Technical Report
2008

from the
Community Reference Laboratory
for Fish Diseases

Technical University of Denmark
National Veterinary Institute
Fish Disease Section,
Aarhus, Denmark

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Introduction

The National Veterinary Institute, Technical University of Denmark (DTU-VET) is appointed as the Community Reference Laboratory for Fish Diseases (CRL), according to Commission Decision of 24 April 2008, 2008/332/EC (notified under document number C (2008) 1570) on financial aid from the Community for the year 2008 for certain Community reference laboratories in the field of animal health and live animals.
The duties of the CRL are described in Council Directive 2006/88/EC of 24. October 2006 introducing minimum Community measures for the control of certain fish diseases (Annex VI). A five year contract was signed in the Framework Partnership Agreement, No. SANCO/2005 FOOD SAFETY/010- Animal Health – Fish and confirmed by Specific Agreement No. 2007/2 to the Framework Partnership Agreement, No. SANCO/2005 FOOD SAFETY/005- Animal Health – Fish Diseases. 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 CRL for 2008, describing activities associated with each point and the status of ongoing projects. The list of functions and duties of the CRL follows this introduction.

The 12th annual meeting of the National Reference Laboratories for fish diseases was held back-to-back with a workshop in diagnosis of the exotic fish diseases EHN and EUS in June 17-20 2008, in Aarhus, Denmark. Colleagues from NRLs in most Member States and several accession- and EFTA countries attended, either by sustaining from EU, TAIEX or on their own account. In total, 64 participants from 32 countries attended over the four-day period. There were six sessions with a total of 40 presentations, 13 of which were given by invited speakers and five working platforms, of which four were run by invited instructors, including two experts from the OIE reference laboratory for EUS in Bangkok. During the workshop establishment and recommendations of standard operation procedures for diagnosis of EHN and EUS were discussed.

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 of EU. A report was submitted in March 2009. Most laboratories performed very well.

During 2008, resources were also used to: 1. Collate data on surveillance and diagnostics in EU; 2. Identify and characterise selected virus isolates; 3. Type, store and update a library of listed virus isolates; 4. Build a database containing information on fish pathogens (www.fishpathogens.eu); 5. Supply reference materials to NRLs; 6.Assess the option for screening of other fish diseases in samples collected as part of the VHSV/IHNV surveillance; 7. Validate molecular techniques for the identification of VHS virus; 8. Provide training courses in laboratory diagnosis and missions to other NRLs; 9. Udating our quality assurance schemes according to our accreditation in ISO 17025 and 10. Establish a new CRL webpage (www.crl-fish.eu)

The permanent staff of the Fish Disease Section in Aarhus, Denmark consists of approx. 20 academic and technical staff, primarily involved in research, diagnostics and consultancy with special focus on fish virology.

Aarhus, 27th March 2009

Niels Jørgen Olesen , Søren Kahns and Nicole Nicolajsen

The functions and duties for the Community Reference Laboratory for Fish Diseases

According to Council Directive 2006/88/EC of 24 October 2006
- Annex VI.
Period: 1 January 2008 – 31 December 2008

Functions and duties of laboratories PART I Community reference laboratories

1. In order to be designated as a Community reference laboratory in accordance with Article 55, laboratories shall fulfil the following requirements. They must:

   (a) have suitably qualified staff with adequate training in diagnostic and analytical techniques applied in their area of competence, including trained personnel available for emergency situations occurring within the Community;

   (b) possess the equipment and products needed to carry out the tasks assigned to them;

   (c) have an appropriate administrative infrastructure;

   (d) ensure that their staff respect the confidential nature of certain subjects, results or communications;

   (e) have sufficient knowledge of international standards and practices;

   (f) have available, as appropriate, an updated list of available reference substances and reagents and an updated list of manufacturers and suppliers of such substances and reagents;

   (g) take account of research activities at national and Community level.

2. However, the Commission may designate only laboratories that operate and are assessed and accredited in accordance with the following European Standards, account being taken of the criteria for different testing methods laid down in this Directive:

   (a) EN ISO/IEC 17025 on ‘General requirements for the competence of testing and calibration laboratories’;

   (b) EN 45002 on ‘General criteria for the assessment of testing laboratories’;

   (c) EN 45003 on ‘Calibration and testing laboratory accreditation system — General requirements for operation and recognition’.

3. The accreditation and assessment of testing laboratories referred to in paragraph 2 may relate to individual tests or groups of tests.

4. For one or more of the diseases under their responsibility, the Community reference laboratories may take advantage of the skills and capacity of laboratories in other Member States or EFTA Member States, provided that the laboratories concerned comply with the requirements laid down in points 1, 2 and 3 of this Annex. Any intention to take advantage of such cooperation shall be part of the information provided as a basis for the designation in accordance with Article 55(1). However, the Community reference laboratory shall remain the

contact point for the National reference laboratories in the Member States, and for the Commission.

5. The Community 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 standardise the tests and reagents used in each Member State, where serological tests are required,

(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 2008

TECHNICAL REPORT

1-2. Organise and prepare for the Annual Meeting for the National Reference Laboratories for Fish Diseases in 2008 and produce a report from the Meeting

Organization of the 12th Annual Meeting

In June 17th-20th 2008, the 12th Annual Meeting of the National Reference Laboratories for fish diseases was held back-to-back with a workshop in diagnosis of the exotic fish diseases Epizootic hematopoietic necrosis (EHN) and Epizootic ulcerative necrosis (EUS) at the National Veterinary Institute in Aarhus, Denmark. A total number of 64 participants from 32 countries attended over the four day period. There were a total of five theoretical sessions and one practical session. In total, there were 40 presentations, 13 of which were given by invited speakers and five working platforms, of which four were run by invited instructors.

