doi: 10.17221/8879-VETMED

Susceptibility of stone loach (*Barbatula barbatula*) and hybrids between sterlet (*Acipenser ruthenus*) and beluga (*Huso huso*) to cyprinid herpesvirus 3

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ABSTRACT: Cyprinid herpesvirus 3 also known as koi herpesvirus is a causative agent of highly contagious disease (koi herpesvirus disease) and can cause significant losses in fish stocks. The disease is restricted to koi and common carp, but recent investigations have shown that other cyprinids as well as non-cyprinid species are asymptomatically susceptible to this virus and can play either a role as a potential carrier or can contribute to biological conservation of this virus. The susceptibility of two non-target species, stone loach (*Barbatula barbatula*) and sterbel – a hybrid between sterlet *(Acipenser ruthenus)* and beluga *(Huso huso)* to cyprinid herpesvirus 3 was tested by means of their co-habitation together with naïve koi and intraperitoneally KHV-infected koi (primary challenge). On the 15 th day post-infection (dpi), a secondary challenge was started (a portion of the surviving stone loach and sterbel were transferred to tanks with other naïve koi). All dead as well as surviving fish were investigated for the presence of KHV DNA in pooled samples of tissue from individual fish by nested PCR. Sampling for PCR from surviving fish was performed on the 15th dpi and on the 30th dpi of the primary challenge, and on the 30th dpi of the secondary challenge. During the primary challenge (up to the 30th dpi), average cumulative mortality in duplicated experimental groups was as follows: koi 100%, sterbel and stone loach both 5%. In the primary challenge, no surviving stone loach or sterbel sampled on the 15th dpi or those that died previously were found to be positive for viral DNA. Results of PCR revealed the presence of KHV DNA in 95% of co-habited naïve koi samples. PCR analysis of tissues taken from surviving fish on the 30th dpi revealed the presence of viral DNA in 77.8% (7/9) of stone loach and in 22.2% (2/9) of sterbel. Cumulative mortality of fish in the secondary challenge was 100% for stone loach and for koi co-habitating with them, and 50% for koi co-habitating with sterbel, which all survived. Despite the high mortality of koi and stone loach in the secondary challenge (probably caused by malfunction of biofilters or bacterial infection), none of them, nor any of the sturgeon hybrids were considered to be positive for KHV DNA. In summary, the hybrid between sterlet and beluga and the stone loach seemed to be susceptible to cyprinid herpesvirus 3, but we could not prove that they can transfer this virus to naïve koi.

Keywords: CyHV-3; KHV; transmission; cohabitation

List of abbreviations

CyHV-3 = cyprinid herpesvirus 3, **CEFAS** = Center for Environment, Fisheries and Aquaculture Science, **dpi** = days post infection, **KHV** = koi herpesvirus, **PCR** = polymerase chain reaction

Supported by the Ministry of Education, Youth and Sports of the Czech Republic (Projects CENAKVA No. CZ.1.05/2.1.00/01.0024 and CENAKVA II No. LO1205 under the NPU I Program), the Ministry of Agriculture of the Czech Republic (Grant No. MZE QJ1210237) and the Grant Agency of USB (Grant No. GAJU 087/2013/Z).

doi: 10.17221/8879-VETMED

Cyprinid herpesvirus 3 (CyHV-3), also known as koi herpesvirus (KHV), is a causative agent of a highly contagious and notifiable disease (Rakus et al. 2013). This disease was initially reported in 1998 in Israel and USA, where the outbreaks resulted in high mortality of koi and common carp (*Cyprinus carpio*) (Pokorova et al. 2005).

Fish affected by CyHV-3 show external signs of disease such as: focal or complete loss of the epidermis with discolouration, haemorrhages (particularly on the caudal fin), transient appearance of the skin (due to excessive or insufficient production of mucus), increased production of gill mucus (at the beginning of disease), severe branchial necrosis (in more protracted course of disease), and sunken eyes (enophthalmus) (Bretzinger et al. 1999; Hedrick et al. 2000; Hedrick et al. 2005). Histological examinations show necrotic gills, fusion of lamellae due to hyperplasia of respiration epithelium and even haemorrhagic patches on the tips of lamellae (Hedrick et al. 2000; Mohi El-Din 2011). The virus is present mainly in the intestine and kidney, but also in droppings of infected fish (Dishon et al. 2005). Moreover, Eide et al. (2011) detected KHV DNA in the brain, eye, spleen, gills and in the haematopoietic (anterior) kidney.

