Detection of infectious salmon anaemia virus by real-time RT–PCR

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Abstract

A one-tube real-time reverse transcription–polymerase chain reaction (RT–PCR) using LightCycler technology and SYBR Green chemistry that quantitatively detects infectious salmon anaemia virus (ISAV) in biological samples is described. The assay utilized primers targeting ISAV RNA segment 8, with ISAV isolate U5575-1 as template. The entire optimized assay, including one cycle of reverse transcription, 50 cycles of complementary DNA amplification, and data acquisition and analysis took only 80 min. The melting curve and gel electrophoresis analyses of real-time RT–PCR showed harmony with each other as a virus-specific single melting peak and a product of the expected size of 211 bp were obtained. A regression line between the mean threshold cycle (Ct) values and viral template concentrations over a $1:10^5$ dilution range with an r^2 value of 0.962 and a slope of -3.771 indicated that the assay was highly reproducible. This assay was 100 times more sensitive than the conventional one-tube RT–PCR assay when compared on the same sample. Analysis of different tissues from fish that survived an ISAV experimental infection further confirmed that real-time RT–PCR was more sensitive than regular RT–PCR for detection of ISAV nucleic acids. Temporal analysis of ISAV-infected TO cell cultures showed that the amount of the specific viral RNA increased more than 100-fold within 32 h post-inoculation (p.i.) and over 1200-fold by 144 h p.i. The melting curve analysis throughout the duration of the infection sampled had a single melting peak suggesting that the virus population was uniform in the targeted region. Quantitative analysis of CHSE-214 cell cultures infected with different ISAV isolates indicated that ISAV isolates, based on their ability to replicate and cause cytopathic effects in CHSE-214 cells, may be differentiated into three CHSE phenotypes: replicating cytopathic, replicating non-cytopathic, and non-replicating. Thus, the SYBR Green real-time RT–PCR is a sensitive, rapid, and highly reproducible assay that can be used to quantitate ISAV in biological samples.

1. Introduction

Infectious salmon anemia (ISA) virus (ISAV) causes an economically important and highly fatal disease of Atlantic salmon (*Salmo salar*) characterized by high mortality, severe anemia, exophthalmia, petechial hemorrhages in the visceral fat and skin, and hemorrhagic hepatic necrosis and kidney tubular necrosis and interstitial hemorrhages (Byrne et al., 1998; Dannevig et al., 1993; Evensen and Thorud, 1991; Falk and Dannevig, 1995; Falk et al., 1997; Hovland et al., 1994; Speilberg et al., 1995). ISAV is the newest member of the family Orthomyxoviridae recently classified in a new genus *Isavirus* (Anon., 2001). It is an enveloped virus with a segmented genome of eight single-stranded RNA segments of negative polarity.

Indirect fluorescent antibody test (IFAT), reverse transcription–polymerase chain reaction (RT–PCR), virus isolation (VI) and virus neutralization (VN) on ASK-2, SHK-1, TO, and CHSE-214 cell lines (Bouchard et al., 1999; Dannevig et al., 1995, 1997; Devold et al., 2000; Kibenge et al., 2000, 2001a; Rolland et al., 2003; Wergeland and Jakobsen, 2001) are the methods most commonly used to detect ISAV infections. However, these methods have several limitations. Some ISAV strains do not grow and/or produce cytopathic effect (CPE) in CHSE-214 and SHK-1 cells (Kibenge et al., 2000, 2001a; Rimstad and Mjaaland, 2002). Joseph et al. (2003) have reported enhanced ISAV infection of macrophage-like (SHK-1 and TO) cells facilitated by ISAV-specific antibodies via Fc-receptor-mediated endocytosis, suggesting reduced sensitivity of these cell

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lines for virus typing by neutralization. RT–PCR, VI, and IFAT also vary in their sensitivity and specificity (Optiz et al., 2000; Snow et al., 2003). Although conventional RT–PCR and nested RT–PCR are currently considered the most sensitive methods for ISAV detection (Devold et al., 2000; Kibenge et al., 2000, 2001b; Lovdal and Enger, 2002; Mjaaland et al., 1997; Optiz et al., 2000; Rimstad et al., 1999), none of the formats routinely used allow the results to be expressed quantitatively. Thus, there is a clear need for a sensitive and rapid method that could quantitatively detect ISAV in biological samples.

We describe below real-time RT–PCR using SYBR Green chemistry for the quantitative detection of ISAV in biological samples. The analytical sensitivity of this technique was compared to that of conventional RT–PCR for ISAV. In order to demonstrate the utility of the assay for samples with varying amounts of ISAV RNA, the technique was used to detect viral RNA in fish tissues as well as to quantitate the replication of several ISAV isolates in TO and CHSE-214 cell lines.

2. Materials and methods

2.1. Cell culture and virus isolates

Field ISAV isolates made from various ISA outbreaks in the Bay of Fundy, Canada, as well as ISAV isolates received from Norway, and Scotland for research purposes have been stored at −80 °C in our laboratory since 1997. Canadian ISAV isolates U5575-1, NBISA01, HKS-36, RPC/NB-970-877-2, RPC/NB-980-414-1, RPC/NB-980-280-2, and Scottish isolate 390/98, and Norwegian isolate 835/9/98 were selected for this study. TO cells and CHSE-214 cells were used to propagate the isolates as described previously (Kibenge et al., 2000, 2001a). Briefly, TO cell monolayers were grown to about 90% confluency at room temperature (22 °C) in HMEM (Eagle's minimum essential medium containing Hanks' salts; BioWhittaker) supplemented with 10% heat inactivated fetal bovine serum (FBS) (Wergeland and Jakobsen, 2001), 292 μg/ml L-glutamine (Invitrogen Life Technologies), 1% non-essential amino acids (Sigma), and 200 μg/ml gentamicin (Sigma) in 25 cm² tissue culture flasks; for maintenance medium, the FBS was reduced to 5%. For infection, the monolayers were washed with phosphate buffered saline (PBS), inoculated with virus, and then incubated for 1 h at room temperature to allow for virus adsorption. Maintenance medium was then added and the flasks were incubated at 16 °C for up to 10 days or until CPE was complete. The tissue culture virus suspensions were harvested, aliquoted, and stored at −80 °C prior to use. For testing ISAV isolate U5575-1 replication in CHSE-214 cells, five flasks were infected and one uninfected flask served used as control. One of the infected flasks was removed from the incubation at time 0 h (i.e. immediately upon adding maintenance

