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Infectious Salmon Anaemia Virus (ISAV) Ringtest: Validation of the ISAV Diagnositic Process using Virus-spiked Fish Tissues and ISAV TaqMan® Real-time RT-PCR

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Summary

Fourteen laboratories validated their procedure for detection of infectious salmon anaemia virus (ISAV) in fish tissues using TaqMan® real-time RT-PCR targeting ISAV RNA segment 8. The participants included 12 laboratories from South America, one from Asia and one from Europe. The OIE Reference Laboratory for ISA at the Atlantic Veterinary College in Canada served as the standard. All laboratories received a panel of 36 blind-coded samples representing six different ISAV preparations in homogenized fish liver or L-15 medium, each in six replicates. A clearly positive control ISAV of known titer was also included. From the results obtained, the 14 laboratories that submitted results reported no false positives. False negatives were mostly observed in the ISAV-spiked fish liver homogenate samples. The lowest virus titer to be detected in the fish liver homogenate was 10^1 $TCID_{50}$/ml, but the virus titer that could be detected accurately by most laboratories was 10^3 $TCID_{50}$/ml in L-15 medium. Within those laboratories that accurately detected presence of virus in a sample, there was great variation in the C_t values making it impossible to recommend a single cut-off C_t value. A significant factor influencing the C_t values obtained and therefore the diagnostic sensitivity might be the thermocycler software used. The repeatability of the test within each laboratory was high, but the reproducibility between laboratories was low. Presumably this could be improved if all the laboratories used the same RNA extraction method since the starting quality and the quantity of the RNA template is the main determinant of the quality of results once reagents have been optimized. The low reproducibility of the test between laboratories is also suggestive of the need to standardize the threshold fluorescence line of the thermocycler software and to use properly trained personnel to perform the test.

Introduction

Infectious salmon anaemia (ISA) virus (ISAV) is a major viral pathogen of marine-farmed Atlantic salmon (*Salmo salar*). ISAV belongs to the genus *Isavirus*, family *Orthomyxoviridae* [1]. The first registered outbreak of ISA occurred in Norway in 1984 [2]. Subsequently the disease was reported in Canada [3], Scotland [4], Faeroe Islands [5], and in Maine, USA [6]. In Chile, ISAV was first detected in 1999 in marine-farmed Coho salmon (*Oncorhynchus kisutch*) and was shown to be of the North American genotype [7], which subsequently became widespread in the Atlantic salmon industry in Chile but without signs of clinical disease [8]. ISAV of the European genotype caused a major epizootic in Chile's massive Atlantic salmon industry starting in June 2007 [8]. This virus was shown by phylogenetic analysis to be most closely related to Norwegian ISAVs isolated in 1997 and to have circulated in Chile for sometime prior to the index case in June 2007 [9]. Since the confirmation of the original ISA outbreak in Chile [8], the principal procedure of ISA laboratory diagnosis has been reverse transcription-polymerase chain reaction (RT-PCR), used directly on fish tissue samples, with primers targeting ISAV RNA segments 6 and 8, and sequencing of the PCR products (Sernapesca). RT-PCR testing in Chile is primarily performed by private diagnostic laboratories following the procedures outlined in the World Organization for Animal Health (OIE) Manual of Diagnostic Tests for Aquatic Animals [10].

The OIE Manual of Diagnostic Tests for Aquatic Animals recommends that when the PCR assay is used as a routine test, it is important to maintain the internal quality control [11]. The assay needs to be consistently monitored for repeatability and accuracy. Reproducibility between laboratories (ringtests) is recommended by the OIE to be estimated at least twice a year [12]. It is also advisable to regularly sequence the selected genomic regions in the viral isolates from the target country. This is especially true for the primer sites, to ensure that they remain stable so that the validation of the assay cannot be questioned.

Materials and Methods

Preparation and shipment of test samples

To simulate field samples, liver tissue was harvested from clinically healthy Atlantic salmon kept at the Atlantic Veterinary College (AVC) Aquatic Animal Facility. The fish were obtained as fingerlings from a local hatchery and were raised as non-challenge controls in strict isolation. The livers were weighed, homogenized, and then a 10% suspension in Leibovitz L-15 medium (L-15) was made. The liver homogenate was confirmed to be free of ISAV by conventional and

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real-time RT-PCR using ISAV RNA segment 8 primers in the OIE Reference Laboratory for ISA at AVC as detailed below (*RNA extraction and TaqMan® real-time RT-PCR*). The homogenate was then spiked with a known concentration of ISAV 10^5 $TCID_{50}$/ml of ISAV strain ADL-PM 3205 ISAV-07 (ADL-ISAV-07) of European genotype [13], which then served as stock for making 10-fold serial dilutions from 10^4 $TCID_{50}$/ml to 10^1 TCID50/ml. Then ISAV-spiked liver samples with ISAV 10^5 $TCID_{50}$/ml, 10^2 $TCID_{50}$/ml and 10^1 $TCID_{50}$/ml dilutions were used in the Ringtest. The liver spiked samples were to mimic field fish tissue samples which when received are macerated and suspended either in phosphate buffered formalin (PBS) or cell culture medium. In addition, two ISAV dilutions of 10^4 $TCID_{50}$/ml and 10^3 $TCID_{50}$/ml in L-15 medium were included in the Ringtest to represent cell culture lysate and/or the clarified supernatant of homogenized fish tissues. The liver homogenate in L-5 medium served as the negative control for the Ringtest panel. Also included were three replicates of ISAV 10^6 $TCID_{50}$/ml in L-15 medium to be used as the positive control. The participating laboratories were instructed to provide their own negative sample, which was to check for ISAV contamination in individual laboratories. For each preparation, six replicates of 200 µl of the sample was mixed with 600 µl of AVL buffer (Qiagen), which inactivated the virus so that the sample was no longer infectious [14]. Samples were shipped from Canada to 12 laboratories in South America, one laboratory in Asia and one laboratory in Europe. Each participating laboratory received a total of 36 blind-coded samples, of which 24 were from experimentally spiked liver homogenate and 12 were experimentally spiked L-15 medium. Three vials were marked "ISAV", and contained ISAV strain ADL-ISAV-07 capable of giving a low cycle threshold (C_t) value (~ C_t of 20) which was to be used as positive control. In total, each participating laboratory received 39 vials of samples, which were shipped frozen. Instructions were provided to each participating laboratory relative to its procedures and response time. Upon receiving the materials, each participating laboratory was instructed to immediately store the materials at -20°C until tested. The reference lab also used similarly handled samples for their subsequent testing.

