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

Running title: A high efficacy DNA vaccine against TiLV

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Abstract

Tilapia lake virus (TiLV) is the main tilapia-infecting virus worldwide, causing serious economic losses to the tilapia aquaculture. 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 then used to evaluate the immune protective effects in Nile tilapia (*Oreochromis niloticus*). RT-PCR and western blot analyses confirmed pcDNA3.1-ORF10 expression in tilapia. Moreover, the transcription levels of immune-related genes such as *immunoglobulin M*, *toll-like receptor 2*, *myeloid differentiation factor 88*, *interleukin 8*, *tumor necrosis factor alpha*, *gamma-IFN*, and *nuclear factor κB* were 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 vaccinated with the DNA vaccine. Moreover, the RPS was significantly higher in fish vaccinated with a high antigen dose of the DNA vaccine

(85.72% RPS at a DNA dose of 45 µg vaccine plasmid). 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 tilapia comparing with pcDNA3.1 vaccination. Thus, pcDNA3.1–ORF10 could induce protective immunity in tilapia and may be a potential vaccine candidate for controlling disease caused by TiLV.

Keywords: tilapia; negative-sense RNA virus; Tilapia lake virus; DNA vaccine; TiLV ORF10

1. Introduction

Tilapia (*Oreochromis* spp.) is the second largest farmed fish and is an important protein source for developing countries (FAO, 2016). Tilapia is primarily farmed in Asia, Africa and America, and its production is increasing yearly (Surachetpong et al., 2020; Thammatorn et al., 2019). However, an emerging viral pathogen, tilapia lake virus (TiLV), causes mass mortality of wild and farmed Nile tilapia (*Oreochromis niloticus*), which has a considerable impact on tilapia aquaculture in several countries (Thawornwattana et al., 2021; Skornik et al., 2019). TiLV is a negative-sense single-stranded RNA virus (-ssRNA virus), the only member in the genus *Tilapinevirus*, of the family *Amnoonviridae* (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 (Al-Hussiney et al., 2018; Subramaniam et al., 2019). The structural characterization and protein functions from the TiLV genome are still under investigation, including TiLV ORF10 (TiLV-ORF10, here and after). Currently, large knowledge gaps exist regarding TiLV and its TiLV-ORF10, which need to be addressed in future studies.

The emergence of this viral disease, termed tilapia lake virus disease (TiLVD), has been reported across tilapia production countries in Southeast Asia, Africa, and South and North America, and it causes high mortalities leading to high economic losses (Kembou Tsofack et al., 2017; Mugimba et al., 2018; Nicholson et al., 2017; Surachetpong et al., 2017; Surachetpong et al., 2020). Therefore, development of biosecurity measures or advanced vaccine tools against TiLV infection is necessary (Bergmann et al., 2017; Zhao et al., 2020). Previously, chemical reagents such as iodine, NaOCl, H₂O₂, formalin, Virkon, etc common disinfectants effectively reduced viral loads and have been used to prevent or control the spread of TiLV (Jaemwimol et al., 2019). In recently, heat-killed (HKV) and formalin-killed TiLV inactivated vaccines have been reported effectively preventing TiLV infection (Mai et al., 2021), however yet no work has been carried out for the characterization of a DNA vaccine against TiLV. Studies have been reported that DNA vaccines expressing viral proteins *in vivo* induced immune response and decreased fish mortality after virus infection (Hu et al., 2019; Chen et al., 2018a). Development of a DNA vaccine will reduce mortality in vaccinated fish and can pave the way for the design of disease control strategy against TiLV.

Tilapia is a valuable fish species in regions of tilapia production worldwide, as well as southern China in Guangdong, Hainan, Guangxi, and Taiwan provinces. TiLV may cause serious economic losses in tilapia production if the virus is epidemic in these regions. In this study, *TiLV-ORF10* was amplified and its encoded protein was characterized by bioinformatics 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 virus amounts were investigated. Our study will establish a DNA vaccine for effective protection the tilapia against the TiLV infection.

2. Materials and Methods

2.1. Fish and Virus

A total of 140 Nile tilapias fish, *Oreochromis niloticus* (118 ± 11.23 mm in body length) were used for vaccination and viral challenge experiments in this study. The fish were maintained in aerated freshwater at room temperature (27 ± 3 °C) and fed once daily with fishing bait. Eight fish were randomly selected and used for RT-PCR detection to ensure that the fish used in the study were free of TiLV infection. Previously, the TiLV-infected tilapias were kept at -80 °C in the laboratory (Chen et al., 2021). Approximately 35 g of TiLV-infected spleen, liver, and kidney tissues of fish were ground in $1 \times$ PBS buffer and subsequently passed through $0.2 \mu\text{m}$ filters. The prepared virus suspension ($\text{LD}_{50} = 10^{1.2}$) was stored at -20 °C for viral challenge experiments. This TiLV 2017A isolate was kindly provided by the Pearl River Fishery Research Institute, Chinese Academy of Fishery Sciences (Wang et al., 2018).

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 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. (Yu et al., 2019a). 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/> (Jespersen et al., 2017).

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 *EcoRI* and *EcoRV* restriction enzyme digestion and bidirectional DNA sequencing using primers TiLVORF10-F2 and TiLVORF10-R2 at Thermo Fisher (Guangzhou, China) (Table 1).

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 amounts 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 fish 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) at 7th day, and a second booster vaccination was conducted using the same dose of DNA vaccine at 15th day, 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) at 7th day, 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 used.

