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Research article

Inter-laboratory ring trial to evaluate real-time reverse transcription polymerase chain reaction methods used for detection of infectious pancreatic necrosis virus in Chile

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ABSTRACT

Background: Infectious Pancreatic Necrosis Virus (IPNV) is the etiological agent of a highly contagious disease that affects salmonids. In Chile, the second worldwide salmon producer, IPNV causes great economic loss and is one of the most frequently detected pathogens. Due to its high level of persistence and the lack of information about the efficiency of its diagnostic techniques, the National Reference Laboratory (NRL) for IPNV in Chile performed the first inter-laboratory ring trial, to evaluate the sensitivity, specificity and repeatability of the qRT-PCR detection methods used in the country.
Results: Results showed 100% in sensitivity and specificity in most of the laboratories. Only three of the twelve participant laboratories presented problems in sensitivity and one in specificity. Problems in specificity (false positives) were most likely caused by cross contamination of the samples, while errors in sensitivity (false negatives) were due to detection problems of the least concentrated viral sample. Regarding repeatability, many of the laboratories presented great dispersion of the results (Ct values) for replicate samples over the three days of the trial. Moreover, large differences in the Ct values for each sample were detected among all the laboratories.
Conclusions: Overall, the ring trial showed high values of sensitivity and specificity, with some problems of repeatability and inter-laboratory variability. This last issue needs to be addressed in order to allow harmonized diagnostic of IPNV within the country. We recommend the use of the NRL methods as validated and reliable qRT-PCR protocols for the detection of IPNV.

1. Introduction

Infectious pancreatic necrosis (IPN) is a highly contagious disease caused by a non-enveloped, bi-segmented, double-stranded RNA virus (infectious pancreatic necrosis virus; IPNV) that affects salmonids reared in intensive culture systems [1,2]. IPN is considered as one of the most important diseases in salmon aquaculture worldwide because it causes high mortality rates in first-feeding fry and in post-smolts shortly after transfer to seawater [3,4]. Additionally, survivor fish from an outbreak can become lifelong asymptomatic carriers of the virus, transmitting it horizontally to other susceptible fish or vertically to their progeny; perpetuating the disease in the population [5]. In Chile, the second major producer and exporter of salmon worldwide [6], IPN is considered an endemic and prevalent disease, that affects mainly Atlantic salmon (*Salmon salar*) fry, producing great economic loss to the salmon farming industry [7,8]. To date, the isolates reported in the country have been classified within genogroups 1 and 5, that correspond to strains from Europe and North America, respectively [9,10,11]. Despite the high prevalence of the disease and the magnitude of its impact in salmon farming, there is not a specific health surveillance and control program for IPN in the country. It is only included in the general health program for fish, which establishes the screening of breeders to identify possible IPNV carriers, with the consequent elimination of their ova, in an effort to prevent vertical transmission [12]. Consequently, rapid diagnostic methods for the control of IPN are needed that ought to be specific and accurate, in order to detect any possible variant of the virus from different sources (e.g. fish in different stages of growth and from both freshwater and marine farms), as well as highly sensitive, to detect the low levels of virus present in asymptomatic carriers when screening valuable broodstock.

Traditionally, the diagnostic method recommended for IPN by the World Organization for Animal Health (OIE) in its Manual of Diagnostic Tests for Aquatic Animals was the isolation of the virus in

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cell culture, followed by antibody-based identification of the agent [13]. Nevertheless, the chapter on IPN was last updated in 2003 without including any molecular methodology. Moreover, because the disease is considered enzootic in most of the regions where salmonid fish are cultivated it is no longer considered in the OIE list, so it has been removed from the last editions of the manual (2009 and forward). Currently, several reverse transcriptase polymerase chain reaction (RT-PCR) based techniques have been described for the detection of IPNV [14,15,16,17], most of which use primers against the capsid protein of the virus (VP2). In Chile, it is well known that diagnostic laboratories use mostly real time RT-PCR (qRT-PCR) for routine diagnosis of IPN, because its application is simple, accurate and offers fast results. However, since there is no standardized methodology, laboratories use different in-house qRT-PCR procedures, with sets of primers and probes targeting different regions of the IPNV genome.

