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Potential role of different fish species as vectors of koi herpesvirus (CyHV-3) infection

Marek Matras, Magdalena Stachnik, Ewa Borzym, Joanna Maj-Paluch, Michał Reichert

Department of Fish Diseases,
National Veterinary Research Institute, 24-100 Puławy, Poland
marek.matras@piwet.pulawy.pl

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Abstract

Introduction: Koi herpesvirus (KHV) has infected farmed common carp in Poland clinically and asymptomatically since 2004. The role of non-carp species as vectors of virus transmission is well known except for in the case of KHV. The aim was to better understand this virus' infection and transmission pathways in common carp, looking at the potential vector role of fishes kept with them. **Material and Methods:** Eight species were experimentally infected with KHV by immersion in a suspension at 20°C ±1 and transferred to a tank after 45 minutes. Specimens were euthanised at intervals up to 56 days post infection (dpi) and tissue was examined for KHV DNA. Surviving infected fishes were introduced at intervals, each time into a separate tank, to naïve common carp for experimental infection. These were observed daily for symptoms, sacrificed along with controls after three months, and dissected to provide tissue samples. Also fish from 14 species collected from a farm with a history of KHV were sampled from 3 to 22 months after disease was confirmed. Organ sections from single fish were collected in a single tube. **Results:** Viral DNA was detected in tench and roach samples up to 49 dpi, but in three-spined stickleback and stone maroko samples only up to 14 dpi. Transmission of KHV to naïve carp occurred after cohabitation. KHV DNA was detected in three fish species three months after the farm outbreak. **Conclusion:** We confirmed that grass and Prussian carp, tench, roach, and brown bullhead can transfer the virus to naïve common carp.

Keywords: fish, common carp, koi herpesvirus, vectors.

Introduction

The problem of losses due to infection with koi herpesvirus (KHV) disease (KHVD) occurred in the 1990s. Since the connection of mass mortality of koi in the United States and Israel with the presence of a new virus of the *Alloherpesviridae* family, there have been an increasing number of reports of prevalence and pathogenicity of the new disease. The associated research performed led to a detailed description of a new pathogen and its occurrence. Koi herpesvirus quickly spreads in the species *Cyprinus carpio*, comprising common carp and its ornamental relative – the koi. Currently, cyprinid herpesvirus-3 (CyHV-3) is isolated in all areas where carp and koi carp are bred (12, 14). The Department of Fish Disease and Pathology Laboratory of the National Veterinary Research Institute diagnosed KHV infection in common carp in Poland for the first time in 2004 (1).

A factor having a huge impact on the rate and extent of spread of the infection has been unrestricted trade in koi, often over very large distances (Fig. 1). Besides the possibility of horizontal transmission of the virus directly from fish to fish, the ability for the virus to spread by vectors like water (it being the major abiotic vector) should also be taken into consideration. However, hypothetically, animate vectors, *e.g.* other fish species, parasitic invertebrates, and piscivorous birds and mammals can also be involved in transmission. The role of different fish species as vectors of virus transmission is well known in the cases of viral haemorrhagic septicaemia virus (VHSV) and infectious haematopoietic necrosis virus (IHNV) (4, 6, 10, 11) but in the case of KHV infection knowledge concerning the scope of vectors is still lacking. In addition, the presence of KHV DNA has been reported (although no infection has been demonstrated) in the following species: Atlantic

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sturgeon (*Acipenser oxyrinchus*), blue back ide (*Leuciscus idus*), common roach (*Rutilus rutilus*), Eurasian ruffe (*Gymnocephalus cernuus*), European perch (*Perca fluviatilis*), hybrid sterlet × beluga (*Acipenser ruthenus* × *Huso huso*), rainbow trout (*Oncorhynchus mykiss*), Russian sturgeon (*Acipenser gueldenstaedtii*), silver carp (*Hypophthalmichthys molitrix*), stone loach (*Barbatula barbatula*), tench (*Tinca tinca*), swan mussel (*Anodonta cygnea*), and scud (a crustacean) (*Gammarus pulex*) (12).

The investigations presented in this article were initiated for better understanding the pathways of CyHV-3 infection transmission in *Cyprinus carpio*. The study focused upon the role of other fish maintained with common carp in ponds as potential KHV vectors.

