Norwegian University of Life Sciences
Faculty of Veterinary Medicine
Institute of Paraclinical Sciences

Philosophiae Doctor (PhD)
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Tilapia Lake Virus (TiLV) – development of PCR-based diagnostic assays and insights into infection mechanisms

Tilapia lake virus (TiLV) – utvikling av PCR-baserte diagnostiske metoder og studier av infeksjonsmekanismer

Augustino Alfred Chengula

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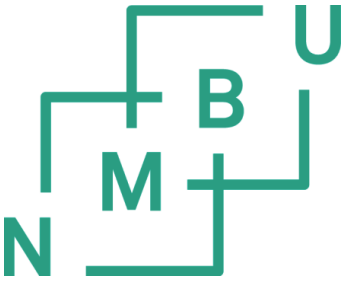

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TABLE OF CONTENTS

TABLE OF CONTENTS 2
ACKNOWLEDGEMENTS 4
LIST OF ABBREVIATIONS 6
SUMMARY 8
SUMMARY IN NORWEGIAN 9
LIST OF PAPERS 12
INTRODUCTION 13
World Tilapiine production and its significance 13
Emerging viral diseases of wild and farmed tilapiines 14
Tilapia lake virus (TiLV) 16
Morphological, biochemical and physiochemical properties of TiLV 16
Nomenclature and classification 19
Viral replication and pathogenicity 19
Tilapia lake virus disease 21
Clinical manifestations and pathological lesions of the infected fish 22
Host range and susceptibility to TiLV 24
Tilapia lake virus transmission and the associated risk factors for TiLVD 25
Geographical distribution of TiLV and countries suggested being potentially at risk 26
Diagnostic methods 27
OBJECTIVES 30
Aims and objectives 30
SUMMARY OF THE PAPERS 31
Paper I: 31
Paper II: 32
Paper III 33
MATERIALS AND METHODS 34
Sample collection and study design 34
Cell culture and virus propagation 35
Ribonucleic acid (RNA) extraction and complementary deoxyribonucleic acid (cDNA) synthesis 35
Primer design, in silico analysis and optimization 36
Polymerase chain reaction (PCR) amplification 37
Hemagglutination assay 38

Inhibition of TiLV replication in E-11 cells with ammonium chloride 38
Data treatment and statistical analysis 39
RESULTS AND DISCUSSION 40
General discussion 40
Tilapia lake virus genome has been detected in Nile tilapia from Lake Victoria in East Africa 41
The developed quantitative real time-PCR is sensitive and specific for TiLV 45
Tilapia lake virus does not cause hemagglutination of erythrocytes from turkey, Nile tilapia or Atlantic salmon 47
Treatment of E-11 cells with ammonium chloride does not affect the replication of TiLV 48
MAIN CONCLUSIONS 49
FUTURE PERSPECTIVES 50
REFERENCE LIST 53

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LIST OF ABBREVIATIONS

µl	-microliter
BLAST	-Basic Local Alignment Search Tool
cDNA	-complementary DNA
CPE	-cytopathic effect
Ct	-the cycle-threshold
CV	-coefficient of variation
DNA	-deoxyribonucleic acid
dpi	-days post-infection
dsDNA	-double-stranded DNA
dT	-deoxythymine
EPC	-endothelial progenitor cell
FAO	- Food and Agriculture Organization of the United Nations
FBS	-fetal bovine serum
HA	-hemagglutinin
HE	-hemagglutinin esterase
HEF	-hemagglutinin esterase-fusion
ICTV	-International Committee on Taxonomy of Viruses
ml	-millilitre
MOI	-multiplicity of infection
NA	-neuraminidase
NCBI	-National Center for Biotechnology Information
NH_4Cl	-ammonium chloride
NMBU	-Norwegian University of Life Sciences
ORF	-open reading frame
PA	-polymerase acidic
PB1	-polymerase basic 1
PB2	-polymerase basic 1
PCR	-polymerase chain reaction
R^2	-the square of the coefficient of correlation

RNA	-ribonucleic acid
RT	-reverse transcriptase
RT-PCR	-reverse transcription PCR
qRT-PCR	-quantitative RT-PCR
SD	-standard deviation
SUA	-Sokoine University of Agriculture
$TCID_{50}$	-tissue culture infective dose by 50%
TFC#10	-Tilapia fin cells number 10
TiLV	-Tilapia lake virus
TiLVD	-Tilapia lake virus disease

SUMMARY

Tilapia lake virus (TiLV) is an emerging virus of wild and farmed tilapiines responsible for causing mortalities and significant economic losses to the aquaculture industry. Since its first report in Israel, the virus has been reported in four continents (Asia, Africa, South and North America) to cause mortalities ranging from the lower extreme of 5-20% and higher extremes of up to 90%. The infection status in many countries is not yet known and implementation of surveillance programs has been recommended. Lake Victoria was selected for TiLV surveillance because it contains a large number of tilapiine species of economic importance to the surrounding East African countries. Well-optimized tools for rapid detection and quantification of the virus are also needed. Moreover, the mode of virus uptake in cells and importance for replication is not known. Therefore, this thesis aimed at understanding the possible presence of TiLV in Lake Victoria in East Africa, to develop tools for detection and quantification of the virus and to shed light of virus uptake mechanisms in permissive cells *in vitro*.

In this thesis, a standard RT-PCR and a quantitative real-time RT-PCR (qRT-PCR) were developed based on all the ten segments of TiLV. The standard PCR was used to screen Nile tilapia (*Oreochromis niloticus*) from Lake Victoria in Eastern Africa. The quantitative real-time RT-PCR was developed based on virus supernatant of known titre and against samples of unknown virus titre originating from infected TFC #10 cells and fish organs. The virus' ability to hemagglutinate avian and piscine erythrocytes was assessed, and the modulation of ammonium chloride on uptake and replication of TiLV in E-11 cells was studied.

The findings reported in study I showed that primers designed from segment two of TiLV for a standard RT-PCR were the best at detecting TiLV in the infected cells and Nile tilapia organs. TiLV genome was detected in 28 Nile tilapias (14. 66%, N = 191) in which 17.78% (N=45) were from wild fish and 13.70% (N=146) from farmed fish (cage farming). The genomes of circulating TiLV in Nile tilapia from Lake Victoria were identical to those detected from Israel (98%), Ecuador (98%), Thailand (96%), Peru (96%) and USA (97%). TiLV was not grown from infected fish and thus its ability to cause disease in Nile tilapia was not studied. Therefore, I am recommending further studies to fulfil Koch's postulates.

The data reported in study II showed that the developed and optimized quantitative real-time RT-PCR detected TiLV in virus supernatants of known titre and in organs of unknown titre from infected Nile tilapia. The developed assay is sensitive and specific to TiLV with all the primers efficiency being within the range of 95-105%, except primers targeting segment ten that gave an efficiency of 93%. The intra- and inter-assay coefficient of variation ranged between 0.00% ~ 2.63% and 0.00% ~ 5.92%, respectively, which is within the recommended range (below 5%) for an assay to be repeatable and reproducible. The detection limit of 2 $TCID_{50}$/ml was found for primers targeting segments 1, 2, 3, 4 and 9 while lower detection limit of 20 TCID50/ml was found for primers targeting segment 5, 6, 7, 8 and 10. Overall primers targeting segment 3 had the highest detection limit and primers targeting segment 7 had the lowest detection limit. Interestingly, despite the two primer sets (for segment 3 and 7) having different TiLV detection limits they had an equal amplification efficiency of 98%. Therefore, primer optimization for qRT-PCR is important to optimize assay sensitivity.

The study reported in paper III was directed at understanding the hemagglutination property of TiLV using avian and piscine erythrocytes, and the infection mechanisms in E-11 cells. TiLV did not hemagglutinate erythrocytes from any of the species tested indicating that the virus lack hemagglutinin. Further, the study has shown that ammonium chloride does not affect the replication of TiLV in E-11 cells indicating that the virus is not using the endocytic pathway during internalization. Taken together, the two observations suggest that, TiLV is not taken up by receptor-mediated endocytosis during internalization into E-11 cells. Thus, further studies are needed to unravel the uptake mechanism(s), which is the important information for controlling the virus by antiviral agents or immunoprophylaxis.

SUMMARY IN NORWEGIAN

Tilapia lake virus (TiLV) er et fremvoksende virus som infiserer ville arter og oppdrettsarter av tilapia og gir dødelighet og betydelige økonomiske tap i oppdrett. Siden den første beskrivelsen av sykdommen fra Israel, har viruset blitt påvist på fire kontinenter (Asia, Afrika, Sør- og Nord-Amerika) og gir dødelighet fra 5-20% opp mot 90%. Forekomst av viruset er ikke kjent i mange av de landene som driver tilapiaoppdrett, og det er nødvendig å etablere bedre overvåknings- og kontrollprogrammer i disse landene.

I denne studien ble Lake Victoria valgt for TiLV-screening fordi den inneholder et stort antall tilapia-arter som er av økonomisk betydning for de omkringliggende østafrikanske landene. Det er også behov for optimaliserte metoder for rask deteksjon og kvantifisering av viruset. Hvordan viruset tas opp i cellene og hvordan det replikerer er ikke kjent. I denne avhandlingen ble det gjennomført studier for å forstå forekomst av TiLV i Lake Victoria i Øst-Afrika, det ble etablert verktøy for påvisning og kvantifisering av viruset med molekylærbiologiske metoder, og det ble gjennomført studier for å bedre forstå opptaksmekanismer i celler under infeksjonen.

Det ble utviklet en standard RT-PCR og en kvantitativ RT-PCR (qRT-PCR) basert på alle de ti segmentene til viruset. Standard PCR ble brukt til å undersøke Nile tilapia (*Oreochromis niloticus*) fra Victoriasjøen. Den kvantitative RT-PCR metoden ble testet mot kjent og ukjent virustiter fra henholdsvis infiserte TFC# 10 celler og organer fra infiserte fisk. Hemagglutinering av røde blodlegemer fra hønsefugl og fisk ble også undersøkt, samt effekten av ammoniumklorid på replikasjonen av TiLV i E-11-celler.

Resultatene i studie I viste at primere designet fra segment 2 benyttet for påvisning med standard RT-PCR var best egnet til å påvise TiLV i infiserte cellene og organer fra infisert fisk. TiLV fra Victoriasjøen ble påvist i 28 fisk (14. 66%, N = 191) hvor 17,78% (N = 45) var fra villfisk og 13,70% (N = 146) fra oppdrettsfisk. De sekvensene som ble påvist i Nil-tilapia fra Victoriasjøen var tilnærmet identiske med de som ble påvist i Israel (98%), Ecuador (98%), Thailand (96%), Peru (96%) og USA (97%). TiLV ble ikke dyrket eller testet med tanke på virulens/evne til å forårsake sykdom i Nil-tilapia, og derfor anbefaler jeg videre studier for å oppfylle Kochs postulater.

I studie II ble det etablert en ny og optimalisert kvantitativ RT-PCR metode for påvisning av TiLV genom i prøver fra infiserte celler med kjent titer (mengde virus) og fra organer fra infisert Nil-tilapia uten kjent titer. Metoden som ble utviklet er sensitiv og spesifikk for TiLV, og primer-effektiviteten var innenfor et akseptabelt område, 95-105%, bortsett fra primere rettet mot segment 10 (93%). Variasjonskoeffisienten for intra- og inter-analyse varierte mellom henholdsvis 0,00% ~ 2,63% og 0,00% ~ 5,92%, som er innenfor det anbefalte området (under 5%) for at en analyse skal anses som repeterbar og reproduserbar. Sensitiviteten til metoden var 2 $TCID_{50}$/ml, og primere spesifikke for segmentene 1, 2, 3, 4 og 9 gav samme resultat. En nedre deteksjonsgrense på 20 $TCID_{50}$/ml ble påvist for primere rettet mot segment 5, 6, 7, 8 og 10. Primere spesifikke for mot segment 3 gav høyest sensitivitet og primere segment 7 den laveste. Begge primersettene hadde en effektivitet på 98%.

I artikkel III var målsettingen å forstå hemagglutinasjonsegenskapen til TiLV ved bruk av erythrocytter fra hønsefugl, tilapia og laks, samt betydningen av endocytose i tidlig fase av infeksjonen i E-11-celler. TiLV gir ikke hemagglutinering av erytrocytter fra noen av de testede artene, noe som indikerer at viruset mangler hemagglutinin. Studien har også vist at ammoniumklorid ikke påvirker, dvs. hemmer eller forsinker replikasjonen av TiLV i E-11-celler, noe som indikerer at viruset ikke tas opp ved endocytose. Samlet antyder de to observasjonene at TiLV ikke blir tatt opp av reseptormediert endocytose i E-11-celler. De gjennomførte studiene viser at det er behov for å forstå opptaksmekanismen(e) til virus, som er en viktig informasjonen for å kontrollere virusinfeksjonen med anti-virale midler eller vaksiner.

LIST OF PAPERS

Paper I:

*Mugimba, K.K., ***Chengula, A.A.**, Wamala, S., Mwega, E.D., Kasanga, C.J., Byarugaba, D.K., Mdegela, R.H., Tal, S., Bornstein, B., Dishon, A., Mutoloki, S., David, L., Evensen, O., Munang'andu, H.M. (2018). Detection of tilapia lake virus (TiLV) infection by PCR in farmed and wild Nile tilapia (*Oreochromis niloticus*) from Lake Victoria. *Journal of fish diseases*, 41: 1181-1189.

*Equal contribution

Paper II:

Augustino Alfred Chengula, Kizito Kahoza Mugimba, Shlomit Tal, Roni Tadmor Levi, Saurabh Dubey, Stephen Mutoloki, Arnon Dishon, Lior David, Øystein Evensen and Hetron Mweemba Munang'andu. (2020). Efficiency, sensitivity and specificity of a quantitative real-time PCR assay for tilapia lake virus (TiLV).

Paper III:

Chengula, A.A., Mutoloki, S., Evensen, Ø., Munang'andu, H.M. (2019). Tilapia lake virus does not hemagglutinate Avian and Piscine erythrocytes and NH_4Cl does not inhibit viral replication in *vitro*. *Viruses*, 11, 1152.

INTRODUCTION

World Tilapiine production and its significance

Fish significantly contributes to human food supply and security by providing about 50 percent of its production for human consumption [1]. In 2018, the contribution of global capture fisheries was 96.4 million tonnes (54.01%) while world aquaculture fish production dominated by finfish (54.3 million tonnes) contributed 82.1 million tonnes (45.99%) [2]. The trend for global fish production in aquaculture shows a strong increase from the early 1990s while capture has remained almost stagnant from the 1990s with an average of 76.9 million tonnes in the last 30 years (1988-2018). About 88% of the overall total fish production in 2018 were used for direct human consumption, equivalent to an estimated annual supply of 20.5 kg per capita with the remaining being used for non-food purposes (examples are fish meal and fish oil) [2]. The global percentage of fish protein consumption by human beings in 2017 was about 17 percent of the total animal protein consumed, and about 7 percent of the total protein consumed. This intake of animal protein from fish is estimated to contribute about 20 percent of average per capita for more than 3.3 billion people globally [2, 3].

In Africa and the Middle East, tilapiines and haplochromines are the two major native lineages (tribes) of cichlids [4, 5] belonging to the family *Cichlidae* of the order Cichliformes. Tilapia is the general name given to all fish in the cichlid group consisting of three genera; *Oreochromis*, *Sarotherodon* and *Tilapia* [6]. The first scientific record of tilapia culture was in Kenya in 1924, later spreading throughout Africa and in the 1940s also towards Far East and the Americas [7]. The genus *Oreochromis* has more than 31 species and contains three extensively cultured aquaculture species; Nile tilapia (*O. niloticus*), Mozambique tilapia (*O. mossambicus*) and blue tilapia (*O. aureus*) introduced to more than 100 countries worldwide [8, 9]. In 2018, Nile tilapia aquaculture production was the leading among tilapiines in the world contributing 4.5 million tonnes of the global tilapia aquaculture production [10]. The global tilapia aquaculture production in 2018 was estimated to be 6.0 million tonnes (Figure 1) and it is expected to increase to more than 7.3 million tonnes by 2030 [11]. The top three tilapia aquaculture producing countries in 2018 were China (1.62 million tonnes), Indonesia (1.22 million tonnes) and Egypt (1.05 million tonnes), which in total produced 64.66% of the global tilapia production [12]. On the other hand, the global human population is expected to grow to more than 9 billion by 2050 of which 2.5 billion will be

in Africa [13, 14]. This increase in the human population boosts demand for sustainable food production to feed the increasing population. Increasing agriculture and aquaculture production should go hand in hand with controlling all factors that hinder production such as diseases.

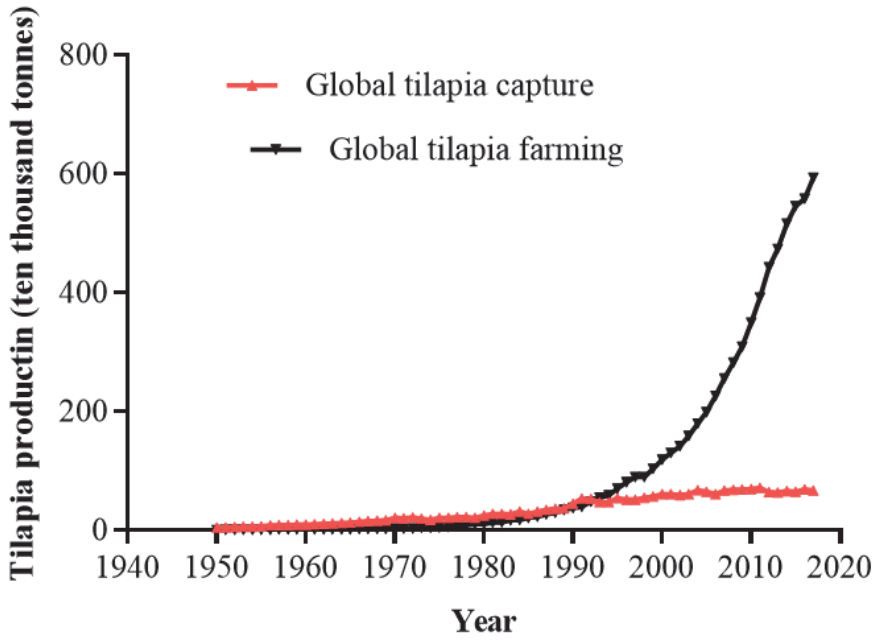

Figure 1. The trend of global tilapia captures and tilapia farming production from the 1950s to 2018. Data for generating the figure were obtained from FAO Fisheries and Aquaculture Department in the FishstartJ Software release 4.00.16 [12].

Emerging viral diseases of wild and farmed tilapiines

The emergence of viral diseases in natural water bodies and aquaculture in different parts of the world has been documented to pose significant ecological and economic threats [15-17]. These viruses may either be new or previously unknown and invade new species of fish or cause disease with new manifestations (increased virulence, incidence or new manifestation of a disease) [18]. Reasons for the increased emerging viral diseases of fish include intensification of aquaculture operations which enhances transmission of viruses between fish and create a stressful environment

to fish, anthropogenic activities and climate changes [19, 20]. Factors facilitating the spread of viral diseases include the trade of fish (for stocks and human consumption) or fish products (for human consumption), natural movement of infected fish and movement of fishing equipment. Other factors that may lead to significant spread with high impacts include delayed diagnosis due to unavailability of detection methods, inadequate knowledge on the host and geographical range and the epidemiological factors of the virus [21]. For a very long time, tilapiines were regarded as 'disease-resistant', but researches have shown that they are susceptible to a number of diseases with devastating consequences. Predisposing factors can be intensive farming, climate change, and other prevailing factors. Table 1 shows emerging viruses of wild and farmed tilapiines reported in different parts of the world with the Tilapia lake virus (TiLV) being the recent one that forms the basis of this thesis.

Table 1. Emerging viral infections of tilapiines and their geographical distributions

Virus	**Genus**	**Family**	**Disease caused**	**Geographical distribution**	**Reference**
Infectious pancreatic necrosis virus	*Aquabirnavirus*	*Birnaviridae*	Infectious pancreatic necrosis	Taiwan and Kenya	[22-24]
Nervous necrosis virus	*Betanodavirus*	*Nodaviridae*	Viral nervous necrosis	France, Thailand, Indonesia	[25-27]
Bohle iridovirus	*Ranavirus*	*Iridoviridae*	Spinning tilapia ('ST') syndrome	Australia	[28]
Lympocystis disease virus	*Lymphocystivirus*	*Iridoviridae*	Lympocystis disease	East African lakes	[29]
Infectious spleen and kidney necrosis virus	*Megalocytivirus*	*Iridoviridae*	Infectious spleen and kidney necrosis	US Midwest, Thailand	[30, 31]
Iridovirus-like		*Iridoviridae*		Canada	
Tilapia larvae encephalitis virus	*Herpesvirus*	*Herpesviridae*	Viral encephalitis of tilapia larvae	Israel	[32]
Tilapia lake virus	*Tilapinevirus*	*Amnoonviridae*	Tilapia lake virus disease	Asia, South America, Africa	[33-37]

Tilapia lake virus (TiLV)

Morphological, biochemical and physiochemical properties of TiLV

The first description of TiLV was in the Sea of Galilee (Kinneret Lake) in Israel [33] where it caused high mortalities in wild tilapia (*Sarotherodon galilaeus,* also referred to as St. Peter's fish). The virus has a genome comprised of a negative-sense single-stranded RNA. In electron micrographs of infected cells and fish tissues, electron-dense virion-like particles were found in the intracytoplasmic membrane or within the cytoplasm (Figure 2). The virions contain rounded or oval, enveloped icosahedral structures of 55-110 nm in diameter with a trilaminar capsid, characteristics very similar to those of *Orthomyxoviridae* [33, 38, 39]. The presence of an envelope was confirmed by ether and chloroform tests [33].

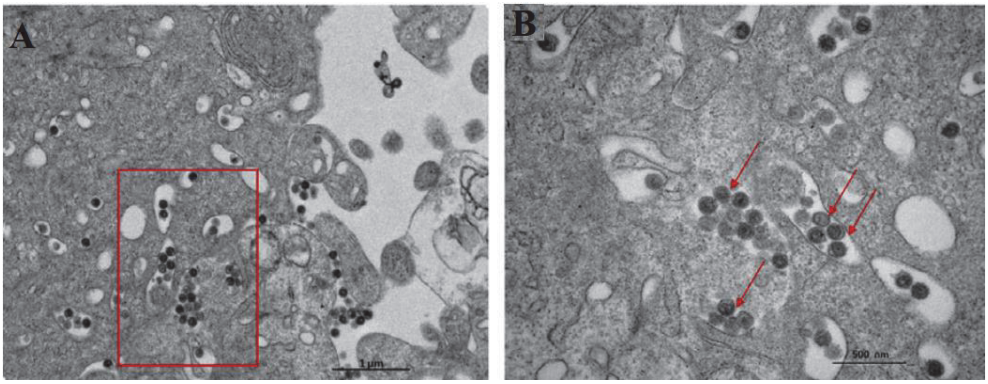

Figure 2. Transmission electron microscopy analysis. (A) TiLV-infected tilapia brain cells showing multiple viral particles in the cytoplasm of infected cells (red box). (B) Intracytoplasmic virions observed at higher magnification showing round-shaped single or aggregated TiLV particles (red arrows). Reused with permission from [40]

The complete or near complete TiLV genomes isolated in Israel, Ecuador [34, 41], Thailand [42, 43] Peru [44], the United States of America (USA) and Bangladesh [45] are publicly available in the GenBank database. The complete genome of the virus is linear consisting of ten unique segments [34] as opposed to most orthomyxoviruses which contain six to eight segments (Table 2). All the ten segments have 13 inverted partially complementary sequences at their 5' and 3' noncoding termini, a genome organization consistent with all known orthomyxoviruses [58, 96-99].

