Active and passive immune responses and protection of Nile tilapia *(Oreochromis niloticus)* immunized with tilapia lake virus inactivated vaccines

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A Dissertation Submitted in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy in Veterinary Science and technology
Common Course
FACULTY OF VETERINARY SCIENCE
Chulalongkorn University
Academic Year 2021
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น.ส.ถาว ธู มาย

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วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
สาขาวิชาวิทยาศาสตร์ทางการสัตวแพทย์และเทคโนโลยี ไม่สังกัดภาควิชา/เทียบเท่า
คณะสัตวแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2564

ลิขสิทธิ์ของจุฬาลงกรณ์มหาวิทยาลัย

Thesis Title	Active and passive immune responses and protection of Nile tilapia *(Oreochromis niloticus)* immunized with tilapia lake virus inactivated vaccines
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ถาว ธู มาย : การตอบสนองของภูมิคุ้มกันแบบแอคทีฟและแบบพาสซีฟ
และการป้องกันตัวของปลานิลที่ถูกกระตุ้นให้สร้างภูมิคุ้มกันด้วยวัคซีนทิลาเปียเลคไวรัสเชื้อตาย. (Active and passive immune responses and protection of Nile tilapia *(Oreochromis niloticus)* immunized with tilapia lake virus inactivated vaccines) อ.ที่ปรึกษาหลัก : ชาญณรงค์ รอดคำ, อ.ที่ปรึกษาร่วม : ฮา ทานห์ ดง

Tilapia tilapinevirus หรือที่เรียกว่า tilapia lake virus (TiLV) เป็นไวรัสก่อโรคที่ติดต่อได้ง่าย ส่งผลให้เกิดอัตราการตายที่สูง และความสูญเสียที่มากต่ออุตสาหกรรมการเลี้ยงปลานิล งานวิจัยนี้เราได้พัฒนาวัคซีนเพื่อการป้องกันการติดเชื้อนี้ขึ้นมาสองชนิด โดยผลิตจากเชื้อไวรัสที่เลี้ยงในเซลล์เพาะเลี้ยงแล้วฆ่าเชื้อด้วยความร้อน และฟอร์มาลิน (HKV และ FKV) ทำการประเมินประสิทธิภาพของวัคซีนโดยการฉีดวัคซีนแต่ละชนิดเข้าช่องท้องปลานิลระยะอนุบาล โดยมีไวรัสที่ถูกฆ่าเชื้อแล้วปริมาณ 1.8×10^6 $TCID_{50}$ จากนั้นทำวัคซีนกระตุ้นในวันที่ 21 หลังการทำวัคซีนครั้งแรกด้วยวิธีการและขนาดเดียวกัน ในวันที่ 28 หลังการทำวัคซีนครั้งแรก ปลาถูกนำมาฉีดเชื้อไวรัส TiLV ในปริมาณที่ทำให้ถึงตาย ผลการทดลองแสดงให้เห็นว่าวัคซีนทั้ง 2 ชนิดให้การป้องกันปลานิลในระยะอนุบาลต่อเชื้อไวรัส TiLV โดยมีอัตราการรอดที่ 71.3% สำหรับ HKV และ 79.6% สำหรับ FKV จากนั้นวัดระดับแอนติบอดี้ที่จำเพาะต่อ TiLV ด้วย ELISA พบว่าวัคซีนทั้งสองชนิดกระตุ้นการสร้างแอนติบอดีที่จำเพาะต่อ TiLV ได้ทั้งในซีรั่ม และในเมือกปลา นอกจากนี้ทำการประเมินการแสดงออกของยีนที่เกี่ยวข้องกับภูมิคุ้มกันจำนวนห้ายีนในเนื้อเยื่อไตส่วนหน้า และม้ามของปลาทดลอง พบการแสดงออกที่สูงขึ้นอย่างมีนัยสำคัญของยีน *IgM* และ ยีน *IgT* ในไตส่วนหน้าของปลาทดลองภายหลังที่ได้รับ HKV ในวันที่ 21 ในขณะที่พบการแสดงออกที่เพิ่มขึ้นของยีน *IgM, IgD* และ *CD4* ในไตส่วนหน้าของปลาทดลองภายหลังที่ได้รับ FKV ในเวลาเดียวกัน แสดงให้เห็นว่าวัคซีนแบบฉีดทั้ง 2 ชนิดนี้สามารถป้องกันการติดเชื้อ TiLV ได้อย่างมีประสิทธิภาพ ต่อจากนั้นเราได้ทำการทดลองฉีดวัคซีนในขนาด 2 เท่า โดยใช้แผนการทดลองแบบเดียวกันกับกล่าวมาข้างต้นให้กับพ่อแม่พันธุ์ปลานิล และดำเนินการผสมพันธุ์ปลาพ่อแม่พันธุ์ที่หนึ่งสัปดาห์หลังจากการทำวัคซีนกระตุ้น ซีรั่มจากพ่อแม่พันธุ์ ไข่ที่ปฏิสนธิแล้ว และตัวอ่อนถูกเก็บในสัปดาห์ที่ 6-14 สัปดาห์ภายหลังการฉีดวัคซีนครั้งแรก เพื่อนำมาวัดระดับแอนติบอดีจำเพาะต่อ TiLV ในขณะเดียวกันได้มีการทดลองการส่งผ่านภูมิคุ้มกันสู่ลูก ด้วยการนำซีรั่มจากแม่พันธุ์ปลานิลที่ทำวัคซีนแล้วมาฉีดให้แก่ปลานิลระยะอนุบาล เพื่อทดสอบว่าภูมิคุ้มกันจากแม่พันธุ์จะส่งผลต่อการป้องกันเชื้อไวรัส TiLV เมื่อผ่านมายังลูกปลาหรือไม่ ผลการศึกษาพบว่าวัคซีนทั้ง 2 ชนิดนี้ส่งผลกระตุ้นให้ทั้งพ่อแม่พันธุ์มีการสร้างแอนติบอดีที่จำเพาะต่อ TiLV ขึ้น และแอนติบอดีที่จำเพาะนี้จากพ่อแม่พันธุ์สามารถส่งผ่านไปยังไข่ที่ปฏิสนธิและตัวอ่อนอายุ 1-3 วันได้ นอกจากนี้จากการทำ passive immunization พิสูจน์ได้ว่าแอนติบอดีที่เกิดจากการทำวัคซีน TiLV สามารถส่งผลต่อการป้องกันการติดเชื้อ TiLV ในปลาระยะอนุบาลได้โดยทำให้มีอัตราการรอดที่ 85-90% สามารถสรุปได้ว่าการทำวัคซีนป้องกัน TiLV ทั้ง 2 ชนิดนี้น่าจะเป็นกลยุทธ์ที่สำคัญในการจัดการสุขภาพปลานิล และการผลิตลูกปลานิลจากพ่อแม่พันธุ์ที่ปราศจากเชื้อไวรัส TiLV ได้

สาขาวิชา	วิทยาศาสตร์ทางการสัตวแพทย์และเทคโนโลยี	ลายมือชื่อนิสิต ....
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6278303131 : MAJOR VETERINARY SCIENCE AND TECHNOLOGY
KEYWORD: Tilapia lake virus inactivated vaccine immune responses broodstock

Thao Thu Mai : Active and passive immune responses and protection of Nile tilapia *(Oreochromis niloticus)* immunized with tilapia lake virus inactivated vaccines. Advisor: Assoc. Prof. Dr. CHANNARONG RODKHUM Co-advisor: Asst. Prof. Ha Thanh Dong

Tilapia tilapinevirus, also known as tilapia lake virus (TiLV), is a contagious viral pathogen, resulting in mass mortalities and economic losses for tilapia industry. Here, we developed two simple cell-culture, heat-killed and formalin-killed vaccines (HKV and FKV) aiming to prevent this disease. The vaccine efficacies were evaluated by intraperitoneal injection in juvenile tilapia with each vaccine containing 1.8×10^6 $TCID_{50}$ inactivated virus, followed by a booster dose at 21-day post primary vaccination (dppv) in the same manner. At 28 dppv, the fish were challenged with a lethal dose of TiLV. Expression of five immune genes in head kidney and spleen of experimental fish was assessed at 14 and 21-dppv and again 7-day post booster. At the same time points, TiLV-specific IgM responses were evaluated by enzyme-linked immunosorbent assay (ELISA). The results showed that both vaccines conferred significant protection, with relative percentage survival of 71.3% and 79.6% for HKV and FKV, respectively. Significant up-regulation of *IgM* and *IgT* was observed in the head kidney of fish vaccinated with HKV at 21 dpv, while *IgM*, *IgD* and *CD4* expression increased in the head kidney of fish receiving FKV at this time point. Both vaccines induced a specific IgM response in both serum and mucus. Then, we run the same vaccination regime on broodstock including four male and eight female fish per treatment with the double vaccine dose. Mating was performed one week later. Broodstock blood sera, fertilized eggs and larvae were collected from 6–14 week post primary vaccination for measurement of TiLV-specific antibody levels. Meanwhile, passive immunization using sera from the immunized female broodstock was administered to naïve tilapia juvenile to assess if antibodies induced in immunized broodstock were protective. The results showed that TiLV-specific antibodies were generated in majority of both male and female broodstock vaccinated with either the HKV or FKV and these antibodies were transferred to the fertilized eggs and 1–3-day-old larvae from vaccinated broodstock. Moreover, passive immunization proved that the antibodies elicited by TiLV vaccination were able to confer protection against TiLV challenge with RPS of 85%-90% in naïve juvenile tilapia. In conclusion, immunization of tilapia broodstock with HKV or FKV might be a potential strategy to reduce the risk of vertical transmission and protect the tilapia fertilized eggs and early stage of larvae from TiLV.

Field of Study:	Veterinary Science and technology	Student's Signature ....
Academic Year:	2021	Advisor's Signature ....
		Co-advisor's Signature ....

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ACKNOWLEDGEMENTS

Foremost, I would like to express my deepest gratitude to my advisor Assoc. Prof. Dr. Channarong Rodkhum and co-advisor Asst. Prof. Dr. Ha Thanh Dong for their continuous support, valuable guidance, and encouragement. I also would like to thank my committee Dr. Kim D. Thompson, Assoc. Prof. Dr. Channarong Rodkhum, Asst. Prof. Dr. Ha Thanh Dong, Assoc. Prof. Dr. Nopadon Pirarat, Asst. Prof. Dr. Patharapol Piamsomboon and Dr. Saengchan Senapin for their valuable comments and suggestions.

My completion of this project could not be accomplished without the support of our collaboration, Dr. Saengchan Senapin, and Ms. Suwimon Taengphu (Center of Excellence for Shrimp Molecular Biology and Biotechnology, Mahidol University), Dr. Kim Thompson and Dr. Janina Z. Costa (Moredun Research Institute, UK), Asst. Prof. Dr. Pattanapon Kayansamruaj, Ms. Chayanit Soontara , Mr. Pattarawit Kerddee and Mr Dinh Hung Nguyen (Faculty of Fisheries, Kasetsart University), Dr. Jorge Del-Pozo (The Royal (Dick) School of Veterinary Studies and The Roslin Institute, University of Edinburgh), Dr. Le Thanh Dien (Center of Excellence in fish infectious diseases), Ms. Pattiya Sangpo (Suan Sunandha Rajabhat University).

I would like to say thank to professors, instructors at Faculty of Veterinary Science, Chulalongkorn University who committed me to valuable scientific foundation, instructors and my friends at the International Graduate Program of Veterinary Science and Technology (VST), my lab mates at Center of Excellence in fish infectious diseases, Faculty of Veterinary Science, Chulalongkorn University, at Laboratory of Aquatic Bioscience, Faculty of Science and Technology, Suan Sunandha Rajabhat University, and at Center of Excellence for Shrimp Molecular Biology and Biotechnology, Mahidol University. This research project is supported by National Research Council of Thailand (NRCT): NRCT5-RGJ63001"Development of vaccines against Tilapia Lake virus (TiLV) in Nile Tilapia (Oreochromis niloticus)"

I would like to say thank the PhD program of Chulalongkorn University for ASEAN and Non-ASEAN countries' students and the financial support from the International Veterinary Vaccinology Network.

Finally, I would like to express my honest gratitude to my parents for their constant support and encouragement as I pursue my PhD.

Thao Thu Mai

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CHAPTER 1

INTRODUCTION

1.1. Importance and rationales

The world's population is projected to be 9.7 billion in 2050 and could reach a peak of roughly 11 billion in 2100 (DESA, 2019), placing an extreme pressure on food production and jeopardizing food security (Askew, 2017). It is estimated that global food yield would increase by 70% by 2050 to meet the demand of 9.1 billion inhabitants (Askew, 2017; Doering & Sorencen, 2018). The increase of global food production could lead to the growth of contagious diseases due to unsustainable management, threatening the food yield and quality (Rohr et al., 2019). Aquaculture has been one of the fastest-growing sectors of food production worldwide, with almost 3 billion people relying on wild caught and farmed seafood as their main protein source. Among fresh-water fish species, tilapia is the second most predominant farmed fish globally after carp. Due to advantageous characteristics such as reaching harvested sizes within 6-month culture, good adaptability with various weather conditions and inexpensive price, tilapia becomes the main source of revenue for farmers in many low- and middle-income countries (LMIC) (FAO, 2020). Nevertheless, diseases caused by multiple bacteria, viruses and parasites has been endangering the tilapia yield annually due to the intensification of rearing system. Furthermore, transboundary

movement of tilapia stock has been resulting in the spreading of severe infectious diseases globally. Starting from 2009, mass mortalities of farmed tilapia were recorded and spreading all over Israel without understanding of the pathogenic reason for these mortalities. In 2013, a newly virus, primarily named tilapia lake virus (TiLV), were discovered to be the etiological agent causing these disease outbreaks, resulting the massive economic losses (Eyngor et al., 2014). At around the same time, a new virus-like particle was determined as the etiological agent responsible for mass mortalities in around 80-90% of farmed tilapia population in Ecuador with many gross lesion, termed syncytial hepatitis of tilapia (SHT), because of the observed characteristic histopathological changes seen in the infected tissue sections (Ferguson et al., 2014). SHT were discovered to be caused by a virus being genetically homologous with TiLV (Del-Pozo et al., 2017). To date, TiLV has spread globally to 17 countries due to the transboundary movement of fish (Surachetpong et al., 2020) and resulted in economic losses for tilapia producers. Studies reported that both wild and farmed tilapia were susceptible with TiLV, showing a variety of clinical signs and cumulative mortalities of up to 90% in field (H. T. Dong, Siriroob, et al., 2017); meanwhile, the mortality ratios of 60% to 95% were recorded from the experimental challenges in the laboratory, indicating that TiLV was a seriously contagious agent threatening tilapia production (Dinh-Hung et al., 2021; Eyngor et al., 2014; Tattiyapong et al., 2017). The presence of TiLV were detected in fertilized egg and fry in a previous report (Dong et al., 2017a). Recent studies have explored the possibility that TiLV was transferred from infected

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broodstock to their offspring, raising a concern for broodstock health management by tilapia hatcheries or multiplication centers to eradicate TiLV-infected seed (Dong et al, 2020; Yamkasem et al., 2019).

Vaccines are one of the best strategies to prevent disease outbreaks for farmed fish. In principle, vaccines are administered to trigger the immune response in fish through the production of specific antibodies and immunologic memory against the pathogenic invasion. The fish's immune system will respond strongly and faster if it is re-stimulated with the same pathogens during a secondary exposure to the pathogen. Furthermore, recent studies have revealed that broodstock immunization could be a viable approach for efficient prevention of infectious diseases, mitigating vertical transmission to offspring, and contributing to specific free pathogen seed production. Among many types of vaccines developed for fish, inactivated vaccines are the most commonly used due to the advantages they offer, such as easy and fast manufacture and good protective efficiency. These vaccines can be simply inactivated using either physical factors such as heat, ultraviolet light and pH adjustment or chemical elements that formalin and -propiolactone are widely used for this purpose (Miccoli et al., 2021). Heat and formalin are two most popular methods to produce inactivated antigens due to their simplicity to manufacture and antigenic maintenance. However, some drawbacks of inactivated vaccine were reported in some cases such as low protection and weak immunogenicity due to lack of ability to trigger the cellular immunity (Ma et

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al., 2019). Many inactivated vaccines have been licensed and commercialized for farmed fish, protecting them from disease outbreaks, and minimizing economic losses. Numerous killed vaccines are available for higher-value fish, such as Atlantic salmon, which are commonly utilized by farmers, and making a great contribution to maintaining fish health and productivity. For example, an inactivated viral vaccine formulated from killed salmonid alphavirus (serotype 3) against pancreas disease in Atlantic salmon can protect fish from illness with an impressive relative percentage survival (RPS) of 98.5% (Karlsen et al., 2012). Numerous killed bacterial vaccines have been patented and widely used for salmonid farms, such as *Aeromonas salmonicida*, *Vibrio anguillarum*, *Vibrio ordalii*, *Vibrio salmonicida* (Ma et al., 2019).

The development of inactivated vaccines is urgently needed for the global tilapia industry to reduce the risk of the pandemic caused by TiLV. Heat and formalin are two easy and fast measures to produce inactivated vaccines. Therefore, in this project, two cell-culture based-killed vaccines were generated using physical (heating) and chemical (formalin solution) methods of inactivation and evaluated if the protection could be conferred after fish have been immunized with the vaccines. In addition, we hypothesized that broodstock tilapia immunized with either heat-killed or formalin-killed vaccines (HKV or FKV) could transfer the TiLV-specific antibody IgM to their offspring.

1.2. Hypothesis

- HKV and FKV can provoke specific immune responses against TiLV in juvenile and broodstock Nile tilapia and protect the fish from TiLV infection
- TiLV-specific antibody can be transferred from either HKV or FKV – immunized broodstock to their offspring
- TiLV-specific antibodies play a role in protecting Nile tilapia from TiLV infection

1.3. Objectives

- To evaluate whether cell culture-based HKV and FKV can enhance the expression of immune – related genes, TiLV-specific IgM antibody and protect juvenile Nile Tilapia from TiLV disease.
- To investigate whether HKV and FKV can generate the TiLV-specific IgM antibody in broodstock tilapia, and if the antibody can be transferred from immunized broodstock to fertilized eggs and larvae.
- To access the role of TiLV-specific antibody in protecting tilapia from TiLV infection throughout passive immunization and neutralization assay.

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CHAPTER 2

LITERATURE REVIEW

2.1. Overview of Tilapia aquaculture and disease challenges

2.1.1. Tilapia aquaculture

Tilapia *(Oreochromis sp.)* become one of the most productive fish and have been being traded globally after carp. With the development of genetic modification and selective breeding, tilapia is the ideal choice for fish farming because of their rapid growth which can help to shorten the culture period and good adaptability to the various weather conditions and rearing systems (FAO, 2020). Global tilapia production reached 6 million tonnes in 2020 (Fletcher, 2020), which was equivalent to a value of US$ 7.9 billion (IMARC, 2020). It is estimated that global tilapia production will able to grow by 6.1% during 2021-2026 (Report tilapia market, 2021). Tilapia is widely cultured in over 90 countries worldwide, with two of the world's largest suppliers including the People's Republic of China and Indonesia, while Bangladesh, the Philippines, Thailand, Egypt, Brazil, and Colombia are also major tilapia producers. In Thailand, tilapia is the most commonly farmed fish species, accounted for 60% of small-scale aquaculture. Nile tilapia *(Oreochromis niloticus)* is the most-produced species, accounting for 38% of annual fish yield (Sampantamit et al., 2020). The genetic selection of breeding program has resulted in an increasing in the annual tilapia yield, contributing to meeting the

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rising demand for animal sourced protein as the population has grown at an accelerating rate over the years (Ponzoni et al., 2011).

2.1.2. Disease challenges

Due to the expansion of rearing systems, annual tilapia production recently has been pressured by the emergence of newly infectious diseases caused by either single or multiple infections of bacteria and viruses. Regarding bacterial outbreaks, large number of fish death and significant economic losses were resulted from streptococcal infection for farmed tilapia (Amal & Zamri-Saad, 2011) with 2 main pathogenic species *Streptococcus iniae* and *Streptococcus agalactiae.* Streptococcosis infection cause the fish to exhibit abnormal behavior such as erratic swimming, lack of appetite and stopping eating, along with gross lesions such as exophthalmia, eye and fin haemorrhages, and scale erosion (Pradeep et al., 2016; Zhang, 2021). Apart from Streptococosis disease, tilapia has been threaten by a variety of other bacteria, such as *Flavobacterium columnare*, which causes the disease outbreak “columnaris disease” with typical clinical signs including gill necrosis and fin erosion or the infection cased by different Aeromonas spp. such as *Aeromonas hydrophila*, which results in haemorrhagic septicaemia, with haemorrhaging on the skin surface (a hallmark characteristic of the disease) (Dong et al., 2015a; Dong et al., 2017c; Lema et al., 2021). In addition, Edwarsiellosis caused by two main species *Edwardsiella ictaluri* and *Edwardsiella tarda* and Francisellosis caused by *Francisella noatunensis* subsp. *orientalis* are endemic occured in farmed tilapia, causing white nodules 1-3 mm on

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the liver, kidney and spleen of affected fish (Dong et al., 2019; Nguyen et al., 2016). More seriously, disease outbreaks frequently occur as concurrent infections involving multi-pathogens, resulting in numerous gross lesions and which have economically devastating effects for tilapia producers (Assis et al., 2017; Basri et al., 2020).

