DEVELOPMENT OF A PCR-BASED ASSAY FOR THE DETECTION OF KOI HERPESVIRUS DNA IN FORMALIN FIXED, WAX EMBEDDED ARCHIVE TISSUES

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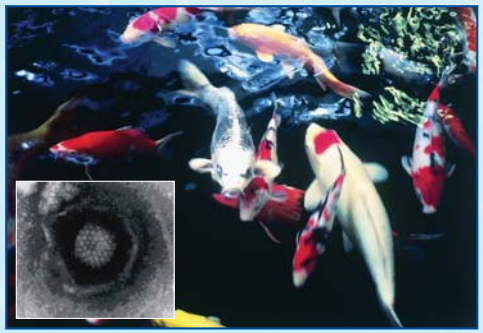
Introduction

The koi herpesvirus (KHV) was initially reported in association with high mortality among common and koi carp stocks in cyprinid-fish farm sites in Israel. Since then, the virus has also been reported in the USA and in several European countries. Indeed, outbreaks of disease associated with KHV have occurred in Germany, the Netherlands, Belgium and the United Kingdom, suggesting that the virus is widespread across Europe. In addition, suspicion of KHV outbreaks has been reported in Asia.



KHV was first isolated in the UK in October 2000, soon after the start of screening suspect samples using the koi fin (KF) cell line (Hedrick *et al.*, 2000) and a single round polymerase chain reaction (PCR) assay (Gilad *et al.*, 2002). However, outbreaks of disease affecting *Cyprinus carpio* varieties had been reported in the UK in previous years. Notably, an unexplained outbreak of disease causing high mortality in koi carp was reported in 1999. The signs of disease observed at that time were consistent with KHV disease.

In order to investigate the possible causes of this outbreak and other unexplained carp mortality events in the UK, we have developed a PCR-based assay to detect KHV DNA in archive formalin-fixed, wax embedded tissues.

Material & Methods

Sectioning.

Wax blocks were sectioned using a microtome that was carefully cleaned with alcohol or Clearene between each sample. A different portion of microtome blade was used for each block within a sample. Five samples per wax block, each of two 20µm sections were recovered in separate Safe Lock (Eppendorf) microfuge tubes, and were stored at 4-8°C until use.

Extraction of total nucleic acids from wax sections and PCR.

The paraffin wax was dissolved by adding 1ml of Clearene or xylene to each tube, vortexing and agitating the tubes at room temperature for 30 minutes. The solvent was removed after centrifugation at 13,000 rpm for 5 minutes and the de-waxing was repeated once. Pellets of tissues were rinsed twice as above but with 1ml of 99-100% ethanol. Tissues were placed at 55°C until dry. Just before use, the required amount of extraction buffer (0.5% Tween 20, 1mM EDTA, 50mM Tris, pH 8.5) was added with the appropriate quantity of proteinase K. 100µl were added to each pellet of tissues, the tubes were vortexed and incubated overnight at 55°C, with agitation. Following incubation, the tubes were vortexed and centrifuged briefly, heated at 95°C in order to inactivate the proteinase K and centrifuged at 13,000 rpm for 5 minutes. The supernatants containing total nucleic acids from tissues were stored at -20°C until use.

The extraction method was tested using various concentrations of proteinase K (20-300µg/ml) on serial sections of infected tissues prepared from three different wax blocks (data not shown) and 200µg/ml was selected for further investigations. The amplification efficiency of various sets of primers was tested at the same time (not shown). All primers were designed to amplify a 200bp fragment. The best performing PCR primer set is given in Figure 1.

PCR conditions.

A master mix was prepared so that each tube contained 5µl 10x reaction buffer (supplied with the Taq polymerase), 5µl $MgCl_2$ 25mM, 0.5µl Taq polymerase 5U/µl (ABgene), 0.5µl dNTPs 25mM each, 0.5µl of each forward and reverse primer 100pmol/µl and 28µl H_2O. 1µl of sample extracted as above was amplified in a 50µl reaction. The mixture was denatured at 94°C for 5 min, followed by 35 cycles of 95°C for 1 min, 55°C for 1 min, 72°C for 1min with a final extension step of 72°C for 10 min.

