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Original Article

First report on the dual infection with emerging viruses, Tilapia Lake Virus (TiLV) and Tilapia Parvovirus (TiPV) in the wild Nile Tilapia (*Oreochromis niloticus*) from Pulicat Lagoon, Tamil Nadu, India

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Abstract

Background: Pulicat Lagoon, Tamil Nadu, India, is a major fish landing site supporting the livelihoods of local fishing communities. Despite its ecological and economic importance, information on viral diseases affecting its fish populations remains limited. **Aims:** This study aimed to investigate the prevalence and seasonal occurrence of viral infections in the tilapia population of Pulicat Lagoon. **Methods:** Active surveillance was conducted from November 2023 to July 2024. Collected tilapia samples were screened for viral pathogens using PCR, followed by sequencing and histopathological analysis for confirmation. **Results:** Among 314 tilapia samples collected and analyzed, 54 (17.19%) tested positive for viral infections, including Tilapia Lake Virus (TiLV) (13.69%), Tilapia parvovirus (TiPV) (9.25%), and co-infections with both TiLV and TiPV (6.68%). PCR-positive samples were predominantly detected from January to March 2024, indicating a higher prevalence during the post-monsoon season. Sequencing and histopathological examinations confirmed the presence of these viruses. **Conclusion:** The findings of this study on the detection of TiLV and TiPV in tilapia populations from Pulicat Lagoon, located in Tiruvallur District, Tamil Nadu, India, can play a crucial role in raising awareness among aquaculture farmers in nearby regions to implement effective biosecurity measures, as well as among researchers and local fishing communities who rely on the lagoon's fish stocks for their livelihoods.

Key words: Fish diseases, Pathology, Phylogenetic analysis, Tilapia, Viruses

Introduction

Fish serves as an important source of nutrition, particularly high-quality animal protein that is more easily digestible than other meat sources. In addition, fish are rich in omega-3 fatty acids, essential vitamins such as vitamin D, A, and B12 and minerals including selenium, iodine and zinc, all of which are vitalfor human health (Mohanty *et al*., 2019). Pulicat Lagoon, located in the Tiruvallur district of Tamil Nadu, India is the second-largest brackish water lagoon in the country and spans the border between Tamil Nadu and Andhra Pradesh. The mouth region of this lagoon, which acts as a buffer zone between the marine environment of the Bay of Bengal and brackish water, lies within Tamil Nadu, while the majority of the lagoon's extends into Andhra Pradesh (Sanjeeva Raj, 2006).

Pulicat Lagoon supports a rich biodiversity, comprising approximately 145 fish species, 8 species of shrimps, 7 species of crabs and 35 species of molluscs. The Lagoon sustains the livelihood of about 50,000 fishermen and their families, who depend on its resources for their daily sustenance. The *Padu* system, a traditional community-based fishing practice, allocates specific fishing grounds to village clusters on a rotational basis. This practice helps prevent overfishing and promotes the sustainable use of aquatic resources. Passed down through generations, the *Padu* system remains an integral component in maintaining the ecological balance of the lagoon's fisheries (Jesintha *et al*., 2022).

Tilapia is a widely farmed freshwater fish known for its adaptability, rapid growth, and ability to thrive in both freshwater and brackish environments. Though primarily cultivated in countries such as China, Indonesia, and

