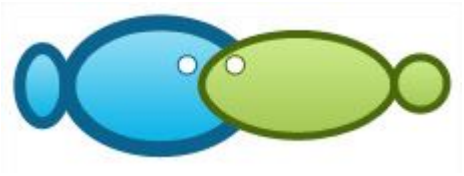

Opportunity plankton as vector transmission of koi herpes virus infection on carp (*Cyprinus carpio*)

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Abstract. Koi Herpes Virus (KHV) is a disease of carp (*Cyprinus carpio*) that is influenced by environmental conditions of water. This study aims to determine the vectors in water pond that influence the spread of KHV infection in carp. The method is experimental exploration research with descriptive analysis of water and fish sampling. KHV analysis is done by Polymerase Chain Reaction analysis. Results show the temperature, brightness, pH, dissolved oxygen (DO), CO_2, nitrate, orthophosphate, biological oxygen demand (BOD), and total organic matter (TOM) have ranges of 25-27°C, 32-33 cm, 8, 7.11-7.76 mg L^{-1}, 3.58-4.63 mg L^{-1}, 0.688-0.762 mg L^{-1}, 0.046-0.051 mg L^{-1}, 3.986-4.729 mg L^{-1}, and 11.376-13.904 mg L^{-1}, respectively. The compositions of the plankton identified in the pond were a type of phytoplankton, from Chlorophyta division with abundance value and relative abundance of 1405.84 cells L^{-1} and 63%, respectively, and the zooplankton from Arthropoda phylum had the highest abundance and the relative abundance value of 225.21 cells L^{-1} and of 71%, respectively. Phytoplankton and zooplankton dominance index are in the range 0.440 to 0.505, and 0.511 to 0.636, respectively. KHV genome on samples of plankton in the water pond and the digestive tract of carp were positively infected with KHV by 292 bp. These results indicate that the planktons are a vector transmission in spreading the KHV in carp in water horizontally.

Key Words: *Cyprinus carpio,* disease vector, KHV, PCR, plankton.

Introduction. The carp (*Cyprinus carpio*) is included in the Cyprinidae family and the order Cypriniformes, which is considered as the largest freshwater fish family (Rahman 2015). The development of carp cultivation has boomed with the various cultivation systems (Tamam 2011). However, based on data from the Directorate General of Aquaculture Indonesia, it knows that the target of increasing the production of the carp decreased. In 2013, it was known that the target of production reached 500,000 tons, while in 2014 production target decreased to 400,000 tons (Subjakto 2012). The decline in the carp production is caused by the mass deaths allegedly caused by Koi Herpes Virus (KHV). KHV produces severe infection in koi and common carp. Outbreaks of infectious diseases among freshwater fish have caused economic loss and global epizootic not only in fish aquaculture, but also in the whole ecosystem, and losses can be immense after an outbreak occurs (Minamoto et al 2015; Haetrakul et al 2018).

KHV is a disease affecting carp in all ages and spread rapidly around the world. The presence of KHV may be caused by poor environmental quality (Subjakto 2012). KHV is most lethal and highly contagious to carp, with disease signs that include skin ulcers, excess mucus, and hemorrhages in the fins (OIE 2013). Fish mortality caused by KHV are still unresolved in many cases and aspects, and require more detailed information and further research on storage, accumulation, appearance, transmission, release, and replication (Kielpinski et al 2010). The horizontal transmissions such as direct from fish to fish or indirectly via infected tissues, water, contaminated equipment or dead fish is general pathway in KHV spreading but the vertical transmission is still ruled out (EFSA 2007). A proper water quality control reduces the risk of disease in fish. KHV is directly transmitted through skin to skin contact with an infected fish to healthy fish. Some of the

factors that influence the spread of KHV are a dead fish, plankton, birds taking fish that are sick from one pond to another (Rakus et al 2013). KHV virus was accumulated through filtration of the active/passive virus inside the scuds (*Gammarus pulex*) and the swan mussels (*Anodonta cygnea*). Invertebrates such as the scuds in the water or the mussels in and on the ground seem to be able to hold the KHV virus for a long time (Kielpinski et al 2010).

