

# The emergence of koi herpesvirus and its significance to European aquaculture

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## Introduction

With the expansion of the European Community (EC) towards the East, the constitution of the aquaculture industry in the EC will change, and along with it the type of fish diseases to be encountered. Until now the EC network of national fish disease laboratories have focused mainly on diseases of salmonids. With the inclusion of Eastern European countries, and more intensive and extensive pond culture of carp and other fish species, we can expect an increase in the proportion of disease problems occurring especially in cyprinids on both sides of the accession line. As a preparation for the necessary carp disease diagnosis, the Community Reference Laboratory (CRL) for Fish Diseases and the UK National Reference Laboratory (NRL) CEFAS in Weymouth organised a workshop on diseases of carp attended by all of the EC NRL's for Fish Diseases in June 2003. One of the topics of major concern at the workshop was Koi Herpesvirus (KHV).

KHV causes severe disease and mortalities in all ages of common carp and koi carp (*Cyprinus carpio*) and is spreading rapidly across the globe. The first known occurrence of the disease described in koi carp in Europe is dated to 1996 but the first major outbreaks of KHV disease were seen in farms culturing common and koi carp in Israel in 1998. Japan experienced its first outbreak of KHV in May 2003, followed by extensive outbreaks of KHV in cage-cultured carp in October 2003. In 2003, German common carp farms also suffered severe mortalities due to KHV after receiving imports of carp from outside the EC. It is now time to seriously reconsider the view, that KHV is a disease limited to the ornamental fish trade, and to evaluate the means with which its impact on European aquaculture can be minimised.

This paper gives a review of the emergence of KHV, the current global situation with special attention to the increased severity of outbreaks in Asia, and a discussion of the dilemma in application of diagnostic methods, with recommendations on combinations of diagnostic techniques and strategies for prevention and control of KHV in Europe.

### Disease characteristics

The disease occurs naturally at temperatures between 17°C and 26°C with an incubation period of 7-21 days depending on water temperature. Morbidity is often 100% with mortality up to 90% at higher temperatures. Behavioural signs of disease include lethargy, fatigue, disorientation, erratic swimming and frequent ventilation (gasping). Fish can die within hours of the first signs appearing, but at lower temperatures the course of the disease is more protracted (Walster, 1999). The most consistent gross clinical sign of disease is an irregular discolouration of the gills consistent with moderate to severe gill necrosis. Other commonly reported clinical signs include anorexia, enophthalmia (sunken eyes), fin erosion, superficial haemorrhaging at the base of the fins, pale, irregular patches on the skin associated with excess mucus secretion and also decreased production of mucus in patches, leaving the epidermis with a sandpaper-like texture. Internal gross pathological signs are inconsistent but enlarged anterior kidney, in early stages of the disease a swollen spleen, and a flaccid and mottled appearance of the heart has been reported. Concomitant parasite infections are commonly reported and include light to heavy infestations by *Ichthyobodo* sp., *Trichodina* sp., *Ichthyophthirius* sp., *Dactylogyrus* sp., *Chilodonella cyprini* and monogenean parasites. Bacterial infections have also been observed with *Aeromonas* sp., *Pseudomonas* sp. and *Shewanella putrefaciens* most predominantly reported. Infection with opportunistic, secondary parasite and bacterial pathogens can present a diagnostic problem for fish veterinarians and pathologists by obscuring virus specific

pathology. Descriptions of behavioural and clinical signs of the disease, internal pathological signs and secondary pathogens have been reported in detail by Bretzinger et al. (1999), Hedrick et al. (2000) and Perelberg et al. (2003).

During KHV disease outbreaks in polyculture systems, mortalities have been restricted to *Cyprinus carpio* and its varieties (Bretzinger et al., 1999; Walster, 1999). A study in Israel showed no clinical transmission of the disease from KHV-infected carp to five commonly cultured fish species, including tilapia (*Oreochromis niloticus*), goldfish (*Carassius auratus*) and silver carp (*Hypophthalmichthys molitrix*). In a subsequent experiment, the virus-exposed resistant species did not transmit the disease to healthy carp (Perelberg et al., 2003). However, no tissue samples were taken from the resistant species to test for the presence of KHV following exposure.

