# Technical Report
# 2010

## from the
## European Union 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 European Union Reference Laboratory for Fish Diseases (EURL), according to Commission Decision of 14 December 2009 on financial aid from the Union for the year 2010 for certain Community reference laboratories in the field of animal health and live animals (2009/961/EU)

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 2010, describing activities associated with each point and the status of ongoing projects. The list of functions and duties of the EURL follows this introduction.

The 14th annual meeting of the National Reference Laboratories for fish diseases was held back-to-back with a Workshop on "Use of Diagnostic Kits for Detection of Fish Diseases" in May 26-28 2010, in Aarhus, Denmark. A total of 61 participants from 35 countries attended over the three day period. There were five sessions with a total of 41 presentations, 9 of which were given by invited speakers. The workshop and meeting was held at rented nearby facilities of Aarhus University, as the premises at our institute in Aarhus are too small for the number of participants. A report was submitted in August 2010.

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. This year, the proficiency was expanded to consist of two tests, PT1 and PT2. PT1 was designed as the previous proficiency tests provided by the EURL, to primarily assess the ability of participating laboratories to identify: VHSV, IHNV and EHNV. PT2 was developed in order to test the proficiency of participating laboratories to identify ISA and KHV. 38 National Reference Laboratories (NRLs) participated in PT1 and 36 NRLs in PT2. A report was submitted in March 2011. Most laboratories performed very well, especially in view of the fact that two new disease pathogens, KHV and ISAV, were included for the first time in the test.

An important focus of the EURL was 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. Preparation of the diagnostic manual for EUS has been delayed as it turned out to be rather difficult to obtain pure *A. Invadans* strains. However, we succeeded in obtaining two different strains which we have been used for setting up diagnostic procedures. The oomycete is thus growing and sporulate well under our laboratory conditions. Production of reference material for standardising diagnostic methods for EUS in EU is in preparation and will be issued in 2011.

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 that the laboratory became accredited for the use of a PCR based diagnostic tool for detection of EHNV. Another was generation of a real-time RT-PCR assay for detection of all genotypes of VHSV.

During 2010, resources were also used to collate data on surveillance 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 and missions to other NRLs; to produce antisera; to prepare SOP´s for detection of fish antibodies; to update the EURL webpage (www.eurl-fish.eu); and finally to attendi 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.

Aarhus, 25th March 2011

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 2010 – 31 December 2010**

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**Functions and duties of Community reference laboratories**

1. The Community reference laboratory shall in accordance with Article 55, 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 2010**

# Technical report

***1-2. Organise and prepare for the Annual Meeting for the National Reference Laboratories for Fish Diseases in 2010 and produce a report from the Meeting***

## Organization of the 14th Annual Meeting

26-28 May 2010 the 14th annual meeting of the National Reference Laboratories for fish diseases was held back-to-back with a Workshop on "Use of Diagnostic Kits for Detection of Fish Diseases". A total of 65 participants from 35 countries attended over the three day period. There were five sessions with a total of 41 presentations, 9 of which were given by invited speakers. The workshop and meeting was held at rented nearby facilities of Aarhus University, as the premises at our institute in Aarhus are too small for the number of participants.

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 primarily from the member states presented new findings from their home countries. Initially an overview of the disease situation and surveillance in Europe 2009 were provided on the basis of the results from the survey and diagnosis questionnaire. Scotland UK updated on the situation after the outbreak of ISA in 2009. Results of a questionnaire send to 12 fish-pathology experts was the basis of a presentation on old and emerging diseases from the Mediterranean aquaculture. Subsequently a talk on multiple infected fish in a Swiss fish farm was presented, followed by a talk on the VHSV eradication program in Denmark. Later in this session presentations about pancreas disease and BKD from Norway and Red Mark Syndrome and the Rosette Agent were given. The session was ended by a talk on bio-security risk associated with EUS and Iridovirus in ornamental fish.

The session on technical issues related to sampling and diagnosis were divided into two parts. The first part of the session focussed on the new EU manuals on sampling and diagnostic procedures that has been uploaded on the www.eurl-fish.eu web page. At the session, the diagnostic procedures for detection of the listed non-exotic fish pathogens VHSV, IHNV, ISAV and KHV as well as the exotic EHNV and EUS were described.
The last part of this section focussed on many different issues. It was initiated with two talks on un-explained increased mortality: how to deal with it from a legislative and a practical perspective, respectively. Later talks were focussed on sensitivity and specificity of test procedures for BKD, non-lethal sampling and KHV detection in latent infected koi carps, identification of a possible novel Cyprinid herpesvirus 3 variant strain and a novel real-time PCR assay to detect VHSV.

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

The last day was opened by an update session on scientific research. At this session, presentations were given on progress in the development in sero-neutralisation test for detection of antibodies against KHV in carp, on vertical transmission of pancreas disease (PD) and infectious salmon anaemia (ISA), respectively, and on the findings that heart and skeletal muscle inflammation (HSMI) most likely is caused by a reovirus. Subsequently two projects were presented: The Club 5 project on EUS diagnostic methods and the NADIR project on access to infection facilities. The session was closed with a presentation on 1) perch rhabdovirus infection in perch and pike-perch and 2) the putative use of miRNA in future diagnostics.

The annual meeting ended with the traditional update from the EURL. The results of

the proficiency test 2009 were presented. A report from year 2009 was given, a year with focus on training of laboratories and thoughts and considerations about preparation of the new EU diagnostic manuals. Furthermore, proposals on the EURL work plans for 2011 were discussed.

Minutes from the meeting were taken by Helle Frank Skall, Søren Peter Jonstrup, Torsten Boutrup and Søren Kahns, and have afterwards been sent to presenters for correcting in order to avoid misunderstandings. The minutes are included in the final report together with abstract and comments from the presentations.

*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 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 2009

The Questionnaire on Surveillance and Diagnosis (S&D) which is collated annually provide 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 also can be obtained. The S&D have evolved over the years and do now comprise 4 parts: 1) General data on production, 2) Epidemiological data on diseases, 3) Laboratory data from NRLs and other laboratories, and as the final part we have for the first time included 4) A questionnaire on commercial diagnostic kits and reagents used in the NRL's and regional laboratories. Previously we also collated data on quality assurance in NRLs and regional laboratories but these questions are now being asked in connection with the inter-laboratory proficiency tests as we found that connection more logical.

The data on the European aquaculture production were obtained from the "Fishery Statistical Collections Global Aquaculture Production (FIGIS)" database. Unfortunately this database does not include information on the number and size of fish farms, which are epidemiologically important data. The production in 2007 and 2008 has risen a bit again after a decrease from 2003-2006. Data from 2009 were not available. The farm sizes vary a lot between countries, e.g. the majority of farms in Germany produced < 5 tonnes, and for Spain the number of farms producing < 5 tonnes, 5-100 tonnes and > 100 tonnes is nearly equal.

In Northern European countries there are mainly salmonid farms, in continental Europe we find a lot of carp farms, and in the Mediterranean area, besides carps, seabream and seabass are also species that many produce. Turkey is a significant producer of rainbow trout with many farms.

Concerning the epidemiological data, the main question is: what is the distribution and amount of infected fish farms in Europe? For the first time ever no farms are considered infected with VHS in Denmark since March 2009. There seems, however, to be severe underreporting of VHS and IHN in many countries. The infection status is known for about ½ of the farms in Europe.

The figures for KHV only reports on carp farms and not outbreaks in private garden ponds. The infection status regarding KHV is unknown for many carp farms, whereas for farms producing Atlantic salmon, the infection status for ISA is known for nearly all farms. For ISA app. 50% of the farms are considered infected at the Faroe Islands, but as HPR0 positives only. Unfortunately, a new outbreak of ISA was observed again at the Shetland Islands after a pause of several years, and ISA is still a problem in Norway.

Many countries have surveillance programmes for SVC, BKD, and IPN, for which they are seeking "additional guaranties". The number of farms in the programmes varies from very few farms to many farms. Fewer countries have surveillance programmes for *Gyrodactylus salaris*.

There are very large differences between countries on how many samples are tested

on cell cultures, ranging from < 100 to several thousands. PCR is really starting to come up in many countries, but the large number of PCR-tests conducted in some countries mostly reflects the KHV and ISA testing.
About a third of the countries have regional laboratories, and of these countries, 8 of 11 organize ring tests for the regional laboratories.
An overview of the current status on how each Member State have categorised their zones and compartments with fish farms according to CD 2006/88/EC was gathered. Very significant variation in the perception on how categorisation should be performed and in the progress of work was observed. A few countries were almost ready whereas most still lack to authorise and categorise a significant number of farms. Risk based surveillance and risk categorisation of farms into low, medium and high risk for the non-exotic diseases have only been implemented in very few places in the Community.

*A summary of the results for 2009 is presented on Our website: http://www.eurl-fish.eu/*

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

## Identification and characterisation of selected virus isolates

Again in 2010 a significant number of virus isolates were received for further characterisation at the EURL and for storing in our virus library:

| Member States/ Countries outside EU | | |
|---|---|---|
| **Material received** | **Laboratories** | **Units** |
| Diagnostic material | 5 | 23 samples |
| Virus isolates | 5 | 13 samples |
| Other material | 5 | 53 specimens |

Table 1: Material received at the EURL from laboratories in Member States and outside EU in 2010

*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:

- **Fish Medicin & Livestock Management, Austria** *(Mansour El-Matbouli)*: 7 samples of purified DNA, pool of organ tissues. KHV not identified (DTU-Vet. 2010-50-95 and 2010-50-149). Diagnostic material from 4 samples from brown trout, VHSV identified in 1 sample (DTU-Vet. 2010-50-99).
- **AFSSA Brest, France** *(Jeannette Castric)***:** Reagent for KHV serologi (DTU-Vet. 2010-50-143) and KHV isolates (DTU-Vet. 2010-50-107).
- **Heilsufrødiliga Starvstovan, Faroe Islands** *(Rakul Biskopstø Joensen)*: Bacteriological material A210-113-1,2 Marineagar 3839 from fish - *Vibrio anguillarum* (vibriose) identified (DTU-Vet. 2010-50-240)
- **Friedrich-Loeffler-Institut (FLI), Germany** *(Dieter Fichtner & Sven bergmann)*: 5 samples of SVCV tested by 50% PNT, All 5 isolates are SVC-like viruses (DTU-Vet. 2010-50-038).
- **FLI, Germany** *(Bernd Hoffmann):* Carp sera (DTU-Vet. 2010-50-142 and CCB cells inoculated with KHV (DTU-Vet. 2010-50-119)
- **IZSVe, Padova, Italy** *(Giuseppe Bovo)*: IPNV supernatant (DTU-Vet. 2010-50-52)

- **National Research Institute of Aquaculture Fisheries Research Agency, Japan** *(Takafumi Ito):* 3 samples KHV (DTU-Vet. 2010-50-120) and 4 samples tested by RT-PCR for VHSV (DTU-Vet. 2010-50-287).
- **Fish Diseases Labratory, NVLU, Japan** *(Kishio Hatai)*: Negative reference material without *Aphanomyces Invadans* (DTU-Vet. 2010-50-236) and positive reference material for *Aphanomyces Invadans* (DTU-Vet. 2010-50-237).
- **National Veterinary Institute, Norway** *(Ingebjørn Modahl)*: A flask with ASK cells with ILAV (DTU-Vet. 2010-50-51).
- **Institute for Diagnosis and Animal Health, Romania** *(Mihaela Costea)*: 2 diagnostic samples for examination for KHV, KHV detected in one sample (DTU-Vet. 2010-50-325).
- **National Veterinary Institute (SVA), Sweden** *(Suzanne Matelius Walter)*: Virological examination of 3 isolates from Perch, Perch rhabdovirus identified (DTU-Vet. 2010-50-147 and 2010-50-178). *(Eva Jansson)* Samples with A. astaci 56/2003 (DTU-Vet. 2010-50-294)
- **Inland Aquatic Animal Health Research Institute (AAHRI), Thailand** (*Somkiat Kanchanakhan)*: *A. Invadans* NJM 9510 from Snakehead, *Aphanomyces invadans* negative (DTU-Vet. 2010-50-43), *A. Invadans* NJM 0002 from Snakehead, did not grow (DTU-Vet. 2010-50-44), NJM 0002 from Snakehead, *Aphanomyces Invadans* negative (DTU-Vet. 2010-50-219) and *A. Invadans* NJM 9701 from Snakehead, *Aphanomyces invadans* positive (DTU-Vet. 2010-50-220).
- **Institute for Animal Science and Health (CVI), The Netherlands** *(Olga Haenen)*: Diagnostic samples from rainbow trout, related to the EPIZONE project (DTU-Vet.2009-50-343).
- **Center for Environment, Fisheries and Aquaculture Science – CEFAS, UK-England** *(Birgit Oidtmann*): *A. Invadans* NJM 0002 from Snake head, *Aphanomyces invadans* positive (DTU-Vet. 2010-50-156).

***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 except KHV were available at the EURL. Therefore rabbit antisera against KHV were produced in 2010.
Additionally antisera against perch rhabdovirus were produced using a purified isolate from grayling in Poland (207237-1).

***6. Assessment and standardisation of Real-time PCR tests for the diagnosis, identification and typing of the listed non-exotic fish diseases.***

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

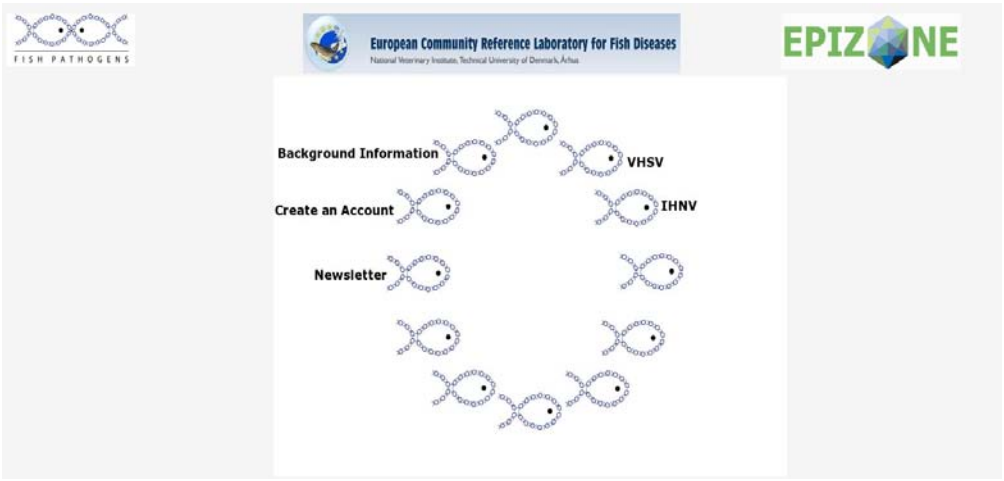
A novel diagnostic real-time RT-PCR assay was designed by the EURL with the ability to detect and quantify all known genotypes and subtypes of VHSV. This was done because assays previously designed by the EURL or assays designed by other laboratories failed to recognize all VHSV genotypes. A VHSV diagnostic real-time RT-PCR will be a faster diagnostic tool than the conventional cell-based diagnostic assay. Furthermore, it seems more sensitive and it is less vulnerable to contaminations than a conventional RT-PCR assay.
An inter-laboratory proficiency test was conducted in order to assess reproducibility and robustness of the newly developed real-time RT-PCR for detection of VHSV as part of the validation procedure according to the recommendations given by the OIE. Samples, probes and primers were shipped to 5 laboratories who all replied with good results.
The experimental part of the validation of the VHSV real-time RT-PCR was finished in 2010. The first draft of a manuscript has been written and the method will be

published in peer-reviewed journal in 2011.
The EURL continued the implementation of real-time PCRs for detection of ISAV and KHV. For ISAV, the assay developed by Mike Snow et al. 2006 was chosen and several diagnostic samples were tested as part of validation.
For KHV detection, validation of the real time PCR described by Gilad et al. 2004 was continued.

***7. Expanding www.fishpathogens.eu with IHNV, SVCV and the inclusion of KHV and ISA will be initiated.***

## 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 database was launched in 2009 with a part on VHSV. In 2010 Fishpathogens.eu was expanded to include IHNV and the work was published in journal of fish diseases (Jonstrup et al. 2010). Furthermore the development of a SVCV database is at the final stage of development. Work on ISAV and KHV databases were initiated. The VHSV database were maintained and expanded. The VHSV database today offers publically available information on 459 isolate reports and 327 sequence reports, while the IHNV database offers publically available information on 82 isolate reports and 84 sequence reports. 106 persons have registered as users of the database and in the last year the database had 6284 visitors.




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

## Virus library

Several isolates of VHSV, IHNV, KHV and ISAV were received and stored in our library during 2010 (listed in annex 2). Furthermore, two isolates of *Aphanomyces Invadans*.
In addition, the EURL recieved 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.

*9. 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 NRL´s 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.
In 2010 the diagnostic manuals for VHS, IHN, ISA, KHV disease and EHN 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, survey and diagnosis etc. are launched at the web page immediately after release.


*10. Update and include standard operating procedures on the EURL web page for the listed exotic and non-exotic diseases*

## Diagnostic manuals

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

*11. Workshop on available kits and reagents for diagnosis of the listed non-exotic diseases VHS, IHN, ISA and KHV including consideration of their sensitivity and specificity.*

## Workshop on kits and reagents for diagnosis of fish diseases.

The workshop on "Use of Diagnostic Kits for Detection of Fish Diseases" was held 26 May, the day before the 14th Annual Meeting in Aarhus. Besides providing a presentation of some of the kits that are currently available on the market, the aim of the workshop was to discuss how laboratories can ensure that commercial as well as own diagnostic kits are properly validated. The workshop started with a presentation on the outcome of the survey and diagnosis questionnaire on what kits are used by the NRLs for detection of fish pathogens. Subsequently, a talk on general needs on development and validation of diagnostic tools were presented. The procedures for tests of different commercial antibody based kits were described in four talks by four speakers from different laboratories/companies. The last two sessions were focused on molecular biological tools. A DNA-array based diagnostic tool followed by the

LAMP originated kits was presented. The workshop was ended by a plenum discussion on how laboratories should ensure properly validation of used diagnostic kits. Discussions continued in the evening where all participants were invited to a reception at the National Veterinary Institute.

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

## Materials supplied by the EURL

On request, the EURL 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 EURL are listed in table 2.

*Further details of the materials are listed in Annex 3*

Table 2: The EURL supplied the following reagents in 2010

| Material | Laboratories | Units |
|---|---|---|
| Cell cultures | 12 | 29 flasks |
| Polyclonal antisera | 6 | 21 vials |
| Monoclonal antibody | 3 | 6 vials |
| Virus isolates | 10 | 50 vials |
| Virus in RNA Safer or extracted RNA | 1 | 4 vials |
| Purified RNA | 5 | 14 vials |
| Other material | 15 | 124 vials |

***13. Inclusion of SOP´s on serological methods for detection of fish antibodies against VHSV, IHNV and KHV on EURL website, and introducing the methods in new Commission Decision on sampling and diagnostic procedures***

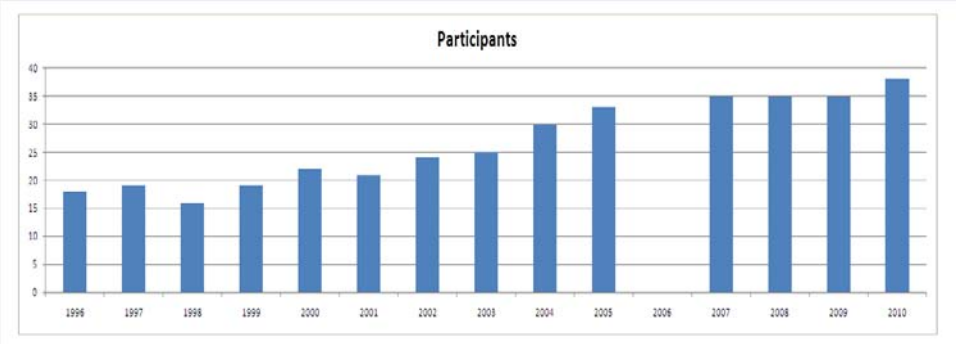
## Standard operating procedures on serology

In 2010 ELISA, immunoflourescence, and seroneutralisation technics were developed to detect antibodies in carp sera against koi herpesvirus. An inter-laboratory proficiency test was conducted and a number of reference sera tested in order to assess the new techniques. The project was successfully conducted as an internal call project in EPIZONE network in a collaboration between ANSES, Brest, FLI, Riems, CVI, Lelystat, IZSVe, Padova, SVA, Uppsala and our institute. The procedures developed should according to our plans have been included on the EURL web page, but due to delays in publishing the SOP´s for virus detection methods this part was postponed to 2011.
The same applies for the methods developed for detection of antibodies in trout against VHSV and IHNV, which have been finally validated in 2010. A scientific review on serological methods is in preparation in order to scientifically justify the inclusion of serological test in surveillance of VHS and IHN.

***14. Prepare the Annual Inter-laboratory Proficiency Test year 2010 for the National Reference Laboratories. The test will be expanded to also include ISAV and KHV.***

## The inter-laboratory Proficiency Test 2010

Since 1996, fourteen inter-laboratory proficiency tests have been organised by the EURL. The number of participants has increased from 18 to 38. 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.




The 2010 test was divided into two: proficiency test 1 (PT1) and proficiency test 2 (PT2). PT1 was designed as the previous tests provided by the EURL, to primarily assess the ability of participating laboratories to identify the viruses: VHS, IHN and epizootic haematopoietic necrosis virus (EHNV). PT2 was a new developed proficiency test with the aim of assessing the ability of participating laboratories to identify the viruses: Infectious salmon anemia virus (ISAV) and *koi herpes virus* **(**KHV). 38 National Reference Laboratories (NRLs) participated in PT1 and 36 NRLs in PT2. The tests were shipped from the EURL in the end of September 2010.

**PT1** contained five coded ampoules (I-V). The ampoules contained VHSV genotype Ia, IHNV genogroup M, EHNV, European catfish virus (ECV) and spring viraemia of carp virus (SVCV). The test was designed to primarily assess the ability to identify VHSV, IHNV and ENHV (all listed in Council Directive 2006/88/EC), if present in the ampoules, bearing in mind that the test ampoules also could contain other viruses (e.g. other fish rhabdoviruses, ranaviruses, or birnaviruses). In addition the participants were asked to quantify the viruses by titration in order to assess the susceptibility of their fish cell cultures for virus infection in the respective laboratories. 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 monolayered cell cultures followed by e.g. neutralisation test, immunofluorescence, ELISA or PCR. If ranaviruses should be present in any of the ampoules, it was mandatory to perform a sequence analysis of the isolate in order to determine if the isolate is EHNV and it was recommended to follow the procedures as 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 that were used for genotyping of isolates.

**PT2** contained five coded ampoules (VI-X). The ampoules contained ISAV and KHV. Furthermore, one ampoule did not contain any virus, only medium. It was decided at the 14$^{th}$ Annual Meeting of the NRLs for Fish Diseases in Aarhus 26-28 May 2010, that testing for ISAV and KHV for the first time should be included in the yearly proficiency test. PT2 was designed to primarily assess the ability of participating laboratories to identify ISAV and KHV if present in the ampoules, bearing in mind that the test ampoules also could contain other viruses. Participants were expected to use their normal PCR or real-time PCR methods for detection of KHV and their normal RT-PCR or real-time RT-PCR methods for detection of ISAV. It was not mandatory to grow KHV and ISAV on cell cultures in this test, but the viruses has not been inactivated and should thus be viable and possible to amplify in cell cultures.

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

## Outcome of Inter-laboratory Proficiency Test 2010

### Proficiency test 1, PT1

Identification of content

17 laboratories correctly identified all viruses in all ampoules.
Furthermore, one laboratory was obliged to identify only VHSV, IHNV and SVCV and did that correct.

Ampoule I – EHNV

- 24 of 37 laboratories correctly identified EHNV.

Ampoule II - IHNV

- 36 of 38 laboratories correctly identified IHNV.

Ampoule III – Ranavirus, ECV (or ESV), not EHNV

- 24 of 37 laboratories correctly identified Ranavirus but not EHNV.

Ampoule IV – SCVC

- 35 of 38 laboratories correctly identified SVCV.

Ampoule V – VHSV

- 33 of 38 laboratories correctly identified VHSV.

### PT1 Conclusion

The inter-laboratory proficiency test 2010 was conducted without major constrains. Most parcels were delivered by the shipping companies within 3 days after submission. It was, however, unfortunate that one parcel made up to 13 days before delivered to the laboratory because of to delay in an Airline “backlog” and that another parcel made op to 22 days before delivering to the laboratory, primarily due to border controls.

In 2009 EHNV was included in the proficiency test for the first time and 28 participants were able to correctly identify the virus. This year EHNV was included again and so was the European Catfish Virus (ECV) that belongs to the ranavirus family. All the 24 laboratories performing sequencing of the isolate in ampoule I identified the virus correctly as being EHNV. 24 laboratories correctly identified the virus in ampoule III as ranavirus but not EHNV. However, 7 laboratories that performed sequencing of the ECV isolate in ampoule III identified the virus as EHNV although the submitted sequences in 6 cases were identical to ECV/ESV. We therefore recommended that laboratories carefully analyse their sequencing results when they identify ranavirus in order to rule out if the virus is the listed EHNV or not.

The EHNV in ampoule I was present in a relative low titre, which likely is the reason why 8 laboratories did not identify any virus in this ampoule. We thus recommended that participant subcultivate the samples as described in Commission Decision 2001/183/EC since subcultivation increases the possibilities of identifying low tittered viruses.

All titres submitted by participants for each cell line and ampoule, respectively are compared to each other in the report. In this way, the titres obtained by each laboratory are plotted in relation to the combined submitted data set and each participating laboratory should be able to compare the sensitivity of their cell lines to the sensitivity of those used by the other participating laboratories. We

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

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

**Proficiency test 2, PT2**

Identification of content

- 23 of 36 laboratories correctly identified all viruses in all five ampoules.

Ampoule VI – ISAV (high titer)

- 31 of 36 laboratories correctly identified ISAV.

Ampoule VII - KHV (high titer)

- 33 of 36 laboratories correctly identified KHV.

Ampoule VIII – medium (no virus)

- 31 of 36 laboratories correctly identified the sample negative for virus

Ampoule IX – ISAV (medium titer)

- 27 of 36 laboratories correctly identified ISAV.

Ampoule X – KHV (low titer)

- 29 laboratories correctly identified KHV.

**PT2 conclusion**

Considering that this was the first time that the EURL provided a proficiency test on ISAV and KHV identification, we find that most participants obtained satisfying results. All 33 laboratories performing KHV identification did correctly identify KHV in the ampoule (VI) containing high tittered KHV. All 31 laboratories performing ISAV identification, except two, did correctly identify ISAV in the ampoule (VII) containing high tittered ISAV.

Lowering the titre of the virus caused three additional laboratories to miss identification of KHV in the low tittered ampoule (X) and three additional laboratories to miss identification of ISAV in the low tittered ampoule (IX). A critical point in PCR based diagnostic tool is avoiding false negative results, e.g. because of low sensitivity of the diagnostic tool. To decrease the risk of having false negative results, it is always recommended that laboratories use the most sensitive tool available, validate the sensitivity of their diagnostic tools and use proper controls.

One ampoule (VII) containing no virus was included in the test. 34 out of 31 laboratories correctly identified that the ampoule was negative for virus. Three laboratories identified KHV in this ampoule, probably due to cross contamination problems. False positive results is a common critical problem in PCR based diagnostics as PCR or RT-PCR can detect very few copies of DNA or RNA, respectively. Therefore, it is extremely important to minimise the risk of cross contaminations. This can be done by optimising the workflow in the laboratory as

e.g. described in the "Report of the workshop "KHV PCR diagnosis and surveillance" 12-13 November 2009, Central Veterinary Institute, Lelystad, The Netherlands". Other ways to minimise the risk of obtaining false positive results is to consider not using nested PCR tools and by using positive controls that can be discriminated from true pathogenic signals.

Many laboratories performed sequencing of ISAV and KHV isolates. However, we did not described according to which notification the genotype of viruses should be performed. In future tests we will clarify this better.

The results will be further presented and discussed at the 15th Annual Meeting of National Reference Laboratories for Fish Diseases to be held 26-27 May 2010 in Århus, Denmark.

*The full report is in Annex 4.*

***16. Establish diagnostic methods for diagnosis of EUS and assess the possibilities for including Aphanomycis invadans in proficiency test in future.***

## Establish diagnostic methods for diagnosis of EUS

In March 2010 two colleagues from the EURL visited the OIE reference laboratory for EUS in Bangkok Thailand for two weeks. The aim of this visit was to be trained in performing diagnostic methods for diagnosis of EUS with the purpose of setting the diagnostic assays up in the laboratories of the EURL. From this visit two strains were brought to the EURL laboratories. Unfortunately one strain could not grow and the other was not *A. Invadans*. Subsequently it turned out that it was not easy to obtain *A. Invadans* strains as it seems like only two are currently present W.W. These two strains have arrived at the EURL, donated by dr. Hatai form Japan and by the OIE reference laboratory for EUS, respectively.
We have implemented diagnostic methods for EUS like storage and cultivation of *A. Invadans*, sporulation, collection of spores, and identification of the fungus by morphology of the spores and currently we are in the process of validation two PCR assays used for detection of *A. Invadans*. We will set up infection trials and finish the diagnostic manuals in 2011.
Because of the delay in obtaining *A. Invadans* strains, the possibility for including *A. Invadans* in future proficiency tests will be made in 2011.

***17. Facilitate and provide training in laboratory diagnosis.***

## Training, missions and scientific collaboration

The EURL have decided that they will offer a yearly training course in diagnostic techniques for identification of listed fish diseases. The course will primarily be for NRL staff. The course will cover training in several diagnostic topics – e.g. PCR, cell culture, antibody based diagnostics - the topics depends on what participants ask for. Therefore other topics may also be offered and it is possible that one or more topics are not offered because of lack of interest. Participants do not have to follow all topics but can choose parts of the program. The course was announced for the first time at the 14th Annual Meeting of the NRLs for fish diseases and the course took place in the laboratories of the EURL in Aarhus in the period between 24 January and 4 February (week 4-5) 2011. Many request for training was postponed to take place at this yearly course.

However, the following colleagues visited the institute during 2010 for scientific meetings, project collaboration or training:

| | |
|---|---|
| Dr. Maria Forlenza, Wageningen University in connection to the integrated EU project IMAQUANIM | 27-30th January |
| Dr. Geert Wiegertjes, Wageningen University in connection to the integrated EU project IMAQUANIM | 27-30th January |
| Takafumi Ito, National Research Institute of Aquaculture, Japan | 4-9th September |
| Study visit on fish health management, diagnostics and research in fish pathology for a delegation from Chile: | 17th September |
| M.Sc. Dennis Bela-Ong, University of the Philippines, Manila, Philippines in connection to the collaborative research project "Co-evolutionary genomics of fish resistance and virulence in an epidemic virus" | 10-11th November |
| M.Sc. Ashish Mittal, Indian Institute of Technology (IIT), Roorkee, India in connection to the collaborative research project "Co-evolutionary genomics of fish resistance and virulence in an epidemic virus" | 17-18th November |

Furthermore, two colleagues from the EURL accomplished visits to the NRLs for fish diseases of Croatia and Greece. These visits were conducted because of requests from the respective laboratories and served as on-site training. For reports from these two visits, please see annex 5 and 6.

***PhD students:***

- M.Sc. Anders Stegmann has been enrolled as PhD student at DTU-VET in association to the ongoing project "Identification of virulence markers in marine VHS virus and use in diagnostics for aquaculture", which is funded by the Danish Research Council. Supervisor: Katja Einer-Jensen, DTU.
- M.Sc. Sekar Larashati, Bandung Institute of Technology, Indonesia. Ph.D. study at DTU-VET Århus in the field of "delivering small RNAs to fish" from November 1st 2009 to October 31st 2012. Supervisor: Niels Lorenzen, DTU; Co-supervisor: Brian Dall Schyth, DTU
- Anna Amanda Schönherz has been enrolled as 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; Co-supervisor: Katja Einer-Jensen, DTU.

***Master students***

- Lasse Bøgelund Juel Kristensen finished his Master study at Aarhus University in collaboration with DTU-Vet Århus. The study was associated with ongoing research activities in the field of small regulatory RNAs. Title of thesis: "MicroRNA expression during viral infection". Supervisor: Finn Skou Pedersen, Aarhus University; Co-supervisor: Brian Dall Schyth, DTU.
- Mikkel Black Christensen finished his Master study at Aarhus University in collaboration with DTU-Vet Århus. The study was associated with ongoing research activities in the field of fish immunology. Title of thesis: "Initiation of the immune response to a protective DNA vaccine in rainbow trout at different temperatures": Supervisor: Finn Skou Pedersen, Aarhus University; Co-supervisor: Jesper Schou Rasmussen, DTU

***18 . Attending missions, international meetings and conferences. Missions will focus on NRLs where on-site communication would be beneficial. And to reference laboratories on listed exotic and non-exotic fish diseases in order to be updated on diagnostic methods.***

## 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. Missions are described under 17. 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 activities in 2010:

**Participation and presentations at international conferences and meetings**

*Annual Meeting of the National Reference Laboratories for fish Disease. Aarhus* and Workshop on Use of Diagnostic kits for the Detection of Fish Diseases, *25th-28th May, 2010*.

- **Niels Jørgen Olesen**. Use of diagnostic kits and reagents in the European NRLs, presentation of data from the "survey and diagnosis" questionnaire 2009
- **Niels Jørgen Olesen**. Diagnostic tools: Development and validation of tests for detection of viral fish diseases.
- **Niels Jørgen Olesen**. Overview of the disease situation and surveillance in Europe in 2009.
- **Niels Jørgen Olesen**. The new EU manuals on sampling and diagnostic procedures and the role of the www.CRL-fish.eu web
- **Helle Frank Skall**. Diagnostic procedures for VHS, IHN, and EHN.
- **Søren Peter Jonstrup** . A novel Real-time PCR assays detecting all VHSV genotypes.
- **Søren Peter Jonstrup**. The Fish Pathogen Database.
- **Torsten Boutrup**. Presentation of the Network of Animal Disease Infectiology and Research Facilities - NADIR
- **BD Schyth.** MicroRNA regulation as a future diagnostic tool.
- **Søren Kahns**. Results and outcome of the Inter-Laboratory Proficiency Test 2009

*Annual Meeting of the National Reference Laboratories for Mollusc Disease IFREMER, Nantes, 23rd-24th March:*

- **N. J. Olesen*, S. Kahns, S.P. Jonstrup, H.F. Skall** (2010) When geographic information meets molecular data. s ,
- **N.J.Olesen** (2010) Fish farm categorisation in some EU Member states.

*Annual Meeting of the National Reference Laboratories for Crustacean Diseases, CEFAS, Weymouth, England 12th-14th October 2010*

- **T. S. Boutrup**. Presentation of the known distribution of aquaculture regarding crustaceans in Denmark, and known disease problems in wild and farmed crustaceans in Denmark.

*NADIR Meeting "Characterization of animal lines" Lodi, Italy, 15th-16th February 2010*

- **T. S. Boutrup, N. J. Olesen.** Presentation on work progress and exchange of fish lines within the NADIR project.

*NADIR First Annual Meeting Paris, France 20th-22th November 2010*

- **T. S. Boutrup, N. J. Olesen**. Presentation of work progress within the workpackages in the NADIR project. Exchange of fish lines, infection trials performed and planned.

*8th International Symposium on Viruses of Lower Vertebrates, Santiago de Compostela, Spain, April 26-29:*

- **Olesen, N.J , S. Kahns, S.P. Jonstrup, H.F. Skall** (2010) Diagnostic tools: Development and validation of tests for detection of viral diseases.
- Ito, T, J. Kurita, M. Sano, **H.Frank Skall, N. Lorenzen, N. J. Olesen** (2010) Assessment of the Epitope Specificity of Monoclonal Antibodies that can Discriminate between the Various Genotypes of VHSV
- **A. Stegmann*, N. Lorenzen**, M. Bremont and **K. Einer-Jensen**. IDENTIFICATION OF GENETIC VIRULENCE MARKERS IN VHS VIRUS (Poster)

*International Symposium on Infectious Salmon Anemia, September 13–15, 2010, Oslo, Norway*

- **Niels Jørgen Olesen** (2010) Classification of fish farms and surveillance of ISA from an EU perspective.

*RNAi2010, Oxford, St. Hildas College, 16th-18th March, 2010*

- **BD Schyth***, JB Bramsen, J Kjems, J Wengel and **N Lorenzen.** In vivo screening of backbone modified siRNAs for their ability to induce interferon based off-target effects. Oral presentation at

*EPIZONE Theme 5 Annual Meeting "Intervention Strategies", Copenhagen, 3-5th November 2010.*

- **BD Schyth**, SAH Jalali, **L Bøgelund Kristensen and Ns Lorenzen**. Viral diseases of fish and a possible role for small regulatory RNAs in the antiviral defence.

*8th International Symposium on Viruses of Lower Vertebrates, April 26-29, 2010, Santiago de Compostela, Spain*

- **K Einer-Jensen*, E Lorenzen, JS Rasmussen and N Lorenzen**. N-LINKED GLYCANS ON THE VIRAL GLYCOPROTEIN ARE NOT REQUIRED FOR INDUCTION OF PROTECTIVE IMMUNITY TO VHSV WHEN DELIVERED AS A DNA VACCINE,
- **N Lorenzen*, E Lorenzen, JS Rasmussen and K Einer-Jensen**. Temperature effects on vaccine induced immunity to fish rhabdoviruses, 8th International Symposium on Viruses of Lower Vertebrates, (Invited speaker).

*First Symposium of the European Organisation of Fish Immunology (EOFFI), May 23-27 2010, Viterbo, Italy*

- **K Einer-Jensen*, JS Rasmussen**, B Collet, C Secombes, **E Lorenzen and Lorenzen**. PROTECTION AGAINST VIRAL HAEMORRHAGIC SEPTICEMIA VIRUS (VHSV) in rainbow trout using a DNA VACCINE with MX1 PROMOTOR controlled expression of the viral G protein.
- **N Lorenzen*** Antiviral immunity – lessens to be learned from DNA vaccines Oral presentation, First Eoffi Symposium (Invited speaker).
- Buchmann, K., **Lorenzen, N**., Martin, S., Secombes, C. & Fischer, U. Temperature-dependent immunity in trout towards viral, bacterial and parasitic pathogens. (Poster)

- **Niels Lorenzen**, Øystein Evensen, Roy A. Dalmo, Bertrand Collet, Uwe Fischer, Giuseppe Scapigliati, Maria C. Alvarez, Victor Mulero, Lluis Tort, Beatriz Novoa, Philipe Roch, Paola Venier, Giuseppe Bovo, Chris Secombes, Geert Wiegertjes, Tomás Vesely, Kurt Buchmann, Bjørn Brudeseth, Alexandra Adams, Niels Hjermitslev. Improved immunity of aquacultured animals (IMAQUANIM) - An integrated research project in the EC FP6 programme. (Poster)
- **JS Rasmussen***, Christensen M. B., **Einer-Jensen K., Lorenzen E., Lorenzen N**. Expression profiling of immune response genes in rainbow trout following DNA vaccination and VHS virus infection, (Poster)
- Wiegertjes, G.F., Forlenza, M., Veselý, T., Pokorová, D., **Lorenzen, N**. Key strategies and molecules for improved immunity to infectious pathogens in carp. (Poster)

*European Aquaculture Society (EAS) in October 2010, Porto, Portugal*

- B. Novoa P. Roch, H. Li, M.G. Parisi, M. Toubiana, P. Venier, A. Pallavicini, G. Lanfranchi, L. Varotto, U. Rosani, M.M. Costa, C. Gestal, S. Dios, **N. Lorenzen**, A. Figueras*. Bivalve genomics used for characterization of the immune defense against microbial infections in mussel.
- Buchmann, K., **Lorenzen, N.,** Martin, S., Secombes, C. & Fischer, U. Temperature-dependent immunity in trout towards viral, bacterial and parasitic pathogens. (Poster)
- **Niels Lorenzen**, Øystein Evensen, Roy A. Dalmo, Bertrand Collet, Uwe Fischer, Giuseppe Scapigliati, Maria C. Alvarez, Victor Mulero, Lluis Tort, Beatriz Novoa, Philipe Roch, Paola Venier, Giuseppe Bovo, Chris Secombes, Geert Wiegertjes, Tomás Vesely, Kurt Buchmann, Bjørn Brudeseth, Alexandra Adams, Niels Hjermitslev. Improved immunity of aquacultured animals (IMAQUANIM) - An integrated research project in the EC FP6 programme. (Poster)
- **JS Rasmussen***, Christensen M. B., **Einer-Jensen K., Lorenzen E., Lorenzen N**. Expression profiling of immune response genes in rainbow trout following DNA vaccination and VHS virus infection, (Poster)
- Wiegertjes, G.F., Forlenza, M., Veselý, T., Pokorová, D., **Lorenzen, N.** Key strategies and molecules for improved immunity to infectious pathogens in carp. (Poster)

*Dafinet / Scofda meeting 9-10 november 2010 KU-Life*

- **E Lorenzen*, JS Rasmussen, TE Kjær, K Einer-Jensen**, K Engell-Sørensen, I Dalsgaard, J Nylén, K Buchmann and **N Lorenzen**. Experimental vaccination of small turbot against bacterial and viral pathogens

*The 4th Annual Meeting EPIZONE, Bridges to the Future, 2010, Saint-Malo, France, from 7-10 June, 2010*

- **BD Schyth* and N Lorenzen**. Using small interfering RNAs (siRNAs) to combat a fish pathogenic virus.
- **Jonstrup, Søren Peter ; Kahns, Søren ; Skall, Helle Frank ; Olesen, Niels Jørgen (2010)** Development of a real-time-RT-PCR suitable for the detection of Viral Haemorrhagic Septicaemia Virus (VHSV). Presented at. (Poster)
- **Jonstrup, Søren Peter** ; Jones, T. Gray **; Olesen, Niels Jørgen (2010).** Fishpathogens.eu: A database based on freeware suitabale for storing isolate and sequence data of pathogens. Presented at. (Poster)

**Participation in international scientific collaborative studies**

- The section is partner in the FP7 EU project: **The Network of Animal Disease Infectiology Research Facilities, NADIR**, that 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, which have to respond to upgraded ethical and safety regulations whilst providing reliable answers in term of physiopathology for emerging infectious diseases (diagnosis, transmission conditions, risk analysis, therapeutic targets) or for vaccines and therapeutic trials. 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.

- 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) and conducted a number of research activities in relation to VHS, e.g. molecular tracing of VHSV, to development of serological tests for KHV, VHS and IHN, and for assessing diagnostic methods for KHV.

- 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. Further information is available at the public project website www.IMAQUANIM.eu where a full list of scientific publications is available along with an informative brochure addressing readers outside the scientific field.

- 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.

- 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 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 trout's 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 Aarhus those are Aarhus University, University of Victoria in Canada and the University of Washington, USA.

- **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.

- Furthermore, we function as **OIE reference laboratory for VHS**. In this function we are responsible for updating the OIE diagnostic manuals for VHS and for validation the corresponding diagnostic methods.

***Scientific publications in peer-reviewed journals***

- Bohle, H., **Lorenzen, N. and Schyth, B. D.** (2010) Species specific inhibition of viral replication using dicer substrate siRNAs (DsiRNAs) targeting the viral nucleoprotein of the fish pathogenic rhabdovirus viral haemorrhagic

septicaemia virus (VHSV) Submitted for Antiviral Research

- **Lorenzen E**., B.E.Brudeseth, T.Wiklund, **N.Lorenzen** (2010) Immersion exposure of rainbow trout (Oncorhynchus mykiss) fry to wildtype Flavobacterium psychrophilum induces no mortality, but protects against later intraperitoneal challenge Fish and Shellfish immunology 28:440-444 (2010)
- **Lorenzen E, Einer-Jensen K, Rasmussen JS,** Kjaer TE, Collet B, Secombes CJ, **Lorenzen N.** (2009) The protective mechanisms induced by a fish rhabdovirus DNA vaccine depend on temperature Vaccine. 2009 Jun 12;27(29):3870-80. Epub 2009 Apr 23
- **Schyth, B. D**., Bramsen, J. B., Pakula, M. M., Wengel, J., Kjems, J. and **Lorenzen, N** (2010) In vivo screening of locked nucleicacid modified siRNAs in a fish model indicates a possible role for duplex stability in triggering non-specific antiviral effects.
- Ito T, **Olesen NJ, Skall HF**, Sano M, Kurita J, Nakajima K & Iida T (2010) Development of a monoclonal antibody against viral haemorrhagic septicaemia virus (VHSV) genotype IVa. *Diseases of Aquatic Organisms* **89**, 17-27.
- VHSV Expert Panel and Working Group1* (2010) Viral hemorrhagic septicemia virus (VHSV IVb) risk factors and association measures derived by expert panel K.H. Amos, R.S. Bakal, M.J. Blair, D.A. Bouchard, P.R. Bowser, P.G. Egrie, S.K. Ellis, M. Faisal, K.A. Garver, C. Giray, A.E. Goodwin, N.L. House, M.J. Kebus, K.C. Klotins, S.E. LaPatra, G.D. Marty, P.L. Merrill, A.D. Noyes, N.J. Olesen, S.M. Saksida, M. Snow, S. St-Hilaire, F.C. Uhland, P. Vennerstrom, B.A. Wagner, J.V. Warg, G.E. Whelan and J.R. Winton. Preventive Veterinary Medicine 94, 128-139.
- T. Ito, **N. J. Olesen, H. F. Skall**, M. Sano, J. Kurita, K. Nakajima, T. Iida (2010) Development of a monoclonal antibody against viral haemorrhagic septicaemia Virus (VHSV) Genotype IVa. *Diseases of Aquatic Organisms , 89: 17-27*
- **S P Jonstrup**, H Schuetze, G Kurath, T Gray, B Bang Jensen, **N J Olesen. (2010)** An isolate and sequence database of infectious haematopoietic necrosis virus (IHNV). Journal of Fish Diseases
- Ellen Ariel, Riikka Holopainen, **Niels Jørgen Olesen**, Hannele Tapiovaara (2010) Comparative study of ranavirus isolates from cod (Gadus morhua) and turbot (Psetta maxima) with reference to other ranaviruses.. Arch Virol 155:1261–1271
- Toplak, I., Peter Hostnik, Danijela Ritharic, **Niels Jørgen Olesen, Helle Frank Skall**, Vlasta Jencic (2010) First isolation and genotyping of viruses from recent outbreaks of viral haemorrhagic septicaemia (VHS) in Slovenia. *Diseases of Aquatic Organisms , 92: 21-29*

**Other communications**

- Project brochure (44 pages): Improved Immunity of Aquacultured Animals (IMAQUANIM): Highlights from the integrated research project IMAQUANIM supported by the European Commission‘s FP6 programme Editors: Geert Wiegertjes, **Katja Einer-Jensen & Niels Lorenzen**. Available freely at www.IMAQUANIM.eu. An informative brochure addressing readers outside the scientific field.

- **Skall HF, Lassen-Nielsen AM & Olesen NJ** (2010) Nyt fra Veterinærinstituttet: Fund af potentielt humanpatogene bakterier i fisk. *Dansk Veterinærtidsskrift* 19, 24

# 14th Annual Meeting of the National Reference Laboratories for Fish Diseases and Workshop on Use of Diagnostic kits for the Detection of Fish Diseases

**Aarhus, Denmark**
**May 26-28, 2010**


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

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# Indhold

## *Introduction and short summary*

26-28 May 2010 the 14th annual meeting of the National Reference Laboratories for fish diseases was held back-to-back with a Workshop on "Use of Diagnostic Kits for Detection of Fish Diseases". A total of 65 participants from 35 countries attended over the three day period. There were five sessions with a total of 41 presentations, 9 of which were given by invited speakers. The workshop and meeting was held at rented nearby facilities of Aarhus University, as the premises at our institute in Aarhus are too small for the number of participants.

The workshop on "Use of Diagnostic Kits for Detection of Fish Diseases" was held the day before the Annual Meeting. Many different kits are available for detection of fish pathogens and in the future more will come. Besides providing a presentation of some of the kits that are currently available on the market, the aim of the workshop was to discuss how laboratories can ensure that commercial as well as own diagnostic kits are properly validated. The workshop started with a presentation on the outcome of the survey and diagnosis questionnaire on what kits are used by the NRLs for detection of fish pathogens. Subsequently, a talk on general needs on development and validation of diagnostic tools were presented. The procedures for tests of different commercial antibody based kits were described in four talks by four speakers from different laboratories/companies. The last two sessions were focused on molecular biological tools. A DNA-array based diagnostic tool followed by the LAMP originated kits was presented. The workshop was ended by a plenum discussion on how laboratories should ensure properly validation of used diagnostic kits. Discussions continued in the evening where all participants were invited to a drinks reception at the National Veterinary Institute.

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 presented new findings from their home countries. Initially an overview of the disease situation and surveillance in Europe 2009 were provided on the basis of the results from the survey and diagnosis questionnaire. Scotland UK updated on the situation after the outbreak of ISA in 2009. Results of a questionnaire send to 12 fish-pathology experts was the basis of a presentation on old and emerging diseases from the Mediterranean aquaculture. Subsequently a talk on multiple infected fish in a Swiss fish farm was presented, followed by a talk on the VHSV eradication program in Denmark. Later in this session presentations about pancreas disease and BKD from Norway and Red Mark Syndrome and the Rosette Agent were given. The session was ended by a talk on bio-security risk associated with EUS and Iridovirus in ornamental fish.

The session on technical issues related to sampling and diagnosis were divided into two parts. The first session focussed on the new EU manuals on sampling and diagnostic procedures that will be uploaded on the www.crl-fish.eu web page later this year. Here diagnostic procedures for detection of the listed non-exotic fish pathogens VHSV, IHNV, ISAV and KHV as well as the exotic EHNV and EUS were described.
The last part of this section focussed on many different issues. It was initiated with two talks on un-explained increased mortality: how to deal with it from a legislative and a practical perspective, respectively. Later talks were focussed on sensitivity and specificity of test procedures for BKD, non-lethal sampling and KHV detection in latent infected koi carps, identification of a possible novel Cyprinid herpesvirus 3 variant strain and a novel real-time PCR assay to detect VHSV.

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

The last day was opened by an update session on scientific research. At this session, presentations were given on progress in the development in sero-neutralisation test for detection of antibodies against KHV in carp, on vertical transmission of pancreas disease (PD) and infectious salmon anaemia (ISA), respectively, and on the findings that heart and skeletal muscle inflammation (HSMI) most likely is caused by a reovirus. Subsequently two projects were presented: the Club 5 project on EUS diagnostic methods and the NADIR project on access to infection facilities. The session was closed with a presentation on 1) perch rhabdovirus infection in perch and pike-perch and 2) the putative use of miRNA in future diagnostics.

The annual meeting ended with the traditional update from the CRL. The results of the proficiency test 2009 were presented. A report from year 2009 was given, a year with focus on training of laboratories and thoughts and considerations about preparation of the new EU diagnostic manuals. Furthermore, proposals on the CRL work plans for 2011 were discussed.

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

We would once again like to thank all the presenters for their great contribution without which the meeting would not have been a success.

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

The meeting next year is tentatively planned to be in week 21, most likely from 24-26 May 2011 but more details will follow.

