*EURL for Fish Diseases*

# Report of the Inter-Laboratory Proficiency Test 2010

# for

# identification of VHSV, IHNV and EHNV (PT1)

# and

# identification of KHV and ISAV (PT2)

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DTU

## Table of contents

## Introduction

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

This report covers both the results of PT1 and PT2.

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

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

this test, but the viruses has not been inactivated and should thus be viable and possible to amplify in cell cultures.

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

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

**Distribution of the test**

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

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

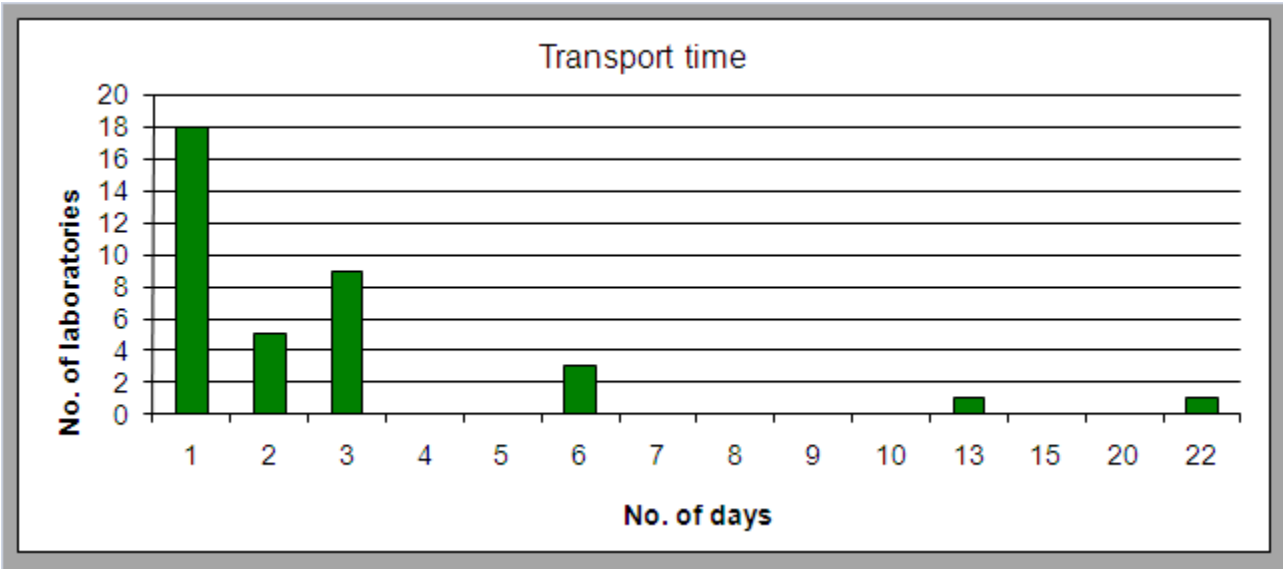
In parcels that had to go through longer transport time, cooling elements were included.

*Shipment and handling*

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

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

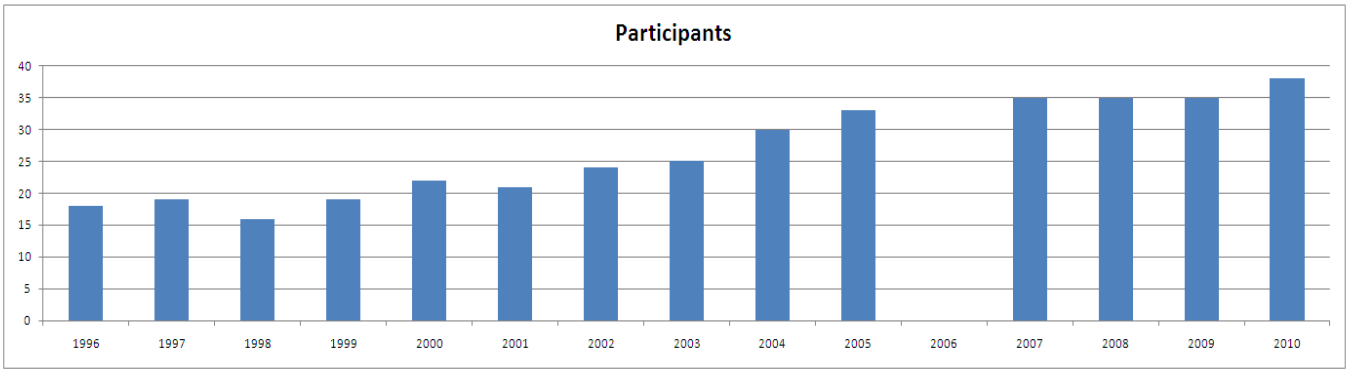
**Figure 1.** Transport time for the parcel to reach the participants.




*Participation*

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

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



## Proficiency test 1, PT1

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

## Content of ampoules

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

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

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

| PT1 | |
|---|---|
| **Code** | **Specifications** |
| **Ampoule III: European Catfish Virus (ECV)** | European catfish virus 562/92.<br><br>Italian isolate from catfish suffering high mortality.<br>Received from Dr. G. Bovo, ISZ-Ve, Padova, Italy.<br>Cell culture passage number: 6.<br><br>Reference:<br>Bovo G, Comuzi M, De Mas S, Ceschia G, Giorgetti G, Giacometti P & Cappellozza E (1993). Isolamento di un agente virale irido-like da pesce gatto (Ictalurus melas) dallevamento. Bollettino Societa Italiana di Patologia Ittica 11, 3–10.<br><br>GenBank accession number:<br>(FJ358606) |
| **Ampoule IV: SVCV** | SVCV 56/70.<br><br>Isolate from carp.<br>Received from Prof. Fijan (January 1979 in a tube named Rhabdo virus carpio 56/70 and given as the reference strain of SVC virus).<br>Cell culture passage number: Unknown.<br>Genotype Id (Stone et al. 2003).<br><br>The isolate is most likely identical to the S/30 isolate described in<br>Fijan N, Petrinec Z, Sulimanovic D & Zwillenberg LO (1971) Isolation of the viral causative agent from the acute form of infectious dropsy of carp. *Veterinarski Archiv* **41,** 125-138.<br><br>Reference:<br>Stone DM, Ahne W, Denham KL, Dixon PF, Liu C-TY, Sheppard AM, Taylor GR & Way K (2003). Nucleotide sequence analysis of the glycoprotein gene of putative spring viraemia of carp virus and pike fry rhabdovirus isolates reveals four genogroups. *Diseases of Aquatic Organisms* **53**, 203-210.<br><br>GenBank accession numbers:<br>Z37505.1 (S30)<br>AJ538061.1 (Fijan) |
| **Ampoule V: VHSV** | VHSV DK-5151 (Rindsholm, 1992)<br><br>Danish freshwater VHSV isolate from rainbow trout.<br>Cell culture passage number: 4 in BF-2 and 6 in EPC.<br>Neutralization pattern III (Olesen et al. 1993).<br>Genotype Ia (Ejner-Jensen et al. 2004).<br><br>References:<br>Olesen NJ, Lorenzen N and Jørgensen PEV (1993). Serological differences among isolates of viral haemorrhagic septicaemia virus detected by neutralizing monoclonal and polyclonal antibodies. *Diseases of Aquatic Organisms* **16**, 163-170.<br><br>Einer-Jensen K, Ahrens P, Forsberg R and Lorenzen N (2004). Evolution of the fish rhabdovirus viral haemorrhagic septicaemia virus. *Journal of General Virology* **85**, 1167-1179.<br><br>GenBank accession number:<br>AF345859.1<br><br>FishPathogens report number:<br>2218 |