Egypt, tilapia is also found in the wild, particularly in rivers, lakes, reservoirs and brackish-water lagoons. Its mild flavour and affordability make it popular in many cuisines. As the second most farmed fish globally, tilapia production has surged in recent years due to its suitability for aquaculture, strong market demand and stable prices. Tilapia Lake Virus (TiLV) is an emerging pathogen that has had a significant impact onthe global tilapia farming industry and food security (Jansen *et al*., 2019). Since the first report of a novel disease in Ecuador, known as syncytial hepatitis of tilapia (Ferguson *et al*., 2014), the disease has been documented in numerous countries. In 2017, TiLV outbreaks were reported in Egypt (Fathi *et al*., 2017), Thailand (Dong *et al*., 2017; Surachetpong *et al*., 2017), and Colombia (Kembou Tsofack *et al*., 2017), India (Behera *et al*., 2018), Malaysia (Abdullah *et al*., 2018; Amal *et al*., 2018), Tanzania and Uganda (Mugimba *et al*., 2018), as well as Indonesia (Koesharyani *et al*., 2018). In 2019, TiLV outbreaks were reported in Peru (Pulido *et al*., 2019) and Ecuador (Al-Hussinee *et al*., 2019). In an earlier study in India (Behera *et al*., 2018), variable levels of morbidity and mortality were noted in the populations of Nile tilapia infected with TiLV from two different geographical regions of India.

In 2019, a novel parvovirus was identified in China from fecal samples collected from crocodiles (*Crocodylus siamensis*) fed with Nile tilapia (*Oreochromis niloticus*). It was tentatively named Ichthyic parvovirus and is believed to belong to the genus *Chaphamaparvovirus* (Du *et al*., 2019). Another study reported the same virus from a severe mortality outbreak affecting adult Nile tilapia in Hubei, China, in 2015. It was renamed Tilapia parvovirus (TiPV). The virus has subsequently been detected in farmed red hybrid tilapia (Yamkasem *et al*., 2021; Piewbang *et al*., 2022) and Nile tilapia (Dong *et al*., 2017) in Thailand. India became the third country to report the presence of Tilapia parvovirus infecting *O. niloticus* and red hybrid tilapia (Badhusha *et al*., 2023; Rajendran *et al*., 2023). Viral diseases have not been reported in the fish population of Pulicat Lagoon. Therefore, TiLV/TiPV are emerging viruses affecting the tilapia fish population worldwide, the present study was designed to understand the prevalence of TiLV/TiPV in Pulicat Lagoon.

Materials and Methods

Sample collection

Tilapia (n=314) were collected fortnightly over a nine-month period from November 2023 to July 2024 in Pulicat Lagoon (Latitude: 13°25'10.4"N, Longitude: 80°19'06.3"E) (Fig. 1), Tiruvallur district, Tamil Nadu, India. Fish samples with clinical signs such as discoloration, tail and fin erosions, scale loss, reddened operculaand surface ulcers were collected for the screening of diseases. Apparently healthy fish were also sampled to account for their potential role as asymptomatic carriers. Fish were collected alive the lagoon using a cast net and transported either in live condition or in sterile plastic bags on ice to the State referral laboratory for Aquatic Animal Health, TNJFU-Madhavaram campus for processing and screening for TiLV and TiPV. Samples of organs including gill, brain, eye, liver and kidney were aseptically collected from each fish for total DNA and RNA extraction. Live fish were anesthetized with MS-222 (100 mg/L) prior to dissection and organ collection.

Screening for viral pathogens

Tilapia samples were screened for Tilapia Lake Virus (TiLV) and Tilapia parvovirus (TiPV) using previously published primers and protocols.

