Technical Report
2009

from the
Community Reference Laboratory
for Fish Diseases

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

Content

Page

Content of Annexes

Introduction

The National Veterinary Institute, Technical University of Denmark (DTU-VET) is appointed as the Community Reference Laboratory for Fish Diseases (CRL), according to Commission Decision of 24 April 2008, 2008/332/EC (notified under document number C (2008) 1570) on financial aid from the Community for the year 2009 for certain Community reference laboratories in the field of animal health and live animals.

The duties of the CRL are described in Council Directive 2006/88/EC of 24. October 2006 introducing minimum Community measures for the control of certain fish diseases (Annex VI). A five year contract was signed in the Framework Partnership Agreement, No. SANCO/2005 FOOD SAFETY/010- Animal Health – Fish and confirmed by Specific Agreement No. 2007/2 to the Framework Partnership Agreement, No. SANCO/2005 FOOD SAFETY/005- Animal Health – Fish Diseases. The duties mainly concern fish diseases listed as exotic diseases: Epizootic haematopoietic necrosis (EHN) and Epizootic ulcerative syndrome (EUS); and fish diseases listed as non-exotic diseases: infectious salmon anaemia (ISA), viral haemorrhagic septicaemia (VHS) infectious haematopoietic necrosis (IHN) and Koi herpes virus (KHV) disease. This report follows the format of the work programme adopted for the CRL for 2009, describing activities associated with each point and the status of ongoing projects. The list of functions and duties of the CRL follows this introduction.

The 13th annual meeting of the National Reference Laboratories for fish diseases was held back-to-back with a mini-workshop on implementation of Council Directive 2006/88/EC in May 26-28 2009, in Copenhagen, Denmark. Colleagues from NRLs in most Member States and several accession- and EFTA countries attended, either by sustaining from EU, TAIEX or on their own account. In total, 61 participants from 35 countries attended over the three-day period. There were five sessions with a total of 32 presentations, 7 of which were given by invited speakers. At the mini-workshop, participants were divided into three groups (Northern European countries, Continental European countries, Mediterranean European countries) where each NRL presented how authorization, categorization and risk management have been implemented in their country/ region. The workshop ended by a summarisation of the outcome of the group discussions. A report was submitted in August 2009.

Again this year an inter-laboratory proficiency test was distributed to the NRLs mainly within the EU but there were also participants from countries outside of EU. A report was submitted in February 2010. Most laboratories performed very well, especially in view of the fact that a new disease pathogen, epizootic hæmatopoietic necrosis virus (EHNV), was included for the first time in the test.

The performance of all CRLs in the veterinary field was evaluated by an external contractor "Veteffect" in 2009. The evaluation of the CRL for fish diseases covered all activities from our appointment in 1994 until 2009. The CRL was evaluated in the areas: fulfilment of duties and tasks, diagnosis and assistance, training, networking and quality issues. In the final report of the evaluation it was concluded that, the CRL for fish diseases have performed satisfactory in all five areas and have met all the requirements asked by the EU commission.

An important focus of the CRL was the development, implementation and validation of diagnostic tools for identification of the listed exotic and non-exotic 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 the detection of KHV.

During 2009, resources were also used to: 1. Collate data on surveillance and diagnostics in EU; 2. Identify and characterise selected virus isolates; 3. Type, store and update a library of listed virus isolates; 4. Develop, update and maintain the database containing information on fish pathogens (www.fishpathogens.eu); 5. Supply reference materials to NRLs; 6. Assess and standardise real-time tests used in diagnostics of listed fish diseases; 7. Preparation and standardisation of control reagents for PCR tests; 8. Provide training courses in laboratory diagnosis and missions to other NRLs; 9. Updating our quality assurance schemes according to our accreditation in ISO 17025 and 10. Update the CRL webpage (www.crl-fish.eu); and 11. Attending international meetings and conferences.

The permanent staff 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, 26th March 2010

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 2009 – 31 December 2009

Functions and duties of laboratories PART I Community reference laboratories

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

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

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

   (c) have an appropriate administrative infrastructure;

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

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

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

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

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

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

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

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

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

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

accordance with Article 55(1). However, the Community reference laboratory shall remain the contact point for the National reference laboratories in the Member States, and for the Commission.

5. The Community reference laboratories shall:

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

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

(ii) supplying standard sera and other reference reagents to the national reference laboratories in order to standardise the tests and reagents used in each Member State, where serological tests are required,

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

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

(b) assist actively in the diagnosis of outbreaks of the relevant disease in Member States by receiving pathogen isolates for confirmatory diagnosis, characterisation and epizootic studies;

(c) facilitate the training or retraining of experts in laboratory diagnosis with a view to harmonising diagnostic techniques throughout the Community;

(d) collaborate, as regards methods of diagnosing animal diseases falling within their areas of competence, with the competent laboratories in third countries where those diseases are prevalent;

(e) collaborate with the relevant OIE reference laboratories with regard to exotic diseases listed in Part II of Annex IV under their responsibility;

(f) collate and forward information on exotic and endemic diseases, that are potentially emerging in Community aquaculture

Work programme for 2009

TECHNICAL REPORT

1-2. Organise and prepare for the Annual Meeting for the National Reference Laboratories for Fish Diseases in 2009 and produce a report from the Meeting

Organization of the 13th Annual Meeting

In 26-28 May 2009 the 13th annual meeting of the National Reference Laboratories for fish diseases was held back-to-back with a mini-workshop on implementation of Council Directive 2006/88/EC. A total of 61 participants from 35 countries attended over the three day period. There were five sessions with a total of 32 presentations, 7 of which were given by invited speakers.

The scientific programme of the Annual Meeting was diverse and covered many topics of current interest. The meeting was opened with the traditional session on update of fish diseases in Europe, where once again participants from the member states presented new findings from their home countries. Scotland UK had experienced outbreak of ISA and presented the investigation done into this. Subsequently, a study on tracing of spread of ISAV in Norway was presented, followed by a talk on the general disease situation in Norway. Later in this session presentations about viral fish diseases from Poland, IPN eradication in Switzerland, isolation of iridovirus from Hungary, a worldwide questionnaire on KHV organised within the EPIZONE project and KHV epidemiology in Germany were given.

The session on technical issues related to sampling and diagnosis were divided into two parts. The first session focussed on diagnostic PCR setup. Here we were informed about the results of the 2008 KHV ring trial, how controls can be used in PCR analyses, application of a new assay for KHV detection and how a real-time PCR can be applied for routine detection of fish pathogens.
The last part of this section focussed on antibody based diagnosis and presentations were given on the ring trial for detection of VHSV and IHNV antibodies, ELISA tests for detection of CyHV-3 antibodies and serological methods for detection of KHV.

The last day was opened by an update session on scientific research. At this session, presentations were given on: 1) susceptible fish species to the listed diseases, 2) the database for fish pathogens, 3) molecular characterisation of VHSV and IHNV in Germany, 4) development of genotype specific monoclonal antibodies against VHSV and 5) status of the RANA-project.

The annual meeting ended with the traditional update from the CRL. The results of the proficiency test 2008 were presented. A report from a year with focus on training of laboratories and the thoughts and considerations about implementing the new listed diseases in our work was given. Furthermore, proposals on the CRL work plans for 2010 were discussed.

Minutes from the meeting were taken by Helle Frank Skall, Søren Peter Jonstrup, Britt Bang Jensen and Søren Kahns, and have afterwards been sent to presenters for correcting in order to avoid misunderstandings. The minutes are included in the report of the meeting 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 2008

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 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 5 parts: 1) General data on production, 2) Epidemiological data on diseases, 3) Laboratory data from NRLs and other laboratories, 4) Data on quality assurance in NRLs and regional laboratories, and as the final part we have for the first time included 5) A questionnaire on how categorisation of fish farms according to the Council Directive 2006/88/EC are made..

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 has risen a bit again after a decrease from 2003-2006. Data from 2008 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, 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 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 is 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.

More and more laboratories are becoming accredited according to the ISO 17025 standards.

For the first time 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 were 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 2008 is presented on Our website: http://www.crl-fish.eu/

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

Identification and characterisation of selected virus isolates

Again in 2009 a significant number of virus isolates were received for further characterisation at the CRL and for storing in our virus library:

Member States/ Countries outside EU		
Material received	**Laboratories**	**Units**
Diagnostic material	5	56 samples
Virus isolate	4	17 samples
Other material	7	65 specimens

Table 1: Material received at the CRL from laboratories in Member States and outside EU in 2009

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:

- **Fisheries and Oceans Molecular biology, Canada** *(Nellie Gagné)*: 4 samples of VHSV Type IVb for inclusion in our collection and for further characterisation. (DTU-Vet. 2009-50-122).
- **University of Prince Edwards Island, Canada** *(Frederick Kibenge)*: ISA virus isolates for identification and characterisation in an inter-laboratory proficiency test (DTU-Vet. 2009-50-123)
- **AFSSA Brest, France** *(Jeannette Castric)***:** Reagents (carp and rabbit sera + KHV isolate) for development of KHV antibody detection test by immunofluorescence (DTU-Vet. 2009-50-082)
- **Teheran University, Iran** (M. Noorozi): Virological examination of 10 virus isolates from rainbow trout (Suspicion of IPN, VHS and IHN). IPNV was identified in all samples, no VHS or IHN. (DTU-Vet. 2009-50-048)
- **IZSVe, Padova, Italy** *(Giuseppe Bovo)*: Rabbit anti Catfish iridovirus Italy (DTU-Vet. 2009-50-349), and virological examination and characterisation of 6 virus isolates. IHNV and VHSV were identified (DTU-Vet. 2009-50-085).
- **IZSVe, Padova, Italy** *(Amadeo Manfrin)*: Bacterial isolates for inter-laboratory proficiency testing (DTU-Vet. 2009-50-58).
- **National Veterinary Institute, Oslo, Norway** *(Ingebjørt Modahl)* **:** 2 Pancreas Disease virus isolate for co-cultivation studies (DTU-Vet. 2009-50-133). 1 MAb against pancreas disease virus (PDV) MAb 4H1 (DTU-Vet. 2009-50-162).
- **National Veterinary Research Institute, Pulawi, Poland** *(Marek Matras)* : 13 purified DNA for KHV diagnosis, all negative (DTU-Vet. 2009-50-318), 2 isolates for virological examination, VHSV identified (DTU-Vet. 2009-50-087), 22 purified DNA, KHV identified in 4 samples, (DTU-Vet. 2009-50-290), 2 samples for virological examination, VHSV identified (DTU-Vet. 2009-50-094).
- **State Veterinary Institute, Slovakia** *(Miroslava Vankusova)*: Virological examination of 1 virus isolate CP: 2131/53, virus not detected. (DTU-Vet. 2009-50-049).
- **Statens Vet.med Anstalt, SVA, Uppsala, Sweden (***Anders Hellström***):** Virological examination of 3 isolates IPNV and VHSV not identified, (DTU-Vet. 2009-50-168 - 172)
- **Veterinary Control and Research Ins., Bornova, Turkey** *(Necdet Akkoca)***:** Virological examination of the following samples for identification and further characterisation: 2 virus isolates for virological

examination (DTU-Vet.2009-50-315), 6 isolates for virological examination, IPNV was detected, (DTU-Vet. 2009-50-251), virological examination of 3 isolates, VHSV was detected, (DTU-Vet. 2009-50-252), virological examination of 4 isolates, IPNV was detected (DTU-Vet. 2009-50- 253).

- **Marine Laboratory Aberdeen, UK-Scotland** *(Alison Mclntosh)*: Kidney supernatant for inter-laboratory proficiency testing for the presence of *Renibacterium salmoninarum* by ELISA (DTU-Vet.2009-50-224).
- **Western Fisheries Research, Biological Resources Division, Seattle, USA** *(Jim R. Winton):* 9 VHSV isolate for inclusion in our VHSV library, (DTU-Vet. 2009-50-013).

5. Production of antisera against selected isolates if necessary

Production of antisera.

Sufficient stocks of poly- and monoclonal antibodies against the listed viral fish pathogens and also against non-listed viruses as SVCV and perch rhabdovirus are available at the CRL.

Rabbit antisera against PFRV and antisera neutralising all VHSV isolates were produced in 2009.

Monoclonal antibodies for discrimination between VHSV genotypes have been developed by Dr. Takafumi Ito from Japan. During his post-doc at the CRL these Mabs were assessed towards large panels of VHSV and heterologous isolates. Based on these result the production of MAbs specifically reacting against VHSV type IVb were produced at the CRL.*(ref: Ito et al* (2009)).

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

A novel diagnostic real-time RT-PCR assay was designed by the CRL with the ability to detect and quantify all known genotypes and subtypes of VHSV. This was done because assays previously designed by the CRL or assays designed by other laboratories failed to recognise all VHSV genotypes. Preliminary testing of the novel assay shows that it is able to detect isolates of each known genotype and subtype without cross reacting with related vira. Finishing and publication of validation of the assay is planned to occur in 2010. A VHSV diagnostic real-time PCR will be much faster than the conventional cell-based diagnostic assay. It also holds the potential to be more sensitive and less vulnerable to contaminations than a conventional RT-PCR assay.

The CRL initiated the implementation of Real-Time PCRs for detection of ISAV, KHV and IHNV. For ISAV, the CRL participated in the ISAV proficiency test provided by the Canadian OIE Reference laboratory for ISA. This proficiency test aimed at comparing different real time PCRs as tools for ISAV detection. The report of this proficiency test will influence on what real time PCR will be implemented for ISAV detection.

For KHV detection, the real time PCR described by Gilad et al. 2004 has proven to be one of the most sensitive tools for KHV detection and especially for survey it is the best tool. This real-time PCR is currently in the process of being validated.

For IHNV some real time PCRs have been tested. However, the assays did not go as expected and more real-time assays will be tested.

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

Develop, update and maintain the new Fish Pathogens Database

We are currently developing a database for storing information on fish pathogens. In 2009 the VHSV database was completed and launched for public access and so far the database contains information of 322 isolates (Jonstrup et al. 2009). In 2009, an expansion of the database with IHNV and SVCV started, IHNV database will be publicly available in the spring of 2010. (www.fishpathogens.eu). The database includes information on relevant publications for a specific isolate, sequence information, geographical information as well as several other features. http://www.fishpathogens.eu

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)

Virus library

Several isolates of VHSV and IHNV and other relevant pathogens (like IPNV. PDV) were received and stored in our library during 2009 (listed in annex 2). Our library is continuously updated and maintained.

9. Preparation and standardisation of control reagents for use in PCR tests. Assessment of viral inactivation

Preparation and standardisation of control reagents for use in PCR tests. Assessment of viral inactivation

The CRL performed viral inactivation experiments using different chemical compounds. These studies showed that the RNASafer Stabilizer Reagent from Omega Biotech: http://www.omegabiotek.com/files/resource/Handbook/70194600.pdf) is able to inactivate the virus. As both RNA and DNA can be extracted from this reagent, RNASafer provides a simple way of storing and distributing control reagents for PCR tests. Furthermore, the CRL implemented a technique for co-measurement of the levels of endogenous fish genes in real time assays, in order to verify that the purification efficiency of the nucleic acids from the sample has been satisfactory.
In conventional PCR assays the CRL implemented the use of DNA-mimics spiked to the samples. These DNA-mimics are purified with the DNA from the sample and are used to control that the overall efficiency of the purification process was acceptable.

10. Organise a workshop on the implementation of Council Directive 2006/88/EC (to be organised back to back with the 13th Annual Meeting).

Organise a workshop on the implementation of Council Directive 2006/88/EC

A mini-workshop on the implementation of Council Directive 2006/88/EC was held right after session two of the Annual Meeting. The workshop started with four presentations dealing with key issues to be addressed when implementing the Directive, on sampling and diagnostic plans for the listed diseases, on how to risk rank fish farms and finally on how fish farms have been categorised in EU according to the answers given in the Annual S&D questionnaire. Subsequently, participants were divided into three groups: 1) participants from Northern Europe, 2) participants from Continental Europe and 3) participants from Mediterranean European countries. Within these groups, participants from each country presented how the implementation process had progressed in their country. At the end of the mini-workshop all participants were gathered together and a summary from the three group discussions were given.

11. Update the webpage of the CRL.

Update the webpage of the CRL.

The website was introduced at the 12th Annual Meeting, in June 2008 and is

currently being updated.
The CRL website (www.crl-fish.eu) is a notice board, where NRL´s and other interested parties can access relevant information and previous reports concerning the activities coordinated by the CRL and relevant upcoming events in the Community.
Reports of the CRL, e.g. of the proficiency test and the Annual Meeting of the NRLs, mission reports etc. are launched at the web page immediately after release.

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

Materials supplied by the CRL

On request, the CRL supplied material to other laboratories in Member States and third countries to aid in the diagnosis and characterisation of fish diseases. The number of laboratories receiving the specific material and the number of units supplied by the CRL are listed in table 2.

Further details of the materials are listed in Annex 3

Table 2: The CRL supplied the following reagents in 2009

Material	Laboratories	Units
Cell cultures	13	57 flasks
Polyclonal antisera	3	7 vials
Monoclonal antibody	2	4 vials
Virus isolates	13	137 vials
Virus in RNASafer or extracted RNA	4	15 vials
Other material	2	2 vials

13. Prepare the Annual Inter-laboratory Proficiency Test year 2009 for the National Reference Laboratories.

The inter-laboratory Proficiency Test 2009

A comparative test of diagnostic procedures was provided by the CRL to 36 NRLs in the first days of September 2009.
The test contained five coded ampoules. Four contained VHSV genotype Ie and IVa, IHNV genogroup L and 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.
In addition the 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. We recommended the participants to follow the procedures described in Chapter 2.3.1 in the 2009 issue of the OIE Manual of Diagnostic Tests for Aquatic Animals.

Laboratories were encouraged to identify VHSV and IHNV isolates by means of genotyping in addition to the standard methods. It was recommended to use the genotype notification described in 2 publications (Einer-Jensen et al. 2004 for VHSV and Kurath et al. 2003 for IHNV). Laboratories were encouraged to submit all the sequencing results that were used for genotyping the isolates.

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 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 a filled scheme electronically or on paper. Furthermore, participants were asked to fill an extended questionnaire in order to obtain more information on the methodology used by the laboratories. Participants were asked to reply latest 13 November 2009

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

Outcome of Inter-laboratory Proficiency Test 2009

Participation

36 laboratories received the annual proficiency test, 34 participants submitted results within the deadline. One participant submitted results 7 days after deadline but before the content of the ampoules were made public available. One participant did not submit results.

Identification of content

- 24 laboratories correctly identified all viruses in all ampoules.

- Ampoule I – EHNV
  - 28 laboratories correctly identified EHNV.
  - 3 laboratories identified ranavirus.
  - 2 laboratories found more isolates than were present.
  - 1 laboratory did not find any virus.
  - 1 laboratory found virus but did not identify it.

- Ampoule II - IHNV
  - 34 laboratories correctly identified IHNV.
  - 1 laboratory did not find any virus.

- Ampoule III - VHSV
  - 35 laboratories correctly identified VHSV.

- Ampoule IV – VHSV
  - 35 laboratories correctly identified VHSV.

- Ampoule V – No virus
  - 30 laboratories correctly identified that there was no virus.
  - 5 laboratories identified a virus.

Starting with proficiency test 2003, a scoring system for the identification part of the proficiency test was provided. In the 2009 proficiency test, a score of 2 for each correct answer was assigned, giving the possibility for obtaining a maximum score of 10. Identification of “ranavirus” as the virus in Ampoule 1 was given the score of 1. If no sequence analysis was performed, the participant would not be able to identify the ranavirus as EHNV and would be given the score of 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.
24 laboratories out of 36 correctly identified all viruses in all ampoules and obtained maximum score. Three laboratories scored 9 because the

virus in ampoule I was identified 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. Serotyping, 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.

Methods applied

The following cell lines were used by the participants:

- 29 laboratories used BF-2 cells
- 34 laboratories used EPC cells
- 12 laboratories used RTG-2 cells
- 10 laboratories used FHM cells
- 5 laboratories used four cell lines
- 5 laboratories used tree cell lines
  - 3 laboratories used BF-2, EPC and FHM
  - 3 laboratories used BF-2, EPC and RTG-2
- 23 laboratories used two cell lines:
  - 17 laboratories used BF-2 cells in combination with EPC cells
  - 4 laboratories used RTG-2 cells in combination with EPC cells
  - 1 laboratory used BF-2 cells in combination with FHM cells
  - 1 laboratory used EPC cells in combination with FHM cells
- 1 laboratory used only EPC cells

Methods used for identification of viruses

- 24 laboratories used ELISA for identification of viruses.
- 21 laboratories used IFAT for identification of viruses.
  11 laboratories used neutralisation tests for identification of viruses.
- 34 laboratories used PCR for identification of viruses.
- 30 laboratories performed sequencing for identification of viruses.

Genotypning and sequencing

- Ampoule I - EHNV
  - 30 laboratories sequenced to identify EHNV.
  - 3 laboratories performed only PCR and no sequence analyses.
  - 1 laboratory did not identify the virus
  - No laboratory reported having performed RFLP analyses.

  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. 30 laboratories used sequencing for identification of EHNV whereas none used RFLP. Of these, 29 used sequencing of the partial MCP gene as recommended by the OIE diagnostic manual for EHN whereas two participants sequenced the polymerase gene as described by Holopainen et al. 2009. Three laboratories identified the virus by PCR but did not perform sequencing analyses.

- Ampoule II-IV
  25 out of 35 laboratories sequenced parts of the genome of either IHNV or VHSV isolates. This is five laboratories less than did sequencing of the EHNV. Both full length and partial N- and G-genes were used for virus/genotype identification.

- Ampoule II - IHNV Genotype L
  - 21 laboratories performed sequencing
  - 11 laboratories genotyped the IHNV isolate as belonging to genogroup L
  - 6 laboratories used alternative genotyping notification or showed blast results
  - 4 laboratories did not give any genotype of the sequences

  11 laboratories genotyped the IHNV isolate as belonging to genogroup L as described in Kurath et al. 2003 (Table 10). This is a more laboratories compared to genotyping IHNV in proficiency test 2008. One laboratory genotyped the isolate according to another publication. Furthermore, five laboratories showed indirect isolate relatedness to genogroup L either by presenting a phylogenetic tree or by showing blast results.

- Ampoule III - VHSV genotype Ie
  - 21 laboratories performed sequencing
  - 14 laboratories identified the VHSV isolate as genotype I
  - 6 laboratories subtyped the isolates as a genotype Ie
  - 2 laboratories subtyped the isolates as a genotype Ib
  - 1 laboratory identified the VHSV isolate as genotype III
  - 4 laboratories showed blast results
  - 2 laboratories did not give any genotype of the sequences

  14 laboratories correctly identified the isolate as belonging to genotype I. 6 laboratories correctly subtyped the isolate as belonging to the Ie subgroup (according to Einer-Jensen et al 2004) whereas two identified it as a genotype Ib. Ib genotype might come up because laboratories uses different genes and if a certain gene is not sequenced for genotype Ie isolates, a blast result will show a related subgenotype. One laboratory identified the isolate as belonging to genotype III. This could be due to the fact that the genotype notification used was according to the method described by Nishizawa et al. 2002, which is different from the generally accepted typing system described by Einer-Jensen et al. and Snow et al.. Four laboratories indicated the genotype of the isolate by showing their blast result.

- Ampoule IV - VHSV genotype IVa
  - 20 laboratories performed sequencing
  - 12 laboratories identified the VHSV isolate as genotype IV
  - 10 laboratories subtyped the isolates as a genotype IVa
  - 1 laboratory identified the VHSV isolate as genotype I
  - 1 laboratory identified the VHSV isolate as genotype Ie
  - 1 laboratory identified the VHSV isolate as a mixture of genotype IVa and III
  - 3 laboratories showed blast results
  - 2 laboratories did not give any genotype of the sequences

12 laboratories correctly identified the isolate as belonging to genotype IV and 10 laboratories correctly subtyped the isolate as belonging to the IVa subgroup. One laboratory identified the isolate as belonging to genotype I which might be due to the same reason using an other typing system than the generally accepted as described above . One laboratory identified the isolate as genotype Ie with identical sequence to the sequence of the isolate in ampoule III and therefore double sequencing of VHSV in ampoule III might have occurred. Four

laboratories indicated the genotype of the isolate by showing blast result.

Concluding remarks

In 2009 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.

The IHNV within this test replicates well on EPC and FHM cells, and less efficiently on BF-2 and RTG-2 cells making all the valid combinations of cell in Commission Decision 2001/183/EC suitable. EHNV replicates well on EPC and BF-2 cells whereas lower titres were observed on RTG-2 cells and FHM cells. Therefore the combination of RTG-2 and FHM cells seems less suitable.
It appears that the two VHSV isolates in this test replicates equally well on BF-2, FHM and EPC cells but less efficient on RTG-2 cells. This is valid for the two VHSV isolates included in this proficiency test but other VHSV isolates prefer BF-2 cells compared to EPC cells and therefore laboratories are still encouraged to use a combination of cells as described in Commission Decision 2001/183/EC. 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, we recommend that laboratories use BF-2 cells and not RTG-2 cells for replication/survey of/for VHSV.
In conclusion we recommend all participants to evaluate the sensitivity of the cells used in their laboratory in relation to the diagnostic purpose.

The CRL 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. This year however, 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 will be further presented and discussed at the 14th Annual Meeting of National Reference Laboratories for Fish Diseases to be held 26-28 May 2009 in Århus, Denmark.

The full report is in Annex 4.

15. Facilitate and provide training in laboratory diagnosis.

Training, missions and scientific collaboration

The following colleagues visited the institute during 2009 for scientific meetings, project collaboration or training:

Tanya Gray (Symantix Ltd, United Kingdom)	22-23. January
Juan Pablo López Classing, Chile	29. January
Training course in molecular diagnosis of Fish Diseases Valentina Panzarin (Italy), Rasvan Motiu (Rumania), Ewa Borzym (Poland), Marine Baud (France).	20-30. April
Eduardo Castro Nallar, Jefe de Laboratorio Diagnotec S.A. Chile	29 May – 7th July 2009

Mohaddes Ghasemi and Somayeh Haghighi Karesidani, Teheran university, Iran. Trainning on diagnosis and characterisation	7 June – 27th September
Dr. Harry Bohle, ADL Diagnostics Chile, project on "using siRNAs for targeting VHS virus in vitro"	August to September 2009
Ph.D. student Seyed Amir Hossien Jalali Hajiabadi, Teheran University in Iran. Engaged in a subproject at DTU-VET Århus on "microRNA and immunology of VHSV infected fish"	August 2009 to April 2010.
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"	November 1st 2009 to October 31st 2012.
Patricia Schiappacasse and F. Victor Agurto Diaz; Departamento Acuicultura; Servicio Nacional de Pesca, Chile, Dorthe Bak Andersen And Jette Skiffard og Henrik Korsholm, FVM, Denmark	11th November
Dr Gael Kurath, Western Fisheries Research Center, Seattle, WA, USA in connection to ongoing research collaboration	6th-8th June

Master and 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 is **Katja Einer-Jensen.**

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**; Co-supervisor: **Brian Dall Schyth**

Lasse Bøgelund Juel Kristensen has been enrolled as Master student at Aarhus University in association to ongoing siRNA research activities. Supervisor is **Brian Dall Schyth**.

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

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.

International meetings and conferences attended.

Meetings and Conferences

Contact with colleagues from other laboratories is a channel for exchange of information in the field of fish diseases, and an opportunity to keep abreast with new developments in the field. Of special interest are of course the activities relating to VHS, IHN, KHV, ISA, EHN and EUS. Scientists at the CRL participated in the following activities in 2009:

Participation and presentations at international conferences and meetings

Jonstrup S.P. Epizone:: Database user forum. Presentation title: Fish Pathogens Database. January 27-28, 2009, Hannover, Germany.

Ito T., Kurita J., Sano M., Iida T., ***Skall H.F., Lorenzen N. & Olesen N.J.*** (2009) O-114: Distinction between genotypes of viral haemorrhagic septicaemia virus (VHSV) using monoclonal antibodies. 14th International Conference of the EAFP on Diseases of Fish and Shellfish 130

Jonstrup S.P*., Gray T.,* ***Kahns S., Skall H.F****., Snow M. &* ***Olesen N.J.*** (2009) P-003: Fishpathogens.eu: a user friendly database for fish pathogens. 14th International Conference of the EAFP on Diseases of Fish and Shellfish 187

Kahns S., Nicolajsen N., Skall H.F. & Olesen N.J. (2009) P-121: Inter-laboratory proficiency tests to detect viral fish diseases. 14th International Conference of the EAFP on Diseases of Fish and Shellfish 309

Kahns S., Jonstrup S.P., Jensen B.B., Skall H.F. & Olesen N.J. (2009) Phylogenetic characterisation of European fresh water viral haemorrhagic septcaemia virus (VHSV) isolates. Presented at: 15th International Bioinformatics Workshop on Virus Evolution and Molecular Epidemiology. Rotterdam, Holland

Type: Conference contribution, Poster presentation.

Kahns S., Skall H.F. EPIZONE meeting on Molecular detection and diagnosis of KHV, November 12-13 2009, Lelystad, The Netherlands

Olesen, N.J., Skall H.F., *Mellergaard S., Korsholm H. & Håstein T.* (2009) Contingency plans for the control and eradication of diseases in aquaculture. International Aquaculture

Biosecurity Conference Proceedings, p. 28-29 Type: Conference contribution, Conference abstract.

Schyth, B.D*., Bramsen, J.B., Kjems, J., Wengel, J. &* ***Lorenzen, N****.* "In vivo screening of chemically modified RNA duplexes for their ability to induce innate immune responses" Poster at the: *RNAi2009 International Conference – ncRNA: Bridging Biology and Therapy*, Oxford, England, March 2009.

Kristensen, L.B.J., ***Schyth, B.D., Lorenzen N.*** *& Pedersen, F.S.* "MicroRNA Expression during Viral Infection or PolyI:C Stimulation in a Fish Model". Poster presented at: *MicroRNA DK 2009*. The Biotech Research & Innovation Centre (BIRC), Copenhagen, 2009.

Schyth, B.D*., Bramsen, J.B., Kjems, J., Wengel, J. &* ***Lorenzen, N****.* "Screening of Modified RNA duplexes". Poster presented at the *RNA interference Summit*. San Francisco, 2009

Lorenzen E., Einer-Jensen K., Rasmussen J. S., *Collet B., Secombes C.J.,* ***Lorenzen N.*** (2009) The protective mechanisms induced by a fish rhabdovirus DNA vaccine depend on temperature. 11th Congress of the International Society of Developmental and Comparative Immunology, June 28 – July 4, Prague, Czech Republic

Lorenzen E., Einer-Jensen K., Rasmussen J.S., Lorenzen N.*, Ellis T.. McLauchlan P., Evensen, Ø.* (2009) Interference of an ERM-vaccine with a VHS-DNA vaccine in rainbow trout. The ontogeny of the fish immune system. University of Copenhagen, Faculty of Life Sciences

Lorenzen E., Kjær T.E., Lorenzen N. (2009) Studies on herd-immunity and primary versus secondary infection of VHSV in challenge and vaccination trials with rainbow trout. Sustainable Control of Fish Diseases in Aquaculture & Danish Fish Immunology Research Network. University of Copenhagen, 2009

Einer-Jensen K, Lorenzen N (2009) Scientific progress and coordination of IMAQUANIM by P1 during the IMAQUANIM progress meeting, April 1-3 2009, Padua, Italy

Lorenzen N., Lorenzen E., Einer-Jensen K., Rasmussen J.S., Kjær T.E*., Vesely, T.* (2009) Functional demonstration of adaptive immunity in zebrafish using DNA vaccination. Scofda and Dafinet Symposium. University of Copenhagen, Faculty of Life Sciences

Schyth B.D, *Bramsen J.B., Kjems J., Wengel J.,* ***Lorenzen, N.*** (2009) *In vivo* screening of chemical modifications of sirnas for effect on the innate immune response in fish. 11th Congress of the International Society of Developmental

and Comparative Immunology, June 28 – July 4, Prague, Czech Republic
***Schyth, B.D.**, Bramsen, J.B., Kjems, J., Wengel, J. &* ***Lorenzen, N**.* (2009) "In vivo screening of chemically modified RNA duplexes for their ability to induce innate immune responses" Poster at the: *RNAi2009 International Conference – ncRNA: Bridging Biology and Therapy,* Oxford, England, March 2009.
Schyth, B.D., Kristensen, L.B.J. & Lorenzen N. (2009) **"**MicroRNA regulation in VHS virus infected rainbow trout". Oral presentation at Hjalet symposium for virologist at DTU-VET.
Stegmann, A., Einer-Jensen K. Pathogenicity markers in VHSV (2009) Meeting for viologists at VET-DTU, October 6-7, Hjalet, Vordingborg, Denmark
Jensen, Ann Britt Bang; Olesen, Niels Jørgen; Ersbøll, A. K. (2009) O-021: A retrospective cluster-analysis of the occurrence of viral haemorrhagic septicaemia in Denmark. Presented at: 14th EAFP International Conference on Diseases of Fish and Shellfish. Prague, Czech Republic, 2009, p. 29
Haenen, Olga; ***Olesen, Niels Jørgen*** (**2009**) P-001: Koi herpes virus world wide: results of the global KHV questionnaire 2007-2009. Presented at: 14th EAFP International Conference on Diseases of Fish and Shellfish. Prague, Czech Republic. p. 185
Olesen, Niels Jørgen*; Castric, Jeanette* (**2009**) Poster: Detection of antibodies against VHSV and IHNV in rainbow trout. Presented at: Annual Meeting EPIZONE. Antalya, Turkey, 2009

Scientific publications in peer-reviewed journals

Jonstrup S.P.*, Gray T****., Kahns S., Skall H. F****, Snow M. &* ***Olesen N. J****.*, 2009, 'FishPathogens.eu/vhsv: a user-friendly viral haemorrhagic septicaemia virus isolate and sequence database' Journal of Fish Diseases vol 32 nr. 11, s. 925-929
Ariel E., Skall H.F. & Olesen N.J. (2009) Susceptibility testing of fish cell lines for virus isolation. *Aquaculture* **298**, 125-130.

Dale O.B., Ørpetveit I., Lyngstad T.M., ***Kahns S., Skall H.F., Olesen N.J.*** *& Dannevig B.H.* (2009) Outbreak of viral haemorrhagic septicaemia (VHS) in seawater-farmed rainbow trout in Norway caused by VHS virus Genotype III. *Diseases of Aquatic Organisms* **85**, 93-103.

Fregeneda-Grandes J.M., ***Skall H.F. & Olesen N.J.*** (2009) Antibody response of rainbow trout with single or double infections involving viral haemorrhagic septicaemia virus and infectious haematopoietic necrosis virus. *Diseases of Aquatic Organisms* **83**, 23-29.

Pedersen K., ***Skall H.F.,*** *Lassen-Nielsen A.M, Friis-Holm L.B. &* ***Olesen N.J.*** (2009) Photobacterium damselae subsp damselae, an emerging pathogen in Danish rainbow trout, Oncorhynchus mykiss (Walbaum), mariculture. *Journal of Fish Diseases* **32**, 465-472.

S. *Ruiz,* ***B.D. Schyth****, P. Encinas, C. Tafalla, A. Estepa,* ***N. Lorenzen*** *and J.M.* Coll. 2009. New tools to study RNA interference to fish viruses: Fish cell lines permanently expressing siRNAs targeting the viral polymerase of viral hemorrhagic septicemia virus. *Antiviral Research*, 82(3): Pages 148-156.

Campbell S., Collet B., ***Einer-Jensen K.****, Secombes C.J., Snow M.* (2009) Identifying potential virulence determinants in viral haemorrhagic septicaemia virus (VHSV) for rainbow trout. *Diseases of Aquatic Organisms* 86: 205–212

Einer-Jensen K*, Delgado L, Lorenzen E, Bovo G, Evensen O, Lapatra S, Lorenzen N.* (2009) Dual DNA vaccination of rainbow trout (Oncorhynchus mykiss) against two different rhabdoviruses, VHSV and IHNV, induces specific

divalent protection. *Vaccine* 27:1248–1253

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

Lorenzen E., ***Einer-Jensen K.***, *Rasmussen J.S., Kjær T.E., Collet B., Secombes C.J., Lorenzen N.* (2009) The protective mechanisms induced by a fish rhabdovirus DNA vaccine depend on temperature *Vaccine* 27: 3870–3880

Lorenzen N., Lorenzen E., Rasmussen J.S., ***Einer-Jensen K.*** (2009). DNA Vaccines against Viral Diseases. Basic Immunological Aspects and Applied Perspectives. *Fish Pathology* 44(1):16-18

Ruiz S., ***Schyth B.D.***, *Encinas P., Tafalla C., Estepa A.*, ***Lorenzen N.***, *Coll J.M.* (2009) New tools to study RNA interference to fish viruses: Fish cell lines permanently expressing siRNAs targeting the viral polymerase of viral hemorrhagic septicemia virus. Antiviral Res. Jun;82(3):148-56. Epub 2009 Mar 9.

VHSV Expert Panel and Working Group[1]* (**2009**) 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. In Preventive Veterinary Medicine.

Tove Johansson and ***Niels Jørgen Olesen*** (**2009**) Detection of infectious pancreatic necrosis virus from rainbow trout (oncorhynchus mykiss) using the macrophage lysis method. Journal of Fish Diseases, vol: 32(6), p. 563-566.

Other communications

Olesen, Niels Jørgen; Skall, Helle Frank ; *Mellergaard, Stig; Korsholm, Henrik; Håstein, Tor*e (**2009**) Contingency plans for the control and eradication of diseases in aquaculture..In: International aquaculture biosecurity conference : Proceedings, p. 28-29

Skall H.F. & Olesen N.J. (**2009)** Nyt fra Veterinærinstituttet. VHS er en trussel for havbrug - særlig om vinteren. *Dansk Veterinærtidsskrift* **92**, 28

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

The group is partner and project coordinator of EU project RANA: Risk assessment of new and emerging systemic iridoviral diseases for European fish and aquatic ecosystems. Proposal/Contract no.: 6459 (http://ranavirus.net/). The project ended in 2009.

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*). The WP consist of the following tasks: 1 Generation of quantitative data on VHS and IHN and implementation of GIS

2 Establishment of the fundaments for molecular epidemiology. This is achieved in collaboration with the CRL fish by establishing the database

www.fispathogens.eu with all relevant information of a large and representative number of virus isolates. 3: Emerging diseases with focus on the identification and characterisation of Koi herpes virus and on the epidemiology of the disease. And finally 4: serology with focus on trout antibodies against VHSV and IHNV.

Within the EPIZONE project the group succesfully applied for an internal call with a proposal named: **Development of serological methods for detection of Koi herpes virus (KHV) antibodies in carp, *Cyprinus carpio*.** The project aim at developing and validate neutralisation tests, ELISA, and immunofluorescence tests for the detection af anti KHV antibodies in carp, and have a duration of 18 months.

A 3-year national research project supported by the Danish Ministry of Food, Agriculture and Fisheries (FØTEK-4 programme), coordinated by the Section for Fish Diseases at DTU-Veterinary Institute and including collaboration with the Danish Fish Farmers Association (Danish Aquaculture), entitled "**Field testing of a DNA vaccine for farmed fish**" has been finalised. Despite variability and limitations in the small scale experimental setup, the overall results indicated that DNA vaccination against VHS in rainbow trout can induce protective immunity against the viral disease under field conditions. No negative side effects on the vaccinated fish were observed and no transfer of vaccine to the environment was detected. The next step towards implementation of DNA vaccines in European Aquaculture should include a full scale clinical testing. This will imply that the vaccinated fish must be allowed to reach the food chain and since no DNA vaccines have been licensed for husbandry animals in Europe yet, this step will require initial acceptance by the food safety authorities. Although a sceptical public opinion against use of gene-modified elements in food production exists in some countries, this should be achievable since all experiments with DNA vaccines in both animals and humans have so far supported the view that the risk of negative side effects is very small, also when compared to those observed for traditional vaccines.

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

A 1.5 year project on EUS: **Epizootic ulcerative syndrome. Development and implementation of diagnostic methods**. The aim of this project is to introduce the best confirmatory methods for EUS. Participants will 1) try out the described EUS methods (fungus isolation, histopathology, PCR) and choose best methods for standardisation and SOPs, 2) develop if needed alternative methods (e.g. other agars for isolation, immunohistochemistry, real-time PCR), and validate and implement them, 3) establish an electronically available slide collection for EUS histopathology and 4) write SOPs for the best diagnostic tests for EUS based on project findings. The project is a club 5 joint project between the coordinator CVI, Netherlands, DTU.Vet., Denmark and SVA, Sweden

A 2+2 year project on ISAV: **Tracing viral disease dissemination in aquaculture: an interdisciplinary approach between molecular virology and dispersal modelling.** The project proposal suggests an approach of molecular virology and stochastic disease modelling to disentangle major transmission

pathways of infectious salmon anaemia virus, and molecular characteristics of virus traits that may affect the probability of disease outbreaks. The project is coordinated by the National Veterinary Institute (NVI), in Oslo, Norway. Other participants are Norwegian Computing Center (NR), Norway, Food, Veterinary and Environmental Agency, FVEA of the Faroe Islands and Norwegian School of Veterinary Sciences, Norway

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 3 year project: Project 190245/S40: **Viral Haemorrhagic septicaemia virus (VHSV) in wild and farmed fish in Norway**, funded by the Norwegian Research Council. The primary objective of the project is to gain knowledge on the prevalence of VHSV in wild and farmed fish species and possible transmission of virus between different fish populations. Secondary objectives: 1. Screening and genotyping of coastal wild fish populations for VHSV with emphasis on fish species shown to be susceptible for VHSV infection. Material will be stored for possible use in future screening also of other infectious agents. 2. Identification of possible genetic virulence markers for VHS. 3. Experimental studies of the pathogenicity of different VHSV strains for various fish species 4. Establish a GIS-based database. 5. Develop a risk-based surveillance system for VHVS in wild and farmed fish populations. The section is primarily involved in objective 3, though help and advice is also provided for the other sub goals.

A new 4-year national collaborative research project "**Co-evolutionary genomics of fish resistance and virulence in an epidemic virus**" has been initiated based on funding from the Danish Research Council. The project aims at 1) identification of genotypes and immunological mechanisms of the trout that confer resistance to diseaseforming variants of VHS, and 2) establish how a subset of susceptible fish allow initially harmless variants of marine VHSV (quasi-species) to become invasive and disease-forming in sea-cage production of rainbow trout. This is an important issue because sea-cage production of rainbow trout represents the greatest growth potential for Danish aquaculture. However, the mere existence of the industry is threatened by emergent outbreaks of disease from VHS variants present in the marine environment. This project will make rainbow trout used in seacage production in Denmark more resilient to disease-forming variants of VHS and reduce the risk of emergent outbreaks of disease.

A new 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.

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

Co-evolutionary genomics of fish resistance and virulence in an epidemic virus. This project seeks a solution to a problem for the expansion of Danish trout farming into the marine environment. Viral haemorrhagic septicemia (VHS) is a viral disease that causes outbreaks with up to 90% mortality in rainbow trout, and the virus is commonly found in wild populations of fish in the coastal waters. We will identify the process of adaptation to the fish host that makes the virus capable of causing epidemic outbreaks in rainbow trout and use the trouts own genetic variants in combination with targeted vaccine development to cope with this adjustment. We can achieve this through a combination of novel technologies that combine genotypning of genetic markers in coding DNA (SNP markers) and regulatory gene sequences (miRNA) with vaccination and infection experiments where we measure gene activity throughout the genome and gene activity in immunological key components. This gives us a unique level of insight into the mechanisms that provide resistance against the virus and effective protection from the vaccine. It is possible to combine these technologies because we have established collaboration between institutions, which have experience in vaccine development, infection experiments, genomic and genetic analysis. Besides the National Veterinary Institute department in Århus those are Aarhus University, University of Victoria in Canada and the University of Washington, USA.

Delivery of small interfering RNAs (siRNAs) for treatment of viral disease in fish aquaculture. The aim of this study is to establish novel delivery strategies for small interfering RNAs including viral and non viral 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 will be used as they provide easy assays for evaluating on gene knockdown efficiency by siRNAs.

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

Beside our function as the CRL for Fish Diseases we are appointed as the **OIE reference laboratory for VHS**. In this function we are actively involved in the development of the OIE Diagnostic Manual for Aquatic Animals, especially on the VHS Chapter and in the corresponding Code. Beside we receive a number of requests for reagents, training and consultancy from countries outside EU.

Report on the 13th Annual Meeting of the National Reference Laboratories for Fish Diseases

Copenhagen, Denmark
May 26-28, 2009

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

Contents

Introduction and short summary

In 26-28 May 2009 the 13th annual meeting of the National Reference Laboratories for fish diseases was held back-to-back with a mini-workshop on implementation of Council Directive 2006/88/EC. A total of 61 participants from 35 countries attended over the three day period. There were five sessions with a total of 32 presentations, 7 of which were given by invited speakers.

The scientific programme of the Annual Meeting was diverse and covered many topics of current interest. The meeting was opened with the traditional session on update of fish diseases in Europe, where once again participants from the member states presented new findings from their home countries. Scotland UK had experienced outbreak of ISA and presented the investigation done into this. Subsequently, a study on tracing of spread of ISAV in Norway was presented, followed by a talk on the general disease situation in Norway. Later in this session presentations about viral fish diseases from Poland, IPN eradication in Switzerland, isolation of iridovirus from Hungary, a worldwide questionnaire on KHV organised within the EPIZONE project, and KHV epidemiology in Germany were given.

The session on technical issues related to sampling and diagnosis were divided into two parts. The first session focussed on diagnostic PCR setup. Here we were informed about the results of the 2008 KHV ring trial, how controls can be used in PCR analyses, application of a new assay for KHV detection and how a real-time PCR can be applied for routine detection of fish pathogens.
The last part of this section focussed on antibody based diagnosis and presentations were given on the ring trial for detection of VHSV and IHNV antibodies, ELISA tests for detection of CyHV-3 antibodies and serological methods for detection of KHV.

