European Union Reference Laboratory for Fish and Crustacean Diseases
NATIONAL INSTITUTE OF AQUATIC RESOURCES, TECHNICAL UNIVERSITY OF DENMARK

EURL for Fish Diseases

Report of the Inter-Laboratory Proficiency Test 2021

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
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Introduction

A comparative test of diagnostic procedures was provided by the European Union Reference Laboratory (EURL) for Fish and Crustacean Diseases. The test was divided into proficiency test 1 (PT1) and proficiency test 2 (PT2).
PT1 was designed to assess the ability of participating laboratories in quantifying and identifying the fish viruses causing 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 other fish pathogenic viruses as pike fry rhabdovirus (PFR), spring viraemia of carp virus (SVCV) and infectious pancreatic necrosis virus (IPNV). The laboratory procedures for isolating and titrating these pathogens is primarily based on cell culture methods, however the use of molecular methods (Real Time PCR based) has been implemented for their detection and identification.
PT2 was designed to assess the ability of participating laboratories to identify the fish viruses: infectious salmon anaemia virus (ISAV), salmonid alphavirus (SAV) and cyprinid herpesvirus 3 (CyHV-3) (otherwise known as koi herpes virus – KHV) by bio molecular methods (PCR based).
Out of the 45 laboratories participating in PT1, 43 performed analysis to identify all viruses included, while out of the 44 laboratories participating in PT2, 40 attempted to identify all fish viral pathogens included.
The tests were sent from the EURL 2th of October 2021.
Both PT1 and PT2 are accredited by DANAK under registration number 515 for provision of proficiency testing according to the quality assurance standard DS/EN ISO/IEC 17043.
The EURL relies on the subcontractor Eurofins Genomics for sequencing the amplicons of viral isolates included in the PTs, DTU – National Food Institute for lyophilisation of the ampoules and the Danish National Reference Laboratory for Fish diseases as provider of cell cultures.
This report covers both the results of PT1 and PT2.

PT1 consisted of five coded ampoules (I-V). These ampoules contained EHNV, IHNV/VHSV, ECV, SVCV, and IHNV, respectively (see table 1).

The proficiency test is designed to primarily assess the ability of participating laboratories to identify fish viral pathogens causing diseases listed in **Commission Implementing Regulation (EU) 2018/1882** [1].

PT1 include the Category A disease EHN for which it is necessary to distinguish by sequencing the causative agent, EHNV, from other ranavirus, and the Category C diseases VHS and IHN. Furthermore the inter-laboratory proficiency test is also suitable for maintaining accreditation for identification of SVCV, and IPNV. Finally, participants have to consider that the test ampoules also could contain other viruses (e.g. other fish rhabdoviruses, ranaviruses, or birnaviruses). The participants were also 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 diagnostic manuals for listed fish diseases available on the EURL website https://www.eurl-fish-crustacean.eu/fish/diagnostic-manuals and on the instruction to participants delivered along with the parcel [2] and by using fish cell cultures followed by e.g. ELISA, PCR or immunofluorescence (IFAT).

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 2.3.2 in the OIE Manual of Diagnostic Tests for Aquatic Animals [3]. Laboratories were encouraged to further

characterize VHSV and IHNV isolates by means of genotyping. It was recommended to use the genotyping procedure described in Einer-Jensen 2004 [4] for VHSV and ; for IHNV, we suggest to follow procedure provided in the latest IHNV chapter of the OIE manual on Aquatic Animal Diseases (primer references are given in Emmenegger et al. (2000) [5], and PCR conditions are given in Garver et al. (2003) [6]. Laboratories were encouraged to submit all sequencing results used for genotyping the isolates.

PT2 consisted of four coded ampoules (VI-IX). These ampoules contained SAV, KHV, ISAV and BF-2 cell supernatant, respectively (see table 11). The test was designed to primarily assess the ability of participating laboratories to identify infection with HPR-deleted ISAV listed as category C disease, , and Koi herpes virus disease listed as category E diseases (Commission Implementing Regulation (EU) 2018/1882**[1]**) 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 40 of 44 laboratories tested for SAV in 2021.
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 Chapter 2.3.8. of the OIE Manual of Diagnostic Tests for Aquatic Animals [7]. It was not mandatory to grow KHV and ISAV on cell cultures in this test, but the viruses were not inactivated and, thus in theory, it should be possible to propagate them in cell cultures.

The EURL has acknowledged the big effort that many participants are putting in sequencing and genotyping the isolates of the PT panel, for this reason, the genotyping results provided by all participants is displayed in Table 10 and 15.

Finally, in the attempt to harmonize the molecular diagnostic methods the EURL has compiled and presented the Ct values reported by the different laboratories (table 9 for PT1; table 14 and figure 13 for PT2).

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 has 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 (https://www.eurl-fish-crustacean.eu/) to be used for reporting results and to be submitted to the EURL electronically. Participants were asked to reply latest December 10th 2021, however due to exceptionally long delivery time to some countries the answering date was extended to December 22th . The results of the inter-laboratory proficiency test for listed fish diseases 2021 and plans and idea for future inter-laboratory tests will be presented at the 26th Annual Workshop of the NRLs for Fish Diseases on May 30th and 31st 2022. Furthermore a specific online meeting on April 8th will be organized to discuss the report and receive comments, inputs and feedback from the participating laboratory.

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 test parcels were delivered by courier, with the exception of participant whom themselves arranged the pick-up of the test. When possible participants were provided with a tracking number so they were able to follow the shipment.

Shipment and handling
The parcels were delivered to 36 participants within the first week; 93% were delivered within the first two 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 proficiency test reports 2007,2010,2011.

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

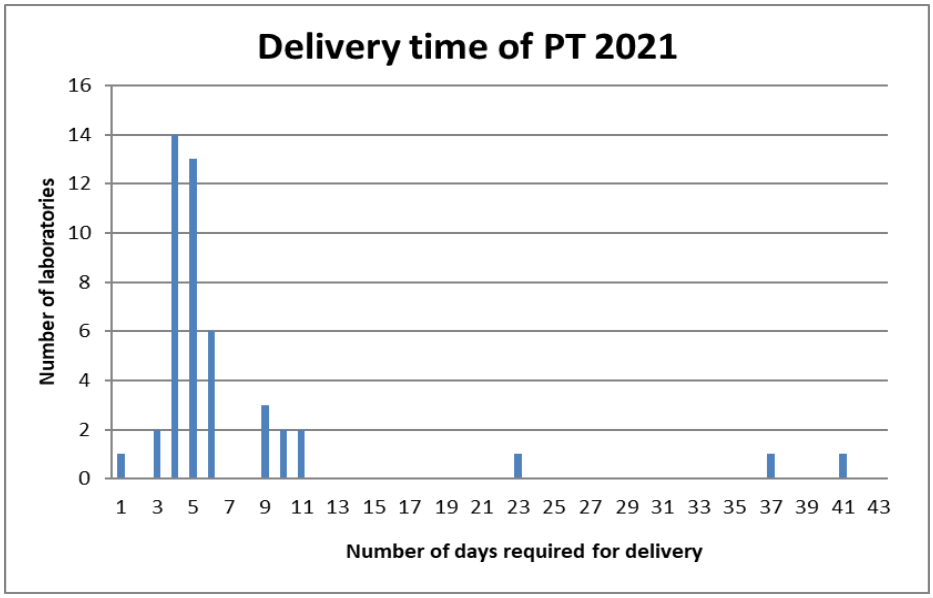


Figure 1. Transport time for the parcels to reach the participants.

Participation

PT1 and PT2: 46 laboratories received the annual proficiency test. 45 participants submitted the full spreadsheet within the deadline; 1 participant did receive the parcel but did not submit the spreadsheet. Figure 2 show the numbers of participants in the proficiency test from 2007 to 2021.

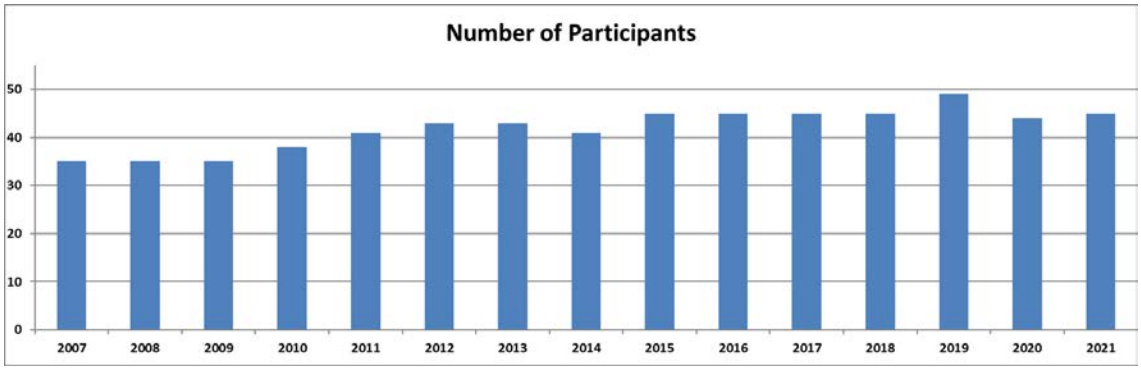


Figure 2. Participants in the EURL proficiency test over the years.

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, Japan, New Zealand, Northern Ireland, Norway, Republic of North Macedonia, Russia, Serbia, Switzerland, Tunisia, Ukraine, the United Kingdom (Scotland, England and Wales) and to two laboratories in South Korea and USA, respectively.
The Belgian NRL covers both Belgium and Luxembourg and the Italian NRL covers Italy and Cyprus for identification of all listed diseases. Figure 3 shows the worldwide distribution of the participating NRLs. This year three participating laboratories which are normally enrolled in this exercise, were not able to participate, possibly in relation to consequences on accessibility to laboratories due to COVID-19 pandemics.

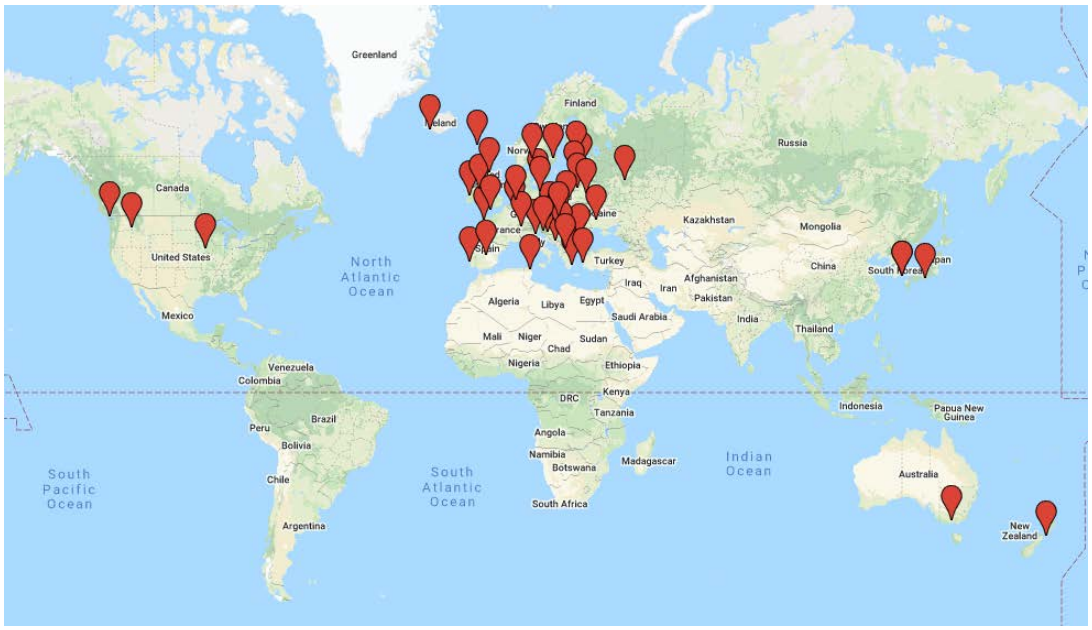


Figure 3. Worldwide distribution of the participants in the EURL proficiency test 2021

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: EHNV	**EHNV Isolate 86/8774 (V017)** Australian freshwater isolate of epizootic haematopoietic necrosis virus from rainbow trout from Adaminaby Trout Farm, NSW obtained in 1986 by Jeremy Langdon. **Received from:** Prof. Whittington, The OIE reference laboratory for EHN, University of Sidney, Australia. **GenBank accession numbers:** FJ433873, AY187045 **Reference on isolate:** Langdon JS, Humphrey JD & Williams LM (1988). Outbreaks of an EHNV-like iridovirus in cultured rainb006Fw trout, *Salmo gairdneri* Richardson, in Australia. *Journal of Fish Diseases* 11, 93-96. **References on sequences:** Hyatt AD, Gould AR, Zupanovic Z, Cunningham AA, Hengstberger S, Whittington RJ, Kattenbelt J & Coupar BEH (2000). Comparative studies of piscine and amphibian iridoviruses. *Archives of Virology* 145, 301-331. Jancovich JK, Bremont M, Touchman JW & Jacobs BL (2010). Evidence for multiple recent host species shifts among the ranaviruses (family Iridoviridae). *Journal of Virology* 84, 2636-2647. Marsh IB, Whittington RJ, O'Rourke B, Hyatt AD & Chisholm O (2002) Rapid differentiation of Australian, European and American ranaviruses based on variation in major capsid protein gene sequence. *Molecular and Cellular Probes* 16, 137-151. Hick,P.M., Subramaniam,K., Thompson,P.M., Waltzek,T.B., Becker,J.A. and Whittington,R.J. Molecular epidemiology of Epizootic haematopoietic necrosis virus (EHNV) JOURNAL Virology 511, 320-329 (2017).

Code	Specifications/References
Ampoule II: **IHNV** **+** **VHSV**	**IHNV Isolate 32/87** First French isolate (April 1987) from rainbow trout. **Genotype:** Geno group E **GenBank accession number:** AY524121 (G-gene), FJ265711 (N-gene). **Reference on isolate:** Baudin Laurencin F (1987) IHN in France. Bulletin of the European Association of Fish Pathologists 7, 104. **Reference on sequence:** 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. Journal of Clinical Microbiology 46, 22-30. Johansson T., Einer-Jensen K., Batts W., Ahrens P., Björkblom C., Kurath G., Björklund H. & Lorenzen N. (2009) Genetic and serological typing of European infectious haematopoietic necrosis virus (IHNV) isolates. Diseases of Aquatic Organisms 86, 213-221. **+** **VHSV 1p8** Marine isolate (1996) from herring (*Clupea harengus*) caught in the Baltic Sea. (Mortensen et al. 1999). **Genotype**: Ib. **GenBank accession numbers:** AY546573 (G-gene) and GQ325430, AY356652 (N-gene) www.fishpathogens.eu ID number: FP.VHSV.52 **Reference on isolate:** Mortensen HF, Heuer OE, Lorenzen N, Otte L and Olesen NJ (1999). Isolation of viralhaemorrhagic septicaemia virus (VHSV) from wild marine fish species in the Baltic Sea,Kattegat, Skagerrak and the North Sea. *Virus Research* 63, 97-108. **References on sequences:** Campbell S., Collet B., Einer-Jensen K., Secombes C.J. & Snow M. (2009) Identifying potential virulence determinants in viral haemorrhagic septicaemia virus (VHSV) for rainbow trout. Diseases of Aquatic Organisms 86, 205-212. Einer-Jensen K, Ahrens P, Forsberg R and Lorenzen N (2004). Evolution of the fish rhabdovirus viral haemorrhagic septicaemia virus. *Journal of General Virology* 85, 1167-1179. Snow M, Bain N, Black J, Taupin V, Cunningham CO, King JA, Skall HF and Raynard RS (2004). Genetic population structure of marine viral haemorrhagic septicaemia virus (VHSV). *Diseases of Aquatic Organisms* 61, 11-21.

Code	Specifications/References
Ampoule III: ECV	**European catfish virus 562/92.** Italian isolate from catfish suffering high mortality. **Received from** Dr. G. Bovo, ISZ-Ve, Padova, Italy. **GenBank accession number**: FJ358608 or KT989884.1 or KT989885.1 or JQ724856.1 **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 IV: SVCV	**SVCV strain 56/70** Spring viraemia of carp virus isolate from carp. **Received from:** Prof. Fijan (January 1979 in a tube named Rhabdo virus carpio 56/70 and given as the reference strain of SVC virus). **Genotype:** Id **GenBank accession numbers:** Z37505.1 (Fijan), AJ538061.1 (S30) 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. **Reference on sequence (S30) and genotype:** Stone DM, Ahne W, Denham KL, Dixon PF, Liu C-TY, Sheppard AM, Taylor GR & Way K (2003). Nucleotide sequence analysis of the glycoprotein gene of putative spring viraemia of carp virus and pike fry rhabdovirus isolates reveals four genogroups. *Diseases of Aquatic Organisms* 53, 203-210.
Ampoule V: IHNV	**IHNV 217/A** First Italian IHNV isolate from rainbow trout. Isolated in 1987. **Received from:** Received from Dr. G. Bovo, ISZ-Ve, Padova, Italy. **Genotype:** E **GenBank accession numbers:** FJ265716.1

Code	Specifications/References
	Reference on isolate: Bovo G, Giorgetti G, Jørgensen PEV and Olesen (1987). Infectious haematopoietic necrosis: first detection in Italy. *Bulletin of the European Association of Fish Pathologists* 7, 124. **References on sequence and genotype:** Johansson T, Einer-Jensen K, Batts W, Ahrens P, Björkblom C, Kurath G, Björklund H & Lorenzen N (2009). Genetic and serological typing of European infectious haematopoietic necrosis virus (IHNV) isolates. *Diseases of Aquatic Organisms* 86, 213-221.

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 in the viral titre especially for VHSV. Previous experience reported during the past Proficiency tests demonstrated a rather high stability for SVCV, EHNV and IPNV serotype Sp. Lyophilised viral supernatant mixed in freeze drying medium preserved in glass sealed ampoules is stable for more than half a year when kept at room temperature (Inter-Laboratory Proficiency Test report 2007); it can survive exposure to 30°C for 24 hours (Inter-Laboratory Proficiency Test report 2010) And a temperature raise from 20 to 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, PCR and/or qPCR and 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 reductions of the titres after lyophilisation were observed. For all of the ampoules, the reduction of the titre was between 1-2 log in the same cell line. No significant reductions (within 1 log from after freeze drying) were observed after long term storage (Table 2 and figure 4)

Ampoul No.	Cell line	Titre before Lyophilisation	Titre after Lyophilisation and before shipment	Titre after deadline for handling in results (112 days of storage 4°C in the dark)
		$TCID_{50}$/ml	$TCID_{50}$/ml	$TCID_{50}$/ml
Ampoule I: EHNV 86/8774	BF-2	5,9E+05	1,3E+05	8,6E+04
	RTG-2	4,0E+04	4,0E+04	1,9E+04
	EPC	2,7E+05	2,7E+04	4,0E+04
	FHM	1,9E+02	< 1,9E+02	< 1,9E+02
Ampoule II: IHNV 32/87 + VHSV 1p8	BF-2	5,9E+04	4,0E+04	1,3E+04
	RTG-2	4,0E+03	2,7E+03	1,9E+02
	EPC	1,3E+05	1,3E+04	1,3E+04
	FHM	1,9E+05	2,7E+04	1,9E+04
Ampoule III: ECV 562/92	BF-2	4,0E+07	5,9E+06	4,0E+06
	RTG-2	4,0E+06	1,3E+06	5,9E+05
	EPC	2,7E+06	8,6E+05	5,9E+05
	FHM	1,3E+03	1,9E+03	1,3E+03
Ampoule IV: SVCV 56/70	BF-2	1,3E+06	5,9E+05	5,9E+05
	RTG-2	1,3E+05	4,0E+05	8,6E+04
	EPC	8,6E+05	2,7E+05	1,9E+05
	FHM	1,9E+05	1,9E+05	1,3E+05
Ampoule V: IHNV 217/A	BF-2	2,7E+02	< 1,9E+02	< 1,9E+02
	RTG-2	4,0E+03	2,7E+03	1,9E+02
	EPC	1,9E+05	4,0E+04	2,7E+04
	FHM	1,3E+04	1,3E+04	1,3E+04

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 approx. 3 months after shipment (1 ampoule), (stored at 4°C).