A number of research studies on KHV have been undertaken at laboratories in the USA, Israel, Germany and the UK. Virus characterisation studies, at the Hebrew University-Hadassah Medical School in Jerusalem, have shown that the virus isolated during disease outbreaks in Israel is morphologically similar to, but has a larger genomic DNA (277 kbp) than that of other known herpesviruses. Also, only small sequences of the genome match any published virus sequences. Based on these observations they have named their unclassified virus carp nephritis and gill necrosis virus (CNGV) rather than adopt the name KHV (Ronen et al., 2003). In contrast, genome characterisation studies at the CEFAS Weymouth laboratory have identified a number of putative genes

and some of these share significant homology with genes found in Channel Catfish Virus (CCV) and other herpesviruses (Way et al., 2004a). More recently, genome studies at the School of Veterinary Medicine, Davis, USA and CEFAS, Weymouth have shown good homology between putative genes of KHV and those of Herpesvirus cyprini (CyHV 1) the carp-pox virus (Hedrick et al., 2004).

### **Emergence and current global situation**

#### *Europe and USA*

The first report of a disease epidemic causing mass mortality of carp in Israel was presented at the 9th International Conference of the European Association of Fish Pathologists (EAFP) in 1999 (Ariav et al., 1999). The first outbreaks of disease in cultured carp occurred near the northwest coast of Israel in early May 1998 and in the following three years regular outbreaks were seen in spring and autumn at water temperatures between 22 and 26°C. By the end of 2000 the disease had spread to 90% of the carp farms in Israel and has been estimated to cost Israeli aquaculture 3 million US\$ every year (Perelberg et al., 2003).

Then followed the first outbreak of disease in the USA, which was seen toward the end of 1998 at a koi show in New York State. Tissue samples from this outbreak and also from an outbreak at an Israeli carp farm were sent to the University of California's School of Veterinary Medicine, Davis, USA and a virus was isolated in a koi fin (KF-1) cell line. Initial characterisation studies indicated a herpesvirus and this was shown to be the causative disease agent by virus transmission studies in carp (Hedrick et al., 2000). Virion

polypeptide and genomic restriction fragment analysis showed the US and Israeli isolates to be identical but the virus was distinct from Herpesvirus cyprini (CyHV 1), a commonly encountered herpesvirus infecting carp. However, the virus did share some characteristics with CyHV 1 and to a lesser extent with another herpesvirus of fish, Channel Catfish Virus, CCV. Also, to allow rapid diagnosis of KHV, a polymerase chain reaction (PCR) assay was developed to detect KHV DNA in clinically infected carp (Gilad et al., 2002). Later in the USA, Gray et al. (2002) reported two further KHV outbreaks that occurred in Ventura county and Los Angeles, California during 1999. Epidemiologically distinct isolates from these outbreaks were shown to be identical by restriction endonuclease (RE) profiling of the virus DNA.

The first published report of KHV disease in Europe described outbreaks of disease with mass mortality that occurred in koi ponds and koi dealerships in Germany in 1997 and 1998 (Bretzinger et al., 1999; Hoffmann, 2000). Gill tissue from affected carp was examined by transmission electron microscopy and herpesvirus-like particles were seen in the nucleus and cytoplasm of respiratory epithelial cells. Also, in a small trial, the disease was transmitted to healthy koi and mirror carp by co-habitation with sick koi carp. Later, Neukirch & Kunz (2001) reported the isolation of a virus from German koi carp suffering mass mortality that they provisionally classified as a herpesvirus. The virus was isolated on two cell lines from carp brain (CCB) and carp fin (Ca F-2) tissue with a cytopathic effect (CPE) characterised by vacuolisation and giant cell formation

(syncytia). In the same publication the authors also mentioned a report, given at a meeting in 2000 of the German branch of the EAFF, on the first outbreak of the disease in mirror carp on a German fish farm. A mass mortality occurred at the farm following the introduction of koi carp 8-10 days previously.

