Article

A high efficacy DNA vaccine against tilapia lake virus in Nile tilapia (Oreochromis niloticus)

Nai-tong Yu*, Wei-wei Zeng1, Jian-hua Wang1, Yu-liang Zhang1, Xiu-chun Zhang1, Zhi-xin Liu1

1 Key Laboratory of Biology and Genetic Resources of Tropical Crops, Ministry of Agriculture and Rural Affairs, Institute of Tropical Bioscience and Biotechnology, Chinese Academy of Tropical Agricultural Sciences, Haikou 571101, China;
2 School of Life Science and Engineering, Foshan University, Foshan 528231, China;
* Correspondence: yunaitong@163.com (N.Y.); Tel.: +86-898-66890770.

Abstract: Tilapia lake virus (TiLV) is the main tilapia-infecting virus worldwide, causing serious economic losses. However, there is no vaccine for this viral disease. Here, TiLV ORF10 (TiLV-ORF10) encoding a protein with abundant epitopes was constructed into the eukaryotic expression vector pcDNA3.1, and used to evaluate the immune protective effects in Nile tilapia (Oreochromis niloticus). RT-PCR and western blot analyses confirmed vaccine plasmid expression in tilapia muscle tissues. Moreover, the transcription levels of immunoglobulin M, toll-like receptor 2, myeloid differentiation factor 88, interleukin 8, tumor necrosis factor alpha, gamma-IFN, and nuclear factor κB immune-related genes were statistically significantly upregulated in the spleen, liver, and kidney of vaccinated tilapias (P < 0.05). TiLV challenge experiments showed that relative percent survival (RPS) was significantly enhanced in fish by this DNA vaccine. Moreover, RPS was enhanced further when using a higher amount of the DNA vaccine (85.72% RPS at a DNA dose of 45 μg pcDNA3.1–ORF10). Vaccination with pcDNA3.1–ORF10 significantly reduced virus replication, as evidenced by the low amount of virus in the spleen, liver, and kidney of vaccinated tilapias after TiLV challenge. Thus, pcDNA3.1–ORF10 could induce protective immunity in tilapia and may be a potential vaccine candidate for controlling diseases caused by TiLV.

Keywords: tilapia; negative-sense RNA virus; Tilapia lake virus; DNA vaccine

1. Introduction

Tilapia (Oreochromis mossambicus) is the second largest farmed fish and is an important protein source for developing countries [1]. Tilapia is primarily distributed in Asia, Africa, and South and North America, and its production is increasing yearly [1,2]. However, an emerging viral pathogen, tilapia lake virus (TiLV), causes mass mortality of wild and farmed Nile tilapia (O. niloticus), which has a considerable impact on tilapia aquaculture in several countries [3,4]. TiLV is a negative-sense single-stranded RNA virus (−ssRNA virus), the only member in the genus Tilapinevirus, of the family Ammonooviridae (ICTV, 2018b). The virus is approximately 60–80 nm in size with a negative-sense RNA genome consisting of 10 segments and is approximately 10,323 kb in length [5,6]. The structural characterization and protein functions of the TiLV genome are still under investigation, including TiLV ORF10 (TiLV-ORF10, here and after). Bioinformatics analysis indicated that TiLV-ORF10 shares a weak amino acid sequence similarity with known viruses. Large knowledge gaps exist regarding TiLV-ORF10, which needs to be addressed in future studies.
The emergence of this viral disease, termed tilapia lake virus disease (TiLVD), has been reported epidemic at a rapid rate, causing global outbreaks [7-10]. Therefore, the development of disinfection methods or advanced vaccine tools against TiLV infection is necessary. Previously, chemical reagents such as iodine, NaOCl, H2O2, formalin, Virkon, and other common disinfectants have been used to prevent or control the spread of TiLV [11]. At present, no information on a possible vaccine exists, and yet no work has been carried out for the characterization of a DNA vaccine against TiLV. It has been reported that DNA vaccines expressing viral proteins induce an immune response and decrease fish mortality after viral infection [12,13]. Development of a DNA vaccine for controlling TiLV can pave the way for the design of disease control strategies.

