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Full Length Research Paper

Four-years surveillance of Tilapia Lake Virus (TiLV) reveals its absence in tilapia farms and hatcheries in southern Mozambique

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In the present paper we report the results of the TiLV active surveillance program carried out during 2019/2020 to 2023. Tilapia is one of the most widely farmed freshwater fish species and currently being cultured in more than 140 countries. Over the last few years, large-scale mortalities have been reported in tilapia due to infection with Tilapia Lake Virus (TiLV), an emerging pathogen affecting both wild and farmed tilapia, with mass mortality events and biosecurity restrictions threating global tilapia industry. In the present study, we report the results of TiLV-targeted surveillance in wild and tilapia farms and hatcheries in southern Mozambique from 2019 to 2023. All 729 samples from wild and farmed fish tested during this period were negative for TiLV. We recommend the continued implementations of strict biosecurity measures, including good management practices and quarantine protocols, preventing the introduction and spread of pathogens both in wild stock and farmed fish.

Key words: Tilapia, TiLV, disease surveillance, Mozambique, aquaculture.

INTRODUCTION

Tilapia are freshwater fishes belong to family *Cichlidae*, native to Africa and Middle East introduced into many tropical, subtropical and temperate regions of the world during the second half of the 20th century. The introduction of tilapia into those areas was for (1) farming as food fish, (2) recreational fishing, (3) aquatic weed control and (4) research purposes. Tilapia have many attributes that make them an ideal candidate for aquaculture, especially in developing countries. These include their fast growth, tolerance to a wide range of environmental conditions (such as temperature, salinity and low dissolved oxygen), resistance to stress and disease, ability to reproduce in captivity and having short generation time and feeding on low trophic levels and acceptance of artificial feeds immediately after yolk-sac absorption (El-Sayed 2020; Pillay 1990).

Nile tilapia (*Oreochromis niloticus*) is among the most important aquaculture species farmed worldwide. According to the Food and Agriculture Organization of the United States (FAO), the world tilapia aquaculture production grew from 380 000 tonnes in 1990 and reached approximately 6.8 million tons during 2022, representing one of the major sources of animal protein for human consumption (FAO, 2022; Fitzsimmons, 2023) particularly in developing countries in Africa, Asia and South America. However, with intensification and increase in tilapia production, infectious disease is one of the main issues threatening the success and sustainability of tilapia production causing multibillion-dollar loss annually (Assefa and Abunna, 2018; Barría et al., 2020).

Compared to other fish, Nile tilapia is considered to be relatively disease-resistant animals, especially to many of the common pathogens that target intensively reared fish. They are still susceptible to protozoan parasites and to some bacteria, notably streptococcal infections. Viral diseases, however, are not common and there are only a few reports in the literature (Bigarre et al., 2009; Shlapobersky et al., 2010). Until 2009, no viral diseases were reported in tilapia (Aich et al., 2022). However, during the summer of 2009, enormous mortalities of both wild and farmed hybrid tilapia (*O. niloticus* × *O. aureus*) were observed in different parts of Israel and the etiological agent was subsequently identified in 2013 as Tilapia Lake Virus (TiLV) (Eyngor et al., 2014; OIE, 2017). Shortly after the first report of a novel disease among tilapia in Ecuador (Ferguson et al., 2014), was discovered as a newly emerging virus that caused mass die-offs in tilapia in Israel (Eyngor, et al., 2014). TiLV is recognized as a significant infectious agent that may threaten the development of the global tilapia industry (Jansen et al., 2018). TiLV outbreaks purportedly caused up to 90% mortality (Behera et al., 2018; Dong et al., 2017; Jansen et al., 2018; Surachetpong et al., 2017). Gross signs include dermal lesions and ulcers, ocular abnormalities, opacity of lens, loss of appetite, slow movement, gathering in the pond bottom and reduced schooling behaviour (CGIAR, 2017). To date, infection with TiLV has been 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, Taiwan Province of China, the Philippines, Malaysia, Peru, Mexico, the United States and Bangladesh (Abbadi, et al., 2023; Behera, et al., 2018; Dong, et al., 2017; Ferguson, et al., 2014; Mugimba et al. 2018; Tang, et al., 2021; Thawornwattana, et al., 2021).