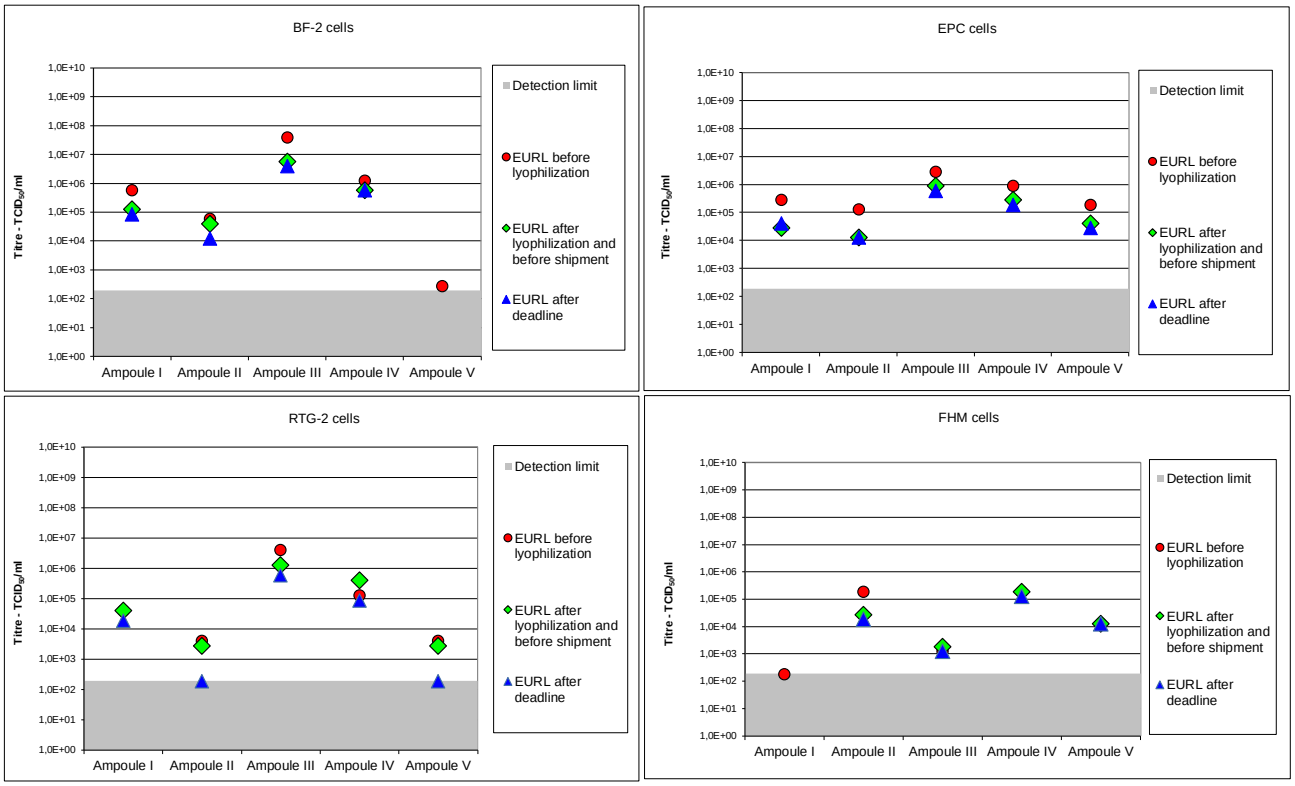


Figure 4. Virus titres in different cell lines: before lyophilisation, before shipment and after deadline for handling in results (storage 4°C in the dark).

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 EURL diagnostic manuals [2], i.e. by cell culture followed by ELISA, IFAT and/or RT-PCR/RT-qPCR. The results of the content in the 5 ampoules as reported by 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). The titration results obtained by 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, the optimal titre will be within these quartiles. 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 8 laboratories used these cells.
Laboratories were encouraged to identify the genotype of the virus isolates.

Table 3. Inter-Laboratory Proficiency Test, PT1, 2021 - Virus identification and score obtained by participants.

Laboratory code number	Score	Ampoule I	Ampoule II	Ampoule III	Ampoule IV	Ampoule V
		EHNV 86/8774	IHNV 32/87 + VHSV 1p8	ECV 562/92	SVCV 56/70	IHNV 217/A
1	10/10	EHNV	IHNV and VHSV	ECV	SVCV	IHNV
2	10/10	EHNV	VHSV + IHNV	Ranavirus - Not EHNV	SVCV	IHNV
3	8/10	EHNV	Nothing	Ranavirus	SVCV	IHNV
4	10/10	EHNV	VHSV / IHNV	Ranavirus - NOT EHNV	SVCV	IHNV
5	10/10	EHNV	VHSV and IHNV	ESV/ECV	SVCV	IHNV
6	10/10	Ranavirus was identified by conventional PCR and than REA was applied as given by OIE manuel to identified EHNV	VHSV, IHNV	Ranavirus was identified by conventional PCR and than REA was applied as given by OIE manuel to identified ECV	SVCV	IHNV
8	10/10	EHNV	VHSV and IHNV	ECV	SVCV	IHNV
9	10/10	EHNV	VHSV & IHNV	ECV/ESV	SVCV	IHNV
10	10/10	EHNV	VHSV, IHNV	Ranavirus-NOT EHNV	SVCV	IHNV
11	10/10	EHNV	VHSV + IHNV	Ranavirus NOT EHNV	SVCV	IHNV
12	7/10	EHNV	IHNV	Ranavirus	SVCV	-
13	10/10	EHNV	IHNV, VHSV	Ranavirus (European catfish virus)	SVCV	IHNV
14	9/10	EHNV	IHNV, VHSV	Ranavirus (not EHNV/other than EHNV)	SVCV	IHNV
15	10/10	EHNV	VHSV, IHNV	Ranavirus - not EHNV	SVCV	IHNV
16	9/10	EHNV	VHSV	Ranavirus – NOT EHNV	SVCV	IHNV
17	9/10	EHNV	IHNV, VHSV	Not VHSV, Not IHNV, Not EHNV, Not IPNV, Not SVCV	SVCV	IHNV
18	9/10	EHNV	VHSV	Ranavirus-NOT EHNV	SVCV	IHNV
19	10/10	EHNV	IHNV, VHSV	Ranavirus- Not EHNV	SVCV	IHNV
20	8/10	EHNV	IHNV + VHSV	Ranavirus – NOT EHNV	0	IHNV
21	10/10	EHNV	VHSV+IHNV	Ranavirus - not EHNV	SVCV	IHNV
22	10/10	EHNV	IHNV and VHSV	Ranavirus - NOT EHNV	SVCV	IHNV
23	6/10	European sheatfish virus	IHNV + VHSV	EHNV	SVCV	IHNV
24	10/10	EHNV	VHS and IHNV	Ranavirus - NOT EHNV	SVCV	IHNV

25	**9/10**	EHNV	IHNV and VHSV	No VHSV, IHNV, EHNV, SVCV, IPNV	SVCV	IHNV
26	**10/10**	Ranavirus	VHSV/IHNV	Ranavirus	SVCV	IHNV
27	**10/10**	EHNV	VHSV, IHNV	Ranavirus	SVCV	IHNV
28	**10/10**	EHN	VHS + IHN	ECV	SVCV	IHN
29	**10/10**	EHNV	VHSV+IHNV	ECV	SVCV	IHNV
30	**10/10**	EHNV	VHSV and IHNV	Ranavirus-NOT EHNV	SVCV	IHNV
31	**10/10**	EHNV	IHNV/VHSV	EHNV	SVCV	IHNV
32	**10/10**	EHNV	VHSV & IHNV	RANAVIRUS	SVCV	IHNV
33	**10/10**	EHNV	IHNV, VHSV	Ranavirus – NOT EHNV	SVCV	IHNV
34	**10/10**	EHNV	VHSV+IHNV	Ranavirus – NOT EHNV	SVCV	IHNV
35	**10/10**	EHNV	VHSV, IHNV	Ranavirus - NOT EHNV	SVCV	IHNV
36	**10/10**	EHNV	IHNV and VHSV	Ranavirus/European catfish virus	SVCV	IHNV
37	**6/6**	no VHSV; no IHNV, no IPNV, no SVCV	VHSV; IHNV	no VHSV; no IHNV, no IPNV, no SVCV	SVCV	IHNV
38	**10/10**	Ranavirus, EHNV	VHS and IHNV	Ranavirus, not EHNV	SVCV	IHNV
39	**10/10**	EHNV	VHSV, IHNV	Ranavirus - NOT EHNV	SVCV	IHNV
40	**10/10**	EHNV	VHSV and IHNV	ECV	SVCV	IHNV
41	**10/10**	EHNV	IHNV and VHSV	Ranavirus - NOT EHNV	SVCV	IHNV
42	**8/10**	Ranavirus	VHSV IHNV	Ranavirus	SVCV	IHNV
43	**4/6**	NO IHNV, VHSV,SVCV, IPNV	IHNV,VHSV	VHSV	SVCV	IHNV
44	**10/10**	EHNV	VHSV IHNV	Ranavirus NOT EHNV	SVCV	IHNV
45	**9/10**	EHNV	VHSV	ECV	SVCV	IHNV
46	**10/10**	EHNV	IHNV and VHSV	Ranavirus - NOT EHNV	SVCV	IHNV

1) Do not test for Ranavirus

2) Did not corroborate the findings in ampoule I and III by sequencing or REA . two laboratories used commercial qPCR kit, the kit is validated in Silico, but there is no documentation of diagnostic validation available, hence these results are considered not sufficient.

Table 4. Inter-Laboratory Proficiency Test, PT1, 2021 – **Results of titration of ampoule I.**

Laboratory Code number	Virus Identification	Titre in			
		BF-2	EPC	RTG-2	FHM
1	EHNV	2,7E+04	1,3E+04	1,3E+04	N/A
2	EHNV	5,9E+04	8,6E+04	N/A	2,7E+03
3	EHNV	N/A	2,7E+05	8,6E+05	N/A
4	EHNV	8,6E+04	5,9E+04	N/A	N/A
5	EHNV	1,9E+05	8,6E+04	N/A	N/A
6	Ranavirus was identified by conventional PCR and then REA was applied as given by OIE manual to identified EHNV	5,9E+04	5,9E+04	N/A	N/A
8	EHNV	4,0E+04	N/A	N/A	< 1,9E+02
9	EHNV	1,9E+05	8,6E+04	N/A	N/A
10	EHNV	8,6E+04	N/A	N/A	4,0E+02
11	EHNV	8,6E+04	4,0E+04	1,3E+05	1,9E+04
12	EHNV	4,0E+04	8,6E+04	N/A	N/A
13	EHNV	1,3E+04	5,9E+03	1,9E+03	1,9E+03
14	EHNV	8,6E+08	8,6E+08	N/A	N/A
15	EHNV	N/A	< 1,9E+02	N/A	N/A
16	EHNV	2,7E+05	1,9E+05	N/A	N/A
17	EHNV	2,7E+06	4,0E+07	1,9E+07	5,9E+06
18	EHNV	1,3E+05	< 1,9E+02	N/A	N/A
19	EHNV	N/A	N/A	N/A	N/A
20	EHNV	N/A	N/A	N/A	N/A
21	EHNV	1,3E+04	1,9E+04	1,3E+03	1,3E+03
22	EHNV	4,0E+04	8,6E+03	N/A	N/A
23	European sheatfish virus	1,9E+04	1,9E+04	N/A	N/A
24	EHNV	2,7E+05	2,7E+04	N/A	N/A
25	EHNV	N/A	1,3E+04	4,0E+02	N/A
26	Ranavirus	1,3E+05	5,9E+03	N/A	N/A
27	EHNV	1,9E+05	4,0E+04	4,0E+04	1,9E+04
28	EHN	4,0E+04	5,9E+04	N/A	N/A
29	EHNV	5,9E+04	5,9E+03	N/A	N/A
30	EHNV	8,6E+02	1,9E+02	N/A	N/A
31	EHNV	1,9E+05	2,7E+04	N/A	N/A
32	EHNV	8,6E+04	8,6E+03	N/A	N/A
33	EHNV	1,9E+04	1,3E+03	8,6E+02	N/A
34	EHNV	8,6E+04	2,7E+04	< 1,9E+02	N/A

35	EHNV	1,3E+04	4,0E+02	N/A	N/A
36	EHNV	N/A	N/A	N/A	N/A
37	no VHSV; no IHNV, no IPNV, no SVCV	4,0E+05	5,9E+03	N/A	N/A
38	Ranavirus, EHNV	8,6E+05	5,9E+04	4,0E+05	< 1,9E+02
39	EHNV	8,6E+04	8,6E+03	N/A	5,9E+03
40	EHNV	5,9E+04	1,3E+05	N/A	N/A
41	EHNV	2,7E+04	4,0E+03	< 1,9E+02	5,9E+02
42	Ranavirus	8,6E+04	1,9E+04	N/A	N/A
43	NO IHNV, VHSV,SVCV, IPNV	4,0E+04	8,6E+03	N/A	N/A
44	EHNV	5,9E+04	1,9E+03	4,0E+03	1,3E+04
45	EHNV	1,9E+05	1,3E+04	N/A	N/A
46	EHNV	2,7E+04	2,7E+04	5,9E+03	N/A

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

EHNV 86/8774	**BF-2**	**EPC**	**RTG-2**	**FHM**
Number of laboratories	39	40	14	12
Median titre	8,6E+04	2,7E+04	7,3E+03	1,9E+03
Maximum titre	8,6E+08	8,6E+08	1,9E+07	5,9E+06
Minimum titre	8,6E+02	<1,9E+02	<1,9E+02	<1,9E+02
25% quartile titre	4,0E+04	5,9E+03	7,5E+02	4,9E+02
75% quartile titre	1,9E+05	7,2E+04	1,9E+05	1,2E+04

Table 5. Inter-Laboratory Proficiency Test, PT1, 2021 – **Results of titration of ampoule II.**

Laboratory Code number	Virus Identification	Titre in			
		BF-2	EPC	RTG-2	FHM
1	IHNV and VHSV	1,9E+02	2,7E+02	< 1,9E+02	N/A
2	VHSV + IHNV	2,7E+02	2,7E+03	N/A	1,9E+03
3	Nothing		< 1,9E+02	< 1,9E+02	N/A
4	VHSV / IHNV	1,9E+03	8,6E+03	N/A	N/A
5	VHSV and IHNV	5,9E+03	8,6E+03	N/A	N/A
6	VHSV, IHNV	1,3E+03	1,3E+03	N/A	N/A
8	VHSV and IHNV	4,0E+03	N/A	N/A	1,9E+03
9	VHSV & IHNV	1,9E+04	8,6E+03	N/A	N/A
10	VHSV, IHNV	1,3E+03	N/A	N/A	1,9E+03
11	VHSV + IHNV	1,3E+04	1,3E+04	8,6E+03	2,7E+03
12	IHNV	8,62E+02	4,00E+03	N/A	N/A
13	IHNV, VHSV	8,62E+02	5,87E+03	< 1,9E+02	1,26E+03
14	IHNV, VHSV	< 1,9E+02	2,7E+07	N/A	N/A
15	VHSV, IHNV	N/A	< 1,9E+02	N/A	N/A
16	VHSV	4,0E+04	1,3E+05	N/A	N/A
17	IHNV, VHSV	1,3E+07	2,7E+08	5,9E+08	4,0E+08
18	VHSV	1,3E+05	< 1,9E+02	N/A	N/A
19	IHNV, VHSV	N/A	N/A	N/A	N/A
20	IHNV + VHSV	N/A	N/A	N/A	N/A
21	VHSV+IHNV	2,7E+03	5,9E+03	2,7E+03	5,9E+03
22	IHNV and VHSV	4,0E+02	8,6E+02	N/A	N/A
23	IHNV + VHSV	1,3E+04	4,0E+04	5,9E+02	2,7E+03
24	VHS and IHNV	1,3E+05	2,7E+03	N/A	N/A
25	IHNV and VHSV	N/A	1,3E+04	4,0E+03	N/A
26	VHSV/IHNV	4,0E+03	1,9E+03	N/A	N/A
27	VHSV, IHNV	1,3E+03	4,0E+03	4,0E+02	2,7E+03
28	VHS + IHN	1,3E+03	1,9E+03	N/A	N/A
29	VHSV+IHNV	1,3E+04	1,3E+03	N/A	N/A
30	VHSV and IHNV	1,3E+03	5,9E+02	N/A	N/A
31	IHNV/VHSV	5,9E+02	< 1,9E+02	N/A	N/A
32	VHSV & IHNV	2,7E+03	2,7E+03	N/A	N/A
33	IHNV, VHSV	4,0E+03	5,9E+03	2,7E+02	N/A
34	VHSV+IHNV	5,9E+02	8,6E+03	< 1,9E+02	N/A
35	VHSV, IHNV	1,9E+03	8,6E+03	N/A	N/A
36	IHNV and VHSV	N/A	N/A	N/A	N/A
37	VHSV; IHNV	8,6E+04	4,0E+04	N/A	N/A
38	VHS and IHNV	1,3E+05	1,3E+05	1,3E+04	1,3E+05
39	VHSV, IHNV	5,9E+03	8,6E+03	N/A	1,3E+04
40	VHSV and IHNV	1,3E+03	1,3E+05	N/A	N/A
41	IHNV and VHSV	5,9E+02	5,9E+03	< 1,9E+02	1,9E+03
42	VHSV IHNV	8,6E+04	1,3E+05	N/A	N/A
43	IHNV,VHSV	1,26E+03	1,26E+03	N/A	N/A

44	VHSV IHNV	1,9E+03	1,9E+04	4,0E+02	8,6E+03
45	VHSV	1,3E+03	4,0E+03	N/A	N/A
46	IHNV and VHSV	1,9E+03	2,7E+03	< 1,9E+02	N/A

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

IHNV 32/87 + VHSV 1p8	**BF-2**	**EPC**	**RTG-2**	**FHM**
Number of laboratories	39	40	15	13
Median titre	1,9E+03	5,9E+03	4,0E+02	2,7E+03
Maximum titre	1,3E+07	2,7E+08	5,9E+08	4,0E+08
Minimum titre	<1,9E+02	<1,9E+02	<1,9E+02	1,3E+03
25% quartile titre	1,3E+03	1,7E+03	<1,9E+02	1,9E+03
75% quartile titre	1,3E+04	1,3E+04	3,4E+03	8,6E+03

