Novel Oral vaccine for Infectious Salmon Anemia

Final Report

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Executive Summary

This project was initiated with an overall goal to developing an oral vaccine against infectious salmon anemia virus (ISAV) of salmon using virus-like particle (VLP) as a platform. Development of oral vaccine against viral diseases in salmonid will be very valuable for aquaculture industries in the US and elsewhere in the globe.

Several milestones were achieved towards our overall goal of developing oral vaccine for fish. These have been described in Chapters 1 to 3. Infectious pancreatic necrosis virus (IPNV) capsid gene, VP2, was successfully cloned and expressed using a yeast expression vector. Transmission electron microscopy showed that the recombinant VP2 protein forms sub virus-like particle (sVLP), 20 nm in diameter. IPNV VP2 sVLPs, when delivered into rainbow trout via injection or oral route, elicited anti-IPNV antibody response. Rainbow trout immunized with IPNV sVLPs, via injection or oral route, were challenged with infectious IPNV. A real-time RT-PCR assay was developed to quantify IPNV load in the virus challenged rainbow trout. Immunized fish showed significant reduction in viral load compared to unimmunized fish indicating that IPNV VP2 sVLPs could be used as oral vaccine.

In order to evaluate the potential of using the IPNV sVLP platform to express foreign epitope, human oncogene *c-myc* epitope was inserted into IPNV VP2 capsid gene and expressed in yeast. The chimeric IPNV-*c-myc* sVLPs, when injected in rainbow trout (*Onorhynchus mykiss*), positive seroconversions were obtained not only against the IPNV VP2 sVLP backbone, but also against the human oncogene epitope *c-myc* epitope) in the injected fish. This demonstrated that IPNV VP2 sVLP can accommodate heterologous epitope (e. g. *c-myc* epitope in our study) without destroying the tertiary structure of the backbone and the antigenicity of the sVLPs, and the foreign epitope retains immunogenecity. Our data indicate that *c-myc* epitope could potentially be replaced with ISAV epitope, and oral vaccine against both ISAV and IPNV could be developed. These findings open up a possibility of developing multivalent vaccine using IPNV VP2 sVLPs platform.

The findings of this research project were presented in national and international conferences, and two manuscripts have been submitted for publication in peer-reviewed journals. Copies of these manuscripts were attached here.

Chapter 1. Develop an oral vaccine using virus-like particle (VLP)-based platform.

The infectious pancreatic necrosis virus (IPNV) capsid protein VP2 was expressed in yeast. Electron microscopy showed that the recombinant IPNV VP2 protein forms sub-virus particle measuring 20 nm in diameter, and reacts to IPNV antibody in western blot analysis. The cesium chloride gradient purified IPNV VP2 sub-virus particles upon injection into rainbow trout elicit antibody response as measured by enzyme-linked immunosorbent assay (ELISA) using IPNV antibody. When recombinant yeast expressing IPNV VP2 protein was fed to rainbow trout, it elicited an anti IPNV antibody response in fish. This indicated that the IPNV VP2 gene expressed in yeast can be used as a potential vaccine (both injection and oral vaccine) against native IPNV.

These findings were described in abstracts #3 and manuscript #1 (see below).

Chapter 2. Develop a quantitative method to measure the efficacy of VLP-based vaccine.

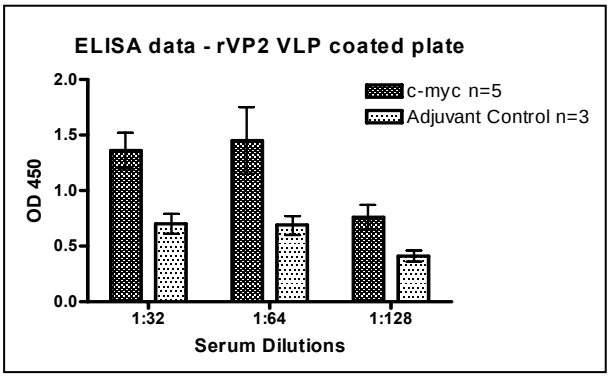
In order to evaluate the efficacy of IPNV sVLP-based vaccine, a real-time RT-PCR method was developed. Rainbow trout were vaccinated with IPNV VP2 sVLP-based vaccine, and then challenged with native IPNV. Real-time RT-PCR data showed that rainbow trout immunized with sVLP-based vaccine had a significant reduction in viral load (approx. 23-fold reduction in injection trial and approx. 13-fold reduction in oral vaccination trial) compared to unimmunized fish. These data clearly indicate that rainbow trout immunized with VLP-based vaccine not only elicit antibody response but also prevent the replication of IPNV when exposed to native infectious IPNV.

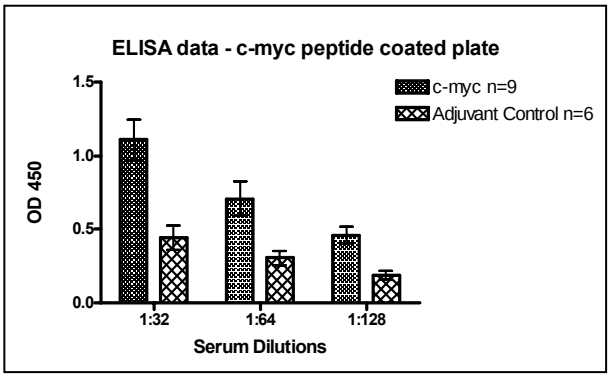
The development of IPNV real-time RT-PCR method was described in abstract #3 and manuscript #2 (see below). The efficacy of the IPNV sVLP-based vaccine in reducing viral load was described in abstracts #3 and manuscript #1 (see below).

Chapter 3. Use IPNV VLP-based platform to express heterologous epitope.

In order to evaluate the potential of using the IPNV sVLP to express foreign epitope, human oncogene *c-myc* epitope was inserted into IPNV VP2 capsid gene and the recombinant plasmid was cloned into yeast expression vector pSC-URA. To determine the antigenicity of the chimeric IPNV-*c-myc* sVLPs, we injected *c-myc* VLPs in rainbow trout (*Onorhynchus mykiss*), and examined the seroconversion (against IPNV VP2 protein and *c-myc* epitope) in the injected fish. Our data showed that there were positive seroconversions against IPNV VP2 and *c-myc* epitopes in the *c-myc* VLP injected fish. This confirmed that IPNV VP2 sVLP can accommodate heterologous epitope (*c-myc* epitope) without destroying the tertiary structure of the backbone and the antigenicity of the sVLPs.

Fig. 1. Mean ELISA values (OD_{450}) of anti-*c-myc* titer in rainbow trout serum following immunization with *c-myc* VLPs by injection. The ELISA plates were coated with (Left panel) IPNV rVP2 sVLP and probed with anti-IPNV antibody or (Right Panel) *c*-myc peptide and probed with anti *c-myc* antibody.





These findings were presented in World Aquaculture Society Meeting in Florence, Italy, May 09-13, 2006 (see below).

Chapter 4. List of abstracts/ papers presented/submitted for publication:

The research findings of this project were in national and international conferences, and two manuscripts have been submitted for publication in peer-reviewed journals. A list of those publications is provided below.

Following papers were presented in international conferences:

1. Arun K. Dhar, Robert Bowers, Christopher Rowe and F. C. Thomas Allnutt. 2006. Formation of non-infective infectious pancreatic necrosis (IPNV) virus-like particles (VLPs) expressing human ONCOGENE *c-myc*. Presented in World Aquaculture Society Meeting in Florence, Italy, May 09-13, 2006.

2. F. C. Thomas Allnutt, Robert M. Bowers, Christopher G. Rowe, Vikram N. Vakharia, Scott E. LaPatra, and Arun K. Dhar. 2007. Antigenicity of infectious pancreatic necrosis virus capsid protein VP2 virus-like particles expressed in yeast. Presented in World Aquaculture Society Meeting in San Antonio, Texas, Feb 26-March 02, 2007.

3. Robert M. Bowers, Scott E. LaPatra, Krista N. Kaizer and Arun K. Dhar. 2007. A real-time reverse transcriptase-polymerase chain method for quantitative detection of infectious pancreatic necrosis virus in rainbow trout (*Oncorhynchus mykiss*). World Aquaculture Society Meeting in

Following manuscripts have been submitted to peer-reviewed journals:

1. F. C. Thomas Allnutt, Robert M. Bowers, Christopher G. Rowe, Vikram N. Vakharia, Scott E. LaPatra and Arun K. Dhar. 2007. Antigenicity of infectious pancreatic necrosis virus VP2 sub-viral particles expressed in yeast. Submitted to Vaccine.

2. Robert M. Bowers, Scott E. La Patra and Arun K. Dhar. 2007. Detection and Quantification of Infectious Pancreatic Necrosis Virus by Real-Time RT-PCR Using Invasive and Non-Invasive Tissue Sampling. Submitted to Journal of Clinical Microbiology.

Chapter 5. Appendix: Copies of manuscripts submitted to peer-reviewed journals.

