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Report on Mortalities of Nile Tilapia (*Oreochromis niloticus*) Due to *Tilapinevirus tilapiae* and Co-infecting Bacteria in Winter Season

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ABSTRACT

Background: *Tilapinevirus tilapiae*, commonly known as Tilapia lake virus, is a major viral infection of cultured tilapia, causing severe rates of fatalites and has evolved as a serious hazard to the tilapia in the world. Predisposing factors, notably temperature and the development stage of the host play a decisive role in influencing the extent of pathogenesis and the mortality rate associated with the disease. The seasonality of TiLV outbreaks has been observed in multiple countries, with a higher frequency occurring during the summer months.

Methods: In the current investigation, the occurrence of TiLV disease was examined in fry and fingerlings of tilapia during the winter season (January - February, 2020) in Pune District, Maharashtra, India. The disease was observed in fish reared in open cages (Case I) and in nursery rearing farm (Case II). Infected tilapia displayed generalized gross clinical signs. TiLV was identified and confirmed in both instances using a semi-nested reverse transcriptase PCR by amplifying TiLV genome segment 3 and further confirmed by histopathogical investigation.

Result: The research demonstrated the vulnerability of early developmemtal stages of *O. niloticus* to TiLV disease, suggesting the potential occurrence of this infection during the winter season, characterized by lower temperatures. Additionally, microbial investigations exposed the prevalence of *Aeromonas* spp., predominantly associated with TiLV-positive fish. These results align with previous publications, underscoring the potential role of bacterial infectious agents (Notably aeromonads), in exacerbating TiLV disease during outbreaks.

Key words: *Aeromonas veronii*, Bacterial co-infection, Nile tilapia, Tilapia lake virus, Winter season.

INTRODUCTION

Tilapines are the next maximum cultured fish variety after cyprinids, being farmed in more than 135 countries (FAO, 2022). The rapid growth of tilapia aquaculture is attributable to its high adaptability to diverse culture conditions, higher disease resistance and faster growh rate, besides their affordability and rich source of protein. In 2020, it was estimated that 6.1 million metric tons of cichilds were produced globally (FAO, 2022). However, the aquaculture industry marked by rapid expansion, is facing significant challenges from diseases (especially bacterial and viral), which are contributing to severe losses (Machimbirike *et al*., 2019).

Tilapinevirus tilapiae, widely known as TiLV, responsible for Tilapia lake virus disease (TiLVD), is a RNA virus under the genus *Tilapinevirus* and the family Amnoonviridae (ICTV, 2023). TiLVD has become a significant disease in all the major tilapia farming nations. TiLV is a devastating infection, exhibiting both horizontal and vertical transmission (Aich *et al*., 2022). Considering the global significance, World Organisation for Animal Health has listed TiLVD as a notifiable disease (WOAH, 2022). Tilapines are often susceptible to TiLV during their whole life cycle, with cumulative mortalities lying between 5-90% (Contreras *et al*., 2021). Nevertheless, early developmental stages exhibit heightened susceptibility due to their nascent immune defences (Tran *et al*., 2022). This virus induces diverse organ impairments. Syncytial hepatitis is considered as the principal pathognomonic manifestation of the viral infection, featuring well-defined cellular necrosis in hepatic cells (Dong *et al*., 2017). TiLV outbreaks occur seasonally in various countries and are noticed more frequently in the summer, in the temperatures between 22-

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32°C (Piewbang *et al.,* 2022). Interestingly, the current study reveals occurrence of the disease during the winter season.

The significance of co-infecting microbial agents is often underestimated due to a concentrated emphasis of the identified principal aetiological agent of the infection. Nonetheless, it is acknowledged that concurrent infections may exert a crucial influence on the disease outcome and spread of pathogens (Piewbang *et al*., 2022). According to earlier studies, *Aeromonas* spp. and *Streptococcus* spp. are consistently identified as predominant coexisting bacteria with TiLV in cultivated tilapia (Nicholson *et al*., 2017; Rao *et al*., 2021; Abdullah *et al*., 2022). In this study, we investigated the occurrence of a TiLV infection in two distinct life stages of Nile tilapia (*Oreochromis niloticus*) that occurred in winter season.