Table 6. Inter-Laboratory Proficiency Test, PT1, 2021 – **Results of titration of ampoule III**

Laboratory Code number	Virus Identification	Titre in			
		BF-2	EPC	RTG-2	FHM
1	ECV	1,3E+07	2,7E+05	4,0E+03	N/A
2	Ranavirus - Not EHNV	1,3E+07	1,3E+06	N/A	1,9E+05
3	Ranavirus	N/A	2,7E+06	1,3E+07	N/A
4	Ranavirus - NOT EHNV	5,9E+07	2,7E+06	N/A	N/A
5	ESV/ECV	1,9E+07	5,9E+07	N/A	N/A
6	Ranavirus was identified by conventional PCR and then REA was applied as given by OIE manual to identify ECV	5,9E+05	5,9E+05	N/A	N/A
8	ECV	4,0E+06	N/A	N/A	4,0E+03
9	ECV/ESV	4,0E+07	2,7E+06	N/A	N/A
10	Ranavirus-NOT EHNV	5,9E+06	N/A	N/A	1,9E+04
11	Ranavirus NOT EHNV	4,0E+06	1,3E+06	5,9E+06	8,6E+05
12	Ranavirus	1,86E+04	1,86E+06	N/A	1,26E+03
13	Ranavirus (European catfish virus)	2,73E+04	1,86E+05	1,26E+04	N/A
14	Ranavirus (not EHNV/other than EHNV)	4,0E+09	4,0E+09	N/A	N/A
15	Ranavirus - not EHNV	N/A	1,3E+04	N/A	N/A
16	Ranavirus – NOT EHNV	2,7E+04	2,7E+04	N/A	N/A
17	Not VHSV, Not IHNV, Not EHNV, Not IPNV, Not SVCV	1,9E+06	1,3E+06	8,6E+06	4,0E+06
18	Ranavirus-NOT EHNV	1,9E+05	< 1,9E+02	N/A	N/A
19	Ranavirus- Not EHNV	N/A	N/A	N/A	N/A
20	Ranavirus – NOT EHNV	N/A	N/A	N/A	N/A
21	Ranavirus - not EHNV	1,9E+05	4,0E+06	1,3E+04	4,0E+03
22	Ranavirus - NOT EHNV	4,0E+05	1,3E+05	N/A	N/A
23	EHNV	1,86E+05	8,62E+04	N/A	2,73E+04
24	Ranavirus - NOT EHNV	4,0E+06	4,0E+05	N/A	N/A
25	No VHSV, IHNV, EHNV, SVCV, IPNV	N/A	1,3E+05	2,7E+04	N/A
26	Ranavirus	5,9E+05	2,7E+05	N/A	N/A
27	Ranavirus	1,9E+06	5,9E+05	1,3E+03	1,9E+06
28	ECV	1,9E+06	5,9E+06	N/A	N/A
29	ECV	1,3E+07	2,7E+05	N/A	N/A
30	Ranavirus-NOT EHNV	1,9E+03	8,6E+02	N/A	N/A
31	EHNV	8,6E+06	2,7E+05	N/A	N/A
32	RANAVIRUS	1,9E+08	1,3E+06	N/A	N/A
33	Ranavirus – NOT EHNV	4,0E+06	1,3E+06	4,0E+04	N/A

34	Ranavirus – NOT EHNV	5,9E+06	5,9E+06	< 1,9E+02	N/A
35	Ranavirus - NOT EHNV	2,7E+05	1,3E+05	N/A	N/A
36	Ranavirus/European catfish virus	N/A	N/A	N/A	N/A
37	no VHSV; no IHNV, no IPNV, no SVCV	2,7E+08	5,9E+05	N/A	N/A
38	Ranavirus, not EHNV	2,7E+07	1,9E+07	1,3E+07	2,7E+04
39	Ranavirus - NOT EHNV	8,6E+04	1,3E+04	N/A	8,6E+03
40	ECV	4,0E+06	5,9E+04	N/A	N/A
41	Ranavirus - NOT EHNV	1,3E+05	1,3E+04	< 1,9E+02	5,9E+03
42	Ranavirus	4,0E+04	1,3E+04	N/A	N/A
43	VHSV	5,87E+06	1,26E+06	N/A	N/A
44	Ranavirus NOT EHNV	2,7E+05	1,3E+04	< 1,9E+02	5,9E+04
45	ECV	5,9E+06	2,7E+06	N/A	N/A
46	Ranavirus - NOT EHNV	2,7E+06	5,9E+05	1,9E+03	N/A

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

ECV 562/92	**BF-2**	**EPC**	**RTG-2**	**FHM**
Number of laboratories	39	40	14	13
Median titre	4,0E+06	5,9E+05	2,0E+04	2,3E+04
Maximum titre	4,0E+09	4,0E+09	1,3E+07	4,0E+06
Minimum titre	1,9E+03	<1,9E+02	<1,9E+02	1,3E+03
25% quartile titre	2,1E+05	1,3E+05	3,3E+03	5,4E+03
75% quartile titre	1,3E+07	2,3E+06	6,6E+06	3,5E+05

Table 7. Inter-Laboratory Proficiency Test, PT1, 2021 – **Results of titration of ampoule IV.**

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

37	SVCV	2,7E+05	5,9E+04	N/A	N/A
38	SVCV	4,0E+06	1,3E+06	1,9E+06	2,7E+06
39	SVCV	1,3E+03	8,6E+03	N/A	1,3E+04
40	SVCV	5,9E+03	2,7E+04	N/A	N/A
41	SVCV	2,7E+03	2,7E+04	< 1,9E+02	4,0E+04
42	SVCV	1,3E+03	2,7E+04	N/A	N/A
43	SVCV	2,7E+03	8,62E+02	N/A	N/A
44	SVCV	2,7E+02	4,0E+04	< 1,9E+02	8,6E+04
45	SVCV	1,9E+04	1,3E+04	N/A	N/A
46	SVCV	4,0E+04	2,7E+04	1,9E+02	N/A

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

SVC 56/70	**BF-2**	**EPC**	**RTG-2**	**FHM**
Number of laboratories	38	40	14	13
Median titre	7,2E+03	2,7E+04	2,3E+02	4,0E+04
Maximum titre	4,0E+07	2,7E+09	1,3E+08	5,9E+07
Minimum titre	<1,9E+02	<1,9E+02	<1,9E+02	1,3E+03
25% quartile titre	1,3E+03	8,6E+03	<1,9E+02	1,3E+04
75% quartile titre	3,7E+04	6,6E+04	2,1E+04	8,6E+04

Table 8. Inter-Laboratory Proficiency Test, PT1, 2021 – Results of titration of **ampoule V**.

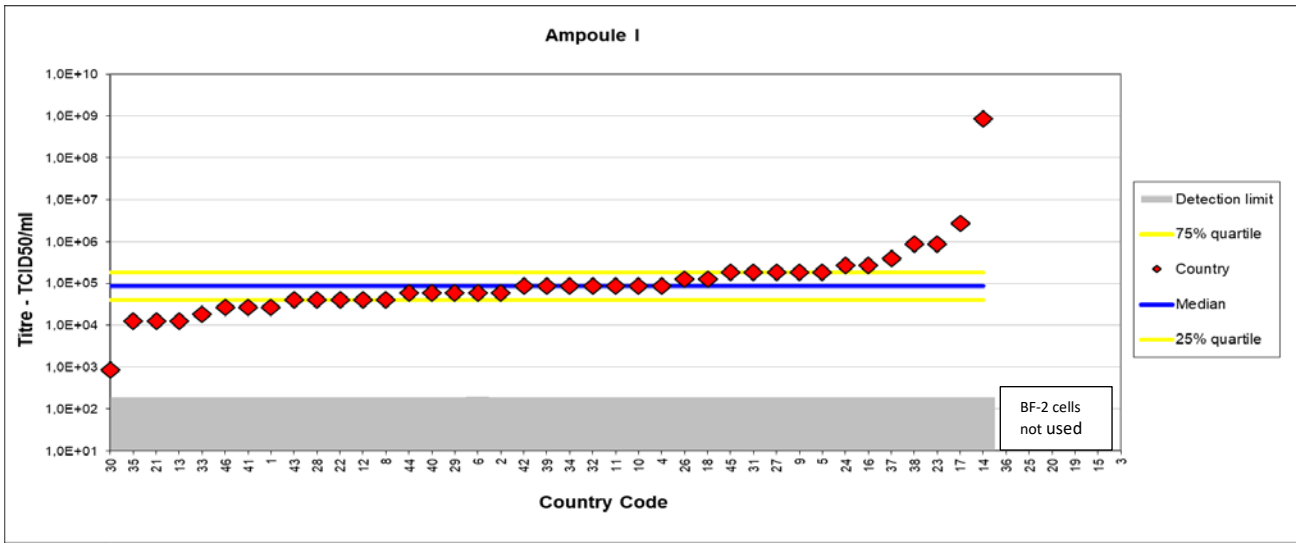
Laboratory Code number	Virus Identification	Titre in			
		BF-2	EPC	RTG-2	FHM
1	IHNV	< 1,9E+02	5,9E+02	8,6E+02	N/A
2	IHNV	< 1,9E+02	5,9E+03	N/A	4,0E+03
3	IHNV	N/A	4,0E+03	< 1,9E+02	N/A
4	IHNV	< 1,9E+02	4,0E+04	N/A	N/A
5	IHNV	< 1,9E+02	8,6E+04	N/A	N/A
6	IHNV	< 1,9E+02	5,9E+03	N/A	N/A
8	IHNV	5,9E+02	N/A	N/A	8,6E+02
9	IHNV	1,9E+03	4,0E+04	N/A	N/A
10	IHNV	< 1,9E+02	N/A	N/A	2,7E+03
11	IHNV	< 1,9E+02	1,3E+04	< 1,9E+02	1,9E+04
12	-	< 1,9E+02	< 1,9E+02	N/A	N/A
13	IHNV	< 1,9E+02	1,3E+04	1,9E+03	1,9E+03
14	IHNV	< 1,9E+02	4,0E+06	N/A	N/A
15	IHNV	N/A	< 1,9E+02	N/A	N/A
16	IHNV	4,0E+05	5,9E+05	N/A	N/A
17	IHNV	2,7E+08	4,0E+08	< 1,9E+02	< 1,9E+02
18	IHNV	1,9E+04	< 1,9E+02	N/A	N/A
19	IHNV	N/A	N/A	N/A	N/A
20	IHNV	N/A	N/A	N/A	N/A
21	IHNV	1,3E+03	1,9E+03	8,6E+02	1,9E+03
22	IHNV	< 1,9E+02	1,9E+03	N/A	N/A
23	IHNV	N/A	1,3E+04	N/A	4,0E+03
24	IHNV	< 1,9E+02	1,3E+04	N/A	N/A
25	IHNV	N/A	1,3E+04	2,7E+03	N/A
26	IHNV	1,9E+02	4,0E+03	N/A	N/A
27	IHNV	< 1,9E+02	2,7E+03	1,9E+02	4,0E+03
28	IHN	< 1,9E+02	1,9E+04	N/A	N/A
29	IHNV	1,3E+03	8,6E+03	N/A	N/A
30	IHNV	< 1,9E+02	4,0E+03	N/A	N/A
31	IHNV	< 1,9E+02	< 1,9E+02	N/A	N/A
32	IHNV	4,0E+02	2,7E+04	N/A	N/A
33	IHNV	< 1,9E+02	1,3E+04	< 1,9E+02	N/A
34	IHNV	< 1,9E+02	1,3E+04	< 1,9E+02	N/A
35	IHNV	< 1,9E+02	1,3E+04	N/A	N/A
36	IHNV	N/A	N/A	N/A	N/A

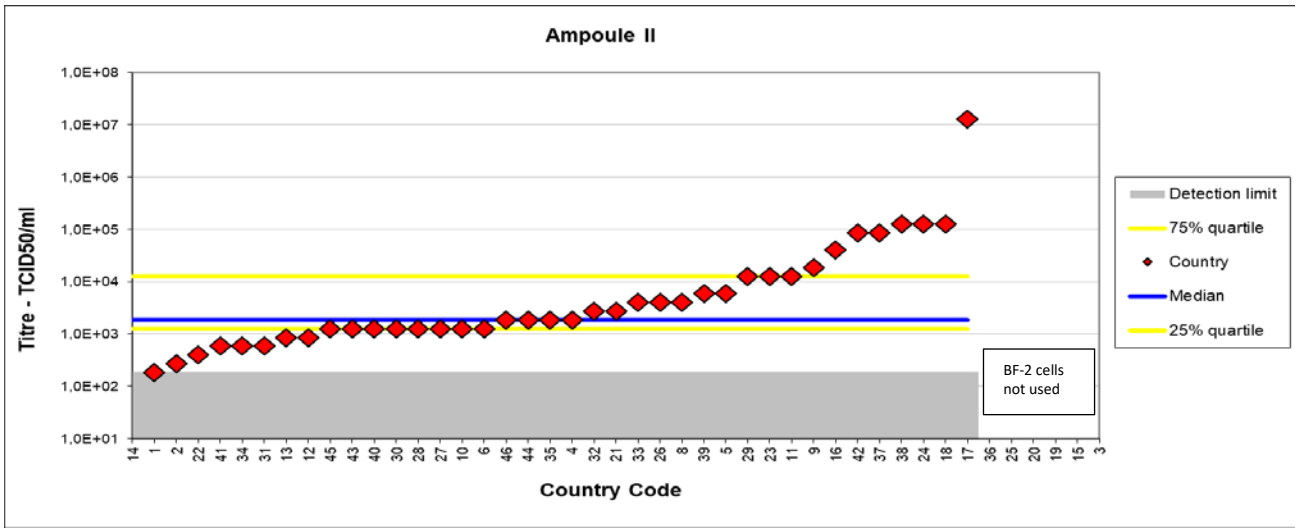
37	IHNV	5,9E+03	4,0E+04	N/A	N/A
38	IHNV	< 1,9E+02	2,7E+05	4,0E+04	1,3E+05
39	IHNV	8,6E+02	1,3E+04	N/A	8,6E+03
40	IHNV	1,3E+04	< 1,9E+02	N/A	N/A
41	IHNV	< 1,9E+02	8,6E+03	< 1,9E+02	8,6E+03
42	IHNV	< 1,9E+02	5,9E+04	N/A	N/A
43	IHNV	< 1,9E+02	2,7E+03	N/A	N/A
44	IHNV	< 1,9E+02	2,7E+04	< 1,9E+02	2,7E+04
45	IHNV	< 1,9E+02	2,7E+03	N/A	N/A
46	IHNV	1,9E+02	8,6E+03	< 1,9E+02	N/A

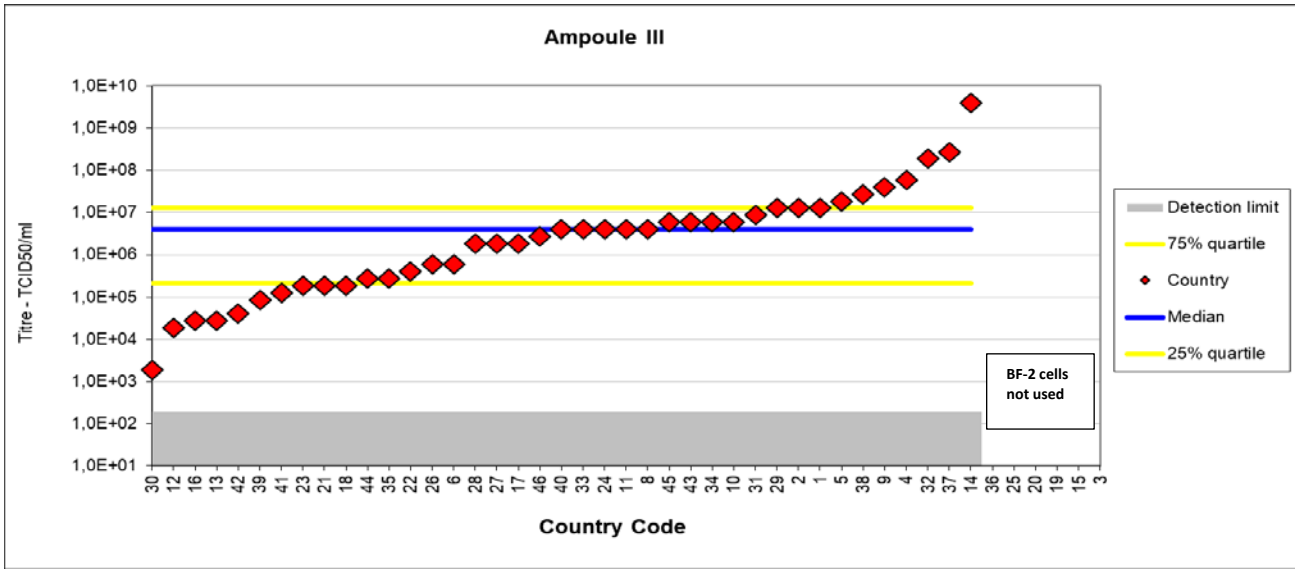
N/A: Cell line not applied by the participating laboratory for titration of the virus

EHNV 86/8774	**BF-2**	**EPC**	**RTG-2**	**FHM**
Number of laboratories	38	40	14	13
Median titre	<1,9E+02	1,3E+04	<1,9E+02	4,0E+03
Maximum titre	2,7E+08	4,0E+08	4,0E+04	1,3E+05
Minimum titre	<1,9E+02	<1,9E+02	<1,9E+02	<1,9E+02
25% quartile titre	<1,9E+02	2,7E+03	<1,9E+02	1,9E+03
75% quartile titre	5,4E+02	2,7E+04	8,6E+02	8,6E+03

Figure 5. Virus titres obtained in BF-2 cells. The titre (red diamond) of each participating laboratory (country code on the x axis) using BF-2 cells illustrated for ampoule I, II, III, IV and V. The detection limit (grey shadow), median (blue line), 75% quartile (upper yellow line) and 25% quartile (lower yellow line) are plotted on all graphs. Participants failing to obtain any titre are listed on the x axis under the grey zone but no red diamond is plotted; participants who did not use a specific cell line are listed under the white text box.