Fig. 1. Shipment of live koi

Material and Methods

Experimental study. Fish from eight different species (30 fish in each species) were experimentally infected with CyHV-3 (replicated in common carp brain cells – CCB) by immersion in 10 L aquaria. Investigations were carried out on specimens of European bitterling (*Rhodeus amarus*), brown bullhead (*Ameiurus nebulosus*), grass carp, Prussian carp (*Carassius gibelio*), roach, stone moroko (*Pseudorasbora parva*), tench, and three-spined stickleback (*Gasterosteus aculaeatus*). The virus suspensions with a titre of 4×10^3 in 2 ml were added to the tank. Control fish (European bitterling, brown bullhead, grass carp, Prussian carp, roach, stone moroko, tench, and three-spined stickleback) were exposed to CCB cell suspension. After a 45 min exposure, both groups were transferred to 350 L tanks with filtered and aerated water at 20°C. Every seven days, two fish per species were euthanised by immersion into a 0.5 g L tricaine solution (Sigma-Aldrich, USA) and samples of the gills, spleen, and kidney were collected. At the same time intervals, samples from control group fish were collected. In the second experimental trial, four times at seven day intervals, 10 infected fish from different species (grass carp, Prussian carp, roach, tench and brown bullhead) were transferred to a separate tank for each time interval with naïve common carp (30 fish per tank). Every day, carp in all four tanks were observed for evidence of any clinical symptoms of KHVD. After three months' observation, the carp were sacrificed. Sections of their gills, spleen, and kidney were collected into sterile tubes. At the same time, samples from control group fish were also collected.

Study on a traditional common carp farm with KHV history. During outbreaks of KHVD on a farm in eastern Poland, 70% cumulative mortality in common carp was reported. Different fish species from this farm were sampled. All collected fish showed no KHVD symptoms. These samples were collected at intervals of 3, 4, 10, 13, 16, and 22 months after confirmation of the KHV outbreak on the farm. Part of the samples were collected from channels which supply water to and drain it from the farm. Fish from 14 different species (bleak, brown bullhead, European perch, freshwater bream (*Abramis brama*), goldfish (*Carassius auratus*), grass carp, ide (*Leuciscus idus*), northern pike (*Esox lucius*), Prussian carp, roach, rudd (*Scardinius erythrophthalmus)*, tench, wels catfish (*Silurus glanis*), and white bream (*Blicca bjoerkna*)) were used for the study and provided a total of 205 samples. Sections of internal organs from single fish were collected in a single tube.

DNA extraction. The tissue samples were homogenised and total DNA was extracted using a QIAamp DNA Mini Kit (Qiagen, Germany) according to the manufacturer's instructions. The DNA was eluted in 100 µL of acetate ethylenediaminetetraacetic acid (AE) buffer and stored at −80°C before testing.

Molecular identification. All samples were initially tested by qPCR as described in Commission Implementing Decision (EU) 2015/1554 of 11 September 2015 (5). Virus DNA detection and quantification were performed using real-time qPCR (TaqMan) modified from the original protocol of Gilad *et al.* (2004) (8). Briefly, qPCR was performed as follows: the forward primer (KHV-86f) was 5′- GACGCC GGAGACCTTGTG -3′; the reverse primer (KHV-163r) was 5′- CGGGTTCTTATTTTGTCCTTGTT -3′; and the probe (KHV-109p) was 5′-FAM- CTT CCTCTGCTCGGCGAGCACG -3′. Cycling conditions were 1 cycle at 95°C for 15 min and 50 cycles at 94°C for 15 sec and 60°C for 60 sec.

Results

Fish from eight different species were experimentally infected with CyHV-3 by immersion and viral DNA was assayed in samples. It could be detected in two species, tench and roach, in samples taken up to 49 days post infection (dpi). We confirmed KHV DNA up to 42 dpi in grass carp and Prussian carp samples. However, viral DNA was found in samples from three-spined stickleback and stone maroko only

up to 14 dpi (Table 1). No clinical symptoms or mortality in fish experimentally infected with KHV were observed to the end of the experiment.

In the second experimental challenge, where fish from different species were transferred at seven dpi to naïve common carp, the first clinical symptoms of KHV occurred after 28 days of cohabitation. Moribund fish presented enophthalmia, over-production of mucus on the skin, and necrosis of the gills (Fig. 1). Carp started dying on the 30th day after transfer of fish from different species and their cumulative mortality reached 90%. In samples from moribund carp the presence of KHV nucleic acids was detected. During three months' observation of carp from the remaining three groups (where fish from different species were transferred after 14, 21, and 28 dpi), neither clinical symptoms nor mortality were observed. In samples from euthanised fish from the three groups mentioned above, the presence of virus DNA was confirmed (Table 2). In both experimental trials, no clinical symptoms or mortality in fish from different species experimentally infected with KHV were observed and in samples originating from control fish, the presence of KHV was not detected.

