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J Vet Res 68, 73-78, 2024
DOI:10.2478/jvetres-2024-0008

Occurrence of herpesvirus in fish

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Received: August 10, 2023 *Accepted: February 5, 2024*

Abstract

Introduction: Herpesviruses are common agents in animals of the aquatic environment. They infect many species of fish but only lead to disease in one or two species. Nevertheless, infected fish without clinical symptoms can actively transfer infectious agents to disease-susceptible species. The aim of the study was to identify and prove the natural presence of different herpesviruses. **Material and Methods:** Koi, Nile tilapia, grass carp, goldfish and crucian carp were infected with a herpesvirus isolate 99% identical to goldfish herpesvirus (GHV) or cyprinid herpesvirus 2 (CyHV-2) obtained from crucian carp. Before and after infection, samples were collected non-lethally at different time points from all five fish species to identify and evaluate the replication of viruses naturally infecting the fish as well as the CyHV-2 experimentally infecting them. Gill swabs and separated leukocytes were subjected to PCR and the results compared. **Results:** These samples yielded DNA of koi herpesvirus (KHV, also referred to as CyHV-3), GHV and a new herpesvirus. While Asian-lineage CyHV-3 DNA was detected in samples from crucian carp and goldfish, CyHV-2 DNA was found in samples from koi and tilapia. A new, hitherto unknown herpesvirus was identified in samples from grass carp, and was confirmed by nested PCR and sequence analysis. The survival rates were 5% for grass carp, 30% for tilapia, 55% for crucian carp, 70% for koi and 100% for goldfish at 20 days post infection. Evolutionary analyses were conducted and five clusters were visible: CyHV-1 (carp pox virus), CyHV-2 with sequences from koi and tilapia, CyHV-3 with sequences from crucian carp and goldfish, probable CyHV-4 from sichel and a newly discovered herpesvirus – CyHV-5 – from grass carp. **Conclusion:** The results obtained with the molecular tools as well as from the animal experiment demonstrated the pluripotency of aquatic herpesviruses to infect different fish species with and without visible clinical signs or mortality.

Keywords: herpesviruses, fish, clinical signs.

Introduction

Herpesviruses of the order Herpesvirales are disease-causing or infectious agents that are common in vertebrates and bivalves (18). After infection, they may induce clinical signs, even episodically, or remain unnoticeable in the characteristic latent or persistent phase of the infection. One of the newly established families is named *Alloherpesviridae* and includes herpesviruses found in fish and amphibians. Within this family, one genus is designated as *Cyprinivirus*. Cyprinid herpesvirus 1 (CyHV-1, carp pox virus), CyHV-2 (goldfish herpesvirus – GHV), CyHV-3 (koi herpesvirus – KHV) and anguillid herpesvirus 1 (AngHV-1, eel herpesvirus) are classified in this group of species (26). In contrast to many mammalian, bird and reptile

herpesviruses, these viruses are not species-specific in terms of infection but sometimes are in terms of which species develop disease in the aquatic environment (2). Specific herpesviruses inducing a disease have so far been identified in samples from carp or koi (*Cyprinus carpio*) (13), tilapia (*Oreochromis niloticus*) (24) and goldfish (*Carassius auratus*) (15), but not in samples from grass carp (*Ctenopharyngodon idella*). Cyprinid herpesvirus-3 DNA was also detected in samples from healthy goldfish and healthy tench (*Tinca tinca*) (10), grass carp and crucian carp (*Carassius carassius*) (21), Prussian carp (*Carassius gibelio*) and brown bullhead (*Ameiurus nebulosus*) (20) besides in samples from common or koi carp. In experiments with rainbow trout (*Oncorhynchus mykiss*) at two different temperatures, CyHV-3 was transferred from infected salmonids to common carp, inducing mortality in the latter species only (2). Similarly to CyHV-3 in having a broad host range, but causing death in more species than goldfish, CyHV-2 was highly lethal when it infected crucian carp and Prussian carp (14). Anguillid herpesvirus 1, the fourth member of the *Cyprinivirus* genus, induced mortality in common carp in Taiwan (25). It seems to be very likely that most of the alloherpesviruses are not host specific by infection but are by disease induction, only doing so in a single species. Herpesviruses present a latent threat not only to aquacultured but also to wild fish. Identifying different new and well-known herpesviruses in different species can help to avoid acute or latent losses. It also seems to be important to know the responses of different fish species to double or even triple infection with species-specific or other herpesviruses.