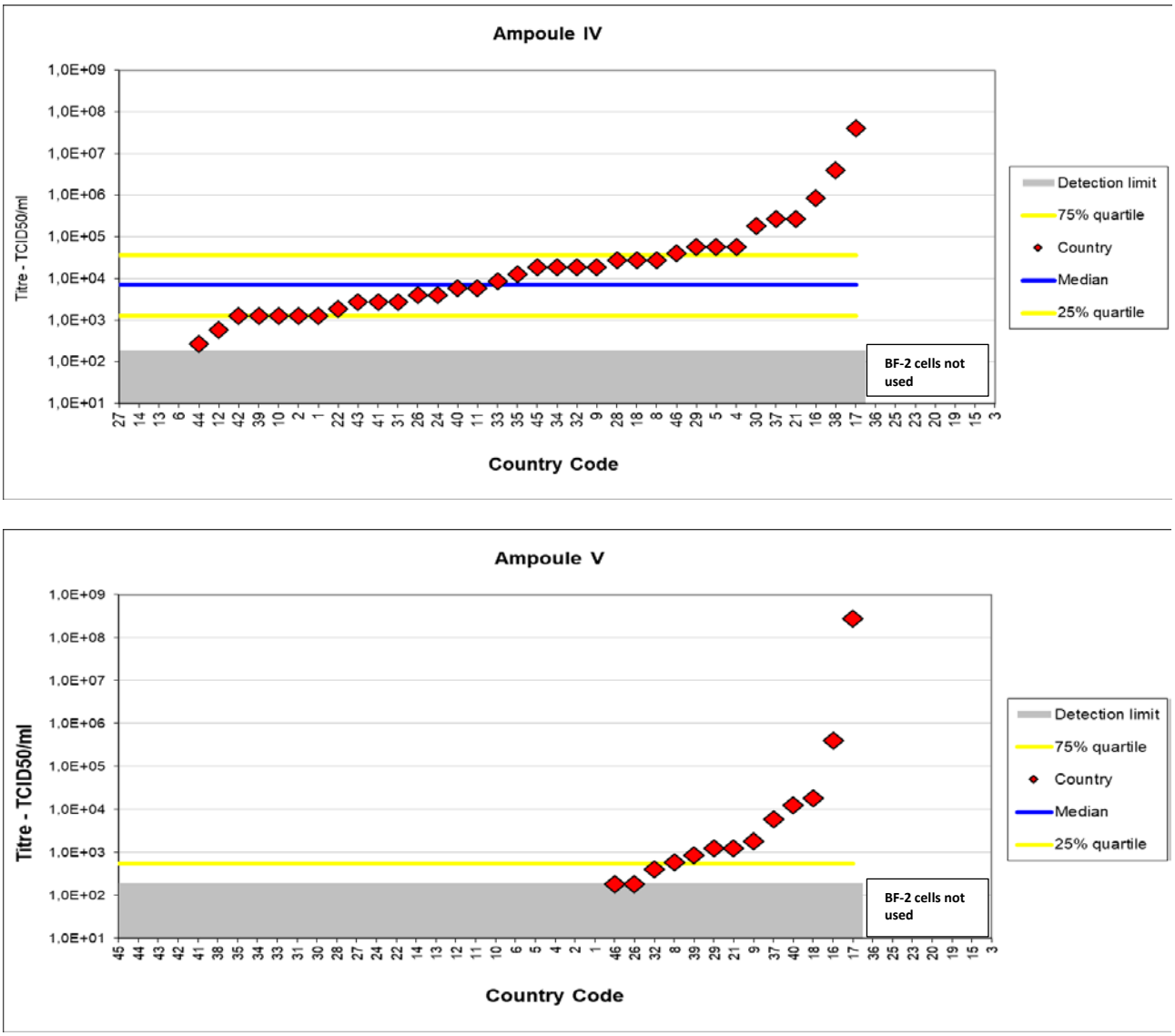
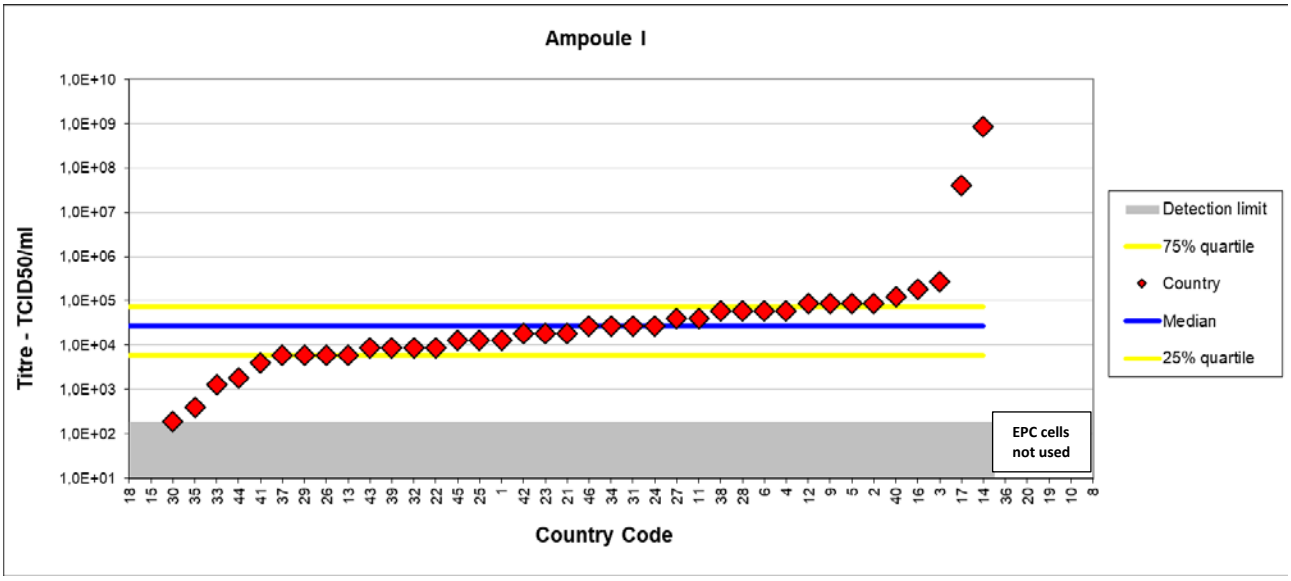
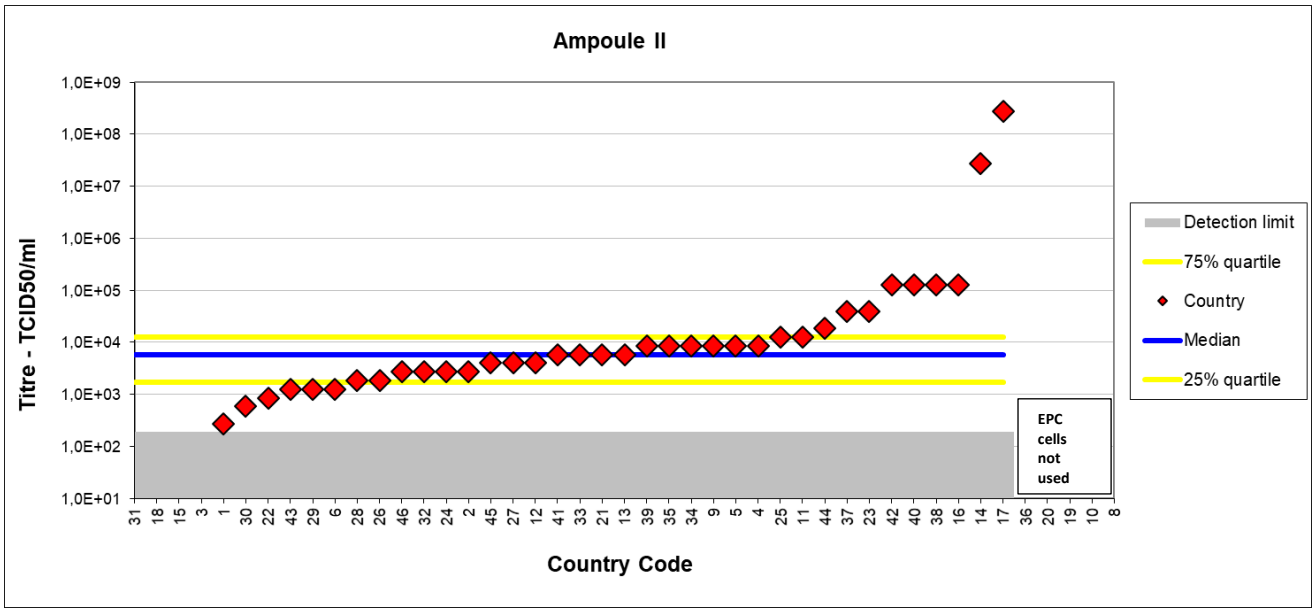
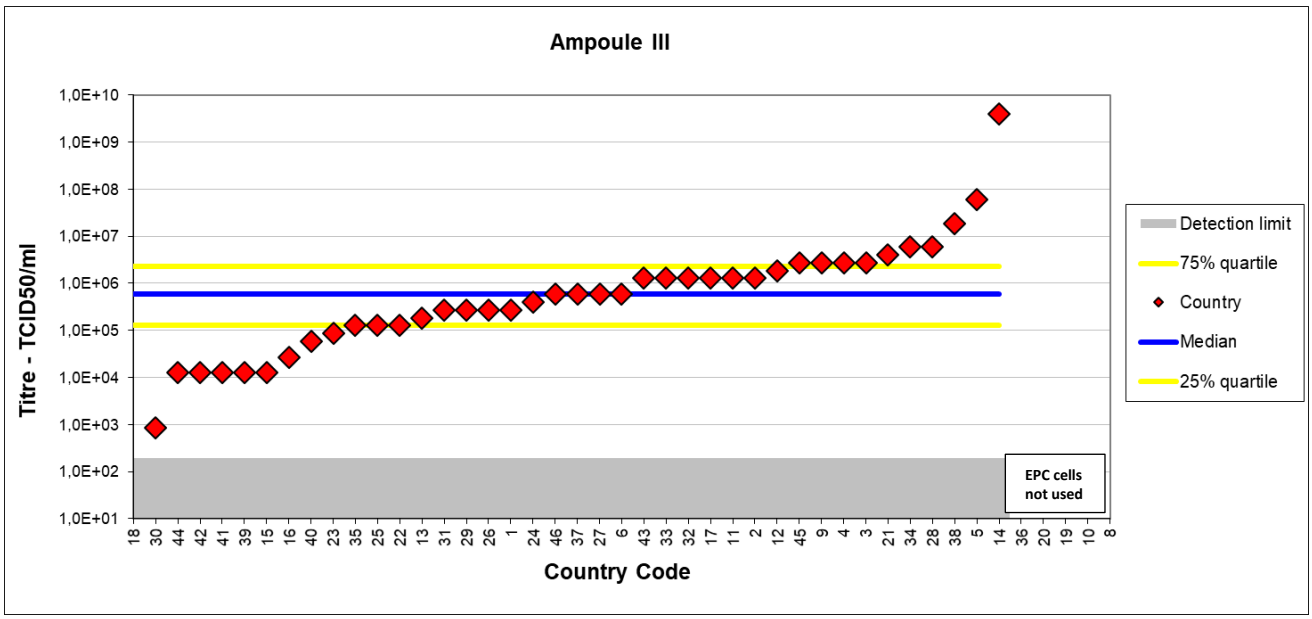
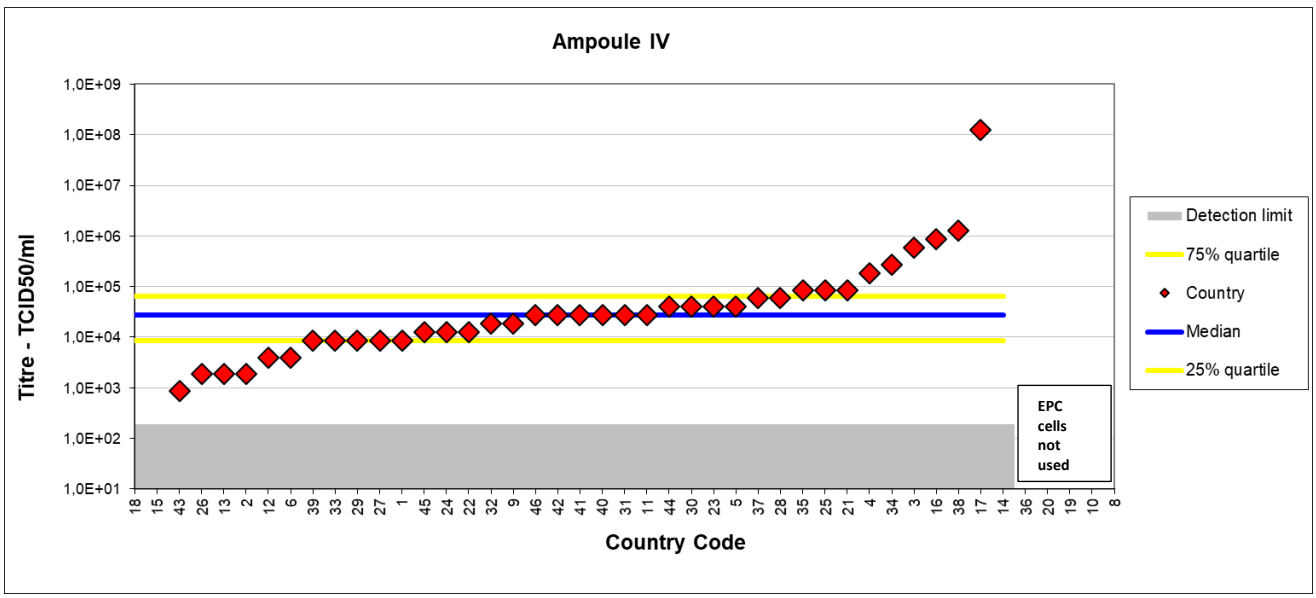


Figure 6. Virus titres obtained in EPC cells. For further details see description at Figure 5




Ampoule II
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
31 18 15 3 1 30 22 43 29 6 28 26 46 32 24 2 45 27 12 41 33 21 13 39 35 34 9 5 4 25 11 44 37 23 42 40 38 16 14 17 36 20 19 10 8
Country Code
EPC cells not used
Detection limit
75% quartile
Country
Median
25% quartile


Ampoule III
Titre - TCID50/ml
1,0E+10
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
18 30 44 42 41 39 15 16 40 23 35 25 22 13 31 29 26 1 24 46 37 27 6 43 33 32 17 11 2 12 45 9 4 3 21 34 28 38 5 14 36 20 19 10 8
Country Code
EPC cells not used
Detection limit
75% quartile
Country
Median
25% quartile


Ampoule IV
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
18 15 43 26 13 2 12 6 39 33 29 27 1 45 24 22 32 9 46 42 41 40 31 11 44 30 23 5 37 28 35 25 21 4 34 3 16 38 17 14 36 20 19 10 8
Country Code
EPC cells not used
Detection limit
75% quartile
Country
Median
25% quartile

