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Maternal Immunity and Immune Improvement of Koi *Cyprinus rubrofuscus* Larvae after DNA Vaccine against Hoi Herpesvirus

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Abstract

Vaccination is an efficient step in preventing disease caused by koi herpesvirus in koi fish (*Cyprinus rubrofuscus*). This study aimed to investigates the impact of maternal broodstock vaccination with ORF81 DNA vaccine on the transmission of maternal immunity to offspring against KHV. The ORF81 DNA vaccine has been shown to increase RPS, but its effect on maternal immunity in koi offspring after vaccination has not been evaluated. The study examined koi broodstocks vaccinated with the ORF81 DNA vaccine 45 and 60 days before spawning (45V and 60V) at a dose of 12.5 µg/100 g. Immunological parameters were assessed in broodstock, eggs, and larvae. Immune gene expression was also analyzed using the qPCR method. The results showed that lysozyme activity and antibody levels in eggs from vaccinated mothers were higher, though not significantly different. Meanwhile, lysozyme activity and antibody levels in mothers were significantly higher ($p<0.05$) compared to controls. Immunoglobulin-M gene (IgM) and recombination-activating gene 1 (RAG1), were significantly induced. The relative survival percentage increased significantly in larvae produced from broodstocks with 60V. These findings indicate that broodstocks treated with the ORF81 DNA vaccine can enhance the immune response of their offspring.

Introduction

In various fish populations, elevated mortality rates during the larval and post-larval stages have been observed worldwide (Swain and Nayak 2009). These fish suffer from a delayed development of lymphoid organs and immune system maturation (Zapata et al., 2006). Relatively limited specific antibody synthesis occurs throughout embryonic development and the early stages of larval development (Magnadottir et al., 2004). Furthermore, the lack of robust fish brrodstocksand susceptibility to disease outbreaks pose constraints on productivity and potential financial losses in aquaculture. Therefore, during these early stages of the life cycle, maternally acquired immunity is crucial. The health and immunity of the broodstock fish are directly tied to their ability to rely on maternally inherited immunity to protect their offspring (Qin et al., 2013). Therefore, the creation of new techniques to enhance the health of brood fish is crucial and can enhance their reproductive efficiency, the caliber and quantity of their progeny, and in the case of species that exhibit parental care, their capacity to defend their progeny (Swain and Nayak 2009).

Koi Herpes Virus (KHV), also recognized as Cyprinid Herpesvirus 3 (CyHV-3), stands as a one of main reasons of the substantial losses in common carp aquaculture (Gotesman et al., 2013). This viral pathogen also impacts ornamental cyprinid species like goldfish and koi carp, leading to considerable economic repercussions. KHV, a member of the Alloherpesviridae family, is characterized by its double-stranded DNA structure and encodes 156 functional Open Reading Frames (ORFs) (Aoki et al., 2007). KHV infection leads to koi herpesvirus disease (KHVD) in carp and koi, typically causing mass mortalities of around 80-100%, with symptoms including red spots and wounds that can progress to tissue death in the gills. This disease is most commonly observed at low water temperatures (18-27 ºC), especially during the rainy season (Eide et al., 2011; McColl et al., 2018). Given the challenging nature of treating KHV outbreaks and also the potential of virus for latency within fish, prevention emerges as the optimal approach. (Aonullah et al., 2016; Eide et al., 2011; Nuryati et al., 2015).

Vaccination stands out as a highly effective measure in preventing KHV disease. Among the most promising vaccine strategies for combating fish diseases is the DNA vaccine, which comprises plasmid DNA inducing the expression of pathogenic proteins in vaccinated fish (Collins et al., 2019). Notably, this vaccine formulation does not induce infection, remains stable, and has the potential to stimulate both humoral and cellular defenses in fish (Chairunnisa et al., 2016; Cui et al., 2015; Zhu et al., 2015). We have developed the DNA vaccine for KHVD utilizing ORF25 and ORF81 from a local strain, which are referred to as the GP-25 and GP-11 vaccines, respectively (Nuryati et al*.,* 2010). Evaluations of both DNA vaccines against KHV have demonstrated enhanced protection in koi fish. Administering our GP-25 vaccine to koi via intramuscular injection at a dosage of 12.5 µg per 100 g resulted in a remarkable increase in fish survival rates to 96.67% post-KHV challenge (Nuryati et al., 2010). Chairunnisa et al. (2016) found that vaccinating koi fish with the GP-11 vaccine at doses of 7.5 µg and 12.5 µg per 100 g significantly increased their survival rate to 93.3% following a challenge with KHV.

