EURL for Fish Diseases

Report of the Inter-Laboratory Proficiency Test 2016

for identification and titration of

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

and identification of

CyHV-3 (KHV), SAV and ISAV (PT2)

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

PT Reg. no.: 515

DANAK

DTU

Table of contents

Introduction

A comparative test of diagnostic procedures was provided by the European Union Reference Laboratory (EURL) for Fish Diseases. The test was divided into proficiency test 1 (PT1) and proficiency test 2 (PT2).
PT1 was designed to primarily assess the identification of the fish viruses causing the notifiable diseases: viral haemorrhagic septicaemia virus (VHSV), infectious hematopoietic necrosis virus (IHNV), and epizootic haematopoietic necrosis virus (EHNV) or related rana-viruses and in addition the fish pathogenic viruses: other fish rhabdoviruses as pike fry rhabdovirus (PFR),spring viraemia of carp virus (SVCV) and infectious pancreatic necrosis virus (IPNV) by cell culture based methods. PT2 was designed for assessing the ability of participating laboratories to identify the fish pathogens: infectious salmon anaemia virus (ISAV), salmon alphavirus (SAV) and cyprinid herpesvirus 3 (CyHV-3) (otherwise known as koi herpes virus – KHV) by biomolecular methods (PCR based). 45 laboratories participated in PT1 while 43 participated in PT2.
Regarding PT2, all 43 participated in identifying ISAV, 42 participated in identifying ISAV and KHV and 37 participated in identifying all three pathogens included, ISAV,KHV and SAV.
The tests were sent from the EURL end of September 2016.
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 PFR, IHNV, VHSV, ECV and IPNV (see table 1). The proficiency test was designed to primarily assess the ability of participating laboratories to identify any of the fish viruses VHSV, IHNV and to be able to discriminate between the exotic listed EHNV from other ranaviruses(Council Directive 2006/88/EC Annex IV part II and Commission Implementing Directive 2014/22/EU of 13 February 2014). Furthermore the interlaboratory proficiency test is also suitable for maintaining accreditation for identification of SVCV, and IPNV; participants have to consider 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 2015-1554 and by 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 2015. Laboratories were encouraged to identify VHSV and IHNV isolates 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 either method as mentioned in the IHN chapter of the 2013 version of the OIE manual on Aquatic Animal Diseases (Emmenegger et al. (2000)) or in Kurath et *al*. (2003) for IHNV. Laboratories were encouraged to submit all sequencing results used for genotyping the isolates. It has to be remarked that although sequencing protocols are recommended in this report and in the instructions included to PT1-parcels, for the two listed rhabdoviruses this procedures relies on a number of different

protocols, targeting different regions of the same pathogen making it difficult to compare results obtained by different participants. Acknowledging that sequencing is an accessory activity of the Interlaboratory Proficiency test and not a demand, this point will be further discussed during the Annual Workshop.

PT2 consisted of four coded ampoules (VI-IX). One ampoule contained CyHV-3 (KHV), one contained SAV, one contained ISAV and one contained sterile cell culture supernatant from BF-2 cells, see table 10. 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, Annex IV and Commission Implementing Directive 2014/22/EU) if present in the ampoules, bearing in mind that the test ampoules could also contain other pathogens. Since SAV is not a listed disease in the European legislation, all participants were free to decide if they would be testing for SAV or not. Each participant was asked to declare whether they would test for SAV or not. The EURL team would then take care of calculating the score accordingly, overall 37 of 42 laboratories tested for SAV in 2016, wich was an increase of three laboratories compared to 2015.
Participants were expected to use their normal PCR or real-time PCR methods for detection of the pathogens. Regarding SAV analysis, participants can refer to the OIE manual Chapter 2.3.5b. — Infection with salmonid alphavirus . 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 EURL-team have included comments to the participants if relevant. An un-coded 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 21th Annual Workshop of the NRLs for Fish Diseases week 22, 2017 in Copenhagen. Participants were asked to reply latest November 25th 2016.

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.

Shipment and handling
Within one day, the tests were delivered to 27 participants; 12 more tests were delivered within the first week; 3 more within the first two weeks; 3 further within three weeks (Figure 1). All the parcels were sent without cooling elements.
A relatively high stability was demonstrated to characterize the lyophilized pathogens in glass ampoules as described in the PT 2012 report.

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

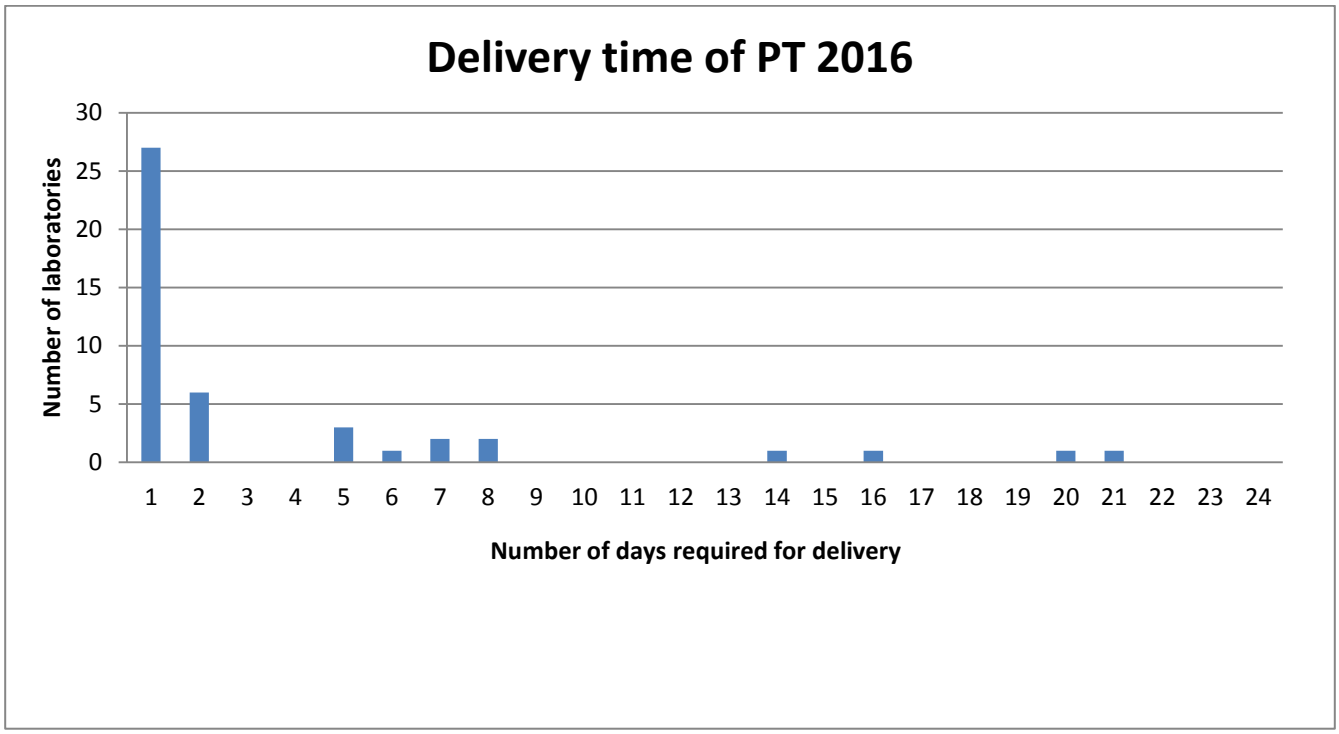


Figure 1. Transport time for the parcels to reach the participants.

Participation

PT1 and PT2: 45 laboratories received the annual proficiency test. 43 of the participants submitted results within the deadline, 1 participants got the deadline extended due to delivery problems or technical problems in the laboratory. Figure 2 show how many laboratories that participated in the proficiency test from 1996 to 2016.

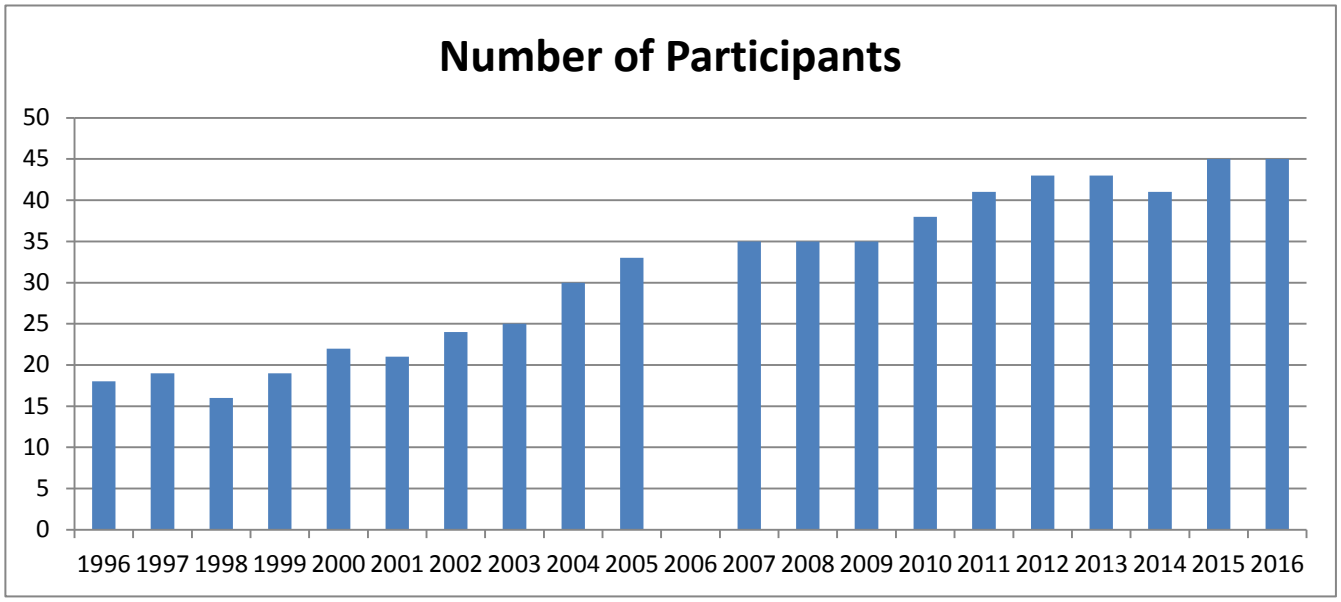


Figure 2. Participants in the EURL proficiency test over the years.

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, Faroe Islands, Iceland, Iran, Japan, New Zealand, Norway, Serbia, Switzerland, Turkey and 2 from: P.R. China, South Korea and 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 2015.

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 2% 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/References
Ampoule I: PFR	**PFR - Pike Fry Rhabdovirus. Reference strain received from Dr. P.de Kinkelin, INRA, 1987** GenBank accession number: FJ872827.1 Reference : de Kinkelin, P., Galimard, B., Bootsma, R. (1973). Isolation and identification of the causative agent of 'red disease' of pike (*Esox lucius* L., 1766). Nature 241: 465-46 Stone D.M., Ahne W., Denham K.L., Dixon P.F., Liu C-T.Y., Sheppard A.M., Taylor G.R. & 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 II: IHNV	**IHNV - isolate BLK94 07699 24:05** American Genotype U Received from Gael Kurath Isolated in 1994 from Sockeye salmon *Oncorhynchus nerka* smolt, in Washington USA. Genogroup U. Kurath et al. 2003, J. General Virology 84:803-814; Mid G USD mG002U refers to Universal sequence designators (USD) defined for North American IHNV isolates as described in the MEAP-IHNV (Molecular Epidemiology of Aquatic Pathogens) database at http://gis.nacse.org/ihnv
Ampoule III: VHSV	**VHSV - Isolate TR-WS13G (= TR-SW13G)** Genotype Ie. Received from Dr. Mamoru Yoshimizu. Turkish isolate (Trabzon coastal area) from turbot (*Psetta maxima*). DTU Vet protocol: 207005-1, recieved as VHS SW 13G – P3 050707. GenBank accession number: AB231160 References: Nishizawa T, Savas H, Isidan H, Üstündag C, Iwamoto H & Yoshimizu M (2006). Genotyping and pathogenicity of viral hemorrhagic septicemia virus from free-living turbot (*Psetta maxima*) in a Turkish coastal area of the Black Sea. *Applied and Environmental Microbiology* **72**, 2373-2378.
Ampoule IV: ECV	**Ranavirus ECV: European catfish virus isolate 562/92.** Italian isolate from catfish suffering high mortality. Received from Dr. G. Bovo, ISZ-Ve, Padova, Italy. GenBank accession number: FJ358608 Reference on isolate: Bovo G, Comuzi M, De Mas S, Ceschia G, Giorgetti G, Giacometti P & Cappellozza E (1993). Isolamento di un agente virale irido-like da pesce gatto (Ictalurus melas) dallevamento. Bollettino Societa Italiana di Patologia Ittica 11, 3–10. Reference on sequence: Holopainen R., Ohlemeyer S., Schütze H., Bergmann S.M. & Tapiovaara H. (2009) Ranavirus phylogeny and differentiation based on major capsid protein, DNA polymerase and neurofilament triplet H1-like protein genes. *Diseases of Aquatic Organisms* 85, 81-91.
Ampoule V: IPNV	**IPNV strain Sp** The Sp (Spjarup) reference strain of infectious pancreatic necrosis (IPN) virus from farmed rainbow trout in Denmark, isolated in 1969 by Dr. Vestergaard Jørgensen. **Received from:** National Veterinary Institute, Technical University of Denmark. **GenBank accession numbers:** AM889221

Code	Specifications/References
	Reference on isolate: Jørgensen PEV & Bregnballe F (1969) Infectious pancreatic necrosis in rainbow trout in Denmark. *Nordisk Veterinærmedicin* **21**, 142-148. Jørgensen PEV & Grauballe PC (1971) Problems in the serological typing of IPN virus. *Acta Veterinaria Scandinavica* **12**, 145-147. **References on sequences:** P. F. Dixon, G.-H. Ngoh, D. M. Stone, S. F. Chang, K. Way, S. L. F. Kueh (2008) Proposal for a fourth aquabirnavirus serogroup Archives of Virology 153:1937–1941

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 and Figure 4).

The lyophilisation procedure is known to determine some reduction especially for VHSV. Previous experience reported during the past Proficiency tests demonstrated a rather high stability for SVCV, EHNV and IPNV serotype 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 (Inter-Laboratory Proficiency Test report 2007).
We have furthermore shown that lyophilised virus in glass sealed ampoules is stable after exposure to 30°C for 24 hours (Inter-Laboratory Proficiency Test report 2010)
In 2011 we have shown that lyophilised virus in glass sealed ampoules is stable when temperature raised from 20-42°C over a period of 5 hours (Inter-Laboratory Proficiency Test 2011)

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/or RT-qPCR. After shipment the stability of the content in the ampoules were assessed by titrating the virus on cell cultures, and identifying it by ELISA, furthermore PCR based tests were performed on the original content of all the ampoules. This year only very limited reductions of titres were observed following lyophilisation and no reduction after long term storage (Table 2 and figure 4)

Table 2. PT1:

Titres in ampoules I to V stored in the dark tested on four cell lines at different time points:

- Before lyophilisation, (stored at -80°C).
- After lyophilisation and before shipment (median titre of 5 replicates), (stored at 4°C), the variation of the titre of the 5 replicates was within 1 log in the same cell line.
- After deadline for handling in results and five months after lyophilisation, (1 ampoule), (stored at 4°C).