MATERIALS AND METHODS

Collection of samples

Nile tilapia (*O. niloticus*) specimens were procured from two distinct sources situated in the Pune district of Maharashtra, India (Fig 1). Live specimens from case I (fingerlings; n = 6) and case II (fish fry; n = 600) were collected and transported to the Aquatic animal Health Management (AAHM) laboratory of ICAR-CIFE, Mumbai and the entire research was conducted between 2020 and 2021. The farm-level observations and details of fish specimens are given in Table 1. In case I, after aseptic dissection, the fish tissues, such as gill, liver and kidney, were pooled (2 samples in 1 pool) for bacteriological studies. Similarly, pooled liver samples were used for TiLV screening and histopathological studies. For case II, fish fry (n = 12) were cut open using Leica CLS 150 X dissection microscope (Germany). Tissues, including gill, liver and kidney, were aseptically sampled and combined (3 samples per pool) for bacteriological studies, while liver samples were collected for TiLV screening. Concurrently, water temperatures, clinical pathognomonic conditions and post-mortem observations were systematically documented in both cases.

PCR confirmation of TiLV using reverse transcriptase PCR

Total RNA was isolated from liver tissues using Trizol reagent (Invitrogen, USA) following the manufacturer's procedure. Subsequently, 2 µg of total RNA was converted into cDNA using the Revert Aid Reverse Transcriptase First Strand cDNA Synthesis kit (Fermentas, USA) following the manufacturer's procedure. The resulting cDNA was preserved at -20°C until subsequent utilization. For the detection of TiLV, a 100 ng cDNA was subjected to amplification through semi-nested reverse transcriptase PCR (RT-PCR) against TiLV genome segment 3 (Dong *et al.,* 2017), besides plasmid DNA targeting *TiLV* segment 3 (415 bp) was used as a positive control. Table 2 gives particulars of primers used for PCR amplification.

Histopathology

Fixation of liver tissues were done using 10% buffered formalin for 24 h. Later, the tissues were washed overnight with running water and transferred to serial ascending grades of ethanol. The tissues were subsequently subjected to processing employing a conventional histopathology procedure. The samples were transferred to a xylene, followed by embedding in the paraffin block, sectioning using a rotary microtome (5 µM) and adequately spread on clean slides. After proper drying, tissue sections were deparaffinised in xylene (1 h) and rehydrated through a descending alcohol series (20 min each) followed by staining with haematoxylin and eosin (H and E), mounting in DPX (Luna, 1968). After drying, stained slides were observed under the light microscope coupled Axiocam 105 colour camera (Carl Zeiss, Germany) for taking photomicrographs.

Bacteriological analysis

Bacterial isolation was conducted in accordance with the previously outlined procedure (Rao *et al.,* 2021). Briefly, a part of the pooled tissue is homogenated in brain heart infusion (BHI) broth and tryptone yeast extract salt (TYES) broth (Himedia, India), later the resulted mixture was serially diluted (10-fold) in the BHI and TYES agar plates, correspondingly. The plates underwent incubation at 30°C for a period of 24-48 hours. Subsequently, 5-10 colonies, representing distinct colony characteristics, were selected and subjected to further purification. The purified colonies were then preserved on BHI and TYES agar slants at 4°C. The bacterial strains were further examined for conventional biochemical tests *viz*., Gram staining, oxidase, esculin hydrolysis, gas from arabinose and glucose, ornithine decarboxylase tests and resistance to O/129 compound (Abbott *et al.,* 2003).

For molecular identification, the bacterial DNA was extractedby means of DNA zol method and the concentration of DNA was measured using Nano Drop (Thermo Fisher Scientific, USA). Subseqently, 100 ng of the DNA was PCR amplified using universal 16SrRNA primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′ (Lane *et al.,* 1991). The resulted products were refined by means of PCR purification kit (Thermo Fisher Scientific, USA) and sequenced (Agrigenome Pvt. Ltd., India). The obtained nucleotide sequences from this investigation were verified are placed in the GenBank with the following accession numbers; ON375295-97, ON375357-64, ON375394-402, ON383329-36, ON383471-78, ON383519-27, ON383822, ON383824.

RESULTS AND DISCUSSION

Confirmation of TiLV

A macroscopic examination of the diseased fish exhibited multifaceted and generalized clinical signs. These encompassed aberrant swimming behavior, anorexia, body darkening, exophthalmia, haemorrhagic skin lesions and uneven loss of scale to severe skin peel off, predominantly at the caudal peduncle portion, fin and tail rot and abdominal swelling (Fig 2 and 4). Internally, enlarged and congested liver, intestinal fluid accumulation, distended gall bladder

(Fig 2-4). The affected fish stock exhibited severe morbidity with mass mortalities. Microscopic observations of both external and internal organs did not indicate any evidence of parasite infestation. A compilation of farm-level observations, inclusive of water temperature and clinical manifestations and symptoms, is presented in Table 1.