RNA extraction

Each participating laboratory was provided a table showing the order in which the samples were to be tested (Table 1). Each laboratory used their own RNA extraction kit to prepare the RNA samples and then performed an ISAV TaqMan® real-time RT-PCR procedure(s) to the 6 samples of one set, including a positive control sample and a negative control sample (lab's own, for example "no template control" or "water") for a total of 8 samples per day. Thus RNA extraction and TaqMan®

Day test performed	1	2	3	4	5	6
Sample code	0A	0B	0C	0L	0J	0K
	0H	0F	0D	0N	0M	0P
	01	0G	0E	0R	0Q	0S
	04	05	02	012	0T	013
	08	06	03	015	014	016
	09	011	07	019	017	018
Positive control	ISAV		ISAV		ISAV	

Table 1: Coded samples provided to each laboratory and the respective day each sample was tested.

Testing Lab	RNA Extraction Kit	RT-PCR Kit	Probe source	Thermocycler		# Cycles	CT cut-off
				Instrument	Software		
OIE Ref Lab, A	QIAamp viral RNA extraction kit Qiagen	LightCycler 480 RNA Master Hydrolysis Probes	IDT	LightCycler 480	LightCycler software release 1.5.0	45	No CT is negative
Lab B	High Pure RNA Tissue kit Roche	LightCycler 480 RNA Master Hydrolysis Probes	IDT or Applied Biosystems	LightCycler 480	LightCycler 480 software release 1.5.0	45	No CT is negative
Lab C	High Pure RNA Tissue kit Roche	LightCycler 480 RNA Master Hydrolysis Probes	Roche	LightCycler 480	LightCycler second derivative max	45	No CT is negative
Lab D	Total RNA kit I (E.Z.N.A)	Express One-Step qPCR SuperScript Mix	Applied Biosystems	StepOne (Applied Biosystems)	StepOne Software v2.0 FAST mode	45	No CT is negative
Lab E	Total RNA kit I (E.Z.N.A)	Invitrogen		Stratagene MX 3000P	MxPro		>38 is inconclusive No CT is negative
Lab F	Total RNA kit I (E.Z.N.A)	Invitrogen		Stratagene MX 3000P	MxPro		>38 is inconclusive No CT is negative
Lab G	Total RNA kit I (E.Z.N.A)	TaqMan® RNA-to-Ct™ 1-Step Kit	Applied Biosystems	StepOnepPlus Real-Time PCR System (Applied Biosystems)	StepOne Software v2.0	45	>37.81 or no CT is negative
Lab H	No report	No report	No report	No report	No report	No report	No report
Lab I	High Pure RNA Tissue kit Roche	LightCycler 480 RNA Master Hydrolysis Probes	Roche	LightCycler 480	LightCycler 480 second derivative max	45	No CT is negative
Lab J	High Pure Viral Nucleic Acid kit Roche	LightCycler RNA Master Hydrolysis Probes		LightCycler 1.5	LightCycler 4.0	45	No CT is negative
Lab K	RNeasy Mini Kit Qiagen	One-step real-time RT-PCR Quantitech Probe Kit Qiagen		Stratagene MX 3000P	MxPro		No CT is negative
Lab L	Total RNA kit I (E.Z.N.A)	SuperScript III Platinum One-Step Quantitative RT-PCR system (Invitrogen)		Stratagene MX 3000P	MxPro	40	>38 or no CT is negative
Lab M	QIAamp viral RNA extraction kit Qiagen	SuperScript III Platinum One-Step Quantitative RT-PCR system (Invitrogen)	IDT	Stratagene MX 3000P	MxPro 3005 V 4.01 build 369	45	Seg 8 >35 & ELF1α <33 is negative
Lab N	Total RNA kit I (E.Z.N.A)	Stratagene Brilliant®II qRT-PCR Master Mix Kit	Applied Biosystems	Stratagene MX 3000P	MxPro QPCR default settings	45	No CT is negative
Lab O	MagMAX-96 Viral RNA Isolation Kit (MagMAX™ Express-96 Magnetic Particle Processors)	VetMAX Multiplex RT-PCR Reagents	confidential	Applied Biosystems 7500 Fast System	7500 System SDS Software Version 1.4.0.25	40	≥38 is negative
Lab V	Total RNA kit I (E.Z.N.A)	SuperScript III Platinum One-Step Quantitative RT-PCR system (Invitrogen)		Stratagene MX 3000P	MxPro	45	>40 is negative

Table 2: Real-time RT-PCR protocols used by each laboratory for amplification of ISAV segment 8.

