Horizontal transmission of koi herpes virus (KHV) from potential vector species to common carp

J. Kempter[1], M. Kiełpiński[1], R. Panicz[1]*, J. Sadowski[1], B. Mysłowski[1] and S. M. Bergmann[2]

[1] Department of Aquaculture, Faculty of Food Sciences and Fisheries, West Pomeranian University of Technology in Szczecin, Kazimierza Królewicza 4, 71-550 Szczecin; [2] Friedrich-Loeffler-Institut (FLI), Federal Research Institute for Animal Health, German Reference Laboratory for KHVD, Südufer10, 17493 Greifswald- Insel Riems, Germany

Abstract

Six fish species, defined as potential vectors in koi herpes virus (KHV) transmission, namely: common roach, European perch, tench, Eurasian ruffe, silver carp and grass carp were included in this study. The fish used to transmit infection originated from a fish culture facility where KHV had been diagnosed and prior to the beginning of the research study the presence of the virus genome was confirmed in each individual fish intended for cohabitation. Specific pathogen free (SPF) carp utilized in the experiment originated from the University of Wageningen. During a four-week period the SPF carp were exposed to infection through cohabitation with vector species previously confirmed as KHV carriers. The obtained results demonstrated the possibility of a horizontal transmission of KHV between selected species, even in the case of species showing no clinical signs of KHV disease (KHVD), while an average water temperature in the tanks ranged from 12°C to 16°C.

Introduction

Koi herpes virus (KHV) disease (KHVD) is a serious viral disease causing mass mortality in the species *Cyprinus carpio*. According to previous research, clinical signs can be observed not only in common carp (*C. carpio*), but also in hybrids: *C. carpio* x *Carassius carassius* and *C. carpio* x *Carassius auratus*. Immersion aquaria challenge experiments, showed severe losses after a waterborne KHV infection of between 35% and 100% in koi x goldfish and koi x crucian carp hybrids as well as in (specific-pathogen-free) SPF common carp (Bergmann et al., 2010). The possibilities of the virus being transferred by fish species other than carp were analysed as part of an experimental study performed by Kempter et al. (2008). According to the results, during experimental infection by immersion, species such as tench (*Tinca tinca*), vimba (*Vimba vimba*), common bream (*Abramis brama*) and grass carp (*Ctenopharyngodon idella*) can transmit KHV to SPF carp when water temperatures range between 18 and 27°C. The four potential

* Corresponding author's email: rpanicz@zut.edu.pl

carrier species were confirmed as being asymptomatic carriers of KHV by PCR analysis of gill swabs prior to the start of the aquaria cohabitation experiment. Virus presence was confirmed in SPF carp in samples of gill or pooled visceral material (Kempter et al., 2008). Samples were analysed according to methods summarised in Table 1. In 2010, carp production in Poland amounted to 15,400 tonnes per annum, and the estimated number of carp farms reported as 500 (Lirski, 2011). Therefore, it would seem advisable to analyze the risk connected with the introduction of accompanying fish species into carp ponds, as they may constitute a reservoir of KHV infection without displaying the clinical signs. The intention of this research study was to discover whether other fish species apart from carp might act as a vector introducing KHV into cyprinid fish farms.

Materials and methods

Experimental infection

Fish adjudged to have the potential to play a role as a vector species for transmitting KHV as well as being species that are important for angling and fisheries in Poland were obtained from farms located in the catchment area of the Oder River in south-western Poland (N50°45'14.364", E18°36'30.1068"). Koi herpes virus (KHV) was initially confirmed in this catchment from cultured common carp in 2004 (Bergmann et al., 2006) and its presence was subsequently confirmed during two breeding seasons, in the spring and summer of 2006 and 2007, prior to the collection of fish for the purpose of this experiment in 2008. The following potential vector fish species were collected for inclusion in experimental trials: common roach (*Rutilus rutilus*), European perch (*Perca fluviatilis*), tench, Eurasian ruffe (*Gymnocephalus cernuus*), silver carp (*Hypophthalmichthys molitrix*) and grass carp. The collected fish were transported to the Fisheries Research Station (RSD) in Nowe Czarnowo (March, 2008) in sealed bags with an appropriate amount of oxygenated pond water. On arrival at RSD, they were placed in 1m^3 tanks, filled with spring water at 12-16°C. During the four-week cohabitation period the tank water was neither replaced nor complemented with fresh water and water filtration was not conducted. The potential vector species intended for experimental infection were placed in duplicate tanks (n = 20 fish per tank) and cohabited with specific-pathogen-free (SPF)

Table 1. Primers utilized in the procedure confirming the presence of the KHV virus in the analysed samples.

