IMPROVEMENT OF NON SPECIFIC IMMUNE SYSTEM ON COMMON CARP (*Cyprinus carpio*) AGAINST KOI HERPESVIRUS DISEASE (KHVD) BY ADDITION OF ASCORBIC ACID ON FISH DIET: A FIELD SCALE STUDY

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ABSTRACT

The research with the aim to know the optimal feeding frequency of supplemented ascorbic acid (microencapsulated vitamin C CFC-90) on the dose of 750 mg/kg feed to control Koi Herpesvirus (KHV) disease infecting common carp has been done in field condition. Fish were reared in floating cages with the size of 3.5 m x 3.5 m x 2.0 m and stocking density of 1,250 fish/cage with the size range of ± 10 g/fish. The treatments applied in the research were: (A) daily application, (B) every 3 days application, and (C) without vitamin C addition as the control. Fish test were challenged to KHV infection on the mid cultivation by cohabitation method in the laboratory scale for 2 weeks. Examination on behavior, clinical sign, and mortality of fish test conducted daily. The results showed that the highest survival rate was found on the application of vitamin C every 3 days (60.16%); and followed by every day (52.00%), and the lowest was found on the control group (47.36%).

KEYWORDS: Koi Herpesvirus, vitamin C, common carp

INTRODUCTION

Koi Herpesvirus (KHV) disease is an infectious disease in carp (*Cyprinus carpio* Linn.) and highly contagious. It negatively affects carp culture and production which is one of the strategic commodities of food and one of the main sources of animal protein for millions of Indonesian people, both rural and urban. The disease can occur in almost any fish age and aquaculture system. Cases of mass mortality due to infection of carp Koi Herpesvirus (KHV) in several centers of cultivation since the mid-year of 2002 has significantly reduced the national carp production by about 40% during the period 2002-2006. Cumulatively, the economic losses because of the disease until the end of 2006 were estimated to be more than IDR 250 billion. Various control measures have been carried out, through the establishment of post outbreak response, dissemination of disease status, quarantine, seminars or workshops, the distribution of aid, etc. including entry into force of special rules through Decree of Minister of Ministry of Maritime Affairs and Fisheries No. 28/Men/2002, No. 40/Men/2002, and Regulation No. 55/Men/2004. Koi herpesvirus is a viral disease and causes fish mortality between 80%-100% of the total fish population, with an incubation period of 1-7 days. KHV infection is triggered by a decrease in environmental temperature similar to attack pattern of a cold virus. Individuals who survived in the event of an outbreak generally will become resistant to a subsequent infec-

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tion. However, the resistance is not transferred to the offspring (maternal immunity). Clinically and visually, KHV infection is often indicated by the gills necrosis, mucus excess, blisters on the skin, and undirectional movement (nervous movement) (Taukhid *et al*., 2005).

Until now, control technology to prevent KHV infection with high level of success and high suitability that can be applied for various culture systems has not available. Medically speaking, a virus infection is very difficult to control either using drugs or chemicals, and the most prospective alternative strategy to control the diseases is through ecological and biological approaches. Ecological approach can be done by exploring the environmental parameters that can block the ability of virus to replicate and reduce its virulence, whereas the biological approach can be made through the immune debriefing as early as possible, both for specific and non-specific immunity, and the use of other biological materials that potentially impede and impair the multiplication ability and virulence of the virus.

Previous research found that cases of KHV could be controlled within carp populations through the provision of vitamin C regularly conducted early before the existence of KHV infection which gives a fairly good protection levels (Taukhid & Lusiastuti, 2010 *in press*). Doses of vitamin C of 750 mg/kg feed were administered once time, every three days give a better protection level than the same elements every day or 5 days once manifested by its higher survival rate after challenge with KHV tested through exposure techniques. The survival of fish populations that were administered with vitamin C once time, every three days amounted to 50%, while the populations were given vitamin C every day for 12.5%, every fifth day by 7.5%, and without administration of vitamin C for 1.3 % (Taukhid & Lusiastuti, 2010 *in press*).

