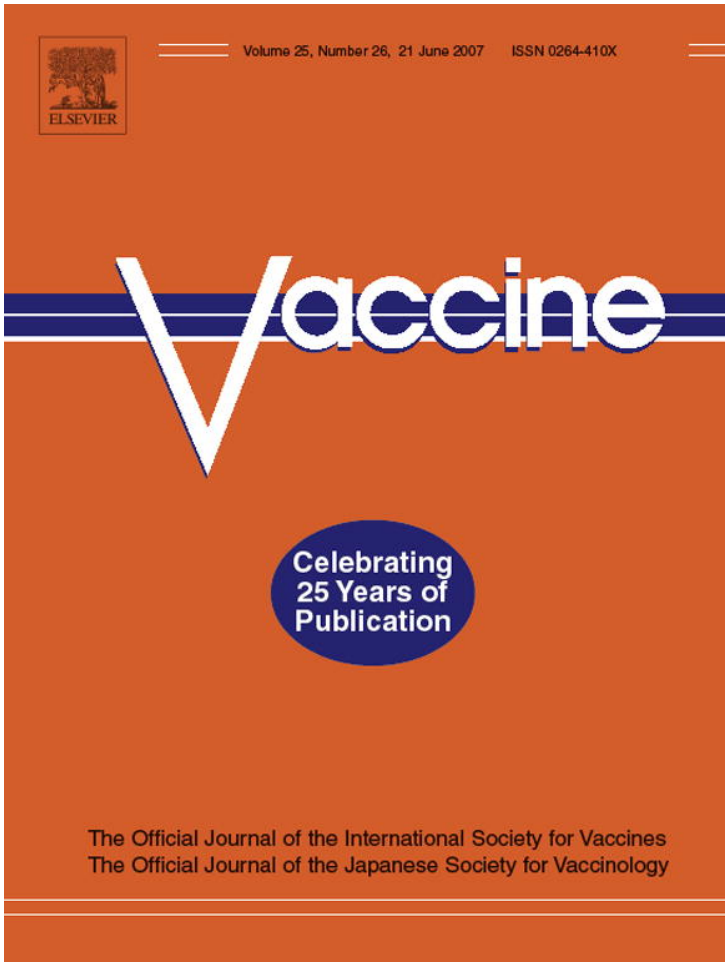

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Antigenicity of infectious pancreatic necrosis virus VP2 subviral particles expressed in yeast

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Abstract

Infectious pancreatic necrosis (IPN) 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 subviral particles composed solely of VP2 protein. Anti-IPNV antibodies were detected in rainbow 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 demonstrated 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.

Keywords: IPNV; VP2 gene; Oral vaccine

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 [23]. This disease is especially destructive in salmonid eggs and fingerlings [27]. 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% [18]. Economic losses due to IPNV in aquaculture were estimated to be over $60 million in 1996 [6,19]. This has been reduced as vaccines for salmonids became available based on killed virus or recombinantly produced viral peptides [15,19]. However, these vaccines are not completely effective and can only be used for fairly large fish due to the reliance on injection for vaccination.

IPNV is a double-stranded RNA virus of the *Birnaviridae* family [7] and is the type species of the Aquabirnavirus genus [8]. 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) [9,13]. Segment A

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(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 [16]. VP2 and VP3 are the major capsid proteins, but the major virus neutralizing epitopes were mapped to VP2 protein [11,14].

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 and 40% of the salmon hatcheries experience an outbreak of IPN [5] 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 of 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. 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 [24]. In fact, Pitcovski et al. [21] reported the development and large-scale use of yeast-derived recombinant Infectious Bursal Disease Virus capsid protein (VP2) based 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 subviral 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 [29]. 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 [25]. 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 [26]. 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 was 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. 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. 1B).

