

Initial Isolation and Characterization of a Herpes-like Virus (KHV) from Koi and Common Carp

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Abstract

In September of 1998 our laboratory was asked to investigate epidemics characterized by high mortality occurring in populations of koi (*Cyprinus carpio*) in both the U.S.A. and Israel. Either live fish or frozen tissues arrived from both locations in September and November of 1998. In addition, tissues fixed for both light and electron microscopy from fish in Israel and the U.S.A. were obtained for examination. The virus observed and then isolated in 1998 from these initial samples has become the focus of a worldwide concern for the health and welfare of both captive and wild populations of common carp and ornamental koi. In this short report we review the initial isolation and characterization of the herpes-like virus referred to as koi herpesvirus or KHV. We also discuss the important role of temperature on in vivo and in vitro infections with the virus, recent comparisons of KHV to other herpes-like viruses from fish and lastly review current methods to detect the virus or evidence that fish have been exposed to the virus.

Key words: koi, common carp, koi herpesvirus, KHV, herpes-like viruses

Introduction

Outbreaks of a new disease among koi and common carp (*Cyprinus carpio*) may have occurred as early 1995 - 1996 but were more formally recognized in Germany in 1997 (Bretzinger *et al.*, 1999). The actual cause of the disease however, was not identified until 1998 following investigations of outbreaks among koi and common carp in Israel and the U.S.A. (Hedrick *et al.*, 1999, 2000). Those investigations demonstrated the presence of a herpes-like virus, designated koi herpesvirus or KHV, in diseased koi from Israel and the U.S.A. that was successfully isolated by use of a newly developed cell line from koi fin (KF-1). The isolated virus was demonstrated to induce the same disease and high mortality

characteristic of the natural outbreaks following experimental infections of koi in the laboratory (Hedrick *et al.*, 2000).

Several viruses have been isolated from koi undergoing high mortality and suffering from gill and skin diseases (Body *et al.*, 2000, Neukirch *et al.*, 1999, Oh *et al.*, 2001). However, the viruses in these reports clearly differ from KHV with respect to virion morphology, cytopathic effects (CPE) induced and types of cell lines that are susceptible, and the inability to cause disease upon experimental exposures of koi. Virus isolations that are similar or identical to KHV have been reported more recently by Neukirch and Kunz (2001), Ronen *et al.* (2003) and Perelberg *et al.* (2003) with the latter two reports preferring the designation for the agent as carp nephritis and gill necrosis virus (CNGV).

KHV has presumably spread through the largely unregulated movements of koi, although other unknown mechanisms may also be involved. The virus now has a global distribution that includes the U.S.A., several European countries, South Africa, Indonesia, China, Taiwan, and most recently Japan (Waltzek and Hedrick, 2004, Tu *et al.*, 2004, Sano *et al.*, 2004). In this short review we discuss the original isolation of the virus, the important effects of temperature on the agent, some initial and more recent characterization of the virus and close with a summary of currently used diagnostic methods.

Signs of the disease

The external signs seen in sick fish include swollen and necrotic gill filaments, excessive mucus production or discolored patches on the skin (Fig. 1), and enophthalmos (Walster, 1999, Hedrick *et al.*, 2000). Internally, the kidney and spleen may be enlarged.

Virus isolation

The development of the koi fin (KF-1) cell line in our laboratory in 1997 made possible the isolation of KHV as most common cell lines used by fish virology laboratories were not susceptible to the new virus. The virus was isolated from numerous tissues of fish with signs of the disease, including the gill, kidney, spleen, gut and liver. The virus induced cell fusion and intense cytoplasmic vacuolation in KF-1 cells within 5 days after inoculation at 20°C (Fig. 2). More complete CPE or cell lysis was evident at 7 – 10 days and progressed to involve all the cells after 14 days.