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

Århus, 28 June 2010

Niels Jørgen Olesen and Søren Kahns

## *Programme*

## *Wednesday 26 May*
## *Workshop on Use of Diagnostic Kits for the Detection of Fish Diseases*

| | |
|---|---|
| *9:00 – 10:00* | **Registration, welcome address and announcements**<br>Chair: *Giuseppe Bovo* |
| *10:00 – 10:20* | Welcome Address and announcements - *Søren Kahns and Niels Jørgen Olesen* |
| *10:20 – 10:50* | Use of diagnostic kits and reagents in the European NRLs, presentation of data from the "survey and diagnosis" questionnaire 2009 - *Niels Jørgen Olesen* |
| *10:50 – 11.20* | Diagnostic tools: Development and validation of tests for detection of viral fish diseases – *Niels Jørgen Olesen* |
| *11:20 – 11:50* | Development and Validation of a Lateral Flow Kit to detect ISAV - *Alexandra Adams* |
| *11:50 – 13:20* | *Lunch Break + Presentation of products by companies* |
| *13:20 – 13:40* | Test-Line kits for the detection of fish pathogens - *Tomas Vesely* |
| *13:40 – 14:00* | Development and assessment of reagents and diagnostic kits from BioX used in the NRL for Fish Diseases in Germany - *Sven M. Bergmann* |
| *14:00 – 14:20* | Specificity and sensitivity testing of diagnostic kits from commercial companies at the CRL - *Helle Frank Skall* |
| *14:20 - 15:00* | *Coffee Break + Presentation of products by companies*<br>*Chair: Søren Kahns* |
| *15:00 – 15:30* | DNA-array based diagnostics: Applications in fish disease diagnosis – *Ingeborg Frans & Bart Lievens* |
| *15:30 – 15:50* | Experiences from using LAMP originated kits in KHV diagnostics in comparison to other tools - *Sven M. Bergmann* |
| *15:50 – 16:20* | Plenum discussion |
| *16:20 – 17:00* | Presentation of products by companies |
| *17.00 – 18.30* | *Drinks reception* |

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## *Thursday 27 May - Annual Meeting of the National Reference Laboratories*

| | |
|---|---|
| *8:30 – 9:00* | **Registration, welcome address and announcements**<br>*Niels Jørgen Olesen and Søren Kahns* |
| **SESSION I:** | **Update on important fish diseases in Europe and their control**<br>Chair - *Brit Hjeltnes* |
| *9:00 – 9:30* | Overview of the disease situation and surveillance in Europe in 2009 – *Niels Jørgen Olesen* |
| *9:30 – 9:50* | An update on the Infectious Salmon Anaemia (ISA) situation in Scotland – *Eann Munro* |
| *9:50 – 10:10* | Old and emerging diseases in Mediterranean aquaculture – *Giuseppe Bovo* |

| | |
|---|---|
| *10:10 – 10:25* | Detection of simultaneous multiple viral infection in a Swiss fish farm – *Thomas Wahli* |
| *10:25 – 10:50* | *Coffee Break* |
| *10:50 – 11:10* | Eradication of VHS from Denmark – surveillance and control – *Henrik Korsholm* |
| *11:10 -11:30* | Pancreas disease (PD; an update on the disease situation and control measures in Norway - *Torunn Taksdal* |
| *11:30- 11:45* | BKD - recent outbreaks in Norway - *Hege Hellberg* |
| *11:45-12:05* | Red mark syndrome – a diagnostic challenge – *Steven Feist* |
| *12:05 - 12:20* | Characterisation of a Rosette Agent (*Sphaerothecum destruens*) from sunbleak (*Leucaspius delineatus*) in the UK – *Richard Paley* |
| *12:20 - 12:35* | Biosecurity risks associated with EUS and Iridovirus in ornamental fish - *Mansour El-Matbouli* |
| *12:35 – 13:30* | *Lunch Break* |

## *Thursday 27 May - Annual Meeting of the National Reference Laboratories*

## SESSION II: Technical issues related to sampling and diagnosis

Chair – Olga Haenen

| | |
|---|---|
| *13.30-13.50* | The new EU manuals on sampling and diagnostic procedures and the role of the www.CRL-fish.eu web page - *Niels Jørgen Olesen* |
| *13.50-14.10* | Diagnostic procedures for VHS, IHN, and EHN – *Helle Frank Skall* |
| *14.10-14.30* | Molecular diagnostic procedures for KHV - conclusions and recommendations from EPIZONE workshop on KHV – *Marc Engelsma* |
| *14.30-14.50* | Standard Diagnostic Procedures for ISA testing used at Marine Laboratory, Scotland – *Eann Munro* |
| *14.50-15.10* | Diagnostic procedures for EUS – *Birgit Oidtmann* |
| *15.10-15.40* | *Coffee break* |
| *15:40 – 16:00* | Council Directive 2006/88/EC: The concept of un-explained increased mortality and how to deal with it from a legislative perspective – *Sigrid Cabot* |
| *16:00 – 16:20* | Un-explained increased mortality in fish farming. A practical approach on how to deal with it. Experiences from Norway – *Hege Hellberg* |
| *16:20 – 16:40* | Diagnostic sensitivity and specificity of test procedures for Renibacterium salmoninarum in rainbow trout - *Malcolm Hall* |
| *16:40 – 17:00* | Non-lethal sampling and virus detection in fish latently infected with koi herpesvirus (KHV) – *Sven M. Bergmann* |
| *17:00 – 17:15* | Cyprinid herpesvirus 3: To be, or not to be – *Marc Engelsma* |
| *17:15 – 17:30* | A novel Real-time PCR assays detecting all VHSV genotypes – *Søren Peter Jonstrup* |
| *19:00* | *Banquet dinner* |

## Friday 28 May

## Annual Meeting of the National Reference Laboratories

### Scientific research update

### SESSION III

Chair – *Hege Hellberg*

| | |
|---|---|
| *9:00 – 9:20* | Progress in the development of seroneutralisation test (SNT) for detection of Cyprinid herpesvirus 3 (CyHV3) antibodies in carp, *Cyprinus carpio* – *Laurent Bigarré* |
| *9:20 – 9:40* | Vertical transmission - PD and ISA – *Brit Hjeltnes* |
| *9:40 – 10:00* | Heart and Skeletal Muscle Inflamation (HSMI) – an emerging disease in salmon, new results indicating a reovirus – *Irene Ørpetveit* |
| *10:00 – 10:15* | Club 5 project: Epizootic ulcerative syndrome. Development and implementation of diagnostic methods – *Olga Haenen* |
| *10:15 – 10:35* | Presentation of the Network of Animal Disease Infectiology and Research Facilities - NADIR – *Torsten Boutrup* |
| *10:35 – 10:55* | Perch Rhabdovirus infection in farmed pike-perch and perch – an emerging disease - *Laurent Bigarré* |
| *10:55 – 11:15* | microRNA regulation as a future diagnostic tool – *Brian Dall Schyth* |
| *11:15 – 11:35* | *Coffee break* |

### SESSION IV: Update from the CRL

Chair – *Niels Jørgen Olesen*

| | |
|---|---|
| *11:35 – 11:50* | CRL achievements in 2009 – *Søren Kahns* |
| *11:50 – 12:05* | The Fish Pathogen Database – *Søren Peter Jonstrup* |
| *12:05 – 12:20* | CRL workplan for 2010 – ideas and plans for 2011 - *Niels Jørgen Olesen* |
| *12:20 – 12:40* | Results and outcome of the Inter-Laboratory Proficiency Test 2009 – *Søren Kahns* |
| *12:40 – 13:00* | Next meeting and end of 14th Annual Meeting - *Niels Jørgen Olesen* *Sandwiches and goodbyes* |

# *Workshop on Use of Diagnostic Kits for the Detection of Fish Diseases*

## Use of diagnostic kits and reagents in the European NRLs

**Niels Jørgen Olesen** and Nicole Nicolajsen
*DTU.VET. Aarhus, Denmark*

Abstract:
This year the questionnaire on Surveillance and Diagnosis of fish diseases in Europe in 2009 included a part requesting data on the use of commercial diagnostic kits in the respective NRL´s and regional laboratories. Based on the replies an overview of the use of kits in the NRL is given.
For diagnosis of VHS and IHN, kits from the companies Bio-X and Test-line are used by a majority of the laboratories using commercial diagnostic kits. For KHV and partly ISA the marked is more spread.
The presentation will in short describe available reagent and kits for detection of some of the listed fish diseases and their use in the NRL´s.

Questions

**Athanasios Prapas:** Is it possible to make revisions to the protocol of a kit to make it work in the laboratory?

**Niels Jørgen Olesen:** Yes but alternative methods and reagents should always be tested and approved before use for diagnostic purpose.

**Olga Haenen:** During validation you test for robustness, and within this a kit should work even though slight chances are made.

**Niels Jørgen Olesen:** We encourage that users ask for documentation on how well the kit perform and how they were validated when you buy kits. The companies are not always presenting these data on their websites and it can therefore be hard to judge whether proper assessment of the tests has been performed.

**Alexandra Adams:** We (Aquatic Diagnostics) send these reports to people who ask for them.

**Olga Haenen:** From the questionnaire it cannot be derived how much people are using a specific kit and what their experience is.

**Niels Jørgen Olesen:** This we can hopefully discuss at this meeting.

**Niels Jørgen Olesen:** During proficiency tests many NRLs use commercial kits for identification of the pathogens and a lot of information from these tests are thus procured that companies might be able to benefit from in regards to providing information on how well their kit performs.

**Neil Ruane:** Will this presentation be available at the CRL website?

**Niels Jørgen Olesen:** Yes. (http://www.crl-fish.eu/Activities/survey_and_diagnosis.aspx)

**Neil Ruane:** I would like to hear more about experiences from laboratories having compared different kits against each other.

**Giuseppe Bovo:** There might be differences between batches of antibodies, kits etc.

**Niels Jørgen Olesen:** Yes, we have also experienced this. We now always test new batches against old ones.

**Giuseppe Bovo:** In the future we might ask which kits are used during the proficiency test?

**Niels Jørgen Olesen:** Good idea.

## Diagnostic tools: Development and validation of tests for detection of viral fish diseases

**N. J. Olesen[1]**, S. Kahns[1], S.P. Jonstrup[1], H.F. Skall[1]
[1]*National Veterinary Institute, Technical University of Denmark*

### Abstract:

The range of diagnostic tools for viruses causing diseases in fish farming is as large as for virology in warm-blooded animals. Development of diagnostic tools has been based on empirical data and knowledge from general virological methods. Looking for differences it is obvious that especially temperature sensitivity always must be taken into consideration when examining for viruses from poikilothermic animals. A significant advantage of working with viruses from cold blooded animals is the relatively easy access and use of permanent cell cultures, these cells are often robust and easy to handle and often have virus susceptibilities that easily match the most sensitive molecular tests. Cell culture based techniques thus still have a significant position in fish virology both for diagnostics, surveillance and research.
When developing a diagnostic test the first step is to determine the purpose of the test. Is it a tool for diagnosis of clinical cases, for justification of virus freedom, or for characterisation and use in e.g. molecular epidemiology? Demands for sensitivity and specificity highly depend on the answer to these questions, e.g. a test for diagnosis of disease in clinically sick fish demands a high specificity, whereas the sensitivity is of less importance. In contrast, for surveillance purposes in symptomless populations a high sensitivity is essential.

After the development of the test it is a prerequisite that the test is validated before taken into general use. This has often been abandoned due to high work load, the cost of validation, the impossibility to fulfil all requirements e.g. due to lacking access to reagents, isolates etc.

In 2004 an RT-PCR was recommended for detection of VHSV in the OIE Diagnostic Manual of Aquatic Animals. The procedure was included before a validation was finalised and it showed up that the recommended primers for VHSV may cross react with BF-2 cell fragments producing a fragment of a size similar to a VHSV fragment. Lacking knowledge of this phenomenon did contribute to the risk of false positive results. This may have grave consequences for the fish farm and the zone status. Due to this phenomenon a new RT-PCR has been included in the 2009 edition of the OIE manual. This new method has reduced the problem with false positives but did not eliminate them totally. Therefore continuous inclusion of the right controls is essential for the reliability of RT-PCR for VHSV detection, in this case non-infected or heterogonous infected cell culture material should act as negative controls. We are currently developing a real time PCR that is specific for VHSV. The validation of this technique include alignments with all published sequences and in-vitro testing against panels of more than 100 viruses representing all genotypes and various rhabdoviruses. In addition the test is validated against tissue material from infected and non infected fish of different species. Validation also include reproducibility, repeatability, efficiency and stability e.g. towards various kits, reagents etc. This validation resulted in a complete redesigning of the first candidate for a test, a test which already had passed intensive test regimes.
Principles of validation of diagnostic test is described focusing on important steps like robustness, repeatability, analytical and diagnostic sensitivity and specificity and reproducibility with examples given from the current validation process of a real-time RT-PCR in our laboratory.

### Questions

**Niels Jørgen Olesen:** Unfortunately there is not much prestige in validation and it is hard to get the necessary funding for this work. Maybe there could be advantages in working together with other NRLs or companies?
**Hege Hellberg:** Be careful about giving isolates etc. to companies. Later problems can arise where you are suddenly not allowed to use your own isolates.
**Niels Jørgen Olesen:** Yes you might need assistance from a lawyer if you want to cooperate with companies in these ways.

**Giuseppe Bovo:** It can be hard to find enough field material for validation.
**Niels Jørgen Olesen:** It is correct, it is especially difficult to obtain enough *A. invadans* (EUS) and EHNV isolates. It could be a task for OIE reference laboratories to have a broad panel of isolates that can be send out to people who need to assess and validate diagnostic tests..

**Richard Paley:** How often do you see unspecific bands in BF-2 cells of the VHSV PCR?
**Niels Jørgen Olesen:** Quite often and other laboratories have also reported the problem.
**Richard Paley:** We see it but only very seldom.
**Heike Schütze:** You can use a digestion enzyme to discriminate between correct band and false positive bands.
**Søren Kahns:** You may also sequence the product. We have done that but found no similar sequences in public databases.

## Development and Validation of a Lateral Flow Kit to detect ISAV

**Alexandra Adams** and Kim D Thompson
*Aquatic Diagnostics Ltd, Institute of Aquaculture, University of Stirling, Stirling, UK*
*E mail: alexandra.adams@stir.ac.uk*

**Abstract:**
Rapid diagnosis and immediate removal of infected fish are needed to implement effective control strategies during disease outbreaks. Lateral-flow immunoassays allow quick and sensitive detection of a pathogen, thus providing time to implement early control measures to avoid the spread of disease. This technology has many advantages over traditional immunoassays, in that the assay is simple to use, very rapid (with results in minutes rather than hours or days), low cost, and does not require skilled operators or expensive equipment. Evaluation of the results is performed by eye and total assay time is less than 15 min. In addition, the kits are stable at room temperature. These assets make the assay very suitable for on-site (or laboratory) sampling to detect pathogens.

A Lateral Flow Kit to detect infectious salmon anaemia virus (ISAV) was developed using two ISAV-specific monoclonal antibodies. Initial kit validation for optimisation and standardisation of reagents, and assay repeatability and reproducibility, were performed in house. Kits were then sent out to independent laboratories in Scotland, Norway and Canada for validation with known positive and negative samples and clinical field samples to evaluate diagnostic sensitivity and specificity. Comparisons were made between the performance of the Lateral Flow Kit and other methods of ISAV detection, i.e. virus isolation, indirect fluorescence antibody test (IFAT), reverse transcriptase (RT-PCR) and real time quantitative reverse transcriptase (qRT-PCR). Laboratory trials were very effective, with the Lateral Flow Kit detecting ISAV in 100% of ISAV-infected samples with no cross reactivity with other pathogens. Field validation data was equally robust: correct diagnoses were obtained in 100% of clinical ISAV cases with the Lateral Flow Kits. No false positives were detected.

The ISAV Lateral Flow Kit was shown to have the highest diagnostic specificity of all the methods used and a higher diagnostic sensitivity than IFAT. The method is easy and fast, and the kits are recommended for use on site to confirm clinical cases of ISAV. It would be useful to include this test in future ring testing for ISAV.

### Questions

**Alexandra Adams:** Companies also see it as very important that their kits are probably validated. It does not give a good reputation if kits that do not function properly are being sold.
**Sven M. Bergmann:** Is there a sterile immunity of ISAV or is there a latent infection?
**Eann Munro and Hege Hellberg:** This is not known.
**Eann Munro:** We demand 3 out of 3 PCR replicates to be positive for ISAV. It has been seen in a farm that 1 out of 3 was positive several weeks before an outbreak.
**Niels Jørgen Olesen:** A fish farmer will be able to use your ISAV lateral flow kit, but will they report outbreaks?
**Alexandra Adams:** The kit is designed for vets, but can of course be used by farmers, so this could be a problem as many farmers are not interested in knowing whether they have ISAV.
**Giuseppe Bovo:** Do you sell mostly to farmers or vets and what is the price?
**Alexandra Adams:** We have sold only few because ISO-certification is not yet ready. Vets won't buy until this is accomplished. Prices are around 10 pounds pr test.

## Test-Line kits for the detection of fish pathogens

Tomas Vesely

Abstract:

It has been reported that about a third of the economically valuable fish population die of some diseases every year. Diseases caused by viral agents significantly contribute to this situation. It is reflected in the EU legislation where four viral diseases are included in the list of non-exotic diseases. The OIE Code contains other three viral diseases of fish.
A rapid and exact diagnostic procedure is very important for the detection of causative agents of the diseases. Application of ELISA tests based on reactions of specific antibody-enzyme conjugates and visualized substrate-chromogen reactions is one possibility.

Regarding the nature of aquaculture in the Czech Republic and the epizootiological situation, three ELISA tests were developed in the early 1990s in collaboration between VRI and Test-Line diagnostics for detection of viral agents causing diseases of salmonid and cyprinid fish, namely for detection of infectious pancreatic necrosis virus (IPNV Ag ELISA), viral haemorrhagic septicaemia virus (VHSV Ag ELISA) and spring viraemia of carp virus (SVCV Ag ELISA).

Principle of the tests
Microtitre wells coated with specific antibodies to a virus are filled with tested samples, positive and negative control antigens. If present in the tested sample, viral antigens bind to antibodies. At the following incubation, rabbit antibodies to appropriate virus bind to the antigens. The binding of rabbit antibodies is detected in further incubation with conjugate solution (SwAR/IgGPx) and visualised by the reaction with substrate (TMB – Complete). After stopping the reaction by adding the Stopping solution, the colour intensity is measured using a photometer at 450 nm.

This ELISA kits are suitable for the demonstration of mentioned viruses in organ homogenates as well as in infected cell cultures. The sensitivity of the methods (approximately $10^3$ $TCID_{50}$ per 0.1 ml of the fluid examined) is satisfactory for routine examinations of field samples. Contamination with bacteria and fungi of samples taken from dead fish has no effect on the results of ELISA. Nevertheless, examination of fresh samples should be preferred.

Questions:

**Neil Ruane:** We have recently had problems with false IPN positives. It was due to the conjugate. Have others experienced problems?

**Eann Munro:** We have also had problems. We contacted Test-Line however they never replied. In my opinion the suggested cut-off value is too low for this kit.

**Giuseppe Bovo:** Is it possible to use the kits directly on fish samples?

**Tomas Vesely:** The kits were originally developed to be used directly on fish, but the current EU legislation demands growth on cells first.

## Development and assessment of reagents and diagnostic kits from BioX (Belgium) used in the NRL for Fish Diseases in Germany

**Sven M. Bergmann*** and Dieter Fichtner
*Friedrich-Loeffler-Institut (FLI), Federal Research Institute for Animal Health, Südufer 10, 17493 Greifswald-Insel Riems, Germany*

Abstract:
The German reference laboratory for fish diseases (NRL-F), located on the Isle of Riems, uses and assesses commercially available kits and monoclonal antibodies (MAbs) from BioX, Belgium, detecting agents of notifiable diseases such as viral haemorrhagic septicaemia virus (VHSV) or infectious haematopoietic necrosis virus (IHNV), but also agents of not notifiable diseases such infectious pancreas haematopoietic virus (IPNV) or spring viraemia of carp virus (SVCV).
Within the NRL-F mainly conjugated and non-conjugated MAbs recognizing IHNV, VHSV, IPNV and SVCV are used for indirect immunofluorescence assay (iFAT), respectively). The specificity, but also the feasibility of these MAbs is assessed with different virus isolates obtained from cell culture. For control reasons, different cell cultures, e.g. EPC, RTG-2, FHM or BF-2, are infected with different viruses for 1 – 2 days, then fixed with methanol-acetone (50:50, v/v) at -20°C and diluted MAbs are added for identification and differentiation as well as for detection of cross-reactions with other perhaps similar viruses by IFAT.
For detection of IHNV and VHSV an antigen ELISA kit (Duo kit) was tested for differentiation between these two viruses using cell culture replicated virus but also with infected tissue materials. Therefore a huge number of virus isolates isolated in different years are included in these tests.
While very stable results are found by iIFAT and antigen ELISA for identification of VHSV, IHNV and IPNV, MAbs recognizing SVCV can cross-react with some other vesiculo-like viruses, e.g. Pike fry Rhabdovirus.

*corresponding author: sven.bergmann@fli.bund.de

### Questions

**Olga Haenen:** Did you ever isolate IPNV VR299 from eel? In The Netherlands this is found a few times in diseased eel.
**Sven M. Bergmann:** No
**Niels Jørgen Olesen:** Birnavirus 2 and IPN are both detected by BIO-X kit and therefore this kit is not specific for IPN.
**Niels Jørgen Olesen:** VHSV detection limit of $10^{5.5}$ $TCID_{50}$/ml is quite high.
**Annita Ginter:** Sensitivity could be made higher by using a 3 step ELISA instead of a 2 step. Tell us if you are interested in this and we can change it
**Niels Jørgen Olesen:** Sensitivity is fine as long as detection is performed on cell culture amplified virus.

## Specificity and sensitivity testing of commercial kits at the CRL

*Helle Frank Skal, Ellen Ariel, Niels Jørgen Olesen*

### Abstract

When having CPE in cell culture it is for many laboratories convenient to buy a diagnostic kit to identify the cause of CPE. In order to avoid false positives and false negatives it is important that these kits are validated properly.

At the CRL we are occasionally approached by commercial companies to help in this process. Some of these tests has already been published (Ariel & Olesen 2001) and resulted afterwards in improvement of one of the test kits.

In 2002 the CRL was asked to test a VHSV ELISA kit from a commercial company and recently we tested the duo (VHSV and IHNV) ELISA kit from another company.

The panel of isolates used to test the VHSV ELISA kit lacked in genotype II and IV isolates. For the tested isolates of genotype I, Ia, Ib, Ic and III the kit detected all isolates, and IHNV, IPNV and SCVC was not detected by the kit. The kit performed equivalent to the CRL in-house ELISA in the tested samples.

The analytical sensitivity was stated as $10^4$ $TCID_{50}$/ml by the producer. This could not be verified.

Recently a duo ELISA KIT was tested against 78 VHSV isolates spanning all known genotypes and isolates from North America, Japan and Europe and 11 IHNV isolates. The kit detected all the tested VHSV and IHNV isolates with correct results. The kit generally produced lower OD values than the CRL in-house ELISA. The reason for this is probably caused by the set-up of the ELISA, as the commercial kit is a direct ELISA, whereas the CRL ELISA is indirect.

The panel lacked of isolates other than VHSV and IHNV.

The testing presented here are only very basic and lacks elements to be a proper validation

References

Ariel E. & Olesen N.J. (2001) Assessment of a commercial kit collection for diagnosis of the fish viruses: IHNV, IPNV, SVCV and VHSV. *Bulletin of the European Association of Fish Pathologists* **21**, 6-11.

### Questions:

**Athanasios Prapas:** What was the sensitivity of the VHSV ELISA?

**Helle Frank Skall:** Sensitivity was comparable to the sensitivity of the BioX kits described by Sven M Bergmann ($10^4 – 10^5$).

## DNA-array based diagnostics: Applications in fish disease diagnosis

*Frans I.*[1,2], *C. Heusdens*[1,2], *K. A. Willems*[1,2], *and B. Lievens*[1,2]

[1]*Scientia Terrae Research Institute, Fortsesteenweg 30A, 2860 Sint-Katelijne-Waver, Belgium;* [2]*Laboratory for Process Microbial Ecology and Bioinspirational Management (PME&BIM), Consortium for Industrial Microbiology and Biotechnology (CIMB), K.U.Leuven Association, Lessius University College, De Nayer Campus, J. De Nayerlaan 5, 2860 Sint-Katelijne-Waver, Belgium.*

Abstract:
The presence of fish pathogens represents a major concern in the international trade of ornamental fish, and fish disease management in general. Fish diseases can be caused by several organisms, e.g. bacteria, fungi, viruses and protozoa. Consequently, identification of potential pathogens requires a diversity of sometimes time-consuming and laborious assays. The advent of molecular biology, in particular polymerase chain reaction (PCR), has lead to alternative means for pathogen detection and identification. Nevertheless, although most of these methods are suitable for the detection of a single pathogen, they are not convenient to simultaneously detect a whole range of pathogens. In contrast, PCR-based DNA array technology allows detection and identification of numerous targets in a single assay. Based on this technology, a diagnostic assay has been developed for the detection and identification of approximately 30 fish pathogenic bacteria and viruses. Each diagnosis can be achieved within 36 hours based on an objective technique utilizing an array of immobilized pathogen-specific DNA fragments. Recently, the assay has been adopted by routine diagnostic laboratories providing diagnostic services to veterinarians, whole sealers, inspection agencies etc. Results illustrating the development and power of the assay for accurate and efficient diagnosis of fish diseases will be discussed in the presentation.

Questions:
**Marc Engelma:** You have 3 independent assays now?
**Ingeborg Frans:** Yes, but we work on combining them to a single assay.
**Sven M. Bergmann:** What is the sensitivity for detection of KHV?
**Ingeborg Frans:** A few picogram of input material is needed.
**Heike Schütze:** Which genes for KHV do you amplify?
**Ingeborg Frans:** Three genes: Thymidine kinase and two others
**Richard Paley:** What is the cost to run the assay?
**Ingeborg Frans**: 175 euro pr. array
**Niels Jørgen Olesen:** Do you look at regulation of host gene expression involved in immune response?
**Ingeborg Frans:** We have been looking at it but there is still a lot of work to do.
**Olga Haenen:** Which organs do you sample?
**Ingeborg Frans:** It depends on the pathogen we want to detect.
**Malcolm Hall:** Cross contamination can be a problem in relation to PCR based diagnostic. Have you experienced contamination problems?
**Ingeborg Frans:** We work in different rooms for different processes and use several controls.
**Malcolm Hall:** Could be a problem if other labs should work with this.
**Svenn M. Bergmann:** Is it possible to examine pools of fish?
**Ingeborg Frans:** We have pooled a few fish but we have not made extensive studies on this.

## Experiences with the use of LAMP originated kits in KHV diagnostics in comparison to other tools

**Sven M. Bergmann***

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## Abstract:

Koi herpesvirus disease (KHVD) has spread world-wide obviously by transfer of infected but non-diseased koi and / or common carp (*Cyprinus carpio*). Due to the limited sensitivity of the available cell cultures, e.g. common carp brain cells (CCB) or koi fin cells (KF-1), for virus isolation, molecular tools are in the focus as "gold standard" for KHV detection. Beside different molecular assays such as PCR, nested PCR, real-time PCR or even *in-situ* hybridization on tissue material, a new method was established. This method, called "loop-mediated isothermal amplification (LAMP)" of DNA, possesses the advantage of amplification at one temperature. No PCR equipment or PCR protocols are necessary.
In the German reference laboratory for KHVD (NRL KHVD) different LAMPs were tested and compared for specificity and sensitivity and compared to other recently used assays.
Additionally, the NRL KHVD supervised a LAMP development which was the subject of a diploma thesis. All these experiences were incorporated into the assessment of tests for detection and differentiation of KHV.
While all tested kits were specific to DNA obtained from KHV infected materials (infected cell cultures, infected fish tissues), the sensitivity did not reach that of PCR or real-time PCR. While LAMPs for agent identification are useful when KHV is present in huge concentrations in or on infected fish during an outbreak of KHVD, as one of our results we could show that LAMP and its commercial variants, e.g. "loopamp" from Eiken Corp. (Japan) or the "i-screen KHV kit" Gene Reach Biotechnolgy Corp. (Taiwan) but also the newly developed LAMP, were never able to identify low KHV concentrations from tissue or droppings of latently (persistently) infected fish.
Some data sheets indicate that a negative result of the LAMP assay might by due to latency. According to our observations, a LAMP will give positive results only at KHV concentrations of more than $10^4$ / $10^5$ genomic copies / ml, comparatively measured by real-time PCR.
A new, much more successful LAMP simultaneously using anti-KHV serum from rabbit for virus attachment in tubes was published in 2009. Due to virus attachment to the tubes, the test sensitivity was increased considerably.
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## Questions

**Marc Engelsma:** I have tried I-screen as well but never got any positive spots.
**Sven M. Bergmann:** You have to use very high concentrations to get a positive result
**Søren Kahns:** Can everybody use this kit test or is it only experienced laboratory personnel?
**Sven Bergmann:** In principle, everyone can perform the test.

Plenum discussion:
**Giuseppe Bovo:** Are we as NRLs obligated to test all the kits the different laboratories in our country uses?
**Niels Jørgen Olesen:** No, but by providing proficiency tests you ensure that laboratories can detect relevant diseases.
**Sven M. Bergmann:** In Germany companies have to pay for testing and approval of their kits at the NRL.
**Richard Paley:** It would be nice to be informed on experiences made by other laboratories. Could we add validation reports to the CRL-website?
**Niels Jørgen Olesen:** Yes, but it will be hard to ensure quality of the reports.
**Sven M. Bergmann:** The reports are often owned by the companies who pay for them.
**Annita Ginter:** We are open about our reports at Bio-X but of course it varies depending on the report in question.
**Niels Jørgen Olesen:** Maybe there should be golden standards to test different kits against?
**Neil Ruane:** The availability of a range of 'Golden standards' would be a powerful tool for the validation of diagnostic kits and method development within the NRL network.
**Annita Ginter:** Often the companies need to do a lot of work for getting our products approved in the different member states.
**Niels Jørgen Olesen:** Are people satisfied with the tests available?
**Athanasios Prapas:** Kits for the new listed diseases could be nice.
**Olga Haenen:** It is hard to validate tests where you only have very few samples.
**Niels Jørgen Olesen:** Maybe in some cases it could be beneficial to license diagnosis of certain diseases to other laboratories receiving more samples.
**Niels Jørgen Olesen:** It could pose a problem that kits are becoming available for fish farmers that thereby can monitor themselves. They might not report listed diseases. The fish farmer will not validate his procedure.
**Niels Jørgen Olesen:** One of the conclusions must be that it is necessary to validate kits in the respective laboratory before use and also to ask companies for documentation of the validation performed when a new kit is to be used.

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

**Brit Hjeltnes:** If you don't know the situation you can't do anything about it, so this session is important.

### Overview of the disease situation and surveillance in Europe in 2009

**N. J. Olesen and N. Nicolajsen**
*National Veterinary Institute, Technical University of Denmark*

**Abstract:**

The Questionnaire on Surveillance and Diagnosis (S&D) which is collated annually is the only comprehensive overview of the disease situation in aquaculture in Europe. The information has been made available on the CRL web site (www.crl-fish.eu), where all raw data can be obtained. The S&D have evolved over the years to now comprise 4 parts: General data on production, epidemiological data on diseases, laboratory data from NRLs and other laboratories, and categorisation of fish farms according to Council directive 2006/88/EC. As we are collating information on quality assurance in NRLs through the annual proficiency test this part of the questionnaire 2009 was omitted. The data on the European aquaculture production were obtained from the FIGIS database. Unfortunately this database does not include information on the number and size of fish farms, which are epidemiologically important data. The production in 2008 has risen a bit again after a decrease from 2003-2006. Data from 2009 is not yet available. The farm sizes vary a lot between countries, e.g. the majority of farms in Germany produced < 5 tonnes, and for Spain the number of farms producing < 5 tonnes, 5-100 tonnes and > 100 tonnes is nearly equal.
In Northern European countries there are mainly salmonid farms, in continental Europe we find a lot of carp farms, and in the Mediterranean area, besides carps, seabream and seabass are also species that many produce. Turkey is a big producer of rainbow trout and lots of rainbow trout farms is found in this country.
Concerning the epidemiological data, there obviously still is a severe underreporting of VHS and IHN in many countries. The infection status is known for about ½ of the farms.
The infection status regarding KHV is unknown for many carp farms, whereas for farms producing Atlantic salmon, the infection status for ISA is known for nearly all farms. ISA is still a problem in Norway.
Many countries have surveillance programmes for SVC, BKD, and IPN, for which they are seeking "additional guaranties". The number of farms in the programmes varies from very few farms to many farms. Fewer countries have surveillance programmes for *Gyrodactylus salaris*.
There is 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, but the large number of PCR-tests conducted in some countries mostly reflects the KHV and ISA testing.
About a third of the countries have regional laboratories, and of these countries, 8 of 11 organize ring tests for the regional laboratories.
A total overview of the current status of the categorisation of fish farms in Europe will be given.

**Minutes:**
Due to the sheer volume, all the results from the Survey & Diagnosis questionnaire have not been inserted in the Annual Meeting booklet this year. Instead all the results will be published on the CRL website (http://www.crl-fish.eu). This presentation will also be available on the CRL website.

An important duty for the CRL is to collate information on the disease status in Europe.

The questionnaire has been divided into different chapters: General data, epidemiologic data, laboratory data, proficiency tests offered to regional labs and a final chapter on the use of kits in the NRLs.
General data: We ask for number of farms according to production size. The production of freshwater fish in Europe has been steady during the last 10 years. This is worrying when compared with the rise in the worldwide production. The marine fish production has risen a bit during the last 10 years.
The distribution of farms according to size varies a lot between the countries. E.g. Germany has mainly small farms, whereas Scotland mainly has large farms. Likewise does the species farmed vary a lot between the different countries.
Health categorisation: For VHS over half of the authorised farms in Europe are in category III and the remaining in category I or II. The picture looks the same for IHN. There is still a lot of underreporting of VHS and IHN. For KHV most carp farms are in category III, unknown status. A lot of farms are not categorised yet. However, categorisation is in good process when comparing to e.g. the molluscs farms in Europe. There are several different views on how categorisation shall be performed.
Epidemiological data: We asked the laboratories to state the number of farms considered to be infected to get a "real" picture, and not just the official picture. For VHS and IHN, we know the exact status for 1/3 of the farms, whereas for KHV we don't know the status for most of the farms. There does not seem to be any real emerging diseases in 2009. For the Mediterranean area nodavirus seems to appear a lot. There was an increase of ISA in Scotland and a decrease of VHS in Denmark with no infected farms. In Austria there was a decrease in the severity of KHV.
Laboratory data: Some countries are doing a lot of examinations. PCR is really coming up as a diagnostic tool.

**Questions:**
**Mansour El-Matbouli:** What is your interpretation that most farms are placed in category III, is it good or of concern?
**Niels Jørgen Olesen:** It was newer the idea the cat. III should be the final stage. When a fish farm is in cat. III there is an obligation to survey actively in order to move on.
**Sigrid Cabot:** I have one comment to categorisation. Some farms which in this overview are put under cat. II, are according to the EU legislation in cat. IV. When a surveillance programme is established with the aim to obtain disease freedom, it is important to declare the programme to the European and the other Member States through SCFCAH.
**Niels Jørgen Olesen:** I think the intention of cat. IV and II has not been followed, most are put in II instead of IV.

## An update on the Infectious Salmon Anaemia (ISA) situation in Scotland

**Eann Munro**
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**Abstract:**
Infectious salmon anaemia (ISA) is a multisystemic contagious disease of farmed Atlantic salmon (*Salmo salar* L.) caused by the orthomyxovirus, infectious salmon anaemia virus (ISAV). The disease is characterised by severe anaemia and haemorrhaging in several organs. Disease outbreaks are predominately associated with Atlantic salmon farmed in the marine environment. A previous ISA epizootic was detected in Scotland in 1998 when the disease spread from a single source of infection. Through implementation of an effective eradication programme this epizootic was eradicated and ISA disease freedom regained. In January 2009 the presence of ISAV was detected within a population of farmed Atlantic salmon in the South West Mainland of the Shetland Islands. In total, 6 sites all of which were in close geographical location to each other were confirmed as ISAV positive.
In accordance with EU Council Directive 2006/88 and commission decision 2003/466 control measures were implemented once suspicion of ISA was placed on the initial site. Notices were served, prohibiting the movement of live and dead fish, personnel, vehicles, equipment, materials or substances that could potentially transmit infection on or off the farm. Control and surveillance zones around the infected sites were also established which placed restrictions on all farms within these areas. All sites within these zones and sites which had significant epidemiological links to the infected sites were inspected and screened for ISAV. A wild fish survey of the surrounding marine and freshwater environments produced negative results.
Scottish Government policy is to eradicate ISA through removal of infected stocks. The speed at which the removal is required takes account of the risk of spread and in the 2009 outbreak the scientific advice was for rapid removal of all fish from ISA confirmed sites. Depopulation of all confirmed sites was achieved within a 7 week period from the date of official confirmation. Once the depopulation and disinfection processes were performed, confirmed sites remained fallow for a minimum of 6 months and all sites within the control zone had to be synchronously fallowed for a minimum period of 6 weeks. Sites within the surveillance zone were required to undergo an asynchronous fallow of 6 weeks. The Atlantic salmon aquaculture facilities within the control zone began restocking in March 2010.
A 2 year targeted surveillance programme within the South West Shetland Islands, to demonstrate negative results for ISA which would enable the lifting of control measures such that the whole of the UK can be an approved zone for ISA in accordance with Council Directive 2006/88/EC will begin in May 2010.
To conclude, 6 sites were confirmed ISA positive from January to November 2009. All sites were located in the South West Shetland Islands; within the one control zone. There is no evidence of ISAV spread out with this control zone and the disease has been eradicated from the South West Shetland Islands. Inspections and testing of the restocked farms within this area will begin in May 2010 to regain ISA disease free status within this area.

**Minutes:**
Rob Raynard gave a presentation on the ISA outbreak last year. This will be an update to this presentation.
ISA is a multisystem disease of Atlantic salmon worldwide caused by an orthomyxovirus. The ISA virus is of 2 independent origins, one in Europe and one in North America.
EU legislation to take into account: CD 2006/88/EC and CD 2003/466/EC
In Scotland we have experienced two outbreaks:

1) May 1998 - May 1999: 11 cases and 2) the 2009 outbreak: The outbreak occurred on the Shetland Isles. This has been very grave for the industry as the Shetland Isles produce 10% of the Atlantic salmon production. The outbreak was notified by a phone call due to high mortality at a fish farm. At visit by the veterinary officers there were clear ISA symptoms. A containment area was established January 2009. Monthly inspections in control zone occurred every 2 months in the surveillance zone. Targeted surveillance (150 fish) in the control zone from each farm. Various assays were used for confirmatory diagnosis. The different farms had big variations in mortality.
The Scottish Government policy is to eradicate the disease. All sites were depopulated within 7 weeks. When a site is confirmed positive it is cleaned and disinfected and fallowed for a minimum period of 6 months. All associated equipment and boats are also cleaned and disinfected. Sites within the confirmed area which were not tested positive were fallowed for 6 weeks. Wild fish testing of 216 freshwater and 1196 marine fish by PCR was performed with negative results.
ISAV in Shetland (2009) differed to the isolate causing the outbreak in Scotland 1998, neither was it similar to Scottish HPR0 (avirulent). Originated from either an import or from a mutation of other HRP0s in the environment.
The epizootic investigation will be published on the marinescotland website within the next few months.
Likely source and spread: The source is at present unknown, the isolate has a novel HRP type. The spread of the disease seems to be hydrodynamic.
To date 7 sites within the control zone have restocked, the first one restocked in March.
In all 6 sites were confirmed positive during the outbreak.

**Questions:**
**Steve Feist:** How did you decide the extent of the zones?
**Eann Munro:** The extents of the zones were based on tidal excursion models developed during the last outbreak. The models cover the whole of Scotland.
**Niels Jørgen Olesen:** When you are surveying you use qPCR assays – what will you do if you find HPR0?
**Eann Munro:** A positive PCR result will only result in suspicion, in addition we always perform sequence analysis on PCR positive samples and we also analyse the results of other assays. Once suspicion is raised we will go back to the site and examine 150 fish and if negative after additional sampling then the farm is deemed ISA negative.

## Old and emerging diseases in the Mediterranean Aquaculture

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**Abstract:** Beside the traditional trout farming existing in some Mediterranean countries, as an industrial activity, since mid-twentieth century, the younger seawater fish farming, consolidated since 1980s, has now reached significant productions. In fact, according to the Federation of European Aquaculture Producers (FEAP) more than 290 thousand tons of seabass and seabream, which represent the most important species, have been produced during 2008. Local pathogens, already present in the wild populations and exotic pathogens introduced through the trade, appeared very soon in farmed population. Nowadays old and emerging diseases play an important role with regard the health of both juveniles and on-growing farmed populations, often representing a limiting factor to the production.

In order to obtain updated information on the most serious diseases affecting the aquaculture industry in the Mediterranean area a questionnaire was sent to 12 fish-pathology experts. 11 reports were obtained back and in five questionnaires data on salmonid diseases were also included. According to the questionnaire old, emerging and re-emerging diseases were reported at Country level as well as their endemic or sporadic *status*. In addition the control methods applied were included for each disease.

The trout industry is still threatened by serious viral diseases as infectious pancreatic necrosis (IPN) and viral haemorrhagic septicaemia, particularly in mountain areas characterized by low water temperature. In other regions where farms are fed with higher water temperatures (14-15°C) viral diseases are less frequent and outbreaks less severe. In these areas bacterial diseases represent the most serious risks, particularly during summer. Infections induced by *Flavobacterium psychrophilum* seem to be widespread in several Countries and difficult to eradicate even in hatcheries where losses may reach more than 50% if no adequate therapy is immediately established. No vaccine is available yet.

The infection due to *Lactococcus garvieae* is endemic in some regions and in hot seasons particularly in association with water scarcity losses may exceed 50%, particularly in market size fish. Antibiotic therapy is not effective mainly because of the early appearance of anorexia. In some Countries a vaccine, produced from inactivated cells is available but complete control of the disease is difficult to obtain because of the limited protective period.

Concerning marine aquaculture, viral encephalopathy and retinopathy (VER) remains one of the most serious problems mainly in seabass farms where losses may vary from 5 to 100 % depending on the age of the affected population. More recently VER outbreaks have been observed in seabream affecting larval and juvenile stages. Furthermore new candidate aquaculture species like *Solea senegalensis* have been seriously affected. During summer 2009 VER was diagnosed in a farm rearing pike-perch (*Sander lucioperca*) and largemouth bass (*Micropterus salmoides*) in freshwater environment. This new finding underlines the large host spectrum of betanodaviruses and new additional species could be affected in the future for this reason attention should be paid to avoid contact with known and unknown susceptible species.

Among emerging diseases reference should be made to furuncolosis and IPN infection detected in sole. The major emerging problem is represented by rash skin syndrome a pathological condition endemic in the Mediterranean Iberian coast and affecting only sea bream. Mortality is very low but affected fish, characterized by the presence of evident ulcers in the skin may not be traded until the complete recover of the clinical signs.

**Minutes:**
A questionnaire was sent out to several experts working on different Countries in the Mediterranean aquaculture.
The production is mainly represented by bream, bass and different trout species but also turbot, tuna, meagre, sturgeon and eel are produced.
Salmonids and related pathogens: In the questionnaire sent out I asked for 2-3 most important diseases: The situation may differ a lot according to where the farm is situated in a country. E.g. VHS in Italy: A farm placed in the mountainous area are in a much greater risk of disease outbreaks due to VHS than farms placed in the lowlands due to the different water temperatures. Rainbow trout fry syndrome (RTFS) is one of the most important diseases; VHS, IPN and *Aeromonas salmonicida* are also very important. RTFS is reported from Spain, Turkey and Italy. Lactococcus was only reported as an important disease from Italy. This means that the disease may be easily controlled in other areas, possibly by means of vaccination. From Greece we received reports of HVA in eels, while from Italy VER was reported once in freshwater fish.
In the marine environment the major problem is still represented by VER (age depending, sporadic, emerging, problem in Spain, Greece, Turkey, Italy); rash skin syndrome, which is very similar to red mark syndrome of rainbow trout, with fish showing skin lesion, disappearing when temperature increase at >17°C. Fish may be advantageously treated with oxytetracycline, maybe correlated to clamydia infection, only seabream is affected; pasteurellosis (reported from Greece, Tunisia and Turkey), Cryptocarion infection, vibriosis, furunculosis, isopods (reported from Greece and Croatia), protozoans, gill trematodes (reported from Greece and Italy). VHS has been reported from turbot in Turkey.
A question is if we should expect further problems due to tuna farming? Frozen fish from all over the world are used for feeding and that may introduce pathogens that are not present in the Mediterranean area.

**Questions:**
**François Lieffrig:** You have seen VNN in freshwater?
**Giuseppe Bovo:** Yes, it started in fry imported from third country**.**

## Detection of simultaneous multiple viral infection in a Swiss fish farm

**Thomas Wahli**
*Centre for Fish and Wildlife Health, Institute of Animal Pathology, University of Bern, 3001 Bern*

**Abstract:**
A fish farm in Southern Switzerland experienced increasing mortalities of rainbow trout in early spring 2010. The events started after increased quantities of snow melting water from an adjacent road flowed into the ponds of the farm. This was firstly considered as the reason for the losses. In the respective facility bacterial gill disease, rainbow trout fry syndrome and the virus of pancreas necrosis had been demonstrated repeatedly in previous years, bacterial gill disease also early in 2010. As mortalities went on, a new lot of fish of the most affected lot was analysed. Besides a marked gill proliferation (no bacteria could be demonstrated but the fish had been disinfected before analysis) IPN could be diagnosed. The viral infection however was not regarded as cause for the increased mortality as the fish measured up to 13 cm. Fish not showing any disease signs nor mortality were transferred to a second farm of the same owner in order to separate them from diseased fish. Despite further disinfections the situation in the first farm did not improve and therefore fish from different ponds, size and age classes were analysed 10 days later. On this occasion, in one sample viral haemorrhagic septicaemia was diagnosed, while fish from a second lot were infected with birnavirus. Trout from a third lot showed neither mortality nor disease symptoms and no virus could be detected. As a consequence fish from 9 tanks from both farms were subjected to virology. In some of the fish few disease symptoms such as pale gills, watery intestinal content, greyish liver was seen but the majority of fish presented without any macroscopic symptoms. Nevertheless VHSV was found in 4 samples. In one sample besides VHSV also IPNV was found. In addition, in two samples infectious haematopoietic necrosis virus (IHNV) was found, among them one where also VHSV had been demonstrated. The results were confirmed by RT-PCR. Thereby in further samples double or even triple infections were detected. Both farms harboured virus-infected fish. Both farms were sequestered and sanitation measures were initiated including stamping out of all stocks.
The results showed that detection of one virus species in cell culture can at least partly be influenced by the growth of another virus present in the same fish.

**Minutes:**
This is a report of a case of multiple infections that we have had for the first time in Switzerland. The case concerns two fish farms belonging to the same owner in the south of Switzerland. Both farms are fed of river water but not of the same river and the production is organic rainbow trout. In farm A there were regular infections with bacterial gill disease, RTFS and in farm IPNV. Farm B was restarted recently after a fallowing period of several years due to a bacterial disease.
In January elevated mortality was observed in farm A. RTFS was diagnosed. In February high mortality was observed after massive inflow of melt water from a nearby road. Diagnosis: proliferation of gill epithelium, protozoan infection of intestines. Transfer of fish to farm B, where no problems with snow water occur. In March: Ongoing mortality, new samples were taken. IPNV was in sample 1, in sample 2 was VHSV. This raised the questions: How widespread is VHSV in the facility? Is the demonstration of VHSV hampered by presence of IPNV? In Switzerland IPN is a notifiable disease so we do not use anti-IPNV antibodies in cell culture. Was VHSV present earlier?
Further investigations: Farm A: 3 samples negative for viral growth, 2 samples positive for VHSV, 1 sample positive for VHSV and IPNV. Only very mild macroscopic symptoms were observed. Farm B: 1 sample negative for viral growth, 1 sample positive for VHSV, 1 sample positive for IHNV, 1 sample double infected with IHNV and VHSV. Macroscopic symptoms in fish were

observed only in one sample. By PCR IHN was also found in farm A.So in fact there were triple infections.
Conclusion: When taking together cell culture results and RT-PCR analyses, we identified double infections in 3 cases, triple infections in 2 cases. In case of growth of IPNV, infection with either VHSV or IHNV is not necessarily detected in cell culture. The origin of virus is not yet determined. Investigation of stock movement in the last several months is needed. Further investigations of wild population in the rivers should be performed. In both farms stamping out of all stocks followed by sanitation has been performed. There is now a discussion on application of detection methods.

**Questions:**
**Torunn Taksdal:** Are there triple or double infections in one single fish?
**Thomas Wahli:** The samples were made from a pool of 3-5 fish from one particular tank.

## Final eradication of VHS in Denmark? Categorisation of and risk based surveillance on Danish fish farms.

**H. Korsholm.**
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**Abstract:**
The preliminary results of the Danish programme for a final eradication of VHS in the period of 2009 -2013 are presented. There have been no outbreaks of VHS since the start of the project (April 1st 2009). The disease preventing measures (main fallowing, auxiliary fallowing and removal of wild living rainbow trout in high risk watercourses) have been carried out as scheduled. Reflections on the categorisation of and the risk based surveillance and testing on Danish fish farms are presented. It is concluded that Danish fish farms are at high to medium risk and that targeted surveillance will be continued.

**Minutes:**
History of VHS in Denmark: 1950s: First observation of VHS. 1960s: Private voluntary eradication. 1970: Official control of VHS eradication. 2009: Final eradication of VHS?
Programme for final eradication 2009-2013: The industry delivered Denmark free of VHS infected fish by 1 April 2009. Application for an eradication programme was sent to EU May 2008 and approved autumn 2008. The eradication programme is financed by a European Fisheries Fund grant.
Preventative measures 2009-2010: Main fallowing of 7 brackish water farms from 1 April 2009 - 16 May 2011. Auxiliary fallowing of 19 (2009)/13 (2010) fresh water farms 1 April – 16 May same year
Removal of wild living rainbow trout in high risk water courses by electro fishing (2009 and 2010). The fish were examined for VHSV with negative results.
Structure of VHS eradication programme: In case of VHS outbreak it is mandatory with immediate stamping out. There is taxation and compensation for the value of the fish.
Advantages for the programme: Favourable epidemic situation. Declared wish form the industry. Financial support. Preventative measures. Immediate eradication is mandatory.
Disadvantages: Limited possibilities for official eradication. Risk of re-infection from anadromous fish
Project runs to 2013 only. What do we do with the sea farms? We will like to block the transfer of marine fish into the freshwater environment.
Categorisation with respect to VHS: Cat I zone: 186 farms. Cat I compartment: 45 farms. Cat II: 66 farms. Cat III: the sea water farms.

Risk based surveillance: Are the farms situated far away or close to infected areas? Far away - low risk
Cat. I compartment within infected area – high risk. In between – medium risk. Marine: high risk
Type of health surveillance: active, targeted
At the moment all farms in DK are high to medium risk with targeted surveillance

**Questions:**
**Giuseppe Bovo:** Will all controls in Denmark be performed by the competent authorities?
**Henrik Korsholm:** Yes.
**Giuseppe Bovo:** And the farmers don't pay?
**Henrik Korsholm:** Yes and no, until now they have been sustained, but this may change in the future.
**Thomas Wahli:** What are the measures taken in the sea water farms?
**Henrik Korsholm:** We will act the same way disregarding which genotype a marine farm is infected with. VHSV is VHSV no matter what.

## Pancreas disease (PD); an update on the disease situation and control measures in Norway

**Torunn Taksdal**
*National Veterinary Institute, Norway*

**Abstract:**

Pancreas disease (PD) and Sleeping disease (SD) are serious viral disease which are caused by *Salmonid alphavirus* (SAV). Until now, PD outbreaks are restricted to farmed salmonid fishes in sea water. In Scotland and Ireland, PD affects only Atlantic salmon in sea water whereas in Norway, both Atlantic salmon and rainbow trout in sea water suffer from PD. In several European countries, SD affects farmed rainbow trout in freshwater.

In western Norway, serious outbreaks of PD have been diagnosed yearly since 1995. The affected area has expanded north- and southwards from this "hotspot". Some outbreaks far north from the endemic zone have been connected to transportation of fish from the endemic zone. The number of outbreaks increased until 2008 when 108 outbreaks were recorded. In 2009, the number of outbreak was reduced to75.

From December 2007, PD was included as a "group B" listed disease in Norway. Preventive measures have been established by the Norwegian Food Safety Authority. The main aims are to prevent further spread from the endemic zone to the non-endemic zone and to reduce the negative impact of the outbreaks inside the endemic zone. More precisely; Norway has now been divided into two zones. In the north, non-endemic zone, the preventive strategy implied from Norwegian Food Safety Authority, is stamping out, whereas inside the endemic zone, the aim is to reduce the negative impact of the disease. This work goes along with important industry initiatives against PD, targeting the same aims. This includes vaccination against SAV, grouping of fish farms giving "fire gates" as well as other hygienic measures. However, the structure of the industry, with several boats still travelling among the fish farms, to and from slaughter houses and even between different countries, still represents a significant risk of spreading the virus and the disease.

**Minutes**
PD and SD are similar diseases caused by salmonid alphaviruses.

SAV subtype 1, 4, 5 and 6 have caused PD in Atlantic salmon in Ireland and Scotland. SAV2 have caused sleeping disease in rainbow trout in fresh water in several European countries. SAV3 is found in Norway.
Lesions: loss of exocrine pancreatic tissue. After an outbreak you see thin PD-runt compared to a healthy fish. They are starving although they eat a lot.
PD is a severe disease in Ireland, Scotland and Norway. Tri-nation cooperation on PD and similar diseases has been established among these countries. The number of PD-outbeaks in Norway has been low from 1998 to 2002, when it started to raise and reached a high levels at the end of the decade (2007, 2008).
Is there SAV subtype 3 infections in fresh water? Freshwater farms were sampled before the fish were released to seawater. No virus was found. The smolts were followed in the seawater at 51 seawater sites. There is a PD-zone where a lot of farms are infected. North of this zone neither SAV nor PD was detected on 15 sites. South of the zone PD is not detected either. Evaluation of risk factors inside the PD zone: No significant difference between sites with and without PD/SAV inside the endemic zone. The infection pressure within the affected area was probably the dominant risk factor.
Is SAV a persistent infection? 14 sites were followed by sampling after first detection. All 14 sited tested positive for SAV at subsequent samplings.
Clinically healthy fish may harbour SAV. Registered time between infection and disease has been up to 18-37 weeks. PD diseased fish have been detected up to 12 months after first diagnosis.
Control strategies: Prevent further spread to new areas. PD is a list 3 disease in Norway since 2007. The industry has control strategies against PD. North of the zone stamping out are used in cooperation with the industry. Last outbreak north of zone was in August 2009. Inside the zone there is mitigation. Information, hygienic measures, vaccination (no control group to validate the vaccine as nearly all are vaccinated), grouping of sites and fire gates, coordinated fallowing, safe transport routes are some of the measures used.
The PD situation in Norway is still serious. The structure of the industry include a lot of boat traffic between fish farms, equipments etc. There have been rumours that such boats also goes to North America and back.

Questions:
Athanasios Prapas: Regarding the vaccine, you are not able to validate the vaccine?
Torunn Taksdal: There have been good results in experiments, but the real challenge is in the field. We have identified PD-diagnosed fish that have been vaccinated.
Niels Jørgen Olesen: Are the PD-viruses all the same for the different outbreaks. Does the vaccine preferably protect against certain forms of the virus?
Torunn Taksdal: There are very few differences in the virus.
Brit Hjeltnes: As far as I know, the lack of efficacy does not correlate to the virus strain.

## BKD – recent outbreaks in Norway

**Hege Hellberg** and Hanne K. Nilsen
*National Veterinary Institute Bergen*

**Abstract:**

Bacterial kidney disease (BKD) is a chronic disease of salmonid fish caused by the bacterium *Renibacterium salmoninarum.* The BKD-situation in Norway has been stable during the last decade, with only occasional outbreaks with limited mortality.

**BKD, number of sites/farms diagnosed**

| | 1998 | 1999 | 2000 | 2001 | 2002 | 2003 | 2004 | 2005 | 2006 | 2007 | 2008 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| BKD | 0 | 3 | 3 | 3 | 1 | 1 | 1 | 2 | 0 | 0 | 1 |

A surveillance programme for BKD was initiated in autumn of 2005. Freshwater sites with salmonids and seawater sites with salmonid broodfish are sampled every other year (coordinated with VHS/IHN surveillance). Sampling is done by the Food Safety Authority. Kidney (+ other internal organs from fingerlings) is tested by ELISA using a monoclonal AB against surface protein p57 (BiosChile). ELISA positive samples are tested by real-time PCR.

**BKD surveillance programme, results**

| | 2005-2006 | 2007 | 2008 | 2009 |
|---|---|---|---|---|
| # Sites | 54 | 150 | 116 | 130 |
| Total samples | 1887 | 4102 | 3817 | 3701 |
| Positives | 0 | 0 | 0 | 0 |

In 2009, *R. salmoninarum* was not detected in the surveillance programme, but BKD was diagnosed in a total of 3 on-growing sites/farms, 2 rainbow trout and 1 Atlantic salmon. These diagnosed were made on material submitted by local fish health services investigating clinical disease and increased mortality at the sites. Both rainbow trout farms were located in a fjord recognised as a recurrent "hot spot" for BKD. Follow up sampling from one rainbow trout farm revealed *R. salmoninarum* in sparse culture from 2 fish. These fish tested negative by ELISA and real-time PCR.

This illustrates the limitations of surveillance programmes in detecting low prevalence disease.

**Minutes**

BKD historical data: BKD has been a considerable problem with the peak of more than 60 outbreaks in one year (1990). 1980: first 5 cases in Norway, wild brood stock was the probable source of the infection. There is a wild reservoir. The most efficient control measure is brood stock screening with elimination of positive brood stock. The disease is notifiable. The last 10 years the situation has been stable with less than 5 outbreaks per year. The surveillance programme was started in 2005 with sampling of 30 fish from ongrowing facilities and 60 fish from broodstock farms. Single fish examined by ELISA. Positives are furthermore tested by in-house PCR. There

have been some problems with incomplete samplings. Details of the surveillance programme are available at the website in the annual reports.
BKD recent outbreaks: BKD detected in 3 on-growing sites in 2009: 2 rainbow trout (in hotspot area close to Bergen) and 1 in Atlantic salmon (import from Iceland).

Case 1: Submission to NVI Bergen by local fish health service; Mortality low-normal; Observed ulcer and boils; Suspect *Flavobacterium psychrophilum;* BKD detected (1 fish sent in); Later samplings: 19 fish all negative; Later sampling again: BKD diagnosed by growth on SKDM agar but not by PCR.
Case 2: The fish had also meningitis where *R. salmoninarum* were detected.
Active surveillance in 2009: 3701 fish tested negative
Passive surveillance: 1 fish selected from a population > 60,000 tested positive

**Questions:**
**Olga Haenen:** Do you have an explanation why PCR is less sensitive than growth on SKDM?
**Hege Hellberg:** Probably because BKD is very unevenly distributed. This is part of the reason.
**Mansour El-Matbouli:** Did the ELISA positives have clinical signs?
**Hege Hellberg:** Yes!
**Niels Jørgen Olesen:** Concerning the risk factors for contracting BKD. We have in Denmark seen big problems in recirculation farms. Have you seen anything similar in Norway?
**Hege Hellberg:** No, we have not seen something similar.
**Brit Hjeltnes:** Recirculation is of limited but growing use in Norway.