The vaccinated 120 fish were challenged with 100 µL LD₅₀ = 10^{1.2} TiLV via intraperitoneal injection at 22nd day. Fish from the same group were randomly distributed into three separate tanks and cultured 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: $RPS = [1 - (\text{death vaccinated group} / \text{total vaccinated group}) / (\text{death control group} / \text{total control group})] \times 100\%$ (Amend, 1981). The statistical significance of RPS in different groups was determined by two-way analysis of variance, and Duncan's multiple range test was used to compare the means (SAS Institute Inc, Campus drive Cary, NC, USA).

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

Total RNA from the injection site in the 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 primers TiLVORF10-F2/R2. *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 RNAs from the spleen, liver, and kidney tissues of tilapias of pcDNA3.1–ORF10 and pcDNA3.1 at 7 dpv 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* (TNF α , NM_001279533), *gamma-IFN* (IFN γ , NM_001287402.1), and *nuclear factor κ B* (NF- κ B, 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 fish vaccinated with pcDNA3.1 was used as reference sample. The transcription level of tilapia immune genes, normalized to the internal control *β -actin*, was evaluated based on the $2^{-\Delta\Delta C_t}$ method (Livak et al., 2001). All data were analyzed using the Student's t-test. Statistical significance was set at $P < 0.05$.

2.7. Determination of the TiLV Amount in vaccinated tilapias Using RT-qPCR

In order to determine the TiLV amount in dead or surviving fish in pcDNA3.1–ORF10(c) group and pcDNA3.1 group, three dead fish (collected at 4–8 dpi) and three survivors (collected at 22 dpi) from the pcDNA3.1–ORF10(c) group, and three dead fish (collected at 4–8 dpi) and three survivors (collected at 22 dpi) from the pcDNA3.1 group were selected from the viral challenge experiments. Then their spleen, liver, and kidney tissues were collected to evaluate the amount of virus. Total RNA was prepared and reverse-transcribed into first-strand cDNA, as described above.

The quantity of virus was determined by TiLV segment 1 (TiLV RNA1) using RT-qPCR. According to the full-length sequence of TiLV RNA1 in NCBI (NC_029926.1), the specific primers TiLVRNA1-F/R targeting TiLV RNA1 were designed for PCR amplification (Table 1). PCR cloning was conducted on first-strand cDNA from TiLV-infected spleen, liver, and kidney tissues as described above, and the PCR product was ligated to pMD18-T (Takara). The virus amount in various tissues of vaccinated fish in two groups was quantified by qPCR using specific primers TiLVRNA1-qF/qR (Table 1). The recombinant plasmid was diluted to 6.50×10^6 copies/ μ L as an initial template. The initial template was further diluted to 10^{-1} , 10^{-2} , 10^{-3} , 10^{-4} and 10^{-5} by ddH $_2$ O, and were used for establishing the standard curves. The qPCR was carried out using the SYBR green real-time PCR master mix reagents kit (CWbio, China) in the StepOne real-time PCR system (Applied Biosystems, United States) as described by Yu et al (Yu et al., 2019b). The copy number of TiLV was calculated by the following formula: Copy number/ μ L = [Concentration_{plasmid} (ng/ μ L) \times 1 μ L] \times 10^{-9} / [(Length_{plasmid} + Length_{insert}) \times 660] \times 6.02×10^{23} . The virus amount of TiLV in the spleen tissues of surviving fish of the pcDNA3.1–ORF10 group was set as 1. All data were analyzed using the Student's t-test. Statistical significance was set at $P < 0.05$.

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. Bioinformatic analysis indicated that TiLV-ORF10 contains the bromodomain, which specifically recognizes acetylated lysine and regulates gene expression (Figure 1A). However, blastn analysis indicated that TiLV-ORF10 shares a weak amino acid sequence similarity with other known aquatic viruses at NCBI.

To establish a highly efficacious DNA vaccine against TiLV, a specific antibody with an abundant epitope is required. The TiLV-ORF10 protein sequence was scanned using the Bepipred Linear Epitope Prediction method and five peptide sequences were predicted to produce specific antibodies. According to BLAST data, these peptide sequences were conserved in all TiLV-ORF10s (Figure 1B), which can be used for preparing the DNA vaccine further.

3.2. The DNA Vaccine Expressed TiLV-ORF10 in Vaccinated Fish

The putative epitopes were 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 protein 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) was 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.

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*, *TNF α* , *IFN γ* , and *NF- κ B* genes in the spleen, liver, and kidney of tilapias from pcDNA3.1-ORF10 and pcDNA3.1. Compared with the pcDNA3.1 group, all seven examined genes were upregulated to different levels 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 *IFN γ* (2.53-fold) and *NF- κ B* (2.41-fold). The upregulation of *TLR2*, *MyD88*, *IL8*, and *TNF α* transcription levels were between 1.53- and 1.89-fold (Figure 3A). Compared with spleen tissues, the fold change of some immune-related genes were further upregulated, while others were downregulated in liver tissues. Transcription levels of *TLR2*, *MyD88*, *IL8*, *TNF α* , and *IFN γ* increased to 2.74-, 1.89-, 2.67-, 1.77-, and 3.53-fold, respectively. Meanwhile, the transcription levels of *IgM* and *NF- κ B* genes decreased to 1.77-

and 1.69-fold, respectively (Figure 3B). Moreover, the transcription levels of these immune-related genes were further changed in kidney tissues comparing with spleen or liver tissues. Notably, the transcription levels of *IL8* and *TNFα* increased into 2.89- and 3.53-fold, respectively; however, upregulation of other immune-related genes transcription levels were between 1.53- and 1.89-fold (Figure 3C). In summary, the DNA vaccine can significantly induce upregulation of immune-related gene expression ($P < 0.05$); however, the degree of upregulation is different in spleen, liver, and kidney tissues.