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

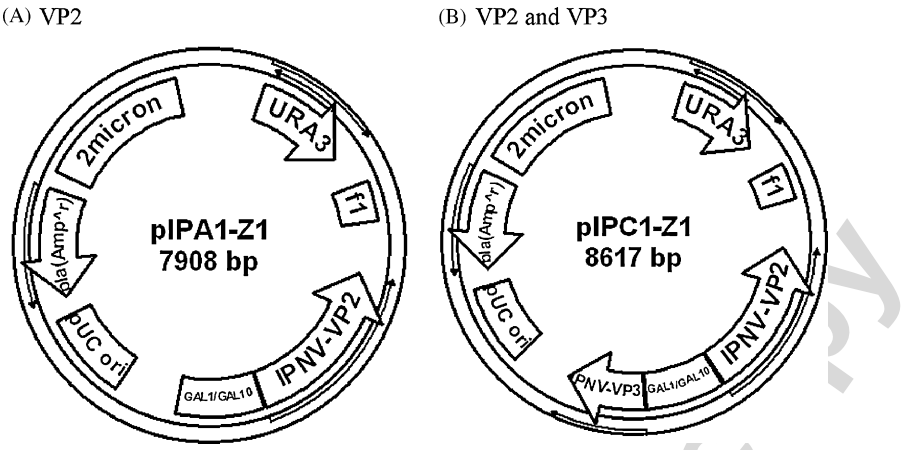

Fig. 1. Plasmid maps for pESC-ura expression vectors containing IPNV genes VP2 and VP3. Panel A illustrates VP2 under the GAL 10 promoter and panel B displays VP2 and VP3 under the GAL 10 and GAL 1 promoters, respectively.

acids, and amino acid dropout mixture (all amino acids plus adenine, no uracil). Mutants were grown at 30 °C for four 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 [20] 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 × *g* (average) for 4 h at 4 °C in a swinging bucket rotor (Beckman SW28), followed by CsCl-gradient centrifugation overnight at 115,584 × *g* (average) at 4 °C 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 4 °C in TN buffer (50 mM Tris and 100 mM NaCl, pH 8.0) to remove CsCl. The cesium chloride gradient purified SVPs were run in a 12% SDS PAGE gel and western immunoblot analysis was performed as described in the Section 2.2. The transmission electron microscopy of the SVPs was performed according to previously published protocols [10].

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 maintained at 15 °C. 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; Sigma Fluka Aldrich, St. Louis, Missouri). There were three groups of fish: naive fish ($n = 9$), fish injected with adjuvants only (sham-injected treatment; Freund's Complete Adjuvant; $n = 8$), 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 a diet containing yeast that had expressed rVP2-SVPs ($n = 13$) or diet containing non-recombinant yeast (control; $n = 10$). At day 63, 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 × *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 4 °C for 16 h. Plates were washed three 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 three 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 three 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. Positive controls consisted of wells coated with purified IPNV rVP2-SVPs and detected using IPNV polyclonal antibody (made against whole IPNV, Strain N1, Microtek International, B.C., Canada).

2.6. IPNV challenge and sample collection

Three days after collecting the blood samples (*i.e.*, at 66 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 [25]. 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 three replicates per sample. The thermal profile for SYBR Green real-time RT-PCR was 95 °C for 10 min, followed by 40 cycles of 95 °C for 10 s and 60 °C for 1 min.

After 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. These particles are of similar size to the native virus but lack associated nucleic acid [17,26]. 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 (Fig. 1B) 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 subviral 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 (Fig. 1). Recombinant yeast containing VP2 or both VP2 and VP3 genes were grown under galactose induction then analyzed by western immunoblot 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 and 31 kDa respectively (Fig. 2, right panel). The immunoblots indicated the presence of both VP2 and VP3 in the yeast mutant (pIPC1-Z1) 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

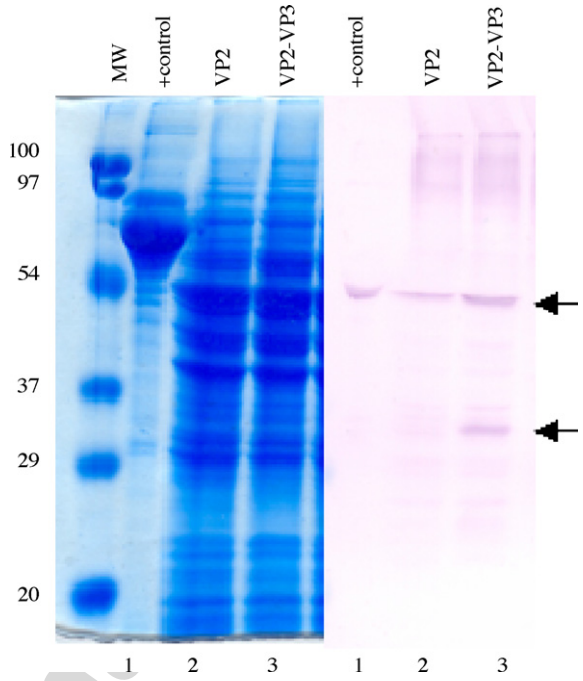

Fig. 2. SDS-PAGE and immunoblot analysis of crude yeast lysates from recombinant yeast clones containing the IPNV VP2 gene or VP2 and VP3 genes. The left hand panel is a Coomassie blue stained gel of IPNV infected CHSE cell culture supernatant (+control, lane 1) and Y-PER extracted total yeast protein from clones expressing VP2 (lane 2) and VP2 + VP3 (lane 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 and 31 kDa, respectively 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.

