Detection of koi herpes virus (KHV) genome in apparently healthy fish

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Abstract

Koi herpesvirus (KHV) induces a lethal disease in species belonging to *Cyprinus carpio,* covering common carp and koi or fancy carp. To date, other cyprinid fish species kept together with KHV infected carp or koi, such as goldfish (*Carassius auratus)* or grass carp (*Ctenopharyngodon idella*) had never shown any sign of KHV infection. Unexplainable outbreaks of KHV infection in common carp or koi led to the suspicion that more disease influencing factors exist, than so far explained. In challenge experiments and by natural routes of infection, it has been demonstrated that naive carp or koi can be infected by the following mechanisms; exposure to water from severe KHV diseased fish, co-habitation with KHV infected carp, by injection or immersion with cell supernatant of KHV infected cultures. Experiments were conducted to determine if apparently healthy koi (carriers/ survivors) or non-*Cyprinus carpio* species stocked with KHV infected carp could become infected. The study proved that these species were able to transfer KHV to naïve carp. As a result scientists suspected that some ornamental fish species could act as reservoirs of KHV infection.

In this study goldfish, grass carp, blue back ide (*Leuciscus idus*) and Ancistrus sp. were screened with routinely used diagnostic methods such as virus isolation in cell cultures and PCR and found to be KHV negative. This suggested that these fish species could not be infected by KHV. However, when using a more sensitive nested PCR, KHV DNA was detected from some of these fish. As confirmative methods sequence analysis of the nested PCR products and *in-situ* hybridization with different KHV probes were used. This study showed that the following cyprinid species; goldfish, grass carp and ide, and also non-cyprinid species *Ancistrus sp*. may act as a carrier of KHV.

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Introduction

Since 1997/ 98, mass mortality due to koi herpesvirus disease (KHVD) has occurred among all ages and sizes of common carp and koi carp (*Cyprinus carpio*) in Europe, USA and Asian countries including Israel (Bretzinger et al. 1999, Neukirch et al. 1999, Hedrick et al. 2000, Haenen et al. 2004). The disease was induced by an agent with herpesviral morphology confirmed by electron microscopy and classified as koi herpesvirus (KHV) or scientifically *Cyprinid herpesvirus 3* (CyHV-3). KHVD only occurs in *C. carpio*. To date, no clinical signs have been reported in other fish species which have been kept with carp or koi populations which have exhibited severe signs of KHVD. As a result, several scientists concluded that only *C. carpio* can become infected and is able to transfer KHV to carp or koi (Gilad et al. 2002, Hedrick et al. 2000, Perelberg et al. 2003, Ronen et al. 2003). Since 2003 many unexplainable cases of KHVD in koi or carp without any epidemiological connection to KHV infected stocks have occurred worldwide. As a consequence of these unexplained KHVD outbreaks apparently healthy carp which had been KHV vaccinated or outbreak survivors or even carp without any KHV history, were under suspicion of spreading KHV. It is very difficult to isolate KHV in cell cultures (Haenen et al. 2004) and almost impossible to obtain a virus isolate from survivor or carrier fish. There is no published report or successful experiment showing the re-activation and re-isolation of herpesvirus from any latent or persistently infected aquatic animal (Dr. Larry Hansen, Mississippi State University, USA, pers. comm.). Using routine PCR (Gilad et al. 2002 or Gray et al. 2002), KHV has not been detected in carrier fish. In this study, KHV DNA was detected using more sensitive methods like nested PCR and *in-situ* hybridization (ISH) (Bergmann et al. 2006) in some carrier hosts.

