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Reservoirs of *Cyprinid herpesvirus 3* (CyHV-3) DNA in sediments of natural lakes and ponds

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ABSTRACT

Cyprinid herpesvirus 3 (CyHV-3) is a lethal DNA virus that infects common carp and koi. It has caused outbreak of the disease within both aquaculture and natural environmental ecosystems. However, there is not enough understanding of the distribution of CyHV-3 in the natural environments, partly because there is no suitable quantification method. In this study, we tested CyHV-3 extraction methods from sediment and then compared its abundance between sediment and water using real-time PCR. Sediment samples were taken from lake and pond, and total viral DNA was extracted using the viral elution method recommended by the US Environmental Protection Agency (manual method), as well as a commercial DNA extraction kit for soil (commercial kit method) before PCR detection. 7 of 12 (58%) and 5 of 10 (50%) sediment samples showed PCR positive signal for CyHV-3 DNA using the manual method and the commercial kit, respectively, and consistent results were obtained from the samples using the manual method between two independent primer sets. The quantification of CyHV-3 DNA in natural sediment using the manual method and external standard virus revealed that its concentration was 1.2×10^4 to 3.3×10^5 copies DNA/kg. The concentration in sediments was 46–1238 times higher than that in water from the same location, suggesting that sediment could act as a reservoir for CyHV-3 in natural freshwater environments. This is the first report of the existence of CyHV-3 in the sediment of a natural lake or pond.

1. Introduction

Cyprinid herpesvirus 3 (CyHV-3, previously described as Koi herpesvirus) causes a lethal disease to common carp *Cyprinus carpio* and koi. The disease was first recognized in Germany in 1997 (Bretzinger et al., 1999), and it rapidly spread among cultured common carp and ornamental koi farms worldwide, including Israel, USA, South Africa, European, and Asian countries (Haenen et al., 2004; Hedrick et al., 2000; Perelberg et al., 2003; Walster, 1999). Great economic losses were caused to the food carp and ornamental koi industry (Haenen et al., 2004; Matsui et al., 2008; Perelberg et al., 2003). Moreover, outbreaks of this disease also occurred among wild carp in natural rivers and lakes (Garver et al., 2010; Grimmett et al., 2006; Hara et al., 2006; Matsui et al., 2008; Nakanishi et al., 2007). In Lake Biwa in Japan, more than 80% of the natural population (100,000 individuals) of wild carp was considered to have died from this disease in 2004. Such massive mortalities of wild carp populations could have lasting impacts on natural ecosystems (Matsui et al., 2008). In addition, the disease could spread to cultured carp and koi in ponds and cages within natural water systems via water transport and/or direct contact with infected fish.

To understand how the disease spread in natural ecosystem, it is important to investigate distribution of CyHV-3 in environmental water and sediment. Recently, detection (Haramoto et al., 2009) and quantification (Honjo et al., 2010) methods for CyHV-3 from environmental water were established, revealing that high levels

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of viral DNA exist (more than 10^5/L) in natural water (Honjo et al., 2010; Minamoto et al., 2009a,b) and suggesting that water is involved in the transmission of CyHV-3 disease. Experiments in aquaria have also shown that CyHV-3 is transmitted horizontally from infected to naive hosts through contaminated water (Costes et al., 2009; Perelberg et al., 2003). On the other hand, a laboratory experiment revealed that the virus is able to survive only for 3 days in environmental water (Shimizu et al., 2006), and a temperature less than 13 °C suppresses the viral propagation (Gilad et al., 2004). Indeed, CyHV-3 concentrations in environmental water have been shown to vary seasonally, with levels lower than quantification thresholds during winter (Minamoto et al., 2009a). Nevertheless, new infection was detected the following spring by seroprevalence surveying of CyHV-3 in newly matured hosts (Uchii et al., 2011). Infectious viruses and/or viral information persisting in fish maintained at a low temperatures supports the assumption that CyHV-3 undergoes latent infection (Gilad et al., 2003, 2004; St-Hilaire et al., 2005); however, there is no evidence that carp becomes carrier in winter for the virus to survive between seasons.