## Red Mark Syndrome – a diagnostic challenge

Stephen W. Feist, David Verner-Jeffreys and Birgit Oidtmann
*Cefas Weymouth Laboratory, Barrack Road, The Nothe,*
*Weymouth, Dorset. DT4 8UB UK*

**Abstract**
Red Mark Syndrome (RMS) has recently been reported in Great Britain and some other European countries and is having a significant impact on the trout aquaculture industry (mainly due to downgrading of carcasses at slaughter). The number of farms affected by RMS in the UK has risen from less than 5 in the winter of 2003/2004, to approximately 80 farms, affecting more than 50% of the rainbow trout industry in the UK. The transmissible nature of the disease strongly suggests that a pathogenic agent is involved but to date this has not been identified. In North America the aetiology of a similar condition called Strawberry Disease is also unresolved but a recent study suggests the possible involvement of a Rickettsia-like organism.
It is known that RMS is transmitted by live fish movements but with an unknown infectious aetiology many risk factors are associated with the condition remain undetermined. Uncertainty regarding the nature of the aetiological agent makes diagnosis of the condition difficult and currently is heavily reliant on histopathological features. Consequently, acquisition of reliable data on the epidemiology of the disease and investigation of potential control methods remains challenging. This presentation provides a summary of the condition and compares the pathology of RMS and similar conditions such as Warm Water Strawberry Disease (WWSD), US Rash and US Strawberry Disease and highlights the urgent need to investigate RMS further as a significant emerging disease in Europe.

**Minutes:**

Background: RMS is a skin condition of rainbow trout. First observed in the UK January 2004. May have a rickettsial involvement. Skin is affected to subdermal layer with moderate to marked inflammatory response. No systemic signs of infection. The diseases causes downgrading of carcasses. The fish can spontaneously recover.
RMS epidemiology: First diagnosed in Scotland late 2003/early 2004. First observed in England in 2005. Within a farm the condition can spread. 50% of annual production is now affected in the UK. The disease is also called coldwater strawberry disease (Warm water strawberry disease in the UK since 1998, summer condition, vitamin C responsive, looks like RMS).
Aetiological agent: Epidemiology strongly suggest an infectious agent
Case definition for a confirmed case of RMS (based on field observations and based on laboratory examination) has been put down by CEFAS.
Epidemiological study: Case-control study performed with the aim to provide information that helps to prevent introduction into unaffected farms, manage the disease etc. Results: Fish pumped between units odds ratio 6.7, gastroenteritis odds ratio 15.22, there is still a lot to do
Conclusions: The disease is caused by an infectious agent. There is a long incubation period. Virulence is questionable

**Questions:**
**Olga Haenen:** Also in Stirling they are working with RMS. One of the pictures you showed also looked quite the same as our first IHN outbreak. Maybe we have red mark syndrome attached to IHN.
**Steven W. Feist:** We work together with Sterling. I am glad you do not suggest that we have IHN!
**Renate Johanson:** When it is so difficult to find the agent I might look for microsporidia. We have a very nice test we are willing to share with you.
**Steven W. Feist:** Thanks a lot. I can assure you I have already looked very hard to find microsporidia.

# Characterisation of a Rosette Agent (*Sphaerothecum destruens*) from sunbleak (*Leucaspius delineatus*) in the UK.

**R. Paley*[1], D. Andreou[2], P.Martin[1], D. Stone[1], K. Bateman[1], S. Irving[1] and S. Feist[1]**
[1]*Cefas, Weymouth, UK*
[2]*Cardiff University, Cardiff, UK*

**Abstract:**
Sunbleak (*Leucaspius delineatus*), a cyprinid fish native to continental Europe is experiencing population decline which appears to be linked to the spread of the invasive Asian cyprinid (*Pseudorasbora parva*). Species interaction studies showed inhibition of spawning, wasting then death in *L. delineatus* cohabited with *P. parva*, or exposed to their holding water (Gozlan *et al.* 2005). Histological examination lead to the identification of an intracellular parasite, similar to the freshwater Mesomycetozoean parasite, Rosette agent (S*phaerothecum destruens*) that infects salmonids in the USA. Subsequent PCR and sequence analysis of a partial 18S rRNA gene demonstrated 100% homology. *S. destruens* is capable of survival in fish in the marine environment and has been associated with sporadic severe infectious disease (occasionally mortalities up to 90%) of cage-reared Chinook salmon (*Oncorhynchus tshawytscha*) in North America (Elston *et al.* 1986; Arkush *et al.* 1998) and in farmed Atlantic salmon (*Salmo salar*) in freshwater in California (Hedrick *et al.* 1989). In the US the disease is usually chronic and does not appear to impair spawning of infected fish. Information on the impact on wild stocks is extremely limited. This is the first identification of this parasite in the UK and from a cyprinid. Given the potential for causing severe disease we have developed cell-culture of the sunbleak rosette agent for use in pathogenicity studies. Sunbleak rosette agent spores are infective to EPC, CHSE and FHM cells replicating most rapidly in EPC cells. Spores can be induced to zoosporulate in water forming motile uni-flagellated zoospores in a temperature dependant manner. Challenge experiments indicated the spores, when injected intraperitonealy, are able to replicate and disperse in Atlantic salmon and sunbleak and contribute to significant mortality.

Arkush, K.D., Frasca, S. (Jr) and Hedrick R.P. (1998) Pathology associated with the Rosette agent, a systemic protest infecting salmonid fishes. *J. Aquat. Anim. Health*. 10:1-11

Elston R.A., Harrell, L. and Wilkinson M.T. (1986) Isolation and *in vitro* characterization of Chinook Salmon (Oncorhynchus tshawytscha) Rosette agent. *Aquaculture*. 56:1-21

Gozlan, R.E., St-Hilaire, S., Feist, S.W., Martin, P. and Kent, M.L. (2005) Biodiversity: disease threat to European fish. *Nature*. 2005 435(7045):1046.

Hedrick, R.P., Friedman, C.S. and Modin, J. (1989) Systemic infection in Atlantic Salmon Salmo salar with a Dermocystidium-like species. *Dis. Aquat. Org*. 7:171-177

## Minutes

Rosette agent do cause severe disease in the USA and it is present in UK, but not severe until now.
Background: Sunbleak populations decline is postulated to be linked to spread of invasive Asian fish. Is rosette agent part of this decline?
The rosette agent, *Sphaerothecum destruens*, is an obligate intracellular parasite of class mesomycetozoea. It has a potential to cause severe disease. It can grow on cell cultures.
How pathogenic is the rosette agent? Challenge experiments: IP injection of spores in salmon or sunbleak. It seems to cause mortality in salmon whereas in sunbleak it produced less mortality. No obvious external signs of disease. Internally nodules in organs (remember IP injection). Gram positive granules. Replication of zoospores in the kidney.
Summary:
First ID of rosette agent in the UK. No apparent species specificity. Capable of replication within salmon and sunbleak and producing mortality

**Questions:**
**Mansour El-Matbouli:** Are there any therapy?
**Richard Paley:** This disease has only caused serious problems few times in the states, so nobody has really worked on this issue.

## Biosecurity risks associated with EUS and Iridovirus in ornamental fishes

**M. El-Matbouli**; M. Saleh; H. Soliman
*Fish Medicine and Livestock Management; Department of Farm Animal and Veterinary Public Health; University of Veterinary Medicine, Vienna, Austria*

**Abstract:**

Global trade of ornamental fishes is unique in that while many countries impose strict requirements on nearly all imported animal and plant products, live ornamental fishes are commonly imported in large volumes without similar controls. The risk associated with importation of gouramis and cichlids, especially in relation to epizootic ulcerative syndrome (EUS) and iridovirus is of concern. We investigated 93 ornamental fish imported from different countries into the EU and concluded there were inadequate procedures in place to regulate importation of live ornamental fish. Irido-virus and *Aphanomyces invadans* were detected in multiple fish, despite these fish being accompanied with certificates from the country of origin to confirm they were free from EUS and Irido-viruses. Both of these organisms are exotic to the EU and there are no reports of their presence in EU-aquaculture. Without urgent action to regulate the importation of live ornamental fish, a significant risk exists to EU-aquaculture from exotic diseases and their subsequent serious economic impacts.

| Fish species | Number tested | Origin | Aphanomyces invadans PCR | Irido virus PCR |
|---|---|---|---|---|
| **Trichogaster** trichopterus | 13 | Thailand | positive | positive |
| **Aplocheilichthys normani** | 10 | Indonesia | negative | positive |
| **Paracheirodon axelrodi** | 10 | Indonesia | negative | positive |
| **Paracheirodon axelrodi** | 10 | Brazil | positive | positive |
| **Colisa lalia** | 15 | Malaysia | negative | positive |
| **Colisa lalia** | 4 | Vietnam | positive | positive |
| **Colisa lalia** | 20 | Singapore | negative | positive |
| **Colisa fasciata** | 6 | Indonesia | negative | positive |
| Macropodus obercularis | 5 | Singapore | negative | positive |

**Minutes**
I will now present a case report that I want to discuss with you.
EUS has been reported from 24 countries on four continents (North America, southern Africa, Asia and Australia). 76 fish species have been confirmed to be naturally affected by EUS. European catfish, rainbow trout, and goldfish are susceptible to the agent causing EUS *Aphanomyces invadans*. European countries are supposed to be free from EUS.
EHNV have not been reported to occur in freshwater ornamental fish.
As ornamental fish dealers do not like us to examine their fish for listed diseases we decided to buy fish and examine them ourselves for EUS and iridoviruses. We bought Trichogaster and brought them to the laboratory. After 14 days they started to show clinical signs corresponding to EUS. Pieces of affected muscle were placed on agar to grow the fungus. The PCR was performed on

isolates grown on agar and on fish tissue. A. Invadans positive samples were found. The PCR protocol used was according to Vandersea et al. 2006.
Detection of iridovirus was done by PCR according to protocol by Go el al. 2006, which cannot identify EHNV. We also tested the samples using the EHNV primers recommended by the OIE. None of the samples were positive by the OIE primers. The iridovirus positive fish were tested according to a protocol that do not detect EHNV.
The fish had health certification that they were free of EUS and EHNV.
Conclusion: The risk associated with the import of gouramis and cichlids especially in relation to EUS and iridovirus is of concern. The import of asymptomatic carriers will continue to be a problem until robust rapid diagnostic techniques are developed and used as basis for health certification. Ensuring that infected fish and water with the infectious agent do not come into contact with fish culture ponds could help prevent the spread of EUS to Europe.

**Questions:**
**Birgit Oidtmann:** How did you select the fish that you bought?
**Mansour El-Matbouli:** I did not select them at all. We just bought the fish. We did not want the sales person to know what we wanted to do. We kept the fish in the laboratory for at least 2 weeks before examining them.
**Heike Schütze:** For iridovirus diagnostics, have you ever isolated the virus on cell culture?
**Mansour El-Matbouli:** We tried to grow them in cell culture without luck.
**Giuseppe Bovo:** Did you identify the iridovirus at genus level or as species?
**Mansour El-Matbouli:** We sequenced the products and we only know it is iridoviruses.
**Olga Haenen:** Did you inform the EU?
**Mansour El-Matbouli:** I cannot keep this information for myself that is why I wanted to share this information with you and discuss what to do. The importers want us to make bacteriological investigations only.
**Sigrid Cabot:** Ornamental fish diseases are of concern. The emphasis put on this is illustrated by the fact that the Food and Veterinary Office of DG SANCO has so far visited only a few third countries as regards Aquatic animal health and the first three ones were Malaysia, Singapore and Thailand, countries which are all important actors in the field of ornamental fish. I would also like to highlight that there is a general obligation for everyone working with aquaculture animals to report suspicions of outbreaks of listed diseases.

# *SESSION II: Technical issues related to sampling and diagnosis*

## The new EU manuals on sampling and diagnostic procedures and the role of the www.CRL-fish.eu web page

**Niels Jørgen Olesen and Helle Frank Skall**
*National Veterinary Institute, Technical University of Denmark*

**Abstract:**
The development of a draft COMMISSION DECISION of Diagnostic Manual for certain aquatic animal diseases has been in process in the recent years. The background for this is

1. A decision in order to ensure uniform procedures for surveillance schemes, including health inspections and sampling, and diagnosis of the diseases listed in Annex IV to Directive 2006/88/EC,
   The Decision sets out:
   (a) minimum requirements for surveillance schemes and diagnostic methods that shall be used by Member States **to obtain disease-free status** for the whole territory of the Member State, zones or compartments;
   (b) minimum requirements for surveillance schemes and diagnostic methods that shall be used by Member States **to maintain the disease-free status** for the whole territory of the Member State, zones or compartments;
   (c) minimum requirements and criteria for the evaluation of the results for **diagnostic methods to be performed in the case of suspicion and to confirm the presence of the listed diseases**.
2. This Decision is directed towards both the authorities responsible for the control of those diseases and the laboratory personnel performing the tests with regard to those diseases. Accordingly, emphasis is put on the sampling procedures, principles and applications of laboratory tests and evaluation of their results.
3. The confirmation of the listed diseases in aquatic animals must be in accordance with the guidelines minimum requirements and criteria for the evaluation of the results for diagnostic methods set out in this diagnostic manual.

The decision on a Diagnostic Manual will thus cover all the exotic and non-exotic fish and molluscs diseases covered in Directive 2006/88/EC and both sampling and diagnostic procedures will be included. In order to reduce and to perform a more flexibel set of guidelines the new decision refers to the websites of the respective CRL for the detailed diagnostic procedures that shall be followed. The procedures will be very similar to the procedures given in previous Commission Decisions on VHS, IHN and ISA, respectively.

**Minutes**
The final working paper of the Comission decision is in process. In comparison with the previous legislation this paper will cover both molluscs and fish within separate annexes and cover both exotic and non exotic diseases. The methods put on the CRL webpage will be closely linked to the methods described in the OIE guidelines. The structure of the parts for the specific diseases will follow a common frame I: Description of the aetiology. II: Provisions on how to obtain and maintain certain health status; surveillance programs, eradication, maintaining and regaining disease free status. III: Diagnostic methods; sampling procedure, methods for surveillance in disease free areas and methods for surveillance/diagnostics in endemic areas or if suspicion of disease.

**Questions.**

**Guiseppe Bovo:** Will the Commission decision be part of the CRL website?
**Sigrid Cabot:** No, the Decision will be adopted by the Commission and published as a Commission Decision in the Official Journal, with links to the CRL webpage.
**Sigrid Cabot:** There will be some fine tuning and then the draft will be sent to Member States for review. Please take active part in this process and comment on the draft Decision.

## Diagnostic procedures for VHS, IHN, and EHN

**Helle Frank Skall, Søren Kahns, Ellen Ariel & Niels Jørgen Olesen**
*National Veterinary Institute, Technical University of Denmark*

### Minutes

VHSV/IHNV

The diagnostic procedures for VHS and IHN are as you know them.
During transport the samples should not exceed at temperature higher than 10°C and ice should still be at least partly present in at least one of the freeze blocks at arrival at the laboratory.
The virological examination should start as soon as possible and no later than 48 h after sampling. In exceptional cases this can be extended to 72 h.
In case of practical difficulties freezing for up to 14 days of organ material in cell culture medium at -20°C can be allowed. Likewise is it allowed to freeze the supernatant after homogenisation for up to 14 days at -80°C in case of e.g. incubator breakdown.
Culture shall be performed on BF-2 or RTG-2 and either EPC or FHM cell lines. At inoculation the cell lines shall be young and actively growing.
The end dilution of inoculation shall be 1:100 and 1:1000, respectively and at least 2 $cm^2$ of cell area for each dilution, this corresponds to the well size in a 24 well plate. Subcultivation after 7-10 days and final reading again after 7-10 days. Toxic effects should not be present at final reading.
If cytopathogen effect is observed identification of VHS or IHN shall be done by neutralisation, IFAT, ELISA or RT-PCR.
Applied diagnostic methods to rule out and confirm VHS and IHN are I: VHSV/IHNV isolation in cell culture. II: VHSV/IHNV detected in tissues by immunoassay. III: VHSV/IHNV detection by RT-PCR followed by sequencing. Disease is considered confirmed if one or more of the diagnostic methods are positive for VHSV or IHNV. Confirmation of the first case of VHS/IHN in an area previously not infected shall be based on method I, whereas diagnosis based solely on method II or III only applies for endemic areas.

EHNV

The CRL diagnostic manual for EHN differs slightly from the manual presented by the OIE. The differences are based on a cost-benefit analysis. If we can't use the same procedures as we use for VHS and IHN it will cost an enormous amount of money to survey for this disease.
The OIE reference laboratory recommends kidney, liver and spleen. As 2 of these organs are already included in the samples taken for VHS and IHN, we believe it will be OK to use these samples for both purposes.
In order to investigate the growth preferences for EHNV the reference isolate was propagated on EPC cells at 20°C and titrated and incubated at 10°C, 15ºC, 20ºC, 24ºC og 28°C, respectively. The trays were read after 3, 5, 7, 10 and 14 days.
On BF-2, EPC and RTG-2 cells the titer reached $10^8$ $TCID_{50}$/ml after 7-10 days, whereas on FHM cells the titer is lower at $10^5$ $TCID_{50}$/ml. The final titer is the same in the temperature interval 15-24°C, but titer rise is fastest at 20-24°C.

In order to examine which organs are best to use for isolation of EHNV and to examine if the culture method used for VHS/IHN is acceptable, an infection trial was performed by Dr. Ellen Ariel.

- EHNV was passed 3 x *in vivo* by IP injection in perch with re-isolation in EPC cells at 20°C.
- Perch were IP injected with 50 µl of EHNV, conc. $10^4$ $TCID_{50}$/ml
- Dead fish were frozen at -20°C till the end of trial
- Brain, heart, head kidney, spleen, liver, gills and tail musculature were collected and homogenized separately and inoculated on cell cultures
- The samples were examined on cell cultures by 3 methods
    1) 1 x 14 days + 1 x 7 days at 22°C
    2) 1 x 14 days + 1 x 7 days at 15°C
    3) 1 x 7 days + 1 x 7 days at 15°C

The single most suitable organ for reisolation of EHNV was kidney and regarding number of reisolations method 1 > method 2 > method 3. So the 2 x 7 days method with incubation at 15°C is not the optimal method for detection of EHN, as only 53% of the positive fish were tested positive.
But since the diagnostics is based on several fish, preferably with symptoms we suggest that the method already used for VHSV/IHNV is appropriate also for EHNV even though it differs from the OIE manual.
For confirmation of EHNV in case of CPE, IFAT and PCR followed by sequencing of amplicon is used. However one should be aware that the available antibodies against EHNV cannot distinguish between this and other rana viruses.

**Questions:**
**Birgit Oidtmann:** What is considered endemic?
**Niels Jørgen Olesen:** Category 3 and worse!
**Guiseppe Bovo:** This should be clarified!
**Sven M. Bergmann:** Why sequence EHNV?
**Helle Frank Skall:** EHNV is notifiable as an exotic disease whereas other rana viruses are not.
**Heike Schütze?** Why this difference in legislation?
No real conclusion, people agreed that the viruses were very close.

## Molecular diagnostic procedures for KHV - conclusions and recommendations from EPIZONE workshop on KHV

**Marc Engelsma**

**Abstract:**

The use of molecular tools is now widespread in life science and PCR based assays are more and more used as diagnostic tools. In general PCR assays are very powerful, sensitive techniques. Because of this specific precautions need to be taken to minimize the risk of cross-contamination, inducing false positive results. This was demonstrated especially during the latest ring trial organised by CEFAS in 2008. The participating laboratories were challenged to detect koi herpesvirus (KHV) in five ampoules by PCR. The results showed a considerable number of labs having problems of false positive samples. During an EPIZONE workshop, November 12-13 2009, at the Central Veterinary Institute (Lelystad, the Netherlands) pitfalls and precautions of using PCR assays in diagnostic laboratories were discussed, with a focus on PCR assays for detecting KHV. The report from the workshop gives an overview and remarks on the currently used PCR techniques and assays for detection of KHV. Furthermore, the reliability of the diagnosis is not only dependent on the test itself; it is also dependent on the processing and handling of the samples in order to prevention of cross-contamination between samples. The report gives a general protocol for handling diagnostic specimens prior to PCR analyses in order to limit the risk of cross-contamination.

### Minutes

The PCR technique is characterised by its high sensitivity and specificity. However pitfalls lie also in the sensitivity since this is prone to cross-contamination, and in the specificity since this only detects agents within a range of primers. Moreover it is detection of an agent not necessarily the disease. During the CEFAS KHV ringtrial 10 of 44 laboratories produced false negative and 19 of 44 laboratories produced false positive results. During an Epizone workshop on the topic in Lelystad, NL, the problems were discussed. The aim of the Workshop was to produce recommendations on handling diagnostic specimens to limit the risk of cross contamination and to review the current PCR assays used. To control the flow of the specimen through; Sampling, DNA extraction, Mix preparation, PCR run and gel electrophoresis is essential to prevent contamination between and within these steps. Keep sampling, mix preparation, DNA extraction and PCR run in separate rooms and use separate coats and equipments and control entrance of people and reagents to each room. Quality of sample material determines parts of the test sensitivity, prolonged time at about $20^{o}$ C, stress before sampling, target organs: gill and kidney. Sensitivity might be reduced when pooling samples. Following the CEFAS ring trial most correct results were after the use of silica matrix based methods. PCR methods available for KHV detection: Isothermal PCR: limitedly used in diagnostic laboratories. Conventional PCR: Bercovier and Gray OIE recommended methods. Nested PCR: High sensitivity but also higher risk of cross-contamination. Semi-nested: Bergmann. Real time PCR: High sensitivity, limited risk of cross-contamination, high costs. In diseased fish the published PCR assays for detection of KHV are suitable; however the most sensitive assay is recommended. For surveillance: Real time PCR seems to be the most optimal.

### Questions

**Søren Kahns**: How do purification kits perform with regard to sensitivity?
**Marc Engelsma:** Phenol Chloroform based kits produce high yields in the extraction process however they seem not to be very constant in their performance!

**Mansour El-Matbouli:** How to interpret cases where a positive result is only seen with nested PCR?
The use of nested PCR was discussed without any conclusion.
**Marc Engelsma:** It depends on the final goal and whether false positive or negative is of more concern. Optimal sampling procedure could overcome the sensitivity issue and optimal laboratory work flow and the use of many negative controls could overcome the specificity issue of the nested PCR.

## Standard Diagnostic Procedures for ISA testing undertaken by Marine Scotland

**Eann Munro**
*Marine Scotland Science, Marine Laboratory, 375 Victoria Road, Aberdeen, Scotland*

**Abstract:**
At Marine Scotland, the diagnosis of infectious salmon anaemia (ISA) is carried out as approved by the World Organisation for Animal Health (OIE 2009), by studying both clinical and pathological features as well as direct methods for detection of the viral pathogen. External clinical signs of disease include pale gills, exophthalmus, ocular haemorrhage and slight abdominal distension. Internally, ascites and a dark coloured liver are common in ISA infected fish. A drop in haematocrit value is also recorded.
Where ISA disease is suspected, MSS fish health inspectors collect tissue samples for the following diagnostic assays:- Histopathology confirmed by immunohistochemistry (IHC); isolation of the virus in cell culture followed by an indirect fluorescent antibody test (IFAT), kidney imprints for IFAT screening and real-time RT-PCR (qRT-PCR) with positive samples confirmed as ISAV by nucleotide sequence analysis.
According to EU Commission Decision 2003/466/EC, ISA is only confirmed if clinical signs and/or pathological changes consistent with the virus are present and/or detection of the agent is verified by two or more of the independent tests described above (Table 1).

Table 1 - Combination of tests that provide official confirmation of ISA

| Test 1 | Test 2 | Result |
|---|---|---|
| Mortality, clinical signs and pathology consistent with ISA | Virus isolation | Confirmed |
| | qPCR | Confirmed |
| | IFAT | Confirmed |
| Virus isolation from 2 independent samples | | Confirmed |
| Virus isolation | qPCR | Confirmed |
| | IFAT | Confirmed |

**Minutes**
Upon inspection of farms a minimum of 5 and up to 30 fish are sampled. If suspect results are obtained but without sufficient data to confirm the results, a second sampling of 150 fish is carried out. Marine Scotland Science has an ongoing project to evaluate and define the sensitivity and specificity of the diagnostic approach.

**Questions:**
**Marc Engelsma:** Does HPR0 give protection?
**Eann Munro:** I don't know, but we have not seen dual infections. They have different tissue/cell tropisms and are fundamentally different strains regarding their appearance in vivo.

## Diagnosis of *Aphanomyces invadans* (Epizootic Ulcerative Syndrome, EUS)

**Birgit Oidtmann,**
*Cefas Weymouth Laboratory, Barrack Road, Weymouth, Dorset, DT4 8UB, UK*

**Abstract:**
Epizootic Ulcerative Syndrome is currently considered exotic to Europe. However, live ornamental fish are imported from countries where the pathogen is known to occur. Therefore there is a real risk that the pathogen will eventually arrive in Europe. In order to detect the disease if introduced, national reference laboratories need to capable of diagnosing the disease.

There are a number of laboratory tests available, including isolation, histopathology and PCR. The advantages and disadvantages of each of the methods and their suitability for purpose will be discussed. Furthermore, competent authorities will need to devise an approach for deciding on when a disease event in a fish population is considered a suspect case that should be investigated by suitable laboratory methods. The issue of developing a suitable case definition of a suspect case and a confirmed case will be discussed.

### Minutes

The Oomycete *Aphanomyces invadans* is suspected to be the causal agent of Epizootic Ulcerative Syndrome (EUS). In the EFSA journal a survey has been published on susceptible species. This definition relies upon recognition of the agent and includes 32 species in 29 genera. However further 190 species in 90 genera is suspected to be susceptible but there has been uncertainty about the correct agent. However the agent certainly seems to have little species specificity, and the list is most likely not complete.

A suspect case of EUS according to the OIE diagnostic manual 2009 is related to typical lesions in susceptible fish or presence of *Aphanomyces* sp. without further identification. A confirmed case is a suspect case presenting typical mycotic granulomas in tissue or where the agent has been identified by PCR or FISH detection techniques in tissue or where *A. invadans* has been isolated and confirmed by either bioassay, PCR or sequence analysis. According to the OIE diagnostic manual from 2006 both pathology and verification of the agent should be present before a definitive diagnosis, in that context the definition of a confirmed case has become somewhat more loose.

Sampling should be done from various lesions and organs and should be done in the edge of a lesion from a clean cut surface. A variation in pathology and clinics is seen in between cases, especially formation of granulomas cannot always be expected - this depends upon variable factors such as stage of infection, age and type of fish etc. There are three PCR methods published “Oidtmann”, “Vandersea” which is specific and “Phadee” which can cross react with other *Aphanomyces* spp. and which also don’t amplify all strains of *A. invadans.* Of the Oidtmann andVandersea, Oidtmann produces a somewhat longer PCR product which is suitable for sequencing.

Following the typical clinics with ulcers and the likely absence or low prevalence of EUS in traditional aquaculture it might not be the most rational way to raise suspicion upon the observation of ulcers. Another approach could be within a limited period of time to do a targeted sampling in farms from fish with skin lesions.

## Council Directive 2006/88/EC: The concept of increased mortality and how to deal with it from a legislative perspective

**Sigrid Cabot,**
*DG SANCO, European Commission*

**Abstract:**

Council Directive 2006/88/EC lays down minimum preventive measures aimed at increasing the awareness and preparedness of competent authorities and the aquaculture industry of diseases in aquaculture animals and minimum control measures to be applied in the case of an outbreak of certain aquatic diseases.

Early detection of disease constitutes an essential part of an efficient and cost effective disease control strategy. For this purpose the Directive require all aquaculture production businesses (APBs) to keep records of mortality in each epidemiological unit as relevant for the type of production (Art. 8). Similar requirements apply to transporters of aquaculture animals. All APBs must also establish a risk based animal health surveillance scheme (Art. 10) which shall aim at the detection of any increased mortality in all farms and mollusc farming areas as appropriate for the type of production and of the listed diseases.

The term increased mortality is defined in Annex I to the Directive as "unexplained moralities significantly above the level of what is considered to be normal for the farm or mollusc farming area in question under the prevailing conditions. What is considered to be increased mortality shall be decided in cooperation between the farmer and the competent authority."

When increased mortality occurs, it must immediately be notified to the competent authority or a private veterinarian for further investigation (Article 26).

If the further investigation leads to a suspicion/confirmation of a listed disease it must be notified to the competent authority (Article 26), which will conduct further investigations as appropriate and take the appropriate measures relevant to the disease status of the area in which the listed disease was detected.

Should the investigation lead to the conclusion that the increased mortalities are caused by an emerging disease, the competent authority shall to take appropriate measures and inform the European Commission, other Member States and EFTA Member States thereof (Article 41).

Should the increased mortalities be caused by other diseases that those listed, Article 43 of the Directive gives a legal basis to under specific conditions to take national measures.

Directive 2006/88/EC lays also down harmonised placing on the market and import requirements for aquaculture animals. According to these rules, aquaculture animals may not be placed on the market for further farming if they are not clinically healthy or originate from a farm or mollusc farming area where there is any unresolved increased mortality (Article 15).

**Minutes**

Everyone with an occupational relationship with aquaculture animals have an obligation to notify increased mortalities and suspicion of listed diseases: farmers, veterinarians, veterinary authorities, transport- and slaughterhouse personnel. What is to be considered as increased mortality should be

determined on a farm-to-farm basis in cooperation between the aquaculture production business and the competent authorities.

**Questions**

Q: What should be done if mortality is seen in a closed ornamental facility?

**Sigrid Cabot:** Only parts of the directive apply to closed ornamental facilities. However, Article 26 on notification of increased mortalities and suspicion of listed diseases does apply.

**Brit Hjeltnes:** There might be differences in fish species and farms with regard to increased mortality. "In cooperation with the farmer" might not always be optimal.

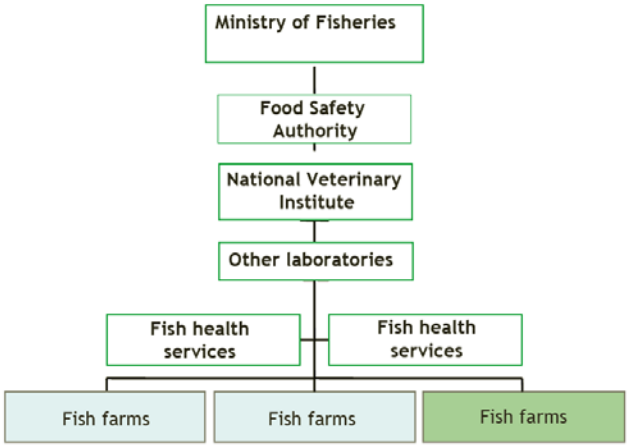
**Sigrid Cabot:** This should be dealt with by the competent authorities. Considerations on the particularities of the farm in question need to be taking into account. However, individual farmers which have high background mortality due to poor management practices should not be rewarded.

## Un-explained increased mortality in fish farming – a practical approach. Experiences from Norway

***Hege Hellberg,***
*National Veterinary Institute*

**Abstract:**
Investigation of un-explained increased mortality in fish farming is crucial to detect and control fish diseases. This presentation will give an overview of the organisation of fish health monitoring in Norway with examples from recent disease cases, including infectious salmon anaemia (ISA) and pancreas disease (PD).

A short review of the development and structure of fish health management in Norway prior to harmonisation with EU legislation will be given to provide background for the present organisation of fish health monitoring in Norway.




As part of licensing requirements, all fish farms must have a contract with an approved fish health service. These are private companies (veterinarians or fish health biologists) specialising in diagnosis, prevention and treatment of fish diseases. On-growing farms with > 50 000 fish must have at least 6 health control visits annually and brood stock farms > 50 fish a minimum of 12 visits a year.

The Norwegian Food Safety Authority is the Competent Authority on aquatic animal diseases. The NFSA monitors mortality in fish farms through monthly, web-based reports supplied by the fish farmers. Increased mortality and suspicion of listed diseases must be reported immediately. In addition, fish health services and laboratories are required to report any suspicion or diagnosis of listed diseases.

In cases of increased mortality, the NFSA (or fish health personnel appointed by the NFSA) inspects the farms. Records are checked, clinical examination and sampling of the fish is performed and samples submitted to the National Veterinary Institute or other approved laboratories for analysis. In addition to these ad-hoc inspections, the NFSA visits all on-growing farms annually in connection with surveillance programmes. Brood stock farms and hatcheries are inspected at a higher frequency.

**Minutes**

The early history of the Norwegian fish health management has its background in a tight network formed under the ministry of agriculture, aimed at supporting production by sampling, surveillance diagnose and control treatable diseases such as furunculosis. This in turn initiated regulations upon the aquaculture business. Today the legislation is placed under the ministry of fisheries and the food safety authorities, and in addition to the direct support for the farmer a broader food-chain and more proactive approach is applied. Several instances is interacting from farmers, private veterinary companies to veterinary authorities, private and official laboratories to survey and diagnose all with the responsibility to report within the national regulative.
Contract with an approved fish health service is required in licensing a fish farm. This includes onside diagnosis and treatment and management advice. Further there is detailed requirements in the regulation on operation an aquaculture establishments including: Keeping records, reporting, qualifications, production plan and contingency plan to be approved by the food safety authorities. The food safety authorities recieves reports from farmers and fish health services and follow up by doing annual inspections checking records and sampling for surveillance programs and also ad hoc inspections based on reports deviant reports or missing reports.
Example VHS: Rainbow trout farm-increased mortality - farmer calls local fish health service for diagnosis – VHS suspicion raised - sampling and diagnosis by the National Veterinary Institute - restrictions – confirmation of diagnosis by the CRL – fallowing of pens.
Example ISA: Salmon farm – reports on increased mortality – food safety authorities initiate inspection – 90% mortality, signs of circulatory disturbances, suspicion of ISA is raised – fish sent to the National Veterinary Institute - following laboratory work pancreas disease was diagnosed but not ISA.
Success of a controle and surveillance system relies upon qualified personnel on all levels and reliable methods.

**Questions.**
**Guiseppe Bovo:** It is a good system which have proven itself. However, in other countries there seems to be big differences in implementing such systems in between countries.
**Olga Haenen:** We had three cases of IHN, however the authorities didn't financially compensate the farmer and had no interest in gaining a IHN free status in our country, so, subsequently no obligatory stamping out occurred.
**Hege Hellberg:** How to deal very much depends on the circumstances in your country, the economic impact etc.

## Diagnostic sensitivity and specificity of test procedures for *Renibacterium salmoninarum* in rainbow trout

**L.M. Hall & A.G. Murray**
*Marine Scotland Science, Aberdeen, UK.*

**Anstract:**

*Renibacterium salmoninarum* is the causative agent of bacterial kidney disease (BKD), a condition which affects rainbow trout and other salmonid fish. Scotland has a surveillance programme which uses bacterial culture, ELISA and qPCR to test for infected farm sites. We have compared the reliability of these test procedures using 2700 rainbow trout sampled from a single farm-site which, although not showing signs of disease at the time of sampling, has a history of BKD. Latent Class Analysis of the categorical test results was used to evaluate the diagnostic sensitivity and specificity of the test procedures for individual and pooled groups of fish. Quantitative values were also used to further investigate the effect of pooling. This work has provided information which will be useful for improving the current surveillance programme and informing policy.

**Minutes**
Specificity for all tests seems to be around 99% on single fish, sensitivity on the other hand varies; culture: 5%, ELISA: 25%, qPCR: 99%. On pooled samples sensitivity decreases; ELISA. 6% qPCR: 36%, culture cannot be done on pooled material. Current practise is ELISA based; 30 fish in 5 fish pools, if ELISA is positive confirmatory growth is performed on 150 fish. However this practice is time and labour consuming and costly. We are considering changing this to a future practice involving qPCR on 30 fish in 5 fish pools and if positive, confirmatory ELISA on 150 fish in pools of 5 fish. The future practice will be more cost effective, confirmatory result will be available more quickly with no compromise in sensitivity and specificity.

**Questions**
**Brit Hjeltnes:** qPCR as a screening – further confirmation, why?
**Malcolm Hall:** We will not make a definitive diagnosis on one test, we need a confirmatory result!
**Hege Hellberg:** Why pool samples?
**Malcolm Hall:** The costs!

## Non-lethal sampling and virus detection in fish latently infected with koi herpesvirus (KHV)

**Sven M. Bergmann*** (D), Niels Jørgen Olesen (DK), Jeannette Castric (F), Eva Jansson (S), Marek Matras (P), Marc Engelsma (NL), Keith Way (UK) and Giuseppe Bovo (I)
**Friedrich-Loeffler-Institut (FLI), German Reference Laboratory for KHVD, Federal Research Institute for Animal Health, Südufer 10, 17493 Greifswald-Insel Riems, Germany*

Within the EU project "EPIZONE" two projects touch farmed aquatic animals. These are:

1. "WP 6.1. Surveillance and Epidemiology of emerging viral diseases in aquaculture", headed by N.J. Olesen (Denmark)
2. "Development of serological methods for detection of Koi herpes virus (KHV) antibodies in carp, *Cyprinus carpio"* (KHV-Sero), headed by J. Castric (France)

**Abstract:**

The first task of the German Reference Laboratory for Koi Herpesvirus Disease (NRL KHVD) was to develop new, highly sensitive methods for KHV detection in latently / persistently infected carrier fish (WP 6.1.) as well as non-lethal sampling methods. The second task was to produce antisera against KHV and other aquatic herpesviruses for assessment by serum neutralization assay (SNT) and antibody ELISA (KHV-Sero).
Especially in latently / persistently infected fish virus concentrations between 5 to 10 KHV DNA genomic equivalents seem to be common in organ tissues, skin and gill swabs as well as in separated leukocytes. To increase virus replication in healthy-looking, but definitely KHV infected carp (60 – 75 d p.i.), these fish were netted for 1 min to simulate transportation and then set back into the same basin. Daily, gill swabs and droppings were tested after DNA extraction for KHV presence by different PCRs, a real-time PCR and different (semi-)-nested PCRs. It was shown that the KHV concentration in gill swabs increased between factors 100 and 1000 after netting for 2 to 4 days. After a period of 5 to 7 days, virus release via the gills stopped. In droppings, KHV was detectable by real-time PCR only on day 3 after netting. These fish did not show any clinical signs of KHVD anymore over this one week period of sampling. As controls, latently KHV infected and non-infected control fish, were caught and sampled once, and the droppings from these basins were tested in the same manner.
In the frame of the "KHV-Sero" project, mainly animal experiments and serological assay developments were carried out. One experiment focussed on sera against different aquatic herpesviruses (KHV, HVA, CCV and carp pox virus). These sera were sent out to 7 project partners for testing. All sera were tested by two different SNT (against homologous and heterologous viruses) and by a KHV antibody ELISA.
While sera against KHV, HVA and carp pox virus reacted only with the homologous agents by SNT, one serum from the CCV group also reacted in KHV antibody ELISA. Additionally, CCV was the only agent which did not induce a neutralizing activity in carp over 8 weeks.
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**Minutes**

Incubation time for development of disease when infected with KHV varies depending on several factors. The time frame can be from a few days to several weeks or months. Following contact infection, disease was observed to occur until two months after exposure. Exposing carps to stress induces viremia and increase in viral load throughout the body within 1-3 days. When sampling possible carriers, inducing stress prior to sampling might increase the chance of finding carrier fish. According to the OIE manual, it is OK to pool up to two fish when fish is diseased and up to 5 when doing surveillance. However, this should be the other way around; a maximum of 2 fish should be pooled when monitoring whether a pool sample of up to 5 fish could be allowed when disease is present, where the amount of KHV virus would be expected to be high.

In 2000 the published PCR's all seemed to perform satisfyingly. From 2005 the Bercovier PCR did not seem to perform in all cases and in 2008 the Gilad and the qPCR didn't seem to work in all cases either. In 2009 our PCR still seem to perform in all cases. I believe the development in sensitivity among published PCR's is attributed to development of genetic strain differences of KHV.

**Questions**
**Mansour El-Matbouli:** How many fish should you sample when dealing with ornamental koi.
**Birgit Oidtmann:** You should report the findings in an objective manner, what did you find and how can this be interpreted with regard to the total population sampled!

## Cyprinid herpesvirus 3: to be, or not to be.

**Marc Engelsma**, Michal Voorbergen-Laarman

**Abstract:**

Cyprinid herpesvirus 3 (CyHV-3), also known as koi herpesvirus (KHV), is a devastating virus in the culture of common carp and ornamental koi. In the last decade the virus has spread rapidly over the globe to major carp culture areas. Thus far the complete genome of three CyHV-3 isolates from different geographical locations has been sequenced showing only very limited differences in the genome sequences of these three isolates. At the Central Veterinary Institute, Lelystad, the Netherlands, an *in house* real time PCR assay is used for the detection of CyHV-3. As part of the validation of the assay, results obtained by the real time PCR assay from a large number of field samples were compared with results of the conventional assay as described by Bercovier *et al*. (2005). Mismatches in the results between the assays (real time PCR positive, conventional PCR negative) were nearly all very weak positive samples and could be attributed to higher sensitivity of the real time PCR assay. Sequencing of amplicons generated with general cyprinid herpesvirus primers (developed by CEFAS), the polymerase gene and major capsid protein gene were confirmed to be identical to the described CyHV-3 isolates. However, in three cases sequences were detected with approximately 96% identity to the polymerase gene and 97% identity to the major capsid protein gene. The koi bearing these viruses were clinically healthy and originated from areas with no actual CyHV-3 outbreak. Thus far we have not been successful to isolate a CyHV-3 variant from these koi. It is therefore difficult to assess the implication of these CyHV-3 variants for the carp and the diagnostics of CyHV-3

**Questions**
**Laurent Bigarré:** Could it be the vaccine strain you are looking at?
**Marc Engelsma:** No, the variation was too big. Furthermore, the koi's in question came directly from Japan where no vaccines are used!
**Sven M. Bergmann:** Did you try to compare with the capsid gene?
**Marc Engelsma:** Yes, it found those as well!
**Søren Kahns:** Do you know anything about the pathogenecity of this strain?
**Marc Engelsma:** I think it is a virus adapted to carp and I don't know if it is pathogenic. It might be the original KHV virus from where the pathogenic KHV strain is developed!
**Richard Paley:** We do not know if it is pathogen but we isolated this strain from diseases fish.

## A novel Real-time PCR assays detecting all VHSV genotypes

**Søren Peter Jonstrup**
[1]National Veterinary Institute, Technical University of Denmark

**Abstract:**

The Rhabdovirus Viral Haemorrhagic Septicaemia Virus (VHSV) is a serious threat to many fish farmers of salmonid fish. To prevent outbreaks it is important to have a fast, sensitive, and specific diagnostic tool to identify infected fish. Traditional diagnosis based on isolation in cell culture followed by ELISA is sensitive and specific but rather slow. PCR based techniques are fast but so far no PCR has been developed that specifically detects all VHSV genotypes with a sensitivity comparable to traditional diagnosis. Here we present a Taqman based real time RT-PCR that detects all VHSV isolates in a panel of 79 VHSV isolates covering all known genotypes and subtypes and not 15 samples of related vira. The sensitivity of the PCR is comparable to traditional cell based diagnosis and it is possible to make a correct diagnosis within one work day.

**Minutes**

There is a demand for a PCR to do VHSV diagnostics directly on tissue samples. Matejusova et al. 2008 have published a qPCR covering genotype I to III. However there is a need to develop a method that performs well on all genotypes to be applicable worldwide. We have produced a qPCR covering all genotypes using a conserved part of the N-gene. Compared with Matejusova our PCR performs equally well for genotype I, comparable or better for genotype II and III, and performs well for genotype IV which Matejusova's does not recognize. When comparing the two PCR's with cell culture our method is comparable to cell culture with regard to sensitivity for all genotype, sometimes being a log less sensitive. Matejusova's were comparable with cell culture for genotype I, but slightly less sensitive for genotype II and III whereas genotype IV was not recognized in our assay.

**Questions:**

**Sven M. Bergmann:** Why does the cell culture perform better than the PCR?
**Søren Peter Jonstrup:** A larger volume of supernatant is used to inoculate cells. When covering many genotypes the PCR might perform less effective on some isolates.

# *SESSION III: Scientific research update*

## Progress in the development of seroneutralisation test (SNT) for detection of Cyprinid herpesvirus 3 (CyHV3) antibodies in carp, *Cyprinus carpio*.

**J. Castric, J. Cabon and L. Bigarré**
*Unité de Pathologie Virale des Poissons, AFSSA-Ploufragan / Plouzané*
*Technopôle Brest-Iroise, BP70, 29280 Plouzané, FRANCE*

**Abstract:**

Koï Herpesvirus disease (KHVD caused by CyHV3) has a high economical impact on the carp farming industry throughout the world. PCR-based methods are recommended for the diagnostic of KHVD in clinically affected carp, but are poorly efficient when applied to the detection of viral genome in asymptomatic carriers. In those fish, the virus may remain latent and cause the spread of the virus to naïve populations. Serological techniques, mainly ELISA, have been developed in order to detect carp populations that have previously been experimentally or naturally exposed to CyHV3 (ref). However, this method lacks specificity as observed by cross-reactions with carp pox herpesvirus (CyHV-1). For this reason, a seroneutralisation test (SNT) has been developed to detect the presence of fish antibodies directed CyHV-3 in experimentally infected carp.

The test was performed in KF-1 cell line cultivated in microplates at 24°C under $CO_2$ atmosphere. The carp plasma were diluted from 1/40 to 1/5120 and the SN reaction conducted for 16 to 18 hours at 24°C with 40 to 50 $TCID_{50}$ of CyHV-3 (isolate 07/108b) per well. The cells were then inoculated with the mixture virus and plasma and incubated for 8 to 10 days at 24°C under $CO_2$ atmosphere before fixation and staining. The reaction was considered specific since no cross reaction was observed with CyHV-1. The SNT was used to study the kinetics of anti-KHV antibodies in the plasma of 40 koï carp experimentaly infected per bath. Six months post-infection, the carps were individually marked and blood samples were then collected regularly during 18 months; 45% of the plasma were found positive for CyHV3 antibodies at the beginning and at the end of the control, 25% remained negative during the 18 months of the study, 25% were found positive in the beginning of the control but negative by the end, and 5% were found doubtful. Those results indicate that antibodies against CyHV3 are still detectable in koï carp 2 years post-exposure to the virus.

A comparison of the SNT used in this study with other serological techniques (ELISA, IFAT, Western-Blot) will be carried out in the frame a proficiency test planned in the SERO-KHV project of the European EPIZONE project.

References

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St Hilaire S., Beevers N., Joiner C., Hedrick R.P. and Way K. (2009) Antibody response of two populations of common carp, *Cyprinus carpio* L., exposed to koi herpesvirus. *Journal of Fish Diseases, 32 (4), 311-320.*

**Minutes:**

KHV can be quite difficult to detect even by the use of molecular methods. Therefore it is desirable to be able to detect KHV antibodies in the fish instead. In order to develop serologic techniques and to study the kinetics an infection trial was performed.

Infection trial: 2 days at permissive temperature of 23°C, then change in temp to 29°C or 10°C which is not permissive for the virus to help the koi to survive the infection and produce antibodies. The developed ELISA test is specific (no neutralisation of CyHV1 using plasma from fish infected with CyHV3 and vice versa). Antibodies can be found in fish for 2 years.

**Questions:**
**François Leiffrig:** Do you use serum or plasma?
**Laurent Bigarré:** We use plasma.
**Sven M. Bergmann:** You get similar results whether you use plasma or serum.
**Tomás Veselý:** Do you keep the koi at the same temperature for 2 years?
**Laurent Bigarré:** No.
**Niels Jørgen Olesen:** The first blood sampling was 5½ month after infection. Did you try earlier?
**Laurent Bigarré:** We could detect antibodies already after 1 month.
**Sven M. Bergmann:** At 20°C you can detect antibodies already after 12 days.

## Vertical transmission - PD and ISA

**Brit Hjeltnes**

**Abstract:**
In 2009 the Norwegian Food Safety Authorities asked the Norwegian Scientific Committee for Food Safety to perform a risk assessment on vertical transmission of notifiable diseases in Norway. In addition the committee was asked to include diseases under consideration for the national list of notifiable diseases and give advice on which diseases should be included in national surveillance programs. To prepare scientific background documents necessarily to answer the questions, the panel on Animal Health and Welfare established an *ad hock* group consisting of four experts. Infectious Salmon Anaemia Virus (ISAV), Infectious Pancreatic Necrosis Virus (IPNV), nodavirus, Salmonid Alphavirus (SAV), *Flavobacterium psychrophilum*, *Piscirickettsia salmonis* and *Francisella philomiragia* were assessed. Heart and Skeletal Muscle Inflammation (HSMI) was excluded from the assessment due to lack of information.

The Committee used the OIE`s Aquatic Animal Health Code (2008) definition for vertical transmission:" Vertical transmission means the transmission of a pathogen from parent aquatic animal to its progeny via sexual products". The risk of vertical transmission was assessed for Norwegian aquaculture and when disinfection procedures are carried out according to official Norwegian requirements and procedures.

Based on the risk assessment, neither ISAV nor SAV were recommended to be included in Norwegian national surveillance programs.

**Minutes:**
The Norwegian scientific committee for food safety www.vkm.no was asked for a risk assessment on vertical transmission. The report in Norwegian can be found on the web. The OIE definition of vertical transmission was used, so it was not taken into account whether the pathogen was situated inside or outside the egg. The committee has looked at the question based on Norwegian procedures and aquaculture and not taken into account aquaculture procedures used in other countries. E.g. it was taken for granted that the farmers follow the procedures for disinfection prescribed by Norwegian law. The information used for this report was scientific papers and open information from the industry.

**Salmonid alphavirus (SAV)**

Sea water: Outbreak and virus isolation from all sizes of fish, including broodstock.

SAV positive broodfish: Eggs from SAV positive broodfish was examined by Bratland & Nylund, 2009:

- 1/220 non fertilized eggs
- 1/220 eyed eggs
- 2/430 fry

were positive for SAV by realtime-PCR.

Detection of SAV in fresh water hatcheries:

- 16/35 hatcheries positive by realtime-PCR (Nylund & Bratland, 2009)
- 0/15 (2007), 0/10 hatcheries (2009), 0/35 (2007-2009) from endemic areas (PatoGen)
- 0/46 hatcheries (Jansen et al, unpublished)
- No positive findings after testing on brood fish and offspring in Ireland (Graham, pers. com.)
- No evidence of SAV in fresh water based on findings at the National Veterinary Institute.
- False positive pcr-results has been observed caused by
    - Vaccinated fish (PatoGen)
    - Contaminated sampling equipment (PatoGen)

Experimental studies:

- Vertical transmission of SAV2 reported after injection of brood fish (Castric pers. com.). Large amount of virus injected.
- No evidence of vertical transmission after screening eggs and offspring of SAV3 positive brood fish. More than2000 samples analysed (Kongtorp et al, unpublished).

Geographical evidence:

- SAV1, 4, 5 and 6 in Atlantic salmon in seawater in Ireland and Scotland
- SAV2 cases (sleeping disease) in rainbow trout in fresh water in several European countries
- SAV3 in Norway
- Different geographical distribution of different subtypes of SAV in Europe despite transportation of eggs during many years
- SAV has not been detected in North America or Chile despite import of eggs from e.g. Norway.

Conclusion:

- In endemic areas in Norway the risk of brood fish being infected with SAV and become carriers are considered to be high
- There are reports of egg and milt testing positive for SAV but this has not been confirmed by several other research groups
- The risk of vertical transmission of SAV is regarded to be low

**Infectious salmon anaemia (ISA)**

Eggs, embryo juveniles smolt and post smolt reported to test pos for ISAV in Norway by one research group who concluded that the most important route of ISA transfer is by vertical transmission. These finding have not been confirmed by other groups.

Conclusion: Vertical transmission of ISAV cannot be excluded but is of minor significance in Norway.

**Questions:**
**Eann Munro:** Are broodfish routinely screened for PD and ISA?
**Hege Hellberg:** No, not routinely but there has been a limited surveillance by the food safety authority. But the broodfish farmers may use private companies for screening.

## Heart and Skeletal Muscle Inflamation (HSMI) – an emerging disease in salmon, new results indicating a reovirus

**Irene Ørpetveit**
*National Veterinary Institute*

**Abstract:**

Heart- and skeletal muscle inflammation (HSMI) is a serious infectious disease of farmed Atlantic salmon, affecting young salmon 5-9 months after seawater transfer with up to 20% mortalities. HMSI was discovered in 1999, and since then, disease outbreaks have occurred in salmonid fish farms along the entire Norwegian coastline. The cause of the disease has long been unknown, although based on results from experimental challenges, a viral aetiology has been suspected. The results from pyrosequencing of total RNA from heart and serum samples from fish experimentally challenged with HSMI indicate that the disease is caused by a novel virus, provisionally called piscine reovirus (PRV). Pyrosequencing resulted in 90% coverage of the viral RNA, and subsequent Sanger sequencing and RACE indicate that the viral genome consists of 10 segments and a total of 23 300 bp. Bioinformatic analysis revealed only 1,5 % identity with known reoviruses at the nucleotide level, and 54 % identity at amino acid level, indicating that the HSMI virus may represent a new genus.

**Minutes:**

Identification of a novel virus associated with HSMI. HSMI was first described in 1999. The disease has not been described in any other countries. All diagnostics is based on histology and pathology.

Determination of disease agents has been pursued by

- Cultivation
- Classical molecular methods – consensus PCR
- Lots of shots in the blind.
- New technology: pyrosequencing.

Pyrosequencing is rather expensive. Extremely large amounts of data must be analyzed. It is difficult to obtain full length sequences.

The project was initiates based on experimental challenge studies. Tissue from fish with HSMI induced clinical signs consistent with HSMI in infected fish. Pre-treatments of tissue from diseased fish had no effect – was that because the pathogen was a virus?

Pyrosequencing:

- Which organs to select?
  - Organs most likely to contain the agent
  - Organs which are likely to contain as few organisms as possible (avoid intestine)
  - For HSMI: heart and blood chosen
- Sequence analysis implied the presence of an aquareovirus.
- Mock-infected fish were negative
- No other viruses were identified.
- Viral genome: 23300 bp on 10 segments.
- A provisional name for the virus is piscine reovirus (PRV).
- Phylogeny: does not cluster with anything known – new genus?

RT-qPCR:

- Very high viral load in fish with HSMI
- Low load in healthy fish
- $10^4$-$10^6$ times more viral load in diseased fish compared to healthy fish

Diagnosis:

- Several pcr methods developed
- Antibodies against the virus are available

The present work has been submitted for publication in PLoS One.

Dr. Torstein Tengs at the NVI will be very interested in testing organs from other salmonids than rainbow trout and Atlantic salmon for this virus.