Table 2. Morphological and biochemical comparison of orthomyxoviruses with TiLV

Genus	Total Segments	Virion size (nm)	Segments size (kb)	Genome length (kb)	Encoded proteins	Surface glycoprotein (s)	Host range
Influenza virus A	8	80-120	0.9 to 2.3	13.6	12 to 14	HA and NA	Human, aquatic birds, Pigs, Horse, Whales
Influenza virus B	8	80-120	0.9 to 2.3	14.5	11	HA and NA	Human and Seals
Influenza virus C	7	80-120	0.9 to 2.3	12.9	9	HEF	Mainly humans, but also found in swine
Isavirus (ISAV)	8	90-130	0.8 to 2.2	14.5	8	HA and HE	Fish
Tilapiine virus (TiLV)	10	55-100	0.5 to 1.6	10.3	10	Unknown	Fish (Tilapiines)
Thogoto virus	6 to 7	80-120	0.9 to 2.3	10	7 to 9	Glycoprotein (G)	Human and mammals
Quaranja virus	6	80-120	0.9 to 2.3	10	6	Glycoprotein (G)	Human

Key: TiLV= Tilapia lake virus, ISAV= Infectious salmon anaemia virus, HA= Hemagglutinin, NA= Neuraminidase, HE= Hemagglutinin-Esterase, HEF= Hemagglutinin Esterase-Fusion

Citations: [34, 46-48]

Each segment of TiLV contains an open reading frame (ORF), which has been confirmed by mass spectrometry with length ranging from 113 to 519 amino acids [34, 43]. Recent studies on structural characterization of ORF encoded functional genes predicted 14 functional genes from the ten segments of TiLV and only six proteins with transmembrane helix region [49]. The evolutionary relationship based on phylogenetic analysis showed that segments 1, 2, 4 and 5 to be very close with gene sequences of Dhori virus. Moreover, the study showed that segment 3 of TiLV to be similar to segment 3 of infectious salmon anemia virus (ISAV), while segments 6, 7 and 8 are close to segments 6, 7 and 8 of influenza virus [49]. Segment 1 has also shown a weak sequence homology to the influenza C virus PB1 subunit (~17% amino acid identity, 37% segment coverage) [50, 51]. This predicted protein PB1 from segment 1 of TiLV possesses low motifs I, II, III, and IV conserved in RNA-dependent RNA and RNA-dependent DNA polymerases of orthomyxoviruses PB1 protein [34, 43]. Fourteen functional genes from the ten segments of TiLV have been predicted, however, their structural layout and their roles are not yet known (Figure 3) and further studies are needed. The glycoproteins (spikes) which are used for attachment to the host cell receptors during entry are not yet determined in TiLV. In influenza A and B viruses, hemagglutinin (HA) encoded by segment 4 (Figure 3) is used for attachment to sialic acid in α2,3 or α2,6 -linkage to galactose on the surface of target cells during virus entry [52]. This protein binds to red blood cells leading to clumping of cells, a phenomenon known as hemagglutination. This property is very useful for the rapid diagnosis of hemagglutinating viruses. The hemagglutinin protein in some hemagglutinating viruses is slightly modified to combine more than one function. In the influenza C virus, there is one unique spike protein, the hemagglutinin-esterase-fusion (HEF) glycoprotein that possesses receptor binding (HA) to the N-acetyl-9-O-acetylneuraminic acid, esterase activity (Neuraminidase) and membrane fusion (F) [53]. Infectious salmon anemia virus (ISAV) contains a distinct hemagglutinin-esterase (HE) protein which binds to the terminal 4-O-acetylsialic acid (4-OAS) glycans on host cells [54]. Paramyxoviruses, on the other hand, have a distinct hemagglutinin-neuraminidase (HN) protein used for attachment to the host sialic acids (SIAs) [55]. These differences have been observed in the sequence identity whereby ISAV HE has more than 10% and influenza C virus HEF has about 12% identity to influenza A and B virus HA [53, 54]. Tilapia lake virus shares some similar characteristics with orthomyxoviruses, but it is not yet known whether it contains hemagglutinin which is used for attachment during virus entry into the host cell. Also, it is not known whether the virus enters the cells via receptor-mediated

endocytosis or other mechanisms. The receptor (assuming it is HA) could be useful in developing rapid diagnostic tools for TiLV such as hemagglutination assay.

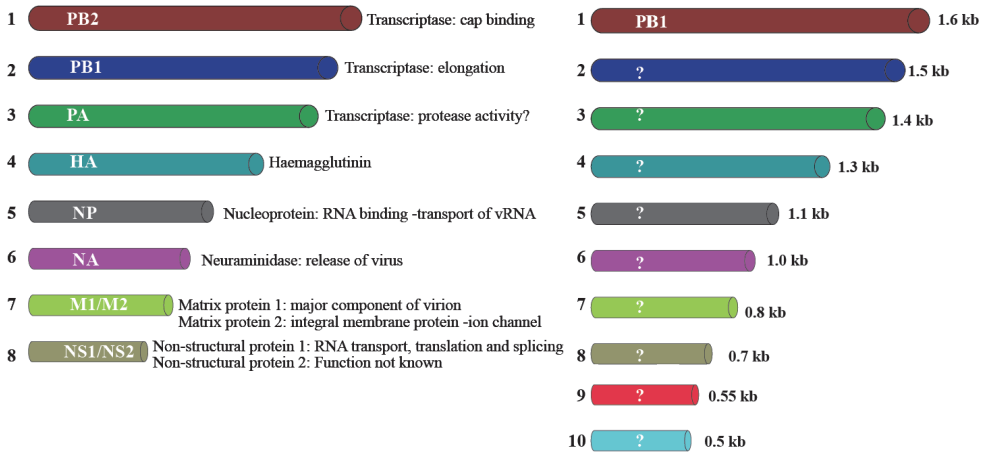

Figure A. Influenza virus genome (https://microbewiki.kenyon.edu)

Figure B. Tilapia lake virus genome (by autor)

Figure 3. Comparison of segments and proteins encoded by influenza viruses to those of TiLV

Nomenclature and classification

The virus was named as TiLV in 2014 [33] based on the host species (tilapia) and the site from which it was detected for the first time (Lake Galilee, Israel). Based on genomic characteristics and ultrastructural features seen, TiLV was classified preliminary in the family *Orthomyxoviridae* [33, 34]. In February 2019, the International Committee on Taxonomy of Viruses (ICTV) ratified the updates on the taxonomy approved during the 50th Executive Committee meeting held in Washington, D.C in July 2018. In this change TiLV has been classified under Realm *Riboviria*, Phylum *Negarnaviricota*, Subphylum *Polyploviricotina*, class *Insthoviricetes*, Order *Articulavirales*, Family *Amnoonviridae*, Genus *Tilapinevirus* and Species *Tilapia tilapinevirus* [56]. Tilapia lake virus is in the same rank as orthomyxoviruses starting from order all the way to ream rank. However, this new classification is based on a limited understanding of the virus and is prone to change upon the availability of more information.

Viral replication and pathogenicity

Based on *in situ* hybridization and pathology of diseased fish, replication and transcription of TiLV takes place in the nucleus of fish cells in the central nervous system and the liver [34]. However,

the mode of replication is not clearly understood and needs to be investigated to help understand the pathogenicity of the virus. Electron micrographs of infected E-11 cells have shown cytoplasmic membrane invagination containing TiLV virions with electron-dense coating suggesting the possibility of virus entry into cells by endocytosis [38]. Most enveloped viruses enter the cell using receptor-mediated endocytosis mechanisms to deliver their cargo (nucleocapsid and accessory proteins) to the cytoplasm and or nucleus. The endocytic mechanisms include clathrin-mediated endocytosis, caveolae-mediated endocytosis, and lipid-mediated endocytosis, clathrin- and caveolin-independent receptor-mediated endocytosis and macropinocytosis [57-59]. Before the virus enters the cell, it has to attach to the susceptible host cell receptor followed by activation of signalling pathways and formation of endocytic vesicles that fuse with the early endosome in the peripheral cytoplasm [60]. Early endosomes containing the virus are transported via microtubules during which they mature to late endosomes triggered by changes in conditions including a drop of pH (<6.0) [60-62]. The drop of intraluminal endosomal pH achieved by proton-pumping vacuolar-ATPase (V-ATPase) also known as H+-ATPase triggers a conformational change in viral fusion proteins (formation of coiled-coils) facilitating fusion, penetration, and uncoating [63-65]. Therefore, interfering with the drop of pH in the endosome will affect the internalization and the ultimate replication of viruses which depend on low pH such as influenza viruses, alphaviruses, retroviruses and rhabdoviruses [66]. The endosomal acidification inhibition has been very useful in understanding the internalization mechanisms of most enveloped viruses. Influenza viruses, for example, have long been used as a model system to understand viral internalization by inhibiting endosomal acidification using weak basic lysosomotropic agents (LA) such as Ammonium chloride (NH_4Cl), Chloroquine and Methylamine [67, 68]. These weak basic compounds inhibit endosomal acidification by penetrating the endosomes and accumulate in protonated form leading to increased pH [68]. Tilapia lake virus, being an enveloped virus, has a high chance of utilizing receptor-mediated endocytosis during entry into susceptible host cells. However, little is known about internalization mechanisms of TILV into host cells using weak basic lysosomotropic agents or other methods. The use of weak basic lysosomotropic agents in studying the entry mechanisms of TiLV into the cells will have two advantages (i) enlighten us on the entry pathway of TiLV and (ii) paves the way for controlling TiLV using antiviral drugs.

The conserved 13 nucleotides in the 3' terminus (noncoding regions) of all the ten segments similar to other orthomyxoviruses is an important area where the promoter binds to regulate viral

replication and transcription [69-71]. However, for the promoter to perform its activity, base pairing between viral RNA 5′- and 3′- terminal sequences is required in the Orthomyxoviruses [72-74]. The observed similarity of the thirteen nucleotides at the 5′- and 3′- termini in all segments of TiLV indicates the possibility of base pairing to allow promoter to perform its function. As opposed to other orthomyxoviruses genomes, which contain short uninterrupted uridine stretch of 5 to 7 bases used by the viral polymerase during the generation of poly (A) tails, the genome of TiLV contains 3 to 5 bases [34]. The implication of this difference in the uridine stretch during replication and pathogenesis of TiLV is yet to be known. The observation of TiLV virions in the perinuclear complex of the Golgi apparatus [38] on the other hand with unknown role and significance in the replication of the virus needs further investigation. Even the role of all the 10 proteins contained in the TiLV genome and how all the ten segments are packaged in a single particle after replication is not known.

Tilapia lake virus disease

Tilapia lake virus is an emerging virus causing TiLV disease (TiLVD) in farmed and wild tilapias worldwide leading to socio-economic losses. The high mortality rate of up to 90% has been observed during different outbreaks. The first outbreak of TiLVD was observed in Kinneret Lake (also known as the Sea of Galilee) and commercial ponds in Israel during the hot season in 2009. The causative agent of the disease was described in detail for the first time in Israel in 2014 and named it the Tilapia lake virus [33]. The disease outbreak with similar manifestation and severe mortality in young farmed Nile tilapia (*O. niloticus*) was also reported in Ecuador and Colombia in 2014 [75]. In Colombia and Ecuador, the disease was called syncytial hepatitis of farmed tilapia (SHT) due to extensive hepatocellular syncytial cell formation seen on histopathological stained tissue sections. Since then, cases of TiLV disease outbreaks have been reported in different countries within the four continents: Asia, North and South America and Africa (Table 3). The source of virus transmission within and between the affected countries remains unknown. However, in Thailand, the source of virus transmission has been proposed to be the importation of red tilapia stocks for selective breeding programs from Ecuador via Vietnam in 2008 [37].

Clinical manifestations and pathological lesions of the infected fish

The clinical presentation of diseased fish includes loss of appetite, lethargy, abnormal behaviours (such as slow swimming movements at the surface and gathering), severe anaemia, abdominal distension, protrusion of eyes and discolouration (darkening), congestion, scale protrusion and detachment, fin rot and finally death (Figure 2) [33, 43, 75-77]. The main pathological lesions seen in the infected fish include pronounced ocular lesions, skin erosions and haemorrhagic patches on the skin (Figure 4) and haemorrhages, abdominal distension and congestions of internal organs [33, 39]. The ocular alterations include opacity of the lens (cataract), ruptured lenses with phacoclastic induced uveitis or endophthalmitis accompanied by the formation of a cyclitic membrane, followed by swelling of inspissated content or shrinkage, and finally leading to loss of ocular functioning (phthisis bulbi) [33, 43].

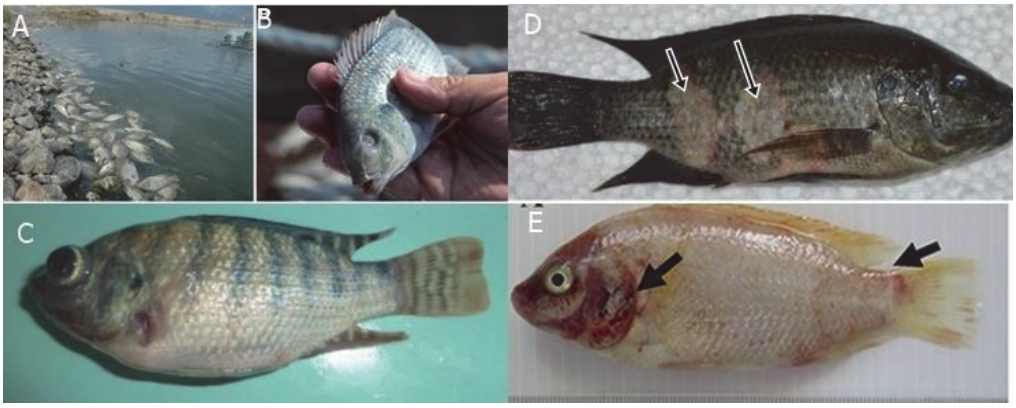

Figure 4. Clinical manifestations and pathological findings of TiLVD in wild and farmed tilapia. (A) Massive mortality of tilapia in a commercial pond. (B) Shrinkage of the eye and loss of ocular functioning (phthisis bulbi). (C) Exophthalmia (eye protrusion) following experimental infection of tilapia. (D) Discolouration of skin along with erosions and loss of scales (arrows) in a moribund Tilapia. (E) Distinct skin redness (black arrows) and skin erosion of diseased red tilapia. Used with permission from [33] (A and B), [36] (C and D) and [39] (E).

Histopathological changes are seen in the brain, liver, spleen, kidney, eyes and multiple other tissues [33, 39, 43, 75, 78] in naturally or experimentally infected fish. In the brain multifocal haemorrhages with severe blood congestion in the leptomeninges and the white and grey matter, the proliferation of glial cells (gliosis), oedema, neuronal degeneration and perivascular cuffing of lymphocytes in the brain cortex have been observed. On the other hand, aggregation of hepatocytes and syncytial cell formation, hepatocyte necrosis, eosinophilic intracytoplasmic inclusion bodies, and multifocal chronic hepatitis have been seen in the liver-stained sections with Haematoxylin and Eosin. Syncytial cell formation in liver-stained sections (Figure 5)

which has been reported in several studies [36, 38, 39, 75, 77, 79, 80] might be a pathognomonic feature of TiLV disease. Syncytium formation is mediated by proteins expressed on the surface of infected cells that attract uninfected cells to fuse with the infected cells at low pH and facilitate the virus to spread within the host [81]. In the spleen, histopathological changes seen include eosinophilic intracytoplasmic inclusion bodies, increased melanomacrophage centres and dispersion of melanin granules. Several histopathological changes have been seen in the anterior kidney with multifocal interstitial haemorrhage, multiple necrotic foci, and infiltration of inflamed cells. Ocular inflammation including endophthalmitis and cataractous changes of the lens and necrosis of gastric glands and diffuse congestion have been observed in TiLV diseased fish.

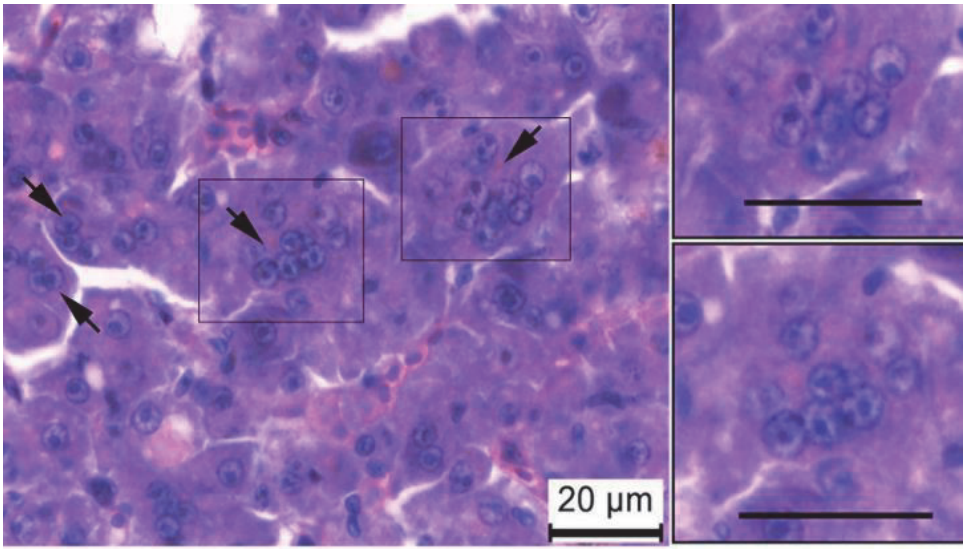

Figure 5. The liver of naturally diseased fish exhibited dissociation, basophilic stained hepatocytes. Syncytial giant cells contained multiple nuclei (arrows). Higher magnification of syncytial giant cells (squared boxes), (H & E staining section, 1000×; scale bars, 20 µm). Used with permission from [77].

The mortality rate in the affected fish farms ranges between 5-20% in the extreme lower levels to 80-90% in the extreme higher levels. The highest mortality rates of up to 90% were commonly observed in fingerlings during the outbreaks [33, 75, 77]. The mortalities of fingerlings are accompanied by the history of the movement from the hatcheries into the ponds or cages within the first month as a result of stress factors [43, 75, 78]. The peak mortality rates are observed within the first two weeks since the onset of mortality. The outbreaks of TiLVD are commonly observed during hot seasons (from May to October) [33, 75, 78]. Experimental

infection studies in Nile tilapia using intraperitoneal injection and co-habitation challenges (Figure 6) produced TiLVD with a cumulative mortality of up to 80% within 4 to 12 days post-infection [33, 39]. Clinical signs, gross and histopathological lesions observed during natural outbreaks and experimental challenges are similar, showing the association of the TiLV with the disease-causing mortalities in tilapia. However, it is not known whether this difference in the pathogenicity of the virus in both the wild and farmed tilapia in the different countries is caused by either the virus or host factors or environmental factors. Also, the cause of the differences in the disease pathological lesions seen during the outbreaks in the Asian (brain lesions) and South American (liver lesions) countries [33, 75] from a similar virus is not known. The presence or absence of co-infections with bacterial pathogens [82-84] on the other hand might be contributing to the differences in the clinical manifestations and mortalities observed in the different outbreaks. It remains unclear for the mechanisms underlying the pathogenesis of the co-infection leading to a synergistic increase of mortalities in the TiLV-infected tilapiines.

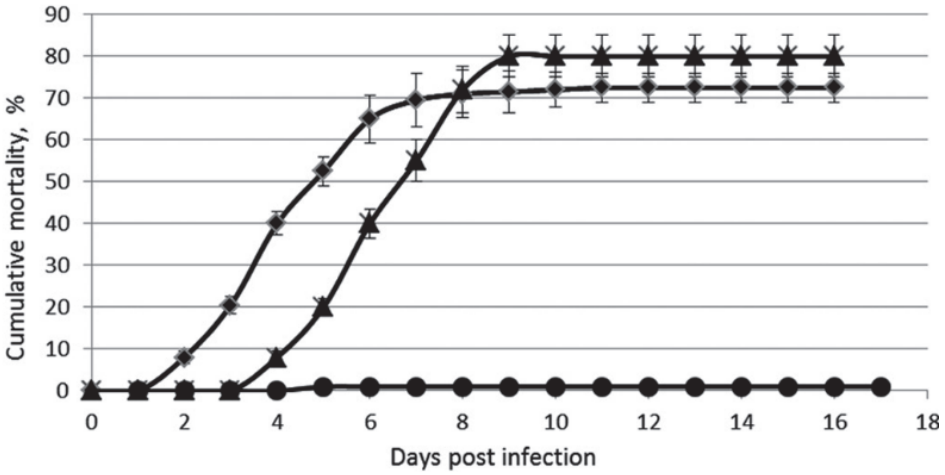

Figure 6. Cumulative mortality in TiLV-challenged Nile tilapia. Two groups were challenged by intraperitoneal injection (♦) or cohabitation (▲) respectively with TiLV at 2.6×10^5 $TCID_{50}$ while the control group (●) was inoculated with the supernatants of naive E-11 cultures. Used with permission from [33].

Host range and susceptibility to TiLV

Tilapia lake virus under natural conditions has been observed to affect fish of all ages in the tilapiine group only [33, 36, 78]. The mortalities have been recorded for Nile tilapia (*Oreochromis niloticus*), red tilapia (Oreochromis sp.), and commercial hybrid tilapia (*O.*

niloticus X *O. aureus* hybrids) in farmed tilapiines [33, 36, 43, 75, 77, 78, 82, 85] and *Sarotherodon galilaeus, Tilapia zilli, Oreochromis aureus*, *Tristamellasimonis intermedia* and black tilapia *(Oreochromis sp.)* in wild tilapiines [33-35, 82]. Controlled laboratory experiments of TiLV infection using warm water fish species to see if other non-tilapiine fish species can be infected and develop TiLV clinical signs have been performed [86]. Clinical signs of TiLV infection were observed only to the red hybrid tilapia and giant gourami with a cumulative mortality rate of 60–100% indicating that most of the non-tilapia species are resistant to TiLV but can transmit the virus to naïve tilapia horizontally through the water. Moreover, TiLV nucleic acids have been detected in Tinfoil barb (*Puntius schwanenfeldii*) [87] and health river barb (*Barbonymus schwanenfeldii*) [88] in Malaysia, but their role in the fish are not known and have not yet been investigated. These three scenarios suggest the possibility of (i) susceptible fish species to TiLV outside the tilapiine group and (ii) non-tilapiine fish species to be the carrier of the virus and should be considered during surveillance of TiLV.

Tilapia lake virus transmission and the associated risk factors for TiLVD

The horizontal waterborne mode of transmission via direct contact from fish to fish and contaminated water is considered the major transmission pathway of TiLV. This mode of transmission has been demonstrated by cohabitation experiments [33] providing proof of the ability of TiLV to spread through a waterborne route. There is a possibility that the TiLV is shed in natural secretions and excretions such as urine, faeces, blood, mucus, and skin to the water where naïve fish get in contact. The occurrence of vertical transmission of TiLV has recently been confirmed based on the natural outbreak and experimental infection emphasizing the importance of biosecurity measures at the level of hatcheries [89, 90]. The question of whether the recovered fish from TiLVD can continuously keep transmitting the virus to the environment has not yet been investigated. The translocation of live fish from one area to the other within or across the international borders for aquaculture, ornamental or direct human consumption is the main risk pathways for TiLV spread. Minor risk pathways of disease spread could be a trade of uncooked fish, frozen whole fish and other fish products such as fillets [91].

The risk factors for the TiLVD outbreaks include the age of fish, the weight of fry at time of transfer to grow-out ponds, high water temperatures, low dissolved oxygen, strain of tilapia and high fish stocking densities [92]. Tilapia lake virus affects all different stages of the life of fish: (i) early developmental stages from fertilized eggs, yolk sac, larvae, fries and fingerlings [36, 37, 43, 75, 77, 79, 82] and (ii) adult tilapia [33, 79]. Tilapia lake virus affects tilapia of

small, medium and large weight where higher mortalities are observed in fish weighing between 1 and 50g than larger sized fish [33, 43, 78]. However, improving the weight of fry at the time of transfer reduces the natural incidences and mortality of fry as a result of TiLV infections probably due to the presence of IgM which depends on the weight of fish [93]. The hot seasons of the year (May to November) where the water temperatures range from 22 to 33 °C [33, 35, 43, 75, 78] have been associated with the outbreaks of TiLVD [78, 92]. Low dissolved oxygen, on the other hand, causes stress to the fish leading to the lowered innate immunity to the cultured fish enhancing susceptibility to TiLV infection [88, 94]. High stocking densities create stress to fish and weakens its immune system making the fish vulnerable to TiLV infections and enables rapid transmission of the virus between the fish and leads to reduced daily weight gain and length of tilapia [95]. All these factors need to be taken into consideration to reduce the chances of TiLV infections.

Geographical distribution of TiLV and countries suggested being potentially at risk

Tilapia lake virus disease outbreaks have been reported in Israel [33], Ecuador [33-35, 38], Colombia [35], Egypt [78], Thailand [43, 77], India [36], Malaysia [82], Taiwan [96, 97], Philippines [98, 99], Indonesia [85], Peru [100] Mexico [101, 102], USA [103, 104] and Bangladesh [45, 105, 106] to cause high mortalities in tilapia. Studies for TiLV infection in archived samples (2012 to 2017) from tilapia hatcheries in Thailand suggested a wide spread of the disease in the world [37]. Based on PCR positive results from archived samples, all countries imported tilapia fry/fingerlings from Thailand between 2012 and 2017 were divided into two groups: forty countries suggested to be potentially at high risk and three countries at lower risk of TiLV spread (Figure 7). This distribution of countries at risk together with the countries which have reported the TiLV outbreaks makes this virus to be of global spread and calls for surveillance in all the countries growing tilapiines in the world.

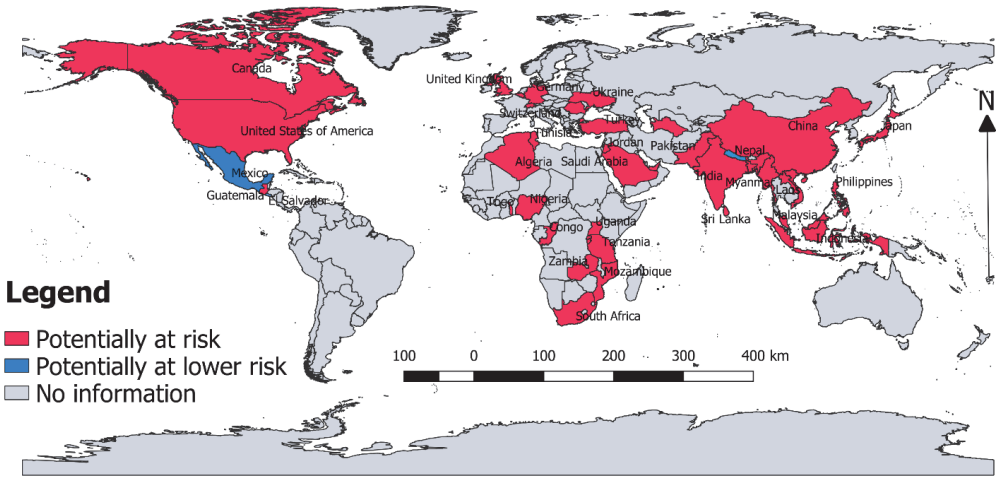

Figure 7. The world map for countries potentially at risk for TiLV. The distribution is divided into three categories based on the recorded destination for tilapia fry/fingerlings exported from Thailand hatcheries between 2012 and 2017: Forty countries reported to be at high risk (red colour), three countries reported to be at lower risk (blue colour) and the rest (grey colour) did not import tilapia fry/fingerings during that period from Thailand. The map was modified by the author from [37].

