Report on the Workshop in diagnosis of the exotic fish diseases EHN and EUS and the 12th Annual Meeting of the National Reference Laboratories for Fish Diseases

Aarhus, Denmark
June 17-20, 2008

Organised by
the Community Reference Laboratory for Fish Diseases
National Veterinary Institute, Technical University of Denmark

Contents

Introduction and short summary

In June 17th-20th 2008, the 12th annual meeting of the National Reference Laboratories for fish diseases was held back-to-back with a workshop in diagnosis of the exotic fish diseases Epizootic hematopoietic necrosis (EHN) and Epizootic ulcerative necrosis (EUS) at the National Veterinary Institute in Aarhus, Denmark. A total number of 64 participants from 32 countries attended over the four day period. There were a total of five theoretical sessions and one practical session. In total, there were 40 presentations, 13 of which were given by invited speakers and five working platforms, of which four were run by invited instructors.
All theoretical sessions were held at Aarhus University close by our laboratory as the number of participants have grown to a size that we do no more have facilities for housing all in-house. The practical session and meals where, however, organised in the laboratory making a very crowded and lively day for all!

At the workshop, the first session focused on providing the participants with a theoretical introduction to the two diseases EHN and EUS that have been listed as exotic diseases in the new Council Directive 2006/88/EC. The introduction to EHNV was provided by four experts from three different laboratories in Europe. The EUS part was organised with the help from two experts from the OIE reference laboratory, in Bangkok, Thailand for EUS and two European experts.
At session II, the practical part of the workshop, five working platforms were designed. Two platforms concerned EHN diagnosis, two concerned EUS diagnosis and one were common for the two diseases. Participants were divided into five groups and each group was circulated between the five platforms. The workshop was terminated with a discussion of the outcome of the workshop followed by a drinks reception, where all the participants had the opportunity to network.
Highly relevant and interesting aspects of the two pathogens were presented in well prepared presentations and a solid theoretical and practical introduction to the methods of diagnosing the diseases was given. Furthermore, the workshop allowed many fruitful discussions.

The scientific programme of the Annual Meeting was diverse and covered many topics of current interest. The annual meeting opened with the traditional session on update of diseases in Europe, where once again participants from the member states presented new findings from their home countries. For the first time, a marine genotype III VHSV strain had caused disease outbreak in rainbow trout – and experiences from this outbreak in Norway was presented. We also heard about the ISAV situation in Faroe Islands, VHS outbreak in Slovenia, SVC outbreak in Romania and several other interesting cases were presented.
This session was followed by a session on technical issues related to sampling and diagnosis with both serological and molecular methods included. In this session we also discussed surveillance, diagnosis and sampling in relation to Council Directive EC 2006/88. On Thursday night the participants were invited to a banquet dinner in the Restaurant Seaside at the Harbour of Aarhus. The last day started with an update on scientific research carried out at some of the participating laboratories and an update was given on the establishment of a database for fish pathogenic viruses.
The annual meeting ended with the traditional update from the CRL, who gave a report from a year with focus on training of laboratories and the preparations and considerations about implementing the new listed diseases within our fields.

Minutes from the meeting were taken by Helle Frank Skall and Søren Kahns, and have afterwards been sent to presenters for correcting in order to avoid misunderstandings. Nicole Nicolajsen helped assembling the report. The minutes are included in this report together with abstract and comments

from the presentations and we would once again like to thank all the presenters for their great contribution without which the meeting would not have been a success.
The workshop and meeting was organised by a team consisting of Niels Jørgen Olesen, Helle Frank Skall, Nicole Nicolajsen and Søren Kahns with the help from the rest of the fish disease section at VET-DTU Aarhus.

The meeting next year is tentatively planned for May 24th -27th and will probably take place at DTU.Vet in Copenhagen, but more details will follow.

We wish to thank all of you for participating and look forward to seeing you next year!

Århus, 18 September 2008

Niels Jørgen Olesen and Søren Kahns

Programme

Workshop in diagnosis of the exotic fish diseases EHN and EUS
& 12th Annual Meeting of the National Reference Laboratories for Fish Diseases
17-20 June 2008
National Veterinary Institute, Technical University of Denmark, Århus, Denmark

Venue address: Aarhus University, Stakladen, Richard Mortensen Stuen, building 1420, Ndr. Ringgade 3, 8000 Århus C

Programme
Tuesday 17 June – **Workshop in diagnosis of the exotic fish diseases EHN and EUS**

REGISTRATION AND WELCOME ADDRESS

10:00 – 11:00	Workshop Registration WELCOME - Søren Kahns and Niels Jørgen Olesen
SESSION IA:	Epizootic haematopoietic necrosis virus (EHNV) Chair - Ellen Ariel
11:00 – 11:30	Epizootic haematopoietic necrosis (EHN) disease – a review - Britt Bang Jensen
11:30 – 12:00	Comparison of isolation procedures for ranavirus in fish organs - Ellen Ariel
12:00 – 12:15	How to detect EHNV and related ranaviruses using IHC and IFAT – Giuseppe Bovo
12:15 – 12:45	Ranaviruses - Molecular detection and differentiation - Riika Holopainen
12:45 – 13:00	Introduction to workshop - Britt Bang Jensen
13:00 – 14:00	Lunch
SESSION IB:	Epizootic Ulcerative Syndrome (EUS) Chair - Birgit Oidtmann
14:00 – 15:00	Introduction to Epizootic Ulcerative Syndrome - EUS – Somkiat Kanchanakhan
15:00 – 15:20	EUS outbreak situations and recent outbreaks in Africa – Somkiat Kanchanakhan
15:20 – 15:35	Morphological differences between *Aphanomyces invadans* and closely related Oomycetes - Birgit Oidtmann
15:35 –16:05	Susceptibility of three European freshwater fish species to *Aphanomyces invadans* - Birgit Oidtmann
16:05 – 16:25	Comparative pathology of fungal and fungal-like infections of fish – Steve Feist
16:25 – 17:00	Coffee break
17:00 – 17:30	Field and laboratory investigation of EUS outbreak - Somkiat Kanchanakhan - Establishing a case definition - Checklist of materials for field sampling - EUS sampling data sheet
18:00 – 18:30	Guidelines - Isolation and identification of Aphanomyces invadans from EUS-affected fish - Varinee Panyawachira
18:30 – 18:45	Introduction to Practical workshop – what to do tomorrow? - Søren Kahns

Wednesday 18 June – Workshop in diagnosis of the exotic fish diseases EHN and EUS

SESSION II:	Procedures for diagnosing EHN and EUS – a practical approach
09:00 – 12:30 13:30- 16:00	Participants will be divided into five groups. Each group will circulate between the following five platforms: Sampling of fish tissue for oomycete isolation and EUS histological slides examination - Varinee Panyawachira/ Birgit Oidtmann Aphanomyces identification; sporulation under microscopy and VDO - Somkiat Kanchanakhan PCR amplification of A. invadans and EHNV DNA - Riika Holopainen/ Søren Kahns Virological examination on cell culture - EHNV - Britt Bang Jensen Immunological methods used for diagnosis of EHNV - Giuseppe Bovo
16:00-17:00	Discussion and summary of the workshop
17:00	Drinks Reception and Goodbye

Thursday 19 June – **Annual Meeting of the National Reference Laboratories**

WELCOME ADDRESS AND ANNOUNCEMENTS

9:00 – 9:30	Annual Meeting Registration WELCOME - Søren Kahns and Niels Jørgen Olesen
SESSION III:	Update on important fish diseases in Europe and their control Chair: Rob Raynard
9:30 – 10:15	Survey & Diagnosis of listed fish diseases in the European Community 2007 Overview of disease situation in Europe - Niels Jørgen Olesen
10:15 – 10.30	Outbreak of viral haemorrhagic septicaemia (VHS) in sea farmed rainbow trout in Norway - Birgit Dannevig.
10:30 – 10:45	VHS outbreak in Slovenia - Vlasta Jencic.
10:45 – 11:00	Eradication of VHS in Denmark – Henrik Korsholm
11:00 – 11:30	Coffee break
11:30 – 11:45	Prevalence of low pathogenic Infectious Salmon Anaemia Virus of the HPR0 subtype in Farmed Atlantic Salmon (Salmo Salar L) in the Faroes - Debes Christiansen.
11:45 – 12:00	The Health Situation in Farmed Fish in Norway 2007 – Brit Hjeltnes
12:00 – 12:15	PD update from Norway – Torunn Taksdal
12:15 – 12:30	Fish diseases situation in Spain - Marta Vigo
12:30 – 13:00	The isolation of spring viraemia of carp virus, in Romania, in a clinical outbreak of disease in carp - Mihaela Costea
13:00 – 14:00	Lunch
SESSION IV:	Technical issues related to sampling and diagnosis Chair: Brit Hjeltnes
14:00 – 14:20	Risk Based surveillance - Pedro Rosado/Niels Jørgen Olesen

14:20 – 14:40	Establishment of a new Commission Decision on sampling and diagnostic procedures of listed diseases - Pedro Rosado/Niels Jørgen Olesen
14:40 – 15:00	Sampling procedures for molecular based methods, as PCR, in practical fish diagnostics – Olga Haenen
15:00 – 15:20	Development and application of real-time PCR assays for the detection of viral haemorrhagic septicaemia virus (VHSV) - Mike Snow
15:20 – 15:40	Serological methods recommended for detection of antibodies against VHSV and IHNV - Jeanette Castric
15:40 – 16:00	Experiences of a micro-dilution method for testing antimicrobial susceptibility of bacteria from - Björn Bengtsson
16:00 – 16:30	Coffee break
16:30 – 16:50	OIE recommended procedures for KHV diagnosis and the KHV PCR ring-trial 2007 – Keith Way
16:50 – 17:10	Infectious salmon anaemia as a non-exotic disease in the EU: Consequences for active surveillance – Rob Raynard
17:10 – 17:40	Health categorisation and implementation of risk based surveillance in Denmark – Henrik Korsholm
17:40 – 18:00	Health categorisation and implementation of risk based surveillance in Italy – Giuseppe Bovo
19:00	Banquet Dinner

Friday 20 June– Annual Meeting of the National Reference Laboratories
Continued

SESSION V	Scientific research update Chair: Giuseppe Bovo
9:00 – 9:30	Establishment of database for fish pathogenic viruses – Tanya Gray
9:30 – 9:50	ICES – Working Group on Pathology and Diseases of Marine Organisms (WGPDMO) – Steve Feist
9:50 – 10:10	Recent advances in fish immunology and virology - Niels Lorenzen
10:10 – 10:30	KHV diagnostics and vaccination against KHVD – Sven Bergman
10:30 – 11:00	Coffee break
SESSION VI:	Update from the CRL Chair: N.J. Olesen
11:00 – 11:10	Presentation of new CRL-fish web page – Helle Frank Skall
11:10 – 11:30	Proficiency tests 2007 - Niels Jørgen Olesen
11:30 – 11:50	A comparative sequence study launched in association with the proficiency test 2007 – Søren Kahns
11:50 – 12:10	Report from Year 2007 and work plan for 2008 and 2009 - Niels Jørgen Olesen
12:10 – 12:30	Next meeting and end of 12th Annual Meeting - Niels Jørgen Olesen
13:00	SANDWICHES AND GOODBYES

SESSION IA: Epizootic haematopoietic necrosis virus (EHNV)

Chair: Ellen Ariel

Epizootic haematopoietic necrosis (EHN) disease – a review

Britt Bang Jensen

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National Food Safety Institute, EVIRA, Finland.

Abstract: Epizootic Haematopoietic Necrosis Virus (EHNV) belongs to the genus ranavirus in the family Iridoviridae. The virus is an enveloped, double-stranded DNA-virus that replicates in haematopoietic tissue, and acquires its envelope by budding from the cell membrane. In ultrathin sections, the virions can be seen as hexagonal structures within the cell lumen. The virions can remain infective at 4°C for extended periods, but replicates best at temperatures between 20 and 28°C. The first outbreak of EHN was detected in red-fin perch (*Perca fluviatilis*) in Australia in 1984 during an outbreak with high mortalities in the summer, and has subsequently been isolated from mortalities in rainbow trout (*Onchorrhynkis mykiss*) in the same area. The virus has not been detected outside Australia, where it is still endemic, but molecularly similar viruses have been isolated from European catfish (*Ictalurus melas*) and European sheatfish (*Siluris glanis*).
The disease EHN is characterized by damage to the haematopoietic tissue in the kidney, spleen, and liver, where extensive necrosis with rounded and dissociated cells with pyknotic nuclei is seen. Clinical findings are associated with vascular damage, and include darkening of skin, bleeding at the fin bases, abdominal swelling, anorexia, apathy and erratic swimming. The virus attacks all ages of fish, but in endemic areas only fry and young fish are affected, whereas surviving adults can serve as reservoirs or carriers.
Virus can be re-isolated using standard procedures, and grows well in fish cell cultures.

Minutes:

The types of ranaviruses: FV3 (Frog virus 3), Tiger salamander ranavirus, BIV (Bohle Iridovirus), EHNV, ECV and ESV (European catfish virus), DFV and GF6 (Santee-Cooper ranavirus)) was described as was locations of outbreaks. In amphibians, all ranaviruses are listed whereas in fish it is only EHNV that is listed. The only reports of naturally susceptible species of EHNV have been in Redfin Perch and Rainbow trout. Experimental results showed that Pike fry and Pikeperch fingerlings were susceptible whereas Perch was not. Furthermore, Rainbow trout was not susceptible (under experimental conditions at VET-DTU). The RANA project was described together with the web page: www.ranavirus.net where more info can be obtained.

Questions/comments:

Sigurdur Helgason: *To what extend is EHNV thought to be spread?*
Britt Bang Jensen: *Maybe it has not been spread, but has always been there. Isolates from Europe and Australia is extremely similar. Only limited DNA sequencing exist for these viruses and we need more to do further genomic characterisation.*
Ellen Ariel: *We can only speculate but there is in some cases a high suspicion towards that the virus has spread. E.g. in England, it spread out as rings in the water – suggesting that it has been spread.*
Steve Feist: *It was stated that there are no antibodies in survivors. How does this relate to carrier state? Non-clinical infections occur but what caused mortality in these fish?*
Somkiat Kanchanakhan: *The spread of ranavirus is new in Thailand. We often find the virus. It is a seasonal disease that spread from frog farms.*

Guiseppe Bovo: *There is no direct evidence of introduction of ECV in catfish. In catfish farms in 1992 there were suddenly a lot of outbreaks. I am convinced that the virus somehow has been introduced.*
Britt Bang Jensen: *We have to remember that there is a high host specificity of the virus – ECV affects only catfish, not rainbow trout.*

Comparison of isolation procedures for ranavirus in fish organs.

E. Ariel[1], N. Nicolajsen[1], H Tapiovaara[2] and B. B. Jensen[1]
National Veterinary Institute, Danish Technical University, Hangøvej 2, 8200 Århus N, Denmark
National Food Safety Institute, EVIRA, Finland.

Abstract: In order to identify the best procedure for isolation of ranavirus in cell-culture, three different virus-isolation methods were tested and compared. An EHNV isolate was passaged in red-fin perch (*Perca fluviatilis*) and re-isolated 3 times in order to simulate natural conditions. Fish were sampled twice weekly and 7 organs were processed separately according to standard virus isolation procedures as described in the OIE Diagnostic Manual where the sample is grown at 22ºC in both EPC and BF2 cells for 2 weeks and then sub-cultivated for one week (method 1), and the same procedure but with cultivation temperature of 15ºC (method 2). These methods were compared to those procedures described in Commission Decision 183/2001/EEC, where the virus is grown for one week and then sub-cultivated for a week at 15ºC (method 3). Subsequently the results were analyzed in a multivariable logistic analysis. (Proc Genmod in the SAS-program). Samples cultured on BF-2 cells at 22°C for 2 weeks + 1 week sub-cultivation (method 1) appeared to provide more positive results than the other 2 methods and the other cell line tested. However, considering that the existing methods of surveillance for fish viruses in Europe also detect fish infected with ranaviruses, albeit at lower sensitivity, and that a sample of 30 fish are generally collected for surveillance purposes (more fish, more chances of finding it), it would be unwise and economically unpractical to recommend alternative procedures for surveillance on the basis of the results reported here alone.

Minutes:
We tested 5 cell lines, 10 isolates at 5 temperatures and registered the titre at intervals over 14 days. We identified the most sensitive isolation methods from organ material in redfin perch to be method 1. BF2 cells were more sensitive than EPC cells. Most sensitive organ for virus isolation is: kidney>brain>muscle> heart> liver>gills>spleen

Questions/comments:
David Stone: *If you check cultures and you can not see the CPE, it could still be that the virus is there – have you checked the negative cultures with IFAT?*
Ellen Ariel: *This would certainly be interesting from a scientific point of view. However, we approached this issue from a practical point of view and therefore tested different existing methods against each other. So if the CPE was not apparent, then the test was not useful. There is also economy to consider.*
Somkiat Kanchanakhan: *Infection experiments are performed at 25°C. Infections of wild or farmed fish often occur at lower temperatures so the disease is induced at lower temperatures and the mortality is higher at lower temperatures. Please specify what is meant by low and high temperatures, this could be very different between Thailand and northern Europe*
Niels Jørgen Olesen: *In Europe we use method 3 – what would it mean if we used method 1? What are your recommendations – can we accept the lower sensitivity.*

Ellen Ariel: *We have to consider the naturally infected fish before making a recommendation for organ samples. If we examine 30 fish in combination with clinics we increase the chance to find the virus and method 3 would then still detect the virus in my opinion.*
Niels Jørgen Olesen: *OIE discriminate between diagnostics and surveillance. For diagnostics we could use method 1. The question is what we should choose for surveillance.*
Britt Bang Jensen: *I am most worried about salmonids as they can have subclinical sign and regular methods will not necessarily find the EHN virus. Have anyone any experiences?*
Olga Haenen: *If there is suspicion of EHN, standard methods plus additional methods should be used, like suggested for KHV when it is first seen.*
Birgit Oidtmann: *What is the prevalence in a population, has the sensitivity of the population been investigated?*
Ellen Ariel: *It would be nice to know – and also to know if it stays constant.*
Niels Jørgen Olesen: *All ranaviruses in Europe has been diagnosed according to method 3.*

How to detect EHNV and related ranaviruses using IHC and IFAT

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Abstract: Epizootic haematopoietic necrosis is included in the list of exotic notifiable diseases of Directive 2006/88/EC . Very soon a diagnostic procedure will be adopted by the Commission, in order to harmonize the differences existing between EU member states.
According to the OIE Manual of Diagnostic Tests for Aquatic Animal, the diagnosis of EHN may be performed by: 1) microscopic methods through indirect detection of inclusion bodies and virus detection; 2) agent isolation and identification through cell culture method or ELISA, or Immunoperoxidase; 3) molecular techniques in particular PCR-REA(restriction endonuclease analysis) and PCR and sequencing. Cell culture is considered the gold standard method for survey and presumptive diagnosis while confirmatory diagnosis should be performed by PCR-REA or PCR-sequencing analysis.
Under the RANAVIRUS EU project two serological techniques have been tested to be applied in rapid diagnosis of EHNV and related viruses . A polyclonal serum produced against ECV virus has been tested for specificity and compared with the reference serum kindly provided by dr. R. Whittington (OIE Reference Laboratory for EHN, Sidney –Australia). An IFAT protocol has been produced and tested on EPC cells infected with selected ranaviruses and on different tissues from catfish (*Ictalurus melas*) experimentally and naturally infected by ECV.
A further protocol has been produced for detection of ranaviruses by means of IHC in tissues from catfish (*Ictalurus melas*) experimentally and naturally infected by ECV.
Both IFAT and IHC have been considered as suitable test for detection of ranavirus infection in clinically affected fish. The results obtained in a proficiency test carried out between the partners of the project revealed some discrepancies between different labs particularly with regard IFAT stained slides, suggesting that this technique requires some more experience in order to avoid false results . No doubts when IFAT is performed as a confirmatory method from cell cultures showing clear CPE.
By IHC it was possible to detect the infection 4-5 days after experimental trials. Positive reaction may be detected in several tissues while kidney seems to be the target organ.
During the workshop positive and negative tissues processed by IHC and IFAT according to the protocol will be shown and discussed.

