DEVELOPMENT AND COMPARISON OF TECHNIQUES FOR THE DIAGNOSIS OF KOI HERPES VIRUS (KHV)

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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) Holland, Belgium, the UK and the USA. A herpes virus (Koi herpes virus, KHV) was isolated from koi carp in the USA and in Israel (Hedrick *et al.* 2000) and on two occasions in 2000 and four in 2001 from koi carp recently imported into the UK. The sequence of a 484bp amplicon obtained from KHV isolates from different UK outbreaks indicate that the UK virus isolates appear to be similar to the KHV isolated in the USA, suggesting the possible emergence of a new viral disease problem.

Material and methods

Samples: Fish sampled from eight disease incidents, represented by 104 pools of gills and viscera of koi and common carp presenting gross symptoms of the KHV infection (Figure 1) were processed by cell culture isolation and PCR. 45 pools of carp gills and viscera samples corresponding to SVC infected and asymptomatic carp were used as negative controls for the tissue culture and the PCR. Additional negative controls for the PCR consisted of channel catfish virus (CCV) and two different isolations of an unknown viral agent from carp (Neukirch *et al.* 1999, Body *et al.* 2000) grown extensively on tissue culture.

Figure 1: External, typical signs of the natural KHV infections range from small patches of discolouration 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 0.45µm filtered to reduce cell toxicity and bacterial contamination.

Cell culture isolation: Koi fin (KF) cell monolayers in 12.5cm^2 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 showing no 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 (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).

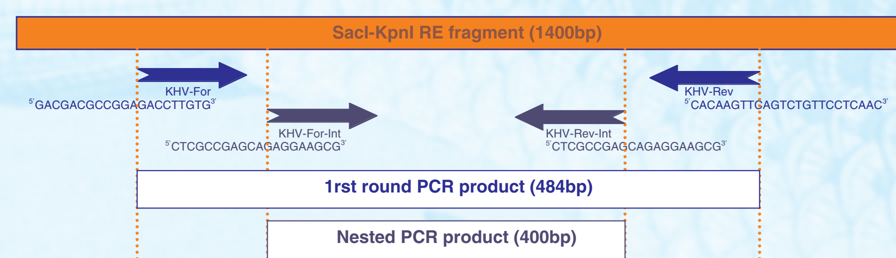


Figure 2: KHV PCR was carried out using the following primers which allowed amplification of a 484bp fragment (single round) or a 400bp fragment (nested) in the 1400bp SacI-KpnI restriction fragment of the KHV genome.

Light and electron microscopy (EM): Tissues were fixed with NBF and processed for standard paraffin embedding and sectioning. 5 µm sections were stained with hematoxylin and eosin and some more specifically with Farley Feulgen DNA stain. For EM, 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, 30', 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; 10' at 42°C) and the specifically bound probe was detected by immunohistochemistry. The BCIP/NBT substrate for the conjugate enzyme alkaline phosphatase stained infected cells blue, while the Bismarck Blue counter-stain produces a light brown background, allowing a good contrast and visualisation of labelling.

Results

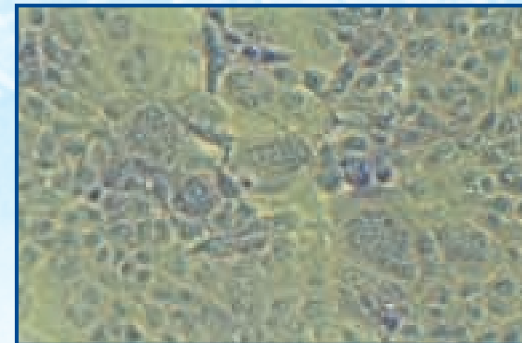


Figure 3: ***Cell culture isolation*** *- KHV produces a distinctive cpe in KF cells. The early cpe (7-9 days) appears as foci of darker, enlarged cells containing pin-point vacuoles. The cpe progresses by fusion to form multinucleate cells and margination of chromatin can be seen in some nuclei on fixed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