Plankton organisms float in the water and serve as a natural food for fish, especially carp. Plankton, as Rotifers, may be related to the spread of the virus (EFSA 2007). It is possible that the virus adheres to the plankton, which then facilitates movement and transmission of the virus into the fish. The virus is an intracellular parasite which lives, multiplies, and only survives inside the host cells such as plankton (Nuryati et al 2013). Plankton associates with the virus when it is inactive and placed at the sediment. As a result, the carps are infected when stirring the sediment in search of food (Ciminiello & Fattorusso 2006). This study is to assign the factors that influence the spread of KHV to carp in the pond.

Material and Method. The method used in this research is descriptive survey with surveillance technique. The materials were the types of plankton that were identified in carp culture pond infected with KHV, and water qualities that will be analyzed include physics, chemistry, and biology parameters. Physical parameters include temperature and brightness; chemical parameters include pH, dissolved oxygen (DO), carbon dioxide (CO_2), nitrate (NO_3), orthophosphate (PO_4), biological oxygen demand (BOD), and total organic matter (TOM).

Fish sampling. The samples used are carps with clinical symptoms and taken at random in the cultivation of the carp in Central Java in mid-December 2016. The fish samples have a length of 27 cm with the number of samples was 10 pieces. All sampling has conducted an observation of clinical symptoms, then a sample of the flesh, brain, gills, skin, spleen, kidneys, intestines, eyes and heart were collected for analysis of virus Polymerase Chain Reaction (PCR) technique.

Water sampling. Water quality parameters were observed: brightness, temperature, pH, DO, CO_2, NO_3, PO_4, BOD_5, and TOM. Monitoring of water quality is done as much as three repetitions with a sampling interval a week. Water samples were taken using the Winkler bottle (600 mL).

Plankton sampling. Plankton samples are taken at fish cultivation pond in East Java. Sampling is done by filtering as much as 25 L of pond water using a plankton net number 25 then put into film bottles and preserved with Lugol (3 drops). Then, it is identified and counted its abundance, relative abundance, diversity index, and the index of the dominance of plankton as shown in the following equations (1), (2), (3), and (4), respectively:

a. Abundance (N) (APHA 2005):

$$N = \frac{T \times V}{L \times v \times p \times W} \times n \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \quad (1)$$

Where:
N = the total number of phytoplankton (ind L^{-1});
n = numbers of phytoplankton in each range of sight;
T = cover glass width (20 x 20 mm);
L = width of the range of sight (πr^2 mm^2);
V = volume of plankton in the film bottle;
v = Volume of one drop of sample water;
p = number of viewing field;
W = filtered water volume (L).

b. Relative abundance (Venrick 1978):

$$KR = \frac{ni}{N} \times 100\% \quad \text{..... (2)}$$

Where:
KR = relative abundance;
ni = number of each species;
N = number of all species.

c. Diversity Index was determined following Shannon-Wiener's Index using the formula (Ludwig & Reynolds 1988):

$$H' = -\sum^{s} pi \log_2 pi \longrightarrow pi = \frac{ni}{N} \quad \text{.....(3)}$$

Where:
H' = Diversity index;
ni = number of each species;
N = number of all species;
s = number of species in the sample;
pi = the proportion of individuals of species i from the total number of individuals (Pi = ni/N).

d. Domination Index (D) (Venrick 1978):

$$D = \Sigma (pi)^2 \quad \text{.....(4)}$$

Where:
D = Domination Index;
pi = relative abundance.

PCR analysis. This method is a standard in the research laboratory test that uses DNA (Joshi & Deshpande 2010). Carp organs tested using PCR are flesh, brain, gills, skin, spleen, kidneys, intestines, eyes and heart. These organs are in isolation and tested in accordance with the procedure of available PCR.

The PCR process was conducted by the protocol of the kit until reaching a final primer concentration of 2.5 µM. PCR reactions were conducted using a thermal cycler (GeneAmp® PCR System 9700, Applied Biosystems). Specific F2 primer Forward F292: 5'-GAC-ACC-ACA-TCT-GCA-AGG-AG-3' and Reverse R292: 5'-GAC-ACA-TGT-TAC-AAT GGT-CGC-3'. PCR cycle conditions follow the kit protocols. In order to verify the presence and the correct size of the PCR products, electrophoresis on agarose gel was performed.

