European Union Reference Laboratory for Fish Diseases

National Veterinary Institute, Technical University of Denmark, Aarhus

Technical Report 2012

from the European Union Reference Laboratory for Fish Diseases

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National Veterinary Institute
Technical University of Denmark
Division of Fisk Diseases
Aarhus, Denmark

Content

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Introduction

The National Veterinary Institute, Technical University of Denmark (DTU-VET) is appointed as the European Union Reference Laboratory for Fish Diseases (EURL), according to Commission Decision of of 21 December 2011 as regards a Union financial aid for the year 2012 to European Union reference laboratories (2011/889/EU) and to Specific Agreement No 6 to Framework Partnership agreement No SANCO/2005/FOOD SAFETY/010- Animal Health-Fish Disease.

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 epizootic ulcerative syndrome (EUS); and fish diseases listed as non-exotic diseases: infectious salmon anaemia (ISA), viral haemorrhagic septicaemia (VHS), infectious haematopoietic necrosis (IHN) and koi herpes virus (KHV) disease. This report follows the format of the work programme adopted for the EURL for 2012, describing activities under each of the categories and the status of on-going projects. The list of functions and duties of the EURL follows this introduction.

The 16th Annual Meeting of the National Reference Laboratories for Fish Diseases was held was held in Aarhus, Denmark, 30-31 May 2012 at the premises of the Section for Fish Diseases at DTU Veterinary. A total of 44 participants from 28 countries attended over the two day period. A report was submitted in August 2011.

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 third 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 + SVCV and IPNV (upon request from laboratories being accredited for these pathogens). PT2 was developed in order to test the proficiency of participating laboratories to identify ISA and KHV and in addition also spores of the oomycete *Aphanomyces invadans* causing EUS. Thereby the proficiency test is covering all 6 listed exotic and non-exotic diseases. 44 National Reference Laboratories (NRLs) participated in the proficiency test, the highest number ever. A report was submitted in February 2012. Most laboratories performed very 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 now available at the EURL web page. Diagnostic manual for EUS has been uploaded in 2012. This disease was, however, delisted by the EU Commission in autumn 2012 and might thus be removed from our web page again. We have nevertheless decided to keep it updated untill discussions on listing/no-listing is finalised. Unfortunately the manual on sampling and diagnostic procedures for the listed diseases has still not been adapted by the EU, the diagnostic methods therefore still relies on the former Commission Decision 2001/183/EC for VHS and IHN and 2003/446/EC for ISA while no legislative text exist for KHV, EHN and EUS.

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

During 2012, resources were also used to collate data on surveillance, health categorisation, and diagnostics in EU; to identify and characterise selected virus isolates; to type, store and update a library of listed virus isolates; to develop, update and maintain the database containing information on fish pathogens (www.fishpathogens.eu); to supply reference materials to NRLs; to provide training courses in laboratory diagnosis; to produce antisera; to update the EURL webpage (***www.eurl-fish.eu***); and finally to attend international meetings and conferences.
The permanent staffs of the Section for Fish Diseases in Aarhus, Denmark consists of approx. 22 academic and technical staff, primarily involved in research, diagnostics and consultancy with special focus on fish virology.

Unfortunately the activities of the EURL were affected in early spring 2011 by the information from the DTU vice-chancellor announcing the movement of our institute facilities in Aarhus to the Copenhagen area in 2013. For this reason our valued coordinator of the EURL Dr. Søren Kahns got another position and left our group 31 December 2011. A new coordinator of the EURL, Niccoló Vendramin, was appointed and started 1sr May 2012. In addition we lost our molecular biologist Søren Peter Jonstrup, April 2012 and from July 2013 all our 6 technicians including laboratory engineer Nicole Nicolajsen who has been an extremely valuable part of the EURL taking care of very significant part of the EURL work. Finally our research group conducted by Dr. Niels Lorenzen and his 6 co-workers will be transferred to Aarhus University and thereby be able to stay in town. We are therefore building up a new team and this does in fact bind many of our resources.. From 1st July our work will be conducted at Bülowsvej 27, Frederiksberg in Copgenhagen where we will be settled until we have to move again in 2016-17 to new premises at DTU Campus in Lyngby North of Copenhagen. It is our intension to keep and continue this important function in our future premises

This report was prepared and collated in a close collaboration between Nicole Nicolajsen, Niccoló Vendramin, Susie Sommer Mikkelsen, Helle Frank Skall, Torsten Snogdal Boutrup and undersigned with contributions from all the academic staff in the Section for Fish Diseases.

Aarhus, 27 March 2013

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 2012 – 31 December 2012

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 2012

Technical report

1. Coordination and training

1-1,1-2 Organise and prepare for the 16th Annual Meeting for the National Reference Laboratories for Fish Diseases (NRLs) and produce a report from the Annual Meeting

Organization of the 16th Annual Meeting

The 16th Annual Meeting of the National Reference Laboratories for Fish Diseases was held in Aarhus, Denmark, 30-31 May 2012 at the premises of the Section for Fish Diseases at DTU Veterinary.
A total of 44 participants from 28 countries attended over the two day period. There were five sessions with a total of 35 presentations, 10 of which were given by invited speakers. The scientific programme of the Annual Meeting was diverse and covered many topics of current interest. The meeting was opened with the traditional session on update of fish diseases in Europe, where once again participants from the member states had the opportunity to present new findings from their home countries. Initially an overview of the disease situation and surveillance in Europe 2011 were provided on the basis of the results from the Survey & Diagnosis questionnaire.

Then the fish disease situation in Norway was presented; a detailed report is available at: http://www.vetinst.no/eng/Publications/Fish-Health-Report.

Recent interesting findings within the Italian territory and outcomes from the Conference of the National Italian Fish Pathologists (SIPI) were delivered as update on the fish disease situation in the Italian and Mediterranean aquaculture.
This was followed by the first presentation from non-EU countries provided by a colleague from the Iranian National Reference Laboratory. This presentation described emerging issues in fish disease management in this country where the aquaculture production is increasing fast. Afterwards two talks on KHV were presented. Firstly the Polish experience with this disease and then an experimental study performed in Germany that aimed at describing KHV pathogenesis in common carp. Then the importance of zoonoses in aquaculture was underlined with the presentation from the Netherlands describing *Vibrio vulinificus* outbreaks in aquaculture and their implication on human health.
This was followed by a presentation on the results of a molecular survey on isolates from VHS outbreaks in Italy. Then a presentation on emerging fish disease in China was given. Finally an update on non/low pathogenic HPR0 ISAV, a current issue for the salmon farming industry, was provided by the expert from the Faroe Islands.
This year the second session was dedicated to a mini-workshop on sampling procedures. Three presentations were delivered describing Standard Operating Procedures, sampling collections protocols and specific screening strategies available in UK and Denmark for notifiable diseases and in Italy for marine viral diseases. These presentations were followed by discussions on strategies.

The third session, on technical issues related to sampling and diagnosis, started by a presentation on health categorization of fish farms in Europe in 2011 based on answers from the questionnaire on surveillance and diagnostic.
A recently funded EFSA project focusing on risk ranking in aquaculture used the questionnaire on surveillance and diagnosis as basis for collating data on the progress of risk management in European aquaculture. The project was presented and the results obtained from the questionnaire were given.

The report from the meeting was prepared and distributed to all participants and interested parties within 3 month after the meeting. The report is uploaded on our website

The final report, including programme and minutes of the meeting is enclosed as Annex 1

1-3 Collect and report 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

Survey and diagnosis of fish diseases in Europe in 2011

The Questionnaire on Surveillance and Diagnosis (S&D) which is collated annually is the only comprehensive overview of the disease situation in aquaculture in Europe. The information has been made available on the EURL web site (www.eurl-fish.eu), where all raw data can be obtained. The S&D have evolved over the years, for 2011 it comprise 4 parts:

1. General data on production type and size, health categorization of fish farms according to Council Directive 2006/88/EC, and information on national surveillance programmes.

2. Epidemiological data on the disease situation in each Member State with focus on the listed diseases but also including other diseases of interest.

3. Laboratory data from the NRLs and other laboratories, including number of samples examined diagnoses of fish diseases made.

4. Status on implementation of the new fish health surveillance legislation (a new part that was included only for 2011 as a deliverable for the EFSA project CFP/EFSA/AHAW/2011/03: Risk categorization for Aquatic Animal Health Surveillance)

1.The data on the European aquaculture production were obtained from the FIGIS database where only data from 2010 were available. Unfortunately this database does not include information on the number and size of fish farms, which are epidemiologically important data. The production in 2010 is almost the same as in 2009 and has for the sixth time in row raised from the previous year and has now passed 2 million ton (Figure 1). The farm sizes vary a lot between countries, e.g. the majority of farms in Germany produced < 5 tonnes, and for Spain the number of farms producing < 5 tonnes, 5-100 tonnes and > 100 tonnes is nearly equal.
The Atlantic salmon production has increased significantly while the rainbow trout production slightly decreased in Europe in 2010. The carp production is still mainly in the Eastern part of Continental Europe and at the same level as the year before. The production of sea bream decreased while the sea bass production increased in the Mediterranean countries. Among other fish species of interest are pike-perch (472t), eel (6845t), sturgeon (3545t), cod (22558t), turbot (8348t), and halibut (1821t). Unfortunately none of these species have observed the foreseen significant increase in production.
The Questionnaire on Surveillance and Diagnosis (S&D) included questions on how fish farms are health categorized according to Council Directive 2006/88/EC in the respective countries. More than half of the authorized farms in Europe are in category III for VHS and IHN and the remaining in category I or II. According to these official data almost no farms are infected with either of these diseases. This might be more due to a significant underreporting than of the de facto situation.
For KHV most carp farms are in category III, unknown status.

