European Union Reference Laboratory for Fish Diseases

National Veterinary Institute, Technical University of Denmark, Copenhagen

Technical Report 2016

from the European Union Reference Laboratory for Fish Diseases

National Veterinary Institute
Technical University of Denmark
Department for Diagnostics and Scientific Advice
Copenhagen, Denmark

Content

Introduction

The National Veterinary Institute, Technical University of Denmark (DTU-VET) is appointed as the European Union Reference Laboratory for Fish Diseases (EURL), in accordance with the Commission Implementing Regulation (EU) No 135/2013 of 18 February 2013, the notification of grant decision for an action regarding the EU Reference Laboratory for Fish Diseases – SI2.725290 and the corresponding grand decision (Ref. Ares(2016)854560 - 18/02/2016) as regards the Union financial aid for the year 2016 and 2017 to the EURL Fish Diseases

The duties of the EURL are described in Council Directive 2006/88/EC of 24 October 2006 (Annex VI). The duties mainly concern fish diseases listed as exotic diseases: epizootic haematopoietic necrosis (EHN); and fish diseases listed as non-exotic diseases: infectious salmon anaemia (ISA), viral haemorrhagic septicaemia (VHS), infectious haematopoietic necrosis (IHN) and koi herpes virus disease (KHVD). This report follows the format of the work programme adopted for the EURL for 2016 and 2017, describing activities and sub-activities and the status of on-going projects. The list of functions and duties of the EURL follows this introduction.

The 20th Annual Meeting of the National Reference Laboratories for Fish Diseases was held in Copenhagen, Denmark, May 31st – June 1st at the premises of the Veterinary Institute. A total of 65 participants from 33 countries attended over the two days period. There were five sessions with a total of 29 presentations, 3 of which were given by invited speakers, a working group session and a round table discussion.

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. For the sixth year, the proficiency test consisted of two tests, PT1 and PT2. PT1 was designed to primarily assess the ability of participating laboratories to identify VHSV, IHNV and EHNV. PT2 was developed in order to test the proficiency of participating laboratories to identify ISA and KHV; also in 2016 the identification of SAV was included in PT2 on a voluntary base. The proficiency test is covering all 5 listed exotic and non-exotic fish diseases. 45 National Reference Laboratories (NRLs) participated in the proficiency test. A report was submitted primo March 2016. The majority of the laboratories performed well.

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 available at the EURL web page based on the finally adopted Commission Decision 2015-1554 on sampling and diagnostic procedures for all non-exotic diseases listed in Council Directive 2006/88/EC, http://www.eurl-fish.eu/Diagnostic_Manuals.

Another important focus area was the development, implementation and validation of diagnostic tools for identification of the listed and emerging diseases and their accreditation. One outcome of these efforts was the implementation of a real time RT-PCR for detection of Calicivirus, a real-time PCR for detection of salmon pox virus and *Renibacterium samoninarum*, and optimization and validation of a real time RT-PCR for surveillance and diagnosis of IHNV.

During 2016, resources were again used to collate data on surveillance, health categorisation, and diagnostics in EU, this year in an amended and more simplified form; 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 training courses in laboratory diagnosis; to update the EURL webpage (www.eurl-fish.eu); and finally to attend international meetings and conferences.

In 2016 our molecular biologist since 2012, Dr. Susie Sommer Mikkelsen, decided to leave her position for a position back in Jutland. She is now replaced by Dr Argelia Cuenca Navarro with a strong background in molecular biology and bioinformatics. In addition our coordinator since 2012 DVM Niccolò Vendramin obtained a 2 year sabbatical leave from the EURL in order to focus on finalizing his PhD study. He is replaced by Dr. Nikolaj Gedsted Andersen with a significant scientific background.

Frederiksberg, 30 March 2017

Niels Jørgen Olesen
Professor, DVM
Head of EURL for Fish Diseases

The functions and duties for the
European Union Reference Laboratory for Fish Diseases
According to Council Directive 2006/88/EC of 24 October 2006 - Annex VI.
Period: 1 January 2016 – 31 December 2016

The functions and duties for the European Union Reference Laboratory for Fish Diseases (EURL)

The European Union reference laboratories shall:

(a) coordinate, in consultation with the Commission, the methods employed in the Member States for diagnosing the disease concerned, specifically by:

(i) typing, storing and, where appropriate, supplying strains of the pathogen of the relevant disease to facilitate the diagnostic service in the Community,

(ii) supplying standard sera and other reference reagents to the national reference laboratories in order to standardize the tests and reagents used in each Member State, where serological tests are required, L 328/48 EN Official Journal of the European Union 24.11.2006

(iii) organising periodic comparative tests (ring tests) of diagnostic procedures at Community level with the national reference laboratories designated by the Member States, in order to provide information on the methods of diagnosis used and the results of tests carried out in the Community;

(iv) retaining expertise on the relevant disease pathogen and other pertinent pathogens to enable rapid differential diagnosis;

(b) assist actively in the diagnosis of outbreaks of the relevant disease in Member States by receiving pathogen isolates for confirmatory diagnosis, characterisation and epizootic studies;
(c) facilitate the training or retraining of experts in laboratory diagnosis with a view to harmonising diagnostic techniques throughout the Community;
(d) collaborate, as regards methods of diagnosing animal diseases falling within their areas of competence, with the competent laboratories in third countries where those diseases are prevalent;
(e) collaborate with the relevant OIE reference laboratories with regard to exotic diseases listed in Part II of Annex IV under their responsibility;
(f) collate and forward information on exotic and endemic diseases, that are potentially emerging in Community aquaculture.

Work programme for 2016

Technical report

1. Coordination and training

1-1,1-2 Organise and prepare for the 20th Annual Workshop for the National Reference Laboratories for Fish Diseases (NRLs) and produce a report from the Annual Workshop

Organization of the 20th Annual Workshop

The 20th Annual Workshop of the National Reference Laboratories for Fish Diseases was held in Copenhagen, Denmark, May 31st – June 1st at the Auditorium of DTU Veterinary Institute in Bülowsvej 27, 1870 Frederiksberg C.

A total of 65 participants from 33 countries attended over the two days period, unfortunately 1 participant cancelled right before the meeting leading to minor re-arrangements of the program. There were five sessions with a total of 29 presentations, 3 of which were given by invited speakers, a working group session and a round table discussion.

The scientific program of the Annual Workshop 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 respective countries.

Initially, an overview of the disease situation and surveillance in Europe 2015 was provided on the basis of the results obtained from the Survey & Diagnosis questionnaire, this year a new questionnaire has been used in order to make data and results more accessible, a report compiling all information has been made available on the EURL website http://www.eurl-fish.eu/Activities/survey_and_diagnosis.

Then the fish diseases situation in Norway was presented; a detailed report in English is available at: http://www.vetinst.no/rapporter-og-publikasjoner/rapporter/2016/fish-health-report-2015

The results of a survey on the impact of fish diseases in the Mediterranean basin, achieved thanks to the collaboration with a panel of acknowledged fish pathologist experts, were presented.

The results of experimental trials conducted in the experimental facilities of DTU VET with the newly described PRV-*Om* in rainbow trout were presented.

The last talk of the first session described results of a survey conducted in Iceland for PRV in wild and farmed salmon.

The second half of the morning was allocated to an interactive activity, introduced for the first time during the Annual Workshop in 2014. Briefly, each participant was asked to consider the relevant infectious diseases for the 2 most important fish species farmed in his country. In this perspective, all participants received on beforehand 4 tables listing the most renowned pathogens for the most important farmed fish species in Europe. Before the WS each participant had the opportunity to interact with different stakeholders and assess impact on production, economy and legislative consequences for the different infectious diseases in 2015.

During the first part of this working group session, the activity was implemented at a country level, meaning that each participant was asked to rank the disease by impact

in 2015. After this first level of investigation, representatives of different macro-areas in Europe were grouped. The regions were Northern Europe, gathering the main salmon producing countries, Eastern Europe focusing mainly on cyprinids and subsequently rainbow trout, Western Europe producing mainly rainbow trout and cyprinids and finally Southern Europe producing mainly the marine species European sea bass and gilthead sea bream and then rainbow trout. Experts had the possibility to discuss and describe the impact of each disease focusing on the 3 most important parameters. The first topic considered was the impact of a given disease on production, meaning the severity of losses in terms of mortalities, reduction of growth, etc. Then impact on the economy, indicating the cost of prevention (i.e. vaccination and biosecurity measures), treatment, reduced value of the product was considered. Finally consequences due to trade restrictions, national plans for control/eradication, suspension time after antibiotic treatment etc. Each group had to finalize its task by selecting and describing the most important diseases.
During the final part of the session a representative of each area described the agreed findings to the whole assembly.

The second session of the WS was dedicated to emerging diseases.
Firstly, the invited speaker from France presented a review of sturgeon viruses and the diagnostic challenges posed by these viruses.
This was followed by two combined presentations describing the detection of two viral pathogens (one Ranavirus and VHSV of genotype IV) in lumpfish in Iceland and the experimental activities conducted at the facilities of the EURL at DTU Vet to assess the risk for farmed salmonids and lumpfish of this new VHSV.
The following presentation addressed the outbreak of nodavirus in gilthead seabream, for many years this fish species has been considered resistant to the virus but re-assortant strains have shown to affect especially larval stage of this host.
This session was closed by presenting the results of an experimental trial conducted in Atlantic salmon looking into the susceptibility of this host for a European strain of IHNV and interaction with infection with PRV.

The third session, on control and surveillance of relevant pathogens in the EU, started by a presentation describing epidemiological investigation and management of VHSV detected in Atlantic salmon in France in 2015.
This was followed by a presentation of the "diagnostic manuals" finally adopted and fully implemented March 2016 and available at http://www.eurl-fish.eu/Diagnostic_Manuals
The third presentation provided interesting overview of the OIE aquatic animal commission.
Finally an overview of the structure of the new aquatic animal health law with focus on criteria for disease listing was given.

In the evening a banquet dinner was held at Restaurant ***"Spiseloppen"*** in Christiania.

The second and last day was opened by the session on scientific research update; this fourth session faced several different topics covering molecular characterization of pathogens, development of new diagnostic techniques

including serology, conventional PCR and Real Time PCR, cell cultures; and characterization and description of new viral fish pathogens belonging to poxvirus group.
The session started with a presentation addressing the use of serology in finfish diagnosis, first a systematic review was presented and subsequently the specific use of serology in KHV diagnosis was presented.
The session continued with a presentation describing if and how molecular characterization and epidemiology has to be combined while addressing the tracing of viral fish disease.
Afterwards the development and validation of a conventional RT-PCR protocol for detection of VHSV that overcome pitfalls of already available methods have been presented. This work has been developed within an OIE twinning project between South Korea and Denmark.
After the coffee break, the topic of infection with poxvirus in farmed fish was presented. First the infection in salmon describing different type of diagnostic cases was presented by invited speaker from Norwegian Veterinary Institute.
Subsequently the outcome of investigations of Carp Edema Virus in cyprinids and networking activities across Europe in regards to this virus were presented by Dutch NRL representative
The session continued presenting the use of salmon embryonic cell lines in detection of fish rhabdovirus.
The presentation were finalised by the description of novel paramyxovirus in UK.
The participant from Faroe Islands cancelled his participation and thereby his presentation was withdrawn from the program.
The section finished with round table discussion where participants representing Scottish, French, Norwegian, Danish and Czech laboratories showed their results on the process of validation of qPCR protocol for surveillance of IHNV.

The annual meeting ended with the traditional update from the EURL. The results of the two proficiency tests sent out in 2015, PT1 and PT2, were presented.
The program and application system for the annual training courses that was provided by the EURL in October 2016 was described. The planned EURL activities in year 2016-2017 were presented and proposals for the EURL work plan for 2017 were discussed.