A mini-workshop on the implementation of Council Directive 2006/88/EC was held right after session two of the Annual Meeting. The workshop started with four presentations dealing with key issues to be addressed when implementing the Directive, on sampling and diagnostic plans for the listed diseases, on how to risk rank fish farms and finally on how fish farms have been categorised in EU according to the answers given in the Annual S&D questionnaire. Subsequently, participants were divided into three groups: 1) participants from Northern Europe, 2) participants from Continental Europe and 3) participants from Mediterranean European countries. Within these groups, participants from each country presented how the implementation process had progressed in their country. At the end of the mini-workshop all participants were gathered together and a summary from the three group discussions were given.

At the evening after the mini-workshop, the participants were invited to a banquet dinner at the Restaurant “Bastionen og løven”, located at one of the old fortifications in Copenhagen.

The last day was opened by an update session on scientific research. At this session, presentations were given on: 1) susceptible fish species to the listed diseases, 2) the database for fish pathogens, 3) molecular characterisation of VHSV and IHNV in Germany, 4) development of genotype specific monoclonal antibodies against VHSV and 5) status of the RANA-project.

The annual meeting ended with the traditional update from the CRL. The results of the proficiency test 2008 were presented. A report from a year with focus on training of laboratories and the thoughts and considerations about implementing the new listed diseases in our work was given. Furthermore, proposals on the CRL work plans for 2010 were discussed.

Minutes from the meeting were taken by Helle Frank Skall, Søren Peter Jonstrup, Britt Bang Jensen 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, Britt Bang Jensen, 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 for May 25-27, 2010, but more details will follow.

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

Århus, 28 August 2009

Niels Jørgen Olesen and Søren Kahns

Programme

Tuesday May 26th – Annual Meeting of the National Reference Laboratories

REGISTRATION AND WELCOME ADDRESS

13:00 – 13:30 **Welcome Address and announcements**
Søren Kahns (Community Reference Laboratory)

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

Chair: *Fiona Geoghegan*

13:30 – 14:00 Overview of the disease situation in Europe – *Niels Jørgen Olesen*
14:00 – 14:30 Occurrence of Infectious Salmon Anaemia (ISA) in Scotland UK – *Rob Raynard*
14:30 – 14:50 Tracing the spread of Infectious Salmon Anaemia virus (ISAV) in salmon farms in Norway – *Trude Marie Lyngstad*
14:50 – 15:10 Fish health trends and developments in Norwegian aquaculture 2008 – *Hege Hellberg*

15:10 – 15:40 Coffee break

14:40 – 14:55 Monitoring of viral fish diseases in Poland in 2004-2008 – *Marek Matras*
14:55 – 15:10 Eradication of IPN in a farm with chronically infected stocks – *Thomas Wahli*
15:10 – 15:25 Total eradication of VHS in Denmark – *Henrik Korsholm*
15:25 – 16:45 Isolation of an Iridovirus (Ranavirus) from catfish *Ameiurus nebulosus* in Hungary – *Tamás Attila Juhász*
16:45 – 17:05 Koi Herpesvirus disease: Data from the global EPIZONE questionnaire 2009 – *Olga Haenen*
17:05 – 17:20 Epidemiology and combat against fish epidemics - thoughts on koi herpesvirus disease (KHVD) – *Sven Bergmann*

Wednesday May 27th – Annual Meeting of the National Reference Laboratories

SESSION II: Technical issues related to sampling and diagnosis

Chair: *Søren Kahns*

8:30 – 10:30 *The first part of this session will focus on diagnostic PCR set-up: Which PCR/real-time PCR to choose, sampling procedures, prevention of contamination, controls, number of replicates, etc*

- The 2008 KHV PCR methods ring trial - *Keith Way*
- Application of controls that minimises the risk of obtaining false-positive or false-negative results in diagnostic PCR assays - *Søren Kahns*
- Implementation of a one-tube assay for koi herpesvirus (KHV) detection adapted to latent infected carrier fish - *Sven Bergmann*
- Development of a robust accredited real-time PCR laboratory system and its application to the routine detection of fish pathogens - *Mike Snow*

Subsequently, a discussion in plenum will follow on how to do PCR diagnostic in "your" lab.

10:30 – 11:00 Coffee break

Chair: *Pia Vennerström*

11:00 – 12:30 The last part of this session will focus on antibody based diagnosis – with the following presentations:

- Results from an inter-laboratory proficiency test on detection of antibodies against VHSV and IHNV in rainbow trout – *Jeanette Castric*
- Optimisation and validation of an ELISA for detection of antibody to cyprinid herpesvirus 3 (CyHV-3, KHV) – *Keith Way*
- Development of serological methods for detection of Koi herpes virus (KHV) antibodies in carp, *Cyprinus carpio* – *Jeanette Castric*

12:30 – 13:30 Lunch

Wednesday May 27th – Mini-Workshop on implementation of Council Directive 2006/88/EC

Chair: *Niels Jørgen Olesen*

13:30 – 13:50 The Key issues to be addressed when implementing the Council Directive 2006/88/EC – *Sigrid Cabot*
13:50 – 14:10 Sampling and diagnostic plans on VHS, IHN, KHV and ISA – *Giuseppe Bovo*
14:10 – 14:40 A model for risk ranking fish farms to inform disease risk-based surveillance – *Birgit Oidtmann*
14:40 – 15:00 Summary of categorisation of Fish Farms in EU according to S&D questionnaire – *Niels Jørgen Olesen*

15:00 – 15:10 Coffee break (in groups)

15:10 – 16:30 In groups: discussion on authorization and categorization and risk management. Each NRL is requested to present how the directive has been implemented in their country – 3 groups

Group 1 Chair: *Birgit Oidtmann*
Northern European countries
Group 2 Chair: *Niels Jørgen Olesen*
Continental European countries
Group 3 Chair: *Giuseppe Bovo*
Mediterranean European countries

16:30 – 17:00 Summarisation of the group discussions

19:00 ***BANQUET DINNER***

Thursday May 28th – Annual Meeting of the National Reference Laboratories Continued

SESSION III Scientific research update

Chair: *Hege Hellberg*

9:00 – 9:20 Susceptible species to listed diseases – EFSA Report – *Ana Afonso*
9:20 – 9:40 Establishment of a CRL-database for fish pathogenic viruses – *Søren Peter Jonstrup*
9:40 –10:00 Molecular characterization of VHSV and IHNV in Germany – *Heike Schuetze*
10:00 – 10:20 Distinction between genotypes of Viral Haemorrhagic Septicaemia virus (VHSV) using Monoclonal Antibodies – *Takafumi Ito*
10:20 – 10:40 Status from the RANA-project – *Britt Bang Jensen*
10:40 – 11:00 Open for presentation from participants

11:00 – 11:30 Coffee break

SESSION IV: Update from the CRL

Chair: *Niels Jørgen Olesen*

11:30 – 11:50 Report from Year 2008 – *Niels Jørgen Olesen*
11:50 – 12:15 Workplan for 2009 and 2010 – *Niels Jørgen Olesen*
12:15 – 12:35 Inter-laboratory Proficiency Test 2008 – *Søren Kahns*
12:35 – 13:00 Next meeting and end of 13th Annual Meeting - *Niels Jørgen Olesen*

13.00 ***Sandwiches and goodbyes***

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

Overview of the disease situation in Europe

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

Minutes

This presentation is a presentation we have had all the years, starting out with each participant presenting data from their own country/region – this took too much time and was actually a boring way of presenting the data. 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 5 parts: General data on production, epidemiological data on diseases, laboratory data from NRLs and other laboratories, quality assurance in NRLs and regional laboratories, and as the final part we have included categorisation of fish farms according to the new legislation in EU. Categorisation will be discussed in detail tomorrow. 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 2007 has risen a bit again after a decrease from 2003-2006. Data from 2008 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, 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 to be severe underreporting of VHS and IHN in many countries. The infection status is known for about ½ of the farms.

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. ½ of the farms are considered infected at the Faroe Islands, but 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 is 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.

More and more laboratories are becoming accredited according to the ISO 17025 standards.

Questions
***Rob Raynard*:** In general, how can we tell that the disease situation is improving?
***Niels Jørgen Olesen*:** The number of category I farms have increased dramatically, compared to the number of farms in VHS and IHN-free zones or compartments under the previous legislation. The new legislation tries to push people to not stay in the unknown category III group. All NRL's could help pushing the veterinary authorities to obtain knowledge on the disease status in the farms in their respective countries.
Stig Mellergaard: Do you think that the SVC surveillance will die out, now it is not listed anymore?
Niels Jørgen Olesen: Yes, for those countries that does not wish to obtain additional guaranties.

Occurrence of Infectious Salmon Anaemia (ISA) in Scotland UK
Rob Raynard*, Charles Allan, Sandy Murray, Mike Snow, Eann Munro, David Bruno, David Smail
Marine Scotland, Marine Laboratory, 375 Victoria Rd, Aberdeen, AB11 9DB Scotland. r.raynard@marlab.ac.uk

Abstract: On the second of January 2009, Infectious Salmon Anaemia (ISA), a disease listed as non-exotic to the EU under Directive 2006/88/EC, was confirmed on an Atlantic salmon ongrowing sea water site in the Shetland Islands of Scotland. A further 2 sites in the same area were officially suspect for ISA due to having received fish from the confirmed site in June 2008.

In accordance with Article 53 of Directive 2006/88/EC the Shetland Islands were temporarily suspended from freedom for ISA based on epidemiological evidence.

This was the first occurrence of ISA in Scotland since the first outbreak in the period 1998-1999 which was successfully eradicated. Measures for containment and eradication were put in place including the establishment of a protection zone and surveillance zone. Of the 17 sites inspected and screened within the ISA containment area, 3 sites were confirmed ISA positive. The first site tested positive by qRT-PCR and virus isolation, the second site was confirmed positive by qRT-PCR, virus isolation and IHC on 30 January and a third site was confirmed by qRT-PCR and clinical and postmortem observations on 20 March. All of the fish held on the confirmed and suspect sites were withdrawn under official supervision.

The conclusion of investigations into the potential spread and origin of infection are that, that infection is restricted to the containment area in South West Shetland, within which it appears to have spread hydrodynamically. The source of infection is unknown. Since the entire UK had previously been free of ISA it is possible that ISA has emerged from a wild source or from import. Genetic analysis carried out on the HE gene of virus from the confirmed cases shows that the sequence is unique representing a novel HPR type. The virus is a member of group EU G1 and the sequence is not closely related to previous sequences identified in Scotland.

Surveillance will continue in the Containment area according to Decision 2003/466/EC. The Government's objective is to eradicate ISA from the area and to regain freedom from ISA for South West Shetland.

Minutes
First I will like to inform you, that there has be a reorganisation in Scotland affecting our laboratory, so we are now merged with the authorities and called Marine Scotland, we still carry out the same functions though.

ISAV is an Orthomyxovirus causing significant mortality in farms. ISA has occurred worldwide in Atlantic salmon aquaculture. ISAV is capable of mutation. Genetics suggest a different origin for European and North American isolates.
In May 1998 to May 1999, Scotland experienced 11 ISA cases, and has been free of ISA since 2004 until last year.
The Shetland Islands are situated halfway between Norway and the Faroe Islands, and they feel quite independent, also from Scotland.
In the first farm diagnosed with ISA, a large number of earlier mortalities were attributed to causes other than ISA. Abnormal behaviour was seen in some fish. During inspection, some signs of disease were observed, but not indicative of ISA. Diagnostic samples were taken. ISA was confirmed 2nd January 2009. Containment area, protection zone and surveillance zones are established. It is an important area as 10% of the national salmon production is produced there corresponding to approximately 13.000 tonnes.
Diagnosis: mainly clinical and post mortem, virus isolation, PCR, histopathology, but also IHC and IFAT. Evidence of circulatory disturbance and damage to endothelial cells, virus identification on TO cells using a haemadsorption test to detect intra-cellular virus. Positive cultures are confirmed by IFAT.
Clinical inspection at least monthly in the control zone, every 2 months in the surveillance zone, or the sites with epidemiological links, weekly reporting of mortalities, and targeted surveillance in the protection zone.
Until now we have 5 cases: 2nd January, 30th January, 20th March, 18th May and 21st May. PCR has contributed to diagnosis in all cases, but also virus isolation, clinical signs, liver pathology, IFAT, and gross signs. Furthermore 2 suspect cases.
On second case initially no clear signs of ISA disease, 4/30 PCR +ve, 6/30 virus +ve. Virus isolation has improved much since the outbreaks in the late 1990-ties.
The farms have to withdraw of fish within 3-6 weeks, and they are slaughtered under strict biosecurity.
Epizootic investigation: all live fish movements and other contacts over 12 months back from 2nd January 2009. There have been no movements of live fish for ongrowing outside the Southwest Shetland containment since mid June 2008, and none outside Shetland. All movements are investigated and spreading ruled out through inspection and testing.
Likely source and spread: The disease appears to have occurred after 27th June and spread hydrodynamically. The source is unknown, genetically the virus belongs to the European genogroup I, the HPR type is unique and novel. We have 2 theories: Either the disease is caused by a possible new emergence or by importation?
We have examined 216 wild freshwater fish and more than 1000 marine fish, all negative by qPCR.
We are aiming at regaining freedom from ISA. The industry have agreed on 6 months synchronous fallowing for the whole area, not just to eradicate ISA, but also to contribute to the control of sea lice.

Questions
***Brit Hjeltnes*:** Is anything previously known on the HPR0 situation in this area?
***Rob Raynard*:** No, not in this area. Mike Snow has tested for this app. 2 years ago. He identified 3 farms with HPR0, but that was in other areas.
***Brit Hjeltnes*:** Has the industry tested themselves?
***Rob Raynard*:** The industry has tested themselves in the autumn in relation to exports to Chile.
***Niels Jørgen Olesen*:** Did you type all the outbreaks, are they all the same HPR type?
Rob Raynard: We have tested 2 isolates until now and they are the same. The type is a completely new type.
Stig Mellergaard: What about vertical transmission?

***Rob Raynard*:** The evidence that vertical transmission of ISA occurs is generally very weak. In this particular case of ISA the eggs came from ISA free fish, and they are disinfected at arrival. The likely date of infection which is after 25 June 2008 does not support vertical transmission in this case.
Stig Mellergaard: Have you considered the human transportation?
***Rob Raynard*:** We have looked into wellboat movements.
***Brit Hjeltnes*:** It could be very interesting to have more information on the HPR0 type.
***Mike Snow*:** It is not a HPR0 type we have seen in Scotland before. Technically it is very difficult to type these isolates.
***Fiona Geoghegan*:** Have you had any resistance among the farmers, now the legislation has changed?
***Rob Raynard*:** Generally there has been good relationships but also some resistance, also because there is no reimbursement.

Tracing the spread of Infectious Salmon Anaemia virus (ISAV) in salmon farms in Norway

T.M. Lyngstad*[1], M.J. Hjortaas [1], P.A. Jansen[1], A.B. Kristoffersen[1], E. Karlsen[2], E.J. Johansen[3], C.M. Jonassen[1]
[1] National Veterinary Institute, Oslo, Norway, [2] Norwegian Food Safety Authority, Harstad, Norway, [3] Norwegian Food Safety Authority, Finnsnes, Norway

Abstract: Outbreaks of infectious salmon anaemia (ISA) were confirmed in 23 sea sites farming salmon in Norway in 2007-2008. Eleven of the outbreaks clustered in a local area in Northern Norway. In this study we present phylogenetic and sequence analyses of the Haemagglutinin-esterase (HE)-gene, including the hypervariable region (HPR), and the fusion protein (F)-gene of Norwegian ISAV isolates from 2007 to 2008. In addition, the study covers investigation for ISAV in 30 farms considered being at risk due to proximate location to outbreak farms (at-risk-farms). Organs from 10 fish from each outbreak farm and 30 fish from each at-risk-farm were screened for ISAV using Real Time RT PCR. The HE- and the F- genes of the virus were sequenced from positive samples. Phylogenetic analysis of the HE- gene, including the deletion pattern in the HPR, and the F-gene showed that 11 of the outbreaks that clustered in a local area in Northern Norway were closely related. ISAV was detected in 41 % of at-risk-farms, and the presumed low virulent HPR0 genotype of ISAV was more common than previously reported from Norway. Two of the eleven clustered outbreaks were sampled as at-risk-farms 3-4 months prior to ISA outbreaks. Phylogenetic comparisons between ISAV HPR0 and subsequent ISAV HPR from these two sites suggest that the different variants of ISAV represented independent infection events. We conclude that ISAV HPR0 is more abundant than previously shown in Norway. It is hypothesised that ISA outbreaks may arise from ISAV HPR0 mutating to virulent ISAV, and that virulent ISAV spreads horizontally resulting in small scale local ISA epidemics.

Minutes

In Norway ISA outbreaks have occurred widespread along the coast, and partly in small space-time clusters of sites. The number of sites included in this project is 23 ISAV outbreak sites and 29 at risk sites; at risk sites are sites in proximity to outbreak sites. Epidemiological data were gathered through standardised questionnaires. For screening, RT-PCR on segment 5 and segment 6 was used and ISAV was detected in 13 at risk sites.
Phylogeny on segment 6 and segment 5 showed nearly no variations in the examined fjord system. The isolates had identical deletions and were of the same HPR-type. One similar HPR-type was identified in an outbreak back in 2004.

HPR0 was isolated in 7 farms. On 3 sites HPR0 was isolated 3-4 months prior to ISA outbreaks.
We discovered it was easier to detect the ISAV on gill samples than in kidneys.
The low virulent sites have more variation than the outbreak sites when looking at segment 6. This is also true for segment 5, with even more variation.
A cluster of 11 outbreaks was identified with closely related HE genes including the depletion pattern in the HRP and F protein gene.
Low virulent HPR0 genotype is more common than previously reported from Norway.

Questions
Stig Mellergaard: Compared to avian influenza, we have a source of low pathogenic ISAV; can it be herring that transport the virus?
***Debes Christiansen*:** At the Faroe Islands we have tested a lot of herring, but have not found ISAV in any of them.
***Stig Mellergaard*:** When we test wild birds, we also seldom find positives.
***Niels Jørgen Olesen*:** What is done when you find HPR0 – is it then a case of suspicion?
***Trude Lyngstad*:** It is reported to competent authority but not treated as a case of suspicion.
***Debes Christiansen*:** Are there other epidemiologic clues, such as stress factors etc?
Trude Lyngstad: We should look more into this. Most outbreaks occur after 1 year at sea. We observed though one outbreak after only 2 months at sea.
***Neil Ruane*:** Have you noticed any effect of these measures on the spread of PD?
Brit Hjeltnes: It is hard to judge as we have no outbreaks of PD in northern Norway.

Fish health trends and developments in Norwegian aquaculture 2008

Hege Helberg* and Irene Ørpetveit
National Veterinary Institute

Abstract: In 2008, Norway produced 740 000 tonnes Atlantic salmon (*Salmo salar* L.), 80 000 tonnes rainbow trout (*Oncorhynchus mykiss*) and 14 000 tonnes Atlantic cod (*Gadus morhua* L.). The trend towards fewer and larger production sites for salmon continues. Viral haemorrhagic septicaemia (VHS) was diagnosed in two rainbow trout sites in 2007 and at two neighbouring sites in 2008. A marked increase in infection with *Flavobacterium psychrophilum* in rainbow trout has been detected. In farmed Atlantic salmon, an increase of outbreaks of ISA is the main finding. Pancreas disease (PD), heart and skeletal muscle inflammation (HSMI) and infectious pancreatic necrosis (IPN) continue to cause large losses, as do cardiomyopathy syndrome (CMS), proliferative gill disease (PGI) and winter ulcer disease. The detection of salmon lice (*Lepeophtheirus salmonis*) resistant to emamectin benzoate is a major concern.

Diseases in salmonids, number of sites diagnosed

	2003	2004	2005	2006	2007	2008
VHS	0	0	0	0	2	2
ISA	8	16	11	4	7	17
IPN	178	172	208	207	165	158
PD	22	43	45	58	98	108
HSMI		54	83	94	162	144
Piscirickettsia	5	0	0	1	1	1
Furunculosis	2	3	1	3	5	0
BKD	1	1	2	0	0	1
F. psychroph		3	1	2	2	16

The disease situation in cod is dominated by bacterial diseases, with francisellosis, vibriosis and atypical furunculosis being the major problems.

Diseases in Atlantic cod, number of sites diagnosed

	2005	2006	2007	2008
IPN	Not detect.	Not detect.	Not detect.	Not detect.
VNN (nodavirus)	Not detect.	3	6	3
Atypical furunculosis	3	13	9	16
Francisellosis	4	7	8	14
Vibriosis (*V. anguillarum*)	18	19	19	20
Cold water vibriosis (*V. salmonicida*)	2	Not detect.	1	1
Infection with *Vibrio ordalii*	1	Not detect.	3	Not detect.
Infection with *Vibrio logei/logei-like*	2	1	2	Not detect.
Infection with *Photobacterium* sp.	3	3	6	4

Minutes
Production: Atlantic salmon 740000 tonnes, rainbow trout 80000 tonnes, in all 1038 production sites. Cod 250 active sites, 13500 tonnes.
Trends: PD going up.
ISA: 17 outbreaks in 2008.
IPN: The disease is delisted, so we probably do not have the full overview. Most fish are vaccinated against IPN. Some outbreaks with large losses. App 45% of cases occurs in the hatchery phase, rainbow trout is almost exclusively affected during the hatchery phase.

PD is of huge concern; the farmers have begged the authority to list the disease, which it has now been since November 2007. In Norway we have SAV3. SAV1 (PD) as well as SAV2 (SD) have not been detected.
The disease has spread from Hordaland, but the middle part of the country is still free.
Heart and skeletal muscle inflammation (HSMI) in Atlantic salmon: infection trials indicates a viral aetiology. The disease is mainly seen in the first year after transfer to sea, but has in 2007 and 2008 also been diagnosed in hatcheries. Mortality 0-20%. Found all over.
VHSV genotype III diagnosed in farmed rainbow trout autumn 2007 and winter 2008. VHSV genotype III also detected in escaped rainbow trout within zone, but not in wild marine fish examined (n=260). 2007: 3 sites, 2008: 2 sites, all belonging to the same company.
There has been a dramatic increase of *F. psychrophilum* in rainbow trout in 2008. Systemic infections in 9 hatcheries with heavy mortalities and in 2 sea growing sites. There may be a common source.
Other disease problems: Cardiomyopathy syndrome (CMS), winter ulcer disease, proliferative gill disease.
Salmon lice resistance to emamectin benzoate confirmed on several sites. Multiresistance suspected on some sites. A new surveillance programme has started.
We have a new, emerging disease. It has been diagnosed in salmon in sea cages on south-western coast. Signs are variable mortality, loss of growth, no major known fish pathogenic agents detected. Macro: swollen and pale gills, yellowish liver, swollen spleen, circulatory failure, empty gut. Histology: gill and kidney lesions, hyperplasia of Bowman's capsule, haemorrhage, macrophage like cells in kidney, in heart epicarditis. Colleagues at Bergen University claim a Microsporidian is involved? Maybe associated with salmon lice as vector? Direct comparison is difficult as university of Bergen has done little histopathology.
Marine fish: mainly cod, 350 submissions from app. 85 sites. The main problem is *Francisella philomiragia* subsp. *noatunensis*. Reservoir for this pathogen can be found in wild fish. *Francisella* was listed in 2008. Íntracellular, chronic, granulomatous infection, all age groups are affected.
In 2009: PD 14 new outbreaks as of April, ISA 5 outbreaks, VHS no reoccurrence. An interactive map showing PD and ISA outbreaks is available at www.vetins.no as well as the annual report.

Questions
***Vlasta Jencic*:** Is piscirickettsiosis not an exotic disease, what have you done with it and how do you diagnose it?
***Hege Hellberg*:** We have had it for several years, but not of major concern and not exotic in Norway. It is diagnosed by histology and confirmed by PCR.
Niels Jørgen Olesen: *F. psychrophilum* what have you done about it? Others have had problem for years.
***Brit Hjeltnes*:** There seems to be a common source, a broodstock farm maybe. Information is put on our website. The Farmers have taken care of it by themselves. There have been proposals for listing of the disease.
***Hege Hellberg*:** The disease has mainly been a rainbow trout problem and there is a shortage of rainbow trout at the moment. Probably transferred from freshwater to the sea, where after horizontal spreading has taken place.

Monitoring of the viral fish diseases in Poland in 2004-2008

Jerzy Antychowicz[1],Marek Matras*[1], Ewa Borzym[1], Michał Reichert[2]
Department of Fish Diseases[1] and Department of Pathology[2] of National Veterinary Research Institute, Pulawy, Poland

Abstract: Fish Diseases Department of National Veterinary Institute exists for more than 70 years. For many years, we have carried out the monitoring of environmental, viral, fungal, and bacterial fish diseases. Since 2000, a regular diagnosis of the etiological agents of the notified fish diseases has been made. The viruses such as VHS, IHN, IPN, and SVC have been isolated at least in two of the following cell lines BF-2, EPC, RTG-2, FHM and identified by at least two of the following methods: ELISA, IFAT, PCR. KHV nuclear acids identification was made with PCR method using at least two of the following modification i.e. Gilad, Grey, and Bercovier. In some random cases, we isolated KHV in CCB cell lines and then we preceded onto virus electron microscopy (TEM) identification. Actually we are investigating on Real Time PCR application to KHV identification in various water temperatures.

The sampling was carried out under the principles presented in EU directives. The sampling was performed by district veterinary officers. The fish for the sample have been chosen according to the instructions prepared by prof. Antychowicz and authorised by Polish General Veterinary Officers. The sample consists of the live or sacrificed and cooled, below 10°C, fish, which were then delivered to our laboratory and immediately subjected to diagnostic investigations.

In 2000, prof. Antychowicz initiated the experiment and for the first time in Poland realized a program of controlling the VHS. It concerned 19 rainbow trout farms situated in one isolated river catchment. We made some achievements at the beginning with a substantial decrease in VHS cases though the region had the worst VHS on record in Poland. Unfortunately, we failed to eradicate the VHS completely, because some fish farmers participating in the programme did not comply with the established guidelines. Nonetheless, during the realisation of this programme we gained a lot of valuable experience, which could be utilised in the fulfilment of official programmes in the future.

Our practical experience gained in the experimental eradication programme and also during eight years of VHS and IHN monitoring, has shown that the main factor contributing to VHS and IHN introduction to Poland and spread throughout the country is unrestricted importation of live rainbow trout and its eyed eggs from other countries and inadequately control of live fish movement between Polish fish farms. It was caused because there were no regions in Poland officially free of VHS and IHN and no official programmes are actually realized. This situation appeared in spite of a great activity of veterinary service official veterinarian survey fish farms, select samples of the fish and eventually perform the eradication of VHS, IHN and KHV cases.

The second important factor involved in spread of VHS was ineffective disinfection of basins used by private proprietors for illegal fish transportation and improper neutralisation of the transport water before pouring it out.

It should also be stressed that the possibility of fish viruses spreading from one infected farm to other farms situated in the same river catchment increases when the distances between them decreases. One of our colleagues Dr. Mazur, a specialist of fish diseases, found that a distance of less than 1 – 2 km between salmonid farms, has a significant effect on the increased susceptibility the of the virus spreading through the water.

Two well-documented first cases of IHN in Poland were detected in 2008. In both cases, the infected fish originated from the fish eggs imported from western European countries, so it is

strongly suggested that the virus originated from the eggs – no cases of IHN were detected for many years in Poland before these eggs were imported.

Our practical experience gained in the experimental eradication programme initiated by Prof. Antychowicz (1999 – 2005) and also during eight years of VHS and IHN monitoring, has shown that the main factors contribiuting to spread of pathogenic salmonid viruses throughout Poland are the following:

- too short distances between salmonid fish farms – that appears as a result of dynamic fish farming development in our country,
- ineffective disinfection of transport tanks used for fish transportation and improper neutralization of the transport water before pouring it out after fish transportation,
- unrestricted importation of live rainbow trout and its eyed eggs from other EU countries and inadequate control of live fish movement between Polish fish farms caused by lack of any EU regulations in the case of the countries or regions that are not officially free of the VHS and IHN.

Concerning KHV, the following factors facilitate the introduction and spread of this disease in Poland:
- uncontrolled movement of live koi carp between the countries throughout EU territory,
- stocking koi carps with carps reared for consumption together in production ponds
- propagation of crossbred between ordinary carp and koi carp for consumption purposes and keeping them together with ordinary carp,
-lack of realistic long-term programmes for the KHV control in the traditional large carp farm environment with complicated systems of water facilities feeding the ponds which make the eradication of the disease in some region in Poland very difficult,
-lack of any EU Directive regulating the movement of live carp and koi carp between the farms that did not have status free of KHV.

As it was stated there were no official programmes realized in Poland for controlling VHS, IHN and KHV for the purpose to obtain official free status in river catchments or in farms. The only way so far to control fish viral diseases applied in Poland is regular monitoring and disease eradication in each infected fish farms, but the other farms in the river catchment are usually not considered in eradication. We found these methods not effective enough as we can see from the presented figures. The regular monitoring of VHS, IHN and KHV could be anyway a good beginning for the implementation of official programmes in the future. The following conclusions could be made:
- the realization of the official programmes is connected first of all with good management fishery practices among others in the proper disinfection of transport vehicle basins used for fish transportation and purchasing by the farmers only certified fish from officially free farms and regions
- the realization of the long-term VHS, IHN and KHV programmes is urgent in order to decrease the spread of these diseases in Poland
- viruses genotyping together with the geographic information system (G.I.S) should be applied in the near future for efficient monitoring of the important fish viral diseases and for tracking the sources of infections especially when there is suspicion that in particular case virus could be introduced from abroad.

I hope that our remarks on the factors, which contribute to the spread of fish viral diseases will be helpful for countries, which like Poland, are going to start with the official programmes of VHS, IHN, and KHV eradication on their territory.

Minutes
There are 442 salmonid fish farms and 1293 carp farms in Poland giving rise to an approximate production of 18.000 tonnes of rainbow trout and 18.000 tonnes of carp. Since 2000, a survey for listed viral fish diseases have occurred in Poland. Sampling has been processed according to EU standards. Most outbreaks of VHS have occurred in the North West of Poland. One factor contributing to VHSV and IHNV introduction to Poland is unrestricted importation of live rainbow trout and eggs. Another is ineffective disinfection of transportation tanks. Furthermore, when distances between farms are less than 2 km an increased risk of VHSV spreading is observed.
Concerning KHV, most cases have been observed in the south of Poland where most carp farms are located. Factors that can contribute to the introduction and spread of the disease are e.g.: uncontrolled import, stocking of koi carp together with consumption carp.

Questions
Giuseppe Bovo: You had two outbreaks of IHN in 2008. Can you import eggs from any kind of farm?
***Marek Matras*:** No, imports only occur from certified disease free farms.
Olga Haenen: Concerning the IHN outbreak in Holland, eggs were bought with certificate from a certified farm. However, the transportation company probably had problems with ineffective disinfection of transportation tanks.
Marek Matras: Import was from certified farms from Western European countries.
Fiona Geoghegan: Does the industry consider legislation as a positive thing?
Marek Matras: Yes, in general they do. It is now easier to get samples and to carry out legislation.

Eradication of IPN in a farm with chronically infected stocks

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Abstract: In rainbow trout from a commercial fish farm producing fish for human consumption IPNV was regularly detected over a period of several years. This resulted in restrictions for the farm as IPN is a notifiable disease in Switzerland. Although the farm received water from a river, it was suspected that the source of virus was the introduction of infected fish imported into the country rather than river water. These imported fish were regularly found to be positive for IPNV. To eradicate the infection from the farm a stepwise procedure was initiated which allowed the farmer to continue production without interruption. To this end series of tanks were disinfected and fallowed before stocking with new fish from a source certified as IPNV-free. This procedure was continued until all fish of the original stock had been replaced. As additional measures newly introduced fish were regularly examined for the presence of IPNV and random samples from all tanks stocked with new fish were tested. All tanks but one stocked with new fish showed to be negative for the whole production period. In the one tank, where the virus had been detected in newly introduced fish no fallowing had been performed after disinfection of the tank. Since 10 months no infected fish could be detected any more. This result clearly indicates that the source of virus was not the river water and that eradication of IPN in a commercial farm is possible, given some conditions are met.

Minutes
IPN is a notifiable disease in Switzerland. In one particular farm - producing market sized rainbow trout (100 t/year) - IPNV positive fish were repeatedly identified. In this farm fish were located in

round tanks/raceways. Two possible sources of infection: from the water system or most probably from imported fish. The consequence was, in agreement with the farmer, that sale of live fish was prohibited and sanitation demanded. A change of fingerling supplier was made and a stepwise sanitation of the farm was set-up. The duration of the sanitation was one year after which all samples were negative, a good growth of new stocks and low mortality rates were observed. All restrictions were lifted and increased production numbers were observed. The absence of new infections at the farm strongly indicated that the cause of infections have been because of import of infected fish.

Questions
Birgit Oidtmann: Does the number of cases reflect those isolated from the same farm?
Thomas Wahli: No, normally we see one or two farms infected
***Olga Haenen*:** IPN can be difficult to get completely rid of. Do you think it could break out again?
***Thomas Wahli*:** The virus may still be present at very low levels. We have not been able to detect it in our laboratory. But we can not be 100 % sure that it is completely gone.
***Sven Bergmann*:** Have you checked if the fish contains antibodies?
***Thomas Wahli*:** No
***Guiseppe Bovo*:** IPN can be vertically transmitted. Can this farm sell fish for restocking?
***Thomas Wahli*:** It is not allowed. This farm can only sell for consumption.
***Niels Jørgen Olesen*:** How long time will it take before this farm can be claimed free of IPNV?
***Thomas Wahli*:** We have not seen IPNV since last year. New fish are growing well. Regular testing for viruses will occur.
***Niels Jørgen Olesen*:** What kind of disinfection have you performed at the farm?
***Thomas Wahli*:** Several, formalin, Virkon S and heating the tanks
Irene Ørpetveit: A comment on sensitivity of IPNV detecting methods: In our lab, we find the sensitivity of real-time PCR and cell lines almost similar.

Total eradication of VHS in Denmark

Henrik Korsholm
Danish Veterinary and Food Administration.

Abstract: Since the first appearance of VHS in the 1950-ties the disease was rapidly spread to all of Denmark by trade of fish. Targeted eradication has been carried out under official supervision and control since 1970. This has resulted in a decrease in the number of infected farm, but not to a total eradication of the disease. A project for final eradication of VHS has been initiated in March 2009 and is planned to run for 5 years. Participation in the project is mandatory for the fish farms. A grant from the European Fishery Fund finances the project, which has a total budget of 6.5 million €.

Minutes
The first observation of VHS in Denmark was in the 1950-ties. In the 1960-ties a private voluntary eradication program was started. In 1970-ties an official control program for VHS eradication was started and in 2009 the final eradication of VHS in Denmark will hopefully occur. For fish farms considered to have a potential risk of being infected by VHSV, the method applied is stamping out (removal of all fish and gametes, cleaning and disinfection, fallowing, restocking with healthy fish). The Skjern Å systems and Ringkjøbing fjord is a special high risk area. According to the new directive, fish farms have to be categorised. Danish fish farms free of VHSV will be put into category I whereas the rest are put into category II. Application for an eradication-programme was

submitted in May 2008 and approved by the European Fisheries Fund in autumn 2008. The total budget is 48.5 mill DKK = 6.5 mill €. In the program: It is mandatory to stamp out; taxation and compensation are provided to the fish farmers according to the value of the fish; cleaning, disinfection and production stop are carried out by the fish farmers.

Questions

Birgit Oidtmann: How did you calculate the amount of food for which the compensation of 0.33 €/kg feed for fallowing was given?

Henrik Korsholm: Each fish farm has been appointed a maximum feed amount allowed to use by the Danish Authorities. When an outbreak occurs, compensation is calculated on behalf on the value of the fish, the day before the outbreak occurred.

Drazen Oraic: How do you remove rainbow trout from a river system?

Henrik Korsholm: By electrofishing. The rainbow trout are removed from the river system whereas other fish are left. Sometime repeated electrofishing occur.

Giuseppe Bovo: Are rainbow trout in the sea coming back to the river systems and do rainbow trout breed in the river systems – if yes it could cause problems.

***Henrik Korsholm*:** Yes, escaping rainbow trout from sea farms are going to freshwater systems and there are indications that the rainbow trout has started breeding in Danish river systems.

***Giuseppe Bovo*:** What is the prevalence of infected feral fish?

***Henrik Korsholm*:** It is low but it has not been determined exactly.

Fiona Geoghegan: The project is financed by a grant from the European Fishery Fund that ends in 2013. Is that why the project runs out in 2013?

***Henrik Korsholm*:** Yes.

Fiona Geoghegan: And the Danish ministry has to supplement by paying 50%?

***Henrik Korsholm*:** Yes.

***Niels Jørgen Olesen*:** Maybe this is the first of such kind of a programme to receive funding from the European Fishery Fond.

***Stig Mellergaard*:** A similar programme from Germany has received support.

Isolation of an Iridovirus (Ranavirus) from catfish *Ameiurus nebulosus* in Hungary

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Abstract: Iridovirus infection was identified in catfish (brown bullhead), *Ameiurus nebulosus* (*Ictalurus nebulosus*), showing severe clinical signs. In May 2008 intense bullhead mortality was observed in a four-hectared lake which belongs to a fishing club. No clinical signs were seen in other fish species living in the same lake, such as carp, pike, pikeperch, and grasscarp. The diseased fish showed distinct clinical and histopatological signs of Iridovirus infection: petechiae on the surface of the body and petechiae and necrosis in the internal organs. A cytopathic virus was isolated from homogenised spleen, liver and kidney on both EPC and BF-2 cells. Inclusion bodies were seen in the cytoplasm of the infected cells using a haemathoxilin-eosin staining method.

The virus was identified by PCR methods. A 580 base pair length PCR fragment spanning the MCP gene was amplified from the organ suspension and tissue culture supernatant by using primers and protocol described by A.D. Hyatt et al. in 2000. Homology searches of GenBank revealed 100% amino acid identity with corresponding MPC sequences of the catfish and sheatfish iridovirus.

Minutes
The presentation was on the first report on the occurence of a disease caused by European Catfish Virus (ECV) in Hungary (sequencing of a PCR fragment of the MCP gene showed 100% identity with ESV and ECV). The virus could induce considerable mortality in Brown bullhead (*Ameiurus nebulosus*) but no disease was seen in other species living in the same lake. Water refill had been performed from backwater 1 week before the outbreak. The source of the infection was not determined. Possible explanations could be that virus carriers could have come into the reservoire from the backwater during the water refilling or might originally be present in the lake, and the changing of water quality have generated the epidemic or maybe both.

Questions
***Sven Bergmann*:** At what temperature started the fish dying?
***Tamas Juhasz*:** At 20–22°C but it was not exactly determined.
***Olga Haenen*:** The rana iridoviruses are warm water viruses with optimum growth at 24°C
Tamas Juhasz: Yes, but we also see that they can grow at 18°C and can induce cytopathic effects at this temperature.
***Giuseppe Bovo*:** Do you have the similar species *Ictalurus melas*?
***Tamas Juhasz*:** No we only have *Ictalurus nebulosus*

Koi Herpesvirus Disease: Data from the global EPIZONE questionnaire 2009

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Abstract: EPIZONE is a big EU network of excellence project within FP6 (www.epizone-eu.net), with 20 partners from Europe, and China, Turkey, FAO and DiVa. Its mission is to improve research on preparedness, prevention, detection, and control of epizootic diseases within Europe to reduce the economic and social impact of future outbreaks of emerging/notifiable diseases, like Foot-and-mouth disease through increased excellence by collaboration.

Within EPIZONE, Work package 6.1 covers emerging diseases of fish, including Koi Herpes virus (KHV), which causes the notifiable KHV disease (KHVD) in koi and carp *(Cyprinus carpio).*

In Sept 2007, at the last EAFP Conference in Grado, results of the detailed EPIZONE questionnaire on KHV disease in 2006-2007 were presented.

In March 2009 a follow up KHV questionnaire was sent to > 65 countries world wide. By the start of May 2009, 40 countries had responded, i.e. > 60%. The results of the KHV questionnaire will be presented, including the trends compared to the 2007 questionnaire.

Questions of the questionnaire were about koi (1), cultured (2) and wild carp (3), all *Cyprinus carpio*:

- Prevalence of KHV in your country? Year of first detection? Number of outbreaks in 2004-2009 in 1, 2 or 3?
- Clinics: what clinical signs were present in KHV outbreaks?
- Outbreaks: Was there disease and mortality in small and/or big fish?

- Diagnosis: Which diagnostic tests were used for KHV detection, screening and confirmation? Did/do you participate in the KHV PCR ring test of CEFAS (UK);
- Susceptible fish species: Was KHV isolated from other species than koi/carp?
- Latent carriers: Do you have any experience with latency of KHV in koi/carp?
- Measures (stamping out, temp change, therapy) and effects in 1, 2, and 3?
- Vaccination: Is a KHV vaccine used in your country?
- Any research on KHV in your country and laboratory?
- National legislation in your country?
- Any Further points?

A full updated literature list on KHV on request: please E-mail olga.haenen@wur.nl

Minutes

The first Epizone KHV disease questionnaire was sent to 67 countries in December 2006 - January 2007; the second was sent to 72 countries in 2009. Many European countries have the disease, some have not and there is also some question marks. On a world wide scale the virus is observed in several continents – Asia, North America and Europe whereas it has not been detected in Australia. Since the first questionnaire, the disease have been identifies in several new countries, e.g. Canada, India, New Zealand, Slovenia and Guatemala. In 2007: 17 countries found KHV in koi carp, 12 in cultured carp and in 4 in wild carp. In 2009: 22 countries found KHV in koi, 10 in cultured carp, and in 5 in wild carp. In 2007: The mortalities varied: in koi 10-100%, cultured carp <10%-100%, and in wild carp mortalities were often high but unknown. In 2009: similar numbers, and 5-95% mortality in wild carp. In 2007: 13 countries had no KHV tests yet; in 2009: 12 have no tests. In such cases, samples may be sent to other countries for KHV testing. KHV may be detected also in species such as crucian & prusian & grass carp, goldfish, ide, *Anacistrus* sp., bream, sturgeon, sheatfish, etc. Possible latency reported from 10 countries. Measures at KHV outbreak: Stamping out, disinfection, stop fish movements, water temperature raised to 28-30°C, vaccination etc. In 2009: 28 countries had legislation on KHV, so many maybe because of 2006/88/EC.

Questions

***Neil Ruane*:** Those countries that are negative for KHV, do they have an active surveillance programme or no surveillance programme?
***Olga Haenen*:** In most cases there is no surveillance programme.
***Sven Bergmann*:** A comment on the vaccine from Japan, Prof. Miyazaki's group, it should work very well.
***Giuseppe Bovo*:** What about the formalin killed vaccine – is that used?
***Olga Haenen*:** It is not officially allowed to use live vaccines in Europe. The reason is that one is afraid they can mutate back to the aggressive type.
***Fiona Geoghegan*:** There is no licence in Europe to use these vaccines.
***Stig Mellergaard*:** An approval to use a vaccine in the EU can be provided in case of an emergency programme.
***Keith Way*:** The EU has to tell which diagnostic test is approved, e.g. which PCR test – is there any news on that?
***Olga Haenen*:** We should ask Sigrid tomorrow.
Birgit Oidtmann: In Scandinavia there is not a big carp industry – what is your opinion?
***Brit Hjeltnes*:** We do care – but it is not a big concern for the industry at the moment but it might be in the future.
***Birgit Oidtmann*:** What about countries where categories are not so important - Can they stay in category 3 or 4?

***Stig Mellergaard*:** It is not meant that countries should stay in these categories - countries can use the opportunity to apply for freedom for historical reasons if it is relevant.
***Olga Haenen*:** If koi import has occurred from Israel – we can not say if fish has KHV, as we cannot distinguish the vaccine strain from the field strain.
***Fiona Geoghegan*:** Is it possible to differentiate your results into cases of KHV in open systems versus cases in closed systems, since this is the way the legislation is now formulated?
***Olga Haenen*:** I will see if that can be done, as it would make the data more meaningful, under the circumstances.

Epidemiology and combat against fish epidemics - thoughts on koi herpesvirus disease (KHVD)

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Abstract: Since December 2005 koi herpesvirus (KHV) disease (KHVD) has been notifiable for carp (*Cyprinus carpio*) and, as a consequence of the severe economic losses especially in cyprinid aquaculture, since 2006 also for koi (*C. carpio*). Carp farmers in different federal states of Germany have no possibility to interfere as a new virus is reducing their animals.
The lack of understanding of the epidemiological correlations of this herpesvirus-induced disease, the limited diagnostic possibilities as well as the extensive form of carp aquaculture in Germany often lead to misinterpretations of the epidemic situation after negative test results.
Every year more farms, pet shops, traders but also wild and non-cyprinid fish are affected by KHV. Nevertheless, clinical symptoms are only found in *C. carpio*.
KHVD seems to be a very seasonal disease. Most cases of KHV and KHVD are detected between June and September. For diagnostics, only molecular tools are suitable because other assays, e.g. virus isolation in cell culture followed by immunofluorescence, are not sensitive enough for virus detection.
To develop an adequate strategy to combat the epidemic it is crucial to assess the actually occurring KHV prevalence in infected or latently infected compartments. At present, there is no legal obligation to combat the disease in Germany. The regional authorities decide individually about the measures to be taken. The options range from stamping out after an acute outbreak with zero prevalence in a farm or pond to immunization strategies which also include escaped animals. The latter measure is most effective when the KHV prevalence is high.
The number of sampled fish and the sampling method (an active targeted sampling is recommended) can strongly influence the results of the diagnostic tests as well as the consequences. False-negative health certificates often lead to the statement "free of KHV" when no positive sample is found by e.g. PCR or *realtime* PCR. In contrast, when only one sample out of 100 is safely considered to be KHV positive, doubts remain.
The general characteristics of herpesviral infections, i.e. the fact that they induce persistence or latency, often are not taken into account. In addition, the situation in possible carrier fish and the education of the hobbyists are neglected.