Outbreaks of disease in UK koi carp had been reported by Walster (1999 and 2000), who described clinical disease signs that were very similar to those seen during the Israeli, US and German outbreaks. Then, at the 10th International Conference of the EAFF, Way and colleagues (2001) reported the first isolation of KHV from koi carp in the UK. The virus was isolated from a disease outbreak in koi imported from Israel in 2000 and characterisation studies showed the isolate to be very similar to KHV isolated in Israel and the USA in 1998. Further isolations were made at UK sites in 2000 and 2001 from koi imported into the UK from the USA, Israel and Malaysia (Way et al., 2001). However, KHV DNA could be amplified by PCR from many samples of koi tissue where no virus could be isolated in cell culture (Le Deuff et al., 2001). Later, a molecular comparison was made of six of the UK isolates, from geographically diverse sources, and they were shown to be very similar to each other by virion polypeptide and genomic restriction fragment analysis. It was suggested that the presence of a high level of homogeneity among KHV isolates indicates a rapid global spread of one prominent virus isolate from a single or limited source (Gilad et al., 2003). At the CEFAS Weymouth laboratory in the UK and at the German reference laboratory for fish diseases, *in situ* hybridisation (ISH) and PCR

methods are being used to analyse archive histological material. Using these methods, KHV DNA has been detected in tissue samples taken in 1996 during an unexplained mass mortality of koi and common carp in the UK (Way et al., 2004b).

Until recently in the UK, KHV had been restricted to ornamental carp. However, during 2003 the CEFAS Weymouth laboratory isolated KHV from common carp during investigations into large mortalities of carp in the wild. It is suggested that the spread of KHV is linked to the rearing or holding of common carp, destined for restocking fisheries, with ornamental varieties of carp. Also, in some cases, fishery owners have stocked their waters with ornamental carp such as ghost koi (common x koi carp) (Denham, 2003).

After reports had been presented at the meeting on KHV disease in Munich in December 2003, nine EC-member countries and 1 non-member had reported KHV disease outbreaks and there was suspicion of a disease outbreak in another new EU-accession country. A summary of reported KHV disease outbreaks in Europe is given in Table 1. Germany, the UK and The Netherlands appear to have recorded the most disease outbreaks. This may be a reflection of the greater trade in ornamental carp in these EC-states but it may also reflect the greater awareness of the disease in these countries.

## **Global spread of KHV**

### *Indonesia*

Further global spread of the disease was feared when, in mid-April 2002, a serious

Country	Current situation	Source of information
<i>European countries with KHV confirmed</i>		
Austria	First outbreak reported summer 2003 - confirmed by PCR.	Reported at Munich KHV Meeting, December 2003.
Belgium	First outbreak 1999 - not confirmed. Outbreaks in 2002 & 2003 - confirmed by PCR.	CER, Marloie, Belgium; CEFAS, Weymouth.
Denmark	First outbreak in 2002 and two further in 2003 - confirmed by PCR.	DFVF, Aarhus.
France	Two suspicions in 2001; first confirmed outbreak in 2003.	Munich meeting, 2003.
Germany	First outbreak in 1997 - over 80 cases in 2002 and 100+ in 2003 - confirmed by PCR, RT-PCR and <i>in situ</i> hybridization.	FLI, Insel-Riems; University of Munich.
Italy	Strong suspicion by EM and Histology.	Munich meeting, 2003.
Luxembourg	Outbreak in 2003.	Munich meeting, 2003.
The Netherlands	First detected in 2001 by PCR; Disease cases confirmed in 2002; Further outbreaks in 2003 and 2004.	CIDC-Lelystad.
Switzerland	First report in 2003 - suspicion before in 2001.	Centre for Fish and Wildlife Health, Univ. of Berne; Munich meeting, 2003.
UK - England & Wales	First isolated in 2000 - suspicion in 1998 and 1999 & earlier in 1996 - confirmed by PCR.	CEFAS Weymouth.
Poland	First detected in 2003 in carp, 2 outbreaks in 2004 in carp farms. Confirmed by PCR's, RT-PCR and <i>in situ</i> hybridization.	Insel-Riems/ Vet.Res.Inst., Pulawy.
<i>Countries where no KHV has been reported (to date)</i>		
Czech Republic, Finland, Greece, Hungary, Republic of Ireland, Russia, Slovenia, Spain, Sweden, parts of UK (Scotland & N.Ireland).	NVFRI (EELA), Helsinki / A.Prapas (pers.comm.) / CVI, Budapest / Marine Inst., Abbotstown/VNIIPRH/ Univerza V.Ljubljani/ J.Barja (pers.comm.) /A.Hellström (pers.comm.)/ FRS, Aberdeen & DARDNI, Belfast.	

