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An Unusual Koi Herpesvirus Associated with a Mortality Event of Common Carp *Cyprinus carpio* in New York State, USA

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ABSTRACT: Koi herpesvirus (KHV), a highly contagious and lethal virus that affects both koi (*Cyprinus carpio koi*) and common carp (*Cyprinus carpio*), was isolated in 1998 from two outbreaks of koi suffering mass mortality in New York State, USA, and in Israel. The disease had been described as early as 1996 in Europe. In July 2004, this virus was found associated with a mass mortality event in wild common carp in the Chadakoin River, New York, USA (42°07′N, 79°W). Affected fish typically showed marked hyperplasia of gill tissues, abdominal adhesions, and severe multifocal to diffuse external hemorrhages. The virus isolated in this outbreak was somewhat unusual in that it initially replicated well in fathead minnow cell cultures, which is typical of spring viremia of carp virus. Testing at the National Veterinary Services Laboratories, Ames, Iowa, USA, confirmed the virus's identity to be KHV. Koi herpesvirus is not currently on the OIE (World Organisation for Animal Health) list of notifiable diseases; however, it is capable of causing mass mortality in susceptible fish at permissive temperatures.

Key words: Carp, common carp, *Cyprinus carpio*, herpesvirus, koi.

On 11 July 2004, eight large adult carp (*Cyprinus carpio*) were presented to the Aquatic Animal Health Program Fish Disease Diagnostic Laboratory at the College of Veterinary Medicine, Cornell University, Ithaca, New York, USA, for assessment of ongoing mortalities in the Chadakoin River (42°07′N, 79°W) near Jamestown, New York. According to New York State Department of Environmental Conservation (NYSDEC) officials, an estimated 6,000 adult carp were reported dying in a mortality event lasting several weeks. Carp were the only fish species reportedly involved in this particular fish kill. All fish were presented dead. All specimens were large adult fish (720–830 mm total length) in good body condition, suggesting recent death. One of the eight fish was too autolyzed to be suitable for assessment.

A skin scraping and gill clipping were taken from one fish (the freshest specimen), and all seven were necropsied with samples collected for histopathology, virology, and bacteriology. Tissue samples including gill, liver, spleen, and kidney were fixed in 10% neutral buffered formalin for routine processing, stained with hematoxylin and eosin stains (Luna, 1968), and examined by light microscopy. Samples for bacteriology were obtained from the posterior region of the kidney of all fish and streaked onto blood agar and tryptic soy agar. Plates positive for bacterial growth were submitted to the New York State Veterinary Diagnostic Laboratory, Cornell University, for identification via fatty acid analysis.

A cell culture inoculate from each fish was made using pooled tissues from the posterior kidney, liver, and spleen in nine volumes of Hank's Balanced Salt Solution, homogenized in a stomacher (model 80, Seward, London), centrifuged at 3,000 × G for 10 min, and the supernatant filtered using 0.2 μm ultra-low-protein binding filters. The samples were each inoculated onto duplicate 25 cm^2 fathead minnow (FHM) cell cultures and allowed to adsorb for 1 hr. Hank's Minimum Essential Media (HMEM) supplemented with 10% fetal bovine serum was then added, cultures were incubated at 20 C, and cytopathic effect (CPE) was noted after two blind passages. A single flask from this passage showing CPE was submitted for virus identification to the USDA, Animal and Plant Health Inspection Service, National Veterinary Services Laboratories

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(NSVL), Ames, Iowa, USA. Frozen tissue specimens from which the virus was isolated and a second flask of the original isolate showing CPE were also submitted to the NVSL at a later date. Fluids from the inoculated flask demonstrating CPE were processed for electron microscopy at the NVSL.

RNA was extracted from 300 µl of cell culture fluids from a flask demonstrating 75–90% CPE in a viewing field using TRIzol® LS (Gibco, Invitrogen, Carlsbad, California, USA). The RNA was evaluated by reverse transcription-polymerase chain reaction with multiple primer sets specific for the phosphoprotein, matrix protein, and glycoprotein genes of spring viremia of carp virus (SVCV).