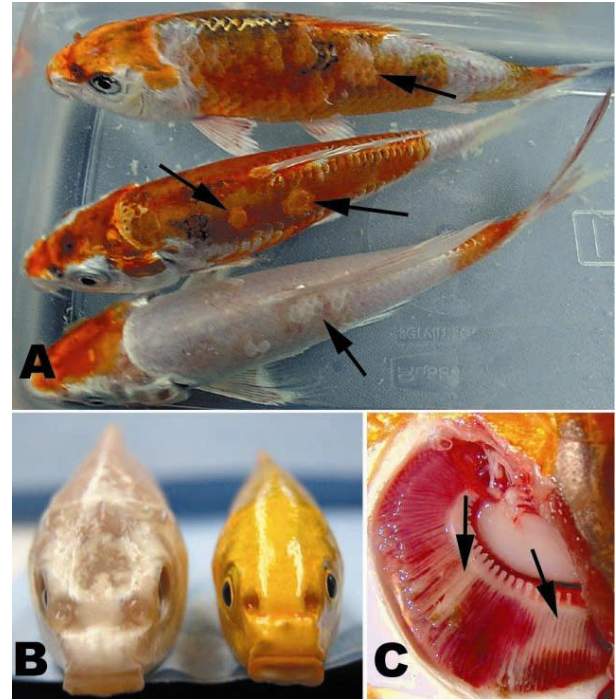


Fig. 1. Juvenile koi with koi herpesvirus disease (KHVD). (A) White patches on the lateral body surface of all three fish are evident (arrows) and (B) bilateral enophthalmos (sunken eyes) in the fish on the left with KHVD compared to a healthy fish on the right and (C) the discoloration and necrosis of gill filaments.



Fig. 2. Koi fin (KF-1) cells 7 days following infection with the koi herpesvirus (KHV) at 20°C.

Large and frequent cytoplasmic vacuolation is present in several cells most of which are involved in syncytium formation.

Bar = 100µm

Effects of temperature

Temperature is a dominant environmental factor that affects the onset and severity of KHV outbreaks. Most outbreaks occur during the spring and autumn (in the Northern Hemisphere) at water temperatures from 18 – 26 °C (Bretzinger *et al.*, 1999, Walster *et al.*, 1999, Hedrick *et al.*, 2000, Perelberg *et al.*, 2003). At lower water temperatures the virus can infect fish without inducing clinical signs of disease but when permissive water temperatures are again experienced the fish undergo typical disease and mortality (Bretzinger *et al.*, 1999, Gilad *et al.*, 2003, Gilad *et al.*, 2004). KHV replicates best in the koi fin cell line (KF-1) at 20 – 25°C but no growth is observed at 30°C or 4°C and only minimal growth occurs at 10°C (Gilad *et al.*, 2003). The known temperature range of KHV has led to experimental means to control the disease which include infecting fish at permissive temperatures and then shifting them to restrictive temperatures to avoid clinical disease and to induce immunity to re-infection. It appears that shifting temperatures either above or below the limits (e.g. 30°C or 13°C) will arrest development of clinical signs (Ronen *et al.*, 2003, Gilad *et al.*, 2004). Regimens of exposure and shifting to high water temperatures have been used in large scale trials in Israel to produce fish that have been called “naturally resistant” to KHV. Concerns remain whether these fish, while resistant to re-infection, may be potential carriers of the virus. Gilad *et al.* (2004) found that fish infected at 13°C did not experience clinical signs but did harbor viral DNA as detected by TaqMan PCR. Upon shifting to water temperatures of 23°C at 30 days post virus infection, the fish underwent disease and mortality. Curiously, fish held for 64 days post infection at 13°C and shifted to 23°C did not experience disease or mortality but whether these fish are immune to re-infection was not tested.

Virion characteristics

Electron microscopy of the virus present in and released from infected KF-1 cells shows a morphology and size consistent with viruses in the family *Herpesviridae*. Virions are composed of an inner capsid with icosadeltahedron symmetry of approximately 100 - 110 nm in diameter (Fig. 3).

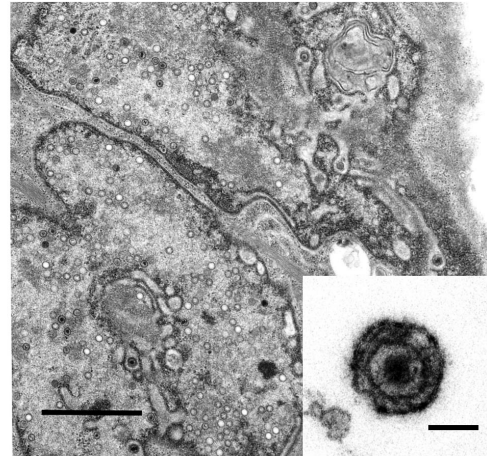


Fig. 3. Virions of the koi herpesvirus (KHV) as present in infected KF-1 cells. The effects of the virus include thickening and distortion of the nuclear membrane. Nucleocapsids of KHV are evident throughout the nucleoplasm. Bar = 500 nm. Inset figure is a complete KHV virion showing the viral envelope, the tegument (lying between the envelope and the nucleocapsid), and the hexagonal nucleocapsid. Bar = 100 nm.

