Development of a serological tool for koi herpesvirus surveillance

Cefas

S. St-Hilaire, N. Beevers, C. Joiner, G. Wood, P. Dunn and K. Way*
Centre for Environment, Fisheries & Aquaculture Science, Weymouth
www.cefas.co.uk

Introduction

Koi herpesvirus (KHV) causes a severe disease and mass mortalities in populations of common carp (*Cyprinus carpio carpio*) and koi carp (*Cyprinus carpio koi*). The disease has spread rapidly around the world and devastating losses have occurred in intensive and extensive carp culture facilities in Europe, Asia and North America. The disease is now notifiable to the World Organisation for Animal Health (Office International des Epizooties, OIE).

The behaviour of the virus in the host makes it very difficult to confirm the presence/absence of the virus in apparently healthy fish with a latent KHV infection. Studies in Israel and the USA have identified serum antibodies to KHV in carp that have survived disease outbreaks (**1**, **2**). If the antibody response is consistently present in survivors and it persists for a long period of time, it may be possible to use KHV-specific antibodies to identify fish populations that have been exposed to the virus. However, antibody assays must be validated and the prevalence and duration of antibodies in fish should be determined.

This study evaluated an ELISA, developed in our laboratory, for detection of serum anti-KHV antibodies in populations of experimentally exposed common carp.

Materials and Methods

The fish used in this study were part of another study to evaluate KHV persistence in common carp (**3**). The same tank identification system used in the published study is maintained here for continuity.

Fish and tanks - Common carp were obtained from a farm in England with no previous history of KHV. All fish tanks were on a flow-through system with 2 L min $^{-1}$ of de-chlorinated bore-hole water. Fish were fed a maintenance diet of 1% bodyweight d^{-1} and monitored 2 times d^{-1}.

Viruses - KHV isolate UK D-132 and Cyprinid Herpesvirus-1 (CyHV-1) isolate F-266. Both viruses were grown in koi fin (KF-1) cells as described previously (**4**). For the ELISA, viruses were purified on sucrose density gradients (**5**).

1st KHV exposure trial - 900 fish (25 to 200g) were exposed to approx.10^4 pfu ml^{-1} of virus for 2 h in a static bath at a temperature of 21°C (tank 1B). 200 carp were maintained in a separate tank (1A) as controls. 300 carp from the exposed tank (1B) were transferred to a separate tank (2B) and the temperature was lowered to 12°C over a period of 4 d and maintained at 12°C for a further 22 weeks. One hundred and ninety-two fish from the original control tank (1A) were also transferred to a new tank (2A) and the temperature in this tank lowered to 12°C as in tank 2B. Tank 2B was subsequently divided into two tanks (2C and 2D). The temperature profiles for each group of tanks are included in figure 1.

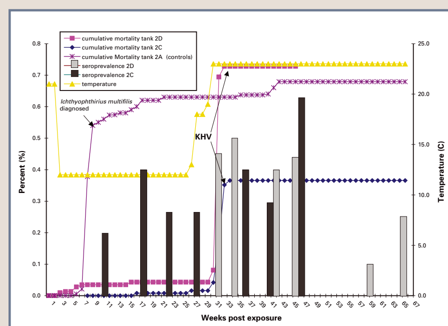

Figure 1: Cumulative mortality rate and seroprevalence of fish in tanks 2A, 2C, and 2D exposed to KHV and maintained at a low temperature for 25 weeks post exposure. Temperature profile is included on the secondary Y axis. Fifty control fish in tank 2A were tested for KHV antibodies over the course of the study (0, 46, 59, and 62 weeks post exposure) and all fish were negative for antibodies.

2nd KHV exposure trial - 30 common carp (25 to 30 g) were injected IP with approx. 10 pfu KHV in 0.2ml of PBSa and returned to a tank (3B) with 500 common carp to serve as the source of virus. 100 carp were maintained in a separate control tank (3A) and 30 of these were injected IP with saline only. All tanks were maintained at 18°C for 20 d and then subsequently the temperature was lowered to 11°C to ensure some fish survived the viral outbreak. Surviving fish in tanks 3A and 3B were used to monitor the antibody response, and both tanks were subject to the temperature profile shown in figure 2.