DNA was extracted from 100 µl of cell culture fluids from a flask demonstrating CPE using the Puregene genomic DNA isolation system (Gentra, Minneapolis, Minnesota, USA). The sample was mixed with 20 µl of proteinase K (10 mg/ml) and 200 µl of cell lysis solution, incubated at 56 C for 3 hr, and the DNA isolated according to the manufacturer's procedure. DNA was extracted from tissue samples associated with the viral isolate using the Qiagen tissue kit (Qiagen, Valencia, California, USA). Briefly, 150 µl of a 20% tissue homogenate was mixed with 200 µl of the ATL buffer and 20 µl of proteinase K solution, incubated overnight at 56 C, and the DNA purified according to the manufacturer's procedures. Nucleic acid was eluted in 100 µl of AE buffer.

The DNA extracted from infected cell culture was evaluated by a polymerase chain reaction (PCR) with five different primer sets for herpesvirus (KHV): consensus viral DNA polymerase primer set (L. Hanson, pers. comm.), consensus fish herpesvirus helicase primer set (D. Stone, pers. comm.), and three KHV primer sets (Gilad et al., 2002; Gray et al., 2002). The different reaction conditions for the different primer sets are outlined in Table 1. Products were visualized with UV light following electrophoresis and ethidium bromide staining. Amplicons produced from all five primer amplicon sets were purified in Microcon™ 100 (Amicon, Pullman, Washington, USA) filters and sequenced in both directions using the corresponding primers. Sequencing services were provided by Iowa State University, Ames, Iowa, USA. Sequences were proofread using the Sequencher program from the Gene Codes computer program package (Gene Codes Corporation, Ann Arbor, Michigan, USA). Alignment with KHV reference sequences was performed using Align Plus 5 (Scientific and Educational Software, Cary, North Carolina, USA).

DNA was extracted from the supernatant of cell cultures showing CPE and from filtered, primary tissue homogenates using the DNeasy® Tissue Kit (Qiagen, Valencia, California, USA). As recommended by the manufacturer, 200 µl of cell culture supernatant or filtered homogenate was mixed with 20 µl proteinase K and 200 µl Buffer AL, vortexed, and incubated at 70 C for 10 min. Further DNA extraction using DNeasy Mini Spin Columns was also performed as per manufacturer's instructions. Samples were assessed with real-time TaqMan PCR using methods, primers, and probes described by Gilad et al. (2004).

Gross necropsy findings in all cases were remarkably similar. All fish showed numerous external petechial to eccymotic hemorrhages that were particularly apparent ventrally and around pectoral, pelvic, and anal fin bases (Fig. 1). No evidence of hemorrhage was detected on the peritoneum, visceral surfaces of the abdominal organs or heart or in the intra-abdominal fat stores. Internal organs were bound together with numerous, fine, fibrous adhesions and fatty tissue, with several of these fibrous adhesions extending between the abdominal viscera and the peritoneal surface of the body wall. Adhesions such as these may be noted in normal, healthy common carp. The gill

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TABLE 1. Reaction conditions for the primer sets used to test for Koi herpesvirus in cell culture fluids from fathead minnow cultures inoculated with cell-free filtrates from moribund common carp from the Chadakoin River, New York, USA.