Despite the suggestion that CyHV-3 is restricted to common carp and koi (Perelberg et al. 2003), transmission of the virus to other cyprinid and non-cyprinid fish is also possible. The virus has been found in numerous other fishes after co-habitation with infected fish, but without obvious symptomatic reactions; these include other cyprinid species, e.g. goldfish *(Carassius auratus)* (Bergmann et al. 2009; Bergmann et al. 2010; El-Matbouli and Soliman 2011; Radosavljevic et al. 2012), grass carp *(Ctenopharyngodon idella)* (Bergmann et al. 2009), silver carp *(Hypophthalmichthys molitrix)* (Kempter et al. 2012; Radosavljevic et al. 2012), common bream *(Abramis brama)* (Kempter et al. 2012), tench *(Tinca tinca)* (Fabian et al. 2012; Kempter et al. 2012; Radosavljevic et al. 2012), some non-cyprinid species such as ruffe *(Gymnocephalus cernua),* spined loach *(Cobitis taenia)* (Kempter et al. 2012), European perch *(Perca fluviatilis)* (Fabian et al. 2012; Kempter et al. 2012), and even Atlantic sturgeon *(Acipenser oxyrinchus)* (Kempter et al. 2009). Some fishes were even able to transmit CyHV-3 to naïve common carp, for example goldfish (Bergmann et al. 2010; El-Matbouli et al. 2011; Radosavljevic et al. 2012), grass carp, silver carp (Kempter et al. 2012; Radosavljevic et al. 2012), common bream (Kempter et al. 2012), tench (Fabian et al. 2012; Kempter et al. 2012; Radosavljevic et al. 2012), ruffe (Kempter et al. 2012) and European perch (Kempter et al. 2012). For this reason, it is necessary to verify susceptibility to CyHV-3, as well as determining species which can act as carriers for susceptible species.

The aim of this study was to determine if two non-cyprinid species, stone loach *(Barbatula barbatula*, family Balitoridae, previously Cobitidae) and sterbel [sterlet (*Acipenser ruthenus*) × beluga *(Huso huso)*], family Acipenseridae), could be potential carriers of CyHV-3 and to establish the presence or absence of CyHV-3 DNA in their tissues after co-habitation using nested PCR.

MATERIAL AND METHODS

Fish. The origin of experimental fish was as follows: stone loach was provided by a private breeder. Hybrids of sterlet and beluga (so-called "sterbel") were obtained from a closed recirculation hatchery system of the Faculty of Fisheries and Protection of Waters. Koi were obtained from a private fish farm without any KHVD history. Before the start of the challenge test, all fish were treated in a long-term FMC bath (formalin, malachite green, methylene blue) for elimination of ectoparasites. After that, all fish were acclimated at 24 °C for three days in experimental aquaria.

Virus. Virus (US isolate F98/50) was provided by S. M. Bergmann, Friedrich-Loeffler-Institut, Germany. Virus was cultivated on CCB (*Cyprinus carpio* brain) cell lines incubated at 24 °C in the Veterinary Research Institute, Brno.

Design of experiment. The study consisted of two challenge experiments – a primary and a secondary one. The primary challenge was conducted to determine the susceptibility of stone loach and sterbel to CyHV-3 during co-habitation. The co-habitation method or communal stocking is based on infected fish and healthy fish sharing the same environment. The secondary challenge was applied to investigate whether fish (potential vectors) exposed to the infection by co-habitation with CyHV-3-infected fish are able to transfer infectious virus to healthy susceptible species (naïve koi).

Primary challenge. During this period, three experimental groups were established: experimental group-1 (E1), experimental group-2 (E2) and negative control group (NC). In each of these groups, koi

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(n = 10, average length 9.2 ± 0.4 cm), stone loach (n = 10, average length 10.1 ± 0.1 cm) and sterbel (n = 10, average length 20.1 ± 0.3 cm) were stocked in 175-L aquaria with three intraperitoneally infected koi. The duration of the primary challenge was 30 days. Fish were observed and fed twice a day and water quality (temperature, pH and concentration of nitrites and nitrates) was measured once a day.