Materials and methods

Fish, samples and controls

Several goldfish (*C. auratus*) varieties such as lion head (7 -10 cm), colored or red goldfish (12-15 cm) and shubunkin (5-7 cm), blue back ide (*Leuciscus idus*, 10-12 cm), grass carp (*Ctenopharyngodon idella*, 10 -15 cm), *Ancistrus sp*. (3-5 cm) and koi (12-15 cm) were directly collected from a wholesaler after importation from an Asian country to Germany without relaying in his facilities or water. These animals (n=10 per species detailed above), appeared clinically healthy. Fish were killed by an overdose of anaesthetic (2% v/v Benzocain) in water. Organ samples (50 mg spleen, kidney and gills, respectively) were individually dissected and put into a sterile Eppendorf tube (2 ml) from each fish for further investigation by PCR. Heparinized blood (0.2-1 ml dependent on the fish sizes) was individually collected into 1 ml vacuette tubes by puncture of the caudal vein for leucocyte separation. Tissue material obtained from specific pathogen free (SPF) common carp (Wageningen, Netherlands) were prepared as negative controls, in addition to 200 µl PCR grade water as negative preparation controls. To include a positive control, the KHV-I isolate (Hedrick et al. 2000) grown in CCB cultures at 20°C with a titre of $10^{3.75}$ TCID50 / ml was prepared with the same master mix (stored at 4°C) and run after the nested PCR of the samples were finished (6-8 hours later). As an additional contamination control, after each

sample a water control was included from the preparation step to nested PCR. For further confirmation of the results, a second nested PCR with the primer pair KHV-2Fn (5'-AAC CCG AGG GGA CTG CTC GC-3') - 2Rn (5'-GAG GGC GGC GGG CCT CCC-3') resulting in a product size of 263 bp was performed. This could not be successfully used by ISH with Bismarck-Brown-Yellow (BBY) counterstaining because of a weak coloration of KHV DNA inside the infected cells.

Nucleic acid extraction, detection and sequencing

DNA was purified from tissue by DNAzol® method (Invitrogen) according to manufacturer's instructions. All DNA samples were primary tested for the presence of KHV DNA fragments by routinely used PCR (Gilad et al. 2002, Gray et al. 2002, Bercovier et al. 2005, Bergmann et al. 2006) slightly modified by using Go Taq Flexi DNA polymerase kit (Promega). Additionally PCR and more sensitive, nested PCR using primer pair KHV 1Fn-1Rn after Gilad's PCR (Bergmann et al. 2006) was performed. Appearing fragments were directly cloned into the vector pGEM-Teasy (Promega) according to manufacturer's instructions and finally sequenced. Consensus sequences were created from three independent analysis. The received sequences were matched with GenBank data (GenBank accession numbers. AF411803 and AB127966) of KHV. For confirmation, *in-situ* hybridization was performed. Also DNA obtained from *Herpesvirus anguillae* (HVA, AngHV-1), *Channel catfish herpesvirus* (CCV, IcHV-1) and *Carp pox virus* (CyHV-1) grown in cell cultures (EK-1, CCO, and CCB respectively) at 20°C were used as heterologous virus controls.

Digoxigenine (DIG) labelling of KHV DNA

Total DNA obtained from KHV infected KF-1 cells (Hedrick et al. 2000) was labelled by Digoxigenin-11-2'-deoxy-uridine-5'-triphosphate (DIG-dUTP, 30%) by substitution of 2'-deoxythymidine 5'-triphosphate (dTTP, 70 %) during PCR (Bergmann et al. 2006) according to "Nonradioactive In Situ Hybridization Application Manual" (Roche). Primer pairs NH1-NH2 (Hutoran et al. 2005) and KHV 1Fn-1Rn (Bergmann et al. 2006) were used to produce DIG-labelled probes. As an additional negative control, an irrelevant DIG-labelled probe was used.

Separation of leucocytes

Fish were anesthetized and blood was collected by puncture of the caudal vein into a syringe previously rinsed with heparin (Sigma) at 1000 U/ml in PBS. Blood was immediately diluted into a five-fold volume of cold cell culture medium used for cultivation of CCB cells (Neukirch et al. 1999) containing 10% fetal calf serum. Prediluted blood was layered onto Ficoll 1077 (Sigma). After centrifugation at 650g at 4 °C for 40 min, cells at the interphase were collected and washed twice with sterile PBS (650g, 4 °C, 10 min) to remove Ficoll. Then cells were adjusted to a maximum of 10^7 cells / ml.