**Testing of the PT1 test**

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

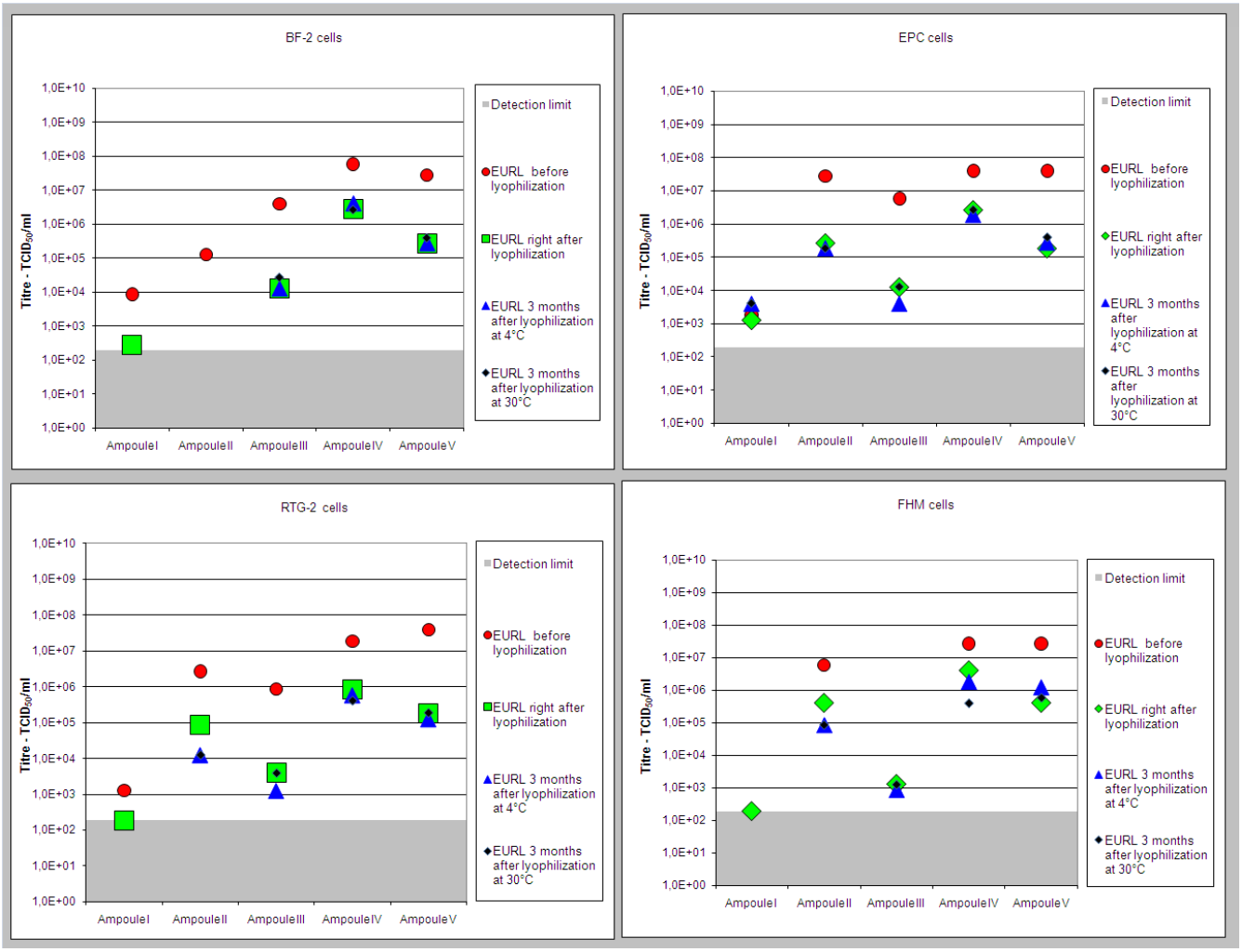
The lyophilisation procedure caused a significant titre reduction, especially for IHNV on BF-2 cells where a 3 log reduction was observed. For the other cell lines a reduction between 1 - 2 log for IHNV was observed. For EHNV a titre reduction between 0 - 1,5 log, for ECV a titre reduction between 0-3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

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

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

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

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



**Virus identification and titration**

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

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

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

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

| Laboratory code number | Score 10/10 | Answer received at EURL | Ampoule I | Ampoule II | Ampoule III | Ampoule IV | Ampoule V |
|---|---|---|---|---|---|---|---|
| | | | EHNV | IHNV | Rana not EHNV or ESV-ECV | SVCV | VHSV |
| | | | IHNV, IPNV and SVCV | | | | |
| 19 | 10 | 26-11-10 | EHNV | IHNV | ECV | SVCV | VHSV |
| 20 | 8 | 29-11-10 | EHNV | IHNV | EHNV | SVCV | VHSV |
| 21 | 10 | 26-11-10 | EHNV | IHNV | ECV or ESV | SVCV | VHSV |
| 22 | 8 | 23-11-10 | Rana | IHNV | Rana | SVCV | VHSV |
| 23 | 10 | 15-11-10 | EHNV | IHNV | ECV | SVCV | VHSV |
| 24 | 10 | 29-11-10 | EHNV | IHNV | Ranavirus not EHNV | SVCV | VHSV |
| 25 | 10 | 22-11-10 | EHNV | IHNV | Iridovirus not EHNV | SVCV | VHSV |
| 26 | 8 | 23-11-10 | EHNV | IHNV | ESV | IHNV/ SVCV | VHSV |
| 27 | 2 | 03-12-2010 * | Virus not found | IHNV | Virus not found | VHSV/ IPNV | Virus not found |
| 28 | 10 | 26-11-10 | EHNV | IHNV | Iridovirus (sheetfish and catfish) | SVCV | VHSV |
| 29 | 8 | 29-11-10 | IHNV/EHNV | IHNV | ESV or ECV | SVCV | VHSV |
| 30 | 10 | 29-11-10 | EHNV | IHNV | Ranavirus (Sheetfish iridovirus) | SVCV | VHSV |
| 31 | 4 | 29-11-10 | VHSV/EHNV | IHNV | EHNV | SVCV | Virus not found |
| 32 | 10 | 26-11-10 | EHNV | IHNV | ECV/ESV | SVCV | VHSV |
| 33 | 6 | 22-11-10 | Virus not found | IHNV | EHNV | SVCV | VHSV |
| 34 | 4 | 29-11-10 | Virus not found | IHNV | EHNV | SVCV | Virus not found |
| 36 | 4 | 29-11-10 | Virus not found | Virus not found | EHNV | SVCV | VHSV |
| 37 | 10 | 23-11-10 | EHNV | IHNV | Ranavirus: ESV / ECV | SVCV | VHSV |
| 38 | 10 | 26-11-10 | EHNV | IHNV | Ranavirus (ECV or ESV or BIV) | SVCV | VHSV |
| 39 | 10 | 06-12-10 | EHNV | IHNV | Ranavirus (sheet fish, doctor fish and catfish iridovirus) | SVCV | VHSV |

