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Distribution of carp edema virus in organs of infected juvenile common carp

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Abstract

Introduction: The disease caused by carp edema virus (CEV) manifests with lethargy as a primary sign; this observation in koi in Japan gained the disease the name koi sleepy disease (KSD). In the years following the discovery of the virus in Japan, KSD cases have been noted in the UK in koi and common carp. Conducting research in order to expand knowledge of the processes of distribution of CEV in infected fish organs will be helpful for eradication and diagnostic purposes. **Material and Methods:** Carp edema virus–affected fish with clinical signs of KSD were experimentally cohabited with common carp fry (30 fish). Three fish were euthanised by bath in a 0.5 g L^{-1} tricaine solution at one week intervals (7, 14, 21 and 28 days post cohabitation). Tissue samples from the brain, gills, spleen, kidney, intestines and skin were collected, and the total DNA was extracted and tested by real-time PCR. **Results:** By the seventh day post infection, CEV DNA was most often found in the skin, gills and brain and less frequently in the kidney and intestines. In many of the common carp fry, CEV DNA could typically be found in several organs of each individual fish, although it was only found in one sample of spleen tissue. **Conclusion:** In this experimental study the pathogenesis of the CEV infection process was shown, the high infectivity of CEV was confirmed and the best organs were determined for sampling in CEV-infection experimentation. The real-time PCR method used in our cohabitation experiments was shown to be useful at the clinical and asymptomatic stage of virus infection.

Keywords: fish, common carp, koi sleepy disease syndrome (KSD), carp edema virus (CEV).

Introduction

Carp edema virus (CEV) was first detected in Japan in the 1970s, when mass mortality was observed in the fry of koi, the coloured variety of *Cyprinus carpio*, during outbreaks of disease (10). In the 1990s, also in Japan, the disease was named koi sleepy disease (KSD) because the main symptom in CEV-infected fish was lethargy. In the years following the discovery of the virus in Japan, KSD cases have been noted in the UK in koi and common carp (15). During a CEV survey based on a PCR method in Poland in 2015–2017, the infection was detected in koi and common carp on many farms in both fish with clinical symptoms and asymptomatic individuals (8). Although the virus has been reported in aquaculture in Europe (1, 5, 6, 8, 14, 15), North America (4, 7), South America (13) and Asia (12, 17) for decades, there are still many information gaps in the scope of pathogenesis of carp edema virus infection. Adamek *et al.* (1, 2) confirmed the gills as the main target organs of CEV infection. In our experimental study, samples from the brain, gills, spleen, kidney, intestines and skin were analysed for the presence of virus genetic material to determine different organs useful for sampling when carp edema virus infection needs investigation in the clinical and asymptomatic stage. Also, a real-time PCR method was confirmed suitable for use for presumptive and confirmatory CEV detection. The research conducted in order to expand knowledge of the processes of distribution of CEV in infected fish organs offers findings which may be helpful for diagnostic purposes.

Material and Methods

Experimental design. Common carp with clinical signs of KSD were collected from a traditional carp farm. After confirmation of carp edema virus by real-time PCR, the fish were experimentally cohabited with common carp fry. In the cohabitation experiment 30 fry (45–50 g) were introduced to tanks with 10 KSD-

affected common carp (320–350 g). As a negative control, another group of 30 fry were put together in a tank with naïve (CEV-negative) carp. After 12 h of cohabitation both groups were moved to separate tanks of 600 L capacity. The water in the tanks was filtered, aerated, and maintained at a temperature of 14 ± 1°C. Three experimental and three control fish were euthanised by immersion in a bath of 0.5 g L^{-1} tricaine solution (Sigma-Aldrich, St. Louis, MO, USA) at one-week intervals (7, 14, 21 and 28 days post cohabitation commencement). Tissue samples of the brain, gills, spleen, kidney, intestines and skin were collected from all euthanised fry. The carp were examined daily for clinical lesions of KSD. The experiment was performed in two identical parallel iterations.

DNA extraction. Total DNA was extracted from samples of the brain, gills, spleen, kidney, intestines and skin using a QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Before testing, genetic material was eluted in 100 µL of acetate ethylenediaminetetraacetic acid buffer and stored at −80°C.

