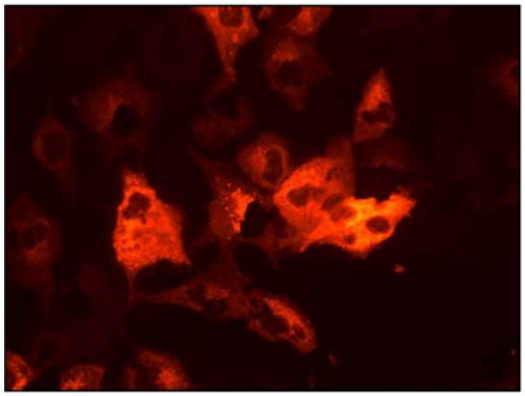
16th Annual Meeting of the National Reference Laboratories for Fish Diseases

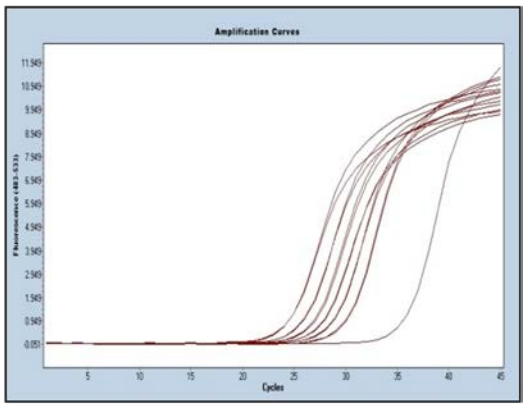
Aarhus, Denmark, May 30-31, 2012

Rainbow trout juveniles

Koi carps ready to be sold



IFAT for KHV antibody detection on CCB cells



Real time PCR output

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

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INTRODUCTION AND SHORT SUMMARY

The 16th Annual Meeting of the National Reference Laboratories for Fish Diseases was held in Aarhus, Denmark, 30-31 May 2012 at the premises of the Section for Fish Diseases at DTU Veterinary.
A total of 44 participants from 28 countries attended over the two day period. There were five sessions with a total of 35 presentations, 10 of which were given by invited speakers.

The scientific program of the Annual Meeting was wide and covered many different topics of current interest. The meeting was opened with the traditional session "Update on important fish diseases in Europe and their control", where participants from the Member States had the opportunity to present new findings from their home countries.
Initially an overview of the disease situation and surveillance in Europe 2011 was provided on the basis of the results obtained from the Survey & Diagnosis questionnaire.
Then the fish disease situation in Norway was presented; a detailed report in Norwegian is available at: http://www.vetinst.no/Publikasjoner/Fiskehelserapporten/Fiskehelserapporten-2011. An English version will be available at: http://www.vetinst.no/eng/Publications/Fish-Health-Report.
Recent interesting findings within the Italian territory and outcomes from the Conference of the National Italian Fish Pathologists (SIPI) were delivered as update on the fish disease situation in the Italian and Mediterranean aquaculture.
This was followed by the first extra-EU talk provided by a colleague from the Iranian National Reference Laboratory. This presentation described emerging issues in fish disease management in this country where the aquaculture production is increasing fast.
Afterwards two talks on KHV were presented, first the Polish experience with this disease and then an experimental study performed in Germany that aimed at describing KHV pathogenesis in common carp.
Then the importance of zoonoses in aquaculture was underlined with the presentation from the Netherlands describing *Vibrio vulinificus* outbreaks in aquaculture and their implication on human health.
This was followed by a presentation on the results of a molecular survey on isolates from VHS outbreaks in Italy.
Then a presentation on emerging fish disease in China was given.
Finally an update on non/low pathogenic HPR0 ISAV, a current issue for the salmon farming industry, was provided by the expert from the Faroe Islands.

This year the second session was dedicated to a mini-workshop on sampling procedures. Three presentations were delivered describing Standard Operating Procedures, sampling collections protocols and specific screening strategies available in UK and Denmark for notifiable diseases and in Italy for marine viral diseases. These presentations were followed by discussions on strategies.

The third session, on technical issues related to sampling and diagnosis, started by a presentation on health categorization of fish farms in Europe in 2011 based on answers from the questionnaire on surveillance and diagnostic.
A recently funded EFSA project focusing on risk ranking in aquaculture used the questionnaire on surveillance and diagnosis as basis for collating data on the progress of risk management in European aquaculture. The project was presented and the results obtained from the questionnaire were given.
Unfortunately it was not possible for the Commission to be present at the meeting but the scheduled presentation on Aquatic Animal Health Law was delivered as a hand out to the participants.

Finally the results of further studies on EUS and the causative agent *Aphanomyces invadans* were presented, describing infection trial models, available diagnostic techniques and a slide collection that soon will be available on the EURL website.

In the evening a banquet dinner was held at Restaurant "Aarhus Folkekøkken", located in downtown Aarhus.

The second and last day was opened by the session on scientific research update, this fourth session was divided into four parts.
The first part focused on Hirame Rhabdovirus, a pathogen detected for the first time in Europe. This virus was found in connection with increased mortality in farmed grayling (*Thymallus thymallus*). The virus was identified in cooperation between laboratories as Hirame Rhabdovirus by use of molecular and immunochemical techniques.
The second part was dedicated to Viral Encephalopathy and Retinopathy (VER), which represents one of the major sanitary constraints for Mediterranean marine aquaculture development. Initially, a presentation on the EPIZONE extension was given describing infection trials performed in order to highlight differences in pathogenicity related to genetically different strains. Then diagnostic techniques were addressed with the validation of a new qRT-PCR developed within the EPIZONE project as well. After that the importance of sharing data was underlined with reference to the newly established, but not yet published, NODA database in the www.fishpathogens.eu database; finally the phenotypic characterization of strains belonging to different genotypes of noda viruses was described.
The third part aimed at updating the meeting participants on KHV. Four presentations were delivered. The first focused on validation of a KHV antibody ELISA. The second highlighted results obtained within the EPIZONE short term mission that took place in Brest for the comparison of KHV antibody detection by various serological techniques. The last two gave the opportunity to the participants to be informed on the results of a specific KHV workshop that took place in Zwettl Austria; this workshop included several issues of this disease from the legislative point of view to diagnostics and control measures.
The fourth and last part was dedicated to update from the Northern part of Europe. Firstly a recent case where Atlantic salmon were affected by PD caused by SAV2 like virus, and not SAV3 as normally in Norway, was presented. Then information was given on MOLTRAQ, a new EMIDA project, describing its organization, the consortium and the expected outputs of the project that aim to increase knowledge on transmission, prevention and control of viral diseases in aquaculture
Finally a pilot experiment from Denmark was presented, describing the efficacy of percolation (the only legal method to sanitize wastewater from fish cutting plants in Denmark) for VHSV inactivation.

The annual meeting ended with the traditional update from the EURL. The results of the two proficiency tests sent out in 2011, PT1 and PT2, were presented. A report from the annual training course provided by the EURL in January/February 2012 was given and topics for next year's training course were discussed. The planned EURL activities in year 2012 were presented and proposals for the EURL work plan for 2013 were discussed.

Minutes from the meeting were taken by Drs. Helle Frank Skall, Susie Sommer Mikkelsen, Torsten Snogdal Boutrup and Niccolò Vendramin, and have afterwards been sent to the presenters for correcting in order to avoid misunderstandings. The minutes are included in this report together

with abstracts delivered by the presenters. Nicole Nicolajsen and Niccolò Vendramin assembled the report.

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 Nicole Nicolajsen, Søren Kahns, Niccolò Vendramin and Niels Jørgen Olesen, with the help from the rest of the fish disease section at DTU Veterinary.

The meeting next year is tentatively planned to be at the end of May 2013, more details will follow.

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

Aarhus, August 21th 2012

Niels Jørgen Olesen and Niccolò Vendramin

PROGRAM

Annual Meeting of the National Reference Laboratories

Wednesday 30 May 2012

8:45 – 9:15	Registration and welcome
9:15 – 09:30	Welcome Address and announcements – Niccolò Vendramin and Niels Jørgen Olesen
SESSION I:	Update on important fish diseases in Europe and their control
	Chair: Olga Haenen. Minutes: Susie Sommer Mikkelsen and Helle Frank Skall
9:30 – 9:50	Overview of disease situation in Europe – *Niels Jørgen Olesen*
9:50 – 10:10	Update on the disease situation in Norwegian fish farming – *Brit Hjeltnes*
10:10 – 10:30	Update on aquatic organisms disease situation in Italy – *Niccolò Vendramin*
10:30 – 10:50	The health and disease situation in aquaculture in Iran – *Mohaddes Ghasemi*
10:50 – 11:10	KHV experience in Poland – *Marek Matras*
11:10 – 11:35	Coffee break
11:35 –11:50	Pathogenesis of KHVD in common carp (*Cyprinus carpio*) – *Sven Bergmann*
11:50 – 12:05	*Vibrio vulnificus* outbreaks in Dutch eel and barramundi culture – *Olga Haenen*
12:05 – 12:20	Molecular epidemiology on VHS in Italy – *Anna Toffan*
12:20 – 12:35	Introduction of some emerging fish diseases in China – *Hong Liu*
12:35 – 12:50	Evidence of ISAV-HPR0 infection and replication in Atlantic salmon gill cells and the ASK cell line – *Debes Christiansen*
12:50 – 13:45	Lunch

SESSION II:	Mini workshop on "Sampling procedures"
	Chair: Torsten Snogdal Boutrup. Minutes – Niccolò Vendramin
13:45 - 14:05	An overview of sampling procedures in England and Wales – *Richard Gardiner*
14:05 - 14:25	Sampling procedures for surveillance and diagnosis of fish diseases in Denmark – *Henrik Korsholm*
14:25 - 14:45	Clinical inspection and sampling in fish farming: practical experiences and guidelines from the Mediterranean point of view – *Niccolò Vendramin*
14:55 – 15:30	Round table discussions on sampling procedures in the respective countries
15:30 – 15.50	Coffee break
SESSION III:	Technical issues related to sampling and diagnosis
	Chair: Anna Toffan. Minutes: Helle Frank Skall
15:50 – 16:10	Health categorisation of fish farms in Europe – *Niels Jørgen Olesen*
16:10 – 16:40	Risk categorisation for Aquatic Animal Health Surveillance. Presentation of the new EFSA project and outcome of the questionnaire spring 2012 – *Britt Bang Jensen*
16:40 – 17:00	The Animal Health Law from an aquatic perspective – *Sigrid Cabot*
17:00 – 17:20	Update on Epizootic ulcerative syndrome (EUS) diagnostics, infection trials and online slide collection – *Torsten Snogdal Boutrup*
19:00 –	BANQUET DINNER at Aarhus Folkekøkken

Thursday 31 May

SESSION IV	Scientific research update
	Chair: Brit Hjeltnes. Minutes: Torsten Snogdal Boutrup
9:00 – 9:15	First detection of Hirame Rhabdovirus (HIRRV) in Europe, part I – *Ewa Borzym*
9:15 – 9:35	First detection of Hirame Rhabdovirus (HIRRV) in Europe, part II – *Laurent Bigarré*
9:35 – 9:55	Outcome on Epizone extension on VER/VNN: Infection trials, pathogenicity and pathology of various VER/VNN isolates – *Niccolò Vendramin*
9:55– 10:15	Outcome on Epizone extension on VER/VNN: Diagnostics, proficiency test and qRT-PCR validation – *Laurent Bigarré*
10:15 – 10:25	Fishpathogens.eu/Noda: a new database for betanodavirus targeted research – *Valentina Panzarin*
10:25 – 10:45	Genotype-phenotype relations among parental betanodavirus genotypes and reassortant strains – *Valentina Panzarin*
10:45 – 11:00	*Coffee break*
11:00 – 11:20	Validation of an antibody ELISA for indirect detection of KHV – *Sven Bergmann*
11:20 – 11:50	Round table discussion on KHV diagnosis: Training session on KHV serological methods – *Thiérry Morin* Zwettl, Austria KHV workshop output – *Oskar Schachner & Sven Bergmann*
11:50– 12:10	Pancreas disease caused by SAV2-like virus in Atlantic salmon in Norway – *Irene Ørpetveit*
12:10 – 12:25	Molecular tracing of viral pathogens in aquaculture (MOLTRAQ) a new EMIDA project – *Britt Bang Jensen*
12:25 – 12:40	Inactivation of VHSV by percolation and salt – *Helle Frank Skall*
12:40 – 13:30	Lunch

SESSION V:	Update from the EURL
	Chair: Niels Jørgen Olesen. Minutes: Niccolò Vendramin
13:30 – 13:45	EURL activities in 2011 – *Niels Jørgen Olesen*
13:45 – 14:05	EURL workplan for 2012; Ideas and plans for 2013 – *Niels Jørgen Olesen*
14:05 – 14:25	EURL Training courses. Report, topics for future courses – *Helle Frank Skall*
14:25 – 15:00	Results of the proficiency test, PT1 and PT2, 2011 – *Niels Jørgen Olesen*
15:00 – 15:15	Next meeting and end of 16th Annual Meeting - *Niels Jørgen Olesen*
15:15	Coffee, cake and goodbyes

Welcome

Niels Jørgen Olesen wished everyone welcome to the 16th Annual Meeting with apologizes for the fact that some colleagues did not receive the invitation in time. Scientists from 28 countries as well as Ph.D. students from the EURL are attending the meeting. After information on technical and practical issues, the situation in Aarhus where the Fish Disease Section has to move to Copenhagen in 2013 was described. Due to these plans 2 colleagues went to other companies as they did not have the possibility to move. Dr. Søren Kahns has left his job as coordinator of the EURL and his work has been taken over by Dr. Niccolò Vendramin while the job of Dr. Søren Peter Jonstrup was taken over by Dr. Susie Sommer Mikkelsen. There are now only fish groups left in Aarhus, as 4 other sections have already been transferred to Copenhagen or moved to other institutes.

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

Chair: Dr. Olga Haenen
Minutes: Drs. Susie Sommer Mikkelsen and Helle Frank Skall

OVERVIEW OF THE DISEASE SITUATION AND SURVEILLANCE IN EUROPE IN 2011

Niels Jørgen Olesen & Nicole Nicolajsen
National Veterinary Institute, EU Reference Laboratory for Fish Diseases, Technical University of Denmark

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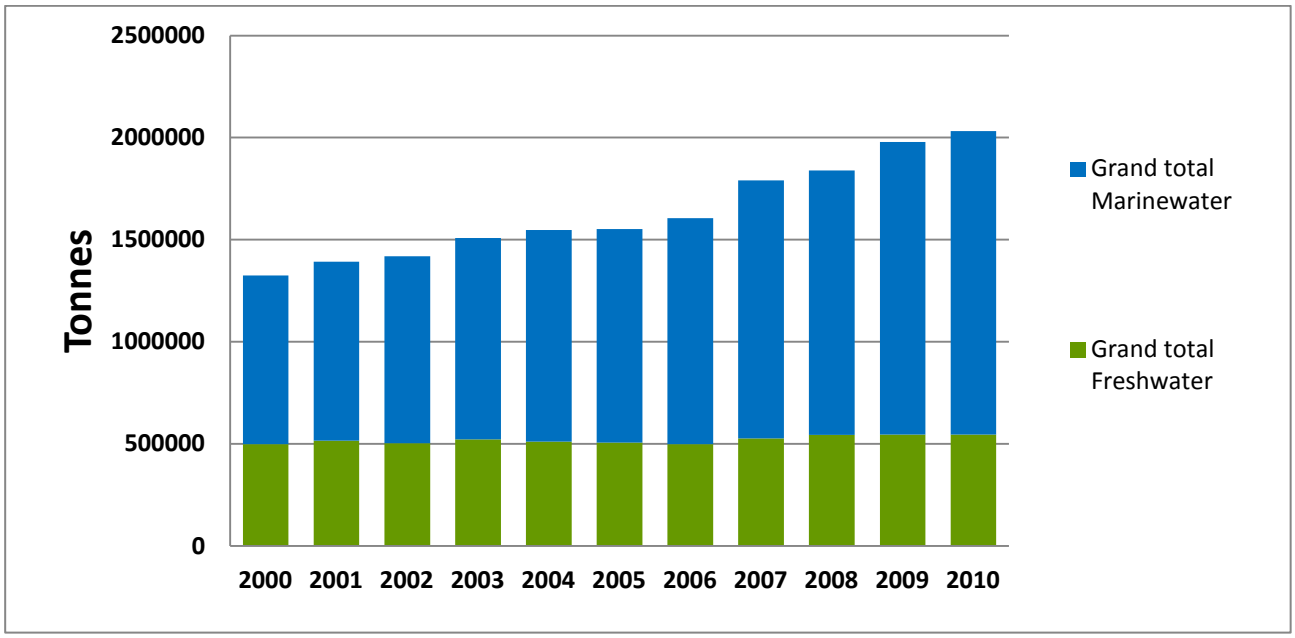
Abstract:
The Questionnaire on Surveillance and Diagnosis (S&D) which is collated annually is the only comprehensive overview of the disease situation in aquaculture in Europe. The information has been made available on the EURL web site (www.eurl-fish.eu), where all raw data can be obtained. The S&D have evolved over the years, for 2011 it comprises 4 parts:
General data on production type and size, health categorization of fish farms according to Council Directive 2006/88/EC, and information on national surveillance programmes.
Epidemiological data on the disease situation in each Member State with focus on the listed diseases but also including other diseases of interest are presented.
Laboratory data from the NRLs and other laboratories, including number of samples examined, diagnoses of fish diseases made.
A new part was included for 2011 as a deliverable for the EFSA project CFP/EFSA/AHAW/2011/03: Risk categorisation for Aquatic Animal Health Surveillance: Status on implementation of the new fish health surveillance legislation.