KHV virions possess a tegument lying between the loosely applied envelope and the nucleocapsid that give the mature virion an overall diameter of 170 – 230 nm. Identical virus particles were observed in tissues from koi involved in epidemics in Israel and the USA. Based on the morphology and size of the virus and the sequential development in the host cell nucleus, the new virus was initially designated KHV for koi herpesvirus (Hedrick *et al.*, 2000). The same agent was isolated subsequently from koi and common carp by Ronen *et al.* (2003) although they preferred the designation of carp nephritis and gill necrosis virus (CNGV) for the agent.

Koch's postulates

The ability to isolate and then grow KHV in the KF-1 cell line provided the first steps in confirming the virus as the cause of the new disease. The virus was first isolated from fish showing signs of the new disease and the virus reproduced the disease in experimentally-infected fish (Hedrick *et al.*, 2000). Re-isolation of the virus from experimentally-infected fish confirmed that KHV was indeed the cause of this new disease. Similar studies for the virus designated CNGV were later described by Perelberg *et al.* (2003) and several other groups have also confirmed the virulent nature of KHV as isolated from infected fish.

KHV Differs from other cyprinid herpesviruses

That the new virus differed from *Cyprinid herpesvirus 1* (CyHV-1) or the agent known to cause carp pox (Sano *et al.*, 1985a, b, 1993b) was suggested by differences in the diseases caused by both agents. Additionally, anti-CyHV-1 antibodies failed to react with KHV in immunofluorescence assays (Hedrick *et al.*, 2000). Subsequently, differences in the virion proteins and genomic sequences provided additional evidence that the two viruses were distinct agents (Gilad *et al.*, 2002). CyHV-1 can cause mortality among koi and common carp but only among fish younger than 2 months in age (Sano *et al.*, 1985b). Also, survivors of CyHV-1 infections develop characteristic papillomatous-like growths commonly known as carp pox (Schubert, 1966). One additional herpes-like virus, *Cyprinid herpesvirus 2* (CyHV-2) originally named goldfish hematopoietic necrosis virus (GFHNV) has been observed by electron microscopy and has been isolated from goldfish (*Carassius auratus*) with severe hematopoietic necrosis (Jung and Miyazaki, 1995). The virus is quite difficult to grow in established cell lines (Groff *et al.*, 1998, H. Fukuda, pers. comm., unpublished

observations). Recent comparisons of the genomic DNA and virion polypeptides of KHV to CyHV-1 indicate sufficient relationships to consider them related but clearly distinct agents and that has allowed the unique detection of each virus by newly developed PCR assays (Gilad *et al.*, 2002, Gray *et al.*, 2002, H. Fukuda, pers. comm., unpublished data). The biological properties of the three cyprinid herpes-like viruses (CyHV-1, CyHV-2 and KHV) also supports the unique nature of the three agents. KHV, unlike CyHV-1, is highly virulent and induces mortality among all sizes of koi and common carp (Bretzinger *et al.*, 1999, Hedrick *et al.*, 2000, Perelberg *et al.*, 2003). CyHV-1 is lethal only for koi and common carp less than 2 months of age (Sano *et al.*, 1985a, b). There is no evidence of papilloma formation in fish surviving KHV infection. Host range is also a clear factor separating the three cyprinid herpes-like viruses. Goldfish are highly susceptible to CyHV-2 but resistant to KHV and CyHV-2 fails to induce disease in koi injected with the virus (Jung and Miyazaki, 1995, Bretzinger *et al.*, 1999, Ronen *et al.*, 2003, Perelberg *et al.*, 2003, unpublished results).

Relationships with other herpes-like viruses from fish

The large size of the genome of KHV, which is estimated at 277 kbp, exceeds that of 250 kbp known for members of the family *Herpesviridae* (Ronen *et al.*, 2003). While genome size is not currently considered a criterion for placement or exclusion of viruses in the family, the differences in sequences so far obtained for KHV have shown little similarities to those known from herpesviruses from birds or mammals. That KHV differs but is closely related to two cyprinid herpesviruses currently classified in the family *Herpesviridae* has recently been established (Waltzek *et al.*, in preparation). Proposals are developing that involve considerable taxonomic changes to the family

Herpesviridae including the creation of higher taxa that would accommodate the large differences seen between herpes-like viruses from fish, amphibians and mollusks from those herpesviruses from birds, reptiles and mammals (A. Davison, pers. comm.). These revisions would formally place the herpes-like viruses from fish into a unique taxon within the family *Herpesviridae*. KHV would then be designated as the third cyprinid herpesvirus, *Cyprinid herpesvirus 3* or CyHV-3 (Waltzek *et al.*, in press). Formal proposals to the International Committee on the Taxonomy of Viruses (ICTV) to establish this nomenclature for KHV or CNGV are in progress.