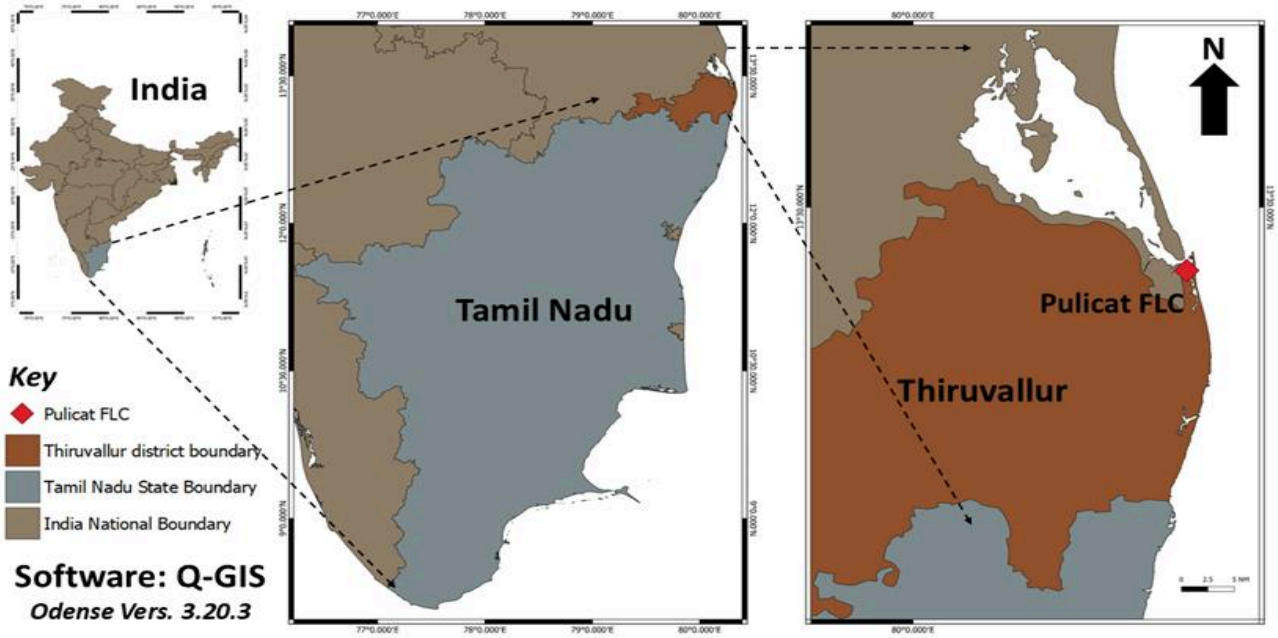

Fig. 1: Map showing the location of the research area

DNA and RNA isolation

Total DNA was extracted from the eye, brain, gill, liver, and kidney tissue of fish samples using the DNeasy Blood & Tissue Kit from Qiagen, Germany. Subsequently, the extracted DNA was subsequently storedat -20°C until it was ready for further use. Total RNA was extracted using RNA-iso Plus (TAKARA, India). Tissue was homogenized in RNAiso Plus, followed by chloroform-mediated phase separation. RNA was precipitated with isopropanol, washed with 75% ethanol and dissolved in RNase-free water. The purity and concentrations of the extracted RNA from each of the samples were determined by a Nanophotometer (Thermo Fisher Scientific, USA). Total RNA extracted from tissue samples was reverse-transcribed into first-strand complementary DNA (cDNA) using the Revert Aid first strand cDNA synthesis kit (Thermo Fisher Scientific, USA). The reaction included Random Hexamer Primer (1 μL), 5x Reaction Buffer (5 μL), RiboLock RNase Inhibitor (1 μL), 10 mM dNTP Mix (2 μL), RevertAid RT (1 μL), nuclease-free water (9 μL) and 1 μL of extracted RNA. The reaction was incubated at 42°C for 60 min, followed by enzyme inactivation at 70°C for 5 min. The synthesized cDNA was stored at -80°C until further use.