In an effort to apply these techniques to be more practical and applicable in the field, yield gaps between laboratory and field condition need to be evaluated on the techniques effectiveness which are highly influenced by various internal and external factors. The aim of this research is to evaluate the application of disease control techniques on carp infected with KHV by improving non-specific immunity through the addition of vitamin C in commercial feed.

METHODOLOGY

The research was conducted in farm level. Procedures for debriefing of non-specific antibodies against KHV in carp population were done using techniques in accordance with the best results obtained from previous research by Taukhid & Lusiastuti (2010 *in press*). Carp samples used came from a batch collected from fish farmers with sizes of ± 10 g/fish. Carp group test was produced by the hatchery that was assumed that the parent population had never been infected with KHV. Confirmation status of the disease, whether negative or positive KHV infected was conducted before the research activities took place through the detection using Polymerase Chain Reaction (PCR) based on the method developed by Gray *et al*. (2002).

Research containers used were six floating net cages (KJA), measuring 3.5 m x 3.5 m. Testing container design, the process of plotting and calculation of the tested fish can be seen in Figures 1 and 2. In each treatment group of at least one container equipped with a maximum-minimum thermometer that is resetting every 24 hours.

Fish density was 1,250 fish per cage. The treatment given was doses of vitamin C type CFC-90 (microencapsulated vitamin C) added into the commercial diet at a dose of 750 mg/kg of feed and fed to:

A. The population of common carp for every day.
B. The population of common carp every three days.

As the control population, common carp fed without the addition of vitamin C. Each treatment was repeated twice. Feed was as much as three times per day (morning, afternoon, and evening). Type of feed used was commercial diet (floating pellets) commonly used by farmers goldfish. No information about the content of vitamin C in the feed, therefore the levels of these vitamins in this research was assumed to be 0 g. Adjustment of the amount of feed (feeding program) was done every two weeks, coinciding with the course of sampling. Amount of feed given during the first 30 days totaled 10% of fish body weight per day and later reduced to 7.5% at 30 the second day, and be 5% at 30 a third day according the age of the carps.

Random sampling was done every two weeks (5 fish/replicate), to detect the exist-

Figure 1. Design of floating net cage sized 3.5 m x 3.5 m x 2.0 m

Figure 2. The process of counting individual tested fish

ence of KHV infection that was detected by PCR according to the method developed by Gray *et. al.* (2002). Haematology profile to identify indicators of non-specific immune responses through differential leucocyte counts was conducted simultaneously at the time of sampling according to the method developed by Anderson & Siwicki (1995). Observation of behavior, clinical symptoms and mortality of fish were conducted every day up to the end of the experiment including daily observation of water temperature (min-max), behavior, mortality, etc. In addition, sampling for detection of KHV by PCR was conducted every two weeks up to the end of observation.

At the fourth week of maintenance period, a challenge test through a process of artificial infection of KHV to each treatment group using the technique of cohabitation in a controlled environment (laboratory). Assurance that fish populations are the source of positively KHV infection is based on case definitions limit KHV by Taukhid *et al.* (2004), and confirmed by laboratories through PCR test according to Gray *et. al.* (2002). Weakness and excellence debriefing of non-specific immunity in the context of the emergence of KHV will be analyzed descriptively to get an alternative strategy of cultivation of carp that have a higher chance of success.

