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Analytical validation of one-step real-time RT-PCR for detection of infectious hematopoietic necrosis virus (IHNV)

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Abstract

Infectious hematopoietic necrosis virus (IHNV) is the causative agent of infectious hematopoietic necrosis, a disease listed by both the European Union (EU) and the World Organisation for Animal Health (OIE). Recently, molecular methods based on real-time RT-PCR have been approved for surveillance of IHNV within the EU, using the two-step real-time RT-PCR developed by Purcell et al. (2013). The two-step procedure has some disadvantages compared to one-step methods due to the higher risk of contamination and it being a more laborious test. Here we show that modifying the original protocol to one-step real-time RT-PCR does not significantly compromise the analytical parameters of the method and it provides a similar detection limit for detection of IHNV when compared with cell culture methods. The modified one-step protocol is robust and reproducible as shown by an inter-laboratory proficiency test carried out in eight laboratories in Europe.

Introduction

Infectious hematopoietic necrosis virus (IHNV) is the causative agent of infectious hematopoietic necrosis, a severe disease affecting mostly wild and farmed salmonids. Infection with IHNV is listed as a notifiable disease in the World Organisation for Animal Health (OIE) Aquatic Animal Health Code (OIE, 2019) and as a Category C disease in the European Union (EU) regulation (Commission implementing regulation (EU) 2018/1882, 2018). IHNV is a negative sense single stranded RNA virus belonging to Novirhabdovirus genus, with a ~11 Kb genome encoding six proteins: a nucleoprotein (N), a phosphoprotein (P), a matrix protein (M), a glycoprotein (G), a non-virion protein (NV) and a RNA dependent RNA polymerase (L) (Kurath et al., 2003). Five major genogroups of IHNV with a relatively low level of nucleotide diversity have been described: the North American U, M, and L, the European E and the Asian J (Enzmann et al., 2005; Kurath et al., 2003; Nishizawa et al., 2006)

The "Gold Standard" for detection of IHNV is the isolation of the virus in cell culture followed by its immunochemical or molecular identification. Although OIE does not recommend other methods for targeted surveillance for obtaining or maintaining freedom from IHNV, the EU has modified its regulations to allow use of either virus isolation in cell culture or real-time RT-PCR for targeted surveillance (Commission Delegated Regulation (EU) 2020/689, 2020). The OIE diagnostic manual stated that the preferred real-time RT-PCR to be used for detection of

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IHNV is the protocol developed by Purcell et al., (2013). This assay has been subjected to analytical and diagnostic validation in North America, showing high sensitivity and specificity to IHNV. However, this assay is a two-step real-time RT-PCR, where RNA is first reverse transcribed to cDNA, and then the qPCR is performed in a separate reaction using target specific primers. The fact that two different reactions need to be set up increases the risk of (cross-) contamination, compared with a procedure where both reverse transcription (RT) and real-time PCR are done in the same tube. Additionally, one-step procedures are generally faster and easier to set up, once they are standardised. In general, one-step RT-PCR procedures are at least as sensitive as the two-steps method, although in particular cases a reduction in sensitivity has been reported (Leutenegger et al., 1999; Wacker and Godard, 2005).

The current structure of the diagnostic manuals which regulate the testing for listed fish diseases allows the diagnostician to introduce some modifications provided they are supported by laboratory documentation showing equal or better performances compared with the initial test. In the particular case of the molecular method used for detection of IHNV, we have modified a fully validated method (Purcell et al., 2013) to perform reverse transcription and qPCR in the same tube (one-step real time RT-PCR). Although this may seem a minor modification, the analytical performance of the new method needs to be evaluated. In our case, the sensitivity and specificity of the new method was compared with the "gold standard" for detection of IHNV, inoculation in EPC (*Epithelioma papulosum cyprini,* Fijan et al., 1983) cell cultures. The reasons for this are that a) inoculation in cell culture is the method that we routinely use for surveillance of IHNV, and b) the two-steps method has not previously been used, and therefore was optimised, in our laboratory.

