TANZANIA VETERINARY ASSOCIATION

PROCEEDING OF THE 35 SCIENTIFIC CONFERENCE OF THE TANZANIA VETERINARY ASSOCIATION HELD AT AICC ARUSHA, TANZANIA ON THE *5TH TO 7TH* DECEMBER, 2017

Volume 35 2017 ISSN 0856 - 1451

All correspondences should be addressed to:
Editor, Tanzania Veterinary Association, P.O. Box 3021, Chuo Kikuu, Morogoro, Tanzania.
e-mail: nongahezron@yahoo.co.uk OR zmakondo@gmail.com

Instruction to authors are provided on the inside of the back cover of every issue of the Tanzania Veterinary Journal

Publishers: Tanzanina Veterinary Association.

Molecular detection of tilapia lake virus (TiLV) genome in Nile tilapia (Oreochromis niloticus) from Lake Victoria

A.A. Chengula[1,3]*, K.K. Mugimba[3,4*], S. Wamala[3,4,] E.D. Mwega[1,3], C.J. Kasanga[1], D.K. Byarugaba[4], R.H. Mdegela[2], A. Dishon[5], S. Mutoloki[3], L. David[6], Ø. Evensen[3] and H.M. Munang'andu[3]

[1]Department of Veterinary Microbiology, Parasitology and Biotechnology, Sokoine University of Agriculture, P.O. Box 3019, Morogoro, Tanzania. [2]Department of Veterinary Medicine and Public Health, Sokoine University of Agriculture, P. O. Box 3021 Morogoro, Tanzania. [3]Norwegian University of Life Sciences, Faculty of Veterinary Medicine and Biosciences, Department of Basic Sciences and Aquatic Medicine, Section of Aquatic Medicine and Nutrition, Ullevålsveien 72, P.O. Box 8146, Dep NO-0033, Oslo, Norway. [4]Makerere University, College of Veterinary Medicine Animal Resources and Biosecurity, Department of Biotechnical and Diagnostic Sciences, P.O Box 7062-Kampala. [5]KOVAX Vaccine Company, P.O. Box 45212, Hartom 19, 97775, Jerusalem, Israel. [6]The Hebrew University of Jerusalem, R.H. Smith Faculty of Agriculture, Food and Environment, Department of Animal Sciences, P.O. Box 12, Rehovot 76100, Israel.
Email: achengula@suanet.ac.tz

SUMMARY

Tilapia lake virus (TiLV) is an emerging pathogen of Tilapiines associated with high mortalities of wild and farmed tilapia posing great threat to the fishery industry worldwide. The virus has been reported in Israel, Ecuador, Colombia, Thailand, Egypt, Taiwan, India and Malaysia. In this study, a reverse transcription polymerase chain reaction (RT-PCR) assay was developed and used to detect TiLV genome in Nile tilapia from Lake Victoria. Nile tilapia samples were collected from the Tanzanian (108 fish) and Ugandan (83 fish) parts of Lake Victoria in 2015 and 2016, respectively. Samples were screened for TiLV by using RT-PCR and the PCR products were sequenced. The findings show that out of the 191 fish examined, 28 had PCR products showing the presence of TiLV genome. The TiLV nucleic acids were detected in the spleen (10.99%, N=191), head kidney (7.69%, N=65), heart (3.45%, N=29) and liver (0.71%, N=140) samples while no PCR amplification was detected in the brain by the developed RT-PCR method. Generally, the findings show that the lymphoid organs, mainly comprising of the head kidney and spleen had the highest number of samples with positive nucleic acids for TiLV followed by heart samples. On the contrary, the liver and brain that have previously been shown to be target organs during acute infection either did not have or had the lowest level of TiLV nucleic acids detected in the present study. All the 28 sequences retrieved had an average length of 768 bp. A blast analysis on NCBI showed that all sequences obtained were homologous to TiLV segment-2 sequences obtained from previous outbreaks in Israel and Thailand. To our knowledge, this is the first detection of TiLV subclinical infections in Nile tilapia in Lake Victoria, a none-outbreak area.

Keywords: Lake Victoria, Nile tilapia, PCR, phylogenetic, surveillance, tilapia lake virus

INTRODUCTION

Tilapia lake virus (TiLV), also known as syncytial hepatitis of tilapia-SHT, was first identified and shown to cause mortalities in Nile tilapia (*Oreochromis niloticus*) in 2012 in Israel by KoVax inc. (Personal communication), following summer mortalities in tilapia fish farms in Israel. Soon after it was reported that the same virus was present in tilapia fish in the Sea of Galilee in Israel and again that this virus was causing disease and mortalities in Nile tilapia (Eyngor *et al.*, 2014b). It has since been associated with outbreaks in Colombia, Ecuador, Egypt, Israel, and Thailand (Kembou Tsofack *et al.*, 2017, Bacharach *et al.*, 2016a, Del-Pozo *et al.*, 2017b, Nicholson *et al.*, 2017, Surachetpong *et al.*, 2017, Fathi *et al.*, 2017). Based on motif alignment of its segment-1 with the PB1 segment of influenza A, B and C, the etiological agent has been characterized as an orthomyxo-like virus (Bacharach *et al.*, 2016b). Classification by the international committee of virus taxonomy (ICTV) puts TiLV as a single new species known as *Tilapia tilapinevirus* in the new genus Tilapinevirus (Adams *et al.*, 2017). It is made of 10 segments unlike other orthomyxoviruses such as influenza that are made up of eight segments (Palese and Schulman, 1976). The length of the total viral genome is about 10,323 kp (Bacharach *et al.*, 2016b, Bacharach *et al.*, 2016a, Del-Pozo *et al.*, 2017b, Eyngor *et al.*, 2014a). *In-vitro* studies show that the virus grows well at 23-30°C in vitro. Studies carried out this far show that mortalities occur at temperatures above 25°C as shown from the summer die-offs associated with TiLV in Egypt and Israel (Fathi *et al.*, 2017). TiLV has so far been reported in Nile tilapia (Egypt, Thailand) (Fathi *et al.*, 2017, Surachetpong *et al.*, 2017, Nicholson *et al.*, 2017), red tilapia (Thailand) *(Surachetpong et al.*, 2017) and the hybrid tilapia *O. niloticus* x *O aureus* (Israel

) (Bacharach *et al*., 2016b) suggesting that the range of the tilapines susceptible to TiLV could be wider.

