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Characterization and Control of the Koi Herpesvirus (KHV), a Newly Recognized Pathogen of Koi (*Cyprinus carpio koi*) and Common Carp (*Cyprinus carpio carpio*)

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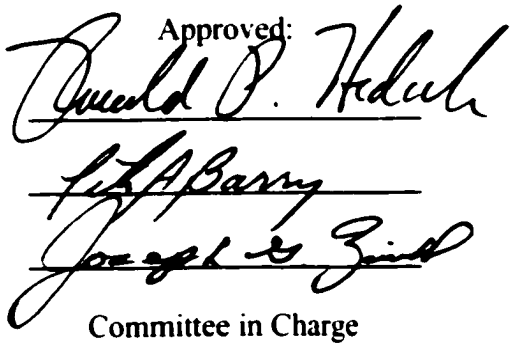

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I would like to express my gratitude to all of the individuals who left their mark on me during my long journey.

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Characterization and Control of the Koi Herpesvirus (KHV), a Newly Recognized Pathogen of Koi (*Cyprinus carpio koi*) and Common Carp (*Cyprinus carpio carpio*)

ABSTRACT

Since 1998, episodes of mass mortality have occurred in populations of common carp *Cyprinus carpio carpio* and koi *Cyprinus carpio koi*. A herpesvirus, termed koi herpesvirus (KHV) isolated from infected fish has been shown to be the cause of the disease which has now been detected in North America, Europe, Israel and Asia.

KHV has 31 virion polypeptides. Both virion polypeptide and restriction fragment length polymorphism (RFLP) analyses of genomic DNA showed that the first two isolates of KHV isolated from koi in Israel and the U.S. were identical; furthermore, KHV was clearly distinguished from *Herpesvirus cyprini* (CHV) the only other herpesvirus isolated from *Cyprinus carpio*.

A polymerase chain reaction (PCR) assay to detect the virus in koi tissues was developed viral sequences obtained from a restriction fragment of KHV genomic DNA. The PCR detected KHV DNA in koi from naturally occurring outbreaks and from koi following experimental infections with KHV induced by intraperitoneal injection or bath challenges.

A comparison of the virion polypeptides and genomic RFLP of 7 geographically diverse isolates of KHV indicated they represent a homogeneous group with the exception of a single isolate from koi in Israel.

Optimal KHV growth occurred in the koi fin (KF-1) cell line at temperatures from 15 – 25°C. Experimental infections of koi by bath exposures to KHV resulted in the greatest cumulative mortality (95.2%) at a water temperature of 23°C. Significant mortality also occurred among virus-exposed koi at water temperatures of 28 and 18°C. No mortality was observed at water temperature of 13°C but, when virus-exposed fish originally held at 13°C were shifted to water at 23°C, a rapid mortality ensued. Survival analyses indicated a significant difference in risk of mortality between the virus-exposed fish at the different temperatures.

A real-time TaqMan PCR assay was developed to detect and quantify KHV DNA for diagnostic and research purposes. Virus concentrations in tissues of experimentally infected koi increased over time at all water temperatures tested (13, 18, 23, and 28°C). High concentrations of the virus at early time points in the mucus suggest that the skin may be an initial site of virus replication. At subsequent time points the virus was found in gill, kidney, spleen, liver, gut and brain. The principal target tissues during active infection are the gill, kidney and spleen. In addition, low copy numbers of KHV DNA were detected in fish that survived KHV at 62 - 64 d post initial virus exposure. There was little evidence for anti-KHV neutralization activity in the serum of koi previously exposed to the virus, thus, detection of KHV DNA may be the most reliable indicator of prior exposures to the virus.

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INTRODUCTION

Koi (*Cyprinus carpio koi*) are beautiful and colorful fish and part of a worldwide hobby that includes holding or rearing of koi in backyard ponds and large display aquaria for personal pleasure or for competitive showing. A significant industry developed around the reproduction and rearing of koi has now spread worldwide. Large numbers of live koi are therefore moved locally, regionally, and internationally to support this hobby. Most movements occur in the complete absence of health inspections or certifications and as a result the spread of serious fish pathogens is a major concern. In the past five years, mass mortality episodes have occurred in both common carp (*Cyprinus carpio carpio*) and koi held by producers, retailers, and individual hobbyists. The cause of many of these outbreaks has now been traced to the presence of a newly recognized viral pathogen.

Through examinations of koi and common carp from the U.S.A. and Israel with the new disease we have identified a herpesvirus, now termed the koi herpesvirus or KHV as the causative agent. The further characterization of KHVand development of improved means to detect the virus are now important next steps in the potential control of the virus.

Studies were needed to establish whether KHV was a newly recognized or a variant a known herpesvirus, the cyprinid herpesvirus (CHV), previously described from koi and common carp. Furthermore, reports of diseases similar to the original outbreaks due to KHV were increasing in frequency and in their geographic range. Whether these outbreaks were due to KHV and whether different strains of the virus might of exist were

important to determine. Upon the discovery of KHV, the only diagnostic methods available were isolation of the agent on a newly developed cell line (KF-1) from koi fin tissues. Isolation of the virus from fish after death or from frozen or otherwise compromised samples was shown to be very difficult. Therefore, the development of improved diagnostic methods was critical and this was approached by developing conventional and real-time polymerase chain reaction (PCR) assays for KHV. Temperature is a key variable controlling virus infections in poikilothermic animals both by influencing the efficacy of the host immune response and by directly influencing the rate of virus replication. The effect of temperature on KHV replication in the KF-1 cell line and in koi following experimental exposures to the virus were examined. Newly developed PCR assays were used to evaluate the tissue sites and concentrations of KHV in koi exposed to the virus at different water temperatures. Lastly, survivors of experimental exposures to KHV were examined to better understand the potential for persistence of the virus in these fish as virus carriers are the suspected means by which KHV has spread with the worldwide trade in koi.

LITERATURE REVIEW

The Importance of Fish

Fish is a major protein source world wide, the projections of world fishery production in 2010 range between 107 and 144 million tones, most of the increase in fish production is expected to come from aquaculture which is growing rapidly (www.fao.org). Fish is a particularly important protein source in regions where livestock is relatively scarce, fish supplies less then 10% of animal protein consumed in North America and Europe, but 17% in Africa, 26% in Asia and 22% in China (www.fao.org). Total aquaculture production reached nearly 36 million tones in 2000 in a total value of 50 billion US$, with carp, barbels and other cyprinids production of nearly 16 million ton in a value of 15 million US$ (www.fao.org). It has been reported that the economical loses in common carp due to KHV infection were estimated as much as 5 million US$ in Israel during 1998 and more recently in Indonesia within three month period (Rukyani 2002). In addition to food fish, aquaculture is also involved in raising fish for the ornamental fish industry in North-America (mainly in the U.S.A), Europe (throughout the continent), the middle-east (mainly Israel), Africa (mainly South-Africa) and Asia (mostly in Japan).

Fish Herpesviruses

Approximately 130 herpesviruses have been identified to date from different animal species; 9 are known to infect humans with most others found among other vertebrates, including fish (Roizman et al. 2002). Although the knowledge on viruses of

poikilothermic animals lags behind that of homoeothermic species, research on viruses of lower verterbrate has steadily increased with growing recognition of the ecologic and economic of aquatic animals (Essbauer & Ahne. 2001). Herpesviruses are commonly identified as the causes of diseases in fish, ranging from rather benign skin conditions to fatal systemic infections (Hedrick & Sano. 1989). The best known herpesviruses are from the channel catfish virus (CCV) from channel catfish (*Ictalurus punctatus*) as originally described by Fijan et al. (1970), the salmonid herpesviruses such as SalHV-1 (Wolf & Taylor. 1975), and the cyprinus herpesvirus (CHV) originally observed associated with cases of carp pox (Schubert 1966). The only information available on genomic properties of known fish herpesviruses are those of CCV and SalHV-1 as the genomes of these two fish herpes-like viruses have been sequenced. The CCV genome size is 134 kbp, with unique and repeat regions containing 97 kbp and 19 kbp, respectively. The DNA G+C nucleotide composition of CCV is 56.2% (Davison 1992). The coding capacity is explained most simply in terms of single exon genes, but three open reading frames (ORF) (62, 69, and 71) may be expressed by splicing (Davison 1992). The CCV genome shares some general characteristics with other herpesviruses including a similar genome size and specific termini which appear not to be covalently blocked (Davison 1992). The genome size of SalHV-1 is 174 kbp, consisting of a long unique region (133 kbp) linked with a short unique region (26 kbp), which is flanked by two inverted repeats (8 kbp) (Davison 1998). SalHV-1 shares at least 18 related genes with CCV. Both viruses have little homology to other mammalian herpesviruses at the nucleotide level but increased homology at the amino acid sequence (Davison 1998).

Channel Catfish Virus (CCV)

Perhaps the most studied herpesvirus from fish is channel catfish virus (CCV) because of its importance to the large aquaculture industry in the southeastern U.S.A. CCV causes an acute, hemorrhagic, and lethal infection among young channel catfish. External signs include hemorrhaging at the base of the fins, abdominal swelling, compromised equilibrium, pale gills, and exophthalmia (Noga 1996). Internal signs include the presence of ascites and petechial hemorrhages in the viscera. Microscopic lesions are characterized by necrosis of numerous cell types of the kidney, liver, spleen and skeletal musculature (Noga 1996). The virions of CCV obtain their envelope at the inner lamellae of the nuclear membrane and by budding into nuclear vacuoles. There is also extensive replication of the virus in the nuclear membrane (Wolf & Darlington. 1971). The nuclear membrane appears ruptured or disintegrated, and as a result, many unenveloped particles appear in the cytoplasm of the cell (Wolf & Darlington. 1971). Most fish herpesviruses have a common morphology and size including CCV, with a nucleocapsid of approximately 100 nm surrounded by an envelope of 175-200 nm in diameter (Wolf & Darlington. 1971).

Cyprinid Herpsvirus (CHV)

CHV is the only herpesvirus isolated from cyprinid fish. It was first recognized in association with the condition called "carp pox" (Schubert 1966, Sano et al. 1985a, 1985b), a seasonal skin disorder, characterized by the appearance of mucoid to waxy epidermal tumors (Sano et al. 1985a, 1985b). Internal signs may include spinal deformities and emaciation (Wolf 1988). Although the carp pox lesions were initially

thought to be the only manifestation of CHV, subsequent studies have revealed a second phase of infection in young (less than 2 mo in age) koi and common carp (Sano et al. 1991). This second and more serious phase is characterized by a systemic infection that can result in significant mortality. The key tissues involved with CHV infection are the brain, gill, liver, kidney, intestine, esophagus and skin with a cumulative mortality as high as 97% among two-week-old common carp, 20% in four-week-old fancy carp and no mortality in both eight-week-old common and fancy carp (Sano et al. 1991). The survivors of these early infections are now known to later demonstrate the more typical carp pox epidermal lesions. Clusters of virions can be found inside and outside inclusion bodies in cells infected with CHV and are more numerous in the karyoplasm of cells undergoing karyorrhexis. Budding immature CHV particles from the nuclear membrane as well as released into the intracellular space can be observed in infected cells (Sano et al. 1985b). The virion of CHV has a nucelocapsid diameter of 113 nm and an envelope diameter of 190 nm (Sano et al. 1985b).

Koi Herpesvirus (KHV)

In contrast to CHV, KHV was initially reported in older fish and was characterized as a gill disease and only later recognized as a more systemic condition (Hedrick et al. 2000). The disease caused by the KHV lacks specific external signs but fish dying from KHV infection have pale and irregularly colored gills which upon microscopic examination reveal severe hyperplasia and necrosis of the respiratory epithelium. In addition to the gill, other organs are affected including the kidney, spleen, liver and intestine. Infected cells in these organs contain enlarged nuclei with marginated

chromatin and containing faint eosinophilic inclusions (Hedrick et al. 2000). Virus particles found in the infected cells or as isolated from infected cell cultures are characterized by hexagonal nucleocapsids (110 nm) surrounded by a loose membrane or envelope with a diameter of 180-230 nm.

In recent years mass mortality has been observed in both common carp and koi in countries throughout the world including the U.S.A., Germany, England, Italy, Netherlands, Israel and most recently Indonesia due to a suspected viral pathogen (Hedrick et al. 2000, Rukyani 2002, Gilad et al. in press). Major losses have occurred both to the aquaculture industry in Israel and to retailers and hobbyists in the U.S.A. The virus isolated from fish involved in these losses has been shown to be a herpesvirus termed the koi herpesvirus or KHV (Hedrick et al. 2000). An active international trade in live fish, including koi, has contributed to the movements of significant fish pathogens, particularly when these movements occur without health certifications or inspections (Hedrick1996, Hoffman 1970, 1990). Unfortunately, this active movement of koi continues to the present and we suspect that KHV will spread unabated.

Virus isolation is the most common diagnostic approach to the detection of fish viruses (Wolf 1988). Virus can generally be isolated during episodes of acute infection and correlated to both gross and microscopic signs of the disease. Chronic and latent infections however, have required the use of additional detection procedures including PCR and in situ hybridization (ISH). A PCR assay developed for CCV was able to detect CCV genomic DNA sequences in catfish during both acute (Gray et al. 1999a) and latent infections (Gray et al. 1999b). In situ hybridization has also been found to be effective in detecting CHV DNA in infected koi tissues (Sano et al. 1992, 1993).

Herpesvirus carriers and latency

During persistent infection there is a limited expression of viral proteins but sufficient to keep the virus in a non-reproductive stage and this may be considered a latent stage. Latency, is considered to be an integral part of the herpesviruses life cycle with the formation of either circular or concatenated molecules, as was demonstrate for α and γ herpesviruses (Lindahl et al. 1976, Rock & Fraser. 1983) Latent infections either in nervous or subcutaneous tissues of koi with CHV or in peripheral blood leukocytes of channel catfish with CCV have been proposed (Sano et al. 1993, Baek & Boyle 1996, Gray et al. 1999b). The potential for latentcy with other fish herpesviruses is presumed but currently unproven.

Anti-viral Immunity

Anti-viral immunity results from contributions of both the humoral and cell-mediated arms of the response in vertebrates (Kuby 1997). The humoral response is characterized by the presence of antibodies that block the attachment and fusion of the virus to the host cell membrane. Antibodies also enhance phagocytosis of viral antigens and can agglutinate viral particles. Complement contributes by formation of the membrane attack complex. IFNα (secreted by leukocytes) and IFNβ (secreted by fibroblasts) both target uninfected cells to inhibit viral replication. IFNγ secreted by Th1, CTL (cytotoxic T leukocytes) and NK (natural killer) cells target uninfected cells (inhibiting future viral replication, increasing MHC-I and MHC-II expression), macrophages (enhancing their activity), and inflammatory cells (affecting delayed-type hypersensitivity) (Kuby 1997). The cell mediated immunity is characterized by the

functions of CTL that actively and specifically kill virus-infected cells and by the less specific functions of NK cells that function to also kill virus-infected cells (Kuby 1997).