Many farms in Europe are not categorized yet, and unfortunately the situation has not improved much from 2010. In the questionnaire we ask for the number of APBs in these areas. There are several different views on how categorization shall be performed, e.g. should VHS free marine rainbow trout farms be placed in Category III or I? If Isavirus HPR0 is found in or in proximity of a farm can it remain its Category I status? Some Member states do not include registered APBs in the categorization but according to 2006/88/EC Annex III health categorization comprise Member states, zone and compartments NOT single APBs. A new Animal Health Law is under preparation and revision and will now include aquatic animals; in this connection the categorization system might be simplified and be made more transparent.

2. Concerning the epidemiological data, obviously, there is still a severe underreporting of VHS and IHN in many countries. For VHS the infection status is only known for 33% of the farms, for IHN the situation is known in 37% of the farms. While for KHV the disease situation is unknown on 95% of the farms! For farms producing Atlantic salmon and categorised for ISA, the infection status for ISA is known for 49% of the farms. The findings of Isavirus HPR0 pose some problems regarding the health categorisation of salmon farms.
Many countries have surveillance programmes for SVC (16 of 35 countries), BKD (14 of 35 countries), IPN (18 of 35 countries) and Gyrodactylus salaries (8 of 35 countries), for which they are seeking “additional guaranties” according to §42 in CD 2006/88/EC. The number of farms in the programmes varies from very few farms to many farms.

3. There are very large differences between countries on how many samples are tested on cell cultures, ranging from < 100 to several thousands. PCR is coming up in many countries, and the large number of PCR-tests conducted in some countries mostly reflects the KHV and ISA testing.

4. Status on implementation of the new fish health surveillance legislation. The Council Directive (CD) 2006/88/EC lays down that Member States shall ensure that a risk-based animal health surveillance scheme is applied in all aquaculture production businesses (APBs), as appropriate for the type of production. It is recognized that the risk of introducing or spreading disease, varies, not only between areas having a different disease status, but also within areas with the same disease status for each particular disease. Each aquaculture production unit therefore needs to be ranked according to the risk of introduction and spread of each of the listed diseases. As the task of risk-ranking is complicated and require considerable resources, the European Food and Safety Authority (EFSA), has funded a project with the objective of describing and critically assess the various factors necessary to categorize fish farms taking into account characteristics of the diseases listed in Part II of Annex IV of the CD.
As a background for this work, one of the tasks of the project is to describe the present level of implementation of the Article 10 of CD 2006/88/EC provisions on risk based surveillance and surveillance for demonstration of disease freedom of fish diseases.

The present level of implementation of the CD 2006/88/EC, and specifically of article 10 (which requires that RBS animal health surveillance schemes is applied in all farms) in the EU MS is largely unknown. In 2010, the Norwegian Veterinary Institute carried out a survey on the implementation of the CD, including article 10. Responses were obtained from 25 member- and EFTA-states and showed that there were clear delays in the implementation of Article 10. The responding countries

stated that delays were due to uncertainties on how to risk rank their APBs, and delays with their registration, which is a pre-requisite for risk-ranking of farms. A handful of countries have presented their strategies for risk-ranking at scientific meetings and seminars, but no overview of methods used is currently available.

In order to further describe the level of implementation of the Article 10 of the CD at the beginning of the project, a section (section 4) with additional questions regarding this was added to the 2011 Survey & Diagnosis sent out by the European reference laboratory for fish diseases.
This section consisted of 7 questions, with several sub-questions, and included a part where respondents could provide comments and clarifications in free text, if desired.
The questionnaire was sent to 36 recipients:
The 27 EU member states, minus Luxembourg and Malta (For the UK, questionnaires were sent to UK-England& Wales, UK-Northern Ireland and UK-Scotland separately. For Denmark, questionnaire was also sent to the Faroe Islands, which is a DK-territory not member of the EU).
6 EU-candidate and potential candidate countries: Albania, Bosnia-Herzegovina, Croatia, Iceland, Republic of Kosovo and Turkey.
2 Non-EU countries: Norway and Switzerland.
The recipients were given two months to reply. By the deadline, answers had been returned by 35 recipients, 33 of whose had answered section 4.

A summary of the results for 2011 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, missions and scientific collaboration

The EURL Fish is offering a yearly 2 wks training course in diagnostic techniques for identification of listed fish diseases.

The training course took in 2012 place at DTU Veterinary, Hangøvej 2, DK-8200 Aarhus N, 24/1-3/2 2012. The course was divided in two parts where one or both parts could be followed. Part 1 “Molecular techniques for identification of listed fish diseases” took place 24/1-27/1 and 12 persons participated. Part two “General Virology” took place 30/1-3/2 and 9 persons participated. 4 persons participated in both parts of the training course.

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.

The 4-day course in molecular techniques was equally devoted to hands-on laboratory work and theoretical workshops.
The experimental work was based on two realistic case stories where molecular based diagnostic methods were used for identification and characterisation of different EC- and OIE listed fish viruses. Participants performed manual purification

of viral RNA and detected the viral agent by RT-PCR. In addition, robot based DNA purification was demonstrated, and participants subsequently performed real time RT-PCR. Gel electrophoresis, purification of PCR products, quantification of the concentration, and sample preparation for sequencing was also included in the hands-on laboratory work.

The techniques used during the practical experiments as well as the participants pre-experience were the starting points of the theoretical workshops. Through lectures, exercises, discussions, and knowledge exchange between participants, the knowledge on molecular techniques and troubleshooting related to these was increased. The facilitated discussions and exercises included focus on the EC/OIE recommended protocols, how to select proper controls, the typical pitfalls, and trouble shooting, retrieving genetic information from relevant databases, and performing phylogenetic analysis of selected sequence data.
As get together, a joint dinner the first evening was included, while an optional dinner event on day 3 was held.

The 5-day course in general virology was primarily based on practical work (hands on) in combination with theoretical presentations.
During the introduction to the course the participants were divided into small groups of 2. As an assignment each group received 2 blinded ampoules containing lyophilized putative fish pathogenic viruses to be identified during the course. All cell culture based and immunochemical methods used for isolation and identification of these viruses was demonstrated and conducted by the participants themselves.
Each group were initially introduced to basic cell culture work, and then produced their own flasks, 24 well trays, and 96-well plates for titration and immunofluorescence. The participants were then introduced to cell freezing- and thawing procedures followed by mycoplasma testing. Inoculation of diagnostic samples on cell cultures was practised. The CPE of different viruses was shown and the participant practised reading of diagnostic trays. Titration procedures was demonstrated for the participants and practised by themselves and titre calculation was practised. Medium production, cell sensitivity tests and test of calf serum batch before general use in cell medium was discussed.

Concerning ELISA and immunofluorescence each group designed and performed the practical testing in order to be able to identify the distributed virus isolates, following theoretical class room teaching on methodologies, pitfalls and error findings.
The course was dialogue based and sufficient time was given for discussion under way and for evaluation of test results.

In addition each group received a collection of slides for studying characteristic IFAT results of ISAV, KHV and other relevant viruses.
Concerning IHC the participants were taught basic methodologies and was given the opportunity to take part in practical performance for staining (like PAP ringing, microwave treatment etc.). A slide collection was distributed to each group for studying pathology and staining patterns in fish tissues subjected to differences in handling during sampling and staining.

Quality assurance, contamination, cleaning and disinfection etc. was an integral part of the practical demonstrations.