Molecular identification. Genetic material of CEV was detected by real-time PCR using a protocol developed originally by the Centre for Environment, Fisheries and Aquaculture Science (CEFAS) in Weymouth, UK, which was successfully used previously in a study concerning infected samples from koi and common carp (8). The assay was performed using CEV qFor1 (5′-AGTTTTGTAKATTGTAGC ATTTCC-3′) and CEV qRev1 (5′-GATTCCTC AAGGAGTTDCAGTAAA-3′) respective forward and reverse primers. The 20 µL reaction volume contained 500 nM of forward and reverse primers, 200 nM of CEV qProbe1 (5′-AGAGT TTGTTTCTTGCCAT ACAAACT-3′), 1 × PCR buffer mix and 5 µL of the template DNA. A CFX Connect Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) was used, and the programme of amplification was initial incubation at 50°C for 2 min, then incubation at 95°C for 10 min, and 50 temperature cycles at 95°C for 15 s and 55°C for 1 min.

Results

In the first experimental trial, the presence of genetic material of carp edema virus was confirmed by the real-time PCR method in tissue samples obtained from the brain, gills, intestines and skin of fish on the 7th day post cohabitation with CEV-affected fish. Carp edema virus nucleic acid was detected in the samples of fry brains, gills and skin on the 14th day of the experiment. At the third sampling interval the presence of carp edema virus in the brain, gills, kidney, intestines and skin was observed, whereas the last sampling confirmed the presence of CEV nucleic acid only in the gills and skin (Table 1). In the second experimental challenge, the presence of CEV DNA was noted in 11 out of 18 samples of the brain, gills, kidney, intestines and skin on the 7th day post cohabitation with CEV-infected common carp. The presence of carp edema virus was also confirmed in all sampled tissue types on day 14 of the second experimental trial. On the 21st day samples were positive in the cases of brain, gill, kidney, intestine and skin samples, whereas 28 days post infection, CEV DNA was only detected in a sample of skin (Table 2).

Clinical symptoms were observed in common carp fry after contact with CEV infected fish, but no mortality was observed in either experiment. The first KSD symptoms were detected on the 8th day post infection in the first experimental trial as skin lesions (Fig. 1) and on the 7th day in the second experiment also as skin lesions and additionally as swollen gills (Fig. 2).

In no samples originating from control fish in either experimental challenge was the presence of carp edema virus genetic material detected, and in clinical examination no signs of disease or mortality in these fish were noted to the end of the experiments.

Fig. 1. Skin lesions (petechiae) in common carp fry experimentally infected with carp edema virus

Fig. 2. Swollen gills and skin petechiae in common carp fry experimentally infected with carp edema virus

Viral DNA was not always found in all organs or the skin of each individual fish. The percentage of positive results in a sample depended on the specific tissue from which the sample was collected. In both iterations of the experiment, the highest percentages of positive results were observed in skin and gill samples, whereas all samples from spleens were negative in the first experiment and only one was positive in the second (Fig. 3).

Table 1. Real-time PCR results in fish experimentally infected with carp edema virus and maintained at 14ºC (first iteration)

Dpi	Fish number	Cycle threshold for a given tissue type					
		Brain	Gills	Kidney	Intestines	Skin	Spleen
	1	+/40.60	–	–	–	+/34.88	–
7	2	+/39.16	+/38.62	–	–	+/38.04	–
	3	+/39.79	+/39.09	–	+/40.77	+/30.89	–
	4	–	+/39.31	–	–	+/32.86	–
14	5	+/40.56	+/40.27	–	–	+/35.84	–
	6	–	+/38.56	–	–	+/40.28	–
	7	+/40.11	+/39.78	+/41.29	+/39.45	+/38.49	–
21	8	–	+/35.84	+/40.09	–	+/32.12	–
	9	–	+/38.56	–	–	+/40.67	–
	10	–	–	–	–	+/32.12	–
28	11	–	+/39.67	–	–	+/40.22	–
	12	–	–	–	–	–	–

Dpi – day post infection; – negative test result; + positive test result

Table 2. Real-time PCR results in fish experimentally infected with carp edema virus and maintained at 14ºC (second iteration)

Dpi	Fish number	Cycle threshold for a given tissue type					
		Brain	Gills	Kidney	Intestines	Skin	Spleen
	1	–	+/34.37	–	–	+/29.98	+/38.01
7	2	+/36.24	+/36.06	–	+/39.97	+/24.19	–
	3	+/31.97	+/34.36	+/36.94	+/40.06	+/26.66	–
	4	–	+/38.13	+/40.83	–	+/32.54	–
14	5	+/38.93	+/39.12	–	–	+/32.33	–
	6	+/32.95	+/33.74	+/35.64	–	+/28.10	–
	7	+/40.21	+/38.52	–	+/38.83	+/37.18	–
21	8	+/38.42	+/37.75	+/34.87	+/37.97	+/28.21	–
	9	+/39.10	+/35.67	–	+/40.57	+/36.79	–
	10	–	–	–	–	–	–
28	11	–	–	–	–	–	–
	12	–	–	–	–	+/33.79	–

