Report of the Inter-Laboratory Proficiency Test for National Reference Laboratories for Fish Diseases

2008

Organised by
the Community Reference Laboratory
for Fish Diseases,
Technical University of Denmark, National Veterinary Institute, Århus, Denmark

Content

(Figure 1-4 is attached as last pages of the report)

Introduction

A comparative test of diagnostic procedures was provided by the Community Reference Laboratory for Fish Diseases (CRL) to 35 National Reference Laboratories (NRLs) in the middle of October 2008.
The test contained five coded ampoules, with viral haemorrhagic septicaemia virus (VHSV), infectious haematopoietic necrosis virus (IHNV), infectious pancreatic necrosis virus (IPNV) or a mixture of VHSV and IPNV, respectively. The proficiency test was designed to primarily assess the ability of participating laboratories to identify the notifiable non-exotic viruses: VHSV and IHNV but also to assess their ability to differentiate other fish viruses, as IPNV, spring viraemia of carp virus (SVCV) perch rhabdovirus etc. In addition the participants were asked to titrate the viruses in order to assess the cell susceptibility for virus infection in the respective laboratories.
Participants were asked to reply latest December 12th 2008.
Laboratories were encouraged to geno- and serotype isolates. Furthermore, due to an ongoing discussion on sequencing as a tool for differentiation between various genotypes of the non-exotic viruses, all laboratories were asked to provide a full-length G-gene sequence of the rhabdovirus identified in the lowest numbered ampoule in the test. The aim of this exercise is to provide a tool for assessing the quality of sequence data, by assessing the homogeneity of the sequences obtained from the same virus isolate.
Each laboratory has been given a code number to ensure discretion. The code number of each participant is supplied to the respective laboratories with this report. An un-encoded version of the report is sent to the Commission.

Participants

Five ampoules with lyophilised tissue culture supernatant were delivered to all NRLs in EU Member States, including Denmark, and likewise to the National reference laboratories in Australia, Croatia, Faroe Islands, Iceland, Israel, Japan, Norway, Switzerland and Turkey according to a special agreement. The NRLs for UK in Aberdeen and in Weymouth received a test each. The Belgian NRL covers both Belgium and Luxembourg and likewise the Italian NRL covers Italy, Cyprus, Malta and Greece. Bosnia & Herzegovina did not participate this year. The reference laboratory of the Jiangsu Province of the P.R.China also received the test.

Content of ampoules

The viruses were propagated on each of their preferred cell line, and when total cytopathic effect (CPE) was observed, the supernatants were collected and filtrated through a 45 µm filter, mixed with equal volumes of 20% w/v lactalbumin hydrolysate solution and lyophilized in glass ampoules. The ampoules were sealed by melting. The details of the virus isolates used in the proficiency test are outlined in table 1.

Table 1. Content of each ampoule with reference to culture conditions and major publications of the included virus.

Ampoule I: VHS virus DK-5151 (Rindsholm) (diluted 10^{-3})	Danish freshwater VHSV isolate (1992) from rainbow trout. Cell culture passage number in BF-2: 4th and in EPC: 5th. Neutralization pattern III (Olesen et al. 1993). Genotype Ia, GenBank accession number: AF345859 (G-gene) (Ejner-Jensen et al. 2004). Olesen NJ, Lorenzen N and Jørgensen PEV (1993). Serological differences among isolates of viral haemorrhagic septicaemia virus detected by neutralizing monoclonal and polyclonal antibodies. *Diseases of Aquatic Organisms* **16**, 163-170. 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.
Ampoule II: VHS virus 1p8 (undiluted)	Marine isolate (1996) from herring (*Clupea harengus*) caught in the Baltic Sea (Mortensen et al. 1999). Cell culture passage number in BF-2: 6th. Genotype Ib, GenBank accession number: AY546573 (G-gene) and AY356652 (N-gene) (Einer-Jensen et al. 2004, Snow et al. 2004). Mortensen HF, Heuer OE, Lorenzen N, Otte L and Olesen NJ (1999). Isolation of viral haemorrhagic septicaemia virus (VHSV) from wild marine fish species in the Baltic Sea, Kattegat, Skagerrak and the North Sea. *Virus Research* **63**, 97-108. 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.
Ampoule III: IHN virus 217/A (DTU Vet protocol no. 4008) (undiluted)	First Italian IHNV isolate from rainbow trout (Bovo et al. 1987). Cell culture passage number in EPC: 10th. 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.
Ampoule IV: VHS virus DK-5151 (Rindsholm) + IPN virus Type Sp (mixed 1:1)	Same VHSV as in ampoule I mixed with same IPNV as in ampoule V.
Ampoule V: IPN virus Type Sp (undiluted)	Type Sp (Spjarup) strain of IPN virus (Jørgensen & Grauballe 1971). Cell culture passage number in BF-2: 17th Jørgensen PEV and Grauballe PC (1971). *Acta Veterinaria Scandinavica* **12**, 145.

Testing of the test

The inter-laboratory test 2008 was prepared and tested according to protocols accredited under DS/EN ISO/IEC 17025 and ILAC-G13: 2000 standards. Prior to distribution the CRL tested 5 ampoules of each virus preparation by titration in 4 cell lines (BF-2, EPC, RTG-2 and FHM), to ascertain a satisfactory titre in the preferred cell line and homogeneity of content of ampoules (Table 2).

The lyophilisation procedure caused a significant titre reduction, especially for the rhabdoviruses where a 2-4 log reduction was observed! However, all titres of the lyophilised viruses were above detection level. Furthermore, when lyophilised the viruses were very stable at storing, tested by titration of one ampoule of each number after 3 months storage in the dark at 4°C. Virus of all ampoules showed similar or no more than one log decrease in titre on their preferred cell line compared to just after lyophilisation (Table 2). We have previously shown that lyophilised virus kept in these ampoules is stable for more than half a year when kept at room temperature (Report of the Inter-Laboratory Proficiency Test for National Reference Laboratories for Fish Diseases 2007; the report is available at http://www.crl-fish.eu/upload/sites/crl-fish/reports/proficiency/report_2007.pdf).

The identities of the viruses in all 5 ampoules were checked and confirmed by ELISA, IFAT, RT-PCR and serum neutralisation tests.

Table 2. Titre of representative ampoules of no. I to V tested at the CRL in four cell lines before lyophilisation, immediately after lyophilisation (median titre of 5 replicates), and after 3 months storage in the dark at 4°C.
(1 replicate), respectively.

Ampoule No.	Content	Cell line	Titre before lyophilisation	Median titre right after lyophilisation	Titre 3 months after lyophilisation (4ºC, dark conditions)
			$TCID_{50}$/ml	$TCID_{50}$/ml	$TCID_{50}$/ml
Ampoule I	VHS virus Rindsholm 5151 Genotype Ia (undiluted "before lyophilisation", diluted 10^{-3} "after lyophilisation")	BF-2	$8.6*10^8$	$1.9*10^3$	$2.7*10^4$
		EPC	$2.7*10^9$	$4.0*10^3$	$4.0*10^3$
		RTG-2	$4.0*10^9$	$2.7*10^3$	$2.7*10^3$
		FHM	$2.7*10^9$	$1.3*10^4$	$8.6*10^3$
Ampoule II	VHS virus 1p8 Genotype Ib	BF-2	$2.7*10^8$	$8.6*10^4$	$1.3*10^5$
		EPC	$< 1.9*10^2$	$1.3*10^3$	$1.9*10^2$
		RTG-2	$2.7*10^5$	$< 1.9*10^2$	$< 1.9*10^2$
		FHM	$5.9*10^3$	$1.3*10^5$	$2.7*10^5$
Ampoule III	IHN virus 217/A (4008)	BF-2	$< 1.9*10^2$	$< 1.9*10^2$	$4.0*10^2$
		EPC	$5.9*10^7$	$2.7*10^4$	$5.9*10^4$
		RTG-2	$1.9*10^7$	$1.3*10^5$	$1.3*10^4$
		FHM	$2.7*10^7$	$4.0*10^4$	$8.6*10^4$
Ampoule IV	VHS virus Rindsholm 5151 + IPN virus Type Sp	BF-2		$1.3*10^7$	$8.6*10^7$
		EPC		$1.3*10^6$	$8.6*10^6$
		RTG-2		$1.9*10^6$	$8.6*10^6$
		FHM		$8.6*10^5$	$2.7*10^6$
Ampoule V	IPN virus Type Sp	BF-2	$4.0*10^9$	$2.7*10^7$	$4.0*10^7$
		EPC	$1.3*10^9$	$2.7*10^6$	$1.3*10^7$
		RTG-2	$4.0*10^9$	$1.9*10^6$	$8.6*10^6$
		FHM	$8.6*10^8$	$1.3*10^6$	$1.9*10^6$

Distribution of the test

The test was sent out according to current international regulations for diagnostic specimens UN 3373, "Biological substance, Category B". Thermo-loggers were included in 14 of the parcels (-40°C to +30ºC) The thermo-loggers were returned immediately upon receipt of the proficiency tests and a computer programme translated the data into a graph, showing the temperature inside the parcel for every 15 minutes during transportation. The loggers were programmed to mark if the temperature had exceeded 30°C at some point during transportation. The average temperature for the transports was 15.3°C; in one laboratory, the temperature rose to 29.5°C. Inclusion of loggers should assure more participants that the temperature encountered during transport has not been detrimental to the viability of the virus in the test.