Primer	Sequence (5'-3')	Product size	References
KHV-F	GACGACGCCGGAGACCTTGTG	486 bp	Gilad et al. (2002)
KHV-R	CACAAGTTCAGTCTGTTCCTCAAC		
KHV-1Fn	CTCGCCGAGCAGAGGAAGCGC	414 bp	Bergmann et al. (2006)
KHV-1Rn	TCATGCTCTCCGAGGCCAGCGG		
KHV-TK-F	GGGTTACCTGTACGAG	409 bp	Bercovier et al. (2005)
KHV-TK-R	CACCCAGTAGATTATGC		
KHV-TK-Fn	CGTCGTGAGGAATACGACG	348 bp	Unpublished Way (2007)
KHV-TK-Rn	ACCGTACAGCTCGTACTGG		

common carp (n = 20 per tank) with a code line $R_8f_{10}xR_3f_{10}$ obtained from the University of Wageningen (The Netherlands). The SPF carp arrived with a certificate declaring that they were free of KHV DNA. Potential vector fish species and SPF carp were of a comparable size, approximately 1.5g. Prior to placing fish into the experimental tanks, 20 fish from each group were screened for KHV. DNA was extracted using DNAzol (Invitrogen) reagent from liver, intestine and pancreas and a standard diagnostic polymerase chain reaction (PCR) assay according to published primers was applied (Bercovier et al., 2005). As expected, the SPF carp screened KHV negative, however, the presence of KHV DNA was confirmed from tissue samples collected from all potential vector species. A negative control group (n = 150) of SPF common carp, code line R_8f_{10} x R_3f_{10}, were maintained in spring water at 12-16 ºC. All tanks were physically separated from each other in order to prevent cross contamination between experimental tanks.

KHV detection

After day 7, 14, 21 and 28 of the experiment, 2 SPF carp were randomly selected from each tank and analysed by PCR in order to detect KHV DNA. Gill samples were aseptically collected from each SPF carp (sample 1), as well as a fragment of the intestine, liver and pancreas as an organ tissue pool (sample 2).

The collected tissues were homogenised with scissors and DNA was isolated according to DNAzol (Invitrogen) reagent following manufacturer's protocol. The confirmation of the presence or absence of the virus involved a standard procedure of PCR and nested PCR using four pairs of KHV specific primers. A second round of PCR was applied in case of low copy numbers of virus genome. The PCR products of KHV-F/KHV-R primers were used as a template for a second nested PCR with KHV-1Fn/KHV-1Rn primers (Gilad et al., 2002; Bergmann et al., 2006). A second set of primers comprised of KHV-TK-F/KHV-TK-R primers used in first round and KHV-TK-Fn/KHV-TK-Rn applied in the nested PCR (Bercovier et al., 2005; Way, 2007 [unpublished]). The PCR assays were performed according to previously published methods and the primers and methods used in this study are detailed in Table 1.

In order to verify the presence and the correct size of the PCR products, electrophoresis on a 1.5% argarose gel (Prona), utilizing PowerPack™ Basic (BioRad) was performed. The amplicons were visualized with the Gel Doc™XR (BioRad) and sized according to a GeneRuler™ 100 bp Plus DNA ladder (Fermentas).

Results

No mortality or clinical signs of KHV disease (KHVD) was observed in any of the fish species constituting the reservoir of KHV nor SPF carp. The water temperatures recorded during the experimental trials ranged from 12-16°C, with an average water temperature of 14°C during the 4 week cohabitation period. Detailed results are summarised in Table 2. In the SPF carp used as a negative control group, no KHVD clinical signs were observed and KHV DNA was not detected at any point during the experimental trial. KHV DNA was detected from all carp sampled at 28 days post (dp) cohabitation without the fish demonstrating any clinical signs consistent with KHVD. The experiment confirmed that KHV might be horizontally transmitted directly from fish to fish or via water even at comparatively

Table 2. KHV DNA detection using nested primers, Bergmann et al. (2006) and Unpublished Way (2007), after cohabitation on 7th, 14th , 21rst and 28th dp cohab.