Ampoul No.	Cell line	Titre before Lyophilisation	Titre after Lyophilisation and before shipment	Titre after deadline for handling in results (and five months after lyophilisation) (storage 4°C in the dark)
		$TCID_{50}$/ml	$TCID_{50}$/ml	$TCID_{50}$/ml
Ampoule I: PFR reference strain	BF-2	5,9E+07	1,3E+07	2,7E+06
	EPC	8,6E+07	1,9E+07	1,9E+07
	RTG-2	1,3E+06	8,6E+06	2,7E+06
	FHM	1,3E+06	1,3E+07	2,7E+07
Ampoule II: IHNV BLK 94	BF-2	4,0E+06	1,3E+04	1,3E+04
	EPC	5,9E+07	8,6E+06	4,0E+06
	RTG-2	5,9E+04	4,0E+04	2,7E+04
	FHM	1,3E+05	2,7E+06	1,3E+06
Ampoule III: VHSV TRW13G	BF-2	4,0E+08	2,7E+07	8,6E+06
	EPC	4,0E+07	1,9E+06	2,7E+05
	RTG-2	8,6E+02	1,3E+03	5,9E+02
	FHM	1,9E+06	8,6E+07	4,0E+07
Ampoule IV: ECV 562/92	BF-2	1,3E+07	2,7E+06	1,9E+06
	EPC	1,9E+07	1,9E+06	4,0E+06
	RTG-2	8,6E+04	4,0E+05	1,3E+06
	FHM	2,7E+03	4,0E+03	1,3E+03
Ampoule V: IPNV Sp	BF-2	1,3E+08	8,6E+07	1,9E+07
	EPC	2,7E+07	1,9E+07	1,9E+07
	RTG-2	8,6E+05	1,3E+07	2,7E+06
	FHM	1,9E+05	5,9E+06	1,9E+06

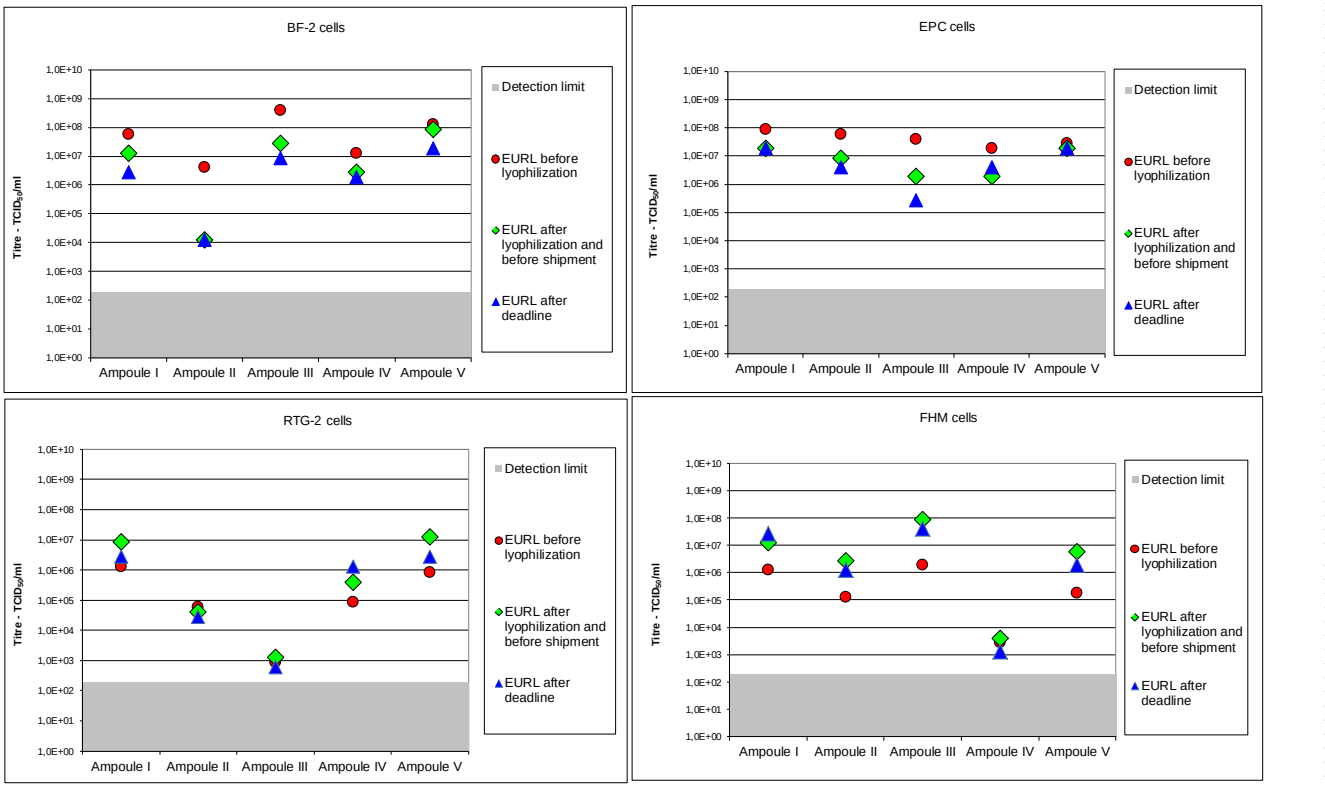


Figure 4. Virus titers in different cell lines:
Before lyophilisation, After lyophilisation-before shipment and After minimum 3 months after lyophilisation (storage 4°C in the dark) (1 ampoule).

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 Commission Decision 2015-1554, i.e. by cell culture followed by ELISA, IFAT, neutralisation test and/or RT-PCR/Q-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 by conducting titrations. The titration procedures were described in the instructions enclosed with the test. All titres were calculated by 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 5-8, all titres submitted by the 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 6 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, 2016 - Virus identification and score obtained by participants.

Laboratory code number	Score	Answer received at EURL	Ampoule I	Ampoule II	Ampoule III	Ampoule IV	Ampoule V
			PFR	IHNV BLK94	VHSV TR-WS13G	ECV 562/92	IPNV Sp
1	**10/10**	24-11-2016	PFRV	IHNV	VHSV	ECV/ESV	IPNV
2	**9/10**	24-11-2016	PFRV	IHNV	VHSV	ECV	IPNV & ECV
3	**9/9**	18-11-2016	no VHSV no IHNV no IPNV no SVCV no RanaV	IHNV	VHSV	Ranavirus ECV/ESV	IPNV
4	**7/8**	29-11-2016	SVCV	INHV	VHSV	No IHNV, VHSV, IPNV, SVCV	IPNV
5	**8/9**	25-11-2016	virus isolated but not identified	IHNV	VHSV	Rana virus	IPNV
6	**9/9**	26-11-2016	Rhabdovirus*	IHNV	VHSV	ECV	IPNV
7	**9/9**	25-11-2016	Negative for VHSV, IHNV, EHNV, Ranavirus, IPNV and SVCV	IHNV	VHSV	ECV/ESV	IPNV
8	**10/10**	24-11-2016	PFRV	IHNV	VHSV	ECV	IPNV
9	**10/10**	25-11-2016	Pike fry rhabdovirus not SVCV	IHNV	VHSV	Ranavirus not EHNV	IPNV
10	**9/9**	24-11-2016	no IHNV , VHSV, IPNV, SVCV	IHNV	VHSV	no IHNV , VHSV, IPNV, SVCV	IPNV
11	**9/9**	23-11-2016	Full CPE on BF-2 and EPC, but none of the listed viruses identified	IHNV	VHSV	Ranavirus (ESV or ECV)	IPNV
12	**10/10**	25-11-2016	Pike fry rhabdovirus	IHNV genogroup U	VHSV genotype Ie	ECV	IPNV genogroup 5
13	**10/10**	27-10-2016	PFRV	IHNV	VHSV	ECV	IPNV
14	**10/10**	10-11-2016	PFRV	IHNV	VHSV	Ranavirus (ESV/ECV)	IPNV
15	**9/9**	25-11-16 (Seq.: 29-11-16)	NO IHN-NO VHS-NO EHNV-NO ECV/ESV- NO IPNV-NO SVC	IHNV	VHSV	ECV/ESV (Ranavirus)	IPNV
16	**9/10**	25-11-2016	SVCV	IHNV	VHSV	Ranavirus	IPNV
17	**9/10**	25-11-2016	SVCV	IHNV	VHSV	ECV	IPNV
18	**6/6**	23-11-2016	Neg	IHN	VHS	Neg	IPN
19	**10/10**	24-11-2016	Pike fry rhabdovirus	IHNV	VHSV	Ranavirus - ECV	IPNV
20	**9/10**	25-11-2016	SVCV	IHNV	VHSV	ECV/ESV	IPNV

21	**10/10**	25-11-2016	Pike fry-like rhabdovirus	IHNV	VHS	Ranavirus	IPN
22	**9/9**	25-11-2016	no VHSV/ no IHNV/no EHNV/ no IPNV	IHNV	VHSV	EHNV	IPNV
23	**9/9**	25-11-2016	Negative	INHV	VHSV	Ranavirus	IPNV
24	**9/9**	24-11-2016	No VHSV, IHNV, Ranavirus, IPNV, SVCV detected	IHNV	VHSV	Ranavirus	IPNV
25	**10/10**	25-11-2016	Pike fry rhabdovirus. IFAT, RT-PCR, sequence analysis	IHNV viable virus detected	VHSV viable virus detected	ECV viable virus detected	IPV viable virus detected
26	**4/10**	25-11-2016	Iridovirus	IHNV	VHSV/ IHNV	Ranavirus other than EHNV	IPNV/ VHSV
27	**10/10**	21-11-2016	SVCV(Pike fry-like rhabdovirus)	IHNV	VHSV	ECV	IPNV
28	**9/9**	21-11-2016	unknow virus	IHNV	VHSV	ECV	IPNV
29	**10/10**	25-11-2016	SVC-like (RT-PCR for PFRV Positive)	IHNV	VHSV	ECV	IPNV
30	**3/10**	25-11-2016	SVCV	IPNV	VHSV	IHNV	Ranavirus (ECV or ESV)
31	**9/9**	25-11-2016	Rhabdovirus	IHNV	VHSV	Not EHNV Ranavirus	IPNV
32	**5/10**	24-11-2016	IHNV		VHSV	EHNV	IPNV
33	**8/10**	25-11-2016	-	IHNV	VHSV	Ranavirus (ECV)	IPNV
34	**9/10**	25-11-2016	Rhabdovirus (SVC)	IHNV	VHSV	ECV/ESV	ECV, IPNV
35	**10/10**	25-11-2016	PFRV(pike fry rhabdovirus)	IHNV	VHSV	ECV (Ranavirus)	IPNV
36	**10/10**	25-11-2016	Pike fry rhabdovirus	IHNV	VHSV	ECV	IPNV
37	**10/10**	25-11-2016	SVC-like virus*	IHNV	VHSV	Ranavirus ECV	IPNV-Sp
38	**9/9**	24-11-2016	CPE in BF-2 and FHM cells	IHNV	VHSV	ECV/ESV	IPNV
39	**10/10**	22-11-2016	Pike fry	IHN	VHS	ECV/ESV	IPN
40	**5/10**	25-11-2016	IPNV	IHNV	VHSV and IPNV	VHSV and ECV	IPNV
41	**9/10**	24-11-2016	SVCV	IHNV	VHSV	ECV	IPNV
42	**10/10**	25-11-2016	Pike Fry Rhabdovirus	IHNV	VHSV	Catfish/sheetfish virus	IPNV
43	**10/10**	24-11-2016	PFRV	IHNV	VHSV	ECV/ESV	IPNV
44	**7/9**	23-11-2016	Negative	IHNV	VHSV	Ranavirus	Negative
45	**10/10**	16-11-2016	pike fry rhabdovirus	IHNV	VHSV	European sheatfish virus	IPNV

Table 4. Inter-Laboratory Proficiency Test, PT1, 2016 – Results of titration of ampoule I.

PFR - Pike Fry Rhabdovirus. Reference strain					
Laboratory Code number	**Virus Identification**	**Titre in**			
		BF-2	**EPC**	**RTG-2**	**FHM**
1	PFRV	2,7E+06	8,6E+06	2,7E+06	N/A
2	PFRV	8,6E+05	5,9E+07	< 1,9E+02	N/A
3	no VHSV no IHNV no IPNV no SVCV no RanaV	1,3E+07	1,3E+07	< 1,9E+02	N/A
4	SVCV	5,9E+06	5,9E+06	N/A	N/A
5	virus isolated but not identified	< 1,9E+02	< 1,9E+02	N/A	N/A
6	Rhabdovirus*	2,7E+03	8,6E+05	< 1,9E+02	4,0E+06
7	Negative for VHSV, IHNV, EHNV, Ranavirus, IPNV and SVCV	2,7E+06	4,0E+06	N/A	N/A
8	PFRV	N/A	5,9E+06	4,0E+06	1,3E+06
9	Pike fry rhabdovirus not SVCV	4,0E+06	4,0E+07	2,7E+06	8,6E+07
10	no IHNV , VHSV, IPNV, SVCV	2,7E+04	8,6E+06	N/A	N/A
11	Full CPE on BF-2 and EPC, but none of the listed viruses identified	5,9E+06	1,9E+07	N/A	N/A
12	Pike fry rhabdovirus	1,3E+06	1,3E+07	N/A	N/A
13	PFRV	1,3E+05	4,0E+07	8,6E+06	N/A
14	PFRV	1,3E+06	1,0E+04	<1,9E+02	<1,9E+02
15	NO IHN-NO VHS-NO EHNV-NO ECV/ESV- NO IPNV-NO SVC	5,9E+06	1,3E+07	N/A	N/A
16	SVCV	8,6E+06	5,9E+06	N/A	N/A
17	SVCV	2,7E+07	1,9E+07	N/A	N/A
18	Neg	<1,9E+02	<1,9E+02	<1,9E+02	<1,9E+02
19	Pike fry rhabdovirus	5,9E+09	5,9E+09	N/A	N/A
20	SVCV	1,9E+07	4,0E+07	N/A	N/A
21	Pike fry-like rhabdovirus	4,0E+05	8,6E+04	8,6E+05	5,9E+04
22	no VHSV/ no IHNV/no EHNV/ no IPNV	4,0E+07	4,0E+07	N/A	N/A
23	Negative	N/A	4,0E+06	<1,9E+02	N/A
24	No VHSV, IHNV, Ranavirus, IPNV, SVCV detected	1,9E+06	8,6E+06	N/A	N/A
25	Pike fry rhabdovirus. IFAT, RT-PCR, sequence analysis	N/A	4,0E+06	N/A	<1,9E+02
26	Iridovirus	5,9E+05	5,9E+05	N/A	N/A
27	SVCV(Pike fry-like rhabdovirus)	1,9E+06	1,9E+08	N/A	N/A
28	unknow virus	N/A	1,3E+07	<1,9E+02	1,9E+06

29	SVC-like (RT-PCR for PFRV Positive)	8,6E+07	5,9E+08	5,9E+07	1,9E+08
30	SVCV	N/A	<1,9E+02	<1,9E+02	N/A
31	Rhabdovirus	8,6E+05	1,9E+05	N/A	N/A
32	IHNV	1,3E+06	1,9E+06	N/A	N/A
33	-	<1,9E+02	<1,9E+02	<1,9E+02	<1,9E+02
34	Rhabdovirus (SVC)	<1,9E+02	4,0E+05	N/A	N/A
35	PFRV(pike fry rhabdovirus)	2,7E+04	4,0E+04	1,9E+04	1,9E+04
36	Pike fry rhabdovirus	<1,9E+02	1,3E+05	<1,9E+02	8,6E+04
37	SVC-like virus*	8,6E+06	8,6E+06	8,6E+06	1,3E+07
38	CPE in BF-2 and FHM cells	4,0E+06	N/A	N/A	4,0E+06
39	Pike fry	1,3E+08	1,3E+08	N/A	N/A
40	IPNV	N/A	<1,9E+02	N/A	2,7E+03
41	SVCV	2,7E+04	4,0E+06	N/A	N/A
42	Pike Fry Rhabdovirus	2,7E+07	5,9E+07	N/A	N/A
43	PFRV	1,3E+07	N/A	N/A	1,3E+07
44	Negative	1,3E+06	8,6E+06	N/A	4,0E+06
45	pike fry rhabdovirus	8,6E+05	2,7E+06	2,7E+05	<1,9E+02

N/A: Cell line not applied by the participating laboratory for titration of the virus

	BF-2	**EPC**	**RTG-2**	**FHM**
Median titre	1,9E+06	7,2E+06	1,5E+05	6,8E+05
Maximum titre	5,9E+09	5,9E+09	5,9E+07	1,9E+08
Minimum titre	<1,9E+02	<1,9E+02	<1,9E+02	<1,9E+02
25% quartile titre	4,5E+05	6,6E+05	<1,9E+02	6,8E+02
75% quartile titre	8,6E+06	1,9E+07	3,0E+06	4,0E+06

Table 5. Inter-Laboratory Proficiency Test, PT1, 2016 – Results of titration of ampoule II.

