EURL for Fish Diseases

Report of the Inter-Laboratory Proficiency Test 2013

for identification of

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

and identification of

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

Organised by the
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PT Reg. no.: 515

DANAK

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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 October 2013.
Both PT1 and PT2 are accredited by the Danish Accreditation and Metrology Fund –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 on cell cultures 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 five coded ampoules (VI-X). The ampoules contained ISAV and KHV. Furthermore, one ampoule contained *Aphanomyces invadans* spores and one sterile pyrogen-free water, see table 9. The test was designed to primarily assess the ability of participating laboratories to identify the notifiable fish pathogens ISAV and KHV (listed in Council Directive 2006/88/EC). *A. invadans* was in 2012 delisted from the Council Directive but was nevertheless included also this year in PT2 following open discussions and agreement at the Annual Workshop held the 29th and 30th of May 2013. 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 have been possible to replicate them in cell cultures.

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. Accreditation data will not be included in this report but will be presented at the 18th Annual Workshop of the NRLs for Fish Diseases June 2014 in Copenhagen. Participants were asked to reply latest November 29th 2013.

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 to 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 during transportation. The loggers were programmed to mark if the temperature had exceeded 30°C at some point during transportation. If the temperature encountered during transport had been detrimental to the viability of the virus in the test, the information in the included loggers should show it.

Shipment and handling

Within two days, the tests were delivered to 26 participants, 18 participants in EU and 8 participants outside EU; due to a mistake in the delivery process by the shipping company 6 laboratories received their own parcels after 7 – 9 days. 2 laboratories (outside EU) received the test after 33 days; 1 participant collected the test directly from Denmark, finally one participant received an extra test because the first package was damaged during transport (Figure 1). 33 parcels were sent without cooling elements while for 10 countries outside EU, the packaging of the parcel was improved using thermic insulated box with cooling elements; the temperature in these special parcels was recorded with loggers. The temperatures in 4 parcels were below 30°C. The temperature exceeded 31°C in one parcel. Five loggers failed to record the temperature during transport.

Considering the high stability that characterize the lyophilized pathogens in glass ampoules as demonstrated in PT 2012, and taking into account that during shipment the 5 working loggers in the parcels never recorded higher temperatures than the one tested after deadline in PT 2012, it is assumed that all parcels were delivered in conditions that did not significantly affect the content in the ampoules.

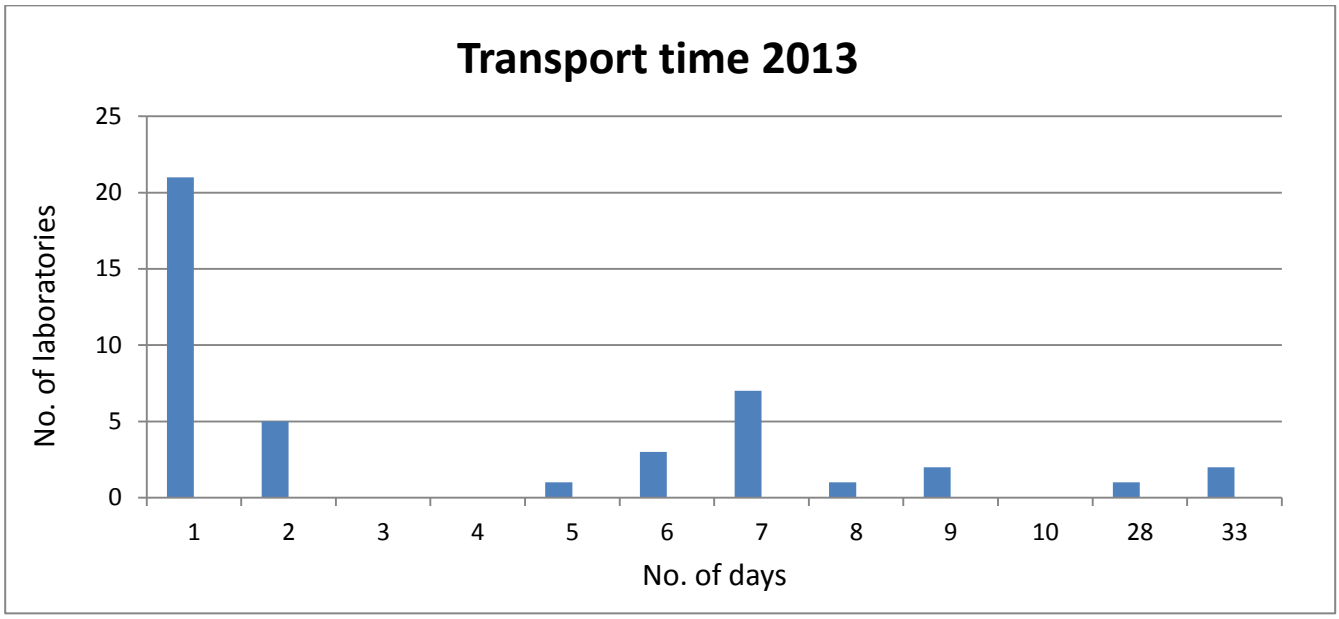

Figure 1. Transport time for the parcels to reach the participants.

Participation

PT1 and PT2: 43 laboratories received the proficiency tests, and all participants submitted results within the deadline.

Figure 2 show how many laboratories that participated in the proficiency test from 1996 to 2013.

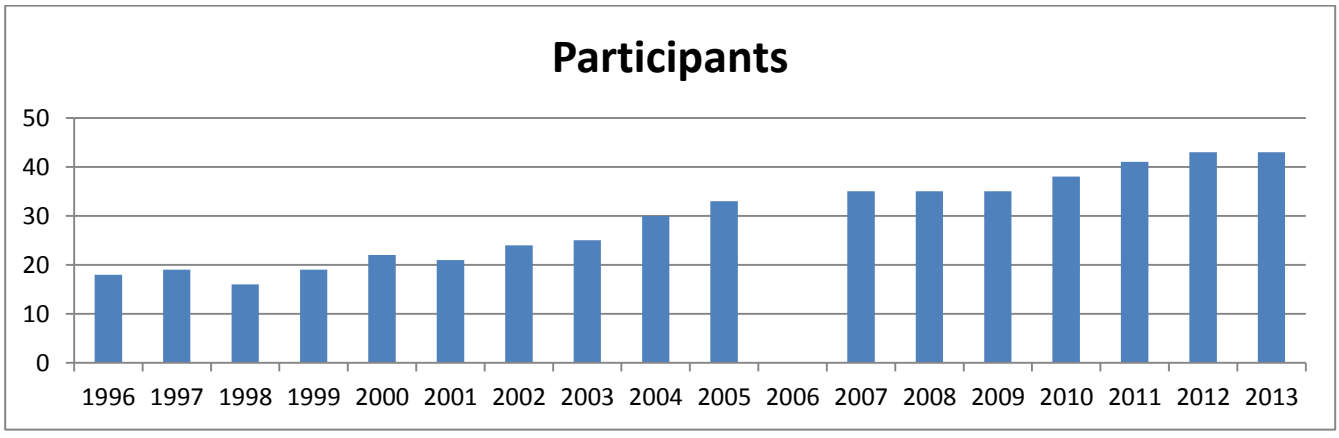

Figure 2. Participants in the EURL proficiency test over the years.

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, Canada, Croatia, Faroe Islands, Iceland, Japan, New Zealand, Norway, 2 from P.R. China, Serbia, Switzerland, Turkey, Republic of Korea, Singapore, and 2 from USA. The Belgian NRL covers both Belgium and Luxembourg and the Italian NRL covers Italy, Cyprus and Malta for identification of all listed diseases. Figure 3 shows the worldwide distribution of the participating NRLs.

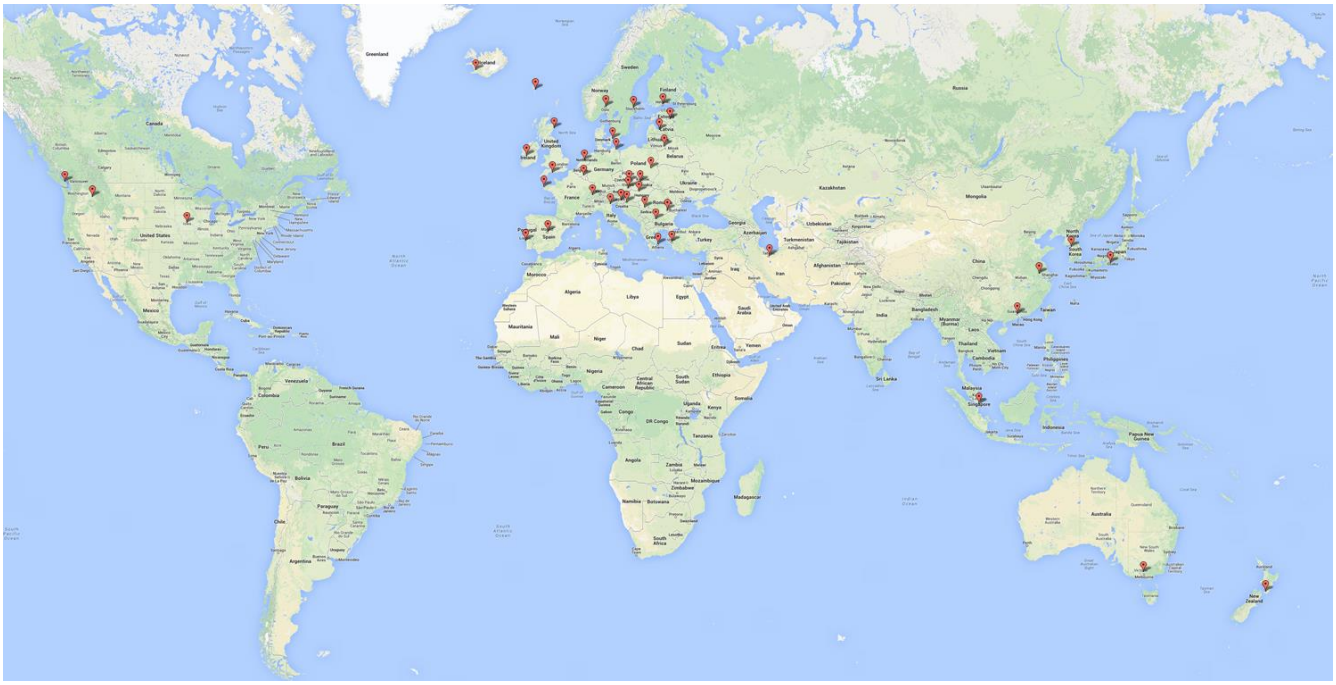

Figure 3. Worldwide distribution of the participants in the EURL proficiency test 2013.

