Koi herpesvirus workshop

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More than 70 international conference participants attended the workshop on Monday 12th Sept 2005. During that afternoon session, the short lectures of several research groups were presented as listed below. As there was no time left for the discussion on Monday, this was postponed to Tuesday afternoon, 13th Sept 2005. During 70 minutes the points listed below were discussed by more than 30 conference participants. The report of the KHV workshop is given below.

Introduction

Olga Haenen stressed the point of importance of KHV at the world level, and the need for this workshop, to aid in prevention and control of the hazard, KHV brings. The O.I.E. had posed some questions, after the request of the EU, to list KHV at the OIE list. These questions would form the basis of a discussion at the end of the workshop. The presentations were meant to communicate current studies by international research groups on KHV to hopefully avoid duplication of research in the future.

KHV short presentations of several research groups

After the introduction, the international KHV experts presented the past and present work of their research groups.

R. Hedrick (University of California, Davis, USA) (presented by O. Haenen):

Current research:

1) *Taxonomy* of cyprinid herpes viruses and herpes viruses of lower vertebrates in general by sequencing of conserved genes with implications for higher taxonomic revisions. Studies in cooperation with Davison and other colleagues (Aoki, Stone and Way, Bercovier).

2) *Mechanisms of pathogenesis* by identification of genes associated with virulence, tumor formation and host range, through genome sequencing and then genome manipulations.

a) Genome sequencing underway:
   - CyHV-3 complete as led by Dr. T. Aoki and others.
   - CyHV-1 approximately 70% sequence complete (Hedrick, Waltzek, Davison).
   - CyHV-2 in initial phases (Hedrick).

b) Genome manipulations – establishment of bacterial artificial chromosomes (BAC) containing the entire genome for each virus and then creation of gene knockouts and selected gene swapping between viruses

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followed by testing of modified viruses for in vitro growth and virulence in koi and or goldfish. CyHV-3 BAC is nearly complete, CyHV-1 underway and CyHV-2 just starting.

3) *Detection methods* for cyprinid herpesviruses.

a) Comparison of virus detection for CyHV-3 by cell culture isolation in KF-1, PCR, and injection of koi,

b) Improved detection of CyHV-2, with Goodwin at University of Arkansas (USA) in lead role, and UC Davis assisting.

4) *Control – by examining carrier state.*

a) Examine correlations between ELISA and RT-PCR among CyHV-3 experimentally-infected koi (Cooperation with Bercovier and Eldar at Hebrew University, Jerusalem).

b) Evaluate criteria for reactivation or shedding of CyHV-3 from carriers.

c) Evaluation of goldfish X common carp hybrids for susceptibility to CyHV-3 and CyHV-2.

M. Yoshimizu (University of Hokkaido, Hakodate, Japan).

Influences of biotic and abiotic factors on KHV were presented. The infectivities of KHV in water and mud from Japanese waters after autoclavation and filtration, at various temperatures were presented. The cumulative mortality of carp infected with KHV in pond water just after and 3 days after mixing were also presented. The effect of heat treatment, and UV irradiation on the KHV infectivity were given. Additionally, the effects of various disinfectants (iodophore, sodium hypochlorite, benzalkonium chloride, ethyl alcohol) on KHV infectivity were presented. These data were also presented in poster no. 2.6 of this conference, and were partly published in Kasai, H. et al., 2005. Virucidal effects of ultraviolet, heat treatment and disinfectants against Koi Herpesvirus (KHV). Fish Pathology 40(3): 137-138, 9. During the discussion, T. Hastings asked about the titers of KHV. Yoshimizu stated, that the detection limit was $10^{1.8}$, and that $10^{3.2}$ was the highest titer found at his laboratory. It is not known, what the highest titer of KHV in fish was. Hastings responded by saying that as the starting titre of virus was not very high, only a small reduction in titre could be measured, and so the effectiveness of the disinfectants was not easy to assess from the data presented.