Sample Identity	Negative control	Liver in L-15 medium	ISAV 10^1 in liver **homogenate**	ISAV 10^2 in liver **homogenate**	ISAV 10^5 in liver **homogenate**	ISAV 10^3 in L-15 medium	ISAV 10^4 in L-15 medium	Positive control **(ISAV 10^6 in L-15 medium)**
Sample Codes	Lab's own	01, 0B, 0D, 012, 0M, 018	0A, 0G, 0E, 015, 0T, 0K	08, 011, 0C, 0L, 0Q, 016	0H, 05, 07, 019, 017, 013	04, 06, 02, 0R, 0J, 0P	09, 0F, 03, 0N, 014, 0S	ISAV
Testing Lab								
OIE Ref Lab, A	0	0	34.98±0.39	31.97±0.38	31.27±0.64 (3/6)	32.55± 0.58	29.63±0.38	22.7±0.55
Lab B	0	0	0	32.0± 0.4 (2/6)	28.89±1.1	31.41±0.37 (2/6)	31.04±0.99	25.98±1.93
Lab C	0	0	35.98±0.34	33.67±0.68	31.8±0.53	32.46±0.98	30.62±0.78	24.14±0.41
Lab D	0	0	30.9 (1/6)	31.4±1.0	24.9±0.99	29.5±0.98	26.18±0.77	19.5±0.28
Lab E	0	0	40.87±1.1	36.85±0.77	30.0 ± 0.59	35.11±1.03	31.78±0.64	24.09±0.44
Lab F	0	0	39.15±0.69	35.62±0.62	30.32± 1.3	34.26±1.0	30.91±0.4	24.16±0.89
Lab G	0	0	0	31.11±0.66	26.59 ±1.18	29.39±0.27	26.58±52	20.58± 0.58
Lab H	No report	No report	No report	No report	No report	No report	No report	No report
Lab I	0	0	35.84±0.56	33.97±0.39	30.81±1.8	33.95±0.56	30.74±0.4	23.82±1.1
Lab J	0	0	29.64 ±2.17 (3/6)	29.06 ±1.69	27.05 ± 3.64	28.96 ±0.83	27.49 ±0.45	21.05±1.1
Lab K	0	0	0	38.12±0.60	29.04±0.85	34.41±0.60	31.76±0.87	25.2±1.27
Lab L	0	0	38.85±0.62	36.18±0.86	28.13±0.62	34.39±0.79	31.28±0.62	23.72±0.78
Lab M	0	0	37.06±0.99	34.2±0.33	28.41±1.4	32.0±0.23	28.94±0.26	22.34±0.19
Lab N	0	0	37.82±1.3 (5/6)	35.22±1.61	28.04±1.18	33.3±1.45	30.14±1.4	24.63±0.98
Lab O	40	39.9±0.2	36.39±1.2	33.57±0.45	24.2 ± 0.59	31.23±1.4	28.08±1.43	22.1±0.11
Lab V	0	42.97±0.51[1] (3/6)[2]	41.02±0.49	31.14±15.37(5/6)	29.9±0.91	35.41±0.33	32.24±0.32	25.13± 0.17

In Blue: denotes mean C_t values that are ≥3 more than expected.
In green: denotes unexpected false negative(s).
In magenta: denotes sensitivity of real-time RT-PCR for ISAV is below that of most laboratories.
In red: denotes poor performing laboratory based on having more than two days (a) with mean Ct values that are ≥3 more than expected, and/or (b) with unexpected false negative(s), and/or (c) unable to detect a virus titer of 10^3 $TCID_{50}$/ml.

Table 3: Overall performance (inter-lab and inter-assay) of different laboratories using real-time RT-PCR protocols for amplification of ISAV segment 8 Mean C_t value ± SD[1] for assays performed on 6 different days. Where a sample had no C_t value (i.e., C_t = 0), the number of days in which it had a C_t value (C_t = >0) out of the six days it was tested is given in brackets[2].

Sample ID	Repeatability of Reference Lab	Overall repeat-ability	Reproducibility	Performance above bench-mark*	Performance at benchmark*	Performance below bench-mark*	Performance above repro-ducibility	Performance above overall repeatability	Same as the overall repeat-ability or below
ISAV 10^1 in liver **homogenate**	1.11	2.04	4.61	D, E, F, I, J, L, M, N, O	A, C, V	B, G, K	D, J	E, M, N, O	A, B, C, F, G, I, K, L,V
ISAV 10^2 in liver **homogenate**	1.07	2.54	5.54	B, C, D, E, F, G, J, K, L, N, V	A, I, M, O	-	B	D, J, N, V	A, C, E, F, G, I, K, L, M, O
ISAV 10^3 in L-15 medium	1.63	2.43	5.46	B, C, D, E, F, J, L, N, O	A, I, K	G, M, V	B, J	C, D, E, F,N, O	A, G, I, K, L, M, V
ISAV 10^4 in L-15 medium	1.08	2.23	4.65	B, C, D,E, F, G, J, K, L, N, O	A, I	M, V	-	B, K, N, O	A, C, D, E, F, G, I, J, L, M, V
ISAV 10^6 in L-15 medium	1.54	1.53	4.51	B, F, I, J, K, L, N	A, G	C, D, E, M, O, V	B	F, I, J, K, L, N	A, C, D, E, G, M, O, V

*The repeatability and repeatability of the reference lab (Lab A) served as the standard. On this basis, most laboratories performed well.

Table 4: Summary of the laboratory performances (repeatability) relative to the repeatability, reproducibility and repeatability of the reference lab for individual ISAV titers.

RT-PCR assays were to be repeated 6 times except for the positive control, which was only extracted on days 1, 3 and 5. The different RNA extraction kits that were used by the different laboratories are listed in Table 2. The reference lab extracted the viral RNA using QiAamp Viral RNA mini extraction kit following the kit manual (Qiagen). RNA was extracted on six different days as shown in Table 1. On days 1, 3 and 5, one tube of the positive control was included. The viral RNA was eluted using 60 µl of AVE buffer (Qiagen) and stored at -80°C until used in ISAV TaqMan® real-time RT-PCR.

