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Research article

Co-immunization with recombinant S5$_{196–272}$ and S6$_{200–317}$ proteins for enhanced protective antibody response against Tilapia lake virus in Nile tilapia, *Oreochromis niloticus* (Linnaeus, 1758)

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Abstract

Importance of the work: Co-immunization with recombinant S5$_{196–272}$ and S6$_{200–317}$ proteins enhances protective immunity and provides insights for future TiLV vaccine development.

Objectives: To evaluate the vaccine potential of combined S5$_{196–272}$ and S6$_{200–317}$ compared to individual immunization.

Materials and Methods: A sample of Nile tilapia was divided into three main groups: immunized with S5$_{196–272}$ and S6$_{200–317}$ individually, or co-immunized. Antibody responses were measured weekly using enzyme-linked immunosorbent assay, with virus neutralization being assessed using a methylthiazolyldiphenyl-tetrazolium bromide (MTT) cell viability assay. A viral challenge test was conducted to determine the relative percentage of survival (RPS).

Results: Co-immunization of the fish with S5$_{196–272}$ and S6$_{200–317}$ resulted in a synergistic effect, leading to the higher production of S6$_{200–317}$-specific antibodies than for immunization with S6$_{200–317}$ alone. A significant increase in serum antibody levels was observed from 7 d, 21 d, 28 d and 35 d post-co-immunization. In contrast, S5$_{196–272}$-specific antibodies were generated at consistently high levels following both individual and co-immunization. The MTT cell viability assay findings demonstrated that antibodies from the co-immunization group had the highest virus-neutralizing effect (87.22% viability). Furthermore, the viral challenge assay revealed that the co-immunization group had the highest RPS (57.14%), whereas individual immunization provided no protection effect against TiLV infection.

Main finding: Co-immunization with S5$_{196–272}$ and S6$_{200–317}$ induced a synergistic antibody response and provided effective protection against TiLV in Nile tilapia.

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Introduction

Nile tilapia, *Oreochromis niloticus* (Linnaeus, 1758) is a globally traded freshwater fish, with its worldwide production reaching 4.407 million t in 2022 (Food and Agriculture Organization of the United Nations, 2022). In 2023, the global tilapia market sustains a favorable growth trend (Food and Agriculture Organization of the United Nations, 2023). Unfortunately, infectious diseases are impacting Nile tilapia farming, specifically Tilapia Lake virus (TiLV), a viral disease that causes mortality in tilapia populations resulting in considerable production losses for tilapia producers globally (Tang et al., 2021). Recently, TiLV has been reported in 17 countries, with China being the most recent addition (He et al., 2023; Kembou-Ringert et al., 2023).

Vaccination is effective in preventing and managing fish viral diseases. Specifically, among vaccine types, subunit vaccines have the advantage of safety because they use only the antigenic components of the pathogen, which cannot replicate in the host to cause disease (Hansson et al., 2000). Several subunit vaccines have been developed to prevent viral infections in fish, including: i) a VP35 protein vaccine that achieved a relative percentage of survival (RPS) of 60% against Grass Carp Reovirus (GCRV) in grass carp (Gao et al., 2018); ii) a SWCNTs-based subunit vaccine that provided up to 69.6% RPS against Infectious Spleen and Kidney Necrosis Virus (ISKNV) in mandarin fish (Zhao et al., 2019); and iii) a VP20 protein-based vaccine that yielded an RPS of 51.3% against TiLV infection in Nile tilapia (Zeng et al., 2021).

Based on this advantage of a subunit vaccine, Lueangyangyuen et al. (2022) explored the injection of TiLV proteins into Nile tilapia and reported that $S5_{196–272}$ induced specific antibodies at high levels, while $S6_{200–317}$ stimulated specific antibodies at lower levels. Typically, TiLV subunit vaccines are developed focusing on a single antigen. However, cocktail-subunit vaccines, which combine more than one antigen, have been reported to provide enhanced protection. For example, a study in mice demonstrated improved efficacy using a combination of HA and M1 proteins from Avian Influenza Virus (Subathra et al., 2014). In cobia (*Rachycentron canadum* [Linnaeus, 1766]) combined vaccines of rHSP60 with either rENOLASE or rGAPDH prodcued greater protection (Ho et al., 2014). Similarly, in tilapia, a vaccine combining TiLV protein segment 9 (Tis9) and segment 10 (Tis10) achieved improved outcomes (Chamtim et al., 2022). Therefore, the current study aimed to evaluate the efficacy of combining the $S5_{196–272}$ and $S6_{200–317}$ proteins in protecting tilapia against TiLV infection, compared with the use of each protein individually. The assessment included measuring specific antibody levels, performing MTT-based cell viability assay and conducting viral-challenging tests. If successful, this approach could be used for future TiLV vaccine development in tilapia aquaculture.