PCR detection and sequence analysis

PCR reactions were performed in a total volume of 25 μL reactions containing 22 μL of 1x red dye PCR master mix (Ampliqon, Denmark), 1 μL each of forward and reverse primers (10 picomoles each) and 1 μL of cDNA/DNA template/positive control. The PCR primers and protocols followed for TiLV were TiLV415F (5´-TAT GCA GTA CTT TCC CTG CC-3´), TiLV415R (5´-GTT GGG CAC AAG GCA TCC TA-3´), TiLV250F (5´-TAT CAC GTG CGT ACT CGT TCA GT-3´) and TiLV250R (5´-GTT GGG CAC AAG GCA TCC TA-3´) (Dong *et al*., 2017). PCR cycling was then carried out for 25 cycles, 30 s at 94°C for denaturation, 30 s at 60°C for annealing and 30 s at 72°C for extension. The PCR primer and protocol followed for TiPV was TiPV534F (5´-GAG ATG GTG TGA AAA TGA ACG GG-3´) and TiPV534R (5´-CTA TCT CCT CGT TGC TCG GTG TAT C-3´) (Liu *et al*., 2020). PCR cycling was then carried out for 35 cycles, 30 s at 94°C for denaturation, 30 s at 55°C for annealing and 60 s at 72°C for extension. PCR amplicons were resolved on a 2.0% agarose gel prepared using 1X Tris-borate-EDTA buffer (TBE buffer) and 0.5 μg/ml of ethidium bromide was added. Approximately 10 μL of amplified PCR products were loaded alongside a 100 bp molecular weight marker (3 μL) (GeneDirex, USA). The products were subjected to electrophoresis at 120 V for 30 min, visualized and documented using a UV gel documentation system (Bio-Rad, USA). Further confirmation of PCR products was done by sequencing with commercial sequencing services (Eurofins, Bengaluru) and compared with sequences published in the National Center for Biotechnology Information (NCBI) GenBank database using BLAST (Basic Local Alignment Search Tool). The confirmed sequences were submitted to the NCBI GenBank and accession numbers were obtained.

Phylogenetic analysis

Phylogenetic analysis of the protein segments of TiLV and TiPV generated in this study was performed by comparing them with sequences from other countries in the GenBank database to assess genetic similarity and divergence. The phylogenetic trees were constructed using the maximum likelihood method (Tamura *et al*., 2021). A bootstrap consensus tree inferred from 1000 replicates was used to represent the evolutionary relationships of the analyzed taxa (MEGA11.0.13).

Histopathological analysis

Fish samples were dissected, and tissues from the brain, liver, and kidney were fixed in neutral buffered formalin and processed for histopathology following standard methods (Roberts, 2012). Briefly, fixed tissues were dehydrated, embedded in paraffin, sectioned at 5 μm, stained with hematoxylin and eosin (H&E) and examined under a microscope (EvosXL, Thermo fisher, USA), with pathological changes documented.

Results

Screening of viruses

A total of 314 tilapia samples were collected from Pulicat lagoon, Tiruvallur district, Tamil Nadu, India, and screened for Tilapia Lake Virus (TiLV) and Tilapia parvovirus (TiPV). Infected fish exhibited symptoms includingskin discoloration, reddish opercula, hemorrhages, and fin erosion (Fig. 2).

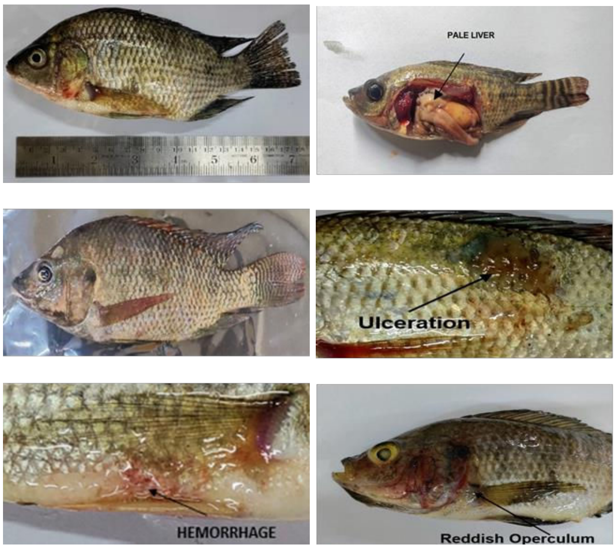

Fig. 2: *Oreochromis niloticus* with the clinical signs of diseases collected in the study

Molecular detection of TiLV and TiPV

Tilapia samples exhibiting disease symptoms were confirmed to be infected with TiLV (Fig. 3) and TiPV

(Fig. 4) by PCR. Semi-nested RT-PCR amplification of cDNA pcrfrom gill, brain, eye, liver, and kidney tissues yielded amplicons of 415 bp amplicons in the first-step PCR, indicating heavy infection with TiLV. Similarly, PCR of gill, brain, eye, liver, and kidney tissues produced 534 bp amplicons, indicating heavy infection with TiPV.

