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CYHV-3 INFECTION DYNAMICS IN COMMON CARP *(CYPRINUS CARPIO)* – EVALUATION OF DIAGNOSTIC METHODS

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Abstract

The KHV free carp were experimentally infected with CyHV-3 suspension by immersion at temperatures of 18ºC±1 and 25ºC±1. Skin, brain, gills, liver, spleen, kidneys, and leukocytes were investigated for the presence of KHV DNA, and sera for the presence of antibodies against the virus. Similar investigations were performed with non-infected carp, designed as control groups. The results of KHV DNA detection in the infected carp kept at 25ºC ±1 were compared with the results of the virus isolation onto common carp brain cells using leukocyte co-cultivation method and results of specific antibody detection. The appearance of specific antibodies at various time intervals after detecting the clinical symptoms of KHVD in naturally infected carp was additionally studied. At 18°C water temperature, KHV DNA was not detected up to the 6th d post infection (dpi). Then it was found in samples from skin, gill, liver, spleen, and kidneys and much later from the brain. At 25°C water temperature, KHV DNA was already detected on the 3rd dpi in samples from skin and gills, and from the 5th dpi it was found in all examined internal organs, except the brain where it was found at 6th dpi. At 25°C, isolations of CyHV-3 have succeeded between the 7th and 11th dpi. The first specific antibodies were found no sooner than on the 21st dpi. The serological examination performed in naturally infected carp in 15 carp pond farms showed that first specific antiviral antibodies can be detected 2 weeks after the appearance of clinical symptoms of KHVD.

Key words: carp, KHV, KHVD, pathogenesis, diagnostics

Cyprinid herpesvirus 3 (CyHV-3) infection, synonym for koi herpesvirus (KHV) disease (KHVD), is recently the most fatal disease ever known in species *Cyprinus carpio* comprising common carp and its ornamental relative the koi. Pathological lesions most often seen in gills (Fig. 1), in and on skin (Fig. 2), and in kidney, accompanied with their dysfunctions. Additionally, secondary infections often lead immediately to a high mortality in fish populations, due to depressed immunity. KHVD was first described in Israel and USA in 1998 (6). The herpesvirus, after many years of coexistence with fish organism weakened by one sided selection (on certain attractive coloration), and probably mutated to the present virulent form, which has caused mass mortality in super intensive koi culture centers, and lately spread all over the world *via* the unrestricted trade of these fish. The Department of Fish Disease and Department of Pathology of the National Veterinary Research Institute diagnosed KHV in common carp for the first time in Poland in 2004 (1). KHV DNA was detected in carp organs by PCR method followed by virus isolation in cell cultures from the samples obtained from naturally infected carp. Koch's postulates were fulfilled when after infection by immersion, the disease was induced and the agent was re-isolation from moribund carp (Figs 4, 5).

The investigations presented in this paper were initiated for better understanding the dynamics of CyHV-3 infection in common carp at temperatures of 18°C and 25°C *i.e.* - the optimal temperature range for the development of the KHVD. The study has been concentrated upon the traceability of KHV DNA, KHV, and specific antibodies in carp at various intervals after the appearance of the first clinical symptoms and pathological lesions. The better understanding of KHVD pathogenesis and critical evaluation of the sensitivity of routinely used diagnostic methods are crucial for diagnostic accuracy, thus for the success in disease eradication. The study was a contribution to understanding the KHV latency.



Fig. 1. Gill necrosis in naturally KHV infected common carp



Fig. 2. "Sand paper" surface of the epidermis experimentally infected *via* water with KHV .

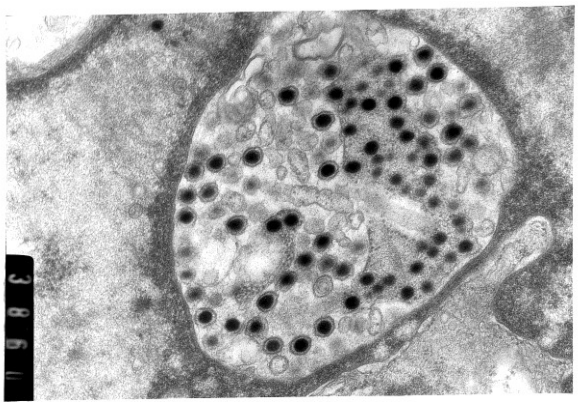


Fig. 3. CyHV-3 viruses in the infected CCB cells, TEM.