Minutes:
The antibodies used were positive for all ranaviruses except DFV and GV6. When IHC and IFAT were performed on symptomatic fish a clear signal was normally detected suggesting that both IHC and IFAT could be useful tools for diagnosis of ranavirus infection. However, from light virus positive tissues, some sections were tested negative or doubtful by IHC and IFAT.

Questions/comments:
Olga Haenen: *Where do you observe fluorescence?*
Guiseppe Bovo: *Mainly in cytoplasm, in cell cultures.*
Sven Bergmann: *Most antibodies recognise the major capside protein which is produced in the cytoplasma so often you see a signal there.*
Ellen Lorenzen: *Have you made a sensitivity comparison of the two methods?*
Guiseppe Bovo: *IHC is easier to perform and you can counter-stain and you can see a single cell. IFAT is not so easy to perform but is good in diagnostics.*
Niels Jørgen Olesen: *Are there different antibodies for these methods and what is the availability of the antibodies?*
Guiseppe Bovo: *We have a good one, there is one from the OIE lab, Sven Bergmanns group also produced one to be used for the RANA project only. The supply is free but limited*
Suzanne Martelius-Walter: *What counter-stain do you use?*
Guiseppe Bovo: *Harris – described in the manual for the workshop.*

Ranaviruses - Molecular detection and differentiation

Riika Holopainen
Finnish Food Safety Authority Evira, Research Department / Veterinary Virology, Finland

Abstract: Ranaviruses are large, icosahedral dsDNA viruses that belong to the family *Iridoviridae*. The viral genome is 150-170 kbp of size and it encodes approximately 30 structural proteins. Ranaviruses can infect a variety of cell types and cause systematic disease in fish, amphibians and reptiles. Epizootic haematopoietic necrosis virus (EHNV) is currently the only ranavirus listed by the OIE. The OIE Manual of Diagnostic Tests for Aquatic Animals (2006) provides PCR methods for amplifying parts of the viral major capsid protein (MCP) gene for detection of EHNV and other ranaviruses.

According to the manual the differentiation of EHNV and other ranaviruses can be done either by sequencing a part of the MCP gene or by digesting a PCR amplified partial MCP gene with different endonucleases. Additional new protocols for molecular detection and differentiation of ranaviruses are being developed under the EU RANA project.

Minutes:
Various PCR methods that can be used for identification of ranaviruses was presented. Furthermore, different endonucleases that could be used to discriminate EHNV from the other ranaviruses were described.

Questions/comments:
David Stone: *From your trees it seems that there are less than 5% divergence between different viruses? What will we do if we find something that fall out of the groups.*
Riikka Holopainen: *Differences are small and it is not clear at the moment.*

Ellen Ariel: *We are not policy makers so we can only report results. I think that it is not only important to look at "genomes" – we also have to consider if there is any differences between the viruses in infection trials.*
Frank Berthe: *How should we discriminate viruses?*
Riikka Holopainen: *Obtain sequences and look in GenBank if it is not EHNV, it is not simple*
Niels Jørgen Olesen: *Were the results obtained by examining a single isolate and would there be a difference if you compare e.g. 10 isolates of approved EHNV?*
Ellen Ariel: *The isolate used could even be an endpoint clone – we don't know.*
Riikka Holopainen: *In the Rana-project we only use one isolate of each virus*

SESSION IB: Epizootic Ulcerative Syndrome (EUS)

Chair - *Birgit Oidtmann*

Introduction to Epizootic Ulcerative Syndrome - EUS

Somkiat Kanchanakhan
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Abstract: Epizootic ulcerative syndrome (EUS) is a disease of freshwater and estuarine water of wild and culture fish that affected large geographical areas including Asia, Australia, North American and recently in Africa. EUS is a seasonal epizootic condition that was first reported in farmed freshwater ayu (*Plecoglossus altivelis*) in Japan in 1971. It was later reported in estuarine fish, particularly grey mullet (*Mugil cephalus*) in eastern Australia in 1972. The outbreak extended its range through Papua New Guinea into South-East- and South Asia, and reached Pakistan in West Asia. Outbreaks were also reported in menhaden (*Brevoortia tyrannus*) in the United States of America (USA). EUS recently spread to the Chobe/Zambezi river system in the African Continent in October 2006.

EUS usually involves complex aetiological agents including parasites, bacteria, fungi, viruses or acid sulphate runoff water. The oomycete fungus that causes EUS is known as *Aphanomyces invadans* or *A. piscicida*. The affected fish exhibit necrotising ulcerative lesions typically leading to a granulomatous response. A suspect case of EUS is described as fish with one or more 'red spot' lesions. For confirmatory case of the EUS consult the current OIE Aquatic Manual.

EUS occurs most often during periods of low temperatures or 18-22°C and after periods of heavy rainfall. These conditions favour sporulation of *Aphanomyces invadans*, and low temperatures have been shown to delay the inflammatory response of fish to oomycete infection. Over 50 species of fish have been confirmed by histological diagnosis to be naturally affected by EUS. The signs of the disease include loss of appetite and float near the surface of the water. Fish usually develop red spots or small to large ulcerative lesions on the body. Red spots may be observed on the body surface, head, operculum or caudal peduncle. Large red or grey shallow ulcers, often with a brown necrosis, are observed in the later stages. Large superficial lesions occur on the flank or dorsum. Control of EUS in natural waters is probably impossible. In outbreaks occurring in small, closed water-bodies, liming water and improving water quality, together with removal of infected fish, is often effective in reducing mortalities and controlling the disease.

Minutes:
EUS is a seasonal epizootic condition, and do not occur all year round.
First hypothesis was that the condition was due to agriculture chemicals (1981), later that it was *Aeromonas* spp. That caused the disease (1983), and later again that it was viruses, as a rhabdovirus was isolated (Snakehead- and ulcerative disease rhabdovirus 1986). In 1992 it was discovered that it was an *Aphanomyces* that caused the syndrome.
Viruses isolated from EUS-affected fish: IPNV Sp (1985, not isolated since), sand goby virus (SGV, birnavirus, 1986), snakehead reovirus (1992), ulcerative disease rhabdovirus and snakehead rhabdovirus (1986).
Fungus species found from EUS-affected fish: *Achlya* and *Aphanomyces*.
Aphanomyces invadans was isolated in February 1992 in Thailand by Dr. G. Willoughby.
For the next OIE manual over 60 species confirmed by histological diagnosis will be listed as susceptible.
Aphanomyces is an oomycete (water mould) and not a fungus, but more like brown algae.
In order to make infection trials it has been difficult to induce the disease without giving the fish skin damage. A group is now able to produce the disease by bath.
Possible way of entrance for producing disease is abrasion, acidified water exposure, viral dermatitis, bacterial dermatitis, skin damage by parasites and then of course the *Aphanomyces.*
Field diagnostic methods: The disease occurs during periods of low temperature (in the tropics) and after periods of heavy rainfall. Fish develop red spots.
Clinical methods: Non-septated hyphae of *Aphanomyces invadans* can be observed in muscle squash preparations. Lesion scrapes generally show secondary fungal, bacterial and/or parasitic infections. Histotpathology must present mycotic granuloma; or isolation of *Aphanomyces*.
Grocott´s staining and H&E staining.
Synonyms: EUS, ERA (EUS-related *Aphanomyces*), RSD (redspot disease), mg, um, EGA (epizootic granulomatous aphanomycosis), ulcerative aphanomycosis.

First outbreaks were observed in Australia in 1971 and the disease has since spread by human activity and by water ways over Asia.

Prevention and control in natural waters is probably impossible. In outbreaks occurring in small, closed water bodies, liming water and improving water quality, together with removal of infected fish, is often effective in reducing the disease.

Questions:
Ellen Ariel: *The OIE definition from 2006 states that both A. invidans and A. piscida are responsible for the disease.*
Somkiat Kanchanakhan: *No difference between the two, they are the same. There are a few publications that genetically show they are the same pathogen.*
Birgit Oidtmann: *It is a Gentleman agreement that both names are used together.*
Olga Haenen: *Thank you for all the pictures. Our country is importing lot of fish from the area, and we see a lot of Saprolegnia. Is there an effect of the temperature? Is the effect of the pathogen decreased if the temperature is decreased?*
Somkiat Kanchanakhan: *Above 25°C the disease starts to diminish, and the disease disappears at 28°C. In cold countries as Bhutan the disease appears when the temperature rises above 12°C.*
Olga Haenen. *Is Aphanomyces a primary or a secondary disease?*
Somkiat Kanchanakhan: *Without Aphanomyces you don't have the disease. But you need predisposing factors to make Aphanomyces invasive.*

Guiseppe Bovo: *The history to reach the definitive diagnosis was very long. The histology shows that the lesions are very severe.*
Somkiat Kanchanakhan: *They saw the granulomas in the start but they did not investigate it further.*
Franck Berthe: *If you are to produce the disease experimentally, do you always have to induce a skin lesion?*
Somkiat Kanchanakhan: *Yes.*
Vlasta Jencic: *This long list of susceptible species is that natural or experimental infections?*
Somkiat Kanchanakhan: *Natural.*
Franck Berthe: *Was the cases always confirmed?*
Somkiat Kanchanakhan: *Yes, by histology showing granulomas.*

EUS Outbreak Situations and Recent Outbreaks in Africa

Somkiat Kanchanakhan
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Abstract: Epizootic ulcerative syndrome (EUS) is a disease of freshwater and estuarine water of wild and culture fish that affected large geographical areas including Asia, Australia, North American and recently in Africa. EUS is a seasonal epizootic condition that first reported in farmed freshwater ayu (*Plecoglossus altivelis*) in Japan in 1971. It was later reported in estuarine fish, particularly grey mullet (*Mugil cephalus*) in eastern Australia in 1972. The outbreak has extended its range through Papua New Guinea into South-East, all South Asia countries, most Asia countries and reached Pakistan in West Asia in 1996. The outbreaks also reported in menhaden (*Brevoortia tyrannus*) in the United States of America (USA) in 1984. EUS recently spread to Chobe/Zambezi river system in Africa continent in October 2006. 7 countries in South Africa region share the border or connect to the Chobe-Zambezi River system. The EUS outbreaks occurred in 3 out of 7 countries, Botswana, Namibia and Zambia in 2006-2007. There is a concern that the EUS will soon spread to the other 4 countries that share the river system. FAO is now developing programs for EUS surveillance and national preparedness to combat emerging aquatic diseases in South Africa region. OIE also develops a program to highlight an importance of aquatic diseases against the growth in the fisheries and aquaculture sector in Southern Africa.

EUS has been reported from >20 countries within 4 continents, Asia, Australia, North America and Southern Africa. Movements of live ornamental fishes from EUS affected countries might spread the disease as in case of the outbreak in Sri Lanka. The water flooding also caused the spread of the EUS as in cases of Bangladesh and Pakistan. Once the outbreaks occurred in the rivers/canals the disease can spread to low-stream as well as to up-stream where the susceptible fish species exist. Stop exchange water from the affected rivers could prevent the EUS from spreading into the culture fish ponds. Currently, the EUS situations in SE Asia and South Asia are getting fewer reports that might be due to global warming effect. As there is no longer cold season in some tropical countries that previously were affected by the EUS.

Minutes:
I have the best information from the Asian-Pacific region through NACA. On www.enaca.org 1998 → quarterly aquatic animal disease reporting system.
The disease has been recorded in 23 countries.

Many countries reported EUS in 1999; in 2005 EUS is only reported from Indian and Australia. One can speculate why it has disappeared, maybe the farmers got used to the disease and did not report it, maybe the disease declined due to climatic changes.
In 2006 only 3 countries, in 2007 4 countries reported: India, Bangladesh, Vietnam, and Australia.
Global warming has been discussed as the reason for the disappearance.
EUS-outbreak in Southern Africa: EUS found in late 2006 in the Chobe-Zambezi river system. The river runs for 2700 km and its basin also drains Angola, Botswana, Malawi, Mozambique, Namibia, Tanzania and Zimbabwe. An unidentified infection brought fears that the disease could be transmitted to humans. Botswana made a request to FAO for help. The EUS OIE reference laboratory went to Botswana. EUS was confirmed from 3 countries sharing the river system.
How EUS appeared in Southern Africa is at the moment unknown.

Questions:
Helle Frank Skall: *The mycotic granulomas observed in the many fish species that will be listed in the new version of the OIE Manual, could they not be caused by another pathogen than Aphanomyces?*
Somkiat Kanchanakhan: *At the moment the mycotic granulomas in fish are only caused by Aphanomyces.*
Olga Haenen: *The OIE Manual gives a PCR test for EUS. Would you recommend to use this test?*
Somkiat Kanchanakhan: *We are not too confident with the PCR-product of the OIE manual. When blasting it match another fungus, meaning the OIE primers are not specific. In the new manual in 2009 new specific primers will be added.*

Morphological differences between *Aphanomyces invadans* and closely related Oomycetes

Birgit Oidtmann
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Abstract: Oomycetes are protista that belong to the Kingdom Stramenopila (Chromista) and comprise many economically important plant and fish pathogens, including *Aphanomyces invadans*, *A. astaci, Saprolegnia parasitica* and *S. diclina*.
A common feature to Oomycetes is their asexual reproduction via biflagellate zoospores, as well as sexual reproduction via oogonia and antheridia (with few exceptions).
Macroscopically, most *Aphanomyces* species grow in velvet-like, white mycelium on agar. Growth velocity of those species relevant to aquatic animals varies and is in comparison slowest in *A. astaci*, and also relatively slow in *A. invadans*. Growth velocity is much higher in some other fish Oomycetes, e.g. *Saprolegnia parasitica*. This often leads to overgrowth of *Aphanomyces astaci* by other Oomycetes.
Oomycetes form a non-septate, vegetative mycelium with cell walls composed primarily of glucans and, to a smaller part, cellulose. Most Oomycetes reproduce both vegetatively and sexually. Sexual reproduction is through oogonia and antheridia. In most Oomycetes, species identification can be achieved based on the morphology of these sexual reproduction organs.
Sexual reproduction is not seen in *Aphanomyces invadans* or *A. astaci.* Therefore the morphology of Oogonia and antheridia cannot be used for species identification.
Oomyetes can be identified down to the genus level by their asexual reproductive stages. The asexual reproduction involves the formation of sporangia, from which zoospores (the asexual reproductive stage) are released. The asexual reproductive stages of *Aphanomyces* and other closely related Oomycetes will be presented.

Minutes:
Aphanomyces are related to brown algae and diatoms, making up a group called the heterokonts (protista). They are NOT fungi. *Aphanomyces* belongs to the Saprolegniales.
For Oomycetes the life cycle consists of both an asexual and a sexual reproductive stage. The sexual reproduction does not exist in *Aphanomyces*.
Identification is based on morphology only down to genus level.
For *Aphanomyces* zoosporangiums have the same diameter as hyphae, sporeball formation takes place at the tip of the zoosporanguim.
Asexual reproduction can take place when growing in agar but mostly by transferring the *Aphanomyces* to water. The *Aphanomyces* grows in medium and is then transferred to water by decanting the media and adding the oomycete to water leaving the oomycete nothing to grow on.
Growth on agar: morphological differences, the growth is very much on the surface of the agar plate, if the mycelium grows high above the agar surface it is not *Aphanomyces invadans*. The *Aphanomyces* grow slowly, and are inhibited by other fungi and bacteria.

Questions:
Olga Haenen: *Have you tried the effect of nystatin or other antimycotic agents?*
Birgit Oidtmann: *No, but we use antibiotics to reduce bacteria. For A. astaci a ring can be placed in the agar, and A. astaci is able to grow below as it is invasive. This may work also for A. invadans. The optimum temperature for A. invadans is about 25°C.*

Susceptibility of three European freshwater fish species to *Aphanomyces invadans*

Birgit Oidtmann[1]*, Peter Steinbauer[2], Sheila Geiger[2], Rudolf Hoffmann[2]
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Abstract: Three fish species, European catfish (*Silurus glanis*), European eel (*Anguilla anguilla*) and rainbow trout (*Oncorhynchus mykiss*)**,** were challenged by intramuscular injection of *Aphanomyces invadans*-zoospores.
Three-spot gouramis (*Trichogaster trichopterus*) are known to be highly susceptible and were used as a positive control. European catfish appeared to be highly susceptible, rainbow trout moderately to little susceptible, whereas the eels appeared largely unaffected. Inflammatory host response in European catfish deviated from the picture seen in most other susceptible fish species and was characterised by a more loosely arranged accumulation of macrophages, small numbers of lymphocytes and multinucleated giant cells without occurrence of EUS-characteristic mycotic granulomas.