Result and Discussion

The carp morphology. The carp sample obtained from cultivation pond was shown in Figure 1. The incubation period of the virus inside the host ranges between 7 and 10 days before the onset of clinical symptoms (Rathore et al 2012). Fish samples taken for testing the presence of KHV has characteristics that normally swim, normal body color, unsunken eyes, and normal head shape. The outer appearance of a sample of this carp looks normal and fine.

The criteria of KHV infection on the fish were the light infection attacks (head and eyes are normal, gills are not white, the skin is not experiencing hemorrhagic lesions). The results of the PCR test showed the band of 290 bp (Masri 2013). The second criteria is the moderate infection attack (head and eyes are normal, gills are white, body skin is hemorrhagic or erosion of the skin color, the test results showed two bands, which are 290 bp and 440 bp. The third criterion is severe infection attack (gills are white, eyes are

sunken into the head lesions, skin is experiencing hemorrhagic or erosion of the skin color, the test results show three bands which are 290 bp, 440 bp, and 630 bp.

Figure 1. Morphology of carp sample infected with KHV.

Analysis of water quality measurement. Analysis of water quality measurements in the carp pond is shown in Table 1. It is known that the maintenance of water quality in the pond was classified as optimal and meet the quality standards to support the life of organisms like fish and plankton. However, the BOD_5 value in a carp fish pond is relatively mild contaminated.

Table 1

Water quality parameters in the carp cultivation pond

No	*Parameter*	*Unit*	*Week of observation*			*Quality standard*
			1st	*2nd*	*3rd*	
1	Temperature	^oC	26	27	25	25-30 (*)
2	Brightness	cm	32.6	33	32	> 30 (*)
3	pH	-	8	8	8	6.5-8.5 (*)
4	DO	mg L^{-1}	7.43	7.76	7.11	≥ 3 (*)
5	CO_2	mg L^{-1}	3.58	4.63	4.59	2-9 (**)
6	NO_3	mg L^{-1}	0.703	0.762	0.688	10 (**)
7	PO_4	mg L^{-1}	0.051	0.047	0.046	≤0.2 (**)
8	BOD_5	mg L^{-1}	3.986	4.662	4.729	3.0-4.9 (***)
9	TOM	mg L^{-1}	12.640	13.904	11.376	≤ 50 (**)

Notes: (*) SNI (2000); (**) Government of Republic Indonesia (2001); (***) EPA (2010).

Measurement of water quality in this study includes physical, chemical and biological parameters. Aquatic organisms including fish and plankton are strongly influenced by environmental conditions (physics, chemistry, and biology) in the vicinity. Temperature is related to the entry of sunlight into the water which will increase the water temperature but varies in value depending on the depth of water and sunlight intensity. The temperature of the pond was measured in a range from 25 to 27°C. The water pond has brightness in the range from 32 to 33 cm. There was a decrease in the brightness by the third week of observation due to the water inlet makes the water level in the pond increases and tends to be cloudy. A high level of brightness in the waters is very useful for phytoplankton in conducting photosynthesis so it can grow well (Radiarta 2013). pH strongly determines the dominance of phytoplankton in the waters so that its presence can act as a limiting factor in the waters. Observation results indicate that the pH of water is eight in all observations. This pH value was in the optimal condition (pH from 6.5 to 8.5) to support the growth of carp (SNI 2015). The DO was measured in a range from

7.11 to 7.76 mg L^{-1}. The main source of oxygen in water is the photosynthesis process. The more fertile water, the more phytoplankton will grow and increase the concentration of DO in the water. Low DO content is caused by higher respiration activity than photosynthesis (Effendi 2003).

