European Union Reference Laboratory for Fish Diseases

National Veterinary Institute, Technical University of Denmark, Aarhus

Technical Report 2011

from the European Union Reference Laboratory for Fish Diseases

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

DTU

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Introduction

The National Veterinary Institute, Technical University of Denmark (DTU-VET) is appointed as the European Union Reference Laboratory for Fish Diseases (EURL), according to Commission Decision of 1 December 2010 on financial aid from the Union for the year 2011 for certain Community reference laboratories in the field of animal health and live animals (2010/735/EU) and to Specific Agreement No 6 to Framework Partnership agreement No SANCO/2005/FOOD SAFETY/010- Animal Health-Fish Disease.

The duties of the EURL are described in Council Directive 2006/88/EC of 24 October 2006 (Annex VI). The duties mainly concern fish diseases listed as exotic diseases: epizootic haematopoietic necrosis (EHN) and epizootic ulcerative syndrome (EUS); and fish diseases listed as non-exotic diseases: infectious salmon anaemia (ISA), viral haemorrhagic septicaemia (VHS), infectious haematopoietic necrosis (IHN) and koi herpes virus (KHV) disease. This report follows the format of the work programme adopted for the EURL for 2011, describing activities associated with each point and the status of ongoing projects. The list of functions and duties of the EURL follows this introduction.

The 15th Annual Meeting of the National Reference Laboratories for Fish Diseases was held 26-27 May 2011, in Aarhus, Denmark. A total of 52 participants from 33 countries attended over the two day period. There were four sessions with a total of 33 presentations, 9 of which were given by invited speakers. The meeting was held at rented nearby facilities (Søauditoriet) of Aarhus University, as the premises at our institute in Aarhus are too small for the number of participants. A report was submitted in August 2011.

In 2011 we were granted financial support to hold a workshop in Surveillance and Epidemiology of Aquatic Animal Diseases. The workshop was organised in close collaboration with the OIE Collaborating Centre for Aquatic Epidemiology and Risk Assessment, constituting of scientists from the Norwegian Veterinary Institute. The Norwegian Veterinary Institute took on the responsibility to plan the scientific programme while the EURL focused on the practical arrangements. The EURLs for Molluscs and Crustacean Diseases were involved as well reflecting that the workshop covered all aquatic animals. The workshop was held 23-24 November, 2011 and took place in the auditorium of the National Veterinary Institute in Copenhagen A total of 50 participants from 25 countries and 5 invited experts attended the workshop,

Again this year an inter-laboratory proficiency test was distributed to the NRLs mainly within the EU but there were also participants from countries outside EU. For the second year, the proficiency test consisted of two tests, PT1 and PT2. PT1 was designed as the previous proficiency tests provided by the EURL, to primarily assess the ability of participating laboratories to identify VHSV, IHNV and EHNV. PT2 was developed in order to test the proficiency of participating laboratories to identify ISA and KHV and in addition for the first time also spores of the oomycete *Aphanomyces invadans* causing EUS. Thereby the proficiency test is now covering all 6 listed exotic and non-exotic diseases. 41 National Reference Laboratories (NRLs) participated in the proficiency test, the highest number ever. A report was submitted in March 2012. Most laboratories performed very well, especially in view of the fact that the non-viral disease EUS was included for the first time in the test.

An important focus of the EURL is to update the standard operating procedures of the non-exotic and exotic listed diseases. Diagnostic manuals for VHS, IHN, ISA, KHV and EHN are now available at the EURL web page. Diagnostic manual for EUS is under preparation and will be included within long. Unfortunately the manual on sampling and diagnostic procedures for the listed diseases has still not been adapted by the EU, the diagnostic methods therefore still relies on the former Commission Decision 2001/183/EC for VHS and IHN and 2003/446/EC for ISA while no legislative text exist for KHV, EHN and EUS. Significant resources were given to the implementation of diagnostic methods for EUS which soon will be in place and updated on our website.

Another important focus area was the development, implementation and validation of diagnostic tools for identification of the listed diseases and their accreditation. One outcome of these efforts was the publication

on the generation of a real-time RT-PCR assay for detection of all genotypes of VHSV that has been proposed for the OIE to be used as an alternative to surveillance for VHS by cell cultivation!
During 2011, resources were also used to collate data on surveillance, health categorisation, and diagnostics in EU; to identify and characterise selected virus isolates; to type, store and update a library of listed virus isolates; to develop, update and maintain the database containing information on fish pathogens (www.fishpathogens.eu); to supply reference materials to NRLs; to provide annual training courses in laboratory diagnosis and missions to other NRLs; to produce antisera; to prepare SOPs for detection of EUS; to update the EURL webpage (***www.eurl-fish.eu***); and finally to attend international meetings and conferences.
The permanent staffs of the Section for Fish Diseases in Aarhus, Denmark consists of approx. 22 academic and technical staff, primarily involved in research, diagnostics and consultancy with special focus on fish virology.

Unfortunately the activities of the EURL were affected in early spring 2011 by the information from the DTU vice-chancellor announcing the movement of our institute facilities in Aarhus to the Copenhagen area in 2012. For this reason our valued coordinator of the EURL Dr. Søren Kahns got another position and left our group 31 December 2011. Our future situation is still unsettled and decisions on the frame and placement of the continued work will be taken spring/summer 2012. We sincerely hope the continuation of this important function will be made possible in our future premises.

This report was prepared and collated in a close collaboration between Søren Kahns, Nicole Nicolajsen and undersigned with contributions from all the academic staff in the Section for Fish Diseases. Especially Dr. Helle Frank Skall is acknowledged for careful review of the report.

Aarhus, 18th March 2012

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

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

According to Council Directive 2006/88/EC of 24 October 2006 - Annex VI.
Period: 1 January 2011 – 31 December 2011

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

The European Union reference laboratories shall:

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

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

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

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

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

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

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

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

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

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

Work programme for 2011

1-2. *Organise and prepare for the 15th Annual Meeting for the National Reference Laboratories for Fish Diseases (NRLs) and produce a report from the Annual Meeting*

Technical report

Organization of the 15th Annual Meeting

26-27 May 2011 the 15th annual meeting of the National Reference Laboratories for Fish Diseases was held in Aarhus, Denmark, at a rented nearby facility of Aarhus University. A total of 52 participants from 33 countries attended over the two day period. There were four sessions with a total of 33 presentations, 9 of which were given by invited speakers.

The scientific programme of the Annual Meeting was diverse and covered many topics of current interest. The meeting was opened with the traditional session on update of fish diseases in Europe, where once again participants from the member states had the opportunity to present new findings from their home countries. Initially an overview of the disease situation and surveillance in Europe 2010 were provided on the basis of the results from the Survey & Diagnosis questionnaire. Results of a questionnaire send to fish pathology experts in the Mediterranean area were the basis of a following talk providing an update on the fish disease situation in Mediterranean aquaculture. Subsequently, needs and requirements for rapid and efficient response to emerging diseases were provided, followed by an update on the disease situation in Norway. Then a talk on IPNV distribution in Austrian aquaculture 1993-2010 and a talk on the VHSV eradication program in Denmark was given. Afterwards, three talks on molecular epidemiology from Denmark, Poland and Germany, respectively were presented. The session was finished with a talk on *Vibrio* infections in Dutch aquaculture.

The session on technical issues related to sampling and diagnosis was started by a presentation on the new EU manuals on sampling and diagnostic procedures and the role of the www.eurl-fish.eu web page. Next were three talks on diagnostic methods; one for identification of EHNV and two for identification of *Aphanomyces invadans*; followed by a plenum discussion on options for surveying for EUS in EU in farmed and ornamental fish. After a short coffee break a talk on health categorisation of fish farms in Europe 2010 based on answers to the questionnaire on Survey & Diagnosis was presented. Then a talk high lightening some of the challenges concerning the implementation of the new legislation on aquatic animal health surveillance in Europe was provided. The session ended with three presentations, one on a new real time LAMP assay for KHV detection, one on the origin of diagnostic fish cells and at last one on challenges in development of real-time PCR diagnostic assays.

In the evening a banquet dinner was held at Restaurant “Koch” located at the harbour side downtown Aarhus.

The second day was opened by an update session on scientific research. The first part of this session focussed on ISA. Initially, a chronicle of events relating to a small scale ISA epidemic situation was described for a specific Norwegian area containing several salmon farms. Subsequently, an overview was provided on ISA and HPR0 strains, followed by presentation of a study on HPR0 appearance in fresh water at the Faroe Island. The last talk on ISA was on HPR0 detection in Denmark in combination with a comparison of the criteria for diagnosis of ISA as described by EU and by OIE. The ISA topic was ended by a discussion in plenum on how the criteria for diagnosis of ISA should be in relation to an identification of HPR0.
At the end of this session a talk on a PD-vaccine that efficiently reduces severity of

disease outbreak in Norwegian aquaculture was presented. This was followed by a talk on an EPIZONE extension project on "Management, control and surveillance of VER in aquaculture" and finally a talk on serological tests for specific antibody detection against betanodavirus ended the session.

The annual meeting ended with the traditional update from the EURL. The results of the two proficiency tests, PT1 and PT2, 2010 were presented. Furthermore, the annual training course provided by the EURL was announced and topics were discussed. A report of EURL activities from year 2010-2011 was given. Finally, proposals on the EURL work plans for 2012 were discussed.

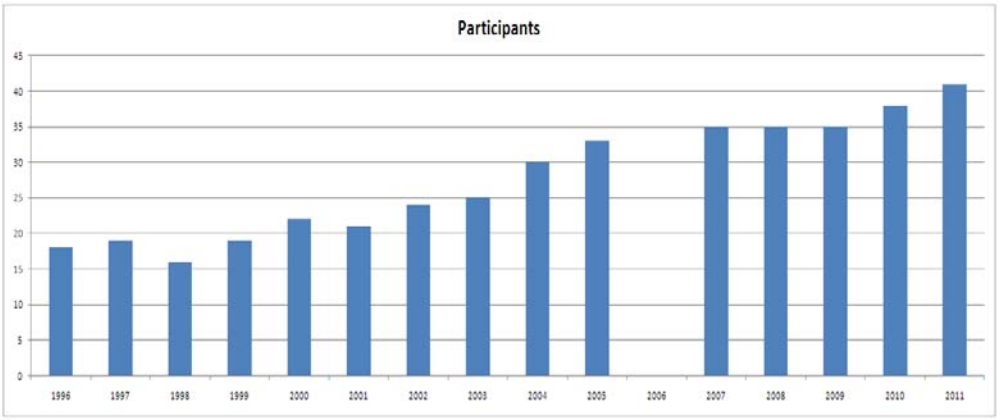
Minutes from the meeting were taken by Helle Frank Skall, Søren Peter Jonstrup and Torsten Boutrup, and have afterwards been sent to presenters for correcting in order to avoid misunderstandings. The minutes are included in this report together with abstracts and comments from the discussions. Nicole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

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

3. Prepare the Annual Inter-laboratory Proficiency Test year 2011 for the NRLs. The test will include VHSV, IHNV, EHNV, ISAV and KHV

The inter-laboratory Proficiency Test 2011

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



PT1 was designed as the proficiency tests provided by the EURL in previous years to primarily assess the identification of the fish viruses viral haemorrhagic septicaemia virus (VHSV), infectious haematopoietic necrosis virus (IHNV) and epizootic haematopoietic necrosis virus (EHNV) by cell culture based methods. PT2 was included for the second time with the aim of assessing the ability of participating laboratories to identify the fish pathogens infectious salmon anaemia virus (ISAV), *koi herpes virus* (KHV) and *Aphanomyces invadans* by PCR based methods. It is the first time *A. invadans* was included in the proficiency test. The number of National Reference Laboratories (NRLs) participating in PT1 and PT2 was 41.
The tests were sent from the EURL in the middle of October 2011.

Proficiency test 1, PT1

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

PT1 consisted of five coded ampoules (I-V). The ampoules contained VHSV, EHNV, European catfish virus (ECV), IHNV+IPNV and IPNV, respectively. The proficiency test was designed to primarily assess the ability of participating laboratories to identify the fish viruses VHSV, IHNV and ENHV (all listed in Council Directive 2006/88/EC) if present in the ampoules, bearing in mind that the test ampoules also could contain other viruses (e.g. other fish rhabdoviruses, ranaviruses, or birnaviruses). In addition the participants were asked to quantify the viruses in PT1 by titration in order to assess the susceptibility of their fish cell lines for virus infection. Participants were encouraged to use their normal standard laboratory procedures. However, the identification should be performed according to the procedures laid down in Commission Decision 2001/183/EC using fish cell cultures followed by e.g. neutralisation test, immunofluorescence, ELISA or PCR. If ranavirus was present in any of the ampoules, it was mandatory to perform a sequence analysis or a restriction endonuclease analysis (REA) of the isolate in order to determine if the isolate was EHNV or another ranavirus and it was recommended to follow the procedures described in Chapter 2.3.1 in the OIE Manual of Diagnostic Tests for Aquatic Animals 2009. Laboratories were encouraged to identify VHSV and IHNV isolates as far as possible by means of genotyping in addition to the standard methods. It was recommended to use the genotype notification described in Einer-Jensen et al. (2004) for VHSV and in Kurath et al. (2003) for IHNV. Laboratories were encouraged to submit all sequencing results used for genotyping the isolates.

PT2 consisted of five coded ampoules (VI-X). The ampoules contained ISAV and KHV. Furthermore, one ampoule contained *Aphanomyces invadans*. It was decided at the 15th Annual Meeting of the NRLs for Fish Diseases in Aarhus 26-27 May 2011, that testing for *A. invadans* for the first time should be included in the yearly proficiency test provided by the EURL. The test was designed to primarily assess the ability of participating laboratories to identify the notifiable fish pathogens ISAV, KHV and *A. invadans* (listed in Council Directive 2006/88/EC) if present in the ampoules, bearing in mind that the test ampoules could also contain other viruses. Participants were expected to use their normal PCR or real-time PCR methods for detection of the pathogens. It was not mandatory to grow KHV and ISAV on cell cultures in this test, but the viruses were not inactivated and they should thus be possible to amplify in cell cultures. If present, only **inactivated** *A. invadans* was included in the ampoules.

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

Outcome of Inter-laboratory Proficiency Test 2011

The inter-laboratory proficiency test 2011 was conducted without major constrains. Most parcels were delivered by the shipping companies within 3 days after submission. It was, however, unfortunate that one parcel was 44 days on the way before delivered to the laboratory primarily due to border controls.

PT1 Conclusion

All laboratories identified VHSV without problems. As IHNV was included as a double infection with IPNV some laboratories failed to correctly identify this virus. IPNV was correctly identified by 36 laboratories.

In 2009 EHNV was included in the proficiency test for the first time and 32 participants were able to correctly identify the virus. This year EHNV was included as well as ECV, both belong to the ranavirus family. Of the laboratories performing PCR based methods, 31 laboratories performed sequencing only for ampoule II and 32 for ampoule III. Of these laboratories all correctly identified the content in ampoule II as EHNV and 31 correctly identified the content in ampoule III as ECV/ESV. One laboratory performed both sequencing and REA for both ampoule II and III without being able to identify which type of ranavirus the isolates belong to. One laboratory performed REA only for both ampoule II and III and was able to identify the isolate as either EHNV or ranavirus, not EHNV.

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

PT2 conclusion

Considering that this was the second time that the EURL provided a proficiency test on ISAV and KHV identification, and the first time that the EURL provided a proficiency test on *A. invadans*, we consider that most participants obtained satisfying results. Out of 36 laboratories performing ISAV identification 32 identified ISAV in ampoule VI containing low titre ISAV and 35 identified ISAV in ampoule VII containing high titre ISAV. All 37 laboratories testing for KHV identified KHV in ampoule VIII containing high titre KHV, and 36 of them identified KHV in ampoule X containing low titre KHV. Out of 31 laboratories testing for *A. invadans* 28 identified the pathogen in ampoule IX.

Lowering the titre of the virus caused only one laboratory to miss identification of KHV in the low titered ampoule X. A reason for the laboratory to miss the correct identification is most likely due to mistaken marking of the ampoule. If this is the reason then all laboratories testing for KHV were able to identify both the high titre and the low titre KHV. For ISAV, one laboratory missed identification in the high titre ampoule and for the low titre ampoule three further laboratories did not succeed in the identification.
A couple of laboratories identified pathogens not present in the ampoules. E.g. one laboratory identified ISAV in all ampoules but only with a weak positive reaction in the ampoules where ISAV was not present.
A critical point in PCR based diagnostic tools is avoiding false positive and false negative results. To decrease the risk of having false negative results, it is always recommended that laboratories use the most sensitive tool available, validate the sensitivity of their diagnostic tools and use proper controls. To decrease the risk of false positive results laboratories have to be very aware of the risk of cross contaminations.

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

It is up to the individual laboratory to assess if they perform according to their own expectations and standards. We also took the opportunity to provide a comment to participants regarding submitted results if relevant. Furthermore we encouraged 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 16th Annual Meeting of National Reference Laboratories for Fish Diseases to be held 30-31 May 2012 in Aarhus, Denmark.

The full report is included as Annex 4

5. Supply reference reagents to the NRLs in Member States

Materials supplied by the EURL

On request, the EURL supplies material to other laboratories in Member States and Third Countries to aid in the diagnosis and characterisation of fish diseases. The number of laboratories receiving material and the specific material and number of units supplied by the EURL in 2011 are listed in Annex 3.

Further details of the supplied materials are listed in Annex 3

6. Production of antisera against selected isolates when necessary

Production of antisera

Rabbit antisera against KHV were produced in 2010 in 2 rabbits (F-1108 and F 1122) and assessed in 2011. The sera can be used in immunofluorescence, immunhistochemistry and in seroneutralisation test.
Rabbit antisera against EVEX were produced by immunizing 2 rabbits (F-102 and F-159) with purified Eel rhabdovirus (EVEX) received from ANSES Brest 4.3.1986. Both sera reacted well in IFAT and very high neutralizing titres were obtained
Additionally the reference strain of *Herpesvirus anguilla* (HVA or AnHV-1) was propagated and purified according to the herpes virus protocol received from Dr. T. Ito. Rabbits will be immunized with the eel virus preparation primo 2012.

7. Update and

Virus library

maintain a library of isolates of ISAV, VHSV, IHNV, KHV, EHNV and Aphanomyces invadans

Several isolates of the listed viruses VHSV, IHNV, KHV and ISAV were received and stored in our library during 2011. Furthermore, two isolates of the oomycete *Aphanomyces invadans* were received.
In addition, the EURL received other relevant pathogens like SVCV, IPNV, perch rhabdovirus and *Aphanomyces astaci*, the causative agent of crayfish plaque. Our library have continuously been updated and maintained.

Further details of the received materials are listed in Annex 2

8. Establish and maintain a library of tissue material from fish infected with listed pathogens

Library of tissue material from fish infected with listed pathogens

Tissue material from VHSV and IHNV infected fish has been collected following infection trials; a panel of organ samples from 75 single fish infected with VHSV and 25 negative control fish has been produced which can be sent to laboratories and used to validate diagnostic tests. A similar infection trial with IHNV has been initiated, and an infection trial with IPNV has been planned. Fish from infection trials with VHSV and IHNV has been collected for use as positive controls in histology.

Rainbow trout and Three Spot Gouramis has been infected with *Aphanomyces invadans* by intra muscular injection, and samples has been collected from fish showing symptoms of Epizootic Ulcerative Syndrome (EUS) to use as positive controls in histology and PCR.

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

Update the webpage of the EURL

The EURL website (***www.eurl-fish.eu***) is a notice board, where NRLs and other interested parties can access relevant information and previous reports concerning the activities coordinated by the EURL and relevant upcoming events in the Community.

The diagnostic manuals for VHS, IHN, ISA, KHV disease and EHN have been uploaded on the web page.

Furthermore, reports of the EURL, e.g. of the results of the proficiency test, the Annual Meeting of the NRLs, result of questionnaire on "Survey & Diagnosis", workshop on surveillance and epidemiology etc. are launched at the web page immediately after release.

10. Update the diagnostic manuals for VHS, IHN, ISA, KHV disease and EHN on the EURL web page, and include diagnostic manuals for EUS

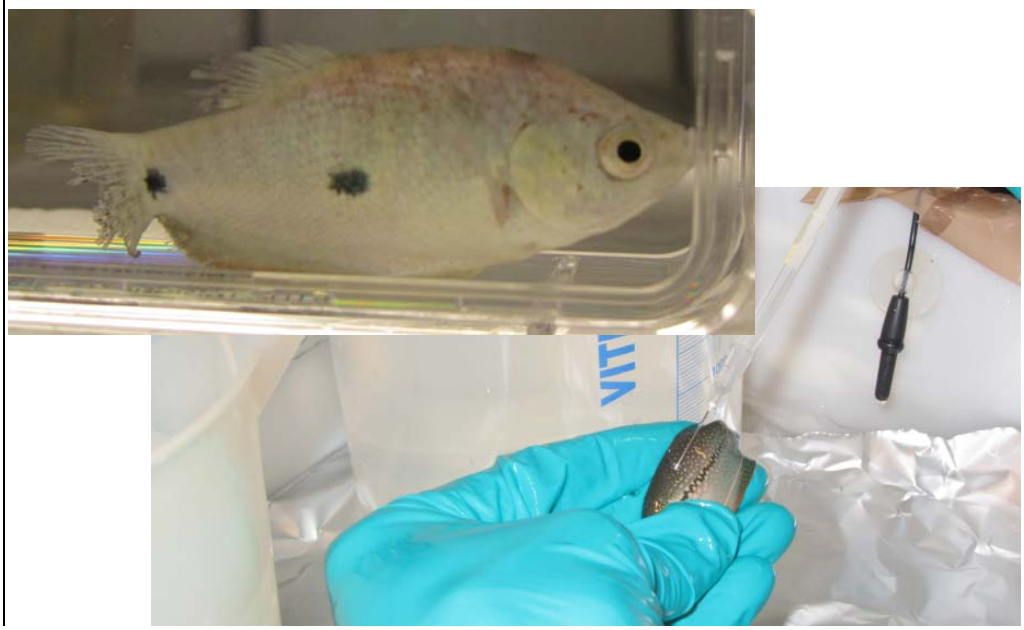
Diagnostic manuals

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

11. Update diagnostic methods for diagnosis of Epizootic Ulcerative Syndrome (EUS) and assess the possibilities for including Aphanomyces invadans in proficiency test in the future

Diagnostic methods for diagnosis of Epizootic Ulcerative Syndrome

Diagnostic methods for detection and cultivation of *Aphanomyces invadans* was implemented, assessed and validated. A validation report on *A. invadans* detection by PCR was produced based on which accreditation was obtained from the Danish accreditation board DANAK.
Isolation and cultivation methods were implemented and demonstrated during the 15th Annual Meeting for NRL.
Disinfection and survival of the oomycetes was tested as was various regimes for sporulation. Infection trials were conducted including 3 species of Gouramis and rainbow trout fingerlings. Reisolation of the oomycete from infected animals was done and samples of infected tissues were collected for distribution to NRLs in the EU.
Lyophilisation under various conditions was tested and the best method for including *A. invadans* in the annual inter laboratory proficiency test assessed



12. Collect and report 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 2010

The Questionnaire on Surveillance and Diagnosis (S&D) which is collated annually is the only comprehensive overview of the disease situation in aquaculture in Europe. The information has been made available on the EURL web site (***www.eurl-fish.eu***), where all raw data can be obtained. The S&D have evolved over the years, for 2010 it comprise 3 parts:

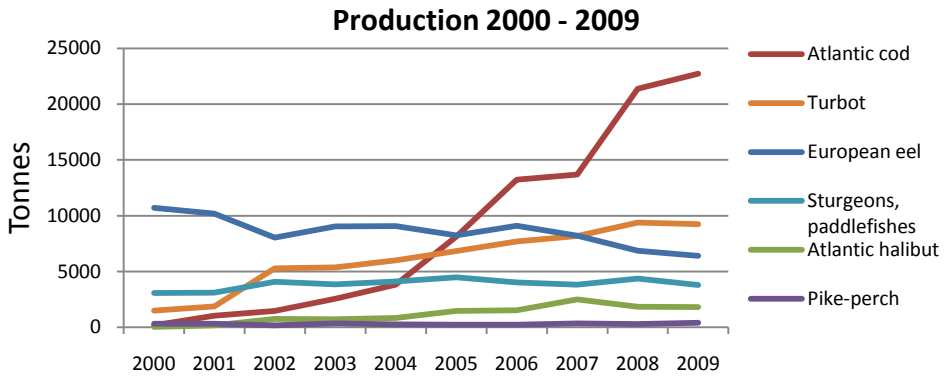
1. General data on production type and size, health categorization of fish farms according to Council Directive 2006/88/EC, and information on national surveillance programmes.

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

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

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. Only data from 2009 were accessible. The production in 2009 is almost the same as in 2008 and has risen after a decrease from 2003-2006. Data from 2010 were not yet available in FIGIS when we collated the data from 2010 from the Member states. 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 almost equal.

The Atlantic salmon production is mainly at the Atlantic Sea coast line of the Northern European countries. Rainbow trout are produced in most European countries, whereas carp production is mainly in the Eastern part of Continental Europe. The production of sea bream, sea bass and tuna has increased significantly in the Mediterranean countries. The production in Turkey rose from 66.000 to 76.000 t last year and Turkey has become one of the countries included with the highest production. Among other fish species of interest are pike-perch (408t), eel (6391t), sturgeon (3789t), cod (22729t), turbot (9238t), and halibut (1806t).



The S&D included questions on how fish farms are health categorised according to Council Directive 2006/88/EC in the respective countries. More than half of the authorised farms in Europe are in category III for VHS and IHN and the remaining in category I or II. According to these official data almost no farms are known infected with either of these diseases. This might be more due to a significant underreporting

than of the de facto situation.
For KHV most carp farms are in category III, the unknown status. Many farms in Europe are not categorised yet. However, categorisation is in good process when comparing to e.g. the molluscs farms in Europe. There are several different views on how categorisation shall be performed, e.g. should VHS free marine rainbow trout farms be placed in category III or I? If ISA virus HPR0 is found in or in proximity of a farm can it retain its category I status? The Council Directive is under revision and in this connection the categorisation system might be simplified and be made more transparent.

Concerning the epidemiological data, obviously, there is still a severe underreporting of VHS and IHN in many countries. For VHS the infection status in only known for 36% of the farms, for IHN the situation is known in 38% of the farms. While for KHV the disease situation is unknown on 94% of the farms! For farms producing Atlantic salmon, the infection status for ISA is known for nearly all farms. ISA is still a problem in Norway. The finding of ISA virus HPR0 poses some problems regarding the health categorisation of salmon farms.
Many countries have surveillance programmes for SVC (20 of 29 countries), BKD (16 of 32 countries), IPN (22 of 32 countries) and *Gyrodactylus salaris* (8 countries), for which they are seeking "additional guaranties" according to §42 in CD 2006/88/EC. The number of farms in the programmes varies from very few farms to many farms.
There are very large differences between countries on how many samples are tested on cell cultures, ranging from < 100 to several thousands. PCR is coming up in many countries, and the large number of PCR-tests conducted in some countries mostly reflects the KHV and ISA testing.
About a third of the countries have regional laboratories, and of these countries, 8 of 11 organize proficiency tests for the regional laboratories.

A summary of the results for 2010 is presented on our website: www.eurl-fish.eu/Activities/survey_and_diagnosis.aspx

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

Identification and characterisation of selected virus isolates

Again in 2011 a significant number of virus isolates were received for further characterisation at the EURL and for storing in our virus library as shown in the table beneath.

Member States/Countries outside EU		
Material received	**Laboratories**	**Units**
Diagnostic material	2/1	7 samples
Virus isolates	2/0	127 samples
Other material	1/0	3 specimens

Further details are listed in Annex 2

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

- **Agence Nationale de Securite Sanitaire (Anses), France (Laurent Bigarre):** Noda proficiency test 11 samples. 4 RNA samples with Noda primer. (DTU-VET 2011-50-192).

- **Agence Nationale de Securite Sanitaire (Anses), France (Jeannette Castric):** Ser Lapin Noda 3 d.10-12-97 CNEVA Brest (1:500 IFAT) and Ser. lap. Anti-Noda CC143 a (0,5ml) d. 6-12-2005 (1:1000 IFAT). (DTU-VET 2011-50-332).

- **Iran Veterinary Organisation, Iran (Mohsen Dastoor):** Diagnostic samples from rainbow trout. Detected positive for IPNV in no. 1-7 and 9. (DTU-VET 2011-50-247).

- **Instituto Zooprofilattico Sperimentale delle Venezia, Italy (Guiseppe Bovo):** 123 vials with IPNV isolates (DTU-VET 2011-50-116).

- **Instituto Zooprofilattico Sperimentale delle Venezia, Italy (Guiseppe Bovo):** Cell culture supernatant marked "IPNV from eel, 2. pass BF-2, 256/V10". (DTU-VET 2011-50-18). Isolate received as lyophilized marked "305/93, 15.04.93". EPC cells Pooled tissues from Black bullhead - Black bullhead rhabdovirus. (DTU-VET 2011-50-19).

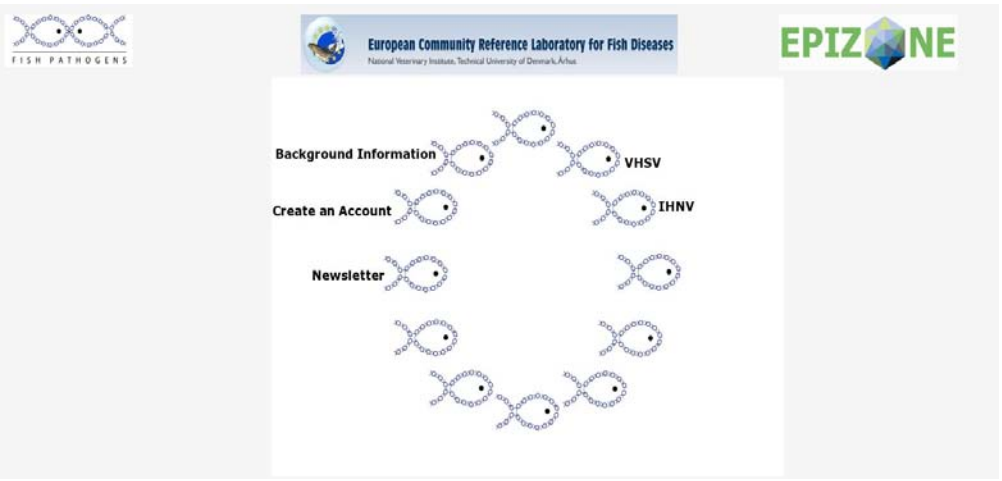
- **Hodowla Ryb, Poland (Mieczyslaw Pelka):** Diagnostic samples from rainbow trout, whole fish, pool of organ tissues. Detected positive for IHNV and IPNV in pool no. 2 (DTU-VET 2011-50-188). Diagnostic samples from rainbow trout. Detected positive for IPNV in no. 1-2. (DTU-VET 2011-50-214). Diagnostic samples from rainbow trout. Detected positive for IPNV in no. 1-2. , (DTU-VET 2011-50-279, 2011-50-280).

- **Veterinary Institute (SVA), Uppsala, Sweden (Eva Blomkvist):** Samples from eel no. 2130, examined for HVA (AnHV-1) by PCR. (DTU-VET 2011-50-288). Detected negative for HVA, IHNV, IPNV, VHSV. Diagnostic samples from cell culture. (DTU-VET 2011-50-341). Detected positive for Perch rhabdovirus.

14. Update and expand www.fishpathogens.eu with more pathogens

www.fishpathogens.eu

The current version of the database *www.fishpathogens.eu* offers a platform for sharing of available information on isolates of fish pathogens and their sequences. The platform contains public available databases on VHSV and IHNV. Furthermore the development of a database on betanodavirus is almost achieved and the database is awaiting publication of a paper describing the database in a scientific journal before it will be public available. An SVCV database has for a while been at the final stage of development, and work on ISAV and KHV databases has been initiated, but the work has been on standby due to other assignments. During 2011 the VHSV and IHNV databases were maintained and expanded. The VHSV database today offers publically available information on 494 isolate reports and 338 sequence reports, while the IHNV database offers publically available information on 82 isolate reports and 84 sequence reports. 117 persons are registered as users of the database and in 2011 the database had 5918 visitors.



15. Perform molecular epidemiology analysis to improve knowledge on diseases spreading mechanisms of viral pathogens

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

VHS virus can be divided into four genotypes with additional sub lineages of which the main source of outbreaks in European rainbow trout farming is caused by sub lineage Ia isolates. Danish aquaculture has been considered endemic infected with VHSV since the first outbreak of the disease was observed in the 1950's until a final eradication program was initiated April 2009. The full length G-genes of all Danish VHSV isolates collected in the period 2004 – 2009 were sequenced. Phylogenetic analyses show that all 58 VHSV isolates sampled from rainbow trout are sub lineage Ia isolates. In the endemic infected Danish fresh water systems, the population of VHSV isolates has evolved into a distinct clade within sub lineage Ia isolates that we designate clade Ia-1. Isolates sampled in other continental European countries clusters in another distinct clade designated clade Ia-2. Through this collection of VHSV sequences, it is possible to characterize likely transmission pathways of VHSV. Within the Ia-1 clade a few VHSV strains have caused outbreaks in Germany and UK, respectively. In these cases it is likely that ancestor viruses may have been transferred out of the Danish environment. Apparently, VHSV strains were also transmitted into Denmark in a few cases. Interestingly, one of the viral strains seems to have survived in the Danish water systems for almost four years between causing outbreaks whereas subclades of highly pathogenic VHSV isolates have disappeared over time.

In another study conducted in collaboration with colleagues in Poland (Michal Reichert and Marek Matras, National Veterinary Research Institute, Pulawy, Poland) we sequenced the full length G-gene sequences of 24 VHSV isolates that caused VHS outbreaks in Polish trout farms between 2005 and 2009. All these isolates were identified as genotype Ia-2, they divided however in to two genetically distinct subgroups, that we name Pol I and Pol II. The Pol I isolates have mainly caused outbreaks in the southern part of Poland, while Pol II isolates predominantly were sampled in the north of Poland although it seems that they have been transmitted to other parts of the country. Molecular epidemiology was used for characterisation of transmission pathways. This study shows that the main cause of virus transmission seems to be movement of fish. Transmission might occur when farmers use the same stocking material and at least in Polish circumstances breeding practices appear to have significant impact on spreading of VHSV infection.

16. Assessment and

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

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

identification

By switching to RT-PCR for surveillance and diagnosis of VHS the time needed before a correct diagnosis can be given will be considerably shortened and the need for maintaining expensive cell culture facilities reduced. A validation according to OIE guidelines of a sensitive and specific Taqman based real time RT-PCR was therefore conducted. The assay detects all VHSV isolates in a panel of 79 VHSV isolates covering all known genotypes and subtypes, with amplification efficiencies of approximately 100%. The analytical and diagnostic specificity of the real time RT-PCR is close to 1. The analytical and diagnostic sensitivity of the real time RT-PCR is comparable to traditional cell based methods. In conclusion, this new real time PCR assay has the necessary quality to be used as a VHSV surveillance tool on par with the cell culture assays.
The EURL continued the implementation of real-time PCRs for detection of ISAV and KHV. For ISAV, the assay developed by Snow et al. 2006 was chosen and several diagnostic samples were tested as part of validation. For KHV detection, validation of the real time PCR described by Gilad et al. 2004 was continued.

17. Facilitate and provide training in laboratory diagnosis. Training courses in methods used for diagnosis of fish diseases will be established and offered at the EURL laboratory facilities. The courses will primarily be for training of staff from NRLs and the content will depend on request from participants

Training, missions and scientific collaboration

The EURL has decided that it will offer a yearly training course in diagnostic techniques for identification of listed fish diseases. The course is primarily for NRL staff. The course covers training in several diagnostic topics – e.g. PCR, cell culture, antibody based diagnostics - the topics depends on what participants ask for. Therefore other topics may also be offered and it is possible that one or more topics are not offered because of lack of interest. Participants do not have to follow all topics but can choose parts of the program. The course was announced for the first time at the 14th Annual Meeting of the NRLs for Fish Diseases and the course took place in the laboratories of the EURL in Aarhus in the period between 24 January and 4 February (week 4-5) 2011. At the 15th Annual Meeting in 2011 a program for the following year was announced. Based on feedback from the participant a course on PCR and cell culture techniques/immunochemistry was organised to take place in January/February 2012.

Many requests from colleagues for training were postponed to take place at this yearly course; however, the following colleagues visited the institute during 2011 for scientific meetings, project collaboration or training:

Christian Fry, Master student of University of Johannesburg South Africa. Focus on EUS diagnostics and pathogenesis.	24/01 2011 to 13/01 2012
EURL training course in fish diagnostics: Tomáš Veselý - Czech Republic; Snjezana Zrncic – Croatia; Marina Eyngor - Israel; Niccolò Vendramin - Italy; Khairi El-Battawy - Austria; Eef Vankerckhove - Belgium; Orinta Rimasaite - Lithuania; Siiri Põldma - Estonia; Robertas Krejaras - Lithuania; Joanna Maj - Poland; Pilar Torres - Spain; Athanasios Prapas - Greece; Elisabetta Capellozza - Italy;	24/01 to 03/02 2011

Mihaela Costea - Romania; Jana Jančeková –Slovakia; Christophe Heusdens - Belgium; Ivana Lojkić - Croatia; Heike Schuetze - Germany; David Swords - Ireland; Maria Aamelfot - Norway; Carmen Alvarez - Spain	
Meritxell Díez Padrisa, DVM, Barcelona. Basic course in fish diagnostics.	31/01 to 18/02 2011
Günter Kotterba from Friedrich-Loeffler-Institut, Bundesforschungsinstitut für Tiergesundheit, Federal Research Institute for Animal Health concerning EUS diagniostics.	22/03 to 23/03 2011
Dr. María Eugenia Cabrejos M. Aqua Innovo S.A. and colleagues from Chile for exchange of views o fish health surveillance	29/03 2011
Guiseppe Bovo, Stefano Maragon and ... Ernst from IZSVe. Italy concerning the establishment of wet lab facilities.	03/05 2011
Susana Martinez Alonso CISA-INIA, Madrid, Spain. VHSV vaccination and infection trial within NADIR project.	07/05 to 10/06 2011
Monika Hjortaas, Brit Hjeltnes and Ole Bendik Dale Norwegian Veterinary Institute. Einar Tormod Karlsen, Norwegian Food Safety Authority Sigrid Cabot, DG SANCO, European Commission. Discussion on ISA and HPR0.	27/05 2011
Jette Skiffard, FVST, training in lab diagnostics	08/07 2011
Dr. Geert Wiegertjes and Dr. Maria Forlenza from Wageningen University The Netherlands visited in connection to coordination of FP7 grant proposal, as well as discussion of ongoing research collaboration.	17/08 to 20/08 2011
Rolando Ibarra, Intesal Salmon Chile, on development of diagnostics	25/08 2011
Nick Moody, CSIRO – Geelong, Australia. Visiting scientist. Research update and scientific exchange	19/09 to 22/09 2011
Erik Jessen Jürgens Bio Aqua on water treatment in aquaculture	27/09 2011
FVO inspection to Denmark: Stefano Sotgia, Georg Czifra, FVO and Richard Gardiner, CEFAS	16/11 2011
Renate Johansen, National Veterinary Institute, Oslo. Infection trials in Atlantic herring with various strains of VHSV- NADIR TA.	16/11 to 18/11 2011 and 07/12 to 09/12 2011

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 in the field of "delivering small RNAs to fish" from 1 November 2009 to 31 October 2012. Supervisor: Niels Lorenzen; Co-supervisor: Brian Dall Schyth

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

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

M.Sc. Nikolaj Gedsted Andersen has been enrolled as PhD student at University of Copenhagen in association to the project “Improved vaccination strategies in marine aquaculture”: Improved vaccination strategies in marine aquaculture (supported by the Strategic Research Council in Denmark). His overall supervisor is Per Juel Hansen (University of Copenhagen). His co-supervisor at DTU-VET, were the experimental challenge experiments including different combinations of algae and virus are performed, are Niels Lorenzen and Ellen Lorenzen.

OIE collaboration: N.J. Olesen was designated expert in the ad hoc group on aquatic animal health surveillance. The chapter on viral haemorrhagic septicaemia was reviewed by H.F. Skall and N.J. Olesen and significant changes were included in the new chapter
Proposal for initiating twinning project with the NRL for Fish Diseases in Turkey was undertaken in order to establish a reference centre for the Middle East

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

Missions to relevant laboratories

Two colleagues from the EURL accomplished two visits.

The first visit was to the NRL for Fish Diseases in Finland located at the Finnish Food Safety Authority, Evira in Helsinki and took place 7-8 November 2011.

The second visit was to the Estonian Veterinary and Food Laboratory (VFL) in Tartu from the 9-10 November 2011. The aim of this mission was to visit the Department of Molecular Analyses in Tartu. The NRL for Fish Diseases in Estonia is located at the Estonian Veterinary and Food Laboratory (VFL) in Tallinn. In 2006 the NRL for Fish Diseases was visited by a delegation from the EURL for Fish Diseases. However, the Department performing the molecular biological diagnostic analyses on fish samples (Department of Molecular Analyses) was not part of the visit in 2006 because this unit is located at the Estonian Veterinary and Food Laboratory (VFL) in Tartu.

Reports from these two visits please see annex 5 and 6

19. Organise a workshop on “Surveillance and Epidemiology of Diseases in Aquaculture” at the premises of the EURL-Fish and in collaboration with the OIE collaborating centre for aquatic epidemiology and the EURLs for Molluscs and Crustaceans

Workshop on “Surveillance and Epidemiology of Diseases in

The Workshop in Surveillance and epidemiology of Aquatic Animal diseases took place in the auditorium of DTU-VET in Copenhagen on 23-24 November, 2011.
A total of 50 participants from 25 countries and 5 invited experts attended. The workshop was organised by the European Union Reference Laboratory for Fish Diseases together with the OIE collaborating centre for Aquatic Epidemiology and Risk assessment, constituting of scientists from the Norwegian Veterinary Institute. The EURLs for molluscs and crustacean diseases were involved as well reflecting that the workshop covered all aquatic animals. The Norwegian Veterinary Institute took on the responsibility to plan the scientific programme while the EURL focused on the practical arrangements.

The overall purpose of the workshop was to give an introduction to some of the

topics of surveillance and epidemiology with a special focus on the challenges for aquatic animal diseases.
After a welcoming and a general introduction the workshop was opened with a talk on the purpose of surveillance, followed by a presentation of concepts for sampling and testing for surveillance. After these basic principles and concepts was presented, two talks were given on risk-ranking of aquaculture farms according to the new legislation on aquatic animal health. A special presentation was given on the challenges regarding surveillance in shellfish and molluscs.

In the afternoon, the workshop participants were divided into groups with the following four topics:

- Screening strategies & tracing of pathogens
- Special challenges regarding surveillance in shell & molluscs
- Design of surveillance programmes
- Risk factors and risk categorization

Within each group, concepts and challenges were discussed, and a summary of the groups' discussions was presented on the second day of the workshop, in order to give all participants insight into the different topics.

On the second day of the workshop, presentations were given on how to use models of risk in space and time when considering surveillance and on molecular epidemiology for tracing the origin of disease outbreaks. A final presentation was given on concepts of economics with regards to surveillance programmes in aquatic animals.

After the workshop, an internet-based evaluation was carried out, with 30 responses. The overall impression is that the participants were satisfied with the outcome of the workshop, and there is a wish for more workshops on epidemiologic topics. The results of the evaluation are presented at the end of this report.

A report on the workshop and results of the evaluation is presented in Annex 7

20. Attending missions, international meetings and conferences in order to be updated on diagnostic methods on listed exotic and non-exotic fish diseases

International meetings and conferences attended.

Contact with colleagues from other laboratories is a channel for exchange of information in the field of fish diseases, and an opportunity to keep abreast with new developments in the field. Missions are described under point 18. Of special interest are of course the activities relating to VHS, IHN, KHV, ISA, EHN and EUS. Scientists at the EURL participated in the following international meetings and conferences in 2011:

Participation and presentations at international conferences and meetings

Annual Meeting of the National Reference Laboratories for Fish Disease. Aarhus, 26-27 May, 2011.

- NJ Olesen. Overview of the disease situation and surveillance in Europe in 2010.
- NJ Olesen. The new EU manuals on sampling and diagnostic procedures and the role of the www.eurl-fish.eu web page.
- NJ Olesen. Options for survey for presence of *A.invadans* in EU in farmed and ornamental fish – discussion in plenum.
- NJ Olesen. Health categorisation of fish farms in Europe.
- S Kahns. Danish genotype Ia VHS viruses constitute a subgroup distinct from

isolates causing outbreaks in other European countries.
- S Kahns Results and outcome of the Inter-Laboratory Proficiency Test 2010.
- **C Fry.** Sporulation of *A. invadans.*
- SP Jonstrup**.** Pitfalls and challenges in development of real-time PCR diagnostic assays.
- HF Skall. Detection of HPR0 in Denmark and Criteria for diagnosis of ISA

15th International Conference on Diseases of Fish and Shellfish, 12-16 September 2011, Split, Croatia:

- SP Jonstrup, S Kahns, HF Skall, TS Boutrup, NJ Olesen. A global Taqman based real time RT-PCR assay suitable for surveillance and diagnosis of viral haemorrhagic septicaemia virus
- T Ito, J Kurita, HF Skall, Lorenzen N, NJ Olesen. Viral haemorrhagic septicaemia virus (VHSV) in rainbow trout: virulence variability within genotype Ib isolates.
- E Lorenzen, TE Kjær, NH Henriksen, I Dalsgaard, LH Andersen, J Nylen, SB Madsen, K Buchmann, N Lorenzen. Insufficient protection of rainbow trout against furunculosis by commercial vaccines under experimental conditions.
- N Lorenzen, JS Rasmussen JS, TE Kjær, E Lorenzen. DNA vaccination of turbot against viral haemorrhagic septicaemia virus (VHSV).
- Schönherz AA; Hansen MH; Jørgensen H; Berg P; Einer-Jensen K. Oral transmission of viral haemorrhagic septicaemia virus in juvenile rainbow trout.
- Schyth, BD; Jalali, SH; Kristensen, LB, Pedersen, FS; Lorenzen, N. Small gene regulating RNAs are themselves regulated during antiviral responses in infected rainbow trout.
- O Haenen, S Kahns, E Jansson, B Oidtmann, I Roozenburg-Hengst, *AM Lassen-Nielsen*, *HB Johansen*, A Aspán, T Hongslo, *J Andersen*, N Nicolajsen, NJ Olesen. Epizootic ulcerative syndrome (EUS): development and implementation of diagnostic methods.

Poster presentations

- SP Jonstrup, S Kahns, HF Skall, TS Boutrup, NJ Olesen. A taqman based real time RT-PCR assay suitable for worldwide surveillance and diagnosis of viral haemorrhagic septicaemia virus -
- SP Jonstrup, T Gray, NJ Olesen. FishPathogens.eu a new database in the research of aquatic animal diseases.
- Bela-Ong, D; Schyth, BD; Jørgensen, H; Hansen, MH; Henryon, M; Berg, P; Lorenzen, N. Correlation of mRNA and micro-RNA profiles and functional immune response in rainbow trout (*Oncorhynchus mykiss*) during infection with viral hemorrhagic septicemia virus (VHS) and in fish vaccinated with a DNA vaccine against VHSV
- Einer-Jensen, K; Biacchesi, S.; Stegmann, A; Bremont, M.; Lorenzen, N. Recombinant hybrid infectious hematopoietic necrosis virus (IHNV) carrying viral haemorrhagic septicaemia virus (VHSV) G or NV genes show different virulence properities.
- Larashati, S; Schyth, BD; Lorenzen, N. Inhibition of reporter genes by small interfering RNAs in cell culture and living fish
- Ito T, J Kurita, HF Skall, N Lorenzen, NJ Olesen (**2011**) Viral haemorrhagic septicaemia virus (VHSV) in rainbow trout: virulence variability within genotype Ib isolates.

DAFINET Workshop, IMMUNE RESPONSE IN EARLY DEVELOPMENTAL STAGES OF FISH, 2-3 November, 2011

- Bela-Ong, D; Schyth, BD; Jørgensen, H; Hansen, MH; Henryon, M; Berg, P; Lorenzen, N. Correlation of mRNA and micro-RNA profiles and functional immune response in rainbow trout (*Oncorhynchus mykiss*) during infection with viral hemorrhagic septicemia virus (VHS) and in fish vaccinated with a DNA vaccine against VHSV
- Einer-Jensen, K; Gautier, L; Rasmussen, JS; Lorenzen, E; Christensen, MB; Villanueva, SA; Martin, S; Evensen, Ø; Schyth, BD; Lorenzen, N Temperature influences the expression profiling of immune response genes in rainbow trout following DNA vaccination and VHS virus infection.
- Larashati, S; Schyth, BD; Lorenzen, N. Inhibition of reporter genes by small interfering RNAs in cell culture and living fish. Presented
- Lorenzen, E; Kjær, TE.; Henriksen, NH; Dalsgaard, I; Andersen, LH.; Nylen, J; Madsen, SB; Buchmann, K; Lorenzen, N. Insufficient protection of rainbow trout against furunculosis by commercial vaccines under experimental conditions.
- Schyth, BD.; Jalali, SAH; Kristensen, LB; Lorenzen, N. Gene regulatory mechanisms in infected fish.
- Stegmann, A; Biacchesi, S.; Bremont, M.; Lorenzen, N; Einer-Jensen, K. Search for genetic virulence markers in viral haemorrhagic septicaemia virus (VHS) using a reverse genetics approach

Joint Western Fish Disease Workshop & AFS fish Health Section meeting. Nanaimo, British Columbia, Canada, 2011

- Einer-Jensen, K; Gautier, L; Rasmussen, JS; Lorenzen, E; Christensen, M Black; Villanueva, SA; Martin, S; Evensen, Ø; Schyth, BD; Lorenzen, N. Temperature influences the expression profiling of immune response genes in rainbow trout following DNA vaccination and VHS virus infection.

Poster presentations

- Schyth BD; Jalali SAH; Kristensen LB, Pedersen, FS; Lorenzen, N. microRNA regulation in rainbow trout infected with a fish pathogenic rhabdovirus.

Annual EPIZONE meeting, Arnhem, The Netherlands, 2011.

- Schyth, BD; Jalali, SAH; Kristensen, LB; Lorenzen, N. Small regulatory RNAs of the RNA interference (RNAi) pathway as a prophylactic treatment against fish pathogenic viruses.

Poster presentations

- SP Jonstrup, T Gray, NJ Olesen. Fishpathogens.eu a new database in the research on aquatic animal diseases

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Scientific publications in peer-reviewed journals

- Bohle, H; Lorenzen, N; Schyth, BD.; Species specific inhibition of viral replication using dicer substrate siRNAs (DsiRNAs) targeting the viral nucleoprotein of the fish pathogenic rhabdovirus viral hemorrhagic septicemia virus (VHSV) In: Antiviral Research 90 (2011) 187–194
- Jørgensen HB, Sørensen P, Cooper GA, Lorenzen E, Lorenzen N, Hansen MH, Koop BF, Henryon M. General and family-specific gene expression responses to viral hemorrhagic septicaemia virus infection in rainbow trout (Oncorhynchus mykiss). In: Mol Immunol. 2011 Apr; 48(8):1046-58.

- Kahns, S.; Skall, H.F.; Kaas, R.S.; Korsholm, H.; Jensen, B.B.; Jonstrup, S.P.; Dodge, M. J.; Einer-Jensen, K.; Stone, D.; Olesen, N.J. European freshwater VHSV genotype Ia isolates divides 1 into two distinct subpopulations In: Diseases of Aquatic Organisms (in press)
- Lorenzen, E; Einer-Jensen, K; Rasmussen, J Skou; Christensen, M Black; Collet, B.; Secombes, C. J.; Lorenzen, N. The Protective Mechanisms Induced by a DNA Vaccine in Fish Depend on Temperature. In: Scandinavian Journal of Immunology, vol: 73(4), p. 392-392 (2011). Wiley-Blackwell Publishing Ltd.
- Scapigliati, G.; Buonocore, F.; Randelli, E.; Casani, D.; Meloni, S.; Zarletti, G.; Tiberi, M.; Pietretti, D.; Boschi, I.; Manchado, M.; Martin-Antonio, B.; Jimenez-Cantizano, R.; Bovo, G.; Borghesan, F.; Lorenzen, N; Einer-Jensen, K; Adams, S.; Thompson, K.; Alonso, C.; Bejar, J.; Borrego, J.J.; Alvarez, M.C. Cellular and molecular immune responses of the sea bass (Dicentrarchus labrax) experimentally infected with betanodavirus. In: Fish and Shellfish Immunology (28), p. 303-311 (2010).
- Schyth, B. D., Ariel, E, Korsholm, H and NJ Olesen (**2011**) Diagnostic capacity for viral haemorrhagic septicaemia virus (VHSV) infection in rainbow trout (*Oncorhynchus mykiss*) is greatly increased by combining viral isolation with specific antibody detection. In press in Fish and Shellfish Immunology – short communication.
- JE Jakobsen, J Li, PM Kragh, B Moldt, L Lin, Y Liu, M Schmidt, K Dahl Winther, B Dall Schyth, IE. Holm, G Vajta, L Bolund, H Callesen, A Lund Jørgensen, A Lade Nielsen, J Giehm Mikkelsen. Pig transgenesis by Sleeping Beauty DNA transposition. In: Transgenic Research (20), p.533-545 (2011).

Participation in international scientific collaborative studies

- 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. The public web site is http://www.epizone-eu.net/default.aspx.

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

- A 5 year 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 been finalized. The work included both basic fish immunology research and applied research and technical development for establishment of a platform of knowledge and tools for better disease prophylaxis in cultured fish and shellfish. Further information is available at the public project website www.IMAQUANIM.eu where a full list of scientific publications is available along with an informative brochure addressing readers outside the scientific field.

- A 3½-year national research project supported by the Danish Research Council focuses on "Identification of virulence markers in marine VHS virus and use in

diagnostics for aquaculture" using in vivo imaging of VHSV propagation in fish, and identification of virulence marker(s) in VHSV by generation and virulence testing of recombinant viruses. Once genetic elements of importance for virulence and/or risk of establishment of virulence have been identified, the information will be used to generate a diagnostic assays based on RT-PCR and gene sequencing for virulence typing of virus isolates. The developed assay will be evaluated by testing on a panel of VHSV isolates with known virulence and will subsequently be distributed to other national EC reference laboratories for extended evaluation. The public web site is http://www.dtu.dk/sites/VHSVIRULENCE.aspx.

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

- The 4-year national collaborative research project "Co-evolutionary genomics of fish resistance and virulence in an epidemic virus" based on funding from the Danish Research Council. This project seeks a solution to a problem for the expansion of Danish trout farming into the marine environment. Viral haemorrhagic septicemia (VHS) is a viral disease that causes outbreaks with up to 90% mortality in rainbow trout, and the virus is commonly found in wild populations of fish in the coastal waters. We will identify the process of adaptation to the fish host that makes the virus capable of causing epidemic outbreaks in rainbow trout and use the trouts own genetic variants in combination with targeted vaccine development to cope with this adjustment. We can achieve this through a combination of novel technologies that combine genotypning of genetic markers in coding DNA (SNP markers) and regulatory gene sequences (miRNA) with vaccination and infection experiments where we measure gene activity throughout the genome and gene activity in immunological key components. This gives us a unique level of insight into the mechanisms that provide resistance against the virus and effective protection from the vaccine. It is possible to combine these technologies because we have established collaboration between institutions, which have experience in vaccine development, infection experiments, genomic and genetic analysis. Besides the National Veterinary Institute department in Århus those are Aarhus University, University of Victoria in Canada and the University of Washington, USA. The public web site is https://djfextranet.agrsci.dk/sites/fishgen/public/Pages/front.aspx

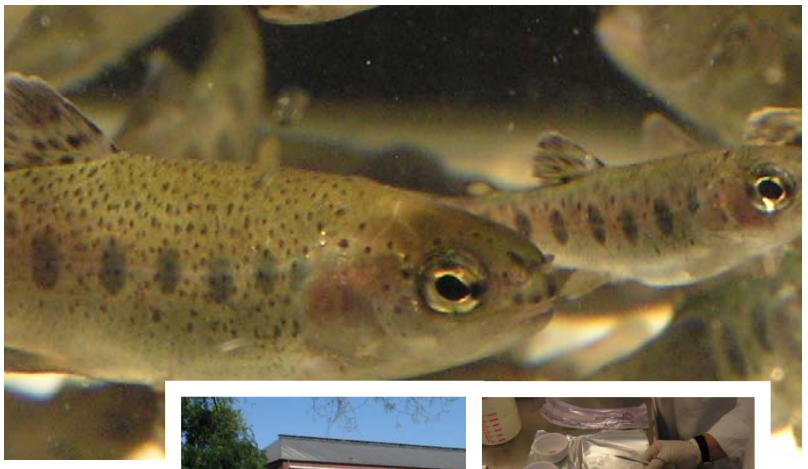
- Delivery of small interfering RNAs (siRNAs) for treatment of viral disease in fish aquaculture – a Ph.D. study funded by the Islamic Development Bank (IDB). The aim of this study is to establish novel delivery strategies for small interfering RNAs including viral and nonviral methods in fish – aiming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

- The 3½-year national collaborative research project "Improved vaccination strategies in marine aquaculture" focuses on fish from the marine environment as they represent an important source for healthy animal food. In Denmark and other countries future plans include considerable expansion of marine aquaculture. This project aims at an integrated strategic research approach towards ensuring healthy fish and fish products from Danish marine aquaculture. By uniting the expertise of 4 research institutes and 4 private companies/organizations (including two international companies) the project will address fish health in scientific and applied terms aiming at improved strategies for disease prophylaxis by vaccination. The project will focus on rainbow trout being the dominating aquacultured fish species in Denmark. For analysing if fish toxic algae may predispose the fish for disease even if vaccinated, the effect of such environmental stress elements will also be studied experimentally. For optimizing the effect of licensed vaccines, these will be tested in new in new time schedules in field trials at 2-3 selected marine fish farms and the immuneresponse of the fish to vaccination and disease will be studied in detail. The overall applied aim is to integrate the obtained results into a new recommendation for disease prophylaxis in marine aquaculture. The project will expire in 2011, but has formed a knowledge platform for an extended new project with increased international collaboration. The public web site is http://www.dtu.dk/sites/marinvac.aspx

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

European Union Reference Laboratory for Fish Diseases

National Veterinary Institute, Technical University of Denmark, Aarhus



15th Annual Meeting of the National Reference Laboratories for Fish Diseases

Aarhus, Denmark May 26-27, 2011

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

Indhold

Introduction and short summary

26-27 May 2011 the 15th annual meeting of the National Reference Laboratories for fish diseases was held in Aarhus, Denmark, at a rented nearby facility of Aarhus University. A total of 52 participants from 33 countries attended over the two day period. There were four sessions with a total of 33 presentations, 9 of which were given by invited speakers.

The scientific programme of the Annual Meeting was diverse and covered many topics of current interest. The meeting was opened with the traditional session on update of fish diseases in Europe, where once again participants from the member states had the opportunity to present new findings from their home countries. Initially an overview of the disease situation and surveillance in Europe 2010 were provided on the basis of the results from the survey and diagnosis questionnaire. Results of a questionnaire send to fish-pathology experts in the Mediterranean area were the basis of a following talk providing an update on fish disease situation in Mediterranean aquaculture. Subsequently, needs and requirements for rapid and efficient responding to emerging diseases were provided followed by an update on the disease situation in Norway. This was followed by a talk on IPNV distribution in Austrian aquaculture 1993-2010 and a talk on the VHSV eradication program in Denmark. Afterwards, three talks on molecular epidemiology from Denmark, Poland and Germany, respectively were presented. The session was finished with a talk on *vibrio* infections in Dutch aquaculture.

The session on technical issues related to sampling and diagnosis were started by a presentation on the new EU manuals on sampling and diagnostic procedures and the role of the www.eurl-fish.eu web page. Next were three talks on diagnostic methods; one for identification of EHNV and two for identification of *Aphanomyces Invadans*; followed by a plenum discussion on options for surveying for EUS in EU in farmed and ornamental fish. After a short coffee break a talk on health categorisation of fish farms in Europe 2010, based on collected answers of the questionnaire on surveillance and diagnostic, was presented. Then a talk high lightening some of the challenges concerning the implementation of the new legislation on aquatic animal health surveillance in Europe, was provided. The session was ended with three presentations, one on a new real time LAMP assay for KHV detection, one on the origin of diagnostic fish cells and at last one on challenges in development of real-time PCR diagnostic assays.

In the evening a banquet dinner was held at Restaurant “Koch”, located at the harbour side, downtown Aarhus.

The last day was opened by an update session on scientific research. The first part of this session was focussed on ISA. Initially, a chronicle of events relating to a small scale ISA epidemic situation was described for a specific Norwegian area containing several salmon farms. Subsequently, an overview was provided on ISA and HPR0 strains, followed by presentation of a study on HPR0 appearance in fresh water at the Faroe Island. The last talk on ISA was on HPR0 detection in Denmark in combination with a comparison of the criteria for diagnosis of ISA as described by EU and by OIE. The ISA topic was ended by a discussion in plenum on how the criteria for diagnosis of ISA should be in relation to an identification of HPR0. At the end of this session a talk on a PD-vaccine that efficiently reduces severity of disease outbreak in Norwegian aquaculture was presented. This was followed by a talk on an Epizone extension project on “Management, control and surveillance of VER in aquaculture” and finally a talk on serological tests for specific antibody detection against VERv ended the session.

The annual meeting ended with the traditional update from the EURL. The results of the two proficiency tests, PT1 and PT2, 2010 were presented. Furthermore, the annual training course provided by the EURL was announced and topics were discussed. A report of EURL activities from year 2011 was given. Finally, proposals on the EURL work plans for 2012 were discussed.

Minutes from the meeting were taken by Helle Frank Skall, Søren Peter Jonstrup and Torsten Boutrup, 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 discussions. 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 Nicole Nicolajsen, Søren Kahns, and Niels Jørgen Olesen, with the help from the rest of the fish disease section at DTU Vet.

The meeting next year is tentatively planned to be in week 21, most likely from 23-25 May 2012 but more details will follow.

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

Århus, 07 September 2011

Niels Jørgen Olesen and Søren Kahns

Programme

Thursday May 26th
Annual Meeting of the National Reference Laboratories

8:45 – 9:15 **Registration and welcome address**

9:15 – 09:30 *Welcome Address and announcements - Søren Kahns and Niels Jørgen Olesen*

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

Chair – *Olga Haenen/Brit Hjeltnes*

9:30 – 9:50 Overview of disease situation in Europe - *Niels Jørgen Olesen*

9:50 – 10:10 Update on fish disease situation in the Mediterranean area – *Giuseppe Bovo*

10:10 – 10:35 Emerging diseases – an overview – *Stephen Feist*

10:35 – 10:55 Update on the Fish Disease Situation in Norway– Brit Hjelt*nes*

10:55 -11:10 Distribution of IPNV in Austria 1993-2010 - *Oskar Schachner*

11:10 - 11:35 Coffee break

11:35 -11:50 Surveillance and eradication of VHS in Denmark – *Stig Mellergaard*

11:50 - 12:15 Danish genotype Ia VHS viruses constitute a subgroup distinct from isolates causing outbreaks in other European countries – *Søren Kahns*

12:15 – 12:30 Characterisation of Polish VHSV isolates (2005-9) – *Marek Matras*

12:30 – 12:45 Characterisation of German IHNV and VHSV isolates – *Heike Schutze*

12:45 – 12:55 Vibrio infections in Dutch fish culture – *Olga Haenen*

12:55 – 13:45 Lunch

SESSION II: Technical issues related to sampling and diagnosis

Chair – *Stephen Feist/Richard Paley*

13:45 - 14:05	The new EU manuals on sampling and diagnostic procedures and the role of the www.EURL-fish.eu web page - *Niels Jørgen Olesen*
14:05 - 14:25	Diagnostic methods for identification of EHNV and other ranaviruses – *Heike Schuetze*
14:25 - 14:55	Epizootic ulcerative syndrome (EUS): Development and implementation of diagnostic methods – *Olga Haenen*
14:55 – 15:10	Sporulation of *A. Invadans* - *Christian Fry*
15:10 – 15:20	Options for survey for presence of *A. Invadans* in EU in farmed and ornamental fish – discussion in plenum – *Niels Jørgen Olesen*
15:20 - 15.50	*Coffee break*
15:50 – 16:10	Health categorisation of fish farms in Europe – *Niels Jørgen Olesen*
16:10 – 16:30	Challenges regarding implementation of the new legislation on Aquatic Animal Health Surveillance in Europe – *Britt Bang Jensen*
16:30 – 16:45	Development and assessment of a real time LAMP assay for KHV– Richard Paley
16:45 – 17:00	Fish cells – some remarks to induce discussion – *Heike Schutze*
17:00 – 17:15	Pitfalls and challenges in development of real-time PCR diagnostic assays – *Søren Peter Jonstrup*
18:30 – 21:30	***BANQUET DINNER at Koch'erier***

Friday 27 May
Annual Meeting of the National Reference Laboratories Continued

SESSION III Scientific research update

Chair – *Søren Kahns*

9:00 – 9:30	Infectious salmon anaemia (ISA) in Norwegian salmon farming, a chronicle of events relating to a small scale epidemic in the Southern and middle parts of Troms county 2007- 2010 – *Einar Karlsen*
9:30 – 9:50	Infectious salmon anaemia and HPR0 strain – an overview – *Eann Munro*
9:50 – 10:10	Detection of low-pathogenic Infectious Salmon Anemia Virus (ISAV-hpr0) in freshwater in the Faroe Islands – *Debes Christiansen*
10:00 – 10:20	Detection of HPR0 in Denmark and Criteria for diagnosis of ISA– *Helle Frank Skall*
10:20 – 10:30	PD-vaccine is effective in reducing severity of disease outbreaks in Norwegian aquaculture – B*ritt Bang Jensen*
10:30 – 10:50	Extension of Epizone – Management, control and surveillance of VNN in aquaculture - *Giuseppe Bovo*
10:50 – 11:05	Serological tests for specific antibody detection in European Sea bass (*D. labrax*) against VERv. - *Niccolo Vendramin*
11:05 – 11:30	Coffee break

SESSION IV Update from the EURL

Chair – *Niels Jørgen Olesen*

11:30 – 11:45	EURL activities in 2010 - *Niels Jørgen Olesen*
11:45 – 12:00	EURL workplan for 2010; Ideas and plans for 2011 - *Niels Jørgen Olesen*
12:00 – 12:05	EURL training course 2011 and Request for ideas for 2012 – *Søren Peter Jonstrup*
12:05 – 12:25	Results of the proficiency test, PT1, 2010 – *Søren Kahns*
12:25 – 12:40	Results of the proficiency test, PT2, 2010 – *Søren Kahns*
12:40 – 13:00	Next meeting and end of 15th Annual Meeting - *Niels Jørgen Olesen*
13.00	***Sandwiches and goodbyes***

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

Chair*: Olga Haenen/ Brit Hjeltnes*
Minutes: *Torsten Boutrup/ Søren Peter Jonstrup*

OVERVIEW OF THE DISEASE SITUATION AND SURVEILLANCE IN EUROPE IN 2010

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

Abstract:
The Questionnaire on Surveillance and Diagnosis (S&D) which is collated annually is the only comprehensive overview of the disease situation in aquaculture in Europe. The information has been made available on the EURL web site (www.eurl-fish.eu), where all raw data can be obtained. The S&D have evolved over the years, for 2010 it comprise 3 parts:

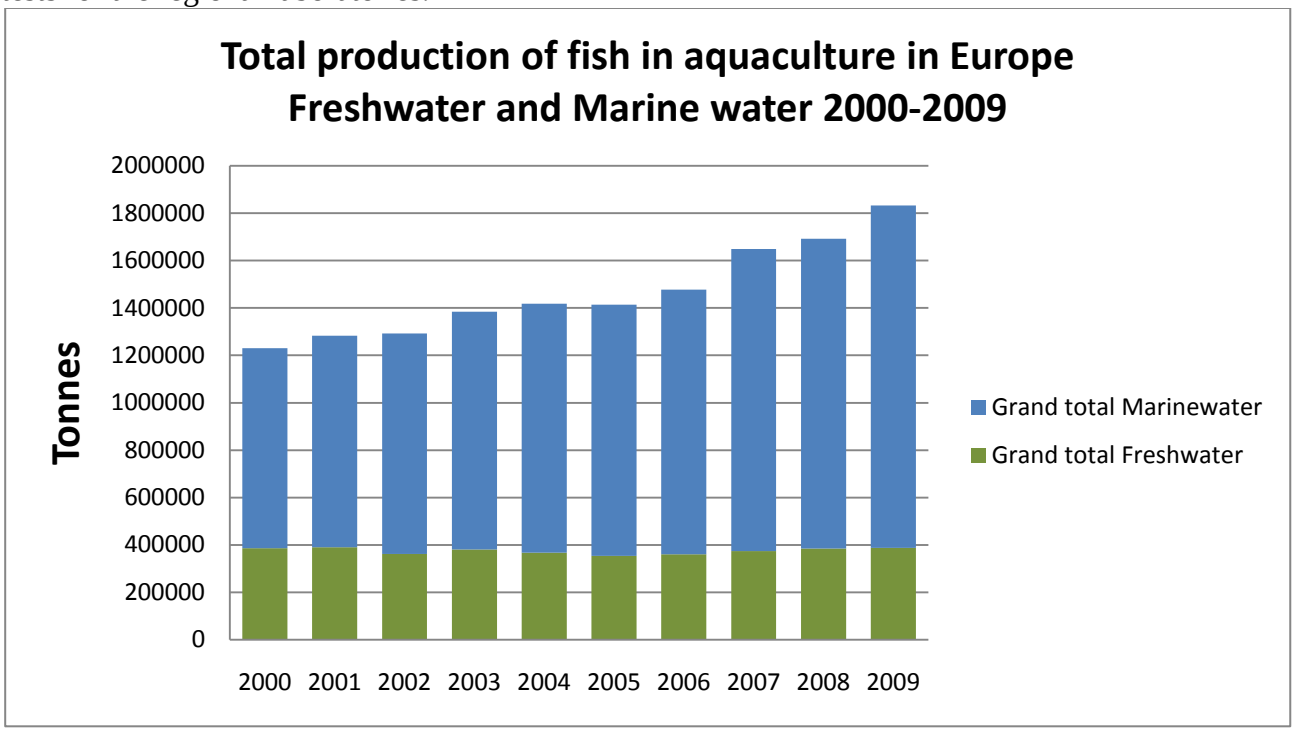
1. General data on production type and size, health categorization of fish farms according to Council Directive 2006/88/EC, and information on national surveillance programmes.
2. Epidemiological data on the disease situation in each Member State with focus on the listed diseases but also including other diseases of interest.
3. Laboratory data from the NRLs and other laboratories, including number of samples examined, diagnoses of fish diseases made

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 2009 is almost the same as in 2008 and has risen after a decrease from 2003-2006. Data from 2010 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.
The Atlantic salmon production is mainly at the Atlantic Sea coast line of the Northern European countries. Rainbow trout are produced in most European countries, whereas carp production is mainly in the Eastern part of Continental Europe. The production of sea bream, sea bass and tuna have increased significantly in the Mediterranean countries. The production in Turkey raised from 66.000 to 76 000 t last year and Turkey has become one of the countries included with the highest production. Among other fish species of interest are pike-perch (408t), eel (6391t), sturgeon (3789t), cod (22729t), turbot (9238t), and halibut (1806t).