IHNV BLK94					
Laboratory code number	**Virus Identification**	**Titre in**			
		BF-2	**EPC**	**RTG-2**	**FHM**
1	IHNV	2,7E+04	8,6E+06	1,3E+03	N/A
2	IHNV	<1,9E+02	1,9E+07	<1,9E+02	N/A
3	IHNV	5,9E+04	5,9E+07	2,7E+04	N/A
4	INHV	<1,9E+02	5,9E+06	N/A	N/A
5	IHNV	<1,9E+02	<1,9E+02	N/A	N/A
6	IHNV	1,3E+03	1,9E+07	4,0E+02	1,9E+06
7	IHNV	4,0E+04	1,3E+06	N/A	N/A
8	IHNV	N/A	4,0E+05	<1,9E+02	<1,9E+02
9	IHNV	1,3E+04	2,7E+07	2,7E+05	1,9E+07
10	IHNV	2,7E+03	1,9E+04	N/A	N/A
11	IHNV	1,3E+03	1,3E+06	N/A	N/A
12	IHNV genogroup U	2,7E+03	8,6E+06	N/A	N/A
13	IHNV	1,3E+03	5,9E+07	1,9E+04	N/A
14	IHNV	<1,9E+02	1,9E+06	1,9E+03	<1,9E+02
15	IHNV	4,0E+04	5,9E+06	N/A	N/A
16	IHNV	1,3E+03	1,3E+06	N/A	N/A
17	IHNV	1,3E+05	1,3E+07	N/A	N/A
18	IHN	<1,9E+02	<1,9E+02	<1,9E+02	<1,9E+02
19	IHNV	1,3E+05	8,6E+06	N/A	N/A
20	IHNV	5,9E+05	4,0E+07	N/A	N/A
21	IHNV	8,6E+03	2,7E+06	2,7E+06	2,7E+05
22	IHNV	2,7E+04	4,0E+06	N/A	N/A
23	INHV	N/A	4,0E+06	1,3E+05	N/A
24	IHNV viable virus detected	8,6E+05	5,9E+06	N/A	N/A
25	IHNV	<1,9E+02	8,6E+05	N/A	1,3E+06
26	IHNV	8,6E+04	5,9E+06	N/A	N/A
27	IHNV	1,3E+06	1,9E+07	N/A	N/A
28	IHNV	N/A	4,0E+05	<1,9E+02	8,6E+02
29	IHNV	1,3E+04	5,9E+06	4,0E+05	8,6E+04
30	IPNV	N/A	<1,9E+02	<1,9E+02	N/A
31	IHNV	1,9E+04	1,3E+03	N/A	N/A
32		4,0E+05	1,9E+06	N/A	N/A
33	IHNV	1,3E+04	2,7E+04	1,3E+03	1,3E+03

34	IHNV	<1,9E+02	5,9E+07	N/A	N/A
35	IHNV	<1,9E+02	1,9E+05	<1,9E+02	<1,9E+02
36	IHNV	<1,9E+02	1,9E+05	<1,9E+02	<1,9E+02
37	IHNV	1,9E+05	1,9E+07	2,7E+04	2,7E+07
38	IHNV	1,3E+03	N/A	N/A	1,9E+06
39	IHN	2,7E+07	4,0E+08	N/A	N/A
40	IHNV	N/A	<1,9E+02	N/A	5,9E+04
41	IHNV	1,3E+03	2,7E+07	N/A	N/A
42	IHNV	2,7E+05	2,7E+07	N/A	N/A
43	IHNV	5,9E+06	N/A	N/A	4,0E+07
44	IHNV	1,3E+04	2,7E+06	N/A	4,0E+06
45	IHNV	1,9E+04	1,9E+06	8,6E+05	<1,9E+02

N/A: Cell line not applied by the participating laboratory for titration of the virus

	BF-2	**EPC**	**RTG-2**	**FHM**
Median titre	1,3E+04	4,0E+06	1,3E+03	7,2E+04
Maximum titre	2,7E+07	4,0E+08	2,7E+06	4,0E+07
Minimum titre	<1,9E+02	<1,9E+02	<1,9E+02	<1,9E+02
25% quartile titre	1,3E+03	6,3E+05	<1,9E+02	<1,9E+02
75% quartile titre	9,6E+04	1,9E+07	7,7E+04	1,9E+06

Table 6. Inter-Laboratory Proficiency Test, PT1, 2016 – Results of titration of ampoule III.

VHSV TR-WS13G					
Laboratory code number	**Virus Identification**	**Titre in**			
		BF-2	**EPC**	**RTG-2**	**FHM**
1	VHSV	4,0E+06	1,9E+05	8,6E+02	N/A
2	VHSV	5,9E+06	1,3E+06	<1,9E+02	N/A
3	VHSV	1,9E+07	1,9E+07	4,0E+03	N/A
4	VHSV	1,26E+06	2,73E+05	N/A	N/A
5	VHSV	<1,9E+02	<1,9E+02	N/A	N/A
6	VHSV	<1,9E+02	2,7E+05	<1,9E+02	1,3E+06
7	VHSV	2,7E+06	2,7E+05	N/A	N/A
8	VHSV	N/A	1,3E+05	1,3E+05	1,3E+06
9	VHSV	5,9E+06	5,9E+05	1,3E+03	2,7E+07
10	VHSV	1,9E+05	8,6E+05	N/A	N/A
11	VHSV	4,0E+06	2,7E+06	N/A	N/A
12	VHSV genotype Ie	2,7E+06	5,9E+05	N/A	N/A
13	VHSV	5,9E+07	4,0E+05	1,9E+03	N/A
14	VHSV	6,8E+03	<1,9E+02	5,9E+02	<1,9E+02
15	VHSV	2,7E+06	8,6E+04	N/A	N/A
16	VHSV	1,3E+06	1,9E+05	N/A	N/A
17	VHSV	1,3E+07	1,3E+06	N/A	N/A
18	VHS	<1,9E+02	<1,9E+02	<1,9E+02	<1,9E+02
19	VHSV	1,9E+07	2,7E+06	N/A	N/A
20	VHSV	1,3E+07	1,9E+06	N/A	N/A
21	VHS	4,0E+05	1,9E+05	1,3E+05	5,9E+04
22	VHSV	2,7E+06	5,9E+04	N/A	N/A
23	VHSV	N/A	4,0E+04	1,3E+04	N/A
24	VHSV	8,6E+07	1,3E+07	N/A	N/A
25	VHSV viable virus detected	1,26E+06	N/A	N/A	N/A
26	VHSV/ IHNV	1,9E+06	1,9E+06	N/A	N/A
27	VHSV	2,7E+04	5,9E+04	N/A	N/A
28	VHSV	N/A	4,0E+03	<1,9E+02	2,7E+05
29	VHSV	1,9E+07	1,9E+07	8,6E+06	5,9E+04
30	VHSV	N/A	<1,9E+02	<1,9E+02	N/A
31	VHSV	2,7E+05	5,9E+03	N/A	N/A
32	VHSV	1,9E+06	1,3E+06	N/A	N/A
33	VHSV	1,3E+05	1,3E+05	1,3E+04	4,0E+03

34	VHSV	5,9E+06	8,6E+05	N/A	N/A
35	VHSV	1,26E+04	5,87E+04	<1,9E+02	1,00E+00
36	VHSV	1,00E+00	2,73E+04	<1,9E+02	4,00E+02
37	VHSV	1,3E+07	2,7E+06	5,9E+06	1,9E+06
38	VHSV	8,6E+05	N/A	N/A	1,9E+06
39	VHS	5,9E+07	8,6E+06	N/A	N/A
40	VHSV and IPNV	N/A	<1,9E+02	N/A	5,9E+06
41	VHSV	4,0E+06	1,9E+06	N/A	N/A
42	VHSV	8,6E+06	8,6E+05	N/A	N/A
43	VHSV	1,9E+07	N/A	N/A	1,9E+06
44	VHSV	1,9E+06	4,0E+05	N/A	1,9E+05
45	VHSV	8,6E+05	4,0E+05	1,9E+04	<1,9E+02

N/A: Cell line not applied by the participating laboratory for titration of the virus

	BF-2	**EPC**	**RTG-2**	**FHM**
Median titre	2,7E+06	3,4E+05	1,3E+03	1,9E+05
Maximum titre	8,6E+07	1,9E+07	8,6E+06	2,7E+07
Minimum titre	<1,9E+02	<1,9E+02	<1,9E+02	<1,9E+02
25% quartile titre	3,7E+05	5,9E+04	<1,9E+02	4,0E+02
75% quartile titre	9,6E+06	1,3E+06	1,6E+04	1,9E+06

Table 7. Inter-Laboratory Proficiency Test, PT1, 2016 – Results of titration of ampoule IV.

ECV 562/92					
Laboratory code number	**Virus Identification**	**Titre in**			
		BF-2	**EPC**	**RTG-2**	**FHM**
1	ECV/ESV	2,7E+06	2,7E+06	1,3E+03	N/A
2	ECV	2,7E+04	5,9E+06	<1,9E+02	N/A
3	Ranavirus ECV/ESV	4,0E+07	4,0E+04	1,9E+04	N/A
4	No IHNV, VHSV, IPNV, SVCV	5,9E+05	4,0E+05	N/A	N/A
5	Rana virus	<1,9E+02	<1,9E+02	N/A	N/A
6	ECV	<1,9E+02	1,3E+05	<1,9E+02	1,9E+04
7	ECV/ESV	2,7E+07	1,3E+07	N/A	N/A
8	ECV	N/A	1,9E+05	1,3E+04	5,9E+03
9	Ranavirus not EHNV	1,3E+07	2,7E+06	2,7E+06	2,7E+04
10	no IHNV , VHSV, IPNV, SVCV	5,9E+03	8,6E+02	N/A	N/A
11	Ranavirus (ESV or ECV)	8,6E+05	5,9E+05	N/A	N/A
12	ECV	2,7E+06	1,3E+06	N/A	N/A
13	ECV	8,6E+07	2,7E+07	4,0E+06	N/A
14	Ranavirus (ESV/ECV)	4,0E+06	1,9E+06	1,3E+03	<1,9E+02
15	ECV/ESV (Ranavirus)	8,6E+06	2,7E+06	N/A	N/A
16	Ranavirus	1,3E+07	1,3E+05	N/A	N/A
17	ECV	5,9E+06	2,7E+04	N/A	N/A
18	Neg	<1,9E+02	<1,9E+02	<1,9E+02	<1,9E+02
19	Ranavirus - ECV	2,7E+07	5,9E+06	N/A	N/A
20	ECV/ESV	2,7E+07	8,6E+06	N/A	N/A
21	Ranavirus	5,9E+05	5,9E+05	8,6E+05	2,7E+04
22	EHNV	1,9E+02	1,9E+02	N/A	N/A
23	Ranavirus	N/A	4,0E+04	5,9E+04	N/A
24	Ranavirus	2,7E+07	1,3E+05	N/A	N/A
25	ECV viable virus detected	5,9E+05	1,9E+05	N/A	<1,9E+02
26	Ranavirus other than EHNV	1,9E+06	1,3E+06	N/A	N/A
27	ECV	2,7E+05	2,7E+04	N/A	N/A
28	ECV	N/A	1,9E+06	4,0E+02	1,3E+05
29	ECV	1,3E+05	4,0E+05	2,7E+05	1,9E+04
30	IHNV	N/A	<1,9E+02	<1,9E+02	N/A
31	Not EHNV Ranavirus	1,9E+06	8,6E+03	N/A	N/A
32	EHNV	1,9E+05	8,6E+04	N/A	N/A

33	Ranavirus (ECV)	1,3E+04	1,3E+05	1,9E+04	1,3E+04
34	ECV/ESV	1,3E+09	1,9E+07	N/A	N/A
35	ECV (Ranavirus)	8,6E+06	8,6E+04	1,3E+04	5,9E+05
36	ECV	<1,9E+02	1,9E+05	<1,9E+02	2,7E+05
37	Ranavirus ECV	8,6E+06	1,9E+07	5,9E+06	8,6E+06
38	ECV/ESV	1,9E+07	N/A	N/A	1,3E+03
39	ECV/ESV	1,3E+08	8,6E+07	N/A	N/A
40	VHSV and ECV	N/A	<1,9E+02	N/A	1,3E+04
41	Ranavirus was identified by conventional RT-PCR and than REA was applied as given by OIE manuel to identified ECV	8,6E+07	5,9E+07	N/A	N/A
42	Catfish/sheetfish virus	1,3E+07	2,7E+07	N/A	N/A
43	ECV/ESV	5,9E+07	N/A	N/A	8,6E+05
44	Ranavirus (see sequencing result)	8,6E+05	1,9E+06	N/A	5,9E+05
45	European sheatfish virus	1,3E+07	1,3E+06	1,3E+05	<1,9E+02

N/A: Cell line not applied by the participating laboratory for titration of the virus

	BF-2	**EPC**	**RTG-2**	**FHM**
Median titre	3,4E+06	4,0E+05	1,3E+04	1,9E+04
Maximum titre	1,3E+09	8,6E+07	5,9E+06	8,6E+06
Minimum titre	<1,9E+02	<1,9E+02	<1,9E+02	<1,9E+02
25% quartile titre	2,5E+05	6,3E+04	2,0E+02	2,4E+03
75% quartile titre	2,1E+07	2,7E+06	2,0E+05	2,4E+05

Table 8. Inter-Laboratory Proficiency Test, PT1, 2016 – Results of titration of ampoule V.

IPNV Sp					
Laboratory code number	**Virus Identification**	**Titre in**			
		BF-2	**EPC**	**RTG-2**	**FHM**
1	IPNV	1,3E+07	8,6E+06	5,9E+06	N/A
2	IPNV & ECV	1,3E+07	2,7E+06	< 1,9E+02	N/A
3	IPNV	8,6E+07	1,3E+08	2,7E+07	N/A
4	IPNV	5,87E+07	8,62E+06	N/A	N/A
5	IPNV	5,9E+04	2,7E+06	N/A	N/A
6	IPNV	< 1,9E+02	1,3E+07	5,9E+04	< 1,9E+02
7	IPNV	8,6E+07	4,0E+07	N/A	N/A
8	IPNV	N/A	1,3E+07	1,9E+07	5,9E+06
9	IPNV	5,9E+08	2,7E+07	1,9E+08	4,0E+07
10	IPNV	1,9E+07	1,9E+06	N/A	N/A
11	IPNV	1,3E+08	1,9E+02	N/A	N/A
12	IPNV genogroup 5	4,0E+07	1,9E+07	N/A	N/A
13	IPNV	4,0E+07	1,9E+08	4,0E+07	N/A
14	IPNV	1,5E+04	4,6E+04	6,8E+03	4,6E+03
15	IPNV	1,3E+07	1,3E+06	N/A	N/A
16	IPNV	1,9E+08	1,3E+07	N/A	N/A
17	IPNV	1,9E+08	2,7E+06	N/A	N/A
18	IPN	< 1,9E+02	< 1,9E+02	< 1,9E+02	< 1,9E+02
19	IPNV	8,6E+07	1,3E+07	N/A	N/A
20	IPNV	5,9E+07	1,9E+07	N/A	N/A
21	IPN	1,9E+03	< 1,9E+02	1,9E+03	< 1,9E+02
22	IPNV	8,6E+06	2,7E+06	N/A	N/A
23	IPNV	N/A	1,3E+06	5,9E+06	N/A
24	IPNV	1,9E+08	4,0E+04	N/A	N/A
25	IPV viable virus detected	1,9E+07	< 1,9E+02	N/A	< 1,9E+02
26	IPNV/ VHSV	2,7E+07	4,0E+07	N/A	N/A
27	IPNV	1,9E+06	2,7E+07	N/A	N/A
28	IPNV	N/A	4,0E+07	< 1,9E+02	1,9E+06
29	IPNV	8,6E+08	1,3E+09	1,9E+09	8,6E+07
30	Ranavirus (ECV or ESV)	N/A	4,0E+06	5,9E+04	N/A
31	IPNV	4,0E+07	5,9E+06	N/A	N/A

32	IPNV	5,9E+05	1,3E+06	N/A	N/A
33	IPNV	1,3E+05	1,3E+05	1,3E+05	5,9E+04
34	ECV, IPNV	2,7E+09	1,9E+09	N/A	N/A
35	IPNV	1,26E+07	8,62E+05	8,62E+04	1,26E+06
36	IPNV	< 1,9E+02	1,86E+04	< 1,9E+02	4,00E+03
37	IPNV-Sp	1,9E+07	4,0E+07	8,6E+06	2,7E+07
38	IPNV	8,6E+07	N/A	N/A	2,7E+07
39	IPN	8,6E+07	1,9E+07	N/A	N/A
40	IPNV	N/A	< 1,9E+02	N/A	2,7E+06
41	IPNV	1,3E+08	1,9E+08	N/A	N/A
42	IPNV	4,0E+07	4,0E+07	N/A	N/A
43	IPNV	1,3E+08	N/A	N/A	4,0E+06
44	Negative	4,0E+07	4,0E+06	N/A	2,7E+07
45	IPNV	1,9E+06	< 1,9E+02	1,9E+05	< 1,9E+02

N/A: cell line not applied by the participating laboratory for titration of the virus

	BF-2	**EPC**	**RTG-2**	**FHM**
Median titre	4,0E+07	1,1E+07	5,9E+06	4,0E+06
Maximum titre	2,7E+09	1,9E+09	1,9E+09	8,6E+07
Minimum titre	1,9E+03	1,9E+02	1,9E+03	4,0E+03
25% quartile titre	1,3E+07	2,1E+06	7,2E+04	1,3E+06
75% quartile titre	8,6E+07	3,7E+07	2,3E+07	2,7E+07

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 (grey shadow), median (blue line), 75% quartile (upper yellow line) and 25% quartile (lower yellow line) are plotted on all graphs. For participants failing to obtain any titre, no red diamond is shown.