Inter-laboratory comparison trials, or ring trials, are studies in which the performance (sensitivity, specificity, repeatability, reproducibility) of a diagnostic method is evaluated using identical samples in several laboratories under control of a supervising laboratory. Ring trials are useful for validation of PCR based diagnostic methods, providing a way for the standardization and harmonization of assay protocols between laboratories [18]. Several ring tests have been carried out to evaluate the performance of different animal pathogen detection methods, as well as the technical competence of the participant laboratories [19,20,21,22,23,24,25,26]; however, at the moment of this study, there are no published reports of ring test for diagnostic assays of salmon viruses.

The Laboratory of Virology from the University of Valparaíso, as the National Reference Laboratory (NRL) for IPN in Chile [27], is responsible for the technical evaluation and standardization of diagnostic methods for the virus in the country. In this context, the NRL carried out the first inter-laboratory comparison trial to evaluate the performance of the in-house qRT-PCR assays used by diagnostic laboratories in Chile to detect IPNV. This paper describes the development of the ring test carried out by the NRL, from the production of the samples, to the evaluation of the laboratories that participated in the trial, assessing the sensitivity, specificity and repeatability of their methods. The aim of this study is to contribute in the standardization and validation of a reliable qRT-PCR protocol for the detection of IPNV that can be recommended for general use in Chile.

2. Material and methods

2.1. Participants

Twelve diagnostic laboratories participated in the trial; their participation was a requirement to continue performing the diagnostic of the IPN disease under the Chilean National Fisheries and Aquaculture Service (SERNAPESCA) authorization. All of the laboratories remained anonymous to avoid any conflict among the participants. The laboratories used their own in-house methods for the detection of IPNV, a summary of the information provided by each one and their identification number is shown in Table 1. The NRL is listed with the number 13 and used two qRT-PCR assays during the trial, 13SG and 13T, which correspond to methods with SYBR-Green and Taqman chemistries, respectively.

2.2. Sample panel composition and distribution

The sample panel was prepared entirely in the NRL and distributed to each participant laboratories. Sets of 8 samples in culture media were prepared. Each set consisted of 5 positive samples and 3 negative samples. The positive samples consisted in viral suspensions at different concentrations obtained from cell culture infected with two strains of IPNV. These strains belonged to genogroup 1, VR299 type strain (UV84 GenBank accession number HQ738519) and genogroup 5, Sp. type strain (ALKA3 GenBank accession number KF954912), and were isolated and sequenced in the NRL. The negative controls were culture medium, cells in culture medium and a suspension of infectious salmon anemia (ISA) virus (Table 2).

Table 1
Information of the qRT-PCR methods used by the laboratories.

Laboratory	Detection chemistry	Real time RT-PCR platform	Genomic target
1	Taqman	MX3000P, Stratagene	VP2 protein
2	Taqman	MX3000P, Stratagene	VP2 protein
3	Taqman	MX3000P, Stratagene	VP2 protein
4	Taqman	StepOne Plus, Applied Biosystems	VP1 protein
5	Taqman	StepOne Plus, Applied Biosystems	VP2 protein
6	Taqman	StepOne Plus, Applied Biosystems	VP2 protein
7	Taqman	LightCycler 480 II, Roche	VP2 protein
8	Taqman	LightCycler 480 II, Roche	VP2 protein
9	Taqman	StepOne Plus, Applied Biosystems	Not informed
10	Taqman	LightCycler 480 II, Roche	Not informed
11	Taqman	MX3000P, Stratagene	Not informed
12	Taqman	MX3000P, Stratagene	VP1 protein
13	Taqman & SYBR	StepOne Plus, Applied Biosystems	VP1 & VP2 proteins

The positive samples were obtained by amplification of the IPNV strains in Chinook salmon embryo cells (CHSE-214) derived from *Oncorhynchus tshawtyscha* embryonic tissue. Once a massive cytopathic effect (CPE) was observed, the cells were subjected to two cycles of freezing and thawing, and centrifuged at 3000 × g for 15 min at 4°C. Supernatants were collected and serially diluted with L-15 (Leibovitz) culture medium, supplemented with 10% fetal bovine serum (FBS, HyClone) and 50 $\mu g \cdot mL^{-1}$ gentamicin, to obtain the different concentrations of each viral strain.