The final report of the 20th Annual Workshop is available on the web site
http://www.eurl-fish.eu/Activities/annual-meetings

1-3 Collect and report **Survey and diagnosis of fish diseases in Europe in 2015**

data on the fish diseases situation in EU, including all the listed non-exotic fish diseases given in Council Directive 2006/88/EC Annex IV Part 2

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 changed significantly since last year. The reason for this is that we have realised that it is not possible to obtain an exact knowledge on the de facto prevalence, spreading and severity of the listed non-exotic fish diseases in Europe. The questionnaire, however, still comprise 3 parts:

1. General data on aquaculture fish production: Number of fish farms, and the health categorization according to Council Directive 2006/88/EC, and information on national surveillance programmes.
2. Epidemiological data on the disease situation in each Member State with focus on the listed diseases (information on number of out breaks and increase or decrease in number of infected farms and severity of outbreaks) but also including other diseases of interest.
3. Laboratory data from the NRLs and other laboratories, including the numbers of samples examined, and diagnoses of fish diseases made.

Production data from FEAP

The data on the European aquaculture production was this year obtained from the ”European aquaculture production report 2005-2014” Prepared by the FEAP secretariat August 2015. We validated the data against the FIGIS database and concluded that they were almost identical except for the common carp production estimated by FEAP to be only 1/3 of the production data we obtained from FIGIS. The report does not include information on the number of fish farms, and therefore these data were obtained directly in the questionnaire. The report only provides data until 2014. The total fish production in aquaculture in Europe increased again in 2014 after a decrease in 2013. The increase however is almost only due to increases in non-EU Member states. Among the Member states the production has been almost horizontal in the past 10 years. In 2014 the Atlantic salmon production, account for 1.55 mill ton against 1.43 mill ton in 2013, and is by far the largest contingency in Europe. The rainbow trout production is again below 400 000 t after steady increases in the previous years. The decrease is primarily due to reduced production of table size rainbow trout. After several years of increased production Turkey have experienced a significant reduction in 2014. The carp production is still mainly in the Eastern part of Continental Europe and is very stable with 57.000 t produced in all. Both the production of sea bream and especially sea bass also increased in the Mediterranean countries with a production of 146.000 t and 148.000 t, respectively. Among other fish species of interest are pike-perch (increase to 573t), eel (increase to 6.507 t from 4.017t in 2013), sturgeon (increase to 2.795t), turbot (decrease from 12.676t to 9.891t in 2013 and increase again to 10.787 in 2014), and halibut (1.600 t) the cod production have almost collapsed from 22.729t in 2009 to 3.310t in 2013. The production of cleaner

fish as lumpfish for lice control is increasing significantly but the total production is not that easy to retrieve due to the many species involved in this industry. Pike-perch have still not yet obtained the expected impact, a large farm in Denmark started in 2015 might give promises of an increase of this production in future, the sturgeon production is still on growing and more attention regarding health management will be given to this species- (see program for the 20th Annual Workshop).

Health categorization of fish farms:

Many countries provided very clear and correct answers and almost all Member States did reply to the questionnaire when compared to the previous year's providing a rather complete overview of the status of fish health categorization in Europe. There was a significant increase in the reported number of farms in categorized zones and compartments (From 8.505 in 2012 to 14.508 in 2015 for VHS and from 7.360 in 2012 to 12.130 in 2015 for KHV!) this was especially due to Germany who successfully included almost all their farms in categorized zones.

76% of the authorised trout farms in Europe are situated in category III zones for VHS and 74% for IHN, with 21% and 23% respectively in Category 1. For both diseases the remaining 3% of the farms are situated in category II, IV or V. In all countries except Norway almost all salmonid farms are in Category I for ISA with 64% in Category I and 35% in category III. Only very few carp farms are approved KHV free in Category I (1%) and almost all are placed in Category III (97%) or nor categorized.

There are still 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 considering the risk of infection with VHSV from the marine environment.

Commission Decision 2015-1554 provide the guidelines for obtaining disease-free health statuses with regard to ISA and to contain infection with HPR deleted ISAV, saying that detection of Isavirus HPR0 will not compromise the health status of a fish farm. Some Member states do not include small registered APBs in the categorisation (e.g. hobby farms) but according to 2006/88/EC Annex III health categorisation comprise all APBs in the Member states, zones and compartments for each category. Only fish species listed as susceptible for the given listed disease shall be included in the categorization. Therefore important aquaculture species as sea bass, sea bream, meagre, eel and pike-perch are not included in the European health surveillance for specific diseases.

The new Animal Health Law has now been adopted and includes all aquatic animals; in this connection the categorisation system might be simplified and be made more transparent.

Outbreaks and severity of listed diseases in Europe

Concerning the epidemiological data on the non-exotic diseases a moderate increase in the number of VHS infected farms were observed in Austria, Belgium, Bavaria, Rhineland-Palatine, Saxony, and Thuringia, whereas a decrease was observed in Czech Republic, no reports of changed severity of VHS was given except for pike infections in Austria. VHSV is regularly isolated from wild marine fish species, but it was anyway a big surprise and worrying that it appeared in wild caught lumpsuckers in Iceland, in brood fish that were caught for breeding of lumpsuckers

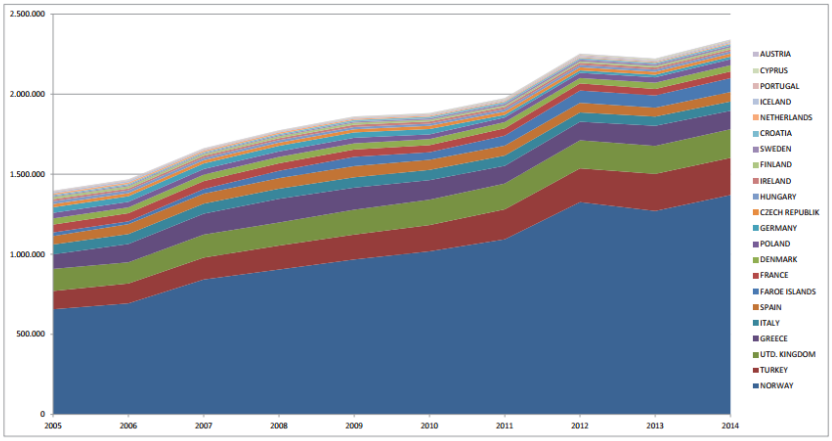
as cleaner fish in the salmon industry. The isolate belong to VHSV genotype IV in a putative new subgroup.
For IHN an increase in number of infected farms were observed in Italy, Bavaria, and Baden-Württemberg. In Italy the severity of IHN seem to increase while a decrease in impact of IHN was observed in Baden-Württemberg.
For ISA Norway reported an increase in number of affected farms. The reason may be that infected smolts from one hatchery were transferred to three sea locations before the infection was detected. ISA was not seen outside Norway in 2015.
Concerning KHV Germany reported increases in number of infected farms in Bavaria, Lower Saxony, Rhineland-Palatina, and Saxony-Anhaltinia and a decrease Saxony. No other information on KHV in Europe was given.

Other fish diseases problems in EU
A whole range of other disease problems 2015 were reported:
• In rainbow trout the major concerns are flavobacteriosis (RTFS), enteric redmouth, and infectious pancreatic necrosis but also, lactococcosis, bacterial kidney disease, proliferative kidney disease, ichthyophtariasis, and furunculosis (especially in brown trout).
• In salmon farming it is sea lice, pancreatic disease, heart and skeletal muscle inflammation, cardiomyopathy syndrome, amoebic gill disease, and moritella.
• In cleaner fish it is primarily vibriosis and *A. salmonicida* infection giving problems
• In seabass and seabream it is primarily VER, Photobacteriosis, and Vibriosis (Annex 3)

In northern European countries the most common problems in the salmon production are thus sea lice, PD, and AGD, in addition several countries reported finding of Winter Ulcer Disease in salmon caused by *Moritella viscosa.* In continental Europe it is primarily bacterial diseases like ERM and Aeromonas infections, AGD and RTFS – but parasite infestations as Ich is still a very serious problem especially in view of the foreseen prohibition of use of formalin, while problems in the Mediterranean countries are the same as in continental except for Lactoccocosis wich is more common in Southern Europe and Nodavirus infection in mariculture which definitely plays an important role and as a bottleneck for especially the seabass production.
There are very large differences between countries on how many samples are tested on cell cultures, ranging from < 100 to several thousands. Annex 5 provide the total number of laboratory examinations conducted in Europe in 2015 on VHSV, IHNV, ISAV, KHV, SVCV, CEV, IPNV, SAV, and Nodavirus, respectively.

Total production of fish in aquaculture in Europe 2005 to 2014 (”European aquaculture production report 2005-2014”)

Development of Fish Farming in Europe (tons) 2005-2014



A summary of the results for 2015 is presented on our website:
www.eurl-fish.eu/Activities/survey_and_diagnosis.aspx

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.

Training

Training courses:

The EURL Fish is yearly offering 2 training courses in diagnostic techniques for identification of listed fish diseases and introduction to histopathology on fish diseases.
The training courses took place in Copenhagen at DTU National Veterinary Institute, Bülowsvej 27, 2700 Frederiksberg C Denmark, from October the 10th to the 20th, 2016. Two courses were prepared: the first one, with 7 trainees, was entitled "Methods for implementation of surveillance procedures for listed fish diseases" and took place from 10th to 14th October 2016.
The second course was entitled "Introduction to histopathology in fish diseases" and took place in Copenhagen 17th to 20th October 2016 with 13 participants. 3 persons participated in both training courses.
The overall purpose of the training course was to provide an opportunity for the NRLs to send employees for training in techniques relevant when working with fish diseases.
Concerning the course on surveillance of listed fish diseases the first course the staff of the EURL provided this training, together with teachers from the Danish Veterinary and Food Administration
For the course focused on Histopathology, staff of EURL and DTU VET, in cooperation with NVI-Oslo and Aquapri DK, constitute the tutor team.
Knowledge-sharing and discussions between participants and teachers were important parts of the courses.

Course 1: Methods for implementation of surveillance procedures for listed fish diseases.
The 5-days course in "Methods for implementation of surveillance procedures for listed fish diseases" was primarily based on practical work (hands on) in combination with theoretical presentations.
Day 1 was dedicated to a fish farm inspection**.**
As procedure does not allow a visit to a fish farm after working in our laboratory it was decided to meet with the participants at the entrance of our institute and to drive to the FVO offices in Vejen, where we were received by Dr. Korsholm. After the training course introduction by Niccoló Vendramin and presentations on Danish surveillance plans for fish diseases held by Dr. Korsholm, the participants visited a rainbow trout farm, Vejen Store Vandmolle, approx 5 km from Vejen.
During the on-site visit, procedures for inspection and sample collection were demonstrated; participants were taught on how fish necropsy techniques are performed in the field. All participants had the opportunity to perform necropsy on diseased fish collected at the farm; they collected relevant samples and brought them back to the laboratory in Copenhagen for further examination.
On day 2 a detailed description of the course program was presented by Niels Jørgen Olesen and discussed with the participants. After the introduction, a lecture on fish virology was given by prof. Niels Jørgen Olesen. The participants were then divided into two groups. As an assignment each participant had to process the samples collected in the fish farm and test it to rule out the presence of the listed disease VHS. Further on, participants were asked to investigate if other pathogens were present in the sample. The processing of fish samples collected the day before was demonstrated before the participants were asked to do it themselves.

