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Detection of cyprinid herpesvirus-3 DNA in lake plankton

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ABSTRACT

The disease caused by cyprinid herpesvirus-3 (CyHV-3) severely impacts the natural freshwater ecosystem and damages carp and koi farming, however, the pathway of CyHV-3 transmission remains unclear. It is possible that the virus adheres to plankton, which then facilitate viral movement and transmission, and therefore, it is hypothesised that plankton are involved in the disease dynamics. In this study, plankton were collected at eight sites in the Iba-naiko lagoon; we detected and quantified CyHV-3 DNA from plankton samples. The results of the correlation analysis showed a significant positive correlation between CyHV-3 copies and the number of Rotifera, suggesting that CyHV-3 binds to and/or is concentrated by Rotifera. Our results suggest that plankton affect viral ecology in the natural environment.

A newly emerging fatal disease of common carp (*Cyprinus carpio carpio*) and koi (*Cyprinus carpio koi*) is known to be caused by cyprinid herpesvirus-3 (CyHV-3). This disease was formerly known as koi herpesvirus (KHV) or carp interstitial nephritis and gill necrosis virus (CNGV). CyHV-3 was first reported in the late 1990s and has subsequently spread worldwide (Hedrick et al., 2000). It has been thought that CyHV-3 infects only carp, however, recently it was reported that other species are infected with this disease (El-Matbouli et al., 2007; Sadler et al., 2008). In some laboratory analyses, more than 80% of infected fish died (Ronen et al., 2003), and this highly contagious and virulent disease severely impacts the natural freshwater ecosystem and damages carp and koi farming.

CyHV-3 belongs to the family *Alloherpesviridae*, and its genome consists of 295-kbp double-stranded DNA (Aoki et al., 2007). CyHV-3 DNA has been detected in the droppings and organs (e.g., gills and brain) of infected fish (Dishon et al., 2005). Furthermore, viral DNA has been detected in water from the surrounding habitat (i.e., environmental water) before, during, and after an outbreak of the disease (Haramoto et al., 2007; Minamoto et al., 2009b). Although it is known that the skin is the major access point for CyHV-3 (Costes et al., 2009), the pathway of CyHV-3 transmission remains unclear.

It is possible that the virus adheres to plankton, which then facilitate viral movement and transmission. Therefore, it is hypothesised that plankton are involved in the disease dynamics of CyHV-3. In this study, we attempted to detect CyHV-3 DNA in plankton collected from Iba-naiko, a lagoon connected to Lake Biwa in Japan, where mass mortality due to CyHV-3 disease was observed in 2004.

Iba-naiko is a muddy-bottomed, shallow lagoon with 49 ha of open water area, which is connected to Lake Biwa by a canal (Fig. 1). The maximum water depth is approximately 3 m and nearly all sections of the lake are shallower than 1.5 m. The lagoon is used by common carp as a feeding and reproductive habitat. A previous study reported that 54% of adult carp (>300 mm in length) caught in this lagoon and the neighbor area show antibody-positiveness, and that infection and transmission of CyHV-3 among natural carp population are still occurring through reproductive behavior of carp (Uchii et al., 2009).

Surface water and plankton were collected on 14 November 2008 at eight sites in the lagoon (Fig. 1). Water quality parameters were measured on site using a multi-parameter water quality sonde (6600EDS; YSI, Yellow Springs, OH, USA). Total plankton were collected from surface water using a NXX17 plankton net (average pore size, 72 μm; Rigo, Tokyo, Japan). For identification of zooplankton and phytoplankton, fresh and fixed (2% Lugol's solution) plankton samples were transported to the laboratory at 4 °C, and plankton were identified under an optical microscope. Prior to further processing, the plankton samples were not washed to prevent dissociation of virus and plankton. For DNA extraction, 25 ml of plankton samples were fixed on site with 50% ethanol and transported to the laboratory. Total DNA was extracted using proteinase K and SDS, and purified using the phenol–chloroform method (Sambrook and Russell, 2001); 500 μl of total DNA was