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

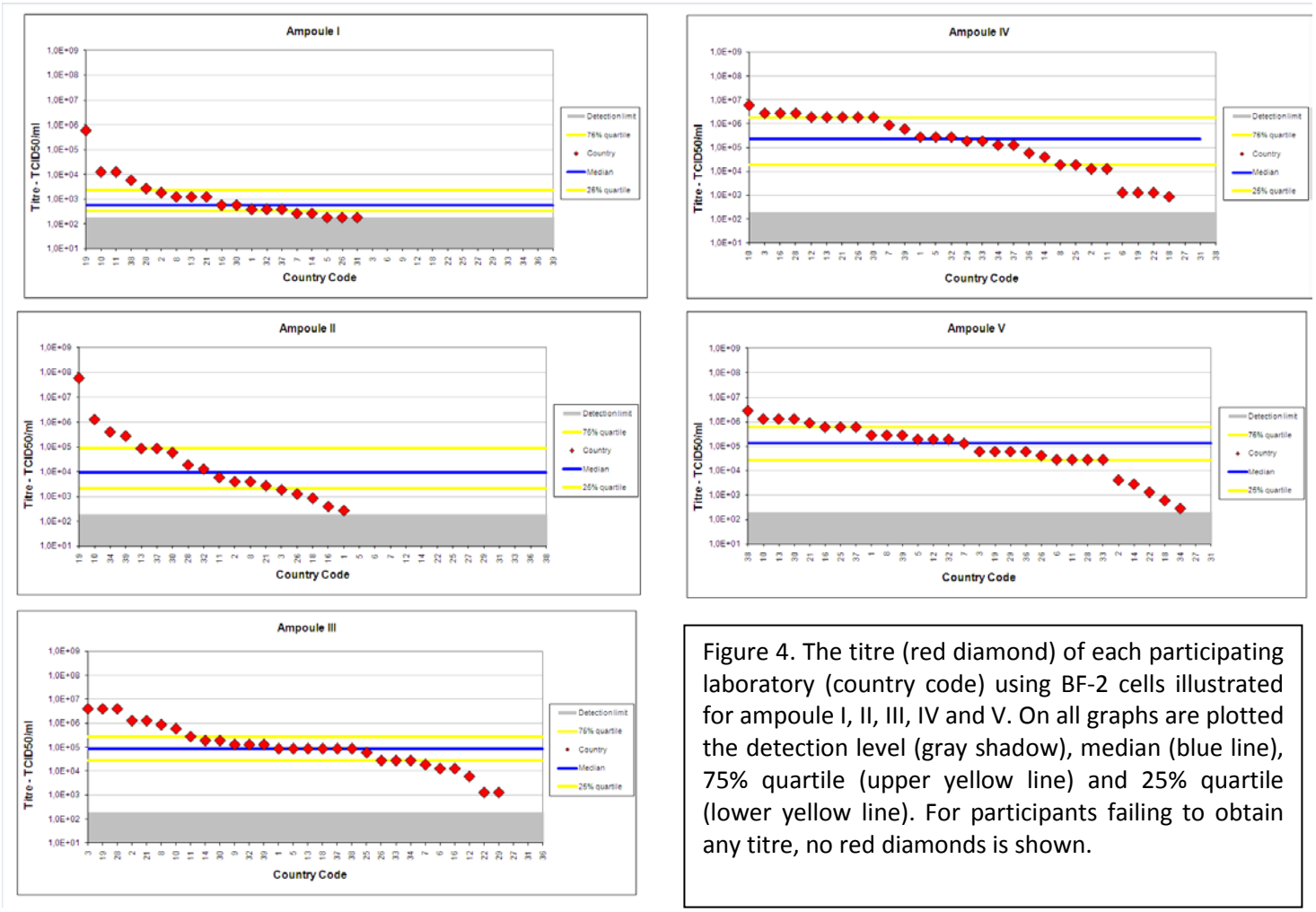



Figure 4. The titre (red diamond) of each participating laboratory (country code) using BF-2 cells illustrated for ampoule I, II, III, IV and V. On all graphs are plotted the detection level (gray shadow), median (blue line), 75% quartile (upper yellow line) and 25% quartile (lower yellow line). For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