Vaccination is a widely utilized method to prevent viral diseases in the aquaculture industry. When brood fish are vaccinated prior to spawning, their offspring receive enhanced immune protection through the transfer of maternal immunity, a phenomenon well-documented in both avian and mammalian species (Pihlaja et al., 2006; Edwards et al., 2021). Maternal immunity is critical in bolstering the immune defenses of fish larvae, whose immune systems are underdeveloped during early life stages. These larvae rely heavily on maternal antibodies and other immune factors transferred from the broodstock to combat pathogenic microorganisms. However, the transfer of maternal immunity in fish is complex, with variability in antibody transmission and limited understanding of the underlying mechanisms. Addressing these complexities is essential for improving larval survival and health in aquaculture. Therefore, this study investigates the impact of vaccinating maternal broodstock with an ORF81 DNA vaccine on the transmission of maternal immunity to offspring against KHV, providing new insights into the potential for enhancing immune protection in fish larvae.

Material and Methods

Broodstock Maintenance

Fish were obtained from the local farmers in Blitar, East Java Indonesia and then transported to the Research Institute for Ornamental Fish Culture, Ministry of Marine and Fisheries, Depok, West Java, Indonesia.

Prior to the experiment, the fish underwent PCR testing to assess the potential for carrying KHV, following OIE guidelines (2019). They were then acclimated in a 60×40×30 cm glass tank at a temperature of 27−28°C for seven days indoor facility, during which they were fed commercial pellets (21% protein) three times daily. No clinical symptoms of KHV or other pathogen infections were observed during this acclimatization period.

DNA Vaccine Preparation

The vaccine was prepared based on our previous research (Nuryati et al*.* 2010). *Escherichia coli* DH5α bacteria carrying the plasmids were cultured in 2xYT broth medium at 37°C and 200 rpm for 18 hours. Following this, the bacterial pellet was collected through centrifugation at 8,000 rpm for 5 minutes at 4°C. The plasmid DNA was subsequently purified using the GeneJET Plasmid Miniprep Kit (Thermofisher Scientific, USA) according to the provided instructions. The concentration and purity of the extracted DNA were determined using spectrophotometry at 260 and 280 nm, and its authenticity was confirmed via PCR using specific primers corresponding to the target ORF. Finally, the plasmid was diluted in TE buffer.

Broodstock Vaccination

Female koi used in this experiment were mixed strains (Taisho, Sanke, Kikokuriy, and Hiutsuri). Female Koi broodstock (n=9), with an average body weight of 551.05±118.35 g, were selected by their gonadal maturity stage using morphological observation and egg diameters (Sivakumaran et al., 2003)) under the microscope. Female koi broodstock was vaccinated using the ORF81 vaccine at different times as the research variable: 45 days before spawning (45V), and 60 days before spawning (60V), each in triplicate. For control, fish were injected by Phosphate Buffered Saline PBS (C) at 45 days before spawning. Koi broodstock was anesthetized before vaccination. 1 mL of ORF81 DNA

vaccine was injected intramuscularly with the dose of 12,5 µg/100 g fish. This dosage was chosen based on the optimum dosage for KHV protection in Koi from our previous study (Chairunnisa et al. 2016). Fish were reared separately from the male in the concrete tank (3×2×1.5 m^3) with recirculated water. Fish were fed with commercial feed (32−36% protein) two times a day at satiation. Temperature was ranged from 27−30°C during the rearing.

Fish Spawning

To obtain the egg, female fish were artificially spawned by Ovaprim™ injection (Syndel, USA) (0.5 mL kg^{-1}) at the dorsal. Then striping the eggs is carried out at intervals of 8 hours. The eggs that come out are collected in a bowl and mixed with the sperm solution. Stir using a chicken feather and add the physiological solution. Fertilized eggs were artificially incubated in a tank measuring 3×2×2 m^3, treated with 1-2 ppm methylene blue as a prophylactic against fungal infections, and equipped with aeration. Eggs were also sampled for the extraction of antibodies for observation. The larvae were fed Artemia from 3 to 7 days post-hatching (dph), followed by ad libitum feeding of Tubifex worms for 10 days, and then satiation feeding with artificial feed containing 40% protein (Prakosa & Ratnayu, 2016). To monitor growth rates, the weight of the larvae was measured at 1 dph as the initial weight and at 30 dph as the final weight. A total of 100 fish per treatment replicate were maintained in a 100×100×50 m^3 aquarium (with three replicates) for 30 days to observe cumulative survival rate. The water quality in the larvae tanks during the study was maintained at a temperature of 24-27°C, pH of 6.61-8.86, dissolved oxygen of 6.7-8.2 ppm, and total ammonia nitrogen levels below 1 mg/L.

Challenge Test

Challenge tests were carried out 7, 14, and 21 days after spawning. The larvae were soaked in KHV filtrate solution with a concentration of 102 copy virus (LD_{50} result) for 1 hour at a density of 30 individuals/100 mL. The fish were moved into an aquarium measuring 20×20×15 cm^3 which was equipped with aeration. Each treatment was carried out 3 times. Fish are maintained for 14 days at a temperature maintained at 18−25°C using the air conditioner. Dead fish were counted daily and stored at -80°C.

