An Enzyme Linked Immunosorbent Assay (ELISA) for Detection of Antibodies to the Koi Herpesvirus (KHV) in the Serum of Koi *Cyprinus carpio*

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ABSTRACT—An enzyme linked immunosorbent assay (ELISA) detected the presence of anti-koi herpesvirus (KHV) antibodies in the serum of koi or colored carp (*Cyprinus carpio*) following either natural or experimental exposures to KHV. Concentrations of anti-KHV antibodies were detected at serum dilutions as great as 1:62,500 in a population of koi kept in virus-free water for 1 year following a naturally occurring outbreak due to KHV. At serum dilutions less than 1:2,500 cross reactions with a second herpes-like viral agent *Cyprinid herpesvirus 1* (CyHV-1) was detected in serum from both experimentally and naturally KHV exposed koi. Passive immunization by administration of anti-KHV antibodies from koi recovered from previous virus infections to naive koi provided only partial and transient protection to waterborne challenges with KHV. Koi that maintained high levels of serum anti-KHV antibodies after 1 year in virus-free water are deemed as suspect carriers of the virus. The identification of suspect carriers by screening of koi and common carp populations, including potential broodstocks, with the KHV ELISA should improve the ability to control this important viral pathogen.

Key words: koi herpesvirus, KHV, *Cyprinus carpio*, ELISA, antibody, koi

The koi herpesvirus (KHV) was first reported as the cause of mass mortality among koi and common carp (*Cyprinus carpio*) in Germany, Israel and the USA in 1998 but retrospective studies indicate the disease was present in common carp in the UK as early as 1996 (Bretzinger *et al.*, 1999; Hedrick *et al.*, 1999; Hedrick *et al.* 2005; Way *et al.*^{*1}). The worldwide movements of koi have presumably contributed to the spread of the virus which has now been recognized in several additional European countries, Indonesia, South Africa, Thailand, Taiwan, Japan and most likely China and Malaysia (Haenen *et al.*, 2004; Tu *et al.*, 2004; Sano *et al.*, 2004).

The virus was isolated from koi originating from Israel and the USA in 1998 and based upon morphologic

and biologic properties was designated as KHV by Hedrick *et al.* (2000). Recent genomic sequence comparisons by Waltzek *et al.* (2005) confirm that KHV is related to a larger group of fish herpes-like viruses in the family *Herpesviridae* and more specifically to *Cyprinid herpesvirus 1* (CyHV-1) from koi and common carp and *Cyprinid herpesvirus 2* (CyHV-2) from gold fish (*Carassius auratus*). Based upon these genetic relationships and that the fish hosts are all restricted to the family *Cyprinidae*, a proposal to formally designate KHV as *Cyprinid herpesvirus 3* has been submitted (Waltzek *et al.*, 2005). Designation of the virus as *Cyprinid herpesvirus 3* (CyHV-3) supplants previous designations for the virus including descriptions of KHV in Israel as carp interstitial nephritis and gill necrosis virus (CNGV) by Ronen *et al.* (2003), Pikarsky *et al.* (2004) and Hutoran *et al.* (2005).

Additional properties of KHV have been described including the important role of temperature on experimental infections of koi and koi cell cultures, major target tissues during infection and improved detection methods for the virus and viral DNA (Gilad *et al.*, 2002, 2003 and

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^{*1} Way, K., N. D. Beevers, C. L. Joiner, C. B. Longshaw, S. St-Hilaire, D. M. Stone, K. L. Denham and P. F. Dixon (2004): Koi herpesvirus in the UK: Detection in archive tissue samples and spread of the virus to wild carp. Abstract 6th International Symposium on Viruses of Lower Vertebrates, Hakodate, Japan, September 2004.

2004; Ronen *et al.*, 2003; Perelberg *et al.*, 2003; Pikarsky *et al.*, 2004). Both natural and experimental infections of koi with KHV at permissive water temperatures 16–28°C result in high mortality (70–100%) over a course of 7–21 days (Gilad *et al.*, 2003). Fish that survive infection, including those subjected to elevated water temperatures following infection, develop some or complete resistance to re-infection (Gilad *et al.*, 2003; Ronen *et al.*, 2003). The basis of this resistance is not known but anti-virus serum antibodies have been shown to increase in survivors post exposure to the virus (Ronen *et al.*, 2003). Tests that detect anti-KHV antibodies are thus important for evaluations of the potential immune status and as an indicator of prior exposure to the virus.

