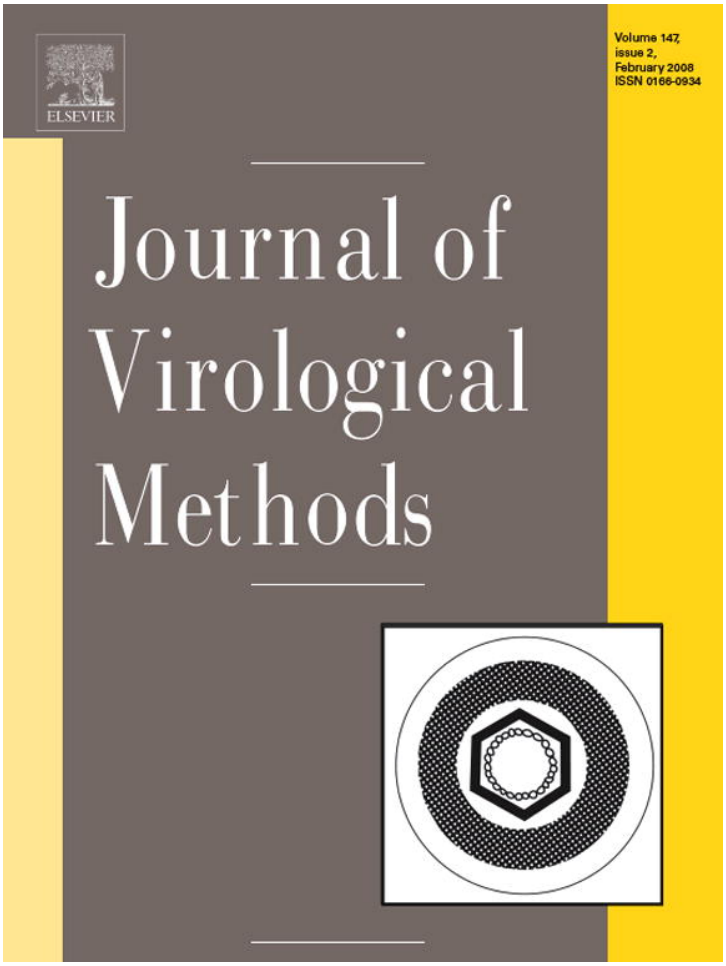

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Journal of Virological Methods 147 (2008) 226–234
Journal of Virological Methods
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Detection and quantitation of infectious pancreatic necrosis virus by real-time reverse transcriptase-polymerase chain reaction using lethal and non-lethal tissue sampling

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Received 22 May 2007; received in revised form 28 August 2007; accepted 5 September 2007

Abstract

Infectious pancreatic necrosis virus (IPNV) is a bisegmented double-stranded RNA virus belonging to the family *Birnaviridae*, genus *Aquabirnavirus*, which is a major viral pathogen of salmonid fish. The virus infects wild and cultured salmonids, causing high mortality in juvenile trout and salmon. A highly sensitive and specific real-time RT-PCR assay using the fluorogenic dye SYBR® Green I was developed for the detection and quantitation of IPNV in rainbow trout (*Oncorhynchus mykiss*). Rainbow trout were infected experimentally with IPNV in the laboratory by injection or immersion and then pectoral fin, spleen, and head kidney samples were collected for analysis. The corresponding cDNA was synthesized using DNase I-treated total RNA and then real-time RT-PCR was performed using primers based on the IPNV non-structural protein gene, designated as either NS or VP4. Rainbow trout β-actin and elongation factor 1α (EF-1α) genes were used as internal controls. Using real-time RT-PCR, the virus was successfully detected in pectoral fin, spleen, and head kidney tissue samples. The dissociation curves for each amplicon showed a single melting peak at 83, 81.5, and 84 °C for IPNV NS, trout β-actin, and EF-1α genes, respectively. The amplicon size and nucleotide sequence was used to confirm the specificity of the products. Using a dilution series of *in vitro* transcribed RNA, IPNV was reliably detected down to 10 RNA copies and had a dynamic range up to 10^7 RNA copies. A time course assay, using immersion challenged samples, revealed that the virus could be detected in pectoral fin, spleen, and head kidney as early as 24 h post-challenge. The average viral load in all three tissues increased over time, reaching its highest level at 21 days post-challenge, which was followed by a slight decrease at 28 days post-challenge. IPNV load in pectoral fin tissue was comparable to the viral load in spleen and head kidney tissues, indicating that pectoral fin could be used for the detection and quantification of IPNV. The development of a non-lethal detection method will be useful for the detection of IPNV and potentially other viruses of finfish in farmed and wild fish.

Keywords: IPNV; Real-time RT-PCR; SYBR Green; Non-lethal detection; Rainbow trout

1. Introduction

Infectious pancreatic necrosis virus (IPNV), the etiological agent of infectious pancreatic necrosis disease (IPN), is a major viral pathogen of wild and cultured salmonids (Wolf, 1988). In salmonid farming, the disease can cause high mortality in both fry and juveniles in freshwater as well as in smolts during their first month after transfer to seawater (Jarp et al., 1994). This virus is prevalent in salmonid hatcheries around the world, from the America to Europe, Asia, and South Africa (Hill, 2000). IPNV is highly contagious and transmitted through a variety of routes including contaminated water (Magyar and Dobos, 1994), ingestion of infected material, direct contact with secretions from infected fish (*e.g.*, feces, sexual fluids), and contact with any contaminated surface. Adult fish can also serve as asymptomatic carriers of the virus, eventually shedding the virus particles into the environment where other fish can become infected (Reno et al., 1978). These carriers may hamper the successful detection of IPNV preceding an outbreak. For this reason, it is critical that highly sensitive detection methods be developed for rapid management of infected stock.