In this manuscript, we report the modification of the Purcell et al., (2013) two-step real-time RT-PCR to a one-step procedure. Since the method is fully validated, we did not attempt to perform a complete diagnostic validation of the modified method, and the analytical data from the one-step version fully corroborated the full validation. In this study, cell cultivation, which is the current recommended method for targeted surveillance in the OIE Aquatic Manual, was used to compare sensitivity and specificity. As we are not attempting viral quantification we will be using the term real-time RT-PCR instead of RT-quantitative PCR (qPCR).

Material and methods

IHNV isolates

Isolates used for method optimisation, PCR efficiency, analytical sensitivity and analytical specificity were part of an IHNV panel (Table 1) kindly provided to the European Reference Laboratory (EURL) for fish and crustacean diseases by Gael Kurath from the Western Fisheries Research Center (US Geological Survey). This panel includes representatives of all recognised genogroups and subgroups of IHNV (Enzmann et al., 2005; Kelley, et al., 2007; Kurath et al., 2003; Mochizuki et al., 2009; Nishizawa et al., 2006; Troyer et al., 2000). In addition, two isolates not present in the G24 IHNV panel: 32/87 (Laurencin and Baudin-Laurencin, 1987), RBH (LaPatra et al., 1993), were used for comparing sensitivity against inoculation in cell culture (see below).

Table 1. Isolates of IHNV used in this study.

Isolate Name	Genogroup	Person who provided the isolate	Sub-group	Used for[a]:
BLk94	U	Joan Thomas, Washington, USA	Up	opt; eff, a.sen; a.sp; rob; pt
Cdr12	U	Joan Thomas, Washington, USA	Up	a.sp
RB1	U	Jo-Ann C. Leong, Oregon, USA	Uc	a.sp
DW10	U	Corie Samson, Idaho, USA	Uc	a.sp
RU1	U	Igor Shchelkunov, Russia	Up	a.sp
RU9	U	Svetlana Rudakova, Kamchatka, Russia	Up	a.sp
LR80	M	Joan Thomas, Washington, USA	MN	eff, a.sen; a.sp, pt
WRAC	M	Clear Springs Foods, Inc., Idaho, USA	MA	a.sp
220-90	M	Scott LaPatra, Idaho, USA	MB	a.sp
MC30	M	Scott LaPatra, Idaho, USA	MC	a.sp
Mer95	M	Joan Thomas, Washington, USA	MD	a.sp
Qts07	M	Jan Gleckler, Washington, USA	MD	a.sp
DW09	M	Corie Samson, Idaho, USA	MD	a.sp
C18	L	Ron Hedrick, Davis, California USA	I	eff, a.sen; a.sp, pt
Col80	L	Jim Winton, USGS Washington, USA	II	a.sp
SRCV	L	Jim Winton, USGS Washington, USA	II	a.sp
FR0031	L	Ron Hedrick, Davis, California USA	II	a.sp
DF04/99	E	Heike Schuetze, Insel-Riems, Germany		eff, a.sen; a.sp
4008	E	Guiseppe Bovo, IZSVe, Italy		opt, a.sen; a.sp, pt
02-248E	E	Vlasta Jencic and Peter Hostnik, Ljubljana, Slovenia		a.sp
RtNag06a	J	Akira Motonishi, Tokyo, Japan	Nagano	eff, a.sen; a.sp, pt
RtUi02	J	Myung-Joo Oh, Gwangju, Korea	Nagano	a.sp
RtShiz06a	J	Mamiko Mochizuki, Yaizu, Japan	Shizuoka	a.sp
RtPy91	J	Jeong-Woo Park, Ulsan, Korea	Shizuoka	a.sp
32/87[b]	E	Laurencin & Baudin-Laurencin, 1987		a.sen
RBH[b]	U	LaPatra, Fryer, et al., 1993		a.sen

[a]Abbreviations: opt = method optimisation; eff = method efficiency; a.sen= analytical sensitivity; a.sp = analytical specificity; rob = robustness to pipetting errors; and pt = proficiency test.
[b]Isolates that are not part of the G24 IHNV panel provided by Dr. Gael Kurath (Western Fisheries Research Center; US Geological Survey).