The negative controls were prepared as follows: Atlantic salmon kidney (ASK) cells were infected with ISA virus, and once a CPE was observed, the same procedure described above was followed to harvest the viruses. In addition, CHSE-214 cells free of infection, were subjected to the same procedure. The third control was L-15 (Leibovitz) culture medium supplemented with FBS and gentamicin.

Aliquots of 0.5 mL of all negative and positive samples were distributed in centrifuge tubes and immediately stored at -20°C. Negative controls and positive samples, were produced and aliquoted in different days to avoid crossed contamination. Finally, all samples were encoded, to blind the trial, and sent refrigerated to each laboratory.

2.3. Results report

The information about the ring trial and specific instructions about reporting the results, were sent via email to all the laboratories two weeks in advance to provide enough time to answer any doubts about the trial. The sample panels were sent to each laboratory via currier and consisted in 3 sets of 8 samples (24 samples in total), to analyze one set per day and thus to measure intra-laboratory repeatability over the three days of the trial.

Laboratories were informed to report the results for each sample set every day via email in a standard form given by the NRL.

Table 2
Samples included in the ring test.

Sample ID	Type of sample	Expected result
M01	IPNV genogroup 5	Positive
M02	IPNV genogroup 5 dilution 10^{-2}	Positive
M03	L-15 Cell culture medium	Negative
M04	CHSE-214 cells in culture medium	Negative
M05	ISAV	Negative
M06	IPNV genoproup 1	Positive
M07	IPNV genoproup 1 dilution 10^{-2}	Positive
M08	IPNV genoproup 1 dilution 10^{-4}	Positive

Positive results for qRT-PCR assays were provided as cycle threshold (Ct) values. Most of the laboratories reported three Ct values per sample each day (3 technical replicates), according to their own protocols to inform results. Nonetheless, some laboratories reported two or just one Ct value per sample each day. Therefore, a mean of these values, or the only value reported for each sample, was used for the analysis. Negative samples were informed with a negative sign or the Ct obtained, indicating the cut-off Ct value determined by the laboratory, in which case the result was annotated with a negative sign as well.

2.4. Real-time RT-PCR assays

To perform the qRT-PCR analysis, the NRL used two previously standardized techniques [14], one targeting segment B of the virus (qRT-PCR VP1, Taqman chemistry) and other to segment A (qRT-PCR VP2, SYBR-Green). Briefly, 200 μL were taken from each sample and viral RNA was extracted with E.Z.N.A.™ Total RNA Kit I (Omega Bio-tek) according to the manufacturer's instructions. The extracted RNA was eluted with molecular biology grade water and stored at -80°C. Concentration and purity of the extracted total RNA was determined by measuring the absorbance ratio at 260 nm over 280 nm using a spectrophotometer (MaestroNano, Maestrogen). To ensure that contamination is strictly controlled during the RNA extraction process, a negative control using molecular biology grade water was always performed.

The extracted RNA was reverse transcribed and amplified by a one-step qRT-PCR using a 48-well plate real-time PCR system Step-One (Applied Biosystems). The two sets of primers and probe used by the NRL for the TaqMan™ and SYBR® green qRT-PCR assays are shown in Table 3.

The AgPath-ID™ One-Step RT-PCR Kit (Applied Biosystems) was used for the amplification of the VP1 protein in segment B. This reaction was carried out in a 15 μL reaction volume containing 7.5 μL of RT-PCR Buffer (2X), 1.35 μL of each forward and reverse primers (0.9 μM), 0.3 μL of the VP1 Taqman probe (0.2 μM), 0.6 μL of RT-PCR Enzyme Mix (25X) and 2 μL of total RNA as template. The thermal profile used was 48°C for 10 min for reverse transcription, pre-denaturation at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 s and annealing/extension at 59°C for 45 s.

The amplification of the VP2 protein in segment B was carried out in a 15 μL reaction volume containing 7.5 μL of 2X Brilliant III Ultra-Fast SYBR® Green QRT-PCR Master Mix (Stratagene), 0.75 μL of each forward and reverse primers (0.5 μM), 0.8 μL of RT/RNAse block, 0.2 μL of ROX (0.3 μM) as passive reference and 2 μL of total RNA as template. The thermal profile used was 50°C for 5 min for reverse transcription, pre-denaturation at 95°C for 3 min, followed by 40 cycles of denaturation at 95°C for 5 s and annealing/extension at 60°C for 10 s. Finally, a melting curve analysis from 70°C to 95°C was performed.