Results

Table 1 shows the results of analysis of wax blocks from twelve different investigations into koi carp mortalities. The results of virus isolation and conventional PCR are included for comparison. Table 2 shows the results of re-analysis of wax-embedded tissues taken during investigations into disease outbreaks in koi carp in 1999 (Table 2a) and koi and common carp in 1996 (Table 2b). During the 1999 outbreak, samples were taken from the site on two occasions. At the time of the samplings KF cells were not available, and PCR was not done on the samples. The PCR results from the five replicate samples from each block were consistently positive or negative. Positive results were confirmed by *in situ* hybridisation (ISH) (Figure 2).

Table 1: Archive samples from previous years were used to validate the method using a range of samples that had been previously analysed using conventional methods. PCR results are scored as (-) Negative, (+) positive but with moderate intensity, (++) positive, strong signal.

Reference & species	Sampling date	Isolation in KF cells	Conventional PCR	Clinical signs	Block No	PCR results obtained for the five replicates from each wax block
Koi carp, batch A	10/00	+	-	KHV suspicion (Typical KHV disease signs)	1A	++ ++ + ++ ++
					1B	- - - - -
					2A	- + + + +
					2B	- - - - -
					3A	+ + + + +
					3B	- - - - -
Koi carp, batch B	07/01	-	-	No signs (sampled when an outbreak was abating)	1	- - - - -
					2	- - - - -
					3	+ + + + +
					4	+ + + + +
Koi carp, batch C	07/01	-	+	KHV suspicion	1.1	- - - - -
					1.2	+ + + + +
					1.3	- - - - -
Koi carp, batch D	08/01	-	-	KHV suspicion	1	+ + + + +
Koi carp, batch E	08/01	-	-	Disease outbreak (no obvious signs of KHV)	1	+ - + + +
					2	+ + + - +
Koi carp, batch F	08/01	+	+	Disease outbreak (no obvious signs of KHV)	1	++ ++ ++ ++ ++
					2	++ ++ ++ ++ ++
					3	++ ++ ++ ++ ++
Koi carp, batch G	08/01	-	-	Disease outbreak	1	- - - - -
					2	- - - - -
Koi carp, batch H	08/01	-	+	KHV suspicion	1	+ + + + +
Koi carp, batch I	06/01	+	+	KHV suspicion	1	+ + + + +
					2	- - - - -
					3	- - - - -
					4	- - - - -
Koi carp, batch J	05/01	-	-	Disease outbreak	1A	- - - - -
					1B	- - - - -
					2	- - - - -
					3	- - - - -
Koi carp, batch K	04/01	-	-	Disease outbreak	1	- - - - -
					2	- - - - -
					3	- - - - -
					4	- - - - -
					5	- - - - -
					6	- - - - -
					7	- - - - -
					8	- - - - -
					9	- - - - -
					10	- - - - -
Koi carp, batch L	11/00	-	-	KHV suspicion	1	- - - - -
					2	- - - - -
					3	- - - - -
					4	- - - - -
					5	- - - - -
					6	- - - - -
					7	- - - - -

Table 2a: Re-assessment of an outbreak of disease that occurred in 1999, for which no aetiological agent was determined. The site was sampled twice during the outbreak. PCR results are scored as (-) Negative, (+) positive but with moderate intensity, (++) positive & intense.

