

IMMUNE RELATED GENES EXPRESSION ANALYSIS IN KOI FISH AFTER VACCINATED WITH KOI HERPES VIRUS DNA VACCINES

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ABSTRACT

Vaccination is a practical step in preventing diseases caused by koi herpes virus (KHV) in koi fish (*Cyprinus carpio haematopterus*). We have developed two DNA vaccines for KHV named as GP-25 and GP-11 from two local isolates coded as ORF25 and ORF81, respectively. Although both vaccines have been reported to increase survival rates, the evaluation of koi fish immune responses at the molecular level has not been done post-vaccinations. The aim of this research was to determine the effects of koi herpesvirus DNA vaccine on the immune-modulation of koi fish at mRNA level. This recent research used the best vaccine doses of both vaccines determined from our previous study: 7.5 and 12.5 μg per 100 g fish of GP-11, and 12.5 μg per 100 g fish of GP-25. The immune gene expression was analyzed using the RT-qPCR method from the fish liver at 0, 1, 7, 14, and 28 days post-vaccination (dpv). The results showed that, in the vaccinated fish, the immune genes viz. tumor necrosis factor α (TNF α), interleukin-1 β (IL1 β), interferon- γ (IFN γ), Mx1, immunoglobulin Mu chain (IgM), and major histocompatibility complex (MHC) class I and class II were induced to significant extents. The higher dose vaccination using the GP-11 vaccine showed higher immune gene expression than that of the lower dose. Furthermore, the GP-25 vaccine had induced lower immune responses than the GP-11 vaccine when using the same dose of vaccination, but relatively the same when the half-dose of GP-11 vaccine was used. In conclusion, the GP-11 and GP-25 vaccine provided the immune-modulatory effects on the koi fish immune response after vaccination.

KEYWORDS: DNA vaccine; gene expression; immune response; KHV; koi

INTRODUCTION

Koi herpes virus (KHV), also known as Cyprinid Herpesvirus 3 (CyHV-3), is one of the pathogens causing high mortality and economic losses in common carp as well as ornamental cyprinid fish such as goldfish and koi carp. KHV is a group of double-stranded DNA viruses from the Alloherpesviridae family that encode 156 functional Open Reading Frames (ORFs) (Aoki *et al.*, 2007). When infecting carp and koi, KHV is referred to as koi herpes virus disease (KHVD). This viral infection can cause a massive death from 80% up to 100% showing clinical symptoms from red-colored spots and wounds to tissue damages in the gills. The infection usually occurs at low water tem-

peratures (18°C-27°C), especially during the rainy season (Eide *et al.*, 2011; Mccoll *et al.*, 2018). When a KHV outbreak has occurred, it is difficult to treat or localize the disease distribution. This means that the best option in KHV disease management is preventing a possible disease outbreak before it happens considering that KHV virus can be latent in fish (Aonullah *et al.*, 2016; Eide *et al.*, 2011; Nuryati *et al.*, 2015).

Vaccination is a practical step in preventing KHV disease. One of the most currently promising vaccine preparations against fish diseases is a DNA vaccine. The vaccine consists of plasmid DNA that will produce the gene expression of the pathogenic proteins in the vaccinated fish (Collins *et al.*, 2018). Moreover, a DNA vaccine does not cause infection, is relatively stable and could activate humoral and cellular defenses in fish (Chairunnisa *et al.*, 2016; Cui *et al.*, 2015; Zhu *et al.*, 2015). We have developed a DNA vaccine for KHVD from the ORF25 and ORF81 of local

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isolates, namely GP-25 and GP-11 vaccine, respectively (Nuryati *et al.*, 2010). Several experiments using both DNA vaccines against KHV showed the alteration of fish protection against KHVD in koi. Vaccination of koi with the GP-25 vaccine at a dose of 12.5 μg per 100 g by intramuscular injection was able to increase fish survival rates to 96.67% after the KHV challenge (Nuryati *et al.*, 2010). Chairunnisa *et al.* (2016) also reported that vaccination of koi fish using the GP-11 vaccine with a dose of 7.5 μg and 12.5 μg per 100 g was able to increase the fish's survival rate to 93.3% after the challenge test with KHV.