Detection of KHV during acute episodes of disease is possible by isolation of the virus using the KF-1 cell line and by one of several PCR assays that detect viral DNA directly from fish tissues (Gilad, *et al.* 2002; Gray, *et al.*, 2002; Yuasa *et al.*, 2005). Detection of KHV or viral genomic DNA has been difficult beyond 64 days post initial exposure even with highly sensitive quantitative PCR approaches (Gilad *et al.*, 2004). Fish that survive infection are viewed as potential carriers and presumably their movements in international trade have spread the virus.

In the current study we describe an enzyme linked immunosorbent assay (ELISA) capable of detecting anti-KHV antibodies in the serum of koi for periods up to 1 year from their last known exposure to the virus. The ELISA allows non-lethal sampling of individual or populations of koi to assess prior exposure and suspect carrier status in the absence of direct detection of infectious virus or viral DNA. In addition, the ability of anti-KHV serum to protect passively immunized koi later challenged with KHV was evaluated.

Materials and Methods

Virus production and purification

The virus isolate (KHV-I) used in this study was obtained from adult koi experiencing mass mortality in Israel in 1998 (Hedrick *et al.*, 2000). The virus was propagated in the KF-1 cell line in minimal essential medium (MEM) supplemented with 7.5% fetal bovine serum (FBS - Hyclone), 50 IU penicillin/mL, 50 µg streptomycin/mL and 2mM L-glutamine as described previously by Hedrick *et al.* (2000). The concentration of FBS in the growth medium was reduced to 2% (v/v) (MEM-2) prior to virus inoculation and cells were placed at 20°C for incubation until complete cytopathic effects (CPE) were observed. *Cyprinid herpesvirus 1* (CyHV-1) was originally isolated from koi in Japan (Sano *et al.*, 1985a) and it was also propagated in KF-1 cells under the same conditions described for KHV. When needed for ELISA antigen or in blotting procedures, both viruses were puri-

fied as previously described by Gilad *et al.* (2002).

Fish

The koi in this study were from four sources representing a local producer and retailer (Supplier 1), a second local retailer (Supplier 2), a population held in Germany (Supplier 3) and a producer (Supplier 4) who maintains and breeds fish in dechlorinated municipal water in fiberglass tanks. Fish ranged from 20 g to 2 kg in weight and in age from approximately 10 mo to > 2 years. Groups of koi from Supplier 1, 2 and 4 were transported live to the University of California at Davis (UCD) Fish Health Containment Laboratory where they were held in variably sized aquaria ranging in capacity from 130 to 650 L that received flow through well water at controlled temperatures of 19–23°C. Fish were fed once daily at 1–2% of body weight with a commercial trout ration. Koi from Supplier 3 were bled, serum collected and sent from the laboratory in Germany to the UCD laboratory for ELISA analysis. Additional information on fish sampled from each of the suppliers is provided below.

Three groups of koi were obtained from Supplier 1 who is a producer and retailer of koi for the local ornamental fish market. One group (S1-1) of 600 fish was approximately 10 mo in age and ranged in size from 20–50 g and came directly from a pond on the farm that showed no signs of KHV infection in 2003. This group was approximately 1.5 year in age and had been held in the UCD laboratory for approximately 6 mo in flow through well water at 19–23°C when they were used for the passive immunity trial described below. Approximately 60 of these fish were tested on several occasions during the 6 mo prior to the passive immunity trial for evidence of KHV by PCR (as controls for other KHV experiments) and no evidence for viral DNA was detected. Also, ELISA evaluations for 41 fish examined after 6 mo post transfer to the laboratory were examined for evidence of anti-KHV antibodies.

A second group (S1-2) of 400 koi of similar size and age to S1-1 were obtained in 2004 again from a second pond with no evidence of KHV infection. The fish were transported to the UCD laboratory and held under conditions described previously. A total of 40 fish were examined for presence of anti-KHV antibodies by ELISA 2 wk following transfer the laboratory. A third group (S1-3) of 300 koi, ranging in size from 50–200 g and approximately 1 year in age were also obtained in 2004 from the retail area where various groups of fish are mixed and held in a recycled water system (21–25°C) prior to sale. These fish were undergoing an active KHV outbreak at the time of transport to the UCD laboratory. The fish were held in the UCD laboratory in a 650 L aquarium receiving 19°C flow through well water. The cumulative mortality over the following week was 75%. A total of 71 fish surviving at 1 wk post

transport were bled and the serum tested for the presence of anti-KHV antibodies by ELISA. An additional 10 fish from this group were examined 47 days later for evidence of anti-KHV antibodies by ELISA.