Minutes
KHV disease is a notifiable disease for carp and it can cause severe loss for the cyprinid aquaculture. It is important to understand the epidemiological relations of the disease. KHV is a seasonal disease, primarily detected between June and September. As diagnostic tools, the

molecular methods are most suitable as they are the most sensitive. It is crucial to assess the actual occurrence of the virus in infected or latent infected compartments.
The level of virus in a fish is unpredictable. Therefore, it is desired that the virus gets a chance to grow up in the fish before sampling. 24 hours in stressing conditions would make the virus grow in the fish. If no stress is applied to the fish, the diagnostic methods might not be sensitive enough to detect the virus. You will never find 100% infected animals in a population. That is a big problem for random sampling approach. Never mix fish with ornamental fish.

Questions
***Stig Mellergaard*:** Concerning latent carriers, it is impossible with eradication unless using uninfected stocks.
***Sven Bergmann*:** Vaccination does not eliminate infection from wild type KHV. Furthermore, the wild type grows faster than the vaccine virus.

SESSION II: Technical issues related to sampling and diagnosis

The 2008 KHV PCR methods ring trial

Keith Way
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Abstract: Following successful KHV PCR methods ring trials in 2006 and 2007 the 2008 trial further expanded to include 44 laboratories in 32 countries. The format was similar to the 2007 trial where participating laboratories were requested to use their preferred DNA extraction method and to trial the two PCR protocols recommended in the OIE diagnostic manual chapter on KHV disease. They were also expected to use a real-time PCR assay or a nested-PCR assay to analyse the samples. The main difference for 2008 was that one of the samples was spiked with a cyprinid herpesvirus (CyHV) that was not KHV and laboratories were then asked to test the samples using a protocol that included generic primers that target the CyHV polymerase gene.
All of the sample vials contained lower amounts of virus DNA and total DNA than in previous samples making the 2008 ring trial more technically demanding than the previous trials. As a result, a much larger proportion of labs reported incorrect results (false positives and negatives) than in previous ring-trials. False positive results were reported by 18 laboratories, with one or more of the assays used. This suggests that they may have cross-contamination and sample handling issues in the labs they use for their molecular virology analyses.

A more detailed analysis of the 2008 ring trial results will be described and the format of future international ring trials or proficiency tests will be discussed.

Minutes
31 of 43 labs achieved clean/correct results with at least 1 PCR assay. 25 of 43 labs reported incorrect results. 19 labs reported false positives. Real-time PCR appeared most reliable. No more funding for doing the ring test is available. Collaboration with VLA in UK on future ring tests is a possibility. The cost of this ring test is to be negotiated.

Questions
***Rob Raynard* and *Søren Kahns*:** Is it generally the more experienced PCR labs that do real-time PCR - could this explain why real-time PCR appeared more reliable?

***Keith Way*:** It is generally the most experienced labs that use real-time PCR but a connection between this fact and the observed reliability of real-time-PCR has not been investigated.
***Debes Christiansen*:** What about results from negative controls do they also show a lot of false positives?
***Keith Way*:** Results from the labs in-house negative controls are not known.

Application of controls that minimises the risk of obtaining false-positive or false-negative results in diagnostic PCR assays

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Abstract: In order to assure a high reliability of the PCR used for routine diagnosis, special precautions should be applied in order to prevent false-positive or false-negative results. False-positive results may arise from cross-contamination from positive samples but most often arise from carry over of PCR products from earlier experiments. False negative results most often arise because of technical (e.g. pipetting) errors or presence of inhibitory factors.

Using the PCR that is based on the Bercovier TK primers for detection of Koi Herpes Virus (KHV) as an example, we will illustrate how controls could be used in a diagnostic PCR setup in order to minimise the risk of obtaining false-positive or false-negative results.

Today, artificial genes are commercial available for reasonable prices. We designed a DNA template (KHV_Pos_2) containing the TK primer sites but flanking a DNA fragment of different size than the region amplified from the KHV genome. In order to reduce the risk of obtaining false positive results, we use this KHV_Pos_2 template as the positive PCR control and the positive purification control because carry over contaminations can be discriminated form a true positive from the sizes of the amplified DNA fragments.

In order to validate the correctness of our negative results, we use a setup, where our samples are spiked with the KHV_Pos_2 template prior to DNA extraction. This allows co-extraction of templates. The identical TK primer sequence in the KHV_Pos_2 template allows co-amplification in the same tube and the size differences allows discrimination. This permits monitoring of the performance of quality of the DNA extraction procedure. However, the presence of two templates in one reaction tube can cause competition that might reduce analytical sensitivity. In order to be sure that a positive signal is not lost because of competition, we analyse our samples in duplicates of which only one of the duplicates is spiked with the KHV_Pos_2 template. Furthermore, in order to minimise reduction in sensitivity, the one duplicate is spiked with the KHV_Pos_2 template in a concentration slightly higher than the detection level.

Questions
***Sven Bergmann*:** Is it wise to use competition with control plasmid when several samples are near detection limit already?
***Søren Kahns*:** We use "duplicates" where only one sample is subjected to competition.
***Birgit Oidtmann*:** It might be that the plasmid serving as extraction control could be easier extracted than KHV DNA.
Søren Kahns: Yes, the control is not bulletproof but in my opinion much better than not using anything.
***Heike Schütze*:** Why not use an internal gene as control?

***Søren Kahns*:** No obvious candidate has been available. It will probably be hard to find a candidate expressed stably and in low enough amounts to avoid substantial competition.

Implementation of a one-tube assay for koi herpesvirus (KHV) detection adapted to latent infected carrier fish

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Abstract: Koi herpesvirus (KHV, syn. CyHV-3) is an emerging disease agent in cyprinid aquaculture world-wide. In Germany, outbreaks of KHV disease (KHVD) and detections of the causative viral agent increased in both edible carps and imported ornamental kois from 2002 to 2008. In extensive carp aquaculture, mortality rates of up to 80 - 100% occurred in 2008 in harvested fish but also in koi (both *Cyprinus carpio*). As a consequence, various fish farms suffered severe financial losses (200.00 and 900.000 €), which in some cases led to the financial collapse of these farms.
One special problem is the behaviour of KHV which obviously induces persistence in infected, but healthy appearing fish. In this phase of viral manifestation, a very weak virus load (5 – 10 particles) can be found in kidney and leukocytes, sometimes also in gill tissue. Most of the currently available diagnostic tools are not sensitive enough to detect these weak virus concentrations. These unrecognized infected animals represent a real threat for naïve, non-infected carps or kois of any size or age.
To overcome this problem, a new molecular assay has been tested and its diagnostic sensitivity and specificity have been compared with other commonly used diagnostic methods. As "golden standard" served a quantitative *duplex realtime* PCR (modified according to Gilad et al. 2004 and Hoffmann et al. 2006) including internal and external controls. This assay permitted an absolute determination of the KHV DNA content in the different sample tissues and controls.
The diagnostic sensitivity/specificity of conventional PCRs, nested PCRs, one commercial LAMP and *realtime* PCRs were directly compared showing the content of "copies/reaction".
In individual samples of latent KHV infected animals we also found that the KHV DNA content often ranged between 5 and 10 genomic equivalents or copies/reaction. To minimize the contamination risk in the laboratory caused by the routine use of nested PCR and to reduce the expenditure of time and effort, a one-tube semi-nested PCR (sn PCR) was established. The test reached an equal diagnostic sensitivity of 5 to 10 copies and is specific for KHV DNA only. As heterologous virus or DNA controls carp pox virus (CyHV-1) DNA, goldfish haematopoietic necrosis virus (CyHV-2) DNA, Channel catfish herpesvirus (CCV, IcHV-1) DNA and *herpesvirus anguillae* (HVA, Ang-HV 1) DNA were utilized. No signals occurred in gels after sn PCR or in *duplex realtime* PCR results with these DNAs.

Keywords: KHV, persistence, DNA content quantification, semi-nested PCR

Main literature:

Gilad O, Yun S, Zagmutt-Vergara FJ, Leutenegger CM, Bercovier H, Hedrick RP (2004) Concentrations of a koi herpesvirus (KHV) in tissues of experimentally infected Cyprinus carpio koi as assessed by real-time TaqMan PCR. Dis Aquat Org 60:179–187

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Minutes
Only one PCR alone seems not to be sufficient. There has been an agglomeration of a Bercovier's TK PCR negative KHV strain in Germany. Latent infected fish are often not detected (a stress model is necessary). KHV- free certificates are often false-negative. Pooling samples are "deadly" for diagnosis of latent/persistent infected fish. Sensitivity and specificity of PCR results after lethal and non-lethal sampling are comparable. Best PCRs: Gilad's realtime, nested PCRs and semi-nested PCR.

Questions
***Søren Peter Jonstrup*:** Did you test the effect of extraction kits?
***Sven Bergmann*:** Yes. Qiagen kit works very well.
***Birgit Oidtmann*:** It would be nice to share information between labs about technical issues.
***Brit Hjeltnes*:** Could such information be collected on the CRL-website?
***Guiseppe Bovo*:** Could you comment on your experience with pooling samples?
Sven Bergmann: Pool no more than 2 fish to discover latent infection.
***Søren Kahns*:** Are the TK primers all right to use?
***Sven Bergmann*:** There could be a population of KHV not recognized by TK-primers.
***Keith Way*:** We recommend the TK primers, but Sven Bergmann may have got information that changes the situation.

Development of a robust accredited real-time PCR laboratory system and its application to the routine detection of fish pathogens

Mike Snow*, Nicola Bain, Fiona Doig, Julia Black, Rebecca McIntosh & Holly McKay
Marine Scotland Marine Laboratory, 375 Victoria Rd, Aberdeen, AB11 9DB Scotland.

Abstract: In recent years, Taqman real-time PCR (qPCR) has increasingly become the molecular method of choice for diagnostic laboratories involved in the routine detection of pathogens of aquaculture. The methodology builds on the principles of conventional PCR but offers significant additional advantages which are of relevance in the diagnostics context including:
i) The specificity conferred by the requirement for binding of both primers and highly specific probe in a single assay.
ii) The sensitivity conferred by the fluorescent detection chemistries employed.
iii) The speed of processing due to the lack of requirement to visualise PCR products.
iv) The potential to gain quantitative data, aiding interpretation of results.
v) The potential to automate and thus include a variety of procedural controls.
Despite the potential advantages of qPCR-based diagnostics, practical implementation and standardisation of the technique in fish health laboratories has not been without its problems. Such problems have been largely focussed around issues such as how to compare, standardise and interpret results (Ct numbers) both within and between laboratories, especially when apparently very low levels of pathogen genetic material are detected. This problem is exacerbated as qPCR tests can often report positive results which may not be supported by alternative tests conducted in parallel.
As with development of any diagnostic test, key to maximising the potential benefits, maintaining credibility and avoiding pitfalls in routine applications is the development of a robust and reliable system. We report here our experience in development of a system for the development and application of qPCR based diagnostics at Marine Scotland. Here at the National Reference Laboratory for fish, shellfish and crustacean diseases in Scotland, we were among the first to develop independent accreditation of qPCR testing based on accreditation of a generic qPCR methodology, encompassing assay design, validation and operational guidelines.

Minutes
qPCR is a potentially sensitive, specific and high throughput diagnostic method. Routine application of qPCR in the diagnostic laboratory requires careful consideration to the prevention of contamination, experienced and trained personnel, inclusion of extensive and appropriate controls to ensure correct interpretation of results, validation and preferably accreditation, and careful interpretation and verification of result where possible/appropriate (e.g. by sequencing). Mike Snow iss happy to share information on how they validated qPCRs.

Questions
***Olga Haenen*:** Do you start by diluting suspected highly concentrated samples?
***Mike Snow*:** No but they are treated differently in the lab to avoid them contaminating other samples.

General discussion on PCR
***Søren Kahns*:** Should we declare conventional PCR dead and only go for real-time PCR?
***Mike Snow*:** Real-time PCR is not the universal solution. There are still cases where a conventional PCR is the best or only available choice.
***Keith Way*:** There are research samples, diagnostic samples, and surveillance samples. Should we go for 3 streams of sampling handling in the lab?
***Mike Snow*:** That would be optimal, but hard to achieve. We have enough work just having two streams.
***Søren Kahns*:** We have seen that factors like qPCR kit, extraction kit etc. can influence the result.
***Sven Bergmann*:** We have also experienced this on KHV PCR.
***Keith Way*:** OIE does not like recommendations of kits even though this may be a quite important factor.
***Sven Bergmann*:** We recommends β-actin as internal standard in qPCR. We have successfully tested this in 25 species.
***Niels Jørgen Olesen*:** We have to decide on good diagnostic method to diagnose KHV for inclusion in guidelines in a new Commission Decision on sampling and diagnostic procedures for KHV. A workshop will be held in NL in November 2009 in EPIZONE regime in order to propose final recommendations.
***Keith Way*:** My favourite is Gilad qPCR. As conventional PCR I recommend nested TK, but Sven Bergmanns results might interfere with this.

Serology Part

Results from an inter-laboratory proficiency test on detection of antibodies against VHSV and IHNV in rainbow trout

J. Castric*[1], C. Quentel[1], J. Cabon[1], F. Lamour[1] and N. J. Olesen[2]
[1]Afssa Ploufragan/Plouzané, Unité de pathologie virale des poisons, France
[2]Community Reference Laboratory for Fish Diseases, Technical University of Denmark, Aarhus, Denmark

Abstract: The main objective of the inter-laboratory proficiency test organized in 2008 by Afssa and DTU Vet in the frame of EPIZONE was the development of robust serological techniques for detection of specific antibodies against VHSV and IHNV. Eight participants from different European laboratories agreed to participate to the test and were asked to check the presence of

antibodies against the two viruses by seroneutralisation techniques (SNT) or by ELISA in thirty rainbow trout sera. Two participants agreed to compare two different SNT, a 50% plaque neutralization test and an end-point serum neutralisation test and five participants applied both SNT and ELISA on all or part of the sera. The sera used in the test were obtained from trout experimentally infected with VHSV and/or IHNV or originated from an infected farm. All participants used the same viral strains, complement, as well as positive and negative control sera.
The results obtained using the two serological methods were in good agreement with those expected, especially in the laboratories where the techniques have been implemented for many years. Differences arose mainly between results concerning sera having low titres but with some exceptions, the same sera were found positive using the two techniques. When comparing the two techniques used in three laboratories for detection of IHNV antibodies, SNT appears more sensitive and more specific that ELISA. With VHSV, SNT was more specific as no false positive was detected by three participants while some negative sera were found positive by ELISA. Additional results obtained using the two techniques applied to the same samples will be necessary before concluding on the sensitivity and specificity of the two techniques.
Both ELISA and SNT can be used to detect specific antibodies against VHSV and IHNV in trout sera at the level of a fish population. The choice of the technique depends on each laboratory practices.

Minutes
Main objective of this inter-laboratory proficiency test is to develop and validate serological techniques (ELISA and SNT) for detection of specific antibodies against VHSV and IHNV. Participants were asked to check for the presence of antibodies against VHS and IHN viruses in the 30 sera. Furthermore, 2 participants agreed to compare 2 seroneutralisation techniques: 50% plaque neutralisation and end-point neutralisation. 5 participants performed ELISA on all or part of the sera. 4 participants performed SNT and ELISA on total or part of the sera. In conclusion both ELISA and SNT can be safely used to detect specific antibodies against VHS and IHN viruses

Questions
***Birgit Oidtmann*:** Regarding the IHNV antibodies – did you have specificity problems?
***Jeanette Castric*:** Only a few labs found false positive samples. In general less concordance between the results with sera having low neutralising titres.
***Brit Hjeltnes*:** For how long time will a population of rainbow trout stay positive for antibodies in the sera?
***Jeanette Castric*:** We have found antibodies at least one year after infection.
***Niels Jørgen Olesen*:** Positive sera can be found in a population at least one year after an outbreak. Infection depends on the temperature. If fish gets infected at low temperature there might be delayed onset, up to three months. This can cause a problem for surveillance.
***Sven Bergmann*:** We sometimes observe problems with our ELISA plates – Nunc Polysorb where some wells work OK whereas some does not. Are there any other having such problems?
***Jeanette Castric*:** We use same NUNC ELISA plates and do not see such problems.
***Sven Bergmann*:** We observe the same problem with other viruses.
***Niels Jørgen Olesen*:** We have tested different ELISA plates, in our hands Macrosorb (Nunc) are the best.

Optimisation and validation of an ELISA for detection of antibody to cyprinid herpesvirus 3 (CyHV-3, KHV)

Keith Way
Cefas Laboratory, The Nothe, Weymouth, Dorset, DT4 8UB, UK.

Abstract: An ELISA was developed for detection of serum anti-KHV antibodies in populations of experimentally exposed common carp (St Hilaire et al. 2009). However, a thorough validation was required before the ELISA could be used to assess previous exposure to KHV in natural populations of carp. During the early stages of the validation, minor improvements were made to the KHV antibody ELISA and further optimisation of the method produced a more robust test that could be performed by less experienced technicians. The test was shown to have good repeatability between technicians in the same laboratory and in different laboratories.
Cross-reaction with Cyprinid herpesvirus-1 (carp pox) antigens was observed at serum dilutions of 1/200 and 1/400. To achieve the necessary analytic specificity higher serum dilutions of 1/800 & 1/1600 were tested in the assay although this did result in a lowering of the analytic sensitivity.

Later stages of the assay validation included ELISA data from tests on 262 serum samples from known sero-positive populations of common and koi carp from 8 sites with a history of KHV disease (KHVD) and from tests on 475 serum samples from sero-negative populations of carp from 20 sites with no history of KHVD. Also included was data from tests on 72 serum samples from 3 sites where a high prevalence of carp pox was observed in the common carp population. This data was then used to establish a more robust positive negative cut-off or threshold.

The use of the sample to positive (S/P) ratio rather than a specific OD value, to determine cut-off values in the test, gave a significantly better overall diagnostic performance. A high positive/negative cut-off was set at a S/P ratio of 13.67% and this gave a diagnostic specificity (D-SP) of 98% and diagnostic sensitivity (D-SN) of 72%. To increase diagnostic sensitivity a low positive/negative cut-off was set at 6.02% and this increased the D-SN to 80% and gave a lower D-SP of 91%. Although the ELISA has only a moderate sensitivity (D-SN 72-80%) the performance characteristics showed that the assay was effective at the population level with a cut-off set to give a specificity of 98% or higher. The assay is not as effective as a tool to determine the exposure of individual fish.

Minutes

Sera from experimentally infected carps were used to develop an ELISA method for detection of KHV. The development has required optimisation at several levels e.g. the dilution of sera. In general the method can effectively be used on a population level whereas it is not that effective to use on individual fish. If the method is used in other laboratories, it may require further optimisation – especially where laboratories works at higher temperatures to ensure high robustness of the method.

Questions

Giuseppe Bovo: Does the temperature affect the results of the ELISA as can happen for e.g. SVCV?
***Keith Way*:** Antibody has been detected in sera sampled in different months – the best season to sample is when temperature increases – see paper in JFD + paper in press on distribution of KHV.
***Sven Bergmann*:** Is it the N-gene that is recognised by the antibodies and what about antibody kinetics – at what time does the antibody arrive in the serum?
Keith Way: Whole viruses have been used in the assays. Concerning the kinetics, the cut off dilution is relatively high. After about 3 weeks you should see antibodies.
***Niels Jørgen Olesen*:** How much purified virus did you use in such assays?

***Keith Way*:** We grow the virus on CCB cells and generate enough antigen for 6 months from 12 (75 cm^2) flasks.
***Sven Bergmann*:** KF-1 cells over passage 100 do not grow virus well.
***Keith Way*:** We have made the same observation but we have not seen that so far with CCB cells.

Development of serological methods for detection of Koi herpes virus (KHV) antibodies in carp, *Cyprinus carpio*

J. Castric*, N. J. Olesen, S. Bergmann, G. Bovo, O. Haenen, E. Jansson, M. Matras, D. Hongan
Afssa Ploufragan/Plouzané, Unité de pathologie virale des poisons, France

Abstract: Due to the difficulties encountered to isolate or identify CyHV-3 in asymptomatic carriers carp, a one year project on KHV serology has started between seven participants from European laboratories and one from China.
The main objective of this EPIZONE project is to develop, validate and implement serological techniques, seroneutralisation, immunofluorescence and ELISA, for detection of antibodies against KHV. The project will aim to compare the three methods regarding sensitivity, specificity and applicability under standard laboratory conditions. Different parameters will be assessed: viral strain, susceptible cell lines, antigen preparations, incubation conditions etc. The project has started in March 2009 by exchange of cells, virus, rabbit and carp sera between the partners. After the best cell line for virus production and titration has been chosen, the serological techniques will be adapted and tested so that standard operation procedures could be made available for all participants. An inter-laboratory proficiency test will then be organized in order to validate the different techniques and evaluate their applicability in the surveillance of carp populations regarding KHV.

Questions
***Olga Haenen*:** It is important to have more methods for KHV diagnostics and to have a method where you don't have to kill the fish.
***Francois Lieffrig*:** In grass carp, are conditions the same?
***Jeanette Castric*:** In general yes.
Keith Way: There is no evidence that KHV replicates in grass carp.
***Rob Raynard*:** Keith Way have already a good ELISA method quite similar to what you are developing - is this project repetition of previous work? Do CEFAS have available some of the reagents already?
***Keith Way*:** All of the reagents apart from the coating antigen of purified KHV are commercially available.
Jeanette Castric: When we started this project the publication was not available and the project was started in Epizone regi.
***Niels Jørgen Olesen*:** There should be collaboration between us and CEFAS. One advantage with more tests as SNT, ELISA and IFAT is that you can compare the tests. Until now CEFAS' work has provided a commercial test that is not publicly available. We would like to develope a test that is publicly available.

Case story from Giuseppe Bovo:
Presentation of surveillance for and detection of IHN Infection. In this case one sample (surveillance) was tested positive by PCR. However, there was no virus isolation and no mortality.

The farm was put under restriction - continued surveillance. Three months later: very few fish showed clinical signs. Sampling was made on symptomatic and non symptomatic fish. No virus positivity has been detected. When we went to serology (50% PNT) we observed 20% positivity in symptomatic fish (1/5) and 41.8% in non symptomatic fish (23/55) These data suggest that in endemic or chronic situations serology could help a lot for a correct diagnosis results

Questions
***Giuseppe Bovo*:** Regarding the negative result from symptomatic fish, an explanation could be that some viruses have a high titer whereas others have a low. If the sample furthermore contain blood – maybe neutralisation in vivo occur?
***Niels Jørgen Olesen*:** Maybe we should look more into serology for surveillance?
***Brit Hjeltnes*:** Were the samples PCR negative?
Giuseppe Bovo: Yes, only the first test was PCR positive. However, the sensitivity of this procedure is low – as stated by the OIE reference laboratory and is mainly used for identification after virus isolation.
Birgit Oidtmann: It is a general questions mark for testing systems if the isolation did not work.
***Giuseppe Bovo*:** It is problematic for IHNV – Serology methods might help.
Brit Hjeltnes: For surveillance – do we have proper tests?
***Niels Jørgen Olesen*:** Serology is most valuable in endemic areas. So we have to look for the purpose of the test.

MINI-WORKSHOP on implementation of Council Directive 2006/88/EC

The Key issues to be addressed when implementing the Council Directive 2006/88/EC

Sigrid Cabot
European Commission, Health & Consumer Protection Directorate-General, Unit D1 - Animal health and Standing Committees

Abstract: Council Directive 2006/88/EC lay down animal health requirements for aquaculture animals and products thereof and contain provisions on the prevention and control of certain diseases in aquatic animals.

The provisions of the Directive can be divided into three elements/pillars:

(a) the animal health requirements to be applied for the placing on the market, the importation and the transit of aquaculture animals and products thereof;

(b) minimum preventive measures aimed at increasing the awareness and preparedness of the competent authorities, aquaculture production business operators and others related to this industry, for diseases in aquaculture animals; and

(c) minimum control measures to be applied in the event of a suspicion of, or an outbreak of certain diseases in aquatic animals.

Compared to the previous legislation on aquatic animal health (Council Directives 91/67/EEC, 95/53/EEC and 95/70/EC) the following elements are either new or strengthened and thus would need special attention in the implementation of the Directive:

- The authorisation of all farms and certain processing establishments

- The establishment of a farm register
- Stronger focus on preventive measures, including risk-based animal health surveillance of all farms and traceability.
- Broader disease notification requirements
- Strengthen contingency plan requirements for emerging and exotic diseases
- New listed diseases: (KHV, EUS and EHN)

Minutes

Scope of the directive:

-Placing on the market, movement, import and transit of aquaculture animals or products thereof

-Minimum preventive measures for awareness and preparedness of diseases

-Minimum control measures in the event of suspicion of, or outbreak of certain diseases.

Aquaculture businesses has to be authorized, so does processing establishments involved in disease control – derogations from this: Animals that are kept without intention to be put on market, put & take, and businesses that will only sell small quantities directly to final consumer or local retail establishments directly supplying the final consumer.

When applying derogation: The directive shall apply mutatis mutandis: take into account the nature, characteristics and situations of the installation in question, and the risk of spreading disease as a result of its operation.

Conditions for authorization; Traceability (keeping of records of movements and mortalities), good hygiene practice, animal health surveillance scheme. Once the authorization is given, controls should be carried out.

Member states shall make a publicly available list of authorized aquaculture production businesses (APB). Internet based information page – decision 2008/392. This is a way to facilitate trade between farmers in different countries. This has to be put in place before August 1st 2009.

Risk based surveillance scheme: All farms and mollusc farming areas. Can be done by competent authorities (CA) or qualified aquatic animal health service. What to do: Advice farmers on animal health issues. Detect mortality and listed diseases. Sampling and laboratory analysis not obligatory but to be decided at inspection. How often the farm should be visited depends on the disease category within which it is listed, and the risk level of the farm (Decision 2008/896/EC – guidelines).

Disease notification: Early warning system; notification to CA of increased mortality or suspicion on listed disease. Notification through ADNS (decision 2008/650/EC)

Disease control

Listed exotic diseases: Import requirements for susceptible species and vectors. Eradication if detected in community.

Listed non-exotic diseases: Suspicion shall be notified. Part of general surveillance. If detected: eradicate or contain, according to disease category and national disease strategy. Disease specific placing on the market requirements.

4 ways to achieve disease freedom:

1. No susceptible species
2. Pathogen can not survive in the area
3. Historical freedom
4. Targeted surveillance

National measures: Member states may take appropriate and necessary measures to prevent the introduction or to control other non-listed diseases, which pose a significant risk. Measures that affect trade between Member States must be approved by the Commission under the Comitology procedure.

NRL
Legislation implementing directive 2006/88/EC: 1251/2008, 1250/2008, 2008/946.

Sampling and diagnostic plans on VHS, IHN, KHV and ISA

Giuseppe Bovo
Istituto Zooprofilattico Sperimentale delle Venezie, Viale dell'Università 10, 35020 Legnaro (PD) Italy.

Abstract: Following the publication of directive 2006/88/EC and because of the experience reached during the last years there is an urgent need to improve the existing legislation concerning sampling methods, surveillance programmes and diagnostic procedures to be adopted both for old and new listed diseases.
No official documents has up to now been issued by the Commission and this presentation is tightly based on the opinions and suggestions of a group of invited private/governmental experts and in any circumstances may be regarded as stating an official position of the European Commission. Contrary to the previous decisions no detailed laboratory procedures will be included in the next manual which will contain only general information while the complete details will be available at the CRL web-site. Besides to basic requirements already introduced in the previous manuals, like the species to be sampled, tissues to be processed, sampling season, interval between two consecutive controls, additional criteria will be introduced as the minimum diagnostic requirements to rule out the infection suspicion or the possibility for immediate reinstatement of the free status.
Different surveillance programmes, to be adopted according to the actual status of compartments/zones will permit to achieve the free status in 2-4 years. In addition the possibility for immediate reinstatement of the free status will also be introduced in the new legislation, only applicable to previously free individual farms which free status has been withdrawn and provided that the sanitary status is independent from surrounding natural waters and if the epizootic investigation concludes that the disease has no further spread to other farms or in the wild.
In some instances, as in the case of KHV, the surveillance plans will be influenced by the density of carp farms inside a catchment basin and when their number is considered limited and targeted surveillance does not provide sufficient epidemiological data of the whole basin sampling would necessary include fish from the wild.

Minutes

The next diagnostic manual will contain sampling details, surveillance programmes, criteria for disease suspicion and confirmation and diagnostic methods. This manual will be available at the CRL website.
Containment areas are defined based on a case to case analysis. The containment area includes the production zone and a 10 km radius surveillance zone.
Sampling criteria set up for VHS & IHN according to the specific disease.
Model A: 2 year surveillance scheme
Model B: Immediate reinstatement of disease free status (previously free, independent from other farms)

Model C: 4 year surveillance programme with reduced sample size.
In order to maintain disease free status, the farms must be in a surveillance programme where sampling is done according to risk level of each farm.

A model for risk ranking fish farms to inform disease risk-based surveillance

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Cefas, Weymouth Laboratory, Barrack Road, Weymouth, Dorset, DT4 8UB, UK

Abstract: Until recently, fish farms involved in disease surveillance programmes in the EU have been visited and samples collected following a prescribed, non-risk based approach. The recent European Council Directive 2006/88/EC on aquatic animal health requires that risk-based animal health surveillance is applied to each aquaculture production business (APB) in the EU. The frequency of visits should take account of the likelihood that a fish farm may contract and spread disease and this requires an assessment of the level of risk applying to each APB. To assist in this process we have developed a model for risk ranking fish farms that can also be applied to other types of APBs (e.g. mollusc farming areas, crustacean farms).

Through stakeholder consultation and input by aquatic animal disease experts and epidemiologists, we have identified a range of risk factor themes: (1) Live fish movements on and off site, (2) Exposure via water, (3) On site processing, (4) Biosecurity, (5) Management practices, (6) Geographical factors, (7) Mechanical transmission and (8) Other routes. We demonstrate how information regarding these risk factors can be assessed and combined to achieve risk scores for introduction and spread.

Minutes

Farms can have different categories for the different diseases.
The presented model is for salmonid farms, with regards to VHS – focus was category 1 farms, using a semi-quantitative approach.

Risk factors: live fish movements, exposure via water on site processing, biosecurity, management practices, geographical factors, mechanical transmission, other routes/ risk

Quantitative factors: Live-fish movements, likelihood of farms to become infected/spread disease within a year.
Semi-quantitative factors: Exposure/spread via water, hazards, flooding. Give a score instead of numbers.

Example: Likelihood of farm becoming infected from live-fish movement: probability of source being infected (number of breakdowns of disease-free farms out of all free farms) x number of sources.

The model is transparent and allows the farmers to see how their score was calculated and how any of their activities influences the score.

Birgit has CD's with the programme for the model.

Workshop on the implementation of the new Fish Health Directive – group Northern Europe
Chair: Birgit Oidtmann. Reporter: Birgit Oidtmann.

Questions discussed:
Have member states completed the process of authorising and registering Aquaculture Production Businesses?
Which types of APBs were authorised opposed to registered?
Have they risk ranked their farms?
Have MS categorised all their farms with regards to their disease category?
For member states that have farms that fall into Cat. III, do they intend to move out of this category, or do they consider Cat. III as a permanent status?

Most MS were well progressed with regards to authorising and registering farms.

- The Faroe Islands have 6 salmonid APBs and 4 processing sites that are authorised. Transporters were authorised and so were the 4 or 5 put and take fisheries in their country.
- England &Wales have about 580 finfish APBs that were authorised. Furthermore, there are about 60 mollusc farming areas and 52 purification centres. There is currently no authorised processing plant. About 2000 fisheries are currently registered, although this does probably not comprise all fisheries yet that exist.
- Denmark has around 345 fin fish farms, 15 processors, 65 mollusc farming areas, and 10 crayfish farms – all authorised.
- Ireland has 99 fish farms, 3 processing plants and 450-500 mollusc farming areas. The authorisation of finfish farms and processing plants is completed. The authorisation of the mollusc farming areas is still in process. Ireland will authorise put and take fisheries. Transporters will be registered.
- Sweden has 140 fin fish farms that will all be authorised. A decision regarding the classification of crayfish farms has not yet been made. The 6 mollusc farming areas will be authorised. The number of processing plants is currently unclear. Transporters will be registered. Sweden has around 300-400 put and take fisheries.
- Finland had to make legal amendments to implement the directive. This was needed in order to have the legal basis to request authorisation of aquaculture farms and processing plants; publish information of the authorised farms/companies on the web
  Finland has around 500 fin fish farms (150 marine, and 350 inland) and 50 crayfish farms. About 80% of fish farming companies have submitted their application for authorisation to the Competent Authority and only 7 of the 50 crayfish farms. Therefore some follow up is still required.
  A threshold by production was set to decide whether APBs were registered or required authorisation. The threshold is 2,000 kg/year for fish farms or less than 2,000 kg of fish feed purchased by the farm and fish only being sold locally.
  Crayfish farms did not require authorisation if they produce less than 2000 animals/year and sell only locally. Derogation from authorisation also applies to angling ponds stocked with farmed fish, shellfish farms (reared for cleaning water) ornamental fish farms not in direct contact with the water systems.
- In Norway, all fish farms have been authorised. Put and take fisheries don't exist.
- In Scotland, the authorisation is still in process. Fisheries have not yet been registered.

Risk ranking of farms and surveillance programme:
Most MS have risk ranked their farms and worked out their surveillance programmes. The surveillance programmes are above the minimum requirements presented by

Guiseppe Bovo earlier that day.

- Ireland has completed the process of risk categorising its farms. Farms were ranked depending on production type. Ireland has decided on 2 visits/year for high risk farms (1 Art. 7, 1 Art. 10), 1 for medium risk farms (alternating in 1 year Art. 7 visit, in next year, Art. 10 visit). Low risk farms will be visited every other year.
- Sweden is still in the process of risk ranking its APBs. Farms are currently grouped by criteria such as size, location, and whether or not they are restocking, but risk ranking criteria to be used still need to be finalised, but focus will be laid on live fish movements.
- Scotland has completed the process of risk ranking its farms.
- Denmark has completed the process of risk ranking its farm during the eradication program for VHS.
- Finland has decided on its surveillance programme and set criteria for increased sampling frequency; these include: live fish movements from the marine to freshwater farms, imports of live eggs from abroad, marine farms located in the vicinity of slaughter houses, and others.

Categorising farms into Cat. I-V

This process was largely completed for all salmonid farms. Most salmonid farms in Northern Europe would have had a status prior to the implementation with regards to VHS/IHN. Therefore, this could easily be transposed into the new FHD.
With regards to cyprinid farms: some countries, such as Norway, don't have cyprinid farms and therefore no need to categorise them. In those countries, that do have cyprinid APBs, virtually all farms are currently in Cat. III with regards to KHV.
There was insufficient time to discuss how this has progressed for crustacean and mollusc farms.

Status of marine RBT farms

The question of how to deal with marine RBT farms was discussed. Niels Jørgen Olesen had earlier mentioned that Denmark had decided to place marine RBT farms into Cat. III. This was based on the assessment that, given the continuous exposure of the fish to VHSV from wild fish, it seemed not reasonable to class these RBT farms as Cat. I. Large RBT may be used for freshwater fisheries, which could jeopardise the status of freshwater sites. It seemed not sensible to put freshwater sites at risk by granting marine RBT sites Cat. I status.

It was felt that if fish farms that are at a high risk of becoming infected, such as the example of a RBT farm in the marine, would obtain Cat. I status, farms of Cat. I status receiving fish from such sources would equally be under a possibly constant threat of becoming infected. Therefore, it was questioned whether farms currently free from a listed pathogen should ever be given Cat. I status, given that these farms may pose a high risk of becoming infected. The consequences for an importing country could be substantial, e.g. a member state might loose its disease free status. Therefore, it was questionable, whether Cat. I status can be granted to APBs free from infection, but at a high risk of becoming infected.

On the question of how to deal with

Duration of Cat. III status:
Different views were expressed on whether or not farms should be allowed to stay for an extended time period in Cat. III.

Scotland felt that farms should be allowed to stay in this category, whereas Ireland took the view that Cat. III was supposed to be a transitional category.

Workshop on the implementation of the new Fish Health Directive – group Continental Europe

Chair: Niels Jørgen Olesen. Reporter: Olga Haenen.

Czech Republic: Farms are under national survey program for VHS, IHN and IPN, and also some for KHV. There are no approved farms according to EC legislation, and the program has not yet been sent to the EC.

Estonia: On historical basis they are Cat. I. Representative was not sure, if the application was sent already to the EC.

Austria: Data are mainly focused on VHS & IHN national program. Since 2006 also KHV and IPN testing. Most facilities are in group III. There is not yet surveillance according to the EC for category III, but there is for cat. II. When Cat. I would be reached is not sure yet. There is only 1 farm in cat. IV, so no cat. V at all, i.e. no infected farms.

Germany: Many farms in cat. I in non approved zones, some in approved zones. No clue about the missing farms in the scheme, the high number is probably due to the high number of non-registered carp farms. Still approx. 2000 rainbow trout farms are missing from the lists? Sven Bergmann: Probably problem with the registration of farms, as there are so many. Also it is unclear whether not-authorized but registered farms shall be categorized. Heike Schütze: 2nd problem: many hobby farms? Where to put the border yes/no registration? Some federal states have governmental service (vet.), but some are private. 12 Farms in cat. V is a too low number, as there were >6 outbreaks of IHN lately already. Sven Bergmann will meet the ministry next week and will ask for clarification. Also the 0 farms for KHV in cat. V is nonsense. Problem: FLI is not involved in the data for this table. Sigrid Cabot: The only application the EC received from the whole EU with a proposed control program on KHV is from Saxony. Category IV is 40x carp farm, according to Sigrid.

Belgium: The directive is not yet implemented in the federal system. Twice a year 30 fish from S-Belgium are under survey, and 3 salmonid farms in S-Belgium are infected (cat. V). The carp production is mainly in the north. In the lab, KHV is often diagnosed in koi, but the sources are unknown (closed/open?). The health situation has improved in S-Belgium. The survey program is not yet approved by the EC.

Netherlands: Niels Jørgen Olesen remarks, that all salmonid farms of the whole EU should be in cat. I for ISA, as the EU is free of that disease. Olga remarks, the missing farms are farms with non-susceptible species. It is confirmed by Sigrid, that only susceptible species should be categorized.

France: Data came from the authorities; Jeannette Castric is surprised that no cat. V farms were given for both IHN and VHS! Cat. I is true, right values. Cat. II: true too, they will move to Cat. I in 2 years. Brittany, Charentes and S-W France are free of VHS and IHN. There is only a surveillance program for salmonids. The KHV data might be right. Cat. I and II are all registered, small farms are difficult to register, and this will not be ready before the end of July 2009. There are problems with risk evaluation, working on data surveillance before submitting the plans to the EC.

Sigrid Cabot, in general:

- The end of June a meeting is foreseen to see, how far the MS are with their implementation.
- The MS will be reminded to have their registers in place, according to EC/2009/177.

- Many data which are presented today are not known yet to the Commission.
- Sigrid explains the trade possibilities between categories, as given in the directive.

Switzerland: The 5 farms in Cat. I and II are regularly tested, but this is not yet approved by the EC. Thomas Wahli doesn't think he could get that approval. Switzerland has: 1) a program to register all farms, and 2) a project to develop a strategy for a risk based surveillance program in accordance of EU regulations. Both have just started.

Lithuania: In April 2008 the implementation was started, and now 28 farms are registered. The data came from the Veterinary Service, so the delegate have no details.

Hungaria: There is a survey program for SVC since 1999-2008. No virus could be found (SVC and other viruses, except iridoviruses). In 2009 an EHN-program has started. For KHV, 120 farms was tested by real time PCR, and they were all negative. There are 1 or 2 surveys per year. The application for disease free status was submitted to the EC by Sept 2008, for KHVD. Hungaria has a risk because of geography, especially for KHV. All carp farms are tested (consumption- and production farms). All lakes should be tested still for KHV.

Latvia: The state surveillance program includes fewer farms (totally 30) than given in the table. For KHV testing there are 20 farms, which produce carp and other species, but for salmonid diseases surveillance - 16 farms, which produce salmonids (farms and state hatcheries, which produce carp, salmonids and/or other freshwater and marine species are included in both surveillances). Small hobby fish farms (totally 143) are not included in surveillance program. The program is not yet approved by the EC. Sigrid Cabot: Yes, the farms should be in cat. III, but only after EC approval they can move. Latvia: The state wants 2 registers: 1) state hatcheries & farms with surveillance, and 2) hobby fish farms. Not sure if that is a good idea.

Slovakia: All cat. I farms are registered and under surveillance. In 2008 there were 2 outbreaks of VHS, and another outbreak last week. A program has been made by the veterinary authority to eradicate the disease. It is not known if we can get cat. I based on historical grounds or only on monitoring. If approved by the EC we can adapt the data.

In general:
Sigrid Cabot: The application is not very complex, and you will soon get an answer from the Commission. Niels Jørgen Olesen: There are many cat. I farms now, so this will influence the trade. Sven Bergmann: You are only cat. I when officially approved by the EC. Niels Jørgen Olesen: Yes, only an official sampling & frequency will be authorized, not many countries will have this already in place.

Poland: Nearly all rainbow trout farms are surveyed, but not officially yet. Some of the cat. V KHVD farms should be Cat. III. Niels Jørgen Olesen: Yes, when you have them all in cat. V, all fish may enter Poland! Sven Bergmann: And when you are cat. V you can only trade with cat. V, whereas there is a lot of trade between Germany, Czech Republic and Poland.

Luxembourg: François Lieffrig tells there are no data, but there are 2 farms under survey by Belgium.

General points for discussion
- Olga Haenen, NL: Our veterinary service has problems with the practice of certificates, especially the import health certificates.
- Tomáš Veselý, CZ: we need 1 system, transparent for all MS.

- Sigrid Cabot, EC: Zones of >75% of the area of the MS are o.k., and smaller sites should be on the website. It is good to see today, that there are many national programs in place.
- Niels Jørgen Olesen, DK: The implementation and zoning should be coordinated through your CVO.
- Sigrid Cabot, EC: If the CRL publishes these data, there should be a disclaimer, because the data may not be correct. It may be misused for trade namely. Take care!
- Niels Jørgen Olesen, DK: What about registration in MS? Tomáš Veselý, CZ: There are hundreds of authorizations still in process, but it goes o.k., including geographical coordinates. Sven Bergmann, GE: The regional fish health services add coordinates in some federal states, in others not. Niels Jørgen Olesen: The coordinates are not obligatory to add, but could be useful. Is the federal law in Germany blocking the EU law? Sigrid Cabot: This should not be the case, as we just need information of business, and not on personal data, it is a community law, so approved by all MS, and it should be followed, the national privacy regulations are overruled by the EC. Sven Bergmann: will ask his government on this the 9th of June 2009.

Workshop on the implementation of the new Fish Health Directive – group Mediterranean Europe
Chair: Giuseppe Bovo. Reporter: Giuseppe Bovo.

Italy: Different registration has been used in some regions. So the first challenge is to unify the coding. The CA is building a database in order to compile this information. The database should be finished by summertime. There has been no categorisation yet. The ministry wants to be declared disease free in to category 1 immediately. Marine farms are mainly rearing non susceptible species like sea bass or sea bream; they will be put in category 1. Put and take fisheries connected with the river system will be considered as farms, registered, authorised and categorised. Those put & take lakes that are not in contact with waterways will not be authorised and categorized, but only registered. They are never visited. The problem with put & take facilities is that they have no interest in getting rid of disease, so they are always infected. It is very good that this directive requires that all farms be registered. Now we can have en exact view of how many farms there are, and their health status. The ministry is not happy about making it public.

Turkey: There is no implementation of this directive, unfortunately. Probably should do so in the future, because of the trade with EU-countries.

Spain: Is similar to Italy. Have a database for fish production units, and is preparing a new database with data on the farms and including their health status to be used for the categorisation. These databases are connected and will be made public. The owner of the database is the Ministry of Agriculture, but every region has their own database that is connected to this one. The registration is in the regions, so it depends on which region.

Romania: Some farms are authorized but within an old system. For the new authorization, we are waiting for the new draft to be approved. There is a register of the farms, and this has to be transferred to the competent authorities.

Serbia: Implementation is at the beginning: the fish farms are centrally registered, in department of agriculture, but it has not been made public yet. The farms have not been categorized. There is a programme for certain diseases; SVC, IPN, VHS and a few bacterial diseases. So there is some data about the health status of some of the fish farms.

Bosnia-Hercegovina: For the last 5 years there has been a surveillance programme. There are 28 salmonid fish farms and 5 carp farms that are centrally registered by the veterinary authorities – they have a veterinary number and are under inspection. In total there is about 120 salmonid farms, 25 cyprinid farms and 2 marine fish farms, but only the previously mentioned are veterinary registered, whereas the others are registered, but not by the veterinary service. They have officially never been visited by the veterinary services. This year monitoring is started on two diseases. Most farms will be included in category II.

Slovenia: Is trying to follow the deadlines. Authorization is in progress, 22 farms are authorized according to the rules of the directive. The majority of farms have been registered with regard to species, trade and so on. We are mostly acquainted with the health status, since there has been surveillance for many years. All fish health management is centralised. We are finishing the application of the website - making the list public before the 1st of August - it is being tested now. Fish farmers are not interested in the surveillance by the Commission Decision. At the moment there are three farms in the category I - you can find them on the website, already. However we have our own surveillance programme paid by VARS (Veterinary Administration Republic of Slovenia). Regarding this programme all hatcheries and fish farms with life fish market are monitored for VHSV and IHNV. Other farms are tested regarding the clinical and pathoanatomical signs presumptive to VHS and IHN or regarding to the epizootiological investigation. Unfortunately fish farms in "our programme" correspond to the category III. In all others the health situation is unknown and is officially infected. In fact we have got 3 VHSV infected fish farms. We are encouraging fish farmers for its eradication. The working team for fish diseases management has prepared a manual for good hygienic measures, and designed a questionnaire for risk ranking.