Day 3 and 4 were replicated so that one group could follow the cell culture part one day and the Real-time PCR the day after and vice versa for the other group. With this organization it was possible for all the trainees to participate in all the practical activities that were demonstrated.
Every activity had a team of tutors in order to provide an effective support to the trainees. For the cell culture activities Niccolò Vendramin, Betina Lynnerup and Christina Flink Desler were assigned, while for the real-time PCR part, Argelia Cuenca Navarro, Troels Secher Rundqvist and Didde Hedegaard Sørensen were the tutors.
The day dedicated to cell culture started with the demonstration of the procedure implemented in the laboratory to prepare diagnostic trays with BF-2 and EPC cell cultures. Subsequently all the participants prepared their own trays and inoculated the trays with the sample prepared the day before. Titration procedure was also demonstrated and a talk on titre calculation and cell susceptibility test was given. The afternoon was allocated to provide the participants with knowledge in reading different fish cell lines inoculated with different viral pathogens, highlighting features of different cytopathogenic effects (CPE). As practical assignment participants were asked to read titration plates previously prepared, calculate the titre of the virus inoculated, and describe and draw different CPE's on infected cell cultures.
The day dedicated to real-Time PCR started with a brief introduction addressing the use of PCR techniques in surveillance and diagnostic, with focus in detection of VHSV. The practical laboratory exercises started with participants being demonstrated how to purify RNA from their samples, with participant processing his own sample. One of the participants of the group prepared the Mastermix, and set up the RT-qPCR for the whole team. Finally samples were loaded in the real-time PCR machine. While the machine was processing the samples, the participants attended a presentation explaining the theoretical principles behind the different PCR techniques and methods of data analysis. Finally, a session addressing troubleshooting and pitfalls in real-time PCR, as well as routines to minimize the risk of (cross-) contamination was provided. At the end of the day, results of the analysis were collected and discussed.
Day 5 was allocated to finalize the course, discuss both results obtained by the participants and different methods for performing surveillance for listed fish diseases in their countries of origin. Finally a questionnaire for the course evaluation was given and participants asked to fill it anonymously.
The methods taught were primarily focused on the protocols given in EU legislation and on the OIE guidelines from the Manual of Aquatic Animal Diseases, and included how to select proper controls, the typical pitfalls, trouble shooting, etc. As get-together, a joint dinner the second evening was included.

Course 2: Introduction to histopathology in Fish Diseases.
The 4-days course in histopathology and immunochemical techniques was divided into theoretical lectures on relevant topics, practical exercises both in necropsy room and microscopy laboratory.
Day 1 started with introduction of course and practical information, each participant had the opportunity to present himself to the tutors and his fellows. Practical demonstration on how to sample fish tissues and organs for proper histological examination followed, and each participant could practically try the technique. Lectures given by Ole Bendik Dahle from NVI-Oslo on the normal histology and

artifacts followed after lunch break.
Day 2 was divided between practical observation of slides from confirmed case at the microscope and theoretical lectures focusing on general pathology. Once again Ole Bendik Dahle took the lead of the teaching supported by Tine Iburg from DTU VET.
The first part of day 3 was dedicated to lectures on Immunehistochemistry-IHC, the different phase of sample preparation for this staining technique and troubleshooting and pitfalls during the process were discussed this part of the program was conducted by Tine Moesgaard Iburg, the fish pathologist employed at the EURL fish team. The day was concluded with presentation and discussion of specific cases in an open forum where participants where welcome to comment the images displayed on the screen.
Day 4, the last day of the course started directly at the microscopy room, diagnostic cases brought by participants were discussed and presented in an open forum, with supervision of tutors Ole Bendik Dahle and Tine Iburg.
After lunch, there was a last round of group work before all the participants met up again for a last discussion about the subjects that had been covered earlier in the day.
At the end of the course the participants were provided with a questionnaire to fill in as part of the evaluation. Furthermore, the participants were invited to give their opinions on the course. All in all, the course was very well-received, and the distribution of practical and theoretical lessons seemed well balanced.
Through lectures, exercises, discussions, and knowledge exchange between participants, the knowledge on histopathology, and troubleshooting related to this technique was increased. There was much fruitful discussion among participants and teachers during the course, both during the lectures, the breaks and the theoretical exercises.
As get-together, an optional dinner event on day 2 was held.

Participant List

Name	Country	Course 1	Course 2
Rud Yuriy	Ukraine	x	
Humberto Gonzalez	Chile	x	x
John Bignell	UK		x
Eva Lewish	Austria		x
Eva Jansson	Sweden		x
Tuja Kantala	Finland	x	
Anna Kycko	Poland		x
Agnieszka Jasik	Poland		x
Marlene Areskog	Sweden	x	
Tamara Dolenšek	Slovenia		x
Moldal Torfinn	Norway	x	
Magdalena Stachnik	Poland		X
Raoul Kuiper	Sweden	x	X
Marta Alarcon	Norway		X

Caroline Wünster	Norway		X
Tomas Myoral Ortega	Spain		X
Panos Kalatsis	Denmark	x	X

The final report is available on the EURL web site
www.eurl-fish.eu/Activities/traning

Master and PhD students:

Anna Luiza Farias Alencar, was from July 1st 2016 enrolled as phd student on a project entitled: Identification of virulence markers in two Novirhabdoviruses causing serious diseases in fish. With Professor Niels Jørgen Olesen as supervisor and Thomas Brun Rasmussen DTU Vet and Michel Bremont INRA, JOUY-EN-JOSAS, France as co-supervisors. Anna have a master in Veterinary medicine and natural science from University of São Paulo, USP

Niccolò Vendramin was from December 1st granted a 2 year sabbatical from his position as coordinator of the EURL for Fish Diseases for finalizing a PhD study entitled "Piscine orthoreovirus in salmonids: geographic distribution, molecular characterization, pathogenesis under experimental conditions" with Professor Niels Jørgen Olesen as main supervisor and Professor Espen Rimstad NMBU, Oslo, Norway as co-supervisor. Niccolò has a degree as DVM from University of Padua, and have been employed as coordinator of the EURL since 2012.

OIE collaboration:

N.J. Olesen is designated expert of the OIE reference laboratory for VHS, and in this function had consultancies and requests worldwide concerning VHS. The OIE chapter on VHS and related diseases in the diagnostic manual for aquatic animal diseases is revised and updated by the OIE reference laboratory for VHS.

In this connection an OIE Twinning project have been established between the National Fishery Products Quality Management Service (NFQS) of the Republic of Korea, as the candidate institute, being the National Reference Laboratory (NRL) for fish diseases in Korea and DTU Vet. The candidate laboratory wants to improve the capabilities of performing its duties as the NRL for Viral Haemorrhagic Septicaemia (VHS). At the end of the twinning Project with the National Veterinary Institute, DTU, Denmark in 2017 the NFQS will apply for obtaining a status as OIE Reference Laboratory or OIE Collaborating Centre for fish diseases with focus on VHS for the Asian region. In this colaboration Dr Hyoung Jun Kim, Ph. D. and his director National Fishery Products Quality Management Service, RoK, visited us 8-10 July 2015.

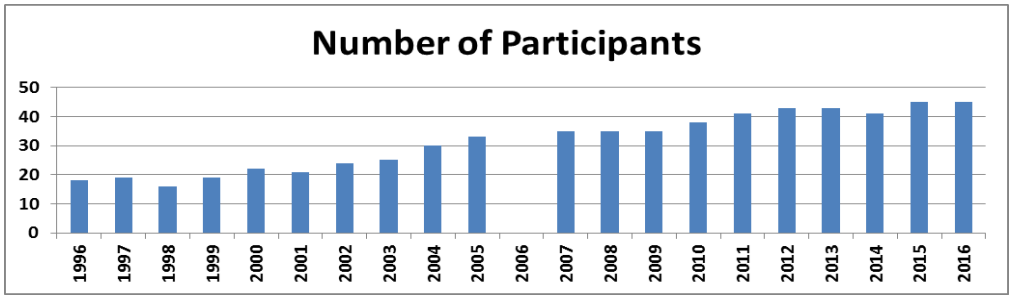
Niels Jørgen Olesen is member of the *ad hoc* group on susceptibility of fish species to infection with OIE listed diseases established by the OIE in 2016 and with regular meetings in Paris.

2. Proficiency tests

2-1 Prepare the Annual Inter-laboratory Proficiency Tests year 2016 for the NRLs. The tests include VHSV, IHNV, EHNV, ISAV, and KHV and in addition address other common viral pathogens in fish farming (IPNV, SVCV etc).

The inter-laboratory Proficiency Tests 2016

Since 1996, 20 inter-laboratory proficiency tests (PTs) have been organised by the EURL. The number of participants has increased from 18 to 45. The goal of these tests is to harmonise diagnostic methods between national reference laboratories and to ensure that the examination of a given sample leads to the same conclusions in any laboratory.



A comparative test of diagnostic procedures was provided by the European Union Reference Laboratory (EURL) for Fish Diseases. The test was divided into proficiency test 1 (PT1) and proficiency test 2 (PT2).
PT1 was designed to primarily assess the identification of the fish viruses causing the notifiable diseases: viral haemorrhagic septicaemia virus (VHSV), infectious hematopoietic necrosis virus (IHNV), and epizootic haematopoietic necrosis virus (EHNV) or related rana-viruses and in addition the fish pathogenic viruses: other fish rhabdoviruses as pike fry rhabdovirus (PFR),spring viraemia of carp virus (SVCV) and infectious pancreatic necrosis virus (IPNV) by cell culture based methods. PT2 was designed for assessing the ability of participating laboratories to identify the fish pathogens: infectious salmon anaemia virus (ISAV), salmon alphavirus (SAV) and cyprinid herpesvirus 3 (CyHV-3) (otherwise known as *koi herpes virus* – KHV) by biomolecular methods (PCR based). 45 laboratories participated in PT1 while 43 participated in PT2.
Regarding PT2, all 43 participated in identifying ISAV, 42 participated in identifying ISAV and KHV and 37 participated in identifying all three pathogens included, ISAV, KHV and SAV.
The tests were sent from the EURL end of September 2016.
Both PT1 and PT2 are accredited by DANAK under registration number 515 for proficiency testing according to the quality assurance standard DS/EN ISO/IEC 17043. This report covers both the results of PT1 and PT2.

PT1 consisted of five coded ampoules (I-V). These ampoules contained PFR, IHNV, VHSV, ECV and IPNV (see table 1). The proficiency test was designed to primarily

assess the ability of participating laboratories to identify any of the fish viruses VHSV, IHNV and to be able to discriminate between the exotic listed EHNV from other ranaviruses(Council Directive 2006/88/EC Annex IV part II and Commission Implementing Directive 2014/22/EU of 13 February 2014). Furthermore the interlaboratory proficiency test is also suitable for maintaining accreditation for identification of SVCV, and IPNV; participants have to consider 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 2015-1554 and by using fish cell cultures followed by e.g. ELISA, PCR, immunofluorescence (IFAT) or neutralisation test.

If ranavirus was present in any of the ampoules, it was mandatory to perform 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 2015. Laboratories were encouraged to identify VHSV and IHNV isolates 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 either method as mentioned in the IHN chapter of the 2013 version of the OIE manual on Aquatic Animal Diseases or in Kurath et *al*. (2003) for IHNV. Laboratories were encouraged to submit all sequencing results used for genotyping the isolates. It has to be remarked that although sequencing protocols are recommended in this report and in the instructions included to PT1-parcels, for the two listed rhabdoviruses this procedures relies on a number of different protocols, targeting different regions of the same pathogen making it difficult to compare results obtained by different participants. Acknowledging that sequencing is an accessory activity of the inter-laboratory Proficiency test and not a demand, this point will be further discussed during the Annual Workshop.

PT2 consisted of four coded ampoules (VI-IX). One ampoule contained CyHV-3 (KHV), one contained SAV, one contained ISAV and one contained sterile cell culture supernatant from BF-2 cells, see table 9. The test was designed to primarily assess the ability of participating laboratories to identify the notifiable fish pathogens ISAV and KHV (listed in Council Directive 2006/88/EC, Annex IV and Commission Implementing Directive 2014/22/EU) if present in the ampoules, bearing in mind that the test ampoules could also contain other pathogens. Since SAV is not a listed disease in the European legislation, all participants were free to decide if they would be testing for SAV or not. Each participant was asked to declare whether they would test for SAV or not. The EURL team would then take care of calculating the score accordingly, overall 37 of 42 laboratories tested for SAV in 2016, wich was an increase of three laboratories compared to 2015.
Participants were expected to use their normal PCR or real-time PCR methods for detection of the pathogens. Regarding SAV analysis, participants could refer to the OIE manual Chapter 2.3.5b. — Infection with salmonid alphavirus . It was not mandatory to grow KHV and ISAV on cell cultures in this test, but the viruses were

not inactivated and, thus, it might had been possible to replicate them in cell cultures.

Participating laboratories in PT1 and PT2 in 2016

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

Outcome of the Inter-laboratory Proficiency Tests 2016

Within one day, the tests were delivered to 27 participants; 12 more tests were delivered within the first week; 3 more within the first two weeks; 3 further within three weeks.

