First detection, confirmation and isolation of koi herpesvirus (KHV) in cultured common carp (*Cyprinus carpio L.*) in Poland

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Abstract

The study involved 30-135 g carp more than one year old, that had been cultured since the stage of summer fry at the Fisheries Research Station operated by the Agricultural University's Department of Aquaculture, University of Szczecin and located in a Dolna Odra power station cooling water canal. The fish selected for analyses showed the following clinical signs in summer: apathy; strong necrotic patches on gills; lustreless and rough skin with numerous deep necrotic spots extending down to the muscles; deposits of thick mucus under the gill covers. On the 4th of June 2004, three carp samples of 15 individuals each were delivered live to the German National Reference Laboratory Insel Riems for analyses. Koi herpesvirus was detected in two out of the three samples using different PCR assays. The PCR results were confirmed by nested PCR and *in situ* hybridization. Assays were performed on gills, brain, and kidney tissues. Samples were also taken from outbreak survivors showing no clinical signs of disease in autumn 2004 and tested by PCR and nested PCR. These results were also confirmed by *in situ* hybridization using different probes. This is the first detection, virus isolation and confirmation of KHV in Poland.

Introduction

Koi herpesvirus (KHV) was first identified in Israel in spring 1998. So far, the virus has been reported in Europe from Belgium, Denmark, United Kingdom, Finland, France, Germany, Austria, Switzerland, Luxembourg, Italy, and the Netherlands (Haenen et al., 2004). In view of the spread into nearly all Western European countries, and considering the fact that two positive incidences of the virus presence were found in our laboratory from fish imported from Poland to Germany, events of mass carp mortality observed in Polish carp farms since 2001 may also be attributable to KHV infections (Figure 1).

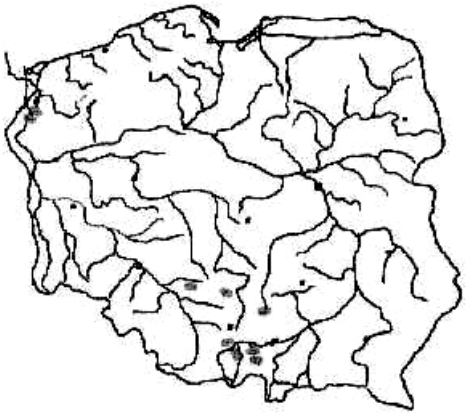

Figure 1. Location of Polish cyprinid fish farms affected by mass mortality of carp with clinical symptoms of KHV between 2002 and 2004.

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In 2001, carp stocks kept at the Fisheries Research Station and other farms located in the Dolna Odra power station cooling water canal were affected by mass mortality. The fish showed anorexia, were apathetic, and gathered close to the water surface. The skin, lacking lustre, showed mucus accumulation and necrotic spots/areas; patches of the external skin layer were observed to peel off. The gills showed necrosis of the tips of the lamellae and deposits of dark thick mucus between the lamellae. In addition, a mass mortality of fish showing no visible disease signs was recorded as well. As the mortality events were repeated in 2002 and 2003, usually in summer and only in carp, it was suspected that KHV was the pathogen responsible. Therefore, it was decided that the following year, when the first signs of disease appeared, samples should be sent for virological examination.

This paper reports the use of different PCR methods and *in situ* hybridisation assays to detect and to confirm KHV infections in Polish aquaculture.

Materials and methods

Fish samples

Tissue samples (spleen, heart, kidney and brain) were taken from carp showing severe signs of KHV infection including mortality (samples KH and KD) and from carp without any clinical sign (sample OP) in summer 2004. In the autumn of 2004, samples were again collected from Fisheries Research Station of Agricultural University in Szczecin (FRSS). These carp had survived a KHV outbreak during summer 2004 and did not show any clinical signs. Part of the tissue samples were ground, centrifuged and tested by cell culture and PCR, and organ parts were also formaline fixed, paraffin embedded and cut for examination by *in situ* hybridisation assays.

