EURL for Fish Diseases

Report of the Inter-Laboratory Proficiency Test 2012

for identification of

VHSV, IHNV, EHNV, SVCV and IPNV (PT1)

and identification of

CyHV-3 (KHV), ISAV and *Aphanomyces invadans* (PT2)

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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 proficiency test 1 (PT1) and proficiency test 2 (PT2).
PT1 was designed to primarily assess the identification of the fish viruses: viral haemorrhagic septicaemia virus (VHSV), infectious hematopoietic necrosis virus (IHNV), epizootic haematopoietic necrosis virus (EHNV), spring viraemia of carp virus (SVCV), and infectious pancreatic necrosis virus (IPNV) by cell culture based methods. PT2 was structured with the aim of assessing the ability of participating laboratories to identify the fish pathogens: infectious salmon anaemia virus (ISAV), Cyprinid herpesvirus 3 (CyHV-3) (otherwise known as koi herpes virus - KHV) and *Aphanomyces invadans* the causative agent of epizootic ulcerative syndrome (EUS) by biomolecular methods (PCR based). The number of National Reference Laboratories (NRLs) participating in PT1 and PT2 was 43.
The tests were sent from the EURL in the beginning of September 2012.
Both PT1 and PT2 are accredited by DANAK under registration number 515 for 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). These ampoules contained IPNV, EHNV, SVCV, IHNV and VHSV, respectively, see table 1. The proficiency test was designed to primarily assess the ability of participating laboratories to identify the listed fish viruses VHSV, IHNV and ENHV (Council Directive 2006/88/EC) and the non-listed viruses SVCV and IPNV 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. ELISA, PCR, immunofluorescence (IFAT) or neutralisation test.
If ranavirus was present in any of the ampoules, it was mandatory to perform 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 four coded ampoules (VI-IX). The ampoules contained ISAV and KHV. Furthermore, one ampoule contained *Aphanomyces invadans* and one sterile pyrogen free water, see table 11. 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 pathogens. 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, thus, it might had been possible to replicate them 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 European Commission.

Participants were asked to download an excel sheet from the EURL web site (http://www.eurl-fish.eu/) to be used for reporting results and to be submitted to the EURL electronically. Additionally, participants were requested 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 17th Annual Meeting of the NRLs for Fish Diseases May 2013 in Copenhagen. Participants were asked to reply latest November 5th 2012.

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 parcels 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 three days, the tests were delivered to 29 participants, 25 participants in EU and 4 participants outside EU; 9 more tests were delivered 8 days; 2 further tests within 20 days and the last within 43 days. (Figure 1). Despite several attempts it was, unfortunately, not possible to send the PT test to the NRL in Iran. All the parcels were sent without cooling elements. 8 countries outside EU had a logger in the parcel. The average temperature was 21,5°C for the transports, in five of those countries the temperature got above 30°C. The temperature exceeded 35°C in four parcels for one hour upon arrival.

Extra parcels were kept at refrigeration temperature in order to be able to provide substitutes in case of damage during transport and to make further investigation on the survival capacity of pathogens maintained at different temperatures (Figure 2).

After receiving results 3 parcels underwent to three different thermical treatments for 10 days period:

- 1 parcel was kept at 4°C
- 1 parcel was kept at 30°C and once a day put at 37°C for 1 hour
- 1 parcel was kept at 37°C for the entire period

After temperature treatment ampoules I-V were titrated on cell cultures and ampoules VI-IX were tested by PCR. No significant decreases in titres were observed in any of the ampoules after 10 days treatment at 4°, 30° or 37°C, except for SVCV where the titre decreased to the detection limit after 10 day at 37°C (Table 2). For ampoules VI-IX it was still possible to identify the pathogens by PCR methods and no reduction in C_t values were observed after heat treatment.

As during shipment loggers in the parcels never recorded higher temperature than the one tested after deadline, it is considered that all parcel were delivered in conditions that did not significantly affect ampoules content.

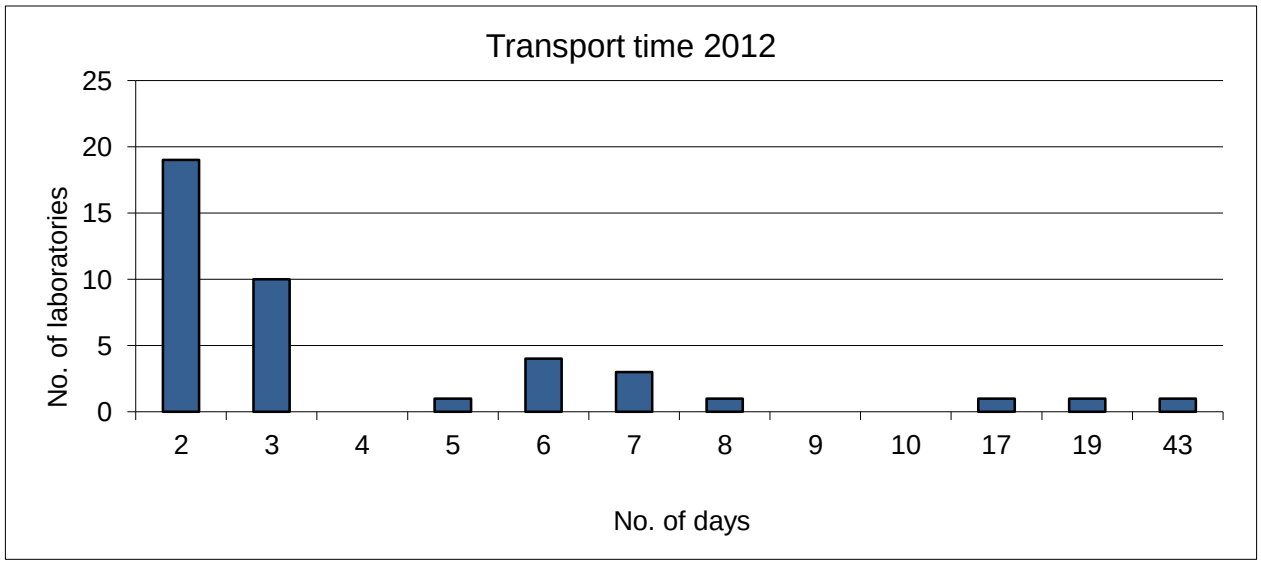

Figure 1. Transport time for the parcels to reach the participants.

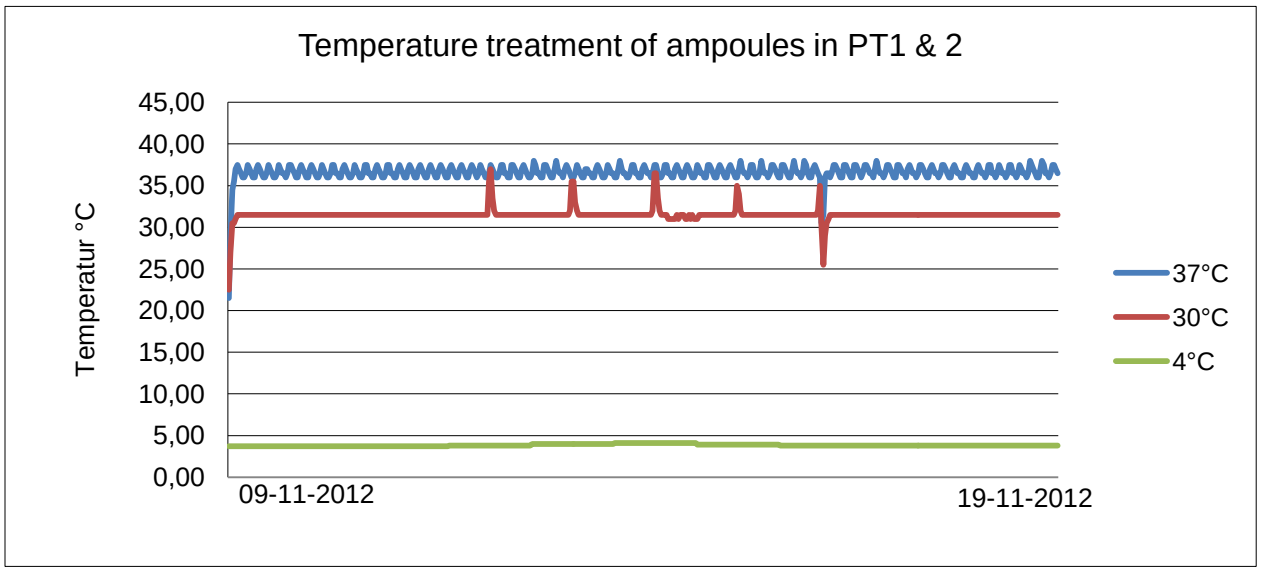

Figure 2. The ampoules were subjected to:

- Green Line- 1 parcel was kept at 4°C
- Red Line - 1 parcel was kept at 30°C and once a day put at 37°C for 1 hour
- Blue line - 1 parcel was kept at 37°C for the entire period.

Participation

PT1 and PT2: 41 laboratories received the annual proficiency test. Of these, 38 participants submitted results within the deadline. One participant submitted results one days after deadline but before the content of the ampoules was made public available. Two participants did not submit results.

Figure 3 show how many laboratories that participated in the proficiency test from 1996 to 2012.

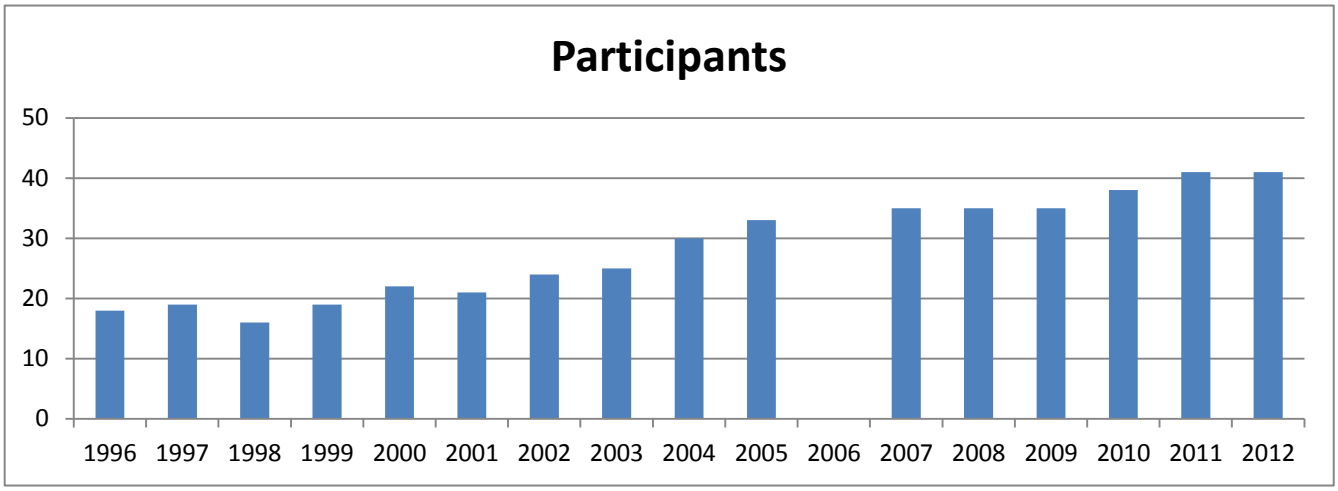

Figure 3. Participants in the EURL proficiency test over the years.

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, Canada, Croatia, Faroe Islands, Iceland, Israel, Japan, New Zealand, Norway, 2 from P.R. China, Serbia, Switzerland, Turkey and 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.

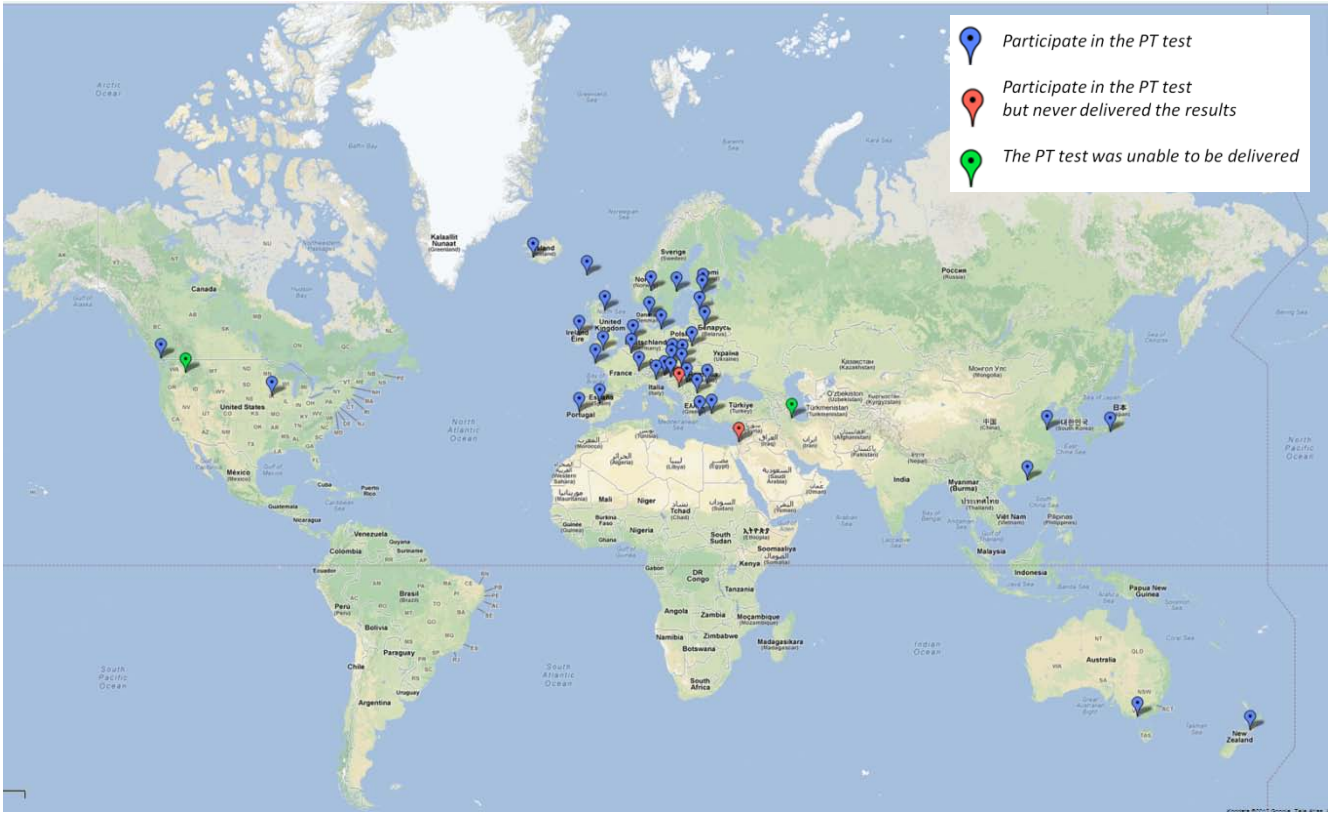

Figure 4. Worldwide distribution of the participants in the EURL proficiency test 2012.

