

Research Article

Detection and quantification of Chilean strains of infectious pancreatic necrosis virus by real-time RT-PCR assays using segment B as a target

Yoanna Eissler¹, María Soledad Pavlov¹, Pablo Conejeros¹,
Juan Carlos Espinoza¹ & Juan Kuznar¹

¹Centro de Investigación y Gestión de Recursos Naturales, Facultad de Ciencias
Universidad de Valparaíso, Gran Bretaña 1111, Valparaíso, Chile

ABSTRACT. Infectious pancreatic necrosis virus (IPNV) is the causal agent of a highly prevalent disease that affects salmonid fish, mostly during their fresh water life period. Like many other viruses, IPNV produces highly heterogeneous populations. Therefore, diagnostic methods need to be checked constantly so that no variants of the virus escape detection. The IPNV genome is composed of two double-stranded RNA segments: A and B, polymerase chain reaction (PCR) methods normally use segment A as a target. In order to develop an optimized protocol to diagnose IPNV, we present a real-time RT-PCR (reverse transcription) technique, using primers designed to recognize segment B of the virus. To validate the ubiquity of the primers used, the IPNV isolates tested were sequenced and compared with previously published cladograms, which include a wide spectrum of genogroups. These primers made it possible to detect viral isolates belonging to genogroups 1 and 5, which were obtained from different locations linked to fish farming. As expected, we were able to detect the virus from distant *Aquabirnavirus* genogroups.

Keywords: infectious pancreatic necrosis virus (IPNV), real-time RT-PCR assay, VP1 gene, phylogenetic tree.

Detección y cuantificación de cepas chilenas del virus de la necrosis pancreática infecciosa por medio de la técnica de RT-PCR en tiempo real usando el segmento B como objetivo

RESUMEN. El virus de la necrosis pancreática infecciosa (IPNV) es el agente causal de una enfermedad altamente prevalente que afecta a peces salmónidos, principalmente durante su período de vida en agua dulce. IPNV, así como muchos otros virus, produce poblaciones altamente heterogéneas. Por lo tanto los métodos de diagnóstico necesitan ser constantemente revisados para evitar que ciertas variantes del virus escapen de la detección. El genoma del IPNV está compuesto por dos segmentos de ARN de doble hebra, A y B, los métodos de PCR (reacción en cadena de la polimerasa) normalmente usan el fragmento A como blanco. Con el propósito de generar un protocolo optimizado para el diagnóstico del IPNV, se presenta una técnica de RT-PCR (transcripción reversa-) en tiempo real, usando partidores diseñados para reconocer el segmento B del virus. Para validar la universalidad de los partidores utilizados, los aislados del IPNV probados fueron secuenciados y comparados con cladogramas previamente publicados, los cuales incluyen un amplio rango de genogrupos. Con estos partidores fue posible detectar aislados virales pertenecientes a los genogrupos 1 y 5, provenientes de distintas localidades relacionadas con el cultivo de peces. Como se esperaba, se logró detectar virus pertenecientes a genogrupos distantes de *Aquabirnavirus*.

Palabras clave: virus de la necrosis pancreática infecciosa (IPNV), ensayo en tiempo real RT-PCR, gen VP1, árbol filogenético.

Corresponding author: Yoanna Eissler (yoanna.eissler@uv.cl)

INTRODUCTION

Infectious pancreatic necrosis virus (IPNV) is an important challenge for the Chilean salmon industry.

It was isolated for the first time in 1984 (Mc Allister & Reyes, 1984) and corresponded to serotype A1, VR-299 that correlates to an American strain (Espinoza *et al.*, 1985). Since then, the virus progre-

ssively becomes one of the major players of sanitary problems affecting salmon industry in Chile. Recent studies indicated that the Sp strain could be considered as a predominant virus as well (Fernández, 2005; Ortega, 2007; Mutoloki & Evensen, 2011) that correlates to an European strain (genogroup 5, serotype A2). However, in spite of the IPNV importance, the information about IPNV epidemiology in Chile is very scanty (Fernández, 2005; Ortega, 2007). In this context diagnostic methods able to detect a broad spectrum of different strains of IPNV virus are crucial to perform an appropriate management of the Infectious Pancreatic Necrosis (IPN) disease.

