European Union Reference Laboratory for Fish Diseases
National Veterinary Institute, Technical University of Denmark, Copenhagen

EURL for Fish Diseases

Report of the Inter-Laboratory Proficiency Test 2014

for identification and titration of

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

and identification of

CyHV-3 (KHV) and ISAV (PT2)

Organised by the
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Copenhagen, Denmark

DANAK

PT Reg. no.: 515

DTU

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Introduction

A comparative test of diagnostic procedures was provided by the European Union Reference Laboratory (EURL) for Fish Diseases. The test was divided into 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) and Cyprinid herpesvirus 3 (CyHV-3) (otherwise known as koi herpes virus – KHV) by biomolecular methods (PCR based). 41 laboratories participated in PT1 while 40 participate in PT2.
The tests were sent from the EURL in the beginning of October 2014.
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 2014. 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). Two ampoules contained KHV, one ampoule contained ISAV and one Sterile Medium, see table 11. 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) 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.

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 19th Annual Workshop of the NRLs for Fish Diseases May 2015 in Copenhagen. Participants were asked to reply latest November 21st 2014.

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 6 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 30 participants; 8 more tests were delivered within 9 days; 1 further test was delivered within 27 days and the last within 57 days (Figure 1). One laboratory did not reported how long time the shipment last, however it is considered to be within 3 days from the shipment. All the parcels were sent without cooling elements. 6 countries outside EU had a logger in the parcel.

A relatively high stability was demonstrated to characterize the lyophilized pathogens in glass ampoules as described in PT 2012.

This year, unfortunately the ampoules containing IPNV Ab were not stable since it was not possible to re-isolate the virus on cell cultures in the stability test, performed 3-months after shipment, it was however possible to detect the specific viral RNA using RT-qPCR. This aspect has been considered when providing score to the participants, and as such, does not affect the integrity of the test as IPN is a non-listed disease.

Extra parcels were kept at 4°C in order to be able to provide fast substitutes in case of damage during transport.

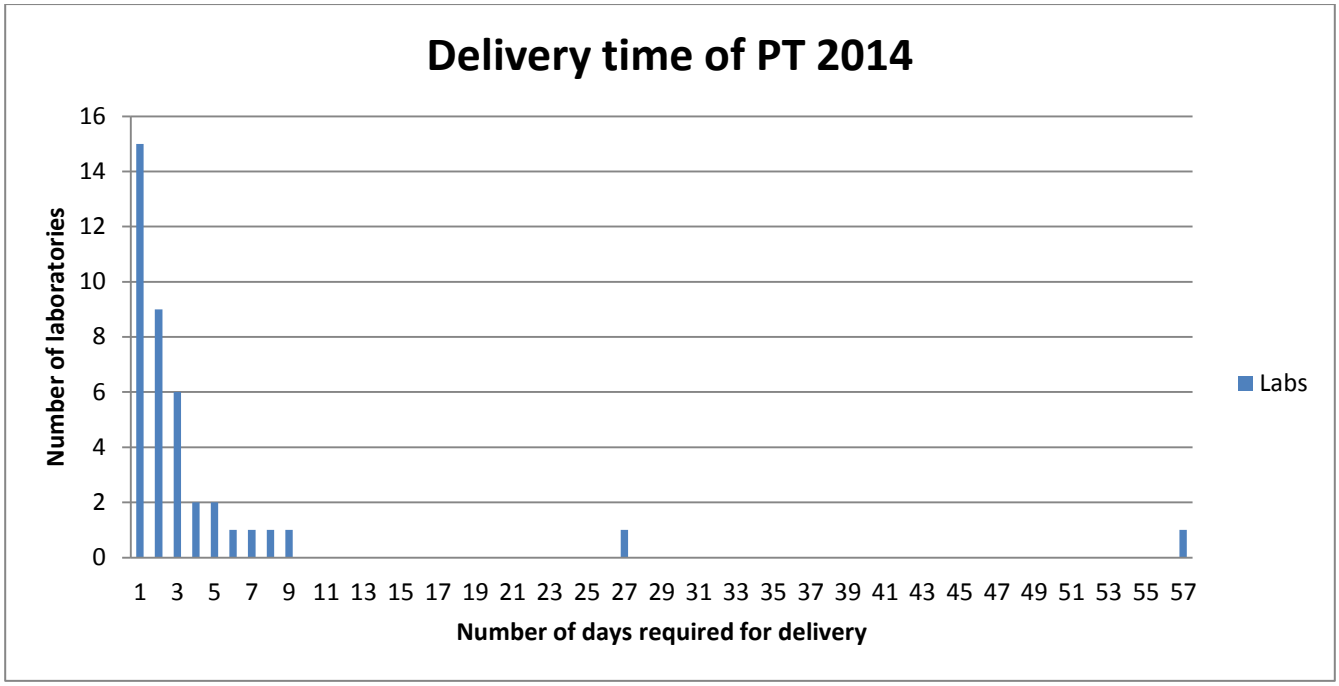


Figure 1. Transport time for the parcels to reach the participants.

Participation

PT1 and PT2: 41 laboratories received the annual proficiency test. All the participants submitted results within the deadline. Figure 2 shows how many laboratories that participated in the proficiency test from 1996 to 2014.

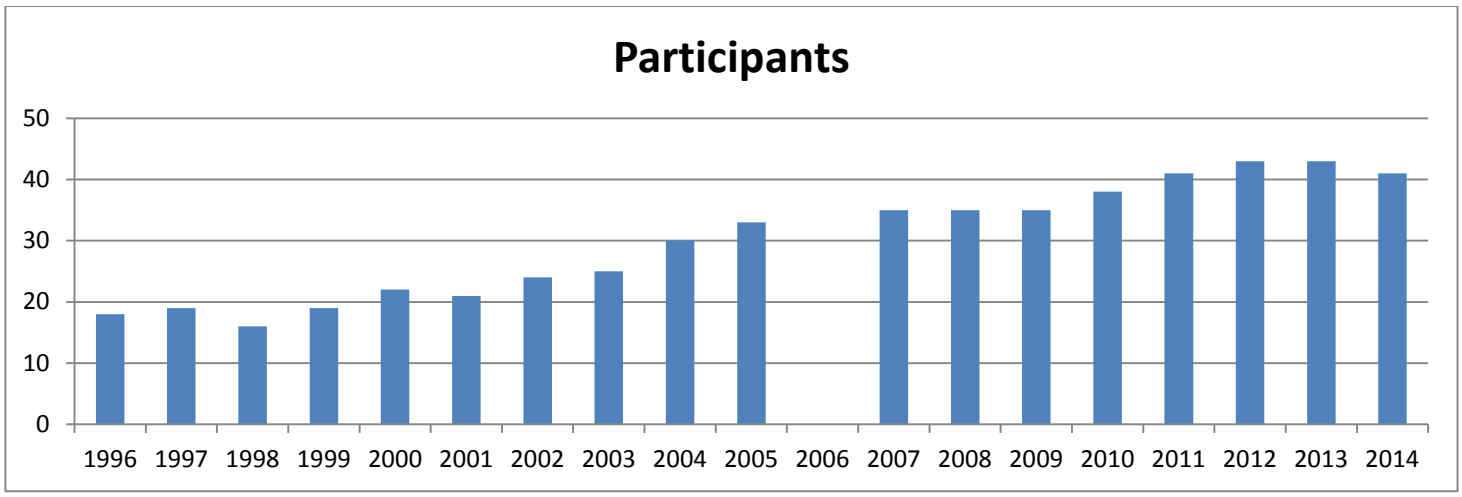


Figure 2. Participants in the EURL proficiency test over the years.

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, Bosnia and Herzegovina, Canada, Croatia, Faroe Islands, Iceland, Japan, New Zealand, Norway, Serbia, Switzerland, Turkey, and 2 from P.R. China and South Korea and USA. A parcel shipped to one laboratory in USA was never delivered due to customer restriction.

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 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 serotype Ab	**IPN virus[1],** serotype Ab, propagated in BF-2 cells. The sequence of the VP2 region of this IPNV isolate is 100% identical to the IPNV polyprotein gene of isolate 578 (serotype Ab) **GenBank accession numbers:** AJ489228.1
Ampoule II: SVCV 56/70	**Received from:** Prof. Fijan (January 1979 in a tube named Rhabdo virus carpio 56/70 and given as the reference strain of SVC virus). Isolate from carp. **Cell culture passage number:** Unknown. **Genotype:** Id (Stone et al. 2003). 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. **GenBank accession numbers:** Z37505.1 (S30), AJ538061.1 (Fijan) **Reference on isolate:** 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 III: EHNV 86/8774	**Received from:** Australia, The EHNV OIE reference laboratory (EURL file number 202213). Australian freshwater isolate from rainbow trout from Adaminaby Trout Farm, NSW obtained in 1986 by Jeremy Langdon. **Cell culture passage number:** 9 **Genotype:** **GenBank accession numbers:** FJ433873, AY187045, AF157667 **Reference on isolate:** Langdon JS, Humphrey JD & Williams LM (1988). Outbreaks of an EHNV-like iridovirus in cultured rainbow trout, *Salmo gairdneri* Richardson, in Australia. *Journal of Fish Diseases* **11**, 93-96. **References on sequences:** Hyatt A.D., Gould A.R., Zupanovic Z., Cunningham A.A., Hengstberger S., Whittington R.J., Kattenbelt J. & Coupar B.E.H. (2000) Comparative studies of piscine and amphibian iridoviruses. Archives of Virology 145, 301-331. Jancovich J.K., Bremont M., Touchman J.W. & Jacobs B.L. (2010) Evidence for multiple recent host species shifts among the ranaviruses (family Iridoviridae). Journal of Virology 84, 2636-2647. Marsh I.B., Whittington R.J., O'Rourke B., Hyatt A.D. & Chisholm O. (2002) Rapid differentiation of Australian, European and American ranaviruses based on variation in major capsid protein gene sequence. Molecular and Cellular Probes 16, 137-151.

Code	Specifications
Ampoule IV: IHNV 32/87	**Received from:** First French isolate (April 1987) from rainbow trout. **Cell culture passage number: 9 passages in EPC.** **GenBank accession numbers:** J265717 AY524121 (G-gene), FJ265711 (N gene) **Reference on isolate:** *Hattenberger-Baudouy AM, Danton M, Merle G, Torchy C, de Kinkelin P* **(1989)** Serological evidence of infectious haematopoietic necrosis in rainbow trout from a French outbreak of disease. *Journal of Aquatic Animal Health* **1**, 126-134. *Baudin Laurencin F* **(1987)** IHN in France. *Bulletin of the European Association of Fish Pathologists* **7**, 104. GenBank accession number:. J265717 AY524121 (G-gene), FJ265711 (N gene).
Ampoule V: VHSV DK-6137 Hjarnø	**Received from:** Denmark, VHS virus, strain DK-6137 (Hjarnø) The isolate originated from an outbreak of VHS with high mortality in sea water aquaculture **Cell culture passage number:** 3 **Serotype:** III **Genotype:** **GenBank accession numbers:** DQ159190 **Reference on isolate:** Ref:*Olesen, N.J., N. Lorenzen & P.E.V. Jørgensen* (**1993**) Serological differences among isolates of viral haemorrhagic septicaemia virus detected by neutralizing monoclonal and polyclonal antibodies. Dis. Aquat. Org. 16, 163-170. Olesen, N. J., Lorenzen, N. & LaPatra, S. (1999). Production of neutralizing antisera against viral haemorrhagic septicaemia (VHS) virus by intravenous injections of rabbits. J Aquat Anim Health 11, 10–16.