Materials and Methods

Experimental fish and virus

Nile tilapias with an average weight of 30 g were sourced from the Manit group, Bangkok, Thailand. Fish were randomly selected to screen for no TiLV infection using a semi-nested reverse transcriptase-polymerase chain reaction technique (Dong et al., 2017). The fish used in the experiment were maintained under controlled laboratory conditions to ensure optimal health and to minimize environmental stress. The fish were reared in aerated tanks at a water temperature of 28–29°C and a dissolved oxygen level of 5.65 ± 0.53 mg/L. Water quality was monitored daily to maintain ammonia levels at 0.41 ± 0.25 mg NH_3-N/L and nitrite concentrations below 0.03 mg NO_2-N/L. A 50% water exchange regimen was performed daily to sustain stable water conditions throughout the experimental period. The fish were acclimatized for 7 d before the experiment and fed a commercial pellet diet containing 30% protein (High grade; Charoen Pokphand Foods; Bangkok, Thailand) twice daily throughout the study. The TiLV strain NV18R used in the MTT assay and the challenge test was povidedby Assistant Professor Dr. Ha Thanh Dong. The original viral stock was $1 \times 10^{7.5}$ Tissue Culture Infectious Dose 50% ($TCID_{50}$), prepared by propagation in the E-11 cell line and quantified using the $TCID_{50}$ assay, as described by Dong et al. (2020).

Antigen preparation for immunization

The recombinant $S5_{196–272}$ or $S6_{200–317}$ proteins were expressed and purified from *Escherichia coli*, following the protocol described by Leungyangyuen et al. (2022). Briefly, recombinant pET15b-thioredoxin expression vectors encoding the peptides $S5_{196–272}$ or $S6_{200–317}$ were introduced into *E. coli* BL21 (DE3) cells. The transformed cells were cultured in 2×YT medium at 37°C with shaking at 200 revolutions per minute (rpm) until the absorbance at an optical density of 600 nm (OD_{600}) reached 0.8. Protein expression was induced with 0.1 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG), followed by incubation at 16°C with shaking at 150 rpm for 16 hr. After centrifugation, the cell pellets were resuspended in lysis buffer and lysed through sonication. The lysates were centrifuged 12,000 × g for 20 min to separate the soluble and insoluble fractions. The proteins were purified from the soluble fraction using nickel-nitrilotriacetic acid affinity chromatography under native conditions and from the insoluble fraction under denaturing conditions (6 M urea). All purified proteins were dialyzed against phosphate-buffered saline (PBS) at pH 7.4. For further purification, the $S5_{196–272}$ and $S6_{200–317}$ were concentrated using Amicon Ultra centrifugal filters (MilliporeSigma; Burlington, MA, USA) with a 10 kDa cutoff and subjected to size-exclusion chromatography using a Superdex 200 16/60 column (Cytiva; Marlborough, MA, USA) equilibrated in PBS at pH 7.4. For immunization, individual proteins ($S5_{196–272}$ or $S6_{200–317}$) were administered at 1.5 µg/ g fish body weight, emulsified with Freund's incomplete adjuvant (FIA; Thermo Fisher Scientific, Waltham, MA, USA) in a water-to-oil ratio of 1:1 using a homogenizer. For co-immunization, equal amounts of $S5_{196–272}$ and $S6_{200–317}$ (each 0.75 µg/g fish body weight) were combined and emulsified with FIA in the same manner. The control vaccine consisted of sterile PBS at pH 7.4 similarly emulsified with FIA. All formulations were freshly prepared, maintained on ice during administration and adjusted to a final volume of 100 µL per fish.