Title of the manuscript: Antigenicity of infectious pancreatic necrosis virus VP2 sub-viral particles expressed in yeast

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Abstract

Infectious pancreatic necrosis virus, the etiologic agent of infectious pancreatic necrosis in salmonid fish, causes significant losses to the aquaculture industry. The gene for the viral capsid protein (VP2) was cloned into a yeast expression vector and expressed in *Saccharomyces cerevisae*. Expression of the capsid gene in yeast resulted in formation of ~20 nm sub-viral particles composed solely of VP2 protein. Anti IPNV antibodies were detected in rain bow trout vaccinated either by injection of purified VP2-subviral particles (rVP2-SVP) or by feeding recombinant yeast expressing rVP2-SVP. Challenge of rVP2-SVP immunized trout with a heterologous IPNV strain and subsequent viral load determination showed that both injection and orally vaccinated fish had lower IPNV loads than naive or sham-vaccinated fish. This study demonstrates the ability of rVP2-SVPs to induce a specific immune response and the ability of immunized fish to reduce the viral load after an experimentally induced IPNV infection.

Key words: IPNV, VP2 gene, oral vaccine

Running Title: Sub-viral particles of IPNV are immunogenic in trout

1. Introduction

Infectious pancreatic necrosis virus is the causative agent of infectious pancreatic necrosis disease (IPN) that infects salmonids and remains a serious problem in the aquaculture industry [1]. IPN is especially contagious and destructive to juvenile trout and salmon. Highly virulent strains may cause greater than 70% mortality in hatchery stocks over a period of two months [21]**.** This disease is especially destructive in salmonid eggs and fingerlings [25]. Survivors of infection can remain lifelong asymptomatic carriers and serve as reservoirs of infection, shedding virus in their feces and reproductive products. Losses due to IPNV on salmon smoltification have been estimated at 5% [16]. Economic losses due to IPNV in aquaculture were estimated to be over $60 million in 1996 [4], [17]. This has been reduced as vaccines for salmonids became available based on killed virus or recombinantly produced viral peptides [13, 17]. However, these vaccines are not completely effective and can only be used in fairly large fish due to the reliance on injection for vaccination.

IPNV is a double-stranded RNA virus of the *Birnaviridae* family [5] and is the type species of the Aquabirnavirus genus [6]. Birnaviruses have a non-enveloped, single-shelled particle structure comprised of a single protein capsid layer with T = 13 icosahedral symmetry [2]. All birnavirus genomes have two dsRNA segments. The IPNV genome's two dsRNA segments are designated segments A and B. Segment B (2777 nucleotides) encodes a minor internal polypeptide VP1 (94 kDa), which is the virion-associated RNA-dependent RNA polymerase (RdRp) [7], [11]. Segment A (3097 nucleotides) encodes a 106-kDa precursor polyprotein composed of pVP2-VP4-VP3, in that order, and a 15-kDa non-structural VP5 protein, found only in infected cells [14]. VP2 and VP3 are the major capsid proteins, but VP2 is the major host-protective antigen of IPNV [9], [12].

There are commercial multivalent vaccines based on inactivated whole virus available as well as those produced with another approach, expressing VP2-derived conserved antigenic epitopes in bacteria for production of a subunit vaccine. In the laboratory, these current vaccines provide impressive protection against bath challenge with IPNV, but the behavior in the field is not predicted by the laboratory studies. This could be due to the lack of a well-defined challenge system with mortality as its endpoint. Results based on viral clearance exist but may not be as rigorous as a standardized challenge model [1]. Another possible explanation could be that the salmon smolts or larger trout being vaccinated are already infected with the virus, as each year between 30-40% of the salmon hatcheries experience an outbreak of IPN [3] and IPN is endemic in many trout rearing areas. The need for better field efficacy could be achieved with improved vaccines that could be economically delivered to young salmonids such that subsequent vaccinations would boost existing immunity instead of trying to combat an existing acute or chronic infection.

An ideal vaccine for IPNV must induce long lasting protection at an early age, prevent carrier formation, and be effective against a large number IPNV serotypes. Injection cannot be used for small fish, therefore either oral delivery or immersion are more preferred routes for early vaccination. These attributes of an ideal IPNV vaccine must be met either by a recombinant subunit vaccine or by an inactivated viral vaccine, as a live attenuated vaccine could potentially lead to carrier formation. The yeast expression system has potential value for oral vaccine development, since yeast is already a component of feeds and is generally regarded as safe. This contrasts with bacterial expression in *Escherichia coli*, which generates pyrogens that would need to be removed before use of any crude preparation as an oral vaccine [22]. The use of yeast is also attractive because production is economical and, through well-developed genetic

systems, can be engineered to provide an abundant supply of the protein or proteins of interest. In fact, Pitcovski et al. [19] reported the development and large-scale use of yeast-derived recombinant VP2 vaccine for the prevention of infectious bursal disease (caused by another birnavirus) of chickens.

Here, we report cloning of the IPNV-VP2 gene into a yeast expression vector, pESC-ura. Expression of the VP2 protein resulted in formation of ~ 20 nm sub-viral particles (SVPs) in yeast, as detected by electron microscopy. Purified recombinant VP2 SVPs (rVP2-SVPs) were used to vaccinate fish by both injection and oral routes and their antigenicity in rainbow trout evaluated by immunoassay. An IPNV challenge trial was also carried out and the effect of vaccination on viral load evaluated.

2. Materials and methods

2.1 Cloning of the VP2 and VP3 genes of IPNV - The West Buxton (WB) strain of IPNV, obtained from American Type Culture Collection (ATCC VR-877), was used for this study. This virulent strain of IPNV is prevalent in Maine and Canada, where the major North American salmon aquaculture industry exists. The WB strain of IPNV was purified as previously described [26]. The virus was propagated in Chinook salmon embryo (CHSE-214) cell cultures (ATCC CRL-1681), maintained at 15°C in Eagle's minimal essential medium (EMEM) and supplemented with 10 % fetal bovine serum (FBS), 100 U/mL penicillin, 100 μg/mL streptomycin and 1 μg/mL fungizone. Total viral RNA was isolated from purified virus by digesting with proteinase K (200 μg/mL final concentration) followed by phenol: chloroform extraction [23]. The IPNV-VP2 and VP3 genes were amplified by reverse transcription-polymerase chain reaction (RT-PCR) and cloned into the pCR2.1 vector (Invitrogen, Carlsbad, California) following previously published protocols [24]. The primer pair used for VP2 cloning was WBABglF (5'-GAGATCTATG AACACAACAA AGGCAACCGC-3'), containing a 5' *Bgl*II site, and WBAVP2R (5'-AAGCTTAAGC CCATGTGTCC ATGAC-3'), containing a 5' *Hin*dIII site. The primer pair used to clone the VP3 gene was WBAVP3F (5'-GGATCCATGT CAGGGATGGAC GAAGAACTG-3') and FA3'NCHindR (5'-ATAAGCTTGG GGGCCCCCTG GGGGGCC-3') with *Bam*HI or *Hin*dIII sites at the 5' ends, respectively. The integrity of the clones were verified by sequencing the plasmid DNA in both directions using an automated DNA sequencer (Applied Biosystems).

To make a yeast expression vector containing the VP2 gene, the VP2-containing plasmid was double digested with *Bgl*II and *Hin*dIII. The VP2 fragment was gel purified, blunt-ended with Klenow enzyme, and inserted between the unique *Eco*RI and *Bgl*II sites of pESC-ura, which had been blunt-ended with Klenow, behind the GAL10 promoter (Fig. 1A). To make the VP3 yeast vector, the VP3-containing plasmid was double digested with *Bam*HI and *Hin*dIII enzymes. The VP3 fragment was gel purified and cloned between the unique *Bam*HI and *Hin*dIII sites of pESC-ura behind the GAL1 promoter (Fig. 1B). Finally, to make the yeast vector that expressed both the VP2 and VP3 capsid protein genes, the VP2 gene was inserted into the unique *Eco*RI and *Bgl*II sites of pESC-ura behind the GAL10 promoter in the VP3-containing constructs (Fig. 1C).

2.2 Expression of VP2 in yeast – Yeast (*Saccharomyces cerivisiae* strain YH501; Stratagene, La Jolla, CA) were transformed using the EZ Yeast Transformation Kit (Zymed, San Francisco, CA). Mutant colonies were selected for growth on autotrophic SG-ura medium containing galactose, yeast extract without amino acids, and amino acid dropout mixture (all amino acids plus adenine, no uracil). Mutants were grown at 30°C for 4 days, collected by centrifugation, then crude protein extracts prepared using Y-PER yeast breaking buffer (Pierce Biotechnology, Rockford, IL). Lysates were electrophoresed on 12% SDS-polyacrylamide gels (BioRad, Richmond, CA) and transferred to nitrocellulose by electroblotting. The blots were probed with sheep-anti-IPNV polyclonal antibody (Microtek International, Inc, Saanichton, B.C., Canada) and detected with rabbit-anti-sheep polyclonal antibody conjugated to HRP (Bethyl Laboratories, Montgomery, TX). Detection was obtained using the colorimetric substrate tetramethyl benzidine (TMB) in a one step solution as described by the manufacturer (Pierce, Rockford, IL).

