OPTIMIZATION OF PCR-BASED ASSAY FOR DETECTION OF TILAPIA LAKE VIRUS IN TANZANIA

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A DISSERTATION SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTERS OF SCIENCE IN HEALTH OF AQUATIC ANIMAL RESOURCES OF SOKOINE UNIVERSITY OF AGRICULTURE. MOROGORO, TANZANIA.

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ABSTRACT

Tilapia Lake Virus disease (TiLVD) is an emerging disease that affects the aquaculture industry world-wide. The disease is caused by the orthomyxo-like virus with 10 segments on its genome called Tilapia Lake Virus (TiLV). The TiLVD causes massive tilapia mortalities, and hence leads to economic loss and food insecurity. The detection of TiLV genome in Lake Victoria from previous study has prompted the need for investigation of TiLV in the region. To understand the infection status of TiLV in Tanzania, the polymerase chain reaction (PCR) - based assay was optimized in the laboratory, followed by a cross-sectional study in Mwanza, Morogoro, Pwani and Iringa regions to unravel the situation of TiLV infection in different aquaculture production systems. The internal fish organs including spleens, kidney, eggs and whole fingerling/fry were analyzed by the optimized PCR-based assay targeting segment-2 of the virus and nucleotide sequencing. The findings from the field study indicated zero prevalence of TiLV genome (N = 120). When the optimized method applied to test the samples from Lake Victoria that used in previous studies, the TiLV genome was detected. Despite the fact that, TiLV genome was not detected in the examined samples, the optimized PCR-based assay and sequencing protocols for TiLV have the potential of being used in surveillance and identification of TiLV. More studies are required to further optimize the protocols and investigate the prevalence and risk factors associated with the occurrence of TiLVD and associated infections.

DECLARATION

I, Mikidadi Issanudia Mtalika, do hereby declare to the Senate of Sokoine University of Agriculture that this dissertation is my own original work done within the period of registration and that it has neither been submitted nor being concurrently submitted in any other institution.

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The above declaration is hereby confirmed by;

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DEDICATION

I dedicate this dissertation to God the almighty for his mercies, strength and grace to persevere to the end of the work. Also, I dedicate this work to my lovely wife, Amina Bitta Burilo and my three sons, Muhammad, Mahmud and Mahdi, for their love, patients and support.

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LIST OF ABBREVIATIONS AND SYMBOLS

%	Percent
µl	Microliter
bp	Base pair
cDNA	Copied deoxyribonucleic acid
FAO	Food and Agriculture Organization of the United Nations
ml	Millilitres
MLFD	Tanzania Ministry of Livestock and Fisheries Development
MORUWASA	Morogoro Urban Water Suply and Sewerage Authority
NACA	Network of Aquaculture Centres in Asia-Pacific
ng	Nonagram
OIE	World Organisation for Animal Health
^{o}C	Degrees of Celcius/Centigrade
PCR	Polymerase Chain Reaction
RNA	Ribonucleic acid
RT-PCR	Reverse transcriptase polymerase chain reaction
SUA	Sokoine University of Agriculture
TiLV	Tilapia Lake Virus
TiLVD	Tilapia Lake Virus disease
U.V	Ultra violet

CHAPTER ONE

1.0 INTRODUCTION

1.2 Background Information

Tilapia Lake Virus disease (TiLVD) is caused by the virus known as Tilapia Lake Virus (TiLV), an *orthomyxo-like* virus belonging to the family *Orthomyxoviridae* (Eyngor *et al.*, 2014). According to CGIAR (2017), the TiLV outbreaks have been reported in Colombia, Ecuador, Israel, Taiwan, Thailand, Egypt and Ghana. Also, Mugimba *et al.* (2018) reported the detection of TiLV genome by RT-PCR in farmed and wild Nile tilapia (*Oreochromis niloticus*) from Lake Victoria. At present, it has been reported in three continents (Asia, Africa and South America) and the number of countries where the agent has been detected is likely to increase rapidly as a result of increased awareness, surveillance and availability of diagnostic methods (Jansen *et al*., 2018). However, a lack of thorough investigation of all mortality incidents means that the geographic distribution of TiLV may be wider than what is currently reported. Investigations rely much on availability of effective methods for detection of virus and its genome.

According to special alert of FAO (2017), Tilapia Lake Virus (TiLV) poses a great threat to the tilapia rearing and production sectors. Tilapia are farmed globally and are the second most important aquaculture species in terms of volumes produced, providing a key source of affordable animal protein, income to fish farmers and fishers, and domestic and export earnings. Tilapines, comprising more than 100 species, are the second most import group of farmed fish worldwide after carp. Global production is estimated at 4.5 million metric tons with a current value in excess of U.S.$7.5 billion (FAO, 2017). Introduction of the virus can cause significant mortality (up to 90%) and thus result in serious economic losses to both farmers and fishers (Eyngor *et al.*, 2014; Dong *et al.*, 2017a).

In Tanzania the aquaculture production was 2700 tons in 2013 (MLFD, 2014) of which over 80% was from tilapia production. According to Ministry of Livestock and Fisheries Development (MLFD) (2014), fish production and value in the Country have indicated oscillating increased trend over the recent past years with the highest quantity being 375 534.6 metric tons of fish landed in 2005. In 2013, the contribution of the sector was estimated at 1.4 % to the national Gross Domestic Product (GDP). During the year of review (2013) fish production was 367,854 metric tons valued at TZS. 1 444 432 520/=, during the same period, the country earned over 6 117 769 193.74US $ million as foreign exchange from export of fish and fishery products.

The TiLV infect both the wild and farmed tilapias (Bacharach *et al.*, 2016; Ferguson *et al.*, 2014; Eyngor *et al.*, 2014; Mugimba *et al.*, 2018; Jansen *et al.*, 2018). Up to date, only tilapines have been shown to be susceptible to TiLV (OIE, 2017). Although it is possible that other species will be found to be susceptible. The mostly susceptible age stage that infected by TiLV are fingerlings as reported by Ferguson *et al.* (2014) and Dong *et al.* (2017a). During this study, adults, fingerlings/fry and eggs were screened for the virus.

The sensitivity for various PCR-based assays has been established so that to understand exactly at what amount of the TiLV genome, the assay can detect its presence. The lower limit of detection (LLOD) has been established by analyzing the lowest concentration of analyte (copies/µl) that would yield the virus. In current studies, Jansen *et al*. (2018); Tattiyapong *et al*. (2018) the SYBR green-based reverse transcription quantitative PCR (RT-qPCR) method targeting the TiLV genome segment 3 was developed for detection of TiLV from clinical samples with a reported sensitivity of two copies/µl. A nested RT-PCR assay using the same primer sets with detailed conditions enabling detection down to seven copies of TiLV, 10 000 times more sensitive than the single RT-PCR (limit

detection of ~70 000 copies) (Tsofack *et al*., 2017; Jansen *et al*., 2018) while semi nested RT-PCR protocol had a detection limit of 7.5 copies (Dong *et al*., 2017a) and was able to detect TiLV from clinically healthy fish (Senapin *et al.*, 2018; Jasen *et al*., 2018).

The RNA yield and purity can affect the sensitivity of the assay due to low amount of template RNA in test sample, inadequate removal of PCR inhibitors, ineffective release of viral RNA content from the host cells and poor RNA/DNA recovery after extraction and purification (Gonçalves-de-Albuquerque *et al*., 2014). Therefore, this leads to false negative results of the test conducted.

1.3 Problem Statement and Justification

The reported TiLV genome in both wild and farmed Nile Tilapia in Lake Victoria (Mugumba *et al*., 2018) as a threat to aquaculture in Tanzania was the information that justified the need for this study on TiLV. Tilapia (*O. niloticus*) from Lake Victoria are not only used directly for consumption, they also serve as source of broodstock for various tilapia seed producers in Tanzania. It is speculated that, not only the broodstock from Lake Victoria may have this challenge, but also the imported broodstock. This was further described by NACA (2017), that Tanzania is one of the 43 countries that are believed to be at high risk of acquiring TiLV, since the virus may have been introduced via direct or indirect translocation of fry/fingerlings from the countries where it has been reported. Lack of an optimized PCR diagnostic assay which is suitable and reliable method for virus and related disease in our laboratories is an important gap that calls for this study. Thus, it was not possible to know the viral distribution in other farming systems out of Lake Victoria. Therefore, this study was conducted in order to optimize and test the PCR assay that could be used to investigate the prevalence of TiLV and associated risk factors. The

main focus of the study was to generate valuable information about the status of the TiLV infection in Tanzania.

1.4 Objectives

1.4.1 Overall objective

The overall objective of the study was to optimize the PCR-based assay that could be used for detection of TiLV infection in Tanzania.