Regarding viral diseases, an array of viruses have been found as aetiological agents causing outbreaks in various tilapia species. The bi-segmented RNA virus Infectious pancreatic necrosis virus (IPNV) was reported to be causing disease outbreaks of *Tilapia mossambicus* in Taiwan (Hedrick et al., 1983). A cumulative mortality of 25% was recorded for fish experimentally challenged. This virus can be contagious through both horizontal and vertical transmission (Garcia et al., 2010; Korsnes et al., 2012; Mangunwiryo & Agius, 1987). Another virus belonging Betanodavirus, termed as nervous necrosis virus (NNV), was discovered as a causative agent targeting the fish nervous system, causing the commonly observed signs such as erratic swimming, unsteadiness, and an increased proclivity for shore swimming (Keawcharoen et al., 2015). NNV – disease in tilapia farms was first reported in France and then spread to other countries (Bigarré et al., 2009; Yanong, 2019). The outbreak occured in Thailand's tilapia farms in 2011, resulting in a high ratio of mortalities of 90%-100% (Keawcharoen et al., 2015). Tilapia larvae encephalitis virus (TLEV) was classified within the Herpeviridae family, resulting in the illness for tilapia at the larvae stage with signs of fin and pigmentation and rotating swimming (Shlapobersky et al., 2010). In addition to these viruses, the

Iridoviridae family is composed of several genrea that cause the disease in fish farming including Ranavirus, Lymphocystivirus, Megalocytivirus, Iridovirus, and Chloriridovirus (Machimbirike et al., 2019; Zhang & Gui, 2015). Bohle virus, a type of Ranavirus, was first identified as causing 100% mortality of blue tilapia (*O. mossambicus*) fry in Australia, whereas the Lymphocystis disease virus (LCDV), a member of Lymphocystivirus genus, was found as a viral aetiological agent inducing disease outbreaks for 4 tilapia species consisting of *T. Esculenta*, *Tilapia amphimelas*, *T. variabilis*, and *Haplochromis sp* (Machimbirike et al., 2019). Regarding Megalocytivirus, infectious spleen and kidney necrosis virus (ISKNV) was discovered to affect both Nile tilapia (*O. niloticus*) and red hydrid tilapia (*Oreochromis* spp.), causing over 50% mortailty in fish stock. Multiple gross lesions such as gill pallor, abdomimal distension, darken body, erratic swimming and lethargy were observed in affected fish (Ramírez-Paredes et al., 2021; Suebsing et al., 2016). In addtion to these viruses, tilapia lake virus (TiLV) has been found primarily in Israel as an aetiological agent causing mass mortality for tilapia farming since 2009 (Eyngor et al., 2014). Up to date, the virus has been spreading through multiple countries over 4 continents due to the transboundary movement of tilapia breeding stock, triggering a TiLV-global pandemic for both wild-type and reared tilapia species (Jansen and Mohan, 2017; Jansen et al., 2018). Recently, a newly emerging virus was discovered in China and named Tilapia parvovirus (TiPV), primarily causing an endemic associated with high mortality of 60% to 70% in adult tilapia. Futhermore, concurrent co-infection between TiPV and other pathogenes,

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including TiLV and *Streptococcus agalactiae,* resulted in more serious tilapia outbreaks with multiple gross lesions and higher cumulative mortality (Liu et al., 2020).

2.2. Overview of *Tilalpia tilapinevirus* and disease it causes

2.2.1. Aetiology

2.2.1.1. Viral properties

Tilapia tilapinevirus, commonly termed Tilapia lake virus (TiLV), is a segmented single-stranded RNA virus with 10 segments, encoding for 14 putative proteins. There are two open reading frames (ORFs) in segments 1 and 9, whereas remaining segments contain one ORF for each. All the segments are enclosed in an icosahedral particle, ranging around 55-100 nm in size (Acharya et al., 2019; Bacharach et al., 2016b; del-Pozo et al., 2017; Eyngor et al., 2014; Ferguson et al., 2014). The virus was initially named as syncytial hepatitis of tilapia due to the histopathological change it causes and described as an orthomyxo-like virus in *Orthomyxoviridae* family due to its morphology. However, there is little homology in genomic sequence between TiLV and orthomyxoviruses in segment 1. Therefore, TiLV was re-classified as a new virus with the name *Tilapia tilapinevirus,* under ***Amnoonviridae*** family, within the order Articulavirales (Bacharach et al., 2016a). Because of the viral envelope 's lipid structure, the virus particles were shown to be susceptible to organic solvents like chloroform or ether (Eyngor et al., 2014). TiLV were detected in the sites of macroscopic lesions including liver, kidney, gill and brain throughout *in-situ* hybridization, suggesting these

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internal organs were the places where the virus can be replicated and transcribed (Bacharach et al., 2016b; Dinh-Hung et al., 2021; Dong et al., 2017b).

2.2.1.2. Genetic diversity

The TiLV's genome is made up by 10 segments. Segment 1 is the largest segment with a length of 1641 bp, sharing the identity of 17% amino acid and 37% coverage sequence to PB1 subunit of the influenza C virus. There was no homologous sequences found between TiLV's remaining segments with any other known viruses (Bacharach et al., 2016c). Most recent studies have analyzed genetic diversity of TiLV based on partial or whole genome sequencing (Jansen et al., 2019; Surachetpong et al., 2020). Taengphu *et al*. (2020) performed the phylogenetic analysis for TiLV strains based on the sequence of segment 1, and found that the 16 isolates orginated from Thailand clustered within one clade, and were closely related with the clade of the Israeli-2012 containing only one isolate from Israeli IL-2012-AD-2016. Two these clades were seperated with the clade of Israeli-2011 which contained three isolates from Israeli, Ecuador and Peru (Taengphu et al., 2020).

The complete genome data of some TiLV isolates, originating Israel (Bacharach et al., 2016c), Thailand (Al-Hussinee et al., 2018; Surachetpong et al., 2017; Thawornwattana et al., 2020), Ecuador (Subramaniam et al., 2019), Peru (Pulido et al., 2019), the United State (Ahasan et al., 2020), and Bangladesh (Chaput et al., 2020) have been publish in Genbank. TiLV isolates were divided into two clades using concatenated genome

sequences, named Thai clade consisting of strains Thailand, Bangladesh and South America and Israeli clade including strains from Israel, Peru and Ecuador (Pulido et al., 2019).

It was estimated that the TiLV's annual rate of site substitutions was $1.81x10^{-3}$–$3.47x10^{-3}$ (Thawornwattana et al., 2020). Recent studies showed that genome re-assortment events occurred between TiLV isolates. For instance, the genome of the Bangladesh isolates was involved in the homologous recombinant between segment 5 and segment 6 of Ecuadorian and Israeli isolates (Chaput et al., 2020). The occurrence of re-assortment events was confirmed from an investigation in Thailand where the Thai isolate TH-2018-K was found to be the genomic reassortment of segment 5 and 6 between Israel-2011 (Til-4-2011) and EC-2012 isolates (Thawornwattana et al., 2020). TiLV genetic diversity may be increased via reassortment, which occurs when two or more isolates infect a single host cell, resulting in the rearrangement of viral segments in the progeny viruses.

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2.2.2. Epidemiology

2.2.2.1. Distribution, host ranges, susceptible stages and transmission

TiLV was primarily reported in Israel and has since spread to 16 countries in four continents, including Ecuador (Ferguson et al., 2014), Colombia (Kembou Tsofack et al., 2017), Peru (Pulido et al., 2019), the United State, Mexico (OIE, 2020), Israel (Eyngor et al., 2014), India (Behera et al., 2018), Bangladesh (Chaput et al., 2020), Thailand

(Dong et al., 2017a; Surachetpong et al., 2017), Indonesia (Koesharyani et al., 2018), Malaysia (Amal et al., 2018), the Philippines, Chinese Taipei (OIE, 2020), Egypt (Fathi et al., 2017), Tanzania and Uganda (Mugimba et al., 2018).

Multiple farmed tilapia species were found to be susceptible with TiLV comprising of hydrid tilapia (*O. niloticus X O. aureus hydrids*) (Eyngor et al., 2014), Nile tilapia (*O. niloticus*) (del-Pozo et al., 2017; Dong et al., 2017a; Surachetpong et al., 2017), red tilapia *(Oreochromis sp*) (Dong et al., 2017b; Surachetpong et al., 2017) and red hydrid tilapia (*O. niloticus x O. mossambicus*) (Amal et al., 2018). Some wild tilapia were reported to be affected by TiLV such as *Sarotherodon galilaeus*, *Tilapia zilli*, *O. aureus*, and *Tristamellasimonis intermedia* (Eyngor et al., 2014). Most other warm water fish were reported to be insusceptible to TiLV such as snakeskin gourami (*Trichogaster pectoralis*), iridescent shark (*Pangasianodon hypophthalmus*), walking catfish (*Clarias macrocephalus*), striped snake-head fish (*Channa striata*), climbing perch (*Anabas testudineus*), common carp (*Cyprinus carpio*), silver barb (*Barbodes gonionotus*), Asian sea bass (*Lates calcarifer*) except giant gourami (*Osphronemus goramy*), which was found to be susceptible with TiLV with cumulative mortality of 100% and gross lesions such as lethargy, erratic swimming, loss of appetide and scale erosion (Jaemwimol et al., 2018). Recently, TiLV has been detected in some fish that were co-cultured or shared the same water source as the tilapia including wild river carp (*Barbonymus schwanenfeldii)* and farmed barramundii (*Lates calcarifer*); however, the fish found

asymptomatic, and this was not concluded they were susceptible to TiLV (Abdullah et al., 2018; Piamsomboon et al., 2021).

All life cycles of fish growth were affected by TiLV infection, from fertilized eggs, yolk-sac fish, and fry (Dong et al., 2017a) to fingerlings, juveniles, and harvestable-size fish (Amal et al., 2018; Behera et al., 2017; Dong et al., 2017a; Ferguson et al., 2014; Fathi et al., 2017; Surachetpong et al., 2017). TiLV was first shown to be transmitted horizontally during cohabitant infection (Eyngor et al., 2014). Direct contact with the fish's skin, mucus, or oral fluids can cause the virus to enter the fish's system, where it can then replicate and spread to various organs (Pierezan et al., 2020). The possibility that TiLV might be vertically transmitted established when it was detected in reproductive organs, fertilized eggs and fry derived from TiLV-challenged broodstock (Dong et al, 2020; Yamkasem et al., 2019).

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2.2.2.2. Diagnosis

Diagnosis of TiLV infection was categorized into three levels (Bondad-Reantaso et al., 2021): (i) level 1, observation of gross lesions and animal behaviors; (ii) level 2, histopathological investigation of infected fish; (iii) level 3, performance of molecular techniques, viral isolations on the cell culture systems and transmission electron microscopy examination.

Level 1

Morbidity and mortality usually occur within one month after stocking the fingerlings to their grow-out cages. Diversity of external and internal gross signs were reported for TiLV-infected fish. Fish start showing some symptoms such as loss of appetite, stopping eating and schooling, erratic swimming, and lethargy. The infected fish then continuously developed multiple lesions such as skin darkening, exophthalmos, abdominal swelling, scale protrusion and erosion, fin and opercular haemorrhages, pallor gill and liver, watery abdomen, and the infected liver sometimes turned into dark or a green colour (Dong et al., 2017b; Jansen et al., 2018; Surachetpong et al., 2020; Tang et al., 2021) before mass mortalities occur **(Fig. 1).**

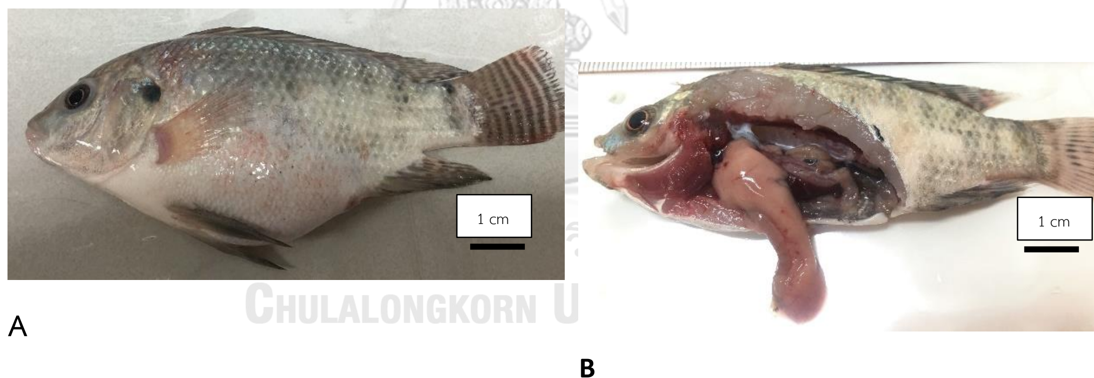

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Figure 1. Multiple gross lesions observed in TiLV-infected fish. (A) Fish showed abdominal swelling, scale protrusion and erosion; (B) Fish showed pallor liver, scale erosion and eye protrusion. (Images by T.T. Mai)

Level 2

Multiple nuclei ranging from 3-4 nuclei to 15-30 nuclei per giant liver cell, called syncytial formation, were observed, and described as one of the most common histopathological changes for fish infected with TiLV, explaining the first syncytial formation of tilapia named for TiLV disease. In addition to the formation of syncytial cells, infected fish liver sections usually showed multiple necrosis cells with pyknotic and karyolitic nuclei, eosinophilic intracytoplasmic inclusion bodies as well as lymphocytic inflammatory cell infiltration (Behera et al., 2018; Ferguson et al., 2014; Senapin et al., 2018; Tattiyapong et al., 2017b). Lymphocyte aggregation, perivascular cuffing and sometime syncytial formation were observed in brain sections (Surachetpong et al., 2017; Behera et al., 2018). There was a rise in melanomacrophage centres, the presence of eosinophilic intracytoplasmic inclusion bodies and numerous necrosis foci observed in spleen and head kidney (Tattiyapong et al., 2017b).

Level 3

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A number of polymerase chain reaction (PCR)-based molecular protocols have been developed to detect different segments of TiLV. Some one-step and two-step semi nested RT-PCR protocols were designed for TiLV detection with a sensitivity of 7-70000 viral copies (Eyngor et al., 2014; Dong et al., 2017b; Tsofack et al., 2017). However, these procedures were found to have low sensitivity and were time-consuming to perform. Some SyBr or Taqman probe-based RT-qPCR assays were then generated to improve the sensitivity up to 2 viral copies (Tattiyapong et al., 2017a; Waiyamitra et al.,

2018). Subsequently, TiLV was detected using RT-loop-Mediated isothermal amplification assays based on colorimetric changes. These tests are simple, fast, and inexpensive (Kampeera et al., 2021; Phusantisampan et al., 2019).

TiLV can be detected by *In situ* hybridazation using specific probes derived from TiLV partial sequences. Positive staining was seen in multiple organs from infected fish including liver, brain, kidney, spleen, gills, connective tissue in the muscle and reproductive organs of broodstock and fertilized eggs (Bacharach et al., 2016b; Dong et al., 2017b; Dong et al, 2020; Dinh-Hung et al., 2021; Yamkasem et al., 2019).

TiLV can be indirectly detected by the TiLV-specific antibody in an indirect enzyme-linked immunosorbent assay (iELISA) (Hu et al., 2019). This assay showed a sensitivity of 80.8% and specificity of 95.6% compared to the semi-nested RT-PCR developed by Dong et al., (2017b). An immunohistochemistry (IHC) procedure was recently developed to detect TiLV antigens in infected tissues including the liver, brain, kidney, spleen, gills, and intestines using a rabbit-derived-polyclonal immunoglobulin G antibody against TiLV, indicating the spacial cellular distribution of TiLV antigens during infection. The IHC signals were increased, correlating with the titer of TiLV inoculation (Piewbang et al., 2021).

The presence of TiLV can be confirmed by isolating the virus from infected tissues in cell culture (Eyngor et al., 2014; Tang et al., 2021). A variety of cell lines have been

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reported to be susceptible to TiLV including E11 (from *Channa striata*), OmB and TmB (from *O. mossambicus*), CFF (from *Pristolepis fasciatus*), OnlB (from *O. niloticus* brain), OnlL (from *O. niloticus* liver), and CAMB (from hydrid snakehead brain *Channa argus* X *Channa maculata*). Infection was confirmed by the appearance of a cytopathic effect (CPE), described as cell structure changes, such as rounding of the infected cell, fusion of adjacent cells to form the syncytia, the appearance of inclusion bodies in the nucleus or cytoplasm of the host cells **(Fig. 2)** (Eyngor et al., 2014; Behera et al., 2017; Kembou Tsofack et al., 2017; Swaminathan et al., 2018; Wang et al., 2020).

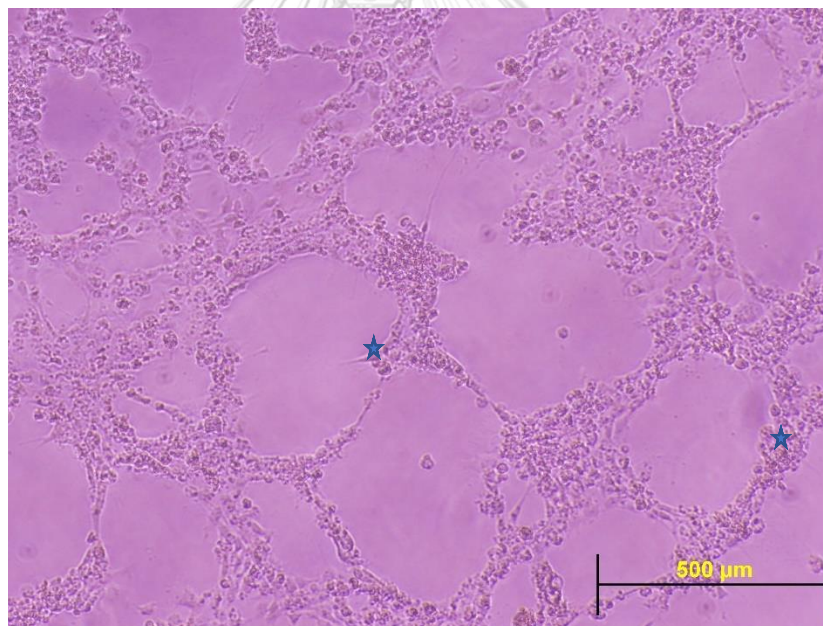

Figure 2. Cytopathic effects, marked with blue star, were observed on E11 cell line 6-day post TiLV inoculation (Image by T.T. Mai).

2.2.2.3. Current status of control measures for TiLV infection

To mitigate the TiLV disease, multiple prophylactic measures were investigated targeting three essential elements, including host, pathogens and environment.

Host

Vaccination is an effective method to prevent disease by eliciting the fish's immune system to fight off invading pathogens. Some vaccines have already been generated to protect tilapia from TiLV in some countries, showing a relative percentage survival over 50%. These vaccines consist of attenuated, DNA and inactivated vaccines. However, most of them are administered by intraperitoneal injection (i.p.), which is labour-intensive, time-consuming and limited for vaccinating for young fish (Bacharach & Eldar, 2016; Zeng et al., 2021a; Zeng et al., 2021b). Up to date, no immersed or oral vaccines have been developed for juvenile fish, resulting in an inability to prevent the spread of TiLV in the early stages. Recent studies have shown that immune gene expression, including CXCL8 (also known as CXCL8), ifn-, mx, rsad-2 (also known as VIPERIN), was up-regulated in red hydrid tilapia supplemented with probiotics *Bacillus* spp., compared to non-probiotic supplementation. In addition, probiotic treatment has been shown to enhance the fish survival after challenge, suggesting that probiotics may be useful in lowering the risk of TiLV-related mortality in small fish (Waiyamitra et al., 2020).

Another strategy is the development of TiLV-resistant tilapia in which tilapia can be resistant to TiLV and become insusceptible although they are exposed to virus. Furthermore, they can get infected by the virus with a very low viral titer and without clinical symptoms and mass mortalities. Natural resistance to TiLV appears to vary

among strains of tilapia. Some tilapia producers in Israel have reported that red tilapia (*O. aureus x O. mossambicus*) is the most resistant strain to TiLV, followed by the Chitralada strain and the least resistant strain is hydrid tilapia (Tang et al., 2021). By contrast, Nile tilapia were found to be more resistant than red tilapia in Thailand (Tattiyapong et al., 2017b). There were a few studies on selective breeding to increase TiLV resistance. Barria et al., (2020) used the data from 1821 pedigreed fish that orginated from 124 Nile tilapia families breeding population of Genetically Improved Farmed Tilapia (GIFT) strain during and after an pond outbreak to evaluate the TiLV resistance with two parameters including binary survival and days to death after an natural TiLV outbreak. Statistical analysis of these two parameters indicated that resistance to TiLV is highly heritable, suggesting the possibility of selective breeding for enhancement of TiLV resistance (Barría et al., 2020). Moreover, Barría analysed the genotyping of 950 GIFT tilipia catergorized as either survival (TiLV resistance) or mortality (TiLV susceptibility) after outbreak with a 65 K single-nucleotide polymorphism (SNP) array. A large effect quantitative trait locus (QTL) associated with resistance was discovered on chromosome Oni22. 43% of average mortality tilapia were found to contain a susceptible allele at the significant SNP (P value = 4.51E-10). The QTL contained several candidate genes for the host response to viral infection, including the *lgals17*, *vps52*, and *trim29e* genes, suggesting some potential genetic markers that can be used for selective breeding to strengthen TiLV resistance for the host (Barría et al., 2021).

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Another strategy for preventing and controlling TiLV disease is the production of TiLV specific pathogen free (SPF) animals. Recently, tilapia producers tend to produce the tilapia offspring from the TiLV-SPF brooders raised in bio-secure facilities where their health is mornitored under health management and diagnostic programs. The TiLV-free status should be declared for two consecutive years with sampling and testing conducted over three months apart (OIE, 2019). Maternal immunity transfer is one strategy to produce SPF aquatic animal, reducing the risk of vertical transmission to the offspring and increasing the larval survival rate (Zhang et al., 2013). This strategy was applied in some fish to produce specific pathogen-free brooders and mitigate the pathogenic transfer from broodstock to offspring (Kai et al., 2010; Pakingking et al., 2018). However, there are no reports on a broodstock vaccination program and maternal antibody transfer against TiLV.

Pathogen

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TiLV contamination of rearing facilities can trigger a TiLV outbreak in farmed tilapia. Eradication of TiLV from water, ponds, and equipment is one of the most important issues before starting a new tilapia culture. Some disinfectants, such as 2.5 ppm iodine, 10 ppm sodium hypochlorite (NaOCl), 300 ppm hydrogen peroxide (H_2O_2), and 5000 ppm (0,5%) Virkon, have been shown to be effective in eradicating TiLV from rearing water and equipment (Jaemwimol et al., 2019). Another study showed that TiLV can be destroyed after 30-minute expose to some common disinfectants such as buffered

povidone iodine (≥ 50 ppm) and household bleach (containing 3%-9% chlorine) (Soto et al., 2019).

Environment

Insufficience of environmental management can result in TiLV endemic. High fish stocking density, poor water quality, and high rearing temperature can stress fish, and promote the spread of TiLV (Wajsbrot et al., 2021). Furthermore, TiLV outbreaks commonly happen during the summer period when the weather is warmer and these mainly affect the small-size fish, rather than the harvest size fish (Fathi et al., 2017). Consequently, to minimise the production loss due to TiLV, tilapia culture should be avoided during the summer months, especially for the vulnerable life stages (Tang et al., 2021).