Virus identification and titration

Participants were asked to identify the content of each ampoule according to the procedures described in the Commission Decision 2001/183/EC, i.e. by a neutralisation test, ELISA, and/or by immunofluorescence. Additional identification by PCR was an option as usual. Identification results of the content of the 5 ampoules for the participating laboratories are summarised in table 3.

Participants were also asked to titrate the contents of the ampoules. The method of titration was described in the instructions enclosed with the test. All titres were calculated at the CRL based on the crude data submitted by each participant and given as Tissue Culture Infective Dose 50% ($TCID_{50}$) per 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)**.** Titration results of the viruses of the 5 ampoules for the participating laboratories are summarised in tables 4 to 8. The titres obtained from each participating laboratory are represented graphically. On figures 1-4 (the number of figures depends on the number of cell lines used by the participant) the titres of an individual participant is displayed for each cell line used by the respective laboratory. Figure 1-4 is attached as last pages of each report. On figures 5-8, all titres submitted by participants for each cell line and ampoule, respectively are compared to each other. On all figures (1-8), 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 as only two laboratories used these cells. Laboratories with the required facilities were encouraged to examine and identify the genotype and the serotype of the virus isolates. However, it was not mandatory to perform these analyses.

Table 3: Inter-Laboratory Proficiency Test 2008 - Virus Identification

Laboratory code number	Score	Answer recived at CRL	Ampoule I	Ampoule II	Ampoule III	Ampoule IV	Ampoule V
			VHSV	VHSV	IHNV	VHSV/IPNV	IPNV
1	8	08-12-08	VHSV	VHSV	IHNV	IPNV	IPNV
2	8	12-12-08	VHSV	VHSV	IHNV	IPNV	IPNV
4	4	01-12-08	Virus not found	Virus not found	IHNV	IPNV	IPNV
5	10	12-12-08	VHSV	VHSV	IHNV	VHSV/IPNV	IPNV
6	10	12-12-08	VHSV	VHSV	IHNV	VHSV/IPNV	IPNV
7	10	27-11-08 and 1-12-2008	VHSV	VHSV	IHNV	VHSV/IPNV	IPNV
8	8	12-12-08	VHSV	VHSV	IHNV	IPNV	IPNV
9	7	12-12-08	VHSV	VHSV	IHNV	VHSV	SVCV
10	10	05-12-08	VHSV	VHSV	IHNV	VHSV/IPNV	IPNV
11	8	02-12-08	VHSV	VHSV	IHNV	IPNV	IPNV
12	10	10-12-08	VHSV	VHSV	IHNV	VHSV/IPNV	IPNV
13	10	12-12-08	VHSV	VHSV	IHNV	VHSV/IPNV	IPNV
14	10	04-12-08	VHSV	VHSV	IHNV	VHSV/IPNV	IPNV
15	10	21-11-08	VHSV	VHSV	IHNV	VHSV/IPNV	IPNV
16	6	12-12-08	VHSV	VHSV	IHNV	VHSV/IPNV/ IHNV	VHSV/IPNV
17	10	11-11-08	VHSV	VHSV	IHNV	VHSV/IPNV	IPNV
18	10	11-12-08	VHSV	VHSV	IHNV	VHSV/IPNV	IPNV
19	10	12-12-08	VHSV	VHSV	IHNV	VHSV/IPNV	IPNV
20	10	12-12-08	VHSV	VHSV	IHNV	VHSV/IPNV	IPNV
21	8	12-12-08	SVCV	VHSV	IHNV	VHSV/IPNV	IPNV
22	8	12-12-08	VHSV	VHSV	IHNV	IPNV	IPNV
23	10	08-12-08	VHSV	VHSV	IHNV	VHSV/IPNV	IPNV
24	8	05-12-08	VHSV	VHSV	IHNV	IPNV	IPNV
25	8	10-12-208	VHSV	VHSV	VHSV/IHNV	VHSV/IPNV	IPNV
26	10	11-12-08	VHSV	VHSV	IHNV	VHSV/IPNV	IPNV
27	10	12-12-08	VHSV	VHSV	IHNV	VHSV/IPNV	IPNV
28	10	10-12-08	VHSV	VHSV	IHNV	VHSV/IPNV	IPNV
29	10	08-12-2008 and 11-12-2008	VHSV	VHSV	IHNV	VHSV/IPNV	IPNV
30	10	11-12-08	VHSV	VHSV	IHNV	VHSV/IPNV	IPNV
31	8	12-12-08	VHSV	VHSV	IHNV	IPNV	IPNV
32	10	09-12-08	VHSV	VHSV	IHNV	VHSV/IPNV	IPNV
33	8	05-12-08	VHSV	VHSV	IHNV	IPNV	IPNV
34	10	12-12-08	VHSV	VHSV	IHNV	VHSV/IPNV	IPNV
35	10	12-12-08	VHSV	VHSV	IHNV	VHSV/IPNV	IPNV
36	10	12-12-08	VHSV	VHSV	IHNV	VHSV/IPNV	IPNV

Correct ID	33	34	34	24	33
No virus	1	1	0	0	0
Wrong ID	1	0	1	11	2
No ID	0	0	0	0	0
No reply	0	0	0	0	0
Total	35	35	35	35	35

Table 4: Inter-Laboratory Proficiency Test 2008 – Identification and titration of ampoule I.

Ampoule I VHSV						
Laboratory code number	**Virus Identification**	**Titre in**				
		BF-2	**EPC**	**RTG-2**	**FHM**	**CHSE-214**
1	VHSV	1.3E+03	1.3E+03			
2	VHSV	1.9E+04	1.3E+05			
4	Virus not found					
5	VHSV	8.6E+02	8.6E+02			
6	VHSV	5.9E+03	4.0E+03			
7	VHSV	1.3E+08	1.3E+03	1.9E+02	8.6E+02	
8	VHSV	2.7E+03	1.9E+03			
9	VHSV		<1.9E+02	<1.9E+02		
10	VHSV	2.7E+02	1.9E+03			
11	VHSV		2.7E+04	<1.9E+02		
12	VHSV	1.9E+04	1.9E+04			
13	VHSV	1.3E+04	1.3E+04			
14	VHSV	8.6E+03	1.3E+03			
15	VHSV	8.6E+03	2.7E+03	4.0E+03	8.6E+03	
16	VHSV	1.9E+03	8.6E+02			
17	VHSV	5.9E+03	1.9E+03	<1.9E+02		<1.9E+02
18	VHSV	1.9E+02	1.9E+02	<1.9E+02	5.9E+02	
19	VHSV	4.0E+03	1.9E+02			
20	VHSV	<1.9E+02	<1.9E+02	<1.9E+02	<1.9E+02	
21	SVCV	1.9E+08	5.9E+07			
22	VHSV	<1.9E+02			1.9E+03	
23	VHSV	5.9E+05	1.3E+05	1.3E+05	1.3E+05	
24	VHSV	5.9E+02	8.6E+02			
25	VHSV		1.9E+03		1.9E+03	
26	VHSV	2.7E+03	1.3E+04	4.0E+02		4.0E+02
27	VHSV	4.0E+02	1.9E+02			
28	VHSV	1.9E+03	2.7E+02			
29	VHSV	8.6E+02	2.7E+03			
30	VHSV	1.9E+03	4.0E+03	1.9E+03	8.6E+03	
31	VHSV	8.6E+02	1.3E+03			
32	VHSV	5.9E+02	1.9E+03			
33	VHSV	1.3E+04	1.9E+03	1.3E+03	1.9E+03	
34	VHSV	2.7E+03	2.7E+02			
35	VHSV		1.9E+02			
36	VHSV		1.3E+03	1.9E+02		

Number of laboratories	29	33	12	9	2
Median titre	2.7E+03	1.9E+03	1..9E+02	1..9E+03	2..0E+02
Maximum titre	1.9E+08	5.9E+07	1..3E+05	1..3E+05	4..0E+02
Minimum titre	<1.9E+02	<1.9E+02	<1..9E+02	<1..9E+02	<1..9E+02
25% quartile titre	8.6E+02	8.6E+02	<1..9E+02	8..6E+02	1..0E+02
75% quartile titre	8.6E+03	4.0E+03	1..5E+03	8..6E+03	3..0E+02

Table 5: Inter-Laboratory Proficiency Test 2008 – Identification and titration of ampoule II.