Material and Methods

Fish used for the experiment and sample collections. Crucian carp (n = 20, 60–80 g), koi (n = 20, 30–50 g), Nile tilapia (n = 20, 20–60 g) and grass carp (n = 20, 10–20 g) were obtained from local ponds and kept for seven days in aquaria without water exchange for acclimatisation. Goldfish (n = 12, 20–30 g) were included from a separated pond of the Pearl-River Fisheries Research Institute after sudden death had occurred twice within 48 h of the arrival of goldfish from a local ornamental fish market. All fish were fed twice a day with commercial food and were kept constantly at 25°C without biosecurity measures to mimic conditions in the field in which a clinically healthy farm with unknown infections would keep apparently healthy fish. Two fish from each species were chosen randomly for primary sample collection without any artificial stress induction. Blood samples were taken from the outside of the body as gill swabs with some blood and from the inside of the body by vene puncture. Leukocytes were separated from the venepuncture samples (5, 6). These were adjusted to not more than 10^7 peripheral blood leukocytes (PBL) per mL before treatment with sample buffers. Infection with non-titrated GHV was performed by intraperitoneal (i.p.) injection (0.2 mL) with virus which had been passaged in *Cyprinus carpio* brain cells (CCB cells) three times. Fish samples were collected before infection with GHV (0 days post infection (dpi)), at 7 dpi and 20 dpi, which was the end of the experiment. The last samples were collected 36 h after simulated transportation of the fish by netting in air for 30 s. Fish were monitored for mortality events twice daily. Dead fish were removed immediately, and kidneys were pooled from each species and tested by different PCRs.

Tools for detection of herpesviruses. For detection of the DNA polymerase genes of CyHV-1 (carp pox virus), CyHV-2 and CyHV-3, a PCR and nested PCR for cypriniviruses according to Engelsma *et al.* (8) were used. The resulting amplicons were sequenced for discrimination. Goldfish herpesvirus DNA was also detected by a conventional PCR according to a qPCR protocol published by Goodwin *et al.* (11) amplifying the viral helicase gene, which was performed in order to test its application for CyHV-2 detection. To verify the presence of KHV DNA, a PCR according to Bercovier *et al.* (1) was used followed by nested PCR by Pokorova *et al.* (23). Additionally, a newly established conventional PCR was tested successfully with primers designed for the GHV polymerase gene, with the forward primer being GHV-DT-F: 5′CGC GGA ACA CGG TGA TG 3′ and the reverse primer GHV-DT-R: 5′CCT CGT TCT TCA TGC GCT TCT 3′. The thermocycler programme was 95 °C for 5 min; 35 cycles at 95°C for 30 s, 58°C for 30 s and 72°C for 20 s; and a final a step at 72°C for 5 min. DNA was extracted from the samples by DNA Mini kit and RNA by RNeasy kit (both Qiagen, Hilden, Germany) according to the manufacturer's protocols, including digestion of the DNA twice for the latter kit. For reverse transcription in PrimeScript RT Master Mix (Perfect Real Time, TaKaRa Bio, Kusatsu, Japan), oligo dT primers were used. The conventional PCRs (8) were carried out with 5 µL of DNA and the nested PCR (23) with 2 µL of complimentary DNA (cDNA). A sequence analysis was performed on the products of all positive reactions.

Results

Fish samples obtained by non-lethal collection were tested on the first day of the experiment prior to the infection and showed a range of herpesviruses (Table 1).

The detected KHV DNA from goldfish and crucian carp samples was subsequently identified according to Klafack *et al.* (17) as an Asian lineage virus. While the detected sequences of viral DNA from KHV were almost identical to the Asian (Chinese) isolate GZ-11 (accession No. MG925488), the GHV sequences were almost identical to the Japanese isolate ST-J1 (accession No. JQ815364). The sequences of grass carp sample–derived strains were not identical to any cyprinivirus sequence in the NCBI database. At 7 dpi, after the i.p. infection of all fish with 0.2 mL GHV which had been isolated from crucian carp and passaged three times on

CCB cells, the results obtained from molecular diagnostics changed (Table 2). For non-lethal sample collection, four fish from each species were selected randomly.