Although both vaccines were able to increase survival rates, the evaluation of koi fish immune responses at the molecular level has not been done post-vaccination. Given the promising results from previous studies, this current study was aimed to investigate the potential use and effects of the GP-25 and GP-11 DNA vaccines to increase the various immune response of fish at the mRNA level.

MATERIALS AND METHODS

Fish and Vaccine Preparation

Koi fish (mixed from Kohaku, Sanke, and Ki bekko strain) with an average body weight of 28.55 ± 0.98 g were obtained from the National Center of Freshwater Aquaculture, Sukabumi, West-Java, Indonesia. Before the experiment, the fish were checked for the possibility of carrying KHV using the PCR method (OIE, 2019). The fish were acclimated for seven days in the aquarium sized 60 cm x 40 cm x 30 cm, and the temperature was maintained between 27°C-28°C. The fish were fed with commercial pellets (21% protein) three times a day. No observed clinical symptoms of KHV or pathogen infection during the acclimatization.

Bacteria *Escherichia coli* DH5 α containing GP-11 and GP-25 KHV vaccine were cultured in 1 L of 2 x YT broth media and spun at 37 and 200 rpm for 18 hours. The bacterial pellet was harvested by centrifugation at 8,000 rpm for 5 min at 4°C. The plasmid DNA was purified using the GeneJET Plasmid Miniprep Kit (ThermoFisher Scientific, USA) following the manual instruction. The concentration and purity of the plasmid DNA were measured using spectrophotometry method at 260 and 280 nm and verified by PCR method using the specific primer for the corresponding ORFs.

Vaccination and Sample Collection

In this study, we used the best vaccine doses of both vaccine types from our previous study, *viz.* 12.5 μg per 100 g fish for GP-25 vaccine, and 7.5 and 12.5 μg per 100 g fish for GP-11 vaccine as presented in Table 1. The vaccination was conducted by injecting

0.1 mL DNA vaccine intramuscularly near the dorsal fin, $n = 15$, with three replications for each treatment. The fish were reared in 12 aquariums (60 cm x 40 cm x 30 cm) for 28 days post-infection (15 fish/aquarium). The fish were fed with commercial feed *at satiation* three times a day.

The samples for the gene expression were collected at 0, 1, 7, 14, and 28 days post-vaccination (dpv). The fish were euthanized using tricaine methanesulfonate (MS222) and then dissected. The fish's kidneys were collected and stored in the GENEzol reagent (Geneaid, Taiwan) at -80°C before the gene expression analysis.

Total RNA Isolation, cDNA Synthesis, and RT-qPCR Analysis

Total RNA was isolated from the sampled kidneys (23.07 ± 2.3 mg) using the GENEzol reagent (Geneaid, Taiwan) following the manufacturer's instruction. RNA concentration and purity were measured using the spectrophotometry method at 260 and 280 nm. The cDNA synthesis was carried out from 100 ng μL^{-1} RNA using the Revertra[®] Ace qPCR RT Mastermix with gDNA removal kit (Toyobo, Japan) following the manual procedure. Real-time quantitative PCR (RT-qPCR) was used to determine the levels of immune-genes after vaccination. The primers were designed based on the references and available sequences in GenBank (ncbi.nlm.nih.gov/genbank) using the Primer3 web program (primer3.ut.ee/). The primers sequence was summarized in Table 2. The qPCR reaction was performed in the Rotor-Gene 6000 (Corbett, USA) machine using 2x SensiFAST SYBR[®] NO-ROX (Bioline, UK) from the 50 ng μL^{-1} xcDNA in the total volume of 20 μL . The reaction consisted of 10 μL qPCR enzyme mix, 0.8 μL of each qPCR primer (10 mMol), 4 μL cDNA, and 14.4 μL nucleases free water. The amplifying program was set at 95°C for two min, and 40 cycles of 95°C for 10 s, 60°C for 15 s, and 72°C for 10 s. The expression levels of all the genes were analyzed according to the $2^{-\Delta\Delta\text{CT}}$ method (Livak & Schmittgen, 2001) after normalized with the β -actin gene and compared to the PBS control at 0 dpv as the expression calibrator. The statistical analysis was conducted in the SPSS v.17 software (IBM, USA) with the one-way ANOVA test and Duncan post-test at $p = 0.05$.

