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MATERNAL IMMUNITY RESPONSE AND LARVAL GROWTH OF ANTI CYHV-3 DNA VACCINATED COMMON CARP (*CYPRINUS CARPIO*) AT DIFFERENT PRE-SPAWNING TIME

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ABSTRACT

This study aimed to evaluate maternal immunity response of common carp broodstock and their progenies. Vaccination of broodstock was performed by injecting GP25 plasmid as anti-CyHV-3 DNA vaccine at 30, 45 and 60 days pre-spawning (dps). Three fish were used for each treatment. Triplicates challenge test at 7, 14, 21 and 28-day-old progenies were performed for 21 days by 1hour immersion of 10^{-3} CyHV-3 filtrate dilution. Serum was collected six times from broodstock, and four times from larvae. The result showed that vaccination could induce the immune response of broodstock. Leukocytes count, and phagocytic activity of the vaccinated broods were significantly higher than that of control ($P<0.05$) at 30 days post vaccination (dpv). Furthermore, broodstock vaccination at different pre-spawning time allowed different immunity response in their larvae. Vaccination at 60 days pre-spawning (dps) had the highest antibody titer in eggs and larvae and offered higher protection on the larvae against CyHV-3 infection. Growth of larvae from vaccinated broodstocks was significantly higher than that of control ($P < 0.05$). This study indicated that broodstock vaccination at a proper pre-spawning time improves its immunity and protection capability is transferred to their immature immunity larvae against pathogens infection.

Keywords: common carp, CyHV-3, DNA vaccine, maternal immunity

INTRODUCTION

Common carp (*Cyprinus carpio*) is prospective commodity in aquaculture which faces a serious outbreaks of Cyprinid herpes virus 3 (CyHV-3). This virus was first identified in Israel 1998, then spreading rapidly around the world and becomes a global problem (Hendrick *et al*., 2000). CyHV-3 belongs to group of double-stranded DNA viruses from *Herpesviridae* family (Yi *et al*. 2014). In condusive environment, CyHV-3 has highly virulent to cause 80-100% death less than 7 days post-infection (Gilad *et al*., 2003) at permissive temperatures of 18-27°C (Hendrick *et al*., 2000). A method known to be effective in preventing CyHV-3 infec- tion is vaccination (Arvin 1996). Some types of CyHV-3 vaccine that have been used were live att-enuated vaccine (Perelberg *et al*., 2005), liposomes entrapping (Miyazaki *et al*. 2008), DNA vaccine (Zhou *et al*., 2014a) and recombinant protein vaccine (Cui *et al*., 2015).

DNA vaccine is the third-generation vaccine after conventional vaccines and recombinant proteins that have advantages over previous vaccine types such as more safe, resistant to temperature changes, modifiable, economical inducing cellular and humoral immune systems simultaneously (Lorenzen & Lapatra 2005). DNA vaccine is the latest innovation form experimental techniques to protect organism by injecting pure DNA (naked DNA). Development of DNA vaccine against Cy-HV-3 infection in carp showed prospective result.

Some research had been reported successfull for maintaining relative percent survival of carp 84.60 % (Nuryati *et al*., 2015) by feeding, 68% by immersion with different density (Aonullah *et al*., 2016), and survival rate up to 61% by immersion method (Nuswantoro *et al*., 2012) and 86.67% by injection (Chairunnisa *et al*., 2016). This vaccine was constr- ucted from DNA of CyHV-3 local isolate encoding glycoprotein-25 (GP25) with β-actin promoter of Japanese medaka fish (Nuryati *et al*., 2015).

Ability of GP25 DNA vaccine protection has not been applied in early stages of carp. In fact, results of cohabitation studies indicated that early stadia of carp were very susceptible to CyHV-3 infection and potentially become main agent (carrier). It is closely related organs which have not been fully proliferated (Perelberg *et al*., 2003), so that protective ability still highly dependent on immunity broodstock transferred (Swain & Nayak 2009). One possible means to prevent CyHV-3 infection in early stages is increasing specific antibody by maternal immunity from broodstock

vaccination. Some research on fish species showed enhancing immunity through broodstock vaccination technique, such as seabass *Sparus aurata* (Hanif *et al*., 2004), Siamese catfish *Pangasianodon hypophtha- lmus* (Hadie *et al*. 2010), and tilapia *Oreochromis niloticus* (Nisaa *et al*., 2017).