IPNV belongs to the Birnaviridae family whose members are characterized by a genome which consists of two segments of double stranded RNA, A and B, and a naked icosahedral single-shelled capsid. Segment A contains two open reading frames (ORF), the largest one is translated into a polyprotein which includes the major protein of the viral capsid (VP2). Segment B contains a single ORF which is translated as the RNA dependent RNA polymerase found as free VP1 or as a genome linked protein (Dobos, 1995).

IPNV displays a variety of strains with differences in virulence. These have been isolated mostly from clinical samples of diseased animals from fish farms (Cutrin *et al.*, 2000; Rodríguez-Saint-Jean *et al.*, 2003). A considerable effort has been done in order to establish virulence factors with the purpose of gaining information that may be useful to develop managing tools for fish industry; for instance, if geographic locations of the most virulent strains are established, preventive measures can be apply with more emphasis at those places. There are a great variety of serotypes in the *Aquabirnavirus* genus including IPNV. It has been proposed that the nine serotypes found may reflect the wide host range of *Aquabirnavirus* (Hill & Way, 1995). A phylogenetic tree was constructed based on VP2 variations, clustering aquatic birnavirus in six genogroups (Blake *et al.*, 2001). The authors also determined that a key factor to have a good correlation between the genogroups and the geographic location and serological properties lies in the amplitude of the VP2 sequence chosen for the analysis.

A good identification of IPNV relies on the availability of diagnostic methods to detect different strains of the virus from different sources. Real-time PCR assay has many advantages as a diagnostic method; is easy to perform, has high sensitivity, great specificity, and provide scope for automation. The method monitors the progress of a PCR reaction in real-time and is based on the detection of the

fluorescence produced as the reaction proceeds. As a reporter fluorescent molecule we used SYBR[®] Green I, which bind to the double-stranded DNA produced in this case after a reverse transcription of the viral RNA. One critical aspect of PCR based diagnostic methods is the selection of appropriate conserved sequences to be primed. Segment A of IPNV, specifically the region coding the protein VP2 (*e.g.*, Rodríguez-Saint-Jean *et al.*, 2001) and NS or VP4 (*e.g.*, Bowers *et al.*, 2008) has been the most used. Within Segment B, VP1 gene codifies to the RNA-dependent RNA polymerase, enzyme which function is essential to the virus replication. It is expected to be less susceptible to change, making this target a good region to be used for primer design. In fact, VP1, as a whole, and compared with VP2, is more conserved than the latter. Taking in account these considerations we decided to optimize a SYBR Green I based one step real-time RT-PCR protocol, aiming to explore if the genomic segment, which codes VP1, could be used as a diagnostic PCR target suitable to detect a wide range of IPNV isolates.

MATERIALS AND METHODS

IPNV isolates and cell line

Five IPNV isolates; *i.e.*, VUV/84, V193/08, V112/06, V33-34/98 and V70/06 (Table 1), were genetically characterized and used for real-time RT-PCR assays. Monolayers of chinook salmon embryo cells (CHSE-214) were used for propagation of IPNV, they were cultured in Leibovitz's L-15 medium (L-15) supplemented with 10% fetal bovine serum and 50 mg L⁻¹ gentamicin in polystyrene bottles (area: 72 cm²) at 19°C.

Virus propagation and RNA extraction

The virus stock was inoculated onto confluent monolayer of CHSE-214 cells. When an advanced cytopathic effect was observed, the cell culture supernatants were harvested as virus source and stored at -20°C until used.

Viral RNA from supernatants of virus stocks was isolated according to the manufacturer's instructions with E.Z.N.A.[™] Total RNA Kit I (OMEGA bio-tek). The concentration and purity of the extracted total RNA was determined by measuring the absorbance ratio at 260 nm over 280 nm using a spectrophotometer (Nanodrop ND-1000 UV/VIS).