Portugal: All farms are in category II, because they are not declared disease free, but there has been a surveillance programme for 15 years. Plan to apply for historical freedom as documentation for placing in category I. The directive is still not implemented in Portuguese legislation, but we are preparing for authorization and categorization. Two fish farms are just now moved to category I.

Bulgaria: The directive has been published in the state gazette. All farms have been registered, and geographical details are also under way. 47 trout farms, 199 carps, 4 crustacean farms. In the register is name, address, type of production, details on water supply. Work is undergoing to include health status and categorisation of farms. Surveillance programme was initiated 3 years ago. 24 trout farms are in category I for VHS, 1 farm is in category IV and one in category V. We intend to implement passive surveillance for KHV.

Greece: The Directive was implemented in national legislation in March 2009. All farms, including marine farms, will be registered before end of July. What happens if someone has sites in both Greece and Turkey? Trout farms and cyprinids will be in category III. The ministry takes care of surveillance.

Cyprus: All the farms (20 in total) are registered. The Directive has been implemented. There is 6 farms with rainbow trout and they are in category I for VHS and EHN. 2 farms with Koi carp are in category 3. Authorization is by veterinary authorization.

Croatia: The legislation is in place. All farms are registered. There is a national surveillance programme for listed diseases including SVC. Farms are not authorized; therefore all farms are put in category III. Internet-site is under preparation. Database with geographic positions is being prepared.

Albania: The directive is not implemented yet, but there is surveillance of farms. The registration is now under Ministry for Environment, but needs to be under VA.
Kosovo: The directive has not been implemented in local legislation yet. There are 50 farms.

There is confusion what is meant with "farms that provide only for human consumption" – Does that mean that this is facilities that only buy living fish and stock them for a few days and then sell them for human consumption? So no "farming" as such, but only stocking?

Until the farms are authorized, they cannot be categorized. So many of the farms that have been noted to be in categories in the questionnaire are based on previous legislation.
Need manual on good hygiene practice and the demonstration that there is a surveillance programme, before farms can be authorized.

In many countries the registration is in different departments, and this has to be coordinated.
First of all; all farms should be registered, then they should be authorized, and then they can be categorized. A lot of work: surveillance schemes, hygienic manual etc.

Workshop on the implementation of the new Fish Health Directive – summary of group discussions

Northern European countries: Authorization: DK, Faeroe Islands and Norway are finished, and rest will finish before summer. All have surveillance programmes in place. There has been discussion about susceptible species. Should those that are not susceptible still be categorized? This does not make sense, only relevant diseases and species should be categorized. Risk-ranking: Denmark, and UK has already done it. The focus has clearly been on trade with live fish.

Continental European countries: There are big differences in the way the member states have categorized the farms. It is important that this is harmonized. Some countries are registered free of KHV, but it is uncertain if this is approved by the commission. In some of the states it is not allowed to put private details like name and address on the internet, but since this is EU legislation, they will have to comply.

Mediterranean countries: In some countries the Directive has not yet been implemented in local legislation. Often the farms are registered in one ministry and have to be transferred to the veterinary authorities. Some countries have several regions that each has their own codes, and it is difficult to harmonise this. Remember that even if the farms have no susceptible species, they still need to be authorized and surveyed every four years.

SESSION III Scientific research update

Susceptible species to listed diseases - EFSA Report

Ana Afonso* and Franck Berthe
EFSA Animal Health and Welfare Panel

Abstract: Following a request from the European Commission, the Panel on Animal Health and Welfare was asked to deliver a scientific opinion on aquatic animal species susceptible to the diseases listed in the Council Directive 2006/88/EC. More specifically, the question was to establish i) which species other than those listed in Part II of Annex IV to Directive 2006/88/EC that could be considered as susceptible and ii) which of the species currently listed as susceptible in Part II of Annex IV to Directive 2006/88/EC cannot be considered as susceptible.

A comprehensive literature review was performed with considerations for: i) reflection of natural pathways provided by the experimental design of reported studies, ii) compliance with four objective criteria pertaining to susceptibility to infection, and iii) thorough identification of the causative agent.

The four criteria used to assess susceptibility of host species were: evidence of replication or growth of the organism (A), presence of a viable organism (B), presence of specific clinicopathological changes (C), and specific location of the pathogen (D).

This led to identification of two main groups: Group I, host species for which the quality of the data provided clear support for susceptibility, and Group II, host species for which incomplete or unclear data prevented a clear conclusion or the only available data was obtained from invasive experiments. Group I (susceptible species) contains i) traded and non-traded species, ii) species belonging to several genera, and iii) many were susceptible to several of the specified pathogens, so may represent different levels of risk.

Within Group I, species were identified that currently are not listed in Directive 2006/88/EC and those species are recommended to be considered for possible inclusion. Partial evidence suggesting susceptibility was obtained for a large number of host species (Group II). Several host species, including some currently listed in Directive 2006/88/EC, were identified as potentially non-susceptible but it was not possible to confirm this status firmly due to the quality of the data.

Further scientific studies are required to resolve the uncertainty concerning the susceptibility of the host species identified in this group. Such studies should apply clear criteria, such as those used in this opinion, to assess susceptibility of host species and clear identification of the pathogen and affected host(s). In addition, the opinion noted that the lack of clear case definition for some of the specified pathogens compromised assessment of the susceptibility of some host species.

Minutes

The objective of this work was to revise the list of susceptible species in the annex of CD 2006/88/EC based on available scientific literature. A risk assessment approach was not followed. Neither was it taken into consideration whether the fish were farmed or imported into EU.
The question of susceptible species was raised by the vector report (for links to the reports, see http://www.crl-fish.eu/useful_links.aspx). Vectors = mechanical carriers.
A problem in the work has been that a clear case definition has not always existed, and the pathogen ID is not always clear, especially for mollusc pathogens.
The list should be reviewed regularly.

Questions

***Sven Bergman*:** Can you explain the difference between whitespot syndrome virus in decapods and KHV in cyprinids? Why are all decapods listed as susceptible for whitespot syndrome virus when all cyprinids are not for KHV?

***Ana Afonso*:** EFSA can not answer to that question since it is a risk managers decision.

***Birgit Oidtman*:** For whitespot syndrome virus, every decapod tested has been found susceptible. I am not sure if this is the case for cyprinids and KHV.

***Ana Afonso*:** The criteria of when to include the whole group or not should be clearly defined based on scientific principles.

***Niels Jørgen Olesen*:** How do you think the Directive will look like after this work? What is your recommendation?

***Ana Afonso*:** EFSA is created to give scientific advice, but the legislators should also take other things into account. It is up to the Commission to decide what to do with this report. The EFSA AHAW panel recommended that the species in group I should be listed as susceptible species, but a risk assessment may have to follow before taking new species into the list.

***Sven Bergman*:** There are no cyprinids which are not infectable and do not grow the virus.

***Olga Haenen*:** The report could only take into account peer reviewed literature, so hopefully the cyprinid-KHV work will be published soon and taken into account when the EFSA reviews it.

Establishment of a CRL-database for fish pathogenic viruses

Søren Peter Jonstrup*[1], Tanya Gray[2], Søren Kahns[1], Helle Frank Skall[1], Mike Snow[3] and Niels Jørgen Olesen[1]

[1]Community Reference Laboratory for Fish Diseases, Section for Fish Diseases, National Veterinary Institute, Technical University of Denmark, Denmark, [2] Symantix Ltd, UK, [3]Fisheries Research Services (FRS) Marine Laboratory, UK

Abstract: A database has been created, www.FishPathogens.eu , with the aim of providing a single repository for collating important information on significant pathogens of aquaculture, relevant to their control and management. This database will be developed, maintained and managed as part of the European Community Reference Laboratory for Fish Diseases function. This concept has been initially developed for VHSV and will be extended in future to include information on other significant aquaculture pathogens. Information included for each isolate comprises sequence, geographic origin, host origin and useful key literature. Various search mechanisms make it easy to find specific groups of isolates. Search results can be presented in several different ways including table based, map based, and graph based outputs. When retrieving sequences, the user is given freedom to obtain data from any selected part of the genome of interest. The output of the sequence search can be readily retrieved as a FASTA file ready to be imported into a sequence alignment tool of choice, facilitating further molecular epidemiological study.

Minutes

We have chosen to use open source software, it is a low cost platform that everybody can access.

You can add virus reports and sequence reports separately, as it is not always the same persons doing the isolation and the sequencing.

Everybody can search the database. You can do map based search, blast based search or a text based search.

Manuals are available on the website.

A publication on the database is accepted and will be published soon in Journal of Fish Diseases.

In the future the database will be extended, first with the other rhabdoviruses, but also including e.g. KHV and ISAV.
At the moment there is app. 230 isolates, most of them are Danish. More isolates will be added in the future, and we invite everybody to add their isolates. If you need help for adding information please contact us, and we will guide you.
It is possible to add private notes that are not publically available, and in case you are not yet ready to release anything in the report yet, the whole report can have restricted assess.

Questions
***Irene Ørpetveit*:** Do you still wish people to publish in GenBank and like?
***Søren Peter Jonstrup*:** At the moment you are still required to publish in GenBank when publishing, but we hope in the future, that we can do this for you, when you put data into the CRL database.

Molecular characterization of VHSV and IHNV in Germany

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Abstract: The fish-pathogenic rhabdoviruses of Infectious Haematopoietic Necrosis (IHN) and Viral Haemorrhagic Septicaemia (VHS) cause substantial losses in German aquaculture. The control of viral pathogens requires intensive studies about the characterization and identification of the respective agent.
The identification of the viral glycoprotein gene sequence makes it possible to distinguish between the different isolates. The nucleotide sequences of the complete glycoprotein genes of German IHNV and VHSV isolates were determined and compared with isolates from other European countries. Comparative studies of the G gene sequences of IHNV and VHSV permit conclusions with regard to their origin, relationship and classification. Phylogenetic analyses illustrate the development and evolution of the viruses as well as the correlation between virus distribution and trade channel.
Main prerequisite for phylogenetic analyses is a close collaboration between the regional, national and international reference laboratories as well as the regional veterinary services. The European Union and the German government demand epidemiological studies of IHNV and VHSV outbreaks in free hatcheries. Until now, the identification and determination of the G gene are not subject to a regulation. In the last year 32 VHSV and 6 IHNV outbreaks were reported in Germany. Phylogenetic analyses were performed for 14 VHSV and 2 IHNV cases only. This situation is far from satisfactory. Therefore, the origin of an isolate often remains unclear. Using classical diagnostic methods a clear differentiation of identified IHNV and VHSV isolates is often impossible. Phylogenetic analyses permit a clear identification of isolates and their evolution as well as the tracing of trading practises.

Minutes
First I will start by bringing greetings from Peter Enzmann who has now retired.
IHNV consist of only 1 serogroup. Differentiation based on serology can only be done with MAbs. The original classification is based on the geographical origin of the isolates: RB (Round Butte; Salmon, Alaska and British Columbia), WRAC (Western Regional Aquaculture Center, freshwater

fish) and SRCV (Sacramento River Chinook Virus, salmon, California) corresponding to U, M and L genogroups.
VHSV, as IHNV, consist also of only 1 serogroup. There is no correlation between serology and pathogenicity.
We sequence the full length G-genes, and sequence a big chunk round the genes to be sure we have the correct G-gene sequence.
Phylogenetic analysis show influence/evolution of Swiss and Danish VHSV isolates and of French, Italian and Swiss IHNV isolates in Germany. The trade plays a big role in the distribution of VHSV in Germany.
In order to improve the epidemiological analysis we need good international cooperation and Gentleman agreements. A common database is a very good idea.

Questions
Very clear talk, no questions.

Distinction between genotypes of Viral Haemorrhagic Septicaemia virus (VHSV) using monoclonal antibodies

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Abstract: VHSV isolates can be divided into 4 major genotypes and a number of subtypes with an almost distinct geographical distribution. Host range and pathogenicity appear to some extent to be linked with genotypes. If once new genotypes of VHSV are introduced into new areas, they can cause severe outbreaks of VHS among susceptible fishes. According to the OIE Aquatic Animal Health Code, even if the same disease agent is present in both the import and the export country, the importing country can demand health certificate of the exporting country for imports when the pathogenicity or host range of the strain in the exporting country is significantly higher or larger than that in the importing country. In order to prevent introduction to or spreading in a country of new genotypes of VHSV and to facilitate the responsibilities of exporting and importing countries, such as issuing health certificates and carry out quarantine and disease control programs, a quick and simple detection method for discriminating between each of the genotypes of VHSV is strongly desired. Monoclonal antibodies (MAbs) VHS-10 and VHS-5.18 specifically recognizing VHSV genotypes IVa and Ib respectively, as well as MAb IP5B11 recognizing all known VHSV isolates, were prepared earlier. In the present study, additional new genotype specific monoclonal antibodies against VHSV were produced, aiming at establishing a complete immunoassay for typing of VHSV according to genotype.

BALb-c mice were immunized with purified preparations of 7 different genotypes (I, Ia, Ib, II, III, IVa and IVb). Six MAbs from these hybridoma clones were selected and their reactivity in IFAT and ELISA tested against a large panel of 79 VHSV isolates. The isolates represent all known geno- and subgenotypes of VHSV.

Among the new MAbs, VHS-1.24, reacted with all types except genotype Ie (the Black Sea variant of VHSV), while MAb VHS-9.23 reacted with all genotypes except genotype III. MAb VHS-3.80 reacted with genotypes Ib, Ic, Id and II, only. MAb VHS-7.57 reacted with genotype II and IVa. Interestingly, MAb VHS-3.75 reacted with all genotype III isolates except the rainbow trout pathogenic isolate from Norway (NO-2007-50-385) (Dale et al. in press), but did react with the New Brunswick VHSV IVb isolate (Oliver 2002, Gagné et al. 2007). Another MAb (VHS-1.88) reacted with genotype IVb only, except with the New Brunswick isolate. The present findings

support a phenotypic difference between NO-2007-50-385 and the other virus representatives in genotype III, and genotype IVb may eventually be split up in two subgroups (the Great Lakes isolates and New Brunswick isolate).

In conclusion, we can now distinguish between all genotypes and some of subtypes of VHSV by testing isolates in IFAT or ELISA with 9 MAbs (Table 1).

Table 1 Reactivity of MAbs for various VHSV genotypes

	Genotype of VHSV								
MAbs	I /Ia	Ib	Ic/Id	Ie	II	III	IVa	IVb-G.L.	IVb-N.B.
IP5B11	+	+	+	+	+	+	+	+	+
VHS-1.24	+	+	+	-	+	+	+	+	+
VHS-9.23	+	+	+	+	+	-	+	+	+
VHS-3.80	-	+	+	-	+	-	-	-	-
VHS-7.57	-	-	-	-	+	-	+	-	-
VHS-5.18	-	+	-	-	-	-	-	-	-
VHS-3.75*	-	-	-	-	-	+	-	-	+
VHS-10	-	-	-	-	-	-	+	-	-
VHS-1.88	-	-	-	-	-	-	-	+	-

+;positive
-;negative
*; The rainbow trout pathogenic genotype III isolate is not reacting.
IVb-G.L.: Genotype IVb , the Greart Lakes isolates
IVb-N.B.: Genotype IVb, New Brunswick isolate

Questions

***Søren Peter Jonstrup*:** Do you think the MAbs recognize a 3D structure and do you think they can discriminate between pathogenicity towards certain species?

***Takafumi Ito*:** I think they recognize a 3D structure, e.g. VHS-10 don't react neither on reduced or non-reduced conditions.

***Mike Snow*:** Are the MAbs neutralizing?

***Takafumi Ito*:** No, they are not neutralizing. Maybe because the epitope is not related to the G-gene. When doing IFAT it does not look like they react with G-proteins.

Status of the RANA-project

Britt Bang Jensen
Community Reference Laboratory for Fish Diseases, Technical University of Denmark, Aarhus, Denmark

Abstract: The high pathogenicity of ranaviruses to fish and amphibians in both the EU and especially also in Australia and the USA have lead to speculations that these ranaviruses might pose a serious threat to both farmed and wild-living freshwater fish and amphibians within the European community. Therefore, the EU-project "Risk assessment of new and emerging systemic iridoviral diseases for European fish and aquatic ecosystems" (RANA, SSPE-CT-2005-006459) was initiated in June 2006 under the 7th framework programme. The RANA-project was carried out by six member institutions in the Czech Republic, Denmark, Finland, Germany, Italy and the United Kingdom and was finalised in February 2009.

The project has focused on the following topics:

-Developing and validating reliable diagnostic methods for identification of ranaviruses

-Establishing host spectrum and pathogenicity of ranaviruses in farmed and wild European fish and amphibians through experimental challenges

-Investigating the occurrence of ranaviruses in European amphibian populations and in imported ornamental fish

-Characterisation and differentiation of different ranaviruses by molecular methods

The project has generated a lot of results with regards to these topics, results which are currently becoming available to the scientific community via publication in peer-reviewed journals and presentations at international conferences.
The final outcome of the RANA-project has been a preliminary risk assessment on the risk of introduction and spread of exotic ranaviruses into the EU plus an extended and a detailed list of recommendations for the European Commission.

Minutes
EHNV, ECV and ESV can infect wild fish, farmed fish, ornamental fish and amphibians.
Both ornamental fish and farmed or wild fish could be infected with amphibian viral isolates.
Holopainen et al. have developed primers that show a very high degree of similarity between EHNV, ECV and ESV – these are 99% similar – and can infect similar fish species.
The RANA-project has looked into validation of detection methods. Giuseppe Bovo tested IHC protocols for identification of Rana viruses and found no discrimination on the different isolates besides doctorfish virus (DFV) and guppy virus 6 (GV6), which are different from the other ranaviruses. One purpose was to survey for presence of RANA viruses within the EU. In a project, 150 samples = imported frogs from the UK were screened for RANA viruses and RANA viruses were identified. A similar study made in the Chech republic on imported ornamental fish did not identify RANA viruses. Interestingly RANA viruses were identified in dead frogs from Denmark. According to the used risk assessment model it will not be possible to introduce EHNV to the EC, especially because no import of fish takes place from Australia. We had trouble confirming that redfin perch and rainbow trout can be infected by EHNV. Our studies questions if it is reasonable to list only EHNV and not the other RANA viruses and phylogenetic analyses illustrates that a new taxonomy might be needed.

Questions
***Birgit Oidtmann*:** What was the number of outbreaks in Australia?
Britt Bang Jensen: There were repeated outbreaks in redfin perch. 5-6 outbreaks occurred with very high mortality. In rainbow trout there was one outbreak showing mortality of about 0.5% in a farm located closely downstream of an infected redfin perch farm. Subsequent Australian infection experiments were made by IP.
***Richard Paley*:** In the pond with the infected Danish frogs, did you find RANA viruses in other animals?
***Britt Bang Jensen*:** There were only these amphibians in the pond. Two carps and snails were OK.
***Ana Afonso*:** The risk assessment model is based on expert opinions - and there are no notes on water contaminations. Was there any information of numbers regarding trade? And what were the origins and the species of imported ornamental fish examined in the Czech study?
***Thomas Vesely*:** Fish came from all continents and there were many different fish species.
***Britt Bang Jensen*:** Purpose to create the model figures and numbers will be included later – we looked at worst case scenario = 100% infected fish being imported.

Inter-laboratory Proficiency Test 2008

Søren Kahns,
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 for Fish Diseases (CRL) to 35 National Reference Laboratories (NRLs) in the middle of October 2008. The test was carried out according to ILAC-G13:2000 Guidelines for the

Requirements for the Competence of Providers of Proficiency Testing Schemes. The test contained five coded ampoules, with viral haemorrhagic septicaemia virus (VHSV), infectious haematopoietic necrosis virus (IHNV), infectious pancreatic necrosis virus (IPNV) or a mixture of VHSV and IPNV, respectively. The proficiency test was designed to primarily assess the ability of participating laboratories to identify the notifiable non-exotic viruses: VHSV and IHNV but also to assess their ability to differentiate other fish viruses, as IPNV, SVCV, perch rhabdovirus etc. The participants were also asked to titrate the viruses in order to assess the cell susceptibility for virus infection in the respective laboratories. In addition participants were encouraged to geno- and serotype isolates, and were asked to provide a full-length G-gene sequence of the rhabdovirus identified in the lowest numbered ampoule in the test. Participants were asked to reply latest December 12th 2008. Each laboratory has been given a code number to ensure discretion.

Outcome of Inter-laboratory Proficiency Test 2008

Identification of content: 22 participating laboratories correctly identified all viruses in all ampoules. 9 laboratories did not identify VHSV in ampoule IV. One laboratory did not identify IPNV in ampoule IV. Two laboratories found more isolates in an ampoule than were actually present. Two laboratories found SVCV in an ampoule when it was not present. 8 laboratories serotyped some isolates. 15 laboratories genotyped some isolates. 17 laboratories submitted sequences.

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. 23 laboratories used ELISA for identification of viruses. 22 laboratories used IFAT for identification of viruses. 11 laboratories used neutralisation tests for identification of viruses. 29 laboratories used PCR for identification of viruses. 9 laboratories used other methods for identification of viruses. 29 laboratories used BF-2 cells. 33 laboratories used EPC cells. 12 laboratories used RTG-2 cells. 9 laboratories used FHM cells. 2 laboratories used CHSE-214 cells

Concluding remarks

In the ampoule containing a mixture of two viruses, only the IPNV and not the VHSV was identified by 9 laboratories. We encourage participants to be aware of the possibility of more viruses being present at the same time and that one can over grow the other on cell cultures, and thereby masking its presence.

The low performance in several laboratories of their RTG-2 cell lines for virus growth 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, we recommend that laboratories use BF-2 cells and not RTG-2 cells for replication/survey of/for VHSV.

PCR was the most frequently used method by participants identifying all viruses but also the method most frequently used by those participants obtaining the lowest scores. Another observation was that neutralisation is used by a relative high proportion of participating laboratories not obtaining highest score. Based on these findings we recommend participants to focus extra on evaluating how these two technologies are used for fish diagnostics.

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

Minutes

Report on the proficiency test 2008 is available at www.crl-fish.eu. Ampoule IV contained a mixture of VHSV and IPNV. 9 laboratories did not identify VHSV in ampoule IV. We encourage laboratories to be aware that more viruses can be present at the same time and that one virus can overgrow the other. Neutralisation tests were used by a relatively high proportion of laboratories

obtaining a low score. We recommend that laboratories focus extra on how these technologies are used for fish diagnostics.
Proficiency test 2009 will be sent out in the beginning of September 2009 – approximately one month earlier than normal. Identification of VHSV, IHNV and EHNV and differentiating from other viruses as IPNV, SVC and other Ranaviruses will be requested. Concerning ISA, KHV and EUS, we are still in the process of designing a proficiency test.

Questions:
Sven Bergmann: How to discriminate between EHNV and related RANA viruses?
Søren Kahns: We use sequencing, but it is difficulty since RANA viruses are very alike.
***Niels Jørgen Olesen*:** It is very much needed that a decision regarding taxonomi within this group of viruses is solved. For sure many laboratories will encounter difficulties in correct identification/ discrimination between EHNV and the other RANA viruses.
***Guiseppe Bovo*:** Do we have to take precautions about the distributions of the test?
***Niels Jørgen Olesen*:** The receiving lab will be responsible for biosecurity after arrival of the proficiency test.
***Guiseppe Bovo*:** I will be able to provide a Ranavirus antibody.
***Amedeo Manfrin*:** It would be nice to have recommendations in comments when receiving the answer on the proficiency test.
***Søren Kahns*:** The proficiency test is a way for the lab to control itself.
***Niels Jørgen Olesen*:** Each lab should decide before receiving how you expect to perform. We would not like to judge between labs.
***Hege Hellberg*:** The mollusc ringtest give recommendations.
***Niels Jørgen Olesen*:** If a lab underperforms for several years a mission to add support is often proposed.
***Søren Kahns*:** It can be difficult to identify the exact problem since we do not have all information.
***Olga Haenen*:** Cross contamination may take place when opening the ampoules with the saw, one after the other. We should realize this. To avoid contaminations, use a new saw, or disinfect the saw and cabinet totally between ampoules.
***Niels Jørgen Olesen*:** The ampoules have been changed in format due to old ampoules gave lower titres.
***Guiseppe Bovo*:** How do you control that all cell lines are what you think?
***Søren Kahns*:** This is a common problem for all cell lines. A lot of cell lines might not be what we think they are. Even if you buy form companies you can not be 100% sure. It can demand a large amount of work to find out the origin of the cell. The most important is that your cell lines are a sensitive tool towards identifying the virus. By participating in the proficiency tests you can test whether your cell lines can detect the pathogens at sensitivities as it should. If this is the case you do not have to worry so much.
***Sven Bergmann*:** As accredited labs we have to report if we work with something else than we state.

Technical report

Niels Jørgen Olesen briefly ran through the technical report 2008, which can be found at www.crl-fish.eu.

Workplan for 2009 and 2010

Niels Jørgen Olesen explained the objectives for 2009 and how these are being fulfilled. Workplan 2009 is available at www.crl-fish.eu/CRL_NRLs/Workplan.aspx. He then presented suggestions for workplan 2010:

- Update and include standard operating procedures on the CRL web page for the listed exotic and non-exotic diseases
- Expanding www.fishpathogens.eu with IHN, KHV and ISA
- Workshop and training courses in most updated diagnostic procedures for VHS, IHN, ISA and KHV
- Include ISAV and KHV in the annual proficiency test
- Assess the possibilities for organising proficiency test on diagnosis of EUS

***Birgit Claudia Oidtmann*:** Could you access specificity and sensitivity of diagnostic tests?
***Niels Jørgen Olesen*:** It's a heavy task to take up, but since it a very important point we will look more into this.

Annual meeting 2010

26-28 May 2010 was suggested.
Both Rumania and Århus (DK) were suggested as venues for next meeting.

***Martin Ruane Neil*:** You could maybe have the meeting in Denmark every second year and somewhere else every second year.

Pictures

Olga Haenen and Vlasta Jencic were excellent photographers during the workshop. For pictures from the workshop please have a look at www.crl-fish.eu/annual_meetings/photo_gallery.aspx.

The CRL received the following reagents in 2009

Country	Name	Date of recieval	Material	Amount	Protocol no
Canada	Nellie Gagné	22-05-2009	CA-NS04-01 2004-175 Brown trout	1 vial	2009-50-122
			CA-NB04-01 2004-81 Striped bass	1 vial	
			CA-NB02-01 2002-118 Stiped bass	1 vial	
			CA-NB00-02 2000-150 Trepigget hundestejle	1 vial	
			CA-NB00-01 200-149 Mummichog	1 vial	
	Frederick S.B. Kibenge	22-05-2009	37 sample for ISA virus	37 vials	2009-50-123
France	Jaennette Catric	1-04-2009	Lyophilized anti-KHV rabbit serum	4 vials	2009-50-82
			Purified anti-KHV rabbit serum		
			Anti-KHV rabbit serum		
			Rabbit serum anti carp IgM		
			2nd group: Decomplemented common carp sera obtained 4 months post-infection K3	10 vials	
			2nd group: Decomplemented common carp sera obtained 4 months post-infection K2		
			1st group: Decomplemented sera from Koï carp C38		
			1st group: Decomplemented sera from Koï carp N38		
			1st group: Decomplemented sera from Koï carp C21		
			1st group: Decomplemented sera from Koï carp N21		
			Negative control carp serum		
			2nd group: Decomplemented common carp sera obtained 4 months post-infection K4		
			3rd group: The same group of common carp as the 2nd group but bled 1 year post-infection. The sera are not decomplemented. N°4		
			3rd group: The same group of common carp as the 2nd group but bled 1 year post-infection. The sera are not decomplemented. N°3		
			CyHV-3 : isolate 07/108b from Afssa	1 vial	
			KF-1 cells	1 vial	
Iran	M. Noorouzi	16-03-2009	CVL/VI/I, sample code 14.6 rainbow trout, supernatant - IPNV	1 sample	2009-50-48
			CVL/VI/I, sample code 14.3 rainbow trout, supernatant - IPNV	1 sample	
			CVL/VI/I, sample code 14.2 rainbow trout, supernatant - IPNV	1 sample	
			CVL/VI/I, sample code 11.1 rainbow trout, supernatant - IPNV	1 sample	
			CVL/VI/I, sample code 15.3 rainbow trout, supernatant - IPNV	1 sample	
			CVL/VI/I, sample code 14.5 rainbow trout, supernatant - IPNV	1 sample	
			CVL/VI/I, sample code 15.3 rainbow trout, supernatant - IPNV	1 sample	
			CVL/VI/I, sample code 14.1 rainbow trout, supernatant - IPNV	1 sample	
			CVL/VI/I, sample code 14.4 rainbow trout, supernatant - IPNV	1 sample	
			CVL/VI/I, sample code 60 rainbow trout, supernatant - IPNV	1 sample	
Italy	Amadeo Manfrin	25-03-2009	IB/05/09	1 sample	2009-50-58
			IB/01/09	1 sample	
			IB/06/09	1 sample	

Country	Name	Date of recieval	Material	Amount	Protocol no
Italy	Amadeo Manfrin	25-03-2009	IB/04/09	1 sample	2009-50-58
			IB/03/09	1 sample	
			IB/02/09	1 sample	
	Guiseppe Bovo	7-04-2009	VHSV isolate 175 - V07	1 vial	2009-50-85
			VHSV isolate 183 - V08	1 vial	
			IHNV isolate 320/2-3 - V07	1 vial	
			VHSV isolate 117/2 - V07	1 vial	
			VHSV isolate 198 - V07	1 vial	
			VHSV isolate 224 - V07	1 vial	
		21-12-2009	Rabbit anti catfish iridovirus anti 562/92 1:100	1 vias	2009-50-349
			Rabbit anti catfish iridovirus anti 562/92 1:800	1 vias	
Norway	Ingebjørn Modahl	4-06-2009	PD virus (SAV-virus) PD, 220409, 1 pass CHSE celler	1 vial	2009-50-133
			PD virus (SAV-virus) PD, 120309, 3 pass CHSE celler	1 vial	
		19-06-2009	PD-antistof MAb 4H1 (SAV)	1 vial	2009-50-162
Poland	Ewa Borzym	15-04-2009	VHSV isolate 101-103	5 vial	2009-50-87
			VHSV isolate Cr-67	5 vial	
		20-04-2009	VHSV isolate 2	1 vial	2009-50-94
	Marek Matras	4-11-2009	KHV not deteced - Purified DNA from gills sample no. 1254,1255,1257,1258,1250,1251,1252,1253,1443,1444,1445,1446,1447,1438,1439,1440,1441,1442	18 vials	2009-50-290
			KHV detected - Purified DNA from gills sample no. 1560,1561,1563,1564	4 vials	
		2-12-2009	KHV not deteced - Purified DNA from gills sample no. 1264,1265,1555,1556,1557,1558,1565,1566,1567,1569	10 vials	2009-50-318
			KHV deteced - Purified DNA from gills sample no. 1266,1267,1268	3 vials	
Slovakia	Julia Habovstiakova	17-03-2009	Sampel no. CP/2131/53 - VHSV	1 sample	2009-50-49
Sweden	Statens Vet. Med. Anstalt, Department Virologi	25-06-2009	Sample no. 1638-1649 from fish farm no. 1 supernatant	12 vials	2009-50-168
			Sample no. 1425-1427 from fish farm no. 2 supernatant	3 vials	2009-50-169
			Sample no. 1428-1430 from fish farm no. 3 supernatant	3 vials	2009-50-170
			Sample no. 1461-1463 from fish farm no. 4 supernatant	3 vials	2009-50-171
			Sample no. 1500-1502 from fish farm no. 5 supernatant	3 vials	2009-50-172
Turkey	Gülnur Kalayci and Serife Incoglu	9-10-2009	IPNV isolate 3 passage BF-2 2947 rainbow trout supernatant from Marmara region - Düzce	1 vial	2009-50-251
			IPNV isolate 3 passage BF-2 2803 rainbow trout supernatant from Blacksea region - Düzce	1 vial	
			IPNV isolate 3 passage BF-2 2112 rainbow trout supernatant from Central Anatolia region - Ankara	1 vial	
			IPNV isolate 3 passage BF-2 2976 rainbow trout supernatant from Blacksea region - Bolu	1 vial	
			IPNV isolate 4 passage BF-2 2926 rainbow trout supernatant from Blacksea region - Zonguldak	1 vial	
			IPNV isolate 4 passage BF-2 3853 rainbow trout supernatant from Blacksea region - Artvin	1 vial	
			VHSV isolate 5 passage BF-2 203 wild turbot supernatant from Blacksea - Trabzon	1 vial	2009-50-252
Turkey	Gülnur Kalayci and Serife	9-10-2009	VHSV isolate 4 passage BF-2 301 wild turbot supernatant from Blacksea - Trabzon	1 vial	2009-50-252

Country	Name	Date of recieval	Material	Amount	Protocol no
	Incoglu		VHSV isolate 4 passage BF-2 702 wild turbot supernatant from Blacksea - Trabzon	1 vial	
			IPNV isolate 4 passage BF-2 604 cultured rainbow trout from Blacksea region of Turkey	1 vial	2009-50-253
			IPNV isolate 4 passage BF-2 601 cultured rainbow trout from Blacksea region of Turkey	1 vial	
			IPNV isolate 5 passage BF-2 303 cultured rainbow trout from Blacksea region of Turkey	1 vial	
			IPNV isolate 4 passage BF-2 302 cultured rainbow trout from Blacksea region of Turkey	1 vial	
	Necdet Akkoca	2-12-2009	VHSV isolate BOLV 18 pas BF-2 18.07.08	1 vial	2009-50-315
			VHSV isolate from Fikret Koksal 2783 14 pas BF-2 18.11.09	1 vial	
UK-Scotland	Marine Laboratory Aberdeen	3-09-2009	6 ampoules containing 1.1 ml kidney supernatant	6 vials	2009-50-224
USA	James R. Winton	26-01-2009	2009-50-13-2 IVa VHSV Isolate Quatsion, BC 02-232-1 Sardines 28-03-2003	1 vial	2009-50-13
			2009-50-13-1 IVa VHSV Isolate p-3 EPC Coco adult 13-12-2002	1 vial	
			2009-50-13-3 IVa VHSV Isolate Tokul Creek,WA p-3 EPC Steelhead 22-02-2006	1 vial	
			2009-50-13-4 IVa VHSV Isolate Port Angels, WA EPC Atlantic salmon #16524 19-03-2008	1 vial	
			2009-50-13-5 IVb VHSV Isolate Lake Ontario, NY p-4 FPL 07-006 Gizzard Shard 17-08-2007	1 vial	
			2009-50-13-6 IVb VHSV Isolate Budd Lake, MI Farcal EPC Bluegill 26-06-2007	1 vial	
			2009-50-13-7 IVb VHSV Isolate Skaneateles Lake, NY Fpl 07-010 Smallmouth bass 17-08-2007	1 vial	
			2009-50-13-8 IVb VHSV Isolate Lake St. Clair, MI EPC MIO3 Muskellunge 15-02-2008	1 vial	
			2009-50-13-9 IVb VHSV Isolate New Brunswick Mummichog 18-01-2008	1 vial	

The CRL supplied the following reagents in 2009

Country	Name	Material	Type	Amount	Date of shipment
Bosnia Herzegovina	Adnan Jazic	2 small tissue cell flasks with BF-2 cells	Cells	2 small flasks	8-09-2009
Bulgaria	Vanya Damyanova Chikova and Marina Ivanova	2 small tissue cell flasks with BF-2 cells	Cells	2 small flasks	3-03-2009
		2 small tissue cell flaskes with EPC cells	Cells	2 small flasks	
		VHS Rindsholm 5151 in RNA Later	Virus i RNA Later	1 vial	27-04-2009
		IHN – 4008 IHN virus 217/A (DTU Vet protocol no. 4008) in RNA Later	Virus i RNA Later	1 vial	
		2 small tissue cell flasks with BF-2 cells	Cells	2 small flasks	8-09-2009
Chile	Eduardo Castro Nallar	2 small tissue cell flasks with FHM cells	Cells	2 small flasks	15-07-2009
		2 small tissue cell flasks with BF-2 cells	Cells	2 small flasks	
	Gerardo Muñoz Perdiguero	RNA extracted from VHSV strain Rindsholm genotype 1a	PCR	1 vial	14-09-2009
		RNA extracted from VHSV US Makah genotype 4a	PCR	1 vial	
		RNA extracted from IHNV 32/87 genotype M	PCR	1 vial	
Faroe Islands	Debes H. Christiansen and Marita Næs	Mab anti VHSV – 1P5B11	Mab	1 vial	27-10-2009
		Mab anti IHNV – Hyb 136-3	Mab	1 vial	
		Pab anti IPN-Ab – F72	Pab	1 vial	
		Pab anti IPN-Sp – F68	Pab	1 vial	
		Pab anti IHNV – F63	Pab	1 vial	
Finland	Tuija Gadd	2 small tissue cell flasks with RTG-2 cells	Cells	2 small flasks	14-05-2009
Iceland	Sigurður Helgason	Pab anti IPN-Sp – F69	Pab	1 vial	8-09-2009
		Pab anti IPN-Ab – F72	Pab	1 vial	
		ICIV European catfish (Italian isolate)	Virus	1 vial	
		EHNV Epizootic Haematopoietic Necrosis Virus	Virus	1 vial	
Iran	Mohaddes Ghasemi	2 small tissue cell flasks with EPC cells	Cells	2 small flasks	17-09-2009
		2 small tissue cell flasks with FHM cells	Cells	2 small flasks	
		2 small tissue cell flasks with BF-2 cells	Cells	2 small flasks	
		2 small tissue cell flasks with SSN-1 cells	Cells	2 small flasks	
		2 small tissue cell flasks with CHSE cells	Cells	2 small flasks	
Ireland	Lorraine McCarthy	2 small tissue cell flasks with BF-2 cells	Cells	2 small flasks	3-03-2009
		2 small tissue cell flasks with EPC cells	Cells	2 small flasks	
Italy	Giuseppe Bovo	Mab anti VHSV – 1P5B11	Mab	1 vial	22-06-2009
		Pab anti IHNV – F63	Pab	1 vial	
		Pab anti VHSV – F45 & F59	Pab	1 vial	
		Ampoule V from the Inter-Laboratory Proficiency Test 2007, VHS virus, 4p101	Virus	1 ampoule	
		Ampoule I from the Inter-Laboratory Proficiency Test 2008, VHS virus, DK-5151 (Rindsholm), (diluted 10 -3)	Virus	1 ampoule	
		Ampoule V from the Inter-Laboratory Proficiency Test 2008, IPN virus Type Sp	Virus	1 ampoule	
		Ampoule II from the Inter-Laboratory Proficiency Test 2008, VHS virus, 1p8, (undiluted)	Virus	1 ampoule	
		Ampoule I from the Inter-Laboratory Proficiency Test 2007, VHS virus, DK-F1, (Undiluted	Virus	1 ampoule	
		9 ml Mab anti trout IgM 4C10, 03.04.1998.	Mab	9 ml	29-06-2009
Japan		DK-2835	Virus	1 vial	12-05-2009
		GE-1.2	Virus	1 vial	
		NO-A163-68 EG46	Virus	1 vial	
		FIN-2ka 66/2000	Virus	1 vial	

Country	Name	Material	Type	Amount	Date of shipment
Japan	Jun Kurita and Takafumi Ito	FIN-ka 422/00	Virus	1 vial	12-05-2009
		DK-5131	Virus	1 vial	
		DK-1p53	Virus	1 vial	
		DK-4p37	Virus	1 vial	
		UK-96-43	Virus	1 vial	
		SE-SVA-1033	Virus	1 vial	
		SE-SVA-14	Virus	1 vial	
		DK-5p276	Virus	1 vial	
		DK-5123	Virus	1 vial	
		TR206239-1	Virus	1 vial	
		DK-1p52	Virus	1 vial	
		DK-1p54	Virus	1 vial	
		DK-4p101	Virus	1 vial	
		DK-4p51	Virus	1 vial	
		UK-H17/5/93	Virus	1 vial	
		UK-860/94	Virus	1 vial	
		UK-H17/2/95	Virus	1 vial	
		FR-L59x	Virus	1 vial	
		NF-GH30	Virus	1 vial	
		DK-9795386 IR-F13.02.97)	Virus	1 vial	
		DK-9695377	Virus	1 vial	
		DK-1p121	Virus	1 vial	
		DK-1p49	Virus	1 vial	
		CZ-R5	Virus	1 vial	
		DK-F1	Virus	1 vial	
		HEDEDAM	Virus	1 vial	
		DK-3592B	Virus	1 vial	
		DK-3971	Virus	1 vial	
		DK-3946	Virus	1 vial	
		DK-5151	Virus	1 vial	
		DK-6137	Virus	1 vial	
		DK-7974	Virus	1 vial	
		DK-4p168	Virus	1 vial	
		DK-200051	Virus	1 vial	
		NO-2007-50-385	Virus	1 vial	
		FR-07-71	Virus	1 vial	
		DK-200149	Virus	1 vial	
		FR-02-84	Virus	1 vial	
		DK-1p120	Virus	1 vial	
		CZ-2077	Virus	1 vial	
		DK-5927	Virus	1 vial	
		AU-8/95	Virus	1 vial	
		CH-FI 262 BFH	Virus	1 vial	
		PL-202473	Virus	1 vial	
		DK-M. Rhabdo	Virus	1 vial	
		DK-1p8	Virus	1 vial	
		DK-1p40	Virus	1 vial	
		DK-1p85	Virus	1 vial	

Country	Name	Material	Type	Amount	Date of shipment
Japan	Jun Kurita and Takafumi Ito	DK-1p86	Virus	1 vial	12-05-2009
		DK-1p93	Virus	1 vial	
		DK-1p116	Virus	1 vial	
		FR-23-75	Virus	1 vial	
		Capione	Virus	1 vial	
		Skaneateles Lake, NY	Virus	1 vial	
		New Brunswick	Virus	1 vial	
		IHNV RBH	Virus	1 vial	
		IHNV 32/87	Virus	1 vial	
		IHNV Coleman	Virus	1 vial	
		IHNV 4008	Virus	1 vial	
		IHNV OSV	Virus	1 vial	
		Budd Lake, MI	Virus	1 vial	
		IHNV Østrig	Virus	1 vial	
		IHNV TR	Virus	1 vial	
		EEV B12	Virus	1 vial	
		SVCV 56/70	Virus	1 vial	
		PFRV S64	Virus	1 vial	
		Tench RV	Virus	1 vial	
		Perch RV	Virus	1 vial	
		USA-MAKAH	Virus	1 vial	
		DK-2p51	Virus	1 vial	
		IHNV ER	Virus	1 vial	
		Quatsino, BC	Virus	1 vial	
		Lake Ontario, NY	Virus	1 vial	
		Minter Creek, WA	Virus	1 vial	
		Tokul Creek, WA	Virus	1 vial	
		Port Angels, WA	Virus	1 vial	
		BC'93	Virus	1 vial	
		CA-3624	Virus	1 vial	
		IHNV HAG	Virus	1 vial	
		USA-Elliott Bay	Virus	1 vial	
		USA-KHV	Virus	1 vial	
		JP-Obama 25	Virus	1 vial	
		JP-JF00Ehi1	Virus	1 vial	
		BR01Ehi1	Virus	1 vial	
		JF01Oit1	Virus	1 vial	
		JSL02Yam1	Virus	1 vial	
		PM05Ehi1	Virus	1 vial	
		Lakes St. Clair, MI	Virus	1 vial	
		GOBY 1-5	Virus	1 vial	
		CA-99-019	Virus	1 vial	
Korea	Milga seo	DK-3592B in RNA Later	PCR	1 vial	2-02-2009
		Makah in RNA Later	PCR	1 vial	
		GB-96-43 in RNA Later	PCR	1 vial	
		1p52 in RNA Later	PCR	1 vial	
		4p101 in RNA Later	PCR	1 vial	

Country	Name	Material	Type	Amount	Date of shipment
Lithuania	Ingrida Jaceviciene	ICIV European catfish (Italian isolate)	Virus	1 vial	14-09-2009
		EHNV Epizootic Haematopoietic Necrosis Virus	Virus	1 vial	
Norway	Duncan Colquhoun	1 vial containing 1 ml of boiled kidney supernatant suitable as positive control for BKD-ELISA and BKD-PCR with batch no. Å-4-AR-061/02.06.2009.	Bakteriologi	1 vial	2-06-2009
	Vidar Aspehaug	Ampoule V from the Inter-Laboratory Proficiency Test 2007, VHS virus, 4p101	Virus	1 ampoule	29-06-2009
		VHSV DK- 1p52 Marine isolate from sprat	Virus	1 vial	
		Ampoule I from the Inter-Laboratory Proficiency Test 2007, VHS virus, DK-F1, (Undiluted)	Virus	1 ampoule	
		Ampoule II from the Inter-Laboratory Proficiency Test 2008, VHS virus, 1p8, (undiluted)	Virus	1 ampoule	
		Ampoule I from the Inter-Laboratory Proficiency Test 2008, VHS virus, DK-5151 (Rindsholm), (diluted 10 -3)	Virus	1 ampoule	
	Birgit Helene Dannevig	EHNV Epizootic Haematopoietic Necrosis Virus	Virus	1 vial	8-09-2009
		ICIV European catfish (Italian isolate)	Virus	1 vial	
P.R.China	Duan Hongan	2 small tissue cell flasks with KF-1 cells	Cells	2 small flasks	14-09-2009
		2 small tissue cell flasks with EPC cells	Cells	2 small flasks	
		2 small tissue cell flasks with CHSE cells	Cells	2 small flasks	
Poland	Marek Matras	2 small tissue cell flasks with FHM cells	Cells	2 small flasks	30-04-2009
		2 small tissue cell flasks with BF-2 cells	Cells	2 small flasks	
	Ewa Borzym	1 small tissue cell flasks with BF-2 cells	Cells	1 small flask	
		1 small tissue cell flasks with FHM cells	Cells	1 small flask	
		1 small tissue cell flasks with EPC cells	Cells	1 small flask	
		1 small tissue cell flasks with RTG-2 cells	Cells	1 small flask	
		3,6 ml Hoechs stock solution	Medium	1 vial	
RANA-Finland	Riikka Holopainen	206113-2518 EHNV organmaterial	Virus	1 vial	13-05-2009
		206113-5510 EHNV organmaterial	Virus	1 vial	
		206113-5508 EHNV organmaterial	Virus	1 vial	
		206113-5506 EHNV organmaterial	Virus	1 vial	
		206113-4518 EHNV organmaterial	Virus	1 vial	
		206113-4516 EHNV organmaterial	Virus	1 vial	
		206113-4514 EHNV organmaterial	Virus	1 vial	
		206113-2521 EHNV organmaterial	Virus	1 vial	
		206113-5521 EHNV organmaterial	Virus	1 vial	
		206113-2516 EHNV organmaterial	Virus	1 vial	
		206113-2514 EHNV organmaterial	Virus	1 vial	
		206113-2512 EHNV organmaterial	Virus	1 vial	
		206113-2510 EHNV organmaterial	Virus	1 vial	
		206113- EHNV organmaterial	Virus	1 vial	
		206113-4512 EHNV organmaterial	Virus	1 vial	
Serbia	Vladimir, Ivan Radosavljevic	1 medium tissue cell flasks with BF-2 cells	Cells	1 small flask	7-07-2009
Slovakia	Julia Habovstiakova	ICIV European catfish (Italian isolate)	Virus	1 vial	29-06-2009
		EHNV Epizootic Haematopoietic Necrosis Virus	Virus	1 vial	
Slovenia	Peter Hostnik	EHNV Epizootic Haematopoietic Necrosis Virus	Virus	1 vial	8-09-2009
		ICIV European catfish (Italian isolate)	Virus	1 vial	
Sri Lanka	M Somarathne	Makah in RNA Later	Virus	1 vial	29-06-2009
		4p101 in RNA Later	Virus	1 vial	
		1p52 in RNA Later	Virus	1 vial	

Country	Name	Material	Type	Amount	Date of shipment
Sri Lanka	M Somarathne	GB-96-43 in RNA Later	Virus	1 vial	29-06-2009
		DK-3592B in RNA Later	Virus	1 vial	
Sweden	Anders Hellström	3 medium tissue cell flasks with BF-2 cells	Cells	3 medium flasks	7-07-2009
	Suzanne Martelius and Anders Hellström	EHNV Epizootic Haematopoietic Necrosis Virus	Virus	1 vial	8-09-2009
		ICIV European catfish (Italian isolate)	Virus	1 vial	
Taiwan (R.O.C.)	Franz Schneefall	Makah VHSV Genotype IVa	Virus	1 vial	22-09-2009
The Netherlands	Olga L.M. Haenen	2 small tissue cell flasks with EPC cells	Cells	2 small flasks	5-05-2009
		EHNV Epizootic Haematopoietic Necrosis Virus	Virus	1 vial	8-09-2009
		ICIV European catfish (Italian isolate)	Virus		
Turkey	Gulnur Kalayci	EHNV Epizootic Haematopoietic Necrosis Virus	Virus	1 vial	29-06-2009
		ICIV European catfish (Italian isolate)	Virus	1 vial	
		2 small tissue cell flasks with ASK cells	Cells	2 small flaskes	3-11-2009
		2 small tissue cell flasks with CHSE cells	Cells	2 small flaskes	
		2 small tissue cell flasks with RTG cells	Cells	2 small flasks	
UK	Keith Way	2 X 250 ml tissue cell flasks with RTG-2 cells	Cells	2 flasks	22-04-2009
	Keith Way and Christopher Pond	2 small tissue cell flasks with BF-2 cells	Cells	2 small flasks	4-11-2009
USA	Jim Winton	860/94	Virus	1 vial	2-02-2009
		1p52	Virus	1 vial	
		1p53	Virus	1 vial	
		1p54	Virus	1 vial	
		1p49	Virus	1 vial	
		2p51	Virus	1 vial	
		4p101	Virus	1 vial	
		4p168	Virus	1 vial	
		F13.02.97	Virus	1 vial	
		H17/5/93	Virus	1 vial	

Report of the Inter-Laboratory Proficiency Test for National Reference Laboratories for Fish Diseases 2009

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

Table of contents

Introduction

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 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.
In addition the 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. We recommend following the procedures described in Chapter 2.3.1 in the OIE Manual of Diagnostic Tests for Aquatic Animals 2009, of which a new version has just been released.

