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Survival of Koi Herpesvirus (KHV) in Environmental Water

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ABSTRACT—The survivability of koi herpesvirus (KHV) in environmental water and sediment was evaluated using CCB cells. Samples were collected from Ibaraki prefecture, Kanagawa prefecture and Hakodate, Hokkaido. Significant reduction in the infectious titer of KHV was observed within 3 days in intact environmental water or sediment. However, KHV infectivity remained for more than 7 days in autoclaved or filtered (0.45 μm) water. In the autoclaved water containing sediment, KHV infectivity dropped below detectable limits within 7 days after inoculation. Ten of the 147 bacterial strains from rivers in Kanagawa, and two of the 62 bacterial strains from water from, Hakodate showed anti-KHV properties. The results suggest that in the absence of hosts, KHV can be rapidly inactivated in environmental water.

Key words: koi herpesvirus, KHV, *Cyprinid herpesvirus* 3, *Cyprinus carpio*, inactivation

Since 1998, a lethal viral disease responsible for mass mortality of *Cyprinus carpio* (koi and common carp) has been reported in North America and Israel (Hedrick *et al*., 2000). Also in Japan, the outbreaks in common carp have occurred since early October 2003 (Sano *et al*., 2004) not only in culture ponds but also in natural environments such as lakes and rivers. A herpesvirus, designated koi herpesvirus (KHV; Hedrick *et al*., 2000) or *Cyprinid herpesvirus* 3 (CyHV-3; Waltzek *et al*., 2005), was isolated from diseased fish and identified as the cause of these mortalities.

This virus has caused severe financial losses to fish breeders, retailers and hobbyists worldwide. Some preventive measures have been established to control the disease, including UV irradiation, heat treatment and disinfectants (Kasai *et al*., 2005). Experimental vaccines, thermal treatment regimes, breeding for increased resistance and production of goldfish *Carassius auratus*-common carp hybrids have also been proposed (Ronen *et al*., 2003; Shapira *et al*., 2005; Hedrick *et al*., 2005, 2006).

However, no method has been established for preventing outbreaks of KHV disease in wild populations. Viral survivability is a crucial factor for that purpose, but little information is available. In this report, the survivability of KHV in an environmental water and water containing sediment was evaluated using a cell line.

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Materials and Methods

Cell lines and virus

The koi fin cell line (KF-1) and a virus isolate (KHV-I) were thankfully provided by Dr. R. P. Hedrick (University of California, Davis) and common carp brain (CCB) cells were generously provided by Haenen OL (CIDC-Lelystad, The Netherlands). These cell lines were propagated in Leibovitz L-15 (GIBCO) supplemented with 10% fetal bovine serum (FBS), 50 IU penicillin/mL, and 50 μg streptomycin/mL at 20°C. KHV was inoculated to sub-confluent KF-1 and CCB cells, and the culture medium containing the virus was collected after 14 days and stored at –80°C until use.

Evaluation of virus survivability using cell lines

Surface water and sediment were collected in sterile plastic bottles from Lake Kasumigaura, Ibaraki Prefecture, Japan in July 2004 (Fig. 1A). In December 2004, water samples were collected from six places in Kanagawa Prefecture, Japan (T-1, T-2, T-3 in Tsurumi River, T-4 in Ookuma River, T-5 in Onda River, T-6 in Asabu River) (Fig. 1B). In December 2005, water samples were collected from five places in Hokkaido, Japan: the moat of Goryokaku fort, Ishikawa River, midstream and upstream area of the Kameda River and Tokiwa River (Fig. 1C). At each sampling station, water temperature, pH, dissolved oxygen, BOD and COD were measured. BOD and COD were measured according to JIS handbook (Nihonkougyoukikaku, 1991). To

