

Report of Carp Edema Virus – CEV Workshop 12th – 13th January 2015



Organized by European Reference Laboratory for Fish Diseases, National Veterinary Institute, Technical University of Denmark Copenhagen Denmark in collaboration with Dr. Olga Haenen Central Veterinary Institute (CVI) of Wageningen UR

Introduction

The Carp Edema Virus- CEV Workshop was held in Copenhagen, Denmark, 12th- 13th January 2015 at the premises of DTU Veterinary Institute in Bülowsvej 27, 1870 Frederiksberg C.

A total of 20 participants from 11 countries attended the meeting over the two days period. The workshop combined different single oral presentations and sessions with general discussions.

The workshop was organized and held due to the increasing amount of diagnostic cases where CEV was detected in diseased cyprinids (both Koi and common carp).

The primary aim of the workshop was to share knowledge, diagnostic protocols and material among participants and evaluate different strategies on how to tackle this issue.

During the first session of the first day representatives of all countries participating in the workshop described their experience and the cases where fish poxvirus was detected.

Subsequently diagnostic procedures available for the detection of this pathogen were described and compared.

In the evening a banquet dinner was held at Restaurant “Cassiopeia”.

During the second and last day a common strategy on how to tackle this pathogen with research project was addressed, looking into funding opportunities and cooperative activities.

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Program

Monday 12th of January 2015

12.15 - 13.00	<i>Arrival and lunch (facultative)</i>
13:00 – 13:15	Welcome address: <i>Niccolò, Niels and Olga</i> Chair: <i>Olga</i> , and Minutes: <i>Niccolò</i>
13:15 – 15:00	<ul style="list-style-type: none">• Representative of each laboratory present their diagnostic findings and relation to CEV, with their experience (approx.10 min each)
15.00 - 15.30	<i>Tea break</i>
15.30 - 17.30	<ul style="list-style-type: none">• Diagnostic techniques currently available for detection of CEV, recommendations for harmonized diagnostic method• Pathogen characterization focusing of differences between viral strains detected in carp and koi• Exchange of materials between labs
19.00	<ul style="list-style-type: none">• Joint dinner

Tuesday 13th of January 2015

8.30 – 10.00	ANIHWA and other calls: discussion, followed by active writing of proposal
10:00 – 10.30	<i>Coffee break</i>
10.30 – 12:30	<ul style="list-style-type: none">• Proposal writing continued• Appointments on a paper on urgency concerning CEV for EAFP Bulletin• EAFP CEV workshop Las Palmas Sept 2015 (Haenen, Way and Waltzek organizers).• Miscellaneous...
12.30 - 13.30	<i>Lunch and goodbyes</i>

Participants list

Name	Institution	E-mail
Sven Bergmann	FLI-Germany	Sven.Bergmann@fli.bund.de
Heike Schütze	FLI-Germany	Heike.Schuetze@fli.bund.de
Laurent Bigarré	Anses	laurent.bigarre@anses.fr
Mikolaj Adamek	University of Veterinary Medicine in Hanover	Mikolaj.Adamek@tiho-hannover.de
Verena Jung-Schroers	University of Veterinary Medicine in Hanover	verena.jung-schroers@tiho-hannover.de
Tomáš Veselý	Veterinary Research Institute, Brno, Czech Republic	vesely@vri.cz
Olga Haenen	CVI, part of WUR, Lelystad	olga.haenen@wur.nl
Thomas Waltzek	University of Florida	tbwaltzek@ufl.edu
Mansour El Matbouli	University of Veterinary Medicine Vienna	Mansour.El-Matbouli@vetmeduni.ac.at
Veronika Piačková	University of South Bohemia	piackova@frov.jcu.cz
Keith Way	CEFAS	keith.way@cefasc.co.uk
David Stone	CEFAS	david.stone@cefasc.co.uk
Marek Matras	Piwet_Poland	marek.matras@piwet.pulawy.pl
Mona Gjessing	NVI	mona.gjessing@vetinst.no
Ole B. Dale	NVI	ole.b.dale@vetinst.no
Anna Toffan	IZSVE	atoffan@izsve.it
Miriam Abbadi	IZSVE	mabbadi@izsve.it
Niels Jørgen Olesen	DTU-VET	njol@vet.dtu.dk
Susie Sommer Mikkelsen	DTU-VET	susmi@vet.dtu.dk
Niccoló Vendramin	DTU-VET	niven@vet.dtu.dk