Tilapia is a valuable fish species in southern China in Guangdong, Hainan, Guangxi, and Taiwan provinces. However, TiLV will cause serious economic losses in tilapia production if the virus is found epidemics in these regions. In this study, TiLV-ORF10 was amplified and its encoded protein was characterized by informatics analysis. Subsequently, a DNA vaccine of pcDNA3.1–ORF10 expressing TiLV-ORF10 was constructed. Furthermore, the effects of the DNA vaccine on tilapia immune-related genes and viral amounts were investigated. Our study will establish a DNA vaccine for effective protective the tilapia against the TiLV infection, which may also be used for control TiLV on tilapia in near future.

2. Materials and Methods

2.1. Fish and Virus

A total of 140 Nile tilapias, Oreochromis niloticus (118 ± 11.23 cm in body length) were used for vaccination and viral challenge experiments in this study. The tilapias were maintained in aerated freshwater at room temperature (27 ± 3 °C) and fed daily with fishing bait. Eight fish were randomly selected and used for RT-PCR detection to ensure that the fish used in the study was free of TiLV infection. Approximately 35 g of TiLV-infected spleen, liver, and kidney tissues of tilapias were ground in 1× PBS buffer and subsequently passed through 0.2 μm filters. The prepared virus suspension was stored at -20 °C for viral challenge experiments. The TiLV 2017A isolate was kindly provided by the Pearl River Fishery Research Institute, Chinese Academy of Fishery Sciences [14].

2.2. TiLV-ORF10 Cloning and Sequence Analysis

Total RNA from TiLV-infected spleen, liver, and kidney tissues of tilapias was extracted using TRIzol reagent (Invitrogen, USA), and reverse transcribed into first-strand cDNA using EasyScript Reverse Transcriptase (Trans, China). According to the full-length sequence of TiLV segment 10 in NCBI (NC_029930.1), the specific pair primers TiLVORF10-F/R targeting the TiLV-ORF10 ORF were designed for PCR amplification (Table 1). PCR cloning was performed as described by Yu et al. [15]. The PCR product was purified and ligated into a pMD18-T vector (Takara, China). Three randomly selected clones were subjected to Sanger sequencing (Invitrogen, China).

The TiLV-ORF10 amino acid sequences of the 2017A-Hainan isolate (2017A-H), two Thailand isolates (AOE22907.1 and AWK60422.1), an Israel isolate (YP_009246485), an Ecuador isolate (QAB07944.1), and a Peru isolate (QDC17502.1) were retrieved from the National Center for Biotechnology Information (NCBI) for computer-assisted analysis. Sequence alignment of the six proteins was performed in Clustal X 1.83, and edited by GeneDoc software. Conserved domains were predicted using a domain search in NCBI (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). The 113 amino acid polypeptide sequence of TiLV-ORF10 (2017A-H) was used to predict an antibody epitope using Bepipred Linear Epitope Prediction (IEDB analysis resource) at http://tools.immuneepitope.org/bcell/.
**Table 1** Primers used in this study.