Positive results were obtained using RT-PCR amplification of cDNA made from liver tissue's RNA using primers specific to TiLV's genome segment 3 (Table 2). Samples obtained from Case I confirmed TiLV infection at both steps of nested PCR, displaying products of 415 bp and 250 bp, correspondingly (Fig 5). Conversely, from Case II specimens, TiLV infection was confirmed in the second step of the PCR, after noticing 250 bp amplicon (Fig 5 and 6). TiLV-infected fish displayed severe pathological changes such as focal to extensive areas of necrosis, typical

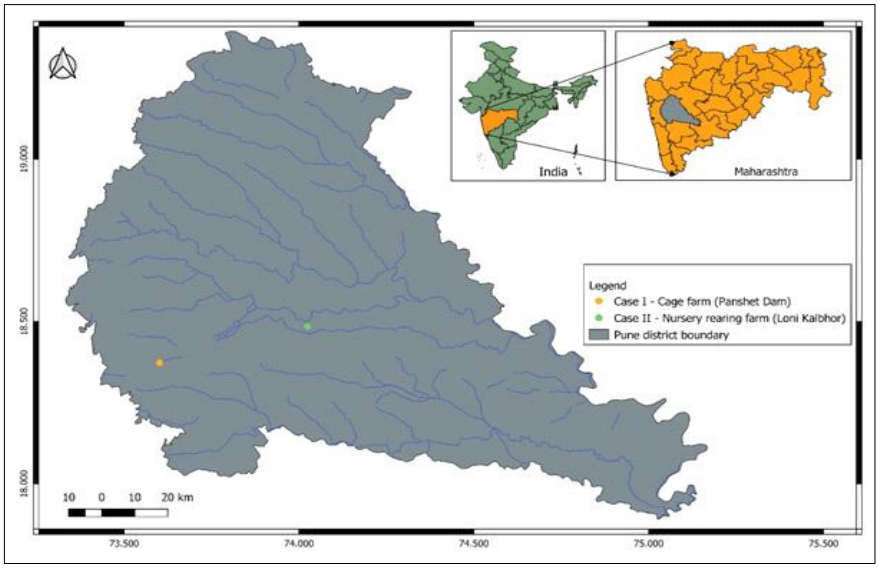
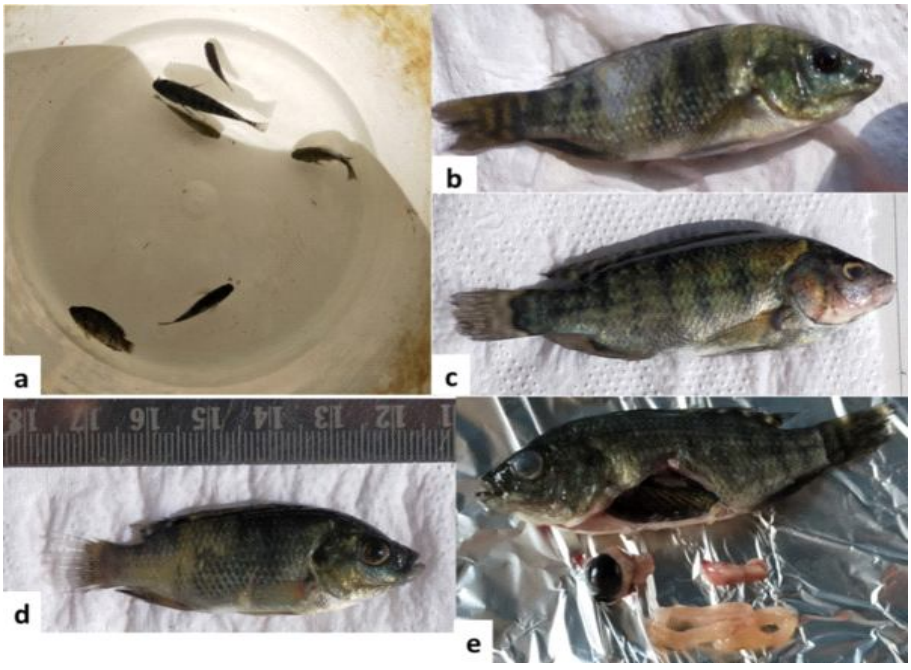


Fig 1: Map depicting the two locations of sampling points (Case I and II) in the present study.