The humoral response to viral infections in fish is poorly understood but presumably involves similar mechanisms to those in higher vertebrates. Fish serum and certain mucus secretions contain lysozyme, complement, interferon and C - reactive protein (CRP) which activate the complement system via the classical pathway (Iwama & Nakanishi. 1996). Studies with fish rabdoviruses suggested that antibodies can have a protective role during late viral infection (Hattenberger-Baudouy et al. 1989, Lorenzen et al. 1999). Vaccination to protect animals from herpesvirus infections has targeted the glycoproteins on the surface of the virion since these proteins are responsible for the recognition, attachment, and entry of the virus into the host cell (Osterrieder et al. 1995). Moreover, DNA vaccines that express selected glycoproteins (gB, gC, gD or gD) of pseudorabies virus have been shown to be effective in protecting pigs after exposures to infectious virus (Gerdts et al. 1999).

Although T-cell subset have yet to be demonstrated, it is well establish that fish posses lymphocyte populations analogous to B and T cells, nonspecific CTL (similar to NK cells), macrophages and granulocytes (Nakanishi et al. 1999). It was also suggested that fish possess distinct populations of NK-like, PBL-derived cytotoxic cells capable of lysing allogeneic and virus-infected target cells (Hogan et al. 1997) and that virus-specific cytotoxic cells have a role in controlling viral infection (Somamoto et al. 2002).

Initial Characteristics of Koi Herpesvirus (KHV) and Development of a Polymerase Chain Reaction (PCR) Assay to Detect the Virus in Koi *Cyprinus carpio koi*

ABSTRACT

Since 1998, episodes of mass mortality have occurred in populations of common carp *Cyprinus carpio carpio* in Israel and in populations of koi *Cyprinus carpio koi* in Israel and the USA. A herpesvirus isolated from infected fish has been shown in experimental studies to induce a disease and mortality similar to those observed in outbreaks at infected farms. Initial characteristics of the virus show that it is clearly different than *Herpesvirus cyprini* (CHV), the most commonly known herpesvirus from cyprinid fish. The koi herpesvirus (KHV) has 31 virion polypeptides. Twelve of the virion polypeptides of KHV have similar molecular weights to those of CHV and 10 are similar to those of channel catfish virus (CCV). Both virion polypeptide and restriction fragment length polymorphisms (RFLP) analyses of genomic DNA showed that the first KHV isolates from Israel and the USA were identical. In contrast, the genomic DNA restriction fragments clearly distinguish KHV from CHV and CCV. A polymerase chain reaction (PCR) assay to detect the virus in koi tissues was developed with sequences obtained from 1 restriction fragment of KHV DNA. The PCR assay effectively detected 484 base pair sequence from KHV but did not amplify genomic DNA from either CHV or CCV. The PCR assay detected as little as 1 pg of KHV DNA as mixed with 100 ng of host DNA. Viral sequences were amplified from koi obtained from field collections and from koi that were experimentally exposed to 10^2 $TCID_{50}$ ml^{-1} KHV via the waterborne route.

All KHV exposed fish dying of infection between 8 – 10 d post-exposure or surviving to 14 d post exposure were found to be positive by PCR, while unexposed control koi were all negative. The assay also showed the presence of KHV DNA in tissues of koi obtained from farms in Israel. The PCR assay should assist virus isolation procedures and histologic and electron microscopic analyses now commonly used to detect KHV infection. Current studies are examining the possibility of using the PCR to detect KHV DNA in live fish and the relative sensitivity and specificity of the KHV PCR assay as compared to other diagnostic tests.

INTRODUCTION

Recent and mass mortality among younger and older common carp (*Cyprinus carpio carpio*) and fancy carp or koi (*Cyprinus carpio koi*) have occurred in the U.S.A, Western Europe, and Israel (Bretzinger et al. 1999, Neukirch et al. 1999, Body et al. 2000, Hedrick et al. 2000). Several of these outbreaks are now suspected to be due to systemic infections with a newly recognized virus, the koi herpesvirus or KHV (Hedrick et al. 2000). Mortality begins typically 7 – 10 d following exposure of previously uninfected koi and common carp to suspected carrier fish and the cumulative mortality in certain populations can approach 100% over a 2 – 3 wk period (Hedrick et al. 2000). Infected fish have pale and irregular coloration of the gills and skin, with few other external or internal signs. Microscopic examinations of the liver, spleen, and kidney demonstrate necrosis of parenchymal cells and numerous macrophages with ingested cellular debris (Hedrick et al. 2000). Intranuclear inclusions may be evident in infected

cells and virions with characteristic herpesvirus morphology can be observed directly in these tissues (Bretzinger et al. 1999, Body et al. 2000, Hedrick et al. 2000). Hedrick et al. (2000) was able to isolate a herpesvirus (KHV) using a koi fin cell line (KF-1) that was identical to the virus found by electron microscopy in infected fish tissues. Furthermore, they were able to demonstrate that juvenile koi exposed to KHV by bath challenges developed disease and experienced mortality that was similar to that observed in the field.

The virus recovered from dying carp by Hedrick et al. (2000) appears to be a newly recognized herpesvirus that differs from cyprinid herpesvirus (CHV) the only other previously isolated herpesvirus from koi and common carp. CHV was initially observed by Schubert (1966) by electron microscopy and later isolated and more thoroughly characterized by Sano et al. (1985a, b). CHV was found to cause both papillomatous skin growths or carp pox lesions in older fish and systemic and lethal infections in koi and common carp less than 2 mo in age (Sano et al.1991).

The rapid spread of KHV is presumably related to the worldwide trade and showing of koi or fancy carp, since most of these movements occur in the absence of any health examinations or certifications. The transport of pathogens with live fish has been a major concern for those responsible for fish health regulations who try to balance the need for trade with disease control programs (Hedrick 1996). Inspection of fish prior to shipment or at the farm sites where they are produced is one effective measure to prevent the spread of serious pathogens. These control programs rely upon sensitive and specific detection procedures for the pathogens of concern. With KHV, the only current diagnostic procedure is isolation of the virus using the KF-1 cell line, a procedure that requires 7 – 10 d incubations at 20°C (Hedrick et al. 2000). This method is effective in

detecting virus during mortality episodes but appears inadequate to detect KHV among carriers, the fish now believed responsible for the spread of KHV. In this study we provide initial characteristics of the viral genome and virion proteins of KHV as isolated from koi in the USA and Israel as compared with CHV. Using the newly obtained DNA sequence data from these comparisons, we developed and have initially tested a polymerase chain reaction (PCR) assay for detecting KHV in fish tissues.

MATERIALS AND METHODS

Source of fish

Koi were obtained from farms in Israel and retailers in the USA that reported suspected KHV outbreaks or from experimental trials in our laboratory. Koi used in the experimental trial were approximately 2 years of age, had a mean weight of 0.25 kg, and a mean fork length of 13 cm. The fish used in the experimental trial were reared in a closed system (i.e., no new fish had entered the facility in the past 10 years) at a commercial ornamental fish producer. Fish were transported to the fish health laboratory at the University of California in Davis, and held in 130 L aquaria receiving 23°C water at 0.8 L/min. The fish were fed a commercial koi ration at 1% body weight per day.

Viruses and cell lines

The KHV from Israel (KHV-I) used in this study was isolated from adult koi experiencing mass mortality in Israel in 1998 (Hedrick et al. 2000). The KHV-U was

recovered from a similar outbreak among adult koi in the eastern USA in 1998 (Hedrick et al. 2000). The CHV in this study was a gift from Dr. T. Sano and Dr. H. Fukuda, Tokyo University of Fisheries. The channel catfish herpesvirus (CCV) strain CA80-5 serving as the unrelated herpesvirus control was isolated from an epizootic among juvenile channel catfish *Ictalurus punctatus* in California in 1980 (Arkush et al. 1992). Both the KHV isolates and CHV were propagated in the KF-1 cell line as described by Hedrick et al. (2000). The channel catfish ovary (CCO) cell line was propagated in minimum essential media (MEM) supplemented with 7.5% fetal bovine serum (FBS), 50 IU penicillin/ml, 50 µg streptomycin/ml, and 2 mM L-glutamine (MEM-7.5). The FBS concentrations of the growth medium were reduced to 2 % (MEM-2) when CCO cells were infected with CCV or when KF-1 cells were inoculated with KHV. Cells were incubated at 25°C following inoculation with CCV or at 20°C for KHV.

The isolation of KHV from fish tissues was that described by Hedrick et al. (2000). Replicate wells of a 12-well tissue culture dish containing monolayers of KF-1 cells were inoculated with 0.2 ml of a 1:50 dilution (volume /volume or weight/volume) of the original tissue extract. After an adsorption period of 1 h, 2 ml of MEM-2 containing 0.015 M N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid (HEPES), 50 IU of penicillin/ml, 50 µg streptomycin/ml, and 2 mM L-glutamine were added to each well and the plates were incubated at 20°C. The plates were observed daily for evidence of cytopathic effects (CPE) over 21 d. Concentrations of KHV in the tissues of experimentally-infected fish were estimated by the method of Reed and Meunch (1938) by 50% end point tissue culture infective dose ($TCID_{50}$) analysis in 96 well plates containing KF-1 cells incubated at 20°C for 14 d.

Virus purification and DNA extraction

After CPE was complete, flasks inoculated with each of the different viruses were frozen at -80°C for at least 24 h. After thawing at room temperature, cells and culture media were collected. Cell debris was separated from the culture medium by centrifugation at 3500 x g for 20 min at 10°C. The pellet was placed into a Dounce homogenizer with 10 ml of MEM-2. After 10-15 strokes, the homogenate was again centrifuged at 3500 x g for 20 min at 10°C. The pellet was discarded and the supernatant centrifuged for 90 min at 95,300 x g at 10°C. The virus pellet was suspended in 1 ml TNE (50 mM Tris-HCl, 150 mM NaCl, 1 mM disodium ethylenediaminetetraacetic acid pH 7.5), placed in a Dounce homogenizer, and after 10-15 strokes the suspension was layered onto a 10-60% (weight/volume) linear sucrose gradient in TNE. After centrifugation at 77,000 x g for 18 h at 10°C two visible bands near the bottom of the tube were collected, diluted in fresh TNE and centrifuged at 151,000 x g for 1 h at 10°C (Arkush et al. 1992). The virus pellet was placed directly into either TNE at a final protein concentration of 1 mg/ml for SDS PAGE analyses (virion polypeptide analyses), or molecular biology grade water and treated with DNaseI and RNaseA at final concentration of 15 µg/ml each (genomic DNA comparisons) for 30 min at 37°C.

Viral genomic DNA was extracted using phenol:chloroform:isoamyl alcohol (IAA) mixture 25:24:1 respectively, followed by ethanol precipitation. Briefly, purified virus that was incubated with proteinase K in the presence of lysis buffer for 3h at 56°C followed by direct temperature shifting to 70°C for 20 min for inactivation. A 0.1 volume of 3M sodium acetate was added and then two volumes of cold 95% ethanol. The mixture was incubated overnight at -70°C, followed by centrifugation at 15000xg to

precipitate the DNA. The supernatant was removed and the pellet was washed with 1 ml of cold 70% ethanol and then air-dried for 5 min. TE buffer was added and the DNA pellet was incubated for 5 min at 37°C to dissolve the DNA. DNA concentrations were determined by spectrophotometer (Pharmacia Biotech, GeneQuant II) and the samples stored at -20°C.

Analysis of virion polypeptides

Purified virus (1 mg protein/ml) in TNE, was mixed 1:1 with 2X sample application buffer, heated to 100°C for 2 min then centrifuged for 2 min at 16,000 x g. Virion polypeptides were separated by SDS-PAGE under reducing conditions in 15% gels or 4-20% Bio-Rad Ready Gels (Bio-Rad, Hercules, CA, USA) according to the system of Laemmli (1975). Novex Mark-12™ molecular weight standards (Novex, San Diego, CA) were included in each gel. After electrophoresis, the gels were stained with Coomassie Blue G-250 and the approximate molecular weight of the virion polypeptides was estimated by their relative mobility as compared to the molecular weight standards.

KHV restriction fragment length polymorphism (RFLP) comparisons to CHV and CCV

For restriction fragment length polymorphism (RFLP) comparisons, a total of 1-2 µg of viral DNA from purified preparations of KHV-U, KHV-I, CHV, and CCV were incubated with 10 U of either K*pn*I or S*ac*I endonuclease for 1 h at 37°C. DNA fragments were separated by electrophoresis on 0.8% agarose gels and observed after staining with 1% ethidium bromide.

Cloning and sequencing of restriction fragments

An aliquot of purified KHV DNA was incubated with 10 U of KpnI and SacI endonucleases. The resulting DNA fragments were inserted into the pGEM®-7Zf(-) plasmid that was then used to transform DH5α *Escherichia coli* cells. The bacterial cells were grown on trypticase yeast extract (TYE) agar at 37°C in the presence of Ampicillin (200 μg/ml), X-gal, and IPTG for screening of clones. White colonies were selected, plated on reference media plates, and the transformants bearing KHV inserts were screened by PCR using SP6/T7 primers flanking the insert site (Sambrook et al. 1989). DNA sequence was obtained by fluorescently labelled dideoxy terminator sequencing using an ABI 377 automated DNA sequencer (Hitachi).

Primer selection and oligonucleotide synthesis

Oligonucleotides to be used as primers in the polymerase chain reaction (PCR) assay were selected and then tested for possible secondary structure and self-complementarity using the 'Amplify' software (University of Wisconsin Genetics, Madison, WI, USA). Oligonucleotides were synthesized by Gibco BRL (Rockville, MD, USA).

Optimization of KHV PCR assay

Initial PCR parameters were established that provided amplification of a 486 bp product corresponding to sequences obtained from the cloned KHV DNA KpnI/SacI fragment. To optimize the PCR assay, varying concentrations: primers from 20 to 40 pmol; Taq DNA polymerase from 1 to 2 U; $MgCl_2$ from 0.5 to 2.5 mM; and tetramethyl

ammonium chloride (TMAC) 5 μM to 20 mM were tested with a known positive sample of purified KHV DNA. A range of annealing temperatures and different number of cycles of amplification were also examined.

Following optimization of the PCR the 486 bp amplified DNA products from both fish collected from the field and following experimentally-induced KHV infections were sequenced and aligned to the original sequence of the KpnI/SacI restriction fragment using Mac DNAsis (Mirabio, Alameda, CA, USA).

Ability of the PCR to discriminate between related herpesviruses

The ability the newly developed PCR to discriminate KHV from other herpesviruses was tested. A total of 50 ng of DNA obtained from purified KHV (I and U), CHV, CCV, or uninfected KF-1 cells were used as templates for the PCR assay. The resulting amplified products were analyzed on agarose gels after staining with ethidium bromide as previously described.

Level of detection

The least amount of target DNA detectable with the optimized PCR assay was determined using 10 fold serial dilutions from 10 ng to 0.001 pg of KHV DNA. This dilution series was tested in the PCR at four different annealing temperatures (e.g., 55°C, 60°C, 65°C, and 68°C). An estimation of the level of detection of KHV DNA sequences that might be expected in infected tissues was evaluated by analyzing amplicons obtained by PCR analyses of dilutions of purified KHV DNA with and without prior mixing with 100 ng of host (koi) DNA.

