

Detection of koi herpesvirus (KHV) after re-activation in persistently infected common carp (*Cyprinus carpio* L.) using non-lethal sampling methods

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Abstract

Surviving carp which had recovered from KHVD were kept for approximately eleven additional weeks at 20°C water temperature. To induce KHV re-activation, carp were subjected to netting stress on day 81 after infection and gill swabs and dropping samples were collected daily for investigation by quantitative real-time PCR. An increase of KHV concentration of up to 1000 KHV genomic equivalents was detected over a three day period post-netting from these non-lethally collected samples.

A considerable decrease in KHV genomic concentration was observed after day four post-netting. KHV DNA was not detected in samples from persistently infected carp on day 10 after stress induction. The results of this study suggest that KHV DNA is more readily detected, in gill swabs, one to three days after stress induced by capture and netting.

Introduction

Koi herpesvirus (syn. Cyprinid herpesvirus 3, CyHV-3) has been responsible for worldwide KHV disease (KHVD) outbreaks, often resulting in high mortality rates (40-100%) and has been reported in both aquacultured and wild common carp in addition to ornamental koi (*Cyprinus carpio*) (Bretzinger et al., 1997; Hedrick et al. 2000). Typical external signs of KHVD in affected carp include gill necrosis, abundant mucus production, distinct circular skin lesions or necrotic areas and enophthalmus. Internally, severe interstitial nephritis and swollen spleen and / or liver may be observed (Gilad et al.,

2003). Due to the nature of the pathogen, an aquatic herpesvirus, taxonomically grouped into the family *Alloherpesviridae* (Waltzek et al., 2009), an infection with KHV results in latency or persistence (Gilad et al., 2003; St-Hilaire et al., 2005). In disease recovered and healthy appearing carp the virus is difficult to detect, due to these fish being able to suppress the KHV concentration to between 5 and 10 particles per 25 – 30 µg tissue material (Bergmann et al., 2010), as quantified by real-time PCR according to Gilad et al. (2004). Other molecular based methods for genomic detection such as

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PCR, nested-PCR or loop-mediated isothermal amplification (Bergmann et al., 2006; Gilad et al., 2002; Gunimaladevi et al., 2004; Bercovier et al., 2005; Bergmann et al., 2010) often fail to detect these low viral concentrations. The actual target tissues for KHV persistence / latency are not known but it has been shown that white blood cells, especially polymorphic granulocytes and enlarged mononuclear cells in interstitial tissue of the kidney can contain the virus (Bergmann et al., 2009). It is known that KHV can be re-activated by different stressors, such as transportation, catching, netting, hormonal maturation or changes in water temperature in the spring or autumn (St-Hilaire et al., 2005; Meyer, 2007). Based on this knowledge, the aim of this study was to create a simple, safe and reproducible procedure for the detection of re-activated KHV by non-lethal sampling that could subsequently be applied as a diagnostic tool. As netting is a procedure that is frequently undertaken during the carp production cycle, this method was utilised as a stress inducer and the KHV genomic concentration from gill and faeces samples determined by real-time PCR.

Materials and methods

Virus and cells

Isolate KHV-E (D 132), kindly provided by Dr. Keith Way (Cefas, UK), was allowed to replicate in common carp brain (CCB) cells (Neukirch et al., 1999). After absorption for two hours onto the CCB monolayer at 20 °C, Minimum Essential Media with Hank's salts (H-MEM) containing 10% fetal calf serum as described by Neukirch et al. (1999) but without antibiotics. KHV infected cultures were incubated for 10 days at 26°C in 25 cm² cell culture flasks (Nunc, Denmark). When a complete cytopathic effect (CPE) was observed, cells and virus were harvested by

one freeze–thaw cycle at –80°C. After virus titration in CCB cells in 96 well plates (Costar, Germany), an adjusted KHV concentration of 10³ TCID₅₀/ml was used for experimental infection by immersion.