(a) Dark discoloration and size variation; (b) Peeled skin; (c) Enophthalmia and petechial hemorrhages; (d) Tail and fin rot and (e) Exophthalmia with ocular opacity.

Fig 2: Naturally infected Nile tilapia from Case I, illustrating macroscopic changes.

Table 1: Details of fish samples collected and the farm-level observations made.

Case	Type of culture system and location	Water temperature	Life-stage and other details	Field-level observations (Clinical manifestations)
II	Cage farm: Panshet dam, Pune District, Maharashtra, India	Winter (January, 2020); Temperature: 20-21°C	Fingerlings: Body weight: 7-12 g; Body size: 6.0-8.0 cm	Symptoms: Unusual behaviour such as lethargy, anorexia, poor growth, gasping, restless swimming near the water surface and size variation. Clinical signs: black discoloration/darkening, pale gills, ocular opacity, typical irregular scale loss to severe skin lesions over the caudal portion, frayed fins, especially over the caudal fin region (mild to complete loss of fins), congested and enlarged liver, haemorrhages, enlarged gall bladder, intestinal necrosis with mucus accumulation (Fig 2 and 3) Mortality: Up to 3 weeks from the day of the first clinical sign observed, fish exhibited severe morbidity with a cumulative mortality of >85%.
III	Nursery rearing system: Pune district, Maharashtra, India	Winter (February, 2020); Temperature: 20-22°C	Fry: Body weight: 0.5-1 g; Body size: 2.1-2.7 cm	Symptoms: lethargy, anorexia, abnormal swimming and swimming at the water surface.Clinical signs: Severe loss of scale and skin of abdomen up to caudal region, In severe cases, deeper erosion revealing caudal skeletal vertebrae, Frayed fins especially caudal fin region, complete loss of fin in severe cases, Haemorrhages on abdomen, lower jaw, operculum and eyes (Fig 4). Mortality: Initially, fish were apparently healthy, after 5 days of stocking nursery rearing system, fish started showing gross clinical signs. Fish exhibited severe morbidity and within 2 weeks, >70% mortality was observed.

g: Grams; cm: Centimeter; °C: Degree celsius.

multinucleate syncytial cell formation, extensive eosinophilic intracytoplasmic inclusion bodies, degeneration of hepatocytes with loss of nuclei, loss of cellular architecture and erythrocytic infiltration (Fig 7).

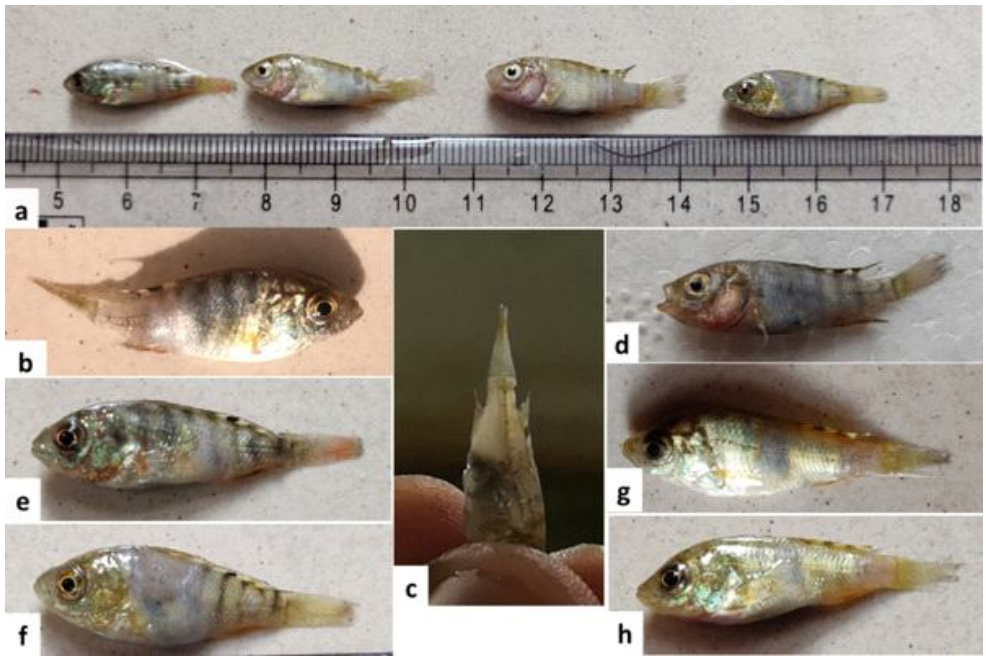
In the TiLV screening process, Case I samples exhibited affirmative amplification in the first step of PCR, affirming the intensity of infection (Fig 5). The PCR outcome aligns with the distinctive severe clinical manifestations