Immunization and challenge testing

In total, 300 fish were divided into four groups (75 fish per group): a $S5_{196–272}$-immunized group, a $S6_{200–317}$-immunized group, a co-immunized group and a control PBS group. Each group was subdivided into three tanks (25 fish per tank). In the two individual immunization groups, each fish was intraperitoneally injected with $S5_{196–272}$ or $S6_{200–317}$ with FIA. In the co-immunized group, each fish was intraperitoneally injected with a mixture of $S5_{196–272}$ and $S6_{200–317}$ with FIA. In the control group, each fish received an injection of a PBS and FIA mixture. After 14 d post immunization (dpi), each immunized fish received a second injection with the same vaccine formulation. Serum was pooled from nine fish per group (n = 3 replicate tanks) at 0 dpi, 7 dpi, 14 dpi, 21 dpi, 28 dpi and 35 dpi, according to the method of Lueangyangyuen et al. (2022). Serum samples were obtained from fish following anesthesia with clove oil (50 mg/L). Blood was collected via the caudal vein using sterile syringes, allowed to clot at room temperature and then centrifuged at 3,000×*g* for 10 min. The resulting serum was aliquoted and stored at –20°C until further analysis. At 42 dpi, the remaining fish (n = 15 per group) were selected randomly and transferred to separate tanks for each treatment group (one tank per group), then challenged intraperitoneally with TiLV isolate NV18R (Dong et al., 2020) at a dose of $1 \times 10^{5.5}$ per fish. Mortality was observed for 14 d post-challenge. The RPS was calculated using the formula RPS = (1 - ratio of mortality percentage in immunized group-to-mortality percentage in control group) ×100% (Amend, 1981). The survival rate of fish was analyzed using the Kaplan-Meier survival curve.

Antibody level detection from immunized fish serum

Enzyme-linked immunosorbent assay (ELISA) was used to evaluate the antibody response of the combined $S5_{196–272}$ and $S6_{200–317}$ proteins, according to the method of Lueangyangyuen et al. (2022). Briefly, $S5_{196–272}$ and $S6_{200–317}$ were coated individually in 96-well plates at 20 µg/well. After incubation and blocking using Bovine Serum Albumin (BSA), serum samples were added and incubated at 25ºC for 2 h, followed by Phosphate-Buffered Saline with Tween 20 (PBST) washing. Next, 100 µL of mouse anti-tilapia IgM purchased from Marine Leader, Bangkok, Thailand (Soonthonsrima et al., 2017). at a ratio of 1:100 in PBS was added and incubated at 4ºC for 2 h, followed by PBST washing Goat anti-mouse antibody conjugated with HRP (Jackson ImmunoResearch; Philadelphia, PA, USA) was added in each well and incubated at 4ºC for 2 hr. After washing with PBST, a ready-to-use solution of 3,3′,5,5′-tetramethylbenzidine substrate (PanReac AppliChem Darmstadt, Germany) was applied at 100 µL per well as a chromogenic reagent for Horseradish Peroxidase HRP detection. After 5 min in the dark at 25ºC, the reaction was stopped using 100 mM of H_2SO_4 and absorbance was measured at 450 nm using a microplate reader (Biorad;Hercules, CA, USA). This experiment was performed in triplicate (n = 3).

Cell viability based on methylthiazolyldiphenyl-tetrazolium bromide assay

The appropriate amount of TiLV used for the MTT assay was determined initially by infecting E11 cells with 10-fold serial dilutions of TiLV stock ($1 \times 10^{7.5}$ $TCID_{50}$). A 96-well plate containing a confluent monolayer of E11 cells was infected with 100 µL of each dilution and incubated at 27°C for 24 hr. Cell viability was assessed using the MTT assay, as described by Angius and Floris (2015). After infection, 450 µM MTT (Sigma-Aldrich; St. Louis, MO, USA) was added to each well and the plates were incubated for an additional 3 hr. Then, the supernatant was removed carefully and 100 µL of dimethyl sulfoxide was added to solubilize the formazan crystals.