Fish

Individual fin clipped common carp (n=10, 1 year old, K 1), were immediately screened after arrival at the laboratory and were proven to be free from KHV (Bergmann et al., 2006; Gilad et al., 2002; Gilad et al., 2004; Bergmann et al., 2010) and carp pox herpesvirus (CyHV-1) by PCR (Gilad et al., 2002). The carp were obtained from a commercial carp farm in Thuringia, Germany, and were adapted to our re-circulating aquaria conditions for four weeks in a 1 m³ tank at 20°C water temperature with a flow rate of 300 L/h. Carp were fed with commercial carp food (Ssniff, Germany) once daily.

Infection experiment and sample collection

After the four week acclimatisation period, the carp (90 – 140 g) were infected by immersion for 2 h in 20 L of water at 20°C, in a 400 L aquarium containing KHV at a concentration of 10³ TCID₅₀/ml. The challenged carp were then transferred to another 400 L aquarium with a flow rate of 100 L/h at 20°C and were observed daily for a total of 4 months. Negative control carp (n = 50) were kept in a separate aquaria room over the same time period and were subjected to the exact same rearing conditions as the challenged individuals.

At 81 days post infection (dpi), the carp (including negative control fish) were netted and subjected to two 30s time periods out of the water to induce stress. After taking gill swabs, the carp were returned to the aquaria that they

originated from and were reared as per the environmental parameters described pre-challenge. Gill swabs, collected on a daily basis from each individual were transferred immediately to Eppendorf tubes containing ATL buffer and proteinase K (100 µl) according to the manufacturer's instructions (Qiagen, Germany). Additionally, droppings (approximately 5 g), were collected daily and placed in an Eppendorf tube containing 200 µl ATL buffer at ambient temperature. This procedure was carried out over a 7 day period and additionally at day 10 post-netting. Negative control samples were collected in the same way.

DNA extraction

Total DNA was extracted from the gill and faeces samples by QIAamp DNA Mini Kit (Qiagen, Germany) according to the manufacturer's instructions.

Quantification of KHV by real-time PCR

All non-lethally collected gill swab and dropping samples were tested by quantitative real-time PCR according to Gilad et al. (2004), modified according to Bergmann et al. (2010), to measure the individual but also the average virus release via gills and gut in direct comparison to PCR reactions obtained with positive KHV DNA containing control plasmids according to Bergmann et al. (2010).

Results

Five days after immersion, several carp demonstrated moderate to severe signs of KHVD (Figure 1). Carp mortalities began at 15 dpi but six out of 10 carp survived the disease and had recovered completely at 35 dpi (Figure 2). At 81 dpi, a netting procedure was carried out to induce stress in the carp. Gill swab samples

were taken from individual carp every day for seven days after the netting stress. Up to 1000 copy numbers of KHV DNA were detected in the samples by real-time PCR (Table 1). From the initial collection, only 1/6 (carp no. 9) gill swab samples contained detectable virus. All other individually collected gill swab samples and droppings, sampled on day 0, were KHV DNA negative by real-time PCR. From days 2-3 post netting (dpm), KHV DNA was detected in all gill swab samples with copy numbers between 10 and 1000. KHV was only detected in droppings at 4 dpm (50 DNA copy numbers). On day 4 pm, a rapid decrease in KHV DNA was observed in gill swab samples with copy numbers between 10 and 100. Lower levels of KHV DNA were detected from 5 dpm to 7 dpm (4/6 carp) with the exception of carp 1 and carp 9 where the copy numbers increased or fluctuated. At 10 dpm no KHV DNA (0/6) was detected in any sample (Figure 3). During this period KHVD was not observed in any of the experimentally challenged carp. In the negative control carp KHVD was not observed nor was KHV detected by real-time PCR from gill swab or dropping samples.