3.4. Protection of Fish Using DNA Vaccination

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 reduced in pcDNA3.1-ORF10 vaccinated groups. Cumulative mortality reached 93.33% in the pcDNA3.1 group at 22 dpi, whereas it reduced 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 high quantity of DNA vaccine (Figure 4). The Duncan's range test showed that cumulative mortality of pcDNA3.1 group is significantly higher than other groups, as well as the groups between pcDNA3.1-ORF10(a) and pcDNA3.1-ORF10(c), but not for the pcDNA3.1-ORF10(b) group with pcDNA3.1-ORF10(a) or pcDNA3.1-ORF10(c) group (Table 2). Compared to the pcDNA3.1 group, RPS value of pcDNA3.1-ORF10(a) reached 60.71% using 15 µg vaccine plasmid, which further increased to 78.57% and 85.72% in the pcDNA3.1-ORF10(b) and pcDNA3.1-ORF10(c) groups, with 30 µg and 45 µg vaccine plasmid respectively (Table 2). Overall, these data indicate that plasmid pcDNA3.1-ORF10, at a dose of 15 µg or more, could function as an effective vaccine against TiLV infection in tilapias.

3.5. The Virus Amount of TiLV in Vaccinated Tilapias

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

4. Discussion

TiLV contains 10 negative-sense single-stranded RNA segments; however, the structural characteristics of these segments and the functions of the viral proteins are unclear. TiLV-ORF10 has richer epitopes, which are distributed throughout the protein; comparatively, the other nine putative viral proteins have fewer epitopes (Supplementary Fig. 1). In this case, *TiLV-ORF10* was used for DNA vaccine preparation and anti-viral effect research. In addition, TiLV-ORF10 contains a bromodomain, which was found to be a class of conserved protein domains that specifically recognize acetylated lysine and form a protein complex that drives transcription active, thereby regulating gene transcriptional activation or transcriptional repression (Fujisawa and Filippakopoulos, 2017; Jain and Barton, 2017). As shown in Figure 1, three epitopes of TiLV-ORF10 were overlapping with this domain. Therefore, we speculate that the bromodomain may have the function of regulating immune gene response. However, the mechanism of regulating immune gene response via specific epitope should be studied further.

In the study, pcDNA3.1-ORF10 was highly transcribed and expressed *in vivo*. As we expected, TiLV-ORF10 could increase transcription levels of fish-related immune genes. In spleen tissue, the most significantly upregulated gene was *IgM*, which was triggered by an innate immune response and plays an important role to against TiLV infection. However, the most significantly upregulated gene in the liver tissue was *IFN γ* , while the *TNF α* in the kidney tissue. The upregulation of immune-related gene transcriptions response to innate immune response, which may play a key role against virus in vertebrates. The study indicates that 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 fish against virus (Chang, 2020), such as mandarin fish against Infectious spleen and kidney necrosis virus (ISKNV) (Zhao et al., 2020). The results showed that the specific immune response in the specific organ. However, the detailed mechanism of immune genes regulation in different tissues and organs responses to DNA vaccine should be further researched.

In the study, the pcDNA3.1-ORF10 could improve tilapia survival rate considerably. The RPS of tilapia was 60.71% when using 15 μ g of pcDNA3.1-ORF10, and its RPS reached 78.57% when using twice the amount of DNA vaccine (30 μ g), an increase of 17.86%. Furthermore, it further increased by only 7.51% at a dosage of 45 μ g, which is far lower than the dosage of 30 μ g. However, the statistical test showed that it is only significant difference between using a dosage of 45 μ g comparing with those of a dosage of 15 μ g. Therefore, more DNA vaccine (>45 μ g) used, RPS can be increased significantly. In addition, to determine this feasibility, the viral challenge experiments have been repeated, and the similar results were obtained. Interestingly, the survived fish injected by control plasmid have less virus amount in their tissues than dead fish injected with the DNA plasmid. We suppose that despite the fish injected with DNA vaccine, the protective effect of the vaccine is not a 100%. Therefore, the dead fish injected with the DNA plasmid have more virus is understandable. DNA vaccine is another new type of vaccine after inactivated vaccine, attenuated vaccine, subunit vaccine and recombinant peptide vaccine. Compared with traditional vaccines, it has many advantages such as simple preparation, stability, relative low cost. At present, DNA vaccine has been used in human, animal and fish for controlling or reducing virus infection (Yu et al., 2019c; Yu et al., 2020; Matamoros et al., 2020; Corbeil et al., 2000). However, considering the cost price and profit, the DNA vaccine could be used by the method of immersion vaccine or other economical ways. The tilapia production center is located in a tropical and subtropical region, which may result in TiLV outbreaks when water temperature

ranges from 22 °C to 32 °C (Eyngor et al., 2014). In short, this study suggested a viral protein from TiLV *ORF10* could be used for against TiLV.

In conclusion, our results showed that pcDNA3.1-ORF10, a DNA vaccine encoding a protein with five epitopes, conferred effective protection against TiLV challenge in tilapia. Moreover, pcDNA3.1-ORF10 expressing TiLV-ORF10 enhanced the innate immune response in tilapia, which is essential for combating TiLV infections.

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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.

Compliance with ethical standards

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).

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Supplementary Materials

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/xxx/fmicb>.