Broodstock vaccination is also relatively easy, safe and efficient in its application although it has weaknesses short protection time (Zhang *et al*., 2013). Therefore, application of GP25 DNA vacc ine is very important to be done on common carp broodstock for early protection against CyHV-3 infection. Objective of present study were to evaluate maternal immunity in broodstocks, eggs and seeds of common carp using GP25 DNA vaccine different pre-spawning times, and to examining performance of seed production in terms of fecundity, egg hatching and growth rate.

MATERIALS AND METHODS

Propagation of anti cyhv-3 DNA vaccine: DNA vaccine that used in this study was GP25 plasmid designed by Nuryati *et al*., (2010). *Escherichia coli* bacteria carrying GP25 vaccine were cultured foll- owing the method of Nuryati *et al*., (2010). Plasmid was isolated using genejet Plasmid Miniprep kit (Thermo Scientific, USA) following the procedure kit manual, then dissolved in 100 µl ion exchange water and stored at -20°C until it will be used. DNA concentration was measured using DNA/RNA calculator (Genquant) referring to method of Brown (1990).

Vaccination and broodstock maintenance: Common carp broodstocks of Majalaya strain were obt ained from fish farmers in Bogor Regency, West Java. Body weight mean of female broodstock was 2.38±0.32 kg, and male was 1.58±0.25 kg. Before vaccination, fish were anesthetized with *Ocean free special arowana stabilizer* (Qian Hu Corporation Ltd., Singapore) of 0.6-1.0 ml/L water following procedure Puteri *et al*. (2016). Gonad maturity (GM) was determined by observing morphological features referring to Jhingran & Pullin (1985) and egg diameter according to FAO (2015). About 9 egg samples were taken by cannulation method and diameter was measured fresh conditions under a microscope-ocular micrometer (0.1 mm precision).

Broodstocks were vaccinated by intramuscular (IM) injected GP25 plasmid with a dose of 12.5 µg 100 gram-1 fish dissolved in 1 ml phosphate buffer saline (PBS) (Nuryati *et al*., 2010). Vaccination was done at different pre-spawning times, namely: 30 (A), 45 (B) and 60 (C) days before broodstocks were mated. As a control, fish were injected with PBS (no vaccination). Three broodstocks were used in each treatment. Female and male broodstocks were kept separately using cage measuring 3×3×1 m3 (density: of 1/fish m3). Female brood-stocks in each treatment were kept one cage installed a concrete pond measuring 20×10 ×1.5 m3. Water quality measurements were done at the beginning, middle and the end of study including ph 7.45-7.98; dissolved oxygen 7.1-7.9 mg/L and total ammonia nitrogen <1 mg L-1. Water temperature was maintained at 27-32 °C. Fish was fed with a commercial diet (32-38% protein content), 2 times daily at satiation.

Broodstock hematology analysis: Blood samp-ling was conducted 15 days before vaccination and 15, 30, 45 and 60 days post vaccination (dpv). Blood was taken from caudal veins using a syringe that has been rinsed with 3.8% sodium citrate. Parameter observations were included total leukocytes (Blaxhall & Daisley 1973) and phagocytic activity (Anderson & Siwicki 1993).

Spawning, egg hatching, and larvae maintenance: Broodstocks were reared until ready for spawning. Induced ovulation of female broodstock was performed by injecting 0.5 ml kg-1 ovaprim disso-lved with 0.9% nacl (Solomon *et al*., 2015). Before spawning, broodstock was carried out in 1.5×3×1.5 m3 cage which equipped with aeration system. After 8 h post-injecting ovaprim, broodstocks were caught and stripped in fish abdomen to collect eggs and sperm. Both eggs and sperm were mixed by adding 0.9% nacl for artificial fertilization refering to Cabrita *et al*., (2009). Eggs were hatched in a 3×2×2 m3 tank. Calculation of fecundity and hatch-ing rate was done by gravimetric method (Effendi 1979).