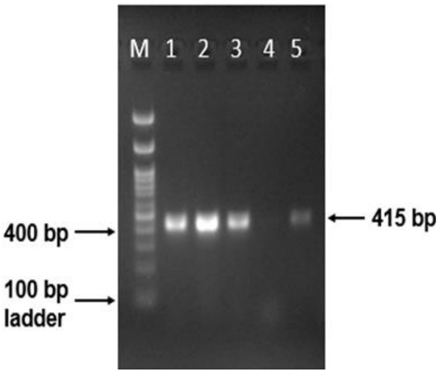

Fig. 3: PCR detection of TiLV in *O. niloticus* samples. Lane M: 100 bp molecular weight marker. Lanes 1-3: Amplified products of TiLV (Liver, Kidney, Brain), Lane 4: Negative control, and Lane 5: Positive control

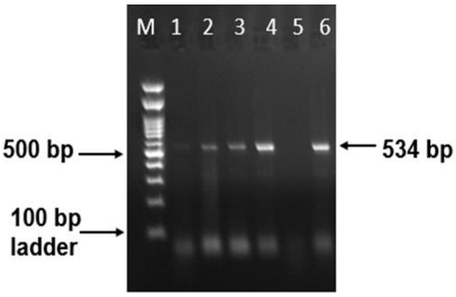

Fig. 4: PCR detection of TiPV in *O. niloticus* samples. Lane M: 100 bp molecular weight marker. Lanes 1-4: Amplified products of TiPV (Liver, Kidney, Brain, Gill), Lane 5: Negative control, and Lane 6: Positive control

Sequencing and phylogenetic analysis

Sequencing of PCR products confirmed their identities and the sequences were submitted to the Genbank under accession numbers PP858046.1, PP858047.1, PQ281793, and PP858045.1. The phylogenetic tree of TiLV, constructed using the segment 3 hypothetical protein gene (Fig. 5) showed that the two TiLV sequences generated in this study (PP858046.1 and PP858047.1) are closely related, indicating minimal genetic divergence and a likely recent common ancestor. This clustering, supported by a high bootstrap value of 100, confirms that they are distinct from geographically distant strains. The phylogenetic tree of TiPV (Fig. 6) revealed that the two Tamil Nadu samples (PP858045.1 and PQ281793) are closely related, showing strong genetic similarity. Forming a distinct branch separate from the Maharashtra isolate (OR158041.1), with a bootstrap value of 100, indicating a significant genetic divergence. These findings suggest that Tamil Nadu strains may represent aunique lineage or recent introduction, highlighting the need for regional surveillance and further research.

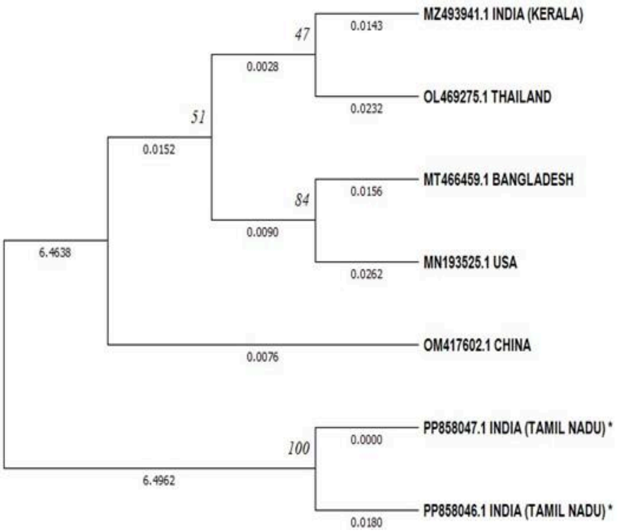

Fig. 5: Phylogenetic tree depicting the relationship of TiLV Tamil Nadu with the reported TiLV strains from other countries