The data on the European aquaculture production were obtained from the FIGIS database. Unfortunately this database does not include information on the number and size of fish farms, which are epidemiologically important data. The production in 2010 is almost the same as in 2009 and has for the sixth time in row raised from the previous year and has now passed 2 million ton (Figur 1). Data from 2011 is not yet available. The farm sizes vary a lot between countries, e.g. the majority of farms in Germany produced < 5 tonnes, and for Spain the number of farms producing < 5 tonnes, 5-100 tonnes and > 100 tonnes is nearly equal.
The Atlantic salmon production has increased significantly while the rainbow trout production slightly decreased in Europe in 2010. The carp production is still mainly in the Eastern part of Continental Europe and at the same level as the year before. The production of sea bream decreased while the sea bass production increased in the Mediterranean countries. Among other fish species of interest are pike-perch (472t), eel (6845t), sturgeon (3545t), cod (22558t), turbot (8348t), and halibut (1821t). Unfortunately none of these species have observed the foreseen significant increase in production.

Data on the health categorisation of fish farms will be given in a later presentation.

Concerning the epidemiological data, obviously, there is still a severe underreporting of VHS and IHN in many countries. For VHS the infection status in only known for 33% of the farms, for IHN the situation is known in 37% of the farms. While for KHV the disease situation is unknown on 95.4% of the farms! For farms producing Atlantic salmon and categorised for ISA, the infection

status for ISA is known for 49% of the farms. The findings of ISA virus HPR0 pose some problems regarding the health categorisation of salmon farms.
Many countries have surveillance programmes for SVC (16 of 35 countries), BKD (14 of 35 countries), IPN (18 of 35 countries) and *Gyrodactylus salaris* (8 of 35 countries), for which they are seeking "additional guaranties" according to §42 in CD 2006/88/EC. The number of farms in the programmes varies from very few farms to many farms.
There are very large differences between countries on how many samples are tested on cell cultures, ranging from < 100 to several thousands. PCR is coming up in many countries, and the large number of PCR-tests conducted in some countries mostly reflects the KHV and ISA testing.



Total production of fish in aquaculture in Europe 2000 to 2010 (http://www.fao.org/figis)

Minutes:
The 16th edition of the Annual Survey and Diagnosis questionnaire (S&D) was presented. A paper version of the complete dataset is included in the folder under part 8 and will be available on the EURL webpage including production data from FIGIS (FAO production tool).The questionnaire structure is composed by:

1. General data
2. Epidemiological data
3. Laboratory data, NRL and regional laboratories
4. New and only for 2011, EFSA project: Status on implementation of the new fish health surveillance legislation.

This is the only comprehensive data collection for the fish diseases situation in Europe, and is therefore important.

Data quality appears to be quite different from one country to another.

FIGIS provided the production data up to 2010 as no data are yet available for 2011. Marine water production is increased, freshwater production is stable. Considering the most important species reared: the Atlantic salmon production increased in 2009 and 2010 while the productions of seabass, seabream and trout have not increased much in recent years. One note on sea bass and bream production, a recent paper (Trujillo P, Piroddi C, Jacquet J (2012) Fish Farms at Sea: The Ground Truth from Google Earth. PLoS ONE 7(2): e30546. doi:10.1371/journal.pone.0030546) shows that a significant percentage of sea farms are not declared/registered so maybe, for the Mediterranean basin the production is much larger than officially recognised. Eel production is decreasing due to the decrease in wild elver population. Pike-perch, considered a new candidate species, demonstrated only small increase. No signs of increase for sturgeon while the production of Atlantic cod increased 2008-2010.

The distribution of size of farms among countries demonstrated to be uneven: while in Denmark there are many large farms, many small farms are present in Germany for example. Fish species distributed differently among different countries. In Greece, seabream is the main species produced, and rainbow trout in DK. Concerning fish health management of non-listed diseases: 21 countries have an active SVC program, 17 countries (especially salmon farming countries) a BKD program, 22 countries have an IPN program (Sweden is working on declaring freedom of IPN). Gyrodactylus is primarily addressed from northern country (8 of 35 countries).

Referring to notifiable diseases, only 75 farms are considered as VHS positive, 61 as IHN positive, 53 as KHVD positive. One new ISA case in 2011. The number of farms in category 3 is extremely high. Concerning non-listed diseases, Slovakia declared to have problems with SVC, while VER/VNN is considered a problem in the Mediterranean Basin, as well as lactoccocosis in trout farming. The general picture shows a decrease in the number of infected farms in Northern Europe, while the reports from the Mediterranean countries and the Continental countries appear to be incomplete.

Concluding, the results of the questionnaire seems to be uneven among different countries and sometimes the questions appeared to cause some confusion, for example there is still an issue in discriminating between authorized and registered fish farms.

Questions:
Brit Hjeltnes: What is the IHN situation in Northern part of Europe?

Niels Jørgen Olesen: there seems to be no changes and IHN do not cause big problems, but we received news that now there is an emerging problem in Canada.

Brit Hjeltnes. There seems to be increasing problems in Russia with IHN.

Vlasta Jencic: This disease represents a problem in Slovenia where it is endemic.

UPDATE ON THE DISEASE SITUATION IN NORVEGIAN FISH FARMING

Brit Hjeltnes
Norwegian Veterinary Institute, Fish and Shellfish Health

Abstract:

For the complete report on fish disease situation in Norway please refer to:

http://www.vetinst.no/Publikasjoner/Fiskehelserapporten/Fiskehelserapporten-2011

Minutes:

An update of disease situation in Norway is provided. The production is quite stable, in EU 700.000 tonnes of finfish were produced, 1 million tonnes in Norway. The yearly report on salmonidae fish and marine fish is uploaded on website (will soon be available in English at http://www.vetinst.no/eng/Publications/Fish-Health-Report).

ISA: 1 site reported in 2011, 7 in 2010, 9 in 2009, 2 weeks ago there was a new outbreak of ISA in brood fish, in the southern part of the country.

PD: Increasing, in 2009 75 reports, 2010 88 reports and 2011 89 reports, mortality has increased possibly because of emergence of a new variant.

HSMI: After a slow decrease from 2008 to 2010 there was a slight increase in 2011.

CMS: 74 cases detected in 2011, it is a problem for big fish (size 3-4 kg), the cumulative mortality rarely reach 20%. In 2011 a slight increase of outbreaks was observed. CMS is associated with a totivirus Piscine Myocarditis virus (PMCV).

IPN: This disease is causing serious problems, mostly in Atlantic salmon. In house strains are causing problems, circling in hatcheries. The possibility to clean up whole farms with success has been demonstrated and there is the need to perform 100% eradication. Infection in sea water seems to be of minor importance. Breeding selection program based on QTL are providing interesting results.

Salmon louse: A major fish health problem is salmon lice. Synchronised treatment in spring is now performed. An increasing level of resistance to anti-lice chemotherapeutants is observed. The more crowded areas suffer of a higher number of lice.

Bacterial infections: There have been no reports on *Renibacterium salmoninarum* and *Aeromonas salmonicida*. Reports are available on *Moritella*, *Tenacibaculum*, *Flavobacterium* and *Pseudomonas fluorescens*.

Marine fish: Problems with vibriosis and atypical furunculosis.

Cleaner fish used for biological control of salmon lice are currently caught in the wild. There are issues about their health status, problems detected are vibriosis and atypical furunculosis.

Questions:

Niels Jørgen Olesen: Are cleaner fish a problem by being a vector for salmon infections?

Brit Hjeltnes: This has not been proven but they can be mechanic vectors. There are discussions if they can be vectors for PD.

UPDATE ON FISH DISEASE SITUATION IN ITALY

Niccolò Vendramin[1] & Anna Toffan[2]
[1]*National Veterinary Institute, EU Reference Laboratory for Fish Diseases, Technical University of Denmark*
[2] *Division of Comparative Biomedical Sciences. Fish Virology Department, Istituto Zooprofilattico Sperimentale delle Venezie, Italy*

Abstract:
In this presentation we provide a general overview of the aquatic animal health issues related to the aquaculture sector and wild environment in Italy in 2011.
Considering saltwater species European sea bass (*Dicentrarchus labrax*) and Gilthead sea bream (*Sparus aurata*) are still the most widely farmed species, nevertheless some "new" candidates are employed often.
The first two species represents more than 95% of the total production while the remaining is obtained by different promising candidates species, including sole (*Solea solea*), meagre (*Argyrosomus regius*), northern blue fin tuna (*Thunnus thynnus*) and amberjack (*Seriola dumerilii*) for which some breeding/reproduction plans have been attempted by some hatcheries.
The farming of sea bass and sea bream is affected by the presence of several important diseases.
Firstly considering bacterial diseases, marine flexibacteriosis caused by *Tenacibaculum maritimum*; vibriosis caused by *Listonella anguillarum* and pasteurellosis caused by *Photobacterium damsela subsp. piscicida* are considered the major bacterial diseases even though for these pathogens exists chemicals and for some of them efficacious vaccines.
Considering viral diseases viral encephalopathy and retinopathy (VER) still plays a key role in some areas where, mortalities ranging from 30-40% can be observed in sea bass rearing units.
Nevertheless in recent years, serious epizootics affecting sea bream larvae, previously considered a resistant species, have been reported. Finally the appearance of the clinical disease has been recorded also in the wild.
Lymphocystis disease (LCD) represents an important disease not for its pathogenicity but for the interferences with strict production plans of farm.
Finally, considering parasites, plus to "old known" protozoans (*Cryptocarion irritans and Amyloodinium ocellatum*) and gill flukes (*Diplectanum aequans* and *Sparicotyle chrysophrii*) mainly present in inland farms (earth ponds and concrete tanks based); isopods crustacea (*Ceratotoa, Anylocra)* and Enteromyxidiosis (*Enteromyxum leei*) represent a treat for offshore cages.
Among the dismetabolic unknown aetiology diseases, Winter Syndrome affects mainly 1-year-old sea bream causing mortalities ranging from 5-15%. The therapeutic treatment addresses great attention to the diet, especially at the end of summer and the approaching winter season.
Considering trout farming which is a well developed industry two main pathological scenarios are present. Farms with low water temperature (mainly located in the mountains) can be more affected and damaged by viral diseases (i.e. viral haemorrhagic septicaemia VHS) which is one of the most important problems.
Rainbow trout fry syndrome (RTFS) is responsible for significant mortalities in salmonids, during juvenile stages, particularly if not treated promptly.
Another bacterial problem that seems to be re-emerging is Enteric Red Mouth (*Yersinia ruckerii*).
Finally considering freshwater wild environment a mortality outbreak in eels is presented.

Minutes:
In Italy there are around 550 mainly small sized fish farms. Two major industries are developed, freshwater production of mainly rainbow trout and saltwater production of sea bass and sea bream. Work is in progress for registering and authorizing all the farms at a national level, on the other hand farm categorization will be done by regional governments. The Italian National Farmer Association (API) signed agreements with NRL and some regional government for enhancing a screening program to be used as starting point for categorization.
In the trout farming sector two different scenarios are present; in farms located in the mountains with cold water VHS and IHN infection can result in huge losses, while in the valley farms with warmer water may suffer less due to viral problems and more due to bacterial diseases.
IPN arise the need for a surveillance tool (PCR based).
About marine fish farms: bacterial diseases are not often detected in the laboratory maybe because they are treated directly at farms after detection. The 3 most important bacterial diseases are vibriosis, photobacteriosis and marine flexibacteriosis.
There are major problems due to the viral diseases VER/VNN and lymphocystis (LCD). Concerning parasites gill flukes, myxidiosis and crustacean isopoda are the most important ones.
In summer 2011, a mortality outbreak occurred in Garda lake affecting mainly eels. Virological and bacteriological test detected HVA, *Aeromonas sobria* and *A. hydrophila*. In autumn an outbreak in wild sea fish occurred in Santa Maria di Leuca area affecting mainly groupers, this was associated with VER/VNN.
One interesting case is presented associating drop of production in rotifers culture with Birnavirus-like particles (TEM detected).

Questions:
Niels Lorenzen: What about the use of antibiotics? Do you have to register them for use? In fish the use of antibiotics is not popular, how is this considered in Italy?

Niccolò Vendramin: There are some antibiotics as well as vaccine, they must be prescribed by a vet and registered, and in Italy also there are some concerns about the antibiotic resistance. One issue with vaccine is the cost in terms of labour and money.

THE HEALTH AND DISEASE SITUATION IN AQUACULTURE IN IRAN

Mohaddes Ghasemi
Iranian fisheries research organization, Fish health and disease

Abstract:
Aquaculture was developed in Iran in the early 1970's with technical assistance from the former Soviet Union for the artificial propagation of sturgeon (Acipenseridae) fingerlings for restocking the Caspian Sea. The Caspian Sea is the main habitat of some important and valuable species like sturgeons which produce about 90% of the world's caviar, also Caspian salmon (*Salmo trutta caspius*), Caspian white fish (*Rutilus frisii kutum*) etc. Since then, propagation of several fish species such as Caspian white fish, Caspian salmon, bream (*Abramis brama*), pike-perch (*Stizostedion lucioperca*), rainbow trout (*Oncorhynchus mykiss*) and four cyprinid species is included in a current national plan undertaken by the Iranian fisheries organization. Then aquaculture was improved in ponds (cyprinids), raceway (rainbow trout) and cage (sea bream, sea bass and newly Caspian salmon and sturgeon, *Huso huso*). In the recent decade, shrimp farming has been developed in the Persian Gulf region with the total production of 6000 tons per year. Also several new species were initially introduced to Iranian aquaculture with the aim of biodiversity such as Indian carps and tilapia. Despite the rapid development of these industries they are faced with several defects such as improper health management, poor environmental condition and risk of exotic diseases that can be imported by eyed eggs, brood stocks and ornamental species such as IPN, IHN, VER/VNN, streptococcosis and white spot syndrome that have become a part of endemic diseases of aquaculture in Iran.

Minutes

Aquaculture activities began in Iran in early 1970's with the technical assistance from Soviet Union for artificial propagation of sturgeon, nowadays this sector has expanded into many species. In the future there will be development for tilapia, sea bass, bunni (*Barbus sharpeyi*) and *Peneaus vannamei*.

There is a plan to increase by 2020 the total fishery output and aquaculture. The cold water fish production was 73.642 tonnes in 2010 and there is hope for a large increase in 2014. Different problems affect the aquaculture development, of these infections are well represented. IHN was firstly identified in rainbow farms in 2002 and many farms are suspected to be infected. Infection with VHS is suspected in trout but not confirmed completely. IPN infection is present in a number of farmed trout. Other diseases that affect trout farming are cytophaga/flexibacter like bacteria and saprolegniosis remains a problem in trout hatcheries.

In warm water fish: Flavobacteria are frequently reported from silver carp farming in winter season associated with *Aeromonas* spp. and poor water condition. Many protozoan and metazoans have been reported from different species both wild and farmed fish. Despite high value very few data are available on sturgeon farming, even though it seems that there are some issues related to water quality. VER/VNN demonstrated to be a problem in golden grey mullet, an outbreak occurred in 2002 with high mortality. Shrimp farming was affected by white spot disease in 2002.

Nutritional health: quality of fish feed is very variable among farms and can cause disease. There are gaps of knowledge about outbreaks, which means one or more strategies are important to combat diseases.

Questions:
Olga Haenen: Do you culture eel?

Mohaddes Ghasemi: No, not in aquaculture, and we haven't much data on it.

KHV EXPERIENCE IN POLAND

Marek Matras[1], Magdalena Stachnik, Ewa Borzym, Joanna Maj
[1] *National Veterinary Research Institute, Department of Fish Diseases, Pulawy Poland*

Abstract:
Koi herpesvirus (KHV) infection causes severe mortalities in carp (*Cyprinus carpio*) and koi carp (*Cyprinus carpio koi*) of all ages. Since the first reports of KHVD in Israel and USA the disease has spread in many carp and koi carp production areas. In 2004 the first outbreaks of the disease were diagnosed in Poland. In these occasions the diagnosis were performed in Department of Fish Diseases of National Veterinary Research Institute using the PCR method published by Gilad et *al*. 2002.
Since 2005, KHV DNA identification was made with PCR method using at least two of the following protocols i.e. Gilad, Grey, and Bercovier (Bercovier et *al*. 2005).
During KHV PCR based survey in Poland in 2004 - 2011 the infection was detected in many farms both in clinical and asymptomatic cases in different fish species.
Investigations carried out on specimens belonging to grass carp (*Ctenopharyngodon idella*), big head carp (*Hypophtalmichthys nobilis*), crucian carp (*Carassius carassius*) and tench (*Tinca tinca*) kept together in ponds with KHV infected carps yielded positive results.
In order to evaluate the potential carrier role of these fish species that share the same habitats with carps an experimental infection has been performed.
Specimens belonging to grass carp, prussian carp, tench and roach were infected with KHV, then analysis performed on collected samples highlighted the KHV DNA presence at different time points.
Moreover, serological techniques for the detection of specific antibodies have been tested at different time points after detecting the clinical symptoms of KHV in naturally infected carps. Noteworthy specific antibodies could be detected firstly 2 weeks after first symptoms of KHVD with water temperature between 18°C to 25°C.
The application of serological methods can be useful in estimating the health status of carp regarding KHV disease.