**Questions:**
**Heike Schütze:** Has the virus been seen by EM?
**Irene Ørpetveit:** The group has not done any work with EM, but others claim they have, but it has not been published.
**Brian Dall Schyth:** Was the sample DNase treated? What about DNA viruses?
**Irene Ørpetveit:** I do not know if the samples were DNase treated. They isolated total RNA because mRNA from DNA viruses should be included.
**Eann Munro:** We have isolated reovirus from a wild returning sea trout.
**Niels Jørgen Olesen:** We have also isolated reovirus by cell cultures from Denmark and Iceland from fish without clinical signs. In China grass carp reovirus is causing severe mortality. It could be interesting to compare the PRV and grass carp reovirus.
**Irene Ørpetveit:** I am sure Torstein will be interested in comparing these two reoviruses, and I will pass on the information.

## Club 5 project: Epizootic Ulcerative Syndrome (EUS): Development and implementation of diagnostic methods

**Olga Haenen (CVI, NL),** Birgit Oidtmann (CEFAS, UK, invited EUS expert), Søren Kahns (DTU Vet, DK), Eva Jansson (SVA, SWE), Thorbjörn Hongslo (SVA, SWE), Marc Engelsma (CVI, NL), Ineke Roozenburg (CVI, NL), Nicole Nicolajsen (DTU Vet, DK), Michal Voorbergen (CVI, NL), Anna Aspán (SVA, SWE), Anne Marie Lassen-Nielsen (DTU Vet, DK), Niels Jørgen Olesen (DTU Vet, DK)

**Abstract:**
Epizootic Ulcerative Syndrome (EUS), caused by the oomycete fungus *Aphanomyces invadans (A. piscicida),* is a notifiable (OIE & EU) emerging (for EU exotic) disease of >60 fish species in Asia, Australia, N-America, with a recent outbreak in the Zambezi River in Africa. EUS must, if introduced into the EU, be eradicated immediately. Since August 2008, all NRL's for Fish Diseases of the EU should be able to diagnose EUS, but most have no Standard Operating Procedures (SOPs) and reference materials yet. Since the EUS workshop for NRL's of Fish Diseases at Aarhus, June 2008, CEFAS and the CRL for Fish Diseases worked together with the EUS OIE Reference laboratory in Bangkok. Although some tests for detection of *A. invadans* and diagnosis of EUS are described in scientific literature and in the OIE Manual, SOPs for the NRL's for Fish Diseases are needed.
This Club 5 project (by CVI, SVA and Vet DTU, with B.Oidtmann (CEFAS) as invited expert, Sept 2009-April 2011), aims to introduce the best confirmatory methods for EUS into the NRL's for Fish Diseases of the EU, in collaboration with the OIE Reference laboratory in Bangkok:

- Culture the reference strain at the participating labs first
- Try out described EUS methods (fresh smears, fungus isolation, histopathology, PCR) and choose best methods for standardisation and SOPs
- Develop alternative methods (e.g. immunohistochemistry, TaqMan PCR), and validate and implement them.
- Establish an electronically available slide collection for EUS histopathology
- Write SOPs for the best diagnostic tests for EUS based on our findings.

A kick off meeting was held March 2010 at CVI of WUR, Lelystad, NL, which resulted in many action points. Division of tasks, activities, progress and plans will be presented in this lecture.

**Minutes:**
Aims of the project: To introduce the best confirmation methods for EUS to the NRLs

**Mansour El-Matbouli:** We got a strain from ATCC that turned out not to be *Aphanomyces invadans*.
**Søren Kahns:** This strain has now been withdrawn. I looked for *A. invadans* in ATCC and could not find it anymore.

## Access to facilities within the Network of Animal Disease Infectiology and Research Facilities – NADIR.

**Torsten Snogdal Boutrup**
*National Veterinary Institute, Technical University of Denmark*

**Abstract:** The NADIR project lies within the Seventh Framework Programme of the European Community, grant agreement n$^{o}$ 228394 and is a project running from 2009-2012. It is focused on unifying and coordinating the interests of- and access to infectiology and research facilities in EU and collaborative states for all major groups of production animals, fish, pig, cattle, sheep, poultry and also mouse as the most widely used laboratory animal.
Within the NADIR consortium fish-groups from three institutions participates. Veterinary Science Opportunities (VESO Vikan) Norway, Institut National de la Recherche Agronomique (INRA) France and National Veterinary Institute, Technical University of Denmark (DTU Vet) Denmark. The first part of the project has been to coordinate interests in respect to the overall NADIR project, and to collaborate on exchanging rainbow trout and salmon strains normally used for infection trials at the partnering institutions to be tested towards susceptibility to VHSV, IHNV, IPNV and PD.
As of November 2009 access to the facilities at VESO Vikan and DTU Vet for European research groups to conduct infection trials at the facilities has been announced on the NADIR homepage; http://www.nadir-project.eu/nadir_project/call_for_access where details about access can be found. Access to the facilities is based on a preliminary application sent via the online application form on the homepage. Upon acceptance of the preliminary proposal a final application should be sent which is assessed by a scientific committee for review. Further the project leader in a proposed activity must be from a different European state than the one in which the activity is applied for.
In between VESO Vikan and DTU Vet there is a broad opportunity to apply for conduction of infection trials on several fish species with a variety of pathogens; both viral, bacterial and parasitic and with manipulation of several physical and chemical water parameters. Access to the facilities typically include all or part of the expenses paid for travelling back and forth, board and wages, help to or performing infection trials, daily care, sampling and termination of the trial and depending on the type of setup some virological or other laboratory testing on sampled fish.

**Minutes:**
The NADIR project is divided into the following activities:
NA, Network activities

- Increase communication between partners
- Exchange of knowledge, materials and methods

RA, Research activities

- To improve the service provided by the facilities
- Characterization of animal and cell lines
- Production of reference material
- Development of animal models

TA, Transnational access

- To provide access for European research groups to infection facilities within the network
- For the fish areas this means VESO Vikan and DTU Vet
- This opportunity have been made available as of November 2009 and has been called for on the NADIR website
- Access include
    - VESO Vikan: 10 tank months (8 trials)

- DTU Vet: approximately 1 month access to facility (3 trials to hand out)
  - Help to or performing infection trials
  - Daily caretaking, sampling and termination of trial
  - Access to laboratory facilities, wet and dry
  - Some laboratory work and testing
  - Travelling expenses and staying for 1 person for 3 weeks

Access is possible for

- Research groups or consortium of research groups
- Project leader must be from a different country than the facility in which the project is applied for

Application

- Application is done by online submission on the NADIR homepage
- Preliminary application followed by
- Final application
- Both applications is reviewed by a scientific committee

VESO Vikan

- Access to different sizes of tanks
- The following fish species can be included:
  - Atlantic salmon
  - Sea bass
  - Cod
  - Rainbow trout
  - Many others
- Experience with a broad range of pathogens

DTU Vet

- Mainly small tanks
- Both fresh and salt water
- Fish species
  - Rainbow trout
  - Also salmon
  - Pikeperch
  - Pike
  - Saltwater species
- Experience with mainly VHSV, IHNV, but also *Aeromonas salmonicida, Yersina ruckeri, Flavobacterium psychrophilum*
- Only limited experience in parasites

**Questions:**
**Brit Hjeltnes:** I am a bit negative towards the limitations of the groups that can apply, e.g. a Danish group cannot apply to do anything at DTU Vet, and a Norwegian group can do nothing in VESO Vikan.
**Torsten Snogdal Boutrup:** This was a very strong point in the EU application. This should be an opportunity for countries not having access to research facilities. This is also to encourage cooperation between countries. It shall, however, be underlined that colleagues from the host country cannot be leader of the proposal, but are most welcome as participant in a consortium.

## Perch Rhabdovirus infection in farmed pike-perch and perch – an emerging disease

**L. Bigarré**, J. Cabon, M. Baud and J. Castric
*Pathologie Virale des Poissons, AFSSA, Technopôle Brest-Iroise, 29280 Plouzané, France*

**Abstract:**
Perch (*Perca fluviatilis*) an pike-perch (*Sander lucioperca*) are increasingly used in aquaculture in Europe since they represent an interesting alternative to diversify the market of fish products. Consequently, infectious diseases should have an increasing impact on the production, unless strategies for control are urgently developed. Among the pathogens of importance, rhabdoviruses are already responsible for serious losses of fry in farms, for instance in France, Ireland and Denmark. Often, the viral contaminations are introduced in the farm by the capture of genitors or eggs from the wild. Specific and sensitive diagnostic tools are urgently needed to select genitors free of virus and prevent vertical dissemination.
However, poor data are available concerning percids rhabdovirus populations (2, 3). Our preliminary results indicate that at least two distinct rhabdoviruses, belonging to the vesiculovirus genus, are found in percids in France, and supposedly in Europe. Furthermore, some molecular relations have been found between perch vesiculoviruses and isolates from other fish species, such as sea trout (*Salmo trutta*)(1, 4), suggesting horizontal transmission between various hosts in the wild.
More isolates from Europe should be studied for a more comprehensive inventory of the viral populations, both in farms and in the wild. This is a prerequisite for developing serological tests and PCR probes.
With several European partners, we propose to tackle this goal in a project called ViPer (Virus of Percids).

1. **Betts, A. M., D. M. Stone, K. Way, C. Torhy, S. Chilmonczyk, A. Benmansour, and P. de Kinkelin.** 2003. Emerging vesiculo-type virus infections of freshwater fishes in Europe. Diseases of Aquatic Organisms **57:**201-12.
2. **Dannevig, B. H., N. J. Olesen, S. Jentoft, A. Kvellestad, T. Taksdal, and T. Håstein.** 2001. The first isolation of a rhabdovirus from perch (*Perca fluviatilis*) in Norway. Bulletin of the european association of fish pathologists **21:**186-194.
3. **Dorson, M., C. Torchy, Chilmonczyk S, P. de Kinkelin , and C. Michel.** 1984. A rhabdovirus pathogenic for perch (*Perca fluviatilis* L.): isolation and preliminary study. Journal of Fish diseases **7:**241-245.
4. **Johansson, T., S. Nylund, N. J. Olesen, and H. Bjorklund.** 2001. Molecular characterisation of the nucleocapsid protein gene, glycoprotein gene and gene junctions of rhabdovirus 903/87, a novel fish pathogenic rhabdovirus. Virus Res **80:**11-22.

**Minutes:**

Perch and pike perch are tasty fish and a traditional high value niche product. The fish are moderately tolerant to environmental conditions. The global production is very small compared to trout, capture 30.000 tons, aquaculture 300 tons per year. There is a need to understand better the reproduction parameters (light, temperature, food etc.). Diseases: bacteria, parasites etc. Rhabdoviruses (mild to heavy losses).
Until now, no complete genome of the percid rhabdoviruses have been sequenced. In the literature there is only poor data concerning percid rhabodoviruses, less than 10 papers are published. The first outbreak caused by percid rhabdovirus occurred in a laboratory in France in 1980 (Dorson et al. 1980)

Outbreaks have also occurred in Ireland, Denmark, Norway and other countries. Percid rhabdovirus belong to the vesiculoviruses.
No efficient PCR test has yet been published. There are serological reagents available but methods are not standardised between laboratories.
Healthy fish from the wild are viral reservoirs. The farmers use wild fish as brood fish or collects eggs in the wild. There may be high mortality in the fry.

The viral population

- Serology
  - Strong relationship between the original isolate from perch in France and pike from Denmark and pike-perch from France. The Danish isolate from pike had moderate relations with perch from Norway and lake trout form Finland.
  - There is a need to develop serologic reagents
- Molecular methods
  - A small domain in the L-gene has been sequenced (Batta et al. 2003) and the perch isolate is different from SVCV, PFRV and lake trout rhabdovirus. Two isolates from pike from Denmark were quite different. More sequences are needed and from other regions.
  - Full G-gene has been sequenced from 8 isolates from 1980 to 1999 from perch and sander. These isolates fall into genogroup IV.
  - Another isolate from France is completely different from the others and fall into genogroup I with lake trout rhabdovirus.
- How many viruses and how many hosts?
  - At least 2 viruses, possibly more and several hosts.
  - There is a need of molecular data to identify the viral population

Viper project: The project has is collaboration between France, Denmark, Sweden and Finland. If granted through EMIDA it will run for 2 years.
The aims are to

- acquire genetic data from European rhabdovirus collections by sequencing.
- design probes for qPCR.
- develop and standardise serologic tools.
- suggest egg disinfection assays not to introduce the virus from the wild to farms

**Questions:**
**Olga Haenen:** About 15 years ago we isolated a rhabdovirus from roach. It was not pathogenic to pike any more, but had fully adapted to cyprinids, which got diseased after artificial induced infection. Are you planning to do infection experiments like this?
**Laurent Bigarré:** Yes, we are also planning to do infection trials.

## MicroRNA regulation as a future diagnostic tool

**Brian Dall Schyth,**
*National Veterinary Institute, Denmark*

**Abstract:**
MicroRNAs belong to a family of small noncoding RNAs which have emerged as important regulators of gene expression. These are initially transcribed as longer single stranded RNA species which fold up into stem loop structures. They are subsequently modified to generate mature 21-25 bp long double stranded microRNAs, of which one or both strands show complementarity to sites in target mRNA(s). In the targeting process the microRNA is used by a large protein assembly called the RNA Induced Silencing Complex (RISC) to find and inhibit transcription by either blocking or cleaving the mRNA target. The fate of the mRNA is probably dependent on the degree of complementarity between the microRNA and the target. Vertebrates have more than 1000 different microRNAs and computational predictions point to that there are single microRNAs having more than 100 different target genes. Accordingly, microRNAs are expected to have a role in gene regulation in various situations including disease and immune response which has been verified in mammals and invertebrates. Our aim is to describe and explain regulation of microRNAs during infections with the rhabdovirus *Viral Hemorrhagic septicaemia virus* (VHSV) in rainbow trout. In addition, we want to describe co-regulation with other genes in combination with bioinformatics and work in cell cultures in order to elucidate the immune state at which microRNAs are regulated and if possible target connections. A perspective of this work is the use of microRNAs as diagnostics for immune or disease state, of vaccine or adjuvant potency and perhaps as a diagnostic of pathogens identity if pathogen specific microRNAs can be identified.

**Minutes**
Same probes can be used for most animals and humans as the microRNAs are very conserved.
When you want to examine for microRNAs you have to extract using kits that will give you these small RNAs (21-25 bp). For doing the PCR you have to extend the size of the microRNA to be able to stuff in some primers.
In rainbow trout during immune response we have a significant up-regulation of microRNAs. More specific diagnosis of pathogen type will probably be restricted to cases where the microRNA is produced by the pathogen itself – evidence from DNA viruses. It seems that the L-polymerase is targeted by the microRNAs associated with VHSV infection by bioinformatics approach.

**Questions:**
**Hege Hellberg:** It would be interesting to determine the point/time of infection?
**Brian Dall Schyth:** Yes - the time of infection and the stage of infection vary for the fish we investigated, but we tried to keep it at a minimum by choosing fish which showed first signs of disease although we do realize that this mean that we will mainly find regulated microRNAs indicative of late stage of infection. But it is a good idea to relate microRNA regulation to the level of virus as an indicator of age of infection. But this can only be an indicator. It is hard to determine directly the time of infection as this may vary between fish being challenged together.

## *SESSION IV: Update from the CRL*

### Results and outcome of the Inter-laboratory Proficiency Test 2009

**Søren Kahns**, Nicole Nicolajsen, Maj-Britt Christophersen, Helle Frank Skall and Niels Jørgen Olesen
*Community Reference Laboratory for Fish Diseases, Technical University of Denmark, Aarhus, Denmark*

**Abstract:**
A comparative test of diagnostic procedures was provided by the Community Reference Laboratory (CRL) for Fish Diseases to 36 National Reference Laboratories (NRLs) in the start of September 2009. The test was prepared and tested according to protocols accredited under DS/EN ISO/IEC 17025 and ILAC-G13:08/2007 standards. The test contained five coded ampoules. Four contained viral haemorrhagic septicaemia virus (VHSV) genotype Ie and IVa, infectious haematopoietic necrosis virus (IHNV) genogroup L and epizootic haematopoietic necrosis virus (EHNV), respectively. Furthermore, one ampoule did not contain any virus, only medium. The proficiency test was designed to primarily assess the ability of participating laboratories to identify the notifiable fish viruses VHSV, IHNV and ENHV (all listed in Council Directive 2006/88/EC). It was decided at the 13th Annual Meeting of the NRLs for Fish Diseases in Copenhagen 26-28 May 2009, that testing for EHNV for the first time should be included in this test.
Participants were asked to titrate the viruses to assess the cell susceptibility for virus infection in the respective laboratories. 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 monolayered cell cultures followed by e.g. neutralisation test, immunofluorescence, ELISA or PCR. If ranaviruses should be present in any of the ampoules, it was mandatory to perform a sequence analysis of the isolate in order to determine if the isolate is EHNV.

Outcome of Inter-laboratory Proficiency Test 2005: Identification of content: 24 laboratories out of 36 correctly identified all viruses in all ampoules. Three laboratories identified the virus in ampoule I only as a ranavirus. 6 laboratories identified a virus in one or more ampoules that were not present. Two laboratories did not identify virus in one or more ampoules where a virus was present. Finally, one laboratory did not submit their results.
*Methods applied:* The general trend was that laboratories which applied more tests to identify samples, scored higher than those, which relied on fewer types of laboratory tests. 24 laboratories used ELISA for identification of viruses, 21 used IFAT, 11 used neutralisation tests, 34 used PCR and 30 laboratories performed sequencing for identification of viruses. 29 laboratories used BF-2 cells, 34 used EPC cells, 12 used RTG-2 cells and 10 used FHM cells.

Concluding remarks: EHNV was included in the proficiency test and 28 participants were able to correctly identify the virus. This is considered to be a relatively large number of participants as it is the first time EHNV is part of the test and because identification of the virus include sequence analyses which has not been mandatory to use in previous tests. Nevertheless, EHN is a listed disease and all laboratories are obliged to implement diagnostic tools for identifying EHNV as soon as possible. Laboratories are encouraged to use a combination of cells as described in Commission Decision 2001/183/EC. However, the bad performance in several laboratories of their RTG-2 cell lines for growth of VHSV is worrying as is it described in Commission Decision 2001/183/EC that RTG-2 cells can be used instead of BF-2 cells. Based on these observations, laboratories are recommended to use BF-2 cells and not RTG-2 cells for replication/survey of/for VHSV. In general, it is recommended that participants evaluate the sensitivity of their cells lines in relation to the diagnostic purpose.

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

**Minutes**
Not many laboratories using PCR methodology are accredited we can only encourage laboratories to become accredited.
In the forms of the proficiency test we asked a number of questions on which methods you used in order to be able to better understand why things sometimes go wrong. Unfortunately it was not possible to make definite conclusions based on this. Many wrong results seem to occur because of cross contamination events.
It was surprising that only few laboratories subcultivated sample V, as it did not contain any viruses. According to the Commission Decision 2001/183 laboratories have to subculture once if no CPE can be observed in cell cultures 7 days after inoculation. For the future we recommend laboratories to do so, also in proficiency tests.
EHNV replicates well on EPC and BF-2 cell lines. VHSV replicates well on BF-2 cells and IHNV on EPC and FHM cells. So the combination of BF-2 and EPC cells seems to be a good combination for VHSV, IHNV and EHNV. But each laboratory has to look on their own cell lines and judge which cell lines will be the best for them to use.
If relevant the CRL provided the laboratories with comments on how they fared in the test. This provided us with a lot of response and good feedback. The possibility for providing comments will also be used in future tests.
The audience was asked if they have any comments, remarks or proposals for improvement of the proficiency test. No direct replies were given and this taken as a sign of satisfying form and performance of the proficiency test 2009.

The next ring test will also include ISAV and KHV.

**Questions:**
**Irene Ørpetveit:** You say VHSV grow better on BF-2 than EPC cells. In Norway we saw that genotype III grew better on EPC cells.
**Niels Jørgen Olesen:** It also seems that genotype IV grows better on EPC cells. It can vary from isolate to isolate but by using a combination of cells you should be able to identify any viruses.
**Niels Jørgen Olesen:** Were cross contaminations mainly seen in laboratories using PCR directly on the ampoules?
**Søren Kahns:** No, cross contaminations were not only caused by PCR as some of the laboratories that were reporting virus in ampoule V did observe CPE and titres on 96-well plates.

## FishPathogens.eu

**Søren Peter Jonstrup**
*Community Reference Laboratory for Fish Diseases, Section for Fish Diseases, National Veterinary Institute, Technical University of Denmark, Denmark*

**Abstract:**
FishPathogens.eu was launched at last annual meeting with a database on VHSV. This database has now grown to include around 400 public available VHSV isolate reports and 250 sequence reports. Almost 100 persons have registered as users and 13 of these have reports stored in the database. Earlier this year an IHNV extension was revealed. In this talk I will give a brief overview on what has happened with the database since last annual meeting.

**Minutes:**
The number of registered users has risen from 14 last year to now 95. The number of visits seems to be stabilizing around 400 per month. Around 1/3 of the visits during the last 3 months has been from USA. Denmark accounts for around 1/5 of the visits,. There are also a lot of visits from Asia. The number of VHSV reports has doubled since last year. The IHNV database has just been launched.
Future databases are planned: SVCV in collaboration with Dr. David Stone from CEFAS, ISAV in collaboration with Dr. Mike Snow, Marine Scotland and KHV in collaboration with Dr. Marc Engelsma from CVI, Lelystad.
We hope soon to be able to include online phylogeny in cooperation with bluetonguevirus.org.

**Questions:**
**Irene Ørpetveit:** The phylogeny will it be using default algorithms?
**Søren Peter Jonstrup:** Yes, the algorithms will be default, but we hope you can be able to choose different parts of the virus genomes. Maybe there will be a possibility to choose between 2 algorithms. Presently only DNA but not proteins are planned to be used for making phylogenetic trees. Proteins are included in the database, though.
**Niels Jørgen Olesen:** In order to secure the existence of database in the future it is linked to the CRL function.

## CRL achievements in 2009

**Søren Kahns**, Nicole Nicolajsen, Helle Frank Skall, Søren Peter Jonstrup and Niels Jørgen Olesen
*Community Reference Laboratory for Fish Diseases, Technical University of Denmark, Aarhus, Denmark*

### I. LEGAL FUNCTIONS AND DUTIES

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

II. CRL OBJECTIVES FOR THE PERIOD JANUARY - DECEMBER 2009

1. Organise and prepare for the 13th Annual Meeting for the National Reference Laboratories for Fish Diseases in 2009 (most likely to be held at the DTU National Veterinary Institute Department in Copenhagen, Denmark in May 2009).
2. Produce a report from the Annual Meeting 2009.
3. Collect data on the fish disease situation in EU, including all the listed non-exotic fish diseases given in Council Directive 2006/88/EC Annex IV Part 2
4. Identify and characterise selected isolates of listed viruses (serological and genetic characterisation)
5. Production of antisera against selected isolates when necessary.
6. Assessment and standardisation of Real-Time PCR tests for the diagnosis, identification and typing of the listed non-exotic fish diseases.
7. Develop, update and maintain the new EU Community Reference Laboratory for Fish Pathogens Database. A database created in order to collate all available information of isolates of listed fish pathogens including their origin, their sequences and their geographical coordinates
8. Update and maintain a library of isolates of Infectious salmon anaemia virus (ISAV), Viral Haemorrhagic Septicaemia virus (VHSV) and Infectious Haematopoietic Necrosis virus (IHNV), Spring Viraemia of Carp virus (SVCV) and Koi Herpes virus (KHV)
9. Preparation and standardisation of control reagents for use in PCR tests. Assessment of viral inactivation and viability of standard reagents for use as reference material in molecular tests.
10. Organise a workshop in the implementation of Council Directive 2006/88/EC (to be organised back to back with the 13th Annual Meeting).
11. Update the new webpage for the CRL.
12. Supply standard antisera and other reference reagents to the National Reference Laboratories in Member States.
13. Prepare the Annual Inter-laboratory Proficiency Test year 2009 for the National Reference Laboratories.
14. Collate and analyse information gained from the Inter-laboratory Proficiency Test
15. Facilitate and provide training in laboratory diagnosis.
16. Attending missions, international meetings and conferences. Missions will focus on accession countries and OIE reference laboratories on listed exotic and non-exotic fish diseases.

## CRL workplan for 2010 – ideas and plans for 2011

**Niels Jørgen Olesen**, Nicole Nicolajsen, Helle Frank Skall, Søren Peter Jonstrup, Torsten Boutrup and Søren Kahns
*Community Reference Laboratory for Fish Diseases, Technical University of Denmark, Aarhus, Denmark*

OBJECTIVES FOR THE PERIOD JANUARY - DECEMBER 2010

1. Organise and prepare for the 14th Annual Meeting for the National Reference Laboratories for Fish Diseases in 2010.
2. Produce a report from the Annual Meeting 2010.
3. Collect data on the fish disease situation in EU, including all the listed non-exotic fish diseases given in Council Directive 2006/88/EC Annex IV Part 2
4. Identify and characterise selected isolates of listed viruses (serological and genetic characterisation)
5. Production of antisera against selected isolates when necessary.
6. Assessment and standardisation of Real-time PCR tests for the diagnosis, identification and typing of the listed non-exotic fish diseases.
7. Expanding www.fishpathogens.eu with IHNV, SVCV and the inclusion af KHV and ISA will be initiated.
8. Update and maintain a library of isolates of Infectious Salmon Anaemia virus (ISAV), Viral Haemorrhagic Septicaemia virus (VHSV) and Infectious Haematopoietic Necrosis virus (IHNV), Koi Herpes virus (KHV) and enzootic haematopoietic necrosis virus (EHNV).
9. Update the webpage for the CRL, www.crl-fish.eu
10. Update and include standard operating procedures on the CRL web page for the listed exotic and non-exotic diseases
11. Workshop on available kits and reagents for diagnosis of the listed non-exotic diseases VHS, IHN, ISA and KHV including consideration of their sensitivity and specificity.
12. Supply standard antisera and other reference reagents to the National Reference Laboratories in Member States.
13. Inclusion of SOP´s on serological methods for detection of fish antibodies against VHSV, IHNV and KHV on CRL website, and introducing the methods in new Commission Decision on sampling and diagnostic procedures
14. Prepare the Annual Inter-laboratory Proficiency Test year 2010 for the National Reference Laboratories. The test will be expanded to also include ISAV and KHV.
15. Collate and analyse information gained from the Inter-laboratory Proficiency Test
16. Establish diagnostic methods for diagnosis of EUS and assess the possibilities for including *Aphanomycis invadans* in proficiency test in future.
17. Facilitate and provide training in laboratory diagnosis.
18. Attending missions, international meetings and conferences. Missions will focus on NRLs where on-site communication would be beneficial. And to reference laboratories on listed exotic and non-exotic fish diseases in order to be updated on diagnostic methods.

**OBJECTIVES FOR THE PERIOD JANUARY - DECEMBER 2011**

1. Organise and prepare for the 15th Annual Meeting for the National Reference Laboratories for Fish Diseases in 2011.
2. Produce a report from the Annual Meeting 2011.
3. Collect data on the fish disease situation in EU, including all the listed non-exotic fish diseases given in Council Directive 2006/88/EC Annex IV Part 2.
4. Identify and characterise selected isolates of listed viruses (serological and genetic characterisation).
5. Production of antisera against selected isolates when necessary.
6. Assessment and standardisation of Real-time PCR tests for the diagnosis, identification and typing of the listed non-exotic fish diseases.
7. Update and expand www.fishpathogens.eu with other pathogens.
8. Update and maintain a library of isolates of Infectious salmon anaemia virus (ISAV), Viral Haemorrhagic Septicaemia virus (VHSV) and Infectious Haematopoietic Necrosis virus (IHNV), Koi Herpes virus (KHV) and enzootic haematopoietic necrosis virus (EHNV).
9. Update the webpage for the CRL, www.crl-fish.eu
10. Establish and maintain a library of tissue material from infected fish.
11. Update the diagnostic manuals for the listed diseases on the CRL web page.
12. Include diagnostic manuals for EUS on the CRL web page.
13. Supply standard antisera and other reference reagents to the National Reference Laboratories in Member States.
14. Perform molecular epidemiology analysis to improve knowledge on diseases spreading mechanisms of viral pathogens
15. Prepare the Annual Inter-laboratory Proficiency Test year 2011 for the National Reference Laboratories. The test will include VHSV, IHNV, EHNV, ISAV and KHV.
16. Collate and analyse information gained from the Inter-laboratory Proficiency Test
17. Update diagnostic methods for diagnosis of EUS and assess the possibilities for including *Aphanomycis invadans* in proficiency test in future.
18. Facilitate and provide training in laboratory diagnosis.
19. Establish and offering a yearly training course in methods used for diagnosis of fish diseases, at the CRL laboratory facilities. The content will depend on request from participants
20. Organizing missions to relevant laboratories. Missions will focus on NRLs where on-site communication would be beneficial.
21. Attending missions, international meetings and conferences in order to be updated on diagnostic methods on listed exotic and non-exotic fish diseases.

**Minutes**

The diagnostic manuals that will be published on the CRL webpage are mandatory to follow and not guidelines as the OIE manuals. Regarding EUS it may end up with that surveillance will be passive and in case of suspicion the recommendation will be to send it to specific laboratories as it is very costly if all laboratories shall be able to diagnose the disease.

The CRL will offer a yearly training course in fish diagnostics primarily to the staff of the NRLs for fish diseases. The course will take place in the laboratories of the CRL. The date has not been decided yet but might be in November 2010 for. The exact date will be advertised before the summer holiday. The topic of the course has not been decided yet, it depends on the needs of the participants - if you have ideas or needs for courses, please tell us.

We also encourage you to provide us with suggestions for the next year's CRL work plan. The NRLs actually have to accept the CRL work plan. The work plan must be approved by the Commission by 1 September. The NRLs can propose and decide plans for the CRL.

Workshop on epidemiology and risk assessment? Should we perform an independent EU workshop on this topic in collaboration with ISAE, e.g. in spring 2011. If there is interest we can hopefully persuade the Commission to support this.

**Questions:**
**Marc Engelsma:** It is quite interesting to hear that you will be obliged to use the tests found on the webpage. I think if we have fixed tests you may have problems after 2 years due to the developments of better diagnostic tests.
**Niels Jørgen Olesen:** You are already obliged to use specific tests, but it is and will also be written that you can use test with proven similar or better standards. It also has to do with trust in trade.
**Giuseppe Bovo:** The official manuals are very important for accreditation. I think there may be a problem if these tests are on the web, and not in the legislation.
**Sigrid Cabot:** We are still working on this decision. If accepted it will be stated in the decision that the manuals can be found on the webpage, and of course it shall be written on the webpage that this is based on the decision. It will be easier to update a homepage than the legislation. Another issue, after the entry into force of the Lisbon treaty, the European Community is replaced by the European Union, which means that we from now on refer to European Union Reference Laboratories (EU-RLs) instead of Community Reference Laboratories (CRLs), and that in the titles of the EU legislation it will be references such as the following: Regulation (EU) No 175/2010.

**Brit Hjeltnes:** The National Veterinary Institute in Norway has together with Prince Edward Island been recognised as an OIE Collaborating Center in Aquaculture Epidemiology. This collaborating centre might be interested in participating in an epidemiology workshop.

**Niels Jørgen Olesen:** What about the CRL training courses – are there any needs?
**Snjezana Zrncic:** We would be interested in a CRL training course in molecular diagnosis.
**Søren Kahns:** We will send out suggestion of the content of the training course and will like you to respond back on your needs.

**Niels Jørgen Olesen:** 15$^{th}$ annual meeting. Are there suggestions for date, place and need for a workshop? It is very important we get feedback on when other meetings, conferences etc. are planned. Please also shout out the date of the Annual Meeting to others when decided so that other meetings are not placed at the same date. The Norwegian participants prefer not to have the meeting on the 17 May. In the autumn is not preferred as there are a lot of conferences.
**Reply:** The general opinion was the same date next year as this meeting was OK.

**Niels Jørgen Olesen:** Regarding venue of the 15$^{th}$ AM, suggestions are Copenhagen, Aarhus, at IZSVe, Padova in Giuseppe Bovos laboratory in Italy or at CVI in Lelystad at Olga Haenen. The nice thing about Århus is that you can all meet all our technicians but we realise that the access to Århus is not so easy. Marine Laboratory, Aberdeen is just about to finish their new lab, so it could be nice to visit them.
**Reply:** Regarding venue, people did not want to go to Brussels. Conclusion was that it was up to the CRL to decide.

**Niels Jørgen Olesen:** Regarding the workshop about kits, how can we be better to share knowledge regarding the usability of the kits? The primary aims of these meetings are to harmonize the diagnostic methods used and to improve the health of European aquaculture. So I am very happy that we covered so many diseases during the meeting.
Lot of thanks to Nicole Nicolajsen for all the work made on arranging this meeting. Thanks to our technicians for organising the drinks reception.

## *Pictures*

Guiseppe Bovo, Olga Haenen, Vlasta Jencic and Nicole Nicolajsen were excellent photographers during the workshop. For pictures from the workshop please have a look at our web page. http://www.crl-fish.eu.

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## The CRL received the following reagents in 2010

| Country | Name | Date of recieval | Material | Amount | Protocol no |
|---|---|---|---|---|---|
| Austria | Mansour El-Matbouli | 27-04-10 | Koicarp, purified DNA, pool og organ tissues 4783 no. 1-7 | 7 samples | 2010-50-95 |
| | | 04-05-10 | Diagnostic material from brown trout | 4 samples | 2010-50-99 |
| | | 10-06-10 | Diagnostic samples from Koi (gills, kidney, liver nad brain) DNA - 3468/10 | 7 samples | 2010-50-149 |
| France | Jeannette Castric | 14-04-10 | KHV - serology Proficiency test | | 2010-50-143 |
| | | 12-05-10 | KHV, strain 07/108b | 4 flasks | 2010-50-107 |
| Færøerne | Rakul Biskopstø Joensen | 20-09-10 | A210-113-2 Marineagar 3839 fish svaberprøve - Vibrio angullarum (vibriose) | 1 sample of each | 2010-50-240 |
| | | | A210-113-1 Marineagar 3839 fish svaberprøve - Vibrio angullarum (vibriose) | | |
| Germany | Dieter Fichtner & Sven bergmann | 25-02-10 | SVCV F04/05 31-03-2009 | 1 sample of each | 2010-50-38 |
| | | | SVCV F24/08 31-03-2009 | | |
| | | | SVCV F06/10 22-02-2010 | | |
| | | | SVCV F34/08 31-03-2009 | | |
| | | | SVCV F01/08 31-03-2009 | | |
| | Bernd Hoffmann | 14-04-10 | Carp sera FK 3702 - 3706 carp infected by KHV isolate from England | 5 vials of each | 2010-50-142 |
| | | | Carp sera FK 3692 - 3696 carp infected by eel herpesvirus | | |
| | | | Carp sera FK 3682 - 3686 carp infected by CCV | | |
| | | | Carp sera FK 3719 - 3723 carp infected by cloned isolate from Taiwan | | |
| | | | Carp sera FK 3672 - 3676 control fish sera | | |
| | | 26-05-10 | 816 KHV 336 KHV-1 | 2 flasks of each | 2010-50-119 |
| | | | 816 KHV HP95Z KHV-T | | |
| Italy | Guiseppe Bovo | 17-03-10 | IPNV isolates, virus is diluted 1:2 with glucerol fro cell culture | 152 | 2010-50-52 |
| Japan | Ito Takafumi | 27-05-10 | KHV NRIA 0308 Okayama | 1 vial of each | 2010-50-120 |
| | | | KHV NRIA 0301 Ibaraki | | |
| | | | KHV NRIA 0312 Nara | | |
| | | 28-10-10 | VHSV cloned isolates SVA1033 | 1 vial of each | 2010-50-287 |
| | | | VHSV cloned isolates SVA14 | | |
| | Kishio Hatai | 16-09-10 | NJM 9510 | 1 vial of each | 2010-50-235 |
| | | | NJM 0002 | | 2010-50-236 |
| | | | NJM 9701 | | 2010-50-237 |
| Norway | Ingebjørn Modahl | 17-03-10 | ISAV Glesvær/2/90 isolate protocol no. ILAV 080310 3 pass SHK cells and 1 pass ASK cells | 1 | 2010-50-51 |
| Romania | Mihaela Costea | 29-11-10 | Diagnostic samples for KHV | 2 samples | 2010-50-325 |
| Sweden | Suzanne Matelius Walter | 09-06-10 | U 100524 - 0394 P1 sampels from Perch - Perch rhabdovirus | 1 | 2010-50-147 |
| | | | U 100623-0281 Y and U 100623-0281 P 2 sampels from Perch - Perch rhabdovirus | 2 | 2010-50-178 |
| | Eva Jansson | 10-11-10 | A. astaci 56/2003 GP-Pox agar from outbrake of crayfish plauge in nobel crayfish in Sweden | 1 plate | 2010-50-294 |
| | | | A. astaci 56/2003 PG1+ab agar from outbrake of crayfish plauge in nobel crayfish in Sweden | | |

| Country | Name | Date of recieval | Material | Amount | Protocol no |
|---|---|---|---|---|---|
| Thailand | Somkiat Kanchanakhan | 08-03-10 | A. Invadans NJM 9510 fra Snake head | 1 tube | 2010-50-43 |
| | | 09-03-10 | A. Invadans NJM 0002 fra Snake head | 1 tube | 2010-50-44 |
| | | 02-09-10 | NJM 0002 | 1 | 2010-50-219 |
| | | | NJM 9701 | 1 | 2010-50-220 |
| The Netherlands | Olga Haenen | 08-12-10 | Diagnostic samples from rainbow trout, related to the EPIZONE project - CVI-NL no. 08016906 | 2x2 tubes | 2010-50-343 |
| | | | Diagnostic samples from rainbow trout, related to the EPIZONE project - CVI-NL no. 09016325 | | |
| | | | Diagnostic samples from rainbow trout, related to the EPIZONE project - CVI-NL no. 08007015 | | |
| UK-England | Veterinary Laboratories Agency | 28-10-10 | KHV - PCR Proficiency test | 5 ampoules | 2010-50-286 |
| | Birgit Oidtmann | 21-06-10 | NJM 0002 | | 2010-50-156 |

## The CRL supplied the following reagents in 2010

| Country | Name | Material | Type | Amount | Date of shipment |
|---|---|---|---|---|---|
| Belgium | Bart Lievens | dk-5131, 1p121, 4p51, 4p168, 4p101, 1p54, 1p53, US-Makah, 1p49, New Brunswick, dk-5123, dk-2835, 1p85, 1p40, 5p276, 1p8, 1p52, IT-217/A, 2p51,1p86, F1, Hededam, DK-9695377, DK-200149, DK-3592b, DK-200051, DK-3971, DK-3946, DK-5151, DK-7974, 1p93, 1p116, DK-M.Rhabdo, 1p120, IHN ER, DK-6137, 4p37 | Purified VHSV RNA | 1 vial of each | 22-06-2010 |
| | | DK-2008-50-238-1, DK-206323-1, DK-204297 | Purified KHV RNA | | |
| | | IHN RBH, Coleman, OSV, IHN HAG, FR-32/87, | Purified IHNV RNA | | |
| | | DK-203311, DK-203273, DK-202276, SVC 56/70 | Purified SVCV RNA | | |
| | | IPN Sp IPN Ab | Purified IPN RNA | | |
| | François LIEFFRIG | 1,2 ml Medium/ASK cells, diluted 1:6 in AVL buffer (Qiagen) | Medium | | 30-08-2010 |
| | | 2 ml ISAV Glesvaer/2/90, diluted 1:6 in AVL buffer (Qiagen)<br>The Glesvaer isolate was originally received from Birgit Dannevig,<br>OIE Reference Laboratory for ISA | Virus | | |
| Bulgaria | Vanya Damyanova Chikova and Marina Ivanova | BF-2 cells | Cells | 1 small falsk | 27-09-2010 |
| Canada | David Groman | Blocks with embedded tissues from VHS infected rainbow trout - PAR 6-3A and 6-3B | IHC | 1 block | 19-11-2010 |
| | | Blocks with embedded tissues from VHS infected rainbow trout - PAR 123-6A and 6B | | | |
| Chile | Paula Miranda | RNA extracted from VHSV strain Rindsholm genotype Ia | Inactivated virus | 1 vial of each | 07-06-2010<br>31-05-2010 |
| | | RNA extracted from VHSV US Makah genotype Iva | | | |
| Denmark | Klara Jensen | EPC celler | cells | 2 flasks | |
| Finland | Tuija Gadd | Pike Fry rhabdovirus (PFR) | Virus | 1 vial of each | 04-02-2010 |
| | | Perch rhabdovirus (PV) | | | |
| | | SDV S49P | | | |
| | | Rabbit anti Monta 3005-2 | PAb | | |
| | | Rabbit anti Perch rhabdovirus, F28 | | | |
| France | Laurent BIGARRE | Purified RNA dissolved in water (sample A) to be used for test of sensitivity | Purified VHSV RNA | 1x1.5 µl tube | 01-11-2010 |
| | | Purified RNA dissolved in water (sample 1-10) of the test for testing specificity | Purified VHSV RNA | 0.2 µl tubes | |
| | | Taqman probe | Probe | 1x1.5 µl tube | |
| | | 5533 LGV | Virus | 1 ampoule | |
| | | Reverse primer | Primer | 1x1.5 µl tube | |
| | | Forward primer | Primer | 1x1.5 µl tube | |
| France - Fisk 3 | Anders Stegmann c/o Dr. Michel Bremont | Plasmider: " pSHV and genome substitution" | plasmid | | 15-09-2010 |
| | | Primers: "genome substitution" | Primers | | |
| | | Phusion PCR kit | PCR Kit | | |
| | | 5e689 | Virus | 40 ml of each | |
| | | V18-Bris3 virus | | | |
| | | E.coli glycerol stocks: "genome substitution" | Bacteria | | |
| | | "genome substitution" PCR reaktions | PCR reaktion | | |
| | | FR-07-71; VHSV isolate from Michel | Virus | 10 ml of each | 02-11-2010 |
| | | FR-07/71; VHSV isolate | | | |
| | | Bris3; plaque cloned VHSV isolate | | | |
| | | 5e689; plaque cloned VHSV isolate | | | |

| Country | Name | Material | Type | Amount | Date of shipment |
|---|---|---|---|---|---|
| Germany | Heike Schuetze | Purified RNA dissolved in water (sample A) to be used for test of sensitivity | Purified VHSV RNA | 1x1.5 µl tube | 01-11-2010 |
| | | Purified RNA dissolved in water (sample 1-10) of the test for testing specificity | | 0.2 µl tubes | |
| | | Taqman probe | Probe | 1x1.5 µl tube | |
| | | Tubes containing purified RNA dissolved in water (sample 1-10) of the test for testing specificity | PCR | 10x0.2 µl | |
| | | Tube containing purified RNA dissolved in water (sample A) to be used for test of sensitivity | | 1x1.5 µl tube | |
| | | Tube containing forward primer | | | |
| | | Tube containing taqman probe | | | |
| | | Tube containing reverse primer | | | |
| | | Reverse primer | Primer | | |
| | | Forward primer | | | |
| | Günter Kotterba | Histology slides PAS from two different tissue blocks of muscle tissue from EUS infection in snakehead. | IHC | 2 slides | 08-06-2010 |
| | | Histology slides H&E from two different tissue blocks of muscle tissue from EUS infection in snakehead. | | | |
| | | Histology slides unstained from two different tissue blocks of muscle tissue from EUS infection in snakehead. | | 6 slides | |
| | | EUS infection in snakehead - And an eppendorf tube containing paraffin embedded tissue scrapings for DNA/RNA extraction for PCR analysis | PCR | 1 vial | |
| | | Aphanomyces Invadans strain: NJM 0002 received from Birgit Oidtmann (Dr. Hatai) | Fungus | 1 vial | 25-10-2010 |
| | | Aphanomyces Invadans strain: NJM 9701 received from Dr. Hatai | | 1 vial | |
| Greece | Athanasios Prapas | DK-5151 (Rindsholm) (diluted 10 -3) | Virus | 1 ampoule of each | 27-09-2010 |
| | | IHNV 32/87 First French isolate | | | |
| | | EPC cells and BF-2 cells | Cells | 1 small flask of each | |
| | | EPC cells | | | 17-11-2010 |
| Hong Kong | Tai Lung | Fungus strain: NJM 9510 received from Dr. Hatai | Fungus | One tube | 11-10-2010 |
| | | Aphanomyces Invadans strain: NJM 0002 received from Birgit Oidtmann (Dr. Hatai) | | | |
| | | Aphanomyces Invadans strain: NJM 9701 received from Dr. Hatai | | | |
| Iceland | Sigridur Gudmundsdottir | SAV negative material in RNAsafer, for accreditation purpose | PCR | 3 vials | 06-12-2010 |
| | | SAV positive material in RNAsafer, for accreditation purpose | | 1 vial | |
| Iran | Mohaddes Ghasemi | SSN-1 and FHM cells | Cells | 2 medium flasks og each | 12-01-2010 |
| | | CHSE-, BF-2 and EPC cells | | 1 medium flask of each | |
| Iran - Lindholm | Nazem Shirazi | IPNV strain Sp and IPNV strain Ab | Virus in RNA Later | 1 vial | 09-08-2010 |
| | | Noda virus strain V26 | | | |
| | | DK- 4008 IHN virus 217/A | | | |
| | | DK-5151 | | | |
| Irland | Neil Ruane | EPC cells | Cells | 2 small flasks | 31-05-2010 |
| Irland | Neil Ruane | BF-2 cells | Cells | 2 small flasks | 04-08-2010 |
| Italy | Giuseppe Bovo | Prot purified Rabbit anti IHN, Rabbit no F32 | PAb | 1 vial | 08-06-2010 |
| | Valentina Panzarin | Purified RNA dissolved in water (sample A) to be used for test of sensitivity | Purified VHSV RNA | 1x1.5 µl tube | 01-11-2010 |
| | | Purified RNA dissolved in water (sample 1-10) of the test for testing specificity | | 0.2 µl tubes | |
| | | Taqman probe | Probe | 1x1.5 µl tube | |
| | | Reverse primer | Primer | 1x1.5 µl tube | |
| | | Forward primer | Primer | 1x1.5 µl tube | |

| Country | Name | Material | Type | Amount | Date of shipment |
|---|---|---|---|---|---|
| Italy | Valentina Panzarin | SE000331098 Often called, (SE-SVA-1033) | Virus | 1 vial of each | 01-11-2010 |
| | | 96-33 | | | |
| | | No name provided. Isolate from Budd Lake, MI | | | |
| | | PM05Ehi1 | | | |
| | | CAN-3624 | | | |
| | | NO-2007-50-385 | | | |
| | | 1p49 | | | |
| | | GE-1.2 | | | |
| | | FiA01A.00 | | | |
| | | 4p37 | | | |
| Lithuania | Ingrida Jaceviciene | Medium/ASK cells, diluted 1:6 in AVL buffer (Qiagen) | PCR | 1 vial | 30-08-2010 |
| | | ISAV Glesvaer/2/90, diluted 1:6 in AVL buffer (Qiagen) | | | |
| | | BF-2-, ASK-, RTG-2-, and EPC cells | Cells | 2 small flasks of each | 18-03-2010 |
| Norway<br>Norway<br>Norway | Irene Ørpetveit | KHV 07/108b | Virus | 1 ampoule | 27-09-2010 |
| | Renate Johansen | IP5B11 | Mab | 2 vials | 22-02-2010 |
| | | 02-232-1, Sardines, Quatsion, BC, genotype Iva (received from Dr. Jim Winton, Seattle) | Virus | 1 vial lyohhilized material of each | |
| | | SVA-14 (Nordblom 1998) (received from Anders Hellström, SVA, Sverige) | | | |
| | | Atlantic salmon #16524, Port Angels, WA, genotype Iva (received from Dr. Jim Winton, Seattle) | | 2 vials of each | |
| | | 4p168 | | | |
| | | DK-3592B | | | |
| | | H16/7/95 (Smail 2000) | | | |
| | | Par 123-5B: lever | IHC Positive material | 1 block | 27-04-2010 |
| | | Par 123-3B: lever | | | |
| | | Par 123-5A: hjerte, nyre og milt | | | |
| | | Par 123-3A: hjerte, nyre og milt | | | |
| | Amund Litlabø | Makah, 4p101, 1p52, DK-3592B | Inactivated VHSV | 1 vial vial of each | 20-04-2010 |
| | Britt Gjerset | Marine VHSV UK-860/94 | Virus | 1 vialof each | 29-11-2010 |
| | | Marine VHSV 5pbris3 | Virus | | |
| P.R.China | Duan Hongan | RTG2 cells | Cells | 2 small flasks | 04-10-2010 |
| Portugal | Miguel Fevereiro and Maria Teresa Duarte | BF-2 cells | Cells | 1 medium flask | 12-01-2010 |
| | | Mab anti VHSV – 1P5B11 | Mab | 1 vial of each | |
| | | Mab anti IHNV – Hyb 136-3 | | | |
| | | Pab anti VHSV – F38 (F1) Serogrupe I, Genotype Id | PAb | | |
| | | Pab anti IPN-Sp – F68 | | | |
| | | Pab anti VHSV – F45 (Hjarnø) Serogrup III*, Genotype Ia | | | |
| | | Pab anti IPN-Ab – F72 | | | |
| | | Pab anti IHNV – F63 | | | |
| Romania | Mihaela Costea and Vlad Serafim | Pab anti SVCV – F75 | Pab | 1 vial | 27-09-2010 |
| | | Pab anti IPN-Sp – F68 | | | |
| | | Pab anti IPN-Ab – F72 | | | |
| | | Pab anti IHNV – F63 | | | |
| | | Pab anti VHSV – F38 (F1) Serogrupe I, Genotype Id | | | |
| | | Pab anti VHSV – F59 | | | |
| | | Pab anti VHSV – F45 (Hjarnø) Serogrup III*, Genotype Ia | | | |
| Serbia | Vladimir, Ivan Radosavljevic | BF-2 cells and EPC cells | Cells | 2 small flasks of each | 04-08-2010 |
| Singapore | Kim Halpin | 1p8, genotype Ib | Virus | 2 vials | 23-08-2010 |
| | | 5p551, genotype II | | | |
| | | 4p101, genotype III | | | |
| | | DK-3592B, genotype Ia, | | 1 vial | |

| Country | Name | Material | Type | Amount | Date of shipment |
|---|---|---|---|---|---|
| Slovenia | Peter Hostnik and Vlasta Jenčič | BF-2 cells | Cells | 2 small flasks of each | 26-01-2010 |
| | | FHM cells | | | |
| | | RTG-2 cells | | | |
| | | Medium 10% FCS | Medium | 500 ml | |
| | Vlasta Jenčič | ISAV Glesvaer/2/90, diluted 1:6 in AVL buffer (Qiagen) | PCR | 1 vial | 30-08-2010 |
| | | Medium/ASK cells, diluted 1:6 in AVL buffer (Qiagen) | | | |
| Spain | Julio Coll Morales | 20 rainbow trout sera diluted 1:10 from Voldbjerg Mølle (206277), collected the 10. 07. 2006 after a VHS outbreak with high mortality. | Trout sera | 20 vials of each | 02-11-2010 |
| | | 20 Rainbow trout sera diluted 1:10 in PBS. The sera originated from Vesterkrog and were collected after an outbreak with medium mortality. (2008-50-362). | | | |
| | Pilar Fernández Somalo & Marta Vigo | Pab anti IHNV – F33 | Pab | 1 vial of each | 22-02-2010 |
| | | Pab anti IPN-Sp – F68 | | | |
| | | Pab anti IPN-Ab – F72 | | | |
| | | Pab anti VHSV – 927 | | | |
| | | Mab anti IHNV – Hyb 136-3 | Mab | | |
| | | Mab anti VHSV – 1P5B11 | | | |
| Sweden | Eva Jansson | Aphanomyces Invadans strain: NJM 0002 received from Birgit Oidtmann (Dr. Hatai) | Fungus | 1 vial | 11-10-2010 |
| | | Aphanomyces Invadans strain: NJM 9701 received from Dr. Hatai | | | |
| | Suzanne Martelius and Anders Hellström | KHV 07/108b, 4 pass KF-1, 1 pass CCB | Virus | 1 ampoule | 27-09-2010 |
| Switzerland | Thomas Wahli | Rabbit anti IPN Sp+Ab | Pab | 1 vial | 30-08-2010 |
| | | ISAV Glesvaer/2/90, diluted 1:6 in AVL buffe | Virus | | |
| | | Medium/ASK cells, diluted 1:6 in AVL buffer | Medium | | |
| The Netherlands | Olga L.M. Haenen | EK-1 cells | Cells | 2 small flask | 22-02-2010 |
| | | Rabbit anti EVXV- DVI: K12 | PAb | 1 vial | 30-08-2010 |
| | | Aphanomyces Invadans strain: NJM 9701 received from Dr. Hatai | Fungus | One tube | 11-10-2010 |
| | | Aphanomyces Invadans strain: NJM 0002 received from Birgit Oidtmann (Dr. Hatai) | | | |
| United Kingdom | Chris Pond | BF-2 cells | Cells | 2 small flasks | 25-10-2010 |
| USA | Andrew E Goodwin | Forward primer | Primer | 1x1.5 µl tube | 01-11-2010 |
| | | Reverse primer | | 1x1.5 µl tube | |
| | | Purified RNA dissolved in water (sample A) to be used for test of sensitivity | Purified VHSV RNA | 1x1.5 µl tube | |
| | | Purified RNA dissolved in water (sample 1-10) of the test for testing specificity | | 0.2 µl tubes | |
| | | Taqman probe | Probe | 1x1.5 µl tube | |
| | | Tube containing purified RNA dissolved in water (sample A) to be used for test of sensitivity | PCR | 1x1.5 µl | |
| | | Tube containing taqman probe | | 1x1.5 µl | |
| | | Tube containing forward primer | | 1x1.5 µl | |
| | | Tubes containing purified RNA dissolved in water (sample 1-10) of the test for testing specificity | | 10x0.2 µl | |
| | | Tube containing reverse primer | | 1x1.5 µl | |

*EURL for Fish Diseases*

# Report of the Inter-Laboratory Proficiency Test 2010

# for

# identification of VHSV, IHNV and EHNV (PT1)

# and

# identification of KHV and ISAV (PT2)

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

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## Introduction

A comparative test of diagnostic procedures was provided by the European Union Reference Laboratory (EURL) for Fish Diseases. The test was divided into two proficiency tests, proficiency test 1 (PT1) and proficiency test 2 (PT2). PT1 was designed as the proficiency tests provided by the EURL in previous years, to primarily assess o the identification of the fish viruses: Viral haemorrhagic septicaemia virus (VHSV), infectious haematopoietic necrosis virus (IHNV) and epizootic haematopoietic necrosis virus (EHNV) by the participating laboratories. PT2 was developed and shipped for the first time this year with the aim of assessing the ability of participating laboratories to identify the fish viruses: Infectious salmon anemia virus (ISAV) and koi herpes virus **(**KHV). 38 National Reference Laboratories (NRLs) participated in PT1 and 36 NRLs in PT2. The tests were sent from the EURL end of September 2010.

This report covers both the results of PT1 and PT2.