Lake Victoria is the world's second largest freshwater lake covering a surface area of 68,000km^2 shared by three countries in East Africa namely Kenya (6%), Uganda (45%) and Tanzania (49%). By the 1960s, it was habitat for several fish species dominated by the tilapiine cichlids such as *O. esculantus* and *O. variabilis* and home to more than 200 haplochromine cichilds (Kudhongania and Cordone, 1974, Ogutu-Ohwayo, 1990, Goudswaard and Witte, 1997, Goudswaard *et al.*, 2002). Nile tilapia and Nile perch (*Lates niloticus*) were introduced in the 1950s to replace the declining tilapine species, which led to disappearance of >50% of the indigenous fish species in Lake Victoria (Ogutu-Ohwayo, 1990). Since then, Nile tilapia and Nile perch species continued to increase although by 2002, the Nile perch population began to decline giving way for the Nile tilapia to become the most dominant fish species in Lake Victoria (Njiru *et al.*, 2012, Ogutu-Ohwayo, 1994, Witte *et* fulfill the Koch's postulates by establishing the disease-causal factor relationship, virus isolation, culture and reinfection is not ideal for surveillance programs especially in situations with high number of samples. This is because the culture and reinfection approach is not only expensive, but could take long to generate results. Hence, there is urgent need for rapid diagnostic tests suitable for surveillance programs in order to expedite the process of establishing the distribution of TiLV. Moreover, developing surveillance diagnostic tools would pave way to designing appropriate disease control measures aimed at preventing the spread of the virus in the aquaculture industry. The aim of this study was twofold: 1) To develop and optimize a PCR-based method for the detection of TiLV and 2) to investigate the possible existence of TiLV in Nile tilapia found in Lake Victoria.

MATERIALS AND METHODS

Sample collection and study sites

Nile tilapia samples were collected from the Ugandan and Tanzanian parts of Lake Victoria in 2016 and 2015, respectively. For Ugandan samples, 83 fish were collected from 14 sampling sites and transported to Makerere University on ice in cool boxes. Among these, seven sites were from cage-farmed fish while the other seven were from wild fish (Table 2). Sampling for the wild sites was done in areas at least 20 – 50 km into the Lake away from the shore with minimum distances of 20 km apart while the cage farms were within 10 km from the *al*., 1991). The recent introduction of cage farming of Nile tilapia further increases its dominance on Lake Victoria. The emergence of viral diseases such as Tilapia lake virus disease (TiLVD) poses a significant threat to the expansion of Nile tilapia production, which has tremendously increased to become one of the leading cultured fish species in the world in the last decade. Furthermore, the rapid rate at which the disease is being reported to cause outbreaks in different continents across the world (Nicholson *et al*., 2017, Surachetpong *et al*., 2017, Fathi *et al*., 2017) calls for development of rapid diagnostic tools for prompt virus identification to pave way for the design of timely disease control strategies. Thus far, diagnosis of reported outbreaks has mainly been based on virus isolation, characterization, culture followed by re-infection in susceptible fish to demonstrate the characteristic syncytial hepatitis and other pathological lesions in susceptible fish (Tsofack *et al*., 2016, Del-Pozo *et al*., 2017a, Tattiyapong *et al*., 2017). Although these steps

Lake shore. Fish were dissected and processed at the Faculty of Veterinary Medicine of Makerere University. All tissues collected were stored in RNAlater and stored at 4°C for 24hrs followed by -80°C until transfer to the Norwegian University of Life Sciences (NMBU) in Oslo, Norway. In Tanzania, a total of 216 samples were collected from 108 wild fish at four sampling sites (Table 2). Two sampling sites approximately 20 km apart were sampled in Maganga beach area and another two sites in the Mchongomani area separated by approximately 25 km apart. Dissections were carried out at the Fisheries Education and Training Authority (FETA) laboratory in Mwanza and the tissues collected were stored in RNAlater at -20 °C for five days. Thereafter, all samples were transported to the College of Veterinary Medicine and Biomedical Sciences at Sokoine University of Agriculture in Morogoro where they were stored at -80°C until shipment to NMBU. Overall, a total of 442 organs from 191 fish were collected from Lake Victoria as summarized in Table 2.

Virus propagation and cell culture

Tilapia cell cultures were generated from hybrid Oreochromis niloticus X Oreochromis aureus. In brief, caudal fins were removed from euthanized 30g fish. Fish were then bathed in 1% sodium hypochlorite solution for 1 min, and then rinsed in 70% ethyl alcohol. Fins were washed three times in phosphate buffer saline (PBS) containing 10% penicillin streptomycin and 2.5% nystatin. The fins

were transferred to Petri dishes, extensively minced pieces of approximately 1mm3 were placed in dry 50 mL culture flasks (Nunc, Denmark). After 24 hours incubation at room temperature, the clumps adhering to the flasks were covered with Leibovitz (L-15) medium (Sigma) supplemented with 10% FBS (Biological Industries, Israel), 1% nystatin and 2.5% penicillin streptomycin. Cells were maintained at 28°C in a CO2 free environment. At 10–14 days incubation cells grew out from the tissue to form a monolayer around each clump. The monolayer cultures were trypsinized and transferred into new flasks with fresh medium. The cells have been passed for over 100 times to form a stable cell line and are referred to as Tilapia Fin Cells - TFC#10.

The virus used as a positive control in this study was provided by KoVax Vaccine Company in Israel. Virus isolation from sick fish was performed as follows: sick fish showing symptoms of apathy, reduced appetite and mortality were collected and frozen at -80°C. Kidney, spleen, intestine, gills and brains were collected and homogenized in PBS. The homogenate was filtered through a 0.2µm filter (Sartorius). Filtered homogenates were used to inoculate naïve TFC#10 cultures, incubated at 28°C and monitored daily. Cytopathic effect (CPE) appeared at 4-7 days post inoculation. Once extensive CPE was evident virus suspension was harvested, aliquoted and stored at -80°C for further use.

For the negative control cells, PBS only was used instead of the virus for adsorption. After seven days of incubation, suspensions for both virus infected and non-infected cells were harvested and used for RNA extraction as described below.