Absorbance was measured at 570 nm using the microplate reader. Absorbance values were used to calculate cell viability, expressed as a relative percentage compared to E11 cells cultured in L-15 medium without infection. A 1:10,000 dilution of TiLV resulted in approximately 50% cell mortality and was selected for use in the neutralization assay. Briefly, serum collected from immunized fish at 7 dpi was heated at 56°C for 30 min to inactivate complement activity. Then, the serum was diluted (1:512), based on the antibody level assay results, to minimize background interference. Equal volumes of the diluted serum and TiLV stock (1:10,000 dilution) were mixed and incubated at room temperature for 1 hr. Then, the virus-serum mixture was added to 96-well plates preloaded with E11 cells and incubated at 27°C for 24 hr. Subsequently, cell viability was assessed based on the MTT assay, as described above. Absorbance values were used to calculate cell viability, expressed as a percentage relative to untreated E11 cells in L-15 medium. All experiments were performed in triplicate ($n = 3$).

Statistical analysis

The statistical analysis was conducted using the SPSS software (version 26.0; IBM Corp.; Armonk, NY, USA). Differences in specific antibody IgM levels and viral neutralizing activity were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. In addition, two-way ANOVA with Tukey's post hoc test was applied to evaluate the effects of immunization, time and their interaction on antibody IgM levels. Statistical significance was set at $p < 0.05$. All assays were performed in triplicate ($n = 3$) and the results were presented as mean ± SD.

Ethics statements

1. Biosafety protocols were approved by the Institutional Biosafety Committee, King Mongkut's University of Technology Thonburi, Thailand (Approval no. IBC-2022-018).

2. Animal care and all experimental procedures were approved by the Institutional Animal Care and Use Committee, King Mongkut's University of Technology Thonburi, Thailand (Approval no. KMUTT-IACUC-2022/003) and the Animal Ethics Committee at the National Center for Genetic Engineering and Biotechnology, Thailand (Approval no. BT-Animal 04/2564).

Results and Discussion

Co-immunization with $S5_{196–272}$ and $S6_{200–317}$ induced higher levels of $S6_{200–317}$-specific antibodies than those of $S5_{196–272}$

Based on Lueangyangyuen et al. (2022), fish immunized with individual $S5_{196–272}$ or $S6_{200–317}$ exhibited different levels of antibody responses. Immunization with the $S6_{200–317}$ protein alone induced lower levels of specific antibodies than from $S5_{196–272}$. In contrast, infection with whole TiLV particles led to the strong production of $S6_{200–317}$-specific antibodies, as indicated using dot blot analysis. These findings suggested that the other viral proteins present in the whole virus may help to enhance the immune response against $S6_{200–317}$. Although the specific antigenic proteins of TiLV have not yet been identified definitively, it could be hypothesized that the $S5_{196–272}$ protein could be one of these contributing factors, as it also contains a signaling peptide similar to that of $S6_{200–317}$ (Bacharach et al., 2016). Based on this evidence, $S5_{196–272}$ may be exposed on the viral surface and could act in coordination with $S6_{200–317}$ to enhance immune activation. Hence, the current study evaluated the antibody response and the neutralizing activity of co-immunization with $S5_{196–272}$ and $S6_{200–317}$, as well as the efficacy of TiLV protection, compared to individual immunization.