medium post-inoculation), and then at 32, 48, 72, 96, 192, and 144 h post-infection (p.i). The flasks were examined for CPE and were then frozen at −80 °C until further use. The CHSE-214 cell monolayers were grown at 16 °C in Hank's MEM (Invitrogen) supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B. The monolayers were similarly inoculated with virus as for the TO cells, and were incubated for up to 15 days at 16 °C. For each ISAV isolate tested for replication in CHSE-214 cells, five flasks were infected and one uninfected flask served as control. One flask was removed from the incubation at time 0 h and then at 72, 120, 192, and 336 h p.i. The flasks were examined for CPE and then were frozen at −80 °C until further use. Virus titration was performed on TO cell monolayers in a 48-well tissue culture plates as previously described (Kibenge et al., 2001a,b). The median tissue culture infectious dose (TCID$_{50}$) was calculated using the procedure described by Reed and Muench (1938).

2.2. Experimental infection of fish with ISAV and tissue sampling

The fish tissues used in our study were collected from Atlantic salmon and rainbow trout populations used in a previous challenge study. The average weight and length of fish at the beginning of this study were approximately 20 g and 10 cm, respectively. The ISAV-negative status of fish was confirmed by attempting virus isolation from tissues of six fish on TO cells and also testing by conventional one-tube RT–PCR at the start of the experiment. For the challenge study, 50 Atlantic salmon and 10 rainbow trout fish were removed from the stock holding tanks and anesthetized by immersion in an aerated solution of tricaine methane sulfonate (TMS-222) (100 mg/l). Each fish was then challenged by intraperitoneal injection of ISAV isolate U5575-1 at a dose of 10⁶ TCID$_{50}$ in 0.2 ml of virus suspension and was then returned to the study tank. Sixty-five uninfected control Atlantic salmon and 55 control rainbow trout were kept in another holding tank in a separate "clean" room. The mortality started 18 days post-ISAV challenge. The fish that survived ISAV challenge up to 76 days were sacrificed and necropsied for evidence of any lesions; their hearts, spleens, trunk kidneys, livers, gills, and pyloric caeca were collected aseptically in separate sterile plastic bags and stored at −80 °C for viral analysis by conventional one-tube RT–PCR and real-time RT–PCR assays.

2.3. RNA extraction

In order to extract total RNA from fish tissues, they were first macerated and homogenized. The homogenates were diluted 1:1 in sterile PBS and clarified by centrifugation at 3000 rpm for 15 min, and the supernatants were used for RNA extraction. Total RNA from infected fish tissues, purified virus, and/or from virus-infected cell culture suspensions was extracted using TRIZOL reagent following the

manufacturer's instructions (Invitrogen Life Technologies) with minor modifications as previously described (Kibenge et al., 2000). The starting sample volume in all cases was 300 μl of infected fish tissue supernatant, purified virus preparation or virus-infected tissue culture suspensions. Duplicate RNA extractions for each sample were performed.

2.4. Virus purification and preparation of ISAV RNA standards

ISAV isolate U5575-1 was purified as previously described (Kibenge et al., 2000). Briefly, the virus-infected TO cell culture suspensions were harvested, pooled, and clarified at 4430 rpm for 30 min at 4 °C. The viral supernatant was concentrated by ammonium sulphate precipitation. The virus in the precipitate was purified on a 10 and 25% Ficoll-400 (Amersham Pharmacia Biotech) step gradient (Kibenge et al., 2000). The RNA from the purified virus was extracted using TRIZOL reagent as previously described. The RNA samples were aliquoted, and immediately frozen at −80 °C. Each aliquot was used only once for the SYBR Green real-time RT–PCR and/or conventional RT–PCR assay. To prepare ISAV RNA standards, the viral RNA was quantitated using a spectrophotometer and serial 10-fold dilutions were then prepared in RNase-free PCR-grade water. Both SYBR Green RT–PCR and conventional RT–PCR assays were performed on three replicas of each dilution of viral RNA in order to determine the detection limit and analytical sensitivity of the two methods, and also to construct a standard curve for the real-time assay.

2.5. Conventional one-tube RT–PCR conditions

The conventional one-tube RT–PCR for ISAV with the F5/R5 primer pair targeting ISAV RNA segment 8 was carried out using the Titan One Tube RT–PCR System Kit (Roche Molecular Biochemicals) following the manufacturer's instructions and as previously described (Kibenge et al., 2000, 2001a,b). The 50 μl reaction volume for RT–PCR contained 0.8 mM deoxynucleotide triphosphates, 0.8 μM of each primer, 5 mM dithiothreitol solution, 0.25 μl RNase inhibitor (40 U/μl), 2 μl template (≥0.632 ng/μl), and 1.0 μl enzyme mix (AMV and Expand High Fidelity PCR-System) and 23.25 μl of PCR-grade water. All RT–PCR reactions were carried out in a PTC-200 DNA Engine Peltier thermal cycler (MJ Research Inc.) with the following thermal profile: a single cycle of reverse transcription for 30 min at 55 °C and a pre-denaturation step for 2 min at 94 °C followed by 40 amplification cycles each consisting of denaturation for 30 s at 94 °C, annealing for 45 s at 61 °C, and extension for 90 s at 72 °C, with a final extension step of 10 min at 72 °C. The amplified PCR products were resolved by 1% agarose gel electrophoresis in 0.5× TBE buffer and visualized by staining with ethidium bromide, and photographed under 304 nm UV light.