Table 2: Details of the primer sets used for the screening TiLV in the present study.

Primer name	Target gene	PCR primer sequence (5′-3′)	Amplicon size (bp)	Reference
Nested ext-1	Segment 3	TATGCAGTAC TTTCCCTGCC	415	Dong *et al*., 2017
ME1		GTTGGGCACAAGGCATCCTA	250	
7450/150R/ME2		TATCACGTGCGTACTCGTTCAGT		

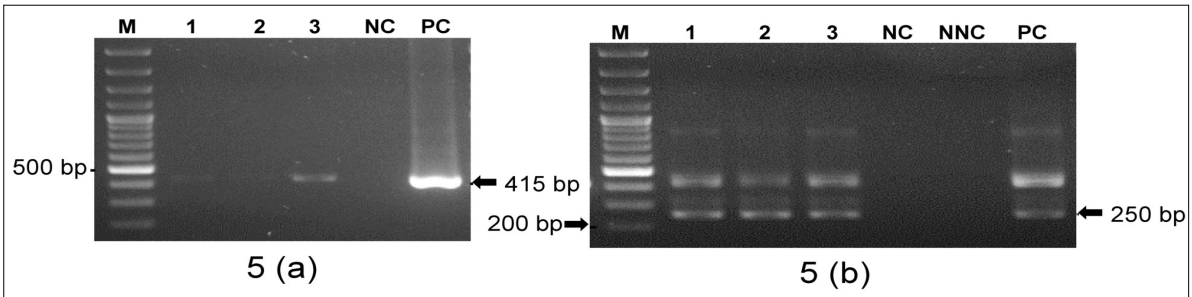
(a) Discolored and enlarged gall bladder with an empty intestine filled with mucus; (b) Pale and enlarged liver; (c) Congested and enlarged liver displaying haemorrhages.

Fig 3: TiLV-infected Nile tilapia from Case I, illustrating internal manifestations.



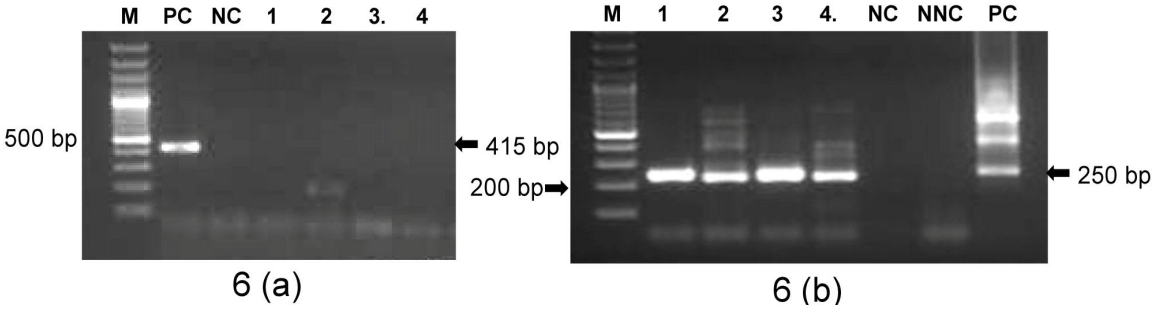
(a) Fin rot; (b and c) Rigorous erosion revealing caudal skeletal vertebrae; (d) Severely infected, freshly died specimen with petechial hemorrhages over the fish; (e) Hemorrhages over fish abdomen, operculum, fins and eyes; (f) Severe scale and skin loss; (g) Abdominal dropsy and (h) Moderate scale loss and tail rot.

Fig 4: Naturally infected Nile tilapia from Case II, illustrating macroscopic changes.