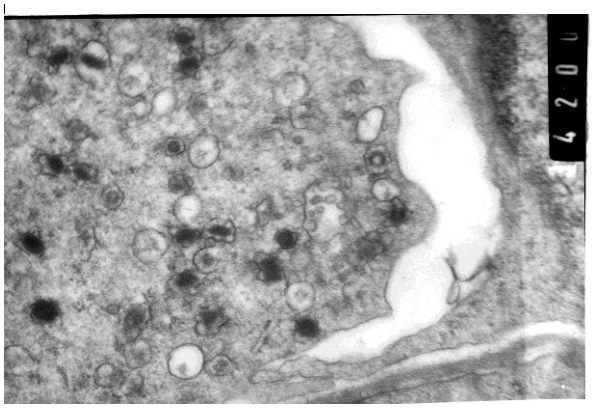


Fig. 4. CyHV-3 in gill epithelium of naturally KHV infected carp, TEM.

Material and Methods

Carp were experimentally infected with CyHV-3, replicated in common carp brain cells (CCB), by immersion in 10-litre aquaria to which 2 ml of virus suspensions with a titer of $4x10^3$ were added. Control carp has been exposed to non-infected CCB cell suspension. Both carp groups were transferred after a 30 min exposure to 350-litre tanks with filtered and aerated water at temperatures of 18°C and 25°C, respectively. Every day several moribund fish were anaesthetised and samples of the brain, gills, liver, spleen, kidney, and skin were collected. These tissues were examined for the presence of KHV DNA by PCR according to Gilad *et al*. (5).

Leukocytes from infected fish, separated according to the method described by Bergmann *et al*. (3), were also used for co-cultivation onto CCB cells for virus re-isolation using the method described by Bergmann (pers. comm.). Sera of these fish were tested by serum neutralisation test (SNT). The results of SNT were compared to the results of PCR as well as with the results of the co-cultivation.

The SNT was also applied to detect antibodies against KHV in sera from naturally infected carp originating from 15 Polish carp farms. These serum samples were collected 2 to 9 weeks after first KHVD symptoms were observed.

Results

At 18°C, KHV DNA was not detected in the tested samples up to the 6^{th} dpi. Afterwards, it was found in samples obtained from skin, gill, liver, spleen, and kidney, and on the 12^{th} dpi from the brain (Table 1). At 25°C, KHV DNA was detected on the 3^{rd} dpi in skin and gills, on the 4^{th} dpi in the liver, and on the 5^{th} dpi it was found in all internal organs except the brain, where it was found on the 6^{th} dpi (Table 2). The first clinical signs of KHVD infection appeared after 9 dpi at 18°C and after 4 dpi at 25°C (Tables 1, 2).

At 18°C, KHVD symptoms and the presence of KHV DNA in examined organs and skin have been detected until the end of the experiment, *i.e.* 26 dpi (Table 1). At 25°C, no disease symptoms were observed from the 9^{th} dpi. Only in one fish, which survived the infection, KHV DNA was detected by PCR (Table 2).

Table 1
PCR results in experimentally infected carp kept at 18ºC

Day post infection	Number of fish	Presence of KHV nucleic acid					
		Brain	Gill	Liver	Spleen	Kidney	Skin
1-5	1-15	-	-	-	-	-	-
6	16	-	-	+	+	-	+
	17	-	+	-	-	-	+
	18	-	-	-	-	-	-
7	19	-	-	-	-	-	+
	20	-	-	-	+	+	+
	21	-	-	-	-	-	+
8	22	-	+	+	-	+	+
	23	-	+	-	+	-	+
	24	-	-	-	+	-	+
9	25	-	-	-	-	-	+
	26	-	+	-	+	+	+
	27	-	-	+	+	-	-
10	28	-	-	-	+	+	+
	29	-	+	-	+	+	+
	30	-	-	+	+	+	+
11	31	-	+	-	+	+	+
	32	-	+	+	+	+	+
	33	-	-	-	-	+	+
12	34	-	+	-	+	+	+
	35	+	+	-	-	-	+
	36	+	+	+	+	-	-
13	37	+	+	+	+	+	+
	38	+	+	+	-	+	+
	39	+	+	-	-	+	+
14	40	-	+	+	-	+	-
	41	-	+	-	-	+	-
	42	-	-	-	-	-	-
15	43	-	+	-	-	-	-
	44	-	-	-	-	-	+
	45	-	+	-	-	-	-
23	46	-	+	-	-	+	+
	47	-	-	-	-	+	+
	48	-	+	+	-	-	+
26	49	+	+	+	-	+	-
	50	+	+	+	-	-	-
	51.	-	-	-	-	-	+

+ positive test results, - negative test results, blue part of the Table – occurrence of clinical symptoms.

