Short communication

Fins Are an Applicable Organ for PCR-based Diagnosis of Koi Herpesvirus Disease in Clinical Fish

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ABSTRACT—We investigated the applicable organ to detect DNA of cyprinid herpesvirus 3 (CyHV-3), the causative agent of koi herpesvirus disease (KHVD). CyHV-3 DNA was detected at the highest positive rate in the caudal fin among several organs including gills, kidney and spleen of the fish sequentially sampled after experimental exposure to the virus. Moreover, CyHV-3 DNA was detected in the caudal and pectoral fins of all dead fish collected in a natural KHVD case. Since fins are an easily accessible organ, they can be an applicable organ for PCR-based diagnosis of KHVD in clinical fish.

Key words: fin, cyprinid herpesvirus 3, KHV, detection, carp

The koi herpesvirus disease (KHVD) has spread to many countries worldwide, with infections reported in at least 28 countries (OIE, 2013) since it was initially reported in 1998. High mortalities of adult common carp (frequently over 30 cm of total length and 0.8 kg) by KHVD have been observed in several cases (Hedrick *et al.*, 2000; Sano *et al.*, 2004; Grimmer *et al.*, 2006; Mabuchi *et al.*, 2008). In the OIE manual of KHVD, the gill, kidney and spleen tissues are recommended for detection of the causative virus, cyprinid her-

pesvirus 3 (CyHV-3), by PCR assay (OIE, 2013). However, the gills of dead fish are readily degenerate to be in a state of advanced tissue decay and sampling representative tissues by dissection from dead fish of more than 30 cm in total length in a putrid state is not easy.

The major portal of entry of CyHV-3 in common carp is the skin (Costes *et al.*, 2009) and CyHV-3 DNA has been detected by real-time PCR from the mucus of carp exposed to the virus (Gilad *et al.*, 2004). These previous reports suggest that CyHV-3 exists in the epithelium cells. Furthermore, Ito *et al.* (2014) reported that CyHV-3 DNA was detected from the caudal fin of all dead fish and the detection rate of the virus DNA from the caudal fin of fish surviving in all infected groups were significantly higher compared with the gill and kidney as the results of experimental infection trials. The results of the study suggest the possibility that fins are an applicable as a detection site of CyHV-3 DNA. The previous study, however, examined only fin, gills, kidney and brain of the experimental infected fish. The organs such as spleen should be compared with fins in the detection of the viral DNA, and also examination of the fish in a natural KHVD case should be done. Therefore the objective of the present study is to clarify the organs with the highest probability for the sequential detection of the viral DNA from experimentally infected fish, and to confirm the detection of viral DNA from the fins of dead fish sampled in the field.

Materials and Methods

Cell line and virus

The koi fin (KF-1) cell line (Hedrick *et al.*, 2000) was used in this study. Cultures of this cell line were maintained in minimum essential medium (Mediatech) supplemented with 10% fetal bovine serum (Equitech-Bio) and antibiotics (penicillin and streptomycin: Life Technologies) at 25°C.

The virus used for the experimental infection was KHV 0308 isolate from diseased common carp in Japan in 2003 (Yuasa *et al.*, 2008). This isolate was propagated in KF-1 cells in 25 cm² flasks (Greiner Bio-one) at 20°C and the cell culture supernatant containing CyHV-3 was stored at –85°C until use as an inoculum.

Experimental infection and sequential sampling

Juvenile common carp were transported from a carp farm with no history of CyHV-3 infection in Kumamoto Prefecture to the Tamaki Laboratory, National Research Institute of Aquaculture. After transportation, these fish were maintained in groundwater to prevent, as far as possible, any infection by pathogens. Thirty-six common carp individuals (mean of total length, 9.51 cm; mean of body weight, 11.7 g) were divided into six groups of six fish each. Fish of five groups were immersed for 1 h in the virus culture supernatant (10^{3.5}

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TCID₅₀/mL) diluted at 1:1000 with groundwater. One of the five groups served as the group for mortality observations. The remaining four groups served as sample groups for sequential detection of CyHV-3. The last group was immersed similarly in diluted cell culture medium but without the virus as a negative control. The initial density of the groups for sampling was set as the same as the group for mortality observations, although surviving fish were randomly sampled after virus exposure. All experimental groups were kept in 60 L tanks of continuously flowing water (0.6 L/min) at 23.5°C (23.2–23.8°C) and fed the commercial diet once a day. The cumulative mortality was observed over a period of 28 days. The caudal fin and gills of all individual fish from groups for mortality observations were tested for CyHV-3 DNA by PCR.

