

# Goldfish (*Carassius auratus auratus*) is a susceptible species for koi herpesvirus (KHV) but not for KHV disease (KHVD)

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## Abstract

In an experiment, koi (*Cyprinus carpio*) and goldfish (*Carassius auratus auratus*) were infected with koi herpesvirus (KHV) by immersion or intraperitoneal (i.p.) injection. Tissue and leucocyte samples were screened for the presence of KHV by PCR at the nucleic acid level and immunofluorescence antibody technique (IFAT) on a protein level at several time points post infection (p.i.). KHV was detected in tissues as well as in separated leucocytes obtained from both koi and goldfish by PCR, *in-situ* hybridization (ISH) and IFAT. Leucopenia occurred in both, koi and goldfish. While koi surviving the infection recovered quickly from leucopenia, it persisted for at least 60 days in goldfish. On day 60 p.i. KHV infected goldfish were cohabitated with naïve koi at 19°C water temperature. Koi were bled for leucocyte separation analysis on day 30 post cohabitation. KHV was found in white blood cells by PCR, ISH and IFAT. It was concluded that goldfish is a susceptible species according to EU Commission Decision 2006 / 88 / EC.

## Introduction

Since 1997, a new disease complex has occurred in *Cyprinus carpio* that has been described as koi herpesvirus (KHV) disease (KHVD) (Bretzinger *et al.*, 1999; Hedrick *et al.*, 2000) or carp nephritis and gill necrosis (Ronen *et al.*, 2003). Severe external clinical signs were found in *C. carpio* only. Besides lethargy and gasping at the water surface, typical external clinical signs include an increase in mucus production on skin and gills, circular or extensive skin lesions or necrotic areas on skin or gills resulting in

the loss of complete skin tissue. Frequently, enophthalmus was also observed. In general, outbreaks occurred when water temperatures between 18 and 29°C were reached. In challenge experiments, other fish species e.g. goldfish (*Carassius auratus auratus*), crucian carp (*Carassius carassius*), golden ide (*Leuciscus idus*), grass carp (*Ctenopharyngodon idella*) (Bergmann *et al.*, 2009), tilapia (*Oreochromis sp.*), silver perch (*Bidyanus bidyanus*) or silver carp (*Hypophthalmichthys molitrix*) did not show any clinical signs after cohabitation

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with severely KHV diseased koi (Ronen *et al.*, 2003). They did not transmit the virus to naïve susceptible fish at a detectable level (Gilad *et al.*, 2002; Ronen *et al.*, 2003; Perelberg *et al.*, 2003). It was concluded that these species were not susceptible to KHV and KHVD.

While Bergmann *et al.* (2004) investigated goldfish leucocytes by PCR and immunofluorescence, Sadler *et al.* (2007) used a real-time PCR for KHV detection in a goldfish population cohabitated with infected koi. El-Matbouli *et al.* (2007) successfully used a loop-mediated isothermal amplification method to detect KHV in goldfish. In this study, it was shown that goldfish can be infected by KHV reproducibly, confirmed by PCRs, IFAT and ISH, and that goldfish are able to transmit the infectious virus to *C. carpio*.

## Materials and methods

### *Fish*

Goldfish (25 – 50 g, n=11) and koi (30 - 50 g, n=27) obtained from hobby ponds without clinical signs of disease and free of ectoparasites or pathogenic bacteria were adapted to our aquarium conditions for 4 weeks. They all were tested for absence of KHV by PCRs (Gilad *et al.*, 2002; Gray *et al.*, 2002; Gilad *et al.*, 2004; Bergmann *et al.*, 2006) using separated leucocytes, gill biopsies and gill swabs. Fish were kept at 20°C (+/- 1°C) water temperature in 300 l tanks in recirculation systems with a water exchange of 50 l per day. Animals were fed with commercial koi food (Tetra) once a day. Fish were divided into three groups: group 1 (four goldfish, six koi) was infected with 100 µl KHV-I (Hedrick *et al.*, 2000), 13<sup>th</sup> cell culture passage in the laboratory, with a dose of 10<sup>3</sup> TCID<sub>50</sub> / ml by intraperitoneal

(i.p.) injection; group 2 (four goldfish, six koi) was challenged with KHV-I by immersion and group 3 (three goldfish, six koi) was kept in separate tanks and were left unchallenged as a negative control group. Additionally, KHV free koi (n=15) were kept separately and used later on in the transmission experiment.