Primer design

The forward and reverse primers VP1 F and VP1 R respectively (Table 2), were designed from the conserved region within the VP1 gene of aquatic

birnaviruses. The conserved regions were identified from alignments of nucleotide sequences available in GenBank (accession numbers AY354523, M58757, M58756, AY823633, EU665685, AY354524, AY354522, D26527, AJ489244, AJ489243, AJ489242, AJ489241, AJ489240, AJ489239, AJ489239, AY780931, AY379741, AY379739, AF078669, AY129664, AY780930, AY780929, AY780928, AY780927, AY780927, AY780925, NC001916, AJ622823, AJ489245, AY780931, AY780926, AY129666, AY129665, AY129663, AY129662, AJ489244, AJ489238, AJ489240, M58757, AY129666, AY129662, AY129663, AJ489245, AJ489243, AY129665). These primers designed for real-time RT-PCR assay were compared with WB1 (forward) and WB2 (reverse) (Table 2) that were designed for conventional PCR (Williams *et al.*, 1999) and adapted here to real-time RT-PCR. These primers recognize a 206-bp cDNA fragment within the VP2 gene of aquatic birnaviruses, that previously have been shown to identify representative isolates of all nine serotypes of aquatic birnavirus serogroup A (Sweeney *et al.*, 1997).

The five isolates were sequenced using the forward AIF and A2 reverse primers (Blake *et al.*, 1995) (Table 2), the forward primer was designed from a conserved region within the VP2 gene of aquatic birnaviruses using sequences available in GenBank (accession numbers AF342727, AF343571, AF343572, AF343573, AY026345, AY026346, AY026347, AY026348, AF343570, AF342728, AY026482, AY026483, AY026484, AY026485, AF342729, AY026486, AY026487, AY026488, AY026489, AF342730, AF342730, AF342731, AY026490, AF342733, AF342734, AF342735).

Real-time RT-PCR assay

Three different primer concentrations (*i.e.*, 0.2, 0.5 and 1 μM) for the real-time RT-PCR assays were tested. The optimized reaction for SYBR Green I real-time RT-PCR contained 7.5 μL of 2X Brilliant III Ultra-Fast SYBR[®] Green QRT-PCR Master Mix (Stratagene), 0.5 μM each of forward and reverse primers, 0.8 μL of RT/RNase block, 0.3 μM of ROX as reference dye and 2 μL of the total RNA in a 15 μL reaction volume. The amplifications were carried out using a 48-well plate real-time PCR system Step-One (Applied Biosystems). The no-template control (NTC) consisted in a reaction mixture without template. The thermal profile included the following steps; 50°C for 5 min for reverse transcription, a pre-denaturation at 95°C for 3 min, followed by 40 cycles of denaturation at 95°C for 5 s and annealing/extension at 55°C (VP1) or 60°C (WB) for 10 s. Finally, a melting curve analysis from 70° to 95°C was performed.

Standard curve

The detection limit of the assay was evaluated using total RNA extracted from the virus isolate named V193/08. The concentrations of the extracted RNA were determined using a spectrophotometer (Nanodrop ND-1000 UV/VIS). After the quantification, total RNA was diluted serially in 10-fold dilutions using V193/08, the dilutions were done in triplicate. The amplification efficiency (%) of the two developed standard curves was calculated based on the formula $[10^{(-1/\text{slope})} - 1] \times 100$.

RT-PCR amplification and purification of cDNA products

The RT-PCR amplification for sequencing was carried out using a PCR Multigene, Labnet, equipment. Seven μL of viral RNA were mixed with AIF (forward) and A2 (reverse) primers at a final concentration of 0.5 μM , 22.5 μL of 2X Brilliant III Ultra-Fast SYBR[®] Green QRT-PCR Master Mix (Stratagene), 2 μL of RT/RNase block, and 9 μL of the RNase free water in 45 μL reaction volume. The reaction was performed as follows; 42°C for 30 min for reverse transcription, a pre-denaturation step at 95°C for 3 min, 35 cycles of denaturation at 95°C for 30 s, annealing at 58°C for 30 s and extension at 72°C for 60 s and final extension at 72°C for 10 min. The amplified fragments of the expected length were cut out of the gel and transferred to microcentrifuge tubes and purified using a E.Z.N.A.[™] Gel Extraction Kit (OMEGA bio-tek). Briefly, the fragments were melted with Binding buffer (XP2) by heating at 60°C for 7 min, the solution was then placed in a Hibind DNA mini column and centrifuged at 10,000 g for 2 min at room temperature. This step was done successively using first Binding buffer (XP2) and later SPW wash buffer to clean the column, finally the obtained cDNA was eluted with 30-50 μL of RNase free water. Purified duplicates of the PCR product were sequenced by Macrogen Inc., Korea using a ABI3730XL DNA Analyzer.