1.4.2 Specific objectives

i. To determine the optimum condition(s) for PCR and sequencing of TiLV genome.
ii. To establish the prevalence of TiLV infection in aquaculture in Tanzania.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Causative Agent

According to Ferguson *et al*. (2014) and Bacharach *et al*. (2016), TiLV is an enveloped, negative sense RNA virus which has 10 genome segments, with a diameter between 55 and 100 nm. With Segments size range from 456 to 1641 nucleotides and genome total size is 10.323 kb. All segments have conserved, complementary sequences at 5′ and 3′ termini. The genome organization and ultrastructural morphology of TiLV resemble other Orthomyxoviruses (Del-Pozo *et al.*, 2017; Eyngor *et al*., 2014). All 10 segments contain an open reading frame (ORF), with the largest segment, segment 1, containing an open reading frame with weak sequence homology to the influenza C virus PB1 subunit (Bacharach *et al.*, 2016). The remaining segments show no homology to other known viruses (Eyngor *et al*., 2014; Bacharach *et al*., 2016). According to Eyngor *et al.* (2014), the viral particles have been found to be sensitive to organic solvents (ether and chloroform), due to their lipid membrane. The virus causes massive deaths on Tilapiine spp from both wild and farmed environment.

2.2 Modes of Transmission

According to OIE (2017a), the co-habitation studies have demonstrated that direct horizontal transmission is an important route of transmission. There is no evidence of vertical transmission. The biophysical characteristics of the virus are not well characterised so it is difficult to determine the significance of indirect transmission by fomites. Also added that, the infected populations of fish both farmed and wild, are the only established reservoirs of infection with unknown original source of TiLV.

2.3 Host Range

Tilapia are the only susceptible species up to date, with mortalities attributed to TiLV been observed in wild tilapia *Sarotherodon (Tilapia) galilaeus*, farmed tilapia *Oreochromis niloticus* and commercial hybrid tilapia (*O. niloticus* X *O. aureus*) (Bacharach *et al.*, 2016; Ferguson *et al.*, 2014; Eyngor *et al.*, 2014).

The affected group of fish reported by Ferguson *et al.* (2014) and Dong *et al.* (2017a) were mainly fingerlings. Dong *et al.* (2017a) reported that, approximately 90% mortality in red tilapia fingerlings within one month of stocking into cages was observed. In medium to large sized Nile tilapia with mortality rate of about 9% (Fathi *et al.*, 2017). Other reports have not commented on different levels of mortality by life stage (Eyngor *et al.*, 2014).

2.4 Clinical Signs and Case Description

The main organs where pathology was observed were the eyes, brain and liver and the hosts manifested lethargy, ocular alterations, skin erosions and discoloration (darkening) (Eyngor *et al.*, 2014). Exophthalmia, discoloration (darkening), abdominal distension, scale protrusion and gill pallor were reported in another study (Ferguson *et al.*, 2014). Loss of appetite, lethargy, abnormal behavior (e.g. swimming at the surface), pallor, anemia, exophthalmia, abdominal swelling, and skin congestion and erosion were also reported by Dong *et al.* (2017a) and Surachetpong *et al.* (2017). Summer mortalities have also been associated with TiLV with affected fish showing haemorrhagic patches, detached scales, open wounds, dark discoloration and fin rot (OIE, 2017).

In subclinical situation, it has been found by Mugimba and his colleagues (2018) that there is a significant difference ($p < .0028$) in tissue distribution among organs examined. The

PCR products were detected in 10.99% (N = 191) spleen, 7.69% (N = 65) head kidney, 3.45% (N = 29) heart and liver 0.71% (N = 140) samples while no PCR products were detected in brain samples (0.0%, N = 17). This implies that, the lymphoid organs, mainly comprising of the head kidney and spleen, are the most susceptible.

The overall population prevalence of 14.66% (N=191) has been established in Lake Victoria tilapia (Mugimba *et al*., 2018) while in Egypt it was 37% of farms infected in 2015 (Fathi *et al*., 2017). In Thailand during the emergence of the disease, three farms had 100% prevalence (N=27) from tested fingerlings (Dong *et al*., 2017a).

2.5 Diagnostic Methods

High levels of mortality in tilapine species, associated with ocular alterations (opacity of the lens or more severe pathology), should be considered suspicious of TiLV. Skin erosions, haemorrhages in the leptomeninges and moderate congestion of the spleen and kidney may be observed on post-mortem (OIE, 2017).

TiLV can be cultured in primary tilapia brain cells or in an E-11 cell line, inducing a cytopathic effect at 5-10 days (Eyngor *et al.*, 2014). Tsofack *et al.* (2016) describe optimal conditions for culturing TiLV.

A PCR primer set has been designed and a reverse transcriptase (RT) PCR has been developed (Eyngor *et al.*, 2014). However, the test was not fully validated. A more highly sensitive, nested RT-PCR has been developed and optimized, suitable for detection of TiLV in clinical cases (Tsofack *et al.*, 2016). Most recently a semi-nest RT-PCR with improved detection sensitivity (7.5 viral copies per reaction) over the nested RT-PCR has been published (Dong *et al.*, 2017a).

However, in subclinical studies performed by Mugimba *et al*. (2018), the PCR method was optimized by testing all ten genome segments to their respective pair of primers. The segment-2 was the only segment detected in positive controls with no bands in negative controls. Therefore, the PCR assay with the use of primer pair targeting segment-2 of the viral genome was finally used in screening samples from Lake Victoria. Also Senapin *et al.* (2018) have detected the segment 3 of TiLV genome in inapparent infection cases of virus in farmed tilapia with the use of semi-nested PCR. Therefore, various molecular based diagnostic assays have been developed, optimized and validated as the diagnostic method for virus and associated disease.

2.6 Control Methods

According to OIE (2017a), restrictions on the movement of live tilapines from farms and fisheries where the virus is known to occur limit the spread of the disease. Generic biosecurity measures to minimize fomite spread via equipment, vehicles or staff (i.e. cleaning and disinfection) should also be implemented. Jansen *et al.* (2018) suggested that, in order to limit the negative impact and to prevent further spread of the virus, combined approaches are required. These include National- and international biosecurity efforts, effective BMPs, capacity building and widespread collaboration between international and national stakeholders must be prioritized.

Currently, there are no published methods shown to be effective in limiting the impact of an outbreak on an infected farm. It has been suggested that breeding for resistance or the development of a vaccine may offer the long-term prospects for managing the disease (Ferguson *et al.*, 2014). A breeding program would need to select and test a range of different strains of tilapia with a view to finding those least susceptible.

CHAPTER THREE

3.0 MATERIALS AND METHODS

The study involved both lab and field works. The lab works conducted were optimization of the assay and screening of the samples collected from the field while field work was mainly sample collection in Mwanza, Morogoro, Pwani and Iringa regions.

3.1 Optimization of the Polymerase Chain Reaction (PCR) Assay

One primer pair (Table 1) from Macrogen Inc. (Korea) targeting segment-2 of the TiLV genome was used. This pair of primer was selected based on its specificity and ability to only detect viral cDNA and not host DNA (Mugimba *et al*., 2018). The primers were used with one positive control and one negative control to optimize the PCR. The positive control was cDNA synthesized from TiLV positive samples obtained from Lake Victoria and prepared at Norwegian University of Life Sciences (NMBU) in Oslo, Norway (Mugimba *et al*., 2018), whereas negative control was the sterile nuclease-free water. The two PCR kits; QIAGEN® OneStep RT-PCR Kit (Qiagen, Hilden, Germany) and Taq DNA Polymerase with ThermoPol Buffer (M0267) (New England Biolabs, USA) were used in the study.

Table 1: Summary of primers for PCR of TiLV

Primer name	Sequence (5’ to 3’)	Length (bp)	Reference
Forward primer (FP) Reverse primer (RP)	GTCCAGGGCGGTATGTATTG CTTACGGCTGACAAGTCTCTAAG	834	Mugimba *et al*. (2018)

The first PCR was optimized by using QIAGEN® OneStep RT-PCR Kit. But the stage for cDNA synthesis was skipped. The PCR was done at 25µl reaction volume with the use of GeneAmp® PCR system 9700 Thermocycler (Applied Biosystems, Foster City, USA), at the following conditions: the first stage involved one cycle of initial denaturation at 95^{o}C for 15 minutes, followed by 35 cycles of the second stage which had denaturation at 95^{o}C for 1 minute. Various annealing temperatures were tested; 56^{o}C, 57^{o}C, 58^{o}C, 59^{o}C and 60^{o}C for 1 minute, extension at 72^{o}C for 2 minutes and completed the third stage at final elongation at 72^{o}C for 5 minutes (Appendix 6).