Method optimization, primers, probes and exogenous control

Primers and probes from Purcell et al., (2013) were used, targeting a conserved region of 97 nucleotides (nt) in the N-gene of IHNV (Table 2). Initial PCR optimisation was carried out using RNA from infected cells supernatants. RNA was isolated from 140 µL of clarified cell supernatant using QIAamp Viral RNA kit (Qiagen, Germany), following the manufacturer's instructions. Different concentrations of primers and probe were tested. Primer tests were done with final concentrations of 150, 300, 400, 600, and 900 nM. Probe concentrations were tested at 50, 100, 200, and 300 nM. All tests were performed in triplicate in a 25 µL reaction, using Quantitect Probe RT-PCR mastermix (Qiagen, Germany) and 5 µL of template, and using ROX as passive reference dye. The thermal profile used was: 50°C x 30 min; 95°C x 15 min, 50 cycles of 95°C x 15 sec and 60°C x 1 min, and fluorescence signal was read at the end of each cycle. All tests reported in this work were carried out in an Mx3005P real-time PCR machine (Stratagene, USA). The primer/probe combination that resulted in the lower Ct value (in this case 900 nM primer and 200 nM probe) was selected and used through subsequent tests. An artificial exogenous PCR control was constructed inserting a 168 nt sequence (including the IHNV target region), following Purcell et al., (2013), into a pEX-A2 plasmid (Eurofins, Germany) backbone. The plasmid control was diluted initially in TE buffer (Tris-EDTA) and serial 10-fold dilutions were done using 10 mg/mL of yeast tRNA (Life Technologies, USA) as matrix. Although the plasmid control is not suitable to validate the reverse transcription (RT) part of the reaction, the method consistently detects from 40 to 4 x 10^8 copies per reaction, with amplification efficiency close to 100%. Dilutions containing approximately 4 x 10^5 and 400 copies of the artificial control, together with a positive RNA sample were used as real-time PCR controls during subsequent tests.

PCR efficiency and analytical sensitivity

The efficiency of the one-step real-time RT-PCR was tested for one isolate of each of the five genogroups of IHNV: BLk94, LR80, C18, DF04/99, RtNag06a from genogroups U, M L, E, and J, respectively. To do so, RNA was extracted from clarified cell supernatant as specified in section above. Each IHNV isolate was used to generate a standard curve consisting in seven serial 10-fold dilutions. Dilution points were tested in triplicate.

The sensitivity of the one-step real-time RT-PCR method was compared with the gold standard for detection of IHNV, inoculation on EPC cells. IHNV isolates BLK94, I-4008, 32/87, RBH, and Col80 were diluted in ten serial 10-fold dilutions using minimum essential media (MEM,

Table 2- Primers and probes used for IHNV detection in the study, obtained from Purcell et al., (2013)

Primer name	Sequence	Final concentration
IHNV N 796F	AGAGCCAAGGCACTGTGCG	900 nM
IHNV N 818MGB	6FAM-TGAGACTGAGCGGGACA-NFQ/MGB	200 nM
IHNV N 875Ra	TTCTTTGCGGCTTGGTTGA	900 nM

Sigma-Aldrich, USA) as diluent. For each dilution point, two replicates were tested by inoculation on EPC cell cultures and two replicates were used to extract RNA and then tested using the modified one-step real-time RT-PCR method.

Congruency between the one-step real-time RT-PCR method and inoculation in EPC cell culture was also evaluated using 20 random samples from an experimental IHNV infection trial carried on in 2018 assessing the virulence of three European IHNV isolates in rainbow trout. In this case, RNA from tissue samples was extracted using the RNAeasy mini kit (Qiagen, Germany), following the manufacturer's instructions.