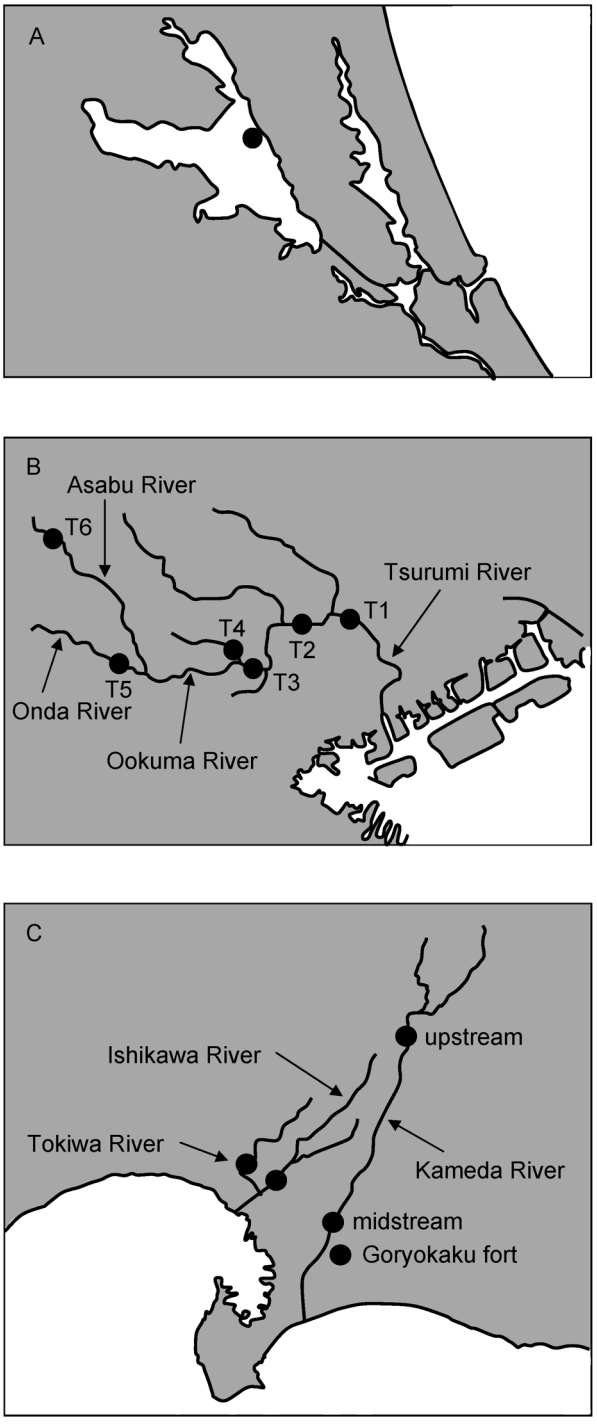

Fig. 1. Location of sampling station. A, Ibaraki Prefecture; B, Kanagawa Prefecture; C, Hakodate, Hokkaido.

determine viable bacterial counts, 0.1 mL of serial dilutions of water was spread on fresh water agar (FWA, Yoshimizu *et al*., 1976) in duplicate. The plates were incubated at 25°C for 5 days. Each water sample was treated in three different ways: filtered through a 0.45 μm membrane filter (Milipore), sterilized by autoclaving at 121°C for 15 min, or kept intact. Sediment preparations were made by mixing 0.27 g of intact sediment with 2.7 mL of autoclaved water sampled from each river. Sterilized sediment was prepared by autoclaving at 121°C for 15 min and mixing 0.27 g of intact sediment with 2.7 mL of autoclaved water sampled from each river. Culture medium containing the virus (0.3 mL) was added to these samples (2.7 mL) under several conditions in a sterile plastic tube. As a control, 0.3 mL of KHV was added to Hanks' BSS (Nissui). From each tube, 0.3 mL of the water samples was obtained to estimate the infectious titer of KHV, as the 50% tissue culture infectious dose ($TCID_{50}$) by inoculating KF-1 cell with a 10-fold dilution series of the original solution in 96-well microtiter plates (BD). The inoculated cells were cultured at 20°C for 14 days. Tests for samples from Lake Kasumigaura were held at 15, 20, 25 and 30°C for 7 days; tests for samples from rivers in Kanagawa were held at 15°C for 7 days; tests for samples from Hokkaido were held at 15°C for 14 days.