2.3. Fish immunology and vaccine

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2.3.1. Overview of fish immune system

Fish possess both innate and adaptive immunity. Innate immunity is considered the first defense to protect fish from initial invading pathogens, and it does not retain the memory for prior pathogen exposure, while adaptive immunity can be triggered subsequently when pathogenic agents are able to evade innate immune responses. The adaptive immunity is able to generate specific protection through humoral and

cell-mediated mechanisms, and it is very important for long-lasting immune responses due to the immune memory from previous infections (Smith et al., 2019).

2.3.1.1. Innate immunity

The epithelial cells of skin, gills and gastrointestinal tract are the early barriers fighting off invasive microorganisms from the aquatic environment. Skin, including scales and mucus, which contain a variety of antimicrobial peptides, lectins, lysozymes, complement proteins, plays a role in preventing bacterial penetration of the fish's body (Angeles Esteban & Cerezuela, 2015). Gills are involved in the function of osmoregulation and gas exchange. Furthermore, the gill's epithelial cell layer and mucus secreted from this organ play an important role in capturing pathogenic agents (Koppang et al., 2015). The gastrointestinal tract (GI) helps absorb nutrients and can also stop microorganisms invading the body through epithelial cells when food is digested (Salinas & Parra, 2015).

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Regarding cellular components, all organs which serve as physical barriers possess their own mucosa-associated lymphoid tissues (MALT) such as skin-associated lymphoid tissue (SALT), gill-associated lymphoid tissue (GIALT) and gut-associated lymphoid tissue (GALT) (Ángeles Esteban & Cerezuela, 2015; Koppang et al., 2015; Salinas & Parra, 2015; Smith et al., 2019). Recently, nasopharynx-associated lymphoid tissue was discovered in teleost fish, also acting as a first line of immune defense against invading pathogens (Tacchi et al., 2015). MALTs contain a wide variety of immune cells such as

secretory cells (e.g. goblet cells) secreting mucus, lymphocytes (B and T cells), macrophages, granulocytes including neutrophils and eosinophils and Langerhans-like cells (Salinas, 2015; Smith et al., 2019). The immune cells recognise pathogen-associated molecular patterns (PAMPs) present on pathogens, such as bacterial lipopolysaccharide, DNA, or viral RNA (Mogensen, 2009) via intracellular or extracellular pattern recognition receptors (PRRs). In fish, TLRs are well-characterized, with over 20 TLRs identified up to date (Kawai & Akira, 2009). Once PAMPs are recognised by PRRs, immune cells are activated and they participate in a variety of immune responses depending on the cell type, such as phagocytosis, which result in direct pathogen destruction, cytokine production to activate multiple immune responses for pathogen elimination, or serving as antigen presenting cells to T cells to stimulate the adaptive immune system (Øverland et al., 2010; Secombes & Belmonte, 2016). Among phagocytes, immune cells including macrophages and neutrophils are the first to arrive in infected tissues and interact with pathogens. Phagocytes stimulate the secretion of cytokines such as interferons (INF), tumour necrosis factor anpha (TNFα) and inteurlekin (IL1β) which initiates the phagocytosis process. Once the phagocytes engulf pathogens, phagosomes containing pathogens bind with lysosomes to form phagolysosomes, where the pathogens are destroyed through mechanisms such as reactive oxygen species (ROS) and nitric oxide (NO) (Hodgkinson et al., 2015). In addition to ROS and NO, neutrophils, the most abundant granulocytes, can kill the pathogenic invaders by

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releasing granules containing cytotoxic, antimicrobial peptides and neutrophil extracellular traps (NETs) (Neumann et al., 2001; Smith et al., 2019).

Humoral components of innate immunity comprise of complement system, lysozyme, and antimicrobial peptides. Approximately 35 serum proteins, which make up the classical, alternative, and lectic pathways, constitute the complement system in fish. They coordinate their activities to mediate some defense mechanisms such as opsonization, phagocytosis, and inflammatory responses (Boshra et al., 2006; Smith et al., 2019). Lysozyme is the enzyme that has been most investigated protein related fish innate immunity. Chicken-type and goose-type lysozymes were detected in various leukocytes such as neutrophils, monocytes, and macrophages of lymphoid organs (kidney, spleen, gills, etc.) in several teleost fish. Lysozyme is able to hydrolyze the 1-4 -link glycoside bonds found in the peptidoglycan layer of the bacterial cell wall, ultimately resulting in bacterial lysis (Saurabh & Sahoo, 2008; Smith et al., 2019). Antimicrobial peptides (AMPs), short oligopeptides with various numbers of amino acids, essentially contribute to fish innate defence by generating pores in bacterial cell membranes, and as a result, they can destroy bacteria. There are over 90 AMPs discovered in fish up to date (Masso-Silva & Diamond, 2014; Smith et al., 2019; Secombes & Wang, 2012). Fish AMPs were found to have broad-spectrum antimicrobial activity against a variety of microorganism. Moreover, they also have an immunomodulatory function, which can activate other immune cells participating in

defence mechanisms (Masso-Silva & Diamond, 2014). For example, hepcidin was found to be able to trigger the inflammatory process through up-regulation of some immune gene expression including MHC-UBA, IL-6, and TNFα (Ghodsi et al., 2020).

2.3.1.2. Adaptive immunity

The second line of defense, known as specific immunity or adaptive immunity, comes later when invaders overcome the first line of defense. Although it comes late, the fish's adaptive immune system is efficient at combating certain diseases and producing long lasting immunity (Secombes & Belmonte, 2016; Smith et al., 2019).

Antigen recognition and presentation

Effective immune responses against pathogenic invaders require efficient antigen recognition and presentation of immune cells through major histocompatibility complex (MHC) molecules. MHC molecules are sets of cell surface proteins encoded by a family of conserved genes that exhibit variability of polymorphism between individuals, allowing different repertoires of antigenic peptides to be presented by different individuals in a population (Goadsby et al., 2016). In teleost fish, there are two MHC classes: I and II (MHC-I and MHC-II), which are found on distinct chromosomes (Kuroda et al., 2002; Goadsby et al., 2016). Most cells express MHC-I molecules, which are composed of three extracellular domains of α1, 2, 3 and the β2-microglobulin light chain, whereas MHC-II, which are constituted by 2α and 2β peptide chains, are expressed by professional antigen-presenting cells (pAPCs) such as macrophages,

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dendritic cells and B-cells, recently characterized as pAPCs. MHC-I molecules present processed antigens to CD8+T-cells (Tcs), activating these cells to destroy infected cells; meanwhile, MHC-II molecules present processed antigens to CD4+T cells, activating these cells for a complex signalling cascade of antibody production to combat invasive pathogens (Kuroda et al., 2002; Goadsby et al., 2016; Secombes & Belmonte, 2016; Smith et al., 2019).

The T-cell immune responses

T-cell receptor

The T-cell receptor (TCR) is a transmembrane glycoprotein heterodimer comprising either the **α** and **β** chains or the **γ** and **δ** chains connected by disulphide linkages. TCR exclusively interacts with processed antigens coupled to MHC-I or MHC-II, triggering differentiation of naive T-cells into CD8+ and CD4+ populations **(Fig. 3)**. The arrangement of V, D, and J during somatic recombinant results in the diversity of TCR and chains or the diversity of TCR repertoires (Castro et al., 2011). Co-receptor CD4 combined with TCR for the recognition of processed antigens delivered by MHC-II. Meanwhile, co-receptor CD8 combined with TCR for the recognition of processed antigens presented by MHC-II (Smith et al., 2019).

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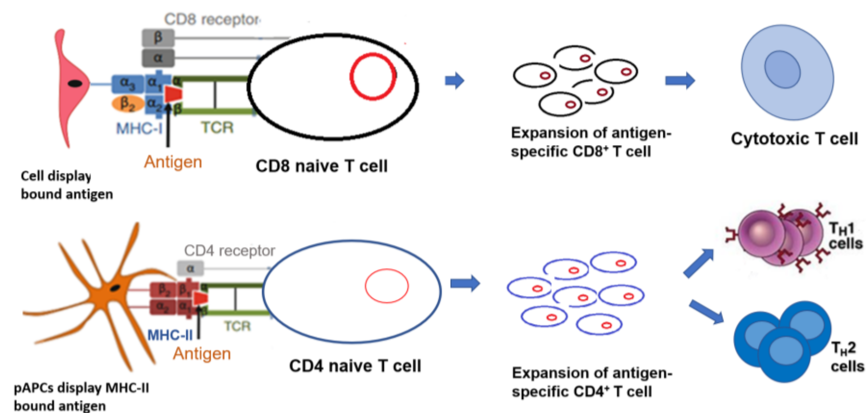

Figure 3. T-cells responses elicited by antigen-presenting cells

T-cell immune responses

CD4$^+$T cells are activated after being presented with the processed antigens delivered by MHC-II, and then they proliferate into one of the four helper T-cell (Th) subsets Th1, Th2, Th17 and Treg. Distinct cytokines are produced by different T-cell subsets as signals to stimulate further immunological responses (Castro et al., 2011; Secombes & Belmonte, 2016; Reyes-Cerpa et al., 2012). The main cytokines secreted by Th1 are INFγ and IL-2, driving the cell-mediated immunity that can destroy intracellular pathogens such as intracellular bacteria and virus. Recombinant IFN can limit viral growth by promoting the expression of anti-viral genes in macrophages and increasing ROS and NO. On the other hand, IL-2 can stimulate the differentiation of Th1 into cytotoxic T cells, which can directly eliminate the viral-infected cells through the apoptosis process (Castro et al., 2011; Secombes & Belmonte, 2016; Zou et al., 2007). The Th2-associated cytokines well characterized in fish to date are IL-4, IL-13 and IL-20, driving the differentiation of B-cells into the antibodies-secreted plasma cells for

specific antibody production (Smith et al., 2019). Immune responses to intraperitoneal injection of IL4 in zebrafish exhibited a rise in IgZ^{+}B-cell populations, indicating IL4 can activate B-cells and hence play an important role in humoral immunity (Hu et al., 2010). Th17's primary cytokine is IL17. Studies demonstrated that IL17 can stimulate the production of the antimicrobial peptide-defensin-3 and other cytokines, such as IL6 and IL8, resulting in bacterial eradication (Secombes et al., 2011). Regarding Treg, the existence of this cell type in fish has been disputed. Recently, researchers discovered Treg-like cells expressing Foxp3 homologous protein, together with CD2, CD25-like receptors in pufferfish (*Tetraodon nigroviridis*) (Wen et al., 2011).

$CD8^{+}$T (Tcs) cells are activated after being processed antigen is presented to them via MHC-I. Then this cell population proliferates and differentiates into cytotoxic T-cells, serving as killing cells to fight off viral infection in fish. They can destroy infected cells through two mechanisms, including secretory and non-secretory pathways. Both of them can result in cell apoptosis (Secombes & Belmonte, 2016; Smith et al., 2019). In the secretory pathway, Tcs emit toxins like granzyme B and perforin, which can cause apoptosis in infected cells (Elmore et al., 2007). Granzyme and perforin have been identified in several fish species. For example, granzyme structure is similar to mammals in ginbuna crucian carp, and its mRNA levels were significantly up-regulated when the fish were infected with *Edwardsiella tarda* (Matsuura et al., 2014). For the non-secretory pathway, it is associated with target cell-death receptors, such as FasL,

which are found on the Tcs. This receptor can bind to the CD95 receptor found on infected cells, triggering the signalling for caspase activation and apoptosis (Elmore et al., 2007).

The B-cell immune responses

B-cell receptors and activation

B-cell receptors (BCRs) are composed of a transmembrane-bound antibody (Ab) and a structure containing Ig **α/β** (CD79a/b) heterodimer with immuno-receptor tyrosine-based activation motifs (ITAMs), which play a role in signal transmission (Secombes & Belmonte, 2016). Following the binding of BCRs with particular antigens, B-cells are initially activated and then they stimulate the CD4+ T-cells to activate and differentiate into Th2, which can secrete IL4 to stimulate the maturation of B cells (Harwood & Batista, 2008; Secombes & Belmonte, 2016; Smith et al., 2019).

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B-cell maturation

Following antigen stimulation, B cells begin the process of maturation, which they can proliferate and differentiate into plasma cells. First of all, B cells will differentiate into short-lived plasma cells (SLPCs) and long-lived plasma cells (LLPCs). In mammals, SLPCs develop rapidly after B-cells contact antigens. Before returning to the bone marrow to become LLPCs, these cells can move to the germline centre and undergo somatic mutation to become plasma cells, serving a cell population mainly producing

specific antibodies. However, teleost fish lack germline center. Some scientific evidence suggests that melano-macrophage centres in the head kidney and spleen of teleost fish can fulfil the same role as the germline centre. Therefore, B-cells mature and differentiate into plasma cells in the posterior kidney and spleen (Harwood & Batista, 2008; Secombes & Belmonte, 2016; Smith et al., 2019; Ye et al., 2011).

Immunoglobulins

Immunoglobulins (Igs), termed antibodies (Abs), are the products of plasma cells and the final step of B-cell responses. The "Y" quaternary structure of abs is made up of two heavy chains (IgH) and two light chains (IgL) held together by a disulfide bond. Both IgH, IgL have one or more constant domains and one variable domain. The antigen-binding sites, known as the Fab region, are formed by one constant and one variable domain from each IgH and IgL in the Y arms. The Y's base is made up of constant domains of two heavy chains known as the Fc region. During B-cell maturation, the somatic rearrangement of V, D, and J segments produces the variable regions of IgH and IgL, resulting in the creation of distinct Abs from B-cells. In mammals, there are five distinct Ig isotypes (IgG, IgM, IgA, IgD, and IgE), whereas teleost fish have three distinct Ig isotypes (IgM, IgD, and IgT, also named as IgZ in zebrafish) (Castro et al., 2013; Hu et al., 2010; Secombes & Belmonte, 2016; Smith et al., 2019). Among Ig serotypes, IgM is the most abundant immunoglobulin found in both secretory and transmembrane forms in plasma. IgM plays an important role in both innate and

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adaptive immune responses, participating in opsonization, complement activation, neutralisation, and antibody-dependent cellular cytotoxicity (Forthal, 2014). Another serotype, IgT, known as IgZ in zebrafish, was first discovered in rainbow trout and zerafish. Studies of IgT in other teleost fish is under investigated. However, it is thought that IgT represents mucosal immunity, which functions similarly to IgA in mammals (Zhang et al., 2011). Studies on rainbow trout revealed that the concentration of IgM in the fish serum is much higher than that of IgT. Nonetheless, the IgT/IgM ratio in the gut is 63 times higher. This finding suggests that IgT plays an important role in mucosal immune responses and this Ig concentrates mostly in mucosa-associated lymphoid tissues such as the skin, gut, and gills (Zhang et al., 2010). Regarding IgD, it has been mostly found in transmembrane form in a variety of vertebrate classes, including teleost fish. However, a study reported that it was found in both membrane and secretory forms in channel catfish (*Ictalurus punctatus*) and Japanese puffer (*Takifugu rubripes*) (Hikima et al., 2011). The function of IgD in fish is still being investigated (Smith et al., 2019).

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Memory B-cells

It is well known that fish possess memory immunological responses, which memory B-cells are produced after the first pathogenic invasion or primary vaccination. Memory B-cells are produced and circulated in the bloodstream before migrating to lymphoid tissues to mature. Memory B-cells will be activated to make specific antibodies for

stronger and faster secondary responses. Once the fish are re-stimulated with the same pathogens, the infections can be quickly cleared up (Secombes & Belmonte, 2016; Smith et al., 2019).

2.3.2. Passive immunity in fish

Passive immunity can be defined as the processing of extraneous immunological components derived from both the innate and adaptive immune systems are transferred to another individuals to produce a transient degree of protection against pathogens. In fish, passive immunity can be the transfer of immune elements from broodstock to their offspring.

2.3.2.1. Passive transfer of maternal innate immune factors

In terms of innate immune-relevant factors, vitellogenin (Vg) is an egg yolk precursor protein. It is produced in the liver of female broodstock and then transported to the ovary via the circulatory system. Vg then is proteolyzed into phosvitin (Pv) and lipovitellin (Lv) in the ovary, where it contributes to oocyte growth (Arukwe and Goksøyr, 2003; Zhang et al., 2013). Studies showed that Pv and Lv, which are transferred from the mother broodstock to the egg, have been shown to be innate-immune molecules involved in host defense against bacteria (Zhang & Zhang, 2011; Zhang et al., 2005) and viruses (Garcia et al., 2010). In addition to Vg, other innate-immune related elements such as complement components, lectin, lysozyme, and cathelicidin are also discovered to be passed from broodstock to their offspring. For complement

components, C3-1, C3-3, C4-C5, C7, C7, Bf, and Df were found to be passed from mother to offspring in rainbow trout (Løvoll et al., 2006; Wang et al., 2009; Shen et al., 2011). Regarding lysozyme, this enzyme was found in the egg cytoplasm, showing an important function in zebrafish bacteriolytic activity (Wang & Zhang, 2010). On the other hand, a study found that unfertilized eggs contained transcripts of cathelicidin, an antimicrobial peptide involved in innate immunity in fish (Seppola et al., 2009). Another innate immune factor lectin, which is the protein involved in recognizing PAMPs on the microbe's surface, was found to have undergone a cytoplamic translocation before arriving at their final destination, which is the egg envelope during fertilisation (Tateno et al., 2002; Dong et al., 2004).

2.3.2.2. Passive transfer of maternal adaptive immune factors

IgM is the humoral immune factor of the adaptive immunity that has been most studied for passive immunity in fish. During the process of vitellogenization, IgM is transmitted to the immature oocytes, and it is subsequently absorbed into the fertilized egg and the larval york sac (Kanlis et al., 1995; Picchietti et al., 2001). Up to date, there has been no report in passive transfer of other immunoglobulin serotypes in fish. According to a study in seabream, IgM can be transmitted to the oocytes via follicle cells. Additionally, maternally derived IgM transcripts were found in seabream oocytes (Picchietti et al., 2006). Nevertheless, detailed mechanisms about how IgM is passed between the broodstock and its progeny has not been elucidated yet. Maternal

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passive immunity is being studied as a potential strategy to reduce vertical transmission and improve the survivals of breeding populations and offspring. For instance, vaccination of grouper and Asian sea bass brood fish was shown to be an efficient method for achieving early defence against the nerve necrosis virus and preventing the risk of vertical transmission for the fish progeny (Kai et al., 2010; Pakingking et al., 2018). Other studies showed that administration of inactivated vaccination against *Streptococus agalactiae* in tilapia broodstock were demonstrated to be a promising measure to accumulate SA-specific antibody in fish egg and larvae with a higher survival percentage after challenging with pathogenic SA strains (Nisaa et al., 2017; Nurani et al., 2020). Polyvalent vaccines made up of *Streptococus agalactiae* and *Aeromonas hydrophila* lowered the mortality of tilapia larvae when administered intraperitoneally into broodstock (Pasaribu et al., 2018).

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2.3.3. Current research in tilapia immune system

Tilapia species, like other teleost fish, have both an innate and an adaptive immune system. There are still many unknown aspects of the tilapia's innate and adaptive immune systems that are yet to be discovered.

2.3.3.1. Innate immune system

Tilapia monocytes, macrophages, neutrophils, and eosinophils were characterized in morphology and phagocytosis activities of these cells were observed both *in vitro* and *in vivo* during experimental intraperitoneally injection of liquid paraffin into tilapia

under light and electron microscope. Monocytes, macrophages and neutrophils showed clear phagocytic functions through formation of phagosome whereas eosinophils came later and less actively phagocytized the foreign materials compared to other phagocytes (Suzuki, 1986). Regarding innate humoral components, study showed that zymosan, a yeast cell wall, usually was used for activation of C3 complement component, a central complement responsible for other immune pathways such as opsonization or inflammation. C3 was well characterized in tilapia serum, being a 190 kDa glycoprotein that can be cleaved into C3a (8 kDa) and C3b (180 kDa). C3a play an important role in recruitment of many leukocytes to the infection tissues, resulting in inflammation whereas C3b can interact with PAMPs on pathogenic surfaces, urging the opsonisation and phagocytosis (Abdel-Salam et al., 2014; Smith et al., 2019). Recent research demonstrated that probiotic administration is an effective strategy for enhancement of complement bactericidal activities in Nile tilapia (Dhanarso et., 2021). Involving in the innate denfense in fish, lysozyme has been well identified in tilapia. It is a 15 kDa molecular, found with the high level in the serum of female broodstock tilapia during oocyte development. It was also found in egg and larvae from the broodstock vaccinated with inactivated *S. agalactiae,* suggesting that lysozyme were maternally transferred (Nurani et al., 2020; Takemura & Takano, 1995). Among AMPs found in fish, **β**-defensins have been well characterized in Nile tilapia. They are small cationic amphiphilic peptide encoded by a sequence of 674 bp from DNA genome. *In vitro* antimicrobial experiments demonstrated that a synthetic **β**-

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defensins polypeptide can inhibit the growth of *E.coli* and *S. agalactiae* (Dong et al., 2015b).

2.3.3.2. Adaptive immune system

The putative genes encoding for major histocompatibility complex class I and class II of Nile tilapia have been available on the Genbank. Studies showed that the high polymorphism characteristic of the *MHC-Iα* gene depended on different strains of Nile tilapia (Poonsawat et al., 2009) whereas 2 subclasses of MHC-II, called MHC-IIA and MHC-IIB were identified in Nile tilapia, in which the characteristics in genomic and expression pattern of each one were determined. The *MHC-IIA* gene consisted of four exons and three introns, which their deduced amino acid shared 25.4%-64.5% identity with those of other teleost fish and mammals. The *MHC-IIB* gene consisted of six exons and five introns, which their deduced amino acid shared 26.9%-74.7% identity with those of other teleost fish and mammals. The deduced sequences of MHC IIA and IIB molecules had all the characteristic elements of MHC class II chain structure, including the leader peptide, α1/β1 and α2/β2 domains, linking peptide, transmembrane and cytoplasmic regions, conserved cysteines and N-glycosylation site (Pang et al., 2013).

The T-cell population has been identified by the specific marker TCR. In tilapia, T-cell receptor's β chain has been characterized in genomic and protein structure. The gene encoding for T-cell receptor's β chain comprises of 942 bp open reading frame, deducing 914 amino acid. The β -chain sequence contains four conserved cysteine

residues that play an important role in the formation of disulphide bridges, as well as a conserved amino acid motif that is thought to be important for TCR/CD3 complex formation and signalling (Nithikulworawong et al., 2012). The putative genes encoding CD4 and CD8 have been identified in GenBank based on the whole Nile tilapia genome sequence, but their proteomic structures have yet to be determined. Therefore, CD4 and CD8 receptors, helping to differentiate between $CD4^+$ and $CD8^+$ T-cell population, have not been elucidated yet.