**PT1** contained five coded ampoules (I-V). The ampoules contained VHSV genotype Ia, IHNV genogroup M, EHNV, European catfish virus (ECV), and spring viraemia of carp virus (SVCV), see table 1. The proficiency test was designed to primarily assess the ability of participating laboratories to identify the fish viruses VHSV, IHNV and ENHV (all listed in Council Directive 2006/88/EC), if present in the ampoules, bearing in mind that the test ampoules also could contain other viruses (e.g. other fish rhabdoviruses, ranaviruses, or birnaviruses). In addition the participants were asked to quantify the viruses in PT1 by titration in order to assess the susceptibility of their fish cell lines for virus infection. Participants were encouraged to use their normal standard laboratory procedures. However, the identification should be performed according to the procedures laid down in Commission Decision 2001/183/EC using fish cell cultures followed by e.g. neutralisation test, immunofluorescence, ELISA or PCR. If ranaviruses should be present in any of the ampoules, it was mandatory to perform a sequence analysis of the isolate in order to determine if the isolate is EHNV or another ranavirus and it was recommended to follow the procedures as 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 that were used for genotyping of isolates.

**PT2** contained five coded ampoules (VI-X). The ampoules contained ISAV and KHV. Furthermore, one ampoule did not contain any virus, only medium, see table 11. It was decided at the 14$^{th}$ Annual Meeting of the NRLs for Fish Diseases in Aarhus 26-28 May 2010, that testing for ISAV and KHV for the first time should be included in the yearly proficiency test provided by the EURL. The proficiency test was designed to primarily assess the ability of participating laboratories to identify the notifiable fish viruses ISAV and KHV (both listed in Council Directive 2006/88/EC) if present in the ampoules, bearing in mind that the test ampoules also could contain other viruses. Participants were expected to use their normal PCR or real-time PCR methods for detection of KHV and their normal RT-PCR or real-time RT-PCR methods for detection of ISAV. It was not mandatory to grow KHV and ISAV on cell cultures in this test, but the viruses has not been inactivated and should thus be viable and possible to amplify in cell cultures.

Each laboratory was given a code number to ensure discretion. The code number of each participant is supplied to the respective laboratories with this report. Furthermore, the providers of the proficiency test have provided comments to participants if relevant. An un-encoded version of the report is sent to the Commission.

In this proficiency test it was possible to download an excel sheet for filling in results. Participants could submit the filled excel sheet electronically. Additionally, participants were asked to fill a questionnaire regarding the accreditation status of their laboratory. Collected accreditation data will not be presented in this report but will be presented at the 15th Annual Meeting of the NRLs for fish diseases in Aarhus in May. Participants were asked to reply latest 29 November 2010.

**Distribution of the test**

The test was sent out according to current international regulations for shipment of diagnostic specimens UN 3373, "Biological substance, Category B". All proficiency tests were delivered by courier and when possible, participants were provided with a tracking number when the test had been shipped.

Thermo-loggers were included in 12 of the parcels (-40°C to +30°C). The thermo-loggers were returned immediately upon receipt of the proficiency tests and a computer programme translated the data into a graph, showing the temperature inside the parcel for every 30 minutes during transportation. The loggers were programmed to mark if the temperature had exceeded 30°C at some point during transportation. Inclusion of loggers should display if the temperature encountered during transport had been detrimental to the viability of the virus in the test.

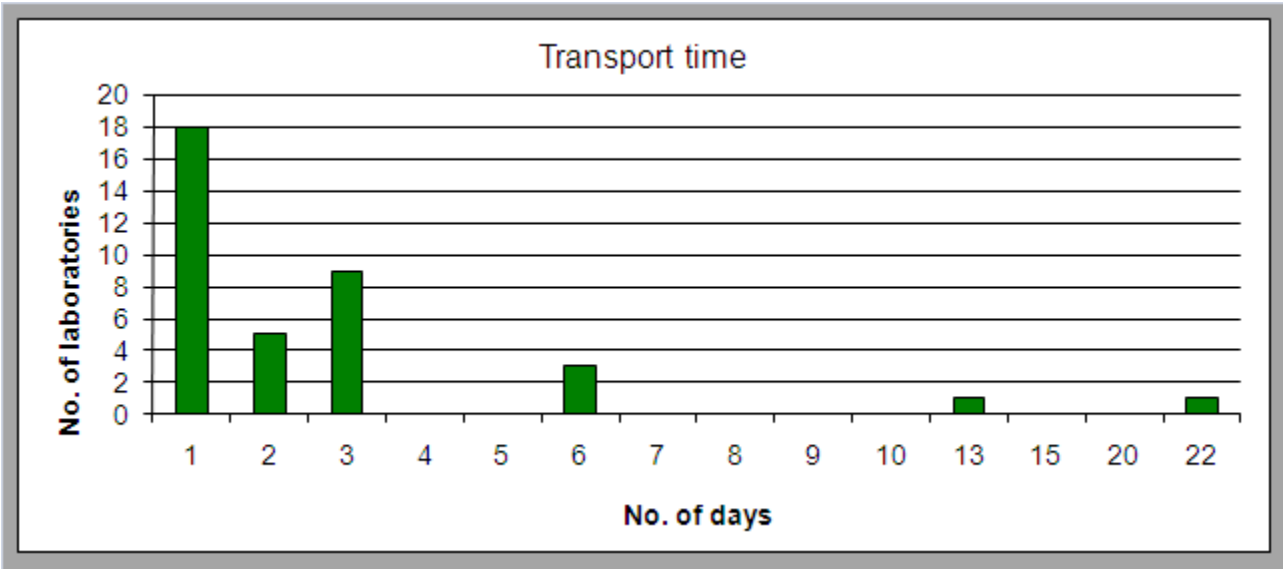
In parcels that had to go through longer transport time, cooling elements were included.

*Shipment and handling*

Within three days, the tests were delivered to 32 participants; 3 tests were delivered within 6 days and 1 test within 2 weeks and 1 test within three weeks (figure 1). The average temperatures for the transports without cooling elements were (for 7 countries) 16.1°C and the temperature only exceeded 28°C for one transports for half an hour upon arrival. The remaining transports (5 countries) were sent with cooling elements because of longer travel time. These transports had an average temperature of 10,7°C.

This year we tested the titres of all ampoules (I-V) after they had been kept for a period of 10 days at temperature from 20,5°C to 30,5°C – with an average temperature of 22,13°C and including a stay at 30°C for 24 hours. No significant decrease in titres was observed for any of these tested ampoules. As the ampoules with loggers at no point exceeded such extreme temperatures during shipment, it is considered that the temperature variation that ampoules experienced during shipment did not influence considerably on virus titres.

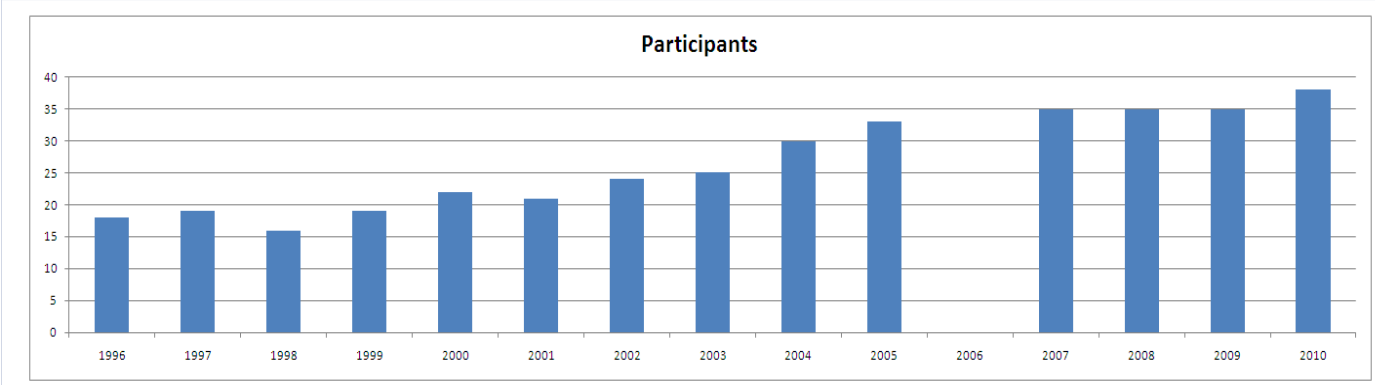
**Figure 1.** Transport time for the parcel to reach the participants.




*Participation*

**PT1:** 38 laboratories received the annual proficiency test, 36 participants' submitted results within the deadline. Two participant submitted results 7 days after deadline but before the content of the ampoules were made public available. **PT2:** 36 laboratories received the annual proficiency test, 35 participants' submitted results within the deadline. One participant submitted results 7 days after deadline but before the content of the ampoules was made public available. Two participants did not submit results. Figure 2 show how many laboratories have been participating in the proficiency test from 1996 to 2010.

**Figure 2.** Participants in the EURL proficiency test over the years. In 2010, the number corresponds to number of participants participating in PT1.



## Proficiency test 1, PT1

Five ampoules with lyophilised tissue culture supernatant were delivered to all NRLs in EU Member States, including Denmark, and likewise to the NRLs in Australia, Bosnia and Herzegovina, Croatia, Faroe Islands, Iceland, Israel, Japan, Norway, P.R China, Serbia and Switzerland, Turkey and USA. The Belgian NRL covers both Belgium and Luxembourg and likewise the Italian NRL covers Italy, Cyprus, and Malta for identification of all listed viruses and Greece for identification of EHNV, KHV and ISAV.

## Content of ampoules

The viruses were propagated on each of their preferred cell line, and when total cytopathic effect (CPE) was observed, the supernatants were collected and filtrated through a 45 µm filter, mixed with equal volumes of 20% w/v lactalbumin hydrolysate solution and lyophilized in glass ampoules. The ampoules were sealed by melting. The details of the virus isolates used in the proficiency test are outlined in table 1.

**Table 1.** Content of each ampoule with reference to culture conditions and major publications of the included virus.

| | **PT1** |
|---|---|
| **Code** | **Specifications** |
| **Ampoule I:**<br>**EHNV**<br>**Low titer** | Reference strain of EHNV.<br><br>Isolate 86/8774 from rainbow trout (Langdon et al. 1988).<br>Received from Dr. R.J. Whittington, EHN OIE reference laboratory, Australia.<br>Cell culture passage number: 7.<br><br>References:<br>Langdon JS, Humphrey JD & Williams LM (1988). Outbreaks of an EHNV-like iridovirus in cultured rainbow trout, *Salmo gairdneri* Richardson, in Australia. *Journal of Fish Diseases* **11**, 93-96.<br><br>Marsh IB, Whittington RJ, O'Rourke B, Hyatt AD & Chisholm O (2002). Rapid differentiation of Australian, European and American ranaviruses based on variation in major capsid protein gene sequence. *Molecular and Cellular Probes* **16**, 137-151.<br><br>GenBank accession number:<br>FJ433873.1 |
| **Ampoule II:**<br>**IHNV** | IHN virus 217/A (DTU Vet protocol no. 4008).<br><br>First Italian IHNV isolate from rainbow trout (Bovo et al. 1987).<br>Received from Dr. G. Bovo, ISZ-Ve, Padova, Italy.<br>Cell culture passage number in EPC: 11.<br>Genotype M (Johansson et al. 2009).<br><br>Reference:<br>Bovo G, Giorgetti G, Jørgensen PEV and Olesen (1987). Infectious haematopoietic necrosis: first detection in Italy. *Bulletin of the European Association of Fish Pathologists* 7, 124.<br><br>Johansson T, Einer-Jensen K, Batts W, Ahrens P, Björkblom C, Kurath G, Björklund H & Lorenzen N (2009). Genetic and serological typing of European infectious haematopoietic necrosis virus (IHNV) isolates. *Diseases of Aquatic Organisms* 86, 213-221.<br><br>GenBank accession number:<br>FJ265716.1 |
| **Ampoule III:**<br>**European Catfish** | European catfish virus 562/92. |

| PT1 | |
|---|---|
| **Code** | **Specifications** |
| **Virus (ECV)** | Italian isolate from catfish suffering high mortality.<br>Received from Dr. G. Bovo, ISZ-Ve, Padova, Italy.<br>Cell culture passage number: 6.<br><br>Reference:<br>Bovo G, Comuzi M, De Mas S, Ceschia G, Giorgetti G, Giacometti P & Cappellozza E (1993). Isolamento di un agente virale irido-like da pesce gatto (Ictalurus melas) dallevamento. Bollettino Societa Italiana di Patologia Ittica 11, 3–10.<br><br>GenBank accession number:<br>(FJ358606) |
| **Ampoule IV: SVCV** | SVCV 56/70.<br><br>Isolate from carp.<br>Received from Prof. Fijan (January 1979 in a tube named Rhabdo virus carpio 56/70 and given as the reference strain of SVC virus).<br>Cell culture passage number: Unknown.<br>Genotype Id (Stone et al. 2003).<br><br>The isolate is most likely identical to the S/30 isolate described in<br>Fijan N, Petrinec Z, Sulimanovic D & Zwillenberg LO (1971) Isolation of the viral causative agent from the acute form of infectious dropsy of carp. *Veterinarski Archiv* **41,** 125-138.<br><br>Reference:<br>Stone DM, Ahne W, Denham KL, Dixon PF, Liu C-TY, Sheppard AM, Taylor GR & Way K (2003). Nucleotide sequence analysis of the glycoprotein gene of putative spring viraemia of carp virus and pike fry rhabdovirus isolates reveals four genogroups. *Diseases of Aquatic Organisms* **53**, 203-210.<br><br>GenBank accession numbers:<br>Z37505.1 (S30)<br>AJ538061.1 (Fijan) |
| **Ampoule V: VHSV** | VHSV DK-5151 (Rindsholm, 1992)<br><br>Danish freshwater VHSV isolate from rainbow trout.<br>Cell culture passage number: 4 in BF-2 and 6 in EPC.<br>Neutralization pattern III (Olesen et al. 1993).<br>Genotype Ia (Ejner-Jensen et al. 2004).<br><br>References:<br>Olesen NJ, Lorenzen N and Jørgensen PEV (1993). Serological differences among isolates of viral haemorrhagic septicaemia virus detected by neutralizing monoclonal and polyclonal antibodies. *Diseases of Aquatic Organisms* **16**, 163-170.<br><br>Einer-Jensen K, Ahrens P, Forsberg R and Lorenzen N (2004). Evolution of the fish rhabdovirus viral haemorrhagic septicaemia virus. *Journal of General Virology* **85**, 1167-1179.<br><br>GenBank accession number:<br>AF345859.1<br><br>FishPathogens report number:<br>2218 |

## Testing of the PT1 test

The PT1 test was prepared and tested according to protocols accredited under DS/EN ISO/IEC 17025 and ILAC-G13:08/2007 standards. Prior to distribution the EURL tested 5 ampoules of each virus preparation by titration in 4 cell lines (BF-2, EPC, RTG-2 and FHM), to ascertain a satisfactory titre in the preferred cell line and homogeneity of content of ampoules (Table 2).

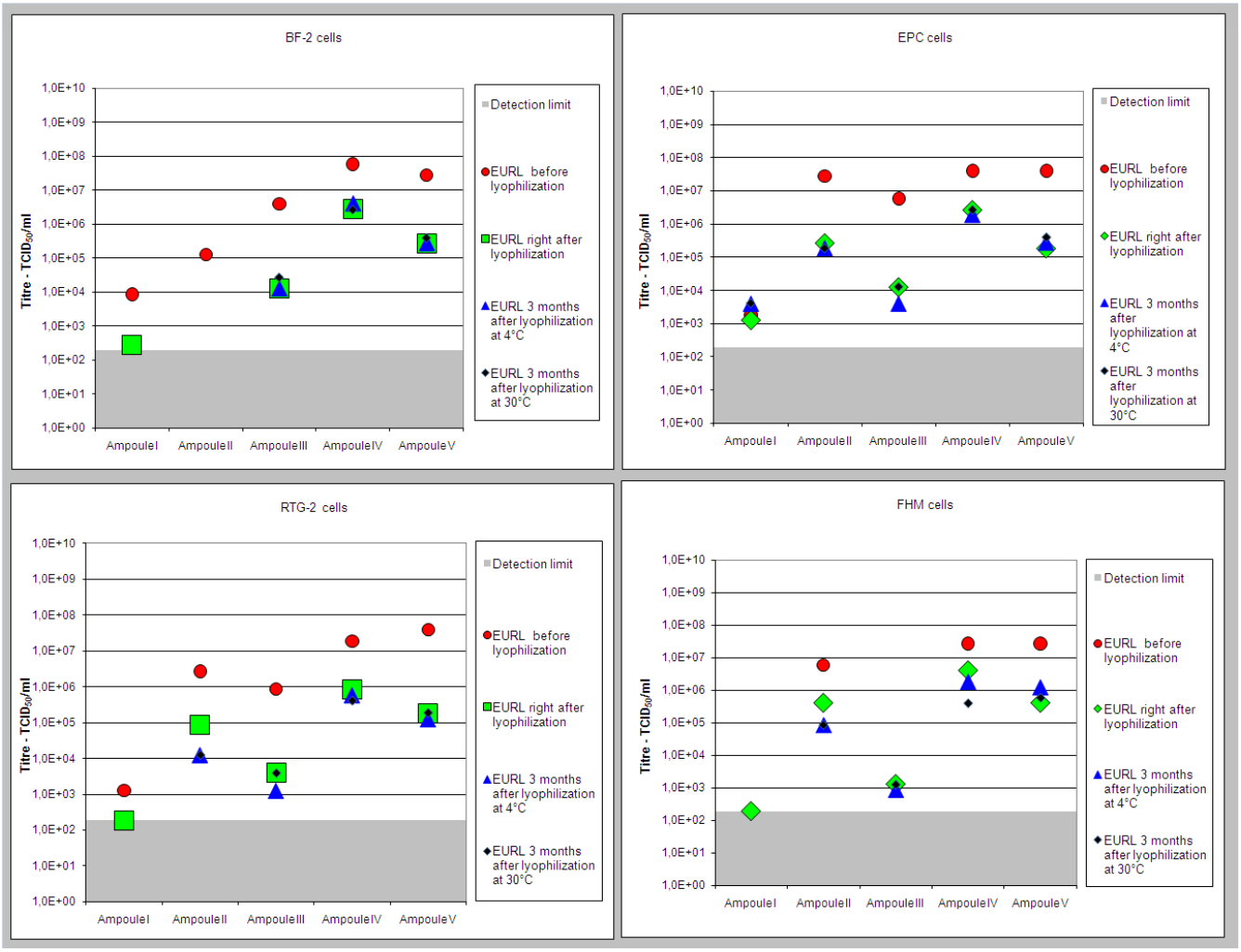
The lyophilisation procedure caused a significant titre reduction, especially for IHNV on BF-2 cells where a 3 log reduction was observed. For the other cell lines a reduction between 1 - 2 log for IHNV was observed. For EHNV a titre reduction between 0 - 1,5 log, for ECV a titre reduction between 0-3 log, for SVCV a titre reduction between 1 - 2 log and for VHSV a 1,5 - 2,5 log reduction was observed (table 2 and figure 3).
However, all titres of the lyophilised viruses were above detection level, except for IHNV on BF-2 cells and EHNV on BF-2, RTG-2 and FHM cells. As participants are expected to use two different cell lines, IHNV should be detected on the other cell line. As EHNV did produce CPE when subcultivated on EPC and BF-2 cells participants should be expected to identify EHNV from ampoule I.

Lyophilised viruses are very stable at storing. We have previously shown that lyophilised virus kept in these ampoules is stable for more than half a year when kept at room temperature (Report of the Inter-Laboratory Proficiency Test for National Reference Laboratories for Fish Diseases 2007; the report is available at
http://www.crl-fish.eu/upload/sites/crl-fish/reports/proficiency/report_2007.pdf).
Besides the test of storing viruses at 30°C for 24 hours, described above, we tested the titre of each virus preparation (ampoule) after 3 months storage in the dark at 4°C and saw no significant decrease in titres. EHNV titre could also be considered to be constant as illustrated in figure 4 on EPC cells. Furthermore, we have previously shown that the EHNV titre is constant during storage three months at 4°C (report from proficiency test 2009). The decrease of EHNV from being exactly at the detection level right after lyophilisation to be below the detection level on BF-2, RTG-2 and FHM cells three month after lyophilisation could be explained by slight statistic variation in titres or cell sensitivity.
The identities of the viruses in all 5 ampoules were checked and confirmed by ELISA, IFAT, RT-PCR and serum neutralisation tests for VHSV, IHNV and SVCV and by PCR, sequencing and IFAT for EHNV and ECV. For each ampoule, presence of viruses other than the expected was not observed.

**Table 2.** Titre of representative ampoules of no. I to V tested at the EURL in four cell lines before lyophilisation, immediately after lyophilisation (median titre of 5 replicates), and after 3 months of storage in the dark at 4°C and at 30°C for 24 hours (1 replicate), respectively.

| Ampoule No. | Content | Cell line | Titre before lyophilisation | Median titre right after lyophilisation | Titre 3 months after lyophilisation (4°C, dark conditions) | Titre 3 months after lyophilisation (30°C, dark conditions) |
|---|---|---|---|---|---|---|
| | | | $TCID_{50}$/ml | $TCID_{50}$/ml | $TCID_{50}$/ml | |
| Ampoule I | EHNV Low titer | BF-2 | $8,6*10^3$ | $2,7*10^2$ | $< 1,9*10^2$ | $< 1,9*10^2$ |
| | | EPC | $1,9*10^3$ | $1,3*10^3$ | $4,0*10^3$ | $4,0*10^3$ |
| | | RTG-2 | $1,3*10^3$ | $1,9*10^2$ | $< 1,9*10^2$ | $< 1,9*10^2$ |
| | | FHM | $< 1,9*10^2$ | $1,9*10^2$ | $< 1,9*10^2$ | $< 1,9*10^2$ |
| Ampoule II | IHNV Genotype M | BF-2 | $1,3*10^5$ | $< 1,9*10^2$ | $< 1,9*10^2$ | $< 1,9*10^2$ |
| | | EPC | $2,7*10^7$ | $2,7*10^5$ | $1,9*10^5$ | $1,9*10^5$ |
| | | RTG-2 | $2,7*10^6$ | $8,6*10^4$ | $1,3*10^4$ | $1,3*10^4$ |
| | | FHM | $5,9*10^6$ | $4,0*10^5$ | $8,6*10^4$ | $8,6*10^4$ |
| Ampoule III | European Catfish Virus (ECV) | BF-2 | $4,0*10^6$ | $1,3*10^4$ | $1,3*10^4$ | $2,7*10^4$ |
| | | EPC | $5,9*10^6$ | $1,3*10^4$ | $4,0*10^3$ | $1,3*10^4$ |
| | | RTG-2 | $8,6*10^5$ | $4,0*10^3$ | $1,3*10^3$ | $4,0*10^3$ |
| | | FHM | $1,3*10^3$ | $1,3*10^3$ | $8,6*10^2$ | $1,3*10^3$ |
| Ampoule IV | SVCV | BF-2 | $5,9*10^7$ | $2,7*10^6$ | $4,0*10^6$ | $2,7*10^6$ |
| | | EPC | $4,0*10^7$ | $2,7*10^6$ | $1,9*10^6$ | $2,7*10^6$ |
| | | RTG-2 | $1,9*10^7$ | $8,6*10^5$ | $5,9*10^5$ | $4,0*10^5$ |
| | | FHM | $2,7*10^7$ | $4,0*10^6$ | $1,9*10^6$ | $4,0*10^5$ |
| Ampoule V | VHSV | BF-2 | $2,7*10^7$ | $2,7*10^5$ | $2,7*10^5$ | $4,0*10^5$ |
| | | EPC | $4,0*10^7$ | $1,9*10^5$ | $2,7*10^5$ | $4,0*10^5$ |
| | | RTG-2 | $4,0*10^7$ | $1,9*10^5$ | $1,3*10^5$ | $1,9*10^5$ |
| | | FHM | $2,7*10^7$ | $4,0*10^5$ | $1,3*10^6$ | $5,9*10^5$ |

**Figure 3.** Virus titers before, right after and 3 months after lyophilisation in different cell lines. Grey area is below detection level.



## Virus identification and titration

Participants were asked to identify the content of each ampoule by the method used in their laboratory which should be according to the procedures described in the Commission Decision 2001/183/EC, i.e. by cell culture followed by ELISA, IFAT, neutralisation test or RT-PCR. Identification results of the content of the 5 ampoules for the participating laboratories are summarised in table 3.

Participants were also asked to titrate the contents of the ampoules. The method of titration was described in the instructions enclosed with the test. All titres were calculated at the EURL based on the crude data submitted by each participant and given as Tissue Culture Infective Dose 50% ($TCID_{50}$) per ml. The titre of the re-dissolved virus was multiplied by a factor of 10 to compensate for the dilution of the original volume of virus in the ampoules (200 µl virus + 200 µl lactalbumin in vials re-dissolved in a total of 2.0 ml cell culture medium)**.** Titration results of the viruses of the 5 ampoules for the participating laboratories are summarised in tables 4 to 8. The titres obtained from each participating laboratory are represented graphically. On figures 4-7, all titres submitted by participants for each cell line and ampoule, respectively are compared to each other. On these figures, the median titre and the 25 and 75% inter quartile range is displayed. In this way, the titres obtained by each laboratory are plotted in relation to the combined submitted data set and each participating laboratory should be able to compare the sensitivity of their cell lines to the sensitivity of those used by the other participating laboratories. CHSE-214 cells are not displayed graphically or commented on in this report as only one laboratory used these cells. Laboratories with the required facilities were encouraged to examine and identify the genotype of the virus isolates. It was not mandatory to perform these analyses for VHSV and IHNV. However, for ranaviruses it is mandatory to perform a sequence analysis of the isolate in order to determine if the isolate is EHNV.

**Table 3**. Inter-Laboratory Proficiency Test, PT1, 2010 - Virus identification.

| Laboratory code number | Score 10/10 | Answer received at EURL | Ampoule I | Ampoule II | Ampoule III | Ampoule IV | Ampoule V |
|---|---|---|---|---|---|---|---|
| | | | EHNV | IHNV | Rana not EHNV or ESV-ECV | SVCV | VHSV |
| 1 | 8 | 29-11-10 | EHNV | IHNV | EHNV | SVCV | VHSV |
| 2 | 10 | 26-11-10 | EHNV | IHNV | ESV-ECV | SVCV | VHSV |
| 3 | 6 | 29-11-10 | Virus not found | IHNV | EHNV | SVCV | VHSV |
| 4 | 10 | 29-11-10 | EHNV | IHNV | ECV/ESV | SVCV | VHSV |
| 5 | 10 | 25-11-10 | EHNV | IHNV | Ranavirus, not EHNV | SVCV | VHSV |
| 6 | 4 | 29-11-10 | Virus not found | IHNV | Not VHSV, IHNV | Not VHSV, IHNV | VHSV |
| 7 | 6 | 26-11-10 | SAV | IHNV | SAV | SVCV | VHSV |
| 8 | 10 | 08-11-10 | EHNV | IHNV | Ranavirus ECV | SVCV | VHSV |
| 9 | 8 | 29-11-10 | EHNV | IHNV | Iridovirus, Ranavirus ESV= ECV= ECV24 | SVCV | VHSV/IHNV |
| 10 | 6/6 | 7-12-2010 * | Identification performed by another NRL | IHNV | Identification performed by another NRL | SVCV | VHSV |
| 11 | 8 | 25-11-10 | EHNV | IHNV | EHNV | SVCV | VHSV |
| 12 | 8 | 29-11-10 | Virus not found | IHNV | Sheetfish/catfish Iridovirus | SVCV | VHSV |
| 13 | 10 | 29-11-10 | EHNV | IHNV | ECV/ESV | SVCV | VHSV |
| 14 | 8 | 29-11-10 | EHNV | IHNV | EHNV | SVCV | VHSV |
| 15 | 8 | 26-11-10 | Virus not found | IHNV | Ranavirus, not EHNV | SVCV | VHSV |
| 16 | 10 | 29-11-10 | EHNV | IHNV | ECV | SVCV | VHSV |
| 17 | 6 | 29-11-10 | EHNV | IHNV/SVCV | ESV or ECV | SVCV | VHSV/SVCV |
| 18 | 8 | 29-11-10 | Not EHNV, VHSV, IHNV, IPNV and SVCV | IHNV | ECV/ESV | SVCV | VHSV |
| 19 | 10 | 26-11-10 | EHNV | IHNV | ECV | SVCV | VHSV |
| 20 | 8 | 29-11-10 | EHNV | IHNV | EHNV | SVCV | VHSV |

**Annex 4**

Technical report from the Community Reference Laboratory for Fish Diseases 2010

| Laboratory code number | Score 10/10 | Answer received at EURL | Ampoule I | Ampoule II | Ampoule III | Ampoule IV | Ampoule V |
|---|---|---|---|---|---|---|---|
| | | | EHNV | IHNV | Rana not EHNV or ESV-ECV | SVCV | VHSV |
| 21 | 10 | 26-11-10 | EHNV | IHNV | ECV or ESV | SVCV | VHSV |
| 22 | 8 | 23-11-10 | Rana | IHNV | Rana | SVCV | VHSV |
| 23 | 10 | 15-11-10 | EHNV | IHNV | ECV | SVCV | VHSV |
| 24 | 10 | 29-11-10 | EHNV | IHNV | Ranavirus not EHNV | SVCV | VHSV |
| 25 | 10 | 22-11-10 | EHNV | IHNV | Iridovirus not EHNV | SVCV | VHSV |
| 26 | 8 | 23-11-10 | EHNV | IHNV | ESV | IHNV/ SVCV | VHSV |
| 27 | 2 | 03-12-2010 * | Virus not found | IHNV | Virus not found | VHSV/ IPNV | Virus not found |
| 28 | 10 | 26-11-10 | EHNV | IHNV | Iridovirus (sheetfish and catfish) | SVCV | VHSV |
| 29 | 8 | 29-11-10 | IHNV/EHNV | IHNV | ESV or ECV | SVCV | VHSV |
| 30 | 10 | 29-11-10 | EHNV | IHNV | Ranavirus (Sheetfish iridovirus) | SVCV | VHSV |
| 31 | 4 | 29-11-10 | VHSV/EHNV | IHNV | EHNV | SVCV | Virus not found |
| 32 | 10 | 26-11-10 | EHNV | IHNV | ECV/ESV | SVCV | VHSV |
| 33 | 6 | 22-11-10 | Virus not found | IHNV | EHNV | SVCV | VHSV |
| 34 | 4 | 29-11-10 | Virus not found | IHNV | EHNV | SVCV | Virus not found |
| 36 | 4 | 29-11-10 | Virus not found | Virus not found | EHNV | SVCV | VHSV |
| 37 | 10 | 23-11-10 | EHNV | IHNV | Ranavirus: ESV / ECV | SVCV | VHSV |
| 38 | 10 | 26-11-10 | EHNV | IHNV | Ranavirus (ECV or ESV or BIV) | SVCV | VHSV |
| 39 | 10 | 06-12-10 | EHNV | IHNV | Ranavirus (sheet fish, doctor fish and catfish iridovirus) | SVCV | VHSV |

| | Ampoule I | Ampoule II | Ampoule III | Ampoule IV | Ampoule V |
|---|---|---|---|---|---|
| | EHNV | IHNV | Rana or ESV-ECV | SVCV | VHSV |
| Correct ID | 24 | 36 | 24 | 35 | 33 |
| No virus | 8 | 1 | 1 | 0 | 3 |
| Wrong ID | 3 | 1 | 10 | 2 | 2 |
| No ID | 2 | | 2 | 1 | |
| Not replied | 0 | 0 | 0 | 0 | 0 |
| Total | 37 | 38 | 37 | 38 | 38 |

* The laboratory submitted results after deadline, but before ampoule content were made public available. The result of this participant is therefore included in this report.

**Table 4.** Inter-Laboratory Proficiency Test, PT1, 2010 – Results of titration of ampoule I.

| ***Ampoule I - EHNV*** | | | | | |
|---|---|---|---|---|---|
| Laboratory code number | Virus Identification | Titre in | | | |
| | | BF-2 | EPC | RTG-2 | FHM |
| 1 | EHNV | $4,0*10^2$ | $1,9*10^2$ | $4,0*10^2$ | |
| 2 | EHNV | $1,9*10^3$ | $1,3*10^3$ | | |
| 3 | Virus not found | $< 1,9*10^2$ | $< 1,9*10^2$ | | |
| 4 | EHNV | | $< 1,9*10^2$ | $1,9*10^2$ | $< 1,9*10^2$ |
| 5 | EHNV | $1,9*10^2$ | $2,7*10^2$ | $< 1,9*10^2$ | $< 1,9*10^2$ |
| 6 | Virus not found | $< 1,9*10^2$ | $< 1,9*10^2$ | | |
| 7 | SAV | $2,7*10^2$ | $< 1,9*10^2$ | | |
| 8 | EHNV | $1,3*10^3$ | $< 1,9*10^2$ | $< 1,9*10^2$ | |
| 9 | EHNV | $< 1,9*10^2$ | $< 1,9*10^2$ | $< 1,9*10^2$ | $< 1,9*10^2$ |
| 10 | Identification performed by another NRL | $1,3*10^4$ | $< 1,9*10^2$ | | |
| 11 | EHNV | $1,3*10^4$ | $1,3*10^3$ | | |
| 12 | Virus not found | $< 1,9*10^2$ | $< 1,9*10^2$ | | |
| 13 | EHNV | $1,3*10^3$ | $< 1,9*10^2$ | | |
| 14 | EHNV | $2,7*10^2$ | $< 1,9*10^2$ | | |
| 15 | Virus not found | | $< 1,9*10^2$ | $< 1,9*10^2$ | |
| 16 | EHNV | $5,9*10^2$ | $2,7*10^2$ | $< 1,9*10^2$ | $1,9*10^2$ |
| 17 | EHNV | | $1,3*10^6$ | $< 1,9*10^2$ | |
| 18 | Not EHNV, VHSV, IHNV, IPNV and SVCV | $< 1,9*10^2$ | | | $< 1,9*10^2$ |
| 19 | EHNV | $5,9*10^5$ | | $5,9*10^4$ | $5,9*10^4$ |
| 20 | EHNV | | $2,7*10^2$ | $< 1,9*10^2$ | |
| 21 | EHNV | $1,3*10^3$ | $2,7*10^2$ | $8,6*10^2$ | $< 1,9*10^2$ |
| 22 | Rana | $< 1,9*10^2$ | | | $< 1,9*10^2$ |
| 23 | EHNV | | $< 1,9*10^2$ | | $< 1,9*10^2$ |
| 24 | EHNV | | $1,9*10^2$ | $1,9*10^2$ | $1,9*10^2$ |
| 25 | EHNV | $< 1,9*10^2$ | | | $< 1,9*10^2$ |
| 26 | EHNV | $1,9*10^2$ | $1,9*10^2$ | | $< 1,9*10^2$ |
| 27 | Virus not found | $< 1,9*10^2$ | $< 1,9*10^2$ | | |
| 28 | EHNV | $2,7*10^3$ | $< 1,9*10^2$ | | |
| 29 | IHNV/EHNV | $< 1,9*10^2$ | $< 1,9*10^2$ | $< 1,9*10^2$ | |
| 30 | EHNV | $5,9*10^2$ | $1,9*10^2$ | | |
| 31 | VHSV/EHNV | $1,9*10^2$ | $< 1,9*10^2$ | | |
| 32 | EHNV | $4,0*10^2$ | $< 1,9*10^2$ | $< 1,9*10^2$ | $< 1,9*10^2$ |
| 33 | Virus not found | $< 1,9*10^2$ | $< 1,9*10^2$ | | |
| 34 | Virus not found | $< 1,9*10^2$ | $< 1,9*10^2$ | | $< 1,9*10^2$ |
| 36 | Virus not found | $< 1,9*10^2$ | $< 1,9*10^2$ | | |
| 37 | EHNV | $4,0*10^2$ | $< 1,9*10^2$ | | |
| 38 | EHNV | $5,9*10^3$ | $< 1,9*10^2$ | | |
| 39 | EHNV | $< 1,9*10^2$ | $< 1,9*10^2$ | | $< 1,9*10^2$ |

| | | | | |
|---|---|---|---|---|
| Number of laboratories | 32 | 34 | 14 | 15 |
| Median titre | $5,9*10^2$ | $2,7*10^2$ | $4,0*10^2$ | $1,9*10^2$ |
| Maximum titre | $5,9*10^5$ | $1,3*10^6$ | $5,9*10^4$ | $5,9*10^4$ |
| Minimum titre | $1,9*10^2$ | $1,9*10^2$ | $1,9*10^2$ | $1,9*10^2$ |
| 25% quartile titre | $3,4*10^2$ | $1,9*10^2$ | $1,9*10^2$ | $1,9*10^2$ |
| 75% quartile titre | $2,3*10^3$ | $7,7*10^2$ | $8,6*10^2$ | 2,9*104 |

**Table 5.** Inter-Laboratory Proficiency Test, PT1, 2010 – Results of titration of ampoule II.

| ***Ampoule II - IHNV*** | | | | | |
|---|---|---|---|---|---|
| Laboratory code number | Virus Identification | Titre in | | | |
| | | BF-2 | EPC | RTG-2 | FHM |
| 1 | IHNV | $2,7*10^2$ | $2,7*10^5$ | $8,6*10^3$ | $1,9*10^5$ |
| 2 | IHNV | $4,0*10^3$ | $2,7*10^6$ | | |
| 3 | IHNV | $1,9*10^3$ | $4,0*10^4$ | | |
| 4 | IHNV | | $4,0*10^5$ | $1,3*10^4$ | $2,7*10^5$ |
| 5 | IHNV | $< 1,9*10^2$ | $5,9*10^5$ | $4,0*10^6$ | $5,9*10^5$ |
| 6 | IHNV | $< 1,9*10^2$ | $1,9*10^6$ | | |
| 7 | IHNV | $< 1,9*10^2$ | $1,9*10^6$ | | |
| 8 | IHNV | $4,0*10^3$ | $5,9*10^5$ | $8,6*10^4$ | |
| 9 | IHNV | | $2,7*10^4$ | $1,3*10^3$ | $2,7*10^3$ |
| 10 | IHNV | $1,3*10^6$ | $4,0*10^5$ | | |
| 11 | IHNV | $5,9*10^3$ | $8,6*10^5$ | | |
| 12 | IHNV | $< 1,9*10^2$ | $1,9*10^5$ | | |
| 13 | IHNV | $8,6*10^4$ | $1,3*10^6$ | | |
| 14 | IHNV | $< 1,9*10^2$ | $2,7*10^5$ | | |
| 15 | IHNV | | $1,3*10^5$ | $1,3*10^6$ | |
| 16 | IHNV | $4,0*10^2$ | $8,6*10^6$ | $1,3*10^5$ | $2,7*10^6$ |
| 17 | IHNV/SVCV | | $4,0*10^5$ | $1,3*10^5$ | |
| 18 | IHNV | $8,6*10^2$ | | | $8,6*10^3$ |
| 19 | IHNV | $5,9*10^7$ | | $1,3*10^5$ | $2,7*10^4$ |
| 20 | IHNV | | $4,0*10^4$ | $8,6*10^3$ | |
| 21 | IHNV | $2,7*10^3$ | $1,9*10^6$ | $1,9*10^4$ | $2,7*10^5$ |
| 22 | IHNV | $< 1,9*10^2$ | | | $1,3*10^3$ |
| 23 | IHNV | | $1,9*10^5$ | | $5,9*10^6$ |
| 24 | IHNV | | $8,6*10^5$ | $2,7*10^5$ | $1,3*10^6$ |
| 25 | IHNV | $< 1,9*10^2$ | | | $2,7*10^6$ |
| 26 | IHNV | $1,3*10^3$ | $4,0*10^5$ | | $4,0*10^5$ |
| 27 | IHNV | $< 1,9*10^2$ | $< 1,9*10^2$ | | |
| 28 | IHNV | $1,9*10^4$ | $2,7*10^5$ | | |
| 29 | IHNV | $< 1,9*10^2$ | $2,7*10^5$ | $2,7*10^4$ | |
| 30 | IHNV | $5,9*10^4$ | $8,6*10^5$ | | |
| 31 | IHNV | $< 1,9*10^2$ | $< 1,9*10^2$ | | |
| 32 | IHNV | $1,3*10^4$ | $4,0*10^5$ | $1,3*10^4$ | $8,6*10^4$ |
| 33 | IHNV | $< 1,9*10^2$ | $1,9*10^5$ | | |
| 34 | IHNV | $4,0*10^5$ | $2,7*10^5$ | | $4,0*10^5$ |
| 36 | Virus not found | $< 1,9*10^2$ | $< 1,9*10^2$ | | |
| 37 | IHNV | $8,6*10^4$ | $1,3*10^6$ | | |
| 38 | IHNV | $< 1,9*10^2$ | $4,0*10^5$ | | |
| 39 | IHNV | $2,7*10^5$ | $8,6*10^5$ | | $5,9*10^5$ |

| | | | | |
|---|---|---|---|---|
| Number of laboratories | 31 | 34 | 14 | 16 |
| Median titre | $9,3*10^3$ | $4,0*10^5$ | $5,7*10^4$ | $3,4*10^5$ |
| Maximum titre | $5,9*10^7$ | $8,6*10^6$ | $4,0*10^6$ | $5,9*10^6$ |
| Minimum titre | $2,7*10^2$ | $2,7*10^4$ | $1,3*10^3$ | $1,3*10^3$ |
| 25% quartile titre | $2,1*10^3$ | $2,7*10^5$ | $1,3*10^4$ | $7,1*10^4$ |
| 75% quartile titre | $8,6*10^4$ | $8,6*10^5$ | $1,3*10^5$ | $7,6*10^5$ |

**Table 6.** Inter-Laboratory Proficiency Test, PT1, 2010 – Results of titration of ampoule III.

| ***Ampoule III - Ranavirus not EHNV; or ESV-ECV*** | | | | | |
|---|---|---|---|---|---|
| Laboratory code number | Virus Identification | Titre in | | | |
| | | BF-2 | EPC | RTG-2 | FHM |
| 1 | ESV | $8,6*10^4$ | $8,6*10^4$ | $4,0*10^4$ | $5,9*10^5$ |
| 2 | ESV-ECV | $1,3*10^6$ | $8,6*10^4$ | | |
| 3 | EHNV | $4,0*10^6$ | $1,9*10^6$ | | |
| 4 | ECV/ESV | | $< 1,9*10^2$ | $< 1,9*10^2$ | $1,3*10^3$ |
| 5 | Ranavirus, not EHNV | $8,6*10^4$ | $5,9*10^4$ | $4,0*10^4$ | $1,3*10^3$ |
| 6 | Not VHSV, IHNV | $1,3*10^4$ | $1,3*10^4$ | | |
| 7 | SAV | $1,9*10^4$ | $1,3*10^4$ | | |
| 8 | Ranavirus ECV | $8,6*10^5$ | $1,3*10^3$ | $1,9*10^3$ | |
| 9 | Iridovirus, Ranavirus ESV= ECV= ECV24 | $1,3*10^5$ | $8,6*10^4$ | $< 1,9*10^2$ | $< 1,9*10^2$ |
| 10 | Identification performed by another NRL | $5,9*10^5$ | $1,3*10^4$ | | |
| 11 | EHNV | $2,7*10^5$ | $2,7*10^6$ | | |
| 12 | Iridovirus | $5,9*10^3$ | $1,9*10^3$ | | |
| 13 | ECV/ESV | $8,6*10^4$ | $4,0*10^4$ | | |
| 14 | EHNV | $1,9*10^5$ | $5,9*10^4$ | | |
| 15 | Ranavirus | | $1,3*10^4$ | $1,3*10^3$ | |
| 16 | ECV | $1,3*10^4$ | $1,3*10^5$ | $1,9*10^3$ | $1,9*10^4$ |
| 17 | ESV or ECV | | $2,7*10^6$ | $1,3*10^3$ | |
| 18 | ECV/ESV | $8,6*10^4$ | | | $4,0*10^3$ |
| 19 | ECV | $4,0*10^6$ | | $2,7*10^3$ | $1,9*10^3$ |
| 20 | EHNV | | $8,6*10^4$ | $< 1,9*10^2$ | |
| 21 | ECV or ESV | $1,3*10^6$ | $1,3*10^5$ | $8,6*10^5$ | $1,3*10^5$ |
| 22 | Rana | $1,3*10^3$ | | | $< 1,9*10^2$ |
| 23 | ECV | | $8,6*10^4$ | | $4,0*10^4$ |
| 24 | Ranavirus | | $4,0*10^5$ | $1,3*10^4$ | $< 1,9*10^2$ |
| 25 | Iridovirus not EHNV | $5,9*10^4$ | | | $1,3*10^4$ |
| 26 | ESV | $2,7*10^4$ | $1,3*10^5$ | | $5,9*10^4$ |
| 27 | Virus not found | $< 1,9*10^2$ | $< 1,9*10^2$ | | |
| 28 | Iridovirus | $4,0*10^6$ | $1,3*10^5$ | | |
| 29 | ESV or ECV | $1,3*10^3$ | $1,3*10^3$ | $< 1,9*10^2$ | |
| 30 | Ranavirus | $1,9*10^5$ | $5,9*10^4$ | | |
| 31 | EHNV | $< 1,9*10^2$ | $< 1,9*10^2$ | | |
| 32 | ECV/ESV | $1,3*10^5$ | $2,7*10^4$ | $< 1,9*10^2$ | $< 1,9*10^2$ |
| 33 | EHNV | $2,7*10^4$ | $2,7*10^3$ | | |
| 34 | EHNV | $2,7*10^4$ | $1,3*10^7$ | | $4,0*10^5$ |
| 36 | EHNV | $< 1,9*10^2$ | $< 1,9*10^2$ | | |
| 37 | Ranavirus: ESV / ECV | $8,6*10^4$ | $1,3*10^5$ | | |
| 38 | Ranavirus (ECV or ESV or BIV) | $8,6*10^4$ | $1,3*10^5$ | | |
| 39 | Ranavirus | $1,3*10^5$ | $8,6*10^4$ | | $1,3*10^5$ |

| | | | | |
|---|---|---|---|---|
| Number of laboratories | 32 | 34 | 14 | 16 |
| Median titre | $8,6*10^4$ | $8,6*10^4$ | $2,7*10^3$ | $2,9*10^4$ |
| Maximum titre | $4,0*10^6$ | $1,3*10^7$ | $8,6*10^5$ | $5,9*10^5$ |
| Minimum titre | $1,3*10^3$ | $1,3*10^3$ | $1,3*10^3$ | $1,3*10^3$ |
| 25% quartile titre | $2,7*10^4$ | $1,6*10^4$ | $1,9*10^3$ | $3,5*10^3$ |
| 75% quartile titre | $2,7*10^5$ | $1,3*10^5$ | $4,0*10^4$ | $1,3*10^5$ |

**Table 7.** Inter-Laboratory Proficiency Test, PT1, 2010 – Results of titration of ampoule IV.

| ***Ampoule IV - SVCV*** | | | | | |
|---|---|---|---|---|---|
| Laboratory code number | Virus Identification | Titre in | | | |
| | | BF-2 | EPC | RTG-2 | FHM |
| 1 | SVCV | $2,7*10^5$ | $2,7*10^5$ | $5,9*10^5$ | $4,0*10^4$ |
| 2 | SVCV | $1,3*10^4$ | $1,9*10^6$ | | |
| 3 | SVCV | $2,7*10^6$ | $5,9*10^5$ | | |
| 4 | SVCV | | $1,9*10^5$ | $2,7*10^4$ | $2,7*10^5$ |
| 5 | SVCV | $2,7*10^5$ | $1,9*10^6$ | $1,9*10^5$ | $4,0*10^5$ |
| 6 | Not VHSV, IHNV | $1,3*10^3$ | $1,3*10^5$ | | |
| 7 | SVCV | $8,6*10^5$ | $4,0*10^5$ | | |
| 8 | SVCV | $1,9*10^4$ | $2,7*10^6$ | $4,0*10^4$ | |
| 9 | SVCV | | $1,3*10^5$ | $< 1,9*10^2$ | $1,9*10^5$ |
| 10 | SVCV | $5,9*10^6$ | $1,3*10^6$ | | |
| 11 | SVCV | $1,3*10^4$ | $1,3*10^6$ | | |
| 12 | SVCV | $1,9*10^6$ | $1,3*10^6$ | | |
| 13 | SVCV | $1,9*10^6$ | $4,0*10^5$ | | |
| 14 | SVCV | $4,0*10^4$ | $5,9*10^4$ | | |
| 15 | SVCV | | $5,9*10^5$ | $2,7*10^4$ | |
| 16 | SVCV | $2,7*10^6$ | $2,7*10^6$ | $4,0*10^4$ | $1,9*10^6$ |
| 17 | SVCV | | $5,9*10^6$ | $< 1,9*10^2$ | |
| 18 | SVCV | $8,6*10^2$ | | | $1,3*10^3$ |
| 19 | SVCV | $1,3*10^3$ | | $2,7*10^4$ | $4,0*10^4$ |
| 20 | SVCV | | $1,3*10^5$ | $< 1,9*10^2$ | |
| 21 | SVCV | $1,9*10^6$ | $1,9*10^6$ | $1,9*10^6$ | $8,6*10^5$ |
| 22 | SVCV | $1,3*10^3$ | | | $1,3*10^3$ |
| 23 | SVCV | | $4,0*10^4$ | | $8,6*10^5$ |
| 24 | SVCV | | $2,7*10^6$ | $1,3*10^5$ | $5,9*10^5$ |
| 25 | SVCV | $1,9*10^4$ | | | $4,0*10^6$ |
| 26 | IHNV/SVCV | $1,9*10^6$ | $1,9*10^6$ | | $8,6*10^5$ |
| 27 | VHSV/IPNV | $< 1,9*10^2$ | $< 1,9*10^2$ | | |
| 28 | SVCV | $2,7*10^6$ | $1,9*10^6$ | | |
| 29 | SVCV | $1,9*10^5$ | $5,9*10^6$ | $4,0*10^4$ | |
| 30 | SVCV | $1,9*10^6$ | $1,9*10^6$ | | |
| 31 | SVCV | $< 1,9*10^2$ | $4,0*10^2$ | | |
| 32 | SVCV | $2,7*10^5$ | $5,9*10^4$ | $1,9*10^4$ | $5,9*10^5$ |
| 33 | SVCV | $1,9*10^5$ | $2,7*10^5$ | | |
| 34 | SVCV | $1,3*10^5$ | $1,3*10^4$ | | $1,9*10^5$ |
| 36 | SVCV | $5,9*10^4$ | $2,7*10^5$ | | |
| 37 | SVCV | $1,3*10^5$ | $8,6*10^5$ | | |
| 38 | SVCV | $< 1,9*10^2$ | $1,3*10^5$ | | |
| 39 | SVCV | $5,9*10^5$ | $4,0*10^5$ | | $2,7*10^5$ |

| | | | | |
|---|---|---|---|---|
| Number of laboratories | 31 | 34 | 14 | 16 |
| Median titre | $2,3*10^5$ | $5,9*10^5$ | $4,0*10^4$ | $3,4*10^5$ |
| Maximum titre | $5,9*10^6$ | $5,9*10^6$ | $1,9*10^6$ | $4,0*10^6$ |
| Minimum titre | $8,6*10^2$ | $4,0*10^2$ | $1,9*10^4$ | $1,3*10^3$ |
| 25% quartile titre | $1,9*10^4$ | $1,3*10^5$ | $2,7*10^4$ | $1,5*10^5$ |
| 75% quartile titre | $1,9*10^6$ | $1,9*10^6$ | $1,6*10^5$ | $8,6*10^5$ |

**Table 8.** Inter-Laboratory Proficiency Test, PT1, 2010 – Results of titration of ampoule V.

| ***Ampoule V - VHSV*** | | | | | |
|---|---|---|---|---|---|
| Laboratory code number | Virus Identification | Titre in | | | |
| | | BF-2 | EPC | RTG-2 | FHM |
| 1 | VHSV | $2,7*10^5$ | $8,6*10^5$ | $5,9*10^5$ | $1,3*10^5$ |
| 2 | VHSV | $4,0*10^3$ | $1,9*10^6$ | | |
| 3 | VHSV | $5,9*10^4$ | $4,0*10^5$ | | |
| 4 | VHSV | | $1,3*10^5$ | $1,9*10^4$ | $8,6*10^4$ |
| 5 | VHSV | $1,9*10^5$ | $4,0*10^5$ | $8,6*10^4$ | $5,9*10^5$ |
| 6 | VHSV | $2,7*10^4$ | $1,9*10^6$ | | |
| 7 | VHSV | $1,3*10^5$ | $5,9*10^5$ | | |
| 8 | VHSV | $2,7*10^5$ | $1,3*10^5$ | $5,9*10^3$ | |
| 9 | VHSV/IHNV | | $8,6*10^4$ | $8,6*10^3$ | $2,7*10^4$ |
| 10 | VHSV | $1,3*10^6$ | $4,0*10^4$ | | |
| 11 | VHSV | $2,7*10^4$ | $4,0*10^4$ | | |
| 12 | VHSV | $1,9*10^5$ | $5,9*10^5$ | | |
| 13 | VHSV | $1,3*10^6$ | $5,9*10^5$ | | |
| 14 | VHSV | $2,7*10^3$ | $1,9*10^3$ | | |
| 15 | VHSV | | $8,6*10^4$ | $5,9*10^5$ | |
| 16 | VHSV | $5,9*10^5$ | $5,9*10^5$ | $2,7*10^5$ | $1,9*10^5$ |
| 17 | VHSV/SVCV | | $8,6*10^6$ | $4,0*10^4$ | |
| 18 | VHSV | $5,9*10^2$ | | | $4,0*10^3$ |
| 19 | VHSV | $5,9*10^4$ | | $4,0*10^4$ | $4,0*10^4$ |
| 20 | VHSV | | $2,7*10^5$ | $5,9*10^4$ | |
| 21 | VHSV | $8,6*10^5$ | $1,3*10^6$ | $8,6*10^5$ | $5,9*10^5$ |
| 22 | VHSV | $1,3*10^3$ | | | $1,3*10^3$ |
| 23 | VHSV | | $5,9*10^4$ | | $8,6*10^5$ |
| 24 | VHSV | | $1,3*10^5$ | $5,9*10^5$ | $5,9*10^5$ |
| 25 | VHSV | $5,9*10^5$ | | | $1,3*10^6$ |
| 26 | VHSV | $4,0*10^4$ | $1,9*10^5$ | | $5,9*10^5$ |
| 27 | Virus not found | $< 1,9*10^2$ | $< 1,9*10^2$ | | |
| 28 | VHSV | $2,7*10^4$ | $1,3*10^4$ | | |
| 29 | VHSV | $5,9*10^4$ | $1,9*10^6$ | $1,9*10^4$ | |
| 30 | VHSV | $1,3*10^6$ | $2,7*10^6$ | | |
| 31 | Virus not found | $< 1,9*10^2$ | $< 1,9*10^2$ | | |
| 32 | VHSV | $1,9*10^5$ | $1,9*10^5$ | $8,6*10^3$ | $2,7*10^5$ |
| 33 | VHSV | $2,7*10^4$ | $8,6*10^4$ | | |
| 34 | Virus not found | $2,7*10^2$ | $1,9*10^2$ | | $8,6*10^2$ |
| 36 | VHSV | $5,9*10^4$ | $1,9*10^5$ | | |
| 37 | VHSV | $5,9*10^5$ | $4,0*10^5$ | | |
| 38 | VHSV | $2,7*10^6$ | $1,9*10^5$ | | |
| 39 | VHSV | $2,7*10^5$ | $2,7*10^5$ | | $1,9*10^5$ |

| | | | | |
|---|---|---|---|---|
| Number of laboratories | 31 | 34 | 14 | 16 |
| Median titre | $1,3*10^5$ | $2,3*10^5$ | $4,9*10^4$ | $1,9*10^5$ |
| Maximum titre | $2,7*10^6$ | $8,6*10^6$ | $8,6*10^5$ | $1,3*10^6$ |
| Minimum titre | $2,7*10^2$ | $1,9*10^2$ | $5,9*10^3$ | $8,6*10^2$ |
| 25% quartile titre | $2,7*10^4$ | $8,6*10^4$ | $1,9*10^4$ | $3,7*10^4$ |
| 75% quartile titre | $5,9*10^5$ | $5,9*10^5$ | $5,1*10^5$ | $5,9*10^5$ |

**Figure 4.** Virus titre obtained in BF-2 cells

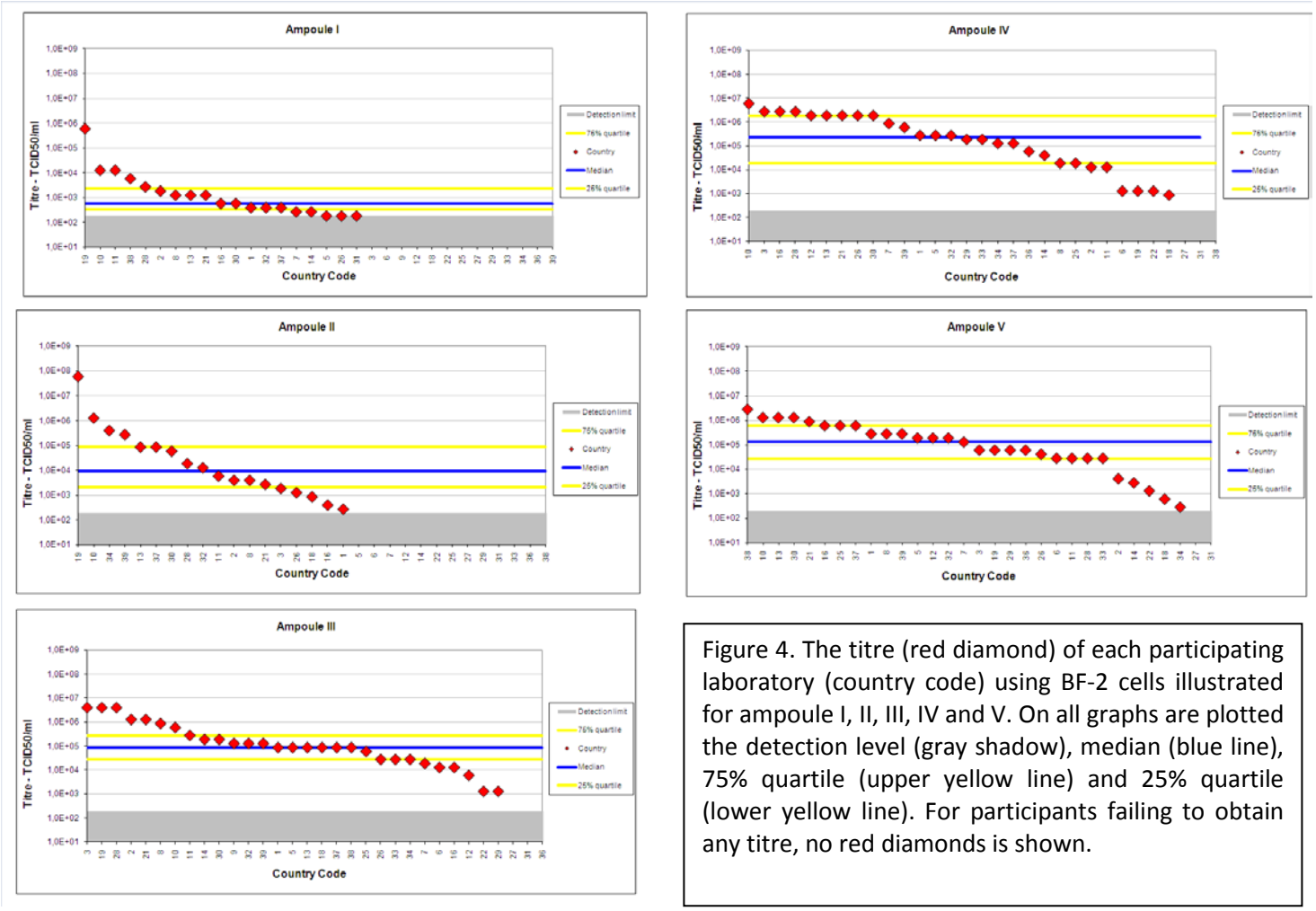



Figure 4. The titre (red diamond) of each participating laboratory (country code) using BF-2 cells illustrated for ampoule I, II, III, IV and V. On all graphs are plotted the detection level (gray shadow), median (blue line), 75% quartile (upper yellow line) and 25% quartile (lower yellow line). For participants failing to obtain any titre, no red diamonds is shown.