The methods teached 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.
The full report is given on our website

Many requests from colleagues for training were postponed to take place at this yearly course; however, the following colleagues visited the institute during 2012 for scientific meetings, project collaboration or training:

EURL training course in fish diagnostics: Vera Deme and Petya Orozova – Bulgaria; Tiago Miguel Baeta Luís – Portugal; Athanasios Prapas and Eleni Papalexiou – Greece, Thierry Morin – France; Siiri Poldma and Ülle Pau – Estonia; Laura Sneitz – Finland; Michelle Geary – Ireland, Magdalena Stachnik – Poland, Eva Blomkvist – Sweden; Rita Granta – Latvia; Kirsten Liland Bottolfsen and Hanne K Nilsen – Norway; Xu Ye – China; Thomas Wahli - Switzerland	24/01 to 03/02 2012
Dr. Li Zhijun, Deputy inspector and Dr. Qian Jin Director at the Fisheries Service, Department Of Ocean And Fisheries Of Liaoning Province; Dr. Zheng Huaidong, Director at the Fisheries Technical Extension Center of Liaoning Province; Dr. Shi Shicheng, Deputy Director of Shenyang Rural Economic Affairs Committee; Dr. Lei Shanmin Director of the Bureau Of Ocean And Fisheries of Jinzhou New Area and Dr. Zhang Xu Director of the Bureau Of Ocean And Fisheries of Dong Gang City	19/06 2012
Diego Vázquez Rodríguez – PhD student, Instituto de Acuicultura- Santiago de Compostella, Spain. Development and assessment of PCR for strain differentiation.	15/5 -15/8 2012
Christian Fry, Master student of University of Johannesburg South Africa. Focus on EUS diagnostics and pathogenesis.	September-December 2012

Master and PhD students:

M.Sc. Sekar Larashati, Bandung Institute of Technology, Indonesia. Ph.D. study at DTU-VET in the field of "delivering small RNAs to fish" from 1 November 2009 to 31 October 2012. Supervisor: Niels Lorenzen; Co-supervisor: Brian Dall Schyth

M.Sc. Dennis Bela-Ong, Manilla University, The Philippines. Ph.D. study at DTU-VET on "The role of RNA interference in host-virus interactions in a fish model" from 1 February 2011 to 28 February 2014. . Supervisor: Niels Lorenzen; Co-supervisor: Brian Dall Schyth

Dagoberto Sepúlveda, Biochemist, Pontifical Catholic University of Valparaíso, Chile. Ph.D. study at DTU-VET Århus on “Functional characterization of protective immunity following DNA vaccination against a lethal viral disease in fish” from June 2012 to May 2015. Supervisor: Niels Lorenzen, PhD; Co-supervisor: Niels Jørgen Olesen, PhD.

Anna Amanda Schönherz has been enrolled as 2+2 MSc/PhD student at Aarhus University with the working title ”Host adaptation mechanisms of the viral haemorrhagic septicaemia virus (VHSV) in rainbow trout”. The study is part of a collaborative research project “Co-evolutionary genomics of fish resistance and virulence in an epidemic virus” funded by the Danish Research Council. Supervisor: Peer Berg, Aarhus University; Anna graduated as MSc August 2011. Co-supervisor: Katja Einer-Jensen.

M.Sc. Nikolaj Gedsted Andersen has been enrolled as PhD student at University of Copenhagen in association to the project “Improved vaccination strategies in marine aquaculture”: Improved vaccination strategies in marine aquaculture (supported by

the Strategic Research Council in Denmark). His overall supervisor is Per Juel Hansen (University of Copenhagen). His co-supervisor at DTU-VET, were the experimental challenge experiments including different combinations of algae and virus are performed, are Niels Lorenzen and Ellen Lorenzen.

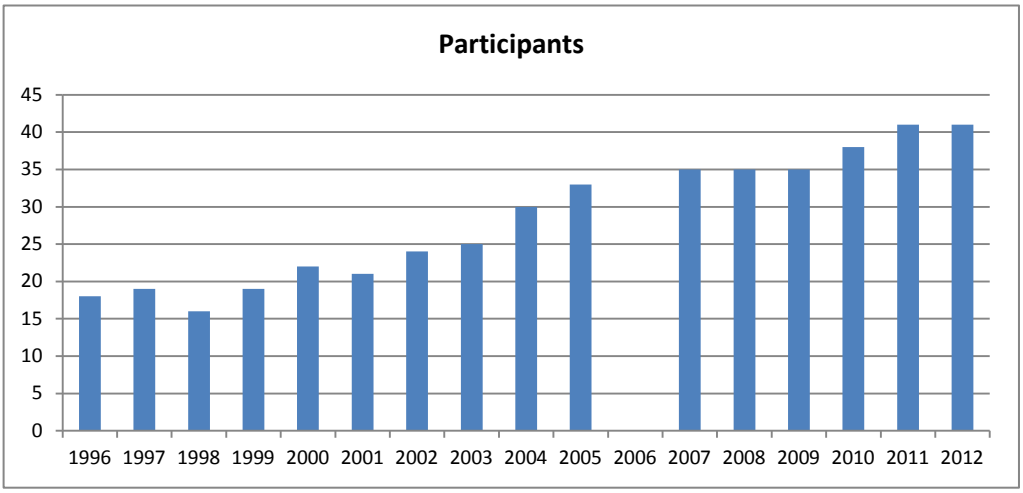
OIE collaboration: N.J. Olesen was designated expert in the ad hoc group on aquatic animal health surveillance and was involved in writing up a paper on surveillance of VHS as a model chapter for the Aquatic Code, that was finalised Autumn 2012.
The chapter on viral haemorrhagic septicaemia in the Diagnostic Manual for Aquatic Animal Health was reviewed by H.F. Skall and N.J. Olesen and significant changes were included in the new chapter
Proposal for initiating twinning project with the NRL for Fish Diseases in Turkey was undertaken in order to establish a reference centre for the Middle East

2. Proficiency test

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

The inter-laboratory Proficiency Test 2012

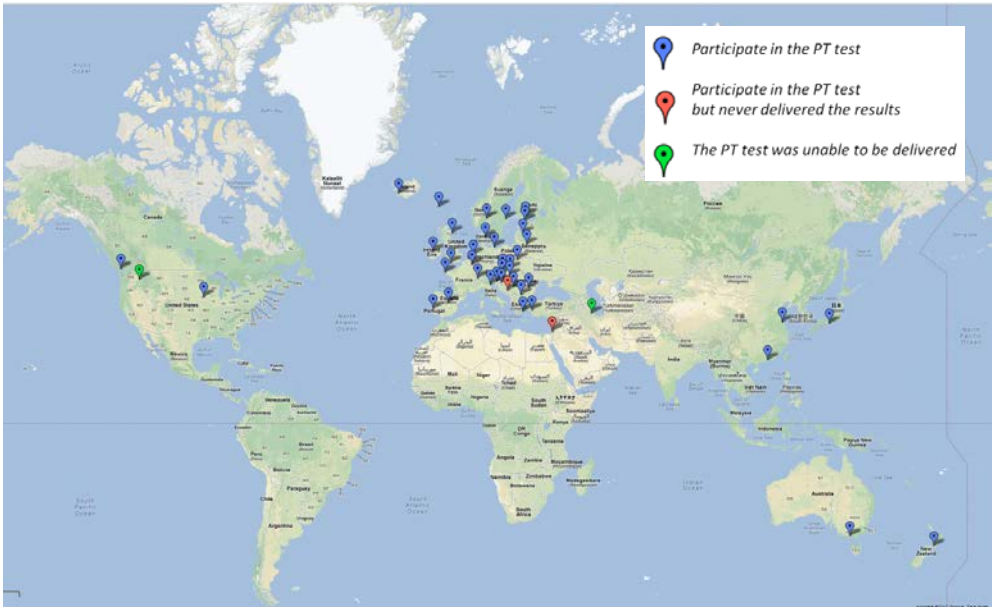
Since 1996, fourteen inter-laboratory proficiency tests have been organised by the EURL. The number of participants has increased from 18 to 41. The goal of these tests is to harmonise diagnostic methods between national reference laboratories and to ensure that the examination of a given sample leads to the same conclusions in any laboratory.



PT1 was designed as the proficiency tests provided by the EURL in previous years to primarily assess the identification of viral haemorrhagic septicaemia virus (VHSV), infectious hematopoietic necrosis virus (IHNV), epizootic haematopoietic necrosis virus (EHNV), spring viraemia of carp virus (SVCV), and infectious pancreatic necrosis virus (IPNV) by cell culture based methods. PT2 was structured with the aim of assessing the ability of participating laboratories to identify the fish pathogens: infectious salmon anaemia virus (ISAV), Cyprinid herpesvirus 3 (CyHV-3) (otherwise known as *koi herpes virus* - KHV) and *Aphanomyces invadans* the causative agent of epizootic ulcerative syndrome (EUS) by biomolecular methods (PCR based). The number of National Reference Laboratories (NRLs) participating in PT1 and PT2 was 41.
The tests were sent from the EURL in the beginning of September 2012.

Proficiency test 1, PT1

Five ampoules with lyophilised cell culture supernatant were delivered to all NRLs in the EU Member States, including Denmark, and likewise to the NRLs in Australia, Bosnia and Herzegovina, Canada, Croatia, Faroe Islands, Iceland, Israel, Japan, New Zealand, Norway, 2 from P.R. China, Serbia, Switzerland, Turkey and from USA. The Belgian NRL covers both Belgium and Luxembourg and the Italian NRL covers Italy, Cyprus and Malta for identification of all listed diseases.
The figure below shows the worldwide distribution of the participating NRLs.