Based on the results from the ELISA, the optimal serum dilution was 1:512 (low background levels at day 0). Based on the detection of $S5_{196–272}$ and $S6_{200–317}$ -specific antibody levels, comparisons among the individual immunization, co-immunization and PBS control groups revealed the following findings. As shown in Fig. 1A, fish immunized with either $S5_{196–272}$ alone or co-immunized with $S5_{196–272}$ and $S6_{200–317}$ produced significantly higher levels of $S5_{196–272}$-specific antibodies than the PBS control group from 7 dpi and these levels remained elevated until 35 dpi. Administration of a second dose at 14 dpi further enhanced antibody production, with the peak response observed at 21 dpi. Notably, at 28 dpi and 35 dpi, the co-immunized group had significantly higher $S5_{196–272}$-specific antibody levels than the $S5_{196–272}$-alone group. These findings indicated that while individual immunization with $S5_{196–272}$ elicited a strong specific antibody response, co-immunization with $S6_{200–317}$ prolonged and enhanced the response. In addition, as shown in Fig. 1B, fish in the co-immunization group had significantly higher levels of $S6_{200–317}$-specific antibodies than both the $S6_{200–317}$-alone and the PBS control groups. Elevated antibody levels were detected as early as 7 dpi in the co-immunization group. Although a transient decline was observed at 14 dpi, the antibody levels increased again following the booster dose, with significantly higher levels maintained from 21 dpi to 35 dpi than for the other groups. These findings indicated that co-immunization elicited a stronger $S6_{200–317}$-specific antibody response than from $S6_{200–317}$ alone. To further assess the effects of immunization and time on antibody levels, two-way ANOVA was performed for both the $S5_{196–272}$ and $S6_{200–317}$ antibody datasets. The analysis revealed highly significant ($p < 0.001$) main effects of immunization and time ($p < 0.001$), as well as a highly significant ($p < 0.001$) immunization-time interaction. As shown in Fig. 1A and 1B, based on these results, both immunization and time post-immunization influenced antibody responses and that the antibody response profile differed among the immunization groups.

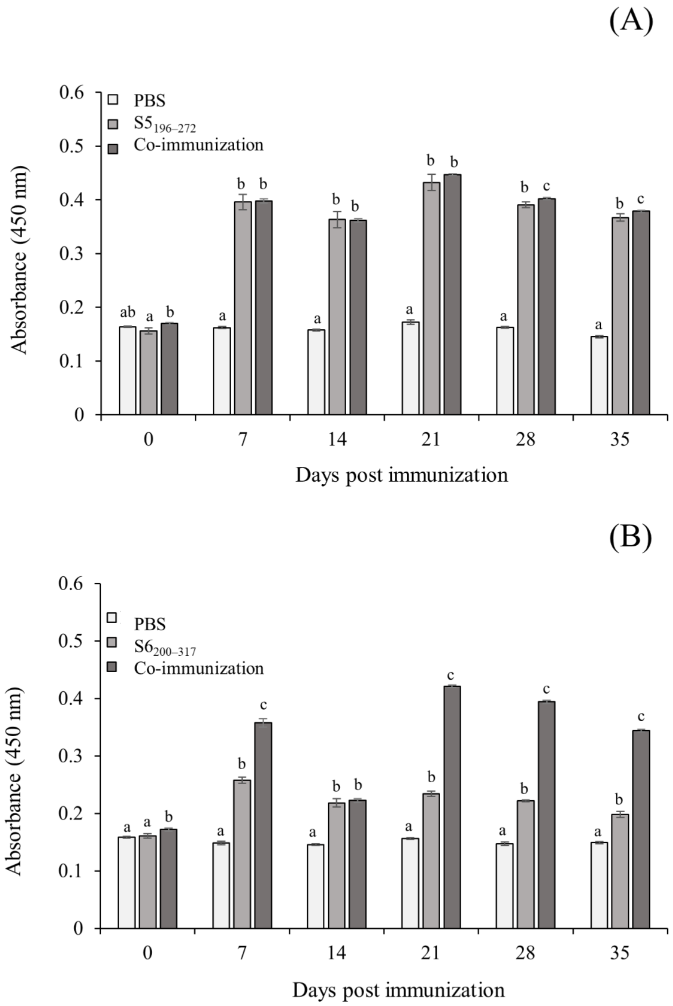


Fig. 1 Antibody response in serum from immunized fish at various days post immunization: (A) enzyme-linked immunosorbent assay (ELISA) plates coated with $S5_{196-272}$; (B) ELISA plates coated with $S6_{200-317}$, where data are presented as mean ± SD values, different lowercase letters (a, b, c) indicate significant ($p < 0.05$) differences among groups and highly significant ($p < 0.001$) effects of immunization, time and their interaction

Notably, co-immunization with $S5_{196–272}$ and $S6_{200–317}$ enhanced the production of $S6_{200–317}$-specific antibodies, resembling the response observed following infection with whole TiLV particles (Lueangyangyuen et al., 2022). This finding supported the hypothesis that the induction of $S6_{200–317}$-specific antibodies requires co-stimulation with other viral proteins, with $S5_{196–272}$ acting synergistically to enhance the antibody response.