Analytical specificity

In order to assure that the one-step real-time RT-PCR method is able to detect IHNV from all the different subgroups, a panel of 24 IHNV reference samples were tested. To assess cross-reactivity with other closely related viruses, a heterologous panel of twenty reference samples of closely related viruses, including isolates of viral hemorrhagic septicemia virus (VHSV), perch rhabdovirus, hirame rhabdovirus, eel rhabdovirus, tench rhabdovirus, snakehead rhabdovirus, pike fry rhabdovirus, spring viremia of carp virus (SVCV), in addition to infectious pancreatic necrosis virus (IPNV), were included in specificity tests. For both, the IHNV panel as well as for the heterologous panel, RNA was extracted using the QIAamp viral RNA kit (Qiagen, Germany) following the manufacturer's instructions. Virus specific diagnostic methods were used to assure that these samples contained detectable amount of viral genetic material (Koutná et al., 2003; Lockhart et al., 2007; Talbi et al., 2011; Jonstrup et al., 2013; Ruane et al., 2014).

Robustness to pipetting errors

The robustness of the one-step method regarding small changes in reactive concentrations was tested modifying the primer/probe concentration ± 20% and master mix concentration ± 25%. This was done using the IHNV isolate, BLK94, in triplicate.

Reproducibility and robustness – Interlaboratory Proficiency Test (ILPT)

In order to test how sensitive the protocol was regarding changes in reagents, equipment, and operators, a interlaboratory proficiency test was organised involving eight National Reference Laboratories for Fish Diseases in Europe.

Five IHNV isolates were propagated in EPC cells until total cytopathic effect has developed. RNA was extracted from the cell supernatant and tested by one-step real-time RT-PCR. Clarified cell supernatant was diluted and 60 µL were inoculated on Whatman FTA cards® (Merck, Germany) in duplicate. Heterologous viruses (VHSV, IPNV, perch rhabdovirus and salmonid alphavirus (SAV)) were propagated in their preferred cell line until development of total cytopathic effect was obtained. Clarified cell supernatant was tested using virus specific diagnostic tests, and 60 µL were inoculated into FTA cards® as above. FTA cards® consist of filter paper impregnated with a mixture of chemicals that lyse cells, denature proteins and capture nucleic acids, allowing long term storage at room temperatures (Cardona-Ospina et al., 2019). This technology was selected as it makes possible shipping of the proficiency test at room temperature.

FTA cards® were stored at room temperature for 24 h, and later placed at -20°C. The presence of IHNV was tested after 24 h post inoculation and after storage for two weeks at -20°C. FTA cards® stored at -20 °C were also tested 6 months after the deadline to submit the proficiency test results, corresponding to approximately 8 months after inoculation. Another test of FTA cards ® was done after three years storage at -20 °C. All testing of samples in FTA cards® were done using a small piece of approximately ¼ of the area where the sample has been adsorbed. In brief, a circle of about 0.5 cm diameter was placed in a 1.5 mL tube, covered with 150 µL of TE buffer and incubated at room temperature for at least 30 min. The tube was centrifuged for 5 min at 10,000 rpm, the TE collected and 140 µL used to extract RNA using the QIAamp Viral RNA Mini Kit (Qiagen, Germany) following the manufacturer's instructions.

After final testing, the ILPT including samples in FTA cards®, letter of instructions and results sheets were shipped to eight laboratories in Europe using regular mail. Participants were instructed to provide the results within one month. FTA cards® stored at -20 °C were tested again six months after the deadline indicated to provide results.

Results

PCR efficiency and analytical sensitivity

PCR efficiency for detection of one isolate of each of the five IHNV genogroups ranged from 98.1 to 104.1%. Ten-fold dilutions for one isolate of each genogroup (starting Ct value from 19.13 to 22.67) consistently detected at least six dilution points, except for isolate DF04/99, genogroup E, where the detection limit was reached after five dilution points (Table 3). However, the stock solution of isolate DF04/99 contained only about ten times less viral RNA than the other isolates (Ct values 19-20.5 for the stock solution of the other isolates, against Ct value close to 23 for the stock solution of DF04/99).