The B-cell population has been identified by the specific marker BCR. To date, there has been no detailed report in characterization of BCR of tilapia. However, the immonoglobulin serotypes including IgM, IgT and IgD secreted by B-cells has been partially discovered in Nile tilapia. Among immunoglobulins, IgM is the most predominant in telost fish. In Nile tilapia, a study revealed that gene expression of secretory (sIgM) and membrane-bound IgM (mIgM) were expressed with high levels in head kidney, spleen and mucosal tissues such as intestine and gill. Both types of IgM were found to be significantly up-regulated in these tissues following a *S. agalactiae* challenge. Serum IgM concentration was found to be incresed after tilapia get infected with *S. agalactiae* using ELISA (Yin et al., 2019). Monoclonal antibody against Nile tilapia IgM has been commercial for monitoring the fish immune respone following challenge or vaccination (Soonthonsrima et al., 2019). Recently, Nile tilapia IgT expression has been characterized from the head kidney tissue. The study showed that the secretory

form of tilapia IgT cDNA comprised of 1344 bp, contain 1122 bp of ORF and deducing for 373 amino acid. On the other hand, the membrane-bound form of tilapia IgT cDNA comprised of 1548 bp, containing of 1239 bp of ORF and deducing 412 amino acid. Analysis of the deduced amino acid sequences with BlastX, the study showed that the sequence of two forms could predict regions including one immunoglobulin domain in variable region, two framework, three complementary determine regions, two immunoglobulin domains in contanst regions and five potential N-glycosylation sites (Velázquez et al., 2018). In term of IgD, a recent research showed that this Ig could play a role in defense responses when the fish get infected with bacteria. Analysis the cDNA encoding for menbrane bound IgD heavy chain revealed this sequence comprised of 3347 bp with 3015 bp of ORF, deducing for 1004 amino acid with the predicted molecular weight of 110.9 kDa. The amino acid sequence was predicted to have a glycosaminoglycan attachment site and major histocompatibility complex protein site. The predicted structure of the menbrane bound IgD heavy chain consisted of the variable (V), diversity (D), and joining (J) regions. The constant region had one μ1 domain, seven CH domains (CH1-CH2-CH3-CH4-CH5-CH6-CH7), and transmembrane regions (Wang et al., 2016).

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2.3.4. Tilapia immunological responses during TiLV infection

To develop an effective vaccine against the disease outbreak, it is very important to understand how the immune system reacts when TiLV is present. A study showed that

TiLV can weaken the immune system of Nile tilapia at the beginning of an infection. The study showed that the gene expression of some pattern recognition receptors, such as TLR3 and TLR4, was down-regulated in the liver and spleen, while TLR3 was significantly up-regulated in the brain and head-kidney. Meanwhile, INF- transcript levels were found to be lower in the spleen and kidney, while they were slightly higher in the brain and liver at the start of the infection. The lower gene expression of some innate genes at the beginning time could explain the reason why viral titer reached its peak and caused mass mortality. However, the study showed that the level of IgM transcript witnessed a gradual increase, which could demonstrate that the mount of TiLV specific antibodies occurred during the infection (Mugimba et al., 2020). This outcome was in line with that of the research conducted by Tattiyapong et al (2020), where the TiLV-challenged fish demonstrated an increase in IgM antibody response 7-day post-challenge (dpc), peaked at 14 dpc, and thereafter dropped until 42 dpc. According to these findings, tilapia that survived after getting infected with TiLV could establish humoral immunity that protects them from subsequent infection with TiLV. The IgM antibody levels in fish re-exposed to TiLV after the primary infection rose more quickly, and no fish perished as a result of the re-exposure (Tattiyapong et al., 2020). Recently, a transcriptome study of the livers of TiLV-infected and uninfected tilapia revealed an increased expression of genes involved in numerous pathways, including antigen processing and presentation; MAPK; necroptosis; apoptosis; chemokine signalling; interferon; and NF-kB. This finding suggested that TiLV infection is a challenge

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for the tilapia and multiple immune responses occurred in the fish to fight off TiLV (Sood et al., 2021).

2.3.5. Vaccine for fish

Vaccines are one of the most effective measures to prevent disease outbreaks in aquaculture. A typical vaccine comprises or generates antigens to boost the fish immune system, including innate and adaptive responses, against a single or many diseases (Adams, 2019). Vaccines are produced by either conventional or biotechnology methods.

The first generation of conventionally developed vaccines for fish consists of inactivated vaccines, which are the most common type of permitted immunisation for fish to date. Inactivated or killed vaccines are those in which the entire bacteria or viruses are rendered incapable of infecting or replicating within or outside of the fish cell by physical (such as UV, heat) or chemical (such as formalin, -propionlactone) methods, without affecting the antigenicity of pathogens. (Ma et al., 2019; Miccoli et al., 2021). Due to the lack of replicate ability, killed vaccinations are deemed safe for fish. They are simple to produce, but they cause modest and transient immune responses. This may be due to the insufficient activation of cellular responses in both innate and adaptive immune systems. Consequently, adjuvants or booster vaccinations should be administered to improve the efficiency of inactivated vaccines (Ma et al., 2019). Many inactivated vaccines has been licenced and are commercial for fish. For

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examples, *Streptococcus* inactivated vaccines were formulated from a single inactivated bacterium of either *S. agalacctae* (biotype 1 or 2) or *S. iniae* and are administered by intraperitoneal injection and booster vaccination 21 days after initial immunisation, which demonstrated an RPS of over 90%. Oral vaccination against *S.agalactiae* was also evaluated with a booster vaccine regimen 14 days following the initial immunisation, revealing a 70% survival rate after virulent strain challenge (Ismail et al., 2016; Kayansamruaj et al., 2020; Pretto-Giordano et al., 2010).

Attenuated vaccines are another type of conventional vaccination developed for fish. They are made up of either bacteria or viruses with attenuated or lost virulence, targeting fish immunity without generating disease, (Adams, 2019). To generate attenuated strains, a variety of techniques such as physical or chemical treatments (e.g., UV, heat, formalin), serial passages on aberrant conditions (for bacteria) or cell culture (for viruses), and genetic modification can be used (Kayansamruaj et al., 2020; Li et al., 2015; Triet et al., 2019). Since they can promote both branches of adaptive immunity, attenuated vaccines are usually more immunogenic than inactivated ones. However, it must be demonstrated that there is no possibility of reverting to virulence. Therefore, the safety of attenuated vaccinations has limited their acceptance for commercial use to date.

The recombinant vaccines for fish are regarded the second generation vaccines. This type of vaccine targets the determinant antigenic proteins that are capable of inducing

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strong immunogenicity (Ma et al., 2019; Miccoli et al., 2021). The genes encoding immunogenic components of microorganisms are introduced into an expression vector. Then the recombinant vectors are cloned into prokaryote or eukaryote expression systems, allowing the production of antigenic proteins based on the host cellular mechanism. The advantage of the recombinant vaccines is its safety due to its inability to infect and its simplicity to create, store, and transport. Their ability to trigger immunological responses, however, is inferior to that of live or inactivated vaccines. This may be the lack of numerous antigens' exposure to the host immune system. Therefore, the effectiveness of subunit vaccines is sometimes reliant on the adjuvant and booster immunisation schedule.

DNA vaccines are manufactured using an expression plasmid carrying one or many genes encoding for antigenic proteins that may be produced in the host cell and elicit the robust immune responses of both innate and adaptive immunity. Recombinant plasmids can be generated by inserting the genes of interest into the numerous cloning sites between the promoter and terminator that can stimulate the expression of proteins in eukaryote cells. The benefit of DNA vaccines is that they can induce both humoral and cellular immune responses, two branches of adaptive immunity. Therefore, DNA vaccines provide superior protection against viruses and intracellular bacteria for fish. Additionally, DNA vaccines can be produced quickly and easily if antigenic proteins are identified. The downside of DNA vaccines is that they require an

efficient delivery method that can carry DNA into the nucleus of the host cell, and the majority of them are administered intramuscularly (Kurath, 2008).

RNA-based technology is a promising new vaccine for humans and animals. RNA vaccines are made from a mammalian alphavirus genome with a single RNA gene encoding for the replication enzyme. The structural genes can be substituted with another gene of interest. RNA recombinants multiply in the cytoplasm of host cells and are destroyed by normal cellular processes; they do not depend on the host replication machine and cannot integrate into the host genome. RNA vaccines boost both arms of cell-mediated immunity, therefore they protect animals from viruses and intracellular bacteria. In fish, RNA vaccines have been being investigated (Adams, 2019; Ma et al., 2019).

2.3.6. Status of vaccine development against *Tilapia tilapinevirus*

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The development of a vaccination against TiLV in tilapia has been reported in a few research to date. First of all, attenuated TiLV strains were generated by passing through 17-20 passages on cell lines, and then they were used for intraperitoneal (i.p) immunization of Nile tilapia and found that RPS values were over 50% (Bacharach & Eldar, 2016). Subsequently, a cell-culture based inactivated vaccine containing β-propiolactone and Montanide IMS 1312 VG adjuvant (Seppic) was created, resulting in a very high protection with a RPS value of 85.7% (Zeng et al., 2021b). In another investigation by the same group, a combination of a DNA vaccine generated by

segment 8, encoding protein VP20, as a primary immunisation and the recombinant protein VP20 as a booster dose at 3 weeks after the initial vaccination were administered by intramuscular (i.m) injection. The VP20 was mixed with adjuvant M402 (China) before performing the booster. This vaccine regime resulted in a survival percentage of 72.5%, compared to 50% and 52.5%, for either DNA vaccine or recombinant protein injection, respectively (Zeng et al., 2021a). In another study, segments 5 and 6 were found to contain some signalling peptides that might presents as viral envelop proteins using bioinformatics predictions. Therefore, some protein fragments were generated from segments 5 and 6 of TiLV and administered into the fish body by i.p injection. Both protein could stimulate the production of specific antibodies against protein expressed from segments 5 and 6, suggesting that they might be potential vaccine candidates for TiLV prevention (Lueangyangyuen et al., 2022). Most recently, a DNA vaccine was generated using the ORF10 enconding a protein of TiLV. It was administered into Nile tilapia by i.m injection and gave relatively high levels of protection depending on vaccine doses, with RPS of 60.71%, 78.57% and 85.72% corresponding to 15 μg, 30 μg and 45 μg of DNA vaccine, respectively. Most of vaccine developed for TiLV prevention were administered by injection (Yu et al., 2022). They are appropriate for vaccine treatment on fish at later stages, such as juveniles and broodstock. Immersion and oral vaccines for TiLV has been researched.

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2.4. Conceptual framework

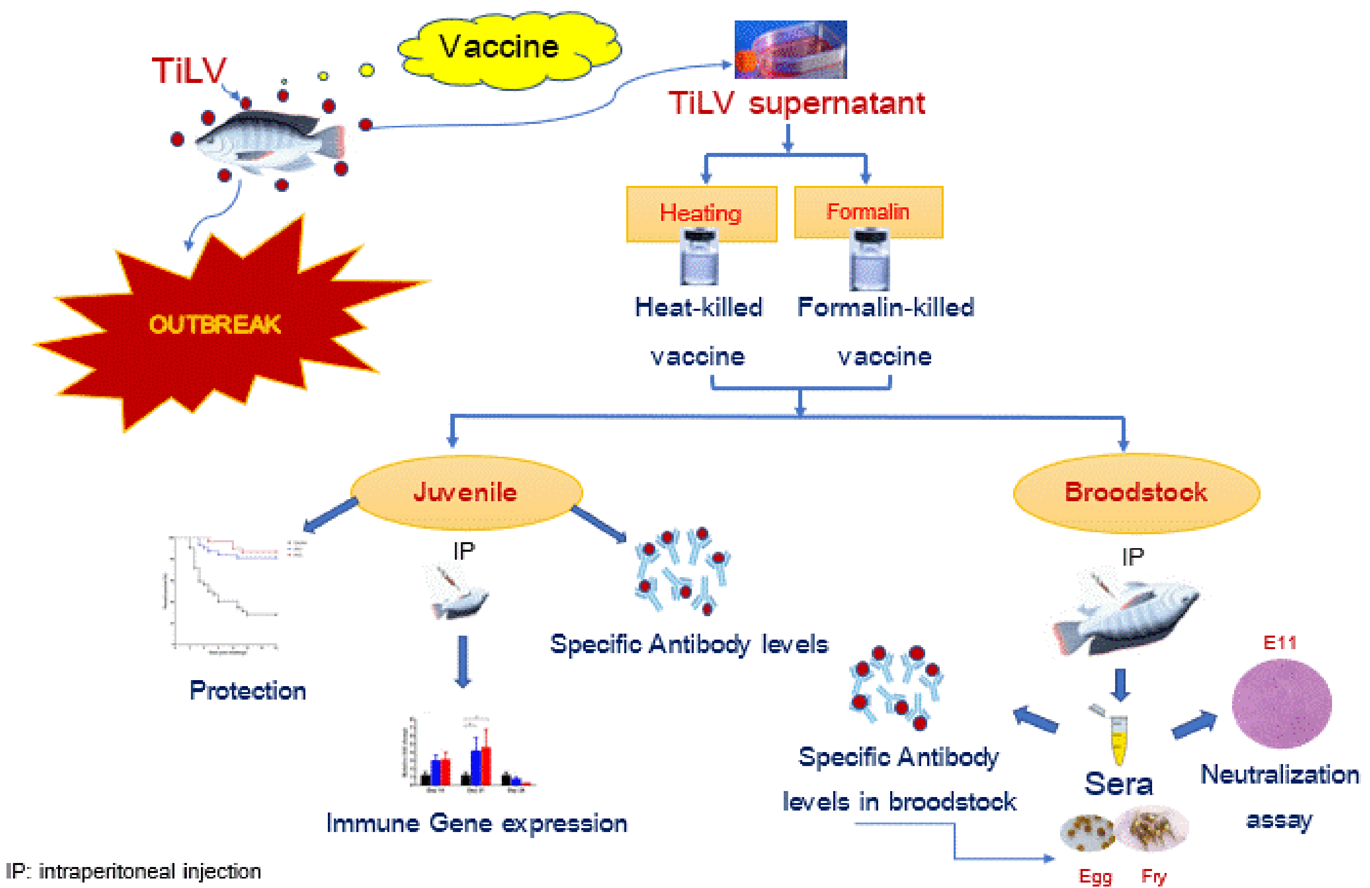

Figure 4. Diagram illustrates the whole experimental design

CHAPTER 3

MATERIALS AND METHODS

3.1. Active immunological responses and protection of juvenile Nile tilapia immunized with TiLV heat-killed and formalin-killed vaccines

This experiment was designed to evaluate if immunization of juvenile Nile tilapia with either HKV or FKV can cause the immune responses and protect fish from TiLV infection. Overall experimental design is presented in **Fig.5**.

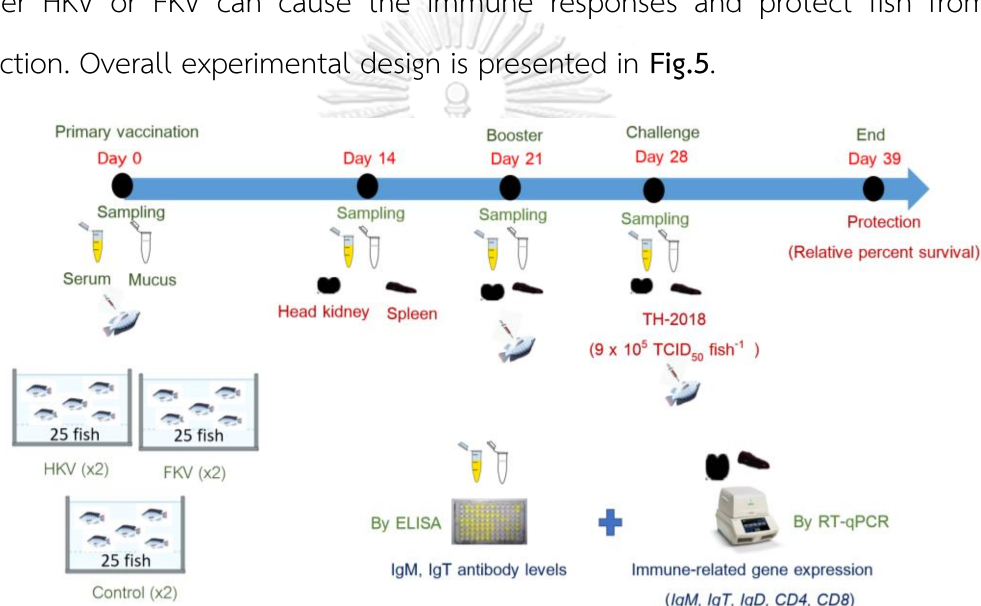

Figure 5. Diagram illustrates immunization of juvenile Nile tilapia with heat-killed and formalin-killed vaccine

3.1.1. Fish

Juvenile Nile tilapia (*Oreochromis niloticus*) (body weight, 7.3 ± 1.2 g; length, 5.9 ± 1.1) were obtained from a commercial tilapia hatchery with no previous record of TiLV infection. The fish were placed in 100-liter containers at a density of 60 fish per tank at around 28°C and fed with a commercial diet daily at 3% of body weight for 15 days

before performing the vaccination trial. Prior to the experiment, 5 fish were randomly selected to screen for the presence of TiLV using a semi-nested PCR (Taengphu et al., 2022) and bacteria using conventional culture method and found to be negative. Water quality parameters including pH, ammonia, and nitrite concentration was monitored every 3 days using a standard Aqua test kit (Sera, Germany), and water was changed twice per week. The vaccination study was approved by Kasetsart University Institutional Animal Care and Use Committee (ACKU62-FIS-008).

3.1.2. Virus preparation

TiLV strain TH-2018-K was isolated from Nile tilapia during a TiLV outbreak in Thailand in 2018 using E11 cell line following the protocol described previously by Eyngor et al., (2014). The virus was cultured in 75 cm^2 flasks containing confluent E11 cells and 15 ml of L15 medium at 25°C for 5-7 days or until the cytopathic effect (CPE) of around 80% was obtained in the cell monolayer. The culture supernatant containing the virus was centrifuged at 4,500g for 5 min at 4°C (Eppendorf 5810R) and stored at -80°C. The concentration of the virus was determined by calculating the virus titre as 50% tissue culture infective dose per milliliter ($TCID_{50}$ mL^{-1}) (Reed & Muench, 1938).

3.1.3. Vaccine preparation

TiLV TH-2018-K (1.8×10^7 $TCID_{50}$ ml^{-1}) was used to prepare both HKV and FKV. Viral inactivation was performed at 60°C for 2, 2.5, and 3 hrs or with formalin (QReC) at a

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final concentration of 0.002%, 0.004%, 0.006%, 0.008% and 0.01% for 24 hrs at 25°C. Viral infectivity was then checked on E11 cells. Successful inactivation of the virus was confirmed by the absence of a cytopathic effect (CPE) after 7 days with all inactivation conditions tested (**Table S1**). Subsequently, inactivation of the virus was performed at 60°C for 2.5 hrs for HKV, while incubation of 0.006% formalin at 25°C for 24 hrs was used for FKV. The inactivated viral solutions were used as vaccine preparations and were not adjuvanted. These were stored at 4°C until used.

3.1.4. Immunization, sampling and challenge test

Before immunization, 6 fish were chosen randomly from the fish population for blood and mucus sampling. The vaccine study comprised of three groups (HKV, FKV and control). Each group consisted of two 100-L replicate tanks with 25 fish each. Prior to vaccination, fish were anaesthetized using clove oil (100 ppm). Fish in the vaccine groups were immunized with either HKV or FKV by intraperitoneal (IP) injection with 100 µL of vaccine using a 28G × 13 mm needle. Booster immunization was carried out at 21 dpv with the same dose of vaccine. Fish in the control group were treated the same, except L15 medium was used in place of the virus solution. Three fish from each tank were randomly collected at 14, 21 and 28 dpv for blood, mucus and tissue sampling (6 biological replicates per treatment). Before sampling, fish were anaesthetized with clove oil at 100 ppm. Mucus samples were collected from each fish by placing the fish into a plastic bag containing 1 mL phosphate-buffered saline

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(PBS, 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1.8 mM KH_2PO_4) followed by gentle rubbing for 30s. These were then centrifuged at 4,000 g for 10 min. The mucus supernatant samples were collected and stored at -20°C until used. Blood (~ 200 µL) was withdrawn from caudal vessel using a 25G × 16 mm needle and allowed to clot for 2 hrs at 4°C. Serum was collected after centrifugation the blood at 4,000 g for 10 mins (Thermo Scientific, UK) and then stored at -20°C. Tissues (head kidney and spleen) were collected, immediately placed in Trizol solution (Invitrogen, UK), and kept at -20°C until RNA extraction. For the challenge test, a viral stock of TiLV strain TH-2018-K (1.8×10^7 $TCID_{50}$ mL^{-1}) was diluted 2 times with sterile distilled water. Each fish was injected IP with 0.1 mL of the diluted TiLV solution (9×10^5 $TCID_{50}$ $fish^{-1}$) at 28 dpv, and mortalities were monitored daily for 21 days. Representative dead fish from each group were subjected for TiLV diagnosis using an in-house RT-qPCR (Taengphu et al., 2022).

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3.1.5. Immune-related gene expression by RT-qPCR

RNA was extracted using Trizol (Invitrogen, UK) following the protocol recommended by the manufacturer. Genomic DNA contamination was removed using DNase I (Ambion, UK) according to the manufacturer's instructions. After DNase I treatment, RNA samples were re-purified using an equal volume of acid phenol:chloroform (5:1, pH 4.7) (Green & Sambrook, 2019) before checking quality and quantity of extracted RNA with Nanodrop ND-1000 Spectrophotometer (Thermo Scientific, UK). DNA

contamination in the treated RNA samples was assessed by performing a qPCR cycling with tilapia elongation factor 1**α** (*EF-1α*) primers using No-RT master mix (absence of reverse transcriptase enzyme provided in iScriptTM Reverse Transcription kit, Bio-Rad, USA). The cDNA synthesis (20 µL reactions) was performed using an iScriptTM Reverse Transcription Supermix (Bio-Rad, USA) containing 100 ng RNA and incubated 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. Immune-related gene expression in the head kidney and spleen were analyzed using a quantitative real-time PCR, with specific primers as listed in **Table 1** and iTaq Universal SYBR Supermix (Bio-Rad, USA). The 10 µL reaction consisted of 5.0 µL 2X Supermix, 0.5 µL forward and reverse primers (10 µM each), 1.0 µL cDNA and 3.0 µL distilled water. The reaction consisted of an initial activation at 95°C for 2 min, followed by 40 amplification cycles of denaturation at 95°C for 30 s, annealing at the optimal temperature of each primer pair (as shown in Table 1), and extension at 72°C for 30 s. Gene expression data for the immune-related genes of vaccinated and control fish were normalized with that of *EF-1α* gene amplification using the $2^{-\triangle\triangle}$Ct method (Livak & Schmittgen, 2001).