RESULTS AND DISCUSSION

The effect of vaccination on the expression of immune genes was examined using RT-qPCR analysis of the transcription of the genes encoding tumor necrosis factor α (TNF α), interleukin-1 β (IL1 β), interferon- γ (IFN γ), Mx1, immunoglobulin Mu chain (IgM), and major histocompatibility complex (MHC) class-I and class-II. The results showed that in the

Table 1. Experimental design of koi fish vaccinated with GP-11 and GP-25 DNA vaccines

| Treatments | DNA vaccine and dose | Number of fish |
|------------|----------------------|----------------|
| A | GP-11; 7.5 µg/100 g | 15 x 3 |
| B | GP-11; 12.5 µg/100 g | 15 x 3 |
| C | GP-25; 12.5 µg/100 g | 15 x 3 |
| Control | PBS control; 0.1 mL | 15 x 3 |

Table 2. Primers sequence used to determine the immune response by RT-qPCR analysis on koi fish after vaccinated with GP11 and GP 25 DNA vaccines

| Gene | Sequence 5'- 3' | PCR product size (bp) | Reference/Genbank accession no. |
|----------------|---|--------------------------|------------------------------------|
| IgM | F: CACAAGGCGGAAATGAAGA R: GGAGGCACTATATCAACAGCA | 86 | Embregts <i>et al.</i> (2018) |
| IFN γ | F: CGATCAAGGAAGATGACCCAGTC R: GTTGCTTCTCTGTAGACACGCTTC | 73 | Embregts <i>et al.</i> (2018) |
| TNF α | F: GCTGTCTGCTTCACGCTCAA R: CCTTGGAAAGTGACATTTGCTTTT | 106 | JX181982.1 |
| MHC1 | F: AGAAAGCTGTGCCGAAGACA R: GGCGGCCAATATCAGTCTGA | 87 | HM372879.1 |
| MHC2 | F: TACTACCAGATTCACTCGG R: CGGGTTCCAGTCAAAGAT | 111 | Zhu <i>et al.</i> (2015) |
| IL1 β | F: ACGCTGAGAGACGGAACAG R: CGACTCGGTACAAGCAAGGT | 141 | MK942107.1 |
| Mx1 | F: TGACATTGCAACCACAGAAGC R: ATCCACCAGATCCGGCTTTG | 97 | KP115357.1 |
| β -actin | F: ACCGGAGTCCATCACAATACC R: GAGCTGCGTGTTGCCCTGAG | 192 | AY395870.2 |

vaccinated fish, all these genes were induced to significant extents (Figure 1-3). Interferon-gamma (IFN γ) or type-II interferon is a cytokine that is critical for innate and adaptive immunity against viral and some bacterial infections. The importance of IFN- γ in the immune system lies in its ability to inhibit viral replication directly and, most importantly, from its immunomodulatory effects (Bedekar *et al.*, 2018; Zou & Secombes, 2016). In this study, IFN γ of vaccinated fish were modulated higher than the control since 1 dpv and maintained its high expression until 28 dpv (Figure 1). GP-11 vaccines gave significantly higher up-regulation than GP-25 with the same dose. Furthermore, the interferon-stimulated gene (ISG), namely Mx protein genes, was also observed in this study (Kim *et al.*, 2000; Leong *et al.*, 1998). The Mx1 gene expression was modulated mainly after 7 dpv in the vaccinated fish and higher in the GP-11 treatment (Figure 1). The modulation of Mx1 gene showed that

the GP-11 and GP-25 vaccines appear to stimulate the IFN-signalling pathway, one of the most important innate immune responses (Kim *et al.*, 2000; Lazarte *et al.*, 2017).