S. Bergmann (FLI, Federal Research Institute for Animal Health, Insel Riems, Germany).

In this presentation, the occurrence of KHV in fish, not belonging to the species *Cyprinus carpio* L., especially goldfish *(Carassius auratus)* and crucian carp *(Carassius carassius)* was presented, based on data of the RT-PCR (modified after Gilad et al., 2002) and nPCR of leukocytes of these fish, 7,14, 45, 60 days and one year after injection with KHV.

This subject gave some discussion. K. Davenport: If goldfish would be susceptible, a genus jump had been made by KHV. Bergmann: The genome is an old one, which changed after 1996 from common carp, to koi, also to goldfish. With Gilad-primers all samples of goldfish had been negative, but if you take another DNA polymerase (not a Taq polymerase), you get many more positives. Hoffmann asked, if survivors of the goldfish experiment could infect naïve carp during

cohabitation, resulting in mortality? Bergmann: This is tested currently, in SPF carp, without stressing them as extra trigger for disease. The best "organs" to test are the leukocytes. The KHV seems to be more lymphotrope than neutrotrope. Ariel: Virus isolation of KHV is not the best method, as carps may be negative, whereas the PCR results are positive. Question: were there other pathogens involved in this mortality? No. El Matbouli: after 4 days post infection KHV is already found in the leukocytes, which is rather early? Bergmann: This is not early, as KHV is taken up easily through the intestine. Hoffmann: are other diseases involved in the mortality? Bergmann: No. Only now and then *Aeromonas hydrophila* was isolated. Eldar: There is no golden standard, so the results on goldfish are speculative. The PCR test is not a proof of presence of infective virus. Therefore, one should be cautious with these results. Bergmann: DNA of KHV, mRNA and specific proteins were found in the goldfish after cohabitation, and virus was isolated after cohabitation, which should be the proof. Further retesting of goldfish for susceptibility to KHV is needed.

The second part of the presentation of Bergmann was about stability of KHV in water and mud. Infected koi from Thailand were cohabitated with SPF carp at 21°C. All fish died within 21 days. The aquarium was cleaned daily over 14 days but not the filter material. After that, SPF carp were placed into the aquarium and the fish died within 10 days under severe clinical signs. Carps in the same experiment with "European type" of KHV did not show any sign of a disease. The third part of his presentation was on latency or persistence of KHV in infected carp. *In situ* hybridization showed, that even after 7 month post infection virus could be found at the basis of the gills and in other organs (kidney, spleen, leukocytes).

See also abstract O-001 of an oral presentation at this conference, entitled: Possible vectors for Koi Herpesvirus (KHV) infection, by Bergmann et al.

P. Dixon (CEFAS, Weymouth, UK).

Past research on KHV at CEFAS, Weymouth was presented: KHV has been isolated from koi carp in Koi Fin cells since 2000. From 2003 KHV was also detected in feral common carp in fisheries. The PCR (Gilad et al., 2002) was used, and was found to be more sensitive than virus isolation. The UK isolates showed 100% homology with the US (ex Israel) isolates. The genome of KHV was partially sequenced. Then the *in situ* hybridization (ISH) and archive PCR were developed, which gave evidence that KHV was present in the UK as early as 1996. Via infection trials, no transmission to goldfish, tench and orfe was found. All isolates had a similar virulence for carp. Analysis of novel virus-induced genes also took place. Published PCR protocols were compared for detection of KHV DNA, and primers showing the highest level of sensitivity were further tested on KHV-infected tissue samples using a range of extraction methods and sample storage conditions. Further optimisation of the PCR included checks on the specificity of the more sensitive primer sets and using optimised protocols. PCR primer sets amplifying a shorter DNA fragment were shown to be more consistent in detecting KHV DNA.