Concluding remarks on PT1:

60% of parcels were delivered by the shipping companies within 1 day after submission and 86% was delivered within 1 week. The remaining six parcels took longer for delivery primarily due to border controls, the maximum time of shipment was 21 days.

This year ECV was included in the Proficiency test. 37 participants provided the correct identification, 1 laboratory identified correctly the isolate but contaminated the ampoule content.

In the Report of the Inter-Laboratory Proficiency Test 2016, all the viral titres submitted by participants are compared to each other. In this way, the titres obtained by each laboratory are plotted in relation to the combined submitted data set and each participating laboratory is able to compare the sensitivity of its cell lines to the sensitivity of those used by the other participants. We recommend all participants to evaluate the sensitivity of the cells used in their laboratory in relation to the diagnostic purpose.

The EURL provides the annual proficiency test, collates the data and process the figures so that individual laboratories can see how they perform in relation to the other participants. It is up to the individual laboratory to assess if they perform according to their own expectations and standards. We will also take the opportunity to provide a comment to participants regarding submitted results if relevant. Furthermore we encourage all participants to contacts us with any questions concerning the test or any other diagnostic matters.

This year pike fry rhabdovirus was included in ampoule I. This virus has generated some challenges to the participants due to its antigenic similarity with SVCV, however the increase implementation of biomolecular techniques has allowed 17 laboratories to identify it correctly and other 17 were able to rule out the presence of VHSV, IHNV, IPNV, SVCV and ranavirus. The scoring system has been adjusted accordingly.

Overall 31 out of 45 participants scored 100% success rate and 8 more than 90%.
It has been a concern that few laboratories have identified the correct virus but not in the right ampoule, meaning that some mistake in traceability of the ampoules during the working flow procedure has occurred. Another critical points that has emerged, is the contamination of ampoule contents. These points will be assessed directly with the single participants that have underperformed.

Concluding remarks PT2:
After the positive experience in 2015, the EURL decided to include SAV in the panel of viruses included in PT2. Considering that 33 laboratories participated in 2015 (of which 32 correctly identified SAV in ampoule VII) this was regarded as a proper initiative that strengthen the diagnostic capacities of the NRLs in detecting emerging pathogens, and it will be included in the coming years as well.
37 laboratories participated in PT2 testing for SAV and all of the 37 correctly identified the virus in Ampoule VI.
42 out of 43 laboratories correctly identified the ISA virus in ampoule VII.
Out of 43 participants, 2 did not test for KHV and 1 did not identify the virus in Ampoule VIII, the other 40 correctly detected KHV in ampoule VIII.
It is an appreciated matter of fact that many laboratories are putting efforts in performing genetic characterization of the isolates through sequence analysis, as it is important that laboratories can discriminate between isolates as e.g. pathogenic ISAV strains with deletions in the HPR region and HPR0 strains, especially after the delisting of ISAV HPR0 (Commission Implementing Directive 2014/22/EU).

The EURL provides the annual proficiency test, collates the data and process the figures so that individual laboratories can see how they fare in relation to the other participants. It is up to the individual laboratory to assess if they perform according to their own expectations and standards. We take the opportunity to provide comments to participants regarding submitted results if relevant. Furthermore we encourage all participants to contacts us with any questions concerning the test or any other diagnostic matters.

The results given in this report will be further presented and discussed at the 21th

Annual Workshop of National Reference Laboratories for Fish Diseases to be held 30th-31st of May 2017 in Copenhagen, Denmark.

The final report is available on the EURL web site www.eurl-fish.eu/Activities/proficiency_tests

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

3. Reagents and products

Materials supplied by the EURL

On request, the EURL supplies material to other laboratories in Member States and Third Countries to aid in the diagnosis and characterisation of fish diseases. The number of laboratories receiving material and the specific material and number of units supplied by the EURL in 2016 are listed in Annex 1.

Further details of the supplied materials are listed in Annex 1

3-2. Production of diagnostic reagents against selected pathogens when necessary

Production of antisera

In 2016 no new productions of antisera were needed and our stocks of supernatants from hybridoma cells producing monoclonal antibodies were sufficient for the year.

3-3. Update and maintain a library of isolates of ISAV, VHSV and IHNV, KHV and EHNV and other relevant putative emerging fish pathogens.

Virus library

Isolates of the listed viruses VHSV, IHNV and KHV were received and stored in our library during 2016. In addition we received Ranavirus isolates from lumpfish in Iceland, Ireland, Faroe Islands and Scotland.
All isolates were further sequences and characterized genetically.

Further details of the received materials are listed in Annex 2

4. Scientific advice and activities

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

Update the webpage of the EURL

The EURL website (www.eurl-fish.eu) is a notice board, where NRLs and other interested parties can access relevant information and previous reports concerning the

activities coordinated by the EURL and relevant upcoming events in the Union.

The diagnostic manuals for VHS, IHN, ISA, KHV, EHN disease and EUS have been updated and uploaded on the web page.
Furthermore, reports of the EURL, e.g. of the results of the proficiency test, the Annual Meeting of the NRLs, result of questionnaire on "Survey & Diagnosis", Training courses, expert group meetings etc. are launched at the web page immediately after release.

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

Diagnostic manuals

The **Commission Decision 2015-1554**, implementing Directive 2006/88/EC as regards requirements for surveillance and diagnostic methods is now adopted and implemented.
The final versions of the surveillance, sampling and diagnostic procedures for each of the listed fish diseases are uploaded on the EURL Fish website for easy and comprehensive access to the details in the Decision.
The decision only comprise the non-exotic diseases listed in the directive

2006/88/EC, therefore a non-approved version of the sampling and diagnostic procedures for the exotic disease Enzootic Hematopoietic Necrosis (EHN) are included as are the methods for detection of EUS which was delisted previously but were the diagnostic procedures are still in force.

4-3 Fishreflabnet: Maintain and further develop the interactive network with the NRLs, in order to promote a proactive data sharing and communication with and between reference laboratories in the Member States.

Fishreflabnet:

An e-mail group was created in 2012: VET-EURL with approximately 100 colleagues subscribing. Official communication and updates of interest to the scientific community are delivered periodically.

Furthermore this tool is used for newsletters, scientific updates and announcements from the EURL Fish like announcements and invitations for the Annual Workshop or publication of content in the ampoules from the proficiency test or on the final Inter-laboratory Proficiency test report. In addition the e-mail group is used for announcing other workshops, training courses and conferences and new publications of interest for the NRL Fish network

4-4 Pathogen characterization: Identify and characterise selected isolates of listed viruses (pathogenicity testing in vivo and in-vitro, serological and genetic characterisation).

Studies conducted on pathogen characterization:

Thirteen different VHSV isolates from the EURL repository were selected based on their virulence pattern and genotype, and were propagated in cell culture and plaque cloned. Fourteen viral clones were collected, propagated and whole genome sequenced and in addition subjected to an experimental infection trial by bath in rainbow trout, in order to assess their respective virulence in this species. The morbidity in the trial varied from 2,7% (VHSV isolate Fin Ka 423/00) to 99,7% (VHSV isolate DK 203490) and were compared with full genome sequence alignments in order to identify possible virulence markers.

Identification and characterisation of selected virus isolates:

In 2016 a number of virus isolates, sera and other reagents were received for further characterisation at the EURL and for storing in our virus library as shown in the table beneath.

Member States and countries outside EU		
Material received	**Laboratories**	**Units**
Diagnostic material for virology	8	210 samples
Diagnostic material for PCR	8	45 samples
Diagnostic material for bacteriology	1	30 samples
Cell cultures	1	1 sample
MAb/PAb	1	4 samples
Other material	1	7 samples

Further details are listed in Annex 2

Below is listed samples, isolates and reagents received for identification, characterization and update of the virus library and diagnostic procedures applied for the relevant cases:

CER Groupe Fish Diseases Laboratory, Belgium (François Lieffrig)

- Sperm + Coelomic liquid from Atlantic salmon to screen for the presence of ISAV within health monitoring in re-stocking program for wild salmon . ISAV was not detected in any of the samples (DTU-VET 16-2045)

Astrid Fishexport AB Sweden

- 175 whole herrings to assess the presence of VHSV in export of frozen wild herring to Canada from Sweden. VHSV genotype Ib was detected in 2 samples (DTU-VET 16-4758)

Istituto Zooprofilattico Sperimentale delle Venezie IZSVe, Italy (Anna Toffan):

- WSSK cells to enhance diagnostic capacities of sturgeon viruses.
- 6 ampoules with freeze dried inactivated nodavirus supernatant to participate in the first proficiency test for nodavirus.(DTU-VET 16-8497)

Veterinary Institute, Croatia (Snjezana Zrncic):

- 19 samples of sea bass organs from suspected cases of *Piscirickettsia salmonis* infection in European sea bass. (DTU-VET 16-7030).The bacterium was isolated and identified by Malditoff and PCR analysis and sequencing.
- 6 samples for KHV investigation. The virus was detected in 4/6 samples by qPCR. (16-9625).

University of Ljubljana, Slovenia (Vlasta Jencic)

- Kidney Tissue of 2 x 150 rainbow trout, grouped in 2 x 30 pools for BKD testing. All samples tested negative (DTU-VET 16-7744)

Institute of Veterinary Medicine, Serbia (Vladimir Radosavljevic):

- 3 samples of homogenate carp tissue (gills and Kidney) to investigate the presence of KHV (DTU-VET 16-12103). Virus was detected in the sample.
- 2 samples of viral isolate from cell culture inoculated with tissue from rainbow trout for confirmation of SAV (DTU-VET 16-12103). Virus was detected in the sample and sequenced.

National Research Institute of Aquaculture, Mie, Japan (Takafumi Ito):

- Monoclonal antibodies against Hirame rhabdovirus (HIRRV) (DTU-VET 16- 14572)

National Veterinary Institute, Norway (Ole Bendik Dale and Torfinn Moldal):

- Pox-Controls, pox-antibodies pox pos. histoslides and pox pos. paraffin blocks received for implementing qPCR and histopathology and for infection trials in Atlantic salmon within H2020 Aquaexcel 2020 project (DTU-VET 16-15593 Antibody ; 16-14800 ref material)

Veterinärmedizinische Universität Wien, Austria (Mansour El-Matbouli):

- 3 samples of homogenated organs and 3 purified DNA from the same samples to confirm presence of CypHV-3 / KHV. The virus was identified by qPCR from purified DNA, not from organs homogenate (DTU-VET 16-17468)

Marine Scotland Science, Marine Laboratory, Scotland (H. Stagg):

- Ranavirus isolates from lumpfish (DTU-VET 16-18335)

Marine Institute Fish Health Unit Rinville, Oranmore, Ireland (Neil Martin Ruane):

- Ranavirus isolates from lumpfish (DTU-VET 16-19667)

Institute for experimental pathology, Iceland (Sigridur Gudmundsdottir):

- Ranavirus isolates from Lumpfish (DTU-VET 16-20738)

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

The pathogen database www.fishpathogens.eu

The database now consisted in 1194 VHSV records, of those 811 are public, and the rest are placed as restricted. Both betanoda and IHNV databases have numbers similar to last year, with 96 records for IHNV and 62 for betanoda. A new SAV database has been established. A number of new features are in the process to be added to all databases. Among them, a batch edit function for add information and review isolates was added. This will allow, for example, to include links to published literature in cases where a group of isolates is mentioned/discussed in an article. Other functions to make the database more useful were added. In particular, we worked with the sequence matcher function in the VHSV database, which has been problematic in the past. This function allows taking a sequence and matching it against our database, obtaining a phylogeny of closely related isolates. A number of bugs were also corrected.

4-6 Perform molecular epidemiology analysis to improve knowledge on diseases spreading mechanisms of viral pathogens.

Molecular epidemiology analysis

In 2015 VHSV was found in Icelandic waters. Molecular tracing of the Icelandic isolates made possible to infer than the Icelandic VHSV isolate is not of European origin, but more closely related to the VHSV isolates found in the Atlantic coast of Canada and USA. In fact, marine isolates of VHSV found in European waters are quite far phylogenetically from the Icelandic one. Using molecular techniques we have inferred the time of separation between isolates and we are proposing the creation of a new subgenotype (IVd) for VHSV. Currently, we are preparing two manuscripts with the molecular tracing and pathogenicity studies of this new VHSV isolate, and results will be presented in the 10th International Symposium of Virus in Lower Vertebrates that will be hold in Budapest on June 2017.