**Table 1.** Koi herpesvirus disease in Europe: the current situation.

disease outbreak causing high mortality in koi and common carp was reported affecting the East of Java Island in Indonesia (Rukyani, 2002). The disease etiology and clinical signs were characteristic of KHV disease. The University of California School of Veterinary Medicine confirmed the presence of KHV DNA in tissue samples by PCR (Hedrick

pers.comm.), and additionally by RT-PCR and *in situ* hybridization at the NRL for Fish Diseases in Germany (Bergmann pers. comm.). An importation of koi from Hong Kong was thought to be a possible source of the infection. By November 2002 the disease had spread to Sumatra, with mortalities averaging 80%, and then further to Bali, East

Kalimantan and Central Sulawesi. During the epizootic, very high mortalities (80-95%) were seen in both koi and common carp, with estimated losses of over 15 million US\$ up to December 2003. Outbreaks occurred after heavy rain, or movements of adult carp or fry. Clinical signs were similar to those reported from Europe, but the fish showed also blister-like lesions on the skin, and haemorrhages in the operculum, fins, tail and abdomen. The Indonesian government declared Java and Bali Islands as an isolated area with movement of all carp from this area to other islands prohibited unless quarantine checks for KHV disease have been carried out. Additionally, imports of koi and common carp are now only allowed from KHV free countries. (Sunarto & Rukyani, 2004).

#### *Japan*

In Japan a survey to detect KHV in koi carp was conducted in October and November 2001 in Niigata prefecture. Fish were sampled from 20 farms, analysed by PCR assay and found negative for KHV (Amita et al., 2002). However, in October 2003 an outbreak of KHV disease was reported affecting food carp cultured in two lakes in Ibaraki prefecture in Japan (Sano, 2004). Over the next two months an estimated 1200 tonnes of carp died in the two lakes and the disease epidemic then spread to 23 of Japan's 47 prefectures (Kimiya, 2004). New legislation was introduced in Japan in July 2003 that included a requirement for an infection-free certificate and Ibaraki prefecture officially prohibited movements of common carp from the affected areas to other areas. The disease threatened the 75 million US\$ ornamental carp (nishikigoi) industry in Japan and all nishikigoi shows were cancelled for November 2003.

Niigata prefecture screened for the presence of KHV by PCR, in December 2002 at 30 breeders, in July-August 2003 at 114 breeders and, so far in 2004, at 150 breeders, that export overseas. Fish were held at 20-25°C and all were found negative for KHV. The export policy has been revised and nishikigoi can be exported overseas once the source is found negative (Yamada, 2004). By the middle of June 2004 the number of prefectures reporting detection of KHV had risen to 38 (Sano pers.comm.). Ten of these had not reported KHV in 2003 but 3 prefectures found positive for KHV in 2003 have not reported detection in 2004. So far in 2004, the KHV positive cases have been mostly in wild populations of carp and aquaculture facilities on river water supply. However, many of the facilities that experienced KHV outbreaks in 2003 have been free of the disease in 2004 and there are no reports of KHV disease in nishikigoi farms in 2004 (Sano pers.comm.).

Several other countries outside Europe have recently reported or have suspected KHV disease outbreaks. A summary of these is listed in Table 2.

#### **Other emerging viral diseases of carp**

Other disease outbreaks causing high mortality in carp populations have also been reported in the years following the first report of KHV in carp. In particular, a disease with clinical signs similar to those seen in KHV outbreaks was reported in cultured carp in Korea (Oh et al., 2001). A virus was isolated on FHM cells and virus particles of 70-80nm diameters were visualised in the cytoplasm of infected cells. The disease was also reproduced in experimental transmission

