

DEVELOPMENT AND VALIDATION OF DIAGNOSTIC TECHNIQUES FOR THE DETECTION OF KOI HERPESVIRUS (KHV)

by R.-M. Le Deuff¹, M.C. Alonso², P.F. Dixon¹, L. Ecclestone³, C.B. Longshaw¹, P.D. Martin¹, E.J. Peeler¹, L.C. Steedman³, D. M. Stone¹ and K. Way¹

Introduction

During 1998, cyprinid-fish farm sites in Israel that regularly supply fish for the world-wide ornamental fish trade suffered high mortalities among their common and koi carp stocks (Ariav *et al.* 1999). Severe gill necrosis was the most distinctive and consistent pathological sign among the affected carp (Figure 1). Mortalities with a similar etiology were also seen in ornamental carp populations in Germany (Bretzinger *et al.* 1999, Neukirch and Kunz 2001) Holland, Belgium, the UK and the USA. A herpesvirus (Koi herpesvirus, KHV) was isolated from koi carp from the USA and Israel (Hedrick *et al.* 2000, Gray *et al.* 2002). From 2000 to date, we have isolated KHV on several occasions from koi carp recently imported into the UK. The sequence similarity of a 484bp amplicon from the polymerase chain reaction (PCR) (Gilad *et al.* 2002) obtained from KHV isolates from different outbreaks in the UK and Belgium indicates that the UK and Belgian virus isolates appear to be similar to the KHV isolated in the USA by Hedrick *et al.* (2000), suggesting the possible emergence of a new viral disease problem. We report here our work on validating diagnostic methods for the detection of KHV.

Material and methods

Samples: Fish sampled from 26 disease incidents in the UK, represented by 172 pools of gills and viscera of koi and common carp presenting gross symptoms of the KHV infection (Figure 1) were processed for cell culture isolation and PCR. Eighty-nine additional pools of carp gills and viscera samples corresponding to spring viraemia of carp virus (SVCV) infected (33 pools) and asymptomatic carp (56 pools) were used as negative controls for the tissue culture and the PCR. Other negative controls for the PCR consisted of channel catfish virus (CCV) and two different isolates of an unknown viral agent from carp (Neukirch *et al.* 1999, Body *et al.* 2000) grown in tissue culture.



Figure 1: External, typical signs of the natural KHV infections range from small patches of discoloration on the gills to extensive gill necrosis [pictured]. Also associated with KHV infection is skin ulceration, which can often be seen as extensive necrotic areas.

Preparation of tissues: Pools of gill tissue and pools of spleen, kidney and liver tissues from each of one to five fish were homogenised with a pestle, mortar and sterile sand. The homogenate was re-suspended in 9 volumes of cold viral transport medium, clarified by centrifugation at 2000 rcf for 20 minutes at 4°C and the supernatant was passed through a 0.45µm filter to reduce cell toxicity and bacterial contamination.

Cell culture isolation: Koi fin (KF) cell monolayers in 12.5cm² flasks containing 4.5ml of culture medium were inoculated with 150µl of filtered tissue homogenate, to give a final dilution of 1:300. After 14 days incubation at 20°C, monolayers not showing a cytopathic effect (cpe) were diluted to 1:10 and subcultured onto fresh cells.

DNA extraction and PCR: Duplicate samples were processed for each pool of tissue homogenate as follows: 100µl of clarified extract was sampled into 1ml DNAzol reagent (Life Technologies) and extracted as recommended by the manufacturer. Extracted DNAs were suspended in 50µl of distilled water and 10µl used for the PCR.

A single round PCR (35 cycles) was carried out on all samples using the primers KHV-For and KHV-Rev (Gilad *et al.* 2002) (Figure 2). A nested PCR (30 cycles for each round) was run on a selection of samples only using the primers KHV-For-Int and KHV-Rev-Int (Figure 2).

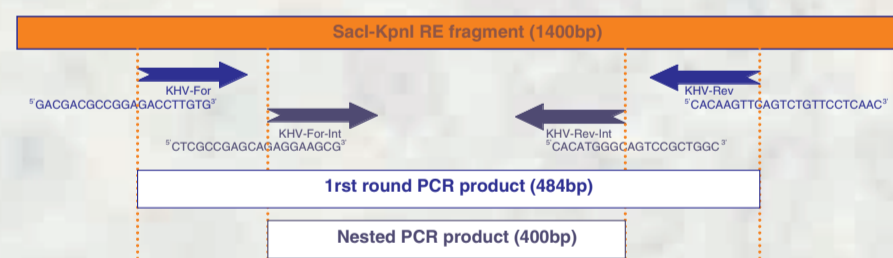


Figure 2: KHV PCR was carried out using the following primers which allowed amplification of a 484bp fragment (single round) or a 400bp fragment (nested) in the 1400bp Sacl-KpnI restriction fragment of the KHV genome.