Minutes:
KHV was detected for the first time in Poland in 2004. Factors enhancing risk of introduction of KHV are related to the growing trade and import of live koi carp, stocking koi together with carps for consumption and lack of monitoring programs. 23 farms were investigated in 2007 and 2008: in 2007 KHV was detected in 6 farms while 17 tested negative, in 2008 13 tested positive and 10 negative. The disease affected farms connected to the same hydrographic basin as well as independent farms. One explanation for the spread of the infection could be the practice of sharing overwintering ponds between farmers.
KHV has been detected also in other fish species often stocked together with carp (i.e. grass carp, bighead carp, crucian carp, prussian carp, tench and brown bullhead). Three months after a big outbreak in one carp (*Cyprinus carpio*) farm resulting in high mortality the infection was detected in other species sharing the same rearing ponds with common carp. Four samples belonging to tench, brown bullhead and prussian carp tested positive while 27 samples tested negative. No positive samples were detected after 4 and 10 months.

Naïve fish belonging to these species were experimentally infected and the put into cohabitation with naïve common carps. After 7 days carps suffered 90% mortality. Samples collected at week 2, 3 and 4 tested PCR positive. Another infection trial in common carps was performed, first symptoms were detected after 2 weeks, blood for serological investigation was collected every week, 30% of samples tested positive after 2 weeks, 100% after 7 weeks.

In Poland it is difficult to make effective eradication of the diseases because of the interconnected hydrographic basins. Carrier species play a role in infection maintenance. Application of serological methods can be useful for estimation of the spread of infection in reared and wild populations.

Questions:
Vlasta Jencic: When you test carp is it because they are ill or for surveillance purpose?

Marek Matras: They are sent both for surveillance and from suspicious farms.

Olga Haenen: Any country where KHV vaccine is registered in Europe?

All participants: No

Niels Jørgen Olesen: How did you classify negative and positive farms?

Marek Matras: By PCR tests.

Niels Jørgen Olesen: Do you suspect they are free or just testing PCR negative?

Marek Matras: It is hard to say because of lack of data as farmers don't send in samples. Farmers are only interested in getting tested if there have problems and they provide clinical samples.

PATHOGENESIS OF KOI HERPESVIRUS DISEASE (KHVD) IN CARP (*Cyprinus carpio*)

Sven M. Bergmann[1], Sean Monaghan[2], Alexandra Adams[2] and Dieter Fichtner[2]
[1]*Friedrich-Loeffler-Institut (FLI), Federal Research Institute for Animal Health, Insel Riems, Germany*
[2]*University of Stirling, Department for Aquaculture*

Abstract:
Koi Herpesvirus (KHV) diagnostics is mainly limited to virological assays. Due to a latent/ persistent phase of the infection, a very weak virus load between five and 10 genomic particles/ml may be found in tissues, smears and leukocytes.
A virus reactivation in fish is possible when a stress situation occurs, e.g. seasonal temperature and hormonal changes, netting for transportation or a rapid food change.
Then KHV is replicated in fish to a reasonable and detectable level between 200 and 1.000 genomic particles measured by quantitative real-time PCR (qPCR) in at least 50 to 70% of the collected carp (*Cyprinus carpio*) samples. When samples are collected directly after catch, often PCR results are false-negative.
At present KHV disease (KHVD) pathogenesis is not fully understood. Those gaps we've tried to close by inclusion of different virological-molecular biological assays with and without induced stress situations and the host immune response by measuring of production of specific antibodies by serum neutralization assay (SNT) and antibody ELISA.
In investigations fish were infected via immersion by a virulent KHV isolate from England (K. Way, CEFAS, isolate D 182). Over a period of 4 month samples were taken lethally and non-lethally for early pathogenesis (1 hour to 10 days post infection) with induced KHVD and for late pathogenesis (7days to 4 month post infection) after surviving and recovering from KHVD.
The developed tools allow the detection but also the exclusion a KHV infection which also can occur silently without any clinical signs.

Minutes:
The disease is found in common carp (including koi) *Cyprinus carpio* only. The virus is characterized by a big genome approximately 297 kbp. KHVD can cause high mortality. Necropsy findings are bleeding in scale, increase in mucous production, necrosis in the gills and enophthalmus. The very early pathogenesis, starting 1 h after infection, was investigated. One fish per hour from each tank was collected for the first 8 hours and then 1 fish each day. Two disease patterns occurred, in one case peracute disease with 100 % mortality within 4 days with no serological reaction took place, in the other case acute disease with 99 % mortality within 11 days 1 survivor.

A huge decrease of the virus occurred in the gills and the skin within the first 2-4 hours in the group with peracute mortality. In the group with acute mortality the drop of virus was not seen.
A huge virus concentration attaches to skin and gill mucus followed by a decrease of virus concentration in the skin and gills (suggesting no replication). After 2 h post infection KHV starts to replicate in gut tissues.

Late pathogenesis.
1 tank with negative controls and 4 tanks with infected fish (infected together and distributed in 4 tanks). Fish were fin clipped to be able to identify each fish. Within 4 weeks all fish tested positive by real time PCR. After 3 months only a very weak concentration of virus was found in the fish.
By netting the fish the virus was reactivated within few days but the amount of KHV went down again within 10 days. The antibody levels were much lower in fish which were in contact with infected fish than in directly infected fish.
KHV is detectable by non-lethal sampling over a period of 4-6 months. Not all fish samples give positive signals. Stress induced KHV reactivation is detectable by real time PCR.

Questions:
Torsten Snogdal Boutrup: What is the difference between the netting for sampling the fish and the netting for stressing fish.

Sven Bergmann: We netted the fish every day so the fish got used to it and did not get stressed.

Niels Jørgen Olesen: Is it a PCR for the transcipt or the genome.

Sven Bergmann: It is for the transcript.

Niels Jørgen Olesen: What about the antibody response, can you find differences between neutralizing antibodies and the whole antibody population?

Sven Bergmann: The neutralizing antibodies dropped down, but the ELISA stayed positive.

Olga Haenen: Did you look on variance between different strains.

Sven Bergmann: I will talk about that tomorrow.

VIBRIO VULNIFICUS OUTBREAKS IN DUTCH EEL AND BARRAMUNDI CULTURE

Olga Haenen[1], Evert van Zanten[2], Ineke Roozenburg[1], Marc Engelsma[1], Angelique Dijkstra[3], Michal Voorbergen-Laarman[1], Lieke Möller[2]
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[2]*Laboratory for Infectious Diseases, Groningen, the Netherlands*
[3]*Sanquin Blood Supply, the Netherlands*

Abstract:
Vibrio vulnificus is a zoonotic bacterial pathogen of fish. In this study, from 1996-2011, *Vibrio vulnificus* was isolated 27 times in the Netherlands, related to aquaculture businesses. Twenty four of the *V.vulnificus* isolates were related to serious disease outbreaks in indoor eel farms, one to a zoonotic infection of an eel farmer (necrotic fasciitis), and two to serious outbreaks in a barramundi farm. Clinical pathology showed severe lesions in fish. The 27 bacterial strains resulted in 10 Diversilab® types. Antimicrobial resistance patterns were diverse amongst biotypes and molecular types of *V.vulnificus* strains. In addition, some strains of the eel farm with the zoonotic case showed multi-resistance to quinolones, probably acquired resistance after prolonged use of flumequine bath and other antibiotics.
Risk assessment and prevention are needed to protect fish farmers and fish processors against *V.vulnificus* infections, particularly from eels, but also from spiny brackish water and marine fish. Furthermore, the medical branch should be informed about the potential of severe zoonotic infections of *V.vulnificus* as a human health hazard for risk individuals in our geographical area.

Minutes:
This work was done together with the human medicine as this is a zoonotic disease. *V. vulnifucus* is a Gram negative, facultative aerobic bacterium, it is a zoonotic invasive fish pathogen. This bacterium occurs in sea water (biotype 1), sediment, crabs, prawns, sewage, shellfish (biotype 2). In man biotype I, II and III have been described. The infection can occur via water and in immune-compromised patients the infection can result in disease.
An eel farmer got his hand infected with biotype II. Firstly occurred a full sepsis and the man was treated with antibiotic and surgery.
Since 1996 *V. vulnificus* was detected in 220 diseased eels and 4 barramundi. Bacteriology is performed using blood agar and sometime marine agar. Typing isolates relied on classical methods (biochemistry) and molecular methods (16S rRNA, VITEK 2® system (BioMérieux)).
12 of 24 isolates were from outbreaks and occurred in 1 eel farm from 2005-2008 (zoonosis in eel farmer in 2007). Preliminary results demonstrated 20 strains belonging to biotype I, and 4 to biotype II. From 1 barramundi farm, 2 isolates of biotype I was identified in 2007 and 2009.
Antibiograms were diverse amongst biotypes and molecular types. Most of the strains were resistant to cefoxitin. All biotype 2 strains belonged to the same Diversilab® type.
V. vulnificus is even found in Doctorfish in wellnes centers in UK (Verner-Jeffreys et al. (2012). Zoonotic disease pathogens in fish used for pedicure. Emerging Infective Diseases, 18(6): 1006-1008).

Questions:
Niels Jørgen Olesen: About the epidemiology of this pathogen, is it everywhere?

Olga Haenen: It is found in wild glass eel but often another biotype. It needs brackish or saltwater.

Niels Jørgen Olesen: Can infected fish be sold for consumption?

Olga Haenen: This disease is not notifiable for fish anywhere in the world. The disease is notified only in USA when detected in human patients. So you can buy this disease in the supermarket.

Torsten Snogdal Bountrup: When you perform bacteriology from a diseased fish it is easy to isolate many different bacteria on the plate, how did you deal with this issue?

Olga Haenen: In most of these cases it was a pure culture. We are improving the vibrio diagnosis at the moment with 16S and 23S rRNA.

MOLECULAR EPIDEMIOLOGY OF VHS IN ITALY

Anna Toffan & Valentina Panzarin
Division of Comparative Biomedical Sciences. Fish Virology Department, Istituto Zooprofilattico Sperimentale delle Venezie, Italy

Abstract:
Viral haemorrhagic septicaemia (VHS) is economically the most relevant viral disease affecting the Italian trout farming. Unfortunately, despite the application of eradication programs, disease outbreaks still occur in Northern Italy, where most of the trout farms are located.
To better understand the epidemiology of VHS, the Fish Virology Department at IZSVe has characterized a selection of VHSV isolates (n=42) mainly deriving from rainbow trout samples collected in Italy during field outbreaks which occurred from 2003 to date. The full length G-gene sequences were obtained according to the protocol described by Einer-Jensen and colleagues (2004). Sequence data were compared with representative nucleotide sequences retrieved from GenBank and phylogenetically analysed. Our analysis revealed that all the Italian VHSV isolates belong to genotype I, sublineage Ia. Within this group, the Italian strains are distributed among 4 different genetic clusters, arbitrarily classified as I, II, III and IV. The coupling of geographical information together with genetic data highlighted interesting epidemiological connections among different fish farms, which will be discussed in the presentation.
This study emphasizes the importance of phylogenetic analysis to describe the epidemiology of fish pathogens and highlights its practical application in molecular monitoring.

Minutes:
The disease can be considered endemic and is mainly a problem in the north of Italy. Of 331 rainbow trout farms, 168 are registered as free of VHSV/IHNV. The work presented here was done to learn more about the distribution of VHSV in Italy. The first sample that tested positive was collected in 1982 from rainbow trout. In the period 2003-2009, 2-4 new strains were isolated per year, in 2010-11 there has been an increase with 8-9 isolates per year due to an outbreak in the North-east of Italy. In total, 42 isolates were analysed by sequencing of the G-gene (full length).
Most of the isolates are coming from the northern part of Italy. All Italian isolates belong to genotype I, sublineage Ia (Kahns et al. 2012). Thanks to this work, 4 main clusters corresponding to 4 different introductions were indentified, 2 of the clusters appear to be eradicated while cluster Ia.I is widespread in the north of Italy. Connections within our country and among European countries are highlighted through this work.
About VHS detected in fish coming from rivers there are no hints about the possibility that they are escapers or wild animals.
Some points will be addressed in the future, considering an improvement for the dataset (nr. of samples, years, farms neighbouring infected sites) that will allow the possibility to perform evolutionary rate analysis, phylogeography, gene flow between farmed and wild animals. Once the national database of fish farms will be finished and the georeference of the Italian farms is implemented more accurate epidemiological investigations will be possible.

Questions:
Niels Lorenzen: What is the cost of VHS in Italy?
Anna Toffan: The cost is quite high for the farms in northern Italy while not so high for those in the valley because of the higher temperature. The farmers are willing to vaccinate the fish, if a vaccine will be available.

INTRODUCTION OF SOME EMERGING FISH DISEASES IN CHINA

Hong Liu
OIE reference laboratory of Spring Viraemia of Carp, Key Laboratory of Aquatic Animal Diseases, Animal and Plant Inspection and Quarantine Centre, Shenzhen Exit-entry Inspection and Quarantine Bureau, AQSIQ, P. R. China

Abstract:
According to the data published, 94 disease outbreaks and nearly 50 cases caused by unknown pathogens occurred in 99 cultured aquatic animals in China 2010. Most of the cases were caused by bacteria and parasites, with average morbidity of 28%, mortality of 7.8%.
This mortality rate caused an estimated direct economic loss ranging between 13 billion to 15 billions of renminbi yuans (up to 1.849.826.286 Euros).
48 cultured fresh water species (mainly *Ctenopharyngodon idellus*, *Ictalurus punctatus*, *Hypophthalmichthys molitrix, Siniperca sp.*, *Tilapia sp*., *Anguilla sp*.) suffered diseases and mortalities.
Major viral problems detected were grass carp haemorrhagic syndrome caused by grass carp reovirus, iridovirus infection and infectious haematopoietic necrosis. Infectious haematopoietic necrosis (IHN) outbreaks were located mainly in the northern provinces, while iridovirus was frequent in the southern China. Other diseases (mostly grass carp haemorrhagic disease) were widespread within 30 provinces territory.
Most frequent bacteria and parasites involved in outbreaks characterized by gill necrosis and enteritis were: *Aeromonas hydrophila*, *Edwardsiella ictaluri*, *Streptococcus* sp., *Vibrio* sp. and *Ichthyophthirius multifiliis*.
18 cultured marine fish species suffered viral nervous necrosis, infection with *Cryptocaryon irritans*, *Vibrio* sp., *Edwardsiella* sp., and other bacteria or parasites. The losses caused by parasites were higher than the ones caused by viruses or bacteria. In some coastal provinces the most seriously affected marine fish species were *Pseudosciaena crocea*, *Paralichthys olivaceus*, *Epinephelus* sp., *Pagrus major* and *Sparus macrocephalus*.
A strain of rhabdovirus was isolated from cultured stone flounder（*Kareius bicoloratus*) and its biological and physico-chemical characteristics were studied. The complete genome of the strain SR080113 was cloned and sequenced with RT-PCR and RACE. The length of the viral genome was 11,037 nt, similar to the HRV Korea strain (Gen-Bank Acc. No. NC_005093) with more than 95% of similarity. Three molecular detection methods including RT-PCR, real-time RT-PCR, reverse-transcription loop-mediated isothermal amplification (RT-LAMP) were set up, with sensitivity of $10^{3.5}TCID_{50}$/100μL, 100 copies per test, and $10^{1.5}TCID_{50}$/100μL, respectively.
13 samples coming from Yellow sea and Bohai sea tested positive for HRV. These fish included *Kareius bicoloratus* and *Psetta maxima*. The detection methods were evaluated during the assay and their diagnostic performance characteristics were analyzed.

Minutes:
In the spring and autumn sometimes the laboratory receives more than 100 samples per day. Samples are also received from the other regional laboratories. A lot of different aquatic animals are bred for food production (48 different freshwater species and 18 different marine species are cultured).
Main diseases are: grass carp haemorragic syndrome by grass carp reovirus (endemic), Iridovirus infection, IHNV, *A. hydrophila*, *E. ictaluri*, *Streptococcus* sp, *Vibrio* sp. and *Ich. multifiliis*.
Several studies are being or have been conducted:
From 2008-11 a hirame rhabdovirus study was initiated, based on incidental findings when surveying for VHSV/IHNV.
In 2004-06 SVCV was studied.
In 2006-2007 VNN was studied.

Questions:
Olga Haenen: How do you search for the goldfish virus?

Hong Liu: We looked after the virus by PCR and real-time PCR.

Katja Einer-Jensen: Is VHSV detected in China?

Hong Liu: BF-2 and FHM cells are used to survey but VHSV has not been found.

Sven Bergmann: You mentioned the finding of a virus like megalocystis virus in freshwater which is quite interesting as this is the first time this virus has been found in freshwater.

Hong Liu: This has been studied for many years especially in cultured mandarin fish (*Siniperca chuatsi*).

Sven Bergmann: How do you look for KHV?

Hong Liu: With PCR and real-time PCR.

EVIDENCE OF ISAV-HPR0 INFECTION AND REPLICATION IN ATLANTIC SALMON GILL CELLS AND THE ASK CELL LINE.