The modulation of the IFN-signalling pathway after GP-11 and GP-25 vaccine is also supported by the result of the inflammatory cytokine expression. The modulation of the IFN-signalling pathway will promote the inflammatory cytokines to trigger other immune responses cascade in fish (Sobhkhez *et al.*, 2018). IL1 β and TNF α are the inflammatory cytokines that have the primary role in the regulation of the immune cells, that enables organisms to respond to microbial invasion, and is involved in a variety of cellular activities (Bird *et al.*, 2002; Zou & Secombes, 2016). In this study, the IL1 β and TLR α expressions were altered rapidly and significantly higher in vaccinated fish, in line with the IFN γ alteration (Figure 2).

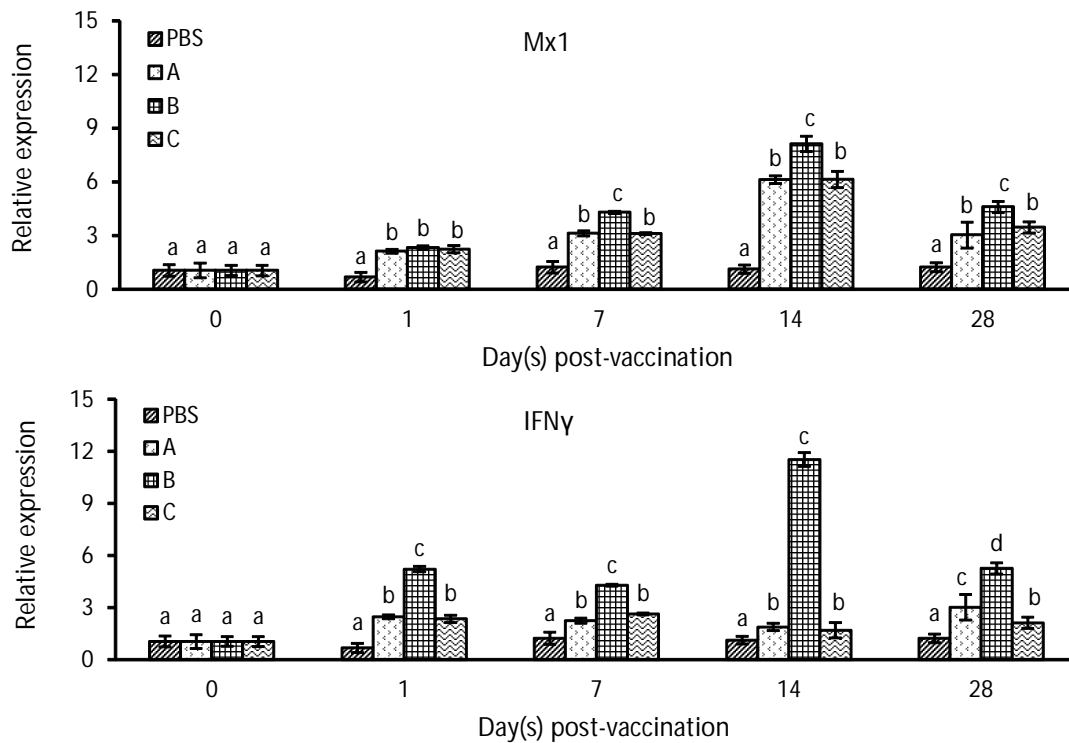


Figure 1. Quantitative relative mRNA expression of IFN γ and Mx1 of koi fish after vaccination with GP-11 and GP-25 DNA vaccine; (A) 7.5 μ g/100 g fish GP-11, (B) 12.5 μ g/100 g fish GP-11, (C) 12.5 μ g/100 g GP-25, (K) PBS control. Data were shown as mean expression \pm SD (n= 3). Different letters indicate the significant difference of expression within the treatments at the same time points.