Diagnostic methods

Several diagnostic tools have been developed and used for the detection of TiLV during outbreaks and experimental infections (Table 3). Electron microscopy and virus isolation in cell culture remain the old gold standards for confirming the presence of the virus from infected materials. However, they are very expensive and labour intensive making them unreliable for providing results within the shortest time enabling quick response. For example, TiLV isolation in the striped snakehead cell line (SSN-1; E-11 subclone) show cytopathic effects (CPE) from 3 to 7 days post-infection (dpi) while other tools like polymerase chain reaction (PCR) and hemagglutination assays provide results within 1 to 3 hours.

A standard RT-PCR has been widely used for detection of the emerging TiLV infections in different countries [33, 35, 37-39, 43, 77, 78, 107-109]. Many have developed and used the RT-PCR without showing that their assays can produce accurate and precise results within the scope of its intended application with few who managed to do it [35, 107]. This could be because of a small window they had to detect and report what is causing deaths to fish for controlling the virus. On the other hand, three types of real-time PCR have been developed and validated for detecting TiLV in clinical and experimental infected materials: SYBR green RT-

qPCR [107], TaqMan RT-qPCR [110] (based on segment 3) and reverse transcription loop-mediated isothermal amplification (RT-LAMP) [111, 112] (based on segment 1 and 3). There is no study which has developed a PCR assay targeting all the ten segments of TiLV to compare their sensitivity, specificity and efficiency. Different segments could have different stability to the host-encoded endonucleases that target and destroy viral RNA leading to different results during amplification.

The use of viral proteins in developing rapid diagnostic tools have been done targeting proteins embedded in the viral envelope. Hemagglutinins in orthomyxoviruses have been used for developing hemagglutination assays used to detect those viruses. The use of hemagglutinins for developing hemagglutination assays for TiLV has not been tried despite being regarded as an orthomyxovirus-like.

Table 3: The strength and weakness of the existing diagnostic tools for TiLV

Technique	Diagnostic tool	Strength	Weakness
Visualization of diseased fish	Naked eye visualization: Clinical signs and gross pathology [33, 36, 43, 78]	Easy to detect the diseased fish	-Not reliable as clinical signs and gross lesions are similar to many conditions
Histopathology	Histopathology: Hematoxylin and eosin (H&E) tissue staining	Quick, inexpensive and allows recognition of significant features under low microscopic magnifications	It is not a confirmatory as it needs other techniques for accomplishments
Cell culture	Primary cells: tilapia brain cells [33]	-The culture provides characteristics of the original tissue making easy for the virus to adapt and grow - A sensitive and reliable method for detecting the virus (confirmatory)	-The cells have a limited lifespan and not good for studying long-term CPE -The technique is expensive, labour intensive and time-consuming
	Continuous cell lines: E-11 from the striped snakehead [33], OmB (brain) and OmL (lip epithelium) from Mozambique tilapia [113], OnlB (brain) and OnlL (liver) from Nile tilapia [114] and	-A sensitive and reliable method for detecting the virus (confirmatory) -It is a gold standard for virus diagnosis	-Cells are not ideal as they have lost the true characteristics of the original tissue from which they were isolated -The technique is expensive, labour intensive and time-consuming

	TiB from the brain of tilapia [40]		
Microscopy	Transmission electron microscopy [33]	-Gives a clear picture of the viral agent -Used to study the viral replication -It is a confirmatory diagnostic tool	-Expensive, slow and cannot be used for daily routine and surveillance
PCR	Standard RT-PCR [33]		-Some of the assays were not confirmed to show that they can produce accurate and precise results [35, 107].
	Nested RT-PCR [35] Semi-nested RT-PCR [109]	-Rapid and trustworthy for use in clinical samples and asymptomatic tilapia	-Nested and semi-nested RT-PCR increases cost in terms of reagents, materials and time to run it
	TaqMan RT-qPCR [110]	-A rapid, accurate and reliable tool	-Expensive because of the use of probes -The use of plasmid does not reflect the actual infected materials
	qRT-PCR [107]	-A rapid, accurate and reliable tool	-The use of plasmid template does not reflect the actual infected materials
	RT-LAMP [111, 112, 115]	-Simple, rapid, effective, sensitive and require no sophisticated instruments	It is suitable for field diagnosis in samples with high template -Less sensitive than real-time PCR assay
Hybridization	In situ hybridization [34, 77]	-It is specific to the sequence of interest due to the use of a probe	-Has not been done in subclinically infected materials -Applicable only to anatomical pathology -Expensive due to the use of a probe
	Northern hybridization [34]	It is specific to the target due to the use of labelled probes	-Time-consuming and expensive due to labelling probes
ELISA	Recombinant protein-based indirect ELISA[116]	-It has high specificity to TiLV antibodies -It is more sensitive than the current IFA technique -It can be used for TiLV surveillance	-If TiLV target antigen is present in extremely small quantities relative to the other proteins, and because all proteins in a sample will bind to the well, the assay may be unable to detect the presence of antigen in a sample

OBJECTIVES

Aims and objectives

The overall aims of this study were to shed light on the viral infection mechanisms, develop tools for rapid virus diagnosis, and investigate the presence of TiLV in Nile tilapia in Lake Victoria.

The work was achieved with the following specific objectives:

1. Develop and optimize a diagnostic PCR method for surveillance of TiLV in farmed and wild Nile tilapia in Lake Victoria, Eastern Africa (Paper I)
2. Validate a quantitative real-time PCR (qRT-PCR) method for TiLV detection (Paper II)
3. Assessment of hemagglutination properties of TiLV and comparison to Influenza A and Infectious Salmon anaemia virus (Paper III)
4. Determining the effect of Ammonium chloride on the replication of TiLV in E-11 cells (Paper III)

SUMMARY OF THE PAPERS

Paper I:

Detection of tilapia lake virus (TiLV) infection by PCR in farmed and wild Nile tilapia (*Oreochromis niloticus*) from Lake Victoria

Mugimba KK*, Chengula AA*, Wamala S, Mwega ED, Kasanga CJ, Byarugaba DK, Mdegela RH, Tal S, Bornstein B, Dishon A, Mutoloki S, David L, Evensen Ø, Munang'andu HM.

*Equal contribution

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https://doi.org/10.1111/jfd.12790.

Tilapia lake virus disease has emerged to be an important viral disease of farmed Nile tilapia (*Oreochromis niloticus*) having the potential to impede the expansion of aquaculture production. There is a need for rapid diagnostic tools to identify infected fish to limit the spread in individual farms. We report the first detection of TiLV infection by PCR in farmed and wild Nile tilapia from Lake Victoria. There was no difference in prevalence between farmed and wild fish samples ($p = .65$), and of the 442 samples examined from 191 fish, 28 were positive for TiLV by PCR. In terms of tissue distribution, the head kidney (7.69%, $N = 65$) and spleen (10.99%, $N = 191$) samples had the highest prevalence ($p < .0028$) followed by heart samples (3.45%, $N = 29$). Conversely, the prevalence was low in the liver (0.71%, $N = 140$) and absent in brain samples (0.0%, $N = 17$), which have previously been shown to be target organs during acute infections. Phylogenetic analysis showed homology between our sequences and those from recent outbreaks in Israel and Thailand. Given that these findings were based on nucleic acid detection by PCR, future studies should seek to isolate the virus from fish in Lake Victoria and show its ability to cause disease and virulence in susceptible fish.

Paper II:

Efficiency, sensitivity and specificity of a quantitative real-time PCR assay for tilapia lake virus (TiLV).

Chengula AA, Mugimba KK, Tal S, Tadmor R, Dubey S, Mutoloki S, Dishon A, David L, Evensen Ø and Munang'andu HM.

Tilapia lake virus (TiLV) is an emerging viral pathogen of tilapiines worldwide in wild and farmed tilapia. TiLV is an orthomyxo-like, negative sense segmented RNA virus, beloning to genus *Tilapinevirus,* family *Amnoonviridae.* Here we aimed at developing a quantitative real-time PCR (qRT-PCR) assay testing primer sets targeting the 10 segments of TiLV. Sensitivity, specificity, efficiency and reproducibility of these assays were examined. Detection sensitivity was equivalent to 2 $TCID_{50}$/ml based when tested on supernatants from cell culture grown TiLV. Specificity tests showed that all primer sets amplified their respective TiLV segments, and standard curves showed linear correlation of R^2 >0.998 and an amplification efficiency between 93% - 98%. Intra- and inter-assay coefficients of variation (CV%) were in the range of 0.0%-2.6% and 0.0%-5.9%, respectively. Sensitivity tests showed that primer sets targeting segments 1, 2, 3 and 4 had the highest detection sensitivity of $10^{0.301}$ $TCID_{50}$/ml. The qRT-PCR used for detection of viral genome in TiLV infected organs, gave calculated virus titers, based on Ct values, of 3.80 $\log_{10}$, 3.94 $\log_{10}$ and 3.52 $\log_{10}$ $TCID_{50}$/ml for brain, kidney and liver tissue parts respectively. These findings suggest that primer optimization for qPCR should not only focus on attaining high amplification efficiency but also sensitivity comparison of primer sets targeting different viral segments in order to develop a method with the highest sensitivity.

Paper III

Tilapia lake virus does not hemagglutinate Avian and Piscine erythrocytes and NH_4Cl does not inhibit viral replication *in vitro*

Chengula AA, Mutoloki S, Evensen Ø and Munang'andu HM.

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Tilapia lake virus (TiLV) is a negative-sense single-stranded RNA (-ssRNA) icosahedral virus classified to be the only member in the family *Amnoonviridae*. Although TiLV segment-1 shares homology with the influenza C virus PB1 and has four conserved motifs similar to influenza A, B, and C polymerases, it is unknown whether there are other properties shared between TiLV and orthomyxovirus. In the present study, we wanted to determine whether TiLV agglutinated avian and piscine erythrocytes and whether its replication was inhibited by lysosomotropic agents, such as ammonium chloride (NH_4Cl), as seen for orthomyxoviruses. Our findings showed that influenza virus strain A/Puerto Rico/8 (PR8) was able to hemagglutinate turkey (*Meleagris gallopavo*), Atlantic salmon (*Salmo salar* L), and Nile tilapia (*Oreochromis niloticus*) red blood cells (RBCs), while infectious salmon anaemia virus (ISAV) only agglutinated Atlantic salmon, but not turkey or tilapia, RBCs. In contrast to PR8 and ISAV, TiLV did not agglutinate turkey, Atlantic salmon, or tilapia RBCs. The qRT-PCR analysis showed that 30 mM NH_4Cl, a basic lysosomotropic agent, neither inhibited nor enhanced TiLV replication in E-11 cells. There was no difference in viral quantities in the infected cells with or without NH_4Cl treatment during virus adsorption or at 1, 2, and 3 h post-infection. Given that hemagglutinin protein that binds RBCs also serve as ligands that bind host cells during virus entry leading to endocytosis in orthomyxoviruses, the data presented here suggest that TiLV may use mechanisms that are different from orthomyxoviruses for entry and replication in host cells. Therefore, future studies should seek to elucidate the mechanisms used by TiLV for entry into host cells and to determine its mode of replication in infected cells.

MATERIALS AND METHODS

Sample collection and study design

This thesis is divided into three main sections (Figure 9): (i) development of diagnostic tools for TiLV, (ii) detection and molecular characterization of TiLV from Nile tilapia in Lake Victoria, Eastern Africa and (iii) assessing the ability of TiLV to hemagglutinate red blood cells of avian and piscine species and the effect of ammonium chloride in the internalization process of TiLV in E-11 cells. In paper I, a standard PCR was developed for use to screen wild and farmed Nile tilapia from Lake Victoria on the Tanzanian and Ugandan sides. A total of 191 Nile tilapias were collected from the two East African countries, 108 fish samples from Tanzania and 83 from the Ugandan sides of the Lake Victoria and transported to Norway at the Norwegian University of Life Sciences (NMBU). In paper II, a SYBR Green-based real-time PCR was developed using TiLV data generated during *in vitro* experiments in cell culture. Confirming the developed SYBR Green-based RT-qPCR was done using TiLV-infected tilapia organs/tissues in the *in vivo* experiments performed in Israel. Study II aimed at comparing the sensitivity, specificity and the efficiency of primers designed from different segments of TiLV. On the other hand, paper III used red blood cells collected from avian and piscine species to assess hemagglutination property of TiLV. Also, paper III used *in vitro* experiments to assess the effect of ammonium chloride in the replication of TiLV in E-11 cells.

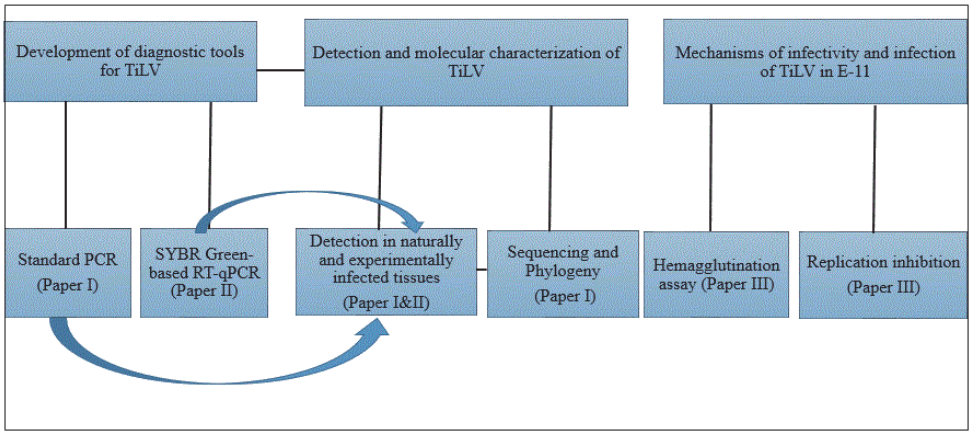

Figure 9: The different methods used in this study to achieve paper I, II and III

Cell culture and virus propagation

Cell culture is the science of growing and manipulating cells *in vitro* from a tissue of a multicellular organism. In virology, cell lines are used to replicate viruses as they are obligate intracellular parasites depending on living cells to produce copies of themselves [117]. Cell culture techniques were used in Paper I to III to propagate viruses. Cell lines used in this thesis to grow the viruses are tilapia fin cells (TFC #10A, paper I and II for TiLV), E-11 from the striped snakehead (*Ophicephalus striatus*) (Paper III for TiLV) and SHK-1 cells from the head kidney of Atlantic salmon (*Salmo salar* L.) (paper III for infectious salmon anaemia virus, ISAV). The influenza virus strain A/Puerto Rico/8 (PR8) was grown in the allantois membranes of 11-day-old embryonated chicken eggs.

Ribonucleic acid (RNA) extraction and complementary deoxyribonucleic acid (cDNA) synthesis

Isolation of total RNA from the infected cells and tissues and converting it to complementary DNA (cDNA) were performed in Paper I-III. Extraction of RNA of high quality from cells grown in culture is far easier than isolating RNA from tissues [118] and therefore requires a good methodology. Also, the performance of PCR depends on the quantity and purity of the template [119]. To obtain good quantity and quality of RNA, TRIzol® (Invitrogen, USA) and the RNeasy® Mini Kit (Qiagen, Hilden, Germany) were combined during the extraction of total RNA. TRIzol® simultaneously solubilizes biological materials and denatures protein [120] and therefore the combination of TRIzol® and RNeasy® Mini Kit increased yield and improved the quality of the RNA. This method for isolating RNA enabled to achieve well solubilized and disrupted cellular membrane, inhibit nuclease activity, deproteinize the sample and concentrate nucleic acids, a worthwhile strategy for RNA purification [121]. Since RNA is naturally labile [122], the extracted RNA was converted to a much more stable cDNA using Transcriptor First Strand cDNA synthesis kit (Roche, Mannheim, Germany) by combining random hexamer and the anchored-oligo deoxythymine $(dT)_{18}$ primers. Random hexamer primers primes throughout the length of RNA for the uniform representation of all RNA sequences allowing reverse transcription of RNAs that do not carry a poly (A) tail. While on the other hand an anchored-

oligo $(dT)_{18}$ primer binds at the beginning of poly (A) tail to generate full-length cDNAs and to prevent priming from internal sites of the poly (A) tail. This approach increased chances of priming all the length of RNA and especially capturing the 5' ends of long mRNAs that are often underrepresented (Transcriptor First Strand cDNA synthesis protocol). To obtain specificity and higher efficiency of reverse transcription, sequence-specific primers would have been better to target specific RNA than using random priming. However, the use of random priming was adopted because the cDNA was to be used in many different experiments targeting different sizes and protein of TiLV viral RNA segments.

Primer design, in silico analysis and optimization

Designing of primers for the overall success of a PCR assay is a critical component which requires much attention. Constructing a PCR assay involving multiple primer sets is even more challenging than an assay involving a single primer set. Primers designed for both developed PCR assays (standard RT-PCR and SYBR Green-based RT-qPCR) targeting all the ten segments of TiLV aimed to achieve two very important goals: desired specificity and efficiency of amplification. A PCR assay with high specificity will generate only one expected amplification product to the intended target gene. The specificity of PCR assay is affected by poor primer designing and inappropriate primer concentration used in the reactions. The annealing step is critical for both high PCR specificity and efficiency. Too low melting temperature (less than 50 ºC) affects the specificity and sensitivity of the PCR assay by amplifying non-specific targets leading to false positive or negative results [123, 124]. On the other hand, at higher melting temperature the high specificity is achieved, but the efficiency is reduced as the primer-template bonds are broken easily [125]. Thus, primer melting temperature ranging between 50 to 65 ºC is recommended to achieve good specificity and efficiency [119]. PCR efficiency depends greatly on the strength of hydrogen bonds formed between the primers and the template. Strong bonds are formed by primers with greater GC (40-60%) that results in stronger anchoring requiring greater energy to break the bonds. At most two strong bases should be on the ends of the primers especially on the 3’ end where the PCR elongation happens and prevents the formation of primer dimers [125]. Therefore, to achieve high specificity and good efficiency of the PCR assay as well as minimize the formation of primer-dimers, designing primers with high melting temperatures and strong bases is very important. Also avoiding high primer concentration in the PCR reactions will reduce self-primer hybridization (primer-dimer formation) or hybridization to non-

complimentary sequences that affect the specificity, sensitivity and efficiency of the PCR assay [124, 126]. All these factors which would affect the specificity, sensitivity and efficiency were considered during the *in-silico* primer design and during the development of the PCR assays.

In both assays (standard and real-time PCR), primers which did not meet the required characteristics were not included in the final assays.

Polymerase chain reaction (PCR) amplification

In this thesis, I have developed, optimized and used to amplify TiLV in cells and infected materials: the standard PCR (paper I) and real-time PCR (paper II). Both PCR assays developed involved two main steps: Reverse transcriptase (RT) dependent conversion of viral RNA into cDNA using random primers combined with anchored-oligo$(dT)_{18}$ and amplification of cDNA using DNA polymerase [127]. Each cycle of PCR amplification consisted of three steps: denaturation of the double-stranded DNA fragment, primer annealing to the respective complementary strands of the DNA and extension of the primer-template hybrid complex by DNA polymerase enzyme activity.

SYBR Green-based technology was preferred over probe-based technology in this thesis due to cost-effectiveness, simple to design, shorter detection time and high sensitivity and specificity of the assay [128] which would allow wider application in TiLV diagnosis and research. The design and synthesis of the probe in the sequence-specific ds DNA binding assay make it more expensive and time-consuming than non-sequence specific ds DNA binding assay [129]. Yet, the correlation coefficient and the amplification efficiency of TaqMan and SYBR Green qPCR methods under optimized conditions are similar [130]. The detection in the SYBR Green-based qPCR is based on the fluorescence produced by a reporter molecule that binds to the double-stranded DNA molecule [131]. The SYBR Green intercalates and binds to the minor groove of the formed double-stranded DNA during amplification emitting a strong fluorescent signal [132]. The intensity of the fluorescence signal increases as the binding to the forming ds DNA takes place in each of the amplification cycles. This increasing intensity of the fluorescence was used to monitor the increasing amount of the amplified DNA in real-time. The data (Ct values) were collected at the end of the elongation step. Since SYBR Green real-time PCR assay lacks specificity by binding of the fluorescent dye to non-specific dsDNA, the melting curve analysis was performed at the end of amplification to increase the specificity of the real-time reaction. The non-specific ds DNA melted at lower temperatures and allowed to

differentiate specific peaks from non-specific dsDNA dissociation and the formation of primer-dimers.

Hemagglutination assay

Hemagglutination (HA) is used to detect the presence of hemagglutinating viruses such as influenza viruses, Newcastle disease virus, infectious salmon anaemia, infectious bronchitis virus, hemagglutinating adenovirus 127, psittacine circovirus [133]. Hemagglutination is based on the ability of these viruses to bind to the receptors found on the surface of red blood cells of their respective hosts. This property has been studied and used for developing diagnostic assays against these viruses. There are two types of hemagglutination assays which have been developed and used for detection of hemagglutinating viruses: hemagglutination (HA) assay and hemagglutination inhibition (HI) assays. The basis of hemagglutination assays is the hemagglutinin found on the surface of some viruses which can bind to sialic acids on the surface of red blood cells leading to agglutination[134]. The principle behind HI is the presence of specific antibodies in the serum from exposed animals to the virus which binds and prevent the ability of the virus from attaching to the red blood cells [133]. In this study, we investigated the hemagglutination property of TiLV using hemagglutination assay.

Inhibition of TiLV replication in E-11 cells with ammonium chloride

Prevention of virus replication *in vitro* for viruses using the endocytic pathway has been done using weak basic agents which can penetrate acidic compartments of the cell and accumulate as protonated forms, leading to an increase in the endosomal pH [67]. Increase in endosomal pH blocks the replication of viruses which utilize low pH (pH<6.0) to fuse their envelope with the endosomal membrane. These weak bases are known as lysosomotropic agents and include ammonium chloride (NH_4C1), chloroquine and methylamine [68]. Inhibition of virus replication is useful in understanding the uptake mechanisms of the virus as well as in designing control measures of the virus. In this study, we used NH_4Cl to study the uptake mechanisms of TiLV (MOI 1.0) in E-11 cells grown at 28° C.

Data treatment and statistical analysis

In both papers I and III, the obtained PCR products were sequenced on a commercial basis using Sanger ABI 3730xl sequencing technology at GATC Biotech Company in Germany (https:// www.gatc-biotech.com). The Basic Local Alignment Search Tool (BLAST) was used to find regions of local identity between sequences obtained from this study and those deposited at NCBI GenBank (https://blast.ncbi.nlm.nih.gov/Blast.cgi). BLAST compares nucleotide sequences to the database and calculates the statistical significance of matches. This search tool is flexible and tractable to mathematical analysis with an order of magnitude faster than existing sequence comparison tools of comparable sensitivity [135]. Nucleotide multiple sequence alignments using the CLUSTAL W algorithm and the reconstruction of phylogenetic trees were performed in MEGA version 7 [136]. The evolutionary history was inferred by using the Maximum Likelihood method (ML) built-in Mega 7 software. To investigate the stability of the reconstructed phylogenetic trees, a bootstrap analysis of 1000 replicates based on the JTT+G matrix-based model [137] was applied. The Maximum Likelihood method was preferred because it is slightly more efficient than the other methods such as Neighbor-Joining, Maximum Parsimony and Bayesian Inference in obtaining the correct topology [138].

Data from real-time RT-PCR (paper II and III) were collected at the end of amplification and melting curve followed by analysis using the LightCycler ® 96 version 1.1.0.1320 software (Roche) compatible to the real-time PCR machine (LightCycler ® 96, Roche) used in the study. The mean (M), standard deviation (SD), coefficient of variation (CV) and the square of the coefficient of correlation (R^2) of the cycle-threshold values (Ct-values) were determined using GraphPad Prism software version 7 (GraphPad Software, La Jolla California USA) (https://www.graphpad.com). The construction of standard curves (paper II) was done in both Microsoft excel™ and Stata15 by plotting the Ct values on the Y-axis and dilutions of the samples ($TCID_{50}$/mL) on the X-axis in $\log_{10}$ scale.

RESULTS AND DISCUSSION

General discussion

Tilapias are the second most farmed fish worldwide after carp [139] and their production have kept increasing due to ease of keeping tilapia in aquaculture and availability of markets. There are three genera of tilapia (*Oreochromis*, *Sarotherodon* and *Tilapia*) and all are native to Africa and the Middle East [6]. Nile tilapia (*Oreochromis niloticus*) of the genus *Oreochromis* are commercially important within Africa and more than 90% of these fish are farmed outside Africa [140]. China is the leading producer of tilapia in the world. Besides access to market and profitability of production, an important limiting factor for the growth and success of tilapia aquaculture globally is fish diseases. This can be linked to and explained by lack of knowledge of disease aetiologies, poor diagnostics management and lack of disease prevention and good biosecurity. In turn, this hampers the economic development of the rapidly growing aquaculture and fishery industries in the world.