Methylthiazolyldiphenyl-tetrazolium bromide-based cell viability assays revealed that co-immunization resulted in the highest TiLV neutralization

After elucidating that co-immunization increases the production of $S6_{200–317}$-specific antibodies, it remained unclear whether these antibodies possess neutralizing properties capable of inhibiting TiLV infection. Therefore, an experiment was conducted to evaluate the viral inhibition efficacy of serum from fish immunized individually with $S5_{196–272}$ and $S6_{200–317}$, as well as from co-immunization. TiLV was pre-incubated with serum from each group and E11 cell line viability was verified using an MTT-based assay.

Based on these results, that serum collected at 7 dpi from the co-immunized fish exhibited the strongest inhibitory effect against TiLV infection, with cell viability reaching 92.00%, which was not significantly different from the negative control (100% cell viability). In contrast, serum from fish immunized individually with either $S5_{196–272}$ or $S6_{200–317}$ conferred only partial protection, with cell viability around 70%. As expected, the PBS control group showed no viral inhibition, resulting in low cell viability (approximately 46%) comparable to the positive control without serum pre-incubation. (Fig. 2).

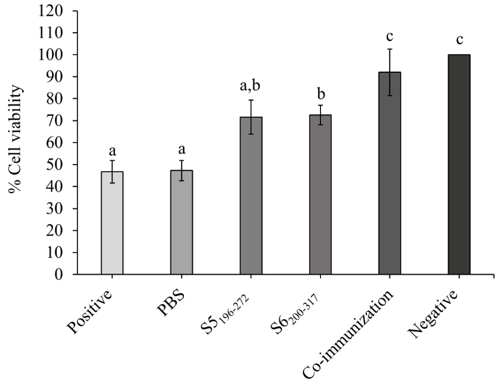


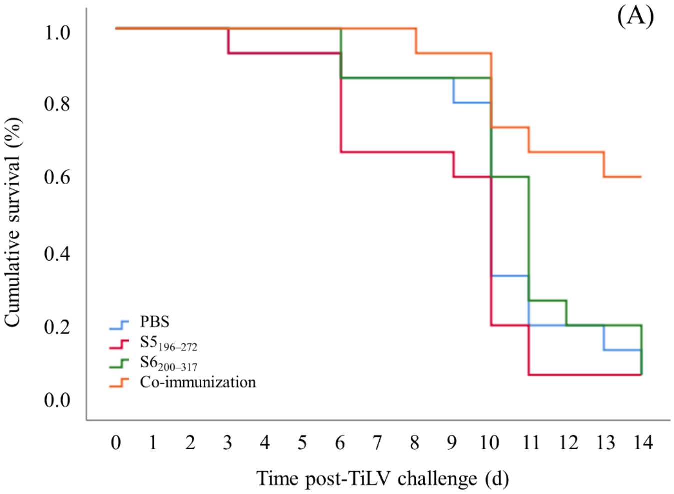
Fig. 2 Cell viability of E11 cell line after being infected with TiLV pre-incubated with serum collected from fish immunized with recombinant proteins or phosphate buffer solution (PBS), where positive control = E11 cells infected with TiLV without pre-incubation, negative control = E11 cells not infected with TiLV, differences among mean values analyzed using one-way analysis of variance, followed by Tukey's post hoc test, difference lowercase letters (a, b, c) indicate significant ($p < 0.05$) differences and error bars indicate mean ± SD