RNA extraction and cDNA synthesis

Extraction of total RNA from the 442 samples was carried out using a combination of the Trizol® (GIBCO, Life Technologies) and RNAeasy Mini kit (Qiagen, Hilden, Germany) techniques as previously described (Munang'andu *et al*., 2013, Munang'andu *et al*., 2012, Munang'andu *et al*., 2013). Briefly, approximately 30 mg of tissue was homogenized in 1mL Trizol followed by centrifugation at 12,000g for 10min at 4°C. Thereafter, the supernatant was transferred into an Eppendorf tube followed by addition of 0.2 mL chloroform to each sample. After vortexing for 15s, samples were left for 5min at room temperature followed by spinning at 12,000g for 15min. The aqueous phase was transferred into another Eppendorf tube. After adding 0.6mL of 70% ethanol, the tubes were vortexed and the contents with scissors, and semi-dry small tissue were transfered to RNeasy spin columns. Thereafter, the Qiagen protocol was used based on the manufacturer's guidelines (Qiagen, Hilden, Germany). RNA quantification was carried out using a spectrophotometer (NanoDrop® ND-1000, Thermo Scientific Inc). The synthesis of cDNA was carried out in 20µl reaction volumes using the Transcriptor First Strand cDNA Synthesis Kit that has an integrated step for the removal of contaminated genomic DNA (Qiagen). The final cDNA was stored at −80°C until use.

Preparation of the negative control samples was done by extracting RNA and cDNA synthesis from the non-infected TFC#10 cells while RNA and cDNA synthesized from infected cells were used to prepare the virus positive controls. In addition, a second negative control was prepared by extracting RNA from headkidney, spleen and liver samples collected from six fish of the 15th Generation of Nile tilapia cultured by the GIFT project cultured at the NMBU followed by cDNA synthesis. The cDNA prepared from the GIFT fish samples was pooled for use as negative control from a population not previously exposed to TiLV. Hence, the negative control samples used in this study were designated as TCF#10 cells and GIFT tissue.

Optimization of the polymerase chain reaction test

A total of 10 primers (Table 1) were designed targeting the 10 segments of the TiLV genome. For PCR optimization, each primer pair was tested against two TiLV positive controls designated as 2V and 5V, two GIFT tissue negative controls designated as 4T and 5T, two TCF#10 cells negative control namely 2C and 3C, and one sterile water negative control (NC). The objective of using two replicates for each control sample was to compare the reproducibility of the PCR products generated after amplification between duplicates. Further, the purpose of using two negative controls (TCF#10 cells and pooled GIFT tissue cDNA) was to compare the reliability of a continuous cell-line and host tissue derived negative control during PCR optimization. Hence, each primer was tested against a total of seven samples in order to identify primers that only detect viral cDNA in order to reduce the chances of producing unspecific PCR products. All PCR reactions for amplification of the segment 1-10 genes were carried out using the Q5 High-Fidelity DNA Polymerase (New England BioLabs inc.). After gel electrophoresis analyses, only primers showing bands in the TiLV positive controls without bands in the TFC#10 cells and GIFT tissue

negative controls were selected for use in the screening of Nile tilapia samples for the presence of TiLV in the next step.

Table 1: Primer sequences

Segment	Primer sequence	Length (bp)	Tm (°C)
Segment-1	FWD-CCTCATTCCTCGTTGTGTAAGT REV-AGGAGTTGCTGTTGGGTTATAG	1000	62
Segment-2	FWD-GTCCAGGGCGGTATGTATTG REV-CTTACGGCTGACAAGTCTCTAAG	834	62
Segment-3	FWD- GTCGAGGCATTCCAGAAGTAAG REV- GAGCTAAGGGAACGGCTATTG	834	62
Segment-4	FWD-GCCTACTTCGTTGCCTATCTC REV-GCCCAATGGTTCCCATATCT	524	62
Sgement-5	FWD-CAACTCTTAGCCTCCGGAATAC REV-CGTTCTGCACTGGGTTACA	696	62
Segment-6	FWD-CCCACACGACAGGACATATAG REV- GAGTTGGCTTAGGGTGATAAGA	948	62
Segment-7	FWD-TCCTTTAGGGATTGGCACTAAC REV-TTCCATCGACTGCTCCTAGA	486	62
Segment-8	FWD-CTTAAGGGCCATCCTGTCATC REV-TGGCTCAAATCCCAACACTAA	476	62
Segment-9	FWD-GATATCCTCCACATGACCCTTC REV-GTACGTCACTTTGTGCCATTAC	261	62
Segment-10	FWD-TCCTCTCTGTCCCTTCTGTT REV-CAGGATGAGTGTGGCAGATTAT	276	62

Screening and sequencing of Nile tilapia samples from Lake Victoria

Once the PCR optimization process was completed, the selected primers were used to screen Nile tilapia samples from Lake Victoria for the presence of TiLV. As shown in Table 2, a total of 442 samples were examined from different organs including heart, liver, brain, head, kidney and spleen. All the 28 PCR products obtained from the screening of Nile tilapia samples shown in Table 2 were extracted and purified using the QIAquick Gel extraction kit according to manufacturer's instruction (Qiagen, Hilden, Germany). Amplification of the TiLV segment-2 genes was done using the Q5 High-Fidelity DNA Polymerase as described above. PCR products were then separated by using 1.5% Agarose gel electrophoresis and extracted using the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany). Sequencing was done on a commercial basis by GATC Biotech (https://www.gatc-biotech.com). The CLC Workbench 6.0 (www.clcbio.com) and Mega7 software (Kumar et al., 2016) were used for sequence alignment and phylogenetic tree analyses. Phylogenetic trees were inferred by the Maximum Likelihood method, bootstrapped 1000 times based on the JTT+G matrix-based model (Jones *et al*., 1992). Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree was drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 36 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There was a total of 270 positions in the final dataset.

Evolutionary analyses were conducted in MEGA7 (Kumar *et al*., 2016). The results obtained for the different groups and organs were analyzed statistically using Fisher's exact test using GraphPad Prism version 7.00 for Windows, GraphPad Software, La Jolla California USA.