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.

Code	Specifications
Ampoule I: IPNV strain Sp	**IPNV strain Sp** The Sp (Spjarup) reference strain of Infectious Pancreatic Necrosis (IPN) virus from farmed rainbow trout in Denmark, isolated in 1969 by Dr. Vestergaard Jørgensen. Received from: National Veterinary Institute, Technical University of Denmark. Cell culture passage number: 18 **GenBank accession numbers: AM889221** **Reference on isolate:** **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.** **References on sequences:** **P. F. Dixon, G.-H. Ngoh, D. M. Stone, S. F. Chang, K. Way, S. L. F. Kueh (2008) Proposal for a fourth aquabirnavirus serogroup Archives of Virology 153:1937–1941**
Ampoule II: EHNV Isolate 86/8774	**EHNV Isolate 86/8774** **Australian freshwater isolate of epizootic haematopoietic necrosis virus from rainbow trout from Adaminaby Trout Farm, NSW obtained in 1986 by Jeremy Langdon.** **Received from: Prof. Whittington, The OIE reference laboratory for EHN, University of Sidney, Australia.** **Cell culture passage number: 9** **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 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.** **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.** **Marsh IB, Whittington RJ, O'Rourke B, Hyatt AD & Chisholm O (2002) Rapid differentiation of Australian, European and American ranaviruses based on variation in major capsid protein gene sequence. Molecular and Cellular Probes 16, 137-151.**
Ampoule III: SVCV strain 56/70	**SVCV strain 56/70** **Spring viraemia of carp virus isolate from carp.** **The isolate is most likely identical to the S/30 isolate described in** **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.** **Received from: Prof. Fijan (January 1979 in a tube named Rhabdo virus carpio 56/70 and given as the reference strain of SVC virus).** **Cell culture passage number: Unknown.** **Genotype: Id** **GenBank accession numbers: Z37505.1 (Fijan), AJ538061.1 (S30)** **Reference on sequence (S30) and genotype:** **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.**

Code	Specifications
Ampoule IV: IHN virus 217/A	**IHNV 217/A** **First Italian IHNV isolate from rainbow trout. . Isolated in 1987.** **Received from: Received from Dr. G. Bovo, ISZ-Ve, Padova, Italy.** **Cell culture passage number: 12** **Genotype: M** **GenBank accession numbers: FJ265716.1** **Reference on isolate:** 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. **References on sequence and genotype:** 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.
Ampoule V: DK-6137 (Hjarnø)	**VHSV strain DK-6137** **The isolate originated from an outbreak of VHS with high mortality in sea water aquaculture in 1991.** **Received from: National Veterinary Institute, Technical University of Denmark.** **Cell culture passage number: 3** **Genotype: Ia** **GenBank accession number: AY546593** **Reference on isolate:** Olesen NJ, Lorenzen N & 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. Olesen NJ, Lorenzen N & LaPatra S (1999). Production of neutralizing antisera against viral hemorrhagic septicaemia (VHS) virus by intravenous injections of rabbits. Journal of Aquatic Animal Health 11, 10-16. **Reference on sequence and genotype:** 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.

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 for some of the virus, especially for VHSV, on all cell lines a 2-3 log reduction was observed. For the IHNV 1-2 log reduction was observed, but for IPNV, SVCV and EHNV almost no reduction was observed. (Table 2 and Figure 5).
However, all titres of the lyophilised viruses were above detection level, except for IHNV on BF-2 cells. As participants are expected to use at least two different cell lines, IHNV would have been 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 after exposure to 30°C for 24 hours (Report of the Inter-Laboratory Proficiency Test for National Reference Laboratories for Fish Diseases 2010).
In 2011 we had shown that lyophilised virus in glass sealed ampoules is stable when temperature raised from 20-42°C over a period of 5 hours (Report of the Inter-Laboratory Proficiency Test for National Reference Laboratories for Fish Diseases 2011).

We tested the titre of each virus preparation (ampoule) after storage in the dark at 4°C after deadline for results submission from participants and observed no significant decrease compared to right after lyophilization. Furthermore we tested virus titres in the ampoules stored 10 days at 30°C and 37°C, respectively without observing any titre decrease except for SVCV at 37°C.

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 ranavirus. For each ampoule, presence of viruses other than the expected was not observed.

Table 2. Titres in ampoules I to V stored in the dark tested on four cell lines at different time points:
- before lyophilisation (T=4°C)
- immediately after lyophilisation (median titre of 5 replicates) (T=4°C),
- after deadline for results submission, stored for 10 days at 4°C held at a temperature at 30°C+37°C one hour per day, for 10 days in the dark, and held at a temperature at 37°C, for 10 days in the dark (1 replicate).
- after deadline for results submission, stored for 10 days at 30°C and once a day put at 37°C for 1 hour (1 replicate).
- after deadline for results submission, stored for 10 days at 37°C (1 replicate).

Ampoule No.	**Cell line**	**Titre before lyophilisation**	**Median titre right after lyophilisation 5 replicates**	**Titre after last date for submission of results (4°C, dark conditions)**	**Titre after last date for submission of results (4°C) and after 30°C+37°C one hour per day, for 10 days, dark conditions**	**Titre after last date for submission of results (4°C) and after 37°C, for 10 days, dark conditions**
		$TCID_{50}$/ml	**$TCID_{50}$/ml**	**$TCID_{50}$/ml**	**$TCID_{50}$/ml**	**$TCID_{50}$/ml**
Ampoule I: IPNV Isolate strain Sp	BF-2	$1,9*10^8$	$2,7*10^7$	$1,9*10^7$	$1,3*10^7$	$2,7*10^7$
	EPC	$4,0*10^7$	$5,9*10^6$	$5,9*10^6$	$8,6*10^6$	$1,9*10^6$
	RTG-2	$5,9*10^7$	$1,9*10^6$	$8,6*10^6$	$4,0*10^6$	$1,9*10^6$
	FHM	$2,7*10^6$	$8,6*10^5$	$1,9*10^6$	$8,6*10^5$	$1,3*10^6$
Ampoule II: EHNV Isolate 86/8774	BF-2	$8,6*10^4$	$1,3*10^5$	$8,6*10^3$	$8,6*10^3$	$4,0*10^4$
	EPC	$2,7*10^4$	$5,9*10^4$	$2,7*10^4$	$1,9*10^4$	$1,9*10^4$
	RTG-2	$5,9*10^3$	$2,7*10^3$	$< 1,9*10^2$	$< 1,9*10^2$	$< 1,9*10^2$
	FHM	$1,3*10^2$	$5,9*10^2$	$< 1,9*10^2$	$< 1,9*10^2$	$< 1,9*10^2$
Ampoule III: Isolate SVCV 56/70	BF-2	$2,7*10^6$	$5,9*10^5$	$4,0*10^5$	$1,9*10^5$	$1,9*10^2$
	EPC	$5,9*10^6$	$8,6*10^5$	$1,9*10^5$	$1,3*10^5$	$8,6*10^2$
	RTG-2	$2,7*10^5$	$4,0*10^4$	$< 1,9*10^2$	$< 1,9*10^2$	$< 1,9*10^2$
	FHM	$1,9*10^6$	$1,3*10^6$	$5,9*10^5$	$1,9*10^5$	$2,7*10^2$
Ampoule IV: Isolate IHNV 217/A	BF-2	$1,3*10^2$	$< 1,9*10^2$	$< 1,9*10^2$	$< 1,9*10^2$	$< 1,9*10^2$
	EPC	$5,9*10^6$	$5,9*10^4$	$1,9*10^5$	$1,9*10^4$	$1,3*10^4$
	RTG-2	$4,0*10^5$	$1,9*10^3$	$< 1,9*10^2$	$< 1,9*10^2$	$< 1,9*10^2$
	FHM	$1,3*10^6$	$2,7*10^5$	$2,7*10^5$	$5,9*10^4$	$1,9*10^4$
Ampoule V: Isolate VHSV DK-6137 Hjarnø	BF-2	$1,3*10^6$	$8,6*10^3$	$4,0*10^3$	$8,6*10^2$	$5,9*10^2$
	EPC	$5,9*10^6$	$5,9*10^3$	$4,0*10^4$	$1,3*10^4$	$5,9*10^3$
	RTG-2	$5,9*10^6$	$5,9*10^3$	$< 1,9*10^2$	$< 1,9*10^2$	$< 1,9*10^2$
	FHM	$8,6*10^6$	$8,6*10^4$	$8,6*10^4$	$8,6*10^3$	$1,3*10^4$

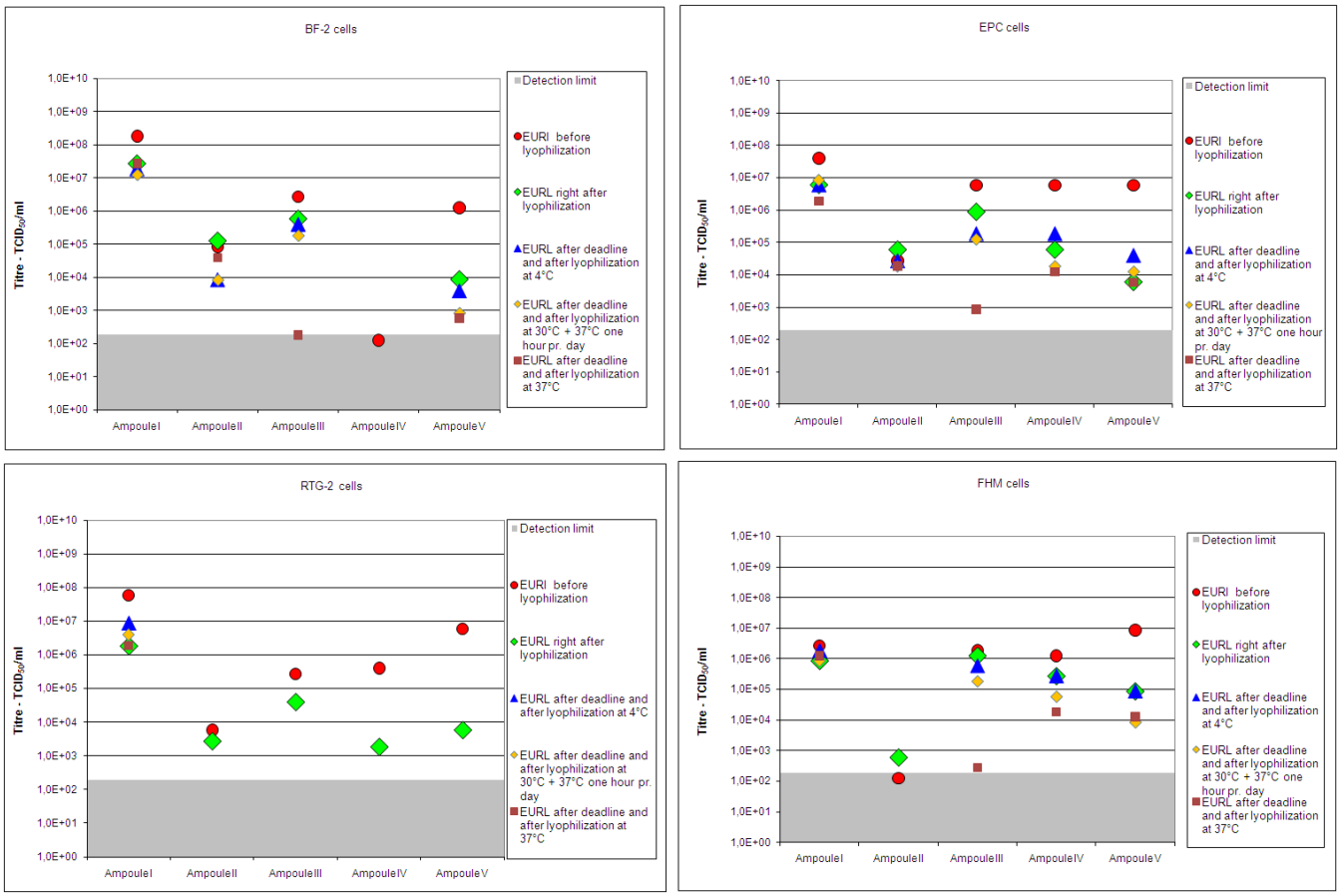

Figure 5. Virus titers in different cell lines before, right after, after deadline, 30°C+37°C, one hour per day, for 10 days and 37°C, for 10 days. Grey area is below detection level.

Virus identification and titration

Participants were asked to identify the content of each ampoule by the methods 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 at the participating laboratories are summarised in table 3.

Participants were also asked to assess the viral load in the ampoules performing titration. The quantification protocol 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% per ml ($TCID_{50}$/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)**.** Viruses titration results obtained in 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 six 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, 2012- Virus identification.