Sequence analysis

Sequences obtained from the five isolates were edited with the software BioEdit version 7.0.5.3 (Hall, 1999) which was also used to define reading frames and to translate them into aminoacid sequences. The obtained sequences were compared and aligned with previously published VP2 IPNV sequences (Blake *et al.*, 2001) by using MEGA 5 (Tamura *et al.*, 2007). MEGA 5 was also used to draw the amino acid based phylogenetic trees using the neighbor-joining method, 1000 bootstrap replicates were performed for each analysis to assess the likelihood of the tree

construction. The genogroup for each of our isolates were defined by direct sequence comparison.

The nucleotide and deduced amino acid sequence data reported in this paper have been deposited in GenBank with the following accession numbers: V70/06, HQ738515, V112/06, HQ738516, V193/08, HQ738517, V33-34/98, HQ738518 and VUV/84, HQ738519 (Table 1).

RESULTS

Genomic characterization of the IPNV isolates

In order to test the ubiquity of the detection method presented here, several isolates of IPNV were selected to have a wide genomic diversity. The comparison of the genomic relationships among viral isolates based on the aminoacid sequences of the VP2 coding region demonstrated identities of 100 and 99% between the VUV/84 and Ja-Dobos (Canada) and VR299 strain (West Virginia, USA), respectively; positioning this isolate in Genogroup 1 and Genotype 3 (Fig. 1). The isolates V193/08 and V112/06 are 98.5 and 97.5% similar to Dry Mills (DM) strain (Maine, USA), respectively, placing them in Genogroup 1 and Genotype 4 (Fig. 1). The isolates V70/06 and V33-34/98 are 98.5 and 98% similar to FR10 strain (France), respectively, placing them in Genogroup 5 (Fig. 1).

Specificity of the real-time RT-PCR

A BLAST (Basic Local Alignment Search Tool) of the primers sequences for this assay showed their specificity towards segment A and B, of aquatic birnaviruses and with no cross-reactivity with other viruses. Primer specificity was further confirmed by using them in real-time RT-PCR against RNA from Infectious salmon anaemia virus (ISAV), no fluorescent signal for SYBR Green I was seen (data not shown).

Table 1. The five isolates of IPNV used in this study. VUV/84 was isolated in our lab (Laboratorio de Virología de la Universidad de Valparaíso) and V193/08, V112/06, V33-34/98 and V70/06 were isolated in Laboratorio de Biotecnología y Patología Acuática, Universidad Austral de Chile.

Tabla 1. Los cinco aislados de IPNV utilizados en este estudio. VUV/84 fue aislado en nuestro laboratorio (Laboratorio de Virología, Universidad de Valparaíso) y V193/08, V112/06, V33-34/98 y V70/06 fueron aislados en el Laboratorio de Biotecnología y Patología Acuática de la Universidad Austral de Chile.

Isolate name	Host of origin	Geographic origin	Access number GenBank
VUV/84	<i>Oncorhynchus mykiss</i>	Llanquihue, Chile	HQ738519
V193/08	<i>Oncorhynchus kisutch</i>	Valdivia, Chile	HQ738517
V112/06	<i>Oncorhynchus mykiss</i>	Temuco, Chile	HQ738516
V33-34/98	<i>Salmo salar</i>	Purranque, Chile	HQ738518
V70/06	<i>Salmo salar</i>	Osorno, Chile	HQ738515

Sensitivity of the real-time RT-PCR

The standard curve of the SYBR Green I real-time RT-PCR assay was constructed independently for both primers sets by using 10-fold serial dilutions of total RNA extracted from V193/08 virus preparation. Amplification of V193/08 RNA at different concentrations showed a linear relationship over a range of six orders of magnitude from a dilution of 10^{-1} to 10^{-5} for VP1 and WB primers sets (Fig. 2).

The regression analysis yielded a correlation coefficient of 0.997 and 0.995 and a y-intercept value of 34.22 and 35.77 for VP1 and WB primers respectively. The slopes were -3.24 and -3.14 for VP1 and WB primers respectively, indicating an amplification efficiency of 104 and 108%, very close to the theoretical maximum amplification efficiency ($100\% = -3.32$ slope). The standard curves showed a high linear correlation between the Ct values of V193/08 RNA (Fig. 2).