IPNV is a bisegmented double-stranded RNA virus belonging to the family *Birnaviridae*, genus *Aquabirnavirus*. The two

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doi:10.1016/j.jviromet.2007.09.003

genome segments are designated Segment A and Segment B. The virions are non-enveloped icosahedrons measuring 60 nm in size. Segment A of the IPNV genome is 3.1 kb and contains a large open reading frame encoding a 106 kDa polyprotein arranged in the order NH_2-preVP2-VP4 (NS-protease)-VP3-COOH. The polyprotein is co-translationally cleaved by the NS-protease to produce preVP2 and VP3. The preVP2 is cleaved further to yield mature VP2, which makes up at least 62% of the capsid protein (Dobos et al., 1979). The Segment A encoded polyprotein also contains a 15-kDa non-structural VP5 protein, which is found only in infected cells (Magyar and Dobos, 1994). Segment B of the IPNV genome is 2.8 kb and presumed to encode the RNA-dependent RNA polymerase, designated VP1. VP1 exists as a free polypeptide and as a genome linked protein in the virion, termed VPg (Dobos, 1995).

Currently, IPNV detection relies on the isolation of virus from suspect fish using established fish cell lines, then confirmed using a variety of immunological assays, such as serum neutralization (Hill, 2000), flow cytometry, immunofluorescense, immunoperoxidase, immunodot-blotting, immunostaphylococcus-protein A (Saint-Jean et al., 2001 #4), and enzyme-linked immunosorbent (ELISA) assays (Nicholson and Caswell, 1982). Virus isolation in cell culture continues to be the gold standard for IPNV detection (OIE, 2003). The major drawback to this approach is the time required to isolate and confirm the replicating agent as IPNV, which may be up to 10–14 days after virus inoculation. Reverse transcriptase-polymerase chain reaction (RT-PCR) (Blake et al., 1995; Saint-Jean et al., 2001; Kerr and Cunningham, 2006) and *in situ* hybridization (ISH) (Alonso et al., 2004) were developed for the detection of IPNV; both are rapid and more sensitive than cell culture assay. A multiplex RT-PCR assay to detect IPNV, infectious hematopoietic necrosis virus and viral hemorrhagic septicemia virus was developed by Williams et al. (1999). In a study by Saint-Jean et al. (2001), the sensitivity of RT-PCR was compared to the immunological assays mentioned above. The RT-PCR and flow cytometry methods were demonstrated to be the most sensitive of the detection methods assessed, detecting virus as early as 4 h post-challenge in CHSE-214 cells. RT-PCR was the only diagnostic test of the six tests evaluated to detect accurately IPNV at the lowest IPNV infective titer of 10 Tissue Culture Infective Doses 50% ($TCID_{50}$)/mL. However, an extensive optimization is required before a fully validated RT-PCR assay could be developed for clinical application (Kerr and Cunningham, 2006).

Alonso and colleagues isolated IPNV from *Solea senegalensis* (Sole), then infected bluegill fibroblast (BF-2), *Epithelioma papulosum cyprinid* (EPC), and Chinook Salmon Embryo (CHSE-214) cell lines to compare the sensitivity of ISH to the detection of IPNV using an immunofluorescence antibody test (IFAT) (Alonso et al., 2004). An effective means for visualizing infected cell lines was developed, but more intriguing were the different levels of IPNV susceptibility observed in these cell lines. These observations demonstrated the need for a standard diagnostic cell culture line or for a new direct detection method sensitive enough to measure low levels of virus, directly from infected tissue, without the need for cell culture amplification.

Recent advances in infectious disease diagnosis in human, plant, and terrestrial agricultural species are being applied gradually to the health and management of economically important aquatic species. One such example is the application of real-time PCR in gene expression studies and viral disease diagnosis in fish and shellfish aquaculture. Real-time PCR was developed in 1992 (Higuchi et al., 1992, 1993) and quickly became a major tool for gene expression studies, and more recently, for disease diagnosis (Bustin, 2000; Mackay et al., 2002; Niesters, 2002). The simplicity, sensitivity, dynamic range of detection, reproducibility, and amenability to high throughput screening makes real-time PCR an attractive tool for viral detection (Mackay et al., 2002; Niesters, 2002). A variety of fluorescently based methods are currently available for the detection of real-time PCR products. These include the non-specific DNA-binding dye SYBR® Green I, target-specific fluorescently labeled linear oligoprobes, 5′-nuclease oligoprobes, molecular becons, and self-fluorescing amplicons (Mackay et al., 2002). Among these, detection by SYBR® Green I is the simplest and least expensive method because it does not require the design of fluorescently labeled oligoprobes. Additionally, the specificity of the amplified product can be determined by examining the melting curve of the amplicon (Ririe and Rasmussen, 1997). Real-time PCR assays employing either SYBR® Green I (Dhar et al., 2001, 2002) or TaqMan probes (Overturf et al., 2001; Tang and Lightner, 2001; Durand and Lightner, 2002; de la Vega et al., 2004; Gilad et al., 2004; Munir and Kibenge, 2004; Tang et al., 2004; Lockhart et al., 2007; Purcell et al., 2006; Rajendran et al., 2006) have been developed to detect and quantify several viral pathogens of fish and shrimp. These tests have potential as routine virus screening tools that would improve the management of viral diseases in commercial hatcheries.

