Egyptian Journal of Aquatic Biology & Fisheries
Zoology Department, Faculty of Science,
Ain Shams University, Cairo, Egypt.
ISSN 1110 – 6131
Vol. 27(5): 185 – 195 (2023)
www.ejabf.journals.ekb.eg

Incidence of tilapia lake virus and megalocytivirus in Qarun Lake, Fayoum Governorate, Egypt

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ARTICLE INFO

Article History:
Received: June 14, 2023
Accepted: Aug. 29, 2023
Online: Sept. 16, 2023

Keywords:
Incidence ;
Tilapia Lake virus;
Water;
Megalocytivirus;
Qarun lake;
Egypt

ABSTRACT

Tilapia Lake virus and megalocytivirus are two major viruses that are causing global fish declines. The detection of these viruses in nearby water samples suggests a non-invasive and consistent method for identifying fish-viral infections and forecasting early viral illnesses in aquaculture systems. The goal of this study was to examine the prevalence of these viruses in water samples taken from Qarun Lake in Egypt's Fayoum Governorate. 72 water samples were collected regularly from two sites from May 2021 to November 2022. The organic flocculation concentration method was combined with molecular analysis to identify fish viral infections. Only 4.1% of the 72 were infected with tilapia lake virus, whereas 9.7% were infected with megalocytivirus. Finally, our results demonstrated that organic flocculation combined with PCR environmental nucleic acid detection is effective for identifying tilapia Lake virus and megalocytivirus in water.

INTRODUCTION

Egypt's fisheries sector is the largest producer of farmed fish on the continent and the world's third largest producer of farmed tilapia **(FAO, 2017)**. There were no current acute illness concerns, although farmers in Egypt reported inexplicable, substantial mortalities of huge Nile tilapia (*Oreochromis niloticus*) throughout the summer months of 2014 (June-October). Several research teams examined these sickness outbreaks, dubbed "summer mortalities," in an effort to identify causal agents, and the findings led to the identification of Tilapia Lake virus (TiLV) and *Aeromonas* species **(Fathi *et al*., 2017; Nicholson *et al*., 2017)**. TiLV was identified in 2014 by scientists who had spent years attempting to determine what was going on in Lake Kinneret, often known as the Sea of Galilee, where wild tilapia output has dropped from 257 to 8 tonnes per year on average **(Eyngar *et al*., 2014)**. Following then, TiLV was discovered in Israel **(Eyngar *et al*., 2014)**, Ecuador **(Bacharach *et al*., 2016)**, Colombia **(Kembou *et al*., 2017)**, Egypt **(Fathi *et al*., 2017)**, Philippines **(OIE 2017a)**, China **(OIE 2017b)**, Thailand **(Sorachetpong *et al*., 2017)**, India **(Behaera *et al*., 2018)**, Indonesia **(Koesharpani *et al*., 2018)**, Malaysia

(Amal *et al*., 2018), Tanzania and Uganda **(Mugimba *et al*., 2018)**, and, more recently, Mexico, Peru and the United States **(OIE 2018a; OIE 2018b; Pulido *et al*., 2019**; and **Ahsan *et al*., 2020,)** and Bangladesh **(Chaput *et al*., 2020)**. TiLV is an Amnoonviridae virus with a negative sense single-stranded RNA genome of 10.323 kb divided into ten segments **(Bacharach *et al*., 2016)**. In most cases, TiLV has a total mortality rate of up to 90% **(Behaera *et al*., 2018)**.

A DNA virus known as megalocytivirus, which belongs to the genus Megalocytivirus in the family Iridoviridae, was found in tilapia as well **(Whttington *et al*., 2010)**. This virus infects both wild and farmed freshwater and saltwater fish, causing systemic illnesses. Megalocytivirus outbreaks are economically significant in aquaculture because they can result in mass death. Megalocytivirus infections are classified into three types based on genetic sequence data: infectious spleen and kidney necrosis virus (ISKNV), red sea bream iridovirus (RSIV), and turbot reddish body iridovirus (TRBIV). The most well-known megalocytiviruses are RSIV and ISKNV **(Song *et al*., 2005; Yanang *et al*., 2010)**. In 2006, Australia had a megalocytivirus outbreak, which was linked to imported gouramis in pet stores **(Go *et al*., 2006)**. In 2009, another megalocytivirus outbreak in 10 aquarium fish species occurred in Korea **(Lee *et al*., 2009)**. Megalocytivirus is thought to spread when a healthy fish consumes tissues from a sick fish or through contaminated water. Most, if not all, previous investigations focused on detecting these viruses in suffering fish while ignoring their immediate surroundings. As a result, our study is one of the few to attempt to detect these viruses in their native habitat. The purpose of this study was to identify TiLV and megalocytivirus utilising an organic flocculation approach in environmental nucleic acids (eNA) extracted from fish-rearing water samples.