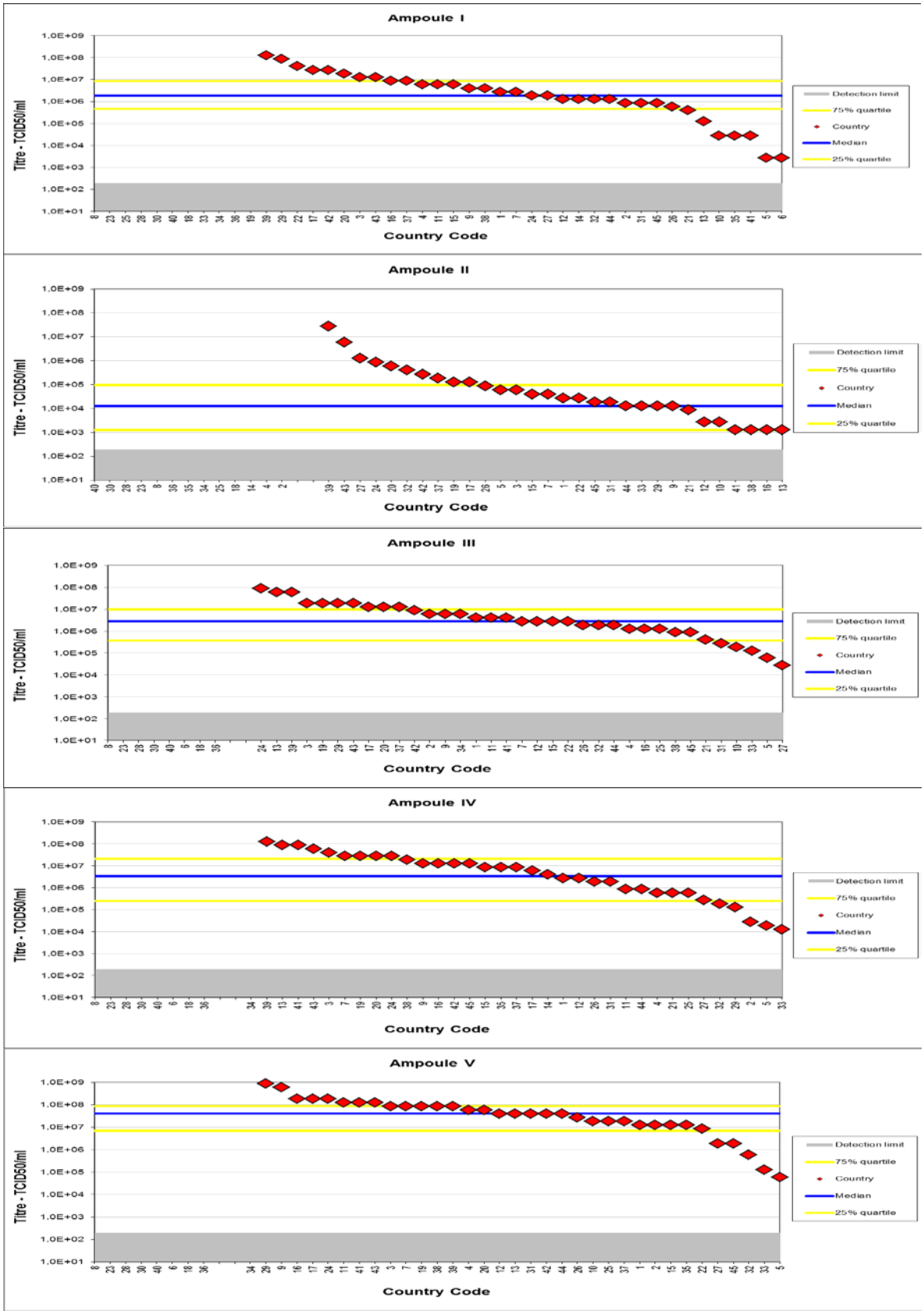


Figure 6. Virus titres obtained in EPC cells. For further details see Figure 5

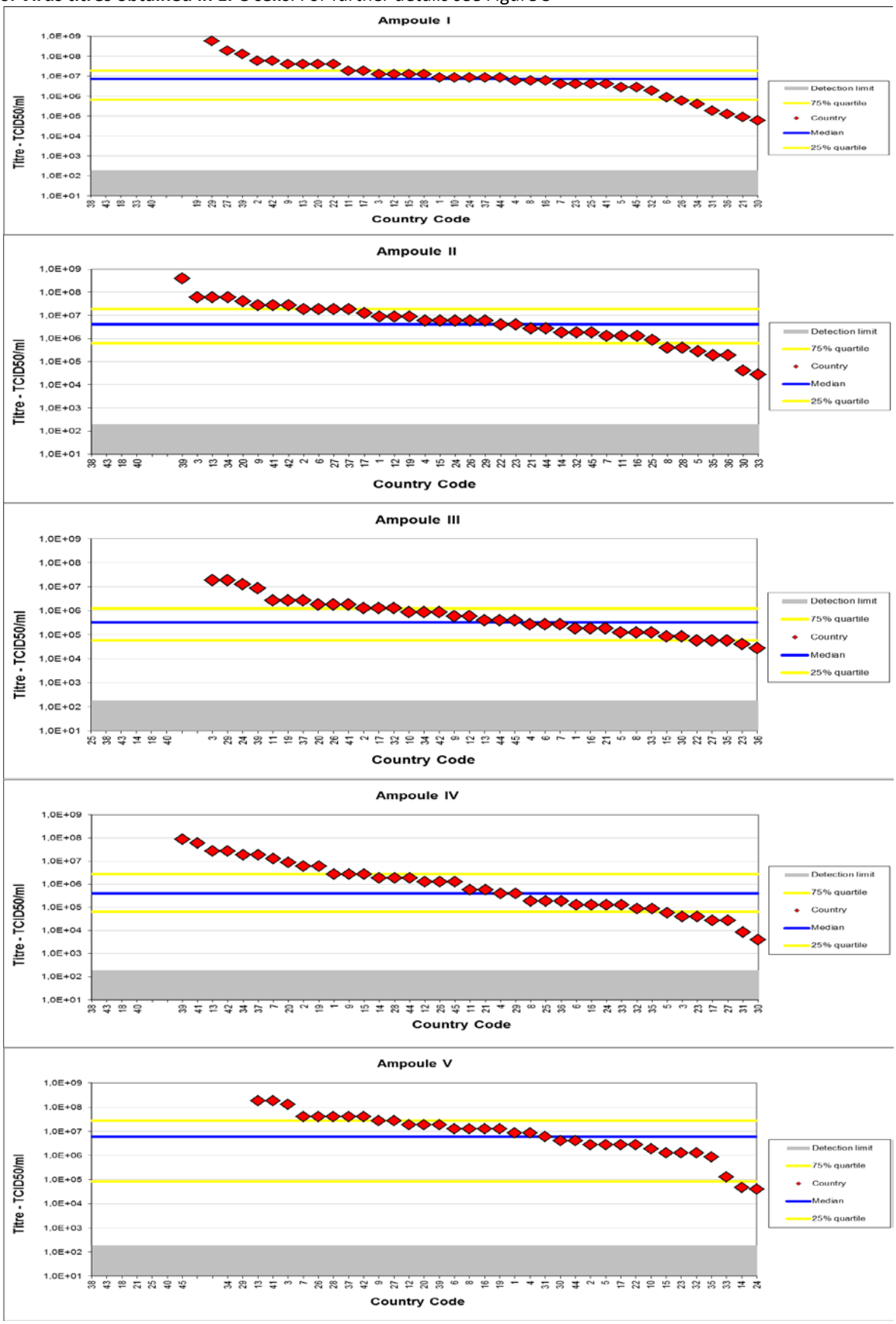


Figure 7. Virus titre obtained in RTG-2 cells. For further details see Figure 5

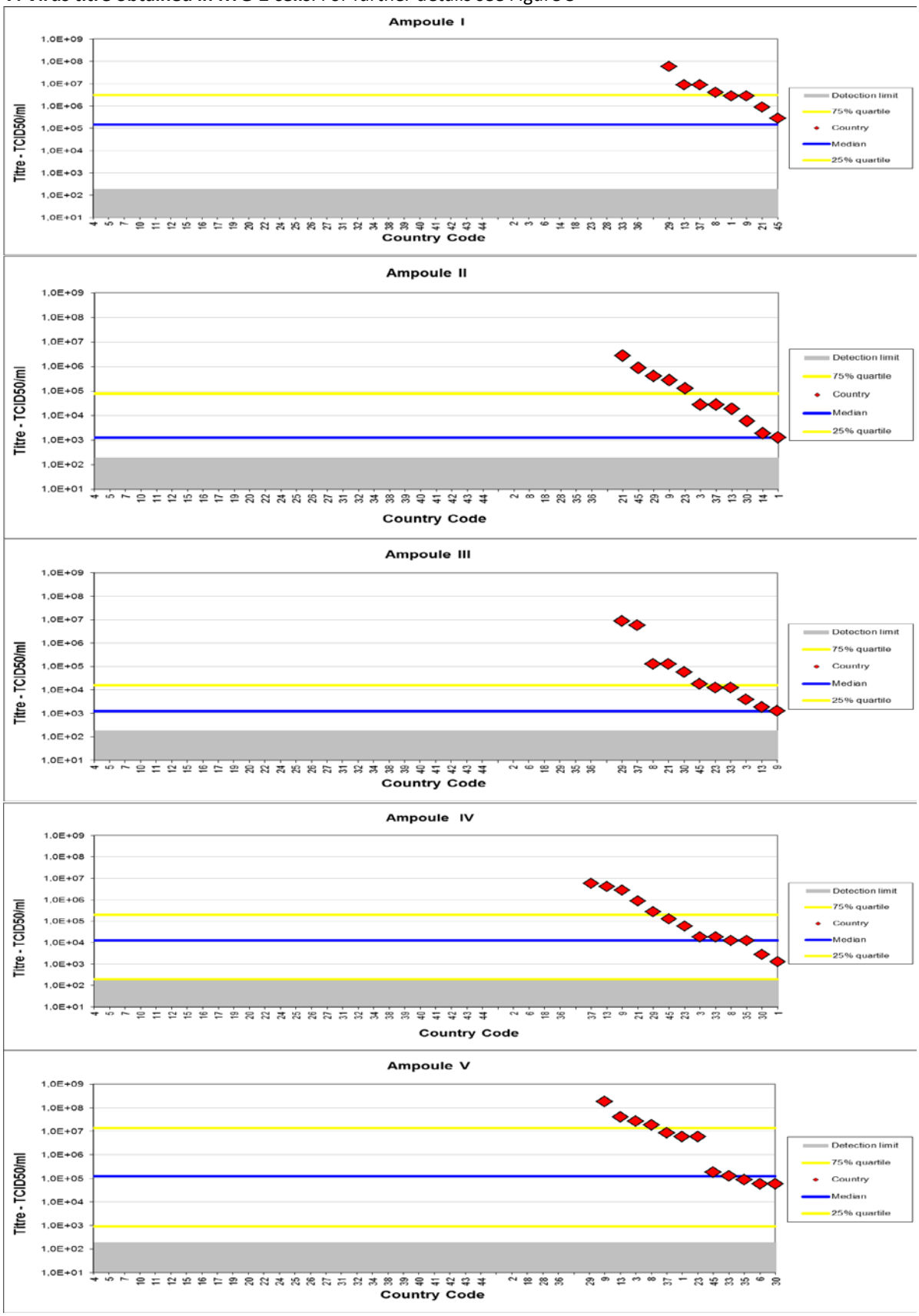
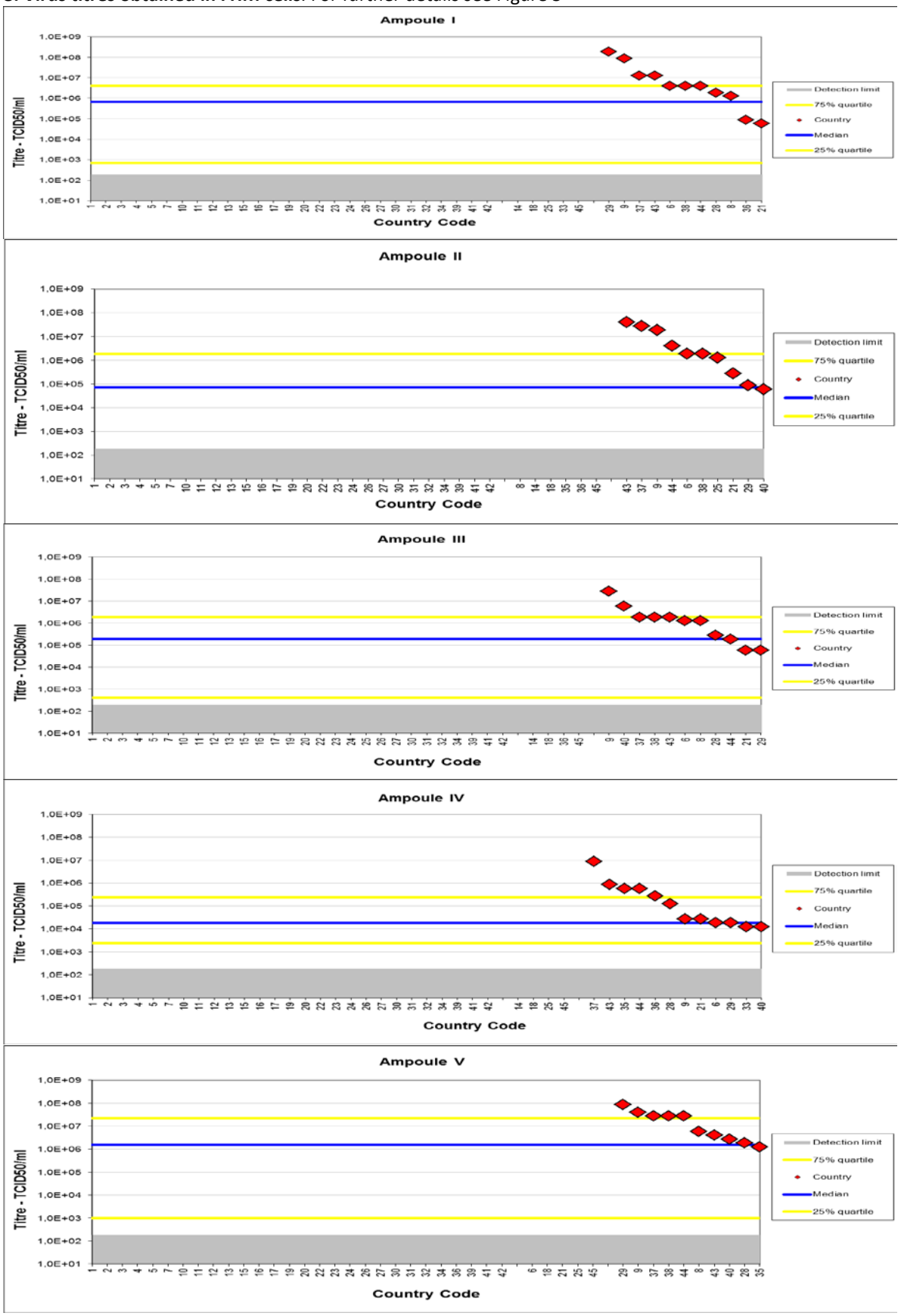


Figure 8. Virus titres obtained in FHM cells. For further details see Figure 5



Identification of content

- 17 laboratories correctly identified all viruses in all ampoules
- All participants submitted the spreadsheet within the deadline, 1 participant needed a small extension to provide sequencing results.