The standard curve showed that the detection limit of the assay was 508 and 14 fg μL^{-1} of total RNA with a cut off value of 30.8 and 32.0 cycles for VP1 and WB, respectively. Therefore samples were interpreted as IPNV positive when presenting an exponential fluorescent curve with a Ct value ≤ 30.8 or 32.0 and a melting curve within a $\pm 0.3^\circ\text{C}$ range, from an average of 82.74 or 82.12 for VP1 and WB respectively.

Quantitative detection of different isolates of IPNV

The performance of the assays was evaluated by using 5 IPNV isolates which are characterized here. All the five isolates showed positive amplification using VP1 and WB primers (Figs. 3a-3d), with Ct values ranging from 11.80 to 16.65 and with melting temperature (T_m) values between 82.39°C and 82.69°C for VP1 primers. For WB primers the Ct values ranged from 14.53 to 18.03, with T_m values

Table 2. Primers for real-time RT-PCR and for sequencing of IPNV isolates.

Tabla 2. Partidores para RT-PCR en tiempo real y para secuenciar los aislados de IPNV.

Primer name	Primer (sequence) 5'-3'*	Amplicon A or B position segment	Amplicon size (bp)	Coding region
AIF A2	(CATACGTCGGVCTWGAGGACGAGAC) (GACAGGATCATCTTGGCATAGT)	518-1190	672	VP2
VP1 F VP1 R	(GTTGATMMASTACACCGGAG) (AGGTCHCKTATGAAGGAGTC)	668-820	152	VP1
WB1 WB2	(CCGCAACTTACTTGAGATCCATTATGC) (CGTCTGGTTCAGATTCCACCTGTAGTG)	20-225	206	VP2

* The position of the primers for VP2 is based on the first nucleotide of the start codon of the viral mRNA encoding the IPNV polyprotein and for VP1 based on the reference of Cutrin *et al.* (2004).

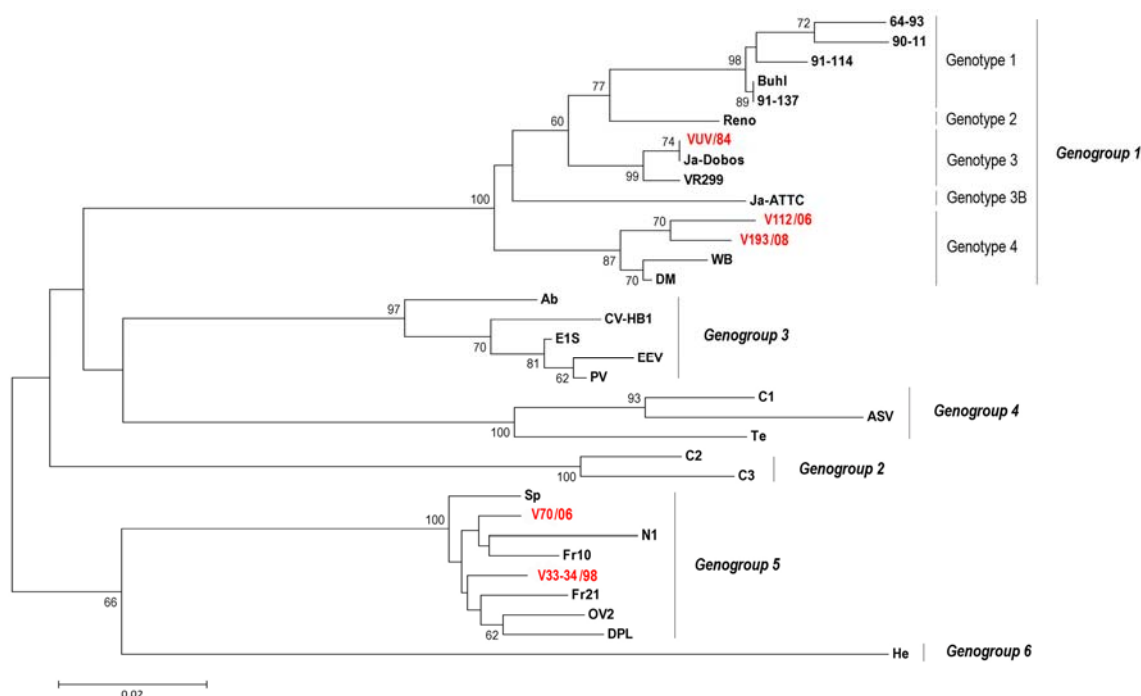


Figure 1. Cladogram representing phylogenetic relationships of aquatic birnaviruses based on deduced amino acid sequences of VP2 using data from Blake *et al.* (2001). The position of the five isolates used in this paper, is presented in the cladogram as red color font (i.e., VUV/84, V193/08, V112/06, V33-34/98 and V70/06).

