

Construction of a DNA Vaccine Using Glycoprotein Gene and Its Expression Towards Increasing Survival Rate of KHV-Infected Common Carp (*Cyprinus carpio*)

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ABSTRACT

Deoxyribonucleic acid (DNA) vaccine has recently been developed as an alternative vaccine against virus infection. This study was the first step of DNA vaccine development to protect cyprinids including common carp (*Cyprinus carpio*) and fancy koi (*Cyprinus carpio*) from KHV (koi herpesvirus) infection in Indonesia. One of KHV glycoprotein genes, i.e. glycoprotein (GP) was ligated with Japanese medaka (*Oryzias latipes*) $\hat{\alpha}$ -actin promoter to generate pAct/GP as a DNA vaccine. Fourty fish in body weight of 10-15 g/fish were individually injected by pAct/GP into muscle in different dosage of 2.5 μ g, 7.5 μ g and 12.5 μ g/100 μ l phosphate buffer saline. Total RNA was extracted from the 12.5 μ g of pAct/GP-injected fish muscle at 24, 48 and 67 hours post-injection to analyze GP expression by RT-PCR method. Potential of pAct/GP as DNA vaccine was examined by injecting KHV into the 30-days-vaccinated fish. Both of possitive and negative control fish group were not vaccinated. Possitive control fish group were injected with KHV, but negative control fish group were not. KHV-challenged fish were reared for 1 month, and the death fish were calculated daily. Result of RT-PCR analysis showed that GP gene expression were detected at 3 d post-injection. Expression of GP in the vaccinated fish groups helped to improve their survival rate after challenged by KHV. All of fish without DNA vaccination had dead 17 days after KHV injection. The results demonstrated that pAct/GP had high potency to be used as a DNA vaccine against KHV infection in cyprinids.

Keywords: common carp, DNA vaccine, glycoprotein, koi herpesvirus (KHV)

INTRODUCTION

DNA vaccination is the direct inoculation of DNA expression vector, such as plasmid containing a specific gene from viruses driven by eukaryotic promoter, to stimulate the *in vivo* synthesis of immunogenic proteins and immune responses (Hirono, 2005). DNA vaccine has some advantages, such as activating both of humoral and cellular mechanisms, good effect when given at an early stage, and inducing protection shortly after vaccination at both low and high water temperature (Lorenzen & LaPatra 2005).

As a poikilotherm organism, immune responses of fish are influenced by environmental water temperature (Vinitnantharat *et al.*, 1999). Water temperature-independent activity of DNA vaccine may meet the need

to induce immune response in order to overcome mass mortality of fish as virus infection. Further, protection of attenuated vaccine is also highly on both humoral and cellular responses. However, attenuated vaccine may cause auto-infection. In contrary, DNA vaccine is most likely far away to be auto-infection agent. In DNA vaccination, just a part of virus genes is introduced to fish (Hirono, 2005).

As an alternative to chemical and antibiotic treatment, a DNA vaccine is the efficacious method for the prevention of infectious diseases in farmed fish, especially viral infectious diseases, because it can not result in anti-medication of fish and the pollution of water (Heppel & Davis, 2000; Zheng *et al.*, 2006). DNA vaccine can induce strong and longlasting humoral and cell-dependent immune responses and posses several practical and economical advantages, such as without

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the need for live organisms, replicating vectors or adjuvants (Tanghe *et al.*, 2000). Immunization with plasmid DNA vector represents a promising new approach to vaccination. It has been shown to elicit humoral and cellular immunity and protection in various infection models (Mollenkopf *et al.*, 2004). This vaccine may be applied in carp farming to KHV infection.