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 + VHSV DK-6137 (Hjarnø)	**IPNV strain Sp:** Infectious Pancreatic Necrosis (IPN) virus isolated from Danish rainbow trout. Reference strain of IPNV serotype Sp. **Isolated by:** National Veterinary Institute, Technical University of Denmark. **Cell culture passage number:** 18 **References 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.
	VHSV strain DK-6137: The isolate originated from an outbreak of VHS with high mortality in sea water aquaculture. **Isolated by**: National Veterinary Institute, Technical University of Denmark. **Cell culture passage number:** 3 **Genotype:** Ia **GenBank accession number:** AY546593 **References 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.
Ampoule II: IHN virus 217/A	**IHNV 217/A:** First Italian IHNV isolate from rainbow trout. **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 III: EHNV Isolate 86/8774	**EHNV Isolate 86/8774:** Australian freshwater isolate 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*

Code	Specifications
	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 IV: SVCV strain 56/70	**SVCV strain 56/70:** 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.
Ampoule V: EMEM	**Sterile medium employed for cell culture cultivation**

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 to participants the EURL tested 5 replicates of each ampoule 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 IHNV with 1-2 log reduction, while for VHSV, IPNV, SVCV and EHNV almost no reduction was observed (table 2 and figure 5).
All titres of the lyophilised viruses were above detection level, except for IHNV on BF-2 cells. As participants, however, 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 during storing. We have previously shown that lyophilised virus, kept in glass-sealed ampoules, are 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 are 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 showed that lyophilised virus in glass-sealed ampoules are stable when the temperature was 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 titres right after lyophilization.

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 tested in four cell lines before lyophilisation, immediately after lyophilisation (median titre of 5 replicates), and after 3 months of storage in the dark at 4°C. (1 replicate).

Ampoule No.	Cell line	Titre before lyophilisation	Median titre right after lyophilisation	Titre 3 months after lyophilisation (4°C, dark conditions)
		$TCID_{50}$/ml	$TCID_{50}$/ml	$TCID_{50}$/ml
Ampoule I: IPNV strain Sp + VHSV DK-6137 (Hjarnø)	BF-2	1,9E+07	8,6E+06	1,3E+07
	EPC	1,9E+07	2,7E+06	8,6E+06
	RTG-2	4,0E+07	4,0E+06	Not performed
	FHM	1,3E+07	2,7E+06	Not performed
Ampoule II: IHN virus 217/A	BF-2	1,9E+04	< 1,9E+02	5,9E+02
	EPC	1,3E+06	1,3E+03	2,7E+04
	RTG-2	8,6E+04	2,7E+02	Not performed
	FHM	5,9E+05	1,9E+03	Not performed
Ampoule III: EHNV Isolate 86/8774	BF-2	1,9E+05	2,7E+05	4,0E+05
	EPC	1,3E+05	4,0E+04	8,6E+04
	RTG-2	5,9E+04	1,3E+04	Not performed
	FHM	< 1,9E+02	< 1,9E+02	Not performed
Ampoule IV: SVCV strain 56/70	BF-2	1,3E+05	1,3E+06	4,0E+05
	EPC	1,9E+05	2,7E+06	2,7E+05
	RTG-2	5,9E+04	1,9E+05	Not performed
	FHM	4,0E+05	2,7E+06	Not performed
Ampoule V: EMEM	BF-2	< 1,9E+02	< 1,9E+02	< 1,9E+02
	EPC	< 1,9E+02	< 1,9E+02	< 1,9E+02
	RTG-2	< 1,9E+02	< 1,9E+02	Not performed
	FHM	< 1,9E+02	< 1,9E+02	Not performed

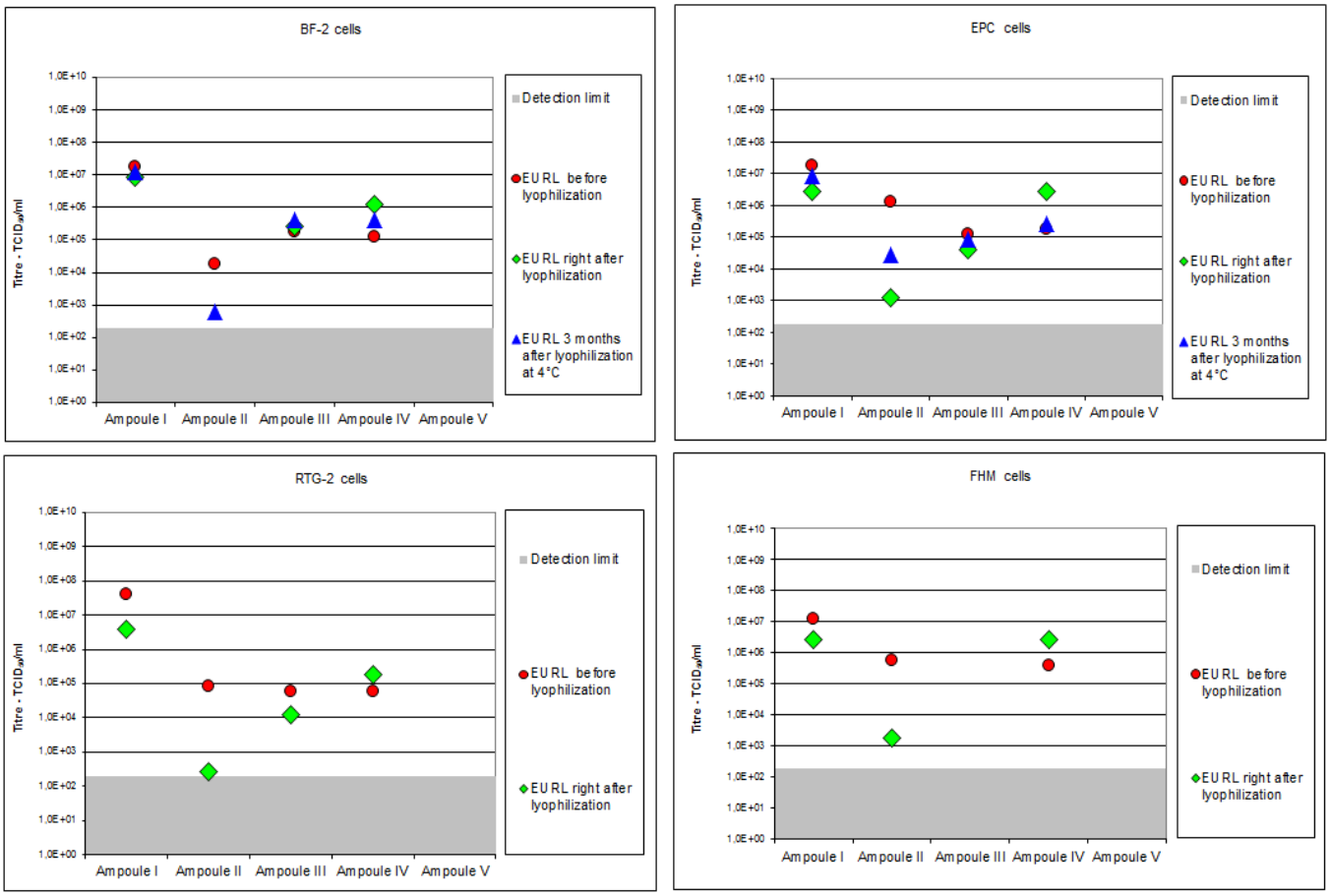

Figure 4. Virus titres in different cell lines before freeze-drying, right after freeze drying and 3 months after deadline. Grey area is below detection level.

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 protocol for quantification 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)**.** Virus 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 5 to 8, 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 five 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, 2013- Virus identification.

Laboratory code number	Score	Answer recived at EURL	Ampoule I	Ampoule II	Ampoule III	Ampoule IV	Ampoule V
			IPNV sp and VHSV DK-6137 Hjarnø	IHN virus 217/A (DTU Vet protocol no. 4008)	EHNV 86/8774	SVCV 56/70	EMEM medium
1	10/10	09-10-13	VHSV and IPNV	IHNV	EHNV	SVCV	No CPE
2	8/10	29-11-13	VHSV & IPNV	IHNV	EHNV	SVCV	SVCV
3	10/10	30-11-13	IPNV and VHSV	IHNV	EHNV	SVCV	No virus detected
4	10/10	30-11-13	VHSV IPNV	IHNV	EHNV	SVCV	Negative
5	10/10	29-11-13	VHSV and IPNV	IHNV	Ranavirus (EHNV)	SVCV	Negative
6	10/10	29-11-13	VHSV IPNV	IHNV	EHNV	SVCV	No virus
7	7/10	29-11-13	VHSV	IHNV	EHNV	Negative (Not: VHSV; IHNV; Ranavirus; IPNV; SVCV)	Negative (Not: VHSV; IHNV; Ranavirus; IPNV; SVCV)
8	10/10	28-11-13	VHSV+IPNV (Sp)	IHNV	EHNV	SVCV	negative
9	10/10	25-11-13	VHSV genotype Ia, IPNV genogroup 5 (sp)	IHNV, genotype M	EHNV	SVCV Id3	no virus
10	10/10	28-11-13	IPNV and VHSV	IHNV	EHNV	SVCV	Negative
11	7/10	30-11-13	IPNV(sp)/VHSV	IHNV	EHNV(Seq)*	SVCV	Ranavirus
12	8/10	29-11-13	VHSV and IPNV	IHNV	EHNV	SVCV	IHNV
13	9/9**	29-11-13	IPNV/VHSV	IHNV	Ranavirus	SVCV	Negativ
14	10/10	29-11-13	VHSV and IPNV	IHNV	EHNV	SVCV	NO VIRUS
15	10/10	29-11-13	VHSV,IPNV	IHNV	EHNV	SVCV	NEG
16	10/10	26-11-13	VHSV, IPNV	IHNV	EHNV	SVCV	-
17	10/10	28-11-13	VHSV; IPNV	IHNV	EHNV	SVCV	neg
18	10/10	20-11-13	IPNV/VHSV	IHNV	EHNV	SVCV	No Viruses isolated by BF2/EPC cell culture
19	9/10	28-11-13	VHSV + Aquabirnavirus	IHNV	EHNV	SVCV	No Virus Detected
20	10/10	27-11-13	VHSV+IPNV	IHNV	EHNV	SVCV	NEGATIVE
21	10/10	28-11-13	VHSV, IPNV	IHNV	EHNV	SVCV	-
22	9/10	29-11-13	VHSV	IHNV	EHNV	SVCV	negative
23	10/10	29-11-13	VHSV and IPNV	IHNV	EHNV	SVC	Virus not detcted
24	9/10	25-11-13	VHSV + IPNV	IHNV	EHNV	SVCV	no VHSV,no IHNV,no SVCV no IPNV,no RANAV
25	10/10	29-11-13	VHSV, IPNV	IHNV	EHNV	SVCV	Negative
26	10/10	12-11-13	VHSV+IPNV	IHNV	EHNV	SVCV	NEGATIVE CONTROL
27	10/10	29-11-13	VHSV / IPNV	IHNV	EHNV	SVCV	no cpe (no VHSV, no IHNV, noIPNV, no SVCV, no EHNV)
28	10/10	12-11-13	VHSV and IPNV	IHNV	EHNV	SVCV	Negative
29	10/10	26-11-13	VHSV+IPNV	IHNV	EHNV	SVCV	-
30	10/10	25-11-13	VHSV + IPNV	IHNV	EHNV	SVCV	Not VHSV, HNV,EHNV, not Ranavirus, IPNV nor SVCV
31	10/10	27-11-13	VHSV + IPNV	IHNV	EHNV	SVCV	Negative
32	7/10	08-11-13	VHSV and IPNV	IHNV	no VHSV, IPNV,IHNV,SVCV	SVCV	no VHSV,IPNV,IHNV,SVCV
33	10/10	29-11-13	VHS-VIRUS & IPN-VIRUS	IHN-VIRUS	EHN-VIRUS	SVC-VIRUS	NOT VHS NOT IHN NOT EHN NOT IPN NOT SVC
34	9/10	27-11-13	IPNV,VHSV	IHNV	EHNV*	SVCV	-
35	10/10	28-11-13	VHSV and IPNV	IHNV	EHNV	SVC	Negative