Laboratory code number	Score 10/10	Answer recived at EURL	Ampoule I	Ampoule II	Ampoule III	Ampoule IV	Ampoule V
			IPN strain Sp	EHNV 86/8774	SCVC 56/70	IHNV 217/A	VHSV DK-6137 Hjarnø
1	10	23-10-12	IPNV	EHNV	SVCV	IHNV	VHSV
2	10	24-10-12	IPNV	EHNV	SVCV	IHNV	VHSV
3	10	05-11-12	IPNV	EHNV	SVCV	IHNV	VHSV
4	10	01-11-12	IPNV	EHNV	SVCV	IHNV	VHSV
5	10	05-11-12	IPNV	EHNV	SVCV	IHNV	VHSV
6 [1]	8	05-11-12	IPNV	Not VHSV, IHNV, IPNV, SVCV	SVCV	IHNV	VHSV
7	10	29-10-12	IPNV Sp	EHNV	SVCV	IHNV	VHSV
8	10	13-10-12	IPNV	EHNV	SVCV	IHNV	VHSV
9	10	05-11-12	IPNV Sp	EHNV	SVCV	IHNV	VHSV
10	10	05-11-12	IPNV	EHNV	SVCV	IHNV	VHSV
11	10	31-10-12	IPNV	EHNV	SVCV	IHNV	VHSV
12	10	18-10-12	IPNV	EHNV	SVCV	IHNV	VHSV
13	10	31-10-12	IPNV	EHNV	SVCV	IHNV	VHSV
14	10	02-11-12	IPNV	EHNV	SVCV	IHNV	VHSV
15 [3]	10	30-10-12	IPNV	EHNV	SVCV	IHNV	VHSV
16	10	05-11-12	IPNV	EHNV	SVCV	IHNV	VHSV
17	10	05-11-12	IPNV	EHNV	SVCV	IHNV	VHSV
18	10	05-11-12	IPNV	EHNV	SVCV	IHNV	VHSV
19	10	05-11-12	IPNV	EHNV	SVCV	IHNV	VHSV
20 [4]	8	05-11-12	IPNV	EHNV	unknown	IHNV	VHSV
21	10	05-11-12	IPNV	EHNV	SVCV	IHNV	VHSV
22	9 Score 9/9	05-11-12	IPNV	Ranavirus *	SVCV	IHNV	VHSV
23	10	19-10-12	IPNV	EHNV	SVCV	IHNV	VHSV
24	10	02-11-12	IPNV	EHNV	SVCV	IHNV	VHSV
25	10	24-10-12	IPNV	EHNV	SVCV	IHNV	VHSV
26	10	02-11-12	IPNV	EHNV	SVCV	IHNV	VHSV
27 [2]	10	05-11-12	IPNV	EHNV	SVCV	IHNV	VHSV
28	10	02-11-12	IPNV	EHNV	SVCV	IHNV	VHSV
29	10	06-11-12	IPNV	EHNV	SVCV	IHNV	VHSV
30	10	02-11-12	IPNV	EHNV	SVCV	IHNV	VHSV
31			They never received the PT				
32	0	n/a	No reply	No reply	No reply	No reply	No reply
33	10	02-11-12	IPNV	EHNV	SVCV	IHNV	VHSV
34	10	01-11-12	IPNV	EHNV	SVCV	IHNV	VHSV
35	10	05-11-12	IPNV	EHNV	SVCV	IHNV	VHSV
36	10	05-11-12	IPNV	EHNV	SVCV	IHNV	VHSV
37	8	05-11-12	IPNV/Ranavirus	EHNV	SVCV	IHNV	VHSV
38	10	31-10-12	IPNV	EHNV	SVCV	IHNV	VHSV
39	8	02-11-12	IPNV/VHSV	EHNV	SVCV	IHNV	VHSV
40	10	05-11-12	IPNV	EHNV	SVCV	IHNV	VHSV
41			They never received the PT				
42	8	05-11-12	Not Ranavirus, VHSV, IHNV, or SVCV	Ranavirus (not determined if EHNV) *	SVCV	IHNV	VHS
43	0	n/a	No reply	No reply	No reply	No reply	No reply

[1] Did not perform test for Ranavirus
[2] The lab performed plaque assay for determining viral titre (PFU) instead of TCID-50
[3] EHNV has been identified directly from the ampoule? No title or result for Cell culture
[4] Examine for SVCV by PCR, but SVCV was not detected
* Genomic analysis not performed
n/a: not applicable

Table 4. Inter-Laboratory Proficiency Test, PT1, 2012 – Results of titration of ampoule I.

Ampoule I - IPNV					
Laboratory code number	Virus Identification	Titre in			
		BF-2	**EPC**	**RTG-2**	**FHM**
1	IPNV	$5,9*10^7$	$8,6*10^6$	$1,9*10^7$	
2	IPNV	$1,3*10^8$	$2,7*10^7$		
3	IPNV	$5,9*10^4$	$2,7*10^4$		
4	IPNV	$1,9*10^8$	$4,0*10^7$	$2,7*10^7$	$1,3*10^7$
5	IPNV	$1,9*10^7$	$1,3*10^8$	$2,7*10^7$	$1,3*10^6$
6 [1]	IPNV	$5,9*10^5$	$8,6*10^6$		
7	IPNV Sp	$5,9*10^8$	$1,9*10^7$		
8	IPNV	$8,6*10^7$	$5,9*10^7$	$1,3*10^7$	
9	IPNV Sp	$1,9*10^7$	$1,9*10^7$	$5,9*10^6$	$5,9*10^6$
10	IPNV	$5,9*10^7$	$8,6*10^6$		
11	IPNV	$8,6*10^7$	$1,9*10^7$		
12	IPNV	$1,9*10^8$	$4,0*10^6$		
13	IPNV	$1,3*10^8$	$4,0*10^7$		
14	IPNV	$1,9*10^7$	$5,9*10^6$		
15 [3]	IPNV		$1,3*10^5$	$1,3*10^6$	
16	IPNV	$1,3*10^8$	$8,6*10^7$	$8,6*10^7$	$5,9*10^7$
17	IPNV		$2,7*10^7$	$< 1,9*10^2$	
18	IPNV	$4,0*10^7$	$8,6*10^6$		
19	IPNV	$1,3*10^7$	$1,3*10^7$	$1,3*10^5$	$1,3*10^6$
20 [4]	IPNV		$1,3*10^4$	$5,9*10^4$	
21	IPNV	$8,6*10^7$	$8,6*10^6$	$4,0*10^7$	$1,3*10^7$
22	IPNV	$4,0*10^7$			$1,9*10^7$
23	IPNV		$1,9*10^7$		$1,3*10^6$
24	IPNV	$5,9*10^9$	$5,9*10^7$		
25	IPNV	$2,7*10^8$			$5,9*10^5$
26	IPNV	$2,7*10^7$	$2,7*10^6$	$2,7*10^6$	
27 [2]	IPNV	PFU	PFU	PFU	
28	IPNV	$1,9*10^9$	$4,0*10^7$		
29	IPNV	$4,0*10^7$	$1,3*10^6$		
30	IPNV	$5,9*10^7$	$1,9*10^7$		
31	They never received the PT				
32	No reply				
33	IPNV	$2,7*10^7$	$2,7*10^6$	$8,6*10^6$	$1,3*10^6$
34	IPNV	$8,6*10^5$	$1,3*10^6$		
35	IPNV	$4,0*10^5$	$4,0*10^6$		
36	IPNV		$5,9*10^7$		$8,6*10^6$
37	IPNV/Ranavirus	$1,3*10^8$	$1,3*10^7$		
38	IPNV	$5,9*10^7$	$1,3*10^7$		
39	IPNV/VHSV	$8,6*10^7$	$4,0*10^7$		
40	IPNV	$5,9*10^6$	$4,0*10^6$	$8,6*10^6$	
41	They never received the PT				
42	Not Ranavirus, VHSV, IHNV, or SVCV	$1,3*10^8$	$4,0*10^7$		$2,7*10^4$
43	No reply				

Number of laboratories	33	36	14	12
Median titre	$5,9*10^7$	$1,3*10^7$	$8,6*10^6$	$3,6*10^6$
Maximum titre	$5,9*10^9$	$1,3*10^8$	$8,6*10^7$	$5,9*10^7$
Minimum titre	$5,9*10^4$	$1,3*10^4$	$5,9*10^4$	$2,7*10^4$
25% quartile titre	$1,9*10^7$	$4,0*10^6$	$2,7*10^6$	$1,3*10^6$
75% quartile titre	$1,3*10^8$	$4,0*10^7$	$2,7*10^7$	$1,3*10^7$

Table 5. Inter-Laboratory Proficiency Test, PT1, 2012 – Results of titration of ampoule II.

Ampoule II - EHNV					
Laboratory code number	Virus Identification	Titre in			
		BF-2	EPC	RTG-2	FHM
1	EHNV	$1,9*10^4$	$1,9*10^4$	$1,9*10^4$	
2	EHNV	$8,6*10^5$	$1,9*10^4$		
3	EHNV	$1,3*10^3$	$4,0*10^5$		
4	EHNV	$4,0*10^4$	$5,9*10^3$	$4,0*10^3$	$< 1,9*10^2$
5	EHNV	$4,0*10^5$	$8,6*10^4$	$1,3*10^5$	$5,9*10^2$
6	Not VHSV, IHNV, IPNV, SVCV	$1,9*10^4$	$8,6*10^4$		
7	EHNV	$4,0*10^4$	$4,0*10^3$		
8	EHNV	$5,9*10^4$	$1,3*10^4$	$< 1,9*10^2$	
9	EHNV	$1,3*10^4$	$5,9*10^3$	$< 1,9*10^2$	$< 1,9*10^2$
10	EHNV	$1,3*10^4$	$1,9*10^4$		
11	EHNV	$4,0*10^4$	$2,7*10^4$		
12	EHNV	$1,9*10^4$	$1,3*10^3$		
13	EHNV	$2,7*10^5$	$1,3*10^5$		
14	EHNV	$8,6*10^2$	$4,0*10^3$		
15	EHNV		$< 1,9*10^2$	$< 1,9*10^2$	
16	EHNV	$1,9*10^5$	$1,9*10^4$	$1,9*10^5$	$1,3*10^5$
17	EHNV		$8,6*10^3$	$< 1,9*10^2$	
18	EHNV	$8,6*10^2$	$< 1,9*10^2$		
19	EHNV	$1,3*10^4$	$1,3*10^3$	$5,9*10^2$	$1,3*10^3$
20	EHNV		$1,9*10^4$	$< 1,9*10^2$	
21	EHNV	$1,9*10^5$	$1,3*10^5$	$5,9*10^4$	$2,7*10^4$
22	Ranavirus *	$2,7*10^4$			$< 1,9*10^2$
23	EHNV		$2,7*10^4$		$1,3*10^3$
24	EHNV	$1,3*10^3$	$4,0*10^4$		
25	EHNV	$1,9*10^5$			$8,6*10^3$
26	EHNV	$2,7*10^4$	$1,9*10^4$	$8,6*10^2$	
27	EHNV	PFU	PFU	PFU	
28	EHNV	$5,9*10^4$	$1,9*10^4$		
29	EHNV	$8,6*10^3$	$1,9*10^4$		
30	EHNV	$1,3*10^4$	$5,9*10^3$		
31	They never received the PT				
32	No reply				
33	EHNV	$2,7*10^4$	$1,3*10^4$	$1,3*10^3$	$< 1,9*10^2$
34	EHNV	$1,3*10^4$	$2,7*10^3$		
35	EHNV	$2,7*10^4$	$2,7*10^4$		
36	EHNV		$1,3*10^3$		$2,7*10^2$
37	EHNV	$8,6*10^4$	$4,0*10^6$		
38	EHNV	$4,0*10^4$	$2,7*10^7$		
39	EHNV	$1,3*10^5$	$1,3*10^4$		
40	EHNV	$4,0*10^4$	$1,3*10^4$	$5,9*10^4$	
41	They never received the PT				
42	Ranavirus (not determined if EHNV) *	$4,0*10^3$	$< 1,9*10^2$		$2,7*10^2$
43	No reply				

Number of laboratories	33	36	14	12
Median titre	$2,7*10^4$	$1,9*10^4$	$1,9*10^4$	$1,3*10^3$
Maximum titre	$8,6*10^5$	$2,7*10^7$	$1,9*10^5$	$1,3*10^5$
Minimum titre	$8,6*10^2$	$1,3*10^3$	$5,9*10^2$	$2,7*10^2$
25% quartile titre	$1,3*10^4$	$5,9*10^3$	$1,3*10^3$	$5,1*10^2$
75% quartile titre	$5,9*10^4$	$2,7*10^4$	$5,9*10^4$	$1,3*10^4$

Table 6. Inter-Laboratory Proficiency Test, PT1, 2012 – Results of titration of ampoule III.

Ampoule III - SVCV					
Laboratory code number	Virus Identification	Titre in			
		BF-2	EPC	RTG-2	FHM
1	SVCV	$5,9*10^2$	$5,9*10^4$	$1,3*10^4$	
2	SVCV	$1,3*10^3$	$2,7*10^5$		
3	SVCV	$4,0*10^4$	$8,6*10^4$		
4	SVCV	$1,3*10^4$	$4,0*10^4$	$5,9*10^3$	$1,9*10^4$
5	SVCV	$1,9*10^5$	$5,9*10^5$	$1,3*10^5$	$5,9*10^5$
6	SVCV	$< 1,9*10^2$	$8,6*10^4$		
7	SVCV	$5,9*10^4$	$1,9*10^5$		
8	SVCV	$1,9*10^3$	$1,3*10^6$	$< 1,9*10^2$	
9	SVCV	$< 1,9*10^2$	$2,7*10^5$	$2,7*10^5$	$2,7*10^5$
10	SVCV	$1,9*10^5$	$2,7*10^4$		
11	SVCV	$1,9*10^4$	$2,7*10^5$		
12	SVCV	$8,6*10^5$	$1,3*10^5$		
13	SVCV	$1,3*10^5$	$1,9*10^5$		
14	SVCV	$1,9*10^5$	$1,9*10^4$		
15	SVCV		$1,3*10^5$	$5,9*10^2$	
16	SVCV	$8,6*10^3$	$8,6*10^4$	$1,9*10^4$	$1,3*10^4$
17	SVCV		$1,3*10^5$	$< 1,9*10^2$	
18	SVCV	$4,0*10^2$	$< 1,9*10^2$		
19	SVCV	$8,6*10^4$	$1,9*10^5$	$1,9*10^4$	$1,9*10^5$
20	unknown		$4,0*10^3$	$< 1,9*10^2$	
21	SVCV	$2,7*10^4$	$1,9*10^4$	$1,9*10^4$	$5,9*10^4$
22	SVCV	$5,9*10^3$			$1,9*10^4$
23	SVCV		$1,9*10^5$		$2,7*10^4$
24	SVCV	$5,9*10^5$	$8,6*10^5$		
25	SVCV	$4,0*10^5$			$1,9*10^5$
26	SVCV	$5,9*10^4$	$2,7*10^4$	$< 1,9*10^2$	
27	SVCV	PFU	PFU	PFU	
28	SVCV	$4,0*10^4$	$2,7*10^6$		
29	SVCV	$1,3*10^5$	$5,9*10^5$		
30	SVCV	$2,7*10^4$	$4,0*10^4$		
31	They never received the PT				
32	No reply				
33	SVCV	$1,9*10^4$	$1,3*10^4$	$1,9*10^3$	$4,0*10^4$
34	SVCV	$1,9*10^4$	$2,7*10^4$		
35	SVCV	$5,9*10^5$	$4,0*10^5$		
36	SVCV		$1,3*10^4$		$2,7*10^4$
37	SVCV	$2,7*10^4$	$1,9*10^6$		
38	SVCV	$1,3*10^4$	$1,9*10^4$		
39	SVCV	$1,9*10^3$	$2,7*10^5$		
40	SVCV	$2,7*10^3$	$8,6*10^3$	$5,9*10^4$	
41	They never received the PT				
42	SVCV	$1,3*10^4$	$4,0*10^4$		$4,0*10^5$
43	No reply				

Number of laboratories	33	36	14	12
Median titre	$2,7*10^4$	$1,3*10^5$	$1,9*10^4$	$4,9*10^4$
Maximum titre	$8,6*10^5$	$2,7*10^6$	$2,7*10^5$	$5,9*10^5$
Minimum titre	$4,0*10^2$	$4,0*10^3$	$5,9*10^2$	$1,3*10^4$
25% quartile titre	$1,1*10^4$	$2,7*10^4$	$7,6*10^3$	$2,5*10^4$
75% quartile titre	$1,3*10^5$	$2,7*10^5$	$4,9*10^4$	$2,1*10^5$

Table 7. Inter-Laboratory Proficiency Test, PT1, 2012 – Results of titration of ampoule IV.

