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Vaccines for Infection Salmon Anemia Virus

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VACCINES FOR INFECTIOUS SALMON ANEMIA VIRUS

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An Abstract of the Thesis Presented
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Infectious salmon anemia (ISA) virus is an emerging pathogen of farmed Atlantic salmon. Due to the massive economic losses inflicted by the ISA virus, effective measures to control future outbreaks are necessary. An attractive method for preventing ISA virus from infecting stocks of Atlantic salmon is vaccination. DNA vaccination is a proven cheap, effective means of protecting fish from aquatic viruses. In this thesis DNA vaccination of Atlantic salmon was investigated. Three different strains of ISA virus were cultured and purified by density gradient ultracentrifugation. ISA virus RNA was isolated and used to amplify full-length cDNAs of five ISA virus segments. These segments were cloned into hCMV vectors and recovered using an ammonium acetate precipitation reaction. DNA vaccines containing full-length ISA virus segments were used to vaccinate rainbow trout and Atlantic salmon. The DNA vaccines were tested for their ability to provide cross-protection, persistent immunity, and a humoral immune response. These vaccines were also compared to whole killed ISA virus vaccines and recombinant peptide vaccines derived from ISA virus surface proteins.

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I did the following work presented in this Thesis: Virus and cell line manipulation, virus RNA purification, reverse transcription and PCR of ISA virus segments, cloning of ISA virus segments into sequencing and vaccine vectors, and purification and preparation of all DNA vaccines. Vaccinations, ELISAs, and western blots were done with Dr. Sharon Clouthier, Microtek Intl., LTD. in collaboration with Dr. Eric Anderson, University of Maine. Additional work performed by me contributed significantly to determining the genomic organization of the ISA virus (Couthier *et al.*, 2002).

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INTRODUCTION

Infectious Salmon Anemia Virus

Classification

Infectious salmon anemia (ISA) is a disease of farmed Atlantic salmon (*Salmo salar*). The etiological agent of ISA has been shown to be a pleomorphic, enveloped virus 100-130 nm in diameter (Hovland *et al.*, 1994). It has been shown that ISA virus (ISAV) possesses a negative sense single stranded RNA genome consisting of eight segments 1-2.3 kb in size which would be consistent with an orthomyxovirus (Mjaaland *et al.*, 1997). Sequence comparisons of the polymerase sequence of ISA virus to that of other orthomyxoviruses suggests that ISA virus should be classified under a fifth, new genus within the *Orthomyxoviridae* family of viruses (Krossoy *et al.*, 1999). Phylogenetic calculations reveal that ISA virus is more closely related to influenza viruses than to Dhori and Thogoto viruses (Krossoy *et al.*, 1999).

Geographic Distribution

ISA was first observed in Norway in 1984 and is believed to have been present as early as the 1970's (Thorud & Djubvik *et al.*, 1988). ISA has been reported in the United Kingdom (Scotland) (Rodger *et al.*, 1998), New Brunswick, Canada (Bouchard *et al.*, 1999) and in the United States off the coast of Maine (Cobscook Bay) (Clancey, 2001). ISA virus has recently been isolated in Chile from Coho salmon (*Oncorhynchus kisutch*) exhibiting symptoms of ISA (Kibenge *et al.*, 2001). The manner in which ISA virus is

introduced from one area to another can be the result of many factors including, transportation of infected fish between fish farms or infection from other species that act as asymptomatic carriers. (Krossoy *et al.*, 2001a).

Host Range and Susceptibility

The primary host of ISA virus is farmed Atlantic salmon (*Salmo salar*) and it has also been shown to replicate in rainbow trout (*Oncorhynchus mykiss*) (Nylund *et al.*, 1997). Although ISA virus does not cause disease in this species, infected rainbow trout exhibit lower levels of red blood cells and petechiae on the liver (Nylund *et al.*, 1997). Brown trout (*Salmo trutta*) can also act as asymptomatic carriers of ISA virus (Nylund *et al.*, 1994). ISA virus has recently been isolated from Coho salmon (*Oncorhynchus kisutch*) exhibiting symptoms of ISA (Kibenge *et al.*, 2001), suggesting that ISA virus can cause disease in species other than Atlantic salmon. Studies have shown that ISA virus can agglutinate erythrocytes from rainbow trout, Atlantic salmon, Atlantic cod (*Gadus morhua*), and crucian carp (*Carassius caraccius*) with varying affinities (Falk *et al.*, 1997).

Transmission

The manner in which ISA virus is transmitted from one area to another is not yet known. Horizontal transmission studies have shown that cohabitation of uninfected Atlantic salmon with ISA virus-infected Atlantic salmon results in the spread of the ISA virus to uninfected fish (Thorud & Djubvik *et al.*, 1988; Jones & Groman, 2001). As stated above, other species of fish can act as reservoirs for ISA virus while not exhibiting signs of disease. Populations of wild Atlantic salmon may also act as virus reservoirs and

mediate the spread of ISA virus through migratory movement. However, the migration patterns of these fish are not well established and the presence of ISA virus within populations of wild fish has not yet been investigated. Studies have suggested that the spread of ISA virus may be linked to transfer of fish from one fish farm to another (Krossoy *et al.*, 2001a). There is also evidence that sea lice (*Lepeophtheirus salmonis*) may play a role in transmission (Nylund *et al.*, 1993).

Vertical transmission of the ISA virus has been investigated (Melville and Griffiths, 1999). Atlantic salmon eggs collected from fish identified as ISA virus-positive were fertilized and reared. The eggs and parr were screened for ISA virus by SHK-1 cell culture and RT-PCR. ISA virus was not detected in eggs or parr and no mortalities due to the virus were apparent. These observations suggest that ISA virus infection does not occur as a result of vertical transmission.

Pathology (signs and symptoms)

The pathology of ISA virus has been characterized (Evensen *et al.*, 1991). Symptoms of infectious salmon anemia include ascites and congestion of the foregut in the early stages. As infection moves from early to mid stage, progressive anemia is observed as indicated by lower haematocrit values. Congestion of the spleen and liver occur with the liver being more severely affected. Dissemination of liver lesions increases gradually as haematocrit values decrease. During later stages of infection, liver degeneration is characterized by lower levels of hepatocytes and haemorrhagic necroses with the majority of lesions located on the parenchyma and the vascular system of the liver.

The presence of ISA virus in the head kidney, liver, spleen, heart, intestine, gill, and muscle organs of Atlantic salmon over the course of infection has been investigated (Rimstad *et al.*, 1999). Organs from ISA virus infected fish have been examined by RT-PCR at 1, 2, 5, 6, 8, 13, 16, 20, 27, 34, and 40 days post infection (DPI). ISA virus is detected as early as 1 dpi in at least one fish and at all other time intervals examined. ISA virus is detected predominantly in the head kidney and mid-kidney from 1 to 8 dpi. At 13 dpi ISA virus is found in all organs sampled indicating that the ISA virus replicates most substantially between 8 and 13 dpi.

Cell Culture of ISA Virus

Isolation and purification of ISA virus was initially hampered by the fact that the virus did not seem to replicate in cell lines routinely used to isolate fish viruses. In 1994 the long term cell line SHK-1 was derived from salmon head kidney cells. SHK-1 cells support ISA virus growth (Dannevig *et al.*, 1995) but the cell line requires precise growing conditions and is difficult to work with. ISA virus can also be propagated in Chinook salmon embryo (CHSE-214) cells, a more commonly used cell line that requires less stringent growth and maintenance conditions (Bouchard *et al.*, 1999; Lovely *et al.*, 1999; Kibenge *et al.*, 2000). Other cells that support ISA virus growth are Atlantic salmon kidney (ASK) cells (Krossoy *et al.*, 1999).

Genome and Genomic Organization

ISA virus possesses a single-stranded, negative sense RNA genome 14.5 kb in length (Mjaaland *et al.*, 1997) consisting of eight segments 1.0-2.3 kb in length that

encode ten different proteins (Clouthier *et al.*, 2002). The ISA viral genome is organized according to the length of each segment from largest to smallest (Table 1.1).

Comparisons of ISA viral sequences to those listed in the Influenza database and in Genbank showed that RNA segments 1 and 5 are unique. RNA segments 2, 3, 4, and 6 encode the putative proteins PB1, NP, PA, and HA, respectively. Segments 7 and 8 each have two open reading frames (ORF). Segment 7 encodes proteins P4 and P5 and segment 8 encodes proteins P6 and P7. A recent study by Biering *et al.* (2002) suggests that the nonstructural (NS) protein of ISA virus is encoded by genome segment 7 and the matrix protein by genome segment 8.

