EPIDEMIOLOGY, PATHOGENICITY AND MOLECULAR BIOLOGY OF KOI HERPESVIRUS ISOLATED IN POLAND

JERZY ANTYCHOWICZ, MICHAŁ REICHERT1, MAREK MATRAS, SVEN M. BERGMANN2 AND OLGA HAENEN3

Department of Fish Diseases, 1Department of Pathology, National Veterinary Research Institute, 24-100 Pulawy, Poland
2Friedrich-Loeffler-Institute, Federal Research Institute for Animal Health, Institute for Infectiology, D-17498 Greifswald-Insel Riems, Germany
3Central Institute for Animal Diseases Control, NL 8203 Lelystad, Netherlands
e-mail: antych@piwet.pulawy.pl

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Abstract

The presence of koi carp herpesvirus (KHV) infection in Poland was confirmed in common carp reared for consumption. Virus was isolated in CCB cells using co-cultivation technique. The carps were experimentally infected and virus was reisolated and identified with PCR method. Monitoring of KHV in 15 carp farms showed the presence of the virus in 4 farms. We have found that lowering of the water temperature to 11-12°C could eventually provoke the recurrence of the disease symptoms in latently infected survivor carp, and thus could help to detect KHV with PCR or co-cultivation methods. Analysis of sequencing data of 484 bp fragments of KHV DNA of 4 Polish isolates revealed the complete identity in 3 cases. One of the Polish isolate differed from the remaining 3 variants by 5 nucleotide substitutions. In order to evaluate fully the importance of small differences in DNA sequences of the KHV isolates, corresponding amino-acid analysis, and subsequent virulence studies are necessary.

Key words: carp, koi herpesvirus, epizootiology.

In Poland, in the last 4 years numerous cases of unexplained mortality in common carp (Cyprinus carpio) with symptoms of gill necrosis were observed (1, 2, 7). The mortality appeared, when water temperature reached values above 18-20°C. The features of this disease were similar to those noted in the course of koi herpesvirus (KHV) infection reported in many countries such as: Austria, Belgium, Denmark, France, Germany, Italy, Luxembourg, Netherlands, Switzerland, UK, Indonesia, Israel, Japan, South Africa, Taiwan, USA, and Thailand (5).

In 2003, a KHV infection was detected by the Friedrich-Loeffler-Institute, Insel Riems in common carps imported from Poland to Germany. In 2004 the first two outbreaks of the disease were diagnosed in Poland (2). In these cases the diagnosis was performed using the PCR method published by Gilad et al. (4).

Koi herpesvirus infection is currently of major concern to common carp and ornamental koi carp breeders in the whole world. The disease causes severe mortalities in carp of all ages and is spreading rapidly across the globe. It is very important not only to test and confirm the presence of KHV infection in Poland by the National Reference Laboratory for Fish Diseases of the National Veterinary Research Institute, but also to determine the spread of the disease in Poland. It is also essential to find out if KHV infection poses a threat to carps at low water temperatures which prevail for half of the year in Poland.

The information will be useful for developing a method to control KHV infection in the future, and to evaluate a risk assessment related to further spreading of this disease in Poland. It is worth noting that Poland is the biggest producer of carps in Europe and KHV infection could seriously jeopardise this branch of national carp production.

Material and Methods

Samples of 10 moribund carps were obtained from 13 traditional carp farms and 2 warm water cage farms. The sampling was done when disease symptoms similar to the ones described in KHV infection had appeared except sampling of one group of symptomless fishes (from a cage farm) which survived the mass mortality connected with a KHV infection. The gills and kidneys of the carps were examined using the PCR method according to Gilad et al. (4).

In case of KHV positive results alignment of the KHV nucleotide sequence was performed and the
results were compared with the data available at the GenBank.

Samples of blood, gills and kidneys obtained from carps from 8 out of 13 farms were used for the virus isolation. Leukocytes were separated from blood and co-cultivated with CCB cells for 10 to 14 d (Bergmann, unpublished data). The CCB cells (received from CIDC-Lelystad) were also inoculated with supernatants prepared from gills and kidneys, respectively.

Infected CCB cells were incubated at 22°C for 2 weeks and then passaged. When CPE developed in this cell line, the PCR method and electron microscopy were applied for virus identification.

A Polish KHV isolate (number 007) was used for the experimental infection of one year old carps (K1). For that purpose 10 carps were exposed to a virus suspension at a titre of 4x10^7 TCID50/ml in little aquaria with water of 22°C ±1°C for 20 min. In the same time the control group consisting also of 10 carps was exposed to cell culture medium, which is routinely used for CCB cell cultivation as a mock infection control. After exposure fishes were placed in 250 l aquaria with aerated, filtrated and heated water of 22°C ±1°C. During the experiment, moribund fishes were euthanized successively, using 0.2% Propiscin, 4 ml/l, then the gills and kidneys were examined for the presence of KHV nucleic acid with the PCR method.