As the number of participants have grown to a size that we do no more have facilities for housing in-house, all theoretical sessions were held at Aarhus University close by our laboratory. The practical session where, however, organised in the laboratory making a very crowded and lively day for all!

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

The annual meeting ended with the traditional update from the CRL, who gave a report from a year with focus on training of laboratories and the preparations and considerations about implementing the new listed diseases within our fields.

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

The workshop and meeting was organised by a team consisting of Niels Jørgen Olesen, Helle Frank Skall, Nicole Nicolajsen and Søren Kahns with the help from the rest of the fish disease section at VET-DTU Aarhus.

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

3.Collect data on the fish disease situation in EU, including the new listed non-exotic diseases in Council Directive 2006/88/EF:

Survey and diagnosis in 2007

Data on survey and diagnosis on fish diseases in Europe in 2007 were collected by submission of a questionnaire. Compared to previous years a few changes were made in the questionnaire. A reason for this is the implementation of the new Council Directive 2006/88/EF Annex IV part B where ISA and KHV were

ISA, SVC and KHV

added on the list of non-exotic diseases together with VHS and IHN In addition, two new diseases, EHN and EUS, were added to the list of exotic diseases. These new diseases therefore got relatively more attention than in the previous questionnaires.

The questionnaire was composed of 4 parts: General data regarding production, and epidemiological data in their home state during 2007, laboratory data and data regarding Proficiency test and Accreditation Situation. The data regarding the aquaculture production during 1995 to 2006 was taken from "Fishery Statistical Collections Global Aquaculture Production (FIGIS)" No data from 2007 were at the time available from FIGIS. A report compiling all the data was distributed during the annual Meeting. The overall conclusion was that.

- Only few changes were observed from 2006 to 2007.
- The European production is increasing again after stagnation
- Many countries reported on the new diseases listed in Dir 2006/88
- New VHS outbreaks were observed in Norway, Bulgaria and Slovenia
- There is still a significant under-reporting of the spread of the diseases in EU.
- The obligation of authorisation of all fish farms in EU including data on their health status will improve the reporting significantly**.**

A summary of the results for 2007 is presented on Our website: http://www.crl-fish.eu/

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

Identification and characterisation of selected virus isolates

Again in 2008 a significant number of virus isolates were received for further characterisation at the CRL and for storing in our virus library:

Table 1: Material received at the CRL from laboratories in Member States and outside EU in 2008

Member States/ Countries outside EU		
Material	**Laboratories**	**Units**
Diagnostic material	8	47 samples
PCR material	2	4 samples
Other material	4	52 specimens

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 Group Division Pisciculture, Belgium** ***(François Lieffrig)*****:** Virological examination of 2 vials lyophilized supernatant of BF-2 culture infected with the 47/07 VHS virus, the virus has been isolated from brook trout fry (DTU-VET 2008-50-377). VHSV was identified and characterised genetically**.**

- **National Diagnostic and Research Veterinary Institute, Bulgaria (Nedelcho Nedelchev):** Virological examination of 4 virus isolates. VHSV was identified from two isolates from Nastan and White river, respectively. Furthermore, IPN was identified in one isolate and Tench rhabdovirus in another (DTU-VET 2008-50-209).

- **AFSSA Ploufragan/Plouzané Unité de pathologie virale des poisons, France** ***(Jeannette Castric)*****:** Participation in serological inter-laboratory proficiency test for detection of antibodies against VHS and IHN in trout sera (DTU-VET 2008-50-124).

- **Mattilsynet, district office for Indre Sunnmøre, Norway** ***(Janitha Ormøy Singdahlsen)*****:** Virological examination of 5 samples from moribund fish with clinical symptoms of VHS (DTU-VET 2008-50-256) VHSV, genotype III was identified in 4 samples.

- **State Veterinary Institute and NRL for Fish diseases, Slovakia** ***(Miroslava Vankusova)*****:** Virological examination of 3 virus isolates: CP: 4479, 365/08 (DTU-VET 2008-50-300) and CP: 10857, 576/08 and CP: 10276, 544/08 (DTU-VET 2008-20-301). VHSV genotype Ia was identified for all three samples.

- **National veterinary Institute NRL for Fish Diseases, Slovenia** ***(Vlasta Nencic)*****:** Virological examination of one virus isolate 1455/07 (DTU-VET 2008-50-22). VHSV genotype Ia was identified.

- **SVA National Veterinary Institute Dept. of Animal Health and Antimicrobial Strategies/ Fish, Sweden** ***(Suzanne Martelius-Walter)*****:** Virological examination/identification of 3 isolates, isolated from Eel (DTU-VET 2008-50-224). VHSV was detected.

- **CVI Central Veterinary Institute of Wageningen, NRL for Fish and Shellfish diseases, Netherlands** ***(Olga Haenen)*****:** Virological examination of 4 isolates, isolated from rainbow trout (DTU-VET 2008-50-263). IHNV was detected in all 4 isolates. Virological examination of 1 isolates, isolated from rainbow trout (DTU-VET 2008-50-268). IHNV was detected.