In addition, data analysis has been performed to molecular trace the epidemiology of VHSV in Demark, from 1962 to the time of its eradication. Using character correlation phylogenetic analysis, we coded each big water catchment in Denmark with a number and treat the water catchments as a phylogenic character. We mapped each character in a ML tree and identify jumps (migrations) from one water catchment to another. As we estimated time for each node in the tree, we can have a good idea of when these events happened. We have identified a number of isolates with identical sequences, some of these persistent for up to two years. One problem was to distinguish truly identical isolates by ancestry from identical isolates caused by back mutations. To address this, we estimated the substitution rate of the G-gene in Danish isolates. Using this information we can estimate the probability of two

isolates having identical sequences after a determined period of time. Currently we have a manuscript in preparation addressing these results, and a presentation will be given at the European Society of Fish Pathologist meeting that will be hold in Belfast on September 2017.

4-7 Assessment and standardization of real-time PCR tests for the diagnosis, identification and typing of emerging and listed non-exotic and exotic fish diseases

Real-time PCR

A standardization and validation of the real-time RT-PCR developed by Purcell et al.: "Universal reverse-transcriptase real-time PCR for infectious hematopoietic necrosis virus (IHNV)" was initiated in 2015 and continued in 2016. The method is being translated and validated to a one-step procedure which is more convenient for use as a tool for surveillance of IHNV. The validation will be finalized and published in 2017. A real-time PCR for detection of Salmon pox-virus and for Cyprinid edema virus (CEV) was implemented. In addition we have implemented a two-step RT-qPCR to detect Atlantic salmon Calicivirus and a conventional PCR for detecting *Onchorynchus mykiss* virus (OMV). In addition, we implemented and are currently testing a real-time PCR for detecting *Renibacterium salmoninarum* the causative agent of bacterial kidney disease (BKD), which will come to be used instead of the nested PCR that we are currently using. We have also implemented a qPCR to detect and quantify Tetracapsuloides bryosalmonae the causative agents of proliferative kidney disease (PKD).

4-8 Emerging diseases: In collaboration with specialised experts WW to review selected emerging fish diseases in Europe and assess their potential listing as exotic or non-exotic diseases

Emerging diseases:

The following studies on emerging diseases have been conducted in 2016:

Red Mark Syndrome (RMS) is an emerging infectious disease affecting rainbow trout in European aquaculture. A *Midichloria*-like organism (MLO) belonging to the family *Midichloriaceae* within the Order *Rickettsiales* has been the main suspect for causing RMS. We have established a repeatable and ongoing cohabitation model of RMS in rainbow trout, where previous cohabitants affected by RMS are seeders for new cohabitants. At present we have successfully made 5 consecutive passages Next generation sequencing of the 16S rRNA gene from skin samples from the cohabitants show a strong correlation of MLO with RMS symptoms.

Salmon gill poxvirus (SGPV) is an emerging pathogen associated with massive mortalities in Norwegian salmon farms. Our colleagues from the Norwegian Veterinary Institute (NVI) and colleagues at National Institute of Health (NIH), Bethesda, USA, had a recent breakthrough on the disease and its causative agent. In order to increase the knowledge on pathways of infection in salmon gill poxvirus disease (SGPVD) infection studies were conducted in our facilities in a transnational access study between NVI and the EURL.

Piscine orthoreovirus (PRV) causing heart and skeletal muscle inflammation (HSMI) in Atlantic salmon (PRV1) and heart inflammation in rainbow trout (PRV3) seem to be widespread in wild Atlantic salmon. The presence of PRV1 and PRV3 was

screened for a large number of fish (salmon and trout) from Denmark and Sweden showing no presence of PRV3 and low prevalence of PRV1.

A novel calicivirus replicating in Atlantic salmon were recently identified and genetically characterized and published in PLOS-ONE. Diagnostic methods and controls were received from the authors at NMBU, Oslo and included in the repository of the EURL for diagnostic and research.

4-9 Producing virtual teaching material (e-learning)

The aim of this task is: Preparing virtual guidelines for conducting proficiency tests, for sampling and shipment of material for laboratory examination; and for receipt and processing fish tissue material for virology (inoculation on cell cultures and for PCR analysis) and histopathology. These tasks will be conducted in 2017.

4-10 Molecular characterization of fish cell lines Perform molecular analysis to "bar-code" and certify cell lines routinely used for viral diagnostics

The cell lines we use as EURL are well characterized in respect of passage numbers and their susceptibility to infection with a range of fish viruses, but have never been characterized properly on their genetics and origin. Over the years several mitch-matches between cell lines of different origin have been observed it was therefore our goal for each cell line and sub lineage to barcode their genetic origin.

As it appears from the table below several of the cell lines in our repository do not have the origin that we expected. Thus originated the carp cell line EPC from fat head minnow and was most likely due to a contamination with FHM cells. This happened however most probably back in the seventies since similar findings was made in another laboratory and the 2 cell lines have evolved in different directions and have now different properties. The channel catfish cells CCO originated from the related fish species brown bullhead, the Atlantic salmon kidney cells ASK originated from rainbow trout and is most likely a derivate of TO cells from Rainbow trout. Surprisingly the sea bass cell in our repository were in fact CHSE cells why we have requested a new batch of this cell line from the laboratory who produced them to assess where the cross contamination originated.

Cell line	Name	Expected origin	De Facto origin
EPC*	*Epithelioma Papullosum Carpio*	Common carp	Fat Head Minnow
BF-2	Bluegill Fry	Bluegill	Green sunfish / bluegill
CHSE-214	Chinook Salmon Embryo	Chinook Salmon	Chinook Salmon
RTG-2	Rainbow trout gonad	Rainbow trout	Rainbow trout
FHM	Fat Head Minnow	Fat Head Minnow	Fat Head Minnow
CCO	Channel Catfish Ovary	Channel Catfish	Brown bullhead
EK-1	Eel Kidney	Pacific eel.	Japanese eel
ASK	Atlantic Salmon Kidney	Atlantic Salmon	Rainbow trout

CCB	Common Carp Brain	Common Carp	Common Carp
SBL	Sea Bass Lymphoid	European seabass	Chinook Salmon
WSSK	White Sturgeon Skin-1	White sturgeon	white sturgeon

5. Missions

5-1 Missions: Organizing missions to relevant laboratories. Missions focus on NRLs where on-site communication would be beneficial. As collaboration with NRLs in 3rd countries from where EU is importing large amount of fish

Missions to relevant laboratories

In 2016 three missions were conducted.

Visit in Korea.
Prof. N.J. Olesen visited the National Fishery Products Quality Management Service, Busan, Republic of Korea in connection with a workshop organized by NFQS on VHS. Since 2011 NFQS participated successfully in the proficiency test organised by the EURL. The laboratory moved in 2016 from Seoul to Busan and was not fully up and running when visited. With very spacious facilities and small but dedicated staff the institute was fully able to cover the requirements for being a National reference laboratory- the facility however still lack access to experimental facilities and the division of tasks between NIFS and NFQS under the same ministry is still unclear with NIFS taking hold on surveillance and NFQS on import/exports.

Visit in China.
The General Administration of Quality Supervision, Inspection and Quarantine (AQSIQ) of P. R. China invited prof. N. J. Olesen for a 1 week workshop and study visit to the National Key Laboratory of Aquatic Animal Health, Animal and Plant Inspection and Quarantine Centre, Shenzhen Exit&entry Inspection and Quarantine Bureau, Guangdong Provine, P. R. China. Since 2012 AQSIQ participated successfully in the proficiency test organised by the EURL. With very spacious facilities and a dedicated staff the institute was fully able to cover the requirements for being a National reference laboratory for the region, the facility however still lack access to experimental facilities

Visit in Switzerland
The National Reference Laboratory for Fish Diseases (NRL-NAFUS) in Switzerland is located at the FIWI-Centre for Fish and Wildlife Health, at the Vetsuisse Faculty in Bern. The laboratory was visited from the 13th to the 15th of December 2016 by Teena Vendel Klinge, Niccolò Vendramin and Nikolaj Gedsted Andersen from the EURL Fish. A program for the visit was set up and a number of persons from the Swiss laboratory met with the EURL representative. The report from the mission describes findings, comments and

recommendations made by the delegation from the EURL.
The report is enclosed as
Annex 3

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

International meetings organized.
Contact with colleagues from other laboratories is a channel for exchange of information in the field of fish diseases, and an opportunity to keep abreast with new developments in the field. Of special interest are of course the activities relating to VHS, IHN, KHV, ISA and EHN, but a number of emerging disease are coming up and need attention as well. Scientists at the EURL organized and/or participated in the following international meetings and conferences in 2016:

Cleaner fish in aquaculture
A scientific working group meeting was organized inviting qualified experts to compile available knowledge and organize it on scientific based advice.
The meeting took place on November 1st and 2nd 2016 at the premises of DTU Veterinary Institute. The meeting was held due to the increasing use of cleaner fish (both wrasse and lumpfish), witch health management and disease control have become issues of central importance especially after the VHS outbreak in Scotland in 2012 and Iceland 2015 and the EURL-Fish was asked by the EU Commission to provide a qualified opinion including guidelines and recommendations for management of these fish in EU and assess the possibilities for including cleaner fish in the legislation.
The following experts attended the meeting:
- Dr. Snorre Gulla DVM PhD from Norwegian Veterinary Institute Oslo
- Dr. Sandy Murray – epidemiologist at Marine Scotland Science
- Prof. Sandra Adams from the Institute of Aquaculture, University of Stirling
- Tamsin Cochrane-Dyet DEFRA- fish health inspector

From the EURL for fish diseases team Prof. Niels Jørgen Olesen, coordinator of the EURL Niccolò Vendramin and Dr. Nikolaj Gedsted Andersen took part in the event.

The meeting aimed to acquire knowledge on current practices in relation to catching, farming, transporting, deployment and re-use of cleaner fish in aquaculture. It was asked how these new farmed species fit into the legislative framework of CD 2006/88 and whether amendments for current legislation are needed.
Prof. Olesen started the meeting presenting the current legislative framework and highlighting the different articles that are relevant for cleaner fish. After this Niccolò Vendramin presented an overview of listed diseases outbreak occurred in cleaner fish in recent years. This presentation included the VHS outbreak in Scotland in 2012 in wild caught wrasse and isolation of VHSV and ranavirus from lumpfish from Iceland in 2015. Dr. Gulla from Norway, who recently defended his PhD thesis focusing on diseases of cleaner fish in

Norway, provided an overview of current practices with cleaner fish focusing on health issues. Afterwards prof. Sandra Adams presented the current practices in Scotland with a focus on use of vaccines for preventing disease outbreak in farmed cleaner fish. Dr. Tamsin Cochrane-Dyet from fish health inspectorate in England and Wales provided interesting suggestion for setting up surveillance program for cleaner fish, looking into current gaps in the legislation

Finally Dr. Murray from Marine Scotland Science in Scotland presented a framework for risk assessment on the use of cleaner fish in aquaculture.

On November 2nd the group decided upon a working plan for drafting and finalizing a report collecting all needed recommendation for the EU commission.

The overarching goal of this report is to investigate if in the current legislation there are gaps in the current legislation with regard to the prevention and control of infectious diseases in aquaculture as well as health surveillance for aquaculture animals, currently regulated by Council directive 2006/88/EC.

The full report with recommendations from the meeting can be downloaded at
http://www.eurl-fish.eu/reports

Participation at international conferences and meetings

20th Annual Workshop of the National Reference Laboratories for Fish Diseases, Copenhagen, Denmark, May 31st- June 1st 2016

Aquatic Animal Health Workshop, AQSIQ Shenzhen City, China; Nov. 7-9th 2016
Aquaculture animal health trends and threats in Europe: Production and diseases Legislation and Reference laboratories; Niels Jørgen Olesen

International workshop on the VHS Research in Korea, Busan, RoK April 2016: Lecture 1: VHS: The current situation or “The disease that never stop surprising us!” Lecture 2. Molecular tracing of VHS outbreaks.