A group (S2-1) of mixed sizes (50 g–500 g) and age koi (1–2 yr) was obtained in 2004 from Supplier 2 who was a second local retailer. The supplier had received a shipment of koi from Japan that upon arrival immediately underwent an outbreak of KHV. Water temperature in the partially recycled system ranged from 21–25°C during the outbreak. Approximately 6 wk following the initiation of the outbreak, mortality ceased and a group of 221 fish were transported to the UC Davis laboratory where they were held under conditions identical to those described for fish from Supplier 1. After 1 wk in the laboratory, 64 fish were bled and the serum was tested by ELISA.

Supplier 3 provided koi (S3-1) that had been shipped to a farm in Germany from Israel. The fish ranged in size from 100–200 g and were approximately 2 years in age at the time of sampling. These koi were survivors of a KHV outbreak experienced following arrival at the farm. Fish surviving the outbreak were transported to the Fish Health Laboratory at the University of Munich where they were held in 500 L aquaria receiving dechlorinated tap water heated to 21°C for approximately 1 year prior to collection of the serum analyzed by ELISA. A total of 17 fish from this group were examined.

Supplier 4 has provided live koi to the UCD laboratory for the past 4 years and these fish have been consistently free of any evidence of KHV. Serum samples were obtained from both the controls and KHV-exposed fish from a trial to evaluate virus replication by PCR at a water temperature of 28°C (Gilad *et al.*, 2004). At 64 days post virus exposure, 8 surviving koi and 8 controls held under the same conditions but not exposed to virus were bled and the serum examined for the presence of anti-KHV antibodies by ELISA and western blotting. The fish were from 200–300 g in size and approximately 2 year in age at the time of sampling. An additional group of 8 koi from S1-1 free of anti-KHV antibodies received intraperitoneal injections with 100 tissue culture infectious doses (TCID₅₀) of CyHV-1 grown in KF-1 cells. After injection, the koi were held in a 130 L aquarium receiving 21°C well water. After 8 wk blood was collected from each fish and the serum separated for western blot analyses with CyHV-1 and KHV proteins.

Koi serum and plasma

Depending on the size of the fish, serum or plasma was collected for ELISA. With smaller fish (<100 g), blood was collected from the caudal vessels into heparinized microhematocrit tubes, centrifuged and the plasma collected. With larger fish, blood was collected

in untreated microhematocrit tubes, vacutainers or syringes, allowed to clot and the serum collected by centrifugation. Serum and plasma were stored at –20°C prior to analysis by ELISA for the presence of anti-KHV antibodies.

The ELISA was developed and optimized similar to the procedures described by Case *et al.* (1983). To optimize each step of the procedures a positive serum pool was created from the serum of 7 koi ranging in size from 0.5–1 kg. The sera were obtained from koi which had survived a bath exposure to KHV that resulted in an 80% cumulative mortality (Gilad *et al.*, 2003). The sera were obtained from survivors at 64 days post initial exposure to KHV. The serum negative pool was made from 7 control fish from the same group of koi used for the KHV exposures for the positive serum pool but never exposed to the virus. Concentrations of each of the following reagents for the ELISA was optimized: coating antigen consisting of gradient purified KHV (Gilad *et al.*, 2002), WCI 12 mouse anti-carp Ig monoclonal antibody (mab) supernatant (Rombout *et al.*, 1993), biotinylated goat anti-mouse IgG (Kirkegaard & Perry) and the horseradish peroxidase (HRP)-streptavidin (SA) conjugate (Kirkegaard & Perry). Optimal conditions were identified by individually titrating each reagent and determining the concentration that generated the maximum positive to negative (P/N) ratio.

The ELISA plates (Falcon) were coated with 50 µL per well of purified virus (2 µg/mL) in 0.1 M carbonate buffer, pH 9.6 for 12 h at 15°C. After blocking the wells with 2% non-fat dried milk in Tris-buffered saline (TBS) (50 mM Tris, 1 mM EDTA, 150 mM NaCl, pH 8.0) for 1 h at 21°C, 50 µL per well of dilutions of koi serum were added and incubated for 2 h. The plates were washed 5 times with TBS with 0.1% Tween-20 (TTBS) and incubated for 1 h at 21°C with 50 µL per well of WCI 12 mouse anti-carp Ig tissue culture supernatant diluted 1:25. The plates were washed 5 times with TTBS and incubated for 1 h at 21°C with 50 µL per well of affinity purified biotin labeled goat anti-mouse IgG antibody at 0.5 mg/mL diluted 1:800. Following five washes, plates were incubated for 30 min at 21°C with 50 µL of HRP-SA diluted 1:500. The plates were washed 9 times and the color reaction was initiated by addition of 100 µL per well of 3,3',5,5'-tetramethyl benzidine (TMB) (50 µg/mL TMB in 50 mM citric acid buffer, pH 4.8, and 0.01% H₂O₂). After 15 min at 21°C, color development was arrested by adding 50 µL per well of 1 M H₂SO₄ and the optical densities (O.D.) determined with a Bio-Tek Ceres 900Hd ELISA reader (Bio-Tek Instruments, Inc.) at a wavelength of 450 nm. All dilutions of koi serum, WCI 12 mab, biotinylated goat anti-mouse and HRP-SA were made in TBS with 0.05% Tween-20 and 1% non-fat dried milk (TTBS-NFDM). All washes were with TBS containing 0.1% Tween-20.

