First clinically apparent koi herpesvirus infection in the Czech Republic

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Abstract
Koi herpesvirus disease (KHVD) is a highly contagious viral illness causing significant morbidity and mortality in common carp (Cyprinus carpio). KHVD has been confirmed in almost all countries around the world except Australia. KHV has been previously detected in the Czech Republic based on PCR screening but with no clinical signs of KHVD. An outbreak of KHVD with clinical signs, and severe mortality was confirmed in July 2009 by histopathology and virological testing of hepatopancreas, anterior kidney, spleen and gill samples.

Introduction
Koi herpesvirus (KHV) is a highly contagious viral illness that can cause significant morbidity and mortality in common carp (Cyprinus carpio) (Hedrick et al., 2000). The aetiological agent is a DNA virus belonging to the virus family Herpesviridae - cyprinid herpesvirus-3 (CyHV-3) commonly known as KHV (Ronen et al., 2003; Waltzek et al., 2005). This virus is closely related to carp pox virus (CyHV-1) and hematopoietic necrosis herpesvirus in goldfish (CyHV-2). The first recognized case of KHV occurred in England in 1996, with subsequent confirmation in almost all countries that culture koi and/or common carp, with the exception of Australia (Hedrick et al., 2000). Other related cyprinid species such as the common goldfish (Carassius auratus) and grass carp (Ctenopharyngodon idella) do not show clinical signs of KHV although DNA of this virus has been identified in tissue of these species using polymerase chain reaction (PCR) (Bergmann et al., 2010). Hybrid goldfish (male goldfish C. auratus x female common carp (C. carpio) was moderately resistant to mortality following experimental infection with KHV (Hedrick et al., 2005). Presence of KHV was also suspected in the Czech Republic based on the screening of PCR results from carp that did not manifest with any clinical signs (Pokorova et al., 2005; Pokorova et al., 2007). No clinical cases of KHVD were reported in the Czech Republic until the occurrence in July 2009.

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Materials and Methods

Origin of the fish

The koi carp originated from a private garden pond (vol. 12 000 l) in Lazne Bohdanec district. Fish stock within the pond consisted of koi carp (n = 48) ranging from 10 to 60 cm body length, goldfish (n = 26), ornamental tench (n = 3) and Caspian sturgeon (n = 2). The water temperature was 22°C. The water purity was maintained by a multi-segment (mechanical and biological) filters. The fish were fed a commercial diet for koi carp. All species in the pond did not show any signs of infection until the described case.

Diagnostic methods

Pathology

Five individuals of koi carp suspected of having KHV infection based on clinical signs, were euthanased with overdosing of tricaine methanesulphonate (MS – 222). Fresh mounts and histopathology samples were taken from gills, hepatopancreas, anterior and posterior kidney, spleen and gut. Samples for histopathology were processed with 10% buffered formalin and fixed for 24 h. Samples were embedded in paraffin, sections cut at 5 μm thickness and stained with haematoxylin and eosin (H&E) or as per the periodic acid-Schiff (PAS) method.

Virology

Sample collection for virological testing

Individual samples of hepatopancreas, anterior kidney, spleen and gills were collected from two fish. After dilution with culture medium (1:5 v/v), organ homogenates from each fish were centrifuged and a part of the supernatant was used for nucleic acid extraction for virus detection by PCR. The other part of supernatant was used for virus culture isolation in the cell line Cyprinus carpio brain (CCB) (Neukirch et al., 1999).

DNA extraction

The nucleic acid extraction was performed using QIAamp Viral DNA Kit (Qiagen, Germany). As recommended by the manufacture protocol, 200 μl of supernatant was mixed with 20 μl proteinase K and 200 μl Buffer AL, vortexed, and incubated at 56 °C for 10 min. After lysis, DNA extraction using ethanol and DNA spin columns was performed as per manufacturer’s instructions. DNA obtained from 200 μl of tested material was dissolved in DNase and RNase – free water and subsequently used in PCR.

PCR assay

Two primer sets (external and internal) based on KHV thymidine kinase (TK) detection were used in this study. The external primer pair, designed by Bercovier (Bercovier et al., 2005): F 5’- GGG TTA CCT GTA CGA G-3’/ R 5’- CAC CCA GTA GAT TAT GC-3’ was used for PCR detection of virus. The internal primer set was provided by Dr. D Stone (Cefas Weymouth Laboratory), the OIE Reference laboratory for Koi herpesvirus disease and was used for nested PCR: F 5’- CGT CTG GAG GAA TAC CCA GTA GAT TAT GC-3’ and R 5’- ACC GTA CAG CTC GTA CTG G -3’. The sizes of the amplification products were 409 bp and 348 bp, respectively. The cycling conditions consisted of 94 °C/5 min, followed by 40 cycles (30 cycles, respectively) of one minute intervals at 95 °C, 55 °C, 72 °C and finally extension was performed at 72 °C for 10 min. A positive control was prepared from tissue homogenates from koi carp.
experimentally infected with KHV (KHV-I), provided by the Friedrich-Loeffler-Institute, Insel Riems, Germany. A negative control was prepared from non-infected CCB cell cultures. Positive and negative controls were examined with each group of analysed samples. PCR products were separated on a 2% ethidium bromide stained agarose gel and visualised by UV.