Debes H. Christiansen
National Reference Laboratory for Fish Diseases, Food & Veterinary Agency (FVA), Faroe Islands

Abstract:
Recently, a low-pathogenic variant of ISAV designated ISAV-HPR0 was identified within farmed Atlantic salmon populations causing an apparent non-clinical transient infection (Christiansen *et al.*, 2011). Genetic analysis showed a close genetic association between ISAV-HPR0 and highly pathogenic ISAV in farmed Atlantic salmon populations within the same hydrographical area. Thus ISAV-HPR0 has been suggested to be the ancestor of pathogenic ISAV variants which through the introduction of mutations by the error-prone RNA polymerase transforms into virulent ISAV. Thus ISAV-HPR0 is a potential risk factor in the emergence of new strains of disease-causing ISAV.
However, little is known about the functional characteristics and infection dynamics of ISAV-HPR0. We have shown that ISAV-HPR0 RNA is mainly detected in gills suggesting that gills are the primary target of ISAV-HPR0 infection and replication. This is supported by our preliminary results showing that gill epithelial cells tested positive for ISAV by immunohistochemistry. However gills are exposed to the environment and gill mucus has been shown to contain large amounts of sialic acid receptors to which ISAV is known to bind. Thus other as yet unidentified organs or cells of the Atlantic salmon could be the primary sites for ISAV-HPR0 replication.
Interestingly, segment 7 has been shown to encode three proteins via an alternative splicing mechanism which is dependent on the cellular splicing machinery. Thus segment 7 can be used as a marker for ISAV-HPR0 replication in cells. The un-spliced mRNA transcript has an open reading frame (ORF) of 900 nucleotides (ORF1) and encodes a putative nonstructural protein with IFN antagonistic properties while the spliced mRNA transcript has an ORF of 477 nucleotides (ORF1/2) suggested to encode for a nuclear export protein. A third spliced mRNA transcript of an ORF of 243 nucleotides (ORF1/3) has been suggested with unknown function.
Therefore we used segment 7 specific RT-PCR and sequencing to test ISAV-HPR0 positive gills for the presence of ORF1, ORF1/2 and ORF1/3. Our preliminary results show that both the un-spliced mRNA transcript (ORF1), the spliced mRNA transcript (ORF 1/2) and possibly also the spliced ORF 1/3 are present in ISAV-HPR0 positive gills. In addition we demonstrate the presence of both transcripts in the ASK cell line inoculated with ISAV-HPR0 positive gill homogenates. Our preliminary results indicate that ISAV-HPR0 can replicate both in gill epithelial cells as well as in the ASK cell line. Thus Atlantic salmon gills seem to be the primary target for ISAV-HPR0 infection and replication.

Christiansen DH et al., Journal of General Virology (2011), 92, 909-918.

Minutes:
I will present preliminary results that HPR0 can replicate in the ASK cell line.
Our results suggest that HPR0 is replicating in the gills and is low pathogenic as only transient infection with no mortality is seen despite Ct values < 20.
The Faroe island ISAV isolates belong to the European genogroup, and cluster in the EU-North American genogroup together with isolates from USA, Canada and a Scottish isolate. The isolates caused the ISA outbreaks.

HPR0 is detected by qPCR in large amounts in gills, whereas only small amount or no virus at all is identified in other organs. The fish have no clinical signs. There is a close genetic link between Faroese HPR0 and ISAV isolates responsible for the Faroese ISA epidemic.
Immunohistochemistry shows that epithelial gill cells are infected, and most likely confined to gills as opposed to highly pathogenic ISAV which also infect other organs.
Segment 7 encodes 3 different proteins by a splicing mechanism. RT-PCR analysis shows that both the big open reading frame 1 and a smaller open reading frame can be seen in gill samples when running the gel.
In ASK cells there is more RNA in the cell fraction of ASK at day 3 and more RNA in the medium fraction at day 7.
Genetics support that the HPR0 is progenitor for the pathogenic ISAV in the Faroe Islands.

Questions:
Niels Jørgen Olesen: PCR is for the transcript or for the genomic RNA?

Debes H. Christiansen: Our segment 7 RT-PCR can distinguish between transcript and genomic RNA because there is an intron in segment 7 spliced out in the transcript

Niels Jørgen Olesen: Can you do swabs from the gills and test the swabs for ISAV?

Debes H. Christiansen: I have tried it and it is possible to do non-lethal sampling but it is nearly impossible to get only mucus, you will also get gill cells.

Debes H. Christiansen: We have tested approximately 5000 rainbow trout with negative results.

Niels Jørgen Olesen: We have not tested rainbow trout.

Brit Hjeltnes: We do now test rainbow trout by PCR, but we have done indirect testing as rainbow trout is tested on ASK cells. App. 25 years ago we tried to infect rainbow trout with ISAV, and we saw that the rainbow trout haematocrit was reduced slightly, but we did not have PCR at the time.

Niels Jørgen Olesen: Shall we consider rainbow trout as susceptible? As it is know we are in fact obliged to examine also rainbow trout.

Sven Bergmann: Do you have an idea how far HPR0 is spread in the wild?

Debes H. Christiansen: We have examined wild fish with negative results, but there is still many wild fish out there we haven't examined.

SESSION II: Mini workshop on "Sampling procedures"

Chair: Dr. Torsten Snogdal Boutrup
Minutes: Dr. Niccolò Vendramin

AN OVERVIEW OF SAMPLING PROCEDURES IN ENGLAND AND WALES

Richard Gardiner
Cefas Weymouth Laboratory, Fish Health Inspectorate, UK-England

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Abstract:
Sampling aquatic organisms for diseases in an effective and repeatable manner is not an easy task. There are a number of restraints on sampling protocol based on size of animal, species, disease being looked for and temperature. In addition there are other factors to consider to ensure that the diagnostic laboratory receives a viable, traceable sample in a standardized format with the correct instructions on what tests are required and why. Based on CD 2001/183/EC, OIE manuals and years of experience, we have developed and refined techniques of sampling that ensure high diagnostic standards. An overview of these techniques will be described, including how the samples are dispatched and received by the diagnostic laboratory. The sampling protocol has been integrated into the compliance and surveillance program of visit to aquatic facilities. Maintaining high standards across of team of inspectors is important; we therefore maintain accreditation and ensure regular training for all involved, from field inspectors to laboratory staff.

Minutes:
Collecting fish samples is a difficult activity and as there are many issues to be considered such as different pathogens, susceptible species, vector species, tissues, temperatures etc. There is the need to collect something that fits for purpose.
Standard operating procedures (SOP) need to be short, simple and easy to use. In our house we do it it that way, that once the draft is issued we conduct several meetings with inspectors to go through the draft so all agree on it. There are many different opinions and the result has to be something that fit field conditions. Compliance with the requirements of CD 2006/88/EC is important. We have one SOP for fish, one for molluscs and one for crustacean. Before the directive was implemented there were 5-6 laws, now the legislation is summed up into ONE regulation for all fish work.
In the authorization of farm there are many indications: what they can do, what they can import, from where, mortality record, biosecurity measure plan, etc. Each farm is visited every year; the inspector performs an effective audit aiming to evaluate if the requirements for the authorizations are fitted correctly.
Great attention is addressed to bio-security measures (once called good hygiene practice), in particular the identification of risk of disease introduction with fish/people, the risk mitigation measures and the contingency plan are checked.
Surveillance plan in action is structured on a database that schedules all visits every year, taking into consideration susceptible species reared in the farm, and water temperature condition in order to correctly address the surveillance purpose.
Generally 5 fish are pooled, if they are very small it is possible to add up to 10 specimens per pools Storing tubes are graduated (1 gram of sample in 9 ml of MEM). Spleen, kidney and brain are collected for virological investigations.

Each sampling is followed by a post mortem form, in order to trace samples from the field to the laboratory. There is a unique reference number for each farm visit.
Samples are stored in cooled boxes that hold temperature for 72 hours. The temperature of the sample is measured each times that a box arrives in the lab (one extra tube with MEM only for measuring temperature in).
Training of new inspectors is one important activity. Training sessions are performed periodically with different scenarios. Sometimes inspections are performed together with laboratory people to get new point of views.

SAMPLING PROCEDURES FOR SURVEILLANCE AND DIAGNOSIS OF FISH DISEASES IN DENMARK

Henrik Korsholm,
Section of Aquaculture, Danish Veterinary and Food Administration, Denmark

Abstract:
Routines applied by Danish fish health inspectors for sampling of laboratory specimens for fish diseases VHS, IHN, IPN and BKD are elucidated. The inspection routine in fresh water and marine farms and the criteria for selection of material are explained. Utensils and packing materials are demonstrated.

Minutes:
As general principle we try to keep the procedures as simple as possible but still complying with all the legal requirements.
Water temperature is one parameter that needs to be always taken into consideration according to the purpose of our investigation.
In fresh water, for VHS/IHN surveillance water temperature needs to be below 14°C and all outlet screens must be inspected at all parts of the farms, for BKD all production area should be screened, while for IPN we focus on fry tanks mainly.
In sea cages, the entire surface needs to be carefully observed, and it may be necessary to use the dead collector connected to the bottom of the net in order sample moribund fish as well as dead ones.
If present, moribund and abnormal specimens should be collected and also fish in the back channel collecting water from the flow through system.
For rainbow trout, fish are pooled in groups of 10 and shall represent fish from the whole farm, both age and place wise.
The fish inspector equipment is composed of wellingtons (preferably belonging to the fish farm), protective clothes (water proof) then can be easily washed at 60°C and dried, for necropsy one sterile pair of scissors for every farm. Tubes where samples are stored are specific for purpose/diseases: they are filled with MEM for VHS/IHN, RNAlater for KHV, empty for BKD and with formalin for histology, swabs are used for bacterial diseases.
Boxes used for delivering samples are cooled with ice packs previously stored in the freezer and isolated from the tubes in order to avoid freezing of samples; each sampling is accompanied by a submission note.

FISH FARM INSPECTIONS AND SAMPLING PROCEDURES: THE MEDITERRANEAN POINT OF VIEW

Niccolò Vendramin
National Veterinary Institute, EU Reference Laboratory for Fish Diseases, Technical University of Denmark

Abstract:
Marine Mediterranean aquaculture meant as intensive rearing systems for zootechnical production has reached the recent level extremely fast.
The development of efficacious breeding protocols, the availability of artificial feeding and principles adopted in facing diseases have lead the growth of an industrial rearing system.
If we consider the health management approach within the context of this complex productive scenario there is the need to discriminate between, at least, two groups of farms that are extremely different and characterised by peculiar health/disease management issues.
First of all there are to be considered the hatcheries, characterised by having an extremely high level of technology and control of the water; in these systems biosecurity measures are generally high and the water quality parameters are monitored constantly.
Secondly there is to be considered all the farms that belong to the category of "ongrowing". These kind of rearing activities, once obtained the seed / juveniles, provide them feeding till the juveniles have reached the market size. Within this group, sea cages have affirmed as the best ones both for economical and technological/environmental aspects.
Sanitary issues to be managed, their evolution, related control strategies and analytical tools are, in this complex, quite different for the two main groups of farms but all these features should be addressed always considering the zootechnical production scenario they belong. In this presentation, mainly based on pictures, different aspects of clinical inspection/sampling protocols are described.

Minutes:
To address an efficient sampling procedure different fish farm typologies have to be considered.
In intensive inland units, both on growing and hatcheries, environmental control is a key point: intended as water renewal, day/night light control and feeding control. On the other hand, in sea cages (inshore, off shore and submersible) the environment can hardly influence the rearing activities. Especially in sea cages sampling can be extremely hard, there is the possibility to use dead collectors placed at the bottom of the nets but also to cooperate with scuba divers.
When is the best moment to sample? It can be at seeding, at harvest or on request when there is a problem. When a fish farm is approached for a problem, which kind of specimens/samples should be collected? Generally the approach shall be wide, firstly it is extremely important to speak to the farmer before/whilst visiting, then during the real visit, a clinical inspection of the whole farm should be performed. The water outlet grids should be checked, the overall impact of outbreak (whole farm/only fry?) should be evaluated and samples representing the whole farm (properly classified) should be collected. Samples in bad conditions should not be investigated as they can lead to non reliable results. All the activities should be planned carefully.
Two major viral marine Mediterranean pathogens (betanodavirus and lymphocystis) screening protocols are then explained as practical examples.

General discussion:

Brit Hjeltnes: Lots of good things have already been done. I think about official procedure, and found out paperwork needs to be done. Can be an opportunity with young people for introducing the use of tablets, so might need to switch to electronic solutions. Screening activity can miss positive samples. Training is needed in order to get always relevant samples. Tutorial videos can be a solution to train people in taking the correct samples.

Anna Toffan: For Dr. Gardiner (UK inspector), if I understood correctly you issue a screening plan on a yearly basis. Do you ask farmers beforehand?

Richard Gardiner: Yes otherwise farmers will not give access if they do not know. We also perform some spot checks.

Sven Bergmann: I would like to underline that screening schedules must be adapted to the different diseases.

Olga Haenen: Numbers of fish should also be adapted to diseases. Koi fish: 1-2 fish per sample. 5-10 fish for other diseases.

Hong Liu: How to take samples from old fish? How to take samples from expensive fish?

Henrik Korsholm: Explain farmers that moribund are not of value anymore. Maybe take samples from the slaughter line.

Richard Gardiner: We can use rejected fish from farms. Imported fish: B grade fish kept together with top grade fish.

Sven Bergmann: Remember that koi herpes virus can only be reactivated in a short window. Use gill swabs with blood and samples 100-150 fish and examine singly. You will maximum find around 20% positive.

Torsten Snogdal Boutrup: If you have imported koi carps that have been infected they will be immune-compromised and you will maximise the chance of finding the virus.

Sven Bergman: You will have to heat up the water in order to reactivate the virus.

Britt Bang Jensen: You showed that by netting the fish you will reactivate the virus. Can you not just stir the net in the water?

Sven Bergman: Your will have to take the fish out of the water.

Britt Bang Jensen: So when you take the samples you take them out of water.

Conclusions

Niels Jørgen Olesen: the reason why we decided to schedule this mini workshop is because we have done nearly nothing on this subject for the last 15 years even though the diagnostic manual is also for sampling. As this discussion has also shown, especially for KHV, there are a lot of different opinions on how sampling should take place. We have sampling plans for VHS/IHN and some for ISA, which was made before HPR0 was taken into account. But we have nearly nothing on KHV. It is really a problem that the member states do not have anything on this to follow.
Many of us are working in the laboratory and do not go to the farms to pick up the samples and so have no direct influence on how the samples are taken. We hope that after this Workshop you will stimulate your competent authorities to raise the question that training should be made available to the inspectors taking the samples including discussions on the pitfalls.

SESSION III: Technical issues related to sampling and diagnosis

Chair: Dr. Anna Toffan
Minutes: Helle Frank Skall

HEALTH CATEGORISATION OF FISH FARMS IN EUROPE IN 2011

Niels Jørgen Olesen & Nicole Nicolajsen
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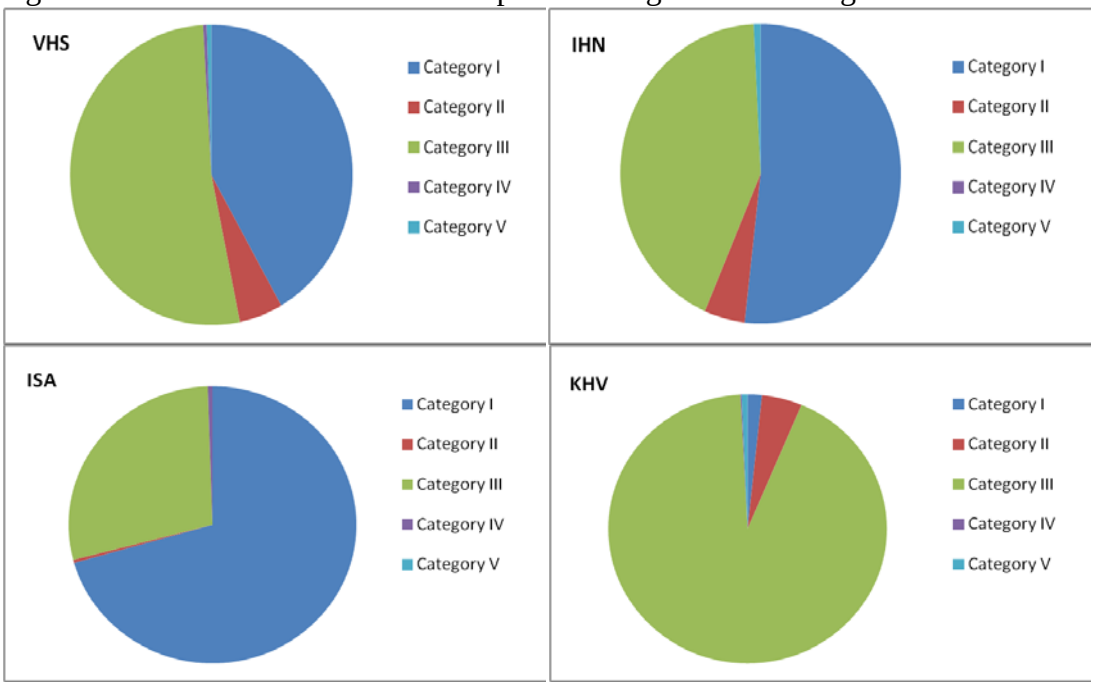
Abstract:
The Questionnaire on Surveillance and Diagnosis (S&D) included questions on how fish farms are health categorised according to Council Directive 2006/88/EC in the respective countries.
More than half of the authorised farms in Europe are in category III for VHS and approximately 45% for IHN, only very few farms are in category IV and V the remaining ones are in category I or II (Figure 1). According to these official data almost no farms are infected with either of these diseases. This might be more due to a significant underreporting than of the de facto situation.
For KHV most carp farms are in category III, unknown status.
Many farms in Europe are not categorised yet, and unfortunately the situation have not improved much from 2010. In the questionnaire we ask for the number of APBs in these areas. There are several different views on how categorisation shall be performed, e.g. should VHS free marine rainbow trout farms be placed in Category III or I? If Isavirus HPR0 is found in or in proximity of a farm can it remain its Category I status? Some Member states do not include registered APBs in the categorisation but according to 2006/88/EC Annex III health categorisation comprise Member states, zone and compartments NOT single APBs. A new Animal Health Law is under preparation and revision and will now include aquatic animals; in this connection the categorisation system might be simplified and be made more transparent.