Country	Current situation	Source
<i>Countries outside Europe with KHV confirmed</i>		
Indonesia - Java	First outbreak reported in 2002 - confirmed by PCR.	Directorate Fish Health & Environment / Fish Health Research Laboratory.
Indonesia - Sumatra & other islands	First outbreak November 2002. Further outbreaks 2003 - and spread to Bali, E-Kalimantan & Central Sulawesi.	As above.
Israel	First outbreaks 1998 & outbreaks in all subsequent years.	Ariav et al. (1999); Perelberg et al. (2003).
Japan	First outbreak in May 2003, confirmed Nov. 2003 by PCR. Further outbreaks in Oct. 2003; 38 of 47 prefectures had seen KHV disease by September 2004.	AAHD, NRIA, Watarai, Mie.
South Africa	Outbreak reported in 2001 - 2003, confirmed by PCR's, RT-PCR and <i>in situ</i> hybridization.	FLI, Insel Riems.
Taiwan	First outbreak reported in 2002 and many further outbreaks seen in 2003 and 2004.	Tu et al. (2004).
USA	First outbreak 1998 - virus isolated on KF cells ; KHV diagnosed in ornamental carp in 14 US states. First outbreak in wild carp in Lake Moultrie, South Carolina in May 2004.	School of Vet.Med., UCAL, Davis ; KHV Workshop, London, UK, February 2004, ProMed Mail (Aug. 2004).
Thailand	Koi exports to Germany in 2004, confirmed by PCR's, RT-PCR and <i>in situ</i> hybridization.	FLI, Insel Riems.
<i>Countries with KHV suspected</i>		
China	KHV outbreak reported in 2001 in Hong Kong; Handpicked koi positive by PCR July 2002 - not confirmed at source.	G.Chu (retail and wholesale of koi, pers.comm.). CEFAS Weymouth, UK.
Malaysia	Koi originating from Malaysia found positive by PCR in 2001 - not confirmed at source.	CEFAS Weymouth, UK.
<i>Countries with no KHV reported (to date)</i>		
Bangladesh, Cambodia, India, Iran, Korea (DPR), Laos PDR, Myanmar, Nepal, Pakistan, Republic of Korea, Singapore, Sri Lanka, Philippines, Vietnam. No KHV reported in any South American country or in Australasia.		Network of Aquaculture Centres in Asia (NACA), Fung et al. (2004), University of Chile, AFFA (Bernoth pers.comm.).

**Table 2.** Koi herpesvirus disease outside of Europe: the current situation.

trials in carp challenged with filtrates from infected FHM cell cultures. In Japan, a corona-like virus was isolated during outbreaks of ulcer disease (ana-aki-byo)

causing high mortality in colour carp (Miyazaki et al., 2000). This virus was of similar size and morphology to the Korean virus, but different disease signs were seen during outbreaks.

Elsewhere, laboratories in Germany and Belgium have reported the isolation of myxo-like viruses during diagnostic investigations of koi carp suffering gill necrosis (Neukirch et al., 1999; Body et al., 2000; Neukirch & Kunz, 2001). In Germany, Neukirch and colleagues isolated the virus in CCB and carp gill cell cultures and also in the EPC cell line. The virus produced a CPE characterised by extensive syncytia formation in all cells and was re-isolated from koi carp experimentally infected by intra-peritoneal injection, but did not cause mortality. They also showed the virus to be sensitive to chloroform but not to IUDR (5-Iodo-2-deoxyuridine), indicating an enveloped virus with an RNA genome. Body and colleagues (2000) reported repeated isolations of virus-like particles from koi carp suffering gill necrosis. The virus was isolated from gill tissue extracts on EPC monolayers at 21°C and also produced a CPE on Fat head minnow (FHM) cells. The CPE description and the virus size (150-200nm) and morphology were very similar to that observed by Neukirch & Kunz (2001).

The CEFAS Weymouth laboratory has isolated a similar virus in EPC cells on a number of occasions since 1996 and also, more recently in KF-1 cells. However, the virus has been difficult to fully characterise because of its slow-growth in cell culture (C. Longshaw pers.comm.).

### **Why has KHV not been regulated in Europe under European Community or national fish health legislation?**

Traditionally, ornamental fish are viewed as pets and therefore not covered by regulations for animals destined for human consumption.