### *Cohabitation experiment*

Goldfish (n=3) infected by KHV (i.p. injection) for 60 days were placed in a tank at 19°C with healthy koi (n = 15), screened KHV negative by PCR and nested PCR (Bergmann *et al.*, 2006).

On day 30 post cohabitation samples were taken from both goldfish and koi.

### *Samples*

From each fish, tissue samples (50 -100 mg) were individually dissected from the spleen, heart, kidney, gill, skin and brain respectively. Tissue samples were homogenised then a low speed centrifugation was performed. Supernatants were collected for cell cultivation and the tissue pellets were used in the PCR assays. Separated leucocytes were used for PCR, *in-situ* hybridization and IFAT (Bergmann *et al.*, 2006; Bergmann *et al.*, 2009).

### *Cells and viruses*

Suspensions of homogenised tissue supernatant samples were adsorbed to common carp brain (CCB) cells (Neukirch *et al.*, 1999) for 1 h at 20°C, carefully washed twice, overlaid with medium and incubated at 20°C for 7 to 14 days. Cell cultures were observed daily for a cytopathic effect (CPE). KHV-I and KHV-E (England, generously provided by Dr. Keith Way, Cefas, Weymouth,

UK) previously cultured on CCB cells were used as positive virus controls according to Gilad *et al.* (2002). As heterologous virus controls, DNA obtained from channel catfish herpesvirus (CCV) propagated in channel catfish ovary cells (CCO) (Bowser and Plumb 1980), herpesvirus anguillae (HVA) in eel kidney (EK-1) cells and carp pox virus in CCB cells were used (Bergmann *et al.*, 2009).

*DNA extraction and identification of KHV nucleic acid*

DNA extraction was carried out by DNAzol® reagent (Invitrogen) according to slightly modified manufacturer’s instructions, in particular - DNA precipitation with ice-cold ethanol, two washing steps with ice cold 70 % ethanol and dilution of the resulting pellet in 50 µl. PCR and ISH for detection and confirmation of KHV DNA were conducted according to assays published by Gilad *et al.* (2002), Hutoran *et al.* (2005) and Bergmann *et al.* (2006) (Table 1). For PCR methods, water (negative) controls were prepared during each step of the process. Amplified KHV DNA was visualized in 1.5 %

agarose gel containing ethidium bromide.

Digoxigenine (DIG) labelling of KHV DNA  
 KHV DNA was labelled by digoxigenin-11-2'-deoxy-uridine-5'-triphosphate (DIG-dUTP, 30%) by substitution of 2'-deoxythymidine 5'-triphosphate (dTTP, 70 %) during PCR according to the “DIG Application Manual for Nonradioactive *In Situ* Hybridization” (Roche). Primer pair NH1-NH2 (Hutoran *et al.*, 2005) was used to produce the probes (Table 1). As additional negative controls, an irrelevant DIG-labelled probe developed against a VHSV fragment and the KHV probe on slides with CyHV-2 containing goldfish tissues, provided by Prof. R. Hedrick (University of Davis, California, USA) were used.