Discussion

Over the past 10 years, KHVD has become an important worldwide threat to aquacultured carp and koi. Under international regulations (OIE, 2010; Anon. 2006), competent authorities within a Member State are required to inform the regulatory authority if KHVD is present or if KHV is detected within their country. In addition, the fact that the development of a persistent / latent phase of infection is one of the main characteristics of all known herpesviruses, calls for the development of safe, rapid and simple diagnostic procedures for virus



Carp 2 demonstrating moderate signs of KHVD (6 dpi); small round skin lesions, bleeding fins and sunken eyes.



Carp 8 showing severe signs of KHVD (12 dpi); Increased mucus production, necrotic of skin and sunken eyes.

Figure 1. Clinical signs of KHVD in two fish.

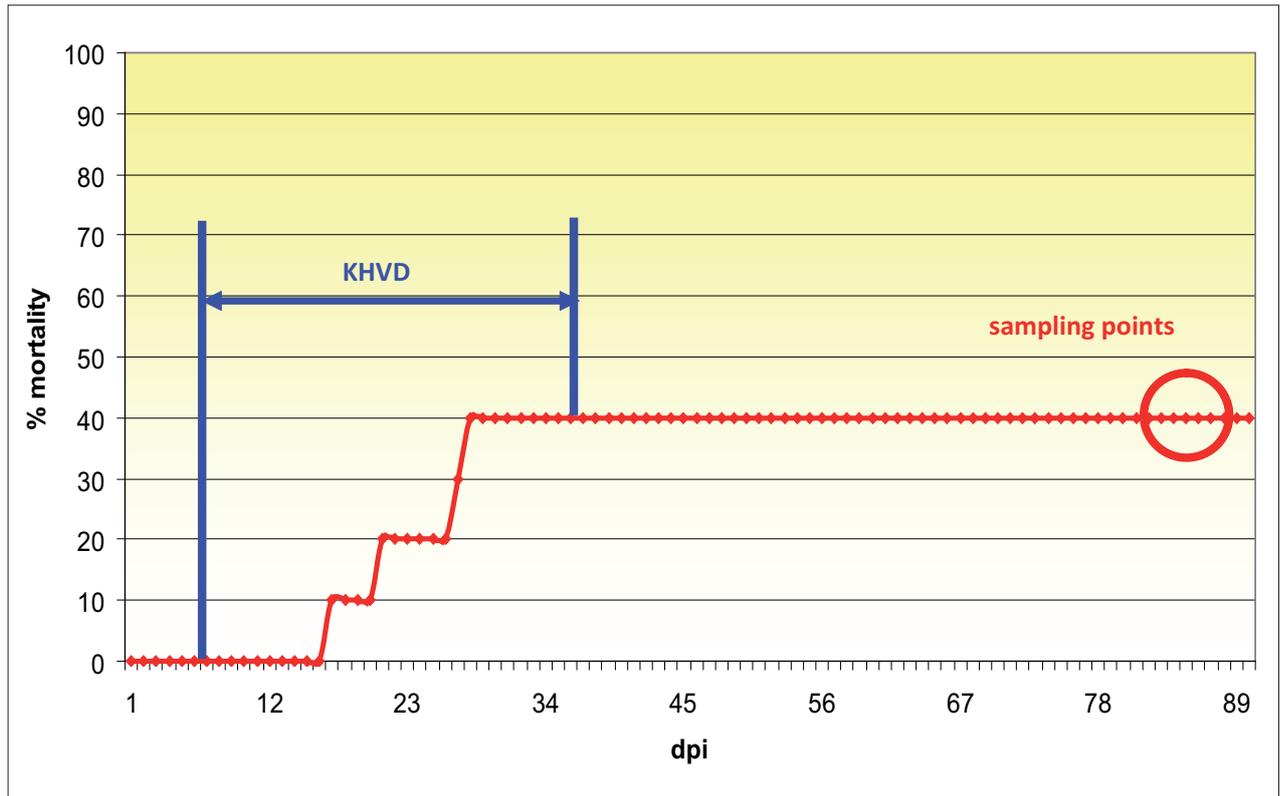


Figure 2. Cumulative mortality induced by KHVD and sampling points after recovering.