RESULTS

Polymerase chain reaction optimization

Figure 1 shows that PCR products obtained for segments 1, 3, 6, 8, 9 and 10 had strong bands in the TiLV positive controls (lanes 2V and 5V) and faint bands in the GIFT tissue (lanes 4T and 5T) and no bands were detected in TCF#10 cells (lanes 2C and 3C) and sterile water (Lane N). Although the presence of strong bands in the virus positive controls show that these primers detected the viral cDNA, the presence of faint bands in the GIFT tissue negative controls show that they produced unspecific amplifications. In addition, there were no PCR products for segment-4 for the positive and negative controls while PCR products for segment 7 had weak bands in the virus control and no bands were seen in the negative controls (Figure 1). However, PCR products for segment-2 were only detected in the virus controls (2V and 5V) and no bands were detected in the GIFT fish (lane 4T and 5T), TCF#10 cells (lanes 2C and 3C) and sterile water (lane N) negative controls. Therefore, the presence of clear bands in the TiLV positive controls (lanes 2V and 5V) and the absence of PCR products in the negative control was indicative that segment-2 primers were only able to detect TiLV cDNA, but not unspecified amplifications. Therefore, segment-2 primers were selected for the screening of Nile tilapia samples from Lake Victoria in the next step based on their ability to only detect viral cDNA and not host DNA, while primers for other segments were considered less suitable because either they gave some levels of unspecific amplifications in the controls or failed to detect the viral cDNA. Finally, the GIFT fish negative control was more reliable at detecting unspecific amplifications (Fig. 1, lanes 4T and 5T) compared to the TFC#10 cells negative control (lanes 2C and 3C) that showed absence of unspecific amplifications for primers tested during the PCR optimization process.

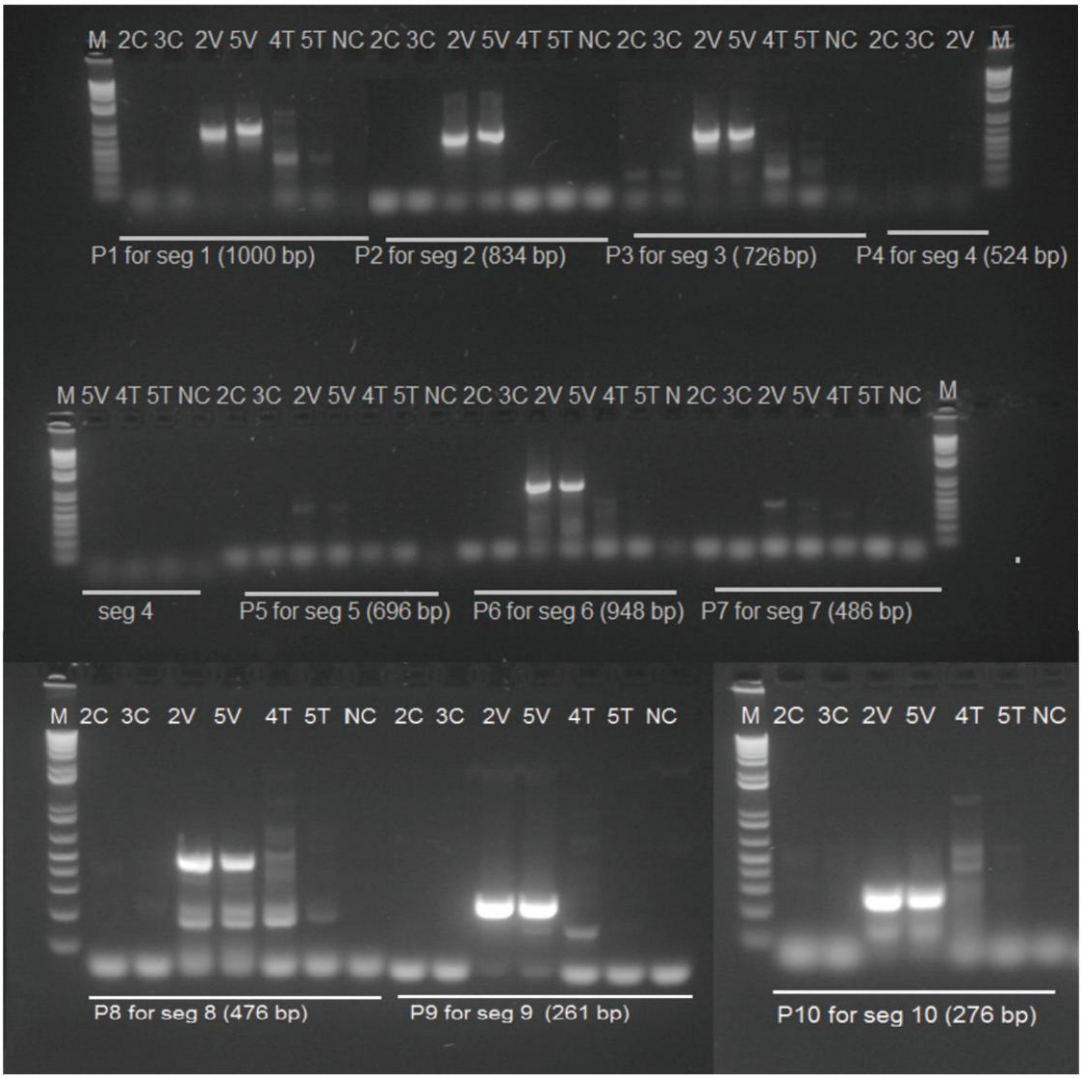

Figure 1: Shows electrophoresis gel analysis of TiLV positive control and the TCF#10 cells and GIFT fish negative control samples tested against TiLV segment 1 -10 for the 10 primers (P1 to P10) enlisted in Table 1. Note that both the positive and negative control samples are tested in duplicates in which the TFC#10 cells cDNA samples are designated as 2C and 3C, GIFT fish cDNA samples are designated as 4T and 5T while the positive virus (TiLV) control samples are designated as 2V and 5V. In addition, a single lane designated as NC for RNase free water was added to each segment tested. The expected amplicon for each primer are shown alongside the name of the segment tested. There were no detectable bands in the TCF#10 cell (lanes 2C and 3C) and RNAse free water (lane NC) negative controls for all the 10 primers tested for segments 1 -10. Note that P1, 2, 3, 6, 8, 9 and 10 showed clear bright bands of the virus positive control (lanes 2V and 5V) while P7 had a faint band in lane 2V and no bands were detected in P4. Finally, the GIFT fish samples showed faint bands in P1, 3, 6, 8, 9 and 10 in lanes 4T and 5T of variable amplicon sizes.