In theory, they are kept in closed systems and any disease outbreak is limited in economic and epidemiological terms (Davenport, 2000), and therefore not a national concern. However, ornamental fish do occasionally escape to the wild, some are deliberately released and in the case of koi carp: some are cultured under the exact same conditions as consumption fish and not necessarily separate from these. Although coldwater ornamentals such as goldfish and koi carp are covered by the EC import legislation, they are still seen as pet fish and checked less stringently for diseases at border crossings, thus allowing KHV to pass freely between countries.

### **International legislation**

In 2002 the OIE and the EC considered the impact of KHV in relation to possible notification of the disease. On these occasions it was recognised that KHV is an untreatable disease of great detrimental potential to the carp industry worldwide, which does merit listing. However, KHV had already spread extensively via the pet-trade and due to shortcomings in the diagnostic methods, absence of the disease could not be ascertained and therefore disease-free areas could not be documented. When KHV-free areas and batches of fish cannot be identified to a high degree of certainty, legislation is of little use to control and prevent incursion of the virus. On these grounds it was decided not to list KHV as a notifiable disease.

The Indonesian and Japanese governments, in contrast, instigated strict national disease control measures in an attempt to contain the outbreaks. Additionally, in Japan, only those producers of koi that have been screened and found negative are permitted to export.



## **Potential impact of KHV on European aquaculture**

In 2003 and 2004 the aquaculture industry in Germany saw major outbreaks of KHV disease in common carp pond farms and KHV is now a very real threat to the large carp industry in Eastern Europe.

Regional koi carp exhibitions, where pet-fish are transported to and from shows and displayed together in tanks or ponds without quarantine considerations, increase the risk of spread of KHV via private garden ponds to wild and farmed carp stocks within the EC. As the EC expands to the east, the trade in live fish in Europe becomes potentially less restricted and as this trade increases so does the risk of disease transfer along with it. There is an intensive koi trade worldwide and as long as KHV disease is not listed as notifiable, national veterinary authorities cannot demand that imported live fish are KHV free. This is an example of unchecked transmission of a serious disease from pet fish to consumption fish culture and the possible further spread to wild populations with the virus becoming endemic in those populations. All of these factors combine to present potential disaster in farmed and wild carp populations from the uncontrolled spread of KHV disease.

## **The diagnostic dilemma**

### *Available techniques for KHV diagnosis*

KHV is still a "young disease", and new tests have only recently been described. During the National Reference Lab workshop on carp diseases at CEFAS, Weymouth, in June 2003, it was advised to use at least two of the available diagnostic methods in parallel to improve the accuracy of KHV diagnosis.

## **Light microscopy & Transmission Electron microscopy (TEM):**

Examination of fixed and stained tissue sections from moribund carp, by light microscopy, can reveal nuclear inclusion bodies in virus-infected cells, particularly in gill, gut and kidney tissue. However, secondary bacterial or parasitic infections can often obscure the virus-specific pathology. For virus to be detected by TEM the glutaraldehyde-fixed tissues need to be in good condition and heavily infected with at least  $10^6$  virus particles. However, this requires the tissues to be sampled at an optimal time in the virus infection cycle and this is not always possible.

## **Virus isolation in cell culture:**

The KF-1 and CCB cell lines have been used to isolate KHV (Hedrick et al., 2000, Neukirch et al., 1999). However, a number of laboratories have had problems in maintaining the KF cell line and some have reported a pseudo-cytopathic effect occurring spontaneously in the negative control cells. Moreover, when the cells and the viruses are in good condition, isolation of KHV often requires 10-12 days. Alternatively, the virus produces a readily identifiable CPE in CCB cells, which takes 5-8 days at 26°C to appear. It should be considered however, that the sensitivity of virus isolations is much lower than that of the PCR test, and a virus negative result is not reliable based on virus isolation alone. In Israel the virus has also been isolated in primary cultures of koi fin cells (Ronen et al., 2003).

## **Detection of KHV DNA:**

The majority of diagnostic laboratories use the fast and sensitive PCR assay for amplification and detection of specific KHV DNA sequences. The published assays available are those

developed in the USA by Gilad et al. (2002) and Gray et al. (2002) and both gills and a pool of brain, spleen and kidney tissue should be tested. It is not known if these assays are suitable for non-lethal testing on blood and faeces. No reports are available on the full validation of these assays, which use primers designed from DNA restriction fragment sequences. However, these sequences are located in non-coding regions of the viral DNA and may not be conserved in newly emerging strains of KHV, which as a result could be undetectable. A number of laboratories are developing PCR protocols that use oligonucleotide primers based on nucleotide sequences from protein-coding regions of the KHV genome.