2.3 Isolation of rVP2-SVPs and transmission electron microscopy - *SVP*s were isolated from yeast cultures expressing recombinant VP2 according to a modified protoplasting protocol [18] to remove the yeast cell wall. The cells were lysed by three freeze thaw cycles then sonicated for five 60-second cycles with 20-second intervals. Lipids were removed by performing two successive Freon extractions. SVPs were then purified by passing them through a 26% sucrose cushion at 82,705 x g (average) for 4 hours at 4ºC in a swinging bucket rotor (Beckman SW28), followed by CsCl-gradient centrifugation overnight at 115,584 x g (average) at 4ºC in a swinging bucket rotor (Beckman SW41). The buoyant density of IPNV is 1.33 g/cm^3. Bands were withdrawn with a syringe and dialyzed overnight at 4ºC in TN buffer (50mM Tris and 100 mM NaCl, pH 8.0) to remove CsCl. SVP's were prepared for negative staining transmission electron microscopy according to the previously published protocols [8].

2.4 ***Immunization and sampling of rainbow trout*** **-** Rainbow trout (*Oncorhynchus mykiss*; ~25 g) originating from the Clear Springs Food, Inc. (Buhl, Idaho) and known to be free of IPNV were used for the immunization experiment. The vaccination and animal work was done at Clear Springs Foods, Inc. while the analytical work was performed at Advanced BioNutrition, Inc. The fish were anesthetized and injected intraperitoneally (IP) with 100 µL of vaccine (50 µL of purified rVP2-SVPs containing 100 µg antigen and 50 µL of Freund's Complete Adjuvant). There were three groups of fish: naive fish (n = 9), fish injected with adjuvants only (sham-injected treatment; Freund's Complete Adjuvant, Sigma, St. Louis, Missouri; n = 8), and a treatment group that was injected with IPNV rVP2-SVPs plus Freud's adjuvant (n = 12). Vaccinations were done at days 1 and 32.

For oral vaccination, recombinant yeast expressing rVP2-SVPs (without prior purification) was mixed with feed. Yeast were ground in liquid nitrogen then incorporated into a fish feed (Clear Spring Foods, Inc., proprietary blend) that was first powdered using a coffee mill then supplemented with 10% wheat gluten as binder. Feed blends were mixed by hand with moisture added as required until a pliable dough was produced. This was then fed through a press to produce ribbons of feed that were chopped to approximately 0.5 cm in length. These were allowed to air dry at room temperature for several hours then spray coated with canola oil and frozen until use. The treatments for the oral vaccination include fish that were fed diet containing yeast expressing rVP2-SVPs (n=13) or diet containing non-recombinant yeast (control, n=10). At day 60, blood was withdrawn from caudal vessels of control and vaccinated fish and allowed to clot overnight at 4°C. Blood samples were centrifuged in a tabletop

centrifuge at 12,568 x g (average) for 5 min., then serum was collected and stored at -75°C until analyzed.

2.5 Enzyme-linked immunosorbent assay (ELISA) - Immuno Breakapart microplates (Nunc, Rochester, NY) were coated with purified IPNV rVP2-SVPs at 150 µg/mL in a 50 mM carbonate coating buffer (pH 9.6) at 4ºC for 16 h. Plates were washed 3 times in TBST (1X Tris Buffered Saline (TBS) + 0.05% Tween 20) for 5 min each wash. The plates were blocked with 1X TBS containing 3% BSA at room temperature. Test sera were diluted 1:32 and 1:64 then 150 µL was added per well and the plates were incubated for 1 h at room temperature. Following incubation with test sera, the microplates were washed again 3 times with TBST for 5 min per wash. The secondary antibody (rabbit anti-rainbow trout IgG; Jackson ImmunoResearch Laboratories Inc, West Grove, PA) was diluted 1:1000 and added to all wells (150 µL/well). The plates were incubated for 1 h at room temperature and then washed 3 times in TBST, 5 min each wash. Horseradish peroxidase-conjugated goat anti-rabbit IgG (Biosource, Camarillo, CA) was added at a 1:1000 dilution and detected by addition of the colorimetric substrate tetramethyl benzidine (TMB, Pierce, Rockford, IL). The absorbance was read at 450 nm using a Spectrafluor Plus fluorescent plate reader (Tecan, Salzburg, Austria). Negative controls consisted of wells that were coated as above, but a 3% BSA solution was added instead of the fish serum at the capture step.

2.6 ***IPNV challenge and sample collection*** - Three days after collecting the blood samples (*i.e.*, at 63 days post-vaccination), IPNV challenge was performed by injecting each fish with approximately 250 µL of 10^7 $TCID_{50}$/mL of IPNV (Buhl strain, LaPatra unpublished). Naive fish injected with buffer served as negative control for the IPNV challenge. Ten days post-injection, animals were sacrificed, spleen samples collected in TRI reagent, then stored at -75°C until RNA isolation was performed.

2.7 Isolation of total RNA and cDNA synthesis - Total RNA was isolated from spleen tissue of control and IPNV-injected rainbow trout using TRI reagent following the manufacturer's protocols (Molecular Research Center, Cincinnati, Ohio). The RNA samples were treated with DNase I (Ambion, Inc., Austin, TX) then the RNA quality assessed by running the samples on a 1% formaldehyde agarose gel [23]. 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 1:10 using DNase and RNase free molecular biology grade water and 2 µL of the diluted cDNA was taken for each reaction.

2.8 Determining IPNV load by SYBR Green real-time RT-PCR - The primers for the SYBR Green real-time RT-PCR were designed based on the nucleotide sequence of segment A of the IPNV genome that encodes the protease protein (VP4) (GenBank Accession no. **NC_001915**, forward primer 1916F: 5' AGGAGATGAC ATGTGCTACA CCG3', and reverse primer 1999R: 5'CCAGCGAATA TTTTCTCCAC CA3'). The rainbow trout elongation factor 1-α (EF-1-α) gene was used as an internal control for normalizing the viral load from sample to sample. The primers for rainbow trout elongation factor 1-α (EF-1-α) were based on the published sequence of these genes (GenBank Accession no **AF498320**, forward primer 136F: 5'TGATCTACAA GTGCGGAGGC A3', and reverse primer 236R: 5'CAGCACCCAG GCATACTTGA A3'). 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 BioRad iCycler iQ (BioRad Laboratories, Inc., Richmond, CA).

The SYBR Green real-time RT-PCR mixture 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 microplate with 3 replicates per sample. The thermal profile for SYBR Green real-time RT-PCR was 95°C 10 min, followed by 40 cycles of 95°C 10 sec and 60°C 1 min.

After a SYBR Green 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). The relative IPNV load in a sample was determined by subtracting the mean C_t values for EF-1α from the mean C_t values of the IPNV amplicon. The differences in the C_t value of the viral genes and the corresponding internal controls were expressed as ΔC_t. The ΔC_t values were plotted using GraphPad Version 4 (Graphpad Software, Inc., San Diego, CA). The difference in the ΔC_t for one vaccine group compared to the ΔC_t of the corresponding control was expressed as a $\Delta\Delta C_t$, and $2^{\Delta\Delta Ct}$ represents the difference in viral load between the two treatments.

3. Results and discussion

The IPNV segment A has previously been cloned and expressed in hamster fibroblast cells, BHK-21, under the Semliki forest virus promoter and in insect cells under the polyhedrin promoter (*pol*h) and were shown to produce virus-like particles (VLPs) that contain both VP2 and VP3 and are of similar size to the native virus but lack associated nucleic acid [15], [24]. However, when we cloned the IPNV segment A in yeast, the polyprotein was expressed but no particles were observed under TEM (data not shown). This might be due to the lack of post-translational processing of the polypeptide in yeast. Therefore, we coexpressed VP2 and VP3 genes under different promoters into the pESC-ura vector so that the post-translational processing of the polyprotein would not be required. For clarity in the following discussion, the authors use the term virus-like particle (VLP) to describe viral-derived particles of similar size to the native virus that lack nucleic acid. For particles that are viral-derived and lack nucleic acid but do not have the same size or shape as the native virus the authors use the term sub-viral particle (SVP) to differentiate the two sets of viral-derived particles.

***3.1 Cloning of VP2 and VP3 genes*-** The predicted mature VP2 and VP3 genes were cloned separately behind GAL10 and GAL1 promoters in pESC-ura. Recombinant yeast containing VP2 or both VP2 and VP3 genes were grown under galactose induction then analyzed by western blot analysis to determine if VP2 and VP3 were expressed (Fig. 2). Two bands were observed that corresponded roughly to the molecular weights predicted for VP2 and VP3 in the co-expression system, 54 kDa and 31 kDa respectively (Fig 2, right panel). The immune blots indicated the presence of both VP2 and VP3 in our yeast mutant designed to express both genes when grown under galactose induction.

