Detection of koi herpesvirus (KHV) in koi carp (*Cyprinus carpio* L.) imported into Ireland.

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Abstract

This report described the first detections of koi herpesvirus (KHV) in the Republic of Ireland in imported koi carp. In both cases the KHV suspicions were confirmed by molecular diagnosis and the infected stocks culled.

A new disease resulting in large mortalities of common and koi carp was first reported following outbreaks in Israel, USA and Germany in the late 1990's (Bretzinger *et al.,* 1999; Hedrick *et al*., 2000). The disease was subsequently identified as being caused by a cyprinid herpesvirus termed koi herpesvirus (KHV; Waltzek *et al*., 2005). The virus has now been reported in Japan (Sano *et al*., 2004), Taiwan (Tu *et al*., 2004), south-east Asia and throughout Europe (Haenan *et al*., 2004; Bergmann et al., 2006). This has led to the disease been added to the list of notifiable diseases to the World Organisation of Animal health (OIE) and it is also listed as a non-exotic disease in the recently enacted fish health regulations in the European Union (EU) (Directive 2006/88/EC). In Ireland, a national KHV surveillance programme commenced in early 2010, as provided for under the above Directive. At least for the duration of the programme, all imports of susceptible species must be certified as originating from a KHV free area.

In August 2005, the Fish Health Unit at the Marine Institute received a report of mortalities of koi carp in a garden pond in Dublin. The owner had reported that five recently purchased fish had been introduced to the pond in mid-July, but had died within a week of the introduction. Subsequently the original stock of koi carp began to exhibit signs of disease and mortalities were observed. An examination of the fish showed severe necrosis of the gills with secondary fungal growth (Figure 1). Tissue samples (gill, heart, and kidney) were taken and fixed in 10% formalin for histological analysis. Kidney swabs were plated onto tryptone soya (TSA) and Colombia blood (CBA) agar plates and incubated at 22^{o}C for bacteriological analysis. For virological analysis, one sample of gill and a pooled sample of kidney, spleen and heart were taken. Additional gill samples were stored in 70% alcohol for molecular analysis according to Bercovier *et al*. (2005).

Histopathological results showed severe gill erosion with hyperplasia of the remaining lamellae. Mats of filamentous bacteria (*Flexibacter*/*Flavobacterium* sp.), low numbers of gill protozoans and fungal hyphae were all observed in the damaged gill tissue. There were also masses of eosinophilic ghost type cells at the tips of the primary lamellae. Further staining (Giemsa and Periodic acid-Schiff) did not show positive reactions in these cells which were believed to be degenerating epithelial and/or eosinophilic granular cells. In addition, a multifocal cardiomyopathy was present in the cardiac ventricle with low numbers of hypertrophied myocytes also observed.

No growth was observed on the TSA and CBA plates. All samples were negative for a range of pathogenic fish viruses including spring viraemia of carp virus (SVCV). Although no viral growth was observed after inoculation of gill homogenates onto Koi Fin (KF-1) cells, analysis of preserved gill tissue samples by PCR and subsequent genetic sequencing of the amplified products confirmed the presence of KHV. This represented the first detection of KHV in koi carp in Ireland. Contact tracing was carried out and all fish from the imported batch were culled.

A second case of KHV in ornamental fish was suspected in koi carp imported into Ireland in August 2007. A consignment of 150 koi carp from Singapore entered Ireland through the Border Inspection Post (BIP) at Dublin Airport. On inspection, the consignment was found to contain a high number of moribund fish. Samples of gill tissue from eleven live fish were sent for histological, virological and molecular analysis as described above. The remainder of the stock was placed in quarantine.

The histopathology results revealed a low level myxosporidean infection in the kidney tubules. The gill had moderate to severe hyperplasia (Figure 2) and low levels of gill flukes. Scattered necrotic hepatocytes with eosinophilic inclusions were observed in liver sections (Figure 3).

The samples were tested for SVCV and KHV by cell culture and were found to be negative. Despite this, four of the eleven gills sampled were positive by nested PCR assay using KHV-TK primers according to Bercovier *et al*. (2005). Sequence analysis further confirmed the pathogen as KHV and the sequenced products from 2005 and 2007 were identical (Figure 4). The gill pathology, which was considered indicative of periods of poor water quality and parasitism, may have contributed to stress in the fish allowing a re-activation of a latent KHV infection within the carp. Alternatively the fish may have succumbed to secondary parasitic and bacterial infection having first been clinically infected with KHV. All of the fish from the imported batch were culled.