Here, we report the development of a real-time RT-PCR assay using SYBR® Green I chemistry for the detection and quantitation of IPNV. Using this method, IPNV was detected and quantified in spleen, head kidney, and pectoral fin tissues of laboratory-challenged rainbow trout (*Oncorhynchus mykiss*). The ability to detect IPNV in pectoral fin samples could be useful as a non-lethal sampling method for IPNV detection in aquaculture operations and in wild populations of salmonids.

2. Materials and methods

2.1. Virus challenge and sample collection

2.1.1. IPNV challenge by injection

Rainbow trout (*O. mykiss*) (average weight ∼50 g) that originated from the Clear Springs Foods, Inc. (CSF, Buhl, ID) Research Hatchery were known to be free of IPNV, and were used for subsequent IPNV challenge work. As part of the Clear Springs Foods Research Hatchery program, the spawning adults and juvenile fish are continually monitored to ensure their virus-free status using standard surveillance and inspection methodologies (USFWS and AFS-FHS, 2005). In addition, all lots of fish used for research are maintained indoor in ultraviolet-light treated spring water with high dissolved oxygen content (9.2 ppm) and at constant temperature (15 °C). The fish were

fed *ad libitum* twice daily the CSF standard rations containing 45% protein and 20% fat.

Each fish ($n = 13$) was injected intraperitoneally with approximately 250 μL of a stock virus lysate of IPNV (Buhl strain) with a titer of $\sim 10^7$ $TCID_{50}$/mL. Fish ($n = 12$) injected with phosphate buffered saline (PBS) served as negative control. This group of fish was designated as the 'healthy' group throughout the rest of this manuscript. Ten days post-injection, animals were sacrificed and approximately 100 mg of head kidney, spleen, and pectoral fin tissue were collected from each fish into microfuge tubes containing TRI reagent, and stored at −75 °C until RNA isolations were performed.

2.1.2. IPNV challenge by immersion

Rainbow trout (mean weight, ∼3 g) were challenged with IPNV *via* the waterborne route. Two groups (Groups #1 and #2) of 36 fish each were infected with IPNV in a volume of water that was 10× the total weight of the fish in grams for 1 h with aeration ($\sim 10^5$ $TCID_{50}$/mL of IPNV). One of the infected groups (Group #1) was monitored for mortality and the other group (Group #2) was randomly sampled for tissue specimens for IPNV real-time RT-PCR assay. A third group (Group #3) of 36 fish was handled similarly but was mock infected. Spleen, head kidney, and pectoral fin specimens from each fish of Group #2 were collected at 1, 3, 7, 14, 21, and 28 days post-challenge and stored in TRI reagent at −75 °C until RNA isolation was performed. There were 6 fish for each of six time points for a total of 36 fish. In addition, six fish were sacrificed prior to IPNV immersion challenge, and tissue (head kidney, spleen, and pectoral fin) samples were collected, which then served as the time 0 h control. All fish were sacrificed by exposing to an overdose of MS-222 prior to tissue sampling. The challenge was terminated after 28 days and kidney and spleen tissues from a subset of fish from Groups #1 and 3 were examined for the presence of IPNV using standard virus isolation techniques (Ganzhorn and La Patra, 1994).

2.2. Isolation of total RNA and cDNA synthesis

Total RNA was isolated from head kidney, spleen, and pectoral fin clip samples from both the IPNV-injected and immersion challenged rainbow trout. The TRI reagent method was used following the manufacturer's protocol (Molecular Research Center, Cincinnati, OH). The purified RNA samples were treated with DNase I (Ambion, Inc., Austin, TX), and the RNA quality was assessed by running the samples on a 1% formaldehyde agarose gel. The cDNA synthesis was carried out in a 40 μL reaction volume containing 1 μg total RNA, 1X RT-PCR buffer, 1 mM dNTPs, 0.75 μM oligo dT, 4 U of RNase inhibitor, and 5 U of MultiScribe reverse transcriptase (Applied Biosystems, Foster City, CA) at 42 °C for 1 h. The cDNA was diluted to 1:10 using DNase and RNase-free molecular biology grade water and 2 μL of the diluted cDNA was taken for each reaction in the real-time PCR assay.

2.3. SYBR® Green real-time RT-PCR assay

The primers for SYBR® Green real-time RT-PCR were designed based on the non-structural protease (NS) gene present in Segment A of the IPNV genome (GenBank accession number NC_001915). The primers for the internal control genes, rainbow trout β-actin and elongation factor 1α (EF-1α), were based on the published sequence of these genes (GenBank accession numbers AF157514 and AF498320, respectively; Table 1). The primers were designed using the Primer Express Software version 1.0 (Perkin Elmer-Applied Biosystem). The real-time RT-PCR amplifications were performed in a Bio-Rad iCycler iQ™ (Bio-Rad Laboratories, Inc., Richmond, CA).

The primer concentrations for the real-time RT-PCR assays were optimized using a checkerboard conformation (all possible combinations of 50, 300, and 900 nM concentrations) of forward and reverse primers. The cDNAs derived from spleen tissue of a healthy and an IPNV-injected fish were used for this RT-PCR optimization. The optimal primer concentration was used for subsequent assays. The optimized reaction for SYBR® Green real-time RT-PCR contained 12.5 μL of 2X SYBR® Green Supermix (iQ SYBR® Green Supermix), 300 nM each of forward and reverse primers, and 2 μL of the 1:10 diluted cDNA in a 25 μL reaction volume. The amplifications were carried out in a 96-well plate and each sample had three replicates. The thermal profile used for the SYBR® Green real-time RT-PCR was 95 °C for 10 min, followed by 40 cycles of 95 °C for 10 s, and 60 °C for 1 min. After the run, the melting curve of each amplicon was examined to determine the specificity of the amplification.

