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Inactivated Tilapia Lake Virus Vaccine Formulated with Montanide™ ISA 763A VG Adjuvant Resulted in Inadequate Protection and Poor Immunity in Vaccinated Nile Tilapia

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Abstract: *Tilapia tilapinevirus*, or tilapia lake virus (TiLV), is a virus that has caused disease outbreaks in many countries worldwide since 2009. We recently developed two simple but effective TiLV vaccines based on heat-killed virus (HKV) and formalin-killed virus (FKV). In the present study, we evaluated the immunological response and efficacy of Montanide™ ISA 763A VG oil-based vaccines (O-HKV and O-FKV) in Nile tilapia (*Oreochromis niloticus*) and compared these responses in fish vaccinated with water-based formulations (W-HKV and W-FKV). Juvenile Nile tilapia were given primary and booster vaccinations with the vaccine formulations. The relative fold change in expression of three immunoglobulin genes (*IgM, IgT*, and *IgD*) was assessed before and after booster vaccination, and a challenge with TiLV was performed to evaluate the efficacy of the vaccines. The results showed that the water-based vaccines induced higher levels of *Ig* transcripts and conferred significantly higher levels of protection compared to the oil-based formulations, with relative percent survival (RPS) values ranging from 54.6% to 59.7% for W-HKV and W-FKV vaccinated fish, and 32% and 0% for the O-HKV and O-FKV vaccinated fish, respectively. These findings suggest that water-based vaccines are more effective at protecting tilapia against TiLV infection, and Montanide™ ISA 763A VG may not be a suitable adjuvant for formulating whole-inactivated TiLV vaccines.

Keywords: tilapia lake virus; TiLV; Nile tilapia; vaccine; adjuvants; Montanide™ ISA 763A VG

1. Introduction

Tilapia (*Oreochromis* spp.) is one of the most common fish species farmed worldwide (over 140 countries) because of their many attributes, including their high protein content, their large size and rapid growth, and their ability to adapt to different geographical locations [1]. The increasing demand for protein from aquaculture has seen an intensification of tilapia farming systems [2]. This has led to increased disease outbreaks caused by bacteria, viruses, parasites, and fungi. Massive die-offs due to disease have resulted in substantial economic losses to tilapia producers and related sectors [3].

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Tilapia lake virus (TiLV) or *Tilapinevirus tilapiae* is an emerging RNA virus affecting both wild and farmed tilapia. The virus has now been reported in 17 countries, with cumulative mortalities due to TiLV infection ranging from 20 to 90% [4]. The initial signs of infection include loss of appetite, erratic swimming, darkening skin, and scale protrusion, followed by more serious symptoms such as abdominal extension, ocular alteration, pale gills, necrosis of internal organs and fin loss [5,6]. The virus has been shown to be transmitted both horizontally and vertically [7,8]. Therefore, initiatives to maintain a TiLV-free status for tilapia broodstock and their progeny are important for controlling TiLV disease in tilapia aquaculture.

Vaccination is one of the effective solutions for fish health management to mitigate against disease outbreaks by triggering a specific immune response against the pathogen [3,9]. Some injectable vaccines have been reported to be efficacious against TiLV infections in tilapia. The first vaccines developed against TiLV were based on attenuated viral strains, which underwent 17 and 20 passages through cell culture, called strains P17 and P20. These vaccines resulted in relative percentage survival (RPS) values of over 50% [10]. Development of DNA and recombinant vaccines based on segment 8, administered with adjuvant M402, resulted in higher survival levels in vaccinated fish compared to unvaccinated fish [11]. Another study by the same research group demonstrated that inactivated whole virus vaccines, combined with adjuvant Montanide IMS 1312VG, were relatively effective in protecting tilapia against TiLV, with RPS values ranging from 32.1% - 85.7%, depending on the virus concentration in the vaccine [12]. Meanwhile, water-based, heat-killed, and formalin-killed vaccines resulted in RPS values of 71.3% and 79.6%, respectively [13]. A subsequent study with these vaccines found that broodstock immunization with water-based inactivated TiLV vaccines produced protective antibodies that were transferred to offspring, indicating that broodstock immunization could potentially prevent vertical transmission of TiLV [14]. Recently, a nano chitosan-based, inactivated whole virus vaccine developed for immersion delivery to small fish, resulted in a relatively high RPS value of 68.17% compared to 25.01% for the group receiving the non-encapsulated inactivated vaccine [15].