RESULTS AND DISCUSSION

The results of clinical observation and detection of KHV genes using PCR in random test of fish populations before being used as test animals obtained negative results as shown

Figure 3. Fish samples population before treatment, healthy looking, and relatively free from pathogen

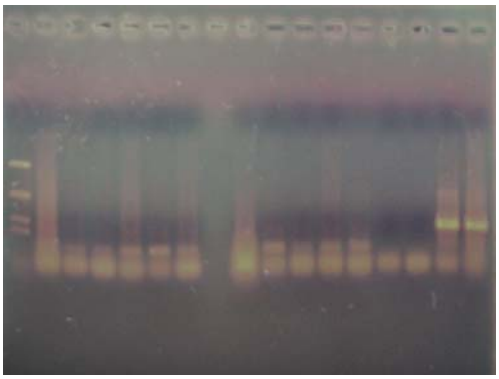

Figure 4. Electrophoresis profile of KHV detection result of the fish samples before kept in the container test, all samples showed negative results

in Figure 3 and 4. Moreover, microscopic and microbiological observations on the fish population who give negative test KHV showed that the populations in a healthy status and free from infectious pathogens, parasitic groups (external protozoa, monogenetic trematodes, and crustaceans), mycotic (*Saprolegniacea*), and bacterial (*Aeromonas hydrophila* and *Flexibacter columnaris*).

The average value of the survival of tested fish from each treatment group at the end of the maintenance period (three months) in floating net cages is presented in Table 1. The data shows that the average value of the highest survival was obtained by group B treatment (populations of common carp fed with the addition of vitamin C as much as 750 mg/kg of feed for every three days) that is equal to 60.16%, followed by group A treatment (populations of common carp fed with the addition of vitamin C as much as 750 mg/kg of feed with the frequency of every day) that is equal to 52.00%, and the average value of the lowest survival was obtained by group C treatment (carp populations fed without the addition of vitamin C) that is equal to 47.36%.

Results from the analysis of variance of survival mean values of each treatment group showed that differences in the frequency of vitamin C application gave different effects on the survival rate of farmed carp in the cage. Duncan test results showed that the average survival rate of goldfish treated every day with vitamin C (treatment A) did not differ with the treatment without vitamin C (treatment C). While the average survival of carp treated once every three days with vitamin C (treatment B) gave a different effect than the other two treatment groups, namely A and C treatments (Table 2).

Cases of fish mortality within the experimental cage started to happen in week-IX (entering month-III) with clinical symptoms and patterns of mortality were observed. Tested fish deaths occurred every day in all treated groups and continued until the end of the trial period with the percentage of deaths continued to rise. Behavior and clinical symptoms observed on the sick fish showed that mortality was mainly caused by KHV infection. Based on the observation of fish behavior, in general, fish showed signs of infection as follows: (1).

Table 1. The average value of the survival of fish at the end of the experiment at KJA from each treatment group

No.	Treatment								
	A			B			C		
	T-0	M	SR	T-0	M	SR	T-0	M	SR
1	1250	211	655/384	1250	117	680/453	1250	708	442/100
2	1250	375	645/230	1250	288	824/138	1250	194	742/314
SR 1		52.40			54.40			35.36	
SR 2		51.60			65.92			59.36	
Average		**52.00**			**60.16**			**47.36**	

Description: T-0: Initial density; M: Mortality; SR: Survival rate

Table 2. The mean survival of tested fish in each treatment group

Treatment vitamin C	Survival rate (%)	Notation
(A) Everyday	52.00	a
(B) Every 3 days	60.16	b
(C) Without vitamin C	47.36	a

Note: Values followed by same letters have no different effect according to Duncan test at 5% level of accuracy

Fish were seen gasping for breath and swim on the surface or in the direction of water flow in, then became weak and gathered near the outlet, and (2). uncoordinated movements (nervous), very slow and separated from the group of healthy fish.

On the other hand, the clinical symptoms of a sick fish showed signs of infection as follows: (1). Production of slime (mucus) excess as a physiological response to the presence of pathogens, production of mucus dropped dramatically so that the fish body was visible. (2). Pale gills and white patches or brown were visible, it then became damaged, geripis filter on the end of the gills and eventually rot (Figure 5). Microscopic observation showed the existence of serious tissue damage and severe cell death (3). Bleeding (hemorrhage) around the base and tip of fins and other body surfaces (4). Often found skin blisters (Figure 6), or even injury followed by secondary infection by bacteria, fungi, and parasites.