TaqMan® real-time RT-PCR

Since the tissue samples in the Ringtest contained whole virus, each laboratory had the option to use a TaqMan® real-time RT-PCR targeting any ISAV RNA segment. All participating laboratories used ISAV TaqMan® probe targeting RNA segment 8; only two laboratories also reported results with probes to RNA segments 5 and 6. Therefore, the results in this report are for TaqMan® real-time RT-PCR targeting ISAV RNA segment 8 [15,16] except for lab O which did not disclose the probe used. The source of the ISAV RNA segment 8 primers, probes and kits were individually procured by the different laboratories. Thus, five different real-time PCR machines were used in this exercise (Table 2). Primers and probes were also ordered from several suppliers. However, all labs used the same primer and probe sequences previously described by Snow et al. (2006) [15]. The reference lab obtained the HPLC purified ISAV RNA segment 8 primers from Invitrogen Life Technologies and the ISAV RNA segment 8 dual-labeled probes from Integrated DNA Technologies Inc (IDT). The real-time RT-PCR with TaqMan® probe and primers targeting ISAV RNA segment 8 was performed as described by Workenhe et al. (2008) [16] using LightCycler 480 RNA Master Hydrolysis Probes (Roche) and LightCycler 480 machine. The data were analyzed by LightCycler software release 1.5.0. The RT-PCR was deemed to be sensitive if there was a 3 C_t difference between 10-fold dilutions or a 7 C_t difference between 100-fold dilutions, since it is generally accepted that a 3.3 C_t difference between two samples is equal to a 10-fold difference in starting sample concentration [17,18].

Statistical analysis

The results were analyzed and a performance report of all participating laboratories prepared. All participating laboratories received all results after analysis, with each laboratory identified only by its Code (to protect their identity) so that they could see how they compared among the other participating laboratories. The analysis

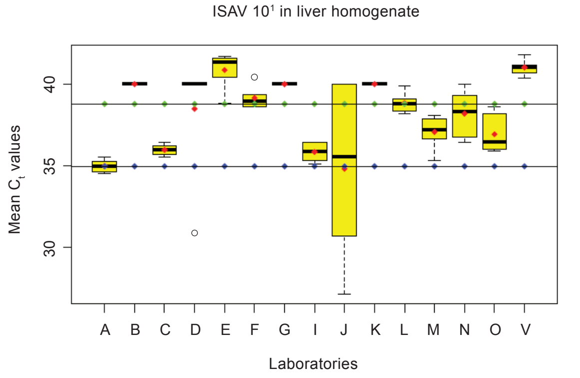

Figure 1: Each box plot represents the relation to the maximum, the minimum, the mean (♦), the median (-) as well as the Inter-Quartile range (■) of the C_t values for each lab, error bars represent the lowest and the highest C_t value. Also shown are the extreme values (○). The line with (-♦-) is the reference lab median C_t value and the line with (-♦-) represents the overall median for all laboratories.

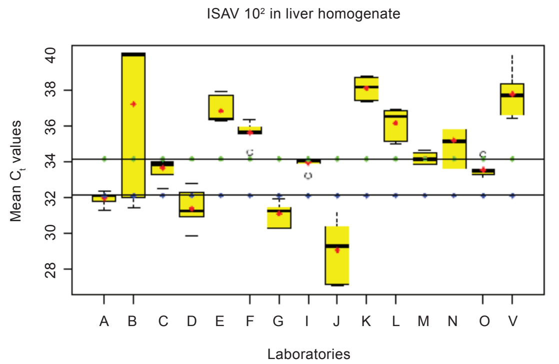

Figure 2: Each box plot represents the relation to the maximum, the minimum, the mean (♦), the median (-) as well as the Inter-Quartile range (■) of the C_t values for each lab, the error bars represent the lowest and the highest C_t value. Also shown are the extreme values (○). The line with (-♦-) is the reference lab for ISA at AVC median C_t value and the line with (-♦-) represents the overall median for all laboratories.

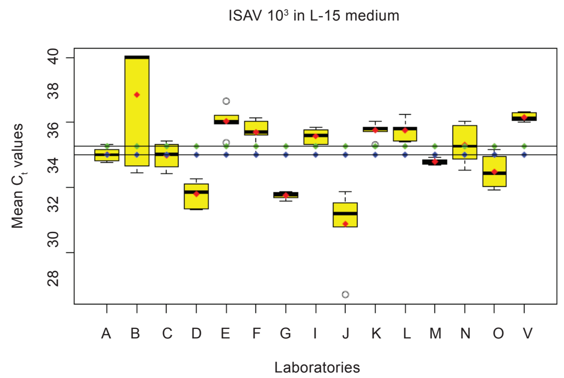

Figure 3: Each box plot represents the relation to the maximum, the minimum, the mean (♦), the median (-) as well as the Inter-Quartile range (■) of the C_t values for each lab, error bars represent the lowest and the highest C_t value. Also shown are the extreme values (○). The line with (-♦-) is the reference lab for ISA at AVC median C_t value and the line with (-♦-) represents the overall median for all laboratories.

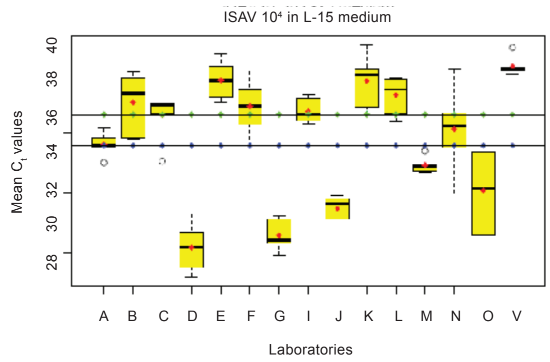

Figure 4: Each box plot represents the relation to the maximum, the minimum, the mean (♦), the median (-) as well as the Inter-Quartile range (■) of the Ct values for each lab, error bars represent the lowest and the highest Ct value. Also shown are the extreme values (○). The line with (-♦-) is the reference lab for ISA at AVC median C_t value and the line with (-♦-) represents the overall median for all laboratories.