Several studies have demonstrated that combining potential antigens with adjuvants can improve co-stimulatory signals, prolong antigen release, and activate lymphocyte proliferation. However, the effectiveness of the resulting vaccines can vary depending on the type of antigen used [16–18]. Water in oil (w/o) emulsion is widely used to formulate vaccines for aquatic animals. The antigen is deposited in the oil phase, which can enhance antigen delivery to antigen-presenting cells, prolong specific immune responses, and attract more effective lymphocytes to the injection site, leading to stronger localized immune responses [19–21]. Various w/o adjuvanted vaccines have been shown to be effective formulations for fish and are often administered via intraperitoneal (IP) injection. Montanide™ ISA 763A VG is one such adjuvant that has been found to be a non-mineral metabolizable oil-based adjuvant. It has been reported to strongly stimulate humoral immunity and be a highly effective adjuvant for bacterin vaccines in a variety of fish species, such as Atlantic salmon (*Salmo salar*), rainbow trout (*Oncorhynchus mykiss*), turbot (*Scophthalmus maximus* L.), tilapia (*O. niloticus*), etc. [20,22–24]. Recently, Montanide™ ISA 763A VG was used as an adjuvant for a recombinant TiLV vaccine expressed from TiLV segments 9 and 10, showing relatively good levels of protection with an RPS value of 55.56% [25]. Although Montanide™ ISA 763A VG is a potential adjuvant and TiLV water-based inactivated vaccines have demonstrated high efficacy, there are currently no reports of its use in TiLV-inactivated vaccinations. Thus, the objective of this study was to investigate whether this adjuvant can enhance immunological responses and the efficacy of TiLV-inactivated vaccines.

2. Materials and Methods

2.1. Fish

Fingerling Nile tilapia weighing 7.3 ± 1.2 g were kindly provided by Kasetsart University, Thailand. The fish were acclimatized for seven days in 100 liter tanks supplied with fresh water at a density of 45 fish per tank. The water temperature ranged from 25 °C to 28 °C. Water parameters (pH,

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NO_2, NH_3) were checked every two day using standard water quality kits (Sera, Germany), and water was changed two times per week. Five fish, randomly selected, were confirmed TiLV-free by real-time PCR [26]. Approval for the use of animals in this study was granted by Kasetsart University Institutional Animal Care and Use Committee (ACKU62- FIS- 008).

2.2. Virus Culture

TiLV propagation was performed as described in a previous study [14]. Briefly, TiLV-KT strain was propagated on E11 cells in Leibovitz's L15 medium (Sigma, Saint Louis, MO, USA) containing 5% fetal bovine serum for 5–7 days or until a cytopathic effect (CPE) of around 80%–90% was obtained. The viral supernatant was clarified by centrifugation at 4500 g for 10 min to remove cell debris. The virus was quantified as a 50% tissue culture infectious dose ($TCID_{50}$ ml^{-1}) [27]. The cell culture supernatant containing the virus was used for vaccine preparation.

2.3. Vaccine Preparation

Water-based heat-killed and formalin-killed vaccines were prepared as described in previous studies [13]. Briefly, the clarified viral supernatant was inactivated by heating at 60 °C for 2.5 h or incubating with 0.006% formaldehyde solution at 25 °C for 24 h to generate W-HKV and W-FKV, respectively. Virus-free cell-culture supernatant was used as a control (W-C) for water-based vaccine treatment. Virus inactivation was confirmed by incubating the inactivated viral solutions on E11 cells and observing for 7 days for the absence of a CPE. Oil-based O-HKV and O-FKV were generated by mixing the water-based vaccines with adjuvant Montanide ISA 763A VG (Seppic, France) at a ratio of 3:7 using a high shear homogenizer (IKA T25 digital Ultra Turrax). Virus-free cell-culture supernatant mixed with adjuvant was used as control (O-C) for oil-based vaccine treatment. Vaccines were stored at 4 °C until used.