Based on the Table 1, CO_2 value was in a range from 3.58 to 4.63 mg L^{-1} that indicates that water was in the normal and suitable state for the carp life. The optimal CO_2 levels for freshwater should contain levels less than 5 mg L^{-1}. The use of CO_2 is directly related to photosynthesis. CO_2 is needed by phytoplankton to carry out photosynthesis (Prasetyaningtyas et al 2012). The NO_3 concentration has a high value in a range from 0.688 to 0.762 mg L^{-1}. It can occur due to ammonia oxidation resulting from the high domestic waste in the water. The pond is located close to the residential and agricultural areas which make the amount of nitrate become higher. Nitrogen is one of the important elements in the formation of protein in the organism. Nitrates are the main form of nitrogen in natural waters and are the main nutrients for the growth of plants and algae (Effendi 2003). The PO_4 concentration was observed in a range from 0.046 to 0.051 mg L^{-1}. The abundance of both nitrate and orthophosphate content in the waters will stimulate phytoplankton growth. When the number of phytoplankton increases and the intensity of sunlight can penetrate the body of water, the photosynthesis process will run optimally and increase DO and organic biomass that are indispensable to aquatic organisms (Bayurini 2006). The value of BOD_5 is between 3.986 and 4.729 mg L^{-1}. The BOD_5 value indicates the level of organic matter in water because the BOD_5 value is a parameter that indicates the oxygen demand by aerobic bacteria to oxidize organic matter in water and can be used as an indication of pollution (Yang et al 2009). Based on the results of these measurements, it is known that BOD_5 of a tilapia pond is classified as lightly polluted. In natural waters, dead plants and animals act as sources of organic matter. In addition, the domestic and industrial waste also affect the BOD_5 value (Effendi 2003). The TOM was observed in the range from 11.376 to 13.904 mg L^{-1}. The TOM naturally comes from the water itself through the decay or decomposition of plants, the dead organisms, and the waste or food leftovers which in the presence of bacteria decomposed into nutrients (Marwan et al 2015).

Identification and abundance of plankton. The composition of the phytoplankton obtained from the identification consists of the division of Chlorophyta (genera *Pediastrum*, *Netrium*), Charophya (genus *Mougeotiopsis*), Bacillariophyta (genera *Melosira*, *Navicula*, and *Nitzschia*), and Cyanophyta (genus *Merismopedia*).

The zooplankton identified belongs to phylum Rotifera (genus *Brachionus*), phylum Arthropoda (genera *Nauplius* and *Calanus*). The high abundance of plankton in the waters indicates fertile waters and vice versa. The result of the calculation of the abundance of phytoplankton and zooplankton in the carp cultivation pond was shown in Table 2. The highest abundance of phytoplankton of Chlorophyta division was in the second week,with 1405.84 cells L^{-1} and 63% relative abundance. Chlorophyta grows well in the environmental conditions that contain quite high nitrates and phosphates. The result is consistent with the conditions in the carp cultivation pond with nitrate values ranged from 0.688 to 0.762 mg L^{-1}. According to the Table 2, the highest abundance of zooplankton is in the 3rd week, with 225.1 cells L^{-1} of the phylum Arthropoda and a relative abundance of 71%. The water of the carp cultivation pond can be indicated as eutrophic.

Table 2

Abundance, Diversity and Domination Index of plankton in the carp cultivation ponds

Division/Phylum	*Week*								
	1st			*2nd*			*3rd*		
	N (ind L^{-1})	*-Pi ln Pi*	*D*	*N (ind L^{-1})*	*-Pi ln Pi*	*D*	*N (ind L^{-1})*	*-Pi ln Pi*	*D*
Phytoplankton									
Chlorophyta	189.65	0.310	0.351	1405.84	0.29	0.39	782.30	0.300	0.380
Charophyta	82.97	0.350	0.067	0.00	0.00	0.00	23.71	0.074	0.000
Bacillariophyta	47.41	0.283	0.022	71.12	0.137	0.001	82.97	0.223	0.002
Cyanophyta	0	0	0	758.60	0.367	0.115	379.30	0.361	0.089
Total	320.03	0.943*	0.440**	2235.56	0.794*	0.506**	1268.28	0.958*	0.471**
Zooplankton									
Rotifera	35.56	0.363	0.184	23.71	0.358	0.082	71.12	0.343	0.058
Arthropoda	47.41	0.000	0.327	59.27	0.000	0.510	225.21	0.209	0.578
Total	82.97	0.363*	0.511**	82.98	0.358*	0.592**	296.33	0.552*	0.636**

Notes: * Diversity index value (H'); ** Domination index value (D).

Diversity index. Diversity index of plankton in the water can determine the fertility of water. Plankton with high diversity shows that the environment is suitable for plankton. When H' < 1 the community biota is unstable; if H' ranges from 1 to 3, the stability of the community of organisms is moderate and if H' > 3, the biota community is in stable condition (Sari et al 2014).