The complete sequence of segment 1 of ISA virus has not yet been reported. The gene product is protein P1. Its function has not yet been determined. Northern blot analysis of this segment indicates that it is approximately 2.4 kb in length (Clouthier *et al.*, 2002).

Segment 2 of ISA virus is 2.4 kb in length with a 2245 bp ORF encoding a 708 amino acid polypeptide with an estimated molecular mass of 80.5 kDa. Sequence data of this segment compared with that of influenza sequences reveals that this protein is the PB1 protein (Krossoy *et al.*, 1999). In influenza viruses, PB1 is one of three subunits that comprise the viral RNA-dependent RNA polymerase. Comparison of the PB1 amino acid sequence of the Charley cove back bay (CCBB) isolate of the ISA virus (AF404346) reveals that it is 82.2% and 84.5% identical to the PB1 amino acid sequence of the Norwegian (AJ002475) and Scottish (AF262392) isolates of the ISA virus, respectively.

Segment 3 is comprised of a 2.2 kb sequence with a 1851 bp ORF encoding a 617 amino acid polypeptide with an estimated molecular mass of 68.0 kDa (Clouthier *et al.*,

Table 1.1 RNA segments of ISA virus and their encoded proteins.

Segment	Length (kb)	Encoded protein*	Predicted molecular mass (kDa)
1	2.4	P1	-
2	2.4	PB1	80.5
3	2.2	NP	68.0
4	1.9	P2	65.3
5	1.6	P3	48.8
6	1.5	HA	43.1
7	1.3	P4/P5	28.6/16.3
8	1.0	P6/P7	26.5/20.3

* Encoded Proteins are as follows: P1 = Protein 1; PB1 = Polymerase Binding Protein 1; NP = Nucleoprotein; P2 = Protein 2; P3 = Protein 3; HA = Haemagglutinin; P4 = Protein 4; P5 = Protein 5; P6 = Protein 6; P7 = Protein 7

2002). Conserved features and amino acid sequence similarities of this segment compared with that of influenza sequences reveal that this segment encodes nucleoprotein (NP) (Snow and Cunningham, 2001). NP of influenza viruses binds to the viral RNA genome forming the nucleocapsid. Comparison of the NP amino acid sequence of the CCBB isolate of ISA virus (AF404345) reveals that it is highly conserved, sharing 96.6% identity to the NP amino acid sequence of the Scottish isolate (AJ276858) (Clouthier *et al.*, 2002).

Segment 4 consists of a 1.9 kb sequence with a 1737 bp ORF that encodes a 579 amino acid polypeptide with an estimated molecular mass of 65.3 kDa (Clouthier *et al.*, 2002). The gene product of ISA virus segment 4 is protein P2. Its function has not yet been determined. Translation of the ORF of segment 4 of ISA virus isolate CCBB (AF404344) reveals that it is highly conserved, sharing 99% identity to the putative PA amino acid sequence (AF306548) described by Ritchie *et al.* (2001) (Clouthier *et al.*, 2002). In influenza viruses, PA is one of three subunits that comprise the viral RNA-dependent RNA polymerase.

Segment 5 is 1.6 kb in length with a 1353 bp ORF that encodes a 451 amino acid polypeptide with an estimated molecular mass of 49.8 kDa (Clouthier *et al.*, 2002). The gene product of ISA virus segment 5 is protein P3. Its function has not yet been determined. However, the P3 protein has a 17 amino acid N-terminal signal sequence and several potential glycosylation sites, suggesting a virion surface location (Clouthier *et al.*, 2002). P3 is likely the acetylesterase involved in receptor-destroying activity. The nucleotide sequence of segment 5 of ISA virus isolate CCBB (AF404343) is 76.0%,

76.4% and 99.7% similar to segment 5 of the Norwegian (AF429987), Scottish (AF429988), and Maine (AF429986) isolates of ISA virus, respectively.

Segment 6 of ISA virus is a 1.5 kb sequence with a 1185 bp ORF that encodes a 395 amino acid polypeptide with an estimated molecular mass of 43.1 kDa (Clouthier *et al.*, 2002). Salmon cell lines engineered to express segment 6 gain the ability to bind salmon erythrocytes, identifying this segment as that encoding the hemagglutinin (HA) (Krossoy *et al.*, 2001b; Rimstad *et al.*, 2001). The HA forms 13-15 nm spikes that coat the surface of the pleomorphic, enveloped virus (Falk *et al.*, 1997). The HA is thought to be involved in ISA virus binding to sialic acid cell surface receptors and fusing with the cell in acidic endosomes (Eliassen *et al.*, 2000). Comparison of the HA amino acid sequence of ISA virus isolate CCBB (AF404342) reveals that it shares 84.8 and 84.3% similarity to the predicted HA protein sequences for ISA virus isolates from Norway (AF302799) and Scotland (AJ276859) and 99.2% similarity to the Maine ISA virus isolate (AY059402).

Segment 7 of ISA virus is a 1.3 kb sequence with two ORFs. The first ORF is 771 bp encoding a 257 amino acid protein with an estimated molecular mass of 28.6 kDa. The second ORF is 441 bp encoding a 147 amino acid protein with an estimated molecular mass of 16.3 kDa (Clouthier *et al.*, 2002). The gene products of the first and second ORFs of segment 7 of the ISA virus are proteins P4 and P5, respectively. Biering *et al.* (2002) suggested that the colinear transcript encodes a non-structural or minor structural protein. The nucleotide sequence for the ISA virus CCBB segment 7 (AF404341) shares 99.6% similarity to a previously reported sequence from a Scottish isolate of ISA virus (AX083264).

Segment 8 of ISA virus is a 1.0 kb sequence with two ORF. The first ORF is 705 bp encoding a 235 amino acid protein with an estimated molecular mass of 26.5 kDa. The second ORF is 552 bp encoding a 184 amino acid protein with an estimated molecular mass of 20.3 kDa. The gene products of the first and second ORFs of segment 8 are proteins P6 and P7, respectively. Biering *et al.* (2002) suggested that segment 8 encodes a structural protein. The nucleotide sequence of segment 8 of CCBB ISA virus isolate (AF404340) shares 88.7 and 99.9% identity to the Norwegian (AF429990) and ME/01 (AF429989) isolates of ISA virus, respectively.

Infectious Hematopoietic Necrosis Virus (IHNV)

Infectious hematopoietic necrosis (IHN) is a disease of salmon and trout. The etiological agent of IHN is a bullet shaped, enveloped virus 110-160 nm in length and 70-90 nm in width. The IHN virus is classified as a member of the *Rhabdoviridae* family of viruses under the genus *Novirhabdovirus* (Walker *et al.*, 2000). The IHN virus has a geographic range from Alaska to California and extending inland to Idaho, it has also been reported in Europe and Asia (Winton, 1991). This virus causes disease in wild and farmed rainbow trout, pacific salmon (*Oncohrynchus keta*), steelhead (*Oncorhynchus mykiss*), and Atlantic salmon, with the highest mortalities usually occurring in juvenile fish. The IHN viral genome is a single-stranded, negative sense RNA, approximately 11,130 bp long (Morzunov et al., 1995). It encodes 6 proteins: the nucleoprotein (N), the matrix proteins (M1 and M2), the non-virion protein (NV), the polymerase protein (L), and the glycoprotein (G). Virus particles of the IHN virus are covered with the G protein, which is a major surface antigen that stimulates a protective immune response in

salmonids (Corbeil et al., 1999; Engelking and Leong, 1989a; Lorenzen and LaPatra, 1999; Winton, 1997).

Control of the ISA virus

Chemical and Physical Inactivation of the ISA virus

The salmon farming industry can incur massive economic losses due to the ISA virus. To prevent this, steps to minimize its impact on the aquaculture industry are being taken. ISA virus survives in sea and fresh water for more than 48 hours, but virus infectivity diminishes between 24 and 48 hours. Since tissue from infected salmon is highly contagious (Flogstad *et al.,* 1991), it is necessary for intake water and wastewater of slaughtering plants to be treated for ISA virus. To this end, the chemical and physical inactivation of ISA virus in free water and wastewater from the slaughtering process has been investigated (Flogstad *et al.*, 1991; Flogstad & Torgersen, 1992). Practical methods for chemical inactivation of ISA virus involve treating water sources with sodium hydroxide, sodium hypochlorite, and formic acid. ISA virus infectivity is lost in the presence of 56 μM formic acid for 8 hours, 1 mM sodium hydroxide at for 48 hours or pH 12.0 for 24 hours, or 1.35 mM sodium hypochlorite for 15 min. Other methods of ISA virus inactivation investigated include ultraviolet (UV) irradiation or heat treatment of water. These methods present an attractive alternative to the use of harmful chemicals. ISA virus infectivity is lost in water heated to temperatures of 50 °C or higher for 2 min or water irradiated with UV light at a dose of 4 mJ/cm^2 or higher.