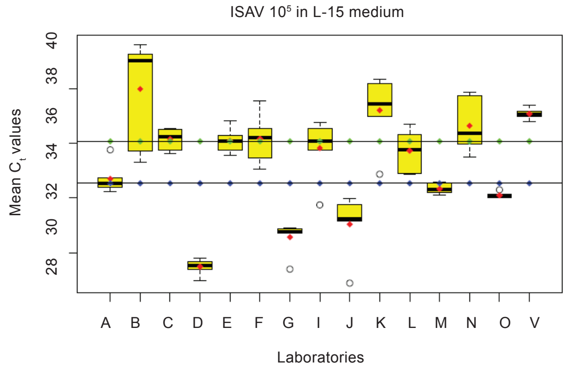

Figure 5: Each box plot represents the relation to the maximum, the minimum, the mean (♦), the median (-) as well as the Inter-Quartile range (■) of the C_t values for each lab, error bars represent the lowest and the highest C_t value. Also shown are the extreme values (○). The line with (-♦-) is the reference lab median C_t value and the line with (-♦-) represents the overall median for all laboratories.

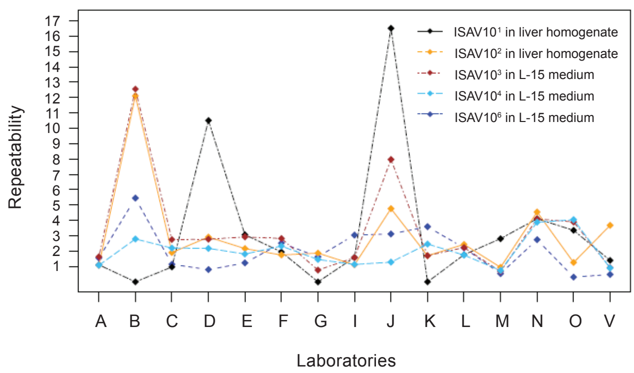

Figure 6: Repeatability from 15 laboratories across 6 dilutions.

included determination of analytical sensitivity and specificity and predictive values of the ISAV TaqMan® RT-PCR procedure for each participating laboratory. By using known varying amounts of ISAV in AVL buffer for RNA extraction, it allowed not only the establishment of the reproducibility of the ISAV TaqMan® RT-PCR procedure, but

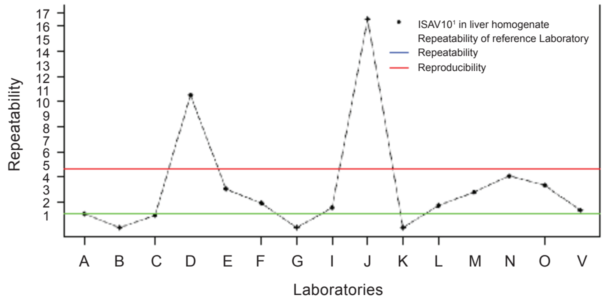

Figure 7: Repeatability from 15 laboratories represents the ISAV10^1 TCID$_{50}$/ml in liver homogenate dilution compared to the repeatability of the reference lab, repeatability and reproducibility.

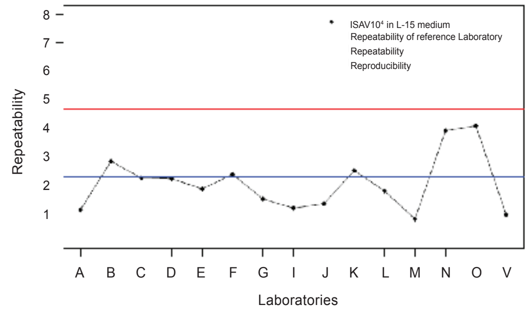

Figure 10: Repeatability from 15 laboratories represents the ISAV 10^4 TCID$_{50}$/ml in L-15 medium dilution compared to the repeatability of the reference lab, repeatability and reproducibility.

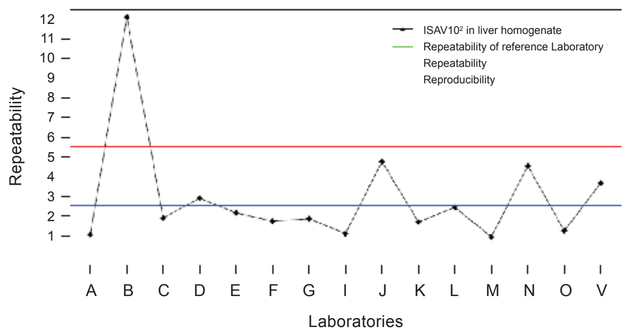

Figure 8: Repeatability from 15 laboratories represents the ISAV 10^2 TCID$_{50}$/ml in liver homogenate dilution compared to the repeatability of the reference lab, repeatability and reproducibility.

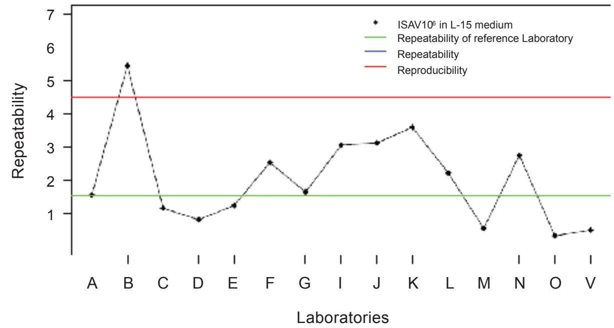

Figure 11: Repeatability from 15 laboratories represents the ISAV 10^6 TCID$_{50}$/ml in L-15 medium dilution compared to the repeatability of the reference lab, repeatability and reproducibility.