2.4. Immunization, Sampling, and Challenge Test

The study consisted of 6 groups, with 45 fish per group. Fish in treatment groups 1, 2 and 3 were IP injected with 100 µL of W-C, W-HKV and W-FKV, while fish in treatment groups 4, 5 and 6 were injected IP with 100 µL of O-C, O-HKV and O-FKV, respectively (Table 1). Booster vaccinations were performed in the same manner 2 weeks post-primary vaccination (wppv). Before sampling, fish were anaesthetized with clove oil at 100 ppm. Three fish were randomly selected from every treatment, followed by sampling the spleen and head kidney for immune gene expression analysis at 7, 14 and 21 days post-primary vaccination (dppv). Tissues were placed immediately into 250 µl RNA later solution (Sigma) and stored at -20°C until RNA extraction. For the challenge test, virus stock ($TCID_{50}$ is $10^{7.25}$ mL^{-1}) was diluted 100X. The remained fish from 6 treatments (see Table 1) were injected IP with 100 µl of diluted viral solution. The cumulative mortality was recorded for the next 15 days. The RPS value was calculated using the following formula:

$$\text{RPS} = \left(1 - \frac{\%\ \text{cumulative mortality of vaccinated group}}{\%\ \text{cumulative mortality of control}}\right) \times\ 100\%$$

Table 1. Details of experiment groups and challenge results.

Treatment	Abbreviation	Number of fish Challenged	Number of Total Dead Fish at 15 dpc	RPS (%) 15 dpc
Water based (W)				
Control (Cell culture supernatant, L15)	W-C	31	5	
Heat-killed vaccine	W-HKV	27	2	54.6
Formalin-killed vaccine	W-FKV	31	2	59.7
Oil-based (O) (Montanide™ ISA 763A VG)				
Control (L15 + Oil adjuvant)	O-C	32	8	

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Heat-killed vaccine	O-HKV	28	5	32.0
Formalin-killed vaccine	O-FKV	24	9	0

dpc, day post challenge; RPS: relative percentage survival.

2.5. Immune Gene Expression by RT-qPCR

The relative fold change of immunoglobulin genes *IgM*, *IgT* and *IgD* was investigated. RNA samples were extracted from head kidney and spleen using Trizol (Invitrogen) following the protocol from the producer. DNA contamination in RNA samples was removed using DNAseI (Ambicon, USA) according to the manufacturer's procedure. RNA samples were re-purified using phenol-chloroform (5:1, pH 4.7) and their qualities were determined by Nanodrop (Thermo Scientific, USA). iScriptTM Reverse Transcription Supermix (Bio- Rad) was used to synthesize cDNA with 100 ng RNA template. The protocol steps included incubation at 25 °C for 5 min for priming, followed by 46 °C for 20 min for reverse transcription and then 95°C for 1 min for inactivation of the reverse transcriptase. Immunoglobulin gene *IgM*, *IgT* and *IgD* expression were analyzed using quantitative real-time PCR (RT-qPCR) using the primers listed in Table 2 and iTaq Universal SYBR Supermix (Bio-rad, USA). The 20 µl reaction consisted of 10.0 µl 2X Supermix, 1.0 µl forward and reverse primers (10 µM each), 1.0 µl cDNA and 7.0 µl distilled water. The protocol for qPCR comprised of one step for initial activation at 95 °C for 2 mins, followed by 40 cycles of denaturation at 95 °C for 30 s, annealing at the optimal temperature of each primer pair for 30 s (Table 2) and extension at 72 °C for 30 s. Melt curves were analyzed from 65 °C to 95 °C during the heating process. *EF-1α* gene was used as a housekeeping gene for normalization in gene expression of vaccinated and control fish using the $2^{-\Delta\Delta Ct}$ method [28].