***3.2 Preparation of SVPs and/or VLPs plus subsequent electron microscopy* -** Using the methods described above, VLP or SVP preparations were prepared on the clones containing both VP2 & VP3 genes. Several areas of high density were observed in the CsCl gradients. The high molecular weight materials pelleted in the ultracentrifuge, and a band of moderate density was observed in the gradient. The moderate density band corresponded to a ~20 nm particle that

contained only VP2 reacting materials (Fig 3). However, 60 nm full sized IPNV virus-like particles, as seen previously in IPNV segment A expression in insect cells [24], were not observed. Similar particles have been previously described for IPNV [10] and are thought to be due to an error in pVP2 processing. Similar particles were also observed and characterized in IBDV [20]. They are formed by 20 VP2 subunit trimers in a T = 1 fashion. VP3 is not involved in their formation. Here, we saw the same thing whether VP2 was expressed in yeast simultaneously with the VP3 gene or alone in yeast. These particles are referred to herein as sub-viral particles (SVPs).

3.3 *Immunization of Rainbow Trout* – Rainbow trout that were free of IPNV were used for a vaccination experiment testing both intraperitoneal injection (IP) with adjuvant and by oral delivery in feed. The rVP2-SVPs were delivered either as purified SVPs (for IP injection) or as crude yeast lysate incorporated into feeds (for oral delivery) to test the antigenicity of these IPNV subunit vaccines in particle form in rainbow trout. The experimental design is outlined in Table 1. To test the ability of rVP2-SVPs to induce anti-IPNV antibody production, the most direct method is to use purified antigen and deliver by injection. Purified rVP2-SVPs were delivered by IP injection with Freud's adjuvant as described in Tables 1 & 2. A booster of the same composition was delivered after 32 days and fish bled at 63 days. All of the injected fish had significantly higher titers of anti-IPNV antibodies than either the naïve or sham-injected controls (Fig. 4A). The naïve fish and the sham-injected fish were not significantly different from each other at the 95% confidence interval when compared using the student's t-test. The purified rVP2-SVP injected fish showed 100% seroconversion (Table 2; Fig. 4A). Student's t-tests were run in Statview Version 5.01 (SAS Institute, Inc.), testing for significant differences between antibody titers of vaccine injected or fed animals compared to both the naïve fish and sham-injected fish (negative controls). At the 1:32 serum dilution, the rVP2-SVP injected fish had a significantly higher seroconversion rate when compared to the naïve fish ($p = 0.013$) and the sham-injected fish ($p = 0.001$). The 1:64 serum dilution also demonstrated significant seroconversion differences between rVP2-SVP injected fish and negative controls ($p = 0.0003$, naïve fish and $p = 0.0007$, sham-injected fish).

Oral vaccination would provide a number of advantages over injection such as ease of use, ability to vaccinate smaller fish, lower cost of vaccine, and easy ability to make multivalent vaccines (through delivery of different clones in the feeds). In order to test the ability of rVP2-SVPs to induce an immune response, recombinant yeast expressing VLPs were incorporated into fish feed and fed to one treatment group for seven days. At day 32 another seven day feeding of the recombinant yeast containing feed was done as a booster (Table 1). At 63 days the fish were bled and the anti-IPNV titers compared to that found in naïve fish and fish fed a control feed supplemented with wild-type yeast in place of the recombinant yeast (Fig. 4B). It was apparent that the orally vaccinated fish had an immune response greater than that observed in either naïve or yeast control fed fish ($p = 0.0002$ for naïve fish and $p = 0.0053$ for yeast control). There appeared to be a higher anti-IPNV titer in the yeast control sera than in the naïve fish, but the difference was not significant ($p = 0.1645$) as determined by the student t-test. Seroconversion of the orally vaccinated fish was slightly less than that observed in the IP injected animals with approximately 75% conversion (Table 2). Oral vaccination with rVP2-SVPs provides an increase, albeit reduced relative to IP injection, in anti-IPNV titer.

While these data do not demonstrate the effectiveness of these vaccination strategies on prevention of disease, they are an indication that oral vaccination could potentially provide an

alternative to IP injection vaccination for the treatment of IPN. A challenge trial would provide definitive evidence that this approach could prevent disease.

3.4 IPNV Challenge/Viral Load- There is no good challenge system for IPNV with mortality as the endpoint [1]. Using IPNV viral load, as determined by real-time RT PCR, could provide a convenient method to track the progress of the disease. In this study, the trout were vaccinated with either rVP2-SVPs delivered in feed or by injection of purified rVP2-SVPs derived from the West Buxton strain of IPNV. After 63 days post-vaccination, fish were injected with the Buhl strain of IPNV that had been isolated from rainbow trout in Idaho (La Patra, unpublished data). This was a different IPNV strain (Buhl) than that from which the rVP2-SVPs vaccine was derived (West Buxton strain). Therefore, the challenge was with a heterologous strain and may help evaluate the specificity of this approach. IP vaccinated rainbow trout had significantly less virus ($p = 0.0280$) (22 fold) than sham-injected control fish (Table 3, Fig. 5). When oral vaccinates were compared to the yeast only controls, a 12-fold reduction in virus was found for IPNV vaccinated fish (Fig. 5B). This difference was visually apparent, but not significant at the 0.05 level ($p = 0.1179$).

These data indicate that rVP2-SVPs produced in yeast could provide a novel means for amplification of a protective immune response in rainbow trout, and by extension to salmonid species like salmon, either by injection or by delivery in feeds. Expression of a rVP2-SVP particle in yeast provides an interesting opportunity for its use as a vaccine for trout and salmon. The ability of these particles to induce the production of IPNV-specific antibodies was demonstrated by both oral and injection routes. The potential for use of the oral route as a vaccine needs further investigation to optimize the immune response and determine if the observed decrease in viral load directly correlates with prevention of IPN. This study sets the foundation for further studies to test in juvenile salmonids the utility of this approach to prevent early onset of IPN.

4. Acknowledgements

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Table 1. Experimental design for vaccination trial on rainbow trout with IPNV rVP2-SVPs delivered by either intraperitoneal injection (IP) or orally in feeds.

Treatment	**Vaccine delivery (Injection/ feeding)***		**No. of Animals/ Treatment**
	Initial (Day)	**Booster (Day)**	
Naïve fish	None (Day 1)	None (Day 32)	9
Injection Control	Injection (Day 1)	Injection (Day 32)	8
Injected rVP2-SVPs	Injection (Day 1)	Injection (Day 32)	12
Control Yeast	Oral (Days 1-7)	Oral (Days 32-38)	10
Oral rVP2-SVPs Yeast	Oral (Day 1-7)	Oral (Days 32-38)	13

Table 2. Effect of intraperitoneal or oral vaccination with IPNV rVP2-SVPs on the titer of anti-IPNV antibodies in rainbow trout.

Treatments*	Vaccine delivery	Serum Dilution	Mean A_{450} value $\pm$ SEM	Seropositives/ Total Number**
Naive fish	None	1:32	0.263 $\pm$ 0.022	0
		1:64	0.235 $\pm$ 0.023	0
Adjuvant Control	Injection	1:32	0.363 $\pm$ 0.049	0
		1:64	0.232 $\pm$ 0.037	0
rVP2-SVPs	Injection	1:32	0.982 $\pm$ 0.128	12 of 12
		1:64	0.701 $\pm$ 0.090	12 of 12
Control yeast	Oral	1:32	0.346 $\pm$ 0.035	0
		1:64	0.295 $\pm$ 0.026	0
rVP2-SVPs Yeast	Oral	1:32	0.530 $\pm$ 0.045	10 of 13
		1:64	0.414 $\pm$ 0.034	9 of 13

*Naive fish were not injected and were fed normal fish feed, adjuvant control fish were IP injected with buffer and adjuvant, rVP2-SVPs fish were injected with 100 μg of antigen plus adjuvant, control yeast fish were fed fish feed supplemented with wild-type yeast, and rVP2-SVPs yeast fish were fed fish feeds containing the recombinant yeast.

**Fish considered seropositive if A_{450} was above the mean adjuvant control plus one standard error.

Table 3. Relative quantification of IPNV load by real-time RT-PCR in rVP2-SVP vaccinated rainbow trout.

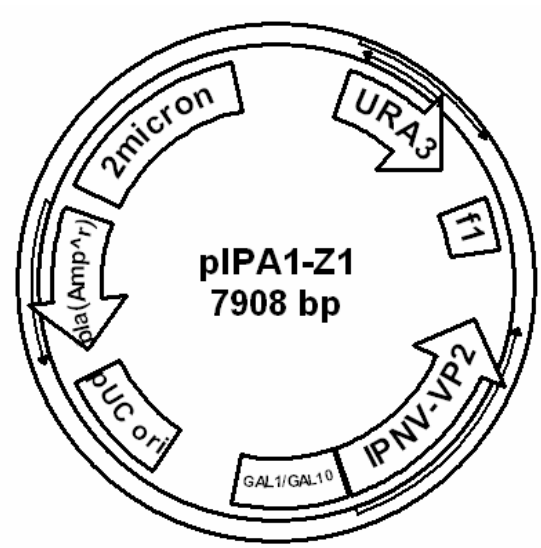
Treatments	Vaccine Delivery Injection/Feeding		Average ΔCt *	ΔΔ Ct**	IPNV Fold reduction ($2^{\Delta\Delta Ct}$)
Adjuvant control	Injection (Day 1)	Injection (Day 32)	9.27		
rVP2-SVPs	Injection (Day 1)	Injection (Day 32)	13.75	4.49	22.40
Control Yeast	Oral (Days 1-7)	Oral (Days 32-38)	5.22		
rVP2-SVPs Yeast	Oral (Days 1-7)	Oral (Days 32-38)	8.83	3.61	12.25

*****ΔCt was first calculated for each fish using the Ct values of IPNV for a fish minus the Ct values of EF-1 alpha gene for the same fish. Then the average ΔCt was calculated taking the Ct value of all the fish in each treatment.