Table 1. Individual examples for the detection of KHV by real-time PCR in gill swab and dropping samples obtained from carp exposed to capture and netting stress 81 days after an experimental KHV challenge. All numbers refer to DNA copy numbers (rounded results).

days after netting	carp 1	carp 2	carp 4	carp 6	carp 7	carp 9	droppings
0	0	0	0	0	0	100	0
1	0	0	0	0	0	50	0
2	100	100	10	10	100	100	0
3	1000	1000	100	1000	10	100	0
4	10	10	10	10	10	100	50
5	50	100	0	0	0	50	0
6	100	0	0	0	0	100	0
7	1000	0	0	0	0	10	0
10	0	0	0	0	0	0	0
neg. controls	0						0

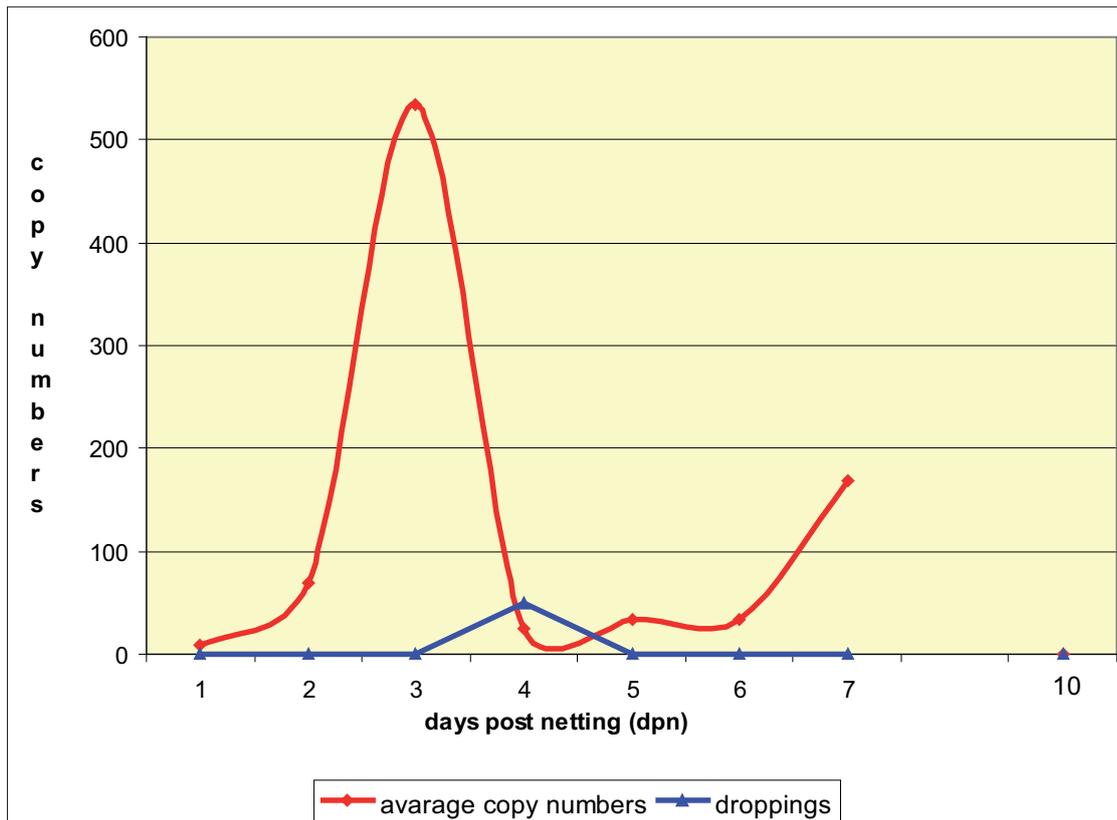


Figure 3. Average KHV copy number obtained from six persistently KHV infected carp after netting induced stress, between days 1 to 7 and day 10.