*Density gradient separation of leucocytes from blood*

Fish were anaesthetized in a benzocaine / water (50 ng / ml, Sigma-Aldrich) bath. Blood was collected by puncture of the caudal vein into a syringe previously rinsed with heparin (1000

**Table 1.** Primer pairs used for PCR and producing the probe for ISH.

Primer	Sequence (5'-3')	Fragment size (ORF)	Reference
KHV-F (KHV9/5F)	GACGACGCCGAGACCTTGTG	486 bp	Gilad <i>et al.</i>
KHV-R (KHV9/5R)	CACAAGTTCAGTCTGTTCCCTCAAC	(89 – 90)	(2002)
KHV-1Fn	CTCGCCGAGCAGAGGAAGCGC	414 bp	Bergmann <i>et al.</i>
KHV-1Rn	TCATGCTCTCCGAGGCCAGCGG	(89 – 90)	(2006)
NH1 Forward	GGATCCAGACGGTGACGGTCACCC	517 bp	Hutoran <i>et al.</i>
NH2 Reverse	GCCCAGAGTCACTTCCAGCTTCG	(139)	(2005)
KHV-JF	CACCACATCTTGCCGGTGTAC	766 bp	Bergmann <i>et al.</i>
KHV-JR	ATGGCAGTCACCAAAGCTCAAC	(81)	(2006)

U/ml in PBS). Blood was diluted immediately in a six-fold volume of cold cell CCB cell culture medium supplemented with 10% fetal bovine serum and layered onto an isotonic Percoll gradient (1.075 g/ml, Sigma-Aldrich) prepared according to the manufacturer's instructions. After centrifugation for 40 min at 650 x g at 4°C the resulting white cell band was washed twice with CCB cell culture medium (200 x g, 4°C, 10 min), resolved in 1 ml CCB cell culture medium and checked (100 µl) for viability by staining with trypan blue and leucocyte composition calculated by cell counting in a Thoma counting chamber (Zeiss) according to manufacturer's instructions.

#### *IFAT with separated leucocytes*

Leucocytes (adjusted to  $10^7$  cells / ml) were dropped on poly-L-lysine treated slides (0.1% w/v in water; Sigma), fixed with methanol-acetone mixture (1:1), air dried, surrounded by PapPen (Merck) and incubated with anti-KHV mab (10A4) and an anti-KHV serum T36 obtained from rabbit (Bergmann *et al.*, 2009, Kempter *et al.*, 2009) for 1 h at room temperature. After washing steps with PBS, cells were incubated with FITC conjugated mouse immunoglobulins (Dako) according to the manufacturer's instructions. Cells were visualised with a fluorescence microscope (IX 51, Olympus).

#### *In-situ hybridization (ISH)*

Separated leucocytes were dropped on Superfrost® microscope slides (Microm International), formalin-fixed according to standard protocols and air dried. Drops were framed by PapPen, 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, leucocyte drops were framed by PapPen again and for equilibration were covered with hybridization mixture (ISH-M) containing 4 x saline-sodium citrate (SSC), 50% formamide, 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 layered onto the leucocyte drops and covered by coverslip, placed on the in-situ plate of a cycler (Eppendorf Mastergardient) and heated to 95°C for 5 min. Subsequently, slides were cooled down on ice for 2 min and incubated overnight at 42°C in a humid chamber. Afterwards, slides were washed twice with 2 x SSC for 10 min. For removal of non-specifically bound probe, slides were incubated with 0.4 x SSC at 42°C for 10 min.

Leukocytes were counterstained with Bismarck-Brown Y (Sigma) to sharpen the possible positive signals which were visible by violet-black foci in the nucleus and cytoplasm of the infected cells.

## **Results**

### *Infection experiment*

During the challenge experiments using KHV-I, no goldfish died, however mortality levels up to 50% were observed in koi in both infected groups, which was observed on day 28 p.i. While in goldfish only weak clinical signs were observed, koi showed "typical" signs of KHVD like increased skin mucus production, enophthalmus and gill necrosis. Goldfish showed a slightly swollen abdomen and a pronounced lateral line with "crater like morphology" in the canal stomas for 10

to 15 days p.i. The surviving koi recovered completely within 40 days. After day 40 p.i., no external sign of KHVD was observed.

#### *Detection of KHV*

Attempts to isolate virus from the tissue samples of dead or surviving fish (koi and goldfish) in CCB cells failed. PCR according to Gilad *et al.* (2002) and Gray *et al.* (2002) were positive when tissue samples, pools of gill and kidney from individual surviving and deceased koi were screened, but were negative when only gill samples from survivors (koi) and goldfish were investigated.