**Figure 5.** Virus titre obtained in EPC cells

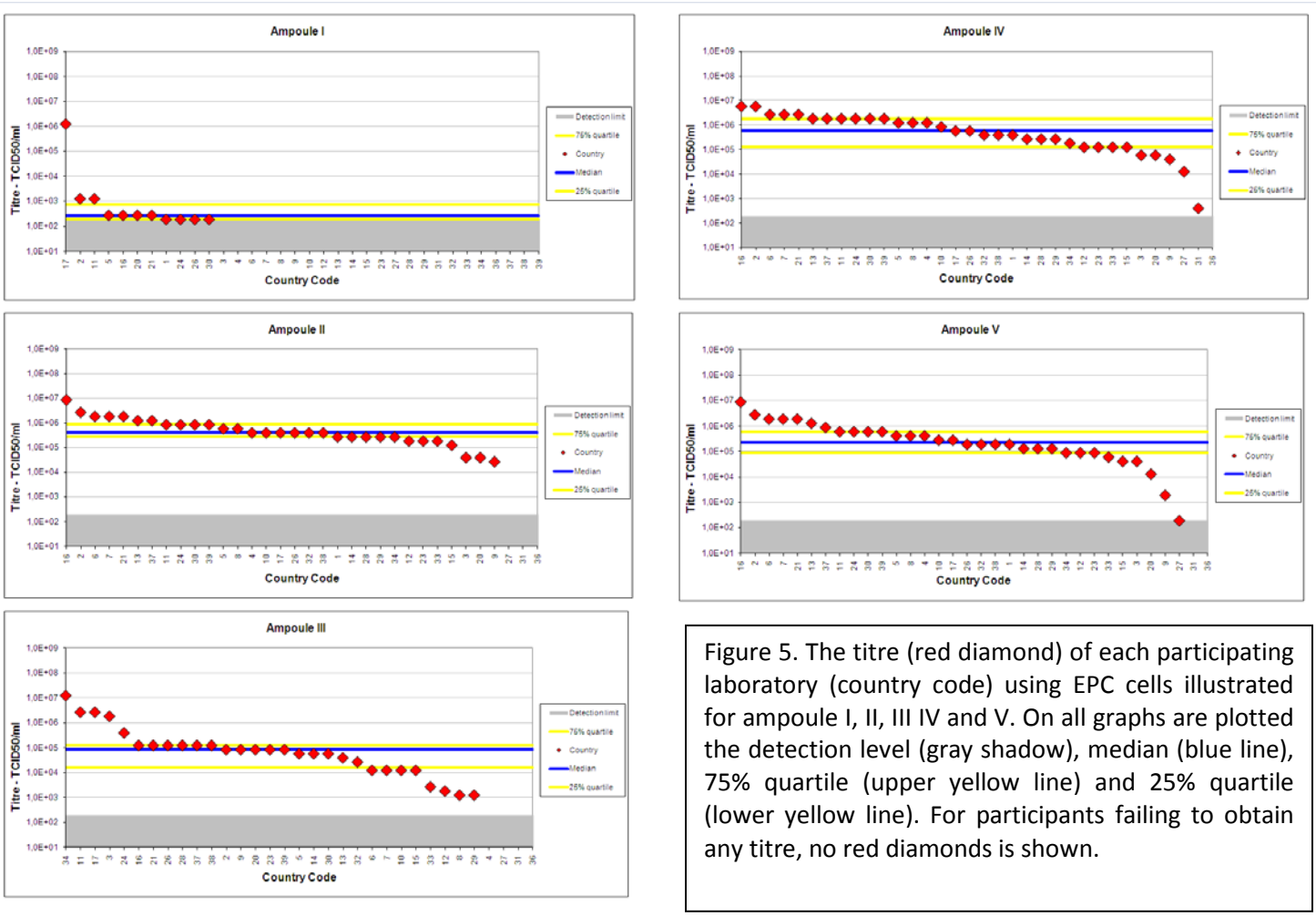



Figure 5. The titre (red diamond) of each participating laboratory (country code) using EPC cells illustrated for ampoule I, II, III IV and V. On all graphs are plotted the detection level (gray shadow), median (blue line), 75% quartile (upper yellow line) and 25% quartile (lower yellow line). For participants failing to obtain any titre, no red diamonds is shown.

**Figure 6.** Virus titre obtained in RTG-2 cells

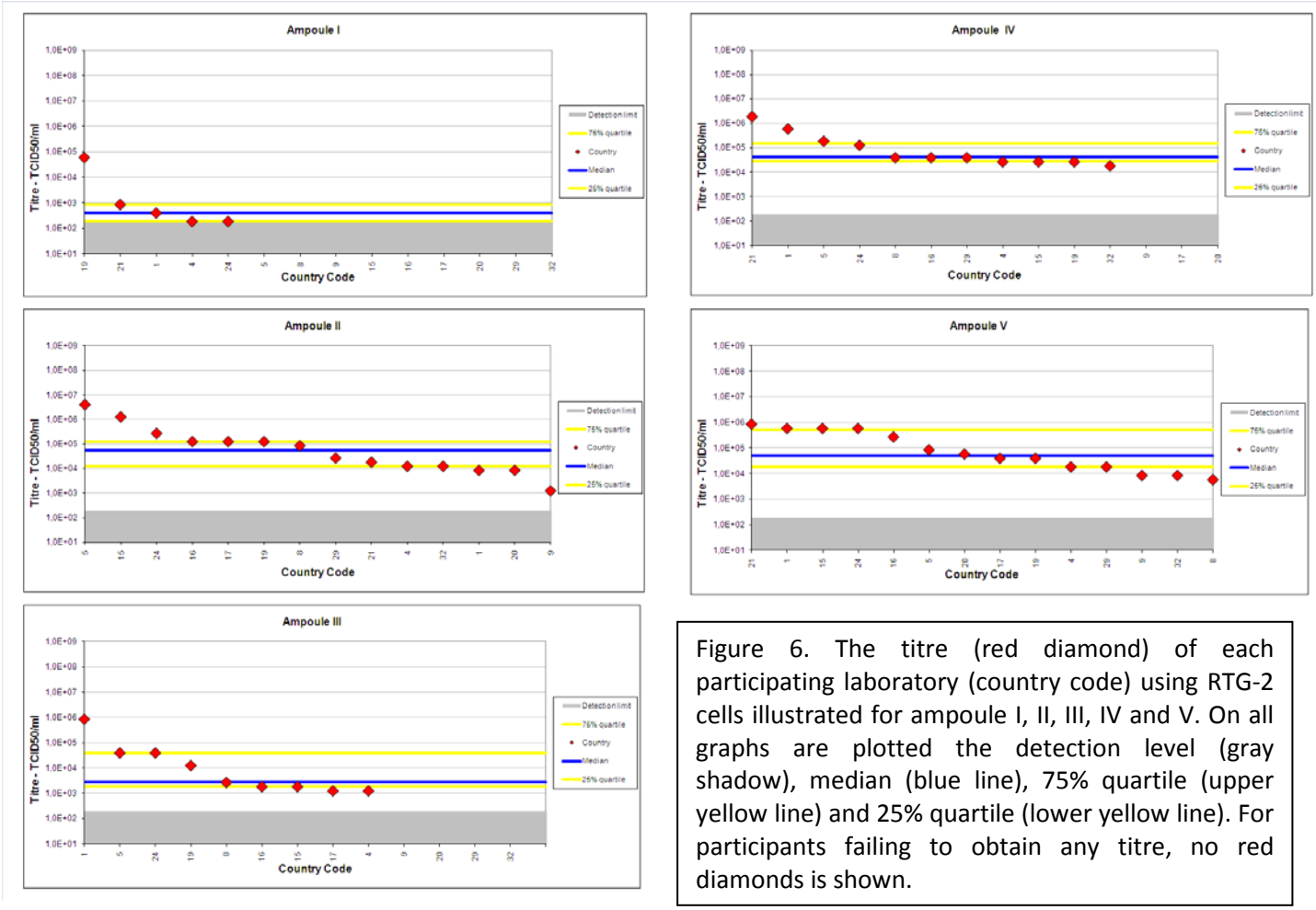



Figure 6. The titre (red diamond) of each participating laboratory (country code) using RTG-2 cells illustrated for ampoule I, II, III, IV and V. On all graphs are plotted the detection level (gray shadow), median (blue line), 75% quartile (upper yellow line) and 25% quartile (lower yellow line). For participants failing to obtain any titre, no red diamonds is shown.

**Figure 7.** Virus titre obtained in FHM cells

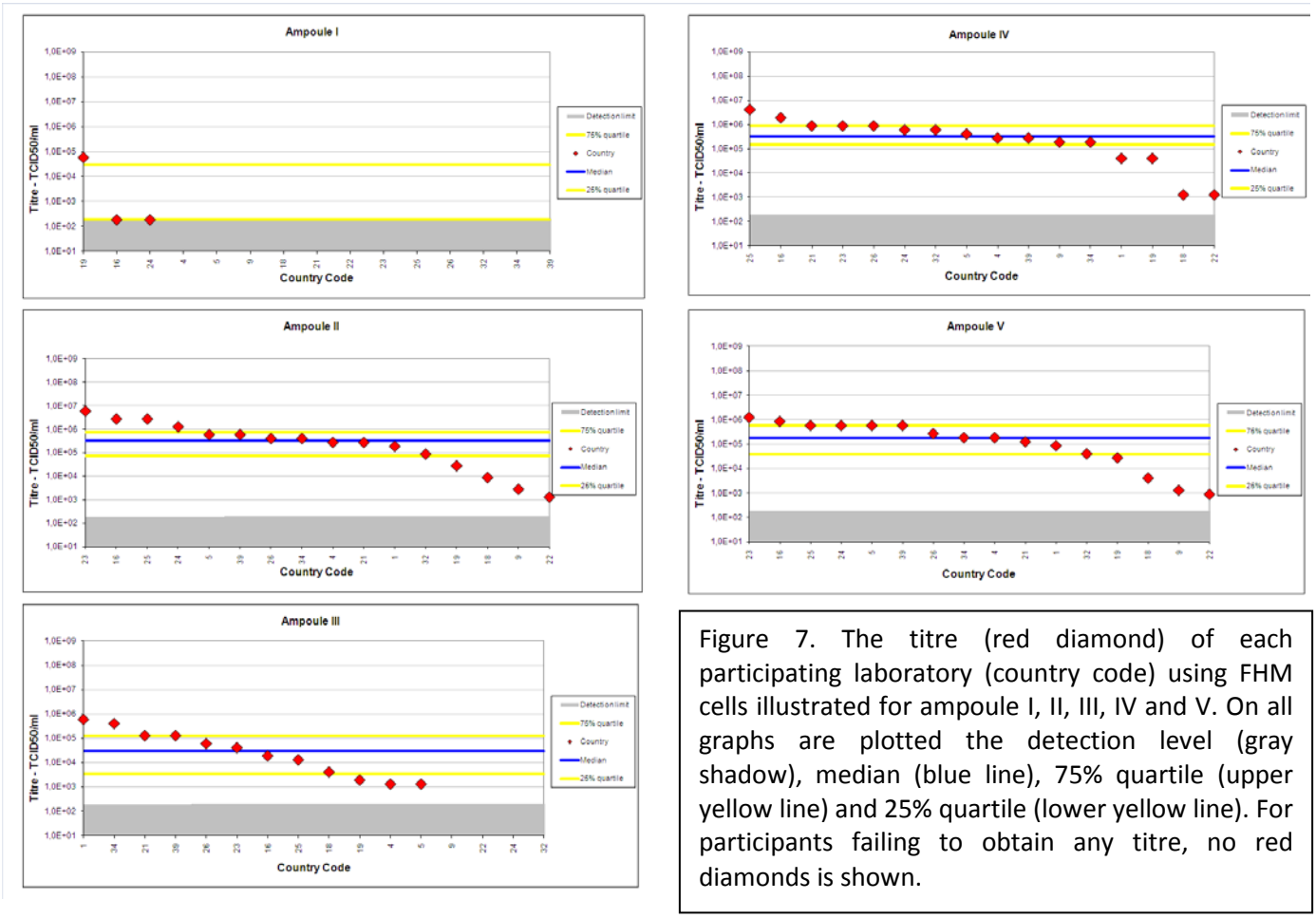



Figure 7. The titre (red diamond) of each participating laboratory (country code) using FHM cells illustrated for ampoule I, II, III, IV and V. On all graphs are plotted the detection level (gray shadow), median (blue line), 75% quartile (upper yellow line) and 25% quartile (lower yellow line). For participants failing to obtain any titre, no red diamonds is shown.

*Identification of content*

- 17 laboratories correctly identified all viruses in all ampoules. Furthermore, one laboratory was only obliged to identify VHSV, IHNV and SVCV and did that correct.

Ampoule I – EHNV

- 24 laboratories correctly identified EHNV.
- 1 laboratory identified ranavirus.
- 2 laboratories identified more isolates than were present.
- 8 laboratories did not find any virus.
- 1 laboratory found virus but did not identify it.
- 1 laboratory identified Salmonid alpha virus (SAV).

Ampoule II - IHNV

- 36 laboratories correctly identified IHNV.
- 1 laboratory identified more isolates than were present.
- 1 laboratory did not identify any virus.

Ampoule III – Ranavirus, ECV (or ESV), not EHNV

- 24 laboratories correctly identified Ranavirus but not EHNV.
- 9 laboratories identified EHNV. 6 of these laboratories submitted sequences that were either identical to or most similar to sheetfish or catfish iridovirus.
- 1 laboratory identified ranavirus.
- 1 laboratory found virus but did not identify it.
- 1 laboratory identified SAV.
- 1 laboratory did not identify any virus.

Ampoule IV – SVCV

- 35 laboratories correctly identified SVCV.
- 2 laboratories identified more isolates than were present.
- 1 laboratory found virus but did not identify it.

Ampoule V – VHSV

- 33 laboratories correctly identified VHSV.
- 2 laboratories identified more isolates than were present.
- 3 laboratories did not identify any virus.

*Scores*
Starting with proficiency test 2003 we have provided a scoring system for the identification part of the proficiency test. This year we have assigned a score of 2 for each correct answer/identification (Table 3), giving the possibility for obtaining a maximum score of 10.

For ampoule I: EHNV identification was given the score of 2. Ranavirus / iridovirus as the only answer for this ampoule was given the score of 1 (e.g. if no sequence analysis was performed)

For ampoule III: Identification of "ranavirus / iridovirus not EHNV" or Catfish/sheatfish iridovirus was given the score of 2. Ranavirus / iridovirus as the only answer for this ampoule was given the score of 1 (e.g. if no sequence analysis was performed)

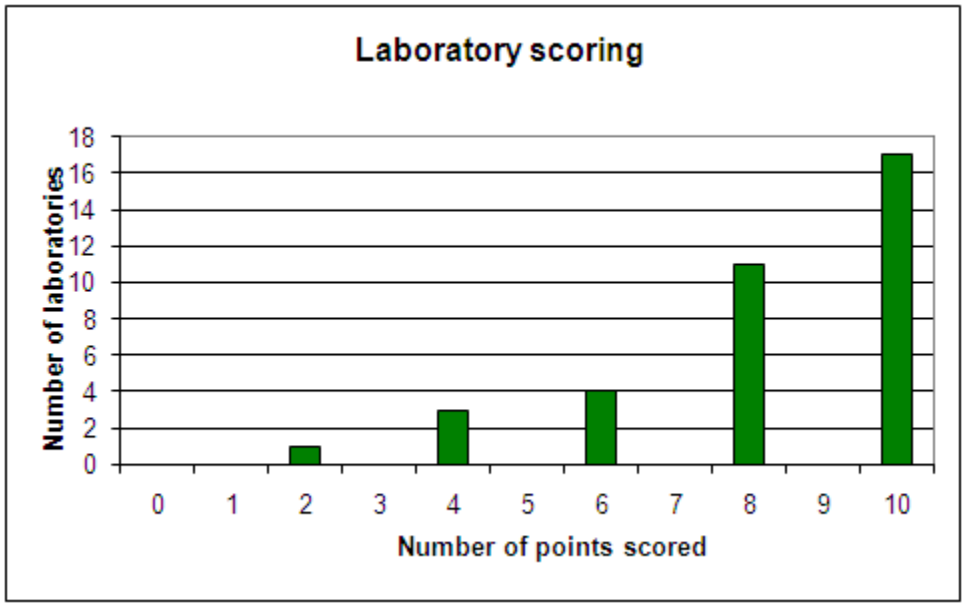
Ampoule IV: Identification of SVCV was given the score 2 and identification of virus as "not VHSV, IHNV or EHNV" was given the score 1.

Incorrectly finding of "no virus" or additional types of viruses than those included in the ampoules scored 0 even though included virus was amongst the identified viruses.

18 laboratories out of 38 correctly identified all viruses in all ampoules correct and obtained maximum score. 10 laboratories did not identify the virus in ampoule III as ECV, ESV or rana but not EHNV. Nine laboratories did not identify virus in one or more ampoules where a virus was present. 5 laboratories observed additional virus than present in the ampoule. A diagram of the scoring obtained by the laboratories is shown in figure 8.

Serotyping and genotyping of VHSV and IHNV and submission of sequencing results is not a mandatory part of the test and is not included in the score of participants.

**Figure 8.** Obtained score by participants.



*Methods applied*

The following cell lines were used by the participants:

- 32 laboratories used BF-2 cells
- 34 laboratories used EPC cells
- 14 laboratories used RTG-2 cells
- 16 laboratories used FHM cells
- 1 laboratory used CHSE cells
- 6 laboratories used four cell lines
- 9 laboratories used tree cell lines
    - 2 laboratories used BF-2, EPC and RTG-2
    - 2 laboratories used EPC, RTG-2 and FHM
    - 3 laboratories used BF-2, EPC and FHM
    - 1 laboratories used BF-2, RTG-2 and FHM

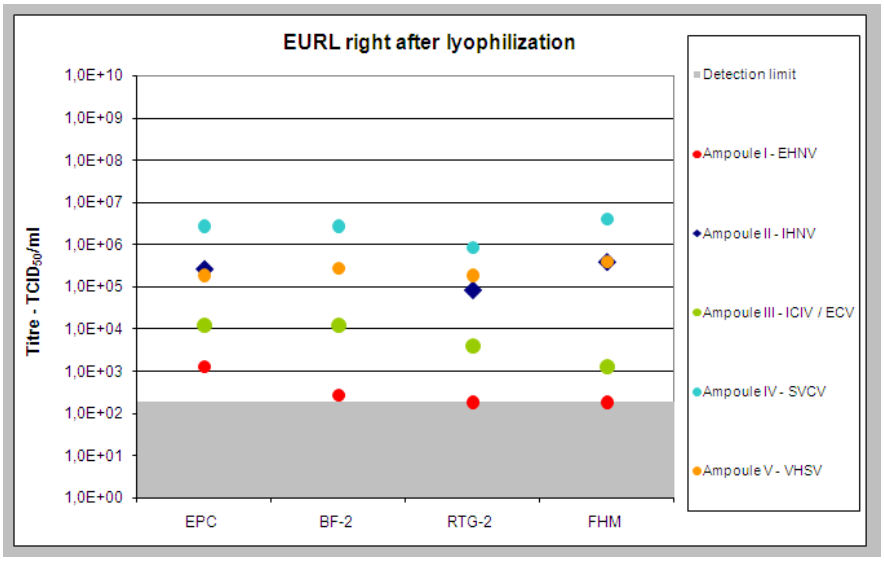
- 23 laboratories used two cell lines:
    - 17 laboratories used BF-2 cells in combination with EPC cells
    - 3 laboratories used RTG-2 cells in combination with EPC cells
    - 3 laboratories used BF-2 cells in combination with FHM cells
    - 1 laboratory used EPC cells in combination with FHM cells

The combination of EPC and FHM cells is not valid according to Commission Decision 2001/183/EC. The laboratories using these combinations are encouraged to include the use of BF-2 cells.

The median titre calculated from the submitted results of each ampoule and in each cell line is illustrated in Figure 9.

It appears that VHSV (Ampoule V) replicates well on BF-2, FHM, RTG-2 and EPC cells. IHNV (ampoule II) replicates most efficiently on EPC and FHM cells. ECV (ampoule III) seems to replicate most efficiently on EPC, and BF-2 cells whereas lower titres were observed on RTG-2 and FHM cells. EHNV seems to replicate best on EPC cells however it is difficult to make a conclusion because of the low titer of the virus in the ampoule (I).

**Figure 9.** Median titre of viruses obtained by participants at different cell lines.



*Methods used for identification of viruses*
(Table 9)

- 25 laboratories used ELISA for identification of viruses.
- 23 laboratories used IFAT for identification of viruses.
- 8 laboratories used neutralisation tests for identification of viruses.
- 35 laboratories used PCR for identification of viruses.
- 30 laboratories performed sequencing for identification of viruses.

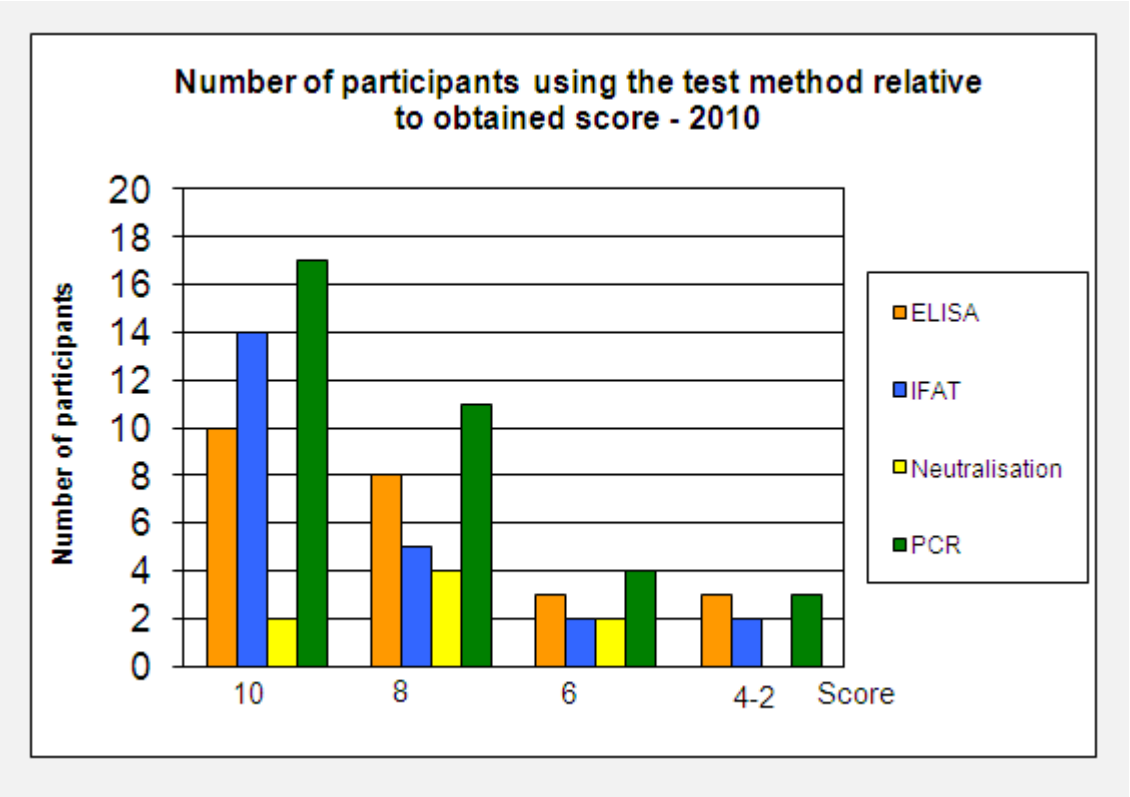
**Table 9.** Results obtained by different test methods in participating laboratories.

| Laboratory code number | Score 10/10 | ELISA | IFAT | Neutralisation | PCR | Sequence | Sequence ampoule no. |
|---|---|---|---|---|---|---|---|
| 1 | 8 | X | X | | X | X | I, II, III and V |
| 2 | 10 | X | X | | X | X | I and III |
| 3 | 6 | X | X | X | X | | |
| 4 | 10 | X | | | X | X | I, II, III, IV and V |
| 5 | 10 | X | X | X | X | X | I, II, III, IV and V |
| 6 | 4 | X | X | | | | |
| 7 | 6 | X | | | X | X | V |
| 8 | 10 | | X | | X | X | I, II, III and V |
| 9 | 8 | X | X | X | X | X | I, II, III, IV and V |
| 10 | 6/6 | X | | | | | |
| 11 | 8 | X | | | X | X | I and III |
| 12 | 8 | X | X | | X | X | II, III, IV and V |
| 13 | 10 | | X | | X | X | I, II, III and V |
| 14 | 8 | X | X | X | X | X | I, II, III and V |
| 15 | 8 | X | | | X | X | II and III |
| 16 | 10 | X | X | | X | X | I, II and III |
| 17 | 6 | X | | X | X | X | I, II, III, IV and V |
| 18 | 8 | X | X | X | X | X | III |
| 19 | 10 | X | X | | X | X | I, II, III, IV and V |
| 20 | 8 | | | | X | X | I, II, III, IV and V |
| 21 | 10 | X | X | X | X | X | I, II, III and V |
| 22 | 8 | X | | X | X | | |
| 23 | 10 | | X | | X | X | I and III |
| 24 | 10 | X | X | | X | X | I, II, III, IV and V |
| 25 | 10 | X | X | | X | X | I, II, III and IV |
| 26 | 8 | | | | X | X | I, II, III, IV and V |
| 27 | 2 | X | | | | | |
| 28 | 10 | X | X | | X | X | I, III, IV and V |
| 29 | 8 | | | | X | X | I, II, III, IV and V |
| 30 | 10 | | X | | X | X | I and III |
| 31 | 4 | | | | X | | |
| 32 | 10 | | | | X | X | I, II, III, IV and V |
| 33 | 6 | | X | | X | | |
| 34 | 4 | | | | X | X | III |
| 36 | 4 | X | X | | X | X | III |
| 37 | 10 | | X | | X | X | I, II,III and V |
| 38 | 10 | X | X | | X | | |
| 39 | 10 | | | | X | X | I, II, III, IV and V |

A graph was constructed to illustrate the association between the methods used by participants for virus identification and the obtained score (Figure 10). The PCR is the most frequently used method by participants and only three participants did not use this method. For participants scoring lower than 10, the deficiency in virus identification cannot directly be assigned to improper use of a single identification method. Rather mistakes might be related to performance of the overall procedure. It is clear, however, that if PCR is not used, a correct answer cannot be made for ampoule I and III.

However, there might be a more general problem related to discrimination of EHNV from the other ranaviruses as 9 participating laboratories have reported identification of EHNV in ampoule III instead of ECV. Another critical point seemed to be the low titer of the EHNV virus in ampoule I as 8 participating laboratories did not identify a virus in the ampoule. We can only recommend that participant subcultivate the samples as it is described in the Commission Decision 2001/183/EC.
Finally, 6 out of the 20 participants scoring lower than 10 identify false positive viruses in the ampoule indicating that cross contamination could have occurred at some point in the diagnostic process.

**Figure 10.** Methods used by participants for identification




*Genotypning and sequencing*
In previous proficiency tests provided by the EURL, we have encouraged participants to genotype the identified viruses though it has not been a mandatory task. As ranaviruses are included in the test, it is mandatory to do sequence analyses in order to discriminate EHNV from the non-listed ranaviruses. For IHNV and VHSV we still encouraged participants to genotype isolates according to the notification described in Einer-Jensen et al. 2004 for VHSV and in Kurath et al. 2003 but this was not an obligatory task.

Ampoule I – EHNV and III – ECV/ESV

- 23 laboratories performed sequencing to identify the virus in ampoule I
- 30 laboratories sequenced to identify the virus in ampoule III
- 1 laboratory performed RFLP for identification of viruses in both ampoules

Within the OIE diagnostic manual for EHN, two sequence based methods are recommended to use in order to discriminate EHNV from the other non-listed ranaviruses. A total of 30 laboratories used

sequencing analyses for identification of EHNV whereas one used RFLP. All laboratories performing sequencing of the isolate in ampoule I, identified the virus correctly as EHNV. However, 7 laboratories that performed sequencing of the ECV isolate in ampoule III identified the virus as EHNV. This was even though that the submitted sequences were in 6 cases identical to ECV/ESV.

Ampoule II - IHNV Genotype M

22 out of 38 laboratories sequenced parts of the genome of either IHNV or VHSV isolates. This is less than did sequencing of the EHNV. Both full length G-gene and partial N- and G-genes were used for virus/genotype identification (see table 10).

- 22 laboratories performed sequencing
- 16 laboratories genotyped the IHNV isolate as belonging to genogroup M
- 1 laboratory genotyped the IHNV isolate as belonging to genogroup U
- 4 laboratories showed blast results
- 1 laboratory did not give any genotype of the sequences

16 laboratories genotyped the IHNV isolate as belonging to genogroup M as described in Kurath et al. 2003 (Table 10). This is more laboratories compared to genotyping of IHNV in proficiency test 2009. One laboratory genotyped the isolate as belonging to genogroup U – the reason for this is not clear. Furthermore, four laboratories showed indirect isolate relatedness to genogroup M by showing blast results.

Ampoule IV - SVCV genotype Id

14 laboratories performed sequencing analyses. Both full length G-gene and partial N- and G-genes were used for virus/genotype identification (see table 10).

- 14 laboratories performed sequencing
- 5 laboratories identified the isolate as being genotype Id
- 3 laboratories showed blast results or used another isolate notification
- 6 laboratories did not give any genotype of the sequences

Ampoule V - VHSV genotype Ia

20 laboratories genotyped the VHSV as described in Einer-Jensen et al. 2004 (Table 10). This is a more laboratories compared to genotyping VHSV in proficiency test 2009.

- 22 laboratories performed sequencing
- 20 laboratories identified the VHSV isolate as genotype I
- 19 laboratories subtyped the isolates as a genotype Ia
- 2 laboratories showed blast results
- 1 laboratory used real-time RT-PCR for genotyping

20 laboratories correctly identified the isolate as belonging to genotype I and 19 laboratories correctly subtyped the isolate as belonging to the Ia subgroup. Two laboratories indicated the genotype of the isolate by showing blast result. Interestingly, one laboratory used a genotype specific real-time RT-PCR to genotype the isolate.

In general, it is positive that more laboratories performed sequencing than at proficiency test 2009 and that sequences were of high quality and usable for genotyping. This high number of laboratories performing sequencing might reflect that EHNV has been included in the test. It is however important that laboratories use their sequencing results to discriminate EHNV from the rest of the much related types of ranaviruses. Furthermore, it is important that the remaining laboratories implement PCR and sequencing techniques in the laboratory as genotyping is the basis for differentiating several listed viruses from others.

**Table 10.** Genotyping, results on viruses in ampoule II-IV submitted by participating laboratories**.**

| Laboratory code number | Score 10/10 | Ampoule I | Ampoule II | Ampoule III | Ampoule IV | Ampoule V |
|---|---|---|---|---|---|---|
| | | EHNV | IHNV | Rana or ESV-ECV | SVCV | VHSV |
| 1 | 8 | | Genogroup M (Partial G) | EHNV (Seq) | | Ia (partial G) |
| 2 | 10 | EHNV (Seq) | | ESV-ECV (Seq) | | |
| 4 | 10 | EHNV (Seq) | (partial G) | ESV-ECV (Seq) | (partial G) | Ia, similar to DK 3345 (partial N) |
| 5 | 10 | EHNV (Seq) | M (partial G) | Ranavirus not EHNV (Seq) | Id (partial G) | Ia (full length G) |
| 7 | 6 | | | | | Ia (partial G) |
| 8 | 10 | EHNV (seq) | identical to FJ265710 (partial N) | European Catfish Virus (seq) | | genotype I a (full length G) |
| 9 | 8 | EHNV (Seq) | M (Europe) (full length G) | ESV-ECV (Seq) | (partial G) | VHSV 1a; IHNV M (Europe) (full length G) |
| 11 | 8 | EHNV (Seq) | | EHNV (Seq) | | |
| 12 | 8 | | U (partial G) | Sheatfish/catfish iridovirus (seq) | (partial G) | Ia (partial G) |
| 13 | 10 | EHNV (Seq) | M (partial G) | ECV/ESV (Seq) | | Ia (partial G) |
| 14 | 8 | EHNV (Seq) | M genogroup (partial N) | EHNV (Seq) | | I (partial N) |
| 15 | 8 | | M genogroup (partial G) | Ranavirus - not EHNV (seq) | | |
| 16 | 10 | EHN 99% homology to FJ 433873.1EHN, AY 187045.1 EHN, AF 157667.1 | IHNV homology to FJ711518.1 IHNV | 99% homology to ECV (AF 157659.1), 99% homology to ESV (AF 157679.1) and 99% homology to AF 157665.1 | | |
| 17 | 6 | EHNV (Seq) | M (partial G) | Ranavirus - not EHNV (seq) | (partial N) | I-a (partial G, N) |
| 18 | 8 | | | ESV-ECV (Seq) | | |
| 19 | 10 | EHNV (Seq) | M (partial N) | ECV (Seq) | (partial G) | I-a (partial N) |
| 20 | 8 | EHNV (Seq) (Acc. No.: AY187045.1; 100%) | (partial G) (Acc. No.: FJ711518.1; 99% identity) | EHNV (Seq) (Acc. No.: FJ433873.1; 99% identity) | (partial G) (Acc. No.: AJ538079.1; 99% identity) | (partial G) (Acc. No.: EU708793.1; 99% identity) |
| 21 | 10 | EHNV (seq) | Genotype M (partial G) | ESV/ECV (seq) | | Genotype Ia (full length G) |
| 23 | 10 | EHNV (seq) | | ECV (seq) | | |
| 24 | 10 | EHNV (seq) | Genogroup M (partial N) | Ranaviruses not EHNV (seq) | Genotype 1d (partial G) | Genotype 1a (partail G) |

| Laboratory code number | Score 10/10 | Ampoule I | Ampoule II | Ampoule III | Ampoule IV | Ampoule V |
|---|---|---|---|---|---|---|
| | | EHNV | IHNV | Rana or ESV-ECV | SVCV | VHSV |
| 25 | 10 | EHNV (seq) | M (partial N) | IRIDOVIRUS NOT EHNV (seq) | 1d (partial G) | 1a (qRT-PCR) |
| 26 | 8 | EHNV (seq) | M-Eur1 (partial G) | ESV (seq) | SVCV (partial G) | 1a (full length G) |
| 28 | 10 | EHNV (seq) | | IRIDOVIRUS (seq) | 99% identical to svcv-arh-98 (partial G) | 98% identical to X73873 (partial N) |
| 29 | 8 | EHNV (seq) | Genogroup M (partial N) | Sheetfish iridovirus (seq) | Z37505 Isolate=Fijan (partial G) | Genotype Ia (partial G) |
| 30 | 10 | EHNV (seq) | | Ranavirus not EHNV (seq) | | |
| 32 | 10 | EHNV (seq) | Genogroup M (full length G)<br><br>European isolates (according to Nishizawa et al. DAO, 2006) | ECV/ESV (seq) | Subgroup Id (full length G) | Genotype Ia (full length G) |
| 33 | 6 | | M | EHNV (seq) | | 97% identity with G-Ia |
| 34 | 4 | | | EHNV (seq) | | |
| 36 | 4 | | | EHNV (seq) | | |
| 37 | 10 | EHNV (seq) | GenBank accession number L40877.1 (partial G) | Ranavirus: ESV / ECV (seq) | | Ia (full length G) |
| 38 | 10 | EHNV (RFLP) | | Ranavirus: ESV / ECV/BIV (RFLP) | | |
| 39 | 10 | EHNV (seq) | M genogroup (partial N) | ranavirus (seq) (99% identity with sheet fish, doctor fish and catfish iridoviruses) | 1d (partial G) | Ia (partial G) |

**Concluding remarks PT1**

The inter-laboratory proficiency test 2010 was conducted without major constrains. Most parcels were delivered by the shipping companies within 3 days after submission. It was, however, unfortunate that one parcel made up to 13 days before delivered to the laboratory due to a delay in an Airline "backlog" and that another parcel made op to 22 days before delivering to the laboratory (primarily due to border controls).

In 2009 EHNV was included in the proficiency test for the first time and 28 participants were able to correctly identify the virus. In this year PT1 EHNV was included and so was the ECV that belongs to the ranavirus family. All the 24 laboratories performing sequencing of the isolate in ampoule I identified the virus correctly as being EHNV. 24 laboratories identified the virus in ampoule III as ranavirus but not EHNV. However, 7 other laboratories that performed sequencing of the ECV isolate in ampoule III identified the virus as EHNV although the submitted sequences in 6 cases were identical to ECV/ESV. We recommend that laboratories carefully analyse their sequencing results when a ranavirus is identified in order to rule out if the virus is the listed EHNV or not.

The EHNV in ampoule was present in a relative low titre, which likely is the reason why 8 laboratories did not identify any virus in this ampoule. We can only recommend that participant subcultivate the samples as it is described in the Commission Decision 2001/183/EC as subcultivation increases the possibilities of isolating low titre viruses.

In this report (figures 4-7), all 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 should be able to compare the sensitivity of their cell lines to the sensitivity of those used by the other participating laboratories. We recommend all participants to evaluate the sensitivity of the cells used in their laboratory in relation to the diagnostic purpose.

The EURL provides the annual proficiency test, collates the data and process the figures so that individual laboratories can see how they perform in relation to the other participants. It is up to the individual laboratory to assess if they perform according to their own expectations and standards. As we did in the proficiency test 2009, we take the opportunity to provide a comment to participants regarding submitted results if relevant. Furthermore we encourage all participants to contacts us with any questions concerning the test or any other diagnostic matters.

The results presented in this report will be further presented and discussed at the 15$^{th}$ Annual Meeting of National Reference Laboratories for Fish Diseases to be held 26-27 May 2011 in Aarhus, Denmark.

# Proficiency test 2, PT2

Five ampoules with lyophilised tissue culture supernatant were delivered to the same laboratories as PT1.

### Content of ampoules

The viruses were propagated on each of their preferred cell line, and when total cytopathic effect (CPE) was observed, the supernatants were collected and filtrated through a 45 µm filter, mixed with equal volumes of 20% w/v lactalbumin hydrolysate solution and lyophilized in glass ampoules. Before the ampoules were sealed by melting, the virus concentration was analysed by real-time PCR (protocol described by Gilad et al. 2004) for KHV and real-time RT-PCR (protocol described by Snow et al- 2006) for ISAV. The details of the virus isolates used in the proficiency test are outlined in table 11.

**Table 11.** Content of each ampoule with reference to culture conditions and major publications of the included virus.

| | **PT2** |
|---|---|
| **Code** | **Specifications** |
| **Ampoule VI: ISAV High titer** | ISAV Glesvaer 2/90<br><br>Received from Dr. B. Dannevig, ISA OIE Reference Laboratory, Oslo, Norway<br><br>References:<br>Mjaaland S, Rimstad E, Falk K & Dannevig BH (1997). Genomic characterization of the virus causing infectious salmon anemia in Atlantic salmon (*Salmo salar* L.): an orthomyxo-like virus in a teleost. Journal of Virology 71, 7681-7686.<br><br>Falk K, Namork E, Rimstad E, Mjaaland S & Dannevig BH (1997). Characterization of infectious salmon anemia virus, an orthomyxo-like virus isolated from Atlantic salmon (Salmo salar L.) Journal of Virology 71, 9016-9023. |
| **Ampoule VII: KHV (CyHV-3) High titer** | KHV 07/108b<br><br>Received from Dr. J. Castric, ANSES, France.<br>Cell culture passage number: 4 in KF-1. |
| **Ampoule VIII: Medium** | Transport medium with 10% fetal bovine serum. No virus. |
| **Ampoule IX: ISAV Medium titer** | ISAV Glesvaer 2/90<br><br>Received from Dr. B. Dannevig, ISA OIE Reference Laboratory, Norway<br><br>References:<br>Mjaaland S, Rimstad E, Falk K & Dannevig BH (1997). Genomic characterization of the virus causing infectious salmon anemia in Atlantic salmon (*Salmo salar* L.): an orthomyxo-like virus in a teleost. Journal of Virology 71, 7681-7686.<br><br>Falk K, Namork E, Rimstad E, Mjaaland S & Dannevig BH (1997). Characterization of infectious salmon anemia virus, an orthomyxo-like virus isolated from Atlantic salmon (Salmo salar L.) Journal of Virology 71, 9016-9023. |
| **Ampoule X: KHV (CyHV-3) Low titer** | KHV 07/108b<br><br>Received from Dr. J. Castric, ANSES, France.<br>Cell culture passage number: 4 in KF-1. |

## Testing of the test

The PT2 test was prepared and tested according to protocols accredited under DS/EN ISO/IEC 17025 and ILAC-G13:08/2007 standards. Prior to distribution we tested 5 ampoules of each virus preparation by PCR (Bercovier et al. 2005) and real-time PCR (Gilad et al. 2004) for KHV and by RT-PCR (Mjaaland et al. 1997) and real-time RT-PCR (Snow et al. 2006) for ISAV, to ascertain identity, a satisfactory titre of the virus and homogeneity of content in the ampoules (Table 12). Furthermore, conventional PCR/RT-PCR fragments were sequenced and so was the HPR region of the ISAV isolate.

The KHV and the ISAV were prepared in different concentrations that were significantly above detection level.

The lyophilisation procedure did not cause a significant virus reduction as detected by real-time PCR or real-time RT-PCR (table 12 and figure 11). The variation is mainly caused by dilution of the viruses. A slight variation can be ascribed the variation in the different assays e.g. the differences in the setting of the threshold Ct values.

Furthermore, after lyophilisation the content of the ampoules were tested for stability. Each virus preparation was stored three months in the dark at 4°C and at 30°C for 24 hours. These conditions did not decrease Ct values of neither KHV nor ISAV.

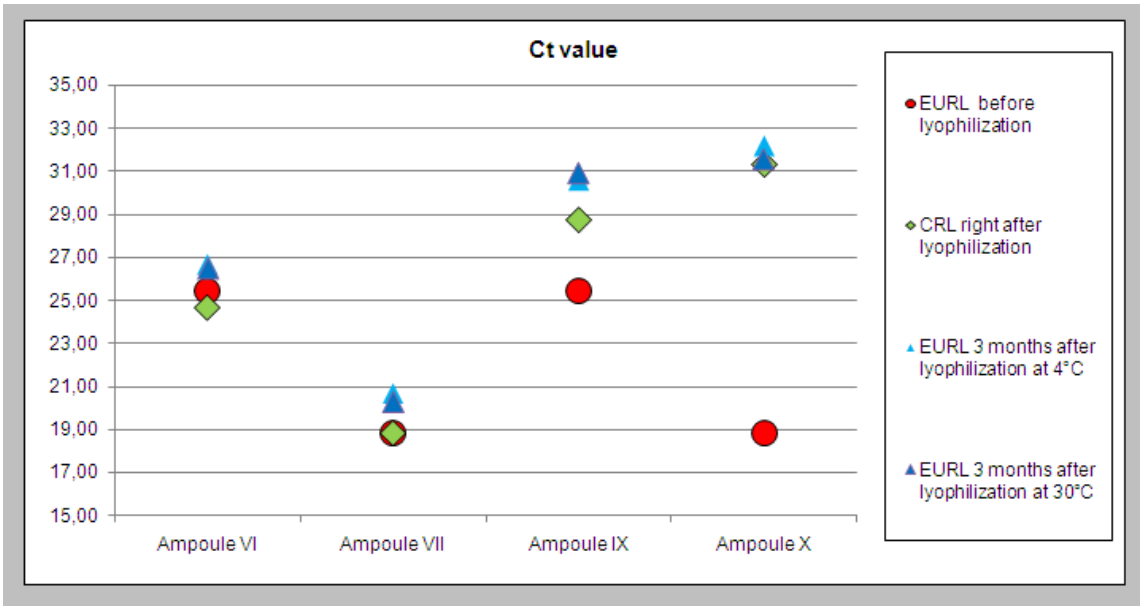
For each ampoule the presence of viruses other than the expected was not detected.

**Table 12.** Ct-value of representative ampoules of no. IV to X tested at the EURL; tested before lyophilisation, immediately after lyophilisation, and after 3 months of storage in the dark at 4°C and at 30°C for 24 hours (1 replicate), respectively.

| Ampoule No. | Content | Ampoule | Ct value before lyophilisation undiluted | Ct value right after lyophilisation | Ct value 3 months after lyophilisation (4ºC, dark conditions) | Ct value 3 months after lyophilisation (30ºC, dark conditions) |
|---|---|---|---|---|---|---|
| **Ampoule VI** | **ISAV High titer (1:4)** | a | 25,33 | 24,68 | 26,71 | 26,50 |
| | | b | 25,65 | 24,85 | | |
| | | c | 25,45 | 24,47 | | |
| | | d | | 24,81 | | |
| | | e | | 24,45 | | |
| **Ampoule VII** | **KHV (CyHV-3) High titer undiluted** | a | 18,96 | 18,66 | 20,71 | 20,28 |
| | | b | 18,62 | 18,82 | | |
| | | c | 18,90 | 18,81 | | |
| | | d | | 18,91 | | |
| | | e | | 18,83 | | |
| **Ampoule VIII** | **Medium** | a | No Ct | No Ct | No Ct | No Ct |
| | | b | | No Ct | | |
| | | c | | No Ct | | |
| | | d | | No Ct | | |
| | | e | | No Ct | | |
| **Ampoule IX** | **ISAV Medium titer (1:64)** | a | 25,33 | 28,64 | 30,62 | 30,95 |
| | | b | 25,65 | 28,79 | | |
| | | c | 25,45 | 28,69 | | |
| | | d | | 28,64 | | |
| | | e | | 28,91 | | |

| Ampoule No. | Content | Ampoule | Ct value before lyophilisation undiluted | Ct value right after lyophilisation | Ct value 3 months after lyophilisation (4ºC, dark conditions) | Ct value 3 months after lyophilisation (30ºC, dark conditions) |
|---|---|---|---|---|---|---|
| **Ampoule X** | **KHV (CyHV-3) Low titer 1:2048** | a | 18,96 | 30,97 | 32,23 | 31,59 |
| | | b | 18,62 | 31,35 | | |
| | | c | 18,90 | 30,64 | | |
| | | d | | 32,13 | | |
| | | e | | 31,48 | | |

**Figure 11.** Ct values before, right after and 3 months after lyophilisation at different cell lines. "EURL before lyophilisation" correspond to the Ct value of the undiluted virus.




## Virus identification

In PT2, Participants were asked to identify any of the fish viruses ISAV and KHV (both listed in Council Directive 2006/88/EC) if present in the ampoules, bearing in mind that the test ampoules also could contain other viruses. Participants were expected to use their normal PCR or real-time PCR methods for detection of KHV and their normal RT-PCR or real-time RT-PCR methods for detection of ISAV. It was not mandatory to grow KHV and ISAV on cell cultures in this test, but the viruses had not been inactivated and should thus be viable in cell cultures. In order to obtain a uniform answer, participants were requested to download a spreadsheet that is available from the EURL web page and insert results in this. A special sheet (Ampoule VI-X) was available for the virus identification results of PT2. The results should be submitted in the spreadsheet by an e-mail. The results from parcipating laboratories are shown in table 13.

All laboratories were encouraged to genotype KHV and ISAV isolates as far as possible. However, this was not a mandatory task.

It was requested that the viruses in the samples were not forwarded to third parts without having contacted the EURL for permission in advance.