A total of 100 larvae were kept in 100×100×50 cm^3 aquarium during 30 days for measurement of daily growth rate and survival rate. Three aquariums were provided for each broodstock as replica tions. Weight of 300 larvae per broodstock was measured at 1-day post-hatch (dph) and 30 dph by an electic balance (accuracy: 0.1 mg). Number of live larvae was calculated at the end of the study.

Other larvae were kept in tank as fish stock for measurement of antibody and challenge test. Over all of larvae were fed with nauplii artemia on 3-7 days post-hatch (dph) followed by a bloodworm (*Tubifex* sp.) on *ad libitum* for 10 days. Afterward, fish were fed with commercial diet (40% protein content) at satiation. Water quality

measurements were done at the beginning, middle and the end of study including pH 6.61-8.86; dissolved oxygen 6.7-8.2 mg/L and total ammonia nitrogen <1 mg L^{-1}. Water temperature was maintained at 24-27 °C.

Analysis of antibody titers: Serum of common carp broodstock was taken before vaccination and 15, 30, 45, 60 and 75 dpv. Serum was obtained by blood centrifugation with a speed of 5000 rpm for 10 min and stored in -20ºC until antibody titer would be measured. About 0.2 grams egg samples were obtained before fertilization and 0.2 grams larvae were also taken at 1, 7, 14, 21 and 28 days post hatch (dph). Samples were washed with aqua-des, crushed, homogenized in PBS solution cont-aining 0.05% Tween-20 (pH 7.2) at a ratio of 1:4, then centrifuged at 6,000 rpm for 15 min. Super-natant was stored in -20ºC until will be used for antibody titer measurement. Antibody titer was measured using enzyme-linked immunosorbent assay (ELISA) method referring to Aonullah *et al*., (2016) with slightly modified. Antigen used for the ELISA test was obtained from the sonication of CyHV-3 filtrate at a frequency of 40 Hz for 5 min (on ice). Sonicated-antigen was measured its protein concentration by Bradford method. Antigen was diluted 1:280 using coating buffer (0.05M carbonate-bicarbonate pH 9.6) to get final concentration 5 µl mL^{-1}. Diluted-antigen was inserted into each microplate as much 100 µl and incubated at 4ºC overnight. Plate was washed four times on each well using 300 µl PBS-T (PBS pH 7.4 + 0.05% Tween-20). Plate was blocked using 100 µl PBS s*kim milk* 5% and incubated 4ºC overnight. Serum samples diluted 1:50 were then inserted into each well microplate 100 µl (duplo) and incubated at 37ºC for one hour. Plate was then washed with same previous step. Anti-carp IgG derived from rabbit (concentration: 1:100) was added as much as 100 µl into each well and incubated 37ºC for 1 h. Plate was then washed with same previous step. Anti-rabbit igG conjugated with Horse Reddish Preoxydase (HRP) was added as much as 100 µl into each well and incubated 37ºC for 1 h. Plate was then washed with same previous step. One-Step Ultra TMB-ELISA was then added to each well as much as 100µl and let it be for 20-30 minutes. ELI-SA reaction was stopped by adding 50 µl of H_2SO_4 3M and an optical density (OD) reading was per-formed at 405 nm (estimated result). Cut of value (CV) was determined based on the equation CV = the mean of negative control + standard deviation.

Challenge test and confirmation of cyhv-3 infection: Challenge test of cyhv-3 was done by immersion method. A total of 30 larvae at 14 days post-hatch was immersed in 100 ml water cont-aining 10-3 cyhv-3 filtrate for 1 h. Cyhv-3 dose was result from LD50 test. Each treatment was set up 3 replications. Furthermore, fish were transferred into 20×20×15 cm3 aquarium. Fish were main-tained for 21 days at a water temperature of 21± 1o C. Dead fish were counted daily and stored at -80 °C. Fish dead as infected by cyhv-3 was charac-terized by clinical symptoms (OATA 2001).