Minutes:
The aim of the study was to assess the risk of European fish species to become infected.
Experimental setup: triplicates with 12 fish in each, one negative control tank.
Granulomas were not shown in European catfish, but giant cells!
The rainbow trout did not develop ulcers, but had only a slight reddening of the skin.
It is important to test the susceptibility of other European fish species.
PCR protocols: Oidman et al 2008, Phadee et al, 2004, Vandersea et al 2006.
Specificity pf PCR protocol: Phadee et al: cross reaction with *A frigidophilus* when high template numbers are present ($\geq 10^5$). Oidtmann and Vandersea primer sets are fully specific. The Vandersea PCR amplicon is short and reaches into the 18S region.
It is important to continue to isolate fungi, as new isolates keeps coming up.

When performing PCR it is important to confirm the PCR product by sequencing.

Questions:
Olga Haenen: *We have a lot of culture of African catfish, barramundi is also being tried. African catfish is "naked" (without scales), would it be interesting to look into "naked" carps? The barramundi have so many spines that they damage each other all the time, and thereby create a port-d'entrée for the Aphanomyces.*
Somkiat Kanchanakhan: *That is an interesting point, to investigate, if naked fish are more susceptible to the infection.*
Birgit Oidtmann: *We have tried to infect goldfish without any luck, even though this species has been reported as susceptible.*
Franck Berthe: *The common carp is accepted as being non-susceptible. Would they have the same reaction as eel in the experiment?*
Birgit Oidtmann: *I can't give an answer to that question.*
Franck Berthe: *Would you conclude that eel is a susceptible species?*
Birgit Oidtmann: *only 1 eel was observed with hyphae, and PCR-positives were only in the start, probably picking up what we had injected.*
Sven Bergman: *There could be a difference on the susceptibility of eels depending on size.*
Vlasta Jencic: *Will rainbow trout be put onto the list of susceptible species?*
Birgit Oidtmann: *That is policy.*

Comparative pathology of fungal and fungal-like infections of fish

Stephen W. Feist
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Abstract: The pathology of fungal and fungal-like infections in fish can be extremely variable depending on the agent involved. Oomycetes ('water moulds') are regarded as fungal-like protists rather than true fungi and appear to be related to brown algae. Members of the genus *Saprolegnia* are common pathogens in farmed and wild fish and are often associated concurrent infections and poor environmental conditions. Epizootic Ulcerative Syndrome (EUS) is characterised by the presence of hyphae of *Aphanomyces invadens*. Hyphal elements are highly efficient at penetrating host tissues and result in extensive dermatitis and necrosis of the epidermis and underlying dermal tissues and musculature. A strong inflammatory and granulomatous response may be seen depending on the fish species involved. Visceral organs can be affected. Infections with *Saprolegnia* are not generally so aggressive but chronic infections produce similar histopathological features and similarly, internal organs can be affected. For both of these agent haemorrhaging is often present. Infections with fungi such as *Exophiala* with septate hyphae are occasionally seen. These are usually chronic infections with pronounced granulomatous lesions with necrosis, frequently with giant cell formation. Similar infections can occur in marine fish.

Some species of *Dermocystidium* can have fungal-like features with hyphae packed full of spores. *D. koi* induces oedematous changes and limited inflammation in the skin of carp. Other species of this genus are cyst forming and usually do not induce serious histopathological changes. However, a systemic form does occur in salmonids where the kidney in particular is affected. Cellular and tissue necrosis is seen, associated with influx of inflammatory cells. Fungal-like agents such as *Sphaerothecum destruens* affecting salmonids and the sunbleak also infect the kidney although in many cases the infection becomes systemic with many organs and connective tissues affected. Chronic inflammation and granuloma formation is common and may be extensive, replacing large

proportions of affected organs. Microsporeans are now recognised as a specific group of fungi. There are extremely common pathogens of fish and invertebrates and a few species infect mammals including man. The histopathological response they induce can be varied. Xenoma forming species are often relatively innocuous whilst others give rise to significant disease associated with tissue necrosis and inflammation and fibroplasias in healing lesions. Neoplasia has not been associated with fungal or fungal-like infections in fish.

Minutes:
For histological slides Silver, PAS, Grocott & Gomori and H&E staining are used..
There exist a large number of fungal or fungal like pathogens. Many have the potential to cause systemic infections. The principle pathologies are inflammation and necrosis. The pathogens are often difficult to diagnose to species. Research is needed for transmission, pathogenesis and control.

Questions:
Olga Haenen: *For EUS, would histochemistry or in situ hybridisation be useful?*
Steve Feist: *Sure, if we had the tools i.e. specific antibodies and molecular probes.*
Franck Berthe: *Do we have any experience of the monoclonals developed against EUS?*
Somkiat Kanchanakhan: *I don't know how many have been produced but published one has low sensitivity.*

Field and Laboratory Investigation of EUS Outbreak

Somkiat Kanchanakhan
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Abstract: EUS outbreak occurs in both finfish aquaculture and wild. Major predisposing factors that associated to the outbreak occurrences are sudden drop of the water temperature, present of susceptible fish species and *Aphanomyces invadans*. There is no record of the EUS occurrence at the water temperature above 28˚C. Usually, the EUS-affected fish co-infect or super-infect with other pathogens such as bacteria, or parasites. The co-infection with viruses is rear. Some small equipment of materials shall be prepared for field examination in order to differentiate the EUS suspect case and normal disease case. The suspect case shall be collected, preserved and transported to the Laboratory for clinical examination, oomycete isolation, histopathology examination or PCR detection.
Field investigation; EUS outbreaks have been associated with mass mortality of various species of freshwater fish in the wild (including rice-fields, estuaries, lakes and rivers) and in farms during periods of low temperatures and after periods of heavy rainfall. The early signs of the disease include loss of appetite and fish become darker. Infected fish may float below the surface of the water, and become hyperactive with a very jerky pattern of movement. Fish usually develop red spots or small to large ulcerative lesions on the body. Red spots may be observed on the body surface, head, operculum or caudal peduncle. Large red or grey shallow ulcers, often with a brown necrosis, are observed in the later stages. Large superficial lesions occur on the flank or dorsum.
Laboratory investigation involves necropsy technique. Simple muscle squash preparation of the infected area around the lesion shall quickly identify non-septate hyphae of *Aphanomyces invadans* (12–25 μm in diameter). The suspect fish specimens shall do further *Aphanomyces* isolation. The same specimens shall be fixed and processed for histopathology examination for the presence of mycotic granulomas in the internal organs or tissue.

Minutes:
Establishment of the case definition:

- suspect EUS fish: a fish showing ulcers or lesion similar to the ones associates with EUS.
- suspect EUS location/farm: al farms where one or more suspect EUS fish have been found.
- mass mortality of various species of freshwater or estuarine fish in the wild.
- periods of low temperatures (18-22 C) and after periods of heavy rainfall.
- red spots or small to large ulcerative lesions on the body.

The estaurine fish gets infected when in freshwater, e.g. after heavy rainfall.

Confirmed EUS case: demonstration of *Aphanomyces* in the internal tissue leading to mycotic ganuloma (and two other points...)

Fixation of tissue samples.
Fish should be killed prior to fixation. With small fish, this can be done by decapitation, this is not suitable for large fish, use instead an overdose of anaesthetics. Keep the fish on ice until fixation. If the fish is small, fix whole fish. For large fish, remove internal organs before adding to fixative. Use min 1-2 days fixation time and store in normal formalin. For transportation of the fixed specimens, use wet transportation or semi-dry transportation.
Use alcohol fixation for PCR.
Be sure that temperature is above 12°C for *Aphanomyces* to grow (on top of a hotel refrigerator the temperature is 22°C – perfect).

In the EUS technical handbook there is an example of a sampling data sheet.
The water quality is very important in regard to the disease, therefore we always check the water also.
Before 1998: over 1900 species recorded as susceptible to EUS.
After 1998: about 50 susceptible species confirmed.
Recently (2008), about 60 species confirmed as susceptible to EUS.

Question:
Olga Haenen: *Do we take the samples from the centre of the lesion or from outside the lesion?*
Somkiat Kanchanakhan: *We prefer to take just outside the lesions, better to use the white meat just next to the red meat.*

Guidelines - Isolation and identification of *Aphanomyces invadans* from EUS affected fish

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Abstract: EUS is a seasonal epizootic condition of great importance in wild and farmed freshwater and estuarine fish; it has a complex infectious aetiology and is clinically characterised by the presence of invasive *Aphanomyces* infection and necrotising ulcerative lesions typically leading to a granulomatous response. Many fungal infections are visible to the naked eyes. Fish body surfaces including gills should be examined carefully for the presence of "tufts", nodules or other epithelial lesions indicative of the presence of fungi.

After the fish abdomen has been opened and bacteriological samples collected, then carefully examine all organs for the presence of cysts, nodules or anything unusual. Gills, brain, and viscera including kidney should be examined with hand lens or dissecting microscope for the presence of fungi and associated lesions. Wet-mount preparations from fresh squashed tissue should be made of all the above tissues as well as airbladder, the contents of stomach and intestine. However, the oomycete can be identified to the genus level by inducing sporogenesis and demonstrating typical asexual characteristics of *Aphanomyces*. *Aphanomyces invadans* is characteristically slow growing in culture and fails to grow at 37°C on GPY agar. Otherwise, diagnosis of EUS is based on clinical signs and confirmed by histopathology. Diagnosis of EUS in clinically affected fish may be achieved by histopathology or by oomycete isolation. Positive diagnosis of EUS is made by demonstrating the presence of mycotic granulomas in histological section. Then confirmed by H&E staining or other techniques staining like Grocott's , PAS and Uvitex CB also. In addition, PCR techniques can be used for confirmation the presence of *Aphanomyces invadans*.

Minutes:
Aphanymyces can utilise the oxygen in the muscle from the host and thereby be invasive.
Incubate at GPY agar plates for 2-3 days at 22-26°C. The *Aphanomyces* will then have grown to 2-3 cm in diameter. *Aphanomyces* does not grow at 37°C.
Aphanomyces have very thin hyphae. Cluster of encysted primary zoospores at the tip.
For sporulation, a piece of agar is taken to the broth e.g. by a cock borer, 2-3 pieces is added to the broth in a petri dish. The oomycete is then washed and left in autoclaved pond water.

Histopathology: H&E, periodic acid shiff reagent (PAS), Grocotts metenamine silver nitrate (GMS), uvitex (need flourescence microscope).
The Grocott's show the hyphae but no details of the tissue.
The *Aphanomyces* can invade all organs.
PCR: we use primers ITS11 and ITS23.

Questions:
Steve Feist: *Can fish showing only superficial lesions recover?*
Varinee Panyawachira: *Yes, if the lesion are not in the head but in the skin, they can recover. The fish treat the oomycete as a foreign body and the reaction makes it difficult for the fish to produce antibodies.*
Niels Jørgen Olesen: *We have got a lot of information today, but what about biosecurity measures and disinfection? What can you use for disinfection and which biosecurity should you use.*
Birgit Oidtmann: *David Alderman has tested several disinfectants and antibiotics. Iodine was very effective. If you deprive the organism of the water it will die.*
Somkiat Kanchanakhan: *General disinfectants and autoclave should get rid of the oomycete.*
Varinee Panyawachira: *It is better to treat things to be autoclaved with chlorine first.*
Birgit Oidtmann: *In the infection trials we autoclaved the water or treated it with iodine (100 ppm for 1 h). There must be a reservoir somewhere, where is it in between outbreaks?*
Somkiat Kanchanakhan: *Maybe in the farm environment, fish with low infection carry the disease until the next disease outbreak.*

SESSION II: Procedures for diagnosing EHN and EUS – a practical approach

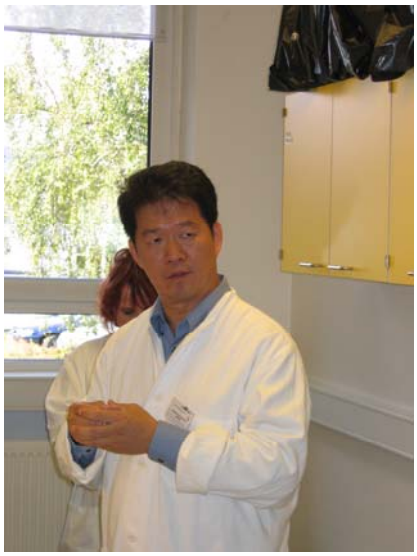
Minutes:
The participants were divided into five groups. Each group was circulated between the following five platforms:

Platform 1: EUS histological slides examination and Video.

Varinee Panyawachira and Niels Jørgen Olesen.
A video demonstrating the sporulation of various oomycetes and other mycotic agents was shown and explained to all groups. Following the participants were given a collection of slides consisting of internal organs, muscles and gills from infected Snake-heads. The slides were stained by Haematoxylin-Eosin (HE), PAS and Grogott-Gomori (GG), respectively - 9 slides in all. Similar slides were placed with 4 microscopes in order for the participants under the supervision of Varinee Panyawachira to examine the classical pathological changes with fungi and granulomas. In addition the participants were invited to examine slides stained with the Uvitex 2B system in an IF microscope, showing very strong and specific fluorescent staining of the hyphae (Switch between normal light microscopy and UV microscopy). All participant were given a colour print of the same tissue slide stained by the 4 staining methods and with the protocol of UVITEX 2B staining of hyphae. It was stated that the microscopy of fish tissue is not always straight forward or easy to interpret!



Platform 2: Sampling of fish tissue for oomycete isolation and sporulation.
Somkiat Kanchanakhan and Birgit Oidtmann.
The technique for isolation of oomycetes was presented using a non-infected fish for demonstration. It was demonstrated how the oomycetes should be isolated from the muscle tissue beneath the ulcerus in a way minimising the risk of obtaining contamination from other secondary patogens associated with the ulcerus and how the oomycetes should be placed and grown on broth agar plates. The demonstration was made in accordance to the EUS OIE manual. Furthermore participants were by microscopy introduced to the morphology of A. Invadans spores that are used for identification of the oomycete.



Platform 3: PCR amplification of *A. invadans* and EHNV DNA
Riikka Holopainen and Søren Kahns.
Participants were presented for general DNA purification methods based on spin column purification kits. It was described how DNA should be isolated from infected fish and in the case of EHNV from inoculated cell cultures and in the case of EUS from growth in broth. Experiences with the PCR methods described in the OIE manuals were presented. Finally, the restriction fragment length polymorphism analysis method for discriminating between the RANA virus species were presented for participants

Platform 4: Virological examination for EHNV on cell culture.
Ellen Ariel
Participants were able to examine BF-2 and EPC cell cultures with different degrees of CPE caused by EHNV. The difference between 15 and 20 degrees C incubation was apparent with a faster development of CPE at 20 degrees C. General virological techniques were discussed, the preparation of 24 well plates were demonstrated in the laboratory and a reference made to Commission Decision 183/2001/EEC.

Platform 5: Immunological methods used for diagnosis of EHNV.

Giuseppe Bovo and Nicole Nicolajsen

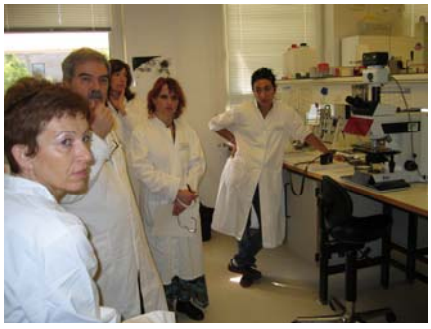
Dr Bovo brought histological slides to all groups. The staining for immunohistochemistry was demonstrated on tissue slides from liver and kidney from experimentally ECV infected catfish. The staining protocol was described and characteristic findings and pathological changes demonstrated. In addition methods for cryo slide preparation were demonstrated together with immunofluorescence staining procedures and patterns. Finally IF procedures on infected fish cell lines were demonstrated. The slides could be examined on site in light and UV microscope and was demonstrated by projection on screen.










Minutes of discussion after Session II

Aphanomyces invadans, where to obtain the pathogen?

Birgit Oidtmann: *My experience to send out A. astaci is that people are not able to keep it going. I don't have a problem to send them out, but I think it will be very difficult to get them going.*
Somkiat Kanchanakhan: *I agree.*
Niels Jørgen Olesen: *The most important is to have the contact with people to verify the isolation. You have today got positive histology slides to take home. The next step will be that you get positive fixed material.*

Ringtest on the diagnosis of EUS?

Niels Jørgen Olesen: *I suggest we do not circulate the live pathogen, but fixed tissue material from infected fish. Maybe a histology slide ringtest*
Steve Feist: *If slides are circulated between the laboratories it will potentially take very long time. And it doesn't test the laboratories ability to undertake histological processing and staining of the tissue sections. Another possibility is to use 'virtual' slides. You download the scanned images (there is free software to use for this) and you 'read' the image via your computer, with the ability to zoom in and out up to the equivalent of using x40 objective lens.*
Sven Bergmann: *I would prefer to have the ringtest as both slides and fixed material, so we can also test our staining ability.*
Steve Feist: You can also send unstained slides.
Niels Jørgen Olesen: *It is also important that you can read the sporulation. Maybe we can also make virtual videos. Then people can look at them and then identify which is the Aphanomyces. It is a list 1 disease so we have to show somehow that we are able to identify the disease.*
Birgit Oidtmann: *I think we should use PCR as this is a method all will be able to perform.*
Olga Haenen: *I agree with Birgit that PCR is one of the first thing we should be able to do.*
Sven Bergmann: *I will prefer both the fixed material and a pcr ringtest.*
Ineke Roozenburg-Hengst: *I once participated in a ring trial where everybody came and looked in the microscope at the same day. This could be done at meetings like this.*
Niels Jørgen Olesen: *I think the conclusion is that we should somehow have a ringtest.*

EHNV, where to obtain the pathogen?

Niels Jørgen Olesen*: One question is where to obtain EHNV from. We suggest contacting the OIE reference laboratory, that is in Australia, where they have the real bug. For ECIV and ESIV from IZSVe, Italy and FLI, Germany and also from J. Castric, France.*
Sven Bergmann: *I got sera from Alex Hyatt and I had to pay for the transportation which was very expensive.*
Olga Haenen: *Who has produced sera in Europe?*
Niels Jørgen Olesen: *Bergmann has a MAb that will take all but the GV6 and the Doctor fish virus and the NZ eel virus. IZSVe and CRL have PAb, primarly for their own use.*
Which organ to collect for EHNV surveillance?

Ellen Ariel: *our test method does not reflect the natural pathway as we had used IP injection. We did not find anything in the gills, whereas Guiseppe Bovo found lot in the gills in natural infections.*

The method for testing for EHNV is, the method used today for VHSV/IHNV, even though it is not as good as the others, my gut feeling is that it is actually okay. If you had sick fish I am quite sure that you will find the virus at 15°C at both BF-2 and EPC cell lines.