[1]The content of Ampoule I was previously been identified as IPNV Ab type strain, sequencing analysis confirmed that it is IPNV belonging to the Ab serotype but not the type strain mentioned in Blake et al. Dis. Aquat. Org. 45 (2), 89-102 (2001). IPNV strain Ab has the Accession number AF342729

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 is known to determine some reduction especially for VHSV. Previous experience reported during past Proficiency tests demonstrated a rather high stability for SVCV, EHNV and IPN Sp. 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).

In PT 2014 it could be observed for the ampoule containing the IPNV Ab that a significant reduction of the viable particles was observed, resulting in a reduction of viable virus particles to below threshold in all ampoules tested 3 months after shipment . The virus was, however, still detectable by RT-qPCR. (Table 2 and Figure 5).

The identities of the viruses in all 5 ampoules were checked and confirmed before shipment by ELISA, IFAT, serum neutralisation tests (SNT), RT-PCR and sequencing for VHSV, IHNV and by ELISA, IFAT, and SNT for IPNV and by PCR, sequencing and IFAT for ranavirus. For each ampoule, no presence of viruses other than the expected was observed.
After shipment stability was assessed by titrating the virus on cell cultures, and performing ELISA on the isolated virus, furthermore PCR based tests were conducted perfomed on the original content of the ampoule.

Table 2. PT1: Titres in ampoules I to V stored in the dark tested on four cell lines at different time points:
- before lyophilisation (T=4°C)
- 2 months- before shipment (median titre of 5 replicates) (T=4°C), The variation of the titre of the 5 replicates was within 1 log.
- 3 months after shipment, stored at 4°C

Ampoule No.	Cell line	Titre before lyophilisation	Median titre 2 months before shipment; 5 replicates	Titre after 3 months after shipment (4°C, dark conditions)
		$TCID_{50}$/ml	$TCID_{50}$/ml	$TCID_{50}$/ml
Ampoule I: IPNV Isolate strain Ab	BF-2	1,3E+06	1,9E+07	< 1,9E+02
	EPC	1,3E+04	8,6E+02	< 1,9E+02
	RTG-2	1,9E+06	8,6E+06	< 1,9E+02
	FHM	< 1,9E+02	1,4E+03	< 1,9E+02
Ampoule II: Isolate SVCV 56/70	BF-2	1,3E+05	8,6E+05	1,3E+05
	EPC	1,9E+05	8,6E+03	5,9E+05
	RTG-2	1,3E+05	2,7E+06	1,9E+05
	FHM	2,7E+06	1,9E+06	2,7E+06
Ampoule III: EHNV Isolate 86/8774	BF-2	5,9E+06	1,3E+06	1,3E+03
	EPC	1,3E+06	8,6E+05	1,3E+04
	RTG-2	1,9E+06	8,6E+04	1,3E+03
	FHM	1,3E+05	1,3E+04	1,9E+03
Ampoule IV: Isolate IHNV 32/87	BF-2	1,3E+05	1,9E+06	< 1,9E+02
	EPC	1,9E+05	5,9E+05	5,9E+05
	RTG-2	5,9E+04	4,0E+05	5,9E+03
	FHM	4,0E+05	4,0E+05	4,0E+05
Ampoule V: Isolate VHSV DK-6137 Hjarnø	BF-2	4,0E+07	4,0E+05	2,7E+05
	EPC	2,7E+07	1,3E+06	2,7E+05
	RTG-2	4,0E+07	2,7E+06	1,3E+06
	FHM	8,6E+07	4,0E+06	4,0E+05

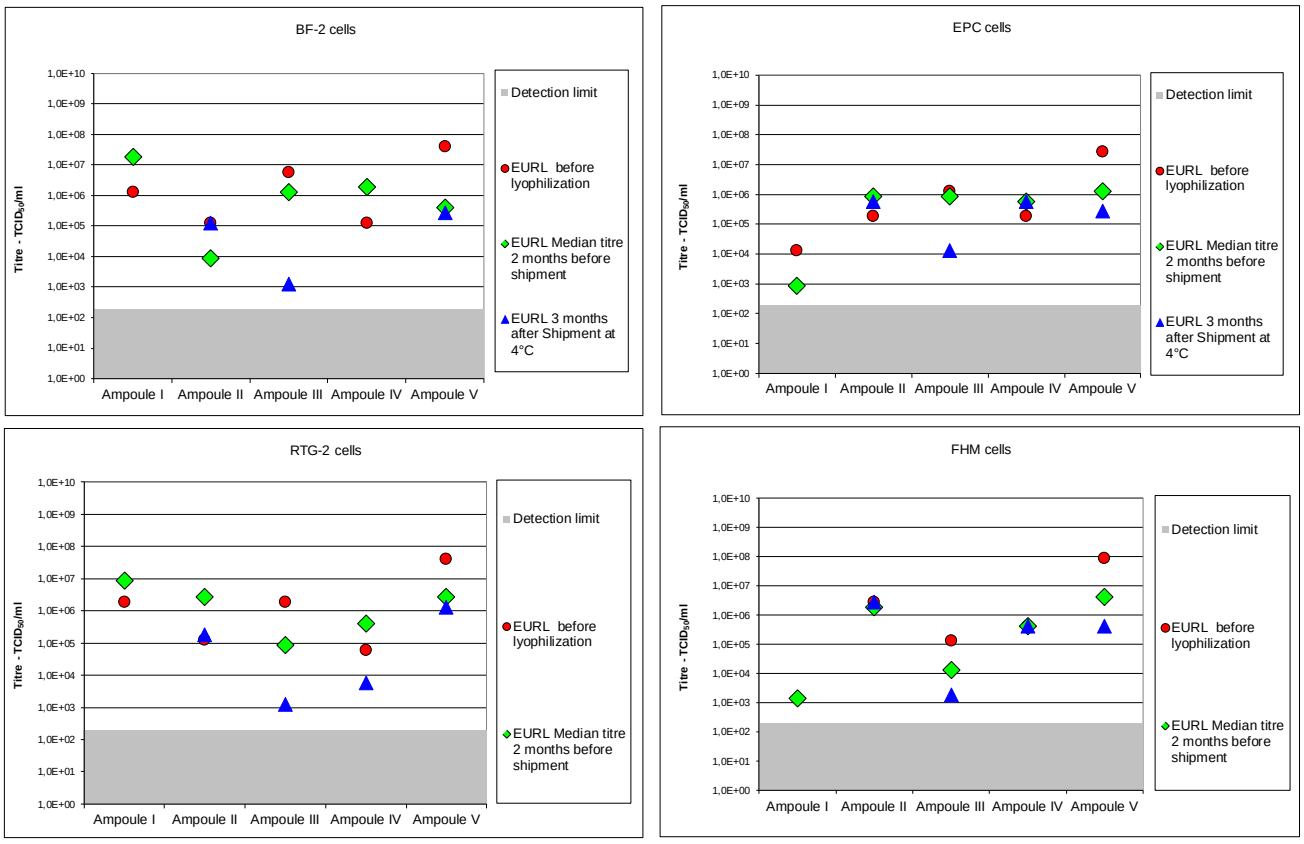


Figure 4. Virus titers in different cell lines before freeze drying, 2 months before- and 3 months after shipment.

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, 2014 - Virus identification and score obtained by participants.

Laboratory code number	Score	Answer recived at EURL	Ampoule I	Ampoule II	Ampoule III	Ampoule IV	Ampoule V
			IPN strain AB	SCVC 56/70	EHNV 86/8774	IHNV 32/87	VHSV DK-6137 Hjarnø
1	10/10	05.12.14	IPNV	SVCV	EHNV	IHNV	VHSV
2	10/10	17.11.14	IPNV	SVCV	EHNV	IHNV	VHSV
3	10/10	01.12.14	IPNV	SVCV	EHNV	IHNV	VHSV
4	8/8	26.11.14	IPNV	SVCV		IHNV	VHSV
5	9/10*	05.12.14	IPNV	SVCV	EHNV	IHNV	VHSV
6	10/10	04.12.14	IPNV	SVCV	EHNV	IHNV	VHSV
7	10/10	04.12.14	IPNV	SVCV	EHNV	IHNV	VHSV
8	10/10	02.12.14	IPNV	SVCV	EHNV	IHNV	VHSV
9	10/10	05.12.14	IPNV	SVCV	EHNV	IHNV	VHSV
10	9/10[1]	26.11.14	IPNV	SVCV	NO IHNV, SVCV, IHNV, VHSV	IHNV	VHSV
11	10/10	05.12.14	IPNV	SVCV	EHNV	IHNV	VHSV
12	6/10	27.11.14	IPNV genogroup 2	SVCV genotype Id	EHNV	VHSV genotype Ia	IHNV genotype M
13	10/10	24.11.14	IPNV	SVCV	EHNV	IHNV	VHSV
14	10/10	03.12.14	IPNV Ab	SVCV Fijan	EHNV	IHNV	VHSV
15	10/10	05.12.14	IPNV	SVCV	EHNV	IHNV	VHSV
16	10/10	27.11.14	IPNV	SVCV	EHNV	IHNV	VHSV
17	10/10	02.12.14	IPNV	SVCV	EHNV	IHNV	VHSV
18	10/10	24.11.14	IPNV	SVCV	EHNV	IHNV	VHSV
19	10/10	02.12.14	IPNV	SVCV	EHNV	IHNV	VHSV
20	10/10	02.11.14	IPNV	SVCV	EHNV	IHNV	VHSV
21	10/10	05.12.14	IPNV	SVCV	EHNV	IHNV	VHSV
22	9/10	04.12.14	IPNV	SVCV	EHNV	EHNV, IHNV	VHSV
23	10/10	05.12.14	IPNV	SVCV	EHNV	IHNV	VHSV
24	10/10	05.12.14	IPNV	SVCV	EHNV	IHNV	VHSV
25	10/10	03.12.14	IPNV	SVCV	EHNV	IHNV	VHSV
26	10/10	08.12.14	IPNV	SVCV	EHNV	IHNV	VHSV
27	10/10	05.12.14	IPNV	SVCV	EHNV	IHNV	VHSV
28	10/10	05.12.14	IPNV	SVCV	EHNV	IHNV	VHSV
29	10/10	05.12.14	IPNV	SVCV	EHNV	IHNV	VHSV
30	9/10	05.12.14	IPNV	SVCV	EHNVs	IHNV	VHSV

31	**10/10**	**27.11.14**	IPNV	SVCV	EHNV	IHNV	VHSV
32	**10/10**	**05.12.14**	IPNV	SVCV	EHNV	IHNV	VHSV
33	**10/10**	**04.12.14**	negative	SVCV	EHNV	IHNV	VHSV
34	**10/10**	**04.12.14**	Negative	SVCV	EHNV	IHNV	VHSV
35	**10/10**	**05.12.14**	IPNV	SVCV	EHNV	IHNV	VHSV
36	**10/10**	**03.12.14**	IPNV	SVCV	EHNV	IHNV	VHSV
37	**8/10**	**03.12.14**	NEG	SVCV	EHNV	IHNV/VHSV	VHSV
38	**10/10**	**21.11.14**	Sterile	SVCV	EHN	IHNV	VHSV
39	**10/10**	**05.12.14**	IPNV	SVCV	EHNV	IHNV	VHSV
40	**10/10**	**03.12.14**	Negative	SVCV	EHNV	IHNV	VHSV
41	**10/10**	**07.12.14**	IPNV	SVCV	EHNV	IHNV	VHSV
42		The PT package was returned due to Customer limitations					

[1] Did not perform test for Ranavirus

* Genomic analysis not performed

Table 4. Inter-Laboratory Proficiency Test, PT1, 2014 – Results of titration of ampoule I.