Ampoule II VHSV						
Laboratory code number	**Virus Identification**	**Titre in**				
		BF-2	**EPC**	**RTG-2**	**FHM**	**CHSE-214**
1	VHSV	2.7E+04	2.7E+03			
2	VHSV	5.9E+02	<1.9E+02			
4	Virus not found					
5	VHSV	4.0E+04	<1.9E+02			
6	VHSV	1.9E+04	4.0E+02			
7	VHSV	1.9E+03	1.9E+02	<1.9E+02	2.7E+03	
8	VHSV	1.3E+04	4.0E+03			
9	VHSV		<1.9E+02	<1.9E+02		
10	VHSV	5.9E+03	5.9E+02			
11	VHSV		1.3E+04	<1.9E+02		
12	VHSV	1.9E+04	<1.9E+02			
13	VHSV	8.6E+04	5.9E+02			
14	VHSV	8.6E+04	5.9E+02			
15	VHSV	5.9E+05	8.6E+02	<1.9E+02	5.9E+05	
16	VHSV	8.6E+03	<1.9E+02			
17	VHSV	2.7E+05	<1.9E+02	1.9E+02		1.9E+02
18	VHSV	2.7E+04	1.9E+03	4.0E+02	5.9E+02	
19	VHSV	4.0E+04	<1.9E+02			
20	VHSV	<1.9E+02	<1.9E+02	<1.9E+02	<1.9E+02	
21	VHSV	1.3E+06	1.3E+07			
22	VHSV	1.3E+03			1.3E+03	
23	VHSV	5.9E+05	5.9E+04	2.7E+04	1.3E+05	
24	VHSV	5.9E+03	<1.9E+02			
25	VHSV		1.9E+03		1.9E+02	
26	VHSV	1.3E+04	8.6E+02	1.9E+04		<1.9E+02
27	VHSV	1.3E+03	<1.9E+02			
28	VHSV	1.9E+05	1.9E+02			
29	VHSV	1.3E+04	4.0E+03			
30	VHSV	5.9E+04	2.7E+03	5.9E+04	1.3E+05	
31	VHSV	1.9E+04	<1.9E+02			
32	VHSV	1.3E+04	4.0E+02			
33	VHSV	1.3E+03	<1.9E+02	<1.9E+02	<1.9E+02	
34	VHSV	8.6E+03	<1.9E+02			
35	VHSV		2.7E+02			
36	VHSV		<1.9E+02	<1.9E+02		

Number of laboratories	29	33	12	9	2
Median titre	1.9E+04	2.7E+02	<1.9E+02	1.3E+03	9.6E+01
Maximum titre	1.3E+06	1.3E+07	5.9E+04	5.9E+05	1.9E+02
Minimum titre	<1.9E+02	<1.9E+02	<1.9E+02	<1.9E+02	<1.9E+02
25% quartile titre	5.9E+03	<1.9E+02	<1.9E+02	1.9E+02	4.8E+01
75% quartile titre	5.9E+04	1.9E+03	5.1E+03	1.3E+05	1.4E+02

Table 6: Inter-Laboratory Proficiency Test 2008 – Identification and titration of ampoule III.

Ampoule III - IHNV						
Laboratory code number	**Virus identification**	**Titre in**				
		BF-2	**EPC**	**RTG-2**	**FHM**	**CHSE-214**
1	IHNV	1.3E+04	1.3E+05			
2	IHNV	<1.9E+02	5.9E+04			
4	IHNV					
5	IHNV	8.6E+03	8.6E+04			
6	IHNV	4.0E+02	1.9E+04			
7	IHNV	<1.9E+02	4.0E+04	<1.9E+02	<1.9E+02	
8	IHNV	2.7E+02	4.0E+03			
9	IHNV		<1.9E+02	<1.9E+02		
10	IHNV	<1.9E+02	8.6E+04			
11	IHNV		8.6E+04	1.9E+04		
12	IHNV	4.0E+03	1.9E+05			
13	IHNV	<1.9E+02	2.7E+04			
14	IHNV	2.7E+03	1.9E+05			
15	IHNV	<1.9E+02	2.7E+04	4.0E+03	2.7E+04	
16	IHNV	<1.9E+02	5.9E+04			
17	IHNV	8.6E+02	8.6E+04	2.7E+04		<1.9E+02
18	IHNV	<1.9E+02	2.7E+05	2.7E+04	4.0E+05	
19	IHNV	4.0E+02	2.7E+04			
20	IHNV	1.3E+03	<1.9E+02	<1.9E+02	<1.9E+02	
21	IHNV	1.3E+04	1.3E+05			
22	IHNV	5.9E+02			1.9E+04	
23	IHNV	1.3E+06	2.7E+05	2.7E+05	5.9E+05	
24	IHNV	1.3E+04	1.3E+04			
25	VHSV/IHNV		2.7E+05		2.7E+05	
26	IHNV	<1.9E+02	5.9E+04	5.9E+02		8.6E+04
27	IHNV	2.7E+02	<1.9E+02			
28	IHNV	<1.9E+02	5.9E+04			
29	IHNV	2.7E+02	8.6E+05			
30	IHNV	5.9E+03	4.0E+06	1.9E+04	5.9E+05	
31	IHNV	<1.9E+02	1.9E+04			
32	IHNV	1.3E+04	5.9E+04			
33	IHNV	<1.9E+02	1.3E+04	1.9E+04	4.0E+04	
34	IHNV	<1.9E+02	1.3E+04			
35	IHNV		1.3E+06			
36	IHNV		8.6E+03	<1.9E+02		

Number of laboratories	29	33	12	9	2
Median titre	2.7E+02	5.9E+04	1.2E+04	4.0E+04	4.3E+04
Maximum titre	1.3E+06	4.0E+06	2.7E+05	5.9E+05	8.6E+04
Minimum titre	<1.9E+02	<1.9E+02	<1.9E+02	<1.9E+02	<1.9E+02
25% quartile titre	<1.9E+02	1.9E+04	<1.9E+02	1.9E+04	2.2E+04
75% quartile titre	4.0E+03	1.3E+05	2.1E+04	4.0E+05	6.5E+04

Table 7: Inter-Laboratory Proficiency Test 2008 – Identification and titration of ampoule IV.

Ampoule IV - VHSV/IPNV						
Laboratory code number	**Virus identification**	**Titre in**				
		BF-2	**EPC**	**RTG-2**	**FHM**	**CHSE-214**
1	IPNV	1.3E+07	1.9E+07			
2	IPNV	8.6E+05	8.6E+06			
4	IPNV					
5	VHSV/IPNV	1.3E+08	1.9E+07			
6	VHSV/IPNV	4.0E+07	2.7E+07			
7	VHSV/IPNV	2.7E+03	8.6E+07	2.7E+07	1.3E+04	
8	IPNV	5.9E+08	1.3E+08			
9	VHSV		1.3E+07	1.3E+07		
10	VHSV/IPNV	1.9E+05	1.3E+06			
11	IPNV		4.0E+06	5.9E+05		
12	VHSV/IPNV	4.0E+07	5.9E+07			
13	VHSV/IPNV	5.9E+07	1.3E+07			
14	VHSV/IPNV	2.7E+09	1.9E+08			
15	VHSV/IPNV	2.7E+07	2.7E+07	4.0E+06	8.6E+06	
16	VHSV/IPNV/IHNV	4.0E+07	8.6E+07			
17	VHSV/IPNV	1.3E+08	8.6E+07	8.6E+07		1.9E+08
18	VHSV/IPNV	1.3E+08	8.6E+07	1.3E+07	8.6E+07	
19	VHSV/IPNV	1.9E+08	1.3E+08			
20	VHSV/IPNV	4.0E+07	8.6E+06	1.9E+07	1.3E+06	
21	VHSV/IPNV	1.9E+07	2.7E+07			
22	IPNV	2.7E+07			2.7E+07	
23	VHSV/IPNV	1.3E+08	5.9E+05	2.7E+07	2.7E+06	
24	IPNV	2.7E+08	5.9E+07			
25	VHSV/IPNV		5.9E+07		1.9E+06	
26	VHSV/IPNV	8.6E+04	1.9E+08	5.9E+07		1.3E+08
27	VHSV/IPNV	1.9E+06	1.9E+04			
28	VHSV/IPNV	1.9E+07	1.9E+07			
29	VHSV/IPNV	5.9E+07	1.9E+07			
30	VHSV/IPNV	8.6E+07	8.6E+07	8.6E+07	1.9E+07	
31	IPNV	2.7E+07	1.9E+07			
32	VHSV/IPNV	4.0E+07	2.7E+07			
33	IPNV	1.3E+05	1.3E+08	1.3E+07	2.7E+08	
34	VHSV/IPNV	8.6E+07	1.9E+07			
35	VHSV/IPNV		5.9E+08			
36	VHSV/IPNV		8.6E+07	5.9E+05		

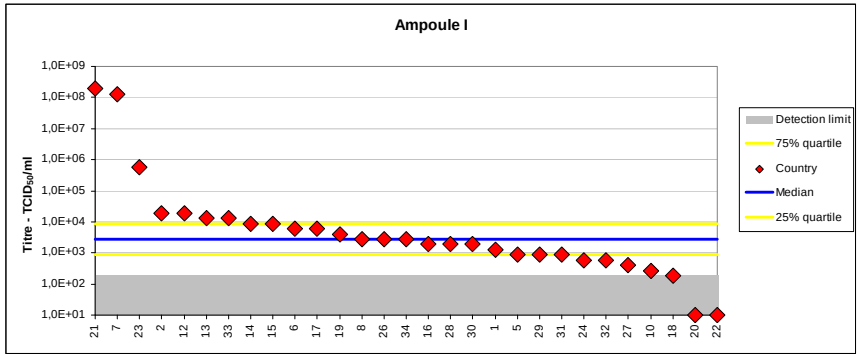
Number of laboratories	29	33	12	9	2
Median titre	4.0E+07	2.7E+07	1.6E+07	8.6E+06	1.6E+08
Maximum titre	2.7E+09	5.9E+08	8.6E+07	2.7E+08	1.9E+08
Minimum titre	2.7E+03	1.9E+04	5.9E+05	1.3E+04	1.3E+08
25% quartile titre	1.9E+07	1.9E+07	1.1E+07	1.9E+06	1.5E+08
75% quartile titre	1.3E+08	8.6E+07	3.5E+07	2.7E+07	1.8E+08