Data on the health categorisation of fish farms will be given in a later presentation.

Concerning the epidemiological data, obviously, there is still a severe underreporting of VHS and IHN in many countries. For VHS the infection status in only known for 36% of the farms, for IHN the situation is known in 38% of the farms. While for KHV the disease situation is unknown on 94% of the farms! For farms producing Atlantic salmon, the infection status for ISA is known for nearly all farms. ISA is still a problem in Norway. The finding of ISAvirus HPR0 poses some problems regarding the health categorisation of salmon farms.

Many countries have surveillance programmes for SVC (20 of 29 countries), BKD (16 of 32 countries), IPN (22 of 32 countries) and *Gyrodactylus salaries* (8 countries), for which they are seeking "additional guaranties" according to §42 in CD 2006/88/EC. The number of farms in the programmes varies from very few farms to many farms.
There are very large differences between countries on how many samples are tested on cell cultures, ranging from < 100 to several thousands. PCR is coming up in many countries, and the large number of PCR-tests conducted in some countries mostly reflects the KHV and ISA testing.
About a third of the countries have regional laboratories, and of these countries, 8 of 11 organize ring tests for the regional laboratories.



Minutes:

The size of production in Europe seems to be stable with a slight increase especially for saltwater species such as cod, turbot and halibut. Within the EU, central plans are to increase aquaculture production. Data can be somewhat misleading especially on farm size since no data has been reported for France and Hungary. Especially for KHV and ISA many farms have been put in category III, for ISA this is to a large extent due to Norway has decided to categorise all farms as III and this also is the case for VHS in Norwegian saltwater facilities rearing rainbow trout. For other diseases than VHS, IHN, KHV, SVC and ISA main problems in northern part of Europe is IPN, HSMI, CMS, PD and ERM. In mid/central Europe ERM, RTFS and furunculosis seems to be the most important diseases, and Noda virus is a pathogen of great importance in Southern Europe/Mediterranean area. For the listed diseases there seems to be a significant underreporting especially on VHS.

Questions:

No questions.

UPDATE ON FISH DISEASE SITUATION IN THE MEDITERRANEAN AREA

Bovo G.
Istituto Zooprofilattico Sperimentale delle Venezie
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e-mail : gbovo@izsvenezie .it

Abstract:
Despite several new species have been introduced, since several years, in Mediterranean aquaculture, in order to diversify the market, European sea bass (*Dicentrarchus labrax*) and gilthead sea bream (*Sparus aurata*) are still the most widely farmed species. The production of these two species represents more than 95% of the total production while the remaining is obtained by different promising candidates species, including Senegalensis sole (*Solea senegalensis*), meagre (*Argyrosomus regius*) and northern blue fin tuna (*Thunnus thynnus*), for which some hatcheries have recently started experimental reproduction. The farming of sea bass and sea bream shows a continuous upward trend despite the presence of several important diseases. Viral encephalopathy and retinopathy (VER) still plays a key role in some areas where, in critical situations, mortalities ranging from 30-40% are usually observed in sea bass cages. Moreover in recent years serious epizootics, affecting sea bream larvae, previously considered a resistant species, have been reported.
Marine Flexibacteriosis, caused by *Tenacibaculum maritimum*; Vibriosis, caused by *Listonella anguillarum* and Pasteurellosis caused by *Photobacterium Damsela subsp. piscicida* are considered the major bacterial diseases.
Gill flukes (*Diplectanum aequans and Sparicotyle chrysophrii*); gill crustacea (*Ceratotoa oestroides*) and traditional "old" protozoans (*Cryptocarion irritans and Amyloodinium ocellatum*) can cause high mortalities particularly in ponds, if not properly and promptly treated.
Among the issues still unresolved Winter Syndrome, affects mainly 1-year-old sea bream causing mortalities ranging from 5-15%. The therapeutic treatment addresses great attention to the diet, especially at the end of Summer and the approaching winter season.
Trout farming which is a well developed industry in some regions bordering the Mediterranean may be affected by several biological risks.
Among viral diseases viral haemorrhagic septicaemia (VHS) is certainly the most important problem causing serious losses in those areas characterized by low water temperature.
Rainbow trout fry syndrome (RTFS) is responsible for significant mortalities in salmonids, during juvenile stages, particularly if not treated promptly. Lactococcosis, due to *Lactococcus garvieae* considered in past years an emerging or re-emerging problem, seems to be now, since a couple of years, in a regressive phase, possibly due to the greater availability of water.
Rainbow trout gastroenteritis syndrome (RTGE), is considered, in some regions, an emerging problem. Mortality is generally low (< 5%) but feed intake and growth performances are significantly affected. The diagnosis is based mostly on clinical signs and detection of the Gram + *Candidatus arthromitus* which has been tentatively associated to the disease.

Minutes:

Compared to previous questionnaires Tunesia has been attributed with an aquaculture production. Marbled trout even though not listed as a susceptible to VHSV is so. In the Mediterranean area 100.000 tons of salmonid species and 300.000 tons of marine species are produced and the presence of certain diseases differs upon geography e.g. mycobacteriosis is considered a problem in Israel. In general some old diseases still affects the production with some modifications, for instance RTFS

seems to affect larger fish than fingerlings and lactococcosis seems to be in regress these years and Noda virus infection has been seen to be able to have serious affect on sea bream larvae. New or unresolved problems include abnormal carp mortality and winter syndrome, the latter might be a combination of several factors, however, *Pseudomonas anguilliseptica* is often isolated in connection with the disease. Furthermore, rainbow trout gastro enteritis RTGE seems to be of increased importance in some regions. An increasingly important aquaculture species is the northern blue fin tuna. Due to the novelty of this production and the management of this production new disease situations might be seen in coming years.

Questions:

Olga Haenen: About the abnormal carp mortality, have you looked for picorna virus?

Giuseppe Bovo: This has not been detected.

Athanasios Prapas: You say that mortality due to Noda virus as high as 20 % can be seen in sea cages with larger specimens. Is that right? I have only seen this in fry and smaller specimens.

Giuseppe Bovo: Yes there are several reports on high mortality in larger fish due to Noda virus.

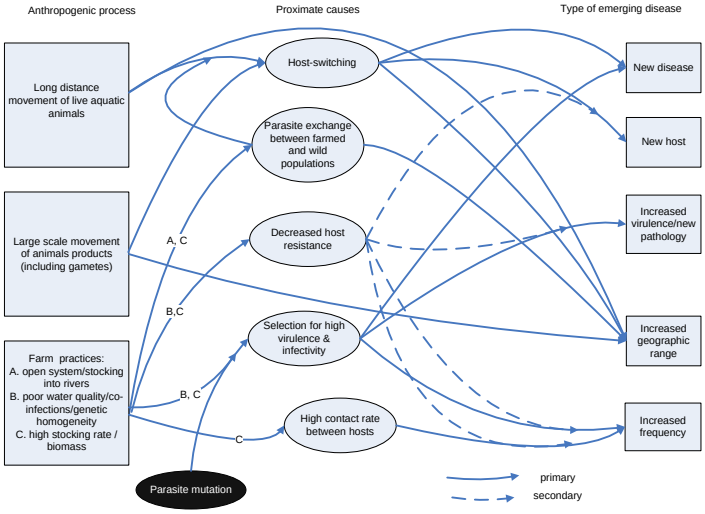
EMERGING DISEASES – AN OVERVIEW

S. W Feist, M Thrush, P Dunn & E Peeler
Cefas Weymouth Laboratory, Barrack Road, The Nothe, Weymouth, Dorset, DT4 8UB. UK

Abstract:
An emerging disease can be defined as 'A newly recognised *infection* resulting from the evolution or change of an existing *pathogenic agent*, a known infection spreading to a new geographic area or population, or a previously unrecognised pathogenic agent or a *disease* diagnosed for the first time and which has a significant impact on aquatic animal or public health' (OIE Aquatic Animal Health Code, 2010). There is a requirement of EC 2006/88 (report & control), OIE (notify) and the new EC Animal Health Regulations for addressing the detection of new diseases. This presentation highlights the regular occurrence of new and emerging diseases in aquaculture, the possible drivers for their emergence such as translocation of non-native species and the processes underpinning disease emergence (e.g. host switching, high contact rate between hosts, pathogen mutation, selection for high virulence and infectivity, lowered host resistance and parasite exchange between farmed and wild populations. An approach for the analysis of global information using electronic sources is described together with an analysis of trends in emerging diseases for the previous eight years. Key messages are that:

Emerging diseases are a natural component of ecosystems, they continue to be a significant threat to aquatic systems
Need for vigilance
Critical need to experienced diagnosticians, especially histopathologists
Need to develop a robust case definition and establish aetiology as soon as possible
Need to understand the drivers for disease emergence
Multidisciplinary approach essential
Rapid response required under EC 2006/88 (report & control) and the new EC Animal Health Regulations

Hypothesised relationships between anthropogenic processes and types of disease emergence in freshwater aquatic animals



From: Peeler, E. J. & Feist, S. W. (2011) Human intervention in freshwater ecosystems drives disease emergence. *Freshwater Biology*, 56, 705-716

Minutes:

To address problem associated with emergence of new diseases, it is of importance to understand the drivers for the appearance of increased potential in an aetiological agent to produce disease, and to acknowledge that this is a naturally occurring process which continually must be taken into consideration to be present and be addressed by fast and rational reporting and swift diagnostic work. Further diagnostic works needs to be multidisciplinary; in this sense histopathology is of importance to establish case definitions on which aetiology, epidemiology and molecular data can be coupled.

Questions:

Stig Mellergaard: The movement of live aquatic animals, especially ornamental fish and molluscs seems to be one of the most serious routes of introducing new disease to an area.

Stephen Feist: I agree about this.

Britt Hjeltnes: Are you able to recall any emerging disease situation that has been handled efficiently?

Stephen Feist: I think that the UK handling of the VHS outbreak was dealt with in the correct manner.

Ole Bendik Dale: Interesting cases where histopathology shows a lesion pattern that indicate a new disease situation often is missed because upon contacting the farmer it turns out that the fish has been destroyed. In some situation where it could be of importance to catch emerging disease situation might be missed due to lack in communication.

UPDATE ON THE FISH DISEASE SITUATION IN NORWAY

Brit Hjeltnes,
Norwegian Veterinary Laboratory

Abstract:
In 2010, 944 000 tons (harvest statistics) of Atlantic salmon, along with an estimated 55 700 tons of rainbow trout, 19 700 tons of cod, 1800 tons of halibut and 1500 tons of other species e.g. coalfish, Arctic char and halibut, were produced (Kontali Analyse AS). Production related losses remain significant, and a large proportion of these losses are related to disease and production conditions.
Pancreas Disease (PD) has been a dominant disease in salmon farming in recent years. Both the industry and the authorities have worked hard towards control of this disease. During 2010 a "min-epidemic" was experienced in the Ry Region, resulting in an increase in the total number of outbreaks from the previous year. Despite this, losses to PD were generally lower. Further spread of the disease northwards was avoided and it appears that the disease has been eradicated from an area in Northern Norway which had experienced repeated outbreaks in recent years.
The number of infectious salmon anaemia (ISA) outbreaks continued to decrease in 2010, although a core area for this disease still exists in Northern Norway.
Infectious Pancreatic Necrosis (IPN) is no longer a notifiable disease, but the losses associated with this disease were again high in 2010, with aggressive outbreaks in some regions. For this disease and the "new" viral diseases like heart and skeletal muscle inflammation (HSMI) and cardiomyopathy syndrome (CMS), the possibility of coordinated eradication strategies should be considered.
AS in 2009, the major fish health challenge during 2010 was again salmon lice infestation. Problems of reduced sensitivity and development of resistance to treatment have increased and as a consequence large numbers of lice were recorded throughout the autumn. Successful salmon louse treatment is dependent on coordinated control strategies requiring extensive cooperation throughout the Norwegian aquaculture industry. While new medications are necessary, they will only provide a breathing space for establishment of other sustainable control strategies. Increased use of wrasse will constitute an important element in such strategies, but will also increase the possibility of other types of disease and disease transmission. Although a vaccine should be an important element in salmon louse control in the longer term, there remains much uncertainty related to both developmental time and effect.
A public surveillance system is under development, and its efficiency will greatly depend on reports of possible resistance development and treatment failure by the fish health services and the industry in general.

Minutes:

This overview is from the yearly report on the disease situation on salmonid species and other marine species in Norway. The report can be found on the Norwegian Veterinary Institutes homepage. HSMI is widespread constituting low to moderate mortality; the aetiological agent piscine reovirus is commonly detected also in healthy fish. CMS is seen in large fish with mortality rates commonly up to 20 %, in 2010 some cases with high mortality were recorded. In conjunction with CMS the new toti virus, piscine myocarditis virus; is found only in diseased fish. The high impact of IPN virus is seen especially in larger fish, the general absence of disease in fry and fingerlings might be due to the possibility for better management procedures in hatching facilities e.g. manipulation with water temperature. Increased gill problems are seen associated with microsporidian parasites. In 2010 two new findings of *Gyrodactylus salaris* were recorded in wild salmon populations. Major health issues in cod are *Fransicella*, *Vibrio* and atypical *Aeromonas salmonicida* infections. In halibut atypical

Aeromonas salmonicida infections are a problem. Wrasse is used as cleaner fish in the battle against sea lice, 30 million of these is expected to be used yearly in near future disease problems that has been reported in these fish is *Vibrio* infections and infections with atypical *Aeromonas salmonicida.*

Questions:

Eann Munro: What is done with the wrasse after use, do they die, are they released or re-used?

Brit Hjeltnes: No definitive strategy. Many will die in cages ant the rest are often released, re-used. Management strategies are getting higher priority as the number of wrasse used is increasing.

Stephen Feist: Your data on the cod production seems to be in opposition to Niels Jørgens presentation where there was a slight increase in cod production and expectation of higher production. Is this due to *Fransicella* infections?

Brit Hjeltnes: The Norwegian production has stabilised at a lower level, this is both due to *Fransicella* and to low market prices.

Richard Paley: For IPN there has been significant progress in selective breeding for resistance – have you seen any had any visible impact of this on the incidences of disease outbreaks?

Brit Hjeltnes: There are eggs available claimed to have increased resistance. However this has not been confirmed by epidemiological data.

DISTRIBUTION OF IPNV IN AUSTRIA 1993-2010

Oskar Schachner, Andrea Dressler, Hatem Soliman, Mansour El-Matbouli
Fishmedicine and Livestock Management
Dept. for Farm Animals and Vet. Public Health
University of Veterinary Medicine
Vienna, Austria

Abstract:

According to the laboratory data covering a period of 18 years IPNV is spread over the whole area of Austria at moderate density. The annual proportion of IPNV-infected facilities never exceeded 16%. By means of 2-4 fish cell lines the virus has been isolated either from visceral organs or gonadal products of mainly clinically healthy fish belonging to 6 salmonid species as well as from grayling and 2 cyprinid species. Most of the isolates exhibited equal infectivity in the different cell lines. Only some of them produced different titers in epitheloid and fibroblastoid cell morphotypes. CHSE-214 cells revealed to be most susceptible to the whole collection of aquabirnaviruses.
A phylogenetic analysis of VP2 gene sequences extending over a 1180 bp segment revealed marked diversity between some of the isolates, which could not be related to different host species or cell culture preferences.

Minutes:

When performing VHSV and IHNV surveillance on cell culture in Austria since more than 18 years all samples from salmon from fish eliciting a CPE are tested for IPNV as well. This practice provides a general insight into the prevalence of IPNV in Austria. IPNV was isolated in 2-4 cell lines and identified using TEST-LINE ELISA and/or BIOX IFAT. According to host species and the proportion of facilities tested positive for IPNV brook trout seemed to be the most susceptible species, rainbow trout intermediate and brown trout the least susceptible. Most of the isolates grew equally well on BF-2, RTG-2, EPC and CHSE cells. Some of them displayed preferences for a distinct cell line. Generally CHSE cells seemed to be most susceptible to the whole range of aquabirnaviruses. Even though CPE appeared sometimes later, titres became highest in CHSE cells. Independent of the fish host species and the cell line used for isolation, phylogenetic analyses revealed significant differences between isolates. The isolates fell in two VP2 groups with as little as 20% identity between groups for some isolates. Some isolates fell into a non-typable group.

Questions:

Guiseppe Bovo: Are there any information on the presence of IPNV in hatcheries?

Oskar Schachner: There is only limited information.

SURVEILLANCE AND ERADICATION OF VHS IN DENMARK

Stig Mellergaard
Danish Veterinary and Food Administration, Division on Animal Health
Denmark

Abstract:

Denmark was granted co-financing from the European Fisheries Fund for an eradication programme for VHS in 2008. The programme was initiated in spring 2009. Apart from a single VHS outbreak in February 2009, just before the start of the eradication programme, no new outbreaks have been observed since then. Hence, Denmark has been free of VHS for more than two years.
A summary of the basic principles in the eradication programme will be presented.

Minutes:

254 Danish fresh water fish farms are category I for VHSV. The marine farms (28) are put in category III to prevent VHSV spreading from marine wildlife to inland farms. 61 inland farms are in category II and are undergoing an eradication program to obtain category I status. Final VHS-eradication program runs from 2009-2013 and is funded by the European Fisheries Fund after national priority. Initially, risk farms were fallowed and wild rainbow trout in risk area caught by electro-fishery. If a new VHS outbreak should occur, a mandatory and immediate stamping-out with compensation will be carried out on the infected farms/sites. So far the project has been very successful and spending has been far below budget.

Questions:

No questions.

DANISH GENOTYPE IA VHS VIRUSES CONSTITUTE A SUBGROUP DISTINCT FROM ISOLATES CAUSING OUTBREAKS IN OTHER EUROPEAN COUNTRIES

S. Kahns[1*], H.F. Skall[1], R.S. Kaas[2], B. Bang Jensen[3], S.P. Jonstrup[1], D. Stone[4], N.J. Olesen[1]
[1] *National Veterinary Institute, Technical University of Denmark, Aarhus, Denmark*
[2] *National Food Institute, Technical University of Denmark, Kgs Lyngby, Denmark*
[3] *Norwegian Veterinary Institute, Oslo, Norway*
[4] *CEFAS Weymouth Laboratory, Weymouth, Dorset, UK-England*

Abstract:

Viral haemorrhagic septicaemia (VHS) is causing significant economical losses in European rainbow trout production. The disease is caused by a rhabdovirus (VHSV). The virus can be divided into four genotypes with additional subgenotypes of which the main source of outbreaks in European rainbow trout farming is caused by genotype Ia isolates. Danish aquaculture has been considered endemic infected with VHSV since the first outbreak of the disease was observed in the 1950's. The number of infected rainbow trout farms was at its highest level displaying ~ 400 infected farms in the mid 1960's, after when the number of infected farms have been significantly reduced. A, hopefully, final eradication program, approved by the European Union, was initiated April 1, 2009 with no outbreaks since then. If the eradication plan is successful, Denmark will be regarded as EU category 1 (officially declared free of VHS) on April 1, 2013.
In order to characterize the population of VHSV in Danish aquaculture we collected strains causing outbreaks in the period 2004 – 2009. Phylogenetic analyses using full length G-gene sequences revealed that all collected isolates belong to the group of genotype Ia isolates. Interestingly, the phylogenetic analyses revealed that the group of genotype Ia isolates can be divided into two subgroups. The group that we designate Ia-1 consists mainly of Danish isolates and seems to have evolved in the endemic infected Danish water systems. Furthermore, the Ia-1 subgroup apparently consists of several sub-clades of which some have been eradicated whereas others have developed into those that were found up until 2009. The other subgroup designated Ia-2, consists mainly of isolates causing outbreaks in rainbow trout farms in continental European countries other than Denmark.
There are a few non-Danish isolates present in the Ia-1 group, as well as there are a few Danish isolates present in the Ia-2 group. This study presents how molecular tracing can be used to elucidate virus transmission pathways between international as well as regional fish farms, and will be important in future work aiming at preventing future spreading of fish pathogens.

Minutes:

All sampled Danish VHSV isolates from 2004-9 were sequenced. All isolates sampled from rainbow trout were characterized as genotype Ia isolates. Phylogenetic analyses including isolates from all over Europe showed that the most of the Danish isolates clusters into a defined subgroup. Some of the Danish subclades of viruses are eradicated from the environment. However, the analyses show that infective VHSV might survive for up to four years between causing outbreaks.

Questions:

Guiseppe Bovo: Did you see any influence related to fish species?

Søren Kahns: We have mainly looked at isolates from rainbow trout. In general the geographic location seems more important than the fish species.

Olga Haenen: Will your sequences be put on fishpathogens.eu?

Søren Kahns: Yes, after publication.

Katja Einer-Jensen: Maybe strains were eradicated because they were stamped out?

Søren Kahns: Yes, it is possible. In 2009 all farms were stamped out at the same time. However, in earlier times, stamping out was not necessary performed synchronous and we cannot tell exactly.

Stephen Feist: Have you looked at your data for the presence of a molecular clock?

Søren Kahns: We have not looked into this in this study.

Katja Einer-Jensen: In a previous publication we addressed the question on when the different genotypes separated from a molecular clock perspective.

CHARACTERISATION OF POLISH VHSV ISOLATES (2005-2009)

Reichert M. [2], **Matras M.** [1], Kahns S.3, Jonstrup SP[3], Olesen NJ[3]
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Abstract:

Abstract: During 2005 – 2009 viral haemorrhagic septicemia virus (VHSV) caused substantial losses in Polish rainbow trout farming. Phylogenetic analyses were performed on 24 selected VHS outbreaks that had occurred in 19 farms. The full G-gene sequences of the VHSV isolates were successfully obtained and analyzed together in with the sequences available in the database www.fishpathogens.eu. Phylogenetic analyses were conducted by the multiple alignment method using the CLUSTAL W program. The analyzed sequences originated from isolates of VHSV collected in the years 2005 – 2009 at different farms in Poland where VHS cases were reported. Eight of these isolates were obtained from three farms (four isolates from two farms collected 2 consecutive yearswith and 4 isolates from one farm collected four consecutive years). The alignment results revealed that some isolates were identical despite quite long distances between the farms from where they were isolated. All remaining VHSV isolates showed varying degree of differences in nucleotide sequence. Altogether it was concluded that the Polish VHSV isolates all belong to genotype Ia like all other freshwater European isolates. But within the Ia group it was possible to divide the isolates into two subgroups: a very homogenous Ia-1 (Pol I) group and a more differentiated Ia-2 (Pol II) group.

Minutes:

Phylogenetic study of 24 VHSV isolates from Poland. Isolates could be divided into two subgroups. Isolates group closely with several German isolates revealing that VHS transmissions across the border between the two countries might occur.

Questions:

Britt Bang Jensen: In the farm where you had reoccurring outbreaks was it the same isolate reappearing or different introduction?

Marek Matras: The 3 of 4 were very similar and could be the same isolate reoccurring while the last was quite different. We will sequence a few more isolates to have a better idea on what is going on.

Guiseppe Bovo: This is a general question to the molecular experts. When you isolate a virus where the disease is endemic should you clone the virus to be sure only one isolate is present?

Soren Kahns: Due to the quasispecies nature, cloning of VHS viruses might not always provide a true picture of the VHSV population. Sometimes you will be able to identify presence of more viruses in a population by analyzing the pictogram of your sequences as multiple double peaks indicate the presence of more isolates

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CHARACTERISATION OF GERMAN IHNV AND VHSV ISOLATES

Heike Schütze
Friedrich-Loeffler-Institut
Federal Research Institute for Animal Health Germany

Abstract:

The fish-pathogenic rhabdoviruses of Infectious Hematopoietic 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. Phylogenetic analyses permit a clear identification of isolates and their evolution as well as the tracing of trading practises.
Last year 24 VHSV and 5 IHNV outbreaks were reported in Germany. Most isolates from Germany were characterised by sequencing analyses of the glycoprotein gene. Analysed German VHSV isolates are clustered within genotype I group and are closely related to isolates from the last 10 years.

Minutes:

Study of recent German IHN and VHS outbreaks reveals evolution of 0-6 nt/year for IHNV and 0-2 nt/year for VHSV. Spreading and evolution of viral strains in Germany was tracked and relationship between German isolates and foreign isolates was investigated revealing possible transmission across borders. One of the isolates made were from burbot (*Lota lota*) feed on trout diet

Questions:

Olga Haenen: Have you looked at your data in the context of clinics and mortality of the outbreaks?

Heike Schütze: No, here we have represented only the phylogenetic analyses.
Nevertheless, all new IHNV and VHSV outbreaks in Germany are registered in the TSN (= Epizootic News Service). The documents include the respective features, like affected species, clinical signs and mortality.

Stig Mellergaard: Have you analyzed trading relationship between the infected farms?

Heike Schütze: We do not have enough information to confirm any relationship. But from the dataset it seems very likely that a relationship exists.

Katja Einer-Jensen: In USA low evolution rates for IHNV and high for VHSV was observed, you seem to observe the opposite?

Heike Schütze: Yes, and I have no explanation for this.

VIBRIO INFECTIONS IN DUTCH FISH CULTURE

O.Haenen[*], I. Roozenburg-Hengst, M. Voorbergen-Laarman, M. Engelsma
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Abstract:

In aquaculture, *Vibrio* infections are diagnosed often. The most important species described in fish are *Vibrio ordalii, V. anguillarum, V. damselae, V. vulnificus, V. alginolyticus, V. parahaemolyticus, V. splendidus – V.lentus* related group, *V. cholerae*, and *V. harveyi*, but also *Aliivibrio fischeri*, and *Aliiv. salmonicida.* Recently, more species are described, like *Vibrio scophthalmi* and *V. ichthyoenteri*, which are genetically related and sometimes difficult to distinguish. *V. scophthalmi* was described as gut bacterium in turbot, and is not in all cases pathogenic. *V. ichthyoenteri* is known to be pathogenic to young flatfish. Vibriosis occurs at low and higher water temperatures, but growth is enhanced at higher temperatures, and in more or less salty water. Some *Vibrio*-species are zoonotic, like for example *V. vulnificus.*

Dutch fish culture occurs mainly indoor, in warm water recirculation farms, some of which are brackish. In The Netherlands, vibriosis has been found for decades mostly in eel, but last few years some cases of vibriosis in newly cultured fish species were diagnosed, like turbot, sole, barramundi and sea bass. Mortalities were various. The affected fish cannot be placed on the market. Antibiotic treatment is no option because of residues in the fish meat.

Vibrio scophthalmi/ichthyoenteri has been isolated a few times from young sole, with fin and muscle haemorrhages, red ulcers and fin rot, and a chronic mortality of 20-30%. Young turbot showed spiral swimming behaviour, petechial haemorrhages in skin and fins, brown red gills, and a high mortality. Another case in turbot resulted in both *Vibrio ichthyoenteri* (from internal organs) and *Pseudomonas anguilliseptica* (from the skin). These fish were lethargic, pale, and had a pale spleen, liver and kidney.

Vibrio vulnificus was isolated several times from eel with severe disease. Additionally, it was found in combination with *Vibrio cholerae/mimicus* in young barramundi, with a red mouth, exophthalmus, tumbling swimming behaviour, and strong fin and gill rot, and a high mortality.

Vibrio anguillarum was isolated from juvenile turbot and sole. Diseased turbot had *V. anguillarum/ordallii* at the skin. Clinics in turbot of 25 cm were superficial and deeper skin lesions with haemorrhages, exophthalmus, fin rot and mortality, by *V. anguillarum*. Sole of 12 cm showed haemorrhages in skin and fins and a chronic mortality, by *V. anguillarum*.

From adult diseased sea bass, *Vibrio harveyi* was isolated. The sea bass showed a grey skin layer, lethargy, anorrhexia, blindness with exophthalmos and a red mouth.
Furthermore, in the past several cases of non typable *Vibrio* species in brackish and marine fish species were diagnosed. These fish showed in general: haemorrhages in skin and fins, with ulcers, and mortality.

It is concluded, that new cultured brackish and marine fish species bring new *Vibrio* infections with disease. It is recommended, to look internationally into vibriosis problems in fish culture, and to improve diagnostic methods for (sub)typing *Vibrio* species.

Minutes:

Presentation on problems with *Vibrio* infections in the Netherlands. The problem is increasing and new cultured brackish and marine fish species cause new *Vibrio* infections with disease.

Questions:

No questions.

SESSION II: Technical issues related to sampling and diagnosis

Chair: *Stephen Feist/ Richard Paley*
Minutes: *Helle Frank Skall*

THE NEW EU MANUALS ON SAMPLING AND DIAGNOSTIC PROCEDURES AND THE ROLE OF THE WWW.EURL-FISH.EU WEB PAGE

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

Abstract:

The final working paper of the Comission decision is still in process and has not been officially adopted yet. The new Commission Decision on sampling and diagnostic procedures will cover molluscs, crustaceae and fish within separate annexes and cover both exotic and non exotic diseases. The methods that were put on the EURL webpage will be closely linked to the methods described in the OIE guidelines, with modifications when judged necessary. The structure of the parts for the specific diseases will follow a common frame I: Description of the aetiology. II: Provisions on how to obtain and maintain certain health status; surveillance programs, eradication, maintaining and regaining disease free status. III: Diagnostic methods; sampling procedure, methods for surveillance in disease free areas and methods for surveillance/diagnostics in endemic areas or if suspicion of disease.
For fish the decision will comprise: VHS, IHN, KHV, ISA, EHN and EUS

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

EHNV

The CRL diagnostic manual for EHN differs slightly from the manual presented by the OIE. The differences are based on a cost-benefit analysis. If we can't use the same procedures as we use for VHS and IHN it will cost an enormous amount of money to survey for this disease.

The OIE reference laboratory recommends kidney, liver and spleen. As 2 of these organs are already included in the samples taken for VHS and IHN, we believe it will be OK to use these samples for both purposes, no need for collecting liver.

In order to investigate the growth preferences for EHNV the reference isolate was titrated and incubated at 10°C, 15ºC, 20ºC, 24ºC og 28°C, respectively. The trays were read after 3, 5, 7, 10 and 14 days. In conclusion the final titre is the same in the temperature interval 15-24°C, but titre rise is fastest at 20-24°C.

In order to examine which organs are best to use for isolation of EHNV and to examine if the culture method used for VHS/IHN is acceptable trials were performed at DTU.Vet:

The single most suitable organ for reisolation of EHNV was kidney and regarding number of reisolations the 2 x 7 days method with incubation at 15°C is not the optimal method for detection of EHN, as only 53% of the positive fish were tested positive that way. But since the diagnostics is based on several fish, preferably with symptoms it was concluded that the method already used for VHSV/IHNV is appropriate also for EHNV even though it differs from the OIE manual.

For confirmation of EHNV in case of CPE, IFAT and PCR followed by sequencing of amplicon is used. However one should be aware that the available antibodies against EHNV cannot distinguish between this and other RANA viruses.

KHV

The diagnosis of KHV is primarily based on PCR analysis either directly on fish tissue material or from supernatant from infected cell cultures.

Following several proficiency tests and workshops the most suitable methods for detection of CyHV-3 was decided and included in the diagnostic manual posted on the EURL web site.

One of the major concerns are the number of false positive and false negative results obtained in the various ring tests, e.g. during the CEFAS KHV ring trial 10 of 44 laboratories produced false negative and 19 of 44 laboratories produced false positive results!

EUS

The Oomycete *Aphanomyces invadans* is regarded as the causal agent of Epizootic Ulcerative Syndrome (EUS).

A suspect case of EUS according to the OIE diagnostic manual 2009 is related to typical lesions in susceptible fish or presence of *Aphanomyces* sp. without further identification. A confirmed case is a suspect case presenting typical mycotic granulomas in tissue or where the agent has been identified by PCR or FISH detection techniques in tissue or where *A. invadans* has been isolated and confirmed by either bioassay, PCR or sequence analysis.

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

finalised and will be uploaded on the EURL web page after final revision. The methods will be described by Club 5, with Dr. Olga Haenen, CVI as coordinator.

ISA
The diagnostic methods are almost the same as the methods described in the latest edition of the OIE manual. The criteria for diagnosis will most likely also include sequencing in order to assess the HPR type of the isolates or alternatively to use HPR0 specific real-time RT-PCR for all findings from fish without clinical symptoms.

Minutes:

The presentation focused on the diagnostic manuals that will be issued. The Commission Decision 2006/88/EC was decided in 2006 and should have been fully implemented by 2008. This has unfortunately not been the case with regard to diagnostic procedures for the listed diseases that has not yet been described and we have to rely on the former 2001/183 for VHS and IHN, and 2004/466 for ISA. There exists no diagnostic manuals for EHN, EUS or KHV and such have to be made.
The new CD is still in preparation and comprises all listed diseases in fish and molluscs. Detailed diagnostic procedures will be put on the EURL web pages.
The template for VHS and IHN will also be used for the "new" diseases.
Surveillance programmes: The quick model, model A, is similar to the one described in the OIE manual with a large sample size for 2 years. In model B the surveillance programme will last for 4 years with a small sample size. Model B will often be used in areas where you have had the disease endemically and I find this model much more valuable in endemically areas as the virus can hide in the environment for at least up to 4 years, as shown in Søren Kahns' presentation earlier today, whereas model A is usable where you get an outbreak in a free areas as in the UK case.
Targeted surveillance is obligatory in cases where the whole country is not free of the disease in question. Also if no clinical symptoms will be present, e.g. ISA in rainbow trout, targeted surveillance is also necessary.
For VHS, IHN and EHN only isolation of the virus is necessary to demonstrate existence, clinical disease is not necessary.
I am quite confident that in the future it will be possible to survey for VHS and IHN by real-time RT-PCR but we need to have validated methods before including real-time RT-PCR in the diagnostic manual. There have been problems with sensitivity and specificity for this method which we have not had with cell culture.
For ISA we have problems that not all isolates are easy to cultivate. For HPR0 it has not been possible and even the high virulent isolates can be difficult to cultivate.
For KHV, surveillance samples should not be pooled, and pooling is only advisable in diseased fish and then from max. 5 fish.
For EUS, in the OIE manual clinical symptoms only are enough to state presence of the disease. I am not sure if this is the correct method and believes that the identity of the pathogen by e.g. PCR should be tested.
At present, manuals for VHS, IHN, EHN and ISA have been uploaded on the EURL webpage but not approved by the Commission.

Questions:

Alexandra Adams: For KHV will serology be included?

Niels Jørgen Olesen: For VHS and IHN we will hopefully be able to include serology as we are close to finish the validation for these two diseases. But for KHV we do not have a validated method yet.

Alexandra Adams: With KHV we sometimes find strange results with PCR and it would be nice to back up with serology.

Niels Jørgen Olesen: Yes, I think we all see these strange results sometime and how we deal with this will have to be solved.

Brit Hjeltnes: Why is it enough for some diseases only to isolate the virus whereas for others you also need clinical signs of the disease?

Niels Jørgen Olesen: I think this is based on historical reasons. For VHS the surveillance for the diseases was started when the causative virus was isolated whereas for ISA the surveillance was started based on clinical symptoms. For VHS, finding of the virus was reason for expecting presence of the disease as all isolates at that time were pathogenic.

DIAGNOSTIC METHODS FOR IDENTIFICATION OF EHNV AND OTHER RANAVIRUSES

Heike Schütze
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Federal Research Institute for Animal Health Germany

Abstract:

Ranaviruses have been isolated worldwide from fish, amphibians and reptiles at an increasing frequency over the last few decades. They cause systemic infection with necrosis of kidney and spleen as well as diffuse subcutaneous and internal haemorrhages and induce high mortalities in host species. Since 2006/2009 the epizootic heamatopoietic necrosis (EHNV) and ranavirus infection of amphibians are listed diseases of the OIE and EU.
Recommended methods for the diagnosis of EHNV are summarized under section 2.3 of the OIE manual. The manual for amphibian ranaviruses is in preparation. Using serological techniques an identification of most ranaviruses is possible. Nevertheless a differentiation has failed. Molecular techniques facilitate the identification as well as the differentiation of ranavirus isolates. Different methods for PCR and restriction enzyme analyses were compared and evaluated. A new method was established to detect and differentiate all published ranaviruses with the exception of the Grouper iridoviruses.

Minutes:

The history of ranavirus started with isolation of frog virus 3, which became the type species.
EHNV and other ranaviruses are extremely resistant. The virus can be inactivated by treatment with 70 % ethanol or with sodium hydrochlorite or by heating to 60 °C for 15 minutes. Recommended cell lines for EHNV propagation are: BF-2, FHM, EPC and CHSE-214. The virus replicates at 15 – 20 °C.

Ranaviruses do not induce neutralising antibodies. Using the available and recommended rabbit or sheep anti-EHNV sera (OIE Manual), a differentiation is not possible, means it is only an identification of a ranavirus isolate. These sera cross react within most ranaviruses with exception of the Santee-Cooper ranaviruses and the grouper iridoviruses.

The MCP is highly conserved within family *Iridoviridae* and also in other viral families. Within an EU project we have identified the complete MCP gene sequence from various ranavirus isolates. Sequences from some isolates were completely or partially available. The aim of this work was to establish a diagnostic tool for identification and differentiation of most ranavirus isolates including the Santee Cooper ranaviruses.

Known and new identified sequences of the MCP gene from several ranaviruses were compared.
The phylogenetic tree demonstrates the closed relation between most ranavirus isolates. The Santee Cooper ranaviruses LMBV, GV6 and DFV cluster in a separate group. The Grouper iridoviruses SGIV and GIV are the most distant from the others.

Based on the sequence alignments a new PCR called Rana MCP PCR was established and validated using 12 different ranavirus isolates. The resulting product is 625 bp in length. After digestion with the restriction enzyme Sal I, EHNV can clearly be discriminated from other ranaviruses.

Some remarks for further discussions: With exception of the grouper iridoviruses and the Santee Cooper ranaviruses all ranaviruses are very strong related. Why EHNV was listed by OIE and the European commission? We have outbreaks of ESV and ECV in farmed sheatfish and catfish in Germany, Italy and France.
Furthermore, within the EU project RANA we have shown that European farmed perch and rainbow trout are not susceptible for EHNV. So a further discussion about listed diseases is indicated.
A final question that I would like to raise: Should all ranaviruses be listed or should EHNV be excluded from the list?

Questions:

Neil Ruane: Will this method of yours be published?

Heike Schütze: Yes, the manuscript has been accepted.

Niels Jørgen Olesen: Remember that in the proficiency test provided by the EURL, detection of ranavirus is included and it might be possible to include the RFLP. Heike, do you think that all ranaviruses should be included or should EHNV be excluded?

Heike Schütze: I think EHNV should be excluded. Outbreaks of similar diseases induced by very closely related ranaviruses e.g. ECV and ESV were observed in Italy, Germany and France.

DIAGNOSTIC PROCEDURES FOR DETECTION OF *APHANOMYCES INVADANS* – THE CAUSATIVE AGENT OF EUS

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Abstract:

Epizootic ulcerative syndrome (EUS), caused by the oomycete *Aphanomyces invadans (A. piscicida),* is a serious emerging notifiable exotic disease, diagnosed in >60 fish species of various families in Asia, Australia, N-America, and Africa. There is no therapy against EUS, and no vaccine. All EU NRL's for Fish Diseases must have EUS diagnosis in place (ref. 2006/88/EC). Although some tests for EUS were described in literature, most tests were not validated.

The aim of this project was to build up knowledge to recommended confirmatory methods for EUS into the EU:

Try out the described EUS methods (fresh smears, fungus isolation, histopathology, PCR), choose methods for validation, and write Operational Procedures (OP's)
Develop if needed alternative methods (e.g. other agars for isolation, real-time PCR), and validate and implement them.
Establish an electronically available slide collection for EUS histopathology.
Write OP's for the recommended diagnostic tests for EUS based on our findings.
Evaluate methods for storage, survival of *A. invadans* outside the host and suitable disinfection.

The four labs kindly received two reference strains from Prof. Kanchanakhan (OIE Reference laboratory in Bangkok), and from Prof. Hatai, Japan.

Results of this project (2010-2011):

- OP´s for 1) selection of fish to sample, 2) sampling for isolation, 3) sampling for PCR & histopathology, 4) cultivation of isolates of *A. invadans*, 5) sporulation of *A. invadans*, 6) (q)PCR's for EUS, and 7) histopathology of EUS.
- An electronically available slide collection for EUS histopathology.
- Recommendations on storage, survival of *A. invadans* outside the host and disinfection. One important finding was that ethanol is not suitable to disinfect *A. invadans*.

The output (OP's, slide collection, recommendations) can be used by the NRL's for Fish Diseases of Europe, and will be accessible via the EURL Fish Diseases website (www.eurl-fish.eu).
This project was funded by Club 5.

Minutes:

I recommend that if it is the first time you isolate EUS in your country, you should also perform PCR and sequencing to confirm that it is *A. invadans* as it is important to be 100% sure before informing OIE and EU.
SOP's will be put on the EURL webpage in the near future.
Ethanol will not disinfect *A. invadans* but can be used to disinfect the skin when taking samples to avoid other microbes.

Questions/comments:

Sigrid Cabot: The Commission has requested an opinion from EFSA on the risk of introduction, spread of EUS into Europe and probable consequences for such a spread.

Olga Haenen: We look forward to see the result of this.

SPORULATION OF APHANOMYCES INVADANS

Christian Fry
Section for Fish Diseases, Division of Poultry, Fish and Fur Animals, National Veterinary Institute, Technical University of Denmark, DK-8200 Århus N, Denmark

Abstract:

Aphanomyces invadans is an oomycete which is associated with epizootic ulcerative syndrome. Over 50 species of fish have been confirmed by histological diagnosis to be naturally affected by EUS. Many of these fish are commercially important, both in the ornamental fish trade and the aquaculture industry. Diagnosis of *A. invadans* usually based on clinical signs and confirmed by histopathology. Demonstrating typical asexual characteristics by inducing sporogenesis allows identification of the oomycete to the genus level. After inducing sporulation, the zoospores can be isolated for use in clinical infection of fish through subcutaneous injection or bath challenge system. The presentation will include a brief description on optimized techniques for inducing sporulation, and footage obtained during the sporulation process.

Minutes:

Using hemp seeds for sporulation is not included in the OIE manual but seems to enhance the sporulation efficiency as the oomycete will attach to the seed and the seed will not give any nutrition to the oomycete. The washing step is crucial for sporulation as it is important removing all the nutrition to induce sporulation.
An interesting video showing the sporulation in real time was presented.

Questions

Steve Feist: Why does the primary zoospore fly up through the evacuation tube?
Nobody could answer this question.

Richard Paley: Have you compared your method to sporulate with the OIE manual which produces the most zoospores?

Christian Fry: We obtains the best sporulation when increasing time of washing

Niels Jørgen Olesen: EUS was found recently in Canada and probably we will also soon find it in Europe, so it is important to be prepared for diagnosing the disease. As I understand it all exporters have to document freedom for EUS?

Sigrid Cabot: Freedom of EUS have to be documented for susceptible and vector species intended for further farming in the Union, but a derogation from this requirement is currently applying for ornamental fish intended for closed ornamental facilities. The Commission has requested EFSA for an opinion on EUS.

Niels Jørgen Olesen: At the proficiency test for 2011 it is our aim to include EUS. Olga raised the question if we should survey for EUS in Europe?

Regarding reference material for the NRLs I suggest that the EURL (or CEFAS) provide you with reference material for PCR.
How many of you are ready to diagnose EUS? Participants indicated that 5-6 countries were ready to diagnose (Norway, DK, UK, Sweden, Holland, Germany ….)

Olga Haenen: I think especially countries that trade a lot with ornamental fish should be prepared to diagnose this disease. As a lot ornamental fish is imported through Schiphol we are prepared.

Niels Jørgen Olesen: I guess also the Czech Republic trade a lot of ornamental fish.

Brit Hjeltnes: Have anyone done infection trials with this pathogen at fish species present in Europe and using temperatures we have here?

Olga Haenen: Birgit Oidtmann has made experiments at permissive temperatures.

Richard Paley: She has tested mullets and other fish species at 18-22°C and found more tested fish species to be infectable.

Stig Mellergaard: If we get the disease, will we ever be able to eradicate it? How much effort should we put into it?

Olga Haenen: I doubt eradication will ever be possible in the wild. We cannot say that we are 100% free of the disease as we have not examined it.

Niels Jørgen Olesen: If we find it in aquariums it will be possible to eradicate it.

Sigrid Cabot: We need to have an assessment of possible routes of introduction into Europe and the risks of the disease to spread within the Union. If the disease is endemic we would have to reconsider the listing of the disease. Since it is a notifiable disease at least a passive surveillance system should be in place in all Member States.

Olga Haenen: Regarding passive surveillance we talk mainly about closed facilities as we will not be able to do passive surveillance in the open waters.

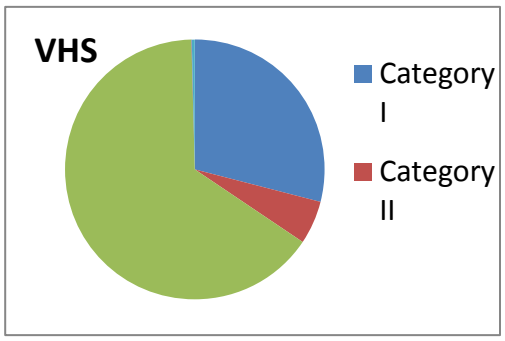
Niels Jørgen Olesen: You will see lot of fish with similar symptoms to those you have seen on the photos today, but as people have not been aware of the disease and the diagnostics have not been in place people have not looked for it. Discussions and distribution of information about the disease should raise the awareness.

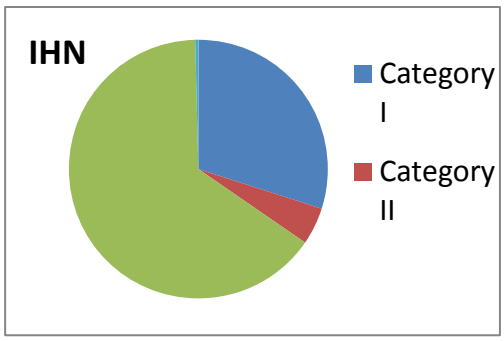
HEALTH CATEGORISATION OF FISH FARMS IN EUROPE IN 2010

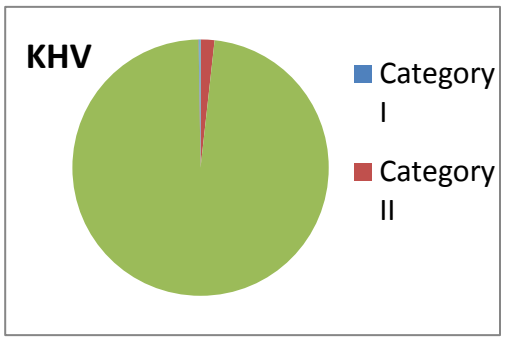
N. J. Olesen and N. Nicolajsen
National Veterinary Institute, Technical University of Denmark

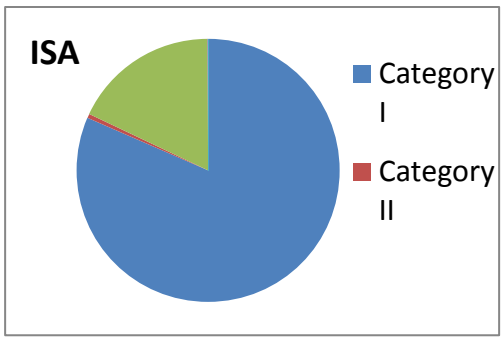
Abstract:

The Questionnaire on Surveillance and Diagnosis (S&D) included questions on how fish farms are health categorised according to Council Directive 2006/88/EC in the respective countries.
More than half of the authorised farms in Europe are in category III for VHS and IHN and the remaining in category I or II. According to these official data almost no farms are infected with either of these diseases. This might be more due to a significant underreporting than of the de facto situation.
For KHV most carp farms are in category III, unknown status.
Many farms in Europe are not categorised yet. However, categorisation is in good process when comparing to e.g. the molluscs farms in Europe. There are several different views on how categorisation shall be performed, e.g. should VHS free marine rainbow trout farms be placed in cat III or I? If ISA virus HPR0 is found in or in proximity of a farm can it remain its cat. I status? The Council Directive is under revision and in this connection the categorisation system might be simplified and be made more transparent.









Minutes:

The health categorisation process in Europe on fish farms is well in progress. The lack of farms in cat 4 and 5 for VHS and IHN most definitely is a question about under-reporting. The high number of farms in category 3 might be explained by the transitatory state the categorisation is in, where difficulties in how to handle the interaction between farmed fish and wildlife with low pathogenic variants of pathogens e.g. ISA HPR0 can be seen. Furthermore this has driven a shift in how category 3 is seen by member states, where category 3 has shifted from an interim category, to a category including uncertainties.

Questions:

Stig Mellergaard: The categorisation in the area of aquaculture lacks simplicity compared to terrestrial animals. Categories could with benefit be reduced to 3; infected, fallowing in process and free.

Sigrid Cabot: In the framework of the Animal Health Law the issue of 3 or 5 categories is being discussed.

CHALLENGES REGARDING IMPLEMENTATION OF THE NEW LEGISLATION ON AQUATIC ANIMAL HEALTH SURVEILLANCE IN EUROPE

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Abstract:
In order to keep up to the SPS-agreement and new knowledge on risk-based surveillance, a new Council Directive (CD) on aquatic animal health surveillance and control was adopted (CD 2006/88/EC) in 2006. One of the intentions of this CD was that disease control should be more cost-effective and risk-based, and focus more on prevention than control. All aquaculture production businesses (APBs) must be registered and authorized, in order to enable an early warning system, with detection of increased mortalities. The risk-based surveillance system for listed diseases must be based on risk-ranking of APBs according to an evaluation of their risk of contracting and spreading disease, and their current status of infection.
In this study, we have asked aquatic animal health professionals in the European Countries how the status is for implementing the legislation in their home country, and what challenges they face in relation to this. A questionnaire was distributed to 34 countries, of which 25 responded.
The survey revealed that there have been delays in establishing registers of APBs, due to the complexity of the industry and lack of resources. 60-80% of the responding countries had ranked farms according to health status, but only 4 countries had drawn up a surveillance program. Furthermore, only 4 of the 25 countries had drawn up contingency plans for handling of exotic and emerging diseases. None of these had yet been approved by the EU.
Thus, many European countries have yet to implement the new legislation concerning aquatic animal health surveillance. Several countries are experiencing problems with inherent complexity of the legislation and the organisation of their production and thus the specific requirements that relate to their within-country scenario. It seems that both in the EC and many of the MS, aquaculture is not prioritized as opposed to terrestrial animal farming.
Thus, as of now, the general aquatic animal health has not yet benefited much from adaptation of new legislation.

Acknowledgements
All respondents are acknowledged for their contribution by answering the questions and providing their opinions in the survey.

Minutes:
The preceding animal health legislation concerning aquatic animals lacked that it was not risk based and that it was not flexible. As of the Council directive 2006/88/EC this should be of more modern animal health program based on: 1) registration/authorisation 2) risk based surveillance and 3) contingency plans. Though good intentions, following the results in our questionnaire there is some way to go to full fill the directive. In most member states initial steps to address the directive have been done, but finalisation of surveillance programs and getting contingency plans approved in the EU has not yet been done. The main problems raised in our questionaire are the complexity of the industry and the lack of dedicated time and resources for implementation.

Questions: No questions.

DEVELOPMENT AND ASSESSMENT OF A REAL TIME LAMP ASSAY FOR KHV

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Abstract:

Loop mediated isothermal amplification (LAMP) is a relatively new and simple method for the amplification of DNA (Notomi *et al*., 2000). The process requires 4 or 6 primers which can provide high specificity. Two of the primers are designed to form loops and are thus self priming meaning the reaction proceeds rapidly and can occur at a single temperature leading to simple and cheap equipment requirements. A number of LAMP assays have previously been developed for Koi herpesvirus (Gunimaldevi *et al*., 2004; Soliman and Matbouli 2005 and 2009; Yoshino *et al*., 2006) with varying sensitivities and length of time to result and all requiring gel electrophoresis or a rather imprecise in tube visual detection. OptiGene Ltd (UK) has developed a novel recombinant polymerase in a proprietary mastermix and an instrument (Genie) for LAMP amplification and analysis. The novel enzyme is reportedly faster than currently used enzymes and the Genie instrument incorporates heating blocks and a fluorimeter providing the conditions for LAMP amplification and allowing the monitoring of these amplification reactions in real time. Furthermore melting curve analysis can be performed on the amplification products to interrogate specificity. We compared a published LAMP assay and newly developed assays using the Genie instrument and OptiGene reagents against nested PCR and realtime qPCR assays on 174 archived diagnostic samples for the presence of KHV. The new LAMP assay detected 10^5 copy number positive control template in a background of negative DNA in less than 10 minutes. The assay consistently detected down to 50 template copies in a reaction from diagnostic samples and occasionally detected as low as 10 copies. There was evidence of some cross reaction with other herpes viruses but not with other non-related viruses. The assay was broadly speaking equivalent to the nested PCR assay with TK specific primers, which is more sensitive than the single round PCR currently recommended by the OIE reference laboratory for KHV detection. Taking PCR as the "gold standard" the LAMP assay showed 89.4% specificity and 86.5% sensitivity with the nested PCR. The LAMP assay is simple and rapid to setup, appears as sensitive as nested PCR, may be less prone to contamination and provides rapid diagnosis and easy interpretation but the potential cross reactivity needs to be assessed further.

Minutes:

LAMP assays have been reported for KHV but are not widespread due to practical issues. We have tested a new commercial real time LAMP method using a small portable machine both to run the amplification procedure and to analyse the product outcome. The initial tests of the system revealed problems with the procedure, but after optimisation done by the commercial company 174 samples were analysed. The test revealed nice results and only a few false positive which was most likely cross contamination. A big advantage with the LAMP procedure is that the specificity can be investigated by analysing the melting point on the products. The overall performance of the test was promising especially as a field tool. Since it was rapid and with comparable sensitivity and specificity as PCR methods.

Questions:

Søren Peter Jonstrup: Would you skip the PCR over LAMP?

Richard Paley: PCR and sequencing still have a big and important role. The LAMP is especially good for use as a quick diagnostic tool.

Stephen Feist: In the future, will we just sequence everything?

Richard Paley: Currently this is still not cost effective enough. However, it might be a possibility in the future.

FISH CELLS – SOME REMARKS TO INDUCE DISCUSSION

Heike Schütze
Friedrich-Loeffler-Institut
Federal Research Institute for Animal Health Germany

Abstract:

A uniform standard, reproducibility and clear references are essential for diagnostics. Sampling plans and diagnostic methods for the detection and confirmation of certain fish diseases are defined in decision 2001/183/EC. Diagnostic methods are also recommended in the diagnostic manuals of the OIE. For the propagation of fish viruses different cell lines are recommended. The development of molecular techniques facilitates the identification of cells and conclusions about their origin. Based on two examples the importance of a clear declaration of cell lines will be demonstrated. The EPC is susceptible to most fish viruses and particularly suited for diagnostics. Genetic analyses have shown that the EPC line is an epithelial cell from skin of fathead minnow and not from carp as originally assumed and published. Based on the activities of the FLI Germany the RTG-2 cell is recommended by the EU (2001/183/EC) for propagation of VHSV. This cell line with cell number CCLV Rie 0088 originates from bluegill fry and not from rainbow trout gonads. Nevertheless, the CCLV 0088 redefined as RT/F is recommended for propagation of VHSV. To establish a uniform standard in all National reference laboratories a clear definition of recommended cell lines is essential. The cell culture library of the FLI Germany provides service and support.

Minutes:

To harmonise diagnostic works it would be advantageous to use cell lines with explicit history. The EU decision from 2001 regulates the sampling plans and diagnostic methods for detection and confirmation of certain fish diseases. Recommended cell lines for diagnostics of IHNV, VHSV, and IPNV are BF-2 or RTG-2 and EPC or FHM cells. Recent evidences have shown, that origin of cell lines can be questioned. If we use and include the catalogue or reference number in OIE documents we have a clear definition of used cell lines.

1. Example: EPC line is an epithelial cell from skin not from carp but from fathead minnow. Since this year EPC is available from ATCC (CRL-2872). Cells were deposited by Winton, 1969 is given as year of isolation and under comments the inconsistency is clarified. In summary: Do we use all the same EPC?

2. Example: Based on the activity and the recommendation of FLI in Germany the RTG-2 cell, listed in our cell library under the number CCLV Rie 0088, was included in the decision 183 from the European Commission for diagnostic of VHSV. But this cell line (CCLV 88) has its origin not from rainbow trout gonade as assumed, but from bluegill fry. In consequence our cell library has declared this line under the abbreviation RT/F. The catalogue number CCLV Rie 0088 remained unchanged. It should be remark, that this cell line is different from BF-2.

A clear declaration of cell lines based on reference numbers/ certification number is essential. The origin of cell lines is not always well defined. Information of the origin of a cell line is based on the respective or actual knowledge we have. Different cell lines exist from same host, but they represent different cell lines or sublines. And, various sublines from the same or similar origin exist in different labs. In conclusion, different results will be achieved. But for diagnostics we need a unique standard, with reproducibility, and clear references.

It is a scientific practice of some journals to proof the reference and certification of used and described cells.
The FLI offers following: FLI will buy EPC, RTG-2 and BF-2 cells from ATCC. These cells will be cultivated and proofed in our cell library. The NRL Germany will check these cells in comparison with other cells for the susceptibility of different viruses. If all is ok, the cell library will send these cells to the EURL and NRL's. Everyone will receive the respective certificate for the cells. EURL and NRL pay only for the transport. The NRL's send this defined material to their regional lab's. At the end we have unique standardized cells in all reference lab's.

Questions:

Brit Hjeltnes: Are you questioning the ATCC?

Heike Schütze: No.

Brit Hjeltnes: Why then, won't the cells just change over time anyway?

Heike Schütze: Every lab has their own cell lines and sub lines. Based on new techniques the origin of some cell lines was identified and corrected. If we talk about a cell line, possibly we talk about different cells or sublines. As NRL we need a standard with well defined cells including the certificate number.

Niels Jørgen Olesen: Thank you for your generous offer which I am sure could be of help for some laboratories. However I think the most important issue here is the sensitivity of the cells, which has been a key issue in sending out the proficiency test where participants can check and standardise sensitivity of the cells used in Europe for diagnostic work. If your cells are not sensitive enough, the EURL can send you cells that are sensitive.

Guiseppe Bovo: I agree with Niels Jørgen. Central acquired cell lines are not better. I have experienced buying cells which from the ATCC had been treated for Mycoplasma, these cells could not be used for diagnostic work. The way to go is getting uniformity in sensitivity among the NRL's by the mean of the proficiency test.

Helle Frank Skall: Getting cells with the same reference number is not a guarantee for similarity as sensitivity can change when cells are passaged.

PITFALLS AND CHALLENGES IN DEVELOPMENT OF REAL-TIME PCR DIAGNOSTIC ASSAYS

SP Jonstrup[1], S Kahns[1], M Christophersen[1], NJ Olesen[1]
1 Section for Fish Diseases, Division of Poultry, Fish and Fur Animals, National Veterinary Institute, Technical University of Denmark, DK-8200 Århus N, Denmark

Abstract:

Late January to early February 2011 a training course was conducted here in Aarhus with participants from all over Europe. Here we had many fruitful discussions on PCR related topics and it was clear that a lot could be learned by sharing non publishable experiences from different laboratories. In this talk I will share some of this knowledge with you. Real-time PCR as a tool for doing diagnostics of fish diseases is getting more and more common. There are many advantages of this technique. It is fast, sensitive, and if designed properly very specific. Compared to conventional PCR there is no need for opening the tubes post PCR, thereby lowering the risk of contamination. In real-time PCR the Ct (cycle threshold) value is defined as the number of cycles required for the fluorescent signal to cross the threshold (i.e. exceeds background level). If two samples in the same assay is compared the sample with the lowest Ct value is regarded most concentrated and if no Ct value is obtained the target is regarded below detection limit. However in some cases interpretations based solely on Ct values can lead to mistakes. This talk will be a presentation of some of the pitfalls we discovered during our investigations and will hopefully illustrate how one should be careful about relaying only in the information contained in the Ct value.

Minutes:

One has to look into the background of the obtained CT values. One should set the baseline of thresh-hold and background signal according to what is appropriate in your set of samples. We have experienced problems with primer dimer which were difficult to see but caused inconsistency in results. By running the samples using Cyber Green instead of Taqman probes we could visualise this problem allowing us to make corrective measures and re-design our protocol whereby the Taqman probes were running as expected. Buffers and kits and commercial products can vary in efficiency, in our newly developed real time RT-PCR detecting all VHSV genotypes we saw that Qiagen kits performed up to 7 Ct values better than Biorad and Stratagene kits. This might be due to that the Qiagen kit can handle small mismatches in probe binding sites better than the others. However, if this is the reason that the Qiagen kits performs better in our assay, then it might not always perform better compared with the other depending on the design of your assay.

Questions:

Debes Christiansen: You can't shift your threshold in the software on a daily basis. However, this must be standardised and uniform between runs.

Søren Peter Jonstrup: All systems include variables, such as machines, days, technicians etc. Therefore it can make sense to look into how your assay has performed and whether the default settings can handle how the assay has been running.

SESSION III: Scientific research update

Chair: *Søren Kahns*
Minutes: *Søren Peter Jonstrup*

INFECTIOUS SALMON ANAEMIA (ISA) IN NORWEGIAN SALMON FARMING, A CHRONICLE OF EVENTS RELATING TO A SMALL SCALE EPIDEMIC

E. Karlsen[a], E.J. Johansen[a], T.M. Lyngstad[b] and P.A. Jansen[b]
[a] The Norwegian Food Safety Authority, Pb 383, 2381 Brumunddal, Norway
[b] National Veterinary Institute, Pb 750 Sentrum, 0106 Oslo

Abstract:
ISA is a viral disease of Atlantic salmon which is subject to a strict control regime in Norway enforced by the Norwegian Food Safety Authorities (NFSA). The annual number of confirmed ISA-outbreaks in Norwegian salmon farms have varied from 1 – 23 during 1993 – 2009. Outbreaks have partly emerged isolated from other outbreaks in space and time, but partly also appeared in small space-time clusters. The aim of the present study was to compile a detailed database describing salmon farming operations and disease progression in a local area experiencing a small scale ISA epidemic. We present a chronological history of events relating to farmed salmon populations during 2007 – 2010, and a total of 22 ISA outbreaks in these populations, occurring in a local area in North Norway. The timing of events has partly been compiled from production plans approved by the local NFSA and partly from information gathered directly from the salmon farmers. The progression of ISA in the area rely on reports from the salmon farmers, surveillance of cage-level mortality on affected farms, and official management decisions made by the NFSA. Maps and animations are used to visualise the dynamics of the salmon farming operations, progression of disease and other events in the area. The chronicle shows how the local structure of the salmon farming industry constantly changes due to; i) fish farm sites being phased into or out of production, ii) populations of fish being moved between sites, iii) fish escaping from cages on farm sites, iv) and finally due to the progression of ISA in the area leading to implementation of control measures. The detailed account of salmon farming operations and disease progression serve as a basis for an epidemiological study, in which genotyping of the ISA-virus have been used to trace dispersal pathways for the virus.

Minutes:

A description of disease progression of an ISA epidemic in the north of Norway (2007-2010) was presented. Escape and transportation of fish seems to be involved in spreading of disease.

Questions:

Niels Jørgen Olesen: Did you make any surveillance of wild fish? Did you find HPR0 in the wild fish population? Was there any genetic relationship between any HPR0 isolates and the isolates causing outbreaks?