Laboratory code number	Virus Identification	Titre in			
		BF-2	**EPC**	**RTG-2**	**FHM**
1	IPNV	8,6E+05	1,9E+02	5,9E+05	N/A
2	IPNV	2,7E+02	< 1,9E+02	< 1,9E+02	1,9E+02
3	IPNV	5,9E+04	1,3E+05	N/A	N/A
4	IPNV	1,3E+06	1,9E+02	N/A	N/A
5	IPNV	1,3E+06	8,6E+02	N/A	N/A
6	IPNV	4,0E+06	< 1,9E+02	8,6E+04	< 1,9E+02
7	IPNV	8,6E+06	2,7E+03	N/A	N/A
8	IPNV	N/A	N/A	< 1,9E+02	< 1,9E+02
9	IPNV	< 1,9E+02	< 1,9E+02	1,9E+02	< 1,9E+02
10	IPNV	< 1,9E+02	1,3E+03	N/A	N/A
11	IPNV	1,9E+06	< 1,9E+02	N/A	N/A
12	IPNV genogroup 2	5,9E+06	5,9E+03	N/A	N/A
13	IPNV	1,3E+03	< 1,9E+02	4,0E+05	N/A
14	IPNV Ab	< 1,9E+02	< 1,9E+02	5,9E+06	N/A
15	IPNV	1,3E+06	< 1,9E+02	N/A	N/A
16	IPNV	1,9E+02	1,3E+03	N/A	N/A
17	IPNV	< 1,9E+02	< 1,9E+02	N/A	N/A
18	IPNV	5,9E+08	8,6E+04	N/A	N/A
19	IPNV	2,7E+08	5,9E+04	N/A	N/A
20	IPNV	5,9E+06	< 1,9E+02	< 1,9E+02	< 1,9E+02
21	IPNV	1,9E+06	< 1,9E+02	N/A	N/A
22	IPNV	< 1,9E+02	4,0E+04	1,9E+07	N/A
23	IPNV	1,9E+07	1,3E+03	N/A	N/A
24	IPNV	8,6E+08	< 1,9E+02	N/A	N/A
25	IPNV	2,7E+05	2,7E+03	N/A	N/A
26	IPNV	N/A	< 1,9E+02	N/A	2,7E+04
27	IPNV	1,3E+06	1,9E+02	1,9E+05	2,7E+02
28	IPNV	N/A	< 1,9E+02	< 1,9E+02	N/A
29	IPNV	8,6E+06	< 1,9E+02	N/A	N/A
30	IPNV	1,9E+05	1,9E+04	N/A	N/A
31	IPNV	1,3E+06	1,3E+04	1,9E+04	1,3E+04
32	IPNV	1,9E+03	< 1,9E+02	N/A	N/A
33	negative	< 1,9E+02	< 1,9E+02	< 1,9E+02	< 1,9E+02
34	Negative	< 1,9E+02	< 1,9E+02	N/A	N/A
35	IPNV	< 1,9E+02	< 1,9E+02	< 1,9E+02	< 1,9E+02
36	IPNV	5,9E+06	N/A	N/A	< 1,9E+02
37	NEG	1,9E+02	< 1,9E+02	N/A	N/A
38	Sterile	N/A	< 1,9E+02	N/A	< 1,9E+02
39	IPNV	4,0E+04	< 1,9E+02	N/A	N/A
40	Negative	< 1,9E+02	N/A	N/A	< 1,9E+02
41	IPNV	2,7E+04	< 1,9E+02	N/A	N/A

Number of laboratories	35	37	14	14
Median titre	1,3E+06	2,7E+03	2,9E+05	2,7E+02
Maximum titre	8,6E+08	1,3E+05	1,9E+07	2,7E+04
Minimum titre	<1,9E+02	<1,9E+02	<1,9E+02	0
25% quartile titre	5,4E+04	1,2E+03	6,9E+04	<1,9E+02
75% quartile titre	5,9E+06	2,4E+04	1,9E+06	1,3E+04

N/A: cell line not applied by the participant laboratory to titrate the virus

Table 5. Inter-Laboratory Proficiency Test, PT1, 2014 – Results of titration of ampoule II.

Laboratory code number	Virus Identification	Titre in			
		BF-2	EPC	RTG-2	FHM
1	SVCV	1,3E+05	8,6E+04	1,3E+03	N/A
2	SVCV	5,9E+03	8,6E+04	2,7E+05	8,6E+05
3	SVCV	4,0E+04	5,9E+05	N/A	N/A
4	SVCV	1,86E+04	4,00E+04	N/A	N/A
5	SVCV	4,0E+05	8,6E+04	N/A	N/A
6	SVCV	1,9E+05	5,9E+06	< 1,9E+02	1,9E+06
7	SVCV	2,7E+04	5,9E+05	N/A	N/A
8	SVCV	N/A	N/A	4,0E+05	1,3E+05
9	SVCV	5,9E+05	1,3E+06	1,9E+05	1,3E+06
10	SVCV	< 1,9E+02	1,9E+05	N/A	N/A
11	SVCV	1,3E+06	1,3E+06	N/A	N/A
12	SVCV genotype Id	4,0E+05	1,9E+06	N/A	N/A
13	SVCV	1,9E+04	4,0E+07	2,7E+04	N/A
14	SVCV Fijan	< 1,9E+02	1,9E+06	< 1,9E+02	N/A
15	SVCV	5,9E+05	1,3E+05	N/A	N/A
16	SVCV	5,9E+04	5,9E+05	N/A	N/A
17	SVCV	1,9E+05	1,9E+05	N/A	N/A
18	SVCV	8,6E+06	8,6E+06	N/A	N/A
19	SVCV	1,3E+07	4,0E+06	N/A	N/A
20	SVCV	1,9E+04	2,7E+05	1,3E+05	2,7E+05
21	SVCV	2,7E+03	2,7E+03	N/A	N/A
22	SVCV	< 1,9E+02	1,9E+06	5,9E+04	N/A
23	SVCV	4,0E+04	4,0E+04	N/A	N/A
24	SVCV	5,9E+06	8,6E+06	N/A	N/A
25	SVCV	8,6E+04	4,0E+02	N/A	N/A
26	SVCV	N/A	1,9E+06	N/A	1,3E+06
27	SVCV	5,9E+06	1,9E+06	1,3E+05	4,0E+05
28	SVCV	N/A	2,7E+05	4,0E+04	N/A
29	SVCV	2,7E+04	< 1,9E+02	N/A	N/A
30	SVCV	1,9E+05	1,3E+05	N/A	N/A
31	SVCV	1,3E+04	2,7E+04	1,3E+04	5,9E+04
32	SVCV	5,9E+06	1,9E+07	N/A	N/A
33	SVCV	4,00E+03	1,86E+05	2,73E+03	2,73E+03
34	SVCV	8,62E+03	1,86E+04	N/A	N/A
35	SVCV	5,9E+04	4,0E+05	4,0E+04	2,7E+05
36	SVCV	1,9E+04	N/A	N/A	2,7E+05
37	SVCV	1,3E+06	4,0E+06	N/A	N/A
38	SVCV	N/A	4,0E+05	N/A	1,9E+05
39	SVCV	2,7E+06	5,9E+06	N/A	N/A
40	SVCV	1,9E+06	N/A	N/A	5,9E+05
41	SVCV	8,6E+02	4,0E+04	N/A	N/A

Number of laboratories	35	38	14	13
Median titre	1,1E+05	4,9E+05	5,9E+04	2,7E+05
Maximum titre	1,3E+07	4,0E+07	4,0E+05	1,9E+06
Minimum titre	8,6E+02	4,0E+02	1,3E+03	2,7E+03
25% quartile titre	1,9E+04	8,6E+04	2,0E+04	1,9E+05
75% quartile titre	1,1E+06	1,9E+06	1,6E+05	8,6E+05

N/A: cell line not applied by the participant laboratory to titrate the virus

Table 6. Inter-Laboratory Proficiency Test, PT1, 2014 – Results of titration of ampoule III.

Laboratory code number	Virus Identification	Titre in			
		BF-2	EPC	RTG-2	FHM
1	EHNV	2,7E+04	4,0E+04	1,9E+04	N/A
2	EHNV	2,7E+04	5,9E+05	5,9E+05	5,9E+03
3	EHNV	1,9E+05	2,7E+05	N/A	N/A
4	No ID	5,87E+04	5,87E+04	N/A	N/A
5	EHNV	8,6E+06	1,9E+04	N/A	N/A
6	EHNV	4,0E+06	4,0E+06	< 1,9E+02	2,7E+04
7	EHNV	2,7E+05	4,0E+04	N/A	N/A
8	EHNV	N/A	N/A	1,9E+04	1,3E+04
9	EHNV	8,6E+05	4,0E+05	4,0E+04	1,3E+04
10	NO IHNV, SVCV, IHNV, VHSV	4,0E+05	2,7E+06	N/A	N/A
11	EHNV	1,9E+05	1,3E+05	N/A	N/A
12	EHNV	8,6E+05	5,9E+05	N/A	N/A
13	EHNV	1,9E+06	5,9E+05	8,6E+04	N/A
14	EHNV	< 1,9E+02	2,7E+06	1,9E+05	N/A
15	EHNV	1,3E+07	1,3E+06	N/A	N/A
16	EHNV	5,9E+05	1,9E+06	N/A	N/A
17	EHNV	8,6E+04	1,3E+05	N/A	N/A
18	EHNV	5,9E+05	1,3E+05	N/A	N/A
19	EHNV	1,9E+07	1,9E+06	N/A	N/A
20	EHNV	4,0E+06	5,9E+06	1,9E+06	1,3E+04
21	EHNV	4,0E+05	4,0E+04	N/A	N/A
22	EHNV	< 1,9E+02	2,7E+03	5,9E+03	N/A
23	EHNV	5,9E+04	1,3E+03	N/A	N/A
24	EHNV	2,7E+08	1,9E+06	N/A	N/A
25	EHNV	1,3E+04	1,3E+04	N/A	N/A
26	EHNV	N/A	8,6E+04	N/A	1,9E+05
27	EHNV	1,9E+04	1,3E+04	1,9E+04	1,3E+03
28	EHNV	N/A	< 1,9E+02	< 1,9E+02	N/A
29	EHNV	1,9E+05	1,9E+03	N/A	N/A
30	EHNV	5,9E+05	5,9E+05	N/A	N/A
31	EHNV	2,7E+04	2,7E+04	2,7E+04	1,3E+05
32	EHNV	4,0E+03	4,0E+05	N/A	N/A
33	EHNV	1,86E+04	2,73E+04	1,86E+03	< 1,9E+02
34	EHNV	1,86E+04	5,87E+04	N/A	N/A
35	EHNV	1,3E+06	1,3E+06	2,7E+06	1,9E+05
36	EHNV	5,9E+05	N/A	N/A	4,0E+03
37	EHNV	2,7E+06	8,6E+05	N/A	N/A
38	EHN	N/A	5,9E+05	N/A	1,9E+05
39	EHNV	5,9E+06	5,9E+06	N/A	N/A
40	EHNV	1,9E+06	N/A	N/A	4,0E+04
41	EHNV	1,3E+04	1,3E+03	N/A	N/A

Number of laboratories	35	38	14	13
Median titre	4,0E+05	2,7E+05	3,4E+04	1,3E+04
Maximum titre	2,7E+08	5,9E+06	2,7E+06	1,9E+05
Minimum titre	4,0E+03	1,3E+03	1,9E+03	1,3E+03
25% quartile titre	4,3E+04	4,0E+04	1,9E+04	5,9E+03
75% quartile titre	1,9E+06	1,3E+06	2,9E+05	1,3E+05

N/A: cell line not applied by the participant laboratory to titrate the virus

Table 7. Inter-Laboratory Proficiency Test, PT1, 2014 – Results of titration of ampoule IV.