Incorporation of ranaviruses in the ringtest?

Niels Jørgen Olesen: *Will you accept to receive live ranaviruses?*
There were no objections to include ranaviruses in the ringtest.
Olga Haenen: *First of all thanks to the CRL for the very fine workshop. I will then ask if it will be possible to share the presentations on the CRL website?*

SESSION III: Update on important fish diseases in Europe and their control

Survey & Diagnosis of listed fish diseases in the European Community 2007

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Abstract: Data on survey and diagnosis on fish diseases in Europe in 2007 were collected again this year and compared to previous years few changes were again made in the questionnaire. By the implementation of the new Council Directive 2006/88/EF with Annex IV part B the questionnaire now concern ISA, SVC and KHV in addition to VHS and IHN on the list of the non-exotic diseases. The new diseases therefore got relatively more attention than in the previous S&D questionnaires. In the new Directive all National Reference Laboratories are requested to be accredited according to quality assurance systems. In order to assess how far the implementation of these accreditations have come the questionnaire was expanded to also include information on quality assurance in the respective laboratories.
In Scandinavia VHS appeared in Norway in 2007 and is still present in Finland and Denmark, despite intensive control measures. In UK the control of the outbreak in 2006 was successful and UK will regain status as approved free this autumn 2008. The Faeroe Islands plan to monitor the disease in order to obtain disease free status in a couple of years. Most of the "old" Member States have a number of farms approved in non-approved zones. While most of the new states still lack any status as VHS or IHN disease free. Bulgaria had the first documented outbreak of VHS in 2007.
The reported occurrence of VHS, IHN, ISA, SVC and KHV in Europe will be shown. As in previous years, however, the de-facto spreading and significance of the non exotic diseases cannot be retrieved from the S&D questionnaire.

Minutes:
The majority of farms in Europe are very small farms with a production of less than 5 t.
More than ½ of the farms are carp farms; this is primarily due to the fact that many of the very small farms produce carp.
When a farm is "considered to be infected" it does not necessarily mean that it is officially declared infected, as well as "considered to be free" does not mean that the farms are officially declared free.
In Norway the first outbreak in a many years happened in the fall 2007.
In Finland only 2 farms are reported being VHS infected out of 203 farms. The 2 farms are situated at the Aaland Islands

In France there are only reports of VHS from Bretagne.
The VHS outbreaks in Denmark have been very severe with high mortality in contrast to the outbreaks in Finland.
In Italy, only VHS reports from the northern part.
Reports of IHN from Germany, France, Italy and Slovenia
The rainbow trout export in Turkey is expanding with a large export to EU. The VHS isolates from Turkey are very similar to older isolates from Georgia placed in genotype Ie.
In Iran they have experienced outbreaks of IHN due to importation of eggs from USA. This is also suspected to be the case of introduction of IHN in Europe, so we may have to reconsider eggs as a safe commodity for the spread of IHN.
Many reports of KHV cases from many countries. Especially many KHV outbreaks have been recorded in Germany. The number of cases often exceed the numbers of carp farms. This is due to the fact that many of the outbreaks are from koi carp garden ponds and retail stores.
ISA have only been reported from Norway.
IPN virus has been isolated from most of the countries. You are not asked specific for this disease in the questionnaire, so it may even be located in the rest of the countries.
BKD: For Denmark it seems that very intensive reproduction units with recirculation of water enhance the disease.
There have been many reports of PD.
HVA has been reported from Holland and Denmark.
Perch Rhabdovirus may be a disease we will have to take more into account in the future due to the increased production of pikeperch.
Reports of VNN and *Lactococcus* from Italy.
There are a very high numbers of farms with unknown status for especially SVC and KHV.
The highest number of fish examined by a single country is done by Italy.
Only a very small number of the samples received are found virus positive reflecting that most of what we do is surveillance.
A lot of reports of *Lactococcus garvieae* in 2007 (104).
11 countries of 37 have regional laboratories and 9 of these provide the regional laboratories with proficiency tests. This is one of the obligations for the NRLs.
21 of the 37 NRLs are accredited. The new legislation asks for accreditation, but it may be allowed not to be accredited.

Conclusion:
Few changes from 2006 to 2007.
Many countries reported data for the new diseases listed in 2006/88.
VHS outbreak in Norway, Bulgaria and Slovenia besides the "typical" countries,
There is still a significant under-reporting.
In the new directive there is an obligation of authorisation of all fish farms in Europe.

Questions:
Sven Bergman: *A lot of KHVD were reported in 2007 in Germany. What is the frequency of outbreaks in the other countries? Do you have problem to combat the disease?*
Rob Raynard: *You said that IHNV in Spain was linked to imports of eggs. Did the outbreaks happen in IHNV free farms? Do you as the CRL carry out any follow up work of this?*
Niels Jørgen Olesen: *this is a question we may take up tomorrow. We may ask others to take up this task.*
Sven Bergman: *Why is ISA a non-exotic diseases for EU, when we don't have any outbreaks?*

Niels Jørgen Olesen: *That is a political decision.*
Pedro Rosado: *We think that this disease is present in the wild. But we have granted the ISA freedom status for the whole EU as the disease has not been detected in farmed populations since the outbreaks in UK and Ireland.*
Snjezana Zrncic: *Do we have to start VHS surveillance in seabream and seabass?*
Niels Jørgen Olesen: *There has been a report of VHS in seabream in Spain and Turkey, but none of these have been confirmed. These species has not been included in the list of susceptible species in the legislation at the moment.*
Franck Berthe: *There is more and more a pressure of the OIE to work on commodities, to assess if the commodities are safe (egg transport).*
Brit Hjeltnes: *There will never be a zero risk when moving commodities; the question is more how big a risk we are willing to take.*
Franck Berthe: *It is quite clear that we are looking for safe commodities where the risk really is zero, so we are really looking for zero risk commodities. There may then not be any commodities to list for us.*
Brit Hjeltnes: *If we are not accepting any kind of risk, then we cannot move anything at all.*

Outbreak of viral haemorrhagic septicaemia (VHS) in sea farmed rainbow trout in Norway

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Abstract: In November 2007, behavioural changes and slightly elevated mortality were observed in a sea farm with rainbow trout (*Oncorhynchus mykiss*) in Storfjorden, Western Norway. Following gross pathological and histopathological examinations, VHS was suspected, and the diagnosis was verified by immunohistochemistry, RT-PCR and virus isolation in cell culture. Parallell samples were examined at CRL for Fish Diseases, Århus, with identical results. The Norwegian Food Safety Authority (NFSA) confirmed the VHS diagnosis in late November. VHS was later diagnosed in three neighbouring sea sites with rainbow trout in the beginning of 2008. The source of infection has not been identified. In a preliminary screening, VHSV was not detected in farms with marine species located nearby the rainbow trout farms.
At the primary outbreak site, the fish showed circular swimming patterns and anorexia. Main autopsy findings were anemia and extensive hemorrhages in the peritoneum. Typical histopathological findings were hepatitis, liver hemorrage, endocarditis and hematopoietic necrosis in kidney and spleen. In some fish, meningitis and meningoencephalitis were observed and VHSV detected by immunohistochemistry in brain tissues, indicating a nervous stage of the disease.
Genotyping of the virus was based on a 286 nucleotide sequence from the VHSV glycoprotein gene. Sequences obtained from the four different farms were identical in this region. In a phylogenetic analysis the sequences clustered with isolates of VHSV Genotype III, which includes isolates originating from a variety of marine species from the North Sea and Skagerrak.
Infection trials with rainbow trout and Atlantic salmon (*Salmo salar*) fingerlings were performed to test the pathogenicity of the Norwegian VHSV strains. The cumulative mortality of rainbow trout reached approximately 70% and 100% within a couple of weeks after infection by immersion and intraperitoneal injection, respectively. The corresponding values for A. salmon were no mortality and a mortality of approximately 50%. Infected fish showed signs typical of VHS, and VHSV isolated from dead fish was genetically identical to the isolate used in the infection trial.

This is to our knowledge the first description of an outbreak of VHS in rainbow trout caused by a pathogenic strain of VHSV genotype III.

Minutes:
The disease was first reported in 1964 and after 1974 we have had no outbreaks. In between 1964 and 1974 we had 13 cases.
VHSV is associated with disease in farmed fish but is also widespread in the marine environment. There are four main genotypes.
It has been discussed if VHSV in marine fish is a threat to farmed salmonids, and the answer is YES.
The recent outbreak is caused by genotype III, that is a marine genotype.
The primary outbreak was in a seafarm with rainbow trout, 1.8 mill fish in 20 net pens of 90-440 g.
Classical VHS disease signs were observed. The diagnosis was based on histopathology, IHC, cell culture with IFAT and RT-PCR.
The outbreak took place in Storfjorden, a long fjord located in Western Norway. Neighbouring outbreak sites were infected in January-February 2008. All the four sites have the same owner. At the primary site mainly young fish, in the secondary sites it was outgrown fish.
Histopathology and IHC: VHSV in CNS – indication of nervous manifestation of the disease.
For RT-PCR a 323 nt fragment of the G-gene was amplified. RNA was extracted from tissue in RNAlater. The isolate cluster within genotype III based on a 286 nt fragment.
Realtime-RT-PCR by Mike Snow has now been established and is used for both surveillance and diagnostics.
The isolate is a unique virus. VHSV from all 4 outbreaks sites are 100% identical in the 286 nt fragment.
By infection trial challenge by immersion app. 70% mortality in rainbow trout. The virus also produced mortality in Atlantic salmon but only by IP injection.
Source of infection: There has been a screening of wild fish by Are Nylund which gave 2 positives in herring by RT-PCR. These were of genotype Ib and therefore not identical to the isolate responsible for the outbreak. The source has not been identified.
The rainbow trout has now been slaughtered after a court trial.

Question:
Katja Einer-Jensen: *Have you sequenced the old Norwegian isolates?*
Birgit Dannevig: *Some of them have been sequenced at the CRL and found to be genotype Id. These old outbreaks occurred in freshwater.*
Birgit Dannevig: *There are so many net pens and so many fish that it has been difficult to follow the disease.*
Brit Hjeltnes: *The fish health service suspected that something was wrong with the fish before the outbreak occurred but which signs I don't know.*
Niels Lorenzen: *How much mortality?*
Birgit Dannevig: *The fish farmers own data is around 2%, but the reliability is unknown.*

VHS outbreak in Slovenia

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Univerza u Ljubljani

Abstract: Slovenia is convenient area for salmonid fish farming. However before the year 1990 there were only few fish farms for the production for human consumption because fish farming was mainly orientated towards production for the repopulation of fishing waters. After the year 1990

number of fish farms increased enormously. We have always been monitoring fish diseases especially for the VHS and IHN. We had VHS outbreak in 1978 in Primorska region when the infection was successfully eradicated. Later on we had neither viral diseases outbreaks nor infections diagnosed. Only after the year 1996 we had several outbreaks of IHN, while we had never had the VHS. Slovenia was an oasis in the middle of Europe without VHSV infection.
However in December 2007 we had an outbreak of VHS in very small fish farm in the North East part of Slovenia. In fact this farm was only the reservoir of couple hundreds fish for the consumption. Affected rainbow trouts weighted more than 500 grams had typical clinical and path anatomical signs for the VHS. The isolation and identification of virus was positive for VHSV type 1. Almost simultaneously was another outbreak of VHS in the fish farm nearby but without water or other epizootiological connection.
A total of five CPE isolates of VHSV obtained from two different origins were included in molecular characterisation. All five VHSV isolates were recognised by the protein G specific primers, which amplified specific products of 695 bp.
Alignment of the 630 bp (210 aa) of two Slovenian VHSV sequences showed that both of them were 100 % identical. The obtained VHS sequences were not identical to any other sequences previously submitted to GenBank. The highest, 98, 6 % (nucleotide and aminoacid), homology was confirmed with VHS genotype Ia, strain CH-FI262BFH sequence from Switzerland.

Minutes:
Slovenia is 20000 km^2 with 10000 hectares of water. We produce 1500 t of rainbow trout but also 500 t of carp and 50 t of seabream. We don't have huge water sources so most of our production sites are smal farms. ¼ of the entire production is produced by one farmer. Our main health problem is IHNV. We were free of VHS until last year.
The first case of VHS occurred in 1978 and was immediately eradicated. The second case occurred in 1995 and was also eradicated. In 2007 we had 2 cases in reservoirs keeping a few 1000s of fish. The temperature was around 7.5°C with typical VHS signs. The samples were CPE positive at day 3 and RT-PCR positive. At the same time the second outbreak occurred. At both places, the fish were slaughtered. The fish farmer told where he bought the fish, but the person selling the fish denied having sold them. The origin of the virus is unknown.
In all we have 5 isolates from the two sites. These isolates are 100% identical and not identical to any sequences in GenBank, closest to isolates from Switcherland, genotype Ia.

Questions:
Niels Lorenzen: *How high was the mortality?*
Vlasta Jenčič: *The mortality was high with serious losses.*

Eradication of VHS in Denmark

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Minutes:
History of VHS in Denmark.
1950's: First observation of the disease in a farm close to the village Egtved, naming the disease Egtved Disease.
1962: First isolation of the causing virus, resulting in the name Egtved virus.
1960's: Voluntary eradiation program initiated.
1970: Official control of VHS eradication.

2009: Final eradication of VHS?

We have in all cases used the stamping out method with a fallowing period of 6 weeks in the warm period and restocked with VHS free fish.

Main structure of national legislation:
Duty of notification
Official approval and control of health status and eradication
Register of VHS-free farms
Rules for transport and slaughter
Rules for stocking of wild waters and put & take facilities
Screening against predating birds
Disinfection of effluent water from cutting plants
Banning of movements of live rainbow trout from seawater into freshwater
Obliged disinfection of transport vehicles at Danish borders.

The numbers of infected farms went down rapidly in the first period of the eradication programme. In the start of the 1980's the number of new infected farms equalled the farms stamped out, levelling the number of infected farms. Later the numbers went down again.
Today VHS is only found at two river systems. At the moment only 3 fish farms are infected with VHS. They will be fallowed. The fish farms at the Holmsland Klit using seawater will slaughter all their fish by New Year 2008-09 and no fish will be restocked for 2 years.
Final eradication: The plan is to have all VHS infected fish out by late 2008. An application for an eradication programme has been applied for in the European Fisheries Fund.

Surveillance and eradication of fish diseases

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Minutes:
It is very important to have a good cooperation between the industry, authorities and laboratories. Otherwise no success will be obtained.
From the industry's point of view we really want a strict legislation as this is necessary to protect the farms and to maintain optimal trade possibilities.
It is very important to get rid of VHS and it has been estimated that the Danish industry looses 3 million € per year.
It is important to remember that the programme only is the finalization of an eradication / surveillance strategy that has been going on since the 1970's.
A total eradication plan has been made this year and the plan has been sent to the EU-commission to be approved according to the legislation.
The plan is that all fish are removed from all the known infected farms (January-March 2009) paid by the farmers themselves. Farms will be fallowed for a certain period: 7 farms for two years and 15-40 farms for 6-8 week during spring time. These are farms not infected but just to be completely sure that we keep no carriers. Electro-fishing in the affected river systems to remove escapees will also be performed.
There will be an upgrading of VHS in DK in the national disease list system with compulsory slaughtering with recompensation.

The expected costs are less than 3 mill € but we have applied for 7 mill €. The gap is money needed if /when reinfection occurs.

Question:
Niels Lorenzen: *What about the marine VHS aspect?*
Niels Henrik Henriksen: *There will be no moving of fish from the marine environment to the freshwater system. The marine farms used to only have production through summertime, but a few farms have started to produce fish the whole year round in seawater and this will be a risk for contracting VHSV from the marine environment.*

Prevalence of low pathogenic Infectious Salmon Anaemia Virus of the HPR0 subtype in Farmed Atlantic Salmon (*Salmo Salar* L) in the Faroes

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Abstract: Vaccination of Atlantic salmon (*Salmo Salar* L.) against Infectious Salmon Anaemia Virus (ISAV) was initiated in April 2005, after the devastating ISA outbreaks from 2000 to 2005 in the Faroe Islands.
To monitor the *in vivo* effect of the ISAV-vaccine test pens with ISAV-vaccinated or sentinel Atlantic salmon were established at each commercial marine grow-out site.
From each of the test pens kidney and/or gill samples were collected once in the freshwater smolt production stage and thereafter monthly from each pen at the marine grow-out site throughout the production cycle. In addition kidney and/or gill samples were collected twice annually from marine farms with Rainbow trout.
From May 2005 to May 2008 a total of 3366 samples from all smolt production sites, 2279 samples from all marine rainbow trout production sites and 20709 samples from all marine Atlantic salmon production sites were screened for ISAV by RT-PCR or real-time RT-PCR.
More than 1250 samples were tested ISAV positive by RT-PCR or real-time RT-PCR. Sequencing of the hemagglutinin (HA) gene of more than 200 ISAV positive samples disclosed the full length HPR0 subtype in all cases.
The findings will be discussed in relation to phylogeny, prevalence, seasonal variation, time to detection and transience of infection.

Minutes:
The first outbreak of ISA was in Norway in 1984, in 1996 in Canada, in Scotland in 1998, in the Faeroes in 2000 and in Chile in 2007.
In the Faeroes we had 1 outbreak in 2000, 2 in 2001, in 2002 5 outbreaks and 10 in 2003 spreading from the north down south.
No importation of eggs, smolts or Atlantic salmon from 1986 to 2003.
One brood stock company, 10-15 fresh water smolt producing companies, 10-15 seawater companies and 30-35 farms.
Importation of eggs and smolts from Norway, Scotland and Iceland in 2005-2008.
The outbreaks occur mainly in spring/early summer followed by a peak in late fall/early winter.
The ISA vaccination project:

ISA contingency plan 2005/86/EEC with efficacy test of two ISAV vaccines. First year we had the following setup - 2 pens with Canadian vaccine, 2 pens with Norwegian vaccines, and 2 sentinels. The second year the Norwegians stopped production of vaccine, leaving only the Canadian vaccine.
A massive amount of samples have been taken during the project.
All samples from 2005 have been screened with a duplex RT-PCR. In 2007 we changed to realtime PCR.
Sea sites are stocked with rainbow trout and ISAV vaccinated Atlantic salmon, 7 sites in 2005, 7 in 2006 and 13 sites in 2007 were restocked.