Based on the physical condition of the fish (the behavior and clinical symptoms) and the pattern of deaths that occurred, then the condition can be considered that a case of KHV was in progress because it was in line with the case definition developed by Taukhid *et al.* (2005) where cases of KHV in field condition can be based on three indicators, namely: that KHV infecting only carp and koi; fish pale-colored fills, there are white spots (white patches) causing damage to fish's gill after a period of time; and there was a mass mortality in a relatively short time (1-7 days).

Challenge test results conducted in a controlled environment for 14 days revealed that the highest average of the survival rate was obtained by group B treatment which was 76.67%, followed by group A treatment with 60.00%, and the lowest survival obtained in group C treatment which is equal to 18.33%.

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The results of analysis of variance of the average mortality values from each group showed that differences in the frequency of vitamin C application gave a different effect on the survival rate of fish. Duncan test results also show that the average mortality of fish in all groups depicts significant differences, either between treatments or between treatments and the control at the level of 5% (Table 3).

Survival rate of common carp fed vitamin C with the frequency of once every three days (treatment B) was higher compared to the other treatments (A and C). The high survival rate in treatment B was achieved because vitamin C application with a frequency of once every

Figure 5. Specific clinical symptoms on infected carp Koi herpesvirus (KHV): pale gills, there are white spots (white patches) continue to damage the gill, geripis at the end of the lamella and eventually rot

Figure 6. Infected tested fish with Koi herpesvirus (KHV) and secondary infection by other pathogenic groups causing necrosis of the skin

Table 3. Cumulative mortality percentage of fish samples in laboratory challenge test during 14 days observation

No.	Treatment		
	A	B	C
1	0.00	10.00	70.00
2	80.00	36.67	93.33
Average	**40.00[a]**	**23.33[b]**	**81.67[c]**

Note: Values followed by same letters have no different effect according to Duncan test at 5% level of accuracy

three days was able to stimulate the immune system of fish, so that in the event of an attack of KHV, carp can survive the infection and able to maintain the optimal condition of the body's defenses. In accordance with the statement by Irianto (2005), that administration of immunostimulant must be appropriate (not too much nor less) both for the dosage and frequency in order to provide a positive influence on the formation of immunity.

Analysis of vitamin C in the fish liver from each treatment was done at the end of the maintenance period using the method of High Performance Liquid Chromatography (HPLC). Results of the analysis showed that the highest average levels of vitamin C found in group A treatment, followed by group B treatment and lowest in group C treatment. The values of vitamin C in the liver are presented in Table 4.

Based on the value of vitamin C that are deposited in the fish liver as shown in Table 4, it can be generally stated that there is a linear relationship between frequency of vitamin C and vitamin levels in fish liver. However, these conditions generally do not show a linear relationship between high levels of vitamin C in the liver with protection ability of the body that is manifested by high levels of survival in the event of infection of KHV. It is proved by the results of the tested fish in the cage or challenge test results in the laboratory (Tables 2 and 3). This phenomenon might be aligned with the opinion by Irianto (2005) which says that the uptake of immunostimulant less than optimal vitamin C which can occur (too low or too high doses) will give an unfavorable influence on the formation of immunity. Giving vitamin C too often or exceeding the level of body defense stimulation will stimulate the kidney to perform excretion continuously. The increasing of excretion activity can worsen the health condition of fish against pathogens.

CONCLUSION

Controlling KHV disease on carp populations can be done through a regular provision of vitamin C as immunostimulant element with the correct dosages and as early as possible before the existence of KHV infection.

Provision of vitamin C on fish diets at a dosage of 750 mg/kg of feed given once every three days gives a better protection level than that of the same amount given every day.

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Table 4. Vitamin C concentration (µg/g) on fish samples liver from each group treatments

No.	Treatment		
	A	B	C
1	60.04	38.44	15.68
2	59.76	37.06	15.05
Average	**59.90**	**37.75**	**15.37**

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