In early 2017, in response to the rapid spread of TiLV, several international organizations issued a disease advisory (NACA, 2017), a fact sheet (CGIAR 2017) and a pathogen information sheet (OIE, 2017). At that time, over 40 countries, including Mozambique, were forecasted to have introduced TiLV *via* direct or indirect translocation of fry/fingerlings from the countries where it has been reported (Dong et al., 2017). The scientific community urged tilapia-producing countries to quickly investigate unusual mortality events and initiate TiLV-targeted surveillance to prevent its spread and the resulting negative consequences. Mozambique, through Aquaculture Research Center (CEPAQ) carried out one-year active surveillance in 10% of all fish farms from Maputo, Gaza and Inhambane provinces that bought fingerlings in 2020. Additionally, fish samples were also collected in main lagoons from Maputo and Gaza. Furthermore, three-years (2021-2023) TiLV-targeted surveillance was carried out in CEPAQ for Mozambique and Nile tilapia. CEPAQ is the largest supplier of tilapia matrices and fingerlings in Mozambique and the biological material (Nile tilapia) used was imported from Thailand in 2014 and 2017. In the present paper we report the results of the TiLV active surveillance program carried out during 2019/2020 to 2023.

MATERIALS AND METHODS

Biological sample collection

The Nile tilapia samples were collected in 2019/2020 at CEPAQ (grandparents, breeders, grow-out fish, fingerlings, genetic enhancement fish generation 2 and 3); 10% of fish farms per province that received fingerlings from CEPAQ (2 in Maputo, 7 in Gaza and 1 in Inhambane) and in wild environment Maputo Province (Corumane Dam and Sotiva Lagoon) and Gaza Province (Massingir Dam), (Table 1). Additionally, from 2021 to 2023 samples were collected only at CEPAQ, fish from hatchery (*Oreochromis niloticus*) and genetic enhancement (*Oreochromis mossambicus*) departments.

Table 1. Place where samples were collected.

Year	Place where samples were collected	Culture system	Number of samples
2019/2020	CEPAQ	Ponds	210
	Maputo province	Ponds and cages	60
	Gaza province	Ponds and cages	210
	Inhambane province	Ponds and cages	30
	Corumana artificial lagoon	Wild	21
	Sotiva lagoon	Wild	30
	Massingir lagoon	Wild	30

2021	CEPAQ	Ponds	30
2022	CEPAQ	Ponds	30
2023	CEPAQ	Ponds	78

For PCR analysis, pieces of the liver, kidney and brain were collected as specimens from each fish immediately after killing and then pooled in RNALater® for preservation.

The analyses were carried out at Istituto Zooprofilattico Sperimentale delle Venezie (IZSVe)-Italy. The sample process workflow is summarized in Figure 1.