**Table 13**. Inter-Laboratory Proficiency Test, PT2, 2010 - Virus identification.

| Laboratory code number | Score | Answer recieved at EURL | Ampoule VI | Ampoule VII | Ampoule VIII | Ampoule IX | Ampoule X |
|---|---|---|---|---|---|---|---|
| | | | ISAV | KHV | No virus | ISAV | KHV |
| 1 | 8 | 29-11-2010 | ISAV | KHV | KHV | ISAV | KHV |
| 2 | 10 | 26-11-2010 | ISAV | KHV | No ISAV no KHV | ISAV | KHV |
| 3 | 10 | 29-11-2010 | ISAV | KHV | NO VIRUS | ISAV | KHV |
| 4 | 10 | 29-11-2010 | ISAV | KHV | No ISAV-, no KHV-, | ISAV | KHV |
| 5 | 10 | 25-11-2010 | ISAV | KHV | no ISAV, no KHV | ISAV | KHV |
| 6[1] | 0 | | | | | | |
| 7 | 6 | 26-11-2010 | ISAV | KHV | KHV | KHV, ISAV | KHV |
| 8 | 8 | 08-11-2010 | ISAV | CyHV3 | No ISAV and no CyHV | No ISAV and no CyHV | CyHV3 |
| 9 | 10 | 29-11-2010 | ISAV | KHV | No ISAV and no KHV | ISAV (weak) | KHV (weak) |
| 10[2] | | | | | | | |
| 11 | 8 | 25-11-2010 | ISAV | KHV | Virus not identified | ISAV | Virus not identified |
| 12 | 10 | 29-11-2010 | ISAV | KHV | No ISAV and no KHV | ISAV | KHV |
| 13 | 10 | 29-11-2010 | ISAV | KHV | No ISAV and no KHV | ISAV | KHV |
| 14 | 10 | 29-11-2010 | ISAV | KHV | No ISAV, no KHV | ISAV | KHV |
| 15 | 10 | 26-11-2010 | ISAV | KHV | No ISAV and no KHV | ISAV | KHV |
| 16 | 10 | 29-11-2010 | ISAV | KHV | No ISAV and no KHV | ISAV | KHV |
| 17 | 10 | 29-11-2010 | ISAV | KHV | No ISAV no and KHV | ISAV | KHV |
| 18[3] | 4 | 29-11-2010 | KHV | KHV | KHV | KHV | KHV |
| 19 | 10 | 26-11-2010 | ISAV | KHV | No ISAV and no KHV | ISAV | KHV |
| 20 | 10 | 29-11-2010 | ISAV | KHV | No ISAV and no KHV | ISAV | KHV |
| 21 | 10 | 26-11-2010 | ISAV | KHV | No ISAV, no KHV | ISAV | KHV |
| 22[3] | 6/6 | 23-11-2010 | No KHV | KHV | No KHV | No KHV | KHV |
| 23 | 10 | 15-11-2010 | ISAV | CyHV-3 (KHV) | No ISAV and no KHV | ISAV | CyHV-3 (KHV) |
| 24 | 8 | 29-11-2010 | ISAV | KHV | Negative | Negative | KHV |
| 25 | 10 | 22-11-2010 | ISAV | KHV | No KHV and no ISA | ISAV | KHV |

| Laboratory code number | Score | Answer recieved at EURL | Ampoule VI | Ampoule VII | Ampoule VIII | Ampoule IX | Ampoule X |
|---|---|---|---|---|---|---|---|
| | | | ISAV | KHV | No virus | ISAV | KHV |
| 26 | 10 | 23-11-2010 | ISAV | KHV | No ISAV and no KHV | ISAV | KHV |
| 27[1] | 0 | | | | | | |
| 28 | 8 | 26-11-2010 | ISAV | KHV | No ISAV and no KHV | ISAV | No ISAV and no KHV |
| 29 | 10 | 29-11-2010 | ISAV | KHV | No KHV and no ISAV | ISAV | KHV |
| 30[4] | 6/6 | 29-11-2010 | ISAV | No ISAV | No ISAV | ISAV | No ISAV |
| 31 | 10 | 29-11-2010 | ISAV | KHV | No ISAV and no KHV | ISAV | KHV |
| 32 | 10 | 26-11-2010 | ISAV | KHV | No ISAV and no KHV | ISAV | KHV |
| 33 | 10 | 22-11-2010 | ISAV | KHV | No ISAV and no KHV | ISAV | KHV |
| 34 | 4 | 29-11-2010 | No ISAV and no KHV | KHV | No ISAV and no KHV | No ISAV and no KHV | No ISAV and no KHV |
| 36 | 6 | 29-11-2010 | ISAV | KHV | No ISAV, no KHV | No ISAV, no KHV | No ISAV, no KHV |
| 37 | 10 | 23-11-2010 | ISAV | KHV | No ISAV, no KHV | ISAV | KHV |
| 38[5] | | | | | | | |
| 39 | 10 | 06-12-2010 | ISAV | KHV | Not ISAV and KHV | ISAV | KHV |

| | Ampoule I | Ampoule II | Ampoule III | Ampoule IV | Ampoule V |
|---|---|---|---|---|---|
| | ISAV | KHV | No virus | ISAV | KHV |
| Correct ID | 31 | 33 | 31 | 27 | 29 |
| No virus | 1 | | | 4 | 4 |
| Wrong ID | 1 | | 3 | 2 | |
| No ID | | | | | |
| Not replied | 3 | 3 | 2 | 3 | 3 |
| Total | 36 | 36 | 36 | 36 | 36 |

[1] Did not submit any results
[2] KHV and ISA identificationis performed by another NRL
[3] Analysed only for the presence of KHV and not ISAV
[4] Analysed only for the presence of ISAV and not KHV
[5] Did not participate in PT2

*Identification of content*

- 23 laboratories correctly identified all viruses in all five ampoules.
- 2 laboratories only examined for KHV and not ISAV
- 1 laboratory only examined for ISAV and not KHV
- 2 laboratories did not submit any results

Ampoule VI – ISAV (high titer)

- 31 laboratories correctly identified ISAV.
- 1 laboratory did not identify any virus.
- 1 laboratory identified KHV.
- 2 laboratories only examined for KHV and not ISAV
- 2 laboratories did not submit any results

Ampoule VII - KHV (high titer)

- 33 laboratories correctly identified KHV.
- 1 laboratory only examined for ISAV and not KHV
- 2 laboratories did not submit any results

Ampoule VIII – medium (no virus)

- 31 laboratories correctly identified the sample negative for virus
- 3 laboratories identified KHV.
- 2 laboratories did not submit any results

Ampoule IX – ISAV (medium titer)

- 27 laboratories correctly identified ISAV.
- 4 laboratories did not identify any virus.
- 1 laboratory identified KHV and ISAV.
- 1 laboratory identified KHV.
- 2 laboratories only examined for KHV and not ISAV
- 2 laboratories did not submit any results

Ampoule X – KHV (low titer)

- 29 laboratories correctly identified KHV.
- 4 laboratories did not identify any virus
- 1 laboratory only examined for ISAV and not KHV
- 2 laboratories did not submit any results

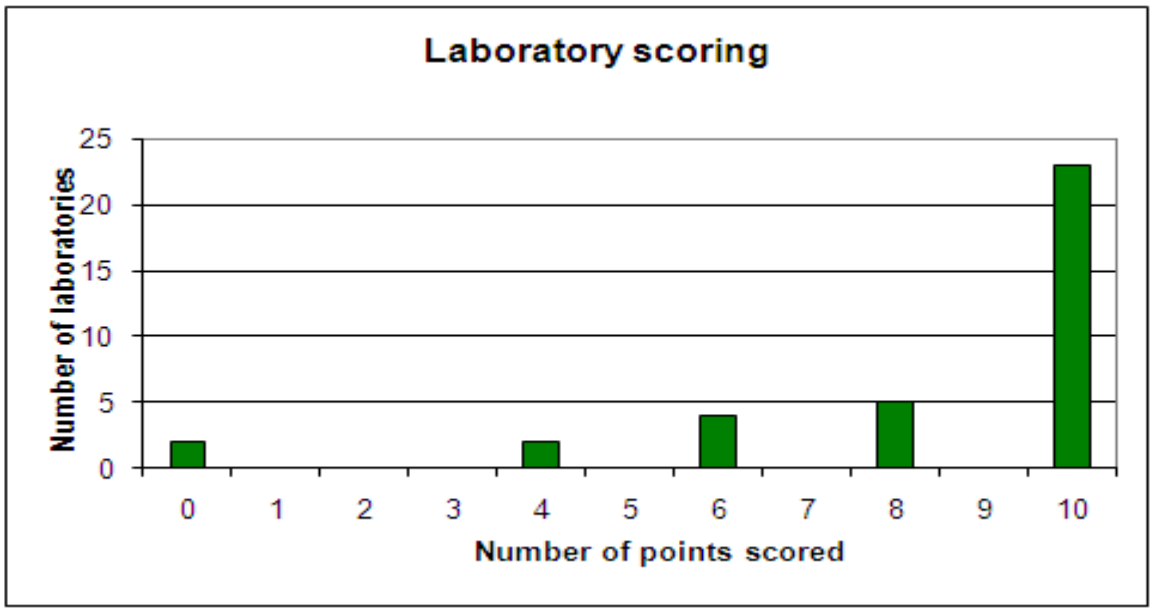
*Scores*

We have assigned a score of 2 for each correct answer (Table 13), giving the possibility for obtaining a maximum score of 10. Incorrectly finding of the viruses in the ampoules gives the scored 0.

23 laboratories out of 36 correctly identified all viruses in all ampoules and obtained maximum score. Two laboratories only examined for KHV and not ISAV and one laboratory only examined for ISAV and not KHV. Of these three laboratories, two laboratories presented correct answers and obtained the score of 6 out of six.
Laboratories scoring 8, 6 or 4 either lacked virus identification or identified additional viruses than those present. Two laboratories did not submit any results and obtained the score of 0.

Genotyping of KHV and ISAV and submission of sequencing results are not a mandatory part of the test and is not included in the score of participants.

**Figure 12.** Obtained score by participants.



*Methods applied*

The following methods were used by the participants:

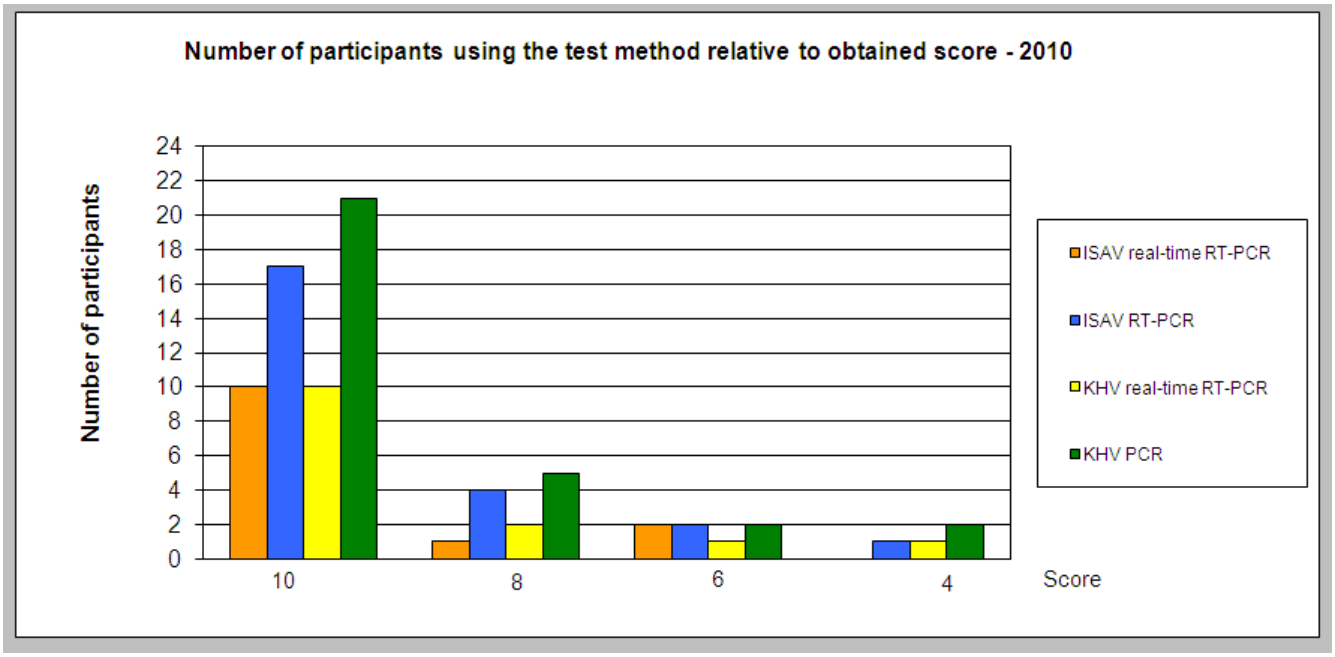
- 12 laboratories used ISAV real-time RT-PCR
- 24 laboratories used ISAV RT-PCR
- 4 laboratories used both ISAV real-time RT-PCR and ISAV RT-PCR
- 14 laboratories used KHV real-time RT-PCR
- 30 laboratories used KHV PCR
- 11 laboratories used both KHV real-time PCR and KHV PCR

| Laboratory code number | Score | Answer recieved at EURL | ISAV real-time RT-PCR | ISAV RT-PCR | KHV real-time RT-PCR | KHV PCR | Sequencing |
|---|---|---|---|---|---|---|---|
| 1 | 8 | 29-11-2010 | | X | | X | ISAV genotype 1(IV)<br>KHV U/I lineage (IIV) |
| 2 | 10 | 26-11-2010 | X | | | X | |
| 3 | 10 | 29-11-2010 | | X | | X | |
| 4 | 10 | 29-11-2010 | | X | | X | ISAV Glesvaer isolate |
| 5 | 10 | 25-11-2010 | X | X | X | X | ISAV HPR2 e.g. glesvaer<br>KHV (no genotype) |
| 7 | 6 | 26-11-2010 | | X | | X | |
| 8 | 8 | 08-11-2010 | | X | X | X | ISAV (AF220606 and DQ785248)<br>CyHV-3 USA/Israel pattern |
| 9 | 10 | 29-11-2010 | | X | X | X | |
| 11 | 8 | 25-11-2010 | X | | X | X | |
| 12 | 10 | 29-11-2010 | X | | X | | ISAV HPR2<br>KHV U/I lineage |
| 13 | 10 | 29-11-2010 | X | X | | X | ISAV<br>CyHV3 |
| 14 | 10 | 29-11-2010 | | X | | X | ISAV European<br>KHV |
| 15 | 10 | 26-11-2010 | X | | X | | |
| 16 | 10 | 29-11-2010 | | X | | X | |
| 17 | 10 | 29-11-2010 | | X | | X | ISAV Genotype 1 (EU-G2)<br>KHV |
| 18 | 4 | 29-11-2010 | | | X | X | |
| 19 | 10 | 26-11-2010 | | X | | X | ISAV<br>CyHV-3 |
| 20 | 10 | 29-11-2010 | | X | | X | ISAV (DQ785276.1)<br>CyHV-3 (HM347113.1) |
| 21 | 10 | 26-11-2010 | | X | X | X | ISAV (DQ785248.1)<br>CyHV-3 (AB375381.1)<br>(genotype/variant: A1, A2) |
| 22 | 6 | 23-11-2010 | | | X | | |
| 23 | 10 | 15-11-2010 | X | | X | X | CyHV-3 |

| Laboratory code number | Score | Answer recieved at EURL | ISAV real-time RT-PCR | ISAV RT-PCR | KHV real-time RT-PCR | KHV PCR | Sequencing |
|---|---|---|---|---|---|---|---|
| 24 | 8 | 29-11-2010 | | X | | X | ISAV (DQ785276.1)<br>CyHV-3 (AP008984.1) |
| 25 | 10 | 22-11-2010 | X | X | X | X | ISAV HPR2<br>CyHV-3 |
| 26 | 10 | 23-11-2010 | X | X | X | X | ISAV HPR2, EU-G2<br>KHV |
| 28 | 8 | 26-11-2010 | | X | | X | ISAV (99% identical to AY971656, AF364881)<br>KHV (100% identical to isolates SF94-xx (EU0539xx)) |
| 29 | 10 | 29-11-2010 | X | | | X | ISAV HPR2, EU-G2<br>KHV (genotype/variant: A1, A2) |
| 30 | 6 | 29-11-2010 | X | | | | |
| 31 | 10 | 29-11-2010 | | X | | X | |
| 32 | 10 | 26-11-2010 | | X | X | X | ISAV European genotype-1 subgroup 2<br>KHV European genotype |
| 33 | 10 | 22-11-2010 | X | | | X | |
| 34 | 4 | 29-11-2010 | | X | | X | |
| 36 | 6 | 29-11-2010 | | X | | X | |
| 37 | 10 | 23-11-2010 | | X | X | X | |
| 39 | 10 | 06-12-2010 | | X | | X | ISAV European<br>KHV |

A graph was constructed to illustrate the association between the methods used by participants for virus identification and the obtained score (Figure 13). The conventional PCR and RT-PCR was the most frequently used method compared to the equivalent real-time assays. For both ISAV and KHV identification, approximate half of the laboratories used real-time assays compared to conventional assays. This approximate ratio seems more or less conserved for laboratories scoring max point as well as for laboratories scoring lower points. Therefore, for participants scoring lower than 10, the deficiency in virus identification cannot directly be assigned to improper use of a single identification method. Rather mistakes might be related to performance of the overall procedure.

**Figure 13.** Methods used by participants for virus identification in PT2




*Genotypning and sequencing*

We have encouraged participants to genotype the identified viruses though it was not a mandatory task.

- 18 laboratories performed sequencing of the ISAV isolate
- 16 laboratories performed genotyping according to various notifications as genotype 1, HPR2 genotype, isolate name, GenBank accession number etc.
- 18 laboratories performed sequencing of the KHV isolate
- 9 laboratories performed genotyping according to various notifications as U/I lineage; genotype/variant A1, A2; GenBank accession number etc.

It is positive that many laboratories performed sequencing of isolates as it is important that laboratories can discriminate between isolates as e.g. pathogenic ISAV strains containing HPR deletion 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.

**Concluding remarks PT2**

The inter-laboratory proficiency test 2010 was conducted without major constrains. Most parcels were delivered by the shipping companies within 3 days after submission. It was, however, unfortunate that one parcel made up to 13 days before delivered to the laboratory because of to delay in an Airline "backlog" and that another parcel made op to 22 days before delivering to the laboratory, primarily due to border controls.

Considering that this was the first time that the EURL provided a proficiency test on ISAV and KHV identification, we think that most participants obtained satisfying results. All 33 laboratories performing KHV identification did correctly identify KHV in the ampoule (VI) containing high titre KHV. All 31 laboratories performing ISAV identification, except two, did correctly identify ISAV in the ampoule (VII) containing high titered ISAV.

Lowering the titre of the virus caused three additional laboratories to miss identification of KHV in the low titered ampoule (X) and three additional laboratories to miss identification of ISAV in the low titered ampoule (IX). A critical point in PCR based diagnostic tool is avoiding of false negative results, e.g. because of low sensitivity of the diagnostic tool. To decrease the risk of having false negative results, it is always recommended that laboratories use the most sensitive tool available, validate the sensitivity of their diagnostic tools and use proper controls.

One ampoule (VII) containing no virus was included in the test. 34 out of 31 laboratories correctly identified that the ampoule was negative for virus. Three laboratories identified KHV in this ampoule, probably due to cross contamination problems. False positive results is a common critical problem in PCR based diagnostics as PCR or RT-PCR can detect very few copies of DNA or RNA, respectively. Therefore, it is extremely important to minimise the risk of cross contaminations. This can be done by optimising the workflow in the laboratory as e.g. described in the "Report of the workshop "KHV PCR diagnosis and surveillance" 12-13 November 2009, Central Veterinary Institute, Lelystad, The Netherlands". Other ways to minimize the risk of obtaining false positive results is to consider not using nested PCR tools and by using positive controls that can be discriminated from true pathogenic signals.

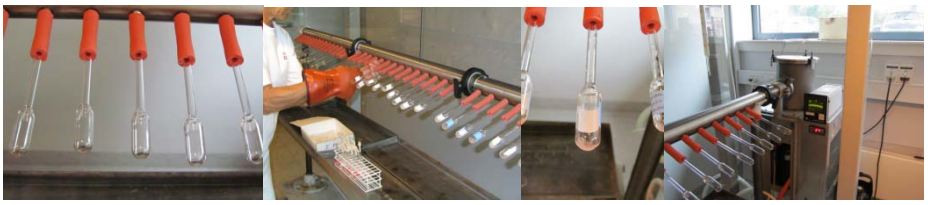
Many laboratories performed sequencing of ISAV and KHV isolates. However, it was not described which notification should be used for genotyping of viruses. This might reflect the various way of reporting isolate genotypes. In future tests we will clarify which notification the genotyping should follow.

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

The results presented in this report will be further presented and discussed at the 15th Annual Meeting of National Reference Laboratories for Fish Diseases to be held 26-27 May 2011 in Aarhus, Denmark.

Nicole Nicolajsen, Niels Jørgen Olesen and Søren Kahns

European Union Reference laboratory for Fish diseases
National Veterinary Institute, Technical University of Denmark, 9 March 2011



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# Laboratory visit at the National Reference Laboratory for Fish Diseases Croatian Veterinary Institute

## Zagreb, Croatia
## 26th – 28th October 2010

Annex 1: Program for the meeting.
Annex 2: Participants at the meeting.
Annex 3: Organisational and Functional Structure.
Annex 4: Proficiency Test
Annex 5: Aquaculture in Croatia
Annex 6: Draft for IFAT 96-well plate

***Introduction***

The National Reference Laboratory for Fish Diseases (NRL) in Croatia is located at the Croatian Veterinary Institute (CVI). The Institute was visited from the 26th to the 28st of October 2010 by Nicole Nicolajsen and Søren Kahns from the European Union Reference Laboratory for Fish Diseases (EURL). The program for the visit is shown in Annex 1 and a list of persons met by the EURL representative is listed in Annex 2. This report describes findings, comments and recommendations made by the delegation from the EURL. This report is sent to the CVI and the EU Commission.

***Organisation***

The CVI is a public governmental institution that was established in 1933. The CVI consists of the Croatian Veterinary Institute in Zagreb, a branch centre for poultry farming in Zagreb and four regional branch centers located in Križevci, Rijeka, Split and Vinkovci.

The CVI obtains approximately 41 % of its funding (2009) form the Ministry of Agriculture, Fisheries and Rural Development and 9 % from the Ministry of Science, Education and Sport. The last 49% is achieved through own activities.

The CVI is managed by Director, Professor, Dr. Željko Cvetnić. The quality policy of CVI is managed by the Quality Manager Dr. Boris Habrun. The CVI in Zagreb is divided into 5 departments: Department for Veterinary Public Health, Department for Bacteriology and Parasitology, Department of Virology, Department for Pathological Morphology and General Department. An organisation plan is shown in Annex 3.

The five departments contain a total of 17 laboratories as shown in Annex 3. The Laboratory for Fish Pathology that is a part of the Department of Pathological Morphology covers the diagnostics of viral fish and mollusc diseases. The Laboratory for Fish Pathology is coordinating Croatian NRL activities for both fish and molluscs diseases.

The CVI employee a total of 244 persons (2009) of which 112 have an university degree.

***Buildings, Furnishing and Access***

The CVI in Zagreb is placed in two main buildings within Zagreb. In 2007 a project of reconstruction was initiated. Since then all buildings, laboratories, administration premises, staircases e.g. have been completely reconstructed and equipped. The reconstruction is finished and the outcome is a very nice institute with well equipped laboratories. The prize for the reconstruction of the CVI have approximately been 4 million Euro, money that have been financed by the institutes own money.

All access to the institute is controlled at the entrance of the site. There is free access between the buildings but entrances to the different laboratories are restricted through locks. All rooms have air-conditioning. Furthermore, fume hoods are located in certain rooms for working with hazardous chemicals.

***Opening of the meeting***

Due to logistic matter the visit was opened already the 26th October in the conference hall of the institute where Quality Manager Dr. Boris Habrun introduced the quality system of the CVI.

The official opening took place 27th October where Director, Professor, Dr. Željko Cvetnić gave a detailed description of the CVI, its history, organization and mission.

The meeting was followed by a tour of the institute where the aim of the tour was to illustrate how processing of fish samples, from it is received and until a diagnostic answer is given, occur. The tour was guided by Dr. Dražen Oraić and Dr. Snježana Zrnčić from the Laboratory for Fish Pathology.

***Sample acceptance and reporting unit***

The visit tour started in the sample acceptance and reporting unit where all samples delivered to the institute is entered. Samples are received from veterinarians of both official and private establishments and/or private persons. Veterinarians receive tubes with transport media for collecting samples. All received material is registered and given a unique number. Providers of samples have to provide information on e.g. fish species, clinical signs, fish farm, water temperature. The sample number and all collected information is entered into an electronically sample registration system "VetLab" that is developed by the CVI. After the samples have been registered, they are sent to the Laboratory for Fish Pathology. If immediate transfer is not possible, the samples will be stored in a refrigerator. Each department has their own refrigerator. The samples can be followed during the analyses in VetLab where there are different levels of access to view and enter sample results. When the results of the diagnostic analyses have been obtained a final report is made in VetLab. This answers has to be signed by the head of the laboratory, the head of the department and the director of CVI before they will be send to the customer.

***The Laboratory for Fish Pathology***

The Laboratory for Fish Pathology is a part of the Department for Pathological Morphology and responsible for diagnostics of diseases of aquatic animals from farms and open water. The Laboratory for Fish Pathology coordinates the NRL for fish diseases and molluscs diseases. The NRL for fish diseases is covered by all the departments within the Institute, e.g. expertise from the Laboratory for Diagnostics of Rabies and General Virology is used for performing of molecular biological based fish diagnostics. The staff of the all these Diagnostic Laboratories is also the staff of NRL.

*Staff of the Laboratory for Fish Pathology*

The Laboratory for Fish Pathology consists of a group two persons: DVM, Dr. Dražen Oraić and DVM, Dr. Snježana Zrnčić who carry out all the viral diagnostics. Dražen Oraić is the Head of the Laboratory for Fish Pathology and the Department for Pathological Morphology and he is overall responsible for the diagnosis of all viral diseases in fish and molluscs. MSc Dr. Ivana Lojkić from the Laboratory for Diagnostics of Rabies and General Virology performs the PCR based fish diagnostic.

*Laboratory for Fish Pathology*

The laboratory was newly reconstructed and equipped. The laboratory was split into three separate working rooms: A sample preparation room, a dirty room and a clean room. Besides the three working rooms, two offices with computer working space were present. All rooms were separated by glass walls. The walls separating the sample room and the dirty room, and the dirty room and the clean room respectively, contained hatches used for sending e.g. samples from the sample preparation room to the dirty room. In this way, the samples can be transferred from one part of the laboratory to another without being introduced to the remaining laboratory and hereby minimizing the risk of contamination.

Equipment was new and adequate. All equipment has a reference number and a logbook. Username, date and sample are registered in the logbook as well as any problems encountered. Repairs, cleaning, calibration and other maintenance such as filter change are likewise registered. Calibration of the pipettes is performed by Dr. Dražen Oraić and Dr. Snježana Zrnčić whereas calibration of other equipment is done by sub-contractors.
Instructions for use of equipment are written down in procedures, one for each piece of equipment. Staffs are required to read and be trained according to these procedures before using the equipment.

| | |
|---|---|
| ***Clean room*** | ***Office*** |
| ***Dirty room*** | ***Office entrance to the area*** |
| ***Sample preparation room*** | |

*Sample preparation room*

After the samples are received and recorded, they are sent directly to the sample preparation room if possible. The registration number of the sample identifies the sample in all subsequent tests. The samples are processed according to the description n in Commission Decision 2001/183/EC. We recommend that mortar and pestle are being kept in the refrigerator until they are being used. Disinfection of the workspace was done by Virkon-S, Asepsol and ethanol.

Equipment in the sample preparation room

- Refrigerator
- Freezer -20°C
- Microscope
- Weight

*Dirty room*

Inoculation of samples on cell cultures is performed in a distinct “dirty room”. Samples and viruses are always inoculated on 96 well plates (in volumes relative to what is described in the Commission Decision) or in flasks. In this room identification of viruses by antibody based methods are also performed and equipment for making the identification is present. Identification of fish viruses is primarily done by ELISA and IFAT using antibodies using kits from Bio-X and Test line. The laboratory do also perform neutralization test but this technique is not often used.
The EURL provided hand outs with protocols used for virus detection by IFAT at the EURL
Disinfection of workspaces is done by Virkon-S and UV light.

Equipment in the dirty room:

- Freezer -80°C
- Centrifuge with cooling
- LAF bench
- Incubator at 15°C
- ELISA reader
- Microscope
- Small oven
- Shaker
- Pipettes

*Clean room*
All cell culturing/preparation work are performed in a “clean room”

*Cell Culture Facilities*
BF-2, EPC, FHM, CHSE cells are continuously cultured in the lab. CCB and SSN-1 cells are available as stocks stored in liquid nitrogen. The BF-2 are passaged every 2 weeks, out growing temperature 21°C; the EPC cells are passage every 4 weeks, out growing temperature 24°C; FHM cells are passage every 4 weeks, out growing temperature 24°C and CHSE are passage every 4 weeks, out growing temperature 21°C. After outgrowth, all cells are kept in incubators at 15°C. To limit occasions where more samples are on the same plate, it is recommended to seed BF-2/EPC cells (half of each) are on the same plate. Cell should still be stored at 24°C for out grow only for 24 hours after passage and then transferred to 15°C.

There is a back-up of cells stored in nitrogen, frozen in Recovery – Cell culture Freezing Medium containing DMSO (10%). Cells are tested for mycoplasma by real-time PCR. There are performing a cell sensitivity test for VHSV, which is followed up with a report. It is recommended that it is being done for the following virus: EHNV, VHSV, IHNV, SVCV and IPNV.

*Cell lines:*

- Cell lines grown in the laboratory: Primarily BF-2 and EPC but the laboratory also has FHM, CHSE, CCB and SSN-1 cells available. The cell has been obtained from the CRL and USA.

*Equipment*

- LAF bench
- Incubator at 15°C, 24°C and 24°C
- Microscope
- Refrigerator with an N2 tank
- Freezer -20$^{o}$ for storage of all required media for cell cultivation

It might be considered to acquire multichannel Electronic Pipettes as this would make pipetting work considerable easier

Disinfection of the workspace is done by 75% Ethanol, Virkon-S and UV.

For all three rooms, sterilization tests using blood agar and Sabouraud agar plates was used to check sterility of refrigerators and LAF benches.

*PCR based diagnostics*
The laboratory for Fish Pathology does not contain equipment for performing PCR based fish diagnostic analyses. Instead diagnostic PCRs and RT-PCRs are performed by Dr. Ivana Lojkić at the Laboratory for Diagnostics of Rabies and General Virology. PCR/RT-PCRs are implemented for detection of VHSV, IHNV, EHNV, KHV, EHNV, IPNV and SVCV, using either OIE recommended or own produced PCR protocols. For comparison, the EURL provided hand outs with protocols used for virus detection at the EURL.
For the listed non-exotic diseases VHS, IHN, the RT-PCR was used to complement cell culture and antibody based analyses. For KHV however, PCR is the main diagnostic tool and we recommend that the laboratory implement the most sensitive PCR based tool available, which for the moment is the real-time PCR described by Gilad et al. 2004. The TK conventional PCR would then be used as a backup diagnostic tool identifying another target sequence the real-time PCR. This would be important since diagnostic of KHV is considered a risk for Carp production in Croatia. For ISA, the main diagnostic tool is the RT-PCR. However, as ISA is not considered a relevant threat to Croatian aquaculture under the present circumstances, we consider implementation of a single RT-PCR to be sufficient. We do however recommend the laboratory to use an RT-PCR targeting segment 8, e.g. the one described in Mjaaland et al. 1997.

Within this laboratory, two rooms are dedicated for performing molecular biological analyses. One separate room acts as a clean room where primers and buffers are stored and master mixes are prepared. In a larger room purification of DNA and RNA is performed along with post PCR analyses. It is in general advisable to perform post PCR analyses in an isolated room as a major source of cross contamination occur from this step. However, this is not always possible and in the Laboratory for Diagnostics of Rabies and General Virology precautions have been established by restricting purifications to occur in a single LAF bench.

Laboratory equipment included is e.g.: PCR machines, a real-time PCR machine, gelelectrophoresis equipment, a nucleic acid purification robot

*Accreditation*

The first day of the visit the Quality Manager DVM, Dr. Boris Habrun of the CVI opened the meeting by presenting the accreditation status of the veterinarian institute. The process of accreditation was started in 2006 by Boris Habrun in collaboration with the Italian Veterinary Institute. The CVI was accredited according to ISO 17025 by the Croatian Accreditation Agency, HAA the 15th of May 2008. The CVI have build up an internal internet portal that is used for distribution of documents to laboratories within the institute. All academic educated employees have been trained to perform internal audits.
The NRL for Fish Diseases have been accredited for viral growth on monolayered fish cell cultures and for identification of VHSV by ELISA. Identification of IHNV by ELISA is expected to be achieved at the coming HAA audit. KHV identification techniques is expected to be accredited in the future whereas there are no plans for accreditation of ISA examination as it is not relevant for Croatia.

*Proficiency Test*

The proficiency tests (PT) allow a laboratory to assess their diagnostic capacity of certain procedures. The Croatian NRL for fish diseases has participated in the PT for identification of notifiable fish diseases organized by EURL, Aarhus, Denmark, since 2001. The NRL used the following tools for identification of viral content in the test: Titration of virus; Isolation of viruses on cell culture; Identification of virus by ELISA, IFAT, RT-PCR, PCR and sequencing. The score obtained by the NRL was 60% correct in 2010 and 80% in 2008 and 90% 2009. For the scores obtained by the NRL in the PT provided by the EURL, see Annex 4. After the result of the PT 2010, the NRL contacted the EURL for advice on implementation of PCR based diagnostic and two staff members will go for the training course offered by the EURL in the beginning of 2011. A general procedure in the laboratory is that when a PT result is less than 100% correct, the NRL will redo the test using all diagnostic procedures to verify that expected results can be obtained.

The Croatian NRL do not organizes PTs for diagnosis of fish diseases for the regional laboratories as there are no regional laboratories for fish diseases in Croatia. However, the NRL have been organizing two workshops for veterinarians working in the field on how fish samples should be prepared.

***Aquaculture in Croatia***

The main fish species cultured in Croatia are carp, rainbow trout, seabream, seabass, and Tuna fish. Croatia have 28 fish farms producing more than 100 tonnes per year, 41 fish farms producing 5 - 100 tonnes per year and 8 fish farms producing less or around 5 tonnes per year (2009). The laboratory received approximately 226 samples in 2009 for diagnostic and surveillance purposes. The samples for virological examinations are mainly submitted from rainbow trout and the samples are submitted in spring and autumn when water temperatures are as described in the council directive. Rainbow trout farms are mainly located in the western part of the country and by tradition, they are located at the spring of the stream and contains hatchery, cultivation of fry and market sized fish. Carp farms are mainly located in the north and eastern part of Croatia. Please see Annex 5 for more details.

*Categorisation of surveillance programs of Croatian fish farms and according to Council Directive 2006/88/EC*

The status of categorisation of Croatian fish farms was presented by Dr. Ivica Sučec from the Ministry of Agriculture, Fisheries and Rural Development, Veterinary Directorate. The Council Directive 2006/88/EC have not been completely implemented in Croatia but implementation is expected to occur very soon. Official surveillance program for VHS, IHN and KHV has been established on fish farms, and surveillance programme for mollusc farms, regarding Infection with *Bonamia Ostreae* and *Marteilia refringens*. Croatian fish farms are surveilled for VHS and IHN in line with what is described for category II farms under a 4-year surveillance program explained by the council Decision 2001/183/EC. All fish farms have been registered, and will be authorized in near future, in line with what is required for authorization according to 2006/88/EC. The data on the fish farms is to be made public available in the very near future when last checks have been made. It was agreed that any questions from Dr. Ivica Sučec could be emailed to the EURL (Søren Kahns) and if the EURL is not able to answer themselves, they would forward the questions to experts in the field of how official EU categorisation of fish farms is obtained according to 2006/88/EC.

***Training needs and future plans***

Diagnostic methods for the exotic methods EUS will have to be implemented. This could be done by implementing one of the PCRs recommended against *A. Invadans*.

The NRL for fish diseases have decided that two staff members will participate in the EURL course on fish diagnostics that will take place in EURL laboratory in January/February 2011. This participation will provide training for the participants in using several diagnostic methods in the fields of antibody based and PCR based fish diagnostics

In view of the available equipment and the educated staff, the laboratory has definitively the potential for performing more research activities. This could include own projects involving own PhD students.

***Summary of recommendations***

- Implementing a second PCR based tool for identification of KHV. If possible the Gilad real-time PCR should be used as first tool and the TK as back up tool.
- Mortar and pestle should be kept at 4ºC before use.
- To seed BF-2/EPC cells (half of each) on the same plate.

***Conclusion***

The visit showed that the Croatian NRL for fish disease has an adequately equipped laboratory. The staff is well educated and very capable and works according to accredited methods and according to EU requirements. No major problems were observed and only minor suggestions were made, as described above. Therefore, the overall conclusion is that the NRL of Croatia is a well functioning laboratory capable of performing its duties as a NRL.

## Annex 1
## Program for the meeting on
## diagnostic procedures of fish diseases and implementation progress of Council Directive 2006/88/EC

**October the 26th**

16:00 – 17:30 Introduction of the quality system of the Croatian National Reference Laboratory for Fish Diseases. Presentation was made by Quality Manager.

**October the 27th**

09:00 – 10:30 Introduction of the Croatian National Reference Laboratory for Fish Diseases. Presentation of the CRL and discussion on the topics of the visit. Participants: All Staff including Head of Institute

10:30 – 13:00 Tour in the lab, looking at all the facilities
The main goal is to learn about how are the Diagnostic procedures according to Commission Decision 2001/183 conducted in the laboratory.

Following issues can be discussed:

- Aquaculture in Croatia and the type of samples received at the laboratory
- Buildings and access
- Staff
- Equipment
- Accreditation
- Registration of Samples
- Sample processing
- Cell Cultivation and cell cultures
- Virus identification by ELISA and IFAT etc
- Molecular techniques (PCR, RT-PCR, Q-PCR)
- Reporting diagnostic tests
- Past (2008 and 2009) Proficiency Test Results

Special attention can be given to molecular biological diagnostic of fish diseases, if you wish.

13:00 – 13:45 Lunch
13:45 – 15:30 In the afternoon we might continue the tour in lab.
15:30 – 17:00 Follow-up (only Søren Kahns and Nicole Nicolajsen). We would be pleased to have the possibility to work together in an office.

**October the 28rd**

08:00 – 11:00 Follow up for from the day before.
11:00 – 12:00 Plans and progress in the implementation of Council Directive 2006/88/EC in Croatia
12:00 – 12:45 Evaluation of the visit. Recommendations and report of the visit
13:30 Departure to airport

## Annex 2

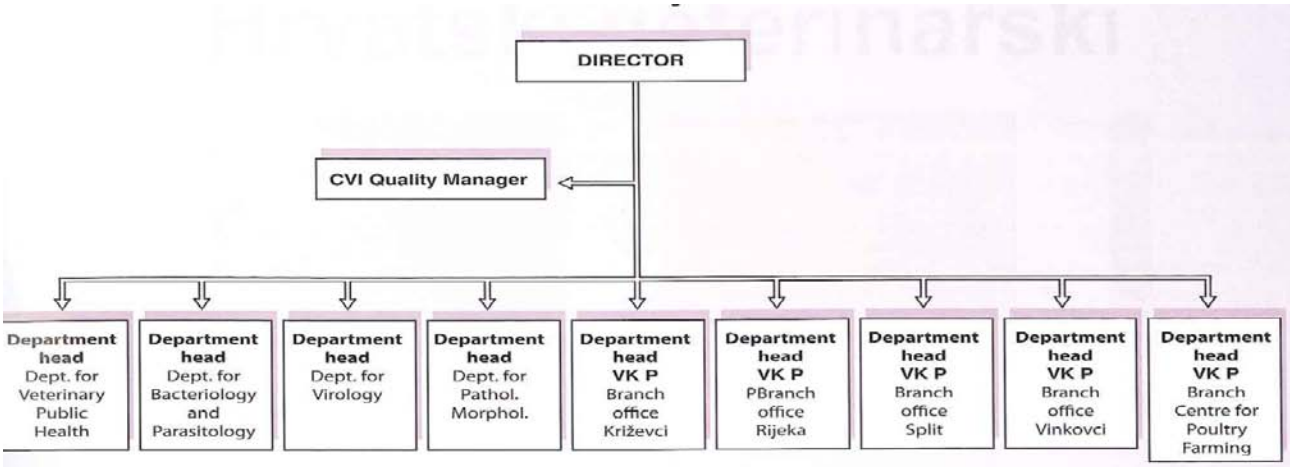
## Persons from CVI participating at the meetings

| Name | Institute | Function |
|---|---|---|
| Professor, Dr. Željko Cvetnić | Croatian Veterinary institute | Director of the Croatian Veterinary Institute Zagreb |
| Dr. Boris Habrun | Croatian Veterinary institute | Head - Department for Bactorology and parasitology and Laboratory for General Bacteriology and mycology |
| Dr. Dražen Oraić | Croatian Veterinary institute | Head – Department for Pathological Morphology<br>and Head of laboratory for Fish Pathology |
| Dr. Snježana Zrnčić | Croatian Veterinary institute | Researcher in laboratory for Fish Pathology |
| Dr. Ivana Lojkić | Croatian Veterinary institute | Researcher in the Laboratory for Diagnostics of Rabies and General Virology |
| Dr. Søren Kahns | EU Reference Laboratory for Fish Diseases, Section for Fish Diseases, National Veterinary Institute, Technical University of Denmark. | Coordinator of the Community Reference Laboratory for Fish Disease |
| Nicole Nicolajsen | EU Reference Laboratory for Fish Diseases, Section for Fish Diseases, National Veterinary Institute, Technical University of Denmark. | |

## Annex 3

## Organisational and Functional Structure

# DIAGRAM of INSTITUTE ORGANISATION




*Integral scheme of the Organisation of the Croatian Veterinary Institute*

Croatian Veterinary Institute employees by qualifications as at 31 December 2009

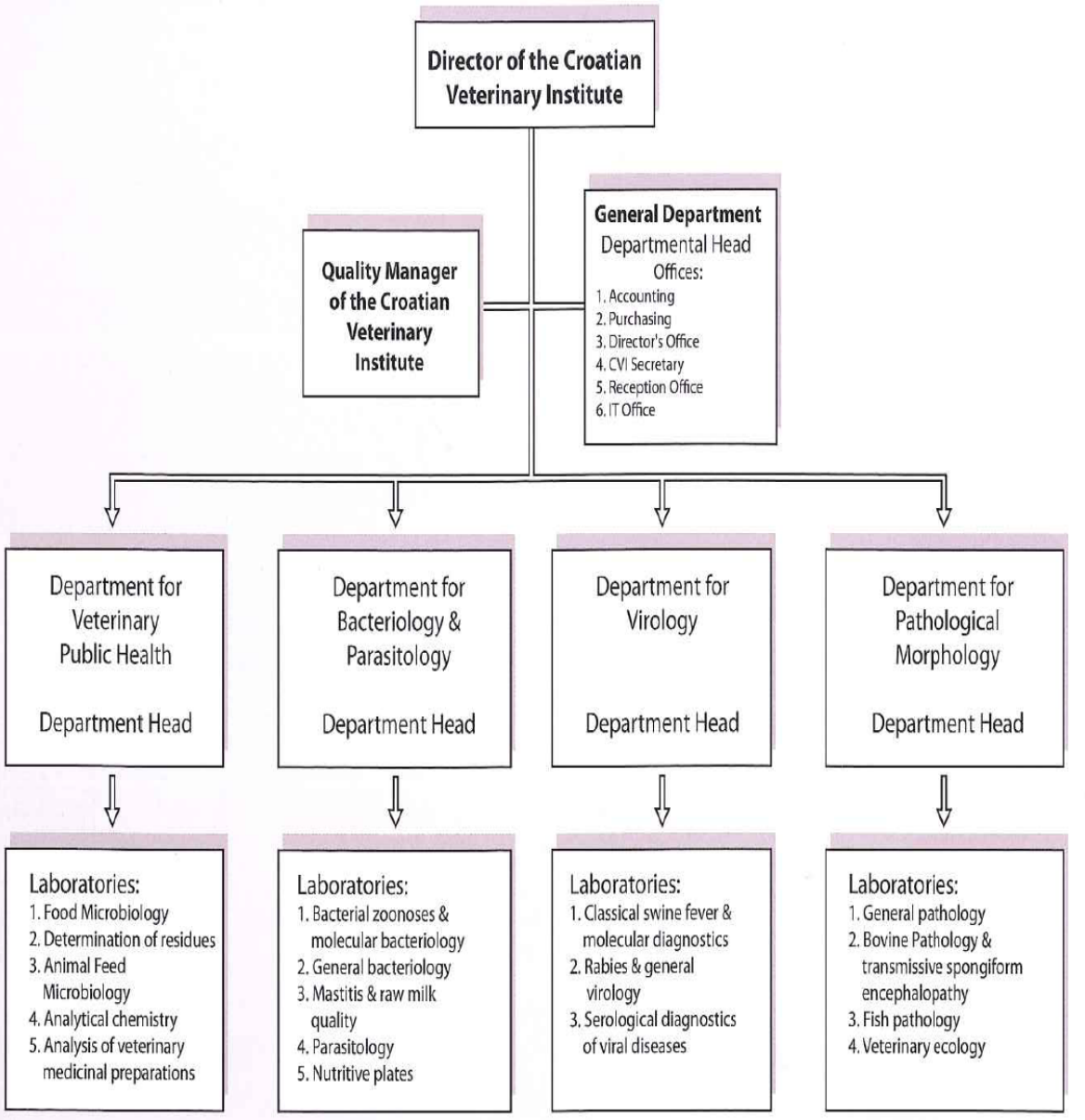
| | | Križevci | Rijeka | Split | Vinkovci | Centar | Zagreb | **Ukupno** |
|---|---|---|---|---|---|---|---|---|
| **1.** | **PROFESSIONAL DEPARTMENTS** | | | | | | | |
| 1.1. | University degree | 10 | 9 | 11 | 13 | 10 | 53 | **106** |
| 1.2. | Associate degree | - | 1 | - | - | - | - | **1** |
| 1.3. | Secondary school | 10 | 6 | 7 | 10 | 8 | 41 | **82** |
| 1.4. | Primary school | 3 | - | - | 3 | - | 2 | **8** |
| **2.** | **GENERAL DEPARTMENTS** | | | | | | | |
| 2.1. | University degree | - | - | - | - | 1 | 5 | **6** |
| 2.2. | Secondary school | 3 | 2 | 3 | 2 | 2 | 7 | **19** |
| 2.3. | Primary school | 1 | 2 | 3 | 4 | 3 | 9 | **22** |
| **3.** | **TOTAL** | **27** | **20** | **24** | **32** | **24** | **117** | **244** |

Croatian Veterinary Institute employees by academic title as at 31 December 2009

| academic title | Križevci | Rijeka | Split | Vinkovci | Centar | Zagreb | **Ukupno** |
|---|---|---|---|---|---|---|---|
| PhD | 1 | - | 1 | 3 | 7 | 25 | **37** |
| MSc | 3 | 2 | 1 | 2 | 1 | 4 | **13** |

# Diagram of Institute Organisation in Zagreb

Croatian Veterinary Institute is organised in 5 departments, one general and 4 professional departments. The professional departments are divided into laboratories or offices.




*Scheme of organisation of the Croatian Veterinary Institute*

## Annex 4

# Proficiency Test

| Croatia | 1996 | 1997 | 1998 | 1999 | 2000 | 2001 | 2002 | 2003 | 2004 | 2005 | 2006 | 2007 | 2008 | 2009 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Participated | | | | | | 1 | 1 | 1 | 1 | 1 | | 1 | 1 | 1 |
| Score % | | | | | | | | 85 | 90 | 100 | | 90 | 80 | 60 |

| **2008**<br><br>**Score 8/10** | **Ampoule I:** | **Ampoule II:** | **Ampoule III:** | **Ampoule IV:** | **Ampoule V:** |
|---|---|---|---|---|---|
| | VHS virus<br>Rindsholm<br>5151 | VHS virus<br>1p8 | IHN virus 217/A<br>(DTU Vet protocol<br>no. 4008) | VHS virus<br>Rindsholm 5151 +<br>IPN virus Type Sp | IPN virus<br>Type Sp |
| Cell line | BF-2/EPC | | | | |
| ELISA, IFAT<br>Neutralisation and<br>PCR | VHSV | VHSV | IHNV | IPNV | IPNV |

| **2007**<br><br>**Score 9/10** | **Ampoule I:** | **Ampoule II:** | **Ampoule III:** | **Ampoule IV:** | **Ampoule V:** |
|---|---|---|---|---|---|
| | VHS virus<br>DK-F1<br>Genotype I<br>(Undiluted ) | VHS virus<br>DK-F1<br>Genotype I<br>(Diluted $10^{-5}$) | SVC virus<br>56/70<br>Genotype Id | IHNV 32/87<br>First French<br>isolate<br>Genotype M | VHSV<br>4p101<br>Genotype III |
| Cell line | BF-2/EPC | | | | |
| ELISA, IFAT<br>Neutralisation | VHSV | Virus not found | SVCV | IHNV | VHSV |

# Inter-laboratory Proficiency Test 2009

**Name of the National Reference Laboratory:**
**Croatian Veterinary Institute Dept. of Pathology, Lab for fish diseases**

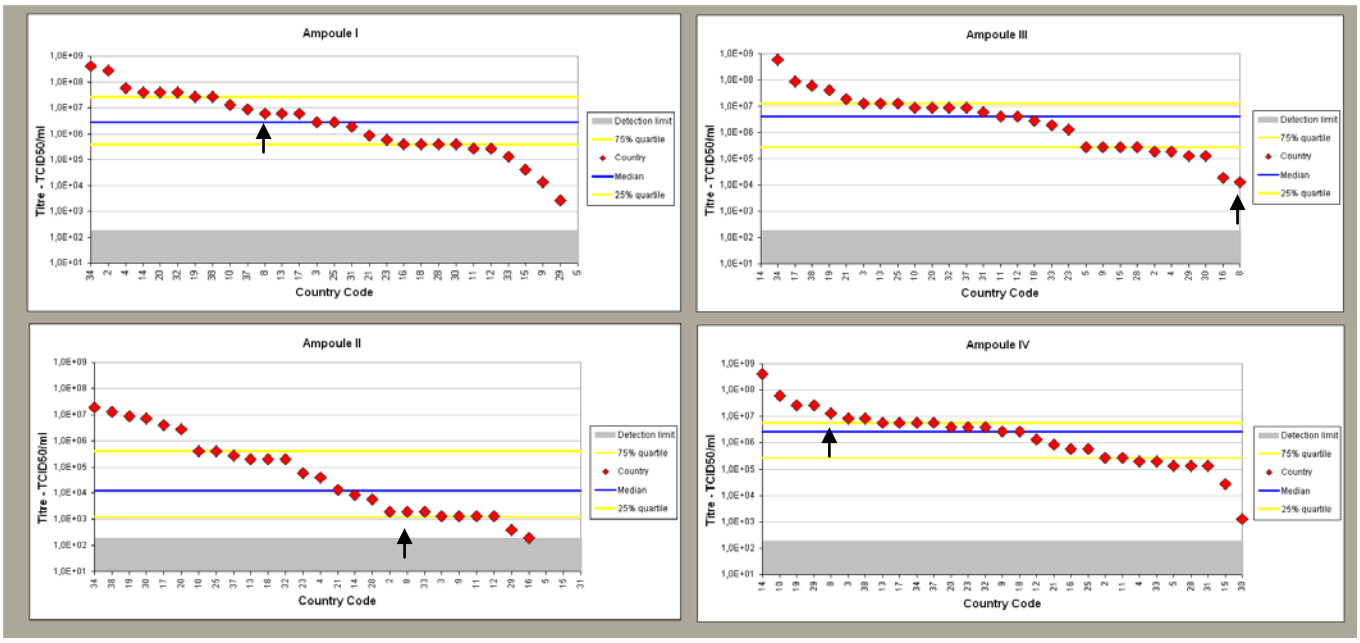
**Country: Croatia**
**Contact name: Drazen Oraic**
**Code: 8**
**Score: 6 (out of 10)**

## LABORATORY RESULTS:

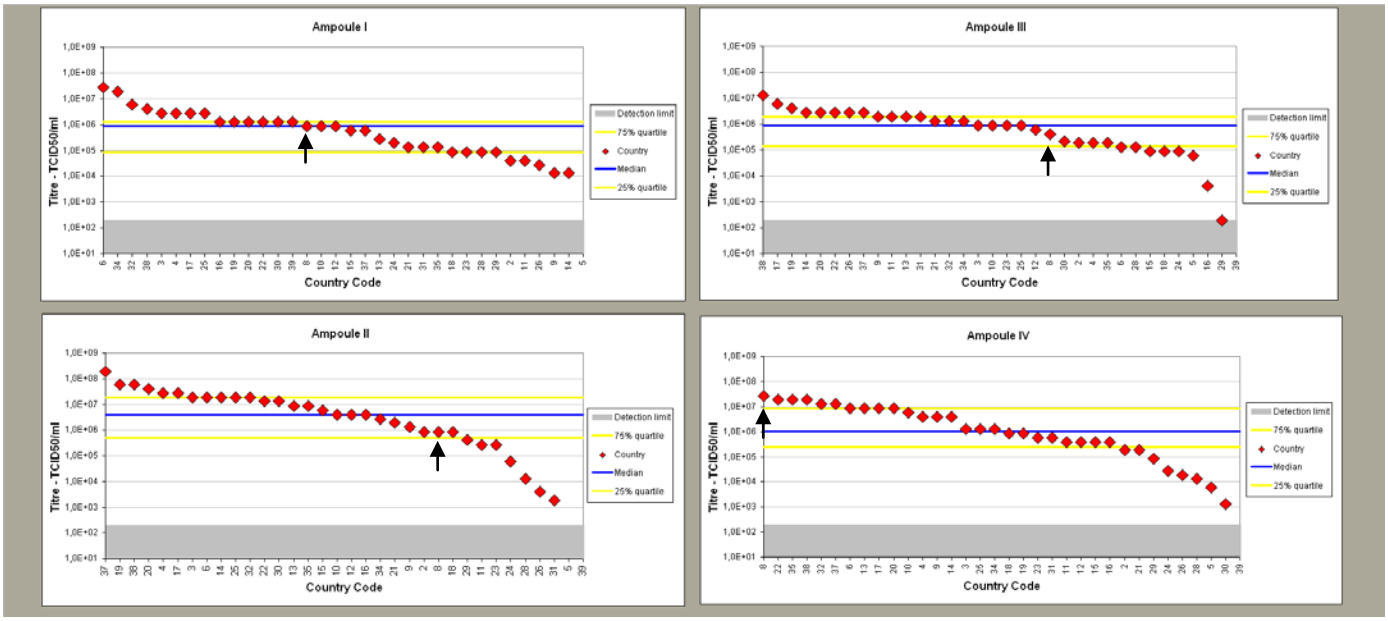
| | ELISA | IFAT | Neutrali-sation | PCR | Other | Final Result |
|---|---|---|---|---|---|---|
| **Ampoule I** Reference strain of EHNV Isolate 86/8774 from rainbow trout | - | - | Not performed | EHNV+ VHSV | Immuno-peroxidase assay VHSV | **EHNV VHSV** |
| **Ampoule II** IHNV genotype L Isolate COL-80 from chinook salmon | - | IHNV | Not performed | IHNV | Immuno-peroxidase assay IHNV | **IHNV** |
| **Ampoule III** VHSV genotype Ie Turkish isolate TR-WS13G | VHSV | VHSV | Not performed | VHSV | Immuno-peroxidase assay VHSV | **VHSV** |
| **Ampoule IV** VHSV genotype IVa Received as RBV | VHSV | VHSV | Not performed | VHSV | Immuno-peroxidase assay VHSV | **VHSV** |
| **Ampoule V** Medium **No virus** | - | VHSV | Not performed | VHSV | Immuno-peroxidase assay VHSV | **VHSV** |

*Comments: The sequences you submit as VHSV in ampoule I and V is not originating from VHSV. We recommend that you "blast" your sequences as that would indicate if you have false positive results. If you have any questions please contact us.*

## Titre obtained in BF-2 cells




## Titre obtained in EPC cells



## Annex 5

# Aquaculture in Croatia

| Land Area | Ocean Area | Environment | Species | Scientific name | 2004 | 2005 | 2006 | 2007 | 2008 |
|---|---|---|---|---|---|---|---|---|---|
| Croatia | Europe - Inland waters | Freshwater | Carps, barbels and other cyprinids | Carps, barbels and other cyprinids | 2 156 | 2 836 | 3 087 | 2 278 | 2 279 |
| | | | Miscellaneous freshwater fishes | Miscellaneous freshwater fishes | 166 | 170 | 115 | 86 | 121 |
| | | | Salmons, trouts, smelts | Salmons, trouts, smelts | 1 075 | 1 301 | 1 885 | 2 031 | 2 058 |
| | | Sub-total Freshwater | | | 3 397 | 4 307 | 5 087 | 4 395 | 4 458 |
| | Sub-total Europe - Inland waters | | | | 3 397 | 4 307 | 5 087 | 4 395 | 4 458 |
| Total Croatia | | | | | 3 397 | 4 307 | 5 087 | 4 395 | 4 458 |

**Data taken from FIGIS**

| **Number of fish farms within country/region, according to size of production (tonnes fish/year)** | | | | |
|---|---|---|---|---|
| | 2009 | 2008 | 2007 | 2006 |
| < 5 tonnes | 8 | 8 | 8 | 8 |
| 5 - 100 tonnes | 41 | 29 | 29 | 29 |
| > 100 tonnes | 28 | 22 | 22 | 22 |

| **Number of fish farms within country/region, according to fish species** | | | | |
|---|---|---|---|---|
| | 2009 | 2008 | 2007 | 2006 |
| Rainbow trout | 25 | 25 | 25 | 12 |
| Atlantic Salmon | 0 | 0 | 0 | 0 |
| Other salmonids | 0 | 0 | 0 | 2 |
| Carp | 17 | 24 | 24 | 19 |
| Eel | 1 | 1 | 1 | 0 |
| Flatfish | 0 | 0 | 0 | 0 |
| Seabream / Seabass | 28 | 22 | 22 | 22 |
| Other marine spp. | 6 | 6 | 6 | 6 |
| Other freshwater spp. | 0 | 0 | 0 | 19 |
| Total | 77 | 78 | 78 | 80 |

| **Number of fish samples (pools of tissue material) examined virologically (in cell cultures and by direct methods without cell cultivation) in NRL and regional laboratories, in total:** | | | | |
|---|---|---|---|---|
| | 2009 | 2008 | 2007 | 2006 |
| No. of samples tested by cell cultivation | 216 | 358 | 198 | 181 |
| No of samples tested by PCR or other direct methods without cell cultivation | 10 | 0 | | |
| No of positive samples | 16 IPNV | 5/210 IPN | | |

| **According to Council Directive 2006/88, please indicate number of farms in your country/region placed in the respective categories according to fish species:** | | | | | |
|---|---|---|---|---|---|
| **Category I** | | **VHS** | **IHN** | **ISA** | **KHV** |
| | Rainbow trout | | | 25 | |

| | | | | | |
|---|---|---|---|---|---|
| **Declared disease-free** | Atlantic Salmon | | | | |
| | Other salmonids | | | | |
| | Carp | | | | |
| | Eel | | | | |
| | Flatfish | | | | |
| | Seabream / Seabass | | | | |
| | Other marine spp. | | | | |
| | Other freshwater spp. | | | | |
| **Category II**<br><br>**Subject to a surveillance programme** | | **VHS** | **IHN** | **ISA** | **KHV** |
| | Rainbow trout | 25 | 25 | | |
| | Atlantic Salmon | | | | |
| | Other salmonids | | | | |
| | Carp | | | | 17 |
| | Eel | | | | |
| | Flatfish | | | | |
| | Seabream / Seabass | | | | |
| | Other marine spp. | | | | |
| | Other freshwater spp. | | | | |
| **Category III**<br><br>**Not known to be infected but not subject to surveillance programme for achieving disease free status** | | **VHS** | **IHN** | **ISA** | **KHV** |
| | Rainbow trout | | | | |
| | Atlantic Salmon | | | | |
| | Other salmonids | | | | |
| | Carp | | | | |
| | Eel | | | | |
| | Flatfish | | | | |
| | Seabream / Seabass | | | | |
| | Other marine spp. | | | | |
| | Other freshwater spp. | | | | |
| **Category IV**<br><br>**Known to be infected but subject to an eradication programme** | | **VHS** | **IHN** | **ISA** | **KHV** |
| | Rainbow trout | | | | |
| | Atlantic Salmon | | | | |
| | Other salmonids | | | | |
| | Carp | | | | |
| | Eel | | | | |
| | Flatfish | | | | |
| | Seabream / Seabass | | | | |
| | Other marine spp. | | | | |
| | Other freshwater spp. | | | | |
| **Category V**<br><br>**Known to be infected. Subject to minimum control measures** | | **VHS** | **IHN** | **ISA** | **KHV** |
| | Rainbow trout | | | | |
| | Atlantic Salmon | | | | |
| | Other salmonids | | | | |
| | Carp | | | | |
| | Eel | | | | |
| | Flatfish | | | | |
| | Seabream / Seabass | | | | |
| | Other marine spp. | | | | |
| | Other freshwater spp. | | | | |

Data from S&D 2009

## Annex 6

## Draft for IFAT 96-well plate

| Antibodies / Cell line | Virus | Anti VHS | | Anti IHN | | Anti SVC | | Anti IPN | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | AMP NO | | | | | | | | VHS pos control | IHN pos control | SVC pos control | IPN pos control |
| BF-2 | Undilute | | | | | | | | | | | | |
| BF-2 | 1:04 | | | | | | | | | | | | |
| BF-2 | 1:16 | | | | | | | | | | | | |
| BF-2 | 1:64 | | | | | | | | | Neg | Neg | Neg | Neg |
| EPC | Undilute | | | | | | | | | | | | |
| EPC | 1:04 | | | | | | | | | | | | |
| EPC | 1:16 | | | | | | | | | | | | |
| EPC | 1:64 | | | | | | | | | Neg | Neg | Neg | Neg |

Each sample and positive controls must be diluted in a dilutions plate in 4-four-fold dilution: undiluted, 1:4, 1:16, 1:64, 1:256, 1:1024 and 1:4096

50 µl of each dilution from each sample transferred from dilutions plate to the plate with cells

The plate shall be fixed with 80% acetone in water.