Ampoule IV - IHNV					
Laboratory code number	Virus Identification	Titre in			
		BF-2	EPC	RTG-2	FHM
1	IHNV	$< 1,9*10^2$	$4,0*10^5$	$2,7*10^2$	
2	IHNV	$1,3*10^4$	$1,9*10^5$		
3	IHNV	$4,0*10^3$	$4,0*10^3$		
4	IHNV	$< 1,9*10^2$	$2,7*10^4$	$4,0*10^4$	$5,9*10^4$
5	IHNV	$< 1,9*10^2$	$4,0*10^5$	$1,9*10^4$	$2,7*10^5$
6	IHNV	$< 1,9*10^2$	$1,3*10^6$		
7	IHNV	$< 1,9*10^2$	$4,0*10^4$		
8	IHNV	$< 1,9*10^2$	$4,0*10^5$	$1,3*10^4$	
9	IHNV	$2,7*10^4$	$1,3*10^5$	$2,7*10^4$	$1,3*10^5$
10	IHNV	$8,6*10^3$	$8,6*10^4$		
11	IHNV	$< 1,9*10^2$	$1,9*10^5$		
12	IHNV	$1,3*10^4$	$5,9*10^5$		
13	IHNV	$8,6*10^4$	$1,3*10^5$		
14	IHNV	$< 1,9*10^2$	$2,7*10^5$		
15	IHNV		$8,6*10^4$	$4,0*10^4$	
16	IHNV	$1,3*10^3$	$5,9*10^4$	$1,3*10^3$	$1,3*10^3$
17	IHNV		$5,9*10^4$	$< 1,9*10^2$	
18	IHNV	$1,9*10^2$	$< 1,9*10^2$		
19	IHNV	$1,9*10^5$	$1,3*10^5$	$5,9*10^4$	$1,3*10^5$
20	IHNV		$5,9*10^4$	$1,9*10^3$	
21	IHNV	$< 1,9*10^2$	$2,7*10^5$	$< 1,9*10^2$	$5,9*10^5$
22	IHNV	$1,3*10^3$			$1,3*10^5$
23	IHNV		$4,0*10^5$		$8,6*10^4$
24	IHNV	$< 1,9*10^2$	$2,7*10^6$		
25	IHNV	$8,6*10^3$			$4,0*10^5$
26	IHNV	$4,0*10^3$	$8,6*10^4$	$< 1,9*10^2$	
27	IHNV	PFU	PFU	PFU	
28	IHNV	$2,7*10^3$	$2,7*10^6$		
29	IHNV	$8,6*10^2$	$1,9*10^4$		
30	IHNV	$2,7*10^3$	$1,9*10^5$		
31	They never received the PT				
32	No reply				
33	IHNV	$< 1,9*10^2$	$2,7*10^3$	$5,9*10^2$	$1,9*10^4$
34	IHNV	$< 1,9*10^2$	$5,9*10^4$		
35	IHNV	$1,9*10^7$	$1,9*10^6$		
36	IHNV		$5,9*10^3$		$2,7*10^3$
37	IHNV	$4,0*10^5$	$8,6*10^5$		
38	IHNV	$5,9*10^4$	$1,9*10^5$		
39	IHNV	$1,3*10^3$	$8,6*10^5$		
40	IHNV	$< 1,9*10^2$	$1,9*10^5$	$1,3*10^5$	
41	They never received the PT				
42	IHNV	$< 1,9*10^2$	$2,7*10^3$		$2,7*10^4$
43	No reply				

Number of laboratories	33	36	14	12
Median titre	$8,6*10^3$	$1,9*10^5$	$1,9*10^4$	$1,1*10^5$
Maximum titre	$1,9*10^7$	$2,7*10^6$	$1,3*10^5$	$5,9*10^5$
Minimum titre	$1,9*10^2$	$2,7*10^3$	$2,7*10^2$	$1,3*10^3$
25% quartile titre	$2,0*10^3$	$5,9*10^4$	$1,6*10^3$	$2,5*10^4$
75% quartile titre	$4,3*10^4$	$4,0*10^5$	$4,0*10^4$	$1,6*10^5$

Table 8. Inter-Laboratory Proficiency Test, PT1, 2012 – Results of titration of ampoule V.

Ampoule V - VHSV					
Laboratory code number	Virus Identification	Titre in			
		BF-2	EPC	RTG-2	FHM
1	VHSV	$2,7*10^3$	$5,9*10^3$	$1,9*10^3$	
2	VHSV	$2,7*10^3$	$2,7*10^4$		
3	VHSV	$5,9*10^3$	$2,7*10^3$		
4	VHSV	$1,9*10^4$	$5,9*10^3$	$1,9*10^4$	$4,0*10^4$
5	VHSV	$5,9*10^3$	$1,3*10^4$	$8,6*10^3$	$1,9*10^4$
6	VHSV	$2,7*10^3$	$2,7*10^4$		
7	VHSV	$4,0*10^2$	$1,3*10^4$		
8	VHSV	$4,0*10^4$	$4,0*10^4$	$4,0*10^3$	
9	VHSV	$4,0*10^3$	$4,0*10^3$	$2,7*10^4$	$1,9*10^3$
10	VHSV	$4,0*10^3$	$8,6*10^2$		
11	VHSV	$2,7*10^2$	$4,0*10^2$		
12	VHSV	$5,9*10^4$	$5,9*10^4$		
13	VHSV	$8,6*10^4$	$8,6*10^3$		
14	VHSV	$1,9*10^3$	$8,6*10^2$		
15	VHSV		$2,7*10^3$	$1,3*10^3$	
16	VHSV	$4,0*10^3$	$4,0*10^3$	$1,9*10^3$	$5,9*10^3$
17	VHSV		$1,9*10^5$	$< 1,9*10^2$	
18	VHSV	$< 1,9*10^2$	$< 1,9*10^2$		
19	VHSV	$1,3*10^6$	$1,9*10^5$	$1,3*10^5$	$2,7*10^5$
20	VHSV		$2,7*10^2$	$1,9*10^4$	
21	VHSV	$1,9*10^3$	$1,3*10^3$	$2,7*10^2$	$4,0*10^3$
22	VHSV	$1,9*10^3$			$8,6*10^3$
23	VHSV		$2,7*10^5$		$2,7*10^4$
24	VHSV	$8,6*10^2$	$1,9*10^4$		
25	VHSV	$5,9*10^3$			$1,3*10^5$
26	VHSV	$1,9*10^3$	$5,9*10^3$	$1,9*10^2$	
27	VHSV	PFU	PFU	PFU	
28	VHSV	$4,0*10^3$	$2,7*10^4$		
29	VHSV	$5,9*10^3$	$1,3*10^4$		
30	VHSV	$1,3*10^4$	$2,7*10^4$		
31	They never received the PT				
32	No reply				
33	VHSV	$2,7*10^2$	$5,9*10^2$	$1,9*10^2$	$8,6*10^2$
34	VHSV	$1,9*10^3$	$1,3*10^4$		
35	VHSV	$1,9*10^4$	$8,6*10^4$		
36	VHSV		$4,0*10^3$		$5,9*10^3$
37	VHSV	$1,9*10^7$	$2,7*10^7$		
38	VHSV	$1,9*10^4$	$8,6*10^3$		
39	VHSV	$2,7*10^3$	$2,7*10^4$		
40	VHSV	$4,0*10^2$	$8,6*10^3$	$1,9*10^5$	
41	They never received the PT				
42	VHS	$< 1,9*10^2$	$1,9*10^3$		4,0*103
43	No reply				

Number of laboratoriums	33	36	14	12
Median titre	$4,0*10^3$	$8,6*10^3$	$4,0*10^3$	$7,2*10^3$
Maximum titre	$1,9*10^7$	$2,7*10^7$	$1,9*10^5$	$2,7*10^5$
Minimum titre	$2,7*10^2$	$2,7*10^2$	$1,9*10^2$	$8,6*10^2$
25% quartile titre	$1,9*10^3$	$3,4*10^3$	$1,3*10^3$	$4,0*10^3$
75% quartile titre	$1,6*10^4$	$2,7*10^4$	$1,9*10^4$	$3,0*10^4$

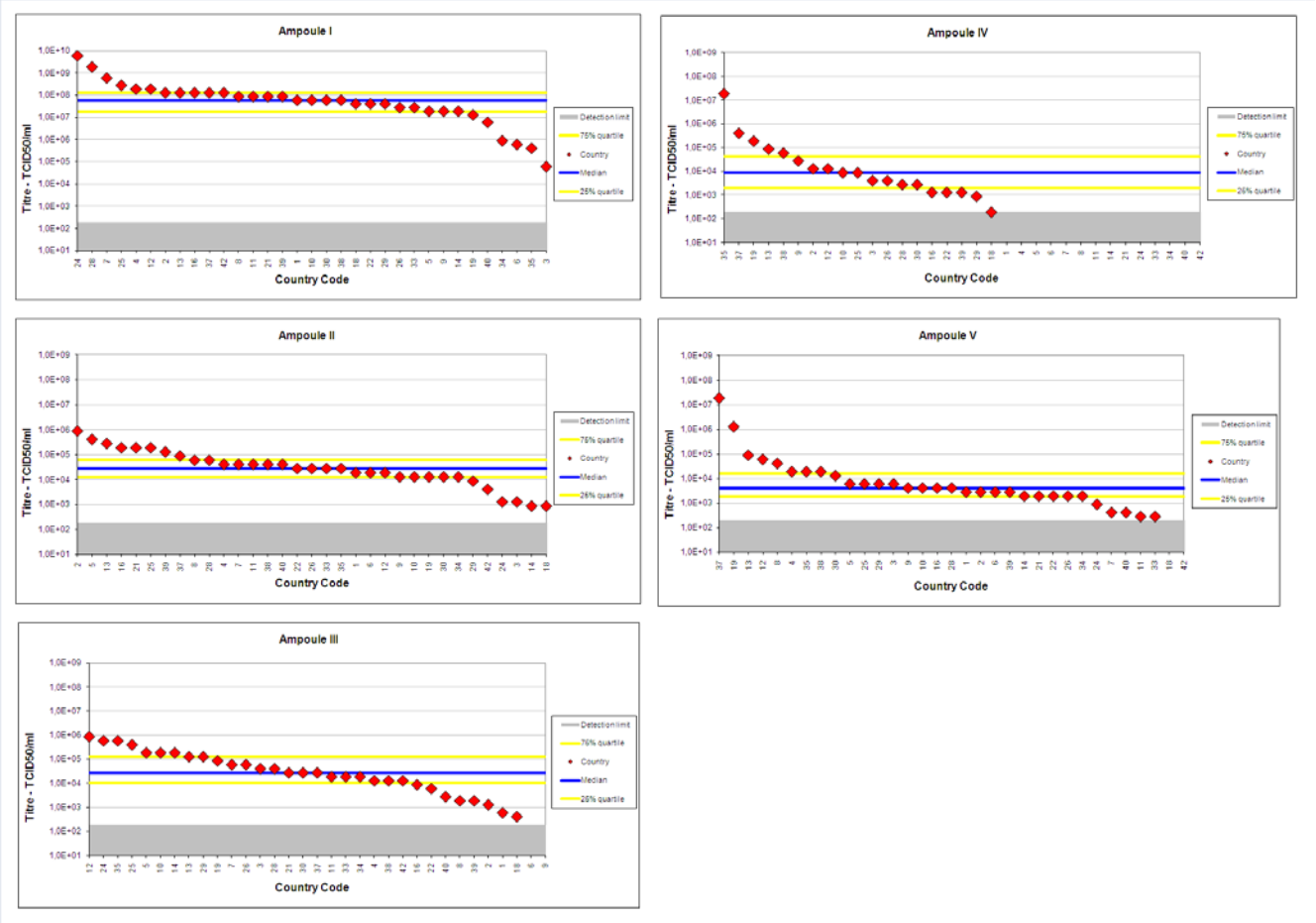

Figure 6. Virus titres obtained in BF-2 cells. The titre (red diamond) of each participating laboratory (country code) using BF-2 cells illustrated for ampoule I, II, III, IV and V. The detection level (gray shadow), median (blue line), 75% quartile (upper yellow line) and 25% quartile (lower yellow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