Figura 1. Cladograma que representa la relación filogenética de birnavirus acuáticos basado en la secuencia de aminoácidos deducidos de VP2, usando la base de datos publicada en Blake *et al.* (2001). La posición de los 5 aislados utilizados en este manuscrito se presenta en el cladograma con letra de color rojo (i.e., VUV/84, V193/08, V112/06, V33-34/98 y V70/06).

between 81.95° and 82.32°C (Table 3). The PCR products using VP1 and WB primers sets were confirmed by agarose gel electrophoresis (data not shown).

DISCUSSION

Real-time PCR is a technique widely used for diagnostic of pathogens and basic research. We

optimized a specific, rapid and sensitive real-time RT-PCR assay by targeting segment B for the detection of IPNV. Five different IPNV isolates were tested (i.e., VUV/84, V193/08, V112/06, V33-34/98 and V70/06), which belong to two very different genogroups, 1 and 5, based on the cladogram presented by Blake *et al.* (2001). The optimized real-time RT-PCR assay using designed primers based on the VP1 region in segment

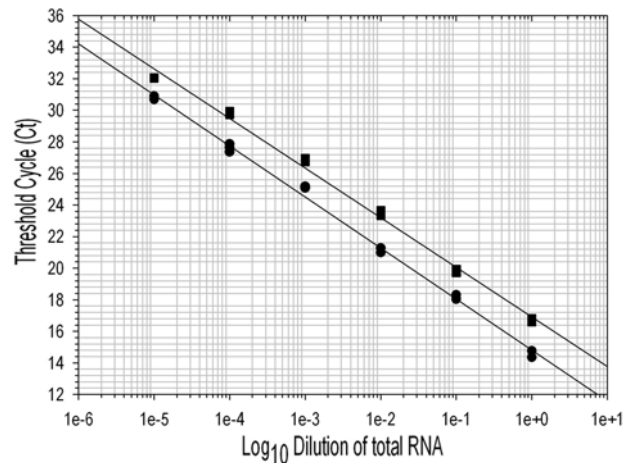


Figure 2. A linear relationship between threshold cycle and serially diluted total RNA of V193/08 isolate. Standard curve was generated from real-time RT-PCR amplification with VP1 (circles) ($r^2 = 0.997$, slope = -3.24) and WB (squares) ($r^2 = 0.995$, slope = -3.14) primers combination.

Figura 2. Relación lineal entre el ciclo umbral y la dilución seriada del RNA total del aislado V193/08. La curva estándar fue generada por la amplificación RT-PCR en tiempo real, con los partidores VP1 (círculos) ($r^2 = 0,997$, pendiente = -3,24) y WB (cuadrados) ($r^2 = 0,995$, pendiente = -3,14).

B, gave consistent results in detecting all five isolates. These results were contrasted with primers sets based on a VP2 gene conserved region, that previously were able to identify representative isolates of all nine serotypes of aquatic birnavirus, serogroup A (Sweeney *et al.*, 1997).

We investigated the genomic differences of five IPNV isolates that represent widely different geographical and host origins from salmon farms in the southern region of Chile (Table 1). We tested a specific amplicon of 672 bp (position 518-1190 bp) from segment A, included within VP2 region. A wider VP2 coding region (1611 bp) was used by Blake *et al.* (2001) to classify 28 aquatic birnaviruses described in the literature, representing all nine serotypes of Serogroup A. We chose this particular amplicon to classify the isolates since it includes a region within a variable zone of VP2 gene. Comparison of the deduced amino acid sequences demonstrated identities of 100 and 99% between VUV/84 and Ja-Dobos and VR299, respectively, both being American strains, isolated from trout. Consequently, confirming the previous finding that VUV/84 belongs to the VR-299 serotype (Espinoza *et al.*, 1985). V193/08 resulted 99.5 and 97.5% similar to DM and WB, respectively, both isolated in Maine, USA, from trout and 98% similar to V112/06, therefore, both Chilean isolates

can be placed in genogroup 1, genotype 4. Interestingly, V70/06 and V33-34/98 resulted to be more similar to Fr10 strain (98.5 and 98%, respectively) isolated in France from trout, which belong to genogroup 5. Including these two distant genogroups such as 1 and 5, (82.8% of similitude) in our segment B based real-time RT-PCR assay, we ensure to incorporate IPNV strains as distant as possible.