Isolation of anti-KHV bacteria from environmental water

Viable bacterial counts of the lake-water and river-water samples were measured by the plate counting method described above. After the plates were incubated at 25°C for 5 days, viable bacterial counts were measured and bacteria from each sample were pure cultured on FWA. A total of 147 bacterial strains were isolated from the Tsurumi River and 62 bacterial strains were isolated from water from Goryokaku. Subsequently, 1 platinum loop of bacterial cells was aerobically grown for 72 h by inoculating into duplicate wells of a 24-well tissue culture plate (BD) containing 1 mL of CYG broth (Casamino acids 5.0 g; Yeast extract 0.5 g; Glucose 1.0 g; NaCl 6.8 g; KCl 0.4 g; $MgSO_4 \cdot 7H_2O$ 0.2 g; $CaCl_2 \cdot 2H_2O$ 0.26 g; DW 1L, pH 7.2, Kamei *et al*., 1988a) on a rotary shaker at 100 rpm at 25°C. The media were filtered (Millipore, 0.45 μm), and the supernatant was mixed with an equal volume of KHV solution (10^3 $TCID_{50}$/mL). After reaction for 1 h at 15°C, 50 μL samples were inoculated into replicate wells of a 48-well tissue culture plate (IWAKI) containing a monolayer of KF-1 or a monolayer of CCB cells. A reaction solution with Hanks' BSS and CYG broth was mixed at a ratio of 1:1 for negative control, and a reaction solution with Hanks' BSS and 10^3 $TCID_{50}$/mL KHV was mixed at a ratio of 1:1 for positive control. These plates were incubated at 20°C for 2 wks to observe the CPE.

Results

Evaluation of survivability of KHV in water samples using cell lines

The environmental conditions at the sampling stations are shown in Table 1. Changes in KHV infectivity in water or sediment samples collected from Lake Kasumigaura are shown in Fig. 2. In the sample of intact water and water containing sediment, KHV infec-

Table 1. Water quality at sampling stations

Station	Date	Water Temperature (°C)	pH	Dissolved Oxygen (mg/mL)	BOD*1 (mg/mL)	COD*2 (mg/mL)	Viable Bacterial Counts (CFU/mL)
Lake Kasumigaura	July 2004	24.6	8.2	7.1	2.3	8.6	NT*3
T-1; Tsurumi River	December 2004	14.6	6.35	6.8	1.7	6.1	NT
T-2; Tsurumi River		14.7	6.95	7.4	5.1	7.0	NT
T-3; Tsurumi River		14.2	7.08	8.0	7.6	7.4	NT
T-4; Ookuma River		12.9	6.72	9.8	1.2	4.4	NT
T-5; Onda River		12.3	6.63	11.5	0.9	2.6	NT
T-6; Asabu River		17.4	6.99	9.1	2.5	5.9	NT
Water from the mort of Goryokaku fort	December 2005	6.4	8.1	12	NT	NT	1.9×10^2
Ishikawa River		6.2	8.1	12.7	NT	NT	2.6×10^5
Kameda River (middle stream)		6.9	8.4	12.4	NT	NT	4.6×10^4
Kameda River (upper stream)		7.9	7.7	11.3	NT	NT	7.8×10^3
Tokiwa River		11.1	7.6	11.5	NT	NT	1.5×10^5