<table>
<thead>
<tr>
<th>Primers</th>
<th>Primers sequences (5’-3’)</th>
<th>Usage</th>
</tr>
</thead>
<tbody>
<tr>
<td>TiLVORF10-F</td>
<td>AAATCTTTCCCTCTGACACCC</td>
<td>RT-PCR</td>
</tr>
<tr>
<td>TiLVORF10-R</td>
<td>CAGGATGAGTGTGGCAGATT</td>
<td></td>
</tr>
<tr>
<td>TiLVORF10-F2</td>
<td>GGgcattcATGACCACTCATCATCATCATCTGAGGTTTTGTC (EcoRI)</td>
<td>pcDNA3.1–ORF10</td>
</tr>
<tr>
<td>TiLVORF10-R2</td>
<td>GGGatatcCTAAGACTGCACGTTACAAGAGAC (EcoRV)</td>
<td></td>
</tr>
<tr>
<td>TiLVRNA1-F</td>
<td>GTGGGTTGTCCAAGCCTAAGGAC</td>
<td>RT-PCR</td>
</tr>
<tr>
<td>TiLVRNA1-R</td>
<td>CACTCTAGTGTGTCCAGGAGG</td>
<td></td>
</tr>
<tr>
<td>TiLVRNA1-qF</td>
<td>GCCATGTGGGGAGAAGTCCTC</td>
<td>RT-qPCR</td>
</tr>
<tr>
<td>TiLVRNA1-qR</td>
<td>CAGGGGACCACTTGTAATCTCTGA</td>
<td></td>
</tr>
<tr>
<td>IgM-qF</td>
<td>AGGAGACAGACTGCACAGAATGCACAA</td>
<td>RT-qPCR</td>
</tr>
<tr>
<td>IgM-qR</td>
<td>GGGAGCAGATAGGTATCATCCTC</td>
<td></td>
</tr>
<tr>
<td>TLR2-qF</td>
<td>GTATCTCAGTCTGCTGCTCA</td>
<td>RT-qPCR</td>
</tr>
<tr>
<td>TLR2-qR</td>
<td>TTTCAATTATCGTCTCCAGTG</td>
<td></td>
</tr>
<tr>
<td>MyD88-qF</td>
<td>TTTCAGCTCCTCACACTTTTAG</td>
<td>RT-qPCR</td>
</tr>
<tr>
<td>MyD88-qR</td>
<td>CCGCCTGCTCACAGTTAT</td>
<td></td>
</tr>
<tr>
<td>IL8-qF</td>
<td>GCACGTCCGCTGCATTAAC</td>
<td>RT-qPCR</td>
</tr>
<tr>
<td>IL8-qR</td>
<td>GCAGTGGGAGTAGGGAAGA</td>
<td></td>
</tr>
<tr>
<td>TNFα-qF</td>
<td>GAGGGTGGGTGCCGCTGGC</td>
<td>RT-qPCR</td>
</tr>
<tr>
<td>TNFα-qR</td>
<td>TGGTTTCCGTCCACAGCGT</td>
<td></td>
</tr>
<tr>
<td>IFNγ-qF</td>
<td>TGACCCATCGTTCAGAGCA</td>
<td>RT-qPCR</td>
</tr>
<tr>
<td>IFNγ-qR</td>
<td>GGCAGCTTTAGCTTCGTT</td>
<td></td>
</tr>
<tr>
<td>NF-kB-qF</td>
<td>CGACACACTACCTACAGC</td>
<td>RT-qPCR</td>
</tr>
<tr>
<td>NF-kB-qR</td>
<td>GATGTCGTTTGGGACATC</td>
<td></td>
</tr>
</tbody>
</table>
2.3. Plasmid Construction

The TiLV-ORF10 ORF was amplified from prepared cDNA as described above using TiLVORF10-F2/R2. The amplified DNA fragment was cloned into the eukaryotic expression vector pcDNA3.1 (Invitrogen, USA) using EcoRI and EcoRV restriction enzyme sites. The recombinant plasmid, designated as pcDNA3.1–ORF10, was confirmed by restriction enzyme digestion and DNA sequencing. The plasmids pcDNA3.1–ORF10 and pcDNA3.1 were purified from the Endo-free Plasmid Midi Kit (Omega Bio-Tek, USA) and used for DNA vaccination.

2.4 DNA Vaccination and Viral Challenge Experiments

For vaccination with different concentrations of DNA vaccine, the plasmid pcDNA3.1–ORF10 was diluted to 150 μg/mL, 300 μg/mL, or 450 μg/mL with sterile 1× PBS buffer, whereas pcDNA3.1 was diluted to 450 μg/mL. A total of 120 healthy tilapias were randomly divided into four groups (30 animals per group). Each group was intramuscularly injected with 100 μL of pcDNA3.1–ORF10 (150 μg/mL), pcDNA3.1–ORF10 (300 μg/mL), pcDNA3.1–ORF10 (450 μg/mL), or pcDNA3.1 (450 μg/mL), and a second booster vaccination was conducted using the same dose of DNA vaccine, designated as pcDNA3.1–ORF10(a), pcDNA3.1–ORF10(b), pcDNA3.1–ORF10(c), and pcDNA3.1 group, respectively. The remaining 12 tilapias were vaccinated with 100 μL of pcDNA3.1–ORF10 (450 μg/mL) or pcDNA3.1 (450 μg/mL), and were necropsied to collect muscle (of injection sites), spleen, liver, and kidney tissues at 7 days post vaccination (dpv). Samples were immediately frozen in liquid nitrogen and stored at −80 °C until use. The 120 tilapias were challenged with 100 μL LD₅₀ = 10¹·² TiLV via intraperitoneal injection at 7 dpv after the second booster vaccination. Tilapias from the same group were randomly distributed into three separate tanks and stored as described above at room temperature (27 ± 3 °C). The dead animals were collected daily, and mortality rates were calculated 22 days post viral challenge. Relative percentage survival (RPS) was calculated according to the following formula: RPS = [1 - (death vaccinated group/total vaccinated group)/(death control group/total control group)] × 100% [16].