Protective immunity against IHN (Infectious hematopoietic necrosis virus) had been achieved through of both non-specific and specific immune responses by vaccination with plasmid DNA encoding a viral glycoprotein (G) (Kim *et al.*, 2000). The distribution and expression of lymphocystis disease virus (LCDV) vaccine, on the basis of DNA vaccine (pEGFP-N2-LCDV0.6 kb) construction, were analyzed by RT-PCR. Results from RT-PCR studies indicated that the *Mcp* gene is expressed in all tissues of vaccinated fish 7-20 hours after vaccination, therefore may have provided an antigen producing specific immune response (Zheng *et al.*, 2006). This observations prompted an investigation into the potency of the glycoprotein to be used as a DNA vaccine and it's dose improving carp resistance to KHV infection.

MATERIALS AND METHODS

Fish rearing. Four populations of 30 common carp were reared as experimental groups either in separately four aquaria (40 x 40 x 60 cm³) with constant aeration and 20% daily water changes. Weight of each fish is approximately 10-15 grams. Fish were fed on dry pellet twice per day *at satiation*.

Plasmid pAct-GP construction. The first step of pAct-GP construction was amplification of open reading frame 25 KHV (GP) using forward primer of GP, F:5'-TTGTCGACATGACGGGTTGTGGGTTTG-3' and reverse primer R:5'-TTAGGGCCTCCGGGAAACCTGG-3'. This sequence was result of genome sequences study of three koi herpesvirus isolates by Aoki *et al.*, 2007). The result of PCR product was ligated with pGEMT-Easy. Sequences of GP were analysed by PCR and sequenced using ABI PRISM 3100-Avant Genetic Analyzer.

Plasmid pAct/GP as a candidate of DNA vaccine was constructed by ligating the KHV glycoprotein gene sequence (GP) at the downstream site of Japanese medaka β -actin promoter (Act). KHV DNA was kindly provided by Research Office on Aquaculture Fisheries (BRPBAT Bogor). GP sequence was isolated by PCR

(polymerase chain reaction) amplification method with two oligonucleotides primers that designed based on GenBank database (accession number: DQ177346). A total of 35 cycles of denaturation for 30 s at 95°C, annealing at 64°C for 30 s, and extension at 72°C for 2 min were performed. The amplified products (approximately 1.8 kb in size) were purified from agarose gel electrophoresis using EZ-10 spin column DNA gel extraction kit (BioBasic Inc., Canada). The purified PCR products were cloned into pGEMT-Easy vector (Promega, Madison, WI). The sequence of GP isolate was confirmed by sequencing analysis using ABI-PRISM 3100-avant with Big-Dye system. Plasmid pActD6 (Alimuddin *et al.*, 2005) were digested with *Sal*I to eliminate D6-desaturase-like gene sequence (D6); results of DNA fragment were named as pAct. GP gene fragment was isolated from pGEM-T easy containing GP gene by digestion with *Sal*I, and then the GP gene fragment were ligated to pAct to design pAct/GP.

GP25 gene expression analysis. Plasmid pAct/GP was injected intramuscularly to examine GP gene expression in muscle of common carp. The dose of injection, i.e. 15 μ g of pAct/Gp in 100 μ l phosphate buffer saline (PBS) was referred to Zheng *et al.* (2006). Ten fish were injected with 12.5 μ g/ 100 μ l pAct-GP. Injected fish were maintained as described above. Total RNA was extracted from the pAct/GP-injected fish muscle at 1 d, 2 d and 3 d post-injection. cDNA synthesis was performed using Ready-To-Go You-Prime First-Strand Beads kit (GE Healthcare, UK). PCR was conducted in 10 μ l of 10x Ex *Taq* buffer, 200 μ M dNTPs, 0,125 U Ex *Taq* polymerase (Takara, Shiga, Japan), 1 μ l of cDNA as template, and 1 pmol of each primer. A total of 35 cycles of denaturation at 95°C for 30 s, annealing at 64°C for 30 s and extension at 72°C for 2 min were applied. Two microliters of the reaction were electrophoretically separated using 0.7% agarose gel, stained with ethidium bromide, and photographed under ultraviolet light. GFP (green fluorescent protein) expression was analyzed as a comparison of foreign gene expression driven by the same promoter in the pAct/GP construct. GFP expression was observed by using fluorescent microscope started at 24 hrs after injection.