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Table 1. Details of primers used for immune-related gene expression in this study.

Gene	Oligo sequences	Annealing (°C)	Product	Gene functions	References
EF-1α	F-5’-CTACAGCCAGGCTCGTTTCG-3’ R-5’-CTTGTCACTGGTCTCCAGCA-3’	56	139	Elongation factor (housekeeping genes)	(Velázquez et al., 2018)

IgM	F-5'-GGATGACGAGGAAGCAGACT-3' R-5'-CATCATCCCTTTGCCACTGG-3'	53	122	Immunoglobulin M (IgM)	(Velázquez et al., 2018)
IgT	F-5'-TGACCAGAAATGGCGAAGTCTG-3' R-5'- [illegible]ACATTCTTTAGAATTACC-3'	53	163	Immunoglobulin T (IgT)	(Velázquez et al., 2018)
IgD	F-5'-AACACCACCCTGTCCCTGAAT-3' R-5'-GGGTGAAAACCACATTCCAAC-3'	61	127	Immunoglobulin D (IgD)	(Wang et al., 2016)
CD4	F-5'- GCTCCAGTGTGACGTGAAA-3' R-5'- TACAGGTTTGAGTTGAGCTG-3'	61	106	Receptor on T-cells	XM_025911776.1, this study
CD8	F-5'- GCTGGTAGCTCTGGCCTTT-3' R'-5'-TGTGATGGTGTGGGCATCTC-3'	49.5	91	Receptor on T-cells	XM_005450353.3*, this study

*Homolog (98% nucleotide sequence identity) of *Oreochromis aureus CD8**α*** (XM_031747820.2).

3.1.6. Measurement of antibody response by ELISA

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Polystyrene 96 well ELISA plates were coated with 0.01% poly-L-lysine solution for 1 hr. The plates were then rinsed 3 times with low salt wash buffer (LSWB, 2 mM Tris; 38 mM NaCl; 0.005% Tween 20, pH 7.3) before the addition of 100 μL of either heat- or formalin-inactivated TiLV (1.8 x 10^7 $TCID_{50}$ mL^{-1}) overnight at 4°C. The plates were washes 3 times with LSWB, followed by a blocking step with PBS + 1% bovine serum albumin (BSA, Sigma) for 2 hrs at room temperature (around 28°C). Then, 100 μL mucus (undiluted) or sera (diluted 1:512 in PBS) were added to each well and incubated overnight at 4°C. The following day, the plates were washed 5 times with high salt

wash buffer (HSWB, 2 mM Tris; 50 mM NaCl; 0.01% Tween 20, pH 7.7) and incubated with anti-tilapia IgM (Soonthonsrima et al., 2019) diluted at the ratio 1:200 in PBS + 1% BSA for 2 hrs at around 28°C. The plates were then washed 5 times with HSWB followed by incubation of goat anti-mouse antibody (Merck, Germany) conjugated with HRP (diluted 1:3000 in LSWB + 1% BSA) for 1 hr at around 28°C. The plates were finally washed 5 times with HSWB before adding 100 µL of TMB (Merck, Germany) to each well. Color was developed in the dark for 5-10 mins before adding 50 µL of 2 M H_2SO_4 stop solution (Merck, Germany). Optical density was read at wavelength 450 nm using the microplate reader (SpectraMax ID3, USA).

3.1.7. Statistical analysis

GraphPad Prism 6 was used to generate the graphs. Kaplan-Meier analysis was performed, and the log-rank test was used to compare the survival curves between vaccinated and control groups. The relative percentage survival (RPS) was calculated using following equation:

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$$RPS = \left(1 - \left[\frac{\text{Average \% mortality of vaccinated}}{\text{Average \% mortality of unvaccinated fish}}\right]\right) \times 100\%$$

The differences in relative fold change of immune-related gene expression and specific antibody IgM level were compared using two-way ANOVA followed by the LSD post

hoc test. The differences are considered at different levels of significance $p < 0.05$, $p<0.01$, $p<0.001$ and $p<0.0001$.

3.2. Immunological responses of broodstock Nile tilapia immunized with HKV and FKV and passive immunity

This experiment was designed to evaluated if immunization of either HKV or FKV can trigger the immune responses against TiLV in broodstock. Furthermore, the objective was to assess whether the passive TiLV- specific antibodies could be transferred from immunized broodstock to their offspring and if these antibodies could protect the fish from TiLV infection. The experimental design is presented in **Fig 6**.

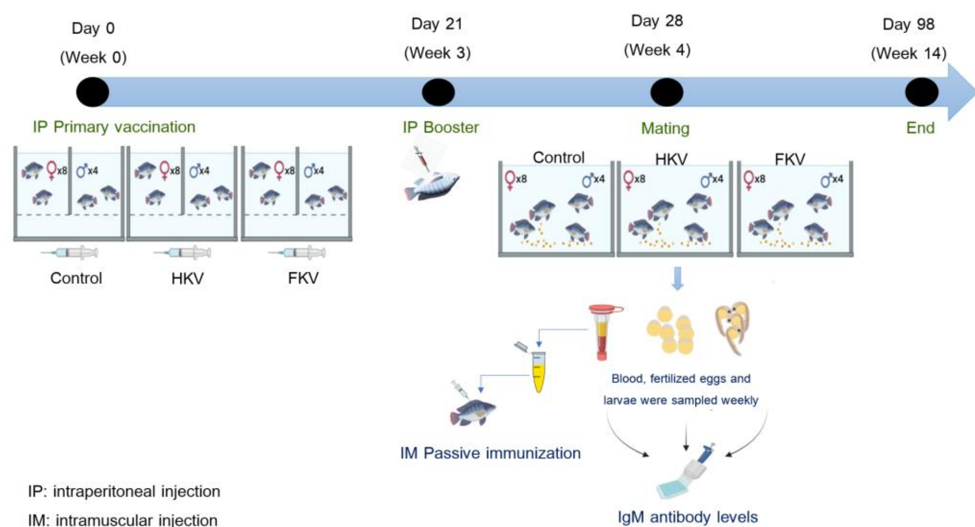

Figure 6. Diagram illustrating the experimental design for broodstock TiLV vaccination, mating and sampling

3.2.1. Fish

Thirty-six Nile tilapia broodstock (12 males and 24 females, body weight 600-800 gram) were kindly provided by the Fisheries Research Station, Faculty of Fisheries, Kasetsart University, Thailand, which were clinical healthy, sexually mature, and ready for breeding. These fish were originally obtained from a hatchery with no previous history of TiLV infection. Fish were separated by gender and acclimated in two 3000 L plastic tanks with aeration. The fish were maintained in an indoor system at a water temperature of 29 ± 1 ^{o}C and fed twice daily at 3% of their body weight with a commercial pellet. Fish were cultured in dechlorinated tap water and half of the water volume was renewed weekly. Prior to the experiment, blood samples were taken from ten randomly selected tilapia and tested for the presence of TiLV using RT-qPCR (Taengphu et al., 2022), and their TiLV-free status at the point of sampling was confirmed. All the animal experiments and procedures used in this study were ethically approved by the Kasetsart University Institutional Animal Care and Use Committee (ACKU62-FIS-008).

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3.2.2. Vaccine preparation

The TiLV inactivated vaccines were prepared as described previously in this chapter. Briefly, TiLV strain TH-2018-K, which was isolated from Nile tilapia during a TiLV outbreak in Thailand in 2018, was propagated on E11 cells, in Leibovitz's L15 medium (Sigma, USA) containing 5% fetal bovine serum, until a cytopathic effect (CPE) of

approximately 80% of the cell monolayer was achieved. The supernatant containing the virus was collected and clarified to remove cell debris, by centrifuging at 4500 g for 5 min at 4°C. Virus concentration was determined using 50% tissue culture infectious dose ($TCID_{50}$/mL) (Reed & Muench, 1938). The virus was inactivated by either heating at 60°C for 2.5 hrs or incubating in a 0.006% formalin solution (16.2 µL formalin 0.37% in 1X phosphate-buffered saline [1X PBS, 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 and 1.8 mM KH_2PO4, pH 7.4] per 1.0 mL viral stock) at 25°C for 24 hrs. Viral infectivity was tested on E11 cells and successful inactivation was confirmed when no CPEs was observed after 7 days. The vaccines were stored at 4°C until used. The virus concentration was determined to be 1.8 $\times 10^{7}$ $TCID_{50}$ per mL before being used. All chemicals used were purchased from Merck (USA).

3.2.3. Immunization, breeding and sampling

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The experimental design for broodstock vaccination is shown in **Fig. 6**. Three groups of broodstock were divided into three tanks, each containing 4 males and 8 females, as previously described (Amadou Ly et al., 2021), where gender groups were separated by a partition. Before vaccination and blood sampling, fish were anaesthetized using clove oil (100 ppm). Fish were immunized by interperioneal (IP) injection with 0.2 mL of either HKV, FKV (3.6 × 10^{6} $TCID_{50}$ per fish) or L-15 medium (control), respectively. Three weeks after the primary vaccination, a booster was administered in the same manner. All fish were clinically healthy after receiving the vaccines. One week after the

booster vaccination, the tank partitions were removed and male and female broodstock were allowed to mix and mate. In each group, blood (~ 500 μL per fish) was collected from one male and one or two female broodstock that did not have fertilized eggs in their mouth weekly after mating from the caudal vessel using a 25G needle. Blood was allowed to clot, and sera were collected by centrifuging the blood samples at 4000 g for 15 min. Sera were stored at -20°C for analysis.

3.2.4. Egg and larvae collection

Fertilized eggs were collected 6 to 14 weeks post primary vaccination (wppv). Approximately 50 fertilized eggs (constituting a batch of fertilized eggs) were collected weekly from mouths of female broodstock that retained eggs in their mouth. Each batch of eggs were kept in a 1.5 mL tube at -20°C to test by ELISA. The remaining eggs were placed in a conical incubation tank where they were continually circulated and thoroughly oxygenated. When eggs began to hatch, the water flow was reduced, allowing the hatched larvae to swim to the surface of the tank, open their mouth and engulf air. The hatched larvae were then transferred to rearing trays where they were allowed to swim freely. Approximately 50 larvae (forming a batch of larvae) were collected between 1 and 14 days post-hatching (dph) and stored at -20 ^{o}C for ELISA analysis.

After mating, not all female broodstock in the 3 groups produced eggs simultaneously for egg sampling. Fertilized eggs were obtained 6, 7, 9, 10, 12-wppv from the control

group; 7, 8, 9, 11, 12 and 14-wppv from the HKV group and 6, 7, 10, 11, 12, 13, 14-wppv from the FKV group. The number of batches of fertilized eggs and larvae that were collected from each batch of hatched eggs at different time points are indicated in **Table 2**.

Time / Treatment	6 wppv	7 wppv	8 wppv	9 wppv	10 wppv	11 wppv	12 wppv	13 wppv	14 wppv
Control	2 FE 1 L (3D-7D-14D)	2 FE 1 L (1D-7D-14D)		1 FE 1L (1D-7D-14D)	2 FE 1 L (1D-7D)		1 FE		
HKV		1 FE 1 L (1D-7D)	1 FE 1 L (1D-7D-14D)	2 FE 1 L (1D)		2 FE 1 L (1D-7D)	1 FE 1 L (1D-7D)		2 FE
FKV	1 FE 1 L (3D-7D)	1 FE 1 L (1D-7D-14D)			2 FE 1 L (1D-7D)	1 FE	1 FE	2 FE	1 FE

Table 2. Batches of fertilized eggs and larvae were collected at different time points post-primary vaccination

Wppv, week post-primary vaccination; HKV: heat-killed vaccine; FKV: formalin-killed vaccine; FE, batch of fertilized eggs (50 fertilized eggs); L, batch of larvae with different day old; D, day post-hatch

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3.2.5. Detection of TiLV-specific antibody by Dot Blot

Five microliters of either heat-killed vaccine or formalin-killed vaccine was dotted in nitrocellulose membranes with 3 dots for every treatment. After being dry in 15 minutes at room temperature (28°C), the membranes were blocked with 1X PBS + 5% skimmed milk and stored at 4°C overnight. Egg supernatants were prepared by homogenizing 100 mg fertilized eggs in 1X PBS containing 0.05% Tween 20 (BioRad, USA) and then the supernatants were collected after samples were centrifuged at 5000 g for 10 min at 4°C. In every treatment, random four female, four male fish sera and four egg supernatants were selected and pooled into 3 samples. The membranes were incubated with fish sera and egg supernatant diluted in 1X PBS at the ratio 1:512 and 1:8 at 4°C overnight, respectively. On the next day, the membranes were washed 3 times with 1X HSBW in 10 mins for each before being incubated with anti-IgM tilapia diluted 1:100 in 1X PBS + 5% dry skimmed milk for 2 hours at RT. After the membranes were washed 3 times with 1X HSBW in 10 mins for each, they were incubated with goat anti-mouse IgG-HRP diluted 1:3000 in 1X LSWB containing 5% skimmed milk for 1 hour at RT. Finally, the membranes were rinsed three times with HSWB before being exposed to enhancing chemiluminescent (ECL) that can react with HRP enzyme, allowing light to be emitted on the X-ray film as a result of a chemical reaction.

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3.2.6. Measurement of TiLV-specific antibody IgM (Anti-TiLV IgM) levels by ELISA

Anti-TiLV IgM levels were measured in broodstock sera, fertilized eggs, and larvae by ELISA. A sample of 100 mg eggs or larvae was homogenized on ice in 400 µL 1X PBS containing 0.05% Tween 20 (BioRad, USA). The samples were then centrifuged at 5,000 g for 10 min at 4°C and the supernatant collected. Ninety-six well polystyrene ELISA plates (Corning, China) were coated with 100 µL of 0.01% poly-L-lysine solution (Sigma, USA) for 1 hr at 28°C. They were rinsed 3 times with low salt wash buffer (LSWB, 2 mM Tris; 38 mM NaCl; 0.005% Tween 20, pH 7.3) before incubating them with 100 µL of either heat- or formalin-killed TiLV (1.8 ×10^7 $TCID_{50}$ per mL) overnight at 4°C. The following day, 50 µl glutaraldehyde 0.05% (EMS, USA) was added and incubated for 20 min at 28°C, then wells were rinsed 3 times with LSWB. Non-specific binding sites were blocked by the addition of 100 µL of 1% bovine serum albumin (BSA, Sigma, USA) in 1X PBS for 2 hrs at 28 ^{o}C. During this time 100 µL of either fish sera (diluted 1:1024 in 1X PBS), egg supernatant (diluted 1:8 in 1X PBS) or larvae supernatant (diluted 1:2 in 1X PBS) were prepared. The blocking reagent was removed from the wells and the diluted samples added to the ELISA plate, which was subsequently incubated overnight at 4°C. The plates were rinsed 5 times with high salt wash buffer (HSWB, 2 mM Tris; 50 mM NaCl; 0.01% Tween 20, pH 7.7) before incubating them with an anti-tilapia IgM monoclonal antibody (Soonthonsrima et al., 2019) (diluted 1:200 in 1X PBS + 1% BSA) for 2 hrs at 28°C. The plates were then rinsed 5 times with HSWB, followed

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by incubation with goat anti-mouse antibody conjugated with horseradish peroxidase (diluted 1:3000 in LSWB + 1% BSA) for 1 h at 28°C. Finally, the plates were rinsed 5 times with HSWB, each well was filled with 100 µL of 3,3′,5,5′-Tetramethylbenzidine (TMB), and the reaction was allowed to develop in the dark for 5-10 mins before adding 50 µL of stop solution (2 M H_2SO_4). Optical density was measured at a wavelength of 450 nm using the microplate reader (SpectraMax ID3, USA). The OD_{450} cut-off value was calculated as standard deviation (SD) × f + mean OD_{450} value of negative control wells, where the f values were the standard deviation multipliers corresponding to the 95% confidence levels at a sample sizes of 2-30 (Frey et al., 1998). Negative controls were the fish sera, fertilized eggs, or larval supernatant from the control group. The cut-off OD_{450} values for broodstock sera, fertilized eggs and larval supernatant were calculated as indicated in **Table 3**. Unless specifically mentioned in the text, all chemicals used were purchased from Merck (USA).

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Table 3. Structures for calculation of ELISA cut-off values (Frey et al., 1998)

Samples	Structures	Cut-off values
Female broodstock sera	Mean OD_{450} + 2.077 × SD	0.070
Male broodstock sera	Mean OD_{450} + 2.01 × SD	0.130
Egg supernatant	Mean OD_{450} + 2.01 × SD	0.104
Larval supernatant	Mean OD_{450} + 1.923 × SD	0.106

Standard deviation multipliers (f) were derived from critical values for a one-tailed *t*-distribution with a confidence level of 95% (Frey et al., 1998), where f = 2.077, 2.01,

2.01 and 1.923 for female broodstock, male broodstock, egg and larval supernatant, respectively.

3.2.7. Passive immunization

Sera pooled from three female broodstock in each group, including HKV with OD_{450} values of 0.911, 1.007, and 0.647, FKV with OD_{450} values of 1.048, 0.889, and 0.944 and the control group with OD450 values of 0.057, 0.058, and 0.060 (1:1024 dilution in 1X PBS before pool) were used for the passive immunization experiment. Clinically healthy tilapia juveniles (body weight 20.3 ± 6.7 g; length 10.9 ± 0.7 cm) were acclimated in dechlorinated tap water using 100-L tanks, with a density of 20 fish per tank. Prior to the experiment, five fish were randomly tested for the presence of TiLV using a RT-qPCR assay (Taengphu et al., 2022) and confirmed as negative. Prior to immunization, fish were anaesthetized using clove oil (100 ppm). Three groups of 20 fish were immunized intramuscularly (IM) in the dorsal musculature with pooling sera (50 µL/fish) from HKV (group 1), FKV (group 2), and control (group 3). Another group of 20 fish (group 4) were IM immunized with L15 as negative control. Twenty-four hours after passive immunization, groups 1, 2 and 3 were IP challenged with TiLV TH-2018 (9×10^5 $TCID_{50}$ per fish) and group 4 were challenged virus-free cell culture media. Cumulative mortalities were recorded for 21 days. Relative percent survival (RPS) was calculated as follows:

$$RPS = \left(1 - \left[\frac{\text{Average \% mortality of vaccinated fish}}{\text{Average \% mortality of unvaccinated fish}}\right]\right) \times 100\%$$

Liver samples from moribund or freshly dead fish, and 5 representative surviving fish from each group were collected at the end of experiment at 21 days post-challenge and placed in RNA later (Sigma) at -20 ^{o}C for viral load determination. RNA samples were isolated using Trizol following the manufacturer's protocol (Invitrogen). Quality and quantity of RNA samples were measured with a Nanodrop ND-1000 Spectrophotometer (Thermo Scientific). TiLV viral load was determined by RT-qPCR, by amplifying 137 bp of TiLV segment 9 using specific Seg9-TaqMan-probe (5'-6-FAM-TGC CGC CGC AGC ACA AGC TCC A-BHQ-1-3'), primers Seg9-TaqMan-F (5'-CTAGAC AAT GTT TTC GAT CCA G-3') and Seg9-TaqMan-R (5'-TTC TGT GTC AGT AAT CTT GAC AG-3') as described by Taengphu et al. (2022). House-keeping gene elongation factor-1**α** (*EF1α*) was used as an internal control for the RT-qPCR. To quantify TiLV copy number, a standard curve was produced using ten-fold dilution of plasmid pSeg9-351 containing 351 bp of TiLV segment 9's open reading frame (Taengphu et al., 2022) (**Fig. S2**).

3.2.8. Neutralization essay (NA)

E11 was prepared on 96-well plate using L15 media complemented with 5% FBS until reaching 90-100% confluent monolayer. The remaining sera of female broodstock were centrifuged 2 times at 5000 g for 10 mins. Collected supernatant were then heated at

50°C in 30 mins for complement inactivation. Every broodstock serum was diluted at the ratio 1:40, 1:60 and 1:80 in L15 to the final volume of 50 **µ**L. Every sample were performed NA 2 times. The number of female broodstock sera that were used for NA were indicated in **Table 5.** Then, every 50 µL of diluted sera were incubated with 50 µl TiLV 10^{2} $TCID_{50}$ ml^{-1} for 2 hours. The mixtures were then transferred to E11 plate in well by well and incubated at 25°C. Three positive control wells were generated by incubation between 50 µL of TiLV $10^{2}TCID_{50}$ ml^{-1} and 50 µL L15, whereas negative control wells are cell line incubated with 100 µL L15 without virus. Changes of E11 cell line were observed at 5-day post inoculation. The neutralized titers were determined as the final dilution levels of fish sera that no CPE appeared (Gauger & Vincent, 2020). The dilutions levels of 40 for the fish sera was considered as the cut-off values since the dilution of 1:40 did not cause the cell lysis for E11 cell line.

3.2.9. Statistical analysis

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GraphPad Prism 6 (GraphPad Software, USA) was used to create graphs. The differences on OD450 readings representing TiLV specific antibody levels were compared with statistically valid cut-off values representing the upper prediction limit using Student's *t*-distribution. Cut-off values were calculated using the structures described in **Table 3**, based on the number of negative control samples and a confidence level of 95%. Kaplan-Meier curves were plotted for cumulative survival rates and the log-rank test was used to compare the differences in survival between groups for the passive

immunization experiment. Nonparametric test Kolmogorov-Smirnov was used to compared the cumulative distribution of neutralizing levels of female broodstock' sera between vaccinated and control samples.

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CHAPTER 4

RESULTS

4.1. Active immunological responses and protection of juvenile Nile tilapia immunized with TiLV heat-killed and formalin-killed vaccines

4.1.1. Efficacy of vaccine

In the challenge experiment, the first mortality occurred at 3-day post challenge (dpc) in the non-vaccinated group (control) and at 5 and 7 dpc in the HKV and FKV groups, respectively (**Fig. 7**). Mortalities continued until 13-15 dpc. Moribund fish showed gross signs of TiLV infection including abdominal distension, skin erosion, exophthalmos, fin rot, gill pallor and pale liver (**Fig. S1**). The dead fish from each group were tested positive for TiLV by RT-qPCR. The survival rates were 81.3 ± 0.0 % and 86.3 ± 0.0% for HKV and FKV groups, respectively, compared to 28.13 ± 30.9% for the control ($p<0.0001$). The survival percentage were analyzed using Kaplan-Meier curves with the log rank test (**Fig. 7**). Average RPS values were 71.3 % for the HKV and 79.6 % for the FKV vaccine (**Table 4**).