CsCl gradients. The high molecular weight materials pelleted in the ultracentrifuge, and a band of moderate density was observed in the gradient. The SDS PAGE electrophoresis and western blot analysis of this preparation are shown in Fig. 3A.

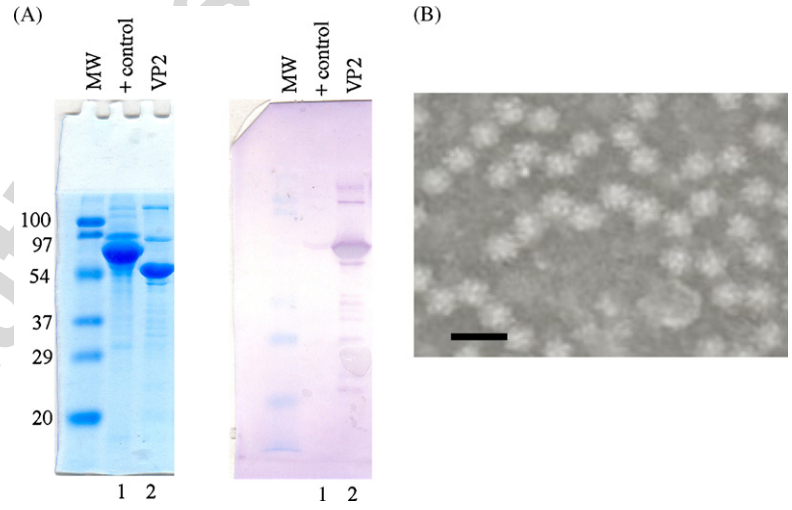

Fig. 3. (A) SDS-PAGE and Immunoblot analysis of purified IPNV SVPs expressed in yeast. The left hand panel shows a Coomassie blue stained gel of the cesium chloride gradient purified preparation (VP2), IPNV infected CHSE cell culture supernatant (+control). The right hand panel shows the immunoblot of the same samples probed with IPNV polyclonal antibody. The molecular weight of VP2 is 54 kDa. (B) Transmission electron micrograph of cesium chloride gradient purified VP2-SVPs negatively stained with sodium phosphotungstate. Marker bar = 40 μm.

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)[a]		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

[a] 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 feed containing the recombinant yeast.

A 54 kDa band corresponding to the molecular mass of the VP2 protein was detected by anti IPNV antibody (Fig. 3A). Transmission electron microscopy of the moderate density band corresponded to a ~20 nm particle that contained only VP2 reacting materials (Fig. 3B). However, 60 nm full sized IPNV virus-like particles, as seen previously in IPNV Segment A expression in insect cells [26], were not observed. Similar particles have been previously described for IPNV [12] and are thought to be due to an error in pVP2 processing. Similar particles were also observed and characterized in IBDV [22]. They are formed by twenty 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 subviral 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-SVP subunit vaccines 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 and 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 improved 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

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

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

[a] 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 feed containing the recombinant yeast.

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

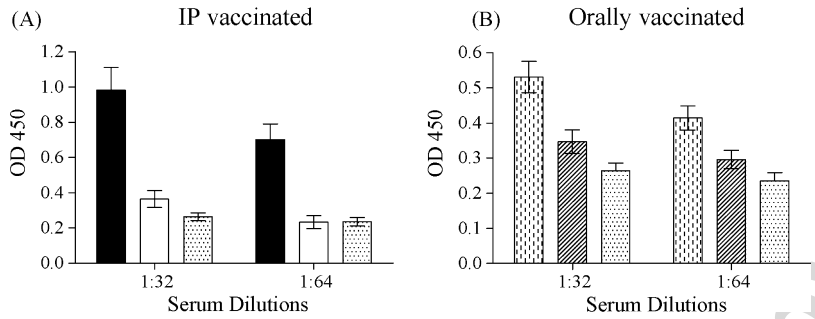

Fig. 4. Mean ELISA values (expressed as the absorbance of the HRP substrate TMB at A_{450}) of serum from responding fish following immunization with IPNV rVP2-SVPs. Panel A represents fish injected with purified rVP2-SVPs by intraperitoneal injection. The treatments for the intraperitoneally vaccinated 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.

expressing SVPs 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 carried out to act 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