KHV is a world wide disease of carp. The global spread of KHV is heavily connected with the worldwide fish trade (Novotny *et al*., 2010), and the control of this spread is often hampered by the illicit transport and trading of koi (Somga *et al*., 2010).' Movement of carp showing no signs of the disease but which harbour persistent or latent viral infections have been strongly implicated in the spread of the virus (Taylor et al., 2010). A recent study by Bergmann *et al*. (2009) also suggests that other ornamental fish species may act as carriers of the virus. With the exception of the cases described above, there have been no recorded outbreaks of KHV in Ireland to date. The described detections of KHV highlight the need for increased surveillance for this disease and also emphasise the importance of accurate certification of susceptible species which are destined for areas which are free of the disease or which are operating KHV disease surveillance programmes.

KHV is now listed as a non-exotic disease under Directive 2006/88/EC and as such, all Member States must have access to appropriate diagnostic tools to detect the presence of the pathogen. Where KHV freedom is not an option in certain Member States, the disease must however, be contained and every effort must be made to minimise disease spread. In that context, molecular methods continue to be the most reliable and sensitive methods for detecting KHV in infected fish (Bergmann *et al*. 2010) and it is important to note that without the use of these methods, KHV would not have been identified in the cases described above. The use of these molecular tools together with the registration of ornamental importers, aquaculture operators and transporters, should allow greater control of this important disease in Ireland and indeed, across Europe.

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Figures and legends

Figure 1. Gill condition of a koi carp from the affected pond in 2005 illustrating severe necrosis of the gill lamellae.

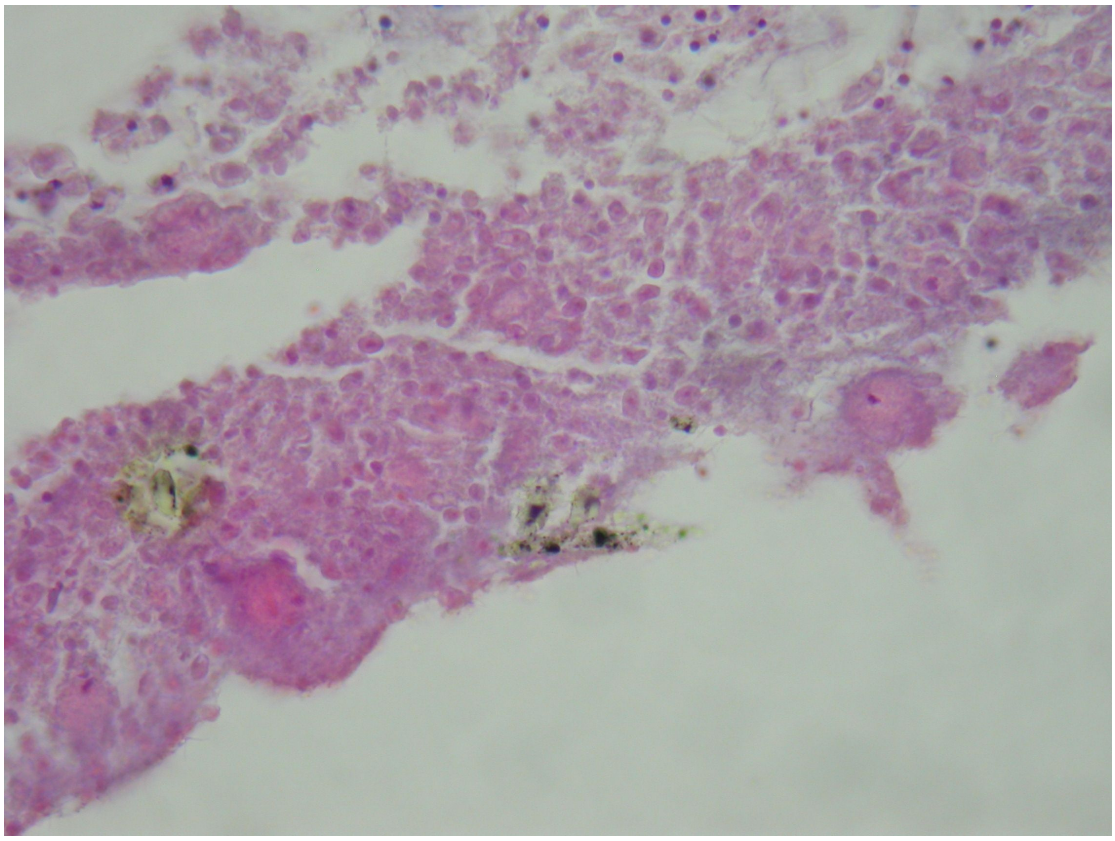

Figure 2. Histological section of koi carp gill tissue from the 2007 case, showing loss of gill structure and severe hyperplasia (H & E x200).