Table 2. Detail of primers used for gene expression.

Gene	Oligo Sequences	Annealing Temperature (°C)	Product Size (bp)	Reference
EF-1α	F-5′-CTACAGCCAGGCTCGTTTCG-3′ R-5′-CTTGTCACTGGTCTCCAGCA-3′	56	139	[30]
IgM	F-5′-GGATGACGAGGAAGCAGACT-3′ R-5′-CATCATCCCTTTGCCACTGG-3′	53	122	[30]
IgT	F-5′-TGACCAGAAATGGCGAAGTCTG-3′ R-5′-GTTATAGTCACATTCTTTAGAATTACC-3′	53	163	[30]
IgD	F-5′- AACACCACCCTGTCCCTGAAT- 3′ R-5′-GGGTGAAAACCACATTCCAAC- 3′	61	127	[31]

2.6. Statistical Analysis

The gene expression data were compared using one way ANOVA and LSD post-hoc test. Kaplan-Meier survival analysis was performed to illustrate the cumulative mortality. Graphpad prism 6 (Graphpad software, USA) was used to generate the graphs.

3. Results

3.1. Immunoglobulin Gene Expression

The mRNA transcript levels of three immunoglobulin genes (*IgM*, *IgD* and *IgT*) were compared between vaccinated and control groups (Table 3). In the O-HKV treatment group, a significant up-regulation of *IgT* and *IgD* transcripts (almost 3-fold) was observed in the head-kidney ($p< 0.05$) 7-dppv, followed by a significant increase in *IgD* mRNA after the booster vaccination (21-dppv) compared to the control. Only *IgD* transcripts were significantly up-regulated (around two times) in the spleen after the booster vaccination ($p<0.05$). In the O-FKV group, mild upregulation of *IgD* was

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observed at 7 and 21-dppv. There was an increase in *IgM* transcript in the O-HKV treatment group at 14 dppv before the booster, but no statistical differences were observed at the time point examined.

Table 3. The fold change of *IgM*, *IgT* and *IgD* expression in head kidney and spleen for the oib-based and water-based vaccine groups at 7, 14, and 21 days post-immunization (↑: the up-regulation of gene with the significant value $p<0.05$; ↑↑: the up-regulation of gene with the significant value $p< 0.01$).

Time Points	Treatments	Head Kidney			Spleen		
		IgM	*IgT*	*IgD*	*IgM*	*IgT*	*IgD*
D7	O-C	1.07 ± 0.46	1.02 ± 0.20	1.07 ± 0.44	1.51 ± 1.60	1.20 ± 0.75	1.05 ± 0.38
	O-HKV	1.75 ± 0.78	**3.14 ± 0.73↑**	**2.93 ± 1.24↑**	1.14 ± 0.45	0.90 ± 0.49	1.00 ± 0.78
	O-FKV	1.53 ± 0.47	1.61 ± 0.99	**1.89 ± 0.11 ↑**	1.51 ± 0.94	0.93 ± 0.75	2.04 ± 1.34
	W-C	1.55 ± 1.77	1.48 ± 1.61	1.07 ± 0.44	1.72 ± 1.97	1.79 ± 2.09	1.24 ± 0.86
	W-HKV	0.96 ± 0.74	0.55 ± 0.32	1.19 ± 0.86	1.04 ±1.02	1.09 ± 0.96	1.23 ± 0.86
	W-FKV	**6.00 ± 2.81↑**	1.43 ± 0.85	**3.80 ± 1.81↑**	0.31 ± 0.12	0.38 ± 0.22	0.42 ± 0.16
D14	O-C	1.58 ± 1.21	1.09 ± 0.48	1.01 ± 0.17	1.19 ± 0.70	1.67 ± 1.72	1.20 ± 0.89
	O-HKV	4.94 ± 4.11	0.77 ± 0.45	1.46 ± 0.92	1.09 ± 0.80	1.00 ± 0.67	1.22 ± 1.04
	O-FKV	1.84 ± 2.25	0.37 ± 0.36	1.08 ±1.14	1.81 ± 0.87	1.11 ± 0.32	2.71 ± 1.71
	W-C	1.44 ± 1.05	0.96 ± 0.80	1.53 ± 1.70	1.43 ± 1.19	2.31 ± 2.80	1.66 ± 1.88
	W-HKV	1.81 ± 0.64	1.09 ± 0.53	1.35 ± 0.45	**6.91 ± 3.32↑**	6.07 ± 4.82	4.12 ± 1.40
	W-FKV	7.61 ± 8.34	2.93 ± 2.19	3.46 ± 2.27	4.30 ± 3.22	**5.89 ± 0.41↑**	**5.69 ± 1.15↑**
D21	O-C	1.16 ± 0.80	1.02 ± 0.22	1.04 ± 0.33	1.02 ± 0.25	1.21 ± 0.94	1.02 ± 0.25
	O-HKV	0.91 ± 0.42	1.44 ± 0.40	**3.22 ± 0.44↑↑**	0.70 ± 0.24	1.15 ± 0.32	**2.12 ± 0.56 ↑**
	O-FKV	1.91 ± 0.26	0.90 ± 0.13	**2.09 ± 0.45↑**	1.60 ± 2.38	0.43 ± 0.52	2.20 ± 3.05
	W-C	1.36 ±1.30	1.29 ± 0.93	1.17 ± 0.82	1.10 ± 0.55	1.70 ± 1.82	1.11 ± 0.65
	W-HKV	1.25 ± 0.94	1.20 ± 1.30	2.01 ±1.45	1.82 ± 1.79	2.76 ± 2.03	2.23 ± 2.55
	W-FKV	0.83 ±0.66	0.66 ± 0.53	0.93 ± 0.61	0.37 ± 0.10	0.79 ± 0.59	0.57 ± 0.19