An experimental cohabitation IPNV challenge model for post-smolts Atlantic salmon (*Salmo salar)* has recently been reported by Bowden et al. [3,4]. The model involves cohabiting IPNV injected and healthy fish together in the same tank and then scoring the mortality as the disease progress. The mortality reached a peak (approximately 60–70%) by 24 days in both groups irrespective of the density of the fish in the tank [4]. However, this model has not been tested in rainbow trout although it is likely that a similar challenge model might work in rainbow trout as much as it works in Atlantic salmon. Since we used rainbow trout in our experiment and an IPNV challenge model similar to post-smolts Atlantic salmon has not been optimized for rainbow trout, we performed the virus challenge by injecting the vaccinated and unvaccinated fish with IPNV and measured the viral load by real-time RT-PCT ten days post-injection to determine the efficacy of the vaccine in reducing the IPNV load. The high throughput nature of kinetic RT-PCR allows to monitoring the progress of the disease and measure the efficacy of the vaccine in real-time.

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

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

Treatments	Vaccine delivery (injection/feeding)		Average ΔC_t[a]	$\Delta\Delta\ C_t$[b]	IPNV Fold reduction ($2^{\Delta\Delta\ Ct}$)
IP Vaccination					
Adjuvant control	Injection (Day 1)	Injection (Day 32)	9.27		
rVP2-SVPs	Injection (Day 1)	Injection (Day 32)	13.75	4.49	22.40
Oral vaccination					
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

[a] ΔC_t was first calculated for each fish using the C_t values of IPNV for a fish minus the C_t values of EF-1 alpha gene for the same fish. Then the average ΔC_t was calculated taking the C_t value of all the fish in each treatment.

[b] $\Delta\Delta\ C_t$ = Average ΔC_t value of a treatment minus the average ΔC_t value of the corresponding control treatment.

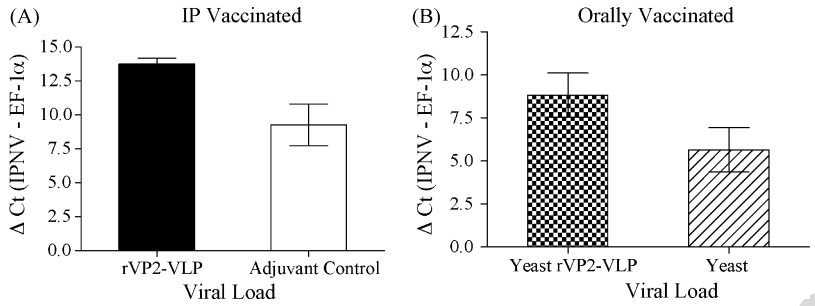

Fig. 5. The relative load of IPNV in spleen tissue of vaccinated and unimmunized rainbow trout as determined by SYBR Green real-time RT-PCR. Panel A represents IPNV load in rVP2-SVP injected (■, $n = 12$), and adjuvant injected (□, $n = 8$) rainbow trout. Panel B represents the IPNV load in rainbow trout 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.

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. IPNV is quite heterogenous and some degree of serological cross reactivity exists among IPNV isolates [28]. Therefore, it was not surprising to see that fish vaccinated with SVPs derived from West Buxton strain showed reduction in viral load when challenged with a heterologous strain, the Buhl Strain. 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 not significant at 0.05 level ($p = 0.1179$), which might be due to the variation in the levels of viral load reduction in individual fish.

It is not known if the recombinant SVPs expressed in yeast had altered epitopes. In the ELISA tests, the SVPs (positive control wells) were detected using IPNV polyclonal antibody (made against whole IPNV, Strain N1, Microtek International, B.C., Canada). This indicated that the even if some epitopes were modified in the SVPs, it could be detected using polyclonal antibody against whole IPNV. This implies that yeast expressed IPNV rVP2-SVPs are antigenic in fish.

The protective immune mechanisms to IPNV are not known. However, our ELISA data (on the antibody titer of the vaccinated fish) and the subsequent demonstration that in the vaccinated fish the IPNV load was approximately 12–25-fold lower (depending on the delivery route of the vaccine; Table 3) than the unimmunized fish, suggest that some sort of protective defense mechanisms are elicited in the vaccinated fish. The defense mechanisms in the vaccinated fish will probably include both virus neutralizing and non-neutralizing antibodies.

These data indicate that rVP2-SVPs produced in yeast could provide a novel means for induction 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 rVP2-SVP particles 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 for vaccination 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 the utility of this approach to prevent early onset of IPN in juvenile salmonids and the potential to provide lasting immunity to the fish during its lifetime.

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