Einar Karlsen: We have not sampled from wild fish. We have no evidence that the outbreaks originated from a mutated HPR0 virus. We have indications that there might have been a not recognised outbreak to start with and that these fish were moved around in the region.

Brit Hjeltnes: If a bigger area was fallowed to start with the problem could have been solved quicker, but politics and economic interests made this solution impossible.

Einar Karlsen: I am not sure that a quicker fallowing would have solved the problem and this would have had a large impact on the local community making it hard to have achieved.

INFECTIOUS SALMON ANAEMIA AND HPR0 STRAIN – AN OVERVIEW

Eann Munro
Marine Scotland Science

Abstract:

Infectious salmon anaemia (ISA) is a multisystemic contagious disease of farmed Atlantic salmon (*Salmo salar* L.) caused by a single stranded enveloped RNA-virus in the family *Orthomyxoviridae*. The virus (infectious salmon anaemia virus; ISAV) contains eight segments within its genome. The disease is characterised by severe anaemia and haemorrhaging in several organs, including the liver, kidney, gut and gill. Disease outbreaks are predominately associated with Atlantic salmon farmed in the marine environment.

ISAV possess two surface proteins, a haemagglutinin (H) and a receptor destroying esterase (E) which are important determinants of virulence (Kibenge et al., 2007). Multiple types based on a highly polymorphic region (HPR) of the haemagglutinin-esterase (HE) gene located on genomic segment 6 have been reported (Markussen et al., 2008). A variant with a longer HPR type, HPR0, was first detected in Scotland in 2002 (Cunningham et al., 2002). Evidence for the HPR0 viral strain comes from PCR and direct sequencing from fish tissues. The putative strain of ISA does not appear to induce disease in Atlantic salmon and is unculturable by *in-vitro* tissue culture. Virulent ISAV strains are hypothesised to have evolved from HPR0 as a consequence of transmission from a viral reservoir to densely populated Atlantic salmon farms.

Recent work by Christiansen (2011) reported that ISAV-HPR0 appeared as a seasonal and transient infection on Faroese Atlantic salmon farms without associated mortality. They suggest that ISAV HPR0 causes a subclinical respiratory infection more like seasonal influenza, compared to the systemic infection induced by pathogenic ISAV.

Factors governing the maintenance of HPR0 in fisheries and the external environment and its role as a risk factor in leading to the emergence of ISA disease must be better understood.

References

Christiansen DH, Østergaard PS, Snow M, Bendik Dale O and Falk K (2011) A low-pathogenic variant of infectious salmon anemia virus (ISAV-HPR0) is highly prevalent and causes a non-clinical transient infection in farmed Atlantic salmon (Salmo salar L.) in the Faroe Islands. . J. Gen. Virol. 92: 909-918.

Cunningham C, Gregory A, Black J, Simpson I and Raynard R (2002) A novel variant of the infectious salmon anaemia virus (ISAV) haemagglutinin gene suggest mechanisms for virus diversity. Bull. Eur. Ass. Fish. Pathol. 22(6): 366-374.

Frederick S. B. Kibenge, Molly J. T. Kibenge, Yingwei Wang, Biao Qian, Shebel Hariharan and Sandi McGeachy (2007) Mapping of putative virulence motifs on infectious salmon anemia virus surface glycoprotein genes. J. Gen. Virol. 88:3100–3111.

Markussen T, Jonassen CM, Numanovic S., Braaen S, Hjortaas M, Nilsen H and Mjaaland S (2008) Evolutionary mechanisms involved in the virulence of infectious salmon anaemia virus (ISAV), a piscine orthomyxovirus.Virology. 374: 515-527.

Minutes:

Surveillance for HPR0 in marine salmon farms in Scotland revealed occurrence of HPR0 in around 10% of samples. None of the found HPR0 viruses seem to be the ancestor of the ISA outbreaks in Scotland. Around same prevalence was found in the Faroe Islands. Attempts to develop challenge models for HPR0 were so far unsuccessful. To date, the detection of HPR0 RNA has not been

associated with ISA disease. It seems that new outbreaks of ISAV to a very large extend is due to horizontal spreading of pathogenic ISAV and that HPR0 evolution into a pathogenic strain is a rare event. Organizing the industry into management areas in Scotland reduced the risk of long term persistence of pathogens and facilitated rapid containment of new emergences of the disease. HPR0 has a tissue tropism different from pathogenic ISAV, with gills as the primary location of HPR0.

Questions:

Olga Haenen: You have to notify OIE even when you find HPR0. What do you think about this?

Eann Munro: If we only detect by PCR as for the HPR0 strains we do not consider it as ISA and therefore do not report it.

Brit Hjeltnes: In infection trials with HPR0 do you think you get replication?

Eann Munro: We think we can get it to replicate in the gills.

DETECTION OF LOW-PATHOGENIC INFECTIOUS SALMON ANEMIA VIRUS (ISAV-HPR0) IN FRESHWATER IN THE FAROE ISLANDS

D.H. Christiansen,
Food and Veterinary Agency, National Reference Laboratory for Fish Diseases, Tórshavn, Faroe Islands

Abstract:

Infectious salmon anemia (ISA) hit the Faroe Islands in 2000 and the following five years the ISA epidemic spread throughout the islands causing an almost total collapse of the industry. A subsequent restocking program with Atlantic salmon vaccinated against the ISA virus and a comprehensive screening program for ISAV provided a unique opportunity to study the risk of re-emergence of ISA in the Faroe Islands. We recently reported that a Faroese ISAV-HPR0 subtype is widespread in seawater farmed Atlantic salmon and causes a transient and seasonal infection without clinical ISA[1].

In our screening program for ISAV six freshwater farms with continues production of Atlantic salmon smolt and Faroese broodfish were screened for the presence of ISAV. During the study period from February 2007 to December 2010 Atlantic salmon smolt from four of the six freshwater farms showed transient infection with ISAV-HPR0. In total 8,7% apparently healthy smolt tested positive for ISAV-HPR0 by RT-PCR and sequencing. The annual prevalence ranged from 0% ISAV-HPR0+ve in 2007 to 14% ISAV-HPR0+ve in 2010. Furthermore, we found a high prevalence of 48% and 93% ISAV-HPR0+ve broodfish stripped in 2008 and 2010, respectively. Since vertical transmission has yet to be definitively demonstrated, the origin of these infections and thus the potential transmission pathways for ISAV-HPR0 remain uncertain. To disentangle potential transmission pathways we have performed genetic analysis of ISAV-HPR0 isolates from broodfish, freshwater smolts and seawater Atlantic salmon. Our preliminary results will be presented.

Minutes:

ISA caused a collapse of the Atlantic salmon industry in the Faroe Islands but the production has now been re-established. HPR0 virus was detected inside gill cells by IHC. Two major HPR0 genotypes are present in the Faroe Islands. The ISAV isolates from outbreaks in the Faroe Islands is closely related to one of these. HPR0 was also found in freshwater farms. HPR0 infection is low pathogenic and transient but highly contagious.

Questions:

Brit Hjeltnes: Interesting that you find HPR0 in freshwater. What could be the source, since I guess the water is treated?

Debes Christiansen: Even though we have treatment of water source then during winter storms sea water might contaminate inlead water.

DETECTION OF HPR0 IN DENMARK AND CRITERIA FOR DIAGNOSIS OF ISA

Helle Frank Skall
National Veterinary Institute, Technical University of Denmark

Abstract:

In August 2010 a surveillance sample from Atlantic salmon sampled in the broodfish section of a Danish farm producing salmon for restocking was tested positive for ISAV by RT-PCR. The fish were offspring of wild salmon and reared from eggs in a closed unit where after they were moved to an open unit containing wild fish (salmon and perch).
The samples consisted of kidney, heart and gills in RNA*later*. They were examined by conventional RT-PCR using the TK primers and by real time RT-PCR using the Gilad primers and 1/9 samples were positive. Sequencing of the HPR region identified the isolate as HPR0.
Samples from Atlantic salmon in the closed unit and from perch in the open unit were examined for ISAV with negative results.
No signs of disease consistent with ISA were observed at the facility.
How should this finding be interpreted?
In the OIE Diagnostic Manual and in the EU Commission Decision 2003/466/EC a number of findings are listed as causing ISA suspicion:

If only RT-PCR positive is this a suspicion?
Another set of criteria is stated for confirmation of ISA:

OIE	EU
Disease or pathological changes consistent with ISA	Disease or pathological changes consistent with ISA
Cell culture positive	Cell culture positive
Evidence for the presence of ISAV from two independent laboratory tests (RT-PCR and IFAT)	Evidence for the presence of ISAV from two independent laboratory tests (RT-PCR and IFAT)
Detection of antibodies to ISAV	Transfer of ISA-infected live fish to farm
	Epidemiological links

Comparison of
OIE (diagnostic manual) and EU (CD 2003/466/EC)

***Confirmation of ISA*:**

Disease **AND** IFAT on tissue preparations

AND

Cell cultivation **OR** RT-PCR

Confirmation of ISAV infection:

Cell cultivation from two independent samples

AND (OR?)

Cell cultivation from one sample **AND** RT-PCR or IFAT on tissue preparations

***Confirmation of ISA*:**

Disease

AND

Cell cultivation **OR** RT-PCR **OR** IFAT on tissue preparations

OR

Cell cultivation from two independent samples

OR

Cell cultivation from one sample **AND** RT-PCR or IFAT on tissue preparations

Confirmation of ISAV infection:

Not described

According to this set of criteria the finding did not lead to confirmation of neither ISA nor ISAV infection as positive cell cultivation is mandatory.

Furthermore, as the isolate was sequenced as HPR0, which has never been detected in ISA diseased fish, the Danish Competent Authorities conclusion was that Denmark is still free of ISA.

Minutes:

HPR0 was detected in Danish salmon produced for restocking purpose. In the OIE – manual of Diagnostic tests for Aquatic animals ISA is in one place defined as infection with salmon anaemia virus, but later the presence of disease is a key point. The Danish Competent Authorities concluded that Denmark is still free of ISA but it is clear that better guidelines are needed.

Questions:

Olga Haenen: Interesting thoughts. I will bring them to OIE. As things are now I think you should notify when you find HPR0.

Brit Hjeltnes: We in Norway are also pressing to get these issues raised.

Einar Karlsen: The problem with the OIE manual is that they do not discriminate between infection and disease but for HPR0 a fish can be infected but the virus will not induce disease.

Søren Kahns: Some of us are meeting later today to discuss this issue in more depth. Participants interested in this topic are welcome to join the discussion.

PD-VACCINE IS EFFECTIVE IN REDUCING SEVERITY OF DISEASE OUTBREAKS IN NORWEGIAN AQUACULTURE

B. Bang Jensen*[1], A.B. Kristoffersen[1], C. Myr[2] and E. Brun[1]
[1]*Norwegian Veterinary Institute, Oslo, Norway*
[2]*PD-fri/Norwegian Seafood Federation, Bergen, Norway*

Abstract:

Pancreas disease (PD) was first discovered in Norway in the mid-eighties. Until 2003, the yearly number of outbreaks was less than twenty, but in 2004 the number of outbreaks doubled, and in 2008, it was more than five times that of 2003. Since the disease is associated with massive losses in salmonid aquaculture, this increase in number of yearly outbreaks led to PD being controlled by national legislation from 2008. Furthermore, the aquaculture industry initiated a parallel project with the aim to reduce the consequences associated with PD (The PD-fri project). Mitigation measures where establishing of a PD-free zone, mandatory vaccinations outside the free zone and movement restrictions outside of and into the free zone. Also, the PD-fri project collated data on management, production and disease for individual seasites outside the PD-free zone.
Data from 201 cohorts of fish from seatransfer to slaughter was collected, from outset from spring 2007 to spring 2009. The data was then analysed in order to investigate the following risk factors: vaccination, strain of fish, other diseases and number of treatments against sea-lice. The effect was analysed with regards to length of PD-outbreaks, cumulative mortality, average weight gain and feed conversion ratio.
Univariat analyses revealed that there was significantly less PD-outbreaks and lower cumulative mortality among fish in vaccinated locations than non-vaccinated locations. Furthermore, the length of outbreaks was significantly shorter in locations with vaccinated fish than locations without.
Multivariate analyses using the probability of getting PD resulted in a model where vaccination and outbreak of Infectious Pancreas Necrosis were significant.
The effect of vaccination against PD has previously been regarded as poor, because it does not seem to reduce the number of outbreaks. This study shows that there are beneficial effects of PD-vaccination, especially in reducing mortality and length of outbreaks. The project would not have been possible without the involvement of the industry, both with regards to containing disease and to obtain data.

Minutes:

PDfri project was initiated to stop further spread of PD, reduce number of outbreaks, and reduce losses. 337 out of 342 sites agreed to participate and pay costs around 1250 euro. Norway was during the project divided into an endemic- and a non-endemic zone. The project showed good results and the fact that this was an industry-driven project meant that the involvement from the industry was high. PDfri has recommended surveillance of all locations in the endemic zone in order to detect infections as early as possible.

Questions:

No question.

EXTENSION OF EPIZONE

Management, control and surveillance of viral encephalopathy and retinopathy in aquaculture

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Abstract:

As part of the research project EPIZONE , a workshop on: " Management , control and surveillance of viral encephalopathy and retinopathy in aquaculture" will be organized during the next EAFP Conference. In order to obtain as much as possible information concerning the impact of the disease, the control measures applied and the results obtained, at global level, a questionnaire has been issued and sent to 26 nodavirus fish pathology experts.

On May, 15 seventeen documents, mainly referring to the Mediterranean region, were returned. Here only data concerning the situation in the Mediterranean area, provided by thirteen experts are presented and discussed. The information obtained confirms that viral encephalopathy and retinopathy (VER), is widespread in the Mediterranean Countries, causing significant economic losses except in Croatia, Turkey and France. In this last Country, in particular, the last VER outbreak dates back to ten years ago. Among all the Mediterranean Countries VER is notifiable only in Israel. In addition to this Country, Croatia also provides trade restrictions, particularly concerning imports from countries outside EU. With regard to bio-security measures, with rare exceptions, their application in hatcheries does not seem particularly strict and effective. The continuous introductions in the hatchery of wild broodfish, without a proper quarantine period and diagnostic screening to avoid potential carrier, represent a high risk of infection as well as the feeding with raw molluscs and fish.

European sea bass (*Dicentrarchus labrax*) is by far the most affected species, but there are other susceptible species such as meagre (*Argyrosomus regius*), Senegalese sole (*Solea senegalensis*), shi drum (*Umbrina cirrosa*), white grouper (*Epinephelus aeneus*), grey mullet (*Mugil cephalus*) and gilthead sea bream (*Sparus aurata*). This last species, which for many years has been considered completely resistant, has suffered, in recent years, serious mortalities even if limited to larval and juvenile stages. The prevalence of the disease varies widely from region to region, showing maximum values above 50%. Morbidity and mortality are age dependent, larvae and juveniles showing the most important losses. In sea bass, however, mortalities ranging between 25 and 50% in on-growing facilities are not uncommon.

Minutes:

In April 2011 a questionnaire was sent out to 26 experts on Nodavirus disease. 17 answers were received with requested information. The 13 answers from laboratories in the Mediterranean area are in focus in this presentation. The disease is only notifiable in Israel. Only 2 countries have an official surveillance plan in force. 8 experts regard VER/VNN as a significant problem for mariculture in their country. 5 experts would like the disease listed on EU level. 8 experts would like the disease listed on OIE level. Losses due to the disease range from below 10% to above 50%.

Questions:
No questions

SEROLOGICAL TESTS FOR SPECIFIC ANTIBODY DETECTION IN EUROPEAN SEA BASS (*D. LABRAX*) AGAINST VERV

Niccolò Vendramin and Elisabetta Cappellozza
IZSVe, Legnaro, Italy, ***E mail:*** *nvendramin@izsvenezie.it*

Abstract:

Viral encephalopathy and retinopathy is a serious disease causing significant economic damages to marine aquaculture industry. This disease has a wide geographical distribution as it has been observed in tropical and temperate climates. More than forty species, mainly of marine origin, have been affected so far and this number is likely to rise in future following the introduction of new species and the increase of aquaculture trade.

According to OIE, all fish mortalities characterized by abnormal swimming behaviours associated with vacuolar lesions in the nervous tissues containing viral particles of the *Nodaviridae* family should be ascribed to one single disease, officially identified as viral encephalopathy and retinopathy (VER), also known as viral nervous necrosis (VNN).

The presence of clinical symptoms depends on several factors such as species, age and temperature; furthermore, acute and sub-acute forms are characterized by different symptoms and mortality rates. The most characteristic and common clinical sign observed in the different species is an abnormal swimming behaviour characterized by a difficulty to maintain the normal static and dynamic equilibrium, speed and swimming direction and to control the swim bladder inflation.

In the Mediterranean Sea the disease appeared in the '90's, nowadays it is considered as an endemic disease and represents one of the major constraint of fish culture all over the world.

European Seabass (*D. labrax*) demonstrated to be one of the most susceptible species to VER in Mediterranean Sea, particularly in larval and juveniles stages. Adult fish may harbour the virus latently and spread the infection to the juveniles present in the farm.The disease proves to be a serious threat also for other species, such as Sea bream (*S. aurata*, particularly larval stage), Sole (*S. solea, S. senegalensis*) and Shi drum (*U. cirrosa*). Diagnostic techniques which have reached high levels of specificity and sensitivity have always aimed at the detection of aetiological agents with both classical viral and biomolecular techniques. Unfortunately these could only partially be applied to non-invasive samples. Few papers report of serological techniques used for the detection of specific antibodies in Sea bass. In this work three different serological techniques are described and compared: ELISA, Serum neutralisation and IFAT. Clinical data, obtained from the field, report of infection and mortality year after year of the same batch of fish reared in particular conditions. Serological data obtained with these tests seem to confirm the suspicion that very low immunitary memory characterise the batch of fish at different time points after the outbreak.

Minutes:

Comparison of three serological tests (ELISA, SNT, and IFAT) for detection of VER. Generally good accordance between different techniques in terms of positive/negative.

Questions:

Athanasios Prapas: You sampled in December and were still able to see clinical signs?

Niccolo Vendramin: Yes.

Athanasios Prapas: In Greece we find that infected fish are very resistant the year after.

Niccolo Vendramin: We find that outbreaks can occur again the year after.

SESSION IV: Update from the EURL

Chair*: Niels Jørgen Olesen*
Minutes: *Helle Frank Skall*

Norway is at present evaluating their surveillance system. In connection to this evaluation Britt Bang Jensen asked the audience about help to filling out a questionnaire regarding specificity and sensitivity of VHSV tests.

Niels Jørgen Olesen: Two of our colleagues will retire this year. One is Jeannette Castric, who unfortunately is not able to be on this meeting. She has been a tremendous help over the years.
The other is the young man, Giuseppe Bovo, who wants to settle in a small house in Sicily. Giuseppe has also done a tremendous work in this field in many years. Let us all applause these two persons and wish them good luck in the future.
In the afternoon we will have a little meeting at the institute at 2 o'clock discussing ISA HPR0 and you are all invited to participate.

I hope you all use the EURL website (www.eurl-fish.eu) where you can find a lot of information.
One of our duties as the EURL is to harmonize diagnosis in the NRL's and the proficiency test are part of that. We also supply a number of standard sera and reagents to other laboratories. We try to produce reagents to be used in various laboratories. Last year we produced antisera against KHV. It is also important to keep and maintain a library of isolates and we are grateful for each isolate provided by you to be included in this library.
We also assist member states on diagnosis and characterization of diseases and isolates, and we are very happy for the collaboration that we have with you all in this respect.
The fish pathogens database now contains both VHSV and IHNV and we hope the database will include more diseases in the future.
We collect information regarding the disease information and production in Europe through S&D. All information can be found at the web page and we encourage you to look at it, and please inform us, if some information should be added or if you do not agree in the data.
We also provide training both through courses at our laboratory and at missions to different laboratories.
We have been working a long time on VHSV qPCR and we are on the steps to send in a manuscript on this subject so the method can be included in the manuals.

WORK PROGRAMME FOR THE EUROPEAN UNION REFERENCE LABORATORY FOR FISH DISEASE, 2010

Søren Kahns, Nicole Nicolajsen, Søren Peter Jonstrup and **Niels Jørgen Olesen**

Every year a work programme of the EURL for the following year is submitted to the Commission for approval. The programme for 2010 was as follows:

I. LEGAL FUNCTIONS AND DUTIES

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

II. OBJECTIVES FOR THE PERIOD JANUARY - DECEMBER 2010

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

WORK PROGRAMME FOR THE EUROPEAN UNION REFERENCE LABORATORY FOR FISH DISEASE, 2011

Niels Jørgen Olesen, Nicole Nicolajsen, Søren Peter Jonstrup, Maj-Britt Christophersen and Søren Kahns

The work plan for the current year is as follows:

OBJECTIVES FOR THE PERIOD JANUARY - DECEMBER 2010

The functions and duties of the European Union Reference Laboratory for Fish Diseases (EURL) are described in the Council Directive 2006//EF Annex VI part I 88

OBJECTIVES FOR THE PERIOD JANUARY - DECEMBER 2011

1. Organise and prepare for the 15th Annual Meeting for the National Reference Laboratories for Fish Diseases (NRLs) in 2011.
2. Produce a report from the Annual Meeting 2011.
3. Prepare the Annual Inter-laboratory Proficiency Test year 2011 for the NRLs. The test will include VHSV, IHNV, EHNV, ISAV and KHV.
4. Collate and analyse information gained from the Inter-laboratory Proficiency Test
5. Supply reference reagents to the NRLs in Member States.
6. Production of antisera against selected isolates when necessary.
7. Update and maintain a library of isolates of Infectious salmon anaemia virus (ISAV), Viral Haemorrhagic Septicaemia virus (VHSV) and Infectious Haematopoietic Necrosis virus (IHNV), Koi Herpes virus (KHV) and enzootic haematopoietic necrosis virus (EHNV) and *Aphanomyces Invadans*.
8. Establish and maintain a library of tissue material from fish infected with listed pathogens.
9. Update the webpage for the EURL, www.crl-fish.eu (the web address will be changed to www.eurl-fish.eu in autumn 2010)
10. Update the diagnostic manuals for VHS, IHN, ISA, KHV disease and EHN on the EURL web page, and include diagnostic manuals for EUS.
11. Update diagnostic methods for diagnosis of Epizootic Ulcerative Syndrome (EUS) and assess the possibilities for including *Aphanomyces invadans* in proficiency test in the future.
12. Collect and report 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.
13. Identify and characterise selected isolates of listed viruses (serological and genetic characterisation).
14. Update and expand www.fishpathogens.eu with more pathogens.
15. Perform molecular epidemiology analysis to improve knowledge on diseases spreading mechanisms of viral pathogens
16. Assessment and standardisation of real-time PCR tests for the diagnosis, identification and typing of the listed non-exotic fish diseases.
17. Facilitate and provide training in laboratory diagnosis. A training courses in methods used for diagnosis of fish diseases will be established and offered at the EURL laboratory facilities. The courses will primarily be for training of staff from NRLs and the content will depend on request from participants.
18. Organizing missions to relevant laboratories. Missions will focus on NRLs where on-site communication would be beneficial.
19. Organise a workshop on “Surveillance and Epidemiology of Diseases in Aquaculture” at the premises of the EURL-Fish and in collaboration with the OIE collaborating centre for aquatic epidemiology and the EURL-Molluscs and –Crustacians, respectively.

20. Attending missions, international meetings and conferences in order to be updated on diagnostic methods on listed exotic and non-exotic fish diseases.

OBJECTIVES FOR THE PERIOD JANUARY - DECEMBER 2012

1. Organise and prepare for the 16th Annual Meeting for the National Reference Laboratories for Fish Diseases in 2012.
2. Produce a report from the Annual Meeting 2012.
3. Collect data on the fish disease situation in EU, including all the listed non-exotic fish diseases given in Council Directive 2006/88/EC Annex IV Part 2.
4. Identify and characterize selected isolates of listed viruses (serological and genetic characterization).
5. Production of antisera against selected isolates when necessary.
6. Assessment and standardization of Real-time PCR tests for the diagnosis, identification and typing of the listed fish diseases.
7. Update and expand www.fishpathogens.eu with other pathogens.
8. Update and maintain a library of isolates of Infectious salmon anaemia virus (ISAV), Viral Haemorrhagic Septicaemia virus (VHSV) and Infectious Haematopoietic Necrosis virus (IHNV), Koi Herpes virus (KHV) and enzootic haematopoietic necrosis virus (EHNV).
9. Update the webpage for the EURL, www.eurl-fish.eu
10. Establish and maintain a library of tissue material from infected fish.
11. Update the diagnostic manuals for the listed diseases on the EURL web page.
12. Supply standard antisera and other reference reagents to the National Reference Laboratories in Member States.
13. Perform molecular epidemiology analysis to improve knowledge on diseases spreading mechanisms of viral pathogens
14. Prepare the Annual Inter-laboratory Proficiency Tests year 2012 for the National Reference Laboratories. The tests will include VHSV, IHNV, EHNV, ISAV, KHV and *Aphanomyces invadans*.
15. Collate and analyse information gained from the Inter-laboratory Proficiency Test
16. Facilitate and provide training in laboratory diagnosis.
17. Offering the yearly training courses in methods used for diagnosis of fish diseases, at the EURL laboratory facilities. The content will depend on request from participants
18. Organizing missions to relevant laboratories. Missions will focus on NRLs where on-site communication would be beneficial.
19. Attending missions, international meetings and conferences in order to be updated on diagnostic methods on listed exotic and non-exotic fish diseases.
20. Any other suggestions?

Minutes:

Apart from all the mandatory tasks for next year, suggestions for other topics to the workprogramme ofthe EURL would be most appreciated

The backbone of our work program for 2011 is similar to our work program for 2010. However extensions have been made. A new thing is to establish and maintain a library of tissue material from fish infected with VHSV, IHNV and IPNV that can be sent to the laboratories for use in validation of methods.

The proficiency test for 2011 will most likely include EUS as well as the viruses included last year. The test will be sent out in October. The test will then comprise all the listed disease pathogens. For EUS we will send out inactivated material to be sure we do not spread the disease! For those of you who need positive control material please contact us. I am sure Birgit Oidtmann will also be helpful providing positive control material.

Regarding diagnostic manuals we have already uploaded manuals for VHS, IHN and EHN. The ISA manual was uploaded but only diagnostic procedures are included not the sampling procedures. The diagnostic manual for KHV will be uploaded soon.

For EUS we have used significant resources on updating and implementing diagnostic procedures.

We have also done a lot of work on epidemiology on VHS.

Later this year we will organize a workshop on Surveillance and Epidemiology of Diseases in Aquaculture. We will host the workshop, but the organization of the workshop is being done in collaboration with the newly appointed OIE collaborating center on aquatic animal epidemiology. You will find an invitation in the folder. The WS will primarily be for colleagues who already have some knowledge on epidemiology. The Commission will invite 1 person from each member state who will have travel and living paid for. But the workshop will be open for all interested and there will be space for app. 60 participants.

We would like to ask you if some of you would like us to make a EURL visit to your laboratory in 2011? Such visit or mission would provide a possibility for exchanging know-how and obtaining experiences in your own laboratory premises in specified diagnostic procedures. We have already talked with the Spanish NRL about a visit to their laboratories but if others are interested please contact us.

In 2012 we will again organize a training course. The course will be advertised in November 2011 and we encourage you to inform us on which topics you will like us to include in the course.

Suggestions for the workplan 2012:
Provide recommendations for strain discrimination of listed pathogens e.g. marine VHS/highly pathogenic VHS strains; ISA HPR0; ranavirus/EHNV; KHV variant strains.
More focus could be added on “emerging” non listed diseases like nodavirus, aquatic alphaviruses. It is a requirement in the legislation to be aware of emerging diseases.
To extend the FishPathogens database by including more pathogens. We hope in 2011 to include nodavirus through an extension of the Epizone project.

If the laboratories have suggestions for tasks to include in the workplan for 2012 please contact us. The workplan has to be sent to the commission by 1 September 2011.

Are there any comments/suggestions?

Olga Haenen: First I would like to congratulate you for a very successful meeting once again. Would you also consider topics beyond virology like e.g. bacteriology.

Niels Jørgen Olesen: We have focused mainly on the listed diseases as this is our task.

Brit Hjeltnes: You have of course to focus on listed diseases, but you also have to be vigilant on the emerging diseases and I think this could be included in the next meeting. We would then have to concentrate on the really important emerging disease like e.g. PD.

Britt Bang Jensen: In the new regulation it is stated that you should focus on increased mortality, how is this handled in the different member states, at what criteria do we recognize it as increased mortality – this is a subject which could be included in next year's meeting.

EURL TRAINING COURSE 2011 AND REQUEST FOR IDEAS FOR 2012

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Abstract:

From late January to early February 23 participants mainly from European NRLs for Fish Diseases participated in an EURL training course in Aarhus, Denmark. Participants could choose from one to three modules each lasting three days. One module focused on PCR, one on cell cultures, and one on Immunochemical methods. Here I present a small update from this course and afterwards we will ask you for ideas for next year's course. So please have your ideas ready.

Minutes:

I will tell you about the training course we had this year and the plans for the coming training course.

We had very good feedback from the participants at the first course. We plan for a course to take place next year and we encourage participants to provide topics for training.

Questions

Olga Haenen: What about double and triple viral infections?

Niels Jørgen Olesen: I think this could be a good idea.

RESULTS AND OUTCOME OF PROFICIENCY TEST, PT1, 2010

Søren Kahns, Nicole Nicolajsen, Maj-Britt Christophersen and Niels Jørgen Olesen
EU Reference Laboratory for Fish Diseases, Technical University of Denmark, Aarhus, Denmark

Abstract:
A comparative test of diagnostic procedures was provided by the EU Reference Laboratory (EURL) for Fish Diseases to 38 National Reference Laboratories (NRLs) in the start of September 2010. The test was prepared and tested according to protocols accredited under DS/EN ISO/IEC 17025 and ILAC-G13:08/2007 standards. The test contained five coded ampoules with the following content VHSV genotype Ia, IHNV genogroup M, EHNV, European catfish virus (ECV), and spring viraemia of carp virus (SVCV), 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).
Participants were asked to titrate the viruses to assess the cell susceptibility for virus infection in the respective laboratories. Participants were encouraged to use their normal standard laboratory procedures. However, the identification should be performed according to the procedures laid down in Commission Decision 2001/183/EC using monolayered cell cultures followed by e.g. neutralisation test, immunofluorescence, ELISA or PCR. If ranaviruses should be present in any of the ampoules, it was mandatory to perform a sequence analysis of the isolate in order to determine if the isolate is EHNV.

Outcome of PT1
18 laboratories out of 38 correctly identified all viruses in all ampoules correctly and obtained maximum score. 10 laboratories did not identify the virus in ampoule III as ECV, ESV or rana but not EHNV. Nine laboratories did not identify virus in one or more ampoules where a virus was present. 5 laboratories observed additional virus than present in the ampoule. 25 laboratories used ELISA for identification of viruses. 23 laboratories used IFAT for identification of viruses. 8 laboratories used neutralisation tests for identification of viruses. 35 laboratories used PCR for identification of viruses. 30 laboratories performed sequencing for identification of viruses. 32 laboratories used BF-2 cells. 34 laboratories used EPC cells. 14 laboratories used RTG-2 cells. 16 laboratories used FHM cells

Concluding remarks
EHNV was included and so was the ECV that belongs to the ranavirus family. All the 24 laboratories performing sequencing of the isolate in ampoule I identified the virus correctly as being EHNV. 24 laboratories identified the virus in ampoule III as ranavirus but not EHNV. However, 7 other laboratories that performed sequencing of the ECV isolate in ampoule III identified the virus as EHNV although the submitted sequences in 6 cases were identical to ECV/ESV. We recommend that laboratories carefully analyse their sequencing results when a ranavirus is identified in order to rule out if the virus is the listed EHNV or not. Furthermore,

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

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

RESULTS AND OUTCOME OF PROFICIENCY TEST, PT2, 2010

Søren Kahns, Nicole Nicolajsen, Maj-Britt Christophersen and Niels Jørgen Olesen
EU Reference Laboratory for Fish Diseases, Technical University of Denmark, Aarhus, Denmark

Abstract:
It was decided at the 14th Annual Meeting of the NRLs for Fish Diseases in Aarhus 26-28 May 2010, that testing for ISAV and KHV (both listed in Council Directive 2006/88/EC) for the first time should be included in the yearly proficiency test provided by the EURL. Therefore, a comparative test, PT2, of diagnostic procedures was provided by the EU Reference Laboratory (EURL) for Fish Diseases to 36 National Reference Laboratories (NRLs) in the start of September 2010. The test contained five coded ampoules of which two contained ISAV, two contained KHV and one did not contain any virus.

Participants were expected to use their normal PCR or real-time PCR methods for detection of KHV and their normal RT-PCR or real-time RT-PCR methods for detection of ISAV. It was not mandatory to grow KHV and ISAV on cell cultures in this test, but the viruses has not been inactivated and should thus be viable and possible to amplify in cell cultures.

Outcome of PT2
23 laboratories out of 36 correctly identified all viruses in all ampoules and obtained maximum score. Two laboratories only examined for KHV and not ISAV and one laboratory only examined for ISAV and not KHV. Of these three laboratories, two laboratories presented correct answers and obtained the score of 6 out of six. Laboratories scoring 8, 6 or 4 either lacked virus identification or identified additional viruses than those present. Two laboratories did not submit any results and obtained the score of 0.

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

Concluding remarks
Considering that this was the first time that the EURL provided a proficiency test on ISAV and KHV identification, we think that most participants obtained satisfying results. All 33 laboratories performing KHV identification did correctly identify KHV in the ampoule containing high titre KHV. All 31 laboratories performing ISAV identification, except two, did correctly identify ISAV in the ampoule containing high titered ISAV. Lowering the titre of the virus caused a few laboratories to miss identification of KHV and ISAV in the low titered ampoules. To decrease the risk of having false negative results, it is always recommended that laboratories use the most sensitive tool available, validate the sensitivity of their diagnostic tools and use proper controls.

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

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

Minutes:

Together with the proficiency test we sent out a questionnaire regarding the accreditation status in the different laboratories both regarding exotic and non exotic diseases.
Many laboratories are accredited for performing cell culture assays for VHS and IHN. Fewer laboratories are accredited for PCR methods that are the main tools for diagnosing KHV and ISA. For EHN some are accredited and only few for EUS. I want to stress that these are results from all participants in the proficiency test and not just the EU member states.

Regarding the proficiency test all EU member states participated or had representatives.
Most parcels were delivered within 3 days after sending. Some were delivered later due to reasons out of our hands. Loggers were included in many of the parcels.

The proficiency tests were prepared according to protocols accredited according to ISO 17025 and ILAG guidelines.

The samples were lyophilized before sending out. We generally see a decrease in titer after lyophilization but all titers were above detection level.

It is important that the laboratories both follow the procedures laid down in the EC regulations but also that the normal procedures in the laboratory as this is a test on the work normally done in the laboratory.

In case of no CPE it is also important to subcultivate the samples as the titer may be low.

PT1
Most laboratories did very well in PT1 but a few laboratories can improve.

If your cell line is performing below the 25% quartile in the titration you should consider if you may need to replace your cell line. If this is the case you are welcome to contact us.

Regarding test methods used by the participants, ELISA and IFAT is used a lot among the participants as seen in the earlier years. But this year PCR and sequencing has been used among a lot of the laboratories also.

Most laboratories used sequencing to discriminate between the ranaviruses and one lab used RFLP.

Generally laboratories performed well on PT1 but there were a few problems with labs not analyzing sequencing results of the ranaviruses properly and thus not being able to tell whether it was EHNV or not. Very few laboratories have not implemented PCR yet, which is a requisite for being able to diagnose all the listed diseases.

PT2
This year KHV and ISA were included in the proficiency test. Each of the isolates was send out in a high titer and a lower titer version and we also sent a blank sample.
Most laboratories were able to identify the virus but a few labs were not able to identify the virus with low titers and a few labs had contaminated the blanks. A few laboratories only examined for one of the viruses.

Regarding the genotyping we will be more specific on which genotyping system we will like you to use in the next proficiency test as the laboratories used different genotyping systems.

Feedback on possible improvements

In general the feedback was very positive, only "critical" comments were shown. Some laboratories would like more time to solve the test and we will try to see if this will be possible this year.
Laboratories were complaining that the glass ampoules are difficult to open. Unfortunately we will not be able to use the rubber ampoules as the titers do not keep well in these.

Questions

Olga Haenen: A remark on ampoule 1. We tried to inoculate on F25 flask and the sample was negative still after 3 passages. On 6 well plates we saw one plaque at the end of the second passage. It seemed that we generally had better growth on 6 well plates than on flasks. The pH seemed to be o.k. in both systems, concerning the color of the medium. Did anyone of you have similar problems? We now always use as well 6 well plates as small flasks, not to miss any cpe.

Sigrid Cabot: I want to congratulate you all for the very good results. Secondly I will like to thank Niels for a very excellent meeting. Regarding priorities the listed diseases are of course the top priority but also the emerging diseases are an important issue.
I would also like to thank Giuseppe for his excellent work and contribution to the work of the Commission through his participation in numerous expert- and ad hoc groups.

CLOSING REMARKS

Where and when should we have our next meeting? Last year we were talking about organizing the meeting in another laboratory as it is nice to visit the other laboratories. Unfortunately it is not allowed to organize the meeting in other countries unless there are very good scientific reasons for this.

Brit Hjeltnes: It is difficult to reach a decision when and where. You should have a look on when and where it is practical. For next year meeting, one could consider including topics that are relevant for the Industry.

Niels Jørgen Olesen: One idea could be to visit the brand new facilities of FLI.

Sigrid Cabot: I cannot promise you it will be possible to go anywhere else than Denmark but if you have reasons to go elsewhere you might apply and we will have a look at it.

Niels Jørgen Olesen: If you know of meetings etc that would coincide with this meeting please tell us these dates so we do not organize overlapping meetings. I will like to thank both the audience and the speakers for participating and thanks to all for having provided abstracts for the booklet. I will like to ask the speakers if it will be OK for us to upload your presentations. Please contact Søren Kahns or Nicole Nicolajsen.
I will also like to thank Sigrid for helping us. And big thanks go to Nicole Nicolajsen for all her work in organizing this meeting and also thanks to Søren Kahns.
Torsten Boutrup, Søren Peter Jonstrup and Helle Frank Skall have been taking the minutes. A report of the meeting will be made and send to all of you.

Pictures

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

The EURL received the following reagents in 2011

Country	Name	Date of receive	Material	Amount	Protocol no
France	Laurent Bigarre	07-07-11	Nodavirus proficiency test; 11 samples + 4 RNA samples with Noda primer	11	2011-50-192
	Jeannette Castric	14-10-11	Rabbit antiserum against Nodavirus "Ser Lapin Noda 3 d.10-12-97 CNEVA Brest (1:500 IFAT)"	1	2011-50-332
			Rabbit antiserum against Nodavirus "Ser. lap. Anti-Noda CC143 a (0,5ml) d. 6-12-2005 (1:1000 IFAT)"	1	
Iran	Mohsen Dastoor	11-09-11	Diagnostic samples from rainbow trout. Detected positive for IPNV in no. 1-7 and 9	10	2011-50-247
Italy	Guiseppe Bovo	20-04-11	IPN virus collection received for the EURL virus library	123	2011-50-116
		25-01-11	Received at cell culture supernatant marked "IPNV from eel, 2. pass BF-2, 256/V10".	1 vials	2011-50-018
		25-01-11	Isolate received as lyophilized marked "305/93, 15.04.93". EPC cells Pooled tissues from Black bullhead - Black bullhead rhabdovirus	2 ampoules	2011-50-019
Norway	Ingeborg Modahl	20-10-11	Virus for experimental infection in herring and rainbow trout in aquarium experiments VHS+72=NO-F/2009 2 pass BF-2	1	2011-50-289
Poland	Mieczyslaw Pelka	28-06-11	Diagnostic samples from rainbow trout, whole fish, pool of organ tissues. Detected positive for IHNV and IPNV in pool no. 2	2	2011-50-188
		22-08-11	Diagnostic samples from rainbow trout, whole fish. Detected positive for IPNV in pool no. 1-2	2	2011-50-214
		13-10-11	Diagnostic samples from rainbow trout. Detected positive for IPNV in no. 1-2	2	2011-50-279
			Diagnostic samples from rainbow trout. Detected positive for IPNV in no. 1-2	2	2011-50-280
Sweden	Eva Blomkvist	20-10-11	Samples from eel no. 2130, examined for HVA (AnHV-1) by PCR. Detected negative for HVA, IHNV, IPNV, VHSV.	2	2011-50-288
		22-11-11	Diagnostic samples from cell culture. Detected positive for Perch rhabdovirus	2	2011-50-341

The EURL supplied the following reagents in 2011

Country	Name	Material	Type	Amount	Date of shipment
Austria	Khairi El Battawy	Eagles medium with tris	Other	50 ml	21-02-11
		RTS11 cell line from rainbow trout originally received from Dr. Niels C. Bols, University of Waterloo, Canada	Cells	1 small tissue cell flasks	
Bosnia Herzegovina	Adnan Jazic	EPC and BF-2 cells	Cells	One small tissue cell flasks of each	23-02-11
Bulgaria	Vanya Damyanova Chikova	BF-2 cells	Cells	Small tissue cell flasks	27-09-11
Denmark	Klara Jensen	EPC celler	Cells	3 medium tissue cell flasks	08-03-2011 23-06-2011 10-11-2011
Faroe Islands	Debes H. Christiansen and Marita Næs	Ampoule I from the Proficiency test 1 in 2010	Virus	1 ampoule	21-02-11
Finland	Tuija Gadd	*Aphanomyces invadans* inactivated spores, NJM 9701 strain	Spores	2 ampoules	17-10-11
France	Jeannette Castric and Laurent BIGARRE	*Aphanomyces invadans* inactivated spores, NJM 9701 strain	Spores	2 ampoules	17-10-11
Germany	Günter Kotterba	*Aphanomyces Invadans strain*: NJM 9701	Fungi	One plate	09-08-11
		Aphanomyces Invadans strain: NJM 0002 and NJM 9701	Fungi	One plate	23-03-11
Greece	Athanasios Prapas	*Aphanomyces Invadans* strain: NJM 9701	Spores	1 ampoule	18-10-11
		ISAV Glesvaer 2/90	Virus	1 ampoule	
		KHV 07/108b	Virus	1 ampoule	
		Fetal bovine serum	Serum	2 bottles	21-11-11
		EPC and BF-2 cells	Cells	1 medium tissue cell flask of each	
Iceland	Sigridur Gudmundsdottir	7 samples, marked 1-7, containing 1 part IPNV positive or negative material in 5 parts RNAsafer.	Virus	6 samples	12-12-11
		6 samples, marked 1-6, containing 1 part IPNV positive or negative material in 5 parts RNAsafer.	Virus	6 samples	07-10-11
	Sigurdur Helgason	EPC and BF-2 cells	Cells	2 small tissue cell flasks of each	
Iran	Mohaddes Ghasemi	Virus: IPNV Sp, IPN Ab, SVCV isolate 56/70, EHNV Isolate 86/8774, ECV isolate 562/92, KHV, ISAV	Virus	1 ampoule of each	17-10-11
		Virus: VHSV isolates DK-5151, VHSV isolates DK-F1	Virus	1 ampoule of each	
		Aphanomyces invadans	Spores	1 ampoule	
		PAb anti SVCV – F75, PAb anti VHSV – K930, IHNV isolate 32/87, PAb anti IHNV – F63, PAb anti IPN-Sp – F68, PAb anti IPN-Ab – F72, PAb anti VHSV – K930	PAb	1 vial of each	
		Mab anti VHSV – 1P5B11, Mab anti IHNV – Hyb 136-3	Mab	1 vial of each	
		BF-2 and SSN-1 cells	Cells	2 small tissue cell flasks	15-09-11
Italy	Amedeo Manfrin	*Aphanomyces invadans* inactivated spores, NJM 9701	Spores	1 ampoul	17-10-11
Mexico	Igor Romero Sosa	VHSV Inactivated – Genotype Ia: DK-3592b (Voldbjerg)	ELISA	1 vials of each	17-08-11
		VHSV Inactivated - Genotype III: 4p101			
		VHSV Inactivated – Genotype Ib: 1p8			
		VHSV Inactivated - Genotype Iva: USA-Makah			
		VHSV Inactivated - Genotype II: 1p49			
		VHSV Inactivated - Genotype Iva: USA-Makah			

Country	Name	Material	Type	Amount	Date of shipment
		VHSV Inactivated - Genotype II: 1p49	PCR	1 vials of each	
		VHSV Inactivated – Genotype Ia: DK-3592b (Voldbjerg)			
		VHSV Inactivated - Genotype III: 4p101			
		VHSV Inactivated – Genotype Ib: 1p8			
Norway	Bjørn Erik Brudeseth	Tubes 22-26 dated the 22.06.2011 from Simon Brøndsgaard Madsen	Plasma	5 vials	10-08-11
		Tubes 1-21 dated the 22.06.2011 from Simon Brøndsgaard Madsen	Plasma	21 vials	10-08-11
	Monika Hjortaas	Ampoule I from the Inter-Laboratory Proficiency Test 2010	Virus	1 ampoule	08-06-11
Poland	Marek Matras	Pike fry rhabdovirus (PFRV).	Virus	1 ampoule	21-02-11
		Anti-IPNV antibody	PAb	1 ampoule	21-02-11
		Infectious Pancreatic Necrosis virus – IPNV strain Ab	Virus	1 vial	08-06-11
		Infectious Pancreatic Necrosis virus – IPNV strain Sp	Virus	1 vial	08-06-11
Portugal	H. Marina Martins	*Aphanomyces invadans* inactivated spores, NJM 9701	Spores	1 ampoule	03-11-11
Romania	Mihaela Costea	BF-2, EPC, ASK and CCB cells	Cells	2 small tissue cell flasks of each	27-09-11
Serbia	Vladimir, Ivan Radosavljevic	EPC, BF-2 cells	Cells	2 small tissue cell flasks	23-06-11
		Pab anti SVCV – F75	PAb	1 vials	22-03-11
Slovenia	Vlasta Jenčič	*Aphanomyces invadans* inactivated spores, NJM 9701	Spores	1 ampoule	17-10-11
Spain	Carolina Tafalla Piñeiro	Tissue samples from oral vaccine trial	IHC	54 paraffin tissue blocks	05-12-11
		Paraffin tissue blocks: 190-2-10 and 190-1-10: gut from infected fish, IgT+		Paraffin tissue blocks	
		Paraffin tissue blocks: Par 96-5 and 90-5: muscle tissue from vhsG DNA-vaccinated fish, IgT+ in injection site and kidney (positive control for IgT).		Paraffin tissue blocks	
		Paraffin tissue blocks: Par 93-7: vhsG-DNA vaccinated fish, positive control for vhsG.		Paraffin tissue blocks	
		Anti IgM monoclonal antibodies (anti IgM 4C10, dilute 1:2)		1 vials	
		Paraffin tissue blocks: Par 6-2A: VHSV infected fish, positive control for VHSV (i.e. G and N-proteins)		Paraffin tissue blocks	
		Anti IgM monoclonal antibodies (anti IgM F1-18, dilute 1:30)		1 vials	
		Anti IgT monoclonal antibodies (anti IgT F1-15, dilute 1:25)		1 vials	
		Virus: 1p52, 1p53 and 1p54	Virus	1 vial	22-03-11
		Fish samples from NADIR TA1 trial: 4 boxes with cDNA from processed samples in storage buffer.	Vaccine	1 vial	28-06-11
		VHSV DNA vaccine		1 vial	28-06-11
		IHNV DNA vaccine		1 vial	28-06-11
		Alginate		1 vial	28-06-11
		Fish samples from NADIR TA1 trial: 8 boxes with organ samples from vaccinated fish in RNA later		1 vial	28-06-11
		Vaccine plasmid		1 vial	28-06-11
	Pilar Fernández Somalo & Marta Vigo	ASK and CCB cells	Cells	2 small tissue cell flasks of each	27-09-11
		Aphanomyces invadans inactivated spores, NJM 9701	Spores	1 ampoule	
		Aphanomyces invadans inactivated spores, NJM 9701	Spores	1 ampoule	17-10-11

Country	Name	Material	Type	Amount	Date of shipment
Sweden	Suzanne Martelius	KHV 07/108b, 4 pass KF-1, 1 pass CCB – Ampoule VII from the Proficiency test 2 in 2010	Virus	1 ampoule	21-02-11
		ISAV Glesvaer 2/90 - Ampoule VI from the Proficiency test 2 in 2010	Virus	1 ampoule	21-02-11
The Netherlands	Olga L.M. Haenen	Ranavirus maxima, Rmax DK-9995205 Turbot (Psetta maxima)	Virus	1 vials of each	22-03-11
		Cod ranavirus Cod (Gadus morhua)	Virus	1 vials of each	22-03-11

EURL for Fish Diseases

Report of the Inter-Laboratory Proficiency Test 2011

for identification of

VHSV, IHNV and EHNV (PT1)

and identification of

KHV, ISAV and *Aphanomyces invadans* (PT2)

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

DANAK
PT Reg. no.: 515

DTU

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Introduction

A comparative test of diagnostic procedures was provided by the European Union Reference Laboratory (EURL) for Fish Diseases. The test was divided into two proficiency tests, proficiency test 1 (PT1) and proficiency test 2 (PT2). PT1 was designed as the proficiency tests provided by the EURL in previous years to primarily assess the identification of the fish viruses viral haemorrhagic septicaemia virus (VHSV), infectious haematopoietic necrosis virus (IHNV) and epizootic haematopoietic necrosis virus (EHNV) by cell culture based methods. PT2 was included for the second time with the aim of assessing the ability of participating laboratories to identify the fish pathogens infectious salmon anaemia virus (ISAV), koi herpes virus (KHV) and *Aphanomyces invadans* by PCR based methods. It is the first time *Aphanomyces invadans* has been included in the proficiency test. The number of National Reference Laboratories (NRLs) participating in PT1 and PT2 was 41.
The tests were sent from the EURL in the middle of October 2011.
Both PT1 and PT2 are accredited by DANAK under registration number 515 to proficiency testing according to the quality assurance standard DS/EN ISO/IEC 17043. This report covers both the results of PT1 and PT2.

PT1 consisted of five coded ampoules (I-V). The ampoules contained VHSV, EHNV, European catfish virus (ECV), IHNV+IPNV and IPNV, respectively, see table 1. The proficiency test was designed to primarily assess the ability of participating laboratories to identify the fish viruses VHSV, IHNV and ENHV (all listed in Council Directive 2006/88/EC) if present in the ampoules, bearing in mind that the test ampoules also could contain other viruses (e.g. other fish rhabdoviruses, ranaviruses, or birnaviruses). In addition the participants were asked to quantify the viruses in PT1 by titration in order to assess the susceptibility of their fish cell lines for virus infection. Participants were encouraged to use their normal standard laboratory procedures. However, the identification should be performed according to the procedures laid down in Commission Decision 2001/183/EC using fish cell cultures followed by e.g. neutralisation test, immunofluorescence, ELISA or PCR. If ranavirus was present in any of the ampoules, it was mandatory to perform a sequence analysis or a restriction endonuclease analysis (REA) of the isolate in order to determine if the isolate was EHNV or another ranavirus and it was recommended to follow the procedures described in Chapter 2.3.1 in the OIE Manual of Diagnostic Tests for Aquatic Animals 2009. Laboratories were encouraged to identify VHSV and IHNV isolates as far as possible by means of genotyping in addition to the standard methods. It was recommended to use the genotype notification described in Einer-Jensen et al. (2004) for VHSV and in Kurath et al. (2003) for IHNV. Laboratories were encouraged to submit all sequencing results used for genotyping the isolates.

PT2 consisted of five coded ampoules (VI-X). The ampoules contained ISAV and KHV. Furthermore, one ampoule contained *Aphanomyces invadans*, see table 11. It was decided at the 15th Annual Meeting of the NRLs for Fish Diseases in Aarhus 26-27 May 2011, that testing for *A. invadans* for the first time should be included in the yearly proficiency test provided by the EURL. The test was designed to primarily assess the ability of participating laboratories to identify the notifiable fish pathogens ISAV, KHV and *A. invadans* (listed in Council Directive 2006/88/EC) if present in the ampoules, bearing in mind that the test ampoules could also contain other viruses. Participants were expected to use their normal PCR or real-time PCR methods for detection of the pathogens. It was not mandatory to grow KHV and ISAV on cell cultures in this test, but the viruses were not inactivated and

they should thus be possible to amplify in cell cultures. If present, only **inactivated** *A. invadans* was included in the ampoules.

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

The participants were requested to download an excel sheet for filling in results and submit the filled out sheet electronically. Additionally, participants were asked to answer a questionnaire regarding the accreditation status of their laboratory. Collected accreditation data will not be presented in this report but will be presented at the 16th Annual Meeting of the NRLs for Fish Diseases in Aarhus 30-31 May 2012. Participants were asked to reply latest 16 December 2011.

Distribution of the test

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

Thermo-loggers were included in 10 of the parcels. The thermo-loggers were returned immediately upon receipt of the proficiency tests and a computer programme translated the data into a graph, showing the temperature inside the parcel for every 30 minutes during transportation. The loggers were programmed to mark if the temperature had exceeded 30°C at some point during transportation. Inclusion of loggers should display if the temperature encountered during transport had been detrimental to the viability of the virus in the test.

Shipment and handling

Within four days, the tests were delivered to 32 participants; 6 tests were delivered within 7 days and 2 test within 2 weeks and 1 test within three weeks (figure 1). All the parcels were sent without cooling elements. The average temperature was 16°C for the transports to the 8 countries were the temperature did not exceed 30°C. The temperature exceeded 30°C for one transport for half an hour upon arrival and for one transport the temperature was 38-42°C for 2 hours.

To test the influence of temperature the ampoules were subjected for a period of 5 hours to temperatures from 20°C to 42°C (figure 2). Then ampoules I-V were titrated on cell cultures and ampoules VI-X were tested by PCR. No significant decrease in the titres was observed for any of the tested ampoules and it was still possible to identify the pathogens by PCR methods. As the ampoules followed by a logger at no point exceeded such extreme temperatures during shipment, it is considered that the temperature variation that the ampoules experienced during shipment did not influence considerably on the pathogens.

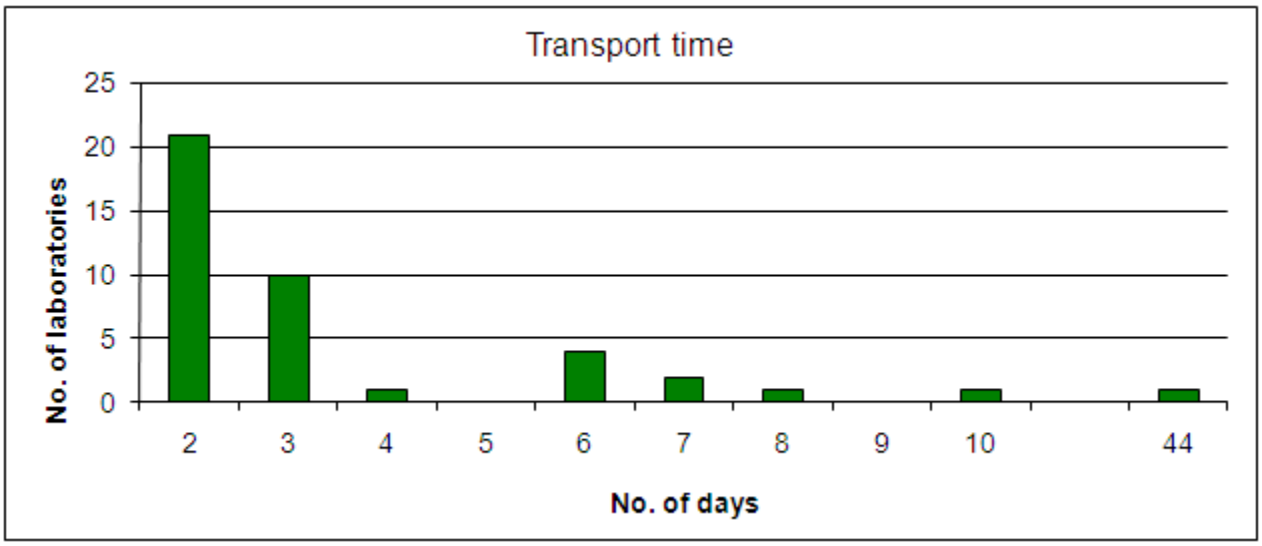


Figure 1. Transport time for the parcels to reach the participants.

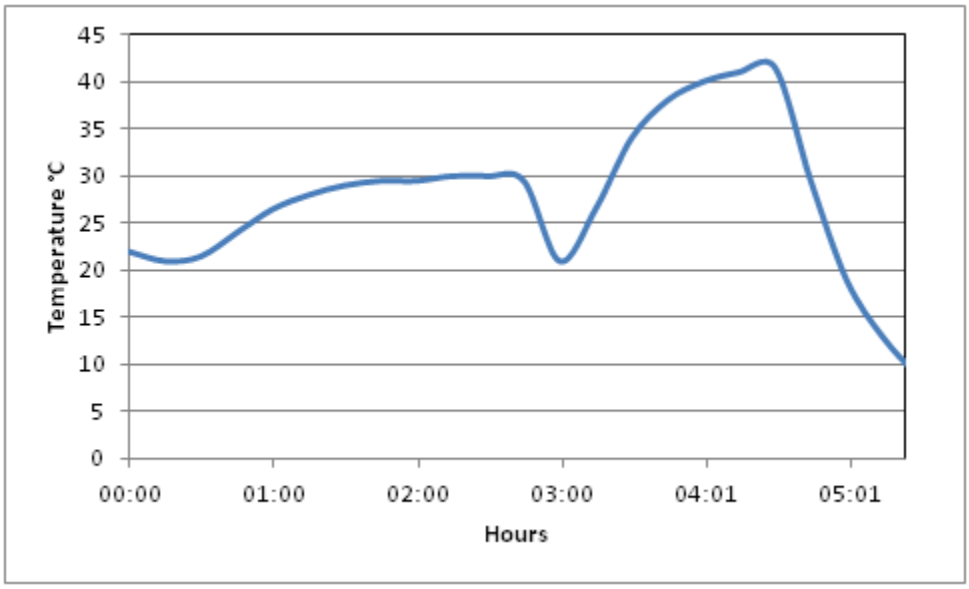


Figure 2. The ampoules were subjected to temperatures of 20-42°C for a period of 5 hours and then tested.

Participation

PT1: 41 laboratories received the annual proficiency test. Of these, 39 participants submitted results within the deadline. One participant submitted sequencing results 5 days after deadline but before the content of the ampoules was made public available. Two participants did not submit results.

PT2: 41 laboratories received the annual proficiency test. Of these, 38 participants submitted results within the deadline. One participant submitted the results for *A. invadans* 7 days after deadline but before the content of the ampoules was made public available. Three participants did not submit results.

Figure 3 show how many laboratories that participated in the proficiency test from 1996 to 2011.

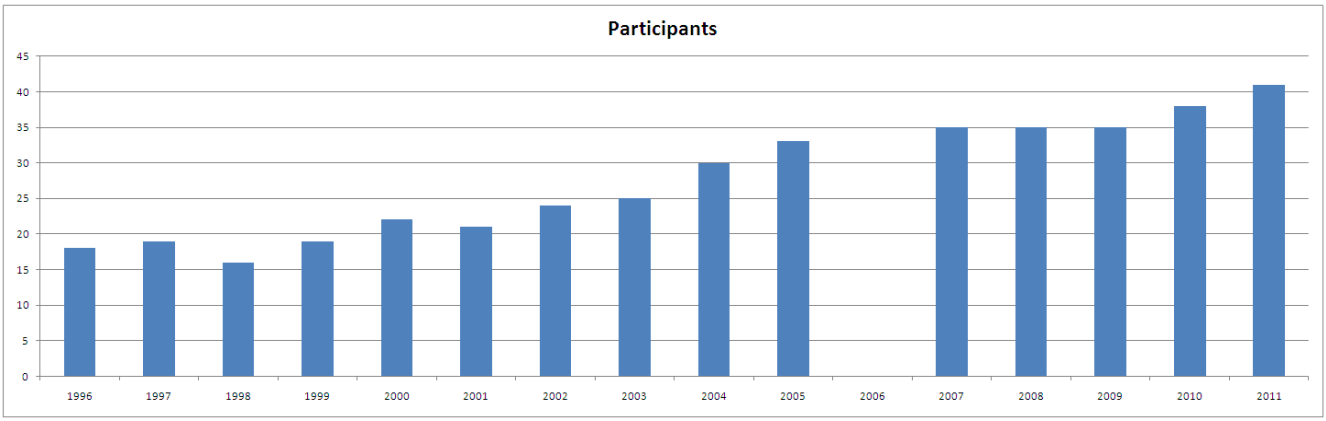


Figure 3. Participants in the EURL proficiency test over the years.

Proficiency test 1, PT1

Five ampoules with lyophilised cell culture supernatant were delivered to all NRLs in the EU Member States, including Denmark, and likewise to the NRLs in Australia, Bosnia and Herzegovina, Croatia, Faroe Islands, Iceland, Israel, Iran, Japan, Norway, P.R. China, Serbia, Switzerland, Turkey and 2 from USA. The Belgian NRL covers both Belgium and Luxembourg and the Italian NRL covers Italy, Cyprus and Malta for identification of all listed diseases. Figure 4 shows the worldwide distribution of the participating NRLs.

Figure 4. Worldwide distribution of the participants in the EURL proficiency test 2011.

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 viruses.

PT1	
Code	**Specifications**
Ampoule I: VHSV Isolate 1p8	**VHSV 1p8** Marine isolate from herring (*Clupea harengus*) caught in the Baltic Sea in 1996. Cell culture passage number: 7 Genotype Ib. GenBank accession numbers: AY546573 (G-gene) and GQ325430, AY356652 (N-gene) www.fishpathogens.eu ID number: 2251 Reference on isolate: Mortensen HF, Heuer OE, Lorenzen N, Otte L and Olesen NJ (1999). Isolation of viralhaemorrhagic septicaemia virus (VHSV) from wild marine fish species in the Baltic Sea,Kattegat, Skagerrak and the North Sea. *Virus Research* **63**, 97-108. References on sequences: 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, Ahrens P, Forsberg R and Lorenzen N (2004). Evolution of the fish rhabdovirus viral haemorrhagic septicaemia virus. *Journal of General Virology* **85**, 1167-1179. Snow M, Bain N, Black J, Taupin V, Cunningham CO, King JA, Skall HF and Raynard RS (2004). Genetic population structure of marine viral haemorrhagic septicaemia virus (VHSV). *Diseases of Aquatic Organisms* **61**, 11-21.
Ampoule II: EHNV Isolate 86/8774	**EHNV Isolate 86/8774** Received from the EHNV OIE reference laboratory (EURL file number 202213). Australian freshwater isolate from rainbow trout from Adaminaby Trout Farm, NSW obtained in 1986 by Jeremy Langdon. Cell culture passage number: 8 GenBank accession numbers: FJ433873, AY187045, AF157667 Reference on isolate: Langdon JS, Humphrey JD & Williams LM (1988). Outbreaks of an EHNV-like iridovirus in cultured rainbow trout, *Salmo gairdneri* Richardson, in Australia. *Journal of Fish Diseases* **11**, 93-96. References on sequences: Hyatt A.D., Gould A.R., Zupanovic Z., Cunningham A.A., Hengstberger S., Whittington R.J., Kattenbelt J. & Coupar B.E.H. (2000) Comparative studies of piscine and amphibian iridoviruses. Archives of Virology 145, 301-331. Jancovich J.K., Bremont M., Touchman J.W. & Jacobs B.L. (2010) Evidence for multiple recent host species shifts among the ranaviruses (family Iridoviridae). Journal of Virology 84, 2636-2647. Marsh I.B., Whittington R.J., O'Rourke B., Hyatt A.D. & 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 III: European Catfish virus (ECV) Isolate 562/92 Low titre	**European catfish virus 562/92.** Italian isolate from catfish suffering high mortality. Received from Dr. G. Bovo, ISZ-Ve, Padova, Italy. Cell culture passage number: 7 GenBank accession number: FJ358608 Reference on isolate: Bovo G, Comuzi M, De Mas S, Ceschia G, Giorgetti G, Giacometti P & Cappellozza E (1993). Isolamento di un agente virale irido-like da pesce gatto (Ictalurus melas) dallevamento. *Bollettino Societa Italiana di Patologia Ittica* **11**, 3–10. Reference on sequence: Holopainen R., Ohlemeyer S., Schütze H., Bergmann S.M. & Tapiovaara H. (2009) Ranavirus phylogeny and differentiation based on major capsid protein, DNA polymerase and neurofilament triplet H1-like protein genes. *Diseases of Aquatic Organisms* **85**, 81-91.

PT1	
Code	**Specifications**
Ampoule IV: IHNV Isolate 32/87 + IPNV Strain Sp	**IHNV 32/87.** First French isolate (April 1987) from rainbow trout. Cell culture passage number: 9 GenBank accession numbers: J265717 and AY524121 (G-gene), FJ265711 (N-gene). Reference on isolate: Baudin Laurencin F**.** (1987) IHN in France. *Bulletin of the European Association of Fish Pathologists* **7**, 104. Reference on sequence: Kolodziejek J., Schachner O., Dürrwald R., Latif M. & Nowotny N. (2008) "Mid-G" region sequences of the glycoprotein gene of Austrian infectious hematopoietic necrosis virus isolates form two lineages within European isolates and are distinct from American and Asian lineages. *Journal of Clinical Microbiology* **46**, 22-30. 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. + **Type strain Sp (Spjarup) of IPN virus**. Cell culture passage number in BF-2: 17 Reference on isolate: Jørgensen P.E.V. & Bregnballe F. (1969) Infectious pancreatic necrosis in rainbow trout in Denmark. *Nordisk Veterinærmedicin* **21**, 142-148. Jørgensen P.E.V. & Grauballe P.C. (1971) Problems in the serological typing of IPN virus. *Acta Veterinaria Scandinavica* **12**, 145-147.
Ampoule V: IPNV Strain Sp	**Type strain Sp (Spjarup) of IPN virus**. Cell culture passage number in BF-2: 17 Reference on isolate: Jørgensen P.E.V. & Bregnballe F. (1969) Infectious pancreatic necrosis in rainbow trout in Denmark. *Nordisk Veterinærmedicin* **21**, 142-148. Jørgensen P.E.V. & Grauballe P.C. (1971) Problems in the serological typing of IPN virus. *Acta Veterinaria Scandinavica* **12**, 145-147.

Testing of the PT1 test

The PT1 test was prepared and tested according to protocols accredited under DS/EN ISO/IEC 17043. Prior to distribution the EURL tested 5 ampoules of each virus preparation by titration in 4 cell lines (BF-2, EPC, RTG-2 and FHM), to ascertain a satisfactory titre in the preferred cell line and homogeneity of content of ampoules (Table 2).