Cell culture and viruses

Clarified suspension in 1:10 and 1:100 dilutions obtained from tissue samples were inoculated onto koi fin-1 (KF-1) (Hedrick et al., 2000) and *Cyprinus carpio* brain (CCB) cell monolayers (Neukirch et al., 1999) for one hour at 20°C, then carefully washed twice, overlaid with medium again and incubated at 20°C for 14 days. Cells were observed daily for the appearance of a cytopathic effect (CPE). KHV-I (Hedrick et al., 2000) was used as positive control virus. As a heterologous virus control, channel catfish herpesvirus (CCV) was propagated in channel catfish ovary cells (CCO) (Gilad et al., 2002).

DNA extraction

DNA was extracted by DNAzol® genomic DNA isolation reagent (Invitrogen), precipitated with cold (-20°C) ethanol (0.5 ml), washed twice with cold ethanol 75% (1 ml) and suspended in 30 – 50 µl water (DNase and RNase free) according to manufacturer's instructions.

PCR and nested PCR

PCR assays for detection of KHV fragments were performed according to protocols published by Gilad et al. (2002), Gray et al. (2002) and Hutoran et al. (2004). Additionally PCR assays were prepared as per Gilad's protocol using newly designed primer pairs (table 1). All samples investigated by the method according to Gilad et al. (2002) were confirmed by nested PCR using primer pair KHV-1Fn-1Rn. KHV-I (homologous virus)

Primer	Sequence (5'-3')	Size	Reference
KHV-F (KHV9/5F)*	GACGACGCCGGAGACCTTGTG	486 bp	Gilad et al
KHV-R (KHV9/5R)*	CACAAGTTCAGTCTGTTCCTCAAC		(2002)
KHV-1Fn*	CTCGCCGAGCAGAGGAAGCGC	392 bp	in this study
KHV-1Rn*	TCATGCTCTCCGAGGCCAGCGG		(AF411803)**
KHV-Gray-1F (BamHI-6)	TCGCATGTGAGGGTTCATGC	365 bp	
KHV-Gray-1R (BamHI-6)	CATCAGCGGCATCAGCATCG		Gray et al.
KHV-Gray-2F (SphI-5)	GACACCACATCTGCAAGGAG	290 bp	(2002)
KHV-Gray-2R (SphI-5)	GACACATGTTACAATGGTGGC		
NH1 Forward*	GGATCCAGACGGTGACGGTCACCC	517 bp	Hutoran et al.
NH2 Reverse*	GCCCAGAGTCACTTCCAGCTTCG		(2004)
KHV-JF	CACCACATCTTGCCGGTGTAC	766 bp	in this study
KHV-JR	ATGGCAGTCACCAAAGCTCAAC		(AB178537)**

* used as probe for ISH
** accession number

Table 1. Primer pairs used in this study.

and CCV (heterologous virus) were used as virus controls. Tissue samples from carp without any KHV history (specific pathogen free) were also used as negative preparation control. For each test, from preparation to the nested PCR, water controls were also included. In positive cases obtained from tissue samples, PCR products were visualized in a 1.5 % agarose gel containing ethidium bromide.

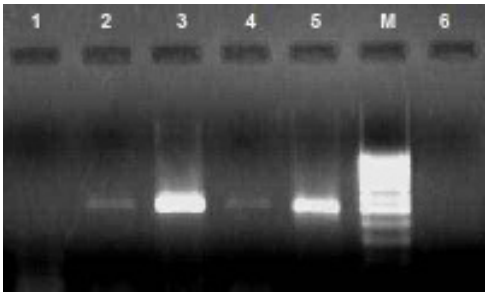
Digoxigenine (DIG) labelling of KHV DNA

Koi herpesvirus 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 "Nonradioactive *In Situ* Hybridization Application Manual" (Roche).

Selected primer pairs were used to produce the probes (Table 1). As an additional negative control, an irrelevant (VHSV) DIG-labelled probe was used.