DAY 1 – January 12th 2015

National reports

Reports describing the detection of poxvirus in Cyprinids are available from The Netherlands, UK, Germany, France, Austria, Italy and Czech Republic.

So far it has not been possible to grow the virus in cell cultures. The virus is suspected to be a primary pathogen in clinical outbreaks associated with gill edema, gill necrosis and enophthalmia and mortality in Cyprinids.

The diagnosis relies on demonstration of viral genetic material by PCR and qPCR protocols. Sequencing analysis and phylogeny describe two rather distinct clusters or lineages, one associated with disease in Koi carp at higher water temperatures, and one with common carp at mostly lower water temperatures.

Histopathological analysis display that gills are often severely affected.

The detection of this pathogen has increased in Europe in recent years. First detections were in 2008 in Germany and 2009 in UK, when samples belonging to imported diseased koi carp tested positive for CEV.

PCR analysis has allowed demonstration of CEV also in several European archive samples dating back to the late 1990's.

Reports (see PPT presentations) were provided by:

- Dr. Olga Haenen – The Netherlands
- Dr. Laurent Bigarré – France
- Dr. Miriam Abbadi – Italy
- Dr. Adamek, Dr. Schütze and Dr. Bergmann- Germany
- Dr. Veselý – Czech republic
- Dr. Way and Dr. Stone- UK
- Dr. El Matbouli – Austria

After reports of diagnostic cases in Europe, Dr. Waltzek from the University of Florida provided interesting inputs on poxviruses in aquatic animals. Four different poxvirus are associated with diseases in fish animals both food and ornamental fish.

The typical appearance of these viruses, once observed with electron microscopy, displays peculiar surface projections and presence of crystalloid. In samples from clinically affected fish, the different stages of viral maturation can be observed, with immature viral particles and mature that has typical bean-shaped core.

It was suggested to use Tartrate gradient to purify the agent from clinical samples.

Finally the presentation of diagnostic cases was concluded with description of poxvirus infection in Atlantic salmon gills. The disease has represented a problem for more than twenty years; it is an acute disease that

affects fish in the freshwater phase of the production cycle. Currently the target for the PCR used for diagnostics is the rifampicin resistance gene.

The clinical signs reported in koi and carp, in which CEV was detected, were:

lethargy, enophthalmus, excess of mucus production, lesions of skin and gills, and mortality.

Diagnostic techniques currently available for CEV

A number of different diagnostic procedures and the experience of the different participants was described.

The panel of diagnostic procedures consisted of:

- Conventional PCR described by Oyamatsu and colleagues in 1997
- Nested PCR designed by CEFAS
- Taq Man Real time PCR designed by CEFAS
- Taq Man Real time PCR designed by University of Hannover starting from Oyamatsu primers
- Real Time PCR with SYBR Green developed by CVI the Netherlands
- Taq Man Real time PCR designed by Florida University

All these protocols target the Core Protein P4.

The need for having sequences of other genes available in order to develop a confirmatory test, targeting another region of the pathogen was underlined.

It was agreed that a single qPCR protocol should be adopted in Europe, in order to guarantee a harmonized effective method all over the Union. The CEFAS Taq Man Real time PCR was the chosen method, and is available on request, via Keith and David. This method could be used also for monitoring.

Gills are the target organ to sample when CEV infection is suspected.

Pathogen Characterization

In this session a number of goals to achieve and ideas to develop were discussed in an open discussion in order to increase the knowledge and diagnostic capacities on this disease.

Key points in this topic are:

1) The establishment of in-vitro system for isolating and cultivation of the aetiological agent.