- **DTU-Aqua National Institute of Aquatic Resources, Denmark** ***(Inger Dalsgaard)*****:** Virological examination of organ material from 5 wild turbot (DTU-VET 2008-50-136). VHSV genotype Ib was detected in all five fish.

- **Heilsufrødiliga Starvstova, Inst. of microbiology, Faroe Islands** ***(Rakul Biskopstø):*** Bacterial culture from 12 samples (DTU-VET 2008-50-295, -296, -297, -313, -381). *Vibrio Spp., Aeromonas salmonicida, Aeromonas hydrophila , Shewanella putrefaciens*, yeast and *Vibrio ordalii* positive

- **Istituto Zooprofilattico Sperimentale delle Venezie (Giuseppe Bovo):** 4 samples from Tropical ornamental fish (DTU-VET 2008-50-208). Virus was not finally identified. Virus was resistant against chloroform and might belong to the birnavirus group (not IPN).

5. Production of antisera against selected isolates if necessary

Production of antisera.

Sufficient stocks of poly- and monoclonal antibodies against the listed viral fish pathogens and also against non-listed viruses as SVCV and perch rhabdovirus are available at the CRL. Therefore no antibodies were produced in 2008.
Monoclonal antibodies for discrimination between VHS genotypes have been developed by Dr. Takafumi Ito in Japan. During his post-doc at the CRL in 2008-2009 these Mabs were assessed towards large panels of VHSV isolates. Based on the result hereof the production of MAbs specifically reacting against VHSV type IVb is planned for 2009.

6. Assess the options for screening of other fish diseases in samples collected as part of a VHS/IHN surveillance programme

Assess the options for screening of other fish diseases in samples collected as part of a VHS/IHN surveillance programme

According to Commission Decision 183/2001/EEC screening for VHSV and IHNV is performed on BF-2 or RTG-2 cells and EPC or FHM cells. Samples are inoculated on cells for one week at 15 ºC and sub-cultivated for another week at same temperature and cell-line. Spring viraemia of carp virus (SVCV), pike-fry rhabdovirus (PFR), perch rhabdoviruses and related rhabdoviruses can be screened for using similar cell culture protocols whereas these protocols are not recommended for screening for KHV and ISAV (OIE manuals).

In order to investigate whether the above mentioned protocol can be used for screening for the recently listed iridovirus Epizootic haematopoietic necrosis virus (EHNV), a study was carried out whit the aim of identifying the optimal cell culture protocol for isolating EHNV. Furthermore, the EU protocol for screening for fish viruses was compared to two other protocols, in order to identify the best protocol for screening for EHNV. These studies were performed in association with RANA-project (FP7 funded STRP). Initially it was tested if EHNV would replicate on cell lines as described in to Commission Decision 183/2001/EEC to be used for screening for VHSV and IHNV. The results of these tests were that low titres were observed using RTG-2 and FHM cells. In contrast, EHNV replicated well on BF-2 and EPC, showing highest titres on BF-2 cells.
Subsequently, an EHNV isolate was passaged in red-fin perch (*Perca fluviatilis*) and re-isolated 3 times in order to eliminate bias caused by virus being adapted to propagation in cell culture and simulate natural infection. Fish were sampled twice weekly and 7 organs were processed separately according to standard virus isolation procedures as described in the OIE Diagnostic Manual where the sample is grown at 22ºC in both EPC and BF2 cells for 2 weeks and then sub-cultivated for one week (method 1), and the same procedure but with cultivation temperature of 15ºC (method 2). These methods were compared to those procedures described in Commission Decision 183/2001/EEC, where the virus is grown for one week and then sub-cultivated for a week at 15ºC (method 3). Subsequently the results were analyzed in a generalized linear mixed model. (Proc GLIMMIX, SAS statistical software). Samples cultured on BF-2 cells at 22°C for 2 weeks + 1 week sub-cultivation (method 1) appeared to provide more positive results than the other 2 methods and the other cell line tested.
The conclusion of these analyses is that the existing methods recommended for surveillance VHSV and IHNV in Europe detect fish infected with ranaviruses, albeit at lower sensitivity than the method recommended by the OIE reference laboratory for EHNV. As a sample of 30 fish are generally collected for surveillance purposes (more fish, more chances of finding it), it would be unwise and economically unpractical to recommend alternative procedures for surveillance on the basis of the results of this study.

This study was presented at the 12th Annual Meeting of NRLs for Fish Diseases by Ellen Ariel. The study will be published in an international peer review journal in the near future.

7. Optimization and standardisation of real-time PCR for the diagnosis and identification of VHS.

Optimization and standardisation of real-time PCR for the diagnosis and identification of VHS

Development of a novel real-time RT-PCR assay for detection of viral haemorrhagic septicaemia virus (VHSV)

A novel diagnostic real-time RT-PCR assay was designed with the aim of having the ability to detect and quantify all known genotypes and subtypes of VHSV. Preliminary testing shows that the assay is able to detect representative isolates of each known genotype and subtype. For this assay, we developed a triplex system (two primersets and three taqman probes) where amplification and detection of an endogenous control is included in each PCR reaction. This triplex system simultaneously measures the quality of the sample (RNA) thereby ensuring that the results of the RT-PCR reaction is reliable. A novel designed positive PCR control further ensures a reliable result of the analysis. This control contains a unique taqman probe binding site and can be distinguished from VHSV and can therefore ensure that test samples are not tested false positive because of contamination by the positive controls. This real-time PCR system will lower the risk of false positives and false negatives substantially. A further validation of the assay is planned in 2009.