Challenge test. Challenge test was carried out at Viral Challenge Installation of Great Office on Aquaculture Fisheries (BBPBAT Sukabumi). DNA vaccine, pAct/GP25 was injected intramuscularly with

2.5 µg; 7.5 µg and 12.5 µg per fish (in 100 il PBS). No injection with pAct/GP for the control fish group was performed. One month after vaccination, fish were challenged with lethal dose of KHV (Santika, 2008). Dead fish were observed daily and then be discarded from aquarium. Survival rate (SR) was considered based on the quantity of lived fish during experiment.

The potency of pAct/GP as a DNA vaccine was determined by comparing the SR of vaccinated and control fish groups after challenged with KHV. Survival data were analyzed descriptively.

RESULTS AND DISCUSSION

GP Sequence Analysis. PCR products amplification using forward and reverse primer of GP, F:5'-TTGTCGACATGACGGGTTGTGGGGTTTG-3' and R:5'-TTAGGGCCTCCGGGAAACCTGG-3' were DNA fragment approximately 1,8 kb (Figure 1a). This sequence (Figure 2) was be used as DNA inserted into pGEMT-Easy.

Results of partial sequencing read from forward direction were showed with following figure.

Results of GP25 similarity analysis with data in GenBank were 99% similar with KHV from Japan, USA and Israel. Results of GP allignment with data sequence at GenBank were showed with following figure 3.

Results of sequences analysis showed that GP gene that had been used as an insert DNA to pGEMT-Easy was glycoprotein target of koi herrpesvirus (KHV). This GP gene could be used to DNA vaccine construction using eukaryotic promoter.

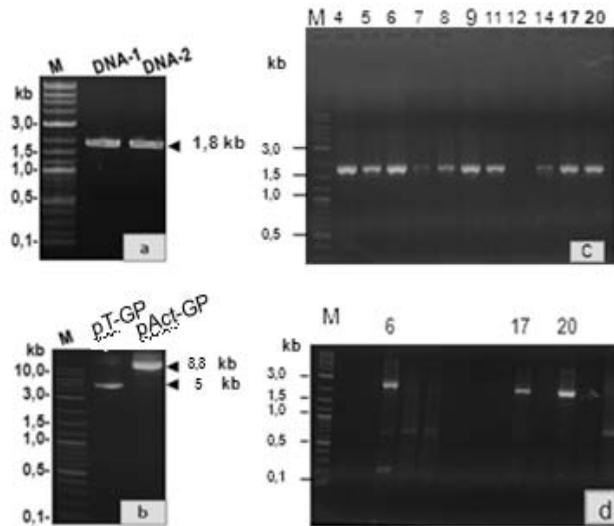


Figure 1. Glycoprotein gene construction. Glycoprotein gene approximately 1,8 kb (a), plasmid pAct-GP (10,5 kb) were compared with pGEMT-GP(5,8 kb) (b). Clones number 6, 17 and 20 have right orientation of ligation.