Additionally, Fig. 2 shows that cell viability in the group immunized with S6$_{200–317}$ alone was comparable to that in the S5$_{196–272}$-immunized group. This suggested that even though S6$_{200–317}$-specific antibodies were produced at lower levels, they had similar neutralizing activity to the higher levels of the S5$_{196–272}$-specific antibodies. Notably, these results highlighted that S6$_{200–317}$ protein might contain strong neutralizing epitopes. Therefore, the enhanced efficacy of TiLV inhibition observed in the co-immunization group could be attributed to the increased production of S6$_{200–317}$-specific antibodies. This finding indicated that promoting antibody production against S6$_{200–317}$ is a key factor in enhancing viral neutralization efficiency, which can be effective through the synergistic effect of the S5$_{196–272}$ protein. Among the theories explaining the enhanced immune stimulation induced by multiple antigens, it has been demonstrated that T-cell receptors can recognize multiple MHC epitopes, with this capability resulting in augmented immune activation, a phenomenon referred to as T-cell cross-reactivity (Petrova et al., 2012). Although the precise mechanisms remain to be fully elucidated, T-cell cross-reactivity has been widely documented (see for example Mateus et al. (2020) and Chaisawangwong et al. (2022)). It may occur through recognition of distinct epitopes on the same pathogen. For example, a study on SARS-CoV-2 reported that protective anti-SARS-CoV-2 S1-IgG responses were enhanced when cross-reactive CD4+ T cells targeting SARS-CoV-2 S-II and spike (S)816–830 epitopes were identified (Loyal et al., 2021). Additionally, cross-reactivity has been observed between epitopes derived from different pathogens, such as the influenza virus epitope (FLU-M1:58–66) and the HIV epitope (HIV-1 p17 GAG:77–85) (Acierno et al., 2003). Structural analyses on S5$_{196–272}$ and S6$_{200–317}$ proteins have identified 4 B-cell epitopes and 2 MHC class II epitopes within each protein (Lueangyangyuen et al., 2022). It could be hypothesized that the enhanced S6$_{200–317}$-specific antibody response may be mediated by T-cell cross-reactivity due to the inclusion of S5$_{196–272}$ epitopes alongside S6$_{200–317}$ epitopes that allowed T-cell receptors to recognize multiple epitope types, promoting T-cell cross-reactivity and thereby enhancing B-cell activation to produce stronger S6$_{200–317}$-specific antibody responses. A promising future direction would be to investigate whether co-immunization induces T-cell cross-reactivity, as hypothesized. This could be examined using MHC class II tetramer staining in combination with flow cytometry to detect T-cells capable of recognizing multiple epitopes, or through T-cell receptor repertoire analysis to identify shared clonotypes across different antigen stimulations. These approaches have been reported in applications to characterize T-cell cross-reactivity in viral infections (Becerra-Artiles, et al., 2022; Dykema et al., 2021).

Although co-immunization with S5$_{196–272}$ and S6$_{200–317}$ demonstrated effective viral neutralization in cell line assays, it remained unknown whether the stimulation of S6$_{200–317}$-specific antibodies at high levels simultaneously leads to better survival of Nile tilapia from TiLV infection compared to the stimulation of antibodies against single proteins. Therefore, the next experiment involved viral challenge testing using fish immunized with S5$_{196–272}$ and S6$_{200–317}$ individually, as well as through co-immunization.

Co-immunization provides protection against TiLV infection

This experiment evaluated the protective efficacy of immunization with S5$_{196–272}$, S6$_{200–317}$ and their combination against TiLV challenge during 14 d. The Kaplan-Meier survival analysis showed that the co-immunized group had the highest survival, with markedly lower survival for both individually immunized groups and the PBS control (Fig. 3A).



(B)

Group	Dead	Alive	Total	Survival (%)
PBS	14	1	15	6.67
S5$_{196–272}$	14	1	15	6.67
S6$_{200–317}$	14	1	15	6.67
Co-immunization	6	9	15	60.00

Fig. 3 Survival of immunized fish after challenging with TiLV ($1 \times 10^{5.5}$ Tissue Culture Infectious Dose 50% (TCID$_{50}$)) at 42 d post immunization: (A) Kaplan-Meier cumulative survival curves of experimental groups during 14 d; (B) final survival status of fish by group at day 14, where PBS = phosphate buffered saline

At day 14, the co-immunized group had 9 survivors and 6 mortalities, corresponding to a survival rate of 60.00%. In contrast, the individually immunized and PBS groups each had only 1 survivor and 14 mortalities, with a survival rate of 6.67% (Fig. 3B). Based on these results, co-immunization provided the most effective protection against TiLV, achieving the highest RPS value (57.14%). However, as these were preliminary findings, further studies are required to validate the protective efficacy of co-immunization using larger sample sizes, additional replicates or field-based trials.