Laboratory code number	Virus Identification	Titre in			
		BF-2	EPC	RTG-2	FHM
1	IHNV	< 1,9E+02	< 1,9E+02	< 1,9E+02	N/A
2	IHNV	< 1,9E+02	4,0E+06	< 1,9E+02	2,7E+06
3	IHNV	5,9E+04	1,3E+07	N/A	N/A
4	IHNV	< 1,9E+02	1,86E+05	N/A	N/A
5	IHNV	2,7E+06	2,7E+03	N/A	N/A
6	IHNV	1,9E+04	1,3E+07	1,3E+04	1,3E+07
7	IHNV	4,0E+02	2,7E+04	N/A	N/A
8	IHNV	N/A	N/A	1,9E+06	1,3E+05
9	IHNV	1,3E+03	4,0E+06	2,7E+06	1,9E+06
10	IHNV	1,3E+04	1,3E+07	N/A	N/A
11	IHNV	2,7E+03	8,6E+06	N/A	N/A
12	VHSV genotype Ia	5,9E+05	1,9E+06	N/A	N/A
13	IHNV	8,6E+03	1,3E+08	2,7E+06	N/A
14	IHNV	< 1,9E+02	4,0E+07	4,0E+05	N/A
15	IHNV	1,3E+04	8,6E+06	N/A	N/A
16	IHNV	1,9E+03	2,7E+05	N/A	N/A
17	IHNV	8,6E+03	8,6E+06	N/A	N/A
18	IHNV	1,9E+03	1,9E+07	N/A	N/A
19	IHNV	2,7E+03	2,7E+07	N/A	N/A
20	IHNV	< 1,9E+02	1,3E+03	1,3E+05	1,3E+03
21	IHNV	< 1,9E+02	1,3E+04	N/A	N/A
22	EHNV, IHNV	< 1,9E+02	5,9E+05	5,9E+05	N/A
23	IHNV	2,7E+03	1,3E+05	N/A	N/A
24	IHNV	1,3E+03	1,3E+09	N/A	N/A
25	IHNV	5,9E+04	4,0E+04	N/A	N/A
26	IHNV	N/A	5,9E+05	N/A	5,9E+05
27	IHNV	4,0E+02	5,9E+06	4,0E+06	2,7E+05
28	IHNV	N/A	2,7E+05	5,9E+04	N/A
29	IHNV	1,3E+03	< 1,9E+02	N/A	N/A
30	IHNV	1,9E+05	4,0E+05	N/A	N/A
31	IHNV	1,3E+05	1,3E+05	1,3E+04	1,3E+04
32	IHNV	4,0E+02	1,9E+04	N/A	N/A
33	IHNV	< 1,9E+02	1,26E+05	5,87E+04	1,26E+05
34	IHNV	1,86E+03	4,00E+03	N/A	N/A
35	IHNV	< 1,9E+02	8,6E+06	< 1,9E+02	4,0E+06
36	IHNV	1,3E+03	N/A	N/A	1,9E+06
37	IHNV/VHSV	1,3E+04	2,7E+08	N/A	N/A
38	IHNV	N/A	5,9E+06	N/A	1,9E+07
39	IHNV	2,7E+03	1,9E+07	N/A	N/A
40	IHNV	2,7E+03	N/A	N/A	5,9E+06
41	IHNV	< 1,9E+02	4,0E+05	N/A	N/A

Number of laboratories	35	38	14	13
Median titre	2,7E+03	4,0E+06	4,9E+05	1,9E+06
Maximum titre	2,7E+06	1,3E+09	4,0E+06	1,9E+07
Minimum titre	4,0E+02	1,3E+03	1,3E+04	1,3E+03
25% quartile titre	1,6E+03	1,3E+05	7,6E+04	1,3E+05
75% quartile titre	1,6E+04	1,3E+07	2,5E+06	4,0E+06

N/A: cell line not applied by the participant laboratory to titrate the virus

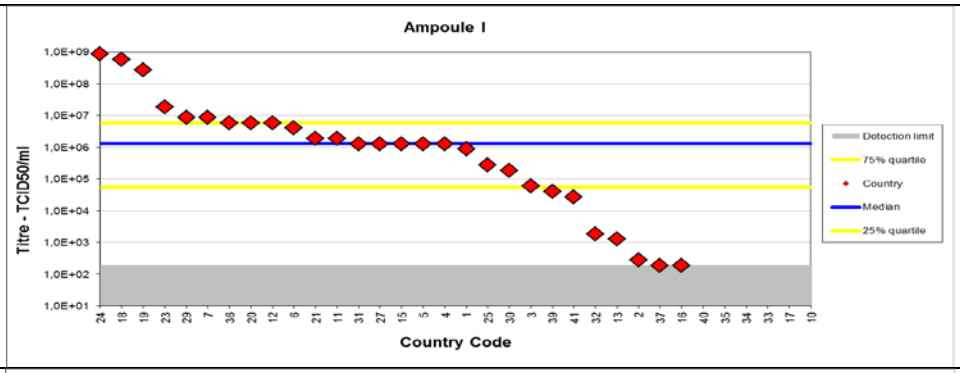
Table 8. Inter-Laboratory Proficiency Test, PT1, 2014 – Results of titration of ampoule V.

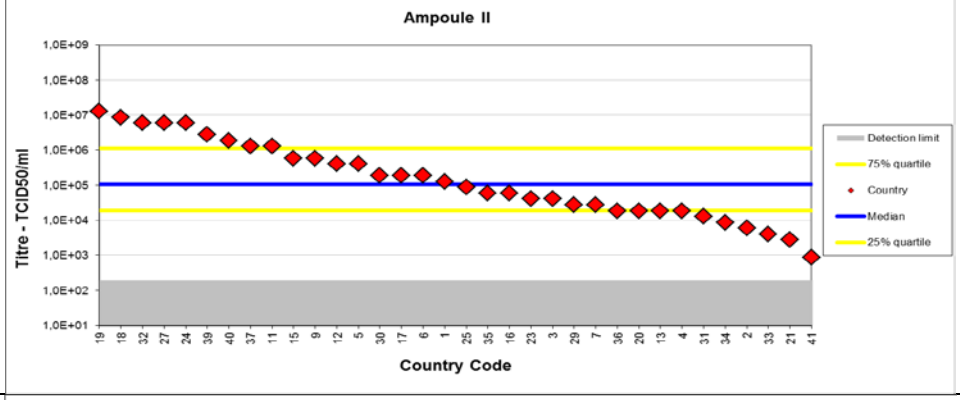
Laboratory code number	Virus Identification	Titre in			
		BF-2	EPC	RTG-2	FHM
1	VHSV	1,3E+04	1,9E+05	1,9E+05	N/A
2	VHSV	2,7E+05	1,9E+05	8,6E+04	4,0E+06
3	VHSV	4,0E+05	4,0E+06	N/A	N/A
4	VHSV	2,73E+04	2,73E+05	N/A	N/A
5	VHSV	1,3E+06	1,9E+03	N/A	N/A
6	VHSV	8,6E+05	5,9E+06	4,0E+05	2,7E+06
7	VHSV	4,0E+04	2,7E+03	N/A	N/A
8	VHSV	N/A	N/A	5,9E+06	5,9E+05
9	VHSV	1,9E+05	1,3E+06	5,9E+06	2,7E+06
10	VHSV	5,9E+05	4,0E+05	N/A	N/A
11	VHSV	1,3E+06	8,6E+06	N/A	N/A
12	IHNV genotype M	< 1,9E+02	1,3E+07	N/A	N/A
13	VHSV	8,6E+06	8,6E+07	1,3E+06	N/A
14	VHSV	< 1,9E+02	4,0E+06	1,9E+05	N/A
15	VHSV	5,9E+05	8,6E+05	N/A	N/A
16	VHSV	5,9E+05	1,9E+06	N/A	N/A
17	VHSV	5,9E+05	4,0E+06	N/A	N/A
18	VHSV	4,0E+06	8,6E+06	N/A	N/A
19	VHSV	1,9E+06	4,0E+06	N/A	N/A
20	VHSV	1,3E+03	4,0E+04	4,0E+04	2,7E+04
21	VHSV	1,3E+06	4,0E+05	N/A	N/A
22	VHSV	< 1,9E+02	1,3E+05	1,9E+06	N/A
23	VHSV	< 1,9E+02	< 1,9E+02	N/A	N/A
24	VHSV	4,0E+06	5,9E+06	N/A	N/A
25	VHSV	1,9E+03	1,3E+05	N/A	N/A
26	VHSV	N/A	1,9E+05	N/A	5,9E+04
27	VHSV	1,9E+06	5,9E+06	4,0E+06	4,0E+06
28	VHSV	N/A	5,9E+06	4,0E+05	N/A
29	VHSV	1,3E+04	< 1,9E+02	N/A	N/A
30	VHSV	1,9E+05	1,9E+05	N/A	N/A
31	VHSV	1,3E+06	5,9E+05	2,7E+04	1,3E+04
32	VHSV	1,3E+05	5,9E+05	N/A	N/A
33	VHSV	1,86E+04	< 1,9E+02	< 1,9E+02	< 1,9E+02
34	VHSV	1,26E+06	1,26E+07	N/A	N/A
35	VHSV	8,6E+05	4,0E+06	8,6E+05	4,0E+06
36	VHSV	1,9E+05	N/A	N/A	8,6E+06
37	VHSV	1,9E+07	2,7E+07	N/A	N/A
38	VHSV	N/A	4,0E+06	N/A	4,0E+06
39	VHSV	4,0E+06	1,9E+07	N/A	N/A
40	VHSV	2,7E+07	N/A	N/A	1,3E+07
41	VHSV	4,0E+03	1,3E+06	N/A	N/A

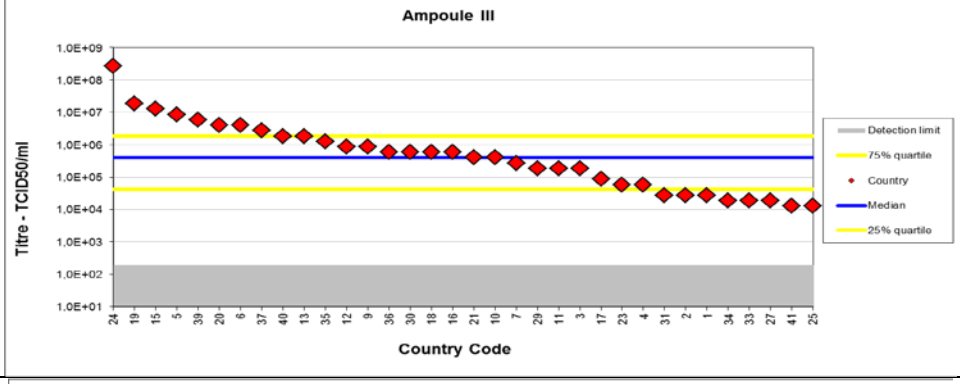
Number of laboratories	35	38	14	13
Median titre	5,9E+05	1,6E+06	6,3E+05	3,4E+06
Maximum titre	2,7E+07	8,6E+07	5,9E+06	1,3E+07
Minimum titre	1,3E+03	1,9E+03	2,7E+04	1,3E+04
25% quartile titre	1,3E+05	2,1E+05	1,6E+05	4,6E+05
75% quartile titre	1,3E+06	5,9E+06	2,4E+06	4,0E+06

