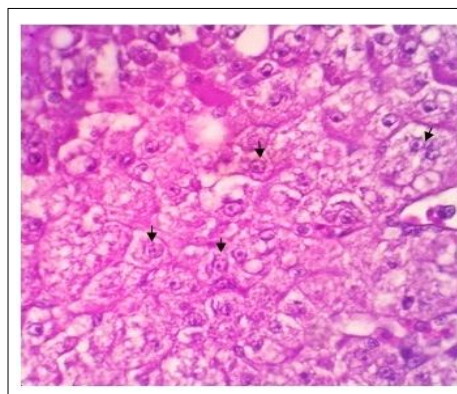


Fig 3: Histopathology of liver section of TiLV infected tilapia showing syncytial multinuclei hepatocytes.

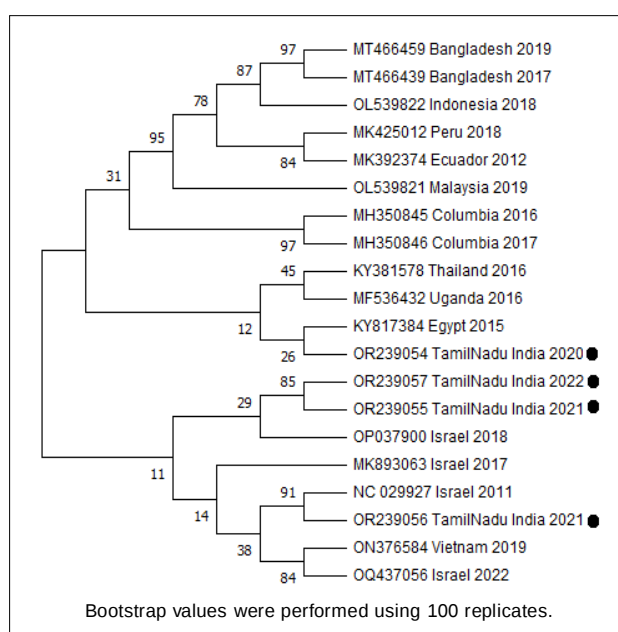


Fig 4: Maximum likelihood phylogenetic trees of the TiLV segment 3 using general time-reversible model (GTR+G).

ranging from low levels 0.71% in Malaysia and 6.4% in Chinese Taipei (OIE, 2017a; OIE, 2017b) to high levels 80% in Israel, 20-90% in Thailand and 80-90% in India (Bacharach *et al.*, 2016; Dong *et al.*, 2017b; Behera *et al.*, 2018).

Molecular phylogeny

The phylogenetic tree constructed based on the TiLV segment 3 from Tamil Nadu isolates (OR239054, OR239055, OR239056 and OR239057) formed a distinct cluster. These isolates exhibited close relationship with sequences from Israel (OP037900, MK893063, NC029927 and OQ437056), suggesting a potential historical transmission link or independent introduction from a common source. The temporal range of Tamil Nadu isolates (2020-2022) overlaps with the Israel isolates which span from 2011-2022. However, Tamil Nadu isolates were highly divergent from the isolates of Uganda, Egypt, Colombia, Peru and Ecuador, suggesting that these strains may have evolved separately (Fig 4). Phylogenetic analyses based on short fragments of one or a few segments are often used to trace TiLV transmission across geographical regions (Nicholsen *et al.*, 2017; Skornik *et al.*, 2020). Other researchers have similarly utilized phylogenetic analysis of partial or complete TiLV genomes to track viral migrations across regions (Ahasan *et al.*, 2020; Chaput *et al.*, 2020; Surachetpong *et al.*, 2017; Pulido *et al.*, 2019; Verma *et al.*, 2021). These findings emphasize the need for continued surveillance and genetic analysis to track TiLV evolution and its potential routes of transmission. These findings were also consistent with other studies (Behera *et al.*, 2018; Saranya and Sudhakaran, 2020; Verma *et al.*, 2021). Interestingly, isolates from Thailand and Peru are more closely related to those from Israel but remain genetically distinct, suggesting that these countries may have been infected by TiLV of Israeli origin (Dong *et al.*, 2017b).

CONCLUSION

In this study, feral and farmed tilapia infected with TiLV was reported and confirmed through molecular detection. The prevalence of TiLV in the samples collected was 67.64%, samples from disease outbreak sites that showed a 100% prevalence of TiLV in Chennai, Krishnagiri and Ranipet districts. Samples from non-outbreak areas showed 91.6% prevalence in Tiruvarur district. These findings highlight the critical need for early diagnosis and timely reporting of TiLV infections, as the advancement of the tilapia industry is impeded by emerging infection of TiLV in India. The prevalence of TiLV in Tamil Nadu contributes valuable insights into the epidemiology of the virus and this potentially may aid in implementing robust bio-security measures in aquaculture to control the TiLV infection in India.

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Disclaimers

The views and conclusions expressed in this article are solely those of the authors and do not necessarily represent the views of their affiliated institutions. The authors are responsible for the accuracy and completeness of the information provided, but do not accept any liability for any direct or indirect losses resulting from the use of this content.

Conflict of interest

The authors declare that there are no conflicts of interest regarding the publication of this article. No funding or sponsorship influenced the design of the study, data collection, analysis, decision to publish or preparation of the manuscript.

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