Husbandry

Atlantic salmon must be raised in an ISA virus free environment to prevent virus propagation. Visitors to an aquaculture facility are required to wear disposable "pull on" shoes to prevent the introduction of contaminants. Visitors are not allowed to come into contact with intake water or food dispensers. When moving from one area of the facility to another visitors and staff are required to step into footbaths containing disinfectant. Equipment entering and leaving the facility are disinfected. Small items such as nets are cleaned using a high-pressure cleaning device to remove visible contaminants and then washed with the appropriate disinfectant. Tires of fish transport vehicles and boats used at sea based facilities are similarly treated. All holding pens and pipes must be non-absorbant. Pipes and holding pens on farms and transport vehicles must be treated by first eliminating visible contaminants such as algae using a high-pressure device and then washing with an appropriate disinfectant by mechanical scrubbing. Seawater installations are dissembled and dried before they are disinfected. Tanks that consist of material that is porous, such as fiberglass and non-painted concrete, are treated with low-pressure devices and cleaned by mechanical scrubbing with an alkaline detergent from the top to the bottom of the tank.

The movement and rearing of fish is highly controlled. Any outbreaks of ISA virus are reported within four days to ensure that proper precautionary measures can be implemented. ISA combat zones have been established in Norway where strict regulations are applied to prevent the spread of the ISA virus. The movement of fish from sites where the ISA virus has been verified into "ISA combat" zones is prohibited. Stocks of fish entering a farm from another facility may be quarantined for a period of

time. Fish eggs are disinfected with Iodophor at a pH of 6-8 to prevent infection of new stocks. Fish are carefully monitored for possible outbreaks. Dead fish are removed, tested for the ISA virus, and processed to prevent possible contamination. Crops of Atlantic salmon exhibiting ISA virus symptoms are immediately slaughtered to prevent further spread of the disease to other fish pens. The fish pens where the ISA virus has been detected are restocked only after being disinfected and quarantined.

Genetic Immunization

DNA Vaccination

Proper treatment of contaminated equipment and close monitoring of the rearing and transport of fish are two methods of controlling ISA virus. Vaccination of farmed Atlantic salmon against ISA virus offers an additional effective method for controlling this pathogen. Inactivated whole virus ISA vaccines and peptide vaccines composed of ISA virus protein subunits or recombinant peptides are being tested.

In 1993, researchers developed a new form of vaccination called the DNA vaccine (Ulmer *et al.*, 1993). DNA vaccination consists of injecting the host with a circular piece of DNA containing the gene for an immunogenic protein. This results in the host cell expressing the foreign protein and the immune system recognizing it. In many cases, this results in a better immune response than peptide or whole killed vaccines, presumably because the antigenic protein is synthesized in the host *in situ* in a manner that more closely mimics its synthesis during a natural infection. DNA vaccines are desirable over other forms of vaccination because they are cheap, easy to synthesize,

and activate both humoral and cell-mediated branches of the immune system (Fynan *et al.*, 1993; Ulmer *et al.*, 1993).

DNA Vaccination of Fish

In 1996, the first DNA vaccine for fish was described (Anderson *et al.*, 1996b). In this study, intramuscular injection of a plasmid containing the gene encoding IHN virus G protein (IHNV-G), under the control of the human cytomegalovirus (hCMV) promoter, protected rainbow trout fry from homologous virus challenge at 28 days post-vaccination (DPV) (Anderson *et al.*, 1996b). The G protein also elicits a nonspecific, cross protective immune response in rainbow trout that begins 4-7 d post-vaccination (LaPatra *et al.*, 2001). Fish antibodies do not mediate the early viral response (EVR) but are detectable in fish approximately 3-4 weeks after vaccination (LaPatra *et al.*, 1993). The appearance of serum antibodies coincides with the loss of the EVR and the appearance of a specific viral response (SVR). The SVR results in 90-100% relative percent survival (RPS; RPS = (1 – (% Mortality of Vaccine/ % Mortality of Negative Control)) X 100) of fish vaccinated with the IHN virus DNA vaccine pIHNw-G, for 1-3 months post-vaccination, 69% RPS 6 mo post-vaccination, and 65% RPS 13 mo post vaccination. Thus, protection at the early time points is more likely the result of a cell-mediated immune response or non-specific anti-viral reaction that functions to protect the host at the onset of infection.

Another example of an aquatic DNA vaccine is the viral haemorrhagic septicaemia virus glycoprotein (VHSV-G) DNA vaccine (Lorenzen *et al.*, 1999). This vaccine protects rainbow trout from infection and stimulates high levels of virus specific

neutralizing antibodies (Boudinot *et al.*, 1998). The VHSV-G DNA vaccine provides protection as early as 7 days post vaccination and stimulates Mx and MHC class II mRNA production (Boudinot *et al.*, 1998). Protection at these early time points and the activation of these genes indicates that the VHSV-G DNA vaccine may stimulate the same non-specific, cell-mediated immune response as the IHNV-G DNA vaccine.

The EVR defense mechanism stimulated by IHNV-G and VHSV-G DNA vaccines has not been characterized. In rainbow trout, both vaccines stimulate production of Mx and MHC class II mRNA as early as 7 dpv (Boudinot *et al.*, 1998). In mammals, synthesis of these proteins is induced by interferon and the products play critical roles in cell-mediated immunity. Other types of early non-specific immune responses in fish have been observed. The VHSV-G DNA vaccine induces synthesis of VHSV induced gene (VIG) 7days post injection. The VIG protein may be involved in the production of enzymatic cofactors of the nitric oxide pathway (Boudinot *et al.*, 1999). The connection between nitrogen metabolism and resistance to viral pathogens is unknown. VIG-2 is a new interferon-responsive gene induced by exposure of rainbow trout to VHS virus (Boudinot *et al.*, 2001). Type 1 interferon-like activity has also been observed in fish cells as early as 48 hours after being exposed to IHN virus or poly I:C (Eaton *et al.*, 1990; Trobridge *et al.*, 1997). The ability of ISA virus segments to stimulate the same type of early non-specific response was investigated as part of my thesis studies.

Vaccine Strategies

The efficacy of DNA vaccination of Atlantic salmon against the ISA virus has not yet been investigated. To this end several DNA vaccines containing ISA virus segments

3, 5, 6, 7, and 8 were created and tested in the following vaccine trials. The ability of these vaccines to protect rainbow trout and Atlantic salmon from lethal challenges of IHN and ISA virus was tested. Serum samples were collected from vaccinates to measure the production of antibodies. The data from these trials helped to determine which ISA virus gene segments produced immunoreactive peptides, which ones are cross protective, which ones produce an antibody response, and the kinetics of that response. These trials also helped to determine if ISA viral genes can induce in Atlantic salmon the same type of early non-specific cell-mediated immune response seen with the IHNV-G and VHSV-G DNA vaccines in rainbow trout. Also included in these trials were different types of whole killed virus and peptide vaccines so that their efficacy could be compared with that of the DNA vaccines.

A further question addressed in these trials is the possibility that ISA virus contains an immunosuppressive protein. Recent studies have shown that the NS1 protein of influenza A virus inhibits alpha/beta interferon production by preventing the activation of NF-κB (Wang *et al.*, 2000). In the mammalian system, secretion of alpha/beta interferon represents the first line of defense against viral infection. Due to the similarities between influenza viruses and ISA virus it is possible that the ISA virus possesses a gene that has a similar immunosuppressive function. It is not yet known which segment of the ISA viral genome encodes a similar NS1 protein. Segment 7 and 8 are the most suspect genes because they are most similar in length and both contain two open reading frames like the NS gene of influenza viruses. The possibility that either ISA virus segments 7 or 8 encode an immunosuppressive protein was investigated using the IHNV-G DNA vaccine as a determinant.

Chapter 2

METHODS

Virus and Cell Line

ISA virus was isolated from farmed Atlantic salmon. The North American ISA virus isolates CCBB and Conner have previously been described (Clouthier *et al.*, 2002). The European isolates (Glesvaer 2/90 Norway, and 390/98 Scotland) were kindly provided by Dr. Knut Falk and Dr. Katrinna Ross, respectively. The ISA virus was grown in Chinook salmon embryo (CHSE-214) cells (Lannan *et al.*, 1984) at 15 °C in minimal essential medium (MEM) containing Hank's salts (Gibco BRL), 10 mM L-glutamine and 10% fetal bovine serum (Gibco BRL).