Screening of Nile tilapia samples from Lake Victoria

Table 2 shows a summary of the number of samples examined for the presence of TiLV nucleic acids by PCR using segment-2 primers. Of the 191 fish examined, 28 were found positive by PCR for TiLV nucleic acids with a prevalence of 14.7% (N=191). The prevalence in caged and wild fish was 17.8% (N=45) and 13.7% (N=146), respectively. There was no significance difference in the prevalence ($p=0.136$) among wild fish from the Tanzania side (16.67%, N=108) compared with the Uganda side (5.3% N=38). However, there was a significant difference ($P<0.0028$) in tissue distribution among organs examined. PCR products were detected in 10.99% (N=191) spleen, 7.7% (N=65) head kidney, 3.5% (N=29) heart and liver 0.71% (N=140) samples while no PCR products were detected in brain samples (0.0%, N=17). In summary, Table 2 shows that the lymphoid organs, mainly comprising

of the head kidney and spleen had the highest prevalence followed by heart samples.

Table 2. Sampling sites and number of fish samples

Country	Sampling site	Culture system	Positive fish/ total	Positive samples/ total	Organs (Positive/total)				
					Liver	Heart	Head kidney	Spleen	Brain
Tanzania	Maganga beach-1	Wild	4/19	4/38	0/19	-	-	4/19	-
	Mchongomani-1		7/28	7/56	0/28	-	-	7/28	-
	Maganga beach-2		6/35	6/70	0/35	-	-	6/35	-
	Mchongomani-2		1/26	1/52	0/26	-	-	1/26	
Uganda	Kigungu	Cage farms	5/8	5/32	0/8	0/8	3/8	2/8	
	Lwera		1/8	1/16			1/8	0/8	
	Kasenyi		0/8	0/8				0/8	
	Entebbe		0/5	0/5				0/5	
	Bukanama		0/5	0/5				0/5	
	SON		1/6	1/23	1/6	0/6	0/5	0/6	
	Kome		1/5	1/11			1/6	0/5	
	Lake Victoria-1	Wild	0/5	0/10			0/5	0/5	
	Lake Victoria-2		0/6	0/12			0/6	0/6	
	Lake Victoria-3		0/6	0/12			0/6	0/6	
	Lake Victoria-4		0/3	0/6			0/3	0/3	
	Lake Victoria-5		1/6	1/30	0/6	0/6	0/6	1/6	0/6
	Lake Victoria-6		1/6	1/30	0/6	1/6	0/6	0/6	0/6
	Lake Victoria-7		0/6	0/26	0/6	0/3	0/6	0/6	0/5
Total			28/191	28/442	1/140	1/29	5/65	21/191	0/17
Prevalence			14.7%	6.3%	0.7%	3.5%	7.7%	10.9%	0.0%

Sequence alignment and phylogenetic analysis

Table 3 provides a summary of sequenced samples showing their origin, organ, size of the sequence product and Genebank accession numbers. The average length of the 28 sequences retrieved was 768 bp. A blast analysis against the NCBI sequence database showed that all sequences obtained were homologous to TiLV segment-2 sequences obtained from viruses isolated from Israel and Thailand. The samples clustered into two groups (Figure 2) that were slightly different from each other and corresponded to different isolates that were already reported in the Genebank database. Group-I comprised of a total 25 Lake Victoria sequences of which nine were from Uganda and 16 from Tanzania that were identical with the Israeli KU552132 sequence deposited by Tal *et al.* (2016) and Thailand KX631922.1 sequence deposited by Surachetpong *et al*. (2017). Group-II consisted of only three Lake Victoria sequences of which one was from Uganda and two were from Tanzania that were closely related to the Israeli KU751815.1 and NC029921 sequences deposited by Eygor et al. (2014b) and Bacharach *et al*. (2016b), respectively

Table 3. Description of samples used for TiLV sequencing

Sample ID	Country	Source	Organ	Size (bp)	Genebank Acc #
UG2016-01	Uganda	Wild	Headkidney	830	MF536423
UG2016-02	Uganda	Wild	Liver	782	MF536429
UG2016-03	Uganda	Wild	Headkidney	332	MF536432
UG2016-04	Uganda	Wild	Heart	805	MF536426
UG2016-05	Uganda	Wild	Spleen	818	MF536427
UG2016-06	Uganda	Wild	Headkidney	830	MF536424
UG2016-07	Uganda	Wild	Spleen	819	MF536425
UG2016-08	Uganda	Wild	Spleen	787	MF536428
UG2016-09	Uganda	Wild	Headkidney	540	MF536430
UG2016-10	Uganda	Wild	Headkidney	724	MF536431
TZ2015-01	Tanzania	Maganga beach	Spleen	777	MF526992
TZ2015-02	Tanzania	Mchongomani	Spleen	767	MF526988
TZ2015-03	Tanzania	Mchongomani	Spleen	827	MF526987
TZ2015-04	Tanzania	Maganga beach	Spleen	755	MF526989
TZ2015-05	Tanzania	Mchongomani	Spleen	828	MF526980
TZ2015-06	Tanzania	Maganga beach	Spleen	669	MF526982
TZ2015-07	Tanzania	Maganga beach	Spleen	706	MF526991
TZ2015-08	Tanzania	Maganga beach	Spleen	675	MF526981
TZ2015-09	Tanzania	Mchongomani	Spleen	725	MF526993
TZ2015-10	Tanzania	Mchongomani	Spleen	827	MF526983
TZ2015-11	Tanzania	Mchongomani	Spleen	578	MF526994
TZ2015-12	Tanzania	Mchongomani	Spleen	758	MF526984
TZ2015-13	Tanzania	Mchongomani	Spleen	792	MF526985
TZ2015-14	Tanzania	Maganga beach	Spleen	576	MF526995
TZ2015-15	Tanzania	Maganga beach	Spleen	731	MF526996
TZ2015-16	Tanzania	Maganga beach	Spleen	765	MF526990
TZ2015-17	Tanzania	Maganga beach	Spleen	794	MF526986
TZ2015-18	Tanzania	Maganga beach	Spleen		