Figure 1: Distribution of farms in Europe according to health categories



Minutes:
This presentation aims to describe how the health categorization has been performed in different countries. In the CD 2006/88/EC it is stated that all farms shall be registered and have a health category. In some countries the concept of health categorization seems to be misunderstood, since some Member states do only health categorize authorised and not registered farms APB´s (derogation in art. 4) . The general purpose of the directive is to evaluate the entire sanitary status of aquaculture industry in Europe thus also the small hobby farms that do not place any live fish on the market shall be categorized.
After this presentation there will be a presentation by Britt Bang Jensen concerning the question that she asked in this year's S&D; comparing the answers given to her questions compared with the questions in the normal S&D totally different answers are sometimes obtained.
Some general remarks, for ISA nearly 1/3 of the farms are in category 3. This is because Norway has taken the status that all their marine salmon farms are put in the unknown category.
Almost all farms are in the undetermined group (cat. 3) for KHV.
The presentation is divided into 3 areas: nordic, continental, and southern area.

Norway: All farms are free of IHN and most free on VHS, due to the outbreak of VHS in 2007 where some farms lost their category I status.
Finland: the farms which had VHS outbreaks are now put into category 4, so they are now subject to an eradication program.
Estonia: Had the most northerly outbreak of VHS caused by genotype Ia in 2011.
Latvia: Has no categorized farms.
Lithuania: Has all its 21 farms in category I and II.
Denmark: The country was nationally declared free for VHS last year, and will be put in Category I in 2013, due to the 4 year program. Farms in the marine environment has been put into category III for VHS, because we do not want farms to be able to return fish from the marine environment to the freshwater environment as we know that there is VHSV in marine wild fish.

Germany: A country like Germany has put a lot of effort into this questionnaire and we are grateful for that. Germany is using all the categories as the only country in Europe. Most of their farms are placed in category III. It also seems that only one third of the farms are categorized yet.

France: 1.248 farms still have to be health categorized.
Belgium: Has initiated a program to obtain freedom for VHS and IHN.
Netherlands: Both VHS and IHN are present now in the country.
Poland: VHS, IHN and KHV are present.
Czech Republic: Half of the farms are categorized.

Austria: Based on the questionnaire most farms in Austria have not been categorized. The same is the case in other countries.
Italy: No data on categorization has been submitted and the country is still in the process of implementing the categorization.
Greece: Has not categorized their seabream and seabass farms.
Turkey: 96 farms are in program for VHS/IHN freedom.

It seems as the process of categorization has stopped in the last 2-3 years. Maybe this is due to the crisis, but it can also be because the issue of the new animal law is in progress and people may be waiting to see how the new law will look. Furthermore the Commission has not been pushing the

countries to follow the rules. I think that we, as NRLs, also have a duty in helping to pushing our competent authorities to finish this work.

There are different views on how categorization shall be performed, e.g. the marine/freshwater question regarding VHS.
There is really many farms in category 3, and remember that these farms have to be inspected by active surveillance.
There are also many farms in category 2. Has all these farms been included in a program approved by the Commission?

Questions:
Niccolò Vendramin: I think that there is a problem with category 3, as it seems to be a way to provide a categorization without doing anything, in the beginning it was supposed to be a short term period category.

Niels Jørgen Olesen: Yes, I agree with you. We have a problem there. As far as I understand in the new legislation there will be 3 health categorisations: 1) free, 2) free, but in a water catchment where there are infected farms and 3) all the rest combining both the unknown and the infected. The problem at present it that nobody wants to be stated as infected.

RISK CATEGORIZATION FOR AQUATIC ANIMAL HEALTH SURVEILLANCE
Presentation of the new EFSA project and Outcome of the questionnaire spring 2012

Britt Bang Jensen[1], Birgit Oidtmann, Angus Cameron, Katharina Staerk, Manuela Dalla Pozza, Edmund Peeler, S Tavornpanich, Trude M. Lyngstad & Edgar Brun.
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Abstract:
The Council Directive (CD) 2006/88/EC lays down that Member States shall ensure that a risk-based animal health surveillance scheme is applied in all aquaculture production businesses (APBs), as appropriate for the type of production. It is recognized that the risk of introducing or spreading disease, varies, not only between areas having a different disease status, but also within areas with the same disease status for each particular disease. Each aquaculture production unit therefore needs to be ranked according to the risk of introduction and spread of each of the listed diseases. As the task of risk-ranking is complicated and require considerable resources, the European Food and Safety Authority (EFSA), has funded a project with the objective of describing and critically assess the various factors necessary to categorize fish farms taking into account characteristics of the diseases listed in Part II of Annex IV of the CD.
As a background for this work, one of the tasks of the project is to describe the present level of implementation of the Article 10 of CD 2006/88/EC provisions on risk based surveillance and surveillance for demonstration of disease freedom of fish diseases.
The present level of implementation of the CD 2006/88/EC, and specifically of article 10 (which requires that Risk Based Surveillance - RBS, animal health surveillance schemes is applied in all farms) in the EU MS is largely unknown. In 2010, the Norwegian Veterinary Institute carried out a survey on the implementation of the CD, including article 10. Responses were obtained from 25 member- and EFTA-states and showed that there were clear delays in the implementation of Article 10. The responding countries stated that delays were due to uncertainties on how to risk rank their APBs, and delays with their registration, which is a pre-requisite for risk-ranking of farms. A handful of countries have presented their strategies for risk-ranking at scientific meetings and seminars, but no overview of methods used is currently available.
In order to further describe the level of implementation of Article 10 of the CD at the beginning of the project, a section (section 4) with additional questions regarding this was added to the 2011 Survey & Diagnosis sent out by the European reference laboratory for fish diseases.
This section consisted of 7 questions, with several sub-questions, and included a part where respondents could provide comments and clarifications in free text, if desired.
The questionnaire was sent to 36 recipients:
The 27 EU member states, minus Luxembourg and Malta (For the UK, questionnaires were sent to UK-England& Wales, UK-Northern Ireland and UK-Scotland separately. For Denmark, the questionnaire was also sent to the Faroe Islands, which is a DK-territory not member of the EU).
6 EU-candidate and potential candidate countries: Albania, Bosnia-Herzegovina, Croatia, Iceland, Republic of Kosovo and Turkey.
2 Non-EU countries: Norway and Switzerland.
The recipients were given two months to reply. By the deadline, answers had been returned by 35 recipients, 33 of whom had answered section 4.
The results of this survey will be presented and discussed at the meeting.

Minutes:
Council directive 2006/88/EC requires risk based surveillance health.
In 2010 EFSA investigated the implementation status and then decided to fund a one year action project in order to figure out which factors are necessary for risk categorisation of fish farm.
The consortium is headed by CEFAS, there are three partners (UK, Norway and Italy), one external advisor (Dr. Angus Cameron, Australia) and one cooperator (EURL for fish diseases).
The project is divided into 3 Work packages:

WP1:
- Task 1: Literature review of risk-based surveillance methods for farmed/wild and terrestrial / aquatic animal surveillance.
- Task 2: Describe the present level of implementation of the Article 10 of Council Directive 2006/88/EC

WP2:
- Task 3: Describe the characteristics of the listed fish diseases
- Task 4: Describe and assess the various factors necessary to categorize fish farms
- Task 5: Develop a methodology for ranking of fish farms

WP3:
- Task 6: Validate the output of the risk categorization and ranking using case studies

This presentation will focus on Task 2. Since the implementation of 2006/88 is largely unknown, there is a need to understand what the problems are (i.e. delation because of delays of registration, how to risk rank). The method was the extra section of the S&D (35 delivered).
Concerning registration of fish farms, this has been finalised in many countries, and is under way in most of the rest. Only few have not begun.
There are difficulties due to the complexity of the industry, with many hobby farms and put-and-take ponds.
Concerning Health categorization this is almost finalised for fish, but some are still lacking for molluscs/crustaceans. It is ok to use area categorization but there are different approaches in different countries since some categorize according to production type, i. e. all marine farms in cat. III, all mollusc farms in cat. III or V; some find categorization easy, whereas others struggle with determining focus.
Concerning the Risk-based surveillance scheme: most of the countries have decided to risk-rank the farms, many of them find the guidelines useful and many have decided on the method to use.
Half of the countries investigated have begun collecting data and half have begun assigning risk-levels.
Approximately one fourth have finished risk-ranking, some have maintained high surveillance, some have few resources available.
Concerning surveillance programs for disease freedom: very few have applied for fish diseases, none for molluscs or crustaceans.
Finally concerning contingency plans: approximately two third have either submitted a contingency plan, or is working on it, but none have been approved.

Questions
Olga Haenen: I think authorities will be very interested in seeing this report, will it be possible to get this report.

Britt Bang Jensen: We need to find out with the respondents if it will be OK to publish this and how anonymous it should be.

THE ANIMAL HEALTH LAW FROM AN AQUATIC PERSPECTIVE

Sigrid Cabot,
European Commission, DG Health and Consumer

We investigated the possibility to show the presentation by Sigrid Cabot by skype or similar, but this was not possible as they did not have the tools in the Commission. The slides of the presentation are included in the folder though. We want to stress the last slide saying "please continue the work to implement CD 2006/88."

UPDATE ON EUS DIAGNOSTICS, INFECTION TRIALS AND ONLINE SLIDE COLLECTION

Torsten S. Boutrup & Christian Fry
National Veterinary Institute, EU Reference Laboratory for Fish Diseases, Technical University of Denmark

Abstract:
Following the presentation with an update on growth and sporulation of *Aphanomyces invadans*, by Christian Fry at last year's annual meeting, we have conducted a series of infection trials. These infection trials have had several functions, both to establish an infection model in our laboratory and getting experiences in this context, but also to use the fish for performing diagnostic procedures from clinical cases, and to collect positive tissue material from infected fish.
We performed a pre-trial with three different species of Gourami's: three spot Gourami, pearl Gourami and dwarf Gourami. Each fish was intramuscularly injected with 1600 spores in 10 µl of milliQ water. We observed that 3 out of 9 three spotted Gouramis got clinical diseased, none of 4 pearl Gouramis got diseased and all of 5 dwarf Gouramis got diseased. From this we chose to use the three spotted Gouramis as the fish of choice, to include as positive control of the pathogenicity of injected spores.
Secondly we set up a confirmatory trial in three spotted Gouramis, here it was also seen that around a third of the fish got clinically diseased. From these and later trials it is our experience that using *A. invadans* grown on agar, around a third of the threspotted Gouramis will show clinical disease ranging from 2-5 fish, some of these might resolve the lesion within a couple of weeks. Further, using an *A. invadans* reisolated from a three spot Gourami we have seen 100% morbidity. In contrast, using an *A. invadans* reisolated from a rainbow trout, with clinically disease, resulted in no mortality in 10 three spotted guramis, with only one fish showing a slight reddening.
We have performed intra muscular injection trials in rainbow trout at temperatures at 10, 15, 18 and 22 degrees Celsius respectively. Here we have seen variable morbidity; at 10 degrees Celsius we have not observed any symptoms, at 15 degrees Celsius we have seen a few fish with clinical disease, at 18 degrees Celsius up to 70 % have been clinically affected and at 22 degrees Celsius almost 100% morbidity was seen. This shows that rainbow trout can support oomycal growth and develop lesions at temperatures present in European rearing conditions, however, at the predominant temperatures, for rearing salmonids in Europe, disease and lesion development most likely will be to a limited degree. Moreover, our preliminary results indicate that oomycal growth in rainbow trout don't support a normal pathogenic potential of the organism, but an attenuated strain with lower virulence.
Following the above described trials we have collected material for histology, oomycete reisolation and PCR. From this material, pictures will be uploaded to the EURL website. The pictures will show different types of lesions using standard H&E stain and special stains, explanatory text will follow each picture. First we will upload tissue from infected Gouramis, following this; pictures of lesions in rainbow trout.

Minutes:
I will try to give an update on the work done on EUS since the last meeting where Christian Fry told you about sporulation.

There have been several presentations over the last year. The basis for this presentation is the Club 5 meeting trying to make a uniform diagnostic manual to be used in the EU, including a slide collection. The presentation is also based on the work done in the project NADIR, which is an EU 7 framework program project, where we here at DTU Vet is trying to establish a infection trial model so we can expect to get a validated result when performing infection trials using *Aphanomyces invadans*.
We started using three spot Gourami in order to see if we could validate the results obtained by others. We then used pearl Gourami, as it seemed to be a bit more susceptible, and we tried dwarf Gourami as this species seem to be notoriously difficult to keep in aquariums.
We validated the trial in three spot Gourami.
We injected the fish intramuscularly with 10 µl of spore suspension.
All the trials we have made in three spot Gouramies are summarized in one figure showing all the mortality curves. As you can see it varies a bit from 1-2 fish getting diseased in a tank of 10 fish up to half getting diseased.
On this other graph you can see that all dwarf Gouramis died in the infection trial. This species demonstrated to be extremely susceptible and if you want to be able to see strain differences among isolates it may not be the best species to use.
When performing infection trials with a strain reisolated after "*in vivo*" infection we saw a much higher mortality than when infections trials were performed with an isolate that had been growing on agar.
The rainbow trout trial showed that there was a difference in mortality based on temperature. At 10°C no fish got sick and the mortality raised as the temperature raised. The pre trial also showed no difference between sizes of fish. We could not validate this last finding in a following trial, where there was differences based on size.
If you are not familiar with looking at histological slides I suggest that you start looking at the slide just up through the light, this will give you lot of information that you can use when you put the slide into the microscope.
Depending of the fish species you may not see granulomas but you will see a granulomatous inflammation. You will also see tissue destruction due to the cytolytic capability of this pathogen.
When you use the special Grocott stain, you have to be aware that other stuctures than fungae will stain.
I tried to use the PAS staining and also tried to optimize the protocol without good results. I also tried to use the uvitek stain without good results. I will work further on this. One reason for the bad results for the uvitex stain may be because I decalcified the samples first.

Questions:
Olga Haenen: Does the uvitex needs a special lamp to be observed in the microscope?

Torsten Snogdal Bountrup: It is the same lamp as for the Hoechst stain.

Richard Paley: You mentioned bath challenge – did you succeed?

Torsten Snogdal Bountrup: No mortality appeared. We used the same spores as we used for injecting which means that the spores may not be motile. We also removed some scales and that did not give even a superficial infection. For sure the bath infection would be the most useful model as this would be more like the natural infection. It would be the way to examine e.g. if pH has an effect etc.

Cohabitation could also be an idea but there are some question marks with cohabitation as it is much more difficult to make sure that everything is standardized in all the tanks. (E.g. may one fish in one tank produce many more spores than in the other tanks).

SESSION IV: Scientific research update

Chair: Dr. Brit Hjeltnes
Minutes: Dr. Torsten Snogdal Boutrup

FIRST DETECTION OF HIRAME RHABDOVIRUS (HIRRV) IN EUROPE

Ewa Borzym[1], Marek Matras[1], Joanna Maj[1] , Agnieszka Sandomierska[1] , Niels Jørgen Olesen[2] , Mette Eliassen[2] , Marine Baud[3], Chiraz Talbi[3] **& Laurent Bigarré[3]**
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[2] *National Veterinary Institute, EU Reference Laboratory for Fish Diseases, Technical University of Denmark*
[3]*ANSES, Agence nationale de la sécurité sanitaire, Unité de pathologie Virale des Poissons, France*

Introduction
Hirame rhabdovirus (HIRRV) is one of the four recognized species within the *Novirhabdovirus* genus, represented by the type species *Infectious Haematopoietic Necrosis Virus* (IHNV). HIRRV was first isolated during an outbreak in cultured flounder (*Paralichthys olivaceus*) and ayu (*Plecoglossus altivelis*) in Japan (1). It was also found in other marine fish in Asia, such as stone flounder (*Kareius bicoloratus*) in China (2). Furthermore, it was shown to be pathogenic for a range of salmonid species, including rainbow trout, which were experimentally challenged in freshwater. The major clinical signs of HIRRV infection were congestion of the gonads, focal haemorrhages of the skeletal muscle and fins, and ascitic fluid collection (3).
Here we report the first identification of HIRRV in Europe, which was isolated from grayling and brown trout in a farm in Poland.

Materials & methods
Thirty adult graylings (*Thymallus thymallus*) with clinical signs and thirty asymptomatic adult brown trouts (*Salmo trutta m. fario*) from the same farm in Poland were tested for the presence of novirhabdoviruses by cell culture. Pools of kidney and spleen from a maximum of 10 fish were homogenized. For virus propagation, epithelioma papulosum cyprini (EPC), fathead minnow (FHM), rainbow trout gonad (RTG) and bluegill fry (BF-2) cell lines were inoculated and incubated at 15°C. Cell supernatant was collected for virus identification when cytopathic effect (CPE) appeared, usually 4 to 7 d later.
Starting from RNA extracted from cell culture supernatant, a random-priming sequence-independent single primer amplification (SISPA) was adopted to search for viral sequences (4). PCR products were cloned and sequenced according to the Sanger method.
Transmission studies were carried out on rainbow trout (*Oncorhynchus mykiss*) fry and grayling fry. Virus was propagated in EPC cells and harvested at maximum CPE, about 4-5 days post inoculation (dpi). Experimental fish were kept in 10 l aquaria supplied with freshwater; the temperature was maintained at 10 or 12°C.

Results

72 hours after inoculation, the homogenates from graylings induced a strong cytopathic effect (CPE), suggesting the presence of a virus.

The virus isolated from cell culture induced mortalities in experimentally infected graylings, reaching 10-25% after 21 days. In moribund graylings, light petechiae and congestions in rump muscles and internal organs were observed.

Although some antigenic similarities with perch rhabdovirus (PRhV) were observed, RT-PCR with several sets of generic primers amplifying all fish vesiculo-like viruses gave consistently negative results. Therefore, we used a sequence-independent single primer amplification (SISPA) strategy to obtain and identify viral genomic fragments with similarities to other viruses in GenBank. Surprisingly, of the 60 clones sequenced two of them showed high sequence similarities (> 99%) with either the L gene or the N gene of HIRRV, a viral species that has been reported only in Japan, China and Korea until now. By specifically amplifying the P gene, we observed that the virus exhibited a higher identity with the Chinese strain compared to the Korean. This data suggests that the virus was imported from China, maybe in frozen food.