Sample Collection

Fish specimens were randomly selected from each group (n=3). Serum samples were aseptically obtained from anesthetized fish and diluted 1:4 with PBS-T (PBS pH 7.4 + 0.05% Tween-20). After centrifugation at 5000 g for 10 minutes, serum was stored at −20°C for analysis of lysozyme activity and antibody levels. Lysozyme activity was assessed by incubating 100 µL of serum with 100 µL of *Micrococcus lysodeikticus* suspension (0.4 mg/mL in 0.1 M PBS at 25°C) and measured at 0 and 30 minutes post-incubation. Antibody titers were determined using an indirect ELISA method as described by (Aonullah et al., 2016). Both lysozyme activity and antibody levels were evaluated spectrophotometrically at an optical density of 450 nm. Tissue samples for gene expression analysis were collected at 0, 1, 7, 14, and 28 days post-vaccination (dpv). Fish were euthanized with MS222 and dissected. Kidney tissues were preserved in GENEzol reagent (Geneaind, Taiwan) at −80°C before gene expression analysis.

Total RNA Isolation and qPCR Analysis

Kidney tissue weighing 23.07±2.3 mg was processed for total RNA extraction using GENEzol reagent (Geneaid, Taiwan) following the manufacturer's instructions. RNA concentration and purity were assessed spectrophotometrically at wavelengths of 260 and 280 nm. Subsequently, cDNA synthesis was performed using Revertra® Ace qPCR RT Mastermix with gDNA removal kit (Toyobo, Japan) following the provided protocol, starting with 100 ng μL^{-1} RNA. Real-time PCR (qPCR) was employed to gauge the expression levels of immune genes post-vaccination. Primers were designed based on available sequences in GenBank (ncbi.nlm.nih.gov/genbank) using the Primer3 web program (primer3.ut.ee/), and their sequences are detailed in Table 2. The qPCR reaction was conducted in a Rotor-Gene 6000 machine (Corbett, USA) using 2× SensiFAST SYBR® NO-ROX (Bioline, UK) with 50 ng μL^{-1} cDNA in a total volume of 20 µL. Each reaction mixture contained 10 µL of qPCR enzyme mix, 0.8 µL of each qPCR primer (10 mMol), 4 µL cDNA, and 14.4 µL nuclease-free water. The amplification protocol consisted of an initial denaturation step at 95°C for 2 min, followed by 40 cycles of denaturation at 95°C for 10 s, annealing at 60°C for 15 s, and extension at 72°C for 10 s. The expression levels of all genes were analyzed using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001) after normalization with the β-actin gene and comparison to the PBS control at 0 days post-vaccination (dpv) as the expression standard.

Data Analysis

The statistical analysis was conducted in the SPSS v.17 software (IBM, USA) with the one-way ANOVA test and Duncan post-test at $p<0.05$. Immune gene expression analysis was conducted according to the $2^{-\Delta\Delta CT}$ method, after normalizing with β-actin genes and measured as the fold-change expression to the expression at PBS treatment.

Results

Fecundity, Fertilization Rate, and Hatching Rate of the Vaccinated Koi Broodstock

Fecundity in the vaccination treatment 60 days before spawning was not significantly different from either the control or the vaccination treatment 45 days before spawning. The percentage level of fertilized eggs in vaccinated mothers ranged from 79% to 82%, not significantly different between treatments. The percentage of fertilized eggs showed higher results in all treatments of vaccinated mothers but was not significantly different compared to controls. Meanwhile, the hatchability of vaccinated mother eggs ranged from 85% to 92% and was significantly different ($p<0.05$) compared to controls which only showed the hatchability of mother eggs around 52%. The results of observing the characteristics of koi fish eggs can be seen in Table 1.

Lysozyme Activity of Koi Broodstock, Eggs, and Larvae

Lysozyme activity observed in the sera of dams vaccinated with the ORF81 DNA vaccine was significantly different ($p<0.05$) compared to unvaccinated broodstock. Lysozyme activity observed in eggs of mothers vaccinated with the ORF81 DNA vaccine was not significantly different compared to eggs of mothers who were not vaccinated. Meanwhile, lysozyme activity in larvae from vaccinated broodstocks was higher than in larvae from unvaccinated broodstocks. Lysozyme activity in broodstocks, eggs, and larvae is shown in Figure 1.

Lysozyme activity of koi larvae after the challenge test showed an increasing trend. At 7, 14, and 21 days post-fertilization, the 60V treatment showed a significant difference ($p<0.05$) compared to the control. Lysozyme activity after the challenge test at different days post-fertilization is shown in Figure 2.

Antibody Level of Koi Broodstock, Eggs, and Larvae

Antibody levels in vaccinated mothers were significantly different ($p<0.05$) compared to unvaccinated mothers (Figure 3). Meanwhile, egg antibody levels showed no significant difference compared to controls. Larval antibody levels from vaccinated mothers showed higher results compared to controls. Koi antibodies after vaccination can be seen in Figure 3.

Antibody levels in fish tested 7, 14, and 21 days after hatching showed a significantly different increase ($p<0.05$) compared to controls. The highest antibody values were seen in fish that were challenged after 21 days after hatching. The antibody levels of fish vaccinated with the KHV ORF81 DNA vaccine after the challenge can be seen in Figure 4.