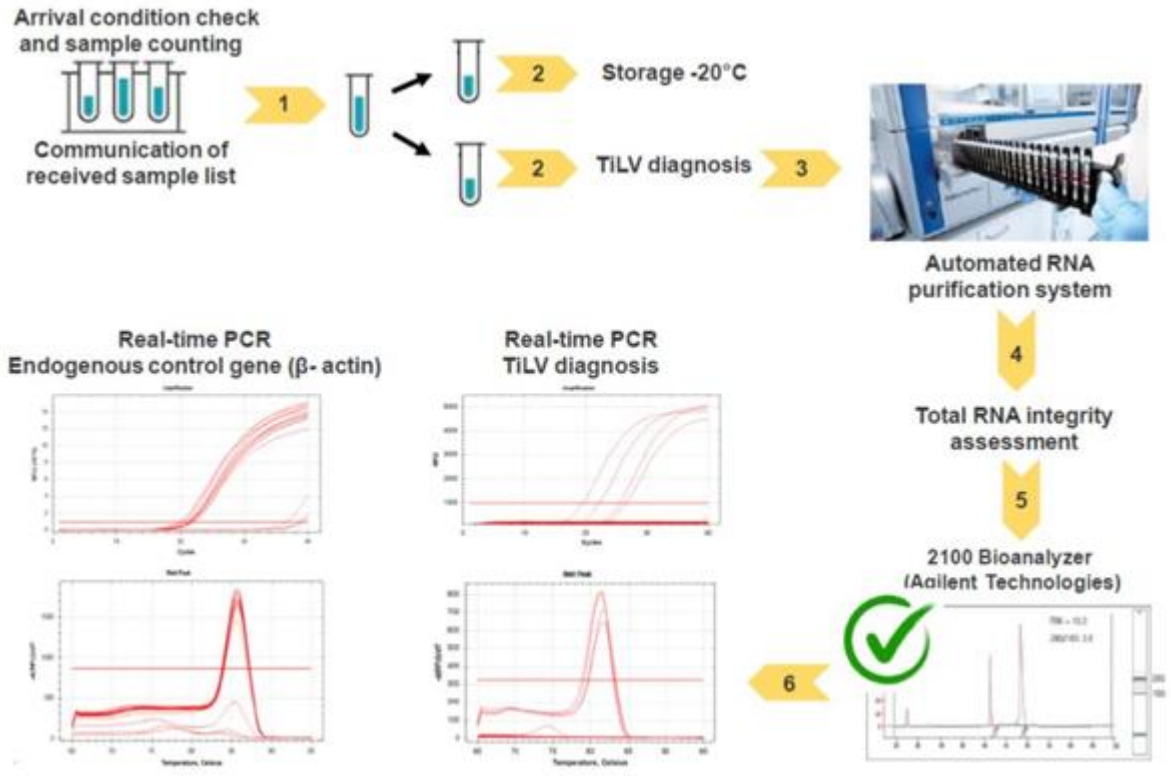

Figure 1. Process workflow.

Briefly, samples were initially visually inspected for integrity. The RNALater® solution was removed and samples were weighted. A small portion of brain and liver (approximately 30 mg-40 mg) were added with 400 µl of RLT Plus Lysis buffer and homogenized through high-speed shaking with sterile stainless-steel beads in the TissueLyser apparatus (Qiagen). The remaining portion of samples was stored a -20°C. After clarification by centrifugation, 400 µl of supernatant was subjected to RNA extraction by using QIAsymphony® RNA kit (Qiagen, Hilden, Germany), in combination with the automated system QIAsymphony SP® (Qiagen, Hilden, Germany). RNA elution was performed in 100 µl of RNase/DNase free water. Quality of the extracted RNA was checked through 2100 Bioanalyzer (Agilent) and the Agilent RNA 6000 nano kit. Then RNA was retro-transcribed with random hexamers (Invitrogen-ThermoFisher Scientific) and the obtained cDNA finally subjected to real time PCR applying the primers developed and described by Tattiyapong et al., (2017) and EvaGreen as nucleic acid dye (SSoFast EvaGreen®Supermix).

During each run 4 positive controls were used to monitor the whole reaction. The positive controls contained a fragment of segment 3 of the TiLV genome and consisted in: i) 2 different dilutions of synthetic RNA, ii) 2 different dilutions of plasmid (pTiLV). As well, two negative controls (reagents without sample and reagents without cDNA) were inserted in each run. A separate real time PCR targeting a host endogenous gene (E actin) was performed and used as amplification control of the synthetized cDNA. The real time PCR applied primers developed by Gigante et al.

Internal validation of TiLV assay

The chosen protocol was standardized internally before being used on samples. The molecular test is a two-step EvaGreen-based real time polymerase chain reaction (RT-rPCR) assay targeting segment 3 of the TiLV. Standardization of the RT-rPCR conditions were performed using synthetic RNA and plasmid DNA (pTiLV) obtained from TiLV RNA.

The sensitivity of the assay was tested by serially diluting the synthetic RNA in water and in fish (healthy cichlids from wholesaler aquarium) liver and brain homogenates. The determined Limit of Detection (LoD) was 1×10^1 copies/reaction in water, while 1×10^2 copies/reaction in fish organs homogenates.

RESULTS

The results of TiLV-targeted surveillance from 2019/2020 to 2023, are presented in Table 2. All samples tested using PCR analysis were negative for TiLV by PCR for TiLV nucleic acids with in all culture systems.

Table 2. TiLV detection in clinical samples using RT-qPCR procedure.