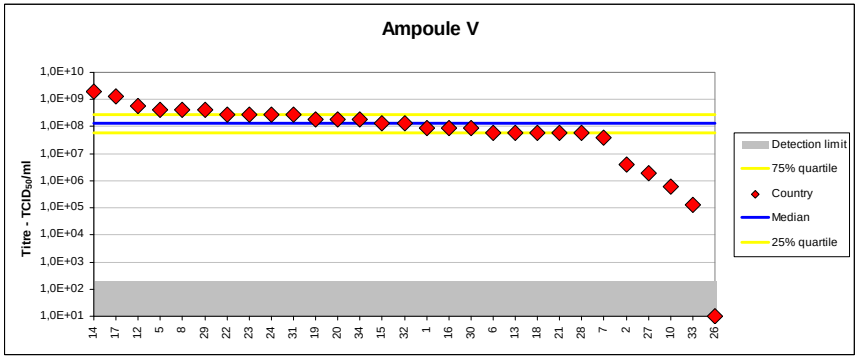
Table 8: Inter-Laboratory Proficiency Test 2008 – Identification and titration of ampoule V.

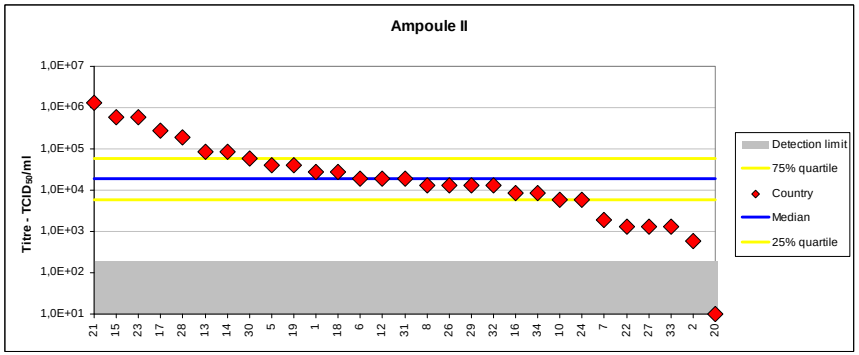
Ampoule V - IPNV						
Laboratory code number	**Virus identification**	**Titre in**				
		BF-2	**EPC**	**RTG-2**	**FHM**	**CHSE-214**
1	IPNV	8.6E+07	1.3E+08			
2	IPNV	4.0E+06	1.9E+08			
4	IPNV					
5	IPNV	4.0E+08	2.7E+07			
6	IPNV	5.9E+07	2.7E+07			
7	IPNV	4.0E+07	4.0E+07	1.9E+07	2.7E+07	
8	IPNV	4.0E+08	8.6E+08			
9	SVCV		1.3E+07	1.3E+07		
10	IPNV	5.9E+05	1.9E+06			
11	IPNV		1.3E+07	5.9E+06		
12	IPNV	5.9E+08	2.7E+08			
13	IPNV	5.9E+07	2.7E+07			
14	IPNV	1.9E+09	2.7E+08			
15	IPNV	1.3E+08	5.9E+07	8.6E+06	1.3E+07	
16	VHSV/IPNV	8.6E+07	4.0E+07			
17	IPNV	1.3E+09	8.6E+08	8.6E+08		1.9E+09
18	IPNV	5.9E+07	8.6E+07	1.9E+07	1.9E+08	
19	IPNV	1.9E+08	1.9E+08			
20	IPNV	1.9E+08	1.9E+07	8.6E+07	8.6E+06	
21	IPNV	5.9E+07	5.9E+07			
22	IPNV	2.7E+08			5.9E+07	
23	IPNV	2.7E+08	1.3E+06	2.7E+07	1.3E+06	
24	IPNV	2.7E+08	4.0E+08			
25	IPNV		4.0E+07		1.3E+06	
26	IPNV	<1.9E+02	5.9E+07	2.7E+07		1.9E+08
27	IPNV	1.9E+06	2.7E+05			
28	IPNV	5.9E+07	8.6E+06			
29	IPNV	4.0E+08	2.7E+08			
30	IPNV	8.6E+07	5.9E+07	2.7E+08	2.7E+07	
31	IPNV	2.7E+08	8.6E+07			
32	IPNV	1.3E+08	8.6E+07			
33	IPNV	1.3E+05	4.0E+08	4.0E+08	8.6E+07	
34	IPNV	1.9E+08	4.0E+07			
35	IPNV		4.0E+07			
36	IPNV		2.7E+08	8.6E+05		

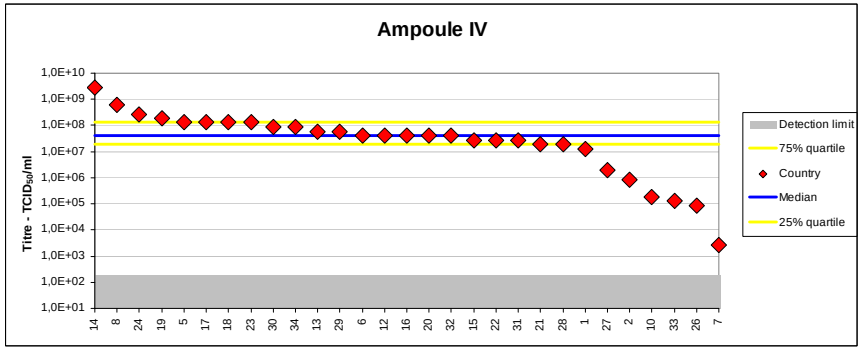
Number of laboratories	29	33	12	9	2
Median titre	1.3E+08	5.9E+07	2.3E+07	2.7E+07	1.0E+09
Maximum titre	1.9E+09	8.6E+08	8.6E+08	1.9E+08	1.9E+09
Minimum titre	<1.9E+02	2.7E+05	8.6E+05	1.3E+06	1.9E+08
25% quartile titre	5.9E+07	2.7E+07	1.2E+07	8.6E+06	6.2E+08
75% quartile titre	2.7E+08	1.9E+08	1.3E+08	5.9E+07	1.5E+09

Figure 5: Titre obtained in BF-2 cells









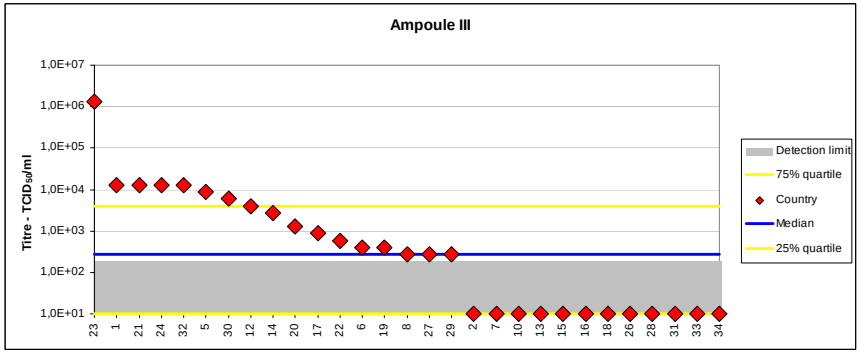
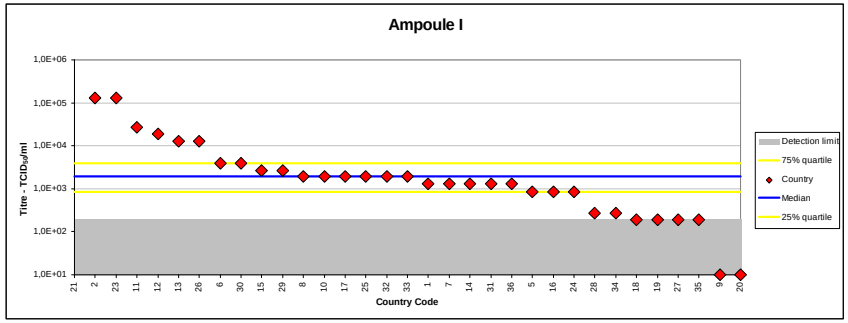
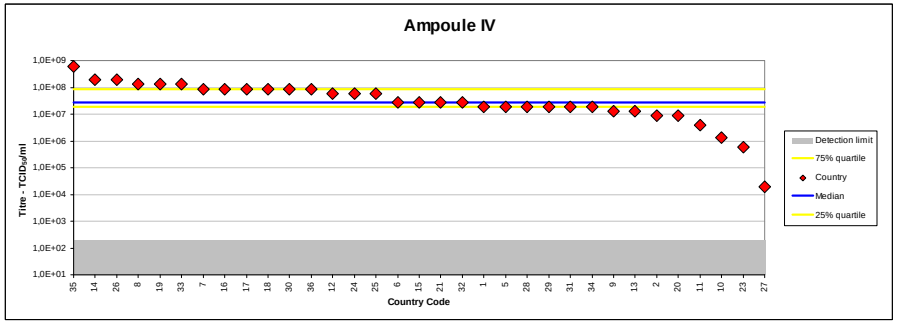
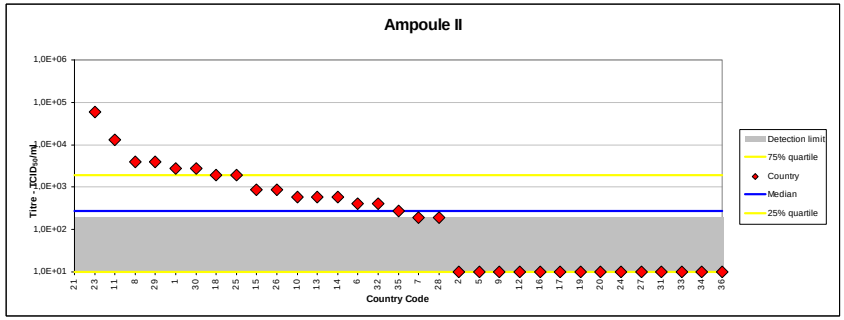