PT1 consisted of five coded ampoules (I-V). These ampoules contained IPNV, EHNV, SVCV, IHNV and VHSV, respectively, see table 1. The proficiency test was designed to primarily assess the ability of participating laboratories to identify the listed fish viruses VHSV, IHNV and ENHV (Council Directive 2006/88/EC) and the non-listed viruses SVCV and IPNV if present in the ampoules, bearing in mind that the test ampoules also could contain other viruses (e.g. other fish rhabdoviruses, ranaviruses, or birnaviruses). In addition the participants were asked to quantify the viruses in PT1 by titration in order to assess the susceptibility of their fish cell lines for virus infection. Participants were encouraged to use their normal standard laboratory procedures. However, the identification should be performed according to the procedures laid down in Commission Decision 2001/183/EC using fish cell cultures followed by e.g. 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 2009. Laboratories were encouraged to identify VHSV and IHNV isolates as far as possible by means of genotyping in addition to the standard methods. It was recommended to use the genotype notification described in Einer-Jensen et *al*. (2004) for VHSV and in Kurath et *al*. (2003) for IHNV. Laboratories were encouraged to submit all sequencing results used for genotyping the isolates.

PT2 consisted of four coded ampoules (VI-IX). The ampoules contained ISAV and KHV. Furthermore, one ampoule contained *Aphanomyces invadans* and one sterile pyrogen free water, see table 11. The test was designed to primarily assess the ability of participating laboratories to identify the notifiable fish pathogens ISAV, KHV and *A. invadans* (listed in Council Directive 2006/88/EC) if present in the ampoules, bearing in mind that the test ampoules could also contain other pathogens. Participants were expected to use their normal PCR or real-time PCR methods for detection of the pathogens. It was not mandatory to grow KHV and ISAV on cell cultures in this test, but the viruses were not inactivated and, thus, it might had been possible to replicate them in cell cultures.
If present, only **inactivated** *A. invadans* was included in the ampoules.

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

Outcome of Inter-laboratory Proficiency Test 2012

The inter-laboratory proficiency test 2012 was conducted without major constraints. 92% of parcels were delivered by the shipping companies within 8 days after submission. It was, however, unfortunate that two parcels were 20 days on the way and one parcel was 43 days on the way before delivered to the laboratory primarily due to border controls. Two parcels never left the EURL. In one case this was due to delivery restriction for such reagents (Iran), in the other case because the fetal bovine serum (FBS) used was from a country not certified free from foot and mouth disease. In the meantime the batch of serum currently used in the EURL for cell culture has tested negative for foot and mouth disease virus (FMDV) following accredited procedures.

PT1 Conclusion
In 2009 EHNV was included in the proficiency test for the first time this year 36 participants were able to correctly identify the virus. Of the laboratories performing PCR based methods, 33 laboratories performed sequencing. Of these laboratories all correctly identified the content. Two laboratories performed REA and one laboratory performed restriction enzyme fragmentation.

All viral titres submitted by participants for each cell line and ampoule, respectively 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 can be 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.

PT2 conclusion
Considering that this was the third time that the EURL provided a proficiency test on ISAV and KHV identification, and the second time that the EURL provided a proficiency test on *A. invadans*, we consider that most participants obtained satisfying results. Out of 34 laboratories testing for *A. invadans* all 34 identified the pathogen in ampoule VI. Out of 38 laboratories performing KHV identification, 36 laboratories identified KHV in ampoule VII. Out of 39 laboratories 32 laboratories identified Not *A.invadans, KHV or* ISAV in ampoule VIII. Out of 38 laboratories performing ISAV identification 36 identified ISAV in ampoule IX. Very significant improvement in the proficiency of identifying and typing these pathogens has been observed during these 3 years. In autumn 2012 the European Commission decided to de-list EUS and it is officially no more considered as an exotic disease in the Union. However we find that a certain level of preparedness for the introduction of this disease in European aquaculture should be maintained. But it is still unclear whether

the pathogen will be included in future inter-laboratory proficiency tests or not and the topic will be discussed at our next Annual Meeting in May 2013.

It is an appreciated matter of fact that many laboratories are putting efforts in performing genetic characterizing 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. It was not described according to what notification the genotype of viruses should be performed reflecting the various way of reporting isolate genotypes. In future tests we will clarify which notification the genotyping should follow.

The results presented in this report will be further presented and discussed at the 17th Annual Meeting of National Reference Laboratories for Fish Diseases to be held 29-30 May 2013 in Copenhagen, Denmark.

The full report is included as Annex 2

3. Reagents and products

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

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 2012 are listed in Annex 3.

Further details of the supplied materials are listed in Annex 3

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

Production of antisera

Rabbit antisera against *Herpesvirus Anguilla* (AnHV-1) were produced and assessed in 2012 in 2 rabbits (F-3015 and F-3253). The reference strain of *Herpesvirus anguilla* (HVA or AnHV-1) was propagated and purified according to the herpes virus protocol received from Dr. T. Ito. The sera can be used in immunofluorescence, immunohistochemistry and in sero-neutralization test. In the latter high specific titres were obtained (1:5120).

3-3 Update and maintain a library of isolates of Infectious salmon anaemia virus (ISAV), Viral Haemorrhagic Septicaemia virus (VHSV) and Infectious Haematopoietic Necrosis virus (IHNV), Koi Herpes virus (KHV) and enzootic haematopoietic necrosis virus (EHNV)

Virus library

Several isolates of the listed viruses VHSV, IHNV and KHV were received and stored in our library during 2012. Furthermore, two isolates of the oomycete *Aphanomyces invadans* were received and 400 vials of VHSV tissue homogenate supernatant for validation of VHS RT PCR and real-time RT-PCR.

In addition, the EURL received other relevant pathogens like SVCV, IPNV, perch rhabdovirus and *Aphanomyces astaci*, the causative agent of crayfish plaque. Our library have continuously been updated and maintained.

Further details of the received materials are listed in Annex 4

and Aphanomyces Invadans.

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

Library of tissue material from fish infected with listed pathogens

Challenge trials planned for VHSV, IHNV and IPNV has been conducted and infected organ material from single fish; for use as tissue library of positive naturally infected tissue has been collected and stored, as well as organ material from negative controls.
Challenge trials with VHSV, IHNV, IPNV, SD virus and OMV of small rainbow trout for positive controls in histology as whole body mount were conducted and samples collected. Infection for VHSV, IHNV and SD virus were successful. For IPNV and OMV no clear classical lesions could be seen upon processing for histology.
Collected tissue material with *Aphanomyces invadans* (EUS) has been processed and evaluated. Histology samples with varying degrees of infection from both gouramis and rainbow trout is being stored, as well as tissue material for use as positive controls in PCR.

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 Community.

The diagnostic manuals for VHS, IHN, ISA, KHV, EHN disease and EUS have been 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 etc. are launched at the web page immediately after release.
In addition a new e-mail group was created in 2012: VET-EURL with approximately 100 colleagues subscribing. The site is used for newsletters, scientific updates and announcements from the EURL Fish.

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

Diagnostic manuals

The diagnostic manuals for VHS, IHN, ISA, KHV, EHN disease and EUS have all been prepared and are available from the EURL web page. The diagnostic manuals for VHS and IHN are updated and modifications of Commission Decision 2001/183/EC made. The diagnostic manual for ISA was prepared based on Commission Decision 2003/446/EC. In all five manuals with the latest information on test developments as analytical sensitivity and specificity are included.
The diagnostic manual for EUS has been uploaded.

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

Strain variation

VHS: Studies have be conducted in order to develop methods to discriminate between VHSV genotypes using monoclonal antibodies (*T. Ito, J. Kurita, M. Sano, H.F. Skall, N. Lorenzen, K. Einer-Jensen and N.J. Olesen* (2012) Typing of viral hemorrhagic septicemia virus by monoclonal antibodies, Journal of General Virology, 93, 2546–2557).
Seven mAbs with specific reaction patterns against each of the four genotypes and eight subtypes of VHSV were produced, aiming to establish an immunoassay for typing VHSV isolates according to their genotype. Among the mAbs, VHS-1.24 reacted with all genotypes except genotype Ie, whilst mAb VHS-9.23 reacted with all genotypes except genotype III. MAb VHS-3.80 reacted with genotypes Ib, Ic, Id and II. MAb VHS-7.57 reacted with genotypes II and IVa, and mAb VHS-5.18 with genotype Ib only. Interestingly, mAb VHS-3.75 reacted with all of the genotype III isolates except a rainbow trout-pathogenic isolate from the west coast of Norway, and reacted in addition with the IVb isolate, CA-NB00-01, from the east coast of the USA. Finally, mAb VHS-1.88 reacted with all genotype IVb isolates from the Great Lakes, but not with CA-NB00-01. In conclusion, we can now distinguish between all four genotypes and between five of eight subtypes of VHSV by testing isolates in immunoassay using a panel of nine mAbs. As an outcome of this study it was observed that some virus isolates had an atypical reaction pattern, and in some cases it was likely that these reactions were linked to pathogenicity traits in rainbow trout. Thus the research is now focused on search for pathogenicity markers in VHSV isolates.
KHV: Another study primarily conducted by the NRLs in NL and UK, respectively, revealed that CyHV-3 might be more widely prevalent than previously anticipated, as the most used specific PCRs do not identify several subtypes regularly present in healthy carp. Only by using generic CyHV primers the viruses could be identified. A manuscript on the topic has been submitted to Veterinary Research: *M. Engelsma, K. Way, M.J. Dodge, M. Voorbergen-Laarman, V. Panzarin, M. Abbadi, M. El-Matbouli, H.F. Skall, S. Kahns, D.M. Stone*: Detection of novel strains of Cyprinid herpesvirus closely related to koi herpesvirus.
Intensive studies on **IHNV** variation is conducted by the OIE reference laboratory for IHNV in Seattle, USA and the EURL keep updated on this topic as well.
ISA HPR0 is still in focus. It has been proven that this non-pathogenic but infective

virus is widespread in both wild and farmed Atlantic salmon. It is therefore very important to gain insight in risk factors associated with presence of ISAV HPR0 in relation to development of the disease ISA. N.J. Olesen thus participated in a EFSA working group giving a Scientific Opinion on infectious salmon anaemia (ISA)