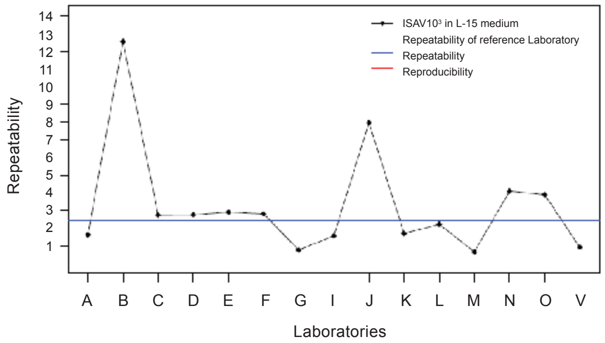

Figure 9: Repeatability from 15 laboratories represents the ISAV 10^3 TCID$_{50}$/ml in L-15 medium dilution compared to the repeatability of the reference lab, repeatability and reproducibility.

also to objectively compare the relative performance of the different laboratories' total RNA extraction step, ISAV TaqMan® assay protocol, thermocyclers, and technologists combination against the reference lab as standard.

Statistical analysis on the data was performed using R software [19] for statistical computing and graphics. Results were compiled and analyzed as per ISAV dilution and each participating laboratory. For the statistical analyses, all negative results (see Table 3) were replaced with a C_t value of 40. Box plot summary statistics were generated. Repeatability and reproducibility were calculated according to the international standard [20] ISO 5725. Random effects model [21] was used to estimate the variance components between laboratories and within laboratories' C_t values for each dilution, which were used to estimate the repeatability and reproducibility. Then the residual variance was used to calculate the repeatability and the total variance was used to calculate the reproducibility [22]. The repeatability is defined by ISO as "the value less than or equal to which the absolute difference between two test results obtained under repeatability conditions may be expected to be with a probability of 95%". The reproducibility is defined by ISO as "the value less than or equal to which the absolute difference between two test results obtained under reproducibility conditions may be expected to be with a probability of 95%". In all cases, two external benchmarks were used. The first benchmark, represented in Figure 7, Figure 8, Figure 9, Figure 10, Figure 11 by the blue line, is based on the international standard ISO 5725 [20], where if the repeatability of a participating laboratory exceeds the repeatability of the corresponding ISAV dilution investigated (i.e., the blue line) then the laboratory's repeatability is not up to standard. The second benchmark, represented in Figure 7, Figure 8, Figure 9, Figure 10, Figure 11 by the green line, is based on the repeatability of the reference lab (Lab A), where if the repeatability of a participating laboratory exceeds the repeatability of the reference lab (i.e., the green line) but is below the repeatability of the corresponding ISAV dilution investigated (i.e., the blue line) then the laboratory's repeatability is considered acceptable. Therefore, the repeatability for each laboratory was calculated and plotted across several dilutions. Repeatability for each laboratory was also plotted against the repeatability of the reference lab, as well as the repeatability and the reproducibility for each dilution.

Results

The study was set up to compare the efficiency of RNA extraction from serial dilutions of ISAV-spiked fish liver tissue, and the sensitivity of TaqMan® real-time RT-PCR assay of several diagnostic laboratories that test fish samples for ISAV. For purposes of this Ringtest, the reference lab used five criteria for interpreting the RT-PCR results (strong positive, weak positive, very weak positive, suspicious, and negative). The sample was considered strong positive if the C_t value was below ≤30, weak positive when C_t was ≥ 30.1 but ≤35 C_t, the sample was very weak positive when the C_t value was between 35.1 and 40, was considered suspicious if C_t was between 40.1 and 45.0, and was negative if there was no C_t value. In this study each participating laboratory set its own cut-off C_t value as shown in Table 2. A sample was considered negative if there was no C_t value but the cut-off C_t value for negative samples ranged between ≥40 and ≥35. One laboratory included the result of ELF1α assay (Snow et al., 2006) [15] in the interpretation of the C_t values (Table 2).

The results of the 15 participating laboratories are summarized in Table 3 as mean C_t plus standard deviation. None of the laboratories reported a false positive result; all liver homogenates without virus and all negative controls were reported negative. In contrast, false negatives were reported in some ISAV dilutions, mostly in liver homogenate samples. The sample ISAV 10^5 $TCID_{50}$/ml in liver homogenate was prepared as the stock from which the two ISAV-spiked liver samples, 10^2 $TCID_{50}$/ml and 10^1 $TCID_{50}$/ml, were prepared as serial 10-fold dilutions. During RNA extractions in the reference lab, it was noted that this stock sample tended to clog the Qiagen column in contrast to the serial dilutions, and gave very variable results. It was included in the Ringtest panel to check on the efficiency of extractions in the participating laboratories. Consequently, the results of the ISAV 10^5 $TCID_{50}$/ml in liver homogenate sample were not included in the statistical analysis.

For most laboratories, with a few exceptions, the C_ts obtained reflected the serial dilution of the samples particularly for ISAV in L-15 medium. The C_ts increased with lower ISAV titer (for example for the reference lab, ISAV at 10^6, 10^4, and 10^3 $TICD_{50}$/ml in L-15 medium had C_ts of 22.7±0.55, 29.63±0.38, and 32.55± 0.58, respectively), and the sensitivity of the RT-PCR was achieved within each laboratory whereby there was a 3 C_t difference between 10-fold dilutions (10^3 $TCID_{50}$/ml and 10^4 $TCID_{50}$/ml in L-15 medium) and a 7 C_t difference between 100-fold dilutions (10^4 $TCID_{50}$/ml and 10^6 TCID50/ml in L-15 medium) (Table 3). The summary statistics results for the sample dilutions are presented in Figures 1-5, showing the mean(◆), the median (-) as well as the Inter-Quartile range (▫) of the C_t values for each laboratory; the error bars represent the lowest and the highest C_t value. Also shown is the extreme value (○) where applicable. The median C_t value (-◆-) is for the reference lab and the overall median C_t value (-◆-) for all laboratories is also shown. The larger the box, the more spread out the C_t values are. The repeatability for all dilutions is shown in Figure 6 while repeatability and reproducibility for individual dilutions for all laboratories are shown in Figure 7, Figure 8, Figure 9, Figure 10, Figure 11. The closer the points appear for the same laboratory in Figure 6 (for example, labs A, C, F, G, L), the better the consistency of the laboratory results across dilutions. Generally, the variations between laboratories (reproducibility) are expected to be larger than the variations within the laboratories (repeatability). Thus, excess variability within a particular laboratory (for example, labs B, D, J) reveals a strong indication of inconsistency in results. Therefore, repeatability of a particular laboratory that is closer or larger than the reproducibility (--) is not detrimental (Figure 7, Figure 8, Figure 9, Figure 10, Figure 11), and vice versa for the overall average (--).