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

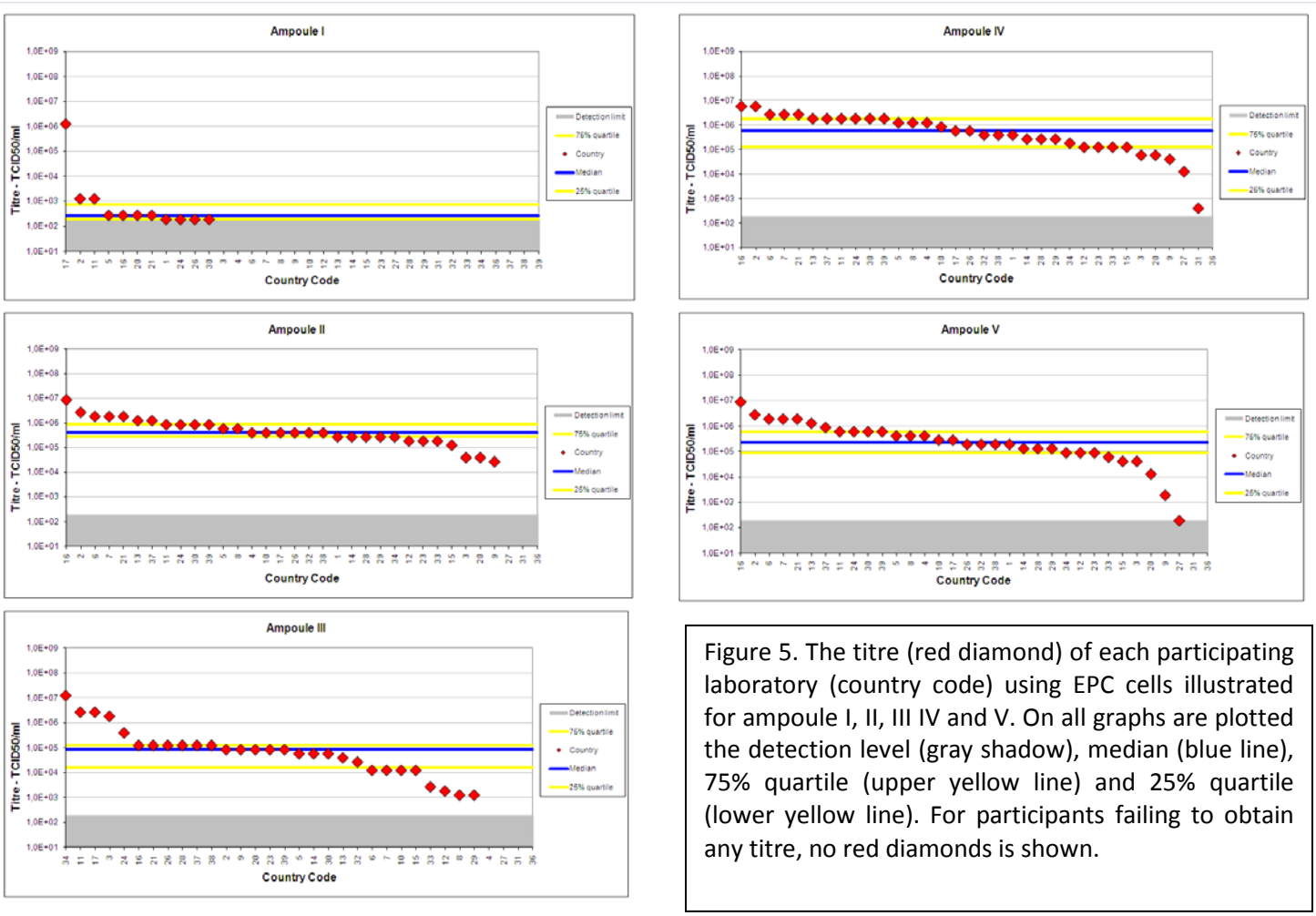



Figure 5. The titre (red diamond) of each participating laboratory (country code) using EPC cells illustrated for ampoule I, II, III IV and V. On all graphs are plotted the detection level (gray shadow), median (blue line), 75% quartile (upper yellow line) and 25% quartile (lower yellow line). For participants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

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

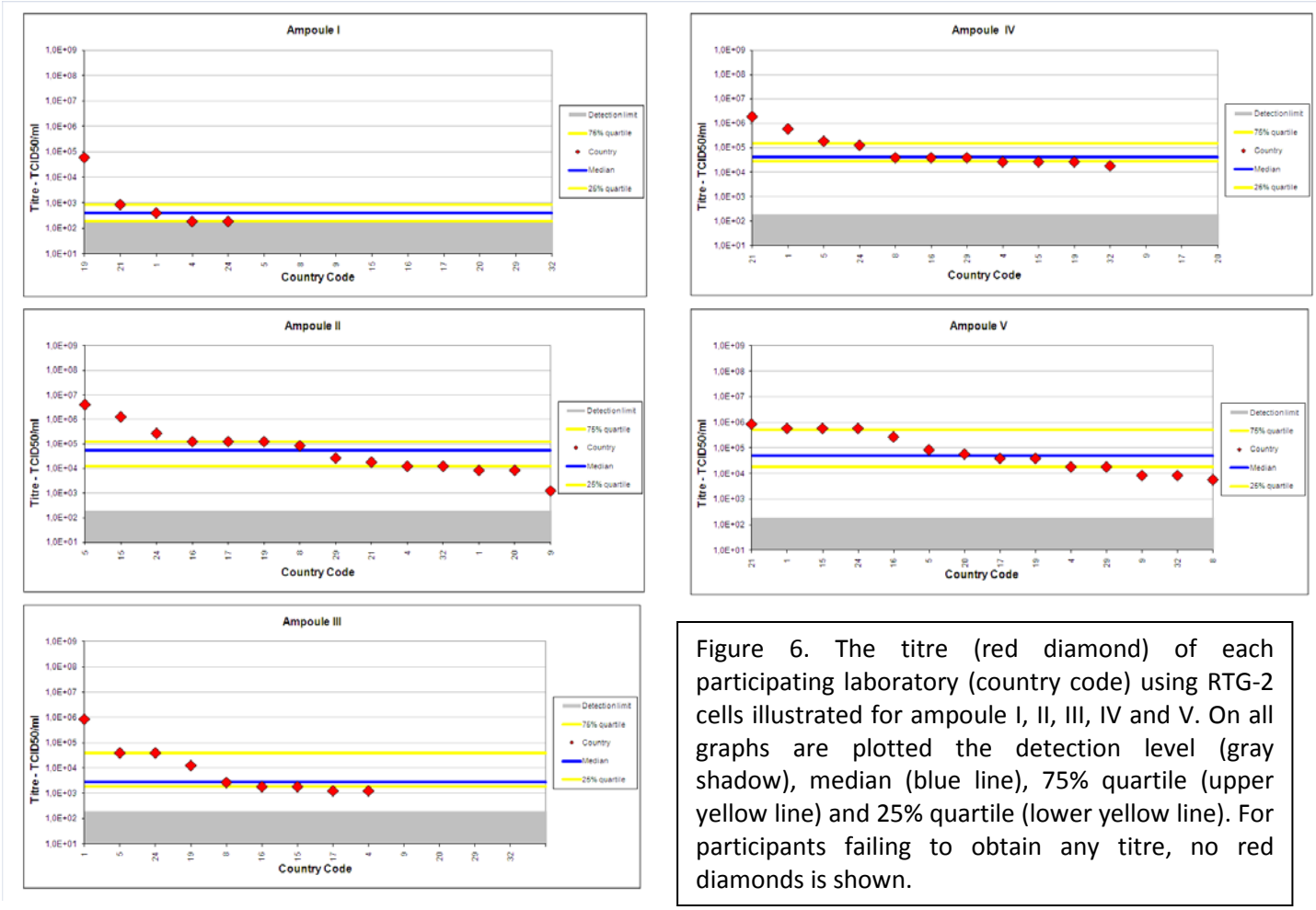



Figure 6. The titre (red diamond) of each participating laboratory (country code) using RTG-2 cells illustrated for ampoule I, II, III, IV and V. On all graphs are plotted the detection level (gray shadow), median (blue line), 75% quartile (upper yellow line) and 25% quartile (lower yellow line). For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