Table 1. Fecundity, fertilization rate, and hatching rate of the vaccinated koi broodstock by ORF81 KHV DNA vaccine

Vaccination Treatment	♀ Broodstock bodyweight	Fecundity (total eggs)	Fertilization rate (%)	Hatching rate (%)
Control	638.67±61.56	50,048±2,417^{b}	66±13.37^{b}	52±13.33^{c}
45V	652.67±102.79	72,565±13,875^{a}	82±9.26^{a}	92±1.97^{a}
60V	635.00±80.71	63,227±21,280^{ab}	79±7.49^{ab}	85±5.28^{b}

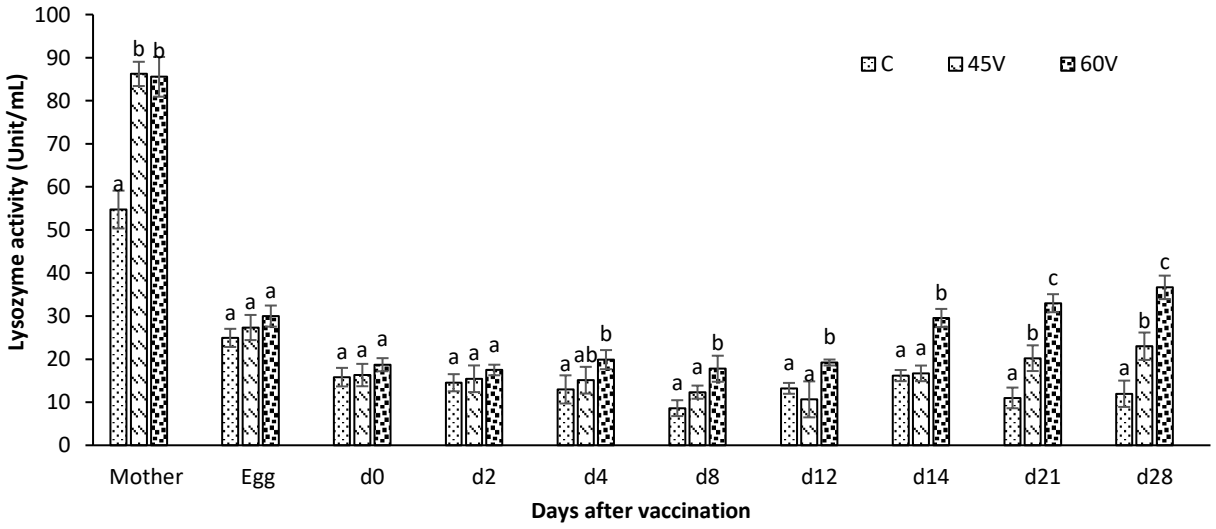

Figure 1. Lysozyme activity of koi broodstock and its offspring after vaccination with ORF81 KHV DNA vaccine. C= control, 45V= Vaccinated at 45 days before spawning, 60V= Vaccinated at 60 days before spawning. Data was presented as mean±S.D (n=3). Different letters indicate the significant difference between treatments at the same time points ($p<0.05$).

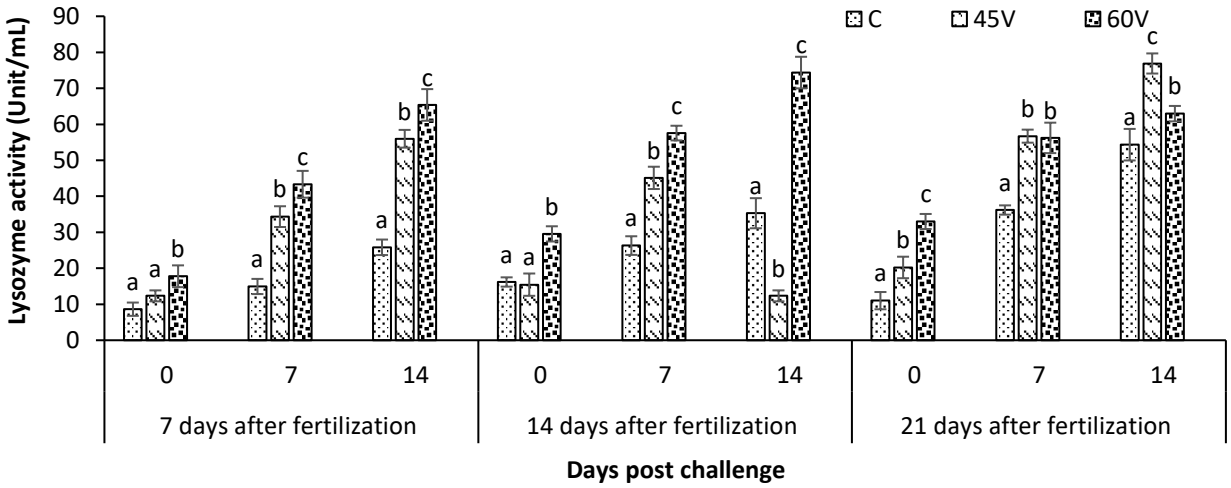

Figure 2. Lysozyme activity of koi larvae after challenge test with KHV. C= control, 45V= Vaccinated at 45 days before spawning, 60V= Vaccinated at 60 days before spawning. Data was presented as mean±S.D (n=3). Different letters indicate the significant difference between treatments at the same time points (p<0.05).