The lyophilisation procedure caused a significant titre reduction, especially for VHSV on BF-2, EPC and RTG-2 cells where a 2-3 log reduction was observed. For the other cell lines a reduction between 1 - 2 log for IHNV/IPNV was observed. For EHNV and ECV a minimal titre reduction of 0 - 1 log was observed. For IPNV the titre reduction was 1-2 log (table 2 and figure 5). However, all titres of the lyophilised viruses were above detection level, except for VHSV on RTG-2 cells. As participants are expected to use two different cell lines, VHSV should be detected on the other cell line.
Lyophilised viruses are very stable at storing. We have previously shown that lyophilised virus kept in glass sealed 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).
We have furthermore shown that lyophilised virus in glass sealed ampoules is stable has after exposed to 30°C for 24 hours (Report of the Inter-Laboratory Proficiency Test for National Reference Laboratories for Fish Diseases 2010).

The identities of the viruses in all 5 ampoules were checked and confirmed by ELISA, IFAT, serum neutralisation tests, RT-PCR and sequencing for VHSV, IHNV and IPNV (RT-PCR/sequencing not performed for this virus) and by PCR, sequencing and IFAT for EHNV and ECV. For each ampoule, presence of viruses other than the expected was not observed.
We tested the titre of each virus preparation (ampoule) after 3 months storage in the dark at 4°C and observed no significant decrease compared to right after lyophilization.

Table 2. Titre of representative ampoules of no. I to V tested at the EURL in four cell lines before lyophilisation, immediately after lyophilisation (median titre of 5 replicates), and after 3 months of storage in the dark at 4°C and after being held at a temperature raised from 20-42°C over a period of 5 hours (1 replicate).

Ampoule No.	Cell line	Titre before lyophilisation	Median titre right after lyophilisation	Titre 3 months after lyophilisation (4°C, dark conditions)	Titre 3 months after lyophilisation (20-42°C, dark conditions)
		$TCID_{50}$/ml	$TCID_{50}$/ml	$TCID_{50}$/ml	$TCID_{50}$/ml
Ampoule I: VHSV Isolate 1p8	BF-2	$2,7*10^8$	$5,9*10^5$	$1,3*10^6$	$2,7*10^5$
	EPC	$8,6*10^5$	$2,7*10^3$	$8,6*10^3$	$5,9*10^3$
	RTG-2	$4,0*10^5$	$< 1,9*10^2$	$< 1,9*10^2$	$< 1,9*10^2$
	FHM	$1,9*10^8$	$1,9*10^6$	$1,3*10^7$	$5,9*10^6$
Ampoule II: EHNV Isolate 86/8774	BF-2	$5,9*10^4$	$5,9*10^5$	$2,7*10^5$	$2,7*10^5$
	EPC	$1,3*10^5$	$1,9*10^4$	$5,9*10^4$	$1,9*10^5$
	RTG-2	$8,6*10^4$	$4,0*10^3$	$4,0*10^4$	$1,9*10^4$
	FHM	$8,6*10^3$	$1,3*10^3$	$1,3*10^3$	$1,3*10^3$
Ampoule III: European Catfish virus (ECV) Isolate 562/92 Low titre	BF-2	$1,3*10^5$	$1,3*10^4$	$1,3*10^4$	$8,6*10^3$
	EPC	$5,9*10^4$	$1,3*10^4$	$8,6*10^3$	$8,6*10^3$
	RTG-2	$8,6*10^4$	$1,3*10^3$	$1,3*10^4$	$1,3*10^4$
	FHM	$1,3*10^3$	$1,3*10^3$	$< 1,9*10^2$	$< 1,9*10^2$
Ampoule IV: IHNV Isolate 32/87 + IPNV Strain Sp	BF-2	$4,0*10^6$	$2,7*10^5$	$1,9*10^6$	$4,0*10^6$
	EPC	$5,9*10^7$	$1,9*10^5$	$1,9*10^6$	$2,7*10^6$
	RTG-2	$2,7*10^5$	$8,6*10^4$	$2,7*10^5$	$8,6*10^5$
	FHM	$4,0*10^6$	$2,7*10^5$	$4,0*10^5$	$4,0*10^5$
Ampoule V: IPNV Strain Sp	BF-2	$1,3*10^8$	$5,9*10^6$	$2,7*10^7$	$4,0*10^5$
	EPC	$2,7*10^8$	$1,9*10^6$	$1,9*10^7$	$5,9*10^5$
	RTG-2	$5,9*10^7$	$8,6*10^5$	$1,3*10^7$	$4,0*10^5$
	FHM	$4,0*10^7$	$1,9*10^5$	$1,3*10^6$	$2,7*10^5$

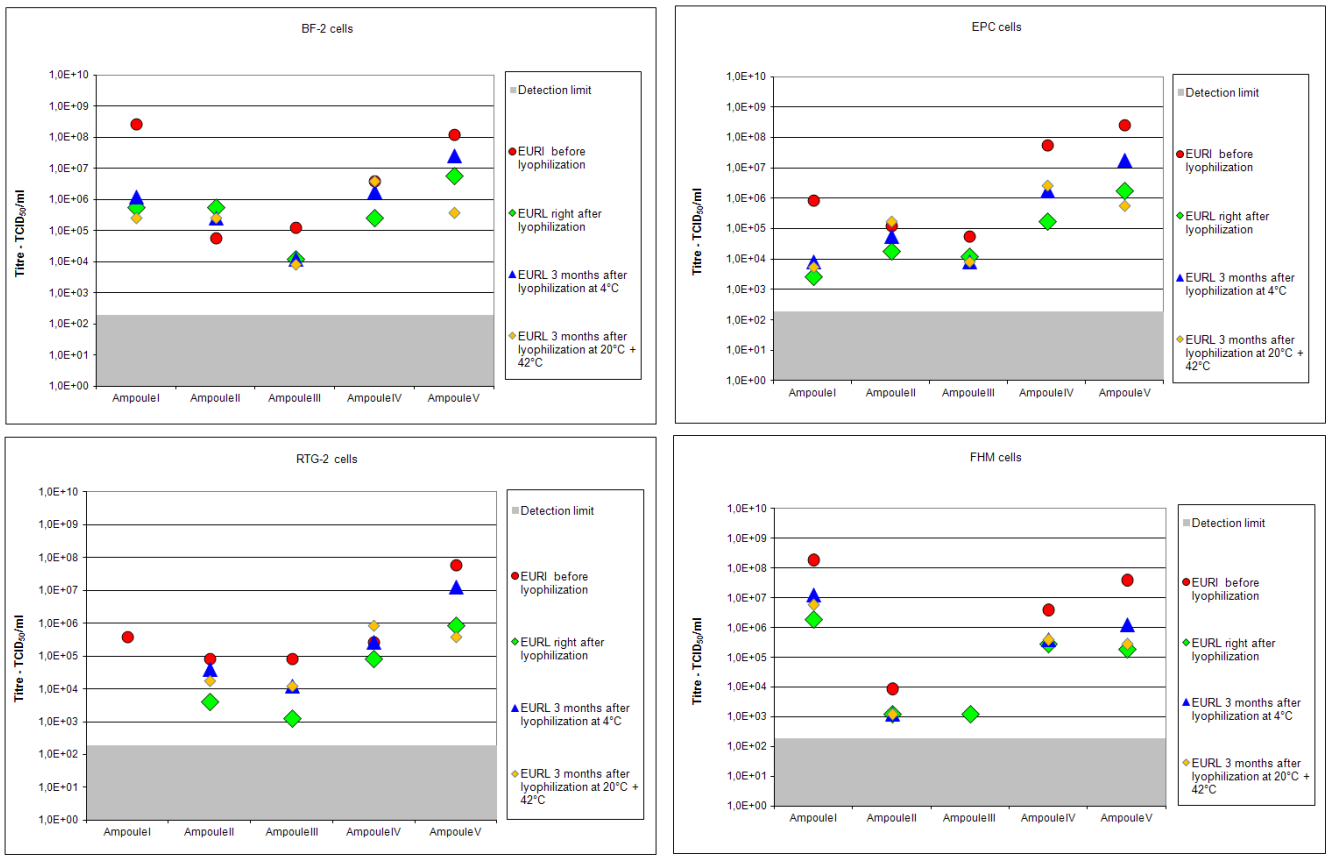


Figure 5. Virus titers in different cell lines before, right after, 3 months after lyophilisation and after staying at a temperature raised from 20-42°C over a period of 5 hours. Grey area is below detection level.

Virus identification and titration

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

Participants were also asked to titrate the contents of the ampoules. The method of titration was described in the instructions enclosed with the test. All titres were calculated at the EURL based on the crude data submitted by each participant and given as Tissue Culture Infective Dose 50% ($TCID_{50}$) per ml. The titre of the re-dissolved virus was multiplied by a factor of 10 to compensate for the dilution of the original volume of virus in the ampoules (200 µl virus + 200 µl lactalbumin in vials re-dissolved in a total of 2.0 ml cell culture medium)**.** Titration results of the viruses of the 5 ampoules for the participating laboratories are summarised in tables 4 to 8. The titres obtained from each participating laboratory are represented graphically. In figures 6-9, 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 three laboratories 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 or restriction endonuclease analysis of the isolate in order to determine if the isolate is EHNV.

Table 3. Inter-Laboratory Proficiency Test, PT1, 2011 - Virus identification.

Laboratory code number	Score 10/10	Answer recived at EURL	Ampoule I	Ampoule II	Ampoule III	Ampoule IV	Ampoule V
			VHSV Isolate 1p8	EHNV Isolate 86/8774	Ranavirus, not EHNV European Catfish virus (ECV) 562/92 Low titre	IHNV and IPNV IHNV 32/87 + IPNV Strain Sp	IPNV IPNV Strain Sp
1	10	13-12-11	VHSV	EHNV	Ranavirus, not EHNV	IHNV and IPNV	IPNV
2[1]	8	29-11-11	VHSV	EHNV	Ranavirus, not EHNV	IHNV	Not VHSV, IHNV and EHNV
3	10	12-12-11	VHSV	EHNV	Ranavirus, not EHNV	IHNV and IPNV	IPNV
4	7	16-12-11	VHSV	Ranavirus*	VHSV	IHNV and IPNV	IPNV
5	10	13-12-11	VHSV	EHNV	Ranavirus, not EHNV	IHNV and IPNV	IPNV
6	10	09-12-11	VHSV	EHNV	Ranavirus, not EHNV	IHNV and IPNV	IPNV
7[2]	6	16-12-11	VHSV	Not VHSV, IHNV, IPNV, SVCV	Not VHSV, IHNV, IPNV, SVCV	IHNV and IPNV	IPNV
8	10	16-12-11	VHSV	EHNV	Ranavirus, not EHNV	IHNV and IPNV	IPNV
9	10	23-11-11	VHSV	EHNV	Ranavirus, not EHNV	IHNV and IPNV	IPNV
10	10	16-12-11	VHSV	EHNV	Ranavirus, not EHNV	IHNV and IPNV	IPNV
11[3]	10	16-12-2011 21-12-2011	VHSV	EHNV	Ranavirus, not EHNV	IHNV and IPNV	IPNV
12	10	08-12-11	VHSV	EHNV	Ranavirus, not EHNV	IHNV and IPNV	IPNV
13	10	15-12-11	VHSV	EHNV	Ranavirus, not EHNV	IHNV and IPNV	IPNV
14	10	16-12-11	VHSV	EHNV	Ranavirus, not EHNV	IHNV and IPNV	IPNV
15	8	16-12-11	VHSV	Ranavirus*	Ranavirus*	IHNV and IPNV	IPNV
16	10	15-12-11	VHSV	EHNV	Ranavirus, not EHNV	IHNV and IPNV	IPNV
17	8	14-12-11	VHSV	EHNV	Ranavirus, not EHNV	IPNV	IPNV
18	0	n/a	No reply	No reply	No reply	No reply	No reply
19	10	16-12-11	VHSV	EHNV	Ranavirus, not EHVN	IHNV and IPNV	IPNV
20	10	16-12-11	VHSV	EHNV	Ranavirus, not EHNV	IHNV and IPNV	IPNV
21	9	16-12-11	VHSV	EHNV	Ranavirus, not EHNV	IHNV	IPNV
22	10	16-12-11	VHSV	EHNV	Ranavirus, not EHNV	IHNV and IPNV	IPNV
23	6	14-12-11	VHSV	Ranavirus*	Ranavirus*	IPNV and Ranavirus*	IPNV
24	10	07-12-11	VHSV	EHNV	Ranavirus, not EHNV	IHNV and IPNV	IPNV
25	10	16-12-11	VHSV	EHNV	Ranavirus, not EHNV	IHNV and IPNV	IPNV
26	10	06-12-11	VHSV	EHNV	Ranavirus not EHNV	IHNV and IPNV	IPNV
27	10	16-12-11	VHSV	EHNV	Ranavirus, not EHNV	IHNV and IPNV	IPNV
28	0	n/a	No reply	No reply	No reply	No reply	No reply
29	10	16-12-11	VHSV	EHNV	Ranavirus, not EHNV	IHNV and IPNV	IPNV
30	10	16-12-11	VHSV	EHNV	Ranavirus, not EHNV	IHNV and IPNV	IPNV
31	8	16-12-11	VHSV	EHNV	Ranavirus, not EHNV	IPNV	IPNV
32	4	15-12-11	VHSV	Ranavirus*	Ranavirus* and SVCV	IHN and SVCV	not ranavirus, not IHNV, VHSV, SVCV
33	10	16-12-11	VHSV	EHNV	Ranavirus, not EHNV	IHNV and IPNV	IPNV
34	10	16-12-11	VHSV	EHNV	Ranavirus, not EHNV	IHNV and IPNV	IPNV
35	9	16-12-11	VHSV	EHNV	Ranavirus, not EHNV	IHNV	IPNV
36	7	16-12-11	VHSV	Virus not identified	Ranavirus*	IHNV and IPNV	IPNV
37	10	13-12-11	VHSV	EHNV	Ranavirus, not EHNV	IHNV and IPNV	IPNV
38	8	16-12-11	VHSV	Ranavirus*	Ranavirus*	IHNV and IPNV	IPNV
39	10	07-12-11	VHSV	EHNV	Ranavirus, not EHNV	IHNV and IPNV	IPNV
40	8	16-12-11	VHSV	EHNV	Ranavirus, not EHNV	IHNV	not EHNV or other ranavirus, not IHNV, VHSV, SVCV
41	10	16-12-11	VHSV	EHNV	Ranavirus, not EHNV	IHNV and IPNV	IPNV

[1] Only tested by PCR

[2] Not tested for Ranavirus

[3] The laboratory submitted sequencing results after deadline but before ampoule contents were made public available. The result of this participant is therefore included in this report.

* Genomic analysis not performed

n/a: not applicable

Table 4. Inter-Laboratory Proficiency Test, PT1, 2011 – Results of titration of ampoule I.

Ampoule I - VHSV					
Laboratory code number	Virus Identification	Titre in			
		BF-2	EPC	RTG-2	FHM
1	VHSV	$2,7*10^4$	$1,3*10^3$	$5,9*10^5$	
2	VHSV	not performed	not performed	not performed	not performed
3	VHSV	$4,0*10^3$	$4,0*10^3$		
4	VHSV	$4,0*10^2$	$2,7*10^2$		
5	VHSV	$5,9*10^5$	$4,0*10^3$	$4,0*10^3$	$1,9*10^3$
6	VHSV	$5,9*10^5$	$1,9*10^3$	$< 1,9*10^2$	$4,0*10^6$
7	VHSV	$4,0*10^4$	$1,3*10^3$		
8	VHSV	$4,0*10^5$	$4,0*10^4$		
9	VHSV	$1,3*10^7$	$4,0*10^4$	$5,9*10^4$	
10	VHSV	$< 1,9*10^2$	$2,7*10^2$	$< 1,9*10^2$	$1,3*10^4$
11	VHSV	$4,0*10^5$	$5,9*10^3$		
12	VHSV	$1,3*10^5$	$1,3*10^5$		
13	VHSV	$1,3*10^5$	$< 1,9*10^2$		
14	VHSV	$1,9*10^5$	$5,9*10^3$		
15	VHSV	$5,9*10^5$	$2,7*10^2$		
16	VHSV		$2,7*10^4$	$1,3*10^3$	
17	VHSV	$1,3*10^6$	$4,0*10^5$	$8,6*10^4$	$1,3*10^6$
18	No reply				
19	VHSV	$1,3*10^4$	$< 1,9*10^2$		$< 1,9*10^2$
20	VHSV	$1,3*10^5$	$1,3*10^5$	$1,3*10^4$	$1,3*10^5$
21	VHSV		$1,9*10^4$	$5,9*10^2$	
22	VHSV	$8,6*10^5$	$2,7*10^4$	$2,7*10^5$	$1,3*10^4$
23	VHSV	$4,0*10^4$			$1,9*10^5$
24	VHSV		$1,9*10^3$		$1,9*10^3$
25	VHSV	$1,3*10^4$	$1,3*10^5$		
26	VHSV	$1,9*10^6$			$8,6*10^4$
27	VHSV	$1,3*10^5$	$5,9*10^3$	$< 1,9*10^2$	
28	No reply				
29	VHSV	$2,7*10^5$	$8,6*10^3$		
30	VHSV	$2,7*10^5$	$1,3*10^4$		
31	VHSV	$5,9*10^5$	$5,9*10^4$		
32	VHSV	$1,9*10^2$	$< 1,9*10^2$		
33	VHSV	$4,0*10^5$	$1,3*10^3$	$< 1,9*10^2$	$1,3*10^5$
34	VHSV	$1,9*10^4$	$1,9*10^2$		
35	VHSV				$< 1,9*10^2$
36	VHSV	$1,3*10^6$	$< 1,9*10^2$		
37	VHSV	$8,6*10^5$	$2,7*10^3$		
38	VHSV	$2,7*10^5$	$1,9*10^4$		
39	VHSV	$5,9*10^3$	$8,6*10^4$	$5,9*10^4$	$< 1,9*10^2$
40	VHSV	$2,7*10^5$	$2,7*10^2$		$4,0*10^3$
41	VHSV	$1,9*10^4$	$2,7*10^4$		$4,0*10^5$

Number of laboratories	34	35	13	15
Median titre	$2,3*10^5$	$5,9*10^3$	$4,0*10^3$	$1,3*10^4$
Maximum titre	$1,3*10^7$	$4,0*10^5$	$5,9*10^5$	$4,0*10^6$
Minimum titre	$<1,9*10^2$	$<1,9*10^2$	$<1,9*10^2$	$<1,9*10^2$
25% quartile titre	$2,1*10^4$	$7,7*10^2$	$<1,9*10^2$	$1,9*10^3$
75% quartile titre	$5,9*10^5$	$2,7*10^4$	$5,9*10^4$	$1,3*10^5$

Table 5. Inter-Laboratory Proficiency Test, PT1, 2011 – Results of titration of ampoule II.

Ampoule II - EHNV					
Laboratory code number	Virus Identification	Titre in			
		BF-2	EPC	RTG-2	FHM
1	EHNV	$1,9*10^5$	$5,9*10^4$	$2,7*10^5$	
2	EHNV	not performed	not performed	not performed	not performed
3	EHNV	$5,9*10^5$	$1,3*10^5$		
4	Ranavirus (not seq)	$1,3*10^3$	$4,0*10^4$		
5	EHNV	$1,3*10^3$	$5,9*10^4$	$4,0*10^3$	$4,0*10^3$
6	EHNV	$1,3*10^6$	$2,7*10^5$	$1,3*10^4$	$1,3*10^3$
7	Not VHSV, IHNV, IPNV, SVCV	$5,9*10^6$	$4,0*10^6$		
8	EHNV	$8,6*10^5$	$1,9*10^4$		
9	EHNV	$5,9*10^6$	$8,6*10^4$	$1,9*10^4$	
10	Ranavirus - EHNV	$4,0*10^4$	$1,9*10^4$	$< 1,9*10^2$	$< 1,9*10^2$
11	EHNV	$1,3*10^4$	$4,0*10^4$		
12	EHNV	$1,3*10^5$	$1,9*10^5$		
13	EHNV	$1,9*10^5$	$1,9*10^4$		
14	EHNV	$2,7*10^6$	$1,3*10^5$		
15	EHNV (not seq, therefore Ranavirus)	$5,9*10^5$	$1,9*10^5$		
16	EHNV		$1,3*10^3$	$4,0*10^3$	
17	EHNV	$1,9*10^6$	$4,0*10^5$	$2,7*10^5$	8,6*104
18	No reply				
19	EHNV	$4,0*10^4$	$< 1,9*10^2$		$< 1,9*10^2$
20	EHNV	$1,3*10^5$	$1,3*10^5$	$1,3*10^5$	$1,3*10^5$
21	EHNV		$4,0*10^6$	$1,9*10^4$	
22	EHNV	$2,7*10^6$	$1,3*10^6$	$4,0*10^6$	$1,9*10^6$
23	Ranavirus (not seq)	$4,0*10^4$			$1,3*10^3$
24	EHNV		$4,0*10^5$		$2,7*10^4$
25	EHNV	$1,3*10^3$	$2,7*10^5$	$4,0*10^4$	
26	EHNV	$1,3*10^5$			$4,0*10^5$
27	EHNV	$4,0*10^6$	$5,9*10^5$	$5,9*10^3$	
28	No reply				
29	EHNV	$4,0*10^4$	$1,9*10^5$		
30	EHNV	$2,7*10^5$	$1,3*10^5$		
31	EHNV	$4,0*10^5$	$2,7*10^4$		
32	EHNV (not seq, therefore Ranavirus)	$1,3*10^5$	$4,0*10^5$		
33	EHNV	$2,7*10^6$	$5,9*10^4$	$8,6*10^2$	$< 1,9*10^2$
34	EHNV	$5,9*10^4$	$4,0*10^3$		
35	EHNV				$1,3*10^4$
36	Virus not identified	$5,9*10^6$	$5,9*10^4$		
37	EHNV	$1,3*10^6$	$1,3*10^5$		
38	Ranavirus	$4,0*10^5$	$1,9*10^6$		
39	EHNV	$4,0*10^5$	$8,6*10^4$	$1,9*10^5$	$8,6*10^4$
40	EHNV	$5,9*10^6$	$4,0*10^4$		$2,7*10^4$
41	EHNV	$4,0*10^4$	$5,9*10^5$		$1,9*10^3$

Number of laboratories	34	35	14	15
Median titre	$3,4*10^5$	$1,3*10^5$	$1,9*10^4$	$1,3*10^4$
Maximum titre	$5,9*10^6$	$4,0*10^6$	$4,0*10^6$	$1,9*10^6$
Minimum titre	$1,3*10^3$	$<1,9*10^2$	$<1,9*10^2$	$<1,9*10^2$
25% quartile titre	$4,5*10^4$	$4,0*10^4$	$4,5*10^3$	$1,3*10^3$
75% quartile titre	$1,7*10^6$	$3,4*10^5$	$1,7*10^5$	$8,6*10^4$

Table 6. Inter-Laboratory Proficiency Test, PT1, 2011 – Results of titration of ampoule III.

Ampoule III - Ranavirus					
Laboratory code number	Virus Identification	Titre in			
		BF-2	EPC	RTG-2	FHM
1	ECV or ESV	$4,0*10^4$	$1,9*10^5$	$4,0*10^4$	
2	ECV or ESV	not performed	not performed	not performed	not performed
3	ECV or ESV	$4,0*10^4$	$4,0*10^4$		
4	VHSV	$4,0*10^2$	$2,7*10^2$		
5	ESV or ECV	$< 1,9*10^2$	$2,7*10^3$	$< 1,9*10^2$	$< 1,9*10^2$
6	ECV or ESV	$4,0*10^5$	$1,3*10^5$	$1,3*10^4$	$< 1,9*10^2$
7	Not VHSV, IHNV, IPNV, SVCV	$8,6*10^4$	$1,3*10^5$		
8	ECV or ESV	$8,6*10^4$	$1,3*10^4$		
9	Ranavirus - ECV	$4,0*10^5$	$1,9*10^5$	$8,6*10^2$	
10	Ranavirus - ESV	$1,9*10^3$	$1,3*10^4$	$< 1,9*10^2$	$< 1,9*10^2$
11	ECV or ESV	$8,6*10^3$	$4,0*10^3$		
12	ESV	$5,9*10^4$	$1,9*10^4$		
13	ECV or ESV	$2,7*10^4$	$4,0*10^3$		
14	ECV orESV	$1,9*10^5$	$2,7*10^5$		
15	EHNV (not seq, therefore Ranavirus)	$1,9*10^3$	$1,9*10^3$		
16	Ranavirus - ECV		$2,7*10^3$	$2,7*10^4$	
17	ECV or ESV	$1,3*10^5$	$4,0*10^4$	$1,3*10^5$	$1,3*10^3$
18	No reply				
19	Ranavirus	$1,9*10^3$	$< 1,9*10^2$		$< 1,9*10^2$
20	ECV or ESV	$1,3*10^5$	$1,3*10^5$	$1,3*10^5$	$1,3*10^5$
21	ECV		$2,7*10^3$	$5,9*10^2$	
22	Ranavirus not EHNV ESV or EC or DFV	$5,9*10^5$	$1,3*10^5$	$4,0*10^5$	$1,9*10^5$
23	Ranavirus (not seq)	$1,3*10^4$			$< 1,9*10^2$
24	ECV		$4,0*10^4$		$5,9*10^3$
25	ECV or ESV	$8,6*10^3$	$4,0*10^5$		
26	Ranavirus not EHNV	$< 1,9*10^2$			$1,3*10^4$
27	ESV	$1,9*10^6$	$1,9*10^5$	$< 1,9*10^2$	
28	No reply				
29	Iridovirus - ESV	$5,9*10^4$	$2,7*10^4$		
30	ECV or ESV	$2,7*10^3$	$1,9*10^4$		
31	ECV or ESV	$4,0*10^4$	$8,6*10^3$		
32	EHNV and SVC (not seq, therefore Ranavirus)	$< 1,9*10^2$	$4,0*10^5$		
33	ECV or ESV	$2,7*10^5$	$4,0*10^4$	$1,9*10^2$	$< 1,9*10^2$
34	Ranavirus - ESV	$2,7*10^3$	$1,3*10^3$		
35	ECV or ESV				$5,9*10^3$
36	Ranavirus	$4,0*10^5$	$8,6*10^4$		
37	ESV	$1,9*10^5$	$1,3*10^4$		
38	Ranavirus	$1,9*10^4$	$5,9*10^4$		
39	Ranavirus	$1,9*10^4$	$1,9*10^4$	$1,3*10^4$	$1,3*10^5$
40	ECV or ESV or Doctor fish virus	$4,0*10^5$	$8,6*10^3$		$1,3*10^4$
41	ECV or ESV	$5,9*10^4$	$1,3*10^5$		$4,0*10^2$

Number of laboratories	34	35	13	15
Median titre	$4,9*10^4$	$2,7*10^4$	$1,3*10^4$	$1,3*10^3$
Maximum titre	$1,9*10^6$	$4,0*10^5$	$4,0*10^5$	$1,9*10^5$
Minimum titre	$<1,9*10^2$	$<1,9*10^2$	$<1,9*10^2$	$<1,9*10^2$
25% quartile titre	$8,6*10^3$	$6,3*10^3$	$1,9*10^2$	$<1,9*10^2$
75% quartile titre	$1,9*10^5$	$1,3*10^5$	$4,0*10^4$	$1,3*10^4$

Table 7. Inter-Laboratory Proficiency Test, PT1, 2011 – Results of titration of ampoule IV.

Ampoule IV - IHNV and IPNV					
Laboratory code number	Virus Identification	Titre in			
		BF-2	EPC	RTG-2	FHM
1	IHNV and IPNV	$1,9*10^5$	$2,7*10^5$	$4,0*10^5$	
2	IHNV	not performed	not performed	not performed	not performed
3	IHNV and IPNV	$1,9*10^5$	$1,9*10^5$		
4	IHNV and IPNV	$1,9*10^4$	$5,9*10^5$		
5	IHNV and IPNV	$1,9*10^6$	$1,9*10^6$	$1,9*10^6$	$5,9*10^5$
6	IHNV and IPNV	$4,0*10^6$	$8,6*10^5$	$1,3*10^6$	$5,9*10^5$
7	IHNV and IPNV	$8,6*10^6$	$8,6*10^6$		
8	IHNV and IPNV	$5,9*10^5$	$8,6*10^5$		
9	IHNV and IPNV	$1,3*10^6$	$5,9*10^6$	$1,3*10^6$	
10	IHNV and IPNV	$1,3*10^6$	$5,9*10^5$	$1,3*10^6$	$2,7*10^6$
11	IHNV and IPNV	$1,3*10^6$	$1,9*10^6$		
12	IHNV and IPNV	$2,7*10^4$	$1,9*10^4$		
13	IHNV and IPNV	$1,3*10^7$	$2,7*10^5$		
14	IHNV and IPNV	$4,0*10^6$	$1,9*10^6$		
15	IHNV and IPNV	$1,3*10^4$	$8,6*10^3$		
16	IHNV and IPNV		$8,6*10^4$	$4,0*10^5$	
17	IPNV	$1,9*10^8$	$1,9*10^6$	$2,7*10^6$	$5,9*10^6$
18	No reply				
19	IHNV and IPNV	$1,9*10^5$	$1,9*10^4$		$1,3*10^4$
20	IHNV and IPNV	$1,3*10^6$	$1,3*10^6$	$1,3*10^5$	$1,3*10^6$
21	IHNV		$1,9*10^6$	$1,3*10^5$	
22	IHNV and IPNV	$1,9*10^6$	$1,3*10^6$	$4,0*10^6$	$1,3*10^6$
23	IPNV and Ranavirus	$4,0*10^6$			$4,0*10^5$
24	IHNV and IPNV		$5,9*10^5$		$1,3*10^5$
25	IHNV and IPNV	$4,0*10^6$	$4,0*10^6$		
26	IHNV and IPNV	$4,0*10^7$			$5,9*10^6$
27	IHNV and IPNV	$5,9*10^6$	$1,9*10^6$	$2,7*10^5$	
28	No reply				
29	IHNV and IPNV	$4,0*10^6$	$1,3*10^4$		
30	IHNV and IPNV	$4,0*10^6$	$4,0*10^5$		
31	IPNV	$5,9*10^6$	$2,7*10^6$		
32	IHN and SVC	$4,0*10^2$	$2,7*10^6$		
33	IHNV and IPNV	$2,7*10^6$	$2,7*10^5$	$2,7*10^5$	$4,0*10^5$
34	IHNV and IPNV	$8,6*10^4$	$2,7*10^4$		
35	IHNV				$4,0*10^4$
36	IHNV and IPNV	$4,0*10^6$	$5,9*10^7$		
37	IHNV and IPNV	$1,9*10^6$	$5,9*10^6$		
38	IHNV and IPNV	$8,6*10^5$	$8,6*10^5$		
39	IHNV and IPNV	$1,3*10^3$	$1,3*10^6$	$8,6*10^4$	$8,6*10^3$
40	IHNV	$2,7*10^6$	$1,9*10^4$		$1,9*10^5$
41	IHNV and IPNV	$8,6*10^6$	$5,9*10^6$		$8,6*10^4$

Number of laboratories	34	35	13	15
Median titre	$1,9*10^6$	$8,6*10^5$	$4,0*10^5$	$4,0*10^5$
Maximum titre	$1,9*10^8$	$5,9*10^7$	$4,0*10^6$	$5,9*10^6$
Minimum titre	$4,0*10^2$	$8,6*10^3$	$8,6*10^4$	$8,6*10^3$
25% quartile titre	$2,9*10^5$	$2,7*10^5$	$2,7*10^5$	$1,1*10^5$
75% quartile titre	$4,0*10^6$	$1,9*10^6$	$1,3*10^6$	$1,3*10^6$

Table 8. Inter-Laboratory Proficiency Test, PT1, 2011 – Results of titration of ampoule V.

Ampoule V - IPNV					
Laboratory code number	Virus Identification	Titre in			
		BF-2	EPC	RTG-2	FHM
1	IPNV	$8,6*10^7$	$1,9*10^7$	$8,6*10^7$	
2	Not VHSV, IHNV and EHNV	not performed	not performed	not performed	not performed
3	IPNV	$8,6*10^6$	$8,6*10^6$		
4	IPNV	$1,3*10^5$	$5,9*10^6$		
5	IPNV	$4,0*10^7$	$2,7*10^7$	$1,3*10^8$	$1,3E+0^7$
6	IPNV	$8,6*10^6$	$1,3*10^6$	$8,6*10^5$	$8,6*10^5$
7	IPNV	$5,9*10^7$	$5,9*10^7$		
8	IPNV	$1,3*10^8$	$1,3*10^7$		
9	IPNV	$2,7*10^6$	$1,3*10^7$	$5,9*10^5$	
10	IPNV	$8,6*10^4$	$1,9*10^5$	$2,7*10^5$	$4,0*10^5$
11	IPNV	$1,3*10^8$	$1,9*10^7$		
12	IPNV	$8,6*10^6$	$5,9*10^6$		
13	IPNV	$2,7*10^7$	$2,7*10^5$		
14	IPNV	$8,6*10^5$	$2,7*10^5$		
15	IPNV	$1,9*10^7$	$5,9*10^6$		
16	IPNV		$5,9*10^5$	$5,9*10^6$	
17	IPNV	$2,7*10^9$	$4,0*10^9$	$5,9*10^8$	$1,9*10^9$
18	No reply				
19	IPNV	$5,9*10^7$	$1,3*10^6$		$8,6*10^6$
20	IPNV	$2,7*10^5$	$1,3*10^6$	$1,3*10^5$	$1,3*10^6$
21	IPNV		$2,7*10^8$	$1,9*10^6$	
22	IPNV	$1,3*10^8$	$5,9*10^7$	$2,7*10^7$	$2,7*10^7$
23	IPNV	$4,0*10^6$			$2,7*10^6$
24	IPNV		$2,7*10^6$		$5,9*10^4$
25	IPNV	$5,9*10^7$	$8,6*10^7$		
26	IPNV	$1,9*10^6$			$1,9*10^4$
27	IPNV	$5,9*10^7$	$4,0*10^7$	$2,7*10^6$	
28	No reply				
29	IPNV	$8,6*10^5$	$5,9*10^3$		
30	IPNV	$1,3*10^3$	$1,3*10^3$		
31	IPNV	$2,7*10^8$	$4,0*10^7$		
32	Virus not identified	$4,0*10^2$	$1,3*10^7$		
33	IPNV	$4,0*10^8$	$1,3*10^7$	$1,9*10^6$	$1,9*10^7$
34	IPNV	$5,9*10^5$	$1,3*10^6$		
35	IPNV				$2,7*10^3$
36	IPNV	$2,7*10^9$	$5,9*10^9$		
37	IPNV	$1,9*10^6$	$1,9*10^6$		
38	IPNV	$2,7*10^7$	$4,0*10^7$		
39	IPNV	$1,9*10^6$	$8,6*10^6$	$1,9*10^6$	$< 1,9*10^2$
40	not EHNV or other ranavirus, not IHNV, VHSV or SVCV	$2,7*10^7$	$1,3*10^4$		$8,6*10^5$
41	IPNV	$1,3*10^7$	$1,3*10^6$		$1,3*10^4$

Number of laboratoriums	34	35	13	15
Median titre	$1,6*10^7$	$8,6*10^6$	$1,9*10^6$	$8,6*10^5$
Maximum titre	$2,7*10^9$	$5,9*10^9$	$5,9*10^8$	$1,9*10^9$
Minimum titre	$4,0*10^2$	$1,3*10^3$	$1,3*10^5$	$<1,9*10^2$
25% quartile titre	$1,9*10^6$	$1,3*10^6$	$8,6*10^5$	$3,9*10^4$
75% quartile titre	$5,9*10^7$	$3,4*10^7$	$2,7*10^7$	$1,1*10^7$

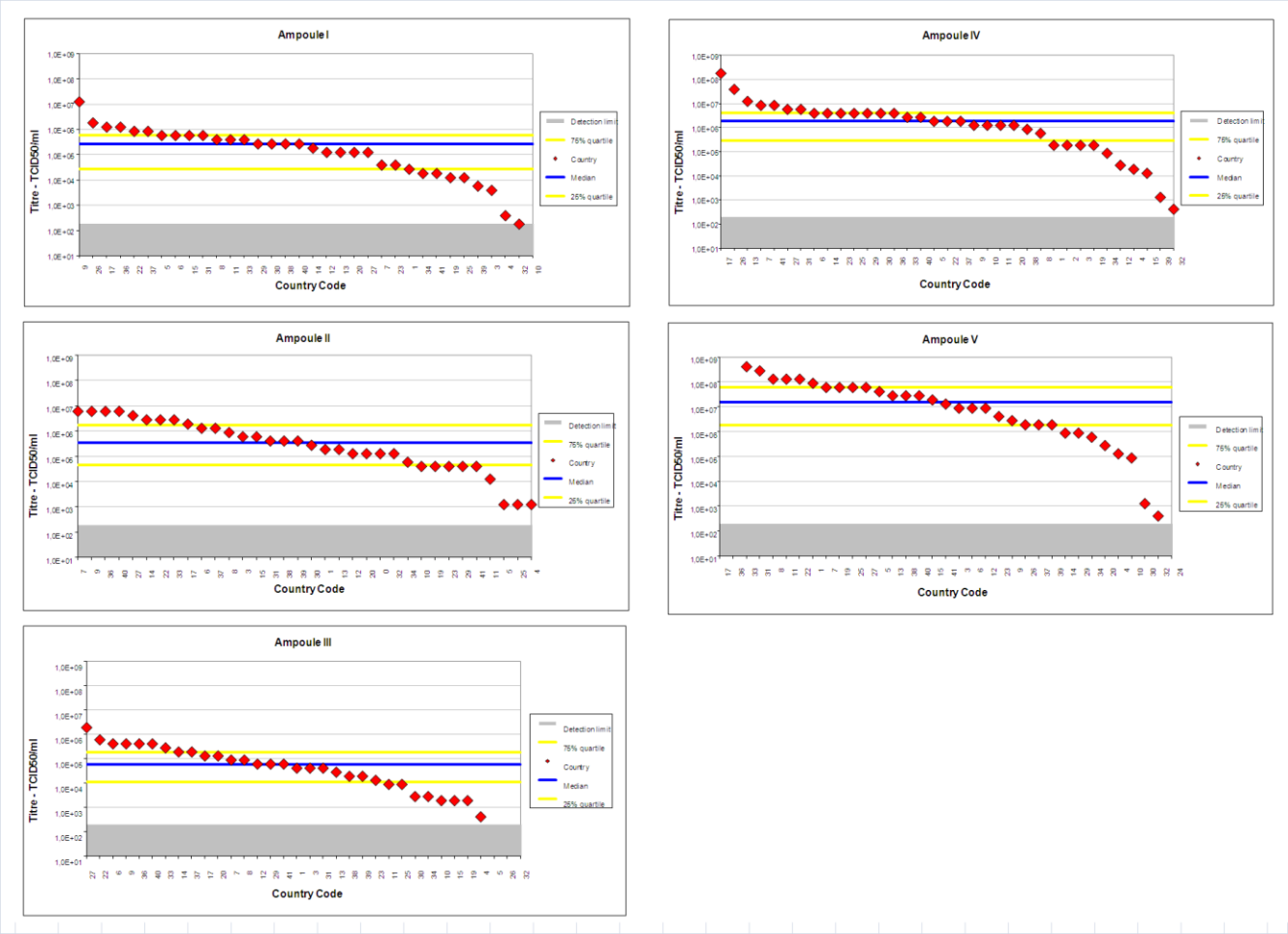


Figure 6. Virus titres obtained in BF-2 cells. The titre (red diamond) of each participating laboratory (country code) using BF-2 cells illustrated for ampoule I, II, III, IV and V. The detection level (gray shadow), median (blue line), 75% quartile (upper yellow line) and 25% quartile (lower yellow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

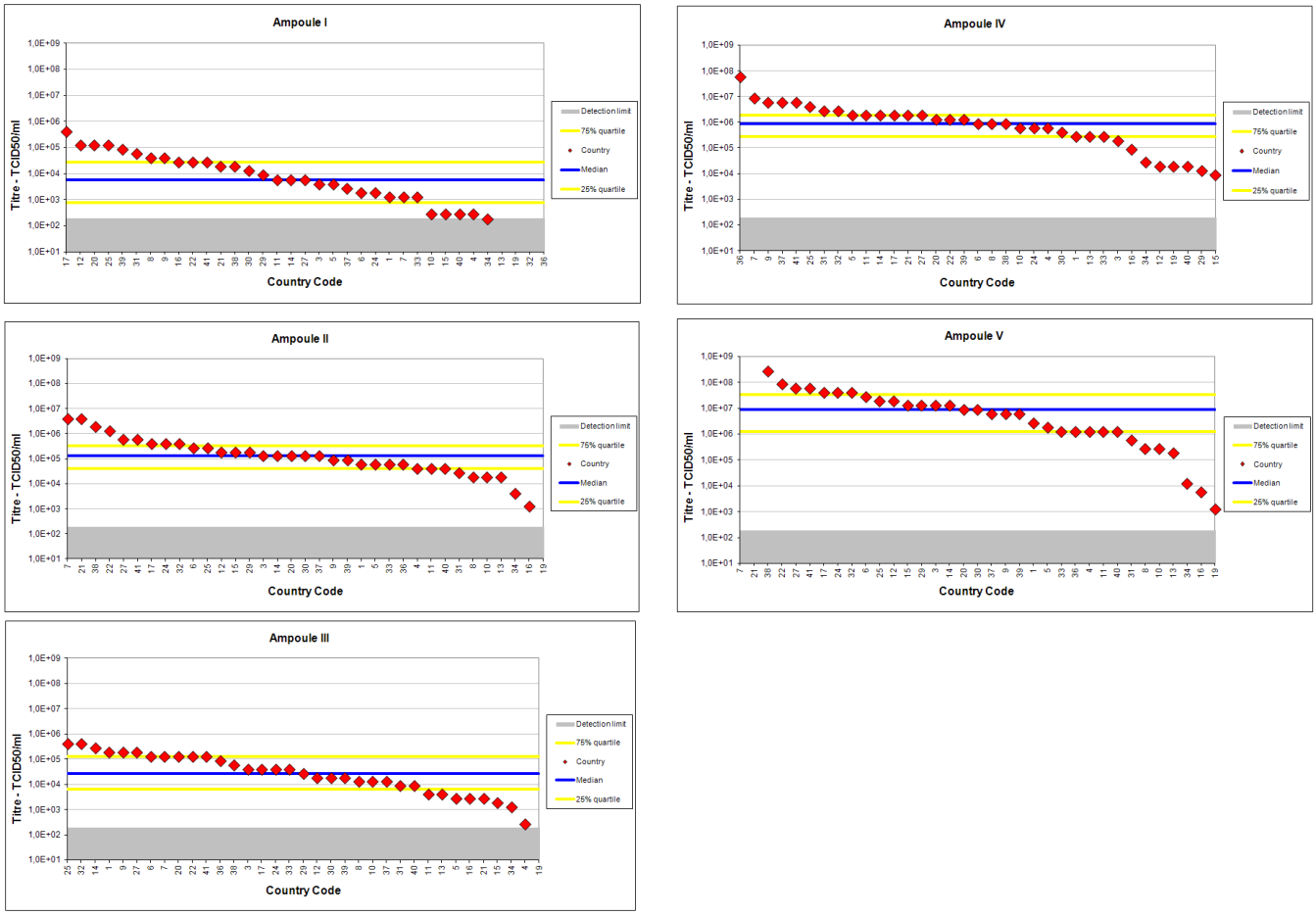


Figure 7. Virus titres obtained in EPC cells. For further details see Figure 6

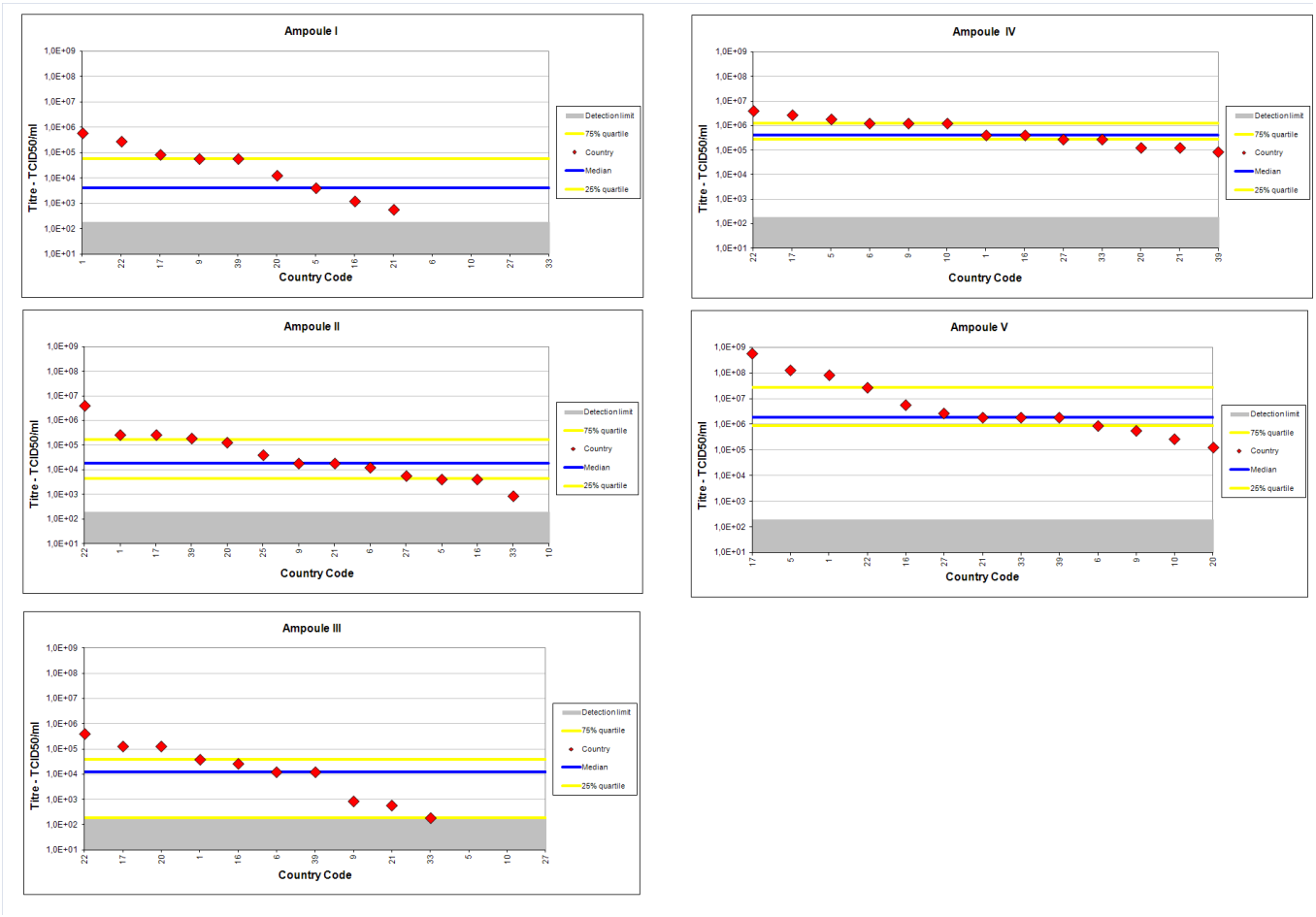


Figure 8. Virus titre obtained in RTG-2 cells. For further details see Figure 6

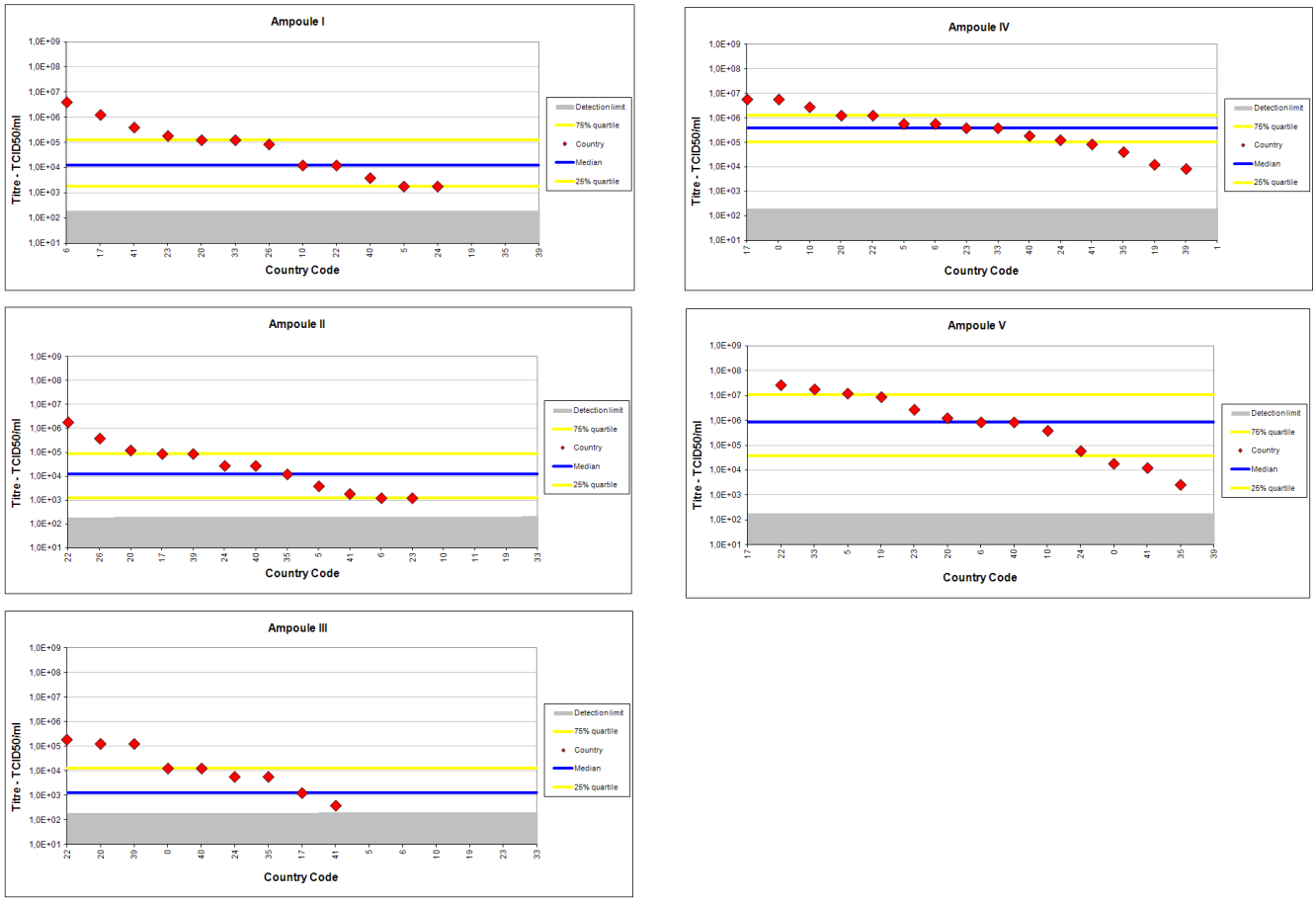


Figure 9. Virus titres obtained in FHM cells. For further details see Figure 6

Identification of content

- 26 laboratories correctly identified all viruses in all ampoules
- 2 laboratories did not reply to this test

Ampoule I – VHSV

- 39 laboratories correctly identified VHSV

Ampoule II – EHNV

- 32 laboratories correctly identified EHNV
- 5 laboratories identified ranavirus, but did not employ genomic analysis
- 2 laboratories found virus but did not identify it

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

- 32 laboratories correctly identified Ranavirus, not EHNV
- 4 laboratories identified ranavirus, but did not employ genomic analysis
- 1 laboratory identified VHSV
- 1 laboratory identified ranavirus (no sequencing) and SVCV
- 1 laboratory found virus but did not identify it

Ampoule IV – IHNV and IPNV

- 31 laboratories correctly identified IHNV and IPNV
- 4 laboratories identified only IHNV
- 2 laboratories identified only IPNV
- 1 laboratory identified IPNV and ranavirus
- 1 laboratory identified IHNV and SVCV

Ampoule V – IPNV

- 36 laboratories correctly identified IPNV
- 2 laboratories identified the virus as not ranavirus, IHNV, VHSV or SVCV
- 1 laboratory examined by PCR only and did not employ IPN RT-PCR

Scores

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

Ampoule I: VHSV identification was given the score 2. VHSV not identified was given the score 0.

Ampoule II: EHNV identification backed up by genomic analysis was given the score 2. EHNV identification not backed up by genomic analysis or ranavirus/iridovirus as the only answer for this ampoule was given the score 1. In case of no genomic analysis the result is stated as ranavirus* in table 3.

Ampoule III: Identification of "ranavirus/iridovirus, not EHNV" or Catfish/sheatfish iridovirus was given the score 2 (stated as ranavirus, not EHNV in table 3). Ranavirus/iridovirus as the only answer for this ampoule was given the score of 1 (if no genomic analysis was performed).

Ampoule IV: Identification of IHNV and IPNV was given the score 2, identification of IHNV only was given the score 1 and identification of IPNV only was given the score 0.

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

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

Of the laboratories that submitted results, 26 out of 39 correctly identified all viruses in all ampoules and obtained the maximum score 10. Of the laboratories that did identify ranavirus in ampoule II and ampoule III, 5 were not able to identify the ranavirus as EHNV in ampoule 2 and 5 were not able to identify the ranavirus as ranavirus, not EHNV in ampoule III. Additional viruses than present in the ampoules were observed by 2 laboratories. A diagram of the scoring obtained by the laboratories is shown in figure 10.

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

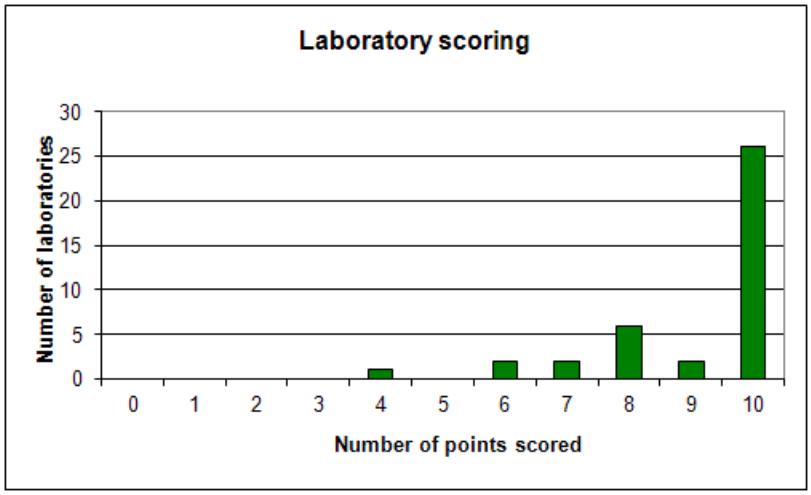


Figure 10. Scores obtained by participants.

Cells applied for solving the test

The following cell lines were used by the participants:

- 34 laboratories used BF-2 cells
- 35 laboratories used EPC cells
- 13 laboratories used RTG-2 cells
- 15 laboratories used FHM cells
- 3 laboratory used CHSE-214 cells
- 8 laboratories used four cell lines
- 6 laboratories used tree cell lines
    - 3 laboratories used BF-2 cells in combination with EPC cells and RTG-2 cells
    - 3 laboratories used BF-2 cells in combination with EPC cells and FHM cells
- 23 laboratories used two cell lines:
    - 18 laboratories used BF-2 cells in combination with EPC cells
    - 2 laboratories used RTG-2 cells in combination with EPC cells
    - 2 laboratories used BF-2 cells in combination with FHM cells
    - 1 laboratory used EPC cells in combination with FHM cells
- 1 laboratory use only FHM cells
- 1 laboratory did not use any cell line, they only performed PCR

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

The median titre calculated from the submitted results of each ampoule and in each cell line is illustrated in Figure 11. It appears that all ampoules replicates on all cell lines, however there is a tendency that BF-2 cells are better for replication of VHSV genotype Ib, and BF-2 and EPC cells are better for ranaviruses and IPNV than the other cell lines. No conclusions regarding IHNV can be made based on this comparison as the ampoule with this virus also included IPNV.

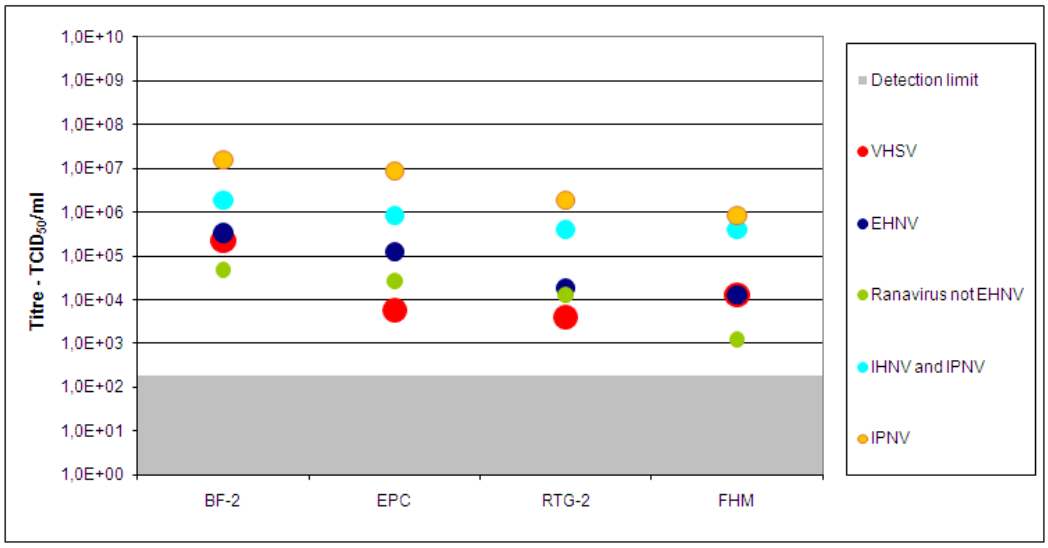


Figure 11. Median virus titres obtained by participants at different cell lines.

Methods used for identification of viruses

(Table 9)

- 22 laboratories used ELISA for identification of viruses.
- 25 laboratories used IFAT for identification of viruses.
- 12 laboratories used neutralisation tests for identification of viruses.
- 39 laboratories used PCR for identification of viruses.
- 33 laboratories performed sequencing for identification of viruses.
- 2 laboratory performed REA according to the OIE Aquatic Animals Manuals for identification of ranaviruses.

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

Laboratory code number	Score (max. 10)	ELISA	IFAT	Neutralisation	PCR	Sequence ampoule no.
1	10	X	X	X	X	I, II, III,IV and V
2*	8				X	II and III
3	10	X	X		X	II and III
4	7	X	X	X	X	
5	10	X			X	I, II, III and IV
6	10	X	X	X	X	I, II, III and IV
7**	6	X	X		X	
8	10	X			X	I, II, III and IV
9	10		X		X	I, II, III and IV
10	10	X	X	X	X	I, II, III and IV
11***	10	X			X	II and III
12	10	X		X	X	II and III
13	10	X	X		X	I, II, III,IV and V
14	10		X		X	I, II, III and IV
15	8	X	X		X	
16	10	X	X		X	II, III and IV
17	8	X	X		X	I, II, III,IV and V
18	n/a					
19	10	X	X	X	X	REA
20	10	X	X		X	I, II, III,IV and V
21	9				X	I, II, III,IV and V
22	10		X		X	I, II, III and IV
23	6	X		X	X	
24	10		X		X	II and III
25	10	X	X		X	I, II, III,IV and V
26	10	X	X		X	I, II, III,IV and V
27	10				X	I, II, III,IV and V
28	n/a					
29	10	X	X		X	I, II, III,IV and V
30	10				X	I, II, III,IV and V
31	8			X	X	II and III
32	4				X	
33	10				X	I, II, III,IV and V
34	10		X	X	X	I, II, III and IV
35	9				X	II and III
36	7	X			X	III
37	10		X		X	I, II, III and IV
38	8	X	X		X	II and III, REA
39	10		X	X	X	I, II, III,IV and V
40	8		X	X	X	I, II and III
41	10		X	X	X	I, II, III,IV and V

A graph was constructed to illustrate the association between the methods used by participants for virus identification and the obtained score (Figure 12). For participants scoring lower than 10, the deficiency in virus identification cannot directly be assigned to improper use of a single identification method. Rather mistakes might be related to performance of the overall procedure. It is clear, however, that if sequencing or REA is not used, a correct answer cannot be made for ampoule II and III. This accounts for 6 of the laboratories where 4 laboratories have reported ranavirus, due to lack of sequencing and 1 laboratory reported ranavirus due to lack of translation of the sequences and one laboratory likewise reported ranavirus due to low quality sequence and no conclusion was possible based on REA results. Finally, 3 out of the 13 participants scoring lower than 10 identified false positive viruses in ampoules indicating that cross contamination could have occurred at some point in the diagnostic process. Furthermore 8 out of the 13 participants scoring lower than 10 only found one out of two viruses in ampoule IV.

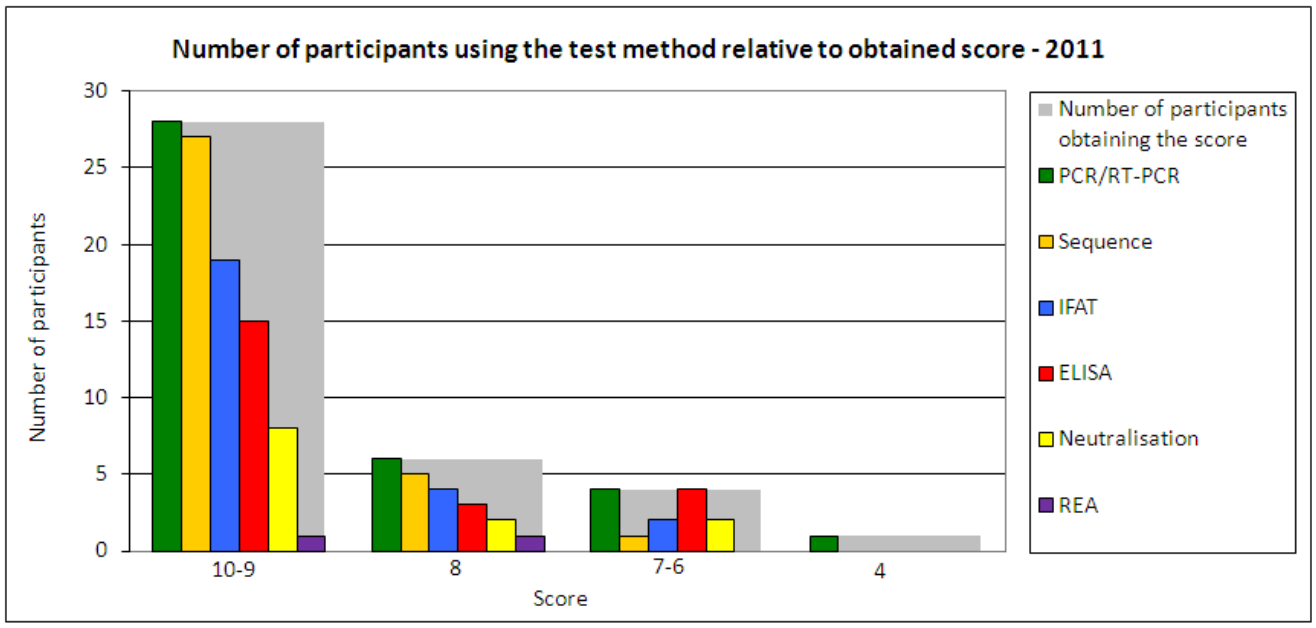


Figure 12. Methods used by participants for identification of viruses in relation to the obtained score.

Genotypning and sequencing

In previous proficiency tests provided by the EURL, we have encouraged participants to genotype the identified viruses though it has not been a mandatory task. As ranaviruses are included in the test, it is mandatory to do sequence or REA analysis 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) for IHNV but this was not an obligatory task.

Ampoule I – VHSV genotype Ib

- 23 laboratories performed sequencing to identify the virus in ampoule I
- 6 laboratories used the primers described in Einer-Jensen et al. (2004)
- 6 laboratories used the primers described in Snow et al. (2004)
- 11 laboratories used primers described in other references or did not report the reference
- 20 laboratories identified the VHSV isolate as genotype Ib
- 1 laboratory identified the VHSV isolate as genotype Ie
- 2 laboratories did not genotype the VHSV

Ampoule II – EHNV

- 31 laboratories performed sequencing to identify the virus in ampoule II with correct result
- 1 laboratory performed REA according to the OIE Manual of Diagnostic Tests for Aquatic Animals. Chapter 2.3.1 with correct result
- 1 laboratory performed both REA and sequencing. From this laboratory it was not possible to identify which type of ranavirus the isolate belong to
- 10 laboratories used primers described in Hyatt et al. (2000)
- 11 laboratories used primers described in the OIE Manual of Diagnostic Tests for Aquatic Animals. Chapter 2.3.1
- 2 laboratories used primers described in Holopainen et al (2009)
- 10 laboratories were using primers described in other references or they did not report the reference

Within the OIE diagnostic manual for EHN, two sequence based methods are recommended to use in order to discriminate EHNV from the other non-listed ranaviruses. A total of 32 laboratories used sequencing analyses for identification of EHNV whereas one used REA. Of the laboratories that sequenced the isolate in ampoule II all but one identified the virus correctly as EHNV.

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

- 31 laboratories submitted sequences that were either identical to or most similar to sheetfish or catfish iridovirus
- 1 laboratory performed REA according to the OIE Manual of Diagnostic Tests for Aquatic Animals. Chapter 2.3.1 with correct result
- 1 laboratory performed both REA and sequencing. From this laboratory it was not possible to identify which type of ranavirus the isolate belong to
- 1 laboratory sequenced the isolate but were not able to tell which type of ranavirus the isolate belong to
- 10 laboratories used primers described in Hyatt et al. (2000)
- 12 laboratories used primers described in OIE Manual of Diagnostic Tests for Aquatic Animals. Chapter 2.3.1
- 4 laboratories used primers described in Holopainen et al. (2009)
- 9 laboratories were using primers described in other references or they did not report the reference

Ampoule IV – IHNV and IPNV

- 23 laboratories submitted sequences
- IHNV
  - 20 laboratories genotyped the IHNV isolate as belonging to genogroup M
  - 1 laboratory did not provide a genogroup despite having sequenced the isolate
- IPNV
  - 4 laboratories genotyped the IPNV isolate as belonging to genogroup 5 (Sp)
  - 1 laboratories genotyped the IPNV isolate as belonging to genogroup 3
  - 2 laboratories did not provide a genogroup despite having sequenced the isolate
- For IHNV 4 laboratories used primers described in Emmenegger et al. (2000)
- For IHNV 5 laboratories used primers described in the OIE Manual of Diagnostic Tests for Aquatic Animals. Chapter 2.3.4
- For IPNV 2 laboratories used the primers described in Taksdal et al. (2001)
- For both IHNV and IPNV, 16 laboratories were using primers described in other references or they did not reported the reference

Ampoule V – IPNV

- 13 laboratories submitted sequences
- 4 laboratories genotyped the IPNV isolate as belonging to genogroup 5 (Sp)
- 1 laboratory genotyped the IPNV isolate as belonging to genogroup 3
- 8 laboratories did not provide a genogroup despite having sequenced the isolate
- 2 laboratories used the primers described in Taksdal et al. (2001)
- 11 laboratories were using other primers or they did not report the primers used

Of the 39 laboratories that delivered results to this PT 32 laboratories used either sequence analysis or REA to identify it the ranavirus was EHNV or not. It is important that the remaining laboratories implement PCR and sequencing techniques in the laboratory as genotyping is the basis for differentiating several listed viruses from others.