Positive signals were only obtained from gill material sampled from survivors by nested PCR (Bergmann *et al.*, 2006).

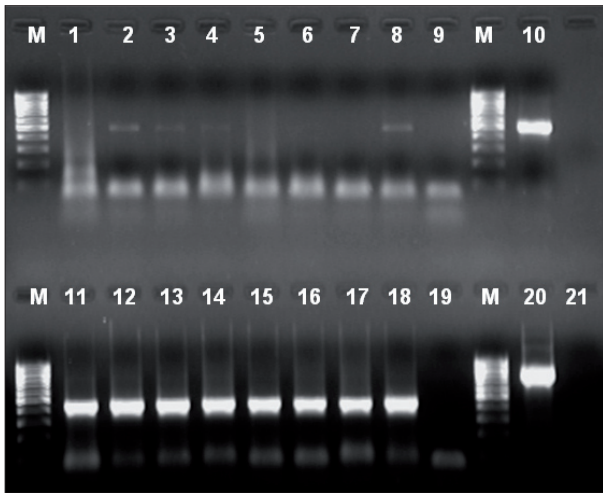
On day 7 p.i., skin, spleen, kidney and leucocyte samples from immersed goldfish were positive for KHV DNA. The confirmatory nested PCR (Bergmann *et al.*, 2006) was positive for all tissues (Figure 1A). By PCR (Gilad *et al.*, 2002) using goldfish samples from day 7 after i.p. injection, skin, kidney, liver, brain and leucocyte samples were considered to be KHV positive. Nested PCR was positive for all investigated tissues (Figure 1B). Using the same template volume (2  $\mu$ l from the samples) from the positive control (Figure 1B, lane 20) for nested PCR, an additional band (approx. 880 bp) occurred because of overloading with specific KHV DNA. Almost identical results were found with koi samples for PCR and nested PCR (data not shown).

On day 14, only leucocytes were included in the investigation. Samples obtained from goldfish and koi were considered to be KHV

positive. Results were confirmed by nested PCR (Figure 2). On day 45 p.i. leucocyte samples obtained from two fish of both species were still positive for KHV by PCR according to Bergmann *et al.*, (2006) (data not shown). In all cases, negative controls (water, leucocytes and tissues from negative fish) and DNA from the heterologous virus controls remained negative by PCR and nested PCR recognizing KHV.

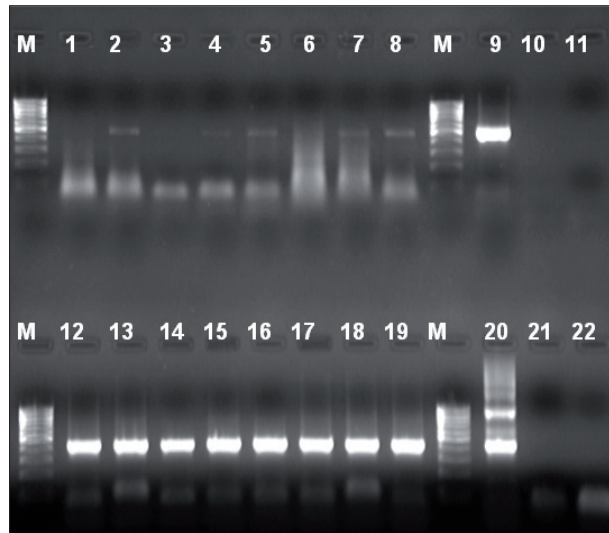
For additional confirmation, ISH with probe NH1-NH2 (Hutoran *et al.*, 2005) and IFAT with mab 10A9 (Bergmann *et al.* 2009) were carried out. ISH was performed on fixed goldfish and koi leucocytes obtained on day 7 p.i. In both samples KHV DNA was identified inside the cells (Figure 3 A and B) which was not found in leucocytes from negative control fish (data not shown). For additional control, IFAT with koi leucocytes from day 7 p.i. was carried out with rabbit anti-KHV serum T36 (Figure 4). It could be shown that, despite the background from rabbit serum T 36 visible in the controls of Figure 4 C, more leucocytes were infected by KHV after immersion compared to i.p. injection (Figures 4 A and B). This was not detected in leucocytes from goldfish where the number of infected leucocytes was always equal in both groups: immersion and i.p. injection. Goldfish leucocytes were also examined on day 60 p.i. by IFAT. In Figure 5 A, KHV bearing cells were identified with mab 10A9 by IFAT. No staining was found in leucocytes from negative controls (Figure 5 B) as well as in leucocytes from infected goldfish but with a negative rabbit serum and an irregular mab (data not shown).