Laboratory code number	Score	Answer recived at EURL	Ampoule I	Ampoule II	Ampoule III	Ampoule IV	Ampoule V
			IPNV sp and VHSV DK-6137 Hjarnø	**IHN virus 217/A (DTU Vet protocol no. 4008)**	**EHNV 86/8774**	**SVCV 56/70**	**EMEM medium**
36	10/10	14-11-13	VHSV & IPNV	IHNV	EHNV	SVCV	no pathogen identified
37	8/10	29-11-13	VHSV,IPNV	IHNV	EHNV	SVCV	VHSV
38	10/10	27-11-13	VHSV & IPNV	IHNV	EHNV	SVCV	No Virus Isolated
39	10/10	29-11-13	VHS+IPN	IHNV	EHNV	SVCV	Negative
40	10/10	25-11-13	VHSV and IPNV	IHNV	EHNV	SVCV	Not VHSV, not IHNV, not Ranavirus, not IPNV, not SVCV
41	10/10	25-11-13	VHSV & IPNV	IHNV	EHNV	SVCV	NO VHSV, IPNV, EHNV and SVCV detectable
42	10/10	27-11-13	VHSV & IPNV	IHNV	EHNV	SVCV	not VHSV, IHNV, EHNV, SVCV
43	10/10	29-11-13	VHSV and IPNV	IHNV	EHNV	SVCV	no virus detected

*Sequence provided does not match with EHNV sequence available on GenBank.
**Did not sequence the ranavirus isolate to identify potential EHNV

Table 4. Inter-Laboratory Proficiency Test, PT1, 2013 – Results of titration of ampoule I.

Ampoule I - IPNV + VHSV

Laboratory code number	Virus Identification	Titre in			
		BF-2	EPC	RTG-2	FHM
1	VHSV and IPNV	1,3E+07	1,9E+07	2,7E+06	1,3E+07
2	VHSV & IPNV	5,9E+04	2,7E+05		
3	IPNV and VHSV	2,7E+06	5,9E+06		
4	VHSV IPNV	4,0E+06	5,9E+05		
5	VHSV and IPNV	1,3E+05	4,0E+05	8,6E+05	
6	VHSV , IPNV	4,0E+06	8,6E+06	1,3E+07	5,9E+06
7	VHSV		1,3E+07	1,3E+06	
8	VHSV+IPNV (Sp)	8,6E+05	8,6E+05	1,3E+06	1,3E+06
9	VHSV genotype Ia, IPNV genogroup 5 (sp)	2,7E+07	2,7E+06		
10	IPNV and VHSV	2,7E+07	1,3E+07	4,0E+06	
11	IPNV(sp)/VHSV	1,3E+07	1,3E+07		
12	VHSV and IPNV		1,3E+07		8,6E+06
13	IPNV/VHSV	8,6E+06			2,7E+06
14	VHSV and IPNV		4,0E+06		1,3E+06
15	VHSV,IPNV	2,7E+07	5,9E+06		
16	VHSV, IPNV	4,0E+06			1,3E+06
17	VHSV; IPNV		1,3E+06	1,3E+06	
18	IPNV/VHSV	1,9E+05	4,0E+06		
19	VHSV + Aquabirnavirus	1,3E+07	5,9E+06		
20	VHSV+IPNV	5,9E+06	5,9E+06		
21	VHSV, IPNV	8,6E+07	5,9E+06		
22	VHSV	1,3E+04	1,3E+04		
23	VHSV and IPNV	1,9E+07	4,0E+07		
24	VHSV + IPNV	5,9E+06	2,7E+06		
25	VHSV, IPNV	1,3E+08	1,9E+08	2,7E+07	1,3E+08
26	VHSV+IPNV	1,9E+07	4,0E+07	1,9E+07	
27	VHSV / IPNV	4,0E+06	4,0E+06		
28	VHSV and IPNV	2,7E+06			5,9E+06
29	VHSV+IPNV	1,3E+04	5,9E+03	5,9E+03	8,6E+03
30	VHSV + IPNV	1,3E+08	4,0E+06		
31	VHSV + IPNV	8,6E+06	1,9E+07		
32	VHSV and IPNV	4,0E+04	1,3E+09		
33	VHS-VIRUS & IPN-VIRUS	1,3E+07	4,0E+06		
34	IPNVVHSV	1,9E+06	5,9E+06		
35	VHSV and IPNV	2,7E+07	5,9E+06		
36	VHSV & IPNV	8,6E+06	4,0E+06	4,0E+06	4,0E+05
37	VHSV,IPNV	8,6E+05			1,9E+06
38	VHSV & IPNV	5,9E+06	2,7E+06		4,0E+06
39	VHS+IPN	2,7E+07	5,9E+06		
40	VHSV and IPNV		1,3E+06	1,9E+06	2,7E+06
41	VHSV & IPNV	2,7E+07	1,9E+08		
42	VHSV & IPNV	5,9E+05	4,0E+06	1,9E+06	1,3E+06
43	VHSV and IPNV	2,7E+06	1,3E+07		

Number of laboratories	**43**	**43**	**43**	**43**
Median titre	5,9E+06	5,9E+06	1,9E+06	2,7E+06
Maximum titre	1,3E+08	1,3E+09	2,7E+07	1,3E+08
Minimum titre	1,3E+04	5,9E+03	5,9E+03	8,6E+03
25% quartile titre	2,1E+06	2,7E+06	1,3E+06	1,3E+06
75% quartile titre	1,9E+07	1,3E+07	4,0E+06	5,9E+06

Table 5. Inter-Laboratory Proficiency Test, PT1, 2013 – Results of titration of ampoule II.

Ampoule II - IHNV

Laboratory code number	Virus Identification	Titre in			
		BF-2	EPC	RTG-2	FHM
1	IHNV		5,9E+03	4,0E+03	1,3E+04
2	IHNV	2,7E+05	1,9E+06		
3	IHNV		1,9E+03		
4	IHNV	1,9E+03	1,3E+03		
5	IHNV		4,0E+04	1,9E+02	
6	IHNV		4,0E+04		1,3E+05
7	IHNV		8,6E+04	8,6E+05	
8	IHNV		5,9E+02	1,9E+02	1,3E+03
9	IHNV, genotype M		8,6E+03		
10	IHNV	1,9E+02	4,0E+04	2,7E+02	
11	IHNV		1,3E+04		
12	IHNV		8,6E+04		4,0E+04
13	IHNV				1,3E+04
14	IHNV		1,9E+04		8,6E+03
15	IHNV		2,7E+03		
16	IHNV	1,9E+03			1,3E+03
17	IHNV		5,9E+04	2,7E+03	
18	IHNV		400		
19	IHNV		1,9E+04		
20	IHNV	1,9E+02	1,3E+04		
21	IHNV		8,6E+03		
22	IHNV				
23	IHNV		4,0E+04		
24	IHNV	1,9E+03	5,9E+03		
25	IHNV	1,3E+03	1,3E+03	1,3E+03	4,0E+03
26	IHNV		4,0E+04	4,0E+03	
27	IHNV		1,3E+04		
28	IHNV		< 1,9E+02		1,9E+04
29	IHNV				
30	IHNV		4,0E+03		
31	IHNV		5,9E+04		
32	IHNV		5,9E+09		
33	IHN-VIRUS		5,9E+04		
34	IHNV	1,9E+04	4,0E+04		
35	IHNV		2,7E+04		
36	IHNV		4,0E+03		2,7E+03
37	IHNV	4,0E+02			5,9E+02
38	IHNV	1,9E+02	1,3E+04		2,7E+04
39	IHNV	5,9E+02	1,3E+04		
40	IHNV				
41	IHNV	2,7E+04	5,9E+04		
42	IHNV	1,3E+03	1,3E+04	2,7E+03	8,6E+03
43	IHNV	2,7E+02	8,6E+03		

Number of laboratories	**43**	**43**	**43**	**43**
Median titre	1,3E+03	1,3E+04	2,7E+03	8,6E+03
Maximum titre	2,7E+05	5,9E+09	8,6E+05	1,3E+05
Minimum titre	1,9E+02	4,0E+02	1,9E+02	5,9E+02
25% quartile titre	3,0E+02	5,9E+03	2,7E+02	2,7E+03
75% quartile titre	1,9E+03	4,0E+04	4,0E+03	1,9E+04

Table 6. Inter-Laboratory Proficiency Test, PT1, 2013 – Results of titration of ampoule III.