This thesis aimed at characterizing an emerging virus (TiLV) causing high mortalities in tilapia, discovered recently in Israel [33], and developing diagnostic tools for the virus. Two tools (conventional RT-PCR and SYBR Green-based RT-qPCR) were developed for the rapid detection of TiLV. The conventional PCR was used for screening Nile tilapia from Lake Victoria against TiLV (Paper I), while the SYBR Green RT-qPCR developed in paper II was confirmed using the experimentally infected Nile tilapia organs/tissues from Israel. Polymerase chain reaction (PCR) is one of the powerful techniques widely used for genetic manipulations [141] and diagnosis of genetic and infectious diseases in human and animals [142-144], genotyping and DNA forensics [145], vaccine development, food control and analysis of environmental specimens [146]. Although PCR is more sensitive than other diagnostic techniques, it needs good designing and optimization for it to be reliable in detecting subclinically and clinically infected materials. Some of the PCR developed previously for detection of TiLV were not fully optimized and were only reliable for clinical and experimentally infected materials but not subclinically infected materials.

At the beginning of this study, there were only four articles published on TiLV by Eyngor et al. (2014), Bacharach et al. (2016), Del-Pozo et al. (2017) and Kembou Tsofack et al. (2017). Since then, more articles are increasingly being published on TiLV every year and by the end

of January 2021, about 86 articles (Original and reviews) were published in peer review journals. The highest article publications for TiLV were in 2020 (25 articles), 2019 (23 articles) and 2018 (22 articles) followed by 2017 (10 articles), 2021 (three articles), 2014 (two articles), 2016 (one article) and with no publications in 2015. Compared with other diseases of tilapiines, there are about 11 papers published among 7 diseases of tilapiines within 45 years [147]. This shows how TiLV has grabbed the attention of many stakeholders from fish farmers, researchers, organizations and the public trying to understand this emerging deadly virus of tilapiines and coming up with a solution as soon as possible. All articles published for TiLV are based on virus detection and characterization, which is crucial information for understanding the virus for control and development of long-term control measures such as vaccines.

Tilapia lake virus genome has been detected in Nile tilapia from Lake Victoria in East Africa

The outbreaks of TiLV in tilapia farms have been reported in several countries since its first report in Israel in 2014. This has created a threat to tilapia farmers and the fish industry at large. Data from archived and newly collected fish samples obtained in Thailand hatcheries from 2012 to 2017 were positive for TiLV and more than 40 countries worldwide imported tilapia fry and fingerlings during that period [37]. Tanzania was among the countries that imported fry and fingerlings from Thailand during that period. Paper I of this study developed a highly sensitive PCR which does not need nesting and was used to screen tilapia samples collected from wild fish and cages in Lake Victoria in East Africa. The developed assay was able to detect TiLV nucleic acids from wild tilapia (20/146) and tilapia kept in the cages (8/45). The detection of TiLV nucleotide sequences from fish in the cages put farming in cages in the Lake Victoria at high risk of this deadly disease which can wipe almost 90% of the fish. Since cages are open systems that allow free movement of pathogens from the Lake to the cages, the control of pathogens through biosecurity measures is something inevitable.

As opposed to most of the previously developed conventional PCR assays that targeted segment three of the virus genome, the developed assay targeted all the ten segments of TiLV. Interestingly, primer pairs designed from segment two were better than others in detecting TiLV in Nile tilapia field samples while all detected better in the infected cells. This difference could be due to the presence of different copy numbers for the segments during chronic and

acute infection. Another possibility could be the differences in stability to host RNA decay machinery among the segments of TiLV during chronic and acute infection. The strategies used by viruses to stabilize their RNA and regulate gene expression include inherent RNA shields, incapacitating the host decay machinery, hijacking host RNA stability factors and by changing the entire landscape of RNA stability in cells using virally-encoded nucleases [148, 149]. Segment two of TiLV could be encoding the viral RNA polymerase (for transcription and replication) as in the other orthomyxoviruses and therefore, well protected from host RNA decay machinery [150]. Above all, primer design and optimization play an important role in achieving the desired sensitivity and specificity of an assay and therefore should be given priority.

The nucleotide sequences of TiLV from thirteen countries (Israel, Ecuador, Colombia, Thailand, Egypt, Tanzania, Uganda, India, Malaysia, Indonesia, Peru, USA and Bangladesh) are available in the GenBank database at the National Centre for Biotechnology Information (NCBI) (https://www.ncbi.nlm.nih.gov/genbank/). Complete genome sequences of TiLV isolated in Israel, Ecuador, Thailand, Peru, USA and Bangladesh are also available in the GenBank database. This study has demonstrated that the TiLV genome sequences detected in Nile tilapia from Lake Victoria (both Tanzanian and Ugandan sides) had high sequence identity (96-98%) to those of Israel, Ecuador, Thailand, Peru and USA isolates (Table 4). Apart from this identity, we did not manage to isolate the virus from Lake Victoria and test its pathogenicity in cells or experimental infection in tilapia so that we can hypothesize its impact to the fish in Lake Victoria. Based on phylogenetic analysis, there are two suggested genetic clades for TiLV; the Israeli-2011 clade and Thai clade [151]. The Israel-2011 clade contains TiLV sequences from Israel, Ecuador, Lake Victoria (Tanzanian and Ugandan sides) and Indian isolates while the Thai clade contains TiLV sequences from Thailand, Egypt, Peru and USA isolates.

Table 4. A summary of pairwise comparison of TiLV segment two nucleotide sequences obtained from Lake Victoria (in Tanzanian and Ugandan sides), Israel, Thailand, Ecuador, Peru and USA.

Countries	Accession number	TiLV nucleotide sequence identity (%)						
		Israel	**Ecuador**	**Thailand**	**Peru**	**USA**	**Tanzania**	**Uganda**
Israel	KU751815	100.00	98.02	96.60	96.60	96.60	98.30	98.30
Ecuador	MK392373	98.02	100.00	95.75	97.45	96.32	98.02	98.02
Thailand	KX631922	96.60	95.75	100.00	95.75	95.18	96.88	96.88
Peru	MK425011	96.60	97.45	95.75	100.00	95.47	96.88	96.88
USA	MN193524	96.60	96.32	95.18	95.47	100.00	97.17	97.17
Tanzania	MF526980-MF526996	98.30	98.02	96.88	96.88	97.17	100.00	100.00
Uganda	MF536423-MF536432	98.30	98.02	96.88	96.88	97.17	100.00	100.00

NB: Nucleotide percent sequence identity was defined as the percentage of overlapping nucleotide alignment positions where the two sequences agree

The emergence of TiLV affecting tilapia poses many questions including whether the virus was there in the countries being detected and now evolved or has jumped borders through importation/exportation of fry and fingerlings. Also, it is not known as to whether the viruses in the different countries have a single origin or multiple and to whether the virus comes from the wild tilapia to farmed ones or the other way around. In any way, three forces might have contributed to the emergence of TiLV in the different countries: (i) virus factors such as rapid virus evolution and environmental adaptability, (ii) host factors such as susceptibility, high population and movement from one location to another and (iii) environmental factors like extreme climatic conditions [152-154]. Most of the emerging and re-emerging diseases are caused by RNA viruses that can quickly adapt to and exploit the mentioned factors above as their polymerases responsible for genome replication make high mistakes due to poor proofreading [155]. The climate change-taking place throughout the world alters the distribution, prevalence and virulence of pathogens (viruses, bacteria, parasites and fungi) and increases the susceptibility of the host species. Climate change also alters water temperature in the aquatic environment favouring either the pathogens (increasing replication) or the host [156]. This could be the case for TiLV whose outbreaks have been reported in different countries to occur during hot seasons (between May and October) [33, 75, 78].

Eight countries (Indonesia, Egypt, Bangladesh, Philippines, Thailand, Taiwan Province of China, Colombia and Mexico) out of the top twelve-tilapia producing countries in the world have reported the outbreaks of TiLVD. Indeed, this is a significant global threat to tilapia production and its contribution to economic growth. Interestingly, nine countries (Philippines, Bangladesh, India, Indonesia, Malaysia, Mexico and USA, Tanzania and Uganda) that were reported in the previous studies to be potentially at risk of TiLV due to importation of tilapia fry and fingerlings from Thailand [37] have investigated and confirmed the presence of TiLVD (red colour) or presence of TiLV infection through PCR techniques (green colour) (Figure 10). This indicates the possibility of a wider distribution of TiLV and calls for more efforts in carrying out surveillance in other countries to enable us to understand better the global distribution of the virus. In most mortality cases in farmed tilapia, farmers and researchers tend to associate the disease with bacterial infections since few viral infections have been reported in tilapia. Nevertheless, the simultaneous occurrence of bacterial infections (*Aeromonas veronii*, *A. ichthiosmia*, *A. enteropelogenes* and *A. hydrophilia*) with TiLV have been reported [76, 82, 83] and should be considered during disease outbreaks in fish.

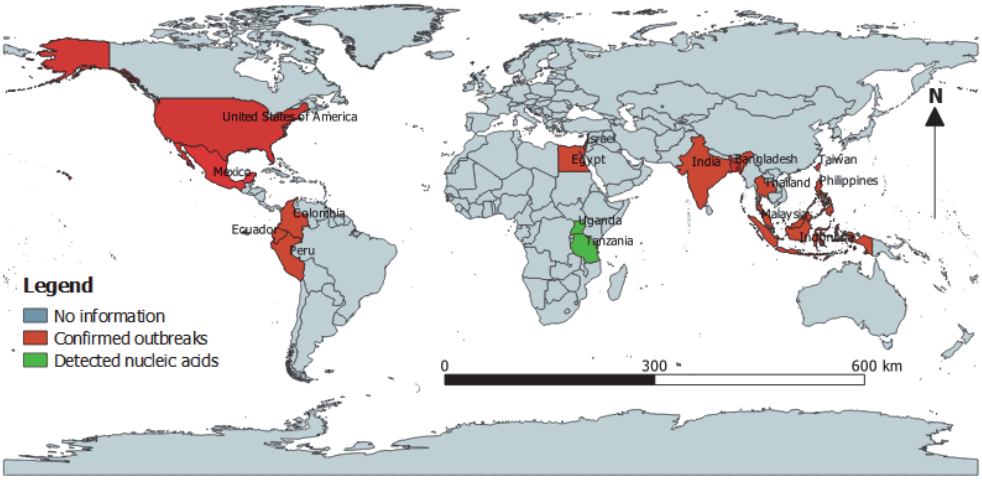

Figure 10: The current worldwide geographical distribution map of TiLV. The distribution is divided into three categories: Fourteen countries which have confirmed the presence of TiLV during the outbreaks (red colour), two countries which have reported TiLV based on nucleic acids (green colour) and the remaining have not yet investigated and reported the presence of the virus or outbreak (grey colour) (Made by the author).

The developed quantitative real time-PCR is sensitive and specific for TiLV

In this study, we developed and optimized a SYBR Green-based real-time RT-PCR assay using a known serially diluted supernatant with titre ranging from $10^{5.301}$ to $10^{0.301}$ $TCID_{50}$/mL ($2x10^5$ $TCID_{50}$/ml to 2 $TCID_{50}$/ml) obtained from TFC #10 cells. The developed assay was then tested using a known virus titre (starting titre of 10^6 $TCID_{50}$/mL) from infected TFC #10 cells and unknown virus titre from infected tilapia organs. The conditions for efficient amplification and quantification of TiLV nucleic acids suitable for all the ten segments using real-time fluorescence measurement were defined in one run. The primers designed for all the ten segments were compared in terms of their efficiency, sensitivity and specificity for detecting TiLV. Since DNA binding dyes can bind to any double-stranded DNA generated during the PCR, we used several approaches to ensure the specificity is maintained at the end of the assays. The approaches included (a) generation of consistent melting peak for each primer in intra- and inter-assays (b) production of expected size of the amplicon for the respective primers (c) sequencing of the PCR products from each primer set and (d) testing the cross-reactivity with heterogenous viruses which are genetically similar to TiLV.

Overall, the developed SYBR Green-based real-time RT-PCR assay had efficiency within the recommended range of 90-105% [157], highly sensitive and specific for TiLV (Figure 11). The coefficient of variation calculated from the mean Ct values for both intra- and inter-assay ranged from 0.00% ~ 2.63%, which are within the acceptable limit of less than 5% [158, 159]. Moreover, the developed assay had a detection limit of 2 $TCID_{50}$/ml implying that the method can be applied for accurate quantification of TiLV in clinical and subclinical cases. Further, the developed assay had good efficiency and reproducibility, which are key requirements for nucleic acid quantification [160, 161]. The primers designed from segment 3 are regarded as primers of choice if one was to select one set of primers for use in real-time PCR for detection and quantification of TiLV. Therefore, the developed assay can generate reproducible results and we recommend using it for TiLV quantification during diagnosis, research and vaccine development.

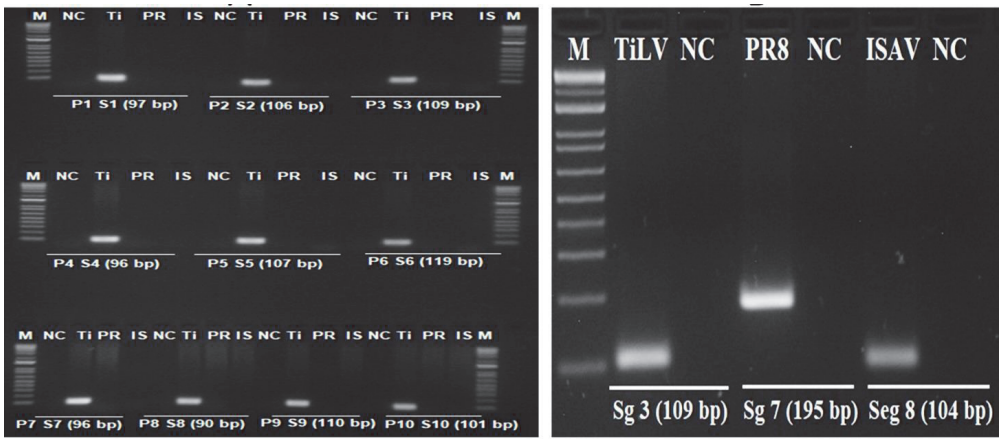

Figure 11: Specificity test. Figure A shows specificity test results of TiLV (Ti), Influenza A virus (PR) and Infectious Salmon anaemia virus (IS) based on gel electrophoresis when tested against TiLV primers (P1 S1-P10 S10). No bands were detected in Lane NC (sterile RNase free water) and the primers for PR8 and ISAV. Figure B shows the confirmation results on gel electrophoresis for the viruses (TiLV, PR8 and ISAV) used to check specificity. Also, no bands were detected in Lane NC (sterile RNase free water).

In the past, a SYBR Green-based quantitative RT-PCR was developed and confirmed for detection of TiLV in clinically and experimentally infected challenged fish [107]. As opposed to our assay, they used primers designed from segment 3 to detect the TiLV in gills, liver, brain, heart, anterior kidney and spleen. A recently developed and confirmed TaqMan RT-qPCR assay for TiLV

detection in tilapia [110] was also developed again based on segment 3 of the virus. Moreover, TaqMan RT-qPCR and SYBR green RT-qPCR assays when both are well designed and optimized perform equally well [110, 130, 162], yet TaqMan RT-qPCR is more expensive. Since it is not known whether designing primers targeting other segments of the virus would do better or not, our assay used primers targeting all the ten segments of the virus so that we can compare their results. Our findings showed that different primers targeting different TiLV segments have variable sensitivity and efficiency in detecting TiLV. This difference could be due to (i) designing and optimization of the primers (ii) differences in expression of the segments during replication and (iii) differences in stability of the segments to the host endonucleases. The latter could be contributed by the ability of the virus to protect more of its important proteins encoded by the respective segments such as segment coding for the polymerase.

The developed assay gives wider applicability such that the tool can be used for diagnostics in disease surveillance and control programs, vaccine developments, replication studies and other research projects.

Tilapia lake virus does not cause hemagglutination of erythrocytes from turkey, Nile tilapia or Atlantic salmon

Orthomyxoviruses and some paramyxoviruses contain hemagglutinin proteins on the surface important for the attachment to the sialic acid receptor (α2-3, α2-6 or α2-8 linkages) on the surface of the host cells [134]. The hemagglutinins are capable of binding to the sialic acid receptors found on the surface of avian, piscine and mammalian erythrocytes leading to the formation of a diffuse lattice (agglutination). In this study we used erythrocytes from turkey, Nile tilapia and Atlantic salmon to investigate the hemagglutination of TiLV by Influenza A virus (PR8) and Infectious salmon anaemia virus (ISAV) as positive controls. The findings indicated that TiLV was unable to agglutinate erythrocytes from all the three species suggesting that there is no hemagglutinin protein on the surface of the virus. Influenza A virus was able to agglutinate all the erythrocytes from the three species while ISAV agglutinated erythrocytes from Atlantic salmon which is well-known [163]. Influenza A virus agglutinated erythrocytes from all the three species due to its ability to bind different sialic receptors (either α2-3 or α2-6 linkages) on erythrocytes [164, 165]. Infectious salmon anaemia, on the other hand, uses 4-O-acetyl sialic acid receptor [166] which has been

reported to be located on endothelial and epithelial cells and RBCs of Atlantic salmon, rainbow trout, horse, donkey and rabbit [167, 168]. The erythrocytes from turkey and tilapia lack the 4-O-acetyl sialic acid receptor, thus ISAV does not hemagglutinate them. Tilapia lake virus might be lacking hemagglutinin important for hemagglutination activity. Further studies are needed to investigate the mechanism(s) used by TiLV for attachment and entry into the cells.

Treatment of E-11 cells with ammonium chloride does not affect the replication of TiLV

Some enveloped viruses have been shown to fuse their envelope with the endosomal membranes under low endosomal pH (<6.0). We wanted to test this property for TiLV by using weak bases that diffuse across the lysosomal membrane due to protonation and raise the acidic pH in the intracellular compartments [67, 169, 170]. To achieve a low pH in the E-11 endosomal compartments, we inhibited endosomal luminal acidification using ammonium chloride (NH_4Cl). The findings indicated that NH_4Cl (30 mM) does not inhibit the replication of TiLV in E-11 as there were no observed differences under light microscopy between the treated and control cells infected with TiLV. The main cytopathic effect (CPE) observed in both treated and control infected cells was the formation of vacuoles (patches) of detached cells from E-11 monolayers. Detection of viral RNA in the replicates of treated and the control infected cells showed no difference in the viral titer based on Ct-values indicating that NH_4Cl did not interfere with TiLV replication in E-11 cells.

Ammonium chloride has been used by researchers [66, 171-173] to study the requirement of low endosomal pH during internalization of viruses that use endocytic pathways. These studies have shown that NH_4Cl can inhibit virus multiplication.

These findings from acidification inhibition of TiLV, suggest that TiLV might be using different mechanism (s) during internalization into E-11. Moreover, it has been reported that some viruses can use more than one of the entry pathways [171] which give a high chance of internalization despite blocking one of the pathways. The mechanisms of internalization vary between viruses but generally, two main pathways exist endocytic and non-endocytic pathways. The endocytic pathway includes phagocytosis, clathrin-mediated endocytosis, caveolae-mediated endocytosis (CvME), lipid-mediated endocytosis, clathrin- and caveolin-independent receptor-mediated endocytosis and macropinocytosis [57, 58]. While the non-endocytic pathway includes the pore formation-mediated

penetration, low pH insertion peptides (non-enveloped viruses) [58, 174], fusion with the plasma membrane and cell-to-cell fusion (enveloped viruses) [81, 175]. This study targeted the receptor-mediated endocytic pathway which ends up by fusion of the virus envelope with the endosomal membranes under low pH (pH <6.0) to release the nucleic acid and accessories into the cytosol for replication. We recommend further studies to elucidate the internalization mechanisms of TiLV, which will pave the way for understanding how the virus infects the cell and its control.

MAIN CONCLUSIONS

In Paper I, nucleic acids of TiLV in Nile tilapia from Tanzanian and Ugandan sides of Lake Victoria in East Africa were detected. Primers designed from segment-2 of TiLV, which were the only primers able to detect TiLV without non-specific PCR products during optimization were used. The nucleic acids of TiLV were detected in both the wild (Tanzanian and Ugandan sides) and farmed (in Ugandan cages) Nile tilapia. High tissue tropism was observed in lymphoid organs (spleen and kidney) followed by the heart and the liver. This suggests that the virus replicates in the lymphoid organs at a low level in apparently healthy Nile tilapia. Blast search in the NCBI database and phylogenetic analyses of these TiLV nucleotide sequences showed high identity with nucleotide sequences from Israeli (98%), Ecuador (98%), Thailand (96%), Peru (96%) and USA (97%) isolates. Although we have not done virus isolation and experimental infection, the findings indicate that there were low levels of viral nucleic acids circulating in the wild and farmed Nile tilapia in Lake Victoria. Follow-up studies to detect and isolate the virus from infected materials and also to demonstrate Koch's postulates need to be done in future to understand the potential threat of the circulating TiLV in the fish.

The developed SYBR Green-based real-time PCR (paper II) based on all the ten segments of TiLV had high specificity, sensitivity, amplification efficiency ranging between 93-98% and low intra- and inter-assay variability. The assay was reproducible with low intra- and inter-assay variations based on low SD and %CV values. Primer 3 had the highest detection limit of TiLV in Nile tilapia infected tissues (liver and kidney), while primer-7 had the lowest detection limit. The findings indicate that primers having equal amplification efficiency but targeting different TiLV segments,

may have different sensitivity. This is only achieved through sensitivity optimization and systematic comparative approach of the primers designed from different viral segments that facilitate selection of the best primers and segment (s) to target during amplification. The overall findings on the developed RT-qPCR suggest the use of primers designed from segment 3 of TiLV in diagnosis and quantification of the virus in infected materials and during surveillance.

In paper III where the main aim was to shed light on the infection and infectivity of TiLV in cells, I examined the ability of the virus to agglutinate red blood cells, attachment and internalization into E-11 cells. Using red blood cells from turkey, Nile tilapia and Atlantic Salmon, TiLV was unable to agglutinate any erythrocytes from all the three species suggesting that the virus does not contain hemagglutinin on its surface. Influenza A virus used as a positive control was able to agglutinate the erythrocytes from all the three species while infectious Salmon anemia virus (another positive control) was able to agglutinate the erythrocytes from Atlantic salmon only. Moreover, treating E-11 cells with NH_4Cl did not alter the internalization of TiLV suggesting that TiLV might not be using receptor-mediated endocytosis where virus release into the cytosol requires acidification. Putting together these observations, we suggest that TiLV may not be in the family *Orthomyxoviridae* as suggested previously. Therefore, further studies leading to the correct classification of the virus are still needed.

FUTURE PERSPECTIVES

Tilapia lake virus has been reported to cause greater economic losses to the tilapia industry leading to many scientists being involved in understanding the virus and finding a solution to prevent virus infection and disease outbreaks. Some work has been done on surveillance and developing diagnostic tools with vaccine development being underway, which will help to protect tilapia from being infected. However, the current understanding of TiLV and factors associated with the emergence in different geographical locations is still limited. Gaps are remaining to be filled in the areas of aetiology, TiLV infection, virus multiplication and pathogenesis, epidemiology and control. These gaps provide new research possibilities which can be studied in the future. Based on my understanding, future studies may try to answer the following questions raised in this thesis.

❖ *What is the role of non-tilapiine fish species in epidemiology and spread of TiLV?*

Two non-tilapiine fish species, river barbs or tinfoil barb (*Barbonymus schwanenfeldii*) and giant gourami (*Osphronemus goramy*) have been found to be susceptible to TiLV under experimental settings in Malaysia and Thailand respectively. However, it is not known whether the two fish species can be susceptible and develop the disease in natural settings. In Lake Victoria with 500 fish species, the potential role of non-tilapiine fish species in transmitting TiLV to naïve susceptible tilapia remains to be unknown. Nile tilapia culture in the cages, as well as wild tilapia in Lake Victoria, were found to have nucleic acid genomes for TiLV. However, it is not known when, how and where did the infection come from to the wild tilapiine type and cultured tilapia in Lake Victoria. In this study, we did neither isolate the virus nor test its pathogenicity *in vivo* and *in vitro* to fulfil Koch's postulates. Therefore, it is important in the future to carry out further studies to confirm the presence of the virus in Lake Victoria and test its pathogenicity in the tilapia.

❖ *What is/are the roles of the different proteins found in the virus?*

Tilapia lake virus genome contains ten segments each with an open reading frame (ORF) coding one protein. Fourteen functional genes have been predicted from the 10 gene segments of TiLV with only six proteins showing transmembrane helix region. However, the names and roles of each of the proteins coded by each segment are not known giving room for further research. Studying the roles of the protein coded by each segment will help us understand the interactions of the virus with the host cell and in designing antiviral vaccines and therapeutics.

❖ *What is the mode of uptake into permissive cells for TiLV?*

In this thesis, we have seen that TiLV does not hemagglutinate erythrocytes from avian and piscine species indicating the lack of hemagglutinin protein. This protein is important for attachment and internalization of influenza viruses. On the other hand, blocking the endosomal acidification with NH_4Cl did not affect replication. This indicates that the virus might not be using the endocytic pathway during internalization. The question as to which mechanism TiLV is using for internalization into the cells remains unanswered. Above all, the whole mode of the infectious cycle of TiLV is not understood which could give the understanding of its pathogenesis and facilitate the

prevention and control of TiLV. The way all the steps of the replication cycle are coordinated and all the proteins involved during interactions with the host cell need to be studied in more detail.