2.6. Optimization of ISAV SYBR Green real-time RT–PCR assay

The thermal profile, reagent conditions, $MgCl_2$ and primer concentrations for SYBR Green real-time RT–PCR assay were optimized using a checkerboard system. The SYBR Green assays were carried out using the same primer pair as for the conventional one-tube RT–PCR. All real-time RT–PCR reactions were performed in the LightCycler (Roche Applied Science) using RNA Amplification Kit SYBR Green I (Roche Applied Science). The size of real-time amplification reaction products was also analyzed by agarose gel electrophoresis similar to the conventional RT–PCR products.

2.7. Data analyses

The quantitation plot and melting curve data for all SYBR Green real-time RT–PCR runs were analyzed using the LightCycler software version 3.5 (Roche Applied Science). Minitab 13 software (Minitab Inc., State College, PA, USA) was subsequently used to calculate and plot a linear regression line between the logarithms of the ISAV RNA standards and the corresponding mean threshold cycle (Ct) values. The absolute ISAV RNA levels in the various biological samples were determined by extrapolating the Ct values from the standard curve. The coefficient of variation (CV) of precision within-run and between-runs was calculated by dividing the standard deviation of Ct values of replicas with mean Ct values of replicas. One-way analysis of variance was carried out using Minitab software in order to compare the mean Ct values of the different ISAV isolate RNA levels in CHSE-214 and/or TO cell lines at different hours post-infection.

3. Results

3.1. Optimization of real-time RT–PCR conditions for ISAV RNA

The specificity of primer pair F5/R5 for ISAV genomic segment 8 in RT–PCR has already been determined (Devold et al., 2000; Kibenge et al., 2000). The optimized concentrations of F5/R5 primers and other reagents for a 20 μl-reaction volume for the SYBR Green-based real-time assay are summarized in Tables 1 and 2. The melting curve and gel electrophoresis analyses of the real-time RT–PCR reactions were harmonious with each other as a virus-specific single melting peak (Fig. 1A) and a 211 bp band (data not shown) were always obtained.

3.2. Viral RNA quantitation

The amplification plot of serial dilutions of the ISAV genomic RNA is shown in Fig. 1B. The increasing number of cycles was proportional to the increasing dilutions of the
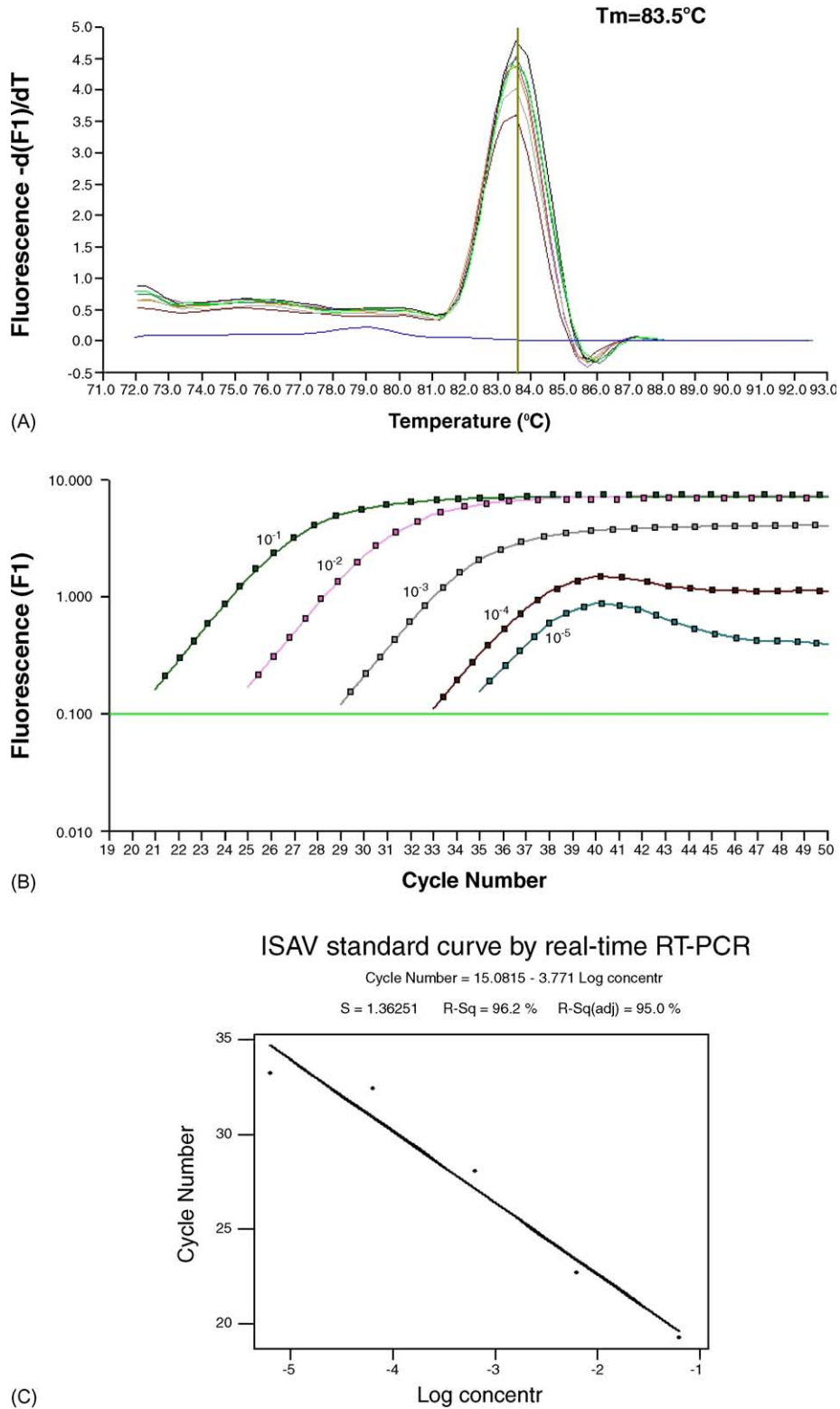
Fig. 1. Development of a one-tube SYBR Green real-time RT–PCR that quantitatively detects ISAV in biological samples. (A) Melting peak of ISAV isolate U5575-1 indicating the melting temperature (T_m) of virus-specific amplicon. Note the virus-specific single melting peak at 83.5 °C. (B) Quantitative analysis of ISAV dilutions. Serial dilutions of ISAV genomic RNA were amplified. The intensity of fluorescence is given on the *y*-axis and Ct values (here mentioned as cycle number) are plotted on *x*-axis. For simplicity, the amplification plot of a single replica of dilutions from 1:10^1 to 1:10^5 is shown. Note that the dilution containing the highest viral RNA concentration exhibits fluorescence earlier than the one with second highest concentration of viral template and so on. (C) Linear relationship between Ct values and log concentrations of the viral RNA. Note r^2 value and slope are 96.2% and −3.771, respectively.