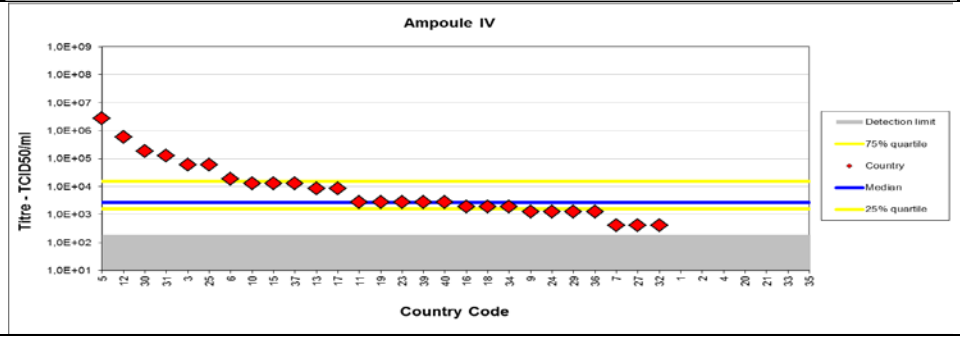
N/A: cell line not applied by the participant laboratory to titrate the virus

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.









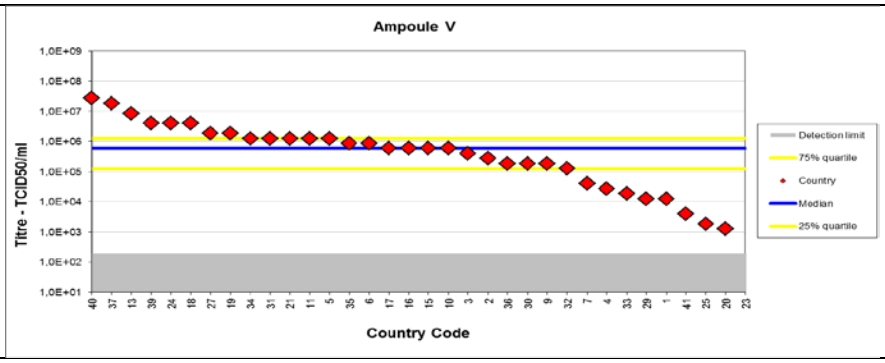
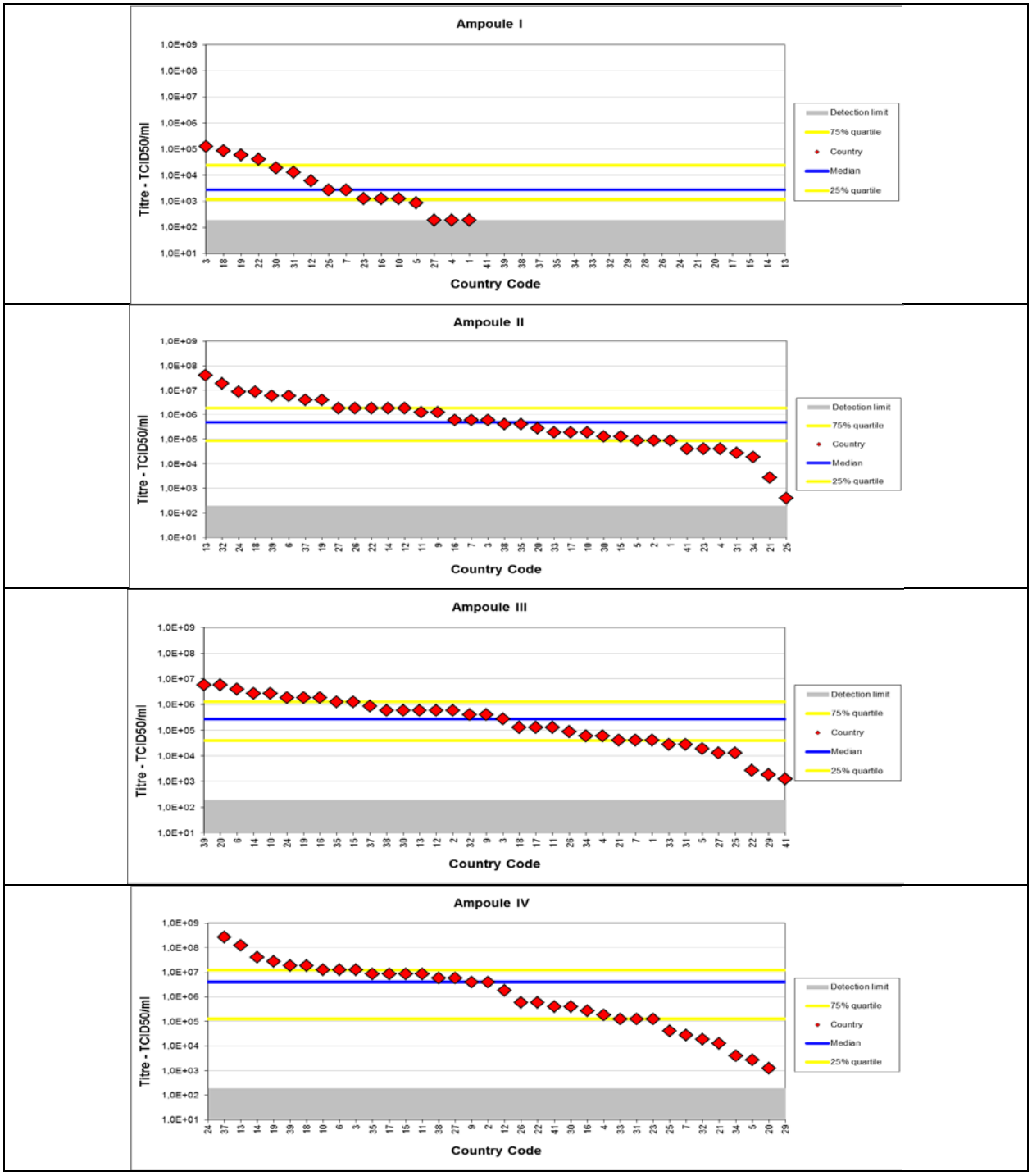

Ampoule V
Titre - TCID50/ml
1,0E+09
1,0E+08
1,0E+07
1,0E+06
1,0E+05
1,0E+04
1,0E+03
1,0E+02
1,0E+01
40 37 13 39 24 18 27 19 34 31 21 11 5 35 6 17 16 15 10 3 2 36 30 9 32 7 4 33 29 1 41 25 20 23
Country Code
Detection limit
75% quartile
Country
Median
25% quartile

Figure 6. Virus titres obtained in EPC cells. For further details see Figure 5



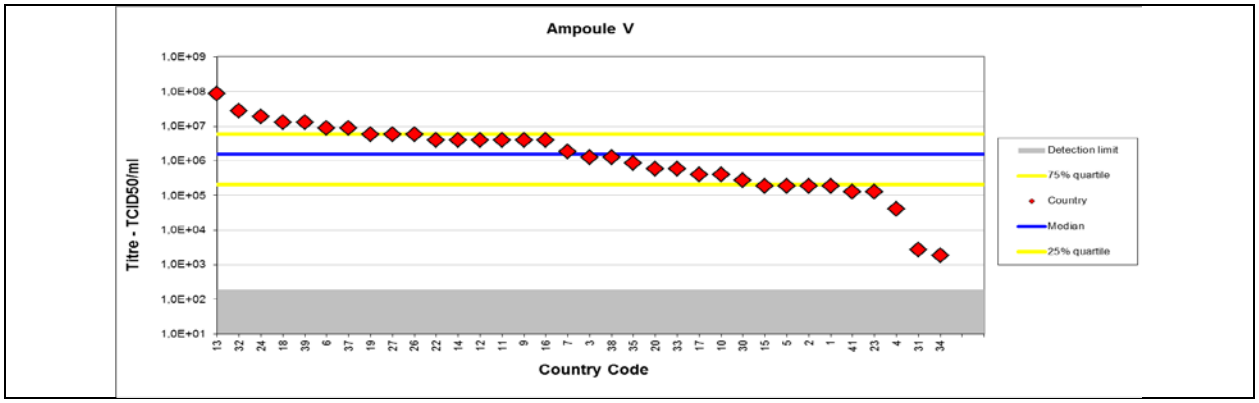
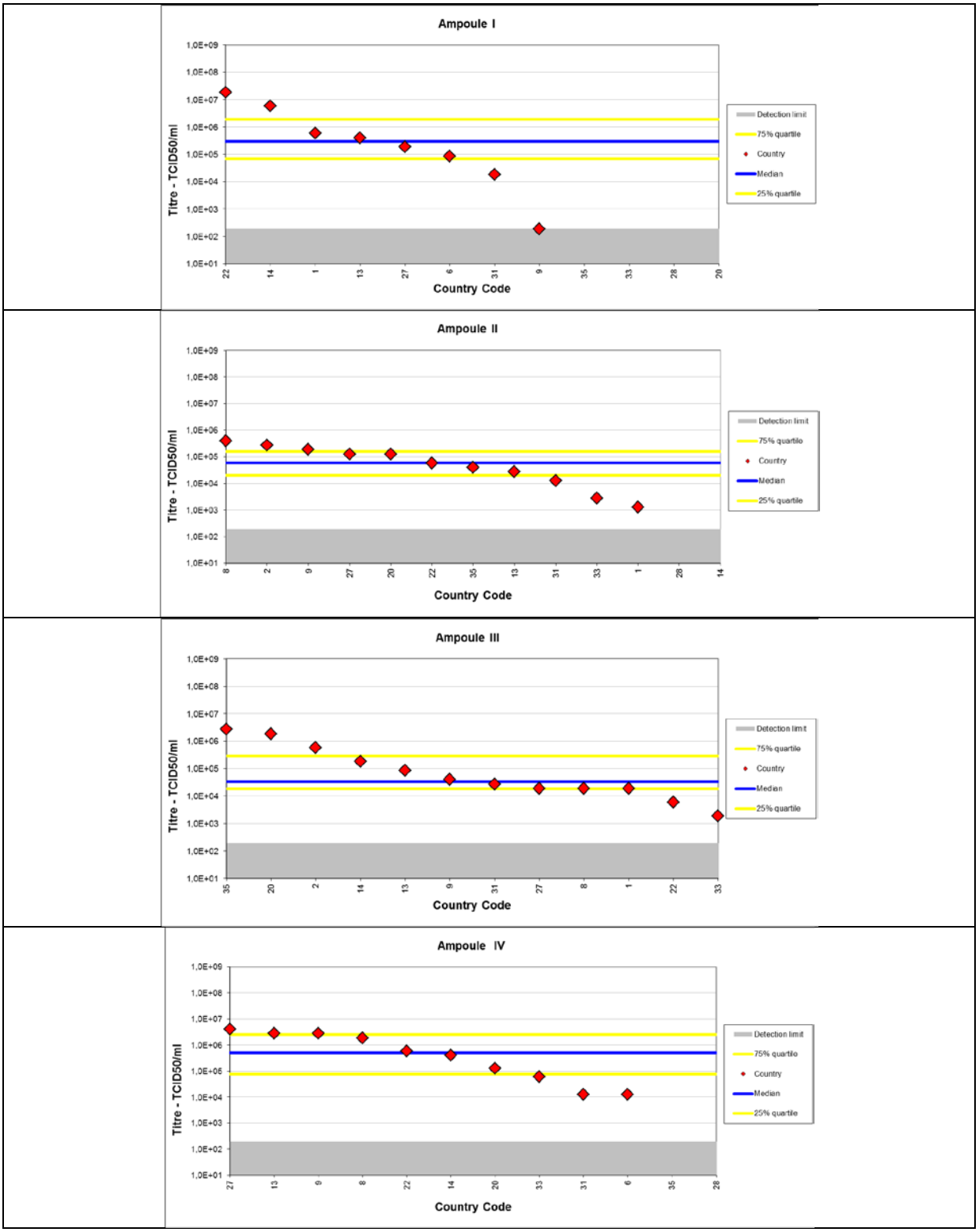