As the viral load of each sample used to generate standard curves was not tested, no direct inference about detection limit on copy number of each isolate can be drawn. Sensitivity of the one-step real-time RT-PCR method was instead estimated in comparison with the "gold standard method" meaning inoculation in cell culture. Detection limit obtained when using real-time RT-PCR was similar to results obtained by inoculation on EPC cells, although this was only measured, in the first instance, using five IHNV isolates (Table 4). In the case of isolates BLk94 (5.9 x 10^8 $TCID_{50}$/mL), and

Table 3. One-step real-time RT-PCR efficiency for each of the IHNV genogroups.

Isolate	Genogroup	Efficiency (%)	R^2	Dynamic range[a]	Ct value stock	Final Ct value
BLk94	U	104.1	0.998	6 logs	21.3	37.5
LR80	M	99.2	0.998	7 logs	19.9	38.2
C18	L	100.6	0.997	6 logs	21.2	36.4
DF04/99	E	98.1	0.999	5 logs	22.7	36.1
RtNag06a	J	98.6	0.996	7 logs	19.2	38.2

[a]Defined as the range in which the efficiency of the method is in the range of 95-105%

32/87 (5.9 x 10^7 $TCID_{50}$/mL), EPC cells are able to detect until 10^{-6} dilution in both duplicates. Similarly, the one-step real-time RT-PCR protocol gives positive results for up to 10^{-6} dilution in both samples of BLK94, and positive and one suspect positive (Ct > 35) for 32/87. In the case of isolates 4008 and RBH, the starting titers were lower (1.9 x 10^6 $TCID_{50}$/mL and 1.9 x 10^5 $TCID_{50}$/mL, respectively), but again, inoculation in EPC cells and the PCR method showed similar results. In general, the last dilution detected as positive in EPC cells is detected as suspect positive by real-time RT-PCR (with Ct values close to 36), indicating roughly the same sensitivity for detection of IHNV. The main exception is isolate Col80, where one of the duplicates was detected as suspect, with Ct values ranging 38-40 in two dilution points more than in the positive results of EPC cells.

Table 4. Analytical sensitivity for detection of five IHNV isolates. Samples were tested in duplicate by inoculation in EPC cells and by one-step real-time RT-PCR. Initial TCID50 /mLl titers of each isolate are shown in parenthesis.

	10^0	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}
BLK94 ($5.9*10^8$)									
EPC cells	++**	++	++	++	++	++	++	+	
One-step	++	++	++	++	++	++	++	(+)	
Average Ct	13.34	16.55	19.99	23.06	26.75	30.40	33.70	36.27*	
I-4008 ($1.9*10^6$)									
EPC cells	++	++	++	++	++	+			
One-step	++	++	++	++	++	+(+)			
Average Ct	18.23	21.68	25.08	28.34	31.78	34.96			
32/87 ($5.9*10^7$)									
EPC cells	++	++	++	++	++	++	++		
One-step	++	++	++	++	++	++	+(+)	(+)*	
Average Ct	17.14	22.07	25.53	29.08	32.02	34.51	36.23	38.7	
RBH (1.9×10^5)									
EPC cells	++	++	++	++	++				
One-step	++	++	++	++	(+)(+)				
Average Ct	23.09	28.01	31.15	34.38	36.47				
C18 ($1.3*10^5$)									
EPC cells	++	++	++	++					
One-step	++	++	++	+(+)	(+)*	(+)*			
Average Ct	25.16	30.18	33.82	36.22	39.83	38.44			

** ++ positive for IHNV in both replicates; (+) detection with a Ct value higher than Ct = 35
*Ct value for a single positive sample, as the other one is negative (no Ct)

Congruence between results obtained by inoculation on EPC cells and the one-step real-time RT-PCR method was evaluated with fish tissue samples (0 passage). From an infection trial assessing the virulence of three European IHNV isolates displaying 50-75% reduced survival in the fish population, samples from 65 fish were taken at the end of the trial and tested by cell culture examination. Of those, 20 samples were taken randomly from fish challenged with one of the three IHNV isolates and tested by the one-step real-time RT-PCR. Results from cell culture examination and real-time RT-PCR examination were fully in agreement regarding the number of positive and negative samples (Table 5). The only difference was that in two samples with negative results in EPC cell culture gave a suspect result in PCR (suspect Ct > 35).