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.

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 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 a filled scheme electronically or on paper. Furthermore, participants were asked to fill an extended questionnaire in order to obtain more information on the methodology used by the laboratories. Participants were asked to reply latest 13 November 2009

Participants

Five ampoules with lyophilised tissue culture supernatant were delivered to all NRLs in EU Member States, including Denmark, and likewise to the National reference laboratories in Australia, Bosnia and Herzegovina, Canada, Croatia, Faroe Islands, Iceland, Israel, Japan, Norway, P.R China, Serbia and Switzerland. The Belgian NRL covers both Belgium and Luxembourg and likewise the Italian NRL covers Italy, Cyprus, Malta and Greece.

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.

Ampoule I: EHNV	Reference strain of EHNV Isolate 86/8774 from rainbow trout Received from Dr. R.J. Whittington, EHNV OIE reference laboratory, Chair Farm Animal Health, Faculty of Veterinary Science, University of Sydney, 425 Werombi Road, Private Bag 3, Camden NSW 2570, Australia Cell culture passage number 6 References: Langdon JS, Humphrey JD & Williams LM (1989). Outbreaks of an EHNV-like iridovirus in cultured rainbow trout, *Salmo gairdneri* Richardson, in Australia. *Journal of Fish Diseases* **11**, 93-96. 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.
Ampoule II: IHNV	IHNV Genotype L Isolate COL-80 from chinook salmon Received from Dr. Jim Winton, U.S. Geological Survey, Western Fisheries Research Center, 6505 NE 65th St., Seattle, WA 98115, USA Cell culture passage number unknown References: Nichol ST, Rowe JE & Winton JR (1995). Molecular epizootiology and evolution of the glycoprotein and non-virion protein genes of infectious hematopoietic necrosis virus, a fish rhabdovirus. *Virus Research* **38**, 159-173. Kurath G, Garver KA, Troyer RM, Emmenegger EJ, Einer-Jensen K & Anderson ED (2003). Phylogeography of infectious haematopoietic necrosis virus in North America. *Journal of General Virology* **84**, 803-814.
Ampoule III: VHSV	VHSV genotype Ie Turkish isolate TR-WS13G (=TR-SW13G) from turbot (*Psetta maxima*) Received from Dr. Toyohiko Nishizawa and Dr. Mamoru Yoshimizu. Graduate School of Fisheries Sciences, Hokkaido University, Hakodate 041-8611 Japan Cell culture passage number 5 References: Nishizawa T, Savas H, Isidan H, Üstündag C, Iwamoto H & Yoshimizu M (2006). Genotyping and pathogenicity of viral hemorrhagic septicemia virus from free-living turbot (*Psetta maxima*) in a Turkish coastal area of the Black Sea. *Applied and Environmental Microbiology* **72**, 2373-2378.
Ampoule IV: VHSV	VHSV genotype IVa Received as RBV (Ray Brunson virus from the Makah hatchery) isolate from coho salmon Received from Dr. Jim Winton, U.S. Geological Survey, Western Fisheries Research Center, 6505 NE 65th St., Seattle, WA 98115, USA Cell culture passage number is unknown References: Brunson R, True K & Yancey J (1989). VHS virus isolated at Makah National Fish Hatchery. *American Fisheries Society Fish Health Section Newsletter* **1**7, 3-4. Winton JR, Batts WN & Nishizawa T (1989). Characterization of the first North American isolates of viral hemorrhagic septicemia virus. *American Fisheries Society Fish Health Section Newsletter* **17**, 2-3. Winton JR, Batts WN, Deering RE, Brunson R, Hopper K, Nishizawa T & Stehr C (1991). Characteristics of the first North American isolates of viral hemorrhagic septicemia virus. *Proceedings of the Second International Symposium on Viruses of Lower Vertebrates*, 43-50.
Ampoule V: No virus	Pure cell culture medium

Testing of the test

The inter-laboratory test 2009 was prepared and tested according to protocols accredited under DS/EN ISO/IEC 17025 and ILAC-G13:08/2007 standards. Prior to distribution the CRL 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).

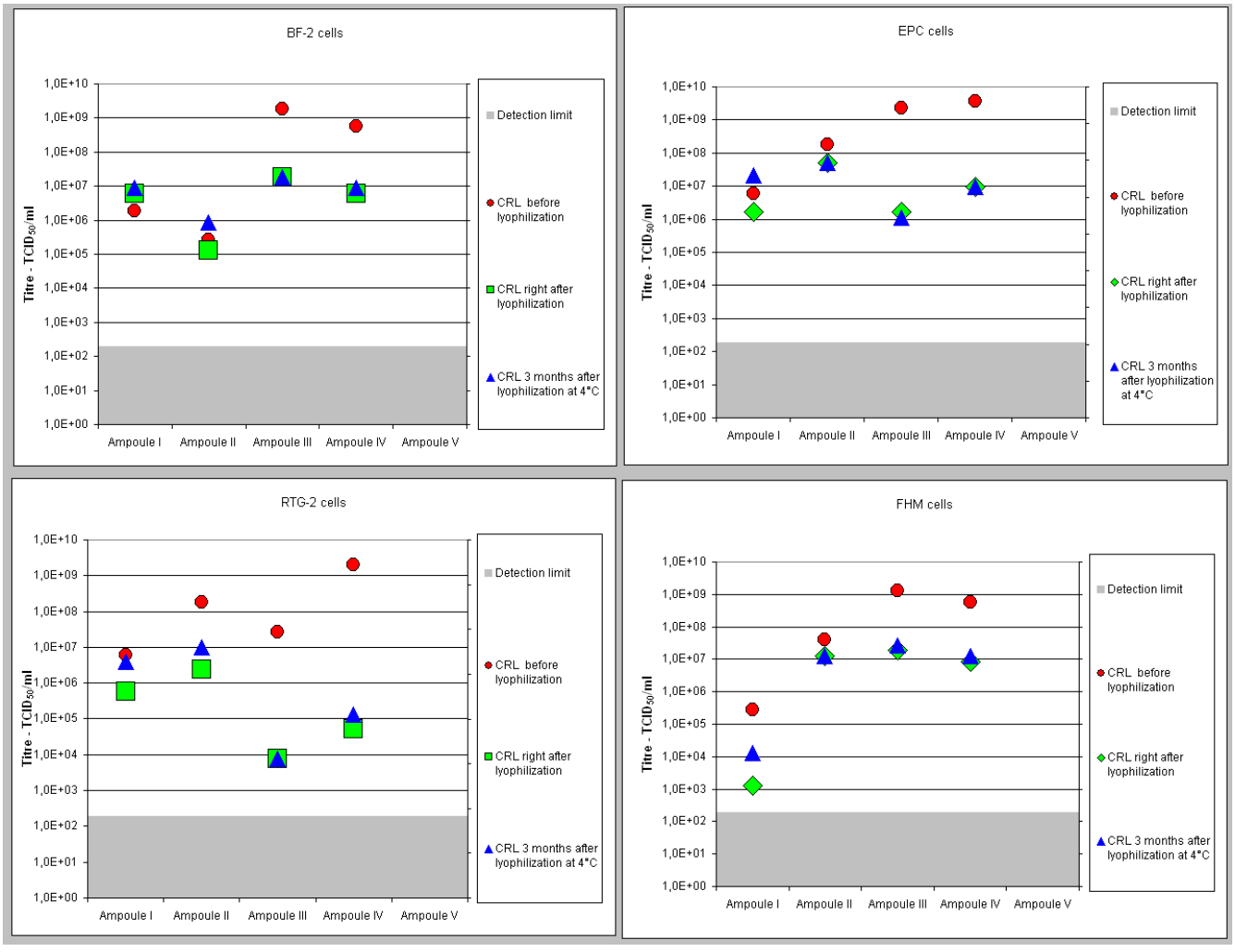
The lyophilisation procedure caused a significant titre reduction, especially for the viral haemorrhagic septicaemia virus (VHSV) where a 2-4 log reduction was observed (figure 1). For the infectious haematopoietic necrosis virus (IHNV) and epizootic haematopoietic necrosis virus (EHNV) a titre reduction between 0-2 log occurred. However, all titres of the lyophilised viruses were above detection level. Furthermore, when lyophilised the viruses were very stable at storing, tested by titration of one ampoule of each virus preparation after 3 months storage in the dark at 4°C. 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).
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, IPNV and SVCV and by PCR and sequencing for EHNV, and IFAT for ranavirus. Presence of viruses other than the expected in each ampoule were not observed.

Table 2. Titre of representative ampoules of no. I to V tested at the CRL 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 (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)
			$TCID_{50}$/ml	$TCID_{50}$/ml	$TCID_{50}$/ml
Ampoule I	Reference strain of EHNV	BF-2	$1,9*10^6$	$5,9*10^6$	$8,6*10^6$
		EPC	$1,3*10^6$	$4,0*10^5$	$4,0*10^6$
		RTG-2	$2,7*10^5$	$4,0*10^4$	$1,9*10^5$
		FHM	$2,7*10^5$	$1,3*10^3$	$1,3*10^4$
Ampoule II	IHNV Genotype L	BF-2	$2,7*10^5$	$1,3*10^5$	$8,6*10^5$
		EPC	$2,7*10^7$	$8,6*10^6$	$8,6*10^6$
		RTG-2	$4,0*10^6$	$1,3*10^5$	$4,0*10^5$
		FHM	$4,0*10^7$	$1,3*10^7$	$1,3*10^7$
Ampoule III	VHSV genotype Ie	BF-2	$1,9*10^9$	$1,9*10^7$	$1,9*10^7$
		EPC	$2,7*10^8$	$4,0*10^5$	$2,7*10^5$
		RTG-2	$8,6*10^5$	$1,3*10^3$	$1,3*10^3$
		FHM	$1,3*10^9$	$1,9*10^7$	$2,7*10^7$
Ampoule IV	VHSV genotype IVa	BF-2	$5,9*10^8$	$5,9*10^6$	$8,6*10^6$
		EPC	$4,0*10^8$	$1,9*10^6$	$1,9*10^6$
		RTG-2	$2,7*10^7$	$5,9*10^3$	$1,3*10^4$
		FHM	$5,9*10^8$	$8,6*10^6$	$1,3*10^7$

Ampoule No.	Content	Cell line	Titre before lyophilisation	Median titre right after lyophilisation	Titre 3 months after lyophilisation (4ºC, dark conditions)
			$TCID_{50}$/ml	$TCID_{50}$/ml	$TCID_{50}$/ml
Ampoule V	Medium	BF-2		$< 1{,}9*10^2$	$< 1{,}9*10^2$
		EPC		$< 1{,}9*10^2$	$< 1{,}9*10^2$
		RTG-2		$< 1{,}9*10^2$	$< 1{,}9*10^2$
		FHM		$< 1{,}9*10^2$	$< 1{,}9*10^2$

Figure 1. Titration before, right after and 3 months after lyophilisation at different cell lines. For ampoule V no CPE was observed when titrated.



Distribution of the test

The test was sent out according to current international regulations for 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 15 of the parcels (-40°C to +30ºC). 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 15 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 assure more participants that the temperature encountered during transport has not 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.

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 a neutralisation test, ELISA, and/or by immunofluorescence. Additional identification by PCR was an option as usual. 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 CRL 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 2-5, 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 2009 - Virus identification.

Laboratory code number	Score	Answer recived at CRL	Ampoule I	Ampoule II	Ampoule III	Ampoule IV	Ampoule V
			EHNV	**IHNV**	**VHSV**	**VHSV**	**No Virus**
2	10	12-11-09	EHNV	IHNV	VHSV	VHSV	No virus
3	10	09-11-09	EHNV	IHNV	VHSV	VHSV	No virus
4	10	10-11-09	EHNV	IHNV	VHSV	VHSV	No virus
5	6	02-11-09	Virus not found	Virus not found	VHSV	VHSV	No virus
6	9	13-11-09	Ranavirus	IHNV	VHSV	VHSV	No virus
7	0	no reply	no reply	no reply	no reply	no reply	no reply
8	6	13-11-09	EHNV and VHSV	IHNV	VHSV	VHSV	VHSV
9	10	12-11-09	EHNV	IHNV	VHSV	VHSV	No virus
10	10	05-10-09	EHNV	IHNV	VHSV	VHSV	No virus
11	8	13-11-09	Virus not identified	IHNV	VHSV	VHSV	No virus
12	10	13-11-09	EHNV	IHNV	VHSV	VHSV	No virus
13	10	06-11-09	EHNV	IHNV	VHSV	VHSV	No virus
14	10	06-10-09	EHNV	IHNV	VHSV	VHSV	No virus
15	10	16-10-2009 21-10-2009	EHNV	IHNV	VHSV	VHSV	No virus
16	10	09-11-09	EHNV	IHNV	VHSV	VHSV	No virus
17	10	13-11-09	EHNV	IHNV	VHSV	VHSV	No virus
18	10	13-11-09	EHNV	IHNV	VHSV	VHSV	No virus
19	8	10-09-09	EHNV	IHNV	VHSV	VHSV	VHSV
20	10	12-11-09	EHNV	IHNV	VHSV	VHSV	No virus
21	10	13-11-09	EHNV	IHNV	VHSV	VHSV	No virus
22	10	12-11-09	EHNV	IHNV	VHSV	VHSV	No virus
23	8	13-11-09	EHNV	IHNV	VHSV	VHSV	VHSV
24	8	13-11-09	EHNV	IHNV	VHSV	VHSV	IPNV
25	10	09-011-09	EHNV	IHNV	VHSV	VHSV	No virus
26	10	12-11-09	EHNV	IHNV	VHSV	VHSV	No virus
28	9	13-11-09	Ranavirus	IHNV	VHSV	VHSV	No virus
29	10	13-11-09	EHNV	IHNV	VHSV	VHSV	No virus
30	10	10-11-09	EHNV	IHNV	VHSV	VHSV	No virus
31	8	20-11-2009*	EHNV	IHNV	VHSV	VHSV	VHSV
32	10	10-11-09	EHNV	IHNV	VHSV	VHSV	No virus
33	9	05-11-2009 12-11-2009	Ranavirus	IHNV	VHSV	VHSV	No virus
34	10	10-11-09	EHNV	IHNV	VHSV	VHSV	No virus
35	10	09-11-09	EHNV	IHNV	VHSV	VHSV	No virus
37	10	12-11-09	EHNV	IHNV	VHSV	VHSV	No virus
38	10	05-11-09	EHNV	IHNV	VHSV	VHSV	No virus
39	8	04-11-09	EHNV / IPNV	IHNV	VHSV	VHSV	No virus

	Ampoule I	Ampoule II	Ampoule III	Ampoule IV	Ampoule V
	EHNV	IHNV	VHSV	VHSV	No Virus
Correct ID	28	34	35	35	30
Correct virus group	3				
No virus	1	1	0	0	0
Wrong ID	2	0	0	0	5
No ID	1	0	0	0	0
Not replied	1	1	1	1	1
Total	36	36	36	36	36

* 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 2009 – Identification and titration of ampoule I.

Ampoule I – EHNV					
Laboratory code number	**Virus Identification**	**Titre in**			
		BF-2	**EPC**	**RTG-2**	**FHM**
2	EHNV	2,7E+08	4,0E+04		4,0E+05
3	EHNV	2,7E+06	2,7E+06	1,3E+06	
4	EHNV	5,9E+07	2,7E+06		
5	Virus not found	<1,9E+02	< 1,9E+02		
6	Ranavirus		2,7E+07		
7	no reply				
8	EHNV and VHSV	5,9E+06	8,6E+05		
9	EHNV	1,3E+04	1,3E+04	1,3E+03	1,3E+03
10	EHNV	1,3E+07	8,6E+05	1,3E+05	4,0E+03
11	Virus not identified	2,7E+05	4,0E+04		
12	EHNV	2,7E+05	8,6E+05	2,7E+04	
13	EHNV	5,9E+06	2,7E+05		
14	EHNV	4,0E+07	1,3E+04	4,0E+03	
15	EHNV	4,0E+04	5,9E+05	1,3E+05	<1,9E+02
16	EHNV	4,0E+05	1,3E+06		
17	EHNV	5,9E+06	2,7E+06		
18	EHNV	4,0E+05	8,6E+04		
19	EHNV	2,7E+07	1,3E+06		
20	EHNV	4,0E+07	1,3E+06		
21	EHNV	8,6E+05	1,3E+05		
22	EHNV		1,3E+06	1,3E+04	
23	EHNV	5,9E+05	8,6E+04		
24	EHNV		1,9E+05	1,9E+03	
25	EHNV	2,7E+06	2,7E+06	5,9E+03	1,9E+03
26	EHNV		2,7E+04	4,0E+04	
28	Ranavirus	4,0E+05	8,6E+04		
29	EHNV	2,7E+03	8,6E+04		
30	EHNV	4,0E+05	1,3E+06		2,2E+05
31	EHNV	1,9E+06	1,3E+05		
32	EHNV	4,0E+07	5,9E+06	1,9E+07	8,6E+05
33	Ranavirus	1,3E+05			1,3E+03
34	EHNV	4,0E+08	1,9E+07		
35	EHNV		1,3E+05		8,6E+04
37	EHNV	8,6E+06	5,9E+05		
38	EHNV	2,7E+07	4,0E+06		2,7E+05
39	EHNV / IPNV		1,3E+06	5,9E+06	
Number of laboratories		29	34	12	10
Median titre		2,7E+06	8,6E+05	3,4E+04	4,5E+04
Maximum titre		4,0E+08	2,7E+07	1,9E+07	8,6E+05
Minimum titre		<1,9E+02	1,3E+04	1,3E+03	<1,9E+02
25% quartile titre		4,0E+05	8,6E+04	5,4E+03	1,4E+03
75% quartile titre		2,7E+07	1,3E+06	4,1E+05	2,6E+05

Table 5. Inter-Laboratory Proficiency Test 2009 – Identification and titration of ampoule II.

Ampoule II - IHNV					
Laboratory code number	**Virus Identification**	**Titre in**			
		BF-2	**EPC**	**RTG-2**	**FHM**
2	IHNV	1,9E+03	8,6E+05		1,9E+05
3	IHNV	1,3E+03	1,9E+07	8,6E+05	
4	IHNV	4,0E+04	2,7E+07		
5	Virus not found	<1,9E+02	<1,9E+02		
6	IHNV		1,9E+07		
7	no reply				
8	IHNV	1,9E+03	8,6E+05		
9	IHNV	1,3E+03	1,3E+06	1,3E+04	1,3E+04
10	IHNV	4,0E+05	4,0E+06	1,9E+06	4,0E+07
11	IHNV	1,3E+03	2,7E+05		
12	IHNV	1,3E+03	4,0E+06	1,9E+06	
13	IHNV	1,9E+05	8,6E+06		
14	IHNV	8,6E+03	1,9E+07	8,6E+04	
15	IHNV	<1,9E+02	5,9E+06	1,3E+03	1,9E+05
16	IHNV	1,9E+02	4,0E+06		
17	IHNV	4,0E+06	2,7E+07		
18	IHNV	1,9E+05	8,6E+05		
19	IHNV	8,6E+06	5,9E+07		
20	IHNV	2,7E+06	4,0E+07		
21	IHNV	1,3E+04	1,9E+06		
22	IHNV		1,3E+07	5,9E+06	
23	IHNV	5,9E+04	2,7E+05		
24	IHNV		5,9E+04	<1,9E+02	
25	IHNV	4,0E+05	1,9E+07	8,6E+04	1,9E+06
26	IHNV		4,0E+03	1,9E+04	
28	IHNV	5,9E+03	1,3E+04		
29	IHNV	4,0E+02	4,0E+05		
30	IHNV	7,1E+06	1,3E+07		2,2E+06
31	IHNV	<1,9E+02	1,9E+03		
32	IHNV	1,9E+05	1,9E+07	2,7E+06	1,9E+07
33	IHNV	1,9E+03			1,3E+06
34	IHNV	1,9E+07	2,7E+06		
35	IHNV		8,6E+06		2,7E+07
37	IHNV	2,7E+05	1,9E+07		
38	IHNV	1,3E+07	5,9E+07		1,9E+08
39	IHNV		<1,9E+02	<1,9E+02	
Number of laboratories		29	34	12	10
Median titre		1,3E+04	4,0E+06	8,6E+04	2,1E+06
Maximum titre		1,9E+07	5,9E+07	5,9E+06	1,9E+08
Minimum titre		<1,9E+02	<1,9E+02	<1,9E+02	1,3E+04
25% quartile titre		1,3E+03	5,2E+05	9,8E+03	4,6E+05
75% quartile titre		4,0E+05	1,9E+07	1,9E+06	2,5E+07

Table 6. Inter-Laboratory Proficiency Test 2009 – Identification and titration of ampoule III.

Ampoule III - VHSV					
Laboratory code number	**Virus Identification**	**Titre in**			
		BF-2	**EPC**	**RTG-2**	**FHM**
2	VHSV	1,9E+05	1,9E+05		5,9E+04
3	VHSV	1,3E+07	8,6E+05	5,9E+06	
4	VHSV	1,9E+05	1,9E+05		
5	VHSV	2,7E+05	5,9E+04		
6	VHSV		1,3E+05		
7	no reply				
8	VHSV	1,3E+04	4,0E+05		
9	VHSV	2,7E+05	1,9E+06	8,6E+06	1,3E+07
10	VHSV	8,6E+06	8,6E+05	1,3E+07	1,3E+07
11	VHSV	4,0E+06	1,9E+06		
12	VHSV	4,0E+06	5,9E+05	4,0E+02	
13	VHSV	1,3E+07	1,9E+06		
14	VHSV	2,7E+09	2,7E+06	2,7E+05	
15	VHSV	2,7E+05	8,6E+04	1,3E+03	5,9E+04
16	VHSV	1,9E+04	4,0E+03		
17	VHSV	8,6E+07	5,9E+06		
18	VHSV	2,7E+06	8,6E+04		
19	VHSV	4,0E+07	4,0E+06		
20	VHSV	8,6E+06	2,7E+06		
21	VHSV	1,9E+07	1,3E+06		
22	VHSV		2,7E+06	<1,9E+02	
23	VHSV	1,3E+06	8,6E+05		
24	VHSV		8,6E+04	<1,9E+02	
25	VHSV	1,3E+07	8,6E+05	5,9E+02	1,3E+07
26	VHSV		2,7E+06	2,7E+04	
28	VHSV	2,7E+05	1,3E+05		
29	VHSV	1,3E+05	1,9E+02		
30	VHSV	1,3E+05	2,2E+05		1,3E+04
31	VHSV	5,9E+06	1,9E+06		
32	VHSV	8,6E+06	1,3E+06	1,3E+07	5,9E+05
33	VHSV	1,9E+06			8,6E+06
34	VHSV	5,9E+08	1,3E+06		
35	VHSV		1,9E+05		2,7E+05
37	VHSV	8,6E+06	2,7E+06		
38	VHSV	5,9E+07	1,3E+07		1,3E+05
39	VHSV		<1,9E+02	<1,9E+02	
Number of laboratories		29	34	12	10
Median titre		4,0E+06	8,6E+05	1,4E+04	4,3E+05
Maximum titre		2,7E+09	1,3E+07	1,3E+07	1,3E+07
Minimum titre		1,3E+04	<1,9E+02	<1,9E+02	1,3E+04
25% quartile titre		2,7E+05	1,4E+05	3,0E+02	7,6E+04
75% quartile titre		1,3E+07	1,9E+06	6,6E+06	1,2E+07

Table 7. Inter-Laboratory Proficiency Test 2009 – Identification and titration of ampoule IV.

Ampoule IV - VHSV					
Laboratory code number	**Virus Identification**	**Titre in**			
		BF-2	**EPC**	**RTG-2**	**FHM**
2	VHSV	2,7E+05	1,9E+05		1,9E+05
3	VHSV	8,6E+06	1,3E+06	5,9E+05	
4	VHSV	1,9E+05	4,0E+06		
5	VHSV	1,3E+05	5,9E+03		
6	VHSV		8,6E+06		
7	no reply				
8	VHSV	1,3E+07	2,7E+07		
9	VHSV	2,7E+06	4,0E+06	2,7E+05	1,9E+07
10	VHSV	5,9E+07	5,9E+06	8,6E+06	5,9E+07
11	VHSV	2,7E+05	4,0E+05		
12	VHSV	1,3E+06	4,0E+05	8,6E+03	
13	VHSV	5,9E+06	8,6E+06		
14	VHSV	4,0E+08	4,0E+06	2,7E+04	
15	VHSV	2,7E+04	4,0E+05	1,3E+03	5,9E+04
16	VHSV	5,9E+05	4,0E+05		
17	VHSV	5,9E+06	8,6E+06		
18	VHSV	2,7E+06	8,6E+05		
19	VHSV	2,7E+07	8,6E+05		
20	VHSV	4,0E+06	8,6E+06		
21	VHSV	8,6E+05	1,9E+05		
22	VHSV		1,9E+07	5,9E+03	
23	VHSV	4,0E+06	5,9E+05		
24	VHSV		2,7E+04	<1,9E+02	
25	VHSV	5,9E+05	1,3E+06	1,9E+03	2,7E+06
26	VHSV		1,9E+04	2,7E+04	
28	VHSV	1,3E+05	1,3E+04		
29	VHSV	2,7E+07	8,6E+04		
30	VHSV	1,3E+03	1,3E+03		1,3E+03
31	VHSV	1,3E+05	5,9E+05		
32	VHSV	4,0E+06	1,3E+07	2,7E+06	2,7E+06
33	VHSV	1,9E+05			1,9E+06
34	VHSV	5,9E+06	1,3E+06		
35	VHSV		1,9E+07		8,6E+06
37	VHSV	5,9E+06	1,3E+07		
38	VHSV	8,6E+06	1,9E+07		1,9E+07
39	VHSV		<1,9E+02	<1,9E+02	
Number of laboratories		29	34	12	10
Median titre		2,7E+06	1,1E+06	1,8E+04	2,7E+06
Maximum titre		4,0E+08	2,7E+07	8,6E+06	5,9E+07
Minimum titre		1,3E+03	<1,9E+02	<1,9E+02	1,3E+03
25% quartile titre		2,7E+05	2,4E+05	1,7E+03	6,0E+05
75% quartile titre		5,9E+06	8,6E+06	3,5E+05	1,6E+07

Table 8. Inter-Laboratory Proficiency Test 2009 – Identification and titration of ampoule V.

Ampoule V					
Laboratory code number	**Virus Identification**	**Titre in**			
		BF-2	**EPC**	**RTG-2**	**FHM**
2	No virus	<1,9E+02	<1,9E+02		<1,9E+02
3	No virus	<1,9E+02	<1,9E+02	<1,9E+02	
4	No virus	<1,9E+02	<1,9E+02		
5	No virus	<1,9E+02	<1,9E+02		
6	No virus		<1,9E+02		
7	no reply				
8	VHSV	2,7E+03	1,3E+03		
9	No virus	<1,9E+02	<1,9E+02	<1,9E+02	<1,9E+02
10	No virus	<1,9E+02	<1,9E+02	<1,9E+02	<1,9E+02
11	No virus	<1,9E+02	<1,9E+02		
12	No virus	<1,9E+02	<1,9E+02	<1,9E+02	
13	No virus	<1,9E+02	<1,9E+02		
14	No virus	<1,9E+02	<1,9E+02	<1,9E+02	
15	No virus	<1,9E+02	<1,9E+02	<1,9E+02	<1,9E+02
16	No virus	<1,9E+02	<1,9E+02		
17	No virus	<1,9E+02	<1,9E+02		
18	No virus	<1,9E+02	<1,9E+02		
19	VHSV	<1,9E+02	<1,9E+02		
20	No virus	<1,9E+02	<1,9E+02		
21	No virus	<1,9E+02	<1,9E+02		
22	No virus		<1,9E+02	<1,9E+02	
23	VHSV	<1,9E+02	<1,9E+02		
24	IPNV		<1,9E+02	<1,9E+02	
25	No virus	<1,9E+02	<1,9E+02	<1,9E+02	<1,9E+02
26	No virus		<1,9E+02	<1,9E+02	
28	No virus	<1,9E+02	<1,9E+02		
29	No virus	<1,9E+02	<1,9E+02		
30	No virus	<1,9E+02	<1,9E+02		<1,9E+02
31	VHSV	<1,9E+02	<1,9E+02		
32	No virus	<1,9E+02	<1,9E+02	<1,9E+02	<1,9E+02
33	No virus	<1,9E+02			<1,9E+02
34	No virus	1,3E+03	<1,9E+02		
35	No virus		<1,9E+02		<1,9E+02
37	No virus	<1,9E+02	<1,9E+02		
38	No virus	<1,9E+02	<1,9E+02		<1,9E+02
39	No virus		<1,9E+02	<1,9E+02	
Number of laboratories		29	34	12	10
Median titre		<1,9E+02	<1,9E+02	<1,9E+02	<1,9E+02
Maximum titre		2,7E+03	1,3E+03	<1,9E+02	<1,9E+02
Minimum titre		<1,9E+02	<1,9E+02	<1,9E+02	<1,9E+02
25% quartile titre		<1,9E+02	<1,9E+02	<1,9E+02	<1,9E+02
75% quartile titre		<1,9E+02	<1,9E+02	<1,9E+02	<1,9E+02

Figure 2. Titre obtained in BF-2 cells

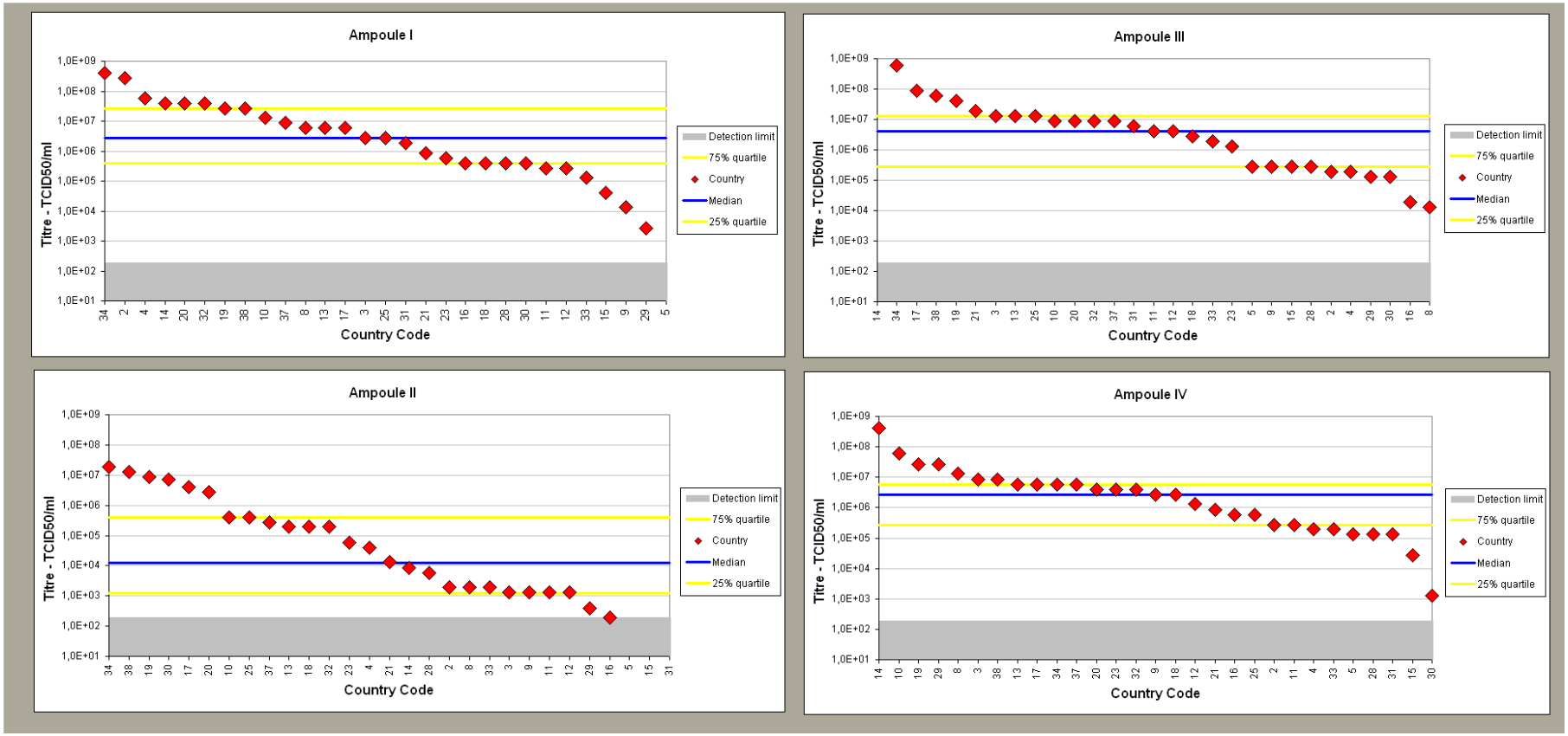


Figure 2. The titre (red diamond) of each participating laboratory (country code) using BF-2 cells illustrated for ampoule I, II, III and IV. On all graphs are plotted the detection level (gray shadow), median (blue line), 75% quartile (upper yellow line) and 25% quartile (lower yellow line). For participants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

Figure 3. Titre obtained in EPC cells

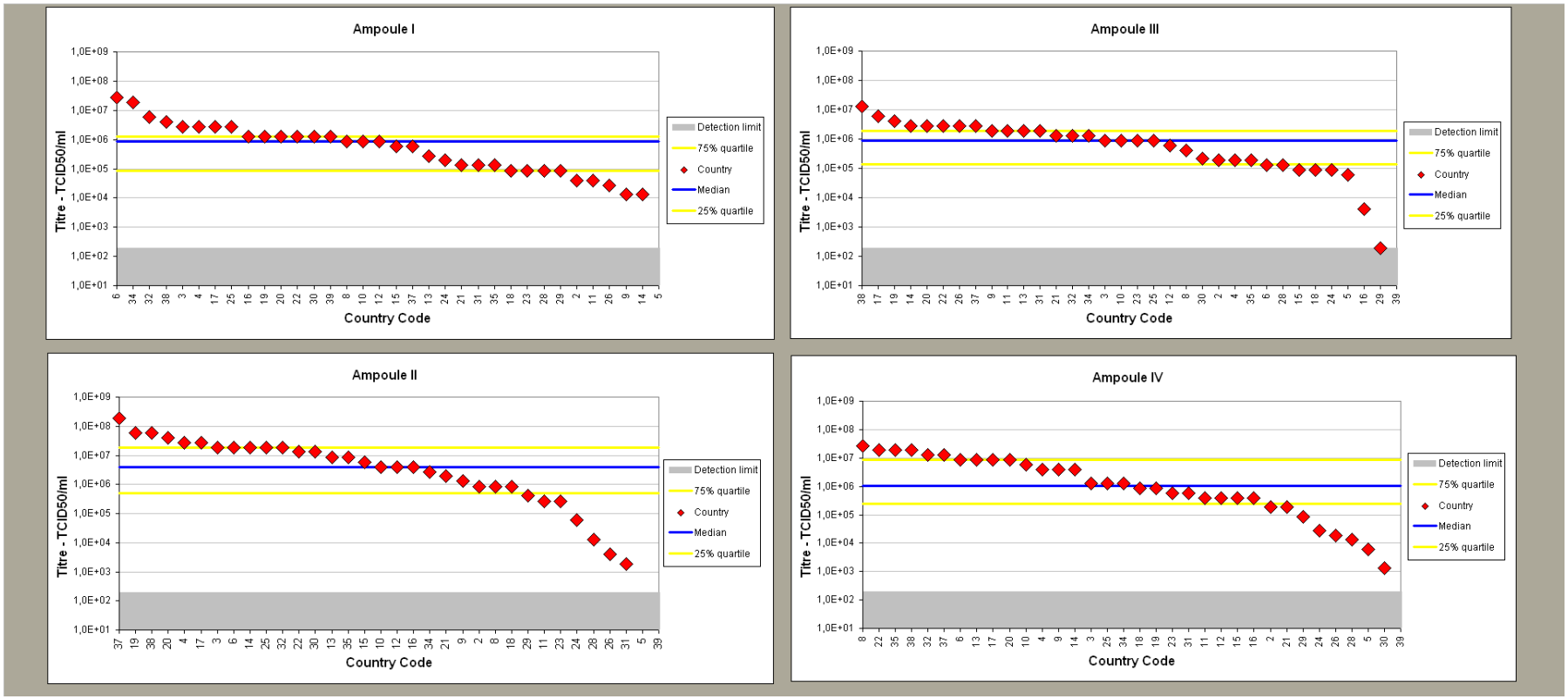


Figure 3. The titre (red diamond) of each participating laboratory (country code) using EPC cells illustrated for ampoule I, II, III and IV On all graphs are plotted the detection level (gray shadow), median (blue line), 75% quartile (upper yellow line) and 25% quartile (lower yellow line). For participants failing to obtain any titre, no red diamonds is shown.