At 25°C, isolations of KHV were successful from the 7th to 11th dpi. This was connected with the occurrence of KHVD symptoms and possibly with high virus concentrations in the blood or leukocytes. From 21st dpi until the end of the experiment, virus isolation using this method failed (Table 3).

In the serological examination of the experimentally infected carp kept in aquarium with water temperature of 25°C, the first positive SNT results were detected not earlier than on the 21st dpi, whereas KHV DNA was found already on 3rd dpi (Table 3).

The serological investigations performed in naturally infected carp showed that first neutralising specific antibodies against KHV were found 2 weeks after the first symptoms of KHVD were reported (Table 4).The percentage of fish with the positive results of serological tests increased in time from 30% on the 2nd week to 100% on the 7th week after appearance of clinical signs (Fig. 5).

Discussion

The results of the experimental infection of carp by immersion confirmed a high infectivity and high virulence of KHV (CyHV-3) for this fish species. Our investigations have also shown that PCR method modified according Gilad *et al*. (5) is very effective in detecting KHV DNA in internal organs and skin of KHV infected fish.

In the incubation period of the disease, KHV DNA is mostly found in or on the skin (Table 1) and relatively rarely in internal organs but never in the brain. In many moribund fish, KHV DNA could be detected usually in several organs of each individual fish but hardly in brain samples.

Table 2
PCR results in experimentally infected carp kept at 25ºC

Day post infection	Number of fish	Presence of KHV nucleic acid					
		Brain	Gill	Liver	Spleen	Kidney	Skin
1-2	1-6	-	-	-	-	-	-
3	7	-	-	-	-	-	+
	8	-	+	-	-	-	-
	9	-	-	-	-	-	-
4	10	-	-	-	-	-	-
	11	-	-	-	-	-	+
	12	-	-	+	-	-	-
5	13	-	+	+	-	+	+
	14	-	+	+	+	+	+
	15	-	+	+	+	+	+
6	16	+	+	+	+	+	+
	17	-	+	+	-	+	+
	18	+	+	+	+	+	+
7	19	+	+	+	+	+	+
	20	+	+	+	+	+	+
	21	-	+	+	+	+	+
8	22	-	+	+	+	-	+
	23	-	+	-	-	+	-
	24	-	-	+	-	+	+
9	25	-	-	-	-	-	-
	26	-	-	-	+	-	-
	27	-	-	-	-	-	-
10-12	28-36	-	-	-	-	-	-

Symbols as in Table 1.

Table 3
Comparison of diagnostic methods for KHV detection in experimentally infected carp kept at 25ºC

Day post infection	Number of fish	Methods		
		PCR	rec. SNT titres	Co–cultivation
3	1	+	-	-
	2	+	-	-
	3	+	-	-
	4	+	-	-
7	5	+	-	+
	6	+	-	+
	7	+	-	+
	8	+	-	-
14	9	+	-	-
	10	+	-	+
	11	+	-	+
	12	+	-	-
21	13	+	160	-
	14	+	640	-
	15	+	320	-
	16	+	320	-
28	17	+	2,560	-
	18	+	640	-
	19	-	640	-
	20	+	320	-
35	21	-	320	-
	22	-	640	-
	23	-	1,280	-
	24	-	640	-
42	25	-	1,280	-
	26	-	640	-
	27	-	2,560	-
	28	-	640	-

+ positive results, - negative results

Table 4
Results of serological examinations of naturally KHV infected carp from commercial farms

No. of farm	Antibody titres in particular fish	Time (weeks) after occurring clinical symptoms	No. of farm	Antibody titres in particular fish	Time (weeks) after occurring clinical symptoms
1	-	2	9.	2,560	
	320				
	-		10.	5,120	4
	5,120			1,280	
	5,120			160	
2	2,560	2		2,560	
	1,280			2,560	
	-			160	
				640	
	-			160	
			11.	-	5
3	-	2		1,280	
	-			80	
	-			640	
	-				
	-			5,120	
	80				
4	-	2	12.	1,280	5
	-			1,280	
	-			-	
	-			640	
	-				
5	-	3		640	
	1,280				
	80				
	640		13.	320	7
	5,120			320	
6	-	3		5,120	
	-			5,120	
	-			5,120	
	-			1,280	
	-			640	
7	-	4	14.	1,280	8
	-			5,120	
	-			5,120	
	-			80	
	5,120			1,280	
8	-	4		5,120	
	-			640	
	-		15.	320	9
	640			5,120	
	160			640	
	5,120				
9	-	4		2,560	
	-			320	
	5,120			2,560	
	5,120			640	

- negative test results

Collected samples consisted of all internal organs, *i.e.* the brain, gill, liver, spleen, kidney, and skin from each fish and their single testing increased the probability for positive result of PCR test. Any pooling would reduce at least positive signals by PCR due to sample dilation effects.