2.5. Detection of Transcription and Expression of Vaccine Plasmids in Tilapias

Total RNA from the injection site muscle was extracted using TRIzol reagent (Invitrogen), following the manufacturer’s instructions. Three micrograms of RNA was pretreated with gDNA Eraser (Takara) and reverse transcribed into the first strand cDNA using EasyScript Reverse Transcriptase (Beijing, China). PCR was conducted to detect the transcripts of the vaccine plasmid using the specific primer TiLVORF10-F/R. β-actin mRNA was used as an internal control.

To prepare total protein lysates, frozen muscle tissues were homogenized in 50 mM Tris-HCl buffer (pH 7.5) with a protease inhibitor cocktail (Roche, USA). Lysates were separated by 12% SDS-PAGE and transferred onto a polyvinylidene difluoride membrane for western blot analysis. Mouse anti-His serum (Vector Laboratories, USA) was used as the primary antibody (1:2000), and peroxidase-conjugated goat anti-mouse IgG (H + L) antibody (Vector Laboratories, USA) was used as the secondary antibody (1: 2000). The signals were detected using a chemiluminescent horseradish peroxidase substrate (Millipore). Simultaneous internal control was performed by detecting the β-actin protein with an anti-β-actin antibody (Boster; 1: 2000).
2.6. Transcription Levels of Immune-Related Genes by Reverse Transcription Quantitative PCR (RT-qPCR)

Total RNA from the spleen, liver, and kidney tissues of tilapias at 7 dpv from pcDNA3.1–ORF10 and pcDNA3.1 were extracted and subjected to first-strand cDNA synthesis as described above. RT-qPCR was used to measure the relative RNA levels of immune-related genes, which included immunoglobulin M (IgM, KC677037.1), toll-like receptor 2 (TLR2, XM_019360109.2), myeloid differentiation factor 88 (MyD88, NM_001311322.1), interleukin 8 (IL8, NM_001279704.1), tumor necrosis factor alpha (TNFα, NM_001279533), gamma-IFN (IFNγ, NM_001287402.1), and nuclear factor κB (NF-κB, XM_019363515.2) genes. RT-qPCR was performed using SYBR green real-time PCR master mix reagent kit (CWBIO, China) in a StepOne real-time PCR system (Applied Biosystems, USA) with a cycle condition of 95 °C for 5 min, followed by 40 cycles of 95 °C for 15 s, and 60 °C for 1 min. All samples were tested in triplicate. The transcription level of tilapia immune genes, normalized to the internal control β-actin, was evaluated based on the 2^−ΔΔCt method [17]. All data were analyzed using the Student’s t-test. Statistical significance was set at P < 0.05.

2.7. Determination of the Amount of TiLV Using RT-qPCR

Three dead and three surviving tilapias were selected from the pcDNA3.1–ORF10(c) or pcDNA3.1, and their spleen, liver, and kidney tissues were collected to evaluate the relative amount of TiLV. Total RNA was extracted and reverse-transcribed into first-strand cDNA, as described above. According to the full-length sequence of TiLV segment 1 (TiLV RNA1) in NCBI (NC_029926.1), the specific primer pair TiLVRNA1-F/R targeting TiLV RNA1 was designed for PCR amplification (Table 1). PCR cloning was conducted, and the PCR product was ligated into pMD18-T (Takara) as described above. The relative amount of TiLV was quantified by RT-qPCR as described previously using specific primers TiLVRNA1-qF/qR (Table 1) that anneal to TiLV RNA1. The mRNA level of TiLV-ORF10 was normalized to that of β-actin mRNA, and the mRNA level of TiLV-ORF10 in the spleen tissues of surviving tilapias in the pcDNA3.1 group was set as 1.

3. Results

3.1. RT-PCR Cloning and Sequence Analysis

RT-PCR results showed that a specific DNA band of expected size (423 bp) was obtained. The complete ORF of TiLV segment 10 is 342 bp long and encodes a protein with a predicted molecular weight of approximately 12.73 kDa and a theoretical isoelectric point of 4.68. Amino acid sequence analysis showed that TiLV-ORF10 (2017A-H isolate) is identical to the protein sequence of the Thailand isolate (AOE22907.1), and has 98.2~99.1% sequence homology with other TiLV-ORF10s of the different isolates. Bioinformatic analysis indicated that TiLV-ORF10 contains the bromo-domain, which specifically recognizes acetylated lysine and regulates gene expression (Figure 1A).