Light and electron microscopy (EM): Tissues were fixed with neutral buffered formalin (NBF) and processed for standard paraffin embedding and sectioning. Five-micron sections were stained with haematoxylin and eosin or with Farley Feulgen DNA stain. For transmission electron microscopy, gill and visceral organ tissues were fixed in 3% glutaraldehyde in 0.06 M cacodylate buffer (pH 7.4) and post-fixed and processed for epoxy resin embedding using standard methods (Hedrick *et al.* 2000). Thin sections (70 - 90 nm) were stained with 6% methanolic uranyl acetate and lead citrate and examined using a Jeol 1210 transmission EM.

In situ hybridisation (ISH): A digoxigenin labelled probe was prepared by PCR using the primer pair KHV-For and KHV-Rev. NBF fixed tissues were processed using a standard ISH protocol. De-waxed sections were treated with proteinase K (10µg/ml, 30min, 37°C) and incubated with the probe diluted in hybridisation buffer (>18h, 42°C), the excess of probe was washed (2x SSC, 6M urea, 0.2% BSA; 10min at 42°C) and the specifically bound probe was detected by immunohistochemistry. The 5-bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium substrate for anti-digoxigenin antibody conjugated with alkaline phosphatase stained infected cells blue, while the Bismarck Blue counter-stain produced a light brown background, allowing a good contrast and visualisation of labelling.

Results

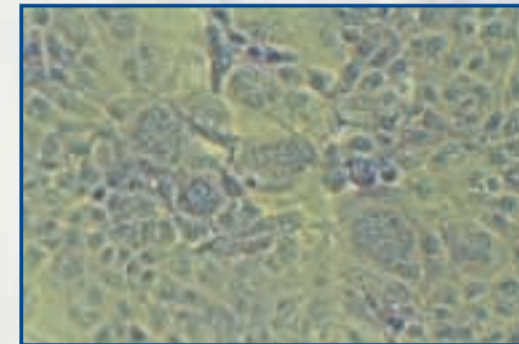


Figure 3: Cell culture isolation - KHV produces a distinctive cpe in KF cells. The early cpe (7-9 days) appears as foci of darker, enlarged cells containing pin-point vacuoles. The cpe progresses by fusion to form multinucleate cells and margination of chromatin can be seen in some nuclei on fixed and Giemsa stained monolayers at higher magnification [pictured].

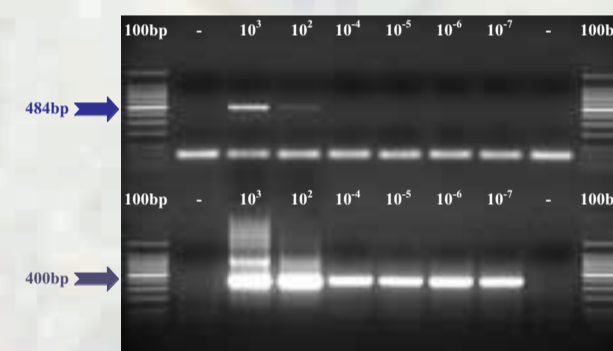


Figure 4: PCR - The sensitivity of the PCR was assessed using serial dilutions of total DNA extracted from a KHV culture of known titre (10² TCID₅₀/ml). The limit of detection of KHV DNA by single round PCR is at the dilution corresponding to 10² TCID₅₀/ml (first lane) and it is at least at 10⁷ TCID₅₀/ml by nested PCR (second lane).

Clinical signs	Number of sites/number of fish	Sites with PCR positive fish (%)	Sites with KHV-positive fish by cell culture (%)
KHV disease	26/132	50 (13/26)	40 (10/25)
Signs other than KHV	15/55	13 (2/15)	0 (0/15)
No signs	8/152	12 (1/8)	0 (0/8)
Total	49/339	33	21

Table 1: Comparison of virus isolation and PCR for detection of KHV. Fish with gross signs of KHV infection from 25 sites, fish showing other clinical signs from 16 further sites, and fish showing no signs of disease from 8 sites were tested in parallel by isolation in KF cells and by PCR. The single round PCR was more sensitive than cell culture for detecting the presence of KHV. Fish with no signs of disease from one site (12%) were positive for KHV by PCR. Virus had been isolated one month earlier from that site from fish with clinical signs of KHV infection.