Western Blotting

Western blots with gradient purified KHV and CyHV-1 were used to demonstrate the protein specificities of the anti-KHV and anti-CyHV-1 antibodies in koi serum. Proteins from purified KHV and CyHV-1 were separated by SDS-PAGE in a 10% or 12% acrylamide gel under reducing conditions as described by Gilad *et al.* (2002). Viral proteins were then transferred to Immobilon P membrane (Millipore) using a transphor wet blotting apparatus (BioRad). A strip of the membrane containing molecular weight markers and viral proteins was stained with 0.1% amido black in 40% methanol and 10% acetic acid for total protein determinations. Unstained strips were blocked for immunoblotting with 2% nonfat milk in TBS for 1 h. Koi serum was diluted in TTBS-NFDM, added to the strips and incubated for 2 h. The strips were washed 3 times for 5 min and then WCI 12 mab tissue culture supernatant was added to the strips. After 1 h at 21°C, the strips were washed with TTBS and alkaline phosphatase coupled to goat anti-mouse IgG (Sigma) in TTBS-NFDM was added. The blots were washed 4 times for 5 min in TTBS followed by 2 washes for 5 min each in TBS. The color reaction was visualized by staining with BCIP/NBT substrate (Sigma).

Passive immunity trial

A total of 60 juvenile koi (10 g) were divided into 6 equal groups ($n = 10$) each held in 130 L aquaria receiving flow through water at 23°C. Three groups of koi received 0.1 mL of a pooled serum sample (positive serum) from 10 koi that were survivors of an experimental exposure to KHV in the laboratory (Gilad *et al.*, 2003). Three other groups received 0.1 mL injections of a pooled serum sample from 10 additional koi from the same source but not exposed to KHV (negative control serum). Two groups of koi receiving the positive serum and two groups receiving the negative serum were then challenged by KHV propagated in the KF-1 cell line and then added to aquarium water to a concentration of 11 TCID₅₀ per mL. The remaining two groups received an equal volume of cell culture medium from the KF-1 line. After 1 h, water flow was resumed to all aquaria which were observed twice daily for dead fish for 24 d when the study was terminated.

Results

ELISA development

For the ELISA development, both positive and negative serum pools were used to determine the optimal conditions for the assay. Optimal reagent concentrations were determined by calculating the reagent concentration that produced the highest P/N ratio for each step. The optimal serum concentration that provided the highest P/N ratio was 1:500 or 1:2500. We used these two serum dilutions of our positive and nega-

tive serum pools for all subsequent reagent titrations and P/N ratio determinations. From these titrations the following optimal concentrations of each reagent for the ELISA was determined; purified KHV coating antigen, 2 mg/mL, WCI-12 mouse anti-carp Ig, 1:25, biotin labeled goat anti-mouse antibody (0.5 mg/mL) diluted to 1:800 and HRP-SA (0.5 mg/mL) diluted to 1:500. The WCI 12 mouse anti-carp Ig was tissue culture supernatant and each batch was tested individually. All subsequent ELISA utilized these reagent concentrations with times for each step as indicated previously. Additionally, each plate used in all assays contained both a positive and negative serum control from which O.D. values for each test sample could be expressed as a % of the positive control. The frequency or number of fish in the group at each % of the positive control serum (reactivity)s were plotted or alternately O.D. values were shown for each serum dilution (1/x) for each individual fish. Optical density values considered as indicating the presence of anti-KHV antibodies were found in serum dilutions as great as 1:62500 with the positive control serum pool.