ABSTRACT

Atlantic salmon is the only species in which the disease infectious salmon anaemia (ISA) has been observed naturally. Initial reports of findings of infectious salmon anaemia virus (ISAV) before 2002, did not distinguish between non virulent HPR0 and virulent HPRΔ viruses, thus making interpretation of older findings difficult in the light of current knowledge. Following a request from the European Commission, EFSA was asked to deliver a scientific opinion on the relationship between HPR0 and HPRΔ, the risk of HPRΔ ISAV emerging from HPR0 ISAV, and possible risk factors for such an emergence. HPR0 ISAV does not cause clinical disease in Atlantic salmon; however, it causes a transient subclinical infection and replicates mainly in gills. There is no evidence for HPR0 ISAV leading to natural infection and replication in fish species other than Atlantic salmon. Virulent ISAV have deletions in the HPR region of the HE gene and they have either an insertion or the Q266L mutation in the F gene. The most plausible hypothesis is that virulent ISAV (HPRΔ) is derived from HPR0 ISAV. This is further supported by the close association between the genetic relatedness and spatio-temporal distances of virus strains in solitary outbreaks. Epidemiological and historical data from solitary disease outbreaks indicates that the risk of HPRΔ ISAV emerging from HPR0 is low, but not negligible. The risk factors for HPRΔ emergence from HPR0 are unknown. Nevertheless, any factor that affects virus replication or host susceptibility could possibly influence the risk of emergence. More research is needed on the drivers for transition from HPR0 to HPRΔ and factors affecting host susceptibility and thereby emergence of clinical disease. A quantitative assessment of the different evolutionary forces for ISA would be useful, as well as the prevalence of ISAV HPR0 in farmed and wild Atlantic salmon.

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

Identification and characterisation of selected virus isolates

Again in 2012 a significant number of virus isolates were received for further characterisation at the EURL and for storing in our virus library as shown in the table beneath.

Member States/Countries outside EU		
Material received	**Laboratories**	**Units**
Diagnostic material for virology	3	22 samples
Diagnostic material for PCR	2	41 samples
Virus isolates	2	27 samples
PCR material	3	442 samples
Other material	2	8 specimens

Further details are listed in Annex 4

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:

- **National Diagnostic Veterinary Inst, Bulgaria (Vanya Chikova and Vera Deme):** VHSV_BG, rainbow trout, virus isolate. The samples was sent in for VHSV confirmation and sequencing. The isolate was identified as VHSV,

genotype Ia. (DTU-VET 2012-50-009).

- **Estonia veterinary and food laboratory, Estonia (Ülle Pau):** VHSV in cell culture supernatant from Rainbow trout. (DTU-VET 2012-50-018) for further characterisation. The isolate was genotype Ia-2 (Continental European type) and was highly pathogenic to Rainbow trout in infection trials conducted at the premises of the EURL..

- **National Food Chain Safety office, Hungary (Adam Dan):** 28 samples of organs for examination of KHV. (DTU-VET 2012-50-208).

- **Iran Veterinary Organisation, Iran (Mohsen Dastoor):** Gill and kidney tissues for KHV and SVCV examination. SVCV was not detected; KHV examination by PCR and real time PCR, KHV was not detected. (DTU-VET 2012-50-143).

- **Instituto Zooprofilattico Sperimentale delle Venezia, Italy (Anna Toffan):** Samples with kidney in RNAlater 204-V12/1-19R/ 204-V12/1-19B. KHV examination by PCR and real time PCR, KHV was detected. (DTU-VET 2012-50-129).

- **Institute of Food safety, Latvia (Veronika Bubovicha):** Organs from Rainbow trout for examination. The samples were tested by bacteriological, histological and virological methods. No specific causal organisms were found. (DTU-VET 2012-50-141).

- **Norwegian School of Veterinary Science, Norway (Øystein Evensen):** A IVa VHSV isolate, JF-9, from Korea, received for pathogenicity testing. (DTU-VET 2012-50-055).

- **Distriktsveterinärerna, Sweden (Matti Ohlsen):** Histology examination in relation to unexplained increased mortality in fish from imported eggs (DTU-VET 2012-50-267).

- **Central veterinary Institute Wageningen, The Neterlands (Olga Haenen):** Cultures of the oomycete *Aphanomyces invadans* from fish on GPY agar Isolate NJM 9701 and NJM 0002. The EUS is used as positive control. (DTU-VET 2012-50-268, 269).

- **Faculty of veterinary Medicine, Turkey (Hakan Isidan):** VHSV Ie isolates for infection trials in rainbow trout and turbot. (DTU-VET 2012-50-232).

- **CEFAS, Kelly Batemann (UK-England):** Proficiency test for White Spot disease by PCR (DTU-VET 2012-50-266).

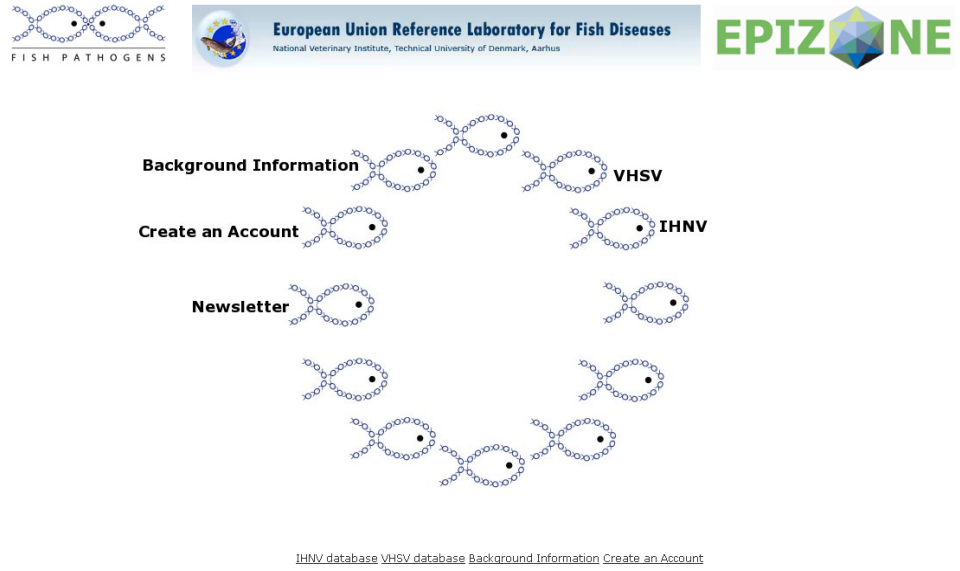
- **National Veterinary and service laboratory, USA:** 400 VHSV tissue homogenate supernatant for validation of VHS RT PCR and realtime RT-PCR. (DTU-VET 2012-50-014).

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

www.fishpathogens.eu

The current version of the database *www.fishpathogens.eu* offers a platform for sharing of available information on isolates of fish pathogens and their sequences. The platform contains public available databases on VHSV and IHNV. Furthermore the development of a database on betanodavirus has been finalised, but the addition

of a new sequence matcher function has delayed the publication of the paper describing the database. Once the sequence matcher function has been fully developed and the article has been publicised, the database will become publicly available. The betanodavirus database currently holds 59 isolate reports and 108 sequence reports. Work on ISAV and KHV databases has begun, but due to technical difficulties further work on these databases has been postponed until these problems have been solved within the wider fish community.
An SVCV database has for a while been at the final stage of development, but the work has been on standby due to other assignments. During 2012 the VHSV and IHNV databases were maintained and expanded. The VHSV database today offers publically available information on 607 isolate reports and 408 sequence reports, while the IHNV database offers publically available information on 92 isolate reports and 84 sequence reports. 134 persons are registered as users of the database and in 2012 the database had 6165 visitors.