Secondary challenge. Two experimental groups were established and placed in two new tanks – "transmission group 1" (T1) and "transmission group 2" (T2). Also, a new negative control group (NC2) was established. Each of these groups contained 10 healthy specimens of naïve koi (average length 10.6 ± 0.7 cm). When the cumulative mortality of koi in the primary challenge reached 60% in the E1 group and 100% in the E2 group (15th dpi), the secondary challenge was started: three stone loach each from experimental groups E1 and E2 (six specimens in total) were transferred to the T1 group and the same number of sterbel from both experimental groups were transferred to the T2 group. Before being transferred, all fish were rinsed with clean water so as to avoid contamination of the new experimental environment with infected water on the body surface of fish. At the same time (on the 15th dpi), two specimens of stone loach were sampled for PCR from both experimental groups E1 and E2 (four specimens in total) and the same number of sterbel were collected from both E1 and E2 groups. Experimental conditions for the secondary challenge were the same as in the primary challenge and the same parameters of water quality were checked daily.

Sampling for PCR assay. Pooled samples of gill, brain, hepatopancreas, kidney and intestine were taken from each deceased as well as surviving fish. Samples were diluted with ultra-pure deionised sterile water (1 : 5) and homogenised (QIAGEN homogeniser, Germany). After homogenisation, organ homogenates were centrifuged and supernatant was collected for total DNA extraction.

Extraction of DNA. The extraction of DNA was performed using the QIAamp DNA Mini Kit (QIAGEN, Germany) following the manufacturer's instructions. During the extraction process a positive control to verify the correctness of extraction (samples from intraperitoneally infected koi) was used.

PCR assay. The nested PCR uses primers based on the sequence of the thymidine kinase gene of CyHV-3 (Bercovier et al. 2005) supplemented with a nested primer set provided by D. Stone (Pokorova et al. 2010) which is still recommended for routine diagnostics. The first set of outer primers was used according to Bercovier et al. (2005) and give a final amplicon of 409 bp. The nested primer set was used according to D. Stone (described in the CyHV-3 protocol distributed by CEFAS for the ring test of reference laboratories), and gives a final amplicon of 348 bp. For preparation of PCR, PPP master mix (TopBio, Czech Republic) was used. The 25 µl master mix, both for conventional and nested PCR, consisted of 12.5 µl of PPP master mix, 9.5 µl of PCR water (TopBio, Czech Republic), 1 µl of DNA template, 1 µl of forward primer (0.1µM) and 1 µl of reverse primer (0.1µM). Cycle conditions for conventional and nested PCR were performed in accordance with Pokorova et al. (2010).

Detection of PCR products. PCR amplicons were detected using gel electrophoresis with 1.5% agarose gel prepared in TBE buffer, stained with GelRed Nucleic Acid Stain (Biotium, USA) and illuminated by UV light. TrackIt 1 Kb Plus DNA Ladder (Invitrogen, USA) was used to determine the size of nested PCR amplicons.

RESULTS

Results of co-habitation tests

During the primary challenge, the mortality rate of intraperitoneally infected koi and co-habited koi reached 100%. The mortality of stone loach reached 10% in E2, while no stone loaches died in E1. The mortality of sterbel reached 10% in E1 and was 0% in E2 (Figure 1). During the secondary challenge, the mortality of all fish in T1 (both stone loach and koi) was 100% on the 14th dpi (Figure 2). In T2, the mortality rate was 50% on the 18th dpi, but only koi died in this group; all sterbel from this group survived.

Results of nested PCR

In the primary challenge, nested PCR revealed that 100% (6/6) of intraperitoneally infected koi were positive for CyHV-3 DNA. 100% (10/10) of co-habited naïve koi from E1 and 90% (9/10) of cohabited koi from E2 were also positive for KHV DNA. Pooled samples obtained from sterbel that died on

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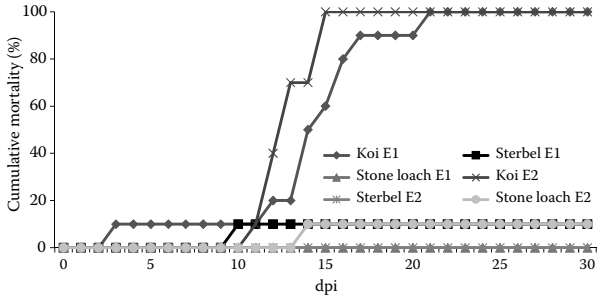