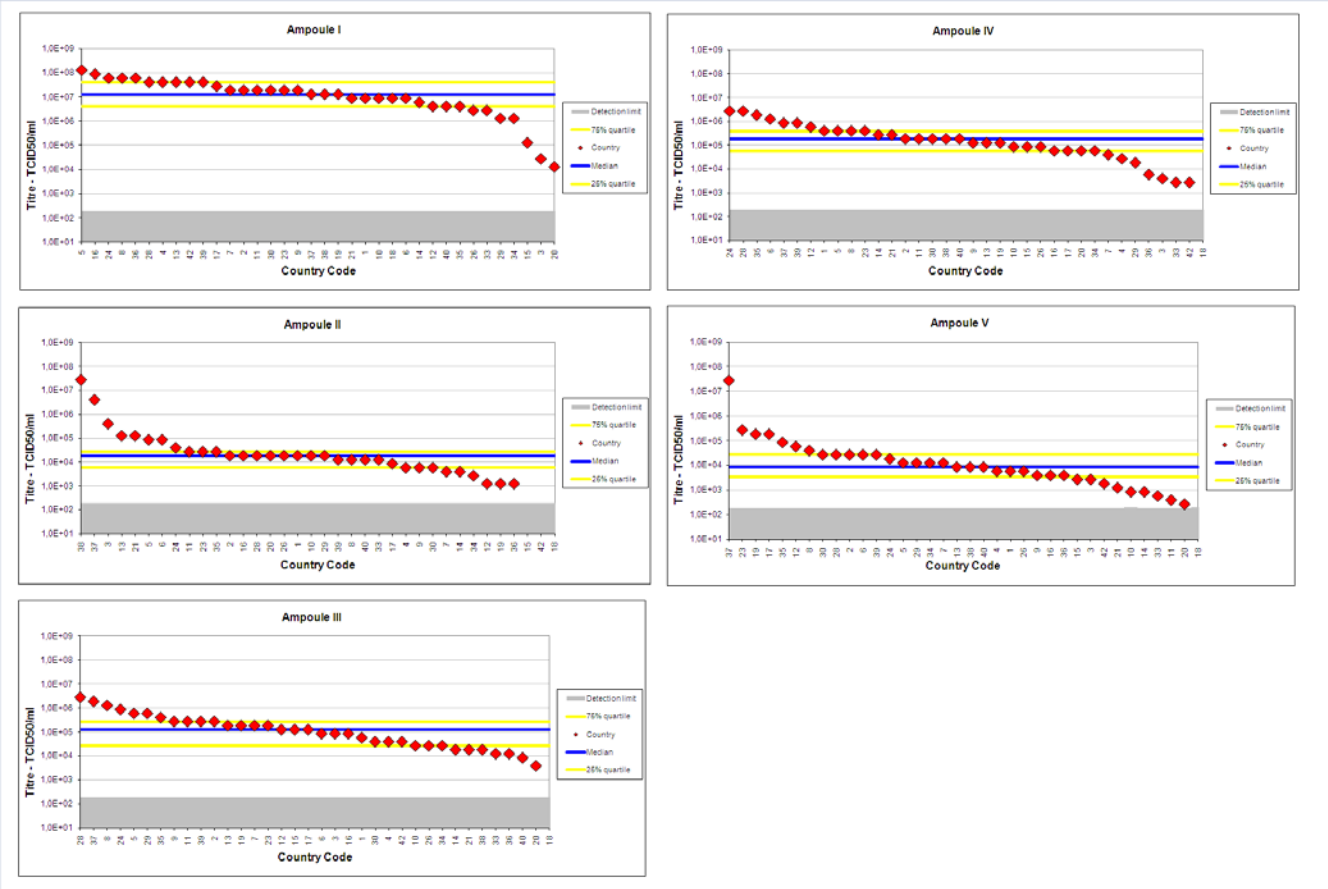

Figure 7. Virus titres obtained in EPC cells. For further details see Figure 6

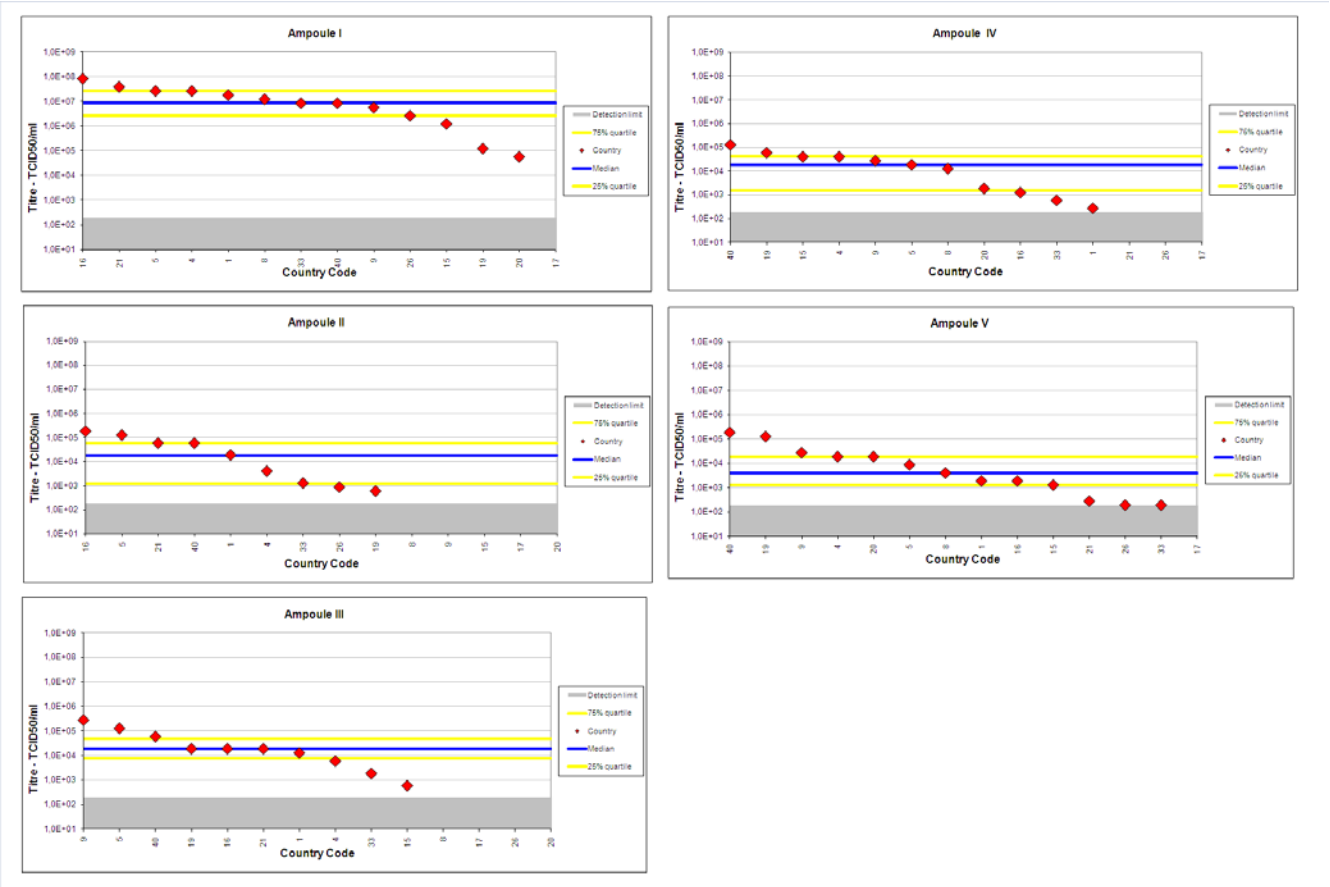

Figure 8. Virus titre obtained in RTG-2 cells. For further details see Figure 6

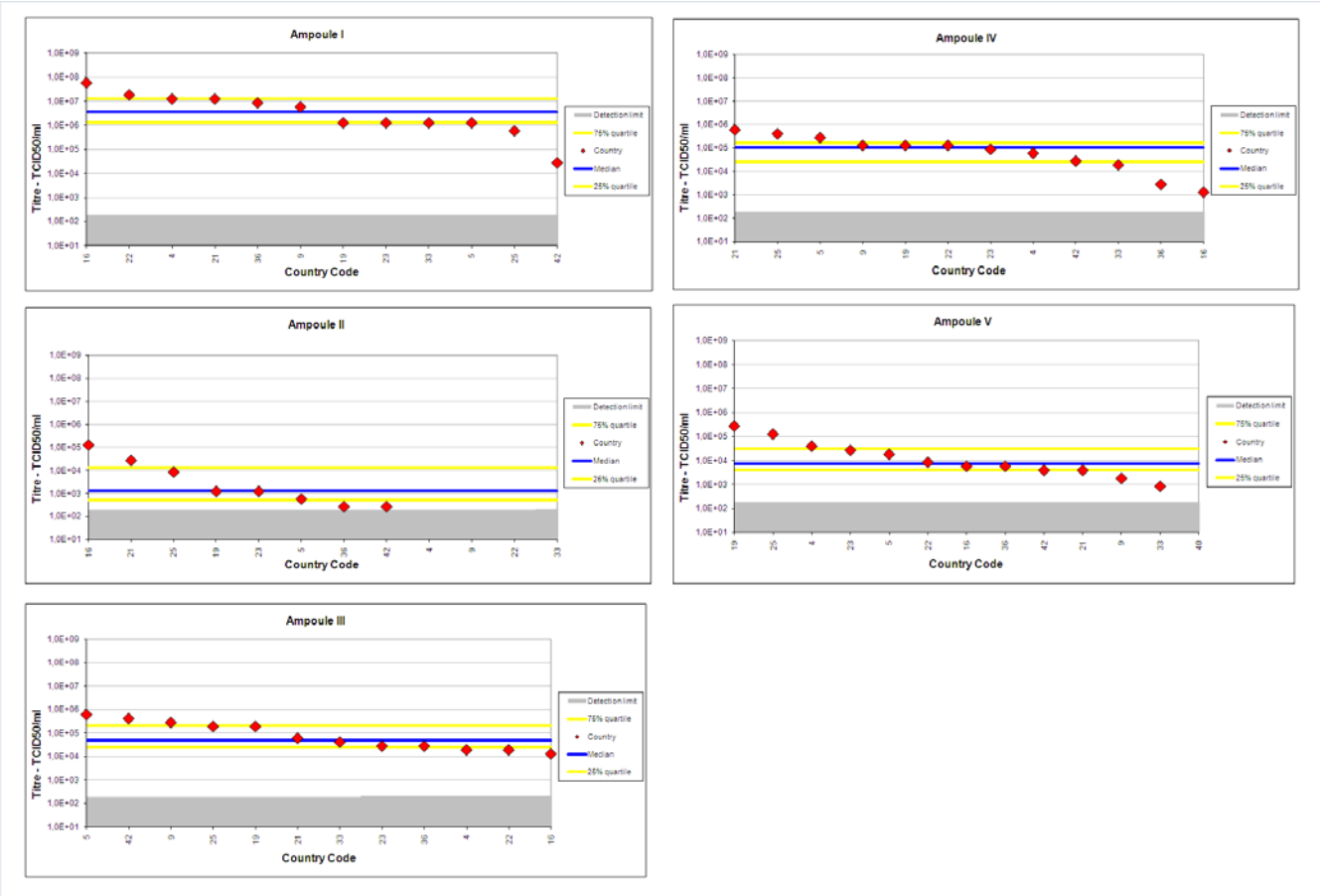

Figure 9. Virus titres obtained in FHM cells. For further details see Figure 6

Identification of content

- 33 laboratories correctly identified all viruses in all ampoules
- 2 laboratories did not submit any results
- 2 laboratories did not receive the parcel

Ampoule I – IPNV

- 36 laboratories correctly identified IPNV
- 2 laboratories found a double infection
- 1 laboratory did not test for IPNV

Ampoule II – EHNV

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

Ampoule III - SVCV

- 38 laboratories correctly identified SVCV
- 1 laboratory found virus but did not identify it

Ampoule IV – IHNV

- 39 laboratories correctly identified IHNV

Ampoule V – VHSV

- 39 laboratories correctly identified VHSV

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, giving the possibility for obtaining a maximum score of 10 (Table 3).

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

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

Ampoule IV: IHNV identification was given the score 2. IHNV not identified was given the score 0.

Ampoule V: VHSV identification was given the score 2. VHSV not identified was given the score 0.
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, 33 out of 39 correctly identified all viruses in all ampoules and obtained the maximum score 10. 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.

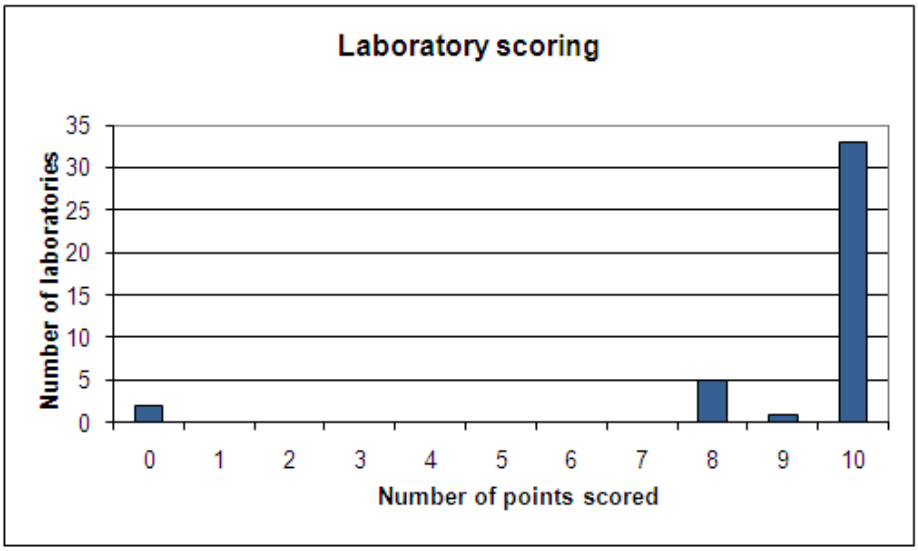

Figure 10. Scores obtained by participants.

Cells applied for solving the test

The following cell lines were used by the participants:

- 34 laboratories used BF-2 cells
- 37 laboratories used EPC cells
- 15 laboratories used RTG-2 cells
- 12 laboratories used FHM cells
- 6 laboratory used CHSE-214 cells
- 7 laboratories used four cell lines
- 6 laboratories used tree cell lines
  - 5 laboratories used BF-2 cells in combination with EPC cells and RTG-2 cells
  - 1 laboratories used BF-2 cells in combination with EPC cells and FHM cells
- 26 laboratories used two cell lines:
  - 19 laboratories used BF-2 cells in combination with EPC cells
  - 3 laboratories used RTG-2 cells in combination with EPC cells
  - 2 laboratories used BF-2 cells in combination with FHM cells
  - 2 laboratory used EPC cells in combination with FHM cells

The combination of EPC and FHM cells or BF-2 and RTG 2 as well 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 FHM cells are less efficient replication of IPNV and EHNV and that BF-2 and RTG-2 cells are less replication of IHNV. SVCV and VHSV grow on all cell lines.