Figure 4. Titre obtained in RTG-2 cells

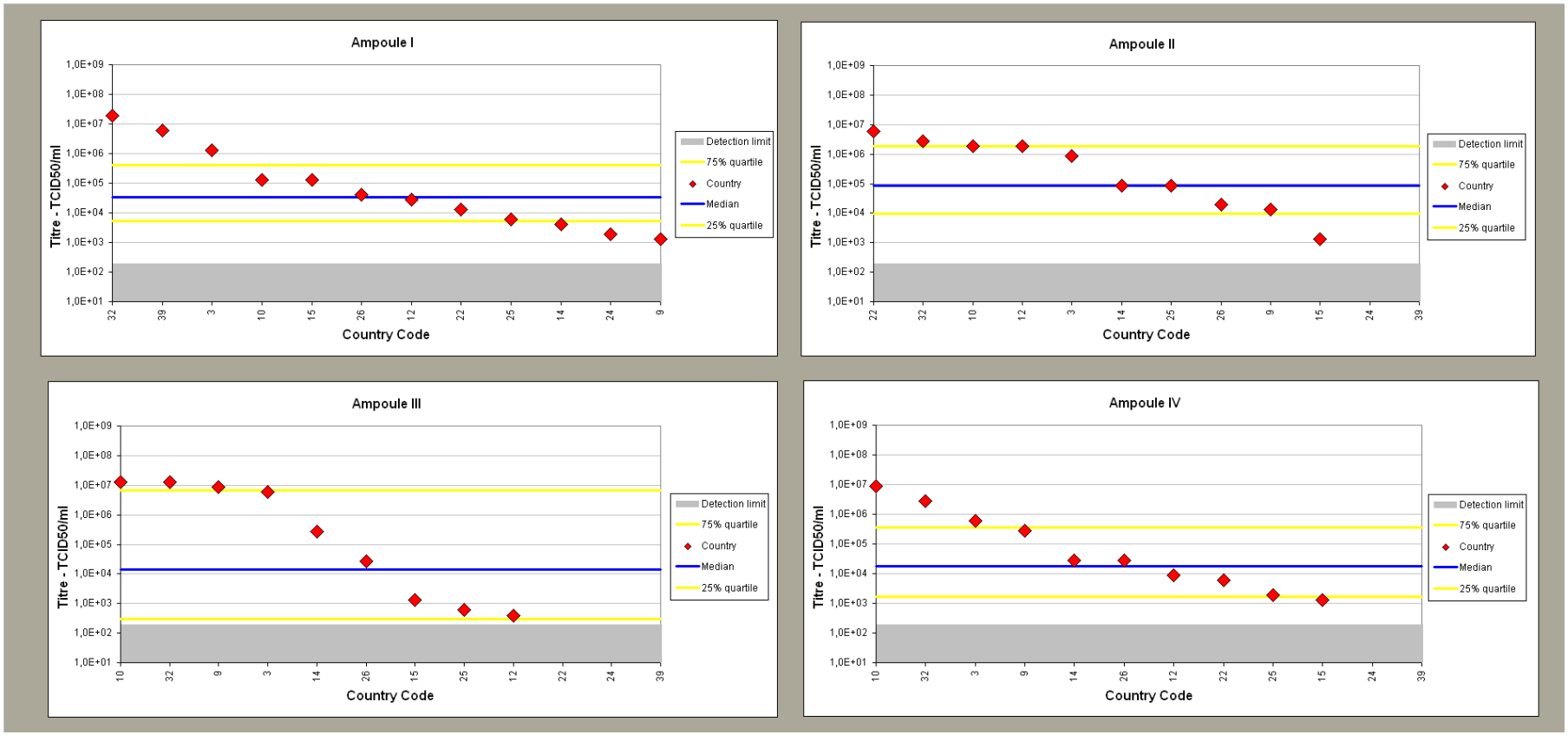


Figure 4. The titre (red diamond) of each participating laboratory (country code) using RTG-2 cells illustrated for ampoule I, II, III and IV. On all graphs are plotted the detection level (gray shadow), median (blue line), 75% quartile (upper yellow line) and 25% quartile (lower yellow line). For participants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

Figure 5. Titre obtained in FHM cells

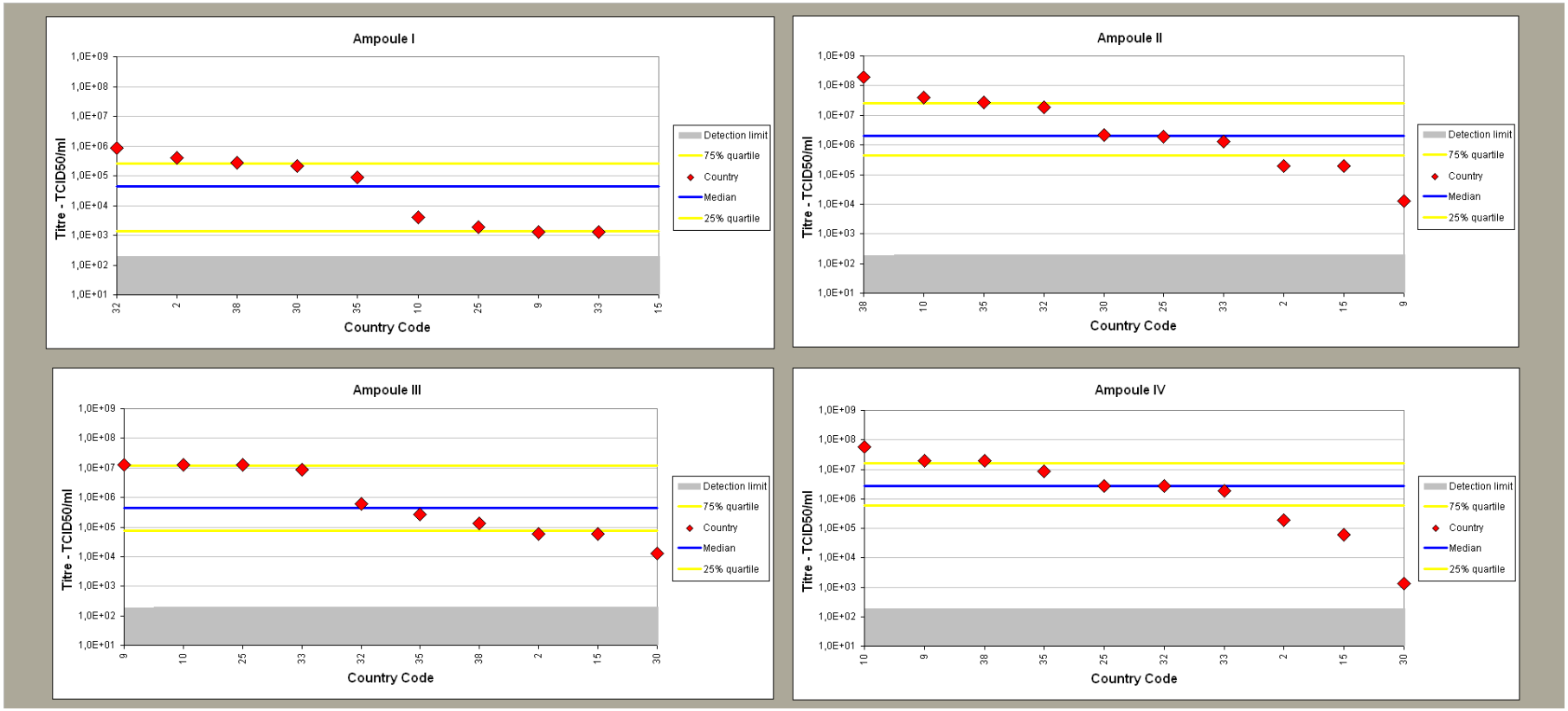


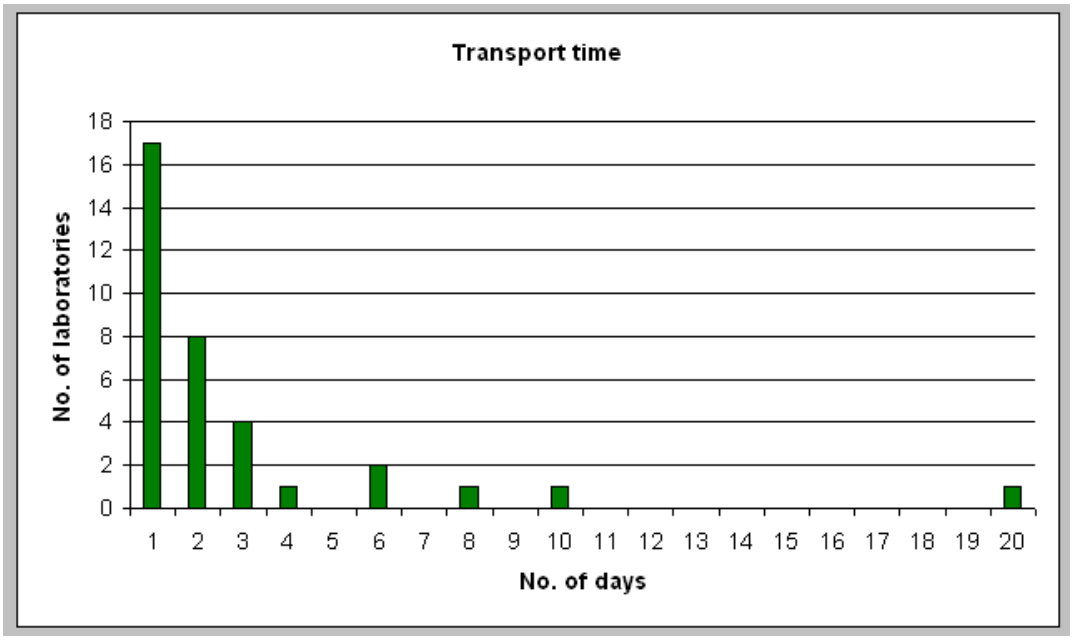
Figure 5. The titre (red diamond) of each participating laboratory (country code) using FHM cells illustrated for ampoule I, II, III, and IV On all graphs are plotted the detection level (gray shadow), median (blue line), 75% quartile (upper yellow line) and 25% quartile (lower yellow line). For participants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

Findings

Participation
36 laboratories received the annual proficiency test, 34 participants submitted results within the deadline. One participant submitted results 7 days after deadline but before the content of the ampoules were made public available. One participant did not submit results.

Shipment and handling
All proficiency tests were delivered by courier and when possible, participants were provided with a tracking number when the test had been shipped. A thermo-logger was included in 15 of the parcels. Within three days, 29 proficiency tests were delivered to participants; 3 tests were delivered within 7 days and 4 tests within three weeks. The average temperatures for the transports without cooling elements were (for 9 countries) 17.8°C and the temperature only exceeded 25.5°C for one transports for two hours upon arrival. The remaining transports (6 countries) were send with cooling elements because of longer travel time. These transports had an average temperature of 13.9°C. The temperature of four of the transports did not exceed 24°C whereas it reached 29.5°C for very short periods of time for the last two. The laboratory receiving ampoules having reached highest temperature for longest exposure time was however, able to obtain virus titres significantly above background. Therefore, the temperature rise during transportation is not considered to have lowered the virus titres considerably.

Figure 6. Transport time for the parcel to reach the participants.



Identification of content

- 24 laboratories correctly identified all viruses in all ampoules.

Ampoule I – EHNV

- 28 laboratories correctly identified EHNV.
- 3 laboratories identified ranavirus.
- 2 laboratories found more isolates than were present.
- 1 laboratory did not find any virus.
- 1 laboratory found virus but did not identify it.

Ampoule II - IHNV

- 34 laboratories correctly identified IHNV.
- 1 laboratory did not find any virus.

Ampoule III - VHSV

- 35 laboratories correctly identified VHSV.

Ampoule IV – VHSV

- 35 laboratories correctly identified VHSV.

Ampoule V – No virus

- 30 laboratories correctly identified that there was no virus.
- 5 laboratories identified a virus.

One laboratory did not submit any results

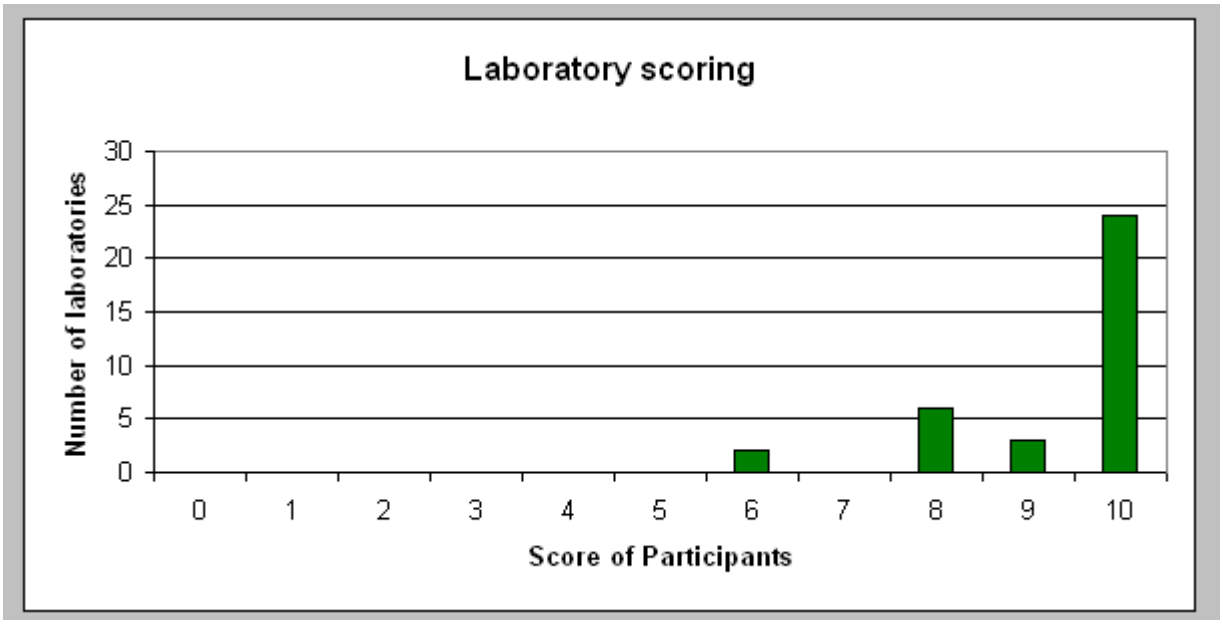
Scores

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 (Table 3), giving the possibility for obtaining a maximum score of 10. Identification of "ranavirus" as the virus in Ampoule 1 was given the score of 1. If no sequence analysis was performed, the participant would not be able to identify the ranavirus as EHNV and would be given the score of 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.

24 laboratories out of 36 correctly identified all viruses in all ampoules and obtained maximum score. Three laboratories scored 9 because the virus in ampoule I was identified 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. A diagram of the scoring obtained by the laboratories is shown in figure 7. Serotyping, 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 7. Obtained score by participants.



Methods applied

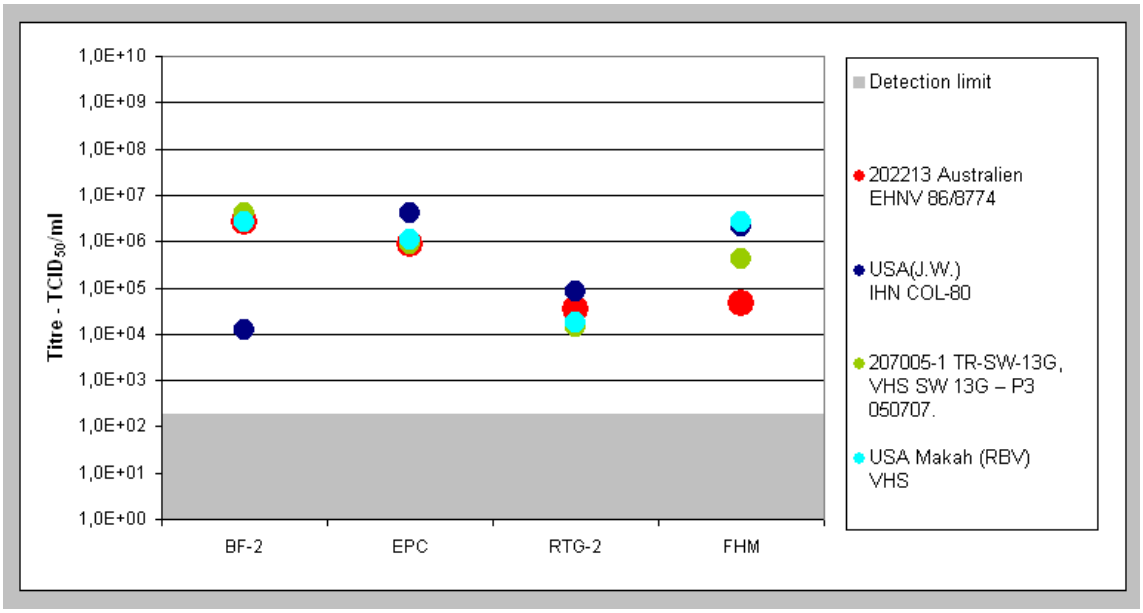
The following cell lines were used by the participants:

- 29 laboratories used BF-2 cells
- 34 laboratories used EPC cells
- 12 laboratories used RTG-2 cells
- 10 laboratories used FHM cells
- 5 laboratories used four cell lines
- 5 laboratories used tree cell lines
  - 3 laboratories used BF-2, EPC and FHM
  - 3 laboratories used BF-2, EPC and RTG-2
- 23 laboratories used two cell lines:
  - 17 laboratories used BF-2 cells in combination with EPC cells
  - 4 laboratories used RTG-2 cells in combination with EPC cells
  - 1 laboratory used BF-2 cells in combination with FHM cells
  - 1 laboratory used EPC cells in combination with FHM cells
- 1 laboratory used only EPC cells

The combination of EPC and FHM cells is not valid according to Commission Decision 2001/183/EC, neither is the use of EPC cells alone. 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 8. It appears that VHSV (Ampoule III and IV) replicates well on BF-2, FHM and EPC cells but less efficient on RTG-2 cells. IHNV (ampoule II) replicates well on EPC and FHM cells, and less efficiently on BF-2 and RTG-2 cells. Finally, EHNV (ampoule I) replicates well on EPC and BF-2 cells whereas lower titres were observed on RTG-2 cells and FHM cells.

Figure 8 Median titre of viruses obtained by participants at different cell lines. For ampoule V the median was below detection level and is not shown.



Methods used for identification of viruses (Table 9)

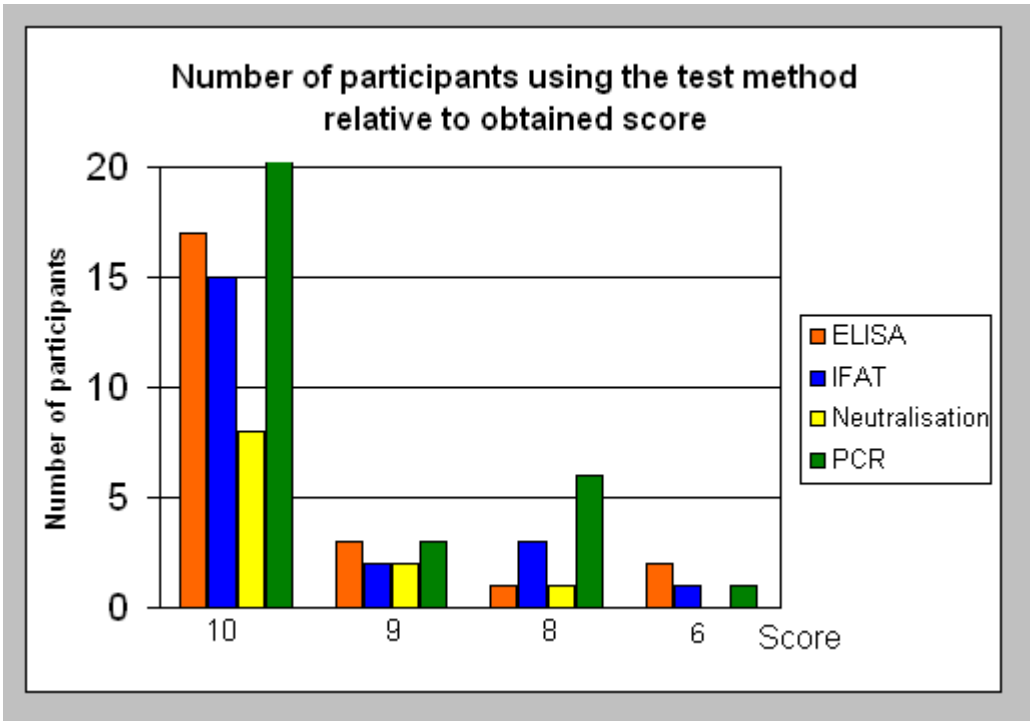
- 24 laboratories used ELISA for identification of viruses.
- 21 laboratories used IFAT for identification of viruses.
- 11 laboratories used neutralisation tests for identification of viruses.
- 34 laboratories used PCR for identification of viruses.
- 30 laboratories performed sequencing for identification of viruses.

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

Laboratory code number	Score Top score 10	ELISA	IFAT	Neutralisation	PCR	Sequence	Sequence ampoule no.
2	10				X	X	I, II, III, IV
3	10	X	X		X	X	I, II, III, IV
4	10	X			X	X	I
5	6	X					
6	9	X	X	X	X		
7							
8	6	X	X		X	X	I, II, III, IV, V
9	10	X			X	X	I
10	10	X	X	X	X	X	I, II, III, IV
11	8	X	X		X		
12	10	X	X		X	X	I, II, III, IV
13	10	X			X	X	I, III, IV
14	10		X	X	X	X	I, II, III, IV
15	10	X	X	X	X	X	I, II, III, IV
16	10	X		X	X	X	I, II, III, IV
17	10			X	X	X	I, III, IV
18	10	X	X		X	X	I, II, III, IV
19	8		X		X	X	I, II
20	10				X	X	I, II, III, IV
21	10	X	X	X	X	X	I, II, III, IV
22	10	X			X	X	I, II
23	8		X	X	X	X	I, III, IV
24	8				X	X	I, II
25	10	X	X		X	X	I, III, IV
26	10			X	X	X	I, II, III, IV
28	9	X	X		X		
29	10	X	X		X	X	I
30	10	X	X		X	X	I, II, III, IV
31	8	X			X	X	I, II, III, IV
32	10	X	X	X	X	X	I, II, III, IV
33	9	X		X	X		
34	10		X		X	X	I, II
35	10		X		X	X	I
37	10	X	X		X	X	I, II, III, IV
38	10	X	X		X	X	I, II, III, IV
39	8				X	X	I
Number of laboratories		24	21	11	34	30	

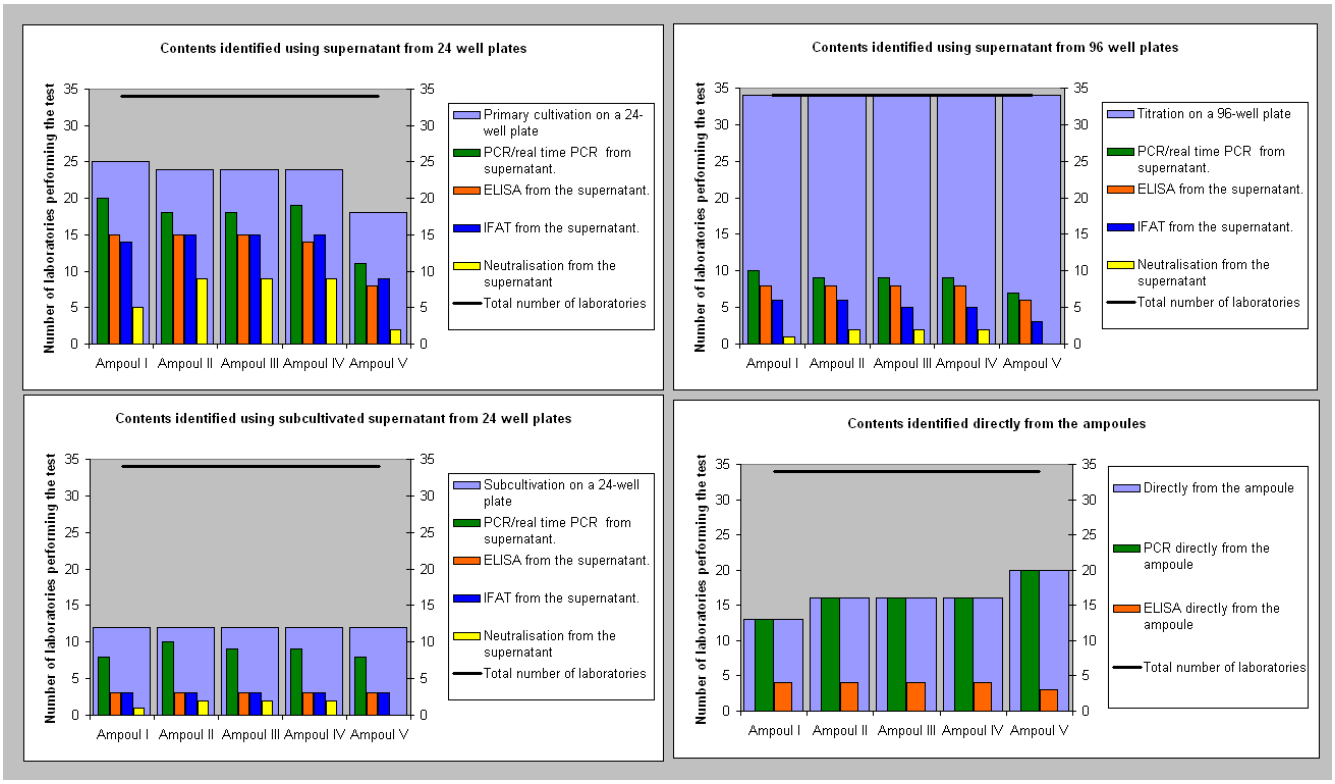
A graph was constructed to illustrate the association between the methods used by participants for virus identification and the obtained score (Figure 9). The PCR is the most frequently used method by participants and only one participant did not use this method. Participants scoring 9 all used PCR for identification of the virus in ampoule 1 as ranavirus but did not perform sequencing analyses to further identify the virus as EHNV. For participants scoring lower than 9, the deficiency in virus identification can not directly be assigned to improper use of an identification method. Rather mistakes might be related to performance of the overall procedure. 6 out of the 8 participants scoring lower than 9 identify false positive viruses in the ampoule indicating that cross contamination could have occurred at some point in the diagnostic process. The last two participants are unable to identify viruses in one or more ampoules. In one of these laboratories this might occur because cells are not sensitive towards detection of these viruses as no titres are reported. In another laboratory there was detectable titre but missing identification which may be due to the identification method (EHNV PCR) has not been implemented.

Figure 9. Methods used by participants for identification



At this year's proficiency test, participants were asked to fill out a questionnaire regarding the diagnostic methods used in the laboratory. The results are summarized in figure 10 A-D. All participating laboratories used 96 well plates for titration. Primary cultivation on 24 well plates was done by 18-25 participants and 12 participants subcultivated on 24 well plates. PCR was the most used method for identification of virus. On supernatant from subcultivated 24 well plates or directly on ampoule content, PCR was used more than double as frequently as any other method. However, when virus was identified from supernatant from primary cultures of either 24 or 96 well plates ELISA and IFAT was used at an only slightly lower frequency than the PCR. The reason for the different pattern in methods used for virus identification on primary and subcultivated cells is unclear but might reflect different usage of identification methods in the lower number of laboratories using subcultivation. The high proportion of laboratories using of PCR for identification of virus directly in the ampoule most likely reflect that PCR is a tool that can be very easily used for this purpose.

Figure 10. Diagnostic methods used for identification of viruses. A) Method used for identification using supernatant from primary cultivation from 24 well plates. B) Same as A but from subcultivated plate. C) Method used for identification using supernatant from primary cultivation from 96 well plates. D) Method used for identification directly on content in the ampoules.



Genotypning and sequencing

In previous proficiency tests provided by the CRL, we have encouraged participants to genotype the identified viruses though it has not been a mandatory task. As ranaviruses was for the first time 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

- 30 laboratories sequenced to identify EHNV.
- 3 laboratories performed only PCR and no sequence analyses.
- 1 laboratory did not identify the virus
- No laboratory reported having performed RFLP analyses.

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. 30 laboratories used sequencing for identification of EHNV whereas none used RFLP. Of these, 29 used sequencing of the partial MCP gene as recommended by the OIE diagnostic manual for EHN whereas two participants sequenced the polymerase gene as described by Holopainen et al. 2009. Three laboratories identified the virus by PCR but not did not perform sequencing analyses.

Ampoule II-IV

25 out of 35 laboratories sequenced parts of the genome of either IHNV or VHSV isolates. This is five laboratories less than did sequencing of the EHNV. Both full length and partial N- and G-genes were used for virus/genotype identification (see table 10).

Ampoule II - IHNV Genotype L

- 21 laboratories performed sequencing
- 11 laboratories genotyped the IHNV isolate as belonging to genogroup L
- 6 laboratories used alternative genotyping notification or showed blast results
- 4 laboratories did not give any genotype of the sequences

11 laboratories genotyped the IHNV isolate as belonging to genogroup L as described in Kurath et al. 2003 (Table 10). This is a more laboratories compared to genotyping IHNV in proficiency test 2008. One laboratory genotyped the isolate according to another publication. Furthermore, five laboratories showed indirect isolate relatedness to genogroup L either by presenting a phylogenetic tree or by showing blast results.

Ampoule III - VHSV genotype Ie

- 21 laboratories performed sequencing
- 14 laboratories identified the VHSV isolate as genotype I
- 6 laboratories subtyped the isolates as a genotype Ie
- 2 laboratories subtyped the isolates as a genotype Ib
- 1 laboratory identified the VHSV isolate as genotype III
- 4 laboratories showed blast results
- 2 laboratories did not give any genotype of the sequences

14 laboratories correctly identified the isolate as belonging to genotype I. 6 laboratories correctly subtyped the isolate as belonging to the Ie subgroup (according to Einer-Jensen et al 2004) whereas two identified it as a genotype Ib. Ib genotype might come up because laboratories uses different genes and if a certain gene is not sequenced for genotype Ie isolates, a blast result will show a related

subgenotype. One laboratory identified the isolate as belonging to genotype III. This could be because the genotype notification is according to Nishizawa et al. 2002? Four laboratories indicated genotype of isolate by showing blast result.

Ampoule IV - VHSV genotype IVa

- 20 laboratories performed sequencing
- 12 laboratories identified the VHSV isolate as genotype IV
- 10 laboratories subtyped the isolates as a genotype IVa
- 1 laboratory identified the VHSV isolate as genotype I
- 1 laboratory identified the VHSV isolate as genotype Ie
- 1 laboratory identified the VHSV isolate as a mixture of genotype IVa and III
- 3 laboratories showed blast results
- 2 laboratories did not give any genotype of the sequences

12 laboratories correctly identified the isolate as belonging to genotype IV and 10 laboratories correctly subtyped the isolate as belonging to the IVa subgroup. One laboratory identified the isolate as belonging to genotype I which might be because the genotype notification in Nishizawa et al. 2002 is used. One laboratory identified the isolate as genotype Ie with identical sequence to the sequence of the isolate in ampoule III and therefore double sequencing of VHSV in ampoule III might have occurred. Four laboratories indicated the genotype of the isolate by showing blast result.
Interestingly, one laboratory report that a mixture of two VHSV isolates is present in ampoule IV. Direct sequencing of a PCR fragment identified the VHSV as a genotype IVa. Cloning of three independent clones identified one genotype IVa isolate and two genotype III isolates. The sequence of the genotype III isolate is very closely related to the marine 4p168 isolate (Einer-Jensen et al. 2004, Mortensen et al. 1999). The reason for this finding is not clear though it seems reasonable that a contamination have taken place. No other laboratories report of genotype III present in ampoule IV, indicating that a contamination might have occurred in the laboratory. However as the reported genotype III was from cloned sequences, it is also a possibility that primers favouring genotype III amplification could have amplified traces of genotype III RNA present as contaminating viruses in the sealed ampoule and that these sequences have been cloned into the sequencing vector.

In general, it is positive that more laboratories performed sequencing than at last year's proficiency test and that sequences were of high quality and usable for genotyping. This high number of laboratories performing sequencing might reflects that EHNV has been included in the test. It is important that the remaining laboratories implement the technique in the laboratory as genotyping is the basis for differentiating notifiable viruses from others. Genotyping of VHSV and IHNV were performed according to different notifications although references were provided on what notification should be used. In future proficiency tests it will again be specified for all listed disease according to which references, the genotyping should be performed.

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

Laboratory code number	Score	Ampoule II - IHNV		Ampoule III - VHSV		Ampoule IV - VHSV	
		Genotype	Gene sequenced	Genotype	Gene sequenced	Genotype	Gene sequenced
2	10	Genogroup L Subgroup 2	Partial G	Genotype Ie	Partial G	Genotype Ie	Full length G
3	10	(98 % similar to Col-80…)	Partial N	(100% identical to TR-SW13G…)	Partial G	(99 % identical to ME03….)	Partial G
8	6		Partial N		Partial N		Partial N
10	10	Genogroup L	Partial G	Genotype Ie	Full G	Genotype IVa	Partial G
12	10	Genogroup L	Partial G	Genotype Ie	Partial G	Genotype IVa	Partial G
13	10			Genotype I	Partial G	Genotype IVa	Partial G
14	10	Phylogenetic tree	Partial N	Genotype I	Partial G = direct sequencing Full length G = 3 clone	2 X Genotype III 2 X Genotype Iva	Partial G = direct sequencing = genotype IV
15	10	Genotype L	Full length G	Genotype I	Full length G	Genotype IVa	Full length G
16	10	(100 % identical to AY442509)		(96% identical to Z93414)			
17	10			Genotype III	Partial N	Genotype I	Partial N
18	10	Genogroup L	Partial N Partial G	Genotype I	Partial N	Genotype IV	Partial N
19	8	Genogroup L	Partial G Partial NV				
20	10	Genogroup L	Partial G	Genotype Ie	Partial G	Genotype Iva	Partial G
21	10		Partial N		Partial N		Partial N
22	10	Genogroup L	Partial G				
23	8			Genotype Ib	Partial G	Genotype IVa	Partial G
24	8		Partial N				
25	10			Similar to AY546619	Full length G	Similar to AB490792	Full G
26	10	Genogroup L	Partial G	Genotype Ib	Partial N	Genotype IVa	Partial N
30	10		Partial N	Genotype Ie	Partial N	Genotype IVa	Partial N
31	8	(100 % homology with L40874)	Partial G	(98% hology to Z93412)	Partial G	(100% homology to DQ401192)	Partial G
32	10	Genogroup U+L	Partial G	Genotype I	Full length G	Genotype IV	Full length G
34	10	(100 % homology with AY442509)	Full length G				
37	10	Genogroup L	Partial N	Genotype I	Partial G	Genotype IVa	Partial G
38	10	Genogroup L	Partial N	Genotype Ie	Partial G	Genotype IVa	Partial G

Concluding remarks

The inter-laboratory proficiency test 2009 was conducted without major constrains. Most parcels were delivered by the shipping companies within 3 days after submission; it was, however, unfortunate that one of the parcels made op to 3 weeks before delivering to the laboratories (primarily due to border controls).

In 2009 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.

The IHNV within this test replicates well on EPC and FHM cells, and less efficiently on BF-2 and RTG-2 cells (figure 8) making all the valid combinations of cell in Commission Decision 2001/183/EC suitable.
EHNV replicates well on EPC and BF-2 cells whereas lower titres were observed on RTG-2 cells and FHM cells (figure 8). Therefore the combination of RTG-2 and FHM cells seems less suitable.
It appears that the two VHSV isolates in this test replicates equally well on BF-2, FHM and EPC cells but less efficient on RTG-2 cells. This is valid for the two VHSV isolates included in this proficiency test but other VHSV isolates prefer BF-2 cells compared to EPC cells and therefore laboratories are still encouraged to use a combination of cells as described in Commission Decision 2001/183/EC. The bad performance in several laboratories of their RTG-2 cell lines for growth of VHSV (Figure 4) 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, we recommend that laboratories use BF-2 cells and not RTG-2 cells for replication/survey of/for VHSV.
In conclusion we recommend all participants to evaluate the sensitivity of the cells used in their laboratory in relation to the diagnostic purpose.

The CRL 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. This year however, 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 will be further presented and discussed at the 14th Annual Meeting of National Reference Laboratories for Fish Diseases to be held 26-28 May 2009 in Århus, Denmark.

Nicole Nicolajsen, Helle Frank Skall and Søren Kahns

European Community Reference Laboratory for Fish Diseases
National Veterinary Institute, Technical University of Denmark, 12 February 2010

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Laboratory visit by the Community Reference Laboratory for Fish Diseases to the Portuguese National Reference Laboratory for Fish Diseases Laboratório Nacional Investigação Veterinária - LNIV

October the 22nd - 23rd 2009

Introduction

The National Reference Laboratory for Fish Diseases (NRL) in Portugal is part of the National Laboratory of Veterinary Research (LNIV). The NRL was visited on the 22nd and the 23rd of October 2009 by Nicole Nicolajsen and Søren Kahns from the European Community Reference Laboratory for Fish Diseases (CRL). The programme for the visit is shown in Annex 1 and a list of participants at the introductory meeting in Annex 2. This report describes findings, comments and recommendations made by the delegation from the CRL. The report is sent to the LNIV and the EU Commision.

Organisation

The LNIV is a part of the Ministry of Agriculture, Rural Development and Fisheries and is managed by one director, Dr. Nuno Canada. The LNIV are physically located at two sites: one part is in Lisbon and the other is located close to Porto. The Unit of Animal Health that is a part of the LNIV is located in Lisbon and is headed by Dr. Alice Amado, consists of five laboratories. One of these, the Laboratory of Virology that is headed by Dr. Miguel Fevereiro, includes the NRL for Fish Diseases. The LNIV deals with notifiable diseases of animals, zoonosis or intoxications. The Laboratory of Virology deals with more than 70 viral diseases. An organisation plan is shown in Annex 3.

The visit was opened by a presentation of the employees from LNIV. Dr. Miguel Fevereiro introduced the NRL for fish diseases, Dr. Nuno Canada gave a historical overview and a presentation of the organization of the LNIV and Dr. Susana Freitas from the Direcção Geral de Veterinária (DGV) - the National Veterinary Sanitary Authority (that ensure the fiscalization of the constant norms in the Directive 2006/88/EC of 24 of October, *on animal health requirements for* aquaculture animals and products thereof and on the prevention and control of certain diseases in aquatic animals) gave a presentation of the aquaculture in Portugal. Subsequently, the functions and duties of the CRL were presented by Søren Kahns.

Aquaculture in Portugal

The main fish species cultured in Portugal are European seabass, Gilthead seabream and rainbow trout, but turbot and carp farms are also present (see Annex 4). In 2008 Portugal had 14 fish farms producing around 5 tonnes per year and 125 fish farms producing 5 - 100 tonnes per year.

Portugal has 23 rainbow trout farms. Two are declared free of VHS and IHN (category I) whereas the rest are under surveillance programme (category II). Turbot farms are in category II for VHS and carp farms are in category II for KHV disease.



Samples from rainbow trout farms (category II) are collected twice a year in the period: October to May where water temperature is less or equal to 14°C. 30 fish are analysed in pools - 5 fish in each pool. Turbot and carp are sampled in a similar manner. Category I rainbow trout farms are sampled once a year.

The marine fish farms (seabass, seabream and other marine species) are in category II for VHSV and IHNV These farms are subjected to a surveillance programme (active and targeted surveillance). Samples are collected in the period: October to May, where water temperature is less or equal to 14°C. 30 fish are analysed in pools - 5 fish in each pool.

The total fish production is increasing in Portugal and it is expected to increase further in the future.

Buildings, Furnishing and Access

A guard is placed at the entrance to the institute controlling people entering the LNIV. When inside of the building, there is free access to enter laboratories.

The laboratories at LNIV in Lisbon is located in older buildings but LNIV plan to move into new facilities within 18 to 24 months. Presently, the laboratory for fish diseases is present in a sepearate building/laboratory shared with laboratory for Bee diseases. Rooms are clean and containing adequate furnishings.

The Portuguese NRL for Fish Diseases

Staff

The Head Dr. Miguel Fevereiro is also Head of the Laboratory of Virology that consists of a group of 17 persons. The NRL is a part of the Laboratory for Virology and consists of three persons. Dr. Miguel Fevereiro is the overall responsible for the NRL, Dr. Teresa Duarte who is responsible for the dayly work on sample processing, and survei for and diagnostic of viral fish diseases. Furthermore, a techniscian, Heather George assists on the dayle work in the laboratory.

New employees receive basic safety and laboratory training before they are able to work independently.

Equipment

Equipment was adequate although it is recommended that one additional LAF bench is obtained in future so that clean and dirty work is not performed in the same working space. It was discussed that such solution would be a possibility when LNIV moves to new buildings.

All equipment has a reference number. As the laboratory is not yet accredited it is not necessary having logbook for registration of activities and eventual problems. The laboratory are in the process of being accredited and will implement these procedures in the future. Calibration of pipettes is performed by an external company once a year. It is recommended that incubators are temperature calibrated.
Instructions for use of equipment are also in the process of being written down in procedures, one for each piece of equipment. The purchase of reagents and apparatus was negotiated with superior employees before ordered.

Large Laboratory Equipment included items as:

- Incubators, temperatures (14, 20 29, 37°C)
- Centrifuges (cooling),
- Freezers (-150, -80, -25, -20)
- Refrigerator
- LAF benches
- Fume cabinet
- Lamina flow
- Safety cabinet for chemicals
- Microscope
- Microscope for IF
- ELISA reader
- ELISA washer
- Microwave
- Genetic analyser
- Spectrophotometer for DNA and RNA
- Thermocycler
- Real-time PCR ICycler
- Autoclave
- Etc.

Small equipment included items as:

- Plastic plates
- Pipettes
- Different kind of tubes

Cell lines:

- Cell lines grown in the laboratory: RTG-2; FHM and EPC. All cell lines have been obtained from the CRL. Media was made in the laboratory.

Laboratory of Fish and Bee diseases

One part of the fish laboratory where samples are processed is air-conditioned. This room also contains a LAF bench and incubators. Furthermore, a fume hoods is present in the laboratory for working with hazardous chemicals.

Neutralisation tests are performed in this laboratory using antibodies made by the NRL. The CRL will send additional antibodies that the NRL can use as reference material.

PCR analyses and microscopy are performed in the laboratory of Virology by Dr. Teresa Duarte.

Laboratory of Virology

The staff of the laboratory consisted of 17 persons, including the head of the laboratory, Dr. Miguel Fevereiro. The laboratory was well equipped and contained e.g. several PCR machines; several real time PCR machines and a sequenator. Furthermore they possessed necessary kits for purifying DNA and RNA, and DNA visualisation equipment. Nucleic acid preparation, reaction mixing and post PCR analysis were performed in separate rooms to avoid contamination. The Laboratory of Virology deals with more than 70 pathogenic viral diseases. Molecular diagnostics of fish diseases have been implemented for listed diseases EHN, VHS, IHN, ISA and KHV diseases, and also for IPN, SVC. For all these diseases, primers and reaction conditions are as described in the OIE diagnostic manual. (If a conventional PCR is chosen as method for diagnosis of KHV disease, it is recommended to use the Bercovier PCR as it shows highest sensitivity).

Registration and processing of Samples

The NRL provides veterinarian with tubes containing transport medium. On the tubes, it is recommended that the NRL mark production date, expiry date and that the tubes should be stored at 4°C.
Samples are normally received as organ material. The organ material is usually sent on ice however there have been few cases where ice has been omitted. The NRL have informed the responsible veterinarians that samples should always be kept on ice. It this becomes a problem in future, it is recommended that the NRL keep a record of the date of receiving sample without ice and the name of the responsible veterinarian in order to follow the problem and immediately takes contact to the responsible veterinarian. The consequence if temperature of samples is too high could be that any active virus is destroyed.



Samples are sent to a direct entrance room where all paperwork and registration of samples takes place. Samples are given a unique number and a barcode. All samples are registered electronically (Nautilus) together with all necessary information. In Nautilus it is possible for the veterinarian at the LNIV to follow where in the diagnostic process the samples are. When registered, the NRL are notified that samples have arrived and employees from the NRL transport samples in boxes to the laboratory.

When samples arrive as living fish, Dr. Teresa Duarte receives the fish in the fish laboratory where she takes out the samples. Fish that are not examined are kept in aquariums until analyses are done. After centrifugation of samples, it is divided in two parts: one is inoculated on cells and the other is analysed by PCR.

The samples (0,1 ml 1:10 diluted sample) are inoculated on monolayer cells (0,5 ml medium) (The Commission Decision 2001/183/EC recommends 1:10 solution and at the CRL we use a total of 1.5 ml medium). It is recommended that mortar and pestle are kept in the fridge or the freezer until they are being used. Samples from one farm are kept on a single plate in a separate box in the incubator to avoid contamination events to occur between "farms". The monolayer cells are carried to the Laboratory for Virology for microscopy.

In addition, all samples are tested for VHSV, IHNV, EHNV and IPNV by PCR. The PCR methods implemented are all described in OIEs diagnostic manual.

Fish samples to be analysed for bacterial diseases are send to Lab of Bacteriology whereas samples to be analysed for fungal diseases are send to Lab of Mycology.

The results of all tests performed for are registered in Nautilus.

Cell Culture Facilities

EPC, FHM and RTG-2 cells are continuously cultured in the laboratory. The RTG-2, FHM and the EPC cells are passaged every 1 to 2 weeks. Furthermore, there is a back-up cell bank at -150ºC. The CRL will send BF-2 cells to the laboratory. It is recommended that cells are tested for mycoplasma regularly. Pictures from and procedures for making mycoplasma tests are shown in Annex 9. Sensitivity test is performed for listed diseases VHSV, IHNV, EHNV and for IPNV.
It is recommended to make the test twice a year. It would be a good idea to make a report of the sensitivity test over time in order to follow the sensitivity of the cells. See Annex 5 for the Danish sensitivity test. Since sensitivity tests were performed, there is no need to include positive controls and only negative controls are included in the tests. Disinfection of the workspace was done by Virkon X and UV light (was turned on 15 min after and before each work in the bench).

Accreditation

The LNIV was accredited according to ISO 17025 in 2008 by the Portuguese Accreditation body APCER. Further two laboratories (Lab of Pathology and lab of Bacteriology) within the Unit of Animal Health have been accredited. Laboratory of Virology is in the process of being accredited and the Fish laboratory is expected to be accredited for cell culturing, PCR and neutralisation techniques by 2010.

Proficiency Test

The proficiency tests (PT) allow a laboratory to assess their diagnostic capacity of certain procedures. The Portuguese NRL for fish diseases has participated in the PT for identification of notifiable fish diseases organized by CRL, Aarhus, Denmark, in 1996, 1997, 2002, 2003, 2004, 2005, 2007 and 2008 (in 2006, no PT was distributed). The Portuguese NRL included the following techniques in the test: Titration of virus; Isolation of viruses on cell culture; Identification of virus by PCR and neutralisation. The score obtained by the NRL are listed in Annex 6.

The results from PT 2007 and 2008 were discussed as the NRL incorrectly identified SVCV in one or more ampoules by both PCR and neutralisation. In 2007, two false positive SVCV identifications could come from contamination from ampoule 3 that contaminated that virus. However, such contamination could not explain the SVCV finding in PT 2008 as no SVCV was included in any of the ampoules. After receiving the results of the 2008 PT it was decided by the laboratory to take action and change the procedures for analyzing the PT. So for the PT 2009, extra precautions were made to avoid cross contaminations. This includes that the ampoules are opened at different locations and the nucleic acid purification is performed separate in a purification robot. It was also recommended that each ampoule is inoculated at a single monolayer plate located in a separate box (as is done for ordinary fish samples) in order to minimize the risk of contamination when the plates are carried to microscopy at the lab of virology. Furthermore it was suggested that ELISA or IFAT could be used as supplement to or instead of the neutralization. If neutralisation is made as the only antibody based tool, it is recommended to do neutralization of a series of sample dilutions and register results for traceability (See Annex 8 for an scheme for registration of results).

For future PTs it is recommended to re-examine the content of the ampoule if it does not correspond to the official content announced by the CRL. If result is repeated the CRL might send an antibody for repeating the result.

The Laboratory for Virology did participate in many other PTs focusing on viral diseases of other animals. None of these addressed fish diseases.

The Portuguese NRL for fish diseases do not organizes PTs as it is the only laboratory in Portugal.

Implementation of the Council Directive 2006/88/EC in Portugal

During the Meeting the model for implementation of the Council Directive 2006/88/EC in Portugal was presented by Dr. Susana Freitas from the Competent Authorities (CA). The discussion took place at the office of the CA that was located in a building at the LNIV.

A surveillance programme for VHSV and IHNV has existed in Portugal since 1992. EHNV have been included in the programme since 2008. VHSV, IHNV and EHNV have never been identified in Portugal during this survey.

The Council Directive 2006/88/EC had been implemented in Portugal and authorisation of fish farms has been accomplished. Two rainbow trout farms have been categorised in category I based on historical reasons. These two rainbow farms are brood stock farm for the Rainbow trout production in Portugal. The other 21 rainbow farms have been categorised in category II. Eight of these farms are located in national river systems and it will be applied for that these farms becomes category I in 2010. The rest of the category II farms that are located in river systems shared with Spain is expected to be in category I from 2010/2011 when they have been in the surveillance programme for four years.

Turbot farms are in category II for VHSV and IHNV. If none of the listed diseases are identified these farms are expected to go into category I, in 2010/2011, after a four year surveillance period.
Carp farms are in category II for KHV. These farms expected to go into category I, in 2011, after a four year surveillance period.

From all rainbow trout, turbot and carp farms, samples for survey are sent to the LNIV twice a year, in April and November – the second sampling occurs approximately four month after the first. 30 fish are sampled in pools of 5 for diagnostic analyses.

The marine fish farms (seabass, seabream and other marine species) are in category II for VHSV and IHNV. These farms are subjected to a surveillance programme where samples for survey are sent to the LNIV once a year.

Training needs and future plans

Diagnostic methods for the listed exotic disease EUS will have to be implemented.

In light of the problems in previous PTs, it was suggested that the NRL could use ELISA or IFAT as supplement to neutralisation. Alternatively, the neutralisation method should be performed on a series of sample dilutions at the result should be recorded at more time points – e.g. once every day.