Dead fish because of CyHV-3 infection were confirmed by PCR method referring to Yuasa *et al.,* (2005). Total DNA was extracted using Puregene® DNA Purification Kit (Minneapolis, USA) by a manual procedure. DNA extract pellets were disso-lved in 30 µL ion exchange water and stored at -20 °C before being used for PCR analysis. CyHV-3 amplification was using forward 5'-GAC ACC ACA TCT GCA AGG AG-3' and reverse 5'-GAC ACA TGT TAC AAT GGT CGC-3'. PCR result were electrophoresed at 1% agarose.

Statistical analysis: Blood profiles, antibody titers, survival (SR), fecundity, egg hatchability, and larvae growth rate were analyzed using IBM SPSS Statistic for Windows, version 16.00 (IBM Corp, Armonk, NY, USA) at significant level of 0.05. Significant differeces between treatments were determined using a *post hoc* Duncan test.

RESULTS

Production performance: Production performances in terms of fecundity, egg hatching rate, survival rate and growth of carp larvae are presented in Table 1. Broodstock fecundity did not differ among treatments. Hatching rate on treatment C (60 days vaccinated pre-spawning: 89.39±4.17%) was higher ($P < 0.05$) than that of other treatments (30, 45 days vaccinated pre-spawning and control). Daily growth rate of larvae from the vaccinated broodstock was higher ($P < 0.05$) than larvae from broodstock without vaccination (control). Mean while, survival rate of larvae was not significantly different ($P > 0.05$).

Table 1: Fecundity, egg hatching rate (HR), daily growth rate (DGR) and survival rate (SR) of larvae from vaccinated common carp broodstocks

Treatment	Egg Fecundity ($\times 10^5$ eggs)	HR (%)	DGR (%/day)	SR (%)
A	2.21±0.24	81.65±4.16^{a}	15.17±0.30^{b}	95.67±2.00^{a}
B	2.24±0.45	78.66±4.85^{a}	15.78±0.80^{bc}	96.00±0.57^{a}
C	2.39±0.31	89.39±4.17^{b}	16.20±0.45^{c}	96.33±1.52^{a}
K	2.17±0.56	74.61±1.69^{a}	14.12±0.14^{a}	94.67±0.58^{a}

Vaccination of GP25 plasmid was applied 30 days (A), 45 (B) and 60 (C) days before broodstocks were spawned. Different superscript letters in the same column showed significantly different values ($P < 0.05$).

Blood profiles of common carp broodstock: Blood profiles of common carp broodstock including total leukocytes and phagocytic activity is presented in Table 2. Results indicated that there was vaccine administration effect to blood profiles. Total leukocyte increased significantly ($P < 0.05$) in vaccinated fish from 15 dpv to 45 dpv, followed by increased phagocytic activity at 30 to 60 dpv.

Table 2: Blood profiles of common carp broodstock before and after vaccination

Parameter	Treatment	Before Vaccination	Times (day post vaccination) 15	30	45	60
Σ Leukocytes ($\times 10^4$ cell/mm^3)	A	6.03±3.54^{a}	9.7±2.40^{b}	13.96±2.91^{c}	14.96±3.93^{b}	12.20±4.35^{a}
	B	6.43±2.17^{a}	8.47±3.43^{ab}	11.16±2.55^{b}	13.96±3.77^{b}	13.76±4.00^{a}
	C	7.93±2.12^{a}	9.03±4.57^{ab}	14.26±3.77^{c}	14.20±3.84^{b}	10.60±2.92^{a}
	K	7.73±3.02^{a}	7.83±3.75^{a}	7.57±4.82^{a}	9.20±1.99^{a}	10.30±4.00^{a}
Phagocytic Activity (%)	A	6.00±1.00^{a}	13.67±4.73^{a}	18.67±3.79^{b}	13.00±4.58^{ab}	9.67±2.08^{ab}
	B	5.33±2.52^{a}	14.67±2.08^{a}	17.67±3.21^{b}	10.67±1.53^{ab}	11.67±1.53^{ab}
	C	8.00±1.00^{a}	11.00±5.29^{a}	20.33±2.08^{b}	15.33±1.53^{b}	13.67±3.06^{b}
	K	5.33±3.21^{a}	8.00±3.00^{a}	9.33±4.93^{a}	9.67±2.08^{a}	8.00±1.00^{a}

The control fish were injected with PBS (K), vaccination of GP25 plasmid was performed 30 days (A), 45 (B) and 60 (C) days before broodstocks were spawned. Different superscript letters on the same columns and parameters showed significantly different values ($P < 0.05$).