To establish a highly efficacious DNA vaccine against TiLV, a specific antibody with a high immunodominant epitope is required. The TiLV-ORF10 protein sequence was scanned using the Bepipred Linear Epitope Prediction method to identify the epitope for antibody production [18]. Five peptide sequences were predicted. According to BLAST data, these peptide sequences were conserved in all TiLV-ORF10s (Figure 1B).
The putative epitope was widely distributed in TiLV-ORF10. Thus, the full-length amino acid sequence of TiLV-ORF10, whose N-terminus is linked with a 6×His tag, was expressed in the eukaryotic expression vector pcDNA3.1–ORF10. RT-PCR was performed at 7 dpv to analyze the transcription of pcDNA3.1–ORF10 in muscle tissues of vaccinated tilapias. The results showed that the transcripts of the TiLV-ORF10 gene were detected by a pair of specific primers TiLVORF10-F2/R2, while no amplification was obtained for the negative control group (Figure 2A). The transcripts of the reference gene, β-actin, were maintained at a similar level in the pcDNA3.1–ORF10 and pcDNA3.1 groups.

To analyze the expression of TiLV-ORF10 in muscle tissues of vaccinated tilapias, western blotting was performed at 7 dpv. The result showed that the specific immunoreactive bands of approximately 14 kDa (His-TiLV-ORF10) were
detected in the pcDNA3.1–ORF10 group; however, no band was observed in the pcDNA3.1 group (Figure 2B). Equal amounts of loading were evident by similar amounts of β-actin protein in the two groups (Figure 2B, lower panel). Collectively, these results demonstrated that the DNA vaccine, pcDNA3.1–ORF10, expressed TiLV-ORF10 in vaccinated tilapias.

![Figure 2](image_url)

**Figure 2.** Transcription and expression of TiLV-ORF10 in vaccinated tilapia muscles. RT-PCR (A) and western blot (B) analysis of transcription and expression of the TiLV-ORF10 gene in muscle tissues from vaccinated tilapias at 7 dpv (n = 3). β-actin mRNA and its protein were used as internal controls.

3.3. Transcription Level of Immune-Related Genes After DNA Vaccine Administration

We evaluated the transcription levels of seven immune-related genes, namely IgM, TLR2, MyD88, IL8, TNFα, IFNγ, and NF-κB genes in the spleen, liver, and kidney of tilapias from pcDNA3.1–ORF10 and pcDNA3.1. Compared with the control group, all seven examined genes were upregulated to different extents in tested organs of vaccinated tilapias (Figure 3). Compared with the pcDNA3.1 group, the transcription levels of IgM were highly upregulated (3.3-fold) in the spleen of the pcDNA3.1–ORF10 group, followed by IFNγ (2.53-fold) and NF-κB (2.41-fold). The upregulation of TLR2, MyD88, IL8, and TNFα transcription levels were between 1.53- and 1.89-fold (Figure 3A). Compared with spleen tissues, some immune-related genes were upregulated, while others were downregulated in liver tissues. Transcription levels of TLR2, MyD88, IL8, TNFα, and IFNγ increased into 2.74-, 1.89-, 2.67-, 1.77-, and 3.53-fold, respectively. The transcription levels of IgM and NF-κB genes decreased to 1.77- and 1.69-fold, respectively (Figure 3B). Moreover, the transcription levels of these immune-related genes were further altered in kidney tissues. Notably, the transcription levels of IL8 and TNFα increased into 2.89- and 3.53-fold, respectively; however, upregulation of other immune-related gene transcription levels were between 1.53- and 1.89-fold (Figure 3C). In summary, the DNA vaccine can statistically significantly induce upregulation of immune-related gene expression (P < 0.05); however, the degree of upregulation is different in spleen, liver, and kidney tissues.
Figure 3. RT-qPCR analysis of transcription levels of IgM, TLR2, MyD88, IL8, TNFα, IFNγ, and NF-κB genes in spleen (A), liver (B), and kidney (C) tissues from vaccinated tilapias. The mRNA level of each gene was normalized to that of β-actin mRNA. For each gene, the mRNA level of the pcDNA3.1 group was set as 1. Asterisks indicate significant differences from the control group. Data are presented as the mean ± SE (n = 3). *P < 0.05.