The second PCR assay employed the use of Taq DNA Polymerase kit with ThermoPol Buffer (M0267), the optimization was done with Initial Denaturation at 95°C for 30 seconds, followed by 30 Cycles involving 95°C for 30 seconds denaturation, 59°C for 1 minute of annealing and 1 minute of extension at 68°C. Final Extension was at 68°C for 5 minutes and Hold at 4°C (Appendix 5). For this kit, 50µl volume was used with some variation of template volume from 0.5µl to 1µl (Appendix 2). Then, the PCR products amplified were separated by 1.5% Agarose gel electrophoresis. The clear bands were observed with 834 bp size (Fig. 3) similar to that published by Mugimba *et al*. (2018).

These protocols were repeated three times each to ensure their consistence and efficiency. Thereafter, the assay was used to detect the TiLV genetic material from the aliquot of previously confirmed positive samples from Lake Victoria used by Mugimba and his colleagues (2018). The pool of those samples was tested and gave the positive results (Fig. 3).

Thereafter, the post PCR product of detected TiLV samples was purified and sequenced by capillary electrophoresis technique using the AB3500 genetic analyzer (HITACHI

High-Technlogies Corporation, Tokyo-Japan). The sequence data were analyzed using BioEdit software (http://www.mbio.ncsu.edu/bioedit/bioedit.html) and compared with similar nucleotide sequences available in GenBank using the Basic Local Alignment Search Tool (BLASTN) (http://blast.ncbi.nlm.nih.gov). The summaries for protocols for cycle sequencing reaction, post-PCR product purification and Sanger sequencing used in this study are shown in Appendix 4, Appendix 7 and Appendix 8, respectively.

3.2 Study Design, Sample Collection and Study Areas

After optimization of the protocol, the cross-sectional study was conducted in order to establish the prevalence using the assay optimized for detection of TiLV. The study involved apparently health fish with no signs of the disease from non-outbreak areas. The samples were collected from three different zones and transported into two different media types. From the randomly selected non-diseased sampled fish, the spleens, kidneys, livers, brain and eggs from mature females were collected.

In Southern highlands of Tanzania, twenty (20) adult fish samples were collected in Kilolo district found in Iringa region. These samples were collected in October 2017 from grow-out farms that also locally produced seeds among themselves. At special prepared site in the field, the fish were dissected aseptically and tissues of brain, kidney, liver and spleen were collected. These tissues were collected in pool in 15ml tubes of 50% glycerol and kept at -20^{o}C before being transferred within 48 hours to the College of Veterinary Medicine and Biomedical sciences, at Sokoine University of Agriculture (SUA), Morogoro; where they were stored at-80^{o}C until processing.

Other group of fish samples was collected in January and February 2018. These samples were collected from Lake Victoria basin and Coastal area of Tanzania. The targeted

groups of farms on these regions were Nile tilapia seeds producers, both with and without hatcheries. A total of ten (10) farms were sampled of which 60% of them were from Lake Victoria basin; Nyamagana (50%) and Misungwi (10%) districts of Mwanza region. The other 40% of the remaining fish samples were collected from Pwani region (Kibaha and Kisarawe 10% each) and Morogoro region (Morogoro Town and Kilosa districts 10% each) in Coastal zone of Tanzania. A total of 57 adult fish and 110 fingerlings/fry were sampled.

About eighty three percent (83.3%) of all farms sampled in Lake Zone used water from Lake Victoria while 16.7% of farms used underground deep well. In Kilosa, Wami River was the source of water; MORUWASA was the supplier in Morogoro town while Ruvu river in Kibaha, deep-drilled well in Kisarawe and small streams from catchment areas in Kilolo were the main source of water in sampled areas.

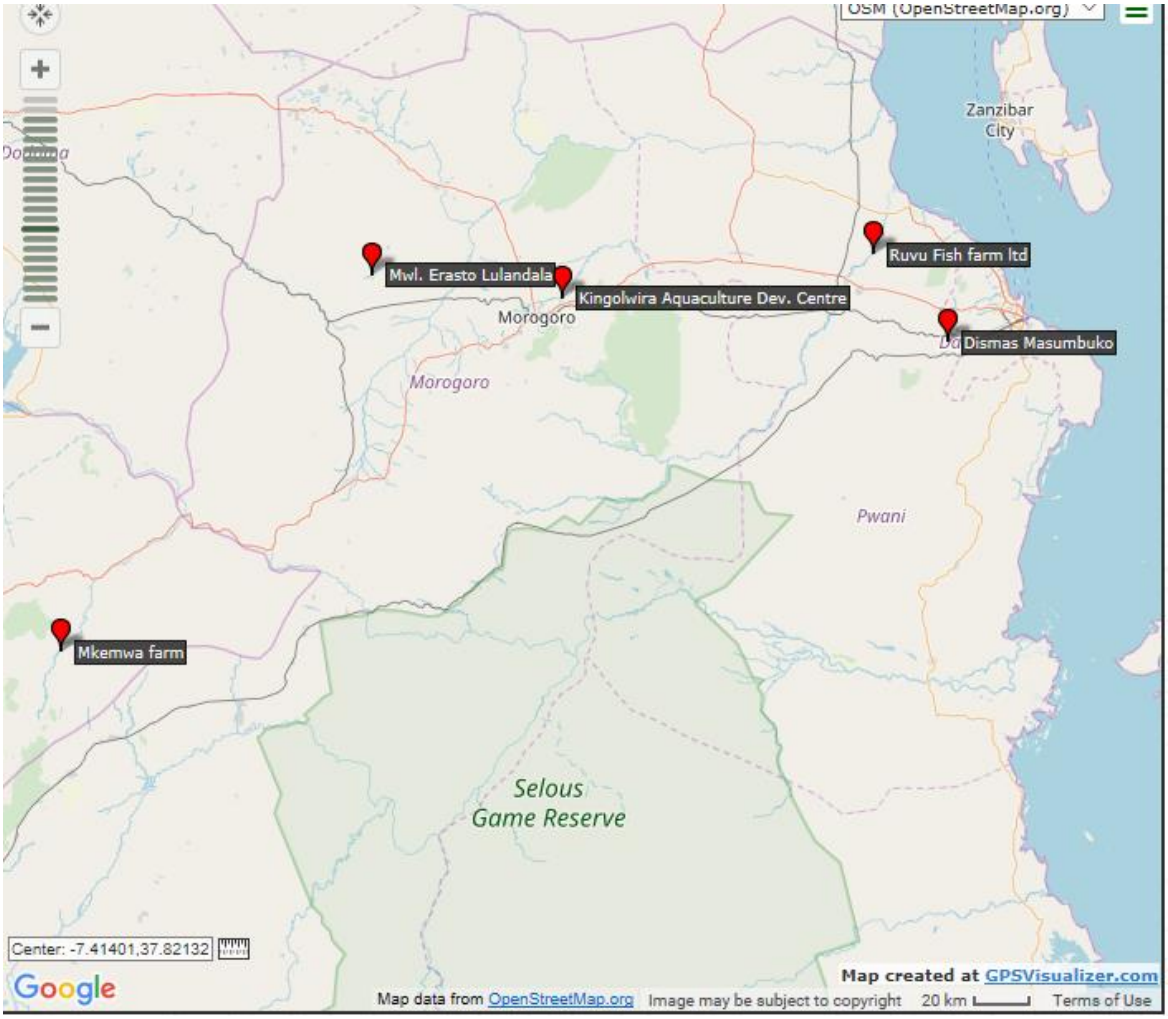

Figure 1: The map showing the distribution of the sampled farms in Morogoro, Pwani and Iringa regions during the study

On simple dissecting table prepared on-site, the adult fish were dissected aseptically with the use of Sodium Hypochlorite as disinfectant. The tissues of brain, liver, kidney, spleen and eggs from mature breeding females were collected in cryo-vial tubes with RNAlater and kept in cool box with ice bags and stored at -20^{o}C within 5 hours. The samples were stored at -20^{o}C for 3 days before transportation and further storage at -80^{o}C at the College of Veterinary Medicine and Biomedical Sciences, at SUA, Morogoro.