Table 10. Genotyping results on viruses in ampoule I-V submitted by participating laboratories**.**

Laboratory code number	Score (max. 10)	Ampoule I VHSV Isolate 1p8	Ampoule II EHNV Isolate 86/8774	Ampoule III Ranavirus not EHNV European Catfish virus (ECV) 562/92 Low titre	Ampoule IV IHNV and IPNV IHNV 32/87 and IPNV strain Sp	Ampoule V IPNV IPNV strain Sp
1	10	VHSV Ib	EHNV (Seq)	ESV-ECV (Seq)	IHNV Genogroup M	IPNV
2*	8		EHNV (Seq)	ESV-ECV (Seq)		
3	10		EHNV (Seq)	ESV-ECV (Seq)		
4	7					
5	10	VHSV Ib	EHNV (Seq)	ESV-ECV (Seq)	IHNV Genogroup M	
6	10	VHSV Ib	EHNV (Seq)	ESV-ECV (Seq)	IHNV Genogroup M	
7**	6					
8	10	VHSV Ib	EHNV (Seq)	ESV-ECV (Seq)	IHNV Genogroup M	
9	10	VHSV Ib	EHNV (Seq)	Ranavirus-ECV (Seq)	IHNV Genogroup M	
10	10	VHSV Ib	EHNV (Seq)	Ranavirus-ECV (Seq)	IHNV Genogroup M	
11***	10		EHNV (Seq)	ESV-ECV (Seq)		
12	10		EHNV (Seq)	ESV (Seq)		
13	10	VHSV Ib	EHNV (Seq)	ESV-ECV (Seq)	IHNV Genogroup M IPNV Genogroup 5 (Sp)	IPNV Genogroup 5 (Sp)
14	10	VHSV Ib	EHNV (Seq)	ESV-ECV (Seq)	IHNV Genogroup M	
15	8					
16	10		EHNV (Seq)	Ranavirus-ECV (Seq)	IHNV Genogroup M	
17	8	VHSV	EHNV (Seq)	ESV-ECV (Seq)	IPNV	IPNV
18	n/a					
19	9		EHNV (REA)	Ranavirus (REA)		
20	10	VHSV Ib	EHNV (Seq)	ESV-ECV (Seq)	IHNV Genogroup M IPNV Genogroup 5 (Sp)	IPNV Genogroup 5 (Sp)
21	9	VHSV	EHNV (Seq)	ESV-ECV (Seq)	IHNV	IPNV
22	10	VHSV Ib	EHNV (Seq)	ESV-ECV-DFV (Seq)	IHNV Genogroup M	
23	6					
24	10		EHNV (Seq)	ECV (Seq)		
25	10	VHSV Ib	EHNV (Seq)	ESV-ECV (Seq)	IHNV Genogroup M IPNV A2	IPNV
26	10	VHSV Ib	EHNV (Seq)	Ranavirus not EHNV (Seq)	IHNV Genogroup M IPNV Genogroup 5 (Sp)	IPNV Genogroup 5 (Sp)
27	10	VHSV Ib	EHNV (Seq)	ESV (Seq)	IHNV Genogroup M	IPNV
28	n/a					
29	10	VHSV Ib	EHNV (Seq)	Ranavirus ESV	IHNV Genogroup M	IPNV
30	10	VHSV Ie	EHNV (Seq)	ESV-ECV (Seq)	IHNV Genogroup M IPNV Genogroup 5 (Sp)	IPNV Genogroup 5 (Sp)
31	8		EHNV (Seq)	ESV-ECV (Seq)		
32	3					
33	10	VHSV Ib	EHNV (Seq)	ESV-ECV (Seq)	IHNV Genogroup M IPNV Genogroup III	IPNV Genogroup III
34	10	VHSV Ib	EHNV (Seq)	Ranavirus ESV (Seq)	IHNV Genogroup M	
35	9		EHNV (Seq)	ESV-ECV (Seq)		
36	7			Ranavirus (Seq)		
37	10	VHSV Ib	EHNV (Seq)	ESV (Seq)	IHNV Genogroup M	
38	8		Ranavirus (Seq, REA)	Ranavirus (Seq, REA)		
39	10	VHSV Ib	EHNV (Seq)	Ranavirus (Seq)	IHNV Genogroup M	IPNV
40	8	VHSV Ib	EHNV (Seq)	ESV-ECV (Seq)		
41	10	VHSV Ib	EHNV (Seq)	ESV-ECV (Seq)	IHNV Genogroup M	IPNV

Concluding remarks PT1

The inter-laboratory proficiency test 2011 was conducted without major constrains. Most parcels were delivered by the shipping companies within 3 days after submission. It was, however, unfortunate that one parcel was 44 days on the way before delivered to the laboratory primarily due to border controls.

In 2009 EHNV was included in the proficiency test for the first time and 32 participants were able to correctly identify the virus. This year EHNV was included as well as ECV, both belong to the ranavirus family. Of the laboratories performing PCR based methods, 31 laboratories performed sequencing only for ampoule II and 32 for ampoule III. Of these laboratories all correctly identified the content in ampoule II as EHNV and 31 correctly identified the content in ampoule III as ECV/ESV. One laboratory performed both sequencing and REA for both ampoule II and III without being able to identify which type of ranavirus the isolates belong to. One laboratory performed REA only for both ampoule II and III and was able to identify the isolate as either EHNV or ranavirus, not EHNV.

In this report (figures 6-9), all titres submitted by participants for each cell line and ampoule, respectively are compared to each other. In this way, the titres obtained by each laboratory are plotted in relation to the combined submitted data set and each participating laboratory should be able to compare the sensitivity of their cell lines to the sensitivity of those used by the other participating laboratories. We recommend all participants to evaluate the sensitivity of the cells used in their laboratory in relation to the diagnostic purpose.

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

The results presented in this report will be further presented and discussed at the 16th Annual Meeting of National Reference Laboratories for Fish Diseases to be held 30-31 May 2012 in Aarhus, Denmark.

Proficiency test 2, PT2

Five ampoules with lyophilised cell culture supernatant or *A. invadans spores* were delivered to the same laboratories as PT1.

Content of ampoules

The viruses were propagated on each of their preferred cell line and when total cytopathic effect (CPE) was observed, the supernatants were collected and filtrated through a 45 µm filter, mixed with equal volumes of 20% w/v lactalbumin hydrolysate solution and lyophilized in glass ampoules. Produced *A. invadans* spores were treated the same way as supernatants. Before the ampoules were sealed by melting, the pathogen concentration was analysed by real-time PCR for KHV (protocol described by Gilad et al. (2004)), real-time RT-PCR for ISAV (protocol described by Snow et al. (2006)) and conventional PCR for *A. invadans* (protocol describe by Kurata et al. (2000)).

The details of the virus isolates used in the proficiency test are outlined in table 11.

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

PT2	
Code	**Specifications**
Ampoule VI: ISAV Low titre	**ISAV Glesvaer/2/90** Received from Dr. B. Dannevig, ISA OIE Reference Laboratory, Oslo, Norway References: Mjaaland S, Rimstad E, Falk K & Dannevig BH (1997). Genomic characterization of the virus causing infectious salmon anemia in Atlantic salmon (*Salmo salar* L.): an orthomyxo-like virus in a teleost. *Journal of Virology* **71**, 7681-7686. Falk K, Namork E, Rimstad E, Mjaaland S & Dannevig BH (1997). Characterization of infectious salmon anemia virus, an orthomyxo-like virus isolated from Atlantic salmon (Salmo salar L.) *Journal of Virology* **71**, 9016-9023.
Ampoule VII: ISAV High titre	Same as Ampoule VI
Ampoule VIII: KHV High titre	**KHV-TP 30** Koi Herpesvirus (CyHV-3): KHV-TP 30 (syn: KHV-T (for Taiwan)). KHV-TP 30 was isolated from koi in Taiwan and cloned for producing large plaques by Dr. Peiyu Lee, Institute of Medical Biotechnology, Central Taiwan University of Science and Technology, Dakeng, BeiTung District, TaiChung City 406, Taiwan in-2005. The isolate was provided by Dr. Sven M. Bergmann, Friedrich-Loeffler-Institut (FLI), Federal Research Institute for Animal Health, Südufer 10, 17393 Greifswald-Insel Riems, Germany
Ampoule IX: *Aphanomyces invadans*	***Aphanomyces piscicida/invadans* spores NJM9701** Received from Dr. Kishio Hatai, Lab Fish Diseases NVLU Tokyo, Japan Reference: Kurata O., Kanai H. & Hatai K. (2000) Hemagglutinating and hemolytic capacities of Aphanomyces piscicida. *Fish Pathology - Gyobyo Kenkyu* **35**, 29-33.
Ampoule X: KHV Low titre	Same as Ampoule VIII

Testing of the test

The PT2 test was prepared and tested according to protocols accredited under DS/EN ISO/IEC 17043. Prior to the distribution we tested 5 ampoules of each virus preparation by PCR (Bercovier et al. (2005)) and real-time PCR (Gilad et al. (2004)) for KHV and by RT-PCR (Mjaaland et al. (1997)) and real-time RT-PCR (Snow et al. (2006)) for ISAV, to ascertain identity, a satisfactory titre of the virus and homogeneity of the content in the ampoules (Table 12). Furthermore, conventional PCR/RT-PCR fragments were sequenced and so was the HPR region of the ISAV isolate.

KHV and ISAV were prepared in different concentrations that were well above detection level.

The lyophilisation procedure caused a significant virus reduction as detected by real-time PCR or real-time RT-PCR by approximately 4-5 Ct values (Table 13). Furthermore, after lyophilisation the content of the ampoules were tested for stability over time. Each virus preparation was stored three months in the dark and then kept for a period of 5 hours at temperatures rising from 20°C to 42°C, furthermore the viruses were tested with and without filtration through a 45 µm filter. These conditions did not decrease Ct values of neither KHV nor ISAV. *A. invadans* went through the same treatment, but only conventional PCR was done, and there was no change in the size of the band.

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

Table 12. Ct-value of representative ampoules of no. VI to VIII and X tested at the EURL; tested before lyophilisation, immediately after lyophilisation, and after 3 months of storage in the dark at 4°C and rising from 20°C to 42°C for 5 hours (1 replicate), respectively. For ampoule IX the presence of a specific band after conventional PCR is stated.

Ampoule No.	**Ampoule**	**Ct value/presence of band before lyophilisation undiluted**	**Ct value presence of band right after lyophilisation**	**Ct value presence of band 3 months after lyophilisation (4°C, dark conditions)**	**Ct value presence of band 3 months after lyophilisation (4°C, dark conditions)**	**Ct value presence of band 3 months after lyophilisation (up to 42°C, dark conditions)**	**Ct value presence of band 3 months after lyophilisation (up to-42°C, dark conditions)**
				+ Filtration	- Filtration	+ Filtration	- Filtration
Ampoule VI: ISAV Glesvaer/2/90 Low titre	a	28,00	33,61	32,56	32,63	32,56	32,43
	b	27,90	33,42				
	c	27,91	33,89				
	d		33,00				
	e		34,03				
	Average	27,94	33,59				
Ampoule VII: ISAV Glesvaer/2/90 High titre	a	24,02	29,79	28,62	28,70	28,82	28,56
	b	24,14	29,56				
	c	24,27	30,08				
	d		29,98				
	e		29,89				
	Average	24,14	29,86				
Ampoule VIII: KHV KHV-TP 30 High titre	a	22,72	25,00	25,70	24,83	24,99	24,67
	b	22,84	25,15				
	c	21,62	24,92				
	d		25,14				
	e		25,28				
	Average	21,73	25,10				
Ampoule IX: *Aphanomyces invadans* NJM 9701	a	+	+	+	+	+	+
	b	+	+				
	c	+	+				
	d		+				
	e		+				
Ampoule X: KHV KHV-TP 30 Low titre	a	25,68	29,36	29,37	29,34	29,12	29,03
	b	25,74	29,60				
	c	26,03	29,73				
	d		29,25				
	e		29,27				
	Average	25,82	29,44				

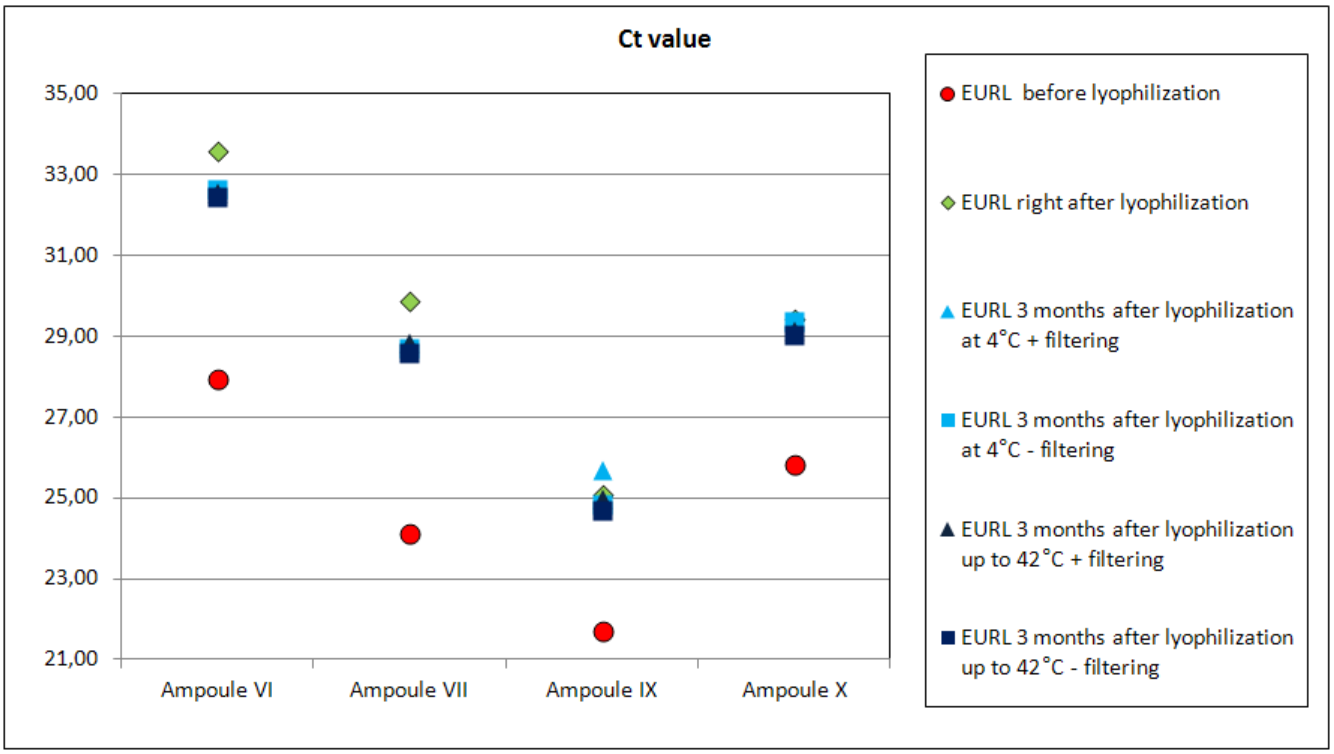


Figure 13. Ct values before, right after and 3 months after lyophilisation at different cell lines. "EURL before lyophilisation" correspond to the Ct value of the undiluted virus.

Virus identification

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

All laboratories were encouraged to sequence the HPR region of ISAV isolates. However, this was not a mandatory task.

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

Table 13. Inter-Laboratory Proficiency Test, PT2, 2011 - Virus identification.

Laboratory code number	Score (max. 10)	Answer recieved at EURL	Ampoule VI ISAV (low titer)	Ampoule VII ISAV (high titer)	Ampoule VIII KHV (high titer)	Ampoule IX *A.invadans*	Ampoule X KHV (low titer)
1	10	13-12-2011	ISAV (Seq)	ISAV (Seq)	KHV (Seq)	*A. invadans* (Seq)	KHV (Seq)
2	4	29-11-2011	ISAV (Seq)	ISAV (Seq)	KHV; ISAV (weak positive) (Seq)	*A. invadans* ISAV (weak positive) (Seq)	KHV, ISAV and *A. invadans* (weak positive) (Seq)
3	8	12-12-2011	ISAV, *A. invadans* (Seq)	ISAV (Seq)	KHV	*A. invadans*	KHV
4	4	16-12-2011	*A. invadans*		KHV		KHV
5	8	13-12-2011	ISAV	ISAV	KHV		KHV
6	10	09-12-2011	ISAV (Seq)	ISAV (Seq)	KHV	*A. invadans*	KHV
7	8	16-12-2011	ISAV	ISAV	KHV	not KHV, ISAV	KHV
8	8	16-12-2011	ISAV	ISAV	KHV	*A. invadans* negative	KHV
9	10	23-11-2011	ISAV (Seq)	ISAV (Seq)	KHV	*A. invadans*	KHV
10	8	16-12-2011	not ISAV, KHV, SVCV, EUS	ISAV (Seq)	KHV	*A. invadans*	KHV
11	10	16-12-2011	ISAV	ISAV	KHV	*A. invadans*	KHV
12	10	08-12-2011	ISAV	ISAV	KHV	*A. invadans*	KHV
13	10	15-12-2011	ISAV (Seq)	ISAV (Seq)	KHV (Seq)	*A. invadans* (Seq)	KHV (Seq)
14	10	16-12-2011	ISAV	ISAV	KHV	*A. invadans*	KHV
15	8	16-12-2011	ISAV	ISAV	KHV	Not ISAV and KHV	KHV
16	8	15-12-2011	ISAV	ISAV	KHV		KHV
17	10	14-12-2011	ISAV	ISAV	KHV	*A. invadans*	KHV
18	0	n/a	No reply	No reply	No reply	No reply	No reply
19	6	16-12-2011	Not IHNV, VHSV, KHV, Ranavirus, CyHV, SVCV and ISAV	ISAV	KHV	Not IHNV, VHSV, KHV, Ranavirus, CyHV, SVCV and ISAV	KHV
20	10	16-12-2011	ISAV (Seq)	ISAV (Seq)	KHV (Seq)	*A. invadans* (Seq)	KHV (Seq)
21	10	16-12-2011	ISAV (Seq)	ISAV (Seq)	KHV (Seq)	*A. invadans* (Seq)	KHV (Seq)
22	10	16-12-2011	ISAV (Seq)	ISAV (Seq)	KHV (Seq)	*A. invadans* (Seq)	KHV (Seq)
23	6	14-12-2011			KHV	*A. invadans*	KHV
24	10	07-12-2011	ISAV	ISAV	KHV (Seq)	*A. invadans*	KHV (Seq)
25	10	16-12-2011	ISAV (Seq)	ISAV (Seq)	KHV (Seq)	*A. invadans* (Seq)	KHV (Seq)
26	10	06-12-2011	ISAV (Seq)	ISAV (Seq)	KHV (Seq)	*A. invadans* (Seq)	KHV (Seq)
27	8	16-12-2011	ISAV (Seq)	ISAV (Seq)	KHV (Seq)	Not ISAV and KHV	KHV (Seq)
28	0	Did not participate	No reply	No reply	No reply	No reply	No reply
29	10	16-12-2011	ISAV (Seq)	ISAV	KHV (Seq)	*A. invadans*	KHV (Seq)
30	10	16-12-2011	ISAV (Seq)	ISAV (Seq)	KHV (Seq)	*A. invadans* (Seq)	KHV (Seq)
31	4	16-12-2011	ISAV	ISAV			
32	6	15-12-2011	*A. invadans*? *ISAV*?	*A. invadans*? ISAV?	KHV	*A. invadans*	KHV
33	10	16-12-2011	ISAV (Seq)	ISAV (Seq)	KHV	*A. invadans*	KHV
34	6	16-12-2011	ISAV	ISAV	KHV	KHV	no virus detected
35	8	16-12-2011	none	ISAV	KHV	*A. invadans*	KHV
36	10	16-12-2011	ISAV	ISAV	KHV	*A. invadans*	KHV
37	10	13-12-2011	ISAV	ISAV	KHV	*A. invadans*	KHV
38	n/a	16-12-2011	No reply	No reply	No reply	No reply	No reply
39	10	07-12-2011	ISAV (Seq)	ISAV (Seq)	KHV	*A. invadans* (Seq)	KHV
40	10	16-12-2011	ISAV	ISAV	KHV	*A. invadans* (Seq)	KHV
41	10	16-12-2011	ISAV (Seq)	ISAV (Seq)	KHV (Seq)	*A. invadans*	KHV (Seq)

[1] ISAV identification is performed by another NRL

[2] Analysed for the presence of ISAV only

[3] The concluding results were not written, the EURL has therefore written the concluding results in the sheet, the *A. invadans* in ampoule VI and VII may be due to a typing mistake in the received sheet

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

[5] Did not participate in PT2

n/a: *not applicable*

Identification of content

- 38 laboratories submitted results
- 22 laboratories correctly identified all five ampoules
- 29 laboratories tested for all three listed pathogens
- 36 laboratories tested for ISAV
- 37 laboratories tested for KHV
- 31 laboratories tested for *A. invadans*
- 1 laboratory tested for ISAV only
- 6 laboratories did not test for *A. invadans* but did for ISAV and KHV
- 2 laboratories did not test for ISAV but did for *A. invadans* and KHV
- 1 laboratory tested for ISAV by cell culture and IFAT, not by RT-PCR methods
- 3 laboratories did not submit any results

Ampoule VI – ISAV Low titre

- 31 laboratories correctly identified ISAV
- 1 laboratory identified ISAV and *A. invadans*
- 2 laboratories identified *A. invadans*
- 4 laboratories tested for and did not identify ISAV, one of these laboratories tested by cell culture only
- 2 laboratories did not examine for ISAV

Ampoule VII – ISAV High titre

- 35 laboratories correctly identified ISAV
- 1 laboratory identified *A. invadans*
- 1 laboratory tested for and did not identify ISAV
- 2 laboratories did not examine for ISAV

Ampoule VIII – KHV High titre

- 36 laboratories correctly identified KHV
- 1 laboratory identified KHV and ISAV (weak positive)
- 1 laboratory did not examine for KHV

Ampoule IX – Aphanomyces invadans

- 27 laboratories correctly identified *A. invadans*
- 1 laboratory identified *A. invadans* and ISAV (weak positive)
- 1 laboratory identified KHV
- 3 laboratories tested for but did not identify *A. invadans*
- 7 laboratories did not examine for *A. invadans*

Ampoule X – KHV Low titre

- 35 laboratories correctly identified KHV
- 1 laboratory identified KHV, *A. invadans* and ISAV (weak positive)
- 1 laboratory tested for and did not identify KHV
- 1 laboratory did not examine for KHV

Scores

We have assigned a score of 2 for each correct answer (Table 13), giving the possibility for obtaining a maximum score of 10. Incorrectly finding of pathogens not present in the ampoules gives the score 0.

Of the 38 laboratories submitting results 22 laboratories correctly identified all ampoules and obtained maximum score. One laboratory examined for ISAV only, this laboratory obtained the score 4 out of 4 possible, and one laboratory did not examined for ISAV, this laboratory obtain the score 6 out of 6 possible. Six laboratories did not examine for *A. invadans*, of these laboratories the maximum score of 8 was obtained by 4 of them. Tree laboratories did not submit any results and obtained the score 0. Laboratories not scoring for them the maximum possible score either lacked identification or identified pathogen(s) not present. Genotyping of ISAV HPR region and submission of sequencing results was not a mandatory part of the test and is not included in the score of participants.

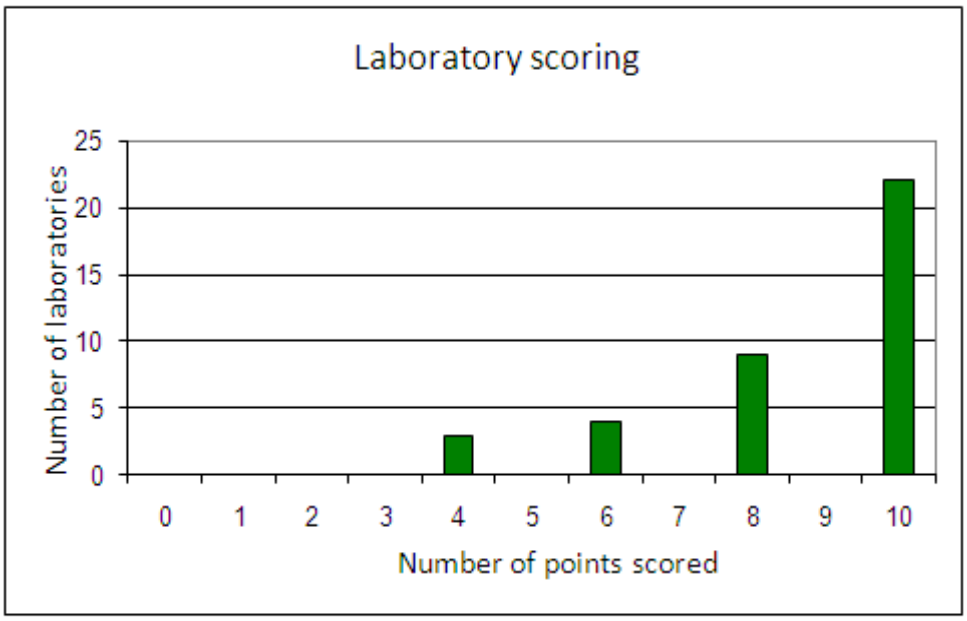


Figure 14. *Obtained score by participants.*

Methods applied

The following methods were used by the participants:

- 15 laboratories used ISAV real-time RT-PCR
- 28 laboratories used ISAV RT-PCR
- 8 laboratories used both ISAV real-time RT-PCR and ISAV RT-PCR
- 13 laboratories used KHV real-time RT-PCR
- 34 laboratories used KHV PCR
- 10 laboratories used both KHV real-time PCR and KHV PCR
- 31 laboratories used *A. invandans* PCR

Laboratory code number	Score (max. 10)	Answer recieved at EURL	ISAV real-time RT-PCR	ISAV RT-PCR	KHV real-time RT-PCR	KHV PCR	*A. invadans* PCR	Sequence ampoule no.
1	10	13-12-2011		X	X	X	X	VI,VII,VIII,IX and X
2	4	29-11-2011		X		X	X	VI,VII,VIII,IX and X
3	8	12-12-2011	X	X		X	X	VI and VII
4	4	16-12-2011		X		X	X	
5	8	13-12-2011		X		X	X	
6	10	09-12-2011	X	X	X	X	X	VI and VII
7	8	16-12-2011	X			X		
8	8	16-12-2011		X		X	X	
9	10	23-11-2011	X	X	X	X	X	VI and VII
10	8	16-12-2011		X		X	X	VII
11	10	16-12-2011		X		X	X	
12	10	08-12-2011	X		X	X	X	
13	10	15-12-2011	X	X	X	X	X	VI,VII,VIII,IX and X
14	10	16-12-2011	X			X	X	
15	8	16-12-2011		X		X		
16	8	15-12-2011	X		X			
17	10	14-12-2011		X		X	X	
18	0	n/a	No reply	No reply	No reply	No reply	No reply	
19	6	16-12-2011				X		
20	10	16-12-2011		X		X	X	VI,VII,VIII,IX and X
21	10	16-12-2011		X		X	X	VI,VII,VIII,IX and X
22	10	16-12-2011		X	X	X	X	VI,VII,VIII,IX and X
23	6	14-12-2011			X		X	VIII and X
24	10	07-12-2011	X		X	X	X	VIII and X
25	10	16-12-2011		X		X	X	VI,VII,VIII,IX and X
26	10	06-12-2011	X	X	X	X	X	VI,VII,VIII,IX and X
27	8	16-12-2011	X	X	X	X		VI,VII,VIII,IX and X
28	0	Did not participate	No reply	No reply	No reply	No reply	No reply	
29	10	16-12-2011		X		X	X	VI, VIII and X
30	10	16-12-2011	X			X	X	VI,VII,VIII,IX and X
31	4	16-12-2011	X					
32	6	15-12-2011						
33	10	16-12-2011		X		X	X	VI and VII
34	6	16-12-2011	X	X		X		
35	8	16-12-2011		X		X	X	
36	10	16-12-2011		X		X	X	
37	10	13-12-2011		X	X	X	X	
38	n/a	16-12-2011	No reply	No reply	No reply	No reply	No reply	
39	10	07-12-2011		X		X	X	VI, VII and IX
40	10	16-12-2011	X	X	X		X	IX
41	10	16-12-2011		X		X	X	VI,VII,VIII and X

A graph was constructed to illustrate the association between the methods used by participants for pathogen identification and the obtained score (Figure 15). The conventional PCR and RT-PCR was the most frequently used method compared to the equivalent real-time assays. For ISAV and KHV identification, approximate half the number of laboratories used real-time assays compared to the number of laboratories using conventional assays. This approximate ratio seems more or less conserved for laboratories scoring max point as well as for laboratories scoring lower points. Therefore, for participants scoring lower than 10, the deficiency in virus identification cannot directly be assigned to improper use of a single identification method. Rather mistakes might be related to performance of the overall procedure.

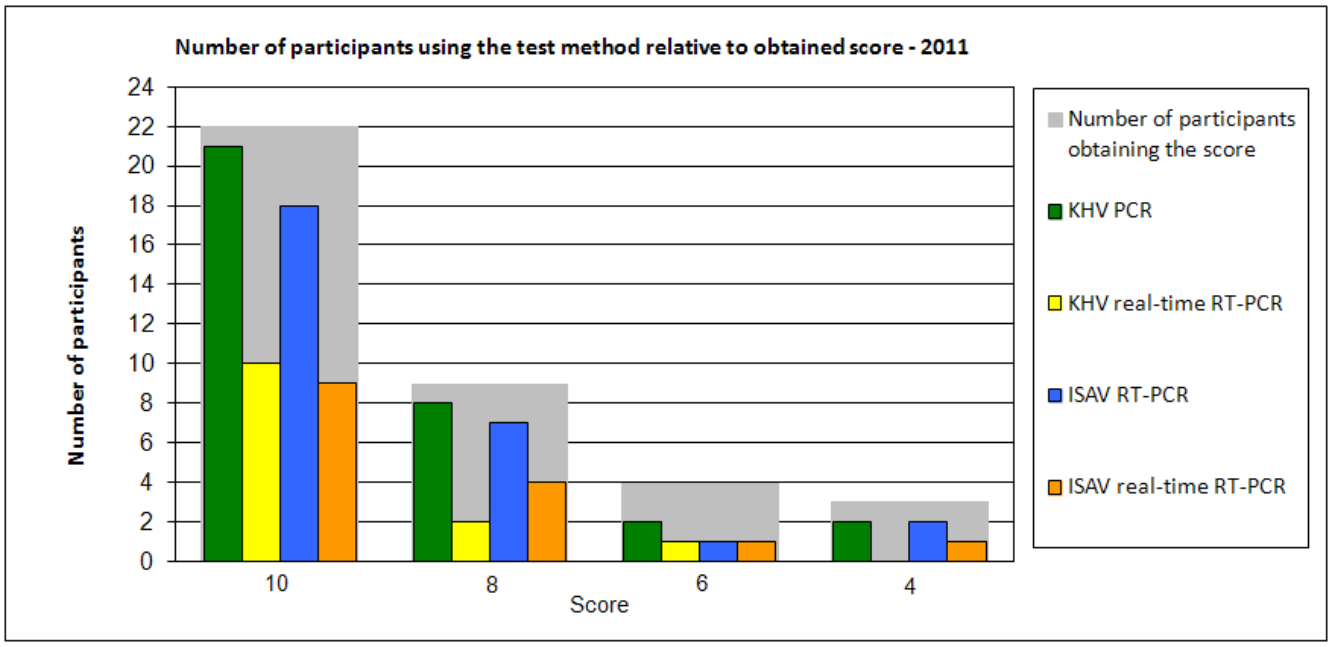


Figure 15. Methods used by participants for virus identification in PT2

Genotypning and sequencing

Participants were encouraged to sequence the HPR region of possible ISAV isolates though it was not a mandatory task.

- 18 laboratories performed sequencing of the ISAV isolate
- 14 laboratories performed sequencing of the KHV isolate
- 12 laboratories performed sequencing of the *A. invadans* isolate

It is positive that many laboratories performed sequencing of isolates as it is important that laboratories can discriminate between isolates as e.g. pathogenic ISAV strains containing deletion in the HPR region and HPR0 strains. It was not described according to what notification the genotype of viruses should be performed reflecting the various way of reporting isolate genotypes. In future tests we will clarify which notification the genotyping should follow.

Concluding remarks PT2

The inter-laboratory proficiency test 2011 was conducted without major constrains. Most parcels were delivered by the shipping companies within 3 days after submission. It was, however, unfortunate that one parcel was on the way for 44 days before delivered to the laboratory primarily due to border controls.

Considering that this was the second time that the EURL provided a proficiency test on ISAV and KHV identification, and the first time that the EURL provided a proficiency test on *A. invadans*, we consider that most participants obtained satisfying results. Out of 36 laboratories performing ISAV identification 32 identified ISAV in ampoule VI containing low titre ISAV, and 35 identified ISAV in ampoule VII containing high titre ISAV. All 37 laboratories testing for KHV identified KHV in ampoule VIII containing high titre KHV, and 36 of them identified KHV in ampoule X containing low titre KHV. Out of 31 laboratories testing for *A. invadans* 28 identified the pathogen in ampoule IX.

Lowering the titre of the virus caused only one laboratory to miss identification of KHV in the low titered ampoule X. As this laboratory stated that KHV was identified in ampoule IX whereas no virus was identified in ampoule X, it may be due to either a typing mistake or a mix-up of ampoule IX and X as this laboratory did not test for *A. invadans* and not that the low titered virus was under detection level in this particular laboratory.. If this is the reason then all laboratories testing for KHV were able to identify both the high titer and the low titer KHV. For ISAV, one laboratory missed identification in the high titer ampoule and for the low titer ampoule three further laboratories did not succeed in the identification.

A couple of laboratories identified pathogens not present in the ampoules. E.g. one laboratory identified ISAV in all ampoules but only with a weak positive reaction in the ampoules where ISAV was not present.

A critical point in PCR based diagnostic tools is avoiding false positive and false negative results. To decrease the risk of having false negative results, it is always recommended that laboratories use the most sensitive tool available, validate the sensitivity of their diagnostic tools and use proper controls. To decrease the risk of false positive results laboratories have to be very aware of the risk of cross contaminations.

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

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

The results presented in this report will be further presented and discussed at the 16th Annual Meeting of National Reference Laboratories for Fish Diseases to be held 30-31 May 2012 in Aarhus, Denmark.

Nicole Nicolajsen and Niels Jørgen Olesen

European Union Reference laboratory for Fish diseases
National Veterinary Institute, Technical University of Denmark, 12 March 2012

References

Baudin Laurencin F (1987) IHN in France. *Bulletin of the European Association of Fish Pathologists* **7**, 104.

Bovo G, Comuzi M, De Mas S, Ceschia G, Giorgetti G, Giacometti P & Cappellozza E (1993). Isolamento di un agente virale irido-like da pesce gatto (Ictalurus melas) dallevamento. Bollettino Societa Italiana di Patologia Ittica 11, 3–10.

Bercovier H, Fishman Y, Nahary R, Sinai S, Zlotkin A, Eyngor M, Gilad O, Eldar A & Hedrick RP (2005) Cloning of the koi herpesvirus (KHV) gene encoding thymidine kinase and its use for a highly sensitive PCR based diagnosis. *BMC Microbiology* **5**, 9 pages

Campbell S, Collet B, Einer-Jensen K, Secombes CJ & 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, Ahrens P, Forsberg R & Lorenzen N (2004). Evolution of the fish rhabdovirus viral haemorrhagic septicaemia virus. *Journal of General Virology* **85**, 1167-1179.

Emmenegger EJ, Meyers TR, Burton TO & Kurath G (2000) Genetic diversity and epidemiology of infectious hematopoietic necrosis virus in Alaska. *Diseases of Aquatic Organisms* **40**, 163-176.

Falk K, Namork E, Rimstad E, Mjaaland S & Dannevig BH (1997). Characterization of infectious salmon anemia virus, an orthomyxo-like virus isolated from Atlantic salmon (Salmo salar L.) Journal of Virology 71, 9016-9023.

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. *Diseases of Aquatic Organisms* **60**, 179-187.

Hyatt AD, Gould AR, Zupanovic Z, Cunningham AA, Hengstberger S, Whittington RJ, Kattenbelt J & Coupar BEH (2000) Comparative studies of piscine and amphibian iridoviruses. *Archives of Virology* **145**, 301-331.

Holopainen R, Ohlemeyer S, Schütze H, Bergmann SM & Tapiovaara H (2009) Ranavirus phylogeny and differentiation based on major capsid protein, DNA polymerase and neurofilament triplet H1-like protein genes. *Diseases of Aquatic Organisms* **85**, 81-91.

Jancovich JK, Bremont M, Touchman JW & Jacobs BL (2010) Evidence for multiple recent host species shifts among the ranaviruses (family Iridoviridae). *Journal of Virology* **84**, 2636-2647.

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.

Jørgensen PEV & Bregnballe F (1969) Infectious pancreatic necrosis in rainbow trout in Denmark. *Nordisk Veterinærmedicin* **21**, 142-148.

Jørgensen PEV & Grauballe PC (1971) Problems in the serological typing of IPN virus. *Acta Veterinaria Scandinavica* **12**, 145-147.

Kolodziejek J, Schachner O, Dürrwald R, Latif M & Nowotny N (2008) "Mid-G" region sequences of the glycoprotein gene of Austrian infectious hematopoietic necrosis virus isolates form two lineages within European isolates and are distinct from American and Asian lineages. *Journal of Clinical Microbiology* **46**, 22-30.

Kurata O, Kanai H & Hatai K (2000) Hemagglutinating and hemolytic capacities of *Aphanomyces piscicida*. *Fish Pathology - Gyobyo Kenkyu* **35**, 29-33.

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.

Langdon JS, Humphrey JD & Williams LM (1988) Outbreaks of an EHNV-like iridovirus in cultured rainbow trout, *Salmo gairdneri* Richardson, in Australia. *Journal of Fish Diseases* **11**, 93-96.

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.

Mjaaland S, Rimstad E, Falk K & Dannevig BH (1997) Genomic characterization of the virus causing infectious salmon anemia in Atlantic salmon (*Salmo salar* L.): an orthomyxo-like virus in a teleost. *Journal of Virology* **71**, 7681-7686.

Mortensen HF, Heuer OE, Lorenzen N, Otte L and Olesen NJ (1999) Isolation of viralhaemorrhagic septicaemia virus (VHSV) from wild marine fish species in the Baltic Sea,Kattegat, Skagerrak and the North Sea. *Virus Research* **63**, 97-108.

Snow M, Bain N, Black J, Taupin V, Cunningham CO, King JA, Skall HF and Raynard RS (2004) Genetic population structure of marine viral haemorrhagic septicaemia virus (VHSV). *Diseases of Aquatic Organisms* **61**, 11-21.

Snow M, McKay P, McBeath AJA, Black J, Doig F, Kerr R, Cunningham CO, Nylund A & Devold M (2006) Development, application and validation of a Taqman® real-time RT-PCR assay for the detection of infectious salmon anaemia virus (ISAV) in Atlantic salmon (*Salmo salar*). In: *New Diagnostic Technology: Applications in Animal Health and Biologics Controls* (ed. by P. Vannier & D. Espeseth), pp. 133-145, Basel.

Laboratory visit at the
Finnish Food Safety Authority, Evira

Helsinki, Finland
7th – 8th November 2011

Introduction

The National Reference Laboratory for Fish Diseases (NRL) in Finland is located at the Finnish Food Safety Authority, Evira in Helsinki. Evira was visited from the 7th to the 8th of November 2011 by Nicole Nicolajsen and Søren Kahns from the European Union Reference Laboratory for Fish Diseases (EURL). The program for the visit is shown in Annex 1 and a list of persons met by the EURL representative is listed in Annex 2. This report describes findings, comments and recommendations made by the delegation from the EURL. This report is sent to the Evira and the EU Commission.

Organisation

The Evira is a public governmental institution under the Ministry of Agriculture and Forestry. The Evira headquarters are located in Helsinki, on Viikki Campus. Eight additional regional offices are located in the following cities: Joensuu, Kouvola, Kuopio, Lappeenranta, Loimaa, Oulu, Seinäjoki and Turku, is shown in Annex 3. Fish samples are accepted only in Helsinki, Oulu and Kuopio. About 750 persons are employed at Evira of which 530 works at the location in Helsinki.

Three operational bodies (Food Safety, Animal Health and Welfare, and Plant Production and Health) are services by the Control Department, the Research and Laboratory Department and the Administration. An organization diagram of Evira is shown in Annex 4.

TASO is the unit responsible for diagnosis of TSE and fish viral diseases. The NRL for Fish Diseases is part of the TASO section. The head of the NRL for fish diseases is Dr. Tuija Gadd and head of TASO is Hannele Tapiovaara. TASO is a part of the Veterinary Virology Research Unit that is headed by Liisa Kaartinen. The Veterinary Virology Research Unit is part of the Research and Laboratory Department that is headed by the director Tuula Honkanen-Buzalski.

Buildings, Furnishing and Access

Evira in Helsinki is located in newly constructed buildings within Helsinki. The buildings were finished in 2006 and they provide a very nice working place with well equipped laboratories. The buildings are owned by Senate Properties which is a government owned enterprise under the aegis of the Finnish Ministry of Finance and is responsible for managing the Finnish state`s property assets and for letting premises. Evira rents the space in the buildings.

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

Opening of the meeting

At the official opening of the visit, Head of the Veterinary Virology Research Unit, Dr. Liisa Kaartinen welcomed the visitors from the EURL and gave a detailed description of the Evira, its history, organization and mission. Subsequently, the head of the NRL, Dr. Tuija Gadd gave a presentation on the Finnish NRL and the disease situation regarding listed diseases in Finland. This was followed by a talk on the functions and duties of the EURL, by Dr. Søren Kahns.

The meeting was followed by a tour at the institute with the aim of illustrating how processing of fish samples occur - from the sample is received and until an answer of the diagnostic analyses is given. The

tour was guided by Dr. Tuija Gadd, Dr. Hannele Tapiovaara and Maria Hautaniemi to the fish virology laboratory and the cell culture unit at day 1. At day 2, Dr. Pia Vennerström and Maria Eriksson-Kallio presented the sample acceptance laboratory, Maria Hautaniemi and Dr. Hannele Tapiovaara presented the molecular biology facilities, and Dr. Tuija Gadd described the process of handling proficiency tests.

Sample acceptance and autopsy

Fish samples were received at the sample acceptance and autopsy rooms at the Fish section (fish diseases, pathology) in Helsinki, Kuopio or Oulu. The Fish section is one part of the Production Animal and Wildlife Health Research Unit. Procedures and equipments were presented by Dr. Pia Vennerström and Maria Eriksson-Kallio in Helsinki. All fish samples delivered to the institute is entered at this unit. All virological samples are sent to Veterinary Virology Unit in Helsinki also from regional laboratories Kuopio and Oulu. Samples are received from veterinarians of both official and private establishments and/or private persons. Veterinarians are not specialized in the "fish" area. Therefore Evira provide education of veterinarians on how to sample fish. Instructions are provided directly to veterinarians at courses arranged by Evira. Furthermore, instructions are also found at the www.Evira.fi webpage. Veterinarians phone the day before Evira receive the samples. Samples are sent in as whole fish or organs. All received material is registered and given a unique number. Providers of samples have to supply information on e.g. fish species, clinical signs, fish farm. The sample number and all collected information is entered into an electronically sample registration system. After the samples have been processed and homogenised, they are sent to the laboratory that will perform the required diagnostic analyses. Samples are analysed for the presence of viruses at the Veterinary Virology Unit. Detection of bacteria is performed at the Veterinary Bacteriology Research Unit in Helsinki or Kuopio or Animal and Wildlife Health Research Unit in Oulu. All these three laboratories are independently responsible for mycological diagnostics also, whereas Kuopio is responsible for the verification of suspected cases of EUS. Finnish Food Safety Authority Evira Kuopio that is also designated as OIE Reference Laboratory for Crayfish plague (*Aphanomyces astaci*).The diagnostic status of the samples can be followed during the analyses in electronically sample registration system. When the results of the diagnostic analyses have been obtained a final report is made in the electronically sample registration system. This answer has to be signed by the persons responsible for fish diagnostics before an answer is send to costumers.

Processing of the fish or organs received takes place in the sample acceptance and autopsy room. For virus diagnostics, brain, kidney, heart and spleen are taken out when samples are received as whole fish. When fish are dissected in the field only kidney, heart and spleen are collected. Organs are homogenized on ice using a homogenisator. A total of one gram of tissue is homogenised. If samples are send in because of suspicion of diseases, extra sample material is collected. Information on suspicion of disease is forwarded to the laboratory performing the identification of the pathogens. Back up of all samples is kept in the freezer. If immediate transfer to the laboratory performing identification of pathogens is not possible, the samples will be stored in a refrigerator.

Disinfection of the workspace was done by UV light and 70% ethanol.

Equipment in the sample preparation room

- Refrigerator
- Freezer -20°C
- Weight
- Centrifuges
- Homogenisor
- Required small laboratory equipment

A recipe for Transport Media/Eagle's mem with tris and 10 % newborn calf serum ph 7.6 (dilution medium) as it is made at the EURL is attached in Annex 5.

The Laboratory for Fish Virology and the NRL for Fish Diseases

The Laboratory for Fish Virology is a part of the TASO section and responsible for diagnostics of viral fish diseases. The Laboratory for Fish Virology coordinates the NRL for fish diseases. The NRL for fish diseases is covered by several units within Evira. The Laboratory for Fish Virology performs cell culture assays and identification of fish viruses by virus identification tests (ELISA, IF) and by the PCR based diagnostics. Analyses for identification of the listed oomycete *Aphanomyces invadans* is performed at Evira Kuopio.

Staff of the Laboratory for Fish Virology and the NRL for Fish Diseases

Hannele Tapiovaara is the Head of the TASO section and Tuija Gadd is responsible for diagnostics of fish viral diseases. The Laboratory for Fish Virology consists of two senior scientists, Hannele Tapiovaara and Tuija Gadd, and technicians Laura Sneitz, Hanna Kauno, Tarja Seppänen and Nura Farah who carry out all diagnostics of fish viruses besides other tasks in Veterinary Virology Research Unit. There are also other scientists and technicians are qualified to carry out diagnostics for viral diseases in fish in the research unit.

Laboratory for Fish Virology

The laboratory was newly reconstructed and well equipped. The laboratory was split into separate working areas: One workbench was used for working with samples suspected for or known to contain virus infections. Two workbenches were used for handling of surveillance samples from fish without clinical signs. Furthermore, computer working space was present for registration of diagnostic results.

Equipment was new and adequate. All equipment has a reference number and a logbook. Username, date and sample are registered in the logbook as well as any problems encountered. Repairs, cleaning, calibration and other maintenance such as filter change are likewise registered. Calibration of the pipettes and other equipment was performed by an internal calibration unit.

Instructions for use of equipment are written down in procedures for each piece of equipment. Staffs are required to read and be trained according to these procedures before using the equipment.

Samples are received as homogenized and they are stored in the fridge in the laboratory until further processing occurs. Inoculation of samples on cell cultures is performed in a distinct LAF-bench depending on the expected infectivity status of the sample. Samples at dilutions of 1:10 and 1:100 are inoculated on 24 well plates (It might be considered to inoculate with an additional dilution at 1:1000 for screening for IPNV). Samples are inoculated on BF-2 and EPC plates. Samples are inoculated on cells on the same day as they are received except for weekends where samples are frozen.
Samples are added antibiotics and sometime anti-IPN. Cell cultures are checked for CPE at two specified dates by a technician. The final "reading" of the result of the cell culture assay is performed by the responsible scientist. The cell culture assays are checked twice a year through cell sensitivity tests for VHSV, IHNV and IPNV but not EHNV.

Identification of viruses by antibody based methods is also performed in the laboratory and adequate equipment for making the identification is present. Identification of fish viruses is primarily done by ELISA using kits from Bio-X and Test line. The laboratory does also perform IFAT tests but this technique is not often used.

Disinfection of the workspace was done by UV light and 70% ethanol every day and once a week by Virkon S.

Equipment in the room

- Centrifuge
- 2 LAF bench surveillance
- 1 LAF benches for dirty/research
- 2 incubators 1 dirty 1 surveillance
- Refrigerator
- Incubator at 16°C
- ELISA reader + washer
- 2 Microscope
- Pipettes
- Safety cabinet
- Required Small laboratory equipment






Cell Culture Laboratory

All work with cell culturing is performed in a Cell Culture laboratory that is a "clean room" only used for this purpose. All cell cultures are produced by this unit for the whole institute.

Fish Cell Culture Facilities

BF-2, EPC, RTG-2, CHSE, ASK, SHK-1 cells are continuously cultured in the lab. The cells are passaged once or twice weeks. EPC cells are grown on Corning CellBind surface Costar plates whereas BF-2 is grown on normal plates. The out growing occurs for one day at room temperature after which BF-2 and EPC is transferred to 16C. RTG-2, CHSE, ASK, SHK-1 is stored at RT. There is a back-up of cells stored in liquid nitrogen and cells are tested for mycoplasma by PCR. Cell culture media is made by a central media laboratory at Evira. Two persons take care of the work in the cell culture laboratory: One academic staff (approximately an hour per day) and one full time technician. The academic staff decides when the cells have to be passaged.

Cell lines

- Fish cell lines grown in the laboratory: Primarily BF-2 and EPC but the laboratory also has SHK-1, ASK, FHM, CHSE and SSN-1 cells available. The cell has been obtained from the CRL and Veterinary Institute in Oslo.

Equipment

- 2 LAF bench
- Incubator at 16°C,
- Microscope
- Refrigerator
- Freezer -20^{o} for storage of all required media for cell cultivation
- Required small laboratory equipment

Disinfection of the workspace was done by UV light and 70% ethanol every day and once a week by Virkon S.

PCR based diagnostics

PCR based diagnostic tools are implemented for detection of VHSV, IHNV, EHNV, KHV and ISAV, using either OIE recommended or own produced PCR protocols. For ISAV detection, the method described in Mjaaland et al. 1997 is used. For EHNV detection, the method described in the OIE manual and an assay developed by Evira is used. For IHNV detection, a nested method described in the OIE manual is used. For KHV detection, a nested TK PCR method is used. For VHSV detection, several conventional methods

are used targeting several genes as well as a real time PCR assay described by Chico et al. For comparison, the EURL provided hand outs with protocols used for virus detection at the EURL.

The molecular biology facilities are divided into several separated rooms in the Veterinary Virology Research Unit. One room acts as a clean room where primers and buffers are stored and master mixes are prepared. This mastermix room contains e.g. two LAF benches, freezer, fridge and an ice machine. In another room, purification of DNA and RNA is performed along with cDNA synthesis. This room contains e.g. one PCR machine for cDNA synthesis and a semi-automatic purification machine. The following Qiagen kits are used for purification of nucleic acids: RNA purification: QIAamp viral RNA purification kit and DNA purification: DNA mini kit. The diagnostic methods are perfomed in a PCR room containing three real-time thermocyclers, 6 PCR machines, gelelectrophoreses apparatuses and gel visualization apparatus. This room contains a separated LAF bench used for nested PCRs only. In addition, a separate room contains an ABI sequencing machine.

Accreditation

Evira is accredited according to ISO 17025 by the Finnish Accreditation Service (FINAS). The NRL for Fish Diseases have been accredited for viral growth of VHSV, IHNV, SVCV and IPNV on monolayered fish cell cultures and for identification of VHSV, IHNV, SVCV, and IPNV by ELISA and IFAT. Accreditation of molecular biological assays is expected to occur in the near future.

Proficiency Test

The proficiency tests (PT) allow a laboratory to assess their diagnostic capacity of certain procedures. The Finnish NRL for fish diseases has participated in the PT for identification of notifiable fish diseases organized by EURL, Aarhus, Denmark, since 1996. The NRL used the following tools for identification of viral content in the test: Titration of virus; Isolation of viruses on cell culture; Identification of virus by ELISA, RT-PCR, PCR and sequencing. The score obtained by the NRL was 60% correct in 2010 and 100% correct in 2008 and 2009. For the scores obtained by the NRL in the PT provided by the EURL, see Annex 6. A general procedure in the laboratory is that when a PT result is less than 100% correct, the NRL will redo the test using all diagnostic procedures to verify that expected results can be obtained. After the result of the PT 2010, the proficiency test was reexamined and the correct results were obtained.

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

Aquaculture in Finland and categorization of fish farms according to Council Directive 2006/88/EC

The main fish species cultured in Finland are salmonids as rainbow trout, whitefish and salmon. In 2010, Finland had circa 34 fish farms producing more than 100 tonnes per year, 190 fish farms producing 5 - 100 tonnes per year and 85 fish farms producing less or around 5 tonnes per year. The laboratory received approximately 1267 samples in 2010 for diagnostic and surveillance purposes. The samples for virological examinations are mainly submitted from rainbow trout. Please see Annex 7 for more details.

The disease situation in Finland was described by Senior Officer, Hanna Kuukka-Anttila. All fish farms have been registered and farms selling juveniles or producing more than 5000 kg a year are authorized. Authorised farms are listed in the public EU Register of aquaculture production businesses and authorised processing establishments. Finnish aquaculture is considered free of IHN and ISA disease. Concerning KHV disease Finnish farms are in category III. In the last 10 years, VHS outbreaks rainbow trout farms have only been observed in three areas in Finland. In the area around Pyhtää in the Gulf of Finland, the last outbreak was observed in 2001 and the area has been considered to be free of VHSV since 2008. In the area around Uusikaupunki in the Gulf of Bothnia, the last outbreak was observed in 2008 and the area has been considered to be free of VHSV since 2011. The Åland Islands experienced 6 farms positive of VHS in 2009 and one in 2010. A restriction zone covering Åland islands was established in 2000. Positive farms inside restriction zone operate under restrictive orders. Fish in positive farms are cultivated until they reach slaughter size. Officially approved eradications are not done. This area is the only area in Finland not considered free of VHS (2011) and is placed in Category V.

Training needs and future plans

The diagnostic methods for detection of VHSV, IHNV, ISAV, KHV and EHNV are fully implemented in the laboratory. The diagnostic methods for identification of EUS are currently being implemented at the Evira Kuopio. The only suggestions made were too include EHNV in the cell sensitivity test.

Conclusion

The visit showed that the Finnish NRL for fish disease has an adequately equipped laboratory. The staff is well educated and very capable and works according to accredited methods and according to EU requirements. No major problems were observed and only minor suggestions were made. Therefore, the overall conclusion is that the NRL of Finland 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

November 7th 2011

13:00 – 15:00	Meet with colleagues/ sandwiches Introduction of the Finnish National Reference Laboratory for Fish Diseases. Presentation of the EURL and discussion on the topics of the visit. Participants: As in accompanying list - meeting room Pirkko with video connection via multipoint 1; Note the time 12:00 – 14:00, colleagues in Evira Oulu and Kuopio - meeting room Esko without video connection 14:00 - 15:00
15:00 – 17:00	Tour in the lab, looking at facilities. We would like to learn about how the diagnostic procedures are conducted in the laboratory. Following issues could be discussed: • Aquaculture in Finland 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 (2009 and 2010) Proficiency Test Results On **day 1** we suggest that we go through the issues listed above from the top and focus on the laboratory and the general handling on samples in the laboratory.

November 8th 2011

08:00 – 11:00	Continuation of the tour in lab. We suggest that we on day 2 split into two groups. Nicole and some of your colleagues could make one group, where handling of the proficiency test 2009 - 2011 could be used as basis for discussion of how viral identification procedures takes place. We suggest that Soren could focus on molecular diagnostics with your colleagues performing these methods. Meeting room Pirkko from 9:00 – 12.00 (room Pirkko with video connection via multipoint 2)
11:00 – 11:30	Plans and progress in the implementation of Council Directive 2006/88/EC in Finland Lunch
11:30 – 12:15	Evaluation of the visit. Recommendations and report of the visit
12:30	Departure to airport Meeting room Pirkko with video connection via multipoint 1 is available 9:00-12:00

Annex 2

Persons from EVIRA participating at the meetings

Name	**Task**
Liisa Kaartinen	Head of Veterinary Virology Research Unit
Hannele Tapiovaara	Senior scientist, head of TASO section. TASO is responsible for diagnostics of TSE and fish viral diseases and maintain cell lines
Tuija Gadd	Senior scientist, TASO section, responsible scientist for diagnostics for fish viral diseases
Maria Hautaniemi	Scientist, TASO section, TSE- and virus diagnostics, molecular biology
Hanna Kauno	Technician, virology
Tarja Seppänen	Technician, virology
Nura Farah	Technician, virology
Hanna Kuukka-Anttila	Senior Officer, Animal Health and Welfare Unit, Control Department
Perttu Koski	Senior scientist, head of fish section in the Production Animal and Wildlife Health Research Unit (Oulu, Finland); via video link
Pia Vennerström	Scientist, Animal and Wildlife Health Research Unit, Fish section, fish diseases, pathology
Maria Eriksson-Kallio	Scientist, Animal and Wildlife Health Research Unit, Fish section, fish diseases, pathology
Satu Viljamaa-Dirks	Scientist, Animal and Wildlife Health Research Unit, Fish section, fish diseases, pathology(Kuopio, Finland); via video link
Sirpa Heinikainen	Scientist, The Veterinary Bacteriology Research Unit, Kuopio laboratory section (Kuopio, Finland); via video link
Varpu Hirvelä-Koski	Senior scientist, head of Wildlife and Laboratory section in the Production Animal and Wildlife Health Research Unit (Oulu, Finland); via video link
Dr. Søren Kahns	Coordinator of the EU Reference Laboratory for Fish Diseases, Section for Fish Diseases, National Veterinary Institute, Technical University of Denmark.
Nicole Nicolajsen	Laboratory Technologist EU Reference Laboratory for Fish Diseases, Section for Fish Diseases, National Veterinary Institute, Technical University of Denmark.

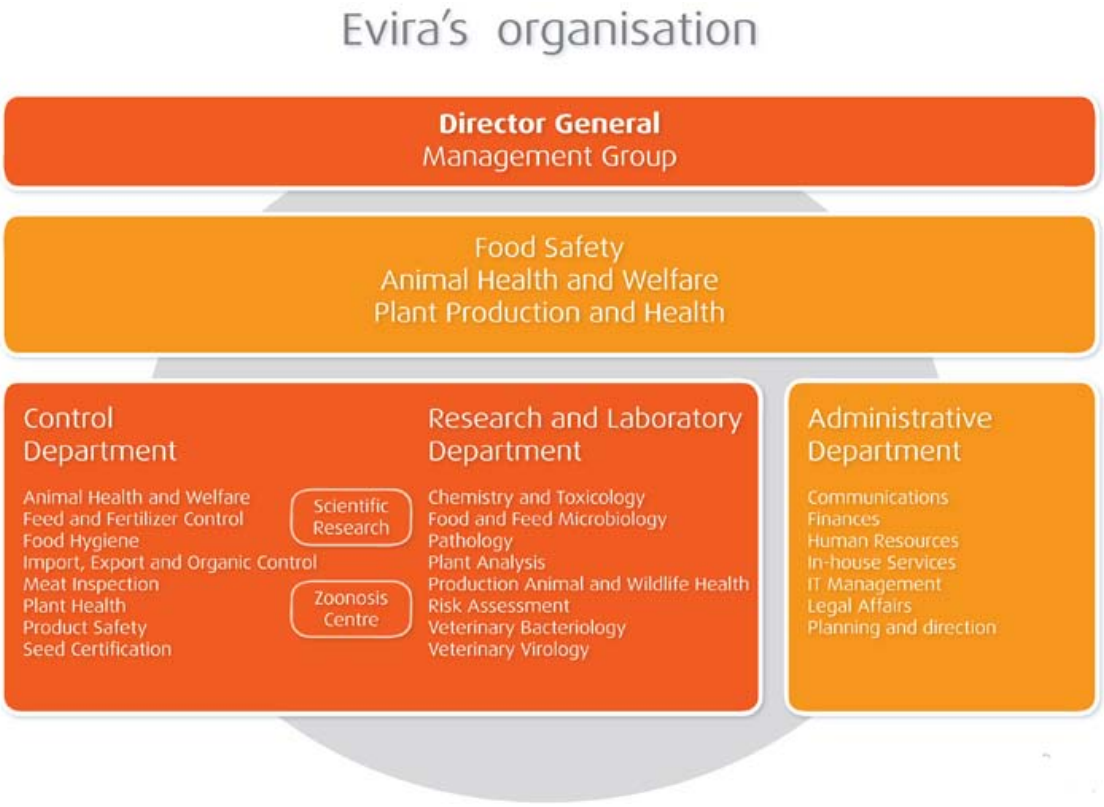
Annex 3

Location of Evira department

Maps of containing 9 cities that are housing Evira departments

Annex 4

Organisational and Functional Structure



Annex 5

Transport Media/Eagle's mem with tris and 10 % newborn calf serum ph 7.6 (dilution medium)

Aim

Eagle's minimal essential medium (Eagle's MEM) with TRIS and 10 % newborn calf serum is used to store diagnostic samples during transport and as dilution medium for e.g. immunofluorescence, neutralisation, simultaneous neutralisation and titrations.

Equipment

- one 2000 ml sterile measuring cylinder
- two 250 ml sterile measuring cylinder
- two 2 L sterile measuring flasks
- one 25 ml sterile pipette
- 250 ml sterile glass bottles with screw-cap lid
- pH meter
- Balance
- Pressure tank and plastic tubes for sterile filtration
- Filter
- Rack
- Laminar airflow cabinet
- Magnetic stirrer

Reagents

1. Milli Q water
2. Dehydrated Eagle's MEM (e.g. Life Technologies, Gibco cat. no. 61100-103, stored in refrigerator)
3. TRIS-HCl buffer pH 7.6, stored in refrigerator
4. Penicillin-streptomycine solution (e.g. Life Technologies, Gibco cat. no.15140-122,, stored in -20°C freezer)
5. Newborn bovine serum (stored in -20°C freezer)

Safety precautions

Eagle's MEM and penicillin-streptomycine can cause allergies. Avoid direct contact by using a fume hood and gloves when handling

Method

Recipe for the production of approx. 2.2 L medium in 2L flask:

Milli Q water.....1750 ml
Dehydrated Eagle's MEM.....19.2 g
TRIS-HCl buffer pH 7.6.....250 ml
Penicillin-streptomycine .....20 ml
Newborn bovine serum .....200 ml

Procedures

1. 1750 ml Milli Q water is measured in a 2000 ml measuring cylinder and poured into the 2 L flask. Eagle's MEM and TRIS-HCl buffer are weighed in a 250 ml measuring cylinder and added to the flask too. Mix on a magnetic stirrer.
2. In the Laminar airflow cabinet (remember to start at least 15 min before use on highest flow) the following reagents are added: penicillin-streptomycine (use 25 ml pipette) and newborn calf serum (use 250 ml measuring cylinder). Mix on a magnetic stirrer.
3. Measure the pH which should have a value of 7.6 ± 0.2. Do not make any adjustments if the pH value is outside these limits but discard the batch. Try to find out what went wrong during the procedures.
4. The non-sterile medium is now transferred to the pressure tank. Put the lid on.
5. All following procedures are carried out in a Laminar Airflow cabinet. Unwrap the autoclaved filter. Be aware of the risks of contamination of flasks and lids where contact with medium is possible. Put the filter in a rack, that has been desinfected by Virkon S, and connect the access valve of the filter with the tube from the pressure tanks exit valve.
6. The 2L flask is placed underneath the filter, which is tightened before and after filtration. Switch on the nitrogen at a rate of 0.8 kg/cm^2 and connect the tube to the access valve on the pressure tank.
7. Monitor the filtration continuously.
8. Pour the solution into sterile 250 ml flasks. Screw lids are placed onto the bottles. Be sure to close them correctly. Wrap flamed alufoil on the lids.
9. Label the bottles and place them in a refrigerator.
10. Medium can be stored for up to 6 months.

Labelling

Eagle's MEM with TRIS and 10% Newborn calf serum
TRIS pH 7.6
(dilution medium)
Production date.
Batch no.
Store at 4°C.

Good advice

If there are problems with the flow of the medium through the filter, try to remove air bubbles in the filter. To test if the medium is sterile, a sample can be smeared on a blood agar plate, which is incubated overnight at 37°C in a plastic container.