A conclusion of our work with the VHSV diagnostic real-time PCR is that it is faster than the conventional cell-based diagnostic assay. Furthermore, it appears to be more sensitive and less vulnerable to contaminations than a conventional RT-PCR assay.

8. Update and maintain a library of Infectious salmon anaemia (ISA), Viral Haemorrhagic Septicaemia (VHS) and Infectious Haematopoietic Necrosis (IHN), Spring Viraemia of Carp (SVC) and Koi Herpes virus (CyHV3) virus isolates (including the sequences and the geographical coordinates of selected isolates) and entering this information into a database

Virus library

Several isolates of VHSV and IHNV were received and stored in our library during 2008 (listed in annex 2). Our library is continuously updated and maintained. Only few isolates of KHV and ISAV were, however, included.

Furthermore, the CRL has built a fish pathogen database. This database under FishPathogens.eu is currently going through a final review by experts and is expected to be ready for submission within the first half of 2009. The database is initially finished for VHSV and will be available on http://www.fishpathogens.eu/vhsv/. In the future, other fish pathogens will be included in the database. This database will serve as a public interface where characteristics as sequences and geographic coordinates of VHSV isolates can be shared by colleagues interested in control and management in aquaculture diseases. Laboratories performing diagnosis of fish diseases will be encouraged to share their knowledge by adding data into the database.

9. In collaboration with OIE reference laboratories to establish and recommend standard operating procedures for the diagnosis of the two exotic fish disease EHN and EUS.

In collaboration with OIE reference laboratories to establish and recommend standard operating procedures for the diagnosis of the two exotic fish disease EHN and EUS.

Standard operating procedures for the diagnosis of EHNV and EUS were discussed with several invited experts at the workshop in the diagnostic of the listed exotic fish diseases EHN and EUS (Please see also: *10. Organise a practical workshop in the diagnosis of the listed exotic fish diseases EHN and EUS (to be organised back to back with the 12th Annual Meeting)).*

Invited experts for discussion of EHNV diagnostics were Ellen Ariel, Denmark; Giuseppe Bovo, Italy; Riika Holopainen, Finland and Britt Bang Jensen; Denmark. All these experts are partners of the RANA project – an EU FP7

funded STRP aiming at assessing the threat of systemic iridoviruses to farm and wild freshwater fish and amphibian wildlife in the EU. For discussion of the EUS diagnostic procedures, two experts, Somkiat Kanchanakhan and Varinee Panyawachira from the OIE reference laboratory for EUS in Bangkok, Thailand were invited together with Birgit Oidtman and Steve Feist from UK.

With the background of the OIE diagnostic manuals for EHN and EUS, the experts provided a theoretical sessions focussing on what tools to be used for diagnosis these diseases. For EHNV, focus was on how cell culture systems, immunogenic methods and PCR detection are to be applied. For EUS, focus was on clinical examination, oomycete isolation, histopathology examination and PCR detection.
Subsequently, a practical part of the workshop, were arranged where the experts showed participants how these diagnostic methods should be applied in practice in the laboratory. All participants were divided into five groups. Each group was circulated between the following five platforms:

Platform 1, entitled “EUS histological slides examination and Video”, was arranged by Varinee Panyawachira and Niels Jørgen Olesen. On this platform sporulation of various oomycetes and other mycotic agents was demonstrated. Participants were given a collection of Haematoxylin-Eosin (HE), PAS and Grogott-Gomori (GG) slides consisting of internal organs, muscles and gills from EUS infected Snake-heads. The slides were stained by Haematoxylin-Eosin (HE), PAS and Grogott-Gomori (GG). Classical pathological changes with fungi and granulomas were studied under microscopes.

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

Platform 3 entitled “PCR amplification of *A. invadans* and EHNV DNA”, was arranged by Riikka Holopainen and Søren Kahns. At this platform it was described how DNA should be isolated from infected fish and in the case of EHNV from virus inoculated cell cultures and in the case of EUS from oomycetes grown in broth. Experiences from the PCR methods described in the OIE manuals were presented. Finally, the restriction fragment length polymorphism analysis method for discriminating between the RANA virus species were presented for participants

Platform 4 entitled “Virological examination for EHNV on cell culture”, was arranged by Ellen Ariel. At this platform it was demonstrated how to use BF-2 and EPC cell cultures for survey for CPE caused by EHNV. The difference between 15 and 20°C incubation was apparent with a faster development of CPE at 20°C . General virological techniques were discussed, the preparation of 24 well plates were demonstrated in the laboratory and a reference made to Commission Decision 183/2001/EEC.

Platform 5 entitled “Immunological methods used for diagnosis of EHNV”, was arranged by Giuseppe Bovo and Nicole Nicolajsen. At this platform the immunohistochemistry staining technique was demonstrated on tissue slides

from liver and kidney from experimentally ECV infected catfish. The staining protocol was described and characteristic findings and pathological changes demonstrated. In addition methods for cryo slide preparation were demonstrated together with immunofluorescence staining procedures and patterns. Finally IF procedures on infected fish cell lines were demonstrated. The slides could be examined on site in light and UV microscope and was demonstrated by projection on screen.
At the end of the workshop, all experts and participants were gathered to discuss the methods demonstrated at the workshop.
On behalf of the recommendations provided at the workshop, special operating procedures for diagnosis of EHN and EUS are currently being prepared and will be submitted to our webpage www.crl-fish.eu.