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

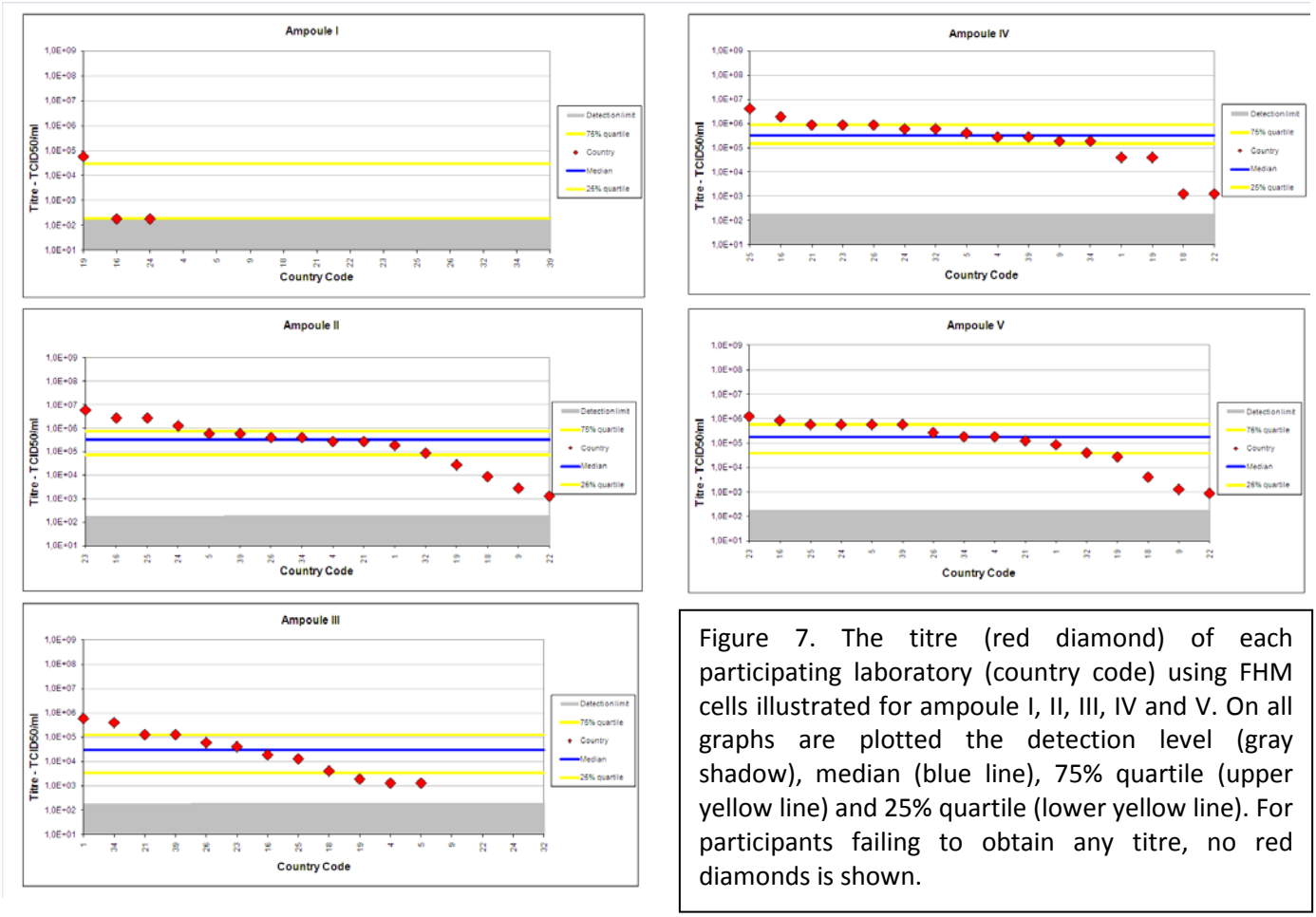



Figure 7. The titre (red diamond) of each participating laboratory (country code) using FHM cells illustrated for ampoule I, II, III, IV and V. On all graphs are plotted the detection level (gray shadow), median (blue line), 75% quartile (upper yellow line) and 25% quartile (lower yellow line). For participants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

*Identification of content*

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

Ampoule I – EHNV

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

Ampoule II - IHNV

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

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

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

Ampoule IV – SVCV

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

Ampoule V – VHSV

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

*Scores*

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

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

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

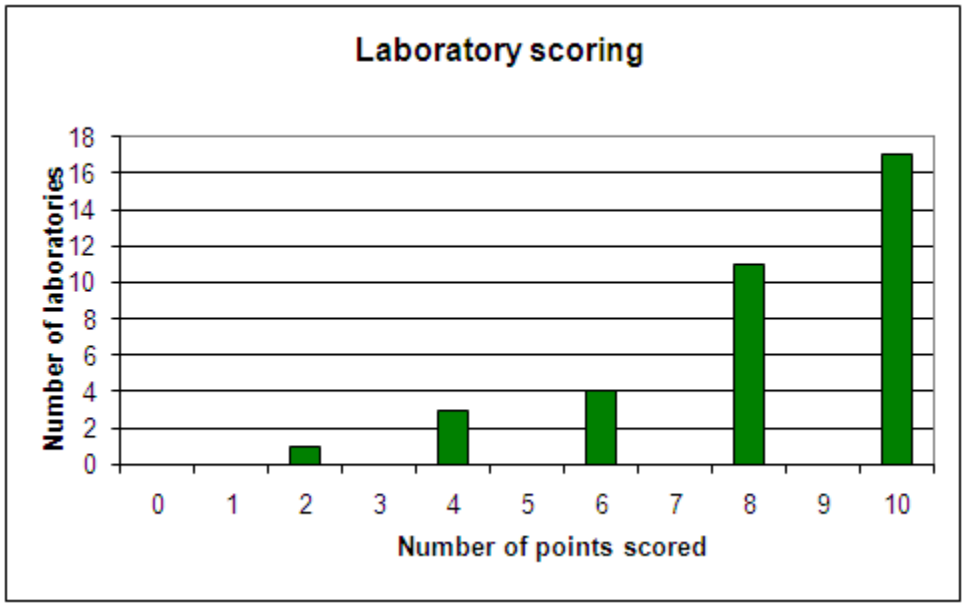
Ampoule IV: Identification of SVCV was given the score 2 and identification of virus as "not VHSV, IHNV or EHNV" was given the score 1.

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

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

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

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



*Methods applied*

The following cell lines were used by the participants:

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

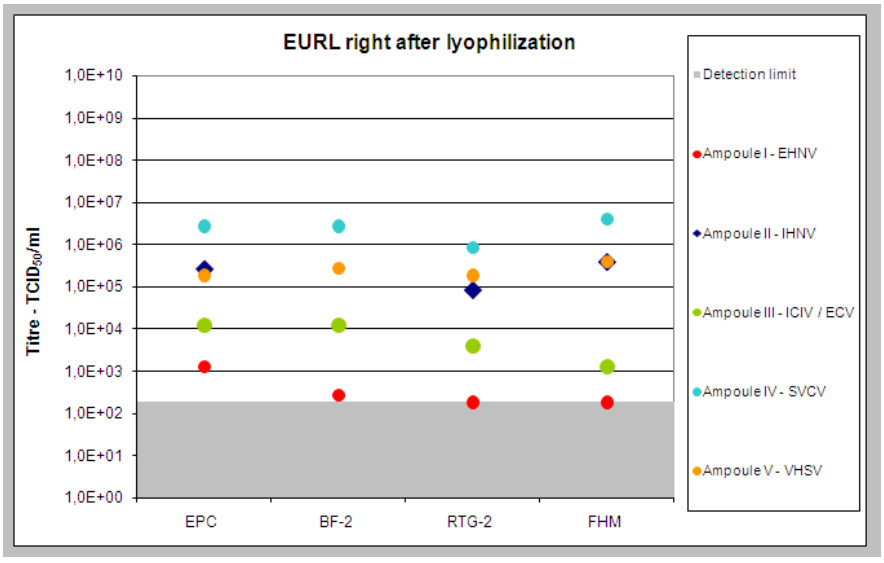
- 23 laboratories used two cell lines:
    - 17 laboratories used BF-2 cells in combination with EPC cells
    - 3 laboratories used RTG-2 cells in combination with EPC cells
    - 3 laboratories used BF-2 cells in combination with FHM cells
    - 1 laboratory used EPC cells in combination with FHM cells