Table 1

Optimized reagent concentrations in 20 µl reaction volume for ISAV SYBR Green real-time RT–PCR

Reagent	Concentration
5× RT–PCR reaction mix SYBR Green I[a]	4.0 µl
5× resolution solution[a]	3.0 µl
MgCl$_2$[a]	5.0 mM
Forward primer-F5 (5′-GAA GAG TCA GGA TGC CAA GAC G-3′)[b]	0.3 µM
Reverse primer-R5 (5′-GAA GTC GAT GAT CTG CAG CGA-3′)[b]	0.3 µM
RT–PCR enzyme mix[a]	0.4 µl
Viral RNA template	≥0.006 ng in 1.0 µl
Sterile PCR-grade water	9.4 µl

[a] Reagents are part of the RNA Amplification Kit SYBR Green I (Roche Diagnostics GmbH).

[b] Primers have been previously used in ISAV RT–PCR assays (Devold et al., 2000; Kibenge et al., 2000).

Table 2

Thermal profile for ISAV SYBR Green real-time RT–PCR

Step	Temperature (°C)	Time (s)
1 cycle of reverse transcription	55	1800
Pre-denaturation	95	30
50 cycles of		
Denaturation	95	5
Annealing	59	10
Elongation	72	10
Fluorescence acquisition	80	2

Melting curve was performed from 70 to 95 °C in 0.1 °C/s increments.

viral RNA. The linear regression analysis yielded a straight line between the mean Ct values and logarithmic concentrations of the viral template over a 1:10^5 dilution range with an r^2 value of 0.962 (Fig. 1C). The slope was −3.771, suggesting an amplification efficiency of 84% for this real-time RT–PCR assay.

3.3. Comparison of SYBR Green real-time RT–PCR and conventional RT–PCR for ISAV

The real-time format was able to detect viral RNA down to a 1:10^5 dilution (Fig. 1B) whereas agarose gel analysis of conventional one-tube RT–PCR product could detect viral RNA only down to a 1:10^3 dilution (Table 3). The detection limits of SYBR Green real-time RT–PCR assay and conventional one-tube RT–PCR on the same sample were 0.006 and

Table 4

Determination of absolute viral RNA levels at various times post-ISAV inoculation in TO cells by extrapolation of Ct values from the standard curve

Post-inoculation (h)	CPE[a]	Mean Ct value[b,c]	ISAV RNA levels (µg/µl)
0	−	25.370	0.001
32	−	18.600	0.117
48	−	16.910	0.331
72	−	16.770	0.363
96	+	16.295	0.478
120	+	15.780	0.653
144	+	14.730	1.238

[a] CPE denotes virus-induced cytopathic effects in cell culture.

[b] Ct values are averages of duplicate samples except for samples harvested at 72 and 144 h that represent Ct values of single samples.

[c] The difference among mean Ct values of ISAV RNA levels in TO cells at different hours post-infection was significant ($P \geq 0.05$) at 5% α level.

0.632 ng of ISAV genomic RNA, respectively. Thus, the analytical sensitivity of the real-time RT–PCR assay was 100 times higher than that of the conventional one-tube RT–PCR.

3.4. Repeatability of the SYBR Green real-time RT–PCR assay

The repeatability (agreement within- and between-runs) of the SYBR Green RT–PCR was assessed by testing the three replicas of the serial 10-fold dilutions of the ISAV genomic RNA within the same run and independently in five different runs on different days. The correlation coefficient (r) for precision-within and -between assays was 0.98 and 0.96, respectively. The mean CV for precision within- and between-runs was 2.78 and 6.40%, respectively.

3.5. Analysis of replication of ISAV isolate U5575-1 in TO and CHSE-214 cell lines by real-time RT–PCR

To verify that quantitation by real-time RT–PCR is reliable, total RNA extracted from TO cell cultures infected with ISAV isolate U5575-1 and harvested at different time intervals after infection was analyzed. Table 4 shows the ISAV genomic RNA levels determined by extrapolating the Ct values from the standard curve. The CPE in TO cells was first observed at day 4 (96 h) post-infection. The quantitation plot of ISAV genomic RNA levels at different hours post-infection is shown in Fig. 2A. The melting curve

Table 3

Analytical sensitivity of the conventional RT–PCR and SYBR Green real-time RT–PCR assays for ISAV in TO cells

Detection format	Template dilution							
	Neat	1:10^1	1:10^2	1:10^3	1:10^4	1:10^5	1:10^6	1:10^7
Gel electrophoresis of conventional one-tube RT–PCR products	3/3[a]	3/3	3/3	3/3	0/3	0/3	0/3	0/3
Real-time RT–PCR format	3/3	3/3	3/3	3/3	3/3	3/3	0/3	0/3
Gel electrophoresis of real-time RT–PCR products	3/3	3/3	3/3	3/3	0/3	0/3	0/3	0/3