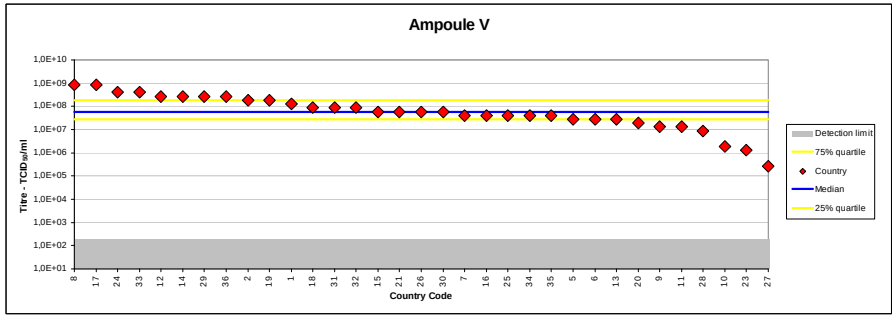
Figure 5. The titre (red diamond) of each participating laboratory (country code) using BF-2 cells illustrated for ampoule I, II, III, IV and V. On all graphs are plotted the detection level (gray shadow), median (blue line), 75% quartile (upper yellow line) and 25% quartile (lower yellow line). Participants failing to obtain any titre are given a $TCID_{50}$/ml value of 10 that is below the detection level.

Figure 6: Titre obtained in EPC cells









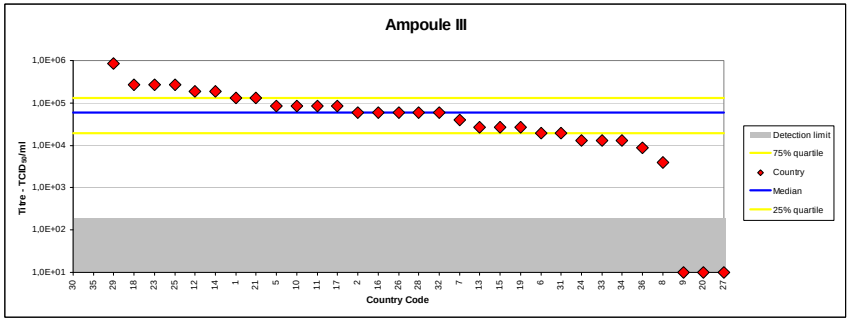
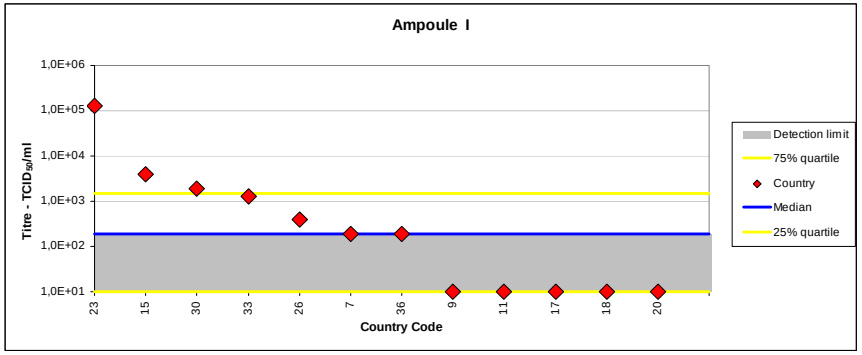
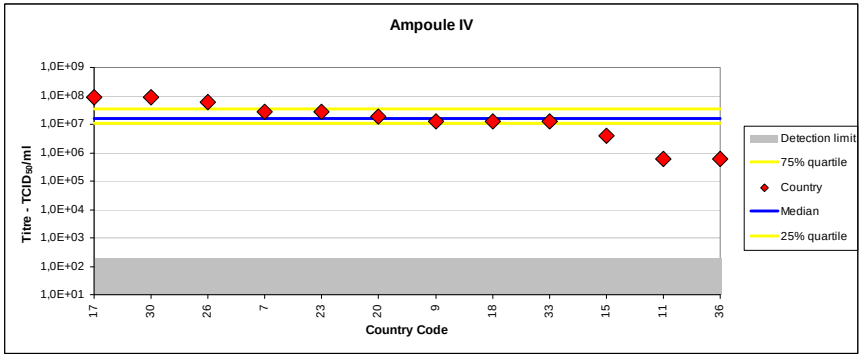
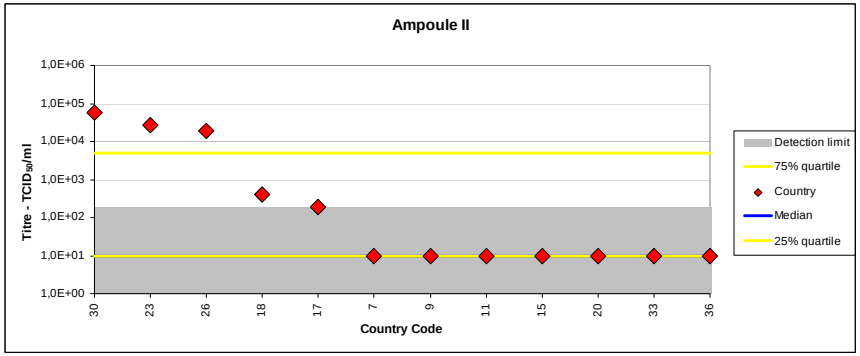


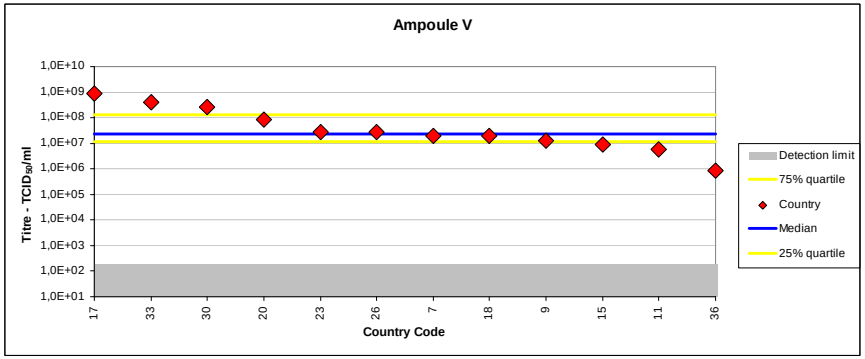
Figure 6. The titre (red diamond) of each participating laboratory (country code) using EPC cells illustrated for ampoule I, II, III, IV and V. On all graphs are plotted the detection level (gray shadow), median (blue line), 75% quartile (upper yellow line) and 25% quartile (lower yellow line). Participants failing to obtain any titre are given a $TCID_{50}$/ml value of 10 that is below the detection level.