DAFINET international workshop, Copenhagen, Denmark November 2016

Peer reviewed publications
Presentations and posters

Presentations and posters

Articles published in peer-reviewed journals:

Haenen O.L.M., H. Schuetze, M. Cieslak, S. Oldenburg, M.A.H. Spierenburg, I. Roozenburg-Hengst, M. Voorbergen-Laarman, M.Y. Engelsma and N.J. Olesen (**2016**) First evidence of Infectious Hematopoietic Necrosis Virus (IHNV) in the Netherlands. Journal of Fish Diseases doi:10.1111/jfd.12434

Takafumi Ito, Jun Kurita, Koh-ichiro Mori, Niels J Olesen (**2016**) Virulence of viral haemorrhagic septicaemia virus (VHSV) genotype III in rainbow trout. Vet Res 47:4

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Abstracts, proceedings and reports at international meetings

20th Annual Workshop of the National Reference Laboratories for Fish Diseases, Copenhagen, Denmark, May 31st- June 1st 2016 with the following presentations:
1: Overview of disease situation in Europe, Niels Jørgen Olesen
2: Update on the disease situation in aquatic organisms in the Mediterranean Niccolò Vendramin
3: Risk assessment of new VHSV from Lumpfish for Rainbow Trout, Atlantic Salmon and Lumpfish, Niccoló

Vendramin
4: Susceptibility of Atlantic Salmon to IHNV E-genotype and interference with PRV infection; Niccoló Vendramin
5: Diagnostic Manual for NON EXOTIC and EXOTIC fish diseases; Niels Jørgen Olesen
6: Development and validation of a conventional RT-PCR for detection of VHSV Niels Jørgen Olesen

International scientific collaborative studies

Participation in international scientific collaborative studies

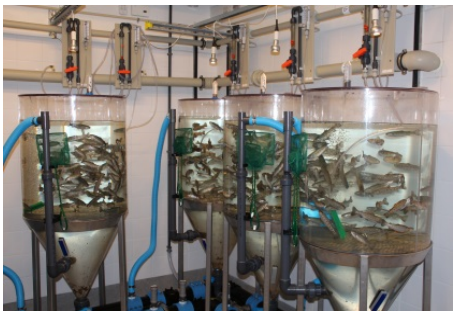
NOVIMARK: Identification of virulence markes in VHSV and development of tests for discriminating between virulence properties
The group is partner in the ANIHWAS-ERA Net project NOVIMARK and work package leader of WP1.
The purpose of the project is to identiy virulence markers in two loss-making Novirhabovirus as a key to improve diagnostic and strategic management in farmed rainbow trout . The participants are Institut National de la Recherche Agronomique (INRA); Agence Nationale de Sécurité Sanitaire (ANSES); Universidad de Santiago de Compostela (USC); Istituto Zooprofilattico Sperimentale delle Venezie (IZSVe) and Centre for Environment Fisheries and Aquculture Sciences (CEFAS). A PhD student, Anna Luiza Farias Alencar was employed in our team from July 1st 2016 and have conducted a number of cloning's and infection trials with selected VHSV isolates from our large repository.

AQUAEXCEL^{2020}
The DTU Fish diseases group is partner in AQUAEXCEL^{2020}.The objectives of AQUAEXCEL^{2020} are to provide a wider and more efficient access to, and use of, the aquaculture research infrastructures existing in the EU. AQUAEXCEL2020 is a key vehicle in the improvement of aquaculture research practices. It will lead to a better management of animal experiments for research according to the 3 R's. One major feature of AQUAEXCEL2020 is its **Transnational access (TNA) program**, allowing external teams to access the partners' infrastructures via submission of research proposals, which are funded or not based on evaluation by an independent selection panel. Detailed information is available at http://www.aquaexcel2020.eu/
In 2016 a transnational access was conducted where the infectiology of pox virus was assessed in Atlantic salmon.

ParaFishControl.
DTU is partner in the H2020 project ParaFishControl under the call "sustainable food security". The aim of the project is to 1) generate new knowledge on the most important parasites in fish farming including genome analysis, life cycles, and host parasite interactions especially in order to improve prophylaxis 2) assess the impact of transfer of parasites between farmed and wild fish. 3) develop an array of new prophylactic measures, including vaccines and functional feed. 4) develop specific and sensitive diagnostic tools 5) risk assessments, 6) identify zoonotic risks 7)

develop catalogue on GMP
The main objectives for DTU is:

1. Validation of diagnostic procedures for detection of parasites based on qPCR, ISH and IHC, by e.g. inter-laboratory proficiency tests.
2. Participate in development of ”point of care” diagnosis
3. Participate in publishing diagnostic protocols and standards.
4. Optimization of use of cleaner fish for controlling ectoparsittes.
5. Treatment and management of infected fish farms.



Annex 1 Reagents supplied by the EURL-Fish in 2016

Country	Name	Institute	Date of receipt	Material	Amount	No.
The Netherlands	Olga L.M. Haenen	Central Veterinary Institute (CVI) of Wageningen UR	20.01.16 + 26.01.16	Freeze dried VHSV	1 ampoule	
Italy	Anna Toffan	Istituto Zooprofilattico Sperimentale delle Venezie	20.01.2016	Cell supernatant. Virus not identified	2 tubes	15-16934
Norway	Bjørn Spilsberg	Norwegian Veterinary Institute	26.01.2016	Blood infected with Virus-Y	2 tubes	14-4566
Iceland	Sigridur Gudmundsdottir	Institute for Experimental Pathology University of Iceland	08.02.2016	Antibodies, IPN, VHS, IHN, SVC	6 tubes	
Spain	Pilar Fernández Somalo	Laboratorio Central de Veterinaria MAGRAMA	11.02.2016	Antibodies, VHS + IHN	2 tubes	
The Netherlands	Olga L.M. Haenen	Central Veterinary Institute (CVI) of Wageningen UR	07.03.2016	EPC and FHM cells	2x 2 flasks	
The Netherlands	Olga L.M. Haenen	Central Veterinary Institute (CVI) of Wageningen UR	29.03.2016	BF-2 cells	4 flasks	
Canada	Dante Mateo	Atlantic Veterinary College University of Prince Edward Island (UPEI)	07.04.2016	SAV II SAV VI	2x 2 tubes	9895349 9895379
Scotland	Eann Munro	Diagnostic Group Leader Marine Scotland – Science Scottish Government	12.04.2016	G24-IHNV panel SHRV HIRRV	24 tubes 1 tube 1 tube	 16-6904 16-5313
Korea	Hyoung Jun KIM	NFQS National Fishery Products Quality Management Service	15.04.2016	BF-2 cells	1 flask	
Italy	Anna Toffan	Istituto Zooprofilattico Sperimentale delle Venezie	20.04.2016	Supernatant of organs homogenate from Sturgeon	2x 1 tube	16-5934
Brasil	Anna Luiza Farias Alencar	Universidade de São Paulo FZEA	20.04.2016	CHSE cells	2 small flasks	
Estonia	Ave-Ly Toomvap	Estonia Veterinary and Food Laboratory	24.05.2016	BF-2 and EPC cells	2x 2 small flasks	
The Netherlands	Olga L.M. Haenen	Central Veterinary Institute (CVI) of Wageningen UR	01.06.2016	BF-2 cells	2 flasks	
Poland	Marek Matras	National Veterinary Research Institute	01.06.2016	CHSE cells	2 flasks	

Country	Name	Institute	Date of receipt	Material	Amount	No.
Belgium	François Lieffrig	CERGroupe Fish Diseases Laboratory	06.06.2016	EK-1 cells	2 flasks	
Belgium	A. Vanderplasschen	University of Liège	06.06.2016	Virus from Eel	5 tubes	
Sweden	Charlotte Axén	National Veterinary Institute	06.06.2016	Supernatant after grinding of organ from Herring	9 tubes	16-4758
The Netherlands	Olga L.M. Haenen	Central Veterinary Institute (CVI) of Wageningen UR	08.06.2016	EPC cells	2 flasks	
UK - England	Richard Keith Paley	CEFAS Weymouth Laboratory	08.06.2016	KF-1 cells	2 flasks	
Italy	Anna Toffan	Istituto Zooprofilattico Sperimentale delle Venezie	22.06.2016	Cell supernatant. Virus not identified	2 tubes	16-7445
Norway	Torfinn Moldal	Norwegian Veterinary Institute	28.06.2016	G24-IHNV panel Infected cell supernatant	24 tubes + 1 tubes	15-16934
Italy	Anna Toffan	Istituto Zooprofilattico Sperimentale delle Venezie	06.07.2016	Fish tissue	4+1 tubes	16-10588
Chile	Ricardo Enríquez Sais	Facultad de Ciencias Veterinarias Universidad Austral de Chile	19.07.2016	FTA Cards (VHSV) EPC cells BF-2 cells	2x 4 cards 2 flasks 2 flasks	
Italy	Anna Toffan	Istituto Zooprofilattico Sperimentale delle Venezie	24.08.2016	Cell supernatant. Virus not identified. RTG-2 cells EK-1 cells	4 tubes 1 flask 2 flasks	16-74450 16-9507
India	Kooloth Valappil Rajendran	Indian Council of Agricultural Research (ICAR)	24.08.2016	FTA Cards (VHSV)	2 cards	
Norway	Espen Rimstad	Veterinærhøgskolen - NMBU	14.09.2016	PRV-*Om* infected blood	9 tubes	15-4566
Serbia	Vladimir, Ivan Radosavljevic	Institute of Veterinary Medicine of Serbia	14.09.2016	BF-2 cells EPC cells CHSE cells	2 flasks 2 flasks 2 flasks	
Norway	Maria Dahle	Norwegian Veterinary Institute	03.10.2016	RNA	118 tubes	15-10833
UK - England	Keith Way	Cefas Weymouth Laboratory	03.10.2016	Mab anti IHNV	1 tube	
Belgium	François Lieffrig	CERGroupe Fish Diseases Laboratory	03.10.2016	SAV VI	1 ampoule	
France	Yannick Blanchard	ANSES Ploufragan-Plouzane Laboratory	12.10.2016	VHSV infected cell supernatant	13 tubes	16-13894
Norway	Maria Dahle l	Norwegian Veterinary Institute	17.10.2016	Full blood (PRVom)	15 tubes	16-15941

Country	Name	Institute	Date of receipt	Material	Amount	No.
Korea	Hyun-Wook KWON	NFQS National Fishery Products Quality Management Service	26.10.2016	VHSV infected supernatant VHSV Ring Test PCR Produkter	25 tubes 2x 5 FTA Cards 24 tubes	
P.R. China	Liu Hong	The National Key labortory of Aquatic Animal Health Animal and Plant Inspection and Quarantine Centre, Shenzhen Exit&entry Inspesction and Quarantine Bureau General AQSIQ	04.11.2016	EPC cells BF-2 cells CHSE cells SSN cells	2 flasks 2 flasks 2 flasks 2 flasks	
Norway	Karine Lindmo Yttredal	PHARMAQ AS	07.11.2016	IHNV, cell culture	3 tubes	
Denmark	Torsten Boutrup	Aquapri A/S	14.11.2016	Eagles MEM	1 flask	
Norway	Anne Berit Olsen	Norwegian Veterinary Institute	16.11.2016	Glass slides (Atlantic salmon with PRV and IHNV)	136 slites	
Norway	Simon Weli	Norwegian Veterinary Institute	16.11.2016	CCB cells. SSN-1 cells. GF cells. WSSK cells.	2 flasks 2 flasks 2 flasks 2 flasks	
China	Tao Sun	Shandong Entry-exit Inspection and Quarantine Bureau	22.11.2016	MAb IP5B11 MAb IHNV Hyb 136-3	1 tube 1 tube	
Serbia	Vladimir, Ivan Radosavljevic	Institute of Veterinary Medicine of Serbia	30.11.2016	CCB cells CHSE cells	2 flasks 2 flasks	
Norway	Maria Dahle	Norwegian Veterinary Institute	30.11.2016	Fish tissue (PRV-IHNV)	118 tubes	15-10833
Peru	Mervin Guevara Torres	Coastal Laboratory of IMARPE, Tumbes headquarters	07.12.2016	FTA Cards - VHSV	2 cards	
Denmark Germany France Italy England Korea	Tine Iburg Heike Schütze Thierry Morin Anna Toffan David Stone Kwon Hyun	NVI - DTU FLI ANSES IZSVe CEFAS NFQS	07.12.2016	VHSV PT2016 on FTA Cards	5 cards 5 cards 5 cards 5 cards 5 cards 5 cards	
Norway	Knut Falk	Norwegian Veterinary Institute	19.12.2016	Mab IHNV	1 tube	