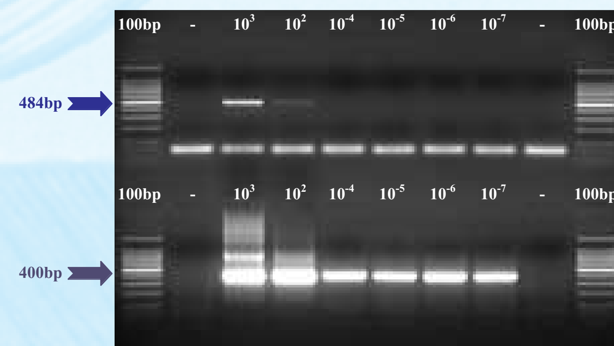
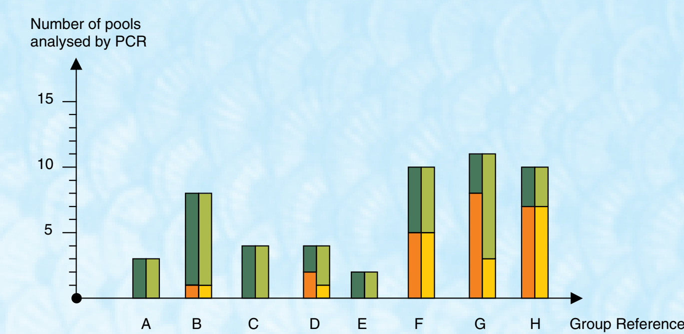


Figure 4: ***PCR*** *- The sensitivity of the PCR was assessed using serial dilutions of total DNA extracted from a KHV culture of known titre (10^5 TCID$_{50}$/ml). The limit of detection of KHV DNA by single round PCR is at the dilution corresponding to 10^2 TCID$_{50}$/ml (first lane) and it is at least at 10^{-7} TCID$_{50}$/ml by nested PCR (second lane).*



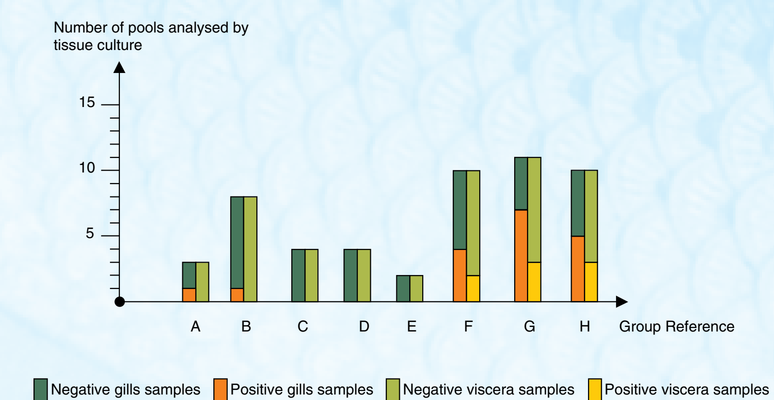


Figure 5: ***Isolation/PCR comparison*** *- 8 groups of carp showing gross symptoms of KHV infection were tested in parallel by culture isolation and PCR. Single round PCR allowed detection of KHV DNA in 6 groups but KHV could be isolated directly from only 5 groups. PCR positive results obtained with group D were confirmed by carp challenge and re-isolation of KHV from mortalities.*