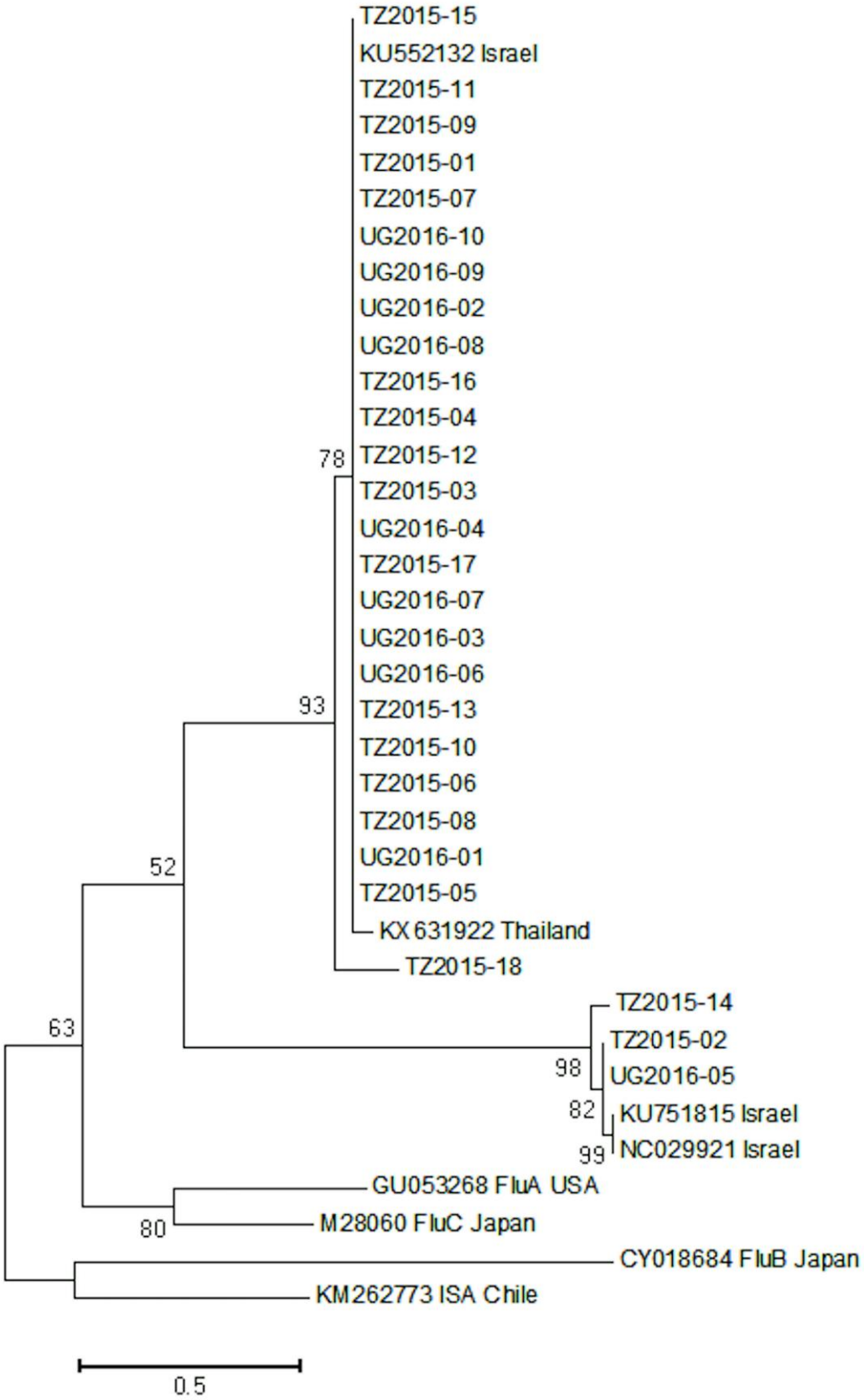

Figure 2: Phylogenetic analysis of the 28 Nile tilapia samples from Lake Victoria (TZ for Tanzanian and UG for Ugandan samples) sequenced using segment -2 primers. The evolutionary history was inferred using the Maximum Likelihood method based on the Tamura-Nei model (Tamura and Nei, 1993)**.**

.

Infection status determined by post-challenge seroconversion

In unvaccinated cattle seroconversion is an indicator of AlHV-1 infection. This can be used in combination with PCR results to determine the proportion of unvaccinated cattle that became infected during challenge. Of 97 initially seronegative unvaccinated cattle, 40 seroconverted during the challenge phase, while a further 26 remained seronegative but were PCR positive (Table 1). In total 66 out of 97 (68%) unvaccinated cattle showed evidence of post-challenge infection, from Lake Victoria using PCR. Although we did not fulfill the Koch's postulate (Evans, 1976, Gradmann, 2014, Fredericks and Relman, 1996) by establishing the disease-casual factor relationship based on isolation, characterization, culture and re-infection to demonstrate the induction of clinical disease in susceptible fish, our data are strongly suggestive of TiLV infecting Nile tilapia in Lake Victoria. These findings have significant implications on tilapia farming and in countries using parent stocks from Lake Victoria. It is likely that as the demand for high production outputs increases, the use of intensive farming systems based on high stocking densities and artificial feeds aimed at enhancing growth rate is also bound to increase. These factors have the propensity to induce stress in cultured fish (Munang'andu, 2016), which could lead to underlying viruses to replicate culminating in disease outbreaks. Moreover, high stocking densities are proponents of a high transmission index (Munang'andu, 2016), which could increase the risk of inducing TiLV outbreaks in farmed Nile tilapia. Therefore, the detected TiLV nucleic acids in this study serve as an early warning system in which future outbreaks should be thoroughly investigated in order to confirm the possible existence of TiLV in Nile tilapia in Lake Victoria.