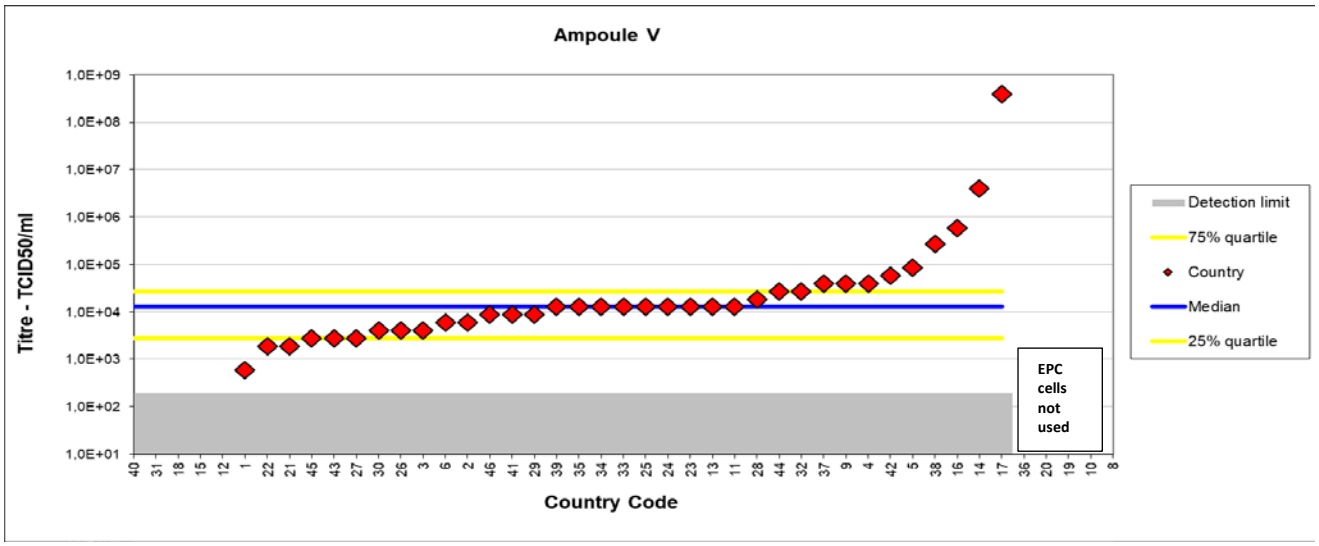
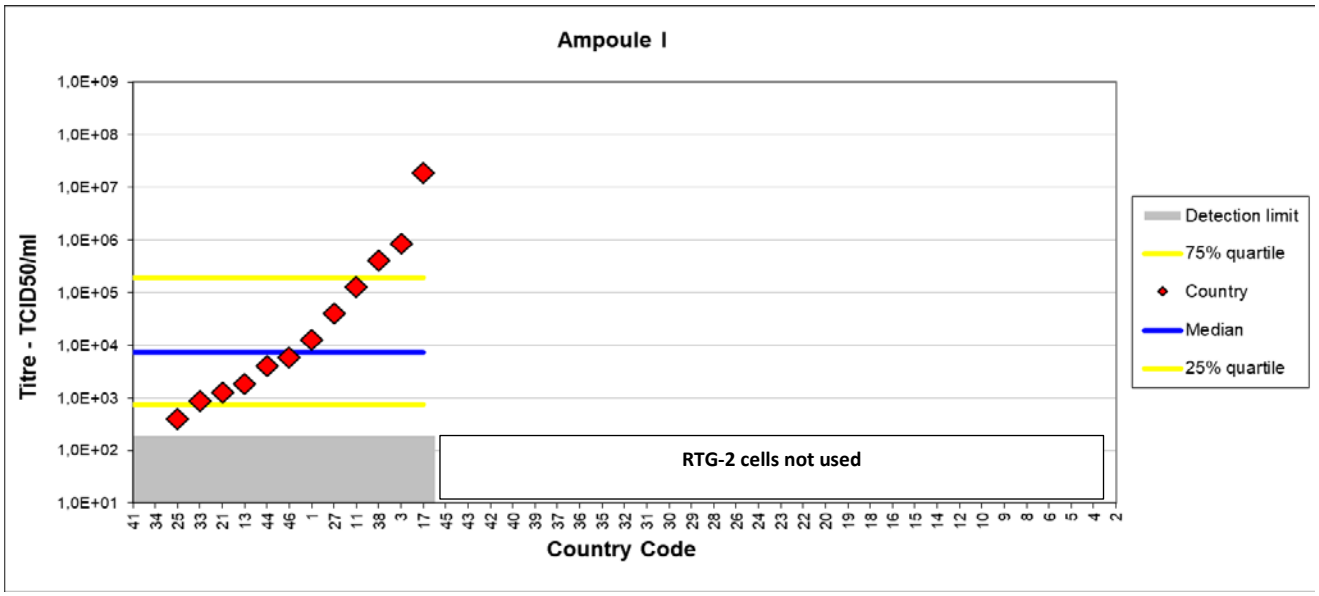
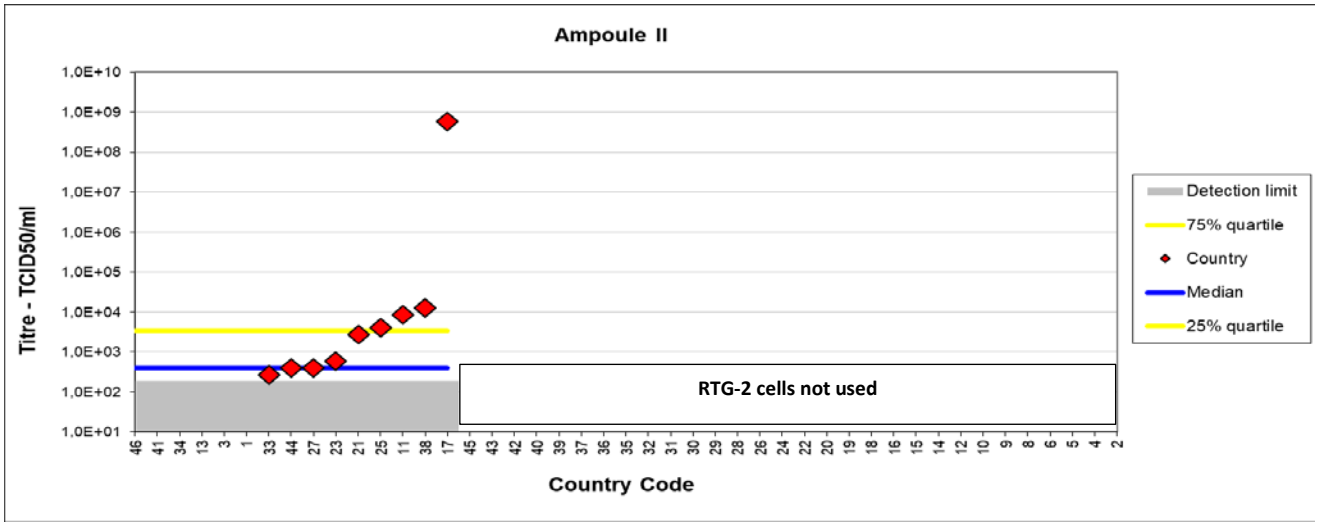
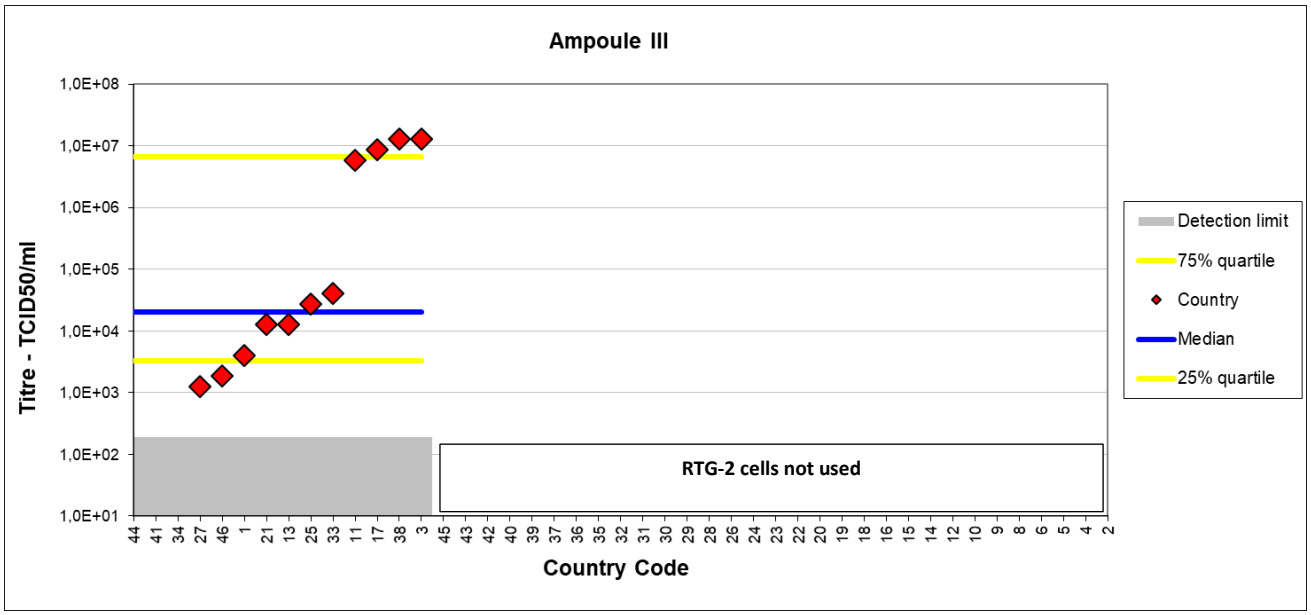
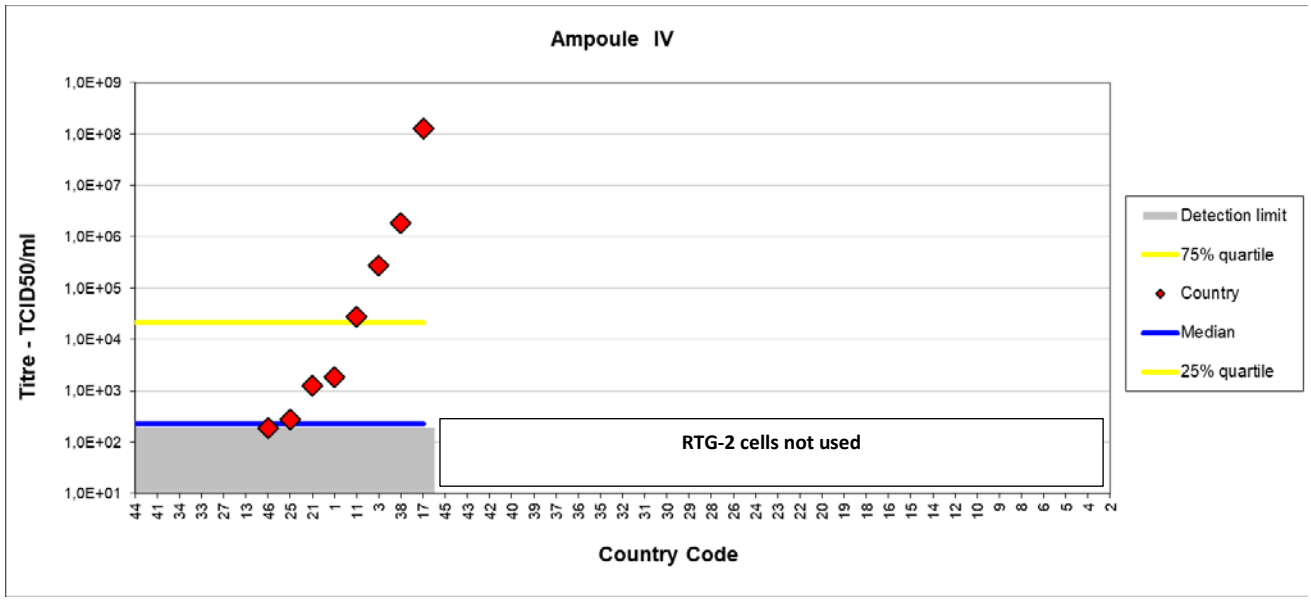


Figure 7. Virus titre obtained in RTG-2 cells. For further details see description at Figure 5






Ampoule III
Titre - TCID50/ml
1,0E+08
1,0E+07
1,0E+06
1,0E+05
1,0E+04
1,0E+03
1,0E+02
1,0E+01
RTG-2 cells not used
Country Code
Detection limit
75% quartile
Country
Median
25% quartile


Ampoule IV
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
RTG-2 cells not used
Country Code
Detection limit
75% quartile
Country
Median
25% quartile

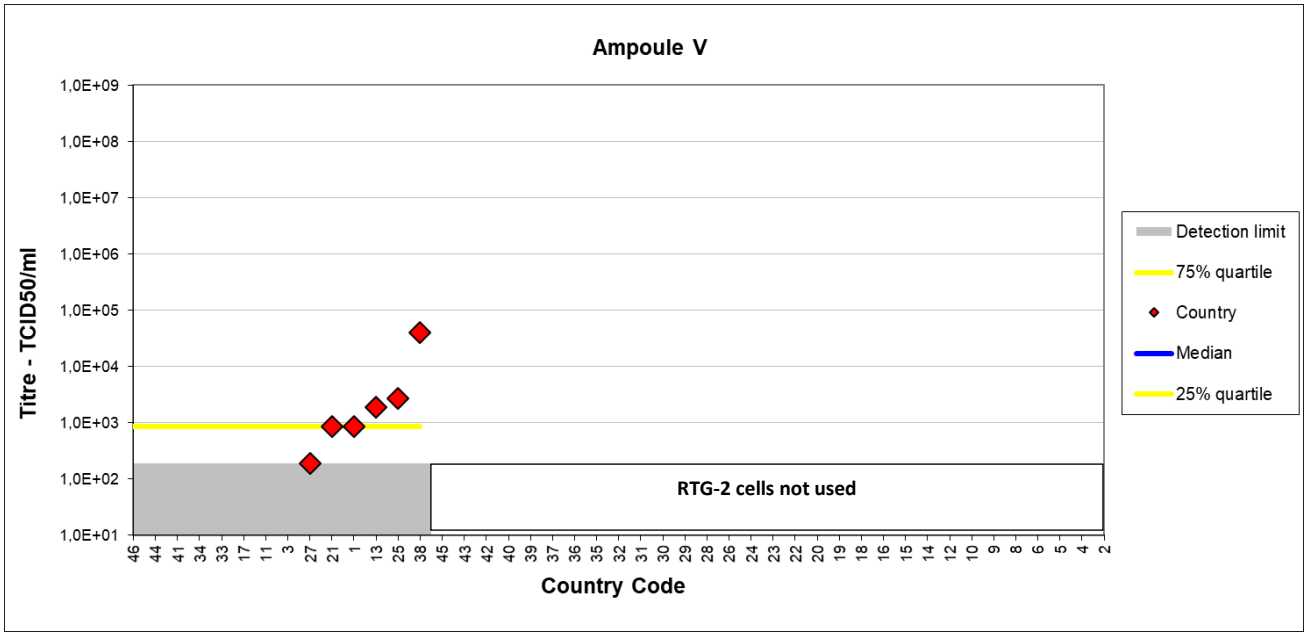
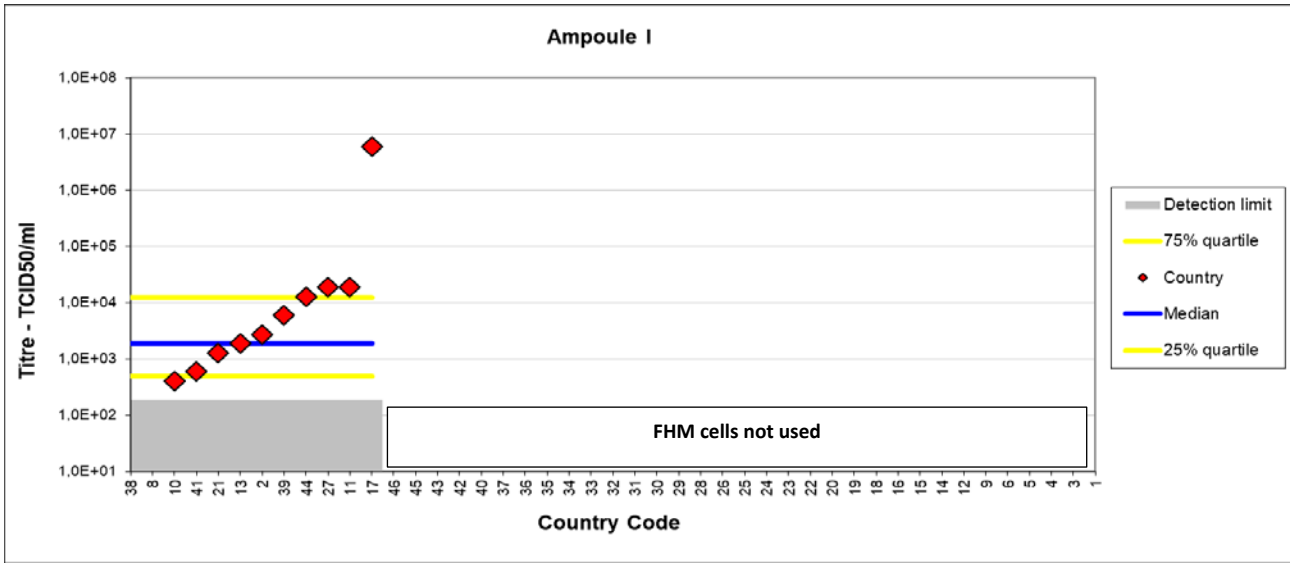
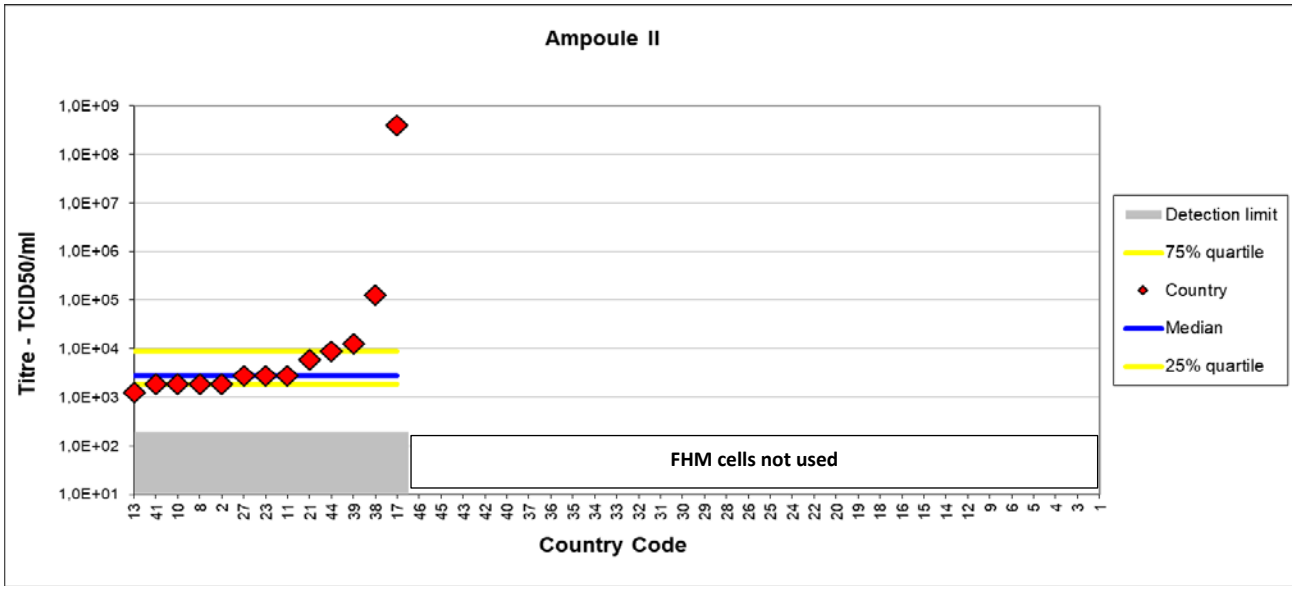
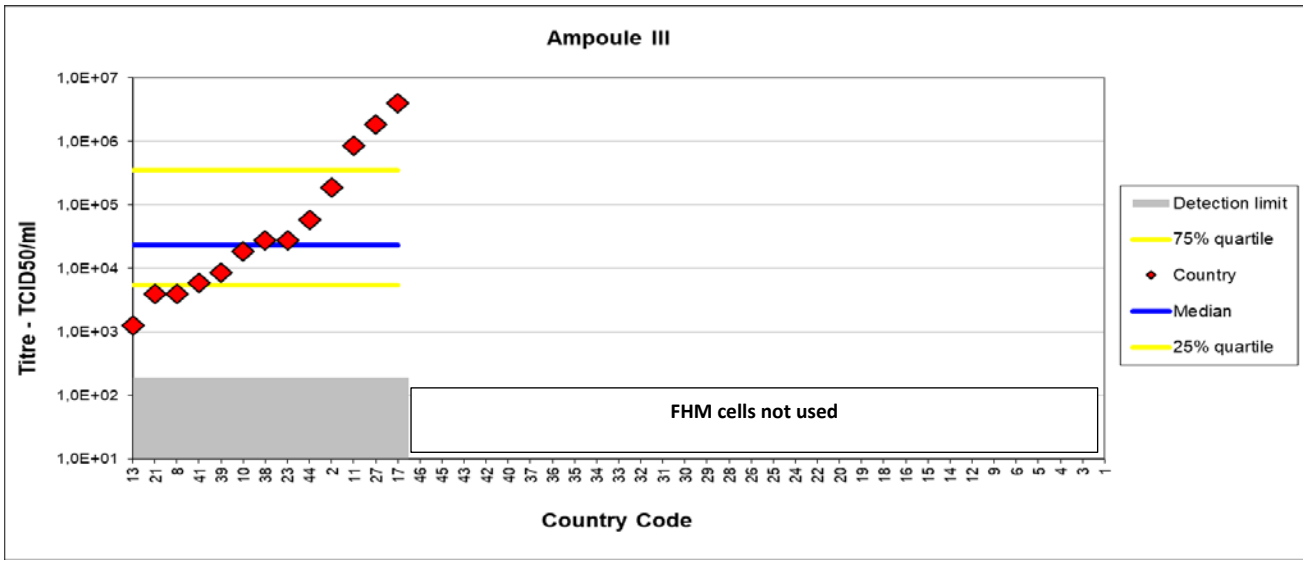
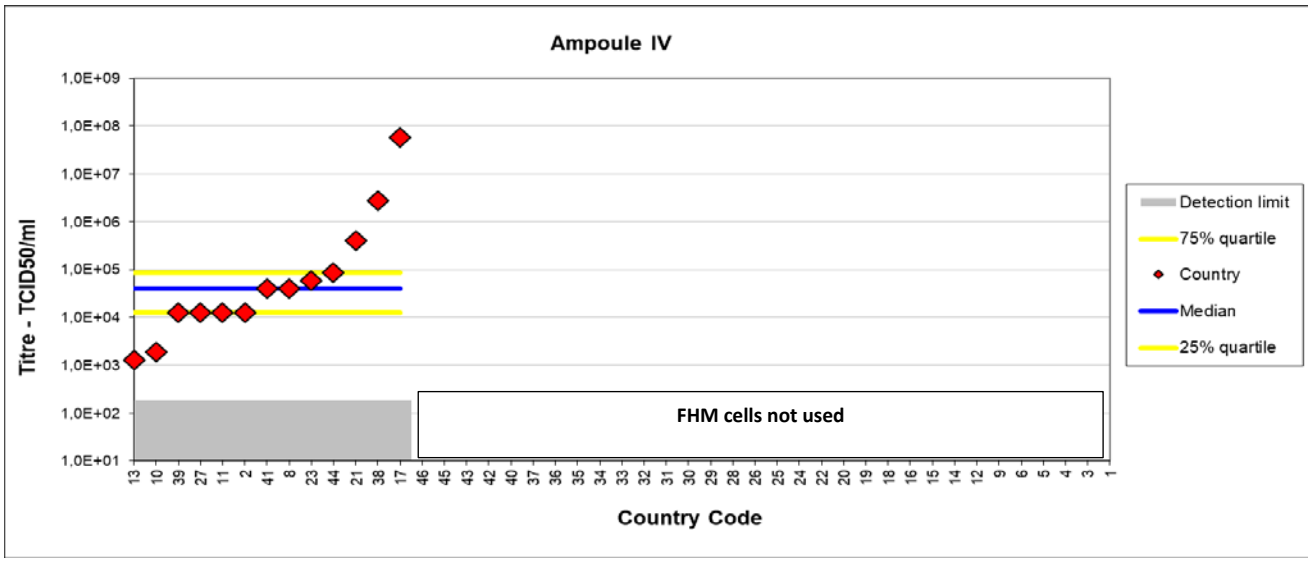