Virus and RNA Purification

ISA virus was purified from the supernatant of virus infected CHSE-214 cells showing complete cytopathic effect (CPE). The virus supernatant was filtered using a sterile Suporcap-50 0.45 micron filter (Gelman). The virus supernatant was placed in Specpore 7 tubing (Spectrum Laboratories) and dialyzed for 8 hr at 4 °C against PEG 8000 (Sigma). The concentrated virus supernatant was centrifuged for 2 hr at 104,000 ***g*** in a Beckman SW-28 rotor using a Beckman L8-70M ultracentrifuge. The crude virus pellet was resuspended in 1 ml TNE (10 mM Tris pH 7.5, 100 mM sodium chloride and 1 mM EDTA, pH 7.5) and layered onto a 25-35-45% discontinuous sucrose gradient and centrifuged for 3 hr at 132,000 ***g*** in a Beckman SW28 rotor using a Beckman L8-70M ultracentrifuge. The virus was collected from the 35-45% interface, diluted in TNE and

centrifuged for 2 hr at 104,000 ***g*** in a Beckman SW-28 rotor using a Beckman L8-70M ultracentrifuge. The virus pellet was then resuspended in 0.5 ml of TNE.

cDNA Synthesis

ISA virus genomic RNA was prepared using Trizol (Invitrogen Corp.), and converted into cDNA by reverse transcription. Purified viral RNA (500 ng), was mixed with 2.5 μM forward and reverse oligonucleotide primers (Table 2.1) and 40 U of RNase Out (Gibco BRL) were combined and incubated at 72 °C for 2 min. A solution of, 625 mM dNTPs, 6.25 mM of dithiothreitol, 100 U of Superscript II reverse transcriptase (all Gibco BRL) were added to the solution and the mixture was incubated at 22 °C for 10 min and then 42 °C for 1 hr.

The polymerase chain reaction (PCR) was used for second strand cDNA synthesis. First strand cDNA was combined with PCR reaction buffer, 10 μmol dNTP, 100 μmol magnesium chloride, 50 pmol forward and reverse oligonucleotide primers (Table 2.1) and 5 U of Taq polymerase (all Gibco BRL). The mixture was incubated at 95 °C for 2 min and then for 35 cycles at 95 °C for 1 min, 50 °C for 1 min and 72 °C for 2 min followed by 72 °C for 10 min. The PCR amplified cDNA was separated in a 1.5% agarose gel (Sigma) in a RAGE-100 (Cascade Biologics) apparatus and purified using a the Qiaquick Gel Extraction Kit (Qiagen Inc.) as described by the manufacturer (Qiagen Inc.).

Table 2.1 Primers used to amplify full-length ISA virus segments.

Segment	Primers	Sequence 5'-3'
Segment 3	pTargeT-NP-For	CGCATAATAGGATCCGCAAAG ATTGCTCAAATCCCAAAAATA
	pTargeT-NP-Rev	CATGCTCAAGGTACCTGTAAA TATCGTCAATACCGTTTC
Segment 5	5E7FullF	AGTTAAAGATGGCTTTTCTAAC
	5E7FullR	CTATTTATACAATTAATAATG CATAATC
Segment 7	2B10F1	TACAAAGAAAATGTTCAGAAC ATGTCTG
	2B10R1	AAAAATTATCATCAACACCAT GTACGAC
Segment 8	Class 2	AAGCAGTGGTAACAACGCAGA CTGTATCTACCATG
	Seg8-3-R-mRNA	TTATTGTACAGAGTCTTCC

DNA Vaccines Plasmids

ISA virus cDNA segments 3, 5, 7 and 8 were ligated into pGEM-T (Table 2.2) as described by the manufacturer (Promega Corp.) and cDNA segments 6, 7, and 8 were ligated into pTargetT (Table 2.3) as described by the manufacturer (Promega Corp.) The ISA virus cDNA clones were amplified using *E. coli* JM109 cells (Sambrook *et al.*, 1989). Verification that the plasmids contained full length ISA virus cDNA was done by DNA sequence analysis. Sequencing was done at the University of Maine Sequencing Facility by Patty Singer. ISA virus cDNAs in the pGEM-T plasmids were subcloned into the DNA vaccine plasmid pcDNA 3.1 (Table 2.4; Invitrogen). Approximately 10 μg of plasmid and 20 U Apa1, restriction enzyme buffer, and 60 μg of bovine serum albumin (all New England Biolabs) were mixed and incubated at 37 °C for 4 hr and then at 65 °C for 20 min. The plasmid was purified using Qiaquick PCR purification kit as described by the manufacturer (Qiagen Inc.) and then treated as described above with the restriction enzyme Not1. The pTargetT ISA virus cDNA plasmids were prepared in a similar manner using the restriction enzymes Nhe1 and Not1. The ISA virus cDNA were separated by agarose gel electrophoresis and purified using the Qiaquick Gel Extraction Kit as described by the manufacturer (Qiagen Inc.). Plasmid pcDNA 3.1 was digested with Not1 and Apa1 or Nhe1 and purified using a Qiaquick PCR purification kit as described by the manufacturer (Qiagen Inc.).

The restriction enzyme digested and purified ISA virus cDNA was ligated into linearized pcDNA 3.1 by combining the DNAs (3:1 insert to vector molar ratio, respectively) with Tris-acetate buffer (33 mM Tris-OH, 66 mM potassium acetate, 10

Table 2.2 pGEM-T-Vector Constructs

Segment and Strain	Plasmid Name
Segment 3 (CCBB)	pGISA-NP_{NA}
Segment 3 (Conner)	pGISA-NP_{ME}
Segment 3 (Norway)	pGISA-NP_{Nor}
Segment 5 (CCBB)	pGISA-Ac_{NA}
Segment 5 (Conner)	pGISA-Ac_{ME}
Segment 5 (Norway)	pGISA-Ac_{Nor}
Segment 7 (CCBB)	pGISA-7_{NA}
Segment 7 (Conner)	pGISA-7_{ME}
Segment 7 (Norway)	pGISA-7_{Nor}
Segment 8 (CCBB)	pGISA-8_{NA}
Segment 8 (Conner)	pGISA-8_{ME}
Segment 8 (Norway)	pGISA-8_{Nor}

Table 2.3 pTargeT-Vector Constructs

Segment and Strain	Plasmid Name
Segment 6 (CCBB)	pTISA-HA_{NA}
Segment 6 (Conner)	pTISA-HA_{ME}
Segment 6 (Norway)	pTISA-HA_{Nor}
Segment 7 (CCBB)	pTISA-7_{NA}
Segment 7 (Conner)	pTISA-7_{ME}
Segment 7 (Norway)	pTISA-7_{Nor}
Segment 8 (CCBB)	pTISA-8_{NA}
Segment 8 (Conner)	pTISA-8_{ME}
Segment 8 (Norway)	pTISA-8_{Nor}

Table 2.4 pcDNA3.1 Vector Constructs

Segment and Strain	Plasmid Name
Segment 3 (CCBB)	pISA-NP_{NA}
Segment 3 (Conner)	pISA-NP_{ME}
Segment 3 (Norway)	pISA-NP_{Nor}
Segment 5 (CCBB)	pISA-Ac_{NA}
Segment 5 (Conner)	pISA-Ac_{ME}
Segment 5 (Norway)	pISA-Ac_{Nor}
Segment 6 (CCBB)	pISA-HA_{NA}
Segment 6 (Conner)	pISA-HA_{ME}
Segment 6 (Norway)	pISA-HA_{Nor}
Segment 7 (CCBB)	pISA-7_{NA}
Segment 7 (Conner)	pISA-7_{ME}
Segment 7 (Norway)	pISA-7_{Nor}
Segment 8 (CCBB)	pISA-8_{NA}
Segment 8 (Conner)	pISA-8_{ME}
Segment 8 (Norway)	pISA-8_{Nor}

mM magnesium acetate, and 10 mM dithiothreitol, pH 7.4) 4 μg bovine serum albumin (New England Biolabs), 8 μM adenosine triphosphate and 1 U T4 ligase (Promega Corp.) and incubated at 12 °C for 12-16 hr. For transformation, the ligation mixture was added to *E. coli* DH5α competent cells and incubated on ice for 15 min, heated at 42 °C for 2 min, and then incubated on ice for 10 min. The solution was added to 2 ml of LB broth (1% Tryptone (Difco), 0.5% Yeast Extract (Difco), and 0.5% NaCl (Fisher)) containing 0.5% glucose and shaken at 225 rpm at 37 °C for 1 hr. The *E. coli* cells were centrifuged at 6000 ***g*** at 4 °C for 3 min using a Beckman J2-21 centrifuge and JA-20 rotor. The cells were resuspended, spread on LB agar (1% Tryptone (Difco), 0.5% Yeast Extract (Difco), 0.5% NaCl (Fisher), and 1.5% Agar (Difco)) containing 100 μg of ampicillin/ml (Sigma) and incubated at 37 °C for 16-24 hr. Plasmid DNA from the transformed *E. coli* was purified using a Qiagen Miniprep Kit as described by the manufacturer (Qiagen Inc.). The presence and orientation of the insert was verified by restriction enonuclease digestion.