Carefully add 150 µl of acetone with multichannel pipette to all the wells.

Let it stand at room temperature for 10 min, then acetone gently sucked off.

Now the plate is ready for use, otherwise keep it cool.

The primary antibody diluted with PBS without Ca + + and Mg + +.

The antibody can be attached with multichannel pipettes. Using 20µl – 50µl / well, incubate for ½ hour at 37 ° C.

Then wash the plate with PBS-TWEEN immediately after incubation.

The secondary antibody diluted with PBS without Ca + + and Mg + +. Using 20µl – 50µl diluted antibody / well.

The secondary antibody can be attached with multichannel pipettes. Using 20µl – 50µl / well, incubate for ½ hour at 37 ° C, keep dark.

Then wash the plate with PBS-TWEEN immediately after incubation.

The plate is ready to be read in the fluorescence microscope, or put into the refrigerator.

# Laboratory visit at the National Reference Laboratory for Fish Diseases Center of Athens Veterinary Institutions Athen, Greece 18th – 19th November 2010

Annex 1: Program for the meeting.
Annex 2: Participants at the meeting.
Annex 3: Organisational and Functional Structure.
Annex 4: Proficiency Test
Annex 5: Aquaculture in Greece
Annex 6: Draft for IFAT 96-well plate
Annex 7: Mycoplasma infected cells and normal cells
Annex 8. Counting Chamber
Annex 9: Sterilization of bottles with red cap
Annex 10: Verification and calibration of Combined pH electrode
Annex 11: PCR kits
Annex 12: Cell sensitivity test

***Introduction***

The National Reference Laboratory for Fish Diseases (NRL) in Greece is located at the Centre of Athens Veterinary Institutions (CAVI). The CAVI was visited from the 18th to the 19st of November 2010 by Nicole Nicolajsen and Søren Kahns from the European Union Reference Laboratory for Fish Diseases (EURL). The program for the visit is shown in Annex 1 and a list of persons met by the EURL representative is listed in Annex 2. This report describes findings, comments and recommendations made by the delegation from the EURL. This report is sent to the CAVI and the EU Commission.

***Organisation***

The CAVI is a public governmental institution under the Ministry of Rural Development and Food and was established initially in 1921. The CAVI is managed by Director, Dr. Michael Patakakis and it consists of 6 institutes: Institute of Infectious and Parasitic Diseases, Institute of foot and mouth disease, Institute of Biochemistry, Toxicology and Animal Nutrition, Institute of Artificial Insemination, Institute of Biological Products and Institute of Food Hygiene. An organization plan is shown in Annex 3.

CAVI contain a total of 57 laboratories of which not all of them active at the moment. The Laboratory for Fish diseases is a part of the Institute of Infectious and Parasitic Diseases and covers the diagnostics of viral and bacterial fish diseases. The Laboratory for Fish diseases is coordinating Greek NRL activities for fish diseases.

A laboratory in Thessaloniki is coordinating Greek NRL activities for molluscs diseases.

***Buildings, Furnishing and Access***

The CAVI is placed in several separated buildings within one site in Athens. All access to the institute is controlled at the entrance of the site. There is free access between the buildings but entrances to the different laboratories are restricted through locks. All rooms have air-conditioning. Furthermore, fume hoods are located in certain rooms for working with hazardous chemicals.

### *Opening of the meeting*

The official opening took place 18th November where Head of the NRL for Fish Diseases, Dr. Athanasios Prappas gave a description of the CAVI, its history, organization and mission.

The meeting was followed by a tour of the institute where the aim of the tour was to illustrate how processing of fish samples occur, from it is received and until a diagnostic answer is given. The tour was guided by Dr. Athanasios Prappas and Dr. Stamatina Arfara from the Laboratory of Fish Diseases.

### *Laboratories/ buildings*

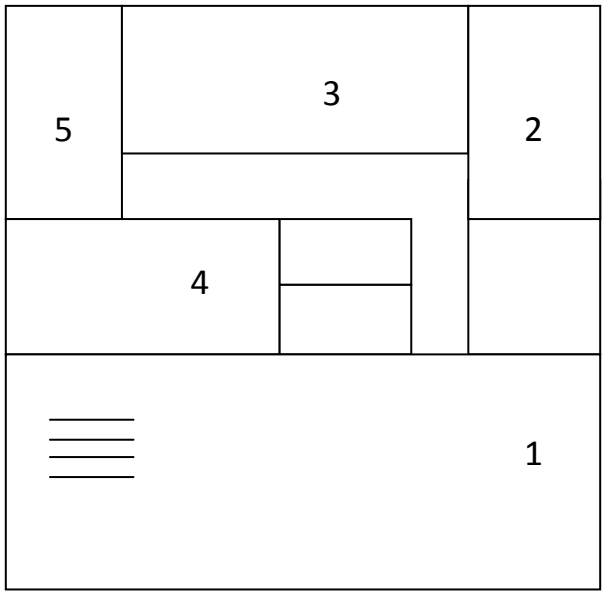
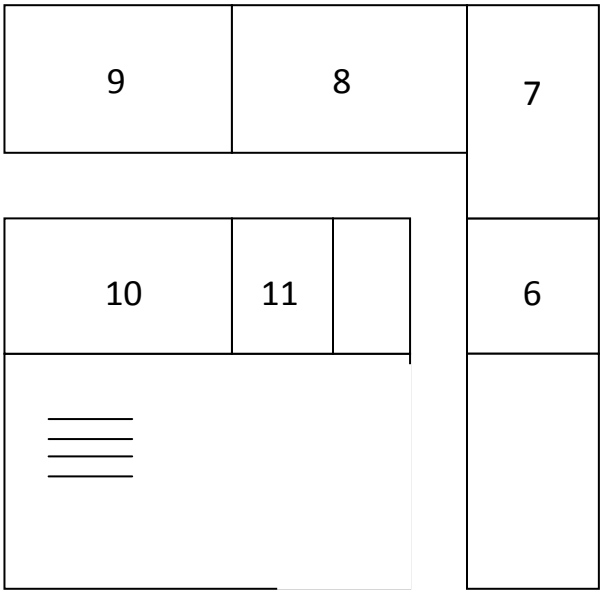
| **First floor** | **Basement** |
|---|---|
|  |  |
| 1. Acceptance room (the hall).<br>2. Laboratory for bacteriological examination and fish necropsy room.<br>3. Laboratory for sample preparation: Grinding and centrifugation of samples, inoculation of samples on cells and performance of ELISA.<br>4. Clean room.<br>5. Cell incubator room. | 6. Distillation and autoclaving room<br>7. Culture media preparation and dishwasher room<br>8. Immunohistochemistry and slide staining room<br>9. Freezer and $N_2$ storage room<br>10. Extra room<br>11. Dark Room with a microscope<br><br>Basement can be used for accredited methods when another floor has been laid. |

Figure 1 – Drawing of the Laboratory for Fish Diseases

***Sample acceptance and reporting unit***

The visit tour started in the fish sample acceptance room (the hall) where all fish samples delivered to the institute is entered. Samples are received from veterinarians of both official and private establishments and/or private persons. Currently, all samples are received as whole fish send on ice by courier companies. All received material is registered and given a unique number. Providers of samples have to provide information on e.g. fish species, clinical signs, fish farm, water temperature. The sample number and all collected information are entered into a registration book. During summer time, it can occur that ice on samples has disappeared before arrival – this will also be registered. After the samples have been registered, they are transferred to the Laboratory for Bacteriological examination. If immediate processing is not possible, the samples will be stored in a refrigerator. When the results of the diagnostic analyses have been obtained a final report is made by Dr. Athanasios Prappas. The answers have to be signed by the head of the Laboratory for Fish Diseases and the director of the Institute of Infectious and Parasitic Diseases before they will be sent to the customer.


***The Laboratory for Fish Diseases***

The Laboratory for Fish Diseases is a part of the Institute of Infectious and Parasitic Diseases. The Laboratory for Fish Diseases is located in a building shared with laboratory for Parasitic Diseases. The Laboratory for Fish Diseases is responsible for diagnostics of all diseases of fish from farms and open water. The Laboratory for Fish Diseases coordinates the NRL for fish diseases. The NRL for fish diseases covers all activities on diagnosis of listed as well as non-listed fish diseases.

*Staff of the Laboratory for Fish Diseases*

The Laboratory for Fish Diseases consists of a group of two persons: DVM, Dr. Athanasios Prappas and DVM Stamatina Arfara who carry out all the viral diagnostics. Athanasios Prappas is the Head of the Laboratory for Fish Diseases and he is overall responsible for the diagnosis of all viral as well as non-viral diseases in fish. All tasks (including diagnostic assays, implementation and validation of novel diagnostic assays, preparation of media, cleaning of rooms etc.) of Laboratory for Fish Diseases are carried out by Athanasios Prappas and Stamatina Arfara.

*Laboratories*

The buildings for Laboratory of Fish Diseases were newly reconstructed. The reconstruction process was started in 2008 and was completed summer 2010 and resulted in the construction of a large building in continuation of the old building. The reconstruction process was funded by the Ministry of Rural Development and Food, and EU funds. The new building is in two floors and includes 7 laboratories/ rooms in the upper floor and 6 laboratories/ rooms in the basement. The Laboratory for Fish Diseases can use all laboratories/rooms in the new building plus an office in the reconstructed old part of the

building. All rooms have a central system for heating and cooling) that keeps the temperature constant at 22°C.

The set-up of the laboratory was not yet completely finished. So far, four separate laboratories in the upper floor were finished and actively used: 1. Laboratory for bacteriological examination that were also used as a fish necropsy room, 2. Laboratory for sample preparation, where grinding and centrifugation of the samples, inoculation of samples on cells and ELISA were performed, 3. A clean room for cell culture work and 4. Cell incubators room. This is illustrated in figure 1.
This arrangement allows samples to be processed according to the description in Commission Decision 2001/183/EC. Further three rooms in the upper floor were reserved for being used as the future diagnostic PCR laboratories: Master mix room, nucleic acid purification room and post PCR room.

Equipment was new and adequate however, it is recommended to acquire some additional equipment as refrigerators, laminar flow benches etc. This is described in the section below on *"PCR based diagnostics".*
Equipment was placed in laboratories but since the reconstruction process had just ended, PCR equipment had not yet been ready installed for use. Equipment used for accredited analyses had a reference number and a logbook. Username, date and sample are registered in a logbook, one for accredited samples and one for none accredited samples, as well as any problems encountered. Repairs, cleaning, calibration and other maintenance such as filter change are likewise registered and according to accreditation. Sub-contractors perform calibration of the pipettes and other equipment externally.
Instructions for use of equipment are written down in procedures, one for each piece of equipment. Staffs are required to read and be trained according to these procedures before using the equipment, or should be according to accreditation.

*Laboratory for bacteriological examination and fish necropsy room*
After the samples are received and recorded, they are sent directly to the fish necropsy room that is also laboratory for bacteriological examinations. Considering the number of available laboratories in the building, we recommend that necropsy is performed in a separate room to minimize risk of any cross contaminations. The registration number of the sample identifies the sample in all subsequent tests. The samples are processed according to the description in Commission Decision 2001/183/EC. We recommend that mortar and pestle are being kept in the refrigerator until they are being used. Disinfection of workspaces is done daily by UV light and ethanol and weekly by Virkon-S *(the last step is applicable only for the laminar flow cabinets).*

*Sample preparation (grinding and centrifugation of samples, inoculation of samples on cell cultures and performance of ELISA*

Inoculation of samples on cell cultures is performed in a distinct "dirty room". Samples and viruses are inoculated on 24 well plates where BF-2/EPC cells (half of each) are seeded on the same plate. Sometimes samples are inoculated in flasks for identification of VNN. In this room identification of viruses by antibody based methods are also performed and equipment for making the identification is present. Identification of the fish viruses VHSV, IHNV, SVCV and IPNV is done by ELISA and IFAT using antibodies using kits from Bio-X. VNN IFAT is performed using antibodies provided by Dr. Giuseppe Bovo.

Disinfection of workspaces is done daily by UV light and ethanol and weekly by Virkon-S.

*Clean room*

All cell culture work are performed in a "clean room"

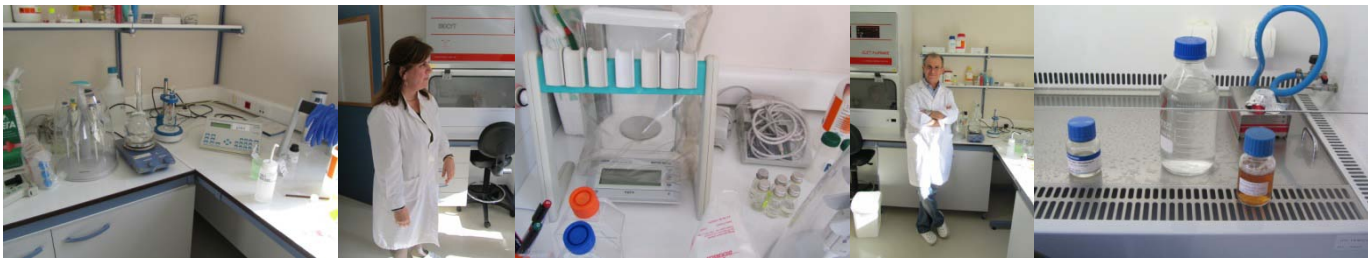
*Cell Culture Facilities*

BF-2, EPC and SSN-1 cells are cultured in the clean lab. New BF-2 and EPC cells and protocols (on how cells are handled at the EURL) were provided by at the visit. SSN-1 cells have been provided by Dr. Giuseppe Bovo. It was recommended that the BF-2 cells is passaged every 2-3 weeks, out growing temperature 24°C, and that the EPC cells is passaged every 3-4 weeks, out growing temperature 24°C. After outgrowth, all cells are kept in incubators at 15°C. Cells are stored in 10% DMSO in liquid nitrogen for long term storage. Cells are tested for Mycoplasma infections at the Bacteriology Department. A description on how mycoplasma tests are performed at the EURL is shown in annex 7. It is recommended to make a sensitivity test of the cells using virus VHSV, IHNV and e.g. IPNV isolates of known titres to ensure that cells stay sensitive towards the respective virus strains. A description of how the sensitivity test at the EURL looks like is shown in annex 12. A manual for counting cells is attached in annex 8.

Disinfection of workspaces is done daily by UV light and ethanol and weekly by Virkon-S.

*Equipment on first floor and Basement*

- Refrigerators
- Freezer -20°C, -40°C and -80°C
- Microscopes
- Fluorescent microscope
- Balance
- Centrifuge with cooling
- LAF benches
- Incubators at 15°C, 22°C, 28°C and one which was under repair
- ELISA reader
- ELISA washer
- Microwave oven
- Shaker
- Pipettes
- $N_2$ tank
- Autoclave
- Equipment for automatic freezing of cells
- Fume cabinet
- Safety cabinets for chemicals
- Histokinette
- Automatic staining devise
- Cryotome
- Microtome
- PCR thermocycler
- Gel electrophoresis equipment
- UV-light table
- Camera



*Pictures from basement*

Room 6

Room 7

Room 8

Room 9

Room 10

Room 11

*PCR based diagnostics*
The Laboratory for Fish Diseases does not yet perform PCR based diagnostic. However, the equipment for performing PCR based fish diagnostic analyses is present and it is the intention that the Laboratory for Fish Diseases will perform these analyses in the near future. The PCR equipment includes a PCR thermo cycler, gel electrophoresis equipment, UV light table and a camera. The equipment is placed in three separate laboratories and it is the intention to arrange the PCR based diagnostics in a Mastermix room (that can act as a clean room where primers and buffers are stored and master mixes are prepared), a nucleic acid purification room and a post PCR room (post PCR analyses is considered to be a major source of cross contamination) as it is recommended for PCR diagnostic laboratories. It is recommended that the master mix room contains a freezer, a fridge and a laminar flow cabinet. It is recommended that a laminar flow cabinet and a fume hood (as mercaptoethanol is used for RNA extraction) is present in the nucleic acid purification room. Furthermore, it is recommended that a fridge is present in both the purification and the post PCR room.
The Laboratory for Fish Diseases has kits for purification of RNA and DNA. A kit for purification of PCR fragments from buffers or agar is required when sequence analyses has to be performed. In Annex 11 is a list of the kits used at the EURL for performing PCR based analyses.
Handouts were provided on recommended PCR, RT-PCR, real-time PCR and real-time RT-PCR assays for identification of VHSV, IHNV, ISA, KHV, EHNV, EUS and SVC.

*Accreditation*
The CAVI is accredited in accordance with ISO 17025. Accreditation was achieved through a private accreditation company in 2008 having the accreditation number: 695. Because of the reconstruction process, the Laboratory for Fish Diseases has not yet obtained accredited methods. An aim is to become accredited for using IHNV and VHSV ELISA – how this could be done was discussed. Accreditation of these methods is planned to occur at the next visit of the accreditation company. Some of the equipment has log books, calibration of pipettes, ELISA reader etc is performed by external companies. Audits are performed by employees that have been trained to perform internal audits

*Proficiency Test*
The proficiency tests (PT) allow a laboratory to assess their diagnostic capacity of certain procedures. The Greek NRL for fish diseases has participated in the PT for identification of notifiable fish diseases organized by EURL, Aarhus, Denmark, since 2000 and until 2007 after when the reconstruction of the laboratory started. The score obtained by the NRL was 100% correct in the last two proficiency tests. For all the scores obtained by the NRL in the PT provided by the EURL, see Annex 4.

The Greek NRL do not organizes PTs for diagnosis of fish diseases for the regional laboratories as there currently are no regional laboratories for fish diseases in Greece.

***Aquaculture in Greece***
The main fish species cultured in Greece are seabream, seabass, other new marine species (like sharpsnout seabream and *Pagrus pagrus*) and rainbow trout but there is also a small production of carp and eel. Greece have 325 fish farms producing more than 100 tonnes per year, 88 fish farms producing 5 - 100 tonnes per year and 5 fish farms producing less or around 5 tonnes per year (2009). The laboratory received approximately 103 samples in 2008 for diagnostic and surveillance purposes on cell cultures. In

2009 samples were submitted for many types of examination except virological testing on cell cultures. The samples for virological examinations are mainly submitted from sea bream and sea bass and examined for VNN/Noda virus on SSN-1 cells. See Annex 5 for more details.

*Categorization of Greek fish farms according to Council Directive 2006/88/EC*
The Council Directive 2006/88/EC have been implemented in Greece but there has not been established an official surveillance program for VHS and IHN for Greek aquaculture. All fish farms have been registered in line with what is required for authorization according to 2006/88/EC. The data on the fish farms is made public available.

***Training needs and future plans***
Diagnostic methods for the non-exotic diseases, ISA and KHV and for the exotic diseases EUS and EHN will have to be implemented. This could be done by implementing some of the PCR assays handed out and recommended by the EURL.

In order to implement these diagnostic methods the Laboratory for Fish Diseases need to finalize the establishment of the PCR facilities. This is a task that will require additional resources, especially for the two employees at the laboratory for fish diseases that already have many daily assignments. Therefore, the possibility to employ an experienced molecular biologist to assist on this task might be considered. Such a person could in case be associated for a longer period of time to be in charge of the molecular biological fish diagnostics. Alternatively it might be considered, to create a PCR based diagnostic platform at the Institute that can be used by all laboratories at the institute as it have been done in other similar veterinary institutes.

The Greek NRL for fish diseases have decided that one staff members will participate in the EURL course on fish diagnostics that will take place in Aarhus, Denmark, in January/February 2011. This participation will provide training in using diagnostic PCR based methods and knowledge on how the PCR facilities should be set up in the laboratories. Furthermore, the course will provide training in cell based and antibody based fish diagnostics.

***Summary of recommendations***
- Necropsy should be performed in a separated room to minimize risk of having cross contaminations.
- Mortar and pestle should be kept at 4ºC before use.
- BF-2 cells should be passaged every 2-3 weeks, out growing temperature 24°C, and EPC cells should be passaged every 3-4 weeks
- A sensitivity test should be made every half year for VHSV and IHNV and EHNV.
- PCR facilities should be set up including a master mix room, a nucleic acid purification room and a post PCR room. It is recommended that the master mix room contains a freezer, a fridge and a laminar flow cabinet. It is recommended that a laminar flow cabinet and a fume hood (as mercaptoethanol is used for RNA extraction) is present in the nucleic acid purification room. Furthermore, it is recommended that a fridge is present in both the purification and the post PCR room.
- A PCR based tool for the identification of KHV, ISAV, EHNV and EUS should be implemented.

***Conclusion***

The visit showed that the Greek NRL for fish disease has an adequately equipped laboratory. The staff is well educated, enthusiastic and very capable. After the reconstruction, the laboratory has some tasks that have to be accomplished: 1) The BF-2 and EPC cells should grow properly, 2) The PCR laboratories should be set up as recommended above and the PCR and RT-PCR assays for KHV, EHNV, ISAV, and EUS should be implemented and 3) the laboratory should participate fully in both proficiency tests submitted by the EURL in 2011. When these tasks have been accomplished the Greek NRL for fish diseases should be capable of performing its duties as a NRL.

## Annex 1
## Program for the meeting on
## diagnostic procedures of fish diseases and implementation progress of Council Directive 2006/88/EC

November 18th:

09:00 – 10:30 Introduction to the Greek National Reference Laboratory for Fish Diseases. Presentation of the CRL and discussion on the topics of the visit. Participants: All Staff including Head of Institute

10:30 – 13:00 Tour in the lab, looking at all the facilities: we would like that the people working with fish diagnostics will be present during the tour.

The main goal is to learn about how are the Diagnostic procedures according to Commission Decision 2001/183 conducted in the laboratory.

Following issues can be discussed:

- Aquaculture in Greece and the type of samples received at the laboratory
- Buildings and access
- Staff
- Equipment
- Accreditation
- Registration of Samples
- Sample processing
- Cell Cultivation and cell cultures
- Virus identification by ELISA and IFAT etc
- Molecular techniques (PCR, RT-PCR, Q-PCR)
- Reporting diagnostic tests
- Past (2008 and 2009) Proficiency Test Results

Special attention will be on molecular biological diagnostic of fish diseases, if you wish.

13:00 – 13:45 Lunch

13:45 – 15:30 In the afternoon we might continue the tour in lab.

15:30 – 17:00 Follow-up (only Søren Kahns and Nicole Nicolajsen) We would be pleased to have the possibility to work together in an office.

November 19th:

08:30 – 10:30 Follow up for from the day before.

10:30 – 11:30 Plans and progress in the implementation of Council Directive 2006/88/EC in Greece.

11:30 – 12:00 Evaluation of the visit. Recommendations and report of the visit

12:15 Departure to airport

## Annex 2

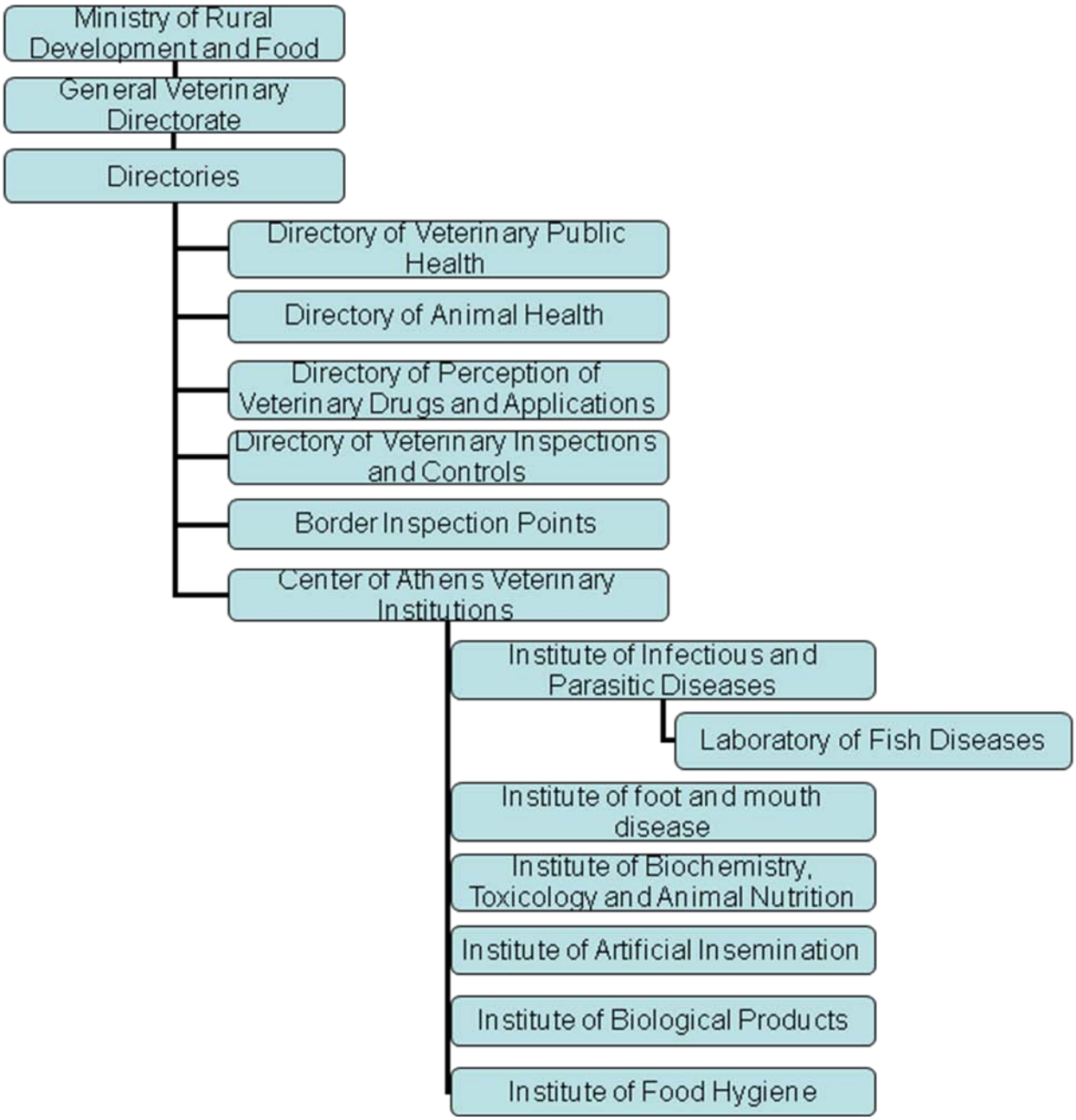
## Participants at the meeting

| Name | Institute | Function |
|---|---|---|
| Mr. Athanasios Prappas | Institute of Infectious and Parasitic Diseases | Head of the Laboratory for Fish Diseases, Head of the NRL for fish diseases; veterinarian |
| Mrs Stamatina Arfara | Institute of Infectious and Parasitic Diseases | Veterinarian |
| Mrs Olga Magana | Institute of Infectious and Parasitic Diseases | Director |
| Mrs Sofia Boutsini | Parasitology Lab | Head |
| Mr Michael Patakakis | CAVI | Director |
| Søren Kahns | EURL for Fish Diseases | Coordinator of the EURL for Fish Diseases |
| Nicole Nicolajsen | EURL for Fish Diseases | Laboratory Technologist |

**Annex 3**

**Organisational and Functional Structure**

## DIAGRAM of INSTITUTE ORGANISATION



# Number of persons at the institutes at CAVI

INSTITUTE OF INFECTIOUS AND PARASITIC DISEASES: 25 PERSONS (veterinarians & technicians)
INSTITUTE OF FOOT AND MOUTH DISEASE: 6 PERSONS
INSTITUTE OF BIOCHEMISTRY TOXICOLOGY AND ANIMAL NUTRITION: 4 PERSONS
INSTITUTE OF ARTIFICIAL INSEMINATION: 2 PERSONS
INSTITUTE OF BIOLOGICAL PRODUCTS: 3 PERSONS
INSTITUTE OF FOOD HYGIENE: 20 PERSONS

## Annex 4

# Proficiency Test

| Greece | 1996 | 1997 | 1998 | 1999 | 2000 | 2001 | 2002 | 2003 | 2004 | 2005 | 2006 | 2007 | 2008 | 2009 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Participated | | | | | 1 | | 1 | 1 | 1 | 1 | | 1 | | |
| Score % | | | | | | | | 70 | 80 | 100 | | 100 | | |

| **2007**<br><br>**Score 10/10** | **Ampoule I:** | **Ampoule II:** | **Ampoule III:** | **Ampoule IV:** | **Ampoule V:** |
|---|---|---|---|---|---|
| | VHS virus<br>DK-F1<br>Genotype I<br>(Undiluted ) | VHS virus<br>DK-F1<br>Genotype I<br>(Diluted $10^{-5}$) | SVC virus<br>56/70<br>Genotype Id | IHNV 32/87<br>First French<br>isolate<br>Genotype M | VHSV<br>4p101<br>Genotype III |
| Cell line | BF-2/EPC | | | | |
| ELISA, IFAT | VHSV | VHSV | SVCV | IHNV | VHSV |

## Annex 5

# Aquaculture in Greece

| Land Area | Ocean Area | Environment | Species | Scientific name | 2004 | 2005 | 2006 | 2007 | 2008 |
|---|---|---|---|---|---|---|---|---|---|
| Greece | Europe - Inland waters | Brackishwater | Miscellaneous freshwater fishes | Miscellaneous freshwater fishes | 312 | 317 | 293 | 305 | 284 |
| | | | River eels | River eels | 52 | 110 | 94 | 81 | 87 |
| | | Sub-total Brackishwater | | | 364 | 427 | 387 | 386 | 371 |
| | | Freshwater | Carps, barbels and other cyprinids | Carps, barbels and other cyprinids | 105 | 107 | 136 | 93 | 113 |
| | | | Marine fishes | Marine fishes | 63 | 31 | 21 | 17 | 22 |
| | | | Miscellaneous freshwater fishes | Miscellaneous freshwater fishes | 3 | 3 | 8 | 18 | 19 |
| | | | River eels | River eels | 429 | 261 | 290 | 365 | 399 |
| | | | Salmons, trouts, smelts | Salmons, trouts, smelts | 2 067 | 2 452 | 3 198 | 2 828 | 3 438 |
| | | Sub-total Freshwater | | | 2 667 | 2 854 | 3 653 | 3 321 | 3 991 |
| | Sub-total Europe - Inland waters | | | | 3 031 | 3 281 | 4 040 | 3 707 | 4 362 |
| Total Greece | | | | | 3 031 | 3 281 | 4 040 | 3 707 | 4 362 |

**Data taken from FIGIS**

| **Number of fish farms within country/region, according to size of production (tonnes fish/year)** | | | | |
|---|---|---|---|---|
| | 2006 | 2007 | 2008 | 2009 |
| < 5 tonnes | 5 | 5 | 5 | 5 |
| 5 - 100 tonnes | 123 | 88 | 88 | 88 |
| > 100 tonnes | 310 | 330 | 325 | 325 |

| **Number of fish farms within country/region, according to fish species** | | | | |
|---|---|---|---|---|
| | 2006 | 2007 | 2008 | 2009 |
| Rainbow trout | 93 | 69 | 69 | 69 |
| Atlantic Salmon | 0 | 0 | 0 | 0 |
| Other salmonids | 5 | 4 | 4 | 4 |
| Carp | 13 | 8 | 8 | 8 |
| Eel | 10 | 9 | 9 | 9 |
| Flatfish | 0 | 0 | 0 | 0 |
| Seabream / Seabass | 309 | 328 | 328 | 328 |
| Other marine spp. | 0 | 0 | 0 | 0 |
| Other freshwater spp. | 8 | 0 | 0 | 0 |
| Total | 438 | 423 | 418 | 418 |

| **According to Council Directive 2006/88, please indicate number of farms in your country/region placed in the respective categories according to fish species:** | | | | | |
|---|---|---|---|---|---|
| **Category I** | | **VHS** | **IHN** | **ISA** | **KHV** |

| | | | | | |
|---|---|---|---|---|---|
| **Declared disease-free** | Rainbow trout | | | 25 | |
| | Atlantic Salmon | | | | |
| | Other salmonids | | | | |
| | Carp | | | | |
| | Eel | | | | |
| | Flatfish | | | | |
| | Seabream / Seabass | | | | |
| | Other marine spp. | | | | |
| | Other freshwater spp. | | | | |
| **Category II**<br><br>**Subject to a surveillance programme** | | **VHS** | **IHN** | **ISA** | **KHV** |
| | Rainbow trout | 69 | 69 | 69 | |
| | Atlantic Salmon | | | | |
| | Other salmonids | 4 | 4 | 4 | |
| | Carp | | | | 9 |
| | Eel | | | | |
| | Flatfish | | | | |
| | Seabream / Seabass | | | | |
| | Other marine spp. | | | | |
| | Other freshwater spp. | | | | |
| **Category III**<br><br>**Not known to be infected but not subject to surveillance programme for achieving disease free status** | | **VHS** | **IHN** | **ISA** | **KHV** |
| | Rainbow trout | | | | |
| | Atlantic Salmon | | | | |
| | Other salmonids | | | | |
| | Carp | | | | |
| | Eel | | | | |
| | Flatfish | | | | |
| | Seabream / Seabass | | | | |
| | Other marine spp. | | | | |
| | Other freshwater spp. | | | | |
| **Category IV**<br><br>**Known to be infected but subject to an eradication programme** | | **VHS** | **IHN** | **ISA** | **KHV** |
| | Rainbow trout | | | | |
| | Atlantic Salmon | | | | |
| | Other salmonids | | | | |
| | Carp | | | | |
| | Eel | | | | |
| | Flatfish | | | | |
| | Seabream / Seabass | | | | |
| | Other marine spp. | | | | |
| | Other freshwater spp. | | | | |
| **Category V**<br><br>**Known to be infected. Subject to minimum control measures** | | **VHS** | **IHN** | **ISA** | **KHV** |
| | Rainbow trout | | | | |
| | Atlantic Salmon | | | | |
| | Other salmonids | | | | |
| | Carp | | | | |
| | Eel | | | | |
| | Flatfish | | | | |
| | Seabream / Seabass | | | | |
| | Other marine spp. | | | | |
| | Other freshwater spp. | | | | |

## Annex 6

## Draft for IFAT 96-well plate

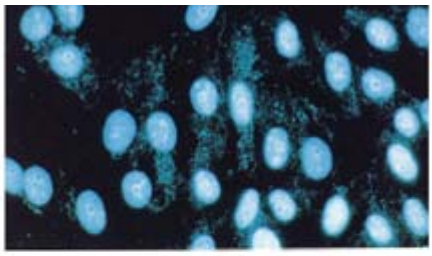
| Antibodies | Anti VHS | Anti IHN | Anti SVC | Anti IPN | | | | |
|---|---|---|---|---|---|---|---|---|

| Cell line | Virus | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | AMP NO | | | | | | | | VHS pos control | IHN pos control | SVC pos control | IPN pos control |
| BF-2 | Undiluted | | | | | | | | | | | | |
| BF-2 | 1:04 | | | | | | | | | | | | |
| BF-2 | 1:16 | | | | | | | | | | | | |
| BF-2 | 1:64 | | | | | | | | | Neg | Neg | Neg | Neg |
| EPC | Undiluted | | | | | | | | | | | | |
| EPC | 1:04 | | | | | | | | | | | | |
| EPC | 1:16 | | | | | | | | | | | | |
| EPC | 1:64 | | | | | | | | | Neg | Neg | Neg | Neg |

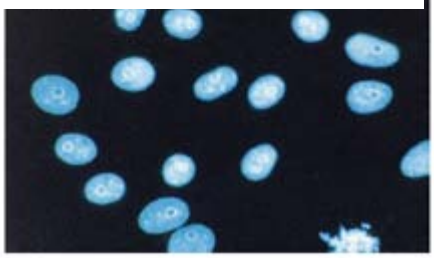
- Each sample and positive controls must be diluted in a dilutions plate in 4-four-fold dilution: undiluted, 1:4, 1:16, 1:64, 1:256, 1:1024 and 1:4096
- 50 µl of each dilution from each sample transferred from dilutions plate to the plate with cells
- The plate shall be fixed with 80% acetone in water.
- Carefully add 150 µl of acetone with multichannel pipette to all the wells.
- Let it stand at room temperature for 10 min, then acetone gently sucked off.
- Now the plate is ready for use, otherwise keep it cool.
- The primary antibody diluted with PBS without Ca + + and Mg + +.
- The antibody can be attached with multichannel pipettes. Using 20µl – 50µl / well, incubate for ½ hour at 37 ° C.
- Then wash the plate with PBS-TWEEN immediately after incubation.
- The secondary antibody diluted with PBS without Ca + + and Mg + +. Using 20µl – 50µl diluted antibody / well.
- The secondary antibody can be attached with multichannel pipettes. Using 20µl – 50µl / well, incubate for ½ hour at 37 ° C, keep dark.
- Then wash the plate with PBS-TWEEN immediately after incubation.
- The plate is ready to be read in the fluorescence microscope, or put into the refrigerator.

# Annex 7
## Mycoplasma infected cells and normal cells

Mycoplasma infected cells



Normal cells



**PROTOCOL FOR MYCOPLASMA-TESTING OF CELL CULTURE BY HOECHST BISBENZAMIDE AND MERTHIOLATE STAI**

**AIM**
This protocol describes the DNA staining of cell cultures for detection mycoplasma.

**BACKGROUND**
Cell cultures used in virological investigations should be free of infected mycoplasma. To detect possible infections the cultures are regularly screened at the laboratory and immediately prior to export.

**PRINCIPLE**
Cells infected with mycoplasma will strongly stain DNA in both cell nuclei and mycoplasms after incubation at low pH of Bisbenzimidazol (Hoechst fluorochrome dye 33258). Mycoplasms can be observed via an immunofluorecence microscope as small bluish or greenish fluorescent dots just around the large fluorescent cell nuclei.

**EQUIPMENT**
Sterile disposable pipettes 2 ml and 10 ml
Sterile disposable petri dishes, 2 cm. in diameter e.g Nunclon no. 153066
Cell culture flasks
Sterile cover slips packed in petri dishes
Glass slides e.g. Super Frost
Device for discarding of medium
Incubator 15°C, 21°C, 24°C, 28°C
Laminar Flow Cabinet
Fluorescence microscope

**REAGENTS**
Eagle´s MEM cell culture medium with 10% fetal bovine serum
Versene with added trypsine
Mycoplasma medium
Stock solution of Hoechst bisbenzamide fluorochrome and merthiolate
Fixation fluid for cells mycoplasma staining
Mounting fluid for mycoplasma staining

**SAFETY PRECAUTIONS**
Bisbenzamide reacts with DNA and is potentially carcinogenic.

Merthiolate is registered under the name Thiomersal or Thimerosal, and belongs to the mercury group and is harmful (Xn) in the used (≤1%) concentration.
R-26/27/28: Very toxic by inhalation, in contact with skin and if swallowed.
R-33: Danger of cumulative effects.
S-13: Keep away from food, drink and animal feeding stuffs.
S-28: After contact with skin, wash immediately with plenty of water.
S-36: Wear suitable protective clothing.
S-45: In case of accident or if you feel unwell, seek medical advice immediately (show the labels where possible).
Use a fume hood and nitrile gloves e.g. Super Glove Finite PF Disposable.

**METHOD**
In continually subcultivated cell cultures, the cells have to be tested approximately every 3rd month for a possible mycoplasma infection.

**All the following procedures have to be carried out in a laminar flow cabinet.**

1. One or two sterile cover slips are placed in a petri dish. 2 ml mycoplasma medium is added. The flask that has to be tested for mycoplasma is trypsinated. A drop of the cell suspension is added to the petri dish. The petri dish is then incubated over night or 24 hrs at the temperature the cell line usually grows at.
   After one day, the petri dish is moved to an incubator at 15°C for 8 days. On Fridays, the petri dish may be placed directly in the incubator 15°C ± 2°C.

   **All the following procedures are carried out in a fume hood.**
3. Medium from the petri dish is removed and 2 ml of fixation fluid is added. The fluid is replaced after 2 min with fresh fixation fluid. (The fixation fluid is poured in the can in the fume hood.) Incubate for 10 min.
4. Fixation fluid is removed and the samples are washed three times with Milli Q water.
5. The working dilution is made from the stock solution Hoechst by taking e.g. 10 ml Milli Q water and adding 0.100 ml stock solution Hoechst. The working dilution has to be mixed carefully and should be protected from light by wrapping the container in foil.
6. The cover slip to be stained is transferred to a new petri dish. Two ml of the working dilution of Hoechst is added. The petri dish is placed in a humid chamber lined with foil, and incubated at 37°C±2°C for 30 minutes.
7. Remove the working solution (fluid is poured in the special can under the fume hood) and wash three times with Milli Q water.
8. The cover slip is airdried or the excess water is removed with filter paper.
9. The cover slip is mounted on an ethanol-cleaned object glass slide with the side that is covered with cells down towards the mounting fluid. The samples are covered with foil and kept at 4°C
10. The samples are now ready for screening in an immunofluorescence microscope. Use objective 1 or 2 and filter 3.

**ADVICE**
Begin the experiment by placing the Hoechst solution on a magnetic stirrer. The stock solution crystalizes during storage at low temperatures and has to be stirred for at least 30 min at room temperature before the working dilution can be made. The longer the stock dilution is stirred the better the final result. The cover slips can be stored at 4°C after fixation and washing with Milli Q water and stained later. The stained cover slips can also be kept at 4°C until microscopy examination.

**WASTE DISPOSAL**
All the equipment that has been in contact with bisbenzamide and/or merthiolate is treated as contaminated. Disposable waste is placed in autoclave buckets inside the plastic autoclave bags. Glass ware is rinsed and washed-up.

# Annex 8
# Counting Chamber

1. Wash Counting Chamber and the associated cover slips (under water) and dry. Cover slip should be placed over the counting chamber and secured with two clamps that are on the counting chamber.

2. A bit of the cell suspension should be transferred with a Pasteur pipette to counting chamber. Do not allow cell suspension to enter completely into Pasteur pipette as a part of the cells easily remains in the pipette. Place the tip of the Pasteur pipette into the space between the counting chamber and cover slip. Cell suspension will distribute itself in counting chamber.

3. The counting takes place using a microscope with 10 x lens. Figure 1 shows graduation in a Burke-Turk counting chamber. The entire network consists of 9 large squares. There are counted in the 4 major squares, located in every corner of the counting chamber.

4. Figure 2 shows one of the squares in the counting chamber. In the example shown is the distance 0.25 mm between the individual lines and 1.0 mm between tripel-lines. When the depth is known, the corresponding volume can be calculated. In the magnified part of the counting chamber is shown the cells to be counted (open circles) and which are not (black circles). Cells that touch the median line of the upper and left tripel-line are counted, while cells that touch the median line in the lower and right tripel-line are not counted. Note that the sizes indicated by the counting chamber may vary from one type of counting chamber to another. Counting chamber will normally be provided with an indication of the dimensions.

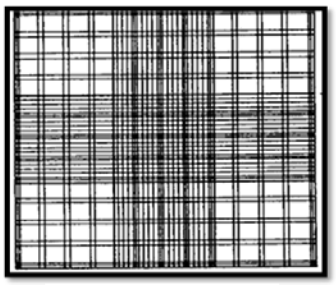


Figure 1

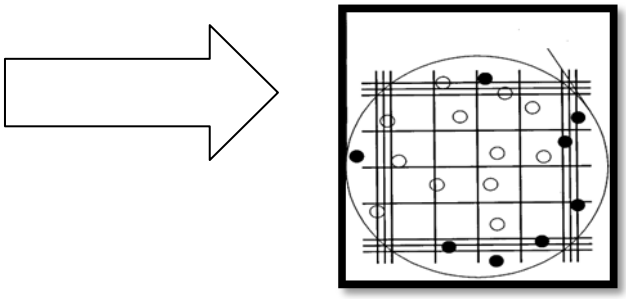


Figure 2

5. Calculation of the cell concentration: A quadrant (large field) consisting of 16 small fields has an area of 1 $mm^2$, and depth can be 0,1 or 0,2 mm depending on the type of
counting chamber. At a depth of 0.1 mm has a quadrant of a volume of 0,1 $mm^3$.

   0,1 $mm^3$ = 0,1x $10^{-3}$ ml = $10^{-4}$ ml = 0.1 µl

6. Cells per. ml = N x F x (1 / V) cells / ml,.
   N = is the average number of cells per quadrant.
   F = is the dilution of the original cell suspension.
   V = is the volume contained in one quadrant.

   Eksampel:
   N = The average number of counted cells per quadrant = 11+12+13+12/4 =12
   F = Undiluted
   V = The volume in one quadrant =
   12 cells x 1 x (1/$10^{-4}$ml) = 12 cells x $10^{-4}$ml = **12 x $10^{-4}$ cells / ml**

## Annex 9
## Sterilization of bottles with red cap

Screw on the bottles screwed and then rotated a half turn back.
After sterilization, screwed the lid on the bottles immediately completely.

Sterilization in dry sterilization at 150 ° C for 3 ⅓ hours.
Sterilization in dry sterilization at 160 ° C for 3 ¾ hours.

## Annex 10
## Verification and calibration of Combined pH electrode

| Date | Elektrode no. | Calibrations buffer | | | Sensitivity | Remarks |
|---|---|---|---|---|---|---|
| | | pH | pH | pH | $97 \leq \% \leq 103$ | |
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## Annex 11
## PCR kits from QIAGEN

Nucleic acid purification kits used at the EURL:

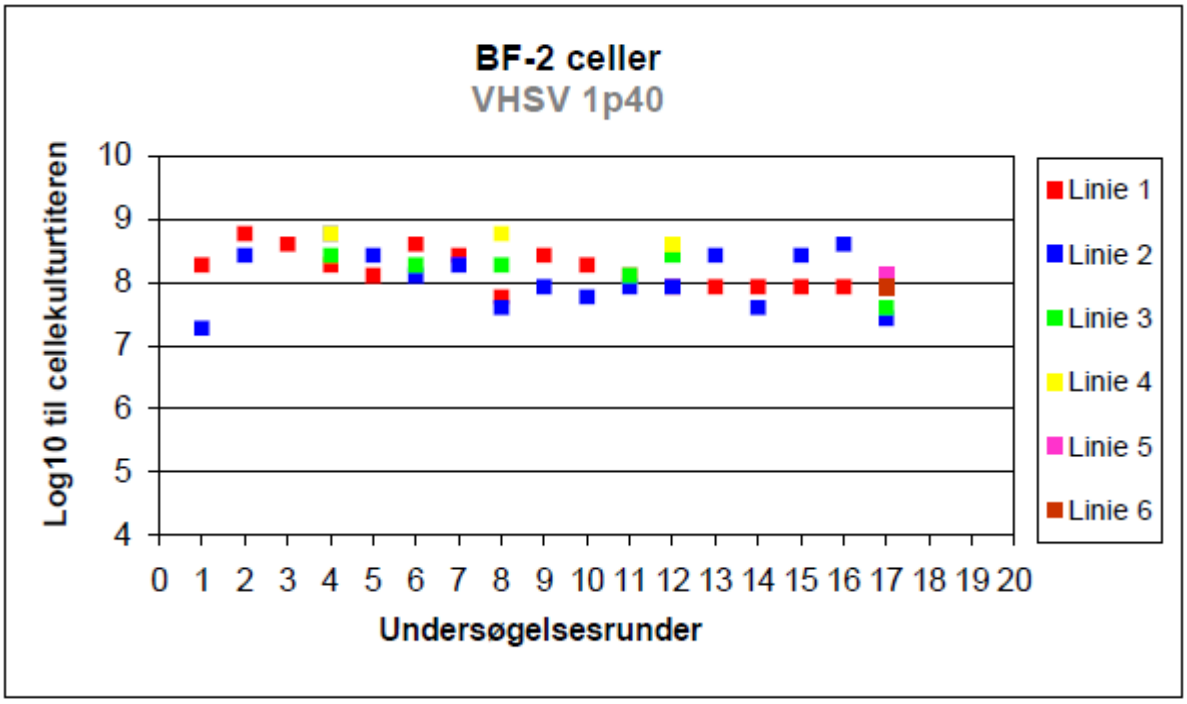
**RNA**: RNeasy mini kit, Cat. No. 74106

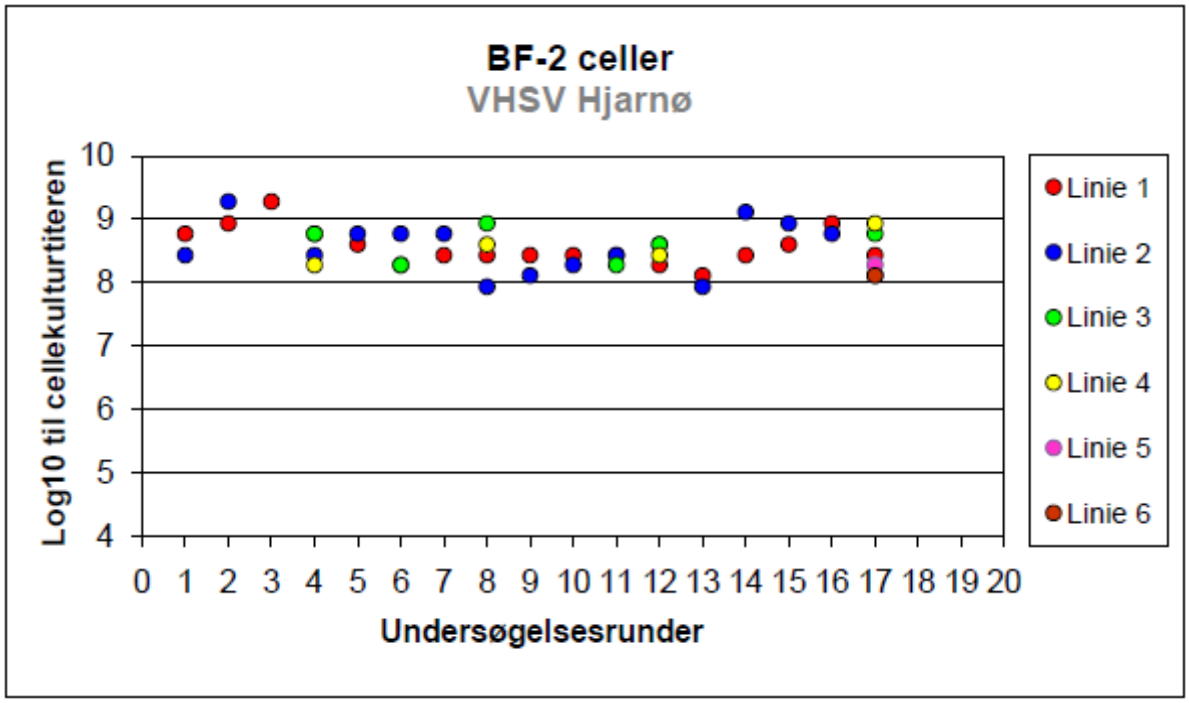
**DNA**: QIAamp DNA blood kit, Cat No. 51106

**PCR fragments**: QIAquick PCR purification kit, Cat No. 28104

**QIAGEN home page**: http://www.qiagen.com/default_qiacube.aspx

## Annex 12
## Cell sensitivity test




This figure show the results of the sensitivity test of EPC and BF-2 cells at the EURL during time using a given reference strain. Linie 1-6 represent different sub cell lines. The test is performed every half year. Therefore the gaps in the X-axis represent half a year and the test have been performed every half year for 10 years (number=20). Y-axis is the titer obtained.