Annex 6

Proficiency Test – PT1

Finland	1996	1997	1998	1999	2000	2001	2002	2003	2004	2005	2007	2008	2009	2010
Participated	X	X	X	X	X	X	X	X	X	X	X	X	X	X
Score %											100	100	100	60

2006 No Proficiency test

2010 **Score 6/10** **Code 7**	**Ampoule I:** **EHNV** **Low titer**	**Ampoule II:** **IHNV**	**Ampoule III:** **European Catfish Virus (ECV)**	**Ampoule IV:** **SVCV**	**Ampoule V:** **VHSV**
Cell line	BF-2/EPC				
ELISA	VHSV - IHNV - IPNV - SVCV -	VHSV - IHNV + IPNV - SVCV -	VHSV - IHNV - IPNV - SVCV -	VHSV - IHNV - IPNV - SVCV +	VHSV + IHNV - IPNV - SVCV -
PCR	VHSV - IHNV - SAV +	VHSV - IHNV +	VHSV - IHNV - SAV +	VHSV - IHNV -	VHSV + IHNV -
Result	**SAV**	**IHNV**	**SAV**	**SVCV**	**VHSV**

2009 **Score 10/10** **Code 13**	**Ampoule I:** **EHNV**	**Ampoule II:** **IHNV**	**Ampoule III:** **VHSV** genotype Ie	**Ampoule IV:** **VHSV** genotype IVa	**Ampoule V:** **No virus**
Cell line	BF-2/EPC				
ELISA	VHSV - IHNV - SVCV - IPNV -	VHSV- IHNV+ SVCV - IPNV -	VHSV + IHNV - SVCV - IPNV -	VHSV + IHNV – SVCV – IPNV -	Not performed
PCR	VHSV - IHNV - EHNV +	VHSV - IHNV + EHNV -	VHSV + IHNV – EHNV -	VHSV + IHNV - EHNV -	Not performed
qPCR:	VHSV-	VHSV-	VHSV+	VHSV+	Not performed
Result	**EHNV**	**IHNV**	**VHSV** genotype I	**VSV** genotype IVa	**Negative**

Inter-laboratory Proficiency Test 2010

Name of the National Reference Laboratory: Finnish Food Safety Authority, Evita

Country: Finland

Contact name: Tuija Kristiina Gadd

Code: 7

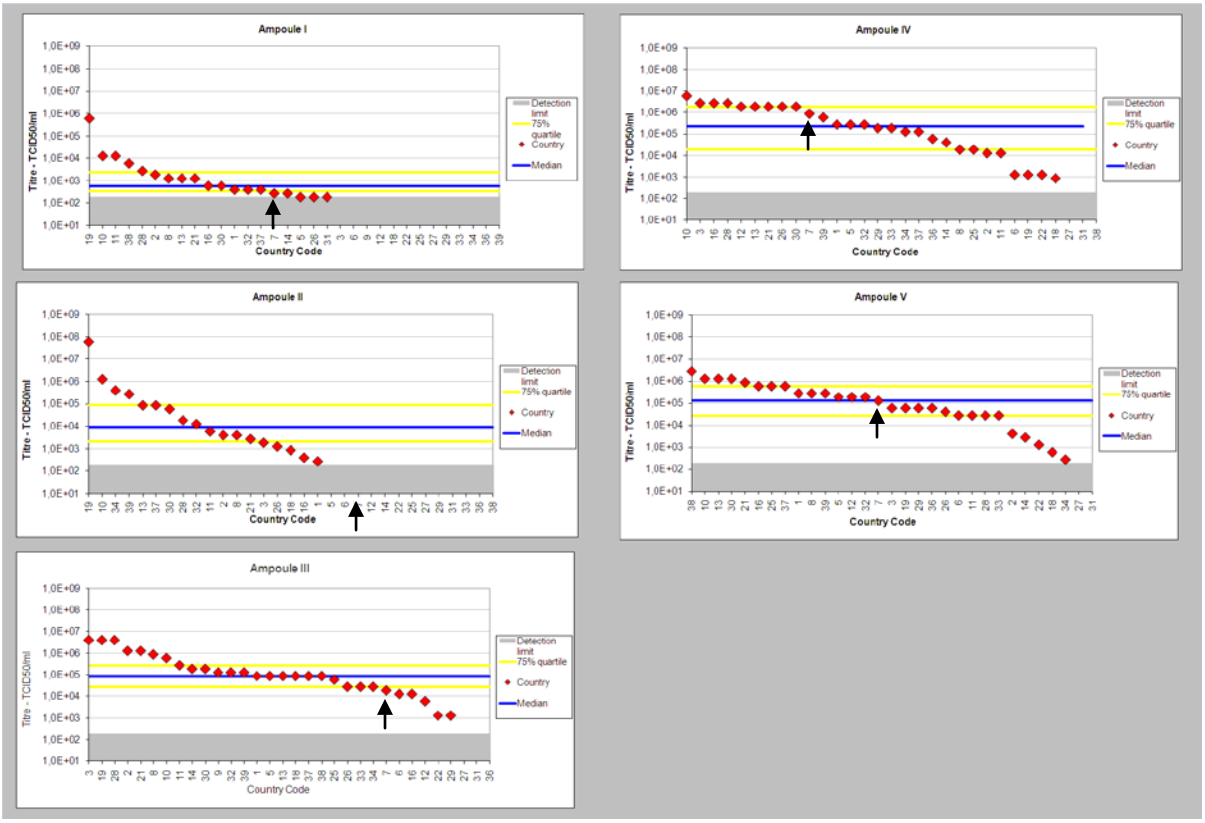
Score: 6 (out of 10)

LABORATORY RESULTS:

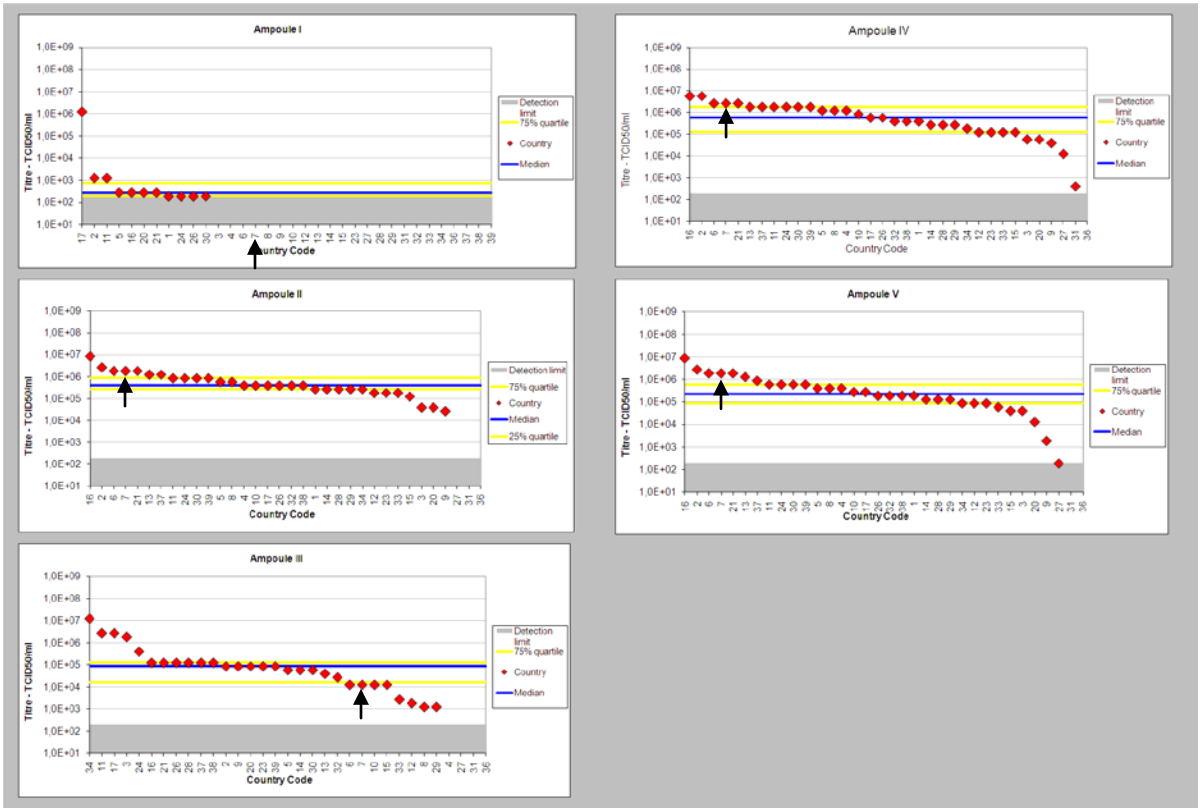
	ELISA	IFAT	Neutralisation	PCR	Other	Final Result
Ampoule I	VHSV – IHNV – IPNV – SVCV –	Not performed	Not performed	VHSV – IHNV – SAV+		**SAV**
Ampoule II	VHSV - IHNV + IPNV – SVCV –	Not performed	Not performed	VHSV – IHNV +		**IHNV**
Ampoule III	VHSV – IHNV – IPNV – SVCV –	Not performed	Not performed	VHSV – IHNV – SAV+		**SAV**
Ampoule IV	VHSV – IHNV – IPNV – SVCV +	Not performed	Not performed	VHSV – IHNV –		**SVCV**
Ampoule V	VHSV + IHNV – IPNV – SVCV –	Not performed	Not performed	VHSV + IHNV –		**VHSV**

Comments: You find SAV instead of ranaviruses in ampoule I and III. We recommend that you check the specificity of your EHNV and SAV identification assays.

Titre obtained in BF-2 cells



Titre obtained in EPC cells



Proficiency Test – PT2

Finland	2010	2011
Participated	X	
Score %	60	

2010 **Score 6/10**	**Ampoule VI:** **ISAV** **High titer**	**Ampoule VII:** **KHV** **(CyHV-3)** **High titer**	**Ampoule VIII:** **Medium**	**Ampoule IX:** **ISAV** **Medium titer**	**Ampoule X:** **KHV** **(CyHV-3)** **Low titer**
RT-PCR	ISAV+	ISAV-	ISAV-	ISAV+	ISAV-
PCR	KHV-	KHV+	KHV+	KHV+	KHV+
Result	**ISAV**	**KHV**	**KHV**	**KHV /ISAV**	**KHV**

Inter-laboratory Proficiency Test 2 2010

Name of the National Reference Laboratory: Finnish Food Safety Authority, Evita

Country: Finland

Contact name: Tuija Kristiina Gadd

Code: 7

Score: 6 (out of 10)

LABORATORY RESULTS:

	RT-PCR	Real-time RT-PCR	PCR	Real-time PCR	Other - specify:	Final Result
Ampoule VI	ISAV+	Not performed	KHV-	Not performed		**ISAV**
Ampoule VII	ISAV-	Not performed	KHV+	Not performed		**KHV**
Ampoule VIII	ISAV-	Not performed	KHV+	Not performed		**KHV**
Ampoule IX	ISAV+	Not performed	KHV+	Not performed		**KHV, ISAV**
Ampoule X	iSAV-	Not performed	KHV+	Not performed		**KHV**

Comments: You find both KHV in ampoules VIII and IX. Please consider if this might be caused by cross-contamination of your samples.

Annex 7

Aquaculture in Finland

Data from S&D 2010

Land Area	Ocean Area	Environment	Species	Scientific name	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009
Finland	Europe - Inland waters	Brackishwater	Miscellaneous freshwater fishes	Miscellaneous freshwater fishes	0-	0-	0-	0-	0-	0-	0-	0-	0-	28
			Salmons, trouts, smelts	Salmons, trouts, smelts	69	157	159	284	383	509	672	702	568	578
		Sub-total Brackishwater			69	157	159	284	383	509	672	702	568	606
		Freshwater	Miscellaneous freshwater fishes	Miscellaneous freshwater fishes	44	49	38	26	54	37	26	56	72	92
			Salmons, trouts, smelts	Salmons, trouts, smelts	2 077	2 500	2 934	2 097	1 798	2 197	2 120	2 173	2 087	2 421
		Sub-total Freshwater			2 121	2 549	2 972	2 123	1 852	2 234	2 146	2 229	2 159	2 513
	Sub-total Europe - Inland waters				2 190	2 706	3 131	2 407	2 235	2 743	2 818	2 931	2 727	3 119
Total Finland					2 190	2 706	3 131	2 407	2 235	2 743	2 818	2 931	2 727	3 119

Data taken from FIGIS

Number of fish farms within country/region, according to size of production (tonnes fish/year)				
	2010	2009	2008	2007
< 5 tonnes	85	85	85	42
5 - 100 tonnes	190	190	190	224
> 100 tonnes	34	34	34	27

Number of fish farms within country/region, according to fish species				
	2010	2009	2008	2007
Rainbow trout	205 (some hatcheries have also some other salmonids	205 (some hatcheries have also some other salmonids	205 (some hatcheries have also some other salmonids	203
Atlantic Salmon	included in other salmonids	included in other salmonids	included in other salmonids	
Other salmonids	100 (some hatcheries have also other salmonids)	100 (some hatcheries have also other salmonids)	100 (some hatcheries have also other salmonids)	96
Carp	1	1	1	1
Eel	0	0	0	
Flatfish	0	0	0	
Seabream / Seabass	0	0	0	
Other marine spp.	0	0	0	
Other freshwater spp.	3	3	3	3
Total	309	309	309	303

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:				
	2010	2009	2008	2007
No. of samples tested by cell cultivation	1161	1180	1206	1291
No of samples tested by PCR or other direct methods without cell cultivation	106	54	53	
No. of samples tested positive by cell cultivation	VHSV=1 /IPNV=6		VHSV=4 / IPNV=1	VHSV=2 / IPNV=9
No of samples tested positive by PCR or other direct methods without cell cultivation				

According to Council Directive 2006/88, please indicate number of farms in your country/region placed in the respective categories according to fish species:					
		VHS	**IHN**	**ISA**	**KHV**
Category I **Declared disease-free**	Rainbow trout	163	205	205	163
	Atlantic Salmon				
	Other salmonids	100	100	100	100
	Carp				
	Eel				
	Flatfish				
	Seabream / Seabass				
	Other marine spp.				
	Other freshwater spp.				
		VHS	**IHN**	**ISA**	**KHV**
Category II **Subject to a surveillance programme**	Rainbow trout				
	Atlantic Salmon				
	Other salmonids				
	Carp				
	Eel				
	Flatfish				
	Seabream / Seabass				
	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.				

Laboratory visit at the
Estonian Veterinary and Food Laboratory (VFL)

Tartu, Estonia
9th November 2011

Introduction

The National Reference Laboratory for Fish Diseases (NRL) in Estonia is located at the Estonian Veterinary and Food Laboratory (VFL) in Tallinn. In 2006 the NRL for Fish Diseases was visited by a delegation from the EURL for Fish Diseases. However, the Department performing the molecular biological diagnostic analyses on fish samples (Department of Molecular Analyses) was not part of the visit in 2006 because this unit is located at the Estonian Veterinary and Food Laboratory (VFL) in Tartu.

The aim of this mission was to visit the Department of Molecular analyses in Tartu. The Department in Tartu was visited the 9th of November 2011 by Nicole Nicolajsen and Søren Kahns from the European Union Reference Laboratory for Fish Diseases (EURL).

The program for the visit is shown in Annex 1 and a list of persons that participated in the visit of the EURL representative is listed in Annex 2. This report describes findings, comments and recommendations made by the delegation from the EURL. This report is sent to the VFL and the EU Commission.

Organisation

The Estonian Veterinary and Food Laboratory (VFL) is a governmental institution under the Ministry of Agriculture. It was established in 1998 by consolidating the former State Veterinary Laboratory and regional Veterinary laboratories that used to be a part of the local Veterinary Centers.

The Central Veterinary and Food Laboratory is situated in Tartu. In addition, VFL have three smaller sections located in Tallinn, Rakvere and Saaremaa. The Central VFL is specialised in the diagnosis of viral diseases in cattle, pigs and sheep whereas the VFL location in Tallinn are more focused on poultry, horse and fish diseases.

The VFL obtains approximately 60 % of its funding from the Ministry of Agriculture, whereas the last 40 % is achieved through own diagnostic activities.

A total of 145 persons are employed at the VFL. The VFL is managed by Director, Olev Peetsu and has furthermore three deputy directors. The VFL in Tartu is divided into 8 departments: Department of Quality Management and development, Management Department, Department of Data Processing, Department of Bacteriological Pathology, Department of Virology-Serology, Department of Molecular Analysis, Chemistry Department and Food Microbiology Department. An organization plan of VFL is shown in Annex 3.

The Department of Molecular Analyses covers the PCR based diagnostics of fish diseases in Estonia.

Buildings, Furnishing and Access

The VFL in Tartu is located in newly reconstructed buildings within Tartu and all laboratories, administration premises appear very nice and well equipped. The reconstruction has been financed through governmental and private funding.

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

Opening of the meeting

At the official opening of the visit, the director Olev Peetsu welcomed the visitors from the EURL. Subsequently, deputy director Külli Must gave a detailed description of the VFL, its history, organization and mission. This was followed by a talk on the functions and duties of the EURL, by Dr. Søren Kahns. Present at the meeting was, besides all persons employed at the Department of Molecular analyses and also Ülle Pau, head of the Estonian NRL for fish diseases in Tallinn.

The opening meeting was followed by a tour at the institute with the aim of illustrating how processing of fish samples occur - from the sample is received and until an answer of the diagnostic analyses is given. The tour was guided by Külli Must, Siiri Põldma, Mihkel Mäesaar, Annika Vilem and Triinu Juurik from the VFL in Tartu. Furthermore, Ülle Pau participated in the tour.

Sample acceptance unit

The visit tour started in the sample acceptance room where all samples delivered to the institute is entered. Fish samples to be analysed for the presence of listed diseases by molecular biological techniques are either sent from Tallinn to Tartu by Cargo-bus service or is sent directly to Tartu by veterinarians or private farmers. When samples are sent from Tallinn, the Department of Molecular Analyses in Tartu is notified by a phone call that samples will arrive. Samples can be received as homogenized samples, as organs or as whole fish. Providers of samples have to provide information on e.g. fish species, clinical signs, fish farm, water temperature. All received material is registered and given a unique number. When samples sent from Tallinn arrives in Tartu, the sample is provided with a unique number. All collected information is entered into an electronically sample registration system.

When samples are received as whole fish, they are sent to the autopsy room for further processing. If samples are received as organs or homogenates they are forwarded directly to nucleic acid purification room for further processing. The laboratory is always contacted when a sample is to be analysed and samples are placed on ice at a table outside of laboratory. If immediate transfer is not possible, the samples will be stored in a refrigerator.

The samples can be followed during the analyses in electronic registration system. When the results of the diagnostic analyses have been obtained a final report is made. This answer has to be signed by the head of department before they will be sent to the customer.

The Department of Molecular Analysis

The Department of Molecular Analysis is responsible for molecular biological diagnostics of several animal diseases including those of the listed aquatic animals. In total, the Department has implemented

more than 40 different PCR based diagnostic analyses detecting different pathogens. These covers the following listed non-exotic fish pathogens: VHSV, IHNV, KHV and ISAV.

Staff of the Department of Molecular Analysis
The Department of Molecular Analysis consists of a group four persons: The Head of Department is Siiri Põldma. Mihkel Mäesaar, Annika Vilem and Triinu Juurik are employed as chief specialists. Together these four persons perform the molecular biological diagnostic analyses of VFL including fish samples.

Laboratories of Department of Molecular Analysis
The laboratories was newly reconstructed and equipped. The laboratories included a clean room, a sample preparation room, a purification room, an analysis room and an electrophoresis room. The clean room was located separately. The other laboratories were divided into several separate working rooms but located next to each other. In this way the analysis started in the sample preparation room and could enter and continue directly in the purification room, going through the analysis room and ending in the electrophoresis room. In this way the workflow was set up to minimizing the risk of contaminating. Besides the working rooms, one office with computer working spaces for all members of the Department was present.

The clean room served as a storage room for primers and kits and was furthermore the place where master mixes was prepared. Once placed in this room, primers and kits were not allowed to be taken out. Furthermore, no samples or pathogenic nucleic acids were allowed to enter the room. The clean room contained freezer, fridge, LAF bench and other necessary equipment.

The sample preparation room contained a LAF bench where organ suspension was made. Lysis of samples was also performed in this room. In this laboratory was also equipment as homogenisator, heat block and centrifuges that is required for processing of samples.

The purification room contained all the kits used for nucleic acid purification and the equipments necessary for performing the purification. The room was separated into two working spaces, one used for purification of DNA and one for purification of RNA. Several different purification kits were present and were used for different purification purposes. E.g. the RTP DNA/RNA virus mini kit (INVITEK), RTP Bacteria DNA mini kit (INVITEK) used for virus and bacteria respectively. For food and Parasites, DNA IQ system kit was used. In addition, the purification room contained a Magnapure Robot (Roche) that was able of purification of 8 simultaneously samples.

Between the purification room and the analyses room was a small room containing a NanoDrop Machine that was used for measuring the amount of nucleic acids collected through the purification process. This measurement was done in order to ensure that a constant amount of nucleic acids were used in each diagnostic assay . and also to consider the quality of nucleic acids (in the case of bad quality of NA it is important to make some dilutions from purified NA to minimize PCR inhibition or false negative results) .

The PCR amplification of the pathogenic target DNA was performed within the analysis room. Realtime PCR was preferred towards conventional PCR if possible. The analysis room contained two real-time thermocyclers and one conventional PCR machine.

The last room of in the pathway was the electrophoresis room where the visualization of the molecular biological diagnostic results. The room contained a Biorad UV-Gel Doc and an UV trans illuminator, used for e.g. cutting out amplified bands. A separate work space was restricted for work with EtBr. The room contained several gelelectrophoresis apparatuses and power supplies.

The laboratories and equipment was new and adequate. All rooms contained air condition. All equipment has a reference number and a logbook. Username, date and sample are registered in the logbook as well as any problems encountered. Repairs, cleaning, calibration and other maintenance such as filter change are likewise registered. Calibration of the pipettes is performed by staff for >10 µl whereas calibration of smaller pipettes was performed by sub-contractors. Instructions for use of equipment are written down in procedures, one for each piece of equipment. Staffs are required to read and be trained according to these procedures before using the equipment. For disinfection, UV light was turned on overnight. Furthermore, laboratory spaces were disinfected using 70% Ethanol.

PCR based methods

PCR/RT-PCRs are implemented for detection of VHSV, IHNV, KHV and ISAV, using OIE recommended PCR protocols. For comparison, the EURL provided hand outs with protocols used for virus detection at the EURL. The laboratory used the assay described by Jonstrup 2010 (unpublisht) for detection of VHSV and the method described by Kurat et al 2003 for detection of IHNV. For ISAV detection the Mjaaland et al. 1997 assay was used and for KHV detection the TK assay developed by Bercovier et al 2005 has been implemented. No PCR based assays was implemented for detection of other fish pathogens, including the two exotic pathogens EHNV and *Aphanomyces invadans*.

Sequencing was performed outside VFL at the nearby university through a collaborative agreement.

Accreditation
All these laboratories of VFL are separately accredited according to EN ISO/IEC 17 025 by the Estonian accreditation Center. Although molecular methods are accredited at VFL, the methods used for detection of fish pathogens have not yet been accredited.

Proficiency Test
The proficiency tests (PT) allow a laboratory to assess their diagnostic capacity of certain procedures. The Estonian NRL for fish diseases has participated in the PT for identification of notifiable fish diseases organized by EURL, Aarhus, Denmark, since 2002. The NRL have used the following tools for identification of viral content in the test: Titration of virus; Isolation of viruses on cell culture; Identification of virus by ELISA, IFAT and RT-PCR. The score obtained by the NRL in PT1 was 40% correct in 2010 and 80% in 2009 and 80% 2008. The laboratory did not participate in PT2 in 2010. For the scores obtained by the NRL in the PT provided by the EURL, see Annex 4. A general procedure in the laboratory is that when a PT result is less than 100% correct, the NRL will redo the test using all diagnostic procedures to verify that expected results can be obtained.

The Estonian NRL do not organize PTs for diagnosis of fish diseases for the regional laboratories as there are no regional laboratories for fish diseases in Estonia.

The main fish species cultured in Estonia are rainbow trout and carp. Estonia have 1 fish farm producing more than 100 tonnes per year, 16 fish farms producing 5 - 100 tonnes per year and 5 fish farms producing less or around 5 tonnes per year (2010). The NRL in Tallinn received approximately 178 samples in 2010 for diagnostic and surveillance purposes. Samples of carp were sent from the laboratory in Tallinn to the molecular department in Tartu for further analyses. Samples of rainbow trout were sent for molecular analysis if there were CPE in cell culture. Rainbow trout and carp farms are located throughout Estonia. Please see Annex 5 for more details.
All fish farms have been registered and authorized, and are listed in the public EU Register of aquaculture production businesses and authorised processing establishments. Estonian aquaculture have 11 salmonid farms located in category II for VHS and IHN. Furthermore, one carp farm is also in category II with respect to KHV disease and there send samples for KHV surveillance.. All other farms are located in category III (Annex 5).

Recommendations
Diagnostic methods for the exotic diseases EHN and EUS will have to be implemented. This could be done by implementing one of the PCR assays recommended in the OIE for detection of *A. Invadans* and implementing the PCR and the sequencing assays for detection of EHNV.
We recommend that the NRL for fish diseases in Tallinn makes a sensitivity test of the monolayered cells used for fish diagnostics towards sensitivity towards VHSV, IHNV and EHNV.

Conclusion

The visit showed that the Department of Molecular Analyses 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 recommendations with respect to implementing additional tool for identification of the exotic fish diseases were made, as described above. Furthermore the communication between the NRL for Fish Diseases in Tallinn and the Department of Molecular Analyses in Tartu seemed fine. Therefore, the overall conclusion is that the Department of Molecular Analyses is a well functioning laboratory capable of performing its duties and is able to full fill the molecular biological requirements on behalf of the Estonian NRL for Fish Diseases.

Annex 1

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

November 9th 2011

09:00 – 10:00 Introduction of the Estonian National Reference Laboratory for Fish Diseases/ Veterinary and Food Laboratory, Central Laboratory, Tartu. Presentation of the EURL and discussion on the topics of the visit. Participants: All Staff including Head of Institute

10:00 – 13:00 Tour in the lab, looking at facilities.

We would like to learn about how the diagnostic procedures related to diagnostics of fish and molecular biological tests are conducted in the laboratory.

Following issues could be discussed:

- Aquaculture in Finland and the type of samples received at the laboratory
- Buildings and access
- Staff
- Equipment
- Accreditation
- Registration of Samples
- Sample processing
- Virus identification by molecular techniques (PCR, RT-PCR, Q-PCR)
- Reporting diagnostic tests
- Past (2009 and 2010) Proficiency Test Results

13:00 – 13:45 Lunch

13:45 – 15:30 Continuation of the tour in lab.

15:30 – 16:00 Plans and progress in the implementation of Council Directive 2006/88/EC in Estonia

16:00 – 16:30 Follow-up - only Søren Kahns and Nicole Nicolajsen - We would be pleased to have the possibility to work together in an office.

16:30 – 17:00 Evaluation of the visit. Recommendations and report of the

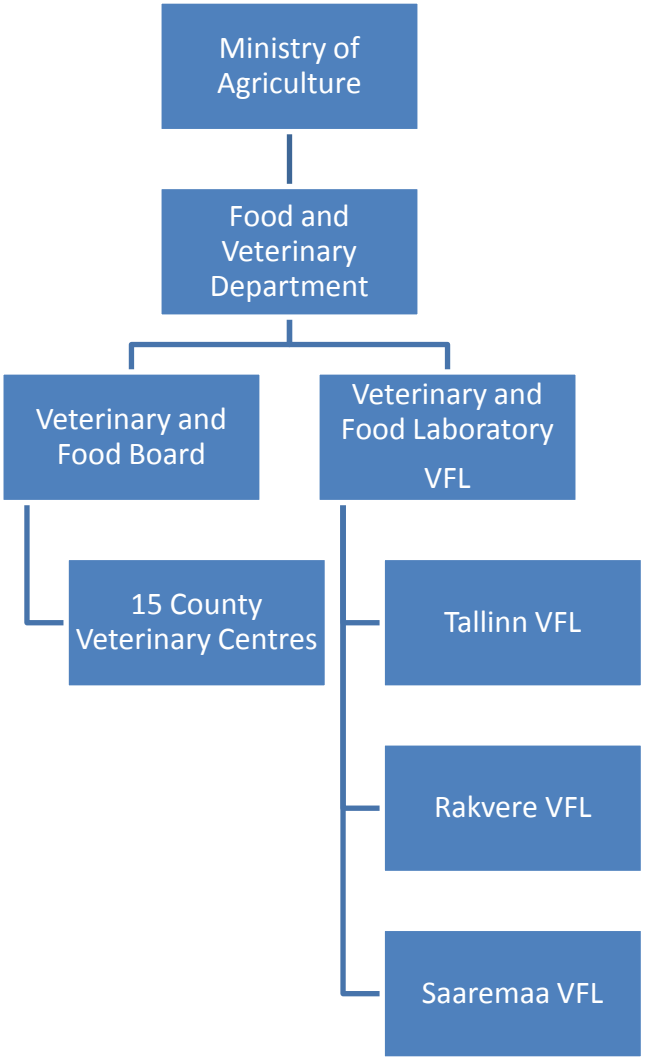
Annex 2

Estonian Veterinary and Food Laboratory (VFL) participating at the visit

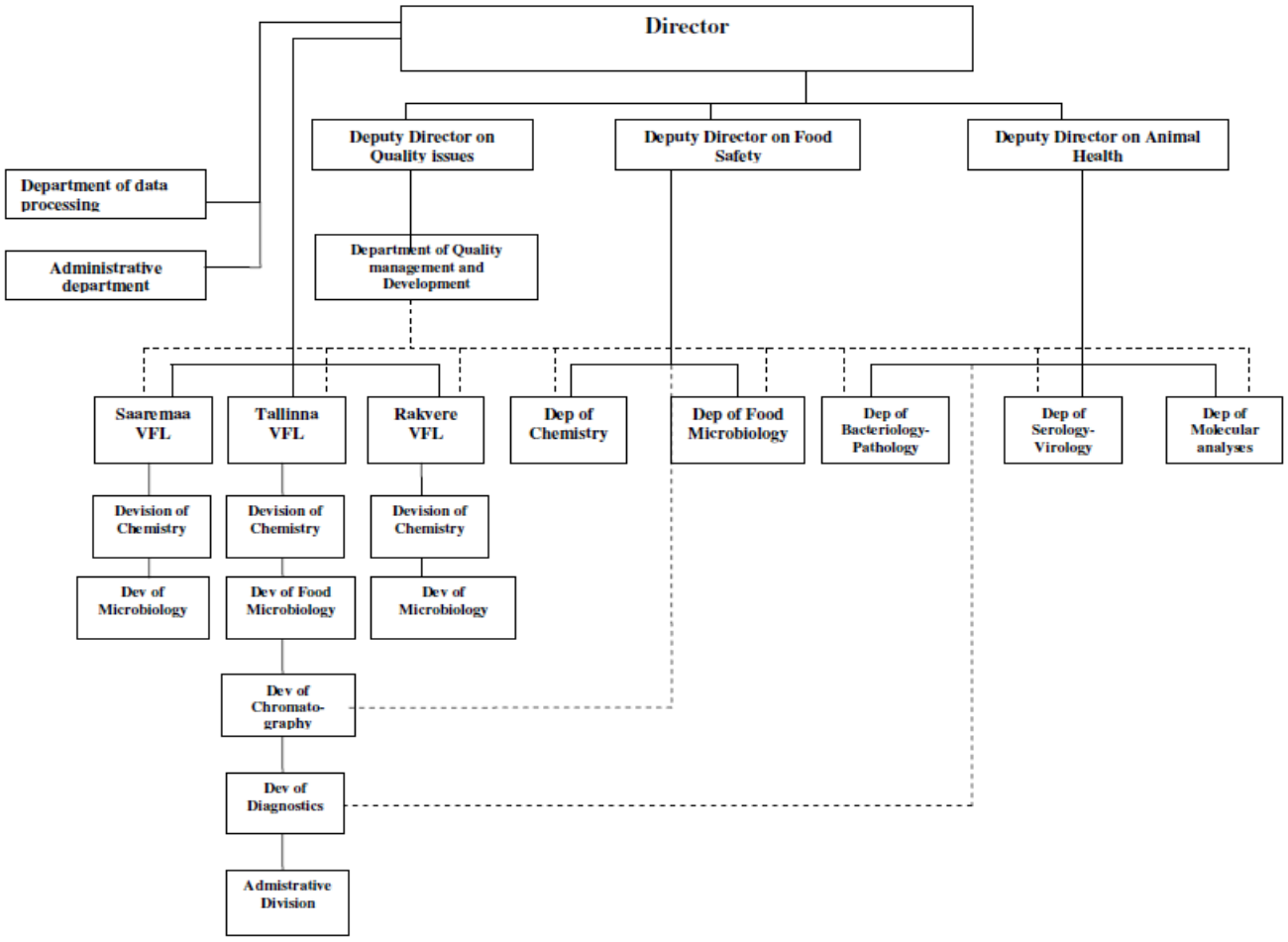
Külli Must	Deputy director on animal health, Department of Molecular analyses in Tartu
Siiri Põldma	Head of department, Department of Molecular analyses in Tartu, Department of molecular analysis
Mihkel Mäesaar	Chief specialist, Department of Molecular analyses in Tartu, Department of molecular analysis
Annika Vilem	Chief specialist,Department of Molecular analyses in Tartu, Department of molecular analysis
Triinu Juurik	Chief specialist, Department of Molecular analyses in Tartu, Department of molecular analysis
Ülle Pau	Head of department, Estonian Veterinary and Food Laboratory (VFL) in Tallinn, Diagnostic department
Dr. Søren Kahns	Coordinator of the EU Reference Laboratory for Fish Diseases, Section for Fish Diseases, National Veterinary Institute, Technical University of Denmark.
Nicole Nicolajsen	Laboratory Technologist EU Reference Laboratory for Fish Diseases, Section for Fish Diseases, National Veterinary Institute, Technical University of Denmark.

Annex 3

Organization of Institute



VFL organization



Annex 4

Proficiency Test – PT1

Estonia	1996-2001	2002	2003	2004	2005	2006	2007	2008	2009	2010
Participated		X	X	X	X	(X)	X	X	X	X
Score %				80	80		90	80	80	40

2010 **Score 4/10** **Code 6**	**Ampoule I:** **EHNV** **Low titer**	**Ampoule II:** **IHNV**	**Ampoule III:** **European Catfish Virus (ECV)**	**Ampoule IV:** **SVCV**	**Ampoule V:** **VHSV**
Cell line	BF-2/EPC				
ELISA	VHSV – HNV-	VHSV – IHNV+	VHSV – IHNV-	VHSV – IHNV-	VHSV + IHNV-
IFAT	VHSV – HNV-	VHSV – IHNV+	VHSV – IHNV-	VHSV – IHNV-	VHSV + IHNV-
PCR	Not performed	Not performed	Not performed	Not performed	Not performed
Result	**No virus**	**IHNV**	**No VHSV - No IHNV-**	**No VHSV - No IHNV-**	**VHSV**

2009 **Score 8/10** **Code 11**	**Ampoule I:** **EHNV**	**Ampoule II:** **IHNV**	**Ampoule III:** **VHSV** genotype Ie	**Ampoule IV:** **VHSV** genotype IVa	**Ampoule V:** **No virus**
Cell line	BF-2/EPC				
ELISA	VHSV- IHNV- IPNV- SVCV-	VHSV- IHNV+ IPNV- SVCV-	VHSV+ IHNV- IPNV- SVCV-	VHSV+ IHNV- IPNV- SVCV-	VHSV- IHNV- IPNV- SVCV-
IFAT	VHSV- IHNV- IPNV- SVCV-	VHSV- IHNV+ IPNV- SVCV-	VHSV+ IHNV- IPNV- SVCV-	VHSV+ IHNV- IPNV- SVCV-	VHSV- IHNV- IPNV- SVCV-
PCR	VHSV- IHNV- SVCV-	VHSV- IHNV+ SVCV-	VHSV+ IHNV- SVCV-	VHSV+ IHNV- SVCV-	VHSV- IHNV- SVCV-
Result	**Virus found, not identified**	**IHNV**	**VHSV**	**VHSV**	**No Virus**

PCR: 2009, 2008, 2007

Inter-laboratory Proficiency Test 1 2010

Name of the National Reference Laboratory: Tallinn Veterinary and Food Laboratory

Country: Estonia

Contact name: Ülle Pau

Code: 6

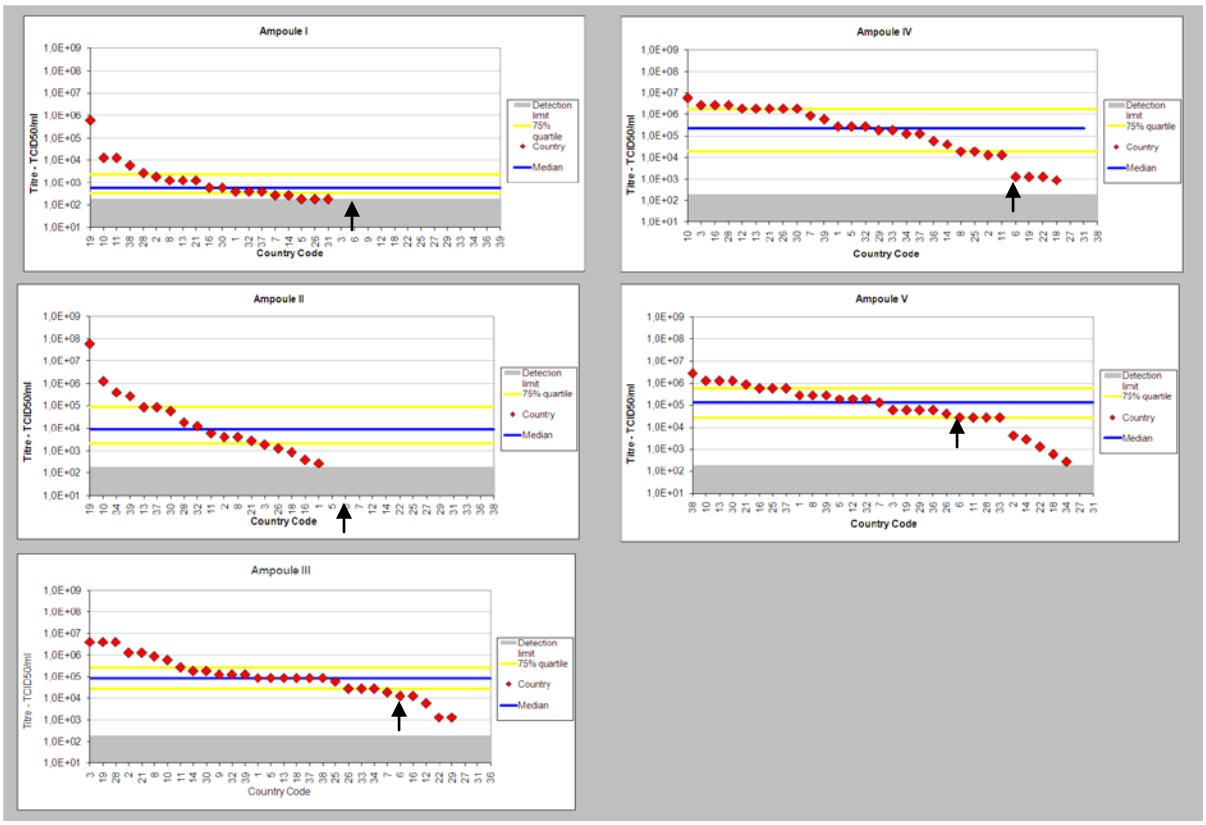
Score: 4 (out of 10)

LABORATORY RESULTS:

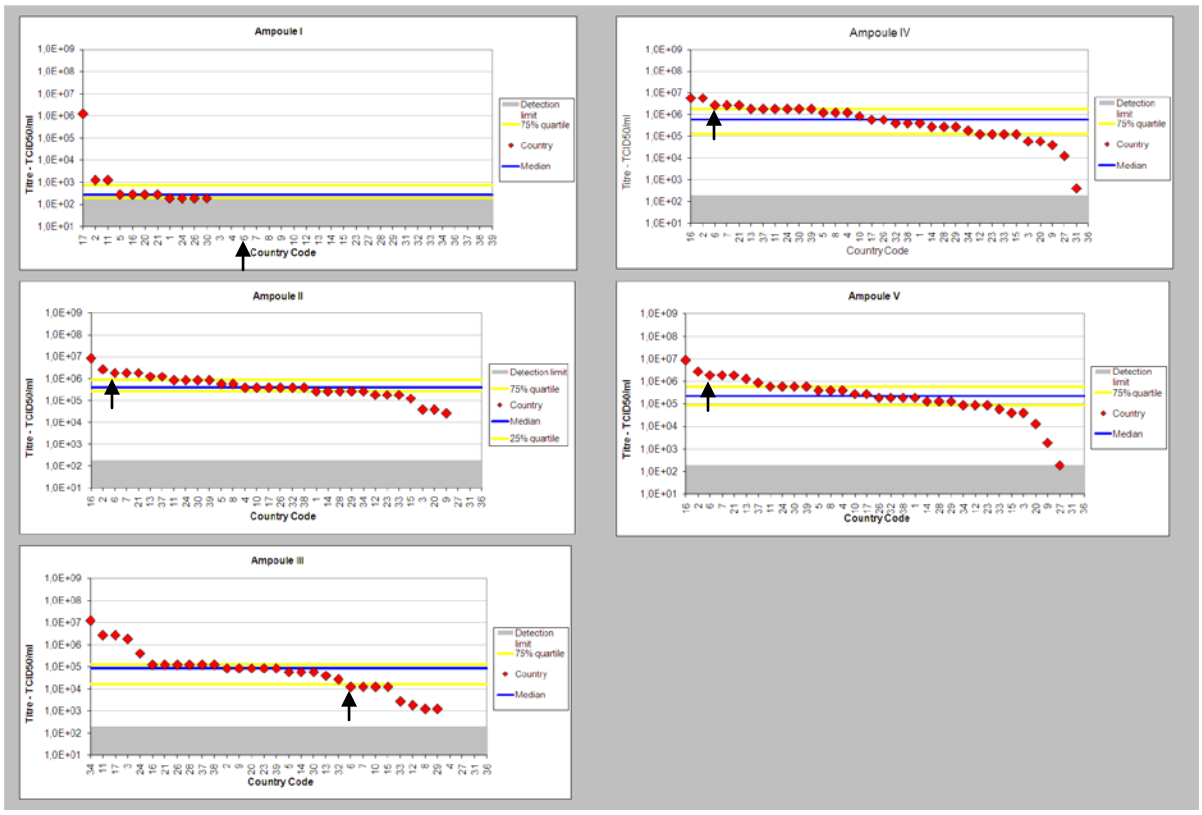
	ELISA	IFAT	Neutralisation	PCR	Other	Final Result
Ampoule I	VHSV - IHNV-	VHSV - IHNV-	Not performed	Not performed	Not performed	**No virus**
Ampoule II	VHSV - IHNV+	VHSV - IHNV+	Not performed	Not performed	Not performed	**IHNV**
Ampoule III	VHSV - IHNV-	VHSV - IHNV-	Not performed	Not performed	Not performed	**No VHSV - No IHNV-**
Ampoule IV	VHSV - IHNV-	VHSV - IHNV-	Not performed	Not performed	Not performed	**No VHSV - No IHNV-**
Ampoule V	VHSV + IHNV-	VHSV + IHNV-	Not performed	Not performed	Not performed	**VHSV**

Comments: You write that you had problems with your PCR methods. This might be solved by now, if not we recommend that you set up your diagnostic PCR assays and if you need any advice from us, please contact us.

Titre obtained in BF-2 cells



Titre obtained in EPC cells



Annex 5
Aquaculture in Estonia

Land Area	Ocean Area	Environment	Species	Scientific name	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009
Estonia	Europe - Inland waters	Freshwater	Carps, barbels and other cyprinids	Carps, barbels and other cyprinids	47	53	42	52	49	46	82	30	72	47
			Miscellaneous freshwater fishes	Miscellaneous freshwater fishes	0-	1	0	0	1	1	1	0	1	2
			River eels	River eels	0-	0-	5	15	7	40	40	45	47	30
			Salmons, trouts, smelts	Salmons, trouts, smelts	178	413	210	304	194	451	520	619	649	549
		Sub-total Freshwater			225	467	257	371	251	538	643	694	769	628
	Sub-total Europe - Inland waters				225	467	257	371	251	538	643	694	769	628
Total Estonia					225	467	257	371	251	538	643	694	769	628

Data taken from FIGIS

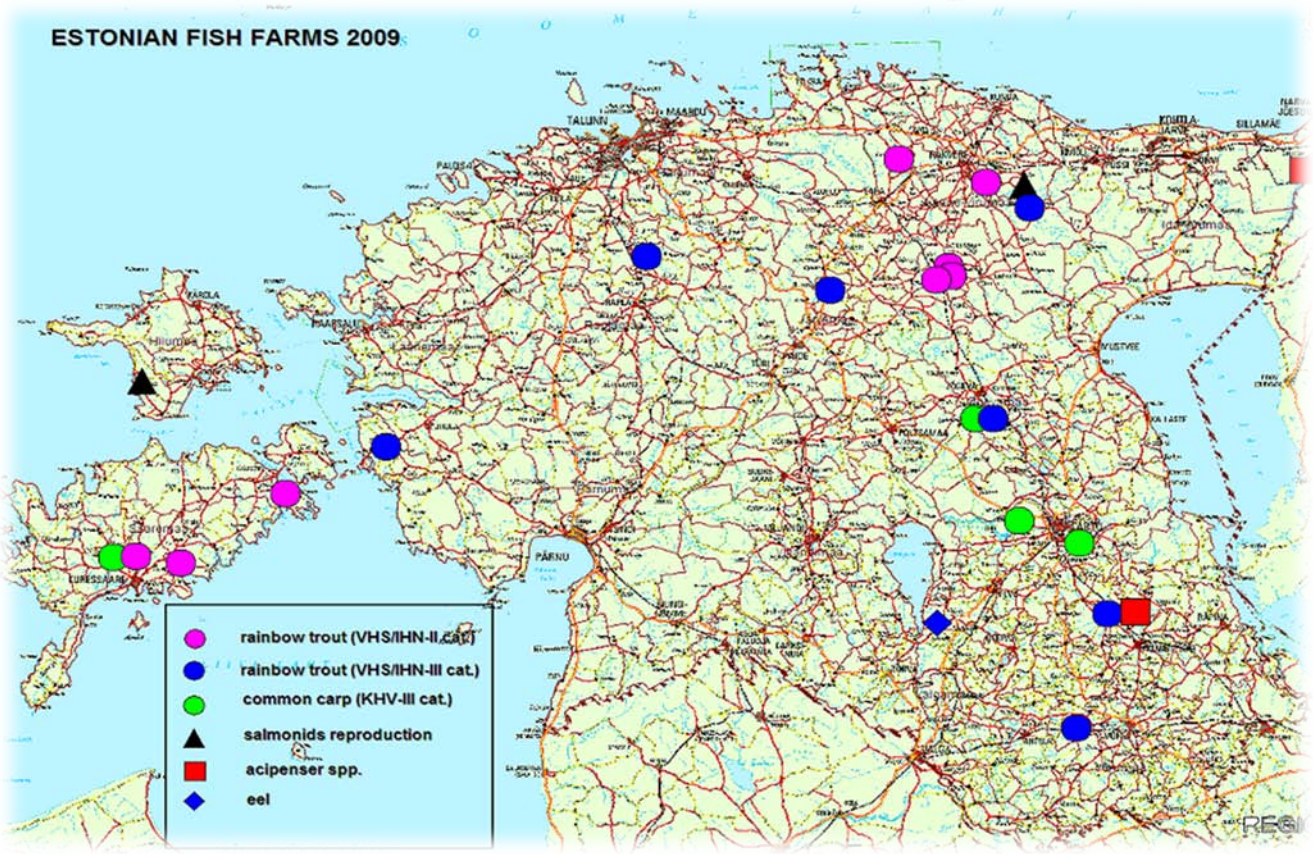
Number of fish farms within country/region, according to size of production (tonnes fish/year)				
	2010	2009	2008	2007
< 5 tonnes	5	15		13
5 - 100 tonnes	16	12	18	10
> 100 tonnes	1	2	1	2

Number of fish farms within country/region, according to fish species				
	2010	2009	2008	2007
Rainbow trout	14	15	9	12
Atlantic Salmon		1	1	
Other salmonids	4	1		
Carp	3	4	4	8
Eel	1	1	1	1
Flatfish		0		
Seabream / Seabass		0		
Other marine spp.		0	2	
Other freshwater spp.	3	3	2	8
Total	25	25	19	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:				
	2010	2009	2008	2007
No. of samples tested by cell cultivation	178	201	244	?
No of samples tested by PCR or other direct methods without cell cultivation	0	0	15	
No. of samples tested positive by cell cultivation	0	0	1/IPNV	
No of samples tested positive by PCR or other direct methods without cell cultivation	0	0	15/IPNV	

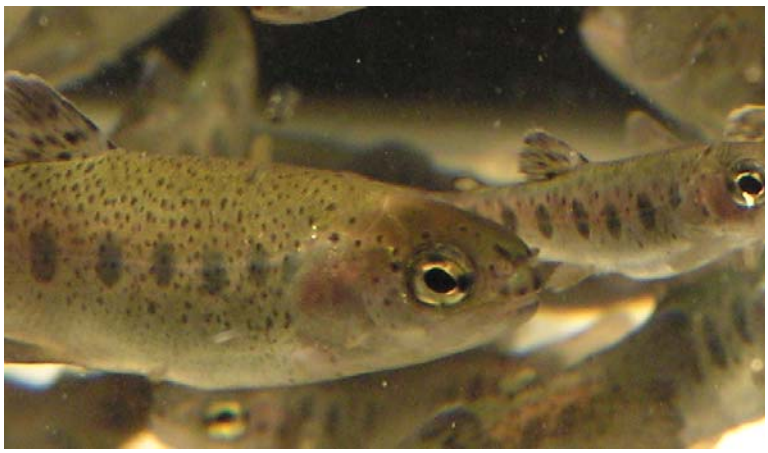
According to Council Directive 2006/88, please indicate number of farms in your country/region placed in the respective categories according to fish species:					
		VHS	**IHN**	**ISA**	**KHV**
	Rainbow trout	0	0	0	0
	Atlantic Salmon				
	Other salmonids				
Category I	Carp				
Declared disease-free	Eel				
	Flatfish				
	Seabream / Seabass				
	Other marine spp.				
	Other freshwater spp.				
		VHS	**IHN**	**ISA**	**KHV**
	Rainbow trout	10	10		
	Atlantic Salmon				
	Other salmonids	1	1		
Category II	Carp				1
Subject to a surveillance programme	Eel				
	Flatfish				
	Seabream / Seabass				
	Other marine spp.				
	Other freshwater spp.				
		VHS	**IHN**	**ISA**	**KHV**
	Rainbow trout	5	5	1	
	Atlantic Salmon				
Category III	Other salmonids	1	1	1	
Not known to be infected but not subject to surveillance programme for achieving disease free status	Carp				2
	Eel				
	Flatfish				
	Seabream / Seabass				
	Other marine spp.				
	Other freshwater spp.	2	2	1	1
		VHS	**IHN**	**ISA**	**KHV**
	Rainbow trout	0	0	0	0
	Atlantic Salmon				
	Other salmonids				
Category IV	Carp				
Known to be infected but subject to an eradication programme	Eel				
	Flatfish				
	Seabream / Seabass				
	Other marine spp.				
	Other freshwater spp.				
		VHS	**IHN**	**ISA**	**KHV**
	Rainbow trout	0	0	0	0
	Atlantic Salmon				
	Other salmonids				
Category V	Carp				
Known to be infected. Subject to minimum control measures	Eel				
	Flatfish				
	Seabream / Seabass				
	Other marine spp.				
	Other freshwater spp.				

Data from S&D 2010


ESTONIAN FISH FARMS 2009
rainbow trout (VHS/IHN-II cat.)
rainbow trout (VHS/IHN-III cat.)
common carp (KHV-III cat.)
salmonids reproduction
acipenser spp.
eel

European Union Reference Laboratory for Fish Disease

National Veterinary Institute, Technical University of Denmark, Aarhus



Workshop in Surveillance and epidemiology of Aquatic Animal diseases

Copenhagen,
November 23-24. 2011

Hosted by the Community reference laboratories for fish, shellfish and mollusc diseases.
Directed by the OIE collaborating centre for Aquatic Epidemiology and Risk Assessment.

OIE Collaborating Centre for Epidemiology & Risk Assessment for Aquatic Animal Diseases (ERAAAD)

Veterinærinstituttet
Norwegian Veterinary Institute

Contents

Introduction and short summary

The Workshop in Surveillance and epidemiology of Aquatic Animal diseases took place in the auditorium of the National Veterinary Institute in Copenhagen on November 23-24, 2011.
A total of 50 participants from 25 countries attended, with 5 invited experts. The workshop was organised by the European Union Reference Laboratory for Fish Diseases, together with the OIE collaborating centre for Aquatic Epidemiology and Risk assessment, constituting of scientists from the Norwegian Veterinary Institute. The EURL´s for molluscs and crustacean diseases were involved as well reflecting that the workshop covered all aquatic animals. The Norwegian Veterinary Institute, took on the responsibility to plan the scientific programme while the EURL focused on the practical arrangements.

The overall purpose of the workshop was to give an introduction to some of the topics of surveillance and epidemiology with a special focus on the challenges for aquatic animal diseases.
After a welcoming and a general introduction the workshop was opened with a talk on the purpose of surveillance, followed by a presentation of concepts for sampling and testing for surveillance. After these basic principles and concepts had been presented, two talks were given on risk-ranking of aquaculture farms according to the new legislation on aquatic animal health. A special presentation was given on the challenges regarding surveillance in shellfish and molluscs.

In the afternoon, the workshop participants were divided into groups with the following four topics:

- Screening strategies & tracing of pathogens
- Special challenges regarding surveillance in shell & molluscs
- Design of surveillance programmes
- Risk factors and risk categorization

Within each group, concepts and challenges were discussed, and a summary of the groups' discussions was presented on the second day of the workshop, in order to give all participants insight into the different topics.

On the second day of the workshop, presentations were given on how to use models of risk in space and time when considering surveillance and on molecular epidemiology for tracing the origin of disease outbreaks. A final presentation was given on concepts of economics with regards to surveillance programmes in aquatic animals.

After the workshop, an internet-based evaluation was carried out, with 30 responses. The overall impression is that the participants were satisfied with the outcome of the workshop, and there is a wish for more workshops on epidemiologic topics. The results of the evaluation are presented at the end of this report.

Presentations from the workshop were collected and are included in this report. For the group work, a synthesis of the discussions is presented. All presenters and facilitators have had the opportunity to correct misunderstandings before the report was finalised.

We would like to thank all the presenters and facilitators for their great contribution, and all the participants for their enthusiasm. Without them, the workshop would not have been a success.

Oslo and Århus, January 2012

Britt Bang Jensen and Niels Jørgen Olesen

Programme

Venue: Auditorium of DTU Vet, Bülowsvej 27,

Day 1	**Wednesday November 23.**			
9.00-9.15	Welcome and introduction. Britt Bang Jensen / Niels Jørgen Olesen			
9.15-10.00	The purpose of surveillance and control of diseases in aquaculture: Angus Cameron			
10.00-10.45	Basic concepts in sampling and testing for aquatic diseases: Charles Caraguel			
10.45-11.15	*Coffee break*			
11.15-12.00	Examples of risk categorization of farms according to EU-legislation • Proposal for a risk based surveillance program of Swiss fish farms: Beat von Siebenthal • Epizootic risk analysis of lower Saxony Aquaculture production businesses: Dirk W. Kleingeld			
12.00-12.30	Special challenges related to shell and mollusc surveillance: Edmund Peeler			
12.30-13.30	*Lunch*			
13.30-13.45	Introduction to practical part of workshop and allocation into groups			
13.45-16.30	Practical part: Parallel sessions with group-work and practical examples			
	Screening strategies & tracing of pathogens Facilitators: Charles Caraguel & Peder Jansen	Special challenges regarding surveillance in shell & molluscs Facilitator: Edmund Peeler	Design of surveillance programmes Facilitator: Angus Cameron	Risk factors and risk categorization Facilitators: Britt Bang Jensen & Trude Lyngstad
16.30-17.00	Preliminary wrap-up of discussions within the groups			
Social event: Dinner at restaurant "Wining and Dining"				
Day 2	**Thursday November 24.**			
9.00-9.15	Introduction to day 2: Britt Bang Jensen			
9.15-10.00	Disease risk in space and time –Implications for surveillance: Peder Jansen			
10.00-10.30	*Coffee break*			
10.30-11.15	Use of molecular epidemiology in tracing disease: Trude Lyngstad			
11.15-12.00	Economic aspects of surveillance programmes: Britt Bang Jensen			
12.00-13.00	*Lunch*			
13.00-15.00	Discussion based on experiences from the practical part of workshop 15 minute presentation from each group.			
15.00-15.30	Workshop wrap-up and goodbyes			

Presenters/facilitators:

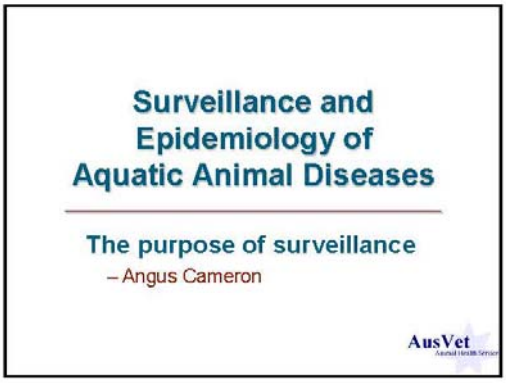
- Dr. Angus Cameron, Director of AusVet Animal Health Services
- Dr. Charles Caraguel, University of Adelaide
- Dr. Edmund Peeler, Centre for Environment, Fisheries and Aquaculture Science
- Trude Lyngstad, Section for epidemiology, Norwegian Veterinary Institute
- Peder Janssen, Section for epidemiology, Norwegian Veterinary Institute
- Britt Bang Jensen, Section for epidemiology, Norwegian Veterinary Institute

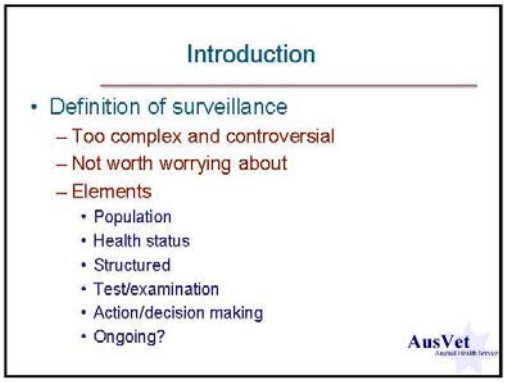
Basic epidemiological principles

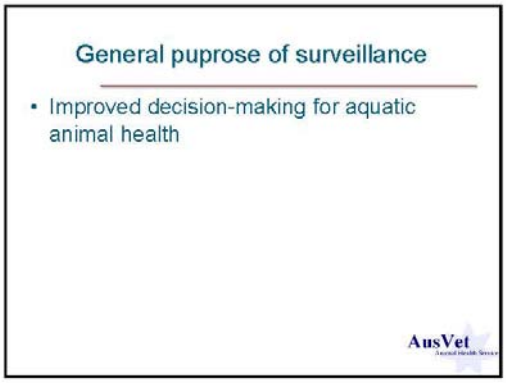
The purpose of surveillance and control of diseases in aquaculture

A. Cameron
AusVet Animal Health Services
140 Falls Road, Wentworth Falls, NSW 2782
Australia
E-mail: angus@ausvet.com.au

Presentation:







Specific purposes of surveillance

- Diseases that are present
  - Describe disease occurrence
  - Find cases of disease
- Diseases that are absent
  - Early detection of disease
  - Demonstrate freedom

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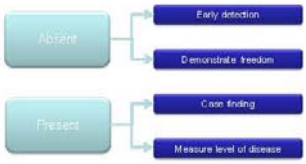

Purpose

- Specific objectives
  - What questions is the surveillance trying to answer?
  - What decisions are made on the basis of the surveillance results?
  - How does the surveillance benefit animal health / human health and well being / the environment?

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Examples

- Is the level of infection in our spat / post-larvae / fingerlings acceptable?
- We have a limited budget for disease control. What should be our priority diseases?
- Have any exotic diseases recently arrived in our country?

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Design of surveillance programs

- Surveillance requirements
  - Understand the surveillance question that is being asked
  - Decide what information is required to answer the question
- Understand range of surveillance tools available
  - Characteristics, strengths, weaknesses
- Select appropriate tools
  - Technical, practical, economic considerations

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Characteristics of a surveillance system

- Example: describing a car
- My wife and I agreed:
  - Red
  - 2 door
  - Italian
  - Couple, no children no need for too much space in the back
  - Sun roof

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What I was thinking


What my wife was thinking


Description
•Included
–Colour
–Doors
–Country
–Users
•Didn't include
–Power
–Cost
–Gearbox
–Fuel efficiency
–etc...
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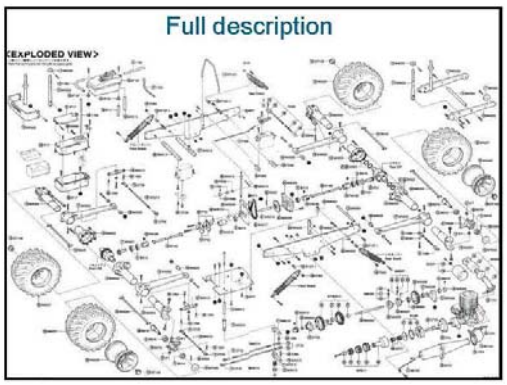
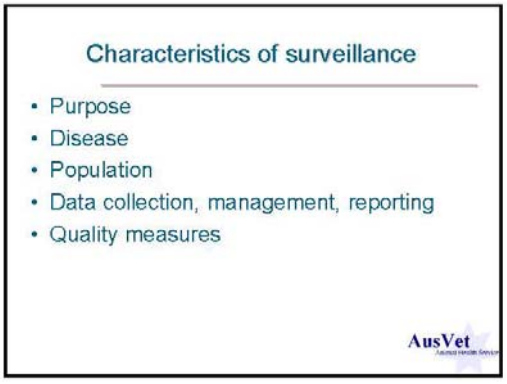

Full description
<EXPLODED VIEW>


Characteristics of surveillance
• Purpose
• Disease
• Population
• Data collection, management, reporting
• Quality measures
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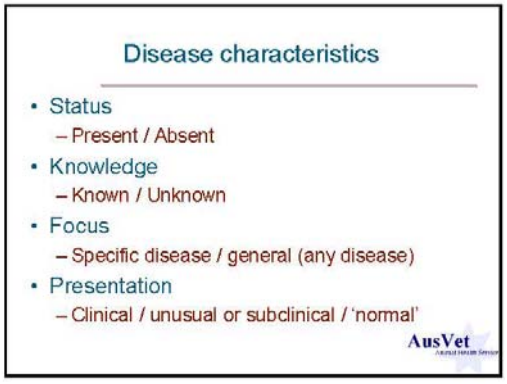
Disease characteristics
• Status
– Present / Absent
• Knowledge
– Known / Unknown
• Focus
– Specific disease / general (any disease)
• Presentation
– Clinical / unusual or subclinical / 'normal'
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Population and sampling

- Target population
- Coverage
  - Census, sample, high or low coverage
- Representativeness
  - Representative (eg random sampling)
  - Biased
  - Risk-based (intentional bias)
- Timing
  - Continuous / intermittent / one-off

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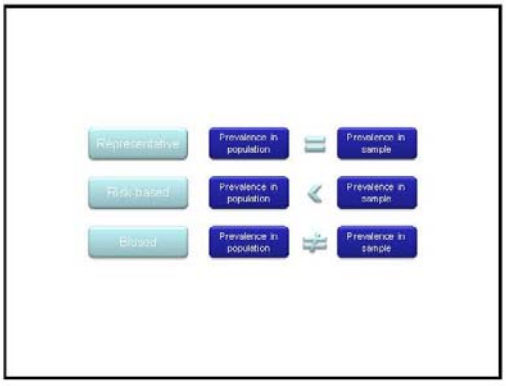

Data collection

- Who generates the data and why? (active / passive)
- Unit of interest / source of data (animal, farmer, etc)
- Form of data (sample, questionnaire, clinical observation...)
- Test or measurement systems (sensitivity, specificity)

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Data management

- Communication systems
- System outputs, feedback systems

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Quality measures

- Quantitative
- Qualitative

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Quantitative quality measures

- Freedom
  - Sensitivity, probability of freedom
- Early detection
  - Sensitivity, time to detection
- Prevalence estimation
  - Precision, bias
- Case finding
  - Detection fraction

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Qualitatavie measures of quality
• Cost-effectiveness
• Timeliness
• Practicality
• Fitness for purpose
• Sustainability
• Acceptability, ownership
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Requirements of surveillance
• Requirements differ according to purpose and context
• Key characteristics
– Coverage
– Representativeness
– Timing
– Testing
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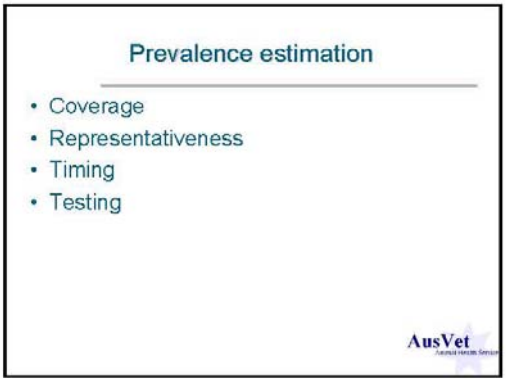
Prevalence estimation
• Coverage
• Representativeness
• Timing
• Testing
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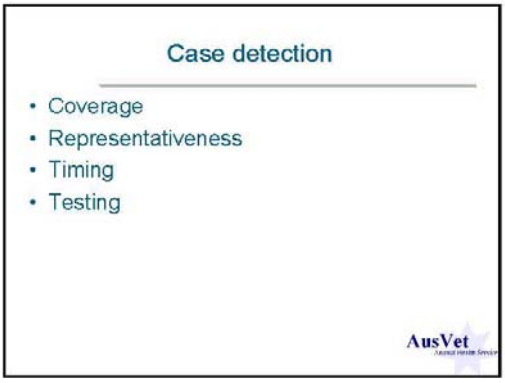
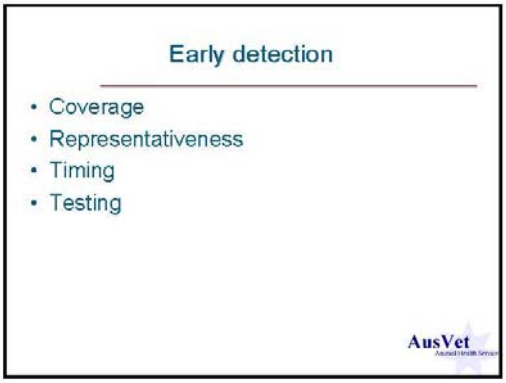
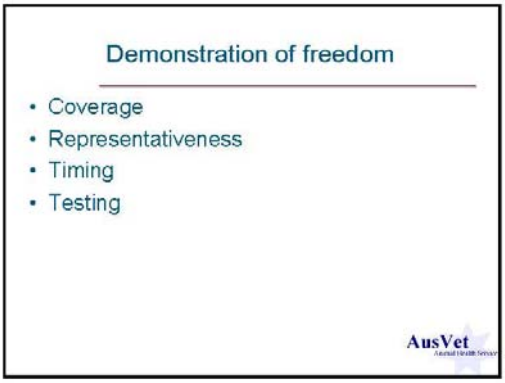

Case detection
• Coverage
• Representativeness
• Timing
• Testing
AusVet


Early detection
• Coverage
• Representativeness
• Timing
• Testing
AusVet


Demonstration of freedom
• Coverage
• Representativeness
• Timing
• Testing
AusVet

Examples of approaches to surveillance

- Farmer reporting system
- Structured representative surveys
- Risk-based sampling surveys
- Sentinel populations
- Indirect surveillance
- Syndromic surveillance
- Participatory disease surveillance
- Processing plant surveillance
- Zero reporting (negative surveillance)
- Sentinel veterinary practices
- Disease hotline / SMS reporting
- Dutch Veekijker helpline

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Rules of thumb

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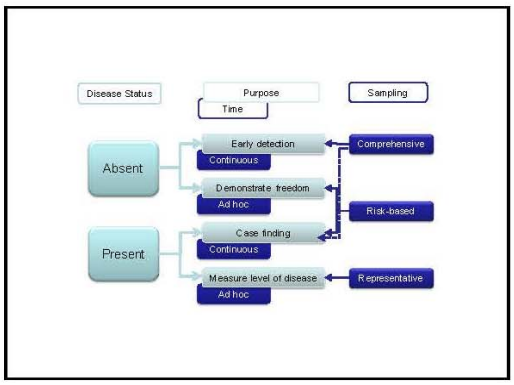
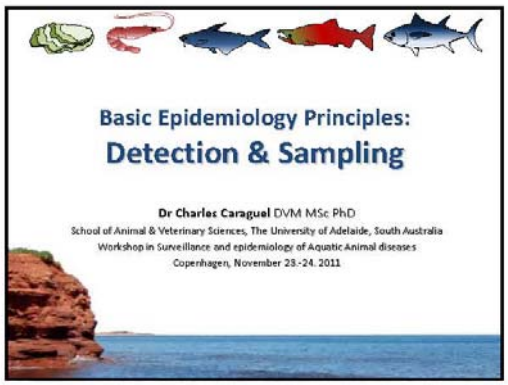
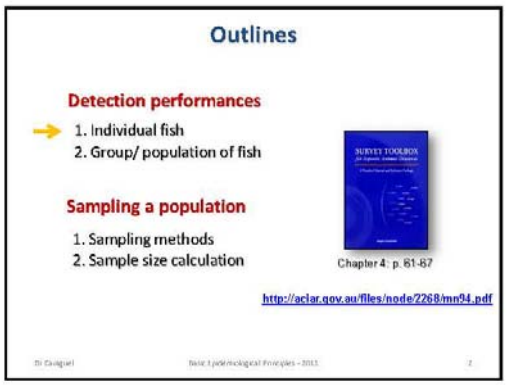


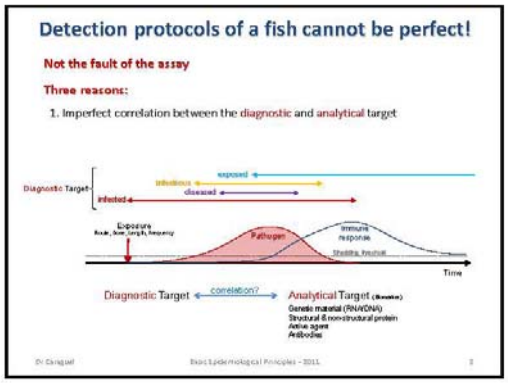
Basic concepts in sampling and testing for aquatic diseases

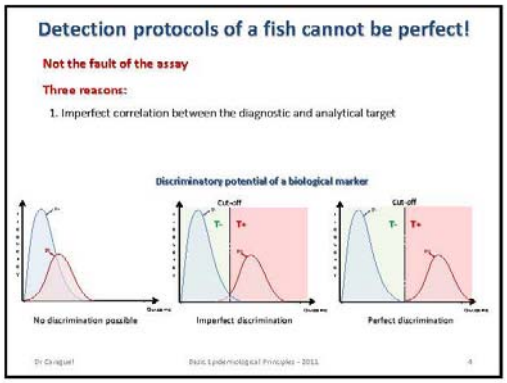
C. Caraguel
University of Adelaide
Roseworthy SA 5371
Australia
E-mail: charles.caraguel@adelaide.edu.au

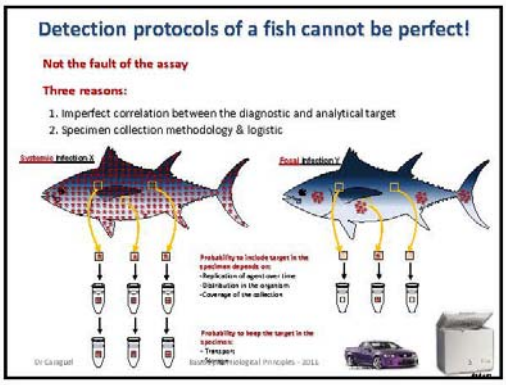
Presentation:

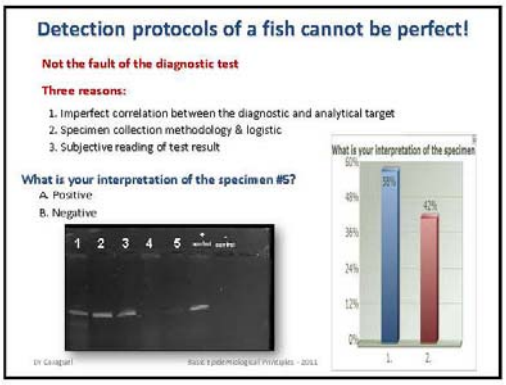


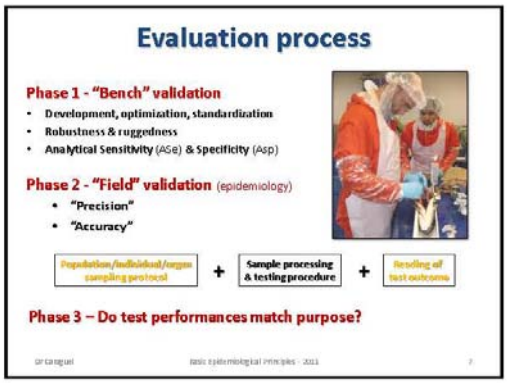


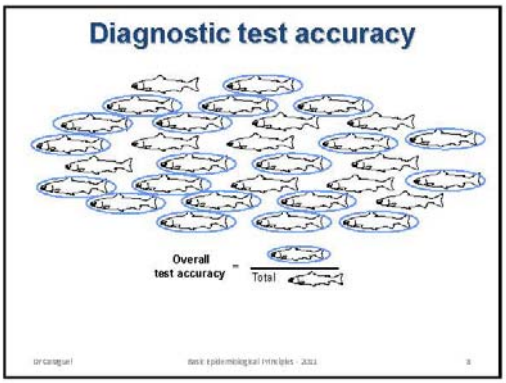


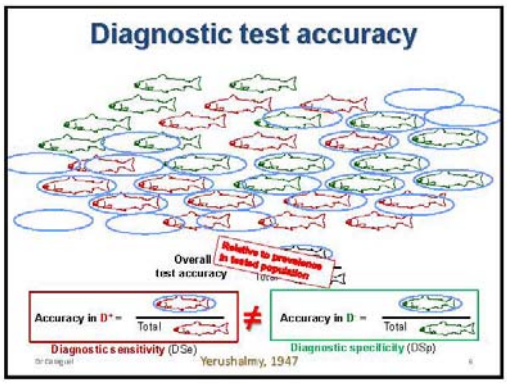


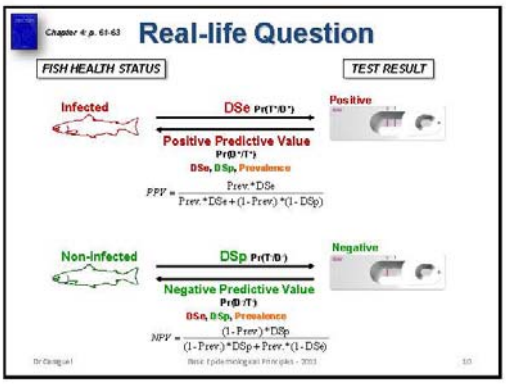

Detection protocols of a fish cannot be perfect!
Not the fault of the assay
Three reasons:
1. Imperfect correlation between the diagnostic and analytical target
2. Specimen collection methodology & logistic

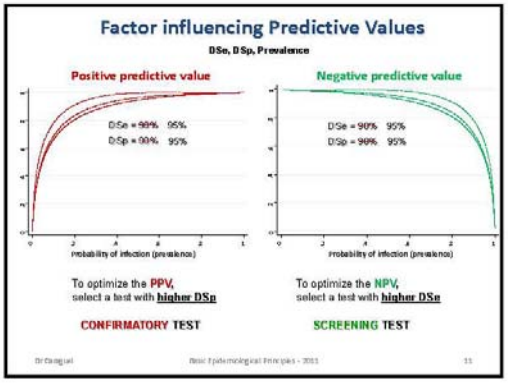

Detection protocols of a fish cannot be perfect!
Not the fault of the diagnostic test
Three reasons:
1. Imperfect correlation between the diagnostic and analytical target
2. Specimen collection methodology & logistic
3. Subjective reading of test result
What is your interpretation of the specimen #5?
A. Positive
B. Negative

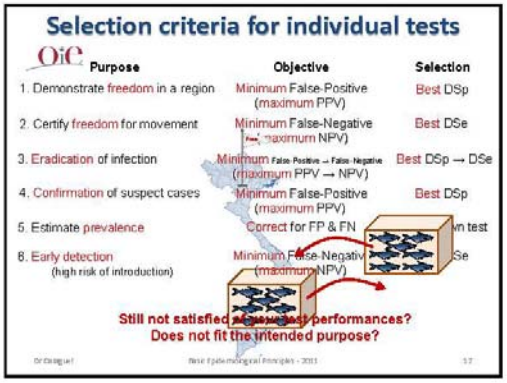

Evaluation process
Phase 1 - "Bench" validation
Development, optimization, standardization
Robustness & ruggedness
Analytical Sensitivity (ASe) & Specificity (Asp)
Phase 2 - "Field" validation (epidemiology)
"Precision"
"Accuracy"
Sample processing & testing procedure
Phase 3 – Do test performances match purpose?