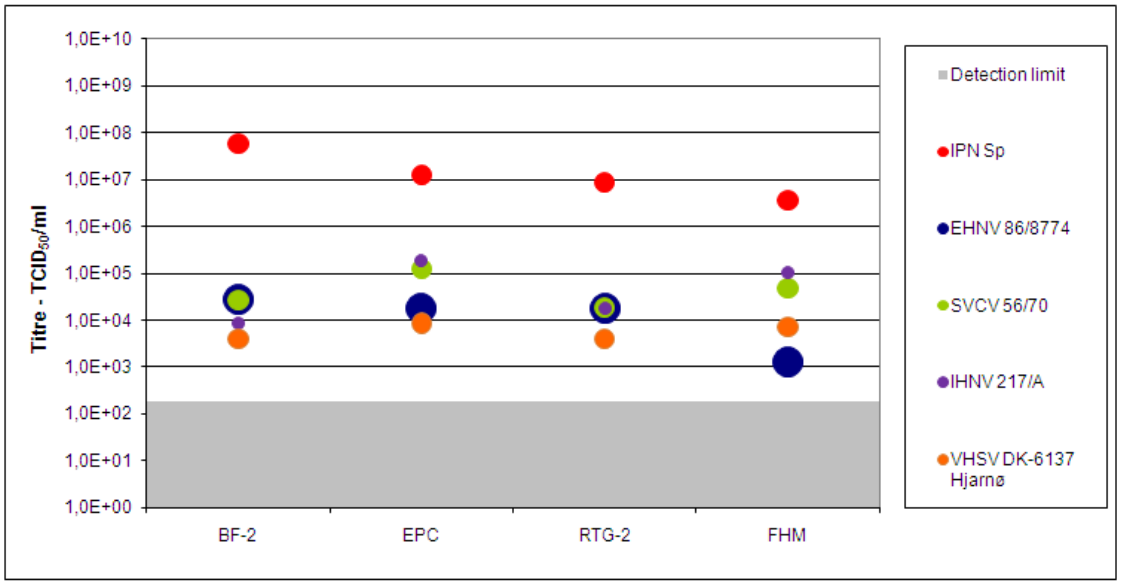

Figure 11. Median virus titres obtained by participants at different cell lines.

Methods used for identification of viruses

(Table 9)

- 22 laboratories used ELISA for identification of viruses
- 23 laboratories used IFAT for identification of viruses
- 12 laboratories used neutralisation tests for identification of viruses
- 36 laboratories used conventional (RT-) PCR for identification of viruses
- 16 laboratories used real-time (RT-) PCR for identification of viruses
- 33 laboratories performed sequencing for identification of viruses.
- 3 laboratory performed REA according to the OIE Aquatic Animals Manuals for identification of ranaviruses
- 1 laboratory performed restriction enzyme fragmentation for identification of ranaviruses

Table 9. Inter-Laboratory Proficiency Test, PT1, 2012 -Results obtained by different test methods in participating laboratories.

Laboratory code number	Score 10/10	ELISA	IFAT	Neutralisation	Conventional (RT-) PCR	Real-time (RT-) PCR	Sequencing	Other	REA
1	10	+	+	+	+	+	+		
2	10	+	+		+		+		
3	10	+	+	+	+			+	+
4	10	+			+		+		
5	10	+	+		+	+	+		
6	8	+	+			+			
7	10	+			+		+		
8	10		+	+	+	+	+		
9	10	+	+	+	+		+	+	
10	10	+	+		+		+		
11	10	+			+		+		
12	10	+	+		+	+	+		
13	10		+		+	+	+		
14	10	+	+		+		+	+	
15	10	+			+		+		
16	10	+	+		+		+	+	
17	10			+	+		+		
18	10	+	+	+	+	+		+	+
19	10	+	+		+		+		
20	8			+	+	+	+		
21	10		+	+	+	+	+		
22	9 Score 9/9	+		+		+			
23	10		+		+		+		
24	10	+	+		+		+		
25	10	+	+		+	+	+		
26	10				+		+		
27	10					+	+		
28	10	+	+	+	+		+		
29	10				+	+	+		
30	10		+	+	+	+	+	+	
31		They never received the PT							
32	0	No reply							
33	10				+			+	

Laboratory code number	Score 10/10	ELISA	IFAT	Neutralisation	Conventional (RT-) PCR	Real-time (RT-) PCR	Sequencing	Other	REA
34	10		+	+	+	+	+		
35	10				+		+		
36	10				+		+		
37	8	+			+		+		
38	10		+		+		+		
39	8	+	+		+		+	+	+
40	10				+		+		
41		They never received the PT							
42	8				+	+			
43	0	No reply							

The methods used for virus identification by participants and the related score obtained were plotted on a graph (Figure 12) to evaluate the presence of correlation. The tendency shown by the graph seems to indicate that participants scoring lower than 10 should focus on the performance of the overall procedure since it is not possible to directly assign the improper use of a single method.
It is clear, however, that if sequencing or REA is not used, a correct answer cannot be made for ampoule II. Two laboratories identified ranavirus but did not type the virus by sequencing or REA for EHNV identification.
2 out of the 6 participants scoring lower than 10 (who delivered results) identified false positive viruses in ampoule I indicating that cross contamination could have occurred at some point in the diagnostic process.
Furthermore 1 out of the 6 participants scoring lower than 10 did not identify SVCV in ampoule III although they isolated the virus on cell cultures.

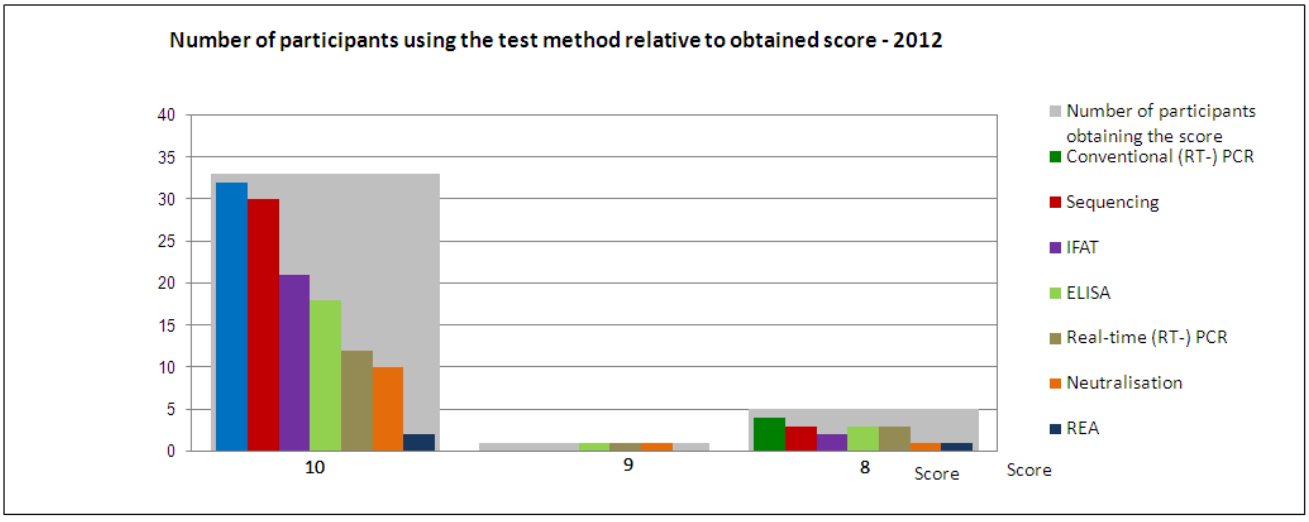

Figure 12. Methods used by participants for identification of viruses in relation to the obtained score.

Genotyping 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 – IPN Sp

- 14 laboratories submitted sequences
- 9 laboratories genotyped the IPNV isolate as belonging to genogroup 5 (Sp)
- 1 laboratory genotyped the IPNV isolate as belonging to genogroup 3
- 4 laboratories did not provide a genogroup despite having sequenced the isolate
- All the laboratories used different references.

Ampoule II – EHNV

- 33 laboratories performed sequencing to identify the virus in ampoule II with correct result
- 2 laboratories performed REA according to the OIE Manual of Diagnostic Tests for Aquatic Animals. Chapter 2.3.1 with correct result
- 1 laboratory performed restriction enzyme fragmentation
- 10 laboratories used primers described in Hyatt et *al*. (2000)
- 13 laboratories used primers described in the OIE Manual of Diagnostic Tests for Aquatic Animals. Chapter 2.3.1
- 3 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 III – SVCV

- 15 laboratories performed sequencing
- 7 laboratories identified the isolate as being genotype Id
- 8 laboratories did not give any genotype of the sequences
- 5 laboratories used primers described in the OIE Manual of Diagnostic Tests for Aquatic Animals. Chapter 2.3.8
- 5 laboratories used primers described in Stone et *al*. (2003)
- 5 laboratories were using primers described in other references or they did not report the reference

Ampoule IV – IHNV

- 23 laboratories performed sequencing to identify the virus in ampoule IV with correct result
- 17 laboratories genotyped the IHNV isolate as belonging to genogroup M
- 1 laboratory genotyped the IHNV isolate as belonging to genogroup M or U
- 1 laboratory genotyped the IHNV isolate as belonging to genogroup U
- 1 laboratory genotyped the IHNV isolate as belonging to genogroup L

- 3 laboratories did not give any genotype of the sequences
- 7 laboratories used primers described in the OIE Manual of Diagnostic Tests for Aquatic Animals, chapter 2.3.4
- 4 laboratories used primers described in Emmenegger et *al*., 2000
- 11 laboratories were using primers described in other references or they did not report the reference

Ampoule V – VHSV genotype Ia

- 22 laboratories performed sequencing to identify the virus in ampoule V with correct result
- 19 laboratories identified the VHSV isolate as genotype Ia
- 2 laboratories identified the VHSV isolate but didn't write the genotype
- 1 laboratory identified the VHSV isolate as genotype I
- 17 laboratories did not genotype the VHSV
- 4 laboratories used the primers described in Einer-Jensen et *al*. (2004)
- 3 laboratories used the primers described in Snow et *al*. (2004)
- 3 laboratories used used primers described in the OIE Manual of Diagnostic Tests for Aquatic Animals, chapter 2.3.9
- 11 laboratories used primers described in other references or did not report the reference

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

Laboratory code number	Score 10/10	Ampoule I	Ampoule II	Ampoule III	Ampoule IV	Ampoule V
		IPN strain Sp	EHNV 86/8774	SCVC 56/70	IHNV 217/A	VHSV DK-6137 Hjarnø
1	10	IPNV	EHNV (Seq)	SVCV 1d	IHNV Genogroup M	VHSV Ia
2	10		EHNV (Seq)			
3	10		EHNV (REA)			
4	10		EHNV (Seq)	SVCV	IHNV Genogroup M	VHSV Ia
5	10		EHNV (Seq)			
6	8					
7	10	IPNV Sp	EHNV (Seq)	SVCV Id3	IHNV Genogroup M	VHSV Ia
8	10		EHNV (Seq)		IHNV Genogroup M or U	VHSV Ia
9	10		EHNV (Seq)	SVCV	IHNV Genogroup M	VHSV Ia
10	10		EHNV (Seq)			
11	10		EHNV (Seq)			
12	10	IPNV Sp	EHNV (Seq)	SVCV	IHNV Genogroup M	VHSV Ia
13	10	IPNV Sp	EHNV (Seq)		IHNV Genogroup M	VHSV Ia
14	10		EHNV (Seq)		IHNV	VHSV I
15	10		EHNV (Seq)			
16	10		EHNV (Seq)		IHNV L	VHSV Ia
17	10		EHNV (Seq)			
18	10		EHNV (REA)			
19	10	IPNV Sp	EHNV (Seq)	SVCV	IHNV Genogroup M	VHSV Ia
20	8	IPNV	EHNV (Seq)		IHNV	VHSV
21	10	IPNV Sp	EHNV (Seq)	SVCV	IHNV Genogroup M	VHSV Ia
22	9 Score 9/9					
23	10		EHNV (Seq)			
24	10	IPNV A2(Sp)	EHNV (Seq)	SVCV Id	IHNV Genogroup M	VHSV Ia
25	10	IPNV Sp	EHNV (Seq)	SVCV Id	IHNV Genogroup M	
26	10	IPNV	EHNV (Seq)	SVCV	IHNV Genogroup M	VHSV Ia
27	10	IPNV III	EHNV (Seq)	SVCV Id	IHNV Genogroup M	VHSV Ia
28	10	IPNV Sp	EHNV (Seq)	SVCV	IHNV Genogroup M	VHSV Ia
29	10	IPNV 5 (Sp)	EHNV (Seq)	SVCV Id	IHNV Genogroup M	VHSV Ia
30	10		EHNV (Seq)			

Laboratory code number	Score 10/10	Ampoule I	Ampoule II	Ampoule III	Ampoule IV	Ampoule V
		IPN strain Sp	EHNV 86/8774	SCVC 56/70	IHNV 217/A	VHSV DK-6137 Hjarnø
31		They never received the PT				
32	0	No reply	No reply	No reply	No reply	No reply
33	10		EHNV (restriction enzyme fragmentation)			
34	10		EHNV (Seq)		IHNV Genogroup M	VHSV Ia
35	10		EHNV (Seq)			
36	10		EHNV (Seq)		IHNV Genogroup M	VHSV Ia
37	8	Ranavirus (Seq)	EHNV (Seq)	SVCV	IHNV	VHSV
38	10		EHNV (Seq)		IHNV Genogroup U	VHSV Ia
39	8		EHNV (Seq)			
40	10	IPNV	EHNV (Seq)	SVCV Id	IHNV Genogroup M	VHSV Ia
41		They never received the PT				
42	8					
43	0	No reply	No reply	No reply	No reply	No reply

Concluding remarks PT1

The inter-laboratory proficiency test 2012 was conducted without major constraints. 92% of parcels were delivered by the shipping companies within 8 days after submission. It was, however, unfortunate that two parcels were 20 days on the way and one parcel was 43 days on the way before delivered to the laboratory primarily due to border controls. Two parcels never left the EURL. In one case this was dued to delivery restriction for such reagents (Iran), in the other case because the fetal bovine serum (FBS) used was from a country not certified free from foot and mouth disease.
In the meantime the batch of serum currently used in the EURL for cell culture has tested negative for foot and mouth disease virus (FMDV) following accredited procedures.

In 2009 EHNV was included in the proficiency test for the first time this year 36 participants were able to correctly identify the virus. Of the laboratories performing PCR based methods, 33 laboratories performed sequencing. Of these laboratories all correctly identified the content. Two laboratories performed REA and one laboratory performed restriction enzyme fragmentation.