Five of 10 carps which had survived the spontaneous KHV infection from a cage farm were sacrificed immediately after delivery and examined by PCR. Five that were left after a short adaptation period were put into an aquarium with filtrated and aerated water, the temperature of which was decreased to 11-12°C.

**Results**

In carps from 4 out of 15 examined farms (from two traditional farms and two cage farms) at least gills or kidneys, sometimes both, were KHV PCR positive (Table 1, Fig. 9).

By using the co-cultivation method the presence of KHV virus in leukocytes of carps from 2 out of 8 farms was demonstrated and with this method the virus was transferred from blood cells into CCB cell (Figs 1, 2). CCB cells inoculated with supernatants from gills and kidneys of these carps did not show any CPE. Examination of CCB cells co-cultivated with leukocytes in which CPE had appeared, showed under transmission electron microscope (TEM) the presence of the icosahedral enveloped virions measuring 110-125 nm (Figs 3a, 3b). Virions were located inside the nucleus and in the cytoplasm, sometimes in large congregations.

At 5 to 8 d post infection, all experimentally infected fishes showed symptoms characteristic of KHV infection, like gill necrosis (Fig. 4), sloughing of the epidermis (Fig. 5), and the black patches of skin discoloration (Fig. 6). Mostly, fishes died within 24-48 h after the first symptoms appeared.

<table>
<thead>
<tr>
<th>Farm number</th>
<th>Sampling date Year of production</th>
<th>Symptoms</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>2004 second</td>
<td>gill necrosis, high mortality</td>
<td>negative</td>
</tr>
<tr>
<td>II</td>
<td>2004 second</td>
<td>gill necrosis, high mortality</td>
<td>negative</td>
</tr>
<tr>
<td>III</td>
<td>2004 second</td>
<td>gill necrosis, high mortality</td>
<td>negative</td>
</tr>
<tr>
<td>IV</td>
<td>2004 second</td>
<td>gill necrosis, high mortality</td>
<td>negative</td>
</tr>
<tr>
<td>V</td>
<td>2004 third</td>
<td>intensive gill necrosis, excess mucus secretion on the skin, very high mortality</td>
<td>positive</td>
</tr>
<tr>
<td>VI</td>
<td>2004 first</td>
<td>slight gill necrosis, medium mortality</td>
<td>negative</td>
</tr>
<tr>
<td>VII</td>
<td>2004 third</td>
<td>intensive gill necrosis, excess mucus secretion on the skin, very high mortality</td>
<td>positive</td>
</tr>
<tr>
<td>VIII</td>
<td>2004 first</td>
<td>slight gill necrosis</td>
<td>negative</td>
</tr>
<tr>
<td>IX</td>
<td>2004 first</td>
<td>paleness of gills, necrosis of the distal regions of gill orches</td>
<td>negative</td>
</tr>
<tr>
<td>X</td>
<td>2004 first</td>
<td>paleness of gills, excessive mucus secretion</td>
<td>negative</td>
</tr>
<tr>
<td>XI</td>
<td>2004 first</td>
<td>gill necrosis, high mortality</td>
<td>positive</td>
</tr>
<tr>
<td>XII</td>
<td>2005 second</td>
<td>high mortality</td>
<td>negative</td>
</tr>
<tr>
<td>XIII</td>
<td>2005 second</td>
<td>high mortality</td>
<td>negative</td>
</tr>
<tr>
<td>XIV</td>
<td>2005 second</td>
<td>lack of symptoms</td>
<td>positive</td>
</tr>
<tr>
<td>XV</td>
<td>2005 spawners</td>
<td>lack of symptoms</td>
<td>negative</td>
</tr>
</tbody>
</table>
**Fig. 1.** CCB cells co-cultivated with leukocytes of control (healthy) fish.

**Fig. 2.** CPE in CCB cells co-cultivated with leukocytes of naturally KHV infected fish.

**Fig. 3a, 3b.** Koi carp herpesvirus in the infected CCB cell, TEM.

**Fig. 4.** Gill necrosis in carp yearling (K₁) induced by experimental KHV infection.

**Fig. 5.** Sloughing of the epidermis in carp yearling (K₁) induced by experimental KHV infection.
**Fig. 6.** Black patches of skin discoloration in carp yearling (K₁) induced by experimental KHV infection.

**Fig. 8.** Phylogenetic tree constructed from 484 nucleotides of the non-coding DNA sequence of KHV. Based on the percentage of identity and divergence (lower table) isolate No. 007 is situated between Polish isolates represented by sequences 4101-1/04, 4101-2/04, 4750-1/05 and the comparison due to short DNA fragment analysed 3 Polish sequences, in particular clones 4101-1/04, 4101-2/04, 4750-1/05 from slightly diverged genetic subgroup in comparison with the abroad isolated variants (AF411803 and AB127966).
Fig. 7. Alignment of the KHV nucleotide sequence (484 bp) in 4 Polish isolates with sequences deposited in GenBank.
The samples of gills and kidneys taken immediately after fish delivery from 5 out of 10 symptomless survivor fishes (from the cage culture) was weakly positive in the KHV PCR (showed very weak positive bands). In five fish from this group left for further observation 7-14 d after adapting them (in aquarium) to water temperature lowered to 11-12°C, the clinical symptoms characteristic of KHV infection have appeared. Examination of the gills and kidneys of these fishes using the PCR method was strongly KHV positive. Co-cultivation of leukocytes obtained from these fishes with CCB cells gave positive results and the examination of these cells with TEM showed typical icosahedral virions.