Previous studies have used primers designed to amplify the segment A encoding the VP2 (Wang *et al.*, 1997; Rodríguez-Saint-Jean *et al.*, 2001, 2010; McBeath *et al.*, 2007; Marroquí *et al.*, 2008), NS or VP4 (non-structural protein gene) (Bowers *et al.*, 2008) or NS/VP3 (Kerr & Cunningham, 2006) regions. These studies have employed traditional RT-PCR (Wang *et al.*, 1997; Rodríguez-Saint-Jean *et al.*, 2001; Kerr & Cunningham, 2006) and real-time RT-PCR based on SYBR Green I (Bowers *et al.*, 2008) or on Taqman probes (McBeath *et al.*, 2007; Marroquí *et al.*, 2008; Rodríguez-Saint-Jean *et al.*, 2010), which have different advantages in relation to their application but some disadvantages as well, for instance, to find an appropriate internal set of primers in a rather variable gene. Segment B should be more suitable to find those internal zones for probing because its lesser variability. The results of real-time RT-PCR assays were compared using VP1 and VP2 based primers, only significant differences between Ct values for V112/06 and V33-34/98 IPNV isolates (t-test analysis, $P < 0.05$) were found regardless the fact that the same amount of total RNA was used as template in the experiments. Despite of the genomic differences present among the five IPNV isolates studied, comparing all Ct and Tm values, for VP1 and VP2 based primers respectively, no significant differences were found (Kruskal-Wallis ANOVA by Ranks $P > 0.05$) and all analysis resulted in a positive detection.

The detection limit of the technique was 508 and 14 fg μL^{-1} of total RNA with cut off values of 30.8 and 32.0 cycles for VP1 and WB, respectively. This is similar to what was reported by Bowers *et al.* (2008) using the same methodology but with different primers sets based on the IPNV NS gene; Ct mean was 31.88 which is equivalent to 10 RNA copies of *in vitro* transcribed RNA. Besides, Bowers *et al.* (2011) compared four different templates representing the IPNV protease gene. Regardless of the template type they did not find any significant differences in copy number calculations for the quantification of IPNV load in experimentally-challenged fish. Irrespective of the use of total RNA as template, our results show that small loads of viral RNA can be detected as well. In fact when compared with those calculated by Bowers

Table 3. Analysis of IPNV isolates by real-time RT-PCR using different primer combinations. Ct: threshold cycle, SD: standard deviation, Tm: melting temperature.

Tabla 3. Análisis de aislados de IPNV por medio de RT-PCR en tiempo real usando diferentes combinaciones de partidores. Ct: ciclo umbral, SD: desviación estándar, Tm: temperatura de fusión.

IPNV isolates	VP1 primers					WB primers						
	Ct (Duplicate measures)	Average Ct	SD	Tm (°C) (Duplicate measures)	Average Tm	SD	Ct (Duplicate measures)	Average Ct	SD	Tm (°C) (Duplicate measures)	Average Tm	SD
VUV/84	15.65 15.69	15.67	0.03	82.69 82.69	82.69	0.00	15.75 15.49	15.62	0.19	82.40 82.25	82.32	0.11
V193/08	17.13 15.98	16.55	0.82	82.69 82.69	82.69	0.00	16.11 16.43	16.27	0.23	81.95 81.95	81.95	0.00
V112/06	12.15 11.45	11.80	0.50	82.54 82.54	82.54	0.00	14.50 14.57	14.53	0.05	82.25 82.25	82.25	0.00
V33-34/98	16.14 15.55	15.85	0.42	82.39 82.39	82.39	0.00	18.16 17.91	18.03	0.17	81.95 81.95	81.95	0.00
V70/06	16.92 16.38	16.65	0.38	82.39 82.39	82.39	0.00	17.40 16.93	17.16	0.34	82.25 82.25	82.25	0.00