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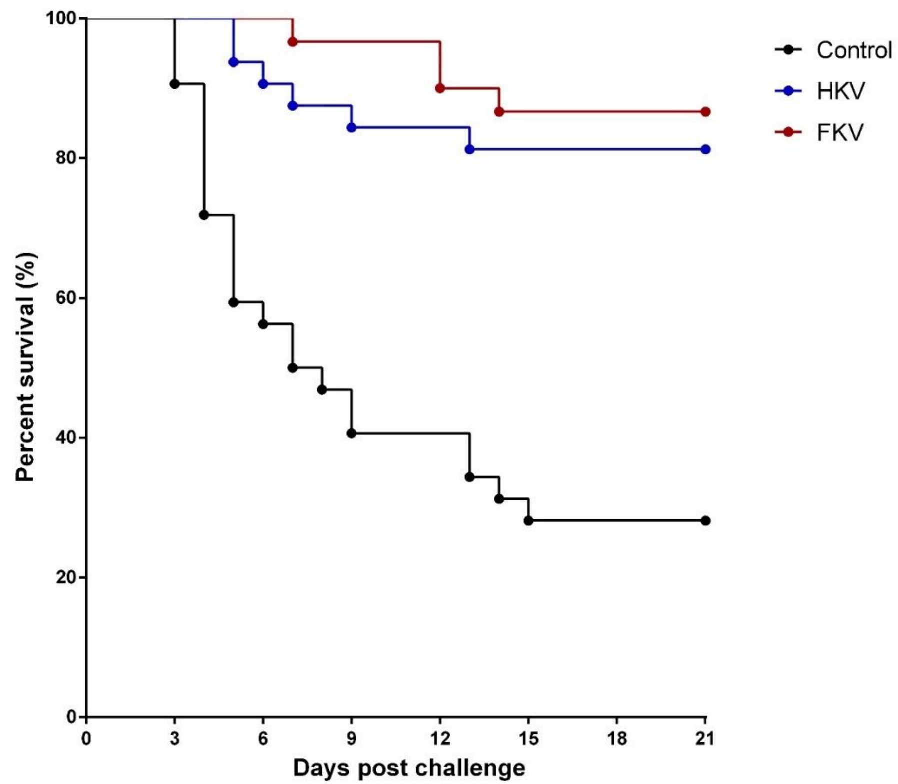

Figure 7. Average percent survival of heat-killed and formaldehyde-killed vaccinated groups (HKV vs. FKV) compared to the non-vaccinated group (Control) at 21-day post challenge with TiLV (strain TH-2018-K). Statistical analysis of cumulative survival between both vaccinated groups and the control were analyzed using Kaplan-Meier curve with log-rank test ($p < 0.0001$)

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Table 4. Details of experimental groups and challenge results

Treatment	Administration route	No of fish challenged	Primary vaccination ($TCID_{50}$ $fish^{-1}$)	Booster vaccination ($TCID_{50}$ $fish^{-1}$)	Challenge ($TCID_{50}$ $fish^{-1}$)	Survival rate (%)	RPS (%)	Significant level (compared to control)
			Day 0	Day 21	Day 28			
Control	IP	16 (× 2 rep.)	0	0	9×10^5	28.13 ± 30.9	NA	
HKV	IP	16 (× 2 rep.)	1.8×10^6	1.8×10^6	9×10^5	81.3 ± 0.0	71.3	$p<0.0001$
FKV	IP	15 (× 2 rep.)	1.8×10^6	1.8×10^6	9×10^5	86.3 ± 0.0	79.6	$p<0.0001$

Control: non vaccinated group; HKV: heat-killed vaccinated group; FKV: formalin-killed vaccinated group; NA: not applicable

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4.1.2. Immune-related gene expression

The relative fold changes of five immune genes (*IgM, IgT, IgD, CD4, CD8*) were compared to that of the control group **(Fig. 8)**. In the head kidney, a non-significant increase of IgM mRNA relative to the control was noted at 14 dpv, which was followed by significant increase relative to the control at 21 dpv for both HKV and FKV groups (**Fig. 8A**, $p<0.05$). A similar trend was observed for IgT at 14 dpv for both vaccine groups, which was followed by significantly higher expression levels at 21 dpv for the HKV group only (**Fig. 8B**, $p<0.05$). Regarding mRNA levels of IgD, there was significant up-regulation of IgD in the FKV group only at 21 dpv (**Fig. 8C**, $p<0.01$). The *CD4* gene was significantly upregulated at 14 dpv in the HKV only (**Fig. 8D**, $p<0.05$) and at 21 dpv in the FKV ($p < 0.001$). No statistical difference was observed in CD8 expression between the vaccinated and control groups at the time point examined (**Fig. 8E**).

In the spleen, non-significant, relative up-regulation of IgM expression was noted in both HKV and FKV groups compared to the control at 14 dpv. **(Fig. 8F)**. There was a slight increase of IgM mRNA level relative to the control in the HKV group after booster (28 dpv), which were not significant. Also, at 28 dpv, IgT expression was over 25 times higher in the HKV group ($p<0.05$) and almost 20 times higher in the FKV group **(Fig. 8G)**. A slight significant increase in IgD expression was seen in the HKV group at 14-dpv (**Fig. 8H**, $p<0.05$). No significant increase of CD4 expression was found at any time point (**Fig.**

8I); meanwhile, an approximately tenfold increase of CD8 expression was observed at 28 dpv in the HKV group (**Fig. 8J**, *p<0.05*).

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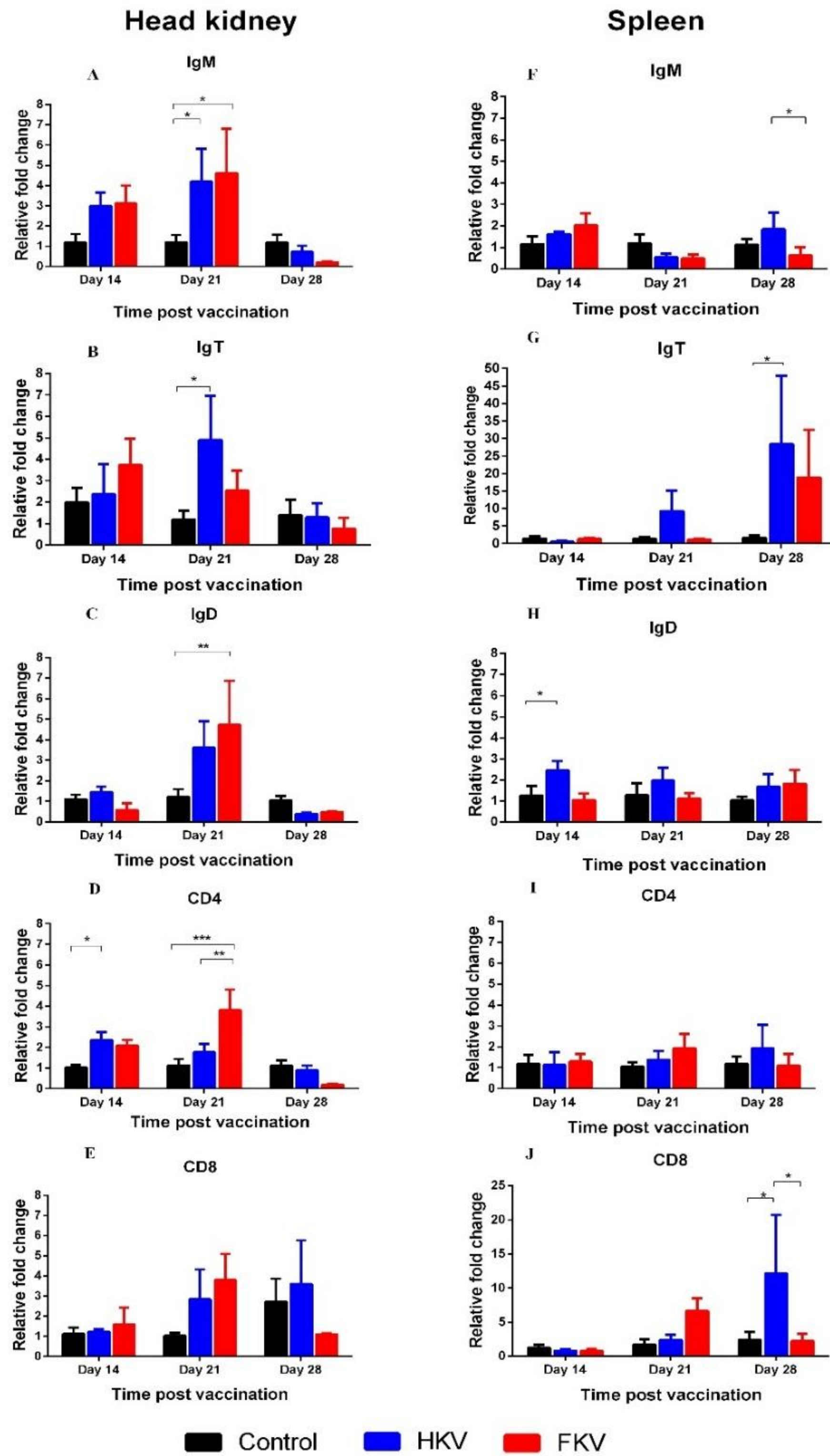
Head kidney
Spleen
A
IgM
B
IgT
C
IgD
D
CD4
E
CD8
F
G
H
I
J
Relative fold change
Day 14
Day 21
Day 28
Time post vaccination
Control
HKV
FKV

Figure 8. Fold change in gene expressions between non-vaccinated and vaccinated fish at 14, 21 and 28 - day post vaccination. Data are presented as the mean ± SE (n=6). Control, non-vaccinated group; HKV, heat-killed vaccine group; FKV, formalin-killed vaccine group. Asterisks show significant levels between groups. * $p<0.05$, ** $p< 0.01$, *** $p<0.001$

4.1.3. Detection of antibody IgM against TiLV in serum and mucus

Systemic TiLV-specific antibody IgM (anti-TiLV IgM) levels pre-vaccination (0 dpv) and at 14, 21 and 28 dpv, as indicated by optical density (OD) at 450 nm, were determined by ELISA (**Fig. 9A, Table S4**). Before immunization, the average OD value of the fish sera was 0.096 ± 0.009. The OD readings for HKV, FKV and control groups were 0.254 ± 0.053, 0.363 ± 0.09 and 0.096 ± 0.015 at 14 dpv, respectively. The OD values showed an increase in antibody levels in both groups of vaccinated fish but were only statistically different in the FKV group ($p<0.01$). A slight decrease was seen in OD readings at 3 wpv in both the HKV and FKV groups relative to the control group (0.249 ± 0.049, 0.317 ± 0.043 and 0.128 ± 0.017, respectively). One week after the booster vaccination at 28 dpv, the anti-TiLV IgM levels had increased considerably in both the HKV ($p<0.001$) and the FKV ($p<0.0001$) groups, reaching the highest values obtained between the different sampling points, compared to that of the non-vaccinated group (average OD readings were 0.438 ± 0.127, 0.483 ± 0.088, and 0.081 ± 0.01 respectively) (**Fig. 9A**).

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A similar pattern was observed with the mucosal anti-TiLV IgM response (**Fig. 9B, Table S5**). Before vaccination, the average OD value of fish mucus was 0.068 ± 0.003. At 14 dpv, the TiLV-specific antibody IgM rose in both vaccinated groups, HKV and FKV, compared to the non-vaccinated group (0.251 ± 0.104, 0.404 ± 0.142, and 0.07 ± 0.005, respectively), but a significant difference was only noted for the FKV group (*p<0.01*). At 3 wpv, the antibody levels were not significantly differed between groups, with OD values of 0.159 ± 0.031 (HKV), 0.290 ± 0.064 (FKV), and 0.083 ± 0.007 (control) being recorded. At 4 wpv (after administering the booster vaccination), a considerable increase in anti-TiLV IgM levels was seen in the mucus of the FKV group (*p<0.001*) (0.585 ± 0.145), whereas the increase measured in HKV fish (0.235 ± 0.044) was not statistically different to that of the control group (0.107 ± 0.018). No significant changes in average OD readings were seen between the non-vaccinated group and pre-immunized fish in either sera or mucus (**Fig. 9 A-B**).

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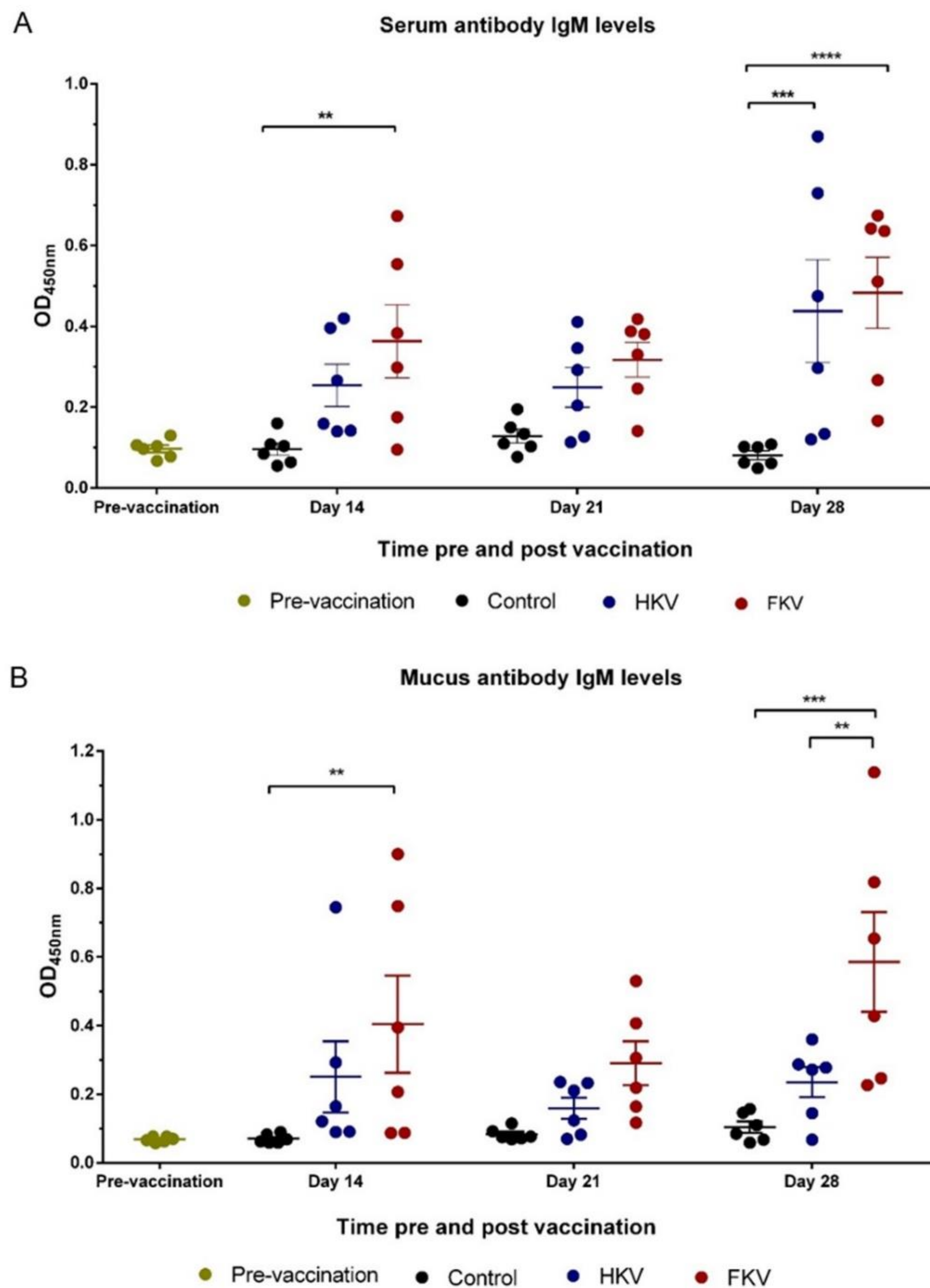

Figure 9. Optical Density (OD) at 450 nm for IgM levels against TiLV in fish sera (diluted 1:512) (A) and mucus (undiluted) (B). Data are presented as the mean ± SE (n=6). Control, non-vaccinated group; HKV, heat-killed vaccine group; FKV, formalin-killed vaccine group. Asterisks indicate significant levels between groups. * *p<0.05*, ** *p< 0.01*, *** *p<0.001*, **** *p<0.0001*.

4.2. Immunological responses of broodstock Nile tilapia immunized with HKV and FKV and passive immunity.

4.2.1. Detection of TiLV-specific antibody by Dot Blot

TiLV-specific antibody were detected in both male and female broodstock vaccinated with HKV and FKV with the dark dots appearing on the membrane, whereas no dots appeared in the control group immunized with virus-free cell culture media. Interestingly, TiLV-specific antibody were found in fertilized egg obtained from both HKV and FKV broodstock, but not in that of control group **(Fig. 10).**

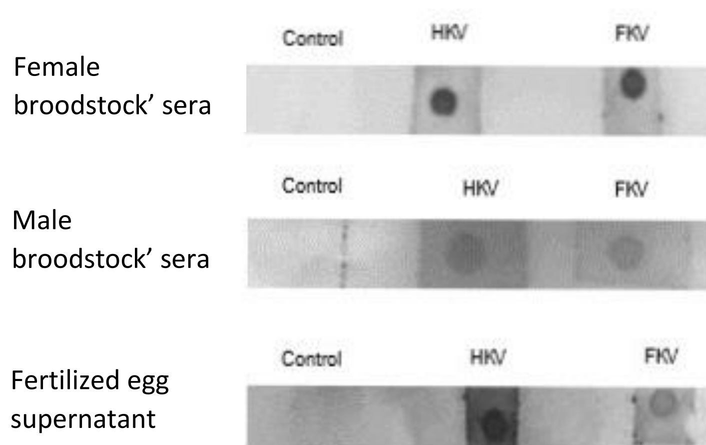

Figure 10. TiLV-specific antibody detected from female and male broodstock vaccinate with either heat-killed vaccine (HKV) or formalin-killed vaccine (FKV) and fertilized eggs derived from every broodstock group.

4.2.2. Measurement of systemic anti-TiLV IgM levels by ELISA

TiLV-specific IgM antibody (anti-TiLV IgM) levels in ELISA were measured as optical density (OD) values at 450 nm **(Fig. 11)** and compared with statistical cut-off values **(Table 3)**. Overall, both male and female broodstock immunized with either HKV or

FKV had OD_{450} values above the cut-off value (0.070 and 0.130, respectively) and higher than that of the control group which were lower than the cut-off value during the period from 6 to 14 wppv. There was one exception, where one female broodstock from the FKV group showed an OD_{450} value below the cut-off value (week 7, **Fig. 11b**). There was wide variation in OD_{450} values between individuals, ranging from 0.230 to 0.497 and 0.089 to 0.398 for male broodstock, and from 0.197 to 1.007 and 0.148 to 1.048 for female broodstock that received HKV and FKV, respectively **(Fig 11a and 11b, Table S6).**

In eggs, TiLV-IgM was detected in fertilized eggs from broodstock immunized with both HKV and FKV over the course of the sampling period, with OD_{450} readings above the statistical cut-off value (0.104) and higher than that of control group **(Fig. 11c).** For the HKV group, the highest TiLV-IgM was detected in the eggs collected at 7 wppv (OD = 0.375), followed by those at 9, 12 and 14 wppv (OD > 0.2), and the lowest values at 8 and 11 wppv with OD values of 0.155 and 0.17, respectively. The FKV group had OD_{450} values much lower than the HKV group, with values ranging from 0.11 to 0.173 **(Fig. 11c, Table S7)**

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In tilapia larvae, TiLV-IgM was detected in 1-day-old larvae derived from the batches of eggs of the HKV-vaccinated female broodstock at 7, 8, 9 and 12 wppv with OD_{450} values ranging from 0.121 to 0.136. On the other hand, TiLV-IgM was detected only in 3-day old larvae derived from the batch of egg of FKV-vaccinated female broodstock

at 6 wppv at OD_{450} of 0.141 **(Fig 11d, Table S8).** All OD_{450} readings of larvae samples from control group were below the cut-off value (0.106).

4.2.3. Passive immunization

After infection with TiLV, the percent survival of the fish receiving sera from HKV and FKV-vaccinated female broodstock (groups 1 and 2) was 85% and 90%, respectively. Conversely, the survival percentage in the group receiving sera from unvaccinated female broodstock (group 3) was only 25%. The differences between groups were statistically significant using a log-rank test ($p < 0.0001$). No mortality was recorded in the negative control (group 4). For the groups vaccinated with HKV sera and FKV sera, an average RPS value of 80% and 86.7%, respectively, was observed **(Fig. 12).**

In all challenge groups, especially in group 3, moribund or dead fish showed a variety of abnormal behaviors and gross lesions. Fish showed loss of appetite, stopped eating, gathered at the corners of the tank and some fish showed erratic swimming. Gross lesions of infected fish showed scale erosion, skin lesions, discoloration. Internally post-mortem changes included gill pallor, liver pallor and ascitic fluid (**Fig. S3**).

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Most dead fish in group 3 were TiLV positive by RT-qPCR. The viral load reached a peak at 6 days post challenge (dpc) with a value of 1.4×10^6/µg for RNA template detected, which gradually declined until 17 dpc with a value of 2.5×10^1/µg RNA template recorded (**Table S2**). There were only 2 and 3 dead fish in groups 1 and 2, respectively. However, only one freshly dead fish from each group was found positive for TiLV by

RT-qPCR. Viral load was undetectable in surviving fish collected at the end of experiment (21 dpc) for all groups.