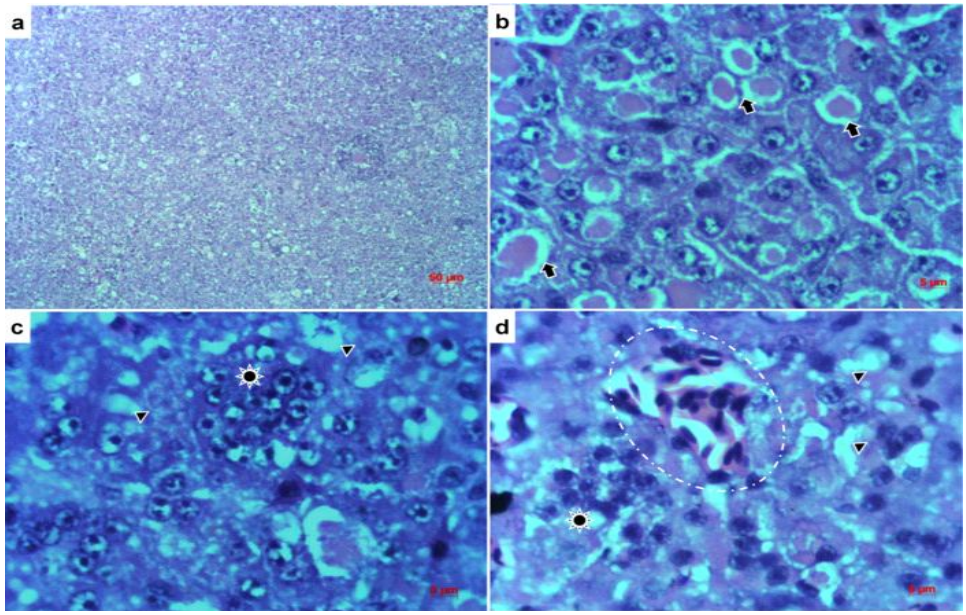
(a) 1st step PCR (415 bp product) - Lane M: 100 bp DNA ladder (Thermo Fisher Scientific), Lanes 1, 2 and 3: Template (Infected samples), Lane NC: Negative control, Lane PC: Positive control; (b): 2nd step PCR (250 bp product) - Lane M: 100 bp DNA ladder, Lanes 1, 2 and 3: Nested template (Infected samples), Lane NC: Negative control, Lane NNC: Nested PCR negative control, Lane NPC: Nested PCR positive control.

Fig 5: Detection of TiLV in liver tissues (Case I).



(a) 1st step PCR (415 bp product) - Lane M: 100 bp DNA ladder (Thermo fisher scientific), Lane NC: Negative control, Lane PC: Positive control, Lanes 1, 2, 3 and 4: Template (Infected samples); (b): 2nd step PCR (250 bp product) - Lane M: 100 bp DNA ladder, Lanes 1, 2, 3 and 4: Nested template (Infected samples), Lane NC: Negative control, Lane NNC: Nested PCR negative control, Lane NPC: Nested PCR positive control.

Fig 6: Detection of TiLV in liver tissues (Case I).



Low (10×) and high magnification (100x) images of sections of liver tissues showing (a-d) Focal to extensive necrosis; (c and d) Multinucleated syncytial giant cell formation in the hepatocytes (star), (b) Eosinophilic intracytoplasmic inclusion-like bodies (black arrow); (c and d) Vacuolation (arrow head); and (d) Erythrocytic infiltration (circle).

Fig 7: Histological section of TiLV-positive liver tissues stained with haematoxylin and eosin (Hand E).

observed in the fingerlings, resulting in a mass mortality rate exceeding 85%. Consistent with previous instances, various signs and symptoms related to TiLV were nonspecific. However, the presence of ocular opacity, identified as an overt clinical sign (Fig 2e) linked to widespread TiLV-induced mortalities, aligns with the findings of Eyngor *et al*. (2014). Conversely, despite the prominent clinical signs and a mass mortality rate exceeding 70% observed in Case II, the specimens exhibit virus prevalence in the 2nd step of nested PCR, indicating a low level of infection (Fig 6).

In this research, we examined two independent disease episodes in cage-reared fingerlings (Case I) and nursery-reared fry (Case II) tilapia and are confirmed to be positive to TiLV disease. The clini-cal signs, histological changes observed (winter season) were agreeing with the earlier publiced naturally infected tilapia cases (Behera *et al*., 2018; Rao *et al*., 2021; Abdullah *et al*., 2022).