**ΔΔ Ct = Average ΔCt value of a treatment minus the average ΔCt value of the corresponding control treatment

Figure 1. pESC-ura expression vector maps containing IPNV genes VP2 and VP3. Panel A illustrates VP2 under the GAL 10 promoter and panel B displays VP2 and VP3 under the GAL 10 and GAL 1 promoters, respectively.

(A) VP2



(B) VP2 and VP3

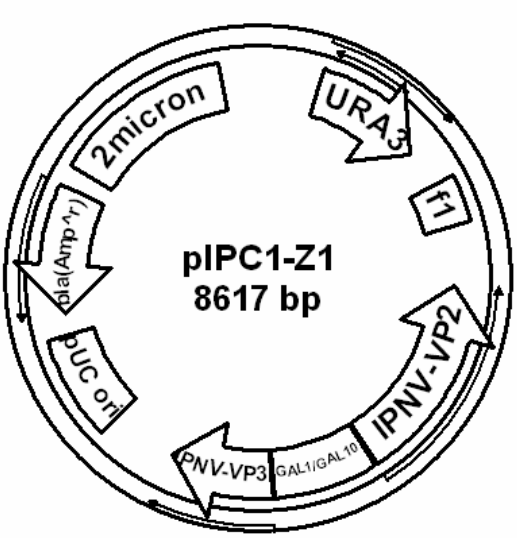


Figure 2. SDS-PAGE and Immunoblot analysis of crude yeast lysates from recombinant yeast clones containing the IPNV VP2 gene or VP2 and VP3 genes. The left hand panel is a Coomassie blue stained gel of IPNV infected CHSE cell culture supernatant (+ control, lane 1), and Y-PER extracted total yeast protein from the two clones expressing VP2 and VP2 + VP3 (lanes 2 and 3). The right hand panel shows the immunoblot of the same samples probed with IPNV polyclonal antibody. The molecular weights of VP2 and VP3 are 54 kDa, and 31 kDa shown by the two arrows. The VP3 band in the positive control (lane 1) was detected at a very low level and is therefore not visible in the scanned photograph.

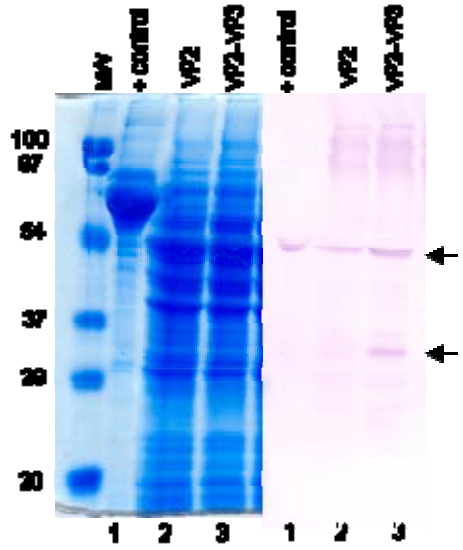


Figure 3. Transmission electron micrograph of cesium chloride gradient purified rVP2-SVPs negatively stained with sodium phosphotungstate. Marker bar, 40µm.

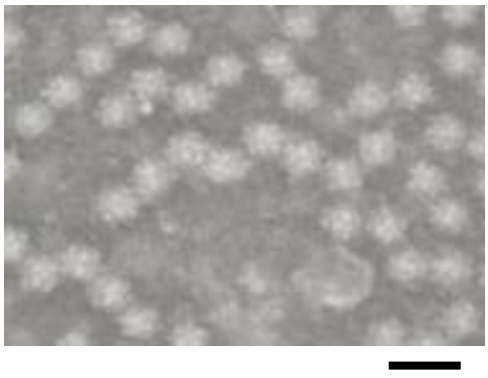
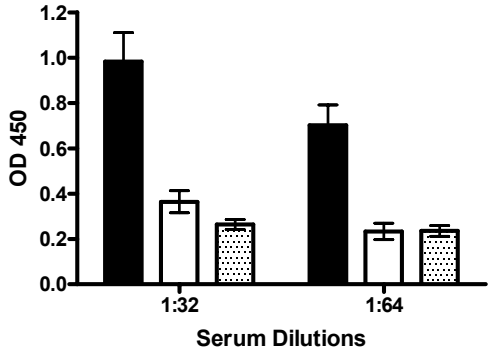


Figure 4. Mean ELISA values (expressed as the absorbance of the HRP substrate TMB at A_{450}) of serum from responding fish following immunization with IPNV rVP2-SVPs. Panel A represents fish injected with purified rVP2-SVPs by intraperitoneal injection. The treatments for the intraperitoneally vaccinated group include fish vaccinated with rVP2-SVPs (■, n=12), adjuvant only control fish (□, n=8), and naïve (unimmunized) fish (⊡, n=9). Panel B represents fish vaccinated orally with yeast expressing rVP2-SVPs. The treatments for the oral vaccinated group include fish fed diets containing recombinant yeast expressing rVP2-SVPs (▩, n= 12), non recombinant yeast (▨, n=11), and naïve (unimmunized) fish (⊡, n=9). The error bars represent 1 SEM.

A. IP vaccinated



B. Orally vaccinated

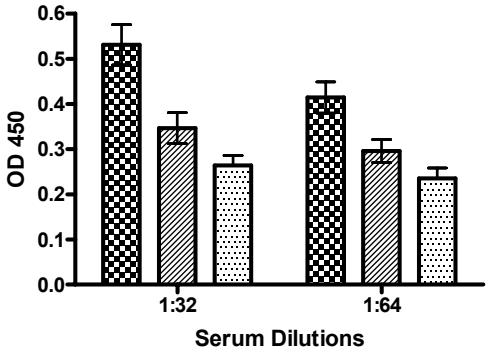
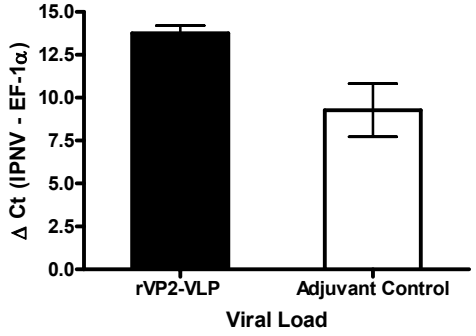
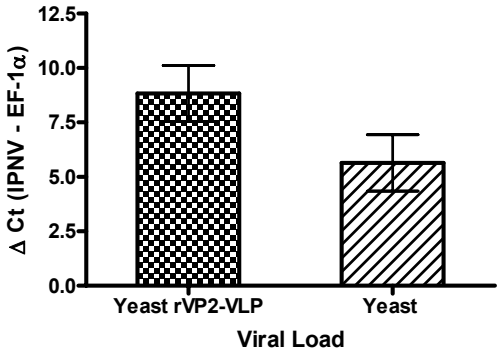


Figure 5. The relative load of IPNV in spleen tissue of vaccinated and unimmunized rainbow trout as determined by SYBR Green real-time RT-PCR. Panel A represents IPNV load in rVP2-SVP injected (■, n=12), and adjuvant injected (□, n=8) rainbow trout. Panel B represents the IPNV load in rainbow trout that were orally vaccinated (diet containing yeast expressing rVP2-SVPs) (▩, n= 12), or control fish (diet containing yeast only) (▨, n=11). The IPNV load was normalized with respect to rainbow trout EF-1-α expression. The ΔC_t values are inversely correlated to IPNV copy number. Therefore, lower the ΔC_t value higher the IPNV load. The error bars represent 1 SEM.

A. IP Vaccinated



B. Orally Vaccinated



Title of the manuscript: Detection and Quantification of Infectious Pancreatic Necrosis Virus by Real-Time RT-PCR Using Invasive and Non-Invasive Tissue Sampling

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ABSTRACT

Infectious pancreatic necrosis virus (IPNV) is a major viral pathogen of wild and cultured salmonids. A highly sensitive and specific real-time RT-PCR assay using the fluorogenic dye SYBR® Green I was developed for the detection and quantification of IPNV in rainbow trout (*Oncorhynchus mykiss*). Rainbow trout were experimentally infected with IPNV via an injection challenge and an immersion challenge. Pectoral fin, spleen, and head kidney tissue samples were collected from the injection challenged fish at 10 days post-challenge, and from the immersion challenged fish at 1, 3, 7, 14, 21, and 28 days post-challenge. Real-time RT-PCR was performed using primers based on the IPNV nonstructural protein gene, designated as either NS or VP4. Using real-time RT-PCR, the virus was successfully detected in pectoral fin, spleen, and head kidney tissue samples as early as 24 hours post-challenge. The melting temperature of the amplicon (Tm = 83 $^{\circ}$C), the amplicon size, and nucleotide sequence confirmed the specificity of the product. 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 revealed that the average IPNV 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 as a non-invasive tissue source for the early detection of IPNV.