A specific qPCR was developed and used to demonstrate that the same virus was also present in cell culture inoculated with brown trout extracts from the same farm.

Discussion & conclusions

This study is the first to identify the presence of HIRRV in grayling and brown trout from a fresh water farm in Europe. The European isolate was highly similar to two other Asiatic strains from Korea and China, respectively. The sequence of the P gene revealed a stronger similarity with the Chinese strain, which would be consistent with the hypothesis that the virus was introduced via frozen food imported from China and used at the farm. This finding raises concerns about the spread of the virus out of Asiatic countries and its potential emergence in freshwater conditions.

The virus, once propagated in cell culture, provoked mortalities during an infectious challenge in graylings and rainbow trouts. The conditions of virulence should be further investigated to estimate the epizootic risks in Europe on grayling and other freshwater fish. It must be mentioned that at the same period of viral isolation, a massive mortality occurred in wild grayling in a river in the same region. Although no samples could be analyzed at that time, there are indications of a possible HIRRV outbreak.

We now have the specific diagnostic tools for routine surveillance and investigation of other mortality events caused by HIRRV.

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Minutes:
In 2007 in Poland, mortalities occurred in a fresh water farm, reaching 90 % of the grayling stock. A rhabdovirus was isolated from these grayling, as well as from brown trouts from the same farm. A cooperative work by Poland, France and Denmark was engaged to characterize this virus. First, an infection trial with this virus isolate was performed on graylings and rainbow trouts. Grayling were more sensitive to the virus suffering, at least, a mortality rate of 10-25% following bath challenge, while no mortality was observed in rainbow trout. Second, using sequence-independent single primer amplification (SISPA), the virus was identified as Hirame Rhabdovirus. Partial sequencing revealed that the two isolates from grayling and brown trout were identical while exhibiting 97 % similarity with a Korean isolate and 99 % with a Chinese isolate. A qPCR targeting the N-gene was developed using virus propagated in cell culture. Following the infection trial it was seen, especially for the rainbow trout, that a transient presence of virus could be seen in internal organs.
HIRRV can be considered an emerging pathogen in Europe. Sequence analysis of affected fish from a European freshwater farm shows that the virus found is closely related to a Chinese isolate. Although it has not been proven, the virus might have been imported by feed or other raw frozen product and afterwards exposed to the environment. We stress that laboratories should consider the use of SISPA as it can be a valuable tool to describe new disease complexes. However, in order to obtain good results, a high concentration and a high purity of the pathogen in question are needed.

Questions:
Katja Einer-Jensen: Have you tested feed used on the farm? Could it be a route?

Ewa Borzym: It is a possibility, and we suspected it, but could not get access to the feed used for feeding the grayling at the farm.

Hong Liu: In our experience it is relatively easy to find this virus in marine cold water fish but not in fresh water, mortality seen among marine fish is low. There is only few isolates so identification to Chinese or Korean type is not certain.

Niels Jørgen Olesen: Only two other novi rhabdoviruses are notifiable, this poses a classic introduction of a new pathogen/disease and discussion how to deal with it legally in the context of an emerging disease. What have been done following this finding?

Ewa Borzym: It has not been notified.

Richard Keith Paley: Is it of economic importance?

Hong Liu: In China we have not seen losses which were attributed to this virus.

OUTCOME OF EPIZONE EXTENSION ON VER/VNN: Pathogenicity study of 10 betanodavirus strains with an *in vivo* challenge in European sea bass (*Dicentrarchus labrax*)

Niccolò Vendramin[1,2], Anna Toffan[2], E. Cappellozza[2], M. Mancin[2] & G. Bovo[2]
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[2]*Division of Comparative Biomedical Sciences. Fish Virology Department, Istituto Zooprofilattico Sperimentale delle Venezie, Italy*

Abstract:
Viral encephalopathy and retinopathy (VER), otherwise known as viral nervous necrosis (VNN), is a severe pathological condition, caused by small RNA viruses belonging to the Nodaviridae family, genus Betanodavirus. The disease, which has been described in more than 45 fish species worldwide, is considered the most serious viral threat affecting marine farmed species in the Mediterranean region, thus representing one of the bottlenecks for further development of aquaculture industry.
Epidemiological investigations carried out in different geographical areas has demonstrated that Betanodavirus can be detected in wild fish as well as other aquatic organisms (artemia, rotifers, molluscs and crustaceans) in addition to farmed fish.
The RGNNV genotype is the most widespread in the Mediterranean region. Interestingly some strains, characterized by containing genetic material belonging to both the RGNNV and the SJNNV genotypes in their genome, have also been identified. The existence and the spread of these genetically different viral agents that share inter-genotype genetic material could be one of the major causes for the differences in observed mortality in the field.
In order to contribute to a better understanding of the pathogenicity of circulating viruses, ten selected VER/VNN strains differing in origin and/or genotype were tested "*in vivo*" by challenging sea bass juveniles in infection trials. The infection was performed under controlled conditions and all the infected groups were monitored for 68 days after infection. The results clearly confirmed the pathogenicity, to different degrees, of all the selected strains, including one strain belonging to the SJNNV genotype and four reassortant strains from SJNNV and RGNNV genotypes and underlined the potential risk represented by sea bream and other apparently resistant species in the transmission of the disease to other highly susceptible species.

Minutes:
Ten betanodavirus strains were tested in an infection trial using sea bass, the isolates originated from different geographical areas and from different fish species. Three isolates were considered to be highly pathogenic. Dead fish were collected continuously, and random sampling were done at 8, 44 and 68 days post infection. At day 8, the day before onset of mortality, the sampling proved positive for VER/VNN. Differences in mortality were seen among isolates, and it was seen that virus isolated from other species than sea bass could be highly pathogenic to sea bass highlighting the possible low host-specific nature of this virus. Growth of fish was measured and this revealed that not only does the virus have an impact on average weight, but the fish showed uneven growth to a high degree.

Questions:
Richard Keith Paley: what was the number of replicates?

Niccolò Vendramin: one group per strain.

Richard Keith Paley: Is that strong enough for conclusions?

Niccolò Vendramin: The statisticians involved in the experimental design agreed that with this set up the test was robust enough.

Katja Einer-Jensen: what is the normal growth temperature of sea bass?

Niccolò Vendramin: around 20 degree.

Katja Einer-Jensen: is 25 degree then stressful for sea bass?

Niccolò Vendramin: not really, the temperature range of this fish is rather wide.

OUTCOME ON EPIZONE EXTENSION ON VER/ VNN: Diagnostics, proficiency test and qRT-PCR validation

Laurent Bigarré[1], Valentina Panzarin[2], Marine Baud[1], Soren Peter Jonstrup[3], Eva Jansson[4], Mats Isaksson[4], Marc Engelsma[5], Niels Jørgen Olesen[3] & Giuseppe Bovo[2]
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Introduction

Betanodaviruses are genetically very diverse with at least five genogroups described and a large range of variants within each group, including reassortants carrying components of two groups (Panzarin *et al.*, 2011; Toffolo *et al.*, 2007). Their genome is composed of two strands of RNA: RNA1 and RNA2. Till now, only one universal real-time RT-PCR method has been published which detects all variants of RNA2 (Panzarin *et al.*, 2010) in clinical cases. An additional method based on detection of RNA1 would be useful to confirm results obtained from RNA2. Another advantage of detecting RNA1 is to bring valuable genetic information on the second genome component of a given isolate. ANSES has developed new DNA probes targeting RNA1 with the goal of detecting all genotypes of nodaviruses. The aim of the project is thus:

- To organize, conduct and report an inter-laboratory proficiency test for detection of aquatic nodaviruses by real time RT-PCR targeting RNA1 and RNA2, respectively.

- To test a newly developed real-time RT-PCR targeting RNA1: sensitivity, specificity, range of detection and genetic information provided by sequencing the PCR product.

Materials & methods

Primers and probes specific for RNA1 and RNA2 were ordered by ANSES and aliquots from the same batch were provided to all partners. IZSVe produced for all partners 11 samples (1-11) of inactivated isolates produced in cell culture and covering 4 genogroups. The samples were forwarded by ANSES and RNA had to be extracted by each partner. In the meantime, ANSES produced and distributed RNA extracted from healthy or infected fish (2 genogroups), or cell culture; one sample from cell culture had to be serially diluted to test the sensitivity of each method in partners' hands. Samples were tested in duplicates and the mean Ct values reported.

Results

A total of 192 virus-containing samples were tested (negative controls excluded), implicating 5 partners performing each two or four methods (16 tubes with virus * 12 methods). For each genetic component (RNA1 or RNA2), the same ratio of detection (80 / 96) was found, indicating that both RNA1 and RNA2 probes are globally equivalent for detection of all genotypes. When the samples sent were considered, the rate of false positives was 5,5% (2 / 36) and the rate of false negative 13,5% (31 / 228). Both RNA1 and RNA2 proved to be valuable targets for viral detection, with advantages and disadvantages depending on the method and the viral isolates.

Conclusions
Both sets of the nodavirus probes proved to be useful for detection of betanodaviruses in each lab's usual conditions. Ideally, both RNA should be targeted for samples exhibiting high Ct values (for instance in healthy carriers) to confirm the presence of low loads of virus.

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Minutes:
Betanodavirus is a highly variable group of viruses, which poses some challenges both with respect to getting the right diagnosis and the genogrouping of the viruses. Depending on the purpose, several methods, have been developed, all targeting RNA2, with low or high genogroup specificity. It would be of interest to target RNA1 too, in a sensitive generic test. We have designed such a qPCR alternative and asked 5 different laboratories to test the new RT- qPCR, in parallel with a generic test targeting RNA2. Both the RNA1 and the RNA2 qPCR showed consistent results in the detection of the various genotypes. However, the RNA1 RT-qPCR is not able to detect the TPNNV genotype and the RNA2 RT-qPCR needs to be in two steps (RT + qPCR).

Questions:
Uwe Fischer: Did you try multiplex PCR?

Laurent Bigarré: No, we will do that later.

Katja Einer-Jensen: what is the level of variation?

Valentina Panzarin: Very high, up to 70-80 %.

Richard Keith Paley: What was the level of degeneration

Laurent Bigarré: 1 degeneration

Katja Einer-Jensen: Is it true reassortment?

Laurent Bigarré: Yes.

FISHPATHOGENS.EU/NODA:
A new database for Betanodavirus targeted research

Valentina Panzarin & Giovanni Cattoli
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Abstract:
In June 2009, the European Union Reference Laboratory for Fish Diseases launched the fishpathogens.eu database for Viral Haemorrhagic Septicaemia Virus (VHSV), which was later extended to also include Infectious Haematopoietic Necrosis Virus (IHNV). These tools have proved valuable for those involved in fish virology, and ongoing works aim at extending the database to also include other fish pathogens e.g. *Betanodavirus*, the causative agent of Viral Nervous Necrosis (VNN). The severity of VNN and its wide extent in terms of both geographical distribution and host range, highlights the need of a platform for epidemiological and molecular data sharing. The DTU, IZSVe and ANSES are working in this direction by developing a freely searchable database which will include both sequence data and epidemiological information (e.g. host species, geographic origin, etc) related to each betanodavirus strain. This contribution is intended to present the main features of the Fishpathogens.eu/noda database and its possible applications.

Minutes:
Part of the EPIZONE extension was aimed at including betanodavirus into the Fishpatogens.eu database. This database has proven of much value in data sharing, storage and research purposes for VHSV and IHNV. Betanodavirus has now been added to the database providing new opportunities to extract data for the pathogen. We would like to stress the importance of input of data from other users into this database as it will improve our knowledge and give valuable information about disease spread.

Questions:
No questions

GENOTYPE-PHENOTYPE RELATIONS AMONG PARENTAL BETANODAVIRUS GENOTYPES AND REASSORTANT STRAINS

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Abstract:
Betanodaviruses are small, naked viruses with a bi-segmented genome: the RNA1 molecule encodes the viral polymerase and the RNA2 segment encodes the coat protein. In Southern Europe the presence of the RGNNV and the SJNNV betanodavirus genotypes has been reported. More recently, the existence of natural reassortants (e.g. RGNNV/SJNNV and RGNNV/SJNNV) containing genomic segments deriving from the two parental genotypes has been reported in the same area. Among the different environmental factors, water temperature seems to play a key role in determining the appearance of the disease and its severity. Interestingly, *in vitro* experiments also highlight that temperature dependency of betanodaviruses correlates to genogrouping, as well as CPE severity and replication efficiency in cell monolayers. However, the phenotypic features of natural reassortants are still poorly known. The results obtained in our laboratory on the genetic and *in vitro* phenotypic characterizations of RGNNV, SJNNV, RGNNV/SJNNV and RGNNV/SJNNV betanodavirus strains will be presented and discussed.

Minutes:
We have looked at 23 reassortment strains of RGNNV and SJNNV and applied reverse genetics to investigate the relationship between genotype and phenotypic characteristics.

Starting from 4 wild isolates (2 parental and 2 reassortants) a growth experiment was performed at various temperatures in order to understand their replication efficacy. The RGNNV genotype showed normal growth at 30 degrees celsius. Reassortant strains showed that the temperature dependent growth is regulated by the RNA1 segment and that higher temperatures enhance virus propagation for betanodavirus with a RGNNV type RNA1.

Questions:
Mohaddes Ghasemi: I have been working with a nodavirus outbreak in mullets living in 15-20 °C water. I could not grow virus at 15-20 °C but at 25°C. Do you have an explanation?

Valentina Panzarin: There are differences between in vivo and in vitro situations. 25°C is preferable for *in vitro* growth.

Mohaddes Ghasemi: I could not find vacuolation in the brain but only in the retina.

Valentina Panzarin: This can also vary however there is a need for a study on tissue tropism in these viruses.

Mohaddes Ghasemi: We have also seen mortality in sturgeon infected with nodavirus**.**

Helle Frank Skall: At which temperature do you do your titration?

Valentina Panzarin: At 25°C.

Katja Einer Jensen: Is it the same isolates Niccolò used for his infection trial?

Valentina Panzarin: Yes.

VALIDATION OF AN ANTIBODY ELISA FOR INDIRECT DETECTION OF KHV

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Abstract:
According to the recommendation published by OIE, we conducted a trial for validation of a serological assay with the main goal of producing an "antibody ELISA" for Koi Herpes Virus. The focus was on:

- preparation and characterization of antigens for ELISA
- choice of ELISA plates
- serum dilution used for antibody detection
- investigation of the assay composition
- determination of the "cut-off" of the assay
- investigations on inter and intra assay and plate variations
- investigation of personal assay and plate variations
- investigations of assay variations (sera inactivation)
- investigations of used control panels (positive, negative, assay) on each plate
- investigations of specificity and sensitivity in comparison to serum neutralization and immunofluorescence
- assessments and their variations

The time and money consuming process was partly carried out by the EU project "Epizone WP 6.1. (Denmark)". Within this project, different European laboratories (Germany, Denmark, Italy, Sweden and France) as well as a Chinese laboratory exchanged materials (sera, viruses, buffers, methods, results) and carried out two serological ring tests (Epizone WP 4.5, KHV serology, France).
It could be shown that serology is a very good tool for investigation and monitoring on the farm level. Suspicious results obtained by serum neutralisation tests or ELISA were confirmed or declined by immunofluorescence.

More investigations are urgently needed to increase the knowledge of different phases of KHV infection or KHV Disease. These are:

- antibody development in different fish
- antibody kinetics in carp
- antibody persistence over seasons, years and fish
- antibody development after superinfection, immunization and viral reactivation.

Minutes:
We have developed an antibody ELISA to test carp sera for antibodies against Koi Herpes Virus. All aspects of critical points which could be of importance for the technique were evaluated for optimization and a list of most optimal procedures, utensils and chemicals were produced. The ELISA was developed within EPIZONE; IFAT and neutralization assays for the use of detecting carp antibodies to KHV were also tested. The overall results show that when having suspicious sera

ELISA complemented with IFAT and neutralization assay, but especially IFAT, is a good tool to screen for KHV infection on a population level. However, we still lack a lot of information about the antibody kinetics in the individual fish and how the level of detectable antibodies will fluctuate over time in a given infected population.

Questions:
Tomas Vesely: I think 5 minutes is a too short time before stopping the reaction.

Sven Bergmann: We have suggested a protocol that is optimal for us, you can of course modify according to your needs and optimal situation in your lab.

Anna Toffan: Did you try the possibility to store plates after coating?

Sven Bergmann: You can keep the plates for months in the fridge.

Niels Jørgen Olesen: There have been some problems with antigen production.

Sven Bergmann: We are looking into that, which parts of the antigen is the best to use, in order to produce recombinant peptides suitable for reagent standardization.

Niels Jørgen Olesen: It is not exactly clear how the ELISA results are interpreted. I will urge you to use your fish antibody as its own negative control.

Sven Bergmann: That is also how the procedure will be recommended; moreover we established a cut off value to discriminate between positive sera, doubtful and negative ones.