ISAV in the ISAV 10^1 $TCID_{50}$/ml in liver homogenate:

Three laboratories (B, G, K) did not detect ISAV at 10^1 $TCID_{50}$/ml in liver homogenate sample while eight laboratories (D, E, F, J, L, M, N, V) reported some false negatives in this sample. All laboratories reported mean C_t values that were more than the reference lab median C_t (Figure 1) and four laboratories (E, F, L, V) C_t values were above the overall median C_ts (Figure 1). The data were analyzed for both repeatability and reproducibility, and with a 95% confidence, the difference of two values (repeatability) from the same laboratory did not exceed 2.04, whereas two values (reproducibility) from different laboratories with 95% confidence do not differ by more than 4.61. Nine laboratories (A, B, C, F, G, I, K, L, V) had the same or lower repeatability than the repeatability of all laboratories whereas for 4 laboratories (E, M, N, O) the repeatability was above the repeatability of all laboratories. Laboratories D and J had repeatability greater than the reproducibility value (Table 4, Figures 6 and 7), which was due to the larger variability that was created by replacing the negative C_t values (Table 3). Also, laboratories B, G, and K showed repeatability below that of the reference lab (Lab A) because they consistently had all negative C_t values (i.e., they did not detect any viral RNA) in this sample dilution (Table 3).

ISAV in the ISAV 10^2 $TCID_{50}$/ml in liver homogenate:

Two laboratories (B, V) reported some false negatives for the ISAV at 10^2 $TCID_{50}$/ml in liver homogenate sample. Another five laboratories (E, F, K, L, N) had C_t values that were ≥3 more (i.e., 10-fold less virus titer) than expected. The estimated repeatability and reproducibility for this sample were 2.54 and 5.54, respectively. Four laboratories showed repeatability above the repeatability (D, J, N, V), and the repeatability of laboratory B exceeded the reproducibility (Table 4, Figures 6 and Figure 8).

ISAV in the ISAV 10^3 $TCID_{50}$/ml in L-15 medium

False negatives were reported by laboratory B (4/6) in ISAV at 10^3 $TCID_{50}$/ml in L-15 medium sample, and for statistical analysis, these were replaced by a C_t value of 40. The repeatability and reproducibility for the sample of this dilution were 2.43 and 5.46, respectively. Six laboratories (C, D, E, F, N, O) showed repeatability above the repeatability but below the reproducibility (Figure 9). The repeatability of two laboratories (B, J) exceeded the reproducibility (Figure 9). The box plot for these laboratories was large indicating larger variability in the C_t values reported (Table 4, Figures 3 and 9).

ISAV in the ISAV 10^4 $TCID_{50}$/ml in L-15 medium:

No laboratory had a missing value in this sample. In the box plot eight laboratories had C_t values above the overall median and five were below the laboratory A median (Figure 4). The repeatability and reproducibility were 2.23 and 4.65, respectively. All laboratories had repeatability below the dilution's reproducibility (Figure 10). However, the repeatability of four laboratories (B, K, N, O) was above the repeatability. The box plot of these laboratories were large (Figure 4) indicating larger variability in the C_t values reported (Table 4, Figures 4 and Figure 10).

ISAV in the ISAV 10^6 $TCID_{50}$/ml in L-15 medium

No laboratory had a negative C_t value in this sample, which was the ISAV positive control. The box plots of nine laboratories (B, C, E, F, I, K, L, N, V) were above or very close to the overall median and four laboratories (D, G, J, O) were below the reference lab median (Figure 5). The estimated repeatability and reproducibility were 1.53 and 4.51, respectively. The repeatability of laboratory B exceeded the reproducibility, while six laboratories (F, I, J, K, L, N) showed repeatability above the repeatability (Table 4, Figure 11).

The ISAV at 10^4 TCID $_{50}$/ml and at 10^6 TCID $_5$0/ml in L-15 medium were identified as positive by all laboratories, although two laboratories (B, K) had C_t values ≥3 more (i.e., 10-fold less virus titer) than expected for the positive control (Table 3). Overall, the repeatability of the reference lab was always below the repeatability except for the ISAV at 10^6 TCID $_5$0/ml in L-15 medium where the two were similar (Table 4, Figure 11). The repeatability of the samples ranged from as low as 1.53 for the ISAV at 10^6 TCID $_{50}$/ml in L-15 medium (positive control) to as high as 2.54 for the ISAV at 10^2 TCID $_{50}$/ml in liver homogenate. Similarly, the reproducibility ranged from 4.51 for the ISAV at 10^6 TCID $_{50}$/ml in L-15 medium (positive control) to 5.54 for the ISAV at 10^2 TCID $_{50}$/ml in liver homogenate.

Discussion

This Ringtest was set up to compare the efficiency of RNA extraction from serial dilutions of ISAV-spiked fish liver tissue, and the sensitivity of Taqman® real-time RT-PCR assay of several laboratories involved with ISAV diagnostic testing. As most private fish diagnostic laboratories in South America have adopted real-time RT-PCR with Taqman® probe assay for detection of ISAV in field samples, a need for standardization and optimization of the test was requested. The purpose was to be sure that all the diagnostic laboratories were performing at the same level and the reported results were accurate. Considering that the private diagnostic laboratories do not normally quantify the RNA used in the RT-PCR and since most of the variation between laboratories is at the RNA extraction step, this Ringtest was designed to use fish tissue spiked with known amounts of ISAV provided in AVL buffer.