The detection limit and the efficiency of the assays were evaluated using 10-fold dilutions of RNA extracted from the virus reference strain, UV84. The amplification efficiencies were 103% for the qRT-PCR targeting segment B (Taqman) and 108% for the segment A qRT-PCR (SYBR green), both calculated with the formula $[10^{(-1/\text{slope})} - 1] \times 100$. In addition, the cut-off Ct values were 30.8 and 32.0, respectively.

No-template controls (NTCs), consisting in a reaction mixture without template, were used in all reactions, alongside with negative controls from the RNA extraction and positive controls from the virus reference strain. All samples and controls were run in triplicate (qRT-PCR technical replicates), and for the statistical analysis, the mean Ct value obtained for each sample was used.

The analysis of the samples using the two methods described, i.e., qRT-PCR Taqman and SYBR green chemistry, targeting segment B and A, respectively, was done twice: 1) before the samples were sent to the laboratories (to confirm the expected results) and 2) in parallel with the participant laboratories. The results of this latter analysis are the ones used in the inter-laboratory comparison.

2.5. Analysis of the results

The analysis of the results and the performance of the qRT-PCR assays for each laboratory were evaluated based on the methodology described by the OIE in the Manual of Diagnostic Tests for Aquatic Animals [28]. Consequently, sensitivity is defined as the proportion of positive IPNV samples that effectively tested positive in the assay and specificity is the proportion of negative samples for IPNV that effectively tested negative in the assay. In total, from the 24 samples sent to the laboratories, 15 samples were positives (contain viral suspensions of IPNV) and 9 were negatives (samples free of IPNV).

Repeatability (i.e. within-laboratory agreement between replicates) was evaluated in relation to the dispersion of the Ct values the three days of the trial for each positive sample. Thus, descriptive statistics (mean, standard deviation and range) were calculated for positive samples according to the data reported by each laboratory, shown in Table 4. In addition, overall statistics were calculated using all the results reported by the laboratories. In order to observe the dispersion of the Ct values for each positive sample, a standard box plot was graphed using the SigmaPlot software version 10.0 in which whiskers indicate the 90th and 10th percentiles.

In order to compare and assess the agreement among the laboratories in-house methods, the Fleiss Kappa statistic from rating scores was calculated using the online software StatsToDo (http://www.statstodo.com/CohenKappa_Pgm.php). The Fleiss Kappa statistic is a measurement of concordance or agreement between two or more raters, where agreement due to chance is factored out. Here, we consider the fourteen different qRT-PCR methods to be the raters, and to calculate the kappa statistic the results of the positive samples were categorized in 5 scores: Negative = 0; Ct >30 = 1; Ct 25–30 = 2; Ct 20–25 = 3; Ct <20 = 4. The scale used to interpret the Kappa statistic was as follows: below 0.01 less than chance agreement, 0.01–0.20 slight agreement, 0.21–0.40 fair agreement, 0.41–0.60 moderate agreement, 0.61–0.80 substantial agreement and 0.81–0.99 indicate almost perfect agreement [29].

3. Results

The results of the qRT-PCR assays from the different participating laboratories (laboratory identification number 1–12) were received mostly as expected, with the majority of the laboratories sending their

Table 3
NRL primers and probe used for the qRT-PCR assays.

Primers/probe	Name	Sequence	Amplicon position	Coding region
TaqMan				
Probe	IPNV VP1	56FAM-TACATAGGC-ZEN-AAAACCAAAGGAGACAC-3IABkFQ	Segment B: 668–820	VP1
Forward primer	VP1F	GTTGATMMASTACACCGGAG		
Reverse primer	VP1R	AGGTCHCKTATGAAGGAGTC		
SYBR-Green				
Forward primer	WB1	CCGCAACTTACTTGAGATCCATTATGC	Segment A: 20–225	VP2
Reverse primer	WB2	CGTCTGGTTCAGATTCCACCTGTAGTG		

Table 4
Results of the mean threshold cycle values during the three days of the ring trial.