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

Molecular epidemiology analysis to improve knowledge on diseases spreading mechanisms of viral pathogens

Two papers from the EURL on molecular epidemiology were published in 2012: *S. Kahns, H.F. Skall, R.S. Kaas, H. Korsholm, B. Bang Jensen, S.P. Jonstrup, M. J. Dodge, K. Einer-Jensen, D. Stone, and N.J. Olesen* (**2012**) European freshwater VHSV genotype Ia isolates divide into two distinct subpopulations. Diseases of Aquatic Organisms 99(1):23-35 and Reichert *M., Matras M., Jonstrup SP, Olesen NJ, Kahns S* (**2013**) Trade practices are main factors involved in the transmission of VHS in Polish aquaculture. Journal of Fish Diseases, volume 36, issue 2, pp. 103-114.
A fundament for studying molecular epidemiology is the access to significant amounts of reliable data on virus isolates and corresponding sequences data, and to connect these with classical epidemiological data such as prevalence, GIS, sampling strategies and number of positive samples in relation to negative samples. Part of this information is most accessible in our database www.fishpathogens.eu and efforts have primarily been devoted to expand and refine this database in order to use the must updated tools for conducting the studies. A close collaboration between partners in Europe on the topic has been established through the “MOLTRAQ” FP7 project.

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

Assessment and standardisation of real-time PCR tests for diagnosis and identification

VHS virus can be divided into four distinct genotypes and additional sub lineages. Genotype I is the main cause for outbreaks in European rainbow trout farms, while genotype II is restricted to the Baltic region and genotype III have been connected to the North Atlantic Ocean. Genotype IV, on the other hand, has been associated with North America and Asia only.
A study was conducted in collaboration with colleagues from Spain (Dr.Carlos Dopazo and Diego Vázquez Rodríguez, Unidad de Ictiopatología Viral, Instituto de Acuicultura, Universidad de Santiago de Compostela, Spain) in order to develop real-time PCR assays able to distinguish between the four genotypes. Such assays will lead to shortened response time in order to trace a current outbreak, especially concerning imported stock.
Assay 1 consisted of a primer/probe set able to recognise and discriminate between genotypes I-III, while assay 2 consisted of a primer/probe set able to recognise gentoype IV. Both assays were tested against a panel of 79 VHSV isolates covering all four genotypes and subtypes as well as a panel of closely related and unrelated viruses used as negative control. Assay 1 was found to be able to pick up and distinguish between genotypes I-III in all cases except 1 for genotype Ia, and in further three cases the ct-value was higher than desired. No genotype IV isolates or any of the isolates from the control group were amplified by assay 1. Assay 2 was able to amplify all genotype IV isolates but not any of the genotype I-III isolates nor any from the control group. Further work is ongoing to improve the specificity of the two assays and two validate it according to OIE guidelines.

4-8 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 (e.g. using discontools and similar tools)

The EURL Fish was invited to submit data on the listed fish diseases to the Discontools EU FP7 project and meetings with NRL representatives were held in connection with the Annual Meeting. Due to restricted possibilities for funding it was however abandoned. All diseases in this program are diseases of warm blooded animals and we realised that people seeking information on fish diseases would go to other sites for this.
It has been questioned whether or not *Aphanomyces invadans* is present in EU and during autumn the EU Commission decided based on the EFSA Report of the technical hearing meeting on Epizootic Ulcerative Syndrome (EUS) to delist EUS from the list of exotic diseases. As the EURL has put considerable efforts into establishment and harmonization of diagnostic procedures for this disease and as the question still is a matter of discussions (OIE have taken the position based on the same report to keep EUS as a listed disease), the EURL have decided to keep upgraded on diagnostic preparedness for this disease if it should enter and give rise to severe problems in EU.
Other diseases of interest is pancreas disease and sleeping disease caused by the salmonid alphaviruses, SAV-1 and SAV-2, respectively, studies are undergoing in order to assess if these diseases should be listed or not (In collaboration with colleagues in Norway)
Betanodavirus is a major threat for marine aquaculture, especially in the Mediterranean basin, management og control procedures are assessed and its putative listing is discussed (in collaboration with IZSVe, and stakeolders in Greece, Spain and Italy)

VHSV strain IVb is a major problem to wild fresh water fish species in Canada and United States and could be a serious threat for European wild fish stocks, therefore measures to reduce the risk of introducing this virus should be taken. The EURL have developed fast methods for discriminating between European and American genotypes.

5. Missions

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

Missions to relevant laboratories

In 2012 no missions were conducted to NRL´s in EU. The reason for this was primarily that our previous coordinator of the EURL Fish, Dr. Søren Kahns, left his position December 2011 and that he was only replaced with Dr. Niccoló Vendramin 1st May, who first should go through and intensive training program in order to be qualified for auditing other laboratories.
However the EURL participated actively in a workshop on strategy for health management and surveillance in carp farming, with focus on KHV. In an international meeting on aquatic alphaviruses as it is considered whether the diseases cause by this virus should be listed or not. And finally in an EURL meeting in Brussels

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

International meetings and conferences attended.

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, EHN and EUS. Scientists at the EURL participated in the following international meetings and conferences in 2012:

Participation and presentations at international conferences and meetings

*16th Annual Meeting of the National Reference Laboratories for Fish Disease. Aarhus, 30-31 May, 2012***.**

5th Annual Meeting of EPIZONE, Brighton, UK, 12-15th June 2012

DAFINET WORKSHOP, Copenhagen 2012:

List of 2012 publications from the EURL Fish

1. *Søren Peter Jonstrup, Søren Kahns, Helle Frank Skall, Torsten Snogdal Boutrup, Niels Jørgen Olesen* (**2013**) Development and validation of a novel Taqman based real time RT-PCR assay suitable for demonstrating freedom from Viral Haemorrhagic Septicaemia Virus. Journal of Fish Diseases, volume 36 , issue 1 , pp. 9-23
2. *Reichert M., Matras M., Jonstrup SP, Olesen NJ, Kahns S* (**2013**) Trade practices are main factors involved in the transmission of VHS in Polish aquaculture. Journal of Fish Diseases, volume 36 , issue 2 , pp. 103-114
3. *S. Kahns, H.F. Skall, R.S. Kaas, H. Korsholm, B. Bang Jensen, S.P. Jonstrup, M. J. Dodge, K. Einer-Jensen, D. Stone, N.J. Olesen* (**2012**) European freshwater VHSV genotype Ia isolates divide into two distinct subpopulations. Diseases of Aquatic Organisms 99(1):23-35
4. *Ito, Takafumi • Kurita, Jun • Sano, Motohiko • Skall, Helle Frank •*

Lorenzen, Niels • Einer-Jensen, Katja • Olesen, Niels Jørgen. **2012** Typing of viral hemorrhagic septicemia virus by monoclonal antibodies. Journal of General Virology —, Volume 93, Issue Pt 12, pp. 2546-2557

5. Hart, L M • Lorenzen, N • LaPatra, S E • Grady, C A • Roon, S E • O'Reilly, J • Gregg, J L • Hershberger, P K. Efficacy of a glycoprotein DNA vaccine against viral haemorrhagic septicaemia (VHS) in Pacific herring, Clupea pallasii Valenciennes. Journal of Fish Diseases — **2012**, Volume 35, Issue 10, pp. 775-779
6. Schönherz, A A • Hansen, M H H • Jørgensen, H B H • Berg, P • Lorenzen, N • Einer-Jensen, K. Oral transmission as a route of infection for viral haemorrhagic septicaemia virus in rainbow trout, Oncorhynchus mykiss (Walbaum). Journal of Fish Diseases — **2012**, Volume 35, Issue 6, pp. 395-406
7. Schyth, Brian Dall • Bramsen, Jesper Bertram • Pakula, Malgorzata Maria • Larashati, Sekar • Kjems, Jorgen • Wengel, Jesper • Lorenzen, Niels. In vivo screening of modified siRNAs for non-specific antiviral effect in a small fish model: number and localization in the strands are important. Nucleic Acids Research — **2012**, Volume 40, Issue 10, pp. 4653-4665
8. Holten-Andersen, Lars • Dalsgaard, Inger • Nylén, Jørgen • Lorenzen, Niels • Buchmann, Kurt. Determining Vaccination Frequency in Farmed Rainbow Trout Using Vibrio anguillarum O1 Specific Serum Antibody Measurements. P L o S One — **2012**, Volume 7, Issue 11,
9. von Gersdorff Jørgensen, Louise • Sigh, Jens • Kania, Per Walter • Holten-Andersen, Lars • Buchmann, Kurt • Clark, Theodore • Rasmussen, Jesper Skou • Einer-Jensen, Katja • Lorenzen, Niels. Approaches towards DNA vaccination against a skin ciliate parasite in fish. PloS one. — **2012**, Volume 7, Issue 11, pp. e48129
10. Schönherz, A.A., Lorenzen, N., Einer-Jensen, K. (**2012**) Inter-species transmission of viral haemorrhagic septicaemia virus (VHSV) from turbot (Scophthalmus maximus) to rainbow trout (Onchorhynchus mykiss). Journal of General Virology, Epub ahead of print 2012 Dec 12.
11. Schyth, B.D., Ariel, E., Korsholm, H., Olesen, N.J. (**2012**). Diagnostic capacity for viral haemorrhagic septicaemia virus (VHSV) infection in rainbow trout (Oncorhynchus mykiss) is greatly increased by combining viral isolation with specific antibody detection. *Fish and shellfish immunology*, 32(4): 593-597.