Ampoule I – PFRV

The inclusion of pike fry rhabdovirus has created some challenges to the participants, being a virus, antigenically related to SVCV

- 17 laboratories correctly identified viruses included in ampoule II,III,IV,V and ruled out VHSV,IHNV,IPNV,Ranavirus,SVCV from ampoule I
- 17 laboratories correctly identified PFR, by means of sequencing
- 6 laboratories identified ampoule I as SVCV by immunochemical techniques (ELISA,IFAT)
- 4 laboratories identified another virus
- 1 laboratory did not provide a reply

Ampoule II - IHNV

- 43 laboratories correctly identified IHNV
- 1 laboratory provide the wrong identification
- 1 laboratory did not provide a reply

Ampoule III – VHSV

- 43 laboratories correctly identified VHSV
- 2 laboratories correctly identified VHSV in Ampoule III but contaminated the ampoule with another virus (1 laboratory with IHNV and 1 with IPNV)

Ampoule IV – ECV

- 37 laboratories correctly identified the isolate as ranavirus and not asthe listed EHNV by sequencing or REA (restriction enzyme analysis)
- 1 Laboratory correctly identified ECV but contaminated the ampoule with VHSV
- 1 laboratory identified another virus
- 1 laboratory identified EHNV
- 5 laboratories did not identify the virus

Ampoule V –IPNV

- 40 laboratories correctly isolated and identified IPNV
- 3 laboratories correctly identified IPNV but contaminated the ampoule with another virus (2 with ECV and 1 with VHSV)
- 1 laboratory did not provide a reply
- 1 did not isolated any virus

Scores

Starting with proficiency test 2003 we have provided a scoring system for the identification part of the proficiency tests. We have assigned a score of 2 for each correct answer/identification, giving the possibility for obtaining a maximum score of 10 (Table 3). This year the inclusion of PFR in the panel has created some challenges due to its antigenic similarity to SVCV. For this reason we have adopted a specific scoring system.

Ampoule I: PFR identification was given the score 2. Virus isolated but not identified providing that the ampoule content was NOT VHSV; IHNV; IPNV; SVCV or RANAvirus 1 point, but reducing the maximum score achievable to 9. In this way if a participant has not identified PFR but has ruled out all other pathogen will get a success rate of 100%. Identification as SVCV 1 point on a total maximum score for PT 1 of 10. Other incorrect findings or "no virus" or additional types of viruses than those included in the ampoule scored 0.

Ampoule II: IHNV identification was given the score 2. IHNV not identified was given the score 0. Incorrectly finding of "no virus" or other type of viruses than the one included in the ampoule scored 0. Finding of additional type of viruses scored 0 if the contamination was with a listed pathogen, and 1 with a non-listed one.

Ampoule III: VHSV identification was given the score 2. VHSV not identified was given the score 0. Incorrectly finding of "no virus" or other type of viruses than the one included in the ampoule scored 0. Finding of additional type of viruses scored 0 if the contamination was with a listed pathogen, and 1 with a non-listed one.

Ampoule IV: ECV identification was given the score 2. ECV not identified was given the score 0. Incorrectly finding of "no virus", EHNV or other type of viruses than the one included in the ampoule scored 0. Finding of additional type of viruses scored 0 if the contamination was with a listed pathogen, and 1 with a non-listed one.

Ampoule V: IPNV identification was given the score 2. IPNV not identified was given the score 0. Incorrectly finding of "no virus" or other type of viruses than the one included in the ampoule scored 0. Finding of additional type of viruses scored 0 if the contamination was with a listed pathogen, and 1 with a non-listed one.

Out of 45 laboratories participating in the PT 1 2016, 17 obtained score 10/10 being able to identify PFR in ampoule I and 12 obtained a score of 9/9.
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:

- 40 laboratories used BF-2 cells
- 43 laboratories used EPC cells
- 19 laboratories used RTG-2 cells
- 18 laboratories used FHM cells
- 6 laboratories used CHSE-214 cells

- 9 laboratories used four cell lines (BF-2, EPC, RTG-2 and FHM)

- 8 laboratories used tree cell lines:
  - 5 laboratories used BF-2 cells in combination with EPC cells and RTG-2 cells
  - 1 laboratory used BF-2 cells in combination with EPC cells and FHM cells
  - 2 laboratories used RTG-2 cells in combination with EPC cells and FHM cells

- 28 laboratories used two cell lines:
  - 22 laboratories used BF-2 cells in combination with EPC cells
  - 2 laboratories used RTG-2 cells in combination with EPC cells
  - 2 laboratories used BF-2 cells in combination with FHM cells
  - 2 laboratory used EPC cells in combination with FHM cells

The combination of EPC and FHM cells or BF-2 and RTG 2 as well is not valid according to Commission Decision 2015-1554. 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 9.
It appears that:
Ampoule I (PFR) replicates equally well on EPC and BF-2 cells, slightly less efficiently on FHM and RTG-2.
Ampoule II (IHNV) replicate well on EPC cells, whereas less efficiently on BF-2, FHM and RTG-2.
Ampoule III (VHSV) replicates well on all four cell lines, however it grows best on BF-2 cells.
Ampoule IV (ECV) replicates equally well on BF-2 and EPC.
Ampoule V (IPNV) replicated best on BF-2 and EPC.

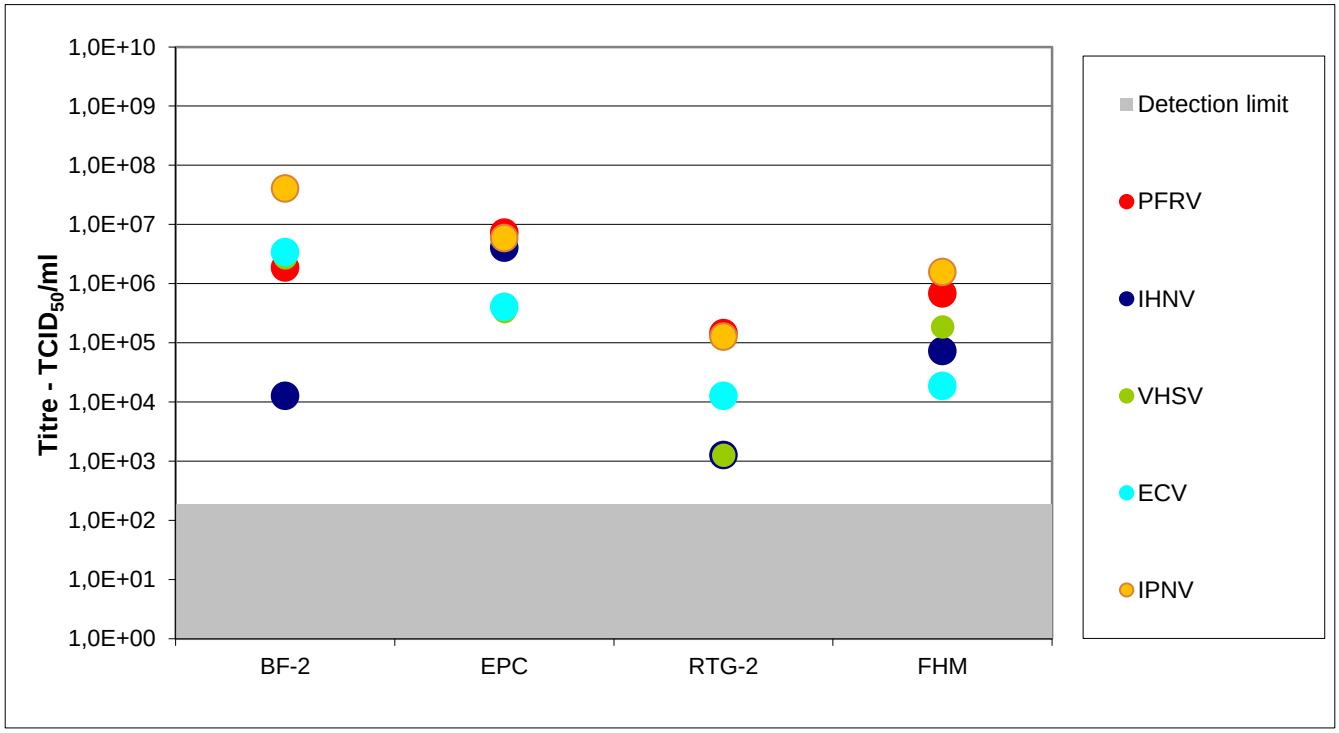


Figure 9. Median virus titres obtained by the participants in 4 different cell lines.

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.

An Overview of the genotyping results obtained for PT1 by all participants is displayed in the following table 9

Laboratory code number	Score	Ampoule I	Ampoule II	Ampoule III	Ampoule IV	Ampoule V
		PFR	IHNV BLK94	VHSV TR-WS13G	ECV 562/92	IPNV Sp
1	**10/10**	Vesiculovirus Genogroup III	IHNV Genogroup U	VHSV Genogroup Ie	ECV/ESV	IPNV Genogroup 5
2	**9/10**	France "1972", originally from The Netherlands	USA, genotype U	Ie, Georgia "1981"	Hungary, strain 13051/2012	IPN: Sp/ EHNV: Hungary, strain 13051/2012
3	**9/9**				ECV/ESV	
4	**7/8**					
5	**8/9**					
6	**9/9**		U	Ie		
7	**9/9**		L	Ie		
8	**10/10**		U	I		Sp
9	**10/10**			III		Sp
10	**9/9**					
11	**9/9**		Genogroup U	Genogroup I		Genogroup V
12	**10/10**		U	Ie		5
13	**10/10**		Genotype U	Genotype Ie		
14	**10/10**		Asia	Ie		Sp
15	**9/9**					
16	**9/10**			1 E		
17	**9/10**				KT989884.1	
18	**6/6**					
19	**10/10**	Genogroup III (from Stone et al., 2003)	U	Genotype Ie		Genogroup 5 (Sp)
20	**9/10**		U	Ie	ECV/ESV	genotype 5 serotype Sp
21	**10/10**	Genogroup III	U	Ie	ECV, ESV	
22	**9/9**					
23	**9/9**		IHNV L genogroup			
24	**9/9**		U	1e	Not EHNV	N/a
25	**10/10**		Genogroup U	Genogroup 1b		-
26	**4/10**		IHNV U	VHSV Ie and IHNV U	ECV or ESV	VHSV Ie

27	**10/10**					
28	**9/9**		U	Ib	ECV	
29	**10/10**			Ib		
30	**3/10**					
31	**9/9**					
32	**5/10**					
33	**8/10**		Genogroup U	Genotype Ie		Sp
34	**9/10**					
35	**10/10**			Ie		
36	**10/10**		Genotype U (North American)	Genotype Ie		
37	**10/10**		U genogroup	Ie genotype		Sp Serotype
38	**9/9**		Genogroup U	Genotyp 1e		Genogroup 5
39	**10/10**		IM2Ws+ID3a-Primer: M, E for X89213 IHN-OIE Primer: U	Depending on primers different subtypes of gentype 1 were found: With GA-Primers Ia, Id & Ie; with the EURL-primer pair Ib and Ie; with both primer pairs isolate GE-1.2 under different accession numbers		
40	**5/10**					
41	**9/10**					
42	**10/10**		Upper genogroup	Genotype Ie	N/A	Genogroup V, Serotype A2
43	**10/10**		U	1e	Not EHNV	Genogroup 5
44	**7/9**					
45	**10/10**		IHNV-U	I-b		SP; Genogroup III

AMPOULE I - PFR

17 laboratories sequenced correctly PFR in ampoule 1

- 10 laboratories sequenced the isolates using protocol provided under OIE manual for SVCV virus, according to primer set of Stone et al., 2003
- 2 laboratories sequenced the isolate according to protocol from Ruane et al,2014
- 1 laboratory used protocol from Talbi et al.,2011
- 3 laboratory used in house developed protocol.

AMPOULE II- IHNV

33 laboratories sequenced IHNV isolate in Ampoule II

- 24 laboratories partially sequenced the glycoprotein gene according to protocol from Emmeneger et al., 2000
- 1 Laboratory sequenced the full G gene with in house developed primer sets
- 2 laboratories targeted the G gene according to the protocol provided by Kolodzejek et al., 2008
- 1 laboratory 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 Emmenegger et al., 2002
- 1 laboratory targeted the G gene according to the protocol provided by Enzmann et al., 2005
- 1 laboratory targeted the G gene according to the protocol provided by Abbadi et al., 2016
- 3 laboratories targeted the N gene according to OIE manual
- 24 of the laboratories that sequenced correctly identified this isolate as belonging to genotype U
- 6 laboratories did not conclude on the genotype
- 2 laboratories genotyped the isolate as belonging to L genotype
- 1 laboratory genotyped the isolate as belonging to Asian genotype

It has to be specified that some participants used more than one protocol for sequencing the isolate.

AMPOULE III- VHSV

31 laboratories sequenced the VHSV isolate included in Ampoule III

- 10 laboratories targeted the G gene according to the protocol from Einer-Jensen et al., 2004
- 3 laboratories targeted the G gene according to the protocol provided by Miller et al., 1998
- 13 targeted the nucleocapsid protein according to protocol from Snow et al.,2004
- 2 laboratories targeted the G gene according to Hedrick et al.,2003
- 2 laboratories provided primer sets without reference and target region
- 1 laboratory targeted the g gene region with an “in house” developed protocol
- 20 laboratories correctly genotyped the isolate as belonging to genotype Ie
- 4 laboratories genotyped the isolate as belonging to genotype Ib
- 1 laboratory genotyped the isolate as belonging to genotype III
- 3 laboratories genotyped the isolate as belonging to genotype I but did non assigned a subgroup
- 3 laboratories did not provide a genotype for the isolate

AMPOULE IV - ECV

36 laboratories sequenced ECV in ampoule III,

- 29 laboratories targeted the major capsid protein MCP according to the protocol provided by Hyatt et al., 2000.
- 1 laboratories targeted the major capsid protein MCP according to protocol provided by Holopainen et al, 2009
- 1 laboratory targeted the major capsid protein MCP according to the protocol provided by Bigarre et al. 2008
- 1 laboratory targeted the major capsid protein MCP according to protocol provided by Marsh et al.,2002
- 1 laboratory targeted the major capsid protein MCP according to protocol provided by Hanson et al.,2006
- 3 laboratories used an in-house developed protocol without providing a protocol.
- 37 laboratories correctly sequenced the content of the ampoule as ECV.