Analysis of phytoplankton diversity index gained from the first, second, and third week is equal to 0.943, 0.794, and 0.958, respectively (Table 2). In the analysis of zooplankton diversity index, it is 0.363 for the first week. While in the second and the third week is 0.358 and 0.552 respectively (Table 2). It is known that for the first and second weeks, the zooplankton diversity is relatively low while in the third week, the diversity is moderate.

Domination index. In Simpson dominance index value, when the dominance index is close to 1, there are certain species dominating the water, while if the index value of the dominance is close to 0, then no species predominates in the water. Table 2 shows the domination index of phytoplankton and zooplankton on the first, second, and third week are 0.440, 0.506 and 0.471, and 0.511, 0.592 and 0.636 respectively. Domination index results obtained from carp cultivation pond belong to the dominance that is closer to 1 so that the community of plankton in carp pond is still in stable condition. A division of Chlorophyta in water is relatively higher when compared to the other division for optimal water conditions with sufficient brightness to support the growth of Chlorophyta. The results of brightness in the carp cultivation pond are ranging between 32-33 cm, while based on the quality standard of good cultivation, the brightness reaches a value of > 30 cm.

Water quality status and the presence of KHV. KHV disease spread by direct contact between infected fish and normal fish, water contamination, and handling as well as the environmental change of temperature fluctuations. Extreme changes in water temperature and poor water quality conditions will cause the immune system of fish to become low, and KHV will so easily infect it. Temperature also has a role in the process of multiplying (replication) of the virus (Zhang et al 2012). Occasionally, this environmental parameter alone can cause a high mortality (Raja & Jithendran 2015). Viruses can only live on vulnerable cells and unwittingly spread through infected fish into the aquatic environment (Matsui et al 2008). Water temperature is one of the main environmental factors affecting the initial spread of viral infections in fish (Rakus et al 2013). Depending on the temperature of the water, fish exposed to the virus have the potential to become infected, develop into diseases and die, or those who survive the early infection will become carriers of the virus (Eide et al 2011).

KHV disease spread at a temperature ranging between 17 and 28°C (Ilouze et al 2006). At temperatures below 13°C or above 30°C, KHV alter to be a dormant phase and clinical signs generally suspend (Cheng et al 2011). The observation of temperature in the carp cultivation pond is ranging between 25 and 27°C. Temperature also affects the replication of KHV which infect host cells.

Besides the temperature, another factor that can affect the spread of KHV in carp is pH. The phase of virus infection occurs in the pH range of less than 3 or more than 11 (Neukirch 2003). The observation of pH in the carp cultivation pond is 8.

Our result suggests that DO values in ponds are in accordance with water quality standards that can be tolerated by aquatic organisms with a standard value of > 3 mg L^{-1} (SNI 2015). Even though DO conditions in ponds are normal, this condition does not improve the health status of fish that have been infected with KHV, because fish are unable to absorb oxygen due to gill damage by infection from KHV (Hedrick et al 2000; OATA 2001).

CO_2 concentrations in carp fish ponds showed values ranging from 3.58 to 4.63 mg L^{-1}. The CO_2 concentration is still classified as normal with a standard value between 2-9 mg L^{-1} (SNI 2015). Under normal conditions, fish health is not affected by CO_2 concentration, but at high CO_2 concentrations, fish biochemistry will be affected, causing

fish to experience environmental stress (Treanor et al 2017). Environmental stress is a trigger for virus attacks in fish (Haenen et al 2016).

Based on Table 1, NO_3 concentrations ranged from 0.688 to 0.762 mg L^{-1}. These values indicate that the NO_3 is at the normal water threshold value (≤10 mg L^{-1}). Normal NO_3 in the water supports the growth of fish and provides sufficient nutrients for pond fertility. However, nitrates with high concentrations will become toxins for fish leading to fish become weak and change in swimming behavior (Davidson et al 2014) but nitrate concentrations do not affect replication of the virus (Inendino et al 2005).

The PO_4 concentration shows normal conditions with values ranging from 0.046 to 0.051 mg L^{-1}. PO_4 is not a toxin for fish (Kim et al 2013), but it is useful as a nutrient used to support the life of plankton in ponds and also the increase in phosphorus can reduce viral proliferation (Maat et al 2016).