*1: Biochemical oxygen demand, *2: Chemical oxygen demand, *3; Not tested

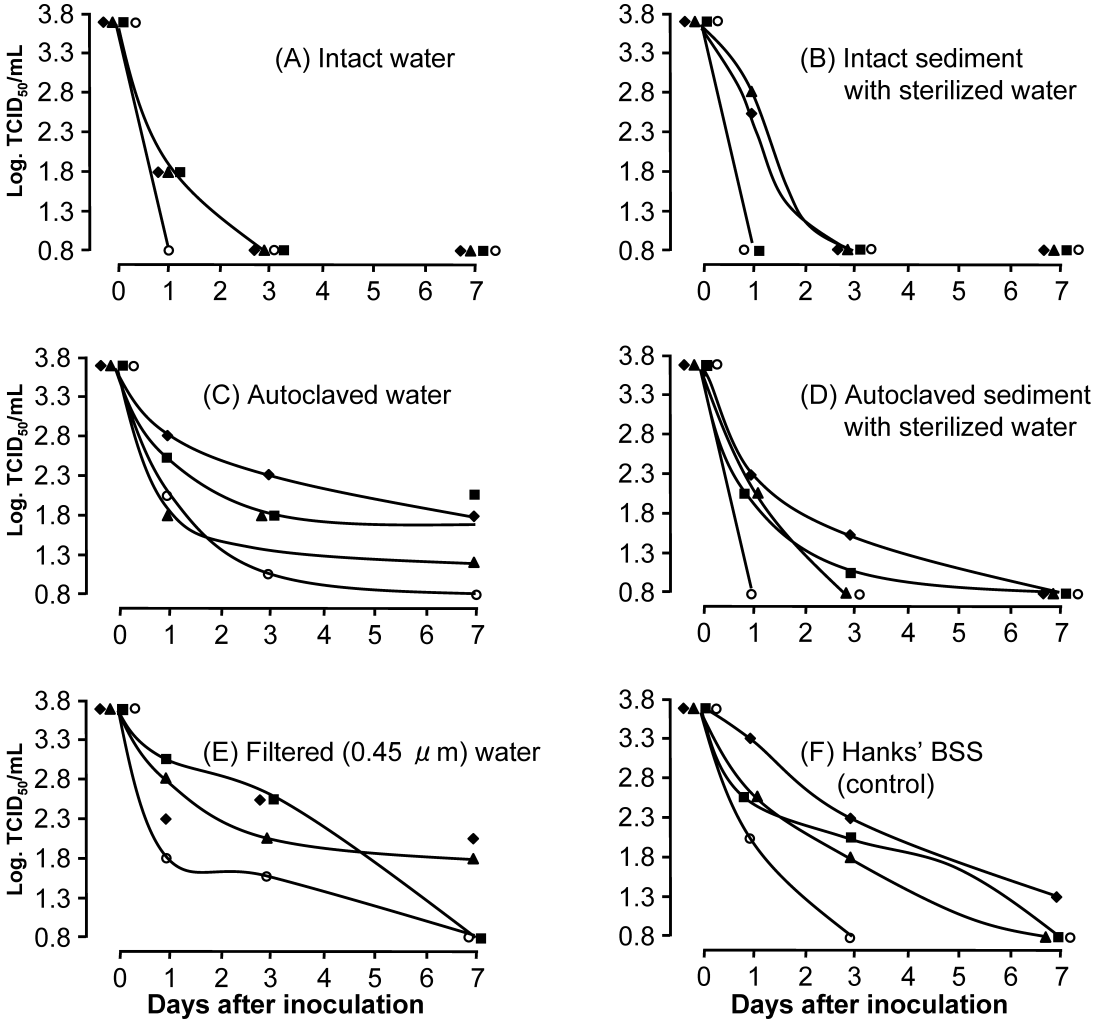

Fig. 2. Changes in infectious titer of KHV in water or sediment collected from Lake Kasumigaura held at 15, 20, 25 or 30°C.
◆ 15°C ▲ 20°C ■ 25°C ○ 30°C