Day	Sample	Expected results	Laboratories													
			1	2	3	4	5	6	7	8	9	10	11	12	13SG	13T
1	M01	+	19.7	20.1	22.7	20.8	21.5	22.3	20.8	20.9	17.7	16.3	18.2	20.7	18.7	14.4
	M02	+	25.7	26.2	28.6	25.9	27.3	29.8	27.8	25.8	24.3	26.5	28.1	26.5	26.7	19.5
	M03	−	−	−	−	−	−	−	−	−	−	−	34.8	−	−	−
	M04	−	−	−	−	−	−	−	−	−	−	−	33.3	−	−	−
	M05	−	−	−	−	−	−	−	−	−	−	−	35.1	−	−	−
	M06	+	19.9	19.6	22.0	31.7	22.3	23.7	21.7	20.4	21.0	20.8	24.7	28.3	16.5	15.0
	M07	+	25.4	25.0	25.7	36.7	29.5	31.0	27.1	27.4	27.3	27.9	32.2	33.4	22.0	19.5
	M08	+	31.5	31.2	32.7	−	35.8	34.5	34.9	33.7	32.5	35.0	33.9	−	29.4	26.5
2	M01	+	20.0	22.0	24.4	20.9	22.0	21.8	20.0	22.2	16.9	16.2	20.5	20.1	15.7	15.2
	M02	+	25.9	29.5	28.9	27.7	28.4	30.4	24.9	27.0	24.3	22.6	29.9	25.5	24.3	23.4
	M03	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
	M04	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
	M05	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
	M06	+	20.5	19.6	21.4	32.4	22.8	21.9	21.1	22.8	21.3	22.4	30.2	25.7	16.4	18.9
	M07	+	25.9	26.4	26.9	36.8	28.3	32.5	24.7	27.3	27.7	25.6	33.1	32.5	22.1	24.5
	M08	+	31.5	31.5	32.3	37.3	34.1	35.7	33.6	32.6	33.2	35.0	−	−	30.0	30.6
3	M01	+	20.9	22.0	21.6	20.3	20.8	21.1	20.6	23.6	17.3	16.8	19.4	18.9	20.5	15.0
	M02	+	26.6	29.2	25.1	27.0	27.7	28.4	27.6	27.7	24.2	27.9	27.4	25.5	25.8	19.5
	M03	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
	M04	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
	M05	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
	M06	+	20.3	21.6	22.4	31.1	20.8	22.5	21.2	23.6	22.2	21.5	25.8	27.6	16.7	15.0
	M07	+	26.5	28.6	25.0	33.4	29.1	30.5	27.3	29.2	28.8	28.8	33.6	32.3	21.8	20.6
	M08	+	31.6	31.7	31.9	−	34.4	34.0	35.0	33.3	32.7	33.6	−	−	31.1	26.5

(−) Negative results.

results daily (starting one or two days after the samples were sent). Only one laboratory (No. 11) did not follow the instructions and sent the results with one week of delay.

Most of the laboratories detected as negative the true negatives samples i.e., with culture medium only (M03), culture medium with cells (M04) and the suspension with ISA virus (M05). Only laboratory No. 11 reported positive values for these 3 samples (i.e., false positives) the first day of the trial. The positive samples to IPN virus: M01, M02, M06 and M07 were interpreted as positive (i.e., true positives) by all laboratories. Only the positive sample with the lower concentration of virus (M08) was reported as negative (i.e., false negative) (Lab No. 4, days 1 and 3; Lab No. 11, days 2 and 3; Lab No. 12 the three days of the trial), or too close to the cut-off Ct value for some laboratories (Lab Nos. 5, 6, 7 and 10) (Table 4).

Table 5 shows the results of sensitivity and specificity calculated for each laboratory. Only three of the twelve laboratories showed values with less than 100% in sensitivity and only one laboratory showed less than 100% in specificity. Of the three laboratories that showed sensitivity problems (Labs No. 4, 11 and 12), just one, laboratory No. 11 presented the lowest value (80%), due to the report of three false negatives. In addition, it was the only one reporting three false positives showing the lowest specificity (66.67%). Overall sensitivity and specificity of the trial were 96.67% and 97.62%, respectively.