Ampoule V
Titre - TCID50/ml
1,0E+09
1,0E+08
1,0E+07
1,0E+06
1,0E+05
1,0E+04
1,0E+03
1,0E+02
1,0E+01
13 32 24 18 39 6 37 19 27 26 22 14 12 11 9 16 7 3 38 35 20 33 17 10 30 15 5 2 1 41 23 4 31 34
Country Code
Detection limit
75% quartile
Country
Median
25% quartile

Figure 7. Virus titre obtained in RTG-2 cells. For further details see Figure 5



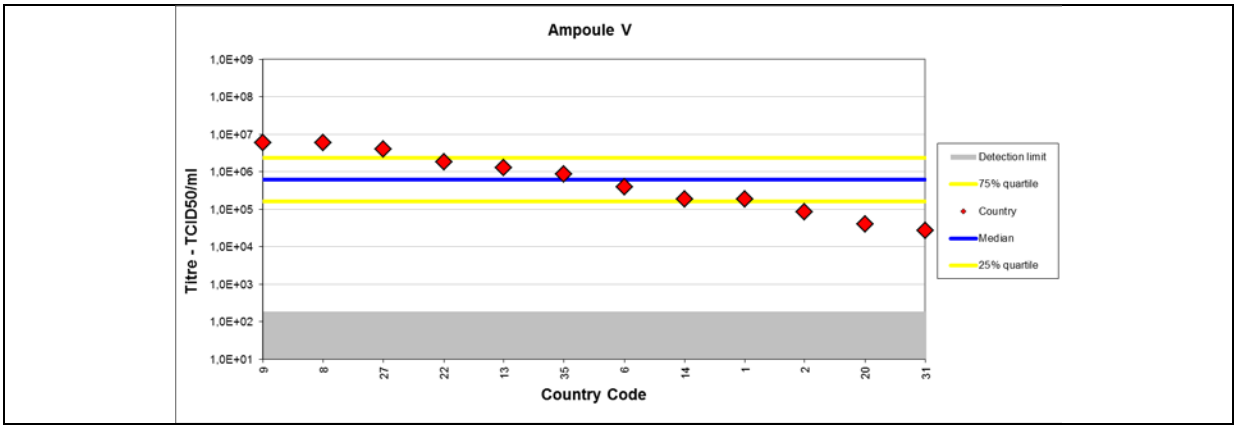
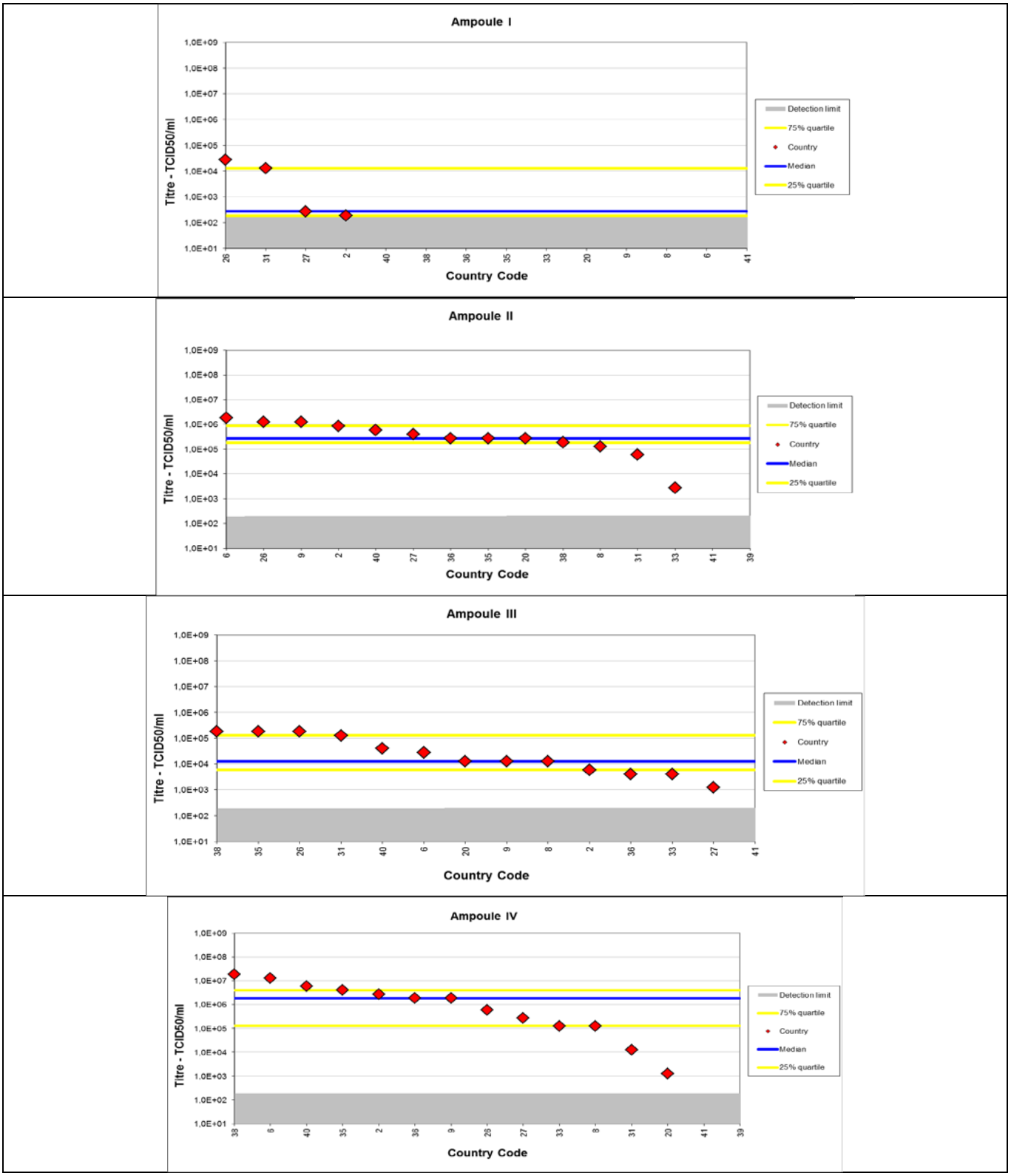
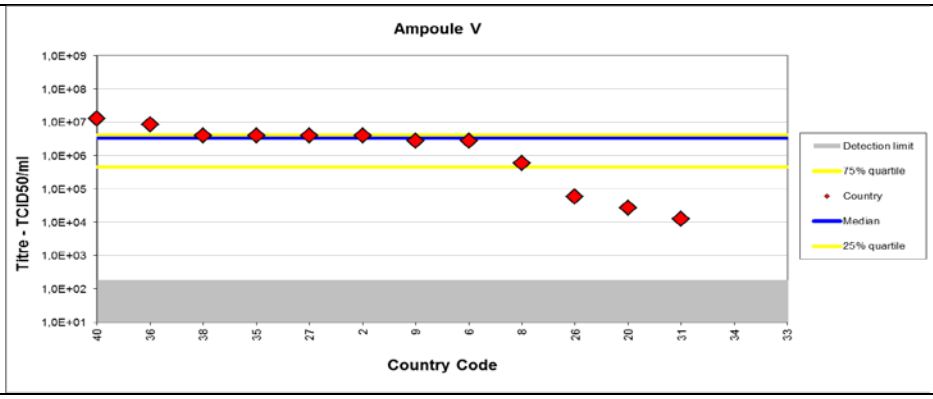

Ampoule V
1,0E+09
1,0E+08
1,0E+07
1,0E+06
1,0E+05
1,0E+04
1,0E+03
1,0E+02
1,0E+01
Titre - TCID50/ml
9
8
27
22
13
35
6
14
1
2
20
31
Country Code
Detection limit
75% quartile
Country
Median
25% quartile

Figure 8. Virus titres obtained in FHM cells. For further details see Figure 5




Ampoule V
1,0E+09
1,0E+08
1,0E+07
1,0E+06
1,0E+05
1,0E+04
1,0E+03
1,0E+02
1,0E+01
Titre - TCID50/ml
40
36
38
35
27
2
9
6
8
26
20
31
34
33
Country Code
Detection limit
75% quartile
Country
Median
25% quartile

Identification of content

- 34 laboratories correctly identified all viruses in all ampoules
- all laboratories submitted the spreadsheet within the deadline

Ampoule I – IPNV

- 32 laboratories correctly isolated and identified IPNV
- 4 laboratories could correctly identify IPNV but the virus amount was below the threshold for titration

  5 laboratories did not isolate any virus

Ampoule II - SVCV

- All 41 participant laboratories correctly identified SVCV

Ampoule III – EHNV

- 40 laboratories correctly identified EHNV
- 1 laboratory found virus but did not identify it

Ampoule IV – IHNV

- 38 laboratories correctly isolated and identified only IHNV in ampoule IV
- 2 laboratories isolated and identified IHNV together with another virus
- 1 Laboratory isolated and identified VHSV

Ampoule V – VHSV

- 40 laboratories correctly identified VHSV
- 1 laboratory isolated and identified IHNV

Scores

Starting with proficiency test 2003 we have provided a scoring system for the identification part of the proficiency test. 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, isolation and identification of another virus was given 0 points. Because of lack of stability in the batch of lyophilized ampoules produced for Ampoule I it was agreed to give 2 points also to participants that did not find any virus.

Ampoule II: 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 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. In case of no genomic analysis the result is stated as ranavirus* in table 3.

Ampoule IV: IHNV identification was given the score 2. IHNV 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.

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.

Out of 41 laboratories participating in the PT 1 2014, 36 obtained maximum score.
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.

Cells applied for solving the test

Within the panel of cell lines available in the legislation the following ones were used by the participants:

- 35 laboratories used BF-2 cells
- 38 laboratories used EPC cells
- 14 laboratories used RTG-2 cells
- 14 laboratories used FHM cells
- 7 laboratories used four cell lines
- 2 laboratories used tree cell lines: BF-2 cells in combination with EPC cells and RTG-2 cells

- 32 laboratories used two cell lines:
  - 24 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
  - 1 laboratory used RTG-2 in combination with 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 laboratories using these combinations are encouraged to include the use of BF-2 cells and EPC or FHM.
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 IPNV and EHNV replicate less efficiently in FHM cells and that IHNV replicate to a lower level in BF-2 cells when compared to EPC and FHM. SVCV and VHSV grow on all cell lines.