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

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

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

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



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

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

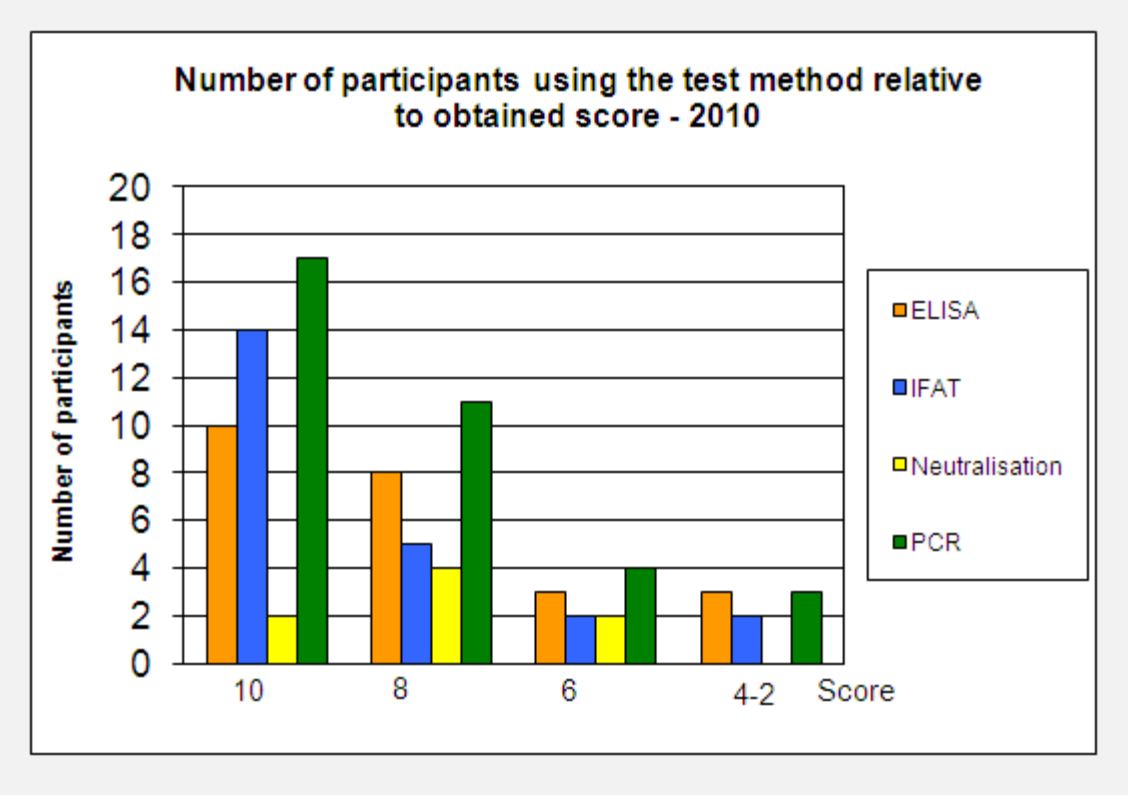
**Table 9.** Results obtained by different test methods in participating laboratories.

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

A graph was constructed to illustrate the association between the methods used by participants for virus identification and the obtained score (Figure 10). The PCR is the most frequently used method by participants and only three participants did not use this method. For participants scoring lower than 10, the deficiency in virus identification cannot directly be assigned to improper use of a single

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

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




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

Ampoule I – EHNV and III – ECV/ESV

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

Within the OIE diagnostic manual for EHN, two sequence based methods are recommended to use in order to discriminate EHNV from the other non-listed ranaviruses. A total of 30 laboratories used sequencing analyses for identification of EHNV whereas one used RFLP. All laboratories performing sequencing of the isolate in ampoule I, identified the virus correctly as EHNV. However, 7 laboratories that performed sequencing of the ECV isolate in ampoule III identified the virus as EHNV. This was even though that the submitted sequences were in 6 cases identical to ECV/ESV.

Ampoule II - IHNV Genotype M

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

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

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

Ampoule IV - SVCV genotype Id

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

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

Ampoule V - VHSV genotype Ia

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

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

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

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

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

| Laboratory code number | Score 10/10 | Ampoule I | Ampoule II | Ampoule III | Ampoule IV | Ampoule V |
|---|---|---|---|---|---|---|
| | | EHNV | IHNV | Rana or ESV-ECV | SVCV | VHSV |
| 1 | 8 | | Genogroup M (Partial G) | EHNV (Seq) | | Ia (partial G) |
| 2 | 10 | EHNV (Seq) | | ESV-ECV (Seq) | | |
| 4 | 10 | EHNV (Seq) | (partial G) | ESV-ECV (Seq) | (partial G) | Ia, similar to DK 3345 (partial N) |
| 5 | 10 | EHNV (Seq) | M (partial G) | Ranavirus not EHNV (Seq) | Id (partial G) | Ia (full length G) |
| 7 | 6 | | | | | Ia (partial G) |
| 8 | 10 | EHNV (seq) | identical to FJ265710 (partial N) | European Catfish Virus (seq) | | genotype I a (full length G) |
| 9 | 8 | EHNV (Seq) | M (Europe) (full length G) | ESV-ECV (Seq) | (partial G) | VHSV 1a; IHNV M (Europe) (full length G) |
| 11 | 8 | EHNV (Seq) | | EHNV (Seq) | | |
| 12 | 8 | | U (partial G) | Sheatfish/catfish iridovirus (seq) | (partial G) | Ia (partial G) |
| 13 | 10 | EHNV (Seq) | M (partial G) | ECV/ESV (Seq) | | Ia (partial G) |
| 14 | 8 | EHNV (Seq) | M genogroup (partial N) | EHNV (Seq) | | I (partial N) |
| 15 | 8 | | M genogroup (partial G) | Ranavirus - not EHNV (seq) | | |
| 16 | 10 | EHN 99% homology to FJ 433873.1EHN, AY 187045.1 EHN, AF 157667.1 | IHNV homology to FJ711518.1 IHNV | 99% homology to ECV (AF 157659.1), 99% homology to ESV (AF 157679.1) and 99% homology to AF 157665.1 | | |
| 17 | 6 | EHNV (Seq) | M (partial G) | Ranavirus - not EHNV (seq) | (partial N) | I-a (partial G, N) |
| 18 | 8 | | | ESV-ECV (Seq) | | |

| Laboratory code number | Score 10/10 | Ampoule I | Ampoule II | Ampoule III | Ampoule IV | Ampoule V |
|---|---|---|---|---|---|---|
| | | EHNV | IHNV | Rana or ESV-ECV | SVCV | VHSV |
| 19 | 10 | EHNV (Seq) | M (partial N) | ECV (Seq) | (partial G) | I-a (partial N) |
| 20 | 8 | EHNV (Seq) (Acc. No.: AY187045.1; 100%) | (partial G) (Acc. No.: FJ711518.1; 99% identity) | EHNV (Seq) (Acc. No.: FJ433873.1; 99% identity) | (partial G) (Acc. No.: AJ538079.1; 99% identity) | (partial G) (Acc. No.: EU708793.1; 99% identity) |
| 21 | 10 | EHNV (seq) | Genotype M (partial G) | ESV/ECV (seq) | | Genotype Ia (full length G) |
| 23 | 10 | EHNV (seq) | | ECV (seq) | | |
| 24 | 10 | EHNV (seq) | Genogroup M (partial N) | Ranaviruses not EHNV (seq) | Genotype 1d (partial G) | Genotype 1a (partail G) |
| 25 | 10 | EHNV (seq) | M (partial N) | IRIDOVIRUS NOT EHNV (seq) | 1d (partial G) | 1a (qRT-PCR) |
| 26 | 8 | EHNV (seq) | M-Eur1 (partial G) | ESV (seq) | SVCV (partial G) | 1a (full length G) |
| 28 | 10 | EHNV (seq) | | IRIDOVIRUS (seq) | 99% identical to svcv-arh-98 (partial G) | 98% identical to X73873 (partial N) |
| 29 | 8 | EHNV (seq) | Genogroup M (partial N) | Sheetfish iridovirus (seq) | Z37505 Isolate=Fijan (partial G) | Genotype Ia (partial G) |
| 30 | 10 | EHNV (seq) | | Ranavirus not EHNV (seq) | | |
| 32 | 10 | EHNV (seq) | Genogroup M (full length G)<br><br>European isolates (according to Nishizawa et al. DAO, 2006) | ECV/ESV (seq) | Subgroup Id (full length G) | Genotype Ia (full length G) |
| 33 | 6 | | M | EHNV (seq) | | 97% identity with G-Ia |
| 34 | 4 | | | EHNV (seq) | | |
| 36 | 4 | | | EHNV (seq) | | |
| 37 | 10 | EHNV (seq) | GenBank accession number L40877.1 (partial G) | Ranavirus: ESV / ECV (seq) | | Ia (full length G) |
| 38 | 10 | EHNV (RFLP) | | Ranavirus: ESV / ECV/BIV (RFLP) | | |
| 39 | 10 | EHNV (seq) | M genogroup (partial N) | ranavirus (seq) (99% identity with sheet fish, doctor fish and catfish iridoviruses) | 1d (partial G) | Ia (partial G) |