Table 1
List of primers used for the real-time RT-PCR assay for the detection and quantification of infectious pancreatic necrosis virus (IPNV)

Gene	Primer name	Primer sequence (5′-3′)	%GC	T_m[a]	Amplicon size (bp)
IPNV	1916F	AGGAGATGAC ATGTGCTACA CCG	52	60	84
	1999R	CCAGCGAATA TTTTCTCCAC CA	45	60	
	2727F	GACTATGTGC GAAAACCGAT AACC	46	60	77
	2803R	AGGCCGTAGA CACTGTTGGC TA	55	60	
Rainbow trout β-actin	1301F	CCCAAACCCA GCTTCTCAGT CT	55	64	113
	1413R	TGCTTCACCG TTCCAGTTGT G	52	64	
Rainbow trout EF-1α	136F	TGATCTACAA GTGCGGAGGC A	52	64	101
	236R	CAGCACCCAG GCATACTTGA A	52	63	

[a] Melting temperature at 50 mM Na^+.

2.4. Standard curve

The real-time RT-PCR amplicon from spleen tissue of an IPNV infected fish amplified using the primers 1916F and 1999R was run in a 2% agarose gel, and gel-purified using QIAQuick gel-extraction kit (Qiagen, Valencia, CA). The gel-purified amplicon was reamplified by PCR using the primers 1916F and a nested 1999R-T_n (5′-TTTTTTTTTT TTTTTTCCAG CG-3′). The PCR amplified product was run in a 2% agarose gel; gel-purified using the QIAQuick gel-extraction kit (Qiagen), and the PCR product was then subsequently cloned into pCR2.1-TOPO vector (Invitrogen, Carlsbad, CA). Then three recombinant clones were sequence verified. The plasmid DNA of a verified recombinant clone was then digested with the restriction enzyme *Bam*H1, run in a 1% agarose gel, and the digested plasmid was gel-purified.

The gel-purified plasmid DNA was used as template to perform *in vitro* transcription using the MEGAscript High Yield transcription Kit (Ambion). The *in vitro* transcribed RNA was used to generate a standard curve for the SYBR® Green real-time RT-PCR assay. The cDNA synthesis and the real-time RT-PCR parameters were the same as described above. Five standard curves using the *in vitro* transcribed RNA were run and the mean cycle threshold (C_t) value at each 10-fold dilution was taken for the final standard curve. The slope and y-intercept were obtained using linear regression. The C_t values of the samples were extrapolated into the standard curve to calculate the absolute copy number of IPNV.

2.5. Data acquisition and analysis

After a SYBR Green real-time RT-PCR run, data acquisition and subsequent data analyses were performed using the iCycler iQ Real-Time PCR Detection System (Biorad iQ Software Version 1.3). Each individual sample was run in duplicate and scored for the presence of the correct melting peak before performing further analysis. The mean C_t values for the IPNV amplicons and for the corresponding trout internal control amplicons from both the IPNV injection challenge and the IPNV immersion challenge were exported into a Microsoft Excel spreadsheet. The variability of internal control gene expression between healthy and IPNV-injected trout in pectoral fin, spleen, and head kidney was assessed with a Student's *t*-test for each tissue between the healthy and infected samples. The absolute copy number of IPNV was calculated based on the linear regression of the *in vitro* transcribed RNA standard curve. All plots and statistics were run in Graphpad, Version 4 (Graphpad Software, Inc., San Diego, CA).

3. Results and discussion

3.1. Primer optimization for real-time RT-PCR

Two sets of IPNV primers (Table 1) were screened using a matrix of all possible combinations of forward and reverse primers at 50, 300, and 900 nM with cDNA derived from spleen tissue of an IPNV-injected fish and a healthy fish. Amplification was obtained with both primer sets with cDNAs derived from IPNV-injected fish, but no amplification was evident in healthy fish. The IPNV primer set 1916F/1999R amplified only the target amplicon, as judged by the melting profile of the cDNAs, whereas the primer set 2727F/2803R provided specific as well as non-specific amplification. The melting curve of the IPNV cDNA amplified using the primer set 1916F/1999R displayed a single peak at 83 °C (Fig. 1). The optimized primer concentrations were 300 nM for both forward and reverse primers. The