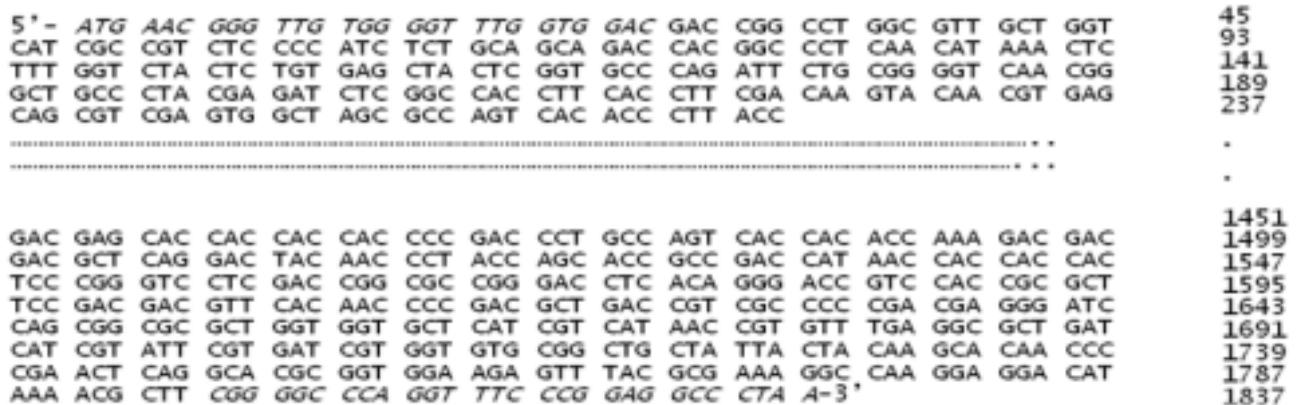


Figure 2. Results of glycoprotein gene sequencing. Italic fonts showed forward dan reverse primer nitrogen base of glycoprotein.

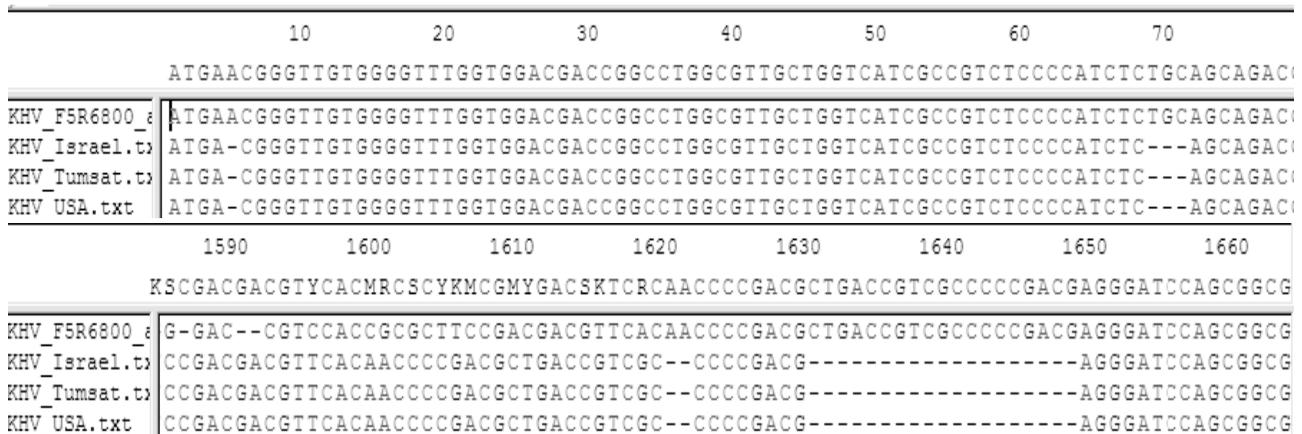


Figure 3. Results of GP allignment with data sequence of KHV from Japan, USA and Israel in GenBank

Construction of DNA vaccine. The first result of making pAct-GP construction was pGEMT-GP (Figure 1b-1). pGEMT-GP was result of ligation between GP and pGEMT-Easy. pGEMT-GP was digested and result of GP sequence analysis was shown in Figure 2. GP is ligated with pAct, so it be formed pAct-GP (Figure 1b-2). PCR analysis of pAct-GP using GP forward and reverse primers was shown in Figure 1c. The right orientation of ligation was analysed using forward primer GP and reverse primer T7 (5'TTGTAATACGACTCACTATAGGGCGAA-3'). Plasmid pAct-GP were used as template of amplification. There were three clones (clones no. 6, 17 and 20) that have right orientation of ligation (Figure 1c). The size of pAct-GP was 8.8 kb consists of 3.7 kb Act, 1,8 kb GP, 0.2 kb BGH fragments and the remains of 3 kb pBluescript as a backbone (Figure 4).

GP expression. GP was expressed at site injection of fish tissue that had sampled and analyzed by using RT-PCR method (Figure 5). GP were expressed at 3 d postinjection.

