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Is There Any Species Specificity in Infections with Aquatic Animal Herpesviruses? – The Koi Herpesvirus (KHV): An *Alloherpesvirus* Model

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Abstract

Most diseases induced by herpesviruses are host-specific; however, exceptions exist within the family Alloherpesviridae. Most members of the Alloherpesviridae are detected in at least two different species, with and without clinical signs of a disease. In the current study the Koi herpesvirus (KHV) was used as a model member of the Alloherpesviridae and rainbow trout as a model salmonid host, which were infected with KHV by immersion. KHV was detected using direct methods (qPCR and semi-nested PCR) and indirect (enzyme-linked immunosorbant assay; ELISA, serum neutralization test; SNT). The non-koi herpesvirus disease (KHVD)-susceptible salmonid fish were demonstrated to transfer KHV to naïve carp at two different temperatures including a temperature most suitable for the salmonid (15°C) and cyprinid (20°C). At 20°C KHVD was induced in carp cohabitated with infected trout. KHV was also detected virologically and serologically at the end of the experiment in both rainbow trout and carp.

Keywords: KHV; KHVD; Transmission; Rainbow trout

Introduction

Herpesviruses constitute a diverse family of widespread pathogens inducing severe diseases of veterinary importance in all animals including animals used for human consumption. It is likely that they evolve within their host species over long periods of time and most of them induce very host-specific disease, although sometime infections occur in the absence of clinical disease signs. As a rule herpesviruses have large genomes and all of them induce a latent or a persistent phase of infection, sometimes in different hosts. For the family Alloherpesviridae it has been demonstrated that most of the virus species have developed a high level of host specificity. In the majority of animal herpesviral infections only mild symptoms (internally and externally) appear under natural conditions. Virulence associated with herpesviral infection is often initially displayed in immunologically weakened hosts or during primary infection of a naïve host. The characteristics of KHV, including the morphology investigated by electron microscopy [1,2], and phylogeny meets the taxonomical criteria for viruses of the family Alloherpesviridae [3], which have been shown to be non-host-specific, but sometimes causing disease in one species. Within this family are four genera grouped as the *Batrachovirus*, *Salmonivirus*, *Ictalurivirus* and *Cyprinivirus*. In *Batrachovirus,* the *ranid herpesvirus* 1 *(RaHV-1)* can be found in different leopard frog (*Rana pipiens*) (sub) species and has been identified as the causative agent of renal adenocarcinoma [4], and the *ranid herpesvirus* 2 (*RaHV-2*), which can cause infections in a lot of species of the family Ranidae (pets and wild) leading to skin lesions and sometimes inducing tumors [5]. The members of the family ictaluriviridae, such as acipenserid herpesvirus 1 (*AcHV-1*), infect sturgeon spp. [6] and at least two sturgeon sp. can be infected with *AcHV-2* [7]. The most previously researched Alloherpesvirus, channel catfish herpesvirus (*Ictalurid herpesvirus* 1 (*IcHV-1*), CCV) infects channel catfish but also blue catfish [8] and possibly other catfish species or subspecies while *IcHV-2* was detected in black bullhead (*Ameiurus melas*) and in channel catfish (Ictalurus punctatus) [9]. Members of the *salmonivirus* group are known to often infect more than one species: *salmonid herpesvirus* 1 (*SaHV-1*) have been detected in rainbow trout (*Ocorhynchus mykiss*) [3] but also in Chum salmon (*O. keta*) and Chinook salmon (*O. kisutch*) [10]. *SaHV-2* was considered to infect all salmonid species [3] and *SaHV-3* was detected in different fish and hybrids of the genus *Salvelinus* [3,11,12]. In addition to *cyprinid herpesviruses* 1,2 and 3 the eel herpesvirus (HVA, *AngHV-1*) is also included in the genus *Cyprinivirus*. HVA was detected during disease outbreaks in Japanese eel (*Anguilla japonica*) and European eel (*A. anguilla*) in Japan [13] and in American eel (*A. rostrata*) in Poland [14]. A variant of the HVA in eels in Taiwan (eel herpesvirus Formosa, FEHV) has also been found to induce mortality in common carp (*Cyprinus carpio*) as well [15]. From investigations conducted in our Lab it was confirmed that HVA may at least persist in artificially infected common carp (immersion) for up to six weeks, which was detectable directly by PCR and indirectly by serum neutralization assay (Bergmann, unpublished data). The cyprinid herpesvirus 1 (*CyHV-1*, carp pox virus) induced typical clinical signs in carp or koi (*C. carpio*), in golden ide (*Leuciscus idus*) [16] or in other cyprinids (carps and minnows) [17]. *CyHV-2* or goldfish herpesvirus was first detected in goldfish (*Carassius auratus)*, inducing severe disease outbreaks in those affected populations [18,19]. Recently severe outbreaks of disease with high mortalities were observed in crucian carp (*Carassius carassius*) [20] and Prussian carp (*C. c. gibelio*) [21] where *CyHV-2* was identified as the disease causing agent. In Germany severe mortality rates were also induced by *CyHV-2* in a wild Prussian carp population