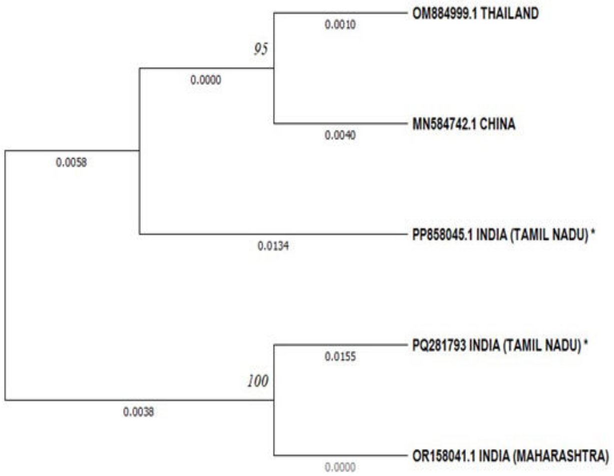

Fig. 6: Phylogenetic tree depicting the relationship of TiPV Tamil Nadu with the reported TiPV strains from other countries

Table 1: GenBank accession numbers of TiLV and TiPV

S. No.	Viral pathogen	Host	GenBank Acc No.
1	Tilapia parvovirus	*Oreochromis niloticus*	PP858045.1
2	Tilapia Lake Virus	*Oreochromis niloticus*	PP858046.1
3	Tilapia Lake Virus	*Oreochromis niloticus*	PP858047.1
4	Tilapia parvovirus	*Oreochromis niloticus*	PQ281793