Intra-laboratory repeatability for the qRT-PCR assays was evaluated in relation to the dispersion of the results (Ct values) reported for the positive samples (i.e., M01, M02, M06, M07 and M08) by each laboratory over the three days of the trial. The Ct average, standard deviation and range values for each positive sample are shown in Table 6. Overall, a large variability was observed in terms of Ct range by each laboratory, with the largest being up to 5.5 Ct of difference (Lab No. 11 sample M06; Table 6). Laboratory Nos. 3, 4, 10, 11 and the NRL, showed the greatest dispersion of data, with standard deviations equal or greater than 2 Ct for at least one of the positive samples during the three days of the ring test. Note that Table 6 shows no standard deviation for the laboratory Nos. 4 and 11, because they presented just one replicate out of three as positive for M08 sample during the whole trial, showing a major deficiency in their repeatability. The laboratory No. 12, meanwhile, did not detect any of the replicas of the sample M08 as positive (Table 6). Laboratory Nos. 1, 5, 6 and 9 showed better repeatability, with standard deviations equal or less than 1 in most of the samples (Table 6).

The overall dispersion of positive samples (Table 6) was calculated using all the results reported by the laboratories (shown in Table 4), to evaluate inter-laboratory variability. When analyzed throughout all the laboratories, each sample presented a wide dispersion of the Ct values, with standard deviations higher than 2.3 and up to 4.1. Furthermore, samples M06 and M07, showed ranges of more than 17 Ct points of difference between their lowest and highest value (Table 6).

In addition, Fig. 1 plots the dispersion of the results for positive samples throughout all laboratories, showing more clearly the high range of Ct values for each sample. Moreover, Fig. 1 shows that the mean Ct values of each sample are well correlated with the dilution of the samples, with substantial differences between the largest (M01, M06) and lowest sample concentration (M02, M08) for each viral strain, respectively. Besides, less dispersion of the data for the European type viral strain (genogroup 5) was found as well (Fig. 1, samples M01 and M02).

Table 5
Number of true positive (TP), false positive (FP), true negative (TN) and false negative (FN) samples, and the sensitivity and specificity calculated by laboratory and for the overall trial.

Labs	TP	FP	TN	FN	Sensitivity %	Specificity %
1	15	0	9	0	100	100
2	15	0	9	0	100	100
3	15	0	9	0	100	100
4	13	0	9	2	86.67	100
5	15	0	9	0	100	100
6	15	0	9	0	100	100
7	15	0	9	0	100	100
8	15	0	9	0	100	100
9	15	0	9	0	100	100
10	15	0	9	0	100	100
11	13	3	6	2	86.67	66.67
12	12	0	9	3	80	100
13SG	15	0	9	0	100	100
13T	15	0	9	0	100	100
Overall	203	3	123	7	96.67	97.62

Table 6
Mean (M), standard deviation (SD) and range (Ra) for positive samples results (Ct values) during the three days of the trial obtained by each laboratory and overall.

Labs	M01			M02			M06			M07			M08		
	M	SD	Ra	M	SD	Ra	M	SD	Ra	M	SD	Ra	M	SD	Ra
1	20.2	0.6	1.2	26.1	0.5	0.9	20.2	0.3	0.7	25.9	0.5	1.1	31.5	0.0	0.1
2	21.4	1.1	1.9	28.3	1.8	3.3	20.2	1.2	2.1	26.7	1.8	3.6	31.5	0.3	0.5
3	22.9	1.4	2.8	27.5	2.1	3.7	21.9	0.5	1.0	25.9	1.0	1.9	32.3	0.4	0.8
4	20.6	0.3	0.6	26.9	0.9	1.8	31.8	0.7	1.3	35.6	2.0	3.4	37.3	–	–
5	21.4	0.6	1.2	27.8	0.6	1.1	21.9	1.0	2.0	29.0	0.6	1.1	34.7	0.9	1.7
6	21.8	0.6	1.3	29.5	1.0	2.0	22.7	0.9	1.8	31.4	1.0	2.0	34.7	0.9	1.7
7	20.5	0.4	0.8	26.8	1.6	2.9	21.4	0.3	0.6	26.3	1.4	2.6	34.5	0.8	1.4
8	22.2	1.4	2.7	26.9	1.0	2.0	22.3	1.6	3.2	27.9	1.1	1.9	33.2	0.6	1.1
9	17.3	0.4	0.8	24.3	0.1	0.1	21.5	0.6	1.1	28.0	0.8	1.5	32.8	0.4	0.7
10	16.4	0.3	0.6	25.7	2.7	5.3	21.6	0.8	1.6	27.4	1.6	3.2	34.5	0.8	1.4
11	19.4	1.2	2.4	28.5	1.3	2.5	26.9	2.9	5.5	33.0	0.7	1.4	33.9	–	–
12	19.9	0.9	1.7	25.8	0.6	1.0	27.2	1.4	2.7	32.7	0.6	1.1	–	–	–
13SG	18.3	2.4	4.7	25.6	1.2	2.4	16.5	0.2	0.3	22.0	0.2	0.3	30.2	0.8	1.7
13T	14.9	0.4	0.8	20.8	2.3	3.9	16.3	2.3	3.9	21.5	2.6	5.0	27.9	2.4	4.1
Overall	19.8	2.4	9.9	26.4	2.4	10.9	22.3	4.1	17.5	28.1	4.1	17.3	32.7	2.3	10.8