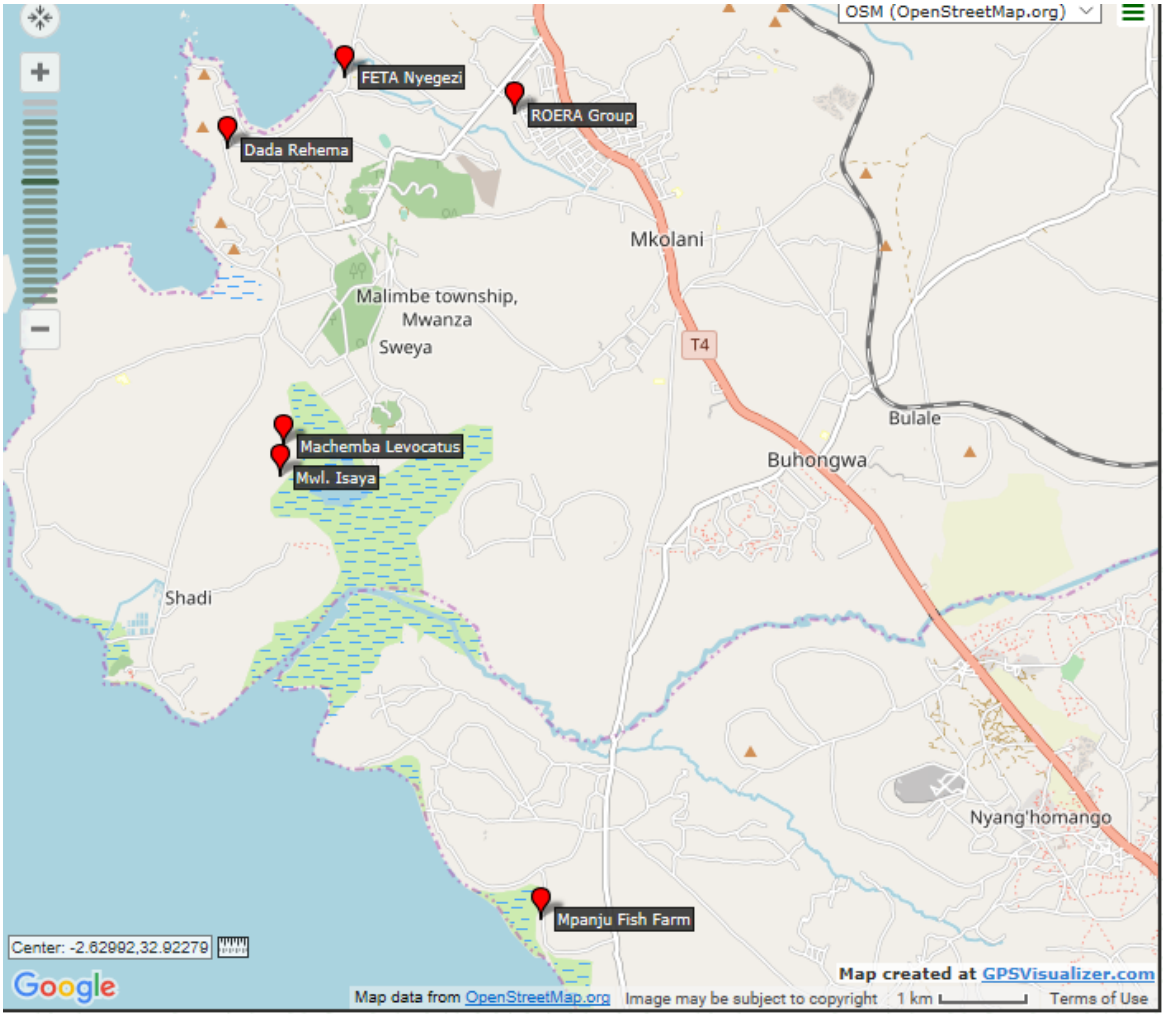

Figure 2: The distribution of sampling sites in Mwanza region

3.3 RNA Extraction and cDNA Synthesis

The extraction of total RNA from 120 samples (Table 1) was carried out using RNeasy® Mini Kit (Qiagen, Hilden, Germany) techniques as previously described by Mugimba *et al*. (2018). The Qiagen extraction protocol (Appendix 1) was used based on manufacturer's guidelines (Qiagen, Hilden, Germany). Thereafter, the RNA quantification was done by nanodrop spectrophotometer (NonaVue Plus®, BIOCHROM LTD, Cambridge, England). The actual concentrations of total RNA and 260/280 ratios for the samples has been attached in Appendix 9. Then, the total RNA extracted was stored at -80^{o}C until further steps.

Table 2: Source and type of samples used during this study

District	Total samples collected	Spleens	Eggs	Fingerlings/fry	Kidney
Nyamagana	42	25	4	12	1
Misungwi	11	5	2	4	
Morogoro	13	6	3	4	
Kilosa	11	10		1	
Kibaha	12	6	4	2	
Kisarawe	11	5	5	1	
Kilolo	20	20			
Total	120	77	18	24	1

The synthesis of cDNA was carried out in 20 µl reaction volumes using the Thermo Scientific RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific Inc., USA). Since there was no sample normalization as well as the RNA integrity checking before cDNA synthesis, the positive control of the kit was used to ensure the effectiveness of the kit on synthesizing the cDNA. Briefly, the reaction mixture included nuclease-free water (9µl), RevertAid RT (1µl), Ribolock RNase Inhibitor (1µl), 5X Reaction Buffer (4µl), dNTP (2µl), Random Hexamer (1µl) and 2µl of total sample RNA. This volume was incubated at 25^{o}C for 5 minutes, followed by 60 minutes at 42^{o}C then terminated the reaction by heating at 70^{o}C for 5 minutes. It was done by using GeneAmp® PCR system 9700 Thermocycler (Applied Biosystems, Foster City, USA).

3.4 Screening of Nile Tilapia Samples

After the process of PCR optimization being completed, the primer pair targeting the segment-2 of the TiLV genome was used to screen Nile tilapia samples from the field for

the presence of TiLV. The total of 120 tissue samples including 77 spleens, 24 fish (a pool of 110 fingerlings/fry), 18 eggs (from mature breeding females) and 1 kidney were screened (Mugimba *et al*., 2018).

The use of fingerlings/fry samples and eggs was to find out and diversify the prevalence in all developmental stages from eggs to adult. This could be used as the chance of finding if there is any vertical transmission among the fish, as well as to estimate the chance of viral spread from the fingerling/fry producers to the grow-out farmers.

The amplification was done by GeneAmp® PCR system 9700 Thermal cycler (Applied Biosystems, Foster City, USA), then the PCR products were separated using 1.5% agarose gel electrophoresis. Thereafter, the gel was evaluated on UV light for documentation.

3.5 Data Analysis

The Microsoft Excel 2010 was used to perform calculations, analyze information and visualize the data and results obtained in the study into spreadsheets.

CHAPTER FOUR

4.0 RESULTS

4.1 Polymerase Chain Reaction Optimization

The PCR products obtained at annealing temperature of 59^{o}C had a strong band in the positive control sample without band on the negative control. This was observed for all kits used. In testing the assay, the bands observed in both positive control and previously diagnosed TiLV positive sample at exactly expected band size (834bp) while the negative control showed no band (Fig. 3). During sample screening, the tested fish samples had no bands (Fig. 6 and Fig. 7) indicating the TiLV negative results. The obtained bands were observed at about 834bp size as that described by Mugimba *et al*. (2018).

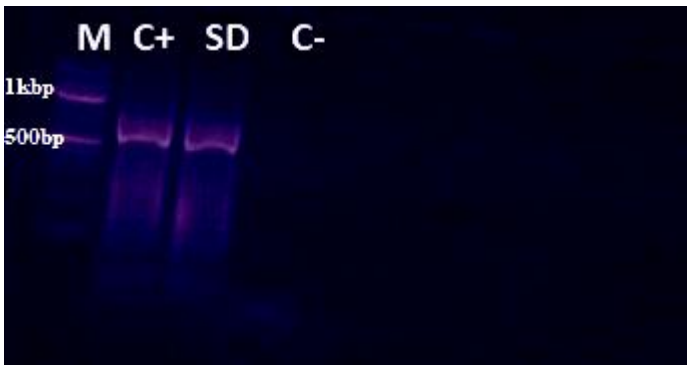

Figure 3: The gel electrophoresis showing the bands of PCR results of positive control (C+) and a positive diagnosed sample (SD) used in the study of Mugimba *et al.* (2018). This indicates the ability of the optimized protocol to detect TiLV in fish samples

4.2 DNA Sequencing and BLAST Search for Previous Positive Sample

The obtained sequence data for the previously diagnosed TiLV positive sample (Fig. 4 and Fig. 5) were analyzed using BioEdit software (http://www.mbio.ncsu.edu/bioedit/

bioedit.html) and compared with similar nucleotide sequences available in GenBank using the Basic Local Alignment Search Tool (BLASTN) (http://blast.ncbi.nlm.nih.gov). With the nucleotide sequence generated from the confirmed positive sample, the BLAST gave 100% query coverage and 99% nucleotide identity to the *Tilapia Lake Virus TZ2015-03 segment-2* that recovered in Lake Victoria and published (GeneBank accession no. MF526980.1). It indicated that, the optimized PCR protocol, primer used and the positive control were effective and capable of screening the TiLV.