3.4. Protection of Tilapias Using DNA Vaccination
Following acclimatization for 14 d in aerated freshwater at room temperature (27 ± 3 °C), tilapias were vaccinated with the DNA vaccine on the 7th and 15th d, respectively. At 7 dpv of the second booster vaccination, vaccinated tilapias used for experimental TiLV infection were intraperitoneally injected with 100 μL of virus suspension at a concentration of LD$_{50}$ = $10^{1.2}$. Fish were monitored daily for clinical signs and mortality after TiLV challenge until 22 days post-inoculation (dpi). As shown in Figure 4, death was first recorded at 3 dpi in the pcDNA3.1 group; however, it was delayed in pcDNA3.1–ORF10 vaccinated groups. Cumulative mortality reached 93.33% in the pcDNA3.1 group at 22 dpi, whereas it was reduce to 36.67% in the pcDNA3.1–ORF10(a) group when using 15 μg pcDNA3.1–ORF10. Furthermore, the cumulative mortality was further reduced to 20.00% and 13.33% in the pcDNA3.1–ORF10(b) and pcDNA3.1–ORF10(c) groups, respectively, when using more DNA vaccine (Figure 4). Compared to the pcDNA3.1 group, RPS values of pcDNA3.1–ORF10(a) were 60.71%, which further increased to 78.57% and 85.72% in the pcDNA3.1–ORF10(b) and pcDNA3.1–ORF10(c) groups, respectively (Table 2). Overall, these data indicate that plasmid pcDNA3.1–ORF10, at an appropriate dose, could function as an effective vaccine against TiLV infection in tilapias.

**Figure 4.** Cumulative mortality curves of vaccinated tilapias upon challenge with tilapia lake virus (TiLV). Vaccinated tilapias of pcDNA3.1, pcDNA3.1–ORF10(a), pcDNA3.1–ORF10(b), pcDNA3.1–ORF10(c) were intraperitoneally challenged with TiLV and monitored daily for mortality over a 22 d observation period. Data are presented as the mean ± SE (n = 3).

**Table 2** Cumulative mortality and relative percentage survival (RPS) of vaccinated tilapia challenged with tilapia lake virus (TiLV).

<table>
<thead>
<tr>
<th>Vaccinated tilapia</th>
<th>Cumulative mortality (death/total)</th>
<th>RPS%</th>
</tr>
</thead>
<tbody>
<tr>
<td>pcDNA3.1–ORF10 (c)</td>
<td>13.33% (4/30)</td>
<td>85.72</td>
</tr>
<tr>
<td>pcDNA3.1–ORF10 (b)</td>
<td>20.00% (6/30)</td>
<td>78.57</td>
</tr>
<tr>
<td>pcDNA3.1–ORF10 (a)</td>
<td>36.67% (11/30)</td>
<td>60.71</td>
</tr>
</tbody>
</table>
3.5. The Relative Amount of TiLV in Vaccinated and Control Tilapias

The relative amount of TiLV in the spleen, liver, and kidney tissues of dead and surviving tilapias in the pcDNA3.1–ORF10(c) or pcDNA3.1 groups was further determined by RT-qPCR. Overall, surviving tilapias had a lower level of TiLV compared with dead tilapias, and the viral amount of pcDNA3.1-vaccinated tilapias was lower than that in pcDNA3.1–ORF10(c) vaccinated tilapias (Figure 5). Comprehensively, the relative amount of TiLV from surviving tilapia spleens of the pcDNA3.1–ORF10(c) group was 7.33-fold higher than that in the pcDNA3.1 group; however, it was 4.51- and 2.92-fold higher in liver and kidney tissues, respectively. The relative amount of TiLV in the spleen, liver, and kidney tissues of dead tilapias in both groups was dramatically increased. The amount of TiLV in dead tilapia spleens of pcDNA3.1–ORF10(c) and pcDNA3.1 was 99.03- and 322.63-fold higher than the surviving tilapia spleens in the pcDNA3.1 group, respectively, which is less than the amount of TiLV in liver tissues, with 633.4- and 580.99-fold higher, respectively. Moreover, the amount of TiLV in dead tilapia kidney tissues in the two groups were 167.11. and 417.5-fold higher, respectively, at medium levels.

Figure 5. RT-qPCR analysis of the relative mRNA level of TiLV-ORF10 in vaccinated tilapias. The mRNA level of TiLV-ORF10 was normalized to that of β-actin mRNA. For each group, the mRNA level of TiLV-ORF10 in spleen tissues of pcDNA3.1–ORF10 vaccinated surviving tilapias was set as 1. Data are presented as the mean ± SE (n = 3).