In support of this taxonomic proposal is data from our initial studies on virion polypeptides and RFLP analyses of the genomes of CyHV-1 and KHV that clearly showed they are related viruses (Gilad *et al.*, 2002). More recently comparisons by Waltzek *et al.* (in press) of the sequences of 3 genes common to CyHV-1, CyHV-2 and KHV show a strong relationship (up to 80% homology at the nucleotide and amino acid level). This relationship provides sufficient justification to designate KHV as a third herpes-like virus from cyprinids. Additional comparisons in our laboratory of the DNA polymerase gene of these 3 cyprinid herpes-like viruses to a range of herpes-like viruses found in eel, sturgeon, trout, catfish, walleye and frogs indicate they form a group of unique viruses quite distant from other members of the family *Herpesviridae* and other viruses in general (unpublished data). Although, this group of herpes-like viruses from fish and amphibians differs significantly from other members of the family *Herpesviridae*, changes to the family mentioned above will accommodate these distantly related groups.

Current detection methods

A presumptive diagnosis of KHV disease includes observation of high mortality despite treatments for bacterial and or external parasites among koi or common carp with characteristic external pathological signs at water temperatures ranging from 18 – 26°C. Confirmatory diagnoses require demonstrations of the presence of virus by isolation using KF-1 or other KHV susceptible cells followed by polymerase chain reaction (PCR) testing of virus isolates or fish tissues directly by one of two PCR assays (Gilad *et al.*, 2002, Gray *et al.*, 2002). Unfortunately, it is often difficult to isolate or to enumerate concentrations of KHV from infected koi using cell culture methods, particularly if the fish have been dead for several hours or have been frozen. Detection may therefore, rely solely on the PCR results obtained from fish tissues.

The principal target tissues for KHV based on microscopic pathological exams and recent quantitative PCR assays of naturally and experimentally-infected koi are the gill, kidney and spleen but other tissues including the skin, brain, gut and liver, are also involved (Hedrick *et al.*, 2000, Gray *et al.*, 2002, Gilad *et al.*, 2003, Gilad *et al.*, 2004). Current diagnostic approaches most often use portions of the gill, kidney and spleen for virus isolation and or PCR analysis. Standard PCR approaches have not demonstrated the ability to routinely detect evidence for the virus in the tissues of suspected carriers of KHV, which is viewed as important to controlling the spread of the virus.

Since the initial isolation of KHV in 1998, a number of new and improved methods have been developed to detect the virus. That isolation of the virus was difficult was shown early and thus other detection methods have been rigorously pursued. The PCR method has proven to be an effective means to detect viral DNA in a number of fish tissues during the acute disease and for a period following

recovery. As many as 7 different PCR tests for KHV are currently in use in different laboratories throughout the world (Gilad *et al.*, 2002, Gray *et al.*, 2002, H. Bercovier, K. Way, M. El-Matbouli, pers. comm.). These tests have greatly improved the ability to detect evidence for the virus but leave unresolved whether carriers with persistent or latent infections occur following acute disease. Under additional development are sensitive and specific enzyme linked immunosorbent assays (ELISA). One such ELISA was described by Ronen *et al.* (2003) and it effectively detected progressive increases in anti-KHV antibodies in naturally exposed koi. A similar assay developed in our laboratory using a monoclonal antibody to carp immunoglobulin is now under field testing and preliminary evidence suggests it is quite effective in detecting prior exposures to the virus, even up to 1 year past the last evidence of clinical signs in the koi population (Adkison *et al.*, in preparation). Lastly, that the serum antibodies detected by the ELISA are at least partially protective to virulent virus challenge was demonstrated in passive immunity trials with juvenile koi (unpublished data). These studies suggest that anti-KHV antibodies may serve as useful diagnostic indicators of prior exposure to the virus and perhaps as good indicators of the immune status following virus exposure or vaccination (Ronen *et al.*, 2003).

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