TRAINING SESSION ON KHV SEROLOGICAL METHODS

Cabon Joëlle, Lamour François, **Morin Thierry**[1] & Castric Jeannette.
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Abstract:
Koi herpesvirus disease, KHVD, is a highly contagious disease responsible for heavy losses in carp ponds throughout the world. The disease is listed as a non-exotic serious disease in EU and as a notifiable disease for the OIE. The virus (KHV or CyHV-3) is suspected to be in a latent phase in asymptomatic carriers but its presence is difficult to put in evidence using the techniques available (PCR). Serological methods were developed during the Epizone project for the detection of antibodies against KHV in order to improve the surveillance and control of KHVD and to establish a more reliable sanitary status of carp populations.
The aim of this presentation is to provide you with information about a training session focused on the transfer of these methods to European laboratories. This session was organized in October 2011 within the Fish Viral Diseases Unit of the French Agency for Food, Environmental and Occupational Health Safety (Anses) located in Brest.
During this session, the two methods developed, a seroneutralisation (SN) test and an indirect ELISA, were presented to the participants. For the SN test, each participant did his own analysis on 10 sera. For the indirect ELISA test, a member of our lab did a demonstration. All the results were then analyzed and discussed.

Minutes:
Following the EPIZONE project a workshop on serological methods was held in Brest with participants from France, Sweden, Italy and Denmark. Before the workshop a ringtest for the participating countries had been performed, testing a panel of sera. At the workshop the ELISA technique and neutralization test were demonstrated on known sera and the participants did parts of the techniques themselves with follow-up discussions about the results. In general there was coherence between the participating laboratories with respect to evaluating the sera positive or negative; however, there are differences between individuals in the quantitative evaluation of positive reaction.

Questions:
No questions.

CD 2006/88/EC – a TOOL BOX FOR the control of CARP Diseases KHV-MEETING IN ZWETTL / Austria, 28.-30. March 2012

Andrea Höflechner-Pöltl[1], Gunnar Graber & **Oskar Schachner[2]**
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[2] *Fish Medicine and Livestock Management Dept. for Farm Animals and Vet. Public HealthUniversity of Veterinary Medicine*

Abstract:
In some carp producing countries there are objections to the practicability of the EU directive CD 2006/88/EC concerning the control of carp diseases as KHV-I. In order to meet some of the concerns a short meeting has been arranged in Lower Austria.
For traditional carp farming countries the actual legislation of KHV control measures and eradication according to the directive is inconsistent with the conservative near-natural method of carp production and contravenes with nature protection. In case of infection the directive can't be fully implemented without high economical and ecological losses.
Together with the representative of the EC various stakeholders and experts from 10 countries concerned should find common proposals for a more appropriate European legislation generally applicable in common carp farming. Furthermore they should discuss issues concerning the global challenge of KHV control in ornamental fish trade as well as the diagnostic confusions around KHV, especially in clinically healthy fish.
Veterinary representatives from 6 countries gave an account of regional fish production, the status of infection and the impact of KHV as well as the control measures in their respective countries. With the exception of Hungary the virus has been detected in all countries already. However in whole Europe the status of most carp farms is unknown. Corresponding to the etiological impact attributed to KHV, the efforts spent in the fight against the virus highly vary between the countries.
Subject-specific presentations concerning fish farming, diagnostics, ornamental fish and legislation of KHV control have been given by experts. Some of the problems and perspectives addressed have been discussed in 4 groups.
Concordantly urgent need has been expressed at 2 different levels: on the one hand the need of a more flexible legislation of control measures taking regional conditions of carp breeding into account and the need of a strict global harmonization of diagnostic tools for the detection of KHV and the surveillance of clinically healthy fish on the other hand.
For KHV prevention in ornamental fish trade, minimal measures in case of a KHV disease outbreak should be harmonized and implemented as should be the prevailing or adapted directives concerning trade and import. For EU fishmongers importing animals from third countries authorization and self control should be obligatory, and an official surveillance is suggested.
Diagnostic tools recommended for the detection of KHV are real-time PCR, and for confirmation nested or semi-nested PCR including a sequence analysis. Organ pools tested should contain material from a maximum of 5 fish showing signs of disease and only 2 fish without pathological findings. Questions concerning the cost-benefit balance of detecting as well as combating diverse variants of virus and the diagnostic troubles concerning the use of serological methods and reliable PCR protocols for detection of all variants of KHV remained open.
However pleading for the EU commission's case Sigrid Cabot was talking about an Animal health strategy in consideration of carp breeding, themed "prevention is better than cure" and presented general tools to increase awareness and preparedness concerning KHV disease.

In general the meeting contributed to a more complex understanding of the special conditions of carp farming and carp disease control.

Questions:
Olga Haenen: Did you discuss cases regarding production of carps for consumption?
Oskar Schachner: Yes, and here the legislation has a huge impact on farm level.
Olga Haenen: Recently also in the Netherlands KHV has been detected in clinically unapparent common carps.

WORKSHOP "KHV DIAGNOSTICS" IN ZWETTL (Austria) on March 30th 2012

Sven M. Bergmann
Friedrich-Loeffler-Institut (FLI), Federal Research Institute for Animal Health, Insel Riems, Germany

Abstract:
From March 29th to 30th 2012 a seminar "New animal health legislation with special attention to carp production in Europe" was held in Zwettl, Austria.

The main goal was to get an agreement within Europe on how to react against the threat KHVD. The presentations ranged from presentation of the recent situation in different European countries to the pathogenesis of KHVD.
My task was to lead a workshop on "KHV diagnostics". At the workshop colleagues from Denmark, Czech Republic, Hungary, UK, Slovenia, Austria and Germany attended. In discussions agreements were achieved on:

1. Suspicion of KHVD and its clarification in a carp population
2. Confirmation of a KHVD case
3. Diagnostic assays for detection of KHV in carp tissue samples
4. Sampling
5. Further possible diagnostic methods for KHV detection
6. Further open questions

Some still open questions were:
What do we understand when we are talking about KHVD?
How we can really combat KHVD?
Is vaccination a helping tool against KHVD?
How far has KHV spread within the carp population in Europe?
Which are the best tools for diagnostics? etc.

Minutes:
Suspicion of a case will be the presence of disease with classic clinical and pathological manifestation of KHVD or an epidemiological connection to an already confirmed case of KHVD. Confirmation of a KHVD case can be by demonstrating genomic DNA by PCR, detecting antigen by antibodies with IFAT or ELISA techniques or isolating virus in cell culture. For diseased fish up to 5 individuals can be pooled for diagnostic purposes, but for monitoring and surveillance a maximum of 2 fish should be pooled. When sampling dead fish, gill and kidney should be included. Alternatively non lethal sampling could be applied by gill swaps or from faecal material, however this most likely needs some activation of virus to be sensitive. Research is needed to evaluate to what level of confidence these methods can supply the correct answer. Finally, serodiagnostics is now a possibility, further research and practical experience is however still needed to evaluate how and when it is timely to use these tests.

Questions:
No questions.

PANCREAS DISEASE CAUSED BY SAV2-like VIRUS IN ATLANTIC SALMON IN NORWAY

Monika J. Hjortaas, Hanne R. Skjelstad, Torunn Taksdal, Anne Berit Olsen, Vik-Mo Frode, Britt Bang Jensen, **Irene Ørpetveit** [1]& Hide Sindre.
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Abstract:
Pancreas disease (PD) affects salmonids and is caused by a salmonid alphavirus (SAV). In Norway, the disease is endemic along the southwest coast, and only sporadically detected north of Hustadvika, near Kristiansund, in the Møre and Romsdal county. There are at least 6 genotypes of SAV, and until very recently, only SAV3 had been detected in Norway.
In April 2011, a sea farm with Atlantic salmon was diagnosed with PD based on histopathological findings and detection of SAV by real-time RT-PCR. This farm was situated just north of the endemic zone. Characterization of the viral genes E2 and nsP3, followed by phylogenetic analysis, revealed that the virus differed from SAV3, rather it was more closely related to a SAV2 variant previously detected in Atlantic salmon from Scotland. However, the new Norwegian, as well as the Scottish, SAV differed from the classical SAV2 found in rainbow trout in connection with sleeping disease.
In the autumn of 2011, new cases of PD caused by the new SAV-variant were diagnosed in farms in both Møre and Romsdal and in South Trøndelag counties. SAV-positive samples submitted in 2010 were examined, and we found that one of these outbreaks, in a fish farm in Møre and Romsdal within the endemic zone, was also caused by the new SAV-variant.
Further studies have been initiated, aiming at mapping the distribution of the virus, determine the time of introduction, and to identify the transmission routes. In addition, an experimental infection study has been initiated, in order to further characterize the new SAV variant with regards to virulence.

Minutes:
No minutes.

Questions:
Helle Frank Skall: Is it possible that well boats are responsible for spreading the disease further north than Hustadvika?

Irene Ørpetveit: Well boats are not allowed to sail further north than Hustadvika

MOLECULAR TRACING OF VIRAL PATHOGEN IN AQUACULTURE (MOLTRAQ): a new EMIDA project

Britt Bang Jensen[1], Magne Aldrin[2], Jean C. Avarre[3], Sven M. Bergmann[4], Laurent Bigarré[5], Edgar Brun[1], Peder A. Jansen[1], Niels J. Olesen[6], Tristan Renault[7] & Heike Schuetze[4]
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Abstract:
Here we present a new research-project funded under the EMIDA-ERA Net under the EU 7th Framework program (For more details about EMIDA: www.emida-era.net).
The purpose of the project is to increase knowledge of transmission, prevention and control of viral diseases in aquaculture and develop a generic approach to viral disease control by using information on epidemiological and phylogenetic attributes from several important aquatic animal viruses.
The project will i) generate and use spatio-temporal epidemiological data, phylogeographic data and gene expression data for important host-viral pathogen systems to identify important factors affecting the spread of diseases in aquaculture, and ii) integrate these in scenario simulation models to assess effects of various control strategies for selected host-pathogen systems.
The project consists of six workpackages: WP 1: Project co-ordination and consortium management; WP 2: Collection of virus sequences and epidemiological data; WP 3: Phylogeny and evolution of viruses; WP 4: Investigation of the effect of temperature on gene expression patterns; WP 5: Scenario simulation models for control options and WP 6: Dissemination and exploitation.
Partners into the project are: Norwegian Veterinary Institute (NO, Coordinator), Technical University of Denmark-National Veterinary Institute (DK), Agence Nationale de Sécurité Sanitaire (FR), Friedrich-Loeffler Institut (DE), Institut Francais de Recherche pour l'Exploitation de la Mer (FR), Institut de Recherche pour le Développement (FR) and Norwegian Computing Center (NO).
The project began on April 1st, 2012, and will run until March 31st, 2015. The total budget is 1.9 M€, of which 1.4€ is funded via the EMIDA-ERA Net.

Minutes:
The workpackages in the project are interwoven in a way that WP2 contribute to WP3 etc. and some of the interaction will also interact across WP's. Output from the project will be implemented into Fishpatogens.eu for further evaluation and consolidation.

Questions:
Valentina Panzarin: How is it planned to perform the evolutionary parts.

Britt Bang Jensen: I don't know, this has not been thoroughly planned in details yet.

Sven Bergmann: I think that one of the things we had in mind was looking at some of the isolates from early days of isolation and until recently.

Niels Jørgen Olesen: In this context I think we need to stress that we in the project group do not necessarily have access to all the relevant data to get the best out of the proposed work. And hopefully there will be interaction with others to get the relevant isolates.

Niels Lorenzen: How are you planning to do WP4, in fish or in cell culture?

Britt Bang Jensen: It will be easiest to work in cell culture. However, it is not really defined yet. It will most likely not be by using infection trials.

Niels Lorenzen: At which level will WP5 be done?

Britt Bang Jensen: On a zone level. Not farm or individual level. However it will be depending on available data.

Olga Haenen: Will there be a website?

Britt Bang Jensen: No, due to budget cuts this was taken out.

Note: After the meeting, we found a way to have a cheap website. The address will be:
http://moltraq.wordpress.com/

INACTIVATION OF VHSV BY PERCOLATION AND SALT UNDER EXPERIMENTAL CONDITIONS

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[2]*DHI Hørsholm, Denmark*

Abstract:
At the moment the only legal method in Denmark to sanitize wastewater from fish cutting plants is by percolation. To evaluate the inactivation effect of percolation on VHSV an experimental examination was initiated. A column packed with gravel as top- and bottom layer (total of 22 cm) and a mid layer consisting of dug sand (76 cm) was used for the trial. Over a period of 18 h 3.9 x 10^{10} $TCID_{50}$ VHSV was supplied to the column, where after normal tap water was supplied for the rest of the trial period, in total 7 days. During the 7 days samples for virological examination was taken. The sampling was most intensive in the period where the risk of VHSV breaking through the column was highest. The sensitivity of the virological examination was 13.9 $TCID_{50}$/ml and no virus was isolated. A reduction of VHSV > 4 log in the outlet water was seen. This experiment suggests that percolation can be a valuable method to sanitize VHSV infected water. Changes in temperature, pH, earth types in the area used for percolation etc. may change the virus reduction, though.
As some of the fish cutting plants are also smoking rainbow trout fillets, the question arose whether a brine solution will inactivate VHSV. In order to answer this question a small trial was set up. VHSV and NaCl was added to cell culture medium with 10% foetal bovine serum, in order to mimic a "dirty" environment, to obtain from 1.9% to 20.9% NaCl and kept in the dark at 4°C. Samples were titrated after 5 min, 1 h and 20 h. No reduction in titre was observed in any of the samples.

Minutes:
Following addition of water with a low NaCl concentration to the column, measuring the conductivity can be used to follow the flow of water through the gravel in the column. Following our trial we only have limited information about virus inactivation within the gravel in the column. The content of the column was investigated 10 months after end of trial by cell culture with negative results. It could be interesting to look into what is happening in the column shortly after the adding virus, whether the virus is trapped or inactivated

Questions:
Uwe Fischer: In real life can there be a risk of flushing of from the surface, contaminated water ending up in a stream?

Helle Frank Skall: In Denmark the water is led out below the earth surface.

Niels Lorenzen: Did you saturate the column with water before adding virus water?

Helle Frank Skall: Yes.

Niels Lorenzen: Did you perform ELISA or which test did you use for evaluating binding of the

virus in the column?

Helle Frank Skall: No we didn't perform any ELISA, but results from Japan suggest that IHNV binds to the surface of clay and can in some cases still be infective after 9 weeks.

??? Does the height of the column correspond to the height in real life?

Helle Frank Skall: under real conditions the column height would be higher.

Tomas Vesely: Did you look into pH?

Helle Frank Skall: No, but it could be done. The pH might change the charge of particles and thereby change how effective virus particles are bound.

SESSION V: Update from the EURL

Chair – Niels Jørgen Olesen and minutes: Niccolò

EURL ACTIVITIES IN 2011

Niels Jørgen Olesen, Helle Frank Skall, Nicole Nicolajsen, Søren Peter Jonstrup,
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The duties of the EURL are described in Council Directive 2006/88/EC (Annex VI). The duties mainly concern fish diseases listed as exotic diseases: EHN and EUS; and fish diseases listed as non-exotic diseases: ISA, VHS, IHN, and KHV disease.

Activity report:

The *15th Annual Meeting* of the National Reference Laboratories for Fish Diseases was held 26-27 May 2011, in Aarhus, Denmark. A total of 52 participants from 33 countries attended over the two day period. There were four sessions with a total of 33 presentations, 9 of which were given by invited speakers. The meeting was held at rented nearby facilities (Søauditoriet) of Aarhus University, as the premises at our institute in Aarhus are too small for the number of participants. A report was submitted in August 2011.

In 2011 we were granted financial support to hold a *workshop in Surveillance and Epidemiology of Aquatic Animal Diseases*. The workshop was organised in close collaboration with the OIE Collaborating Centre for Aquatic Epidemiology and Risk Assessment, constituting of scientists from the Norwegian Veterinary Institute. The Norwegian Veterinary Institute took on the responsibility to plan the scientific programme while the EURL focused on the practical arrangements. The EURLs for Molluscs and Crustacean Diseases were involved as well reflecting that the workshop covered all aquatic animals. The workshop was held 23-24 November, 2011 and took place in the auditorium of the National Veterinary Institute in Copenhagen. A total of 50 participants from 25 countries and 5 invited experts attended the workshop.

Again this year an *inter-laboratory proficiency test* was distributed to the NRLs mainly within the EU but there were also participants from countries outside EU. See following report.

An important focus of the EURL is *to update the standard operating procedures of the non-exotic and exotic listed diseases*. Diagnostic manuals for VHS, IHN, ISA, KHV and EHN are now available at the EURL web page. A diagnostic manual for EUS is under preparation and will be included within the coming months. Unfortunately the manual on sampling and diagnostic procedures for the listed diseases has still not been adapted by the EU, the diagnostic methods therefore still relies on the former Commission Decision 2001/183/EC for VHS and IHN and 2003/446/EC for ISA while no legislative text exist for KHV, EHN and EUS. Significant resources were given to the implementation of diagnostic methods for EUS which will soon be in place and updated on our website.

Another important focus area was the *development, implementation and validation of diagnostic tools for identification of the listed diseases and their accreditatio*n. One outcome of these efforts was the publication on the generation of a real-time RT-PCR assay for detection of all genotypes of VHSV that has been proposed for the OIE to be used as an alternative to surveillance for VHS by cell cultivation.

During 2011, resources were also used to *collate data on surveillance, health categorisation, and diagnostics in EU*; to *identify and characterise selected virus isolates*; to *type, store and update a*

library of listed virus isolates; to *develop, update and maintain the database containing information on fish pathogens (www.fishpathogens.eu)*; to *supply reference materials to NRLs*; to *provide annual training courses in laboratory diagnosis and missions to other NRLs*; to *produce antisera; to prepare SOPs for detection of EUS; to update the EURL webpage (**www.eurl-fish.eu**)*; and finally to *attend international meetings and conferences*.
The permanent staffs of the Section for Fish Diseases in Aarhus, Denmark consists of approx. 22 academic and technical staff, primarily involved in research, diagnostics and consultancy with special focus on fish virology.
Unfortunately the activities of the EURL were affected in early spring 2011 by the information from the DTU vice-chancellor announcing the movement of our institute facilities in Aarhus to the Copenhagen area in 2012. For this reason our valued coordinator of the EURL Dr. Søren Kahns got another position and left our group 31 December 2011. Our future situation is still unsettled and decisions on the frame and placement of the continued work will be taken spring/summer 2012. We sincerely hope the continuation of this important function will be made possible in our future premises.