DNA Vaccine Preparation

DNA vaccines were prepared using an ammonium acetate precipitation protocol (Saporito-Irwin *et al.*, 1997). LB containing 100 μg of ampicillin/μl (Sigma) was inoculated with a fully-grown culture at a 1:100 ratio and grown 14-16 hours at 37 °C. The cells were collected by centrifugation for 5 min at 10000 ***g*** at 4 °C in a Beckman J2-21 centrifuge using a JA10 rotor and resuspended in solution 1 (25 mM Tris-HCl, pH 7.6, 10 mM EDTA, pH 8.0, 50 mM glucose). The cells were mixed by gentle rocking and incubated on ice for 20 min and solution 2 (200 mM NaOH, 1% SDS) was added. The

cells were incubated on ice for 10 min and mixed with ice-cold 7.5 M ammonium acetate (pH 7.6). The samples were centrifuged for 20 min at 25000 ***g*** at 4 °C in a Beckman J2-21 centrifuge using a JA14 rotor. The supernatant was transferred to a fresh tube and 0.6 vol of isopropanol was added, samples were incubated at room temperature prior to centrifugation for 10 min at 10000 ***g*** at 4 °C in a Beckman J2-21 centrifuge using a JA14 rotor. The supernatant was poured off and the pellet containing the crude plasmid DNA was resuspended in 2.0 M ammonium acetate followed by a 10 min incubation on ice. The plasmid DNA was centrifuged for 10 min at 10000 ***g*** at 4 °C in a Beckman J2-21 centrifuge using a JA20 rotor. The supernatant was transferred to a fresh tube and 0.6 vol of isopropanol was added followed by a 10 min incubation at RT. The resulting pellet was resuspended sterile water and 0.3 mg of RNase A (Invitrogen) was added prior to a 20 min incubation at 37 °C. A total of 0.3 vol of ice-cold 7.5 M ammonium acetate was added followed by a 5 min incubation at room temperature. The plasmid DNA was centrifuged for 10 min at 10000 ***g*** at 4 °C in a Beckman J2-21 centrifuge using a JA20 rotor. The supernatant was transferred to a new tube and 0.6 vol of isopropanol was added followed by a 10 min incubation at room temperature. Centrifugation was performed at 10000 ***g*** for 10 min at 4 °C in a Beckman J2-21 centrifuge using a JA20 rotor. The resulting pellet was washed in 70% ethanol, dried at room temperature, and resuspended in sterile water.

Transfection and Immunohistochemistry

The capacity of the plasmids pISA-HA_{NA} and pIHNw-G (Anderson and Leong, 1999) to express the HA or G protein in fish cells was assessed in transfected epithelial

papillosum cyprini (EPC) cells as described previously (Anderson and Leong, 1999). Briefly, EPC cells seeded in six-well plates to 90% confluency were transfected with 1-10 μg of the appropriate DNA vector in lipofectamine prepared according to the manufacturer's instructions (Invitrogen). The DNA-lipid mixture was incubated with the cells for 24 hours at 22°C after which MEM-Hanks containing 5% FBS was added to each well. Cells were incubated at 22°C for 72 hours and then fixed with 10% formaldehyde. Expression of the HA and G proteins in EPC cells was confirmed by staining with the monoclonal antibodies 10A3 (Clouthier *et al.*, 2002) and 1H8/6A7/5A6 (Huang *et al.*, 1994, 1996), respectively, as described by Drolet *et al.* (1994).

Vaccine Trials

Trial #1

An antibody study was performed with 150-160 g specific-pathogen free Rainbow trout (*Oncorhynchus mykiss*) (Clear Springs Foods, Inc., Buhl, Idaho). Groups of 30 fish were anaesthetized by immersion in of tricaine methane sulfonate (100 μg/ml) and injected intramuscularly at the base of the dorsal fin with a 200 μl dose of one of four different DNA vaccines (Table 2.5) suspended in phosphate buffered saline (PBS). Experimental groups were held separately in UV-treated single-pass spring water (15 °C). Serum was collected from 10 naive fish at the time of vaccination and at 4, 6, 8, 10, and 12 weeks post-vaccination from 5 fish per treatment per time point by lethal bleeding from the caudal vein.

Table 2.5. Antibody study in rainbow trout: vaccines and dose.

Group	Vaccine and Dose
1	pTISA-HA_{NA} (10 μg)
2	pTISA-HA_{Nor} (10 μg)
3	pTISA-7_{ME} (10 μg)
4	pTISA-8_{NA} (10 μg)

Trial #2

An IHN virus challenge study to establish vaccine efficacy was performed with 1-10 g (mean weight 2 g) specific-pathogen free Rainbow trout (*Oncorhynchus mykiss*) (Clear Springs Foods, Inc., Buhl, Idaho). Groups of 150 fish were anaesthetized by immersion in 100 μg/ml of tricaine methane sulfonate and injected intramuscularly at the base of the dorsal fin with a 25 μl dose of one of nine vaccines (Table 2.6) suspended in PBS. At 7 days post-vaccination (dpv) duplicate groups of 25 fish from each group were challenged by waterborne exposure to 10^5 plaque forming units (PFU)/ml of IHN virus isolate 220-90 (LaPatra *et al.*, 2001) for 60 min with aeration in a volume of water 10 x the total weight of the fish. The remaining fish were used as controls. Control groups

Table 2.6 Efficacy study in rainbow trout: vaccines and dose.

Group	Vaccine and Dose
1	pIHNw-G (1 μg)
2	pISA-7_{NA} (1 μg)
3	pISA-7_{NA} (10 μg) + pIHNw-G (1 μg)
4	pISA-8_{NA} (1 μg)
5	pISA-8_{NA} (10 μg) + pIHNw-G (1 μg)
6	pISA-HA_{NA} (1 μg)
7	pISA-HA_{Nor} (1 μg)
8	pISA-NP_{NA} (10 μg) + pISA-Ac_{NA} (10 μg) + pISA-HA_{NA} (10 μg)
9	pLuc (10 μg)

were not exposed to IHN virus but were otherwise treated identically. Experimental groups were held separately in UV-treated single-pass spring water (15 °C) and monitored for 21 days for mortality. RPS rates were calculated (RPS = 1 – [% mortality of vaccine/% mortality of control] x 100). Significant differences in mortalities were detected by the Chi square test using a probability of error threshold (P) value of < 0.05.

Trial #3

A study to establish antibody levels during the EVR was performed on 50-100 g specific-pathogen free Atlantic salmon (*Salmo salar*). Groups of 45 fish were anaesthetized by immersion in 100 µg/ml of tricaine methane sulfonate and injected intramuscularly at the base of the dorsal fin with a 200 µl dose of one of four different DNA vaccine treatments (Table 2.7) suspended in PBS. Serum was collected from 5 naive fish at the time of vaccination and at 7 and 21 dpv from 5 fish/treatment/time point by lethal bleeding from the caudal vein.

Trial #4

An ISA virus challenge study to establish vaccine efficacy was performed on 50-100 g specific-pathogen free Atlantic salmon (*Salmo salar*). Groups of 225 fish were anaesthetized by immersion in 100 µg/ml of tricaine methane sulfonate. One group was vaccinated intramuscularly with a 200 µl dose of a trivalent DNA vaccine (Table 2.8) suspended in PBS. The remaining groups were vaccinated by intraperitoneal with a 200 µl dose of one of the remaining three vaccine treatments (Table 2.8). Serum was collected from 5 naive fish at the time of vaccination and at 700 and 1,400 degree days

Table 2.7 Antibody production in Atlantic salmon during EVR: vaccines and dose

Group	Vaccine and Dose
1	pISA-HA_{NA} (1 μg)
2	pISA-HA_{Nor} (1 μg)
3	pISA-7_{NA} (1 μg)
4	pISA-8_{NA} (1 μg)
5	pβ-Gal (1 μg)

Table 2.8 ISA virus efficacy trial: vaccines and dose

Group	Vaccine and Dose
1	pISA-NP_{NA} + pISA-Ac_{NA} + pISA-HA_{NA} *
2	whole killed ISA (1 x dose) **
3	recombinant ISA virus peptide **
4	Saline

* 10 μg of each vaccine

** Product of Maine BioTek, Inc. and Microtek Int. Ltd.

(dd) post-vaccination from 10 fish per treatment per time point by lethal bleeding from the caudal vein. At 700 and 1,400 dd post-vaccination 100 fish/vaccine group were anaesthetized and challenged by IP injection of 1 ml live ISA virus isolate CCBB at $1x10^6$ $TCID_{50}$/ml. RPS rates were calculated (RPS = 1 – [% mortality of vaccine/% mortality of control] x 100). Significant differences in mortalities were detected by the Chi square test using a probability of error threshold (P) value of < 0.05.