Table 2. Survivability of KHV in environmental water at 15°C

Sample	Date (Month, year)	KHV infectivity (log. $TCID_{50}$/mL)				
		Days after inoculation				
		0	1	3	7	14
T-1; Tsurumi River	December, 2004	2.5	< 1.8*1	< 1.8	< 1.8	NT*2
T-2; Tsurumi River		2.5	< 1.8*1	< 1.8	< 1.8	NT
T-3; Tsurumi River		2.5	< 1.8*1	< 1.8	< 1.8	NT
T-4; Ookuma River		2.5	< 1.8*1	< 1.8	< 1.8	NT
T-5; Onda River		2.5	< 2.1	< 1.8*1	< 1.8	NT
T-6; Asabu River		2.5	< 2.1	< 2.1	< 1.8*1	NT
Water from the mort of Goryokaku fort	December, 2005	3.3	2.1	< 0.8*1	< 0.8	< 0.8
Ishikawa River		2.8	< 0.8*1	< 0.8	< 0.8	< 0.8
Kameda River (midstream)		2.8	< 0.8*1	< 0.8	< 0.8	< 0.8
Kameda River (upstream)		3.3	3.3	< 0.8*1	< 0.8	< 0.8
Tokiwa River		3.3	< 0.8*1	< 0.8	< 0.8	< 0.8

*1; Detection limit, *2; Not tested

tivity dropped below detectable limits within 3 days after inoculation (Fig. 2A, B). In the autoclaved water, however, KHV infectivity was higher than 10^1 $TCID_{50}$/mL at 3 days after inoculation (Fig. 2C). Substantial infectivity also remained in the filtered water samples at 3 days after inoculation (Fig. 2E), and in the autoclaved water containing sediment, KHV infectivity dropped below detectable limits within 7 days after inoculation (Fig. 2D).

Changes in infectious titers of KHV in intact water samples from Kanagawa Prefecture and from Hokkaido are shown in Table 2. Similarly, the infectivity dropped below detectable limits within 3 days after inoculation.

Isolation of anti-KHV bacteria from environmental water

Anti-KHV properties were determined by observation of CPE due to KHV. Ten of the 147 bacterial strains from rivers in Kanagawa and two of the 62 bacterial strains from water from Goryokaku showed anti-KHV properties.

Discussion

Results in this study suggest that KHV loses its infectivity within a few days in environmental water above 15°C. KHV was unstable compared with other fish herpesvirus and rhabdovirus. In fish rearing water, for *Oncorhynchus masou* virus (OMV) strain OO-7812, virus infectivity dropped from $10^{3.8}$ to $10^{0.8}$ $TCID_{50}$/mL at 15°C within 3 days after inoculation, and for infectious hematopoietic necrosis virus (IHNV) strain ChAb, infectivity dropped from $10^{3.0}$ to $10^{1.0}$ $TCID_{50}$/mL at 15°C within 7 days (Yoshimizu *et al*., 1986).

KHV lost infectivity within 3 days in natural environmental water, however, KHV infectivity remained for more than 7 days in filter-sterilized or autoclaved water. The results suggest that inactivation of KHV was due to bacteria existing in environmental water. Actually 5.7% of isolated bacteria from environmental water showed anti-KHV activity. We have reported that many bacteria showing anti-viral activity were isolated from fish rearing water and played an important role in the microbial ecosystem (Yoshimizu *et al*., 1976; Kamei *et al*., 1987, 1988a, b; Kimura *et al*., 1990; Yoshimizu and Ezura, 1999). Anti-viral mechanisms of the bacteria isolated from Tsurumi River and from Goryokaku are unknown, but these bacteria were assumed to produce anti-viral substances that reduce of KHV infectivity. It is also conceivable that bacteria in intact water can directly inactivate KHV.

Recent mass mortality among koi and common carp due to KHV in many locations suggests that the virus is now widely distributed. The results in this study showed that KHV could be inactivated within 3 days in natural environments without common carp or koi. The use of anti-viral bacteria in natural environments may be effective as an additional control strategy for fish viral diseases. Particularly, the plan can be effective to inactivate the KHV in places that are difficult to be disinfected. Currently control measures involve the use of hypochlorite. This has disadvantages because of its cost and pungent smell. From the results of this study, removal of the hosts (koi and carp) from the water for a period, will result in loss of infectivity of KHV in the water. This is an important and practical solution to the KHV problem.

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