Identification of TiLV-associated bacterial isolates

Microbiological examination of representative specimens led to the retrival of 48 bacterial isolates. Among them, 21 isolates were obtained from Case I and 27 isolates were derived from Case II. The predominant bacterial taxa from Case-I samples, were identified to be *Aeromonas* species, of which, *Aeromonas veronii* and *A. jandaei* possess major share (24% each; 5/21 each), followed by *A.hydrophila* (19%; 4/21). Among all the bacterial isolates recovered from Case-I, *Aeromonas* spp. (15/21) alone (*A. veronii*, *A. hydrophila, A. jandaei and A. dhakensis*) constitutes of 72% (Table 3). A similar bacteriological composition was observed in TiLV-infected samples from Case II. Among the 27 bacterial isolates, *A. veronii* and *Plesiomonas shigelloides* were the prevailing taxa, each constituting 30% (8/27 isolates) followed by, *A. hydrophila* accounted for 18.5% (5/27), of all the isolates recovered from Case-II, *Aeromonas* spp. (14/27) alone (*A. veronii*, *A. hydrophila and A. dhakensis*) constitutes of 52% (Table 3). The complete list of tissue-wise co-infecting bacteria from the Case I and Case II specimens are detailed in Table 3.

The simultaneous infection of a fish by multiple pathogens is termed as co-infection. Typically, studies conducted during disease outbreaks primarily focus on the primary etiological agent, which may lead to missing out the secondary pathogens that contributes equally to the disease (Kotob *et al*., 2017). In view of the above, after PCR confirmation of the virus infection, the samples were processed further to understand the possible association of bacteria as secondary pathogens. *A. veronii* and *A. hydrophila* were consistently isolated from both cases of TiLV-positive tilapia.

As per the earlier studies, Aeromonads are the common co-infecting bacteria with *Tilapinevirus tilapiae* (Nicholson *et al*., 2017; Amal *et al*., 2018; Rao *et al*., 2021; Suresh *et al*., 2023). Similarly, in this investigation, Aeromonads were detected in all screened fish tissues (Table 3). Aeromonads are prevalent in inland waters and are recognized as a significant challenge in tilapia farming. *Aeromonas* spp. constituted 72% of all the isolates from case I samples and 52% in case II samples. It must be noted that the case I samples were severely infected with TiLV and a high incidence of *Aeromonas* spp. in these samples suggest their vulnerability to colonization by bacterial pathogens following viral infection.

The findings of this study further recommend the combined impact of bacterial and viral infections in rapid development and progression of disease leading to high mortality rates (Nicholson *et al.,* 2020). Instances of natural coinfection involving TiLV and *A. veronii* in Malaysia and India has led to elevated mortality rates in both cultured and wild tilapines (Amal *et al*., 2018; Suresh *et al*., 2023). Furthermore, *in vivo* studies carried out by Nicholson *et al.* (2020) showed that *Tilapinevirus tilapiae* - *A. hydrophila* co-infection has a synergistic effect that resulted in higher mortalities (93%) in comparison to individual pathogen challenges. According to Lu *et al.* (2021), the concentration of *A. hydrophila* might be the key environmental sign in fish ponds of TiLV-positive fish during concurrent infection leading to mass mortalities. Compared to Nile tilapia

Table 3: Description on bacteria isolated from TiLV infected tilapia from Case I and II.

Aeromonads (n = 15)	Tissues/organ	Others (n = 6)	Tissues/organ
Case I- Bacteria from TiLV-infected cage farmed tilapia (n = 21)			
A. veronii (5)	Gill, liver and kidney	*Vogesella urethralis* (1)	Gill
A. hydrophila (4)	Gill, liver and kidney	*Vogesella* spp.(2)	Gill and liver
A. jandaei (5)	Gill, liver and kidney	*Undibacterium oligocarboniphilum* (1)	Gill
A. dhakensis (1)	Gill	*Undibacterium* spp.(2)	Gill and kidney
Case II- Bacteria from TiLV-infected nursery reared tilapia (n = 21)			
Aeromonads (n = 14)	Tissues/organ	Others (n = 13)	Tissues/organ
A. veronii (8)	Gill, liver and kidney	*Plesiomonas shigelloides* (8)	Gill, liver and kidney
A. hydrophila (5)	Gill, liver and kidney	*Flavobacterium columnare* (1)	Gill
A. dhakensis (1)	Gill	*Staphylococcus* spp.(1)	Liver
		Acinetobacter spp. (1)	Kidney
		Vogesella spp.(1)	Gill
		Bacillus paramycodies (1)	Gill

experimentally infected with TiLV alone; co-infected fish were observed to have more severe gross lesions (Nicholson *et al*., 2020). In this investigation, the pronounced clinical manifestations may arise from the synergistic impact of both viral and *Aeromonas* spp. infections, contributing to an escalation of the disease severity (Basri *et al*., 2020).