Supplementary Figure 1 | The antibody epitope prediction of 10 putative proteins encoded by TiLV.

References

- Al-Hussine, L., Subramaniam, K., Ahasan, M.S., Keleher, B., and Waltzek, T.B. (2018). Complete genome sequence of a Tilapia lake virus isolate obtained from Nile tilapia (*Oreochromis niloticus*), *Genome Announc.* 6, e00580-18. doi: 10.1128/genomeA.00580-18
- Amend, D.F. (1981). Potency testing of fishing vaccines [C]//International Symposium on Fish Biologies: Serodiagnostics and Vaccines. Leetown: Developments in biological standardization, 49: 447–454.

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- Bergmann, S.M., Monro, E.S., and Kempter, J. (2017). Can water disinfection prevent the transmission of infectious koi herpesvirus to naive carp? - a case report. *J Fish Dis.* 40, 885-893. doi: 10.1111/jfd.12568.
- Chang, C.J. (2020). Immune sensing of DNA and strategies for fish DNA vaccine development. *Fish Shellfish Immunol.* 101, 252–260. doi: 10.1016/j.fsi.2020.03.064.
- Chen, D.D., Yao, Y.Y., Cui, Z.W., Zhang, X.Y., Peng, K.S., Guo, X., et al. (2018a). Comparative study of the immunoprotective effect of two DNA vaccines against grass carp reovirus. *Fish Shellfish Immunol.* 75, 66–73. doi: 10.1016/j.fsi.2018.01.047
- Chen, Z.Y., Wang, R.H., Liu, Z.X., Yu, N.T. (2021). Subcellular localization of Tilapia Lake Virus ORF10 protein and its tissue expression analysis. *Journal of Fishery Sciences of China.* 28, 896–902. doi: 10.12264/JFSC2020-0583
- Corbeil, S., LaPatra, S.E., Anderson, E.D., and Kurath, G. (2000). Nanogram quantities of a DNA vaccine protect rainbow trout fry against heterologous strains of infectious hematopoietic necrosis virus. *Vaccine.* 18: 2817-24. doi: 10.1016/s0264-410x(00)00078-5
- Eyngor, M., Zamostiano, R., Kembou Tsofack, J.E., Berkowitz, A., Bercovier, H., Tinman, S., et al. (2014). Identification of a novel RNA virus lethal to tilapia. *J. Clin. Microbiol.* 52, 4137–4146. doi: 10.1128/JCM.00827-14
- FAO. (2016). The state of world fisheries and aquaculture, 2016. Contributing to food security and nutrition for all. Retrieved from Rome: <http://www.fao.org/3/a-i5555e.pdf>
- Fujisawa, T., and Filippakopoulos, P. (2017). Functions of bromodomain-containing proteins and their roles in homeostasis and cancer. *Nat Rev Mol Cell Biol.* 18, 246–262. doi: 10.1038/nrm.2016.143
- Hu, F., Li, Y., Wang, Q., Wang, G., Zhu, B., Wang, Y., et al. (2019). Carbon nanotube-based DNA vaccine against koi herpesvirus given by intramuscular injection. *Fish Shellfish Immunol.* 98, 810–818. doi: 10.1016/j.fsi.2019.11.035
- Jaemwimol, P., Sirikanchana, K., Tattiyapong, P., Mongkolsuk, S., and Surachetpong, W. (2019). Virucidal effects of common disinfectants against tilapia lake virus. *J. Fish Dis.* 42, 1383–1389. doi: 10.1111/jfd.13060
- Jain, A.K., and Barton, M.C. (2017). Bromodomain Histone Readers and Cancer. *J. Mol. Biol.* 429, 2003–2010. doi: 10.1016/j.jmb.2016.11.020.
- Jespersen, M.C., Peters, B., Nielsen, M., and Marcatili, P. (2017). BepiPred-2.0: Improving sequence-based B-cell epitope prediction using conformational epitopes. *Nucleic Acids Res.* 45, W24–W29. doi: 10.1093/nar/gkx346