In the first phase of the development of the disease, 2 dpi at 25°C and 5th dpi at 18°C, KHV DNA was not detectable by PCR according to Gilad *et al*. (5). It seems very probable that at temperatures lower than 18°C this phase may be elongated, which causes diagnostic problems *i.e.* false-negative PCR results from fish being in an early phase of the virus infection. The examination by PCR in the second phase of this nearly symptomless incubation period led to the assumption that KHV DNA is detectable more often in or on the skin rather than in internal organs. This also might be due to a weak KHV replication connected to a weak virus load in these organs.

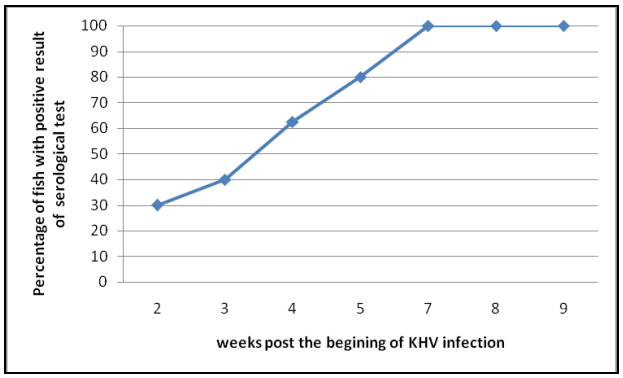


Fig. 5 The cumulative results of serological examinations performed in 15 KHV infected carp from farms with water temperature between 18°C and 25°C

In survivor fish it was not possible to detect viral DNA using PCR according Gilad *et al.* (5), except one case out of 12 fish, which survived the infection at 25°C *i.e.* on the 9th, 10th,11th, and 12th dpi. This observation shows that a very specific PCR also should to be very sensitive to detect the virus in symptomless carriers/survivors.

The disease incubation period (time interval since experimental infection up to appearance of the clinical symptoms) according to our observations is 5 d at 25°C and 10 d at 18°C, which confirms the findings of other authors (7, 8).

The results of serum neutralisation test showed that the test can only assist the PCR method and seems to be useful for indirect detection of KHV survivors. In carp, it is possible at least 15-20 dpi when specific antibodies against the virus are formed. At the lower water temperatures (18°C and lower) the lag time would be longer and antibodies could appear less regularly and obviously in weaker concentration in fish population. The experiments performed by St. Hilarie *et al*. (9, 10) with koi showed that after a rapid temperature decrease at the outset of clinical symptoms fish mortality stopped and a lot of fish survived. In such cases, the antibodies against KHV will be detectable at least for 125 dpi. According to Eide *et al*. (4), CyHV-3 infection could transform to latent stage and then the infective material localises inside leukocytes and some other fish cells. Even slight thermal shock, namely the temperature rise with the rate of 1.5°C could reactivate the virus.

According to Bergman *et al*. (3), KHV is a lymphoid virus and the virus can survive in leukocytes (in carp, sturgeon, and goldfish), mainly in polymorphic granulocytes, with a half life time between 15 and 45 d, depending on the water temperature, as shown by *in-situ* hybridisation (DNA level) and immunofluorescence (protein level). Eide *et al.* (4) suggested that KHV DNA is localised most often in B limpfocytes but without a real proof for this hypothesis.

Co-cultivation methods, which enable virus isolation from fish leukocytes, could only be applied as supplemental diagnostic methods and the positive results could be expected only from moribund fish with high virus loads. In the latent/persistent phase, *e.g.* in disease survivors, no virus isolation using these methods is possible.

In conclusion, the results of our investigations show that to increase the ability of detection of CyHV-3, various PCR methods as well as serological methods should be applied simultaneously. Each of these methods proved to be useful in different stages of virus infection. Further investigation should be devoted to the development of sensitive methods enabling the detection of the virus or its DNA in asymptomatic, eventually latent stage of the infection. The improvement of the sensitivity of serological methods would also be important in terms of accurate and effective KHV diagnosis, which may help in KHV eradication.

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