In this report (Figures 6-9), all viral 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 can be able to compare the sensitivity of its cell lines to the sensitivity of those used by the other participats. We recommend all participants to evaluate the sensitivity of the cells used in their laboratory in relation to the diagnostic purpose.

In figure 13 it is possible to see the percentage success rate during the last 16 years.
It has to be underlined that the trend of laboratories that are achieving better results is increasing as a consequence of training courses held by the EURL every year and specific missions performed by the EURL team in laboratories that have been performing poor results.

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 17th Annual Meeting of National Reference Laboratories for Fish Diseases to be held 29-30 May 2013 in Copenhagen, Denmark.

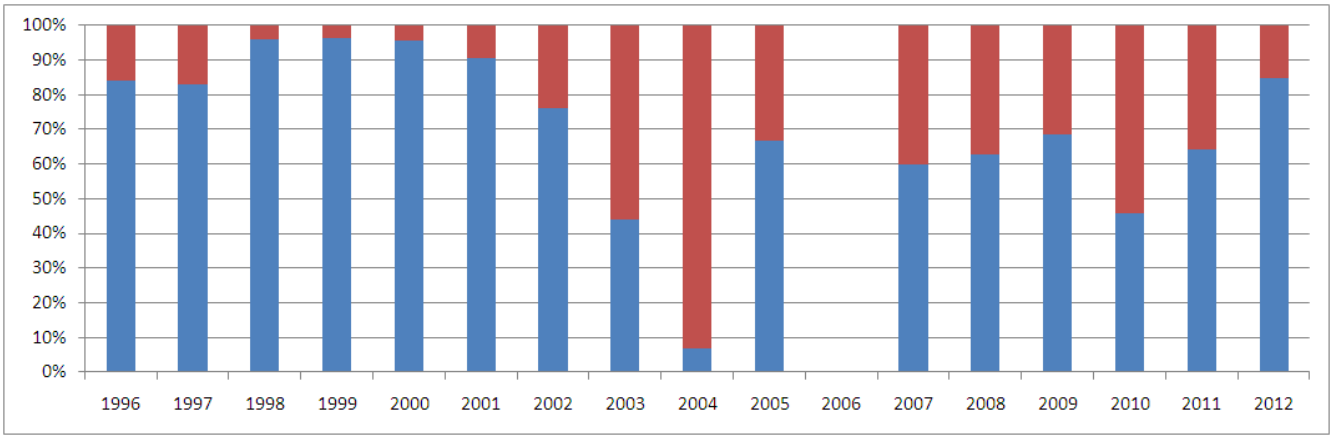

Figure 13 Success-rate of participating laboratories 1996 – 2012

Proficiency test 2, PT2

Four ampoules containing 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.

Code	**Specifications**
Ampoule VI: Aphanomyces piscicida/ invadans spores NJM9701	**Aphanomyces piscicida/invadans spores NJM9701** **Received from:** **Dr. Kishio Hatai, Lab Fish Diseases NVLU Tokyo, Japan** **Reference on isolate:** Kurata O, Kanai H & Hatai K (2000). Hemagglutinating and hemolytic capacities of Aphanomyces piscicida. Fish Pathology - Gyobyo Kenkyu 35, 29-33.
Ampoule VII: KHV-TP 30	**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.** **Cell culture passage number:** **Unknown** **Received from:** **Dr. Sven M. Bergmann, Friedrich-Loeffler-Institut (FLI), Federal Research Institute for Animal Health, Südufer 10, 17393 Greifswald-Insel Riems, Germany**
Ampoule VIII: Sterile pyrogenfree water	**Sterile pyrogen free water**
Ampoule IX: ISAV Glesvaer/2/90	**ISAV Glesvaer/2/90** **Received from:** **Dr. B. Dannevig, OIE Reference Laboratory for ISA, Oslo, Norway** **Cell culture passage number:** **Unknown** **HPR Genotype:** **2** **GenBank accession numbers:** **HQ259676,** **References on isolate:** Dannevig BH, Falk K & Namork E (1995). Isolation of the causal virus of infectious salmon anaemia (ISA) in a long-term cell line from Atlantic salmon head kidney. Journal of General Virology 76, 1353–1359. 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. **References on sequence:** Mérour E, LeBerre M, Lamoureux A, Bernard J, Brémont M & Biacchesi S (2011). Completion of the full-length genome sequence of the infectious salmon anemia virus, an aquatic orthomyxovirus-like, and characterization of mAbs. Journal of General Virology 92, 528-533. **References on genotype:** Table 15. Opinion of the Panel on Animal Health and Welfare of the Norwegian Scientific Committee for Food Safety 26.01.07. Which risk factors relating to spread of Infectious Salmon Anaemia (ISA) require development of management strategies? Dok.nr.06/804, 68 pages.

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 virus reduction as detected by real-time PCR or real-time RT-PCR by approximately 2 Ct values (Table 13). Furthermore, after lyophilisation the content of the ampoules were tested for stability over time.

We tested the C_t values of each virus preparation (ampoule) after the deadline for results submission and after storage at 4°C in the dark and no significant decrease compared to right after lyophilisation was observed. Furthermore we tested the ampoules stored at 30°C and 37°C, respectively, for 10 days without seeing any significant increase in Ct values (approx 1 Ct)except for ISAV where a 2.6 Ct increase was observed after 10 days at 37°C. *A. invadans* went through the same treatment, but only conventional PCR was done, and there was no changes in the size of the band. (Figure 14)

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

Table 12. Ct-value of ampoules VII and IX tested before lyophilisation, immediately after lyophilisation, and last date for submission, storage in the dark at 4°C and 30°C+ 37°C one hour per day, for 10 days, dark conditions and 37°C for 10 days, dark conditions (1 replicate), respectively. For ampoule VI 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 after last date for submission of results (4°C, dark conditions)	Ct value/ presence of band after last date for submission of results (4°C) and after 30°C+37°C one hour per day, for 10 days, dark conditions	Ct value/ presence of band after last date for submission of results (4°C) and after 37°C, for 10 days, dark conditions
Ampoule VI: *Aphanomyces piscicida/invadans* spores NJM9701	a	+	+	+	+	+
	b		+			
	c					
	d					
	e					
	Average					
Ampoule VII: KHV-TP 30	a	17,42	17,96	21,23	21,70	22,45
	b	16,15	18,04			
	c	16,93	18,69			
	d		19,23			
	e		18,07			
	Average	16,83	18,40	21,23	21,70	22,45
Ampoule VIII: Sterile pyrogenfree water	a	-	-	-	-	-
	b	-	-			
	c	-	-			
	d		-			
	e		-			
Ampoule IX: ISAV Glesvaer/2/90	a	22,26	25,59	24,92	25,10	27,60
	b	24,37	24,34			
	c	22,43	23,94			
	d	21,74	25,08			
	e		24,91			
	Average	22,70	24,77	24,92	25,10	27,60

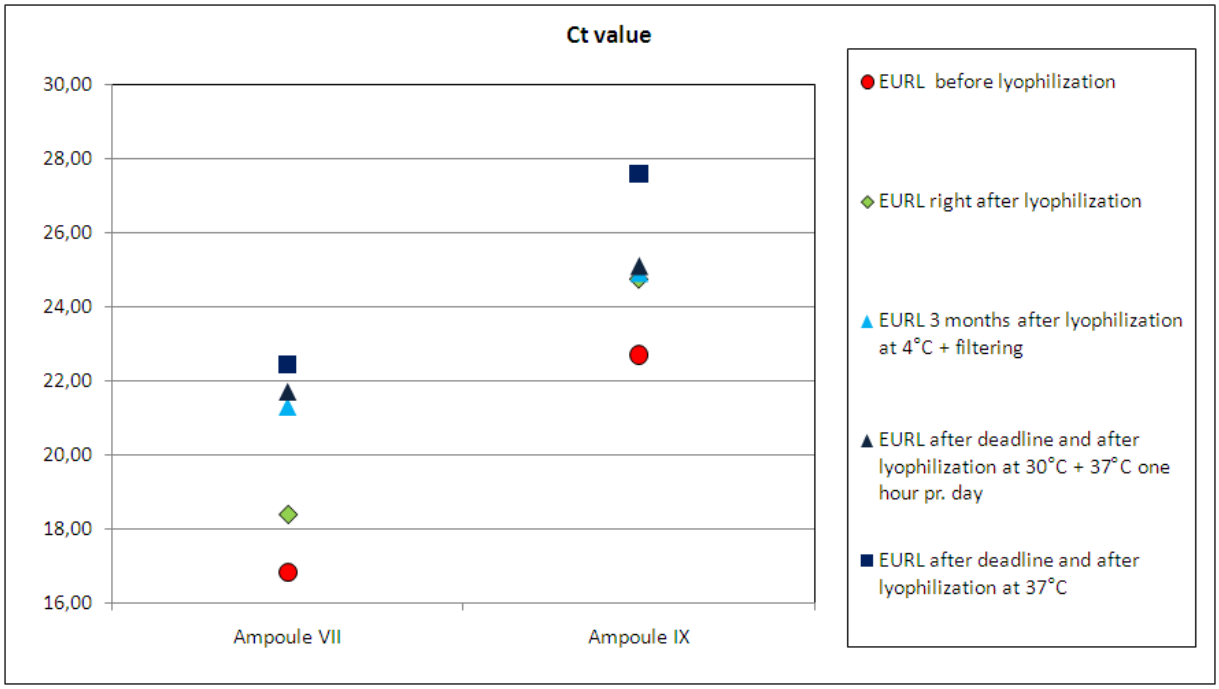

Figure 14. Ct values before and submission results deadline and after lyophilisation. The red dot (EURL before lyophilisation in the legend) corresponds to the Ct value of the undiluted virus.

Pathogen 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.

In addition the participants were asked to identify the oomycete *Aphanomyces invadans* the causative agent of EUS if present in the ampoules

It was requested that the pathogens 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, 2012 - Virus identification.

Laboratory code number	Score (Maximum 8/8)	Answer recived at EURL	Ampoule VI	Ampoule VII	Ampoule VIII	Ampoule IX
			A.invadans	KHV	Sterile pyrogenfree water	ISAV
1	8	23-10-2012	*A.invadans*	KHV (Seq)	Not *A.invadans*, KHV or ISAV	ISAV (Seq)
2	8	24-10-2012	*A.invadans*	KHV	Not *A.invadans* KHV or ISAV	ISAV (Seq) Ct=30,9
3	8	05-11-2012	*A.invadans*	KHV	Not *A.invadans*, KHV or ISAV	ISAV
4	8	01-11-2012	*A.invadans* (Seq)	KHV	Not *A.invadans*, KHV or ISAV	ISAV (Seq)
5	8	05-11-2012	*A.invadans* (Seq)	KHV (Seq) Engelsma: Ct=20,98 Gilad Ct=18,85	Not *A.invadans*, KHV or ISAV	ISAV (Seq) Ct=26,08
6	4	05-11-2012	Not ISAV or KHV	KHV	Not ISAV or KHV	ISAV
7	8	29-10-2012	*A.invadans*	KHV (Seq)	Not *A.invadans*, KHV or ISAV	ISAV (Seq)
8	8	13-10-2012	*A.invadans*	KHV Ct=24,9	Not *A.invadans*, KHV or ISAV	ISAV (Seq)
9	8	05-11-2012	*A.invadans*	KHV Ct=18-19	Not *A.invadans*, KHV or ISAV	ISAV (Seq)
10	8	05-11-2012	*A.invadans*	KHV	Not *A.invadans*, KHV or ISAV	ISAV
11	8	31-10-2012	*A.invadans*	KHV Ct=21,8	Not *A.invadans*, KHV or ISAV	ISAV Ct=25,8
12	8	18-10-2012	*A.invadans* (Seq)	KHV (Seq) Ct=19,6	Not *A.invadans*, KHV or ISAV	ISAV (Seq) Ct=30,5
13	8	31-10-2012	*A.invadans* (Seq)	KHV (Seq) Ct=26,3	Not *A.invadans*, KHV or ISAV	ISAV (Seq) Ct24,4
14	8	02-11-2012	*A.invadans* (Seq)	KHV (Seq)	Not *A.invadans*, KHV or ISAV	ISAV (Seq)
15	4	30-10-2012	Not ISAV or KHV	KHV Ct=21,02	Not ISAV or KHV	ISAV Ct=29,42
16	8	05-11-2012	*A.invadans*	KHV Ct=17,49	Not *A.invadans*, KHV or ISAV	ISAV
17	8	05-11-2012	*A.invadans*	KHV	Not *A.invadans*, KHV or ISAV	ISAV (Seq)
18	8	05-11-2012	*A.invadans*	KHV	Not *A.invadans*, KHV or ISAV	ISAV
19	8	05-11-2012	*A.invadans* (Seq)	KHV (Seq)	Not *A.invadans*, KHV or ISAV	ISAV (Seq)
20	8	05-11-2012	*A.invadans* (Seq)	KHV (Seq) Ct=20,29	Not *A.invadans*, KHV or ISAV	ISAV (Seq)
21	8	05-11-2012	*A.invadans* (Seq)	KHV (Seq) Ct=16,58	Not *A.invadans*, KHV or ISAV	ISAV (Seq)
22[1]	6/6	05-11-2012	*A.invadans*	KHV Ct=20,56	Not *A.invadans*, or KHV	Not *A.invadans*, KHV
23	8	19-10-2012	*A.invadans* Ct=31,19	KHV Ct=19,2	Not *A.invadans*, KHV or ISAV	ISAV Ct=31,91
24	8	02-11-2012	*A.invadans* (Seq)	KHV (Seq)	Not *A.invadans*, KHV or ISAV	ISAV (Seq)
25	8	24-10-2012	*A.invadans* (Seq)	KHV (Seq) Ct=24,88	Not *A.invadans*, KHV or ISAV	ISAV (Seq) Ct=27,72
26[3]	6/6	02-11-2012	Not KHV or ISAV	KHV (Seq) Ct=20,7	Not KHV or ISAV	ISAV (Seq) Ct=28,76
27	8	05-11-2012	*A.invadans*	KHV (Seq) Ct=19,84	Not *A.invadans*, KHV or ISAV	ISAV (Seq) Ct=24,61
28	4	02-11-2012	Not KHV or ISAV	KHV (Seq)	Not KHV or ISAV	ISA (Seq)
29	4	06-11-2012	*A.invadans* (Seq)	Not *A.invadans*, KHV or ISAV	Not *A.invadans*, KHV or ISAV	ISAV (Seq) Ct=22,49
30[2]	4/4	02-11-2012	Negative	Negative	Negative	ISAV Ct - (29,58 and 29,82)
31			They never received the PT			
32	0	n/a	No reply	No reply	No reply	No reply
33	8	02-11-2012	*A.invadans*	KHV	Not *A.invadans*,	ISAV (Seq)