Ampoule V
Titre - TCID50/ml
1,0E+09
1,0E+08
1,0E+07
1,0E+06
1,0E+05
1,0E+04
1,0E+03
1,0E+02
1,0E+01
RTG-2 cells not used
Country Code
Detection limit
75% quartile
Country
Median
25% quartile

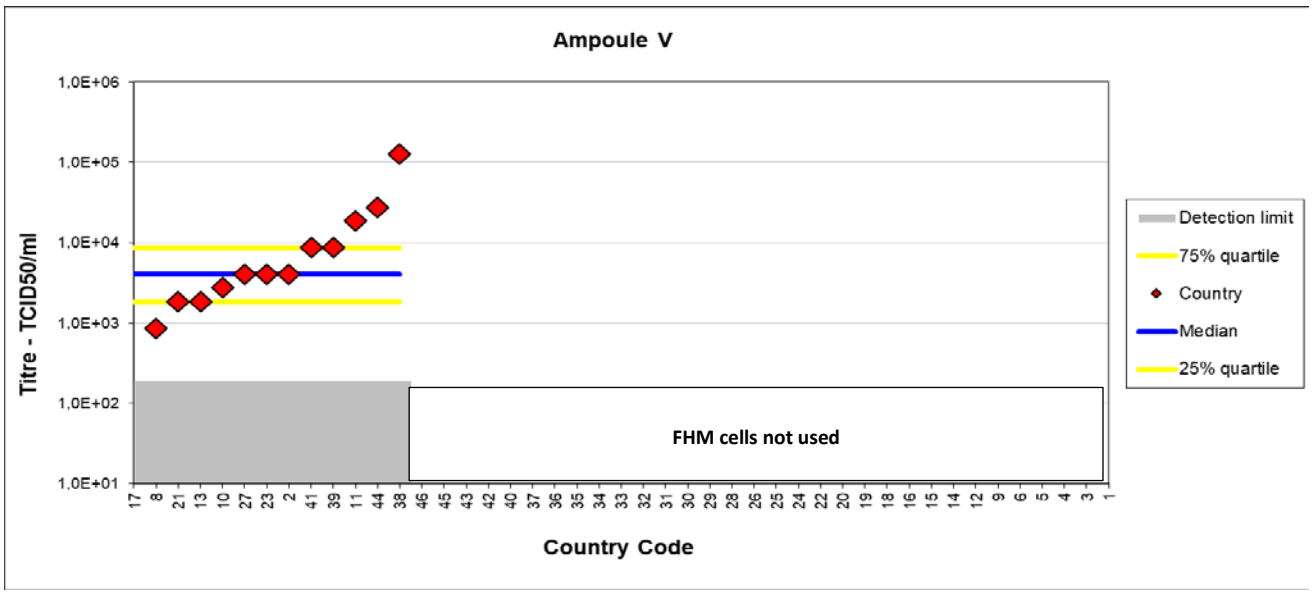
Figure 8. Virus titres obtained in FHM cells. For further details see description at Figure 5











Identification of content

- 43 laboratories out of 45 participants analysed for all viruses; 32 of these laboratories correctly identified all viruses in all ampoules.
- 2 laboratories out of 45 participants did not test for Ranavirus; one laboratory correctly identified all other viruses in the ampoules.

Ampoule I – EHNV (86/8774)

- 38 laboratories correctly identified the isolate as the listed EHNV in ampoule I by sequencing or REA.
- 2 laboratories reported the use of pathogen specific qPCR kit, but documentation for validation of the kit has not been made accessible.
- 2 laboratories identified Ranavirus but did not specify if the isolate was the listed EHNV or not by sequencing or REA.
- 1 laboratory switch ampoule I and III
- 2 laboratories do not test for Ranavirus

Ampoule II – IHNV (32/87) + VHSV (1p8)

- 40 laboratories correctly identified both IHNV and VHSV in ampoule II.
- 3 laboratories did only identify VHSV
- 1 laboratory did only identify IHNV
- 1 laboratory did not identify either IHNV or VHSV

Ampoule III – ECV (562/92)

- 38 laboratories correctly identified the isolate as ECV or Not EHNV in ampoule III by sequencing or REA.
- 2 laboratories answered "Not VHSV, IHNV, EHNV, SVCV, IPNV" (reported the use of pathogen (EHNV) specific qPCR kit).
- 2 laboratory identified Ranavirus but did not specify if the isolate was the listed EHNV or not by sequencing or REA.
- 1 laboratory switch ampoule I and III
- 2 laboratories do not test for Ranavirus

Ampoule IV – SVCV (56/70)

- 44 laboratories correctly identified the isolate as SVCV in ampoule IV
- 1 laboratory did not find any virus in the ampoule.

Ampoule V – IHNV (217/A)

- 44 laboratories correctly identified the isolate as IHNV in ampoule IV
- 1 laboratory did not find any virus in the ampoule.

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).

- Ampoule I: EHNV identification was given the score 2. No identification of the Ranavirus by sequencing or REA was given the score 1.
- Ampoule II: IHNV and VHSV identification was given the score 2, identification of only IHNV would have given 1 point and identification of VHSV only would have given 1 points to this ampoule.
- Ampoule III: ECV or Not EHNV identification was given the score 2. No identification of the Ranavirus by sequencing or REA was given the score 1.
- Ampoule IV: SVCV identification was given the score 2
- Ampoule V: IHNV identification was given the score 2.

In relation to the ranaviruses included in the ILPT, full score was given only in case one laboratory could isolate the virus and fully identify the isolate by means of sequencing or REA analysis.
Although it is acknowledged that, theoretically, other methods can be used to discriminate (e.g. specific qPCR assay) these have not been fully validated or the data of such validation are not available, hence we have considered that the result is not corroborated and fully supported from the diagnostic method used.

Out of 45 laboratories participating in the PT 1 2021, 32 obtained score 10/10.
The score 6/6 was assigned to one participant as they did not test for Ranavirus.
12 laboratories scored below 100% due to no identification by either sequencing or REA of the Ranavirus or due to not finding all virus in the ampoule or finding the wrong virus.

Cells applied for solving the test

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

- 39 laboratories used BF-2 cells
- 40 laboratories used EPC cells
- 15 laboratories used RTG-2 cells
- 13 laboratories used FHM cells
- 8 laboratories used CHSE-214 cells
- 3 laboratories did not titrate and 1 laboratory did only use EPC cells

The combination of EPC and FHM cells or BF-2 and RTG 2 alone is not valid according to EURL diagnostic manuals [2] The laboratories are encouraged to include the use of BF-2 cells or RTG 2 cells and EPC cells or FHM cells.

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

It appears that:

- Ampoule I (EHNV 86/8774) replicates on all four cell lines however it grows a little less efficiently on RTG-2 and FHM but within two log difference.
- Ampoule II (IHNV 32/87 + VHSV 1p8) replicate on all four cell lines but a little less efficient on RTG-2
- Ampoule III (ECV 562/92) replicates on all four cell lines however it grows equally less efficiently on RTG-2 and FHM.
- Ampoule IV (SVCV 56/70) replicates on all four cell lines however it grows poorly on RTG-2.
- Ampoule V (IHNV 217/A) replicates equally on EPC and FHM and does not replicates or grow poorly on BF-2 and RTG-2.

As from Table 3-8 the variations in titres between laboratories was high – with more than 7 log differences from the lowest to the most sensitive performances. Laboratories scoring low titres should definitely consider to exchange their cell lines with more sensitive ones or assess if the performance of their cells could be improved and the ones with a high titre should ensure to follow the correct titration procedure.

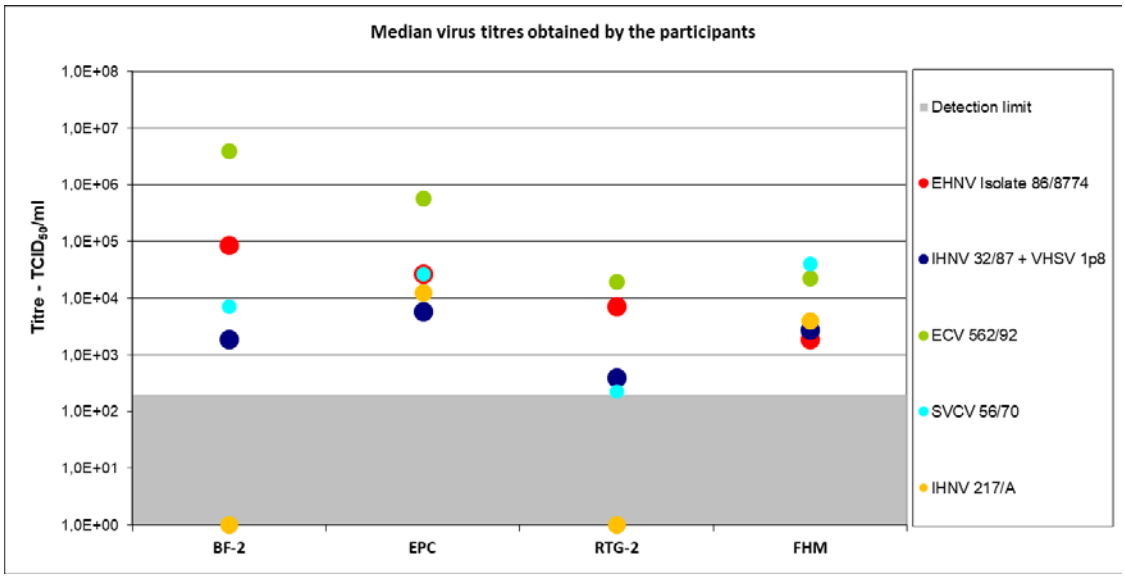


Figure 9. Median virus titres obtained by the participants in 4 different cell lines.

Ct. values

We have encouraged participants to insert the Ct value in the spreadsheet if they have performed a real-time (RT-) PCR.
The Ct values obtained by the participating laboratories are summarised in tables 9.

The Ct values cannot be directly compared due to the use of different methods, reagents and equipment for nucleic acid extraction and (RT)-qPCR. Additionally, some participants may have tested directly from the ampoule provided whereas others used cell supernatant from inoculation on cell culture. Participants will be asked to test the ampoules by molecular methods directly from the re-suspended material and not from the viral isolates from the next proficiency test, to streamline comparison of the results.

Table 9. Inter-Laboratory Proficiency Test, PT1, 2021 – Ct.-values.

Laboratory Code number	Ct. value Ampoule I (EHNV)	Ct. value Ampoule II (IHNV)	Ct. value Ampoule II (VHSV)	Ct. value Ampoule III (ECV)	Ct. value Ampoule IV (SVCV)	Ct. value Ampoule V (IHNV)
EURL	**24,85**	**29,9**	**28,7**	**21,6**	**26,4**	**28,78**
1	N/A	N/A	37,3	N/A	N/A	N/A
2	N/A	N/A	15,2	N/A	N/A	N/A
3	N/A	N/A	N/A	N/A	N/A	N/A
4	25,6	36,0	32,4	21,9	30,9	31,1
5	N/A	N/A	N/A	N/A	N/A	N/A
6	N/A	N/A	16,8	N/A	N/A	N/A
8	N/A	30,0	>36,9	N/A	32,0	30,0
9	N/A	36,2	37,3	N/A	N/A	33,2
10	12,5	38,1	15,2	12,5	N/A	18,1
11	N/A	23,7	27,9	N/A	N/A	22,0
12	N/A	N/A	N/A	N/A	N/A	N/A
13	N/A	N/A	N/A	N/A	N/A	N/A
14	N/A	16,0	25,1	N/A	N/A	14,9
15	N/A	33,4	32,4	N/A	N/A	30,7
16	N/A	N/A	31,6	N/A	28,7	31,7
17	25,2	33,0	33,0	N/A	15,9	29,5

18	N/A	N/A	36,9	N/A	31,0	27,5
19	N/A	29,1	27,4	N/A	25,8	27,1
20	N/A	N/A	29,4	N/A	N/A	N/A
21	N/A	20,4	18,9	N/A	N/A	13,6
22	N/A	35,5	33,2	N/A	N/A	34,4
23	21,1	30,9	33,7	16,7	29,4	30,6
24	N/A	N/A	18,4	20,1	N/A	N/A
25	N/A	22,5	33,1	N/A	17,0	19,5
26	N/A	32,4	28,6	N/A	N/A	28,3
27	N/A	N/A	N/A	N/A	N/A	N/A
28	N/A	24,7	22,8	N/A	N/A	18,9
29	N/A	N/A	N/A	N/A	N/A	N/A
30	19,2	18,9	14,4	15,9	N/A	16,8
31	N/A	32,9	30,8	N/A	N/A	29,2
32	N/A	N/A	N/A	N/A	N/A	N/A
33	N/A	33,7	31,5	N/A	N/A	30,9
34	N/A	31,7	32,0	N/A	N/A	28,7
35	N/A	26,3	17,5	N/A	N/A	21,2
36	N/A	32,0	31,7	N/A	N/A	29,7
37	N/A	17,4	17,5	N/A	N/A	15,4
38	N/A	29,1	28,8	N/A	27,0	27,6
39	N/A	N/A	19,3	N/A	N/A	N/A
40	N/A	33,6	16,3	N/A	N/A	29,2
41	N/A	12,3	21,9	N/A	26,4	11,4
42	N/A	N/A	N/A	N/A	N/A	N/A
43	N/A	N/A	N/A	N/A	N/A	N/A
44	N/A	N/A	13,1	N/A	N/A	N/A
45	N/A	N/A	18,6	N/A	20,2	21,1
46	15,5	19,4	34,2	15,0	14,2	18,2

N/A: No Ct-value given by the participating laboratory.

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. An overview of the genotyping results obtained for PT1 by all participants is displayed in the following table 10. The EURL has disclosed the content of the ampoules after deadline for delivering results.

Table 10. The genotyping results obtained for PT1 by all 44 participants

Code number	Ampoule I	Ampoule II	Ampoule III	Ampoule IV	Ampoule V
	EHNV 86/8774	**IHNV 32/87 Genotype E + VHSV 1p8 Genotype 1b**	**ECV 562/92**	**SVCV 56/70 Genotype Id**	**IHNV 217/A Genotype E**
1	EHNV	E: Ib	ECV	Genogroup Id	0
2	EHNV	VHSV genotype Ib IHNV genotype M	Ranavirus - Not EHNV	Type Id	genotype U
3	EHNV	0	Ranavirus	Fijan	0
4	EHNV	IHNV - E, VHSV - 1b	Ranavirus - NOT EHNV	1d	U
5	EHNV	VHSV Ib & IHNV M(European)	ESV/ECV	SVCV 1d	U/L intermediate
6	Ranavirus was identified by conventional PCR and than REA was applied as given by OIE manuel to identified EHNV	0	Ranavirus was identified by conventional PCR and than REA was applied as given by OIE manuel to identified ECV	0	0
8	EHNV	0	ECV	0	0
9	EHNV	VHSV: Genotype I, Subtype Ib; IHNV: Genogrooupe-M-EUR-1 (Genogroupe E)	ECV/ESV	Genogroupe I	Genogroupe U
10	EHNV	1b, E	Ranavirus-NOT EHNV	0	0
11	EHNV	VHSV: Ib IHNV: E	Ranavirus NOT EHNV	Genogroup 1d	E
12	EHNV	E	Ranavirus	Id	0
13	EHNV	IHNV : E VHSV : Ib	Ranavirus (European catfish virus)	Id	IHNV : E
14	EHNV	0	Ranavirus (not EHNV/other than EHNV)	0	0
15	EHNV	Ib	Ranavirus - not EHNV	genogroup Id	0
16	EHNV	0	Ranavirus – NOT EHNV	0	0
17	EHNV	0	Not VHSV, Not IHNV, Not EHNV,	0	0

			Not IPNV, Not SVCV		
18	EHNV	I(b)	Ranavirus-NOT EHNV	Genogroup 1(d)	L
19	EHNV	VHSVgen. Ib; IHNV gen. E	Ranavirus- Not EHNV	0	E
20	EHNV	VHSV - Ib	Ranavirus – NOT EHNV	0	0
21	EHNV	VHS: Ia	Ranavirus - not EHNV	0	0
22	EHNV	IHNV: E VHSV: Ib	Ranavirus - NOT EHNV	0	U
23	European sheatfish virus	IHN M VHS 1b	EHNV	SVC 1d	IHN M
24	EHNV	I (b) , M	Ranavirus - NOT EHNV	Genogroup 1	L
25	EHNV	0	No VHSV, IHNV, EHNV, SVCV, IPNV	0	0
26	Ranavirus	VHSV Ib/ IHNV E	Ranavirus	1d	E
27	EHNV	VHS: I-b	Ranavirus	Id	European isolate
28	EHN	IHN: E; VHS: Ib	ECV	Id	E
29	EHNV	VHS: Geno 1b: N-gene;isolate Ip8; IHNV Geno E; G-gene	ECV	0	Geno M;G gene
30	EHNV	VHSV:1b IHNV:M	Ranavirus-NOT EHNV	Genogroup 1d	U
31	EHNV	0	EHNV	SVCV	0
32	EHNV	VHS: Ib IHN: E	RANAVIRUS	Id	E
33	EHNV	IHNV: E (Europe; former genogroup M); VHSV: Ib	Ranavirus – NOT EHNV	SVCV: Id	IHNV: E (Europe; former genogroup M)
34	EHNV	VHSV: Ib IHNV: E	Ranavirus – NOT EHNV	Id	Mix of 2 genotypes: seq A: L seq B: E
35	EHNV	VHSV: Ib, IHNV: E	Ranavirus - NOT EHNV	0	E
36	EHNV	0	Ranavirus/European catfish virus	0	0
37	no VHSV; no IHNV, no IPNV, no SVCV	0	no VHSV; no IHNV, no IPNV, no SVCV	0	0
38	Ranavirus, EHNV	VHS: Genotype Ib IHNV: E	Ranavirus, not EHNV	SVCV: Id	IHN: U
39	EHNV	VHSV: Ib, IHNV: E	Ranavirus - NOT EHNV	Id	E
40	EHNV	VHSV:Genotype Ib IHNV: Genogroup E	ECV	Genotype Id	Genogroup E
41	EHNV	E Ib	Ranavirus - NOT EHNV	1d	U
42	Ranavirus	0	Ranavirus	0	0
43	NO IHNV, VHSV,SVCV, IPNV	0	VHSV	0	0

44	EHNV	0	Ranavirus NOT EHNV	0	0
45	EHNV	Genotype: Ia-1; Denmark	ECV	Genotype: Id; Hungary	Genotype: E, Germany
46	EHNV	E ; 1b	Ranavirus - NOT EHNV	1d	U

[1] This laboratory doesn't test for ranavirus
[2] This laboratory has used commercial PCR kit to identify EHNV
[3] This laboratory has not provided corroborating data to support the finding of EHNV in ampoule I and Ranavirus in ampoule III

Résumé and concluding remarks PT1

The parcels were submitted on a Friday and 78% of parcels were delivered by the shipping companies within one week, 93% was delivered within two week and 100% was delivered within 41 days.
Overall 33 out of 45 participants scored 100% success rate; out of the 12 laboratories which underperformed four participants scored <100% for the sole reason that they did not back up their concluding results of ampoule I and/or III (EHNV/ECV) with sequencing. Suggestions to improve on underperformance will be provided individually to each laboratory.