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ORIGINAL ARTICLE

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Detection of tilapia lake virus (TiLV) infection by PCR in farmed and wild Nile tilapia (*Oreochromis niloticus*) from Lake Victoria

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Abstract

Tilapia lake virus disease (TiLVD) has emerged to be an important viral disease of farmed Nile tilapia (*Oreochromis niloticus*) having the potential to impede expansion of aquaculture production. There is a need for rapid diagnostic tools to identify infected fish to limit the spread in individual farms. We report the first detection of TiLV infection by PCR in farmed and wild Nile tilapia from Lake Victoria. There was no difference in prevalence between farmed and wild fish samples ($p = .65$), and of the 442 samples examined from 191 fish, 28 were positive for TiLV by PCR. In terms of tissue distribution, the head kidney (7.69%, $N = 65$) and spleen (10.99%, $N = 191$), samples had the highest prevalence ($p < .0028$) followed by heart samples (3.45%, $N = 29$). Conversely, the prevalence was low in the liver (0.71%, $N = 140$) and absent in brain samples (0.0%, $N = 17$), which have previously been shown to be target organs during acute infections. Phylogenetic analysis showed homology between our sequences and those from recent outbreaks in Israel and Thailand. Given that these findings were based on nucleic acid detection by PCR, future studies should seek to isolate the virus from fish in Lake Victoria and show its ability to cause disease and virulence in susceptible fish.

KEYWORDS
Lake Victoria, Nile tilapia, PCR, phylogenetic, surveillance, tilapia lake virus

1 | 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, (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., 2014). It has since been associated with outbreaks in Colombia, Ecuador, Egypt, Israel and Thailand (Bacharach et al., 2016; Del-Pozo et al., 2017; Fathi et al., 2017, Kembou Tsofack et al., 2017; Nicholson et al., 2017; Surachetpong et al., 2017). Based on motif alignment of its segment-1 with the PB1 segment of influenza A, B and C, the

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aetiological agent has been characterized as an orthomyxo-like virus (Bacharach et al., 2016). Classification by the International Committee of Virus Taxonomy (ICVT) 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 & Schulman, 1976). The length of the total viral genome is about 10,323 kp (Bacharach et al., 2016, Bacharach et al., 2016; Del-Pozo et al., 2017, Eyngor et al., 2014). 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; Nicholson et al., 2017; Surachetpong et al., 2017), red tilapia (Thailand) (Surachetpong et al., 2017) and the hybrid tilapia *O. niloticus* × *O. aureus* (Israel) (Bacharach et al., 2016), 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 68,000 km^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 cichlids (Goudswaard & Witte, 1997; Goudswaard, Witte, & Katunzi, 2002; Kudhongania & Cordone, 1974; Ogutu-Ohwayo, 1990). 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 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 (Fathi et al., 2017; Nicholson et al., 2017; Surachetpong et al., 2017) calls for the 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 reinfection in susceptible fish to demonstrate the characteristic syncytial hepatitis and other pathological lesions in susceptible fish (Del-Pozo et al., 2017; Tattiyapong, Dachavichitlead, & Surachetpong, 2017; Tsofack et al., 2016). Although these steps fulfil the Koch's postulates by establishing the disease–causal factor relationship, virus isolation, culture and reinfection are not ideal for surveillance programmes 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 programmes 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: (i) to develop and optimize a PCR-based method for the detection of TiLV and (ii) to investigate the possible existence of TiLV in Nile tilapia found in Lake Victoria.

2 | METHODS AND MATERIALS

2.1 | 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 1). Sampling for the wild sites was performed 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 lakeshore. 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 24 hr 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 1). Two sampling sites approximately 20 km apart were sampled in Maganga beach area and another two sites in the Mchongomani area separated by a distance of 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 5 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 1.

2.2 | Virus propagation and cell culture

Tilapia cell cultures were generated from hybrid *Oreochromis niloticus* × *Oreochromis aureus*. In brief, caudal fins were removed from killed fish that weighed 30 g. 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 with scissors, and semi-dry small tissue pieces of approximately 1 mm^3 were placed in dry 50-ml culture flasks (Nunc, Denmark). After 24-hr 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%

TABLE 1 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			14.66%	6.33%	0.71%	3.45%	7.69%	10.99%	0.00

penicillin–streptomycin. Cells were maintained at 28°C in a CO_2-free environment. At 10- to 14-day 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 signs 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 0.2-μm 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 7 days of incubation, suspensions for both virus-infected and virus-non-infected cells were harvested and used for RNA extraction as described below.

2.3 | RNA extraction and cDNA synthesis

Extraction of total RNA from the 442 samples (Table 1) was carried out using a combination of the TRIzol® (GIBCO, Life Technologies) and RNeasy Mini kit (Qiagen, Hilden, Germany) techniques as previously described (Munang'andu, Fredriksen, Mutoloki, Dalmo, & Evensen, 2013; Munang'andu et al., 2012; Munang'andu, Sandtrø, et al., 2013). Briefly, approximately 30 mg of tissue was homogenized in 1 mL TRIzol followed by centrifugation at 12,000 *g* for 10 min 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 5 min at room temperature followed by spinning at 12,000 *g* for 15 min. The aqueous phase was transferred into another Eppendorf tube. After adding 0.6 ml of 70% ethanol, the tubes were vortexed and the contents were transferred 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 20 μl 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 made 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 head kidney, 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

TABLE 2 Primer sequences

Segment	Primer sequence	Length (bp)	Tm (°C)
Segment-1	FWD-CCTCATTCCTCGTTGTGTAAGT	1,000	62
	REV-AGGAGTTGCTGTTGGGTTATAG		
Segment-2	FWD-GTCCAGGGCGGTATGTATTG	834	62
	REV-CTTACGGCTGACAAGTCTCTAAG		
Segment-3	FWD-GTCGAGGCATTCCAGAAGTAAG	834	62
	REV-GAGCTAAGGGAACGGCTATTG		
Segment-4	FWD-GCCTACTTCGTTGCCTATCTC	524	62
	REV-GCCCAATGGTTCCCATATCT		
Segment-5	FWD-CAACTCTTAGCCTCCGGAATAC	696	62
	REV-CGTTCTGCACTGGGTTACA		
Segment-6	FWD-CCCACACGACAGGACATATAG	948	62
	REV-GAGTTGGCTTAGGGTGATAAGA		
Segment-7	FWD-TCCTTTAGGGATTGGCACTAAC	486	62
	REV-TTCCATCGACTGCTCCTAGA		
Segment-8	FWD-CTTAAGGGCCATCCTGTCATC	476	62
	REV-TGGCTCAAATCCCAACACTAA		
Segment-9	FWD-GATATCCTCCACATGACCCTTC	261	62
	REV-GTACGTCACTTTGTGCCATTAC		
Segment-10	FWD-TCCTCTCTGTCCCTTCTGTT	276	62
	REV-CAGGATGAGTGTGGCAGATTAT		

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.

2.4 | Optimization of the polymerase chain reaction test

A total of 10 primers (Table 2) 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 TFC#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 (TFC#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 PCRs 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.

2.5 | 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 1, 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 1 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 performed using the Q5 High-Fidelity DNA Polymerase as described above. PCR products were then separated using 1.5% agarose gel electrophoresis and extracted using the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany). Sequencing was performed on a commercial basis by GATC Biotech (https://www.gatc-biotech.com). The CLC Workbench 6.0 (www.clcbio.com) and Mega7 software (Kumar, Stecher, & Tamura, 2016) were used for sequence alignment and phylogenetic tree analyses. Phylogenetic trees were inferred by the maximum-likelihood method, bootstrapped 1,000 times based on the JTT+G matrix-based model (Jones, Taylor, & Thornton, 1992). Initial tree(s) for the heuristic search were obtained automatically by applying neighbour-joining 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+non-coding. All positions containing gaps and missing data were eliminated. There were a total of 270 positions in the final data set. Evolutionary analyses were conducted in MEGA7 (Kumar et al., 2016). The results obtained for the different groups and organs were analysed statistically using Fisher's exact test using GraphPad Prism version 7.00 for Windows, GraphPad Software, La Jolla, California, USA.

3 | RESULTS

3.1 | 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 TFC#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 shows 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 (lanes 4T and 5T), TFC#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 controls were indicative that segment-2 primers were only able to detected 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 (Figure 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.

3.2 | Screening of Nile tilapia samples from Lake Victoria

Table 1 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 14.66% (N = 191). The prevalence in caged and wild fish was 17.78% (N = 45) and 13.70% (N = 146), respectively. There was no significance difference in the prevalence (p = .136) among wild fish from the Tanzania side (16.67%, N = 108) compared with the Uganda side (5.30% N = 38). However, there was a significant difference (p < .0028) in tissue distribution among organs examined. PCR products were detected in 10.99% (N = 191) spleen, 7.69% (N = 65) head kidney, 3.45% (N = 29) heart and liver

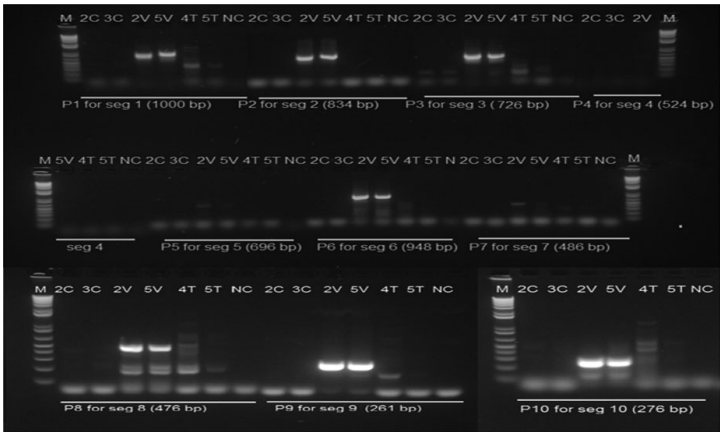

FIGURE 1 Shows electrophoresis gel analysis of TiLV positive control and the TFC#10 cells and GIFT fish negative control samples tested against TiLV segment 1–10 for the 10 primers (P1–P10) enlisted in Table 1. Note that both the positive and negative control samples are tested in duplicates in which 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 is shown alongside the name of the segment tested. There were no detectable bands in the TFC#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

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.

3.3 | Sequence alignment and phylogenetic analysis

Table 3 provides a summary of sequenced samples showing their origin, organ, size of the sequence product and GenBank 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 that were slightly different from each other and corresponded to different isolates that were already reported in the GenBank database (Figure 2). 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 Eyngor et al., (2014) and (Bacharach et al., (2016) , respectively.

4 | DISCUSSION

In this study, we detected TiLV nucleic acids in an area with no record of previous outbreaks. In line with Louws, Rademaker, & de Bruijn, (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 from Lake Victoria using PCR. Although we did not fulfil the Koch's postulate (Evans, 1976; Fredericks & Relman, 1996; Gradmann, 2014) by establishing the disease–casual factor relationship based on isolation, characterization, culture and

TABLE 3 Description of samples used for TiLV sequencing

Sample ID	Country	Source	Organ	Size (bp)	GenBank Acc #
UG2016-01	Uganda	Wild	Head kidney	830	MF536423
UG2016-02	Uganda	Wild	Liver	782	MF536429
UG2016-03	Uganda	Wild	Head kidney	332	MF536432
UG2016-04	Uganda	Wild	Heart	805	MF536426
UG2016-05	Uganda	Wild	Spleen	818	MF536427
UG2016-06	Uganda	Wild	Head kidney	830	MF536424
UG2016-07	Uganda	Wild	Spleen	819	MF536425
UG2016-08	Uganda	Wild	Spleen	787	MF536428
UG2016-09	Uganda	Wild	Head kidney	540	MF536430
UG2016-10	Uganda	Wild	Head kidney	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		

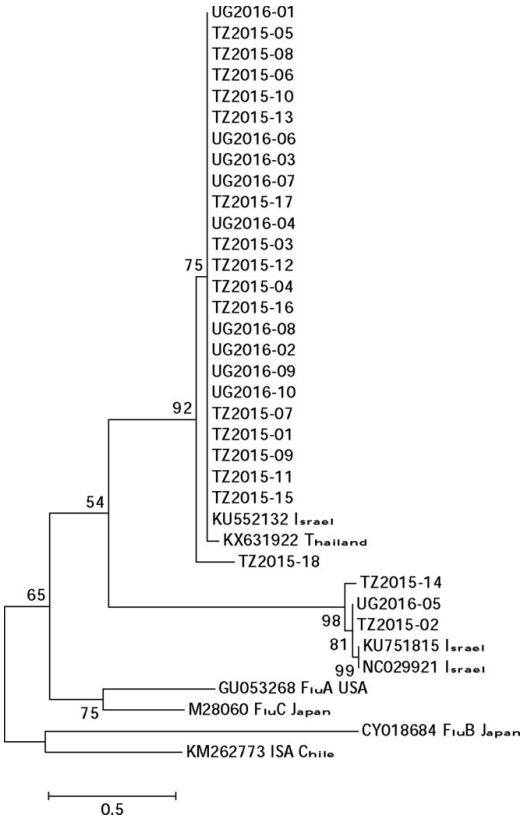

FIGURE 2 Phylogenetic analysis of the 28 Nile tilapia samples from Lake Victoria (TZ for Tanzanian and UG for Ugandan samples) sequenced using segment-2 primers. The evolutionary history was inferred using the maximum-likelihood method based on the Tamura–Nei model (Tamura & Nei, 1993)

reinfection 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, Mutoloki, & Evensen, 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 et al., 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 to confirm the possible existence of TiLV in Nile tilapia in Lake Victoria.

Our findings show an overall population prevalence of 14.66% (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., 2016, 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 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., 2017). The high prevalence of TiLV nucleic acids in the head kidney 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; Dong, Ataguba, Khunrae, Rattanarojpong, & Senapin, 2017; Molnar, Gamboa, Revenga, & Spalding, 2008), 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 reinfection in future studies.

In summary, this is the first documentation of TiLV genomes in a none-outbreak area (Lake Victoria). The findings clearly demonstrate that viral nucleic acids are 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.

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CONFLICT OF INTEREST

Authors declare no competing interests.

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Efficiency, sensitivity and specificity of a quantitative real-time PCR assay for tilapia lake virus (TiLV)

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Abstract

Tilapia lake virus (TiLV) is an emerging viral pathogen of tilapiines worldwide in wild and farmed tilapia. TiLV is an orthomyxo-like, negative sense segmented RNA virus, beloning to genus *Tilapinevirus,* family *Amnoonviridae.* Here we aimed at developing a quantitative real-time PCR (qRT-PCR) assay testing primer sets targeting the 10 segments of TiLV. Sensitivity, specificity, efficiency and reproducibility of these assays were examined. Detection sensitivity was equivalent to 2 $TCID_{50}$/ml when tested on supernatants from cell culture grown TiLV. Specificity tests showed that all primer sets amplified their respective TiLV segments, and standard curves showed linear correlation of R^2 >0.998 and an amplification efficiency between 93% - 98%. Intra- and inter-assay coefficients of variation (CV%) were in the range of 0.0%-2.6% and 0.0%-5.9%, respectively. Sensitivity tests showed that primer sets targeting segments 1, 2, 3 and 4 had the highest detection sensitivity of $10^{0.301}$ $TCID_{50}$/ml. The qRT-PCR used for detection of viral genome in TiLV infected organs, gave calculated virus titers, based on Ct values, of 3.80 log_{10}, 3.94 log_{10} and 3.52 log_{10} $TCID_{50}$/ml for brain, kidney and liver tissue parts respectively. These findings suggest that primer optimization for qPCR should not only focus on attaining high amplification efficiency but also sensitivity comparison of primer sets targeting different viral segments in order to develop a method with the highest sensitivity.

1.0 Introduction

Tilapia lake virus (TiLV) is an emerging viral pathogen of tilapiines causing high mortality in wild and farmed tilapia (1). Tilapiines are the second most important farmed fish species after carp in the world and are a prime source of protein in developing countries (2). TiLV is an orthomyxo-like, negative-segmented RNA virus belonging to the genus *Tilapinevirus* under the family *Amnoonviridae* (3). Its genome consists of 10 segments with variable sizes ranging from 465 to 1641 nucleotides with a genome total length of about 10.323 kb. The first segment encodes a protein with weak homology to polymerase PB1 subunit of influenza virus C (4), and no other segments have been found to contain genes with homology to known viruses or any host genes. Based on computational biology analysis, ORF-encoding 14 functional genes have been predicted from the 10 segments of TiLV (5) but their functions are not yet studied. TiLV has been reported from farmed and wild tilapia in Israel (1), Ecuador (4, 6), Colombia (7), Egypt (8), Thailand (9, 10), India (11), Malaysia (12), Taiwan (13), China (14), Philippines (15, 16), Indonesia (17), Peru (18), Lake Victoria in Eastern Africa (19), Mexico (20, 21) and USA (22, 23). Detection of the virus has been carried out using transmission electron microscope (1, 9), isolation in cell culture (1, 7), polymerase chain reaction (1, 8, 9), sequencing (4, 9), Northern hybridization (4), and *in situ* hybridization (4, 9). Most of these diagnostic methods are centered on virus or genome detection but no real comparison has been made as regards the optimal target for quantitative real-time diagnosis of infection.

Quantitative real-time PCR is a tool widely used for virus detection (24-26), and can be used to quantify absolute copy numbers of the target gene with high sensitivity, down to 1 - 10 copies in infected organs (27). In disease surveillance, it is used to determine the presence of virus infections and prevalence. It can be used to study establishment of infection at portals of virus entry, tissue

tropism in primary replication sites and localization in target organs (28). It can also be used to determine TiLV persistence and predisposing factors of recrudescence during outbreaks.

One of the major constraints in optimizing RT-qPCR, is selection of targets of detection since various viral genes can be expressed at different levels during the replication cycle. Their relative abundance can vary, and some genes are expressed early while others are expressed late in the replication cycle. In the case of TiLV which consist of 10 segments, little is known about functional genes and their relative expression levels during replication in infected cells or organ tissues. Because the functions of the transcribed products are not well known for this virus, optimal design focusing on viral gene expression during parts of the infection cycle or during persistence are not known. Our objective in this study was to develop an optimized RT-qPCR assay that can be used to detect TiLV genome during *in vitro* and *in vivo* infection.

2.0 Materials and Methods

2.1 Virus and virus culture

Tilapia lake virus Til-4-2011 isolate from Israel (1) was used in the experiments in this study. Viruses for testing specificity included infectious pancreatic necrosis virus (IPNV) (dsRNA) Norwegian Sp strain NVI015 (Acc. No AY397940), piscine reovirus (PRV) isolate strain 050607 (dsRNA) and Salmonid alphavirus subtype 3 isolate H10 (SAV-3) (+ssRNA).

Tilapia fin cells # 10 (TFC#10) (19) were grown in L-15 medium (Leibovitz) containing Glutamax® (Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS)

(Sigma Aldrich, St. Louis, MO, USA) at 28 ºC (19). When the cells were 80%–90% confluent, TiLV was inoculated in triplicates at a multiplicity of infection (MOI) 0.1 and, thereafter, cells were incubated at 28 ºC. Cells were inspected by microscopy daily post inoculation and at 7-8 days when cytopathic effect (CPE) exceeded 80%, the supernatant was harvested followed by centrifugation at 700 g for 10 min. Virus concentration in the supernatant was determined by 10-fold dilution and propagation in TFC#10 cells cultured in 96-well plates. This was followed by estimating the $TCID_{50}$/ml using Spearman and Karber's method (29) after seven days of incubation at 28 °C. Pelleted virus from supernatants after centrifugation 700 g for 10 min was stored at -80 °C and used for RNA extraction.

2.2 RNA extraction and cDNA synthesis

Total RNA from 10-fold serially diluted supernatant of TiLV (grown in cell culture) with a known virus concentration ($TCID_{50}$/ml), infected and uninfected tilapia tissues (head kidney, spleen and liver tissues from the GIFT project at NMBU), and uninfected cell pellets, was extracted using a modification of the TRIzol (Invitrogen, USA) method as previously described (26, 30). Briefly, cell pellets and tilapia tissues were homogenized in 1ml Trizol and centrifuged at 12,000g for 10 min at 4°C followed by transferring the supernatant into Eppendorf tube and addition of 0.2 ml chloroform, vortexing for 15 minutes and spinning at 12,000g for 15min. The aqueous phase was transferred into another Eppendorf tube, 0.6ml of 70% ethanol added and vortexed. The purification of total RNA from the supernatant was carried out using the RNeasy® Mini Kit following manufacturer's protocol (Qiagen, Hilden, Germany). After elution using RNase-free water, RNA was quantified by using Gen5 take3™ (BioTek®, USA).

cDNA synthesis was performed using Transcriptor First Strand cDNA synthesis kit following the manufacturer's instructions (Roche, Mannheim, Germany). The concentration of RNA used to synthesize cDNA in a 20µl final reaction mixture was 500 ng. The reaction conditions for cDNA synthesis included heating the tubes at 65 °C for 10 min to denature the template-primer mixture followed by cooling at 4 °C. The second reaction mixture was performed at 25 ºC for 10 min, 50 ºC for 60 min followed by inactivation of the Transcriptor Reverse Transcriptase by heating at 85 ºC for 5 min and cooling at 4 ºC. The synthesized cDNA was stored at -20 ºC until use. RNA extraction and cDNA synthesis were also carried out on three RNA viruses namely infectious pancreatic necrosis virus (IPNV) (dsRNA), piscine reovirus (PRV) (dsRNA) and salmonid alphavirus subtype (SAV-3) (+ssRNA) that were subsequently used for specificity tests together with sterile RNase free water.

2.3 Quantitative real-time PCR assay

Four sets of primer pairs were originally designed for each of the 10 segments of TiLV based on deposited sequences in the GenBank using CLC Main Workbench version 6.9.2 (http://www.clcbio.com). At the beginning of this study there were only three complete sequences of TiLV from Israel, Ecuador and Thailand which were aligned using the Qiagen CLC Main Workbench and consensus areas with 100% homology for all the ten segments were used to design the primers. The designed primers were blasted in the NCBI GenBank to check for non-specificity binding. The melting temperatures for the designed primers were around 62 and 63 °C. Quantitative real-time PCR was performed using the LightCycler® 480 SYBR Green I Master kit following manufacture's protocol (Roche, Mannheim, Germany). The total volume for each reaction contained 20 µl of the following: 2x SYBR Green I PCR Master Mix, 0.5 µM of each

forward and reverse primer, 4 µl of cDNA template, giving a 1/5 dilution of the original cDNA and correspondingly, highest concentration was $10^{5.301}$ $TCID_{50}$/ml and lowest $10^{0.301}$ $TCID_{50}$/ml. In addition, comes negative controls that included uninfected tilapia tissue, non-infected cells, and RNase-free water. All reactions of the qPCR assay were performed in triplicates using a LightCycler® 96 real-time PCR detection system (Roche, Germany). PCR cycle conditions included initial denaturation at 95 ºC for 5 min followed by 30 cycles of 95 ºC for 10 s, 55 ºC for 20 s and 72 ºC for 10 s. Melting curve analyses were performed for specificity verification of each amplified product. The criteria for selecting 1 primer set (from the original 4) was based on the best generated standard curves for the primer set for each segment; absence of matches with non-specific sequences upon blasting in the GenBank; absence of primer-dimers; presence of only one product and consistency of the melting peaks of the products from different samples. Table 1 shows the primer sets selected for each segment and their sequences.

Table 1. Primer sequences used in this study targeting TiLV segments

Primer/ Segment	Segment length (bp)	GenBank Acc no.	Primer name	Primer sequence	Position	bp
1			TiLV-SG1-F1	GTCGCTGAAAGACAGGAATCT	727-748	97
	1 641	KU751814	TiLV-SG1-R1	AGTAGAGTGTCCAGGAGGTATG	802-824	
			TiLV-SG2-F1	GAACCTGGCCCACAACATA	926-945	101
2	1 471	KU751814	TiLV-SG2-R1	GGGTACACTTTGGCTGATAGAG	1005-1027	
			TiLV-SG3-F4	TCCAGATCACCCTTCCTACTT	314-335	109
3	1 371	KU751816	TiLV-SG3-R4	ATCCCAAGCAATCGGCTAAT	403-423	
			TiLV-SG4-F4	GCGCAGTATTCAGTCCATCT	829-849	96
4	1 250	KU751817	TiLV-SG4-R4	GAGGGAGAACGCTAAGAAATCA	903-825	
			TiLV-SG5-F2	GATAATGGAAGCAGAGGGACTT	68-90	110
5	1 099	KU751818	TiLV-SG5-R2	GTATTCCGGAGGCTAAGAGTTG	156-178	
			TiLV-SG6-F3	ACGCTTGACGTGTAGTTTGA	881-901	119
6	1 044	KU751819	TiLV-SG6-R3	ATGAGTTGGCTTAGGGTGATAAG	977-1000	
			TiLV-SG7-F1	CCAGGTTGTGCCATTTGTATTG	551-573	96
7	777	KU751820	TiLV-SG7-R1	TTCCATCGACTGCTCCTAGA	627-647	
			TiLV-SG8-F2	GCCTTTCCTCTATCCGCTTTC	364-385	110
8	657	KU751821	TiLV-SG8-R2	TCCGGATTTGCTTTCCCTAAC	453-474	
			TiLV-SG9-F3	CCCTATCACCATTGCACTCTC	273-294	119
9	548	KU751822	TiLV-SG9-R3	CATCACAACCTGGTCAAGTCT	371-392	
			TiLV-SG10-F2	CAGAATCACAGTCGTCCATCTC	208-230	101
10	465	KU751823	TiLV-SG10-R2	GCGAAGTTGGAAGAATGAAAGG	287-309	

NB: Sequences used to design the primers were generated by (4)

2.3.1 Sensitivity assays and amplification efficiency

Tilapia lake virus cDNA from ten-fold serially diluted supernatants (from neat concentration of 10^6 $TCID_{50}$/ml to 10^1 $TCID_{50}$/ml of supernatant) was used to establish the standard curve for each primer, and the minimum virus concentration (sensitivity) detected by the different primer sets. All the diluted samples were subjected to PCR conditions described in Section 2.3 above. Electrophoresis gel (1.5%) analysis was used to determine the dilution effect on the samples and PCR products were confirmed by sequencing (data not shown). Standard curves were generated using www.mycurvefit.com or Stata15 based on linear regression estimates. Amplification efficiency (E) was calculated using the formula $E = [(10^{-1/slope}-1) \times 100]$ as previously described (31, 32). Sensitivity was given as an equivalent to the number of virus particles ($TCID_{50}$/ml) in the highest dilution giving a positive reaction by RT-qPCR of the serially diluted samples.