#### *Cohabitation experiment*



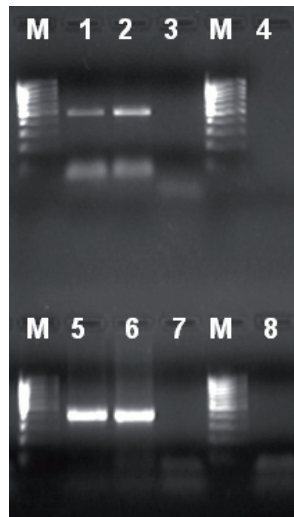
lane M 100 bp marker (peqlab); lane 1 gills; lane 2 skin; lane 3 spleen; lane 4 kidney; lane 5 liver; lane 6 gut; lane 7 brain; lane 8 leucocytes; lane 9 negative preparation control; lane 10 positive control (KHV-I, CCB cells); lanes 11-19 nested PCR from products above; lane 20 additional PCR with primers KHV-JF-JR (Bergmann *et al.*, 2006); lane 21 negative control KHV-JF-JR.

**Figure 1A.** KHV detection in samples from goldfish infected by immersion from day 7 p.i. by PCR (Gilad *et al.*, 2002) and nested PCR (Bergmann *et al.*, 2006).



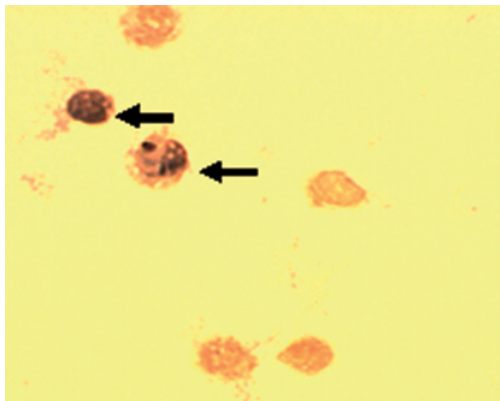
lane M 100 bp marker (peqlab); lane 1 gills; lane 2 skin; lane 3 spleen; lane 4 kidney; lane 5 liver; lane 6 gut; lane 7 brain; lane 8 leucocytes; lane 9 positive control (KHV-I); lane 10 negative preparation control; lane 11 negative PCR control; lanes 12 - 22 nested PCR from products above.

**Figure 1B.** KHV detection in samples from goldfish infected by intraperitoneal injection from day 7 p.i. by PCR (Gilad *et al.*, 2002) and nested PCR (Bergmann *et al.*, 2006).

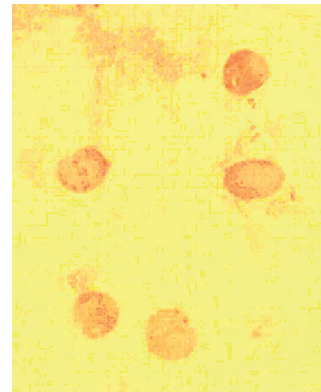


lane M 100 bp marker (peqlab); lane 1 goldfish; lane 2 koi; lane 3 negative preparation control; lane 4 negative control (water); lanes 5-8 nested PCR from products above.

**Figure 2.** KHV detection in leucocyte samples from day 14 after i.p. infection by PCR (Gilad *et al.*, 2002) and nested PCR (Bergmann *et al.*, 2006).