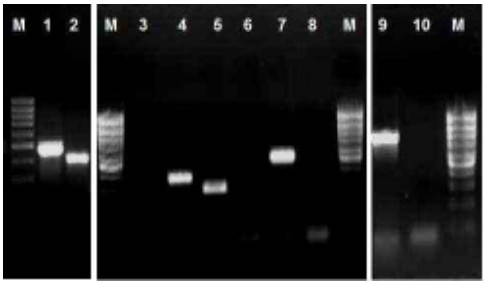
In situ hybridization (ISH)

Organ specimens were formalin-fixed and paraffin-embedded according to standard protocols. Sections of 5 – 7 µm were placed on Superfrost ® microscope slides (Microm International) for 18 h at 62°C, dewaxed by 2 x Rotihistol (Roth) for 10 minutes, followed by 2 x 100% ethanol for 10 minutes and air dried. Sections were framed by PapPen (Merck), treated with proteinase K (100 µg proteinase K / ml) in TE buffer (50mM Tris, 10 mM EDTA) for 20 minutes at 37°C and fixed again by 95% ethanol followed by 100% ethanol for 1 minute, respectively. After air drying, sections were framed by Pap Pen again and, for equilibration, covered by a hybridization mixture (ISH-M) containing 4 x standard saline citrate (SSC), 50% formamide, 1 x Denhardt's reagent, 250 µg yeast



M 100 bp marker (peqlab), lane 1 OP, lane 2 KH, lane 3 KD, lane 4 KH 1:10, lane 5 KD 1:20, lane 6 negative control

Figure 2. PCR for detection of KHV in samples obtained from common carp (according to Gilad et al. 2002).



M 100 bp marker (peqlab), lane 1 KHV-F-R, lane 2 KHV-1Fn-1Rn, lane 3 negative control KHV-Gray 1F-1R, lane 4 KHV-Gray 1F-1R, lane 5 KHV-Gray 2F-2R, lane 6 negative control KHV-Gray 2F-2R, lane 7 NH 1-2, lane 8 negative control NH 1-2, lane 9 KHV-JF-JR, lane 10 negative control -JF-JR

Figure 3. PCR reactions of sample KD using additional primer pairs recognizing KHV (according to the protocols of Gilad et al. 2002).

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 added to the sections and covered by an ordinary cover slip, placed on the *in situ* plate of a thermal cycler (Eppendorf Mastergradient) and heated to 95°C for 5 min for denaturation of DNA. Then slides were cooled on ice for 2 minutes and then incubated overnight at 42°C in a humid chamber for hybridization. Next day, cover slips were removed by washing in 2 x SSC twice for 10 minutes. For removal of non-specifically bound probes, slides were incubated in 0.4 x SSC at 42°C for 10 minutes. Sections were counterstained with Bismarck-Brown Y for sharpening a possible positive signal, which will be visible as violet-black foci in infected cells.