Key words: IPNV, real-time RT-PCR, SYBR Green I, non-invasive virus detection

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 (40). 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 (17). This virus is prevalent in salmonid hatcheries around the world, from the Americas to Europe, Asia, and South Africa (15). IPNV is highly contagious and transmitted through a variety of routes including contaminated water (21), 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 (33). 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 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, containing one 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 (7). The Segment A encoded polyprotein also contains a 15 kDa non-structural VP5 protein, which is found only in infected cells (21). 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 (8).

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 (15), flow cytometry, immunofluorescense, immunoperoxidase, immunodot-bloting, immunostaphylococcus-protein A (35), and enzyme-linked immunosorbent (ELISA) assays (24). Virus isolation in cell culture continues to be the gold standard for IPNV detection (26). 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-transcription polymerase chain reaction (RT-PCR) (2, 35) and *in situ* hybridization (ISH) (1) were developed for the detection of IPNV; both are rapid and more sensitive than cell culture assay. However, RT-PCR and ISH still rely on the lengthy propagation of virus in susceptible cell lines (2), which is the major time component of these assays. In a study by Saint-Jean and colleagues (35), 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 hours post-challenge in CHSE-214 cells. RT-PCR was the only diagnostic test of the six tests evaluated to accurately detect IPNV at the lowest IPNV infective titer of 10 Tissue Culture Infective Doses 50% ($TCID_{50}$)/mL. Alonso and colleagues (2004) 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). 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 diagnostics in human, plant, and terrestrial agricultural species are gradually being applied 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 diagnostics in fish and shellfish aquaculture. Real-time PCR was developed in 1992 (13, 14) and quickly became a major tool for gene expression studies, and more recently, for disease diagnostics (3, 20, 25). The simplicity, sensitivity, dynamic range of detection, reproducibility, and amenability to high throughput screening makes real-time PCR an attractive tool for viral detection (20, 25). 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 (20). 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 (34). Real-time PCR assays employing either SYBR® Green I (5, 6) or TaqMan probes (4, 9, 11, 19, 23, 28, 29, 31, 37, 38) 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 quantification 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 not only as a non-invasive sampling method for IPNV screening in aquaculture operations, but also for epidemiological studies in wild populations of salmonids.

MATERIALS AND METHODS

Virus challenge and sample collection

IPNV challenge by injection - Rainbow trout (*Onorhynchus mykiss*) (average weight ~50 g) that originated from the Clear Springs Foods, Inc. (Buhl, Idaho) Research Hatchery were known to be free of IPNV, and were used for subsequent IPNV challenge work. Each fish (n = 13) was injected intraperitoneally with approximately 250 µL of a stock virus lysate of IPNV (Buhl strain) with a titer of ~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.

IPNV challenge by immersion - Rainbow trout (mean weight, ~3 g) were challenged with IPNV *via* the waterborne route. A total of 36 fish were challenged in a volume of water that was 10X the total biomass for 1 hour with aeration (~10^5 $TCID_{50}$/ ml of IPNV). Six fish were collected at 1, 3, 7, 14, 21, and 28 days post challenge. Spleen, head kidney, and pectoral fin tissues were sampled from each fish immediately after removal from the tank and stored in TRI reagent at -75°C until RNA isolation was performed. Six additional fish were sacrificed prior to the IPNV

immersion challenge. The same tissue samples were collected and subsequently served as the time 0 hour (uninfected) control.

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, Ohio). 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 42^0C for 1 hour. 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.

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 1-α (EF-1-α), 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 3 replicates. The thermal profile used for the SYBR® Green real-time RT-PCR was 95°C for 10 minutes, followed by 40 cycles of 95°C for 10 seconds, and 60°C for 1 minute. After the run, the melting curve of each amplicon was examined to determine the specificity of the amplification.

Standard curve

The real-time RT-PCR amplicon from spleen tissue of an IPNV infected fish was amplified using the primers 1916F and 1999R, 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 CG3'). The PCR amplified product was run in a 2% agarose gel, gel-purified using the QIAQuick gel-extraction kit (Qiagen, Valencia, CA), 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, *Bam*H1 plasmid DNA was used as template to perform *in vitro* transcription using the MEGAscript High Yield transcription Kit (Ambion, Austin, TX). 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.

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 Ct 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.

RESULTS AND DISCUSSION

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 83ºC (Fig. 1). The optimized primer concentrations were 300 nM for both forward and reverse primers. The amplicons derived from the IPNV NS gene, rainbow trout β-actin, and EF-1-α genes were gel-purified and sequenced. The nucleotide sequence of the cDNAs showed 100% similarity with the corresponding GenBank entries (IPNV **NC_001915**; 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).

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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 – 5.2% for IPNV, 0.2 – 8.4% for β-actin, and 0.3 – 9.2% for EF-1α amplicons (Table 2). 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.

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 (39). 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 and Table 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) (18) 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 authors 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 (16, 27, 37) 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.

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 5 times and the mean A_{260} value was taken to calculate the RNA concentration. A ten-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 5 standard curves, run on 3 separate plates, using cDNAs derived from 5 independently prepared dilution series demonstrated that this real-time RT-PCR assay could consistently detect as few as 10 RNA copies (mean Ct value range 31.14 – 32.69, Table 3, Fig. 3). A linear relationship was observed between 10^7 and 10 *in vitro* transcribed IPNV RNA equivalents (r^2 = 0.999, average slope = -3.406 for the 5 standard curves) as shown in Figure 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 (6, 11, 31, 38). 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.

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 mean viral load reached the highest level in spleen tissue (2.9 x 10^5 copies / μg of total RNA) followed by pectoral fin (1.1 x 10^3 copies / μg of total RNA) and head kidney (5.3 x 10^2 copies / μg of total RNA; Fig. 4A). The viral load ranged from 1.2 x 10^2 to 3.4 x 10^6 copies in spleen, 39 to 3.7 x 10^3 copies in pectoral fin, and 31 to 5.9 x 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-invasive tissue sampling for the detection of the virus by real-time RT-PCR.

In the IPNV immersion challenge experiment, tissue samples were collected at 1, 3, 7, 14, 21, and 28 days post-challenge with 6 fish sampled per time point. Although different fish were sampled at each time point, the average viral load in all three tissues 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 x 10^3 to 1.4 x 10^5 copies per μg of total RNA (average = 3.2 x 10^4 copies), 1.3 x 10^2 to 8.4 x 10^6 copies (average = 1.5 x 10^6 copies) in spleen, and 4.2 x 10^3 to 1.7 x 10^5 copies (average = 4.1 x 10^4 copies) in head kidney. Although the average viral load was highest in spleen at 21 days, load values were highly variable in this tissue. 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 Figure 5. The dissociation curves indicated that the melting temperature (T_m) of the IPNV amplicons generated from pectoral fin, spleen, and head kidney are the same (Tm = 83°C) indicating the specificity of the products (Fig. 5, Column 2). The agarose gel electrophoresis photographs of the amplicons exhibited a single band at the expected size (~100bp, Fig5, Column 3).

It was interesting to note that IPNV could be detected as early as 24 hours 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 possibility of

using pectoral fin as a non-invasive tissue for the early detection of IPNV without the need of cell culture for virus propagation.

Detection methods utilizing nested PCR (1) or RT-PCR with end-point detection (2, 22, 35) 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-invasive detection method will circumvent this need. Recently, Harmache and colleagues (12) 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 (30), 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., unpublished), we decided to examine if IPNV could be detected in pectoral fin 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 hours 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-invasive 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 (32). 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-invasive sampling strategies could be very useful for IPNV screening in aquaculture operations and for epidemiological studies in wild populations of salmonids.

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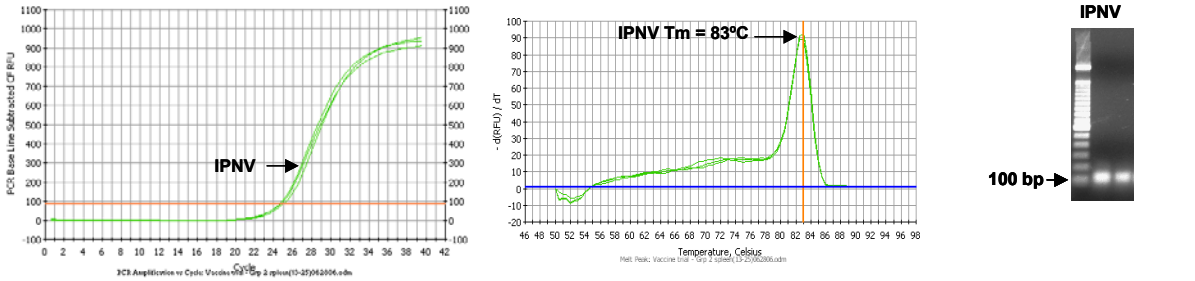
Figure 1. Amplification profiles (Column 1), dissociation curves (Column 2), and agarose gel electrophoresis photographs (Column 3) of the IPNV NS gene (77 bp, Row A), trout β-actin (112 bp, Row B), and EF-1α (100 bp, Row B) genes generated by SYBR Green® real-time RT-PCR using cDNAs derived from spleen tissue of an IPNV injected rainbow trout.

