Gene Expression of Infectious Salmon Anemia Virus Relevant to Understanding its Pathogenesis in Fish

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ABSTRACT

Infectious salmon anemia virus (ISAV) is an orthomyxovirus recently assigned to the genus *Isavirus*. It is currently the most important viral pathogen threatening commercial salmonid aquaculture in the Northern Hemisphere. The virus causes infectious salmon anemia (ISA) a highly fatal disease in marine farmed Atlantic salmon (AS). The disease is characterized epidemiologically by variable mortalities, grossly by exopthalmia, pale gills and ascites, and microscopically by congestion and hemorrhagic necrosis of internal organs. Difficulties have been reported in reproducing the lesions associated with the infection experimentally and in some cases infected AS are asymptomatic. Also the virus seems to persist in recovered or asymptomatic fish, a phenomenon unusual for other orthomyxoviruses. In this study, *in situ* hybridization (ISH) was used to monitor the virus gene expression *in vitro* and *in vivo,* while histology was used to correlate lesions with presence of viral mRNA.

ISH conditions were established for the detection of mRNA transcripts of ISAV RNA segments 6 - 8 in infected TO, SHK-1 and CHSE-214 cell lines. Hybridization signals were observed first in the nucleus and then in the cytoplasm of infected cells, which is consistent with the nuclear transcription and replication of other orthomyxoviruses. Transcription signals were intense and widespread for all three mRNAs in TO cells and in few cells in SHK-1 cells from one day post infection (dpi). In CHSE-214 cells, maximum hybridization signals were seen in the nucleus at 2, 4 and 5 dpi with segments 8, 7 and 6 riboprobes respectively. The earlier transcription of segment 8 and later transcription of segment 7 might suggest that there is a difference in the time requirement and amount of proteins encoded by these segments. ISH with segments 7 and 8 riboprobes, and histology were used to study the relationship between the presence of lesions and viral mRNA in AS liver, spleen, kidney, heart, gills and pyloric caeca collected during clinical phases of the infection. Three groups of AS were infected with three ISAV isolates, two belonging to North American (RPC and CH7) and one to European (NSC) HA genotypes. Lesions consistent with ISA were observed in 100% of spleen, 95% of livers and pyloric caeca, 82% of hearts, 60% of gills and 27% of kidneys. Of the tissues examined, the heart and liver consistently showed the strongest hybridization signals and therefore the most *in situ* viral mRNA which was located in the endothelium of blood vessels and in leucocytes. Signals were observed in 100% of livers and hearts, 93% of gills, 84% of spleen, 79% of pyloric caeca, and 56% of kidneys. Lesions were most correlated with viral mRNA in the liver and least in the kidney. Viral RNA was not detected in parenchymatous cells undergoing degeneration and necrosis. Attempts at identifying the viral mRNA in tissues of fish that survived an experimental challenge by ISH were unsuccessful. However, the segment 8 RNA was detected in all organs of infected rainbow trout, coho and AS up to 6 weeks post natural mortality by RT-PCR. The absence of signals by ISH, indicated that the viral RNA might be present at very low levels detectable only by a more sensitive method like RT-PCR.

The gene expression of segments 7 and 8 mRNA of three ISAV isolates (RPC, NSC, and NOR) were further studied in a TO cell line. Distinct hybridization signals were detected at 24 hours post infection (hpi) with both riboprobes in all isolates, while CPE was observed

from 48 hpi in the RPC and NOR and later in NSC infected cells. The NOR and NSC isolates belong to the European hemagglutinin genotype. The NOR and RPC infected cells showed similar intensity and frequency of hybridization signals up to 96 hpi, unlike the NSC infected cells which were significantly lower (P= 0.004). Overall, signal intensity appeared to be higher with segment 7 riboprobe. This result suggested that the frequency and intensity of signals are dependent on the isolate rather than the genotype. The difference in hybridization signals between isolates was further characterized in infected AS using segment 7 riboprobe and histologic examination of heart, liver and kidney. Signals were observed first at 3 dpi in heart endothelial cells of NOR infected group and at 6 and 10 dpi in RPC and NSC infected groups respectively and thereafter in other organs. Severe histopathological lesions were observed beginning with the onset of mortality in the three infected groups. The severity of lesions correlated with maximum intensity and frequency of ISH signals ($P < 0.001$) in all groups. There was a strong association between ISH signals and severity of lesions in the three organs ($R = 0.70 - 0.81$ respectively, $P < 0.001$). The ISH signals were indicative of viremia as they were observed predominantly in individual blood cells and endothelial cells of the three organs, but not in the necrotic areas. These findings further support the view that while the endothelial lesions are a direct result of virus multiplication, the hepatocellular and renal tubular necrosis are probably secondary to ischemia. The NSC isolate appeared to be the least virulent isolate compared to the NOR and RPC isolates (P= 0.001).