PCR examinations of fish exposed to KHV in the field and laboratory

A total of 31 koi from farms or retailers that experienced unexplained mortality were examined for the presence of KHV by isolation of the virus on the KF-1 cell line and detection of KHV DNA sequences by the PCR assay. In the laboratory trial, the presence of KHV was examined by the same procedures among koi exposed to the virus or control koi not exposed to the virus. A total of 40 koi were divided into two equal groups. The experimental group received 6.3 x 10^3 $TCID_{50}$/ml of KHV in MEM-2 via a waterborne exposure in 100 L of 23°C well water for 1 h. The second group of fish was treated in the same manner but only with MEM-2. After exposure, fish were transferred to 130 L aquaria receiving flow through 23°C well water at a rate of 1.8 L min. Fish were fed a commercial diet each day. Fish were observed twice daily following virus exposure. The kidney, spleen and gill were collected from experimentally infected fish and processed by standard virological methods (Ganzhorn & LaPatra 1994). For PCR analyses 1g of the kidney, spleen, and gill were removed and DNA from the tissues was extracted as previously described. A total of 100 ng of this DNA was used in each PCR assay. Dead fish were examined for virus isolation immediately and samples for PCR were stored at −70°C. At 21 d a total of 10 control fish were examined for presence of KHV by virus isolation and PCR analysis. At 4 mo post exposure all remaining fish in the exposed and control groups were sacrificed by overdose with 500 ppm of tricaine methanosulfonate (MS-222) and examined for KHV by virus isolations and by PCR assays.

RESULTS

Virion polypeptides of KHV-I, KHV-U, CHV and CCV

The virion polypeptide profiles of KHV isolates were distinctly different from those of CHV and CCV (Fig.1). Depending on the virus, a total of 23 to 35 polypeptides were present. KHV possessed 31 polypeptides with 8 sharing the same molecular weight as those found in CHV while only 4 polypeptides of KHV shared a similar size to those of CCV. A comparison of the KHV-I and KHV-U isolates showed no significant differences in the number or size of virion polypeptides.

RFLP Analysis of KHV-I, KHV-U, CHV and CCV

The genomic DNA of KHV was found to provide a unique and clearly distinguishable RFLP from that of CHV or CCV when cut with KpnI or SacI with these two enzymes (Fig.2). There were no apparent differences between the RFLP of KHV-U and KHV-I (Fig.2). The largest molecular weight KpnI fragments were from KHV while the lowest molecular weight fragments were found with CCV. Similar sized high molecular weight restriction fragments were generated by SacI treatments of both KHV and CHV and these fragments were generally larger than those of CCV (Fig.2).

Optimization of KHV PCR assay

The optimal parameters for the PCR assay were obtained by sequential testing of target DNA from purified virus and experimentally and naturally exposed koi. The conditions that provided the strongest amplification of the specific 486 bp fragment were:

Cocktail: 2 mM $MgCl_2$, 1X buffer, 400 µM dNTP, 30 pmoles primers, 1U Taq polymerase.

Template: 70 - 100 ng DNA

Cycling Conditions: 95^0C for 5 min, 94^0C for 1 min, 68^0C for 1 min, 72^0C for 30 sec,39 cycles,72^0C for 7 min.

Forward primer-KHV9/5F 5'-GACGACGCCGGAGACCTTGTG-3'

Reverse primer –KHV9/5R 5'-CACAAGTTCAGTCTGTTCCTCAAC-3'

The ability of the PCR to discriminate KHV from other herpesviruses

The PCR assay under the optimal conditions amplified only DNA from KHV-I and KHV-U but not from CHV, CCV or the KF-1 cell line (Fig.3). At lower annealing temperatures (e.g., 55°C) there was a weak amplification of CCV DNA. However, when annealing temperatures were increased to between 60°C and 68°C, there was no amplification of CCV DNA.

Level of detection of KHV DNA in fish tissues

The lowest amount of KHV DNA detected was 1 pg when the annealing temperature in the PCR was 68°C. The viral DNA was detected when the DNA sample was from purified virus or when 100 ng of DNA from uninfected fish tissues were included in the reaction (Figure 4).

Detection of KHV in koi by the PCR assay

Koi from one source in the USA showed no evidence for KHV by either virus isolation or PCR assay. Koi from a second site yielded one KHV isolate using the KF-1 cells and 3 of 8 fish examined were positive by PCR (Table 1). KHV was not isolated from frozen koi tissues obtained from 7 sites in Israel although a PCR analysis demonstrated that 4 of the sites had a least one positive fish (Table 1). We examined the sequence of the PCR products from 3 fish from the field (two fish from Israel and one from the USA) and 2 fish from the experimental exposure study and all were identical to that of the original KHV DNA fragment used to develop the PCR.

A total of 11 fish exposed to KHV in the laboratory trial died between 10 and 22 d post exposure. The virus was isolated from 9 of 11 exposed fish that died but not from 2 dead fish that were severely autolyzed at the time of collection. All KHV exposed fish that died were positive by PCR assay (11 of 11). KHV was not isolated nor were survivors of the KHV exposure positive by PCR at 4 mo. There was no evidence of KHV by virus isolation or PCR assay among 10 control fish examined at 21 d nor among the remaining control fish examined at 4 mo.

DISCUSSION

Recent and mass mortality among koi and common carp in both the USA and Israel has been associated with the presence of KHV, a newly recognized herpesvirus (Hedrick et al. 2000). Initial characteristics of KHV, principally differences in antigenic

properties and the pathogenesis of the disease in larger koi and common carp, suggested the virus was not CHV, the most commonly encountered herpesvirus in carp (Hedrick et al. 2000). Further characterization of KHV from both Israel (KHV-I) and the USA (KHV-U) by examination of virion polypeptides and RFLP analyses of genomic DNA indicate that the agents are identical, but clearly different from CHV and CCV. In addition, sequence data from restriction fragments of KHV have been utilized to develop a PCR assay that can detect the agent in naturally and experimentally exposed koi. The PCR provides a new approach to detecting KHV that appears to be more effective than routine virus isolation on the KF-1 cell line. The PCR also detects both KHV-I and KHV-U and this further indicates the similarity of the two isolates.

The virion polypeptide profiles of KHV-I and KHV-U were indistinguishable and this provides further support that the agents are identical. There were similarities among several virion polypeptides between KHV and CHV that suggest that these agents are more similar to each other than either was to a third herpesvirus (CCV) from channel catfish. The presence of certain common polypeptides may be due in part to nonstructural components (some of which may be of cellular origin) that may copurify or be trapped by virions (Spear & Roizman 1972).

The RFLP analyses further confirmed the identity of KHV from the USA and Israel and that KHV differs significantly from CHV and CCV. Analyses with a total of 5 restriction enzymes demonstrated no fragment polymorphism between KHV-I and KHV-U. This provides strong evidence that there is significant DNA sequence homology shared by the two isolates. RFLP analyses of genomic DNA have been used to compare related viruses or isolates of the same fish herpesviruses (Colyer et al. 1986, Sano et al.

1991a). Although the profiles for a single isolate of KHV from Israel and the USA were identical, we presume that a comparison of additional KHV isolates will indicate some potential polymorphisms. Since both geographically distant isolates appear to be identical, this argues for a common origin of the virus together with a rapid spread from that source. Additional studies of more isolates of KHV may show patterns of spread for the virus as has been demonstrated for certain mammalian herpesvirus (Banks 1993).

Estimates for the genome size of KHV ranged from 107,507 to 244,940 bp. The wide range of the estimates obtained for KHV may have resulted from the methylation of some restriction sites. Incompletely digested fragments, particularly larger fragments, may have co-migrated in the gels resulting in underestimates of the total genome size. If we consider the largest estimate of the genome size for KHV obtained with K*pn*I (data not shown) which is 208,265 bp or a molecular weight of 137 x 10^6, it is very similar to that of CHV 220,909 bp or a molecular weight of 145.8 x 10^6 (Sano et al. 1991a), but considerably larger than estimates of 84 - 88 x 10^6 for CCV (Chousterman et al. 1979, Dixon & Farber. 1980). The complete genome sequencing of CCV yielded 134,226 bp (Davison 1992). Due to the large range in the KHV genome size obtained from all of the restriction endonucleases used (i.e., B*gl*I, B*gl*II, S*ac*I, K*pn*I and B*am*HI), we are not able to accurately determine the KHV genome size. However, sequencing of the KHV genome now underway (H. Bercovier, unpublished data) will provide the most accurate estimate. Restriction mapping (with B*am*HI) and some sequencing determined that a second herpesvirus from fish, the salmonid herpesvirus I (SalHV-1), had a genome size of 174,400 bp and curiously shares open reading frames (ORFs) showing homology with 18 genes from CCV (Davison 1998). We suspect, but must determine, that KHV and

CHV both from cyprinid fish will share several genes in common, just as has been shown with the two herpesviruses (SalHV-1 and SalHV-2) examined from salmonid fish (Davison 1998).

The similarities between the disease caused by KHV in larger koi and that due to CHV in koi and common carp less than 2 mo in age are remarkable (Sano et al. 1991b, Hedrick et al. 2000). This has led to some speculation that KHV might be a more pathogenic variant of CHV now capable of attacking and killing larger fish. While we presume the two viruses will share certain genes or gene sequences, the PCR assay developed in this study and both the RFLP and virion polypeptide analyses argue against KHV being a variant of CHV. More complete comparisons of the two viruses that consider genome arrangement and sequence homologies that are in progress should further clarify their relationships. Both viruses share the similar property of being rather difficult or impossible to isolate using most commonly available cell lines (Sano et al. 1985a, Hedrick et al. 2000) and this has severely compromised diagnostic tests to detect the agents.

Current diagnostic procedures for KHV include detection of typical clinical signs, characteristic microscopic lesions, and isolation of the agent in the KF-1 cell line (Hedrick et al. 2000). These methods are effective when fish are dying of infection but even then, virus isolation may be difficult or impossible if tissues are frozen. Detecting the virus among fish surviving KHV infections has not been possible but these fish are presumably carriers since co-habitation with previously unexposed fish has been the principal means for spread of the virus. Development of a PCR assay was viewed as one method to overcome these diagnostic problems.

The PCR developed in this study was effective in detecting KHV in koi tissues from fish collected from the field and from controlled laboratory exposures to the virus. In contrast, virus isolation using the KF-1 cell line was less reliable as demonstrated particularly with the field samples from both the USA and Israel (Table 2). We suspect that KHV survives poorly in frozen tissues and virus isolations are best conducted on fresh tissues. This may explain why the frozen tissues sent from Israel to our laboratory yielded no isolates of KHV while live fish received from a USA source did provide an isolate on the KF-1 cell line. An examination of freshly dead fish from the laboratory trial also provided isolates of KHV. The only exceptions were the two exposed fish that died and from which virus could not be recovered. These fish had been dead for at least 16 h (and were severely autolyzed) and this may explain the failed attempt to recover KHV on the KF-1 cell line. However, the PCR was effective in detecting KHV DNA from both of those dead fish and from frozen tissues received from Israel. Unfortunately, KHV could not be detected in virus-exposed koi surviving for 4 mo post exposure by either virus isolation or PCR assay. This may indicate that kidney, spleen, and gill are not sites where latent KHV is found, or alternatively, that our PCR assay does not have the needed level of detection for the amount of viral DNA that might be present in these tissues. Additional studies that subject previously exposed fish to stress and or examination of additional tissues by PCR for KHV are currently underway. Although further testing is needed, these initial results suggest that the PCR assay for KHV is much more effective than virus isolation on tissue samples obtained from fish experiencing outbreaks even if the samples are compromised by autolysis or freezing.

The latent phase of infection with herpesviruses, including those from fish, can make it difficult or impossible to isolate the virus from fish tissues (Wise et al. 1985, Boyle & Blackwell 1991, Sano et al. 1993). However, the use of the PCR or in situ hybridization has demonstrated that DNA of CCV and CHV can be detected among suspected carrier fish in a range of tissues that may change over the course of infection (Boyle & Blackwell 1991, Gray et al. 1999 a,b, Sano et al. 1993). We presume that KHV, as seen with CCV and CHV, establishes a latent infection among exposed koi and common carp and these fish, perhaps through periodic shedding of infectious virus, serve as the carriers by which virus is spread. Unfortunately, our first attempts to detect these carriers using the three target tissues (kidney, spleen, gill) in previously exposed koi in the laboratory were unsuccessful. We are currently examining in more detail the course of infection and other potential sites for KHV latency in exposed koi.

The ability to detect KHV carriers will be a critical step in controlling KHV outbreaks. We are hopeful that this PCR assay can be used to detect KHV carriers using nonlethal sampling methods. If these approaches are effective with individual fish and the results can be combined with those obtained from health inspections of populations at the producer and retailer, control of KHV becomes possible. Additional laboratory studies are examining the pathogenesis of experimental infections of koi with KHV and the potential to utilize vaccination as a preventive measure to control the virus.

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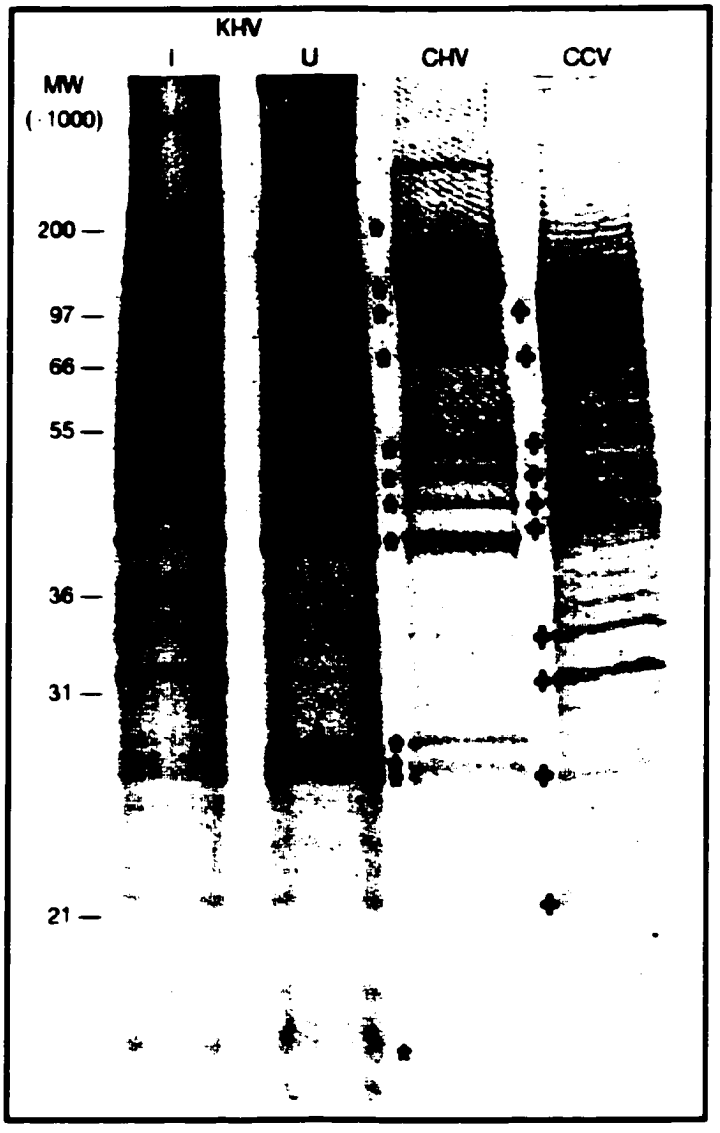