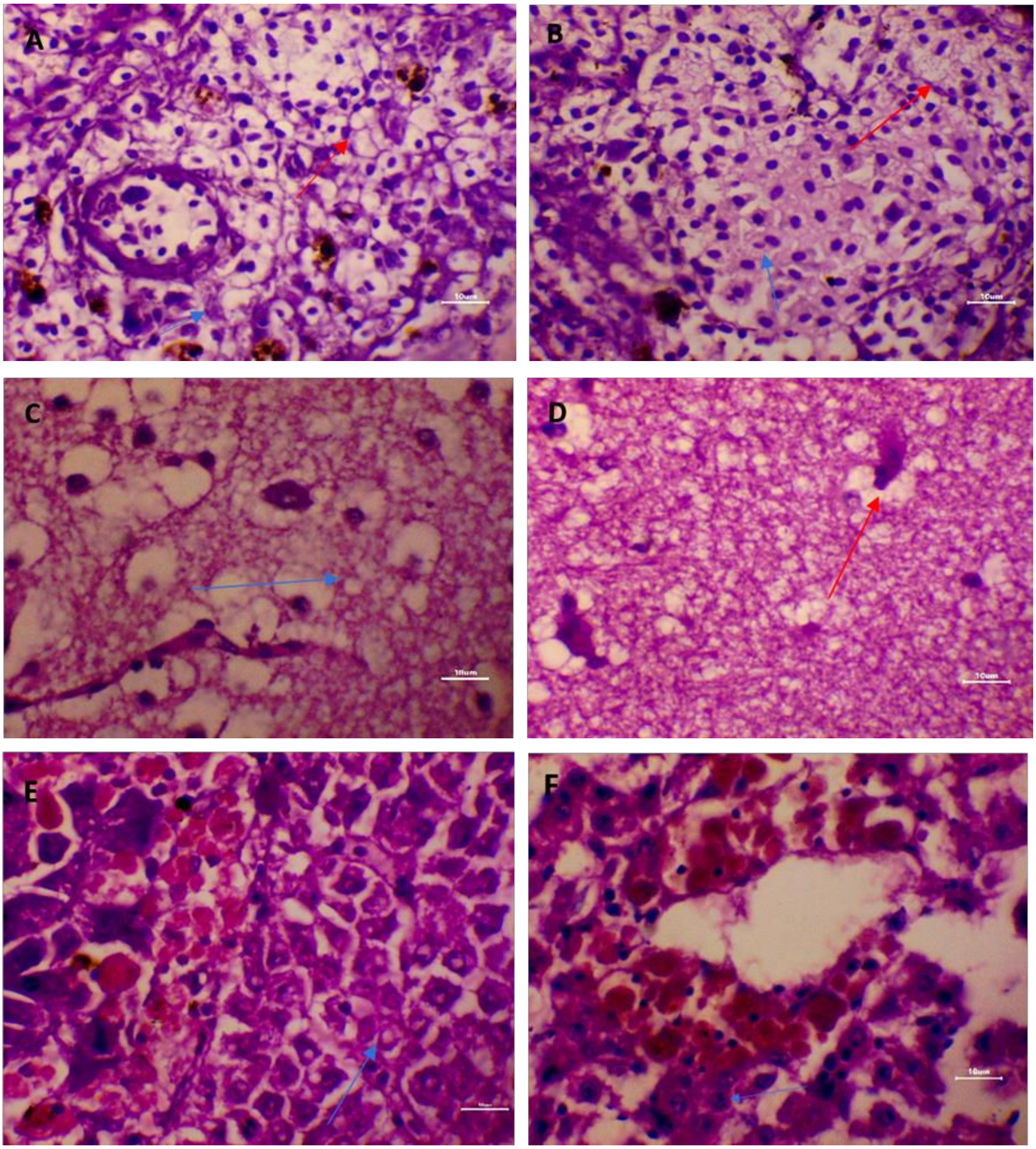

Fig. 7: Histopathological changes observed in the organs of *O. niloticus* with TiLV and TiPV dual-infection ((H&E). (**A** and **B**) Sections of kidney tissue showing degeneration of renal tubules with vacuolation (red arrow) and tubular necrosis (blue arrow), (**C**) Section of brain showing vacuolation (blue arrow), (**D**) The section of brain with neuronal necrosis (red arrow), (**E**) Section of liver showing vacuolation (blue arrow), and (**F**) The section of liver showing the giant cell with multiple nuclei (blue arrow). Scale bars: 10 µm (A–F).

Histopathology of TiLV and TiPV infected fish

Histopathological examination of the liver in Nile tilapia (*Oreochromis niloticus*) with dual infection of TiLV and TiPV revealed hepatocyte vacuolation (intracytoplasmic vacuoles), indicating liver damage due to infection. In the kidney, degeneration of renal tubules with vacuolation and tubular necrosis was observed. Brain tissue exhibited neuronal necrosis and vacuolation.