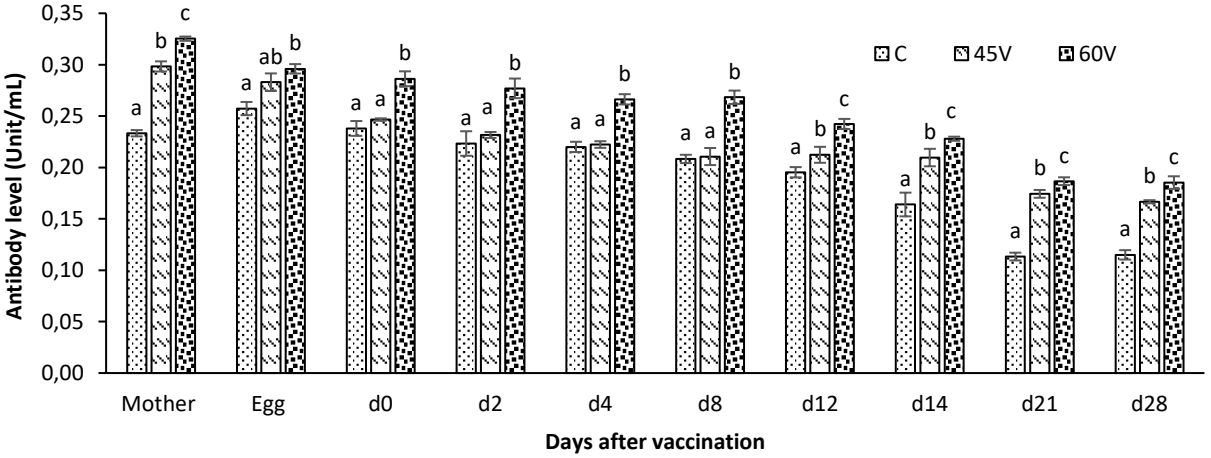

Figure 3. Antibody levels of koi broodstock and its offspring after vaccination with ORF81 KHV DNA vaccine. C= control, 45V= Vaccinated at 45 days before spawning, 60V= Vaccinated at 60 days before spawning. Data was presented as mean±S.D (n=3). Different letters indicate the significant different between treatment at the same time points (p < 0.05).

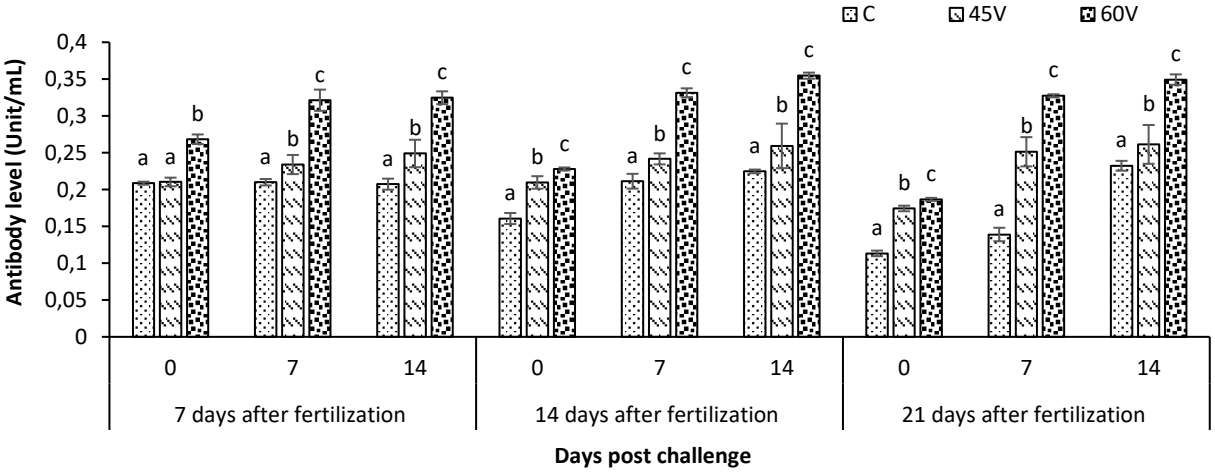

Figure 4. Antibody levels of koi of koi larvae after challenge test with KHV. C= control, 45V= Vaccinated at 45 days before spawning, 60V= Vaccinated at 60 days before spawning. Data was presented as mean±S.D (n=3). Different letters indicate the significant different between treatment at the same time points (p<0.05).