Antibody titers of common carp broodstock: Antibody titers of common carp broodstock that has been vaccinated by anti- CyHV-3 DNA are presented in Fig. 1. Absorbance value of all vaccinated fish was higher ($P < 0.05$) than that of controls at 30-60 dpv, except treatment of B (45 days vaccinated pre-spawning) was like control at 60 dpv. Furthermore, antibody titers of the vaccinated and unvaccinated broodstocks were the same at 75 dpv ($P > 0.05$).

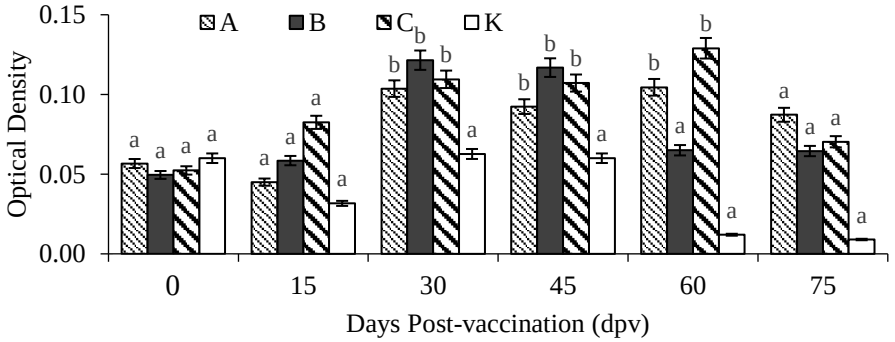

Figure 1: Antibody titers on common carp broodstocks before and after vaccination with anti CyHV-3 DNA vaccine (GP25). The DNA vaccine was administered at 30 (A), 45 (B) and 60 days pre-spawning (C). K is the control without vaccination. CV value = 0.103. Different superscript letters on the same dpv indicate significantly different results ($P < 0.05$).

Antibody titer on common carp egg: Antibody titers in eggs are presented in Fig. 2. Results showed that antibody titer at treatment C (60 days vac- cinated pre-spawning) was higher than that of other treatments ($P < 0.05$), while antibody titers on treatment A (30 days vaccinated pre-spawning) and B (45 days vaccinated pre-spawning) were same ($P > 0.05$).

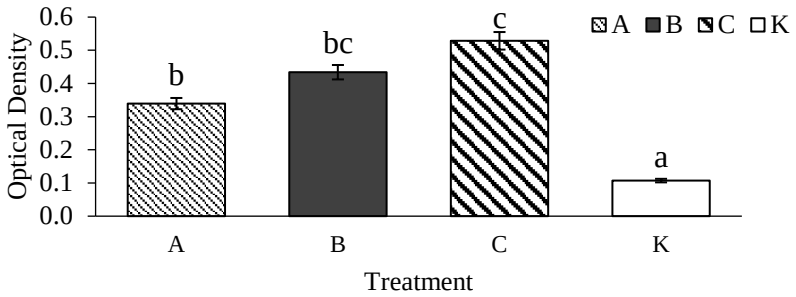

Figure 2: Antibody titer on eggs from the vaccinated broodstocks with and without anti- CyHV-3 DNA vaccination. The administration of GP25 DNA vaccine was performed at 30 (A), 45 (B) and 60 days pre-spawning (C). K is the control without vaccination. CV value = 0.206. Different superscript letters on the same dpv indicate significantly different results (P < 0.05).