To investigate the occurrence of the CyHV-3 DNA among the organs after infection, the immersed KHV 0308 fish were sampled periodically at 1, 4, 7, 9, 10, 11, 13, 14, 19, 22 and 28 days post-exposure (dpe) to the virus, and the caudal fin, gill, heart, intestine, spleen, brain, kidney and hepatopancreas of two or three of the surviving fish from the 4 tanks were randomly sampled and all dead individuals were tested for CyHV-3 DNA by PCR. Surviving fish were sacrificed, and then the occurrence of the viral DNA from the caudal fin, gill, heart, intestine, spleen, brain, kidney and hepatopancreas were tested by PCR. These organs from all dead individuals from the sample groups were likewise tested for CyHV-3 DNA.

Samples from KHVD outbreak case in a river

For detection of CyHV-3 from fish collected in a natural KHVD case, the caudal fin, pectoral fin and gill were collected from 30 dead individual common carp caused by CyHV-3 infection in the Asakumi River (lat. 35° 28' N and long. 133° 4' E), Shimane Prefecture, Japan in May 2005. All collected samples were tested for CyHV-3 DNA by PCR.

PCR detection of CyHV-3 DNA

For samples of each organ of the experimentally infected fish and dead fish from natural waters, the total DNA was extracted from tissues (approximately 20 mg each) using Gentra Puregene Tissue Kit (Qiagen). For the specific detection of CyHV-3, a PCR assay was employed (Gilad *et al.*, 2002). The PCR reaction was performed with *TaKaRa Ex Taq*® Hot Start Version (TaKaRa) according to the manufacturer's protocol.

Results and Discussion

Cumulative mortality rates of experimentally infected and control fish and results of CyHV-3 DNA detection by PCR of the mortality observation groups are shown in Table 1 and Fig. 1. The mortality rate in

Table 1. Results of cumulative mortality in infected and control groups for mortality observations and detection of CyHV-3 DNA by PCR from the experimental fish

Group	Cumulative mortality (%)	PCR detection for CyHV-3			
		Dead fish		Surviving fish	
		Caudal fin	Gill	Caudal fin	Gill
Infected group	100	6/6*	6/6	–	–
Control group	0	–	–	0/6	0/6

*Number of positive fish / number of tested fish.

Fig. 1. Cumulative survival curves of common carp; (●) Common carp infected by immersion with KHV 0308 isolate, (○) Negative control common carp.

the mortality observation group immersed with KHV-0308 isolate was 100% at 15 dpe. CyHV-3 DNA was detected in the caudal fin and gills of all infected dead fish. There was no mortality in the negative control and viral DNA was not detected in the caudal fin and gill of the negative control fish.

The sequential detection of CyHV-3 DNA by PCR from the caudal fin, gill, heart, intestine, spleen, brain, kidney and hepatopancreas of the infected common carp sampled at certain intervals is shown in Table 2. CyHV-3 DNA was detected in two of the three fish from the caudal fin and gill, and caudal fin and spleen on 1 dpe from sub-clinical fish. On 4 dpe, one fish showed fin congestion and the viral DNA was detected in all three fish from almost all tested organs but not commonly from the brain. The viral DNA was detected in all tested organs of the dead or moribund fish during 7 dpe to 11 dpe. On 13, 14 and 22 dpe, the viral DNA was not detected in some tested organs. The DNA was not detected from all of the tested organs of two surviving fish on 22 dpe. On 28 dpe which was the final day of the infection trial, CyHV-3 DNA was detected from the caudal fin, gill, heart, intestine, spleen and kidney of one of the two fish, and was detected from the brain of the other fish.