This is one of the major constraints for the development of more research activities and diagnostic capacity.

Despite a systematic approach in trying to cultivate the virus is still missing, it seems that fish cell lines currently used for viral diagnostics are not susceptible to the virus. In this perspective one explanation is that this virus has tropism for epithelia while most available fish cell lines are fibroblastic. The establishment of primary cell lines from gills and skin is thereby seen as a promising activity. This will allow, also, to increase the knowledge on the immunological features that characterize this infection. In this connection our Japanese colleague Dr. Takafumi Ito has a large number of still unexplored self developed fish cell lines available in his repository, cell lines that might contribute to CEV detection on new sensitive cells. In addition the capacity of generating primary cell lines at the NVI in Norway and work performed at University of Hannover will provide major contributions towards development of tools for poxvirus cultivation. Furthermore some work on how to enrich viral inoculum to enhance viral isolation in Vitro and to use antibodies to better purify the virus from naturally infected tissues has been made available. In a poster of Dr. M. Sano at Vietnam, end of Nov 2014, it was further emphasized, that adding a kidney cell suspension to cell lines for culture of CyHV-2 increases the titer – a possibility for CEV with gill cells (Haenen, pers. comm.).

The importance to save and collect as much positive material as possible from diagnostic cases for various tests, like virus isolation, histology, E.M. etc., was underlined.

2) In vivo challenge model

Despite this pathogen is frequently detected in association with disease outbreaks, and histological lesions are consistent with disease pattern associated to poxviruses, the demonstration of Koch's postulates is still missing. Once a protocol for cultivating the pathogen or for purifying it from the infected tissue of a natural outbreak is made available, the set up and implementation of experimental infections will provide crucial information on the role of this virus as primary or secondary pathogen.

3) Epidemiological and genetic characterisation of strains and further discrimination of different lineages

One of the key issues to investigate is an epidemiological survey, looking at reinfection in the farms that experience the outbreak in the past years.

It will also be important to assess the role of parasites often detected on carps as vector of this virus. With regards to the genetic characterisation of the viruses detected, it will be important to boost and strengthen the sequencing of the viruses, in order to better elucidate if the clustering in two lineages is consistent among a bigger case numbers.

4) Treatment of the infection

For this viral disease, treatment with 0.5% NaCl of the water in which the infected fish grow seems to provide good results enhancing survival of the treated koi and carp stocks. Further investigations are needed to understand the role of the osmotic pressure on the host tissues and on the pathogen to optimise the protocols when applicable (NB the use of salt is not applicable for big ponds frequently used for carp production in central Europe)

Further points from the presentations:

- Dr. Abbadi and Dr. Toffan – In Italy, a carp CEV case was detected with mortality < 10%, at 23°C. Analysis in archive samples detected 1 positive sample from a case in common carp in 2010 combined with CyHV-1, at 14°C. Another case from samples collected from symptomatic carps at 6-7°C combined with secondary infection from *Aeromonas* spp. For what concern koi carps one positive sample in 2014 with CyHV-1; one suspicious case of CEV in 2015 is under analysis, in this case koi displayed edema in CNS, gill and muscle and enophthalmus.
- Dr. Haenen, NL: The Netherlands detected CEV at temperatures around 20-23°C in koi, and in 6-9°C but also once at 18°C in carp. The SybrGreen qPCR is functional, but sometimes the positive control is not positive. Therefore we will step over to the qPCR developed by CEFAS soon. CVI will try to isolate CEV at fish cells kindly obtained from Dr. Ito.
- Dr. Bigarré, F: France has detected strains belonging to 2 genetic lineages of CEV since 2010. The qPCR is functional, but needs validation. A CEV ring test would be needed.
- Dr. Adamek, GE: Outbreaks detected at 15-20°C. Use of primary epithelium cells needed probably for virus replication. They try to make a skin cell line. Pathogenesis studies planned, and interest in immune response, type I IFN? Mucosal response? Population genetics, markers?
- Dr. Schütze, GE: Used EcoR1 restriction. Concluded, that the Oyamatsu et al., PCR was not suitable, but the CEFAS PCR was. Don't use Go Taq DNA polymerase. For monitoring use qPCR. We need a good positive control, organotropism, complete sequence of the virus, alternative diagnostic PCR or qPCR, isolation possibilities for CEV.
- Dr. Bergmann, GE: showed videos of sleepy behaviour of CEV positive and diseased koi, with enophthalmus and excessive slime production, at 13°C. Will work on serology of CEV. Good SPF carps needed for experimental infections.
- Dr. Way, UK: In Israel, KSD was detected in 2009 and in 2011 again. Purification of CEV was not yet successful. It was questioned, if CEV is related to Spring Carp Mortality Syndrome (SCMS). This is seen since 1980'es and occurs at 8-12°C. Increasing the water temperature suppressed the clinical signs.
- Dr. Stone, UK: There have probably been various introductions into the UK, as proven by sequences. CyPP-3 is the Japanese strain. ISH has been done on formaline-fixed samples, especially the gills were found CEV positive.
- Dr. Veselý, CZ: In CZ Republic, the 2nd case of CEV was found in koi, in a polyculture with *ide* (*Leuciscus idus*), showing uncoordinated swimming and sleepy behavior (!), swollen gall bladder and enophthalmus, but no gill lesions. Koi was qPCR CEV positive, ide was negative. Diameter of the virus was 300 nm. The koi detection was like M141 strain, the carp virus differed a bit.
- Dr. El Matbouli, Austria: Tested 9 x koi, of which 1x positive; and 13 x common carp, of which 4x positive; 1 detection was combined with SVCV. They saw in CEV positive carp in Austria as well many ectoparasites, and tested various cell lines, without cpe, but cells became CEV PCR+ after 3 passages. They saw edema in

carp and an empty gut with sticky, orange mucus, infiltrations in kidney and spleen. Is CEV immunosuppressing, and is it a chronic disease? Are there carriers? It seems so. Dr. Waltzek, USA: since 1996 CEV in USA (1st detection in California). T.E.M. performed epithelial cells of koi displayed in the cytoplasm both virions and square structures, probably cristalloids (often the case with pox viruses). Compares the 4A protein of Oyamatsu with the international received DNA sequences. Works also on molecular typing of other pox viruses of fish.

- Dr. O.B. Dale, NO: pox virus in salmon at 10-15°C, with apoptosis in gill epithelial cells. Oyamatsu CEV PCR was negative. Poxvirus of salmon seems to be a primary pathogen. Also here, salt adding inhibits clinics. It is a gill epithelial disease, like with CEV. Not replicable at cell cultures. Add gill cell suspension, see poster Dr. M.Sano? No giant cells observed and no inclusion bodies.
- Dr. Matras, POL: No detections of CEV in Poland so far: tested 6 batches of 15 carps from various carp farms.
- Hungary and other central European countries: no detections yet, no testing yet? Bergmann detected one positive CEV carp group imported into Italy from Hungary.
- Belgium: Dr. Lieffrig detected CEV.

DAY 2 -January 13th 2015

Funding opportunities

A list of funding opportunity was discussed in order to provide support to the research activities related to this initiative.

- ANHIWA deadline for pre-proposal 12 February 2015 (IT, F, SP, IS only can apply)
- Continuous call ANHIWA-ERA
- EMIDA: no new call coming in 2015
- H2020 - FeHRiDi pending. If approved in this project there are some money available for meeting/workshop also for CEV
- H2020 - ITN (innovative training network): money available for research and student, can include 3rd countries partners and industry/SME/companies but there is huge competition, writing one of this project is really time consuming. Next call in March and September 2015
- H2020 call in spring 2015 addressing fish health will be screened
- AQUAEXCEL 2020 pending : DK, CZ
- Norwegian Research Council and collaboration with Norwegian industry could find opportunities in cooperating on similar topics with other European countries
- Koi societies to be contacted
- EPIZONE II
- CoVetLab: a proposal have been submitted but unfortunately rejected (see Annex 1)
- COST project: money only for meeting and exchange personnel. Next call in March 2015

It is agreed to keep an eye and start interaction for funding sources such as: H2020 ITN, Norwegian Research Council, koi societies, EPIZONE II, CoVetLab, COST actions.