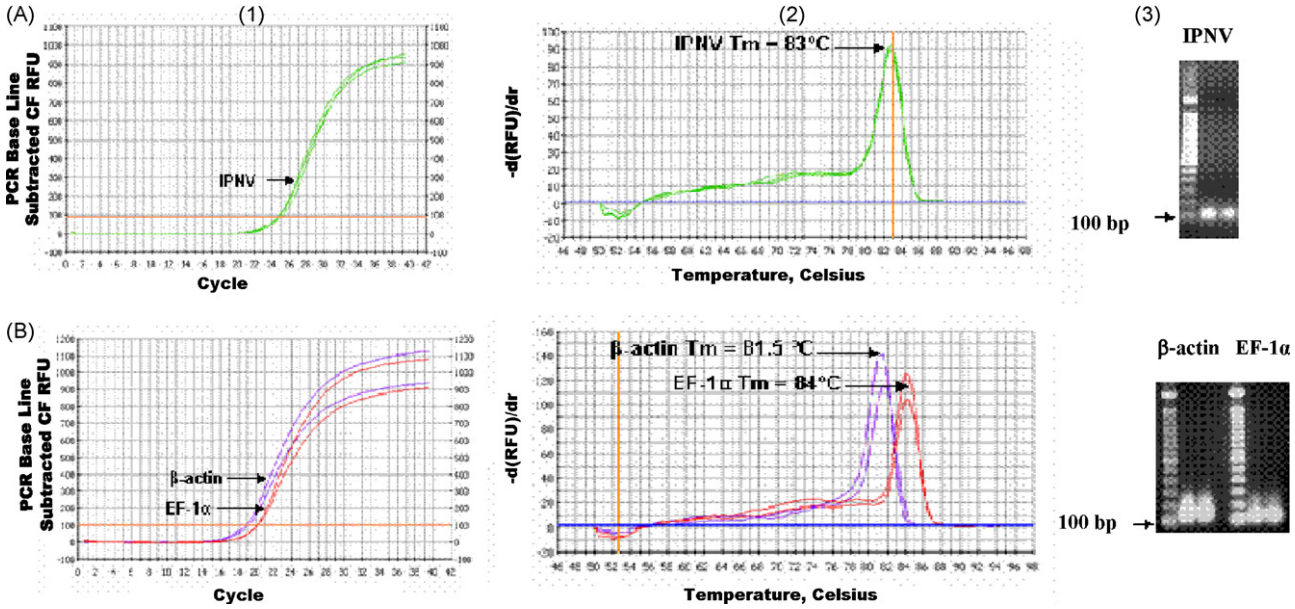

Fig. 1. Physical properties of IPNV and control amplicons generated by the real-time RT-PCR assay from spleen tissues of an IPNV-injected rainbow trout. Amplification profiles (Column 1), dissociation curves (Column 2), and agarose gel electrophoresis photographs (Column 3) of the IPNV amplicons (77 bp, Row A), β-actin (112 bp, Row B) and EF-1α (100 bp) (Row B).

amplicons derived from the IPNV NS gene, rainbow trout β-actin, and EF-1α genes were gel-purified and sequenced. The nucleotide sequence of the cDNAs showed 100% similarity with the corresponding GenBank entries (IPNV AF498320; β-actin, AF157514, and EF-1α, AF498320) on which the primers were originally based. This confirmed the specificity of the product generated by the IPNV primer set (1916F/1999R)) and the two control gene primer sets (1301F/1413R for β-actin and 136F/236R for EF-1α).

3.2. Reproducibility of the real-time RT-PCR assay

To determine the reproducibility of the real-time RT-PCR assay, three separate real-time RT-PCR runs were performed using cDNAs derived from different tissues of IPNV-injected fish ($n = 13$) and the optimized primer sets (1916F/1999R, 1301F/1413R, and 136F/236R). The C_t values for triplicate assays were reproducible with coefficients of variation ranging from 0.2 to 5.2% for IPNV, 0.2 to 8.4% for β-actin, and 0.3 to 9.2% for EF-1α amplicons. This indicated that the SYBR® Green I real-time RT-PCR assay was reproducible and comparisons between independent assays could be made when determining the IPNV load in rainbow trout.

3.3. Internal control for IPNV real-time RT-PCR assay

Internal control genes are typically used to normalize for varying amounts of starting RNA, differences in cDNA synthesis efficiency, PCR amplification efficiency, and differences in overall gene expression across tissue types (Vandesompele et al., 2002). The expression of internal control genes can also indicate the integrity of the total RNA, as the internal control genes are usually abundantly expressed genes across a variety of experimental conditions. Ideally, there would be no statistically significant difference in the expression of an internal control gene in the sampled tissue between experimental conditions. In the present study, we surveyed the expression of EF-1α and β-actin in healthy ($n = 12$) and IPNV-injected ($n = 13$) rainbow trout in pectoral fin, spleen, and head kidney tissues (Fig. 2) to determine which gene is more stable across tissues and between treatments.

Overall, upon IPNV injection, the expression of both EF-1α and β-actin genes were up-regulated in spleen (EF-1α average $C_t = 21.7 \pm 0.71$ in healthy *vs.* 19.8 ± 0.26 in infected animals; β-actin average $C_t = 20.0 \pm 0.64$ in healthy *vs.* 18.3 ± 0.23 in infected), but down-regulated in head kidney (EF-1α average $C_t = 21.0 \pm 0.50$ in healthy *vs.* 23.8 ± 0.38 in infected animals; β-actin average $C_t = 18.6 \pm 0.48$ in healthy *vs.* 22.3 ± 0.25 in infected), whereas the expression remained static in pectoral fins (EF-1α average $C_t = 21.1 \pm 0.52$ in healthy *vs.* 21.6 ± 0.36 in infected animals; β-actin average $C_t = 20.9 \pm 0.50$ in healthy *vs.* 20.3 ± 0.27 in infected). Therefore, the expression of these two genes demonstrated significant differences between healthy and IPNV-injected fish in spleen and head kidney but not in pectoral fin. This is probably due to spleen and head kidney being transcriptionally more active organs than pectoral fin tissue, and the viral infection modulates the transcriptome profiles in spleen and head kidney much more than in pectoral fin. Recently, Jorgensen et al. (2006) evaluated the expression of seven reference genes (β-actin, EF-1α, 18S rRNA, β2-microglobulin, RNA polymerase I and II, and glycerol 6-phosphate dehydrogenase) in different tissues (gill, liver, spleen, head kidney, hind gut, midgut and heart) of healthy and infectious salmon anemia virus (ISAV) infected salmon. These investigators reported that the expressions of all genes were affected by viral infection with the C_t values of 18S rRNA and EF-1α being the most stable. The RNA polymerase I and II showed intermediate variability and the rest of the genes including β-actin demonstrated high variability. This study, along with several other published studies (Suzuki et al., 2000; Olsvik et al., 2005; Ingerslev et al., 2005), suggests that internal control genes need to be optimized for each study. Additional reference genes, such as those evaluated in the ISAV-salmon study, need to be examined in IPNV infected rainbow trout to determine if any of those genes show less variation than EF-1α. Until then, EF-1α could be used as a reference gene for IPNV pathogenesis studies in rainbow trout.