Common carp was the only fish species involved in this mortality outbreak in the Asakumi River. The

mean total length was 57.3 cm (ranging from 42.5 to 70.0 cm) and the sampled fish consisted of seven females, 22 males and one individual that the gender was unclear due to decay of the internal organs. Water temperature at the sampling point was 18.8°C. Many adult size common carp died, and then they floated on the surface of the river. The decay of the internal organs of all collected dead fish was advanced and these fish had a strong putrid smell. CyHV-3 DNA was detected in the caudal fin, pectoral fin and gills of all collected dead fish.

CyHV-3 DNA was detected in the caudal fin of 20 of 24 samples consisting of surviving and dead fish in the infection trial (Table 2). As a result, the caudal fin had the highest detection rate of the viral DNA among the tested organs including spleen from the early stage through convalescence of the KHVD. Ito *et al.* (2014) reported that CyHV-3 DNA was detected with a high positive rate from the caudal fin and brain compared with gill and kidney from surviving fish. In this study, however, CyHV-3 DNA was only detected in the brain of one of four surviving fish. This difference might be caused by the number of the samples since the previous result was obtained from more than 100 survivors.

At present the gills have been mainly employed for the diagnosis of KHVD in Japan. However, the gills of

dead fish are readily degenerate to be in a state of advanced tissue decay and sampling representative tissues by dissection from dead fish of more than 30 cm in total length in a putrid state is not easy. In addition, the dissection may lead to the risk of viral contamination at the sampling field sites. In this study, CyHV-3 DNA was detected in the caudal and pectoral fin of all sampled dead fish from the field. Since fins can be easily collected using tweezers and scissors, the fins are more applicable than the gills and internal organs for detection of CyHV-3 DNA from infected dead fish. Eide *et al.* (2011) reported that CyHV-3 DNA was detected in gill swabs by day 8 following temperature stress. In this sense, for harvest of the epidermal cells of fins, the swab method may be available for fins sampled in the field as a more convenient method, although the present study used tweezers and scissors. Evaluation of the fin swab method for the detection of CyHV-3 from the diseased fish is necessary as a future study.

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Table 2. Detection of viral DNA from common carp experimentally exposed to CyHV-3

Days post exposure	Sample no.	Disease stage	Organ							
			Caudal fin	Gill	Heart	Intestine	Spleen	Brain	Kidney	Hepatopancreas
1	1	Sub-clinical	–	–	–	–	–	–	–	–
	2	Sub-clinical	++	++	–	–	–	–	–	–
	3	Sub-clinical	++	–	–	–	++	–	–	–
4	1	Sub-clinical	++	++	–	+	–	–	+	–
	2	Clinical (fin congestion)	++	++	+	+	++	–	++	–
	3	Sub-clinical	++	++	+	+	+	–	+	++
7	1	Sub-clinical	+++	+++	+++	+++	+++	++	+++	++
	2	Clinical (fin congestion)	+++	+++	+++	+++	+++	++	+++	+++
	3	Sub-clinical	+++	+++	+++	+++	+++	+++	+++	+++
9	1	Dead	++	++	+	++	++	+	++	++
10	1	Dead	+++	+++	++	+++	+++	+++	+++	+++
11	1	Dead	++	++	++	++	++	++	++	++
	2	Moribund	++	++	++	++	++	+	++	++
	3	Dead	++	++	++	++	++	++	++	++
13	1	Dead	++	++	+	+	++	–	++	+
14	1	Dead	++	++	–	–	–	+	++	–
	2	Dead	+++	+++	+++	+++	++	+++	+++	+++
	3	Dead	++	++	++	++	+	+	++	+
19	1	Dead	+	–	–	++	+	+	++	–
	2	Dead	++	–	–	–	–	–	+	–
22	1	Surviving	–	–	–	–	–	–	–	–
	2	Surviving	–	–	–	–	–	–	–	–
28	1	Surviving	++	++	+	+	+	–	++	–
	2	Surviving	–	–	–	–	–	++	–	–

–; negative, +; positive (number of plus marks (+) indicate the brightness and density of the bands after electrophoresis)

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