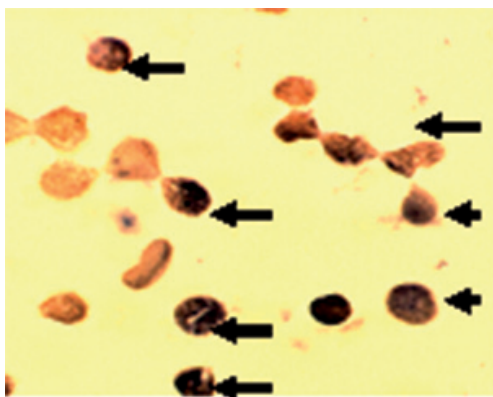


positive signals (arrows)

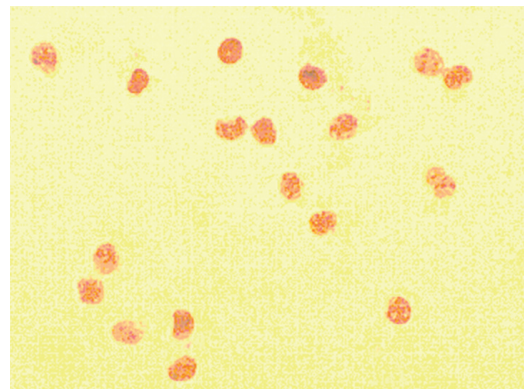


negative control

**Figure 3A.** ISH with separated koi leucocytes from day 7 p.i. after immersion using probe NH1-NH2 (Hutoran *et al.*, 2005).

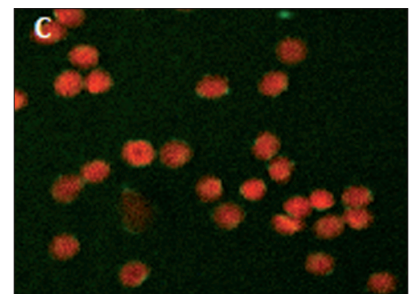
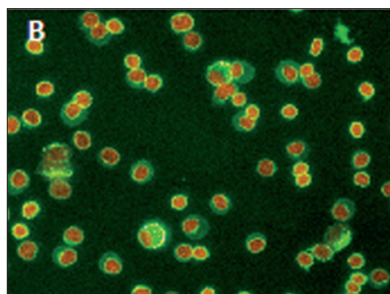
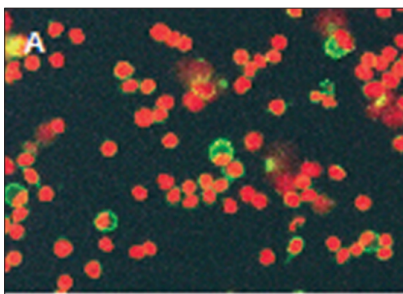


positive signals (arrows)