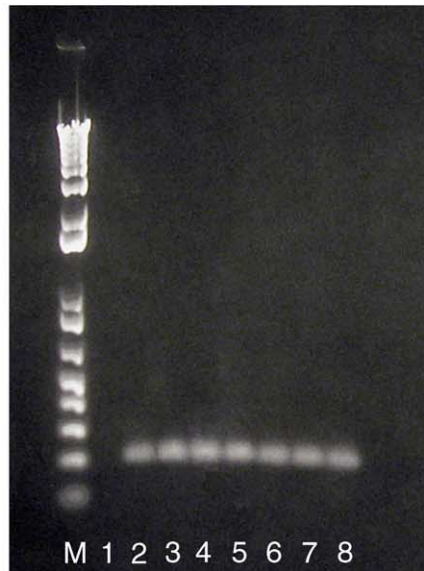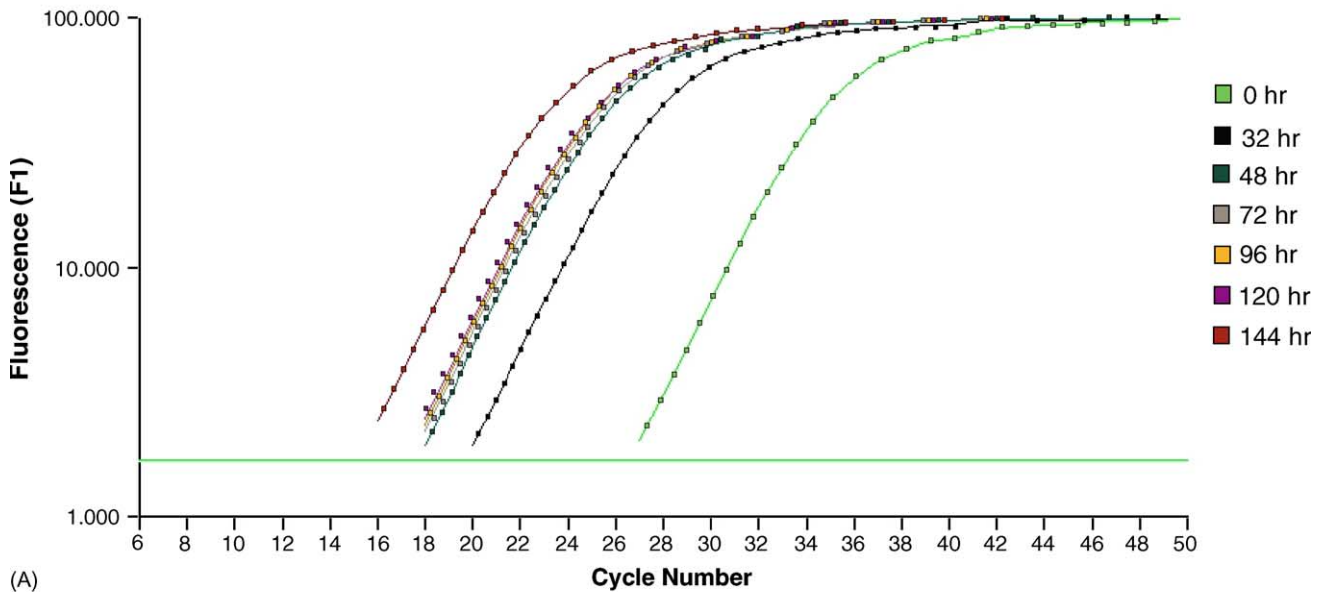[a] Number of replicas positive out of total tested.

Fig. 2. Quantitation of viral RNA in ISAV-infected TO cells at different times post-inoculation. (A) Quantitative analysis of viral RNA in different samples. For simplicity, the amplification plot of a single replica of genomic RNA of the virus at various post-inoculation hours is shown. The green, black, dark green, gray, golden yellow, purple, and brown lines represent 0, 32, 48, 72, 96, 120, and 144 h p.i. samples, respectively. (B) Gel electrophoresis of real-time RT–PCR reactions of RNA extracted from TO cells at various post-ISAV inoculation hours. Lanes M and 1 contain 1 kbp plus DNA ladder and water, respectively. Lanes 2–8 represent viral RNA levels at 0, 32, 48, 72, 96, 120, and 144 h, respectively. The 1% agarose gel was stained with ethidium bromide and visualized under 304 nm UV light.

analysis indicated a single peak similar to the one in Fig. 1A, suggesting that amplification products were specific and the virus population was uniform throughout the sampling times. The agarose gel electrophoresis also revealed a band of the expected size (Fig. 2B) indicating that the RT–PCR products are virus-specific. The difference among the mean Ct values of viral RNA in different samples was significant ($P \leq 0.05$) indicating that there was a real increase in viral RNA levels in ISAV-infected TO cells over the course of the infection. To determine if ISAV isolate U5575-1 replicates in CHSE-214 cells, total RNA from virus-infected CHSE-214