Presentations and posters

1. Olesen, Niels Jørgen • Nicolajsen, Nicole — 2012 Overview of the Disease Situation and Surveillance in Europe In 2011, 16th Annual Meeting of the National Reference Laboratories for Fish Diseases
2. Olesen, Niels Jørgen • Nicolajsen, Nicole — 2012 Health Categorisation of Fish Farms in Europe In 2011, 16th Annual Meeting of the National Reference Laboratories for Fish Diseases
3. Jensen, B. Bang • Aldrin, M. • Avarre, M. C. • Bergmann, S. M. • Bigarre, L. • Brun, E. • Jansen, P. A. • Olesen, Niels Jørgen • Renault, T. • Schuetze, H 2012 Molecular Tracing of Viral Pathogen in Aquaculture (MOLTRAQ): a new EMIDA project, 16th Annual Meeting of the National Reference Laboratories for Fish Diseases
4. Olesen, Niels Jørgen • Skall, Helle Frank • Nicolajsen, Nicole • Jonstrup, Søren Peter • Kahns, Søren EURL activities in 2011 — 2012 16th Annual Meeting of the National Reference Laboratories for Fish Diseases
5. Kahns, Søren • Nicolajsen, Nicole • Christophersen, Maj-Britt • Olesen, Niels Jørgen— 2012 Results of the Proficiency Test, PT1 and PT2, 2011,

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

6. *Olesen, Niels Jørgen • Skall, Helle Frank • Nicolajsen, Nicole • Jonstrup, Søren Peter • Kahns, Søren* EURL activities in 2011 - 2012.
7. *Torsten S Boustrup*. Update on Epizootic ulcerative syndrome (EUS) diagnostics, infection trials and online slide collection.
8. *Niccolò Vendramin* - Update on aquatic organisms disease situation in Italy.
9. *Niccolò Vendramin* - Clinical inspection and sampling in fish farming: practical experiences and guidelines from the Mediterranean point of view.
10. *Niccolò Vendramin* - Outcome on Epizone extension on VER/VNN: Infection trials, pathogenicity and pathology of various VER/VNN isolates.
11. Bigarré, Laurent • Panzarin, Valentina • Baud, Marine • Jonstrup, Søren Peter • Jansson, Eva • Isaksson, Mats • Engelsma, Marc • Olesen, Niels Jørgen • Bovo, Giuseppe 2012 Outcome on EPIZONE Extension on VER/ VNN: Diagnostics, proficiency test and qRT-PCR validation, 16th Annual Meeting of the National Reference Laboratories for Fish Diseases
12. Borzym, Ewa • Matras, Marek • Maj, Joanna • Sandomierska, Agnieszka • Olesen, Niels Jørgen • Eliassen, Mette • Baud, Marine • Talbi, Chiraz • Bigarré, Laurent— 2012 First Detection of Hirame Rhabdovirus (HIRRV) in Europe 16th Annual Meeting of the National Reference Laboratories for Fish Diseases
13. Skall, Helle Frank • Olesen, Niels Jørgen • Jørgensen, Claus — 2012 Inactivation of VHSV by Percolation and Salt Under Experimental Conditions, 16th Annual Meeting of the National Reference Laboratories for Fish Diseases
14. Bela-Ong, Dennis • Schyth, Brian Dall • Lorenzen, Niels. Expression of micro-RNAs and immune-relevant genes in rainbow trout (Oncorhynchus mykiss Walbaum) upon vaccination with a Viral Haemorrhagic Septicemia Virus. DAFINET Workshop — 2012,
15. Schyth, Brian Dall • Bramsen, Jesper Bertram • Kjems, Jørgen • Wengel, Jesper • Lorenzen, Niels. Chemical modification of RNA-based medicine can be used to reduce its induction of the innate immune response. DAFINET Workshop — 2012,
16. Lorenzen, Ellen • Kjær, Torben Egil • Henriksen, Niels Henrik • Dalsgaard, Inger • Holten-Andersen, Lars • Madsen, S. B. • Krossøy, B. • Buchmann, Kurt • Lorenzen, Niels. Improved Protection of Rainbow Trout Against Furunculosis by an Autologous Vaccine Under Experimental Conditions. DAFINET Workshop : Immune Responses in Fish — 2012, pp. 20
17. Schönherz, A. A. • Lorenzen, Niels • Einer-Jensen, Katja. Inter-Species Transmission of Viral Haemorrhagic Septicaemia Virus Between Turbot (Scophthalmus Maximus) and Rainbow Trout (Onchorhynchus Mykiss). DAFINET Workshop : Immune Responses in Fish — 2012, pp. 24
18. Niels Lorenzen[1], Ellen Lorenzen[1], Katja Einer-Jensen[1], Jesper S. Rasmussen[1], and S.E. LaPatra[2.] Fish as models for understanding DNA vaccine efficacy. Poster at "Viral Immunity and Host gene influence" Symposium, Keystone March 21-26, USA.
19. Jesper Skou Rasmussen, Ellen Lorenzen, Torben Egil Kjær, Katja Einer-Jensen & Niels Lorenzen. DNA vaccination of small rainbow trout fry against VHSV. Danish Fish Immunology Research Centre and Network (DAFINET) Workshop on Ontogeny of the Immune System of Fish, Copenhagen, Denmark, April 24-25, 2012.
20. Lorenzen, Ellen; Kjær, Torben Eigil; Henriksen, Niels Henrik; Dalsgaard, Inger; Holten-Andersen, Lars; Madsen, Simon B; Krossøy, B;, Buchmann, K; Lorenzen, Niels. Improved protection of rainbow trout against furunculosis by an autologous vaccine under experimental conditions.

Presented at: Dafinet workshop: Immune Responses in fish, November 6th and 7th, 2012.

21. Bela-ong DB, Schyth BD, and Lorenzen N. Expression of microRNAs and interferon-related genes in rainbow trout (*Oncorhynchus mykiss* Walbaum) infected with *Viral hemorrhagic septicemia virus*. International Workshop on Small RNAs in Cancer, Inflammation, and Aging, Copenhagen, Denmark, September 3-4, 2012.
22. Bela-ong DB, Schyth BD, and Lorenzen N. Expression of microRNAs and immune-relevant genes in rainbow trout (*Oncorhynchus mykiss* Walbaum) upon vaccination with a *Viral hemorrhagic septicemia virus* glycoprotein gene (*G*)-encoding DNA vaccine. Danish Fish Immunology Research Centre and Network (DAFINET) Workshop on Ontogeny of the Immune System of Fish, Copenhagen, Denmark, April 24-25, 2012.
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24. Larashati, Sekar; Schyth, Brian D.; Lorenzen, Niels Inhibition of reporter genes by small interfering RNAs in rainbow trout (*Oncorhyncus mykiss*) Presented at: International Conference on Gene Regulation by Small RNAs. Oxford, England, 27-29 March 2012
25. Larashati, Sekar; Schyth, Brian D.; Lorenzen, Niels Inhibition of reporter genes by small interfering RNAs in rainbow trout (*Oncorhyncus mykiss*) Presented at: International Conference on Small RNA in Cancer, Aging, and Inflamation. Copenhagen, Denmark, 2-4 September 2012
26. Schönherz, Anna Amanda; Lorenzen, Niels; Einer-Jensen, Katja Inter-species transmission of viral haemorrhagic septicaemia virus between turbot (Scophthalmus maximus) and rainbow trout (Onchorhynchus mykiss), 10th Annual Meeting, Department of Molecular Biology and Genetics - Aarhus University
27. Schönherz, A.A., Lorenzen, N., Einer-Jensen, K. Inter-Species Transmission of viral haemorrhagic septicaemia virus between marine and freshwater fishes. SMBE 2012 - Society of Molecular Biology and Evolution 2012, Dublin, Ireland, June 23rd-26th, 2012
28. Schönherz, A.A., Einer-Jensen, K., Hansen, M.H., Buitenhuis, B. Genetic markers of virulence in a fish rhabdovirus. EMBO practical course on Analysis of high-throughput sequencing data, UK, Hinxton, October 29th - November 3rd, 2012
29. Schyth, B. D. Learning Virology by playing games. Active Learning in Engineering Education (ALE) workshop, June 2012, Copenhagen
30. Schyth, B.D., Jalali, S.A.H., Kristensen, L.B., Pedersen, F.S. and Lorenzen, N. MicroRNA regulation in rainbow trout infected with a fish pathogenic rhabdovirus. RNAi 2012 – 7th international conference and exhibition, 27-29th March 2012, St. Hildas College, Oxford.
31. Einer-Jensen, K. Reverse genetics as a tool to study the pathogenesis of fish rhabdoviruses Dansk Virologisk Selskab: Virusdagen October 4th, 2012
32. Niels Lorenzen, Ellen Lorenzen, Katja Einer-Jensen, Jesper S. Rasmussen, and S.E. Fish as models for understanding DNA vaccine efficacy. Keystone
33. LaPatra. S.L., Schyth, B.D., Jalali, S.A.H., Kristensen, L.B., Pedersen, F.S.

and Lorenzen, N. Chemical modification of RNA-based medicine can be used to reduce its induction of the innate immune response. Dafinet meeting, April 24-25th, 2012.