Ampoule III - EHNV

Laboratory code number	Virus Identification	Titre in			
		BF-2	EPC	RTG-2	FHM
1	EHNV	8,6E+04	1,3E+04	2,7E+04	
2	EHNV	1,9E+06	2,7E+06		
3	EHNV	1,3E+05	1,3E+04		
4	EHNV	5,9E+04	2,7E+03		
5	Ranavirus (EHNV)	5,9E+04	1,3E+05	5,9E+03	
6	EHNV	4,0E+05	1,3E+05		
7	EHNV				
8	EHNV	1,3E+04	5,9E+04	5,9E+03	
9	EHNV	5,9E+03	5,9E+03		
10	EHNV	1,3E+06	4,0E+05	5,9E+02	
11	EHNV(Seq)	8,6E+04	1,3E+05		
12	EHNV		1,9E+05		5,9E+04
13	Ranavirus	8,6E+04			2,7E+04
14	EHNV		8,6E+04		1,9E+05
15	EHNV	2,7E+05	1,9E+04		
16	EHNV	2,7E+04			1,3E+04
17	EHNV		4,0E+04		
18	EHNV	4,0E+05	4,0E+04		
19	EHNV	1,9E+05	4,0E+04		
20	EHNV	8,6E+04	1,3E+04		
21	EHNV	1,3E+05	2,7E+05		
22	EHNV	5,9E+03	5,9E+03		
23	EHNV	1,9E+04	8,6E+03		
24	EHNV	2,7E+06	4,0E+04		
25	EHNV	4,0E+04	1,9E+05	1,9E+05	4,0E+03
26	EHNV	2,7E+06	1,3E+03	1,3E+04	
27	EHNV	1,3E+05	4,0E+04		
28	EHNV	5,9E+04			2,7E+04
29	EHNV	8,6E+04	5,9E+04	1,9E+04	2,7E+02
30	EHNV	4,0E+03	2,7E+04		
31	EHNV	2,7E+05	4,0E+05		
32	no VHSV, IPNV,IHNV,SVCV	1,9E+04	1,3E+09		
33	EHN-VIRUS	1,3E+05	1,9E+04		
34	EHNV	5,9E+04	2,7E+05		
35	EHNV	4,0E+05	4,0E+04		
36	EHNV	1,9E+05	8,6E+04	4,0E+04	
37	EHNV	4,0E+02			1,9E+03
38	EHNV	4,0E+04	1,9E+03		1,3E+03
39	EHNV	1,3E+06	1,3E+05		
40	EHNV				
41	EHNV	5,9E+05	8,6E+04		
42	EHNV	2,7E+05	5,9E+04	2,7E+05	4,0E+04
43	EHNV	2,7E+05	2,7E+04		

Number of laboratories	**43**	**43**	**43**	**43**
Median titre	1,1E+05	4,0E+04	1,9E+04	2,0E+04
Maximum titre	2,7E+06	1,3E+09	2,7E+05	1,9E+05
Minimum titre	4,0E+02	1,3E+03	5,9E+02	2,7E+02
25% quartile titre	4,5E+04	1,9E+04	5,9E+03	2,4E+03
75% quartile titre	2,7E+05	1,3E+05	4,0E+04	3,7E+04

Table 7. Inter-Laboratory Proficiency Test, PT1, 2013 – Results of titration of ampoule IV.

Ampoule IV - SVCV

Laboratory code number	Virus Identification	Titre in			
		BF-2	EPC	RTG-2	FHM
1	SVCV	8,6E+05	8,6E+05	4,0E+05	1,9E+06
2	SVCV	4,0E+05	8,6E+06		
3	SVCV	4,0E+04	4,0E+05		
4	SVCV	2,7E+04	1,3E+03		
5	SVCV	1,3E+03	2,7E+05	4,0E+03	
6	SVCV	1,9E+04	5,9E+04		
7	Negative (Not: VHSV; IHNV; Ranavirus; IPNV; SVCV)				
8	SVCV		1,9E+04		4,0E+04
9	SVCV Id3	2,7E+05	2,7E+05		
10	SVCV	4,0E+06	5,9E+05	1,3E+03	
11	SVCV	1,3E+04	1,9E+05		
12	SVCV		8,6E+05		5,9E+05
13	SVCV	2,7E+04			2,7E+04
14	SVCV		8,6E+04		1,9E+05
15	SVCV	4,0E+04	2,7E+05		
16	SVCV	2,7E+04	< 1,9E+02		2,7E+03
17	SVCV		1,3E+05		
18	SVCV		5,9E+04		
19	SVCV	1,9E+05	1,3E+05		
20	SVCV	1,9E+04	5,9E+04		
21	SVCV		4,0E+05		
22	SVCV	2,7E+02	2,7E+03		
23	SVC	2,7E+04	2,7E+05		
24	SVCV	1,3E+03	1,9E+05		
25	SVCV	8,6E+03	5,9E+04	1,3E+04	5,9E+04
26	SVCV	4,0E+04	1,3E+07	5,9E+04	
27	SVCV	5,9E+04	4,0E+04		
28	SVCV	5,9E+04			1,9E+06
29	SVCV	8,6E+03	1,3E+04	4,0E+03	1,9E+05
30	SVCV	5,9E+04	4,0E+04		
31	SVCV	1,3E+05	2,7E+06		
32	SVCV		1,3E+09		
33	SVC-VIRUS	1,3E+05	8,6E+04		
34	SVCV	8,6E+04	2,7E+05		
35	SVC	2,7E+06	1,3E+06		
36	SVCV	8,6E+04	1,3E+05	8,6E+04	5,9E+04
37	SVCV	4,0E+02			1,9E+03
38	SVCV	2,7E+03	1,9E+04		1,3E+05
39	SVCV	8,6E+05	8,6E+04		
40	SVCV		1,3E+03	1,9E+03	2,7E+02
41	SVCV	5,9E+05	1,3E+06		
42	SVCV	8,6E+03	5,9E+05	8,6E+03	2,7E+05
43	SVCV	8,6E+05	4,0E+04		

Number of laboratories	**43**	**43**	**43**	**43**
Median titre	4,0E+04	1,6E+05	8,6E+03	9,3E+04
Maximum titre	4,0E+06	1,3E+09	4,0E+05	1,9E+06
Minimum titre	2,7E+02	1,3E+03	1,3E+03	2,7E+02
25% quartile titre	1,4E+04	5,9E+04	4,0E+03	3,0E+04
75% quartile titre	1,7E+05	5,4E+05	5,9E+04	2,5E+05

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

Ampoule V - Medium

Laboratory code number	Virus Identification	Titre in			
		BF-2	EPC	RTG-2	FHM
1	No CPE				
2	SVCV	2,7E+03	2,7E+04		
3	No virus detected				
4	Negative				
5	Negative				
6	No virus				
7	Negative (Not: VHSV; IHNV; Ranavirus; IPNV; SVCV)				
8	negative				
9	no virus				
10	Negative				
11	Ranavirus	5,9E+02	1,9E+03		
12	IHNV				
13	Negativ				
14	NO VIRUS				
15	NEG				
16	-				
17	neg				
18	No Viruses isolated by BF2/EPC				
19	No Virus Detected				
20	NEGATIVE				
21	-				
22	negative				
23	Virus not detcted				
24	no VHSV,no IHNV,no SVCV no IPNV,no RANAV	1,9E+04			
25	Negative				
26	NEGATIVE CONTROL				
27	no cpe (no VHSV, no IHNV, noIPNV, no SVCV, no EHNV)				
28	Negative				
29	-				
30	Not VHSV, IHNV,EHNV, not Ranavirus, IPNV nor SVCV				
31	Negative				
32	no VHSV,IPNV,IHNV,SVCV				
33	NOT VHS NOT IHN NOT EHN NOT IPN NOT SVC				
34	-				
35	Negative				
36	no pathogen identified				
37	VHSV	4,0E+02			5,9E+02
38	No Virus Isolated				
39	Negative				
40	Not VHSV, not IHNV, not Ranavirus, not IPNV, not SVCV				
41	NO VHSV, IPNV, EHNV and SVCV detectable				
42	not VHSV, IHNV, EHNV, SVCV				
43	no virus detected				

Number of laboratories	**43**	**43**	**43**	**43**
Median titre	1,7E+03	2,7E+04	< 1,9E+02	5,9E+02
Maximum titre	1,9E+04	1,3E+09	< 1,9E+02	5,9E+02
Minimum titre	4,0E+02	1,9E+03	< 1,9E+02	5,9E+02
25% quartile titre	5,4E+02	1,5E+04	< 1,9E+02	5,9E+02
75% quartile titre	6,7E+03	6,3E+08	< 1,9E+02	5,9E+02