Another cause of KHV spread in ponds is through feces and secretion of virus particles in water (Michel et al 2010). Viruses that are spread through the feces will accumulate in the sediments. The accumulation of feces increases the content of organic matter in the waters, resulting in an increase in waters biological activity. Based on the results of the BOD_5 measurement, the values are 3.986-4.729 mg L^{-1}.

Based on Table 1, the TOM value ranged from 11.376 to 13.904 mg L^{-1} so that the pool has a normal TOM value (<50 mg L^{-1}). In accordance with TOM, it is assumed that zooplankton serves as virus transmitter which the organism infected with KHV will die and settle to the bottom. The zooplankton has a filter feeder nature, zooplankton that filter food from the base indicated will carry the virus in their body and zooplankton will be eaten by fish so that the organic material becomes a transfer of KHV to higher trophic levels (Honjo et al 2010).

Indication of the presence of plankton and KHV in carp cultivation pond. PCR test results showed that DNA virus can not be detected in the skin, tissue, brain, but detected on the plankton in the gastrointestinal tract, as shown in Figure 2, Figure 3, Figure 4, and Figure 5, respectively.

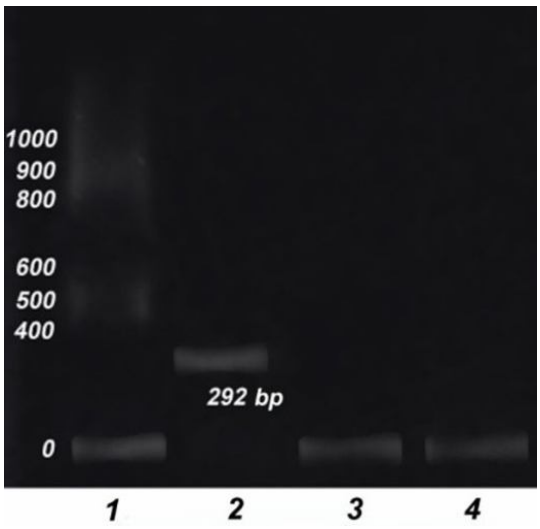

Figure 2. Amplification genome of KHV-infected carp skin sample: (1) marker 1 kb; (2) positive control of KHV 292 bp; (3) negative control; (4) negative KHV sample.

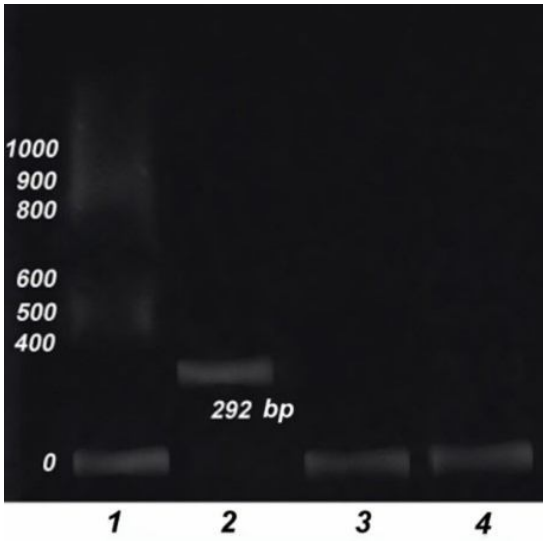

Figure 3. Amplification genome of KHV-infected carp tissue sample: (1) marker 1 kb; (2) positive control of KHV 292 bp; (3) negative control; (4) negative KHV sample.

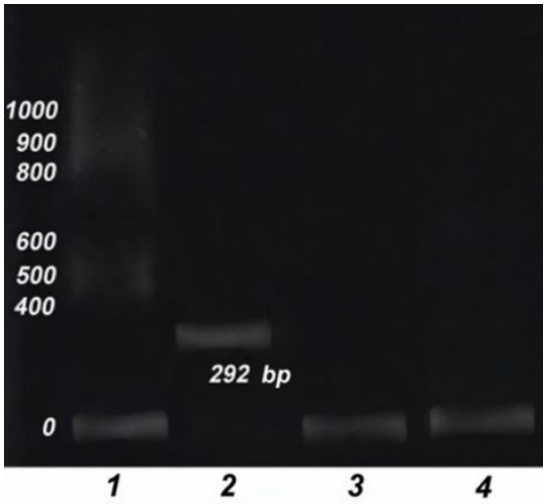

Figure 4. Amplification genome of KHV-infected carp brain sample: (1) marker 1 kb; (2) positive control of KHV 292 bp; (3) negative control; (4) negative KHV sample.