The Fleiss's Kappa analysis for fourteen raters, when assessing all the positive samples the three days of the trial, indicated a fair agreement among all the qRT-PCR in-house methods analyzed, Kappa = 0.3170, SE = 0.0154.

4. Discussion

According to the sanitary reports of Chilean salmon aquaculture, IPNV is one of the most detected pathogens of prevalent diseases in the country [30]. Diagnostic laboratories use mostly qRT-PCR for routine diagnosis; however, unlike other viral diseases affecting salmon farming in the country (e.g. Infectious Salmon Anemia, ISA), there is not a standard protocol officially recommended for qRT-PCR detection of IPNV. The technical norm for IPN only recommends the primers reported by Blake et al. [31] for conventional RT-PCR; mentioning that qRT-PCR in-house primers could be used prior validation or through internationally recognized bibliographic support [32]. Hence, as a first approach, the goal of the ring trial was to assess the efficiency of the in-house diagnostic techniques used by the laboratories in the country.

To our knowledge no ring test for IPNV detection has been previously published and this is the first report performed to compare the qRT-PCR in-house methods used by diagnostic laboratories in Chile.

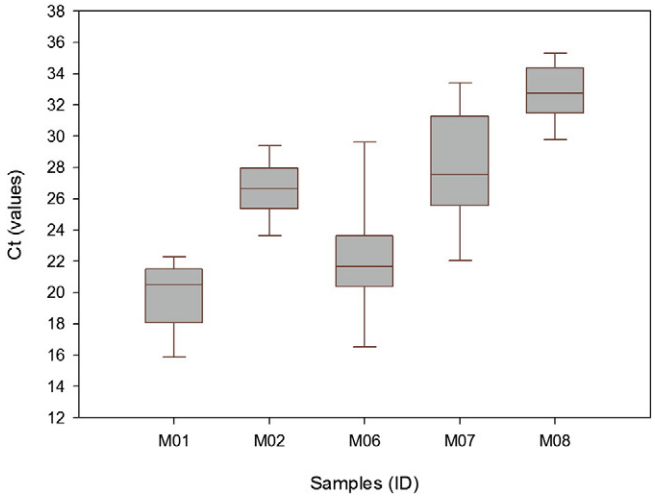

Fig. 1. Boxplot graph of the Ct values for positive samples obtained from all the laboratories. Whiskers indicate the 90th and 10th percentiles.

In general, most of the participant laboratories obtained the expected results using their in-house methods in the ring trial, despite the differences in the platforms and the assay protocols used. Regarding this, it is important to emphasize that since the laboratories are private, and therefore not obligated to disclose details of the in-house methods, they only provided general information about the genomic target in their assays (i.e., protein VP1 or VP2), or no information at all.