**Concluding remarks PT1**

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

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

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

In this report (figures 4-7), all titres submitted by participants for each cell line and ampoule, respectively are compared to each other. In this way, the titres obtained by each laboratory are plotted in relation to the combined submitted data set and each participating laboratory should be able to compare the sensitivity of their cell lines to the sensitivity of those used by the other participating laboratories. We recommend all participants to evaluate the sensitivity of the cells used in their laboratory in relation to the diagnostic purpose.

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

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

# Proficiency test 2, PT2

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

**Content of ampoules**

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

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

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

**Testing of the test**

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

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

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

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

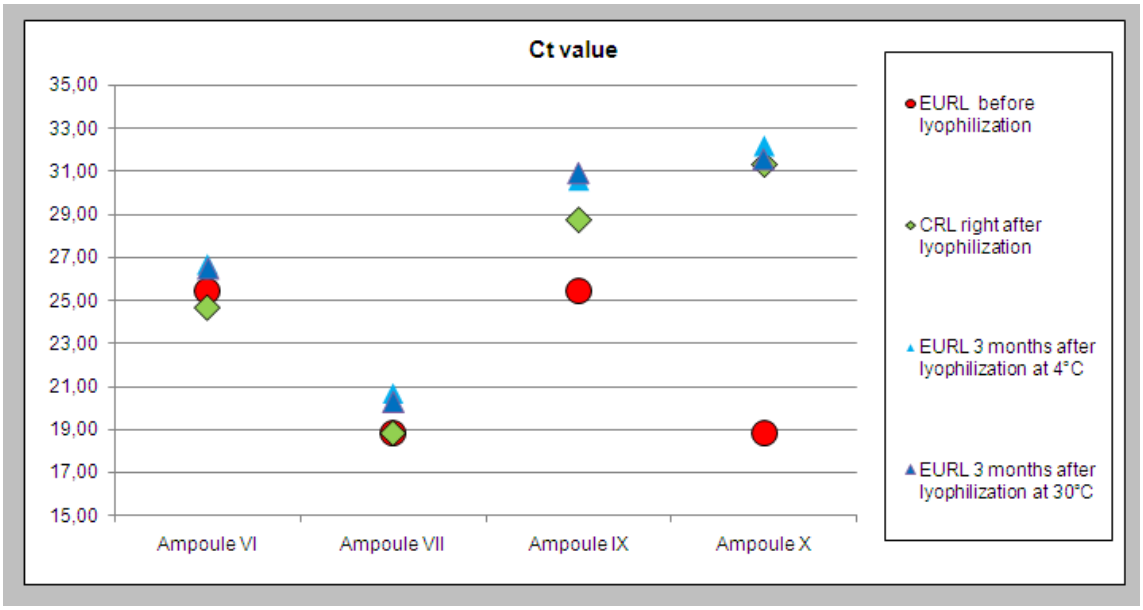
For each ampoule the presence of viruses other than the expected was not detected.

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

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

| Ampoule No. | Content | Ampoule | Ct value before lyophilisation undiluted | Ct value right after lyophilisation | Ct value 3 months after lyophilisation (4ºC, dark conditions) | Ct value 3 months after lyophilisation (30ºC, dark conditions) |
|---|---|---|---|---|---|---|
| | (1:64) | d | | 28,64 | | |
| | | e | | 28,91 | | |
| Ampoule X | KHV (CyHV-3) Low titer 1:2048 | a | 18,96 | 30,97 | 32,23 | 31,59 |
| | | b | 18,62 | 31,35 | | |
| | | c | 18,90 | 30,64 | | |
| | | d | | 32,13 | | |
| | | e | | 31,48 | | |

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




## Virus identification

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

All laboratories were encouraged to genotype KHV and ISAV isolates as far as possible. However, this was not a mandatory task.
It was requested that the viruses in the samples were not forwarded to third parts without having contacted the EURL for permission in advance.

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

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

| Laboratory code number | Score | Answer recieved at EURL | Ampoule VI | Ampoule VII | Ampoule VIII | Ampoule IX | Ampoule X |
|---|---|---|---|---|---|---|---|
| | | | ISAV | KHV | No virus | ISAV | KHV |
| 24 | 8 | 29-11-2010 | ISAV | KHV | Negative | Negative | KHV |
| 25 | 10 | 22-11-2010 | ISAV | KHV | No KHV and no ISA | ISAV | KHV |
| 26 | 10 | 23-11-2010 | ISAV | KHV | No ISAV and no KHV | ISAV | KHV |
| 27[1] | 0 | | | | | | |
| 28 | 8 | 26-11-2010 | ISAV | KHV | No ISAV and no KHV | ISAV | No ISAV and no KHV |
| 29 | 10 | 29-11-2010 | ISAV | KHV | No KHV and no ISAV | ISAV | KHV |
| 30[4] | 6/6 | 29-11-2010 | ISAV | No ISAV | No ISAV | ISAV | No ISAV |
| 31 | 10 | 29-11-2010 | ISAV | KHV | No ISAV and no KHV | ISAV | KHV |
| 32 | 10 | 26-11-2010 | ISAV | KHV | No ISAV and no KHV | ISAV | KHV |
| 33 | 10 | 22-11-2010 | ISAV | KHV | No ISAV and no KHV | ISAV | KHV |
| 34 | 4 | 29-11-2010 | No ISAV and no KHV | KHV | No ISAV and no KHV | No ISAV and no KHV | No ISAV and no KHV |
| 36 | 6 | 29-11-2010 | ISAV | KHV | No ISAV, no KHV | No ISAV, no KHV | No ISAV, no KHV |
| 37 | 10 | 23-11-2010 | ISAV | KHV | No ISAV, no KHV | ISAV | KHV |
| 38[5] | | | | | | | |
| 39 | 10 | 06-12-2010 | ISAV | KHV | Not ISAV and KHV | ISAV | KHV |

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

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

*Identification of content*

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

Ampoule VI – ISAV (high titer)

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

Ampoule VII - KHV (high titer)

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

Ampoule VIII – medium (no virus)

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

Ampoule IX – ISAV (medium titer)

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

Ampoule X – KHV (low titer)