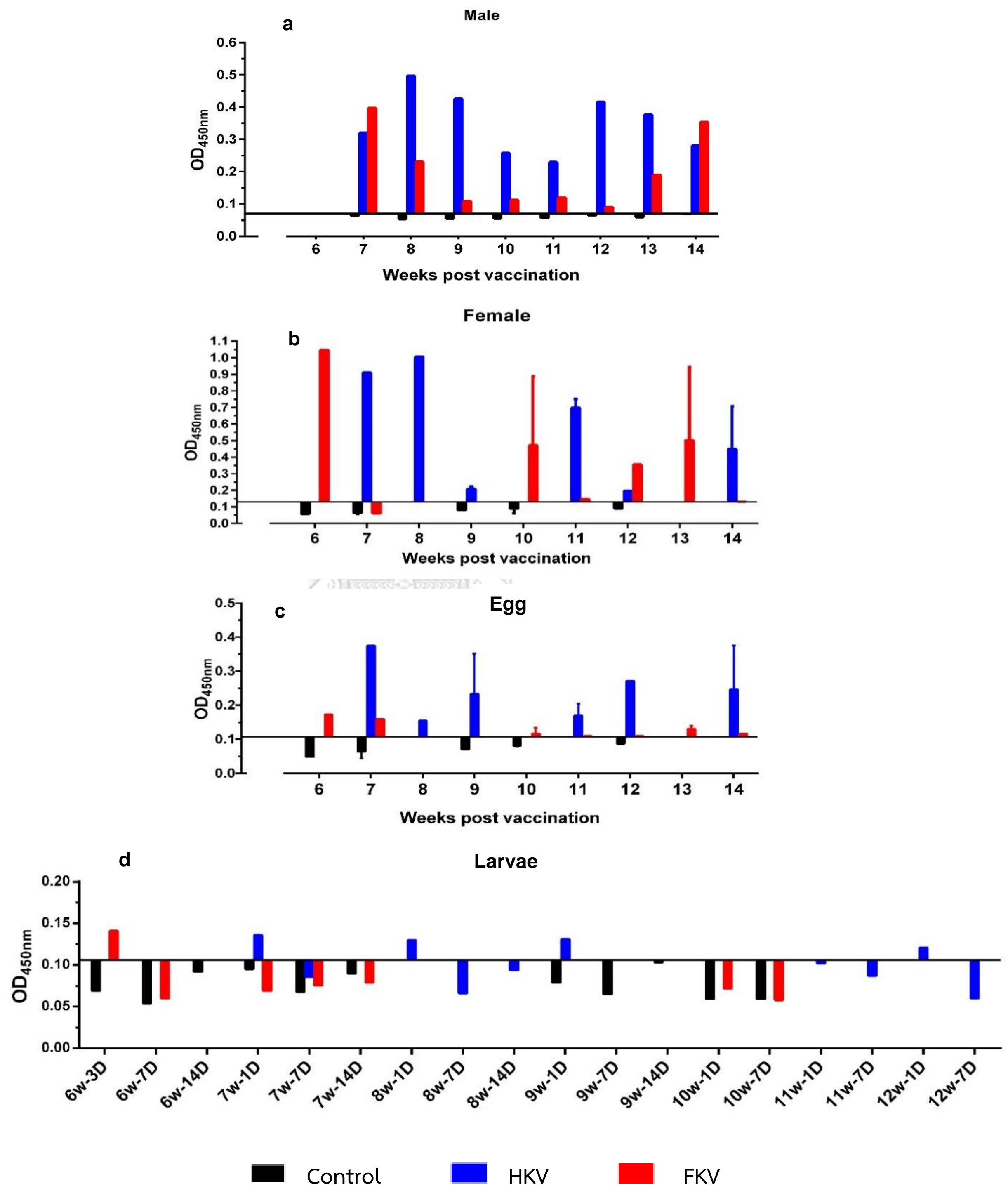

Figure 11. TiLV-specific IgM levels (OD_{450}) from 6 to 14 week post primary vaccination in (a) vaccinated male broodstock (diluted 1:1024, n = 1 per treatment

weekly), (b) vaccinated female broodstock (diluted 1:1024, n = 1-2 per treatment weekly), (c) egg supernatant (diluted 1:8, n = 1-2 per treatment weekly) and (d) larval supernatant (diluted 1:2, n = 1 per treatment at different sampling time points). The OD_{450} values were compared with significant statistical cut-off values. HKV, FKV, control mean that the broodstock, eggs or larvae originate from the formalin-killed vaccine group, the heat-killed vaccine group and the control group, respectively.

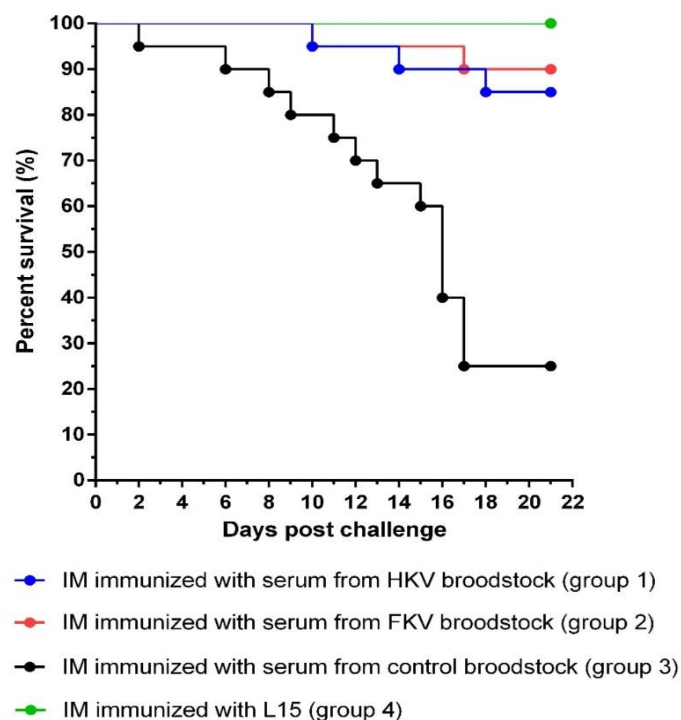

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Figure 12. Average percent survival of Nile tilapia juveniles passively immunized with pooled sera from female broodstock by intramuscular injection (IM) and then challenged with TiLV TH-2018 at 9×10^5 $TCID_{50}$ per fish. Group 1, 2, 4 were significantly different from group 3 (Log Rank test: $p < 0.0001$). HKV, FKV, control means broodstock fish were immunized with formalin-killed vaccine, heat-killed vaccine, and virus-free media (n = 20 per group), respectively. Group 4 is negative control group treated with L15 medium without virus (n = 20).

4.2.4. Neutralization assays (NA)

The result showed that 5/7 (71.4%) and 2/5 (40%) samples was found to be able to neutralize TiLV at the dilution levels ≥ 40 for HKV and FKV treatments, respectively, compared to most of the control samples showed the CPE at the dilution level of 40, except only one sample FC1-7wk did not show CPE at this dilution (16.67%) **(Table 5)**. However, only sera from HKV-broodstock showed the statistical difference in capacity of neutralizing TiLV with that of control ($p<0.05$). The neutralizing titer of all samples were presented in **Table S3**. Positive wells showed very clear CPE 5-day post inoculation whereas no CPE appeared in negative wells **(Fig. S4)**.

Table 5. Neutralization assay for female broodstock sera

Treatment	No of samples run NA	No of samples showed NT ≥ cut-off value (40)	% samples showed NT≥ 40	Significant levels (compared to control)
Control	6	1/6	16.67	
HKV	7	5/7	71.4	$p<0.05$
FKV	5	2/5	40	Not significant

FC: female control group; FH; female HKV group; FF: female FKV group, NT: neutralizing titer

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CHAPTER 5

DISCUSSION

5.1. Active immunological responses and protection of juvenile Nile tilapia immunized with TiLV heat-killed and formalin-killed vaccines

5.1.1. Both HKV and FKV were effective in protecting tilapia from TiLV infection

Although many different types of vaccines have been developed for aquaculture in recent years, whole-cell inactivated vaccines remain the major type of vaccine licensed for use by the aquaculture industry (Kayansamruaj et al., 2020; Ma et al., 2019). They are safe, relatively simple to produce, and are affordable for farmers, especially for species that are intensively cultured, but low in price like tilapia in LMICs. In this study, we prepared two versions of simple water-based inactivated vaccine (HKV and FKV) for TiLV and assessed the ability of both to protective tilapia against the virus. Both HKV and FKV were able to confer relatively high levels of protection (RPS, 71.3% vs. 79.6%) in vaccinated fish. Differences in methods used to inactivate the virus, vaccine formulation, viral strains, antigen concentration, route of vaccine administration and the population of fish can all contribute to the level of protection obtained from a vaccine (**Table 6**). Despite this, vaccination is still considered as a promising strategy to protect tilapia from TiLV infection, although the design of the vaccine should be carefully considered to optimize the level of protection obtained. Other inactivated vaccines have shown relatively high levels of protection in fish. For example, other

formalin-killed vaccines resulted in RPS values of 79%, 81.9% and 74% for infectious hematopoietic necrosis virus in rainbow trout (*Oncorhynchus mykiss*) (Tang et al., 2016), *Betanodavirus* in European sea bass (*Dicentrarchus labrax*) (Nuñez-Ortiz et al., 2016), and scale drop disease virus (SDDV) in Asian sea bass (*Lates calcarifer*) (de Groof et al., 2015), respectively. In addition, a heat-killed *Aeromonas hydrophila* vaccine gave 84% protection in rain-bow trout (Dehghani et al., 2012). Although the efficacy of these and the current vaccines were not tested against heterologous strains of TiLV, the high level of protection elicited against the homologous strain suggests that autogenous inactivated vaccines may be effective as an emergency vaccine to reduce the risk of production losses in affected tilapia farms.

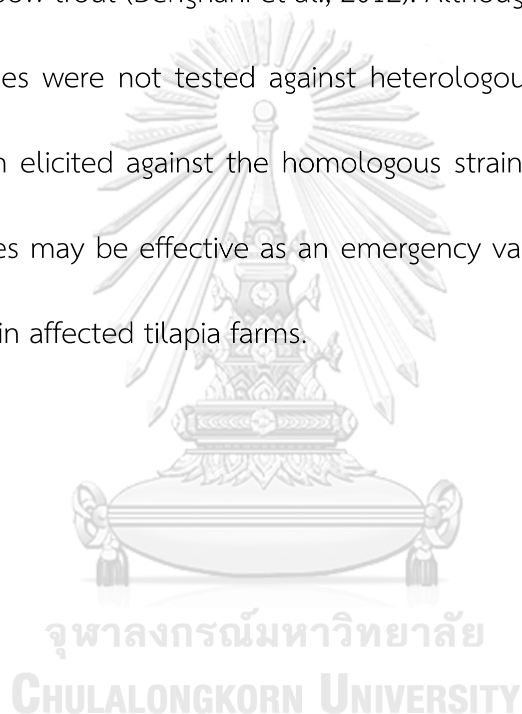
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Table 6. Summary of TiLV vaccines and their efficacies

Vaccine type	Description	Country of origin	Dose ($TCID_{50}$ $fish^{-1}$)	Administration route	Adjuvant	% RPS or survival rate	Reference
Live attenuated	17 and 20 passages (P17 & P20) on cell culture	Israel	1.2×10^7 (P17) and 8.9×10^6 (P20)	IP	No	> 50^a	Bacharach & Eldar et al., 2016
DNA vaccine	Segment 8 (VP20)	China	5 µg	IM	No	50^b	Zeng et al., 2021b
Recombinant vaccine			20 µg	IM	M402 (China)	52.5^b	
DNA + recombinant vaccine			5 µg of DNA vaccine (prime) + 20 µg of recombinant vaccine (booster)	IM	M402 for booster	72.5^b	
Inactivated	β-propiolactone	China	10^5; 10^6; 10^7	IM	Montanide IMS 1312 VG (Seppic, France)	32.1^a - 85.7^a	Zeng et al., 2021a
				IM	No	14.3^a - 42.9^a	
	Heat	Thailand	1.8×10^6	IP	No	71.3^a	This study
	Formalin		1.8×10^6	IP	No	79.6^a	

IP, intraperitoneal injection; IM: intramuscular injection; a: Relative percentage survival (RPS); b: survival rate

5.1.2. Immunization with HKV or FKV activated both branches of the tilapia's specific immune system

Upregulation in the expression of *IgM*, *IgD* and *IgT* and *CD4* (genes encoding proteins involved in humoral immunity) and *CD8* (cell-mediated immunity) following immunization with HKV and FKV suggests that the vaccines are able to activate both arms of the specific immune response in Nile tilapia. Protection from these vaccines is, therefore, likely to result from a synergistic effect of humoral (B cell) and cellular immune (T cell) responses. This is similar to the recent report by Zeng et al (2021a), showing that **β**-propiolactone-inactivated TiLV vaccines induced up-regulation of *MHC-I* and *MHC-II/CD4,* which belong to different arms of the immune system.

The increase in *CD4* transcripts at 14 and 21 dpv in fish vaccinated with HKV or FKV may reflect activated naïve CD4+ cells differentiating into helper T-cell subsets, Th1 and Th2. The Th1 cells produce cytokines that stimulate the expression of anti-viral and inflammatory genes, whereas cytokines secreted by Th2 cells stimulate the differentiation of B-cells into plasma cells to produce specific antibody (Secombes & Wang, 2012; Secombes & Belmonte; 2016; Smith et al., 2019). On the other hand, CD8 transcription was only seen to be significantly up-regulated in the spleen of the HKV group after booster vaccination, indicating that the HKV may stimulate CD8+ cell activation, which then differentiate into cytotoxic T-cells. These cells play a crucial

role in cell-mediated immunity (Bo et al., 2012; Somamoto et al., 2002; Smith et al., 2019).

As well as assessing the expression of *IgM* transcripts, this study also examined the expression of two additional immunoglobulins IgD and IgT. Similar patterns of up-regulation were found in head kidney of fish after the primary immunization, suggesting that all three antibodies may be involved in the protective response elicited by the vaccines. Interestingly, significant increases in mRNA *IgT* levels were seen in the head kidney before booster vaccination and in the spleen after the booster vaccination for both the HKV and FKV groups, suggesting that IgT may be strongly associated with the protective response against TiLV. Unfortunately, the function of IgT in tilapia remains poorly understood. Functional localization studies in other fish species have shown that IgT plays an important role against infectious pathogens on mucosal surfaces, such as skin, gills and gut (Smith et al., 2019; Salinas et al., 2021; Zhang et al., 2011). Nevertheless, further studies are required to gain a better understanding on the role of IgT in tilapia's defense system, especially in response to infection.

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Although immune genes were significantly upregulated in the head kidney after primary immunization, this pattern of expression was not observed in the spleen. This suggest that the head kidney, apart from being a primary lymphoid organ, also act as an important secondary lymphoid organ where specific immune responses to the TiLV vaccine occurred. Studies in other fish have shown that the head kidney, containing

blast cells, plasma cells and melano macrophages, is an important site for antigen presentation and antibody production (Kumar et al., 2016; Soulliere & Dixon, 2017). This might be similar in tilapia. However, it was unexpected to find no significant up-regulation of *IgM, IgT, IgD and CD4* in the head kidney at 7 days after the booster vaccination. It is possible that the increase in gene expression occurred later than 7 days after the booster vaccination or in other secondary lymphoid organs (not assessed in this study). Therefore, future studies should investigate a longer time course for gene expression to better understand the dynamics of immune gene responses after booster vaccination.

5.1.3. HKV and FKV induce both systemic and mucosal IgM

In present study, HKV and FKV were shown to trigger both systemic and mucosal IgM responses, with similar patterns observed between the two vaccines. The increase in systemic and mucosal IgM in teleost is usually derived from the major lymphoid organs, such as head kidney and spleen (Zapata et al., 2006), but also from the mucosa-associated lymphoid organs located in the skin, gills, gut, or nasopharynx (not investigated in this study) (Smith et al., 2019; Salinas et al., 2021). In the present study, up-regulation of IgM expression occurred mainly in the head kidney, and to a less extent in the spleen, suggesting head kidney to be one of the main organs for IgM production in response to the TiLV vaccines. Although the pathway of IgM secretion in the mucosal compartment (mucus) is unclear, it is possible that mucosal antibodies

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are produced locally in the mucosa-associated lymphoid organs and/or by the systemic immune system (Esteban & Cerezuela, 2015; Koppang et al., 2015; Salinas et al., 2011; Salinas & Parra, 2015; Salinas et al., 2021). In other research using Asian seabass, monovalent and bivalent bacterial vaccines induced both systemic and mucosal IgM (Thu-Lan et al., 2021). Similar kinetics have been reported for IgM secretion in the serum of red hybrid tilapia, infected IP with TiLV (Tattiyapong et al., 2020). The levels of serum IgM increased significantly in Nile tilapia after immunization with **β**-propiolactone-inactivated virus (Zeng et al. 2021a) or with a recombinant vaccine based on segment 8 of TiLV (Zeng et al., 2021b). Mucosal IgM was not investigated in these studies, however. The presence of TiLV-specific IgM in the mucus of vaccinated fish suggests that these vaccines may be able to generate a primary immune response in multiple mucosal organs such as skin and gills, which are crucial sites to prevent the initial invasion of pathogenic agents (Esteban & Cerezuela, 2015; Koppang et al., 2015). The IgM levels produced by FKV was always slightly higher than HKV in both serum and mucus at all sampling points analyzed, indicating that FKV induces stronger systemic and mucosal IgM responses than HKV. This could be one of the factors explaining for slightly higher level of protection conferred by FKV.

In this study, increased levels of TiLV specific IgM after booster vaccination in both serum and mucus indicate successful induction of specific immune memory after first immunization. However, low levels of *IgM* mRNA detected at 28 dpv did not reflect

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the IgM levels measure by ELISA at this time point. It was likely that the earlier *IgM* transcripts had already degraded, while its translated products (antibody) remained. B-cells are the major component involved in humoral adaptive immunity. They are activated by specific antigen binding to the B-cell receptors on the cell, followed by presentation of processed antigens to naïve CD4-Tcells, which then differentiate into helper T-cells. With T cells' help, B-cells differentiate into plasma cells and memory B-cells. Plasma cells are committed to antibody secretion, whereas memory B-cells are responsible for the long-lasting protection from subsequent exposure to the same pathogens (Secombes & Belmonte, 2016; Smith et al., 2019).

Although systemic and mucosal IgM levels were assessed in the study, we were unable to measure levels of other antibodies i.e. IgD and IgT by ELISA due to a lack of monoclonal antibodies for these immunoglobulin classes in tilapia. Further studies should investigate the cost of the vaccine for commercial production, the persistence of the immune response in vaccinated fish, duration of protection and efficacy testing these vaccines in a commercial setting.

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5.2. Immunological responses of broodstock Nile tilapia immunized with HKV and FKV and passive immunity

In the previous results, vaccination of tilapia juveniles with HKV and FKV resulted in a significant increase in systemic TiLV-specific IgM and high level of protection against TiLV challenge (RPS = 71.3% to 79.6%). However, persistence of specific antibody was

not evaluated. In the current study, we used the same vaccination protocol for the tilapia broodstock, using double doses of antigen (3.6×10^6 $TCID_{50}$ per fish compared to 1.8×10^6 $TCID_{50}$ per fish in our previous study) for both primary immunization and the booster vaccination. Relatively high levels of TiLV-IgM were detected from 6 to 14-wppv, suggesting that both HKV and FKV elicited relatively long persistence (98 days) of TiLV-IgM in vaccinated broodstock. This finding is consistent with a previous observation in tilapia juveniles challenged with TiLV, where a specific antibody response was maintained for 6 to 16 weeks post infection (Tattiyapong et al., 2020).

Although the protective efficacy of several TiLV vaccines has been reported recently, the specific role of anti-TiLV antibody against TiLV challenge is still unclear, since several studies reported that TiLV vaccines can stimulate both humoral immunity and cell-mediated immunity (Zeng et al., 2021a; Zeng et al., 2021b). In this study, the high survival of passive immunized tilapia (85% - 90%) after receiving sera from the vaccinated broodstock (both HKV and FKV), suggests that humoral immunity plays an important role in protecting against TiLV infection through anti-TiLV antibodies. The reduction in TiLV load during the course of infection, which decreased to undetectable levels in surviving fish by the end of the experiment, reinforces a putative role of protective antibodies in virus clearance. Theoretically, these antibodies could be capable of removing TiLV from the body of the fish by various mechanisms such as neutralization, phagocytosis, antibody-dependent cellular cytotoxicity and

complement-mediated lysis of infected cells (Forthal, 2014). The results of neutralization assay could support for the finding of passive immunization that TiLV specific antibodies can inhibit the TiLV penetration into E11 cell throughout the absence of CPE observed after 5-day inoculation with TiLV. Statistical analysis of the neutralizing titers indicated that the sera derived from HKV-female broodstock might inhibit the virus better than those of FKV-female broodstock. Several studies have shown that passive immunization can protect fish from viral infection. For example, intraperitoneal injection of plasma obtained from Pacific herring (*Clupea pallasii*) recovering from a viral hemorrhagic septicemia virus (VHSV) showed that neutralizing antibodies produced against VHSV after infection could protect fish from this virus (Hershberger et al., 2011). Since tilapia broodstock are usually kept in the hatchery for 3 to 5 years (Towers, 2015), vaccination would be an effective strategy to prevent TiLV infection in the broodstock, minimizing economic loss and maintaining good health of the broodstock during the breeding period.

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Evidence was provided in the current study that maternal antibodies from TiLV-vaccinated tilapia broodstock are transferred to their offspring. Interestingly, these antibodies were found to be protective during passive immunization in tilapia juveniles challenged with the virus. Furthermore, neutralization assay showed that these antibodies could inhibit the viral penetration into the E11 cell line. Together, these results suggests that anti-TiLV antibodies may not only help to reduce the risk of

infection in broodstock but may also reduce the risk of vertical TiLV transmission. Several studies reported that vaccination of broodstock is an effective strategy to enhance the maternal transfer of immunity from mother to offspring and reduce the risk of vertical transmission of the pathogen. For example, tilapia broodstock vaccinated with inactivated vaccines against *Streptococcus agalactiae* and *Aeromonas hydrophila* were able to induce passive transfer of specific antibodies to eggs and larvae, thus improving the quality and survivability of the offspring (Abu-Elala et al., 2019; Nurani et al., 2020; Pasaribu et al., 2018). A bivalent inactivated vaccine against NNV and grouper iridovirus (GIV) administered to grouper (*Epinephelus tukula*) prior to spawning, induced neutralizing antibodies against both NNV and GIV (Huang et al., 2017). These antibodies were vertically transferred to the eggs and reduced the risk of vertical infection. In another study in grouper (*E. tukula*), antibodies against NNV persisted for up to 17 months following vaccination with a NNV-inactivated vaccine. Five months after vaccination, NNV was no longer detectable in the eggs of the vaccinated group, but was detected in the eggs of the unvaccinated group (Kai et al., 2010).