While only one sample (collected from crucian carp) of all samples from live fish of all species at the time points before stress induction tested positive for the GHV injected i.p., a number of positive results for KHV DNA were registered. The additional analysis of viral RNA at 7 dpi also confirmed the predominance of KHV DNA in these samples. There were hardly any sequence differences to both GHV obtained at 0 and 7 dpi. In grass carp samples, no grass carp-specific herpesviral DNA was present in a sufficient concentration for a positive signal to be obtained by PCR. At 4 dpi pseudo faeces were visible in the aquarium with koi, crucian carp and goldfish showing that an infection was developing in these species. During the experiment, mortality was observed in all fish groups except the goldfish. Mortality started at 6 dpi in the aquarium with crucian carp and reached its maximum of 45% at 12 dpi. The first dead fish were observed at 10 dpi in the aquaria with koi and tilapia, and at 16 dpi in the aquarium with grass carp, where almost all fish died within three days (Fig. 1). The group with the second highest mortality was tilapia, while no mortality over the entire experimental period was registered for goldfish.

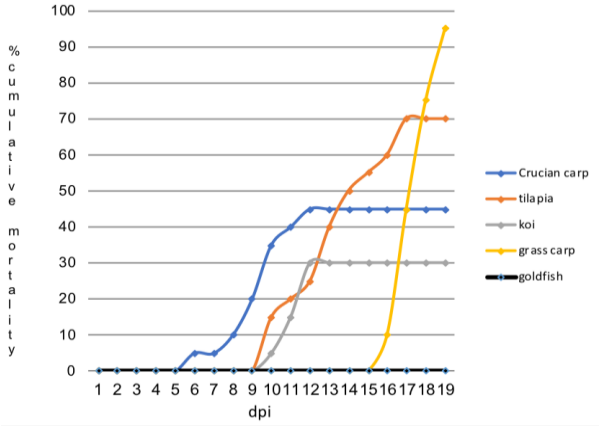

Fig. 1. Cumulative mortality in five species of fish after intraperitoneal infection with goldfish herpesvirus
dpi – days post infection

Table 1. Herpesviruses detected at 0 days post infection (prior to the cyprinid herpesvirus 2 infection)

Fish species	Sample	PCRs*	Nested PCRs**	Sequence analysis (300 base pairs)	Remarks
Goldfish	Gill swabs and leukocytes	All negative	+/+	99% koi herpesvirus	Herpesvirus-positive
Crucian carp			+/+	99% koi herpesvirus	
Koi			+/–	99% goldfish herpesvirus	
Tilapia			+/–	98% goldfish herpesvirus	
Grass carp	Gill swabs only***		+/–	82% carp pox virus 78% koi herpesvirus 78% goldfish herpesvirus 79% sichel herpesvirus	

* – newly established PCR for goldfish herpesvirus according to Bercovier *et al.* (1) and Engelsma *et al.* (8); ** – nested PCR according to Pokorova *et al.* (23); *** – these fish were too small for a 0.5 mL blood sample to be collected for leukocyte separation

Table 2. Herpesvirus detection by different PCRs at 7 days post infection after intraperitoneal injection with goldfish herpesvirus (GHV) isolated from crucian carp

Fish species (n=4 for each)	New PCR for GHV	Bercovier *et al.* nested PCR (1)	Engelsma *et al.* nested PCR (8)	Assessments
Goldfish	0/4	2/4	2/4	KHV
Crucian carp	1/4	4/4	4/4	GHV / KHV
Koi	0/4	3/4	1/4	KHV
Tilapia	0/4	4/4	1/4	KHV
Grass carp	0/4	3/4	3/4	KHV

KHV – koi herpesvirus

Table 3. Investigation of pooled samples from fish that died during the experiment

Fish species	Detected at 0 dpi	Detected in pooled samples	Overall assessments
Goldfish	KHV	GHV and KHV	Double infection
Crucian carp	KHV	GHV and KHV	Double infection
Koi	GHV	negative*	Double infection despite negative result
Tilapia	GHV	GHV	Double infection
Grass carp	new HV	GHV	Triple infection

KHV – koi herpesvirus; GHV – goldfish herpesvirus; HV – herpesvirus; * – below the detection limit after testing three pooled samples from two fish

Table 4. Detection of herpesviruses at 20 days post infection after intraperitoneal injection with goldfish herpesvirus (GHV) isolated from crucian carp