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, especially as it appears that the variations in titres between laboratories this year was rather high – with more than 7 log differences from the lowest to the most sensitive performances. Laboratories scoring low titres should definitely consider to exchange their cell lines with more sensitive strains or assess if the performance of their cells could be improved and the laboratories scoring very high titres should ensure that the titration procedure is properly implemented.

Although the direct comparison of Ct Values cannot be done due to specific differences in laboratory, reagents, assay setup etc. the tables included in this report may provide valuable information for the participating laboratories, in assessing their results with other laboratories as well as with the EURL, and evaluate the working pipeline in the molecular laboratory, in case of significant differences in the results are obtained. Further specifications both on the assay set up and on the working pipeline will be provided at the specific meeting on April 8th.

This year we have not provided the score for the sequencing, there is still a large variations in sequencing efforts. Thanks to the table provided, each laboratory can compare its own sequence analysis and genotyping.
Compared to previous years there is an increased homogeneity within the nomenclature used by participants to define the isolates detected within the ampoules. The next step to further harmonize the analysis, is to provide a list of reference sequence to perform genotyping and a list of reference genotypes/genogroups to each viral species.
The sequencing and genotyping of VHSV is well implemented in the network of laboratory participating in this Inter-Laboratory proficiency test , 30 laboratories have sequenced VHSV, and 28 have correctly genotyped the isolate in ampoule II as Genotype 1b.
The sequencing of the IHNV isolate in ampoule V has posed some more challenges, 23 laboratories have sequenced the isolate but 10 have indicated a genogroup different from “E”, being this isolate one of the first discovered in Europe, it likely to belong to M Genogroup, hence “M” could also be considered correct answer
Interestingly one laboratory has initiated the use of Whole Genome Sequencing (WGS) to identify the isolates and provide genotyping.

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. We will also take the opportunity to provide a comment to participants regarding submitted results if relevant. Furthermore we encourage all participants to contacts us with any questions concerning the test or any other diagnostic matters.

The results presented in this report will be further presented and discussed at the 26th Annual Workshop of National Reference Laboratories for Fish Diseases to be held 30th and 31st of May , 2022.

73% of the participating laboratories obtained 100% success rate in PT1.

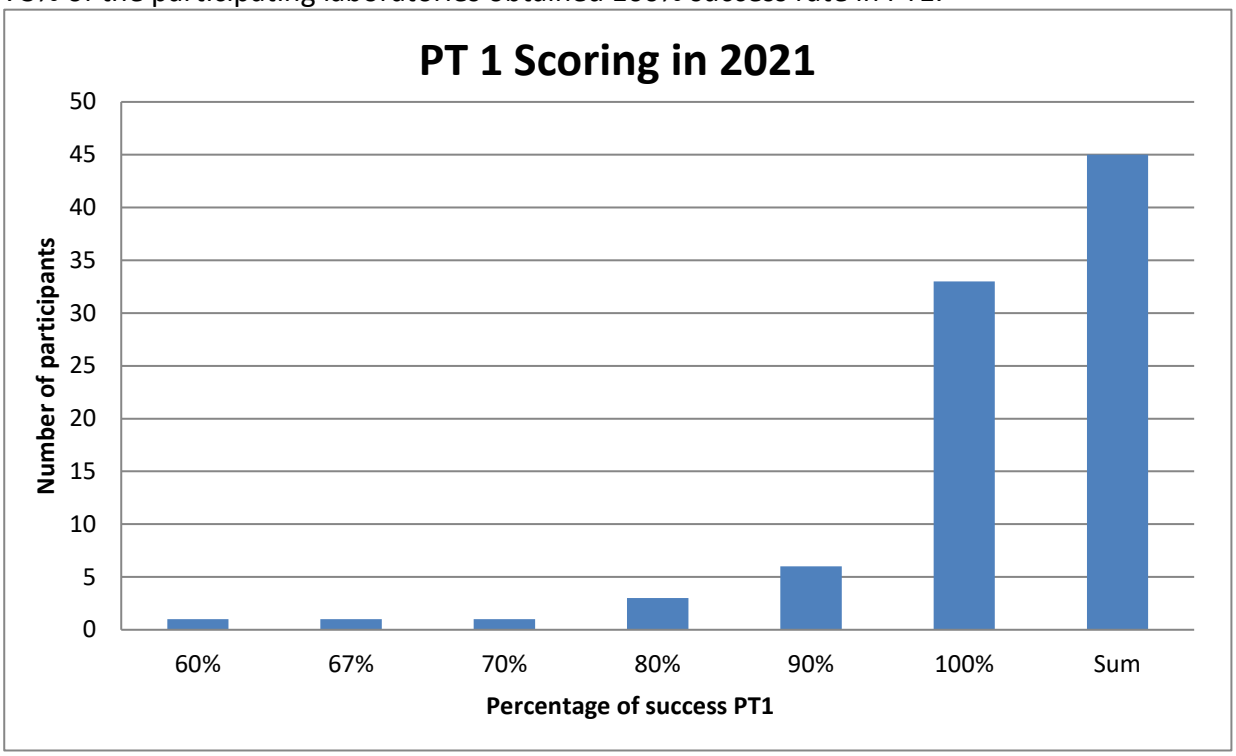


Figure 10 Success-rate of participating laboratories 2021 for PT1

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 laboratory 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) [8]and the conventional PCR protocol described by Bercovier et *al*. (2005) [9], the SAV real-time RT-PCR protocol described by Hodneland et al. (2006) [10], and the conventional PCR targeting segment E2 described by Fringuelli et al. (2008) [11] and the ISAV real-time RT-PCR protocol described by Snow et *al*. (2006) [12] and conventional RT-PCR protocol described by Mjaaland et *al*. (2002) [13].

The details of the virus isolates used in the proficiency test 2 are outlined in table 11.

Table 11. 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 19. as isolate S49p **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

Code	Specifications/References
Ampoule VII: CyHV-3/KHV	**KHV-TP 30 (syn: KHV-T (for Taiwan))** KHV-TP 30 was isolated from koi in Taiwan and cloned for producing large plaques by Dr. Peiyu Lee, Institute of Medical Biotechnology, Central Taiwan University of Science and Technology, Dakeng, BeiTung District, TaiChung City 406, Taiwan in 2005. **Received from:** Dr. Sven M. Bergmann, Friedrich-Loeffler-Institut (FLI), Federal Research Institute for Animal Health, Südufer 10, 17393 Greifswald-Insel Riems, Germany **Reference on isolate:** The TK gene is 100% identical to serval isolates present in the Genbank e.g. KX609547.1
Ampoule VIII: ISAV	**ISAV Glesvaer/2/90** **Received from:** Dr. B. Dannevig, OIE Reference Laboratory for ISA, Oslo, Norway **HPR Genotype:** 2 **GenBank accession numbers:** HQ259676, or AF220607.1 or DQ785248.1 **References on isolate:** Dannevig BH, Falk K & Namork E (1995). Isolation of the causal virus of infectious salmon anaemia (ISA) in a long-term cell line from Atlantic salmon head kidney. *Journal of General Virology* 76, 1353–1359. Falk K, Namork E, Rimstad E, Mjaaland S & Dannevig BH (1997). Characterization of infectious salmon anemia virus, an orthomyxo-like virus isolated from Atlantic salmon (Salmo salar L.) *Journal of Virology* 71, 9016-9023. **References on sequence:** 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 IX: Blank	**Cell supernatant from BF-2 cells** **03/16** **Passage No.: 28.**

Testing of the PT2 test

The PT2 test was prepared and tested according to protocols accredited under DS/EN ISO/IEC 17043. Prior to the distribution we tested 5 ampoules of each virus preparation, by real-time PCR (Gilad et *al*. (2004))[8] for KHV, by real-time RT-PCR (Snow et *al*. (2006)) [12]for ISAV and by real-time RT PCR (Hodneland et al. (2006))[10] 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, after deadline for handling in results and 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 12 and Figure 12). Conventional PCR/RT-PCR fragments were sequenced and so was the HPR region in segment 6 of the ISAV isolates.

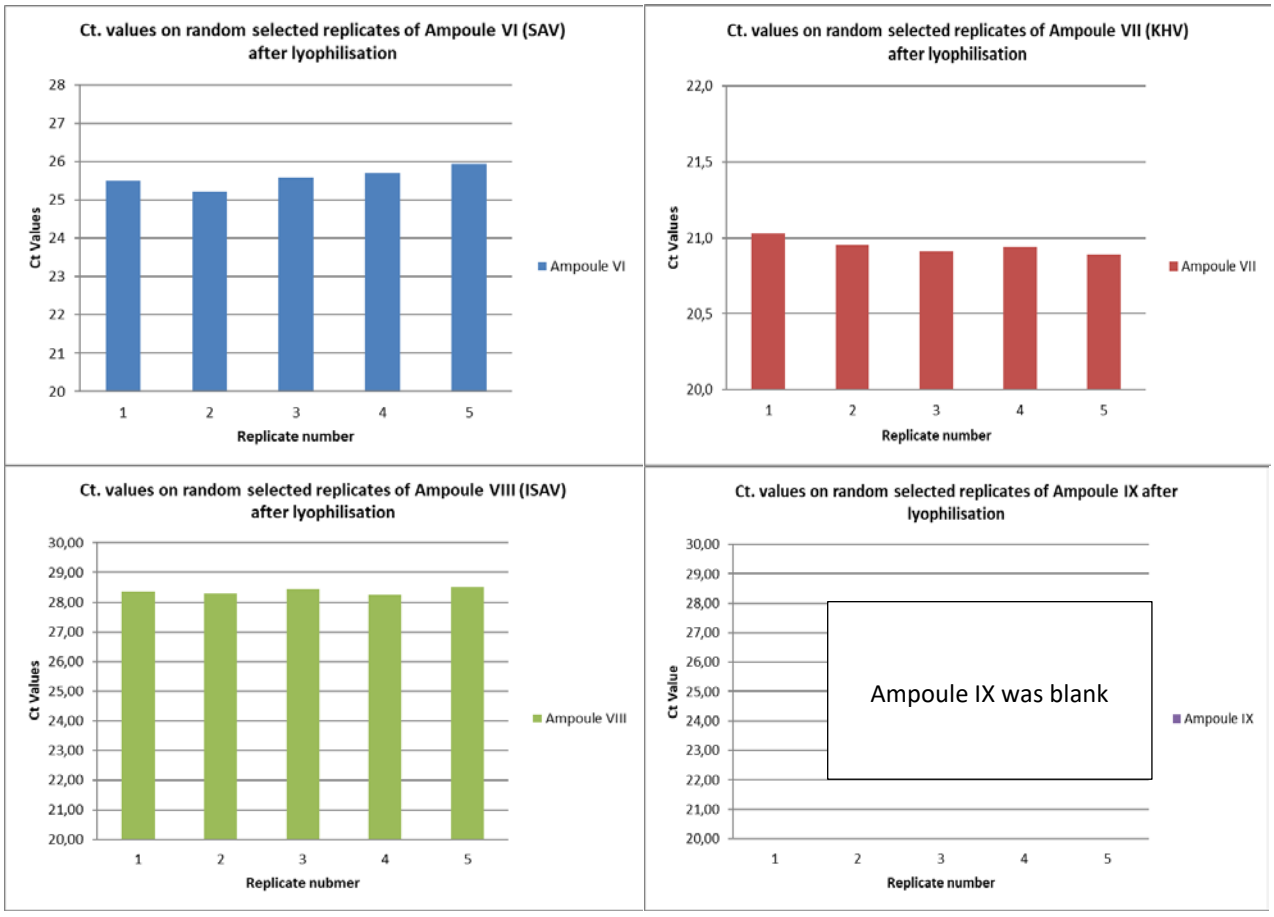


Figure 11, Ampule VI (SAV), VII (KHV), VIII (ISAV) tested shortly after lyophilisation to assess homogeneity of the content.

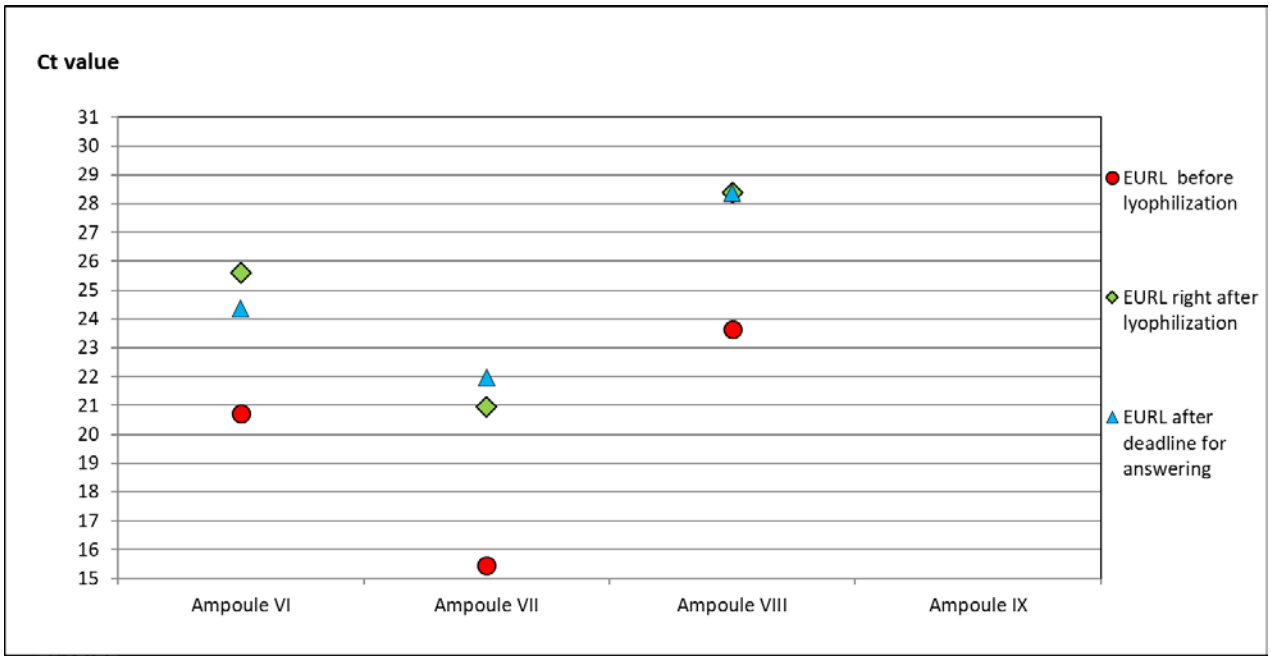


Figure 12, Ampoule VI, VII, VIII and IX tested before and after lyophilisation and after deadline for handling in results.

Table 12: Ct-value of ampoules VI to 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	20,72	25,50	24,36
		b		25,23	
		c		25,58	
		d		25,69	
		e		25,94	
			20,72	**25,59**	**24,36**
Ampoule VII	**KHV**	a	15,45	21,03	21,95
		b		20,95	
		c		20,91	
		d		20,94	
		e		20,89	
			15,45	**20,94**	**21,95**
Ampoule VIII	**ISAV**	a	23,63	28,37	28,35
		b		28,29	
		c		28,43	
		d		28,23	
		e		28,51	
			23,63	**28,37**	**28,35**
Ampoule IX	**Blank**	a	No Ct	No Ct	No Ct.
		b		No Ct	
		c		No Ct	
		d		No Ct	
		e		No Ct	
			No Ct	**No Ct**	**No Ct**

The lyophilisation procedure caused a significant virus reduction in all four ampoules (between 4-6 Ct. values) 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 according to diagnostic procedures described in the EURL diagnostic manuals [2]. 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, so, theoretically, the isolates should be viable.
Also this year, the panel of pathogens to be investigated included 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.
In order to obtain uniform answers, participants were requested to download a spreadsheet available from the https://www.eurl-fish-crustacean.eu, insert results in this and return by email.
The results from participating laboratories are shown in table 13.

Table 13. Inter-Laboratory Proficiency Test, PT2, 2021 - Virus identification.

Laboratory code number	Score	Ampoule VI	Ampoule VII	Ampoule VIII	Ampoule IX
		SAV2 , SD	KHV, (CyHV-3)	HPRΔ ISAV	BF-2 cell supernatant
1	8/8	SAV	KHV	ISAV	Not ISAV, KHV, or SAV
2	8/8	SAV	KHV	HPR-deleted ISAV	Blank
3	8/8	SAV	KHV	HPR-deleted ISAV	Nothing
4	8/8	SAV	KHV	HPR-deleted ISAV	-
5	8/8	SAV	KHV	HPR deleted ISAV	Negative
6	8/8	SAV	KHV	HPR-deleted ISAV	Pathogen not detected
8	8/8	SAV	KHV	HPR-deleted ISAV	Negative
9	8/8	SDV	KHV	ISAV	NO KHV, ISAV, SAV
10	8/8	SAV	KHV	HPR-deleted ISAV	no virus
11	8/8	SAV	KHV	HPR-deleted ISAV	NEG.
12	8/8	SAV	KHV	ISAV	-
13	8/8	SAV	KHV	ISAV	Negative
14	8/8	SAV	KHV	ISAV	Negative/Not detected
15	8/8	SAV	KHV	ISAV	0
16	6/8	SAV	KHV	NEG	NEG
17	7/8	SAV	KHV	ISAV	Not ISAV, Not KHV, Not SAV
18	8/8	SAV	KHV	HRP-deleted ISAV	NOT ISAV, KHV, SAV
19	8/8	SAV	KHV	HPR-deleted ISAV	Not KHV; Not SAV; Not ISAV
20	6/6	0	KHV	HPR-deleted ISAV	0
21	8/8	SAV	KHV	HPR-deleted ISAV	-
22	8/8	SAV	KHV	HPR-deleted ISAV	No virus detected
23	7/8	SAV	KHV + IPNV	HPR-deleted ISAV	Negative
24	8/8	SAV	KHV	ISAV	No virus detected
25	7/8	SAV	KHV	ISAV	_
26	6/6	0	KHV	HPR-deleted ISAV	0
27	8/8	SAV	KHV	ISAV	0

28	8/8	SAV	KHV	ISAV	NEG
29	8/8	SAV	KHV	ISAV	BLANK
30	8/8	SAV	KHV	HPR-deleted ISAV	Not ISAV, Not SAV, Not KHV
31	6/6	0	KHV	ISAV	0
32	8/8	SAV	KHV	ISAV-HPRΔ	NO KHV NO ISAV NO SAV
33	8/8	SAV	KHV	HPR-deleted ISAV	negative
34	8/8	SAV	"KHV Japanese lineage"	HPR-deleted ISAV	no SAV, no KHV, no ISAV
35	8/8	SAV	KHV	HPR-deleted ISAV	No virus
36	7/8	SDV	KHV	ISAV and SPDV	No virus detected
37	6/6	no ISAV; no KHV	KHV	HPR-deleted ISAV	no ISAV; no KHV
38	8/8	SAV	KHV	ISAV	Not ISAV, not KHV, not SAV
39	8/8	SAV	KHV	ISAV	negative
40	8/8	SAV	KHV	ISAV	not KHV, not ISAV nor SAV
41	8/8	SAV	KHV	HPR-deleted ISAV	0
42	7/8	SAV	KHV	ISAV	no virus
43	0	0	0	0	0
44	8/8	SAV	KHV	HPR deleted ISAV	No SAV No KHV No ISAV
45	8/8	SAV	KHV	ISAV	no virus detected
46	8/8	SAV	KHV	HPR-deleted ISAV	Negative

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

All laboratories are asked to sequence the HPR region of ISAV isolates to distinguish from the pathogenic HPRΔ variant from ISAV HPR0 .