cells harvested sequentially at different times post-infection was analyzed using the SYBR Green real-time RT–PCR assay. The amplification plot of viral RNA levels at different times post-inoculation is shown in Fig. 3B. The mean Ct values of viral RNA in CHSE-214 cells at different hours post-infection are shown in Table 5. The difference among the mean Ct values of different samples was not statistically significant ($P \geq 0.05$) indicating that there was no change in the amount of ISAV RNA over time in the different samples. A single melting peak specific of the viral product was observed in all the samples (Fig. 3B). No
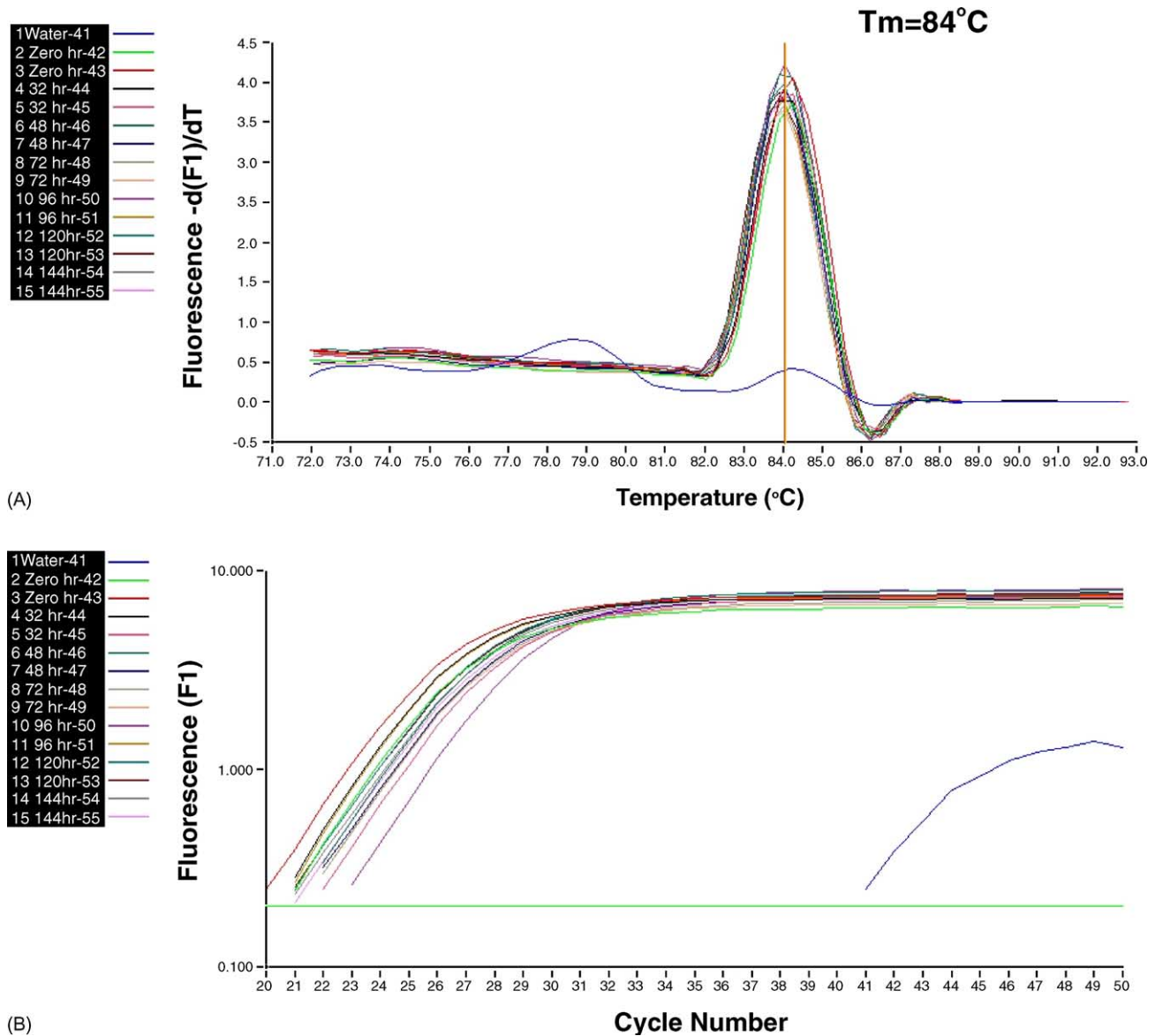
Fig. 3. Quantitation of viral RNA in ISAV-infected CHSE-214 cells at different times post-inoculation. (A) Single melting peak of ISAV strain U5575-1 indicating that the real-time RT–PCR products were virus-specific. (B) Amplification plot of ISAV strain U5575-1 indicating absence of virus replication in CHSE-214 cells.

CPE was observed in any of virus-infected CHSE-214 cell monolayers for the duration of the sample period (15 days). These results confirm that ISAV isolate U5575-1 does not replicate in the CHSE-214 cell line.

3.6. Detection of ISAV RNA in fish tissues

To demonstrate the utility of the SYBR Green real-time RT–PCR assay for fish tissues with varying amounts of ISAV RNA, the tissue samples from fish that survived an experimental ISAV infection were tested. Both conventional one-tube RT–PCR and SYBR Green-based real-time RT–PCR assays were compared on these samples. The results are summarized in Table 6. The real-time RT–PCR assay detected ISAV RNA in more tissues that the conven-

tional one-tube RT–PCR assay. This was expected since real-time RT–PCR was shown to be $100\times$ more sensitive than the conventional one-tube RT–PCR. Moreover, with the real-time RT–PCR assay, it was possible to obtain an indication of the relative amount of viral RNA in the sample from the Ct values, where possible from absolute RNA amounts (in ng). Since all the fish tested were survivors of a lethal experimental ISAV challenge, the results demonstrate that the real-time RT–PCR assay would be useful in detecting subclinical ISAV infections in fish.

3.7. Replication of ISAV isolates in CHSE-214 cells

The finding that ISAV isolate U5575-1 replicated in TO cell line but not in CHSE-214 cells indicated to us that the

Table 5
Ct values of ISAV U5575-1 in CHSE-214 cells at various hours post-inoculation[a]

Post-inoculation (h)[b]	Mean Ct value[c]	Standard deviation
0	20.105	0.757
32	21.005	0.856
48	21.000	0.042
72	21.200	0.000
96	21.485	1.407
120	20.565	0.035
144	20.805	0.163

[a] The difference between the mean Ct values of ISAV in CHSE-214 cells at various hours post-inoculation was not statistically significant ($P \leq 0.05$).