2.3.2 RT-qPCR specificity tests

Specificity tests for all primers targeting different TiLV segments were carried out using cDNA from TiLV infected cells in order to test the ability of the primers to only detect their targeted viral segments. In addition, all 10 primer sets were tested against IPNV, SAV-3 and PRV in order to assess their specificity. Similar primer concentrations and qPCR conditions as described in Section 2.3 above were used in this test. All PCR products detected by gel electrophoresis for all specificity tests were verified by sequencing.

2.3.3 Intra- and inter-assay variability

For intra-assay variation test (CV%), qPCR performed in Section 2.3 above was done in triplicates for all 10 primers at each time-point in order to determine consistency. For inter-assay variability, qPCR (Section 2.3) was repeated thrice on different days in order to determine whether the results obtained were reproducible among replicates.

2.4 Detecting TiLV in samples from infected fish

A total of twenty Nile tilapia (*Oreochromis niloticus*) weighing approximately 8 grams were used in this study. All fish were acclimatized for seven days in a re-circulation airflow system (RAS) at 28 °C. Six fish were injected with TiLV Til-4-2011 isolate from Israel (1) at a concentration of 1 x 10^5 $TCID_{50}$/fish while negative control fish (n=6) were injected with phosphate buffered saline (PBS), intraperitoneally. The fish used were obtained from Nir David Hatchery in Israel. The experiments were approved by the Animal research ethics committee of the Hebrew University of Jerusalem under permit number AG-19-15763-5. The TiLV and PBS injected fish were reared separately in different tanks at 28 °C in a recirculation airflow system (RAS). All fish were fed *ad-libitum* daily using commercial feed (Raanan fish feed, Israel). Fish were monitored daily for clinical signs and mortalities after TiLV challenge. The fish tissues were collected at the point of death from six infected fish and controls. The organs (kidney, liver, and brain) were preserved in RNAlater and used for RNA extraction by a modification of the TRIzol and Qiagen protocols as described in section 2.2. RNA quality was assessed by gel electrophoresis and Gen5 take 3^{TM} (Bio Tek®, USA). cDNA synthesis was prepared as described in section 2.2 above. The cDNA was then used for qPCR analysis. The qPCR conditions used were as described in Section 2.3. Virus concentrations in the different organs were calculated on the basis of the standard curves using

non-linear regression, symmetrical sigmoidal curves using the online MyCurve-fit webpage (www.mycurvefit.com) for the different primer sets (1-10).

2.5 Statistical analysis

Standard curves for serially diluted TiLV (10^6 $TCID_{50}$/ml to 10^1 $TCID_{50}$/ml) dilutions were generated in both Microsoft excel™ and Stata15 by plotting the Ct values on the Y-axis and dilutions of the samples ($TCID_{50}$/ml) on the X-axis in log_{10} scale. The linear regression equation (R^2) for each standard curve was used to determine the primer amplification efficiency (E). GraphPad Prism software version 7 (http://www.graphpad.com) was used to determine the mean, standard deviation (SD) and percentage (%) of coefficient of variation (CV%) for intra- and inter-assay variability analysis as a measure of reproducibility. Back-calculation of virus titers in fish organs was done by use of online (www.mycurvefit.com) tool after generating the regression curves using www.mycurvefit.com or Stata15 based on linear regression estimates.

3.0 Results

3.1 Quantitative real-time PCR assay

3.1.1 Sensitivity for standard PCR and electrophoresis analysis

The results of gel electrophoresis analysis for all primers tested on 10-fold serially diluted TiLV supernatant from TFC#10 infected cells are shown in Fig. 1. Diluted TiLV samples were sequentially placed in a decreasing order with the highest number of virus particles ($10^{5.301}$ $TCID_{50}$/ml) in lane 1 and the lowest in lane 8 ($10^{-1.699}$ $TCID_{50}$/ml). Lane NC shows results of reactions with sterile RNase free water (negative control) of which all 10 primers did not produce any PCR products (no bands detected). All primer sets showed a decreasing amount of PCR products by dilution as shown by the band thickness and intensity (Fig. 1). Primer sets 1, 2, 3, 4, 5, 6, 8, 9 and 10 had clear bands in lane 5, corresponding to a detection sensitivity of $10^{1.301}$ $TCID_{50}$/ml. Further, there was a faint band in lane 6 for primer sets 1, 2, 3, 4, 9, and 10, corresponding to $10^{0.301}$ $TCID_{50}$/ml. Primer set 7 had a detection limit of $10^{2.301}$ $TCID_{50}$/ml (Fig. 1, lane 4), and a faint band in lane 5.

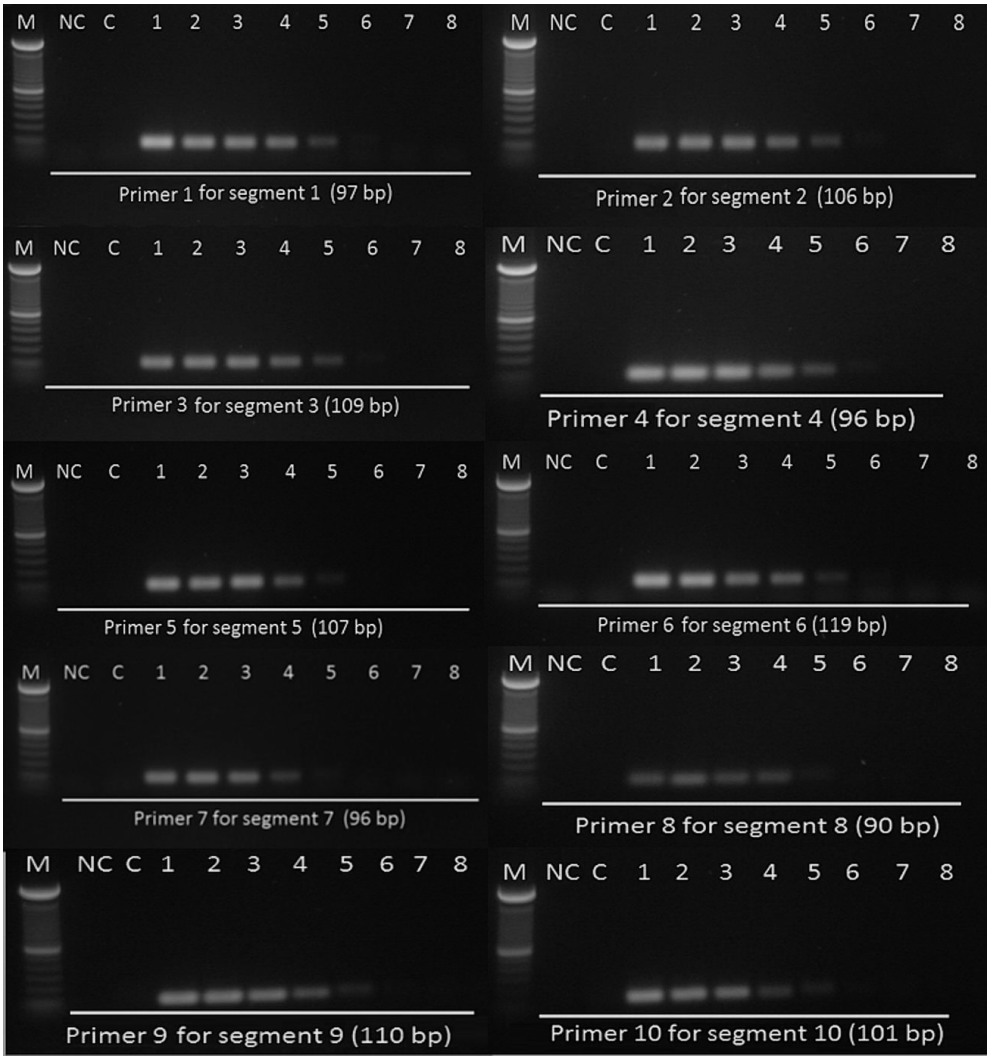


Fig. 1. Gel electrophoresis analysis for different primers targeting TiLV segments. Lane NC is sterile RNase free water; C is non-infected cell lines. Lanes 1 to 8 are PCR products from each primer tested against 10-fold serially diluted TiLV.

3.1.2 Detection of sensitivity for RT-qPCR - virus supernatant

The different primers gave different end point detection using virus supernatant as template (Fig. 2). The dilutions were the same as above. Primer pairs 1, 2, 3, 4 and 9 had a detection limit equivalent to 2 $TCID_{50}$/ml while primers 5, 6, 7, 8 and 10 had a detection limit corresponding to 20 $TCID_{50}$/ml (Fig. 2, Table 2).

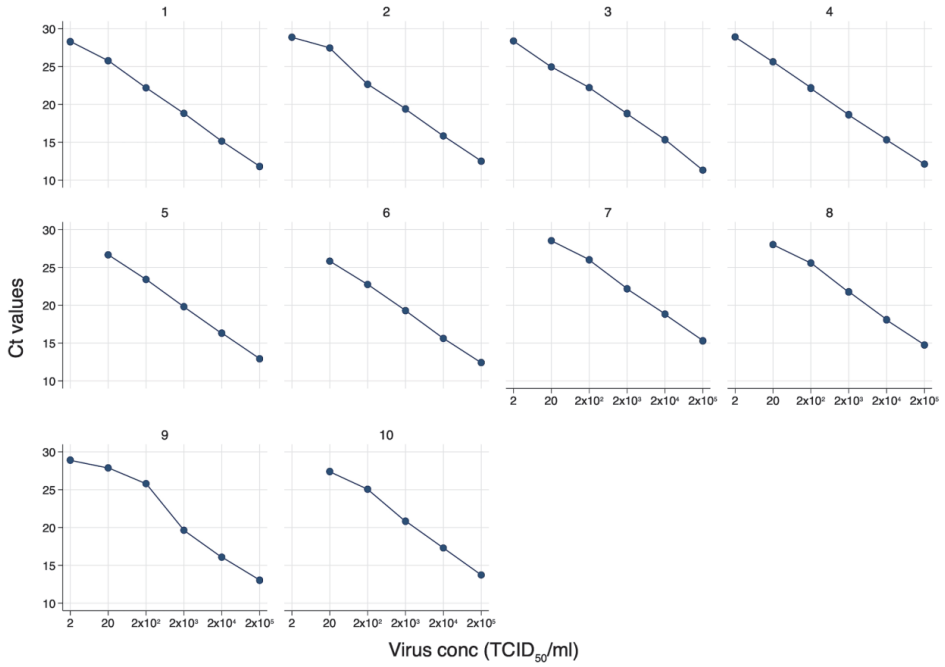
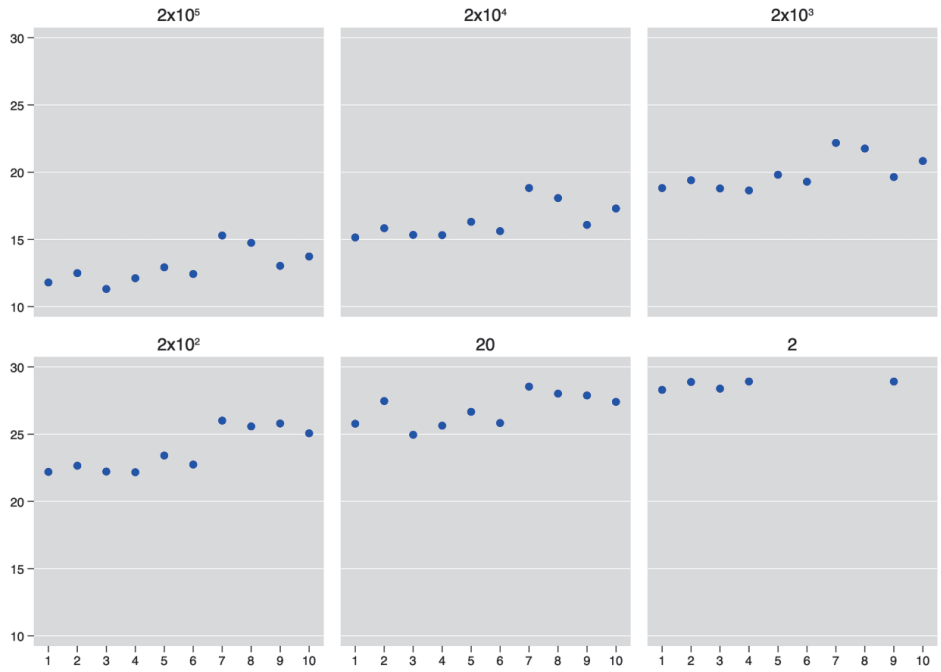


Fig. 2. Threshold cycle (Ct) values plotted against virus titers for primers targeting all 10 TiLV segments. The Ct values on the Y-axis against log_{10} of serially diluted virus ($10^{0.301}$ $TCID_{50}$/ml to $10^{5.301}$ $TCID_{50}$/ml) on the X-axis were generated using the SYBR green RT-qPCR method. The virus concentrations ($TCID_{50}$/ml) for x-axes were the same for all the axes above.

Table 2. TiLV detection sensitivity for the different primers

Primers	Detection limit ($TCID_{50}$)	Ct value
Primer 1	2	28.29
Primer 2	2	28.88
Primer 3	2	28.39
Primer 4	2	28.92
Primer 5	20	26.66
Primer 6	20	25.83
Primer 7	20	28.54
Primer 8	20	28.01
Primer 9	2	28.91
Primer 10	20	27.4

The Ct values obtained for each primer set was then plotted against virus concentration for the different dilutions of the virus supernatant (Fig. 3). The crossing point values for the different primers sets vary across all virus dilutions (Fig. 3, S1 Fig). For primer set 3, the Ct value was 11.31 at highest virus concentration ($10^{5.301}$), while primer set 7 had crossing point at 15.39 at this virus copy number (Fig. 3), a difference of 4.08 Ct. The difference for primer sets 3 and 7 (having equal amplification efficiency, 98%), points to a potential difference in template levels for the two segments or other factors like secondary RNA structures or impact from inhibitors that might vary between primer sets (33). Note that only primer sets 1, 2, 3, 4, and 9 gave positive reaction at 2 $TCID_{50}$/ml.



243

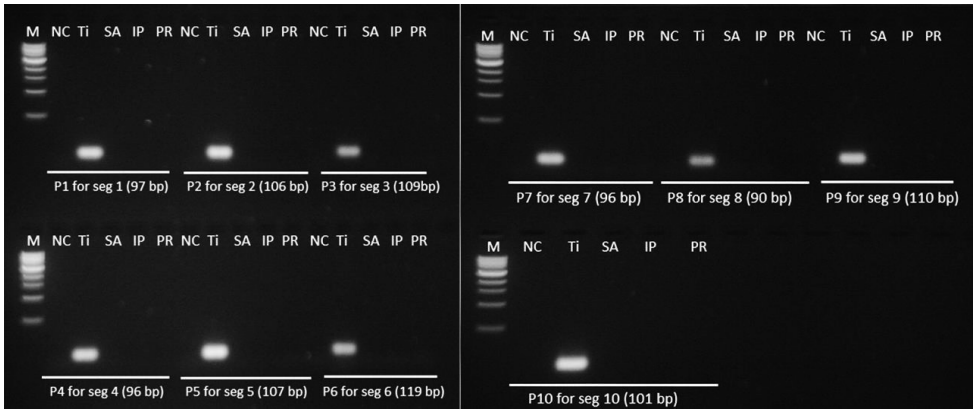
244 **Fig. 3. Ct values (y-axis) for the different primer sets (x-axis) tested using dilutions with virus**
245 **copy numbers.** The virus copy numbers as indicated ($2x10^5$ down to 2 $TCID_{50}$/ml) in the
246 supernatant were examined by RT-qPCR.

247

248 ### 3.1.3 Primer specificity test

249 All 10 primer sets (P1-P10) tested against TiLV, SAV-3, IPNV and PRV were positive for TiLV
250 RNA templates, and negative for SAV-3, IPNV and PRV (Fig 4a).

251

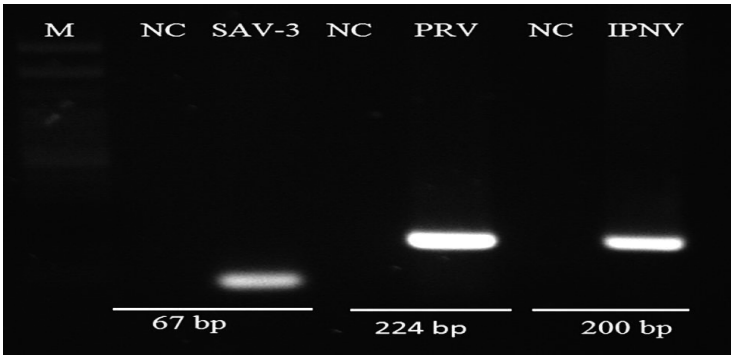


252

253 **Fig 4a. Specificity test based on gel electrophoresis analysis.** Gel electrophoresis of primers
254 (P1-P10) tested against targeted TiLV segments (seg 1 -10) and other RNA viruses, salmonid
255 alphavirus subtype 3 (SAV-3), infectious pancreatic necrosis virus (IPNV), and piscine reovirus
256 (PRV). Lanes NC, Ti, SA, IP and PR show reactions of each primer (P1-P10) tested against sterile
257 RNase free water (NC), TiLV (Ti), SAV-3 (SA), IPNV (IP), and PRV (PR), respectively.

258

259 Primers specific for SAV-3, PRV and IPNV viruses, respectively, produced PCR products from
260 their homologous viruses only (Fig. 4b). No PCR products were generated from RNase-free sterile
261 water (Fig 4b).



262

Fig. 4b. Specificity test based on gel electrophoresis analysis for SAV-3, PRV and IPNV against their respective primers.

3.1.4 Primer efficiency

Obtained Ct values are plotted against cDNA from 10-fold serial dilutions of TiLV supernatants using Stata15 and efficiencies were calculated on the basis of obtained slopes for the different primer pairs (Table 3). The slopes for standard curves varied between 3.361 ± 0.045 and 3.511 ± 0.074 for all 10 primers, giving amplification efficiencies for primers 1-9 ≥95%, while primer 10 had a 93% efficiency. Primer pairs 1, 3 and 7 had the highest efficiency followed by primers 4, 6 and 8, while primers 5 and 9 had equal efficiencies.

Table 3. The linear slopes, coefficient of determination (R^2) and the amplification efficiency of TiLV primers

Segment/ primer	Slope	1/slope	10^-(1/slope)	95% Conf Interv	R^2	Tm°C	Equation	Efficiency (%)
Segment 1	-3.364 ± 0.04051	-0,2972	1,98	-3.45 to -3.279	0,9977	83.0	Y = -3.364*X + 32.11	98
Segment 2	-3.430 ± 0.08778	-0,2915	1,96	-3.616 to -3.244	0,9896	82.5	Y = -3.430*X + 33.13	96
Segment 3	-3.361 ± 0.04552	-0,2975	1,98	-3.458 to -3.265	0,9971	81.0	Y = -3.361*X + 31.93	98
Segment 4	-3.385 ± 0.01304	-0,2954	1,97	-3.413 to -3.358	0,9998	83.0	Y = -3.385*X + 32.31	97
Segment 5	-3.457 ± 0.01478	-0,2892	1,95	-3.489 to -3.425	0,9998	81.0	Y = -3.457*X + 33.65	95
Segment 6	-3.393 ± 0.02627	-0,2947	1,97	-3.45 to -3.336	0,9992	83.5	Y = -3.393*X + 32.75	97
Segment 7	-3.368 ± 0.05406	-0,2969	1,98	-3.484 to -3.251	0,9967	83.5	Y = -3.368*X + 35.64	98
Segment 8	-3.403 ± 0.06338	-0,2938	1,97	-3.54 to -3.266	0,9955	84.5	Y = -3.403*X + 35.25	97
Segment 9	-3.456 ± 0.1765	-0,2894	1,95	-3.83 to -3.082	0,9599	83.5	Y = -3.456*X + 33.99	95
Segment 10	-3.511 ± 0.07465	-0,2848	1,93	-3.672 to -3.35	0,9942	84.0	Y = -3.511*X + 34.91	93

3.2 Intra- and Inter- assay coefficients of Variation

The reproducibility of the RT-qPCR assay was analyzed basing on the Ct values obtained from testing TiLV for all 10 primers at each time-point in triplicates within each run (intra-assay, S 1 Table) and also in three different runs on different days (inter-assay, S1 Table). The intra-assay coefficient of variation (CV) varied from 0.00% ~ 2.63% while the inter-assay CVs ranged from 0.00% to 5.92%. The calculated CV for both intra- and inter-assay were less than 5%, which is considered to be very good, while CV between 5% and 10% is considered to be good (34). The calculated SD values were also low, Ct≤0.05±0.03 and Ct≤0.05±0.03 for intra- and inter-assay estimates, respectively (S1 Table). These results show that the RT-qPCR is consistent and reproducible for all the ten primers in the detection of TiLV.

3.4 Sensitivity for RT-qPCR of TiLV in Nile tilapia infected organs

We next tested the different primers for the presence of TiLV genomes in liver, kidney and brain tissues of tilapia that had been infected with 1 x 10^5 $TCID_{50}$/fish, 10 days prior to sampling. Based on the obtained Ct values for the different primers for the 3 organs (Fig. 5), we (back) calculated the $TCID_{50}$ titer based on obtained Ct values using the standard curves obtained for the different primer sets with the help of linear or multinomial regression analysis. The anticipation was that calculated $TCID_{50}$ should be within the same range for the different primer sets although the Ct values would differ.

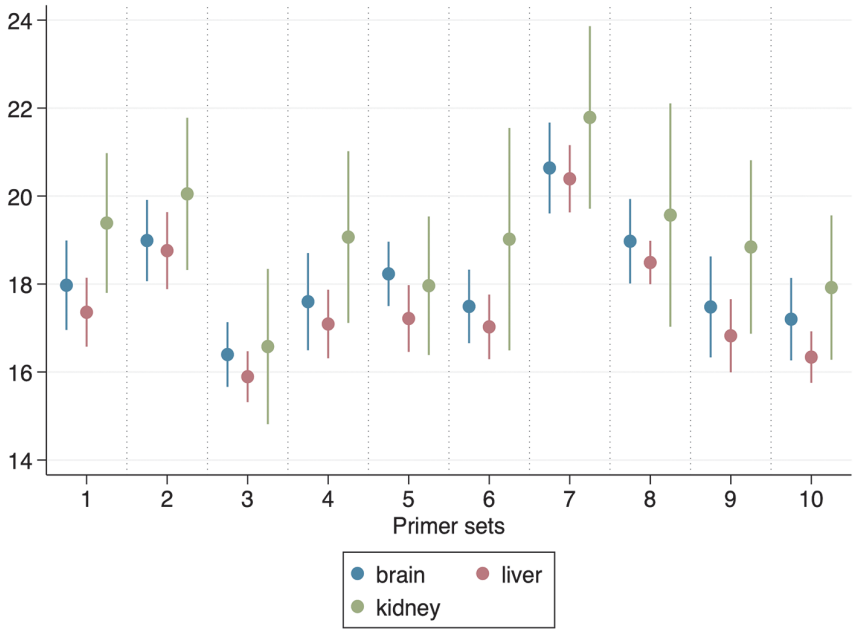
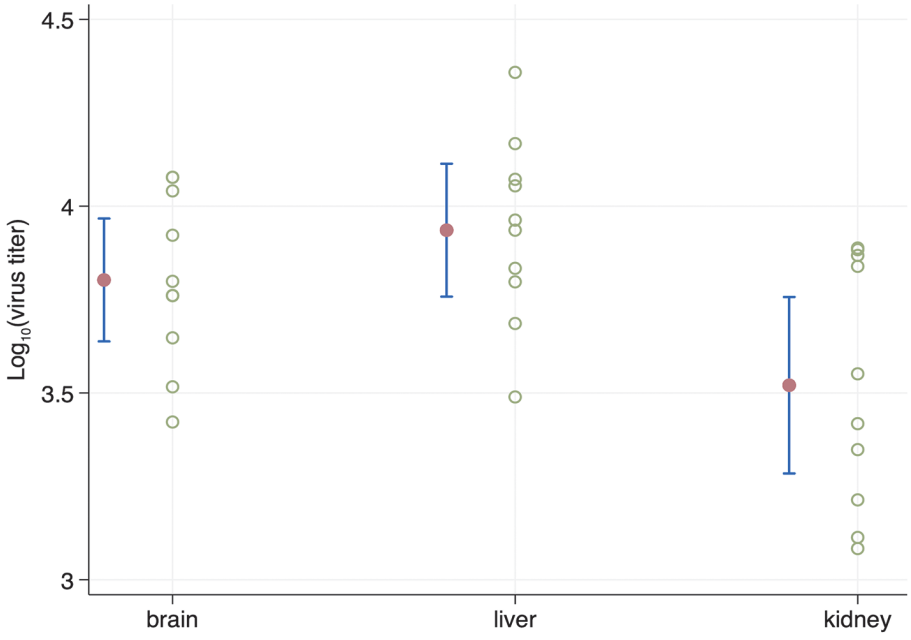


Fig 5. Average Ct values (± 95% confidence limits) for each primer set per organ (n=6), for brain, liver, and kidney. The pattern is the same for all organs with primer set 3 giving the lowest Ct value and primer set 7 being the highest (numerically).