ELISA of exposed and control fish

Koi from Supplier 1 that were from farm ponds in both 2003 (S1-1) and 2004 (S1-2) that had no evidence of KHV infections were found to have little anti-KHV antibody in their serum (Fig. 1). All but 4 fish in these two groups had O.D. values that were below 10% of that for the positive control. Smaller lots of fish from both of these groups used as controls in several other KHV studies that were analyzed by PCR and ELISA provided additional evidence that they were a population with little or no exposure to KHV. A third group (S1-3) of koi obtained from the retail area of Supplier 1 that was undergoing a KHV outbreak contained numerous fish with O.D. values from 10 to 95% that of the positive control (Fig. 2). An increased number of anti-KHV positive fish were found upon examination of 10 fish from this same group 47 days later with all fish at O.D. values of

Fig. 1. Enzyme-linked immunosorbent assay (ELISA) detection of anti-koi herpesvirus (KHV) antibodies in the serum of two populations (S1-1 $n = 40$ and S1-2 $n = 41$) of koi from two ponds with no evidence of KHV disease. Frequency or number of fish at that ELISA value for each serum sample is expressed as a percent of the positive control included in each assay.

Fig. 2. Enzyme-linked immunosorbent assay (ELISA) detection of anti-koi herpesvirus (KHV) antibodies in the serum of a population (S1-3 $n = 71$) of koi from the retail area of Supplier 1 that were in the early stages of an outbreak due to KHV and from koi from the same group (S1-4 $n = 10$) sampled 47 days later. Note: black bars, during outbreak; dotted bars, 47 days later. Frequency or number of fish at that ELISA value for each serum sample is expressed as a percent of the positive control included in each assay.

Fig. 3. Enzyme-linked immunosorbent assay (ELISA) detection of anti-koi herpesvirus (KHV) antibodies in the serum of a population (S2-1 $n = 64$) of koi from a retailer (Supplier 2) that were examined 6 wk after recovery from a KHV outbreak. Frequency or number of fish at that ELISA value for each serum sample is expressed as a percent of the positive control included in each assay.

>70% that of the positive control (Fig. 2).

Koi from Supplier 2 (S2-1) were survivors of a recent KHV outbreak that were examined approximately 6 wk following the cessation of mortality. Most fish in this group had O.D. values of > 60% that of the positive control with many fish clustering between 70 and 89% of the positive control (Fig. 3).

Serum from a group of 14 fish examined from Supplier 3 (S3-1) demonstrated a similar distribution of O.D. values to that from S2-1 with all fish but one at >40% of the positive control serum (Fig. 4). An examination of these sera as tested by ELISA with both KHV and CyHV-1 as coating antigens provided the first evidence of shared antigens between the two virus agents (Fig. 5). At serum dilutions of 1:20 to 1:500 there were substantial cross reactions with CyHV-1 as O.D. values reached up to 80% of the positive control (KHV) serum. The extent of cross reaction of the serum with CyHV-1 antigen was reduced or eliminated at serum

Fig. 4. Enzyme-linked immunosorbent assay (ELISA) detection of anti-koi herpesvirus (KHV) antibodies in the serum of a population (S3-1 $n = 17$) of koi from Germany that had been held in dechlorinated municipal water for 1 yr following recovery from a KHV outbreak. Frequency or number of fish at that ELISA value for each serum sample is expressed as a percent of the positive control included in each assay.

dilutions of 1:2500 or greater (Fig. 5).

Serum from koi from Supplier 4 (S4-1) were obtained from an experimental and a control group from a KHV laboratory exposure trial (Gilad *et al.*, 2004). The 8 unexposed fish showed little anti-KHV activity at dilutions of 1:500 or greater while 8 fish surviving a KHV exposure had consistently higher O.D. values at all serum dilutions tested (Fig. 6). Sera from this same group of fish were used in western blots prepared to examine potential KHV proteins recognized by the serum antibodies.

Based upon examinations of the different populations of naturally and experimentally exposed koi to the KHV some general guidelines were established for O.D. values considered as indicating the presence of anti-KHV antibodies in a given serum sample. At serum dilutions of 1:2500, O.D. values greater than 15% of the positive control (in our study O.D. value of approximately 0.18 and above) were considered as a strong indication of the presence of anti-KHV antibodies. Also, at this serum dilution potential cross reactions with anti-CyHV-1 antibodies is minimized.