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(Bergmann, pers. obs.). For *CyHV-3* or koi herpesvirus (KHV), although KHV disease (KHVD) is induced only in the species *C. carpio* (common carp and koi), it has been demonstrated that this virus can be present, and sometimes replicated, in a lot different fish species living in fresh and brackish water. KHV DNA has been identified by different PCR methods in a lot of members of the families Acipenseridae [22], Cyprinidae, including common carp or koi hybrids [23-25], and Percidae in many wild fish populations within close vicinity to, or in carp ponds [26,27]. Furthermore KHV has also been detected in fresh water mussels and crustaceans [28]. In this study we have shown that a common salmonid species, rainbow trout, is able to transfer KHV to naive carp inducing KHVD under certain circumstances.

Materials and Methods

Fish

Rainbow trout, steelhead strain (n=30, 80-100 g) were obtained from a commercial farm and kept under a re-circulating system with a volume of 450 l and fed once a day with commercial trout food. The daily water exchange was 100-120 l/h. The rainbow trout were divided into four groups including fish kept at 15°C (n=10) and 20°C (n=10) water temperature for the infection trial and two additional tanks containing fish (n=5, respectively) kept as negative controls at the same temperatures. The fish were confirmed free from notifiable pathogens like viral hemorrhagic septicemia virus (VHSV) and infectious hematopoietic necrosis virus (IHNV) by RT-PCR [29] and additionally with the conventional PCR and nested PCR [30] according to Engelsma et al.

Carp (n=15, 80-100 g) were obtained from a commercial farm in German federal state Thuringia and tested free for CyHV-1, -2 and -3 by PCR [24,30] for 10 years. They were divided into three groups (2×n=5, for the infection trial at 15°C and 20°C, respectively) and as a negative control (n=5). They were kept at 18°C in the same re-circulating system as the rainbow trout and fed with commercial carp food.

Scheme of animal experiment

After a 14 day adaptation period rainbow trout were kept at 15 and 20°C for one further week. Samples were then collected non-lethally (swabs, leukocyte separation, serum) and rainbow trout were infected with KHV by immersion at 15 and 20°C, respectively, for 1 h with 10^4 $TCID_{50}$/ml in water (v/v) in a separate aquarium. After the challenge period fish were caught and replaced in to the aquaria and maintained there for another 7 days. After 7 days post infection (dpi) samples were collected non-lethally but also lethally (gill and kidney tissue parts) from two rainbow trout from both aquaria (15°C and 20°C) as well as from negative controls from tanks set at both temperatures. All rainbow trout were caught and transportation was stimulated (30 seconds in the air, placed in a new aquarium for 10 minutes and returned to the original aquaria). After a water exchange the following day, the aquaria were divided using a water permeable wall with a water flow from the rainbow trout to the carp in circulation. Before carp were placed into the aquaria, samples were collected non-lethally. All fish were observed for another 21 days. At the end of the experiment samples were collected lethally and non-lethally.