detection in infected fish is urgently necessary. Persistently infected but healthy appearing carp or koi pose a great risk to naïve fish as KHV is often not detectable by routinely used diagnostic methods due to very low viral loads being present in samples (Bigarré et al., 2009; Bergmann et al., 2009, Bergmann et al., 2010). False-negative results and the absence of clinical signs of KHVD have led to the assumption that KHV is not present in these fish (Meyer, 2007; Bigarré et al., 2009; Bergmann et al., 2010). The results of the present study show that there is a simple, reproducible and safe procedure that can be performed to re-activate KHV in persistently infected carp by inducing stress using a netting procedure commonly applied in the fish trade. A non-lethal sampling regime (collection of gill swabs and droppings) was carried out and the samples were screening by

quantitative real-time PCR. In this study it was demonstrated that apparently healthy KHV infected carp which survive KHVD, release very low quantities of virus via gills or droppings after stress induction. However, in samples taken within one day after capture, KHV DNA was detected in only one of six carp. This seems to be the situation when carp or koi are caught and sampled immediately. In similar netting experiments it was also shown that the majority of samples taken immediately after capture were negative (Meyer, 2007), although it was known that these carp or koi had gone through KHVD earlier. Using production steps such as size sorting or even normal transportation, it might be possible to re-activate KHV by commonly applied stress-inducing procedures. In such a stress situation, KHV forces infected cells to produce viral proteins and genomes forming

infectious virus particles that are then released (Meyer, 2007). In some cases this can lead to a re-occurrence of KHVD when the usually stable anti-KHV immunity collapses due to huge amounts of internally produced virus particles (Dr. Grit Bräuer and Dr. Kerstin Böttcher for carp, pers. comm.; Dr. Sandra Lechleiter for koi, pers. comm.). Other known and unknown co-factors might influence the re-outbreak of disease. Most commonly, KHV will not have been replicated at such high levels and the carp or koi may survive without displaying any clinical signs of KHVD, as seen in this experiment. To follow the EU strategy of KHVD eradication, a safe KHV detection protocol from persistently infected fish, including a non-lethal sampling, seems to be essential. The induction of stress by commonly applied capture and handling methods will open a small time window for successful application of diagnostic assays. In this study, KHV was exponentially re-activated up to 1000 genomic KHV equivalents. Samples collected immediately after the fish have been caught or more than seven days post-stress induction may produce false-negative results which could lead to incorrect veterinary certifications of carp or koi populations. These results indicate that fish should be caught and separated at least for 24 – 48 hours before sampling, but samples are best collected no later than four to five days after capture. The best period of sampling seems to be between 48 and 72 hours after capture. Additionally it does not appear to be necessary to perform a lethal sampling procedure. Most unexpected, carp got used to the daily catch and gill swab collection without a sustained increase of virus replication, at least not in the gills or droppings over the 10 day period. Comparative tests with different diagnostic assays did not reveal any difference

in the results between lethally (sampling of gill and kidney tissue) and non-lethally (swabs) collected samples from the same fish (results not included). The combination of fish separation for three to four days post-stress induction and non-lethal gill swab sampling appears to be a good tool for detecting KHV by real-time PCR, especially in persistently infected carp or koi. Recent EU and OIE recommendations to keep carp at a permissive temperature for three to four weeks prior to sampling may lead to a stabilisation of the fish in their new environments with subsequent suppression of KHV to a non-detectable level. Due to the persistent nature of KHV infection, this problem cannot be solved by increasing sample numbers because low KHV concentrations may remain undetectable. The stress inducing procedure presented in this study might be a good tool to reduce the reporting of false-negative KHV results at the population level.

Beside molecular virological assays for virus quantification, further investigations should include serological tests such as serum neutralization assay or antibody enzyme-linked immunosorbent assay for quantification of the humoral immune response before, during and after infection, re-activation, re-infection and / or vaccination. This could lead to a more accurate indirect detection of KHV infection by antibody based assays compared to results of the molecular assays (St-Hilaire et al., 2009).

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