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Is Koi Herpesvirus (KHV) Related to the Mass Mortality Occurring among Cultured Carp, *Cyprinus carpio*, in Korea?

Wi-Sik Kim, Sung-Ju Jung, Duwoon Kim[1], Seok-Ryel Kim[2], Jeong-Ho Kim[3] and Myung-Joo Oh*

Department of Aqualife Medicine, College of Fisheries and Ocean Science, Chonnam National University, Yeosu 550-749, Korea

[1]Division of Food Science and Technology and Functional Food Research Center, Chonnam National University, Gwangju 500-757, Korea

[2]Pathology Division, National Fisheries Research and Development Institute, Busan 619-902, Korea

[3]Faculty of Marine Bioscience and Technology, Gangneung-Wonju National University, Gangneung 210-702, Korea

Since 1998, a new viral disease with high mortality has been consistently recorded in Korea in cultured carp, *Cyprinus carpio*. In this study, we investigated an epizootic of the disease that caused high mortality rates in carp obtained from 11 farms in Korea between 1999 and 2007. Assessment of koi herpesvirus (KHV) levels in diseased carp was carried out to determine if this virus was the etiologic agent of disease in this instance. High mortality rates in carp were recorded mainly in the spring and autumn at water temperatures between 19°C and 24°C. Diseased fish typically showed surface discoloration, with a thick opaque mucus covering the body and gills. Protozoan parasites and bacteria were recovered from 7/29 (24%) and 2/26 (8%) of fish, respectively. Evidence of viral infection was marked; cytopathic effects (CPEs), characterized by cell rounding and an extended cytoplasm in fathead minnow (FHM) cells, were detected in 40/41 fish (98%). A high mortality rate (80%) resulted when supernatants of cell cultures showing CPEs were applied to previously healthy fish. KHV was detected by polymerase chain reaction in 6/41 fish (15%), but was not detected in supernatants obtained from cell cultures showing CPEs. These results suggest that KHV may not be the etiologic agent of the high mortality occurring among cultured carp in Korea; therefore, some other—as yet unidentified—infective agent must be responsible.

Key words: Viral disease, Unknown virus, *Cyprinus carpio*, Koi herpesvirus, Korea

Introduction

Koi herpesvirus disease (KHVD), a relatively new viral disease, has recently become a major problem in the global carp aquaculture industry. The geographical range of this disease has become extensive since KHVD was first reported in Israel and the United States in 1998 (Hedrick et al., 2000). It is now known to occur in several countries, including the United States, along with several in Europe and Asia (Hedrick et al., 2000; Haenen et al., 2004; Sano et al., 2004; Pokorova et al., 2005). The causative agent of the disease is known as koi herpesvirus (KHV) and is classified in the family *Herpesviridae* (Hedrick et al., 2000). KHV is also known as carp nephritis and gill necrosis virus (CNGV), in accordance with its pathology (Hutoran et al., 2005). More recently, evidence supporting its classification as a herpesvirus has given rise to the name cyprinid herpesvirus 3 (CyHV-3) (Waltzek et al., 2005).

Disease caused by KHV is highly contagious and extremely virulent in the common carp *Cyprinus carpio carpio* and koi *Cyprinus carpio koi*. Fish of all ages are susceptible to KHV infection. The disease course is typically rapid, particularly so at optimal temperatures (23-25°C), but tends to be slower at temperatures below 23°C. Discoloration and severe necrosis of the gills are the most consistent signs of

*Corresponding author: ohmj@chonmam.ac.kr

the disease, though fish generally do not exhibit skin lesions (Hedrick et al., 2000). Microscopic necrotic lesions are prominent in the gills and in several internal organs. A characteristic pathognomonic feature of cells infected with KHV is formation of intranuclear inclusion bodies with marginal hyperchromatosis (Hedrick et al., 2000; Miyazaki et al., 2008).