Comparisons between PCR primer sets based on non-coding regions of the virus genome and those based on coding (gene) regions have shown that a published primer set based on the thymidine kinase (TK) gene is most sensitive for detection of KHV. Validation of the PCR methods will be carried out by proficiency testing of the selected protocols by ring-trials in other diagnostic laboratories around the world. My laboratory is willing to organise or co-ordinate these studies.

Related to long-term KHV-carrier studies, a carrier state was established in koi carp by manipulating water temperature. There were 2 scenarios – high and low initial mortality, and it was confirmed that KHV can become latent and can be re-activated by increasing the water temperature. Furthermore, reactivation was confirmed by virus detection and transmission of virus by co-habitation with naïve carp, introduced 150 days after the initial virus challenge. Levels of antibody to KHV were monitored with an ELISA developed at CEFAS (accepted for publication in Diseases of Aquatic Organisms: *St-Hilaire S, Beevers N, Way K, Le Deuff RM, Martin P, Joiner C* - Reactivation of KHV infections in common carp *Cyprinus carpio* [D 1544]).

The ELISA to detect antibodies to KHV in carp serum was based on a published assay developed for detection of channel catfish virus (IcHV-1) antibody (*Crawford, S.A., Gardner, I.A., & Hedrick, R.P.* (1999). An enzyme-linked immunosorbent assay (ELISA) for detection of antibodies to channel catfish virus (CCV) in channel catfish. J. Aquat. Anim. Health, 11: 148-153 and uses a commercial anti-carp-Ig monoclonal antibody. Antibodies to KHV have been detected after at least 12 months following exposure to the virus. This test has a very good potential as a tool to identify groups of carp previously exposed to KHV. It has detected antibody in Israeli carp vaccinated with KHV. ELISA validation has shown low-level cross-reaction with antibodies to the carp poxvirus; the specificity is being compared with the ELISA developed at UC Davis.

There has been collaborative work with UC Davis and others, where KHV was compared with CyHV-1 (Carp pox herpesvirus), CyHV-2 (haematopoietic necrosis herpesvirus of goldfish) and IcHV-1 (channel catfish virus). Complete genes were sequenced – including Helicase, Intercapsomeric triplex protein, DNA polymerase and the major capsid protein. Sequence analysis showed KHV to be closely related to CyHV-1 and CyHV-2, and the 3 are distantly related to IcHV-1. It is suggested that KHV joins the group of related lower vertebrate viruses in the family *Herpesviridae* and is designated as CyHV-3 (see Waltzek et al. (9 co-authors): J.Gen.Virol. (2005) 86, 1659-1667).

A new EU project: EUROCARP: Disease and stress resistant common carp: combining quantitative, genomic and proteomic and immunological marker technologies to identify high performance strains, families and individuals starts Dec 2005, in cooperation with HAKI, Hungary, Institute of Aquaculture, UK, CEFAS, UK, University of Liverpool, UK, Akvaforsk, Norway, VNIRO, Russia, and FCFGS, Russia.

Bergmann asked if the cohabited fish in the long-term carrier study showed clinical disease. Answer: Yes, and all naïve cohabited

fish died. And was there any disease in the original fish after 150 days following the increase in temperature? Answer: Yes, a small number of them died as well.

T. Aoki (Tokyo University of Marine Science and Technology, Japan).

Complete sequences and comparative analysis of the koi herpesvirus genome isolated in Japan, USA and Israel from koi carp, *Cyprinus carpio* were presented. First, clinical signs of KHV disease were shown, and histories of KHV infections were given. The objective of this study was, the development of a preventive method against KHV infection. Therefore, understanding the KHV biology at the molecular level was necessary. In this study, the complete nucleotide sequences of KHV isolated in Japan, USA and Israel were determined. The KHV virus particles were purified and the KHV genomic DNA was isolated by the phenol extraction method. The purified genomic DNAs were digested with *Not*I or *Xba*I and then all digested fragments were separated by pulse-field gel electrophoresis (PFGE). The complete KHV genomic sequences were determined by shotgun sequencing at approximately 8 times coverage of their genome sizes. The determined sequences were annotated by computer software "Genome Gambler". Further genome features were presented.