Trial #5

An antibody study was performed on 50-100 g specific-pathogen free Atlantic salmon (*Salmo salar*). Groups of 28 fish were anaesthetized by immersion in 100 µg/ml of tricaine methane sulfonate. Vaccine groups 1, 2, 3 and 7 were vaccinated intramuscularly with a 200 µl dose of DNA vaccine suspended in PBS (Table 2.9). Vaccine groups 4, 5, 6 and 8 were vaccinated by intraperitoneal injection with a 200 µl dose of vaccine (Table 2.9). Serum was collected from 25 naive fish at the time of vaccination and at 4, 6, 10, and 12 dpv from 5 fish/treatment/time point by lethal bleeding from the caudal vein.

Enzyme Linked Immunosorbent Assay (ELISA)

ELISA was used to measure the binding antibody titer in serum from vaccinated fish. The target antigen, untreated IHN or ISA virus, was dried onto the wells of an ELISA plate (Costar) overnight at 37°C. After blocking nonspecific binding sites, serial dilutions of the primary antisera collected from vaccinated rainbow trout or Atlantic

Table 2.9 Antibody study in Atlantic salmon: vaccines and dose

Group	Vaccine and Dose
1	pISA-NP_{NA} (10 μg)
2	pISA-Ac_{NA} (10 μg)
3	pISA-HA_{NA} (10 μg)
4	recombinant ISA peptide *
5	whole killed ISA (2 x dose) **
6	whole killed ISA (4 x dose) **
7	pLuc (10 μg)
8	Saline

* Product of Maine BioTek, Inc. and Microtek Int. Ltd.

** Product of Microtek Int. Ltd.

salmon were added to the target antigen and detected with mouse anti-salmonid fish immunoglobulin monoclonal antibody (Immuno-Precise Antibodies, LTD). Goat anti-mouse IgG or IgM conjugated to alkaline phosphatase (Southern Biotechnology Associates, Inc) were added followed by 5-Bromo-4-chloro-3-indoyl phosphate (66 pg/ml) and Nitrotetrazolium Blue chloride (59 pg/ml). ELISA measurements were expressed as a percentage of IHN virus or ISA virus specific mAb reactivity recorded at an absorbance 405 nm. Using this technique we typically detected IHN virus or ISA virus specific antibodies in the serum dilution range 1:20 to 1:2560.

Chapter 3

RESULTS

ISA Virus DNA Vaccine Transfected Cells.

The synthesis of HA encoded by the DNA vaccine pISA-HA_{NA} was examined by immunohistochemical analysis of transfected EPC cells. The pISA-HA_{NA} transfected cells reacted with ISA virus specific mAb10A3 (Figure 3.1).

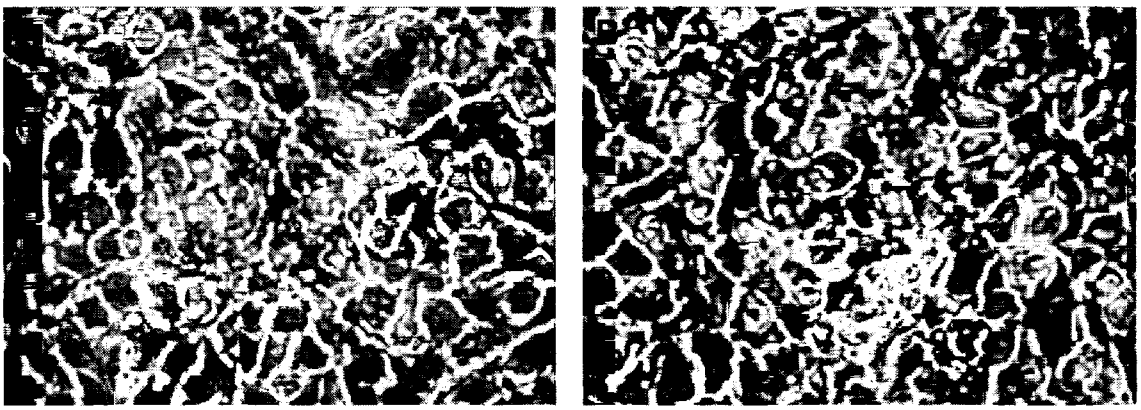

Figure 3.1 Expression of the ISA virus HA protein in cells transfected with pISA-HA_{NA}. EPC cells were transfected with pISA-HA_{NA} and then probed with 10A3, a HA-specific monoclonal antibody (A). The cells expressing the HA protein were identified using alkaline phosphatase immunocytochemical staining which stains the cells red in the photographs. Also shown are cells that were transfected with pβGal and then stained with the HA-specific mAb (B).

ISA Virus DNA Vaccine: Efficacy & Humoral Immune Response

The efficacy of the ISA virus DNA vaccines was determined in Trial #4 and the results are presented in Table 3.1. Atlantic salmon (50-100 g) were vaccinated a trivalent DNA vaccine and challenged by intraperitoneal injection with $10^{7.5}$ tissue culture infectious dose (TCID) at 700 dd or 10^{6} TCID at 1,400 dd of ISA virus. Also included in the study were whole killed (wk) and recombinant ISA virus vaccinated fish as well as fish inoculated with saline. All of the vaccines were more efficacious at 1,400 dd post-vaccination compared to 700 dd. The efficacy of the trivalent ISA virus DNA vaccine in Atlantic salmon was less than that observed using an IHN virus G DNA vaccine in Atlantic salmon and rainbow trout. In addition, the ISA virus DNA vaccine was less efficacious than the whole killed, and recombinant vaccines as determined by the Chi square test (Table 3.1).

The antibody response elicited by the DNA, killed and recombinant ISA vaccines was determined in Trial #4 and #5 and measured by ELISA. ISA virus-specific antibodies were present 700 and 1,400 dd post vaccination in the DNA vaccinated fish, though antibody binding was 23-31% of that obtained using an ISA virus specific monoclonal antibody (Figure 3.2). The serum from vaccinated fish that survived ISA virus challenge 700 or 1,400 dd post-vaccination had relatively high levels of ISA virus specific antibodies.

Table 3.1 ISA virus efficacy trial: mortality and RPS values

Vaccine	Challenge 700 dd		Challenge 1400 dd	
	% Mortality	RPS_{60}	% Mortality	RPS_{40}
pISA-NP_{NA}, pISA-Ac_{NA}, pISA-HA_{NA}*	45	25	22	45
wk ISAV	29	52	n.d.**	n.d.**
rISA	31	48	15	63
Saline	60	-	40	-

* 10 μg each vaccine

** Not done

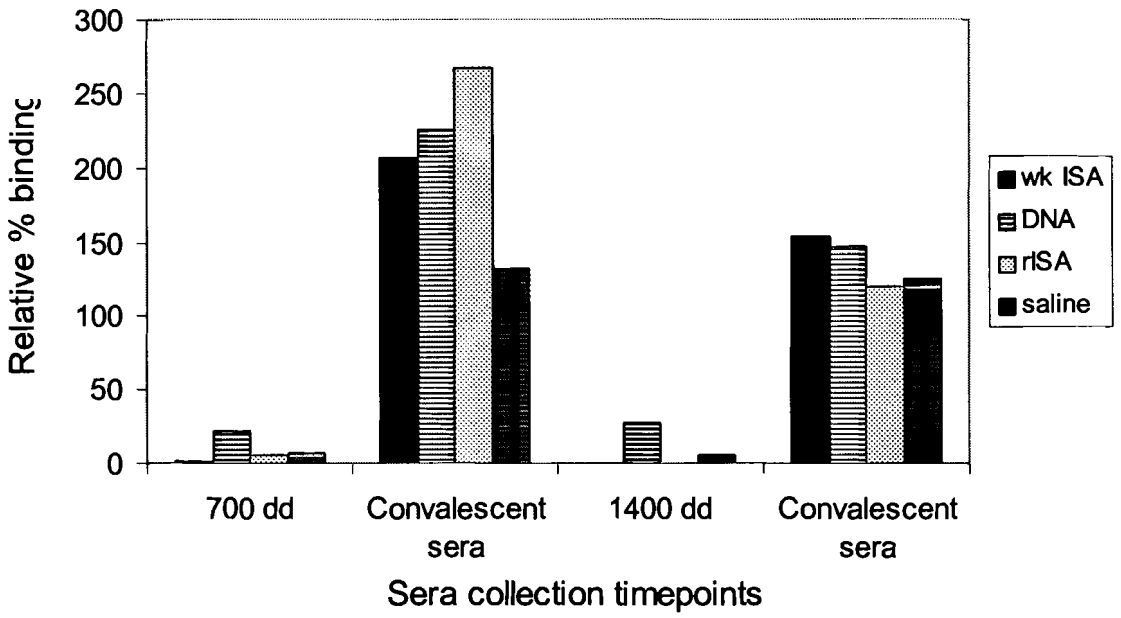

Figure 3.2. Antibody response to ISA virus vaccines in Atlantic salmon. Measured by ELISA before ISA virus challenge and at the end of the efficacy trial. Vaccines are as follows: 1. wk ISA: whole killed ISA virus vaccine; 2. DNA: trivalent DNA vaccine pISA-NP_{NA}, pISA-Ac_{NA}, pISA-HA_{NA}; 3. rISA: recombinant ISA virus vaccine (product of Maine BioTek, Inc. and Microtek Int. Ltd); 4. saline