Carp aquaculture in Korea began in the 1970s. From this time until 1998, no viral diseases were observed in these fish populations. However, a new viral disease has been recorded consistently since 1998 at water temperatures between 20°C and 24°C in cultured carp, the common carp, and the Israel carp *Cyprinus carpio nudus*, resulting in drastic reductions in carp production (Oh et al., 2001). Oh and coauthors reported that discolorations and a thick opaque mucus covering the body and gills were the main external signs in affected fish. Histologically, the kidney and spleen showed severe necrotic changes. Tissue filtrates from diseased fish produced cytopathic effects (CPEs) in fathead minnow (FHM) cells, while electron microscopy analysis revealed viruslike particles with a diameter of 70-80 nm. Experimental infection trials using a viral suspension prepared from infected FHM cells resulted in a high mortality (80-100%) within a period of 15 days postinfection. Therefore, these data suggested that a new, and as yet unidentified, virus was causing mass morality in cultured carp in Korea. We hypothesized that the causative agent may be KHV due to the similarity of disease clinical signs, outbreak time, and outbreak temperature to those seen in outbreaks of KHV disease (KHVD). However, as yet no evidence exists in the published literature to support this hypothesis. Thus, in the present study, we investigated an epizootic of the disease which caused high mortality in carp obtained from various farms in Korea between 1999 and 2007. A KHV polymerase chain reaction (PCR) assay was performed to determine the relationship of this virus to the mass mortality of carp in Korea.

Materials and Methods

Fish samples and examinations of diseased carp

Samples of moribund common carp and Israel carp were obtained from 11 private farms in Jangseong in 1999; Pyeongchang and Hadong in 2000; Sancheong, Jeongeup, and Cheongdo in 2001; Jinju, Uisung, and Jeongeup in 2002; Jinju in 2005; and Jeongeup in 2007 (Table 1). Also, samples from two farms in Jecheon in 1998 and in Jangseong in 1999 that were infected with a new virus, as reported by Oh et al. (2001), were used. Samples were transported on ice and immediately subjected to parasitological, bacteriological, and virological examination. The gills and body surface were examined microscopically for the presence of parasites. Pieces of the kidney and spleen were cultured for bacterial isolation on brain heart infusion (Difco, USA) agar plates supplemented with 0.5% NaCl and incubated at 20°C for 7 days. Kidney and spleen tissues were homogenized using a stomacher, diluted 1:10 in Hanks' balanced salt solution (Gibco, USA), and centrifuged at 3,000×*g* for 20 min. Supernatants were then passed through a 450-nm membrane filter and 100 μL was inoculated onto three fish cell lines in 24-well tissue culture plates (Nunc, The Netherlands): fathead minnow (FHM), chinook salmon embryo (CHSE-214), and epithelioma papulosum cyprini (EPC). Cell cultures were incubated at 20°C and examined for development of CPEs. Spleen and kidney tissue homogenates and supernatants of cell cultures showing CPEs were examined for the presence of KHV using PCR.

Extraction of DNA

DNA was extracted from tissue homogenates of carp spleen and kidney and supernatants from those FHM cell cultures showing CPEs. Briefly, Proteinase K (20 μL; 1 mg/mL, TaKaRa, Japan) was added to 200 μL aliquots of each sample followed by incubation at 55°C for 2 h. DNA was isolated using the phenol/chloroform method. Nucleic acids were precipitated with isopropanol, resuspended in distilled water, and stored at -20°C until required.

Detection of KHV by PCR and sequencing

PCR was performed to produce a predicted 484-bp amplicon using the primers KHV9/5F (5'-GACGA CGCCGGAGACCTTGTG-3') and KHV9/5R (5'-CA CAAGTTCAGTCTGTTCCTCAAC-3'), which target sequences within the KHV DNA *Kpn*I/*Sac*I fragment, as described by Gilad et al. (2002). PCR conditions were the following: 20 μL reaction volume containing 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 1.5 mM $MgCl_2$, 0.1% Triton X-100, 100 pM of each primer, 0.2 mM of each dNTP, 1U *Taq* DNA polymerase, and template DNA. Amplification was performed using a GeneAmp 2400 thermal cycler (Perkin Elmer, USA) and 39 cycles (94°C for 1 min, 64°C for 1 min, and 72°C for 1 min). PCR products were resolved on 1.5% agarose gels and bands excised and purified using a QIAquick gel extraction kit (Qiagen, USA). Amplified products were subjected to nucleotide sequence analysis using an ABI

Table 1. Isolation of pathogen and detection of KHV by PCR from diseased *Cyprinus carpio*