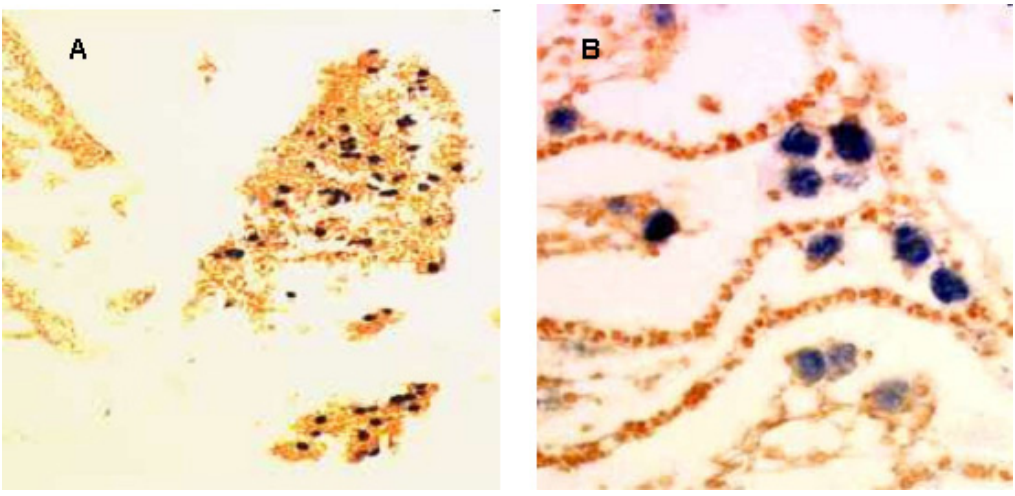
Results

Cell culture and viruses

While control viruses induced a CPE in CCO (CCV), KF-1 and CCB (KHV-I) at least 7 days after incubation at 20°C, tissue samples obtained from carp showed a "pseudo" CPE, visible as round, degenerated cells with or without cytolysis, on KF-1 and CCB in the first passage. The second passage was completely CPE negative and only in passages incubated with a pool of sample KD CPE occurred which was just visible after 14 days at 20°C. Virus isolation was only successful using this sample KD. All other samples obtained from an acute outbreak (sample KH), symptomless carp (sample OP) or from the survivors (samples FRSS) did not show any CPE after five passages in cell cultures.

PCR and nested PCR

Original ground tissue materials were first tested by PCR according to Gilad et al. (2002). Samples KH and KD from the acute outbreaks were considered to be PCR positive (Figure 2), sample OP always stayed negative in all PCR assays. These results were confirmed by nested PCR (KHV-1Fn-1Rn, KHV-2Fn-2Rn) and an additional PCR with KHV-JF-JR (this investigation) and NH1-2 primer pairs (Hutoran et al. 2004). The best results obtained from PCR reactions were reached when DNA from sample KD was used (Figure 3). PCR described by Gray et al. (2002) was always positive when the primer pair designed from the *Bam*HI-6 fragment (365 bp) was used. In



A: KHV-1Fn-1Rn probe **B:** NH 1-2 probe

Figure 4. *In situ* hybridization for confirmation of KHV in samples obtained from acute outbreaks.

some of the samples, the PCR with the primer pair designed from the sequence of the *Sph*I-5 fragment (290 bp) did not amplify any fragment.

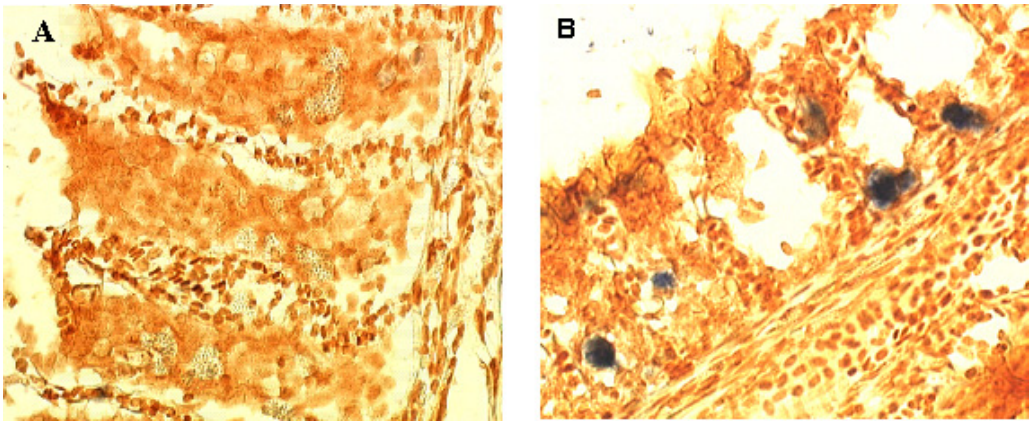
Control KHV-I virus was identified by PCR according to Gilad et al. (2002), Gray et al. (2002) and confirmed by PCR using different primer pairs in table 1 and by immuno-fluorescence assay (Bergmann et al., 2004). CCV was identified by PCR according to Gray et al. (1999). No cross-reaction was found with KHV primer pairs and CCV DNA or CCV primer pairs and KHV DNA.