From this, we found that the calculated virus titers differed by 0.66 $\log_{10}$ in brain (maximum and minimum values), 0.87 $\log_{10}$ in kidney and 0.81 $\log_{10}$ also in liver (Fig. 6). Average calculated titers were 3.80 $\log_{10}$, 3.94 $\log_{10}$ and 3.52 $\log_{10}$ for brain, liver and kidney, respectively, and 95% confidence limit plots for all primer sets per organ are shown (Fig. 6).



303

304 **Fig 6. Calculated virus titers (log_{10}) for the different primer sets (1-10) for brain, liver and**
305 **kidney.** Each primer set is displayed as an individual dot per organ, and average and 95%
306 confidence limits are also shown.

307

Discussion

Quantitative real time PCR was used to test the primers' ability to detect different TiLV concentrations with the view to determine the lowest concentration detected as a measure of sensitivity while specificity was measured by the ability of the primers to only bind their targeted viral proteins without binding proteins of other different viruses. The main conclusion from this study is that a sensitive RT-qPCR assay has been developed for detection of TiLV infection in tilapia. The RT-qPCR assay is a SYBR green-based method and several primer sets targeting different segments are well suited for diagnosis of infection. We started off with four sets per segment and only primers with no dimerization were selected for RT-qPCR optimization. The specificity test showed that the selected primers were able to amplify their targeted TiLV segments and did not produce PCR products from heterologous viruses.

Hallmarks of reliable and reproducible RT-qPCR methods are high primer amplification efficiency (95-105%), linear standard curves $R^2 \geq 0.980$, high precision between experiments, reproducibility across replicate experiments, and a wide dynamic range (35). We found that several of these requirements were fulfilled for the optimizing the RT-qPCR setup developed in this study. Primer amplification efficiency is assessed by generating a standard curve of serially diluted template plotted as a linear regression slope of Ct-values versus log template concentration, and a slope of -3.32 translates to 100% amplification efficiency (36). Given that RT-qPCR optimization requires a minimum of three replicates, variability in dilution often leads to inefficiencies amplification efficiency deviating from 100% ± 5% as an acceptable range (35, 36). In this study, the slopes for primers 1-9 varied between -3.36 and -3.45 with corresponding 95%-98% efficiencies being within optimal range (35) but primer set 10 gave a slope of -3.51 with resulting low amplification

efficiency (93%). Overall, our findings show that nine of the 10 primers designed had >95% efficiency that could reliably be used for detection TiLV with minimal variability.

Another important parameter essential for RT-qPCR optimization is R^2, which measures how successful a fit is in determining data variability (36), and the closer it is to 1, the greater the reduction of variability (35, 36). R^2>0.980 provides >95% correlation between threshold cycles (Ct) and target template copy numbers (35). We found that except for primer-9 with an R^2=0.960, the other nine primers used in the present study, R^2 varied between 0.994 and 0.999 resulting in 95%-98% efficiency befitting the 100% ± 5% optimal range (37-39).

Reproducibility across replicates is a crucial factor in developing reliable RT-qPCR assays (40). The most widely used measure of intra- and inter-assay variation is CV%, which has been shown to be reliable at evaluating the reproducibility of data generated using the same assay. Komurian-Pradel et al (41) found a low intra- and inter-assay CV% of 0.7 and 3.7%, respectively, for a RT-qPCR method developed for detection of hepatitis C virus while Kim et al (42) found an intra- and inter-assay CV% of 3.43% and 3.33%, respectively, for qPCR developed for porcine epidemic diarrhea virus. In this study, intra-and inter-assay CV% were <2.63% and <5.92%, respectively, corresponding with R^2>0.960 for all primers tested. And concluding, the low CV% obtained from triplicate experiments showed that the RT-qPCR method developed detection of TiLV has a high precision and high reproducibility.

In line with several scientists who have shown that primers with a wider detection range yield higher detection sensitivity than primers with a narrow detection range (36-39). Primers with wider detection range will have the ability to detect low quantities of TiLV RNA template in the sample while those with narrow range will not detect. This finding could be of importance when PCR is used for TiLV surveillance given that highly sensitive assays are required for the detection of the

lowest quantity of virus present in carrier fish. From the studies performed, primer set 3 would be the most suitable for detection of low quantities of virus in carrier fish. In vaccine development, this would be useful in determining the level at which highly protective vaccines can suppress virus replication by determining the lowest quantity of virus present in vaccinated fish of which primers with low detection sensitivity such as primer set 7 could be at risk of failing to detect low levels of virus present in infected fish after challenge.

One aspect to consider is that primer sets with different detection sensitivity can have an equal amplification efficiency with primers giving less sensitivity. For this reason, we advocate that primer optimization should not only aim at attaining high amplification efficiency but should include a comparison of primers targeting different viral genes in order to find optimal primers giving highest detection sensitivity.

Conclusion

Here we have developed a SYBR green-based RT-qPCR for the detection of TiLV targeting all 10 TiLV segments, and the assay developed has shown high specificity, amplification efficiency >90%, R^2>0.960 and very low intra- and inter-assay variability. We have shown the importance of comparing the detection sensitivity for the different primer sets in developing an optimal RT-qPCR method. Primer sets with high amplification efficiency >93% yield variation in method sensitivity. We advocate that optimization of detection sensitivity should not only focus on high amplification efficiencies but include a comparison of a number of primers sets in order to find optimal primers with the lowest detection limit. We anticipate that the approach used in developing

and optimizing the RT-qPCR for the diagnosis of TiLV in this study will contribute to developing highly sensitive diagnostic assays for diagnosis of subclinical infections of TiLV.

Acknowledgements

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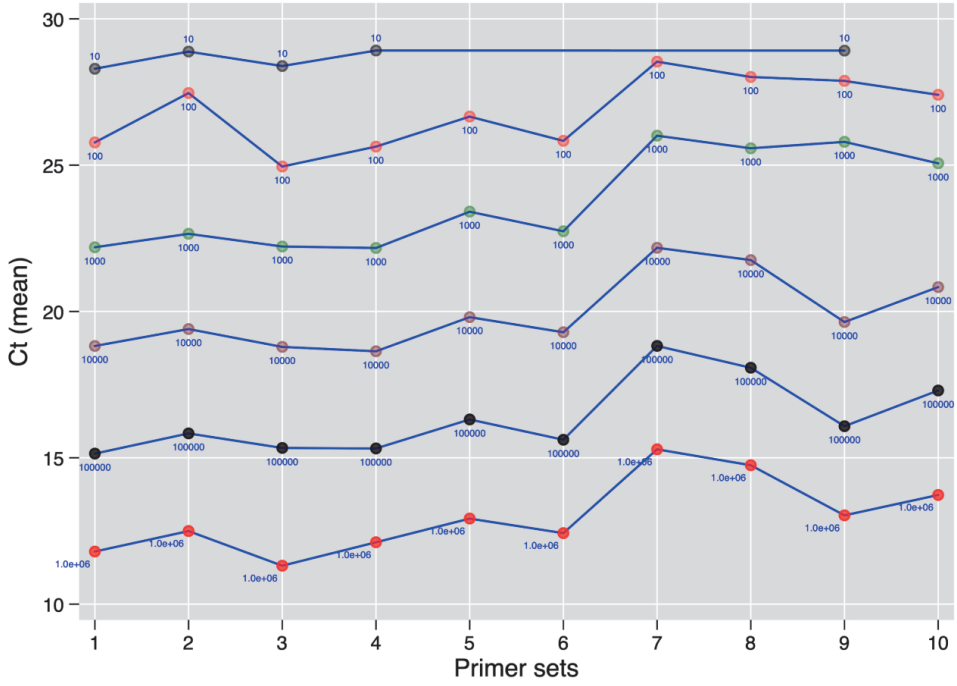
Supporting informationS1 Fig. The crossing point values for the different primers sets.



S1 Fig. The crossing point values for the different primers sets.

S1 Table 3. Intra- and inter-assay means, standard deviations and coefficient of variations of RT-qPCR

Primer	Dilution ($TCID_{50}$/ml)	TEST 1			TEST 2			TEST 3			AVERAGE TEST 1-3		
		Mean*	SD	%CV	Mean*	SD	%CV	Mean*	SD	%CV	Mean#	SD	%CV
Primer 1	10^6	11,80	0,03	0,21	11,99	0,00	0,00	12,06	0,01	0,08	11,95	0,13	1,13
	10^5	15,14	0,01	0,04	15,40	0,02	0,11	15,47	0,03	0,22	15,34	0,17	1,13
	10^4	18,82	0,02	0,11	18,36	0,02	0,09	19,36	0,02	0,10	18,85	0,50	2,66
	10^3	22,19	0,02	0,07	23,03	0,03	0,11	24,46	0,01	0,04	23,23	1,15	4,94
	10^2	25,78	0,03	0,10	26,19	0,04	0,15	27,32	0,05	0,17	26,43	0,80	3,02
	10^1	28,29	0,04	0,14	28,73	0,03	0,09	28,83	0,03	0,09	28,62	0,29	1,00
Primer 2	10^6	12,50	0,03	0,20	12,74	0,04	0,28	12,64	0,02	0,12	12.63	0.12	0.95
	10^5	15,83	0,02	0,10	16,04	0,03	0,16	15,93	0,03	0,16	15,93	0,11	0,66
	10^4	19,40	0,03	0,15	19,14	0,02	0,10	19,75	0,02	0,08	19,43	0,31	1,58
	10^3	22,65	0,03	0,11	24,25	0,01	0,05	24,81	0,05	0,19	23,90	1,12	4,69
	10^2	27,46	0,02	0,06	27,13	0,01	0,02	27,61	0,03	0,11	27,40	0,25	0,90
	10^1	28,88	0,02	0,06	28,88	0,02	0,06	28,93	0,02	0,05	28,90	0,03	0,10
Primer 3	10^6	11,31	0,01	0,05	11,89	0,03	0,25	12,02	0,03	0,25	11,74	0,38	3,22
	10^5	15,34	0,04	0,23	15,04	0,03	0,18	15,31	0,00	0,00	15,23	0,17	1,08
	10^4	18,79	0,05	0,24	18,22	0,03	0,16	19,20	0,02	0,11	18,74	0,49	2,63
	10^3	22,22	0,01	0,05	22,62	0,04	0,18	24,32	0,03	0,11	23,05	1,12	4,84
	10^2	24,95	0,02	0,06	26,05	0,02	0,08	27,29	0,02	0,07	26,10	1,17	4,49
	10^1	28,39	0,01	0,04	28,39	0,01	0,04	28,43	0,02	0,07	28,40	0,02	0,08
Primer 4	10^6	12,11	0,02	0,13	12,35	0,03	0,25	12,07	0,07	0,55	12,18	0,15	1,24
	10^5	15,32	0,03	0,17	15,42	0,03	0,17	15,47	0,01	0,07	15,40	0,08	0,50
	10^4	18,64	0,04	0,21	18,99	0,03	0,14	19,16	0,02	0,10	18,93	0,27	1,40
	10^3	22,17	0,06	0,28	23,57	0,02	0,08	24,14	0,03	0,11	23,29	1,01	4,35
	10^2	25,63	0,02	0,08	26,97	0,03	0,09	27,01	0,06	0,20	26,54	0,79	2,96
	10^1	28,92	0,01	0,04	28,92	0,01	0,04	28,76	0,03	0,09	28,87	0,09	0,32
Primer 5	10^6	12,92	0,02	0,16	12,89	0,01	0,04	13,97	0,02	0,15	13,26	0,62	4,64
	10^5	16,31	0,04	0,22	16,35	0,02	0,09	17,36	0,04	0,21	16,67	0,60	3,57
	10^4	19,81	0,02	0,11	19,45	0,01	0,06	20,86	0,02	0,10	20,04	0,73	3,66
	10^3	23,41	0,02	0,09	24,13	0,02	0,06	25,46	0,02	0,08	24,33	1,04	4,27
	10^2	26,66	0,02	0,08	26,95	0,00	0,00	27,71	0,02	0,07	27,11	0,54	2,00

Note: low SD and %CV depicting low intra- and inter-assay variability

Intra-assay variation

Inter-assay variation

***Mean** = Average of three replicates
#**Mean** = Average of three tests

S1 Table. Intra- and inter-assay means, standard deviations and coefficient of variations of RT-qPCR continued

Primer	Dilution ($TCID_{50}$/ml)	TEST 1			TEST 2			TEST 3			Mean test 1-3		
		Mean	SD	%CV	Mean	SD	%CV	Mean	SD	%CV	Mean	SD	%CV
Primer 6	10^6	12,43	0,03	0,20	12,52	0,00	0,00	12,43	0,03	0,20	12,46	0,05	0,42
	10^5	15,62	0,02	0,10	15,65	0,06	0,35	15,62	0,02	0,10	15,63	0,02	0,11
	10^4	19,29	0,02	0,10	19,48	0,02	0,11	19,29	0,02	0,10	19,35	0,11	0,57
	10^3	22,74	0,02	0,09	24,14	0,02	0,06	22,74	0,02	0,09	23,21	0,81	3,48
	10^2	25,83	0,01	0,04	26,24	0,01	0,04	25,83	0,01	0,04	25,97	0,24	0,91
Primer 7	10^6	15,29	0,04	0,24	15,12	0,00	0,00	14,43	0,03	0,17	14,95	0,46	3,05
	10^5	18,82	0,04	0,19	19,84	0,03	0,13	17,62	0,02	0,09	18,76	1,11	5,92
	10^4	22,18	0,03	0,11	22,63	0,06	2,63	21,29	0,02	0,09	22,03	0,68	3,10
	10^3	26,01	0,03	0,10	26,93	0,04	0,13	24,74	0,02	0,08	25,89	1,10	4,25
	10^2	28,54	0,02	0,05	28,94	0,02	0,05	28,83	0,01	0,03	28,77	0,21	0,72
Primer 8	10^6	14,75	0,03	0,17	14,61	0,04	0,26	14,09	0,04	0,26	14,48	0,35	2,40
	10^5	18,08	0,04	0,22	17,96	0,04	0,20	17,33	0,03	0,15	17,79	0,40	2,26
	10^4	21,76	0,04	0,19	21,66	0,03	0,12	21,22	0,03	0,14	21,55	0,29	1,33
	10^3	25,58	0,03	0,12	26,53	0,05	0,17	25,28	0,02	0,08	25,80	0,65	2,53
	10^2	28,01	0,02	0,05	28,01	0,02	0,05	28,01	0,02	0,07	28,01	0,00	0,00
Primer 9	10^6	12,98	0,02	0,15	13,03	0,03	0,23	12,27	0,02	0,16	12,76	0,43	3,33
	10^5	16,26	0,04	0,25	15,07	0,03	0,20	16,20	0,02	0,13	15,84	0,67	4,23
	10^4	19,83	0,08	0,41	19,14	0,01	0,05	19,90	0,03	0,13	19,62	0,42	2,14
	10^3	25,54	0,05	0,18	26,36	0,04	0,14	25,01	0,03	0,12	25,64	0,68	2,65
	10^2	27,57	0,03	0,11	27,68	0,02	0,06	27,62	0,02	0,08	27,62	0,06	0,20
	10^1	28,83	0,01	0,04	28,55	0,03	0,11	28,81	0,03	0,11	28,73	0,16	0,54
Primer 10	10^6	14,54	0,02	0,11	13,73	0,00	0,00	13,63	0,02	0,11	13,97	0,50	3,57
	10^5	16,94	0,04	0,24	17,30	0,00	0,00	16,87	0,01	0,03	17,04	0,23	1,35
	10^4	19,50	0,01	0,05	20,83	0,03	0,15	20,49	0,01	0,05	20,27	0,69	3,41
	10^3	24,53	0,06	0,25	25,95	0,07	0,25	25,85	0,01	0,04	25,44	0,79	3,11
	10^2	28,00	0,10	0,34	27,40	0,03	0,11	26,72	0,02	0,06	27,37	0,64	2,34

viruses

MDPI

Article

Tilapia Lake Virus Does Not Hemagglutinate Avian and Piscine Erythrocytes and NH_4Cl Does Not Inhibit Viral Replication In Vitro

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Abstract: Tilapia lake virus (TiLV) is a negative-sense single-stranded RNA (-ssRNA) icosahedral virus classified to be the only member in the family Amnoonviridae. Although TiLV segment-1 shares homology with the influenza C virus PB1 and has four conserved motifs similar to influenza A, B, and C polymerases, it is unknown whether there are other properties shared between TiLV and orthomyxovirus. In the present study, we wanted to determine whether TiLV agglutinated avian and piscine erythrocytes, and whether its replication was inhibited by lysosomotropic agents, such as ammonium chloride (NH_4Cl), as seen for orthomyxoviruses. Our findings showed that influenza virus strain A/Puerto Rico/8 (PR8) was able to hemagglutinate turkey (*Meleagris gallopavo*), Atlantic salmon (*Salmo salar* L), and Nile tilapia (*Oreochromis niloticus*) red blood cells (RBCs), while infectious salmon anemia virus (ISAV) only agglutinated Atlantic salmon, but not turkey or tilapia, RBCs. In contrast to PR8 and ISAV, TiLV did not agglutinate turkey, Atlantic salmon, or tilapia RBCs. qRT-PCR analysis showed that 30 mM NH_4Cl, a basic lysosomotropic agent, neither inhibited nor enhanced TiLV replication in E-11 cells. There was no difference in viral quantities in the infected cells with or without NH_4Cl treatment during virus adsorption or at 1, 2, and 3 h post-infection. Given that hemagglutinin proteins that bind RBCs also serve as ligands that bind host cells during virus entry leading to endocytosis in orthomyxoviruses, the data presented here suggest that TiLV may use mechanisms that are different from orthomyxoviruses for entry and replication in host cells. Therefore, future studies should seek to elucidate the mechanisms used by TiLV for entry into host cells and to determine its mode of replication in infected cells.

Keywords: red blood cells; Tilapia lake virus; hemagglutination; ammonia chloride; inhibition

1. Introduction

Tilapia lake virus (TiLV) causes high mortality rates in wild and farmed tilapia. The disease caused by TiLV was first reported in Israel in 2009, while the virus was first characterized in 2014 [1]. Since then, TiLV infections have been reported from different countries around the world [2–5]. It is an icosahedral virus made of a trilaminar capsid with a diameter of 55–110 nm, which is morphologically similar to orthomyxoviruses [1]. Its genome is made of -ssRNA consisting of 10 segments, of which segment 1 has a weak homology with the influenza virus C PB1 subunit (~17% amino acid identity, 37% segment coverage). In addition, it has four motifs (I–IV) conserved in RNA-dependent RNA and RNA-dependent DNA polymerases that are homologous with influenza A, B, and C viruses [6].

As such, TiLV was initially proposed to be an orthomyxo-like virus belonging to the genus *Tilapinevirus* based on ultrastructural, chemical, and weak sequence similarities with orthomyxoviruses, but was recently classified as the only genus of the family *Amnoonviridae* of the order Articulavirales. It is transmitted horizontally from infected to susceptible fish [1], although vertical transmission has also been reported [7]. Clinical signs include lethargy, exophthalmia, haemorrhages, irregular swimming, and skin erosions [1]. Mortality ranges between 10% and 90% in TiLV-infected tilapia. Mortality caused by TiLV has been reported in various countries such Egypt, Thailand, Peru, Malaysia, India, and Ecuador [1,2,5,8–13]. Current control measures are mostly focused on the implementation of biosecurity measures.

Influenza A and B viruses contain envelope-associated proteins, namely, hemagglutinins (HA) and neuraminidase (NA), inserted into the lipid bilayer, which protrude from the outer surface as spikes. HAs play vital roles in virus entry by binding to sialic acid (Sia) on epithelial cell surfaces, which promotes fusion of the envelope to the cell membrane [14,15]. On the other hand, NA cleaves sialic acid on virion proteins to prevent clumping and facilitates virus release from infected cells [16]. During viral infection or budding, HA is activated when its precursor, HA0, is cleaved by membrane-bound host trypsin-like proteases into HA1 (50 kDa) and HA2 (25 kDa) subunits [15,17,18]. The HA1 subunits of the influenza A and C viruses contain a shallow pocket of conserved amino acids at the distal tip (receptor-binding site) that binds to Sia receptors, either α2-3 or α2-6 linkages [19]. The HA2 subunit contains the hydrophobic peptide required for membrane fusion [15,20]. Sialic acids (Sia) are among the class of monosaccharides containing a nine-carbon backbone, which normally attach to the glycans of animal cells [17,21]. In general, red blood cells (RBCs) from different host species possess both α2-3 and α2-6 linkages, where viruses containing HAs bind [22]. For example, influenza viruses agglutinate avian, mammalian, and piscine RBCs, while infectious salmon anemia virus (ISAV) agglutinate RBCs from cold-water-adapted piscine species [23]. The term hemagglutinin was coined due to the binding ability and aggregation (hemagglutination) of RBCs by the virus [16]. This viral property led to use of the HA assay, first introduced by Hirst [16] in the early 1940s, as a method to identify and quantify viruses that agglutinate RBCs. The HA assay is based on the principle of hemagglutination, in which HA binds to Sia receptors on RBCs, forming lattices that keep RBCs in suspension and appearing as a diffuse suspension. The ability of TiLV to agglutinate RBCs from tilapia or other host species has not been determined, despite the identified similarities with orthomyxoviruses.

Following HA binding to Sia receptors, virus internalization into host cells is mostly dependent on the endocytic pathway, leading to virus localization in endosomal vesicles, where uncoating is facilitated by fusion of the viral envelope with endosomal membranes, further leading to viral cargo delivery into cytoplasm [24]. The fusion of viral envelopes with endosomal membranes is highly dependent on the low endosomal pH (<6.0). Inhibiting endosomal acidification using weak basic lysosomotropic agents such as ammonium chloride (NH_4Cl) and chloroquine that diffuse across lysosomal membranes due to protonation have been shown to interfere with orthomyxovirus replication [25,26]. Hence, in the present study, we wanted to determine whether the replication of TiLV could be inhibited by endosomal membrane fusion inhibitors, such as NH_4Cl. Given that TiLV has been shown to have some properties that are similar to influenza viruses, we also wanted to determine whether TiLV agglutinated piscine and avian RBCs, as seen in influenza viruses and ISAV. We found that TiLV did not agglutinate RBCs from any of the species included in the present study, thereby suggesting an absence of surface proteins with hemagglutination properties, pointing toward receptors other than sialic acid types for uptake. Resistance toward lysosomotropic agents indicated that the viral uptake mechanisms were independent of acidification.

2. Materials and Methods

2.1. Viruses and Cells

E-11 cells (ECACC 01110916, European Collection of Authenticated Cell Cultures, Salisbury, UK) and SHK-1 cells (ECACC 97111106, Salisbury, UK) were cultivated in Leibovitz L-15 medium Glutamax® (Glutamax, Gibco, Gaithersburg, MD, USA) and supplemented with 10% (*v*/*v*) fetal bovine serum (FBS) (Sigma-Aldrich, St. Louis, MO, USA) at 28 °C and 20 °C, respectively, until formation of near confluent monolayers (80–90%). E-11 cells were used to propagate TiLV, while SHK-1 cells were used to grow ISAV. Near confluent monolayers were washed twice with phosphate-buffered saline (PBS), followed by adding the virus suspension and incubation for 1 h at 28 °C (TiLV) and 15 °C (ISAV) to allow virus adsorption onto the cells. The cell culture flasks were gently tilted to ensure uniform distribution of the virus solution on the cell monolayer. After 1 h of virus adsorption, the unbound virus was washed off three times using PBS and freshly prepared growth media containing 2.5% FBS was added to the cells. Cell culture flasks infected with TiLV were cultured at 28 °C, while ISAV-infected flasks were cultured at 15 °C. The virus was harvested when the cytopathic effect (CPE) was fully developed by centrifugation of the supernatant from each flask at 2500 rpm for 10 min. The virus supernatant harvested from each flask was filtered at a size of 22 µm to remove cell debris. Each virus was stored at −80 °C until use. The influenza virus strain A/Puerto Rico/8, designated as PR8 in this study, was grown in the allantois membranes of 11-day-old embryonated chicken eggs and harvested after 3 days. The PR8 virus was kindly provided by Professor Espen Rimstad of Norwegian University of Life Sciences (NMBU) in Norway.