In the current study, based on the challenging test results, it was clear that individual immunization with $S5_{196–272}$ or $S6_{200–317}$ failed to prevent TiLV infection in Nile tilapia, likely due to insufficient induction of neutralizing antibodies. Based on the neutralization assay (Fig. 2), there was no significant difference between the $S5_{196–272}$ group and the PBS control, suggesting limited neutralizing activity. Therefore, the failure of $S5_{196–272}$ immunization to protect fish against TiLV infection, comparable to the PBS group, was not unexpected. The higher mortality observed in the $S5_{196–272}$ immunization group than in the PBS group on day 6 post-challenge was likely due to uncontrollable external factors, such as stress from injection or excessive immune stimulation in some fish (Martos-Sitcha et al., 2020; Zornu et al., 2023). In addition, Fig. 2 that the $S6_{200–317}$ immunization group exhibited significantly higher cell viability than the PBS group, indicating that the $S6_{200–317}$ protein may contain epitopes capable of inducing neutralizing antibodies, leading to virus inhibition in cell lines. However, $S6_{200–317}$ immunization failed to protect fish against TiLV infection. Based on this observation, it could be postulated that $S6_{200–317}$ immunization induced insufficient levels of neutralizing antibodies to produce effective inhibition of viral infection in fish, consistent with the antibody level results in Fig. 1B, showing low levels of $S6_{200–317}$-specific antibodies. Nevertheless, the current study demonstrated an effective approach for preventing TiLV infection in tilapia through co-immunization. When the proteins $S5_{196–272}$ and $S6_{200–317}$ are administered together, they may induce T-cell cross-reactivity leading to a significantly higher production of neutralizing antibodies than the individual immunization groups. This was supported by the cell viability results from the neutralization assay, as shown in Fig. 2. This enhanced antibody response from co-immunization may contribute to more effective protection against TiLV infection in fish. Notably Chamtim et al. (2022) reported on recombinant Tis9 and Tis10 immunization and demonstrated that co-immunization resulted in a higher survival rate (RPS 55.56%) than the lower protective efficacy observed with individual protein immunization. This indicated a synergistic effect between TiLV proteins, consistent with the results of the current study. Therefore, the current findings, along with the investigation of the Tis9 and Tis10 proteins, provide valuable insights for the future development of vaccines against TiLV.

Furthermore, recombinant protein vaccines formulated with adjuvants effectively induce $CD4^+$ T cell and antibody responses (Rattan, et al., 2019; Wørzner, et al., 2021). In contrast, their ability to elicit $CD8^+$ cytotoxic T lymphocytes (CTLs) responses is limited (Han, et al., 2021; Baljon and Wilson, 2022). According to fundamental immunological principles, protein-based vaccines are generally inefficient at activating CTLs due to extracellular proteins being processed typically via the MHC class II pathway and not accessing the MHC class I presentation pathway (Rock, et al., 2016). The elimination of virus-infected cells by CTLs represents a critical mechanism for viral clearance (Wherry and Ahmed, 2004; Plotkin, 2010). Therefore, enhancing the co-immunization of $S5_{196–272}$ and $S6_{200–317}$ to stimulate CTL responses may improve protective efficacy. This can be achieved by incorporating an appropriate delivery system, such as protein encapsulation, which facilitates intracellular delivery and promotes cross-presentation of $S5_{196–272}$ and $S6_{200–317}$ proteins through the MHC class I pathway (Shen, et al., 2006; Song, et al., 2016).

Conclusions

There was a synergistic effect from combining $S5_{196–272}$ and $S6_{200–317}$. Co-immunization led to higher production of $S6_{200–317}$-specific antibodies than from immunization with $S6_{200–317}$ alone. In terms of protection, the co-immunized group had the highest cell viability (92.00%) in the neutralization assay and the highest RPS value (57.14%) in the TiLV challenge test. In contrast, the groups immunized with a single protein had lower values for cell viability and RPS. These findings suggested that co-immunization with $S5_{196–272}$ and $S6_{200–317}$ enhanced the induction of neutralizing antibodies and provided effective protection for Nile tilapia against TiLV infection.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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