10. Organise a practical workshop in the diagnosis of the listed exotic fish diseases EHN and EUS (to be organised back to back with the 12th Annual Meeting).

Organise a practical workshop in the diagnosis of the listed exotic fish diseases EHN and EUS (to be organised back to back with the 12th Annual Meeting).

As described above, a workshop in diagnosis of the exotic fish diseases Epizootic hematopoietic necrosis (EHN) and Epizootic ulcerative necrosis (EUS) was held back-to-back with the 12th annual meeting of the National Reference Laboratories for fish diseases at the National Veterinary Institute in Aarhus, Denmark, June 17th-20th 2008.
The workshop started with a theoretical session focused on providing the participants with a theoretical introduction to the two diseases EHN and EUS that have been listed as exotic diseases in the new Council Directive 2006/88/EC and what diagnostic toll to use. The introduction to EHNV was provided by four experts from three different laboratories in Europe. The EUS part was organised with the help from two experts from the OIE reference laboratory for EUS, in Bangkok, Thailand and two European experts.
At the practical part of the workshop, five working platforms were designed. Two platforms concerned EHN diagnosis, two concerned EUS diagnosis and one were common for the two diseases. For a more detailed description of the platforms, please see 9. above. Participants were divided into five groups and each group was circulated between the five platforms. The workshop was terminated with a discussion in plenum of the outcome of the training.
In conclusion: A highly relevant and interesting aspects of the two pathogens were presented in well prepared presentations and a solid theoretical and practical introduction to the methods of diagnosing the diseases was given. Furthermore, the workshop allowed many fruitful discussions.
The workshop was followed by a drinks reception, where all the participants had the opportunity to network.

11. Establish and update a new webpage for the CRL. Maintain and update the webpage for the CRL.

Establish and update a new webpage for the CRL.

This new website was introduced at the 12th Annual Meeting, in June 2008 and is currently being updated.
The CRL website (http://www.crl-fish.eu/) is a notice board, where NRL´s and other interested parties can access relevant information and previous reports concerning the activities coordinated by the CRL and relevant upcoming events in the Community.

12. Supply standard antisera and other reference reagents to the National Reference Laboratories in Member States.

Materials supplied by the CRL

On request, the CRL supplied 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 the specific material and the number of units supplied by the CRL are listed in table 2.

Further details of the materials are listed in Annex 3

Table 2: The CRL supplied the following reagents in 2008

Material	Laboratories	Units
Cell cultures	2	8 flasks
Polyclonal antisera	5	7 vials
Monoclonal antisera	10	17 vials
Virus isolates	7	14 vials
Protein A	4	8 vials
Other material	7	58 vials

13. Prepare the Annual Inter-laboratory Proficiency Test year 2008 for the National Reference Laboratories.

Preparation of Inter-laboratory Proficiency Test 2008

A comparative test of diagnostic procedures was provided by the Community Reference Laboratory for Fish Diseases (CRL) to 35 National Reference Laboratories (NRLs) in the middle of October 2008.
The test contained five coded ampoules, with viral haemorrhagic septicaemia virus (VHSV), infectious haematopoietic necrosis virus (IHNV), infectious pancreatic necrosis virus (IPNV) or a mixture of VHSV and IPNV, respectively. The proficiency test was designed to primarily assess the ability of participating laboratories to identify the notifiable non-exotic viruses: VHSV and IHNV but also to assess their ability to differentiate other fish viruses, as IPNV, spring viraemia of carp virus (SVCV) perch rhabdovirus etc. In addition the participants were asked to titrate the viruses in order to assess the cell susceptibility for virus infection in the respective laboratories.
Participants were asked to reply latest December 12th 2008.
Laboratories were encouraged to geno- and serotype isolates. Furthermore, due to an ongoing discussion on sequencing as a tool for differentiation between various genotypes of the non-exotic viruses, all laboratories were asked to provide a full-length G-gene sequence of the rhabdovirus identified in the lowest numbered ampoule in the test. The aim of this exercise is to provide a tool for assessing the quality of sequence data, by assessing the homogeneity of the sequences obtained from the same virus isolate.
Each laboratory has been given a code number to ensure discretion.

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

Outcome of Inter-laboratory Proficiency Test 2008

Identification of content

- 22 laboratories correctly identified all viruses in all ampoules.
- 33 laboratories correctly identified the virus in ampoule I and V.
- 34 laboratories correctly identified the virus in ampoule II and III.
- 24 laboratories correctly identified the two viruses VHSV and IPNV in ampoule IV.
- 9 laboratories did not identify VHSV in ampoule IV.
- 1 laboratory did not identify IPNV in ampoule IV.
- 2 laboratories found more isolates in an ampoule than were actually present.
- 2 laboratories found SVCV in an ampoule when it was not present.

Genotypning and sequencing

- 8 laboratories serotyped some isolates.
- 15 laboratories genotyped some isolates.
- 17 laboratories submitted sequences.