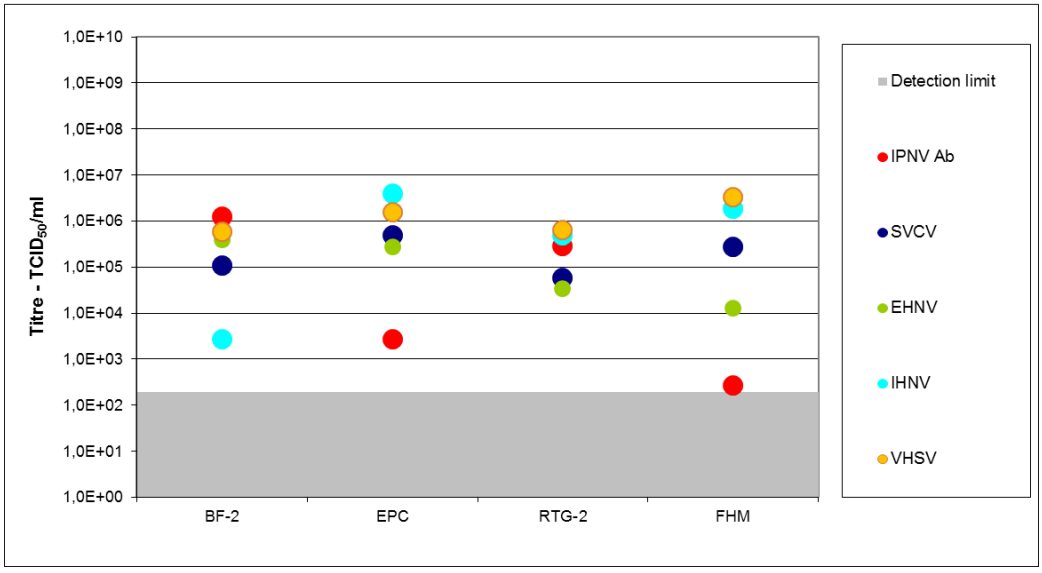


Figure 9. Median virus titres obtained by participants at different cell lines.

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) and Emmenegger et *al*., 2000 for IHNV but this was not an obligatory task.

AMPOULE I – IPNV sequence

18 laboratories sequenced the IPNV isolates in ampoule I.

3 laboratories targeted the VP 3 region . Among these:

- 1 laboratory used an in house elaborated protocol
- 1 laboratory referred to Taksdal T et al., . Bull. Eur. Ass. Fish Pathol., 5, 214-219 (2001).
- 1 laboratory referred to Kerr et al. Letters in Applied Microbiology 2006, 43, 98-104

9 laboratories targeted the VP 2 region

- 1 laboratory referred to Heppel et al.,1992J Gen Virol73:2863-
- 1 laboratory to Nishizawa et al JGV 86: 1973-1978 (2005)
- 1 laboratory to Santi N. et al. 2004:322,31-40 Virology
- 1 laboratory to Blake et al. 1995. J Clin Microbiol 33:835-839;
- 1 laboratory to Williams, K. et al. (1999). J. Clin. Microbiol. Vol 37, Nº 12, p. 4139-4141.
- 1 laboratory to Wang et al. 1997, Dis Aquat. Org. 28, 229-233
- 1 laboratory to Blake et al., 2004
- 1 laboratory to Davies et al. (2010) Dis Aquat Org 93:1-15

1 Laboratory targeted the VP 1 region without providing a reference

5 laboratories did not specify which region was targeted by their sequencing analysis

AMPOULE II – SVCV sequence

24 laboratories sequence correctly SVCV in ampoule II

20 laboratories targeted the G protein (some participants used more than one protocol)

- 3 laboratories referred to KOUTNA M., et al. Diseases of Aquatic Organisms. 55, 229-235 (2003).
- 1 laboratory to Oreshkova S.F. et al Virus Res. 63:3-10 (1999)
- 1 laboratory with In house developed primers
- 17 laboratories referring to Stone et al. 2003 Stone,. Diseases of Aquatic Organisms, 53, 203-210 cosndiering that the same primers are recommended in the OIE manual

1 laboratory targeted the RNA polymerase gene using a protocol provided in Ruane et al., Genetic diversity and associated pathology of rhabdovirus infections in farmed and wild perch *Perca fluviatilis* in Ireland. Diseases of aquatic organisms (In press)

1 laboratory targeted the L gene referring to the protocol from Ruane et al. Dis. Aquat. Org. Doi: 10.3354/dao02801

1 laboratory did not specify which protocol was used.

AMPOULE III - EHNV

37 laboratories sequenced EHNV in ampoule III1 lab. Submitted 2 sequences

32 laboratories targeted the Major Capsid Protein Gene

- 28 laboratories targeted the Major Capsid Protein MCP according to the protocol provided by Hyatt et al., 2000.
- 1 laboratory targeted the Major Capsid Protein MCP according to the protocol provided by Holopainen et al, 2009
- 1 laboratory targeted the Major Capsid Protein MCP according to the protocol provided by Ohlemeyer et al., 2011
- 2 laboratories targeted the Major Capsid Protein MCP according to the protocol provided by OIE Manual 2011

1 laboratory targeted the Neurofilament according to the protocol provided by Holopainen et al, 2009

5 laboratories did not specify which protocol was used.

AMPOULE IV- IHNV

29 laboratories sequenced correctly IHNV in ampoule 4

18 laboratories targeted the Glycoprotein Gene

- 12 Laboratories targeted the G gene according to the protocol provided by Emmeneger et al.,2000
- 2 laboratories targeted the G gene according to the protocol provided by Kolodzejek et al., 2008
- 1 laboratory targeted the G gene fully sequencing the gene with in house developed method
- 2 laboratories targeted the G gene according to the protocol provided by Miller et al., 1998
- 1 laboratory targeted the G gene according to the protocol provided by Enzmann et al
- 1 laboratory targeted the G gene according to Williams, K. et al. (1999). J. Clin. Microbiol. Vol 37, Nº 12, p. 4139-4141

8 laboratories targeted the Nucleocapsid protein Gene

- 1 laboratory targeted the Nuceloprotein according to Purcell et al.,2013
- 2 laboratories targeted the Nucleocapsidprotein according to protocol by Bergmann et al 2000
- 5 laboratories targeted the Nucleocapsid protein according to the protocol provided in the OIE manual 2006

1 laboratory followed protocol from OIE manual in 2009

1 laboratory did not specify which protocol was used

AMPOULE V- VHSV

28 laboratories sequenced correctly VHSV in ampoule 4

17 laboratories targeted the Glycoprotein gene

- 1 laboratories targeted the G gene according to the protocol from Einer-Jensen et al., 2004
- 1 laboratory targeted the Nucleoprotein gene according to protocol form Bergmann et al.,2000
- 3 laboratory targeted the G gene according to protocol form Hedrick et al.,2003
- 1 laboratory targeted the G gene according to Miller et al. 1998
- 1 laboratory targeted the G gene according to protocol from Raja Halli et al.,2006
- 2 laboratories used unpublished results targeting the G gene
- 1 laboratory targeted the G gene according to Williams, K. et al. (1999). J. Clin. Microbiol. Vol 37, Nº 12, p. 4139-4141

1 laboratory sequenced the Non-structural protein NV isolate according to Schuetze et al., 1999

5 laboratories targeted the Nucleo-capsid protein

- 2 laboratories targeted the Nucleocapsid protein according to protocol from Snow et al.,2004
- 3 laboratories targeted the Nucleocapsid protein gene according to protocol reported in Manual of Diagnostic Tests for Aquatic Animals 2012

1 laboratory did not reported which protocol was used

Concluding remarks PT1

The inter-laboratory proficiency test 2014 was conducted without major constraints. 91% of parcels were delivered by the shipping companies within 8 days after submission. It was, however, unfortunate that one parcel was 27 days on the way and one parcel was 57 days on the way before delivered to the laboratory primarily due to border controls.

EHNV was included in the proficiency test for the first time in 2009. This year 40 participants were able to correctly identify the virus. Of the laboratories performing PCR based methods, 38 laboratories performed sequencing. Of these laboratories all correctly identified the content.

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 participants. We recommend all participants to evaluate the sensitivity of the cells used in their laboratory in relation to the diagnostic purpose. This year it has to be remarked that a problem with the batch of ampoules containing IPNV Ab has appeared, this has been taken into consideration in the process of giving score to participants. This year variation between virus titres obtain in the various laboratories was more pronounced than usually with up to 6 log differences between highest and lowest titre. It might reflect variation in the stability of the virus in the respective batches. Special precautions will therefore be taken in the following PT´s to ensure uniformity of the amount of viable viruses in the ampoules.

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.

This year a problem with the IPNV strain was observed. The issue has been managed both with the participants and DANAK the accreditation body that audit the QA system at DTU. As the freeze-drying process is suspected to be the critical point further precautions will be implemented in future PT preparations such as UV inspection of ampoules to verify correct vacuum in the glass vials.

The results presented in this report will be further presented and discussed at the 19th Annual Workshop of National Reference Laboratories for Fish Diseases to be held 27-28 May 2015 in Copenhagen, Denmark.