Laboratory code number	Score (Maximum 8/8)	Answer recived at EURL	Ampoule VI	Ampoule VII	Ampoule VIII	Ampoule IX
			A.invadans	KHV	Sterile pyrogenfree water	ISAV
					KHV or ISAV	
34	8	01-11-2012	*A.invadans* (Seq)	KHV	Not *A.invadans*, KHV or ISAV	ISAV (Seq) Ct=22
35	8	05-11-2012	*A.invadans*	KHV	Not *A.invadans*, KHV or ISAV	ISAV
36	8	05-11-2012	*A.invadans*	KHV (Seq)	Not *A.invadans*, KHV or ISAV	ISAV (Seq)
37	8	05-11-2012	*A.invadans*	KHV	Not *A.invadans*, KHV or ISAV	ISAV (Seq)
38	8	31-10-2012	*A.invadans*	KHV Ct=21,89	Not *A.invadans*, KHV or ISAV	ISAV
39	6	02-11-2012	*A.invadans*	KHV	Not *A.invadans*, KHV or ISAV	ISAV,KHV
40	8	05-11-2012	*A.invadans* (Seq)	KHV (Seq)	Not *A.invadans*, KHV or ISAV	ISAV (Seq)
41			They never received the PT			
42	2	05-11-2012	*A.invadans*	Not ISAV, KHV or A.invadans	KHV	KHV, ISAV (Ct=30,33 (ISA)
43	0	n/a	No reply	No reply	No reply	No reply

[1] ISAV identification is performed by another NRL
[2] Analysed for the presence of ISAV only
[3] *A. invadans* is enzootic, as such, the Laboratory does not have responsibility for its diagnosis and so A. invadans testing was not undertaken.
n/a: *not applicable*

Identification of content

- 39 laboratories submitted results
- 30 laboratories correctly identified all four ampoules
- 33 laboratories tested for all three listed pathogens
- 38 laboratories tested for ISAV
- 38 laboratories tested for KHV
- 34 laboratories tested for *A. invadans*
- 1 laboratory tested for ISAV only
- 5 laboratories did not test for *A. invadans* but did for ISAV and KHV
- 1 laboratory did not test for ISAV but did for *A. invadans* and KHV
- 2 laboratories did not submit any results
- 2 laboratories did not receive the parcel

Ampoule VI – *Aphanomyces invadans*

- 34 laboratories correctly identified *A. invadans*
- 5 laboratories did not examine for *A. invadans*

Ampoule VII – KHV

- 36 laboratories correctly identified KHV
- 2 laboratory tested for but did not identify KHV
- 1 laboratories did not examine for KHV

Ampoule VIII – Sterile pyrogenfree water

- 32 laboratories correctly identified Not *A.invadans, KHV or* ISAV
- 1 laboratory found KHV

Ampoule IX – ISAV

- 36 laboratories correctly identified only ISAV
- 2 laboratories identified ISAV and KHV
- 1 laboratories did not examine for ISAV

Scores

We have assigned a score of 2 for each correct answer (Table 13), giving the possibility for obtaining a maximum score of 8. Incorrectly finding of pathogens not present in the ampoules gives the score 0.

Of the 38 laboratories submitting results 30 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, another laboratory did not examined for *A. invadans,* this laboratory obtain the score 6 out of 6 possible. Three laboratories did not examine for *A. Invadans,* but they did not inform us about that, these three laboratories obtained a score of 4. Two laboratories did not submit any results and obtained the score 0. 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. A diagram of the scoring obtained by the laboratories is shown in Figure 15.

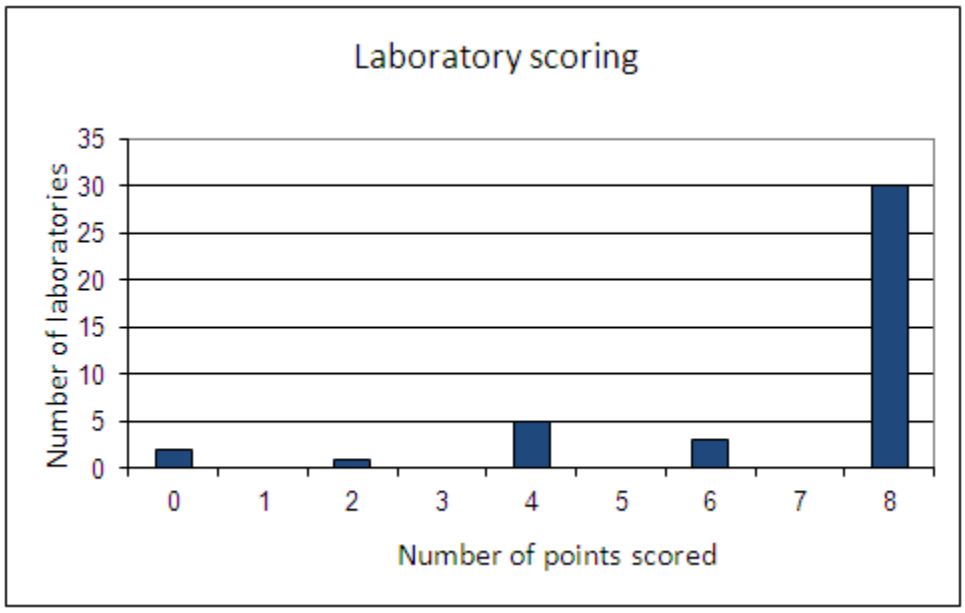

Figure 14. Obtained score by participants.

Methods applied

The following methods were used by the participants:

- 30 laboratories used *A. Invadans* PCR
- 2 laboratory used *A. Invadans* Real-time PCR
- 1 laboratory found the Ct value for *A. Invadans*
- 12 laboratories performed sequencing for *A. Invadans*

- 29 laboratories used KHV PCR
- 17 laboratories used KHV Real-time PCR
- 11 laboratories used both KHV real-time PCR and KHV PCR

- 30 laboratories used ISAV RT-PCR
- 16 laboratories used ISAV real-time RT-PCR
- 9 laboratories used both ISAV real-time RT-PCR and ISAV RT-PCR

Table 14. Inter-Laboratory Proficiency Test, PT2, 2012 - Results obtained by different test methods in participating laboratories.

Laboratory code number	Score 8/8	PCR	Real-time PCR	Ct	Sequencing	PCR	Real-time PCR	Ct	Sequencing	RT-PCR	Real-time RT-PCR	Ct	Sequencing	Other
		A. Invadans				KHV				ISAV				
1	8	+				+			+	+			+	
2	8	+				+				+	+	+	+	
3	8	+				+				+				
4	8	+			+	+				+			+	
5	8	+			+	+	+	+	+	+	+	+	+	
6	4						+				+			
7	8	+				+			+	+			+	IFAT
8	8	+					+	+		+			+	
9	8	+				+	+	+		+			+	
10	8	+				+				+				
11	8	+				+	+	+			+	+		
12	8	+			+	+	+	+	+	+	+	+	+	
13	8	+				+	+	+	+	+	+	+	+	
14	8				+				+	+			+	
15	4						+	+			+	+		
16	8	+				+	+	+		+				Cultivation and nested PCR
17	8	+				+				+			+	
18	8	+					+				+			Cell cultivation and IFAT
19	8	+			+	+			+	+			+	
20	8	+			+	+	+	+	+	+			+	
21	8	+			+	+	+	+	+	+			+	Cultivation
22[1]	6 Score 6/6		+				+	+						
23	8		+	+		+	+	+		+	+	+		
24	8	+			+	+			+	+			+	Cultivation and IFAT
25	8				+			+	+				+	Cultivation and IFAT
26[3]	6 Score 6/6					+	+	+	+	+	+	+	+	

Laboratory code number	Score 8/8	PCR	Real-time PCR	Ct	Sequencing	PCR	Real-time PCR	Ct	Sequencing	RT-PCR	Real-time RT-PCR	Ct	Sequencing	Other
		A. Invadans				KHV				ISAV				
27	8	+					+	+	+	+	+	+	+	Cultivation
28	4					+			+	+			+	
29	4	+			+						+	+	+	
30[2]	4 Score 4/4										+	+		
31														
32	0													
33	8	+				+				+			+	
34	8	+			+	+				+	+	+	+	
35	8	+				+				+				
36	8	+				+			+	+			+	
37	8	+				+				+			+	
38	8	+				+	+	+		+				
39	6	+				+				+				
40	8	+			+	+			+	+	+		+	
41														
42	2	+				+					+	+		
43	0													

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.

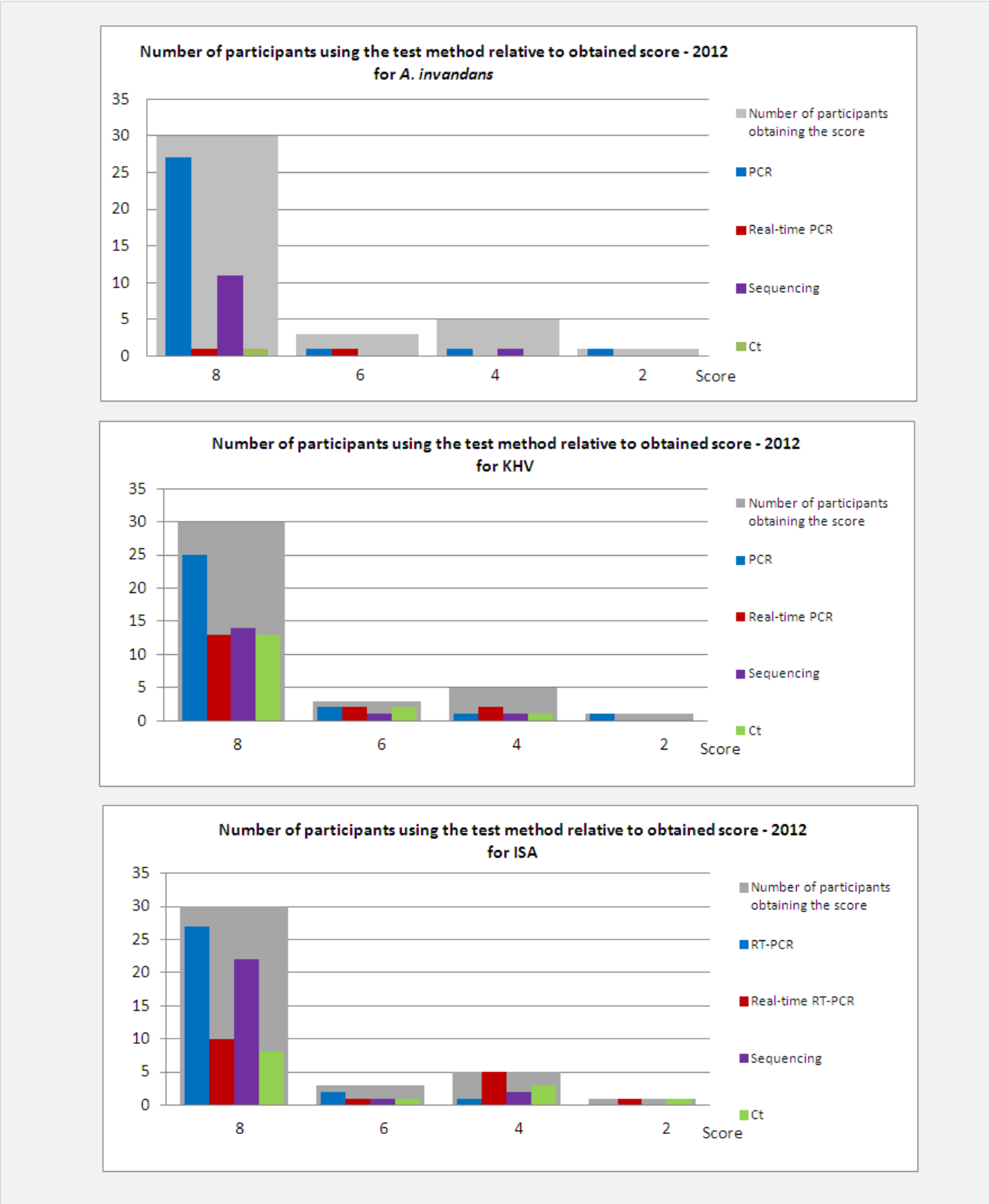

Figure 15. Use of various methods by the participants in relation to their scores

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

- 1 laboratory found the Ct value for *A. Invadans*
- 12 laboratories performed sequencing for *A. Invadans*

- 16 laboratories found the Ct value for KHV
- 16 laboratories performed sequencing for KHV

- 13 laboratories found the Ct value for ISAV
- 25 laboratories performed sequencing for ISAV

Concluding remarks PT2

Considering that this was the third time that the EURL provided a proficiency test on ISAV and KHV identification, and the second time that the EURL provided a proficiency test on *A. invadans*, we consider that most participants obtained satisfying results. Out of 34 laboratories testing for *A. invadans* all 34 identified the pathogen in ampoule VI. Out of 38 laboratories performing KHV identification, 36 laboratories identified KHV in ampoule VII. Out of 39 laboratories 32 laboratories identified Not *A.invadans, KHV or* ISAV in ampoule VIII. Out of 38 laboratories performing ISAV identification 36 identified ISAV in ampoule IX. Very significant improvement in the proficiency of identifying and typing these pathogens has been observed during these 3 years. In autumn 2012 the European Commission decided to de-list EUS and it is officially no more considered as an exotic disease in the Union. However we find that a certain level of preparedness for the introduction of this disease in European aquaculture should be maintained. But it is still unclear whether the pathogen will be included in future inter-laboratory proficiency tests or not and the topic will be discussed at our next Annual Meeting in May 2013.

It is an appreciated matter of fact that many laboratories are putting efforts in performing genetic characterizing the isolates through sequence analysis as it is important that laboratories can discriminate between isolates as e.g. pathogenic ISAV strains with deletions 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.

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 17th Annual Meeting of National Reference Laboratories for Fish Diseases to be held 29-30 May 2013 in Copenhagen, Denmark.

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European Union Reference laboratory for Fish diseases
National Veterinary Institute, Technical University of Denmark, 4 February 2013

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