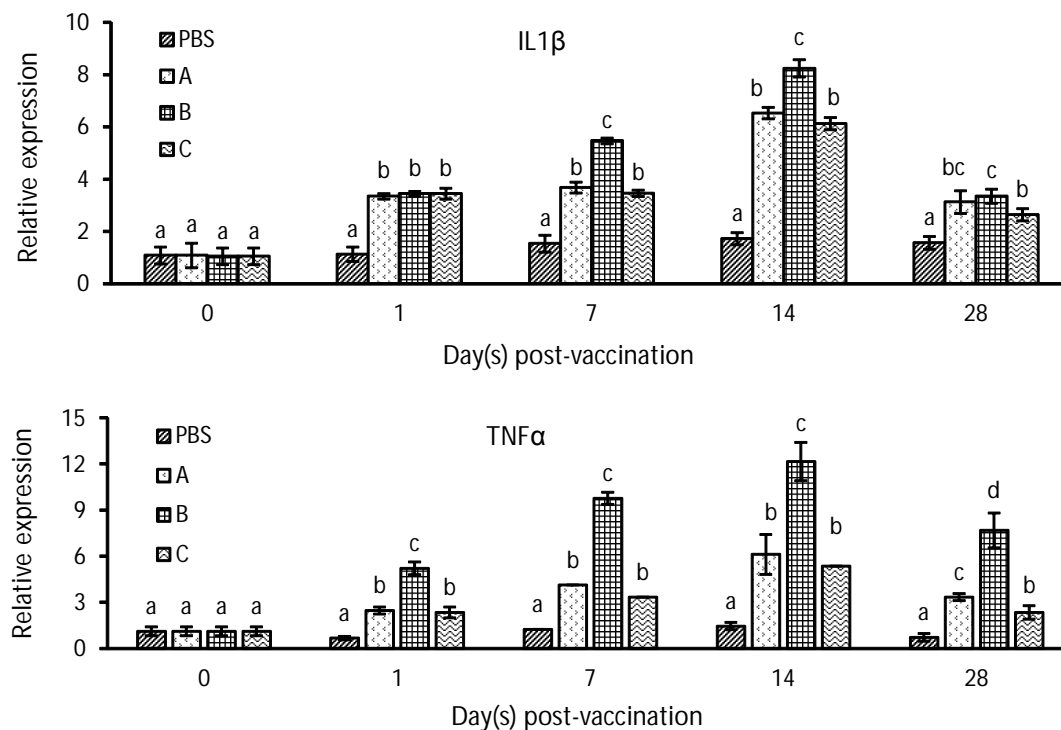


Figure 2. Quantitative relative mRNA expression of IL1 β and TNF α of koi fish after vaccination with GP-11 and GP-25 DNA vaccine; (A) 7.5 μ g/100 g fish GP-11, (B) 12.5 μ g/100 g fish GP-11, (C) 12.5 μ g/100 g GP-25, (K) PBS control. Data were shown as mean expression \pm SD (n= 3). Different letters indicate the significant difference of expression within the treatments at the same time points.

The expression of both genes has up-regulated since 1 dpv and may be resulted due to a combined effect between the vaccine and injection stress. The cytokines expression was modulated in high-level and peaked at the 14 dpv with the highest expression found at the 12.5 $\mu\text{g}/100\text{ g}$ of GP-11 vaccination treatment.

DNA vaccines have been used to stimulate protective immunity against many infectious pathogens. Plasmid DNA encoding antigen DNA vaccine is injected into a host. It enters host cells and serves as template for the antigen protein translation (Collins *et al.*, 2018; Zhu *et al.*, 2015). MHC molecules have important roles in viral and bacterial antigen presenta-

tion in immune cells such as dendritic cells, B cells, and macrophages (Rakus *et al.*, 2009; Sobhkhez *et al.*, 2018). In this study, The MHC1 and MHC2 expression was up-regulated significantly on the vaccinated fish at 7 dpv (Figure 3). Their expression increased and reached a peak at 28 dpv. Consistent with the expression of other immune genes, the MHC1 and MHC2 expressions were also highest in the higher dose of GP-11, compared with the same doses of GP25 vaccine. This is marked by the immunomodulatory effect of the GP11 and GP25 vaccines. This study also showed that the GP11 and GP25 DNA vaccines appeared to be successfully detected as antigens, from their structure or their expressed protein.

