

REPORT OF KOI HERPESVIRUS (KHV) OPEN WORKSHOP
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During the 13th EAFP Conference, an open koi herpesvirus (KHV) workshop was organized. About 60 people attended the workshop. The first part (A) of the workshop consisted of 8 short presentations by invited speakers. Some of these presentations are available as PDF files at the EAFP website. After the presentations, a discussion (B) on a list of topics followed, with conclusions.

A) SHORT PRESENTATIONS

1. The EPIZONE questionnaire: Global spread of Koi Herpes Virus by 2007 :

O. Haenen* & N. Olesen (see PDF).

Results from a global KHV questionnaire, held from end 2006 to mid 2007, were presented. The data were also presented in poster no. 10 at the conference and the PDF is available on the EAFP website. The questionnaire on KHV was returned by 45 countries. KHV has been detected and reported in 26 countries and more than 33 countries tested for KHV. Of these 33, >28 used PCR-based tests and 15 of these laboratories participated in the PCR ring test organised by Cefas, UK in 2006. KHV disease was reported to occur in all sizes of koi and common carp. More studies of virus latency and susceptibility of goldfish and other fish species to KHV are urgently needed. Measures taken to control KHVD included stamping out, disinfection, temperature changes, and/or vaccination and the latter is still under development. Although KHV is listed as notifiable to the OIE and EU, only 10 countries had national legislation to control KHV. The need for international training in diagnostic methods was also stressed.

2. Current status of KHV infection in Japan and our research activity: M. Sano*, S. Miwa & T. Iida (see PDF).

An overview of KHV disease cases diagnosed by the Fisheries Research Agency and NRIA in Japan was presented. Following the first outbreaks in 2003, 465 cases were diagnosed in 2004, 222 cases in 2005 and 121 cases in 2006. The research activity on KHVD at NRIA was also outlined, project outputs included: improvement of Gray's PCR protocol and development of a LAMP protocol, an Asian strain of KHV could be distinguished from European, USA and Israeli strains by PCR and latent KHV can be detected in brain tissue of surviving carp. Trials on reactivation of KHV and susceptibility studies in goldfish, crucian carp, indigenous common carp and carp larvae were also summarised. The future research projects at NRIA for 2007-2010 include: development of detection methods for asymptomatic, potential carriers of KHV, prevention of KHV spread from farmed carp/koi and monitoring the status of KHV in lakes where outbreaks of KHV have occurred.

Question (Q). - G. Bovo: Are the carp in your rivers native or introduced?

Answer (A). - There is a small proportion of localised indigenous carp, which are highly susceptible, the rest are introduced and less susceptible.

Q. - N. Taylor: Is the increase in cases of KHV seen in aquaculture an increase in real terms or an increase relative to the other sectors?

A. – increase in cultured (ornamental and food) carp populations compared to the wild carp.

3. Possible new variant KHV found in the UK: D.M. Stone*, M.J. Dodge, G. Woolford and K.Way. (see PDF).

Reported the discovery of a new KHV variant during diagnostic investigations at Cefas Weymouth. The variant has been detected in common carp at 4 sites in the southern UK. Variant DNA was amplified using a nested PCR assay with generic primers that target the polymerase gene of cyprinid herpesviruses. The variant was not amplified by the Bercovier TK PCR assay and no virus was isolated in KF cell culture. The variant shares 96% nucleotide identity with the published, partial, DNA polymerase gene sequence. Also, 97% identity with the intercapsomeric triplex protein and 94% with the major capsid protein partial gene sequences. This translated into 98,93 and 98% identity at the amino acid level for the three genes. Further studies include development of more specific PCR assays to detect the variant and circulation of the new primer sequences to European NRL's as soon as possible.

Q. - S. Bergmann - Were these variant isolates from experiments or wild fish and if the latter could it be due to a vaccinated fish?

A.- The variant was detected in wild fish and yes it could be. We have asked for sequence information from the Israelis to compare but this has not yet been sent.

Q. - B. Oidtmann: Were these isolates from clinical outbreaks?

A. – Yes. The PCR products were of very low intensity and it is possible the KHV variant was not the cause of the clinical signs.