Figure 1. Cumulative mortality of fish during the primary challenge

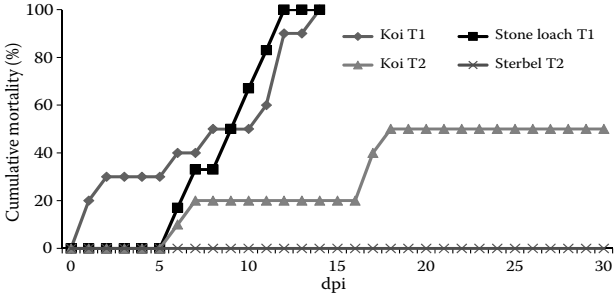

Figure 2. Cumulative mortality of fish during the secondary challenge

the 10th dpi in E1 and from stone loach that died on the 14th dpi in E2 were CyHV-3 DNA-negative as were four specimens of stone loach and sterbel sampled from E1 and E2 on the 15th dpi. PCR testing of tissues taken from surviving fish sampled on the 30th dpi revealed the presence of CyHV-3 DNA in 60% (3/5) of stone loach from E1 and 100% (4/4) of stone loach from E2. In the case of sterbel, 25% (1/4) from E1 and 20% (1/5) from E2 were CyHV-3 DNA-positive (Figure 3).

Even though the mortality was high during the secondary challenge, neither stone loach nor koi from T1 were found to be positive for viral DNA. Likewise in T2, none of the sterbel nor any of the dead or surviving koi were CyHV-3 DNA-positive. The total number of positive fish detected by nested PCR is listed in Table 1.

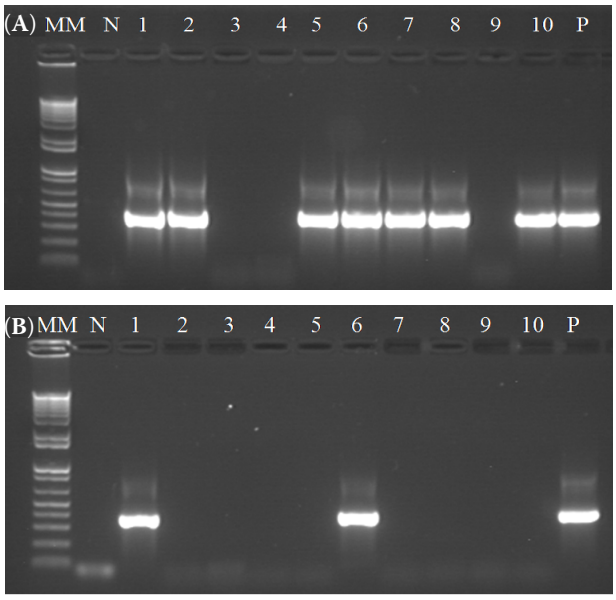

Figure 3. Electrophoresis of the final amplicon (348 bp) of nested PCR: **A** – stone loach (1, 2, 5: positive fish from E1; 3, 4: negative fish from E1; 6, 7, 8, 10: positive fish from E2; 9: negative fish – † 14 dpi). **B** – sterbel (1: positive fish from E1; 2, 3, 4, 5 – † 10 dpi: negative fish from E1; 6: positive fish from E2; 7, 8, 9, 10: negative fish from E2). N = negative control of PCR, P = positive control of PCR, MM = molecular marker

DISCUSSION

Kempter et al. (2009) reported that Atlantic sturgeon and Russian sturgeon are susceptible to CyHV-3; similarly, we found sterbel to be positive for CyHV-3 DNA. The susceptibility of stone loach (previously member of family Cobitidae, now Balitoridae) might suggest that species of the family Cobitidae could be generally susceptible to CyHV 3; for instance, Kempter et al. (2012) reported susceptibility of spined loach. Nevertheless, we did not find that those fish are able to transmit CyHV-3.

The fish which were transferred for secondary challenge (start of this challenge on the 15th dpi in the primary challenge) were found to be CyHV-3 DNA-negative as were fish that were sacrificed for PCR on the 15th dpi. One potential reason for these observations is that the fish were exposed to virus for too short a time. Sterbel from E1 and stone loach from E2 which died on the 10th dpi and 14th dpi, respectively, were also found to be negative for the presence of viral DNA.