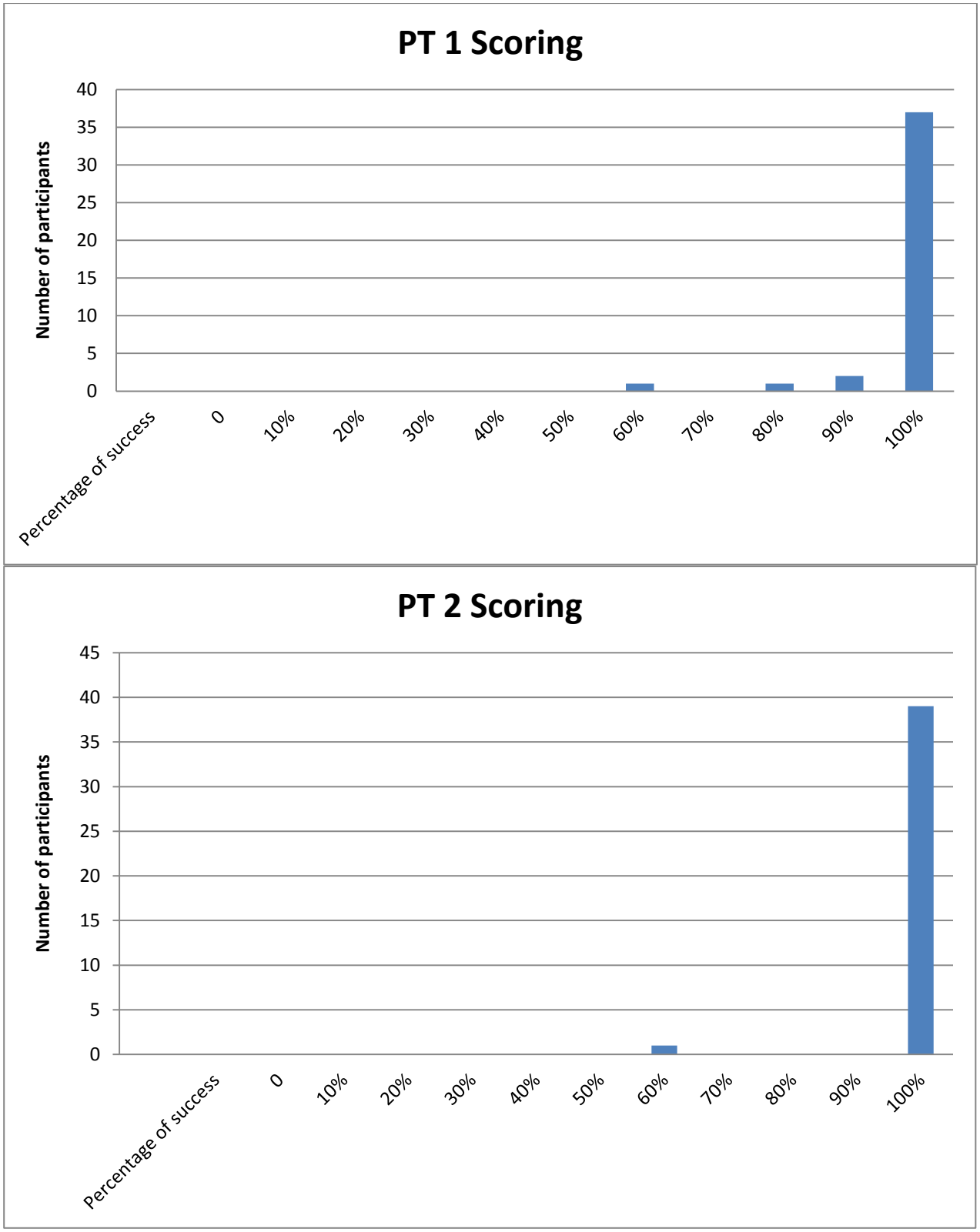


Figure 10 "a" and "b" Success-rate of participating laboratories 2014

Proficiency test 2, PT2

Four ampoules containing lyophilised cell culture supernatant were delivered to the same laboratories that participated in PT1 with the exception of one "new entry" in the network that participated only in PT1.

Content of ampoules

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

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: KHV-TP 30 Diluted 1:3	**Reference on isolate:** Koi Herpesvirus (CyHV-3): KHV-TP 30 (syn: KHV-T (for Taiwan)). KHV-TP 30 was isolated from koi in Taiwan and cloned for producing large plaques by Dr. Peiyu Lee, Institute of Medical Biotechnology, Central Taiwan University of Science and Technology, Dakeng, BeiTung District, TaiChung City 406, Taiwan in-2005. The isolate was provided by Dr. Sven M. Bergmann, Friedrich-Loeffler-Institut (FLI), Federal Research Institute for Animal Health, Südufer 10, 17393 Greifswald-Insel Riems, Germany
Ampoule VII: Blank	**EMEM**
Ampoule VIII: KHV-TP 30 Undiluted	**Reference on isolate:** Koi Herpesvirus (CyHV-3): KHV-TP 30 (syn: KHV-T (for Taiwan)). Same as ampoule VI
Ampoule IX: ISAV FO/01/01/HPR13	**Genotype: HPR13** **GenBank accession numbers:** AJ440970 **Reference on isolate:** Cunningham CO et al. Bull. Eur. Assoc. Fish Pathol 22 (6), 366-374 (2002) Christiansen et al. J. General Virology 2011:92;909-918 **The isolates was** The isolate was provided by Dr. Debes Christiansen, Deildarleiðari & Granskari Føroyska tilvísingarkanningarstovan fyri fiska- & djórasjúkur Heilsufrøðiliga starvstovan V.U. Hammershaimbsgøta 11 FO-100 Tórshavn, Faroe Island

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, 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 (Table 10). 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) 3 months after shipment and after storage at 4°C in the dark and no significant decrease compared to right after lyophilisation was observed. (Table 10)

For each ampoule no other pathogens than the expected were detected

Table 10. Ct-value of ampoules VII and IX tested before lyophilisation, immediately after lyophilisation, and 3 months after shipment storage in the dark at 4°C

Ampoule No.	Ampoule	Ct value/presence of band before lyophilisation undiluted	Ct value/ presence of band right after lyophilisation	Ct value/ presence of band 3 months after shipment (4°C, dark conditions)
Ampoule VI: KHV-TP 30	a	Ct 14,52	29,88	24.72
	b		27,75	
	c		26,64	
	d		26,92	
	e		27,29	
	Average		27,70	
Ampoule VII: Blank	a			
	b			
	c			
	d			
	e			
	Average			
Ampoule VIII: KHV-TP 30	a	Ct 15,84	21,72	23.30
	b		23,29	
	c		22,42	
	d		23,73	
	e		23,17	
	Average		22,87	
Ampoule IX: ISAV FO/01/01/HPR13	a	Ct 27.06	33,94	35.14
	b		33,87	
	c		33,71	
	d		33,10	
	e		33,81	
	Average		33,69	

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.

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, 2014 - Virus identification.

Laboratory code number	Score (Maximum 8/8)	Answer received at EURL	Ampoule VI	Ampoule VII	Ampoule VIII	Ampoule IX
			KHV	Blank	KHV	ISAV
1	8	**05.12.14**	KHV	Negative	KHV	ISAV
2	8	**17.11.14**	KHV	no pathogen identified	KHV	ISAV
3	8	**01.12.14**	KHV	no pathogen found	KHV	ISAV
4		**26.11.14**	0	0	0	0
5	8	**05.12.14**	KHV	NO	KHV	ISAV
6	8	**04.12.14**	KHV	Negative	KHV	ISAV
7	8	**04.12.14**	KHV	Not KHV, not ISA	KHV	ISA
8	8	**02.12.14**	KHV	0	KHV	ISAV
9	8	**05.12.14**	KHV	Not ISAV, not KHV	KHV	ISAV
10	8	**26.11.14**	KHV	no KHV and ISAV	KHV	ISAV
11	8	**05.12.14**	KHV	No virus detected	KHV	ISAV
12	8	**27.11.14**	KHV	No virus	KHV	ISAV HPR13
13	8	**24.11.14**	KHV	negative	KHV	ISAV
14	8	**03.12.14**	KHV Europe	0	KHV Asia	ISAV
15	8	**05.12.14**	KHV	NO KHV NO ISAV	KHV	ISAV
16	8	**27.11.14**	KHV	NEGATIVE	KHV	ISAV
17	4/4	**02.12.14**	0	0	0	ISAV
18	8	**24.11.14**	KHV	Negative	KHV	ISAV
20	8	**02.12.14**	KHV	Negative	KHV	ISAV
20	8	**02.11.14**	KHV	-	KHV	ISAV
21	8	**05.12.14**	KHV	not KHV, not ISAV	KHV	ISAV
22	8	**04.12.14**	KHV	0	KHV	ISAV
23	8	**05.12.14**	KHV	No ISAV or KHV detected	KHV	ISAV
22	8	**05.12.14**	KHV	0	KHV	ISAV
25	8	**03.12.14**	KHV	/	KHV	ISAV
26	8	**08.12.14**	KHV	NO VIRUS	KHV	ISAV
26	8	**05.12.14**	KHV	-	KHV	ISAV
25	8	**05.12.14**	KHV	0	KHV	ISAV
29	8	**05.12.14**	KHV	Negative	KHV	ISAV
30	5	**05.12.14**	KHV	KHV	KHV	ISAV / KHV
29	8	**27.11.14**	KHV			
28	8	**05.12.14**	KHV	Not detected	KHV	ISAV
33	8	**04.12.14**	KHV	negative	KHV	ISAV
34	8	**04.12.14**	KHV	0	KHV	ISA
32	8	**05.12.14**	KHV	Not virus	KHV	ISAV
31	8	**03.12.14**	KHV	Negativ	KHV	ISAV
37	8	**03.12.14**	KHV	NO KHV or ISAV	KHV	ISAV
38	8	**21.11.14**	KHV	negative	KHV	ISAV
39	8	**05.12.14**	KHV	NEG	KHV	ISA
40	8	**03.12.14**	KHV	Negative	KHV	ISAV
41	8	**07.12.14**	KHV	0	KHV	ISAV

*Did test only for ISAV

Identification of content

- 40 laboratories submitted results
- 38 laboratories correctly identified all four ampoules
- 39 laboratories tested for both two listed pathogens
- 40 laboratories tested for ISAV
- 39 laboratories tested for KHV
- 1 laboratory tested for ISAV only
- 1 laboratory that did participate in PT 1 did not participate in PT2

Ampoule VI – *KHV*

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

Ampoule VII – Blank

- 39 laboratories correctly identified Not *KHV or* ISAV
- 1 laboratory found KHV

Ampoule VIII – KHV

- 39 laboratories correctly identified KHV
- 1 laboratory did not examined for KHV

Ampoule IX – ISAV

- 39 laboratories correctly identified only ISAV
- 1 laboratories identified ISAV and KHV

Scores

We have assigned a score of 2 for each correct answer (Table 11), 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 40 laboratories submitting results 39 laboratories obtained maximum score. One laboratory examined for ISAV only, this laboratory obtained the score 4 out of 4 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.

Methods applied
The following methods were used by the participants:

- 18 laboratories used KHV PCR, among these the most used protocol was the one provided by Bercovier et al 2005
- 20 laboratories used KHV Real-time PCR among these the most used protocol was the one from Gilad et al 2004
- 9 laboratories used both KHV real-time PCR and KHV PCR

- 27 laboratories used ISAV RT-PCR among these the two most used protocols were the ones from Mjaaland et al 2002 and OIE Manual for diagnostic tests for aquatic animals 2009.
- 20 laboratories used ISAV real-time RT-PCR among these the two most used protocols were the ones from Snow et al.,2006 and method recommended in OIE Manual for diagnostic tests for aquatic animals 2009.
- 11 laboratories used both ISAV real-time RT-PCR and ISAV RT-PCR

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

- 19 laboratories performed sequencing for KHV
- 24 laboratories performed sequencing for ISAV

Concluding remarks PT2

Considering that this was the fifth time that the EURL provided a proficiency test on ISAV and KHV identification, we consider that most participants obtained very good results. All 39 laboratories testing for KHV identified the virus in ampoule VI and VIII! Out of 40 laboratories 39 laboratories identified Not *KHV or* ISAV in ampoule VII. With only one false positive this is much less than observed in the PTs from previous years. All 40 laboratories testing for ISAV identified the virus in ampoule IX. Thereby very significant improvement in the proficiency of identifying and typing these pathogens has been observed during these past 5 years, especially in relation to the sensitivity, as this year the viral content in the ampoule was low. After the European Commission in autumn 2012 de-listed Epizootic Ulcerative Syndrome cause by *Aphanomyces invadans* it has been agreed not anymore to include this pathogen in the PT for fish diseases.
It is an appreciated matter of fact that many laboratories are putting efforts in performing genetic characterization of 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.
Of the 24 laboratories sequencing the ISAV virus all found that the isolate was with deletion in segment 6 and thus not belong to HPR0. Some of the participant also noticed that this year the HPR13 isolate from the Faroes was used instead of the Gleasvaer isolate that we have included in all the former PT´s.

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 given in this report will be further presented and discussed at the 19th Annual Workshop of National Reference Laboratories for Fish Diseases to be held 27-28 May 2015 in Copenhagen, Denmark.

Niccolò Vendramin and Niels Jørgen Olesen

European Union Reference laboratory for Fish diseases
National Veterinary Institute, Technical University of Denmark, February 2015

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