MATERIALS AND METHODS

Water sample collection

From May 2021 to November 2022, 72 surface water samples were taken biweekly from the centre and shoreline of Qarun Lake in Egypt's Fayoum Governorate. Qarun Lake is about 40 kilometres long from east to west and 6.7 km wide at its widest point. At 43 metres below sea level, it has a surface area of around 243 km^2 and a water volume of approximately 924 million m^3 **(Shalloof, 2020)**. Five litres of each water sample were collected in plastic bottles and sent to the National Research Centre's Environmental Virology lab in an ice box within four hours.

Sample Concentration

Water samples were concentrated, according to the US-EPA **(Williams *et al*., 2001)**. To boost viral stability during delivery, 2.5 mL of 1 M magnesium chloride per liter of sample was added. Before filtering using a nitrocellulose membrane filter (0.2 m pore size, 142 mm diameter), the pH of wastewater samples was corrected to 3.5 using 1M HCl. The viruses were then eluted and re-concentrated in 100 mL of 3% beef extract-0.05 M glycine solution, as described previously **(Katazanelson *et al*., 1976; Allayeh *et al*., 2022; El-Gyar *et al*., 2022)**. The elution was then adjusted to pH 3.5 and stirred for

30 minutes before centrifugation and dissolving the pellet in 1 ml of dihydrogen orthophosphate.

Nucleic Acids Extraction

Using a Qiagen Viral DNA Extraction Kit (QIAGEN, Hilden, Düsseldorf, Germany) and TRIzol^{TM} Reagent (ThermoFisher Scientific, Waltham, Massachusetts, U.S.), we extracted viral DNA and RNA from 200 µL of concentrated samples according to the manufacturer's instructions. The nucleic acids were either tested immediately by PCR or kept at -80^{o}C for subsequent examination.

TiLV identification using RT-PCR

The presence of TiLV was determined using a previously reported RT-PCR approach (**Eyngar *et al.*, 2014**). In a single step RT-PCR reaction utilizing the HiSenScriptTM RH (-) RT PreMix Kit (iNtRON Biotechnology, Hong Kong, China), about 20 ng of pure RNA were used. To detect TiLV virus, we employed primer sequences described in **Table 1** that were designed using the TiLV isolate Til-4-2011 reference genomes (KU751817 and KU751822) and were positioned on the coding portions of segments 4 and 9. PCR products (15 µL) were separated by gel electrophoresis in 1.5% agarose after amplification. After being treated with ethidium bromide, the fragments were visible under UV light.

Megalocytivirus identification using PCR

Megalocytivirus was detected using a previously described method **(Choi *et al.*, 2006)**. PCR reactions were carried out according to the manufacturer's instructions using primer sequences in **Table 1**, DreamTaq Green PCR Master Mix (2X) (ThermoFisher Scientific, Waltham, Massachusetts, U.S.). In a Bio-Rad T100 Thermal Cycler (California, U.S.), the amplification profile was initial denaturation at 95 °C for 2 minutes, followed by 35 cycles of 94 °C for 30 s, 55 °C for 1 minute, and 72 °C for 1 minute, and a final extension at 72 °C for 10 minutes. Amplicons were electrophorized in 1.5% agarose gel, stained with ethidium bromide, and visualized under UV light.

Table 1: Primer sequences used in this study

Virus	Primer	Sequence	Fragment size	Reference
TiLV	Seg4_F	AGCAGCAGCAGGAGAAAGAG	358-bp	Eyngor *et al.*, 2014
	Seg4_R	ACCGTCCTGTTTCTGAA		
	Seg9_F	TTGGTGATGTCACGATGGATA	351-bp	
	Seg9_R	AGTTCTATCGCCAGCCATGT		
Megalocytivirus	MV_F	ATGTCTGCAATCTCAGGTG	1,362 bp	Choi *et al.*, 2006
	MV_R	TTACAGGATAGGGAAGCCTGC		
	nMV_F	CACCGCAACGTGCAAAGCAA	369 bp	
	nMV_R	TTGACTGCAATAACGACCAGTTCAAAC		

Sequencing and phylogenetic analysis

All amplicons were purified using a MEGA total fragment DNA purification kit (iNtRON Biotechnology, Hong Kong, China) and sanger sequenced (https://macrogen.com/). The sequences were compared to the sequences of TiLV and megalocytivirus in Genebank (https://ncbi.nlm.nih.gov/).