The research activities of the laboratory are limited due to lack of funding and priority. In view of the available equipment and the educated staff, the laboratory has the potential for performing more research activities. This could include projects involving e.g. PhD students. Alternatively, there would be possibilities for e.g. involvement in applications for research projects funded by EC.

Some staff members of the NRL could be expected to be retiring within next 5 years. If the NRL find it necessary to employ and educate new staff, they should be welcome to make a training visit to the CRL.

Conclusion

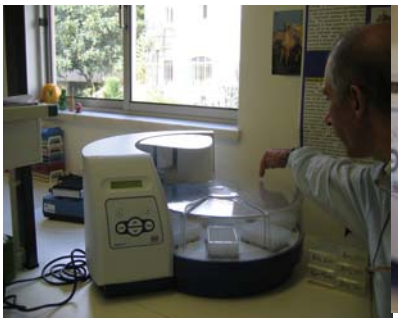
The visit showed that the NRL of Portugal has an adequately well equipped laboratory to perform the necessary analyses. The LNIV will move into new buildings within the next 18 to 24 months. In the new laboratories it is recommended that the NRL for fish diseases obtains two separate working space/LAF-benches, one for clean work as well as one for dirty work. Such could be implemented in one Fish laboratory or in a cell culturing unit. Furthermore it is recommended that working spaces are made so that cells are not to be carried between buildings for microscopy.

The staff is well educated and very capable and works are in general performed according to the described methods according to EU requirements.

The lower score obtained in the previous two PTs had made the NRL to decide to take action and change the procedure in the laboratory in a manner that decreases the possibility of obtaining cross contamination.

No major problems were observed and only minor suggestions were made, described above.

The overall conclusion is that the NRL of Portugal 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 22nd

8:30	Pick up at hotel
09:00 – 10:30	Presentation of the employees from LNIV and introduced the Portuguese NRL for fish diseases by Dr. Miguel Fevereiro. Historical overview and a presentation of the organization of the NLIV by Director Dr. Nuno Canada. Presentation of aquaculture in Portugal by Dr. Susana Freitas. Presentation of the functions and duties the CRL by Dr. Søren Kahns. Discussion on the topics of the visit.
10:30 – 13:00	Tour in the lab, looking at all the facilities The main goal is to discuss how the diagnostic procedures according to Commission Decision 2001/183 conducted in the laboratory. Following issues can be discussed: • Aquaculture in Portugal 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 (2007 and 2008) Proficiency Test Results*
13:00 – 13:45	Lunch
13:45 – 15:30	In the afternoon we 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.

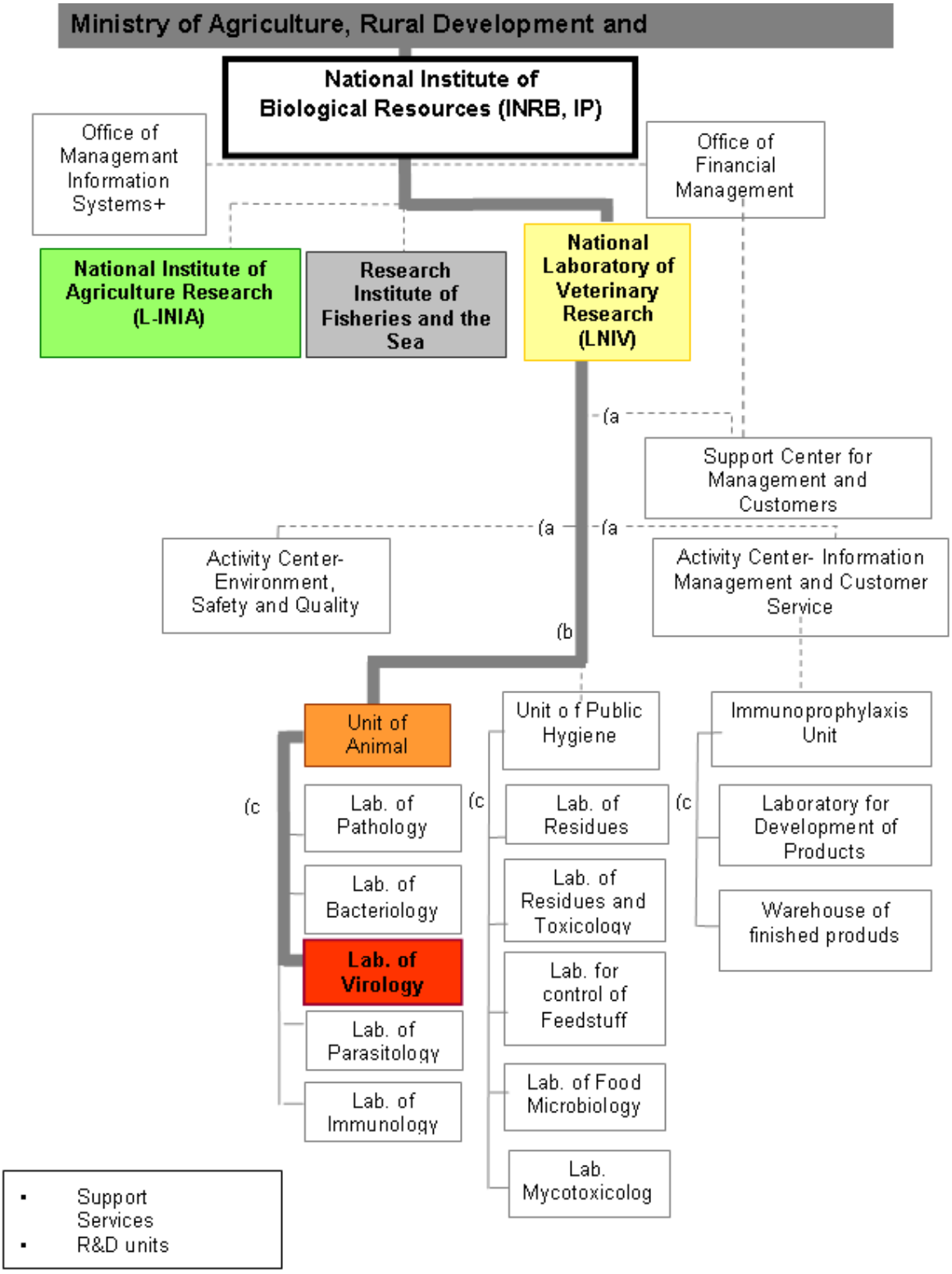
October the 23rd

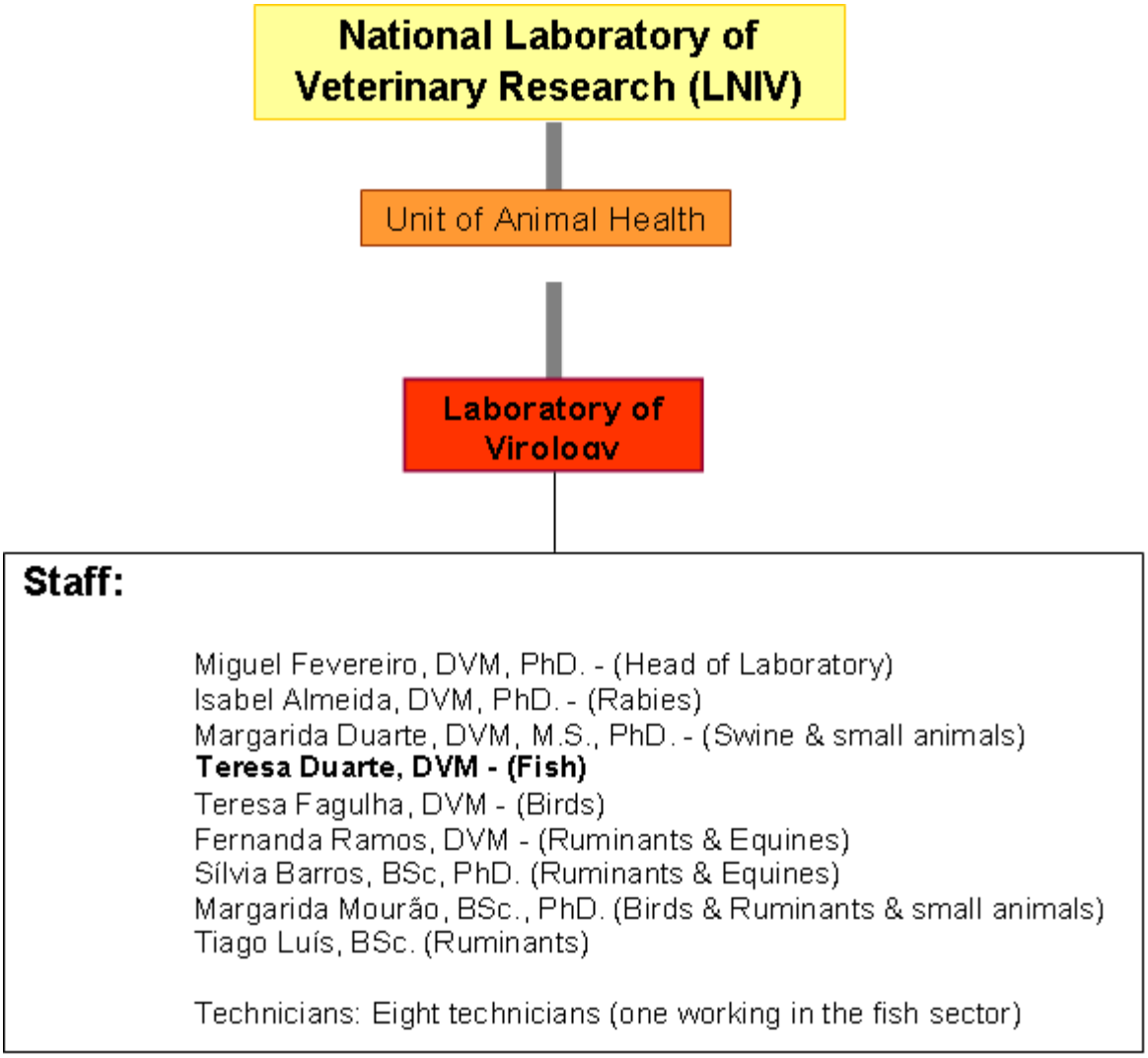
8:30	Pick up at hotel
09:00 – 12:00	Follow up for from the day before.
12:00 – 12:45	Evaluation of the visit. Recommendations and report of the visit
12:45	Departure to airport

Annex 2
Participants at the meeting

Name	Institute	Funktion
Miguel Fevereiro	LNIV / Laboratório Nacional Investigação Veterinária Virologia	Head of laboratory
Teresa Duate	LNIV / Laboratório Nacional Investigação Veterinária Virologia	Responsible of Diagnosis for Fish diseases
Susana Freitas	DGV / National Veterinary Sanitary Authority	
Nuno Canada	INRB, I.P./LNIV	Director
Alice Amado	LNIV / Animal Health Unit	Head of Animal Health Unit
Fátima loja	LNIV / Environment, Safety and Quality	Coordinator
Fernanda Mascarenhas	LNIV / LIMS and Customer Service	Coordinator
Søren Kahns	Community Reference Laboratory for Fish Diseases, Section for Fish Diseases, National Veterinary Institute, Technical University of Denmark.	
Nicole Nicolajsen	Community Reference Laboratory for Fish Diseases, Section for Fish Diseases, National Veterinary Institute, Technical University of Denmark.	

Annex 3
Organisational and Functional Structure





Diagnostic of Virus Infections of Fish:

Teresa Duarte:	Virus isolation; Virus neutralization assay; PCR & RT-PCR.
Miguel Fevereiro:	PCR, RT-PCR & Sequencing
Heather George:	Sampling, sample preparation & inoculation; cell culture. Maintenance & cleaning

Annex 4
Aquaculture in Portugal

Land Area	Ocean Area	Environment	Species	Scientific name	2005	2006	2007
Portugal	Inlands waters	Freshwater	Rainbow trout	Oncorhynchus mykiss	843	942	892
			Sea trout	Salmo trutta	2	1	11
		Sub-total Freshwater			845	947	903
	Sub-total Inland waters				845	947	903
	Marine areas	Brackishwater	Mullets nei	Mugilidae	0 *0*	0 *0*	1
		Marine	European seabass	Dicentrarchus labrax	1 530	1 584	1 391
			Gilthead seabream	Sparus aurata	1 519	1 623	1 930
			Marine fishes not identified	Marine fishes not identified	2	1	20
			Meagre	Argyrosomus regius	47	23	25
			Sargo breams nei	Diplodus spp	0 *0*	2	0 *0*
			Tunas, bonitos, billfishes	Tunas, bonitos, billfishes	1	11	13
		Sub-total Marine			3 099	3 244	3 379
	Sub-total Marine areas				3 099	3 244	3 380
Total Portugal					3 944	4 191	4 283
Grand total					3 944	4 191	4 283

Data taken from FIGIS

Number of fish farms within country/region, according to size of production (tonnes fish/year)				
	2008	2007	2006	2005
< 5 tonnes	14	21	142	16
5 - 100 tonnes	125			12
> 100 tonnes				1

Number of fish farms within country/region, according to fish species				
	2008	2007	2006	2005
Rainbow trout	23	21	19	23
Atlantic Salmon	0	1	0	0
Other salmonids	0	4	5	3
Carp	2	1	1	1
Eel	0	0	0	0
Flatfish	4	3	3	2
Seabream / Seabass	111	116	114	0
Other marine spp.	0	3	0	0
Other freshwater spp.	0	0	0	0
Total	140	149	142	29

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:				
	2008	2007	2006	2005
No. of samples tested by cell cultivation	715	472	384	307
No of samples tested by PCR or other direct methods without cell cultivation	300			

According to Council Directive 2006/88, please indicate number of farms in your country/region placed in the respective catagories according to fish species:					
		VHS	**IHN**	**ISA**	**KHV**
Category I **Declared disease-free**	Rainbow trout				
	Atlantic Salmon				
	Other salmonids				
	Carp				
	Eel				
	Flatfish				
	Seabream / Seabass				
	Other marine spp.				
	Other freshwater spp.				
		VHS	**IHN**	**ISA**	**KHV**
Category II **Subject to a surveillance programme**	Rainbow trout	23	23		
	Atlantic Salmon				
	Other salmonids				
	Carp				2
	Eel				
	Flatfish	4			
	Seabream / Seabass	113	113		
	Other marine spp.				
	Other freshwater spp.				
		VHS	**IHN**	**ISA**	**KHV**
Category III **Not known to be infected but not subject to surveillance programme for achieving disease free status**	Rainbow trout				
	Atlantic Salmon				
	Other salmonids				
	Carp				
	Eel				
	Flatfish				
	Seabream / Seabass				
	Other marine spp.				
	Other freshwater spp.				
		VHS	**IHN**	**ISA**	**KHV**
Category IV **Known to be infected but subject to an eradication programme**	Rainbow trout				
	Atlantic Salmon				
	Other salmonids				
	Carp				
	Eel				
	Flatfish				
	Seabream / Seabass				
	Other marine spp.				
	Other freshwater spp.				
		VHS	**IHN**	**ISA**	**KHV**
Category V **Known to be infected. Subject to minimum control measures**	Rainbow trout				
	Atlantic Salmon				
	Other salmonids				
	Carp				
	Eel				
	Flatfish				
	Seabream / Seabass				
	Other marine spp.				
	Other freshwater spp.				

Data from S&D

Annex 5
The Danish sensitivity test

Report of the 13th cell susceptibility test performed at the NRL Fish Diseases, Denmark. The report is in Danish but the figures might be useful giving an example on how it can be done. The report describes the testing of 5 cell lines and in all 10 sublineages in use in our laboratory.

Rapport for celleføIsomhedstesten
Januar 2008

Veterinærinstituttet, Århus

Sektion for Fiskesygdomme

Formål
At teste følsomheden af cellelinierne BF-2, EPC, RTG-2 og FHM overfor VHS virus, følsomheden af EPC og FHM celler overfor IHN virus og cellelinierne BF-2 og CHSE-214 overfor IPN virus.

Metode
Se instruks Å-4-AR-031 Afprøvning af celleliniers sensitivitet overfor fiskepatogene virus. Bemærk at der fra efteråret 2002 anvendes en ny instruks, og at der derfor ikke medtages resultater fra før 09-10-2002. Resultater afbildes og vurderes som beskrevet i instruksen.

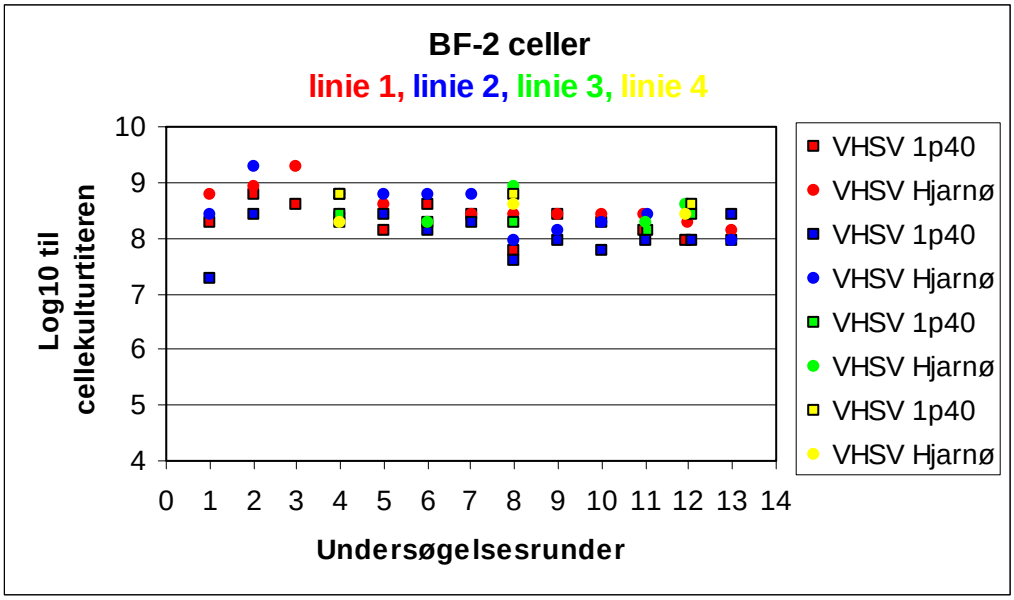
Bemærk at opdelingen i underlinier af en given cellelinie (eks. BF-2 linie 1 og 2) udelukkende bør betragtes som en måde at skelne mellem flere linier indenfor den enkelte testrunde. Celler deles op i flere linier, når det vurderes, at der er grund til det. BF-2 linie 1 i en testrunde er derfor ikke nødvendigvis identisk med linie 1 i en næste testrunde. Når følsomheden skal sammenlignes mellem testrunder må man derfor sammenligne den fulde variation hidrørende fra alle underlinier i en given testrunde med samme fra tidligere runder.

Materialer
Der anvendes to isolater af VHS virus: et marint isolat (1p40) og et ferskvandsisolat (Hjarnø), ét ferskvandsisolat af IHN virus (32/87) samt to serotyper af IPN virus (Sp og Ab). Disse er alle velkarakteriserede isolater.

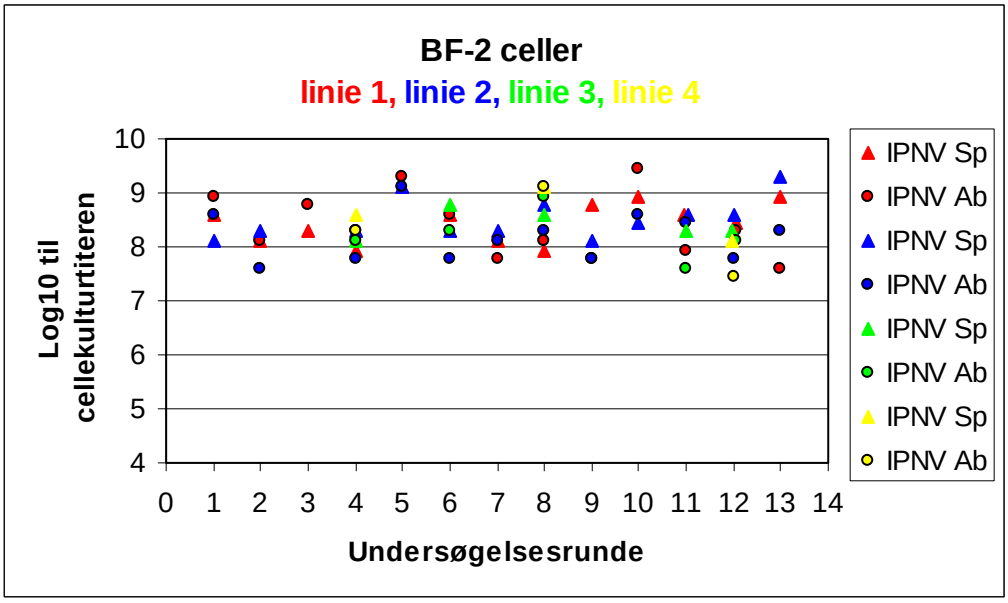
I testen undersøges følsomheden af de cellelinier der anvendes i diagnostikken. De anvendte cellelinier er beskrevet i nedenstående tabel:

Cellelinier anvendt i celleføl somhedstest januar 2008		**Batch nr. for 96-huls bakker**	**Cellelinie ID**					
			Navn	**Passage nr.**	**Passage nr. efter optøning**	**Passage dato**	**Mykoplasma test**	**Bemærkninger**
BF-2	Linie 1	AR-018-44	01/08	1 (368)	32	16-06-08	26-02-08	Fra diagnostikskab. Stammer fra cellebankflaske med følgende ID: 367/B1, 31 p.e.opt., (26/2-08)
	Linie 2	AR-018-44	72/06(3)	28 (463 i alt)	59	16-06-08	03-03-08	Fra diagnostikskab. Stammer fra cellebankflaske med følgende ID: 435/1A, 31 p.e.opt., (20.09.06), -myk 20.09.06 **samme cellebankflaske som linie 2 i l2. testrunde**
EPC	Linie 1	AR-018-44	61/06	22 (i alt 314)	44	09-06-08	19-02-08	Fra diagnostikskab. Stammer fra cellebankflaske med følgende ID: 292, 22 p.e.opt., (17.10.06), -myk 02.08.08 **samme cellebankflaske som linie 1 i l2. testrunde**
	Linie 2	AR-018-44	73/06	21 (258 i alt)	33	09-06-08	19-02-08	Fra diagnostikskab. Stammer fra cellebankflaske med følgende ID: 237/1, 12 p.e.opt., (07.10.06), -myk 12.09.06 -
CHSE-214	Linie 1	AR-018-44	11-06	32 (395 i alt)	36	09-06-08	19-02-08	Fra diagnostikskab. Stammer fra cellebankflaske med følgende ID: 373, 4 p.e.opt., (23-01-06), -myk 05-10-05
	Linie 2	AR-018-44		393	7	02-06-08	08-01-08	Direkte fra cellebank - **samme sublinie som linie 4 i l2. testrunde**
RTG-2	Linie 1	AR-018-44	652/2	652	35	28-05-08	03-03-08	Direkte fra cellebank
	Linie 2	AR-018-44	121C/1	121	28	24-06-08	11-03-08	Direkte fra cellebank.
FHM	Linie 1	AR-018-44		333	15	14-05-08	01-04-08	Direkte fra cellebank - **samme sublinie som linie 2 i l2. testrunde**
	Linie 2	AR-018-44		333	36	27-05-08	15-03-08	Direkte fra cellebank - **samme sublinie som linie 1 i l2. testrunde**

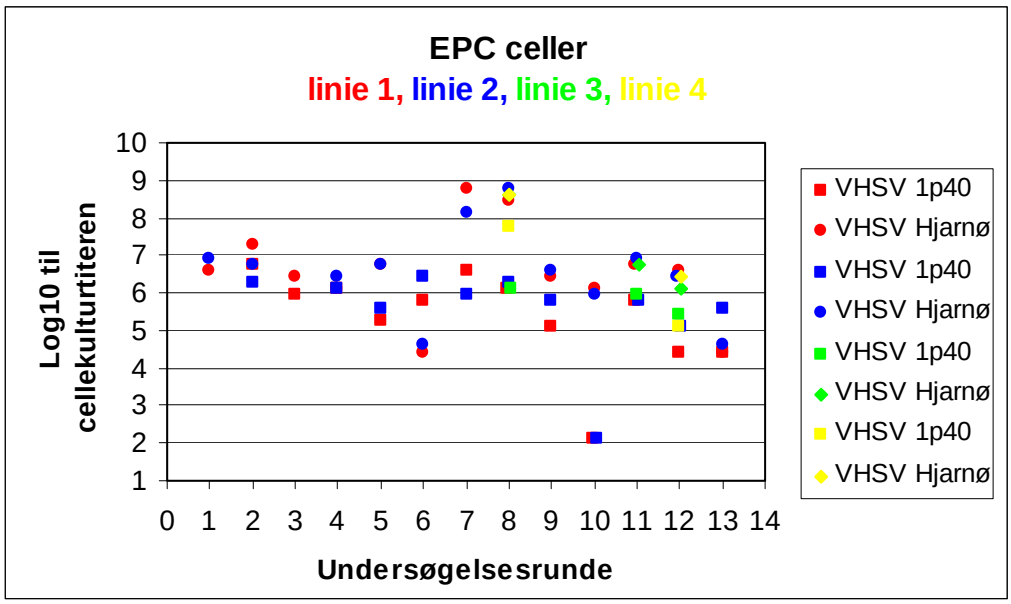
Resultater



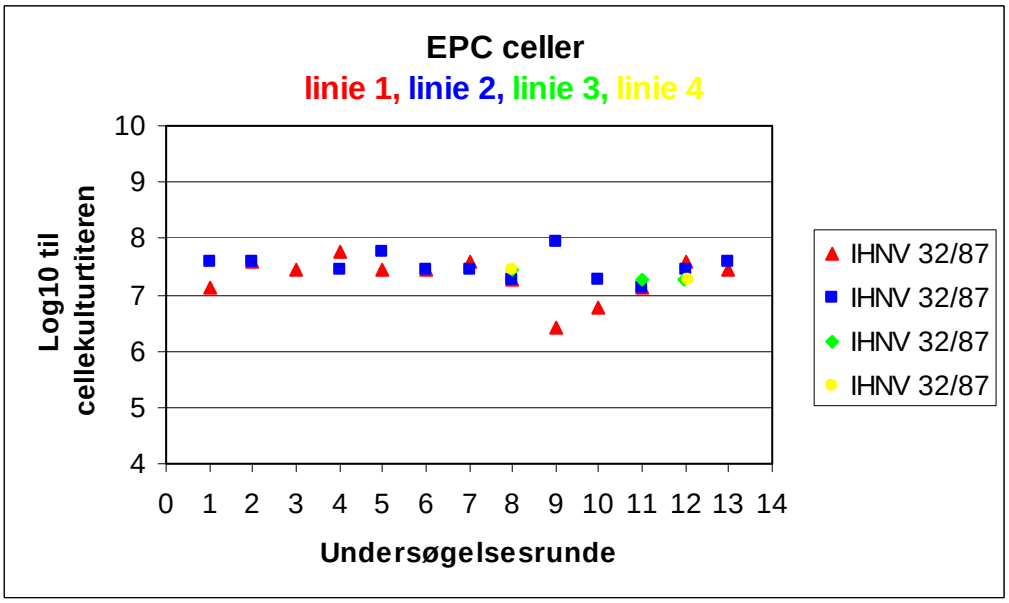
Vurdering:
Begge BF-2 cellelinier opererer med en høj følsomhed overfor VHSV i området $10^{7,9}$-$10^{8,4}$ $TCID_{50}$/ml. Følsomheden ligger meget jævnt i forhold til de tidligere testrunder. Testfølsomheden for både det marine VHSV isolat og for ferskvandsisolatet er ens i denne testrunde. Følsomheden i alle testrunder ligger jævnt ens.



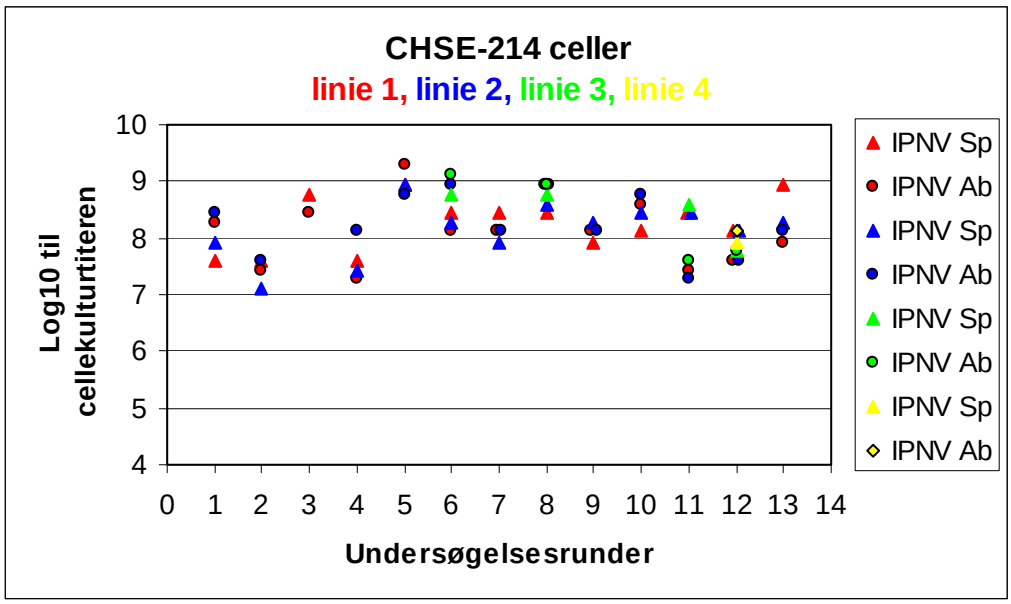
Vurdering:
Alle BF-2 cellelinierne opererer med en høj følsomhed overfor IPNV i området $10^{7,6}$-$10^{9,3}$ $TCID_{50}$/ml, en anelse højere end testrunde 11 og 12. Følsomheden i alle testrunder ligger jævnt ens.



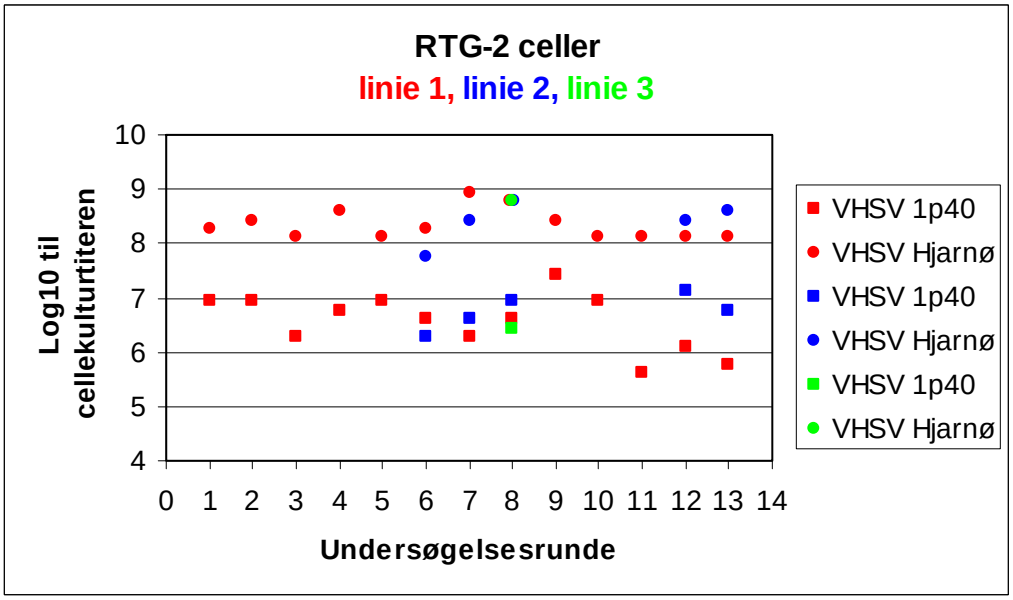
Vurdering:
Følsomheden er i denne testrunde ens for det marine isolat 1p40 og ferskvandsisolatet Hjarnø med en titerværdi på $10^{4,4}$-$10^{5,6}$ $TCID_{50}$/ml. Følsomheden for VHSV har igennem runderne været svingende. I runde 13 er følsomheden på linie med de andre runder, dog med en tendens til at ligge i den lave ende.



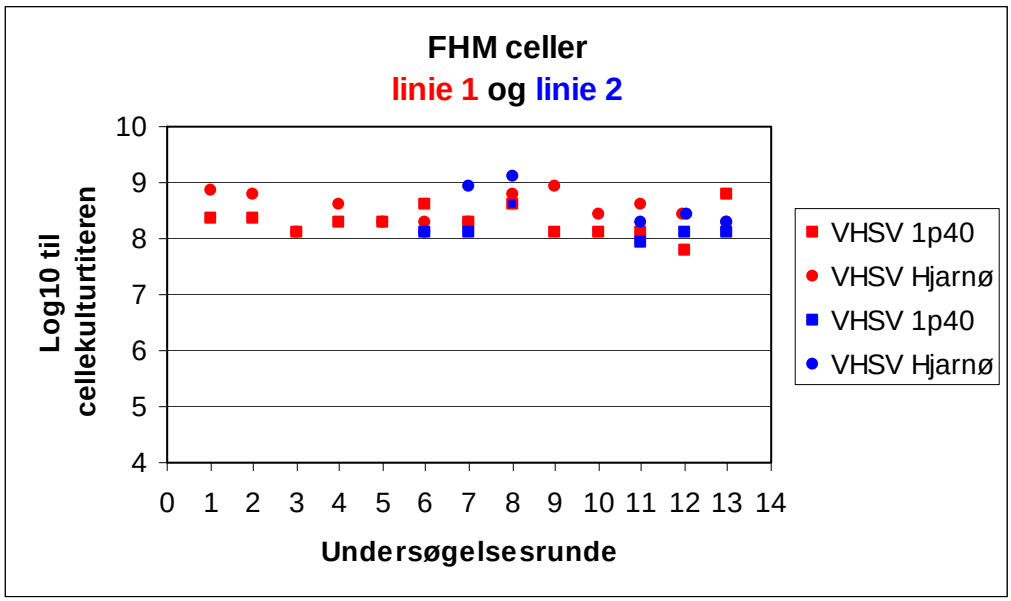
Vurdering:
EPC liniernes følsomhed overfor IHNV er i runde 13 på linie med de tidligere runder med en følsomhed i området $10^{7,4}$-$10^{7,6}$ $TCID_{50}$/ml. Bortser fra linie 1 i runde 9 har følsomheden overfor IHNV har været konstant over testrunderne.



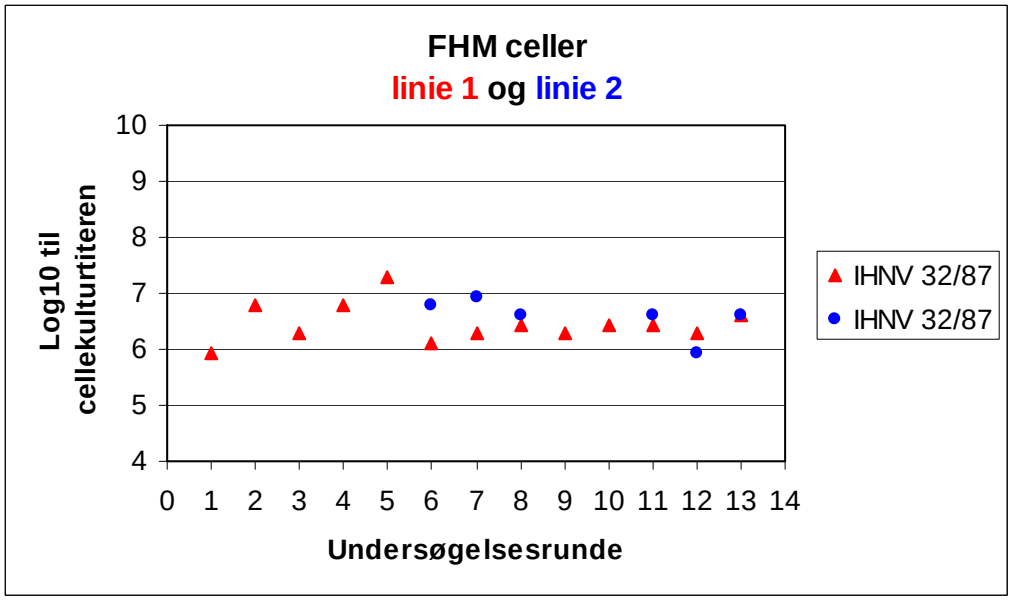
Vurdering:
CHSE-214 cellerne udviser i 13. testrunde følsomhed overfor IPNV Sp og IPNV Ab isolaterne i området $10^{7,9}$-$10^{8,9}$ $TCID_{50}$/ml. Følsomheden afviger ikke fra den opnået i tidligere testrunder.



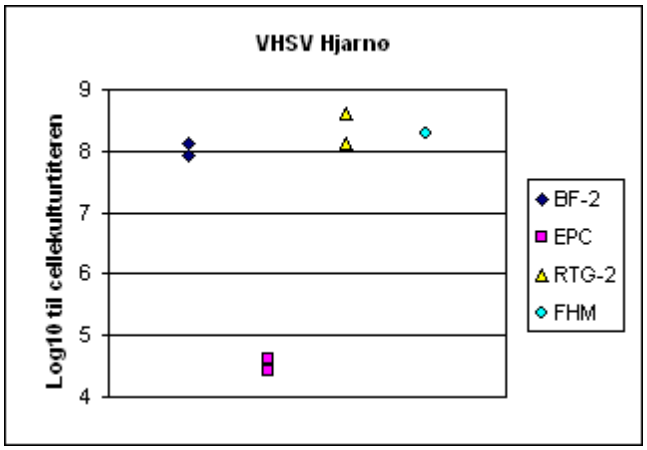
Vurdering:
RTG-2 linien udviser en høj følsomhed overfor VHSV isolatet Hjarnø med en titer på $10^{8,1}$ – $10^{8,6}$ $TCID_{50}$/ml og følsomhed overfor 1p40 med en titer på $10^{5,8}$ – $10^{6,8}$ $TCID_{50}$/ml. Følsomheden ligger på linie med de tidligere runder. Det ses at VHSV Hjarnø i alle testrunder giver højere titerværdi end VHSV 1p40.

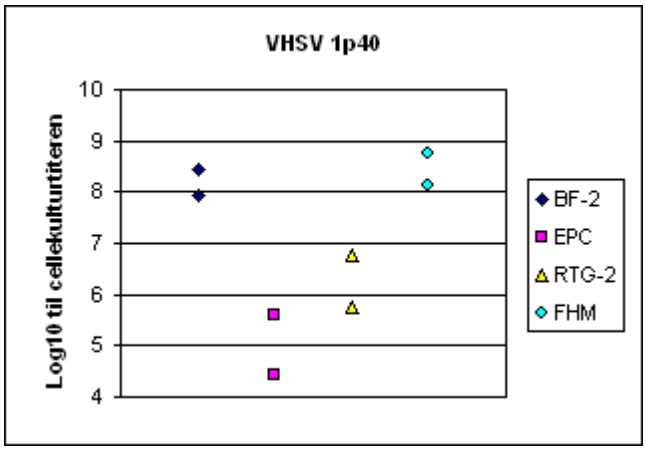


Vurdering:
FHM cellerne udviser en høj og konstant følsomhed overfor begge VHSV isolater i området 10^8-10^9 $TCID_{50}$/ml.

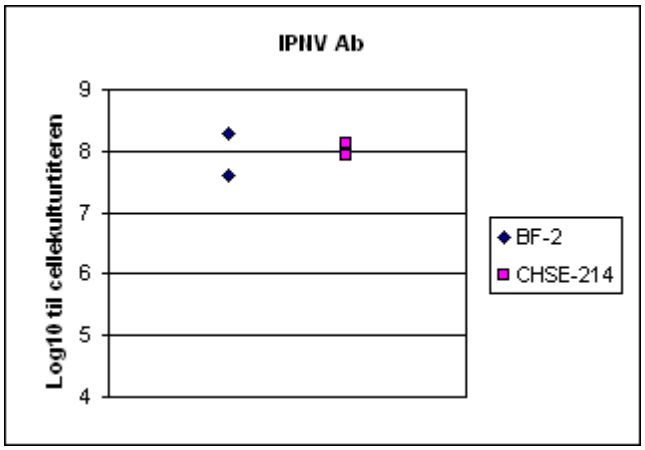


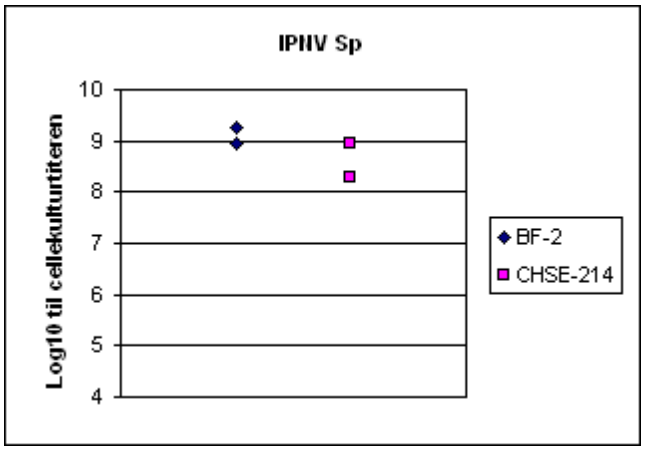
Vurdering:
Følsomheden for IHNV ligger ret konstant i området 10^6-10^7 $TCID_{50}$/ml.



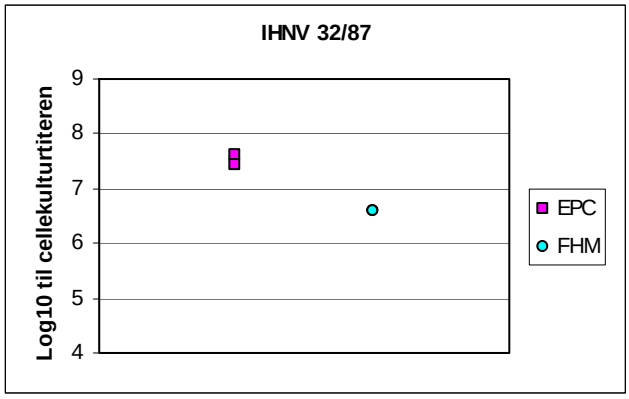


Vurdering:
For VHSV Hjarnø ses den bedste cellelinie at være BF-2, FHM og RTG-2 på en delt førsteplads. For 1p40 ses den bedste cellelinie at være BF-2 og FHM celler at være på linie. For begge vira viser EPC cellerne den laveste titer.





Vurdering:
For IPNV Sp og Ab set under et ses BF-2 at være en anelse bedre end CHSE-214.



Vurdering:
For IHNV 32/87 ses det, at EPC er bedre end FHM.

Konklusioner
Generelt viste cellelinierne et højt titer for både VHSV, IHNV og IPNV, på linie med tidligere runder. Følsomheden i denne cellerunde var generelt på højde med følsomheden i tidligere runder, og der er således ikke påvist fald i celleliniernes følsomhed over tid.

Ud fra denne celleføl somhedstest anbefales det at bruge følgende cellelinier:

VHSV 1p40: BF-2 = FHM > RTG-2 > EPC
VHSV Hjarnø: BF-2 = FHM = RTG-2 > EPC
IHNV 32/87: EPC > FHM
IPNV Sp: BF-2 ≥ CHSE-214
IPNV Ab: BF-2 = CHSE-214

Der kan således fortsættes med at benytte BF-2 og EPC celler i de diagnostiske bakker som sædvanlig.
Det er ligeledes forsvarligt at benytte alle de testede celleunderlinier.