In samples from different fish species from a traditional common carp farm with a history of KHV, KHV DNA was detected in common carp three months after confirmation of a KHV outbreak. Viral nucleic acids were detected in samples from four fish (two Prussian carp, one tench, and one brown bullhead). All samples collected at the 4, 10, 13, 16, and 22 month time points were negative (Table 3), and in samples collected from channels which supply water to and drain it from the farm no presence of KHV DNA was detected.

Fig. 2. Clinical symptoms of KHVD in common carp

Table 1. Results of experimental infection of fish from eight different species with CyHV-3

Dpi	Species							
	Three-spined stickleback	Stone moroko	European bitterling	Grass carp	Prussian carp	Tench	Roach	Brown bullhead
7	+	+	+	+	+	+	+	+
14	+	+	+	+	+	+	+	+
21	–	+	–	+	–	+	–	–
28	–	–	–	–	+	+	–	+
35	–	–	–	+	–	–	+	–
42	–	–	–	+	+	+	+	–
49	–	–	–	–	–	+	+	–
56	–	–	–	–	–	–	–	–

+ positive test results, – negative test results

Table 2. Species of fish experimentally infected with KHV and results of virus transfer trial to naïve carp

Species of fish experimentally infected with KHV *via* water:	Grass carp, Prussian carp, tench, roach, brown bullhead			
	Time of transfer of different fish species (two fish per species) to naïve common carp (dpi)			
	7	14	21	28
Mortality in common carp (%)	90	0	0	0
qPCR results	+	+	+	+

Symbols as in Table 1

Table 3. Results of examination of different fish species taken from farm with KHV history

Sampled species:	Bleak, brown bullhead, European perch, freshwater bream, goldfish, grass carp, ide, northern pike, Prussian carp, roach, rudd, tench, wels catfish, white bream	
Time of sampling after KHV outbreak (months)	Number of positive samples	Number of negative samples
3	4 (tench, brown bullhead, Prussian carp)	27
4	0	25
10	0	48
13	0	45
16	0	56
22	0	28

Discussion

The results of our experimental and field investigations showed the presence of KHV DNA in species of fish which had contact with infected common carp. According to published data (7), we have confirmed the presence of KHV DNA in 10 different fish species. Bergmann *et al.* (3) detected KHV by nested PCR in several different varieties of goldfish as well as grass carp, ide, and ornamental catfish (*Ancistrus* sp.). KHV was detected by PCR in Russian sturgeon and Atlantic sturgeon from fish farms in northern Poland (9). We also confirmed the presence of virus nucleic acids in other fish species reared in polyculture with carp but we also did so in fish that can migrate in and out of a farm. Interestingly, Bergmann *et al*. (2) reported the replication of KHV in goldfish after experimental infection by immersion, but clearly visible clinical symptoms of KHVD did not occur. In our experimental infection and field findings, we also did not observe any clinical symptoms or mortality caused by CyHV-3 in fish from different species than *Cyprinus carpio*. We have evidence that other fish species are potential vectors of KHV from literature and our data. In eight different fish species experimentally infected with KHV, we observed that after a short contact with the virus, viral DNA could subsequently be found until 49 dpi in two sampled fishes (tench and roach). However, this period of viral DNA presence was different for each species: for example in samples from three-spined stickleback and stone moroko we had positive results only until 14 dpi. This investigation concerning transfer of the pathogen from vectors to naïve common carp showed that after a long period (four weeks), other fish species can play a role in virus transmission. In the study by Bergmann *et al*. (2), transfer of infectious virus from goldfish to naïve koi was possible after 60 dpi.

Concluding, *in vitro* and *in vivo* studies confirmed that in samples from European bitterling, brown bullhead, grass carp, Prussian carp, roach, stone moroko, tench, and three-spined stickleback which had had short contact time with CyHV-3, viral DNA was detected for a long period of time (from two up to seven weeks). Even after a short contact time of with KHV, other fish can transfer the virus to a naïve population for four weeks. The potential virus transmission periods are different and depend on the fish species.

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