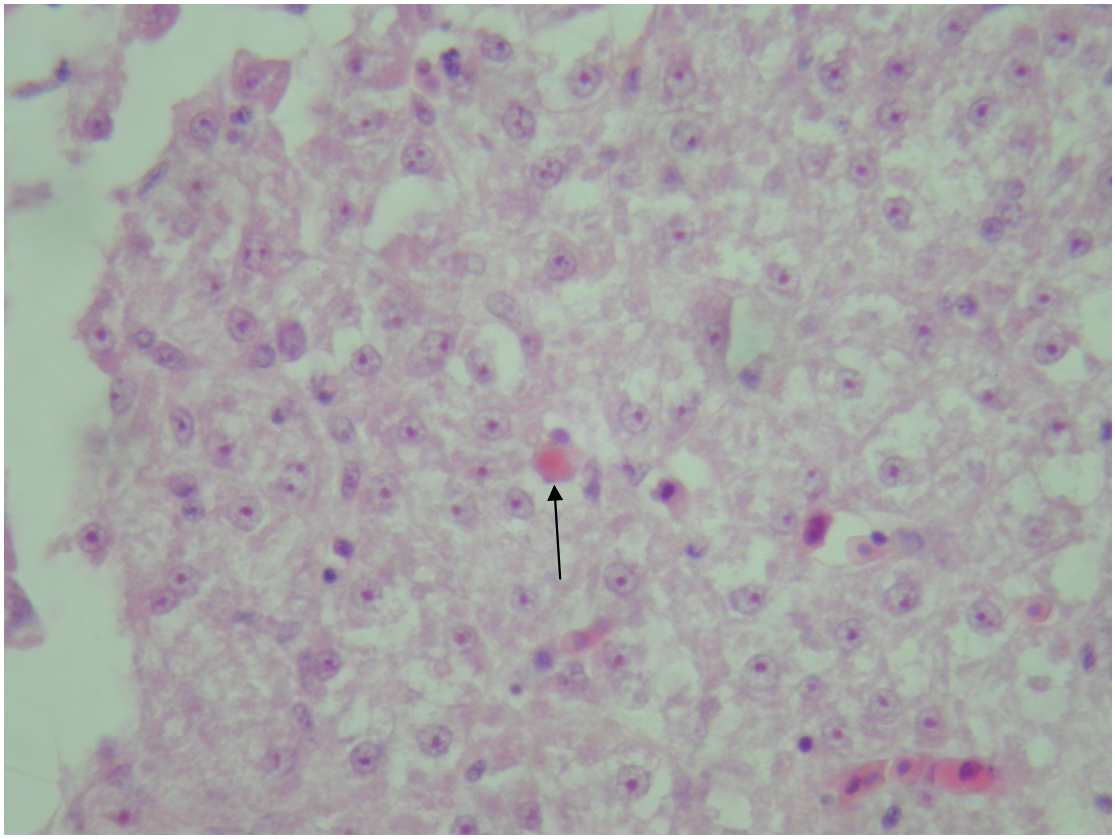

Figure 3. Histological section of koi carp liver from the 2007 case, showing scattered necrotic hepatocytes and eosinophilic inclusions (arrow) (H & E x200).

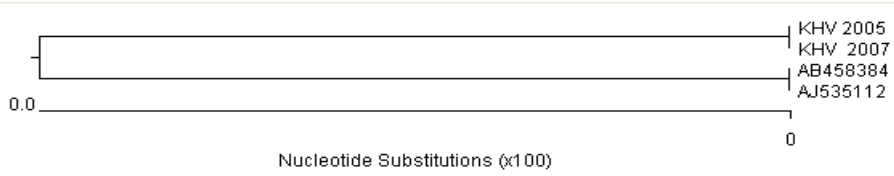

Figure 4. Phylogenic tree showing genetic relatedness of KHV isolates from 2005 and 2007 with representative GenBank published sequences at the nucleotide level.