1 **2** **3**

A.



B.

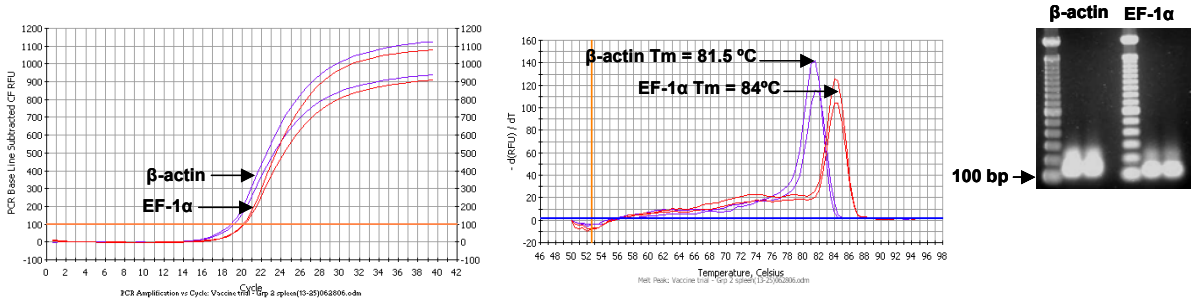
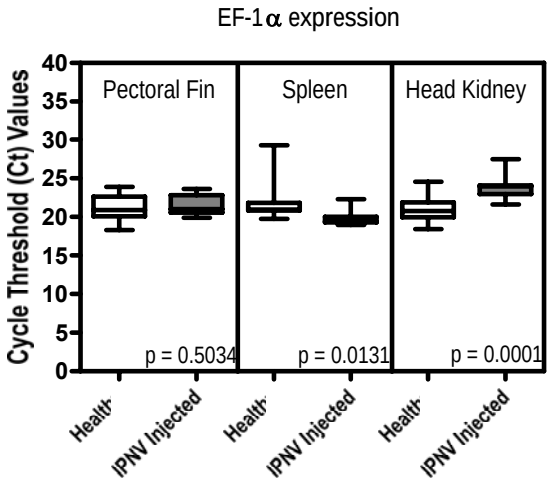


Figure 2. Box plot analysis of rainbow trout internal control genes, EF-1α (A) and β-actin (B) from different tissues of healthy (N=12) and IPNV injected (N=13) rainbow trout. The *P* values correspond to an unpaired student's t-test run for each tissue type between healthy and IPNV injected rainbow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

A.



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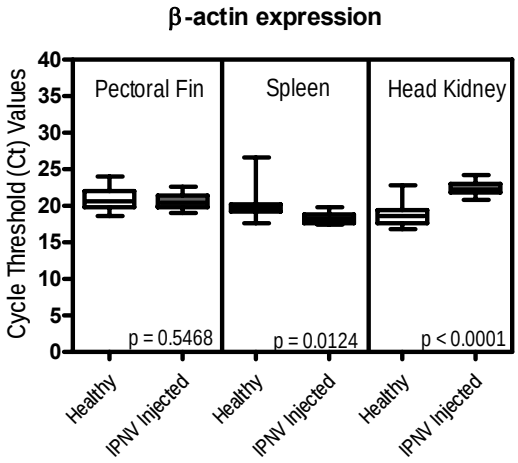


Figure 3. IPNV standard curve generated using a 10-fold dilution series (1 x 10^7 to 10 cDNA/RNA equivalents per 25 µl reaction) of *in vitro* transcribed IPNV RNA. The standard curve represents the mean standard curve generated from five independently run RNA dilution series. Slope = -3.41, *Y*-intercept = 35.47, R^2 = 0.9994.

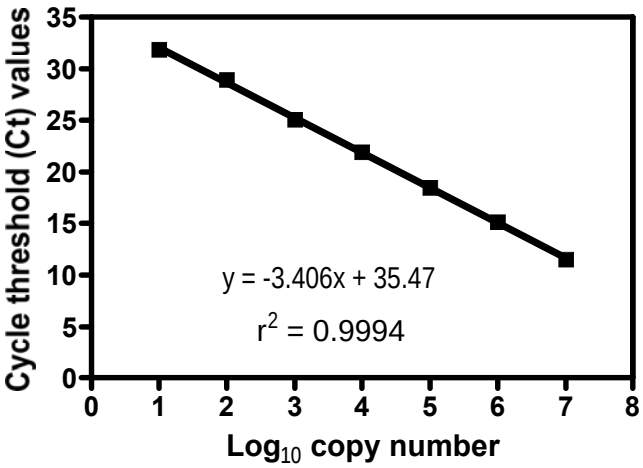
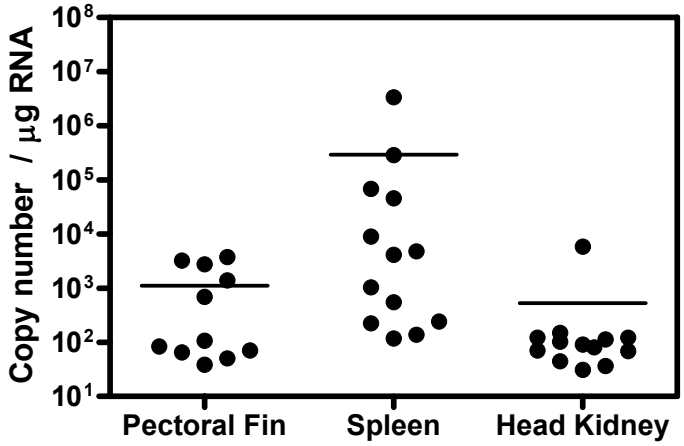


Figure 4. Dot plot analyses representing the IPNV load in pectoral fin, spleen, and head kidney samples of rainbow trout. Panel A represents fish (N=13) challenged in the laboratory via injection and samples collected at 10 days post-injection. Panel B represent fish from an IPNV immersion challenged 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.

A. IPNV Injection challenge



B. IPNV Immersion challenge

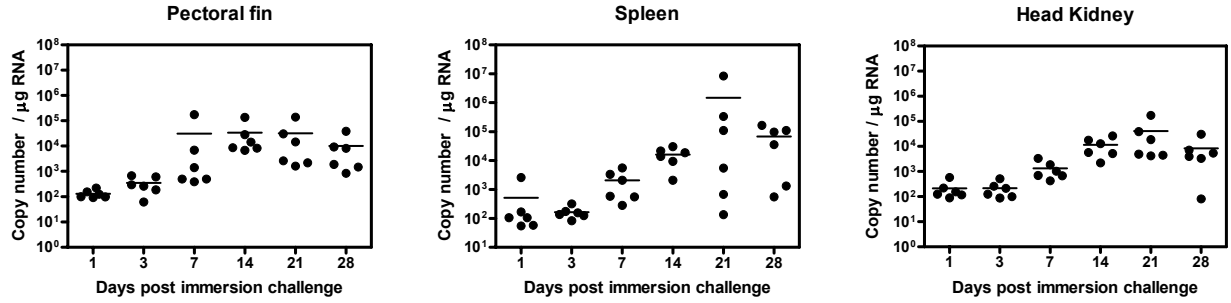


Figure 5. Amplification profiles and dissociation curves of IPNV NS gene generated by SYBR Green® real-time RT-PCR using cDNAs derived from different tissues (pectoral fin, spleen and head kidney) of an immersion challenged rainbow trout collected at 21 days post-challenge. Amplification profiles (Column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

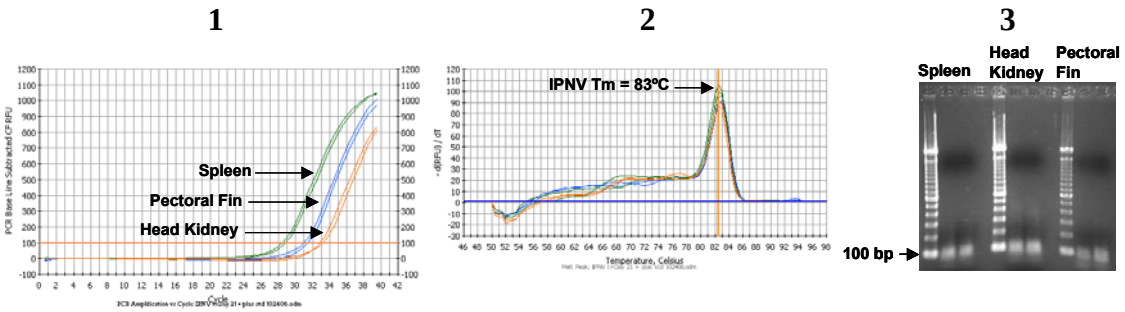


Table 1. List of primers used for the real-time RT-PCR assay for the detection and quantification of infectious pancreatic necrosis virus (IPNV).