It was concluded, that the total genome length of the Japanese, American and Israel KHVs is approximately 295 kb. These sizes and *Not*I restriction enzyme sites of all KHVs were conformed to the results of the PFGE analysis. The identities among these three KHVs were over 99% with some single nucleotide polymorphism and insertion or deletion mutations. Approximately 22 kb of the terminal sequences at both ends were highly identical, and there were many different repetitive sequences in their genome. There were 183-185 open reading frames (ORF) in these KHV genome, almost all of which have no significant identity or homology with known sequences available in GenBank. Some ORFs showed homology with cytokines and cytokine receptors.

Bergmann asked about the sequences for different KHV strains, carp poxvirus and goldfish herpesvirus which are older than one year now and nothing is published.

S. Tinman (Central Fish Health Laboratory, Nir David, Israel).

Israel had its first KHV outbreak in 1998. The disease, KHV causes have decreased the last few years in seriousness: less outbreaks and lower mortalities (from 80% towards 30-40%) in common carp and koi ponds are seen. The past 2 years, erratic swimming is seen, but often with no mortality at all. The topics under study: The carrier stage: what are the most important reasons for an outbreak, with stress induction? No results were found sofar. Goldfish were injected with KHV, which resulted in no clinical signs and no mortality. Cohabitation of these fish with naïve carp also gave no mortality. Other food fish were also injected with KHV, but no transfer took place to naïve common carp. They therefore assume, *Cyprinus carpio* is the only susceptible fish species to KHV. Serological testing is done. The kilo price of carp has decreased, as there is more carp available, as KHV disease has

become less serious. El Matbouli: was there mortality in all sizes of carp? Tinman: Yes. If we see disease, we use the PCR test, which is then mostly positive. We do not know, why the KHV problem has decreased – is it through genetic factors, cohabitation, vaccination?

A. Goodwin (University of Arkansas, Pine Bluff, USA).

In this presentation on KHV in the USA, first some major outbreaks in wild fish were reviewed: In Wisconsin, 5,000 fish died in 2005 and KHV was found in some of the fish by PCR, in Lake Chautauqua, NY, 10,000 fish died in 2004, and 25,000 fish in 2005, and in Santee-Cooper, SC, 50,000 fish died in 2004. KHV is still a huge problem in cultured carp and koi in private ponds and for dealers. It is very rare on koi farms, but the koi market is still down 15-20 % from pre-KHV levels. Problems that dealers have, correlate well with reliance on imports from multiple sources.

To test latency of KHV, a group of 600 common carp were infected with KHV and held at 21-22° C for 12 months. The mortality was 90%. By qPCR (Gilad), KHV was detected in 17% of the carps at 4 months post infection, but in none from 8 months post infection on. During the meeting of the AKCA (Associated Koi Clubs of America), July 2005, the following questions were raised as the most important to hobbyists. : Are survivors of KHV still dangerous? Can survivors be detected? What about those Israeli fish? Is inspection paperwork trustworthy? When will we have a vaccine? There was also a lively discussion about inspection ethics: If a farmer brings a koi sample to the lab in July (water temps 35°C) and asks for KHV testing by culture, do you culture the fish for virus and issue a report? What about by PCR? Is inspection of a "lot" any use? They were also extremely interested in vaccines and even pursuing the financing of their own effort. According to publications and Internet reports, labs working on vaccines include Yoshimura & Miyazaki, Mie University, Japan; Aoki, Tokyo University, Japan; Perelberg & Kotler, Hebrew University-Hadassah Medical School, Israel; North Carolina State University, and at least one commercial company.

R. Hoffmann (University of Munich, Institute of Zoology, Fish Biology and Fish Diseases, Munich, Germany).

The KHV-working group Munich includes cooperation with colleagues from CEFAS, Weymouth, UK.