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

- 44 laboratories submitted results
- 34 laboratories correctly identified all four ampoules (SAV, KHV, ISAV, Blank)
- All 44 laboratories tested for the two listed pathogens (KHV, ISAV)
- 40 laboratories tested for SAV
- 1 laboratory that did participate in PT 1 did not participate in PT2

Ampoule VI – SAV

- 40 laboratories correctly identified SAV
- 4 laboratories did not participate for SAV and answered ‘0’ or ‘no ISAV; no KHV’

Ampoule VII – KHV

- All 44 laboratories correctly identified KHV hereof one laboratory had a contamination with another pathogen.

Ampoule VIII – ISAV

- 43 laboratories correctly identified ISAV hereof three laboratories did not sequenced and one laboratory had a contamination with another pathogen.
- 1 laboratories did not find ISAV and answered ‘NEG’

Ampoule IX – Blank

- All 44 laboratories ruled out the presence of pathogens they were testing for, the answers varied from 'Not KHV, Not ISAV, Not SAV' to leaving the field empty.

Scores

We have assigned a score of 2 points for each ampoule (Table 13), giving the possibility for obtaining a maximum score of 8. Identifying the correct pathogen or the correct combination of pathogens in one ampoule gives score of 2 points.
For the ISAV isolate in ampoule VIII, full score was given if ISAV virus was detected by molecular methods, and if the isolate was sequenced to discriminate between listed HPRΔ ISAV and non listed HPR0 ISAV.
Of the 44 laboratories submitting results 38 laboratories obtained maximum score. The maximum score was calculated according to the number of pathogen tested by the laboratory.
A laboratory could obtain a maximum score of 8 if tested for all three pathogens included (ISAV, KHV and SAV). A maximum score of 6 is given if only tested for ISAV and KHV.

Ct. values

We have encouraged participants to insert the Ct value in the spreadsheet if they have performed a real-time (RT-) PCR.
The Ct. values obtained by the participating laboratories are summarised in tables 14. The Ct. values obtained from each participating laboratory are represented graphically in Figures 13. On these figures, the median Ct value and the 25% and 75% inter-quartile range is displayed.

The Ct-values cannot be directly compared due to the use of different methods, reagents and equipment nucleic acid extraction and (RT)-qPCR.

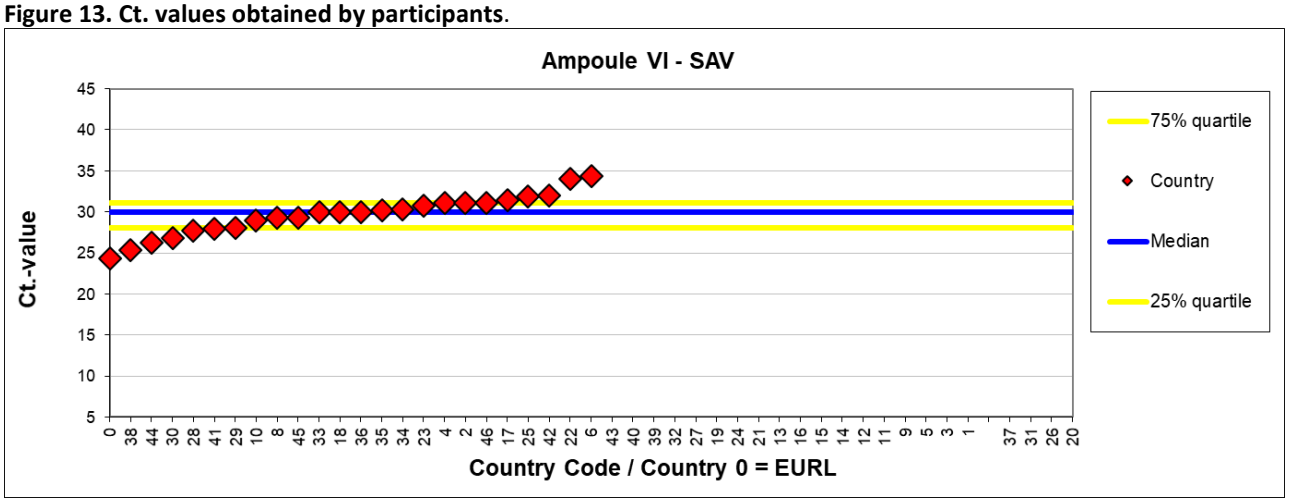
Table 14. Inter-Laboratory Proficiency Test, PT2, 2021 – Ct.-values.

Laboratory Code number	Ct. value Ampoule VI (SAV)	Ct. value Ampoule VII (KHV)	Ct. value Ampoule VIII (ISAV)
EURL	24,4	22,0	28,35
1	N/A	N/A	32,7
2	31,1	23,9	31,7
3	N/A	N/A	N/A
4	31,1	23,7	32,9
5	N/A	N/A	N/A
6	34,4	N/A	N/A
8	29,3	20,7	34,7
9	N/A	21,6	33,3
10	28,9	20,8	31,2
11	N/A	16,6	20,4
12	N/A	N/A	N/A
13	N/A	N/A	N/A
14	N/A	19,3	N/A
15	N/A	25,2	32,8
16	N/A	22,3	N/A
17	31,5	21,4	32,6

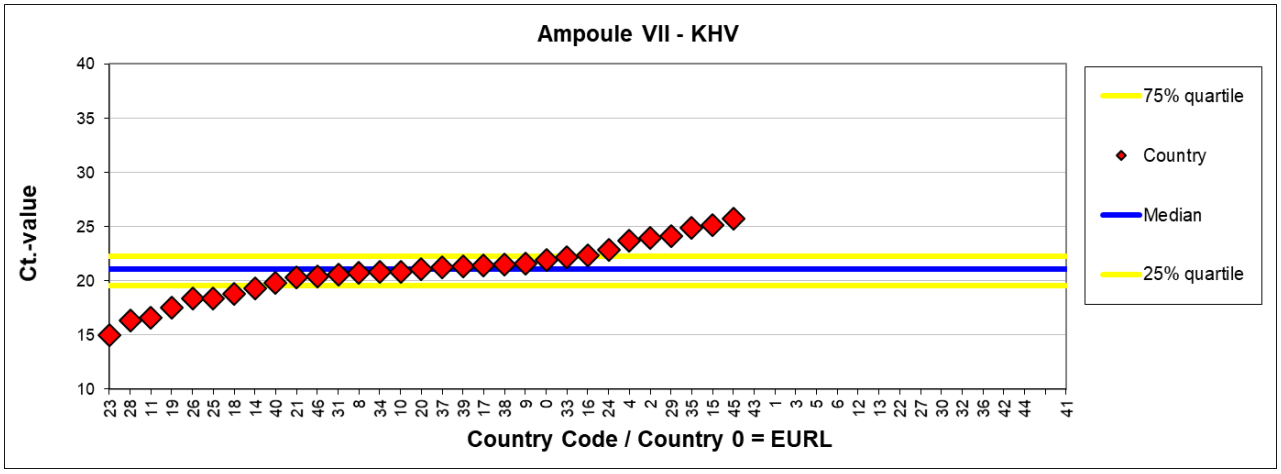
18	30,0	18,8	32,4
19	N/A	17,5	N/A
20	N/A	21,0	28,7
21	N/A	20,3	35,1
22	34,0	N/A	38,6
23	30,7	15,0	32,7
24	N/A	22,8	25,1
25	31,9	18,4	32,6
26	N/A	18,3	26,6
27	N/A	N/A	N/A
28	27,7	16,4	31,3
29	28,0	24,1	34,6
30	26,8	N/A	33,1
31	N/A	20,6	30,6
32	N/A	N/A	N/A
33	29,9	22,2	32,9
34	30,3	20,8	N/A
35	30,2	24,9	29,7
36	30,0	N/A	27,6
37	N/A	21,3	36,3
38	25,4	21,5	28,4
39	N/A	21,3	21,3
40	N/A	19,8	N/A
41	27,9	N/A	N/A
42	32,0	N/A	30,0
43	-	-	-
44	26,2	N/A	33,5
45	29,3	25,7	30,9
46	31,1	20,4	29,7

N/A: No Ct-value given by the participating laboratory. [1)] Did not participate in PT2.
Amp.IX is blank and thereby not included

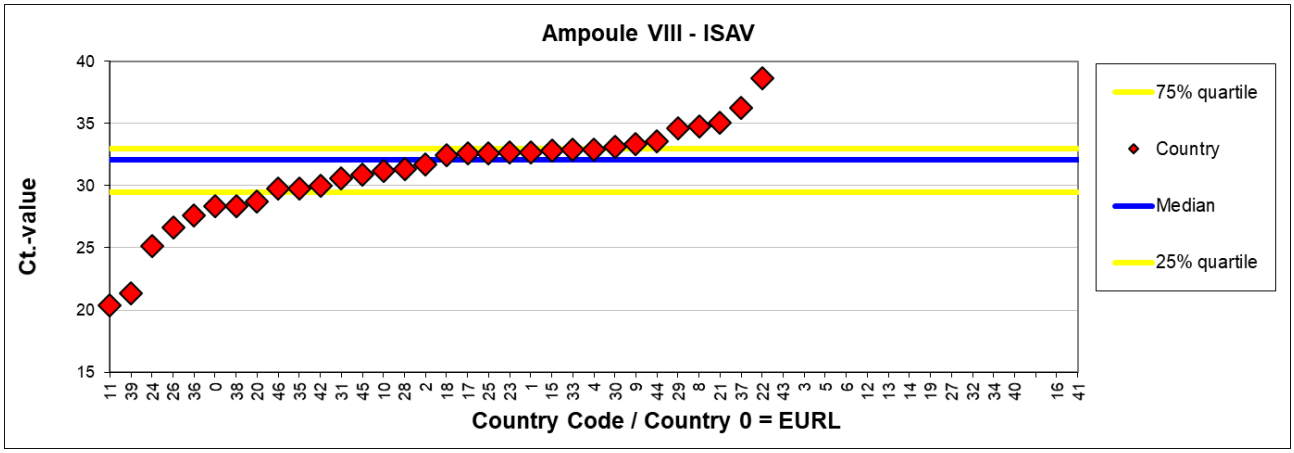
Figure 13. Ct. values obtained by participants.



Number of laboratories	47
Median Ct.-value	30,0
Maximum Ct.-value	34,4
Minimum Ct.-value	24,4
25% quartile Ct.-value	28,0
75% quartile Ct.-value	31,1



Number of laboratories	47
Median Ct.-value	21,0
Maximum Ct.-value	25,7
Minimum Ct.-value	15,0
25% quartile Ct.-value	19,6
75% quartile Ct.-value	22,3



Number of laboratories	47
Median Ct.-value	32,1
Maximum Ct.-value	38,6
Minimum Ct.-value	20,4
25% quartile Ct.-value	29,5
75% quartile Ct.-value	33,0

Genotyping and sequencing

Participants were asked to sequence the HPR region of possible ISAV isolates and determine whether isolates included in the ampoules were HPRΔ ISAV currently listed in EU legislation or non-listed HPR0 ISAV, the correct characterization of HPRΔ ISAV has been calculated in the general score. Three laboratories did not sequence the ISAV isolate in ampoule VIII; importantly all the laboratories that sequenced the HPR segment concluded that the isolated was HPRΔ ISAV. The identification of KHV didn't pose particular issues. Finally, regarding sequencing of SAV isolate in ampoule VI, only 2 out 29 of laboratories which performed genotyping of SAV, assigned the incorrect genotype.
An Overview of the genotyping results obtained for PT2 by all participants is displayed in the following table 15.

Table 15 The genotyping results obtained for PT2 by all 44 participants

Code number	Ampoule VI	Ampoule VII	Ampoule VIII
	SAV2	KHV, CyHV-3	HPRΔ ISAV
1	2	CyHV3 - Asian	European- deleted
2	0	0	HPR Genotype 2, HPR deleted
3	0	0	ISAV4 (segment 6)
4	2	CyHV-3	HPR-deleted
5	2	CyHV-3	HPR 2
6	0	0	HPR-deleted
8	0	0	HPR-deleted, HPR-2 (genotype)
9	SAV2	CyHV-3	ISAV_HPR-deleted
10	2	0	HPR-deleted
11	SAV 2	CyHV 3	HPR-deleted
12	SAV3	CyHV-3	HPR genotype 2
13	II	CyHV-3	HPR4
14	SAV (SD)	CyHV3	HPR deleted (HPR2)
15	2	CyHV 3	HPR-deleted
16	2	0	0
17	0	CyHV-3	0
18	0	CyHV3	HPR-deleted
19	2	CyHV 3	HPR- deleted
20	0	CyHV-3	HPR-deleted
21	SAV2	0	HPR Genotype2, HPRΔ
22	2	CyHV3	HPR-deleted
23	SAV 2	CyHV 3	HPR-deleted ISAV

		IPNV - Genogroup: 5, serotype: Sp, serogroup: A	
24	2	CyHV 3	ISAV HPR-deleted
25	0	0	0
26	0	CyHV3	HPR2
27	2	CyHV3	ISAV HPRΔ, CIIIa
28	II (SAV2)	CyHV3	HRP-deleted
29	SAV2	CyHV-3	HPR-deleted
30	3	CyHV3	HPR-deleted
31	0	Cyprinid herpesvirus 3	Salmon Isavirus
32	SAV2	CyHV-3	ISA-HPRΔ
33	2	no genotypes but lineages	HPR-deleted
34	2	CyHV3 Japanese lineage	ISAV: HPR-deleted
35	0	CyHV-3	HPR-deleted
36	0	0	SPDV subtype=SAV3
37	0	0	HPR2
38	SAV 2	CyHV3	HPR-deleted, PR4
39	2	CyHV3	HPR-deleted
40	SAV Subtype 2	CyHV3	HPR deleted
41	2	CyHV-3	HPR-deleted
42	0	0	0
43	0	0	0
44	0	0	HPR deleted
45	SAV2; France	CyHV-3; European E, CROATIA	HPR6
46	2	CyHV3	HPR-deleted

Concluding remarks PT2

44 laboratories participated in PT2, 38 obtained 100% success rate. Out of the 6 laboratories which underperformed, three obtained a lower score because did not provide sequencing for the ISAV isolate in ampoule VIII. This point will be addressed directly with the participants that has underperformed.

43 out of the 44 laboratories correctly identified the ISA virus in ampoule VIII, hereof three laboratories did not sequenced. One laboratory did not find ISAV and one had a contamination with another pathogen.

44 laboratories correctly identified the CyHV-3 (KHV) in ampoule VI, hereof one laboratory had a contamination with another pathogen.

40 laboratories tested for SAV and all correctly identified the virus in Ampoule VI, four laboratories did not test for SAV.

It is highly appreciated that many laboratories are putting efforts in performing genetic analysis and further 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.

Regardless instructions provided with the proficiency test on the nomenclature there is still some heterogeneity on how the genotype is provided from each participants. These points will be addressed again at the upcoming ad hoc meeting on the Proficiency test results and at the annual workshop

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. 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 26th Annual Workshop of National Reference Laboratories for Fish Diseases to be held May 30th and 31st 2022

86% of the participating laboratories obtained 100% success rate in PT2.

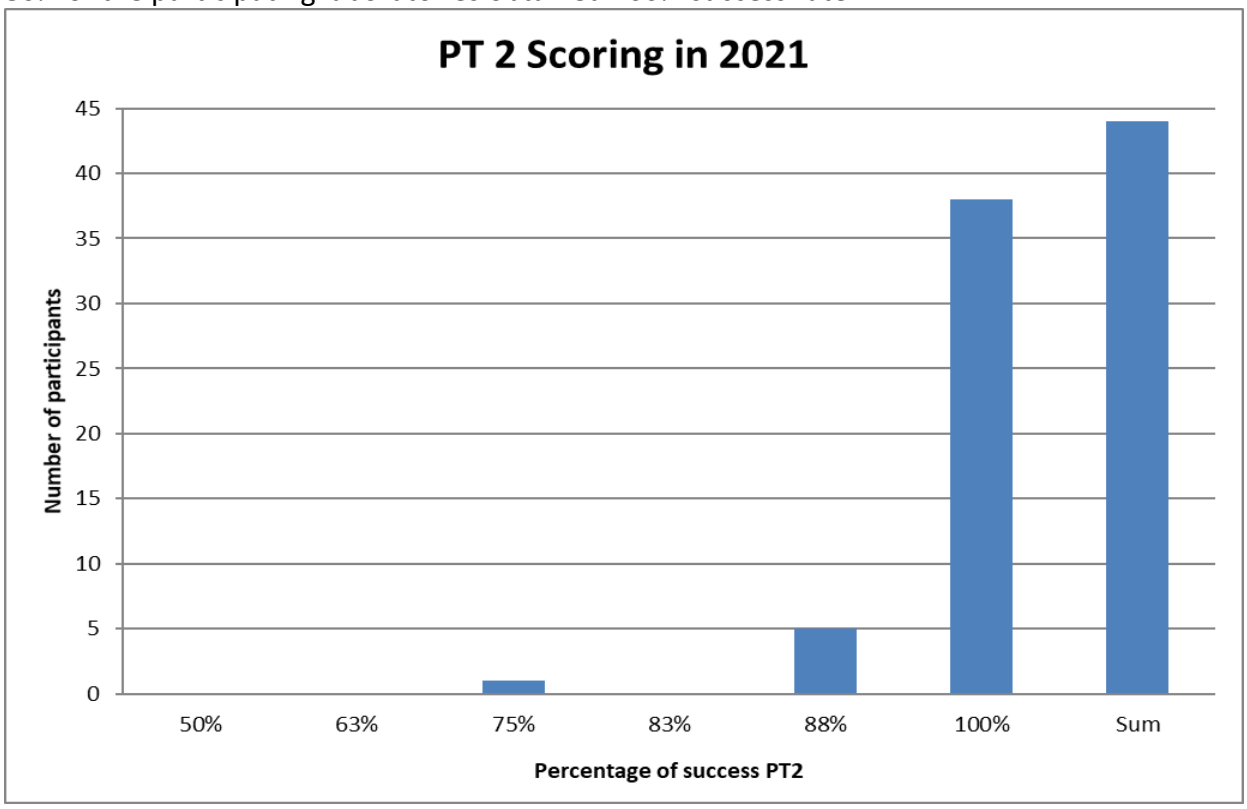


Figure 14 Success-rate of participating laboratories 2021 for PT2

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