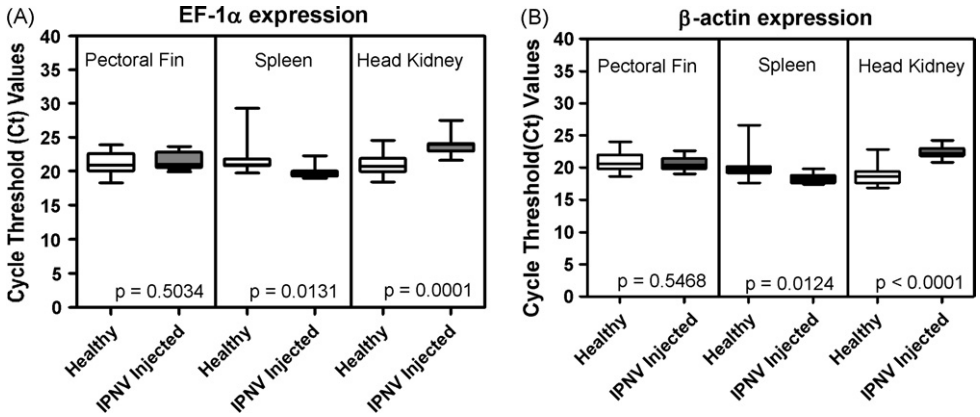

Fig. 2. Box plot analysis of rainbow trout internal control genes, EF-1α (A) and β-actin (B) from different tissues of healthy ($n = 12$) and IPNV-injected ($n = 13$) rainbow trout. The *p*-values correspond to an unpaired student's *t*-test run for each tissue type between healthy and IPNV-injected rainbow trout.

Table 2
Within sample reproducibility of the IPNV real-time RT-PCR assay using purified *in vitro* transcribed RNA as template

RNA standard	Replicate C_t values					Mean	S.E.M.
	1	2	3	4	5		
1.0×10^7	11.89	11.15	11.24	11.42	11.89	11.52	0.158
1.0×10^6	15.64	14.54	14.80	15.16	15.61	15.15	0.218
1.0×10^5	19.14	17.88	18.28	18.30	18.72	18.46	0.215
1.0×10^4	22.71	21.36	21.49	21.64	22.21	21.88	0.253
1.0×10^3	26.10	24.54	24.82	24.42	25.43	25.06	0.313
1.0×10^2	29.36	27.79	28.58	29.58	29.64	28.99	0.355
1.0×10^1	31.78	31.14	31.78	32.00	32.69	31.88	0.249

3.4. Generating IPNV standard curves using in vitro transcribed RNA

Following DNase I treatment of the *in vitro* transcribed RNA, the absorbance at 260 nm (A_{260}) was taken five times and the mean A_{260} value was taken to calculate the RNA concentration. A 10-fold RNA dilution series of *in vitro* transcribed RNA ranging from 10 pg (1×10^7 copies) to 0.01 fg (10 copies) was made using total RNA at 10 ng/μL from healthy spleen as diluent. The *in vitro* transcribed RNA dilution series was used to generate the IPNV standard curves. Data from five standard curves, run on three separate plates, using cDNAs derived from five independently prepared dilution series demonstrated that this real-time RT-PCR assay could consistently detect as few as 10 RNA copies (mean C_t value range 31.14–32.69, Table 2, Fig. 3). A linear relationship was observed between 10^7 and 10 *in vitro* transcribed IPNV RNA equivalents (coefficients of regression, $r^2 = 0.999$, average slope = −3.406 for the five standard curves) as shown in Fig. 3. The dynamic range of IPNV detection (10^7 to 10 copies) is comparable to those reported for real-time RT-PCR assays of DNA and RNA viruses in other fish and shellfish (Dhar et al., 2002; Tang et al., 2004; Gilad et al., 2004; Rajendran et al., 2006). The high sensitivity and wide dynamic range of IPNV real-time RT-PCR makes it ideal for the detection of low levels of IPNV infection in asymptomatic carrier fish.

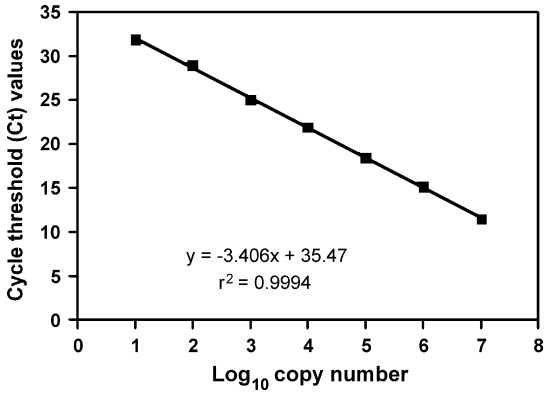

Fig. 3. IPNV standard curve generated using a 10-fold dilution series (1×10^7 to 10 cDNA/RNA equivalents per 25 μL reaction) of *in vitro* transcribed IPNV RNA. The standard curve represents the mean standard curve generated from five independently run RNA dilution series. Slope = −3.41, *y*-intercept = 35.47, $r^2 = 0.9994$.