KHV isolation procedure

CCB cells and KF-1 cells were prepared according to Neukirch et al. (1999) and Hedrick at al. (2000), respectively. Cells were allowed to replicate at 20°C in an incubator with 5% CO_2 atmosphere. Confluent cells, 24 hours old in 12 well plates (Costar), were used for isolation investigations. Supernatants (200 – 500 µl) obtained from ground tissues and separated

leucocytes (10^7 cell / ml, 200 µl) were adsorbed for one hour onto cell monolayer without the medium at 20°C. After carefully washing with sterile PBS, 1 ml CCB cell culture medium was added to each well. Wells without samples were treated in the same manner but with 200 µl medium as negative controls. Additionally, one well on each plate was not treated (no absorption) for growth control. KHV-I was used as positive control.

In-situ hybridization (ISH)

Formalin fixed and paraffin embedded tissue sections were transferred onto Superfrost ® microscope slides (Microm International) and framed by PapPen (Merck), treated with proteinase K (100 µg proteinase K/ ml) in TE buffer (50mM Tris, 10 mM EDTA) for 20 min at 37°C and fixed again by 95% ethanol followed by 100% ethanol for 1 min, respectively. After air drying, sections were framed by Pap Pen again and, for equilibration, covered by hybridization mixture (ISH-M) containing 4 x standard saline citrate (SSC), 50% formamide (v / v), 1 x Denhardt's reagent, 250 µg yeast tRNA / ml and 10% dextran sulphate and incubated for one hour at 42°C in a humid chamber. DIG-labelled probes (5 µl in 200 µl ISH-M) were applied to each section, covered by an ordinary cover slip, placed on the *in-situ* plate of a thermal cycler (Eppendorf Mastergradient), and heated up to 95°C for 5 min for denaturation of the DNA. The slides were cooled down on ice for 2 min and then incubated overnight at 42°C in a humid chamber for hybridization. Next day, cover slips were removed by washing in 2x SSC for 10 min. For removing of unspecific bound probes, slides were incubated in 0.4 x SSC at 42°C for 10 min. Sections were counterstained with Bismarck-Brown Y for sharpening a possible positive signal which would be visible as violet-black (high homologous DNA concentration) or red-violet foci (low homologous DNA concentration) in infected cells.

Results

Between five and 10 single fish samples as single organs or tissue pools (spleen, gill, kidney) or separated leucocytes from each fish obtained from apparently healthy ornamentals were screened for KHV by PCR and nested PCR. Investigations for virus isolation in KF-1 cell (Hedrick et al. 2000) and CCB cells (Neukirch et al. 1999) were all negative.

In first round PCR using different primer pairs (Gilad et al. 2002, Gray et al. 2002, Bercovier et al. 2005, Bergmann et al. 2006) and tissues or leucocytes from the different ornamental fish species, all samples tested KHV PCR negative. Detections were only successful when nested PCR was used. Leucocytes, kidney, spleen and gill tissue of exemplarily red goldfish were found to be KHV DNA positive (Figure 1).

Generally, KHV DNA fragments were detected in samples from red goldfish, shubunkin goldfish, lion head goldfish, blue back ide, *Ancistrus* sp., koi and grass carp by nested PCR only (Table 1). In all preparations an additional negative control after each single sample always tested negative, even when screened by nested PCR. There was no clinical sign of any disease on these fish.