Survival and Relative Percent Survival of Koi Offspring after KHV Challenge Test

The survival of larvae from vaccinated broodstockshowed a significant increase compared to controls ($p<0.05$). Meanwhile, the relative percent survival value of larvae that were challenged with KHV showed a significant difference ($p<0.05$) in the 60V treatment 7 days after the challenge test compared to 45V. The survival results of the larvae challenged with KHV are shown in Table 2.

Expression Profiles of Immune-related Genes

Data on relative gene expression levels showed that 45V and 60V had increased immunoglobulin-M (IgM) and were significantly different ($p<0.05$) compared to controls. The 60V treatment showed higher IgM expression levels compared to the 45V treatment starting from the 8th day of observation until the final day of observation ($p<0.05$). Recombination-activating gene (RAG1) showed a significant increase at 4 days until the end of observation after fertilization ($p<0.05$). The relative gene expression of Immunoglobulin-M (IgM) and Recombination-activating gene (RAG1) can be seen in Figure 5. Based on the data obtained, over time, the expression of IgM and RAG1 continued to increase, providing greater protection for the fish.

Table 2. Vaccinated Koi's offspring survival after KHV challenge test

	RPS(%)	
	45V	60V
7 days	37.33±2.31[a]	38.67±6.11[a]
14 days	25.64±4.44[a]	73.08±6.67[b]
21 days	11.11±6.42[a]	50.62±7.71[b]

Note: 45V= Vaccinated at 45 days before spawning, 60V= Vaccinated at 60 days before spawning. Data was presented as mean±S.D (n=3). Different letters indicate the significant difference between treatments at the same time points ($p<0.05$).

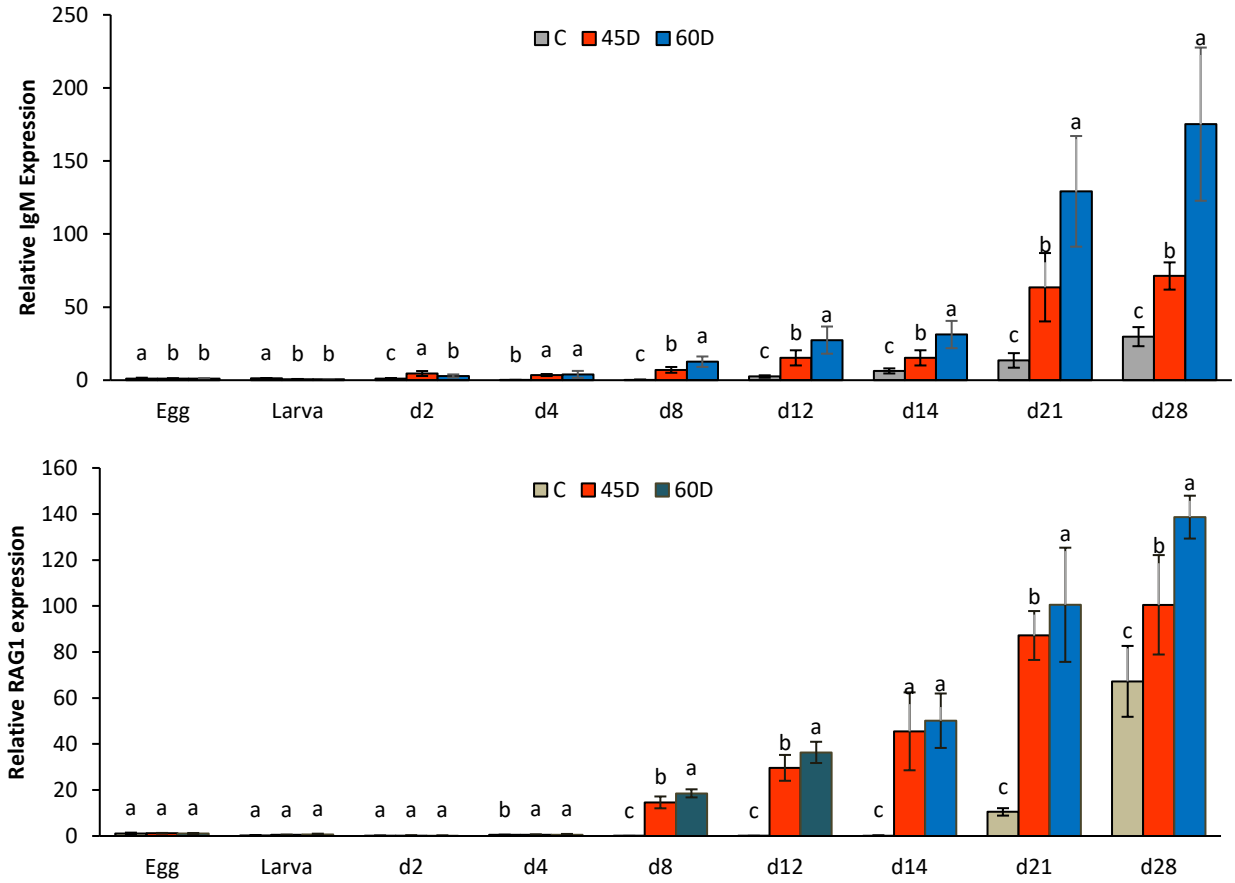

Figure 5. The pattern of mRNA expression of IgM and RAG1 from the vaccinated Koi's offspring after vaccination with ORF81 KHV DNA vaccine. C= control, 45V= Vaccinated at 45 days before spawning, 60V= Vaccinated at 60 days before spawning. Data was presented as mean±S.D (n=3). Different letters indicate the significant difference between treatments at the same time points ($p<0.05$).