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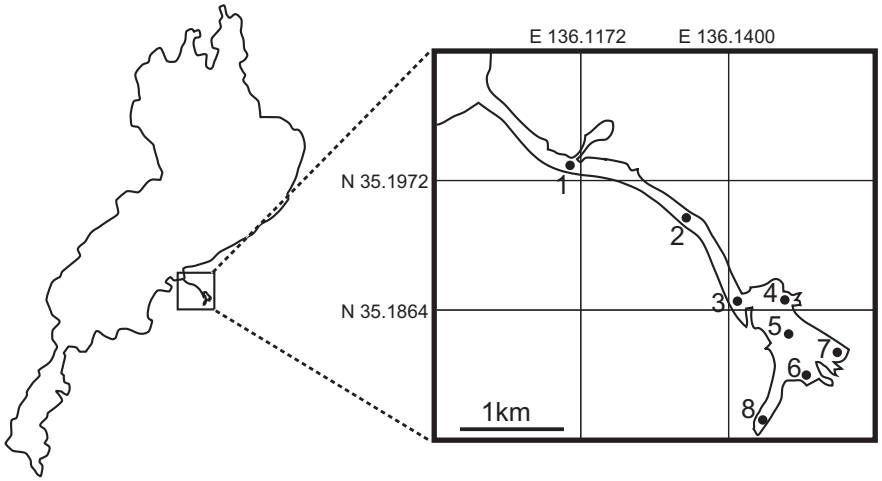

Fig. 1. Study sites. Iba-naiko is a lagoon connected to Lake Biwa by a canal. Surface water and plankton samples were collected at the eight sites indicated by closed circles.

obtained. The DNeasy Blood and Tissue kit (Qiagen, Hilden, Germany) was used to further purify 100 μl DNA solution according to a previously described method for the quantification of CyHV-3 from water samples (Honjo et al., 2010; Minamoto et al., 2009a); 11.1, 154.3, 2.9, 7.4, 8.0, 22.5, 26.8 and 145.7 μg total DNA were isolated from plankton collected at sites 1–8. Finally, CyHV-3 DNA was quantified using real-time polymerase chain reaction (PCR; Gilad et al., 2004) with 150 ng DNA derived from each plankton sample as a template. The real-time PCR was performed in triplicate, and a mean value was obtained; if any of the triplicates showed a negative result it was assigned a value of zero. The sequences of the amplified products were sub-cloned and confirmed as targets by a commercial sequencing service (Takara-Bio, Otsu, Japan). The concentration of CyHV-3 DNA in the environmental water was determined using a modified version of the cation-coated filter method and quantitative real-time PCR (Honjo et al., 2010; Minamoto et al., 2009a).

Despite the short distances between sampling sites, plankton composition was highly variable among samples, reflecting the heterogeneous environment of the lagoon. When we identified zooplankton, Cladocera and Cyclopoida were most frequently observed, and Rotifera and Arcellinida were also identified. Regarding phytoplankton, Oscillatoriales, Zygnematales, Centrales, and Pennales were present (Table 1). A comparison between the logarithm of total zooplankton and the logarithm of total phytoplankton showed a significant negative correlation (Pearson's correlation coefficient $r = -0.848$, $p = 0.00782$). Particles other than plankton, such as detritus, were hardly observed in the samples.

We detected and quantified CyHV-3 in seven of eight samples (sites 1–7), and no positive signal was obtained for plankton samples from site 8. The CyHV-3 copy numbers in total plankton samples (25 ml) are shown in Table 1. For quantification of CyHV-3 from environmental water, the CyHV-3 concentration was successfully determined at seven sites (Table 1). We found that CyHV-3 concentration was approximately 100–1000 copies/l, which is lower than the concentrations observed at Lake Biwa in October 2008 (Minamoto et al., 2009a). Because the concentration of CyHV-3 DNA in the water was low (<2% of the plankton samples), we con-

Table 1
Plankton composition and CyHV-3 quantity at each site.