Several laboratories are working on the development of more sensitive assays for the screening of potential virus carriers. This includes real-time quantitative PCR assays (e.g. Taq-man PCR) (Bercovier et al., 2004, Gilad et al., 2004) and two-round, nested-PCR assays (Stone pers.comm.). It is not known if these more sensitive PCR assays will be able to detect latent KHV in carrier fish. Studies of the virus genome are required to identify genes that may be expressed in a latent stage or possibly code for latency.

**Detection of antibodies to KHV:** Enzyme Linked-Immunosorbent Assays (ELISA) to detect antibodies to KHV in carp serum have been developed at the Hebrew University, Jerusalem (Ronen et al., 2003) and at the University of California's School of Veterinary Medicine (Hedrick pers.comm.). Studies on non-lethal sampling techniques for KHV diagnosis are also underway at a number of laboratories. It is hoped that KHV may be

reliably detected in blood samples to avoid lethal sampling of expensive koi brood stock.

**Other detection methods:** *In-situ* hybridisation (ISH) has been used to confirm the presence and location of KHV DNA in fixed, paraffin-embedded tissues (Le Deuff et al., 2001) or cryo sections (Bergmann pers.comm.) and PCR methods have been developed to analyse archive histological material (Way et al., 2004b).

Polyclonal antibodies in rabbits and mouse monoclonal antibodies have been raised at the Federal Research Institute for Animal Health in Insel Riems, Germany, and an immunofluorescence assay (IFAT) has been developed. Additionally, investigations at Insel Riems are exploring the possibility of direct DNA transfection by a transfection reagent or electroporation into CCB cells. Antibodies to KHV have also been produced at the Hebrew University-Hadassah Medical School and at FLI Insel Riems and used in the development of immunohistochemical staining methods and in the development of an immuno-diagnostic kit for KHV (Kotler pers.comm.). New tests focusing on confirmation of PCR results are in development at the University of Munich, CEFAS Weymouth and Insel Riems. Currently, the CEFAS Weymouth laboratory is using a reverse hybridization technique (Stone pers.comm.), as an alternative to sequencing, for confirmation of products amplified by the KHV PCR.

**Recommended techniques for diagnosis:** Presumptive KHV diagnosis can be achieved by observations of clinical pathology (described earlier) and histopathology in gill,

gut and kidney tissues. Confirmation of KHV infection is achieved by virus isolation or, more reliably, by PCR detection of KHV DNA in gill and kidney tissue homogenates.

#### **Screening fish populations for virus carriers:**

Virus screening of fish stocks might be achieved, by using the standard sampling procedure from the EC Decision 96/240, but only when the water temperature has reached 18-27°C for at least four weeks. However, the EC procedure was written for VHS and IHN, and not for KHV and the choice of diagnostic technique is crucial: It is very important to choose the most sensitive test, which is currently the PCR. Moreover, lethal sampling of 150 koi carp from all sizes and ages present on the site could be a very costly test, depending on the source and quality of the koi carp that are sampled.

#### **Management and control measures**

##### *Industry measures to counteract the impact of KHV*

Faced with great economic loss and possible collapse of an otherwise lucrative industry, the wholesalers of koi in Germany initiated a network to inform one another of KHV entry points. In this way exporters of diseased batches of koi were made known to the rest of the network, which could then avoid these sources. The exporters of disease-free batches were likewise listed in the network and they in turn receive more business from the German importers. This arrangement is an example of industry working together to combat a disease problem where no disease control legislation exists. During a meeting between koi producers and wholesalers, scientists and policymakers in London

February 2004, this idea was expanded to cover the international network of koi producers and formalised in a web-based register ([www.koibiosecurity](http://www.koibiosecurity)) where producers can provide independent verification of their KHV status.