Acknowledgements

This work was conducted under DEFRA contracts FI 136 and CI043.

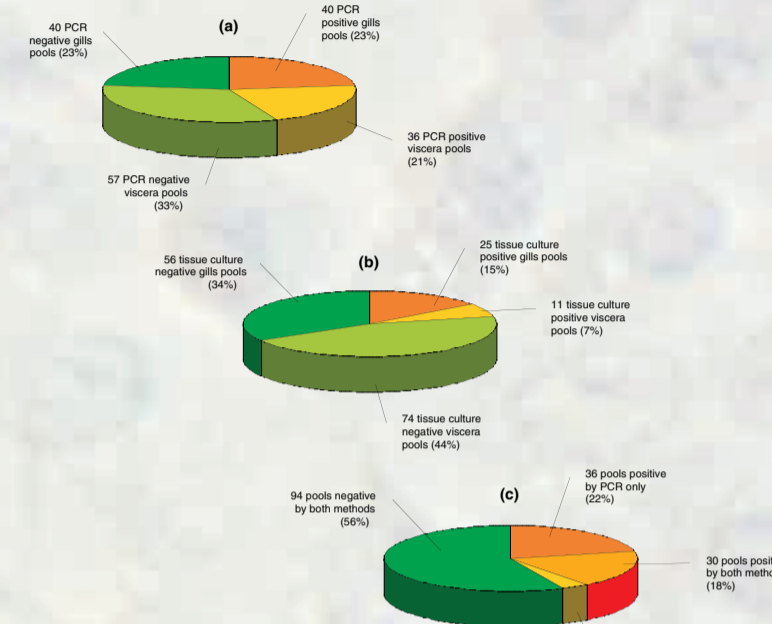


Figure 5: The total number of pools analysed suggests that the single round PCR (a) is better than culture isolation (b) for the diagnosis of KHV. This is confirmed when results of individual pools are compared (c). Although positive results of both methods partly overlap, the PCR allows detecting more positive samples than culture isolation.

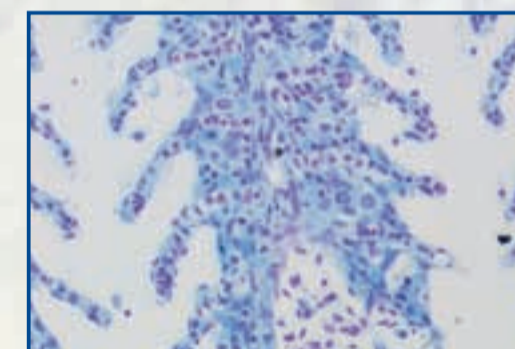


Figure 6: Histopathology - Abnormal nuclei are most easily found in gill tissues [pictured, x200 Farley-Feulgen], but also in other affected tissues including the liver, kidney and spleen. Enlarged KHV-infected nuclei are clearly seen, characterised by chromatin margination, forming a signet ring appearance. EM - Visualisation of KHV particles in the same infected tissues proved to be very difficult and is not recommended for rapid diagnosis.

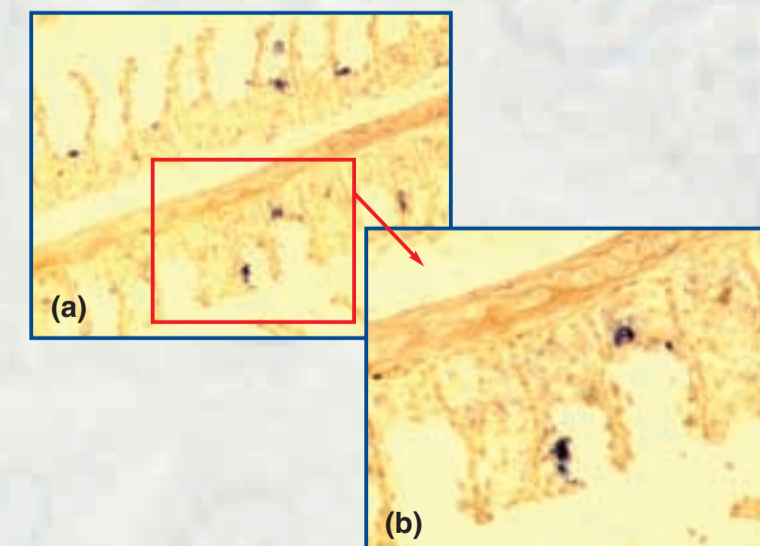


Figure 7: ISH allowed specific labelling of infected cells in gills (a and b). A probe specific for another fish virus (SVCV) was used as negative control on serial sections of the same material. Labelling was not observed with the latter probe (not shown).