Our findings show an overall population prevalence of 14.7% (N=191) suggesting that in subclinical infection, TiLV could be present in a population at low prevalence only increasing to high levels reaching up to 90% mortality during massive die-offs (Fathi *et al*., 2017, Surachetpong *et al*., 2017). Detection of TiLV nucleic acids in the liver, heart, head kidney and spleen in this study is in line with previous studies in which it was shown that TiLV has a tropism for different organs inclusive of the liver, brain, spleen and head kidney when clinical signs of disease are observed (Bacharach *et al*., 2016b, Fathi *et al*., 2017, Ferguson *et al*., 2014, Surachetpong *et al*., 2017, Tsofack *et al*., 2016). Previous studies have pointed to the brain and liver of which fourdied of MCF. The proportion of infected unvaccinated cattle that died of MCF was 6%.

DISCUSSION

In this study, we detected TiLV nucleic acids in an area with no record of previous outbreaks. In line with Louws et al (Louws *et al.*, 1999) who pointed out that the three Ds of PCR analyses are detection, diversity, and diagnosis, we have; (i) detected; (ii) shown phylogenetic diversity; and, (iii) diagnosed the presence of TiLV nucleic acids in Nile tilapia as target organs of which it has been associated with syncytial formation in the liver as a pathognomonic feature of the disease, at least in one study (Del-Pozo *et al*., 2017a). The high prevalence of TiLV nucleic acids in the headkidney and spleen coupled with a low presence in the liver and absence in brain samples shown in this study suggests that lymphoid organs could be ideal for screening the presence of TiLV nucleic acids during surveillance. However, there is a need for more studies to consolidate this observation.

Phylogenetic analysis clustered our sequences in two similar groups. It is interesting to note that based on segment 2 fragments, these groups correspond with Israeli isolates as shown that group-I sequences were clustered together with the Israeli KU552132 sequence while group-II sequences were clustered with Israeli KU751815 and NCO29921 sequences suggesting that TiLV sequences found in Lake Victoria are similar to strains found in the Sea of Galilee in Israel. In addition, group-I sequences that formed the largest cluster were similar to the Thailand isolate KX631992 suggesting that TiLV sequences in Thailand, Israel and Lake Victoria might have a common origin. Given that Nile tilapia is originally a freshwater teleost species native to the Nilo-Sudanian ecoregion of Africa (McAndrew, 2000), which in recent decades has been introduced into more than 85 countries in the world (Casal, 2006, Molnar *et al*., 2008, Dong *et al*., 2017), it is likely that its dispersal could have contributed to the spread of TiLV. The existence of TiLV sequences shown in our findings suggests that the virus could have been in existence for a long time such that as tilapia were being dispersed across the world, they carried the virus unnoticed. Its emergence as a fish pathogen is most likely due to stress related factors induced by current intensified aquaculture systems as well as the increasing environment changes that stress fish in natural waterbodies. However, there is need for detailed studies to determine its distribution and to identify factors linked to its dispersal in aquaculture. Moreover, future studies should seek to

establish whether genomic differences seen between group I and II strains in this study account for differences in virulence and persistence linked to subclinical infection, tissue tropism or other factors. Although the homology between sequences obtained in this study and those from previous outbreaks in Israel and Thailand suggests that Lake Victoria sequences could be originating from a virus having the potential to cause outbreaks in Nile tilapia it is important that these findings are supported with virus isolation, culture and re-infection in future studies.

Conclusions

This is the first documentation of TiLV genomes in a none-outbreak area (Lake Victoria). The findings clearly demonstrate that viral nucleic acids were present at low-level in seemingly healthy fish. Future studies should focus on isolating the virus from Nile tilapia and demonstrate its ability to cause disease in susceptible fish.

ACKNOWLEDGEMENTS

This study was supported in part by the TRAHESA project, which is the Capacity building for training and research in aquatic and environmental health in Eastern and Southern Africa, funded by the Norwegian Development Agency, Project No. TAN/13/0027.

REFERENCES

Adams MJ, Lefkowitz EJ, King AM, Harrach B, Harrison RL, Knowles NJ, Kropinski AM, Krupovic M, Kuhn JH, Mushegian AR. Changes to taxonomy and the International Code of Virus Classification and Nomenclature ratified by the International Committee on Taxonomy of Viruses. *Arch Virol* 162:2505-2538, 2017.

Bacharach E, Mishra N, Briese T, Zody MC, Kembou Tsofack JE, Zamostiano R, Berkowitz A, Ng J, Nitido A, Corvelo A, Toussaint NC, Abel Nielsen SC, Hornig M, Del Pozo J, Bloom T, Ferguson H, Eldar A, Lipkin WI. Characterization of a novel Orthomyxo-like virus causing mass die-offs of tilapia. *MBio*, 7: e00431-16, 2016.

Casal CMV. Global documentation of fish introductions: the growing crisis and recommendations for action. *Biol Inv*, 8:3-11, 2006.

Del-Pozo J, Mishra N, Kabuusu R, Cheetham S, Eldar A, Bacharach E, Lipkin WI, Ferguson HW. Syncytial Hepatitis of Tilapia (*Oreochromis niloticus* L.) is associated with Orthomyxovirus-like virions in hepatocytes. *Vet Pathol*, 54:164-170, 2017.

Dong HT, Ataguba GA, Khunrae P, Rattanarojpong T, Senapin S. Evidence of TiLV infection in tilapia hatcheries from 2012 to 2017 reveals probable global spread of the disease. *Aquaculture*, 479:579-583, 2017.

Evans AS. Causation and disease: the Henle-Koch postulates revisited. *The Yale J Biol Med*, 49:175, 1976.

Eyngor M, Zamostiano R, Kembou Tsofack JE, Berkowitz A, Bercovier H, Tinman S, Lev M, Hurvitz A, Galeotti M, Bacharach E, Eldar A. Identification of a novel RNA virus lethal to tilapia. *J Clin Microbiol*, 52:4137-46, 2014.

Fathi M, Dickson C, Dickson M, Leschen W, Baily J, Muir F, Ulrich K, Weidmann M. Identification of Tilapia lake virus in Egypt in Nile tilapia affected by 'summer mortality'syndrome. *Aquaculture*, 473:430-432, 2017.

Ferguson H, Kabuusu R, Beltran S, Reyes E, Lince J, del Pozo J. Syncytial hepatitis of farmed tilapia, Oreochromis niloticus (L.): a case report. *J Fish Dis*, 37:583-589, 2014.

Fredericks D, Relman DA. Sequence-based identification of microbial pathogens: a reconsideration of Koch's postulates. *Clin Microb Rev*, 9:18-33, 1996.