Figure 7: Titre obtained in RTG-2 cells









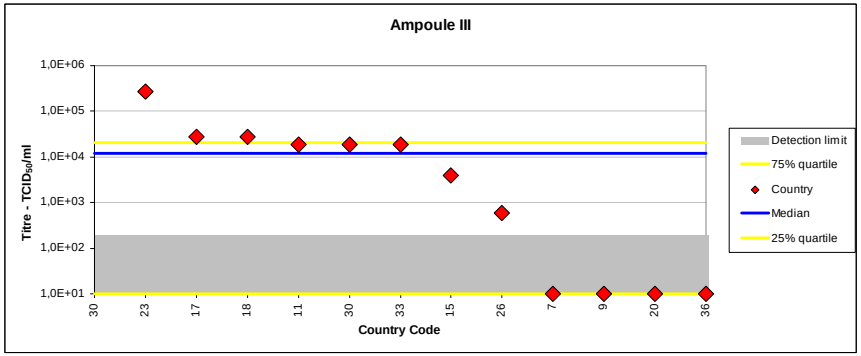
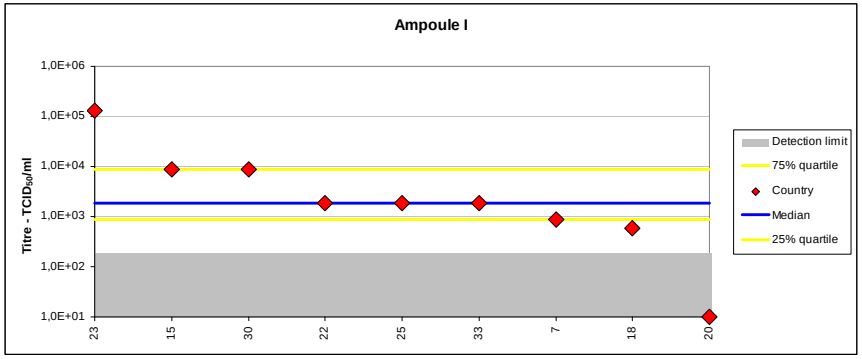
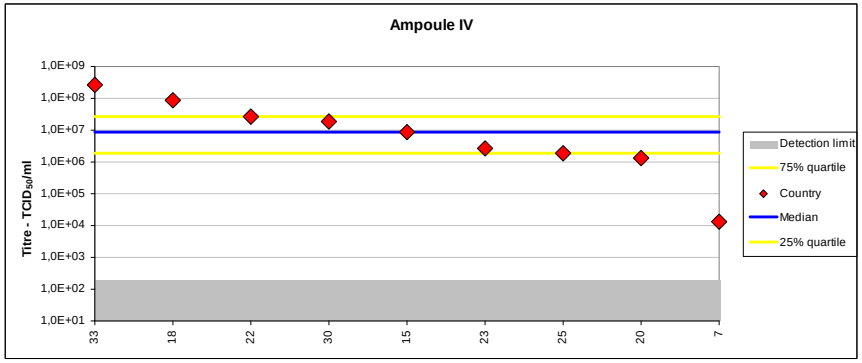
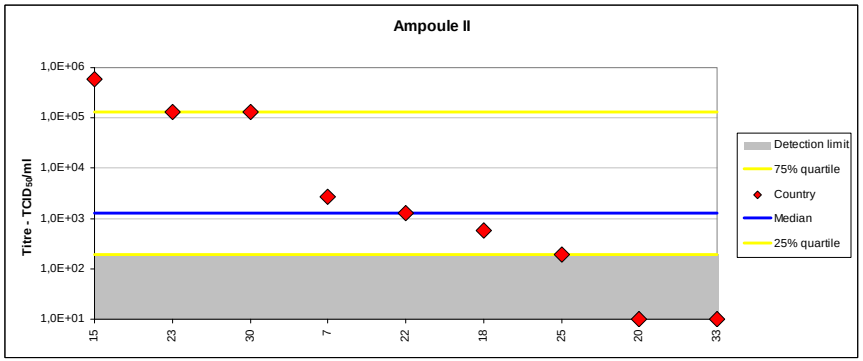


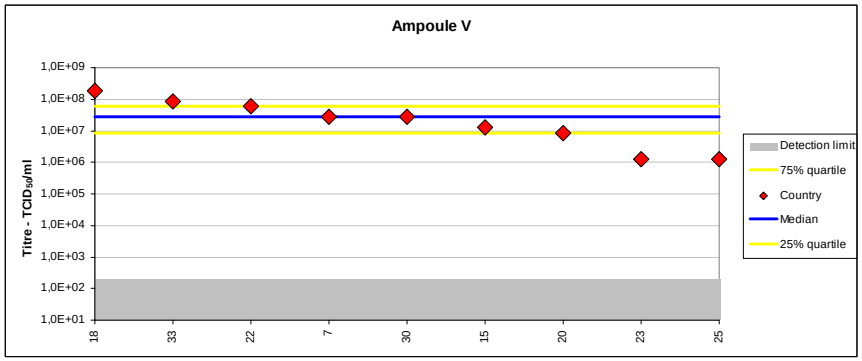
Figure 7. The titre (red diamond) of each participating laboratory (country code) using RTG-2 cells illustrated for ampoule I, II, III, IV and V. On all graphs are plotted the detection level (gray shadow), median (blue line), 75% quartile (upper yellow line) and 25% quartile (lower yellow line). Participants failing to obtain any titre are given a $TCID_{50}$/ml value of 10 that is below the detection level.

Figure 8: Titre obtained in FHM cells









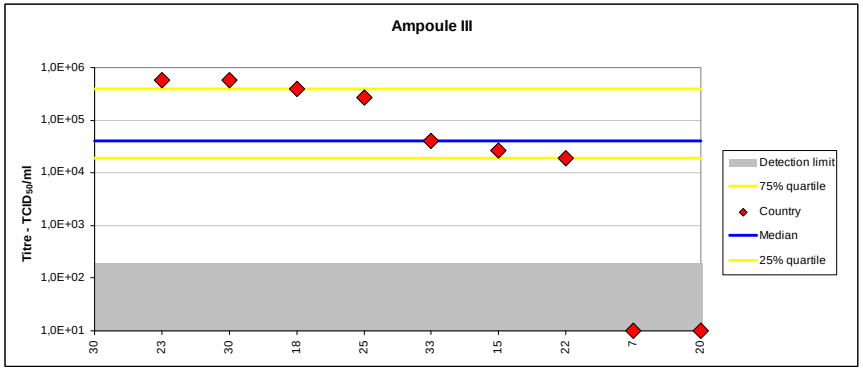


Figure 8. The titre (red diamond) of each participating laboratory (country code) using FHM cells illustrated for ampoule I, II, III, IV and V. On all graphs are plotted the detection level (gray shadow), median (blue line), 75% quartile (upper yellow line) and 25% quartile (lower yellow line). Participants failing to obtain any titre are given a $TCID_{50}$/ml value of 10 that is below the detection level.

Findings

The individual code numbers are supplied to the respective laboratories with this report. An un-encoded version of the report will be sent to the Commission.

Participation
35 laboratories received the annual proficiency test, in 14 of the parcels a thermo-logger was included. All participants replied within the deadline.

Shipment and handling
All proficiency tests were delivered by courier and when possible, participants were provided with a tracking number when the test had been shipped. Within three days, 26 proficiency tests were delivered to participants; four tests were delivered within 7 days and three tests within three weeks.

One parcel/proficiency test had been destroyed during transportation. The participant was immediately sent a new test.

Two participants received one ampoule with wrong ID code. Ampoules with correct ID code were immediately sent to the participants.

Two participants received one empty ampoule. New ampoules were immediately sent to the participants.

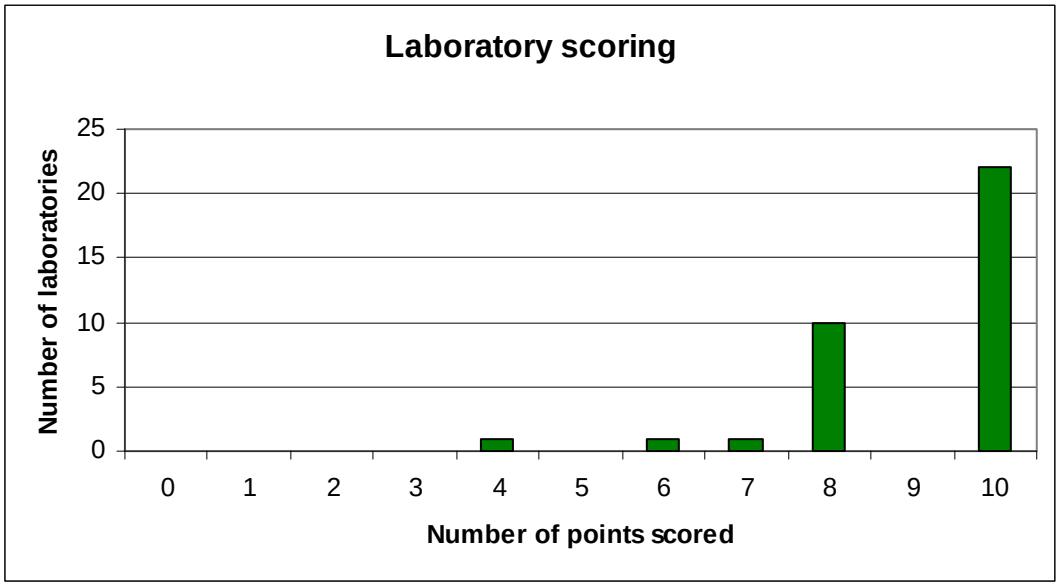
Identification of content

- 22 laboratories correctly identified all viruses in all ampoules.
- 33 laboratories correctly identified the virus in ampoule I and V.
- 34 laboratories correctly identified the virus in ampoule II and III.
- 24 laboratories correctly identified the two viruses VHSV and IPNV in ampoule IV.
- 9 laboratories did not identify VHSV in ampoule IV.
- 1 laboratory did not identify IPNV in ampoule IV.
- 2 laboratories found more isolates in an ampoule than were actually present.
- 2 laboratories found SVCV in an ampoule when it was not present.