Figure 1. Comparison of the virion polypeptides of 2 isolates of koi herpesvirus (KHV), one from Israel (KHV-I) and the second from the USA (KHV-U), with those of the cyprinid herpesvirus (CHV) and channel catfish herpesvirus (CCV). The bands marked with (*) are shared between KHV and CHV and those with (+) are similar between KHV and CCV. MW: molecular weight

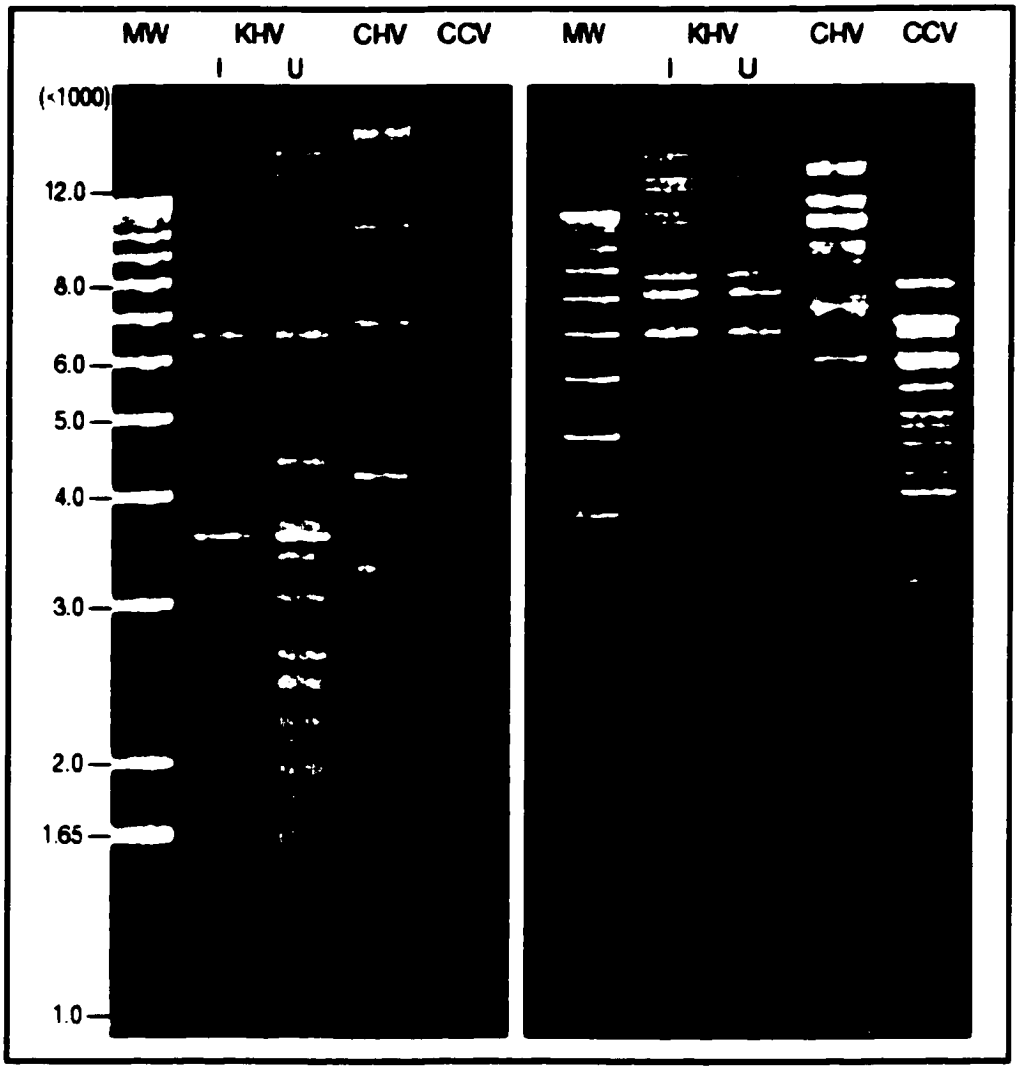

Figure 2. Restriction fragment length polymorphisms (RFLP) resulting from digestion of the viral DNA from KHV from Israel (KHV-I) and the USA (KHV-U), CHV and CCV with either (left) *Sac* I or (right) *Kpn* I. DNA molecular weight (MW) standards are found in the far left lanes of each agarose gel.

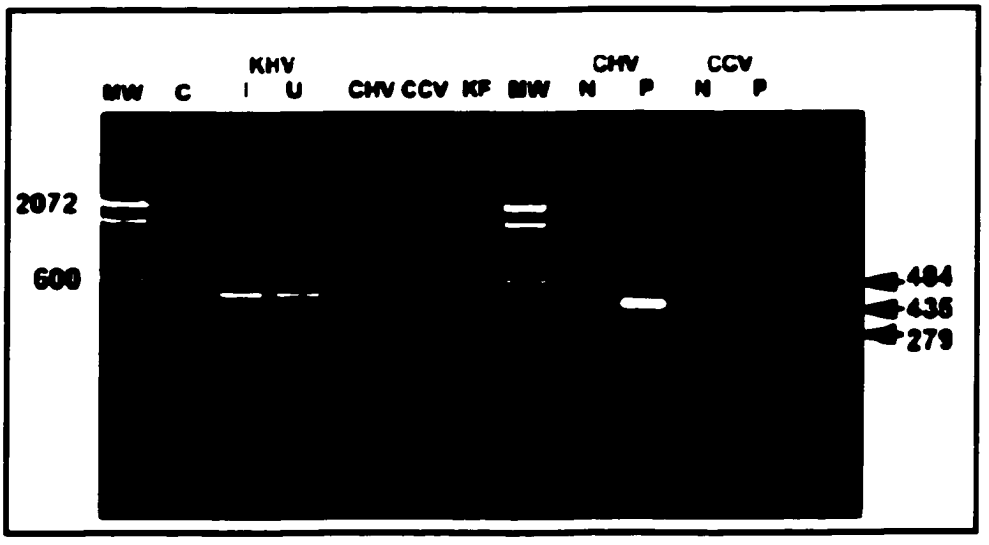

Figure 3. Results from the polymerase chain reaction (PCR) assay for KHV when purified viral DNA was examined from KHV isolates from Israel (KHV-I) and the USA (KHV-U) with purified viral DNA from CHV and CCV. On the same gel are the results from the amplification of purified viral DNA from CHV and CCV used in the KHV PCR as tested with specific PCR assays for CHV and CCV, respectively. The negative controls (C or N) were water only and the positive control (P) was DNA from purified virus. Molecular weight (MW) markers were included in the gel and the sizes of the specific PCR products are indicated by arrows.

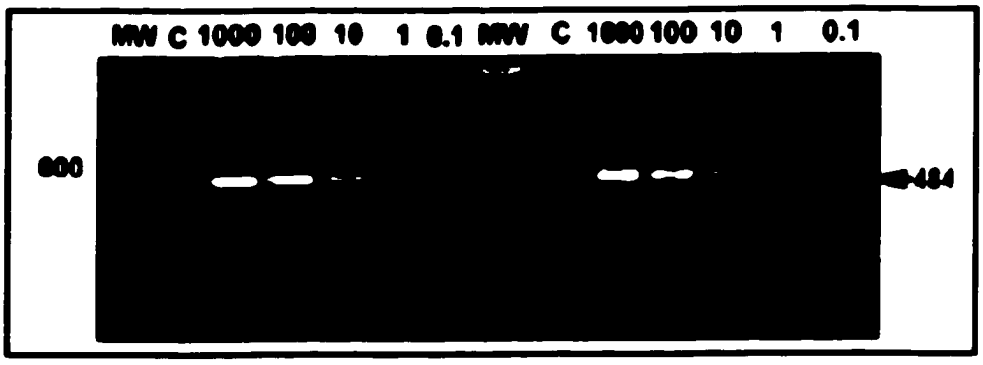

Figure 4. KHV PCR assay as applied to serial 10-fold dilutions of KHV DNA beginning with 1000 pg and ending with 0.1 pg. The KHV DNA was either amplified without host DNA (right half of the gel) or mixed with 100 ng of host DNA (left half of the gel)

Table 1. Detection of koi herpesvirus (KHV) by virus isolation on koi fin (KF-1) cells or by polymerase chain reaction (PCR) assay. Fish were obtained from sources in the USA or Israel or from controlled laboratory exposures to KHV. Virus isolation was attempted from the kidney, spleen and gill of each fish. A fish was considered virus positive if virus was isolated from any tissue. For PCR assays DNA was extracted from the gill, kidney and spleen of each fish. A fish was considered positive if any 1 of the 3 individual tissue samples provided a positive signal.

Origin of fish	Number of fish	Virus isolation	PCR
USA #1	5	0	0
USA #2	8	13% (1/8)	38% (3/8)
USA #3	2	50% (1/2)	50% (1/2)
USA #4	2	50% (1/2)	100% (2/2)
Israel #1	3	0	33% (1/3)
Israel #2	4	0	25% (1/4)
Israel #3	2	0	50% (1/2)
Israel #4	3	0	67% (2/3)
Israel #5	2	0	0
Israel #6	3	0	0
Israel #7	1	0	0
Laboratory: KHV exposed (dead)	11	82% (9/11)	100% (11/11)
Laboratory: KHV exposed (survivors at 4 mo)	9	0	0

Molecular Comparison of Isolates of a New Emerging Fish Pathogen, the Koi Herpesvirus (KHV) and the Effect of Water Temperature on Mortality of Experimentally Infected Koi

ABSTRACT

The koi herpesvirus (KHV) has been associated with devastating losses of both common carp (*Cyprinus carpio carpio)* and koi *(Cyprinus carpio koi)* in North America, Europe, Israel and Asia. A comparison of the virion polypeptides and genomic restriction fragments of 7 geographically diverse isolates of KHV indicated that with one exception they represented a homogeneous group. A principal environmental factor influencing the onset and severity of disease is water temperature. Optimal growth of KHV in the koi fin (KF-1) cell line occurred at temperatures from 15 – 25°C. There was no or minimal growth at 4, 10, 30 or 37°C. Experimental infections of koi with KHV at a water temperature of 23°C resulted in a 95.2% cumulative mortality. Disease progressed rapidly but with less mortality (95.2 – 89.4%) at 28°C. Mortality (85.0%) also occurred at 18°C but not at 13°C. Shifting virus-exposed fish from 13°C to 23°C resulted in a rapid onset of mortality.

INTRODUCTION

Common carp *Cyprinus carpio carpio* are a widely cultivated fish species for human food with 1.5 million metric tons harvested annually principally from China and other Asian and European countries and Israel (1). Fish is a particularly important protein source in regions where livestock is relatively scarce, supplying less then 10% of animal

protein consumed in North America and Europe, but 17% in Africa, 26% in Asia and 22% in China (1). Total aquaculture production reached nearly 36 million tons in 2000 with a value of $50 x 10^9 USD, with carp, barbels and other cyprinid production of nearly 16 million tons in a value of $15 x 10^6 USD (1). In contrast to common carp, the subspecies *Cyprinus carpio koi* are beautiful and colorful fish and part of a worldwide hobby that includes holding in backyard ponds and large display aquaria for personal pleasure or for competitive showing. The hobby originated with the first century A.D. Romans, matured into the present science and art practiced in Japan, and then subsequently spread world wide (2).

Beginning in 1998, mass mortality suspected to be of a viral etiology was observed in both common carp and koi in countries throughout the world including the U.S.A., Germany, England, Italy, Netherlands, Israel and most recently Indonesia (3). These losses tended to occur seasonally during periods when water temperatures ranged from 18 to 25°C. The cumulative mortality associated with outbreaks has had major negative impacts on the koi aquaculture industry and retailers and hobbyists. A herpes-like virus referred to as the koi herpesvirus (KHV) (4), has been isolated or identified from koi and common carp in many of these mass mortality episodes. In both field and laboratory studies, KHV has caused significant losses among all ages of koi or common carp (4). In contrast, the cyprinid herpesvirus or CHV, a previously known viral pathogen of koi and common carp, causes losses principally among fish less than 2 mo in age (5,7).

Approximately 130 herpesviruses have been identified to date from different animal species; 9 are known to infect humans with most others found among other

vertebrates, including fish (8). In fish, herpes-like viruses are commonly identified as the causes of diseases ranging from rather benign skin conditions to fatal systemic infections (9).

We believe that intensive fish culture, koi shows, and regional domestic and international trading are the three main mechanisms that have contributed to the rather rapid global spread of KHV. The movements of fish pathogens with ornamental fish and the active international trade in live fish, including koi, has been recognized as a key pathway for the spread of emerging fish diseases (10). Unfortunately, as with most ornamental fish, these unrestricted movements of koi continue, nearly all without any health inspections or implementation of quarantine programs at the wholesale or individual hobbyist level. The importance of fish viruses is recognized by the Office International des Epizooties (OIE) with all five notifiable fish diseases being of viral etiology. Each of the five viruses is considered to be capable of causing significant negative socio-economic and ecological impacts if introduced from one zone to another (11).

Diagnostic methods for the detection of fish viruses continues to rely on virus isolation but newer techniques including the polymerase chain reaction (PCR) assay have been developed for the most known agents (12). A widely used PCR assay for KHV was developed by Gilad et al., (2002) (13). A second PCR assay for KHV has also been described by Gray et al., (2002) (14). These PCR assays have significantly increased the ability to detect KHV infections in koi and common carp.

In this study we compared 7 KHV isolates obtained from diverse geographic regions for genomic variations by restriction fragment length polymorphisms (RFLP) of

genomic DNA and differences in the number or size of structural polypeptides. In addition, we examined the role of temperature on growth of the virus in cell culture and as a key variable influencing the onset and severity of disease among koi following experimental exposures to the virus.

MATERIAL AND METHODS

Viruses and cell line

The KHV (KHV-I) isolate used in this study was obtained from adult koi experiencing mass mortality in Israel in 1998 (4). The virus was passed four times on the koi fin (KF-1) cell line prior to genome and polypeptide analyses, growth studies in vitro or experimental infections of koi. An additional 6 isolates of KHV were obtained from other geographic areas (Table 1). The KF-1 cells were grown in minimum essential media (MEM) supplemented with 7.5% fetal bovine serum (FBS), 50 IU penicillin/ml, 50 μg streptomycin/ml, and 2 mM L-glutamine (MEM-7.5). The FBS concentration of the growth medium for the KF-1 cells was reduced to 2 % (MEM-2) prior to virus inoculation and cells were placed at 20°C for incubation until complete cytopathic effects (CPE) were observed. The medium was buffered with 0.15 M N-2-hydroxyethyl-piperazine-N-2-ethane sulfonic acid (HEPES) when KF-1 cells were propagated in tissue culture plates (15). Virus concentrations were estimated by tissue culture infective dose ($TCID_{50}$) using the method of Reed and Meunch (1938) (16) as described by Hedrick et al. (2000) (4).

Virus purification and DNA extraction

Production and purification of KHV for virion polypeptide and genomic restriction fragment evaluations were similar to those described for the channel catfish herpesvirus (17) with minor modifications (13).