3.5. Determining IPNV load in laboratory challenged rainbow trout by real-time RT-PCR

The real-time RT-PCR assay was used to quantify the viral load in different tissues of rainbow trout in two major experiments: (1) following an IPNV challenge *via* injection and subsequent sample collection at a single time point, and (2) following an IPNV immersion challenge with sample collection occurring over a 28 day time course. For the injection challenge, pectoral fin, spleen, and head kidney were sampled from 12 healthy and 13 IPNV-injected fish. No IPNV specific amplicons were present in any of the healthy fish tested. Among the different positive tissues, the geometric mean viral load reached the highest level in spleen tissue (4.5×10^3 copies/μg of total RNA) followed by pectoral fin (3.1×10^2 copies/μg of total RNA) and head kidney (1.1×10^2 copies/μg of total RNA; Fig. 4A). The viral load ranged from 1.2×10^2 to 3.4×10^6 copies in spleen, 39 to 3.7×10^3 copies in pectoral fin, and 31 to 5.9×10^3 copies in head kidney (Fig. 4A). It was surprising to find such a high viral load in pectoral fin compared to the two other tissues tested. This indicated that there are enough IPN virions present in the pectoral fin to permit non-lethal tissue sampling for the detection of the virus by real-time RT-PCR.

In the IPNV immersion challenge experiment, there were three groups of fish designated Groups #1–3 with 36 fish in each group. Group #1 was maintained throughout the experiment to record mortality due to IPNV, Group #2 was sampled at different time points post-infection to measure the IPNV load in different tissues, and Group #3 was kept as mock infected control. The animals did not show any clinical sign of infection and there was no mortality in any of these groups of fish during the duration of the study. At the end of the study a subset of the fish in Group #1 was examined by cell culture-based diagnostic methods and was shown to be infected with IPNV. No IPNV was detected in a similar number of animals that were examined from the mock-infected control group.

IPNV load was measured in different tissues of Group #2 animals that were collected at 1, 3, 7, 14, 21, and 28 days post-challenge. There were six fish sampled per time point, and the virus was successfully detected in all the samples. None of these fish showed any clinical sign of IPNV infection yet the virus was successfully detected and quantitated by real-time RT-PCR. This suggests that real-time RT-PCR could be used to detect the virus in asymptomatic carriers. The average viral load in different tissues of all six fish sampled increased over time, reaching the highest level at 21 days post-challenge followed by a slight decrease in viral load at day 28 (Fig. 4B). At 21 days, the IPNV load in pectoral fin ranged from 1.6×10^3 to 1.4×10^5 copies per μg of total RNA (geometric mean = 9.1×10^3 copies), 1.3×10^2 to 8.4×10^6 copies (geometric mean = 2.3×10^4 copies) in spleen, and 4.2×10^3 to 1.7×10^5 copies (geometric mean = 1.5×10^4 copies) in head kidney. The amplification profiles and dissociation curves of IPNV amplicons derived from pectoral fin, spleen, and head kidney of a representative fish collected at 21 days post-challenge are shown in Fig. 5. Although the average (geometric mean)

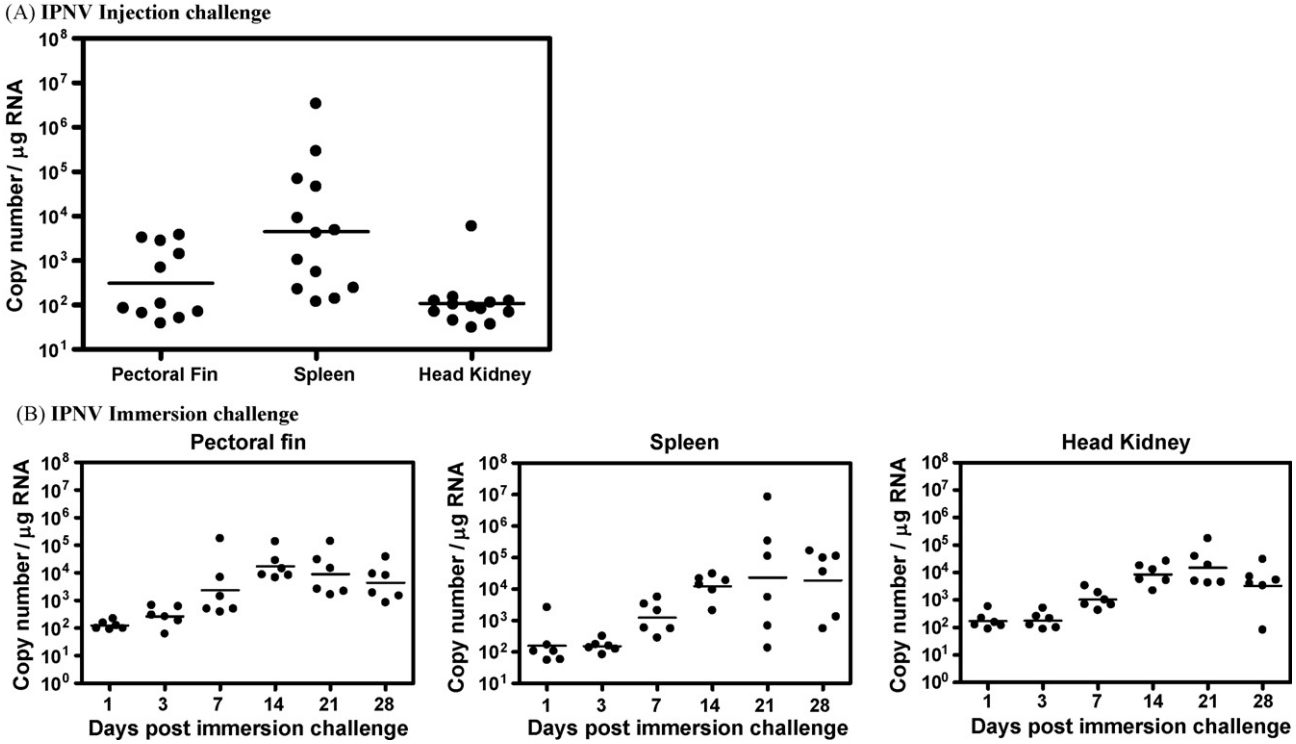

Fig. 4. Dot plot analyses representing the IPNV load in pectoral fin, spleen, and head kidney samples of rainbow trout. Panel A represents fish ($n = 13$) challenged in the laboratory *via* injection and samples collected at 10 days post-injection. Panel B represent fish from an IPNV immersion challenged study where samples were collected at 1, 3, 7, 14, 21 and 28 days post-challenge. There were six fish for each time point in the time course study. Each dot in both panels A and B represents data from a single fish, and the horizontal bar is the mean viral load for each treatment.

viral load was highest in spleen at 21 days, the viral load was highly variable in this tissue.