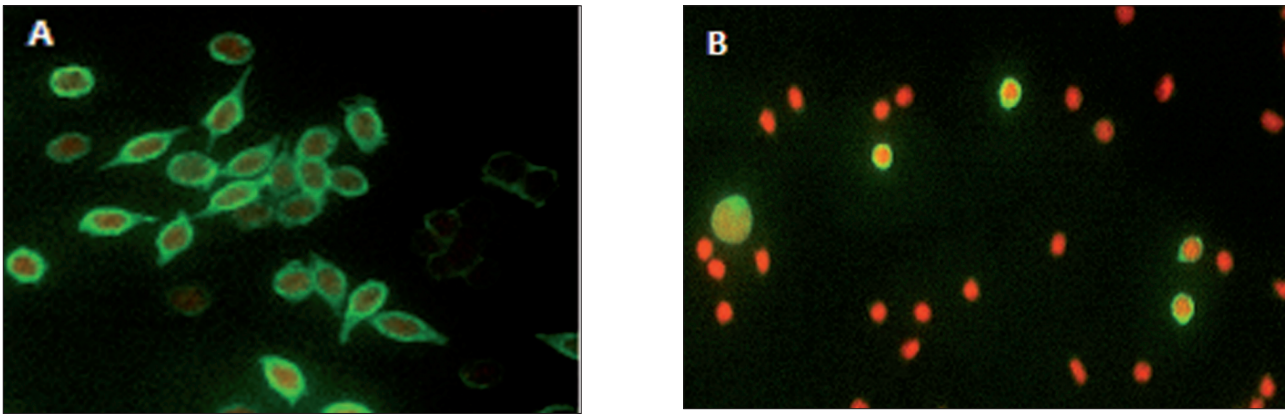


negative control

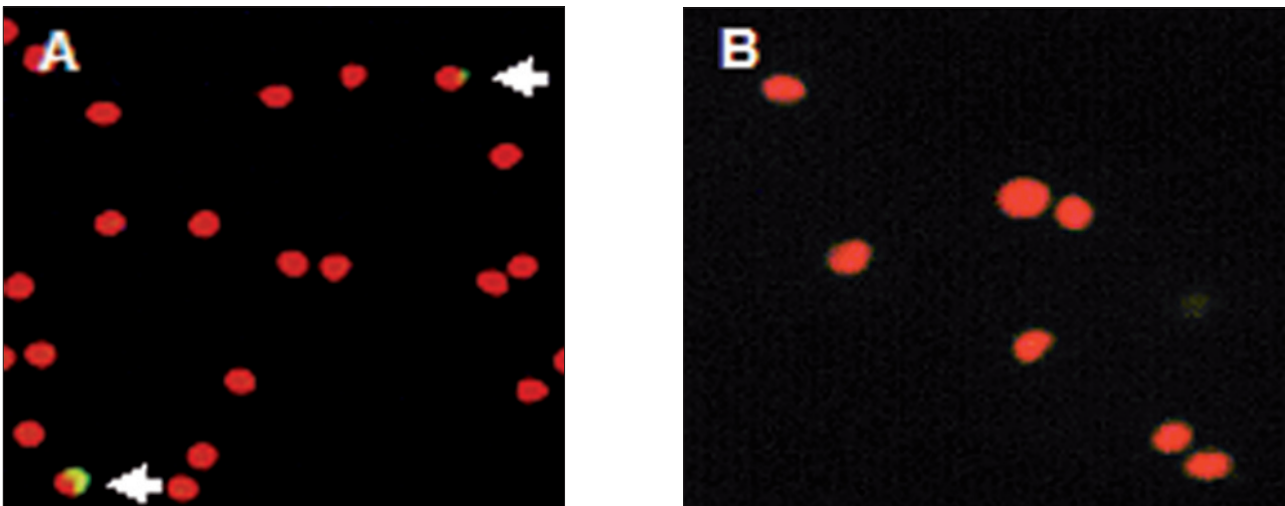
**Figure 3B.** ISH with separated goldfish leucocytes from day 7 p.i. after immersion using probe NH1-NH2 (Hutoran *et al.*, 2005).



**Figure 4.** IFAT on separated koi leucocytes from day 7 p.i. after injection (A), immersion (B) and negative control leukocytes (C) using rabbit anti-KHV serum T 36.



**Figure 6.** IFAT with leucocytes from koi cohabitated with KHV infected goldfish using rabbit anti-KHV serum T 36 (A) and mab 10A9 (B).



**Figure 5.** IFAT with mab 10A9 on goldfish leucocytes from day 60 p.i. by injection (A) and leucocytes from negative controls (B).

After 30 days of cohabitation, no fish showed any external clinical sign of KHVD but leucocytes from these koi and goldfish were considered positive mainly by nested PCR and IFAT using mab 10A9 as well as rabbit anti-KHV T36 (Figure 6).

#### *Investigation of peripheral blood leucocytes (PBL) composition*

The infection experiment was accompanied by investigation of the PBLs of two fish from

each group at each day. On day 0, PBLs of goldfish, koi and of negative controls moved in a normal frame. Special attention was paid to lymphocytes and thrombocytes. In infected fish, lymphocytes decreased rapidly from 41 to 10 on day 7 in goldfish and from 60 to 20 on day 7 in koi. On day 14, lymphopenia of both fish intensified. While koi blood cells recovered from day 45 on, goldfish lymphocytes remained on the level of day 14 (Table 2). This was also found on day 60



p.i. (data not shown). An unusual feature was found when thrombocytes of goldfish were analysed. On day 0, 40 cells were identified by their spindle-shaped morphology. This cell type could not be identified morphologically later on, i.e. on days 14, 45 or 60 p.i. (Table 2).