Year	Place where samples were collected	Culture system	Number of samples	Number of TiLV-positive/tested samples	Organs (positive/total)		
					Liver	Kidney	Brain
2019/2020	CEPAQ	Ponds	210	0/210 (100%)	0/210	0/210	0/210
	Maputo province	Ponds and cages	60	0/60 (100%)	0/60	0/60	0/60
	Gaza province	Ponds and cages	210	0/210 (100%)	0/210	0/210	0/210
	Inhambane province	Ponds and cages	30	0/30 (100%)	0/30	0/30	0/30
	Corumana artificial lagoon	Wild	21	0/21 (100%)	0/21	0/21	0/21
	Sotiva lagoon	Wild	30	0/30 (100%)	0/30	0/30	0/30
	Massingir lagoon	Wild	30	0/30 (100%)	0/30	0/30	0/30
2021	CEPAQ	Ponds	30	0/30 (100%)	0/30	0/30	0/30
2022	CEPAQ	Ponds	30	0/30 (100%)	0/30	0/30	0/30
2023	CEPAQ	Ponds	78	0/78 (100%)	0/78	0/78	0/78
Total			729	0/729	0/729	0/729	0/729

DISCUSSION

Nile Tilapia is the most farmed fish in Mozambique, with production ranging from extensive backyard ponds to large, commercial operations. In this study, we conducted a TiLV-targeted surveillance of the virus in Southern of Mozambique targeting wild and farmed tilapia and the potential circulation of the virus among tilapia broodstocks and fingerlings.

Globally, fish diseases are among the major challenging factors that limit aquaculture industry with subsequent impact on livelihood of farmers, loss of job, reduced incomes and food insecurity (Ali et al., 2020). TiLV is an emerging and transboundary recently described virus affecting tilapia industry and it has been reported in Asia, Africa and American countries (Aich et al., 2022). TiLV poses a major threat to fish supplies and the nutritional status in populations that eat tilapia on a regular basis and likely constitutes a food security issue (Hounmanou et al., 2018).

No estimate on the socio-economic impact of TiLV in a national or global context has been published (Jansen et al., 2018). High levels of mortality have been reported from field cases on all three continents (Ali, et al., 2020; Behera, et al., 2018; Debnath, et al. 2020; Dong, et al., 2017; Fathi, et al. 2017; Ferguson, et al., 2014; Mugimba, et al., 2018) suggesting that the impact may be significant.

Currently, ten sub-Saharan Africa countries (including Mozambique) have imported fish from Thailand (Dong et al. 2017) and represent risk of TiLV spread through translocation of tilapia fry/fingerlings. Investigations of TiLV is lacking in most Sub-Saharan Africa countries (Hounmanou, et al., 2018) and the actual distribution status of the virus in the region remain unknow despite the only one and first report of TiLV infection in Lake Victoria (Mugimba, et al., 2018).

CEPAQ is the main center for the production and distribution of tilapia fingerlings and broodstocks for hatcheries distributed throughout in South and Center of Mozambique and the current biological material was imported from Thailand in 2014 and 2017. The TiLV-targeted surveillance carried out in Thailand from 2012 to 2017 (Dong et al., 2017) revealed the presence of TiLV in fertilized eggs, yolk-sac larvae, fries, and fingerlings in Thai hatcheries and therefore,

the countries that imported tilapia from Thailand during this period might also have a potential risk of viral transmission. The negative results obtained during the surveillance program during 2020 to 2023 indicate that there was no introduction of TiLV through the biological material imported from Thailand as forecasted.

However, the transboundary spread of TiLV and other pathogens, reinforces the importance of strict biosecurity measures implemented at CEPAQ during the production of fingerlings and matrices of tilapia, including uses of improved husbandry/management practices, vehicles and staff and movement restrictions. Additionaly, there is a lack of any systematic aquatic diseases surveillance systems in place for the Mozambique tilapia industry, as well as limited diagnostic investigations or collection of baseline information regarding adverse events. The current development of aquaculture industry and internal circulation of aquaculture species highlights the need for developing such systems.

CONCLUSION

In summary, our findings from 2019/2020 to 2023 TiLV-targeted surveillance revealed the absence of TiLV in both wild and farmed fish in Southern of Mozambique. Nevertheless, we recommend the continued implementations of strict biosecurity measures and brought to the attention of national competent authority to include the TiLV in the national list of diseases for consideration under the national surveillance programme.

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