For the water-based vaccine groups, all immunoglobulin transcript levels were up-regulated in the head kidney of fish vaccinated with W-FKV with significant increases in *IgM* and *IgD* at 7- dppv in the head-kidney (with 6- and 3-fold changes, respectively). In the spleen, at 14-dppv, up-regulation of *IgM* transcripts was observed ($p < 0.05$) in fish vaccinated with W-HKV (almost a 7-fold change), and an increase in *IgT* and *IgD* mRNA levels were found in fish vaccinated with W-FKV (a 6-time increase) ($p < 0.05$).

3.2. Vaccine Efficacy

After the challenge with TiLV, mortalities started to appear in the oil-based groups before the water-based groups. The first mortality was seen in O-C at 4-day post-challenge (dpc), followed by the O-FHV and O-HKV groups with moribund or dead fish found at 6- and 7-dpc, respectively (Figure 1). Meanwhile, the first mortality in the W-C group was recorded 7-dpc, whereas mortalities in the W-HKV and W-FKV groups were first observed at 8-9 dpc (Figure 1). The RPS values were 54.6% and 59.7% for W-HKV and W-FKV treatments, compared to 32.0% and 0% for O-HKV and O-FKV ($p < 0.01$) treatments, respectively (Table 1).

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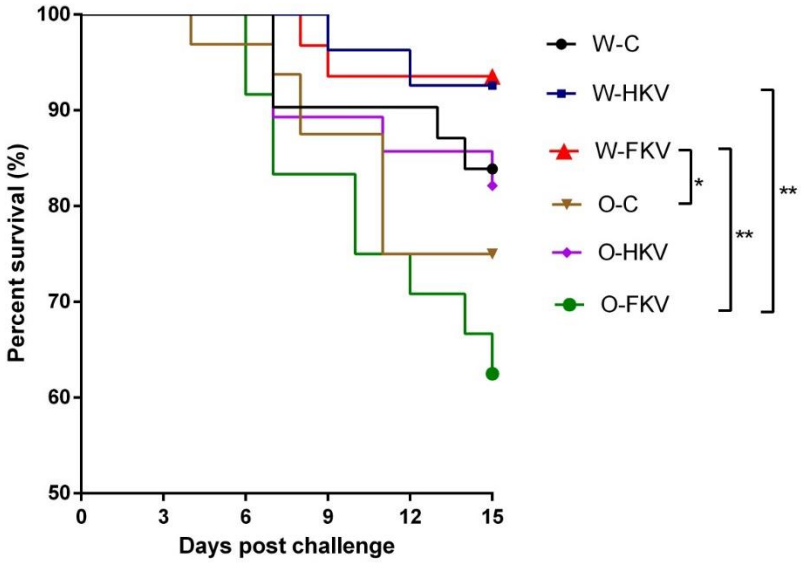