During 2005-2007, 70% of the sites have been tested positive. All ISAV positive salmon had been in contact with seawater. The fish had been in sea at median 8 months before isolation of HPR0.
80% of the farms became positive during the winter. The infections have been transient. The isolates cluster in two separate groups. One group cluster close to the Norwegian group and the other cluster with a North American group.

Conclusion:
We have no clinical signs and only the low pathogenic HRP0 variant has been identified.
HPR0 is highly infectious and proliferative in salmon gills.
The vaccine seems not to protect against infection with and spread of low pathogenic ISAV.
Fast clearance of the virus in the fish.
The Atlantic salmon is infected with low pathogenic ISAV likely from a natural reservoir in a wild host.
Stress can induce the disease.
The spread is horizontal.

The Health situation in Farmed Fish in Norway 2007

Brit Hjeltnes
National Veterinary institute

The total number of pancreas disease (PD) and heart and skeletal muscle inflammation (HSMI) outbreaks has increased dramatically over the last year. IPN remains the largest disease problem in salmon farming, and is diagnosed from start feeding through the first year in sea. The number of farms diagnosed with IPN during 2007 was, however, significantly lower than during 2006, and while some farms experienced high losses, IPN was generally considered to be less of a problem in 2007.

Cardiomyopathy syndrome (CMS) is, as before, a significant cause of loss in larger salmon. Proliferative gill inflammation (PGI) continues to contribute to large scale mortalities on a number of farms, particularly in the first autumn following spring transfer of smolts.

The frequency of infectious salmon anaemia (ISA) outbreaks remains at a fairly stable, low level. All outbreaks during 2007 were located within a relatively small area in Troms and Nordland. During 2007, for the first time since 1974, viral hemorrhagic septicaemia (VHS), a group A notifiable disease, was diagnosed in rainbow trout in Western Norway.

The various bacterial diseases pathogenic for salmon continue to be largely unproblematic, which is due in the main to good vaccines and vaccination strategies. Furunculosis is only diagnosed intermittently. A minimal increase in yersiniosis (infection with *Yersinia ruckeri*) was registered

during 2007. Winter ulcer, related to infection with *Moritella viscosa* continues to be problematic in some areas, both in salmon and rainbow trout.

The situation in 2007 is considered undramatic regarding salmon louse infection in farmed fish and an infection pattern similar to that reported for 2006 was observed. However, as the total number of farmed fish in the sea increased during 2007, there are grounds to believe that the total infection pressure may have increased.

On a national basis the salmon lice situation in relation to wild salmon is considered worse than during 2006.

During 2007, approximately 300 cases involving disease in farmed marine fish were investigated by the National Veterinary Institute. Over 80% of these cases involved cod, with halibut, coalfish and other species accounting for the remainder. Bacterial infections comprised the most significant disease problems in cod farming during 2007, with francisellosis (aetiological agent: *Francisella philomiragia* subsp. *noatunensis*) and vibriosis (aetiological agent *Vibrio* (*Listonella*) *anguillarum*) most prominent. A reduced antibiotic sensitivity level (oxolinic acid) was registered in *Vibrio anguillarum*. The notifiable diseases infectious pancreatic necrosis (IPN) and viral nervous necrosis (VNN) were also registered during 2007. VNN was diagnosed in 6 cod farms during 2007 compared with 3 in 2006. IPN was diagnosed on one halibut farm

Minutes:
Regarding ISA in Norway we are a bit worried about a cluster of ISA in the middle of Norway.
IPN does not cause much disease in the single farm but in total it is the disease causing most mortality. IPN is one of the most important disease problems in Norwegian fish farming.
PD is on the rise.
HSMI is a new disease in Atlantic salmon in seawater, mortality 0-20% in attacked cages. It is infectious and most probably a viral disease. It has until now not been possible to characterise the virus. In 2004 we had 54 cases, 2005: 83 cases, 2006: 94 and in 2007 162 cases. This may reflect we are getting better in diagnosing the disease. We think it is an emerging disease. Most cases are found in mid Norway but the disease can be found all over the country.
CMS is infectious and a viral aetiology is suspected. Hits just before the fish go to the marked. Low or moderate mortality. In 2007 we had 85 cases. The disease may be increasing?
Winter ulcer caused by *Moritella viscosa* is one of the big killers in Norwegian fish farming. The disease has a complex aetiology and is associated with low water temperatures, moderate mortality, chronic disease, down grading at slaughter, fish can swim around with big ulcers. This disease causes a fish welfare problem.
There is a National action plan against salmon lice on salmonids.
In cod the biggest problem is *Vibrio (Listonella) anguillarum*. The disease is found in juveniles, ongrowers and in broodstock. Another problem is *Francisella*, a saltwater disease. This disease will probably be a notifiable disease. VNN was diagnosed for the first time in Norwegian farmed cod in 2006. Deformities are still a problem in cod juveniles. Mortality in sexually matrue female cod "egg-binding" is still a problem, this problem is probably not infections.
There is a good situation for the use of antibiotics, with only limited amounts used. Most of the antibiotics used are used in cod farming.
You can find the fish health report at our website www.vetinst.no under publications.

Question:
Franck Berthe: *For HSMI the diagnosis is based on histology – and you are probably getting better trained. Have you done any retrospective studies?*
Brit Hjeltnes: *No, probably some of the rise is because we do get better in diagnosing, but we do believe that the disease is on the way up.*

PD update from Norway

Anne Berit Olsen and **Torunn Taksdal,**
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Abstract: Pancreas disease (PD) is a serious viral disease which in Norway affects Atlantic salmon and rainbow trout in sea water. It is caused by *Salmonid alphavirus* (SAV) subtype 3. In western Norway, serious outbreaks of PD have been diagnosed yearly since 1995. The affected area has expanded north- and southwards from this "hotspot", and the number of outbreaks has increased.

From December 2007, PD was included as a "group B" listed disease in Norway. The Norwegian Food Safety Authority has established preventive measures. The main aims are to prevent further spread from the endemic zone to yet unaffected areas and to reduce the number of outbreaks inside the endemic zone.

Minutes:
Salmonid alphavirus subtype 3 (SAV3), affects Atlantic salmon and rainbow trout in seawater. The spread is horizontal, but a vertical transmission cannot be excluded. The disease is notifiable since December 2007 under group B. The disease is also a problem in Ireland and Scotland, they deal with subtype 1. In inland Europe they struggle with a similar freshwater disease caused by subtype II.
The hot spots have emerged slowly northwards. The number of cases has increased significantly during the years.
Why is PD so difficult to stop? PD is usually diagnosed in a late phase. Initially the farmer may register a small rise in mortality which drops again, with vague disease signs. Then later the mortality will rise significantly.
Criteria for PD diagnosis is: 1) histopathological changes characteristic for PD in at least 2 fish. 2) SAV in the same individuals by RT-PCR or cell culture or IHC.
Regulation:
Group B listed disease: a geographical zone which includes the endemic area is established. The border of the zone is placed where there is no fish farms with long borders of open sea.
Rules for preventive measure:
For preventing spread of SAV testing is done before transport out of the zone, transport of positives are forbidden. If smolts are transported out of the zone they shall be tested 2 months after transfer to the sea.
Industry initiatives to fight PD can be seen at www.pdfri.no.
In 2008 we have had 1 PD infection north of the zone. There was no increased mortality. Sampling was done for PD diagnosis, but it was difficult to find diseased fish. Early PD was diagnosed. The farmer chose to kill of all fish within one week, before the PD diagnosis was confirmed.
Conclusion: the PD situation is serious, the regulations by the authorities and the industry initiatives may reduce the negative impact of PD and other infectious diseases. However at lot of traffic and transport of fish between farms seems to continue.

Question
Sven Bergmann: *How often do you have IPNV contamination when you test by cell cultivation?*
Birgit Dannevig: *We always neutralise for IPNV before inoculation.*

Fish diseases situation in Spain

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Abstract: Spain is a country with a great variety of pisciculture species, so to speak about the status of fish diseases it is convenient to differ between marine and freshwater aquaculture, although some diseases are common for both.

In freshwater, among diseases that affect to salmonids (Rainbow trout and Brown trout) it can stand out those that are produced by virus as IPN (Infectious Pancreatic Necrosis) and SD (Sleeping Disease) that cause some problems in fish farms. It is possible to isolate the viruses in the routine analysis for the survey and control for other diseases. Among bacterial diseases the most common are Furunculosis (*Aeromonoas salmonicida*), Lactococcosis (*Lactococcus spp.*) and Enteric redmouth (*Yersinia ruckeri*). Of the parasites, the one that is more common find is (*Ichthyophtitius multifilis*) that produces White spot disease.
In marine aquaculture it must be remarked that there are two zones in Spain with different species of farmed fish, the Mediterranean area where the main aquaculture fish are Sea bream (*Sparus aurata*) and Sea bass (*Dicentrarchus labrax*) whose mainly diseases are Pateurellosis and Lymphocystis. And in the north of Spain the main fish farmed species are Turbot *(Psetta maxima*), Common sole (*Solea senegalensis*) and Pollac (*Pollachius pollachius*) where the most important virus disease registered is IPN and among bacterial diseases it is found Vibriosis *(Vibrio spp.)* Myxobacteriosis (*Tenacibaculum sp)*, and Aeromonas *spp*

Minutes:
In Spain we have nearly 8000 km of coastline, with a wide variety of aquaculture especially marine. Most important fish are rainbow trout, brown trout, and tench in freshwater. In the marine the most important fish are turbot, sea bass, sea bream and tuna.
IPNV was present in 10% of the farms that were sampled at routine surveillance for VHS in 2007. IPNV has also been isolated in turbot.
Sleeping disease has been suspected based on the clinical signs. SDV was first isolated in 2003, and was isolated from 4 farms in 2007.
IHNV/VHSV has not been detected in 2007.
The most important bacterial disease is furunculosis, but is more or less controlled by vaccination.
Lactococcosis is widely spread all over the country.
ERM has been reported but is controlled by vaccination.
RTFS provokes huge losses as there are no available vaccines.
In seawater the principal disease is VNN/VER and is a huge problem for seabass, lymphocystis disease affects seabream and is not considered to be a major concern. *Listonella anguillarum* as well as *Photobacterium damselae* subsp *piscicida* are controlled by the use of vaccines. *Flexibacter maritimus* is a problem in turbot nowadays but the use of vaccines has improved the survival.

Amyloodinium ocellatum: massive infection are frequently associated to mortalities in fish reared in lagoons, mainly at high temperatures.

Questions:
Niels Jørgen Olesen: *Last year (2007) there was a publication of finding VHS in seabream by RT-PCR, can you comment on that?*
Marta Vigo: *We have no information, they have been asked to send us samples, but they have sent us nothing at the moment. There has been an official request to give information.*

The isolation of Spring Viraemia of carp virus, in Romania, in a clinical outbreak of disease in carp

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Abstract: In Romania, the production of cyprinids comprise about 85% of the whole fish production. The carp (*Cyprinus carpio*) grow together with other species of cyprinids (crucian carp - *Carassius carassius,* red eye – *Scardinus erythrophthalmus,* bream – *Abramis brama ,* tench – *Tinca tinca, etc*) in natural and artificial lakes, rivers and in the Danube Delta waters. In farms the carp is grown together with silver carp – *H. molitrix*, bighead - *Aristichthys nobilis,* grass carp - *Ctenopharingodon idella*. The total surface of water is estimated at 400.000 ha and the length of rivers at 66.000 km.
The affected farm produce carp reared in policulture with other cyprinid species (crucian carp and grass carp) and with other species of fish as pike, sheat-fish, red eye and tench.
The mortality appeared following introduction of fish in the farm. The fish had clinical and pathological changes similar to SVC infected fish: exophthalmia, hemorrhages in eyes, paleness of gills, hemorrhages of internal organs, ectasis of blood vessels in the fat tissue. They were investigated by laboratory examination as: virological, bacteriological, parasitological and cytomorphology of blood cells.
The results of the virological examinations (isolation of virus in cell culture and identification by ELISA kit and indirect imunofluorescence) showed the presence of spring viraemia of carp virus.
This is the first case of SVC identified by laboratory means with virus isolation in Romania.

Minutes:
The total surface of water in Romania is 400000 hectares and the length of the rivers are 66000 km. 85% of the production is cyprinids. The farm has 2.5 ha surface of water. In 2008 there was an introduction of 2 years old carp. The water temperature was 16.5°C, pH 7.0, dissolved O_2 8.5 mg/l.
CPE at EPC cells +++, CPE at BF-2 cells ++.
Positive ELISA and IFAT for SVCV.
The mortality was 21% in 14 days.
First signs of disease and mortality show up at 2 years old specimen.

Questions:
Guiseppe Bovo: *Congratulations on this very thorough approach. It seems to be a very serious disease.*
Mihaela Costea: *Yes, but only in the fish stressed by movement etc.*

First outbreak of IHN in the Netherlands.

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31 March 2008 rainbow trout in a put and take fishery (closed, no connection with open water) showed a strange red bulb at one side, whitish appearance, and there were some dead fish in the storage basins. The farm uses well water, with no drain or connection to open water. The water temperature was 12°C. It was a new farm which started in July 2007 with rainbow trout of 350-500g and salmon trout of 1.2 kg present. The fish had very light pink fat. No bacteria were isolated.
The rainbow trout came from France by a German lorry. Clinics were reported by a visiting angler, not by the owner.
RT-PCR was IHNV positive, and virus cultivation and isolation was made and showed positive after 3 days. Dutch contact farms were tested by virus isolation and found negative.
No samples from Germany or France have been tested, nor has the health certificate from France been seen yet. No stamping out, no measures at all were taken, that was it. The Netherlands does not stamp out EU non-exotic diseases, neither will it in the nearby future. This fact might inhibit farmers to notify disease.
Put&take farms or lakes are the most risky because they bring in the cheapest fish, which might be infected.

SESSION IV: Technical issues related to sampling and diagnosis

Chair: *Brit Hjeltnes*

Risk Based surveillance

Pedro Rosado Martín
EUROPEAN COMMISSION
HEALTH & CONSUMERS DIRECTORATE-GENERAL
Unit D1 - Animal health and Standing Committees

Abstract: Council Directive 2006/88/EC lays down animal health requirements for aquaculture animals and products thereof and contains provisions on the prevention and control of certain diseases in aquatic animals.
Chapter II of the Directive introduces a series of minimum preventive measures aimed at increasing the awareness and preparedness of the competent authorities, aquaculture production business operators and others related to this industry, for diseases in aquaculture animals. These measures include obligations for farms to apply animal health surveillance as appropriate for the type of production (Article 10).
The Commission has drafted guidelines to provide the Member States with guidance on how to implement the requirements of Article 10 on the establishment of a risk-based animal health surveillance scheme, in particular on the risk classification of aquaculture farms.
This general animal health surveillance scheme shall aim at the detection of:

- any increased mortality (relevant for all farms);
- listed diseases (relevant for farms areas keeping species susceptible to the listed diseases).

In addition to conduct a general check of the health status of the animals of the farm, the purpose of Article 10 is to advise the aquaculture production business operators on aquatic animal health issues, and where necessary, undertake the necessary veterinary measures.

In the framework of this scheme, inspections must be carried out, either by the competent authorities or by any private veterinarian or other qualified aquatic animal health service. Part B of Annex III to the Directive lays down recommended frequencies of inspection. The frequencies are determined by two factors:

- The health status of the concerned zone or compartment in relation to the listed non-exotic diseases (categories I – V).
- The general risk the farm poses in relation to spreading and contracting diseases.

At each inspection it may be appropriate to go through the records of the farm in particular the mortality records, to get a picture of the health status evolution and health history of the farm. A representative selection of all production unites should be inspected. If the outcome of this examination leads to suspicion of infection with a listed disease, the animals should be subject to laboratory testing. This examination should in particular aim at detecting infection with the suspected listed diseases.

Minutes:
Risk Based surveillance according to Council Directive 2006/88/EC, was presented and explained.

Question/comments:
Birgit Oidtman: *Risk assessment systems could vary between member states – can we have confidence that there will not be different levels of the disease status in the different member states.*
Pedro Rosado Martin: *Such a situation may only occur in category III farms where the risk based surveillance scheme mostly applies. In the other categories e.g. category I member states have to fulfil same requirements. If a member state is free of a disease, this status is maintained by similar basic requirements.*

Establishment of a new Commission Decision on sampling and diagnostic procedures of listed diseases

Pedro Rosado Martín
EUROPEAN COMMISSION
HEALTH & CONSUMERS DIRECTORATE-GENERAL
Unit D1 - Animal health and Standing Committees

Abstract: Council Directive 2006/88/EC lays down animal health requirements for aquaculture animals and products thereof and contains provisions on the prevention and control of certain diseases in aquatic animals.

Chapter II of the Directive on disease free status establishes the pathways for a Member State, zone or compartment to obtain the freedom status with regard to the non-exotic diseases listed in Annex IV part II to the Directive (VHS, IHN, KHV and ISA). These pathways are:

- absence of the species susceptible to the disease in question in the area to be qualified as free from the disease;
- the pathogen is known not to be able to survive in the area to be qualified as free from the disease;
- based on historical grounds (before 1st November 2008);
- based on targeted surveillance.

The Commission is drafting a Commission Decision implementing Directive 2006/88/EC laying down the requirements to achieve the freedom status following a targeted surveillance scheme. This draft Decision will cover:

- case definition for each listed disease (including exotic diseases);
- diagnostic methods that could lead to a suspicion or confirmation (including exotic diseases) and for surveillance;
- sampling plans to achieve the freedom status;
- sampling plans to maintain the freedom status.

Minutes:
As a direct continuation of the presentation made by Pedro Rosado Martin, Niels Jørgen Olesen gave a presentation on the subject: Proposal for a draft Commission Decision on Sampling plans and diagnostic methods for the exotic and non-exotic fish diseases. The goal is to give specific guidelines in order to harmonise the sampling and diagnostic procedures. It should create trust in trading fish; The Decision will be in compliance with the OIE Diagnostic Manual for Aquatic Animal Diseases and includes the diseases VHS and IHN (2001/183), ISA and the new diseases: KHV, EHN and EUS. With respect to surveillance, clinical inspection is important for all diseases and targeted surveillance with laboratory examination will have to be applied for proving disease freedom**.** Sampling plans and diagnostic methods for VHS and IHN surveillance in order to obtain disease free status of a zone or a compartment was presented and explained. Furthermore, inspection and sampling schemes for achievement and maintenance of disease free status for VHS and/or IHN in zones and compartments were presented and explained.