Reference & species	Sampling date	Isolation in KF cells	Conventional PCR	Clinical signs	Block No	PCR results obtained for the five replicates from each wax block
Koi carp, first batch sampled	1999	Not done	Not done	Disease outbreak (no obvious signs of KHV)	1	+ + + + +
					2	++ ++ + ++ ++
					3	+ + + + +
					4	+ ++ ++ ++ +
					5A	++ ++ ++ ++ ++
					5B	++ ++ ++ ++ ++
					6	++ ++ ++ ++ ++
					7	- - - - -
					8	- - - - -
Koi carp, re-sampled	1999	Not done	Not done	Disease outbreak (no obvious signs of KHV)	1	+ + + + +
					2	++ ++ + ++ ++
					3	- - - - -
					4	+ + + + +

Table 2b: Re-assessment of an outbreak of disease that occurred in 1996, for which no aetiological agent was determined. The site was sampled by the Environment Agency during the outbreak. PCR results are scored as (-) Negative, (+) positive but with moderate intensity, (++) positive & intense.

Reference & species	Sampling date	Isolation in KF cells	Conventional PCR	Clinical signs	Block No	PCR results obtained for the five replicates from each wax block
Koi carp	1996	Not done	Not done	Typical KHV disease signs	1	- - - - -
					2	+ + + + +
Common carp	1996	Not done	Not done	Typical KHV disease signs	3	- - - - -
					4	- - - - -

Figure 1: PCR primers were designed to amplify a 200bp fragment in the genome of KHV. A pair of 24-mers was selected for further investigations

Forward primer:

5'-TggCCACCTCgCCgCTCTTCTCCg-3'

Reverse primer:

5'-ggCACgACgCCgAggTCgCTCAgg-3'

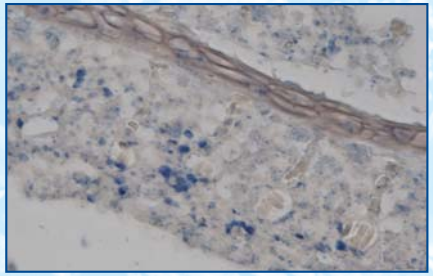


Figure 2: PCR on archive wax-embedded tissues are confirmed using in situ hybridisation (ISH) - koi gill section showing KHV DNA detected by ISH and staining intense blue

Discussion & further prospects

- The quality of fixed samples is an essential key to success.
  - ☞ Over-fixation can significantly affect results and can lead to false negatives.
  - ☞ The quality of samples can be assessed using a universal probe.
- Potential as a confirmatory diagnostic technique (Table 1).
  - ☞ Positive results were obtained for samples (A, C, F, H & I) that were diagnosed KHV positive by isolation in KF cells and/or conventional PCR.
  - ☞ Negative results were obtained for three samples (G, J & K) corresponding to outbreaks of disease not related to KHV. The isolation on KF cells and conventional PCR was also negative.
- The technique is highly sensitive & complementary to other diagnostic techniques (Table 1).
  - ☞ This is dependent on the quality of the samples (i.e. not over-fixed).
  - ☞ A sample of koi carp presenting no clinical signs of disease (B) was found positive (i.e.: two negative and two moderately positive wax blocks). These carp were sampled on a site four weeks after an outbreak of KHV. This suggests that the technique can detect sub-clinical levels of infection or latency in healthy carrier fish.
  - ☞ Positive results were obtained for two samples (D & E) corresponding to outbreaks of disease, one of which was suspected to be a KHV outbreak on the basis of the observed signs of disease. Both samples failed to be diagnosed by PCR and isolation in KF cells.
- Potential to investigate archive material (Table 2).
  - ☞ Can give insights into the occurrence of KHV in previous years.
  - ☞ Frozen archive material may not be available.
  - ☞ UK outbreaks of the disease in 1996 and 1999 could be associated with KHV using this method This was subsequently confirmed by ISH.
  - ☞ Histopathology and ISH are complementary techniques to the PCR on fixed archive material.
- On-going work.
  - ☞ We are currently carrying out investigations on wild carp sampled in the UK in previous years.
  - ☞ KHV might represent one of the factors involved in the occurrence of the syndrome of spring carp mortality in the UK.

References

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