Other initiatives are underway in Europe, where wholesalers attempt to establish disease-free broodstock, which in time can provide disease-free batches of koi. Should this enterprise become successful, koi can be sold certified free of KHV, which is currently a product in high demand in Europe. However, the cost of production of fish in Europe is much higher compared to some of the countries that currently mass produce and export koi, which will render the certified koi an expensive product – perhaps too expensive for the market! The downside of this idea is that KHV may already be so widely established in Europe that these expensive disease-free koi will sooner or later come into contact with the virus and succumb. Breeding koi carp for resistance to KHV or vaccinating them at an early age could be a better strategy in an environment where the virus has become endemic.

#### **Vaccination experiments**

Research into vaccine development has been conducted at the Hebrew University in Jerusalem. Ronen et al. (2003) described a vaccine against carp nephritis and gill necrosis disease (CNG) of koi. The authors named the virus from the clinical disease manifestations because they found no evidence to classifying the virus as a herpesvirus. However, researchers at the Hebrew University have subsequently confirmed that CNGV and KHV

is the same virus (Kotler pers.comm.). The vaccine is in a live attenuated form and vaccinated koi (50 fish/tank) experimentally challenged with KHV suffered 39% mortality, whereas non-vaccinated koi carp showed 82% mortality.

Following the disease outbreaks in Japan, researchers there have also announced plans to develop a vaccine (Miwa, 2004).

### **Disease management strategy**

There is currently no therapy against KHV. Furthermore, by the very nature of the family herpesviridae (from Greek: "hidden virus"), it is difficult to detect in its latent stage, and therefore control and prevention requires an effort on several levels.

Following outbreaks in aquaculture ponds, veterinary authorities may advise on, and supervise, eradication procedures, similar to those recommended in some EC member states for notifiable diseases such as SVC, VHS and IHN. Although increasing the water temperature of affected koi populations to 30°C may in some cases stop mortalities during outbreaks (Ronen et al., 2003), the virus may be transferred to naïve hosts by surviving carriers, which appear clinically healthy. It is uncertain, whether some form of latency develops in KHV-exposed fish held for longer periods at temperatures below 15°C (Gilad et al., 2003). Recent experiments did show that the virus was present for longer than 12 months in apparently healthy koi (Bergmann, unpublished data).

The industry has in certain countries already taken measures to record the source of entry of the virus and in this manner avoid further

imports from infected areas or farms. Furthermore, the koi carp industry and veterinary authorities would do well to work together to increase public awareness of the need for quarantine of pet-fish, especially during the pet-fish exhibitions.

Diagnostic laboratories throughout Europe need to sharpen their skills in the techniques recommended herein, to be equipped for the challenge of diagnosing an infection with KHV and, of equal importance, to be able to declare freedom from this disease with a reasonable certainty.

Some wholesalers have considered local production of disease-free stock rather than importation, even though the cost of production is magnified. Imports of susceptible fish species should be quarantined at virus-permissive temperatures until KHV infection have been ruled out by clinical and laboratory examination. To enhance the diagnosis, cohabitation with naïve fish during quarantine is also very effective as the naïve carp are more likely to show disease signs. Scientists are working on vaccine development, which together with breeding programmes to select for disease resistance in koi may be a future avenue of disease prevention.

### **Conclusions**

KHV is spreading fast within Europe. As more koi mortalities occur in private ponds, the infection pressure on koi and common carp increases. Europe has already seen major KHV disease outbreaks in German (Schlotfeldt, 2004) and now also in Polish carp farms (Kempter & Sadowski 2004). Such news

warns of an emerging threat to pond carp culture in East-European countries and wild carp populations all over Europe. Measures should be introduced to prevent entrance and spread of KHV on several levels, including quarantine arrangements and fast and reliable detection and identification by diagnostic laboratories. National authorities are encouraged to participate in integrated chain management with the industry and to increase public awareness of the transmission routes of KHV among pet-fish. Ornamental koi should be kept strictly separate from common carp culture for consumption.

Notification, to competent authorities and the industry, would be helpful for those countries that are currently regarded as free of KHV or have only seen the disease in imported ornamental carp, and can continue to be protected against the disease. However, this would implicate, that those countries have reliable tools for diagnosis of KHV in place, and their policy concerning KHV in a similar state of readiness.

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