Of 35 laboratories, 17 submitted sequence data. Full length G-gene sequence were submitted by 8 laboratories, five laboratories submitted partial G-gene sequence and four laboratories partial N-gene sequences. Compared to proficiency test 2007, three more laboratories submitted full length G-gene sequences this time. Of the 8 submitted full length G-gene sequences, four

sequences were 100% identical with the published sequence (GenBank accession number AF345859). Three out of five partial G-gene sequences were 100% identical with the published sequence, whereas none of the partial N-gene sequences were 100% identical at overlapping sequence stretches. This shows that at least 8 of 17 laboratories submitted sequences containing mismatches.
One conclusion drawn of this study is that more laboratories submitted full length G-gene sequences this year compared to last year and sequences were in general of better quality. We hope that these exercises have improved the awareness of the importance of submitting correct nucleotide sequences.

Concluding remarks
One ampoule contained 2 viruses as ampoule IV contained a mixture of IPNV and VHSV. Only the IPNV and not the VHSV was identified by 9 laboratories, despite the fact that VHSV was present in a relative high titre in the ampoule (undiluted). We encourage participants to be aware of the possibility of more viruses being present at the same time and that one can over grow the other on cell cultures, and thereby masking its presence.

The results will be further presented and discussed at the 13th Annual Meeting of National Reference Laboratories for Fish Diseases to be held 26-28 May 2009 in Copenhagen, Denmark.

The full report is in Annex 4.

15. Facilitate and provide training in laboratory diagnosis.

Training, missions and scientific collaboration

The following colleagues visited the institute during 2008 for scientific meetings, project collaboration or training:

Nermina Vejzagic and Dejan Stojanovic, Veterinary Faculty of Sarajevo, Department of Parasitology; Department of Aquaculture, Bosnia and Herzegovina Training course on fish diagnostics	28. January - 15. February
Tanya Gray, Company Name: Symantix Ltd, United Kingdom. Database development	29 - 30. April 22 - 24 October
Khong Thi My, University of Bern, Switzerland. Training in serological methods.	26th March- 3rd April 2008
Takafumi Ito, National Research Institute of Aquaculture, Japan. Visiting research scientist with focus on development and characterisation of MAb´s against VHSV.	1st October 2008- 31st May 2009
Study visit on fish health management, diagnostics and research in fish pathology.Daniel Woywood W., Gerente Técnico, Aquabench,, Puerto Montt, Chile	18th November 2008
Study visit on fish health management, diagnostics and research in fish pathology for a delegation from Chile: Erwin Serón – Etecma; Gerardo Muñoz - Fundación Chile; Angelica Lisperguer - Marine Harvest; Roxana Arriagada – Genexpress; Marco Rosas – ADL; Javier Moya – Diagnotec; Jorge Ríos – Intesal; Rolando del Rio - Comercial Galilea; Gonzalo Romero - Corfo: Integrated Territorial Program for salmon cluster; Marcos Godoy – Biovac ; Patricia Aillapán - EuroChile	9. December 2008
The CRL conducted a mission to Bulgaria.: Laboratory visit at the National Diagnostic & Research Veterinary Institute National Reference Laboratory for Fish and Mollusc Diseases in Bulgaria. *The full report is in Annex 5.*	17th-19th November 2008

The CRL conducted a mission to Romania.: Laboratory visit at the Institute of Diagnosis and Animal Health Department of Aquatic Animals and Usefull Insects Health National Reference Laboratory for Fish Diseases in Romania *The full report is in Annex 6*.	19th-21st November 2008
Workshop on VHS and IHN serology : The group co-organised and participated in an international workshop on VHS and IHN serology together with Dr. J. Castric and her group at AFSSA, Brest, France	24-27th March 2008.
A 1 week course on fish diseases was conducted in Iran 21.May – 1st June 2008 and we participated in a workshop on EU-legislation in Tirana, Albania	13-15th April 2008.

16. Attending international meetings and conferences

International meetings and conferences attended.

Meetings and Conferences

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 CRL participated in the following activities in 2008:

Participation and presentations at international conferences and meetings

Olesen N.J.: Participated in the EPIZONE FP6-2004-Food-3-A ½-year meeting in Lelystadt, NL, January 09-11 2008.
Olesen N.J.: Co-organiser of, and participant in EPIZONE WP 6.1 Workshop on VHS and IHN serology, Brest, France 24-27th March 2008.
Olesen N.J., Kahns S.: Workshop on risk management with focus on ISA, Oslo, 3-4th April 2008
Olesen N.J.: Invited speaker in TAIEX workshop on fish health management, 12-14th April 2008, Tirana, Albania.
Olesen N.J.: Visit to Iran including teaching all lectures at a 5 day course in fish pathology and fish health management, 21-31st May 2008, Gilan Province, Iran
Olesen N.J., Jensen B. B.: Participated in the 2nd Annual Meeting of EPIZONE, Brescia, Italy 03- 06th June 2008.
Olesen N.J.: Invited speaker at the VHS workshop 26th June 2008 in Oslo. Norway
Olesen N.J.: Is member of two working groups in the European Commission on risk based surveillance and diagnostic methods, respectively, with 4 meetings in Brussels.
Olesen N.J.: Invited speaker at ECM WORKSHOP "DIRECTIVE 2006/88/EC: a further tool for improving aquaculture" 25-26th September 2008, Treviso, Italy
Olesen N.J, Kahns S. and Nikolajsen N.: Mission to the National Reference Laboratories for Fish Diseases in Sofia, Bulgaria, and Bucharest, Rumania, 17-21st November 2008
Kahns S., Olesen N.J., Skall H.F and Nicolajsen N. Arranging the Workshop in Diagnosis of the Exotic Fish Diseases EHN and EUS and the 12th Annual Meeting of the National Reference Laboratories for Fish Diseases. June 17-20, 2008, Aarhus Denmark
Kahns S. 6th workshop on Proficiency Testing in Analytical Chemistry, Microbiology and Laboratory Medicine: Current Practice and Future Directions. Poster presentation title: A proficiency testing scheme to detect viral fish diseases. August 7-8, 2008, Rom, Italy