-
- Kembou Tsofack, J.E., Zamostiano, R., Watted, S., Berkowitz, A., Rosenbluth, E., Mishra, N., et al. (2017). Detection of Tilapia lake virus in clinical samples by culturing and nested reverse transcription-PCR. *J. Clin. Microbiol.* 55, 759–767. doi: 10.1128/JCM.01808-16
- Livak, K.J., and Schmittgen, T.D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C_t}$. *Method.* 25, 402–408. doi: 10.1006/meth.2001.1262
- Mai, T.T., Kayansamruaj, P., Taengphu, S., Senapin, S., Costa, J.Z., Del-Pozo, J., et al. (2021). Efficacy of heat-killed and formalin-killed vaccines against Tilapia tilapinevirus in juvenile Nile tilapia (*Oreochromis niloticus*). *J Fish Dis.* doi: 10.1111/jfd.13523.
- Matamoros, T., Alejo, A., Rodríguez, J.M., Hernáez, B., Guerra, M., Fraile-Ramos, A., et al. (2020). African Swine Fever Virus Protein pE199L Mediates Virus Entry by Enabling Membrane Fusion and Core Penetration. *mBio.* 11, e00789-20. doi: 10.1128/mBio.00789-20
- Mugimba, K.K., Chengula, A.A., Wamala, S., Mwega, E.D., Kasanga, C.J., Byarugaba, D.K., et al. (2018). Detection of tilapia lake virus (TiLV) infection by PCR in farmed and wild Nile tilapia (*Oreochromis niloticus*) from Lake Victoria. *J. Fish Dis.* 41, 1181–1189. doi: 10.1111/jfd.12790
- Nicholson, P., Fathi, M.A., Fischer, A., Mohan, C., Schieck, E., Mishra, N., et al. (2017). Detection of Tilapia lake virus in Egyptian fish farms experiencing high mortalities in 2015. *J. Fish Dis.* 40, 1925–1928. doi: 10.1111/jfd.12650
- Skornik, R., Eyngor, M., Behar, A., Markovich, M.P., Wajsbrot, N., Klement, E., et al. (2019). Tilapia lake virus disease: Phylogenetic analysis reveals that two distinct clades are circulating in Israel simultaneously. *Transbound Emerg. Dis.* 67, 494–501. doi: 10.1111/tbed.13407
- Subramaniam, K., Ferguson, H.W., Kabuusu, R., and Waltzek, T.B. (2019). Genome sequence of Tilapia lake virus associated with syncytial hepatitis of tilapia in an Ecuadorian aquaculture facility. *Microbiol. Resour. Announc.* 8, e00084-19. doi: 10.1128/MRA.00084-19
- Surachetpong, W., Janetanakit, T., Nonthabenjawan, N., Tattiyapong, P., Sirikanchana, K., and Amonsin, A. (2017). Outbreaks of Tilapia lake virus infection, Thailand, 2015-2016. *Emerg. Infect. Dis.* 23, 1031–1033. doi: 10.3201/eid2306.161278
- Surachetpong, W., Roy, S.R.K., and Nicholson, P. (2020). Tilapia lake virus: The story so far. *J. Fish Dis.* 43, 1115–1132. doi: 10.1111/jfd.13237
- Thammatorn, W., Rawiwan, P., and Surachetpong, W. (2019). Minimal risk of tilapia lake virus transmission via frozen tilapia fillets. *J. Fish Dis.* 42, 3–9. doi: 10.1111/jfd.12924
- Thawornwattana, Y., Dong, H.T., Phiwsaiya, K., Sangsuriya, P., Senapin, S., and Aiewsakun, P. (2021). Tilapia lake virus (TiLV): Genomic epidemiology and its early origin. *Transbound Emerg. Dis.* 68, 435–444. doi: 10.1111/tbed.13693

-
- Wang, Y., Wang, Q., Zeng, W., Yin, J., Li, Y., Ren, Y., et al. (2018). Establishment and characterization of a cell line from tilapia brain for detection of tilapia lake virus. *J. Fish Dis.* 41, 1803–1809. doi: 10.1111/jfd.12889
- Yu, J., Tostanoski, L.H., Peter, L., Mercado, N.B., McMahan, K., Mahrokhian, S.H., et al. (2020). DNA vaccine protection against SARS-CoV-2 in rhesus macaques. *Science.* 369, 806–811. doi: 10.1126/science.abc6284
- Yu, N.T., Zhang, Y.L., Xiong, Z., Qu, L., and Liu, Z.X. (2019a). A simplified method for the simultaneous detection of nervous necrosis virus and iridovirus in grouper *Epinephelus* spp. *Acta Virol.* 63, 80–87. doi: 10.4149/av_2019_107
- Yu, N.T., Xie, H.M., Zhang, Y.L., Wang, J.H., Xiong, Z., and Liu, Z.X. (2019b). Independent modulation of individual genomic component transcription and a cis-acting element related to high transcriptional activity in a multipartite DNA virus. *BMC Genomics* 20, 573. doi: 10.1186/s12864-019-5901-0
- Yu, N.T., Zheng, X.B., and Liu, Z.X. (2019c). Protective immunity induced by DNA vaccine encoding viral membrane protein against SGIV infection in grouper. *Fish Shellfish Immunol.* 92, 649–654. doi: 10.1016/j.fsi.2019.06.051
- Zhao, Z., Zhang, C., Lin, Q., Li, N.Q., Huang, Z.B., Zhao, M., et al. (2020) Single-walled carbon nanotubes as delivery vehicles enhance the immunoprotective effect of an immersion DNA vaccine against infectious spleen and kidney necrosis virus in mandarin fish. *Fish Shellfish Immunol.* 97, 432–439. doi: 10.1016/j.fsi.2019.12.072

Table 1 Primers used in this study.

Primers	Primers sequences (5'-3')	Usage
TiLVORF10-F	AAATCTTCCCTCTGACACCC	RT-PCR
TiLVORF10-R	CAGGATGAGTGTGGCAGATT	
TiLVORF10-F2	GGgaattcATGCACCATCATCATCATATGAG-TGTGGCAGATTATTTGTC (<i>EcoRI</i>)	pcDNA3.1-ORF10
TiLVORF10-R2	GGgatatcCTAAGACTGCACGTCAAGAGAC (<i>EcoRV</i>)	
TiLVRNA1-F	GTGGGTTGTTCCAAGCCTAAGGAC	RT-PCR
TiLVRNA1-R	CACTCTAGTAGAGTGTCCAGGAGG	
TiLVRNA1-qF	GGCATGTGGGGAGGAAGTCCTC	RT-qPCR
TiLVRNA1-qR	CAGGGGACCACTTGTGACTCTGA	
IgM-qF	AGGAGACAGGACTGGAATGCACAA	RT-qPCR
IgM-qR	GGAGGCAGTATAGGTATCATCCTC	
TLR2-qF	GTATCTCAGTGCTCGTCGCTCA	RT-qPCR
TLR2-qR	TTTCATTATCGTCTCCAGTGCG	
MyD88-qF	TTTACGCTTCCTCACCATTGT	RT-qPCR
MyD88-qR	CCGCCTGCTCCACAGTTAT	
IL8-qF	GCACTGCCGCTGCATTAAG	RT-qPCR
IL8-qR	GCAGTGGGAGTTGGGAAGAA	
TNF α -qF	GAGGTCGGCGTGCCAAGA	RT-qPCR
TNF α -qR	TGGTTTCCGTCCACAGCGT	
IFN γ -qF	TGACCACATCGTTCAGAGCA	RT-qPCR
IFN γ -qR	GGCGACCTTTAGCCTTTGT	
NF-kB-qF	CGACCACTACCTACACGCTC	RT-qPCR
NF-kB-qR	GATGTCGTTTGAGGCATCGC	
β -actin-qF	AACAACCACACACCACACATTTTC	RT-PCR, RT-qPCR