Overall, the ring trial showed satisfactory values of sensitivity and specificity, with most laboratories obtaining the highest score. The only drawbacks observed in sensitivity among the laboratories involved the detection of the most diluted sample (M08, American strain UV84), reported as a false negative (Lab Nos. 4, 11 and 12). It is worth noting that, Laboratories 4 and 12 were the only ones that reported VP1 protein as genomic target; however, their problems in sensitivity could not strictly be related to this since the NLR using the same protein as target had no problems in sensitivity and showed the lowest Ct values for M08. Overall, the problems in the sensitivity results could be explained by methodological differences such as unsuited primers, extraction methods and not fully optimized PCR systems. In addition, the experience of the operator may play an important role in the sensitivity of the diagnostic techniques as pointed out by other authors in inter-laboratory studies [19,24]. Although the extraction RNA method used might had an impact in the diagnostic performance of the test, this aspect was beyond the scope of this inter-laboratory comparison. Regarding the unsuitability of the primers, false negative results could be due to the lack of specificity of the primers and probes used to recognize a given genogroup, i.e., they might have been designed to detect the European IPNV strains, which are the most prevalent in the country [11,33]. Hence, the methods using these primers might not detect lower concentrations of American viral strains. In addition, comparing the results of the European (M01, M02) and American strain samples (M06, M07) that are within the same dilution factor range, the former ones presented lower dispersion of the results among the laboratories, showing a higher consistency for the detection of Sp type strains. Wernike et al. [26] found that when comparing the detection of European genotypes for the porcine reproductive and respiratory syndrome virus (PRRSV), inconsistencies were observed that were mainly explained by the large genetic diversity within the viral strains, which can also be observed when European and American IPNV Chilean strains are compared [11]. For the detection of classical swine fever virus (CSFV), Hoffmann et al. [34] also found that some in-house systems presented unspecific reactions or suboptimal sensitivity even with only a single CSFV genotype. Furthermore, it was recently shown that IPNV genomic variability also includes changes in the primer-target binding

sites [35]. Therefore, negative results must be carefully interpreted and private laboratories should take into account that hermeticism in-house methods has its own risks.

Only one laboratory had problems of specificity, reporting false positives during the first day of the trial. This is most likely a result of cross-contamination, since the same laboratory tested negative the same sample in the following days of the ring trial. These kinds of problems are not exceptional as have been reported in other ring trials [19,26,36] and should be addressed since could be potentially a hazard in routine diagnostics. Nonetheless, the laboratories that presented false negatives and positives had to repeat the ring test in order to maintain SERNAPESCA's authorization to perform the diagnostic of IPN, improving their results in sensitivity and specificity the second time of the trial (results not shown).

The repeatability for each laboratory, observed in the standard deviation and range values for positive samples throughout the three days of the trial, was variable and sometimes deficient; with some laboratories showing great differences in the Ct values for the same sample over the three days. As was mentioned before, operator expertise can be an important factor in the outcome of the techniques; therefore, to improve repeatability, it is of critical importance to implement a good training and a strict supervision of the personal in charge of the diagnostics, as well as to maintain a continuous quality check of all the instruments involved in the protocols.

On the other hand, when each positive sample was compared throughout all the laboratories, an even greater dispersion in the results was found. This inter-laboratory variability was further examined with the Fleiss Kappa coefficient, which showed only a fair agreement among the laboratories when assessing the positive samples. Variability between the results of the participants is understandable, since every laboratory uses their own in-house methods, and as discussed before, it could be related to several methodological differences. However, such large differences between the Ct values reported for a sample among all the laboratories were higher than anticipated. It is expected that if the laboratories would use the same standard test for IPNV detection, the level of agreement would increase. This has been shown for other inter-laboratory comparisons, in which it was possible to determine the reproducibility of a standard method [21]. It can be accepted that, for practical and competitive reasons, the laboratories use in-house methods; however, the noteworthy differences found need to be addressed in order to allow harmonized diagnostic activity within the country. The OIE recommends the continue assessment of a validated assay to ensure the maintenance of its fitness, especially during routine use in the targeted population [28]. Complementary, ring trials to determine how the in-house techniques work, regarding the RNA extraction methods, should soon be done.

In conclusion, the inter-laboratory comparison showed high values of sensitivity and specificity, but some problems of repeatability were presented. Besides, the variability observed for the Ct values of each sample among the laboratories was higher than expected. Therefore, the use of a standardized and generalized method is needed to ensure inter-laboratory reproducibility of qRT-PCR detection of IPNV. The methods used by the NRL in this study can be recommended to be used as a validated and reliable qRT-PCR protocol for the detection of IPNV for general use in Chile.

Conflict of interest

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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