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

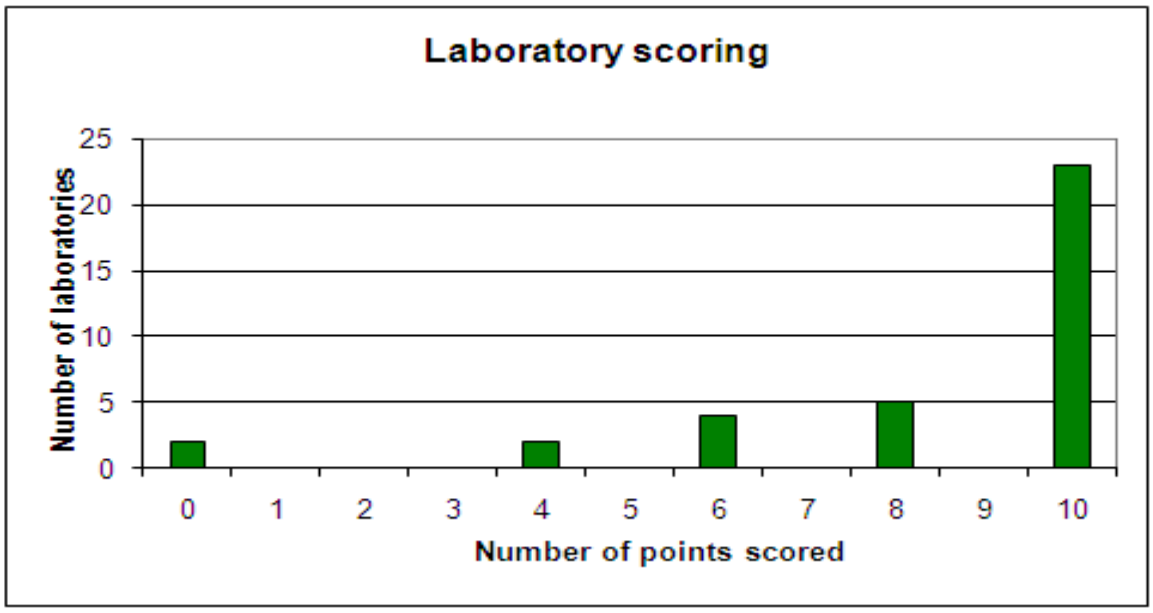
*Scores*

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

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

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

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



*Methods applied*

The following methods were used by the participants:

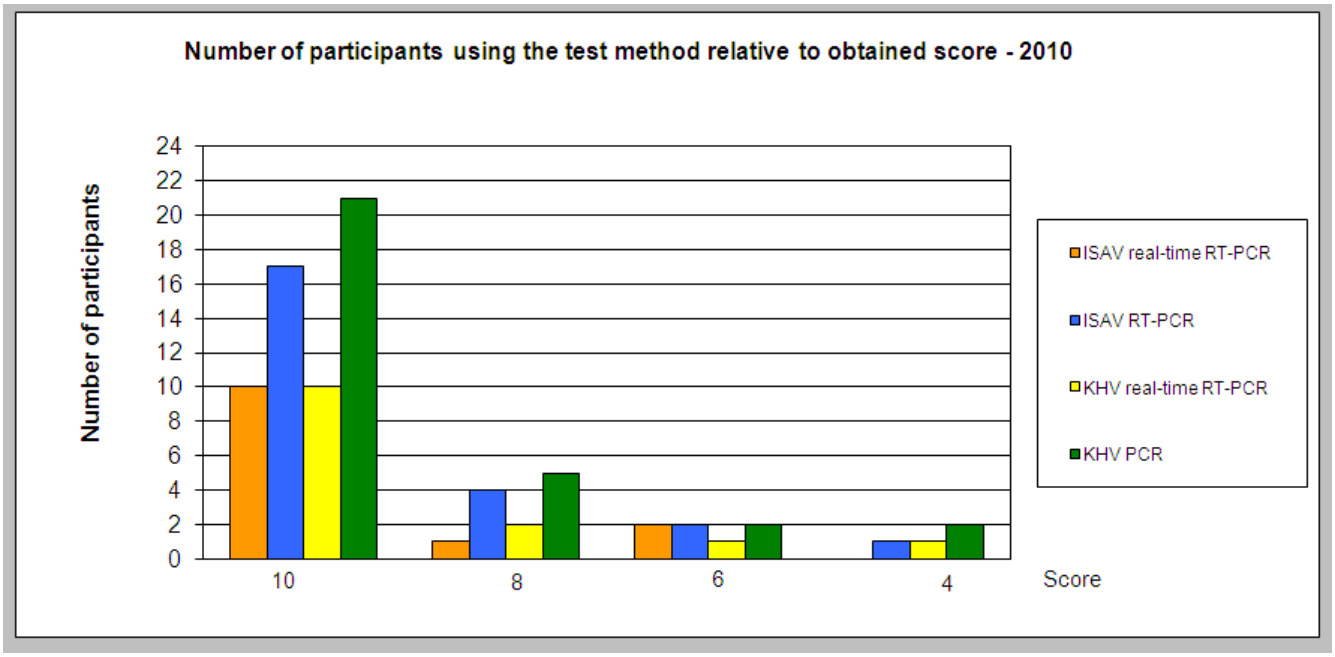
- 12 laboratories used ISAV real-time RT-PCR
- 24 laboratories used ISAV RT-PCR
- 4 laboratories used both ISAV real-time RT-PCR and ISAV RT-PCR
- 14 laboratories used KHV real-time RT-PCR
- 30 laboratories used KHV PCR
- 11 laboratories used both KHV real-time PCR and KHV PCR

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

| Laboratory code number | Score | Answer recieved at EURL | ISAV real-time RT-PCR | ISAV RT-PCR | KHV real-time RT-PCR | KHV PCR | Sequencing |
|---|---|---|---|---|---|---|---|
| 23 | 10 | 15-11-2010 | X | | X | X | CyHV-3 |
| 24 | 8 | 29-11-2010 | | X | | X | ISAV (DQ785276.1)<br>CyHV-3 (AP008984.1) |
| 25 | 10 | 22-11-2010 | X | X | X | X | ISAV HPR2<br>CyHV-3 |
| 26 | 10 | 23-11-2010 | X | X | X | X | ISAV HPR2, EU-G2<br>KHV |
| 28 | 8 | 26-11-2010 | | X | | X | ISAV (99% identical to AY971656, AF364881)<br>KHV (100% identical to isolates SF94-xx (EU0539xx)) |
| 29 | 10 | 29-11-2010 | X | | | X | ISAV HPR2, EU-G2<br>KHV (genotype/variant: A1, A2) |
| 30 | 6 | 29-11-2010 | X | | | | |
| 31 | 10 | 29-11-2010 | | X | | X | |
| 32 | 10 | 26-11-2010 | | X | X | X | ISAV European genotype-1 subgroup 2<br>KHV European genotype |
| 33 | 10 | 22-11-2010 | X | | | X | |
| 34 | 4 | 29-11-2010 | | X | | X | |
| 36 | 6 | 29-11-2010 | | X | | X | |
| 37 | 10 | 23-11-2010 | | X | X | X | |
| 39 | 10 | 06-12-2010 | | X | | X | ISAV European<br>KHV |

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

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




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

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

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

**Concluding remarks PT2**

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

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

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

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

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

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

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

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

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