β -actin-qR	TGTCTCCTTCATCGTTCCAGTTT
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Table 2 Cumulative mortality and relative percentage survival (RPS) of vaccinated tilapia challenged with tilapia lake virus (TiLV).

Vaccinated tilapia	Cumulative mortality (death/total)	RPS%	Duncan's range test
pcDNA3.1–ORF10 (c)	13.33% (4/30)	85.72	cd
pcDNA3.1–ORF10 (b)	20.00% (6/30)	78.57	bc
pcDNA3.1–ORF10 (a)	36.67% (11/30)	60.71	b
pcDNA3.1	93.33% (28/30)	-	a

RPS = [1 - (death vaccinated group/total vaccinated group)/(death control group/total control group)] × 100%. ^{a, b, c, d} Different letters in the same column indicate significant differences (p < 0.05).

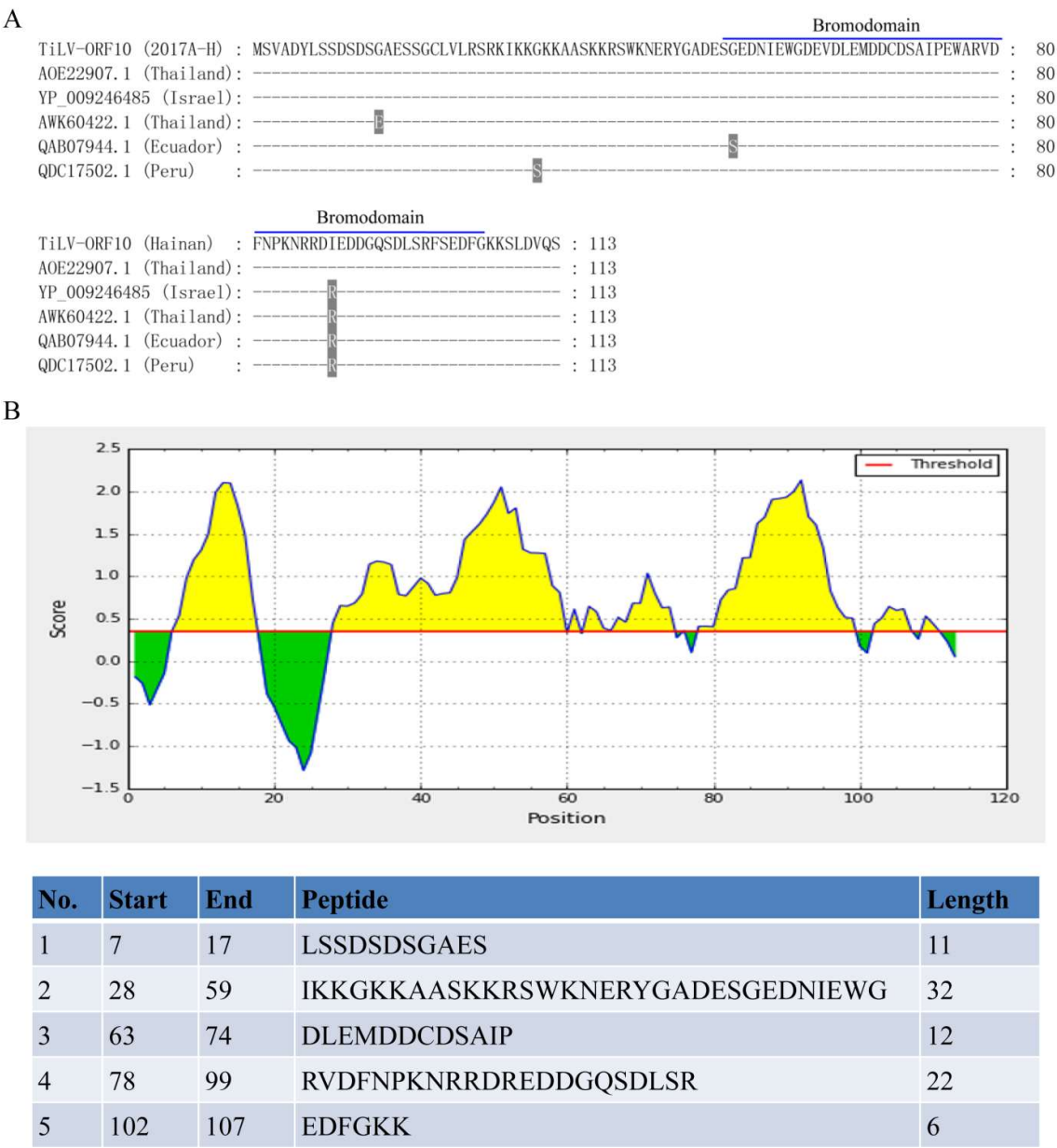


Figure 1. Multiple sequences alignment of TiLV-ORF10 and its antibody epitope prediction. **(A)**, multiple sequences alignment of TiLV-ORF10 and its homologues. Bromodomains, 51~105 aa, are shown under the blue line. **(B)**, the antibody epitope prediction of TiLV-ORF10. The five-peptide sequences with antigenicity are shown.