Effect of DNA immunization against KHV challenge 42 d post vaccination. Other groups of common carp that have vaccinated with pAct-GP showed increasing of survival rate (SR) of the experimental group after challenge test with lethal dose of KHV (Figure 6). The dose of vaccination 12.5 µg in 100 µl phosphate buffer saline gave survival rate 96,7 % at end experiment. All fish of control group that was not vaccinated with pAct-GP died at 17 d after challenge

test. Fish vaccination with 7.5 µg in 100 µl phosphate buffer saline increased resistance of KHV infection, although it's level was low so all of fish had died at 20 d post vaccination.

Plasmid vaccine costruction of pAct-GP were DNA vaccine candidate to control KHV infection in common carp and koi carp. This DNA vaccine was using medaka *α-actin* promoter. Using of this promoter was different with other promoter of DNA vaccine. DNA vaccine usually used CMV (*cytomegalovirus*) promoter (Zheng et al., 2006; LaPatra et al., 2001; Kim et al., 2000).

Some kinds of promoter that had been isolated and tested at some fish species by researcher were CMV from human virus, medaka elongation factor-1á (EF-1á), medaka *α-actin* and myosin light chain-2 (Mylz-2) from zebra fish (Alimuddin, 2003). Medaka *α-actin* promoter was active in same species, same family or other species, like rainbow trout (Yoshizaki, 2001), zebra fish (Alimuddin et al., 2005), Nile tilapia (Kobayashi et al., 2007), catfish (Ath-thar, 2007) and common carp (Purwanti, 2007). Using of *α-actin* promoter in DNA vaccine of KHV had great chance to produce glycoprotein of KHV in common carp. The functional of glycoprotein gene in common carp can produce glycoprotein as an antigen. This antigen will induce immune respons in fish.

DNA vaccine encoding glycoprotein of KHV was expressed at 2 days after injection and still expressed 2 weeks later. This results were different with research of Zheng et al., (2006) that had expressed *Mcp* gene at 7 days until 20 days after vaccination with DNA vaccine for lymphocystis disease cirus (LCDV). The construction of LCDV vaccine used CMV promoter.

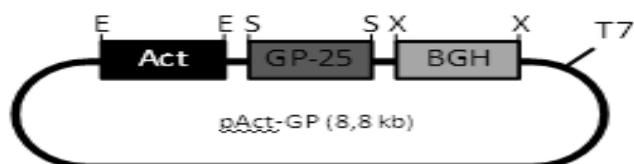


Figure 4. The map of pAct-GP construction (8.8 kb). Site restrictions of plasmid: E: *Eco* RI, S: *Sal* I, X: *Xho* I, T7: T7 primer sequence.

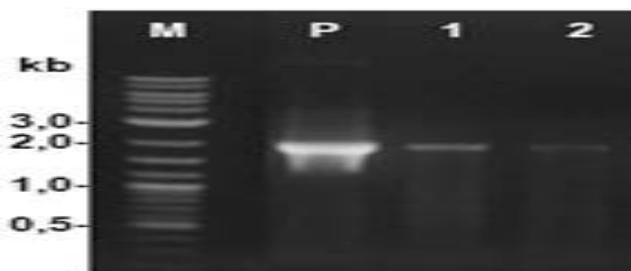


Figure 5. Results of GP expression analysis 1 d postinjection. Possitive control of plasmid contains GP gene (P), results of pAct-GP25 expression at 1 d post injection from the same sample (1,2).

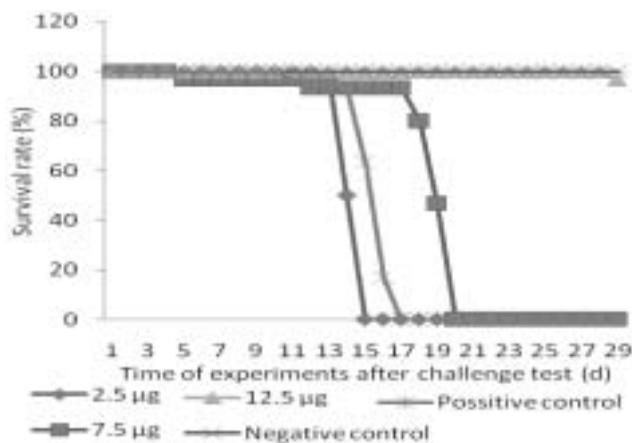


Figure 6. Survival rate of vaccinated and control fish after challenge test with KHV. Both of control fish were not vaccinated; negative control was not infected with KHV, but possitive control was infected with KHV.