[b] Another CHSE-214 monolayer inoculated with ISAV U5575-1 strain was incubated until day 15 post-inoculation, to monitor for evidence of CPE.

[c] Each mean Ct value is representative of two replicas.

Table 7
Ct values of ISAV genomic RNA levels at different post-infection hours in CHSE-214 cells

ISAV isolate	Mean Ct values[a]				
	0 h	72 h	120 h	192 h	336 h
U5575-1[b]	25.865	29.265	28.785	26.595	26.835
NBISA01[c]	34.235	35.135	34.730	31.065	26.100
HKS-36[c]	ND[d]	ND	35.04	34.545	29.37
RPC/NB-970-877-2[b]	31.959	34.950	33.870	35.725	32.540
390/98[b]	40.535	ND	40.965	40.640	38.050
RPC/NB-980-414-1[b]	38.100	38.135	37.535	36.500	35.620
RPC/NB-980-280-2[b]	34.160	35.630	35.280	33.877	36.535
835/9/98[b]	32.125	31.545	30.950	ND	32.005

[a] Each mean Ct value is representative of two replicas.

[b] The difference between the mean Ct values of viral RNA levels in CHSE-214 cells at various post-inoculation hours was not statistically significant ($P \leq 0.05$).

[c] The difference between the mean Ct values of viral RNA levels in CHSE-214 cells at various post-inoculation hours was statistically significant ($P \geq 0.05$).

[d] ND denotes not done.

real-time RT–PCR assay could be used to differentiate replicating non-cytopathic ISAV strains from non-replicating strains. In order to demonstrate this and also prove that the optimized real-time RT–PCR conditions can detect different ISAV isolates, we tested eight ISAV isolates inoculated in CHSE-214 cells and harvested at different hours post-infection. The results are summarized in Table 7. ISAV isolate NBISA01 was used as the positive control for virus replication with CPE production in CHSE-214 cells (Kibenge et al., 2000). ISAV isolate U5575-1 served as the negative control for virus replication in CHSE-214 cells as demonstrated above. NBISA01 was the only ISAV isolate that produced CPE in CHSE-214 cells; the CPE was first observed on day 8 post-infection. The melting curve analysis indicated that the real-time RT–PCR products were virus-specific. The results further showed that there was a significant difference ($P \leq 0.05$) among the mean Ct values of genomic RNA levels in CHSE-214 cells at different times post-infection for NBISA01 and HKS-36 suggesting that these two isolates do replicate in the CHSE-214 cell line. The differences among the mean Ct values of genomic RNA levels for ISAV isolates U5575-1, RPC/NB-970-877-2, 390/98,

RPC/NB-980-414-1, RPC/NB-980-280-2, and 835/9/98 were not statistically significant ($P \geq 0.05$), implying that these ISAV isolates do not replicate in the CHSE-214 cell line. These observations allowed us to recognize three different CHSE phenotypes of ISAV: replicating cytopathic (e.g. NBISA01), replicating non-cytopathic (e.g. HKS-36), and non-replicating (e.g. U5575-1, RPC/NB-970-877-2, 390/98, RPC/NB-980-414-1, RPC/NB-980-280-2, and 835/9/98).

4. Discussion

Rapid and sensitive molecular methods that can quantitate viruses in vitro and in vivo are an essential requirement not only for disease diagnosis, but also for studying various aspects of viral infections including epidemiology, pathogenesis, and virus–host interactions at the molecular level (Mackay et al., 2002; Niesters, 2001, 2002).

Table 6
Detection of ISAV RNA segment 8 by conventional RT–PCR and SYBR Green real-time RT–PCR in different tissues of fish at 76 days post-ISAV challenge

Method	Fish species	Fish tissues					
		Liver	Spleen	Kidney	Heart	Caeca	Gill
Conventional RT–PCR	Atlantic salmon	+[a]	−[b]	+	+	−	+
	Rainbow trout	+	+	−	−	+	−
Real-time RT–PCR	Atlantic salmon	+ (36.44,[c] 0.0022[d])	+ (37.92, 0.001)	+ (37.71, 0.001)	+ (35.61, 0.0036)	+ (35.99, 0.0029)	+ (39.71, NC[e])
	Rainbow trout	+ (35.75, 0.0033)	+ (37.98, 0.001)	−	+ (36.61, 0.0020)	+ (36.43, 0.0022)	+ (38.04, NC)

[a] + Denotes positive by conventional one-tube RT–PCR or real-time RT–PCR detection format.

[b] − Denotes negative by either agarose gel electrophoresis for conventional one-tube RT–PCR or real-time RT–PCR detection format.

[c] Ct value.

[d] ISAV RNA concentration (ng/μl) extrapolated from the standard curve

[e] NC denotes not calculated.

Quantitative PCRs (and RT–PCRs) have been available for a long time and are known for their advantages over the conventional PCR (and RT–PCR) assays. The real-time PCR (and RT–PCR) assays have a number of advantages over the conventional PCR (and RT–PCR); they are rapid, sensitive, and capable of displaying the real-time quantitation of the viral load in samples because they do not require post-PCR detection procedures (Mackay et al., 2002). The real-time PCRs are also considered more accurate than the conventional PCR because in the former, the amplification product is measured/detected during the log-linear phase of amplification when the conditions are optimum, whereas in the later, reaction products are analyzed by the gel electrophoresis of end-point products; the amplification of the nucleic acid target especially during the later rounds of amplification may be affected by various factors including reagent limitations, inhibitory PCR by-products, and competition between the DNA strands and primers for the complementary sequences (Mackay et al., 2002); moreover, the end-point quantification of PCR products based on gel electrophoresis may not yield consistent results because measurement of band intensities is very subjective.