Discussion & Future Prospects

Although progress has been made to improve the diagnosis of KHV, some results are still ambiguous and we may still underestimate the real importance of KHV in outbreaks of ornamental carp in the UK and in other countries.

It should be possible to apply optimised molecular based diagnostic techniques in the following areas :

- to detect and identify virus in fixed and frozen archive material. Although, the successful application of ISH may depend on the length of fixation time
- screening of virus carrier fish & epidemiological surveys of carp stocks
- virus pathogenicity studies, particularly primary infection of naive carp and chronic infection of carrier fish. Also, examining latent and replication phases of the virus life cycle and factors influencing re-emergence
- routine diagnosis of new disease outbreaks in carp populations

Further improvement of diagnostic methods?

Indirect isolation of KHV from challenged fish?

Challenging fish with tissue extracts from suspect samples has been successful in isolating virus from infected fish that are positive by PCR and negative in initial cell culture isolation (data not shown). Such PCR positive tissue pools were injected into virus-free ghost carp. Other carp were co-habited in the same tank and mortality was observed in both injected fish and co-habitants and KHV was isolated from the mortalities.

Apply systematically the nested PCR procedure?

A nested PCR procedure was tested on DNA extracted from tissue culture with good success. It will also be applied to a selection of tissue samples that were either badly preserved or sampled late after the beginning of the outbreak and to screen asymptomatic fish.

Improve cell culture isolation?

Fish challenge and tissue culture remain the only methods capable of demonstrating the presence of infectious virus in the samples.

Candidate approaches currently being investigated are:

- Supplementing the culture medium with trypsin to enhance infectivity.
- Improve virus-membrane fusion by pre-treatment of cells with PEG.
- Compare the infection of KF cells in suspension with monolayers.
- Develop new, more virus sensitive carp cell lines

Continue the standardisation of the ISH procedure

As this procedure is relatively lengthy and expensive, ISH is unsuitable as a rapid routine diagnostic method. However, it represents a very useful tool in understanding the pathogenesis of the virus, to assess archive material or to confirm PCR results.

Use PCR on archive material

A new PCR protocol that would allow detection of KHV DNA in fixed, wax embedded carp tissues is under development. Initial results (not shown) are promising and after validation, the technique could be applied to analyse fixed archive material corresponding to previous unexplained carp mortality.

References

- Ariav R., Tinman S. and Bejerano I. (1999) First report of newly emerging viral disease of *Cyprinus carpio* species in Israel. In: Abstracts of 9th International EAFP Conference, Rhodes, Greece. Sept. 1999., P-151.
- Body A., Loeffrig F., Charlier G. and Collard A. (2000) Isolation of virus-like particles from koi (*Cyprinus carpio*) suffering gill necrosis. Bull.Eur.Ass.Fish Pathol. 20: 87-88.
- Bretzinger A., Fischer-Scherl T., Oumouna M., Hoffman R. and Truyen U. (1999) Mass mortalities in koi carp, *Cyprinus carpio*, associated with gill and skin disease. Bull.Eur.Ass.Fish Pathol. 19: 182-185.
- Gilad O., Yun S., Andree K.B., Adkison M.A., Zlotkin A., Bercovier H., Eldar A. and Hedrick R.P. (2002) Initial characteristics of koi herpesvirus and development of a polymerase chain reaction assay to detect the virus in koi, *Cyprinus carpio* koi. Dis. aquat. Org. 48: 101-108.
- Gray W.L., Mullis L., LaPatra S.E., Groff J.M. and Goodwin A. (2002) Detection of koi herpesvirus DNA in tissues of infected fish. J. Fish Dis. 25: 171-178.
- Hedrick R.P., Gilad O., Yun S., Spangenberg J.V., Marty G.D., Nordhausen R.W., Kebus M.J., Bercovier H. and Eldar A. (2000) A herpesvirus associated with mass mortality of juvenile and adult koi, a strain of common carp. J. aquat. Anim. Health 12: 44-57.
- Neukirch M., Botcher K. and Bunnajirakul S. Isolation of a virus from koi with altered gills. Bull.Eur.Ass.Fish Pathol. 19: 221-224.
- Neukirch M. and Kunz U. (2001) Isolation and preliminary characterization of several viruses from koi (*Cyprinus carpio*) suffering gill necrosis and mortality. Bull. Eur.Ass. Fish Pathol. 21: 125-135.