2.2. Virus Titrations

Quantification of TiLV and ISAV was done in E-11 and SHK-1 cells cultured in 96-well plates, respectively, using the tissue culture infective dose ($TCID_{50}$/mL) method. The titer for influenza virus strain A/Puerto Rico/8 (PR8) was determined using the HA assay with chicken red blood cells.

2.3. Verification of Virus Species Used for the Hemagglutination Assays

Verification of virus species used for the HA assay was carried out using total RNA extracted from cell culture supernatants for the TiLV and ISAV, whereas the allantois fluid was used for the extraction of total RNA for influenza virus PR8 using a modification of the Trizol (GIBCO, Life Technologies, Gaithersburg, MD, USA) and RNAeasy Mini kits (Qiagen, Hilden, Germany), as previously described [27]. cDNA synthesis was carried out in 20 µL reaction volumes using the Qiagen quantiTect® reverse transcription kit that involved an integrated step for the removal of contaminated genomic DNA (Qiagen), as previously described in our studies [28]. TiLV, PR8, and ISAV nucleic acids were amplified using primers specific to each virus, as shown in Table 1, targeting segment 3 for TiLV, segment 7 for PR8, and segment 8 for ISAV. PCR reactions were carried out using the Qiagen kit according to the manufacturer's guidelines. RNase-free water was used as a negative control.

Table 1. List of primers used for verifying the viruses (tilapia lake virus (TiLV), influenza A/Puerto Rico/8 (PR8), and infectious salmon anemia virus (ISAV)).

Primer Name	Primer Sequence	Expected Product (bp)	Virus/Segment	Reference
TiLV-SG3-F4	TCCAGATCACCCTTCCTACTT	109	TiLV/3	This study
TiLV-SG3-R4	ATCCCAAGCAATCGGCTAAT			
Flu A-F	TAACCGAGGTCGAAACGTA	195	PR8/7	[29]
Flu A-R	GCACGGTGAGCGTGAA			
H520-F	CTACACAGCAGGATGCAGATGT	104	ISAV/8	[30]
H534-R	CAGGATGCCGGAAGTCGAT			

2.4. Collection and Preparation of Erythrocytes

Red blood cells (RBCs) used for the HA assay were collected from turkey (*Meleagris gallopavo*), Nile tilapia (*Oreochromis niloticus*), and Atlantic salmon (*Salmo salar* L.). Whole blood from Nile tilapia and Atlantic salmon cultured at the Norwegian University of Life Sciences aquarium was collected from the caudal vein of each fish and immediately mixed with an equal volume of Alsever's solution (A3551, Sigma-Aldrich). Fish used for blood collection were anesthetized using benzocaine (20 mg/L). Blood from turkey was obtained from NAISER Company (Oslo, Norway), provided on a commercial basis. The blood from turkey was collected and immediately mixed with an equal volume of Alsever's solution. Thereafter, whole blood in the Alsever's solution from turkey, tilapia, and salmon was washed in PBS to remove plasma, thereby leaving RBCs for the HA assay. For washing, 5 mL of RBCs was diluted in 45 mL PBS at room temperature, followed by centrifugation at 2000 rpm for 10 min at 4 °C; the supernatant was discarded. The washing in PBS was repeated three times and the pellet containing the RBCs was estimated using the graduated marks on the tubes after the final washing and centrifugation. This was followed by 10% dilution of RBCs in PBS (1:10) to prepare a stock solution. Finally, the 0.5% RBC working solution for HA test was prepared by diluting the RBC suspension in PBS at a ratio of 1:20, giving a final suspension of 0.5% RBCs from the stock solution. The 0.5% RBC working solution was prepared every day, while the RBC stock solution was only used up to a maximum of three days.

2.5. Hemagglutination Assay

Viruses used for the HA assay were TiLV (in four concentrations of 10^7, 10^6, 10^5 and 10^4 $TCID_{50}$/mL), ISAV (10^6 $TCID_{50}$/mL), and PR8 (512 HA/50 µL). The HA assays were performed in V-shaped 96-well plates (ThermoFisher Scientific, Waltham, MA, USA), as previously described [31]. Briefly, 50 µL of PBS was added to all wells. Thereafter, 50 µL of virus was added to the first column (column-1), giving a total of 100 µL. After mixing, 50 µL was transferred to wells in the next column (column-2). This two-fold serial dilution of mixing and transferring of 50 µL into the next column was continued until the last column on the plate, at which 50 µL, after mixing in the last column (column-12), was discarded. Thereafter, 50 µL of 0.5% RBC was added to all wells, followed by gentle mixing. The plate was monitored for hemagglutination at room temperature for up to 60 min. Wells showing a uniform reddish color of RBC suspension were regarded as positive for the HA assay, while wells with a red dot in the center at the bottom of each V-shaped well after one hour of incubation at room temperature were regarded as negative. As a measure of non-agglutination (negative results), stripping of unbound RBCs settled at the bottom of each well was performed by tilting the plate at a 60° angle. The highest dilution showing a uniform reddish color of RBCs was regarded as the HA titer of the virus tested.

2.6. Evaluating the Effect of Ammonium Chloride on TiLV Replication in E-11 Cells

E-11 cells were seeded into T-25 culture-flasks in L-15 growth media, followed by incubation at 28 °C until 95% confluence. Confluent monolayers were washed twice using PBS before treatment using various concentrations of NH_4Cl. Experiments using NH_4Cl treatment of E-11 cells were done in two parts, of which the first part was to determine the toxicity of different concentrations of NH_4Cl on E-11 cells. To do this, the concentrations of NH_4Cl tested were 1, 5, 20, 25, 30, and 50 mM. Once the different NH_4Cl concentrations mixed with growth media were put on the cells followed by incubation at 28 °C, the cells were observed every hour to check for toxicity based on morphological changes and cell death. The second part was to test the impact of NH_4Cl on replication of TiLV in E-11 cells once the optimal duration that did not cause toxicity on the cells was determined. To do this, cells were treated with NH_4Cl during virus adsorption, while post-adsorption treatment was done at 1, 2, or 3 h post-infection (hpi). Cells were infected with TiLV at multiplicity of infection (MOI) 1.0 and adsorbed for 1 h at 28 °C to allow for virus binding onto E-11 cells. The cell culture flasks were gently tilted to ensure uniform distribution of the virus solution on the cell monolayer. After

1 h of virus adsorption, any unbound virus was washed off three times using PBS. The cells were cultured in normal growth media containing 5% FBS after NH_4Cl treatment. Three negative control cell culture flasks without NH_4Cl treatment and cells treated with NH_4Cl but without virus infection were included in the study. The positive control group consisted of cells infected with the virus at MOI 1.0 without NH_4Cl treatment. All cells were incubated at 28 °C and monitored daily for cytopathic effect (CPE) development. Once CPE was observed, total RNA was extracted for virus quantification from all treatment groups using a modification of the Trizol (GIBCO, Life Technologies) and RNAeasy Mini kit (Qiagen) methods, as previously described [32,33]. Quantitative real time PCR (qRT-PCR) was performed on triplicates of the uninfected and infected cells using primers targeting TiLV segment 3 (Table 1).

3. Results

3.1. Verification of the Viruses Used in the Hemagglutination Assay

Species-specific primers were used to detect the different viruses used for the hemagglutination assay by PCR followed by gel electrophoresis, and the amplified products were specific for each of the viruses (Figure S1). Detection of the 109 bp band for TiLV (lane TiLV), the 195 bp band for PR8 (lane PR8), and the 104 bp band for ISAV (lane ISAV), as shown in Figure S1, was in concordance with the expected amplicon products for each virus, as shown in Table 1. Note that no bands were detected in the RNase free water in the NC lanes tested against TiLV, PR8, and ISAV primers, thereby indicating that there was no unspecific binding for the primers used in the gel electrophoresis analysis.

3.2. TiLV Hemagglutination Test Using Avian Erythrocytes

Red blood cells from turkey were used as representative of avian species for the HA assay. The viruses used in the assay were PR8 titrated in a two-fold dilution in row A, starting with the highest concentration of 512 HA units, while ISAV was titrated in a two-fold dilution in rows B and C, starting with the highest concentration of 10^6 $TCID_{50}$/mL in column 1. The HA titer used for PR8 had no direct link to the TiLV and ISAV $TCID_{50}$/mL. As shown in Figure 1, four concentrations of TiLV, starting with 10^7, 10^6, 10^5, and 10^4 $TCID_{50}$/mL, were diluted two-fold in rows D, E, F, and G, respectively, while row H was used as a negative PBS control. Note that the highest dilution factor at which PR8 agglutinated turkey erythrocytes was 1:128/50 µL, as shown in Figure 1, while the weak stripping of unbound turkey RBCs for the same plate were held at a 60° angle, as shown in Figure S2. Elution of the PR8 from turkey erythrocytes was not observed even after 4 h of incubation at room temperature and 12 h incubation at 4 °C. However, there was no agglutination of turkey erythrocytes by ISAV and TiLV (Figure 2) and no stripping of RBCs, as shown in Figure S2, when the same plate was held at a 60° angle in order to detect the stripping of non-agglutinated RBCs, similar to observations seen in the PBS negative control in row H. In summary, only PR8 agglutinated turkey erythrocytes, while neither of the piscine viruses (TiLV and ISAV) agglutinated the turkey RBCs (Table 2).

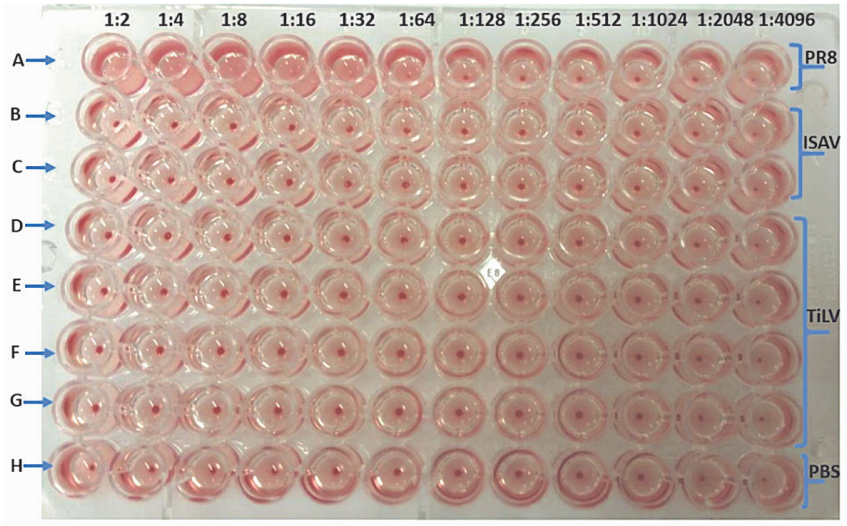

Figure 1. Hemagglutination of turkey red blood cells by PR8 (512HA/50 μL), ISAV (10^6 $TCID_{50}$/mL), TiLV (10^7, 10^6, 10^5, and 10^4 $TCID_{50}$/mL in rows D, E, F, and G, respectively) after 1 h of incubation at room temperature.

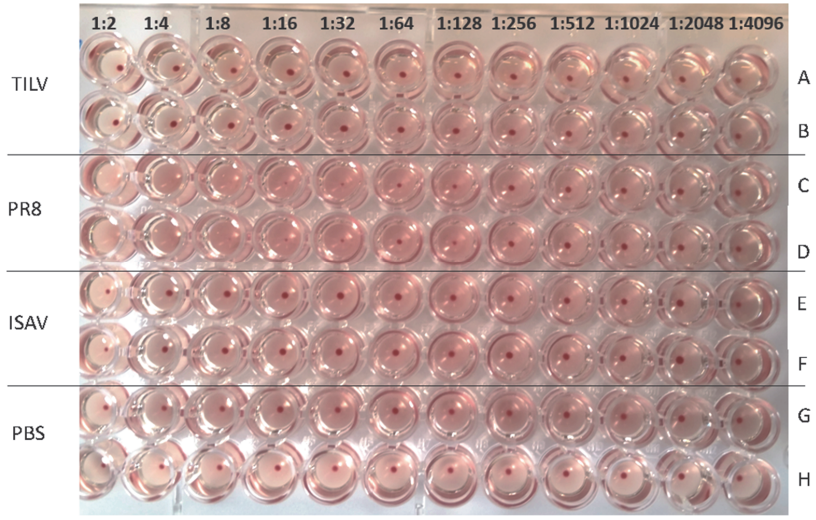

Figure 2. Hemagglutination of tilapia red blood cells by PR8 (512HA/50 μL), ISAV (10^6 $TCID_{50}$/mL), and TiLV (10^7 $TCID_{50}$/mL) after 1 h of incubation at room temperature.

Table 2. Hemagglutination of TiLV, PR8, and ISAV of avian and piscine red blood cells (RBC).

Viruses	Hemagglutination Titre (Dilution Factor/50 μL) of Avian and Piscine Erythrocytes		
	Turkey RBCs	Tilapia RBCs	Atlantic Salmon RBCs
PR8	1:128	1:64	1:512
ISAV	0	1:4	1:16
TiLV	0	0	0

3.3. TiLV Hemagglutination Test Using Piscine Erythrocytes

Tilapia and Atlantic salmon RBCs were used as representatives of piscine species for the HA assays. Figure 2 shows the layout of the viruses used in the assay, of which TiLV was used starting with a concentration of 10^7 $TCID_{50}$/mL diluted two-fold in rows A and B, PR8 (512 HA/50 µL) in rows C and D, and ISAV (10^6 $TCID_{50}$/mL) in rows E and F, while rows G and H contained the PBS negative control. Figure 2 shows that there was no agglutination of tilapia erythrocytes by TiLV, as demonstrated by rows A and B being similar to the PBS negative control in rows G and H. Rows E and F in Figure 2 showed weak agglutination of tilapia RBCs by ISAV at a 1:4 dilution, while Figure S3 shows that there was no stripping of RBCs at the 1:4 dilution (rows E and F) on the hemagglutination plate. The stripping became more prominent at the 1:8 dilution onward, which was similar to the PBS negative control. However, rows C and D showed that the highest dilution factor at which PR8 agglutinated tilapia RBCs was 1:64/50 µL.

Rows A and B, as seen in Figure 3 and Figure S4, showed that the highest dilution factor at which ISAV agglutinated Atlantic salmon erythrocytes was 1:16/50 µL, whereas the highest dilution factor for PR8 was 1:512/50 µL. Elution of Atlantic salmon agglutinated RBCs by PR8 and ISAV was not observed after 4 h of incubation at room temperature and 12 h of incubation at 4 °C. Rows E and F (Figure 3) showed that there was no agglutination of Atlantic salmon erythrocytes by TiLV, which was a similar observation as that seen in rows G and H for the PBS negative control.

In summary, these findings showed that PR8 was able to agglutinate turkey, Atlantic salmon, and tilapia RBCs, while ISAV only agglutinated Atlantic salmon and tilapia RBCs. In contrast, TiLV was not able to agglutinate turkey, tilapia, or Atlantic salmon RBCs (Table 2).

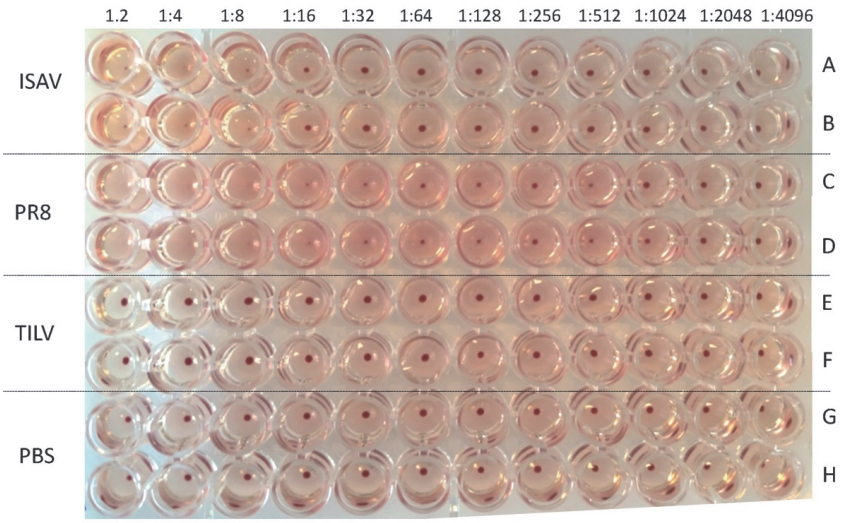

Figure 3. Hemagglutination assay of Atlantic salmon red blood cells by, ISAV (10^6 $TCID_{50}$/mL), PR8 (512HA/50 µL), and TiLV (10^7 $TCID_{50}$/mL) after 1 h of incubation at room temperature.

3.4. Evaluating the Effect of Ammonium Chloride on TiLV Replication in E-11 Cells

Treatment of E-11 cells with different concentrations of NH_4Cl showed that leaving the NH_4Cl media for more than 24 h on the cells was toxic for concentrations of ≥50 mM NH_4Cl. Lower concentrations, i.e., between 1 and 30 mM, had no toxic effect even when left on the cells until the end of the experiments. When the NH_4Cl media was replaced by maintenance media (L15 plus 2.5% FBS) after 7 h of NH_4Cl treatment had no toxic effect on the cells for high concentrations (≥50 mM NH_4Cl).

Hence, a concentration of 30 mM was selected to test the effect of NH_4Cl on TiLV replication, which was replaced with maintenance media after NH_4Cl treatment. The choice of 30 mM was in the range of NH_4Cl concentration used in several previous studies [34–36]. Therefore, in the pre-treatment group, NH_4Cl-containing media was added 30 min before virus adsorption, NH_4Cl treatment was performed during virus adsorption in the group designated as 0 hpi, and NH_4Cl media was added after 1, 2, and 3 hpi and was later replaced with maintenance media in the post-infection groups. The cytopathic effects (CPE) seen at 3 days post-infection (dpi) included the formation of vacuoles (patches) of detached cells from E-11 monolayers infected with TiLV (Figure S5A–H). Figure 4 shows that there was no significant difference in the mean viral titer based on *C*t-values detected by qRT-PCR from three replicates of TiLV-infected cells treated with 30 mM NH_4Cl at 0, 1, 2, and 3 hpi. In addition, there was no significant difference in the mean virus titer detected in the positive control (without NH_4Cl treatment) and the groups treated with NH_4Cl at 0, 1, 2, and 3 hpi.

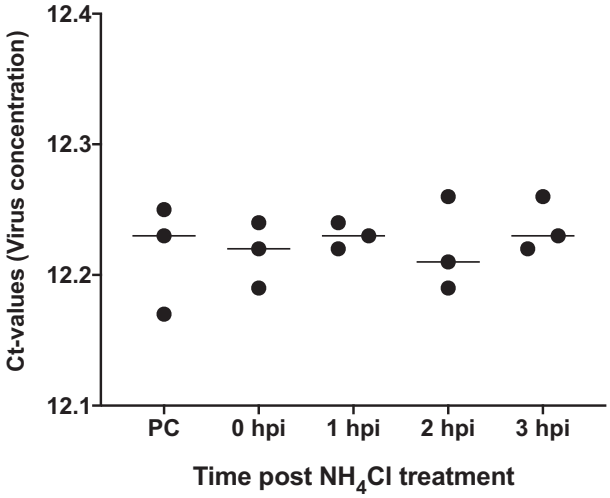

Figure 4. Mean viral loads of NH_4Cl-treated E-11 cells infected with TiLV at 0, 1, 2, and 3 h. Quantification of tilapia lake virus (TiLV) in E-11 cells nontreated and treated with NH_4Cl using quantitative real-time PCR (qRT-PCR). *C*t-values depict the quantity of viral RNA detected by qRT-PCR at 3 days post-infection (dpi) from three replicates of E-11 cells treated with NH_4Cl at 0, 1, 2, and 3 h post-infection with TiLV.

4. Discussion

The main finding in this study was that TiLV did not agglutinate tilapia or Atlantic salmon RBCs, and that NH_4Cl treatment neither inhibited nor enhanced TiLV replication in E-11 cells. The influenza virus strain A/Puerto Rico/8 (PR8) agglutinated avian and piscine RBCs, while ISAV only agglutinated Atlantic salmon and tilapia RBCs. The agglutination of tilapia RBCs by ISAV observed in this study was weak. The inability of TiLV to agglutinate RBCs from any of the species included suggests an absence of surface proteins able to agglutinate RBCs. A lack of an observed effect of lysosomotropic agents indicates an acidification-independent uptake mechanism, although additional studies should be carried out to understand the details of the mechanisms involved.

The observed ISAV properties are in line with observations made by Falk et al. [25], showing that ISAV only agglutinated piscine RBCs and not avian RBCs. Similarly, it was shown by the same authors that influenza virus A agglutinated both avian and piscine RBCs, which corroborated our findings.

Different orthomyxoviruses bind to different Sia receptors on host cells. In human influenza A and B viruses, the HA1 subunit binds to Sia receptors attached to either α2-3 or α2-6 linkages [19,21], avian

and equine influenza viruses bind Sia containing the α2-3 linkage [17], and swine influenza viruses bind Sia attached to the α2-3 and α2-6 linkages [37,38]. Erythrocytes from several species, such as chicken, turkey, equine, guinea pigs, and humans, possess both α2-3 and α2-6 linkages [22]. Hence, it is likely that the influenza virus strain PR8 used here binds α2-3 and α2-6 linkages on RBCs found in different host species, including avian and piscine species. Paramyxoviruses, such as the Newcastle disease virus (NDV), share the Neu5,9Ac_2 antigenic determinant with orthomyxoviruses [39]. Other viruses that use the Neu5,9Ac_2 receptor include coronaviruses. This receptor has been reported in various species, such as humans, rabbits, rats, guinea pigs, horses, chicken, turkey, goose, salmonids, crucian carp, mollusks, and sea urchins [40–50]. Unlike influenza C, ISAV specifically binds Neu4,5Ac_2 glycans on host cells using its hemagglutinin-esterase (HE) protein. This HE is unique when compared with the HA or hemagglutinin-esterase-fusion (HEF) proteins of other orthomyxoviruses, where the sequence identity is very low (<10%). The ISAV HE sequences shares sequence similarity with non-orthomyxoviruses, such as toroviruses and coronaviruses, by <25% [51–53]. It is this high specificity of the ISAV HE protein for Neu4,5Ac_2 glycans which make it different from other orthomyxoviruses, and may be what accounts for ISAV having lower tropism and mostly binding to fish RBCs, unlike other orthomyxoviruses which bind a wide range of RBCs from different avian, mammalian, and piscine species.

Given that the HA and HE proteins that bind RBCs in hemagglutination assays also serve as ligands that bind Sia receptors used for virus entry into host cells, the inability of TiLV to bind RBCs suggests that it uses mechanisms that are different from orthomyxoviruses for entry into and replication within host cells. This observation is supported by our findings regarding the NH_4Cl treatment of E-11 cells infected by TiLV. Studies into cell attachment and replication mechanisms of orthomyxoviruses showed that after HA binding to Sia receptors on host cells, the virus is taken up into endosomal compartments by endocytosis. The low endosomal pH mediates the fusion of viral envelopes with endosomal membranes to facilitate virus uncoating, leading to release of nucleoproteins required for virus replication by acid-dependent cellular proteases. The pH dependence of viral uncoating has been completely paralleled with the fusion of viral envelopes with endosomal membranes [35]. As such, raising the endosomal or lysosomal pH by adding weak lysosomotropic bases like chloroquine and NH_4Cl has been shown to prevent virus uncoating, thereby blocking virus replication [35,54]. This phenomenon is supported by several studies [55–59] including Matlin et al. [60], who showed that NH_4Cl treatment of Madin–Darby canine kidney (MDCK) cells was able to block the uncoating of the influenza virus in endosomal compartments, leading to replication failure and culminating in the prevention of infection. Other viruses where replication was shown to be inhibited by NH_4Cl treatment include alphaviruses [55], herpes simplex virus type 1 [56], reoviruses [57], and coronaviruses [58,59]. In the present study, NH_4Cl treatment neither inhibited nor enhanced TiLV replication in E-11 cells, as there was no difference in virus replication in NH_4Cl-treated or -nontreated cells.

Altogether, the data presented here show that TiLV does not agglutinate avian and piscine RBCs and its ability to replicate in NH_4Cl-treated cells suggests that it could use uptake mechanisms that are different from orthomyxoviruses. Therefore, future studies should seek to elucidate the mechanisms by which TiLV binds to host cells and its mode of entry into infected cells.

Supplementary Materials: The following are available online at http://www.mdpi.com/1999-4915/11/12/1152/s1.

Author Contributions: Conceptualization, Ø.E., S.M., and H.M.M.; formal analysis A.A.C., S.M., Ø.E., and H.M.M.; funding acquisition, S.M. and Ø.E.; investigation, A.A.C., S.M., Ø.E., and H.M.M.; methodology, A.A.C., S.M., Ø.E., and H.M.M.; project administration, S.M. and Ø.E.; resources S.M. and Ø.E.; supervision S.M, Ø.E., and H.M.M.; validation, A.A.C., S.M., Ø.E., and H.M.M.; visualization, A.A.C and H.M.M.; writing—original draft A.A.C.; writing—review and editing, A.A.C., S.M., Ø.E., and H.M.M.

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