Analysis of virion polypeptides

An equal volume of purified virus (1 mg protein/ml) in TNE was mixed with 2X sample application buffer, heated to 100°C for 2 min then centrifuged for 2 min at 16000 x g. Virion polypeptides were separated by SDS-PAGE under reducing conditions in 10% gels according to the system of Laemmli (1975) (18). Invitrogen Mark-12™ molecular weight standards (Invitrogen, Carlsbad, CA) were included in each gel. After electrophoresis, the gels were stained with Coomassie Blue G-250 and the approximate molecular weights of the virion polypeptides were estimated by their relative mobility as compared to the molecular weight standards.

Restriction Fragment Length Polymorphism (RFLP) comparisons of KHV isolates

For RFLP comparisons, a total of 1-2 μg of viral DNA purified from each of the KHV isolates were incubated with 10 U of KpnI endonuclease for 1 h at 37°C. DNA fragments were separated by electrophoresis on 0.8% agarose gels and observed after staining with 1% ethidium bromide.

Temperature effects on replication of KHV in KF-1 cells

Each well on a total of seven 24-well tissue culture plates containing KF-1 cell monolayers was inoculated with 0.1 ml of KHV at a multiplicity of infection (MOI) of 0.008. As a cell control, 0.1 ml of sterile MEM-2-HEPES, not containing KHV, was added to all wells of a second set of seven plates. One inoculated plate and one control plate were then placed into incubators at each of the following temperatures: 4, 10, 15, 20, 25, 30 and 37°C for virus adsorption. After 1 h, each well was rinsed twice with 1 ml of MEM-0. After the second rinse with MEM-0, 1 ml of MEM-2-HEPES was added to each well and the plates were returned to their respective incubation temperatures. Two replicate wells from each virus-inoculated plate at each temperature were used to evaluate virus concentrations as present in each of the cell-free and cell-associated fractions at 6, 12, 24, 36, 48 and 96 h, and 7, 13, and 25 d post inoculation. At each sample period, the cells were scraped from each well into the media that was then centrifuged at 4°C for 10 min at 15000 x g. These centrifugation conditions were used for all subsequent samples collected in the growth temperature study. After centrifugation, the supernatant was collected and stored on ice and the remaining cells were resuspended in 1 ml of MEM-0 and centrifuged again. The cells were rinsed one additional time and collected by centrifugation. A 1 ml aliquot of MEM-2-HEPES was added and the cells were disrupted by 10 – 15 strokes with a Dounce tissue homogenizer. The cell debris was subsequently removed by centrifugation and the supernatant retained. Serial 10-fold dilutions of the cell extract (cell-associated) or the original cell culture medium (cell-free) were prepared in MEM-2 and used to inoculate KF-1 cells to determine virus concentrations by $TCID_{50}$ assay. In addition to estimates of virus concentrations by $TCID_{50}$ analyses, at each

sampling point 150 µl of supernatant from one of the two replicate wells sampled at each temperature was collected and DNA was extracted and tested for the presence of KHV DNA by PCR. Viral DNA was extracted using a low salt lysis buffer (20 mM Tris HCl pH 8, 10 mM EDTA, 1% SDS), followed by a phenol:chloroform:isoamyl alcohol (IAA) purification. The PCR assay for KHV utilized was that previously described by Gilad et al., (2002) (13).

Source of fish

Koi used in the experimental trial were from a closed-system commercial ornamental fish producer in Northern California, with no history of KHV infection. Fish were transported live to the Fish Health Laboratory at the University of California Davis and held in 130 L flow through aquaria receiving 18°C well-water at 1.8 L/min, and were fed a commercial koi ration at 1% body weight per day. At the time of experimentation these fish were approximately 2 years in age with a mean weight of 0.274 kg and a mean length of 23 cm.

Effect of water temperature on mortality following KHV exposure

All fish were combined into one 800 L aquarium before being randomly distributed to a total of 12 aquaria of 130 L capacity for the temperature trials. Fish were randomly assigned to aquarium by drawing numbers from a common pool such that each aquarium eventually contained 19 to 21 fish. All fish were initially at a water temperature of 18°C. Acclimation to each of the three other water temperatures (13, 23, or 28°C) for the trial was accomplished by lowering or increasing the water temperature in increments of 3°C per day until the desired temperature was obtained. There were three replicate

aquaria at each water temperature. Fish in one aquarium at each temperature received a bath exposure to 12 $TCID_{50}$/ml KHV. Fish in a second aquarium at each temperature received a bath exposure to 1.2 $TCID_{50}$/ml of KHV. The third aquarium was treated identically to the virus-exposed groups but received only MEM with no virus. During the exposure period to virus or MEM (no virus) the water flow was stopped and oxygen was bubbled into each aquarium for a period of 1 h. After the exposure, the flow of the water to the aquaria was resumed. Fish that died during the study were removed daily and selected tissues (gill, kidney, and spleen) were examined for the presence of virus by isolation with KF-1 cells (4). Tissues from dead fish from which KHV could not be isolated in KF-1 cells were tested by the PCR (13). Tissues that tested negative by both virus isolation and PCR were further examined for the presence of KHV DNA by a newly developed real time TaqMan PCR assay (unpublished data). At 30 d post initial exposure (p.e.) to KHV, 6 fish from each of the three aquaria at the 13°C water temperature were sacrificed and examined for the presence of the virus as described above. Also at 30 d p.e. 6 additional fish from the same aquaria at 13°C, were moved to new aquaria (keeping them as three separate groups) and the water temperature was shifted to 23°C by increments of 3 degrees each day. At 63 d p.e. 5 fish from each of the aquaria that had remained at 13°C were sacrificed and examined for the presence of KHV. At 64 d p.e. 2 control unexposed fish, 1 exposed high-dose, and 2 exposed low-dose fish, previously held at 13°C were shifted to water at 23°C. An equal number of fish representing these same three groups were kept at 13°C. All fish remaining at 13°C or shifted from 13 to 23°C were sacrificed and sampled for the presence of KHV at 100 d p.e. At 30 d p.e. 6 fish from the control unexposed fish from the 28°C, 23°C and 18°C

groups were sacrificed and sampled for the presence of the virus. At 64 d p.e. 5 fish from the control unexposed groups at 28°C, 23°C and 18°C were sacrificed and sampled for the presence of the virus. Also, at 64 d p.e. all remaining virus exposed fish (survivors) at 28°C, 23°C and 18°C were sacrificed and sampled for presence of KHV. The mean day to death for each treatment was calculated as the sum of the days when each fish died divided by the total number of fish that died in that treatment.

Virus testing of fish in the temperature trial

Our primary method for detecting the presence of KHV in the tissues of fish was by isolation in KF-1 cells. Approximately $2x10^6$ cells in each well of a 12-well plate received 0.2 ml of a 1:50 dilution (weight/volume) of the original fish tissue extract prepared as described by Gilad et al. (2002) (13). After an adsorption period of 1 h, 2 ml of MEM-2-HEPES were added to each well and the plates were incubated at 20°C. The plates were observed daily for evidence of CPE for 30 d. To confirm that the CPE was due to KHV, the supernatant from selected wells was tested for KHV DNA by PCR.

KHV DNA extraction from fish tissues

Portions (approximately 0.1 g) of the gill, kidney and spleen were sampled from individual fish and the DNA was extracted by using Qiagen kit, DNeasy, Tissue protocol (Qiagen, Valencia, CA). The DNA was stored in buffer at 4°C until tested by conventional (13) or Taqman PCR.

Statistical analyses

Differences in treatment groups were evaluated by survival analysis using the Cox proportional hazard model (19). The data was analyzed for the effects of water temperature, dose, or shifting from one water temperature to another (13 to 23°C). A second analysis excluded temperature shifted fish and a third analysis examine survivorship as related to water temperature. The accelerated life model (20) was used as an alternate approach to the same three analyses conducted with the Cox proportional hazard model and it yielded qualitatively similar results.

RESULTS

Analysis of virion polypeptides

Six of 7 isolates showed no significant differences in the number or size of the virion polypeptides (Figure 1). Two additional polypeptides of approximately 162 kD and 41 kD molecular weight were observed with the D-081 isolate from koi in Israel.

RFLP comparison

An RFLP comparison of the viral genomic DNA of the 7 isolates revealed no significant difference between six of the seven isolates (Figure 2). Two additional restriction fragments observed with the KHV isolate D-081 were not seen in any other isolates.

Temperature effects on replication of KHV in KF-1 cells

Optimal virus growth in the KF-1 cell line was observed at temperatures between 15 and 25°C (Table 2). The highest virus concentrations were observed in the cell-free fraction at 20°C at 7 d. Virus detected at 4°C and 10°C at early time points and 7 d and 13 d was just above the limits of detection (42 $TCID_{50}$/ml) of the assay. There was no evidence of virus growth at 30 or 37°C.

Experimental effect of water temperature on fish mortality

Of the fish held at a water temperature of 28°C, 17 of 20 died in both the low and high dose virus-exposed groups (Figure 3). Of the fish held at 23°C, 19 of 21 fish died in the high dose group and all 20 fish died in the low dose group. Of the fish held at 18°C, 20 of 21 and 17 of 19 fish died in both the high and low dose virus-exposed groups, respectively. No mortality was observed among fish in virus exposed fish held continuously at 13°C. Of the first group of fish held at 13°C and later shifted to 23°C, 5 of 6 and 5 of 6 fish died in both the high and low-dose groups, respectively. No mortality was observed in the second group of fish held at 13°C and later shifted to 23°C. Also, no mortality was observed in any of the control groups not exposed to KHV held constantly at one water temperature or after shifting from 13° to 23°C.

Mean Day of Death

The mean day of death was least at the highest water temperature and then progressively longer as water temperatures declined. At a water temperature of 28°C the mean day of death was 7.7 and 9.2 d for the high and low dose groups, respectively. In

contrast, at a water temperature of 18°C, the mean day of death was 18.2 d and 23.6 d, for the high and low dose groups, respectively. The mean day of death for the fish after shifting from 13 to 23°C on day 30 p.e. was 7.4 d and 12.8 d for the high and low dose, respectively.

Virus detection among fish from the temperature study

Of the fish held at 28°C, virus was recovered from only 9 dead fish in both the high and low dose groups. However, PCR and TaqMan PCR detected the viral DNA in 7 and 6 of the fish that were negative by virus isolation from the high and low dose groups, respectively. Of the fish held at 23°C, virus was recovered from 16 dead fish from both the high and low dose groups. PCR and TaqMan PCR detected the viral DNA in 1 of 3 and 4 of 4 of the fish that were negative by virus isolation from the high and low dose groups, respectively. Of the fish held at 18°C, virus was recovered from all dead fish in the high dose group and from 15 of 17 dead fish from the low dose group. PCR and TaqMan PCR detected KHV DNA in 1 fish that was negative by virus isolation from the low dose group. Of the fish held at 13°C and later shifted to 23°C on 30 d p.e., virus was recovered from all dead fish from the high dose group and from 4 of 5 dead fish from the low dose group. PCR and TaqMan PCR detected the viral DNA in the 1 fish that was negative by virus isolation from the low dose group. No virus was isolated or detected by PCR in any unexposed fish examined at any water temperature at 30 d, 63 d or 100 d after the experiment began.

Statistical analyses indicated that water temperature, including shifting from 13°C to 23°C, had significant ($p < 0.0001$) effects on survival. There were no differences in

survival observed between virus-exposed fish held continuously at 23°C compared to those originally at 13°C that were then shifted to 23°C. A second analysis included only fish that were not temperature shifted and again water temperature and not dose was the only significant factor in survivorship ($p < 0.0001$). The third analysis demonstrated that survivorship was significantly greater at 13°C than any of the other temperatures tested and that survival at 18°C differed from that at 23°C and 28°C. No differences were observed between survivorship among virus-exposed fish at 23°C and 28°C. When the same three survivorship analyses were conducted with the accelerated life model, the results were the same as those obtained with the Cox proportional hazard model with the sole exception that in the third analysis (effects of temperature alone) the increase in survivorship at 28°C was significantly different from that at 23°C.

DISCUSSION

Since the initial discovery, KHV has spread rapidly, presumably through the unregulated movements of live koi and common carp. KHV is known to cause significant mortality during production phases and then subsequently in older fish during or after local, regional or international shipments (21). The similarities in virion polypeptides and RFLP analyses of genomic DNA found among isolates of KHV from diverse geographic regions are consistent with a rapid spread of the virus from a single or limited source (22). Most outbreaks in the Northern Hemisphere begin in the spring and last through the summer which our experimental trials would suggest is controlled in part

by temperature that influences both virus replication in cell culture and the onset and severity of mortality in virus-exposed koi.

Virion polypeptide and RFLP analyses of genomic DNA have been used to compare related viruses or isolates in many animal species including fish (22,23,24,25,26,27,28,29). Our earlier comparison of the first two isolates of KHV from Israel (KHV-I) and the U.S.A. (KHV-U) by SDS-PAGE and RFLP assays indicated the two isolates were identical but quite different from CHV a second herpesvirus-like virus from koi and common carp (13). The virion polypeptides and RFLP for the new geographically diverse isolates of KHV were identical or similar to those observed previously with KHV-I and KHV-U (13) (Figures 1,2). Considering the minor variation seen with the D-081 isolate, KHV isolates from rather diverse geographic locations form a relatively homogeneous group. RFLP analyses have been used with other viral diseases (e.g. Aujeszky's disease in pigs) to record movements of known strains and emergence of new virus isolates (22). The homogeneity among KHV isolates using this procedure suggests that one prominent virus isolate has spread worldwide most likely with the regional, domestic and international trade of koi. Since routine health inspections of koi are not often required for region or international shipments, we have little ability to trace the exact origin and the temporal spread of KHV. That all of these isolates can be detected during outbreaks was indicated by the production of identical size amplicons with the single round PCR (13) (data not shown). Sequencing of these amplicons and more variable regions of the viral genome in the future may allow more precision in distinguishing between geographically diverse isolates (30). Alternately, a very rapid spread of a single strain of the virus (suggested by RFLP) may have occurred and only

over time will we begin to see more genetic variation between independently evolving strains.

Viral infections in ectothermic vertebrates can be greatly influenced by temperature (31). Water temperature is known to influence the onset and severity of fish viral infections directly by altering virus replication and indirectly by augmenting the efficacy of the host immune response (32,40). The optimal temperature range for KHV growth in KF-1 cells ranged between 15°C and 25°C which is similar to that found for the growth of the CHV, the other herpesvirus isolated from koi and common carp, in the EPC cell line from common carp (33). No replication of KHV or CHV was observed at 30°C (Table 2) (33) suggesting that both viruses are adapted to their host water temperature range from 2 to 30°C (34). In general, most studies examining the influence of temperature on the growth of fish viruses in cell culture have demonstrated that optimal replication in vitro occurs at a wider range than that found in the fish host, most often by 2 - 3°C (35,36,37) which may indicate that a water temperature of 28°C is perhaps the maximum tolerated by the virus in koi. An additional study that examines infections of koi above in the 28°C to 30°C range is needed to confirm or deny the upper water temperature threshold for the virus. This is critical since water temperatures at 28°C and above are currently used in attempts to control virus infections and to confirm immunity among survivors shifted to these higher water temperatures by koi producers (41).