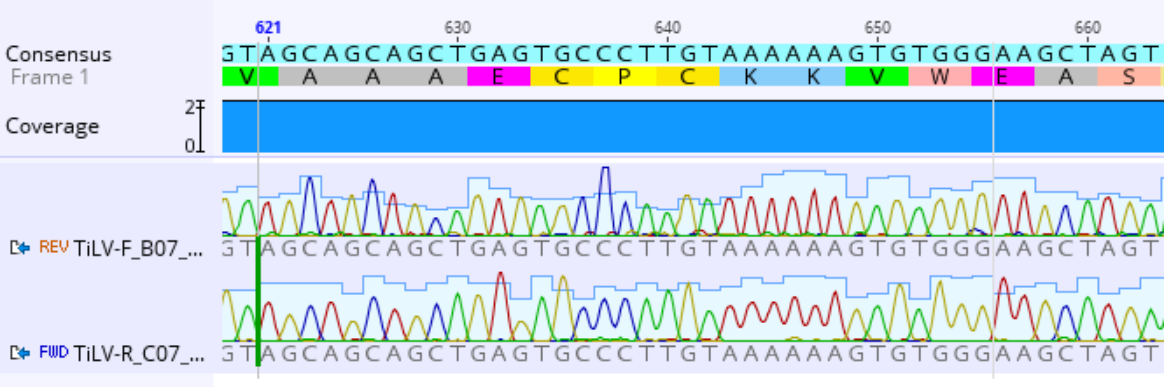

Figure 4: The sequence trace chromatogram showing peaks corresponding to individual nucleotides

4.3 Screening Nile Tilapia Samples

After extraction of total RNA, the quantification was done and revealed the average concentration of total RNA for 120 samples was 188.86ng/µl while for tissue specific; the spleens had 246.03ng/µl, for fingerlings/fry were 113.84ng/µl and 34.37ng/µl for eggs. The samples used for validation had 160 and 71.2ng/µl respectively. The RNA extracted were evaluated for their purity by examining the 260/280 and 260/230 ratios of each sample. Seventy one were pure at 260/280 ratio while forty four were pure at 260/230 ratio out of 115 measured samples (Appendix 9).

All screened samples from the three zones of Tanzania including Southern Highlands, Lake Zone and Coastal zone, were diagnosed TiLV negative and there was no evidence for being infected with TiLV.

GACAAGTCTCTAAGGAAGTCATTCTGCTTTAGGAACGCCCTAAACCAGTTCTTGGAT
AAAGATTTGCCTCTTTTGCCCATTCGGCCAAAATTAGAGTCCAGGGTTGCTGTGAAA
AAGTCTAAGCTGAGGAGTCAGCTGTCGTTCAGACCCGGTTTGACTCAGGAGGAAGC
AATTGATCTTTACAACAAGGGCTATGATGGTGACAGCGTCTCAGGTGCCTTACAAGA
CAGGGTAGTCAATGAGCCTGTAGCTTACTCGAGTGCAGATAATGACAAATTTCACAG
GGGCTTGGCGGCTCTAGGGTACACTTTGGCTGATAGAGCATTTGATACGTGCGAATC
CGGCTTCGTGAGAGCAATTCCTACTACTCCATGTGGGTTCATATGTTGTGGGCCAGG
TTCTTTCAAAGATTCACTTGGATTTGTGATAAAAATCGGCGAATTCTGGCACATGTA
TGACGGGTTCCAACACTTCGTCGCTGTCGAAGATGCTAAGTTCTTAGCAAGTAAGTC
TCCTTCGTTTTGGTTGGCAAAACGTCTTGCAAAGAGGCTGAATCTGGTCCCAAAAGA
GGATCCATCTGTAGCAGCAGCTGAGTGCCCTTGTAAAAAAGTGTGGGAAGCTAGTTT
TGCTAGGGCACCTACTGCACTAGATCCATTTGGAGGCAGGGCCTTCTGCGACCAGGG
TTGGGTGTACCACAGGGACGTAGGGTATGCAACAGCTAACCACATATCACAGGAGA
CACTTTTTCAACAGGCGCTTTCAGTGAGGAACCTTGGACCGCAAGGTAGTGCAAATG
TCTCAGGCTCAATACATACCGCCCCTGGACAA

Figure 5: The sequence data shown on the sequence results for previously diagnosed TiLV positive sample. The nucleotide sequences were assembled using Geneious bioinformatics software after editing.

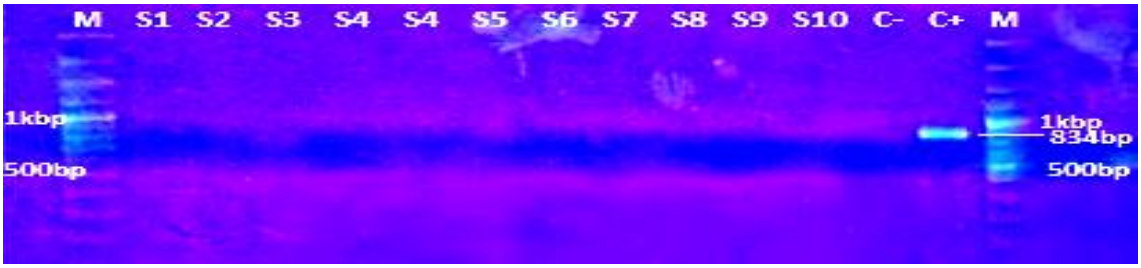

Figure 6: The image shows the PCR results on gel electrophoresis of tested samples using the optimized QIAGEN® One-Step RT-PCR. The samples (S1-S10)

and negative control (C-) has no bands while the positive control (C+) has a clear band of 834 bp in size.

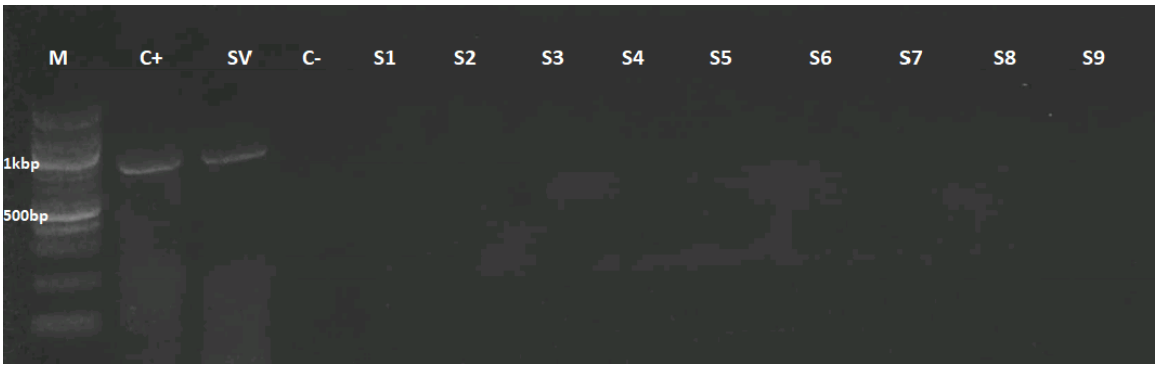

Figure 7: The results of Taq DNA Polymerase with ThermoPol® Buffer (M0267) on gel electrophoresis, showing bands on positive control (C+) and previously diagnosed positive sample (SV) while no bands on negative control (C-) and tested samples (S1-S9). The band of C+ is 834 bp in size and slightly located below to that of SV band.

CHAPTER FIVE

5.0 DISCUSSION

The protocols for PCR-based assays for both QIAGEN® OneStep RT-PCR and Taq DNA Polymerase with ThermoPol® Buffer (M0267) kits were optimized and established. Between the two PCR kits, the use of Taq DNA Polymerase with ThermoPol® Buffer (M0267) was found to be better and could be used for detection of TiLV genome during both active and passive surveillance of TiLVD. With this kit, the two steps for PCR are done separately start with cDNA synthesis from RNA template followed by their amplification as compared to QIAGEN® OneStep RT-PCR. This is because the cDNA synthesized by using Thermo Scientific RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific Inc., USA) used as the template in Taq DNA Polymerase with ThermoPol® Buffer (M0267) while the QIAGEN® OneStep RT-PCR normally combines both steps starting with synthesis of cDNA from RNA template. Therefore, the QIAGEN® OneStep RT-PCR has the extra enzyme for RNA preparation and cDNA synthesis that increases its cost too, compared to Taq DNA Polymerase kit.

Also, the reaction conditions for routine PCR between the two kits are different in terms of temperature and time duration (Appendix 5 and Appendix 6). The Taq DNA Polymerase with ThermoPol® Buffer (M0267) PCR has shorter total time of about 85 minutes compared to 164 minutes taken by QIAGEN® OneStep RT-PCR. Despite these variations, both protocols gave similar results. But the Taq DNA Polymerase with ThermoPol® Buffer (M0267) PCR has high efficiency in terms of its relatively low cost for purchase and short time taken in generation of data.