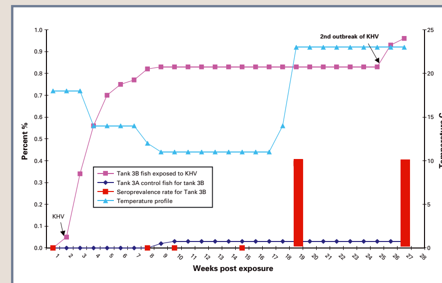

Figure 2: Cumulative mortality rate and sero-prevalence of fish in tanks 3A and 3B exposed to KHV and maintained at an elevated temperature of 18°C for three weeks. The temperature profile is included on the secondary Y axis. Seventy seven control fish in tank 3A were tested for KHV antibodies over the course of the study (weeks 0, 4,10,15, 19, 23, and 27 post exposure) and all fish were negative for antibodies.

Serum collection and handling - Blood was drawn from the caudal vein of carp, after they had received a lethal dose of anaesthetic, using 2ml non-heparinised Vacutainers™ (B&D Biosciences). Blood was allowed to clot overnight at 4°C and serum harvested and stored at -20°C until tested for KHV-specific antibodies.

Serum samples were taken from 10 to 15 fish prior to KHV exposure and then every 4 to 6 weeks (tanks 2C/2D and 3B) until an antibody response was detected. Then the number and interval between samplings varied to ensure adequate fish until the end of the study. Fish in the control tanks (1A, 2A and 3A) were bled periodically during the study.

ELISA for KHV-specific antibodies – Briefly, 96-well ELISA microplates were coated with purified KHV and then blocked with skim milk powder. Test serum and negative and positive control serum were diluted 1/400, 1/800, 1/1600 & 1/3200 and incubated on the plates, in duplicate, for 1 hour. Carp Ig was detected with mouse anti-carp Ig monoclonal antibody (Aquatic Diagnostics Ltd., 45 min. incubation) and bound mouse Ig detected with a goat anti-mouse Ig polyclonal antibody conjugated to peroxidase (Sigma, 45 min. incubation). Bound peroxidase was then visualised by adding tetramethylbenzidine (TMB) in phosphate-citrate sodium perborate buffer (all Sigma). The colour reaction was stopped with H_2SO_4 and absorbance read at 450nm in a microplate spectrophotometer.

Cross reaction of antibodies to Cyprinid herpesvirus 1 (CyHV-1) - Serum samples from 10 koi experimentally exposed to CyHV-1 were tested for antibodies to CyHV-1 and antibody cross -reaction with KHV using the ELISA technique described above. The ELISA for CyHV-1 antibodies was identical to the protocol above with the exception that plates were coated with the homologous virus, CyHV-1 instead of KHV.

Acknowledgement

This work was funded by Defra (Contracts FC1150, FC1136 and FA001) and the English Carp Heritage Organization (ECHO).

References

1. Ronen A, Perelberg A, Abramowitz J, Hutoran M, Tinman S, Bejerano I, Steinitz M, Kotler M (2003) Efficient vaccine against the virus causing a lethal disease in cultured *Cyprinus carpio*. Vaccine 21: 4677-4684.
2. Adkison MA, Gilad O, Hedrick RP (2005) An enzyme linked immunosorbent assay (ELISA) for detection of antibodies to the koi herpesvirus (KHV) in the serum of koi *Cyprinus carpio*. Fish Pathol 40: 53-62.
3. St-Hilaire S, Beevers N, Way K, Le Deuff R-M, Martin P, Joiner C (2005) Reactivation of koi herpesvirus infections in common carp (*Cyprinus carpio*). Dis Aquat Org 67: 15-23.
4. Hedrick RP, Gilad O, Yun S, Spangenberg JV, Marty GD, Nordhausen RW, Kebus MJ, Bercovier H, Eldar A, (2000) A herpesvirus associated with mass mortality of juvenile and adult koi, a strain of common carp. J Aquat Anim Health 12: 44-57.
5. Hutoran M, Ronen A, Perelberg A, Ilouze M, Dishon A, Bejerano I, Chen N, Kotler M (2005) Description of an as yet unclassified DNA virus from diseased *Cyprinus carpio species*. J Virol 79: 1983-1991.