RESULTS AND DISCUSSION

The global understanding has grown, particularly in tilapia-producing countries, and policymakers have put in place regulations to prevent the spread of viral infections in aquaculture systems. The majority, if not all, published papers emphasised on finding fish viruses in sick fish while ignoring their habitat as a water surrounding. A thorough strategy for detecting TiLV and megalocytivirus tilapia-associated water samples over 18 months utilising an organic flocculation combined with PCR for concentration and identification of fish viruses is presented here. Methods for concentrating viral particles from surrounding water samples have been employed in human health research, particularly in studies of waterborne illnesses caused by enteric viruses **(Choi *et al*., 2006, Haramoto *et al*., 2018; Gad *et al*., 2018; Nehal *et al*., 2019; Rizk and allayeh, 2020;)**. Coagulation and flocculation, filtration and ultrafiltration, and centrifugation and ultracentrifugation have all been employed to isolate viruses from aquatic environments **(Mostafa-hedeaba *et al*., 2022)**. Our study employed an organic flocculation approach initially presented for viral concentration from water samples **(katazenelson *et al*., 1976; Watanabe *et al*., 1988)**. It has been adapted to detect viral hemorrhagic septicemia virus (VHSV) in water that is both fresh and saltwater. Electron microscopy revealed that the recovery rate of VHSV in freshwater and saltwater was 57% and 59%, respectively **(Pinto *et al*., 1993)**. In the present study, the recovery rate of TiLV (an RNA virus) and megalocytivirus (an DNA virus) from surface water in the current study was much lower (9.7%) and (4.1%), respectively, as indicated in **Table 2**.This poor recovery rate from water samples in the present study might be attributed to the nature of fish viruses and their capacity to persist outside of their hosts, as well as the sensitivity of the concentration and detection procedure utilized. As a result, additional viral concentration approaches should be investigated in order to increase fish viral recovery from water.

Table 2: Positive samples percentages of TilV and Megalocytivirus in water samples

Qarun Lake /Site	Total Samples	TiLV virus		Megalocytivirus	
		No. of Positive	% of positivity	No. of Positive	% of positivity
Site 1/ The coast	36	5	13.8	2	5.5
Site 2/Middle of lake	36	2	5.5	1	2.7
Total	**72**	**7**	**9.7**	**3**	**4.1**

Following viral concentration, viral identification is often accomplished using PCR-based assays. In this study, we used the RT-PCR and nested-PCR methods to identify TiLV and megalocytivirus, respectively. For TiLV detection, we employed two sets of primers

constructed based on two viral genomic sequences, Seg4 and Seg9, which were highly specific to TiLV; only one sample was positive by Seg4 primers and six samples were positive by Seg9 primers. As a result, in the current study, Seg9 primer sets appear to be more potent than Seg4 primer set. This might be because Seg9 has high levels of genetic similarity (99.52%) with all TiLV isolates **(Chamtim *et al*., 2022)**, allowing it to identify a wide range of TiLV strains from a variety of field samples, including infected-fish and water samples **(Taengphu *et al*., 2022).** However, when we examined the monthly distribution of both viruses' positive samples, we observed that both viruses were only detected in warmer months (May to October). These findings are congruent with the findings of **Eyngor *et al*. 2014**. He declared that the frequency of TiLV in hot seasons is defined by waves of tilapia mortality. This is also comparable with the findings of a previously published article **(kembou *et al*., 2017)**, which suggested that the hot season is the best time for TiLV growth *in vitro*. Finally, the positive samples' nucleotide sequences were compared to those of other known TiLV and megalocytiviruses acquired from the GenBank databases. Clustal W **(Thompson *et al*., 1994)** was used to align several nucleotide sequences. Then, using Molecular Evolutionary Genetics Analysis Version X (MEGA X), a maximum likelihood (ML) phylogenetic tree based on nucleotide sequences was created to evaluate the affiliations of our isolates to known viruses in GeneBank **(Kumar *et al*., 2018)**. Using the BLAST program, all Egyptian positive sequences showed 97.62% and 96.43% identity with the Segment 9 gene of TiLV isolated previously from Egypt and Israil (GenBank #JQ38383), respectively, and 99% identity with the major protein capsid of Megalocytivirus, which belongs to Infectious spleen and kidney necrosis viruses and was isolated from Brazil (GenBank #JQ31377), respectively (Figure 1). All of the Egyptian sequences formed a single clade with the previously isolated sequences from Egypt's Nile Delta in 2015, while the only sequence of megalocytivirus identified in this study formed a single clade with the previously isolated sequence of infectious spleen and kidney necrosis virus from Brazil.