On the validation of the assay, the sensitivity, specificity, false negative rate and false positive rate are the key elements of accuracy that should be addressed (AMP, 2009). In

this study, the sensitivity and specificity were considered in order to avoid the false negative results. The sensitivity is the ability of a test to detect a mutation or disease when that mutation/disease is present (AMP, 2009) and the sensitivity can be affected by RNA/DNA yield and its purity. It also refers to the lower limit of detection for the analyte of interest (i.e., the lowest concentration of analyte that the assay can detect) (AMP, 2009). For instance, from published papers (Mugimba *et al.*, 2018) it was assumed that a sensitivity of less than 100 copies per reaction would be needed to obtain a positive result. The sensitivity in terms of copies per reaction was not established; instead the optimized assay was used to detect the TiLV genome in the aliquot of samples diagnosed TiLV positive. Further studies are required to establish the sensitivity and specificity of the assay so that it could be used for routine surveillance of TiLVD in the region.

During the study, the samples screened were sampled, preserved and processed based on protocol in order to ensure the high quality and avoiding possible losses of the minimal amounts of the targeted viral genome. This was followed by RNA quantification that gave the status of the RNA yield and purity. The RNA yield were expressed into Total RNA concentration in ng/µl basis and it was found that the spleens had average of 246.03, fingerlings/fry 113.84 and eggs 34.37 while the validating samples had 160 and 71.2ng/µl respectively. This indicated that the assay was capable of picking up the RNA during extraction. Normally, the concentration of RNA can be influenced by starting amount of tissues, homogenization, amount loaded in the purification columns, type of tissue, etc. Furthermore, the purity of the RNA were analyzed in 260/280 and 260/230 ratios. This is done since nucleotides, RNA, ssDNA, and dsDNA all absorb at 260 nm, and therefore they will contribute to the total absorbance of the sample. To ensure accurate results when using a Nanodrop Spectrophotometer, nucleic acid samples will require purification prior

to measurement that in this procedure has been done during extraction with the use of RNAeasy technology for RNA purification. Purification was also done on post-PCR products to purify the DNA materials (Appendix 7). Normally, the 260/280 ratio used to assess the DNA and RNA purity while the 260/230 ratio used as the secondary measure of purity (Sandy, 2013). The accepted ratios are of 1.8-2.0 and 2.0 - 2.2 for 260/280 and 260/230 respectively. These are considered as the pure. During the study, the pure samples were 71 out of 115 measured 260/280 ratio while for the 260/230 ratio, 44 out of 115 samples were pure. Both pure and non-pure-considered samples were taken for analysis and provide negative results. Also the two previously diagnosed TiLV positive samples were pure and non-pure respectively but provided a positive result for the TiLV genome.

According to AMP (2009), the specificity of the assay is the ability of that test to give a normal (negative) result in specimens without the mutation or disease being tested. It is also referred to the ability of a test to detect the analyte without cross-reacting with other substances or genetically or biologically similar microbes. The ability of the procedure to distinguish the target sequence(s), allele, or mutations(s), from other sequences/alleles/ mutations in the genome is also termed as the specificity; and this was analyzed by sequencing the PCR products of the validation sample. The results from the sequence with 100% query cover and 99% genome identity indicated the specificity of the protocol to identify the TiLV genome was 99%.

The variation of different annealing temperatures in the assay optimization was used to establish the specificity of the PCR assay. This is because the specificity of the PCR assay is also controlled by the length of the primer and the annealing temperature of the PCR reaction (Dieffenbach *et al*., 1993). In the current study, the primers used had length of 20

and 23 bases for forward and reverse primers respectively. Also the annealing temperature variations of 56^{o}C, 57^{o}C, 58^{o}C, 59^{o}C and 60^{o}C were used for optimization process. This was performed and the findings lied on previous studies observations, including that of Dieffenbach *et al*. (1993) which revealed that oligonucleotides between 18 and 24 bases tend to be very sequence specific if the annealing temperature of the PCR reaction is set within a few degrees of the primer dissociation temperature. They added that, in order to optimize PCR, the utilization of primers of a minimal length that ensures melting temperatures of 54^{o}C or higher will provide the best chance for maintenance of specificity and efficiency. This has been proved also by Phillips (2018), who stated that the best primers have 18 to 24 bases and melting temperatures (Tm) about 5-10 degrees Celcius below the annealing temperature, which is usually between 55 and 65 degrees Celcius. Therefore, the annealing temperature of 59^{o}C that optimize the PCR assay was in agreement with the length of the primers used in assay to improve its specificity.

In order to ensure the results obtained were not false negative, the previously confirmed TiLV positive samples were used. The samples were processed starting with RNA extraction up to sequencing. This was done as a measure of assessing the assay by testing against the positive proved samples by 'standard gold' assay in non-outbreak area used by Mugimba *et al*. (2018).

After optimizing and using the optimized assay to screen the collected samples from the field, the prevalence of the TiLV infection was established. Since the TiLV genome was not detected in any of the screened samples, from all sample areas included. Hence, the prevalence of TiLV in the aquaculture operations included was estimated to 0.0% (n=120). The negative results could be influenced by absence the TiLV infection that contributes to missing of the viral genome and also the unknown sensitivity of the method used.

On the other hand, the main sources of TiLV infection in the farms are the infected incoming biological materials specifically fry and water used for fish rearing if comes from contaminated source (OIE, 2017). The missing of TiLV genome indicates that the randomly selected farms in this study did not import/use the broodstock fish that were infected and also the used water sources were TiLV free.

On water sources, the deep-drilled well is the main safe water source that greatly considered in aquaculture biosecurity, but still other sources used in these farms seem to be safe. Precautions and frequent screening should be done on farms along the rivers and lakes where the risk for the microbial transmission and other aquatic contaminants is high. Therefore, the screened farms were free against TiLV infection when the study was conducted.

The prevalence of 0.0% of TiLV infection in current study, has both social and economic implication, provided that the presence of TiLV in aquaculture could compromise the aquaculture sector, socially and economically by threatening the food security and market for both local & international trade. By missing the TiLV in aquaculture seeds producers, it ensures the safety of grow-out fish farms collecting the seeds from those producers.

This optimization provides the initial information that could lead to future investigation of TiLV in the region. The protocol established with additional improvement on its sensitivity and specificity, will give our laboratory the capacity and capability to test and diagnose the TiLV at any time when needed, and hence improved the efficiency of the lab.

In future studies other PCR assays especially the semi-nested RT-PCR targeting t segment three of the TiLV should be adopted, optimized and validated too, based on our

environment. This is because the protocol has shown the potential of also detecting the TiLV infection in healthy fish (both adult and fingerlings) from commercial farms in Thailand (Senapin *et al*., 2018). Also, further studies involving histopathological investigation of the liver for signs of syncytial hepatitis of tilapia (SHT) should be considered. This will enhance the capacity of our laboratory to detect and characterize the TiLV infection with variable PCR and DNA sequencing assays, respectively.

CHAPTER SIX

6.0 CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

In the current study, the prevalence of TiLV infection was 0.0%. Subsequently, the PCR assay and partial sequencing protocols for TiLV segment-2 genome were optimized and used for screening the TiLV infection in farmed tilapia in Tanzania.

6.2 Recommandations

(i) Further surveillance studies on TiLV infection and spread should be conducted. It should include all potential hatcheries and farms in Tanzania producing the fish seeds; and the TiLV free screened stock should be reserved for production of seeds, followed by strictly regulations on supply of fry and fingerlings.

(ii) Epidemiological studies to determine risk factors associated with the occurrence of TiLVD in Tanzania and the region should be conducted so that the appropriate disease control measures can be recommended.

(iii) Aquatic Animal Health (AAH) Management and Transboundary Movement of Live Aquatic Animals and Aquatic animal products control measures should be observed and practiced from national level to farm level so as to improve the biosecurity in aquatic production chain.