The imported ornamentals from Asia (red, lion head and shubunkin goldfish, as well as grass carp, *Ancistrus sp.* and koi) were

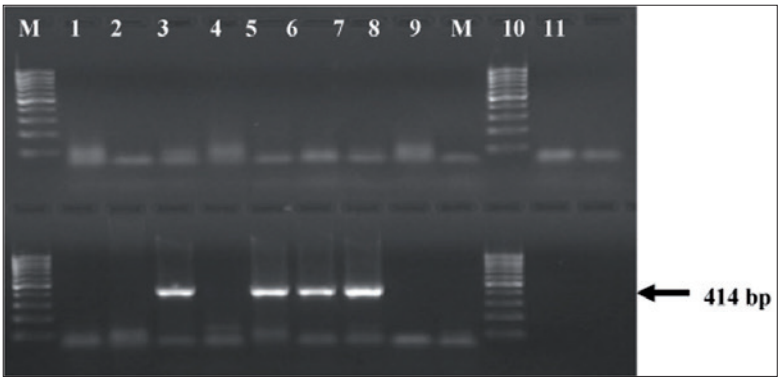

Figure 1. KHV DNA detection in red goldfish (*Carassius auratus* L.) tissue samples by nested PCR. Upper lane: PCR according to Gilad et al. 2002; lower lane: nested PCR (Bergmann et al 2006): M 100 bp marker (peqlab); lane 1 control; lane 2 gill swab; lane 3 leucocytes; lane 4 brain; lane 5 kidney; lane 6 spleen; lane 7 gill; lane 8 preparation negative control 1 (water); lane 9 preparation negative control 2 (SPF carp tissue), lanes 10 and 11 additional negative controls (water).

Table 1. Results of investigations on pooled tissue samples (gills and kidney) by PCR and nested PCR for KHV DNA detection.

	PCR		**Number of positive samples by nested PCR**
Fish species	Gray et al. (2002)	Gilad et al. (2002)	Bergmann et al. (2006)
red goldfish	-	-	4/10
shubunkin	-	-	2/10
lionhead	-	-	5/10
blue back ide	-	-	2/10
Ancistrus sp.	-	-	2/10
grass carp	-	-	3/10
SPF carp kidney	-	-	-
water controls	-	-	-

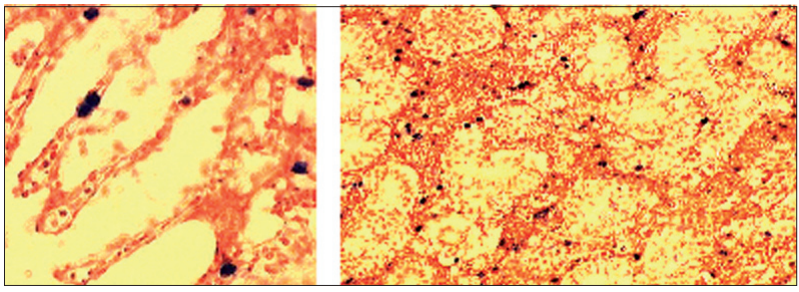

Figure 2. In-situ hybridization with KHV probe (NH1-NH2) after Bismarck-Brown-Yellow counterstaining recognising KHV in grass carp tissue.

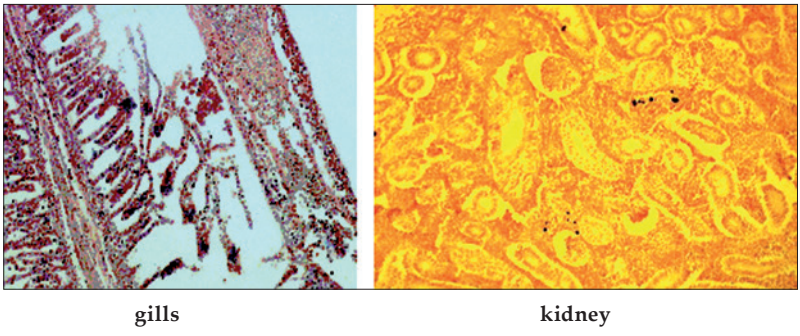

Figure 3. In-situ hybridization with KHV probe (KHV 1Fn-1Rn) after Bismarck-Brown-Yellow counterstaining recognising KHV in tissues from red goldfish.