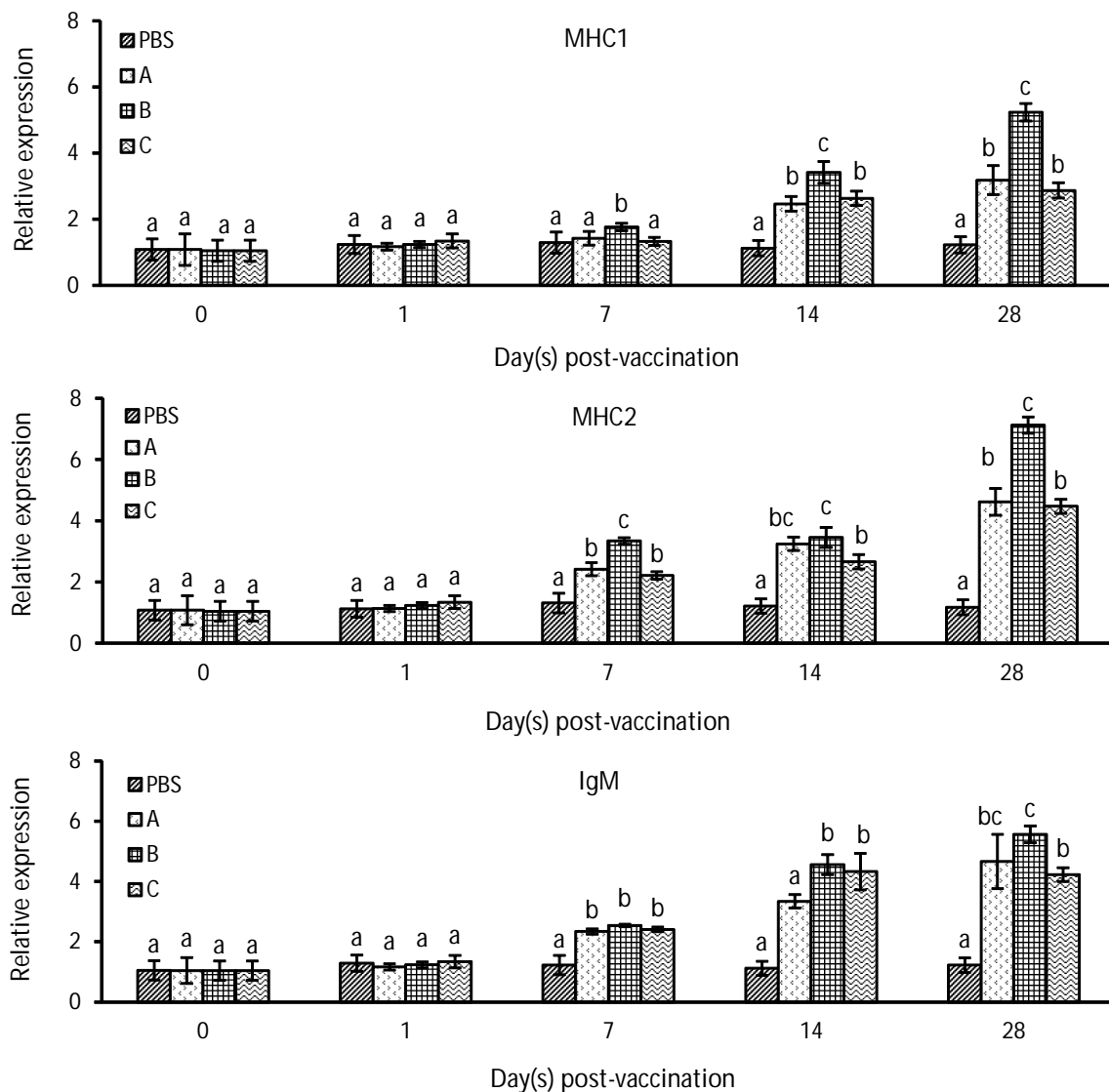


Figure 3. Quantitative relative mRNA expression of MHC1, MHC2, and IgM of koi fish after vaccination with GP-11 and GP-25 DNA vaccine; (A)= 7.5 $\mu\text{g}/100\text{ g}$ fish GP-11, (B)= 12.5 $\mu\text{g}/100\text{ g}$ fish GP-11, (C)= 12.5 $\mu\text{g}/100\text{ g}$ GP-25, (K)= PBS control. Data were shown as mean expression \pm SD (n= 3). Different letters indicate the significant difference of expression within the treatments at the same time points.