Table 5. Comparison between detection of IHNV in fish tissue samples using EPC cells or one-step real-time RT-PCR.

		One-step RT-PCR		
		positive	negative	suspect
EPC cells	positive	3	0	0
	negative	0	15	2
	suspect	0	0	0

Analytical specificity

All IHNV isolates from the G24 panel, representing all genogroups and subtypes within IHNV were tested as positive using the one-step real-time RT-PCR method (Table 6). No amplification was obtained performing the IHNV one-step real-time RT-PCR test of related rhabdoviruses and IPNV samples (Table 6). All heterologous viruses tested positive when diagnostic procedures specific for each virus were used.

Table 6. Results of analytical specificity for IHNV and heterologous virus isolates.

Isolate Name	Ct[a]
IHNV BLk94	21.47
IHNV Cdr12	19.62
IHNV RB1	19.12
IHNV DW10	21.63
IHNV RU1	20.29
IHNV RU9	21.39
IHNV LR80	19.99
IHNV WRAC	22.89
IHNV 220-90	23.67
IHNV MC30	20.84
IHNV Mer95	22.35
IHNV Qts07	23.27
IHNV DW09	19.69
IHNV C18	20.76
IHNV Col80	22.27
IHNV SRCV	23.38
IHNV FR0031	23.82
IHNV DF04/99	22.83
IHNV I-4008	23.45
IHNV 02-248E	20.43
IHNV RtNag06a	18.08
IHNV RtUi02	20.26
IHNV RtShiz06a	20.1
IHNV RtPy91	19.11
VHSV F1	no ct
VHSV 1p8	no ct
VHSV DK-2835	no ct
VHSV 4p168	no ct
VHSV GE 1.2	no ct
VHSV 1p52	no ct
VHSV JFF00Ehi1	no ct
VHSV Goby 1-5	no ct
IPNV serotype Ab	no ct
IPNV serotype VR	no ct
IPNV serotype Sp	no ct
SVCV 56/70	no ct
Perch rhabdovirus 8389	no ct
Perch rhabdovirus 204419 Japan	no ct
Eel rhabdovirus B12	no ct
Eel rhabdovirus EVEX Brest	no ct
Hirame Rhabdovirus 8401	no ct
Tench Rhabdovirus	no ct
Snake head rhabdovirus SHRV6904	no ct
Pike fry rhabdovirus PFRS64	no ct

[a]Ct value obtained using the one-step real-time RT-PCR method for detection of IHNV

Robustness to pipetting errors

Changes in concentration of probe and primers

and in amount of master mix were tested using the IHNV isolate BLK94. Regardless the tested condition considered, variations inferior to one cycle were observed, except when primer and probe concentrations were increased by 20% and simultaneously the master mix volume was reduced with 25%, resulting in an average difference of 1.7 Ct values higher than in the recommended protocol (data not shown). This indicates that small pipetting errors have very little effect on the real-time RT-PCR results.

Interlaboratory proficiency test (ILPT)

Eight European laboratories participated in the ILPT for detection of IHNV in FTA cards®. Four different RNA extraction methods were used by participants in the ILPT: QIAamp® Viral RNA Mini Kit (Qiagen, Germany), NucleoSpin® RNA (Macherey Nagel, Germany), RNAeasy mini kit (Qiagen, Germany), and Nucleospin Virus (Macherey Nagel, Germany). In addition, one participant eluted the FTA card® in RTL buffer (Qiagen, Germany) and not in TE as recommended.

Five different RT-qPCR kits were used among the participants of the proficiency test: Quanti-Tect Probe RT-PCR Master Mix (Qiagen, Germany), OneStep RT-PCR Kit (Qiagen, Germany), AgPath-ID One-step RT-PCR Kit (Applied Biosystems, Ambion, USA), Super-Script III Platinium One-Step qRT-PCR Kit (Invitrogen, USA), and Quantifast pathogen RT-PCR+IC kit (Qiagen, Germany). In some cases, the participants also made changes in the thermal profile and primer/probe concentration

Table 7. Content of the samples provided in the IHNV proficiency test. Samples were tested before inoculation on FTA card, after storage at room temperature for 24 h after inoculation, and after two weeks, after 8 months and after three years of storage at -20°C. Specific diagnostic protocols were used for heterologous viruses.