The mortality observed in our water temperature study indicates that koi are susceptible to very low concentrations of the virus within the temperature range tested, and have little or no ability to overcome infection once initiated at permissive

temperatures. Both the Cox proportional hazard and the accelerated life models indicated that water temperature was the significant factor influencing risk of mortality. Virus dose was not a significant factor, at least between the two doses used in our study. The water temperatures we tested were chosen to represent the seasonal changes that might be anticipated at a fish farm. As an example, during winter in Israel water temperatures can fall to as low as 13°C and then begin to rise in spring to 18°C and then reach 23°C to 28°C or greater in the summer. Fall temperatures would resemble those in spring but be declining into the winter. In general, koi and common carp, whether in backyard ponds or larger production facilities, experience most severe disease episodes, including those with KHV as water temperatures begin to increase in the spring (4,38). This increased disease susceptibility is presumed to result from a lag in the activation of the immune response which declines during the colder winter months. Water temperatures can directly affect the function of both the cellular and humoral arms of the immune response (32,39). KHV outbreaks in Israel increase in the spring as water temperatures approach 18°C. Our experimental trials demonstrated that KHV mortality experienced in spring or summer could represent activation of virus infections that were contracted earlier but were dormant at lower temperatures (e.g. 13°C). Shifting of these infected fish from cooler to higher water temperatures (e.g. 23°C) rapidly induces mortality (mean day to death of 7 – 12 d), an effect that may mimic the over wintering and spring time occurrences of KHV mortality that occur in larger farms in Israel.

Recent mass mortality events among koi and common carp due to KHV suggest a broad geographic distribution of the agent has already occurred. The severity of these outbreaks has caused an increased awareness of the virus and certain state and national

regulatory agencies have instituted or are considering instituting inspection and certification programs. Due to the importance of carp as a major protein source in regions where livestock is relatively scarce (e.g. Asia and the Middle-East) and to the value of individual fish (koi), disease controls are warranted. In addition, programs that include quarantine under permissive water temperatures and sampling by new molecular diagnostic tools should be immediately implemented to control the spread of the virus. Lastly, concerns over the potential spread of the virus from ornamental or farmed fish to wild cyprinid fish, as may have occurred recently with the spring viremia of carp rhabdovirus (6), further supports the need for health controls for this emerging viral disease.

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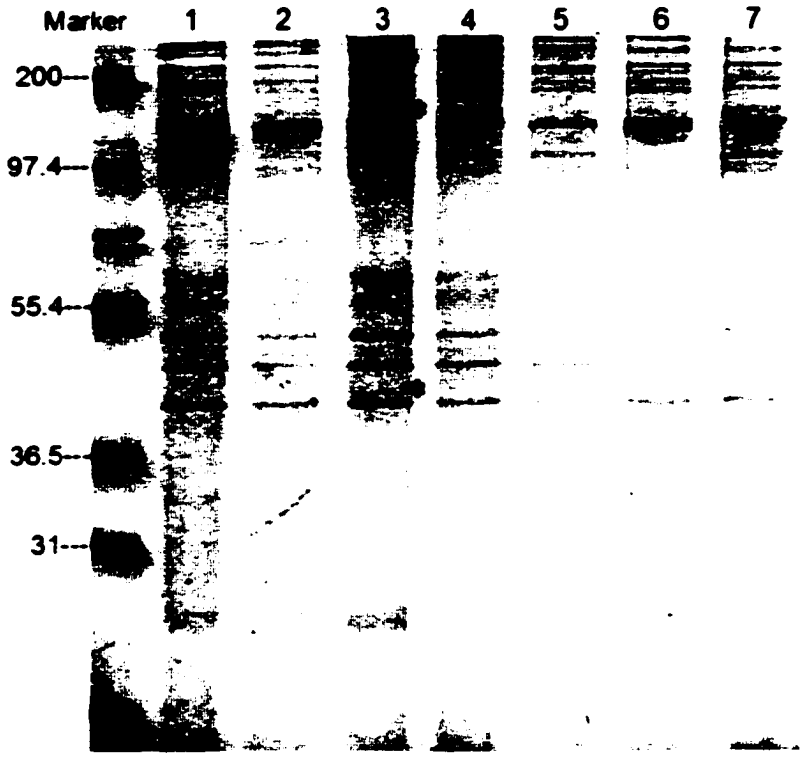

Figure 1. SDS-PAGE comparison of KHV isolates from diverse geographic areas. Lanes 1-7 represent the KHV cases report in this study. The * indicate two additional polypeptides found with D-081 but not the other isolates.

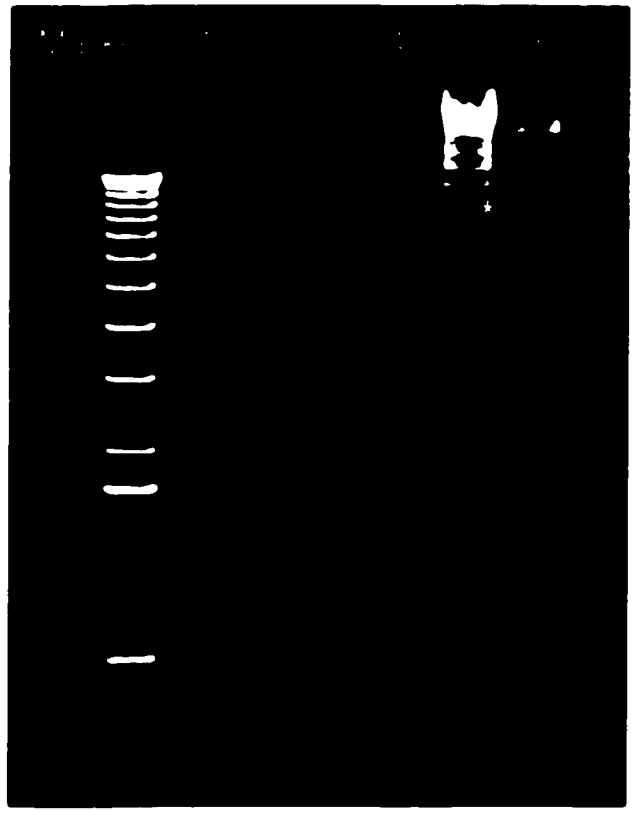

Figure 2. A comparison of KpnI restriction fragments of genomic DNA from KHV isolates. Lanes 1-7 represent the KHV cases report in this study. The * indicates the additional two fragments observed with D-081 not found with other isolates.

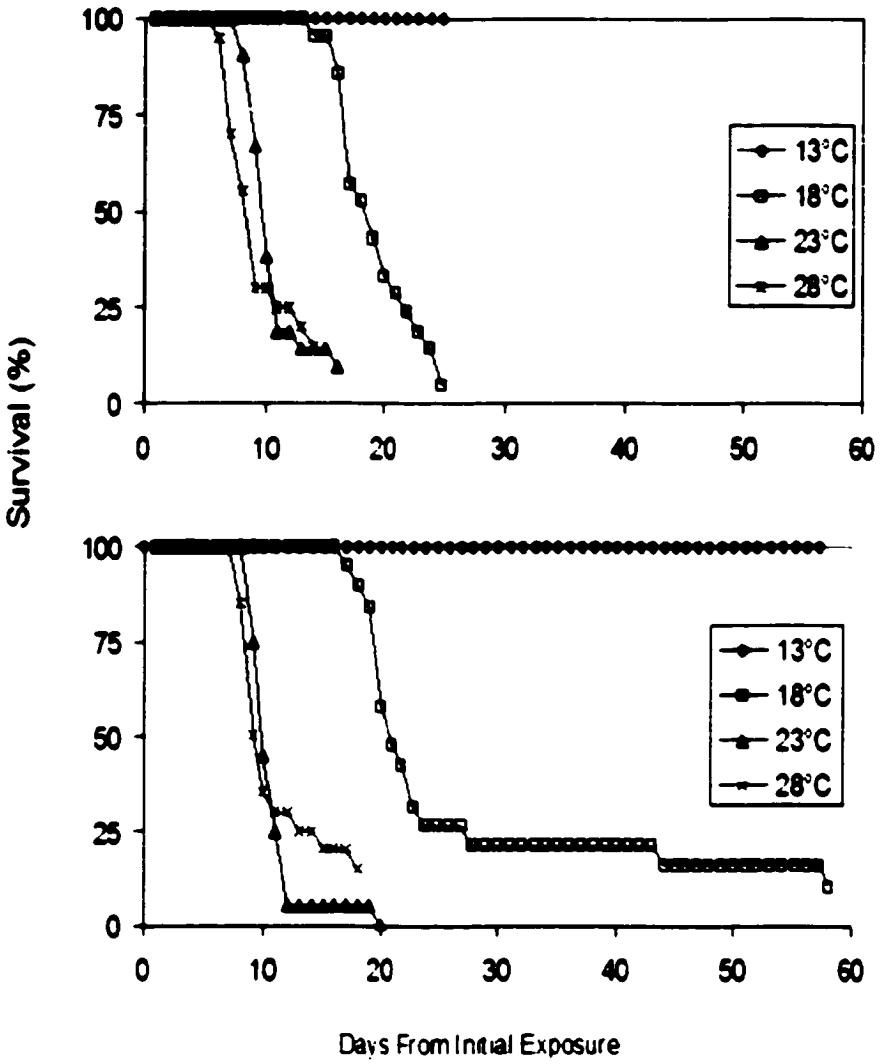

Figure 3. Survival among groups of juvenile koi following waterborne exposures to KHV at different water temperatures. Two experiments were conducted one at a higher dose ($TCID_{50}$/ml = 12) (top figure) and the second at a lower virus dose ($TCID_{50}$/ml = 1.2) (bottom figure). There was no mortality among virus exposed koi in either trial at 13°C or among unexposed groups of koi held at each water temperature.

Table 1. Information on isolates of KHV used in this study including, the water temperatures at the time of outbreaks when the virus isolates were obtained, the fish host species, and the country of origin.

Isolate	Year of isolation	Water Temperature (°C)	Species	Origin
KHV-I	1998	22-24	koi	Israel
C-361	2000	18	koi	Malaysia
D-081	2001	23	koi	Israel
D-164	2001	20	koi	Malaysia
D-060	2001	18 and 23	koi	Mix (Japan/Israel)
D-132	2001	22-25	koi	USA
C-250	2000	21	koi	Israel

Table 2. The effects of incubation temperature on the growth of KHV in the KF-1 cell line. Virus concentrations were evaluated at selected time points (T) post virus exposure in the cell free and cell associated fractions. All values expressed at the $TCID_{50}$/ml.

Cell Free Fraction

T	4°C	10°C	15°C	20°C	25°C	30°C	37°C
6h	<42	0	0	<42	<42	0	0
12h	0	<42	0	0	0	0	0
1d	0	0	<42	<42	<42	0	0
36h	0	<84	<42	<109	38	0	0
2d	0	<42	0	<67	24	0	0
4d	0	0	<67	511	2121	0	0
7d	0	0	776	46077	6736	0	0
13d	0	0	6665	1590	274	0	0
25d	0	0	123	31	1685	0	0

Cell Associated Fraction

T	4°C	10°C	15°C	20°C	25°C	30°C	37°C
6h	0	0	0	0	0	0	0
12h	0	<42	0	0	0	0	0
1d	<42	0	0	<84	<84	0	0
36h	0	<42	<109	<67	<52	0	0
2d	0	0	<42	<98	<56	0	0
4d	0	0	45	226	109	0	0
7d	<42	<42	<135	3542	718	0	0
13d	<42	0	9922	98	<56	0	0
25d	0	0	55	0	<84	0	0

Table 3. Mean days to death for juvenile koi following waterborne exposures to KHV at different water temperatures and two virus doses. KHV exposed koi at 13°C did not undergo mortality until shifted to 23°C at 30 d post initial exposure. The mean day to death was calculated the sum of the days to death for individual fish in a group divided by the total number of dead fish in the group.

Temperatures	$TCID_{50}$=12	$TCID_{50}$=1.2
28°C	7.7	9.2
23°C	9.2	9.9
18°C	18.2	23.6
13°C	No Dead Fish	No Dead Fish
13°C to 23°C	7.4	12.8

Table 4. Isolation of KHV on the KF-1 cell line from tissues of dead juvenile koi following waterborne exposures to either a 12 or 1.2 $TCID_{50}$ of KHV. Virus-exposed koi at 13°C at either virus dose did not undergo mortality. Mortality did occur following shifting of the high dose KHV exposed from 13°C to 23°C at 30 d post initial exposure. Dead fish from which virus was not isolated with the KF-1 cell line were examined by PCR assays for evidence of virus infection. NA = not applicable.

$TCID_{50}$=12

Temperature	No. Dead Fish	Virus Isolation Positive	PCR Positive
28°C	17	9/17	7/8
23°C	19	16/19	1/3
18°C	20	20/20	
13°C	0	NA	
13°C to 23°C	5	5/5	

$TCID_{50}$=1.2

Temperature	No. Dead Fish	Virus Isolation Positives	PCR Positives
28°C	17	9/17	6/8
23°C	20	16/20	4/4
18°C	17	15/17	1/2
13°C	0	No dead fish	
13°C to 23°C	5	4/5	1/1

Quantitative Assessment of the Concentrations of Koi Herpesvirus (KHV) in *Cyprinus carpio koi* Using real-time TaqMan PCR

ABSTRACT:

The koi herpesvirus (KHV) is a major pathogen causing mortality in populations of common carp *Cyprinus carpio carpio* and koi *Cyprinus carpio koi*. Temperature is a key factor influencing both virus replication in cell culture and the onset and severity of mortality in virus-exposed koi. Known KHV genomic DNA sequences were used to optimize a rapid real-time TaqMan PCR assay to detect viral DNA for diagnostic and research purposes. The real-time TaqMan PCR assay recognizes KHV infected cell lines and fish tissues but not host DNA isolated from *Cyprinus carpio* or the KF-1 cell line derived from *C. carpio*. The real-time PCR did not react with either the cyprinid herpesvirus (CHV) or channel catfish virus (CCV). The analytical level of target KHV genome copies ranged from 10 to 10^7 molecules. Time post exposure and water temperature were found to have a significant effect on the replication of KHV as determined by the genome equivalents per 10^6 host cells in experimental infections of koi. The principal target tissues for KHV replication were the gill, kidney and spleen, with virus genome equivalents up 10^{10} per 10^6 host cells. High genome copies of KHV were also found in the mucus, liver, gut, and brain. Lower KHV genome copies (up to 199 per 10^6 host cells) were detected in gill, kidney or brain of fish surviving infection at 62 to 64 d post virus exposure. Anti-KHV neutralizing antibodies were detected at only the lowest serum dilutions tested in 6 of 45 of koi surviving experimental infections as evaluated at 64 d post virus exposure.