The KHV diagnostics from 2001 until July 2005 were presented, based on PCR test results: The total numbers tested/positives/negatives in 2001 were 67/26/41, in 2002 152/69/83, in 2003 448/90/358, and in 2004 256/41/215. In total, 1275 samples were tested, of which 253 were positive, and 1031 were negative. They were mainly koi and a few were common carp. In 2001 and 2002 also koi from the Netherlands were tested. The latest tendency was increasing numbers of KHV outbreaks in 2005. Present and future activities around KHV are: Field studies in Bavarian carp farms and water systems, further development of methods: PCR, cultivation (virus) and ELISA (ab), and epidemiological studies in positive cases. The latest news was the end of August 2005:

detection of a mass mortality of carp by KHV-infection in a canal between Rhine and Danube, from which carp and other cyprinid fish would be tested. The project is supported by the Bavarian Fisheries Association. Haenen asked, the water temperature of the current KHV outbreak in the wild, and if there were typical clinical signs of KHV? Answer: 22-23°C, and there were typical clinical signs.

M. El-Matbouli (University of Munich, Institute of Zoology, Fish Biology and Fish Diseases, Munich, Germany).

Data on an inexpensive and rapid diagnostic method of Koi Herpesvirus (KHV) infection by loop-mediated isothermal amplification were presented. All known diagnostic methods have the disadvantage of long time requirements, instrument for amplification or time consuming methods for detection of the agent. Therefore, in this study they applied and evaluated the LAMP technology as a novel method for KHV diagnosis. The design of KHV-specific LAMP primers was demonstrated. The basic principle of LAMP (Eiken Chemical & Co) was schematically explained. The KHV DNA detection limit was shown, and results were shown of the LAMP assay. The KHV-LAMP assay developed in this study was rapid, specific in its application and was more sensitive compared to the commonly used PCR test in diagnosis of KHV. The assay can be used in small laboratories or private clinics, as only a water bath for DNA amplification is needed. It also requires no further post amplification work, since by using 1µl of the 1:10 diluted SAYBER Green I the result appears at once.

The advantages of LAMP were summarized, compared to the PCR: There is no need for a step to denature DNA, the amplification occurs under isothermal condition, it has a higher amplification efficiency (10^9-10^{10} time in 15-60 min.). By designing 4 primers the assay can recognize 6 distinct regions, and by using 6 primers it recognizes 8 distinct regions. LAMP can specifically amplify the target gene, it is cheap, and can be applied for DNA and RNA.

The method was given: *Diagnosis of KHV by using Loop-mediated isothermal amplification (LAMP) of DNA method:*

DNA isolation from KHV infected tissue by boiling for 15 min.

Isothermal amplification of the KHV at 65°C for one hour by using 4 primers.

Using 6 primers decreases the amplification duration to 35 min.

The LAMP test sensitivity and detection limit are equal to those of the PCR test.

The LAMP test is more specific as the 6 primers recognize 8 regions from the target DNA.

Detection of the amplification product by the naked eye without a need for gel electrophoresis.

The test duration from DNA extraction to the end result takes only 1.30 hour.

LAMP test requires only a regular water bath or heating block.

Bergmann asked, which other viruses were tested for cross reaction in the LAMP assay? Answer: See the poster, no. P.15.14 of this conference, An inexpensive and rapid

diagnostic method of Koi Herpesvirus (KHV) infection by loop-mediated isothermal amplification, by Soliman and El Matbouli.