Fish species	New GHV PCR	Bercovier *et al.* nested PCR (1)	Engelsma *et al.* nested PCR (8)	Assessments
Goldfish	0/6	1/6	0/6	KHV
Crucian carp	5/6	1/6	5/6	GHV / KHV
Koi	0/7	0/7	0/7	negative*
Tilapia	0/6	1/6	1/6	KHV
Grass carp	0/2	0/2	2/2	new HV

* – below the detection limit

From the pooled kidney samples of each species that died during the experiment, only DNA was extracted and tested by different PCRs. Surprisingly, not all samples collected from dead fish contained herpesviral DNA (Table 3). Summarising the results from the three sampling time points with the samples from the deceased fish, in goldfish and crucian carp, KHV and GHV were detected; in koi and tilapia GHV and KHV; and in samples from grass carp KHV, GHV and a new herpesvirus were detected.

At 20 dpi, after the mortality events had stopped in all groups, samples were collected from surviving fish after their subjection to stress by netting. All fish were euthanised by an overdose of 2% benzocaine followed by decapitation. Pools containing kidney tissue of two fish were prepared from goldfish, koi and crucian carp, but one crucian carp sample was tested individually. Samples obtained from tilapia were tested individually. Two samples were prepared from the surviving grass carp (a gill swab and separated leukocytes) (Table 4).

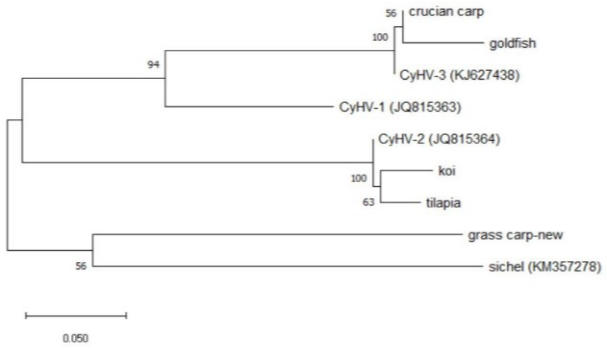

Fig. 2. Evolutionary analysis by maximum-likelihood method comparing sequences from the NCBI database with the sequences obtained in this study

While the results of the sequence analysis obtained from DNA and RNA samples for the single viruses were very similar, a differentiation of the sequences between KHV, GHV and the new herpesvirus based on the viral DNA polymerase was clearly visible (Fig. 2). The latter was also distinct from sichel (*Pelecus cultratus*) herpesvirus detected in Hungary (7). The sequences were compared to those from CyHV-1, CyHV-2, CyHV-3 and CyHV-4 obtained from the NCBI database (Fig. 2). This was achieved by using the maximum likelihood method and Hasegawa–Kishino–Yano model (12). The tree with the highest log likelihood (-1,373.33) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial trees for the heuristic search were obtained automatically by applying neighbour-joining and BIONJ algorithms to a matrix of pairwise distances estimated using the maximum composite likelihood approach, and then selecting the topology with superior log-likelihood value. The rate variation model allowed for some sites (42.03%) to be evolutionarily invariable (+I). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved nine nucleotide sequences and included the 1st+2nd+3rd+noncoding codon positions.

There were a total of 320 positions in the final dataset. Evolutionary analyses were conducted in MEGA X (19). Five clusters are clearly visible: CyHV-1 (carp pox virus), CyHV-2 with sequences from koi and tilapia, CyHV-3 with sequences from crucian carp and goldfish, the herpesvirus from sichel or sabrefish (probably CyHV-4) and the newly discovered herpesvirus from grass carp.

Discussion

While the World Organisation for Animal Health (WOAH, former OIE, 27) equate infections with pathogens in aquatic animals with the existence of disease in those animals, the EU favours clinical observations and prevention followed by pathogen detection and eradication (9). Both strategies have advantages and disadvantages. The approach of WOAH is that a disease is present at all developmental stages, even when there is only infection and no visible clinical signs of disease. If there are no clinical signs and fish appear healthy, there is no obligation to screen for pathogens. The presented study shows that latent or chronic infections may indeed be present without any clinical signs. When KHV was discovered by Hedrick *et al.* (13), koi and common carp were the target fish for the virus but also for the associated KHVD. Perelberg *et al.* (22) showed that other fish than *C. carpio* cannot be infected by KHV and that when such other fish were exposed to KHV, it cannot be transferred by them to disease-susceptible naïve fish, even if they were carp or koi. In contrast, Bergman *et al.* (3) discovered that KHV can infect goldfish and that goldfish release virus particles infectious to common carp. In goldfish, KHV DNA was detected by PCR from kidney and spleen samples and