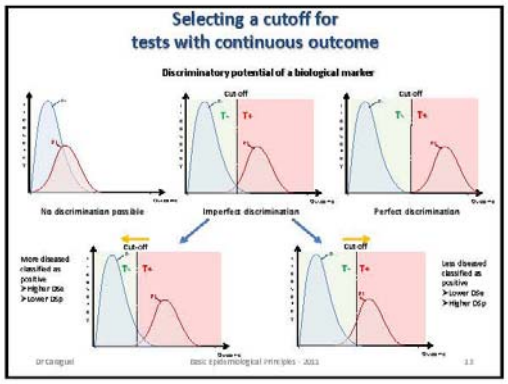

Diagnostic test accuracy
Overall test accuracy
Total

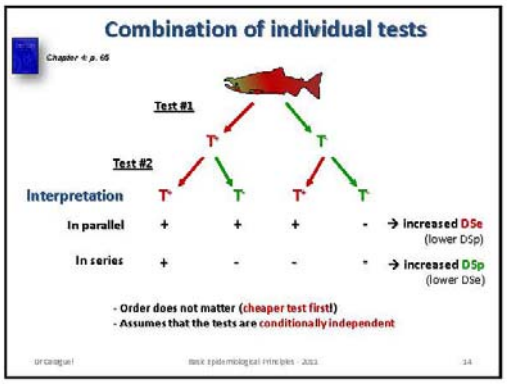

Diagnostic test accuracy
Overall test accuracy
Relative to prevalence in tested population
Accuracy in D+ =
Total
Accuracy in D- =
Total
Diagnostic sensitivity (DSe)
Diagnostic specificity (DSp)
Yerushalmy, 1947

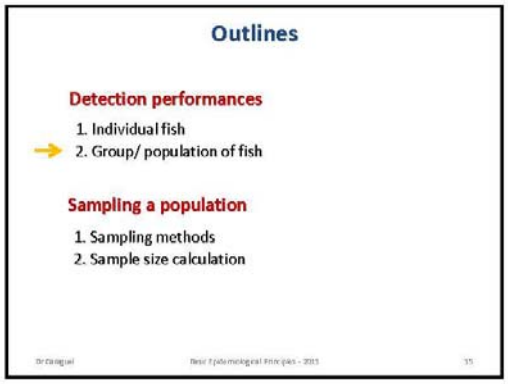

Real-life Question
FISH HEALTH STATUS
TEST RESULT
Infected
DSe
Positive
Positive Predictive Value
DSe, DSp, Prevalence
Non-infected
DSp
Negative
Negative Predictive Value
DSe, DSp, Prevalence

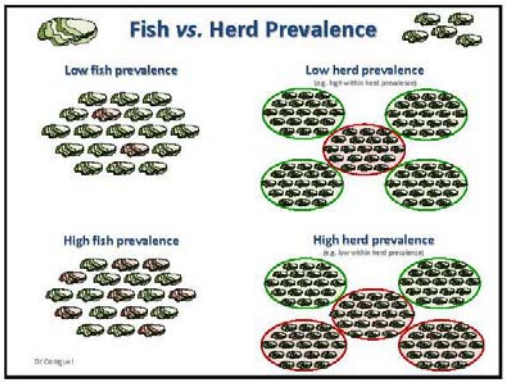

Factor influencing Predictive Values
DSe, DSp, Prevalence
Positive predictive value
Negative predictive value
DSe = 90% 95%
DSp = 90% 95%
Probability of infection (prevalence)
To optimize the PPV, select a test with higher DSp
To optimize the NPV, select a test with higher DSe
CONFIRMATORY TEST
SCREENING TEST

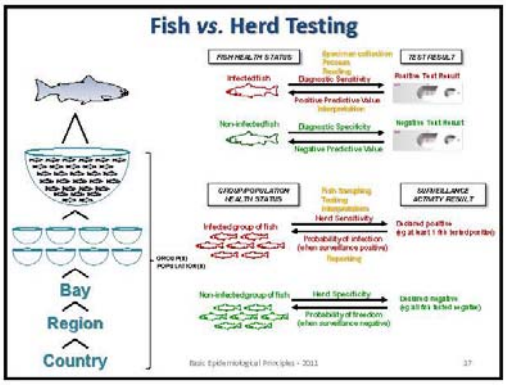

Selection criteria for individual tests
Purpose
Objective
Selection
1. Demonstrate freedom in a region
Minimum False-Positive (maximum PPV)
Best DSp
2. Certify freedom for movement
Minimum False-Negative (maximum NPV)
Best DSe
3. Eradication of infection
Best DSp → DSe
4. Confirmation of suspect cases
Minimum False-Positive (maximum PPV)
Best DSp
5. Estimate prevalence
Correct for FP & FN
6. Early detection (high risk of introduction)
Still not satisfied with low test performances?
Does not fit the intended purpose?

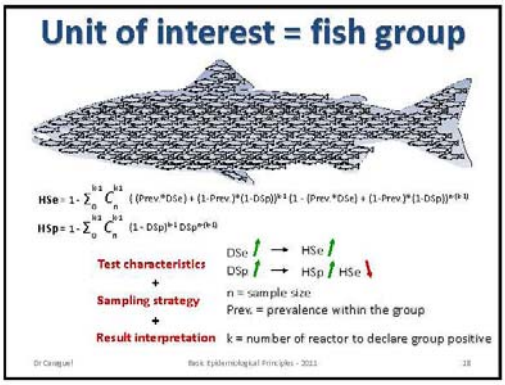

Selecting a cutoff for tests with continuous outcome
Discriminatory potential of a biological marker
Cut-off
No discrimination possible
Imperfect discrimination
Perfect discrimination
More diseased classified as positive
>Higher DSe
>Lower DSp
Less diseased classified as positive
>Lower DSe
>Higher DSp

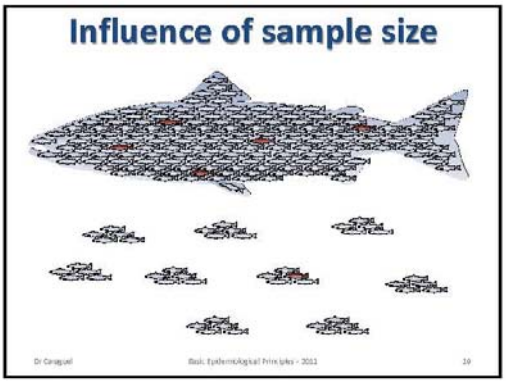

Combination of individual tests
Test #1
Test #2
Interpretation
In parallel
In series
→ Increased DSe (lower DSp)
→ Increased DSp (lower DSe)
- Order does not matter (cheaper test first!)
- Assumes that the tests are conditionally independent

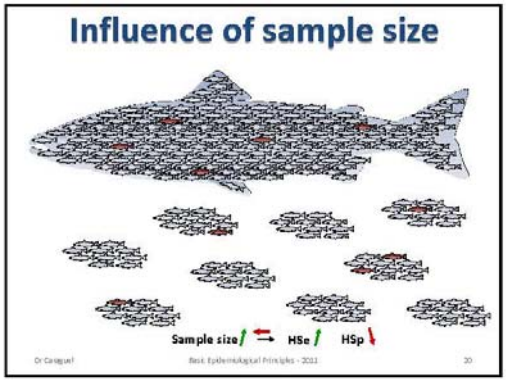

Outlines
Detection performances
1. Individual fish
2. Group/ population of fish
Sampling a population
1. Sampling methods
2. Sample size calculation

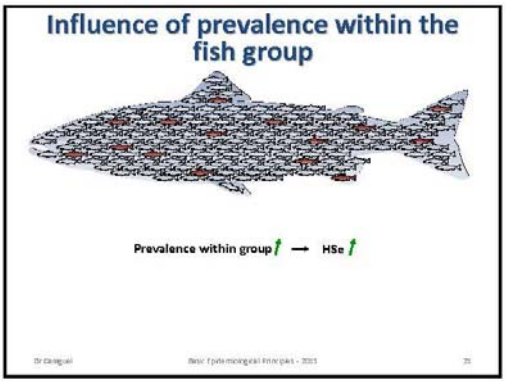

Fish vs. Herd Prevalence
Low fish prevalence
Low herd prevalence
High fish prevalence
High herd prevalence

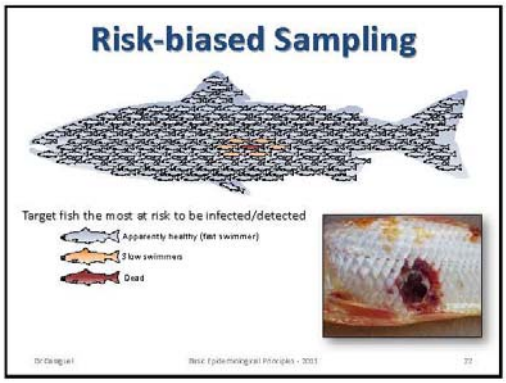

Fish vs. Herd Testing
Bay
Region
Country

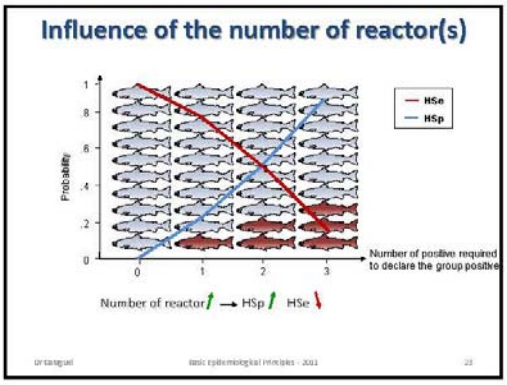

Unit of interest = fish group
Test characteristics
Sampling strategy
Result interpretation
n = sample size
Prev. = prevalence within the group
k = number of reactor to declare group positive

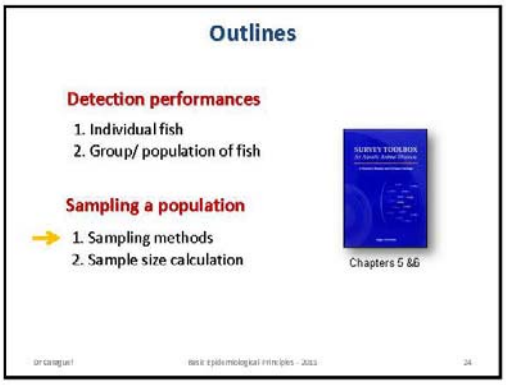

Influence of sample size


Influence of sample size
Sample size
HSe
HSp


Influence of prevalence within the fish group
Prevalence within group
HSe


Risk-biased Sampling
Target fish the most at risk to be infected/detected
Apparently healthy (fast swimmer)
Slow swimmers
Dead


Influence of the number of reactor(s)
HSe
HSp
Probability
Number of positive required to declare the group positive
Number of reactor ↑ → HSp ↑ HSe ↓


Outlines
Detection performances
1. Individual fish
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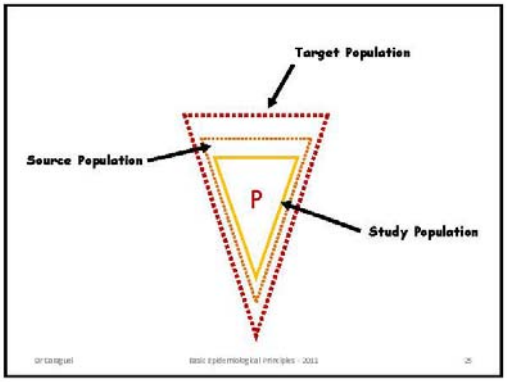
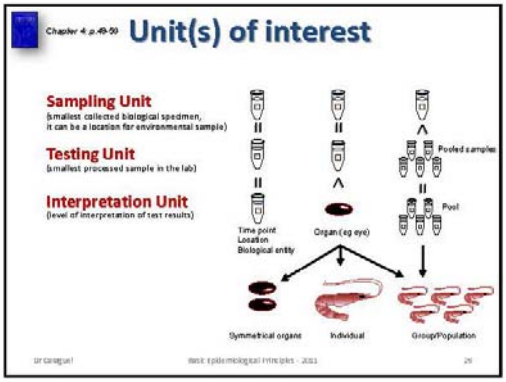
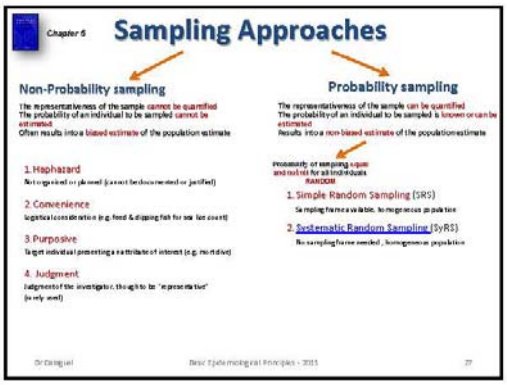
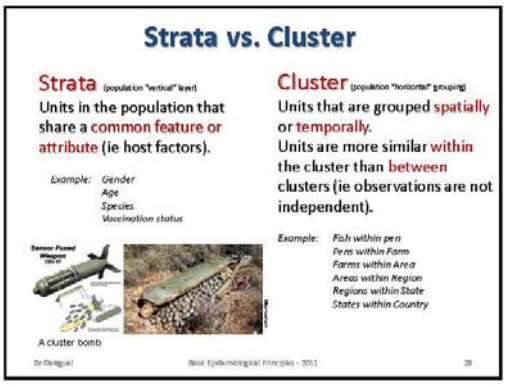
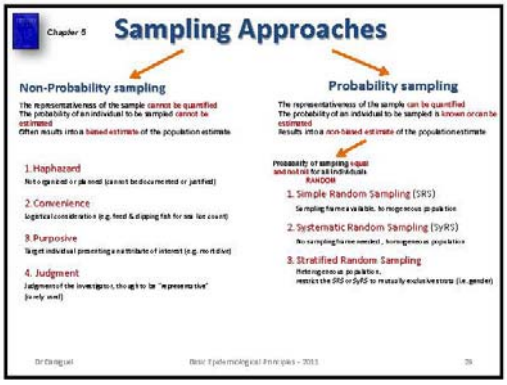
Sampling a population
1. Sampling methods
2. Sample size calculation
Chapters 5 &6

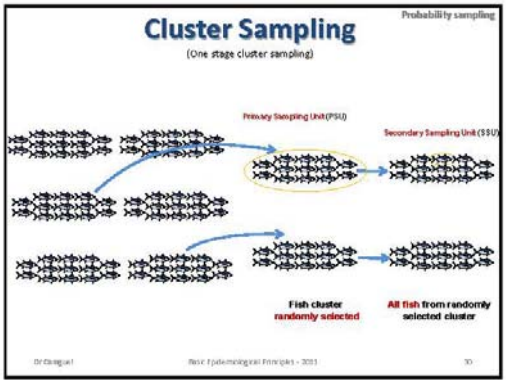

Target Population
Source Population
P
Study Population

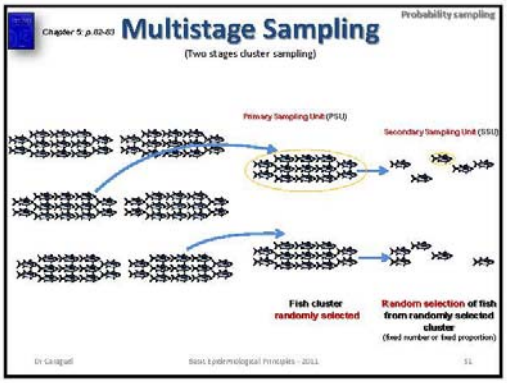

Unit(s) of interest
Sampling Unit
Testing Unit
Interpretation Unit
Pooled samples
Pool
Time point
Location
Biological entity
Organ (eg eye)
Symmetrical organs
Individual
Group/Population

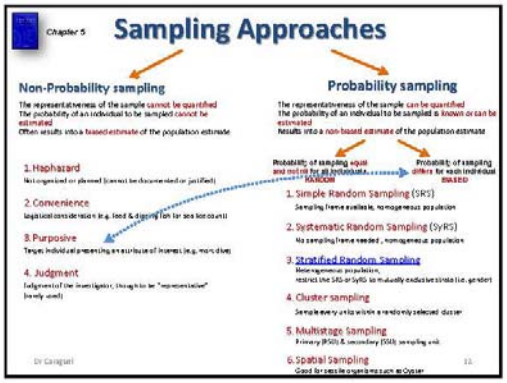

Sampling Approaches
Non-Probability sampling
Probability sampling
1. Haphazard
2. Convenience
3. Purposive
4. Judgment
1. Simple Random Sampling (SRS)
2. Systematic Random Sampling (SyRS)


Strata vs. Cluster
Strata (population "vertical" layer)
Units in the population that share a common feature or attribute (ie host factors).
Example: Gender
Age
Species
Vaccination status
Cluster (population "horizontal" grouping)
Units that are grouped spatially or temporally.
Units are more similar within the cluster than between clusters (ie observations are not independent).
Example: Fish within pen
Pens within Farm
Farms within Area
Areas within Region
Regions within State
States within Country
A cluster bomb


Chapter 5
Sampling Approaches
Non-Probability sampling
Probability sampling
1. Haphazard
2. Convenience
3. Purposive
4. Judgment
1. Simple Random Sampling (SRS)
2. Systematic Random Sampling (SyRS)
3. Stratified Random Sampling


Probability sampling
Cluster Sampling
(One stage cluster sampling)
Primary Sampling Unit (PSU)
Secondary Sampling Unit (SSU)
Fish cluster randomly selected
All fish from randomly selected cluster


Chapter 5: p.82-83
Multistage Sampling
Probability sampling
(Two stages cluster sampling)
Primary Sampling Unit (PSU)
Secondary Sampling Unit (SSU)
Fish cluster randomly selected
Random selection of fish from randomly selected cluster
(fixed number or fixed proportion)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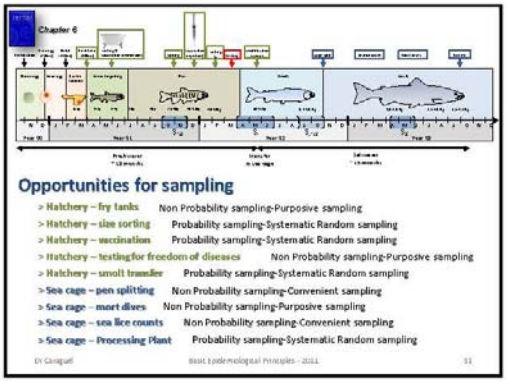
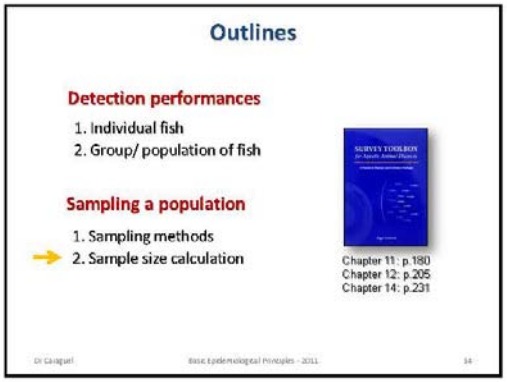
Probability sampling
1. Haphazard
2. Convenience
3. Purposive
4. Judgment
1. Simple Random Sampling (SRS)
2. Systematic Random Sampling (SyRS)
3. Stratified Random Sampling
4. Cluster sampling
5. Multistage Sampling
6. Spatial Sampling


Chapter 6
Opportunities for sampling
> Hatchery – fry tanks Non Probability sampling-Purposive sampling
> Hatchery – size sorting Probability sampling-Systematic Random sampling
> Hatchery – vaccination Probability sampling-Systematic Random sampling
> Hatchery – testing for freedom of diseases Non Probability sampling-Purposive sampling
> Hatchery – smolt transfer Probability sampling-Systematic Random sampling
> Sea cage – pen splitting Non Probability sampling-Convenient sampling
> Sea cage – mort dives Non Probability sampling-Purposive sampling
> Sea cage – sea lice counts Non Probability sampling-Convenient sampling
> Sea cage – Processing Plant Probability sampling-Systematic Random sampling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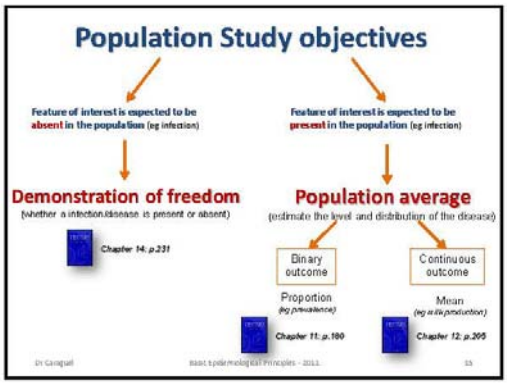
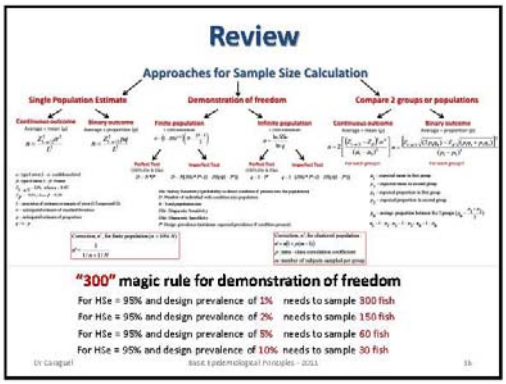
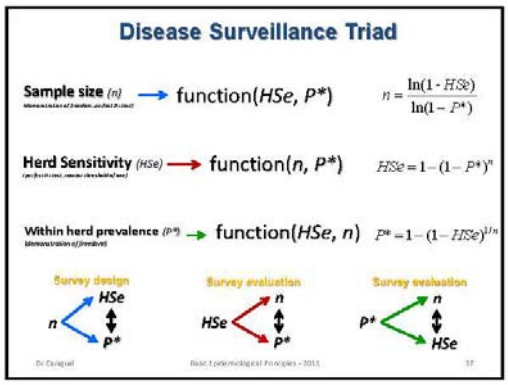
Chapter 11: p.180
Chapter 12: p.205
Chapter 14: p.231


Population Study objectives
Feature of interest is expected to be absent in the population (eg infection)
Feature of interest is expected to be present in the population (eg infection)
Demonstration of freedom
(whether a infection/disease is present or absent)
Chapter 14: p.231
Population average
(estimate the level and distribution of the disease)
Binary outcome
Continuous outcome
Proportion
(eg prevalence)
Mean
(eg milk production)
Chapter 11: p.180
Chapter 12: p.205


Review
Approaches for Sample Size Calculation
Single Population Estimate
Demonstration of freedom
Compare 2 groups or populations
"300" magic rule for demonstration of freedom
For HSe = 95% and design prevalence of 1% needs to sample 300 fish
For HSe = 95% and design prevalence of 2% needs to sample 150 fish
For HSe = 95% and design prevalence of 5% needs to sample 60 fish
For HSe = 95% and design prevalence of 10% needs to sample 30 fish


Disease Surveillance Triad
Sample size (n) → function(HSe, P*)
$n = \frac{\ln(1 - HSe)}{\ln(1 - P^*)}$
Herd Sensitivity (HSe) → function(n, P*)
$HSe = 1 - (1 - P^*)^n$
Within herd prevalence (P*) → function(HSe, n)
$P^* = 1 - (1 - HSe)^{1/n}$
Survey design
Survey evaluation
Survey evaluation


Discussion

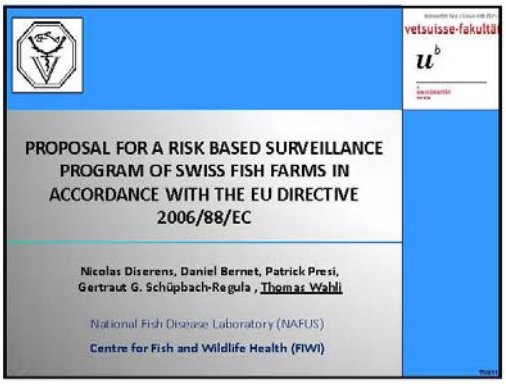
Examples of risk categorization of farms according to EU-legislation

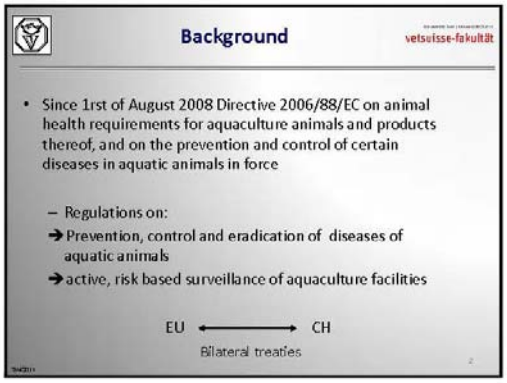
Proposal for a risk based surveillance program of Swiss fish farms

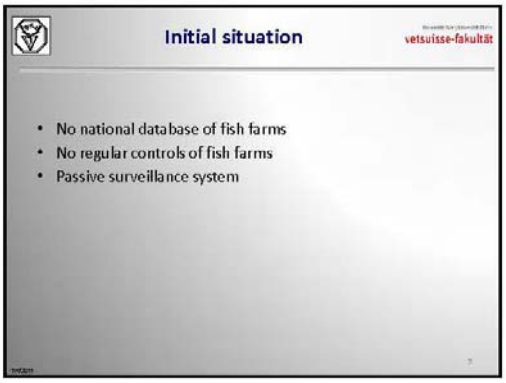
B. von Siebenthal
N. Diserens[1], D. Bernet[2], G. Schupbach[3], P. Presi[3], T. Wahli[1]

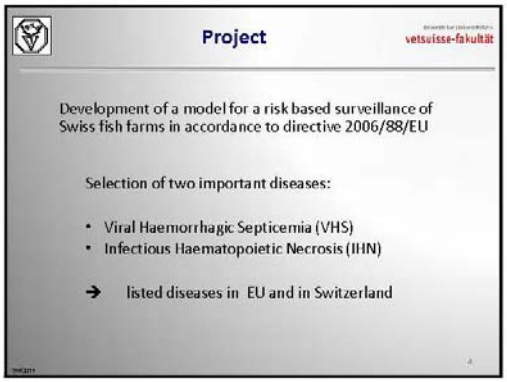
1 Centre for Fish and Wildlife Health (FIWI), Berne, Switzerland
2 Inspectorate of Fisheries, Münsingen, Switzerland
3 Veterinary Public Health Institute, Liebefeld, Switzerland

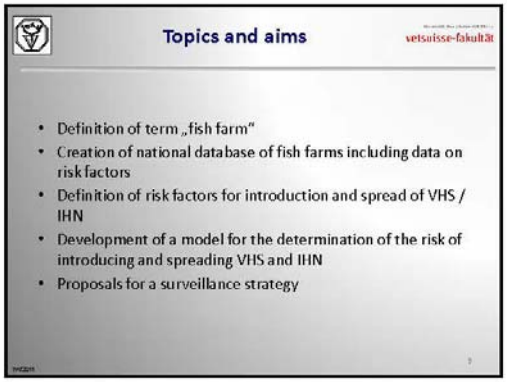
Presentation:

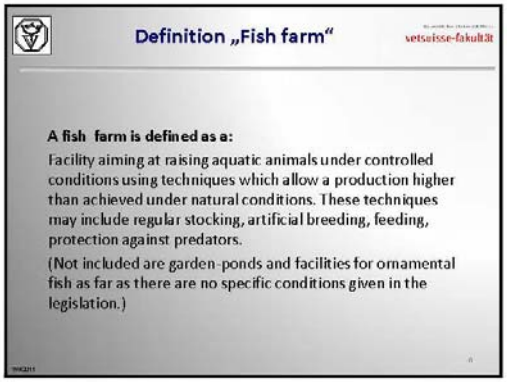


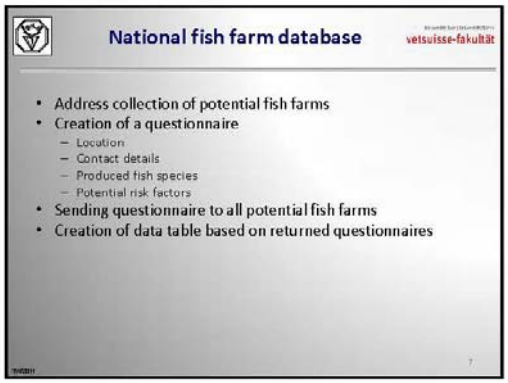


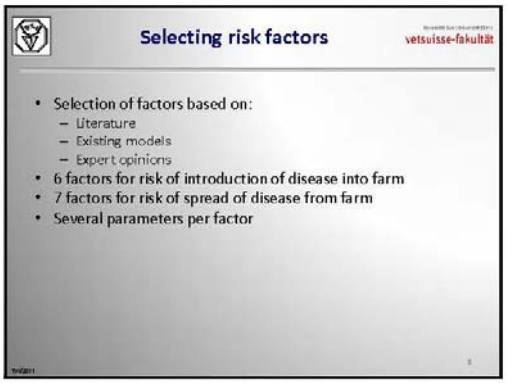


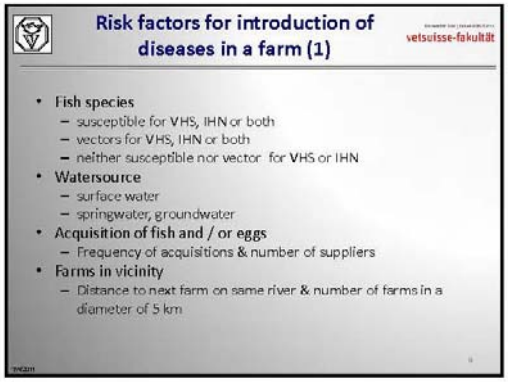


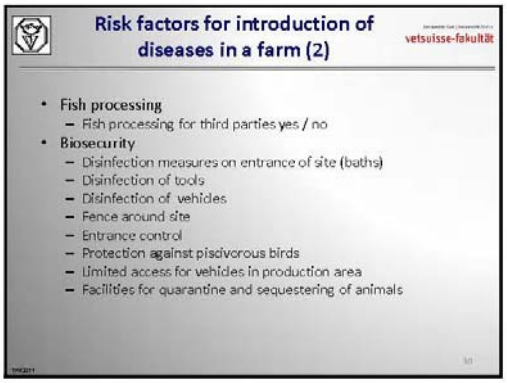

Topics and aims
vetsuisse-fakultät
• Definition of term „fish farm"
• Creation of national database of fish farms including data on risk factors
• Definition of risk factors for introduction and spread of VHS / IHN
• Development of a model for the determination of the risk of introducing and spreading VHS and IHN
• Proposals for a surveillance strategy


Definition „Fish farm"
vetsuisse-fakultät
A fish farm is defined as a:
Facility aiming at raising aquatic animals under controlled conditions using techniques which allow a production higher than achieved under natural conditions. These techniques may include regular stocking, artificial breeding, feeding, protection against predators.
(Not included are garden-ponds and facilities for ornamental fish as far as there are no specific conditions given in the legislation.)


National fish farm database
vetsuisse-fakultät
• Address collection of potential fish farms
• Creation of a questionnaire
– Location
– Contact details
– Produced fish species
– Potential risk factors
• Sending questionnaire to all potential fish farms
• Creation of data table based on returned questionnaires


Selecting risk factors
vetsuisse-fakultät
• Selection of factors based on:
– Literature
– Existing models
– Expert opinions
• 6 factors for risk of introduction of disease into farm
• 7 factors for risk of spread of disease from farm
• Several parameters per factor


Risk factors for introduction of diseases in a farm (1)
vetsuisse-fakultät
• Fish species
– susceptible for VHS, IHN or both
– vectors for VHS, IHN or both
– neither susceptible nor vector for VHS or IHN
• Watersource
– surface water
– springwater, groundwater
• Acquisition of fish and / or eggs
– Frequency of acquisitions & number of suppliers
• Farms in vicinity
– Distance to next farm on same river & number of farms in a diameter of 5 km


Risk factors for introduction of diseases in a farm (2)
vetsuisse-fakultät
• Fish processing
– Fish processing for third parties yes / no
• Biosecurity
– Disinfection measures on entrance of site (baths)
– Disinfection of tools
– Disinfection of vehicles
– Fence around site
– Entrance control
– Protection against piscivorous birds
– Limited access for vehicles in production area
– Facilities for quarantine and sequestering of animals

Risk factors for spread of diseases from a farm (1)

vetsuisse-fakultät

- Fish species
  - Susceptible for VHS, IHN or both
  - Vectors for VHS, IHN or both
  - Neither susceptible nor vector VHS or IHN
- Water effluent
  - Surface water
  - Sewage treatment facility
- Sale
  - For stocking of water bodies or fish farms
  - Direct, wholesale, gastronomy (live fish)
  - Direct, wholesale, gastronomy (dead fish)
  - No sale

Risk factors for spread of diseases from a farm(2)

vetsuisse-fakultät

- Other farms in vicinity
  - Distance to next farm on same river & number of farms in a diameter of 5 km
- Flooding
  - Farm ever flooded yes / no
- Type of ponds
  - Natural
  - Concrete, synthetic
- Biosecurity
  - Same as for introduction

Development of model (1)

vetsuisse-fakultät

Construction of tables to determine risk of every factor e.g. source of water

Risk level	Spring water / ground water	Surface water	Spring, ground & surface water
Zero	x		
Low			
Medium		x	x
High			

Development of a model (2)

vetsuisse-fakultät

Construction of a table with combined parameters e.g. acquisition of fish and / or eggs

Risk „Frequency of acquisition"	Risk „Number of suppliers" Zero (0)	Low (1)	Medium (2-3)	High (≥ 4)
Zero (0)	Zero	-	-	-
Low (1-2)	-	Low	Low	Medium
Medium (3-5)	-	Low	Medium	High
High (≥ 6)	-	Medium	High	High

Development of a model (3)

vetsuisse-fakultät

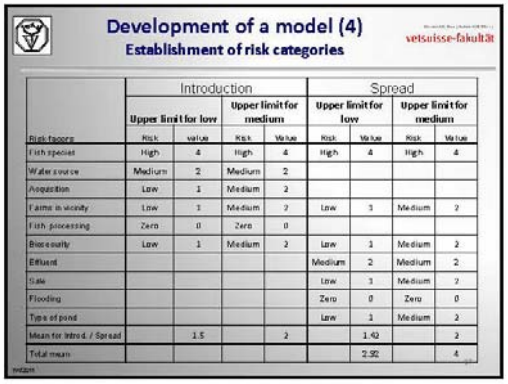
- Attribution of values to risk levels:
  - Zero = 0
  - Low = 1
  - Medium = 2
  - High = 4
- Sum of all values for introduction / number of factors
- Sum of all values for spread / number of factors
- Total risk = sum of mean risk for introduction + mean risk for spread

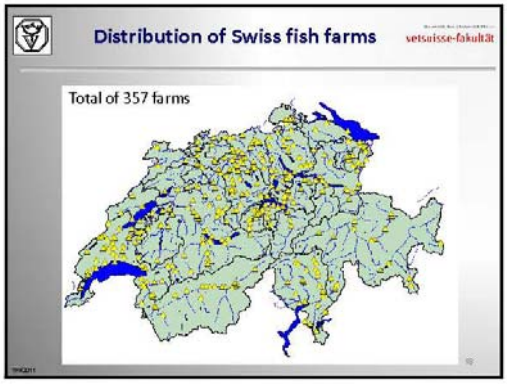
Development of a model (4)

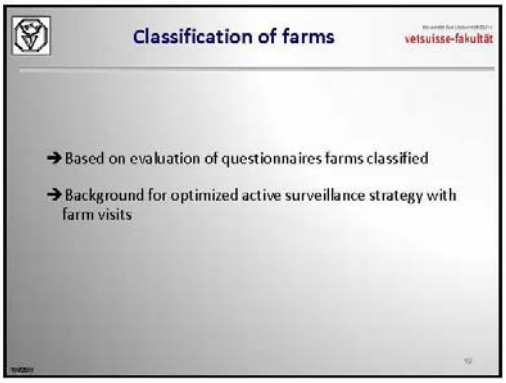
Establishment of risk categories

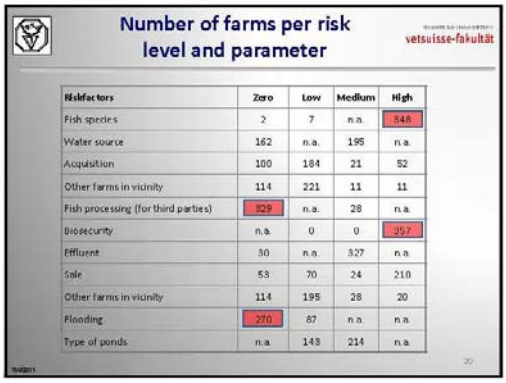
vetsuisse-fakultät

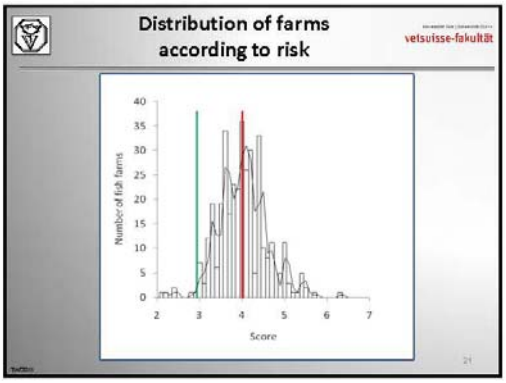
- Two artificial model farms for determination of upper levels for low risk farm and medium risk farm

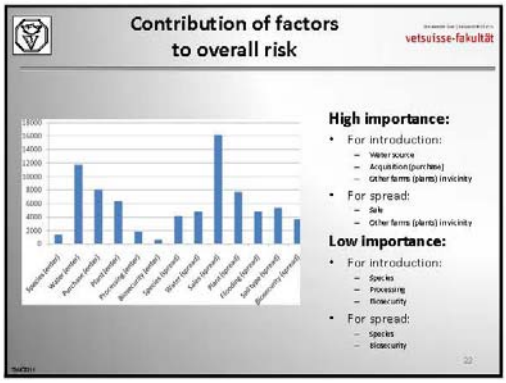

Development of a model (4)
Establishment of risk categories
vetsuisse-fakultät
Introduction
Spread
Upper limit for low
Upper limit for medium
Upper limit for low
Upper limit for medium
Risk factors
Risk
value
Risk
Value
Risk
Value
Risk
Value
Fish species
High 4 High 4 High 4 High 4
Water source
Medium 2 Medium 2
Acquisition
Low 1 Medium 2
Farms in vicinity
Low 1 Medium 2 Low 1 Medium 2
Fish processing
Zero 0 Zero 0
Biosecurity
Low 1 Medium 2 Low 1 Medium 2
Effluent
Medium 2 Medium 2
Sale
Low 1 Medium 2
Flooding
Zero 0 Zero 0
Type of pond
Low 1 Medium 2
Mean for introd. / Spread
1.5 2 1.43 2
Total mean
2.92 4

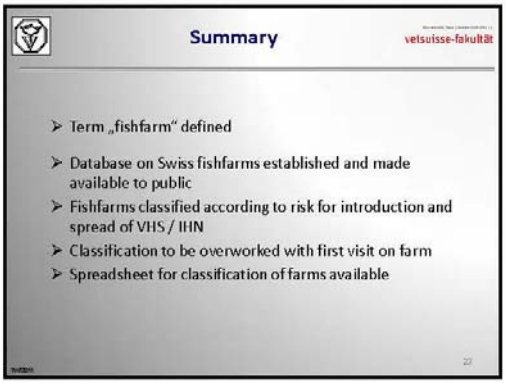

Distribution of Swiss fish farms
vetsuisse-fakultät
Total of 357 farms

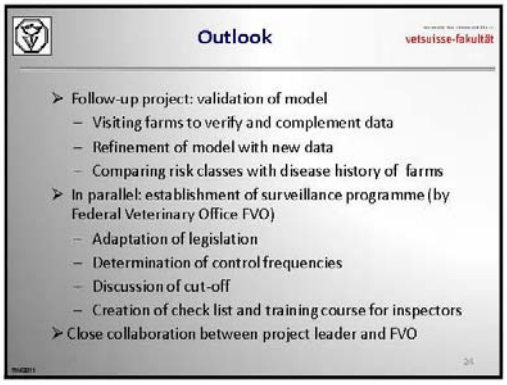

Classification of farms
vetsuisse-fakultät
Based on evaluation of questionnaires farms classified
Background for optimized active surveillance strategy with farm visits


Number of farms per risk level and parameter
vetsuisse-fakultät
Riskfactors Zero Low Medium High
Fish species 2 7 n.a. 348
Water source 162 n.a. 195 n.a.
Acquisition 100 184 21 52
Other farms in vicinity 114 221 11 11
Fish processing (for third parties) 329 n.a. 28 n.a.
Biosecurity n.a. 0 0 357
Effluent 30 n.a. 327 n.a.
Sale 53 70 24 210
Other farms in vicinity 114 195 28 20
Flooding 270 87 n.a. n.a.
Type of ponds n.a. 143 214 n.a.


Distribution of farms according to risk
vetsuisse-fakultät
Number of fish farms
Score


Contribution of factors to overall risk
vetsuisse-fakultät
High importance:
For introduction:
Water source
Acquisition (purchase)
Other farms (plants) in vicinity
For spread:
Sale
Other farms (plants) in vicinity
Low importance:
For introduction:
Species
Processing
Biosecurity
For spread:
Species
Biosecurity


Summary
vetsuisse-fakultät
Term „fishfarm" defined
Database on Swiss fishfarms established and made available to public
Fishfarms classified according to risk for introduction and spread of VHS / IHN
Classification to be overworked with first visit on farm
Spreadsheet for classification of farms available


Outlook
vetsuisse-fakultät
Follow-up project: validation of model
Visiting farms to verify and complement data
Refinement of model with new data
Comparing risk classes with disease history of farms
In parallel: establishment of surveillance programme (by Federal Veterinary Office FVO)
Adaptation of legislation
Determination of control frequencies
Discussion of cut-off
Creation of check list and training course for inspectors
Close collaboration between project leader and FVO


vetsuisse-fakultät
Thank you for your attention!
Questions?

Epizootic risk analysis of lower Saxony Aquaculture production businesses

D.W. Kleingeld[1], G. Hörstgen-Schwark[2] und C.-P. Czerny[3]

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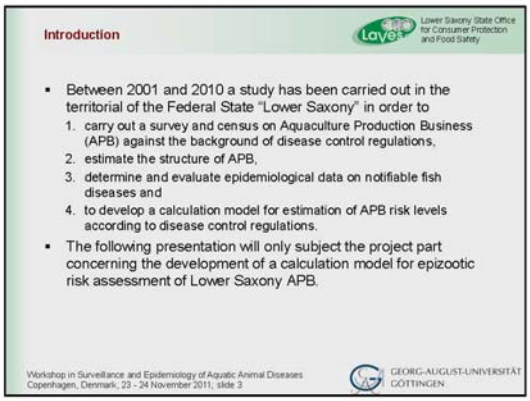
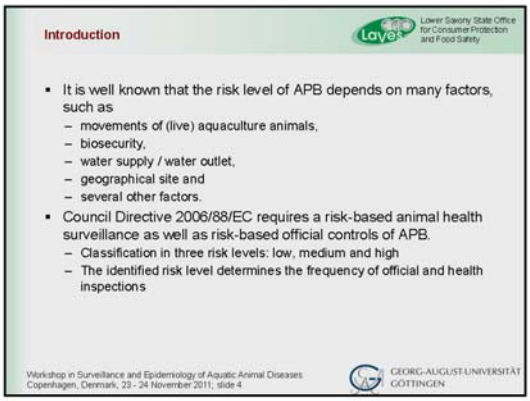
2 Georg-August-University of Göttingen, Department of Animal Sciences, Division of Aquaculture and Aquatic Ecology, Göttingen, Germany
3 Georg-August-University of Göttingen, Department of Animal Sciences, Division of Microbiology and Animal Hygiene, Göttingen, Germany

Presentation:





Introduction

- Commission Decision 2008/896/EC provides a simplified procedure to determine the risk level of APB.
  - Three-step non-linear risk group classification, without any weighting
  - Only few risk factors with respect to the introduction and spread of disease are taken into account:
    1. Introduction or spread of disease via water and due to geographical proximity of farms
    2. Introduction or spread of disease through movements of (live) aquaculture animals
- It can be assumed that a (semi-) quantitative determination of risk levels will result in a better conformity with the actual situation.
  - However more risk factors have to be taken into account
  - Proportional weighting of risk factors is necessary
- Aim of the study was to develop a calculation model to determine risk-levels in a semi-quantitative way and which can be used in practice by the competent authorities.

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APB in Lower Saxony and Germany

Annual Production of Fish in Germany (mt, 2008)

	Lower Saxony	Germany
Salmonids	2.330	27.117
Cyprinids	385	15.432
RAS fish	862	1.431
Total No. APB	2.370	22.881

Source: Jahresbericht Binnenfischerei, 2009

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Contents

- Introduction
- Material and methods
- Outcomes
- Discussion and conclusions

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Material and methods

- A 22-page questionnaire has been carried out among 166 APB in Lower Saxony between 2006 and 2008 in order to collect comprehensive farm data *inter alia* for risk assessment.
- The questionnaire data have been used for determination of risk levels by
  1. non-linear group classification according to Dec. 2008/896/EC,
  2. semi-quantitative linear calculation of risk levels using proportional weightings within several risk groups and risk sub groups; 50:50 weighting of the risk of introduction vs. spread of disease and
  3. semi-quantitative linear calculation of risk levels using proportional weightings within several risk groups and risk sub groups; 70:30 weighting of the risk of introduction vs. spread of disease → more emphasis on the risk of introduction of disease
- Computation has been carried out by using a MS Excel© spreadsheet

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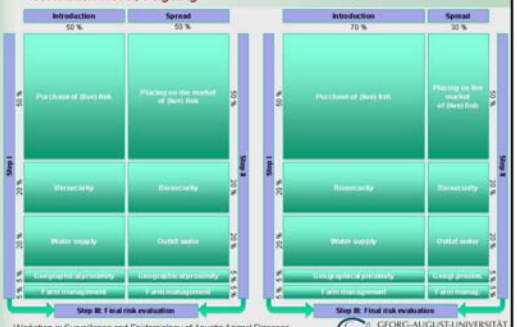
Material and methods
Calculation model, weighting



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Material and methods
Sub risk group weighting

II. Biosecurity	Disinfection measures (personell and visitors)	15 %	Disinfection of hands (0 or 100)	30 %	0 - 100	20 %
			Disinfection of boots / shoes (0 or 100)	20 %		
			Cloth changing (0 or 100)	50 %		
	Other disinfection measures	10 %	Disinfection of equipment (0 or 100)	50 %		
			Disinfection of holding units (0 or 100)	50 %		
	Deterrance	10 %	Complete closed premises (0)			
			Wired premises (20)			
			Partially wired premises (60)			
			No deterrance (100)			

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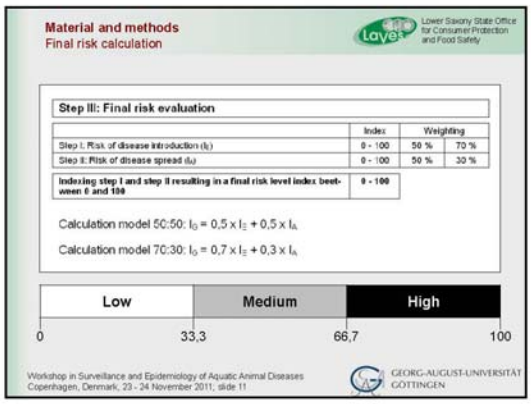
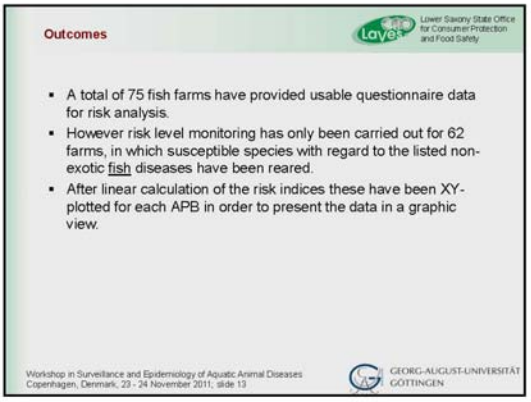
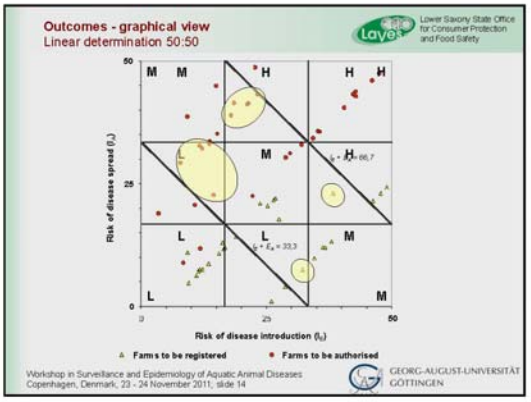
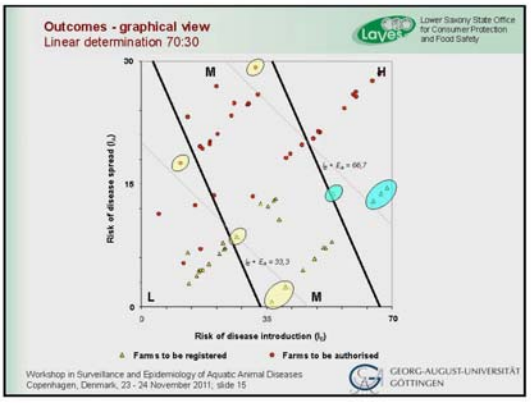
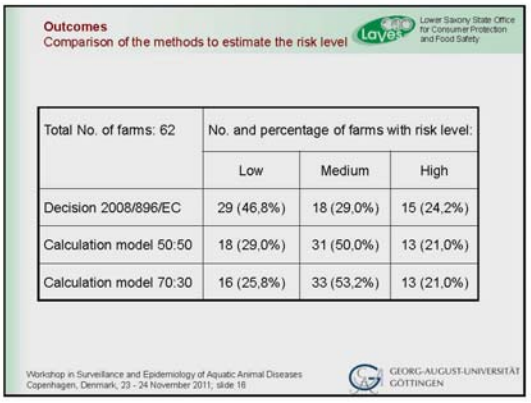




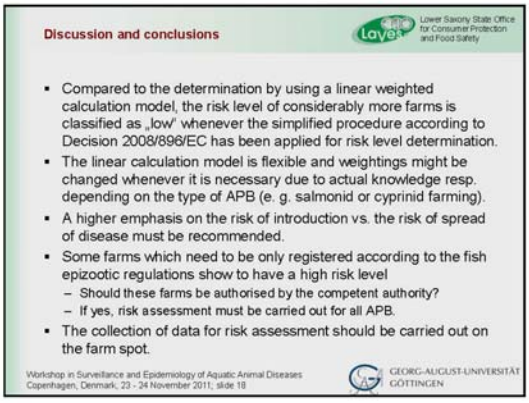






Total No. of farms: 62	No. and percentage of farms with risk level:		
	Low	Medium	High
Decision 2008/896/EC	29 (46,8%)	18 (29,0%)	15 (24,2%)
Calculation model 50:50	18 (29,0%)	31 (50,0%)	13 (21,0%)
Calculation model 70:30	16 (25,8%)	33 (53,2%)	13 (21,0%)


Contents
Lower Saxony State Office for Consumer Protection and Food Safety
Introduction
Material and methods
Outcomes
Discussion and conclusions
Workshop in Surveillance and Epidemiology of Aquatic Animal Diseases Copenhagen, Denmark, 23 - 24 November 2011; slide 17
GEORG-AUGUST-UNIVERSITÄT GÖTTINGEN


Discussion and conclusions
Lower Saxony State Office for Consumer Protection and Food Safety
Compared to the determination by using a linear weighted calculation model, the risk level of considerably more farms is classified as „low" whenever the simplified procedure according to Decision 2008/896/EC has been applied for risk level determination.
The linear calculation model is flexible and weightings might be changed whenever it is necessary due to actual knowledge resp. depending on the type of APB (e. g. salmonid or cyprinid farming).
A higher emphasis on the risk of introduction vs. the risk of spread of disease must be recommended.
Some farms which need to be only registered according to the fish epizootic regulations show to have a high risk level
– Should these farms be authorised by the competent authority?
– If yes, risk assessment must be carried out for all APB.
The collection of data for risk assessment should be carried out on the farm spot.
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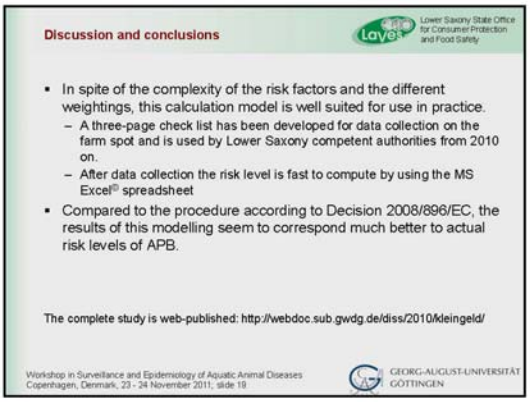
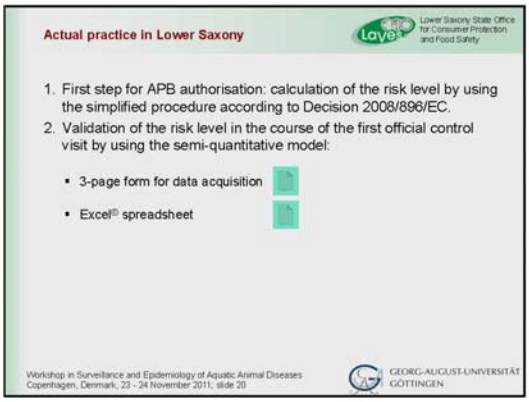
GEORG-AUGUST-UNIVERSITÄT GÖTTINGEN


Discussion and conclusions
Lower Saxony State Office for Consumer Protection and Food Safety
In spite of the complexity of the risk factors and the different weightings, this calculation model is well suited for use in practice.
– A three-page check list has been developed for data collection on the farm spot and is used by Lower Saxony competent authorities from 2010 on.
– After data collection the risk level is fast to compute by using the MS Excel© spreadsheet
Compared to the procedure according to Decision 2008/896/EC, the results of this modelling seem to correspond much better to actual risk levels of APB.
The complete study is web-published: http://webdoc.sub.gwdg.de/diss/2010/kleingeld/
Workshop in Surveillance and Epidemiology of Aquatic Animal Diseases Copenhagen, Denmark, 23 - 24 November 2011; slide 19
GEORG-AUGUST-UNIVERSITÄT GÖTTINGEN


Actual practice in Lower Saxony
Lower Saxony State Office for Consumer Protection and Food Safety
1. First step for APB authorisation: calculation of the risk level by using the simplified procedure according to Decision 2008/896/EC.
2. Validation of the risk level in the course of the first official control visit by using the semi-quantitative model:
3-page form for data acquisition
Excel© spreadsheet
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GEORG-AUGUST-UNIVERSITÄT GÖTTINGEN


EPIZOOTIC RISK ANALYSIS OF LOWER SAXONY AQUACULTURE PRODUCTION BUSINESS
Acknowledgements
- Prof. Gabriele Hörstgen-Schwark and Prof. Claus-Peter Czerny, Univ. of Göttingen
- Fish farmers participating in the project
- Competent veterinary authorities of the rural districts in Lower Saxony
Thanks to you for your kind attention

Special challenges related to shell and mollusc surveillance

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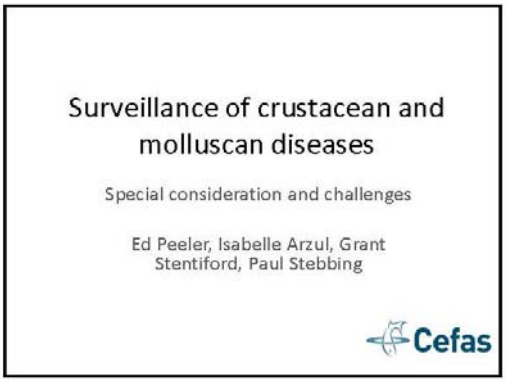
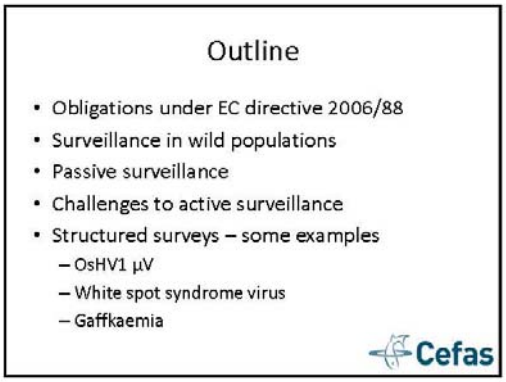
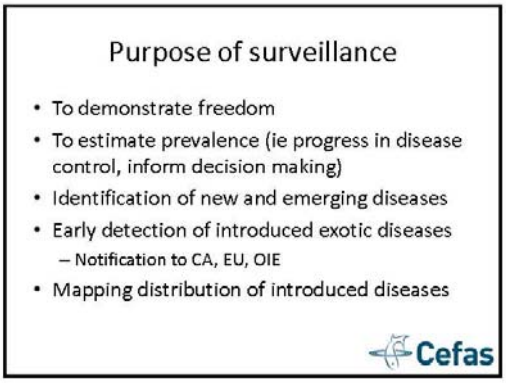
Presentation:







Other diseases of interest

- Crayfish plague (*Aphanomyces astaci*)
  - Listed by OIE
- Gaffkaemia (*Aerococcus vividans*)
- Haematodinium infections in crabs

Cefas

Major European Shellfish Spp.

	Wild	Farmed
Molluscs	•Oysters (Ostrea edulis, Crassostrea gigas) •Mussels (Mytilis edulis, M. galloprovincialis) •Scallops (e.g. Pecten maximus) •Cockles (Cerastoderma edule) •Clams (e.g. Venerupis decussata)	
Crustacea	•Crayfish •native (e.g. Austropotamobius pallipes) • non-native (Pacifastacus leniusculus) •Crabs (e.g. Cancer pagurus) •Lobsters (Homarus gammarus) •Norway lobster (Nephrops norvegicus) •Brown shrimp (Crangon crangon)	•Signal crayfish •Shrimp

Cefas

Disease surveillance in wild animal aquatic animal populations

- Wild animal populations generally not physically constrained, able to move over large areas
- Limited information on population ecology (movement, behaviour, structure, density, distribution)
- Limited information on denominators
- Geographically diverse populations
- Not easily observed
- Diseased animals are quickly removed through predation

Cefas

Passive surveillance – detection of disease

- The most important system for the detection of new and introduced pathogens
- Time to detection relies on farmer-reporting
  - Farmed molluscs less easily observed than fish
  - Routine observation at grading / harvesting
- Clinical signs generally not disease specific

Cefas

Passive surveillance – detection of mortality

- Mortality ascribed by farmers to environmental causes
- Mortality in wild populations only noticed if large-scale and sudden
  - dead animals washed up
- More subtle change in mortality will be detected only through
  - long term population monitoring,
  - analysis of wild capture landings

Cefas

Active surveillance

- To demonstrate freedom
- To map distribution of a disease
  - e.g. during an outbreak
- Assess prevalence
  - Monitor progress in disease control/eradication

Cefas

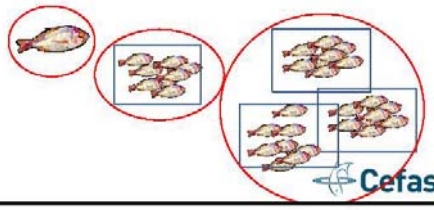
Sampling considerations

- Representative sampling of molluscs most easily done at grading or harvest BUT
- Timing need to take account of
  - intensity of infection
    - Water temp
    - Spawning
  - Transfers of organisms
- Extensive production areas require spatial sampling
  - Random selection of points (defined by coordinates)

Cefas

Epidemiological unit

- Group of animals sharing the share level of exposure
- Need to define the epidemiological unit(s) that will be used as sampling units so that a sampling frame can be constructed



Cefas

Epidemiological units

- Physical barriers define within farm clusters (e.g. ponds, raceways)
- No physical barriers between
  - Adjacent wild populations
  - Farmed marine shellfish animals
  - Farmed and wild shellfish populations
- Epidemiological units for farmed marine and wild populations may be difficult to define

Cefas

Epidemiological unit - molluscs

Farmed

- Bag / Rope
- Trestle
- Farm
- Production area / Bay
  - Defined by hydrology and anthropogenic links (ie animal movements)

Epidemiological unit - molluscs

Wild

- Discrete bed
- Shellfish farming area / shellfish harvesters
- Bay
  - Defined by hydrology and anthropogenic links (ie animal movements)

Epidemiological unit - crustaceans

Farmed

- Pond
- Farm
- Farms with a river / bay

Wild (freshwater)

- Discrete population with a river
- Part of river (tributary)
- River catchment

Wild (marine)

- Harvesting vessels
- Vivier (holding facility)
- Bay

Epidemiology has been called 'the study of denominators'

a lack of information on populations for many wild crustacean and molluscan populations constrains the design and analysis of structured surveillance

Cefas

Clustering

- Surveillance must take account of clustering in survey design (multi-stage sampling) and statistical analysis of data
- Farmed shrimp / crayfish clustered within
  - ponds which are clustered within
  - farms which are clustered within
  - rivers / bays
- Farmed and wild mollusc populations cluster within estuaries / bays
- Wild crayfish populations clustered within rivers or tributaries within rivers

Cefas

Surveillance following disease incursion

- Following detection of a disease surveillance based on forward and backwards tracing to map distribution requires reliable, up to date live animal movement data
  - Often not available for farmed molluscs
- Tracing and sampling may be extended to include 'vector species'
  - Capable of mechanically transferring pathogens
    - Commission Regulation (EC) 1251/2008
  - Reliability of detection?