Sample	Content	Ct value stock[1]	Ct value (24 h)[2]	Ct value (2 weeks)[1]	Ct value (8 months)[2,3]	Ct values (3 years)[2]
1	IHNV BLk94	21.5	24.6	25.4	25.7	27.0
2	IHNV LR80	20.0	26.7	27.2	28.6	27.9
3	VHSV	19.8	-	22.6		
4	IPN SP	26.5		29.7		
5	IHNV C18	20.8	27.0	27.8	29.5	29.7
6	IHNV 4008	23.5	29.5	30.3	31.8	31.1
7	MEM media	-	-	-		
8	Perch rhabdovirus[4]	Positive[4]	-	Positive[4]		
9	IHNV RtNag06a	18.1	24.6	25.3	26.9	27.1
10	SAV	24.4	-	28.0		

[1]Ct values obtained using preferred diagnostic method for each virus
[2] 24 h samples as well as samples stored by eight months and by three years at -20°C were tested only for IHNV
[3]Corresponding to six months after the deadline for submitting answers in the inter-laboratory proficiency test
[4]tested using end point RT-PCR

Table 8. Results of the inter-laboratory proficiency test for detection of IHNV. Different Ct values represent replicates

Sample	Laboratory							
	A	**B**	**C**[1*]	**D**[2*]	**E**[3*]	**F**	**G**	**H**[4]
1- IHNV BlK94	+ 21.9	+ 22.7	+ 23.54 24.84	+ 27.78 27.83 27.66	+ 27.90 25.84	+ 20.6	+ 20.7	+ 22.1 (20.4)
2- IHNV LR80	+ 22.6	+ 23.7	+ 23.60 22.89	+ 29.12 29.51 29.59	+ 29.14 26.00	+ 21.9	+ 22.6	+ 23.0 (21.0)
3- VHSV	-	-	-	-	-	-	-	Doubt 41.2
4- IPN SP	-	-	-	-	-	-	-	-
5- IHNV C18	+ 25.8	+ 24.2	+ 24.5 26.7	+ 30.1 30.2 30.6	+ 30.5	+ 22.8	+ 23.3	+ 24.2 (22.7)
6- IHNV 4008	+ 26.9	+ 27.1	+ 28.7 29.0	+ 32.3 33.5 33.1	+ 31.2 29.1	+ 24.7	+ 25.1	+ 26.8 (25.2)
7- MEM	-	-	-	-	-	-	-	-
8 – Perch Rhabdovirus	-	-	-	-	-	-	-	Doubt 39.6 (35.0)
9 – IHNV Nag06a	+ 23.1	+ 21.6	+ 21.8 21.4	+ 27.9 27.6 28.1	+ 27.5 24.7	+ 19.7	+ 20.8	+ 22.7 (21.9)
10 - SAV	-	-	-	-	-	-	-	Doubt 38.0 (35.0)

[1]Two different RNA extractions and testing.
[2]Different template amount and primer/probe concentration than the recommended.
[3]This laboratory did not followed the recommended RNA extraction method.
[4]In parenthesis Ct values obtained using Purcell et al. 2013 two-step protocol

to adjust the protocol to the different kits used. Additionally, one laboratory also tested using the original two-steps protocol.

Content of the samples in the ILPT and their stability following storage for up to three years are given in Table 7. All five IHNV positive samples were identified as positive by all the participating laboratories (Table 8). Laboratory H, however, scored samples of the heterologous viruses, VHSV, Perch rhabdovirus and Salmonid alphavirus (SAV), as doubtful with high Ct values (Ct 38 and above for the one-step method) (Table 8). For SAV and perch

rhabdovirus, the results were corroborated as doubtful with the original two-steps protocol from Purcellet al. (2013), in both cases with Ct value of 35. This may indicate contamination problems, more than a lack of specificity of the PCR method since no other laboratory reported unspecific amplifications at high Ct values.