High levels of CyHV-3 are found in the intestines and gills of infected carp (Gilad et al., 2004) and their droppings (Dishon et al., 2005). This suggests that environmental sediment may contain CyHV-3 and that sediment may be a virus reservoir. The major portal of entry for CyHV-3 in carp is the skin (Costes et al., 2009), and carp tend to be in contact with sediment during feeding, re-suspension of sediment (Breukelaar et al., 1994; Cline et al., 1994), and spawning in shallow water. Moreover, breeding habitats can become hot spots for transmission of this disease in spring (Uchii et al., 2011). However, the existence of CyHV-3 in the sediment of natural environments has not been examined. This is partly due to a lack of reliable detection and quantification methods for CyHV-3 from environmental sediment (Matsui et al., 2008).

It is difficult to quantitatively extract viruses from sediment because the viruses associate sediment particles. Viral extraction methods from sewage sludge and sediment have been developed for the public health field (Metcalf et al., 1995). An extraction method based on the elution of viruses from beef extract has been recommended by the US Environmental Protection Agency (EPA) (USEPA, 1992) and is the most frequently used method for detecting viruses in environmental samples. Increasing the sample volume can increase the detection sensitivity of this method. In recent years, commercial direct DNA extraction kits that use a surface activating agent and/or bead-mill homogenization have become available for microbial DNA extraction from soils. These kits are relatively quick and straight-forward to use; however, the most suitable method for obtaining the highest recovery yield differs with the type of virus as well as with the sample (LaBelle and Gerba, 1979).

The aims of this paper are (1) establishing the quantification method of CyHV-3 DNA in natural sediment using an external standard virus, (2) examining the existence of CyHV-3 DNA in natural sediment, and (3) comparison of CyHV-3 DNA concentration between sediment and water. Our results demonstrated that sediment contained CyHV-3 DNA at an approximately 100 times higher concentration than water, suggesting that sediment is a potential reservoir of CyHV-3 in natural environments.

2. Materials and methods

2.1. Sediment and water samples

Sediment and water samples were collected from eight sites in Lake Biwa, three sites in Iba-naiko, a lagoon connected to Lake Biwa, and Takaragaike Pond (Fig. 1) from 26 October 2006 to 13 April 2009. Sediment samples were taken using an Ekman dredge and stored at 4 °C until processing the next day or at −20 °C for more than 2 days of storage. Water samples taken from the water surface were stored at 4 °C until processing the next day. Water temperature and pH were measured on site.

2.2. Standard virus

CyHV-3 (NRIA 0301 strain) was propagated using the common carp brain (CCB) cell line, which was established from carp (*C. carpio*) brain tissues (Neukirch et al., 1999). Lambda phage (Promega, Tokyo, Japan) was propagated in *Escherichia coli* LE 392 cells (Maniatis et al., 1982) for use as an external standard. These viruses were purified by the polyethylene glycol (PEG) method (Sambrook and Russell, 2001) and then counted under an epifluorescence microscope using the SYBR Green I staining method (Noble and Fuhrman, 1998).

2.3. Comparison of viral DNA extraction methods from sediment

Viral DNA was extracted from sediments using two methods: a manual method based on the US EPA method (Belguith et al., 2006; USEPA, 1992) followed by DNA extraction, and a commercial DNA extraction kit for soil.

For the manual method, 1% (v/w) of 0.05 M aluminum chloride was added to 10-g sediment samples (except for samples taken from Iba-naiko lagoon, which were 20 g) and adjusted to pH 3.5 using HCl. The mixture was homogenized and centrifuged at 2500 × *g* for 15 min at 4 °C. After discarding the supernatant, 10% (v/w) of 10% beef extract (Remel, Lenexa, KS, USA, 451052) was added to sediment, and the pH was adjusted to 7.0 using NaOH. The mixture was homogenized again and centrifuged at 10,000 × *g* for 30 min at 4 °C. Next, 8% PEG6000 (w/v) was added to the supernatant, which was stirred for 30 min, incubated overnight at 4 °C, and then centrifuged at 10,000 × *g* for 1 h at 4 °C (Lewis and Metcalf, 1988). The pellet was suspended in 10 mL of SMC buffer [50 mM Tris–HCl (pH 7.0), 0.1 M NaCl, 8 mM $MgSO_4$, 1 mM $CaCl_2$, 0.01% (w/v) gelatin]. Viral DNA was extracted using proteinase K and SDS and purified by phenol–chloroform extraction and ethanol precipitation (Sambrook and Russell, 2001). A half-volume of DNA extract solution was further purified with the PowerClean® DNA Clean-Up Kit (MO BIO Laboratories, Carlsbad, CA, USA).