4. Host specificity of KHV infection: S. Bergmann* & J. Kempter. (no PDF available).

Data were presented on host specificity of KHV. By PCR, goldfish were found weak positive, and strongly positive by nested PCR. Goldfish, sheatfish, sturgeon, and grass carp kidney, and sturgeon gills were KHV positive by in-situ hybridisation (ISH). Sturgeon leucocytes stained KHV positive in IFAT testing and gill swabs from *Acipenser gueldenstaedtii* and *A. oxyrinchus* tested positive by nested PCR. Bergmann concluded, that at least the following fish species can be infected with KHV:

- goldfish (*Carassius auratus auratus*) ● crucian carp (*C. carassius*)
- grass carp (*Ctenopharyngodon idella*) ● bighead carp (*Aristichthys nobilis*)
- silver carp (*Hypophthalmichthys molitrix*) ● tench (*Tinca tinca*)
- sheatfish (*Silurus glanis*) ● wild common carp (*Cyprinus carpio*)
- vimba (*Vimba vimba*) ● sturgeon (acipenseridae, *A. gueldenstaedtii*, *A. oxyrinchus*, *A. ruthenus*)

Of the above mentioned fish species, all transmitted KHV infection to common carp, except for bighead carp, silver carp, sheatfish, vimba, and acipenseridae.

Q. - K. Way: From your last slide, were the mortalities indicated in the various species in the species themselves or in susceptible carp cohabited with these species?

A. – Cohabited carp

Q. - K. Way: In your ISH and IFAT testing do you see the same presentation in carp as you do in these other species?

A. – Yes, but many more cells are positive in carp. I believe it is a leukotropic virus.

Q. – K. Way: Did you detect KHV in the brains of carp ?

A. - only in S-African carp, in the granular layer only.

Q. – G. Bovo: When you say wild carp can transmit KHV to farmed carp do you observe mortality in wild carp?

A. - This is not so easy to observe.

Q. – E. Ariel: How was the transmission done from these species to the SPF carp?

A. – Exposure to effluent.

Comment – K. Davenport: We have heard about this work for over two years now yet we still have not heard from the industry that goldfish cause a problem (not even anecdotal evidence).

Q. – K. Davenport: Were other fish species than carp experimentally infected with KHV?

A. - Goldfish, sturgeon, wild carp, crucian carp, silver carp, and bighead carp were naturally infected.

Q. – K. Way: Could you clarify when you have transmitted KHV from other species to carp, whether these other species were naturally or experimentally infected?

A. – For goldfish both, and many of the other species were also both.

5. Investigation on polymerase chain reactions for the detection of KHV:

S. Bergmann* & J. Kempter (see PDF).

This presented the results of an evaluation of several detection methods for KHV. It was concluded that the nested PCR is the most sensitive method for detection of KHV: 0.01 fg DNA can be detected.

Q. – M. Engelsma: Could the sequence difference between Asian and European KHV types be due to cell culture adaption?

Ans. – Maybe, or perhaps temperature adaption?

6. Can goldfish get infected with CyHV-3 and permit propagation of the virus without developing the disease? M. El-Matbouli & H. Soliman (see PDF)

(presented by O. Haenen*).

In this lecture, a cohabitation study of koi and goldfish with KHV was presented. Although the koi died, the goldfish showed no symptoms of disease, but by LAMP method, the presence of CyHV-3 (KHV) DNA was detected in goldfish by PCR (using Bercovier TK primers) and by LAMP method. The amplicon sequenced from

goldfish was 100% similar to the published TK gene sequence for CyHV-3. This might implicate that goldfish are possible vectors of KHV.

7. Evaluation of the KHV PCR ring trial organised by Cefas: K.Way (see PDF).

A report of the 2006 KHV PCR ring-trial was presented. Participating laboratories were asked to incorporate the PCR primers recommended by Cefas into their existing standard PCR assays and compare them with the primers that they currently use. They were also urged to trial the standardised PCR protocols used at Cefas, Weymouth.