Humoral Immune Response to ISA Virus Vaccines

ISA virus specific antibodies in Atlantic salmon immunized with ISA virus DNA, recombinant and whole killed vaccines were measured using an ELISA in Trial #5. Relative to mAb 10A3 (Clouthier *et al.*, 2002) and convalescent serum (see Figure 3.2) all of the vaccines in this study resulted in low binding titers of the respective sera (Figure 3.3). The HA, NP, AC and putative NS encoding DNA vaccines as well as the whole killed (4 x dose) ISA virus vaccine elicited ISA virus specific antibodies in Atlantic salmon 10-12 weeks post-vaccination. The presence of ISA virus specific

antibodies in Atlantic salmon in response to the recombinant and whole killed vaccine (2 x dose) was transient, antibodies being present 4-6 weeks post-vaccination (Figure 3.3).

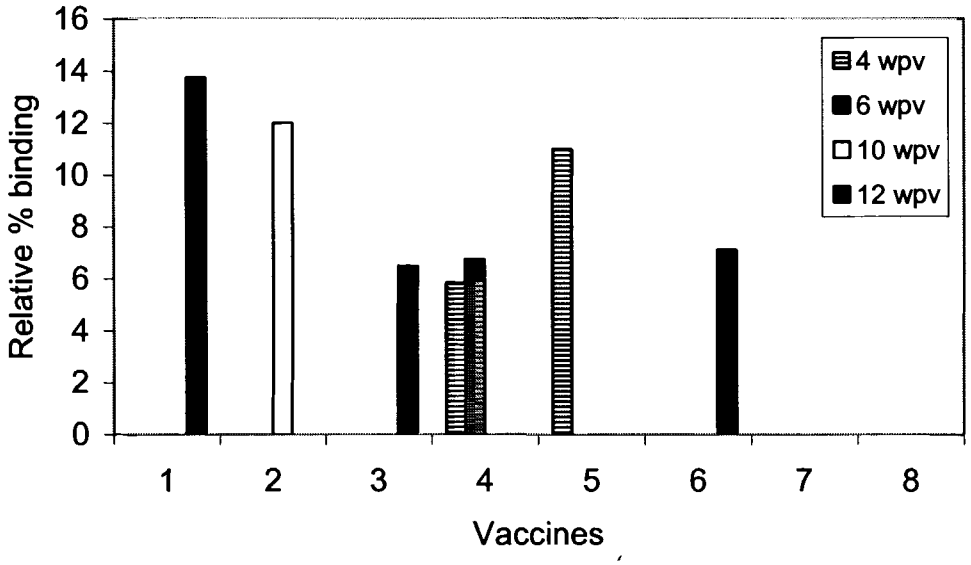

Figure 3.3 Antibody study: antibody production to ISA virus vaccines in Atlantic salmon as measured by ELISA. Sera samples were collected at 4, 6, 10 and 12 weeks post-vaccination. Vaccines 1-8 are as follows: 1, pISA-NP_{NA}; 2, pISA-Ac_{NA}; 3, pISA-HA_{NA}; 4, rISA; 5, wk ISA (2 x dose); 6, wk ISA (4 x dose); 7, pLuc; 8, Saline.

ISA virus specific antibodies in rainbow trout immunized with ISA virus DNA vaccines encoding the HA from North America or Norway were measured in Trial #1 using an ELISA (Figure 3.4). The HA from Norway pISA-HA_{Nor} and pISA-8_{NA}, encoding the putative matrix protein, elicited the highest antibody binding titer 8 weeks post-vaccination (wpv). The HA from North America, pISA-HA_{NA}, elicited antibody titers beginning 4 wpv and lasting for at least 12 wpv.

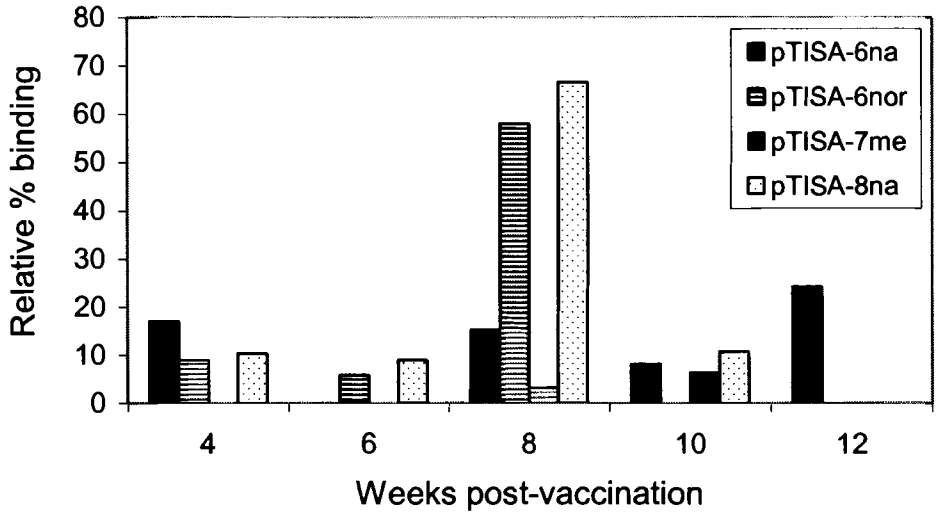

Figure 3.4 Antibody study: antibody production to ISA virus vaccines in rainbow trout as measured by ELISA. Sera samples were collected at 4, 6, 8, 10 and 12 weeks post-vaccination.

EVR: IHN and ISA Virus DNA Vaccines

The ISA and IHN virus vaccines did not elicit a measurable antibody response in Atlantic salmon or rainbow trout at 7 or 21 d post-vaccination as determined in Trial #3 by ELISA (data not shown). These data extend the observations of LaPatra et al. that fish antibodies do not mediate the early antiviral response (EVR) since they are not detectable in fish sera until 3-4 weeks after vaccination. The NS protein of influenza A, the prototypic orthomyxovirus, prevents activation of NF-kB and induction of alpha/beta interferon (Wang *et al.*, 2000). An antiviral interferon response may be an important mechanism of immunity elicited by the IHN virus G DNA vaccine. To determine if the NS protein of ISA virus could modulate the EVR by interfering with the putative interferon response elicited by the pIHNw-G vaccine we co-administered pISA-7_{NA} or

pISA-8_{NA} with pIHNw-G and challenged with IHN virus 7 and 28 d post-vaccination. The ISA virus proteins had no effect on the EVR elicited by pIHNw-G (Table 3.2).

Table 3.2. IHN virus efficacy trial: % mortality and RPS values

Vaccine and Dose	Challenge 7 dpv		Challenge 28 dpv	
	% Mortality	RPS	% Mortality	RPS
pIHNw-G (1 μg)	8	90	10	89
pISA-7_{NA} (1 μg)	71	8	80	9
pISA-7_{NA} (10 μg) + pIHNw-G (1 μg)	12	84	6	93
pISA-8_{NA} (1 μg)	78	0	73	17
pISA-8_{NA} (10 μg) + pIHNw-G (1 μg)	22	71	24	73
pISA-HA_{NA} (1 μg)	76	1	76	14
pISA-HA_{Nor} (1 μg)	71	8	63	28
pISA-NP_{NA} + pISA-Ac_{NA} + pISA-HA_{NA}*	75	3	80	9
pLuc (10 μg)	77	-	88	-

* 10 μg each vaccine

Chapter 4

DISCUSSION

This study was designed to measure the efficacy of ISA virus DNA vaccines encoding the HA, AC or NP (derived from North American or Norwegian ISA virus isolates) and the antibody response these vaccines elicited in Atlantic salmon and rainbow trout. The efficacy of the trivalent DNA vaccine was compared to the efficacy of a prototype recombinant ISA virus vaccine and a whole killed ISA virus vaccine. The ISA virus HA and NP were selected as vaccine target antigens based on the finding that Atlantic salmon that survive ISA virus challenge produce relatively large amounts of anti-HA and NP antibodies (Clouthier *et al.*, 2002). The DNA vaccine encoding the ISA virus HA was active in transfected EPC cells, serving as the template for HA protein synthesis (Figure 3.1). Passive transfer of anti-ISA virus antibodies in serum collected from convalescent fish to naïve Atlantic salmon protects the fish from ISA virus infection (unpublished data). The results suggest that HA specific virus-neutralizing antibodies are important in a successful immune response to ISA virus (Falk & Dannevig, 1995). ISA virus AC (P3) was included in the trivalent vaccine because the protein may be located on the surface of the virion and as such might be an important antigen functioning similar to the NA of influenza virus.