Western Blotting

Twenty-one KHV proteins of 31 kDa or larger were visible in Coomassie blue stained SDS-PAGE gels. After electrophoretic transfer from the gel to the PVDF membrane and blotting, 14 of the 21 KHV virus proteins were visible by amido black total protein staining. Thirteen of the 14 proteins were recognized by serum from at least 1 of the 8 fish that were survivors of laboratory-induced KHV infections (Fig. 7). Five of the KHV proteins of 166, 133, 97, 50 and 43 kDa, were recognized by at least 3 fish and the 97 kDa protein was recognized by all but one fish. Most sera collected from 8 juvenile koi injected with CyHV-1 8 wk prior did not react with KHV proteins (Fig. 7). However, serum from one fish reacted strongly with a protein of approximately

Fig. 5. Enzyme-linked immunosorbent assay (ELISA) detection of anti-koi herpesvirus (KHV) antibodies in the serum of a population (S2-1) of koi from a retailer (Supplier 2) that were examined 6 wk after recovery from a KHV outbreak. A serial titration of each individual serum is shown with KHV (—) or CyHV-1 (-----) as coating antigen.

Fig. 6. Enzyme-linked immunosorbent assay (ELISA) detection of anti-koi herpesvirus (KHV) antibodies in the serum of a population (S4-1) of koi surviving experimentally-induced KHV disease. Koi were examined 64 days following initial exposure to the virus (—). An additional eight control koi not exposed to the virus (-----).

130 kDa and a second fish sera recognized two proteins of a 170 and 56 kDa. Control koi serum failed to react with virus proteins of KHV or CyHV-1 (not shown).

Eleven CyHV-1 proteins were visible by amido black staining on PVDF following transfer from the SDS-PAGE gels. When these membranes were probed with serum from 8 individual CyHV-1 exposed fish 8 of the 11 proteins were recognized by at least 1 fish (Fig. 8). An additional 6 CyHV-1 proteins, not visible by amido black staining, were identified by probing with the anti-CyHV-1 sera. Serum from 8 koi surviving KHV infection recog-

nized several CyHV-1 proteins ranging from 35 to over 200 kDa but in most cases serum from individual fish recognized only one CyHV-1 protein and the size of that protein differed for each fish.

Passive immunity trial

A pooled serum sample from 10 fish surviving experimental KHV infections had high levels of anti-KHV antibodies as determined by ELISA (Fig. 9). In contrast, a serum pool from 10 fish from the same population but not exposed to KHV had low levels of

Fig. 7. Virion polypeptides of the herpesvirus (KHV) recognized by antibodies in the serum of koi recovering from experimental exposures (lanes 1–8) to KHV or following experimental exposures to *Cyprinid herpesvirus 1* (lanes 9–16). Each lane represents serum from an individual fish.

Fig. 8. Virion polypeptides of the *Cyprinid herpesvirus 1* (CyHV-1) recognized by antibodies in the serum of koi following experimental exposures to *Cyprinid herpesvirus 1* (lanes 1–8) or recovering from experimental exposures (lanes 9–16) to KHV. Each lane represents serum from an individual fish.

activity. Intraperitoneal administration of serum with or without anti-KHV activity to groups of koi provided either partial or no protection from experimental exposures to KHV, respectively (Fig. 10). Koi administered serum with anti-KHV activity demonstrated a delayed onset and substantially reduced mortality (40%) compared to koi administered control serum (80%) by 12 days post virus exposure. Following a short cessation in mortality at day 10 to 12 post virus exposure among fish receiving the positive serum, a second mortality episode began resulting in a final cumulative mortality of 80%. Fish

Fig. 9. Enzyme-linked immunosorbent assay (ELISA) detection of anti-koi herpesvirus (KHV) antibodies in a pooled serum sample from a population (S4-1) of koi following recovery from experimental exposures to KHV. The serum was collected 64 days following initial virus exposure. A serial titration of the pooled serum from ten fish is shown (positive – ■) and ten additional koi from the same population not exposed to KHV (negative – ▲).

Fig. 10. Cumulative mortality following KHV exposures of juvenile koi receiving intraperitoneal injections with serum from koi surviving experimental KHV infections (■) or serum from koi with no previous exposures to KHV (▲).

receiving the negative serum pool continued to die until the cumulative mortality reached 100% at 24 days post virus exposure. Virus isolations were positive from a total of 5 dead fish from each of the virus-exposed groups (negative or positive serum) examined at the peak of mortality. All other dead fish displayed the same external signs but were not examined for virus. There was no mortality in groups of koi not exposed to KHV.

Discussion

The presence of serum anti-KHV antibodies was detected by ELISA among populations of koi following experimental or natural exposures to the virus. Serum anti-KHV antibodies were detected in nearly all fish surviving at 42 to 47 days after losses due to naturally occurring outbreaks or experimentally induced KHV disease. High concentrations of anti-KHV antibodies in

the serum of these koi were indicated by elevated O.D. values at serum dilutions as great as 1:62,500. One population of koi (S3-1) held in an aquarium receiving virus-free water (21°C) also had similarly high anti-KHV antibody concentrations when examined 1 year from the last known exogenous contact with KHV. Examinations of serum from koi following natural and experimental infections with KHV by ELISA and western blotting demonstrated the potential for cross reactions with a second related herpes-like virus from cyprinid fish, CyHV-1. These cross reactions were eliminated if sera were diluted to 1:2500 or greater. The passive administration of anti-KHV serum to naive koi failed to provide adequate protection to KHV challenge suggesting that both cellular and humoral components of the immune response are likely needed for immunity to KHV.