Antibody titer on common carp larvae: Anti-body titers on common carp larvae from vaccinated and unvaccinated broodstocks with anti CyHV-3 DNA are presented in Fig. 3. Results showed that larvae of C treatment broodstock (60 days vacci-nated pre-spawning) had higher antibody titers at 1-14 dph than in other treatments (P < 0.05). Treatment B (45 days vaccinated pre-spawning) was only different from treatment A (30 days vaccinated pre-spawning) and control (without vaccination) at 1 dph, subsequently it had same value (P > 0.05). Antibody titer in larvae from vaccinated broodstock became same as unvaccinated brood stock after 14 dph.

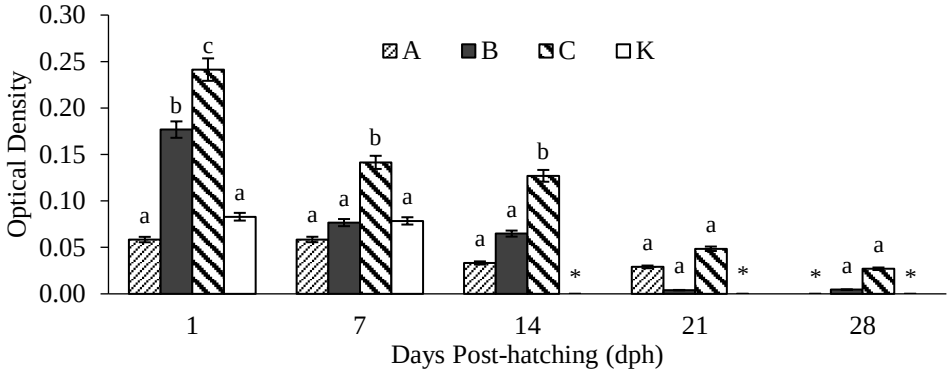

Figure 3. Antibody titer on larvae from broodstock before and after vaccination. The administration of GP25 DNA vaccine was performed at 30 (A), 45 (B) and 60 days pre-spawning (C). K was the control without vaccination. CV value = 0.206. The mark (*) is an undetectable antibody level. Different superscript letters on the same dpv indicate significantly different results (P < 0.05).

Survival rate of common carp larvae: Survival rate of the common carp larvae by post-challenge test with CyHV-3 is presented in Fig. 4. Results showed that the highest survival rate of larvae was obtained by treatment C (60 days vaccinated pre-spawning), followed by treatment A (30 days vaccinated pre-spawning) and B (45 days vaccinated pre-spawning), and the lowest was control (P < 0.05).

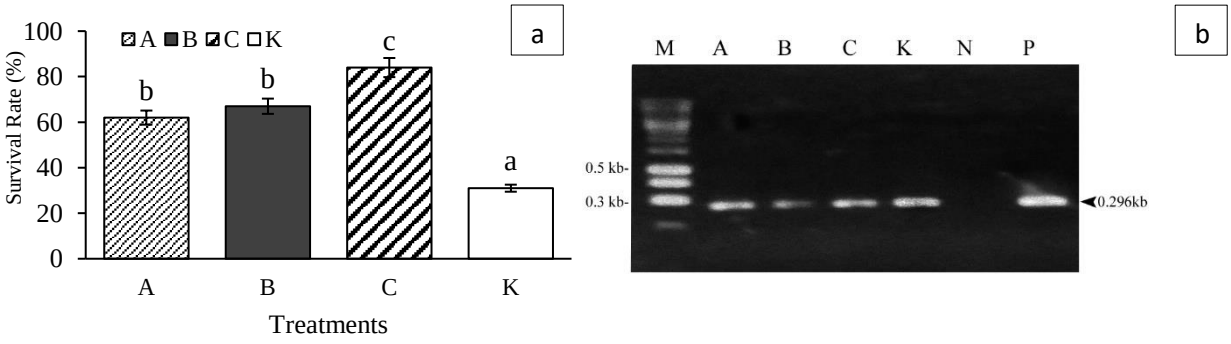

Figure 4: (a) The survival rate of common carp larvae which was challenged with koi herpesvirus at 14 days post-hatching. (b) Electrophoregram of PCR amplification product for confirmation of fish mortality during the period of

challenge test with CyHV-3. The administration of GP25 DNA vaccine was performed at 30 (A), 45 (B) and 60 days pre-spawning (C). K is the control without vaccination. KAPA universal DNA ladder (M), negative control (N), positive control (P). CyHV-3 DNA fragment target (296-kbp) was indicated by an arrowhead. Different superscript letters on the same dpv indicate significantly different results ($P < 0.05$).