Goudswaard KP, Witte F. The catfish fauna of Lake Victoria after the Nile perch upsurge. *Environ Biol Fishes*, 49:21-43, 1997.

Goudswaard P, Witte F, Katunzi E. The tilapiine fish stock of Lake Victoria before and after the Nile perch upsurge. *J Fish Biol* 60:838-856, 2002.

Gradmann C. A spirit of scientific rigour: Koch's postulates in twentieth-century medicine. *Microb Infec*, 16:885-892, 2014.

Jones DT, Taylor WR, Thornton JM. The rapid generation of mutation data matrices from protein sequences. Computer applications in the biosciences: *CABIOS*, 8:275-282, 1992.

Kembou Tsofack JE, Zamostiano R, Watted S, Berkowitz A, Rosenbluth E, Mishra N, Briese T, Lipkin WI, Kabuusu RM, Ferguson H, Del Pozo J, Eldar A, Bacharach E. Detection of Tilapia Lake Virus in Clinical Samples by Culturing and Nested Reverse Transcription-PCR. *J Clin Microbiol*, 55:759-767, 2017.

Kudhongania A, Cordone AJ. Batho-spatial distribution pattern and biomass estimate of the major demersal fishes in Lake Victoria. *African J T Hydrob Fisheries*, 3:15-31, 1974.

Kumar S, Stecher G, Tamura K. MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets. *Molec Biol Evol msw*, 054, 2016.

Louws F, Rademaker J, De Bruijn F. The three Ds of PCR-based genomic analysis of phytobacteria: diversity, detection, and disease diagnosis. *Ann Rev Phytopathol*, 37:81-125, 1999.

McAndrew B. Evolution, phylogenetic relationships and biogeography. *Fish and Fisheries Series*, 25:1-32, 2000.

Molnar JL, Gamboa RL, Revenga C, Spalding MD. Assessing the global threat of invasive species to marine biodiversity. *Front Ecol Environ*, 6:485-492, 2008.

Munang'andu HM, Fredriksen BN, Mutoloki S, Brudeseth B, Kuo T-Y, Marjara IS, Dalmo RA, Evensen Ø. Comparison of vaccine efficacy for different antigen delivery systems for infectious pancreatic necrosis virus vaccines in Atlantic salmon (Salmo salar L.) in a cohabitation challenge model. *Vaccine*, 30:4007-4016, 2012.

Munang'andu HM, Fredriksen BN, Mutoloki S, Dalmo RA, Evensen Ø. The kinetics of CD4+ and CD8+ T-cell gene expression correlate with protection in Atlantic

salmon (Salmo salar L) vaccinated against infectious pancreatic necrosis. *Vaccine*, 31:1956-1963, 2013.

Munang'andu HM, Mutoloki, S., Evensen, O. Prevention and Control of Viral Diseases in Aquaculture. Aquaculture virology, Edited by Kibenge FSB and MG Godoy, Academic Pres, Elsevier, London, UK:17, 2016.

Munang'andu HM, Sandtrø A, Mutoloki S, Brudeseth BE, Santi N, Evensen Ø. Immunogenicity and cross protective ability of the central VP2 amino acids of infectious pancreatic necrosis virus in Atlantic salmon (Salmo salar L.). *PloS one,* 8: e54263, 2013.

Nicholson P, Fathi M, Fischer A, Mohan C, Schieck E, Mishra N, Heinimann A, Frey J, Wieland B, Jores J. Detection of Tilapia lake virus in Egyptian fish farms experiencing high mortalities in 2015. *J Fish Dis,* 40: 1925–1928, 2017.

Njiru M, Nyamweya C, Gichuki J, Mugidde R, Mkumbo O, Witte F. Increase in anoxia in Lake Victoria and its effects on the fishery, Anoxia. *InTech*, 100-128, 2012.

Ogutu-Ohwayo R. The decline of the native fishes of lakes Victoria and Kyoga (East Africa) and the impact of introduced species, especially the Nile perch, Lates niloticus, and the Nile tilapia, *Oreochromis niloticus*. *Environ Biol Fishes*, 27:81-96, 1990

Ogutu-Ohwayo R. Adjustments in fish stocks and in life history characteristics of the Nile perch, *Lates niloticus* L. in lakes Victoria, Kyoga and Nabugabo, 1994. [https://mspace.lib.umanitoba.ca/handle/1993/18221].

Palese P, Schulman JL. Mapping of the influenza virus genome: identification of the hemagglutinin and the neuraminidase genes. *Proceed Nation Acad Sci*, 73:2142-2146, 1976.

Surachetpong W, Janetanakit T, Nonthabenjawan N, Tattiyapong P, Sirikanchana K, Amonsin A. Outbreaks of Tilapia lake virus infection, Thailand, 2015–2016. *Emerg Infect Dis*, 23:1031, 2017.

Tal S, Bornestein, B., Ben-Moshe, E., Nevo, Y., Bronstein, M., Bar, N., Elgavish, S., Tadmor, R., David, L., Diamant, A., Kotler, M. and Dishon, A. Isolation and primary characterization of a virus causing mortality in tilapia. Unpublished NCBI KU5521321 (Research and Development, KoVax Ltd, 19 Hartom ST, Jerusalem 45212, Israel), 2016.

Tamura K, Nei M. Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. *Mol Biol Evol*, 10:512-526, 1993.

Tattiyapong P, Dachavichitlead W, Surachetpong W. Experimental infection of Tilapia Lake Virus (TiLV) in Nile tilapia (*Oreochromis niloticus*) and red tilapia (*Oreochromis* spp.). *Vet Microb*, 207:170-177, 2017.

Tsofack JEK, Zamostiano R, Watted S, Berkowitz A, Rosenbluth E, Mishra N, Briese T, Lipkin WI, Kabuusu RM, Ferguson H. Detection of Tilapia lake virus (TiLV) in Clinical samples by culturing and nested RT-PCR. *J Clin Microb*, 01808-16, 2016.

Witte F, Goldschmidt T, Goudswaard P, Ligtvoet W, Van Oijen M, Wanink J. Species extinction and concomitant ecological changes in Lake Victoria. *Netherlands J Zool*, 42:214-232, 1991.