Fish that survive exposures to viruses whose life cycle involves systemic invasion of tissues most often produce anti-virus antibodies detected in the serum (Plumb 1973; Hattenberger-Badouy *et al.*, 1989; Kibenge *et al.*, 2002). These anti-virus antibodies can be detected by several methods including neutralization, complement fixation, co-agglutination or most commonly by ELISA (LaPatra, 1996). The first descriptions of anti-KHV antibodies detected by ELISA in koi serum were by Ronen *et al.* (2003). They utilized an ELISA to demonstrate that experimentally-infected koi developed serum anti-KHV antibodies that increased in concentration over time and which remained present in fish up to 50 days post virus exposure.

Our studies on four populations of koi provide additional insights into the presence of serum anti-KHV antibodies following experimental or natural exposures to KHV. Anti-KHV serum antibodies were generally absent in two populations of koi with no history of KHV disease (S1-1 and S1-2). In these populations we detected only 3 fish of a total of 81 examined that had O.D. values above 15% of the positive control (19–40%). These positive responding fish may actually have represented low level exposure as later interviews with farm personnel indicated some movements of fish among ponds had occurred unknowingly. In contrast to the few low responding fish in S1-1 and S1-2 fish, a much higher prevalence of positive responding koi were found in a group of koi (S3-1) that had survived an outbreak of KHV disease and were then held for 1 year in a virus-free water source. In a population of koi from Supplier 1 (S1-3) a conversion from low to high prevalence of responders (anti-KHV antibodies found) coincided with an outbreak due to KHV (Fig. 2). Although serum samples were not available from a second population of koi from Supplier 2 (S2-1) before a KHV outbreak, samples examined 47 days later demonstrated that a majority of fish were high anti-KHV antibody responders (Fig. 2). As reported by Ronen *et al.* (2003), a high prevalence of anti-KHV antibody response

was present in a population of koi (S4-1) following experimental exposures to KHV in the laboratory (Fig. 6). Our examination of these 4 koi populations demonstrated a good relationship between the presence of anti-KHV antibody detected by ELISA and a history of exposure to natural or experimental sources of the virus.

Most studies of anti-herpesvirus antibodies in fish are from natural or experimental infections of channel catfish with *Ictalurid herpesvirus 1* (IcHV-1). Plumb (1973) was the first to demonstrate that catfish recovering from natural outbreaks due to the virus contained serum anti-virus antibodies detected by neutralization assays. Later studies demonstrated that the concentrations of serum anti-IcHV-1 showed variability over time that was attributed to seasonal or other environmental factors including changing water temperatures (Amend and McDowell, 1984; Bowser and Munson, 1986; Hedrick and McDowell, 1987; Hedrick *et al.*, 1987). An ELISA developed to detect both the presence of anti-IcHV-1 serum antibodies in naturally occurring channel catfish populations and the kinetics of the response following experimental exposures has been described (Crawford *et al.*, 1999). The prevalence of adult catfish with anti-IcHV-1 antibodies and the detection of DNA specific to IcHV-1 in the adults and progeny from such adults suggest that latent carriers exist with the capability to vertically transmit the virus (Wise *et al.*, 1988; Boyle and Blackwell, 1991; Gray *et al.*, 1999; Thompson *et al.*, 2005).

Evidence from both natural and experimental exposures of koi to KHV suggests that latent infections or a carrier state may be established in fish recovering from initial infections (Gilad *et al.* 2004; Hedrick *et al.*, 2005; K. Way, pers. comm.). That a second herpes-like virus from koi (CyHV-1) related to KHV can establish a latent and recrudescence infection has been demonstrated (Sano *et al.*, 1985a, b, 1991). Several tissues of koi recovering from experimental exposures of CyHV-1 contain virus-specific DNA as detected by hybridization methods (Sano *et al.*, 1993a). In addition, the association and recurrence of the characteristic “pox” lesions in cyprinid fish attributed to CyHV-1 is linked to environmental factors favorable to recrudescence of latent virus (Sano *et al.*, 1985b, 1991; Sano *et al.*, 1993b). That virus-specific DNA of KHV can persist in koi tissues for at least 64 days following initial virus exposure was demonstrated by Gilad *et al.* (2003). KHV may persist for even longer periods in koi or common carp in the absence of disease at lower water temperatures (e.g. 13°C) and these infections may manifest as water temperatures rise with the changing season (Gilad *et al.*, 2002). The lower temperatures most likely directly affect the ability of KHV to replicate efficiently in the fish rather than a strong immune response which would be expected to be functioning at less than optimal capacity at these water temperatures (Bly and Clem, 1992). With CyHV-1, low