5. august 2008

Helle Frank Skall

Annex 6
Proficiency Test

Portugal	1996	1997	1998	1999	2000	2001	2002	2003	2004	2005	2006	2007	2008
Participated	1	1					1	1	1	1		1	1
Score %								85	90	100		50	80

	Ampoule I:	**Ampoule II:**	**Ampoule III:**	**Ampoule IV:**	**Ampoule V:**
2008 Score 8/10	VHS virus Rindsholm 5151	VHS virus 1p8	IHN virus 217/A (DTU Vet protocol no. 4008)	VHS virus Rindsholm 5151 + IPN virus Type Sp	IPN virus Type Sp
EPC/RTG-2 celler	SVCV	VHSV	IHNV	VHSV/IPNV	IPNV
Neutralisation	SVCV	VHSV	IHNV		IPNV
PCR	SVCV	VHSV	IHNV	VHSV/IPNV	IPNV

	Ampoule I:	**Ampoule II:**	**Ampoule III:**	**Ampoule IV:**	**Ampoule V:**
2007 Score 5/10	VHS virus DK-F1 Genotype I (Undiluted)	VHS virus DK-F1 Genotype I (Diluted 10^{-5})	SVC virus 56/70 Genotype Id	IHNV 32/87 First French isolate Genotype M	VHSV 4p101 Genotype III
EPC/RTG-2 celler	VHSV	Virus not found	SVCV	IHNV/SVCV	VHSV/SVCV
Neutralisation	VHSV	Virus not found	SVCV	IHNV/SVCV	VHSV/SVCV
PCR	VHSV	Virus not found	SVCV	IHNV/SVCV	VHSV/SVCV

2005 Score 10/10	Ampoule I	Ampoule II	Ampoule III	Ampoule IV	Ampoule V
	SVCV- and PFR-like	VHSV DK-4p101	IHNV 32/87	VHSV D-5151 Rindsholm	VHSV DK-1p52
EPC/RTG-2 celler	SVCV	VHSV	IHNV	VHSV	Not conclusive
Neutralisation	SVCV	VHSV	IHNV	VHSV	VHSV
PCR					

2004 Score 9/10	Ampoule I	Ampoule II	Ampoule III	Ampoule IV	Ampoule V
	VHSV DK-5151 Rindsholm	VHSV DK-5151 Rindsholm (Diluted 10^{-7})	IPNV IPNV serotype Sp	VHSV DK-5151 Rindsholm (Diluted 10^{-4})	IHNV 32/87, First French
EPC/RTG-2 celler	VHSV	Virus not found	IPNV	VHSV	IHNV
Neutralisation			IPNV		
PCR	VHSV		IPNV	VHSV	IHNV

2003 5,5/6,5	Ampoule I	Ampoule II	Ampoule III	Ampoule IV	Ampoule V
	IHNV 32/87 First French	VHSV DK-5151	IHNV/SVCV **IHNV** Isolate 4008 **SVCV** Isolate 56/70	VHSV 1p8	VHSV/IHNV **VHSV** DK-5131 Klapmølle **IHNV** Isolate 32/87
EPC/RTG-2 celler	IHNV	VHSV/IHNV	IHNV/SVCV	VHSV	VHSV/IHNV
Neutralisation	IHNV	VHSV		VHSV	VHSV/IHNV
PCR	IHNV	VHSV/IHNV	IHNV/SVCV	VHSV	VHSV/IHNV

2002	Ampoule I	Ampoule II	Ampoule III	Ampoule IV	Ampoule V
	IHNV Isolate 32/87 First French	VHSV/IHNV Isolate 1p8 Isolate 32/87 First French	SVCV Isolate 56/70	VHSV/IHNV **VHS virus**, DK-3592B "Voldbjerg Isolate 32/87 First French	VHSV Isolate DK-5151
EPC/RTG-2 celler	IHNV	VHSV/IHNV	SVCV	VHSV	VHSV
IFAT					VHSV
Neutralisation	IHNV	VHSV/IHNV		VHSV	
PCR	IHNV	VHSV/IHNV	SVCV	VHSV	VHSV

Annex 7
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	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

Swine Anti Rabbit TRITC: DAKO Code no. R0156
Rabbit Anti Mouse TRITC: DAKO Code no R0270

Annex 7

Draft for Neutralisation

2nd day	Virus: Ampoule Celler:					
Dilution	Medium	1: Anti VHS	1: Anti IHN	1: Anti IPN	1: Anti SVC	1: Anti
10^{-1}						
10^{-2}						
10^{-3}						

5th day	Virus: Ampoule Celler:					
Dilution	Medium	1: Anti VHS	1: Anti IHN	1: Anti IPN	1: Anti SVC	
10^{-1}						
10^{-2}						
10^{-3}						

3st day	Virus: Ampoule Celler:					
Dilution	Medium	1: Anti VHS	1: Anti IHN	1: Anti IPN	1: Anti SVC	1: Anti
10^{-1}						
10^{-2}						
10^{-3}						

6th day	Virus: Ampoule Celler:					
Dilution	Medium	1: Anti VHS	1: Anti IHN	1: Anti IPN	1: Anti SVC	
10^{-1}						
10^{-2}						
10^{-3}						

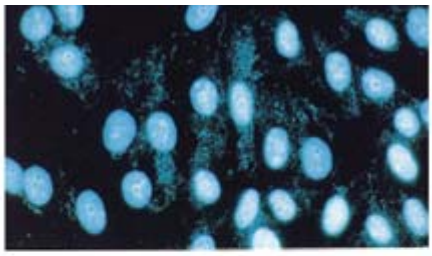
4rd day	Virus: Ampoule Celler:					
Dilution	Medium	1: Anti VHS	1: Anti IHN	1: Anti IPN	1: Anti SVC	1: Anti
10^{-1}						
10^{-2}						
10^{-3}						

7th day	Virus: Ampoule Celler:					
Dilution	Medium	1: Anti VHS	1: Anti IHN	1: Anti IPN	1: Anti SVC	
10^{-1}						
10^{-2}						
10^{-3}						

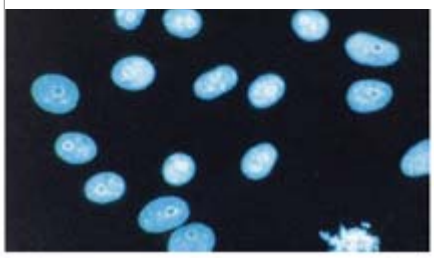
Conclusion:

Annex 9
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 5

Technical report from the Community Reference Laboratory for Fish Diseases 2009

Laboratory visit at the National Reference Laboratory for Fish Diseases Bornova Veterinary Control and Research Institute

Izmir, Turkey
9th – 10th November 2009

Introduction

The National Reference Laboratory for Fish Diseases (NRL) in Turkey is located at the Bornova Veterinary Control and Research Institute (BVCRI). The Institute was visited on the 9th and the 10st of November by Søren Kahns and Niels Jørgen Olesen from the European Community Reference Laboratory for Fish Diseases (CRL). The programme is shown in annex 1 and a list of participants at the meetings in Annex 2. This report describes findings, comments and recommendations made by the delegation from the CRL. The report is sent to the BVCIR and the EU Commision.

Organisation

The BVCRI is founded by the Turkish state and is a public governmental institution. The Institute is situated in the large garden neighbor to the campus of the Ege University of Izmir, and collaborate in several topics with this university.

The BVCRI is managed by director, Dr. Neclet Akkoca, and two deputy directors, Mr. Hasan Aktar and Dr Aysen Beyazit, also head of the Department for Parasitology. The BVCRI has 6 technical departments:

1. National Reference Laboratories (Fish Diseases, Molluscs Diseases, Crustean diseases, Antimicrobial Residues on animal products, and AI & ND in Poultry)
2. Diagnostic services
3. Control Services
4. Laboratory support
5. Financial aid and administrative affairs
6. General support services

The Department for Diagnostic Services are further sub-divided into 6 Departments:

1. Pathology
2. Bacteriology
3. Parasitology
4. Virology
5. Toxicology
6. Poultry (Avian) diseases

The Virology Department covers the diagnostics of viral diseases of farm animals (including fish – excluding avian diseases) and companion animals. The NRL for fish diseases is covered by several departments within the Institute, such as Virology, Bacteriology, Parasitology and Pathology Departments. Virology department is the coordinator of the NRL for fish diseases. The BVCRI is also NRL for Avian Influenza and Newcastle disease. An organisation plan is shown on annex 3.

Buildings, Furnishing and Access

The Institute is placed in several buildings in a large park back to back with the Ege University campus. All access to the institute is controlled at the entrance of the site. Within the Institute there is free access

between the buildings. All rooms have air-conditioning. Furthermore, fume hoods are located in certain rooms for working with hazardous chemicals.
The Department of Virology is located in one building (shared with Department of Parasitology) where all diagnostics of fish viruses is performed. Bacteriological, parasitological, histopathological and toxicological exams are performed in the respective departments at BVCRI.

Opening of the meeting
The visit was opened by an introduction in the conference hall of the institute. The meeting was attended by the senior scientists and head of sections of the institute. The list of participants is given in Annex 2. Dr. N. Akkoca welcomed us and Dr. N.J. Olesen gave a short presentation on the aim of the visit and the outcome that we hoped to achieve. Dr. N. Akkoca then gave a detailed description of the Institute, its history, organisation, mission, the NRL for fish diseases and about aquaculture production in Turkey, and finally on associated research tasks in progress. Finally Dr S. Kahns presented the function and duties of the CRL and the NRL´s for Fish Diseases. The meeting was followed by a visit tour of the institute.

Visit Tour at BVCRI
Sample acceptance and reporting unit
The visit tour followed the samples and started in the sample acceptance and reporting unit where all samples to the Institute is entered. All material is registered and given a unique number which is informed to the customers with a notice on contact for following up. Samples are received from veterinarians of both official and private establishments and/or private persons. When fish samples are delivered, notes on fish species, clinical signs, fish farm, water temperature etc. are collected.

Large animals (including large fish) are sent to the necropsy room for sampling whereas most fish and molluscs are forwarded directly to the coordinator department which provide the distribution of the materials to the diagnostic department/departments according to the requirements of the customer. Molluscs are sampled at the Pathology department and fish at the Parasitology department.

When the diagnostic analyses are completed, the sample acceptance and reporting unit receives a report from the person responsible of the analysis. From this, the unit writes an answering letter for the costumer. Before the letter is send to the costumer, it is signed by the head of the department and the director.

Pathology Department
The Pathology Department was presented by head of department Dr. Öznur Yazicioglu. The department is placed in the same building as the acceptance unit (on the first floor). The department is accredited for detection of Bonamia, Martelia and Perkinsus in molluscs. Samples are divided in specimens for PCR, histopathology and electron microscopy. The department was equipped with all equipment needed for histology and microscopy




Toxicology department
The Toxicology department is placed in a separate building together with departments of control services. The department was presented by Dr. Yasemin Coskun. The department was very well equipped with large amounts of instruments for all toxicological analyses. It consists of 4 veterinarians, 1 biologist, 2 aquaculture engineers and 3 chemists. The department had a separate unit for toxicology and antibiotic residues. The department conducts a function as NRL for antibiotic residues and is testing residues of all commonly used antibiotics in aquaculture. Also pesticides and toxins are tested for, as well as chemical compounds like malachite green.

Poultry Diseases Department
The separate building of this department was not visited, due to lack of time. The department conducts necropsy, virology (including Q-PCR), bacteriology and parasitology in poultry. The department is the Turkish NRL for AI and ND.

Bacteriology
The Department of bacteriology conducts all bacteriological examinations for mammals and fish (including brucellosis, E.coli infection, mycoplasma infection, clostridiosis, botulism, mastitis etc.) using classical bacteriological testing.
Molecular based research is conducted on *Vibrio haemolyticus*. In fish focus is primarily on Sea Bream and Sea Bass farming. Disease problems consider Vibriosis, Aeromonas infection (furunculosis), gram positive infection like *Lactococcus garvieae*, *Photobacteriae sp*., *flavobacteriae* (maritimus and columnare). *Renibacterium salmoninarum* (BKD) was detected from *Salmo trutta labrax* on KDM- and KDM-2. *Vibrio cholera sp*. was isolated in fresh water and *Aphanomycis astac*i in crayfish. The Department is headed by Dr. Seza Eskiizmirliler. The group consist of 5 veterinarians, 1 biologist, 2 laboratory technicians and 2 other technicians. Necla Türk (DVM, specialist in fish bacteriology), Dr. Lüfti Avsever and Dr. Serra Tunaligil conducted studies in fish bacteriology.

Parasitology
The Department of parasitology conducts all parasitological examinations for mammals and fish. All small animal samples and tissue materials were received in this department for necropsy and further distributed from here to the respective specialized laboratories, in specific tubes. Microscopy was conducted directly on site. For necropsy colleagues from the other groups were often invited to participate. The group was presented by the head Dr. Aysen Beyazit. The group consists of 3 veterinarians and 3 technical personel. Dr. Mevlüt Melih Selver presented his work in fish parasitology and the parasites diagnosed at the laboratory.

Control services
The Food Control Department covers red meat, milk, chicken, water and fish. The group consists of 3 veterinarians, 1 biologist and 2 laboratory technicians. Also this laboratory is very well equipped including ICPMS for heavy metal analysis and GCMS for pesticides and formaldehyde. Testing for microbial activity in food and water can be conducted here in separate laboratory facilities. Processing of fish meet testing for microbial activity and residues (farmed Sea Bream) was explained.

PCR laboratory
A new laboratory for conducting PCR in the diagnostic department is under construction in the basement of the building. This facility will be at the service for the virology, bacteriology and parasitology groups. The facilities are not yet adequate, and will need better separation between clean and not-clean laboratories. It is e.g. recommended to install a separate master mix room as well as a room only for post PCR use.

Virology department and the NRL for fish diseases
The Department of Virology is responsible for diagnostics of viral diseases of farm animals - including fish and companion animals. The NRL for fish diseases is covered by all the departments within the Institute, such as Virology, Bacteriology, Parasitology and Pathology Departments. The Virology department is the coordinator of the NRL for fish diseases. The staff of the all these Diagnostic Departments is also the staff of NRL.

Staff of the Virology Department
The NRL for Fish Diseases is coordinated by Dr. Gülnur Kalayci who is also head of the Virology Department. This department consists of a group of four people: Dr. Gülnur Kalayci, who is the head of the section and is overall responsible for the diagnosis of all viral diseases in mammals and fish. Poultry virology is conducted in the laboratory of avian diseases. Furthermore, Dr. Buket Özkan Özyer (veterinarian, with a PhD in fish virology (IPN)), Dr. Serife Incoglu (Biologist) and Yener Küçükali (Technician) is part of the team that carries out the viral diagnostics.

Laboratory of the NRL for viral fish diseases
The laboratory was split into four separate working rooms. All cell culturing/preparation work were performed in a "clean room" where there was a LAF-bench, centrifuges and incubators at 15°C, 24 °C and 37°C. Inoculation of samples on cell cultures was performed in another "dirty room" where also the identification of viruses by antibody based methods was performed. In this room was an incubator at 15°C and equipment for making the identification analyses. Identification of fish viruses is primarily done by ELISA and IFAT using antibodies in kits from Bio-X but the laboratory also performs neutralization. A third room (entrance to the area) was used as the office for the staff. The necropsy facilities are shared with the department of parasitology.
The virology department is currently setting up a facility for performing molecular biological analyses. For that purpose, PCR and real-time PCR machines have been achieved together with kits for purifying DNA and RNA, and DNA visualisation equipment. When setting up such a facility it was discussed that it is recommended that purification of nucleic acid, master mix mixing, reaction mixing and post PCR analysis were performed in separate room to avoid contamination. This is not always possible because of space limits. However an isolated master mix room where no contact with pathogens or PCR fragments must be made available. As PCR fragments are the highest risk factor of causing false positive results, it is strongly recommended that there is a separate post PCR /DNA visualisation room so that PCR fragments are never in contact with any pre-PCR reaction content. PCR methods to be implemented are those recommended by the OIE. Advantages and disadvantages of these methods were discussed.




Equipment
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. Calibration status of the equipment is followed/controlled by the calibration unit at the institute. Calibration of the equipment is done by specialists of the private company.
Instructions for use of equipment are written down in procedures, one for each piece of equipment. Staffs are required to read and train according to these procedures before using it. Also, for large equipment there is a responsible person who receives training from company's representative at the installation of equipment.

Large Laboratory Equipment included items as:

- Incubators, temperatures (15, 24, 37 °C)
- Centrifuges (cooling),
- Freezers (-150, -80 and -20 °C),
- LAF benches
- ELISA washer
- ELISA reader
- Microscope for IF
- Plates shaker

Small equipment included items as:

- Plastic plates
- Pipettes
- Kits from Bio-X

Cell lines:

- Cell lines grown in the laboratory: Primarily BF-2 and EPC but the laboratory also have FHM, RTG-2 and SSN-1 cells available. All cell lines have been obtained from the CRL. Media was ready made from Sigma.

Cell Culture Facilities
BF-2 and EPC cells are continuously cultured in the lab. RTG-2, FHM, and SSN-1 cells are available as stocks in liquid nitrogen. The BF-2 and EPC cells are kept at 24°C and passaged 1 to 2 times per week. It is recommended that the cells are stored at 24°C for out grow only for 24 hours after passage and then transferred to and kept at 15°C. Most likely this will decrease growth rate, causing less stress to cells and make passage needs lees frequent saving time and materials/medium. Passaging once every 3 weeks should be sufficient for maintenance of cell stocks. The saved time, medium, and flasks might then be used for expanding the cell stocks, as these appeared relatively small in view of the obligations of the NRL.

There is a back-up of cells, frozen in 10% DMSO, at -150ºC. Cells are not tested for mycoplasma, which are recommended to be implemented in the future. Pictures from and a protocol for a mycoplasma test are shown in Annex 6. Instead of performing a sensitivity test, the laboratory includes a positive control infection. The control is on a separate plate in a separate box. Disinfection of the workspace was done by Virkon-S.

Accreditation
The BVCRI was accredited according to ISO 17025 by the Turkish Accreditation body TURKAK the 26th of August 2009 with accreditation number AB-0272-T. The NRL for Fish Diseases have accredited tests for VHS ELISA, VHS IFAT, IPN ELISA and IPN IFAT. In future SVC, IHN and KHV will be accredited as well in that priority. There are no plans for accreditation of ISA examination as it is not relevant for Turkey.

Proficiency Test
The proficiency tests (PT) allow a laboratory to assess their diagnostic capacity of certain procedures. The Turkish NRL for fish diseases has participated in the PT for identification of notifiable fish diseases organized by CRL, Aarhus, Denmark, since 2005. The test included the following techniques: Titration of virus; Isolation of viruses on cell culture; Identification of virus by ELISA and IFAT. The score obtained by the NRL was 100% correct for the last three PTs (score is listed in annex 4).
In 2009 the NRL decided not to participate in the PT since the diagnostic methods for identification of EHNV were not implemented yet.

For future PTs, the PCR group will be involved for detection of EHNV, and also KHV and ISA.

The Turkish NRL do not yet organizes PTs for diagnosis of fish diseases for the regional laboratories. However, the NRL have been at a mission to the laboratory in Samsun for assisting on setting up a fish disease laboratory performing cell based diagnostics. When this and other regional laboratories for fish diseases are ready, the Turkish NRL will send out a PT.

Aquaculture in Turkey
The main fish species cultured in Turkey are rainbow trout, seabream, seabass and carp. Turkey have 289 fish farms producing more than 100 tonnes per year, 1086 fish farms producing 5 - 100 tonnes per year and 370 fish farms producing less or around 5 tonnes per year. The laboratory received approximately 202 samples in 2008 for diagnostic and surveillance purposes. The samples for virological examinations are mainly submitted from rainbow trout and the samples are mainly submitted in spring and autumn. Please see annex 5 for more details.

There has not been established an official surveillance programme in Turkish aquaculture. However in 2006 and subsequently in 2007 outbreaks were observed in two independent fish farms in the Bolu region. One connection between these fish farms is that they have shared feed producer.
After removal of all fish, cleaning, disinfection, fallowing and restocking at the infected fish farms, they were put under a surveillance programme where they are obliged to send 150 samples for two years to the

NRL for diagnosis. Farms positioned close to or downstream of the infected farms are obliged to send in 30 samples pr. year in two years. Furthermore, fish farms associated with these farms e.g. by common ownership is also obliged to send in 30 samples pr year for two years for targeted surveillance.

The total production of farmed fish is increasing in Turkey and it is expected to increase further in the future.

Turkish legislation and its synchronising with the Council Directive 2006/88/EC
At the Workshop on Aquaculture disease control and legislation, AGR 33165 organised in cooperation with the Turkish Ministry of Agriculture and Rural Affairs, and held in Ankara 5-6th November it was informed that a new legislation in Turkey on aquaculture which will be in line with the EU legislation will be finalized and implemented in 2012.

Visit to the group of Dr. Hasmet Cagirgan at the Ege University, Fisheries Faculty, Iskele-Urla. Izmir
The second day of the visit the representative of the CRL and the Staff of the NRL visited the group of Dr. Hasmet Cagirgan. The aim of this visit was to extend the research collaborations between the two Turkish groups and the CRL. The visit was opened by a tour around the laboratories, including the wet facilities.
The visit was finished by a discussion on putative future collaborative research projects. Several projects were discussed: IPNV in rainbow trout – general situation in Turkey; IPNV isolation from seabass, Different Serotypes of IPNV; Isolation of VHSV from rainbow trout; NODA virus appearance in Turkey. Most focus was on the isolation of VHSV from rainbow trout as a collaborative project was already running. It was decided that the project should be finished as soon as possible and a joint publication should be made. The NRL will send the isolates of the second outbreak in 2007 to the CRL for sequencing and phylogenetic analyses. It was decided that the group of Dr. Hasmet Cagirgan should be in charge of writing the project into a paper.

Visit to the Sea Bream and Sea Bass hatchery and fish farm of *ÇAMLI YEM BES.SAN.TİC.A.Ş.*
The visit was ended by a visit to the fish hatchery and fish farm located app 80 km south east of Izmir. At this farm seabream and seabass was produced. It was the largest fish farm in turkey producing 3.500 tonnes pr. Year. The visit was very informative and useful to us and gave us the impression of a very competent management. At the end of the visit we had the possibility to ask questions and discuss with Dr Yasemin Özgün on production and management issue, as well as on health issues. Obviously the farm experienced very few disease problems.

Training needs and future plans
Diagnostic methods for the non-exotic diseases KHV and ISA as well as for the exotic methods EUS and EHN will have to be implemented. This is planned to be implemented as soon as the facility for performing molecular biological diagnostic have been properly setup in the very near future.
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.

Alternatively, there would be possibilities for extend the collaboration with Dr. Hasmet Cagirgan at the Ege University.
The institute of the CRL is also the OIE Reference Laboratory for VHS. It was discussed if the BVCRI might have the option to be a future OIE Twinning Laboratory for VHS and other viral disease in fish for the Middle East region. This would be a possibility but would have to be discussed further. An "OIE Conference for reference laboratories and collaborating centres" will be held in Paris in June 2010 where the options for such a twinning project can be presented for the OIE Committee.

Summary of recommendations

- New procedures for cell cultivation with less frequent passaging
- Increase cell stocks
- Include mycoplasma test of cell lines.
- PCR facilities to be finally established with separate laboratory facilities
- Increase focused research activities within fish health issues.

Conclusion

The visit showed that the NRL of Turkey 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 Turkey 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 legislation in line with Council Directive 2006/88/EC

Program for visit, November the 9th – 10th 2009

Laboratory: National Reference Laboratory for Fish Diseases
Bornova Veterinary Control and Research Institute-
Virology Department.
Izmir
TURKEY

Visiting: November the 9th at 9:00 pm – 17:00 pm and November 10th 9:00 am – 17:00 am

Program: **November the 9th**

8:30 Pick up at hotel

09:00 – 10:30 Introduction of the Turkish 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 Turkey 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 (2007 and 2008) Proficiency Test Results

13:00 – 13:45 Lunch

13:45 – 15:30 Continuing the tour in lab.

15:30 – 17:00 Fish diseases legislation in Turkey and harmonization with legislation in EU with a view on Council Directive 2006/88/EC. Strategies for fish health control and management in Turkey

November 10th

8:30 Pick up at hotel

09:00 – 10:00 Follow up for from the day before.

10:00 – 13:00 Joint research activities eventually combined with a visit to Ege University Fisheries Faculty, Iskele-Urla Izmir and including Dr. Ugur Degirmenci and Dr. Hasmet Cagirgan in the discussions

13:00 – 17:00 Visit to the Sea Bream and Sea Bass hatchery and fish farm of ÇAMLI YEM BES.SAN.TİC.A.Ş.

17:00 – 18:00 Evaluation of the visit. Recommendations and report of the visit

Annex 2

Participants at the meeting

Name	Institute	Function
Dr. Necdet Akkoca		Director of BVCRI
Dr. Hasan Aktar		Deputy director
Dr Aysen Beyazit	Parasitology Department	Deputy director and head of Parasitology Department
Dr. Gülnur Kalayci,	Virology Department	Head of Virology Department and Head of the Turkish NRL for fish diseases
Dr. Buket Özkan Özyer	Virology Department	Veterinarian – Fish virology
Dr. Serife Incoglu	Virology Department	Biologist – Fish Virology
Yener Küçükali	Virology Department	Technician – Fish virology
Dr. Öznur Yazicioglu	Pathology Department	Head of Pathology Department
Dr. Ahmet Turan Erdoğdu	Toxicology Department	Head of Toxicology department
Dr. Yasemin Coskun	Toxicology Department	Veterinarian
Dr. Olcay Türe Göksu	Biological Production Control Department	Head of Biological Production Control Department
Dr. Özhan Türkyilmaz	Food Control Department	Head of Food Control Department
Dr. Fethiye Çöven	Poultry Diseases Department	Head of Poultry Diseases Department
Dr. Seza Eskiizmirliler	Bacteriology Department	Head of Bacteriology Department
Dr. Necla Türk	Bacteriology Department	Veterinarian - works with fish bacteriology
Dr. Lütfi Avsever	Bacteriology Department	Veterinarian
Dr. Serra Tunaligil	Bacteriology Department	Veterinarian
Dr. Mevlüt Melih Selver	Parasitology Department	Veterinarian
Sami Tay	Parasitology Department	Aquaculture engineer

Annex 3

Organisational and Functional Structure

DIAGRAM of INSTITUTE ORGANISATION

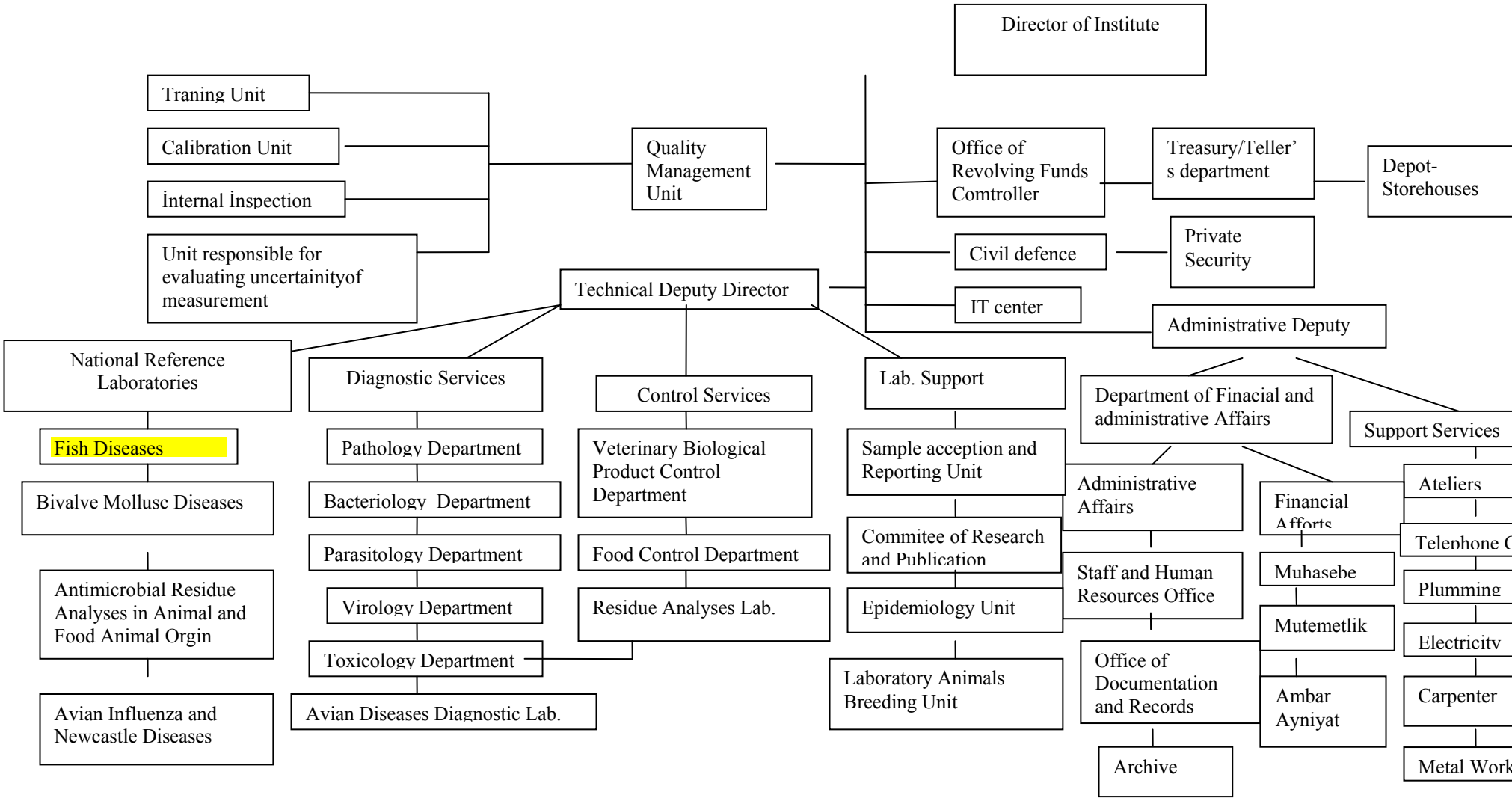
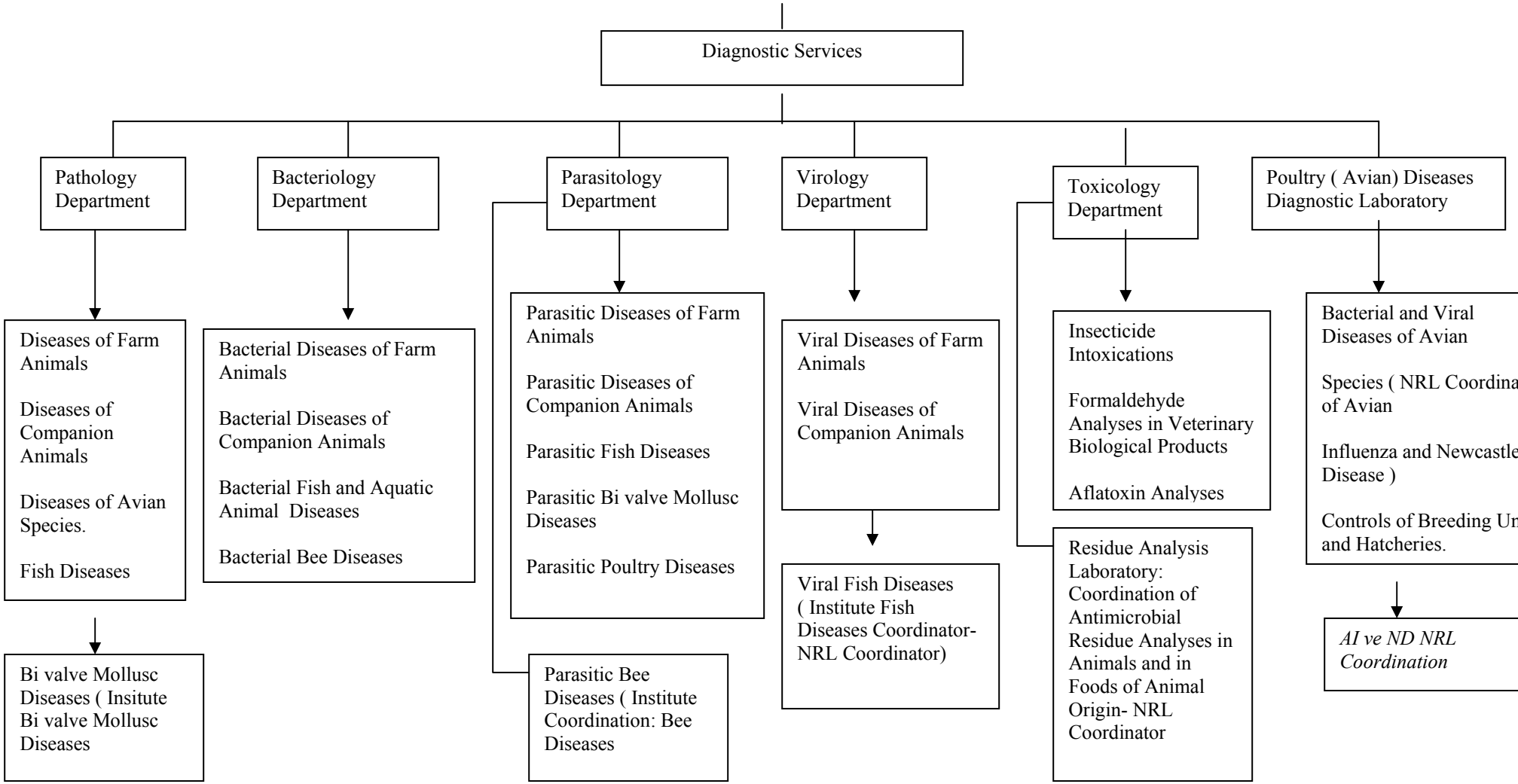


Diagram of Institute Organisation



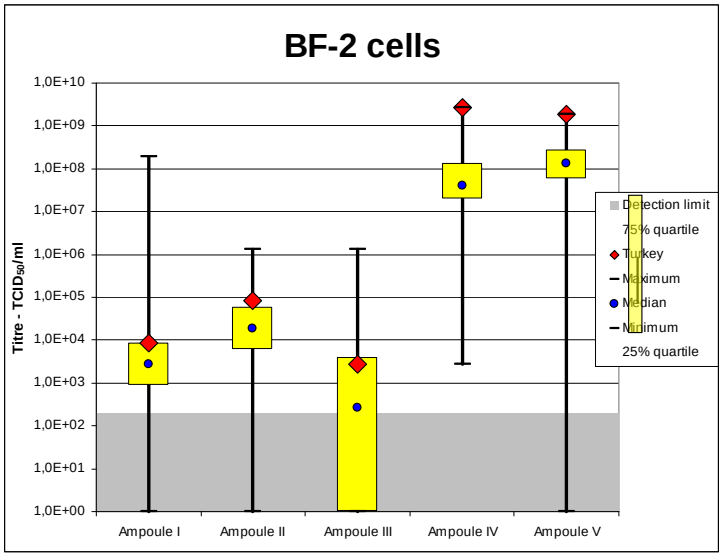
Annex 4 - Proficiency test

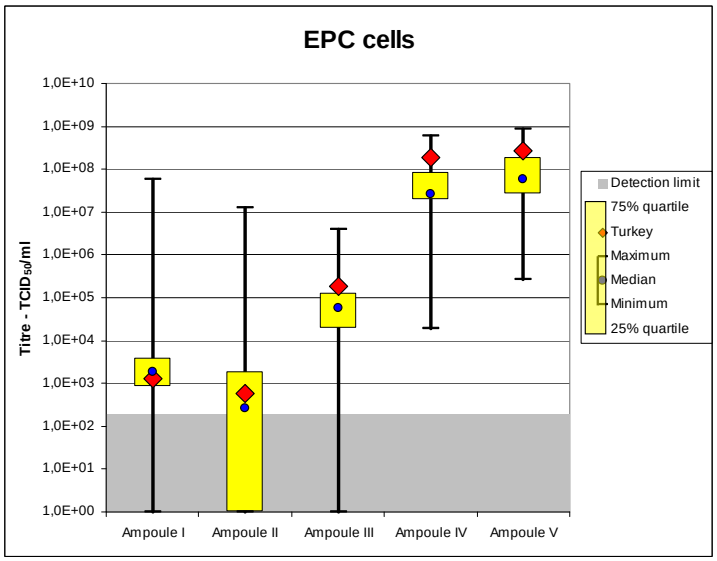
Score at different PTs

Turkey	1996	1997	1998	1999	2000	2001	2002	2003	2004	2005	2006	2007	2008
Participated									1	1		1	1
Score %									90	100		100	100

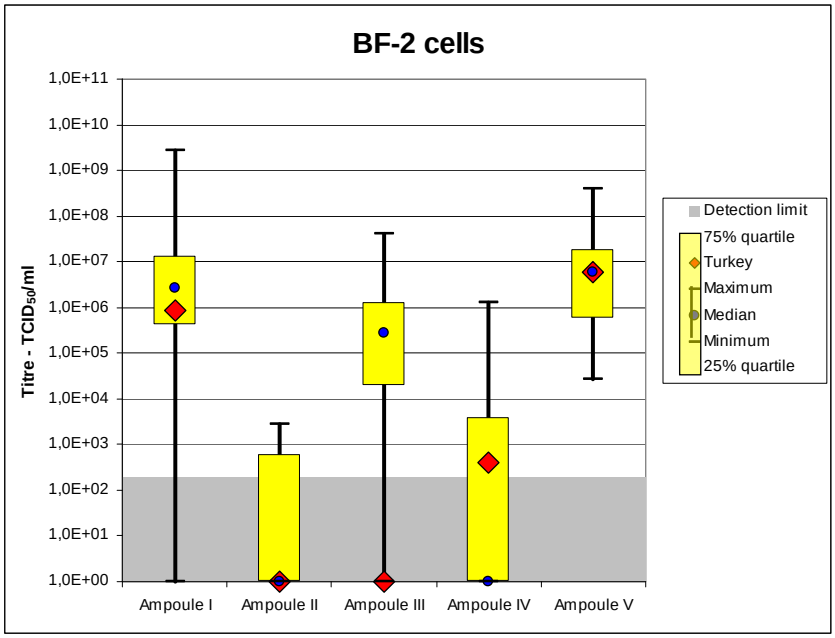
Score at individual PTs

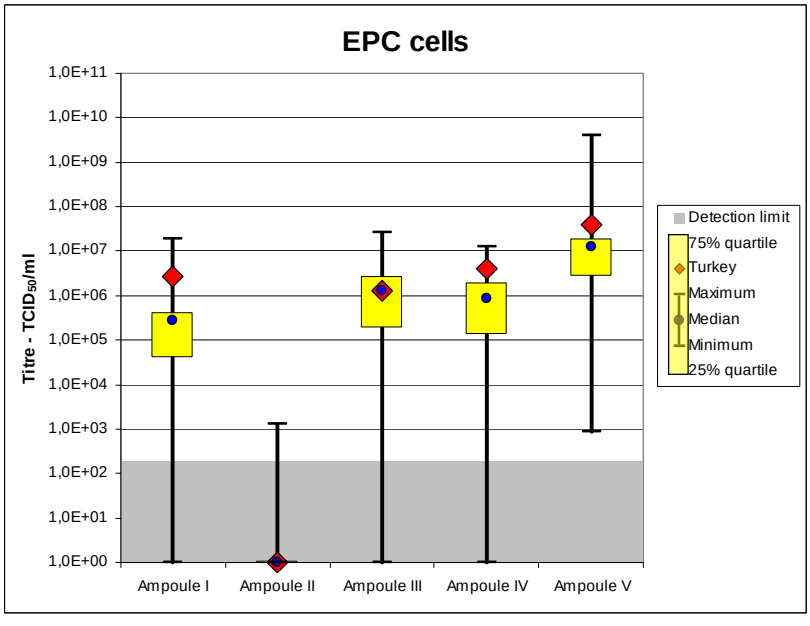
	Ampoule I:	**Ampoule II:**	**Ampoule III:**	**Ampoule IV:**	**Ampoule V:**
2008 Score 10/10	VHS virus Rindsholm 5151	VHS virus 1p8	IHN virus 217/A (DTU Vet protocol no. 4008)	VHS virus Rindsholm 5151 + IPN virus Type Sp	IPN virus Type Sp
BF-2/EPC Celler	VHSV	VHSV	IHNV	VHSV/IPNV	IPNV
ELISA	VHSV	VHSV	IHNV	VHSV/IPNV	IPNV
IFAT	VHSV	VHSV	IHNV	VHSV/IPNV	IPNV





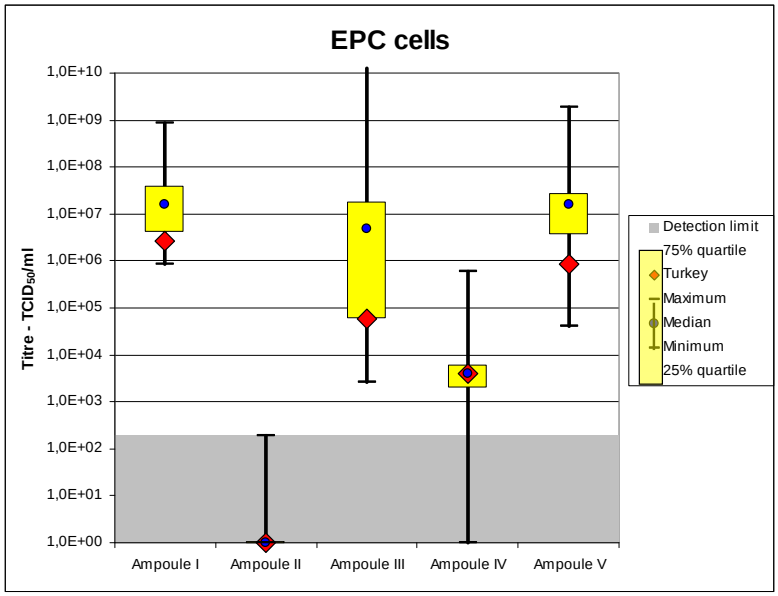
2007 Score 10/10	**Ampoule I:**	**Ampoule II:**	**Ampoule III:**	**Ampoule IV:**	**Ampoule V:**
	VHS virus DK-F1 Genotype I (Undiluted)	VHS virus DK-F1 Genotype I (Diluted 10^{-5})	SVC virus 56/70 Genotype Id	IHNV 32/87 First French isolate Genotype M	VHSV 4p101 Genotype III
BF-2/EPC celler	VHSV	VHSV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

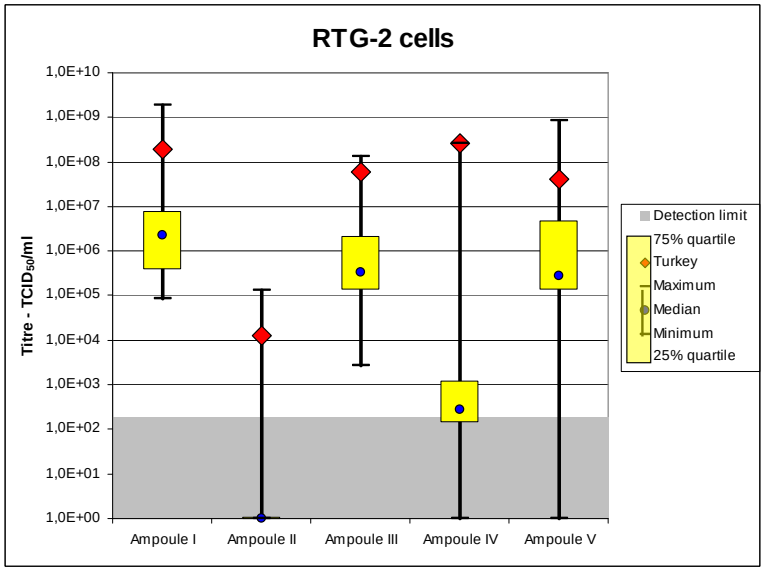




2005 Score 10/10	Ampoule I	Ampoule II	Ampoule III	Ampoule IV	Ampoule V
	SVCV- and PFR-like	VHSV DK-4p101	IHNV 32/87	VHSV D-5151 Rindsholm	VHSV DK-1p52
BF-2/EPC celler	SVCV	VHSV	IHNV	VHSV	VHSV
ELISA	SVCV	VHSV	IHNV	VHSV	VHSV
IFAT	SVCV	VHSV	IHNV	VHSV	VHSV
Neutralisation		VHSV	IHNV	VHSV	VHSV

2004 Score	Ampoule I	Ampoule II	Ampoule III	Ampoule IV	Ampoule V
	VHSV DK-5151 Rindsholm	VHSV DK-5151 Rindsholm (Diluted 10^{-7})	IPNV IPNV serotype Sp	VHSV DK-5151 Rindsholm (Diluted 10^{-4})	IHNV 32/87, First French
EPC/RTG-2 celler	VHSV	Virus not idenfified	IPNV	VHSV	IHNV
ELISA	VHSV		IPNV	VHSV	
IFAT	VHSV		IPNV	VHSV	IHNV
Neutralisation	VHSV		IPNV	VHSV	IHNV





Annex 5 – Aquaculture in Turkey

Number of fish farms within country/region, according to size of production (tonnes fish/year)		
	2008	2007
< 5 tonnes	370	230
5 - 100 tonnes	1086	873
> 100 tonnes	289	211

Number of fish farms within country/region, according to fish species		
	2008	2007
Rainbow trout	1365	1107
Atlantic Salmon	1	
Other salmonids		
Carp	30	31
Eel		
Flatfish		
Seabream / Seabass	305	288
Other marine spp.	39	7
Other freshwater spp.	5	

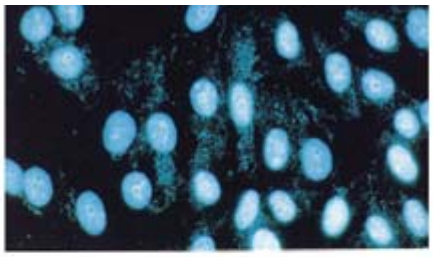
According to Council Directive 2006/88, please indicate number of farms in your country/region placed in the respective catagories according to fish species: **S&D 2008**					
Category I **Declared disease-free**		**VHS**	**IHN**	**ISA**	**KHV**
	Rainbow trout				
	Atlantic Salmon				
	Other salmonids				
	Carp				
	Eel				
	Flatfish				
	Seabream / Seabass				
	Other marine spp.				
	Other freshwater spp.				
Category II **Subject to a surveillance programme**		**VHS**	**IHN**	**ISA**	**KHV**
	Rainbow trout				
	Atlantic Salmon				
	Other salmonids				
	Carp				
	Eel				
	Flatfish				
	Seabream / Seabass				
	Other marine spp.				
	Other freshwater spp.				
Category III **Not known to be infected but not subject to surveillance programme for achieving disease free status**		**VHS**	**IHN**	**ISA**	**KHV**
	Rainbow trout	1363	1365	1365	
	Atlantic Salmon	1	1	1	
	Other salmonids				
	Carp				30
	Eel				
	Flatfish				
	Seabream / Seabass				
	Other marine spp.				
	Other freshwater spp.				
Category IV **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.				
		VHS	**IHN**	**ISA**	**KHV**
	Rainbow trout	2			
Category V	Atlantic Salmon				
	Other salmonids				
Known to be infected. Subject to minimum control measures	Carp				
	Eel				
	Flatfish				
	Seabream / Seabass				
	Other marine spp.				
	Other freshwater spp.				

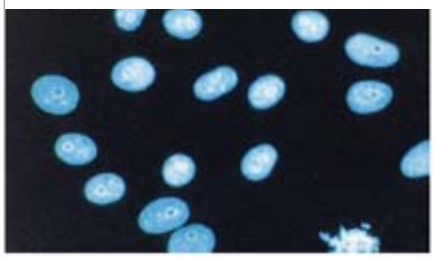
Number of fish samples (pools of tissue material) examined virologically (in cell cultures and by direct methods without cell cultivation) in NRL and regional laboratories, in total:		
	2008	2007
No. of samples tested by cell cultivation	202	738
No of samples tested by PCR or other direct methods without cell cultivation	0	

Annex 6
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.