T. Ito (National Research Institute of Aquaculture (NRIA), Japan).

In this presentation, research by a project group, consisting of NRIA, SEAFDEC, Hokkaido University, Tokyo University of Marine and Technology, Nippon Veterinary and Animal Science University, Eiken Chemical Co., Ltd., and Kyoritsu Seiyaku was presented. The major 3 research directions in this project are: 1) Pathology and epizootiology, especially pathogenesis studies (Nippon Veterinary and Animal Science University), determination of sensitivities of other fish species to KHV, vertical transmission, and virus carrier studies (NRIA), and gene analyses and comparison among different viral strains (NRIA and SEAFDEC); 2) Development of new diagnostic methods, especially improvement of the present PCR technique (NRIA), and development of rapid and/or sensitive diagnostic methods (NRIA and Eiken Chemical); 3) Prevention and disinfection of the disease, especially establishment of disinfection methods (Hokkaido University), vaccine development (Kyoritsu Seiyaku), and search for curative means (Tokyo University of Marine and Technology).

The major results of this project were, that a) The fins and kidney as well as gills were suitable to detect KHV for PCR-based diagnosis. (Ito et al., Abstracts of 7th Asian Fisheries Forum 04, 409, 2004, Nov.), b) KHV did not induce any clinical signs in juvenile and young goldfish and young crucian carp. Furthermore, KHV was not detected in those fish by PCR. (Unpublished), and c) Although carp juveniles are susceptible to KHV as well as adult fish, the larvae are probably not susceptible. (Ito et al., Abstracts of for the Annual Meeting of the Japanese Society of Fisheries Science, 69, 2005, Apr. in Japanese); d) Nucleotide sequences showed KHV in the Asia, USA, Israel and the Netherlands were distinct from each other. (Ito et al., Abstracts of 7th Asian Fisheries Forum 04, 409, 2004, Nov.); e) The PCR method with the *Sph* I-5 primer set was improved. (K. Yuasa et al, Fish Pathol., 40, 37-39, 2005) ; f) the ELISA method by Adkison et al.. (Fish Pathol., 40, 53-62, 2005) revealed that anti-KHV antibodies were found in the sera of many survivors from KHV outbreaks, explaining the absence of a second outbreak in most of the places (unpublished); g) The virucidal effects of UV irradiation, heat treatment and disinfectants against KHV were evaluated. (H. Kasai et al., Fish Pathol., 40(3): 137-138, 2005, 9)

The major ongoing studies of this project are 1) Investigation of viral behavior in infected fish (Do survival fish become carriers? Does KHV spread out through vertical transmission or not?); 2) Development and evaluation of diagnostic tools such as a loop-mediated isothermal amplification (LAMP) method and immunocytochemistry; and 3) Trial of control measures of the infection including vaccination and elevation of the water temperature.

Tinman asked, if they also infected larvae and fingerlings with KHV, and if this resulted in mortality? Ito: Yes, we did. The infection in fingerlings resulted in mortality, and they were positive in the PCR by Gilad et al. (2002). The infection in larvae of 1-3 days did not

result in mortality, and no KHV was diagnosed in the PCR. So, although carp juveniles are susceptible to KHV, the larvae immediately after hatching are probably not susceptible.

H.J. Schlotfeldt (DVM in Aquaculture & Fish Pathology, Ahlten, Germany).

In this presentation, six KHV cases in common (consumption) carp in the summer of 2005 in Germany were summarized very briefly. Because these case histories were preliminary and still confidential no names, no locations and even no Federal areas could be given, with the exception of case 6. Ponds varied from small to 300 ha facilities. Mortalities varied from low to high. In most cases, the epidemiology was unclear. Case no. 6 was the wild carp disease outbreak due to KHV, mentioned by Hoffmann in his lecture, see above. Case 6 happened in the Rhine-Danube section of the transeuropean North Sea-Black Sea canal and was the only one case with a possible "farm to wild" implication. In the mean time three sources of infection were postulated: a) through routine carp restocking from a large scale restocking fish producer (but also dealer), b) release of sick koi into the canal from private holders/hobbyists because of summer holidays and the popular assumption "sick fish will recover in such a large water body", and c) fish predating birds. Because KHV is not yet fully notifiable in Germany it was not feasible to carry out a compulsory sampling in the restocking dealers facility, which was denied by the owner (not a good reference for him). The postulates b) and c) remain speculative and unproven. At the end of the day a disappointing result indeed.