For the first ring trial 21 laboratories, in 20 countries world-wide, were sent a number of vials of freeze-dried tissue homogenate spiked with KHV. Of the 21 laboratories 20 used the Bercovier TK primers and 17 labs obtained clean, correct results for all 5 samples tested. 18 of the 21 labs also used the modified Gray SpH primers and clean, correct results were obtained by 12 of the 18. Some labs encountered problems when using particular primer sets including failure to amplify the target sequence and smearing and extra bands on their PCR gels. Some labs also reported problems with the DNazol extraction protocol. Possible solutions to these problems were highlighted in a report sent to all of the participating laboratories.

The 2007 ring-trial is already underway and is very similar to that in 2006, but includes some modifications to the PCR protocols and more laboratories have agreed to participate.

8. Resistance of carp strains to KHV. P.F. Dixon & K.Way*

KW reported preliminary results from KHV challenge trials at Cefas, Weymouth as part of the E.U. funded EUROCARP project. The challenge experiments were carried out on 96 families from 4 strains of Hungarian carp with the objective to identify carp with enhanced disease resistance response and growth performance. The results showed that a number of families exhibited increased resistance to KHV with the most resistant families showing 42-55% survival rates. The 20 most resistant and 20 most sensitive families were selected for further challenge studies and genomic, proteomic and immunological analysis of plasma and tissue samples.

B. General Discussion

• Evidence for different strains of KHV

H. Frank-Skall: They have had one case in Denmark from a Danish retailer that they were not able to find positive by PCR. A retailer in Sweden (linked to the same importer) also had a similar case.

K. Way: What type of samples were they?

H. Frank-Skall: Gills

K. Way: Were they very necrotic? – if so it may be that the virus was no longer present (it is shed very quickly from the gills).

A. Goodwin: Or was it a bacterial infection that mimics KHV? (eg *Flexibacter*).

O. Haenen: But this gives more yellowish gills.

K. Way: There is a need to get more specific primer sets out to more laboratories in case it is a variant.

S. Bergmann: It is also possible that the PCR reaction was overloaded you could try diluting the extracted sample.

B. Oidtmann: Has anyone sequenced KHV from vaccinated carp?

S. Bergmann: Yes – it is the same as other strains.

K. Way: We have also sequenced KHV at Cefas from vaccinated carp and it was the same, but don't forget the fish may be double infected with vaccine and wild type strains.

N. J. Olesen: The importance of KHV has increased now because it is EU listed, we need a good single test, but when will we have one in the OIE manual.

J. Warg: Are the polymerase primers specific for KHV or will they pick up CyHV 1 and 2?

D. Stone: They are generic, hence sequencing is required.

- **Diagnosis: which test(s) to use in which case (screening, confirmation, etc.), and their value.**

K. Davenport: For how long in a survivor are you able to identify PCR products?

S. Bergmann: So far the longest tested is 3 yrs, and approximately 10-30% of fish from an infected group tested positive. This does not seem to be affected by temperature, but may be affected by strain of carp.

B. Oidtmann: Was this a consistent finding?

S. Bergmann: Yes.

N. Taylor: In which tissues?

S. Bergmann: Kidney and gills more often than in the rest of the fish.

O. Haenen: Do we have enough tests available?

K. Way: Yes for clinical disease and confirmation, we are less confident for carrier/latent fish.

S. Adams: Has anyone tested antibodies on these new variants? Don't forget the use of antibodies for diagnostics. Some antibody-based detection techniques are capable of detecting sub-clinical infection.

K. Davenport: Research needs to be collated and communicated to the industry (EU CRL or OIE).

K. Way: EU is currently funding very little research on KHV.

K. Davenport: Simple clear communication may make a difference to buying decisions. It may be better than more research or control policies.

H. Herikstad: The fact, that the EU is currently not paying much for KHV research means, that you as researchers should convince the EU, and the Aquaculture Technology Platform to get it, via DG SANCO.

- **Latency / carrier state**

O. Haenen: There are sufficient confirmative methods for KHV, but not for screening. The nested PCR is the most sensitive method for screening. Detection of latency is still a problem.

K. Yuasa: a poster on sensitivity of KHV testing is present in the poster room (see book of abstracts, P-26). Also, believes the extraction method is more important than PCR method.