A variety of chemistries including SYBR Green, hydrolysis probes, molecular beacons, and hybridization probes are commercially available that can be coupled to real-time PCR assays (Niesters, 2001). The SYBR Green dye binds to double-stranded DNA in the minor-groove and exhibits increased fluorescence upon binding (Niesters, 2001). SYBR Green is the simplest and least expensive of the chemistries that are commercially available.

The real-time PCR technology provides quantitative detection of target nucleic acids during the progression of amplification cycles. The amplification cycle at which the increase in fluorescence is significantly higher than the mean baseline fluorescence is called the threshold cycle (Ct). The Ct value is inversely related to the amount of target templates in the samples (Mackay et al., 2002; Niesters, 2001). The Ct values (drawn on the x-axis) correlate to the measurements of the fluorescent signal (the y-axis) and provide the quantification of the target template. The melting curve analysis is performed by plotting the loss of fluorescence as the first negative derivative ($-d(F1)$) against the temperature change (dT) (Jackwood and Sommer, 2002); the resulting peak loss of fluorescence corresponds to the melting temperature (T_m) of the virus-specific amplicons.

The development of real-time PCR technology has revolutionized the scientific study on the epidemiology, pathogenesis, immunology, prophylaxis and treatment, and diagnosis of viral infections (Mackay et al., 2002; Niesters, 2001, 2002; Jackwood and Sommer, 2002). The dramatic increase in the potential of viral investigations through the use of quantitative amplification techniques including real-time PCR is likely to contribute towards the ultimate goal for the formulation of better strategies for the diagnosis and control of animal and human viral diseases. The real-time RT–PCR (and PCR) assays have been developed for a variety of viruses including feline calicivirus (Helps et al., 2002), shrimp hematopoietic necrosis virus and white spot virus (Dhar et al., 2001), chicken infectious bursal disease virus (Jackwood and Sommer, 2002; Moody et al., 2000), human immunodeficiency virus type 1 (Lewin et al., 1999), feline coronaviruses (Gut et al., 1999), and influenza A and B viruses (van Elden et al., 2001).

We describe here a one-tube SYBR Green-based real-time RT–PCR that quantitatively detects ISAV in biological samples. The assay is more sensitive and has a higher detection limit than the conventional one-tube RT–PCR for ISAV. The increased sensitivity of the assay may be due to two possible reasons either in combination or alone: (1) agarose gels have limited dynamic range (Mackay et al., 2002; Niesters, 2001); this is supported by our observation that like the gel electrophoresis of conventional one-tube RT–PCR products, the agarose gel analysis of SYBR Green RT–PCR products also shows the virus-specific band only down to a 1:10^3 dilution (Table 3); and (2) SYBR Green I is more sensitive than ethidium bromide for detecting DNA (Karlsen et al., 1995). Because the SYBR Green RT–PCR assay for ISAV is 100 times more sensitive than the conventional one-tube RT–PCR, it might be useful in detecting sub-clinical ISAV infections in carrier fish, since Atlantic salmon that recover from clinical ISA and are negative by the conventional RT–PCR assay, can still transmit ISAV to healthy Atlantic salmon through cohabitation (McAllister et al., 2003).

In addition to having higher sensitivity, the SYBR Green real-time RT–PCR assay is also more rapid than the conventional RT–PCR; the entire assay could be completed within 80 min. We used TRIZOL reagent, a procedure that takes over 1 h for extracting viral RNA from the biological samples; however, we believe that the use of column purification method(s) for recovering viral RNA from infected cell cultures or fish tissues can further reduce the time required for template preparation, in addition to improving the sensitivity of the assay.

Although non-specificity due to primer–dimers has been associated frequently with real-time RT–PCR assays utilizing DNA-binding dyes (Niesters, 2001), the monitoring of SYBR Green real-time RT–PCR reactions by melting curve and gel electrophoresis analyses showed that the assay was very specific for ISAV. The addition of a short, high temperature incubation step after the elongation step (Table 2) conferred further specificity to the fluorescent signal. The correlation coefficient (r) values of 0.98 and 0.96 for precision within- and between-assays, respectively, suggest a strong linear relationship between viral RNA levels and Ct values. An r^2 value of 0.962 implies that most of the variation in log concentrations of viral RNA can be explained by the straight-line relationship with Ct values. The high value for the linear regression line and CV values of 2.78 and 6.40% for precision within- and between-runs, respectively, suggest that the assay is highly reproducible. A slope of -3.771 indicates an amplification efficiency of 84% for ISAV real-time RT–PCR; this is acceptable considering the

fact that the reverse transcription step is not always 100% efficient (Helps et al., 2002).

The finding that ISAV isolate U5575-1 replicates in TO cells but not in CHSE-214 cells (Tables 4 and 5; Figs. 2A and 3B) are in agreement with previous reports (Kibenge et al., 2000, 2001a). Because of the ability of real-time RT–PCR to quantitate the viral RNA, it was possible to differentiate the static viral RNA levels of the residual inocula in the different CHSE-214 cell culture harvests (Table 5; Fig. 3B) from the increasing viral RNA levels associated with replicating virus such as in the ISAV-infected TO cells (Table 4, Fig. 2A). The finding that ISAV isolate NBISA01 is a CHSE-positive phenotype, i.e. it produces CPE, has also been reported previously (Kibenge et al., 2000); similarly, the further finding that viral isolate HKS-36 replicates without causing CPE in CHSE-214 cells is also in agreement with our previous observations (Kibenge et al., 2000). Our data now suggest that the real-time RT–PCR assay may be used to group ISAV isolates into one of three CHSE phenotypes: replicating cytopathic, replicating non-cytopathic, and non-replicating.

In conclusion, we have developed a highly reproducible and flexible real-time RT–PCR assay using SYBR Green chemistry that can quantitate ISAV loads in biological samples. The assay is more sensitive and rapid than conventional one-tube RT–PCR for ISAV and might be useful in detecting subclinical ISAV infections in carrier fish, and for studying aspects of viral infections such as pathogenesis.

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