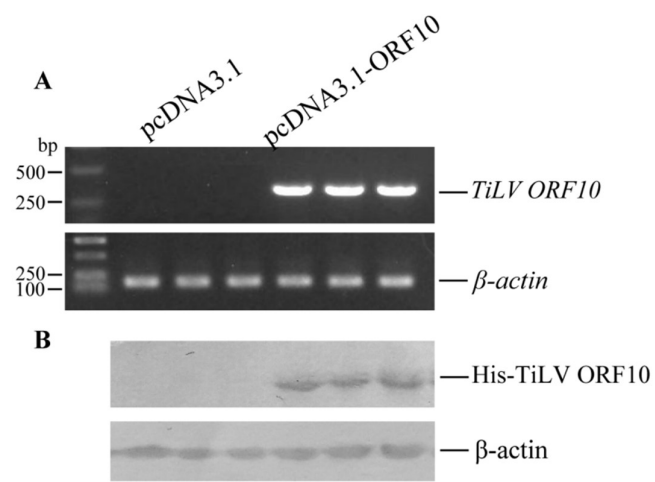


Figure 2. Transcription and expression of *TiLV-ORF10* in vaccinated tilapia muscles. RT-PCR (A) and western blot (B) analysis of transcription and expression of the *TiLV-ORF10* gene in muscle tissues from vaccinated tilapias at 7 dpv (n = 3). β -actin mRNA and its protein were used as internal controls.

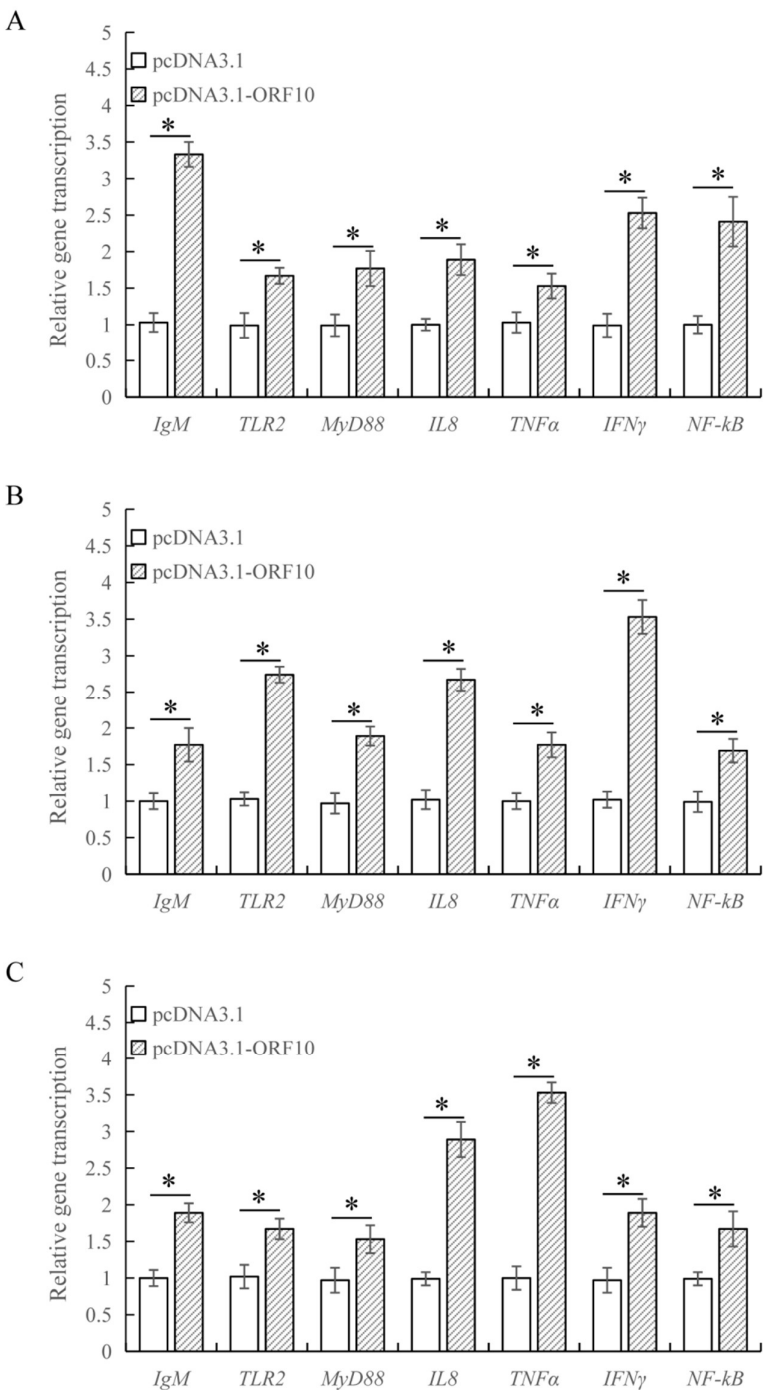


Figure 3. RT-qPCR analysis of transcription levels of *IgM*, *TLR2*, *MyD88*, *IL8*, *TNFα*, *IFNγ*, and *NF-κB* genes in spleen (A), liver (B), and kidney (C) tissues from vaccinated tilapias. The mRNA level of each gene was normalized to that of *β-actin* mRNA. For each gene, the mRNA level of the 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.