Discussion

The immune system in newly hatched larvae has not yet developed perfectly, its immunological capacity is very limited at a certain stage and will develop if invaded by pathogens. In this study, female broodstocks vaccinated with the ORF81 KHV DNA vaccine produced seeds with higher immunity. The survival of koi fish seeds after the challenge test also showed higher results compared to the control treatment. Vaccination with ORF81 KHV DNA can increase fish resistance to KHV infection. Increased resistance to KHV infection can be seen through increased lysozyme activity, antibody levels, and total Ig and IgM levels. The results of this study indicate that the immunity formed in koi larvae is well correlated with increased function of the humoral immune system. Maternal immunity inherited in eggs has been developed to be associated with early defense against pathogens in developing embryos and larvae (Zhang et al., 2013).

The results of this study show that the application of the ORF81 KHV DNA vaccine influences fecundity, the percentage of fertilized eggs, and egg hatchability. The number of fecundities which were not significantly different between the treatments of vaccinated and unvaccinated koi fish broodstocks indicated that giving the vaccine to the broodstocks did not affect egg production so it was safe to use. Research by Kurniaji et al. (2019) stated that fecundity did not differ between treatments of broodstock given the ORF25 KHV DNA vaccine. Vaccinated salmon showed relative fecundity, egg survival, and normal embryo development were not significantly different compared to unvaccinated fish (Treasurer and Zox 2008). This shows that vaccination of koi broodstocks using an anti-koi herpesvirus DNA vaccine does not reduce the fecundity of the broodstocks. Thus, the vaccine does not have a negative effect on fish reproductive performance. Giving broodstock vaccines affects the percentage of hatchability of brood eggs. It is suspected that 60V can produce eggs that contain higher immune components so that they can be protected from pathogen invasion. Several research results show that the presence of immune factors in eggs such as antibodies (Hanif et al. 2004), lysozyme (Yousif et al., 1994; Saino et al., 2002), complement (Huttenhuis et al. 2006) and anti-protease (Ellis, 1987) can function to protect fish eggs from pathogen invasion. In Ghaedi et al.'s research. (2015), giving ß-glucan to broodstock rainbow trout showed a positive effect in increasing seed immunity and resistance to *Y. ruckeri*.

Pre-spawning maternal vaccination has been shown to enhance the offspring's ability to combat pathogens (Mingming et al., 2014). The findings indicate that lysozyme activity increased two days after vaccination and challenge. Lysozyme, a component of the immune system, is a protein crucial for nonspecific immunity (Hanif et al., 2004), playing a significant role in embryonic and larval development (Seppola et al., 2009). Lysozyme and antibodies passed on from vaccinated broodstock can confer protection to the resulting larvae (Mingming et al., 2014; Sukenda et al., 2017). Maternally transferred lysozyme to the eggs serves to prevent vertical transmission of diseases from various pathogens (Zhang et al., 2013). Furthermore, broodstock-derived lysozyme transferred to the larvae has demonstrated the ability to disrupt bacterial cells by hydrolyzing 1,4-glycoside bonds found in their cell walls (Huttenhuis et al. 2006; Zhang et al. 2013). Additionally, lysozyme contains the enzyme muramidase, which can degrade bonds between N-acetyl glucosamine and N-acetyl muramate in bacterial cell walls (Marsh and Rice 2010).

The increase in antibody levels is strongly influenced by the use of vaccines in vaccinated mothers and their offspring. Maternally transferred antibodies from vaccinated mothers are capable of activating specific immunity, but this does not occur in unvaccinated mothers. In conjunction with lysozyme, the primary function of antibodies inherited from parent fish is to shield eggs and larvae from vertical transmission. The results showed that antibody levels decreased slowly as the larvae aged. However, antibody levels increased significantly after challenge (Figure 4). The decline in larval antibody levels from vaccinated mothers occurs gradually (Nurani et al., 2019). Specifically transferred antibodies from the mother via the egg yolk diminish as larvae progress in age (Zhang et al., 2013). Antibody levels in fish larvae diminish progressively due to the natural metabolic breakdown of specific antibodies transferred by the parent fish along with the egg yolk (Sukenda et al., 2018).