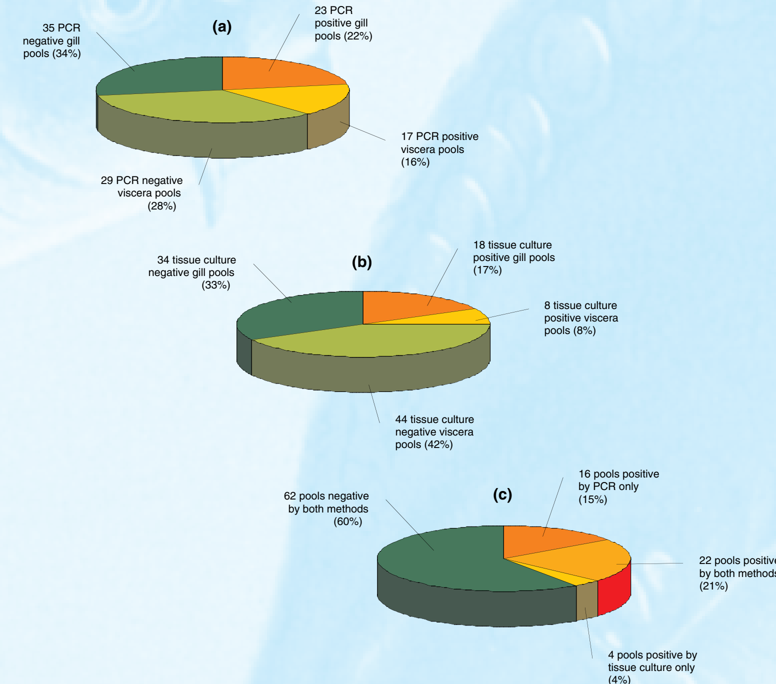


Figure 6: ***Isolation/PCR comparison*** *- The total number of pools analysed suggests that the single round PCR (a) is better than culture isolation (b) for the diagnosis of KHV. However, when results of individual pools are compared (c), the positive results of both methods overlap and the techniques appear to be of comparable accuracy.*

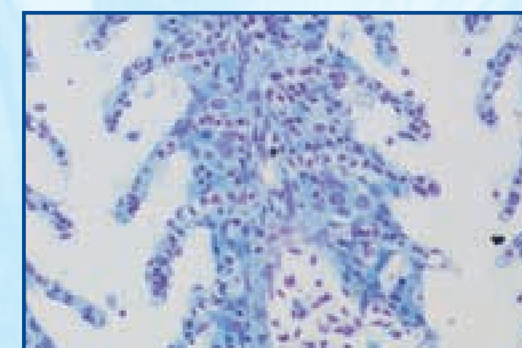


Figure 7: ***Histopathology*** *- Abnormal nuclei are most easily found in gill tissues [pictured, x200 Farley-Feulgen], but also in other affected tissues including the liver, kidney and spleen. Enlarged KHV-infected nuclei are clearly seen, characterised by chromatin margination, forming a signet ring appearance.* ***EM*** *- Visualisation of KHV particles in the same infected tissues proved to be very difficult and is not recommended for rapid diagnosis.*

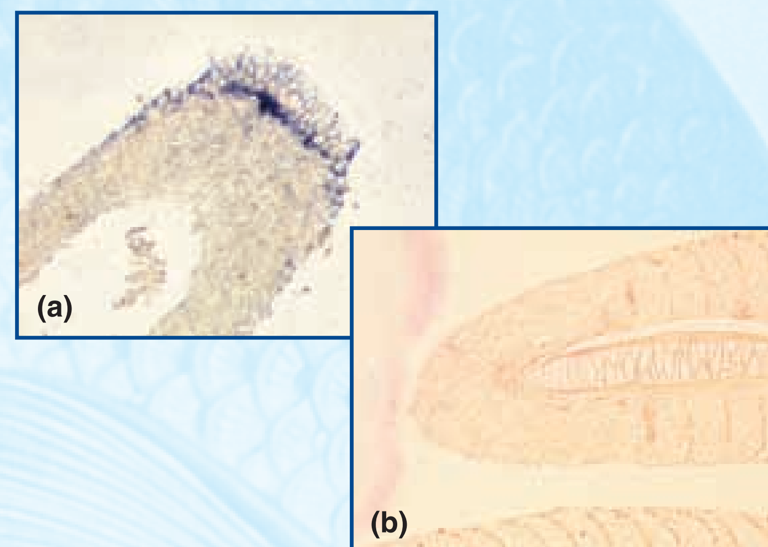


Figure 8: ***ISH*** *allowed specific labelling of infected cells. (a) The extremity of primary lamellae is swollen and labelled infected cells are present. (b) Gills of non-infected fish were used as control and were not labelled.*

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 :

  a) 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

  b) screening of virus carrier fish & epidemiological surveys of carp stocks

  c) virus pathogenicity studies, particularly primary infection of naïve carp and chronic infection of carrier fish. Also, examining latent and replication phases of the virus life cycle and factors influencing re-emergence

  d) 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. PCR positive tissue pools from batch D 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 (e.g., here to batches A, C and E) 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.

References

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Acknowledgements

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