directly transferred from the wholesaler to the laboratory. Proceeding PCR from samples, some (mainly pools of kidney and gill parts or separated leucocytes) gave positive signals for KHV DNA by nested PCR only (Table 1). Resulting fragments were sequenced and consensus sequence of three independent sequence analysis did show 100% similarity to the published sequences with all analyzed fragments. Formalin fixed and paraffin embedded tissue was use for further confirmation. KHV infected cells were detected in both gill and kidney material sectioned from grass carp and goldfish by ISH (Figures 2 and 3). All other fish species screened ISH negative for KHV.

In all cases where negative or heterologous controls were used, no positive signals were found, neither by PCR nor by ISH.

Discussion

The results of this study confirmed the presence of KHV DNA in other fish species than *C. carpio*, also in fish species imported from Asian countries to German pet shops and wholesalers. While many authors deny the presence of KHV in other fish species

than *C. carpio* (Hedrick et al. 2000, Gilad et al. 2002, Ronen et al. 2003, Perelberg et al. 2003, Hutoran et al. 2005) because of the lack of KHVD symptoms, KHV DNA was found by sensitive methods of DNA detection in carrier fish. It seems to be a question of test sensitivity since in our experiments KHV DNA was also not detectable by routine methods such as virus isolation by cell cultivation or PCR (Gilad et al. 2002, Gilad et al. 2004, Gray et al. 2002, Hutoran et al 2005, Bercovier et al. 2005, Bergmann et al. 2006). Isolation of KHV by cell culture methods is often difficult, also when the fish is in the acute phase of infection. It is extremely difficult to find KHV or KHV DNA in carrier fish and it seems not to be possible to re-activate KHV using organ explantates or leucocytes from known KHV infected but "healthy" fish in cell cultures (Dr. Larry Hansen, Mississippi State University, USA, pers. comm.). Nevertheless, there are publications with a successful reactivation of obviously persistent KHV by different stress models like netting as "transportation stress" or with administration of stress hormones (Meyer 2007). In our study, KHV isolation from ornamental fish produced negative results from all the fish screened. This could indicate that the KF-1 or CCB cells are not susceptible enough to KHV, either due to low virus concentrations, or the virus is not able to replicate because of incomplete perhaps non infectious particles. However, KHV DNA was detected by nested PCR only from samples of fish species that had never presented any clinical signs of KHV. In this study, first round PCR using the primer sets described by Gilad et al. (2002), Gray et al. (2002), Bercovier et al. (2005) and Bergmann et al. (2006) also using different DNA polymerase enzymes (Promega, Invitrogen or Qiagen) produced negative results, probably due to a low test sensitivity. The use of a nested PCR (Bergmann et al. 2006) produced positive signals for KHV DNA. It is known that nested PCR is much more sensitive compared to the first round PCR and it seems to be both, low viral concentration and low test sensitivity, that led to negative results. For diagnostic screening using nested PCR, the danger of contamination from the samples itself and/or from positive control material is very high. For this reason, no positive control was prepared and used in the same PCR assays but proceeded with the same master mix and thermal cycler afterwards. Due to this protecting measurement, contamination of the samples in nested PCR by positive control was as much as possible excluded. Further investigations have to focus on improved virus isolation techniques, characterization of different KHV specific genes for comparison as well as on transmission experiments from goldfish, grass carp, ide or other with *C. carpio* co-housed ornamental fish to naïve koi and carp. In conclusion it was shown that more fish species than carp or koi, such as goldfish, ide, *Ancistrus sp*. and grass carp may carry KHV genetic material, especially from areas with endemic KHVD in eastern Asia, which was confirmed by sequence analysis of the PCR products and ISH with different fish tissues.

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