In the reference lab, RNA extracted from some fish tissues has sometimes given a negative real-time RT-PCR result when extracted at high concentration of tissue homogenate, but will give a positive result when either a higher dilution is extracted or conventional RT-PCR is used. Thus, this Ringtest included the ISAV at 10^5 TCID $_{50}$/ml in liver homogenate sample (Table 3) for purposes of evaluating the RNA extraction protocols used in the different diagnostic laboratories. This sample was the initial preparation that was made, and was then diluted down using L-15 medium to generate the ISAV at 10^2 TCID$_{50}$/ml and ISAV at 10^1 TCID$_{50}$/ml in liver homogenate samples; the lower virus titer samples also contained less liver tissue. Consequently, the results of the ISAV at 10^5 TCID$_{50}$/ml in liver homogenate sample were not included in the statistical analysis.

We objectively compared RT-PCR protocols and thermocylers between laboratories in their ability to reproducibly amplify segment 8 of ISAV in comparison to a reference laboratory. We avoided the risk of "importing" the virus by having all samples (including the spiked liver tissue) mixed with AVL buffer (Qiagen), which inactivated the virus so that the samples were no longer infectious [14]. Fourteen international laboratories (in South America, Asia and Europe) requested to participate in order to validate their ISAV assays. This report is only of results by all laboratories using ISAV RNA segment 8 primers and probes [15] except for lab O which did not disclose the probe used and had different working dilution solutions.

There was no standardized RNA extraction protocol; each laboratory used their protocol and their thermocyler as described in Table 2. Whether these are the reasons for the variation observed in the results is not clear because even those laboratories that used the same kits and/or thermocyler had variable results. In general, there were no false positives reported by any laboratory, even though variable C_t cut-offs were used by different laboratories to call a sample negative. Within an individual laboratory, this should increase confidence that there was no cross contamination of samples.

On the basis of these results, it was more likely to obtain a false negative than a false positive result, regardless of the virus concentration in the sample. This was particularly true for the ISAV dilutions in liver tissue homogenate as compared to the dilutions in L-15 medium. All laboratories were able to detect ISAV in L-15 medium samples while there was difficulty in detecting virus in the liver homogenate samples. This could be a result of poor RNA quality (degraded or very limited quantity in the sample) or may be due to presence of RT-PCR inhibitory factors in the liver tissue homogenate. Overall, samples of ISAV in the liver homogenate had poor reproducibility. However, the fact that only one lab reported some false negatives for the ISAV at 10^5 TCID$_{50}$/ml in liver homogenate sample implies that RNA extraction is probably not a major issue for most laboratories, and that all RNA extraction kits used in this study perform well in the diagnostic laboratory. In any case, as a safeguard, it is recommended that when a fish tissue tests negative on the initial screen with real-time RT-PCR, the test should be repeated either on the same RNA preparation or a repeat extraction of RNA. It is also a good practice to attempt virus isolation using permissive cell lines [23] and confirm the virus isolate with real-time RT-PCR before reaching a conclusive diagnosis.

Within those laboratories that accurately detected presence of virus in a sample, there was great variation in the C_t values. The reference lab considered the C_t values to be off if there was at least a 3 C_t difference as this reflected a 10-fold difference in the template concentration [17, 18] and therefore virus titer. However, the difference could be due to the use of different thermocylers, and different software for data analysis, and this has to be accounted for. Regardless of the method of RNA extraction and DNA thermocyler used, the lowest amount of ISAV titer that was detected by almost all laboratories was 10^3 TCID$_{50}$/ml in L-15 medium.

Because there was overlap in both methods of RNA extraction and the thermocylers used in the laboratories that did well in the Ringtest and those that performed poorly, the variability in the results could also be reflective of the level of training and competence of the individuals who performed the test. In addition, the accuracy of the equipments (pipettes), RNA extraction kits, and thermocylers in the different laboratories should be considered. Even with variable equipments, most laboratories were able to accurately detect ISAV in the correct samples. Those that performed poorly on this Ringtest may require more training of their technical staff or to upgrade their thermocylers.

An interesting observation that could easily be overlooked is the effect of the software in the different thermocyclers on the threshold fluorescence; the value that the fluorescence intensity has to exceed in order to register a C_t value. From Tables 2 and 3, it is apparent that the seven laboratories that used the Stratagene software MxPro (Lab E, F, K, L, M, N, V) all reported relatively high C_t values compared to the other participating laboratories for the same samples, and in fact had the highest C_t values for the samples with the lowest amounts of virus (ISAV at 10^1 TCID$_{50}$/ml and 10^2 TCID$_{50}$/ml in liver homogenates) except for Lab V "ISAV 10^2 TCID$_{50}$/ml in liver homogenate", but the lower mean C_t value in this case had a very high standard deviation. The consequence is that these seven laboratories were flagged in Table 3 for having mean C_t values that were ≥3 more than expected and/or having unexpected false negative(s) for the ISAV-spiked liver samples that had the lowest amounts of virus. This indicated to us that a significant factor influencing the C_t values obtained and therefore the diagnostic sensitivity might be the software used. In this particular case, adjusting the threshold fluorescence line in the software appropriately may address the problem of variation in the C_t values, which might allow the generation of a single cut-off C_t value for all laboratories irrespective of the thermocycler and software used.

Conclusions

In conclusion, this Ringtest showed that there are various RNA extraction kits as well as thermocylers that are used in the laboratory diagnosis of ISAV, resulting in poor reproducibility of the test result as a C_t value. The variation in performance of different laboratories could also result in false reporting of the fish tissue sample status. The sensitivity of TaqMan® real-time RT-PCR for ISAV for most laboratories is 10^3 $TCID_{50}$/ml in L-15 medium, although a significant factor influencing the C_t value and therefore the diagnostic sensitivity might be the thermocycler software used. It is considered that exercises such as this one if carried out regularly would encourage individual laboratories to assess their performance in comparison with the other laboratories.

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