Minutes:
Activities achieved in 2011/2012 and proposal for 2013 have been explained and detailed.
2011 has been a difficult year because of the decision of closing the house down, this caused some changes in personnel: Susie Sommer Mikkelsen and Niccolò Vendramin replaced Søren Peter Jonstrup and Søren Kahns, respectively.

Between the EURL function the activity related to TRAINING COURSES have been underlined and will be further detailed in the following presentation.

In the Proficiency Test (PT) this year has been included *A. Invadans* for the first time.
Planning and organization of PT: in 2012 it will also include SVCV as well as VHSV, IHNV, EHNV, ISAV and KHV.

When it comes to the specific activity of establishing and maintaining a library of collected isolates and samples from Europe, in 2011 a tissue collection from infected fish was stored. Torsten Snogdal Boutrup infected rainbow trout and collected organs individually and stored them in tubes for assay development.

Concerning molecular epidemiology a paper on how Danish VHS isolates belonging to genotype Ia clusters into two different populations has been published.

A Workshop in surveillance and epidemiology, organised primarily by OIE collaborating office for Aquatic animal health epidemiology (Dr. Britt Bang Jansen) has been held during 2011.

When it comes to diagnostic manuals the VHS, IHN and ISA manuals follow OIE recommendations, but there are so many different ways to perform their diagnosis, so there is the need to harmonize methods and techniques within the EU.
Manuals related to VHS (with a new qPCR for VHSV), IHN and EHN were uploaded on the EURL website.
The KHV manual has been uploaded but will be improved with further recommendations.
The EUS manual, improved in collaboration with club 5, is almost ready, and will be uploaded during the next few weeks.

Sampling procedures is a subject that will need to be addressed and focused on in the future.
Also, the fishpathogen.eu database will be updated and expanded.

The program for 2012 will be very similar. There are some new chapters as the budget issue has been reorganized; the program will not be as ambitious as in 2011 as there will be problems with moving laboratories.

The new qPCR for VHSV detection now implements the new version from the OIE manual and will also be included in the EU diagnostic manual. This is a big milestone we are passing through, we are very close to getting this method accepted by the OIE for surveillance purposes.
More time will be required in order to establish harmonised validated protocols to use qPCR for surveillance purposes of other viral fish pathogens.

Suggestions from the participants are welcome:
Sweden: no real suggestion, the course in 2012 was really appreciated. Maybe it would be interesting to get more into the deep of some of the subjects like risk analysis.

Norway: What about a workshop on molecular tracing?

UK and Germany: the topic we would like to address is tissue sampling in connection with an harmonised diagnostic manual.

The Netherlands: UTM universal transport medium, increase tracing work from the fish to results to avoid contamination and to make it more efficient and cheaper. Efficiency in sampling and processing after sampling.

Denmark: One topic could be non lethal sampling.
This year TAIEX didn´t cover expenses, this fact reduced the number of the AM participants.

We would underline that is important to PROMOTE this meeting so that as many as possible can take part next year.

TRAINING COURSES

Two courses (molecular biology and classical virology) increase knowledge for fish diagnosis, provide a forum for discussion etc.

PCR
The aim should be more critical, evaluate the work with a hands on and theoretical session. Evaluation was really good, all the teachers were seen as very good, providing relevant information and the teacher was really prepared. The course increased participants´ knowledge on PCR, qPCR and phylogeny.
One remark was asking for more phylogeny and sequencing.

For general virology and immunochemical methods
Cell culture, ELISA, IHC and IFAT have been demonstrated.
Participants got the possibility to try a small PT within the course.
For all methods demonstrated good marks were given in the evaluation (ELISA IFAT and IHC).
General remark is that more time is needed.

Ideas for topics and subject to address in the new course:

Olga Haenen: Eel virus workshop, willing to cooperate and prepare it.

Niels Jørgen Olesen: The commission wants us to focus on notifiable diseases.

Vlasta Jencic and **Thomas Wahli**: We want to ask for more serology.

Niels Jørgen Olesen: I think there are available quite validated methods.

Sven Bergmann: Why don't we include serology in workshop about non-lethal sampling?

Niels Jørgen Olesen: This topic will be for training courses (held week 4 and 5).We will start planning it after summer, in September you will receive some indications.

Britt Bang Jansen: What about a training course on challenge trials? It is actually a huge topic, it would be useful to harmonise protocols in order to compare results.

Niels Jørgen Olesen: It could be also in collaboration with VESO Vikan, maybe could be a good idea.

EURL WORKPLAN FOR 2012

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National Veterinary Institute, EU Reference Laboratory for Fish Diseases, Technical University of Denmark

The work plan for the current year is as follows:

WORK PROGRAMME FOR THE EUROPEAN UNION REFERENCE LABORATORY FOR FISH DISEASES, 2012

The functions and duties of the European Union Reference Laboratory for Fish Diseases (EURL) are described in the Council Directive 2006/88/EF Annex VI part I

1. Coordination and training

1-1 Organise and prepare for the 16th Annual Workshop for the National Reference Laboratories for Fish Diseases (NRLs) in 2012.
1-2 Produce a report from the Annual Workshop 2012.
1-3 Collect and report data on the fish disease situation in EU, including all the listed non-exotic fish diseases given in Council Directive 2006/88/EC Annex IV Part 2.
1-4 Facilitate and provide training in laboratory diagnosis. The yearly training courses in methods used for diagnosis of fish diseases will be offered at the EURL laboratory facilities. The courses will primarily be for training of staff from NRLs and the content will depend on requests from participants.

2. Proficiency test

2-1 Prepare the Annual Inter-laboratory Proficiency Test year 2012 for the NRLs. The test will include VHSV, IHNV, EHNV, ISAV, KHV and *Aphanomyces Invadans*.
2-2 Collate and analyse information gained from the Inter-laboratory Proficiency Test.

3. Reagents and products

3-1 Supply reference reagents to the NRLs in member states.
3-2 Production of antisera against selected isolates when necessary.
3-3 Update and maintain a library of isolates of Infectious Salmon Anaemia virus (ISAV), Viral Haemorrhagic Septicaemia virus (VHSV) and Infectious Haematopoietic Necrosis virus (IHNV), Koi Herpes virus (KHV) and Enzootic Haematopoietic Necrosis virus (EHNV) and *Aphanomyces Invadans*.
3-4 Maintain a library of tissue material from fish infected with listed pathogens.

4. Scientific advice and activities

4-1 Update the webpage for the EURL, www.eurl-fish.eu.
4-2 Update the diagnostic manuals for VHS, IHN, ISA, KHV disease, EHN and EUS on the EURL web page.
4-3 Collect information on strain variation occurring within pathogens causing the listed diseases VHS, ISA, EHN and KHV disease and provide recommendations on how to discriminate between various strains.
4-4 Identify and characterise selected isolates of listed viruses (serological and genetic characterisation).
4-5 Update and expand www.fishpathogens.eu with more pathogens.
4-6 Perform molecular epidemiology analysis to improve knowledge on disease-spreading mechanisms of viral pathogens.
4-7 Assessment and standardisation of real-time PCR tests for the diagnosis, identification and typing of the listed non-exotic fish diseases.

4-8 In collaboration with specialised experts worldwide to review selected emerging fish diseases in Europe and assess their potential listing as exotic or non-exotic diseases (e.g. using discontools and similar tools)

5. Missions

5-1 Organizing missions to relevant laboratories. Missions will focus on NRLs where on-site communication would be beneficial.

5-2 Attending missions, international meetings and conferences in order to be updated on emerging and listed exotic and non-exotic fish diseases.

EURL FOR FISH DISEASES, 2013
OBJECTIVES FOR THE PERIOD JANUARY - DECEMBER 2013

1. Coordination and training

1-1 Organise and prepare for the 17th Annual Workshop for the National Reference Laboratories for Fish Diseases (NRLs) in 2013.

1-2 Produce a report from the Annual Workshop 2013.

1-3 Collect and report data on the fish disease situation in EU, including all the listed non-exotic fish diseases.

1-4 Facilitate and provide training in laboratory diagnosis. The yearly training courses in methods used for diagnosis of fish diseases will be offered at the EURL laboratory facilities. The courses will primarily be for training of staff from NRLs and the content will depend on request from participants.

2. Proficiency test

2-1 Prepare the Annual Inter-laboratory Proficiency Test year 2013 for the NRLs. The test will include testing for VHSV, IHNV, EHNV, ISAV, KHV and *Aphanomyces Invadans* and can include several other fish pathogens.

2-2 Collate and analyse information gained from the Inter-laboratory Proficiency Test.

3. Reagents and products

3-1 Supply reference reagents to the NRLs in Member States.

3-2 Production of antisera against selected isolates when necessary.

3-3 Update and maintain a library of isolates of ISAV, VHSV, IHN, KHV, EHNV and *Aphanomyces Invadans*.

3-4 Maintain a library of tissue material from fish infected with listed pathogens.

4. Scientific advice and activities

4-1 Update the webpage for the EURL, www.eurl-fish.eu.

4-2 Update the diagnostic manuals for VHS, IHN, ISA, KHV disease, EHN and EUS on the EURL web page.

4-3 Collect information on strain variation occurring within pathogens causing the listed diseases VHS, ISA, EHN and KHV disease and provide recommendations on how to discriminate between various strains.

4-4 Identify and characterise selected isolates of listed viruses (serological and genetic characterisation).

4-5 Update and expand www.fishpathogens.eu with more pathogens.

4-6 Assessment and standardisation of real-time PCR tests for the diagnosis, identification and typing of the

listed non-exotic fish diseases.

4-7 In collaboration with specialised experts worldwide to review selected emerging fish diseases in Europe and assess their potential listing as exotic or non-exotic diseases.

5. Missions

5-1 Organizing missions to relevant laboratories. Missions will focus on NRLs where on-site communication would be beneficial.

5-2 Attending missions, international meetings and conferences in order to be updated on emerging and listed exotic and non-exotic fish diseases.

Apart from all the mandatory plans for next year suggestions for other topics to work on for the EURL would be most appreciated.

RESULTS OF THE PROFICIENCY TEST, PT1 AND PT2, 2011

Søren Kahns, Nicole Nicolajsen, Maj-Britt Christophersen & **Niels Jørgen Olesen**
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A comparative test of diagnostic procedures was provided by the EU Reference Laboratory (EURL) for Fish Diseases to 41 National Reference Laboratories (NRLs) in the middle of October 2011. The test was prepared and tested according to protocols accredited by DANAK under registration number 515 to proficiency testing according to the quality assurance standard DS/EN ISO/IEC 17043. The test consisted of 2 tests: PT1 and PT2.

PT1 Introduction

PT1 consisted of five coded ampoules (I-V). The ampoules contained VHSV, EHNV, European catfish virus (ECV), IHNV+IPNV and IPNV, respectively. The proficiency test was designed to primarily assess the ability of participating laboratories to identify the fish viruses VHSV, IHNV and ENHV (all listed in Council Directive 2006/88/EC) if present in the ampoules, bearing in mind that the test ampoules also could contain other viruses (e.g. other fish rhabdoviruses, ranaviruses, or birnaviruses). In addition the participants were asked to quantify the viruses in PT1 by titration in order to assess the susceptibility of their fish cell lines for virus infection. Participants were encouraged to use their normal standard laboratory procedures. However, the identification should be performed according to the procedures laid down in Commission Decision 2001/183/EC using fish cell cultures followed by e.g. neutralisation test, immunofluorescence, ELISA or PCR. If ranavirus was present in any of the ampoules, it was mandatory to perform a sequence analysis or a restriction endonuclease analysis (REA) of the isolate in order to determine if the isolate was EHNV or another ranavirus and it was recommended to follow the procedures described in Chapter 2.3.1 in the OIE Manual of Diagnostic Tests for Aquatic Animals 2009. Laboratories were encouraged to identify VHSV and IHNV isolates as far as possible by means of genotyping in addition to the standard methods. It was recommended to use the genotype notification described in Einer-Jensen et al. (2004) for VHSV and in Kurath et al. (2003) for IHNV. Laboratories were encouraged to submit all sequencing results used for genotyping the isolates.

PT1 Conclusion

All laboratories identified VHSV without problems. As IHNV was included as a double infection with IPNV some laboratories failed to correctly identify this virus. IPNV was correctly identified by 36 of the 41 laboratories. In 2009 EHNV was included in the proficiency test for the first time and 32 participants were able to correctly identify the virus. This year EHNV was included as well as ECV, both belong to the ranavirus family. Of the laboratories performing PCR based methods, 31 laboratories performed sequencing for ampoule II and 32 for ampoule III. Of these laboratories all correctly identified the content in ampoule II as EHNV and 31 correctly identified the content in ampoule III as ECV/ESV. One laboratory performed both sequencing and REA for both ampoule II and III without being able to identify which type of ranavirus the isolates belong to. One laboratory performed REA only for both ampoule II and III and was able to identify the isolate as either EHNV or ranavirus, not EHNV.
All titres submitted by participants for each cell line and ampoule, respectively, were compared to each other. In this way, the titres obtained by each laboratory were plotted in relation to the combined submitted data set and each participating laboratory should be able to compare the sensitivity of their cell lines to the sensitivity of those used by the other participating laboratories.

We recommend all participants to evaluate the sensitivity of the cells used in their laboratory in relation to the diagnostic purpose.

PT2 Introduction

PT2 also consisted of five coded ampoules (VI-X). The ampoules contained ISAV and KHV. Furthermore, one ampoule contained *Aphanomyces invadans*. It was decided at the 15th Annual Meeting of the NRLs for Fish Diseases in Aarhus 26-27 May 2011, that testing for *A. invadans* for the first time should be included in the yearly proficiency test provided by the EURL. The test was designed to primarily assess the ability of participating laboratories to identify the notifiable fish pathogens ISAV, KHV and *A. invadans* if present in the ampoules, bearing in mind that the test ampoules could also contain other viruses. Participants were expected to use their normal PCR or real-time PCR methods for detection of the pathogens. It was not mandatory to grow KHV and ISAV on cell cultures in this test, but the viruses were not inactivated and they should thus be possible to amplify in cell cultures. If present, only **inactivated** *A. invadans* was included in the ampoules.

PT2 conclusion

Considering that this was the second time that the EURL provided a proficiency test on ISAV and KHV identification, and the first time that the EURL provided a proficiency test on *A. invadans*, we consider that most participants obtained satisfying results. Out of 36 laboratories performing ISAV identification 32 identified ISAV in ampoule VI containing low titre ISAV and 35 identified ISAV in ampoule VII containing high titre ISAV. All 37 laboratories testing for KHV identified KHV in ampoule VIII containing high titre KHV, and 36 of them identified KHV in ampoule X containing low titre KHV. Out of 31 laboratories testing for *A. invadans* 28 identified the pathogen in ampoule IX.

Lowering the titre of the virus caused only one laboratory to miss identification of KHV in the low titered ampoule X. A reason for the laboratory to miss the correct identification is most likely due to mistaken marking of the ampoule. If this is the reason then all laboratories testing for KHV were able to identify both the high titre and the low titre KHV. For ISAV, one laboratory missed identification in the high titre ampoule and for the low titre ampoule three further laboratories did not succeed in the identification.

A couple of laboratories identified pathogens not present in the ampoules. E.g. one laboratory identified ISAV in all ampoules but only with a weak positive reaction in the ampoules where ISAV was not present.

A critical point in PCR based diagnostic tools is avoiding false positive and false negative results. To decrease the risk of having false negative results, it is always recommended that laboratories use the most sensitive tool available, validate the sensitivity of their diagnostic tools and use proper controls. To decrease the risk of false positive results laboratories have to be very aware of the risk of cross contaminations.

The results of the proficiency tests will be further discussed at this presentation.

Questions:

Complaint about the ampoules, demonstration on how to open them, no changes planned for the future.

Suggestion for PT1 o PT2

In the questionnaire delivered after the final report it was asked about complaints and suggestions. The accreditation body stressed the point that there is a need to get feedback. Some claim there was not enough time, and other didn´t receive safe ampoules.

Sven Bergmann: Is it possible to divide RNA virus and DNA virus.

Niels Jørgen Olesen: we will think about. I am not sure we will do it, the division at present is based on whether the virus can grow on the common cell lines (PT1) or not (PT2). An idea would be to use lyophilised organ material from infected fish or spike tissue as alternative to cell culture supernatants.

Sven Bergmann: Forwarded 3 questions asked by Heike Schuetze (FLI):

- ISA PCR: which is recommended?
- Sensitivity test recommended for diagnostic (rt pcr against cell culture etc)
- ISA PCR for cell culture ok but spiked material in pcr always negative.

1: Use the method recommended in the most recent ISA chapter of the OIE manual or in the diagnostic manual on the www.eurl-fish.eu website. Both real time RT-PCR and conventional RT-PCR can be used, with the real time RT PCR as the most sensitive.
2: RT-PCR is generally considered as more sensitive than cell cultivation.
3: Interesting, this should be dealt with more in details

Next meeting will probably take place in the end of May-early June 2013; in Bülowsvej in Copenhagen or Poland (depending on location site availability and Commission decision).

Pictures

Olga Haenen, Nicole Nicolajsen, Helle Frank Skall and Susie Sommer Mikkelsen were excellent photographers during the workshop. For pictures from the Annual Meeting please have a look at our web page. http://www.eurl-fish.eu.