Variants	Day 7		Day 14		Day 21		Day 28	
Detection by means of KHV followed by KHVn primers								
	Gills	Pool	Gills	Pool	Gills	Pool	Gills	Pool
SPF carp + Cr	3/4	nr	4/4	nr	4/4	4/4	nr	nr
SPF carp + Ep	4/4	nr	4/4	nr	4/4	4/4	nr	nr
SPF carp + T	2/4	nr	nr	nr	3/4	nr	1/4	nr
SPF carp + Er	nr	nr	4/4	nr	2/4	nr	nr	nr
SPF carp + Sc	2/4	nr	nr	nr	4/4	4/4	1/4	nr
SPF carp + Gc	nr	nr	nr	nr	4/4	4/4	nr	4/4
SPF carp (k-)	nr	nr	nr	nr	nr	nr	nr	nr
Detection by means of KHV-TK followed by KHV-TKn primers								
	Gills	Pool	Gills	Pool	Gills	Pool	Gills	Pool
SPF carp + Cr	nr	1/4	nr	4/4	nr	nr	4/4	4/4
SPF carp + Ep	nr	4/4	nr	4/4	nr	nr	4/4	4/4
SPF carp + T	nr	1/4	nr	nr	nr	nr	1/4	4/4
SPF carp + Er	nr	nr	nr	nr	nr	2/4	1/4	4/4
SPF carp + Sc	nr	nr	nr	nr	nr	nr	1/4	4/4
SPF carp + Gc	nr	nr	nr	nr	nr	nr	1/4	4/4
SPF carp (k-)	nr	nr	nr	nr	nr	nr	nr	nr

Cr - Common roach, Ep - European perch, T – Tench, Er - Eurasian ruffe, Sc - Silver carp, Gc - Grass carp, (k-) – negative control, figures present +ve/number sampled, nr = negative results.

low temperatures. All the fish species included in the study were able to transmit KHV DNA to naïve carp, in our case Dutch SPF carp.

Discussion

In order to determine the epidemiological risk connected with KHV, it is important to specify unequivocally whether certain fish species identified as possible vectors, which do not display KHVD clinical signs, may infect cyprinid populations. These vector species are often not representatives of the family Cyprinidae. This information is relevant both for fish used for relay or re-stocking in natural waters and for fish in polyculture populations in carp farms. Research conducted in order to create an epidemiological map of the Oder catchment area raises the question as to whether certain species which are not considered to be typical KHV carriers, but whose carrier status have been described, such as pike (*Esox lucius*), bullhead (*Cottus gobio*) or tench (*T. tinca*), may take part in spreading the virus as a vector (Kempter et al., 2008). The authors of this report previously

listed additional fish species common in Polish waters (Table 3) which had been confirmed as KHV carriers due to KHV DNA detection by PCR. All fish used in this study were collected from fish farms, streams or brooks connected with the Odra River. From each fish, a sample of tissue homogenate (liver, spleen, skin, kidney and gills) was used to extract DNA (DNAzol Reagent). Four PCR runs, according to references listed in Table 1, were applied to detect KHV DNA. It is important to mention that apart from carp, this list included tench, perch and pike, i.e. species of significant importance from the point of view of fishery management. Taking into account the ratio of fish confirmed as virus carriers for all species sampled, the largest proportion of carriers were Prussian carp (*Carassius gibelio*) (52.3%), common bream (40%), tench (34%) and pike (20%). It was also important to recognize the species described in this study as non-carriers, i.e. common rudd (*Scardinius erythrophthalmus*), stone moroko (*Pseudorasbora parva*), common carp x crucian carp hybrid (*C. carpio x C. carassius*), common bleak (*Alburnus alburnus*), silver bream (*Blicca bjoerkna*), grass carp (*C. idella*), three-spined stickleblack (*Gaster-*

Table 3. List of fish species confirmed to be a KHV carrier (Kempter et al., 2008).