Kahns S. Training Course: Statistics for PT/EQA Schemes and Selection, use and interpretation of PT/EQA Schemes. August 6 2008, Rom, Italy

Kahns S. Presentation at the Second Annual Meeting of Epizone, 2008, "A comparative sequence study launched in association with the proficiency test 2007" June 3-7, 2008 Brescia, Italy.

Schyth, B.D. Small interfering RNAs and their use in targeting of viral genes in fish cells. Abildgaard Symposium organised by the research school SCOFDA, April 29-30, 2008, Copenhagen, Denmark.

Schyth, B.D., Lorenzen, N. Delivery and effect of small interfering RNAs targeting a fish pathogenic virus. 3rd EPIZONE theme 5 meeting, October 22-24, 2008, El Escorial, Spain.

Schyth, B.D., Lorenzen, N. DNA vaccines and other molecular tools in fish health research. SCOFDA workshop: Diagnosis and Control of Fish Diseases, November 4-5, 2008, Copenhagen, Denmark.

Einer-Jensen, K. Identification of virulence markers in marine VHSV and use in diagnostics for aquaculture. SCOFDA workshop: Diagnosis and Control of Fish Diseases, November 4-5, 2008, Copenhagen, Denmark.

Einer-Jensen K, Rasmussen JS, Lorenzen N Scientific progress and coordination of IMAQUANIM by P1 during the IMAQUANIM progress meeting, April 1-4th 2008, Barcelona, Spain.

Einer-Jensen, K. Identification of virulence markers in marine VHSV and use in diagnostics for aquaculture. Meeting for viologists at VET.DTU, Hjalet October 7-8, 2008, Denmark.

Jensen, B.B 6th Progress meeting for the RANA-consortium (Risk assessment of new and emerging systemic iridoviral diseases for European fish and aquatic ecosystems). February 18-19. Bangkok, Thailand

Jensen, B.B Workshop in Risk assessment for RANA-project partners and invited experts. February 20-22. Bangkok, Thailand,

Jensen, B.B Annual meeting and conference for The Society for Veterinary Epidemiology and Preventive Medicine. March 26-28. Liverpool, UK

Jensen, B.B Workshop in Sustainable Control of Diseases in Aquaculture. April 29-30. Copenhagen, Denmark

Jensen, B.B Workshop in diagnosis of the exotic fish diseases EHN and EUS & 12th annual meeting of the national reference laboratories for fish diseases. June 17-20. Aarhus, Denmark

Jensen, B.B 7th Progress meeting for the RANA-consortium, and workshop in Risk assessment. September 17th-19th .Berlin, Germany.

Jensen, B.B Workshop in Disease surveillance. September 25-26. Copenhagen, Denmark.

Skall H.F. ODA-meeting, Marts 6, 2008. October 2, 2008. Denmark

Skall H.F. EFSA meeting. Working group on aquatic species susceptible to diseases listed in Directive 2006/88/EC. Marts 17, 2008 and April 24-25, 2008 Paris, France. June 2-4, 2008, Parma, Italy. June 17-20, 208 Barcelona, Spain.

Skall H.F. SCOFDA meeting - Sustainable Control of Fish Diseases in Aquaculture: Pedersen K, Skall HF, Lassen-Nielsen AM, Bjerrum L & Olesen NJ: *Photobacterium damselae* subsp. *Damselae*, en emerging pathogen in Danish marine rainbow trout and Olesen N.J & Skall H.F. Model trout farms: surveys of viral and BKD infections. November 4-5, 2008

Scientific publications in peer-reviewed journals

Pedersen K; ***Skall H F****; Lassen-Nielsen A M; Nielsen T F; Henriksen N H;* ***Olesen N J*** Surveillance of health status on eight marine rainbow trout, Oncorhynchus mykiss (Walbaum), farms in Denmark in 2006. Journal of fish diseases 2008;31(9):659-67.

Bjørn E. Brudeseth, ***Helle F. Skall****, and Øystein Evensen* Differences in Virulence of Marine and Freshwater Isolates of Viral Hemorrhagic Septicemia

Virus In Vivo Correlate with In Vitro Ability To Infect Gill Epithelial Cells and Macrophages of Rainbow Trout (*Oncorhynchus mykiss*) J. Virol. 2008 82: 10359-10365.

Tore Håstein, Martin Binde, Mike Hine, Stian Johnsen, Atle Lillehaug , ***Niels Jørgen Olesen***, *Neil Purvis, A. David Scarfe and Belinda Wright*. (**2008**) National biosecurity approaches, plans and programmes in response to diseases in farmed aquatic animals – Evolution, effectiveness and the way forward. Rev.sci. tech. Off. Int. Epiz., 27 (1), 125-145

Ruiz, S., Schyth, B.D., Tafalla, C., Estepa, A., ***Lorenzen, N****. and Coll, J.M.* New tools to study RNA interference to fish viruses: Fish cell lines permanently expressing siRNAs targeting the viral polymerase of viral haemorrhagic septicaemia virus. (Submitted)

Schyth, B.D. 2008. RNAi mediated gene silencing in fishes? Review. *Journal of Fish Biology*, 72, 1890-1906.