Pooling effects should not play a role as a potential reason for the negative results of PCR testing of fish which died on the 10th and 14th dpi and also of those which were sacrificed for PCR on the 15th dpi. We pooled tissues, such as the intestine and kidney, that had previously been demonstrated to harbour viral particles (Dishon et al. 2005), and to contain viral DNA (brain, spleen and gill; Eide et al. 2011). Moreover, all samples satisfied weight parameters (minimum 25 mg of tissue samples) as

doi: 10.17221/8879-VETMED

Table 1. Ratio of positive fish as determined by the used primers. 1st round (single round PCR) using primer pair of Bercovier et al. (2005) and 2nd round (nested PCR) using the internal primer pair according to D. Stone (CEFAS protocol)

Final amplicon	1st round – 409 bp						2nd round – 348 bp					
Group	E1	E2	NC	T1	T2	NC2	E1	E2	NC	T1	T2	NC2
Fish	Number of positive fish from all fish tested											
Koi	10/10	9/10	0/10	0/10	0/10	0/10	10/10	9/10	0/10	0/10	0/10	0/10
I. p. infected koi	3/3	3/3	n.p.	n.p.	n.p.	n.p.	3/3	3/3	n.p.	n.p.	n.p.	n.p.
Stone loach												
Transfer time (15th dpi)	0/2	0/2	–	–	n.p.	–	0/2	0/2	–	–	n.p.	–
Final time (30th dpi)	3/5	3/5*	0/10	0/6	n.p.	0/10	3/5	4/5*	0/10	0/6	n.p.	0/10
Hybrid – sterbel												
Transfer time (15th dpi)	0/2	0/2	–	n.p.	–	–	0/2	0/2	–	n.p.	–	–
Final time (30th dpi)	1/5*	1/5	0/10	n.p.	0/6	0/10	1/5*	1/5	0/10	n.p.	0/6	0/10

*stone loach († 14 dpi) and sterbel († 10 dpi) included, n.p. = not present in this group

well as the dilution factor (acceptable ratio is 1 + 9 w/v) (EURL 2015).

The results of the nested PCR revealed the presence of viral DNA only in the stone loach and sterbel sacrificed and sampled at the end of the primary challenge (on the 30th dpi). Although the mortality of koi and stone loach reached 100% in T1 and 50% for koi in T2, CyHV-3 DNA was not found in any tissue samples. The most probable explanation for this mortality is impaired quality of the water in tanks caused by compromised function of biological filters which resulted in a higher concentration of nitrites (1.73 ± 1.65 mg/l for T1). Even if these parameters might lead to stressing and weakening of fish (Carballo and Munoz 1991) and, consequently, to a heightened sensitivity to bacterial (Decostere et al. 1999) or viral (Inendino et al. 2005) infections, nested PCR did not reveal the presence of viral DNA in samples taken during the secondary challenge. Therefore, even though stone loach and sterbel were found to be CyHV-3 DNA-positive at the end of the primary challenge (on the 30th dpi), the fact that fish that were removed from the infected tanks (E1 and E2) on the 15th dpi (sampled or transferred to the transmission tanks and sampled after a further 30 days) were CyHV-3-negative could suggest that 15 days of cohabitation with KHV-infected fish is not enough for infection of stone loach and sterbel.

Finally, for future investigations it might be useful and suitable to determine the positivity of remaining stone loach and sterbel survivors from experimental groups after the time of transfer and until the end of the primary challenge period. This would include PCR testing for the presence of viral DNA in tissues of target species over the course of the entire period until the end of the co-habitation test. In such a protocol, each day after the death of the last koi (or the majority of koi), samples of stone loach and sterbel would be taken for PCR or, more suitably, for qPCR examination, which could help us to explain not only the status of the virus after mass mortality of susceptible species (virus can be detected even three months subsequently (Minamoto et al. 2009), but also if the virus could still be infective for non-susceptible species. The use of various form of PCR for this purpose would be more suitable then serological assays such as iFAT or SNT. They are not considered to be reliable because CyHV-3 growth is slow and unpredictable even in susceptible cell cultures (OIE 2015). On the other hand, ELISA-based tests detecting antibodies in infected koi or common carp exist (Adkison et al. 2005; St-Hilaire et al. 2009), and ELISA focused on the direct detection of CyHV-3 antigen is under development (OIE 2015). However, no serological assays for determining the presence of CyHV-3 in non-susceptible fish species are currently available. Moreover, such techniques are still not accepted as a routine screening method mainly due to insufficient understanding of the serological responses of fish to viral infections (EURL 2015; OIE 2015).

Acknowledgement

The authors are greatful also to D. Luhanova and C. Steinbach for technical assistance and to

doi: 10.17221/8879-VETMED

Professor W.L. Shelton for help with language correction.

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Received: 2015–07–15
Accepted after corrections: 2016–04–04

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