What was disturbing and alarming and rather unusual in these cases? Cases 1-4, where none of the epidemiological data could be identified as causative, the question arises, if KHV is far more disseminated as presumed? Is there an area and regional dependence? Did a contamination or introduction of the KHV infection happen several years before the first outbreaks due to KHV started? The outbreaks did not arise during the hot weather period of end July-first week of Aug (25 to 30°C air temperature), but arose when the temperature sunk during the very rainy and fresh (18-24°C air temperature) rest of August 2005. This is unusual and doesn't reflect the field experience of earlier years.

Oidtmann asked, what densities were present at the farms, and why, only low mortalities were seen? Schlotfeldt: The densities were those of extensive culture of carp in ponds. Sometimes, the real mortalities are only seen during harvesting of the carps in the autumn, when the ponds are emptied.

A. Eldar (Dept. of Poultry and Fish Diseases, the Kimron Veterinary Institute, Beit Dagan, Israel).

In a DNA microchip consortium on KHV, different sets of genes of the viral cycle are evaluated. This forms a useful start for understanding the pathogenesis, and maybe also vaccine development. They will test several isolates with various virulences for this.

The RT-PCR results are in between those of Goodwin and Bergmann. Carriers are believed to exist, but it is not known, in which organs the virus persists. The TK-PCR traces 100 fentogram DNA. The PCR test is now

quantitative, and used to test several fish species in Israel for KHV.

Bergmann asked, if the RT-PCR was also used to test other fish species than koi and carp? Eldar: No, we didn't. Bergmann: we did use the RT-PCR for other fish species and they were found positive. Eldar: The need for a KHV proficiency test for especially the PCR method was stressed. Davenport: PCR tests give various results at various laboratories. What happens, if KHV is listed? Haenen: Who would organize such a proficiency test, and who would like to join? A central person or group of experts should be responsible for it. **Colleagues are hereby invited to show their interest in participating in a proficiency test, by sending an E-mail to Olga Haenen** *(olga.haenen@wur.nl)*. J.B. Jones: In Australia, a ring test on PCR tests of White Sturgeon Irido Virus is organized. In every PCR you'll find differences between laboratories. Barramundi nodavirus in that context was also not right. Every step in the protocol is crucial, so it is a very difficult problem, to standardize this at all laboratories. If the result of the PCR is positive, it is positive. If is is negative, one should go back to virus isolation, or test more fish of the lot. But, what is the golden standard? Sensitivity is a difficult one, as the PCR sofar is the most sensitive. Ariel: If the PCR test is not the right one, should we use an antibody detection method? Dixon: No, they are still in development. Haenen: It also depends on if you are screening for KHV absence (you need a very sensitive method and enough samples), or if you want confirmation of KHV suspicion (a less sensitive but highly specific test is needed). Eldar: in virus isolation, sometimes 4 blind passages are needed before cytopathic effect occurs, which is not practical. Davenport: What about koi and carps of the Czech Republic? Vesely: Czech koi are being tested now, and still found negative for KHV.

M. Engelsma (NRL for Fish and Shellfish Diseases, CIDC-Lelystad, Lelystad, the Netherlands).

The CIDC-Lelystad works on diagnostics of KHV, especially they use the conventional PCR (Gilad et al., 2002), and are busy with the implementation of a Taqman PCR (Gilad et al., 2002). In The Netherlands, KHV has been present since 2001. The PCR positive samples tested sofar are: in 2001: 2 positive cases out of 6; in 2002: 6 positive of 30, in 2003: 27 positive of 63, and in 2004: 38 positive of 86. The positives were all ornamental koi. Until now no positive cases were found in carp in natural waters. The research on KHV deals with experimental infection of koi and carp, to produce defined negative and positive samples for test development, optimisation of an ELISA for detection of antibodies against KHV in carp and koi sera, and molecular identification of KHV strains isolated in The Netherlands (in cooperation with M.Sano).