Annex 2 Reagents received in 2016

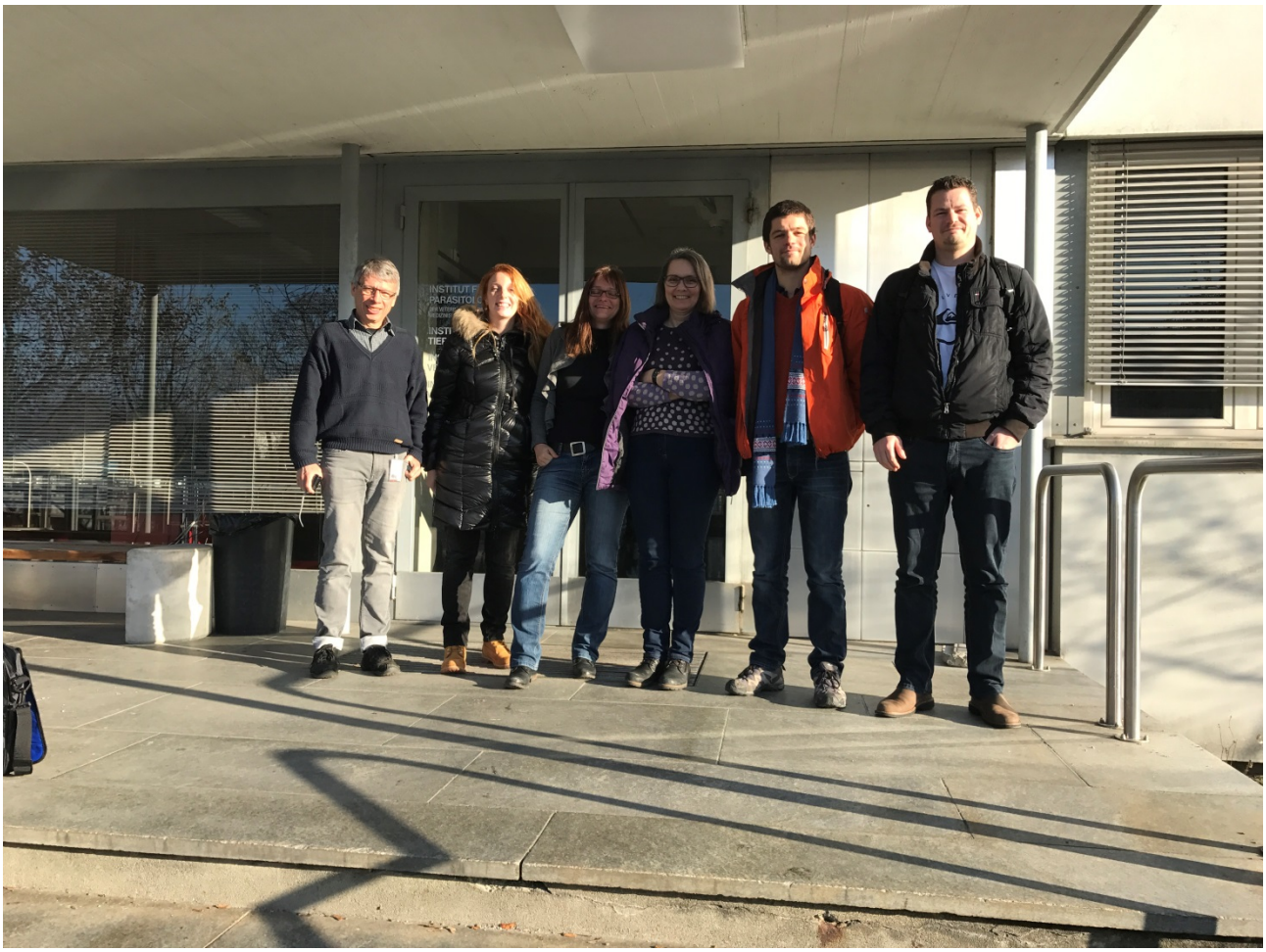
Country	Name	Institute	Date of receipt	Material	Amount	Protocol No.
Belgium	François Lieffrig	CERGroupe Fish Diseases Laboratory	26.01.16	Sperm + Coelomic liquid	1 + 2 tubes	16-2045
Sweden	Astrid Fishexport AB		22.03.2016	Whole herrings	175 fish	16-4758
Italy	Anna Toffan	Istituto Zooprofilattico Sperimentale delle Venezie	19.04.16	WSSK cells	1 small flask	
Croatia	Snjezana Zrncic	Veterinary Institute Lab. of Fish and Molluscs Pathology	28.04.16	Organs Seabass	13 samples	16-7030
Slovenia	Vlasta Jencic	University of Ljubljana, Veterinary Faculty	18.05.16	Kidney	30 units	16-7744
Italy	Anna Toffan	Istituto Zooprofilattico Sperimentale delle Venezie	27.05.16	Freeze dried inactivated viral supernatant.	6 vials	16-8497
Croatia	Snjezana Zrncic	Veterinary Institute Lab. of Fish and Molluscs Pathology	17.06.16	Homogenate organ supernatant	6 samples	16-9625
Serbia	Vladimir, Ivan Radosavljevic	Institute of Veterinary Medicine of Serbia	02.08.16	Homogenate carp tissue (gills and Kidney). Homogenate trout tissue.	3 samples 2 samples	16-12103
Japan	Takafumi Ito		08.09.16	Monoclonal HIRRV antibodies	4 tubes	16-14572
Norway	Torfinn Moldal	Norwegian Veterinary Institute	14.09.16	Pox-control	2 tubes	16-14800
Norway	Ole Bendik Dale	Norwegian Veterinary Institute	27.09.17	Pox-antibodies Pox pos. histoslides Pox pos. Paraffin blocks	1 tube 4 slides 2 blocks	16-15593
Austria	Mansour El-Matbouli	Veterinärmedizinische Universität Wien	26.10.16	Homogenated organs + DNA	3 samples 3 samples	16-17468
Scotland	Hannah Stagg	Disease Diagnostic Dept. Marine Scotland Science Marine Laboratory	08.11.16	Cell supernatant	2 tubes	16-18335

Country	Name	Institute	Date of receipt	Material	Amount	Protocol No.
Ireland	Neil Martin Ruane	Marine Institute Fish Health Unit Rinville, Oranmore	23.11.16	Cell supernatant	2 tubes	16-19667
Iceland	Sigridur Gudmundsdottir	Institute for Experimental Pathology University of Iceland	13.12.16	Cell supernatant	3 vials	16-20738

Annex 3 – Mission in Switzerland

Laboratory visit at the
National Reference Laboratory for Fish Diseases
Vetsuisse Faculty, Bern
Switzerland

Bern, Switzerland
13th – 15th December 2016



Introduction

The National Reference Laboratory for Fish Diseases (NRL-NAFUS) in Switzerland is located at the *FIWI-Centre for Fish and Wildlife Health, at the Vetsuisse Faculty in Bern.* The laboratory was visited from the 13th to the 15th of December 2016 by Teena Vendel Klinge, Niccolò Vendramin and Nikolaj Gedsted Andersen from the European Union Reference Laboratory for Fish Diseases (EURL). The program for the visit is shown in Annex 1 and the list of participants is found in Annex 2. This report describes findings, comments and recommendations made by the delegation from the EURL. This report is sent to the NRL and the EU Commission.

Organisation

The Centre for Fish and Wildlife Health (FIWI) is appointed as national reference laboratory for fish diseases in Switzerland. The group is located in the Bern branch to the Vetsuisse Faculty. Vetsuisse is the veterinary faculty in Switzerland and includes the branch of veterinary sciences of Bern and Zurich.
FIWI tasks are divided into three main clusters of activities; education, research, diagnostic services and scientific advice.
The area where these tasks are conducted are related to the specific needs of different institutional bodies. First the University of Bern has demands in terms of research, teaching (undergraduate, graduate students, thesis students, master students, residents and trainees) and administration. The Federal Food Safety and Veterinary Office (FSVO) sustains activities in the field of diagnostics of fish diseases (accreditation and Swiss reference laboratory for fish diseases), research (fish), advisory service (authorities, private veterinarians and fish farmers) and continuous education (authorities). Finally Federal Office for the Environment (FOEN) inquire demands in terms of diagnostics of wildlife diseases (pathology), research (wildlife), advisory service (authorities and private persons) and further education (authorities).

The Centre for Fish and Wildlife Health (FIWI) is managed by professor Helmut Segner. The National Fish Disease Laboratory is directed by Professor Thomas Wahli, in total the group is composed of 16 members including academics, technicians and students.

Buildings, Furnishing and Access

FIWI is located at the Veterinary Faculty of Bern close to the center of Bern in Switzerland.

All access to the laboratories is controlled through keys. There is free access to the faculty room for the students, whereas the access to laboratories, offices, and wet lab facilities is restricted. Laboratories for cell cultivation have controlled temperature in order to maintain the environment cooled.

Meeting Organization

The meeting was divided into three sessions.

On the 13th after the introduction to the laboratory the following topics were discussed:

- Introduction to the laboratory, main activities carried out, presentation of the staff involved in the area
- Laboratory procedures and proficiency test results
- Visit to the facilities, description of working flows, quality insurance system, traceability system for diagnostic samples receive

On the 14th a seminar on PKD (a relevant parasitic disease) was organized.
On the 15th in the morning there was a wrap up of the activities, and discussion for future cooperation.

December 13th - opening of the meeting

The official opening took place on the 13th December at 13:00 where Thomas Wahli, head of the National Fish Disease Laboratory welcomed the EURL team at the NRL for fish diseases in Switzerland.
Niccoló Vendramin, started with a presentation of the EURL for fish diseases, its activities and tasks and

the background for organizing the visit.
Afterwards Thomas Wahli provided a presentation of the activities run at the laboratory in Switzerland (a handout version of the presentation is added in Annex 6).
The meeting continued dialoguing on performances of Inter-laboratory Proficiency Tests (PT) and the results obtained during the PT2016.
During this first session, an interesting overview of fish farms in Switzerland was provided by Nicolas Diserens who also described his project on surveillance of viral fish diseases in Switzerland.
After a coffee break a short tour through the institute, including a description of the institute and structure of the laboratory for fish diseases, was effected. The tour was guided by Thomas Wahli.

Aquaculture in Switzerland and related surveillance and diagnostic activity for fish diseases
As described in Survey and Diagnosis (report 2016) and in the presentation given by Postdoc Nicolas Diserens, aquaculture is developed in Switzerland to a medium degree. The country counts approximately 350 farms. Most relevant farmed species are Rainbow trout and Brown trout or Lake trout. A first very important data to remark is the number of facilities for re-stocking purposes, in fact in Switzerland approximately a third of the registered farms, have only seasonal activity, and breed salmonids for re-stocking purposes, the remaining farms mostly produce fish as food resource, and a small proportion produce fish both for food and restocking. It is common for smaller facilities that they are side activities of other agricultural businesses, while farms with an annual production of $\geq$ 50 tonnes most often rely exclusively on fish production.
Looking into the distribution of production classes, 84 % of registered farms in Switzerland produce less than 5 tonnes of fish per year, 11 % between 5 and 100 tonnes, and only 1 % more than 100 tonnes (the remaining 4 % is unknown).

Half of the farms purchase juvenile fish from other Swiss producers and the other half purchase eggs, most often from abroad. Very few large farms produce eggs to be sold for the on-growing, thus the production heavily relies on importation of fertilized eggs.