Primer identification	Primer sequence	Reaction and cycle conditions[a] (GenBank accession number)
Consensus DNA polymerase (Hanson, pers. comm.)	5′-CGG AAT TCT AGA YTT YGC NWS NYT NTA YCC-3′ 5′-CCC GAA TTC AGA TCT CNG TRT CNC CRT A-3′ H=A,C,T; Y=C,T; N=A,C,G,T; W=A,T; S=C,G; R=A,G.	A (DQ128163)
Helicase of fish herpesviruses (Stone, pers. comm.)	5′-GAG GAC CCG GAG TAY AGY GAR-3′ 5′-TAG CAG CTT GGC TTD ATN GGR-3′	A (DQ128162)
KHV9/5F	5′-GAC GAC GCC GGA GAC CTT GTG-3′	B (DQ128166)
KHV9/5R (Gilad)	5′- CAC AAG TTC AGT CTG TTC CTC AAC-3′	
BamHI-6 (Gray)	5′-TCG CAT GTG AGG GTT CAT GC-3′ 5′-CAT CAG CGG CAT CAG CAT CG-3′	B (DQ128164)
SphI-5 (Gray)	5′- GAC ACC ACA TCT GCA AGG AG-3′ 5′-GAC ACA TGT TAC AAT GGT GGC-3′	B (DQ128165)

[a] A = 1 μM each primer; 10% glycerol; 1X GeneAmp® PCR buffer; 2 mM $MgCl_2$; 200 μM each dNTP; 1.25 units AmpliTaq Gold™. Cycle conditions: 94 C for 9 min; 40 cycles of 94 C for 30 sec, 46 C for 2 min, 72 C for 3 min; 72 C for 7 min; hold at 4 C; B = 0.6 μM of each primer was used in these reactions. Cycle conditions: 94 C for 9 min; 40 cycles of 94 C for 30 sec, 55 C for 1 min, 72 C for 1 min; 72 C for 7 min; hold at 4 C.

tissue was markedly autolyzed in several cases. In the remaining cases the gills appeared to be slightly swollen, with those lamellae closest to the operculum being slightly pale in appearance. The skin scrape from the freshest fish showed no detectable abnormalities, although a single monogenean parasite was noted on the gill clipping.



FIGURE 1. Petechial and eccymotic haemorrhages ventrally around the base of the fins of a moribund common carp collected from the Chadakoin River, New York, USA.

Histologically, the gill tissues showed low-level infestation with monogenean parasites, epithelial hyperplasia with filling of interlamellar troughs, and fusion of adjacent primary lamellae (Fig. 2). The degree of organization in some areas indicated a degree of severity and chronicity that was in excess of what would be expected due to the low-level monogenean infestation alone. No other notable lesions were observed during microscopic evaluation of tissues from the carp submitted for examination.

A pure culture of *Aeromonas sobria* was cultured from the posterior kidney of all fish sampled.

Cytopathic effects (cell lysis and syncytia formation) were noted after 15 days and two blind passages on FHM cultures in duplicate flasks in samples from four out of seven fish. Samples from the remaining fish were culture negative. Electron microscopic examination of fluids off of infected cell culture revealed parti-

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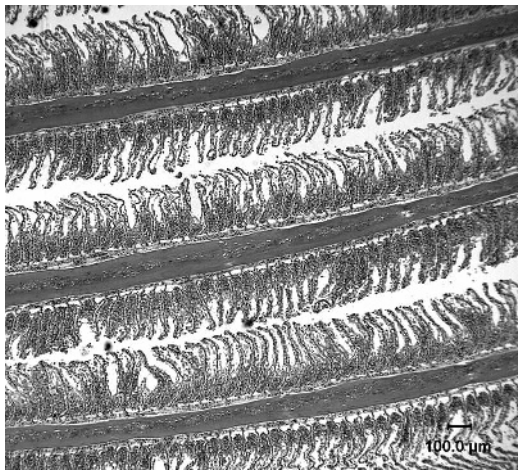


FIGURE 2. Gills of common carp *Cyprinus carpio* infected with koi herpesvirus. Interlamellar epithelial proliferation and fusion of adjacent lamellae can be seen.

cles with the morphologic characteristics of herpesviruses (Fig. 3), an icosahedral nucleocapsid approximately 100 nm in diameter (Hirsh and Zee, 1999).