Scores

Starting with proficiency test 2003 we have provided a scoring system for the identification part of the proficiency test. This year we have assigned 2 points to each correct answer (Table 3), giving the possibility for obtaining a maximum of 10 points. For Ampoule IV containing both VHSV and IPNV, the result: "VHSV" was scored as 1 point whereas the result: "IPNV" was scored as 0 points. Incorrectly finding of additional types of viruses than those included in the ampoules scored 0 even though included virus was amongst the identified viruses. Twenty-two laboratories out of 35 correctly identified all viruses in all ampoules and obtained full points. Serotyping, genotyping and submission of sequencing results is not a mandatory part of the test and is not included in the score of participants. A diagram of the scoring obtained by the laboratories is shown in Figure 9.

Figure 9.



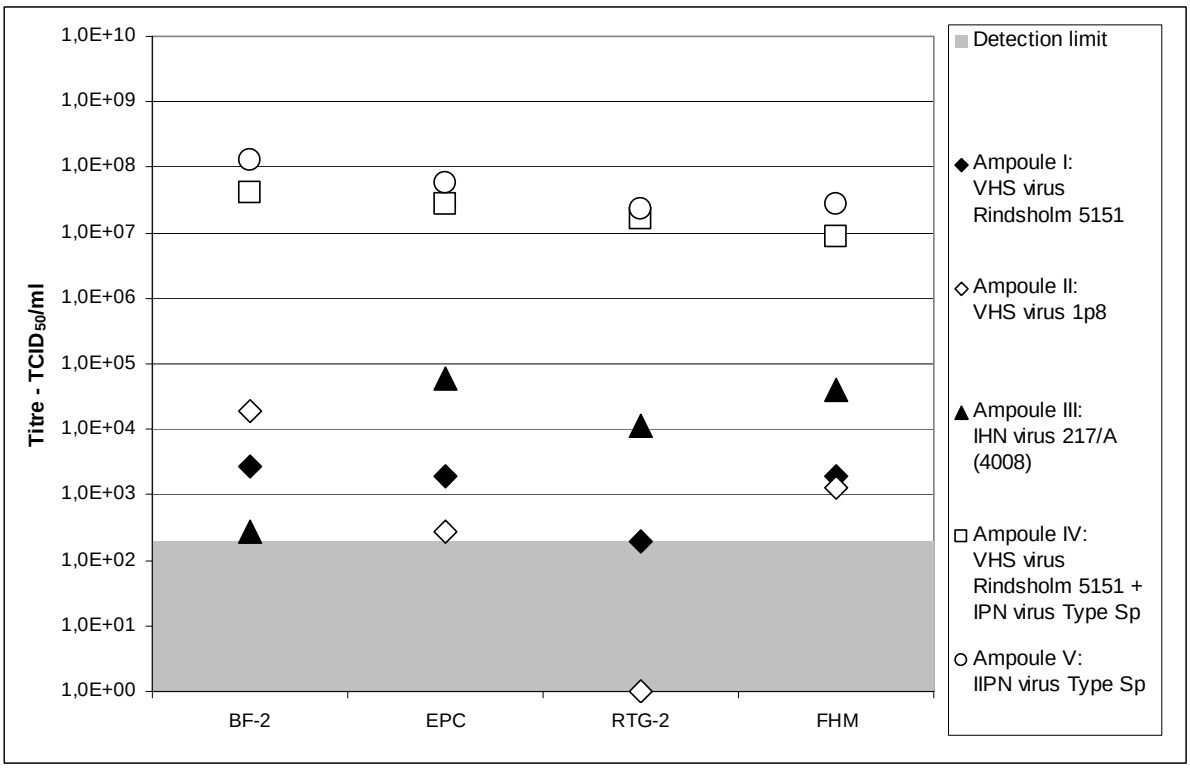
Methods applied

The following cell lines were used by the participants:

- 29 laboratories used BF-2 cells
- 33 laboratories used EPC cells
- 12 laboratories used RTG-2 cells
- 9 laboratories used FHM cells
- 2 laboratories used CHSE-214 cells
- 9 laboratories used four cell lines
- 25 laboratories used two cell lines:
    - 20 laboratories used BF-2 cells in combination with EPC cells
    - 3 laboratories used RTG-2 cells in combination with EPC cells
    - 1 laboratory used BF-2 cells in combination with FHM cells
    - 1 laboratory used EPC cells in combination with FHM cells
- 1 laboratory used only EPC cells
- 1 laboratory did not apply any cells for the test

The median titre calculated from the submitted results of each ampoule and in each cell line is illustrated in Figure 10. It appears that the IPNV replicates efficiently on BF-2, EPC, RTG-2 and FHM cells. The IHNV appeared to replicate efficiently on EPC, FHM, RTG-2 compared to on BF-2 cells. Both VHSV isolates replicated efficiently on BF-2 and FHM cells but not efficiently on RTG-2 cells.

Figure 10 Median titre of each ampoule in each cell line



Methods used for identification of viruses
(Table 9)

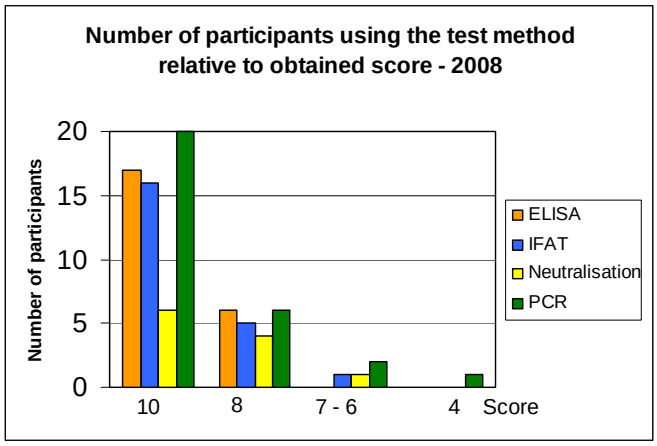
- 23 laboratories used ELISA for identification of viruses.
- 22 laboratories used IFAT for identification of viruses.
- 11 laboratories used neutralisation tests for identification of viruses.
- 29 laboratories used PCR for identification of viruses.
- 9 laboratories used other methods for identification of viruses.

Table 9: Results obtained by different test methods in participating laboratories.

Laboratory code number	ELISA	IFAT	Neutralisation	PCR	Other methods
1	X		X		
2	X	X		X	
4				X	
5		X	X	X	
6	X	X	X	X	
7	X	X		X	X
8	X	X	X	X	X
9				X	
10	X	X		X	
11	X				
12	X			X	X
13		X		X	
14	X	X			
15	X			X	X
16		X	X	X	
17		X	X	X	
18	X	X		X	
19	X	X		X	
20				X	X
21			X	X	
22	X	X			
23	X	X		X	
24				X	X
25		X		X	
26	X	X		X	
27	X			X	X
28	X	X		X	
29	X	X		X	
30	X	X	X	X	
31		X	X		
32		X		X	
33	X			X	
34	X		X	X	
35	X	X	X		X
36	X			X	

Two graphs were constructed to analyse if there could be a connection between the methods used by participants for virus identification and the obtained score (Figure 11, A and B). The results show that PCR is the most frequently used method by participants scoring 10 points but also the method most frequently used by those participants obtaining the lowest scores (4-7 points). Another observation is that neutralisation is used by a relative high proportion of participating laboratories not obtaining highest score.

A



B

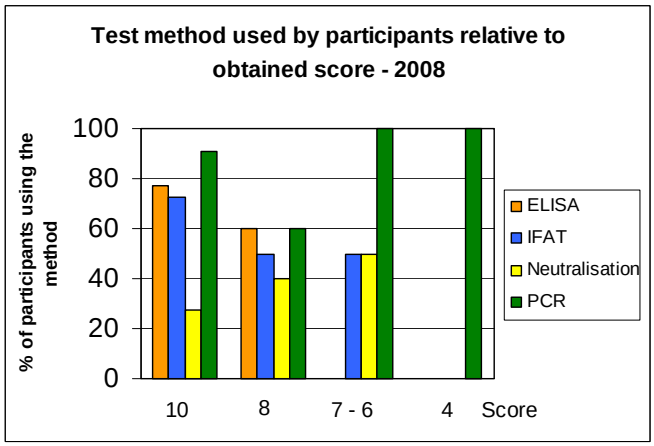


Figure 11: A) The number of participating laboratories using a particular method is illustrated relative to the score obtained by the participant. B) The percentage of the participating laboratories obtaining a certain score that are using the specified technique.

Genotypning and sequencing

- 8 laboratories serotyped some isolates.
- 15 laboratories genotyped some isolates.
- 17 laboratories submitted sequences.

Participating laboratories were encouraged to examine and identify the serotype and genotype of the isolated viruses (Table 10). Serotyping was performed by 8 laboratories. Genotyping was performed by 15 laboratories. All 15 laboratories genotyped one or more of the VHSV isolates and three of the laboratories furthermore genotyped the IHNV. Additional, two of the laboratories compared sequences to sequences of known isolates. It is positive that more laboratories performed genotyping compared to the proficiency test 2007. However, it is less than half of participants performing the genotyping. If the low number reflects a technical inability in the remaining laboratories it is concerning in light of the ongoing discussion on genotyping as a basis for differentiation of notifiable viruses from others, and on including such typing in future legislation. Genotyping were performed according to different notifications. In future proficiency tests it will be specified for all listed disease according to which references, the genotyping should be performed.