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APPENDICES

Appendix 1: Procedure for RNA extraction using RNeasy Mini kit

- Mix the sample with equal volume of Lysis buffer RLT (containing 1% β-Mercaptoethanol) (460µl of virus sample in 460µl lysis buffer).
- Add 460µl of 70% ethanol to tube.
- Mix using a vortex mixer.
- Apply to RNeasy spin column (700µl maximum loading volume).
- Spin in a microfuge for 10-15 seconds at 10,000-12,000rpm (7,000-10,000xg).
- Discard flow-through and reuse collection tube.
- Repeat with remaining volume.
- Wash with 700µl wash buffer RW1. Centrifuge for 10-15 seconds at 10,000-12,000rpm (7,000-10,000xg). Discard flow-through.
- Wash with 500µl wash buffer RPE. Centrifuge for 10-15 seconds at 10,000-12,000rpm (7,000-10,000xg).
- Discard flow-through and reuse collection tube.
- Repeat wash with 500µl wash buffer RPE.
- Centrifuge at maximum speed for 2 minutes to dry membrane.
- Discard the flow-through and collection tube.
- Transfer column to a new collection tube and centrifuge at maximum speed for 1 minute to remove any traces of ethanol.
- Elute RNA with 50µl nuclease-free water into a new 1.5ml tube.
- Spin a microfuge for 60 seconds at 10,000-12,000rpm (7,000-10,000xg).
- Store in clean tube in freezer between -30^{o}C and -5^{o}C or -50^{o}C and -90^{o}C. Label the tube clearly with “RNA”, the virus name/passage history, the date and your name (initials).

Appendix 2: PCR reaction for Taq DNA polymerase with Thermopol® Buffer (M0267)

Component	Volume/Reaction (µl)
10X ThermoPol Reaction Buffer	5
10 mM dNTPs	1
10 µM Forward Primer	1
10 µM Reverse Primer	1
Template DNA	1
Taq DNA Polymerase	0.25
Nuclease-free water	40.75
Total reaction volume	50

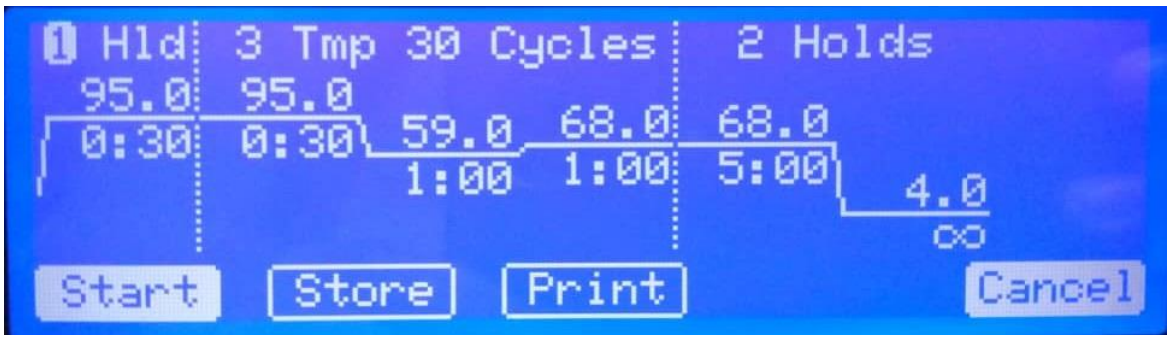
Appendix 3: PCR reaction for RT-PCR

Component	Volume/reaction (µl)
QIAGEN OneStep RT-PCR Buffer, 5X	5
dNTP mix (10 mM each)	1
Forward primer	1
Reverse primer	1
QIAGEN OneStep RT-PCR Enzyme mix	1
Template cDNA	2
RNAse-free water	14
Total reaction volume	25

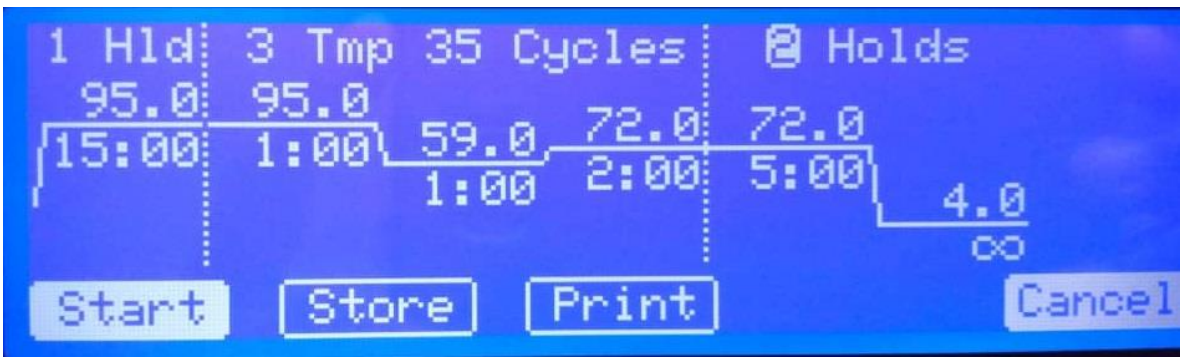
Appendix 4: Cycle sequencing reaction

Component	Volume/reaction (µl)
Water	3.5
Buffer	2
Big dye	0.5
Primer (added separately)	3
DNA	1
Total reaction volume	10

Appendix 5: The reaction conditions for PCR using Taq DNA polymerase with Thermopol® Buffer (M0267) kit



Appendix 6: The reaction conditions for PCR using QIAGEN® OneStep RT-PCR kit



Appendix 7: The purification protocol that used during purification of the post-PCR products

Method / Protocol Title:
GFX PCR product purification

Authors: Jemma Wadsworth (jemma.wadsworth@pirbright.ac.uk)
Nick Knowles (nick.knowles@pirbright.ac.uk)

1. Introduction

This protocol is designed to purify single- or double-stranded DNA fragments from PCR and other enzymatic reactions. Fragments ranging from 100 bp to 10 kb are purified from primers, nucleotides, polymerases, and salts using GFX spin columns in a micro centrifuge.

2. Materials

a. Equipment

Appropriate PPE (Lab coat, disposable gloves)
Sterile tips for single channel pipettes-suitable volume
Calibrated pipettes
Marker pens waterproof
Sterile plastic ware-a selection of suitable volume eppendorfs or screw top tubes
Bench top micro centrifuge

b. Reagents

Illustra GFX PCR purification kit	VWR International
Ethanol	Any manufacturer

3. Procedure

Note 1: Add ethanol (100%) to Wash Buffer Type1 before use (see bottle label for volume).

Note 2: All centrifuge steps are at 13,000 rpm (~17,900 x g) in a conventional bench-top micro centrifuge.

3.1 Add 5 volumes of Capture Buffer to 1 volume of the PCR sample and mix. It is not necessary to remove mineral oil or kerosene. For example, add 500 µl of Buffer PB to 100 µl PCR sample (not including oil).

3.2 Place a GFX spin column in a provided 2 ml collection tube.

3.3 To bind DNA, apply the sample to the GFX column and centrifuge for 60 s.

3.4 Discard flow-through. Place the GFX column back into the same tube.

3.5 To wash, add 0.5 ml Wash Buffer Type1 to the GFX column and centrifuge for 60s.

3.6 Place GFX column in a clean 1.5 ml micro centrifuge tube.

3.7 To elute DNA, add 50 µl Elution Buffer Type 6 or H2O to the center of the GFX membrane, let the column stand for 1 min, and then centrifuge the column for 1 min.

IMPORTANT: Ensure that the elution buffer is dispensed directly onto the GFX membrane for complete elution of bound DNA. The average eluate volume is 48 µl from 50 µl elution buffer volume, and 28 µl from 30 µl elution buffer.

Appendix 8: The method/protocol that was followed during sample sequencing by using Sanger sequencing

Method / Protocol Title:
Sanger Sequencing

Authors: Jemma Wadsworth (jemma.wadsworth@pirbright.ac.uk)
Nick Knowles (nick.knowles@pirbright.ac.uk)

n) If you are loading the samples immediately onto the sequencing machine, add 20 µL Hi-Di Formamide and allow the pellets to resuspend for 10-15min (Keep in the dark). Otherwise, seal the wells for storage and keep in the dark at –20 °C
o) Before adding the plate to the sequencer, put the plate into the centrifuge and spin up to 185 × g (quick pulsing) to make sure the liquid is in the bottom of the well.

IMPORTANT! add 20 µL Hi-Di Formamide to blank wells

3.2.2: Ethanol precipitation in tubes
a) To each reaction add 5µl 125mM EDTA and 60µl 100 % EtOH
b) Vortex tubes and leave in the dark for 15mins (room temp)
c) Spin for 30 mins
d) Remove all of the supernatant (Very Important)
e) Add 60µl 70 % EtOH and vortex the tubes.
f) Spin for 30 mins 15
g) Remove all of the supernatant (Very Important)
h) Vacu-dry for 15mins in the dark. If you are not loading the samples immediately, then they can be stored at this stage.
i) Add 20ul Hi-Di Formamide before loading.