BF-2 cells

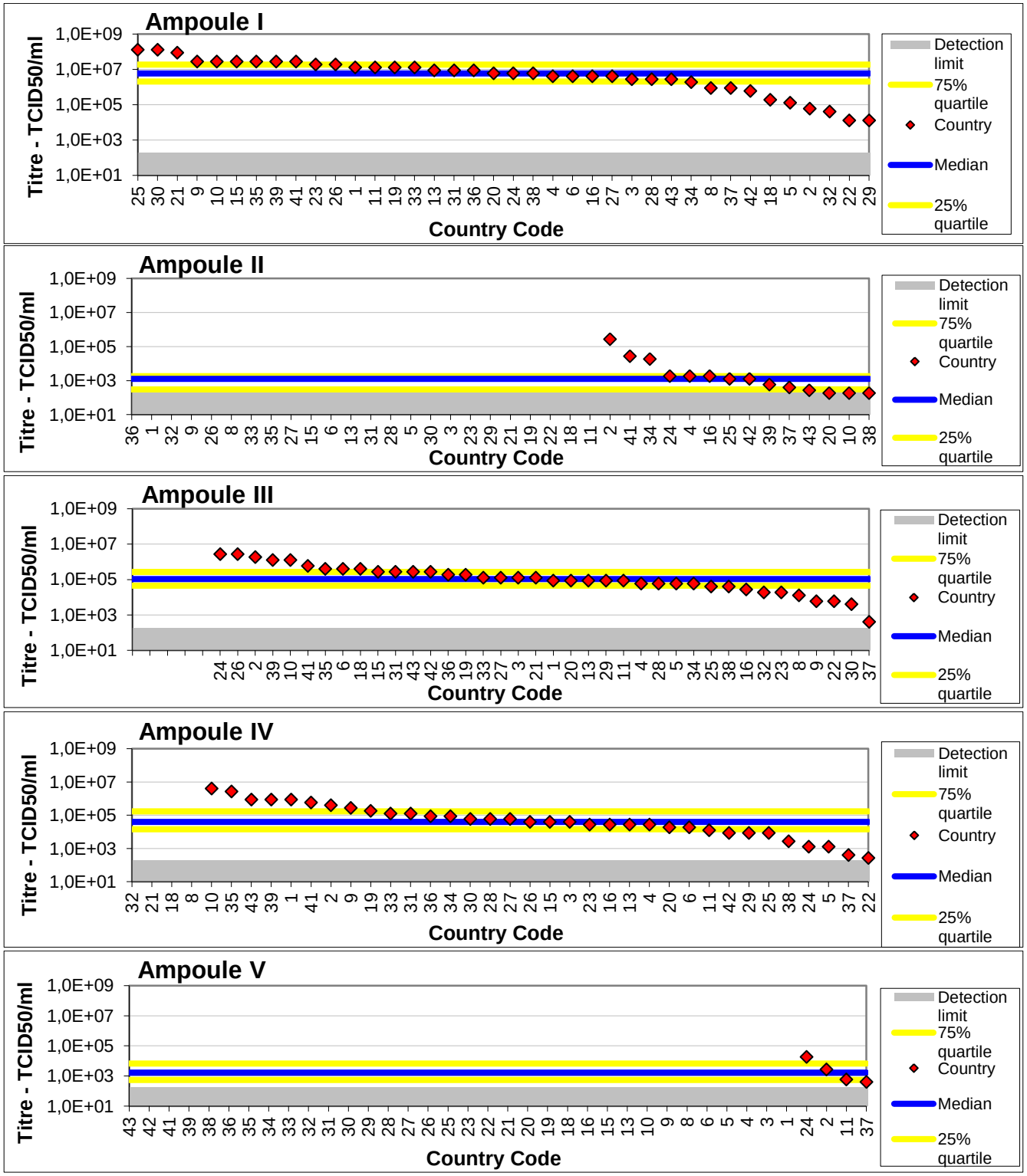

Figure 5. Virus titres obtained in BF-2 cells. The titre (red diamond) of each participating laboratory (country code) using BF-2 cells illustrated for ampoule I, II, III, IV and V. The detection level (gray shadow), median (blue line), 75% quartile (upper yellow line) and 25% quartile (lower yellow line) are plotted on all graphs. For participants failing to obtain any titre, no red diamond is shown.