Cefas

Risk based surveillance

Member States shall ensure that a risk-based animal health surveillance scheme is applied in all farms and mollusc farming areas, as appropriate for the type of production

- Lack of traceability of live molluscs movements constrain application of RBS
- Lack of physical barriers between farms means that mollusc farming areas is a more appropriate level to classify risk than mollusc farm

Council directive 2006/88/EC Article 10

Cefas

Structured surveys

- Survey to demonstrate freedom from OsHV1 new variant
- Survey to demonstrate freedom from WSSV
- Survey to demonstrate distribution of gaffkaemia

Cefas

Freedom from OsHV1µV

- Article 43 disease
- MS may demonstrate freedom through structured surveillance
  - Allows MS to restrict imports for relaying to other areas with same status
- UK and Ireland have approved surveillance programmes
  - Specified by EC guidance document (SANCO/7004/2011/rev5)
  - Not risk- based

Cefas

Guidance document SANCO/7004/2011/rev5

- All farms or mollusc farming areas keeping Pacific oysters in the Member State or compartment covered by the programme should be sampled

Not RBS

Target population

Cefas

Sample size and collection

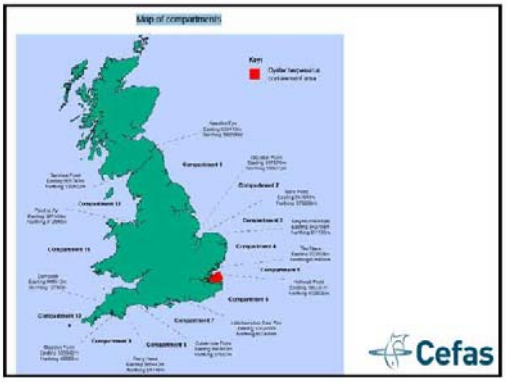
- 3*150 oysters per mollusc farming area
- Sample should reflect entire area
- Sample should be collected when water temp > 16 º C (or maximal)
- Select weak, gaping or freshly dead, if not present select juveniles

Input based sampling requirement

Epidemiological unit

Maximise chance of detection

Cefas



Alternative approach – output based

- Undertake surveillance to demonstrate absence of OsHV1µV with 95% confidence if the prevalence
  - Within farm is ≥ 5% (design prevalence)
  - Farm/farming area level is ≥ 2% (design prevalence)
- And assuming test characteristics, e.g.
  - Sensitivity 98%
  - Specificity 95%
- Highest risk farms and areas should be selected

Cefas

WSSV surveillance to demonstrate freedom in the UK

- Active surveillance
  - Susceptible species
    - All decapods
  - Populations
    - Marine
      - Shore crabs
    - Freshwater
    - Signal crayfish (wild and 2 farms)
- Investigation of mortalities
- Testing imported animals

Combining different sources of data

Cefas

Sampling strategy

- Only 2 farms
- Collection of wild signal crayfish by the Environment Agency
  - Unwanted introduced species
  - Samples available from control measures and regulated harvesting (licensed operators)
- Collection of crabs from fisherman

Cefas

- Many holding facilities of wild caught marine decapods prior to dispatch
- Imports of many crustacea
  - Human consumption (products)
  - Further processing (and exportation)
  - Aquarium trade

Cefas

Diagnostic manual requirements for obtaining disease free status

- Wild populations to be sampled where number of farms are limited
- The number of sampling points should provide
  - 95% confidence of detecting at least one infected sampling point if
  - the proportion of infected sampling points exceeds 10% (design prevalence) and minimum prevalence of infection is 2% (design prevalence)
- 30 samples of 150 animals each
- Representative of different ecosystems where wild susceptible populations are located
- Widespread geographic coverage

Cefas

Maximising the likelihood of detection

- Sampling near to ports and processors in warmer regions whilst also sampling from
  - Wide geographic area
  - All ecosystems (estuarine, coastal, river, still)
- Sampling carried out when water temperatures at highest annual point
- Weak abnormally behaving or freshly dead selected, whilst also sampling from all life stages
- Sample areas selected using ICES subdivisions or river catchments as epidemiological units
- Samples points selected within samples areas (convenience sampling)

Cefas

UK site selection

Marine sites

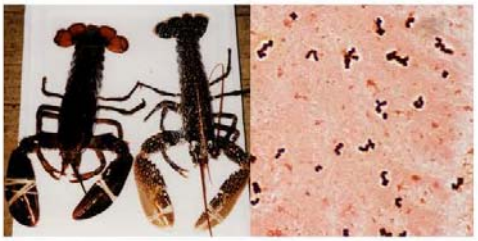
Freshwater sites

Cefas

Practical issues

- No attempt to randomly select
  - Sites where animals were sampled
  - Animals within sites
- Random sampling may considerably increase the complexity and cost of surveillance
- Harvested or trapped animals will not be representative of the population
  - sample was not representative of the population from which they were drawn

Cefas



Survey to assess geographic distribution of gaffkaemia in E&W

Cefas

Lobster fishing in the UK

- Population – exploited European lobster population
- Held in storage facilities before shipment to market, can be held for long periods
- Market mainland Europe
- Mortalities of lobsters in storage facilities

Cefas

Sampling

- 31 sites around E&W coastline, selected based on lobster potting activity
- Animals bought off boats (or from holding facilities)
- Non-lethal sampling of haemolymph

Non-lethal sampling of haemolmph for culture

Cefas

Sampling strategy

- Lobster boats regularly set pots in the same locations
- They are a reasonably proxy for a geographic region
- Harvested animals represent only one part of the populations
  - Egg bearing females may be returned
  - Juveniles not trapped or returned

Is the harvested lobster population susceptible to WSSV?

Cefas

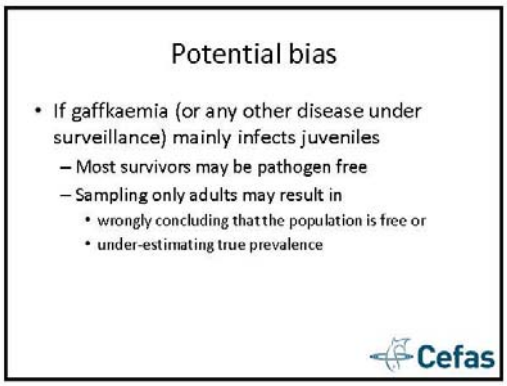
Results

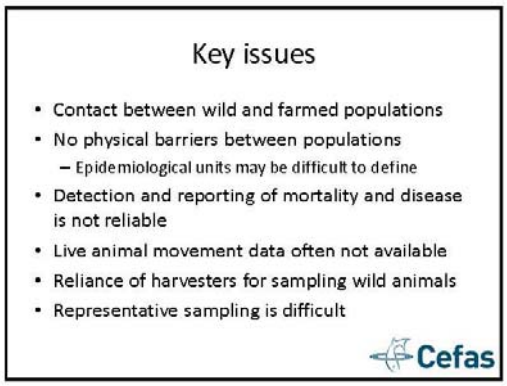
- Approximately 30 animals sample per site
- Total 952 animals samples
- 9 animals from 4 sites tested positive
- Overall prevalence 0.95%

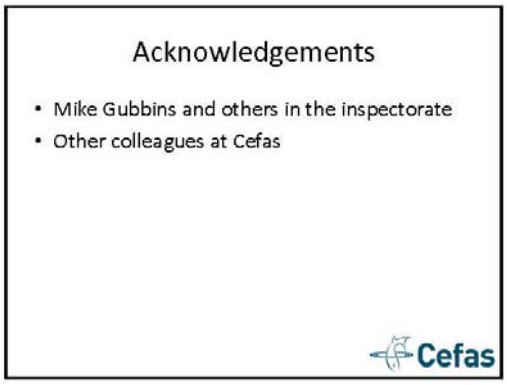
Interpretation

- Sampling 30 animals gives a 95% likelihood of detecting at least one infected animal if the prevalence is above 10% (assuming perfect sensitivity).
- It is not possible to determine whether the pathogen is widespread at a very low prevalence or whether the distribution is patchy.

Cefas


Potential bias
• If gaffkaemia (or any other disease under surveillance) mainly infects juveniles
– Most survivors may be pathogen free
– Sampling only adults may result in
• wrongly concluding that the population is free or
• under-estimating true prevalence
Cefas


Key issues
• Contact between wild and farmed populations
• No physical barriers between populations
– Epidemiological units may be difficult to define
• Detection and reporting of mortality and disease is not reliable
• Live animal movement data often not available
• Reliance of harvesters for sampling wild animals
• Representative sampling is difficult
Cefas


Acknowledgements
• Mike Gubbins and others in the inspectorate
• Other colleagues at Cefas
Cefas

Practical part: Parallel sessions with group-work and practical examples

For this part, the participants were divided into four groups:

- Screening strategies & tracing of pathogens
- Special challenges regarding surveillance in shell & molluscs
- Design of surveillance programmes
- Risk factors and risk categorization

Within each group, several topics or scenarios were discussed, and a synthesis of the discussions was shared with the rest of the workshop in the discussion session on the second day. For ease of reading, the description of the group works, the discussions and comments from both days are all collected under each group below.

Screening strategies & tracing of pathogens

Facilitators: C. Caraguel & P. Jansen

In this group there were 12 participants.
The participants were presented with three scenarios, which were discussed within the group.

Scenario 1: *A new disease syndrome emerges in salmon farming. Mortalities are very high. This situation needs to be managed, and this is up to you.*
Question: What initial information would you seek?
Answers:

- Case definition (host/pathogen/environment)
- Mortality pattern
- Description of spatial and temporal distribution
- Outbreak investigation (risk factor analysis)

All with the purpose of seeking the disease natural history

Scenario 2: *The disease turns out to be infectious and caused by a known virus. Spatial patterns of outbreaks are drawn, marking only farms that have had an outbreak of the disease recently. This shows a pattern with some clusters of diseased farms but also some isolated farms.*
Question: What are the most likely transmission pathways, and how would you find out which it is?
Answers:

- Vertical transmission: Investigate association with hatcheries/smolt supplier. Screen broodstock & egg. Upstream (sea sites to hatchery) and downstream (hatchery to sea sites) investigation
- Fish transfer: Temporal. Spatial pattern. Identification of index case. Index case is the link among cases. Contact network analysis.
- Waterborne: Spatial pattern. Association with distance. Hydrodynamics modelling. Sentinel fish

Scenario 3: *Local epidemics emerge – all outbreaks with the same virulent virus strain => horizontal spread between neighboring farms. Low-virulent ISAV found commonly and in all aquatic environments. No associations between smolt origin, brood stock origin and virulent ISAV found*
Question: How would you manage this? I.e. with reference to:
- Restrictions on diseased farms
- Criteria for restrictions (diagnosis)

- Risk based surveillance
- Notification of non-virulent strains

Answers:
Regarding restrictions on diseased farms:
-Close the factory
-Isolate naive fish
-Lower the susceptibility of the host
Regarding surveillance:
If ISAV is not present (early detection):

- "Complete" coverage (population/space/time)
- Representativeness (given with coverage, except if resources are limited)
- Dx test as sensitive as possible (maximize NPV)
- Investigate potential false positive with confirmatory test: series interpretation (increase Dx and herd specificity to perfection?)

ISAV is common (case detection):

- "Complete" coverage (population/space/time)
- Representativeness (given with coverage, except if resources are limited eg harvest boat)
- "Only clinical outbreak"
- Dx test as specific as possible (maximize PPV)

Special challenges regarding surveillance in shell & molluscs

Facilitator: E. Peeler

In this group, the following three topics were addressed:

1. Risk-based surveillance (RBS) for mollusc diseases
2. Improving farmer-reporting of mortality
3. Determining a cut-off for qPCR

Ad 1) RBS main issues for molluscs are

- Biosecurity
- Interface with wild
- Definition of epidemiological units
- Traceability live animal movements

Attitudes towards biosecurity and disease: Shellfish farmers have less interest in biosecurity and disease control compared with finfish farmers. Disease in farmed shellfish is often viewed as a natural event and cannot be prevented. Biosecurity in open water production is inherently poor, but hatcheries can maintain good biosecurity.
Authorisation / registration of aquaculture production businesses: Register of APBs in EU MS is incomplete. RECC review current situation of authorisation/registration in the EU, including methods of requiring data, main problems in implementation (it is difficult to harmonise divergent data sources (regional, different ministries), there is a large number of farms), and can the database of APBs be regularly updated (costs)?
Surveillance could include other businesses: There are advantages to include other businesses in surveillance programmes. RECC need flexibility in to include businesses currently not authorized as APBs in RBS programmes.
Pathways of spread:
Introduction of mollusc diseases into a country:

- Illegal movements of juveniles across border easily done, lack of enforcement

- Diversion of animals imported for human consumption to aquaculture
- Import of used aquaculture equipment (note recent Australian reg re: disinfection)

Spread within a country:

- RECC: undertake risk ranking at level of the shellfish farming area (SFA)
- Focus attention on recording movements between SFA
- Currently data on movement of molluscs is poor e.g. movements between sites in different SFA owned by the same business often not recorded

Other factors influencing risk:

- Type of farm
    - Mix of age classes (Farms keeping all age classes higher risk (classify farms by type of animal sold))
    - Source of spat (Currently not clear if hatchery sources spat higher or lower risk than wild sourced)
    - Size of farm (Number of sites)
- Presence of vectors and susceptible in zone

Ad 2) Improving farmer reporting:

- Compensation
    - Lack of compensation discourages reporting of notifiable disease
    - Compensation may result in misreporting
    - Tie compensation to assessment of biosecurity
- Communication by CA about importance of reporting mortality
- Guidance on stock inspection
- Guidelines on definition of abnormal mortality (eg number of bags affected)
- Benefits to farmers: feedback results relies on a system to analyse data on annual or regional basis

Approaches: Work through producer organizations. Benefits to industry of early reporting, no benefit to the individual who reports. Need farmer funded insurance schemes

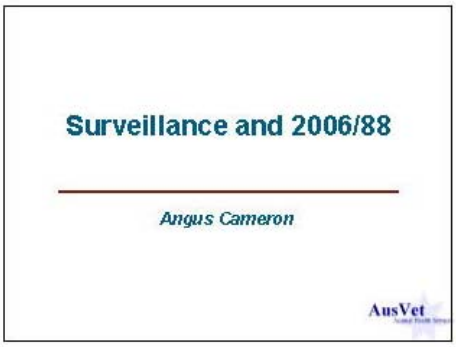
Ad 3) Setting a cut-off for a qPCR

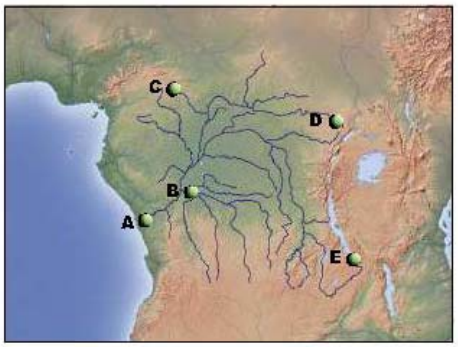
- From OsHV-1 qPCR ring trial labs seem to use different cutt-off values.
- When conducting ring trials.Ask labs for information on cut-off points and ct values.
- Cutt-off depend on goal of test:
    - Declare freedom of disease
    - Estimate prevalence of pathogen

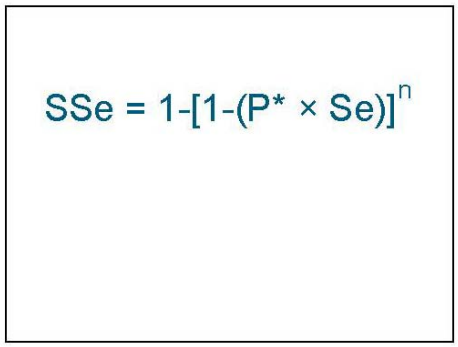
Design of surveillance programmes

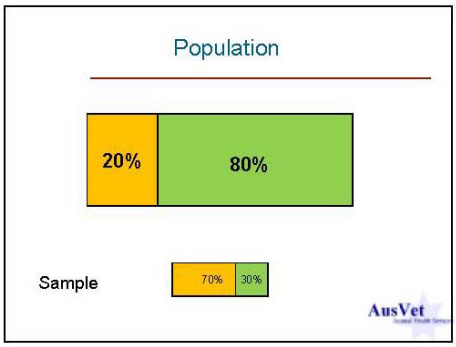
Facilitator: A. Cameron

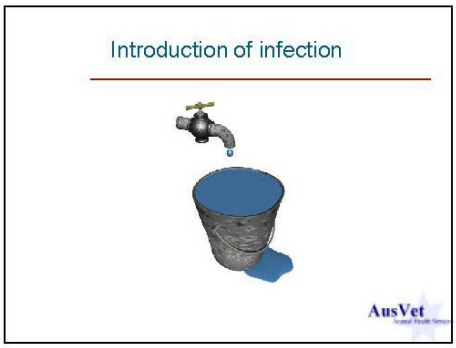
The content of the group work are included in the following presentation, which was given to the whole workshop on day 2:

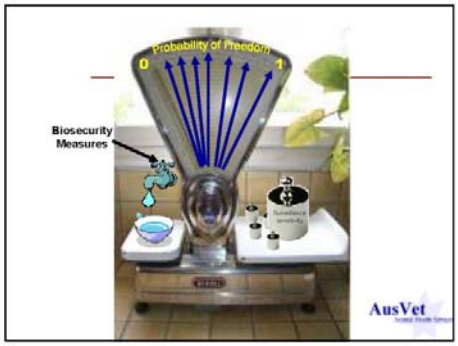


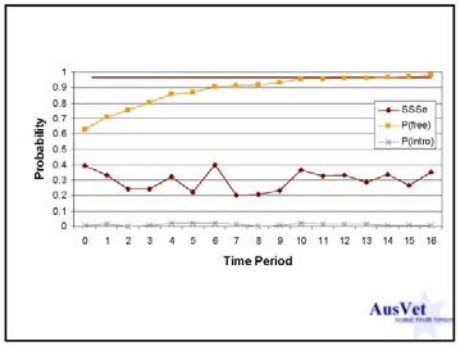












2006/88 and surveillance

- Purpose
  - Disease control
- Mechanism
  - Prevention of spread of disease
  - Classification of disease status
    - Country / zone / compartment
    - Farm
  - Movement restrictions between farms/zones of different disease status
  - High status to equal or lower

AusVet

Status categories

- Categorical interepretation of Pr(free)
  - Cat I: Surveillance, Pr(free) > threshold
  - Cat II: Surveillance, Pr(free) < threshold
  - Cat III: No surveillance, no evidence
    - Infection status unknown
  - Cat IV: Infected, eradication program
    - Assumed low prevalence
  - Cat V: Infected, no program
    - Assumed high prevalence

AusVet

Conclusion

- Control measures based on P(free)

AusVet

Risk-based surveillance (2006/88)

- Different surveillance activities
  - Type of surveillance (based on category)
    - Passive
    - Active
    - Targeted
  - Different frequencies (based on risk)
    - Every 1, 2 or 4 years

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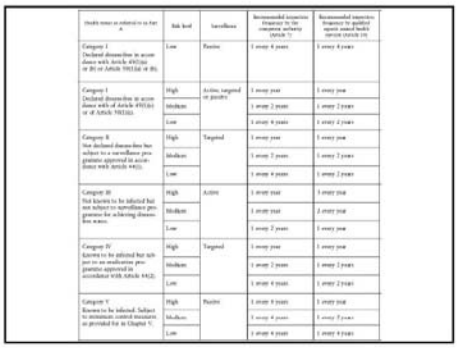
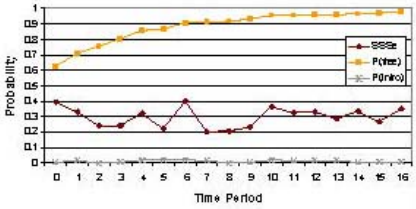


Factors contributing to P(free)

- Surveillance sensitivity
- Probability of introduction
- Cumulating evidence over time (frequency)

AusVet



Surveillance activities

- Passive (farmer reporting)
  - Notification of mortalities
  - High sensitivity (clinical disease)
  - Continuous
  - Very cost effective
  - Risk of non-compliance
  - Good for early detection, freedom (clinical)

AusVet

Surveillance activities

- Active
  - Farm visits, clinical exam
  - Sampling sick fish
  - High sensitivity (clinical disease)
  - Intermittent
  - Moderate cost-effectiveness
  - Required to cover risk of non-compliance
  - Good for freedom (clinical), poor for early detection

AusVet

Surveillance activities

- Targeted
  - Farm visit
  - Sampling healthy fish
  - Low sensitivity (but can detect subclinical)
  - Poor cost-effectiveness
  - Poor for early detection
  - Poor for freedom
  - Required for subclinical diseases

AusVet

What about other surveillance approaches

- Indirect
- Syndromic
- Sentinel
- Etc…

AusVet

Risk categorisation

- Important part of directive
- Requires enormous amount of effort
- Only used to determine frequency of inspections

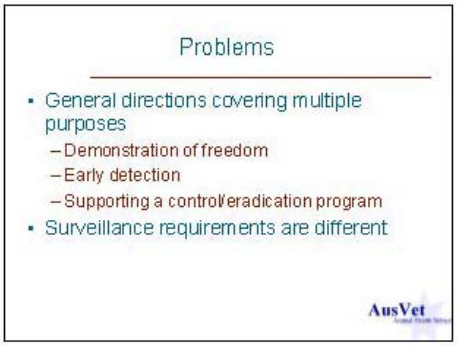
AusVet

Components

1. Probability of being infected (if infection is present)
2. Probability of new introduction of infection (between sampling)
3. Probability of spreading infection (part of consequences)

2 ⇨ 1 ⇨ 3

AusVet



Risk factors and risk categorization

Facilitators: B. Bang Jensen & T. Lyngstad

The purpose of the groupwork was to collate and discuss the most important challenges to risk-ranking of farms within the member states according to the directive 2006/88/EC.

There were 11 participants in the group (plus the facilitators), and they first got to get acquainted by having to present the person sitting next to them after having conducted a short interview. Thereafter, Birgit Oidtmann from Cefas, UK was invited to give a presentation of the model used for risk-ranking in the UK (Ref: Oidtmann, BC., Crane, CN., Thrush, MA., Hill, BJ & Peeler, EJ. (2011): Ranking freshwater fish farms for the risk of pathogen introduction and spread. Preventive Veterinary Medicine, 102, 329-340.)

Then the participants were divided into 4 groups, and given the following questions:

- Would it be possible to apply one or more of the methods presented today in your country? Why/ why not?
- What data are available in your country for risk-ranking of farms?
- How should any method be altered in order to make it useable to your country?
- How do you make sure, that the risk-ranking is performed equally to all farms within your country?
- How could it be ascertained that the risk-rank of a farm in one country, corresponds to the rank in another?
- (Optional) Could this risk-ranking be applied for all diseases? What would it take to make it applicable to all?

Subsequently, the participants discussed the topics in plenum within the group, and presented a summary of these to the rest of the workshop on the following day.

Comments from the discussions:

What are the challenges to risk-ranking?

- Variations in administration, both within and between member states
- Geographical challenges, including river systems shared with other administrative units
- Many different farming systems
- Reliability of data received from fish farmers
- Fish is low priority in many countries
- Lack of competence/ knowledge on risk factors/ fish diseases
- Comparison between countries is difficult - but not necessary according to guidelines

Which methods can be used for risk-ranking?

- Start with the basic level –as provided in the 2008/896/EC. It is up to the individual MS to decide the level of complexity that they want to use.
- The models from Lower-Saxony, Switzerland and UK are good inspiration for taking it to the next step

What are the incentives for risk-ranking –within each MS and across the countries?

- Economic aspects, cost benefit relationship
- Incentives for surveillance, importance of industry varies between and within countries
- Is it necessary to risk-rank farms that are within health category V (Known to be infected, not subject to surveillance program)? This is maybe not clear.

About the directive:

- The 2006/88/EC gives basic biosecurity requirements. But it is up to the individual MS to decide if they want a higher level.
- The directive is made so that it is applicable in all MS, therefore, there is room for variation and refinement within each MS.
- Be aware that surveillance is used in different contexts in the directive

Disease risk in space and time –Implications for surveillance

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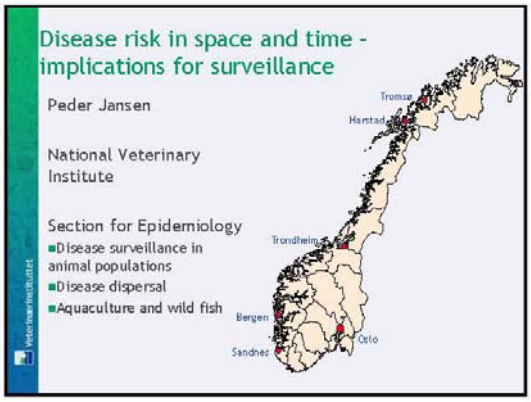
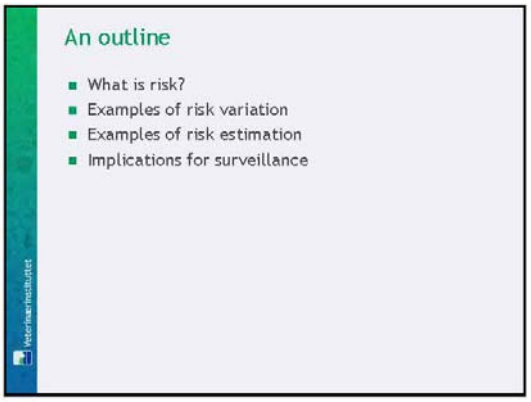
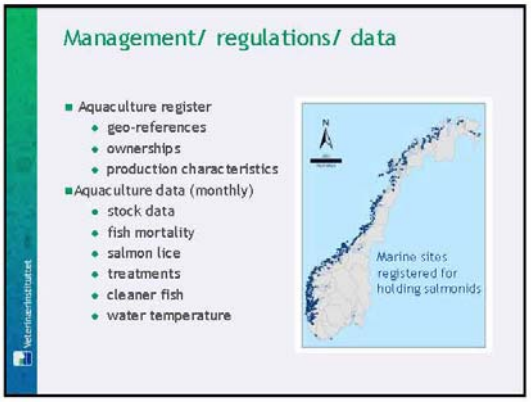
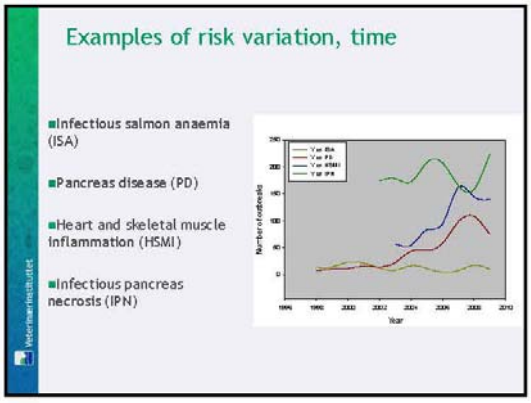
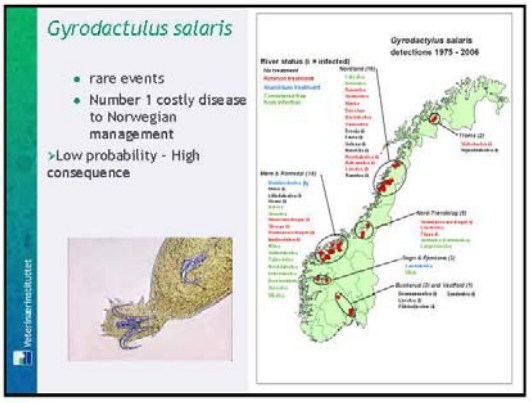
Presentation:

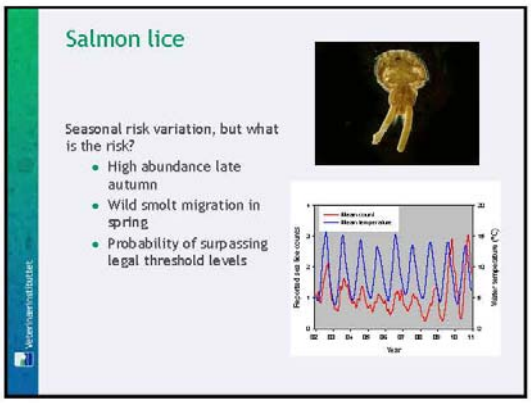


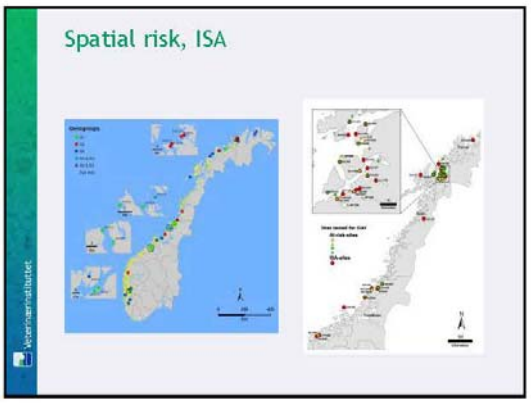


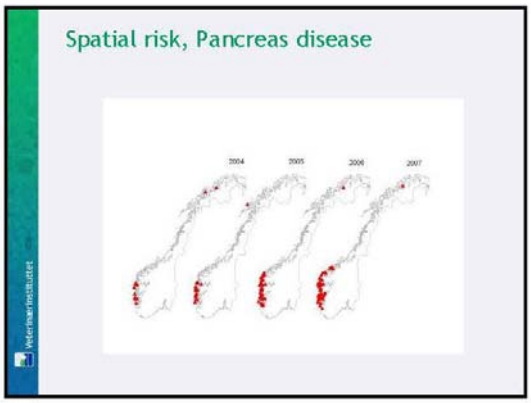


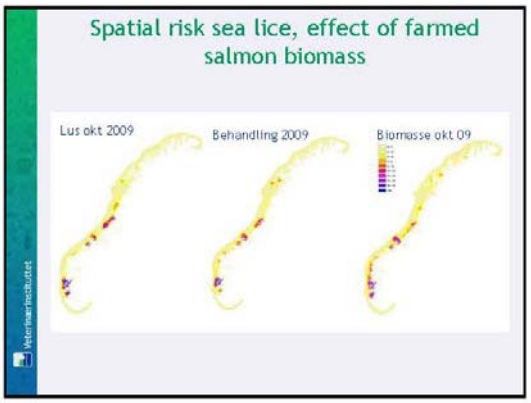

Examples of risk variation, time
Infectious salmon anaemia (ISA)
Pancreas disease (PD)
Heart and skeletal muscle inflammation (HSMI)
Infectious pancreas necrosis (IPN)
Year


Gyrodactulus salaris
rare events
Number 1 costly disease to Norwegian management
>Low probability - High consequence
Gyrodactylus salaris detections 1975 - 2006


Salmon lice
Seasonal risk variation, but what is the risk?
High abundance late autumn
Wild smolt migration in spring
Probability of surpassing legal threshold levels
Year


Spatial risk, ISA

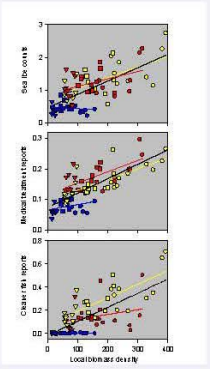

Spatial risk, Pancreas disease
2004
2005
2006
2007


Spatial risk sea lice, effect of farmed salmon biomass
Lus okt 2009
Behandling 2009
Biomasse okt 09

Spatial risk sea lice

- Density of farmed fish in the surroundings
  - affects transmission
- Fish size
  - exposure time and large surface for contact
- Temperature
  - lower lice transmission in the north

➢Increasing biomass density associated with high sea lice abundance and high treatment efforts

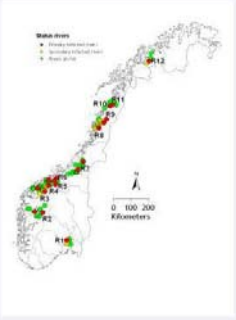


Gyrodactylus salaris spatial risk, importance of long jumps?

- *G. salaris* was introduced to a research fish farm on the west coast in the early 1970's
- Salmon juveniles were produced for cultivation purposes and stocked in salmon rivers
- Close to perfect match between stocked rivers and where the parasite originally was found

A model for this:

- Primary infected rivers (18), Johnsen et al. 1999
  - Stocking fish 9
  - Fishfarms 6
  - Fish transport 1
  - Unknown 2
- Secondary infected rivers (28)
- Rivers at risk (54), database on salmon rivers DN (population category > 2)

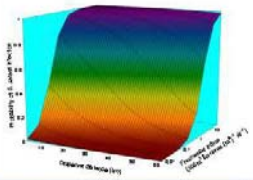


Model for secondary infected rivers:

Logit (*G.inf*) = $\alpha + \beta_1$freshwater inflow + $\beta_2$dispersal distance

Figure shows the probability of *G. salaris* infection in rivers as a function of freshwater inflow and dispersal distance

This model, combined with anthropogenic spread, explains it all?



Parameter	Estimate	Wald Chi-square	Wald 95% CI	p
Intercept (α)	3.779	13.21	(1.741; 5.817)	< 0.001
Dispersal distance	-0.082	6.48	(-0.146; -0.019)	0.011
Log_{10} freshwater inflow	3.923	9.93	(1.483; 6.364)	0.002

Risk estimation (modelling): What should things look like given.....?

If the parasite spreads through fjord systems on infected fish => expectations regarding the probability p(spread) of such dispersal?

G. salaris is a freshwater parasite:

- p(spread) related to freshwater inflow to fjords
- p(spread) related to distance between river outlets

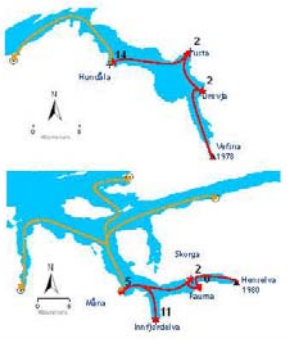
p(spread) could also depend on:

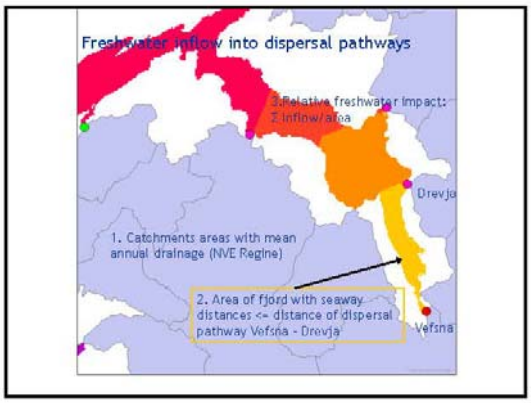
- The number of infected fish that migrates from infected rivers
- The amount of time a river is exposed for infection

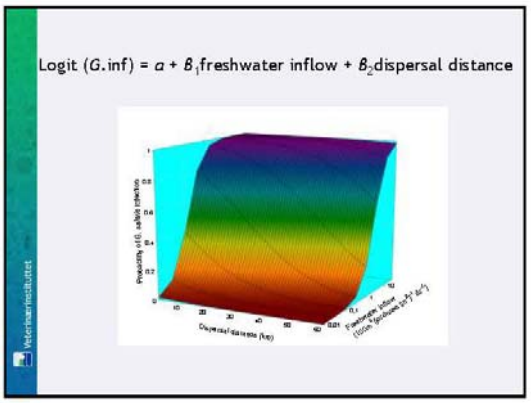
The trick is: how do you formalise your model into something testable, given data?

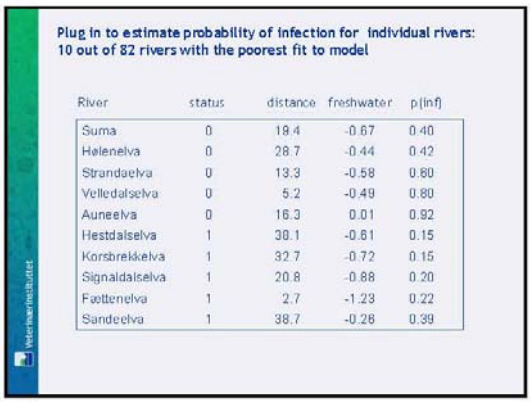
Model assumptions:
Dispersal from a source river - the closest infected river regarding seaway distance between outlets, and where infection was confirmed earlier than in the given secondary infected river

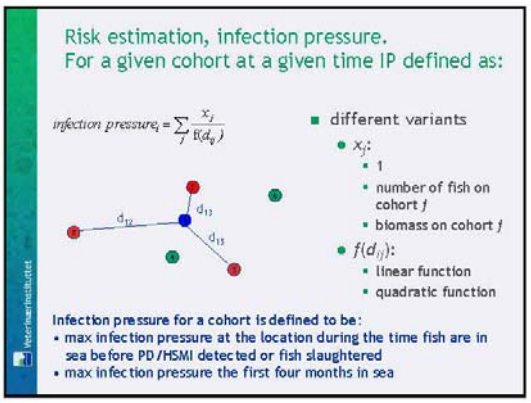
Dispersal pathway is the shortest path between outlets

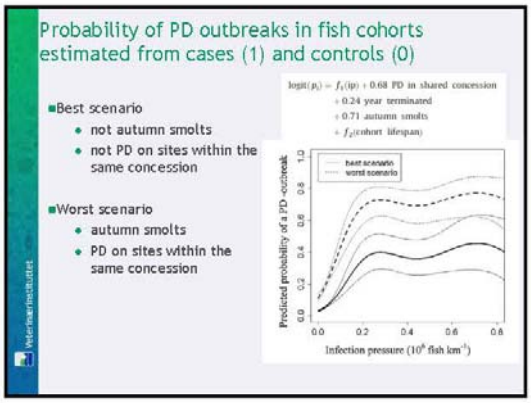


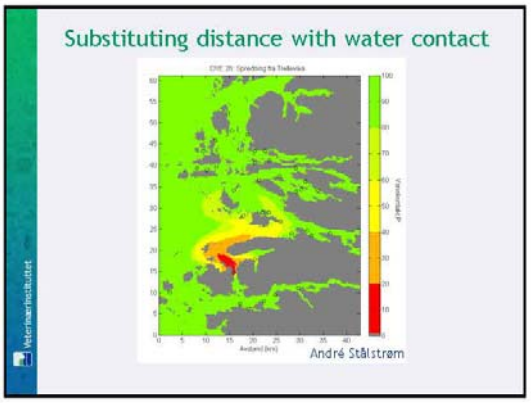

Freshwater inflow into dispersal pathways
3.Relative freshwater impact:
Σ inflow/area
Drevja
1. Catchments areas with mean
annual drainage (NVE Regine)
2. Area of fjord with seaway
distances <= distance of dispersal
pathway Vefsna - Drevja
Vefsna


Logit (G.inf) = α + β1freshwater inflow + β2dispersal distance


Plug in to estimate probability of infection for individual rivers:
10 out of 82 rivers with the poorest fit to model
River status distance freshwater p(inf)
Suma 0 19.4 -0.67 0.40
Hølenelva 0 28.7 -0.44 0.42
Strandaelva 0 19.3 -0.58 0.80
Velledalselva 0 5.2 -0.49 0.80
Auneelva 0 16.3 0.01 0.92
Hestdalselva 1 30.1 -0.61 0.15
Korsbrekkelva 1 32.7 -0.72 0.15
Signaldalselva 1 20.8 -0.88 0.20
Fættenelva 1 2.7 -1.23 0.22
Sandeelva 1 38.7 -0.28 0.39


Risk estimation, infection pressure.
For a given cohort at a given time IP defined as:
different variants
xj:
1
number of fish on cohort f
biomass on cohort f
f(dij):
linear function
quadratic function
Infection pressure for a cohort is defined to be:
max infection pressure at the location during the time fish are in sea before PD/HSMI detected or fish slaughtered
max infection pressure the first four months in sea


Probability of PD outbreaks in fish cohorts
estimated from cases (1) and controls (0)
Best scenario
not autumn smolts
not PD on sites within the same concession
Worst scenario
autumn smolts
PD on sites within the same concession
Predicted probability of a PD -outbreak
Infection pressure

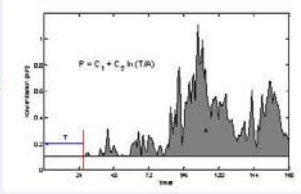

Substituting distance with water contact
André Stålstrøm

Estimation of relative water contact, P, from site *i* to site *j*

P: relative water contact (a small number indicates high contact)

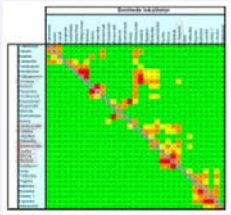
T: time from start discharge site *i* to the front hits site *j*

A: sum of the concentration that hits *j* over seven days

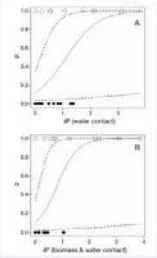


Disease model - infection pressure (IP) as the sum of exposures from infected neighbours

- IP from PD-site *j* at month *t*:
  - i) n_j/distance = number of fish on PD-site *j*/distance
  - ii) b_j/distance = biomass on PD site *j*/distance
  - iii) 1/distance
  - iv) 1/distance^2
- Distance:
  - Euclidean
  - Seaway
  - Water contact



Test of the power to explain cases and controls: water contact outperforms other measures of distance => supports passive spread of PD



The model has been useful in revising *G. salaris* surveillance:

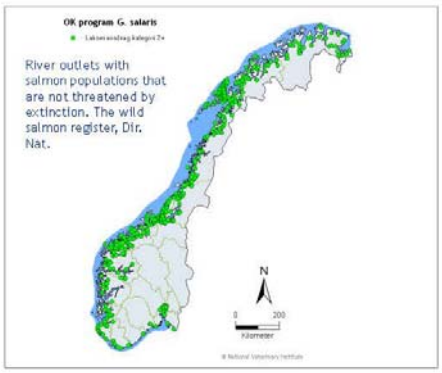
A risk based surveillance and control program for *G. salaris* in Norwegian rivers

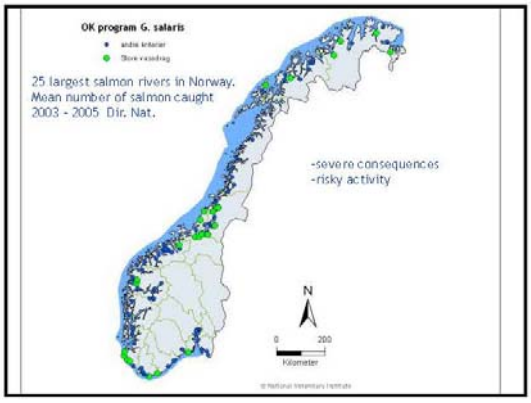
Aims for the surveillance program for *G. salaris*:

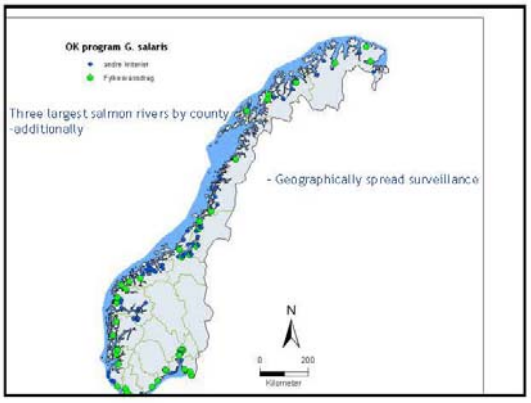
1. Document freedom from disease
2. Early detection of dispersal to new rivers

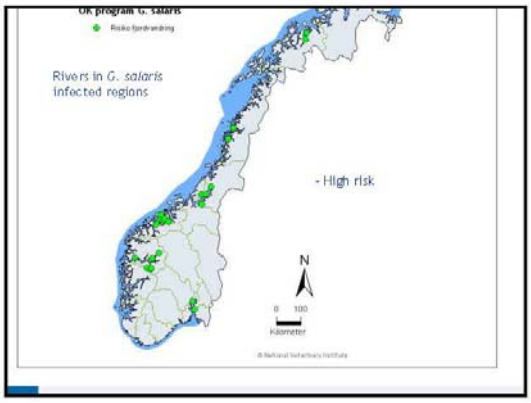
Risk based:

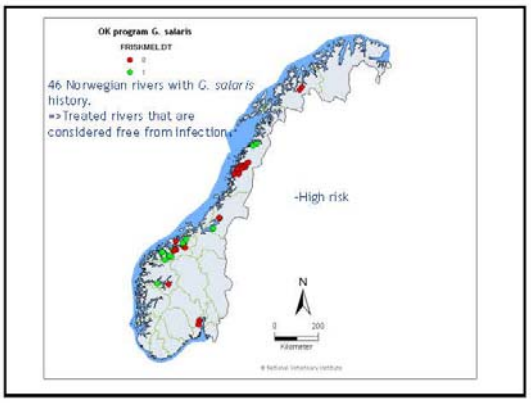
- Rivers where the probability of dispersal is relatively high
- Consequences are severe
- Surveillance must be geographically spread

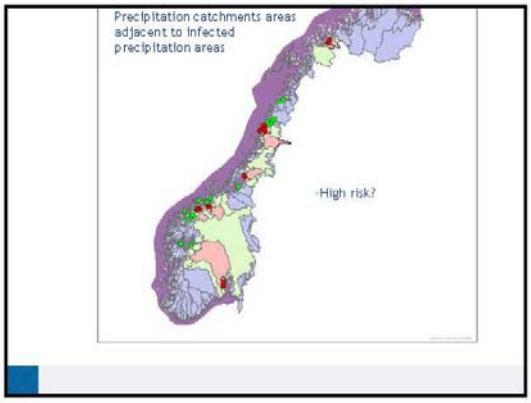


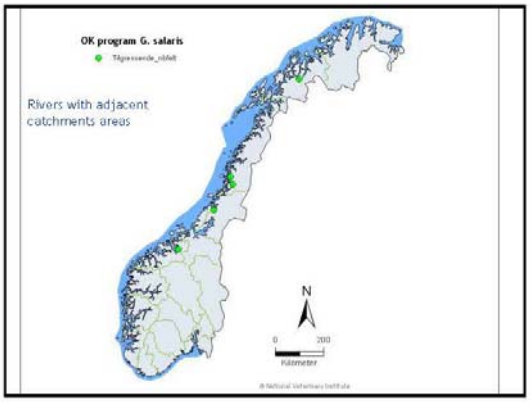

OK program G. salaris
25 largest salmon rivers in Norway.
Mean number of salmon caught
2003 - 2005 Dir. Nat.
-severe consequences
-risky activity
N
0 200
Kilometer


OK program G. salaris
Three largest salmon rivers by county
-additionally
- Geographically spread surveillance
N
0 200
Kilometer


OK program G. salaris
Rivers in G. salaris
infected regions
- High risk
N
0 100
Kilometer


OK program G. salaris
FRISKMELDT
46 Norwegian rivers with G. salaris
history.
=> Treated rivers that are
considered free from infection
-High risk
N
0 200
Kilometer


Precipitation catchments areas
adjacent to infected
precipitation areas
-High risk?


OK program G. salaris
Rivers with adjacent
catchments areas
N
0 200
Kilometer


Thank you for your attention!
Veterinærinstituttet

Use of molecular epidemiology in tracing disease

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Abstract:

Use of molecular epidemiology in tracing disease: Tracing transmission pathways of Infectious salmon Anaemia (ISA) virus

Trude M Lyngstad, Section of epidemiology, Norwegian Veterinary Institute, Oslo, Norway

Here, we present examples of using molecular epidemiology in tracing transmission pathways of Infectious Salmon Anaemia (ISA) virus. We aim to disentangle important transmission pathways of ISA virus by combining epidemiological and gene sequence data. The study examples are based on fish samples and epidemiological data from Norwegian salmon farms including farms with ISA and farms with apparently healthy fish. ISA virus was detected by Real Time RT PCR, and ISA virus segment 5 and 6 were characterized from positive samples.

References

Aldrin, M, Lyngstad TM, Kristoffersen AB, Storvik, B, Borgan Ø, Jansen PA, 2011. Modelling the spread of infectious salmon farms based on seaway distances between ISA virus isolates. Journal of the Royal Society Interface 00; 1-11.

Lyngstad T.M., Kristoffersen A.B., Hjortaas M.J., Devold M., Larssen R.B., Jansen P.A. Phylogeographic analyses of low virulent Infectious salmon anaemia (ISA) virus (HPR0) in farmed Atlantic salmon in Norway. In preparation.

Lyngstad, TM, Hjortaas, MJ, Kristoffersen, AB, Markussen, T, Karlsen, ET, Jonassen, CM & Jansen, PA, 2011. Use of molecular epidemiology to trace transmission pathways for infectious salmon anaemia virus (ISAV) in Norwegian salmon farming. Epidemics 3; 1-11.

Lyngstad TM, Jansen PA, Sindre H, Jonassen CM, Hjortaas MJ, Johnsen S, Brun E, 2008. Epidemiological Investigation of Infectious Salmon Anaemia (ISA) Outbreaks in Norway 2003-2005. Preventive Veterinary Medicine 84; 213-227

Economic aspects of surveillance programmes

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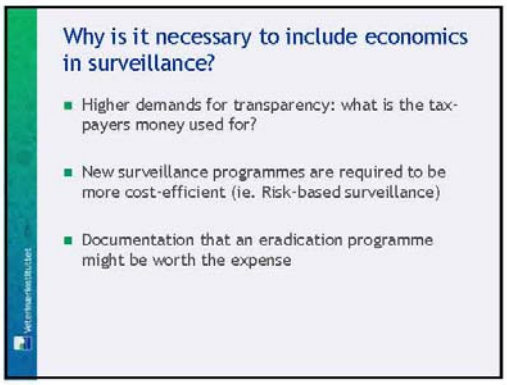
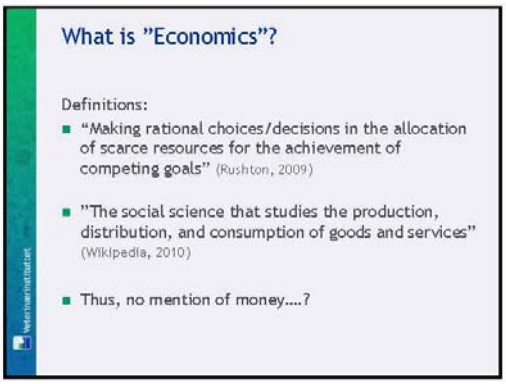
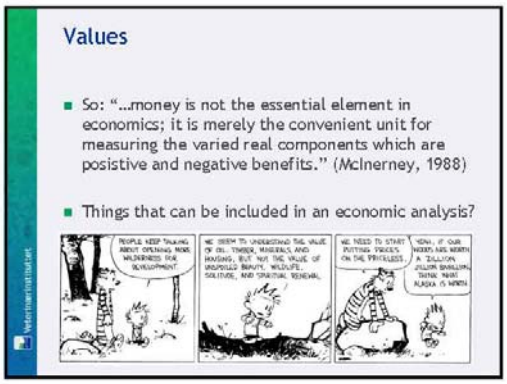
Presentation:





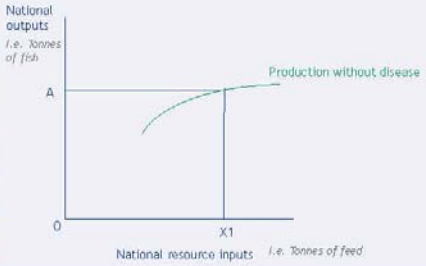


Economic versus financial

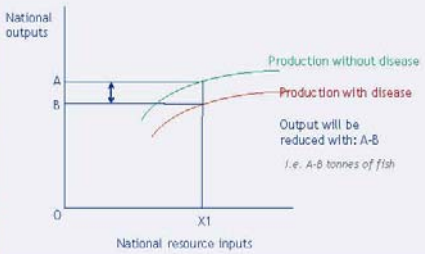
- So, economics includes much more than just monetary values, but we often try to transform values into money, so we can calculate and compare.
- For disease surveillance, a benefit of reducing disease can be increased animal welfare -but how do you measure that?

Lets look into disease costs, and how to measure that.

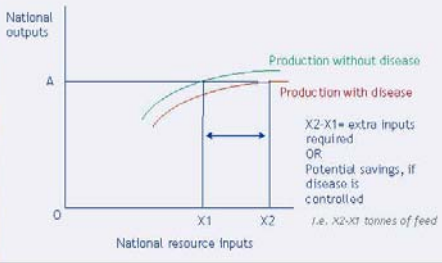
Production and disease relationship



Production and disease relationship



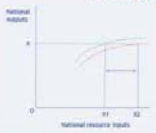
Production and disease relationship



Production and disease relationship

X2-X1= extra inputs required can be:

- Production inputs like feed, manhours, equipment
- Veterinary treatment



So, we can either:

- accept a loss in production
- Put in extra inputs

Or

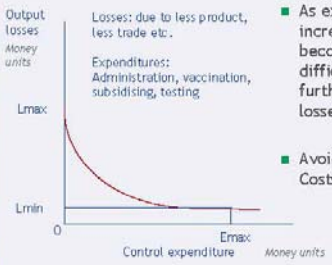
- Try to shift the curve back, by getting rid of the disease
- Avoid disease in the first place

Options

- Of course, shifting the curve back (=no disease), comes at a cost.
- Choosing between options:

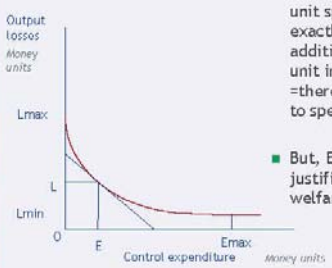
- Goal is to achieve the «Break-even»

Break-even -The loss-expenditure frontier



- Disease cost=L+E
- As expenditure increase, it becomes more difficult to get a further reduction in losses
- Avoidable Costs=Lmax-Lmin

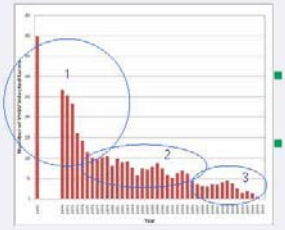
Break-even



- The break-even is achieved, when an additional money unit spent earns exactly the same additional money unit in return =there is no reason to spend it.
- But, Emax can be justified out of i.e. welfare concerns

How does this look in real-life?

- Control of VHS in Denmark

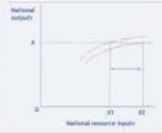


- At 1 few measures necessary to reduce disease
- At 2 The same measures, plus more
- At 3 Level for disease control with current techniques was achieved

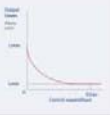
Benefits and costs of surveillance programmes

- Control of VHS in Denmark

- By increasing expenditure, it was possible to push the curve back
- The CA decided, that the extra costs would be worth the extra outputs achieved by gaining disease-free status.



- In this case, Emax was justified



Benefits and costs of surveillance programmes

- Economic justification on a national level is different from that on farm level
- Resposibility towards the community (both inside and outside country borders):
  - Keep industry going
  - Mind animal welfare
  - Consider consumers
  - Remember trade options

Different systems for illustrating and analysing options

- Benefit-cost analysis
- Decision tree analysis
- Partial budgeting
- Gross margin analysis
- Simulation models
- Etc.

Examples of applications:

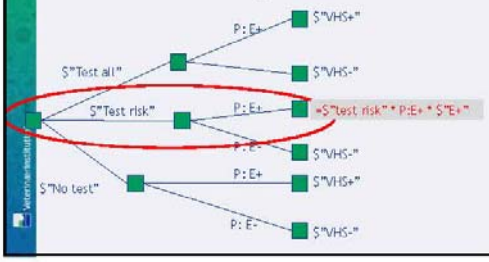
Benefit-cost analysis

- Vaccination for Pancreas Disease on a national level:
- Benefits: Minimise losses, less impact on animal welfare
- Costs: Vaccine, administration
- If B/C>1, do, if B/C<1, don't
- Industry advised to do, so they did. Benefit to the whole industry.

Examples of applications:

Decision tree analysis:

- Control of VHS in Norway:



In summary:

- It is important to consider economic aspects of surveillance
  - For transparency
  - To make rational decisions
- Economic sense is to aim for the Break-even
- Sometimes, it can be justified to spend more
- Commonly, Benefit-cost analysis are used
- Other methods exists
- The question is: What is the true cost of disease?

Thank you for the attention

Workshop wrap-up

At the end of the second day, we tried to draw up the "take-home messages" from the workshop, based on the topics that had been discussed most.

These included:

Incentives: It is important to understand the incentives for implementing surveillance, for implementing biosecurity or for developing eradication programs both from farmers, competent authorities and scientists.

Fit for purpose: It is crucial to understand that the appropriate regime including sampling, testing etc., depends on the purpose.

Definition of risk: There are at least two different definitions of risk: Probability of an event occurring or probability of an event is happening combined with the consequences of the event. When talking about and working with risk, it is important to know which definition is being used.

Council Directive 2006/88/EC: Specifically, the directive needs to cover many different animal species, in many different areas and administrative units and with many different types of APBs (intensive, semi-intensive, extensive, large and small scale etc.). Therefore, the Directive is using a more general approach. But the individual Member States are free to modify, as long as they apply to the basic instructions.

Risk based surveillance: Knowledge on risk factors is necessary for design of surveillance systems. Tracing of pathogens / spread of disease are important tools for detecting risk factors, and should be included in modeling of risk factors for surveillance purposes.

Farmer reporting: The inherent systems for reporting could be better used when designing surveillance programs etc. But again, the incentives need to be clear.

Aquatic epidemiology: Aquatic epidemiologists should be involved in fish meetings/issues –this should be recognized by the CA. There is a need for education of aquatic epidemiologists. The OIE collaborating centre can help with that (http://eraaad.ca/)

Evaluation of workshop

In the week immediately after the workshop, an evaluation was carried out, using an e-survey. 30 participants responded (response rate 60%). The questions and answers are presented below.

1. Was the information given prior to the workshop appropriate?

Too little	Appropriate	Too much	Total
1	26	3	30

Comments to Q1:

- A short abstract for the talks would have been helpful (but not completely necessary)
- The reading material should have been distributed earlier

- It was very good having documents/scientific papers before the workshop in order to be able to have an idea of the major topics

2. Was the program appropriate?

Not at all	Too few topics	Appropriate	Too many topics	Total
0	2	23	5	30

Comments to Q2:

- Given the short time of 2 days, it might have been good to focus on a limited number of aspects of epidemiology, or to have longer time.
- I hope it would be the first one of a series of workshop in order to have the possibility to go more in depth or the different topics and to have more time to think about them

3. How did the reading material fit with the programme?

Not at all	Bad	Well	Very well	Total
0	4	18	8	30

Comments to Q3:

- The background reading was very helpful, and a good amount

4. How much did you get out of the group work?

Group	Nothing	A little	Appropriate	A lot	Total
Screening strategies & tracing of pathogens	0	2	4	3	9
Special challenges regarding surveillance in shell & molluscs	0	0	1	1	2
Design of surveillance programmes	0	2	3	6	11
Risk factors and risk categorization	0	3	4	1	8

5. How did the workshop live up to your expectations?

Not at all	A little	Appropriate	Better	Much better	Total
0	3	11	11	5	30

Comments to Q5:

- There could have been more about the diseases and risks concerning a few diseases as examples
- Too short
- It might be good for the next time to give Caraguel and Cameron more time for their parts while the case studies on the second day could be shorter

- This was the first time we have a joint workshop on the topic, so for me it feels natural that both programs and content was a bit "spiky".
- I was hoping for more/longer group work time.
- I loved it / it was very interesting and helpful
- New practical knowledge to be directly implemented

6. What was the worst part of the workshop?

- Group work time was much too short and group work organization was a bit chaotic (at least in our group)
- Short duration
- The location
- In the group work sessions we were divided into very small groups but I feel it would have been better to have more time discussing with the whole group work
- The rush presentations, a lots of slides skipped. Not all the presentations were given on paper
- I was forced to attend the workshop on my own support, but it was worth it.
- Too little time for preparing the tasks in the practical part.
- I think it was superbly organized. The worst part was that the EU requires a copy of the boarding pass, which is impossible for us who travel with an electronic
- The end of workshop was the worst part of the workshop.
- In my workgroup there were few people interested and therefore they didn't participate to the discussion

7. What was the best part of the workshop?

- In general, I got a good overview over the relevant topics, and I liked that most talks were interactive.
- Opportunities to discuss practical challenges with participants
- Targeted audiences
- The group work (4 respondents)
- Cameron's presentations (5 respondents)
- The talks by the delegates from Switzerland and Germany on their risk ranking
- Dr. Lyngstads presentation (1 respondent)
- Dr. Bang Jensens presentation (2 respondents)
- Dr. Caraguels presentation (2 respondents)

8. Do you have any suggestions for future workshops?

- A future workshop should be more focused, longer (4 days), fewer presenters, more consistent and in depth treatment of the topics. Based on most of the participants' apparent level of experience, a 'training workshop' rather than 'workshop' would be more useful (4 respondents)
- More time for group work / more time for discussion

- An element of future workshops should be to review the exchange of communications / improvements member states have made since previous workshops - the workshops should continue to be about networking and dialogue as well as elements of training in tools of epidemiology
- It would be useful to have a rotation of the participants to all the working groups. It was hard to choose (2 respondents)
- More focus to each member states specific problems
- It would be good to have more time spent discussing solutions rather than just problems, so people could gain ideas to implement in their countries
- Perhaps is better to give more time reserved to presenters and presentations and also it will be nice to have all the presentation on paper
- More practical training concerning fish farming and factors affecting early detection of diseases. To learn how to motivate fish farmers and competent authority to make inspections in practice. Field courses arranged in different part of EU regarding type of fish farming practices.
- In future workshops, there should be three topics: Educational (basic epidemiology and statistics), an important focus area (whatever is important in the EU at the moment), good examples of solutions or approaches
- A continuation to this one would be great
- Better selection of participants

9. Do you have any other feedback you would like to share?

- The workshop was very well organized and planned. (4 respondents)
- My overall impression is good and I thank for the invitation and the opportunity that I have heard some interesting lectures and I have met colleagues and also some new scientists in this field.
- The workshop offered a good introduction into a complex and broad field.
- I think that every Institute who deals with aquaculture should give a name of an epidemiologist who should take part to these workshops. Then a mailing list could be created.
- Keep up the good work. Thank you for your hospitality (2 respondents)
- Nice workshop, but not much new for me personally. I was surprised there were so many with very much experience. I think the workshop was planned for people that have little experience or have just started working in this field.
- Venue was good - nice refreshments and lunches. Coming from a non-aquatics background, I found the delegates very friendly and welcoming, and it was a great opportunity to get to know a few people working in an area in which I am becoming increasingly involved in.

References

Cameron, A. (2002) Survey Toolbox for Aquatic Animal Diseases. A Practical Manual and Software Package. Recommended reading: P.61-66 of chapter 4 & entire chapter 5. Link: http://www.ausvet.com.au/resources/AquaToolbox.pdf (Background for the presentation: “Basic concepts in sampling and testing for aquatic diseases)

Commission Decision 2008/896/EC of 20 November 2008 on guidelines for the purpose of the risk-based animal health surveillance schemes provided for in Council Directive 2006/88/EC. Official Journal of the European Union, 322, 30-38 (Background for presentations and groupwork on risk categorization)

Council Directive 2006/88/EC of 24 October 2006 on animal health requirements for aquaculture animals and products thereof, and on the prevention and control of certain diseases in aquatic animals, Official Journal of the European Union, 328 (2006), 14-56. Recommended reading: Chapter II, V, VI and annex III. 38 (Background for presentations and groupwork on risk categorization)

Oidtmann, BC, Crane, CN, Thrush, MA, Hill, BJ, Peeler, EJ (2011) Ranking freshwater fish farms for the risk of pathogen introduction and spread. Preventive Veterinary Medicine, 102, 329-340. (Especially for the group: Risk factors and risk categorization)

Stentiford, GD, Bonami, J-R, Alday-Sanz, V (2009) A critical review of susceptibility of crustaceans to Taura syndrome, Yellowhead disease and White Spot Disease and implications of inclusion of these diseases in European legislation. Aquaculture, 291, 1-17 (Especially for the group: Special challenges regarding surveillance in shellfish and molluscs)

Stentiford, GD, Oidtmann, B, Scott, A, Peeler, EJ (2010) Crustacean diseases in European legislation: Implications for importing and exporting nations. Aquaculture, 306, 27-34 (Especially for the group: Special challenges regarding surveillance in shellfish and molluscs)

Stärk, KDC, Regula, G, Hernandez, J, Knopf, L, Fuchs,K, Morris, RS, Davies,P (2006) Concepts for risk-based surveillance in the field of veterinary medicine and veterinary public health: Review of current approaches. BMC Health Services Research, 6,20 (Supplement for presentations and groupwork on surveillance)

Closing remarks

Since the implementation of the Council Directive 2006/88/EC with new demands concerning authorisation, health categorisation and risk based surveillance in aquatic animal production units, a significant need for upgrading knowledge and harmonisation has been recognised by the EU Reference Laboratories for aquatic animals. The opportunity arose when the OIE collaborating centre for Aquatic Epidemiology and Risk Assessment was established offering superb knowledge and teaching opportunities. Thus with the sustaining of the European Commission, the EURL fish was, in collaboration with the OIE reference laboratory, given the chance to organise this workshop in epidemiology and risk assessment. It was apparent, however, that the number of topics to be covered was very large in relation to the time given. Thus the workshop should rather be recognised as an introduction to the field rather than a full training course. The comprehensive teaching material distributed prior to the workshop together with all the presentations given in the report should provide the participants with sufficient knowledge and inspiration to implement risk based surveillance schemes in their home country, as most participant were selected based on their working areas, skills and field of interests.

Hopefully this workshop is the first of a series of courses in epidemiology and risk assessment in aquatic animals that will provide a strong scientific background for expanding epidemiology in aquatic animals in Europe.

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And finally but not least Dr. Britt Bang Jensen, NVI, for her very competent and efficient organisation of this workshop including the program, contacts with all speakers, report writing etc. and for keeping us all busy and in time for the workshop.

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