**Results**

**Clinical history**
Clinical signs were observed by the owner 3 days after adding 10 new koi carp imported from Thailand. The koi carp remained near the surface, swam lethargically, exhibited respiratory distress and uncoordinated swimming. The first fish died the next morning and over the next 4 days 38 koi carp died. Other species in the pond did not exhibit any signs of infection.

**Post mortem**
Immediate post mortem observations of the fish were sunken eyes, pale skin patches approximately 1 cm in diameter, severe necrosis of gill lamellae with loss of epithelium and also gill hyperaemia (Figure 1). Numbers of *Trichodina* sp., *Ichthyobodo necator* and monogenean were observed in the fresh mount samples of the skin and gills and also severe necrosis of secondary gill lamellae. Histopathology confirmed severe gill necrosis with the secondary infection by *Trichodina* sp. and occasional intranuclear inclusions in the respiratory epithelial cells (Figure 2). An inflammatory infiltration with lymphocytes and heterophils was observed in the interstitial tissue of the posterior kidney and collecting ducts.

**Virology**
Samples of fish tissues and gills were examined by two-round PCR for KHV virus detection. The samples were found to be positive in both the 1-step and nested PCR. Specific bands of expected size were detected (Figure 3).

![Figure 1. Koi carp, operculum removed. Severe necrosis of gill lamellae with loss of tissue.](image-url)
Figure 2. Occasional inclusion bodies have been observed in the gills epithelial cells. Haematoxylin and eosin.

Figure 3. Electrophoresis of KHV-DNA in tissue homogenate sample

<table>
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<tr>
<th>PCR with external primers (Bercovier)</th>
<th>PCR with internal primers (nested PCR)</th>
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<td>MM 1 2 3</td>
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< 409 bp <- 348 bp

**MM** - The Truckit™1kb Plus DNA Ladder, lane 1- positive KHV control, lane 2- negative control, lane 3- examined sample
Virological examination consisted of inoculation of homogenate prepared from pooled organs and gills of infected koi carp onto the CCB cell line. Inoculated tissue cultures did not show any CPE even after two additional subcultivations.

**Discussion**

KHVD is an ubiquitous worldwide disease. Until July 2009 KHVD had not been reported in the Czech Republic. Clinical signs and post mortem and virological examination of diseased koi carps led to the diagnosis of KHVD in a Czech garden pond.

There are several diseases which can result in gill necrosis in carp: toxic gill necrosis, bacterial gill disease, branchiomycosis and infection with *Sanguinicola inermis*. Branchimycosis is easy to diagnose using optical microscopy of fresh mounts or histological slides of the gills as there are many hyphae in the branchial tissue (Khoo, 2000). Infection with *Sanguinicola inermis* is also deferrable by gill histopathology (Kirk and Lewis, 1998). *Flavobacterium columnare* infection can also causes gill necrosis, but lesions are usually restricted to skin and fins. This infection can be detected by cultivation or PCR diagnostic techniques (Tripathi et al., 2005; Triyanto et al., 1999). Toxic gill necrosis, caused by highly increased levels of ammonium requires the assessment of blood ammonium (Svobodova et al., 1995). Excessive proliferation of gill epithelium as well as degeneration and necrosis is often observed in KHVD. Infected cells can be identified by intranuclear inclusion bodies. Necrosis is also observed in the liver, kidney and gastrointestinal tract together with infiltration of macrophages (Hedrick et al., 2000). Gill necrosis and enophthalmos are frequently seen in KHVD but are not pathognomonic. The only method to ensure a precise diagnosis of KHVD is by using PCR (OIE Manual of Diagnostic Tests for Aquatic Animals, 2009). The PCR method used in this case showed the presence of a thymidine kinase and a specific band of 409 bp (Bercovier et al., 2005). It is recommended to use at least two PCR methods (OIE Manual of Diagnostic Tests for Aquatic Animals, 2009) so nested PCR was also used and a specific band of 348 bp was detected. Virus isolation on the CCB cell line was not successful in this case which was not unexpected as it is difficult to infect cell lines with KHV (Haenen et al., 2004).

The introduction of koi carp from Thailand resulted in a high mortality of koi carps in a Czech garden pond. Based on clinical observation and histopathology an infection of KHVD was suspected. The presumptive diagnosis was confirmed using PCR methods as the presence of KHV DNA was shown.

KHVD is on the list of dangerous infections in the Czech Republic and the outbreak described in this case was contained and decontaminated. There is currently no cure for KHVD however there is an active programme for vaccine development which was initiated after discovery of KHVD (Ronen et al., 2003; Perelberg et al., 2005). The results of experimental vaccine studies have shown that immunized fish, even with no detectable antibodies, are resistant to virus infection. This is probably due to the subsequent rapid response of high affinity anti-virus antibodies (Perelberg et al., 2008). The quarantine, prevention, appropriate diagnosis and
immediate response to an outbreak are the most important tools controlling the spread of KHVD.

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References


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