Gene	Primer Name	Primer Sequence (5'-3')	%GC	T_m*	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	

*Melting temperature done at 50 mM Na^+.

Table 2. Assay to assay reproducibility of the SYBR® Green I real-time RT-PCR method for detection and quantification of IPNV and internal control genes, rainbow trout β-actin and EF-1α in IPNV injected rainbow trout. Samples 13-25 were amplified in three separate real-time RT-PCR reactions. Missing values (ND) from the IPNV amplicon data correspond to samples that failed to show the presence of IPNV through the melting peak profile.

Amplicon	Tissue	Sample #	C_t Values Expt 1	Expt 2	Expt 3	Mean	SD	CV (%)
IPNV	Spleen	13	30.78	31.29	31.04	31.04	0.255	0.8
		14	35.31	34.90	36.50	35.57	0.831	2.3
		15	29.84	30.74	32.22	30.93	1.202	3.9
		16	36.26	35.75	37.15	36.39	0.709	1.9
		17	37.15	36.56	38.29	37.33	0.879	2.4
		18	37.46	37.81	37.46	37.58	0.202	0.5
		19	ND	ND	ND	ND	ND	ND
		20	26.84	28.24	28.03	27.70	0.755	2.7
		21	27.43	28.64	28.47	28.18	0.378	2.3
		22	30.99	31.84	32.72	31.85	0.865	2.7
		23	ND	ND	ND	ND	ND	ND
		24	33.03	35.25	35.53	34.61	1.370	4.0
		25	31.48	31.31	31.91	31.56	0.309	1.0
	Head Kidney	13	37.01	37.66	37.01	37.22	0.345	1.0
		14	35.90	36.68	35.62	36.07	0.450	1.2
		15	37.04	37.17	36.29	36.84	0.075	0.2

		16	ND	ND	ND	ND	ND	ND
		17	36.20	37.09	36.54	36.61	0.514	1.4
		18	30.48	30.65	30.00	30.37	0.098	0.3
		19	36.45	37.09	37.04	36.86	0.370	1.0
		20	36.21	35.75	36.30	36.09	0.266	0.7
		21	36.64	35.72	34.82	35.73	0.307	1.5
		22	38.23	37.42	35.86	37.17	0.468	1.2
		23	37.69	37.40	37.51	37.53	0.167	0.4
		24	36.31	38.20	36.90	37.14	1.091	2.9
		25	36.82	38.13	37.51	37.49	0.756	2.0
	Pectoral Fin	13	35.42	36.01	35.90	35.78	0.031	0.9
		14	32.34	31.58	31.99	31.97	0.380	1.2
		15	36.45	36.78	37.58	36.94	0.581	1.6
		16	ND	ND	ND	ND	ND	ND
		17	ND	ND	ND	ND	ND	ND
		18	36.46	34.49	35.38	35.44	0.987	2.8
		19	ND	ND	ND	ND	ND	ND
		20	33.86	34.45	34.09	34.13	0.297	0.9
		21	32.03	31.65	32.03	31.90	0.219	0.7
		22	35.41	35.69	35.65	35.58	0.151	0.4
		23	37.07	37.59	37.29	37.32	0.261	0.7
		24	35.71	35.52	37.01	36.08	0.811	2.2
		25	36.18	32.90	36.45	35.18	1.980	5.6
β-actin	Spleen	13	17.65	17.71	17.79	17.72	0.070	0.4
		14	18.18	18.66	18.81	18.55	0.329	1.8
		15	17.77	18.13	18.19	18.03	0.227	1.3
		16	19.72	17.57	17.65	18.31	1.219	6.1
		17	19.72	19.71	19.84	19.75	0.072	0.4
		18	18.71	18.16	18.23	18.36	0.299	1.6
		19	17.28	17.62	17.75	17.55	0.243	1.4
		20	17.45	17.08	17.24	17.26	0.186	1.1
		21	18.52	18.37	18.34	18.41	0.096	0.5
		22	18.67	18.58	18.62	18.62	0.045	0.2
		23	18.71	17.81	17.92	18.15	0.491	2.7
		24	17.70	18.07	19.73	18.50	1.081	5.8
		25	17.50	18.02	19.82	18.45	1.217	6.6
	Head Kidney	13	20.75	20.07	20.81	20.54	0.441	0.2
		14	19.09	19.00	19.75	19.28	0.410	2.1
		15	21.11	20.66	21.24	21.00	0.304	4.1
		16	20.12	19.66	20.32	20.03	0.338	1.7
		17	19.67	19.31	19.97	19.65	0.330	1.7
		18	19.80	19.49	20.15	19.81	0.330	1.7
		19	19.66	19.62	20.18	19.82	0.312	1.6
		20	19.99	19.89	20.51	20.13	0.333	1.7
		21	21.53	21.52	21.78	21.61	0.147	0.7
		22	19.32	19.17	19.85	19.44	0.357	1.8
		23	20.21	20.08	20.94	20.41	0.464	2.3
		24	18.34	18.37	19.19	18.63	0.482	2.6
		25	19.58	19.58	20.17	19.77	0.341	1.7
	Pectoral Fin	13	17.97	17.91	18.11	17.99	0.103	0.6
		14	19.08	19.10	19.28	19.15	0.110	0.6
		15	19.38	19.57	19.51	19.49	0.097	0.5

		16	19.02	19.10	19.15	19.09	0.066	0.3
		17	20.50	20.66	20.72	20.63	0.114	0.6
		18	20.39	20.46	20.60	20.48	0.107	0.5
		19	20.89	21.05	20.99	20.97	0.081	0.4
		20	18.54	18.81	18.76	18.70	0.144	0.8
		21	20.67	20.77	21.03	20.82	0.186	0.9
		22	19.20	19.23	19.41	19.28	0.114	0.6
		23	21.16	21.30	21.42	21.29	0.130	0.6
		24	19.71	19.78	19.72	19.73	0.038	0.2
		25	19.92	17.28	20.16	19.12	1.598	8.4
EF-1α	Spleen	13	19.31	19.50	19.62	19.48	0.156	0.8
		14	19.31	21.60	21.59	20.83	1.319	6.3
		15	18.95	19.67	19.74	19.45	0.437	2.2
		16	22.25	19.01	19.11	20.12	1.842	9.2
		17	19.34	22.72	19.73	20.59	1.849	9.0
		18	21.18	19.39	19.82	20.13	0.934	4.6
		19	19.85	20.34	20.38	20.19	0.295	1.5
		20	19.38	19.65	19.69	19.57	0.169	0.9
		21	19.14	19.42	19.46	19.34	0.174	0.9
		22	20.20	20.33	20.28	20.27	0.066	0.3
		23	19.29	19.54	19.38	19.40	0.127	0.7
		24	19.46	19.76	19.65	19.62	0.152	0.8
		25	19.69	20.57	20.62	20.29	0.523	2.6
	Head Kidney	13	22.03	21.83	22.57	22.14	0.383	1.7
		14	19.99	19.86	20.37	20.07	0.265	1.3
		15	21.45	21.20	22.28	21.64	0.565	2.6
		16	21.50	21.23	21.91	21.54	0.342	1.6
		17	20.57	20.39	21.04	20.66	0.336	1.6
		18	21.41	21.20	22.12	21.58	0.482	2.2
		19	21.56	21.41	22.44	21.80	0.556	2.6
		20	21.44	21.18	21.97	21.53	0.403	1.9
		21	24.97	24.78	25.64	25.13	0.452	1.8
		22	20.81	20.68	21.37	20.95	0.367	1.7
		23	21.02	21.13	21.51	21.22	0.257	1.2
		24	19.20	19.13	19.79	19.37	0.363	1.9
		25	21.66	21.36	22.03	21.68	0.336	1.5
	Pectoral Fin	13	18.81	18.85	18.95	18.87	0.072	0.4
		14	19.15	19.17	19.15	19.16	0.012	0.1
		15	20.05	20.27	20.05	20.12	0.127	0.6
		16	19.97	19.99	19.95	19.97	0.020	0.1
		17	21.21	21.17	21.12	21.17	0.045	0.2
		18	21.92	22.28	22.10	22.10	0.180	0.8
		19	21.87	21.87	21.84	21.86	0.017	0.1
		20	19.21	19.61	19.35	19.39	0.203	1.0
		21	22.18	22.47	22.33	22.33	0.145	0.6
		22	19.76	19.82	19.73	19.77	0.046	0.2
		23	22.55	22.59	22.58	22.57	0.021	0.1
		24	20.85	20.82	20.83	20.83	0.015	0.1
		25	21.03	19.29	21.10	20.47	1.025	5.0
		25	21.03	19.29	21.10	20.47	1.025	5.0

Table 3. Within sample reproducibility of the IPNV real-time RT-PCR assay using purified *in vitro* transcribed RNA as template.

RNA standard	Replicate Ct values					Mean	SEM
IPNV copy no.	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