Pathogens and related diseases investigated at the fish disease laboratory
Fish health surveillance
There are some remarkable features in Swiss legislation regarding fish health surveillance and management of fish diseases in Switzerland.
The surveillance carried out is only passive surveillance, currently there is no zoning system and Switzerland does not export any live fish to other European countries. Currently seven diseases are listed in the national legislation and different control measures are implemented according to which disease is involved in a given outbreak. Three viral diseases; ISA, VHS and IHN are considered major threats and upon demonstration of the pathogen, eradication procedures are implemented to prevent an outbreak. Switzerland is considered free of ISA, while VHS and IHN are considered to be endemic in the country.
IPN is also a listed disease in the legislation but in case of an outbreak, the farm can finalize the production cycle and sell the animals to slaughtering, where introduction of new animals is not allowed until the outbreak is cleared.
Finally PKD and SVC are also listed diseases, but these need only to be notified to the authorities. Other diseases provoking major losses in European countries such as Alphavirus infections have only be

detected occasionally so far in Switzerland. A major threat to the aquaculture are bacterial diseases with Flavobacterial infections being particularly wide spread.

Fish diagnostics

NAFUS/FIWI is also the only diagnostic laboratory in Switzerland having important role in monitoring fish diseases in the country and being acknowledged by the federal authorities for performing diagnostics on notifiable fish diseases.

Since 1978, when the group was started, approximately 15.000 cases have been analysed, mostly Rainbow trout and Brown trout originating from private farms. A significant amount of samples originate also from ornamental fish in fresh water. More than 40 % of the diagnoses performed refer to parasites, approximately 26 % to bacteria and 5 % to viruses. Within parasites, ciliates have been diagnosed in 30 % of the cases, monogeneans in 25 % and zoomastigophora in 23 %.

Within bacteria, a part from known major pathogens for salmonids as *Aeromonas salmonicida*, *Yersinia Ruckerii* and *Flavobacterium psychrophilum*, an important number of gill diseases have been diagnosed.

Finally within viral diseases, VHS has been diagnosed in almost 60 % of viral diseases diagnoses followed by IPN.

The Fish Disease Laboratory

The fish disease laboratory is responsible for diagnostics of diseases of aquatic animals from farms and open water. The division for fish diseases coordinates the NRL for fish, crustacean and mollusk diseases. The NRL for fish diseases include molecular based fish diagnostics, cell culture facilities for viral isolation, histopathology and bacteriology laboratory.

Staff of the Fish Disease Laboratory

The fish disease laboratory consists of a group of 16 persons differently involved in the laboratory.

Professor Helmut Segner is head of FIWI and supervises PhD students, Professor Thomas Wahli is the head of the NRL for fish diseases and is involved in research and teaching activities. Heike Schmidt-Posthaus and Beat Von Siebenthal are mainly in charge of diagnostics activities. Four postdocs and four PhD students currently conduct research projects. Finally a team of three technicians is employed to take care of different tasks in the laboratories. The laboratory gets support from other employees at the institute partly allocated to fish diseases and employ students (MSc and PhD) to carry out research projects.

The Fish Disease Laboratory

The laboratory is well equipped, and the space allocated to laboratory is appropriate, it positively impress how the work flow is organized, keeping space separation between different parts of the laboratories (sample preparation, “dirty room” for inoculation of cells, virus identification and clean room for pathogen free cell preparation).

Cell culture facilities

Two separate rooms are allocated to the cell culture facility. One room is allocated to maintenance and production of cell culture for diagnostic purposes, so to speak a “clean” cell culture facility. This room has several incubators and a LAF bench. A second room is allocated to inoculation of cell culture monolayer with supernatant obtained from diagnostic samples, this activity is conducted in a separate

LAF bench, and cells are incubated in individual incubators. Cell cultures are stocked in -150 °C freezers, with high safety systems and alarms which provide liquid nitrogen in case of technical failure.

Freezers at -150 °C and a liquid nitrogen safety system in case of technical failure.

PCR based diagnostics

The laboratory for fish diseases contains equipment for performing PCR based fish diagnostic analyses. Diagnostic PCRs and RT-PCRs are performed by trained technicians to identify and characterize viral, bacterial (mycobacteria, *Renibacterium salmoninarum*, *Flavobacterium psychrophilum*), fungal (*Pseudoloma neurophilia*) and parasitic (*Tetracapsuloides bryosalmonae*, scuticociliates) pathogens. Currently, the implementation of diagnostic RT-qPCR for VHSV, IHNV and IPNV has been a task through research screening programs conducted in 2015-2016.

Laboratory procedures and Proficiency Test results

The proficiency tests (PT) allow a laboratory to assess their diagnostic capacity of certain procedures. The Swiss NRL for fish diseases has participated in the PT for identification of notifiable fish diseases organized by EURL, DTU since 1996. The NRL used the following tools for identification of viral content in the test: Titration of virus; Isolation of viruses on cell culture; Identification of virus by ImmunoFluorescence, RT-PCR, Real Time PCR and sequencing.

Sample acceptance and reporting unit

The visit tour started in the room used for acceptance of samples, fish necropsy and ELISA for BKD. Samples from fish farms are generally delivered directly to the fish disease laboratory either through post service from the different cantons or personally by the farmers. All samples are after acceptance registered and given a unique number which allows tracing back samples and analyses when the report is issued to the authorities or the fish farmer. The laboratory for fish diseases at VetSuisse relies on an IT system named Polypoint, which is also used in the institute of Animal Pathology and all clinics, for tracing samples and analyses. When the results of the diagnostic analyses have been obtained a final report is made with the same system. These answers have to be signed by the leading researcher or the head of the laboratory, before it is send to the customer.

PCR Facility

PCR facilities are partly shared within the building with other institutes of the same department (Department of infectious diseases and pathobiology = DIP) of the faculty.

One Separate room is allocated for RNA/DNA Purification, one for preparation of the master mix, and a further room contains PCR machines and gel facilities.

Tank facilities

At the laboratory for fish diseases in Bern, a number of in vivo trials are organized yearly. Live fish are normally acquired from farms and stocked and quarantined in a tank facility located outdoors, with restricted access. The management of facilities is done by the employees of the group. In a close by building, there are two rooms with flow through freshwater systems where in vivo challenges are conducted. Water discharged from the facility is disinfected before being released in the municipality waste system.

December 14th – PKD seminar

Time: 9:00 to 16:00

Participants: Thomas Wahli, Heike Schmidt-Posthaus, Nicole Strepparava, Teena Vendel Klinge, Niccólo Vendramin and Nikolaj G. Andersen (minutes)

Aim of the meeting: The aim of the meeting was to discuss and plan laboratory experiments with rainbow trout and PKD, to be conducted at the National Veterinary Institute (DTU, Denmark), and to discuss possibilities for future cooperation on the topic. The DTU team had beforehand sent out a list of questions to be discussed at the meeting (Appendix 1, page 3).

Summary: The meeting was opened with a presentation by Nicole who gave a thorough introduction to PKD and the experimental results from her postdoc. The results were discussed continuously during the presentation. In the afternoon there was a short introduction to the laboratory facilities and fish stable, before the meeting continued. During the afternoon a presentation was given by Elena Wernicke von Siebenthal and Kristina Rehberger on their future PhD projects concerning PKD.

Bryozoans: A culture of Bryozoans is not established at the University of Bern. Colonies of bryozoans are collected from nearby rivers and brought to the lab, where they can be used for experiments for a maximum of four days before they die. Bryozoans are collected from stones and roots by scraping them off the substrate they are attached to. It is not easy to locate bryozoans. They can have a patchy distribution and are mainly found in slow running parts of the river that are partly shaded and have a high content of oxygen. A plan for locating bryozoans in Denmark could be to organize a field trip to a nearby river system (where freshwater biologist before have found bryozoans and where PKD is found in local brown trout), where the participants would be members from the DTU group and possibly Heike or Nicole (e-DNA method might be used to locate rivers inhabited by bryozoans), equipped with waders and “water binoculars”. If infected bryozoans cannot be collected in Denmark, we agreed to try shipping them from Switzerland to Denmark for future experiments.

Life cycle in the lab: The literature describes an established life cycle of *Tetracapsuloides bryosalmonae* in the lab. Thomas believes that such a life cycle system will be difficult to maintain. There is a high risk for fungi infections of the bryozoans. The temperature preferences are also not the same for bryozoans and trout, with optimal temperatures at 20 and < 15 °C, respectively. Bryozoans need to be fed daily with living algal cultures (or other particles e.g. goldfish feces) to be maintained. The parasite spores will leave the bryozoan naturally when the bryozoans are kept in the lab or the bryozoans can be opened (or grinded) to artificially release the parasite spores. A spore sac contains 500-4000 spores. When the spores are out of the bryozoan they are viable for ca. 24 h. The shed spores can be up-concentrated on a

3-5 µm filter. The size of the covert infection stage of bryozoan is not known. At University of Bern, the spores are not counted but quantified by qPCR.
Fish experiments: Nicole has performed a number of long term experiments with brown trout (4-6 cm) and PKD. No fish died during the experiments, probably since brown trout can shed the parasite spores, contrary to rainbow trout or the temperature in the experiment (ca. 15°C) did not provoke a lethal infection. The spores are believed to enter via the gills and move to the head kidney, and thereafter to the trunk kidney. Naturally released spores have a higher infection rate than spores from "grinded" bryozoans. The degree of pathology can be determined by a "kidney damage score" while the intensity of infection can be assessed by qPCR. When fish are challenged with a high and low dose of spores, they start to shed the spores at approximately the same time, possibly meaning that the time of shedding is a matter of maturation of the spores. The brown trout were shedding whole year round but had two peak values, shedding during a year never reaches zero (at constant temperature and light).
Future cooperation: There were interest from both DTU and University of Bern to make a future cooperation on PKD. The scope of this cooperation is somehow depending on, whether DTU can locate infected bryozoans in Danish water systems in the spring of 2017. We agreed that it is important to know the exact concentration of infective spores in the future, both for lab experiments and for field surveillance. Therefore a **standard curve** between the qPCR and light microscopy counting of spores has to be developed. Very little is also known about the **storage of infective spores**, this could be investigated too. The research on the standard curve and storage of spores can be conducted at DTU with material sent from Switzerland. A number of further tasks/experiments to be conducted at DTU were discussed, but they are dependent on the supply of infected bryozoans from Danish rivers;

- Establishment of a culture of bryozoans
- Dose-response experiments with more doses of spores
- Reinfection of fish that have cleared the infection – possibility for developing vaccines
- The mechanism of gill entry of the spores
- The effect of ultrasound on the spores

The next step is to locate infected bryozoans in Danish water systems in April-May 2017.

Appendix 1:
Agenda for designing/planning experiments with *Tetracapsuloides bryosalmonae*

1) Setting up the life cycle of *T. bryosalmonae* in the lab
    a) Is there a dose response relationship between spores and infected fish?
    b) How long are the overt spores of *T. bryosalmonae* viable?
    c) How to count and up-concentrate overt/covert spores of *T. bryosalmonae*?
    d) Are the spores more virulent if they are naturally released from the bryozoa*n?*
    e) Can the spores be stored in -80 degrees?
2) Natural environment
    a) What is the field concentration of overt/covert spores in the water and in the bryozoans?
    b) What is the yearly relationship between overt and covert spores?
    c) How does temperature affect the development of the disease?

d) How to locate bryozoans?

3) What is the size of the covert spores?
   Prophylactic measures
4) On the practical side, opportunities for cooperation (AE2020, exchange of reagents/material for diagnostic purposes and research experiment etc.

December 15th

During the morning of December 15th, Niccoló Vendramin visited the laboratory. This last part of the visit was used to discuss further cooperation in the field of diagnostics. In 2015 a screening program for viral diseases in Switzerland has been conducted, no clear positives have been detected, whereas a few suspicious samples will be sent to the EURL for re-testing. Furthermore, cooperation will be established to investigate diseases in Brown trout for identification of emerging viral pathogens.

Final conclusions

The visit to the Swiss laboratory was regarded as success. As demonstrated by high scores obtained in Proficiency Tests of the past years, diagnostic procedures for listed fish diseases are performed adequately. The visit has also been successful to boost the cooperative activities going on between Swiss NRL and EURL under the perspective of developing research activities in relation to PKD infection, and further enhance the cooperation on diagnostic cases with unclear results.