In situ hybridization (ISH)

Sections from gill samples of KD and KH and from FRSS that were investigated by DIG-labelled probes with primers KHV-F-R, KHV-1Fn-1Rn and NH1-2 showing a positive violet-black signal, giving evidence to the existence of KHV DNA in the cells. In the gill from samples KH and primarily KD (Figure 4), massive KHV DNA bearing cells were found in the gill mucus (Figure 4A) but also in between the secondary lamellae (Figure 4B). Successive sections did not show any differences using the three probes but the picture was most intensive when probes KHV-1Fn-1Rn and NH1-2 were used compared to the KHV-F-R probe. Sections from gills of KHV negative carp hybridized with the three probes (Figure 5A), respectively, and sections from gills of KHV-positive carps hybridized with the irrelevant probe, did not show any labelled cells. In contrast to the samples from the acute outbreak, the detection of KHV DNA in gills of carp that survived the outbreak was much weaker. In our study, the KHV DNA bearing cells were located singly on the base of the secondary lamellae (figure 5A). No differences were found using the three KHV probes and no non-specific binding of the irrelevant probe was observed.

Discussion

KHV infection seems to be a much larger problem in Polish cyprinid culture than assumed. Mass mortality with and without



A: Gill of KHV free carp

B: Virus infected gill cells from carp

Figure 5. *In situ* hybridization using NH1-2 probe and sample carp 3 (FRSS) after Bismarck-Brown Y counterstaining.

clinical signs reminiscent of KHV in aqua-cultured common carp has occurred since at least 2001. No investigation has been done on fancy or koi carp, the ornamental type of the species *Cyprinus carpio*. Clinical signs observed in infected fish were very similar to those described by Hedrick et al. (2000) in USA or in Israel (Ronen et al., 2003) for koi, and also for the first KHV outbreaks in 2003 in Germany (Bräuer, pers. comm.).

In this study, we detected, isolated and confirmed KHV in Polish carp by different methods: cell cultivation, PCR, nested PCR and for the first time *in situ* hybridization. One of the largest problems in KHV diagnostics is to isolate the virus. Cell lines like KF-1 or CCB are useful and available but very often not susceptible enough. Out of 12 samples of single and pooled organs, gained from acute outbreaks, only one isolate was obtained from sample KD in KF-1 cells. The virus titre was still very low and it is hard to passage it. From all other samples no isolate was obtained.

In contrast, PCR according to Gilad et al. (2002), Gray et al. (2002) and Hutoran et al. (2004) and newly designed primer pairs (KHV-1Fn-1Rn and KHVJF-JR) recognized KHV DNA immediately in cell debris of tissues. In sample KD which seems to contain the largest amount of virus, all PCR assays worked perfectly. PCR products resulting from Gilad's and Gray's methods (290 bp fragment only) were weak when sample KH was investigated. Much better and clearer results were reached when primers KHV-JF-JR or NH1-2 were used. Changing annealing temperatures, $MgCl_2$ or template concentration, thermal cyclers (MJ Research, Eppendorf, Progene or Biometra) did not solve this sensitivity problem. The enzymes used for PCR proved to be an important factor influencing the effectiveness of that diagnostic method. The PCR was greatly improved when Platinum Pfx ® DNA Polymerase (Invitrogen) was used. This is helpful when organ samples are investigated where the virus content is extremely low as it was in FRSS specimens. Samples from completely asymptomatic carp were investigated by a routinely used PCR

according to Gilad et al. (2002). Only two gill samples appeared as KHV positive with a weak band in the gel. Primers KHV-1Fn-1Rn, used in nested PCR, increased the detection level for KHV enormously. All FRSS samples were considered to be KHV positive because all controls (tissue from negative carp, preparation water controls, PCR water control, nested PCR water control) stayed negative. In the case of the negative FRSS samples it was a question of specific KHV DNA concentration or a question of test sensitivity.

For confirmation of PCR results we applied on one hand the nested PCR or other primer pairs and on the other hand the ISH with different DIG-labelled probes. Organ samples considered to be PCR or nested PCR positive were all confirmed by ISH. There were no differences between the three probes used in the labelling of the KHV positive cells in the gill.

Based on this information and finding, it can be presumed that KHV appeared in Poland as early as 2001. The lack of relevant information and of diagnostic procedures to detect the virus was probably one of the causes of its rapid spread over various fish farms in all parts of Poland with cyprinid cultures.

Using these possibilities for detection and confirmation of KHV is a powerful diagnostic tool, which can help to find carrier fish or latently infected animals.

Our investigation also shows that the Polish carp industry is endangered. Of course further fundamental examinations are necessary for the exact determination of that danger.

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