AMPOULE V-IPNV

- 22 laboratories sequenced the IPNV strain included in Ampoule V.
- 3 laboratories targeted the VP2/NS junction region according to protocol from Nishizawa et al., 2005
- 2 laboratories sequenced NS/VP3 region the isolate according to Taksdal et al., 2001
- 1 laboratory sequenced the GABF2/P10 amplicon according to Davies et al.,2010
- 1 laboratory seuquenced the isolate according to Wang et al.,1997
- 1 laboratory sequenced the VP2/NS junction region according to Heppell et al. 1992
- 2 laboratory sequenced the isolate according to Blake et al.,1995
- 1 laboratory combined 2 protocols Santi et al. Virology 2004:322;31-40 and Bain et al. J Fish Dis. 2008:31;37-47
- 1 laboratory combined 2 protocols McColl et al. (2009) and Bain et al. 2008
- 1 laboratory sequenced the VP2 Gene according to Santi et al. 2004
- 1 laboratory sequenced the NS/VP3 segment A for IPN according to Kerr et al. 2006
- 1 laboratory sequenced the VP2 gene in segment A according to protocol from Crane et al.,2004
- 1 laboratory followed protocol from Williams et al.,1999
- 1 laboratory followed protocol from Tapia et al.,2015
- 5 laboratories did not provide a reference for the primer they used.
- 15 laboratories classified the isolate as belonging to Genotype V and or Serotype Sp.
- 1 laboratory classified the isolate as genotype III
- 6 laboratories did no provide conclusive results on the genotype

Résumé and concluding remarks PT1

60% of parcels were delivered by the shipping companies within 1 day after submission and 86% was delivered within 1 week. The remaining six parcels took longer for delivery primarily due to border controls, the maximum time of shipment was 21 days.

This year ECV was included in the Proficiency test. 37 participants provided the correct identification, 1 laboratory identified correctly the isolate but contaminated the ampoule content.

In this report (Figures 5-8), all the viral titres submitted by participants 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 is 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.

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 pike fry rhabdovirus was included in ampoule I. This virus has generated some challenges to the participants due to its antigenic similarity with SVCV, however the increase implementation of biomolecular techniques has allowed 17 laboratories to identify it correctly and other 17 were able to rule out the presence of VHSV,IHNV,IPNV,SVCV and ranavirus. The scoring system has been adjusted accordingly.

Overall 31 out of 45 participants scored 100% success rate and 8 more than 90%.
It has been a concern that few laboratories has identified the correct virus but not in the right ampoule, meaning that some mistake in traceability of the ampoules during the working flow procedure has occurred. Another critical points that has emerged, is the contamination of ampoule contents. These points will be assessed directly with the single participants that has underperformed.

The results presented in this report will be further presented and discussed at the 21th Annual Workshop of National Reference Laboratories for Fish Diseases to be held 30th and 31st of May ,2017 in Copenhagen, Denmark.

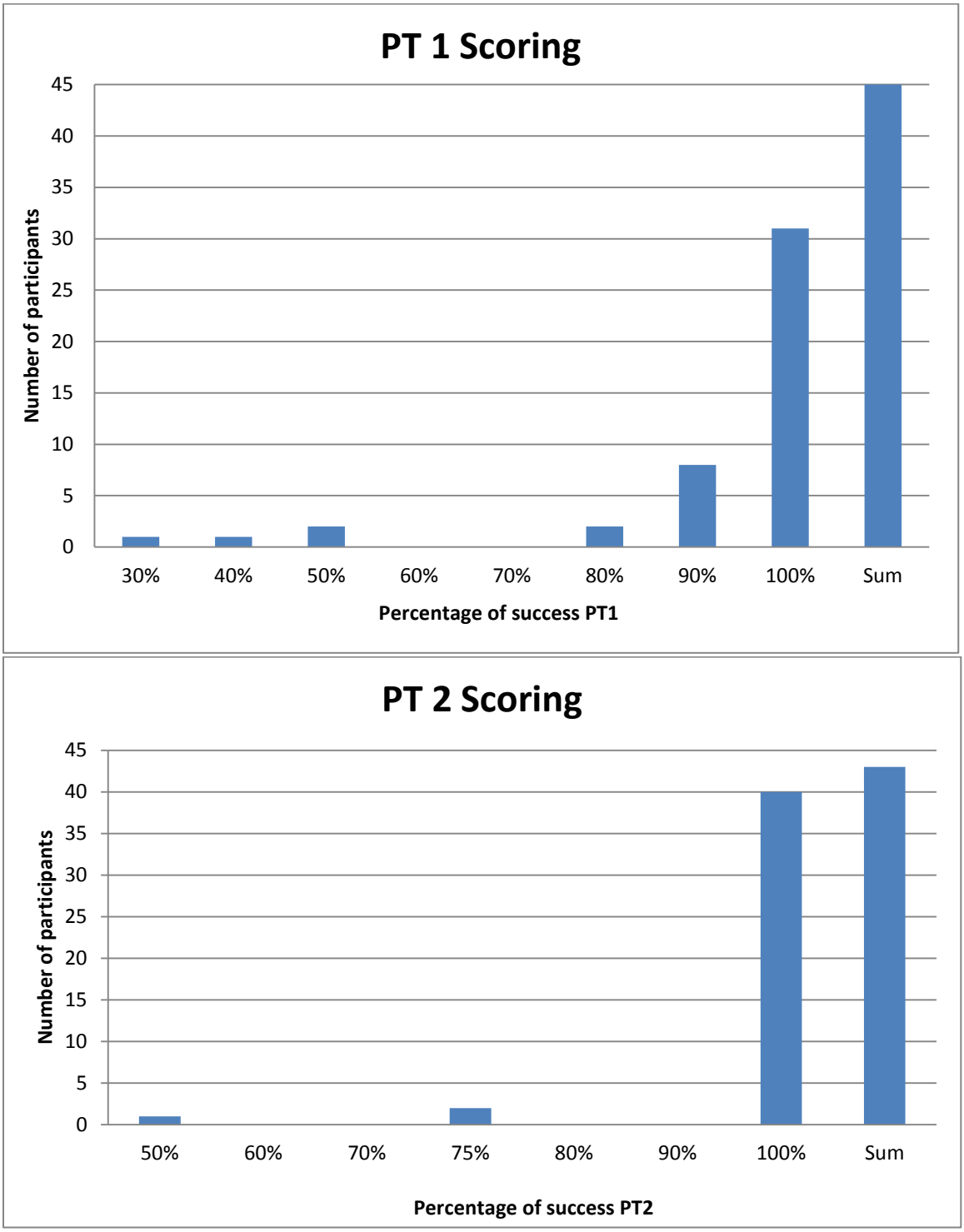


Figure 10 "a" and "b" Success-rate of participating laboratories 2016

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 two 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 2% w/v lactalbumin hydrolysate solution and lyophilized in glass ampoules.
Before the ampoules were sealed by melting, the pathogen concentration was analysed by the KHV real-time PCR protocol described by Gilad et *al*. (2004) and the conventional PCR protocol described by Bercovier et *al*. (2005), the SAV real-time RT-PCR protocol described by Hodneland et al. (2006), and the conventional PCR targeting segment E2 described by Fringuelli et al. (2008) and the ISAV real-time RT-PCR protocol described by Snow et *al*. (2006) and conventional RT-PCR protocol described by Mjaaland et *al*. (2002).

The details of the virus isolates used in the proficiency test 2 are outlined in table 10.

Table 10. Content in each ampoule with reference to culture conditions and major publications of the included pathogens.

Code	Specifications/References
Ampoule VI: SAV	**Salmonid alpha virus (SAV) 2, Sleeping disease virus (SD) received from Dr. J. Castric, ANSES, France in 1998 as isolate sp49** **Genotype: 2** **GenBank accession number:** KC593283.1. **References on isolate:** Castric J., Baudin Laurencin F., Brémont M., Jeffroy J., Le Ven A. & Béarzotti M. (1997) Isolation of the virus responsible for sleeping disease in experimentally infected rainbow trout (Oncorhynchus mykiss). *Bulletin of the European Association of Fish Pathologists* **17**, 27–30. Villoing S., Béarzotti M., Chilmonczyk J.C. & Brémont M. (2000) Rainbow trout sleeping disease virus is an atypical alphavirus. *Journal of Virology* **74**, 173–183. Reference on sequence: E Fringuelli, H M Rowley, J C Wilson, R Hunter, H Rodger, D A GrahamPhylogenetic analyses and molecular epidemiology of European salmonid alphaviruses (SAV) based on partial E2 and nsP3 gene nucleotide sequences Journal of fish diseases Volume 31, Issue 11 November 2008 Pages 811–823
Ampoule VII: ISAV	**ISAV Glesvaer/2/90** **Received from:** Dr. B. Dannevig, OIE Reference Laboratory for ISA, Oslo, Norway **Cell culture passage number:** Unknown **HPR Genotype:** 2 **GenBank accession numbers:** HQ259676, **References on isolate:** Dannevig BH, Falk K & Namork E (1995). Isolation of the causal virus of infectious salmon anaemia (ISA) in a long-term cell line from Atlantic salmon head kidney. *Journal of General Virology* 76, 1353–1359. Falk K, Namork E, Rimstad E, Mjaaland S & Dannevig BH (1997). Characterization of infectious salmon anemia virus, an orthomyxo-like virus isolated from Atlantic salmon (Salmo salar L.) *Journal of Virology* 71, 9016-9023. **References on sequence:** Mérour E, LeBerre M, Lamoureux A, Bernard J, Brémont M & Biacchesi S (2011). Completion of the full-length genome sequence of the infectious salmon anemia virus, an aquatic orthomyxovirus-like, and characterization of mAbs. *Journal of General Virology* 92, 528-533. **References on genotype:** Table 15. Opinion of the Panel on Animal Health and Welfare of the Norwegian Scientific Committee for Food Safety 26.01.07. Which risk factors relating to spread of Infectious Salmon Anaemia (ISA) require development of management strategies? Dok.nr.06/804, 68 pages.
Ampoule VIII: KHV	**Cyprinid herpes virus 3 CyHV-3 – isolate KHV-TP 30 (syn: KHV-T (for Taiwan))** **Received from Dr. Sven Begmann** Koi Herpesvirus (CyHV-3): KHV-TP 30 (syn: KHV-T (for Taiwan)). KHV-TP 30 was isolated from koi in Taiwan and cloned for producing large plaques by Dr. Peiyu Lee, Institute of Medical Biotechnology, Central Taiwan University of Science and Technology, Dakeng, BeiTung District, TaiChung City 406, Taiwan in-2005. The isolate was provided by Dr. Sven M. Bergmann, Friedrich-Loeffler-Institut (FLI), Federal Research Institute for Animal Health, Südufer 10, 17393 Greifswald-Insel Riems, Germany
Ampoule IX : Blank	BF-2 NON infected cell culture supernatant

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, by RT-PCR (Mjaaland et *al.* (1997)) and real-time RT-PCR (Snow et *al.* (2006)) for ISAV and by RT-PCR (Fringuelli et al. (2008)) and real-time RT PCR (Hodneland et al. (2006)) for SAV, to ascertain identity and homogeneity of the content in the ampoules (Figure 11). As a result all the standard deviations were below 1 Ct. value. Furthermore, minimum 3 months after lyophilisation and storage in the dark at 4°C, the content of the ampoules were tested to assess their stability (Table 11 and Figure 12).
Conventional PCR/RT-PCR fragments were sequenced and so was the HPR region in segment 6 of the ISAV isolate.

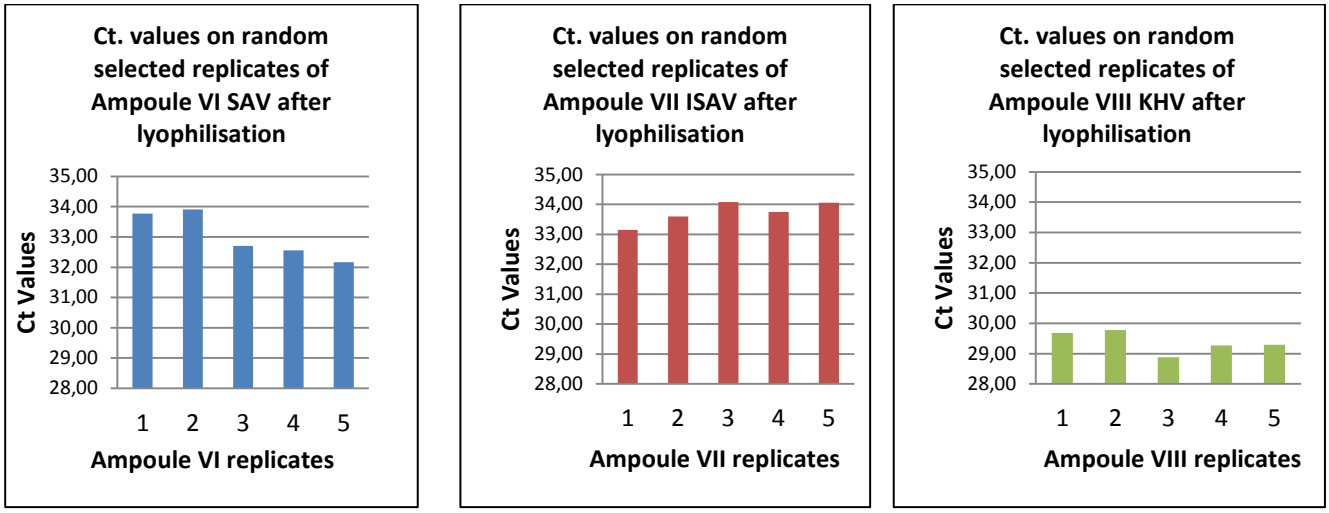


Figure 11, Ampule VI (SAV), VII (ISAV) and VIII (CyHV-3) tested shortly after lyophilisation to assess homogeneity of the content.

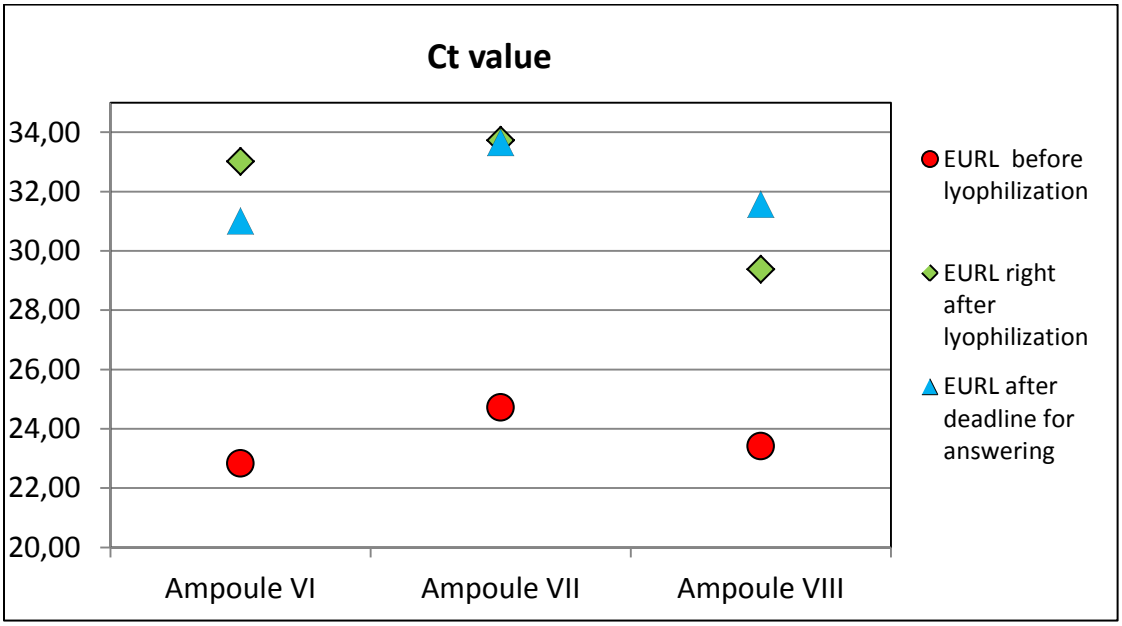


Figure 12, Ampoule VI, VII and VIII tested before and after lyophilisation and after deadline for handling in results.