Discussion

Diseases can significantly disrupt wild fisheries, resulting insubstantial ecological and economic consequences. Infected fish often exhibits lower growth rates and higher mortality, which can alter population dynamics and threaten the long-term viability of fish stocks. Numerous outbreaks have been associated with drastic declines in fish populations, highlighting the vulnerability of wild species to infections. Additionally, infected fish may display abnormal behaviours, such as lethargy and disorientation, increasing their susceptibility to predation and further contributing to population declines (Murray and Peeler, 2005). Tilapia samples collected in the study exhibited symptoms such as skin discoloration, reddish opercula, hemorrhages, and fin erosion, which are preliminary signs of the disease. No internal or external parasitic infections were observed upon microscopic examination. Samples collected between January 2024 to March 2024 were confirmed to be infected with viral pathogens, namely TiLV and TiPV, suggesting that viral disease outbreaks in tilapia predominantly occur during the post-monsoon season. Of the total fish examined, 54 specimens (17.19%) were infected with viral pathogens. The prevalence of viral infections was as follows: Tilapia Lake Virus (TiLV) (13.69%), Tilapia parvovirus (TiPV) (9.25%) and dual infection with TiLV and TiPV (6.68%). Sequencing of PCR products confirmed their identities, and the sequences were submitted to Genbank with accession numbers provided in Table 1. In the phylogenetic tree of TiLV, constructed using the segment 3 hypothetical protein gene, Tamil Nadu samples formed distinct lineage separate from isolates in China (OM417602.1), the USA (MN193525.1), Bangladesh (MT466459.1), Thailand (OL469275.1) and Kerala, India (MZ493941.1). Samples from Kerala and Thailand were more closely related, whereas the China isolate was more divergent. These results suggest that Tamil Nadu strains may represent a unique or recent introduction, highlighting the need for regional surveillance. In the phylogenetic tree of TiPV, the Tamil Nadu isolates share a more recent common ancestor compared with the Maharashtra isolate. Isolates from Thailand (OM884999.1) and China (MN584742.1) also form a closely related group, with a bootstrap value of 95, highlighting a strong relationship among these Asian samples. The genetic distance between the Asian and Indian isolates suggests possible geographic or evolutionary divergence. The histopathological changes in this study are consistent with previous reports of TiLV infection by Saranya and Sudhakaran (2020) and dual infections of TiPV and TiLV by Piewbang *et al.* (2022). Histological changes due to TiLV infection have been primarily described in the liver, with syncytial cell formation (Ferguson *et al.*, 2014) and in the central nervous system of infected tilapia (Eyngor *et al.*, 2014). Our observations of syncytial giant cells in the hepatocytes further confirm the TiLV infection in tilapia. Disease transmission can have cascading effects throughout aquatic ecosystems. Migratory species often act as vectors for pathogens, spreading infections to new regions and exposing previously unaffected populations. This spillover poses serious risks to biodiversity and the overall health of aquatic ecosystems, as well as to the economic stability of fisheries dependent on healthy fish populations. Effective management strategies, including enhanced disease surveillance and biosecurity measures, are essential to mitigate the impact of diseases on fisheries and ensure the sustainability of aquatic resources (Murray and Peeler, 2005; Ashade *et al.*, 2010).

Tilapia Lake Virus (TiLV) has the potential to spread from infected fish in Pulicat Lagoon to nearby fish farms through water currents, contaminated equipment, or the movement of infected fish. Without adequate biosecurity measures, the virus could infiltrate farmed populations, potentially triggering outbreaks that may severely impact local aquaculture operations. The spread of TiLV in wild populations may also have significant ecological consequences, potentially reducing wild tilapia numbers and disrupting the natural balance of the lagoon ecosystem. This ecological disruption could, in turn, affect the livelihoods of local fishermen and threaten the sustainability of fisheries in the area. As Pulicat Lagoon is an open water system, there is a risk that TiLV could become a transboundary issue, potentially spreading to neighbouring regions or even across state or national borders. Tilapia culture is currently practiced on a commercial scale in many South Indian states; therefore, targeted surveillance for early detection is essential to enable timely management decisions.

This study represents the first report of dual infection by TiPV and TiLV in wild Nile tilapia (*Oreochromis niloticus*) from Pulicat Lagoon, Tamil Nadu, South India. The findings underscore the importance of regional surveillance and targeted research to better understand the evolution and spread of TiLV and TiPV. The genetic distinctiveness of the Tamil Nadu isolates, together with the observed histopathological changes, highlights the need for ongoing monitoring and research to mitigate the impact of these viral pathogens on tilapia aquaculture.

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Conflict of interest

The authors declare that they have no conflicts of interest.

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