EPC cells

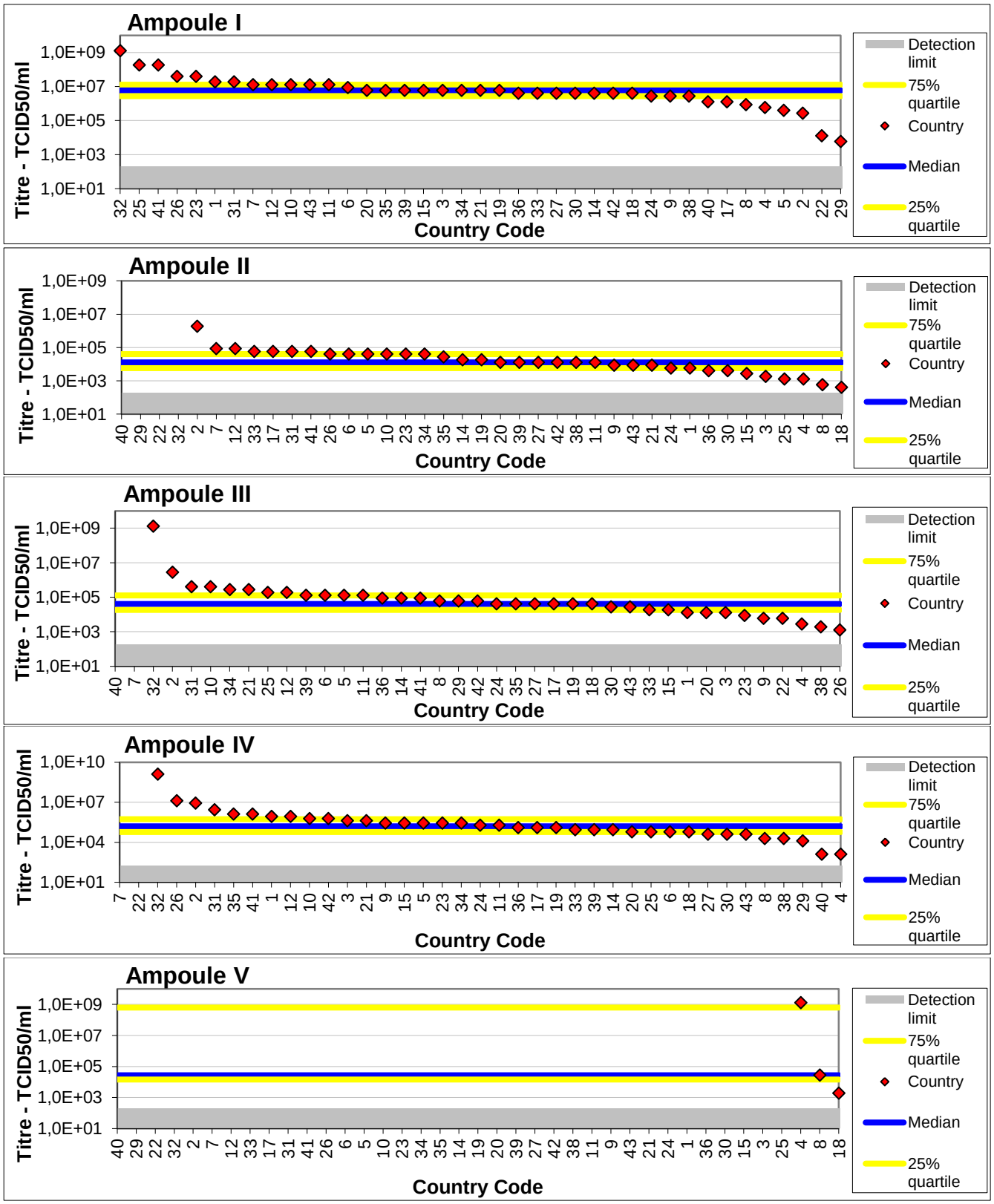

Figure 6. Virus titres obtained in EPC cells. For further details see Figure 6

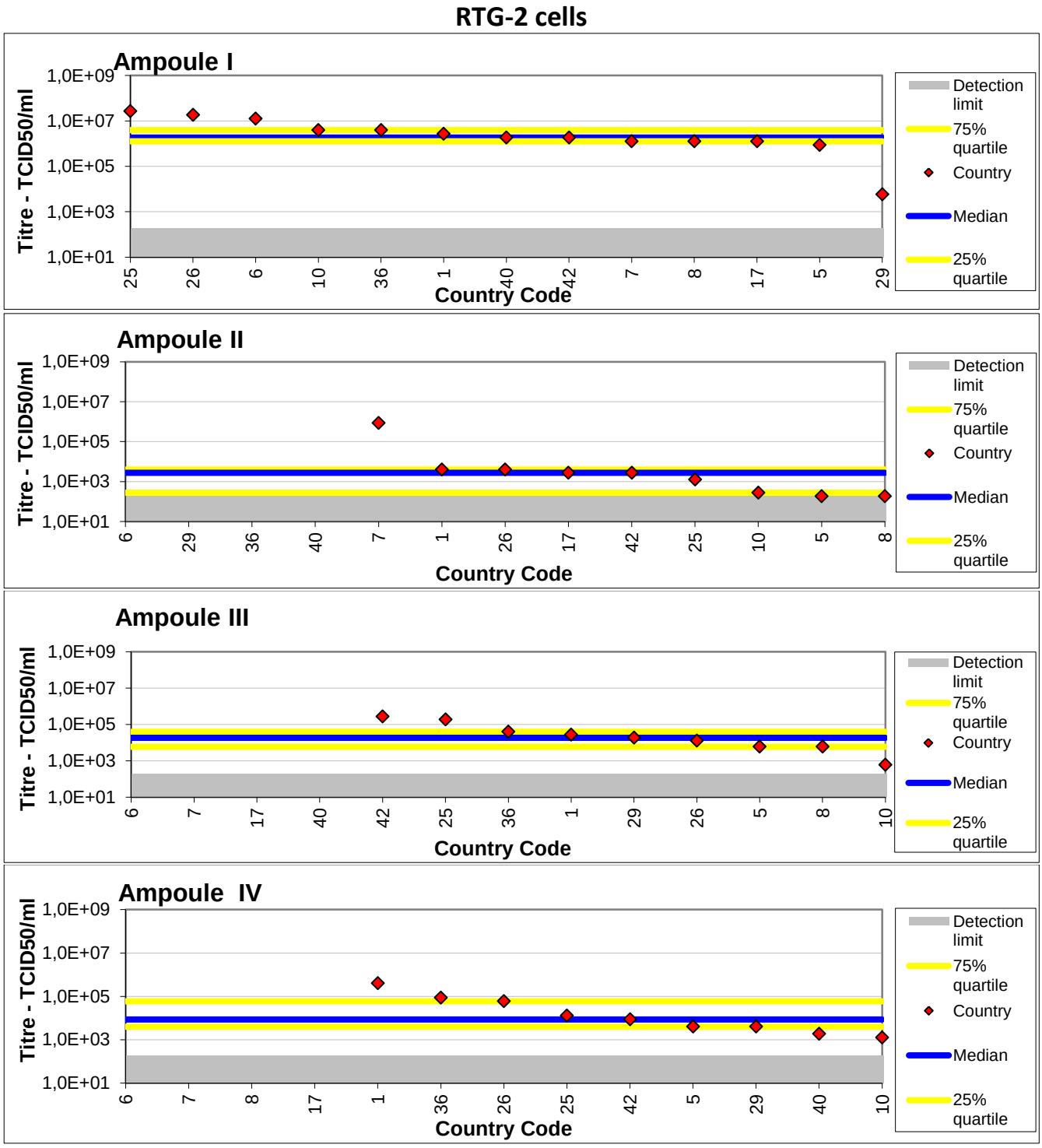

Figure 7. Virus titre obtained in RTG-2 cells. For further details see Figure 6

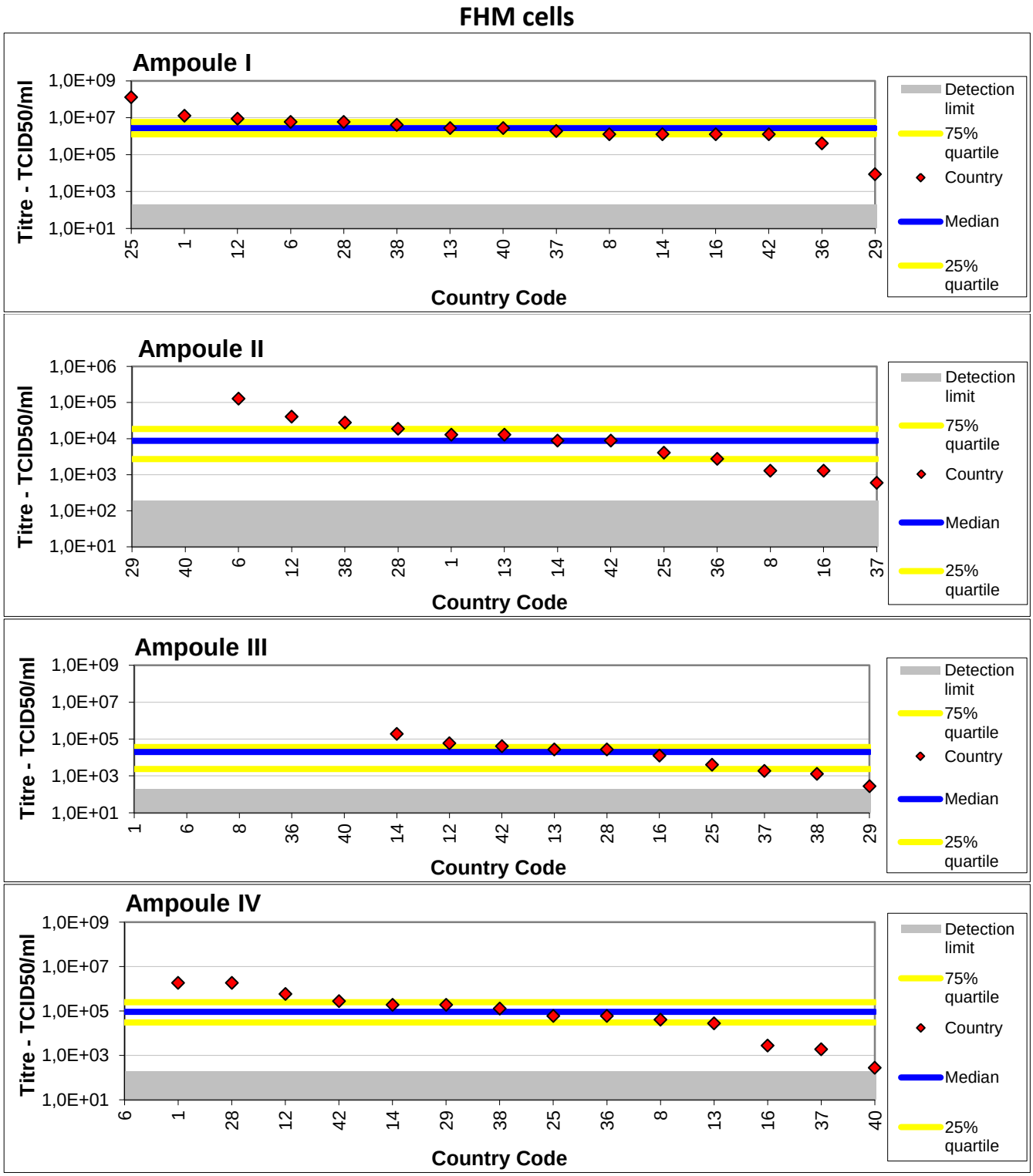

Figure 8. Virus titres obtained in FHM cells. For further details see Figure 6

Identification of content

- 33 laboratories correctly identified all viruses in all ampoules

Ampoule I – IPNV and VHSV

- 40 laboratories correctly identified IPNV and VHSV
- 2 laboratories identified VHSV only and not IPNV
- 1 laboratory did not test for IPNV but correctly identified Aquabirnavirus.

Ampoule II – IHNV

- All 43 laboratories correctly identified IHNV

Ampoule III - EHNV

- 39 laboratories correctly identified EHNV
- 2 laboratory found virus but did not identify it
- 2 laboratories found virus and sequenced it but sequences retrieved were not correct

Ampoule IV – SVCV

- 42 laboratories correctly identified SVCV
- 1 laboratory did not isolate the virus

Ampoule V – MEM

- 37 laboratories correctly did not isolate any virus
- 2 laboratories isolated a virus but did not identify it
- 1 laboratory detected Ranavirus
- 1 laboratory detected IHNV
- 1 laboratory detected VHSV
- 1 laboratory detected SVCV

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 and VHSV was given the score 2, only identification of VHSV was given the score 1.

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

Ampoule III: 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.

Ampoule IV: 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 V: No virus isolated was given the score 2.Virus isolated but not identified was given score 1, virus isolated and identified was given score 0.

All the participants submitted results, 34 out of 43 got a success rate of 100% as they were able to identify all the pathogens they were supposed to. 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.

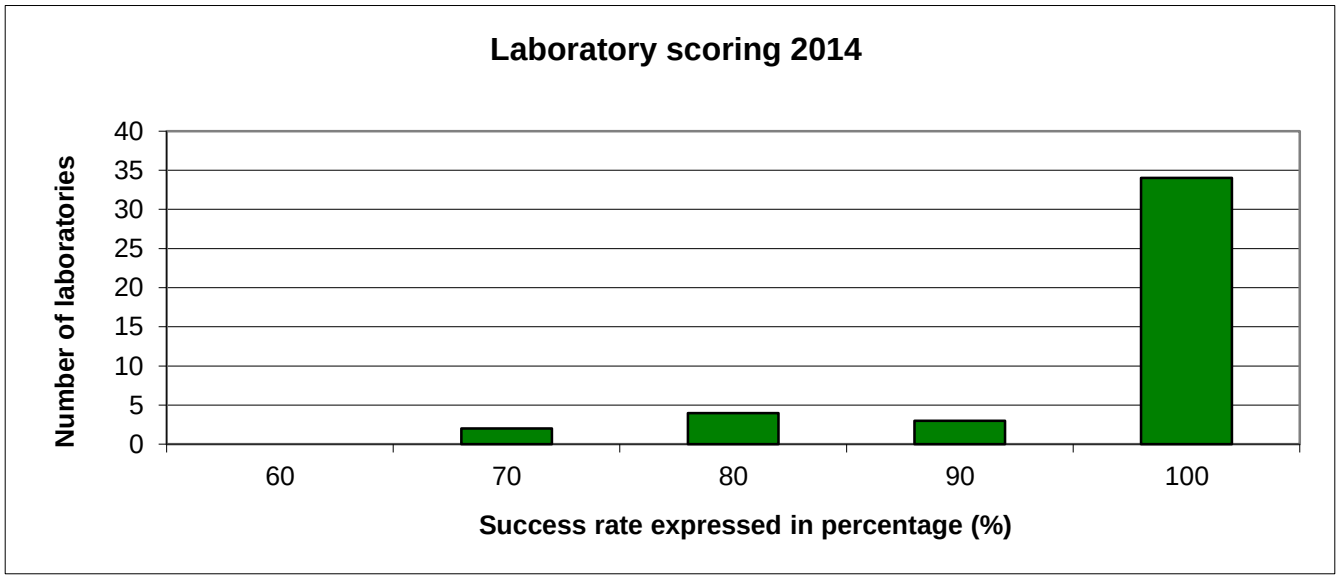

Figure 9. Scores obtained by participants.

Cells applied for solving the test

The following cell lines were used by the participants:

- 38 laboratories used BF-2 cells
- 39 laboratories used EPC cells
- 13 laboratories used RTG-2 cells
- 15 laboratories used FHM cells
- 5 laboratory used CHSE-214 cells

- 9 laboratories used four cell lines

- 5 laboratories used tree cell lines

- 29 laboratories used two cell lines:
  - 22 laboratories used BF-2 cells in combination with EPC cells
  - 2 laboratory used EPC and RTG-2
  - 1 laboratory used EPC and FHM
  - 4 laboratories used BF-2 and FHM

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 laboratory using these combinations is 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.

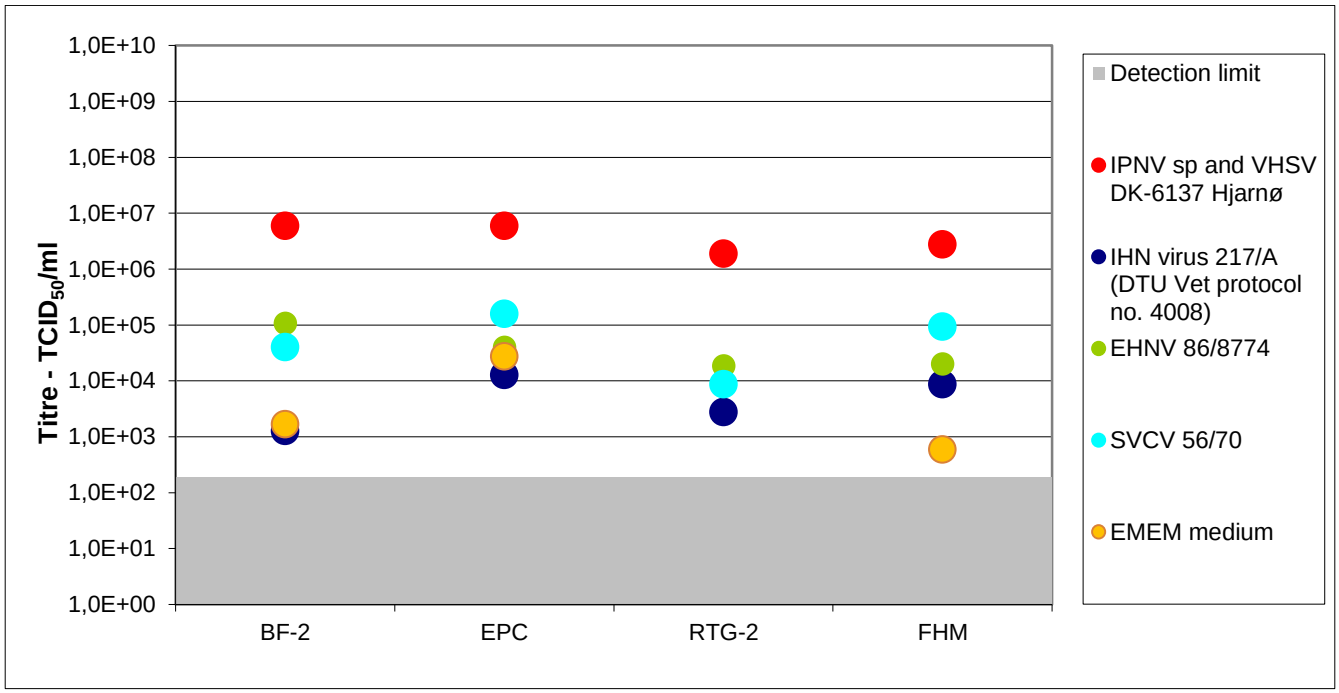

Figure 10. Median virus titres obtained by participants in different cell lines.