The mother's immunity which is passed on to her offspring will decay quickly as the fish larvae age and the immune system develops (Firdausi et al., 2017). According to Saselah et al., (2012), the early stages of koi fish are very susceptible to KHV infection and have the potential to become the main carriers of KHV spread. This is because the body's immune organs are not yet perfect (Lund et al., 2019) so the body's protective ability still depends on maternal immunity transferred by the mother (Akbary et al., 2015). In the research results, the relative expression of the immunoglobulin-M (IgM) gene increased significantly from eggs up to 28 days after fertilization. This shows that the ORF81 KHV DNA vaccine in the broodstock affects the offspring.

The body's protection is provided through humoral immunity and cell-mediated immunity. Additionally, immunological memory produced after vaccination provides protection when fish are exposed to the same pathogen at a later date (Adams 2016). In contrast, passive immunity usually refers to the transfer of antibodies from one individual to another to protect against infectious agents (Zhang et al., 2013). Passive immunity in fish can be obtained through the transfer of protective antibodies from vaccinated broodstocks to their offspring (Rajan et al., 2017). IgM classification in fish can be transferred maternally into immature

oocytes during the process of vitellogenin formation and then absorbed throughout the egg cells in the follicular cells (Mai et al., 2022). In the research results, IgM was detected at the beginning of the observation and continued to increase until the end of the observation (Figure 5). This is in line with research by Kai et al. (2014) who showed that administering the NNV vaccine to 40-day-old grouper larvae gave rise to IgM expression on day 14 after vaccination in the intestines and gills. In the research of Saravanan et al. (2017), the highest increase in IgM expression in Rohu fish began at 24 days of fry. Meanwhile, in zebrafish, IgM gene expression was highest when the seeds were 21 and 28 days old (Lam et al., 2004). Significant IgM gene expression at a certain age can be an indication of immunocompetent development in fish (Saravanan et al., 2013).

Lymphocyte cells in lymphoid organs determine the development of the immune system in several fish species with the RAG-1 gene and IgM gene as markers of the maturation of the body's immune system (Lee et al., 2014). The results of this study (Figure 5) show that the highest RAG-1 gene was detected from 4 days after fertilization to 28 days after fertilization. According to Huttenhuis et al. (2005), RAG-1 gene expression was highest when fish were 2 weeks old and decreased from 4-8 weeks in goldfish. Meanwhile, RAG-1 expression was reported to increase to a maximum in haddock fish at the age of 21−33 days (Corripio-Miyar et al., 2007), striped trumpeter larvae at the age of 50 days (Covello et al., 2013) and flounder fish at the age of 50 days (Lee et al., 2014). The RAG-1 gene is a marker of B cell maturation, and antibody (Ig) expression in lymphoid organs so that it can estimate the time of immunological competence in the body. The highest level of RAG-1 gene expression occurs in the early stages of B cell development and decreases as the cells mature (Covello et al., 2013). Expression of the RAG-1 gene during fish development stages can determine the optimization of vaccination timing.

The heritability of innate resistance to infection and the transfer of maternal immunity to offspring can be used to prevent mass deaths in the early phases of life (Swain and Nayak 2009). RPS results show that the ORF81 KHV DNA vaccine in broodstocks can reduce larval mortality up to the 21st day after hatching (Table 2). Giving vaccines to broodstocks can minimize larval death (Rahman et al., 2022). Vaccine application showed relative survival percentage (RPS) values between 56 to 95% after the challenge at 7 or 21 days post-vaccination (Abu-Elala et al., 2019). In research by Wang et al. (2015) DNA vaccines can promote significantly higher antibody levels, relative expression levels of immune genes, and relative survival percentages.

The use of DNA vaccines, as demonstrated in this study, represents a more sustainable approach to disease management in aquaculture compared to conventional methods such as the use of antibiotics or chemicals. This aligns with global trends towards more sustainable and eco-friendly farming practices. By adopting maternal vaccination strategies, koi farmers can contribute to the reduction of environmental impacts associated with traditional disease management practices, promoting a healthier ecosystem.

Conclusion

Koi broodstock vaccinated with the DNA ORF81 vaccine 60 days before spawning (60V) could significantly modulate the innate and adaptive immunity of the fish, resulting in higher survival rates after KHV infection. However, it is important to investigate the duration of enhanced immunity in offspring over their lifespan, as this could provide insights into the long-term benefits of maternal vaccination. Understanding how long the transferred immunity persists could help optimize vaccination schedules and strategies in breeding programs.

Ethical Statement

All experiments in this study related to fish have found animal welfare and were carried out according to SNI 7734:2017. Which has been approved by the Ethics Committee on Animal Use of the IPB University.

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Author Contribution

SN, EK, SSA conducted the experiment, analyzed the data, and drafted the first version of the manuscript. DH, YPH, OC designed the research and drafted the first version of the manuscript. SN, HN, and MG reviewed the first version of the manuscript, and it was approved for publication. DH, SSA, HN, and MG analyzed the data, prepared of research results, and drafted the manuscript.

Conflict of Interest

All authors declare that they have no conflicts of interest.

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