Results

Anti-KHV antibodies were detected in all populations of virus exposed fish regardless of whether the population experienced high initial mortalities or not (Figures 2 and 3). There was no evidence of anti-KHV antibodies in fish in any of the control groups not exposed to the virus. In the experimental groups, antibodies to KHV were first noted at 10 (tank 2C) and 19 weeks (tank 3B) post exposure and antibodies were still detectable at high titres (>1:1600) in 25% of the fish after 25 weeks (Figure 2). All fish considered seropositive in this study had titres at a serum dilution of 1:1600 or greater. Elevated mortality associated with reactivation of KHV occurred in tanks 2C and 2D (at 30 weeks post exposure) and in 3B (at 25 weeks post exposure) despite a high proportion of antibody positive fish in the population (Figures 1 and 2).

Six of the 10 koi exposed to CyHV-1 and positive for anti-CyHV-1 antibodies had no detectable anti-KHV antibodies by ELISA. Four fish with antibody titres to CyHV-1 of 1:1600 or greater (carp #1, 4, 6, and 7) had cross-reactions with KHV at lower serum dilutions (1:200 and 1:400).

Discussion

Following exposures to KHV, a high proportion of fish in the two populations produced detectable antibodies to KHV. There was a reduction in the number of fish with detectable antibodies over time but after 65 weeks 25% of the carp in tank 2D were seropositive (titre 1:1600) (Figure 1). Also, regardless of whether fish were maintained at a high or low temperature or experienced high virus-associated mortality, a large proportion of the fish seroconverted (Figures 1 and 2).

Antibodies to KHV were not detected in all exposed fish sampled in our study suggesting that this test is more useful in identifying the exposure status of the population rather than individual fish. Negative results when fish are sampled at only one point in time may be inconclusive. To avoid this, screening programs could collect serial samples from fish populations to provide evidence of their KHV exposure history. Also, if the fish are large enough (i.e. >25 g) the sample could be collected non-lethally.

The time required for an antibody response to be detectable in the carp, post -exposure to the virus, was longer in this study (between 10 and 19 weeks) than the 3 to 6 weeks reported by others (**1**,**2**). This lengthy response time was certainly influenced by the temperature manipulations and further studies at Cefas have shown detectable antibodies in carp at 3 weeks post-exposure when held at a constant 22°C.

There appears to be little cross-reaction with CyHV-1 antibodies at high serum dilutions but only ten fish were evaluated in this study. Carp with antibodies to KHV were not tested for their cross-reaction to CyHV-1 and this needs to be investigated further. To validate this assay, naturally infected populations need to be evaluated. Results of tests on populations of common and koi carp that have survived KHV disease outbreaks are summarised in Table 1.

Table 1: Detection of antibody to KHV by ELISA in populations of koi and common carp that have survived natural KHV infections and in carp vaccinated against KHV.

Site	Type	Sample date	Status	No. of Seropositives
A	Farm	November 2005	Vaccinated carp	22 of 40
B	Fishery	March 2006	Survivors of KHV outbreak in 2005	19 of 26
C	Koi dealer	June 2007	Long-term survivors of KHV outbreak	9 of 42

The results of this study indicate that carp populations with persistently infected fish (virus carrier fish) have a high seroconversion rate and seropositives produce high titres of detectable antibody. Our results support those from previous studies (**1**,**2**) demonstrating that the ELISA provides a useful non-lethal tool for identifying fish populations that may have been previously exposed to the virus and are not showing clinical signs of disease.

* keith.way@cefas.co.uk

© Crown Copyright 2007