Virus and cells

Koi herpesvirus isolate KHV-E (D 132), kindly provided by Dr. Keith Way (CEFAS, UK), was allowed to replicate in CCB cells [30,31]. The virus was characterized at the protein level by immunofluorescence assays [24], at the genomic level for quantification by qPCR [32] modified according [31] and at the infectious dose level by titrations onto CCB cells according to Spearman and Kärber [33].

Direct methods of KHV detection in fish samples

Non-lethally collected samples (gill and skin swabs, separated leukocytes) and lethally taken samples (gill and kidney tissue) were investigated regularly by qPCR at different time points of the experiment: rainbow trout on 1st day shortly before infection, on day 7 dpi and 37 dpi and carp on 1st day shortly before cohabitation with rainbow trout and on day 21 post cohabitation (dcoh).

Indirect methods of KHV detection in serum (plasma) samples

Fish (rainbow trout and carp) were bled from the caudal vein at the same time points of the experiment described for direct detection methods. Blood was allowed to clot at 4°C in a BD Microtainer® tube (Becton and Dickinson) over night. The tubes were then centrifuged for serum separation at 1000 × g for 1 min at 4°C. Sera was stored at -20°C until use.

For serum neutralization assays (SNT) all sera obtained from rainbow trout and from carp were pre-diluted 1:4 and 1:16 with CCB cell culture medium, respectively and then 2-fold diluted to 1:1048. Those dilutions were mixed with the same volume of KHV with a titre between $10^{0.5}$ and $10^{1.5}$ $TCID_{50}$/ml. These mixtures were incubated overnight at 4°C. The next day those preparations were adsorbed for 1 h at 20°C directly on the CCB cell monolayer. The medium was re-supplemented and the cell culture plates were incubated at 20°C for 10 days. After 5, 7 and 10 days post inoculation the plates were assessed for an occurring cytopathic effect (CPE) due to KHV replication in CCB cells (this study).

The KHV antibody enzyme-linked immunosorbant assay (ELISA) was prepared in combination of the assays according to [34,35] with some modifications. Briefly, the Medisorp ELISA plate (Nunc) was used for coating the plate with 3 µg purified KHV/ml [36], blocked with Roti Block® (Roth), and as secondary antibody a monoclonal anti-carp IgM (F 16, Aquatic Diagnostics) at a dilution 1:64 and the anti-trout IgM monoclonal antibody 4C10 at a dilution of 1:1000 were used. As ELISA substrate ready to use TMB (Pierce) was used. The plates were measured in an ELISA Reader (iMark, BioRad) at 450 nm. All reactions were carried out at room temperature in duplicates. All dilution and washing steps were proceeded with PBS-Tween 20 (0.05%) without any additional substances.

Results

Infection experiment at 15°C

At 15°C water temperature neither clinical signs of KHVD nor mortality were observed in rainbow trout or in cohabitated carp. The behaviour of both fish species was typical at this temperature. All animals stayed completely healthy throughout the period of the experiment.

Infection experiment at 20°C

At 20°C rainbow trout always stayed away from the heating source in the aquarium. The majority of the rainbow trout exhibited darkening of their skin and moved much slower compared to the animals in the 15°C experiment. While rainbow trout did not show any clinical signs characteristic of an infectious disease, all five carp became sick on 5th d pcoh. On the 10th d pcoh one carp died with severe symptoms of

KHVD. All surviving carp recovered till the 18th d pcoh. At the end of the experiment carp still displayed signs of KHVD like patches in the skin but their behaviour returned back to a normal including typical swimming activity and food uptake.