INTRODUCTION

In 1998, a herpes-like virus was isolated from koi (*Cyprinus carpio koi*) in Israel and the U.S.A. (Hedrick et al. 2000). The virus, termed koi herpesvirus (KHV), was later found to be associated with losses among common carp (*Cyprinus carpio carp*) in several European countries and Indonesia (Rukyani 2002, Gilad et al. in press). Comparisons of the genomic DNA and virion polypeptides have clearly shown that KHV differs from the only other herpes-like virus, cyprinid herpesvirus (CHV, also referred to as *Herpesvirus cyprini*), isolated from koi (Gilad et al. 2002, Sano et al. 1985a,b). CHV can cause mortality among koi and common carp younger than 2 mo in age and survivors may develop the characteristic papillomatous-like growths commonly known as carp pox (Schubert 1966). In contrast to CHV, KHV has been associated with mortality among all ages of koi and common carp (Hedrick et al. 2000). Koi that recover from KHV infections have been shown to transmit the virus to susceptible populations of koi with mortality approaching 100% among these naive fish. The virus has spread rapidly, perhaps from a few initial locations to its current more broad geographic distribution (Gilad et al. in press). The principal means by which the virus has spread is the very active international trade in live koi for the ornamental hobby. Sales and shows that bring koi from distant locations together for competitive judging, have encouraged this trade and contributed to the emergence of this newly recognized viral pathogen (Gilad et al. in press).

Outbreaks in koi and common carp due to KHV tend to occur principally in the spring and autumn when water temperatures range from 18 – 24°C (Hedrick et al. 2000).

A basis for the temperature dependency of KHV outbreaks may relate directly to temperature requirements for the virus. Recent experimental studies have demonstrated that KHV grows optimally in the koi fin cell line (KF-1) at 20 – 25°C and in juvenile koi at water temperatures from 18 – 23°C (Gilad et al. in press). The principal target tissues for KHV based on microscopic pathological exams of naturally and experimentally infected koi are suspected to be the gill, kidney and spleen but other tissues are most likely involved (Hedrick et al. 2000, Gilad et al. in press). Currently, presumptive KHV outbreaks are characterized by observation of the characteristic gill pathology (swollen and necrotic filaments) in gross exams and continuing mortality despite treatments for bacterial and or external parasites occurring at water temperatures between 18 – 25°C. KHV infections are confirmed by isolation of the virus using KF-1 cells followed by PCR testing of virus isolates or fish tissues directly by one of two PCR assays (Gilad et al. 2002, Gray et al. 2002). KHV is often difficult to isolate with the KF-1 line from infected fish, particularly if the fish has been dead for several hours or have been frozen. Detection may therefore, rely solely on the PCR results. In addition to being difficult to isolate, attempts to determine virus concentrations in fish tissues have been unsuccessful. Thus, better procedures are needed to assess the distribution and concentrations of virus found in tissues of infected koi and common carp.

The real-time TaqMan PCR has been shown to detect and quantitatively assess very low copy numbers of target molecules (Clementi 2000, Mackay et al. 2002). The real-time TaqMan PCR has more recently moved from a research to a common diagnostic procedure in mammals (Leutteneger et al. 1999, Berger & Preiser 2002) and fish (Dhar et al. 2001, Overturf et al. 2001, del Cerro et al. 2002).

Here we describe the development of a real-time TaqMan PCR for KHV that can be used to detect and quantify KHV DNA in infected fish. The assay was then used to assess concentrations of target viral DNA as found in tissues of experimentally infected koi at water temperatures of 13, 18, 23 and 28°C.

MATERIAL AND METHODS

Virus strains

The KHV (KHV-I) used in this study was isolated from adult koi experiencing mass mortality in Israel in 1998 (Hedrick et al. 2000). The CHV in this study was a gift from Dr. T. Sano and Dr. H. Fukuda, Tokyo University of Fisheries. The channel catfish herpesvirus (CCV) strain CA80-5 isolated from an epizootic among juvenile channel catfish *Ictalurus punctatus* in California in 1980 (Arkush et al.1992) was used as a control herpes-like virus from fish.

Nucleic acid extraction from purified virions

KHV grown in KF-1 cells was purified and genomic DNA extracted as described by Gilad et al. (2002).

Plasmid preparation for TaqMan quantitation

A plasmid carrying a KHV genome insert was prepared as described by Gilad et al. (2002). Briefly, genomic DNA purified from KHV virions was incubated with 10 U of *Kpn*I and *Sac*I endonucleases. The resulting DNA fragments were inserted into the

pGEM®-7Zf(+) plasmid that was then used to transform DH5α *Escherichia coli* cells. Bacterial cells harboring KHV inserts were selected and screened by PCR using SP6/T7 primers flanking the insert site (Sambrook et al. 1989). The sequence of inserts was obtained by fluorescently labeled dideoxy terminator sequencing using an ABI 377 automated DNA sequencer (Applied Biosystems). The KHV TaqMan assay was developed from the insert designated AF411803.

Real time quantitative PCR system

For each target sequence, two primers and an internal fluorescently labeled TaqMan probe (Table 1) were designed as described by Leutteneger et al. (1999). In addition, to a TaqMan PCR detecting sequences of KHV insert (AF411803), a second assay was developed targeting an exonic sequence of the koi glucokinase (AF053332), a known single copy gene from common carp (Panserat et al. 2000). This second TaqMan assay served as a genomic DNA extraction control for enumeration of the number of host cells present in each assay. Exon positions were extrapolated from human glucokinase sequences (XM_041002) and verified on genomic DNA from koi. The length of the PCR products were kept purposely short (between 69 and 78 bp) to enable high amplification efficiencies. Primer and probe sequences and relevant information to the TaqMan PCR for KHV are listed in Table 1. The least amount of target DNA detectable with the TaqMan PCR assay was determined using 10 folds serial dilutions from 10^8 to 0 copies of the plasmid bearing the KHV insert.

Sample preparation for TaqMan PCR

Tissue samples (approximately 20 – 50 mg) obtained from freshly euthanized koi were stored at –80°C. Before DNA extraction, the tissues were transferred frozen into 96 deep well plates containing 800 µL of 1x ABI lysis buffer (Applied Biosystems, Foster City, CA) and two grinding beads (4 mm diameter, SpexCertiprep, Metuchen, NJ). Tissue samples were ground in a GenoGrinder2000 (SpexCertiprep) for 2 min at 1000 strokes per min. After 30 min at 4°C, genomic DNA was extracted from the tissue lysates with a 6700 automated nucleic acid (ANA) workstation (Applied Biosystems) according to the manufacturer's instructions.

Source of fish

Koi used in the experimental trial were from a closed-system commercial ornamental fish producer in Northern California with no history of KHV infection. The fish were transported live to the Fish Health Pathogen Containment Laboratory at the University of California Davis. The fish were held in 130 L flow through aquaria receiving 18°C well-water at 1.8 L/min, and were fed a commercial koi ration at 1% body weight per day. At the time of experimentation these fish were approximately 2 years in age with a mean weight of 0.274 kg and a mean length of 23 cm.

Experimental design for virus exposures of fish

Just prior to the initiation of the experiment all fish were combined into one 800 L aquarium. Fish were randomly assigned to 8 aquaria by drawing numbers from a common pool such that each 130 L aquarium eventually contained 20 or 21 fish. All fish

were initially at a water temperature of 18°C. Acclimation to each of the three other water temperatures (13, 23, or 28°C) for the trial was accomplished by lowering or increasing the water temperature in increments of 3°C per day until the desired temperature was obtained. There were two replicate aquaria at each water temperature. Fish in one aquarium at each temperature received a bath exposure to 12 $TCID_{50}$/ml of KHV grown in KF-1 cells, whereas the fish in the second aquaria at each temperature were treated identically to the virus-exposed groups but received only minimal essential media (MEM) with no virus. During the virus or MEM exposure period the water flow was stopped and oxygen was bubbled into each aquarium for a period of 1 h. After the exposure, the flow of water to all aquaria was resumed. Two fish were removed for sampling at the following time points post exposure: 1d, 2d, 3d, 6d, 10d, 16d, and 62 or 64d. Fish were euthanized by placement into a container with 500 mg benzocaine per liter of water. Blood was withdrawn from the caudal vein and the serum was collected for neutralization assays as described below. Selected tissues (mucus, gill, gut, liver, spleen, kidney, and brain) from each fish were examined for the presence of the KHV by isolation on cell culture (Hedrick et al. 2000), and for concentrations of target KHV DNA by the real-time TaqMan PCR. Not all samples collected at selected time points were examined by the TaqMan PCR assay. In most cases the mean value KHV genome equivalents for 10^6 host cells for two fish were calculated at each time point but in some instances tissues were from a single fish. Fish that died at or between sampling periods were removed and tested as described above.

Quantification of KHV target in tissue samples

The quantity of KHV target DNA in koi tissues was determined with a modification of the comparative CT method (User Bulletin #2, Applied Biosystems) and is reported as the absolute number of KHV genomes per 10^6 host cells from a given tissue. The TaqMan assay targeting the carp glucokinase gene was used to enumerate the cell number by extrapolation from a standard curve. KHV concentrations were determined in a parallel reaction with the same DNA as the glucokinase assay and calculated from a standard curve generated using dilutions of a plasmid with a cloned fragment of the KHV genomic DNA target region.

Statistical analyses

The KHV genome copy number (expressed as TaqMan values) at selected time points post virus exposure were compared with or without respect to temperature or tissues sampled by repeated measures analysis of variance (ANOVA) followed by multiple comparison analyses using the Tukey method adjusted for unequal sample sizes (SPSS™ release 11.0, Standard Version for Windows SPSS Inc., Chicago, Ill). TaqMan quantification values were log transformed to enhance normality and to fulfill the assumption of the ANOVA model.

Serum neutralization tests

Serum was collected from clotted blood by centrifugation at 3000 x g for 10 min at 10°C and then stored at -20°C until tested. The serum was heat-inactivated for 10 min at 45°C and examined for evidence of anti-KHV neutralizing activity. Serial ten fold

dilutions of serum from each fish in MEM was prepared and then mixed with an equal volume of MEM-2 HEPES containing a total of 760 $TCID_{50/ml}$ of KHV grown in KF-1 cells. The mixture was then left for 1 h at 20°C. After the incubation period, the mixture was distributed into 5 wells (0.1 ml per well) of a 96 well plate followed by an addition of an equal volume of MEM-2-HEPES containing KF-1 cells. Virus neutralizing activity was evaluated by the dilution of serum at which 50% of the wells were protected from viral induced cytopathic effects. End points were calculated using the method of Reed and Meunch (1938).

RESULTS

Optimization of the Real-time TaqMan PCR assay

The optimal parameters for the real-time TaqMan PCR assay were obtained by sequential testing of target DNA from purified plasmid and later from previous experimentally and naturally KHV-exposed koi (Hedrick et al. 2000, Gilad et al. 2002). Each PCR reaction contained 400 nM of each primer, 80 nM of the TaqMan probe in the commercially available PCR mastermix (TaqMan Universal PCR Mastermix, Applied Biosystems) containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 5 mM $MgCl_2$, 2.5 mM deoxynucleotide triphosphates, 0.625 U AmpliTaq Gold DNA polymerase per reaction, 0.25 U AmpErase UNG per reaction and 5 µl of the diluted DNA sample in a final volume of 25 µl. The samples were placed in 96 well plates and amplified in an automated fluorometer (ABI PRISM 7700 Sequence Detection System, Applied

Biosystems). Amplification conditions were 2 min at 50°C, 10 min at 95°C, 40 cycles of 15 s at 95°C and 60 s at 60°C.

Ability of real-time TaqMan PCR to discriminate KHV from other herpesviruses

The real-time TaqMan PCR assay under optimal conditions amplified a 78 bp fragment of DNA from KHV and not from CHV, CCV, control koi tissues or the KF-1 cell line.

Real-Time TaqMan PCR examination for the level of detection

The lowest copy number of KHV plasmid DNA detected was 10. The highest copy number of KHV plasmid DNA detected was 10^7 (Figure 1).

Quantification of KHV DNA in experimentally exposed fish during active infection

The amount of KHV DNA was assessed from a total of 216 tissues collected from koi exposed to the virus in the experimental trial. No KHV DNA was detected in any of the tissues sampled from the control unexposed fish. Statistical analyses indicated that over all, time had significant effects ($p < 0.0001$) on genome copy number (expressed as TaqMan values) regardless of the water temperature or tissues examined (Table 2). Mortality was first observed among KHV exposed koi at a water temperature of 28°C at 5 d, at 8 d for 23°C and at 14 d for 18°C. There was no mortality among virus exposed koi at 13°C or in any of the control unexposed groups at each water temperature. TaqMan values for KHV DNA were generally greatest in the mucus on tissues examined at each temperature at 1 d post exposure. Compared to other tissues, the gill, kidney and spleen,

consistently had high genome copies of KHV at a water temperature of 23°C, although the greatest single KHV genome copy number was detected in the gut of fish sampled on day 9 post virus exposure (Table 3). At the peak of infection at water temperatures of 18, 23, and 28°C KHV genome copies in most tissues examined ranged from 10^7 to 10^9 per 10^6 host cells.

Quantification of KHV DNA in survivors

Mortality had ceased in all groups by day 24 post virus exposure and the fish remaining in each group were kept in their respective aquaria. Fish began actively feeding at this time and were normal in appearance thereafter. The experiment was terminated between days 62 and 64 post virus exposure and the kidney, spleen and gill tissues of survivors were examined for KHV DNA with the TaqMan assay. The greatest KHV DNA values were 92 and 199 genome copies per 10^6 host cells in the gill and kidney, respectively of survivors at the 28°C water temperature (Table 3).

Serum testing for neutralization:

There was evidence of anti-KHV neutralizing activity at the lowest dilution (1:50) of serum tested in only 6 of the 45 serum samples tested.

DISCUSSION

Experimentally infected koi at all water temperatures tested (13 – 28°C) were found to contain KHV DNA detected by the real-time TaqMan PCR within 1 h and then at selected times up to at least 64 d post virus exposure. Detection of viral DNA in the

mucus early in infection suggests that the skin is an initial site of entry and replication of the KHV. The virus spread rapidly, particularly at water temperatures of 18 - 28°C, to other organs, including the gill, kidney, liver, intestine and brain. The gill, kidney and spleen were the principal target tissues with consistently high concentrations of viral DNA that reached levels of 10^{10} copies per 10^6 host cells between 6 and 10 d post virus exposure, a time when mortality was greatest among fish at 28 to 18°C. Although no mortality occurred among virus-exposed koi at 13°C, KHV DNA was detected in several organs but at lower concentrations than virus exposed fish at higher water temperatures. Viral DNA was detected among all virus exposed fish (n = 5) examined at 62 to 64 d and we suspect these fish to be carriers. However, confirmation that these previously KHV exposed koi can undergo periods of virus reactivation and shedding of infectious virus will require additional studies. Sera collected from koi at 62 to 64 d post KHV exposure contained low levels of anti-virus neutralizing antibodies and in only 6 of 45 virus exposed fish examined. The TaqMan PCR may therefore be the current test with the most potential for detecting carriers of KHV since serum neutralization assays and conventional PCR tests routinely are negative for previously virus exposed fish from field and laboratory trials (unpublished data).