DISCUSSION

Results showed that application of GP25 DNA vaccine influenced egg hatching rate, larval growth and immune response on broodstock, egg and larvae. Meanwhile, fecundity of female broodstock was about 2.17-2.39 ($x10^5$) in all treatments (Table 1), thus, administration of vaccine to broodstock did not affect the development and production of eggs. This is in line with statement of Zanchi *et al.,* (2012) that influence of maternal immunity can not always be linear with fecundity and some studies are found a negative relationship.

Egg hatching rate from broodstock of treatment C (60 days vaccinated pre-spawning) was higher than other treatments (Table 1). This is assumed by presence of vaccination on broodstock at 60 days pre-spawning which can generate eggs containing higher immune components (Hanif *et al*., 2004). These immune components can protect eggs from pathogenic infections. Presence of maternal immunity from broodstock has been known to play a role in protection of embryo from pathogen attack (Zanchi *et al*., 2012; Zhang *et al*., 2013).

Growth rate of larvae from vaccinated broodstock was higher (Table 1) which shows role of maternal immunity. Existence of maternal immunity can streamline using of nutrients and energy, so that it can be allocated for growth (Grindstaff 2008; Raberg *et al*., 2002). This is also in line with statement of Huttenhuis *et al.,* (2006) that maternal immunity has a variety of components that can pro-vide protection against pathogen infection.

Vaccination on broodstock affects blood profiles. Total leukocyte significantly increased in all treatments from 15 dpv to 45 dpv ($P < 0.05$). This increasing is most likely be body's response to presence of antigens from injected DNA vaccine expression (Kanellos *et al.,* 2006). Leucocytes differentiate into macrophage cells for phagocytic process (Tonheim *et al.,* 2008) so that increasing of leukocyte is followed by increasing of phagocytic activity (Table 2). DNA vaccine encoding glycoprotein 25 was modified from plasmid pActD6 using B-actin fish promoter medaka and terminator/polyA for expression purposes in fish (Lorenzen & Lapatra 2005). Modified plasmid was propagated in *Escericia coli* bacteria. Purified plasmids that are injected into the fish's body will insert to cell and then translated to immunogenic protein (Tonheim et al., 2008) and be recognized as antigens (Rawat *et al.,* 2007). Immunogenic protein produced as glycoproteins-25 (GP25) is responded by immunity system both non-specific (innate immunity) and spesific (adaptive immune) (Aonallah *et al.,* 2016). Non-spesific immunity its showed at leucocyte changes.

This study shows that prior to vaccination whole broodstocks had the same antibody titer up to 15 dpv ($P > 0.05$) and increasing at 30-60 dpv than control (Fig. 1). This is in line with the efficacy of GP25 DNA vaccine reported by Aonullah *et al.,* (2016) which reached optimal amount for protection against CyHV-3 infection at 28-50 dpv. Optimum level of specific immunity protection at 30-60 dpv was also found in several DNA vaccine tests such as rhabdovirus vaccine in rainbow fish (Lorenzen *et al.* 2005), *A. hydrophila* and *reovirus* vaccine in grass carp (Liu *et al*. 2016; Wang *et al*., 2015), *Mycobacterium* vaccine in hybrid-striped bass fish (David *et al.,* 2005), and DNA-ORF81 CyHV-3 vaccine in carp (Zhou *et al.,* 2014b). This shows range of antibody production time which was commonly found from DNA vaccine applications.