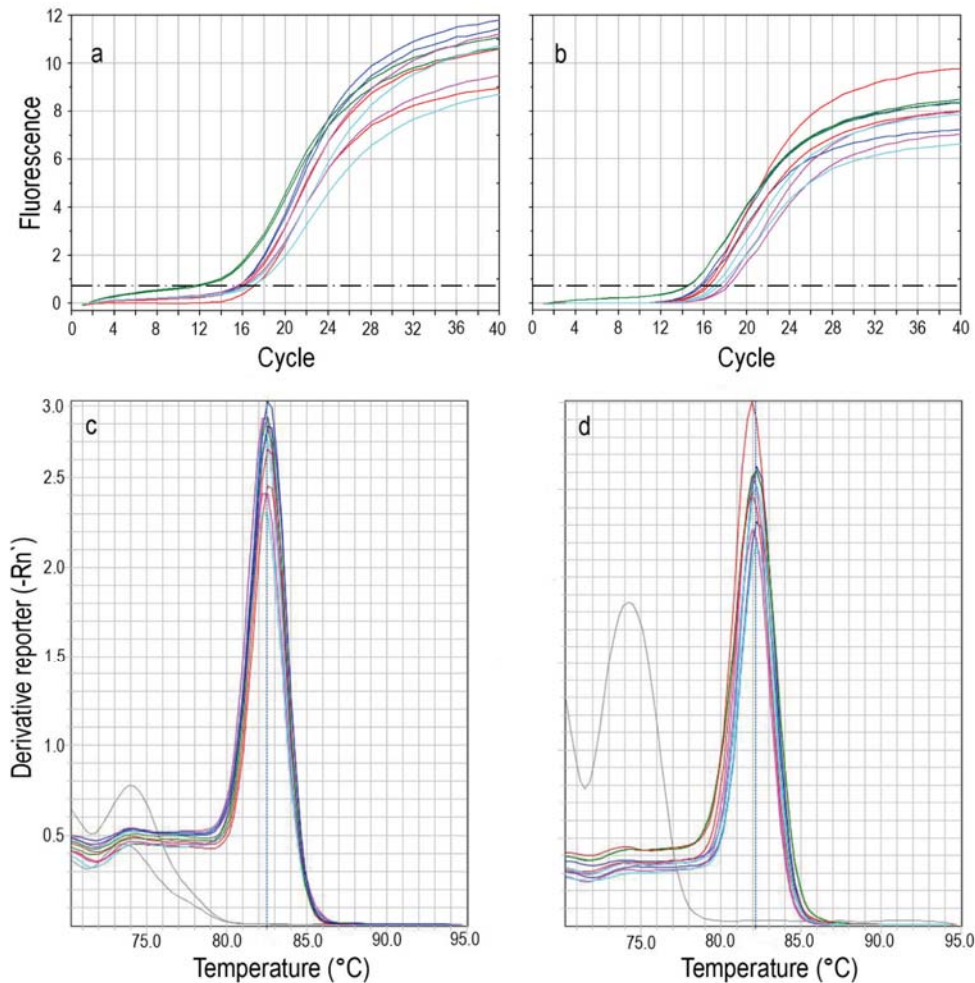


Figure 3. Curves generated by SYBR Green I real-time RT-PCR for the five IPNV isolates a) amplification and c) dissociation using VP1; b) amplification and d) dissociation using WB. Curves are: VUV/84: blue, V193/08: red, V112/06: green, V33-34/98: pink, V70/06: cyan, NTC: grey, Ct threshold: dash-dot line and melting temperature average: blue dot line.

Figura 3. Curvas generadas por SYBR Green I RT-PCR en tiempo real para los cinco aislados de IPNV. a) amplificación y c) disociación usando VP1; b) amplificación y d) disociación usando WB. Las curvas son: VUV/84: azul, V193/08: rojo, V112/06: verde, V33-34/98: rosado, V70/06: cian, NTC: gris, Umbral del Ct: línea guión-punto y promedio de la temperatura de fusión = línea en azul punteada.

et al. (2011), whose slopes of the standard curves were obtained with purified preparations of IPNV RNA, slight differences found between the slopes of both kind of templates are just those expected as caused by the nature of the starting RNA source.

Finally, segment B based real-time RT-PCR assay proposed here was suitable to detect IPNV from the two main genogroups present in Chile (*e.i.*, genogroups 1 American and 5 European strains). This real-time RT-PCR assay should be further validated with more field samples, however our results brings this approach as a promissory alternative to improve the accuracy of the diagnostic results targeting a broad spectrum of IPNV strains.

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