The MHC positive cells and IFN α expressions also trigger the specific B- and T-cells responses, leading to antibody production (Schoenborn & Wilson, 2007; Zhu *et al.*, 2015) as also shown in this study. The mRNA expression of IgM was significantly higher at 7 dpv in all vaccinated fish, indicating that the fish were able to produce the specific antibody for the KHV antigen (Figure 3). The levels of IgM mRNA of the high dose of GP-11 and GP-25 were relatively similar at 7 and 14 dpv. But at 28 dpv, the expression of IgM was highest in the high dose vaccination using GP-11. The alteration of IgM mRNA may lead to the increasing production of IgM, which was found to be the major targets for the antibody response of carp against KHV (Fuchs *et al.*, 2014).

These immunomodulatory effects of DNA vaccine also have been previously reported in other fish species. The vaccination of Japanese flounder *Paralichthys olivaceus* with viral hemorrhagic septicemia virus (VHSV) recombinant glycoprotein DNA vaccine resulted in the elevated expression of immune genes (Byon *et al.*, 2006). The IgM and MHC expression were observed to be up-regulated at 1 or 21 dpv in the fish kidney. The vaccination of salmon with the polyprotein salmonid alpha virus (SAV) DNA vaccine resulted in the large increase of proinflammatory cytokines IL1 β and TNF α , IFNs genes, Mx gene, and also MHC and IgM gene after one and two weeks post-injection (Sobhkhez *et al.*, 2017; Sobhkhez *et al.*, 2018). The protective immunity also has shown by the vp7 gene of grass carp reovirus DNA vaccine injection in grass carp (Zhu *et al.*, 2015). The grass carp IL1 β , TNF α , IFN γ , complement-3, MHC1, MHC2, and IgM gene transcript was significantly modulated 28 days after vaccination. The similar findings in our current study could further explain our previous studies' results related to the protective effects of both vaccines in koi after KHV infection (Aonullah *et al.*, 2016; Chairunnisa *et al.*, 2016; Nuryati *et al.*, 2010; 2015). These differences in the immune gene expressions may help to identify the important components of the koi immune system for conferring an immune response against KHV disease.

CONCLUSIONS

In conclusion, the GP-11 and GP-25, encoding the ORF81 and ORF25 of the KHV glycoprotein gene, provided the immune-modulatory effects on the koi fish immune response after vaccination. The higher dose vaccination using the GP-11 vaccine showed a higher immune response than its lower dose. Furthermore, the GP-25 vaccination resulting in the lower immune responses than the GP-11 vaccine using the same dose

of vaccination, but relatively the same to that of the half-dose of GP-11 vaccine.

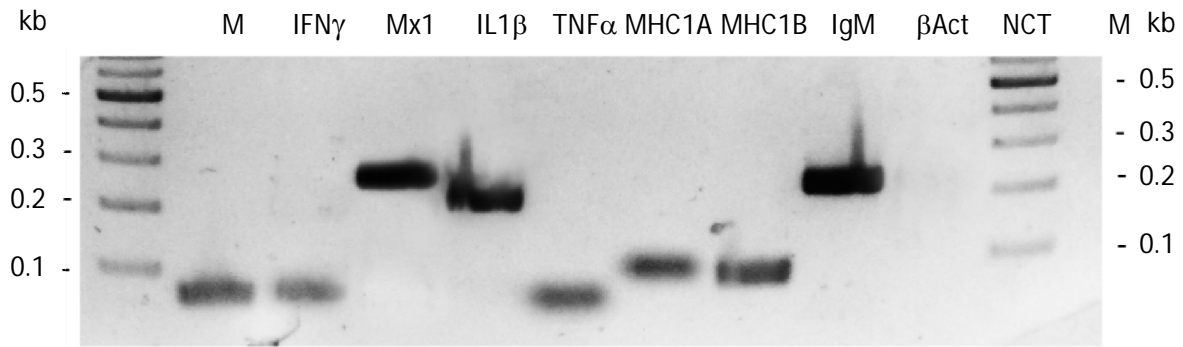
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Supplementary Figure. Agarose gel electrophoresis of various immune-related genes expression of koi fish. kb= kilobasepairs, M= 100 bp DNA markers.