Common name	Scientific name	Collected individuals	Confirmed by PCR
Common carp	*Cyprinus carpio*	81	22
Prussian carp	*Carassius gibelio*	21	11
Tench	*Tinca tinca*	23	8
European perch	*Perca fluviatilis*	70	6
Commonroach	*Rutilus rutilus*	93	5
Northern pike	*Esox lucius*	25	5
Common dace	*Leuciscus leuciscus*	20	4
Gudgeon	*Gobio gobio*	31	4
Common bream	*Abramis brama*	10	4
Ide	*Leuciscus idus*	10	2
Crucian carp	*Carassius carassius*	23	2
European chub	*Leuciscu scephalus*	7	2
Common barbel	*Barbus barbus*	1	1
Vimba bream	*Vimba vimba*	1	1
European bullhead	*Cottus gobio*	1	1
Koi carp	*Cyprinus carpio koi*	4	1
Spined loach	*Cobitis taenia*	5	1
Belica	*Leucaspius delineatus*	3	1
Common nase	*Chondrostoma nasus*	1	1

osteus aculeatus), crucian carp (*C. carassius*) and zander (*Sander lucioperca*). What is especially surprising is the presence in this group of fish species which belong to the Cyprinidae, such as common rudd, common bleak and silver bream. In those cases, a lack of PCR assay sensitivity might also play an important role, knowing that in the latent phase of the KHV infection the viral load is incredibly low (1–5 copies ml^{-1}) (Bergmann and Kempter, 2011). To determine whether such populations are KHV carriers, the fish should be stress induced one or two days before sample collection.

Initially, in 2003, it was believed that the optimum temperature for KHV replication and shedding ranged between 22-26°C (Perelberg et al., 2003). However, KHV induced mass mortality in common carp can also take place when the water temperature is as low as 8°C (Bergmann, pers. comm.). In the present experiment, the water temperature was much lower than in the experiments mentioned above, i.e. 14°C on average, and this resulted in a lack of mortality in SPF carp. However, the authors hypothesize that after approximately 6 years of KHV presence in Poland, the virus has systematically adapted itself to the Polish system of carp breeding (i.e. 3-year cycle at lower water temperatures compared to warm water conditions, e.g. in Israel or Asia). This situation may be due to modifications at the genome level which have taken place in Polish or European KHV variants modified to lower temperatures. Such a situation was described for betanodaviruses that pose a threat for a broad spectrum of fish species (Panzarin et al., 2012), therefore additional studies should be applied for KHV to verify the adaptation hypothesis.

The study of pathogen transfer within the same fish species or among various fish species can provide interesting and useful information about the viral transmission mode. Other species within the Cyprinidae family, such as goldfish (*C. auratus*), have been previously studied in experimental cohabitation trials (El Matbouli et al., 2007; Sadler et al., 2008). According to these authors, field cases indicated that the virus may persist in goldfish for long periods following an outbreak and that they may act as a carrier population. Duration of KHV carriage in goldfish is unknown therefore caution should be taken when newly bought fish are introduced to a fish farm with koi or common carp. In the present study, the experimental cohabitation of SPF carp with 6 fish species (confirmed as KHV carriers prior to the experimental start up) representing two families, Cyprinidae and Percidae, showed that fish to fish horizontal transfer of KHV DNA can occur between selected species and SPF carp.

Another problem is the possibility that wild fish may come into contact with KHV infected farmed fish. In order to increase their profit, individual fish farms breed several fish species simultaneously (polycultures). The possibility exists when KHV vector species, like tench or pike, are sold to another farm or used in restocking programs the virus will be transferred horizontally and spread beyond the farm either to wild fish or neighbouring sites. A reverse situation may take place when infected individuals from the natural environment are introduced into fish farms as spawners. It should be mentioned that no vertical transfer of KHV has been observed to date, however, the presence of the virus particles in gonads has been confirmed (Gomez et al., 2011). The presence

of pathogenic KHV, both when KHVD clinical signs are visible and during the period of latency, constitutes a serious problem for species which are either the subject of aquaculture or inhabit open waters. What is more, as shown by the results of experimental cohabitation, water temperature of ca. 14°C does not restrict the horizontal transmission of the virus. The dynamics of adaptive changes in the survival strategy of KHV shows the necessity of conducting further studies on cohabitation. This would specify not only the paths of pathogen transmission but also changes in its genomic character and carrier status.

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