Field observations and recent laboratory trials have demonstrated the importance of water temperature as a factor influencing the onset and severity of disease in koi exposed to KHV (Hedrick et al., 2000, Gilad et al. in press). During periods of colder water (e.g. winter) KHV disease is rare among koi and common carp and correspondingly our study showed that even though fish become infected at these lower temperatures (13°C) that virus replication occurs, although in a diminished capacity compared to higher

temperatures (Table 3) and no mortality ensues (Gilad et al. in press). At water temperatures of 18 - 23°C, which are more optimal for virus replication, KHV DNA was detected by 1 d post exposure and had reached high levels by 6 to 10 d. KHV genome copies up to 10^8 to 10^{10} per 10^6 host cells were detected in tissues of exposed koi and the trend was to reach these levels more rapidly as water temperature increased. Mortality, although not evaluated as an endpoint in this study was more rapid in onset with increasing temperature, a pattern found in a previous study evaluating the effects of water temperature on mortality among koi exposed to KHV (Gilad et al. in press).

The epidermal tissues may be an initial site of virus replication for KHV as the mucus consistently contained KHV genome copy numbers at early time points in the study. That mucus can harbor infectious virus has been demonstrated by LaPatra (1989) who isolated infectious virus directly from this site from rainbow trout (*Oncorhynchus mykiss*) exposed to infectious hematopoietic necrosis virus (IHNV). Later studies by Yamamoto et al. (1992) demonstrated that IHNV replicated efficiently in excised epidermal tissues from rainbow trout thus directly indicating the important role of the skin in the pathogenesis of IHNV infections. The affinity for epidermal tissues is a feature of many herpesviruses including those found in fish (Hedrick & Sano 1988). Studies of the progress of infections of koi and channel catfish with the cyprinid herpesvirus (CHV) and the channel catfish virus (CCV), respectively emphasize the important role of the skin and gills early in infection (Nusbaum & Grizzle 1987, Sano et al. 1993). A similar initial and then persistent involvement of the skin and gills has been observed among Japanese eels (*Anguilla japonica*) infected with anguillid herpesvirus (Kobayashi & Miyazaki 1997, Lee et al. 1999). Viral DNA is also present in several internal organs including the

brain, heart, liver, kidney, spleen and gut during active infections with CHV and CCV (Wise & Boyle 1985, Sano et al. 1993, Gray et al. 1999a). This same systemic spread of virus, perhaps from initial sites in the skin and gills to internal organs also occurs following exposure of koi to KHV. Based on consistently high copy numbers of KHV genomic DNA, the gill, kidney and spleen are the principal target organs and are the preferred tissues for virus isolation during active infections in koi (Hedrick et al. 2000).

At colder water temperatures (e.g. 13°C) KHV has been shown to establish infections in koi but with no evidence of clinical signs of disease or mortality (Gilad et al. in press). That these fish harbor virus was shown by shifting them to water temperatures of 23°C which resulted in disease and mortality and the ability isolate infectious virus from their tissues. The TaqMan PCR effectively demonstrated that virus is present in fish following exposure to KHV at a water temperature of 13°C although it is considerably less than that found at similar time points among fish at higher water temperatures (Table 3). Fish that have acquired infections during declining water temperature periods (e.g. autumn) may therefore not demonstrate any clinical signs until the following spring when water temperatures again rise to a more permissive range for the virus. The inactivity of KHV at nonpermissive water temperatures most likely differs from the more true latent state that can be found with most herpesviruses, including those from fish (Wolf 1988).

Koi that recover from infections at permissive water temperatures resist re-infection. Programs have been developed to control KHV by utilizing this acquired immunity coupled with screening by single round PCR testing to clear fish for movements. However, if latent infections occur and these infections can be reactivated, survivors of previous infections may be a source of the spread of the virus. Evidence that KHV

persists, at least for periods up to 64 d post virus exposure, was detected with the TaqMan PCR in the gill, kidney and brain of experimentally exposed koi (Table 3). That these fish represent true latent carriers of KHV has not been proven by our studies. This would require a demonstration that active virus can be produced and shed at some point from fish in which KHV DNA was detected. Latent infections either in nervous or subcutaneous tissues of koi with CHV or in peripheral blood leukocytes of channel catfish with CCV have been proposed (Sano et al. 1993, Baek & Boyle 1996, Gray et al. 1999).

Detection of KHV, even during an epidemic, can often be problematic (Hedrick et al. 2000, Gilad et al. 2002) as virus can be difficult to isolate in cell culture from fish dead for several hours or if frozen prior to analysis. Current procedures rely on virus isolation but are supplemented by PCR to either confirm virus found in cell culture or present directly in the tissues of fish with presumptive KHV infections (Gilad et al. 2002, Gray et al. 2002). Direct comparisons of the single and nested PCR assays with the TaqMan PCR are pending but our initial trials suggest that the TaqMan has the ability to detect the lowest concentrations of target DNA and thus may become the preferred method for demonstrating prior exposures to KHV. Attempts to detect neutralizing antibodies in sera of previously exposed fish in our trials were a less reliable indicator of prior virus exposure. That anti-KHV antibodies may be present in koi have been demonstrated by enzyme linked immunosorbent assays (ELISA) with purified KHV as the coating antigen (unpublished data). Cross reactions with CHV detected in the initial ELISA trials however, indicate that this test will require further modifications before being an effective method to evaluate prior exposures of koi to KHV.

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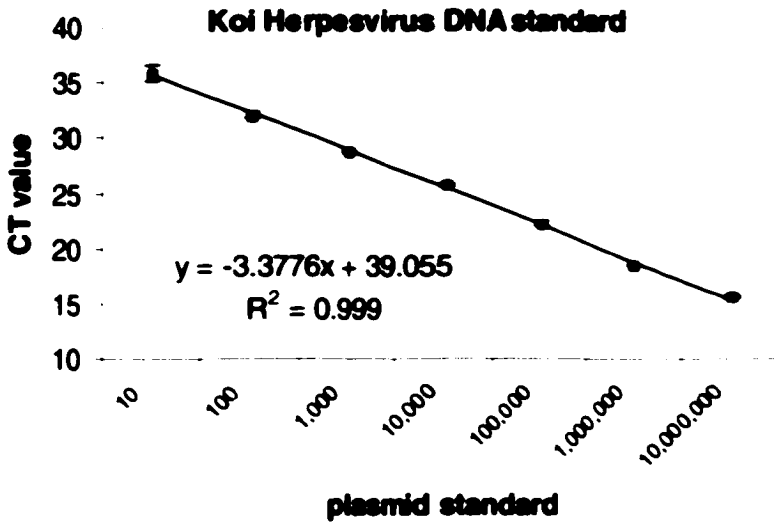

Figure 1: Standard curve using CT values to calculate the analytic sensitivity of KHV genome equivalents with the TaqMan PCR. The assay detects a range from 10 to 10^7 copies of a plasmid bearing the KHV target sequences which can be used as a standard curve.

Table 1. Primer sequences and TaqMan probes specific for a newly developed real-time PCR to detect KHV and the *Cyprinus carpio* glucokinase gene (as an internal control).

Target	Primer	Sequence (5'->3')	Length	Probe	Probe sequence (5'->3')
KHV	KHV-86f	GACGCCGGAGACCTTGTG	78	KHV-109p	CTTCCTCTGCTCGGCGAGCACG
	KHV-163r	CGGGTTCTTATTTTTGTCCTTGTT			
Glucokinase	CgGluc-162f	ACTGCGAGTGGAGACACATGAT	69	cgGluc-185p	AAGCCAGTGTCAAAATGCTGCCCACT
	CgGluc-230r	TCAGGTGTGGAGCGGACAT			

Table 2. Mean TaqMan values for KHV genome copies as found in tissues of koi following experimental infections with the virus at 4 water temperatures. The values are presented as the mean KHV genome equivalents per 10^6 host cells for all tissues sampled at all temperatures combined at each time point post virus exposure. Superscripts in common indicate no differences between those groups following Tukey's pairwise multiple comparisons at a 5% level of significance.

Time (p.e.)	Number of samples	Mean Values	Minimum Values	Maximum Values	Standard Deviation
2 h	3	800 [a]	0	2400	1386
1 d	31	6.51e+5 [a,b]	0	1.20e+7	2.38e+6
3 d	23	1.16e+7 [b]	0	1.24e+8	3.18e+7
6 d	25	1.70e+8 [c]	3835	9.17e+8	2.98e+8
8 d	19	9.14e+8 [d]	1.86e+6	4.93e+9	1.31e+9
9 d	6	4.67e+9 [c,d,e]	1.53e+6	2.31e+10	9.08e+9
10 d	27	2.10e+9 [c,e,f]	1.00e+0	3.53e+10	6.85e+9
15 d	6	1.09e+9 [c,d,e,f]	2708415	3.79e+9	1.44e+9

Table 3. KHV genome equivalents per 10^6 host cells as found in koi following experimental exposures to the virus at 4 water temperatures using the newly developed real-time TaqMan PCR. Not all tissues (dt) were tested at all times post virus exposure (T). Values marked with an "*" represent the mean of tissues from 2 fish otherwise the value is from single fish. Fish that were dead at the time of sampling are indicated by a "m", otherwise fish were alive and sacrificed for sampling. In some cases the TaqMan PCR failed to provide a value (nd).

Temperature	T	Mucus	Gill	Liver	Gut	Spleen	Kidney	Brain
28°C	2h	0	nd	dt	0	dt	dt	dt
28°C	1d	1.64E+05	1.26E+05	6.13E+06	1.20E+07	3.25E+10	5.08E+04	1.52E+09
28°C	3d	nd	0.00E+00	5.68E+04	0.00E+00	9.54E+06	nd	0.00E+00
28°C	6d	8.87E+08	9.18E+08	1.74E+07	5.87E+06	1.16E+08	6.38E+07	3.26E+07
28°C	8d	7.77E+07	6.87E+08	2.02E+08	6.86E+07	8.89E+08	2.17E+09	1.66E+07
28°C	62d	dt	9.20E+01	dt	dt	0.00E+00	1.99E+02	dt
23°C	2h	dt	0	0	0	nd	nd	nd
23°C	1d	3.00E+05*	1.62E+03*	2.69E+04*	8.05E+01*	1.23E+03*	6.83E+03*	3.31E+05*
23°C	3d	3.87E+06*	6.24E+07*	4.80E+06*	4.62E+05*	9.78E+04	1.28E+04	1.17E+05
23°C	6d	8.97E+07*	4.26E+08*	1.22E+05	1.27E+06*	3.90E+08*	1.28E+06	3.90E+06*
23°C	8d[m]	2.24E+08*	1.73E+09*	6.52E+08*	4.97E+08	7.01E+08	2.49E+09*	9.66E+07*
23°C	9d[m]	1.02E+08	1.25E+09*	nd	1.15E+11*	nd	2.31E+10	1.54E+06
23°C	10d[m]	2.10E+08*	3.64E+09*	1.90E+10*	3.45E+08*	1.78E+09*	2.87E+09*	1.80E+08
23°C	64d	dt	13.00E+00 *	dt	dt	0.00E+00*	2.00E+00*	25.00E+0*
18°C	2h	nd	nd	dt	0	dt	dt	dt
18°C	1d	2.72E+04	2.11E+04	1.14E+03	7.94E+03	8.55E+04	1.68E+04	1.03E+05
18°C	3d	4.81E+04	1.58E+05	1.81E+05	2.02E+06	3.23E+04	4.75E+03	2.63E+07
18°C	6d	4.08E+08	6.36E+05	1.00E+00	1.26E+04	5.60E+04	3.07E+05	8.33E+05
18°C	10d	2.88E+08	1.47E+08	8.52E+04	6.79E+07	4.95E+07	5.84E+08	4.33E+06
18°C	15d[m]	2.71E+06	3.48E+08	1.00E+00	9.18E+08	1.48E+09	3.80E+09	5.59E+06
18°C	62d	dt	6.00E+01	dt	dt	0.00E+00	2.80E+01	dt
13°C	1d	0.00E+00	nd	nd	1	nd	0.00E+00	0.00E+00
13°C	10d	3.50E+05	1.28E+04	1.00E+00	6.40E+03	3.51E+03	1.10E+04	3.85E+05
13°C	64d	dt	5.00E+00	dt	dt	nd	1.00E+00	0.00E+00

CONCLUSSIONS:

KHV is an important pathogen causing mass mortality in koi (*Cyprinus carpio koi*) and common carp (*Cyprinus carpio carp*). Since the initial discovery KHV has rapidly spread and the disease it causes has been observed in North America, Europe, Israel and Asia. Mortality due to KHV infection has caused significant economic losses to fish production for food and to the world wide ornamental fish industry. The work conducted in this thesis provides the following conclusions on this new viral agent:

1. KHV is clearly different than *Herpesvirus cyprini* (CHV), the most commonly known herpesvirus from cyprinid fish.

2. KHV has 31 virion polypeptides. Twelve have similar molecular weights to those of CHV and ten are similar to those of the channel catfish virus (CCV).

3. Both virion polypeptide analyses and restriction fragment length polymorphisms (RFLP) of genomic DNA demonstrated that the first two KHV isolates from Israel and the USA were identical.

4. A conventional polymerase chain reaction (PCR) assay to detect the virus in koi tissues was developed with sequences obtained from one restriction fragment of KHV DNA. The PCR assay effectively detected KHV but did not amplify genomic DNA from either

CHV or CCV. The PCR assay detected as little as 1 pg of KHV DNA as mixed with 100 ng of host DNA.

5. The PCR assay should complement virus isolation as an effective diagnostic method for KHV because often it is difficult to isolate the virus with the KF-1 line from infected fish, particularly if the fish has been dead for several hours or has been frozen.

6. KHV has spread rapidly, perhaps from a few initial locations to a world wide distribution. The isolates characterized to date represent a homogeneous group with the exception of a single isolate from koi in Israel.

7. In-vitro, optimal KHV growth occurred in the koi fin (KF-1) cell line at temperatures from 15 – 25°C with the greatest virus concentrations detected in cell free medium after 7 d of incubation at 20°C. There was no or minimal growth at 4, 10, 30 or 37°C.

8. KHV outbreaks are water temperature dependent and experimental studies demonstrate the virus can cause mortality within a range from 18 to 28°C. The greatest cumulative mortality (95.2%) was observed at a water temperature of 23°C.

9. Survival analyses indicated a significant difference in risk of mortality between the virus-exposed fish at 13°C compared to all other water temperatures and between fish held at 18°C compared to those at 23°C.

10. A real-time TaqMan PCR for KHV was developed to detect and quantify viral DNA in infected fish for diagnostic and research purposes. The real-time TaqMan PCR assay specifically recognizes KHV from CHV and CCV and does not react with DNA extracted from the cell line used to grow and isolate the KHV (KF-1) or control koi tissues. The analytical level of detection of the TaqMan PCR ranged from 10 to 10^{-} molecules.

11. KHV establishes systemic infection in koi as illustrated by the DNA distribution among the tissues tested (i.e. mucus, gill, spleen, gut, liver, kidney and brain).

12. KHV DNA concentrations increased significantly ($p < 0.0001$) over time regardless of the temperature and tissues, but as demonstrated by mortality due to the virus water temperature has an effect on KHV replication during active infections.

13. KHV DNA found to be present in low copy number in asymptomatic fish following exposure to the virus suggests the formation of carrier state that coincides with field reports that KHV spreads with asymptomatic fish introduced into naïve populations.

14. The role of the humoral immune response to KHV is most likely less important the cell mediate responses as there was little to no evidence of anti-KHV neutralizing activity in the sera of koi surviving experimental infections with the virus.

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