Antigenic drift and shift were also considered when selecting vaccine candidates for an ISA virus vaccine. Influenza virus undergoes antigenic shifts resulting in disease pandemics and antigenic drift that constitutes microheterogeneity within virus strains (Webster *et al.*, 1981). Antigenic shift has not been reported for ISA virus but distinct differences in nucleic acid and amino acid sequence have been observed upon comparison of the HA of North American and European ISA virus isolates (Krossoy *et al.*, 2001b). Further, ISA virus isolates from Nova Scotia, Canada, have been reported to be more closely related to European isolates than they are to viral isolates from New Brunswick, Canada and Maine (Ritchie *et al.*, 2001b). The potential for antigenic shift in ISA virus isolates was addressed with the inclusion of NP in the ISA virus trivalent DNA vaccine, the protein most likely to be involved in eliciting protection against heterologous virus. In influenza infections, the primary antiviral activity of the helper T cell is to provide help to B cells to produce antiviral antibodies (Scherle *et al.*, 1992) whereas the primary role of the CTL response is in clearance of an established infection (McMichael *et al.*, 1983). The latter is directed mainly against the highly conserved NP and is the basis for the cross-reactivity generated against all influenza virus subtypes in mice and man (MacKenzie *et al.*, 1989). The amino acid sequence of the putative nucleocapsid protein from the ISA virus isolate CCBB shares 96.6% identity to the NP protein sequence from Scottish isolate 390/98 (Clouthier *et al.*, 2002). The high sequence conservation (compared to 70-85% for the other proteins) suggests that the ISA virus NP protein may be a type-specific, cross-protective antigen, like the NP protein of influenza viruses (Lamb & Krug, 1996).

The relative percentage survival of fish immunized with the trivalent DNA vaccine, pISA-NP_{NA}, pISA-Ac_{NA}, pISA-HA_{NA} was 25% when the fish were inoculated with live virus 700 dd post-vaccination and 46% 1,400 dd post-vaccination (Table 3.1). The efficacy of the ISA virus vaccine is much less than has been reported for influenza virus DNA vaccines encoding the HA, NA or NP (Ulmer *et al.*, 1993; Chen *et al.*, 1999). The efficacy of the influenza virus DNA vaccine is dose dependent; increasing the vaccine dose increases the efficacy of the vaccine (Ulmer *et al.*, 1994). Whether increasing the dose or number of boost would increase the efficacy of the ISA virus DNA vaccine must be determined in future studies. The ISA virus DNA vaccine is considerably less efficacious than reported for DNA vaccines encoding the *Novirhabdovirus* G protein. For example, the efficacy of a single dose (0.1 - 1 μg) of the IHN virus G protein DNA vaccine pIHNw-G in Atlantic salmon has been shown previously to be 90-100% (Kurath *et al.*, 2001) and in this study was 90%. More studies will be required to determine the optimum ISA virus DNA vaccine dose.

The trivalent ISA virus DNA vaccine was not as efficacious as the whole killed or recombinant ISA virus vaccine. The reason for the differences in efficacy of the vaccine types is multivariant and the data presented here do not address the question. However, the amount of serum antibody in Atlantic salmon vaccinated with the DNA, whole killed or recombinant ISA virus vaccines was low at 700 and 1,400 dd post vaccination. In contrast, a greater amount of anti-ISA virus antibodies are present in convalescent fish (Figure 3.2). These results indicate that the humoral immune response is important for protection against ISA but the relative contribution of binding and neutralizing antibodies to the immunoprotective response is unknown and will be addressed in future studies.

Other studies have shown that convalescent fish are resistant to live ISA virus challenge and that the serum from convalescent fish, when injected into naïve fish, confers protection to subsequent exposure to ISA virus (unpublished results). Studies with whole killed and recombinant influenza virus vaccines show that the HA and NA elicit an immunoprotective response. The mediators of resistance to influenza virus in mice include binding and neutralizing antibodies (Epstein *et al.*, 1993). The titer of the HA or NA-specific antibodies is directly proportional to efficacy of influenza vaccines (Clements *et al.*, 1986; Murphy *et al.*, 1972). The relatively low antibody binding titers elicited by the vaccines in this study may account for their performance and suggests that strategies to boost the antibody titer in vaccinated fish may improve the efficacy of this vaccine.

The data indicate that IHN and ISA virus DNA vaccines encoding viral glycoproteins are not equivalent in their ability to stimulate an immunoprotective response in Atlantic salmon. The IHN virus G protein encoding DNA vaccine, pIHNw-G, elicits a non-specific, cross-reactive, immunoprotective response (EVR) 4-7 d post-vaccination (LaPatra *et al.*, 2001). Neutralizing or binding antibodies are not required for the EVR. In addition, the pIHNw-G vaccine elicits a specific viral response (SVR) beginning approximately 4 wk post-vaccination. The appearance of serum antibodies coincides with the loss of the EVR elicited by pIHNw-G and the appearance of the SVR. The SVR elicited by pIHNw-G provides 90-100% RPS 1-3 months post-vaccination, 69% RPS 6 mo post-vaccination and 65% RPS 13 mo post vaccination (Kurath *et al.*, 2001). To determine if ISA virus proteins can elicit an EVR or a SVR, rainbow trout were vaccinated with ISA virus DNA vaccines and challenged with IHN virus 7 or 28 d

post-vaccination (Table 3.2). The ISA virus DNA vaccines encoding the HA, NP or AC proteins did not elicit an EVR that was immunoprotective against heterologous challenge with IHN virus. The data from these studies support the idea that the *Novirrhabdovirus* G protein in the DNA vaccine plays an important, possibly unique role in eliciting an EVR.

The NS protein of influenza A, the prototypic orthomyxovirus, prevents activation of NF-kB and induction of alpha/beta interferon (Wang *et al.*, 2000). An antiviral interferon response may be an important mechanism of immunity elicited by the IHN virus G DNA vaccine. For example, there is an increase in proteins mediating cellular responses to interferon in fish vaccinated with DNA vaccines encoding novirhabdovirus G proteins (Boudinot *et al.*, 1999; 2001; Collet & Secombes, 2001; Hansen & LaPatra, 2002; Trobridge & Leong, 1995; Trobridge *et al.*, 1997). Due to the similarities between influenza viruses and the ISA virus it is possible that the ISA virus possesses a gene that has a similar immunosuppressive function. A recent study by Biering *et al.* (2002) suggests that the NS protein of ISA virus may possibly be encoded by genome segment 7 and the matrix protein by genome segment 8. Co-administration of pISA-7_{NA} or pISA-8_{NA} with pIHNw-G had little effect on the EVR upon challenge with IHN virus. In the framework of this study, the putative NS protein of ISA virus does not seem to affect the EVR. Further studies will be required to determine if interferon is involved in the EVR.

In summary, a group of Atlantic salmon vaccinated with the trivalent ISA virus DNA vaccine we developed, resulted in an RPS of 25% when the fish were inoculated with live virus 700 dd post-vaccination and 46% 1,400 dd post-vaccination. The efficacy of the trivalent ISA virus DNA vaccine was modest when compared to the efficacy of a

whole killed or recombinant ISA virus vaccine. In addition, the efficacy of the ISA virus DNA vaccines was much less than the immunoprotection conferred by the vaccine pIHNw-G in rainbow trout against IHN virus challenge. None of the vaccines elicited a pronounced antibody response. The poor antibody response may be related to the relatively modest efficacy of the ISA virus vaccine, which contrasts with IHN virus vaccines for which little correlation exist between immunoprotection and seroconversion. Finally, the trivalent ISA virus vaccine did not elicit an immunoprotective EVR in rainbow trout suggesting that the fish *Novirhabdovirus* G protein may have intrinsic molecular patterns recognized by the innate and adaptive arms of the immune system.

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