viral proteins were also detected by immunofluorescence in separated leukocytes using polyclonal and monoclonal antibodies. KHV was also found in koi leukocytes after a four-week co-habitation experiment with KHV-infected goldfish. Since 2004, many researchers have discovered KHV in different fish species belonging to different families, e.g. *Cyprinidae* (5, 6, 10, 21), *Acipenseridae* (16), and *Salmonidae* (2). In contrast to herpesvirus-induced diseases of mammals, it seems that diseases with aquatic herpesviruses are species specific while the infections are not. These viruses can infect many species without any clinical signs (2). This was also confirmed by the present study. Healthy-appearing fish from local ponds without any sign of a disease were tested for the presence of different herpesviruses, and surprisingly, KHV DNA was found in naturally infected goldfish and crucian carp samples, while GHV DNA was detected in koi and tilapia samples. Additionally, positive PCR results were obtained from grass carp samples, the amplified sequences being genomic parts of a herpesviral DNA polymerase which were less than 80% identical to KHV, GHV or sichel herpesvirus. These samples may represent the situation in the field, where proper laboratory diagnostics and investigation techniques for viral agents cannot be applied and virus variety could be a challenge for sample analysis. Clinical observation or farm inspections alone do not seem to be good recommendations for measures to combat dangerous viral diseases in aquaculture.

In this study, it was confirmed that different fish can be infected with different aquatic herpesviruses without any disease outbreaks. When the fish were additionally infected by GHV, all species besides goldfish developed disease and mortality. The DNA and RNA results showed that KHV was most prominently detectable in the samples of all species except those from crucian carp, where GHV DNA was also traceable. While the published RT-PCR for KHV mRNA detection always yielded negative results for all fish species at all time points, it was decided to divide the assay into firstly separate reverse transcription using oligo-dT primers and then secondly the reaction with the cDNA using the same primer pairs as for a conventional PCR, mainly a PCR and nested PCR according to Engelsma *et al.* (8). After sequence analysis, these results were compared with sequences in the NCBI database. It seems that KHV was transferred by human, equipment or even by water to all fish during the experiment. The results also showed characteristics of an Asian lineage of KHV at higher water temperatures to be very fast replication and suppression of the GHV which was used for infection. Two routes of KHV infection were possible: firstly direct transmission by equipment, water and/or personnel and secondly transmission during the experimental infection with GHV. The actual content in the water was not investigated. All the experimental fish were more susceptible to KHV than to GHV because of the induced stress. In koi, no virus was detectable for unknown reasons, although they suffered almost 50% mortality. Additionally, all the negative controls used for RT-PCRs and PCRs, *e.g.* organs from confirmed-negative carp and non-template water controls were always verified to be negative. Therefore, laboratory contamination with materials from the samples or from the positive test controls was excluded. At the end of the experiment, all fish were infected with at least two herpesviruses. A third herpesvirus was clearly present in the grass carp samples but in no samples from other species. While mortality in crucian carp may have been induced by GHV, the mortality in tilapia and grass carp were induced by a double infection in connection with the induced stress. However, in the grass carp, it may have been the triple infection which led to this massive, unexpected mortality at the end of the experiment. In tilapia, only KHV DNA was detectable at the end of the experiment. Also in this case, the cause of the 70% mortality might not have been KHV alone but the double infection with KHV and GHV. In all groups clinical symptoms were difficult to discern, and it appeared that most of the fish died of peracute disease.

Conclusion

This study showed that infection presenting no visible signs in a latent or persistent stage may occur in all fish species. It might be useful to investigate the status of a farm in regards to virus presence before any transfer of fish to another farm takes place. More investigations are necessary to solve the problem of latent infection detection by virology and molecular biology diagnostics but may be mainly by additional serology.

Conflict of Interests Statement: The authors declare that there is no conflict of interests regarding the publication of this article.

Financial Disclosure Statement: This research did not receive any specific grant from funding agencies in the public, commercial or not-for-profit sectors.

Animal Rights Statement: The experiment was carried out in 2015 in China. At this time point no ethical committee was established. The authors declare the experiments on animals were conducted at the time in a manner in accordance with subsequently introduced local Ethical Committee laws and regulations as regards care and use of laboratory animals in PR China.

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