Figure 1. Percent survival of oil-based vaccine and water-based-vaccine groups compared to the control during 15-day post-challenge with TiLV (strain TiLV-KT). Level of Significant differences were indicated by the number of star with * and ** standing for the level of $p<0.05$ and $p<0.01$, respectively. Significant differences were found between O-FKV and W-HKV ($p < 0.01$), between O-FKV and W-FKV ($p < 0.01$), and between W-FKV and O-C ($p< 0.05$).

4. Discussion

Emulsification of antigens in oil-based adjuvants is commonly used in vaccine formulations for fish. These types of formulations have been shown to be effective in protecting farmed fish against certain bacterial diseases such as *Edwardsiella ictaluri*, *Edwardsiella tarda, Aeromonas salmonicida, Vibrio harveyi,* and *Streptococcus agalactiae,* for example [20,22,24,29]. However, the oil adjuvant Montanide™ ISA 763A VG might not be suitable for formulation with antigen derived from inactivated virus, such as TiLV in the current study, as this vaccine resulted in very low or no protection with RPS values of 32% and 0%, respectively, compared to the corresponding water-based vaccines, W-HKV and W-FKV (54.6 and 59.7%). This outcome may have resulted from lower concentrations of viral particles in the oil-based vaccines after formulating them with the oil adjuvant or the slower release of antigens due to the oil coating covering the virus. Veenstra et al (2021) showed that different adjuvants can elicit different degrees of responsiveness [20], suggesting that the efficacy of other adjuvants should be investigated in the TiLV vaccine to establish if they produce a more efficacious vaccine formulation. Further research should also focus on whether different antigen concentrations formulated with adjuvant affect vaccine efficacy and whether the duration of immunity post-vaccination can impact the challenge results.

This study found a correlation between the level of immunoglobulin mRNA expression and the effectiveness of vaccines. The W-HKV and W-FKV were found to stimulate the expression of three classes of *IgM, IgD* and *IgT* at 7 to 14 dppv. On the other hand, the O-HKV and O-FKV triggered slight up-regulation of *IgT* and *IgD*. The highest expression level of *IgM* mRNA (6 to 7-fold increase) was observed with the water-based vaccines, suggesting that IgM may be more important than the other immunoglobulins in protecting fish from TiLV infection. This assumption is supported by the higher protection offered by the water-based TiLV vaccines compared to the oil-based TiLV vaccines. Although levels of secreted IgM were not performed in this study, our previous studies showed that the same TiLV water-based vaccines formulations were effective in eliciting an IgM antibody response in both the serum and the mucus and provided protection when vaccinated fish were challenged with TiLV [13,14]. The level of IgM and neutralizing antibodies in fish serum after vaccination with a TiLV vaccine were also reported by Zeng et al., (2021).

In summary, these findings suggest several key considerations for developing TiLV vaccines. Firstly, water-based vaccines showed high potential for the development of injectable autogenous vaccines against TiLV. Secondly, the Montanide™ ISA 763A VG oil adjuvant may not be suitable for formulating TiLV inactivated vaccines. Lastly, the booster dose given at 3 weeks post-primary vaccination (as reported by Mai et al., 2021) is more effective in terms of immunological responses and efficacy than a booster given at 2-weeks post-primary vaccination in this study. This knowledge can be used to improve the design of future vaccination strategies against TiLV and possibly other fish pathogens.

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Data Availability Statement: The data that support the findings of this study are available on request.

Conflicts of Interest: The authors declare no conflict of interest.

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