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 – IPNV sp and VHSV DK-6137 Hjarnø

- 9 laboratories submitted sequences for VHSV isolate
- 22 laboratories submitted sequences for IPNV and VHSV isolates
- 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

Ampoule II - IHN virus 217/A (DTU Vet protocol no. 4008)

- 30 laboratories performed sequencing to identify the virus in ampoule II with correct results
- 18 Laboratories provided sequence of the G gene using alternative protocols described by Emmenegger et *al*., 2000, protocols provided in OIE Manual of Diagnostic Tests for Aquatic Animals, chapter 2.3.4 or in-house protocols
- 11 laboratories provided sequence of the N gene using alternative protocols described by Enzmann et al., or unpublished protocols
- One laboratory identified different IHNV strains in the ampoule
- 20 laboratories genotyped the IHNV isolate as belonging to genogroup M
- 1 laboratory described it as European type
- 1 laboratory genotyped the IHNV isolate as belonging to genogroup M or U
- 2 laboratory genotyped the IHNV isolate as belonging to genogroup U

- 7 laboratories did not give any genotype of the sequences

Ampoule III – EHNV

The diagnosis of EHNV rely on sequencing analysis for this reason a specific analysis of sequence retrieved was performed and sequences delivered labelled with country code are attached as annex. For ampoule III 43 sequences where sent in from 41 different laboratories. 1 sequence mapped to the Neurofilament triplet H1-like protein gene, while 41 mapped to the MCP gene. 1 Sequence did not map to EHNV nor any related viruses.

28 sequences contained no errors, while 6 sequences (*) contained 1 error and 7 sequences contained more than 1 error (**). Common for many errors was that they were situated at either the 5’ end or the 3’end. This could be due to several factors:

- If primersites had not been deleted
- If isolates had been sequenced only in one direction. The EURL advises that isolates be sequenced in both the forward and reverse direction. If isolates are cloned it is recommended that at least 3 clones are sequenced.

- Improper reading/analysis of trace files.

Errors consisted of nucleotide substitutions and gaps. No deletions where detected.
Some sequences contained errors further in to the sequence. This could be due to bad trace files or improper reading of the trace files.
The EHNV MCP gene sequence is 99% identical to the European sheatfish virus MCP gene. Sequence 34 was 99% identical to European sheatfish virus and only 98% identical to EHNV. This could be due to a bad trace file or wrong analysis of the trace, but a contamination of the sample is also likely.
Sequence 11 did not map to any gene.

- 39 laboratories performed sequencing to identify the virus in ampoule III with correct result
- 1 laboratory performed only REA according to the OIE Manual of Diagnostic Tests for Aquatic Animals. Chapter 2.3.1 with correct result; 2 laboratory performed REA for confirming sequencing results.

Ampoule IV – SVCV

- 20 laboratories performed sequencing
- 12 laboratories identified the isolate as being genotype Id
- 8 laboratories did not give any genotype of the sequences
- 1 laboratory sequenced L gene
- 19 laboratories sequenced G gene using either protocols described by Stone et al.(2003) or in house made protocols

Concluding remarks PT1

The inter-laboratory proficiency test 2013 was conducted without major constraints. Despite the fact that the shipping company caused a delay in the delivery of the parcels 93% of parcels reached the respective laboratories of destination within 9 days after submission. Once again shipment to China demonstrated to be difficult and laborious taking about a month to reach the laboratories primarily due to border controls.

The overall performance of the participating laboratories was very high, and the fact that we this year included an ampoule with both IPNV and VHSV did not trouble most of the laboratories. It was, however, quite worrying that 6 of 43 laboratories detected virus in Ampoule V that only contain MEM without virus, and these laboratories should consider revising their procedures in order not to cross-contaminate their samples.

This year 40 participants were able to identify the EHNV isolate correctly using either sequencing or REA; however 2 laboratories sequenced the isolate but retrieved a sequence that was not correct. One laboratory did correctly isolate the virus but did not characterize it as they have indicated in their contingency plan that in case of EHNV suspicion they will forward the isolate to the EURL for Fish diseases.

All the viral titres submitted were compared according to cell line and ampoule, respectively. The titres obtained by each laboratory are plotted in relation to the combined submitted data set and each participating laboratory can thereby compare the sensitivity of its cell lines to the sensitivity of those used by the other participants. We recommend all participants to evaluate the sensitivity of the cells used in their laboratory in relation to the diagnostic purpose.

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

The results presented in this report will be further presented and discussed at the 18th Annual Workshop of National Reference Laboratories for Fish Diseases to be held 3rd and 4th of June 2014 in Copenhagen, Denmark.

Proficiency test 2, PT2

Five 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 9.

Table 9. Content of each ampoule with reference to culture conditions and major publications of the included pathogens.

Code	Specifications
Ampoule VI: **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:** Y10404.1, HQ259676, AF283998.1, **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:** 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. 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. Kibenge FS, Kibenge MJ, McKenna PK, Stothard P,Marshall R, Cusack RR & McGeachy S (2001). Antigenic variation among isolates of infectious salmon anaemia virus correlates with genetic variation of the viral haemagglutinin gene. *Journal of General Virology* **82**, 2869-2879. **References on genotype:** 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.

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. **Received from:** Dr. Sven M. Bergmann, Friedrich-Loeffler-Institut (FLI), Federal Research Institute for Animal Health, Südufer 10, 17393 Greifswald-Insel Riems, Germany **Cell culture passage number:** Unknown
Ampoule VIII: **KHV**	**KHV 07/108b** **Received from** Dr. J. Castric, ANSES, France. **Reference on the isolate** Bigarre L., Baud M., Cabon J., Antychowicz J., Bergmann S.M.,Engelsma M., Pozet F., Reichert M. & Castric J. (2009) Differentiation between Cyprinid herpesvirus type-3 lineages using duplex PCR. Journal of Virological Methods 158, 51–57. **Cell culture passage number**: 4 in KF-1.
Ampoule IX: **Sterile pyrogen free water**	**Sterile pyrogen free water**
Ampoule X: ***Aphanomyces 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.

Testing of the PT2 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 replicates of each ampoule:

- For KHV by PCR (Bercovier et *al*. (2005)) and real-time PCR (Gilad et *al*. (2004))
- For ISAV by RT-PCR (Mjaaland et *al*. (1997)) and real-time RT-PCR (Snow et *al*. (2006))
- For EUS by PCR (Kurata et al., 2000)
-

This testing procedure was done in order 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.

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. 3 months after lyophilization ampoule VI was tested by RT-qPCR for ISAV, Ampoule VII and VIII were tested by PCR for KHV (Bercovier et al.,2005) , ampoule X was tested for *A. invadans* (Kurata et al.,2000). Ampoule IX (negative sample) was tested once again for all the three pathogens.

No pathogens other than the expected were detected.

Table 10. Ct-value of ampoules VI, VII and VIII tested before lyophilisation, immediately after lyophilisation, and 3 months after the last date for submission of results. For ampoule X 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)
Ampoule VI: ISAV Glesvaer/2/90	a	21.77/+	26,42 /+	26.31
	b		27,77 /+	
	c		26,00 /+	
	d		26,11 /+	26.33
	e		25,79 / +	
	Average		26.42	26.32
Ampoule VII: KHV-TP 30	a	14.52/+	20.68 /+	+
	b		21.21 /+	
	c		21.24 /+	
	d		21.28 /+	
	e		21.47 /+	
	Average		21.18	
Ampoule VIII: KHV	a	15.84/+	24.44 /+	+
	b		26.25 /+	
	c		26.12 /+	
	d		27.97 /+	
	e		26.98 /+	
	Average		26.35	
Ampoule IX: Sterile pyrogenfree water	a	-	-	-
	b	-	-	
	c	-	-	
	d	-	-	
	e	-	-	
Ampoule X: *Aphanomyces piscicida/invadans* spores NJM9701	a	+	+	+
	b		+	
	c		+	
	d		+	
	e		+	

Pathogen identification

In PT2, participants were asked to identify the fish viruses ISAV and KHV (both listed in Council Directive 2006/88/EC) and the oomycete *A.invadans* 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 11. Inter-Laboratory Proficiency Test, PT2, 2013 - Virus identification.