Participation in international scientific collaborative studies

- The group was partner and work package leader of EU project EPIZONE FP6-2004-Food-3-A WP 6.1: Surveillance & Epidemiology of emerging viral diseases in aquaculture The public web site is http://www.epizone-eu.net/default.aspx. N.J.Olesen is still connected to the official EPIZONE network that have been established in 2011 and who are holding annual meetings and conferences.

- The section is partner in the FP7 EU project: The Network of Animal Disease Infectiology Research Facilities, NADIR, aims to facilitate the development of Europe's high level bio-containment facilities for which there is a strong demand from both the public and private sectors in the field of medical and veterinarian research. The project is divided into network and research activities and gives possibility for transnational access to research facilities. Our team provide access to experimental tank facilities, and aim at characterising experimental fish with respect to different traits.

- A 5-year international network "Danish Fish Immunology Research Network DAFINET" has been established based on funding from the Danish Council for Strategic Research. The project aims at creating an international research network based in Denmark which will take a coordinated action towards the production of highly needed immunological tools for studying the immunity of rainbow trout, a significant cultured fish in most countries throughout the world. The work will elevate the international fish immunological level to standards found in human immunology. Specifically the project will make it feasible to determine the ontogenetic development and function of the immune system in rainbow trout with a well characterised genetic background by using a combination of novel molecular and immunological techniques. The immune protection against the most important viral, bacterial and parasitic pathogens following vaccination/immuno-stimulation procedures will be determined at different developmental and environmental conditions. This basic knowledge will first of all contribute to considerably improved procedures of vaccination and immuno-prophylaxis in rainbow trout farming by pinpointing the developmental stages where vaccination can be performed optimally. This will provide the basis for a sustainable development of rainbow trout aquaculture by reducing the need for antibiotics and chemicals in disease control. The public web site is http://dafinet.dk/DAFINET/Home.html.

- The 4-year national collaborative research project "Co-evolutionary genomics of fish resistance and virulence in an epidemic virus" based on funding from the Danish Research Council. This project seeks a solution to a problem for the expansion of Danish trout farming into the marine environment. Viral haemorrhagic septicemia (VHS) is a viral disease that causes outbreaks with up to 90% mortality in rainbow trout, and the virus is commonly found in wild populations of fish in the coastal waters. We will identify the process of adaptation to the fish host that makes the virus capable of causing epidemic outbreaks in rainbow trout and use the trouts own genetic variants in combination with targeted vaccine development to cope with this adjustment. We can achieve this through a combination of novel technologies that combine

genotypning of genetic markers in coding DNA (SNP markers) and regulatory gene sequences (miRNA) with vaccination and infection experiments where we measure gene activity throughout the genome and gene activity in immunological key components. This gives us a unique level of insight into the mechanisms that provide resistance against the virus and effective protection from the vaccine. It is possible to combine these technologies because we have established collaboration between institutions, which have experience in vaccine development, infection experiments, genomic and genetic analysis. Besides the National Veterinary Institute department in Århus those are Aarhus University, University of Victoria in Canada and the University of Washington, USA. The public web site is
https://djfextranet.agrsci.dk/sites/fishgen/public/Pages/front.aspx

- Delivery of small interfering RNAs (siRNAs) for treatment of viral disease in fish aquaculture – a Ph.D. study funded by the Islamic Development Bank (IDB). The aim of this study is to establish novel delivery strategies for small interfering RNAs including viral and nonviral methods in fish – aiming at achieving systemic delivery of siRNAs. This study will use the rainbow trout as a fish model and viral hemorrhaegic septicemia virus (VHSV). VHSV is an important pathogen which is highly contagious and can cause high mortality in some of the aquaculture fishes such as rainbow trout and turbot. Both RNAi studies on cell culture and in animal will be carried out. For this purpose, reporter genes are used as they provide easy assays for evaluating on gene knock down efficiency by siRNAs.

- The 3½-year national collaborative research project "Improved vaccination strategies in marine aquaculture" focuses on fish from the marine environment as they represent an important source for healthy animal food. In Denmark and other countries future plans include considerable expansion of marine aquaculture. This project aims at an integrated strategic research approach towards ensuring healthy fish and fish products from Danish marine aquaculture. By uniting the expertise of 4 research institutes and 4 private companies/organizations (including two international companies) the project will address fish health in scientific and applied terms aiming at improved strategies for disease prophylaxis by vaccination. The project will focus on rainbow trout being the dominating aquacultured fish species in Denmark. For analysing if fish toxic algae may predispose the fish for disease even if vaccinated, the effect of such environmental stress elements will also be studied experimentally. For optimizing the effect of licensed vaccines, these will be tested in new in new time schedules in field trials at 2-3 selected marine fish farms and the immuneresponse of the fish to vaccination and disease will be studied in detail. The overall applied aim is to integrate the obtained results into a new recommendation for disease prophylaxis in marine aquaculture. The project expired by March 2012, but has formed a knowledge platform for an extended new project with increased international collaboration. The public web site is http://www.dtu.dk/sites/marinvac.aspx

- A 3½-year national research project supported by the Danish Research Council focuses on "Identification of virulence markers in marine VHS virus and use in diagnostics for aquaculture" using in vivo imaging of VHSV propagation in fish, and identification of virulence marker(s) in VHSV by generation and virulence testing of recombinant viruses. Once genetic elements of importance for virulence and/or risk of establishment of virulence have been identified, the information will be used to generate a diagnostic assays based on RT-PCR and gene sequencing for virulence typing of virus isolates. The developed assay will

be evaluated by testing on a panel of VHSV isolates with known virulence and will subsequently be distributed to other national EC reference laboratories for extended evaluation. The project expired by May 2012 but the public web site is http://www.dtu.dk/sites/VHSVIRULENCE.aspx.

- TargetFish - Targeted Disease Prophylaxis in European Fish Farming, is a 5 year collaborative research project funded by The European Commission 7th Framework programme. The TargetFish project is co-ordinated by Geert Wiegertjes from Wageningen UR, has a budget of 6 million Euro and consists of 30 European partners including Section for Fish Diseases at DTU National Veterinary Institute. TargetFish will advance the development of existing and new prototype vaccines against socio-economically important viral or bacterial pathogens of Atlantic salmon, rainbow trout, common carp, sea bass, seabream and turbot. TargetFish will also establish a knowledge- and technology-base for rational development of next generation fish vaccines. The project will develop targeted vaccination strategies for currently sub-optimal and for novel vaccines. Improved vaccines will be brought closer to industrial application by addressing practical issues such as efficacy, safety and delivery route. The public website is http://www.wageningenur.nl/en/show/TARGETFISH.htm

- HabFish – The project is funded by the Programme Committee for Health, Food and Welfare under the Danish Council for Strategic Research, and runs for 5 years. The project is co-ordinated by Associate Professor Per Juel Hansen, Marine Biological Section, Department of Biology, University of Copenhagen. The project consists of 10 Danish and international partners including Section for Fish Diseases at DTU National Veterinary Institute. The present project aims at identifying and quantifying algal toxins associated with fish kills, their effects and mechanisms of actions on fish, and fish fry. The project will also provide tools for contemporary molecular identification and abundance estimates of the implicated algal species. Finally, the project will explore the mechanisms behind possible acclimation of fish to ichthyotoxic algae and potential accumulation of toxins in fish. Thus, the overall objective of the project is to provide a highly improved monitoring and risk assessment program for identification, enumeration of ichthyotoxic algae and their toxin profiles in Danish waters. The public website is http://www.habfish.dk/

- ProFish – A 5 year international research project funded by the Programme Committee for Health, Food and Welfare under the Danish Council for Strategic Research. The 9 project partners represents Danish and international researchers, the aquaculture industry, vaccine and feed producers. The project has a budget of 12 million DKK and is co-ordinated by the Section for Fish Diseases at DTU National Veterinary Institute. The projects´ overall objective is development of a tailor-made vaccination strategy for marine production of rainbow trout in Denmark and hereby minimizing the needs for antibiotics. The main focus will be on preventing disease caused by the bacterium *Aeromonas salmonicida*(*A.s.*), for which currently available vaccines have failed to provide sufficient protection. Based on results obtained in the MarinVac project, this will be achieved by implementing a holistic approach in the sense of covering the whole lifetime of the fish and taking both important infections, management procedures and environmental effects into account along with *A.s* epidemiology and variability. The work will span from understanding the essential elements of protective immunity to development and testing of new vaccination strategies. Specific objectives are: Development and testing of autologous vaccine candidates, characterization of protective immune mechanisms in functional terms and development of related assays for non-lethal monitoring of vaccine

efficacy, and establishment and evaluation of a management strategy including vaccination before exposure to pathogens. Dissemination of the project results to the fish farmers along with recommendations on how the results can be implemented in the current management practice will also represent an important objective.

- Beside our function as the European Union Reference Laboratory for Fish Diseases we are appointed as OIE reference laboratory for VHS.