Reverse transcription-polymerase chain reaction tests of fluids from infected cell cultures using SVCV-specific primers were negative. Polymerase chain reaction testing with KHV-specific primers yielded PCR amplicons of the predicted size. Sequencing of these PCR amplicons revealed 99–100% homology with KHV sequences deposited in GenBank (Table 1). Testing using the real-time Taqman PCR methods described by Gilad et al. (2004) yielded positive results for KHV.

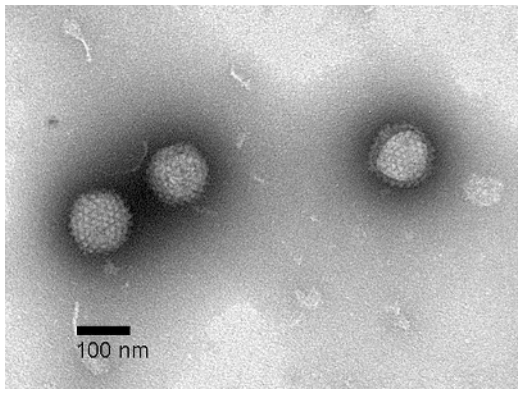


FIGURE 3. Electronmicrograph of herpesvirus particles in fluid from infected fathead minnow cell culture. The cell culture had been inoculated with a cell-free filtrate of pooled posterior kidney, liver, and spleen and had undergone two blind passages.

Koi herpesvirus has been reported to causes losses in all ages of common carp and koi (Hedrick et al., 2000). However, closely related species, such as grass carp (*Ctenopharyngodon idella*), do not appear susceptible to the virus (Ronen et al., 2003). Temperature appears to be a principal environmental factor in virus replication both in vivo and in vitro (Gilad et al., 2004). Maximal replication of the virus in koi fin (KF-1) cell cultures appears to occur between 15 and 25 C (Gilad et al., 2003). Koi herpesvirus induced mortalities on fish farms are seen in both spring and autumn when water temperatures range from 18 to 28 C (Pikarsky et al., 2004).

Koi herpesvirus is not considered to produce sustained CPE in either epithelioma papulosum cyprini or FHM cell lines (Hedrick et al., 2000). Virus isolation normally is carried out using KF-1 cell cultures with CPE detected within 7–10 days post-inoculation (Hedrick et al., 2000). Koi herpesvirus is often difficult to isolate using conventional cell culture methods, particularly if the fish has been dead for a long period of time or has been frozen. Diagnosis often relies solely on PCR results (Gilad et al., 2004). The isolate from carp recovered from the Chadakoin River was unusual because the virus replicated in the FHM cell line causing obvious CPE.

The inclusion of KHV in the family *Herpesviridae* has been somewhat controversial. Recent work has shown that two structural genes contributing to capsid architecture in other known herpesviruses are conserved in KHV, and two other genes encoding proteins involved in DNA replication are closely related to those seen in other cyprinid herpesviruses (CyHV-1 and CyHV-2) (Waltzek et al., 2005). Interestingly, since the worldwide emergence of KHV, several other viruses

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have been isolated from fish showing clinical signs of gill necrosis and mortality. The Centre for Environment, Fisheries and Aquaculture Science (CEFAS), Weymouth, UK, has isolated a paramyxo-like virus on several occasions (Haenan et al., 2004). Several different viruses have been identified in koi suffering gill necrosis and mortalities in Germany (Neukirch and Kunz, 2001). A disease outbreak in Korea in 2001 causing mortality and signs of respiratory distress yielded a virus that grew in the FHM cell line, and disease was reproduced experimentally; however, this virus was not definitively identified in the subsequent report by Oh et al. (2001).

In the Chadakoin River case, given that herpesvirus type particles were visualized by electron microscopy, and that sequence data of PCR amplicons shows 99–100% homology to KHV, we conclude that the isolate was, in fact, KHV. Transmission trials to fulfill River's postulates have yet to be performed. Further work using transmission trials will help determine the pathogenicity of this KHV isolate as well as clarify the relative roles of the virus and *A. sobria* in the carp mortality and the hemorrhagic condition observed in the fish.

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