Farm	Place	Year	Month	Water temperature (°C)	Mean fish weight (g)	Pathogen detection rate % (detection no./ total no.)					Reference
						Parasite	Bacterium	CPEs in FHM cells	KHV PCR		
									TH	S	
JC98	Jecheon	1998	July	24	179	-	-	100% (6/6)	0% (0/6)	0% (0/2)	Oh et al., 2001
JS99	Jangseong	1999	June	21	250	-	-	100% (3/3)	0% (0/3)	0% (0/2)	Oh et al., 2001
JS99-2	Jangseong	1999	July	NT	15	0% (0/2)	0% (0/2)	100% (2/2)	100% (2/2)	0% (0/2)	This study
PC00	Pyeongchang	2000	June	NT	300	0% (0/3)	NT	100% (3/3)	0% (0/3)	NT	This study
HD00	Hadong	2000	July	20	110	0% (0/4)	0% (0/4)	100% (4/4)	50% (2/4)	0% (0/4)	This study
SC01	Sancheong	2001	June	19	166	*67% (2/3)	0% (0/3)	100% (3/3)	0% (0/3)	0% (0/2)	This study
JE01	Jeongeup	2001	June	20	490	0% (0/3)	0% (0/3)	100% (3/3)	0% (0/3)	NT	This study
CD01	Cheongdo	2001	September	20	58	0% (0/3)	0% (0/3)	100% (3/3)	0% (0/3)	NT	This study
JJ02	Jinju	2002	May	23	600	**75% (3/4)	0% (0/4)	75% (3/4)	0% (0/4)	NT	This study
US02	Uisung	2002	May	NT	270	***100% (2/2)	****100% (2/2)	100% (2/2)	100% (2/2)	0% (0/1)	This study
JE02	Jeongeup	2002	June	23	230	NT	NT	100% (3/3)	0% (0/3)	NT	This study
JJ05	Jinju	2005	April	NT	250	0% (0/2)	0% (0/2)	100% (2/2)	0% (0/2)	NT	This study
JE07	Jeongeup	2007	October	NT	1000	0% (0/3)	0% (0/3)	100% (3/3)	0% (0/3)	NT	This study
						24% (7/29)	8% (2/26)	98% (40/41)	15% (6/41)	0% (0/13)	

Parasite: *Dactylogyrus sp. infection; **Myxobolus sp. infection; *** Trichodina sp. infection.
Bacterium: ****Aeromonas sp. infection.
NT: not tested; TH: tissue homogenates; S: supernatants of cell cultures showing CPEs.

PRISM dye terminator sequencing chemistry (Applied Biosystems, USA) with KHV9/5F and KHV9/5R primers, according to the manufacturer's instructions. The resulting sequences were assembled with Genetyx Win Ver. 5.1 software and compared to the KHV gene (GenBank accession no. AF411803; Gilad et al., 2002).

Challenge experiments

Supernatants of FHM cell cultures that showed CPEs after inoculation with tissue homogenates from KHV PCR-negative fish were examined for pathogenicity in Israel carps (8-10 cm in total length). Twenty fish were maintained in each of two tanks, each containing 30 L freshwater maintained at 21± 1°C. Twenty fish from one tank were injected intraperioneally with 50 μL supernatant/fish. As a control, 20 fish were injected with 50 μL HBSS/fish. Kidney and spleen homogenates of moribund and dead fish were assessed for their ability to cause CPEs in FHM cell culture.

Results and Discussion

A high mortality rate (30-70%) in cultured carp was recorded in 11 private farms during the spring and autumn (water temperatures between 19°C and 24°C) of 1999 to 2007 (Table 1). Most diseased fish displayed surface discoloration together with overproduction of mucus by the skin and gills. In a few cases, gills were autolyzed with dermal ulcers, and skin and fin hemorrhages. Upon examination, the gills of 7/29 fish (24%) were found to be infected with *Dactylogyrus* sp., *Myxobolus* sp., or *Trichodina* sp., and the kidneys and spleens of 2/26 fish (8%) were infected with *Aeromonas* sp. (Table 1). CPEs, characterized by cell rounding and an extended cytoplasm, were observed in FHM cells when tissue homogenates of 40/41 fish (98%) were applied (Table 1, Fig. 1). In experimental trials using supernatants obtained from cell cultures showing CPEs, the cumulative mortality rate of the supernatant-injected Israel carp reached 80% 30 days postinfection (Fig. 2). In contrast, no mortality was observed in the control groups. CPEs (with cell rounding and an extended cytoplasm) were observed in FHM cells treated with supernatants derived from moribund and dead fish, while no CPEs were observed in the control groups. The occurrence of CPEs in the majority of the tested samples, the high mortality of carp in the supernatant mortality trials, and the low prevalence of parasites and bacteria in diseased fish strongly suggest a viral etiology for this disease. Oh et al. (2001) reported a novel viral disease responsible for a similarly high mortality in cultured carp in Korea. The principal signs of this disease also included surface discoloration along with a thick and opaque mucus covering the body and gills. Inoculation of tissue filtrates from diseased fish led to CPEs in FHM, generating a clinical picture almost identical to that presented herein. These results suggest that the unknown virus suspected to be the causative agent in this study may be closely related to the as yet unidentified virus reported by Oh et al. (2001).