## Discussion

New investigations on cyprinid herpesviruses in comparison to KHV show similarities in almost all sequenced genome parts (Hedrick *et al.*, 2006; Aoki *et al.*, 2007). Moreover, it was proposed to create within the new order *Herpesvirales* the new family *Alloherpesviridae*. This family comprise fish and amphibian herpesviruses (Aoki *et al.*, 2007; Davison *et al.*, 2005).

According to the literature, it is generally agreed that carp is the only susceptible species for KHVD. Goldfish, grass carp, crucian carp and others are not susceptible (Gilad *et al.*, 2002; Ronen *et al.*, 2003; Perelberg *et al.*, 2003) as no clinical signs occur after infection or cohabitation with severely KHVD infected carp or koi. Sadler *et al.* (2007), El-Matbouli *et al.* (2007) and Meyer (2007) found KHV in goldfish by a variety of molecular methods. In experiments performed by Hedrick *et al.* (2006), goldfish and carp x goldfish hybrids did not show severe clinical signs after infection with KHV.

However, due to intensive work on diagnostic method sophistication, it was possible to show that goldfish can carry KHV (Bergmann, 2004; Meyer, 2007).

In our experiments, clearly visible clinical symptoms of KHV disease did not occur in goldfish but no histological examination was

performed. In addition, PCR according to Gilad *et al.* (2002) and Gray *et al.* (2002) always gave negative results when samples were taken from "healthy appearing" koi and goldfish later than day 21 p.i. In contrast, using nested PCR (Bergmann *et al.*, 2006), KHV was present in different organs of goldfish between days 7 and 60 p.i. However, the actual proof of the presence of KHV in tissues of infected fish was provided by nested PCR and by ISH (mainly used for confirmation of the results) at the nucleic acid level and by IFAT on leucocytes at the viral protein level. These results allow the conclusions that goldfish can be infected with KHV and can spread the virus to naïve koi.

In the cohabitation trial, no clinical signs were observed after transfer of KHV from goldfish to naïve koi. This may be due to the fact that the water temperature only reached 19°C and, additionally, the koi were not stressed. Nevertheless, KHV was identified in leucocyte samples from these koi by PCR and IFAT. These studies also led to the hypothesis that KHV rather seems to be a lymphotropic than a neurotropic virus. A section of koi brain tissue from the only fish observed demonstrating abnormal swimming behaviour was tested by ISH. KHV DNA was detected in uncharacterized cells of the granular layer of mesencephalon but further investigation of the viral tissue tropism is necessary.

From this study it appears that KHV may be replicated in goldfish and infectious virus can be transferred after 60 days p.i. to naïve koi. According to EU legislation (CD 2006 / 88 / EC, Anonymous 2006) and OIE recommendation (Anonymous, 2009), goldfish is a susceptible species for KHV but not for KHVD. The

**Table 2.** Composition of the separated blood cells during the KHV experiment (mean of two fish sampled per day).

Cells	Carp (control)	I.p. injected goldfish (days p.i.)				I.p. injected koi (days p.i.)			
		0	7	14	45	0	7	14	45
Erythrocytes	1	1	-	2	-	-	-	-	1
Lymphocytes	50	41	10	4	7	60	20	6	68
Monocytes	1	-	1	1	1	-	1	1	-
Granulocytes	1	3	-	2	-	2	2	-	4
Thrombocytes	-	40	?	?	?	1	-	1	3
Dead cells	-	-	-	-	-	-	1	-	1

disease can only be observed in *C. carpio*.

Ongoing work shall focus on infection experiments at higher water temperatures as well as including KHV isolates from different areas of the world and other fish species.

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