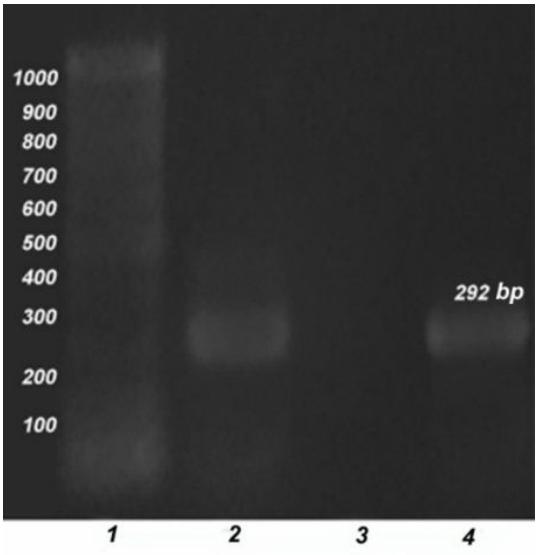

Figure 5. Amplification genome of plankton in carp digestive tract: (1) marker 1 kb; (2) positive control of KHV 292 bp; (3) negative control; (4) positive KHV sample.

Mode of KHV transmission is horizontal, directly from infected fish or through the contaminated water to susceptible fish (Rathore et al 2012). The pattern of the spread of the disease is influenced by temperature of water, the concentration of the virus, the condition and age of the fish, stress factors and fish density (OIE 2016). Some of the potential KHV virus vectors for indirectly distributing is through a dead fish, plankton, sediment, aquatic invertebrates that filter water, and water as the main abiotic vector (Boutier et al 2015). The spread of KHV caused by plankton can occur through plankton predation by carp. KHV can be proved in the plankton on infected fish. The observation of phytoplankton and zooplankton showed that there is a relationship between Rotifera (zooplankton) and the virus. The carp will be infected with the virus because it stirred the bottom of the pond as part of their eating habits (Minamoto et al 2011).

The digestive tract of infected carp contains the plankton with the composition from the division of Chlorophya (genera *Pediastrum,* and *Netrium*) Charophyta (genus *Mougeotiopsis*), Bacillariophyta (genera *Navicula, Melosira,* and *Nitzschia*) and Cyanophyta (genus *Merismopedia*). It also contains zooplankton from phylum Rotifera (genus *Brachionus*), and phylum Arthropoda (genera *Nauplius* and *Calanus*). As shown in Figure 5, positive KHV sample was obtained from the plankton in carp digestion. Allegedly, KHV becomes associated with plankton and can be potentially involved in viral transmission. Proteins that have been encoded by the genetic information in the DNA of the virus will be used to construct the body of the virus. Protein coding sequence contained several genes in KHV is ribonucleotide reductase (RNR), thymidine kinase (TK) and the present form of serine obtained from additional genes through horizontal gene transfer (Donohoe 2013). The virus is indicated by the presence of proteins in the plankton body. In general, the virus consists of either a nucleic acid DNA or RNA (but not both) and a protein layer. Viruses cannot synthesize proteins so for the body's shaping material, the virus requires amino acids and nucleotides (Davidson 2015). Amino acids were observed from the genera *Pediastrum, Melosira, Navicula, Nitzschia* (phytoplankton) and genera *Brachionus* and *Calanus* (zooplankton). The viral membrane formation indicates the serine amino acid used in its body. Our results suggest that phytoplankton and zooplankton act as a transmission vector to spread KHV horizontally.

Conclusions. Result show that the water environment in fish culture was indicated in normal condition. Fish's genome analysis using PCR with specific primers indicate that the organs of the carps have positive infected with KHV with DNA molecule of 292 bp. However, PCR analysis of the plankton in the digestive tract found that plankton contains the KHV virus. The plankton has a role in spreading of KHV infection in the carp.

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