Direct detection of KHV in rainbow trout samples

On the first dpi prior to immersion no KHV DNA was detected in the samples from the 15°C and 20°C aquarium (gill swabs and separated leukocytes) from five rainbow trout, respectively (Table 1). On the 7th dpi before simulated transportation, KHV DNA was detected in leukocyte preparations but also in samples from organ tissues of two fish from both aquaria. In the aquarium with the KHV infection at 15°C ct values between 28 and 33 were detected, and at 20°C ct values between 24 and 28 were observed (Table 1). In both cases the gill swabs were negative by qPCR [32] but positive by the semi-nested PCR [31]. Those two fish from each aquarium were anesthetized, killed by decapitation and gill and kidney tissues were collected for qPCR. In samples from both aquaria a similar ct values between 27 to 33 were detected. At the end of the experiment on the 29th dpi, KHV DNA was detected in all remaining rainbow trout samples. There was no difference between the samples collected lethally or non-lethally and no differences in samples from the aquaria with the different temperatures. The values varied only between ct 27 and ct 33 corresponding to 100.000 to 50 genomic equivalents compared to the positive KHV plasmid controls [31].

Direct detection of KHV in carp samples

Samples were collected from carp before cohabitation to rainbow trout and on 21st dcoh. While samples were negative on 1st dcoh, on 21st dcoh in samples from carp of the aquarium with 15°C four of five (ct 27-ct 33) fish were positive and from aquarium wit 20°C three of four (ct 27-ct 33) were positive for KHV DNA (Table 1). There was no difference in the results from lethal and non-lethal sample collection. All samples obtained from carp that had died from KHVD were positive for KHV DNA (ct 23 in gill tissue-ct 26 in kidney tissue and gill swab) by qPCR.

Detection of KHV antibodies (indirect detection of KHV)

Aquarium with KHV infection at 15°C water temperature: While the sera obtained from rainbow trout were negative for anti-KHV antibodies on 1st and 7th dpi, on 29th dpi four of eight sera were positive for anti-KHV antibodies by ELISA (titres 1:100 to 1:400) and, interestingly, six of eight by SNT (titres 1:16 to 1:32) (Table 2). All sera obtained from carp contained KHV antibodies in large quantities as detected by ELISA but only in one serum sample were neutralizing antibodies detected by SNT (titre 1:64).

Aquarium with KHV infection at 20°C water temperature: All rainbow trout developed antibodies against KHV at this temperature as determined by ELISA (Table 2). However, at this temperature no neutralizing antibodies against KHV were detectable. Also all carp developed antibodies against KHV as detected by ELISA. Interestingly those titres were much lower compared to the aquarium with 15°C. Additionally, in three of the four sera from the remaining carp, neutralizing antibodies were found by SNT (titres between 1:64 to 1:128) (Table 2). In the serum from the carp that had died of KHVD, no antibody was found neither by ELISA nor by SNT.

All sera were tested with both secondary antibodies, anti-carp-IgM and anti-trout-IgM. No cross-reaction occurred by ELISA when carp sera were used for the rainbow trout ELISA with anti-trout IgM antibodies nor when trout sera were used for the carp ELISA with anti-carp IgM antibodies.

Discussion

The strong host specificity of Herpesviruses may be dependent on the infection and disease that they cause. This is indeed the case for most members of the family Alloherpesviridae, but based on the results of the current study, the Alloherpesviridae appear to deviate from this rule. While the diseases induced by members of the Alloherpesviridae occur in most cases in only a single species, infection with the virus in the absence of clinical signs has been be detected in many more species of the same family, e.g. Cyprinidae [23], but also in other fish families, e.g. acipenseridae [26] or percidae [26,27]. Earlier studies on KHV led [37] and [38] to conclude that KHVD cannot be transferred to fish other than *C. carpio*. The virus was therefore defined as host specific. The fact that the disease has never been found within other fish than *C. carpio*, namely in common carp and koi, did not detract from the