Role of pre-disposing factors

Disease caused by TiLV leading to large-scale mortalities is generally encountered during summer, due to which TiLV has been speculated to be associated with the Summer Mortality Syndrome (SMS) in tilapines (Surachetpong *et al*., 2020). According to previous studies from various countries, outbreaks of TiLV infection were reported from higher water temperatures, ranging between 22-32°C. In Israel, at 22-32°C (Eyngor *et al.,* 2014), in Ecuador at 25 -27°C (Ferguson *et al.,* 2014), in Egypt at >25°C (Fathi *et al.,* 2017), in Colombia at 27.5-32°C (Contreras *et al.,* 2021), in Thailand at 28-32°C (Piewbang *et al.,* 2022), in Malaysia at 28.6-30.4°C (Abdullah *et al.,* 2022) and in Vietnam at 26-28°C (Tran *et al.,* 2022). Nevertheless, a water with 25°C are generally linked with disease transmission in communities (Fathi *et al*., 2017) and elevated temperatures are conducive to TiLV outbreaks (Surachetpong *et al*., 2020). Surprisingly, in the present study, mortalities are recorded during the winter season, characterized by a reasonably lower water temperature range of 20-22°C. Stress plays a vital role in the spread of diseases and in this regard the decreasing water temperatures have dropped cellular and humoral immune system and may have predisposed fish to viral infection (Wang *et al.,* 2020). Additionally, due to the lower tolerance of tilapia to low water temperatures (Tang *et al*., 2021), there is a potential for a heightened occurrence of diseases such as TiLV, coupled with co-infections. In addition to the above, Bergmann *et al.* (2021) in an experimental study, recorded 100% mortality in tilapia infected with TiLV at 12°C, however, no mortality was occurred at 17°C. Studies involving natural outbreaks and artifical infection studies with *Tilapinevirus tilapiae* suggest that the early stages of tilapia, starting from eggs to fingerling stage are more susceptible to infections (Dong *et al.,* 2020; Surachetpong *et al.,* 2020). Typically, fish weighing between 1-50 g encounter significant mortality rates attributed to high viral loads and pathological manifestations (Roy *et al.,* 2021).

Low water temperatures and other predisposing conditions, such as low initial weight (during transporation to culture tanks), enhance the prevalence and infectivity of TiLVD in cultured tilapia (Kabuusu *et al.,* 2018). Ferguson *et al.* (2014) observed clinical signs of TiLVD with fatalities within 4-7 days after transferring to culture ponds. Likewise, in this study, clinical signs and mortalities manifested in fish fry within 5 days of transitioning to the nursery rearing system. Rearing tilapia fry and fingerlings in winter poses a risk of TiLV-associated mortalities. It's crucial to highlight that seemingly healthy tilapia can carry latent TiLV infections, which may emerge during suboptimal conditions, resulting in mass mortalities (Senapin *et al.,* 2018).

CONCLUSION

A latent infection (TiLV) in early fish life stages, coupled with low temperature and bacterial co-infection, likely contributed to severe clinical symptoms and mass die-off in the current outbreak. *Aeromonas* spp. is concerning co-infecting pathogen in farmed Nile tilapia, capable of causing significant fatalities in initial life stages. The interaction of viral and bacterial pathogens might have accelerated the disease leading to significant mortalities. This indicates the need for PCR-based screening of early larval stages for TiLV before stocking into the grow out system. Consequently, the study elucidates certain risk factors associated with TiLV disease outbreaks, offering essential insights for the formulation of effective management strategies for Tilapia lake virus disease (TiLVD). Our results reveal TiLV's impact on Nile tilapia across various life stages, even in low-temperature seasons, often co-occurring with aeromonad bacterial pathogens. Experimental infection studies at different low-temperature conditions (15-22°C) are essential for comprehending TiLV infection dynamics with respect to water temperatures.

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Conflict of interest

The authors assert the absence of any conflicts of interest.

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