- **Vaccines: current progress in development**

Q. – O. Haenen: Does anybody have news concerning KHV vaccines?

A. - No response.

Q. - E. Hudson: Will the new the directive allow vaccination?

A. - S. Cabot: This is currently on the agenda for discussion.

The directive states that countries are not allowed to vaccinate, or import vaccinated fish into disease free areas. Since we already have such fish in the EU this is a complex situation. Methods to detect vaccinated fish are needed.

N. Olesen : In KHV-free zones no vaccination should take place.

K. Way: The Israeli protocol is to keep carp until the vaccine strain is no longer detectable by PCR (according to the Israelis). Most sites in Israel are able to vaccinate carp and are also able to export these to the EU – how many of these fish also carry wild type KHV and pose a threat?

K. Davenport: At a meeting in Israel he was told that after a specified time after vaccination (which was quite short) the Israelis were not able to find live virus or virus by PCR.

B. Oidtmann: This has been said a lot but the evidence never really provided.

- **Biosecurity measures: Effective disinfection of KHV**

There was no response to the question – are there any incidences of inadequacy in current disinfection methods available ?

K. Way: It is easy to disinfect for KHV, in contrast to some other viruses.

O. Haenen: Kasai et al., 2005 have tested various disinfectants for KHV; more studies are available.

- **Prevention of KHVD in line with new notifiable status (Codes of practice)**

E. Hudson: Suggested to the meeting to view poster no. 28 (Statutory control of KHV in England and Wales, Denham *et al.*) and efishbusiness.co.uk website.

There was no further discussion.

- **The roles of the OIE reference laboratories for KHV (test validation, training)**

K. Way: Please note there are two reference labs, Cefas (UK) and NIRA (Japan) – training in diagnostic methods and test validation are a high priority. Twinning between National Reference Laboratories and the OIE reference labs is also encouraged (see also www.oie.int).

M. Sano: NIRA cooperates with Network of Aquaculture Centres in Asia-Pacific (NACA) and SEAFDEC in Asia offering training courses. SEAFDEC organizes a meeting on emerging diseases in Thailand in December 2007 (<http://rfdp.seafdec.org.ph/workshop2007>)

O. Haenen: Last year we had a twinning with the NRL's of Slovenia and this year with the NRL of Croatia: experts of our colleague labs participate in a training in KHV testing during a week at our lab, via TAIEX (EU) funding.

G. Bovo: Commented on the 2 year time span in updating the printed OIE diagnostic manual.

K. Way: Reminded the audience that the website is current and amendments are rapidly updated on the website between printed release dates.

S. Bergmann: Noted he is assessing nested real time PCR methods for screening.

K. Way: Noted that testing for latency by PCR may require different primer sets.

CONCLUSIONS :

- There is evidence for the existence of different strains of KHV. Asian strains can be distinguished from European/USA/Israel strains by PCR. For confirmation sequencing is necessary.
- Diagnostic methods are given in the OIE Manual (www.oie.int), but screening (surveillance) tests have not been validated sufficiently to be included. More sensitive PCR-based methods are being developed and nested PCR is of use to detect a very low infection level in koi or common carp. There are a number of confirmatory tests available and the on-line OIE Manual is updated regularly.
- Detection of latency is still a problem and is the subject of studies at a number of laboratories.
- No news on alternative vaccines to the attenuated vaccine in use in Israel. The EU has a non-vaccination policy, but it is in debate.
- Effective disinfection, as part of biosecurity measures, for KHV presents no problems, and is similar to other viral pathogens (e.g. SVCV).
- There are 2 OIE reference laboratories for KHV and they work together to provide advice and training in diagnostic methods for KHV. Twinning between labs for diagnostic training is encouraged and already practiced in Europe, funded by the EC. In Asia, SEAFDEC and NACA assist in training.

All speakers, reporters and attendees are thanked for their active participation in this workshop. This report will be available on the EAFF website soon, together with some of the presentations as PDF files.

Forthcoming events : There was a recommendation to participate in a **two-day International Workshop on CyHV-3 (KHV)**, to be held in the Dan - Caesarea Hotel, Caesarea, Israel: *Cyprinid herpes viruses: Basic and applied aspects*, 17-18 February 2008. email: mkotler@cc.huji.ac.il