water temperature effects on the immune response in koi were cited as one key factor in the recurrence of the skin lesions common to carp pox (Sano *et al.*, 1993b).

Most outbreaks of KHV disease are associated with movement of healthy appearing fish from sites known for presence of the virus which are then mixed with virus naive populations of koi or common carp (Gilad *et al.*, 2002; Haenen *et al.*, 2004; Sano *et al.*, 2004). Although convincing laboratory transmission trials have yet to be reported, the epidemiologic associations between fish movements and virus outbreaks provides strong evidence that carriers and virus latency are characteristic of KHV infections. Investigations in several laboratories are now aimed at understanding these important aspects of the biology of KHV.

Recent genetic studies comparing KHV with two other cyprinid herpesviruses (CyHV-1 and 2) demonstrate they are group of closely related viruses (Waltzek *et al.*, 2005; Hedrick *et al.*, 2005). Initial genome sequencing of KHV and CyHV-1 reveal they possess many similar genes and gene arrangements (T. Aoki, pers comm., unpublished data). Related proteins or glycoproteins of CyHV-1 and KHV that are immunogenic in koi and common carp may interfere with estimates of specific anti-virus antibody levels. Evidence that such cross reacting antibodies are present in koi serum was demonstrated in reciprocal ELISA and western blot examinations of serum from koi infected or inoculated with CyHV-1 or KHV (Fig. 5 and 7). The western blot analyses under denaturing conditions suggested that only a few proteins are cross-reactive and the immunoreactivity may vary considerably between different fish. Proteins of 170, 130, and 56 kDa of KHV were involved in some of the cross reactions with anti-CyHV-1 serum while CyHV-1 proteins in the range of 200 to 35 kDa were involved with anti-KHV serum. At present, we are uncertain of the role of these viral proteins but they may represent key components of the virus envelop or capsid involved in potential anti-viral immunity. Due to the cross reactions observed between KHV and CyHV-1, we suggest using serum dilutions of 1:2,500 or greater in the ELISA. Under these conditions, the ELISA is able to effectively discriminate between anti-KHV and anti-CyHV-1 antibodies (Fig. 5).

That anti-KHV antibodies develop following natural or experimental exposures to KHV have been shown in our current study and that of Ronen *et al.* (2003). Ronen *et al.* (2003) also demonstrated that KHV infected koi subjected to thermal regimes were resistance to subsequent challenge and that these fish possessed high anti-KHV antibody levels by ELISA. Our passive immunity trial suggests that anti-KHV antibodies provide some but only minimal protection to acute KHV disease (Fig. 10). This result contrasts to previous studies with anti-IcHV-1 serum that completely protected juvenile channel catfish from acute infections with IcHV-1 (Hedrick *et al.*,

1987). The pooled anti-KHV serum used in our trials was deemed to contain high levels of anti-virus antibodies. Considering the half-life of carp Ig at nearly 22 days (Overath *et al.*, 1999) ample anti-KHV antibodies should have been present to provide a protective effect. That little protection was observed suggests that anti-KHV antibodies alone are insufficient and other immune mechanisms, including cell-mediated responses are required to provide complete protection to KHV challenge.

In conclusion, an ELISA is described that was effective in detecting anti-KHV antibodies in the serum of several populations of koi. Because samples for ELISA analysis can be taken nonlethally, they offer an alternative to current lethal sampling procedures. The presence of anti-KHV antibodies in the serum of koi or common carp in the absence of disease is a good indicator of prior exposure to the virus and thus the potential for risk of movement of the virus with antibody positive fish. However, more convincing studies linking antibody positive fish to transmission (carrier status) are needed. In the interim and prior to wide scale applications of vaccines that may complicate serological analyses, the ELISA provides additional information to evaluate potential risks of virus transmission when PCR or other virological methods fail to reveal evidence for KHV. Lastly, anti-KHV antibodies present in the serum administered to naive koi provide only a transient and incomplete protection from KHV challenge suggesting that development of effective resistance to KHV will most likely involve stimulation of cell-mediated immune responses.

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