Table 10: Genotyping, serotyping and sequencing results submitted by participating laboratories.

Labora-tory code number	Ampoule	Serotype	Genotype	Sequencing
1	Ampoule IV	IPNV Sp		
5	Ampoule I		VHSV genotype Ia	Full length G
	Ampoule II		VHSV genotype Ib	
	Ampoule III		IHNV (100% homology with X73872 and X89213 N gene in 287 bp PCR-product)	
	Ampoule IV		VHSV genotype Ia	
6	Ampoule I		VHSV subtype III strain DK-5151 Rindsholm	Full length G
7	Ampoule I		VHSV genotype Ia	Full length G
	Ampoule II		VHSV genotype Ib	
	Ampoule III		IHNV isolate from Central Europe	
	Ampoule IV		VHSV genotype Ia	
	Ampoule V	IPNV Sp		
9	Ampoule I		98% Identities with Y18263.1,sequence of the N gene product	Partial N
	Ampoule II		98% Identities with Y18263.1,sequence of the N gene product	
	Ampoule III		98% Identities with X89213.1,sequence of the N gene products	
	Ampoule V		99% Identities with emb|Z37505.1|SVCVGLYC ,G gene sequence	
10	Ampoule I		VHSV genotype Ia	Partial G
	Ampoule II		VHSV genotype Ib	
	Ampoule IV		VHSV genotype Ia	
15	Ampoule I		VHSV genotype Ia	Full length G
17	Ampoule I	III	VHSV genotype Ia	Full length G
	Ampoule II	I	VHSV genotype Ib	
	Ampoule IV	IPNV Sp, VHSV III	VHSV genotype Ia	
	Ampoule V	IPNV Sp		
19	Ampoule I		VHSV genotype Ia	Full length G

Labora-tory code number	Ampoule	Serotype	Genotype	Sequencing
	Ampoule II		VHSV genotype Ib	
	Ampoule IV	IPNV Sp	VHSV genotype Ia, IPNV genogroup 5	
	Ampoule V	IPNV Sp	IPNV genogroup 5	
20	Ampoule I		VHSV genotype Ia	Full length G
	Ampoule II		VHSV genotype Ib	
	Ampoule IV		VHSV genotype Ia	
23	Ampoule I		VHSV genotype Ia	Partial N
	Ampoule II		VHSV genotype Ib	
	Ampoule VI	IPNV A2	VHSV genotype Ia, IPNV genotype III.1	
	Ampoule VI	IPNV A2	IPNV genotype III.1	
24	Ampoule I		VHSV genotype Ia	Partial G
	Ampoule II		VHSV genotype Ib	
	Ampoule III		IHNV genogroup M	
	Ampoule VI	IPNV Sp	VHSV genotype Ia, IPNV genogroup 5	
	Ampoule VI	IPNV Sp	IPNV genogroup 5	
25	Ampoule VI	IPNV Sp		
	Ampoule VI	IPNV Sp		
27	Ampoule I			Partial N
28	Ampoule I		VHSV genotype Ia	Partial G
	Ampoule II		VHSV genotype Ib	
	Ampoule VI		VHSV genotype Ia	
29	Ampoule I		VHSV genotype I	Partial G
	Ampoule II		VHSV genotype I	
	Ampoule III		99,3% id to HV7601 in L gene	
	Ampoule VI		VHSV genotype I + IPNV (99,6% id to NVI-016)	
	Ampoule VI		(99,2% id to NVI-016)	
30	Ampoule I	VHSV III	VHSV genotype Ia	Partial G
	Ampoule II	VHSV I	VHSV genotype Ib	
	Ampoule III		IHNV genogroup M	
	Ampoule VI	VHSV III, IPNV Sp	VHSV genotype Ia, IPNV genogroup III	
	Ampoule VI	IPNV Sp	IPNV genogroup III	
33	Ampoule I		VHSV genotype Ia	
	Ampoule II		VHSV genotype Ib	
34	Ampoule I		VHSV genotype Ia	Full length G
	Ampoule II		VHSV genotype Ib	
	Ampoule VI		VHSV genotype Ia	
36	Ampoule I		VHSV strain Fil3	Partial N

Furthermore, participants were asked to provide the full-length G-gene sequence of the rhabdovirus identified in the ampoule with the lowest ampoule number in a quality applied when submitting to GenBank or performing phylogenetic analysis, in this case, the VHSV DK-5151 isolate (GenBank accession number AF345859) in ampoule I. The aim of this study was to assess the homology of the sequencing results between laboratories when using the same viruses in all laboratories. The reliability of and confidence in these sequence data are crucial for correct interpretation in epidemiology. Thus the assessment of the variability primarily due to technical differences must be recognised and taken into account. Of 35 laboratories, 17 submitted sequence data. Full length G-gene sequence were submitted by 8 laboratories, five laboratories submitted partial G-gene sequence and four laboratories partial N-gene sequences. Compared to proficiency test 2007, three more laboratories submitted full length G-gene sequences this time.
Of the 8 submitted full length G-gene sequences, four sequences were 100% identical with the published sequence (GenBank accession number AF345859). Three out of five partial G-gene sequences were 100% identical with the published sequence, whereas none of the partial N-gene sequences were 100% identical at overlapping sequence stretches. This shows that at least 8 of 17 laboratories submitted sequences containing mismatches. The RNA genome of VHSV is known to mutate quite frequently. However it is believed that at least 4 – 5 passages of a virus on cell cultures is required for a mutation to take over. Therefore the mismatches identified here are not considered as being caused but mutations but rather by misreading. This is supported by the nature of the mismatches: Three sequences (18%) contained insertions shifting the reading frames of the gene. Another four sequences (24%) contained mismatches at the very ends of the submitted sequences. Of these at least two submitted sequence contained mismatching primer sequence. The two other sequences contained several mistakes as is often observed at the ends of a sequence reaction. These mistakes appear most likely because chromatograms can not be read reliable at the very end positions.

It is difficult to compare the quality of these sequencing results obtained here with those submitted to databases as e.g. GenBank as many of the mistakes observed here is not seen at such high frequencies in GenBank. 18% of all VHVS gene sequences submitted to GenBank do not contain frame shift mutations and 24% of sequences do not contain bulk amount of mutation at the end of the genes. Analysis of the chromatograms behind the submitted sequences associated with proficiency test 2007 showed that mismatches in the VHSV F1 isolate could be assigned to improper reading of the chromatograms or inclusion of primers sequences in submitted sequences (presented at the 12th Annual Meeting of NRLs for Fish Diseases). Furthermore, a few errors could be assigned to inclusion of primer sequences in the submitted sequence.

The sequencing reaction is a very robust technology and if analysed carefully, it is possible to submit sequences that do not contain any incorrect nucleotides. As discussed at the Annual Meeting 2007, most errors can be avoided by sequencing the PCR fragment in both directions; sequence three independent clones in both directions; align the sequence to a known reference sequence and double checks nucleotides at positions of mismatches.

More laboratories submitted full length G-gene sequences this year compared to last year and sequences were in general of better quality. We hope that these exercises have improved the awareness of the importance of submitting correct nucleotide sequences.

Concluding remarks

The inter-laboratory proficiency test 2008 was conducted without major constrains. Most parcels were delivered by the shipping companies within 3 days after submission, it was, however, unfortunate that some of the parcels made op to 3 weeks before delivering to the laboratories (primarily due to border controls). Some mistakes were made regarding the ampoules, in these cases new ampoule were immediately forwarded.

In 2008 one ampoule contained 2 viruses as ampoule IV contained a mixture of IPNV and VHSV. Only the IPNV and not the VHSV was identified by 9 laboratories, despite the fact that VHSV was present in a relative high titre in the ampoule (undiluted). We encourage participants to be aware of the possibility of more viruses being present at the same time and that one can over grow the other on cell cultures, and thereby masking its presence.

The bad performance in several laboratories of their RTG-2 cell lines for growth of VHSV (Figure 10) is worrying as is it described in Commission Decision 2001/183/EC that RTG-2 cells can be used instead of BF-2 cells. Based on these observations, we recommend that laboratories use BF-2 cells and not RTG-2 cells for replication/survey of/for VHSV.

PCR was the most frequently used method by participants scoring 10 points but also the method most frequently used by those participants obtaining the lowest scores (4-7 points). Another observation was that neutralisation is used by a relative high proportion of participating laboratories not obtaining highest score. Based on these findings we recommend participants to focus extra on evaluating how these two technologies are used for fish diagnostics. At the 13th Annual Meeting, a session will be aimed at discussing how to set up a diagnostic PCR.

The CRL 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.

The results will be further presented and discussed at the 13th Annual Meeting of National Reference Laboratories for Fish Diseases to be held 26-28 May 2009 in Copenhagen, Denmark.

Nicole Nicolajsen, Søren Kahns and Niels Jørgen Olesen

European Community Reference Laboratory for Fish Diseases
National Veterinary Institute, Technical University of Denmark, March 2009