	Phylum/division	Order	Sampling site							
			1	2	3	4	5	6	7	8
Zooplankton	Arthropoda	Cladocera	243 (0.30)	438 (0.39)	74 (0.19)	1 (0.00)	8 (0.00)	201 (0.16)	127 (0.18)	390 (0.37)
		Cyclopoida	176 (0.22)	190 (0.17)	33 (0.09)	1 (0.00)	13 (0.01)	43 (0.03)	36 (0.05)	473 (0.45)
	Rotifera		60 (0.07)	371 (0.33)	43 (0.11)	2 (0.00)	5 (0.00)	228 (0.19)	22 (0.03)	78 (0.08)
	Sarcomastigophora	Arcellinida	3 (0.00)	2 (0.00)	9 (0.02)	30 (0.02)	8 (0.00)	10 (0.01)	26 (0.04)	7 (0.01)
Total zooplankton			482 (0.59)	1001 (0.90)	159 (0.42)	33 (0.02)	34 (0.02)	482 (0.39)	211 (0.31)	948 (0.91)
Phytoplankton	Cyanobacteria	Oscillatoriales	0 (0.00)	0 (0.00)	0 (0.00)	4 (0.00)	0 (0.00)	8 (0.01)	19 (0.03)	0 (0.00)
	Chlorophyta	Zygnematales	131 (0.16)	19 (0.02)	106 (0.28)	461 (0.30)	1822 (0.93)	504 (0.41)	294 (0.43)	7 (0.01)
	Heterokontophyta	Centrales	163 (0.20)	33 (0.03)	10 (0.03)	120 (0.08)	55 (0.03)	20 (0.02)	28 (0.04)	29 (0.03)
		Pennales	35 (0.04)	63 (0.06)	106 (0.28)	942 (0.60)	56 (0.03)	218 (0.18)	136 (0.20)	57 (0.05)
Total phytoplankton			329 (0.41)	115 (0.1)	222 (0.58)	1527 (0.98)	1933 (0.98)	750 (0.61)	477 (0.69)	93 (0.09)
CyHV-3 in 150 ng plankton DNA solution			3.1	0.4	8.7	6.0	2.2	2.2	0.9	–[a]
CyHV-3 in total plankton samples (25 ml)			1127	2273	827	1481	579	1618	791	–[a]
CyHV-3 conc. in water (copies/l)			–[a]	645	455	1009	134	585	505	302

Each plankton number includes living and dead plankton. Values indicate the number of plankton per 1 ml sample collected using a plankton net. Values in parentheses are each taxonomical group of plankton expressed as a proportion of the total population.

[a] Under detection limit.

cluded that the CyHV-3 DNA detected in plankton samples was not from contaminating water, but rather from the plankton. Future studies are required to identify the mechanisms involved in the association of CyHV-3 with plankton. They may include binding to the cell membrane or cell wall, or some form of internalization.

We performed a Pearson's correlation analysis between the CyHV-3 numbers in plankton samples and numbers in each plankton taxon, and in the total phytoplankton, total zooplankton, and total plankton populations. This analysis revealed a significant positive correlation between CyHV-3 in plankton and the numbers of Rotifera ($r = 0.71$, $p = 0.047$), suggesting that CyHV-3 binds to and/or is concentrated by the filter feeding behavior of Rotifera species.

In sites 1 and 8, no association was observed between the concentrations of CyHV-3 in water and in plankton samples. Although an explanation is uncertain, some plankton may reduce CyHV-3 in water by binding or grazing. The correlations between the concentrations of the CyHV-3 in water and the plankton compositions were also analysed; the CyHV-3 in water was positively correlated with ratio of Centrales ($r = 0.71$, $p = 0.048$) and Pennales ($r = 0.81$, $p = 0.014$), both of which belong to Heterokontophyta (diatoms). However, these results are difficult to interpret from the present data set.

Generally, carp do not feed on living plankton; however, they eat bivalves inhabiting the sediments (Sibbing, 1988). Plankton-associated viruses may settle in the sediment when plankton become inactive. As a result, carp would be infected when stirring the sediment for feeding. Alternatively, virus transmission via bivalves may occur. Bivalves are known to feed indiscriminatingly on organic matter, including plankton, via filter feeding, which may then concentrate viruses in the digestive tube (Ciminiello and Fattorusso, 2006; Lees, 2000). Thus, it is possible that plankton are involved in CyHV-3 transmission. The above hypothesis is also established when the plankton is replaced with suspended solids in the water. When considering dynamics of the CyHV-3 in the water, we should pay attention to the possibility that such particles play roles as carriers in the transmission route.

The degradation process of CyHV-3 in the natural environment has not been clarified. The previous reports indicated that CyHV-3 was released from the infected fish to the environmental water (Haramoto et al., 2007; Minamoto et al., 2009a; Minamoto et al., 2009b); we suggest that the virus then associates with plankton. Thus, plankton may affect viral degradation process in the natural environment.

As discussed above, it is possible that plankton are involved in the transmission cycles of viral diseases and, more generally, in viral ecology. In addition to fish pathogens, a similar transmission cycle may occur for water-borne viruses that cause human disease, such as caliciviruses, enteroviruses, or adenoviruses. Thus, it is important to consider viral concentrations and/or transmission via plankton when examining the dynamics of such viruses/diseases in the natural environments.

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