Sampling procedures for molecular based methods, as PCR, in practical fish diagnostics

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Abstract: In diagnosis of fish diseases, more and more, highly sensitive and specific molecular based methods have been applied to trace DNA or RNA from fish pathogenic organisms as bacteria, virus and parasites. Different kinds of techniques as regular PCR, real-time PCR and nested PCR are routinely used as screening and confirmatory tests, for instance to classify fish populations as infected or not. We, as laboratory employees validate these tests, make considerable efforts to organize laboratory facilities to avoid cross-contaminations, and perform the tests in an optimal manner to work towards a reliable test result.

The reliability of the diagnosis is however not only dependent on the test itself. For this we need to realize, the basis for all testing depends on the sampling. How were the fish or samples taken in the field? How were the fish or samples transported to the laboratory? How was the fish necroptized to obtain the organ material? Were the sample vessels from the necropsy room externally disinfected before being transported to the laboratory or storage room? Were there any traces of nucleic acids remaining on the instruments used for sampling? In other words: how sure can we be that there was no cross contamination of pathogens and/or their DNA or RNA during sampling, making the final results of our very sensitive and specific tests useless?
In the current lecture, we discuss practical considerations to take into account in order to deliver reliable diagnostic samples for further testing. If we realize the need of utmost good laboratory practice from the very start of the sampling chain, we can indeed rely on the results of our highly sensitive and specific validated tests.

Minutes:
Examples were presented on how sampling procedures could be performed in order to obtain a reliable diagnostic result. Recommendations included several steps along the diagnostic pathway including: Sampling in a separate necropsy room using decontaminated lids & cutlery - 1 set per set of fish. Euthanization of fish to occur in the bag and new gloves should be used for each bag of fish. When performing the sampling of fish, samples are taken directly into the vessel. Waste fish are disposed separately and buckets and lids are disinfected overnight in chloramin-T. Cleaning the necropsy table and the cutlery are performed using Chlorine and a DNA/RNA destroying detergent e.g. LTK 008 and UV. Furthermore, recommendations for field sampling at the farm were given.

Development and application of real-time PCR assays for the detection of viral haemorrhagic septicaemia virus (VHSV).

Mike Snow
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Abstract: Viral haemorrhagic septicaemia is an internationally significant disease, exemplified by recent serious new outbreaks in North America, the UK and Norway. Rapid and efficient detection of the causative agent VHSV is fundamental to effective disease control. Real-time PCR or quantitative PCR (qPCR) offers significant advantages over conventional PCR methodologies for detection of the presence VHSV RNA. Such advantages include increased specificity of detection, increased sensitivity, ease of results interpretation, higher throughput and the potential to obtain quantitative data. Real-time PCR methods also facilitate the inclusion of appropriate controls.

The development and application of qPCR to the detection of VHSV will be described. This method has been developed in our laboratory alongside appropriate internal (endogenous) and positive controls and has been validated to the standards required by ISO/IEC 17025. Approaches for accreditation of qPCR methods and work in progress to discriminate different subtypes of VHSV will be discussed.

Minutes:
A VHSV specific qPCR was described that has been accredited according to the principle of flexible scope – based on guidelines in OIE manual. The design of primers and probe was made according to an analysis of the N-gene sequences from 128 isolates. The qPCR identifies genotypes I, II and III. Furthermore, the ongoing development of a genotype specific qPCR was described.

Questions/comments:
Sven Bergmann*: What is the Ct limit within and between the qPCR analyses?*
Mike Snow: *We do no operate with such limits but prefer to look at results of triplicates and when they are similar then we consider the result to be OK*
David Stone: *At a certain level, the Ct values become unreliable – at what Ct values do you consider the result to be VHSV negative?*
Mike Snow: *It is difficult to say only from the Ct values – We do not make diagnostic based on PCR alone. qPCR is a quantitative method that can be used together with clinical studies.*
Niels Jørgen Olesen: *How is the sensitivity of qPCR compared with conventional PCR?*
Mike Snow: *Based on a range of considerations - the qPCR is at least as sensitive as conventional.*
Niels Jørgen Olesen: *What about qPCR compared to cell culture techniques?*
Mike Snow: *We have not performed such a study.*

Serological methods recommended for detection of antibodies against VHSV and IHNV

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Abstract: The need to have available and reliable tools for indirect diagnosis of VHS and IHN, together with the results previously obtained from a proficiency test in fish serology, have led us to validate diagnostic assays for the detection of specific antibodies against those two viruses.
Many authors have already suggested using serology to establish the sanitary status of salmonid farms regarding VHS and IHN. However, the use of such methods has not got the agreement of the authorities in charge of the regulation concerning fish diseases. Nevertheless, in other vertebrates the detection of specific antibodies in the sera is officially recognised as a useful indication of a previous exposure to viruses, especially when the virus cannot be isolated. In order to convince the scientists and authorities that serological methods can help in the detection of VHSV or IHNV infected trout population, the validation of seroneutralisation (SNT) and ELISA techniques has been carried out using sera from trout experimentally or naturally infected with the two viruses. The techniques used were adapted from those described many years ago (N. J. Olesen *et al*., 1991; A.M., Hattenberger *et al*., 1995) and the validation performed as described in the Manual of diagnostic tests for aquatic animals of OIE.
Comparison of the results obtained with the two techniques applied to the same trout sera, indicates that both SNT and ELISA can be used to detect specific antibodies against VHS and IHN viruses. The two techniques can also be used whatever the European genotype of VHSV (I, II or III) infecting the trout. If ELISA appears more sensitive but less specific that SNT, the two tests can be used independently depending on the expertise of each laboratory and of the reagents available.
A proficiency test in VHS and IHN serology, organised within the framework of EPIZONE network is now going on between eight participants. If the results are in agreement, the last step of the validation procedure will be the application of the tests to farmed infected trout.
References:
Olesen N.J., Lorenzen N., Jorgensen P.E.V., 1991. Detection of rainbow trout antibody to Egtved virus by enzyme-linked immunosorbent assay (ELISA), immunofluorescence (IF), and plaque neutralization tests (50% PNT). *Diseases of Aquatic Organisms, vol. 10: 31-38.*
Hattenberger-Baudouy A.M., Danton M., Merle G., De Kinkelin P., 1995. Serum neutralization test for epidemiological studies of salmonid rhabdoviroses in France. *Veterinary research, 26, 512-520.*

Questions/comments:
Giuseppe Bovo: *It should be underlined how important the use of these techniques can be in surveillance as it is easier to detect the presence of antibodies in certain areas especially in "hot"areas.*
Ellen Lorenzen: *Are these antibodies functional in vivo – can they protect the fish?*
Jeanette Gastric: *Yes, positive sera can protect the fish at low titres.*

Experiences of a micro-dilution method for testing antimicrobial susceptibility of bacteria from fish

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Abstract: To test antimicrobial susceptibility of bacteria from fish is a valuable tool to guide therapy in single incidents of disease but it is also important to compile available data and monitor susceptibility over time. Thereby trends in resistance that could be cause for re-evaluation of therapeutic recommendations or other interventions can be detected. However, until recently there have been no accepted standard reference methods for testing antimicrobial susceptibility of bacteria from fish and no accepted criteria (breakpoints/cut-off values) for interpretation of such data. Obviously published data on antimicrobial resistance in bacteria from fish is therefore scarce.

The lack of harmonized methodology was recognised in the late 90s and through an initiative within the EU a workshop on this issue was organised, eventually publishing draft protocols for antimicrobial susceptibility testing of bacteria associated with fish diseases (Alderman & Smith, 2001). The drafts were based on methods for testing bacteria from terrestrial animals issued by The Clinical and Laboratory Standards Institute (CLSI, formerly NCCLS). The drafts from the workshop were further elaborated by CLSI and recently guidelines on disc diffusion (CLSI 2006a) and broth dilution (CLSI 2006b) for susceptibility testing of bacteria from aquatic animals were issued. Interpretive criteria are still lacking, however.

In an effort to standardize susceptibility testing of bacteria from fish at SVA, a microdilution system for testing bacteria from warm-blooded animals, VetMIC™ (SVA, Uppsala, Sweden), was adapted to bacteria from fish according to the first recommendations issued by Alderman & Smith (2001). The methodology has been used since 2005 for routine testing of isolates from samples of clinical submissions. Data on minimum inhibitory concentrations (MIC) of *A. salmonicida* subsp. *achromogenes*, *Flavobacter columnare* and *Flavobacter psychrophilum* from these analyses are presented in Table AQ III. Most isolates represent a unique batch of fish but occasional isolates are duplicates within the same batch. The majority of *A. salmonicida* subsp. *achromogenes* and *F. columnare* are from brown trout, 57 and 72 %, respectively, whereas the majority of *F. psychrophilum* are from rainbow trout (71%). Bacteriological culture and susceptibility testing was performed at the Department for Fish diseases, SVA. For antimicrobials tested and range of dilutions see Table AQ III.

Interpretation of the data is hampered by the lack of accepted interpretative criteria. However the shapes of the distributions of MICs can be evaluated for indications of the presence isolates with deviating MICs, possibly due to acquired reduced susceptibility (resistance). In the present material, the distributions of gentamicin and streptomycin MICs for *A. salmonicida* subsp. *achromogenes* are examples of well defined unimodal distributions (Table AQ III). Such distributions indicate that none of the isolates tested have acquired resistance to these antimicrobials, which is likely since the two antimicrobials are not used in fish in Sweden (Table AQ I).

In contrast, MIC distributions for the quinolones, i.e. nalidixic acid and ciprofloxacin or enrofloxacin, are bimodal in all three bacterial species. Moreover, isolates with an elevated MIC to nalidixic acid (≥16 mg/L) also had elevated MICs to ciprofloxacin or enrofloxacin (data not shown). This indicates the presence of acquired resistance to quinolones in about 15% of the isolates of all three bacterial species, probably due to the past and present use of the quinolone oxolinic acid in aquaculture (Table AQ I).

The tendency for bimodal MIC distributions for sulphonamide, trimethoprim and ampicillin in *A. salmonicida* subsp. *achromogenes* also indicate that some isolates have acquired resistance to these antimicrobials (Table AQ III). The same may apply for tetracycline in *Flavobacter* but for this antimicrobial MICs are truncated at the lower end of the range of concentrations tested. This makes interpretation difficult in the absence of accepted breakpoints for resistance. However, elevated MICs in some isolates of *Flavobacter* may indicate acquired tetracycline resistance. In Sweden infections with *Flavobacter* are often treated with tetracycline and occurrence of resistance could be a consequence of this use.

Truncation of MICs in the upper or lower part of the range of concentrations tested can be avoided by an appropriate design of the test panel. Since the panel used was not designed for testing bacteria from fish it is not optimal for some combinations of antimicrobial and pathogen, e.g. tetracyclines in all three species. Also for florphenicol, distributions are truncated at the lower end of the range tested making interpretation difficult.

Obviously the method used at SVA needs further adaptation and evaluation to be a reliable tool for routine as well as monitoring purposes of bacteria from fish. However, using microdilution according to harmonized methodology and with panels designed for testing bacteria from fish in a wider perspective would make it possible to compile data on MIC for relevant combinations of bacteria and antimicrobials. Thereby interpretive criteria for reduced susceptibility could be determined according to the principles used by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (www.eucast.org) for bacteria from humans and warm blooded animals. This would be of great importance for routine clinical use as well as for monitoring purposes.

Table AQ III. Distribution of MICs for *Aeromonas salmonicida* subsp. *achromogenes* (ASA, n=67), *Flavobacter columnare* (FP, n=30) and *Flavobacter psychrophilum* (FC, n=42). Isolates from 2005-2007.

Antimicrobial	Species	Distribution (%) of MICs[a] (mg/L)																		
		≤0.004	0.008	0.016	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128	256	512	1024	>1024
Ampicillin	ASA										32.8	6.0	7.5		53.7						
	FP						100.0														
	FC						100.0														
Chloramphenicol	ASA								93.9	6.1											
	FP								23.8	33.3	38.1	2.4	2.4								
	FC								33.3	60.0	3.3		3.3								
Ciprofloxacin	ASA	2.5	25.0	40.0	17.5		10.0	5.0													
	FP		16.0	32.0	32.0	4.0	4.0			12.0											
	FC	23.5	58.8	5.9						11.8											
Enrofloxacin	ASA			25.9	48.1	11.1	7.4	7.4													
	FP			58.8	17.6	11.8			5.9	5.9											
	FC			92.3				7.7													
Florfenicol	ASA										97.0	3.0									
	FP										100.0										
	FC										96.7	3.3									
Gentamicin	ASA							3.0	7.5	73.1	16.4										
	FP							92.9	2.4	4.8											
	FC							26.7	60.0	10.0	3.3										
Nalidixic acid	ASA								82.1	4.5					3.0	6.0	4.5				
	FP								11.9		45.2	26.2		2.4	2.4		11.9				
	FC								76.7	6.7	3.3				3.3	3.3	6.7				
Streptomycin	ASA										1.5	4.5	29.9	61.2	3.0						
	FP									100.0											
	FC									100.0											
Sulphonamide	ASA												4.5	1.5	18.2	6.1	7.6	7.6	4.5	6.1	43.9
	FP												53.7	14.6	22.0	7.3	2.4				
	FC												70.0		6.7	10.0					13.3
Tetracycline	ASA							89.6	10.4												
	FP							76.2	7.1	2.4	4.8	9.5									
	FC							90.0	6.7			3.3									
Trimethoprim	ASA						3.0	4.5	28.4	16.4	1.5	3.0	20.9	13.4	9.0						
	FP											2.4		2.4	95.2						
	FC						3.3					13.3	13.3	30.0	40.0						

[a] White fields denote range of dilutions tested for each substance. MICs above the range are given as the concentration closest to the range. MICs equal to or lower than the lowest concentration tested are given as the lowest tested concentration. Bold vertical lines indicate epidemiological cut-off values for resistance

References:

Alderman, DJ. and Smith, P. Development of draft protocols of standard reference methods for antimicrobial agent susceptibility testing of bacteria associated with fish diseases. *Aquaculture,* 2001, 196:211-243.

CLSI. Methods for Antimicrobial Disk Susceptibility Testing of Bacteria Isolated From Aquatic Animals; Approved Guideline. CLSI document M42-A (ISBN 1-56238-611-5). Clinical and Laboratory Standards Institute, Wayne, Pennsylvania, USA, 2006a.

CLSI. Methods for Broth Dilution Susceptibility Testing of Bacteria Isolated From Aquatic Animals; Approved Guideline. CLSI document M49-A (ISBN 1-56238-612-3). Clinical and Laboratory Standards Institute, Wayne.

Questions/comments:
Olga Haenen: *Did you see a lot of multi resistant Bacteria?*
Björn Bengtsson: *No, we haven't seen many but haven't examined many bacteria either.*
Anders Hellstrøm: *We have some data on multi resistant bacteria that you can have*
Snjezana Zrncic: *What is the time period for collecting data?*
Björn Bengtsson: *2005-2007*
Niels Jørgen Olesen: *Do you make end point titration for each test?*
Björn Bengtsson: *No, procedures have been standardized – as described in the guidelines*
Helle Frank Skall: *Why haven't you looked at the sensititer method?*
Björn Bengtsson: *We have made our own panel.*

OIE recommended procedures for KHV diagnosis and the KHV PCR ring-trial 2007

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Abstract: The Office International des Epizooties (OIE) - the World Organisation for Animal Health - has added KHVD to the list of serious diseases whose occurrence must be reported to the OIE by the official services of its member countries. This followed expert assessment that the characteristics of the disease met the OIE listing criteria. Guidelines for safe international trade in KHVD-susceptible species are now given in the OIE Aquatic Animal Health Code (2007), and methods for surveillance and diagnosis of the disease are given in the OIE Manual of Diagnostic Tests for Aquatic Animals (2006). Cefas, Weymouth is one of two OIE reference laboratories for KHVD, the other being the Fisheries Research Agency in Yokohama, Japan.

The selection of the diagnostic methods detailed in the manual has been informed by PCR method ring-trial organized by the Cefas Weymouth Laboratory. The ring-trial has compared standardized PCR protocols developed at Cefas, using published primer sets, with protocols used in other laboratories.

For the first trial in 2006, laboratories were asked to incorporate the Cefas recommended primers in their current KHV PCR protocol using their usual assay parameters and compare them with the primers that they currently use. All laboratories then tested these PCR assays on KHV-spiked tissue homogenates supplied by Cefas. In 2006 21 laboratories from 19 countries around the world participated in the first ring-trial.

For 2007 the format of the ring-trial was changed slightly. As in 2006, laboratories were requested to perform single-round PCR and compare two different sets of primers. The PCR protocols tested were the Yuasa modification of Gray's protocol with SpH primers and a Cefas protocol using Bercovier TK primers. The latter was slightly modified from the Cefas protocol tested in 2006. A further change was in the amount of KHV used to spike the tissue homogenates where at least one of the samples contained very low (target was <20) copy numbers of KHV DNA. Participating laboratories were asked to use a real-time PCR assay or a nested-PCR assay to detect the KHV in the sample.

Thirty-three laboratories from 29 countries participated in the 2007 ring-test and results have, so far, been received from 32 labs. As in 2006 all of the laboratories obtained correct results with at least one of the assay protocols used. The great majority of incorrect results were seen with the two single round assays. As expected, most of these failed to identify KHV in the 2 samples with the lowest genome copy numbers. The more sensitive nested PCR and real-time PCR assays that were used identified KHV in these samples with only two laboratories reporting problems.

Minutes:
KHV-carriers may not be reliably detected using methods currently available however, PCR-based methods recommended as most reliable for diagnosis of and surveillance for KHV . From the ring-test it was observed that Bercovier TK primers have performed well and was reliable; Yuasa mod.Gray primers performed better than Cefas mod. Gray primers; Gilad 9/5 primers are rather inconsistent; DNAzol extraction can be unreliable – silica-based extraction kits are more consistent; Nested PCR assays are more sensitive but cross-contamination can occur; Real-time PCR appears to be very consistent – e.g. the Gilad taqman, is sensitive and reliable.

Infectious salmon aneamia as a non-exotic disease in the EU: Consequences for active surveillance

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Abstract: Member states shall apply the provisions of the EU fish health directive 2006/88/EC http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2006:328:0014:0056:EN:PDF, from 1 August 2008. Infectious salmon anaemia (ISA) is listed as a disease that is non-exotic to the EU. Member states declared free of ISA will not be required to undertake active surveillance provided that conditions conducive to expression of clinical disease occur where susceptible species are present. The implications of this, for example, for rainbow trout which is listed as a susceptible species will be presented.