Dpi – day post infection; – negative test result; + positive test result

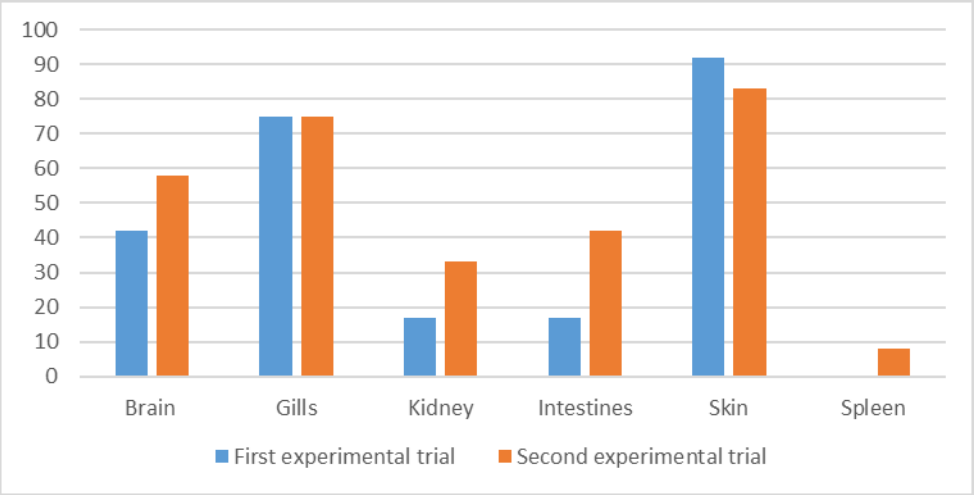

Fig. 3. Percentages of positive real-time PCR results in tissue samples of carp edema virus–infected common carp fry

Discussion

The results of our study on cohabitation of common carp fry with infected carp showed CEV to have high infectivity for this fish species. Common carp with confirmed koi sleepy disease syndrome were able to infect naïve counterparts, and the infection caused similar clinical signs, *e.g.* swollen gills and

lesions on the skin and fins. Similar lesions in the gills and skin were observed in a case of KSD in Germany (5). According to data published by Haenen *et al.* (3), six hours' cohabitation was enough for carp edema virus to be transmitted from infected fish to other specific pathogen–free koi or common carp. Our results were aligned with these, and indicated 12 hours of cohabitation to be a sufficient period of time for virus transmission. Moreover, a short (six-hour) time of cohabitation was adequate for transmission of carp edema virus from affected carp to many vector species such as *Alburnus alburnus*, *Carassius carassius*, *Carassius gibelio*, *Perca fluviatilis*, *Rutilus rutilus* and *Tinca tinca* (9).

The disease incubation period, or in other words the time interval from experimental infection until instances of clinical signs could be observed, was seven days (with the fish maintained at 14°C) according to observations made in our experiment. This result confirms the findings of other authors (2).

Our experiments also showed that the real-time PCR method using CEFAS primers (8) is very efficient in identifying CEV genetic material in tissue samples from moribund and asymptomatic KSD-affected fish. According to the "Infection with carp edema virus (CEV)" technical disease card published by the World Organisation for Animal Health, this diagnostic method can be used for presumptive and confirmatory purposes (16).

In our experimental cohabitation trials, we particularly sought to investigate the distribution of CEV DNA in the tissues of infected common carp in order to determine which organs were useful for sampling during CEV infection research. On the seventh day post infection, CEV DNA was most often found in the skin, gills and brain, and less frequently in the kidneys and intestines. Generally in infected common carp, CEV DNA could usually be found in several organs of each individual fish, although it was only detected in one sample of spleen tissue. The investigations by real-time PCR after the 28th day post infection led to the conclusion that carp edema virus genetic material is detectable more frequently in the skin and gills than in samples of the brain, kidney, intestines or spleen. The lowest cycle threshold value of 24.19 was noted in the skin. This contrasts with the finding of the study by Adamek *et al.* (2) concerning carp edema virus transmission, in which common carp gill tissue showed higher virus loads than intestine, kidney, skin or spleen tissue; the highest levels of viral nucleic acid were also found in the gills of infected koi in research by Ouyang *et al.* (11).

Concluding, in our cohabitation experiment focused on the pathogenesis of the CEV infection process, we confirmed the high infectivity of CEV. We nominated the most relevant organs which should be taken into consideration during sampling to establish CEV infection prevalence. The real-time PCR method used in the experiments was shown to be useful at the clinical and asymptomatic stage of virus infection and it can be used for presumptive and confirmatory purposes. This knowledge concerning the pathogenesis of CEV may be useful in diagnosis, an essential element in disease control.

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