3.2: Ethanol precipitation:
3.2.1: Ethanol precipitation in a plate:
a) Switch on a microcentrifuge
b) Prepare a fresh 125mM EDTA (from 500mM stock, Sigma) with nuclease free water
c) Add the following to each 10 µL reaction: (i) 5 µL of 125 mM EDTA; (ii) 60 µL of 100% ethanol
d) Seal plate with adhesive tape. gently vortex for 15 sec to mix.
e) Leave the reaction plate in the dark at room temperature for at least 15 min to precipitate the extension products.
f) Place the reaction plate in a centrifuge with a plate adaptor and spin at: (i) 1400 to 2000 rpm for 45 min or (ii)2000 to 3000 rpm for 30 min
g) Discard the supernatant as follows
- Without disturbing the precipitate, remove the adhesive tape
- Invert the reaction plate onto a paper towel
- Place the inverted reaction plate onto a folded paper towel (to the size of the plate) and then spin up to 185 rpm (quick pulsing)

h) Remove plate from the centrifuge.
i) Perform a 70% wash by adding 60 µL of 70% ethanol to each pellet.
j) Place the reaction plate in the centrifuge and spin for 15 min at 1650 rpm.
k) Discard the supernatant as in step g
l) Remove the reaction plate from the centrifuge and discard the paper towel
m) Vacuum dry (or air dry) the plate until no ethanol is present (30 min)

IMPORTANT! Make sure the samples are protected from light while they are drying.
IMPORTANT! Make sure the wells are dry.

Appendix 9: The individual sample RNA Concentrations with their respective 260/280 ratios and remarks on their purity

LAB #	FIELD ID	SAMPLE TYPE	RNA CONC. (ng/µl)	260/280 Ratio	Remarks
1	F1-D1	Spleen	350	1.815	PURE
2	F1-D2	Spleen	390.8	1.806	PURE
3	F1-D3	Spleen	518.8	1.837	PURE
4	F1-D4	Spleen	392.4	1.823	PURE
5	F1-D5	Spleen	545.2	1.849	PURE
6	F1-D6	Spleen	404.4	1.883	PURE
7	F1-D7	Spleen	419.6	1.86	PURE
8	F1-F11-15	Fish	259.6	1.788	NOT PURE
9	F1-F16-20	Fish	230.9	1.838	PURE
10	F2-D1	Spleen	262.4	1.853	PURE
11	F2-D2	Spleen	472	1.855	PURE
12	F2-D3	Spleen	511.6	1.827	PURE
13	F2-D4	Spleen	311.2	1.902	PURE
14	F2-E4	Eggs	35.2	1.956	PURE
15	F2-D5	Spleen	354.8	1.852	PURE
16	F2-E5	Eggs	11.7	1.518	NOT PURE
17	F2-F5-10	Fish	28	2	PURE
18	F2-F11-15	Fish	6.2	1.867	PURE
19	F9-E13	Eggs	7.5	1.176	NOT PURE
20	F3-D1	Spleen	205.2	1.813	PURE
21	F3-E1	Eggs	7.2	1.304	NOT PURE
22	F3-F6-10	Fish	8.8	1.228	NOT PURE
23	F3-D2	Spleen	181.2	1.805	PURE
24	F3-D3	Spleen	193.2	1.879	PURE
25	F3-D4	Spleen	217.6	1.902	PURE
26	F3-D5	Spleen	215.6	1.932	PURE
27	F3-F11-15	Fish	8.2	1.609	NOT PURE
28	F4-D1	Spleen	426	1.833	PURE
29	F4-D2	Spleen	324.8	1.833	PURE
30	F4-D3	Spleen	585.2	1.838	PURE
31	F4-D4	Spleen	334.8	1.92	PURE
32	F4-D5	Spleen	370.4	1.819	PURE
33	F4-D6	Spleen	359.2	1.883	PURE

34	F4-E6	Eggs	11	1.165	NOT PURE
35	F4-D7	Spleen	354.8	1.81	PURE
36	F5-C1	Kidney	257.6	1.794	NOT PURE
37	F5-D1	Spleen	360.8	1.887	PURE
38	F5-F2-6	Fish	74.4	2.022	PURE
39	F5-F7-11	Fish	108	1.862	PURE
40	F5-F12-15	Fish	1.4	1.889	PURE
41	F5-F16-20	Fish	116.8	1.814	PURE
42	F6-D1	Spleen	389.6	1.866	PURE
43	F6-D2	Spleen	570.4	1.838	PURE
44	F6-D3	Spleen	280	1.818	PURE
45	F6-E3	Eggs	9.1	1.727	NOT PURE
46	F6-D4	Spleen	363.6	1.789	NOT PURE
47	F6-D5	Spleen	365.6	1.813	PURE
48	F6-E6	Eggs	9.6	1.513	NOT PURE
49	F6-F11-15	Fish	660	1.986	PURE
50	F9-E4	Eggs	260.8	1.952	PURE
51	F6-F16-20	Fish	50	1.689	NOT PURE
52	F7-D11	Spleen	254.8	1.799	NOT PURE
53	F7-D12	Spleen	548.8	1.817	PURE
54	F7-D13	Spleen	344.8	1.815	PURE
55	F7-D14	Spleen	338.8	1.791	NOT PURE
56	F7-D15	Spleen	122.4	1.8	PURE
57	F7-E11	Eggs	35.2	1.313	NOT PURE
58	F7-D28	Spleen	297.6	1.842	PURE
59	F7-E28	Eggs	60.8	1.708	NOT PURE
60	F7-F16-17	Fish	24.8	1.771	NOT PURE
61	F7-F25-27	Fish	3.4	0.885	NOT PURE
62	F7-E14	Eggs	13	1.36	NOT PURE
63	F8-D11	Spleen	311.6	1.807	PURE
64	F8-D12	Spleen	160.4	1.579	NOT PURE
65	F8-D13	Spleen	51.2	1.969	PURE

66	F8-D14	Spleen	249.2	1.688	NOT PURE
67	F8-D15	Spleen	193.6	1.813	PURE
68	F8-D16	Spleen	283.2	1.802	PURE
69	F8-D17	Spleen	252	1.821	PURE
70	F8-D18	Spleen	175.6	1.702	NOT PURE
71	F8-D19	Spleen	121.2	1.741	NOT PURE
72	F8-D20	Spleen	144.4	1.842	PURE
73	F9-D11	Spleen	560.4	1.819	PURE
74	F9-D12	Spleen	304.8	1.814	PURE
75	F9-D13	Spleen	331.6	1.79	NOT PURE
76	MML1	Spleen	12.3	1.203	NOT PURE
77	MML3	Spleen	15.2	1.21	NOT PURE
78	MML8	Spleen	56.8	1.392	NOT PURE
79	LOM1	Spleen	6.6	3.417	PURE
80	LOM3	Spleen	7.2	1.705	NOT PURE
81	LOM2	Spleen	9.6	1.285	NOT PURE
82	MMG1	Spleen	8.8	1.063	NOT PURE
83	MMG3	Spleen	3.4	3	PURE
84	IHM1	Spleen	72	1.748	NOT PURE
85	IHM2	Spleen	18.4	2.46	PURE
86	IMKE1	Spleen	2.9	-3.789	NOT PURE
87	MKK8	Spleen	13.2	3.028	PURE
88	MKK6	Spleen	1.2	-1.933	NOT PURE
89	UJM1	Spleen	12.1	1.094	NOT PURE
90	UJM2	Spleen	14	2.381	PURE
91	IAL3	Spleen	0.2	-0.161	NOT PURE
92	MJG1	Spleen	16	2.778	PURE
93	MKK7	Spleen	27.6	0.233	NOT PURE
94	F9-D14	Spleen	270	1.815	PURE
95	F9-D15	Spleen	309.2	1.798	NOT PURE

96	F9-D16	Spleen	269.1	1.817	PURE
97	F9-F17-26	Fish	229.6	1.882	PURE
98	F9-F27-36	Fish	146.4	1.896	PURE
99	F9-E1	Eggs	22.8	2.676	PURE
100	F9-E12	Eggs	8	2.41	PURE
101	F10-D11	Spleen	396	1.847	PURE
102	F10-E11	Eggs	59.6	2.014	PURE
103	F10-D12	Spleen	223.2	1.866	PURE
104	F10-D13	Spleen	191.6	1.871	PURE
105	F10-E13	Eggs	36.6	1.314	NOT PURE
106	F10-D14	Spleen	173.2	1.789	NOT PURE
107	F10-D15	Spleen	244.8	1.789	NOT PURE
108	F10-E3	Eggs	14	2.593	PURE
109	F10-E10	Eggs	7	1.411	NOT PURE
110	F10-E15	Eggs	9.6	1.491	NOT PURE
111	F10-F17-26	Fish	181.6	1.809	PURE
112	F4-F8	Fish	4.6	1.292	NOT PURE
113	F6-F21	Fish	16.8	1.235	NOT PURE
114	F7-F19	Fish	160	1.86	PURE
115	F8-F21	Fish	71.2	2.171	PURE