Species		dpi	15°C						20°C					
			qPCR (ct)			Semi-nested PCR			qPCR (ct)			Semi-nested PCR		
	Fish		s[3]	L[4]	o[5]	s	L	o	s	L	o	s	L	o
rt[1]	1-5[2]	1st	-	-	-	-	-	-	-	-	-	-	-	-
	1	7th	-	30	28	+	+	+	-	25	28	+	+	+
	2		-	31	29	+	+	+	-	24	28	+	+	+
	1	29th	33	-	32	+	-	+	27	32	31	+	+	+
	2		31	-	-	+	-	-	-	-	33	+	-	+
	3		29	nt[6]	33	+	nt	+	29	nt	30	+	nt	+
	4		29	nt	31	+	nt	+	28	nt	31	+	nt	+
	5		32	nt	-	+	nt	-	29	nt	29	+	nt	+
	6		29	nt	30	+	nt	+	29	nt	31	+	nt	-
	7		28	nt	30	+	nt	+	-	nt	-	-	nt	-
	8		32	nt	-	+	nt	-	30	nt	33	+	nt	+
Carp	1-5[2]	1st	-	-	-	-	-	-	-	-	-	-	-	-
	1	21st	29	-	32	+	-	+	-	-	-	-	-	+
	2		-	-	-	-	-	-	-	28	28	-	+	+
	3		29	-	30	+	-	+	31	29	29	+	+	+
	4		31	-	-	+	-	-	30	28	28	+	+	+
	5		28	-	-	+	-	-	-	-	-	-	-	-

[1]rt: Rainbow trout; [2]Tested before immersion with KHV; [3]s: Gill swab; [4]L: Leukocyte separations; [5]o: Organ tissues; [6]nt: Not tested.

Table 1: Direct detection of KHV DNA by qPCR and semi-nested PCR in samples from rainbow trout and carp [31,32].

Species		dpi	15°C		20°C	
	Fish		SNT[3/4]	ELISA[5/6]	SNT[3/4]	ELISA[5/6]
rt[1]	1-5[2]	1st	-	-	-	-
	1	7th	-	-	-	-
	2		-	-	-	-
	1	29th	1:16	1:400	-	1:800
	2		1:16	1:400	-	1:200
	3		1:16	1:200	-	1:200
	4		-	-	-	1:200
	5		1:16	-	-	1:100
	6		1:16	-	-	1:200
	7		-	-	-	-
	8		1:16	1:100	-	1:200
Carp	1-5[2]	1st	-	-	-	-
	1	21st	-	1:4.800	1:64	1:1.200
	2		-	1:4.800	1:128	1:300
	3		1:64	1:9.600	1:64	1:600
	4		-	1:4.800	-	1:300
	5		-	1:2.400		

[1]rt: Rainbow trout; [2]Tested before immersion with KHV; [3]SNT basic dilution rainbow trout sera 1:4; [4]SNT basic dilution carp sera 1:32; [5]Basic dilution of rainbow trout sera 1:100 and anti-trout-IgM mab 4C10 (FLI Insel Riems) used as secondary antibody for ELISA; [6]Basic dilution of carp sera 1:300 and anti-carp-IgM mab F 16 (Aquatic Diagnostics) used as secondary antibody for ELISA.

Table 2: Indirect detection of KHV by ELISA and SNT for antibody detection in serum samples from rainbow trout and carp.

possibility of virus replication in other fish species and investigations were not conducted to investigate the role of KHVD transmission through vector fish species, i.e., its transfer as an infectious agent to naive carp detected by a combination of virological and serological methods other than in carp or koi [38]. It has since been found that a lot of other fish species such as goldfish, crucian carp, different sturgeon species, grass carp (*Ctenopharyngodon idella*), silver carp (*Hypophthalmichthys molitrix*) or tench (*Tinca tinca*) may be act as a healthy appearing carrier hosts which can transfer the KHV to naive carp or koi [22,24,26,27,39]. KHVD has never occurred in any of those fish species when inoculated with KHV. In contrast, carp hybrids with goldfish and crucian carp were also found to be susceptible for the virus infection but expressed a severe KHVD [31]. In all those studies the results following virological examination has never been compared with the serological/antibody response against the virus. The first indication of vector-facilitated KHVD was apparent when fish such as tench or roach (*Rutilus rutilus*) from ponds with KHVD outbreaks, occurring in cohabitated common carp, were tested for the presence of antibodies against KHV by SNT. The antibody titres against this virus ranged between 1:64 to 1:128 (Fichtner, pers. comm.).