Discussion

Modifying the two-step real-time RT-PCR protocol for detection of IHNV to a one-step procedure including the similar primers and probe did not affect the sensitivity or specificity of the test. We have modified the fully validated method by Purcell et al. (2013) to perform reverse transcription and real-time PCR in the same tube. Although this may seem as a minor modification, the analytical performance of the new method (modified) needs to be evaluated. In our case, the new method was compared with the "gold standard" for detection of IHNV, inoculation on EPC cell cultures. The reason to this is that the two-steps method in our hands gave suboptimal results, as it was not optimised to our conditions. We have shown that the modification done to the protocol do not compromise analytical sensitivity and specificity, with a sensitivity very close to the detection limit using inoculation on EPC cells. The main disagreement between detection of IHNV using EPC cell cultures and the one-step real-time RT-PCR was found for isolate Col80, where the PCR method is able to detect two serial dilutions more than inoculation on EPC cells. However, in both cases the Ct value was quite high (>35). As no strict cut-off value was determined using a diagnostic validation, in practical terms we consider Ct values > 35 as suspects. In this particular case it is clear that the samples were real positive with very low viral load, but one has to be aware of the possibility of unspecific amplification at such high Ct values.

In this manuscript we also provide evidence that the method is robust and reproducible, as it has been shown by the results of an ILPT performed by eight European laboratories. All participant laboratories correctly identified the IHNV samples. Only one participant obtained late positive Ct values for heterologous samples, regardless of the method of amplification used (one-step as well as two-steps). A cross-contamination could explain these results. Additionally, two laboratories reported Ct values that were 3 to 6 units higher that the average reported by the other 6 laboratories. This could partially be explained as laboratory D used different primer/probe concentrations that was suggested in the protocol, as well as the PCR kit not being designed for real-time PCR assays. Laboratory E, on the other hand, used a suboptimal protocol for RNA extraction from FTA cards®. The protocol used by laboratory E consisted in elute FTA cards® in RLT buffer (Qiagen, Germany) for posterior RNA isolation using the RNAeasy mini kit (Qiagen, Germany), This protocol was previously recommended in an ILPT for detection of VHSV in FTA cards® (Kim et al., 2018). However, further testing in our laboratory has shown that elution in TE buffer (as recommended in the IHNV ILPT) provides better RNA recovery than elution in RTL buffer (data not shown), and that cell supernatant samples extracted using the QIAamp® Viral RNA Mini Kit (Qiagen, Germany) show ten-fold more viral RNA compared to samples extracted using the RNA easy mini kit. We believe that these differences in FTA card® elution and RNA extraction caused the high Ct values obtained by this laboratory (Laboratory D).

The one-step real time RT-PCR method was not an attempt to calculate viral load, but to provide an analytical result in the form of positive, negative or dubious. In this sense, results were highly consistent among participant laboratories, where seven out of eight laboratories scored 100%. The present protocol is robust to different master mixes, thermocyclers, RNA extractions methods, and general laboratory set up differences. This ILPT, together with a similar test carried on by the Viral Fish Pathology Unit in ANSES, indicates that the one-step real time RT-PCR protocol is sensitive, specific, and robust for the detection of IHNV.

A National study conducted in Germany (Hoferer et al., 2019) have indicated that some of the German genotype E isolates performed less optimal following the Purcell et al. (2013) protocol due to a mismatch in the region complementary to the hydrolysis probe used. Unfortunately, we were not able to test the one-step protocol against these isolates, as they were not available in our laboratory. This reflects some of the limitations of PCR based tools to detect pathogens, as natural genetic evolution could potentially lead to circulation of variants with mismatches in the primers and probes used for amplification and detection, potentially compromising the sensitivity of the method. A future initiative could include the generation of a larger repository covering the genetic variability of European IHNV isolates, which could be accessible to European laboratories interested in validate their diagnostic procedures.

In this manuscript, we also showed that inoculation on Whatman FTA cards® is a suitable method for shipment and long-term storage of inactivated viral samples without requiring a cold chain during the whole transport.

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