Capacities and skills and the interest of each lab has been listed and discussed in order to investigate the preferred topic/best expertise of each lab for a future project.

Italy had a specific request on use of non-lethal based sample techniques: Dr. Matbouli- University of Vienna found positive results from gill biopsy, skin biopsy and whole blood. This topic deserves to be better investigated in future.

Appointments on alert paper on CEV

In the past an “alert paper” on KHV has been written. It helped in raising awareness and finding financial support for KHV research activities. The structure of the paper (chapter, tables) has been addressed. It should be a brief paper, to let politicians know what is going on and what is needed for the future.

Journal of fish disease or DAO (the opportunity of having Open access paper will be investigated). Dr. Way - CEFAS is designed as “literature keeper”, and would be first author of the alert paper.

A table summarizing data from EU countries will be drafted and made circulated amongst CEV workshop participants (co-authors) to be filled in, and all other person contributing will be acknowledged at the end.

Timetable: Dr. Way-CEFAS and Dr. Haenen- CVI will start working on this paper. By mid February a draft paper will start to circulate, feedback from coauthors has to be provided by the end of March. The paper should be refined and submitted by mid April 2015.

Further events related to CEV

- **Annual workshop for National Reference Laboratories for Fish diseases:** as CEV disease is not a listed disease and there is not enough knowledge to categorize it as emerging disease, two talks will be allocated in the program.
- **EAFP meeting, Sept 2015 at Las Palmas:** a workshop “Novel viral infections of cyprinid fish” is going to be organized by Dr. Haenen-CVI, Dr. Way-CEFAS and Dr. Waltzek-Uni Florida. The workshop will last 1:45-2:00 hours, Dr. Haenen- CVI invited all experts present to participate. Set up of a program was discussed by the three organizers directly after the CEV meeting: After an intro from Olga Haenen, Tom Waltzek and Keith Way will present papers on other cyprinid viruses, and CEV respectively. Moreover, Dr. Ito will be asked to present a paper on CyHV-2. After this, there will be 4 other lectures of 6 min + 4 min discussion. Suggestions are welcome. A detailed program will be placed at the EAFP website, and

a joint publication would result from the workshop, for the EAFP Bulletin. This workshop will help in informing fish pathologists and others in an efficient way on novel cyprinid viruses, including CEV. To be planned at end of a session to allow good discussion (Olga).

Miscellaneous

Dr. Stone – CEFAS is going to publish CEV sequences and disseminate qPCR protocols. The 4a protein from koi virus will be cloned and the sequences made available. The qPCR protocol should be published as soon as possible.

Publications on CEV:

- Dr. Adamek- University of Hannover has already submitted a paper dealing with qPCR in which the Oyamatsu primers' limitations are listed, answer is still pending. Another paper has been sent by the same authors on the first detection of CEV in Germany.
- Dr. Matbouli- University of Vienna is preparing a paper on LAMP for CEV.
- Dr. Vesely- Czech republic expects to publish a paper on 2 CEV detections in common carp in ponds.
- Dr. Haenen- CVI is going to write the joint paper of the EAFP workshop 2015, Dr. Waltzek- University of Florida is preparing a paper on phylogeny on fish DNA viruses.

Dr. Olesen- DTU Vet remembers that for the future there is the need for a laboratory technique for surveillance, in order to decide whether this disease is really emerging or not, and therefore if CEV should be listed or not. Could be useful for a CEV survey in Europe? This point should also be addressed in the CEV paper.

Dr. Way-CEFAS underlined that prevalence in countries could be biased due to population management (release of koi carp is forbidden in open water systems in UK).



Picture with all participants at the Workshop