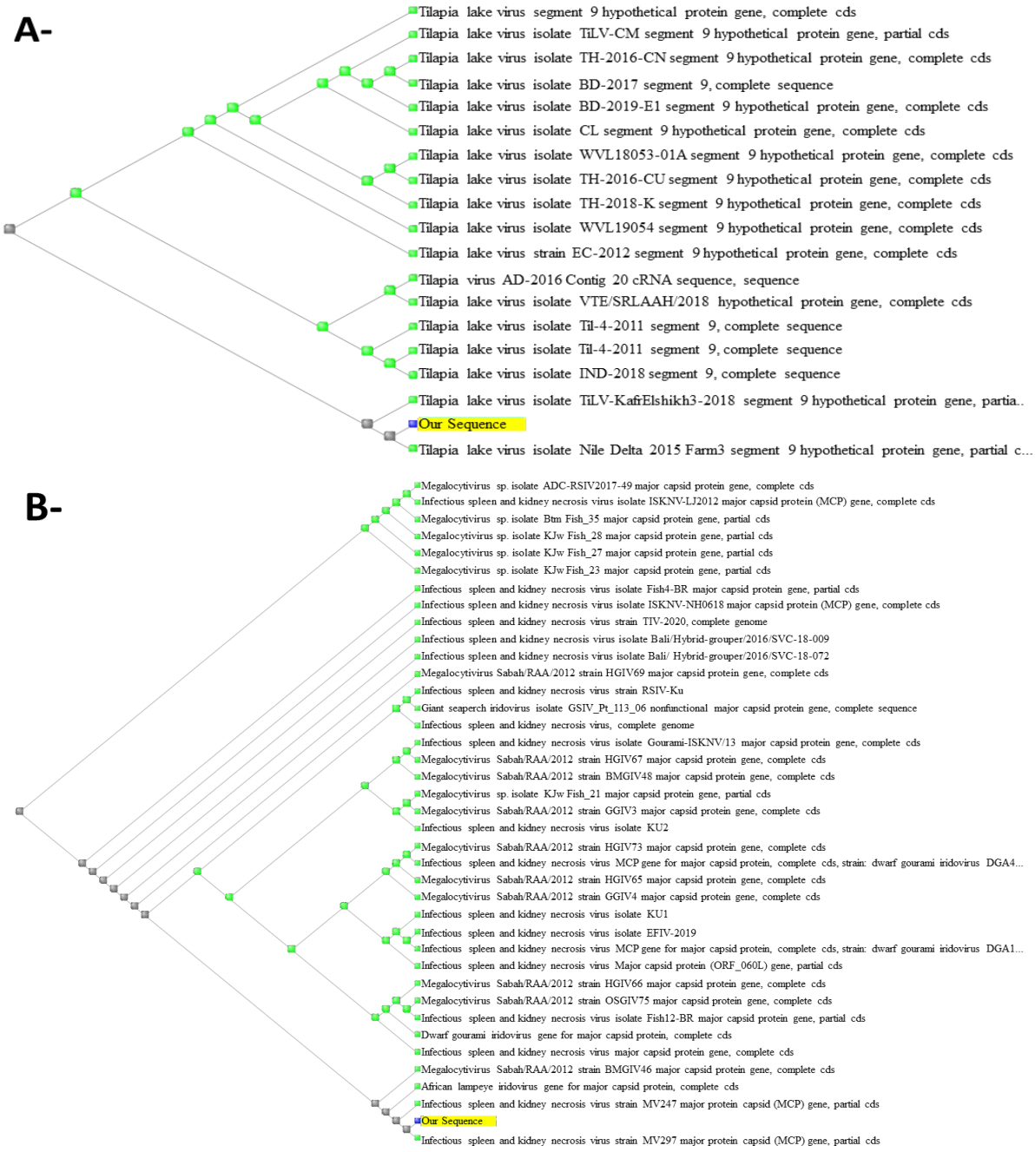

Fig. 1: Phylogenetic tree for positive sequences in the present study. (**A**) TiLV; (**B**) Megalocytivirus. All of the Egyptian sequences formed a single clade with the previously isolated sequences from Egypt's Nile Delta in 2015, while the only sequence of megalocytivirus identified in this study formed a single clade with the previously isolated sequence of infectious spleen and kidney necrosis virus from Brazil.

CONCLUSION

The presence of TiLV and megalocytiviruses in Egyptian fish-rearing water samples was established in this work, as was the application of an eRNA or eDNA detection method based on organic flocculation for the concentration and detection of TiLV and megalocytiviruses in water. This method might be useful for non-invasive viral surveillance in tilapia aquaculture systems, as well as for supporting evidence-based biosecurity choices.

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