Table 11, Ct-value of ampoules VI, VII and IX tested before and immediately after lyophilisation and after deadline for handling in results.

Ampoule	Content	Cell line	EURL before lyophilization	EURL right after lyophilization	EURL after deadline for answering
Ampoule VI	SAV	a	22,83	33,77	31,01
		b		33,91	
		c		32,70	
		d		32,56	
		e		32,16	
	Median Value		**22,83**	**33,02**	**31,01**
Ampoule VII	ISAV	a	24,72	33,15	33,65
		b		33,6	
		c		34,08	
		d		33,75	
		e		34,06	
	Median Value		**24,72**	**33,73**	**33,65**
Ampoule VIII	KHV	a	23,41	29,68	31,57
		b		29,78	
		c		28,88	
		d		29,27	
		e		29,29	
	Median Value		**23,41**	**29,38**	**31,57**
Ampoule IX	BF-2 cells	a	0	0	0
		b		0	
		c		0	
		d		0	
		e		0,00	
	Median Value		**0,00**	**0,00**	**0,00**

The lyophilisation procedure caused a significant virus reduction (mainly in ampule VI and VII) as detected by real-time PCR or real-time RT-PCR.

For each ampoule no other pathogens than the expected were detected.

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) according to diagnostic procedures described in Council implementing directive 2015-1554. 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. This year the panel of pathogens to be investigated were expanded to include SAV – salmonid alpha virus. Since this is not a listed disease in the European legislation the participation was voluntary and therefore the participants were asked to declare if the ampoules were tested for SAV or not. Regarding methods for detection of SAV the participants were notified that they could refer to the

OIE manual chapter 2.3.5b – Infection with salmonid alpha virus. 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 12.

Table 12. Inter-Laboratory Proficiency Test, PT2, 2016 - Virus identification.

Laboratory code number	Score	Answer received at EURL	Ampoule VI	Ampoule VII	Ampoule VIII	Ampoule IX
			SAV 2, Sleeping disease virus (SD)	**ISAV ISAV Glesvaer/2/90**	**KHV- CyHV-3 – isolate KHV-TP 30**	BF-2 NON infected cell culture supernatant
1	8/8	24-11-2016	SAV	ISAV	KHV	Negative
2	8/8	24-11-2016	SAV	ISAV	KHV	no virus detected
3	8/8	18-11-2016	SAV	ISAV	KHV	no KHV no ISAV no SAV
4[2]		29-11-2016	0	0	0	0
5	6/8	25-11-2016	SAV	ISAV	KHV	KHV
6	8/8	26-11-2016	SAV	ISAV	KHV	Neg
7[1]	8/8	25-11-2016	Negative for ISAV and KHV	ISAV	KHV	Negative for ISAV and KHV
8	8/8	24-11-2016	SAV	ISAV	KHV	0
9	8/8	25-11-2016	SAV	ISAV	KHV	Not SAV, not ISAV, not KHV
10[1]	8/8	24-11-2016	no ISA, no KHV	ISAV	KHV	no ISA, no KHV
11	8/8	23-11-2016	SDV (SAV 2)	ISAV	KHV	no virus detected
12	8/8	25-11-2016	SAV	ISAV HPR2	KHV	No virus
13	8/8	27-10-2016	SAV	ISAV (HPR deleted)	KHV	negative for all viruses tested
14	8/8	10-11-2016	SDV	ISAV HPRdel	KHV	neg.
15	8/8	25-11-16 (Seq.: 29-11-16)	SAV	ISAV	KHV	NO ISA,NO KHV,NO SAV
16	8/8	25-11-2016	SAV	ISAV	KHV	NEGATIV
17[3]	6/6	25-11-2016	SAV	ISAV	0	0
18[2]		23-11-2016	0	0	0	0
19	8/8	24-11-2016	SAV	ISAV	KHV	Negative
20	8/8	25-11-2016	SAV	ISAV	KHV	Negative
21	8/8	25-11-2016	SAV	ISAV	KHV	-
22[1]	8/8	25-11-2016	Not ISAV, not KHV	ISAV	KHV	Not ISAV, not KHV
23	8/8	25-11-2016	0	ISAV	KHV	0
24	4/8	24-11-2016	SAV	KHV	ISAV	No ISAV, KHV or SAV detected
25	8/8	25-11-2016	SAV viable virus detected	ISAV viable virus detected	KHV detected	No viruses detected
26	8/8	25-11-2016	SAV	ISAV	KHV	No virus detected
27	8/8	21-11-2016	SAV	ISAV	KHV	0
28	8/8	21-11-2016	SAV	ISAV	KHV	no virus
29	8/8	25-11-2016	SAV	ISAV	KHV	-
30	8/8	25-11-2016	0	ISAV	KHV	0
31[1]	8/8	25-11-2016	Not ISAV Not KHV	ISAV	KHV	Not ISAV Not KHV
32	8/8	24-11-2016	SAV	ISAV	KHV	Negative
33	8/8	25-11-2016	SAV	ISAV	KHV	-
34	6/8	25-11-2016	SAV	ISAV	KHV,ISAV	Not detected
35	8/8	25-11-2016	SAV	ISA	KHV	negative
36	8/8	25-11-2016	SAV	ISAV	KHV	Nagative
37	8/8	25-11-2016	SAV	ISAV	KHV	No virus detected

38	8/8	24-11-2016	SAV	ISAV	KHV	Negative
39	8/8	22-11-2016	SAV	ISA	KHV	no SAV, ISAV or KHV detected
40	8/8	25-11-2016	SAV	ISAV	KHV	Negative: No ISAV, KHV, or SAV
41	8/8	24-11-2016	SAV	ISAV	KHV	Virus was not detected.
42	8/8	25-11-2016	SAV	ISAV	KHV	Negative
43	8/8	24-11-2016	SAV	ISAV	KHV	Negative
44	8/8	23-11-2016	SAV	ISAV	KHV	Negative
45	8/8	16-11-2016	SAV	ISAV	KHV	not ISAV, KHV, SAV

[1]Did not test for SAV, [2] Did not participate in PT2, [3] Did not test for KHV,

All laboratories were asked to sequence the HPR region of ISAV isolates un a voluntary basis. However, since ISAV HPR0 have been delisted in Council Directive 2006/88/EC Annex IV , this will be a mandatory task in future.

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

Identification of content

- 43 laboratories submitted results
- 40 laboratories correctly identified all four ampoules
- 42 laboratories tested for the two listed pathogens
- 43 laboratories tested for ISAV
- 42 laboratories tested for KHV
- 37 laboratories tested for SAV
- 2 laboratory that did participate in PT 1 did not participate in PT2

Ampoule VI – *SAV*

- 37 laboratories correctly identified SAV
- 6 laboratories did not participated in identifying SAV but correctly ruled out the other 2 listed pathogens (KHV and ISAV) in this ampoule

Ampoule VII – ISAV

- 42 laboratories correctly identified ISAV
- 1 laboratory identified KHV

Ampoule VIII – KHV

- 40 laboratories correctly identified KHV
- 1 laboratory correctly identified KHV but contaminated the ampoule with ISAV
- 1 laboratory identified ISAV
- 1 laboratory did not participate for KHV but correctly ruled out the presence of the SAV and ISAV from this ampoule

Ampoule IX – Blank

- 42 laboratories ruled out the presence of pathogens they were testing for
- 1 laboratory identified KHV

Scores

We have assigned a score of 2 for each correct answer (Table 12), 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 43 laboratories submitting results 40 laboratories obtained maximum score. The maximum score was calculated according to the number of pathogen tested by the laboratory.
One laboratory could obtain a maximum score of 8 if tested for all three pathogens included (ISAV;KHV and SAV) or the two listed pathogens (ISAV and KHV) .
If one laboratory did not test for KHV or ISAV the maximum score was 6 points.
Genotyping of ISAV HPR region and submission of sequencing results was this year not a mandatory part of the test and is therefore not included in the score of participants, but it will be in future

Methods applied

The following methods were used by the participants:

KHV detection

- 24 laboratories used Real Time PCR protocols for KHV detection
- 20 laboratories used KHV Real-time PCR referring to the protocol from Gilad et al 2004.
- 3 laboratories used KHV Real-time PCR referring to the protocol from Engelsma et al from CVI
- 1 laboratory used KHV real-time PCR referring to protocol from Rakus et al.,2012
- 31 laboratories used KHV PCR,
- 21 laboratories performed PCR according to protocol from Bercovier et al.,2005
- 2 laboratories performed PCR according to protocol from Engelsma et al.,2014
- 1 laboratory performed PCR according to protocol from Yuasa et al., 2005
- 1 laboratory performed PCR according to protocol from Bigarré et al., 2009
- 1 laboratory performed PCR according to protocol from Garver et al., 2010
- 1 laboratory performed PCR according to protocol from Gray et al., 2002
- 4 laboratories performed PCR according to unpublished protocols

ISAV detection

- 22 laboratories used ISAV real-time RT-PCR referring to the protocol from Snow et al., 2006.
- 1 laboratory used ISAV real-time RT-PCR referring to the protocol from Christiansen et al.,2011
- 1 laboratory used ISAV real-time RT-PCR referring to the protocol from LeBlanc et al.,2012
- 19 laboratories used conventional RT-PCR from Mjaaland et al.,2002
- 3 laboratories used conventional RT-PCR from Kibenge et al.,2009
- 1 laboratory used conventional RT-PCR from McBeath et al.,2009
- 1laboratory used conventional RT-PCR from Cunningam et al.,2002
- 1laboratory used conventional RT-PCR from Christiansen et al.,2011
- 2 laboratories used conventional RT-PCR referring to unpublished protocols

SAV detection

- 20 laboratories used SAV real-time RT-PCR

- 17 laboratories referred the protocol from Hodneland et al. 2006.
- 1 laboratory referred to the protocol from Fringuelli et al.,2008
- 2 laboratories did not provide reference to the protocol used
- 27 laboratories used SAV RT-PCR
- 20 laboratories used the protocol from Fringuelli et al. 2008.
- 1 laboratory referred to the protocol from Hodneland et al.,2006
- 1 laboratory referred to the protocol from Hjortaas et al.,2013
- 1 laboratory referred to the protocol from Villoing et al.,2000
- 1 laboratory referred to the protocol from Cano et al.,2014

Genotypning and sequencing

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

- 24 laboratories performed sequencing for KHV
- 27 laboratories performed sequencing for SAV
- 29 laboratories performed sequencing for ISAV

An Overview of the genotyping results obtained for PT2 by all participants is displayed in the following table 13

Laboratory code number	Score	Ampoule VI	Ampoule VII	Ampoule VIII
		SAV 2, Sleeping disease virus (SD)	ISAV ISAV Glesvaer/2/90	KHV- CyHV-3 – isolate KHV-TP 30
1	8/8	SAV Subtype II	ISAV HPR2	KHV
2	8/8	France, isolate S49P	Faroe Islands, isolate F72b/02	Indonesia, isolate PP3_070411
3	8/8	0	0	0
4[2]		0	0	0
5	6/8	0	0	0
6	8/8	II	HPR2	3
7[1]	8/8	0	0	0
8	8/8	SAV 2	0	0
9	8/8	0	0	0
10[1]	8/8	0	0	0
11	8/8	Subtype II	EU-H1	0
12	8/8	0	HPR2	0
13	8/8	0	Genotype HPR deleted	0
14	8/8	SAV 2 FW (SD) (according to OIE)	2	0
15	8/8	0	0	0
16	8/8	0	0	0
17[3]	6/6	0	0	0
18[2]		0	0	0
19	8/8	SAV2	HPR deleted	0
20	8/8	type 2	0	CyHV-3
21	8/8	subtype II	PR4	0
22[1]	8/8	0	0	0
23	8/8	0	0	0
24	4/8	N/a	N/a	HPR Genotype 2
25	8/8	Subtype 2	G2 (HPR/deleted)	-
26	8/8	SAV2	ISAV HPR-deleted	CyHV3 (KHV)
27	8/8	0	0	0
28	8/8	SAV2	HPR Deleted	E
29	8/8	0	0	0
30	8/8	0	EU-G2	0
31[1]	8/8	0	0	0
32	8/8	0	0	0
33	8/8	0	0	0
34	6/8	0	0	0
35	8/8	-	HPR-4	-
36	8/8	-	HPR deleted(HPR4)	-
37	8/8	SAV 2 FW (SD)	ISAV (HPR2)	CyHV 3
38	8/8	Genogroup 2	HPR deleted variant	0
39	8/8	0	0	0
40	8/8	0	0	0
41	8/8	0	0	0
42	8/8	Subtype 2	HPR2	Wild type KHV
43	8/8	TYPE II	HPR2	CyHv-3
44	8/8	0	North American	0
45	8/8	0	European; HPR-deleted	0

AMPOULE VI SAV:
37 laboratories participated in testing ampoules for SAV, which was included in PT2 2016 on a volunteer basis.
Of these, 26 laboratories sequenced the SAV isolate included in Ampoule VI; 22 participants genotyped the isolated at SAV Genotype 2 – SDV; 4 did not provide a genotype.

- 21 laboratories sequenced the E2 and nsP3 gene using primer sets described in Fringuelli et al.,2008
- 3 laboratories sequenced the E2 region according to Hodneland et al., 2006
- 1 laboratory targeted the RNA polymerase without specifying the protocol
- 1 laboratory used primer sets provided in Villoing et al., 2000
- 2 laboratories provided primer sets without reporting the protocol

One laboratory used more than one protocol for sequencing SAV.

AMPOULE VII ISAV:
25 laboratories sequenced the ISAV isolate included in Ampoule VII
8 laboratories described this isolate as HPR deleted, 7 laboratories as HPR 2 /genotype 2, 2 laboratories as HPR4, 1 laboratory as genotype “north American”, 1 laboratory as EU genotype and 1 laboratory as Faroese Islands. 6 laboratories did not provide a genotype.

- 9 laboratories targeted the HPR region using primer sets described in Mjaaland et al.,2002
- 1 laboratory targeted the HPR region using primer set described in Cunningham et al.,2002
- 3 laboratories sequenced the partial HA gene according to Kibenge et al.,2009
- 1 laboratory sequenced the HA region according to McBeath et al.,2009
- 3 laboratories sequenced the region using primer set by Christiansen et al. 2011
- 2 laboratories referred to in house protocol developed by Warg in USA
- 1 laboratory referred to in house protocol developed by Gagne
- 6 laboratories provided primer sequences without referring to a protocol

1 laboratory used 2 protocols for sequencing ISAV.

AMPOULE VIII KHV:
22 laboratories sequenced the KHV isolate included in Ampoule VIII
4 laboratories genotyped the isolate as Genotype 3- KHV, 1 laboratory described the isolate as Wild Type KHV, 1 laboratory as E genogroup, 1 as Indonesian isolate.

- 9 laboratories sequenced the Thymidine kinase region using primer sets described in Bercovier et al.2005
- 2 laboratories used primer sets described in Engelsma et al.,2013
- 1 laboratory sequenced the sphl gene using primer sets described in Gray et al. 2002
- 1 laboratory sequenced the polymerase gene using primersets from a protocol given by Stone and Way from CEFAS 2010
- 10 laboratories used primer sets without describing the protocol

Concluding remarks PT2

After the positive experience in 2015, the EURL decided to include SAV in the panel of viruses included in PT2. Considering that 33 laboratories participated in 2015 (of which 32 correctly identified SAV in ampoule VII) this was regarded as a proper initiative that strengthen the diagnostic capacities of the NRLs in detecting emerging pathogens, and it will be included in the coming years as well.
37 laboratories participated in PT2 testing for SAV and all of the 37 correctly identified the virus in Ampoule VI.
42 out of 43 laboratories correctly identified the ISA virus in ampoule VII.
Out of 43 participants, 2 did not test for KHV and 1 did not identify the virus in Ampoule VIII, the other 40 correctly detected KHV in ampoule VIII.
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, especially after the delisting of ISAV HPR0 (Commission Implementing Directive 2014/22/EU).