Einer-Jensen K*, Delgado L,* ***Lorenzen E****, Bovo G, Evensen O, Lapatra S,* ***Lorenzen N***. Dual DNA vaccination of rainbow trout (Oncorhynchus mykiss) against two different rhabdoviruses, VHSV and IHNV, induces specific divalent protection. *Vaccine* (In press)

Utke K, Kock H, Schuetze H, Bergmann SM, ***Lorenzen N, Einer-Jensen K****, Köllner B, Dalmo RA, Vesely T, Ototake M, Fischer U.* (2008) Cell-mediated immune responses in rainbow trout after DNA immunization against the viral hemorrhagic septicemia virus. *Developmental & Comparative Immunology.* 32(3):239-52

Other communications

Einer-Jensen, K., Dansk Veterinærtidsskrift (2008; 15. august, nummer 15/16, Årgang 91). "Bedre identifikation af VHS-virus i havet"

Hoffmann, P. DTU Avisen (October 6, 2008 No8, p 1-2): "Jagten på virus-genet".

Hoffmann, P. DTU Avisen (DTU in English, Section 2, October 6, 2008 No8, p 1): "Searching for a deadly gene".

Jensen, B.B. and Ariel, E. (2008): First steps towards a risk assessment of the introduction and spread of fish and amphibian ranaviruses into the EU. Poster presented at Society for Veterinary Epidemiology and Preventive Medicine. Liverpool, March 26-28.

Research relating to fish disease taking place at DTU-VET.

The group is partner and project coordinator of EU project RANA: Risk assessment of new and emerging systemic iridoviral diseases for European fish and aquatic ecosystems. Proposal/Contract no.: 6459 (http://ranavirus.net/).

The group is 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 (http://www.epizone-eu.net/default.aspx).

A 3-year national research project supported by the Danish Ministry of Food, Agriculture and Fisheries (FØTEK-4 programme), coordinated by the Section for Fish Diseases at DTU-Veterinary Institute and including collaboration with the Danish Fish Farmers Association (Danish Aquaculture), entitled "Field testing of a DNA vaccine for farmed fish" has been finalised. Despite variability and limitations in the small scale experimental setup, the overall results indicated that DNA vaccination against VHS in rainbow trout can induce protective immunity against the viral disease under field conditions. No negative side effects on the vaccinated fish were observed and no transfer of vaccine to the environment was detected. The next step towards implementation of DNA vaccines in European Aquaculture should include a full scale clinical testing. This will imply that the vaccinated fish must be allowed to reach the

food chain and since no DNA vaccines have been licensed for husbandry animals in Europe yet, this step will require initial acceptance by the food safety authorities. Although a sceptical public opinion against use of gene-modified elements in food production exists in some countries, this should be achievable since all experiments with DNA vaccines in both animals and humans have so far supported the view that the risk of negative side effects is very small, also when compared to those observed for traditional vaccines.

A 5 year EC-supported FP6 integrated project coordinated by the Section for Fish Diseases at DTU-Veterinary Institute and including 22 participants in nine European countries is entitled “Improved immunity of aquacultured animals” (IMAQUANIM) and has successfully passed the midterm evaluation. The work includes both basic fish immunology research and applied research and technical development for establishment of a platform of knowledge and tools for better disease prophylaxis in cultured fish and shellfish. A report summary of the research activities and results including publications is available at the project website http://imaquanim.dfvf.dk/info/ .

A 3½-year national research project supported by the Danish Research Council has recently been initiated. The project “Identification of virulence markers in marine VHS virus and use in diagnostics for aquaculture” is focused on 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.

A 3½ year project funded by the Danish Research Council for Technology and Production Science "Delivery of small interfering RNAs in vivo". The DTU group involved is partner in the siRNA delivery center www.sirna.dk hosted by Department of Molecular Biology at University of Aarhus. Small interfering RNAs (siRNAs) are small regulatory molecules, which can downregulate the activity of specific genes by the RNA interference pathway of the cell. For this reason siRNAs have a potential as a novel type of gene medicine. The RNA interference mechanism is essentially the cells' own way of reducing expression of unwanted protein. By producing small double stranded RNAs with one string being homologous to a specific target messenger RNA (mRNA), the cell is able to program a cellular enzyme complex, known as RISC, to destroy this mRNA and inhibit its translation into protein. Transfection with three different siRNAs specific to the viral glycoprotein gene of VHS virus efficiently inhibited viral multiplication in infected cell cultures, while two of three corresponding mismatched siRNAs did not have this effect. This suggested specific interference, but similar results were obtained when the same siRNAs were tested against the heterologous virus. Further analyses revealed that the siRNAs induced a non-target-specific anti-viral effect correlating with up-regulation of the Mx gene. The models are now be used for screening combinations of siRNAs, new commercial and non-commercial transfection reagents and delivery routes for their ability to specifically suppress viral replication without activating the innate antiviral defence mechanisms. There are two aims of these studies: 1) investigation of the host-pathogen interaction and diagnosis of the stage of disease and 2) development of treatment strategies based on the natural defence of the host.