Laboratory code number	Score	Answer recived at EURL	Ampoule VI	Ampoule VII	Ampoule VIII	Ampoule IX	Ampoule X
			Ampoule VI: ISAV Glesvaer/2/90	**Ampoule VII: KHV-TP 30**	**Ampoule VIII: KHV**	**Ampoule IX: Sterile pyrogenfree water**	**Ampoule X: *Aphanomyces invadans* spores NJM9701**
1	10/10	09-10-13	ISAV	KHV	KHV	Not A. Invadans, not ISA, not KHV.	A. invadans
2	10/10	29-11-13	ISAV	KHV	KHV	NO VIRUS	A. invadans
3	8/10	30-11-13	ISAV	KHV	KHV	negative for ISAV, KHV and A. Invadens	negative for ISAV, KHV and A. Invadens
4	10/10	30-11-13	ISAV	KHV	KHV	Negative	Aphanomyces invadans
5***	8/8	29-11-13	ISAV	KHV	KHV	Negative	Negative
6	10/10	29-11-13	ISAV	KHV	KHV	No virus, No A. invadans	A. invadans
7	8/8	29-11-13	ISAV	KHV	KHV	Negative (Not: ISAV; KHV)	Negative (Not: ISAV; KHV)
8	10/10	28-11-13	ISAV	KHV	KHV	negative	EUS
9	6/8	25-11-13	ISAV	KHV	KHV	KHV	_
10	10/10	28-11-13	ISAV	KHV	KHV	Negative	A. invadans
11	10/10	30-11-13	ISAV(Seq)	KHV(Seq)	KHV(Seq)	Not A. invadance, KHV or ISAV	A. invadans(Seq)
12	10/10	29-11-13	ISAV	KHV	KHV	negative	A.invadans
13	8/8	29-11-13	Negativ	KHV	KHV	Negativ	A. invadans
14	10/10	29-11-13	ISAV	KHV	KHV	NO VIRUS	A.invadans
15	10/10	29-11-13	ISAV	KHV	KHV	Not detected	EUS
16	10/10	26-11-13	ISAV	KHV	KHV	-	A. invadans
17	8/8	28-11-13	ISAV	KHV	KHV	neg	neg
18*	6/6	20-11-13	KHV negative	KHV	KHV	KHV negative	KHV negative
19	10/10	28-11-13	ISAV	KHV	KHV	No Virus Detetcted	A. invadans
20	10/10	27-11-13	ISAV	KHV	KHV	NEGATIVE	A. invadans
21	10/10	28-11-13	ISAV	KHV	KHV	Virus was not detected.	A.invadans
22	10/10	29-11-13	ISAV	KHV	KHV	negative	A. invadans
23**	4/4	29-11-13	ISAV	ISAV not detected	ISAV not detected	ISAV not detected	ISAV not detected
24	10/10	25-11-13	ISAV	KHV	KHV	no KHV, no ISA, no A.invadans	A. invadans
25	10/10	29-11-13	ISAV	KHV	KHV	Negative	A. invadans
26	10/10	12-11-13	ISAV	KHV	KHV	NEGATIVE CONTROL	A. invadans
27	10/10	29-11-13	ISAV	KHV	KHV	no ISAV, no KHV, no A.invadans	A.invadans
28	10/10	12-11-13	ISAV	KHV	KHV	Negative	A.Invadans
29	10/10	26-11-13	ISAV	KHV	KHV	-	A. invadans
30	10/10	25-11-13	ISAV	KHV	KHV	Not ISAV, KHV,A. invadans	A. invadans
31	10/10	27-11-13	ISAV	KHV	KHV	-	A.invadans
32	8/8	08-11-13	ISAV	KHV	KHV	KHV and ISAV not detected	KHV and ISAV not detected
33	7/10	29-11-13	ISA-VIRUS & KHV VIRUS	NOT ISAV NOT KHV NOT APHANOMYCES INVADANS	KHV VIRUS	NOT ISAV NOT KHV NOT APHANOMYCES INVADANS	APHANOMUCES INVADANS
34	6/10	27-11-13	ISAV	KHV	KHV	A. invadans	-
35	8/10	28-11-13	ISAV	KHV	KHV	Negative	Negative
36	10/10	14-11-13	ISAV	KHV	KHV	no pathogen identified	A. invadans
37	8/10	29-11-13	ISAV	KHV	KHV	A. invadans	A. invadans
38	10/10	27-11-13	ISAV	KHV	KHV	no pathogen	A. invadans

Laboratory code number	Score	Answer recived at EURL	Ampoule VI	Ampoule VII	Ampoule VIII	Ampoule IX	Ampoule X
			Ampoule VI: ISAV Glesvaer/2/90	**Ampoule VII: KHV-TP 30**	**Ampoule VIII: KHV**	**Ampoule IX: Sterile pyrogenfree water**	**Ampoule X: *Aphanomyces invadans* spores NJM9701**
						detected	
39	10/10	29-11-13	ISAV	KHV	KHV	Negative	A. invadans
40	8/10	25-11-13	ISAV	KHV	KHV	Not ISAV, not KHV, not A. invadans	Not ISAV, not KHV, not A. invadans
41	10/10	25-11-13	ISAV	KHV	KHV	NEGATIVE	A.invadans / EUS
42	10/10	27-11-13	ISAV	KHV	KHV	not ISAV, KHV, or A. invadans	A. invadans
43	10/10	29-11-13	ISAV	KHV	KHV	No agent detected	A. invadans

* Analyzed for the presence of KHV only

**Analysed for the presence of ISAV only

****A. invadans* is enzootic, as such, the Laboratory does not have responsibility for its diagnosis and so A. invadans testing was not undertaken.

Identification of content

- 43 laboratories submitted results
- 36 obtained 100% success rate (the score is expressed in percentage as some laboratories do not test for all pathogens)
- 35 laboratories tested for all three pathogens included in PT 2
- 41 laboratories tested for ISAV
- 42 laboratories tested for KHV
- 36 laboratories tested for *A. invadans*
- 1 laboratory tested for ISAV only
- 1 laboratory tested for KHV only
- 1 laboratory did not test for ISAV but did test for KHV and *A.invadans*
- 4 laboratories did not test for *A.invadans* but did test for ISAV and KHV

Ampoule VI – *ISAV*

- 40 laboratories correctly identified *ISAV*
- 1 laboratory detected ISAV and KHV
- 2 laboratories did not examine for *ISAV*

Ampoule VII – KHV

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

Ampoule VIII – KHV

- 42 laboratories correctly identified KHV
- 1 laboratory did not examine for KHV

Ampoule IX – Sterile pyrogenfree wate

- 40 laboratories correctly did not identify any of the 3 pathogens incuded in PT2
- 1 laboratory found KHV
- 2 laboratory found A. invadans

Ampoule X – *Aphanomyces invadans*

- 32 laboratories correctly identified only *A. invadans*
- 7 laboratories did not examine for *A. invadans*
- 4 laboratories tested for but did not find *A.invadans*

Scores

We have assigned a score of 2 for each correct answer (Table 13), giving the possibility for obtaining a maximum score of 10. Correct identification of the pathogen included in the ampoule together with another pathogen gives score of 1; incorrectly finding of pathogens not present in the ampoules gives the score 0.

Of the 43 laboratories submitting results 35 laboratories correctly identified all ampoules and obtained maximum score. Two participants examined for one pathogen only: one laboratory examined for ISAV only and therefore obtained the score 4 out of 4 possible, another laboratory examined for KHV only and this laboratory obtained therefore the score 6 out of 6 possible. 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 14.

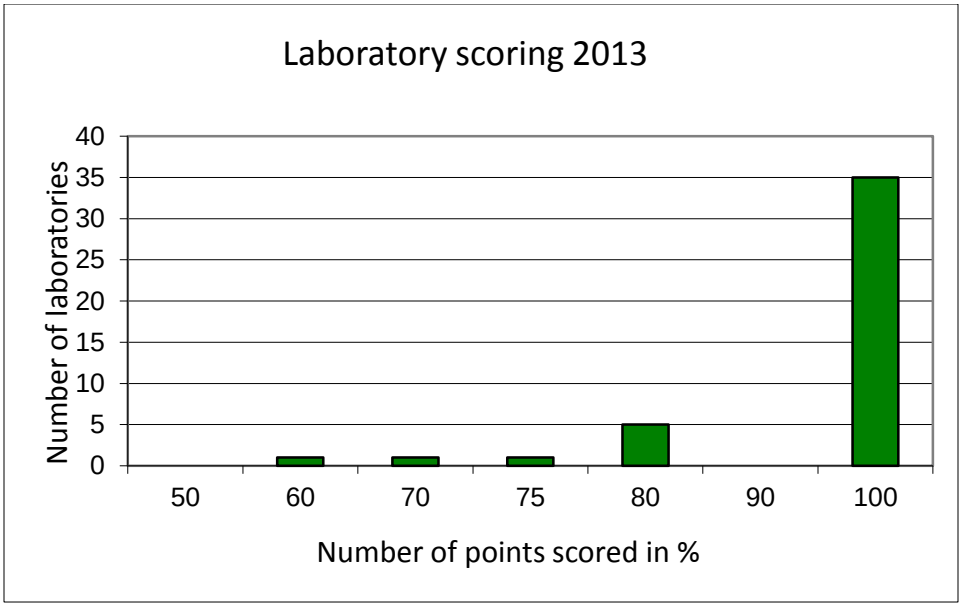

Figure 11. Obtained score by participants.

Methods applied

The methods applied for the molecular diagnosis of the pathogen included in PT 2 by participants are quite different and widespread. Most of the laboratories rely on published and recommended diagnostic methods, while others select in house validated or unpublished methods. In this perspective the Proficiency test provides an added value allowing the possibility to compare the efficacy of different techniques on the same sample.

Furthermore, it is expected that with the future publication of European diagnostic manuals, the process of harmonisation of the diagnostic methods within laboratories will be improved.

It is however possible to specify trends for each pathogen and diagnostic methods:

- ISAV Real time RT PCR: protocols based on Snow et al. 2006 are the most employed
- ISAV RT-PCR protocols based on Mjaaland et al.,2002 are the most employed
- KHV Real time PCR: protocols based on Gilad et al., 2004 are the most employedKHV PCR: protocols based on Bercovier et al., 2005 are the most employed
- A.invadans/EUS PCR: protcols based on Vandersea et al.,2006 are the most employed.

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

- 15 laboratories provided sequences for 18sRNA region of *A. Invadans*

- 13 laboratories provided sequences for TK region of KHV for both Ampoule VII and Ampoule VIII
- 4 laboratories provided sequences for Sphl-5 region of KHV for both Ampoule VII and Ampoule VIII
- 4 laboratories provided sequences for TK region of KHV for both Ampoule VII and Ampoule VIII
- 29 laboratories performed sequencing for ISAV genotyping the isolate based on the HPR region

Concluding remarks PT2

Considering that this was the fourth time that the EURL provided a proficiency test on ISAV and KHV identification, and the third time that the EURL provided a proficiency test on *A. invadans*, we consider that most participants obtained satisfying results.
Out of 36 laboratories testing for *A. invadans* 32 identified the pathogen in ampoule VI.
Out of 42 laboratories performing KHV identification, 41 laboratories identified KHV in ampoule VII and 42 correctly identified KHV in ampoule VIII.
Out of 43 laboratories 40 laboratories identified Not *A.invadans, KHV or* ISAV in ampoule IX.
Out of 42 laboratories performing ISAV identification 40 correctly identified ISAV in ampoule VI while 1 laboratory described coinfection ISAV and KHV scoring 1 point for this ampoule. Very significant improvement in the proficiency of identifying and typing these pathogens has been observed during these 4 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. As agreed at the Annual Workshop held in May 2013 this pathogen was included in 2013 but will not be included in 2014.

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 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 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 18th Annual Workshop of National Reference Laboratories for Fish Diseases to be held 3rd and 4th of June 2014 in Copenhagen, Denmark.

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European Union Reference laboratory for Fish diseases
National Veterinary Institute, Technical University of Denmark, 28February 2014

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