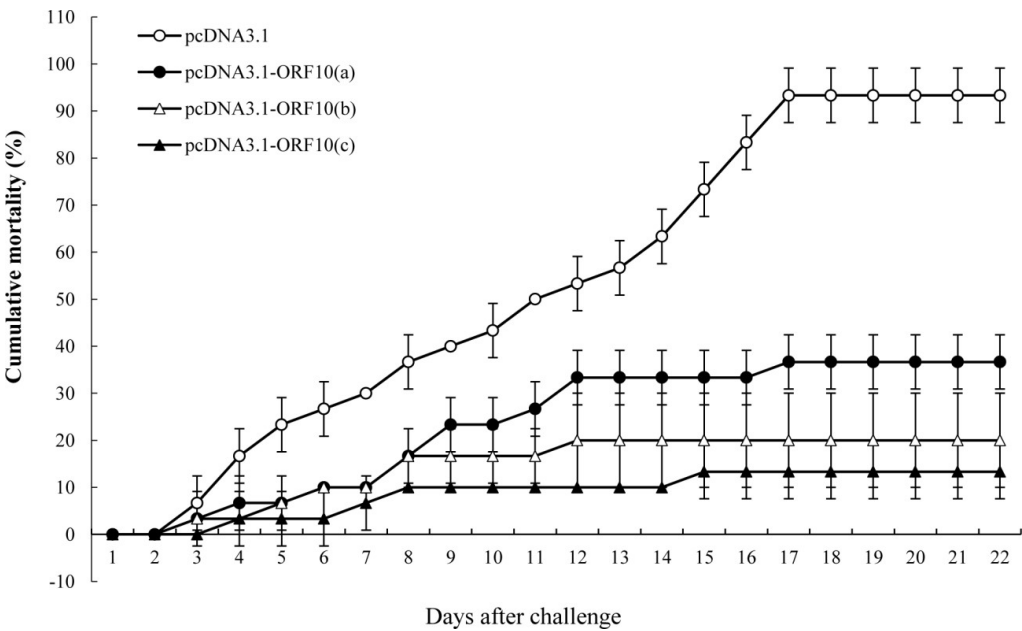


Figure 4. Cumulative mortality curves of vaccinated tilapias upon challenge with tilapia lake virus (TiLV). Tilapias vaccinated with pcDNA3.1, pcDNA3.1–ORF10(a), pcDNA3.1–ORF10(b), pcDNA3.1–ORF10(c) were intraperitoneally challenged with TiLV and monitored daily for mortality over a 22 dpi observation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

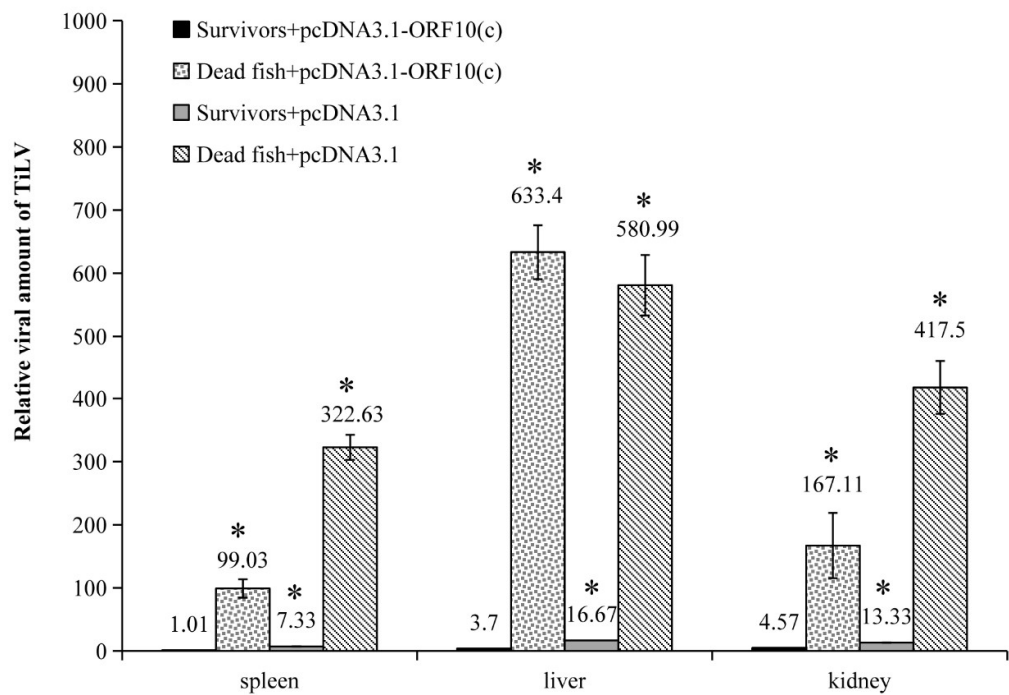


Figure 5. RT-qPCR analysis of the virus amount of TiLV. RT-qPCR analysis of the virus amount of TiLV in spleen, liver, and kidney tissues of dead fish and survivors from the pcDNA3.1-ORF10(c) group and pcDNA3.1 group. The virus amount of TiLV in spleen tissues of pcDNA3.1-ORF10 vaccinated surviving tilapias was set as 1. Data are presented as the mean \pm SE (n = 3). *P < 0.05.