J.B. Jones mentioned, that KHV is a listed pathogen in Australia. Aoki declared, they have fish species specific primers, which are very sensitive. Data will be published next year.

KHV Discussion

The pathogen, disease and its distribution

Chair: Olga Haenen

This is a short version of the KHV discussion report, because of restricted space in the Bulletin.

To obtain the detailed report of this KHV discussion: *Please contact Olga Haenen,by E-mail: olga.haenen@wur.nl, and she will mail it to you.*

1) Phylogenetic affiliation of KHV.

After short reactions from the audience, it was *concluded;* we keep the name KHV as it is, as this name is internationally best known, without any doubt. Hedrick: I agree that KHV remains the common name for the virus. However, whenever the scientific name is called for we should use *Cyprinid herpesvirus* 3 (CyHV-3) as we use *Ictalurid herpesvirus* 1 (IcHV-1) for CCV. This is just proper viral nomenclature according to the International Committee on Taxonomy of Viruses (ICTV).

2) A case definition for the disease (can we define KHV disease clearly?).

Conclusion: We call it KHV infection, and then it is clearly definable.

3) A better understanding of the current and potentially broad distribution of the associated agent (is the current distribution already too broad to control it?).

Conclusion: It is not yet too late to control KHV, if we use good and reliable test methods.

4) Factors in the complex leading to a KHV outbreak in koi (in Thailand f.i.) role of Flexibacteria, parasites, monogenea, other factors?

Conclusion: We should not ignore other factors or pathogens other than KHV, but KHV is the leading factor of KHV infection resulting in disease.

5) Resolution of the apparently conflicting laboratory data emerging for the role of cyprinids other than *Cyprinus carpio* (koi or common carp) in the virus life cycle including virus transmission (f.i. are goldfish potential carriers?).

Conclusion: Preliminary results of infection experiments from one research group suggest, that goldfish seems to be carrier of KHV, but possibly also susceptible to KHV. This latter point requires more research for evidence.

6) Review of past and more currently developed serological tests as sufficient indicators of potential virus carriers: ELISA e.g. Are these tests acceptable for indicating prior exposure and potential carriers?

Conclusion: The serological tests are of value to indicate prior exposure to KHV, when used at the population level. The ELISA used in combination with the most sensitive DNA detection methods is currently the best approach to detect KHV at the population level. Differences in serological methods however are a concern and the formation of a working group on KHV diagnosis and standardization of diagnostic tests is needed.

7) *Dependence on PCR* as the primary method of confirmation for presence of the associated agent, role of Taq Man?

Conclusion: We need to move towards a standardized, published PCR standard

applied internationally, to get uniform results. At the start we may need to use 2 tests in parallel, in an effort to validate the tests used.

8) Capability of member countries to meet the logistical challenges associated with the surveillance programs to demonstrate freedom from KHV infection (there will be many countries with no controls on ornamental fish: will this be a problem for a more global control of the disease?).

Conclusion: Ornamental and consumption fish should be separately treated in the legislation. Surveillance programs for KHV should not be obligatory related to possible EU or O.I.E. listing of KHV. Those countries able to demonstrate freedom of KHV based on surveillance however could do so. It is better, to look to the reality of outbreaks of KHV, in carp. If there are no reasons to suspect other fish species, monitoring is of no value, and/ or not practical to many countries.

9) Vaccination against KHV.

Conclusion: Vaccines are being developed at several laboratories. Which kind of vaccines is not clear. The vaccine should be distinguishable from the field KHV strain in diagnostic testing.

10) Should KHV be listed by the O.I.E., related to the criteria of the O.I.E. for listing?

Conclusion: > 25 people voted for listing of KHV by the O.I.E., and 3 were against listing. Most criteria of the O.I.E. for listing are fulfilled. See the whole discussion.

Acknowledgements

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