Historically, surveillance for ISA has been predominantly achieved through clinical inspections where tissue sampling and diagnostic testing for ISA virus was carried out on the basis of observed clinical disease (2003/466/EC) . The suitability for different methods in surveillance for ISA is described in the OIE Manual of Diagnostic Tests for Aquatic Animals OIE http://www.oie.int/eng/normes/fmanual/A_00026.htm. 2006/88/EC states that where active surveillance for ISA is required, for example, in establishing and maintaining freedom for a zone or compartment then targeted surveillance (sampling and testing) must be carried out. The sampling requirements and diagnostic methods for this surveillance will be provided in a future commission decision but the implications for targeted surveillance will be presented together with a review of some options as to how this could be achieved.

Minutes:
According to the new Council Directive targeted surveillance will include ISA if countries will state freedom for the disease. Currently, the surveillance for the disease is passive. In case of suspicion clinical inspection and laboratory examinations are made according to EU legislation where various methods are described for fish with/without clinical signs. When clinical signs are observed it is acceptable to examine few fish – more fish will have to be examined for targeted surveillance. In Scotland there is currently active surveillance for ISA. A working group has been made to discuss ideas on how to do active surveillance and what is required for sampling and testing.

Questions/comments:
Sven Bergmann: *Can serology be used to confirm ISA – does salmon develop immunity towards ISA?*
Brit Hjeltnes: *They do develop an immune response but we don't know if the fish get rid of the virus.*

Rob Raynard: *If targeted surveillance is used to demonstrate freedom and a positive response is made based on serological analyses then you have to go back to the farm to confirm the serological result – is it there or is it cross reactions that we observe?*
Brit Hjeltnes: *Cross reactions is a concern and it has to be evaluated if the disease been there earlier and is responsible for the positive serology. Should be considered in reaction to claim disease freedom.*
Rob Raynard: *You would still have to carry out all the other tests.*

Health categorisation and implementation of risk based surveillance in Denmark

H. **Korsholm**

Minutes:
In Demark listed diseases will be categorised as follows: IHN: Category I; ISA: Category I; KHV: Category V; SVC: Additional guaranties; VHS: Category I and II or IV. For VHS Category I farm includes farms from approved zones. Farms from non-approved zones might be included in category I on a historical base (10 years disease freedom) or based on compartment considerations.The rest of the farms goes into II or IV. One consideration to choose II versus IV is that category II does less harm trade. The marine farms were tentatively placed in III but their final categorization has not been decided yet. Types of surveillance: For category II or IV it will be targeted surveillance. For category II this implies 2 years with two times 15 pools or 4 years with at least two years with two times 3 pools. For category I areas, surveillance will be targeted or active.

Questions/comments:
Giuseppe Bovo: *Who pays the cost of the survey in Denmark?*
Henrik Korsholm: *In principle, the fish farms have to pay. However, at the moment the fish farms do not pay and the government pays the fee for sampling and laboratory examination. If this dispensation disappears the farms have to pay.*

Health categorisation and implementation of risk based surveillance in Italy

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Abstract: In Italy the registration of fish as well as other animal farms have been adopted by the veterinary authorities since several years. Unfortunately, in the case of fish farms, these data have been produced and collected only at local or regional level. For this reason the implementation of directive 2006/88/EC into the Italian legislation requires the adoption of a national data base under the responsibility of the CVO, already routinely used for several different species.
Beside the centralization of data concerning farms registration, each farm and establishment will receive by the regional veterinary services the requested authorization provided they fulfil all the requirements listed in art. 5. Suspension of the authorization will be adopted if some of those requirements will discontinue. In practice, due to the serious consequences, we believe this measure will be adopted only after all risk reduction tools have been applied with no results, at least for pond trout farms. On the other hand this policy measure could be very interesting in order to prevent the spreading of important pathogens from establishments particularly in geographical areas in which fish farms are located .

According to farm categorization foreseen by Annex III, part A, VHS and IHN approved farms will be automatically placed in category 1, while infected farms will be, in the same way, included in cat. V . The remaining salmonid farms which are not approved or in program but in which no viral infection has been identified since the adoption of the first Council Directive (91/67/EEC) will be temporarily placed in cat III, waiting for their definitive position .
No decision has been so far adopted concerning ISA and KHV status. Cyprinid farms will most likely be regarded as cat. III farms while concerning the ISA status an EU-wide general regulation is expected.
Because put and take lakes may represent a serious infection source particularly in free zones, they will be regarded as fish farms and included in cat V, because they have routinely introduced fish coming from infected farms . This decision will facilitate the market of infected farms which will continue their activity selling their production for human consumption or for infected put and take lakes. Of course put and take lakes located in free zones will be obliged to introduce susceptible species only from free farms. Put and take lakes not connected with the public waters will be regarded in a different way and no control will be adopted by the official veterinary service.
According to our national legislation river and lake restocking has been permitted, since several years, only with fish originating from approved farms and the same requirement will be maintained in the future. This disposition which is more restrictive than the directive will possibly give rise to serious obstacles from a consistent part of fish farmer which are excluded from the restocking job. We believe the decision of the Ministry represents a correct tool to improve the health status of reared and wild populations and furthermore represents an important incentive for infected farms to improve their health status.
Concerning the animal health surveillance, no decision has been taken so far on who will take care of the control but, in any case, all the cost will be charged to the farmers. According to our Ministry in fact no expenses will be supported by the public system except in the case of eradication programs planned at regional level .

Question/comments:
Sven Bergmann: *What will you do with the ECV infected farms?*
Giuseppe Bovo: *We have some small ECV infected farms that we hope will be eradicated.*
Niels Jørgen Olesen: *We are 6 weeks ahead of the implementation date of the new Council Directive. It will be interesting to see how different member states will implement the new directive.*
Brit Hjeltness: *For future Annual Meetings, I suggest that there will be more time for discussions and important questions.*

SESSION V: Scientific research update

Chair: *Giuseppe Bovo*

Establishment of database for fish pathogenic viruses

Tanya Gray
Symantix Ltd, 91 Berkeley Road, Wroughton, Swindon, Wiltshire, SN4 9BN, UK

Abstract: The fish pathogens of aquaculture database (http://fishpathogens.eu) is an online resource concerning pathogens of aquaculture in Europe. The database offers a high quality curated dataset, including isolate and sequence data, freely available to the community for molecular epidemiological studies. In the first instance, the database will hold data on viral pathogens, including: VHS, IHN, ISA, and KHV.

Searches against the database return sequence data in a FASTA format that can be downloaded for further analyses. Isolate and sequence reports can be downloaded in spreadsheet format, based on the user requirements. Geographical functions map the geographical distribution of isolates.

The database has a pathogen expert role whose responsibility is to ensure the accuracy and relevance of data held in the database. All reports are reviewed and approved by the pathogen expert before they are made publically available. Reports are uploaded from spreadsheets or by using an input form. Non-registered users can complete the input form and their report will be emailed to the pathogen expert for review.

The values allowed in the database are restricted using input form validation and a controlled vocabulary. This helps to increase the accuracy of queries against the database using keywords. Use of a controlled vocabulary is also a first step in the development of a data standard to describe isolates and related sequences for the purpose of molecular epidemiological studies. A data standard enables integration of data from different sources, thus allowing combined queries against related datasets, for example, isolate, sequence, geographical and environmental datasets.
The fish pathogens database is the result of a collaboration between the CRL Fish and the European project, EPIZONE. Some ideas on how to implement the database application have their origin in a review of the mlstdbNet database software. The database application itself is easily configurable, and uses high quality freely-available software such as the Apache web server, PHP, Google widgets, and MySQL database. As such, the database application has potential applications beyond the fish pathogens database.

Minutes:
Unfortunately Tanya was sick, so she could not attend the meeting, but she has sent her presentation, which was shortly presented by Niels Jørgen Olesen
For many years we have talked about the establishment of a database, that is in fact one of our obligations to get ahead of these things.
Gael Kurath and Jim Winton in Seattle has established a very nice database for IHNV, but due to copyrights it has been difficult to use the same database, but we are in close contact with them, so we will be able to include also the American VHSV isolates.
Some years ago there was an EU project called EUROPAS. This project started out developing the database, but as the money stopped, the database "fell asleep". Last year Mike Snow, Marine Laboratory tried to revive the database and we went into cooperation. It has now been decided that the database will follow the CRL wherever that goes in order to assure its life in the future. We have employed Tanya to continue the development of the database.
The software used for the database is mySQL which is open software, so everybody will be able to access it.
All the entries in the database will go through a pathogen expert, to ensure the quality of the data, in contrast to e.g. GenBank. We will start with entering data for VHS.
An important thing is how to name the virus isolates. Many of the virus isolates have different names circulating, making things quite difficult.
We are now in the development and test phase, and we expect that the database will be launched in August this year.
We will like to include a lot of background information on the different isolates.
There will be a geographical distribution of the isolates with maps showing the place of origin.

In the beginning we thought that we would only have very restricted sequences included to force people to sequence specific parts of the genomes. But we have now decided that we will put sequences of all parts of the genome into the database in order to collect data also for the future..
This is a tool we as CRL gives high priority, as we are convinced that it will be a very strong tool for both research and epidemiology. We hope you will all use it and adopt it as your own database, and not just the CRL database.
We have also had contact with the CRL-mollusc to also include the mollusc diseases.
The PowerPoint presentation will be put on the CRL website, so all can have a closer look at it.

Question/comments:
Brit Hjeltnes: *It is vary valuable to have a common database. I also think that the CRL will be a very good way to promote it. A slight problem may arise with the non-notifiable diseases with the GIS due to confidentiality.*
Niels Jørgen Olesen: *The federal law in Germany prohibits us to put the name of the farm in the database. So we may have to restrict some of the GIS data, e.g. making them less detailed. I would have preferred a completely open database, but in reality we may have to restrict some of the data, e.g. non-published data.*
Franck Berthe: *Thanks for also allowing mollusc into the database. You can have different parts of the database protected with passwords. What is the capacity to add proteins, other genes etc. to the database?*
Niels Jørgen Olesen: *I don't think there will be limitations to include this in the database, e.g. the HPR0 system for ISA. For the genomes that are very large, in e.g. the mollusc pathogens there may have to be some restrictions.*
Franck Berthe: *We all have our own databases for own purpose. I am concerned about the possibility of exporting data to other database.*
Niels Jørgen Olesen: *The purpose of this database is that it should be very easy to retrieve and use for own purpose.*
Birgit Dannevig: *Will it only be isolates isolated in cell culture or also sequences obtained by PCR?*
Niels Jørgen Olesen: *I am aware that isolation in cell culture will not be possible for all pathogens.*

ICES – Working Group on Pathology and Diseases of Marine Organisms (WGPDMO)

Stephen W. Feist
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(stephen.feist@cefas.co.uk)

Abstract: The International Council for the Exploration of the Seas (ICES) is the oldest inter-governmental organisation in the world concerned with marine and fisheries science. The headquarters are in Copenhagen, Denmark. It co-ordinates and promotes marine research in the North Atlantic region. There are twenty member countries each of which has two national delegates sitting on the council. The main purpose is to provide unbiased advice for management. For example, provision of data to the Commission for the Protection of the Marine Environment of the NE Atlantic (OSPAR), advice on new disease trends in wild and cultured fish, molluscs and crustaceans (ICES website May 2006) and 'advice' following EC or OSPAR requests. It also acts as an international depository of fisheries and environmental data, including fish disease for the ICES region. There are numerous working groups dealing with fisheries and environmental science areas. The WGPDMO operates predominantly in response to mariculture issues but also to environmental monitoring using fish and shellfish health and increasingly disease as a factor affecting commercial stocks.

The WGPDMO works to a set of 'Terms of References' (ToR's):
Standing ToR – *Update on new disease trends in wild and cultured fish, molluscs and crustaceans, based on national reports.*
ToR's on current issues related to mariculture: *e.g. Review current information on HSMI* and *Compile information on the distribution, causes and significance of Summer Mortality in C. gigas and other bivalve species.*
ToR's on environmental assessments using fish and shellfish health: *e.g Evaluate available data for a risk assessment pilot study on population effects of diseases in wild fish using epidemiological and population dynamics modelling* and *Compile a report on effects of climate change on host pathogen interactions.*
ToR's derived from requests by ICES/OSPAR/EC/member states: *e.g. Assess results of the ICES/OSPAR Workshop on Integrated Monitoring of Contaminants…(WKIMON) to resolve outstanding issues.*

The WGPDMO produces an annual report with extensive working documents attached as annexes. ICES publications in the 'disease' area include:
1) Web-based report on diseases and parasites of wild and farmed marine fish and shellfish as part of the ICES Environmental Status report.
2) ICES Disease Leaflet Series (S W Feist Ed.).
3) ICES Techniques In Marine Environmental Science series
4) ICES website with WGPDMO reports

Minutes:
There is certainly an overlap of interest between this group here and the ICES. ICES is the prime source of advice on the marine ecosystem to government and international regulatory bodies that manage the North Atlantic Ocean and adjacent seas. The main activity in ICES concerns fishery data e.g for stock evaluation etc. But environmental monitoring, including disease status is also an important part of ICES work.
A key component of ICES work is undertaken by the expert groups. There are working groups and study groups. A study group will have to answer a specific question and then their work may be completed, whereas the working groups have a more permanent character.
The WG on Pathology and Disease of Marine Organisms (WGPDMO): update on new disease trends in wild and cultured fish, molluscs and crustaceans based on national reports. An annual report is produced and placed on the ICES website.
We also run ring tests under the QA programme for marine fish disease (Biological Effects Quality Assurance in Monitoring, BEQUALM) e.g. on histopathology using virtual slides in order to ensure that everybody is working to the same standard.
An important area of work currently in progress is the development of the fish disease index (FDI)- the assessment tool for using fish disease data in environmental assessments, a tool to provide an assessment of fish disease in the OSPAR maritime area for inclusion in the Quality Status Report 2010. Acceptance by OSPAR will mean the fish disease monitoring will be CEMP 1, mandatory.
There is fish disease information on our website. We also links to CRL/NRLs, permanent advisory groups etc.
Disease leaflets are produced providing brief information on a wide variety of marine fish diseases.
Future activities and possibilities include integration of health information with fish stock assessments - effect on recruitment and adult survival, international initiatives underway for integrated assessments of the OSPAR maritime area, fish disease monitoring, increased awareness of

the importance of health in aquatic organisms in sustainability. During the ICES Annual Science Conference in September 2009 there is a theme session on 'New trends in diseases of marine organisms: causes and effects.

Question/comments:
Guiseppe Bovo: *In the Mediterranean we don't have something similar, unfortunately.*
Franck Berthe: *There is a transfer of letters between the OIE and ICES. This is done to reach an agreement on how to do the work and not to duplicate the work.*
Niels Jørgen Olesen: *The development in the recent years where we have found a lot of the pathogens in the marine environment that we originally thought was only farmed fish diseases indicate the need for more and closer collaboration between ICES and the networks of fish- and molluscs pathologists.*
Steve Feist: *There is a lot of information in the reports regarding this.*
Franck Berthe: *You stress the fact that you work on trends, how sure are we on the quality of these data.*
Steve Feist: *This mainly concerns data derived from international monitoring programmes. The methodology has been published as ICES Techniques in Marine Environmental Science (TIMES) papers and for quality assurance; all aspects of disease diagnosis are covered under the BEQUALM programme. Assessment of trends in diseases and parasites affecting aquaculture species is more difficult.*

Recent advances in fish immunology and virology

Niels Lorenzen
National Veterinary Institute, Technical University of Denmark, Aarhus, Denmark

Abstract: The rapid advance in full genome sequencing and gene array technology represents a major advance in our ability to analyse host-pathogen interactions at a molecular level. However, to fully understand the functional aspects, *in vivo* studies are equally important. Recombinant DNA technologies have here provided some very powerful tools.
DNA vaccines encoding the glycoproteins of fish rhabdoviruses like VHSV and IHNV have proved very efficient under experimental conditions and can induce long-lasting protective immunity when delivered by intramuscular injection in rainbow trout. Immunity is established already a few days after vaccination. In this early phase protection is non-specific and related to interferon induced defence systems whereas specific antibodies and cellular components both play a role in the long-lasting protection. The protective capacity of antibodies can be analysed in passive immunization experiments, which in the same time are very useful for identification of protective antigens. Cloning and manipulation of antibody genes provides further insight to the underlying mechanisms. Similarly, for understanding pathogenesis, work on recombinant pathogens represents a very potent technology. By integrating reporter genes or other visualisation markers into a pathogen genome it is thus possible to monitor propagation of an infection in live fish. RNA interference technology has demonstrated high potential for functional knock down studies of gene expression *in vitro*, but still has to be optimised for *in vivo* use in fish.

Minutes:
Fish vaccinated using the VHS DNA vaccine have a high degree of protection already 4 days post vaccination. But they are also protected against IHNV showing this to be a non specific protection. Later on the vaccination will induce a specific protection and works at least up till 2 years after the

vaccination. The advantage with the DNA vaccine is that there is no virus that could revert to a pathogenic virus and it cannot spread to other fish.
The vaccination induces an inflammatory reaction when injected intramuscularly meaning that it actually carries its own adjuvant.
Can the VHS vaccine be used in the field? An experiment was initiated. We were not allowed to release the fish in the field so they were put into field cages that could be submerged in ponds where a VHS outbreak was occurring. The results were very different in the different cages. In some cages the vaccine worked nicely, in other cages it did not work at all. The reason can be that we had problems with a lot of other diseases, e.g. parasites, so the fish may have died from other reasons than VHS. As we are now aiming at total eradication of VHS it may be impossible to do field tests in Denmark anymore, I will be very happy if someone would be interested in cooperation.
In France they have been working with bioluminescence to see how IHNV spreads in the fish. Tissues where the virus do propagate will be luminized. It would be very interesting to do the same kind of work with VHS. One of the things showed for IHNV is that actually the fins are one of the places where the virus replicates in the start of the infection.