4. Discussion

TiLV contains 10 negative-sense single-stranded RNA segments; however, the structural characteristics of each segment and the function of the viral proteins are unclear. In this study, a DNA vaccine was prepared and used to immunize the tilapia muscle. Before preparing the DNA vaccine, 10 putative proteins of TiLV was performed to predict the epitope using Bepipred Linear Epitope Prediction. The results showed that the TiLV-ORF10 protein has richer epitopes, which are distributed throughout the protein; however, the other nine putative viral proteins have fewer
epitopes (Supplementary Fig. 1). In this case, \textit{TiLV-ORF10} was used for DNA vaccine preparation and antiviral effect research.

Bioinformatics analysis showed that TiLV-ORF10 contains a bromodomain domain. The bromodomain was found to be a class of conserved protein domains that specifically recognize acetylated lysine and form a protein complex that drives active transcription, thereby regulating gene transcriptional activation or transcriptional repression \cite{19, 20}. However, its regulatory functions require further verification.

RT-PCR and western blot results showed that pcDNA3.1–ORF10 was highly transcribed and expressed \textit{in vivo}. Further analysis showed that it could increase transcription levels of fish-related immune genes. In spleen tissue, the most significantly upregulated gene was \textit{IgM}, which mediates an innate immune response and plays an important role to against TiLV infection. However, the significantly upregulated genes in the liver tissue were \textit{IFNγ}, \textit{TLR2} and \textit{IL8}, while the \textit{TNFα} and \textit{IL8} were also significantly increased in the kidney tissue (fold change > 2). These upregulated genes mediated to innate immune response, that plays a key role in innate antiviral immunity in vertebrates. The study indicate that different immune-related genes derived from different tissues and organs may be diverse in response to DNA vaccines. Similar results have been reported using DNA vaccines to protect the Chinese giant salamander against \textit{Andrias davidianus} ranavirus (ADRV) \cite{21}. However, the detailed mechanism of immune regulation in different tissues and organ responses to DNA vaccines should be further researched.

The challenge experiment of TiLV showed that tilapia immunized with pcDNA3.1–ORF10 could improve its survival rate considerably. The RPS of tilapia was 60.71% when using 15 μg of pcDNA3.1–ORF10, and its RPS reached 78.57% when using twice the amount of DNA vaccine (30 μg), an increase of 17.86%. However, it further increased by only 7.51% at a dosage of 45 μg, which is far lower than the dosage of 30 μg. Therefore, if the amount of DNA vaccine (>30 μg) used increase, RPS cannot be increased in equal proportions. The southern region of China is located in a tropical and subtropical region, which may result in TiLV outbreaks when water temperatures range from 22 °C to 32 °C \cite{22}. Therefore, this study suggested the appropriate amount of DNA vaccine pcDNA3.1–ORF10 that should be used for tilapia against TiLV.

5. Conclusions

In conclusion, our results showed that pcDNA3.1–ORF10, a DNA vaccine encoding an abundant epitope protein, conferred effective protection against TiLV challenge in tilapia. Moreover, pcDNA3.1–ORF10 expressing TiLV-ORF10 enhanced the innate immune response and adaptive immune response in tilapia, which are essential for combating TiLV infections.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, Figure S1: The antibody epitope prediction of 10 putative proteins encoded by TiLV.

Author Contributions: N.T. Yu conceived and designed the experiments; N.T. Yu performed the experiments; N.T. Yu, W.W. Zeng, J.H. Wang, Y.L. Zhang, X.C. Zhang, and Z.X. Liu analyzed the data and wrote the manuscript.

Funding: This work was supported by the Young Elite Scientists Sponsorship Program of the Chinese Academy of Tropical Agricultural Sciences (CSTC-QN201704).

Institutional Review Board Statement: All animal procedures were performed in accordance with the recommendations in the Regulations for the Administration of Affairs Concerning Experimental Animals of China. The protocol was approved on September 30th, 2020 by the Institutional Animal Care and Use Committee of the Institute of Tropical Bioscience and Biotechnology, Chinese Academy of Tropical Agricultural Sciences (ITBB[2020]63).
Informed Consent Statement: Not Applicable.

Data Availability Statement: Not Applicable.

Acknowledgments: We would like to thank Editage (www.editage.com) for English language editing.

Conflicts of Interest: The authors declare that they have no conflicts of interest.

References