KHV is known to be the pathogen responsible for mass mortality in common carp and koi in the United States as well as in several European and Asian countries (Hedrick et al., 2000; Haenen et al., 2004;

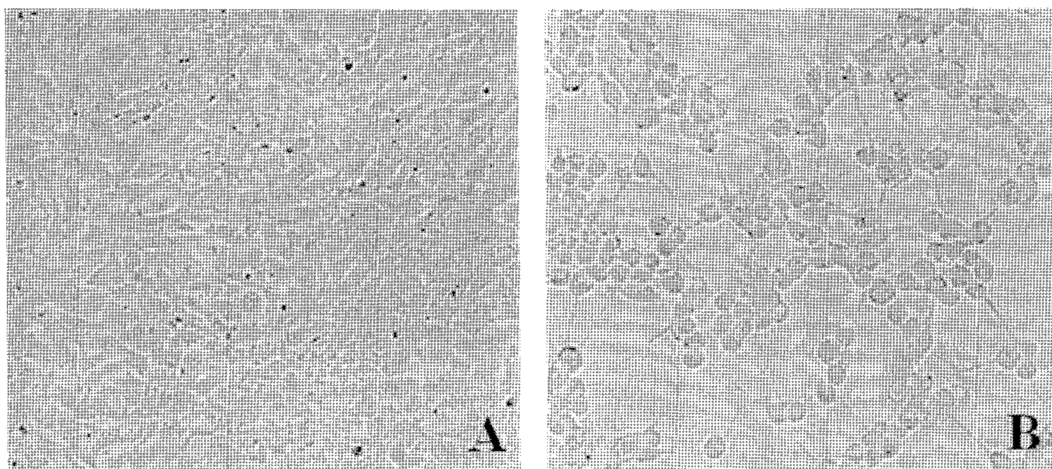

Fig. 1. Cytopathic effects produced by inoculation of the tissue filtrate from diseased carp in FHM cells (5 days post-inoculation). A, normal cells; B, infected cells.

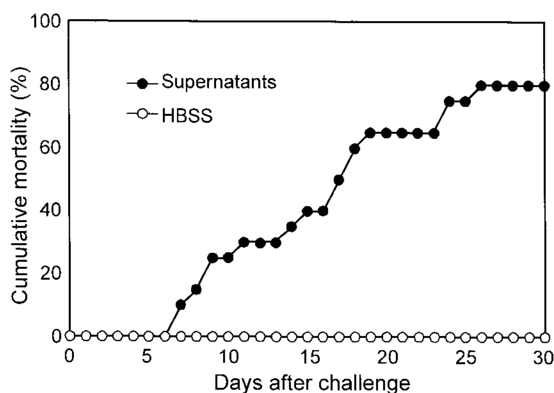

Fig. 2. Cumulative mortality of Israel carp that were injected intraperitoneally with supernatants obtained from cell cultures showing CPE (●) and HBSS (○).

Sano et al., 2004; Pokorova et al., 2005). To investigate any possible relationship between the disease we report here and KHV, PCR assays targeting this virus were performed using the 41 tissue homogenates and 13 supernatants obtained from cell cultures showing CPEs (Table 1). Six of 41 fish (15%) were KHV-positive. Samples from the JS99-2 and US02 farms were all positive, while 2/4 fish (50%) from the HD00 farm were KHV-positive. Furthermore, sequencing of the PCR products amplified from the JS99, HD00, and US02 samples had 100% homology with KHV. In this study, we hypothesized that KHV was responsible for the mass mortality of carp in Korea. However, KHV was detected in only 6/41 fish (15%), suggesting that the majority of diseased fish were not infected with this virus. Moreover, KHV was not detected in supernatants from cell cultures showing CPEs. Eosinophilic intranuclear inclusion, a characteristic of KHV infection, was not detected when histopathological analysis of diseased carp was undertaken (Seo et al., 2010). These data, taken together, suggest that KHV may not be responsible for the observed high mortality in cultured carp in Korea, despite its demonstrable presence in Korean carp farms. Thus, an as yet unknown virus is likely the etiologic agent of this mass mortality. Further studies to be performed in our laboratory will attempt to identify the new virus and discover its pathogenetic mechanisms together with its relationship, if any, to KHV.

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