In this study rainbow trout was selected as a model member of the family salmonidae to determine it is susceptibility to KHV infection and to investigate for the first time the potential for trout to transfer infectious virus to common carp by virological and serological methods. After immersion KHV was detected after 7 dpi inside but not outside the rainbow trout (leukocytes and gill swabs). Furthermore, the faeces were tested every 7 days for the presence of KHV by qPCR, but were always negative. Whilst at 15°C, the most suitable temperature for rainbow trout which is also tolerated by carp, rainbow trout developed antibodies against KHV after 29 dpi. Amazingly, higher titres were observed by SNT compared to antibody ELISA. This phenomenon was previously found in carp sera in the field where sera samples always contained neutralizing antibodies against KHV, but a lack of detectable antibodies by ELISA, even where the sera was strongly SNT positive. This also was reported in Italy where the same immunological difference was observed, but in sera obtained from mirror carp and scaled carp where the scaled carp sera reacted positive in SNT but not by ELISA (Vendramin, pers. comm.). To determine the underlying factors behind this phenomenon basic immunological research is required. Alternatively, it is possible that the immunological differences are influenced by temperature and/or the genetic background of the fish. At 15°C water temperature carp do not normally exhibit a serologically strong response due to the fact that their optimum water temperatures, especially under European conditions, is between 18 and 22°C. This was confirmed by the fact that all carp sera reacted positive by ELISA but only one by SNT, as at this sub-optimal temperature, limited neutralizing antibodies were produced.

Nonetheless, it was concluded from the study that at 15°C, a water temperature suitable for salmonids, that rainbow trout are not only able to replicate KHV, provoking a serological and immunological reaction at this temperature, but they are also capable of transferring it without clinical signs to carp. This was confirmed by the transfer of infectious KHV from rainbow trout to carp at 20°C where clinical symptoms with mortality in carp were induced. Temperatures of 20°C may be tolerated by rainbow trout, where they are also able to react serologically against KHV. This was detected by ELISA where all rainbow trout sera contained antibodies against the virus. In the same sera no neutralizing antibodies were found by SNT. This confirmed the earlier hypotheses that rainbow trout may not produce an optimal antibody response above 17°C, which was in fact around 2-fold lower. In the challenge experiment at 20°C rainbow trout developed antibodies detectable only by ELISA. In contrast, to the observed responses of rainbow trout, the majority of carp developed neutralizing antibodies and other antibodies detected by ELISA and SNT following the disappearance of clinical signs. Carp do not develop antibodies when there is a productive infection with KHV ensuing, which was evident by the negative reaction found with carp serum from the animal that had died of KHVD. Moreover, those reactions were confirmed with 20 sera from carp that displayed severe clinical signs after infection with KHV. No antibodies against KHV were found on the 7th and 14th dpi, neither by ELISA nor by SNT. After an additional 14 days, all survivors had produced antibodies against KHV, as indicated by SNT and ELISA at 22°C water temperature (Fichtner and Bergmann, pers. comm).

In conclusion, from the experiments presented in the current study, the previous observations reported, and the assessment of the literature, it has to be stated that the majority of the members of the family Alloherpesviridae are not species-specific in terms of infection, but are specific with regards to disease. Additionally, it has been demonstrated that rainbow trout is able to replicate KHV and transmit the virus to naive carp at different temperatures. This information will be vital for controlling outbreaks of KHVD. Further investigations on immunogenesis after infection with KHV are urgently needed.

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