It was interesting to note that the virus could be detected as early as 24 h post-challenge in all three tissues. Our data indicated that pectoral fin, like spleen and head kidney could support IPNV replication, and the viral load in pectoral fin of a fish was comparable to the viral load in spleen and head kidney samples of the same fish (Fig. 4B). This demonstrated the usefulness of non-lethal tissue sampling using pectoral fin for the early detection of IPNV.

Detection methods utilizing nested PCR (Alonso et al., 2004) or RT-PCR with end-point detection (Blake et al., 1995; Milne et al., 2006; Saint-Jean et al., 2001) have been developed for IPNV diagnosis but continue to rely on sacrificing a subset of the fish population to obtain organs known to be conducive to IPNV replication. The development of a non-lethal detection method will circumvent this need. Recently, Harmache et al. (2006) used a recombinant IHNV clone expressing the *Renilla* luciferase gene to demonstrate that the portal of entry for the *Novirhabdovirus*, infectious hematopoietic necrosis virus (IHNV), is through the fin bases in rainbow trout. This work not only demonstrated that the fin bases are potential entry routes for IHNV, but also revealed that these tissues are sites of viral replication. The base of the fins has also been shown to be the site of replication for viral hemorrhagic septicaemia (Quillet et al., 2001), another member of the genus *Novirhabdovirus* that infects a very diverse array of freshwater and marine fish. Based on these published results and our own real-time RT-PCR detection of IHNV in pectoral fin tissue (Dhar et al., 2008), we decided to examine if IPNV could be detected in pectoral fin

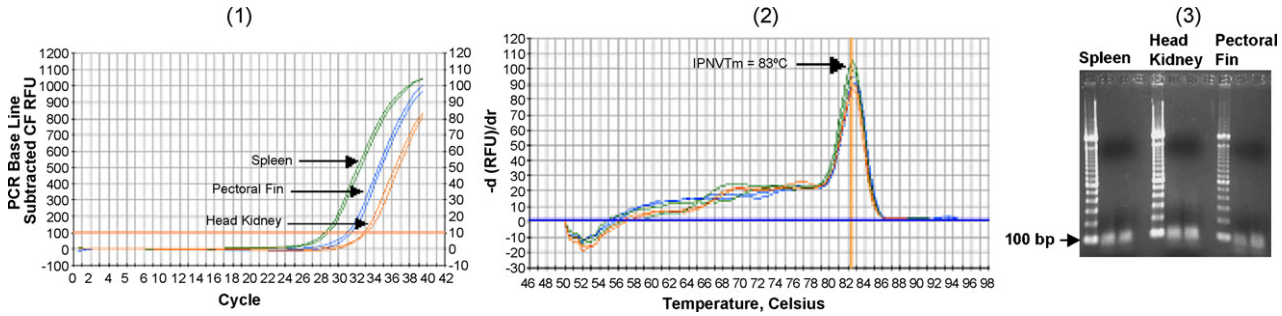

Fig. 5. Amplification profiles and dissociation curves of IPNV NS gene generated by SYBR® Green real-time RT-PCR using cDNAs derived from different tissues (pectoral fin, spleen and head kidney) of an immersion challenged rainbow trout collected at 21 days post-challenge. Amplification profiles (Column 1), dissociation curves (Column 2), and agarose gel electrophoresis photographs (Column 3) corresponding to the IPNV present in each of the three tissues.

samples in laboratory challenged rainbow trout. Our data clearly demonstrates that using a real-time RT-PCR assay; IPNV can indeed be detected in pectoral fin samples as early as 24 h post-challenge. This opens up the possibility for the development of a highly sensitive, high throughput detection method for IPNV, and potentially other viral pathogens in finfish using non-lethal tissue sampling.

Real-time PCR-based detection methods are still in their infancy with regards to field application. Real-time PCR, like other PCR-based detection methods, cannot differentiate between infectious and non-infectious virus particles; therefore, cell culture-based assays are still needed to confirm the pathogenicity of the detected virus. In addition, the utility of real-time PCR for viral detection in commercial application is currently very limited as the highly specific nature of this assay makes it extremely susceptible to false positives when the diagnostic procedures are not being performed in an area free of viral contamination (Reddington, 1995). In spite of these limitations, and as easy to use kits and established primer sets for IPNV and other viruses become available, real-time nucleic acid-based detection methods using non-lethal tissue sampling strategies could be very useful for IPNV screening in aquaculture operations and in wild populations of salmonids.

Acknowledgements

The research was funded by Salton Kennedy Grant no. NA03NMF4270163, 02-NER-030 and Advanced BioNutrition Corp. The authors would like to thank Dr. F.C. Thomas Allnutt for reviewing the manuscript and Ms. Krista N. Kaizer, Advanced BioNutrition Corp. and Mr. William Shewmaker and Mr. Aaron Weighall, Clear Springs Food, Inc. for their excellent technical assistance.

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