Formation of antibodies on broodstock was not constantly increasing until the end of observation. Presence of maternal spawning activity was thought to cause a decreasing in number of antibodies at 15 days post-spawn so that fluctuations of anti-bodies occurred in all treatments. Antibody titer was decreased after spawning; decreased by day 15 for control, day 45 for treatment A, day 60 for treat-ment B and day 75 for treatment C. The reincreasing of antibody titer on treatment A at 60 day was assumed due to continuous antibody production by responding presence of antigens in body that was produced from DNA vaccine. This is in line with Joosten *et al.,* (1997) stated that if the antigen has not been neutralized, antibody production will still continue. Decreasing in antibodies in all post-spawning treatments is due to transfer of anti-bodies from broodstock to larvae through egg yolk during vitellogenesis (Mulero *et al.,* 2007). Vitellogenesis is

process of forming vitellogenin as an egg yolk which includes localization of antibodies bro-ught into the ooplasm; thus, antibodies can join in oocytes along with vitellogenin through transcytosis passing through cell follicles (Swain & Nayak, 2009; Iromo *et al.,* 2014). Facts in this study showed that the difference in pre-spawning vaccine time influenced antibody level transferred to the egg, namely longer vaccination time of pre-spawn, the higher antibody titer produced (Fig. 2). This result is in line with Hanif *et al.,* (2004) who stated that eggs produced from female broodstocks immunized had higher antibody levels than those were not vaccinated.

Eggs and larvae of treatment C (60 days vaccinated pre-spawning) had the highest levels of antibodies. It is evident that larvae from broodstock of treatment C had number of antibodies transferred more than other treatments. Sin *et al.,* (1994) also reported that the number and duration of antibody protection transferred from broodstock to larvae depend on number of antibodies formed on the broodstock during spawning. Broodstocks vaccinated at 60 days pre-spawning were still in vitellogenesis stage, so that the accumulation of antibody proteins that were formed after vaccination was more in egg yolks. This can be seen from size of cannulated egg. Eggs diameter of broodstock of treatment C (60 days vaccinated pre-spawning) at vaccination ranged from 0.57±0.06 mm which was still classified as egg development stage (vitelloge-nesis) (FAO 2015). In contrast to broodstocks vaccinated treatment B (45 days vaccinated pre-spawning) which egg development phase was more mature because egg diameter was 0.76± 0.06 mm. Similarly of broodstock at treatment A (30 days pre-spawning) that has entered final phase of gonad development or approaching dormant phase with egg diameter of 0.97±0.13 mm; thus, the accumu-lation of antibody proteins was fewer in yolk. This is in line with Nisaa *et al.,* (2017) which stated that broodstock vaccination in degree of gonad maturity 2 (DGM 2) was more protective than broodstock vaccination in DGM 3.

Furthermore, whole antibodies observed in all treatments also decreased with increasing age of carp larvae (Fig. 3). This suggests that antibodies in larvae can not survive and will be re-established after active exposure of pathogen (Akbary *et al*., 2015). Presence of antibodies in larvae that were passively received from broodstocks provided protection against CyHV-3 infection. This is indicated by higher survival rate of larvae from vaccinated broodstock ($P < 0.05$) than that of control (Fig. 4a). The highest survival rate was obtained at treatment C (60 days vaccinated pre-spawning), and this was consistent with highest antibody titer found in trea-tment C during challenge test, that was 14 dph (Fig. 3). Swain & Nayak (2009) also reported that high broodstock antibody affects several antibodies transferred to larvae, so protection against pathogen infection is also high. Larvae death of challenge test was caused by CyHV-3 infection. This was confirmed by presence of DNA bands of PCR products in all treatments which were same size as plasmid control (P), whereas in N control there was no PCR product (Fig. 4b). A significantly higher antibody titer and survival rate in larvae from vaccination broodstock demonstrated the ability of DNA vaccines to be applied to maternal immunity. Zaher & Ahmed (2016) stated that among the advantages of DNA vaccine is its ability to induce a maternal immunity that can be transferred to larvae.

CONCLUSION

Application of anti CyHV-3 GP25 DNA vacc-ine in common carp broodstocks enhanced non-specific and specific immune responses to broodstocks and larvae. The highest accumulation of anti-bodies to eggs and larvae was obtained by brood-stocks vaccination at 60 days pre-spawning, resu-lted in high protection against CyHV-3 infection. Broodstocks vaccination was also able to increase the egg hatching rate and larval growth.

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