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Higher levels of TiLV-IgM were found in the fertilized eggs of the group vaccinated with HKV than in those of the fish vaccinated with FKV, suggesting that HKV is more promising for successful maternal vaccination. However, TiLV-IgM transfer only persisted for 1 to 3-day post-hatch and was undetectable by 7 and 14-day post hatch.

Because TiLV challenge was unsuccessful at the larval stage of tilapia, we did not evaluate passive antibody protection in the offspring. However, these findings suggest that maternal antibody transfer in larvae does not last long and may be insufficient to protect offspring after 1-3 days post-hatch. This result is in agreement with the results observed in grouper vaccinated against NNV, where NNV-specific antibodies were found to gradually decreased within 48 h after hatching (Kai et al., 2010). Such short persistence can be explained by the gradual decline in IgM during yolk-sac absorption observed in tilapia (Takemura, 1993), and other fish such as European sea bass (*Dicentrarchus labrax*) (Breuil et al., 1997) and Atlantic salmon (*Salmo salar L.*) (Olsen & Press, 1997). Therefore, in addition to vaccination, biosecurity measures remain essential to prevent the introduction of pathogens into tilapia hatcheries, especially during seed production.

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CHAPTER 6

GENERAL CONCLUSION

6.1. Conclusion

The current study has showed that two water-based cell culture heat-killed and formalin-killed TiLV vaccines can protect the juvenile Nile tilapia from TiLV infection. These vaccines are able to activate both branches of adaptive immunity, trigger expression of three immunoglobulin classes and elicit both systemic and mucosal IgM responses. Most importantly, these vaccines showed relatively high levels of protection against TiLV infection, and therefore they seem very promising to be used as injected vaccines targeting large-sized fish for prevention of disease associated with TiLV. Subsequently, we tried both of these vaccines with tilapia broodstock. The conclusion drawn from this study was that vaccination of tilapia broodstock with HKV and FKV resulted in the production of specific antibodies against TiLV in both male and female fish, and that these antibodies can be transferred to the fertilized eggs and larvae to induce maternal immunity. The antibodies were demonstrated to be protective through passive immunization with the pooled sera from female broodfish in juvenile tilapia and challenging with virulent TiLV later. In comparison to FKV, HKV appears to have a stronger capacity for transmitting antibodies from brooders to offspring. Protective antibodies, however, had a short persistence in the larvae, which can be detected 1–3 days after hatching, leaving a gap between maternal immunity

and immunocompetency. Further vaccination is therefore likely to be needed to protect fish from TiLV infection during this gap as well as the later stages of development. In conclusion, vaccine administration with water based either HKV or FKV is a potential strategy aiming to prevent and protect fish from TiLV infection, minimize the risk of TiLV vertical transmission and contribute to specific pathogen free seed production.

6.2. Recommendations

The output of this project hopefully can contribute considerably to tilapia industry and lessen the devastating economic losses. To help tilapia farmers better manage their broodstock health, generate high-quality tilapia seed, and bridge the gaps left by this study, some recommendations are made for additional research that could increase the efficacy of this technique.

- Vaccination for tilapia broodstock should be scaled up at big hatcheries with a greater number of broodstock. More tilapia broodstock in larger hatcheries means a greater need for vaccination. Therefore, greater research into virus scaling-up is needed in order to meet the hatchery's vaccine demand through mass production. Furthermore, tilapia broodstock can be kept in big hatcheries for three to five years, which means that they may require a considerable volume of vaccine in order to maintain their immunity throughout this period. By delivering specific immune responses against TiLV, this technique

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can assist farmers prevent TiLV infection in their brooders, reduce the danger of TiLV transmission from infected broodstock to their progeny, and contribute to the generation of TiLV-free tilapia seed.

- The results demonstrated that the TiLV-specific antibodies may be sustained for three months. Further research is needed to determine how long these antibodies can remain in the fish body and protect them from TiLV infection, which could lead to an appropriate time for booster(s) scheme to maintain protective antibody levels during risky farming times.

- Further research should investigate on immune responses in term of non-specific elements, and cell-mediated immunity when immunization of tilapia broodstock with TiLV inactivated vaccines. Moreover, more research could also be performed to elucidate whether other immunological components from both innate and adaptive immune system can be maternally transferred from TiLV vaccine-immunized broostock to their progeny.

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- Further immersed or oral vaccination should be investigated for small fish in order to bridge the gap between maternal immunity transfer and immunocompetency.

APPENDIX

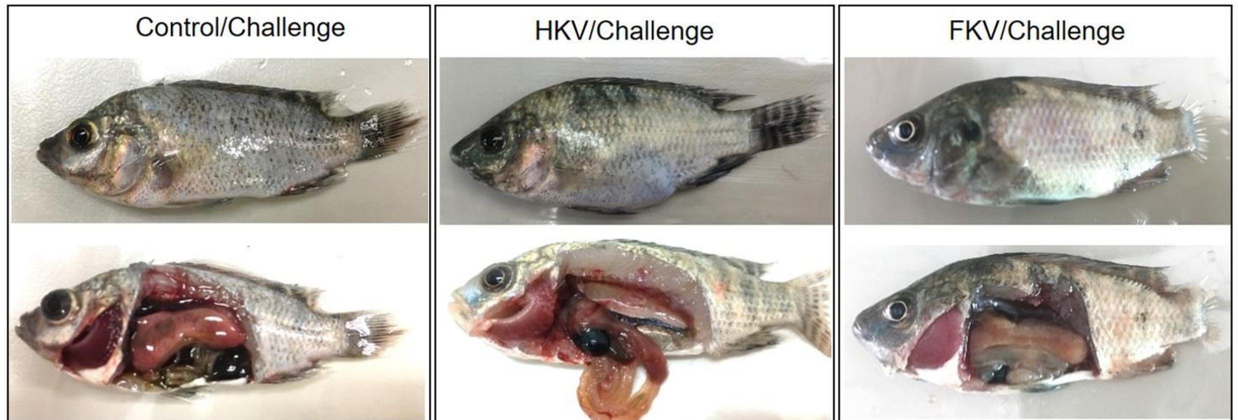

Figure S1. Gross signs of representative dead juvenile fish from control, HKV and FKH groups following challenge with TiLV. The fish showed common signs of TiLV infection, including abdominal distension, skin erosion, exophthalmos, fin rot, gill pallor and pale liver.

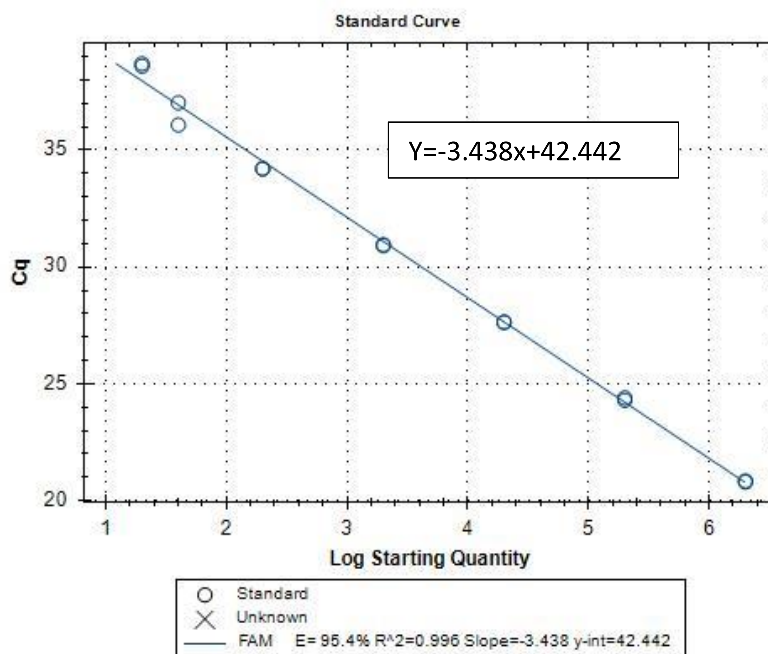

Figure S2. Standard curve for viral load calculation

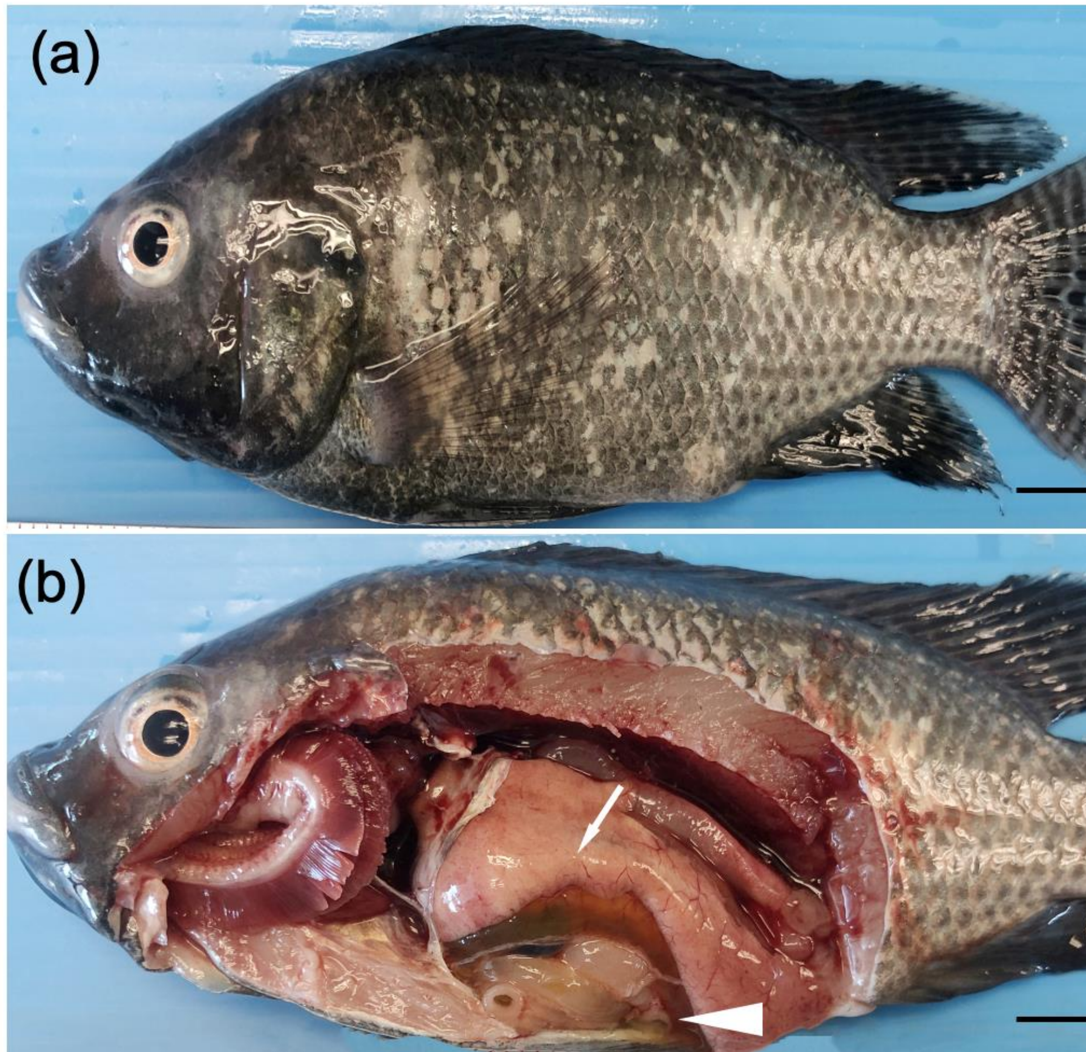

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Figure S3. (a) Gross lesions of infected fish in passive immunization experiment showed scale erosion, skin lesions, discoloration. (b) Internal postmortem changes including gill pallor, liver pallor (arrow) and ascitic fluid (head arrow). Sample was taken on 6 dpc from group 3. Scale bar = 1 cm.

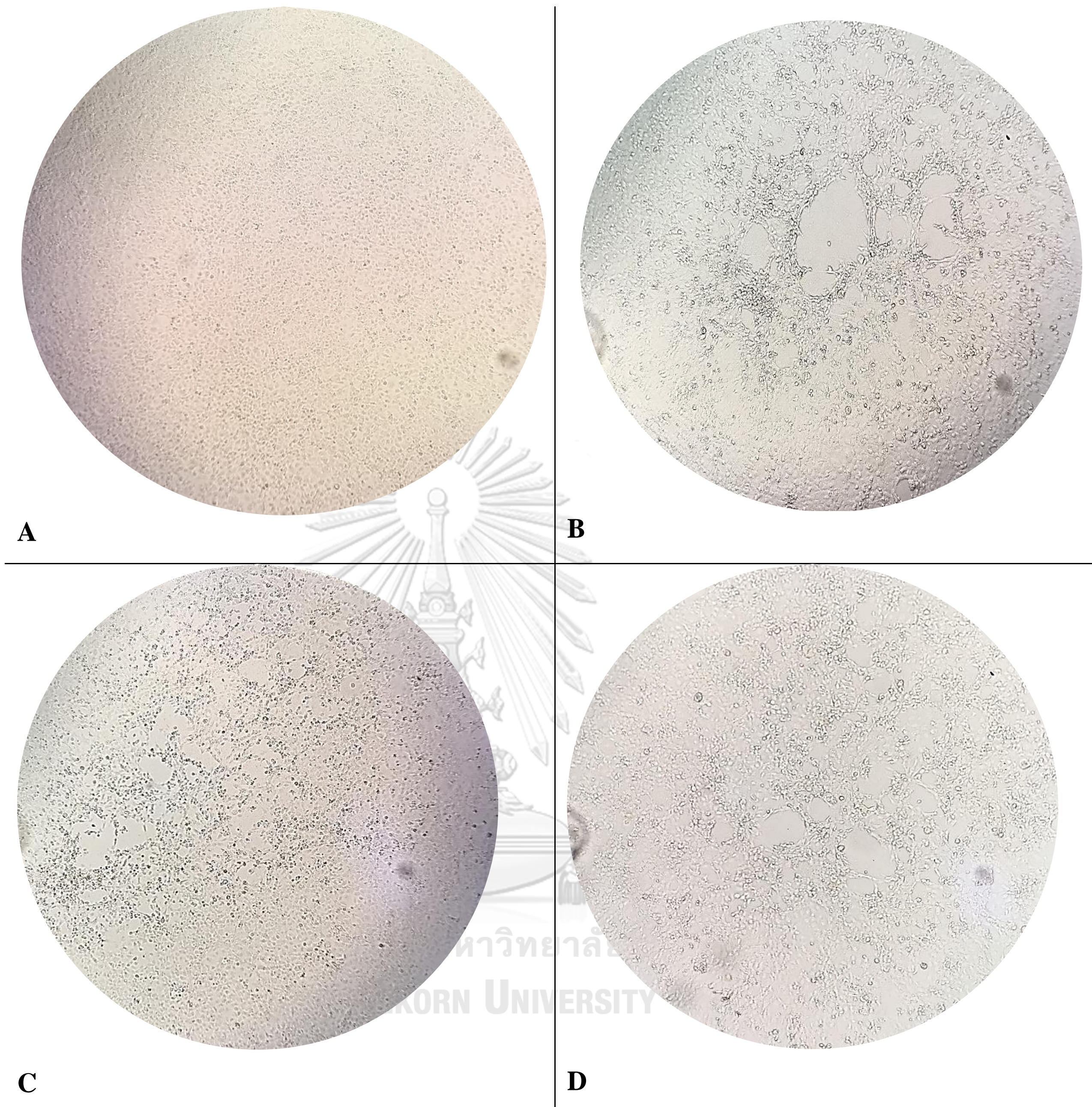

Figure S4. Representative observed CPE in neutralization assay. A- negative control; B- positive control; C and D- representative CPE appeared in neutralization assay plate. Image by T.T. Mai.

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Table S1. Inactivation of TiLV using different methods

Inactivated methods	Concentration (%)	Temperature (°C)	Time (hours)	CPE observation		
				Rep.1	Rep.2	Rep.3
Heat		25	2	+	+	+
		60	2	-	-	-
			2.5	-	-	-
		65	2	-	-	-
			2.5	-	-	-
Formaldehyde solution	0	25	24	+	+	+
	0.002			-	-	-
	0.004			-	-	-
	0.006			-	-	-
	0.008			-	-	-
	0.01			-	-	-

Rep, replicate; CPE: cytopathic effect.

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Table S2. TiLV copy number measured by RT-qPCR targeting RNA segment 9

Fish No	Group	Time of death	C_q for TiLV segment 9	C_q for *EF1α*	Viral copy number/ µg RNA template
1	3	Day 2	31.54	19.95	7.4×10^3
2	3	Day 6	22.7	19.52	1.4×10^6
3	3	Day 8	37.27	20.16	1.6×10^2
4	3	Day 9	ND	19.52	0
5	3	Day 11	38.37	19.62	7.5×10

6	3	Day 12	36.15	20.76	3.4×10^{2}
7	3	Day 16	36.99	21.15	1.8×10^{2}
8	3	Day 16	34.22	21.95	1.2×10^{3}
9	3	Day 16	ND	19.43	0
10	3	Day 16	36.74	21.01	2.3×10^{2}
11	3	Day 17	39.93	19.41	2.5×10
12	1	Day 10	ND	19.81	0
13	2	Day 10	ND	20.11	0
14	1	Day 21	ND	21.01	0
15	1	Day 21	ND	20.91	0
16	1	Day 21	ND	21.04	0
17	1	Day 21	ND	21.21	0
18	1	Day 21	ND	20.90	0
19	2	Day 21	ND	21.79	0
20	2	Day 21	ND	22.40	0
21	2	Day 21	ND	20.24	0
22	2	Day 21	ND	21.58	0
23	2	Day 21	ND	21.25	0
24	3	Day 21	ND	22.44	0
25	3	Day 21	ND	20.37	0
26	3	Day 21	ND	19.06	0
27	3	Day 21	ND	19.59	0
28	3	Day 21	ND	22.31	0

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ND: not detectable; C_q: quantification cycle; *EF1α*: *elongation factor-1α* gene

Table S3. Neutralizing titer of female broodstock sera

Treatment	Fish sera code	OD value	Diluted levels (1:)			Neutrializing titer (NT)
			40	60	80	
Control	FC1-6wk	0.076	+	+	+	<40
	FC1-7wk	0.07	-	+	+	40
	FC2-7wk	0.058	+	+	+	<40
	FC1-9wk	0.081	+	+	+	<40
	FC1-10w	0.06	+	+	+	<40
	FC1-12w	0.09	+	+	+	<40
HKV	FH1-7wk	0.911	-	+	+	40
	FH1-9wk	0.223	-	-	-	80
	FH2-9wk	0.195	+	+	+	<40
	FH1-11wk	0.647	-	-	-	80
	FH1-12wk	0.197	-	-	-	80
	FH1-14wk	0.752	+	+	+	80
	FH2-14wk	0.191	-	-	-	40
FKV	FF1-6wk	1.048	-	-	+	60
	FF1-7wk	0.063	+	+	+	<40
	FF1-11wk	0.148	+	+	+	<40
	FF1-12wk	0.356	-	-	-	80
	FF2-13wk	0.944	+	+	+	<40

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Table S4. Average OD reading values of juvenile fish sera

Number of fish	Time post vaccination								
	Day 14			Day 21			Day 28		
	Control	HKV	FKV	Control	HKV	FKV	Control	HKV	FKV
Fish 1	0.108	0.140	0.175	0.195	0.204	0.381	0.061	0.120	0.267
Fish 2	0.064	0.142	0.383	0.150	0.127	0.388	0.108	0.870	0.167
Fish 3	0.055	0.396	0.298	0.077	0.411	0.141	0.063	0.475	0.643

Fish 4	0.085	0.420	0.673	0.110	0.346	0.330	0.101	0.297	0.511
Fish 5	0.104	0.159	0.554	0.103	0.292	0.246	0.049	0.730	0.636
Fish 6	0.160	0.266	0.095	0.134	0.113	0.418	0.102	0.134	0.675

Table S5. Average OD reading values of juvenile fish mucus

Number of fish	Time post vaccination								
	Day 14			Day 21			Day 28		
	Control	HKV	FKV	Control	HKV	FKV	Control	HKV	FKV
Fish 1	0.083	0.091	0.088	0.115	0.124	0.164	0.068	0.068	0.227
Fish 2	0.063	0.090	0.900	0.092	0.082	0.306	0.113	0.360	0.247
Fish 3	0.059	0.745	0.748	0.075	0.236	0.219	0.059	0.287	1.138
Fish 4	0.069	0.293	0.207	0.069	0.211	0.407	0.167	0.272	0.654
Fish 5	0.059	0.121	0.395	0.072	0.233	0.117	0.085	0.145	0.818
Fish 6	0.090	0.165	0.087	0.077	0.070	0.530	0.150	0.278	0.428

Table S6. Average OD reading values of broodstock fish sera

Week post primary vaccination	**Female**			**Male**		
	Control	HKV	FKV	Control	HKV	FKV
6	0.057		1.048			
7	0.064	0.911	0.063	0.064	0.32	0.398
8		1.007		0.054	0.497	0.231
9	0.081	0.209		0.055	0.426	0.108
10	0.0885		0.472	0.056	0.258	0.112
11		0.6995	0.148	0.057	0.23	0.12
12	0.09	0.197	0.356	0.065	0.415	0.089
13			0.5025	0.059	0.377	0.189
14		0.449	0.132	0.069	0.281	0.354

Table S7. Average OD reading values of egg supernatant

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Week post primary vaccination	Egg supernatant		
	Control	HKV	FKV
6	0.047		0.173
7	0.064	0.375	0.160
8		0.155	
9	0.070	0.234	
10	0.081		0.116
11		0.170	0.110
12	0.087	0.272	0.110
13			0.131
14		0.247	0.116

Table S8. Average OD reading values of larval supernatant

Week post primary vaccination	Control	HKV	FKV
6w-3D	0.069		0.141
6w-7D	0.054		0.06
6w-14	0.092		
7w-1D	0.095	0.136	0.069
7w-7D	0.068	0.086	0.076
7w-14D	0.09		0.079
8w-1D		0.13	
8w-7D		0.066	
8w-14D		0.094	
9w-1D	0.079	0.131	
9w-7D	0.065		
9w-14D	0.103		
10w-1D	0.059		0.072
10w-7D	0.059		0.058
11w-1D		0.102	
11w-7D		0.087	
12w-1D		0.121	
12w-7D		0.06	

w: week post primary vaccination; D: day-old

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