DNA vaccine contained glycoprotein gene and α -actin promoter could increase survival rate of vaccinated fish. Vaccination dose 12,5 μ g gave highest survival rate 96,7% during one month after challenge test with KHV. Patterns of survival rate of experimental fish were successive doses of 7,5 μ g and 2,5 μ g. Mortality of vaccinated fish with 2,5

μ g was faster than control fish. This fact was caused by that vaccination was done by injection. Side effect of this method was stress in vaccinated fish. Control fish were not injected by vaccine, so they didn't undergo stress. Stress in fish could press fish immune system, so their resistance to KHV slope down.

Protein production using glycoprotein DNA had protected fry up to 80 d after immunization and induced protective neutralizing antibodies (Corbeil *et al.*, 1999). Vaccination using G protein (glycoprotein) protected rainbow trout from IHNV infection. Nucleoprotein (N protein), phosphoprotein (P protein), matrix protein (M protein) and non-virion protein (NV protein) had not given protection to rainbow trout after challenge test with IHNV. So, Glycoprotein were immunogenic protein because they could induced immune responses of fish and gave protection from virus infection.

DNA vaccine dose ever applied to prevent IHNV in 2 g rainbow trout were 0,1, 1, and 2,5 μ g. All of those doses gave significant protection after challenge test with IHNV (LaPatra *et al.*, 2001). That dose of vaccination in this study 12,5 μ g was too high, so it needed to reduce. The reduction of this dose had been to get involve of weight fish that would be vaccinated. DNA vaccination to prevent KHV infection can use low dose of DNA vaccine and fry stage of fish. The lower dose of vaccine could reduce cost production of fish farming.

Challenge test of this study did not use LD50 of KHV. Based on an earlier study (data not shown), challenge test of carp using LD50 was not effective because of depending on water temperature. The change of water temperature could change of virus pathogenicity.

As a comparison, vaccinated fish were challenged at 30 or 70 days post-DNA vaccination using lethal dose of virus (Kim *et al.*, 2000). At 30 days post-vaccination, only 5% of fish that had received any of the G vaccine died, whereas more than 50% of the control fish succumbed to virus challenge. When fish were vaccinated and challenged at 70 days post-vaccination,

only 12% of the IHNV-G vaccinated died. Based on Mx protein assay, DNA vaccine in fish induces an early, non-specific antiviral protection mediated by an alpha/beta interferon and, later, a specific immune response.

To administer DNA vaccine using injection method could cause stress in fish. This method need alternative to avoid stress. Stress could cause infection by organisms. The alternative method is application of bacteria containing glycoprotein gene to fish through live feed from Crustacean class like *Daphnia*. Application of DNA vaccine using live feed *Artemia* had been studied by Lin *et al.*, (2007). NNV (nervous necrosis virus) vaccine composed of *Artemia* – encapsulated recombinant *E.coli* could be delivered to, and absorbed in, the hindgut of grouper and that it induced anti NNV VP specific antibodies 7 days after vaccination, as assayed by ELISA. *Daphnia* were bacterial feeder. They could be fed by recombinant *E.coli* containing pAct/GP. Fry of fish will feed *Daphnia*, so pAct-GP25 may transfer from live feed to fry fish.

CONCLUSIONS

Plasmid of pAct-GP has high potency to be used as a DNA vaccine improving carp resistance to KHV infection. Vaccination of fish using 12.5 μ g pAct-GP gave highest survival rate after challenge test with lethal dose of KHV compared to 2.5 and 7.5 μ g.

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