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 21th Annual Workshop of National Reference Laboratories for Fish Diseases to be held 30th-31st of May 2017 in Copenhagen, Denmark.

Teena Vendel Klinge, Niccolò Vendramin and Niels Jørgen Olesen

European Union Reference laboratory for Fish diseases
National Veterinary Institute, Technical University of Denmark, February 2017

References

1. Abbadi M, Fusaro A, Ceolin C, Casarotto C, Quartesan R, Dalla Pozza M, Cattoli G, Toffan A, Holmes EC and Panzarin V (2016) Molecular Evolution and Phylogeography of Co-circulating IHNV and VHSV in Italy. Front. Microbiol. 7:1306. doi: 10.3389/fmicb.2016.01306

2. Bain N, Gregory A, Raynard RS (2008) Genetic analysis of infectious pancreatic necrosis virus from Scotland. J Fish Dis 31: 37–47
3. *Baudin Laurencin F* (1987) IHN in France. *Bulletin of the European Association of Fish Pathologists* 7, 104.

4. Bercovier H., Fishman Y., Nahari R., Sharon S., Zlotkin A., Eyngor M., Gilad O., Eldar A. and Hedrick R. P. 2005 Cloning of the koi herpesvirus (KHV) gene encoding thymidine kinase and its use for a highly sensitive PCR based diagnosis *BMC Microbiology* 2005, 5:13

5. Bigarré L, Baud M, Cabon J, Antychowicz J, et al. Differentiation between cyprinid herpesvirus type-3 lineages using duplex PCR. J Virol Methods. 2009;158:51–57.

6. Bigarre L, Cabon J, Baud M, Pozet F, Castric J (2008) Ranaviruses associated with high mortalities in catfish in France. Bull Eur Assoc Fish Pathol 28: 163-168

7. Blake SL. Schill WB, McAllister PE, Lee M, Singer JT, Nicholson BL (1995) Detection and identification of aquatic birnaviruses by PCR assay. J Clin Microbiol33:835-839

8. Bovo G., Comuzi M., DeMas S., Ceschia G., Giorgetti G., Giacometti P., Cappellozza E. (1993) Isolamento di un agente virale irido-like da pesce gatto (Ictalurus melas) d'àllevamento. Boll Soc Ital Patol Ittica 11:3-10

9. Campbell S., Collet B., Einer-Jensen K., Secombes C. J., M. Snow Identifying potential virulence determinants in viral haemorrhagic septicaemia virus (VHSV) for rainbow trout Diseases of Aquatic Organisms Vol. 86: 205–212, 2009 doi: 10.3354/dao02127

10. Cano I, Joiner C, Bayley A, Rimmer G, Bateman K, Feist SW et al. (2014). An experimental means of transmitting pancreas disease in Atlantic salmon Salmo salar L. fry in freshwater. J Fish Dis 38: 271–281.

11. Christiansen DH, Østergaard PS, Snow M, Dale OB, Falk K. A low-pathogenic variant of infectious salmon anemia virus (ISAV-HPR0) is highly prevalent and causes a non-clinical transient infection in farmed Atlantic salmon (Salmo salar L.) in the Faroe Islands. J Gen Virol. 2011;92:909–918. doi: 10.1099/vir.0.027094-0

12. COMMISSION IMPLEMENTING DECISION (EU) 2015/1554 of 11 September 2015 laying down rules for the application of Directive 2006/88/EC as regards requirements for surveillance and diagnostic methods

13. Commission Implementing Directive 2014/22/EU of 13 February 2014 amending Annex IV to Council Directive 2006/88/EC as regards infectious salmon anaemia (ISA) Text with EEA relevance

14. Council Directive 2006/88/EC of 24 October 2006 on animal health requirements for aquaculture animals and products thereof, and on the prevention and control of certain diseases in aquatic animals

15. Crane, M., Hardy-Smith, P., Williams, L.M., Hyatt, A.D., Eaton, L.M., Gould, A., Handlinger, J., Kattenbelt, J. and Gudkovs, N., 2000. First isolation of an aquatic birnavirus from farmed and wild fish species in Australia. Diseases of Aquatic Organism, 43, 1-14.

16. Cunningham CO, Gregory A, Black J, Simpson I, Raynard RS (2002) A novel variant of the infectious salmon anaemia virus (ISAV) haemagglutinin gene suggests mechanisms for virus diversity. Bull Eur Assoc Fish Pathol 22(6):366–374

17. Davies, K.R., Mccoll, K.A., Wang, L.F., Yu, M., Williams, L.M. and Crane, M.S.J., 2010. Molecular characterisation of Australasian isolates of aquatic birnaviruses. Diseases of Aquatic Organisms, 93, 1- 15.

18. 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.

19. Emmenegger E. J, Meyers T. R., Burton T. O., Kurath G. Genetic diversity and epidemiology of infectious hematopoietic necrosis virus in Alaska. Dis Aquat Organ. 2000 Apr 20;40(3):163-76

20. Engelsma M. Y., Way K., Dodge M. J., Voorbergen-Laarman M., Panzarin Valentina, Abbadi Miriam, El-Matbouli M., Stone D. M., Skall H. F.,Kahns S., Detection of novel strains of cyprinid herpesvirus closely related to koi herpesvirus Diseases of Aquatic Organisms — 2013, Volume 107, Issue 2, pp. 113-120

21. Enzmann, P. J., Kurath, G., Fichtner, D., and Bergmann, S. M. (2005). Infectious hematopoietic necrosis virus: monophyletic origin of European isolates from North American genogroup M. Dis. Aquat. Organ. 66, 187–195. doi: 10.3354/ dao066187

22. Fringuelli, E., Rowley, H. M., Wilson, J. C., Hunter, R., Rodger, H. and Graham, D. A. (2008), Phylogenetic analyses and molecular epidemiology of European salmonid alphaviruses (SAV) based on partial E2 and nsP3 gene nucleotide sequences. Journal of Fish Diseases, 31: 811–823. doi: 10.1111/j.1365-2761.2008.00944.x

23. Garver KA, Al-Hussinee L, Hawley LM, Schroeder T, et al. Mass mortality associated with koi herpesvirus in wild common carp in Canada. J Wildl Dis. 2010;46:1242–1251.

24. Gilad O, Yun S, Andree KB, Adkison MA, Way K, Willits NH, Bercovier H, Hedrick RP: Molecular comparison of isolates of an emerging fish pathogen, the koi herpesvirus, and the effect of water temperature on mortality of experimentally infected koi. *J Gen Virol* 2003, 84:1-8

25. Gray, W. L., Mullis, L., LaPatra, S. E., Groff, J. M. and Goodwin, A. (2002), Detection of koi herpesvirus DNA in tissues of infected fish. Journal of Fish Diseases, 25: 171–178. doi: 10.1046/j.1365-2761.2002.00355.x

26. Hanson LA, Rudis MR, Vasquez-Lee M, Montgomery RD. A broadly applicable method to characterize large DNA viruses and adenoviruses based on the DNA polymerase gene. Virol J. 2006;3:28.

27. *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.

28. Hedrick RP, Batts WN, Yun S, Traxler GS, Kaufman J, Winton JR (2003) Host and geographic range extensions of the North American strain of viral hemorrhagic septicemia virus. Dis Aquat Org 55:211–220

29. Heppell J, Berthiaume L, Tarrab E, Lecomte J, Arella M (1992) Evidence of genomic variations between infectious pancreatic necrosis virus strains determined by restriction fragment profiles. J Gen Virol 73:2863–2870

30. Hjortaas MJ, Skjelstad HR, Taksdal T, Olsen AB and others (2013) The first detections of subtype 2-related salmonid alphavirus (SAV2) in Atlantic salmon, Salmo salar L., in Norway. J Fish Dis 36: 71−74

31. Hodneland K. & Endresen C. (2006). Sensitive and specific detection of salmonid alphavirus using real-time PCR (TaqMan). J. Virol. Methods, 131, 184–192.

32. Holopainen R., Ohlemeyer S., Schütze H., Bergmann S.M. & Tapiovaara H. (2009). Ranavirus phylogeny and differentiation based on major capsid protein, DNA polymerase and neurofilament triplet H1-like protein genes. *Dis. Aquat. Org.,* 85, 81–91.

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

34. Jonstrup S P, Kahns S, Skall H F, Boutrup T S and Olesen N J * 2012Development and validation of a novel Taqman-based real-time RT-PCR assay suitable for demonstrating freedom from viral haemorrhagic septicaemia virus Volume

36, Issue 1, pages 9–23, January 2013

35. Jørgensen, P. E. V., Olesen, N. J., Ahne, W., Lorenzen, N. (1989) SVCV and PFR viruses: Serological examination of 22 strains indicates close relationship between the two rhabdoviruses. In Ahne, W., and Kurstak, E. (eds.): Viruses of lower vertebrates. 349- 366. Springer Verlag, Berlin, Heidelberg

36. Kerr, C.R.C. and Cunningham, C.O. (2006), Moving molecular diagnostics from laboratory to clinical application: a case study using infectious pancreatic necrosis virus serotype A. Letters in Applied Microbiology, 43: 98–104. doi:10.1111/j.1472-765X.2006.01885.x

37. Kibenge, F. S., Godoy, M. G., Wang, Y., Kibenge, M. J., Gherardelli, V., Mansilla, S., Lisperger, A., Jarpa, M., Larroquete, G. & other authors (2009). Infectious salmon anaemia virus (ISAV) isolated from the ISA disease outbreaks in Chile diverged from ISAV isolates from Norway around 1996 and was disseminated around 2005, based on surface glycoprotein gene sequences. Virol J 6, 88.

38. Kolodziejek J, Schachner O, Dürrwald R, Latif M, Nowotny N (2008) “Mid-G” region sequences of the glycoprotein gene of Austrian infectious hematopoietic necrosis virus isolates form two lineages within European isolates and are distinct from American and Asian lineages. J Clin Microbiol 46:22–30

39. Koutna M., Vesely T., Psikal I., Hulova J.: Identification of spring viraemia of carp virus (SVCV) by combined RT-PCR and nested PCR. Dis. Of Aquatic Org. 55, 229-235 (2003).

40. Kurath G, Garver KA, Troyer RM, Emmenegger EJ, Einer Jensen K, Anderson ED (2003) Phylogeography of infectious haematopoietic necrosis virus in North America. J Gen Virol 84: 803−814

41. Kurath G., Garver K. A., Troyer R. M., Emmenegger E. J., Einer-Jensen K. and Anderson E. D. (2003), Phylogeography of infectious haematopoietic necrosis virus in North America Journal of General Virology (2003), 84, 803–814

42. LeBlanc F., Arseneau J.R., Leadbeater S., Glebe B., Laflamme M., Gagné N.Transcriptional response of Atlantic salmon (Salmo salar) after primary versus secondary exposure to infectious salmon anemia virus (ISAV)” Mol. Immunol. 51 (2) (June 2012) 197–209

43. 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.

44. McBeath AJA, Bain N, Snow M, 2009. Surveillance for infectious salmon anaemia virus HPR0 in marine Atlantic salmon farms across Scotland. Diseases of Aquatic Organisms, 87, 161–169.

45. Miller TA, Rapp J, Wastlhuber U, Hoffmann RW, Enzmann PJ (1998) Rapid and sensitive reverse transcriptase-polymerase chain reaction based detection and differential diagnosis of fish pathogenic rhabdoviruses in organ samples and cultured cells. Dis Aquat Org 34:13–20

46. Mjaaland S., Hungnes O.,Teig A., Dannevig, B. H., Thorud K., Rimstad E. Polymorphism in the Infectious Salmon Anemia Virus Hemagglutinin Gene: Importance and Possible Implications for Evolution and Ecology of Infectious Salmon Anemia Disease Virology Volume 304, Issue 2, 20 December 2002, Pages 379–391

47. Mjaaland S., Rimstad E., Falk K, and Dannevig B. H. 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, Oct. 1997, p. 7681–768

48. Ohlemeyer, S., Holopainen, R., Tapiovaara, H., Bergmann, S.M., & Schütze, H. 2011. Major capsid protein gene sequence analysis of the Santee-Cooper ranaviruses DFV, GV6, and LMBV. *Dis Aquat Org.* 96:195-207.

49. OIE Manual of Diagnostic Tests for Aquatic Animals 2015

50. 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.

51. 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.

52. Purcell M. K., Thompson R. L., Garver K. A., Hawley L. M., Batts W. N., Sprague L., Sampson C., Winton J. R., Universal reverse-transcriptase real-time PCR for infectious hematopoietic necrosis virus (IHNV) Dis Aquat Org Vol. 106: 103–115, 2013 doi: 10.3354/dao02644

53. Raja-Halli, M., Vehmas, T.K., Rimaila-Pärnänen, E., Sainmaa, S., Skall, H.F., Olesen, N.J., Tapiovaara, H., 2006. Viral haemorrhagic septicaemia (VHS) outbreaks in Finnish rainbow trout farms. Dis. Aqua. Organ. 72, 201-211

54. Rakus KŁ, Irnazarow I, Adamek M, Palmeira L, et al. Gene expression analysis of common carp (**Cyprinus carpio** L.) lines during Cyprinid herpesvirus 3 infection yields insights into differential immune responses. Dev Comp Immunol. 2012;37:65–76.

55. Ruane N. M., Rodger H. D., McCarthy L. J., Swords D., Dodge M., Kerr R. C., Henshilwood K., Stone D. M., Genetic diversity and associated pathology of rhabdovirus infections in farmed and wild perch Perca fluviatilis in Ireland 2014 DISEASES OF AQUATIC ORGANISMS Dis Aquat Org Vol. 112: 121–130, 2014 doi: 10.3354/dao02801

56. Santi N, Vakharia VN, Evensen Ø (2004) Identification of putative motifs involved in the virulence of infectious pancreatic necrosis virus. Virology 322: 31−40

57. Snow M, McKay P, McBeath AJ, Black J and others (2006) Development, application and validation of a Taqman real-time RT-PCR assay for the detection of infectious salmon anaemia virus (ISAV) in Atlantic salmon (Salmo salar). Dev Biol 126:133–145

58. Snow M., Bain N., Black J., Taupin V., Cunningham C. O., King J. a., Skall H. F., Raynard R. S. Genetic population structure of marine viral haemorrhagic septicaemia virus (VHSV)‡ DAO 61:11-21 (2004)doi:10.3354/dao061011

59. Stone D.M., Ahne W., Denham K.L., Dixon P.F., Liu C-T.Y., Sheppard A.M., Taylor G.R. & 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.

60. Taksdal, T., Dannevig, B.H. & Rimstad, E. 2001. Detection of infectious pancreatic necrosis (IPN)-virus in experimentally infected Atlantic salmon parr by RT-PCR and cell culture isolation. Bulletin of the European Association of Fish Pathologists 21, 214-219.

61. Talbi C[1], Cabon J, Baud M, Bourjaily M, de Boisséson C, Castric J, Bigarré L. Genetic diversity of perch rhabdoviruses isolates based on the nucleoprotein and glycoprotein genes. Arch Virol. 2011 Dec;156(12):2133-44. doi: 10.1007/s00705-011-1103-z. Epub 2011 Sep 17.

62. Tapia D., Eissler Y., Torres P., Jorquera E., Espinoza J. C., Kuznar J. (2015) Detection and phylogenetic analysis of infectious pancreatic necrosis virus in Chile DISEASES OF AQUATIC ORGANISMS Vol. 116: 173–184, 2015 doi: 10.3354/dao02912

63. Villoing S, Castric J, Jeffroy J, Le Ven A, Thiery R, Bremont M (2000b) An RT-PCR-based method for the diagnosis of the sleeping disease virus in experimentally and naturally infected salmonids. Dis Aquat Org 40: 19−27

64. Williams K., Blake S., Sweeney A., Singer J., Nicholson B.L., 1999. Multiples reverse transcriptase PCR assay for simultaneous detection of three fish viruses. J. Clin. Microbiol. 37, 4139–4141.

65. Yuasa K, Sano M, Kurita J, Ito T, Iida T (2005) Improvement of a PCR method with the Sph 1-5 primer set for the detection of koi herpesvirus (KHV). Fish Pathol 40: 37−39