Questions:
Guiseppe Bovo: *Are there problems with the DNA vaccination is the field application.*
Niels Lorenzen: *Yes, there may be problems. One is to be allowed to use it in Europe, but DNA vaccination is in use in other countries, e.g. in Canada where most of the farmed Atlantic salmon industry have been DNA vaccinated against IHNV.*
Niels Lorenzen: *The temperature is very important for the innate and specific immune response.*
Franck Berthe*: Why is it not possible to license the vaccine?*
Niels Lorenzen: *well, I guess it is possible, but there are all these consumer safety aspects, but as there are no DNA vaccines licensed in the EU at the moment it will demand a company that is willing to put a lot of work and money into this.*

KHV diagnostics and vaccination against KHVD

S. M. Bergmann*, Meike Riechardt and Dieter Fichtner
Friedrich-Loeffler-Institut, Federal Research Institute for Animal Health, Institut of Infectology, NRLs for KHV, ISA and Mollusc Diseases, 17493 Greifswald-Insel Riems, Germany

Abstract: Koi herpesvirus (KHV, Cyprinid herpesvirus 3) infection in *Cyprinus carpio* represents a danger for the European cyprinid aquaculture. Now KHV infection is a notifiable disease in Germany, in EU and also by OIE.

One of the most important characteristics of herpesviruses is the development of a latent infection. Infected fish will become a livelong carrier of the virus and reactivate and spread KHV.
Recently, the strategy of the European Community has been to eradicate the disease by a good diagnostic and farm praxis. To cover this, a very sensitive and specific diagnostic tool which is accepted worldwide, should be the basis of virus detection.
We developed a nested PCR (Bergmann et al. 2006) in comparison to the published real-time PCR (Gilad et al. 2004). Both tests are able to find very low amount of viral DNA in fish tissues, swabs or leucocyte preparations. The advantage of real-time PCR is an enormous reduction of contamination risk in the laboratories or in the field. Additionally, real time PCR allows the relative and absolute quantification of the virus DNA content of the samples.

Vaccination against KHV is a possible means to fight against the disease and its spread. We tested the "KV3" vaccine from KoVax (Israel). Vaccinated fish did not develop KHV disease (KHVD) after a challenge with 10^4 $TCID_{50}$ / ml by immersion for 1 hour. Also contact controls kept together with immunised fish which were stressed by catching, did not develop any mortality. Only the very susceptible SPF carp (Netherlands) became sick and some of them died (20 to 30 %) within three weeks post challenge. SPF carp were also used as a positive control. These fish showed classical signs of KHVD and 95% died.

Keywords: KHV diagnostics, sensitivity, vaccination against KHV

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Minutes:
On the surface of KHV there are discovered app. 25 different glycoproteins. In latent infected fish we have found virions, proteins and genomes from KHV.
The development of the disease is dependent of the temperature. At 13°C you will not have any disease at all. At 18°C you will see the disease.
By antibody ELISA it has been shown that a lot of fish can be infected by KHV but they will not show the disease.
The most sensitive methods to find the virus are realtime PCR and nested PCR, but not the Bercovier PCR.
Methods for confirmation: sequence analyses, and in-situ hybridisation. When an infected fish is inserted into a pond it can take up to years before disease breaks out in the other fish. If the fish are stressed, the fish will often within 14 days be positive. After 21 days it may again be impossible to find the virus.
The stress can be transport, changes in food, temperature, new fish groups etc.
The detection safety after an outbreak is going down fast.
When vaccinated, the vaccine strain can be reactivated and the fish can carry both the vaccine virus and the wildtype virus.
The virus is attenuated and when reactivated it will immunise the other fish.

SESSION VI: Update from the CRL

Chair: N.J. Olesen

Presentation of new CRL-fish web page

Helle Frank Skall
National Veterinary Institute, Danish Technical University, Hangøvej 2, 8200 Århus N, Denmark

Minutes:
The CRL web-address has been changed to: WWW.crl-fish.eu. On the webpage you will find contact information. Furthermore, we will add several links e.g. to EU legislation and we will use the web-page to upload different reports e.g. the ringtest report and the report of the Annual Meeting, including minutes if no one objects – No objections. Anyone having usable links to be present on the webpage is encouraged to send them to Helle Frank Skall. Any ideas to improving the webpage are also welcomed.

Questions/comments:
Birgit Oidtmann: *It would be nice having the S&D questionnaires on the webpage. If presentations should be available from the web page they should be launched as PDF files*

The CRL Inter-laboratory Proficiency tests 2007

Niels Jørgen Olesen

The test was designed to assess the ability of participating laboratories to identify the non-exotic viruses:VHSV, IHNV, SVCV. The test was sent out in the end of September. Participants were asked to reply 8 week after at the 16th of November 2007. The results of the sequencing and genotyping were set 2 weeks after the first deadline. Each laboratory was given a code number. An un-coded version of the report was sent to the Commission.
Viruses were propagated in each their preferred cell line. At total CPE the supernatants were lyophilised with equal volumes of 20% w/v lactalbumin hydrolysate solution. Prior to sending out the test, the CRL tested 5 ampoules of each virus preparation by titration in 4 cell lines, to ascertain a satisfactory titre in the preferred cell line.
The lyophilization procedures did cause a decrease in virus titre. The identity of the virus in the 5 ampoules was also checked in ELISA, IFAT, PCR and serum neutralisation.
Results of testing the test:
All expected virus were found and no double infections or cross contamination were observed.
Test was sent according to national transport regulations
The test were sent in the end of September 2007
Titration after 3 and 6 months storage in the dark at 4°C and at room temperature at the CRL: Very stable independent of time and temperature!

The inter-laboratory test 2007 was prepared and checked according to accredited protocols (DS/EN ISO/IEC 17025 and *ILAC – G13:2000)*

Result of the test:

20 laboratories correctly identified all viruses in all ampoules. 11 laboratories did not isolate virus from ampoule II. 33 laboratories correctly identified the virus in ampoule I, IV and V.
25 laboratories correctly identified the virus in ampoule II. 34 laboratories correctly identified the virus in ampoule III. 4 laboratories found double infection in one or two of the ampoules.
24 laboratories tested the ampoules with ELISA. 25 laboratories tested the ampoules with IFAT. 13 laboratories tested the ampoules with neutralisation tests. 26 laboratories tested the ampoules with PCR. 16 laboratories carried out sequencing and/or genotyping of the isolates.

Minutes:
The test was designed to assess the ability of participating laboratories to identify the non-exotic viruses: VHSV; IHN; and SVCV. The inter-laboratory test 2007 was prepared and checked according to accredited protocols (DS/EN ISO/IEC 17025 and *ILAC – G13:2000*). Prior to sending out the test, the CRL tested 5 ampoules of each virus preparation by titration in 4 cell lines, to ascertain a satisfactory titre in the preferred cell line. The lyophilization procedures did cause a decrease in virus titre. The identity of the virus in the 5 ampoules was also checked by ELISA, IFAT, PCR and serum neutralisation. All expected virus were found and no double infections or cross contamination were observed. The test was sent in the end of September 2007. Participants were

asked to examine the content of each ampoule virologically according to the procedures described in the Commission Decision 183/2001/EC (Neutralisation test, ELISA, IFAT and/or PCR).
35 laboratories participated of which 20 correctly identified all viruses in all ampoules. 11 laboratories did not isolate virus from ampoule II containing VHSV close to detection level. The Proficiency test 2008 will be send out in autumn and concern identification of VHSV, SVCV and IHNV and other related viruses.

Ampoule	Virus	Correct ID	Wrong ID	No ID
I (Undiluted)	VHSV	33 / 35	1 / 35	1 / 35
II (Diluted 10^{-5})	VHSV	25 / 35	0 / 35	10 / 35
III	SVCV	34 / 35	1 / 35	0 /35
IV	IHNV	33 / 35	2 / 35	0 /35
V	VHSV	33 / 35	2 /35	0 / 35

Questions/comments:
Niels Jørgen Olesen: *How should we provide ringtests for the other listed diseases? Keith Way and CEFAS will organise a KHV ringtest this year. Maybe Norway would like to be involved in the arrangement of an ISA ringtest?*
Brit Hjeltness: *We have a heavy work load and will not promise anything.*
Niels Jørgen Olesen: *How should we make proficiency test for the exotic diseases? EHN might be included in the Proficiency test 2009 – no objections! A EUS proficiency test have to be performed in discussion with Bangkok and CEFAS*

A comparative sequence study launched in association with the proficiency test 2007

Søren Kahns,
Community Reference Laboratory for Fish Diseases, Technical University of Denmark, Aarhus, Denmark

Abstract: A comparative test of diagnostic procedures (Inter-Laboratory Proficiency Test for National Reference Laboratories for Fish Diseases) was provided to 35 laboratories in autumn 2007. The test contained five coded ampoules with either viral haemorrhagic septicaemia virus (VHSV), infectious haematopoietic necrosis virus (IHNV) or spring viraemia of carp virus (SVCV). In association with the test, we asked participating laboratories to submit full-length G-gene sequences of the provided piscine rhabdoviruses. The aim of this study was to compare inter-laboratory homologies of the full length G-gene sequences submitted. Such results are important for assessing how homogenous sequence data from the same source of virus are and how they can be used e.g. in molecular tracing.
Of 35 participating laboratories, 16 submitted sequences. An analysis of the genomic location from where sequences were obtained and the methods used for preparation of template and for performing the sequencing reactions, will be presented. For comparison of the homology of the submitted sequencing results, we focused on sequences from the VHSV isolate in ampoule 1 and 2 as these

ampoules contained identical virus isolates and therefore allowed us to compare both inter- and intra-laboratory sequences. A consensus sequences were build from all submitted sequences. About half of the submitted sequences contained one or more nucleotide mismatch as compared to the consensus sequence. The origin of these nucleotide conflicts will be discussed.

Minutes:
Of 35 participating laboratories, 16 submitted sequences. For Ampoule 1, 15 sequences were submitted. 5 participants submitted full-length G-gene sequences, 5 submitted partial G-gene sequences, 6 submitted partial N-gene sequences and 1 submitted a partial M-gene sequence. For ampoule 2, 13 sequences were submitted of which distributions were 4, 6, 5 and 0, respectively. 11 partial G-gene sequences from the SVC isolate of ampoule 3 were submitted. For the IHNV isolate of ampoule 4, 13 laboratories submitted sequences of which one was full length G-gene, 5 were partial G-gene and 7 was partial N-gene sequence. From Ampoule 5, 14 sequences were submitted of which 3 were full length G-gene, 5 contained partial G-gene sequence, 5 contained partial N-gene sequence whereas one laboratory submitted partial M-gene sequence.

The further study of submitted sequences was focused on ampoule 1 and 2 that contained identical isolates but at different titres. 6 labs did PCR directly on the isolate in ampoule I, while 8 performed the PCR after passage of the isolate on monolayered cell cultures. For ampoule II the numbers were 4 and 8 respectively. Concerning methods used for sequencing of the PCR fragments, 2 participants sequenced three independent clones whereas 11 participants directly sequenced the PCR fragment (ampoule 1). The corresponding numbers were 2 and 10 for ampoule 2.

All submitted sequences of ampoule 1 and 2 were used for creating a consensus sequence. Some mismatches were seen when. In ampoule 1 there were mismatches in 8 of the 15 submitted sequences as compared to the consensus sequence and in ampoule II mismatches were found in 6 out of 13 sequences. Most mismatches have risen because of improper reading of sequence/chromatograms. A study of the corresponding trace files revealed that 29 of a total of 35 mismatches simply appeared because the trace files were not properly read. Sequencing had only been performed in one direction and mismatches were located next to primer binding site or very far from the primer biding site. Such examples were not presented. Instead, two observed mismatches that could not be assigned to simple misreading were presented. The first example showed the presence of an A where other laboratories reported a T. This mismatch introduced a stop codon in the Glycoprotein amino acid sequence and was therefore not likely to produce a functional virus particle. As the A peak in the chromatogram also displayed the presence of a smaller T peak, the mismatch could possibly come from a putative compression within the sequence reaction. Sequencing in both directions could clarify the conflict.
Another mismatch was observed from two submitted sequences from the N-gene Sequences of ampoule 1 and 2 were identical within each lab but a mismatch was observed between the labs. In each of these cases, there was only one peak present in the respective chromatograms. The course of the mismatch was unclear. However, as the mismatch was located near the ends of the PCR fragment, the course of the mismatch could be because primers with different primer sequences had been used by the participants (this was suggested by David Stone).

It is difficult to conclude on the quality of submitted sequences as the majority of mismatches occurred because laboratories submitted sequence data of low quality - all mismatches' occurred where sequences were read only in one direction – illustrating that when sequencing mistakes occur and that it is necessary to sequence in both directions.. Another problem is that some participants

mentioned that the quality of submitted sequences in this study was lower compared to what they used in normal phylogenetic analyses.

Questions/comment:
David Stone: *The reason for the mismatch on the submitted N-gene sequence may be that they have not removed the primers so it is primer seq.*
Frank Berthe: *Is cloning a recommendation?*
Søren Kahns: *You can clone but only if you sequence at least 3 independent clones otherwise you risk having errors introduced by the polymerase. I do prefer sequencing of the PCR fragment in both directions.*
Frank Berthe: *You were lucky you had the chromatographs, so you could se the misreadings. Will this have influence on the database.*
Søren Kahns: *For recommendations for submitters on how sequence data should be prepared*
Debes Christiansen: *I don't recommend cloning, I would prefer direct sequencing of the PCR fragment. Cloning is too time consuming.*
David Stone: *I would also recommend direct sequencing of two independent PCR fragments.*
Mike Snow: *We have to be clear on what we want to use the sequencing exercise for. Some have used it for confirmation of the diagnosis only (and all have given the correct genotypes) and may have applied a lower level of stringency than they would normally use for preparation of data for eg phylogenetic analysis.*
Niels Jørgen Olesen: *We would like to repeat this test in the next proficiency test, now that people are aware of the pitfalls. This was started by the VHS outbreak in UK where we discussed how alike sequences has to be before you call it the same isolate.*
Mike Snow: *I think then you will have to restrict the work to e.g. only one isolate. Clear guidelines and methodology should be given*

Report from Year 2007 and work plan for 2008 and 2009

Niels Jørgen Olesen
Community Reference Laboratory for Fish Diseases, Technical University of Denmark, Aarhus, Denmark

DRAFT WORK PROGRAMME FOR THE COMMUNITY REFERENCE LABORATORY FOR FISH DISEASE, 2009

I. LEGAL FUNCTIONS AND DUTIES

The functions and duties of the Community Reference Laboratory are described in the Council Directive 2006/88/EF Annex VI part I

II. OBJECTIVES FOR THE PERIOD JANUARY - DECEMBER 2009

1. Organise and prepare for the 13th Annual Meeting for the National Reference Laboratories for Fish Diseases in 2009 (most likely to be held at the DTU.National Veterinary Institute Department in Copenhagen, Denmark in Mai 2009).
2. Produce a report from the Annual Meeting 2009.
3. Collect data on the fish disease situation in EU, including all the listed non-exotic fish diseases given in Council Directive 2006/88/EF Annex IV Part 2
4. Identify and characterise selected isolates of listed viruses (serological and genetic characterisation)
5. Production of antisera against selected isolates when necessary.
6. Assessment and standardisation of Real-time PCR tests for the diagnosis, identification and typing of the listed non-exotic fish diseases.

7. Develop, update and maintain the new EU Community Reference Laboratory for Fish Pathogens Database. A database created in order to collate all available information of isolates of listed fish pathogens including their origin, their sequences and their geographical coordinates
8. Update and maintain a library of isolates of Infectious salmon anaemia virus (ISAV), Viral Haemorrhagic Septicaemia virus (VHSV) and Infectious Haematopoietic Necrosis virus (IHNV), Spring Viraemia of Carp virus (SVCV) and Koi Herpes virus (KHV)
9. Preparation and standardisation of control reagents for use in PCR tests. Assessment of viral inactivation and viability of standard reagents for use as reference material in molecular tests.
10. Organise a workshop in the implementation of Council Directive 2006/88/EF (to be organised back to back with the 13th Annual Meeting).
11. Update the new webpage for the CRL, crl-fish.eu
12. Supply standard antisera and other reference reagents to the National Reference Laboratories in Member States.
13. Prepare the Annual Inter-laboratory Proficiency Test year 2009 for the National Reference Laboratories.
14. Collate and analyse information gained from the Inter-laboratory Proficiency Test
15. Facilitate and provide training in laboratory diagnosis.
16. Attending missions, international meetings and conferences. Missions will focus on accession countries and OIE reference laboratories on listed exotic and non-exotic fish diseases.

Minutes:
The function and duties of the CRL was described and suggestions for new duties in 2009 requested. Our objectives is a mixture of fixed goals and new topics. The recurrent topics are Annual Meetings and reports, webpage updating, Collection of data through the "survey and diagnosis", organising proficiency tests, virus characterisation etc.
New topics will be: Assessment and standardisation of Real-time PCR tests for the diagnosis, identification and typing of VHSV and IHNV. Develop, update and maintain the new EU Community Reference Laboratory for Fish Pathogens Database. Preparation and standardisation of control reagents for use in PCR tests. Assessment of viral inactivation and viability of standard reagents for use as reference material in molecular tests.
Other suggestions were: How to do serology in EU – and how to avoid use of live virus. How to assess targeted versus random sampling in a scientific set up? Can the CRL establish a DNA library control of listed pathogens?

Question/comments:
Birgit Oidtmann: *There is a great need for test validation and for assess the test sensitivity ands specificity of those tests currently in use – it is a too large job for the CRL – are anyone aware of how this could be funded*
Niels Jørgen Olesen: *It is important but it is difficult to find funding. Maybe the EU fisheries funds could be a possibility?*

Next meeting and end of 12th Annual Meeting

Niels Jørgen Olesen
Community Reference Laboratory for Fish Diseases, Technical University of Denmark, Aarhus, Denmark

Minutes/question/comments:

Niels Jørgen Olesen: *In principle there will not be a workshop back to back with the Annual Meeting in 2009. However an EU workshop could still be arranged. There have been request for a workshop on harmonisation of KHV diagnosis - such workshop could be arranged in EU context. Maybe Keith Way and CEFAS would arrange such a workshop?*
Keith Way: *I would prefer to visit such workshop!:-).*
Niels Jørgen Olesen: *Another theme for a workshop could be on QPCR for multiple purposes. However, it could be difficulty to arrange in practice.*
Franck Berthe: *QPCR is a useful technique that will be used as a kind of basic method. You have different variants of the method – sybr green/probes/etc. – It would be nice attending lectures rather than training*
Niels Jørgen Olesen: *The date of the next Annual Meeting will be: May 24-27 2009. The meeting will be over two nights as it covers a broad spectrum of diseases and at that time the different countries have implemented the new directive. Discussions on how implementation has occurred in the different countries could be organised as a kind of workshop. We suggest next meeting to be held at DTU.Vet in Copenhagen.*

The Meeting ended at 13 p.m. with a sandwich and a soft drink to go home.