Analysis of the sequencing data of 484 bp fragments of KHV DNA of 4 Polish isolates revealed the complete identity in 3 cases (isolates No. 4750-1/05, 4101-1/04 and 4101-7/04). The fourth case (isolate No. 007) differed from the remaining 3 variants by 5 nucleotide substitutions (Figs 7, 8).

Alignment of the 484 bp fragments of KHV DNA sequence of 4 Polish isolates with corresponding fragments available in GenBank (isolates No. AB127966 and AF411803) revealed also very small differences (only 3 single nucleotide substitutions) between clones 4750-1/05, 4101-1/04, 4101-7/04, 007, and AF411803, and 2 more substitutions in case of clone AB127966. In case of clone 007 we found 4 nucleotide substitutions in comparison with the abroad isolated variant AF411803, and 6 nucleotide substitutions compared to clone AB127966 (Figs 7, 8).

**Discussion**

The results of our investigations confirmed the presence of KHV infections in Poland in traditional carp farms and in cage farms. The preliminary monitoring for KHV infections in 2004-2005 showed that the disease is wide spread and causes serious carp mortality at water temperatures above 18-20°C, which occur in Poland usually in August and September. The presence of KHV was demonstrated in 4 out of 15 farms suspected of KHV infection. In 11 negative farms the cause of mortality was probably due to environmental factors. Necrotic processes in the gills could appear as a result of environmental branchionecrosis, and eventually as a result of gill blockade with sediments and algae.

During screening of the first 7 farms we were able to use only one modification of the PCR method and only gills and kidneys of carp were examined. According to Haenen *et al.* (5) KHV is still a "young disease" and new tests have only recently been described, but it was advised to use at least two of the available diagnostic methods in parallel, to improve the accuracy of KHV diagnosis.

Co-cultivation of the leukocytes with CCB cells used for the screening of the 8 other farms proved to be more sensitive compared to cell inoculation with supernatants from carp organ suspensions which all considered to be KHV negative.

In our experiments 20 min exposure to the virus suspension at 4x10³ TCID₅₀/ml was sufficient to infect carp. This phenomenon gives evidence to a great infectivity and virulence of this virus. It is also worth noting that the experimental infection was performed in optimal conditions for carp. These fish were well adapted to aquarium conditions (3 months prior to the infection) and properly fed, water was well aerated and filtrated all the time. All control fishes were healthy and robust during the experiment and also many months after and checked afterwards were negative in the PCR tests.
The results of our experiment with carps, which survived spontaneous infection of KHV and developed again the disease symptoms in an aquarium with cold water (11-12°C), correspond well with field observations. Many Polish carp breeders have the opinion that KHV survivors die during the winter, although the disease symptoms are observed usually at temperatures between 17°C and 26°C. According to the results of KHV monitoring in Poland and other reference data the disease appears in August and September (4). According to Ronen et al. (8) in Israel KHV infection appeared predominantly in spring and in autumn at temperatures of 18-25°C. Hoffman (6) stated that in carp kept in the basins with (artificially) heated water there is no relationship between the season and the appearance of KHV infection cases. Single disease cases were observed also at 16°C (3) but there were no reports of KHV clinical symptoms below this temperature. Considering the fact that survivors of a summer KHV infections perish during the winter, the cumulative mortality due to KHV infection in traditional carp farms in Poland (during the summer and winter) often reaches 100% in single ponds. Based on these data it could be stated that KHV infection is the most dangerous carp infection in Poland. A programme of KHV control adapted to traditional carp cultivation in earthen large ponds and cage culture should be established. For official realization of such a programme it is indispensable to place KHV infection on the list of notifiable diseases as it was already done in proposal of Council Directive SEC(2005)1047.

The significance of the small differences in sequence data is difficult to interpret because corresponding amino acid sequence was not available. It is quite possible that single nucleotide deletions or substitutions present in the analysed sequences do not have any consequences on the phenotype of the corresponding virus variants due to fact that they are located in the non-coding fragments of KHV DNA. Also eventual differences in the pathogenicity of the isolates were not analysed in detail due to abundance of other possible factors which might have influenced the course of the infection, for instance different immunological status of the virus hosts, different environmental conditions (water temperature) and facultative pathogens possibly present in the place from which affected fishes originated.

In conclusion, the lack of significant differences in DNA sequences between KHV isolates originating from Poland and abroad confirms either a high degree of homogeneity in this group of viruses or a common place of origin. Nevertheless, if the observed differences at DNA level are to be further confirmed at amino acid level such observation could be helpful in the creation of tools for epidemiological tracking of the pathogen, and can also be explored further in virulence studies.

References