The presence of viral mRNA in blood cells was further studied in blood samples collected from NOR infected AS. Fixed blood smears on glass slides were used for cytology and ISH. Hybridization signals were observed only in leucocytes from 8 - 12 dpi. This supports the inclusion of leucocytes among the viral target cells. The absence of signals in the erythrocytes indicates their inability to harbor the virus and therefore are not part of the viral target cells.

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DEDICATION

To my loving uncle late Benjamin Moneke, my parents and Ijeoma

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LIST OF ABBREVIATIONS

Abbreviations	Term
Standard units of measurements	
Amp	amplitude
bp	base pair
^0C	degree Celsius
cm	centimeter
≥	equal to or greater than
≤	equal to or less than
g	grams
hr	hour
kb	kilo base pair
kDa	kilo Dalton
M	molarity
ng	nanogram
µg	microgram
µl	microliter
µm	micrometer
m^2	meters (square)
mAmps	milliamplitude
min	minutes
ml	milliliter
mM	millimole
nm	nanometer
N	normal (concentration)
pH	hydrogen ion activity (negative logarithm)
rpm	revolutions per minute
RT	room temperature
UV	ultraviolet
W	watts
Others	
aa	amino acid
AS	Atlantic salmon
AP	alkaline phophatase
cDNA	complimentary deoxyribonucleic acid
CHSE-214	Chinook salmon embryo cell line
CS	coho salmon
CPE	cytopathic effect
dATP	deoxyadenosine triphosphate
dCTP	deoxycytosine triphosphate
DEPC	diethyl pyrocarbonate
DIG	digoxigenin
DIG-11-UTP	digoxigenin-11-deoxyuridine triphosphate

dGTP	deoxyguanine triphosphate
DNA	deoxyribonucleic acid
DNAase	deoxyribonuclease
dNTPs	mixture of dATP, dCTP, dGTP and dTTP
dsDNA	double stranded DNA
dpi	days post infection
dTTP	deoxythymidine triphosphate
dUTP	deoxyuridine triphosphate
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
Fab	fragment antigen binding
FBS	foetal bovine serum
GC	guanine-cytosine
^{3}H	radioactive hydrogen isotope
H & E	hematoxylin and eosin stain
HA	hemagglutinin protein
HE	hemagglutinin-esterase
HPR	highly polymorphic region
hpi	hours post infection
IFAT	indirect fluorescent antibody test
IHNV	Infectious hemopoietic necrosis virus
Ig	immunoglobulin
IPNV	Infectious pancreatic necrosis virus
ISA	Infectious salmon anemia
ISAV	Infectious salmon anemia virus
ISH	*in situ* hybridization
Mab	monoclonal antibody
M1, M2	matrix protein
MDBK	Madin-Darby bovine kidney
mRNA	messenger ribonucleic acid
NA	neuraminidase
NBT	nitroblue tetrazolium
NEP	nuclear export protein
NP	nuclear protein
NS1	non-structural protein
ORF	open reading frame
^{32}P	radioactive phosphate isotope
PBS	phosphate buffered saline
PB1, PB2, PA	polymerase proteins
PCR	polymerase chain reaction
pnm	post natural mortality
RDE	receptor destroying enzyme
RNA	ribonucleic acid
RNAse	ribonuclease

RNP	ribonucleoprotein
RbT	rainbow trout
RT-PCR	reverse transcriptase polymerase chain reaction
^{35}S	radioactive sulphur isotope
SDS	sodium dodecyl sulfate
SHK-1	salmon head kidney cell line
ssDNA	single stranded deoxyribonucleic acid
sscRNA	single stranded complimentary ribonucleic acid
vRNA	viral ribonucleic acid
TAE	tris, acetic acid, EDTA buffer
$TCID_{50}$	median tissue culture infective dose
TO	head kidney leucocyte cell line
TE	tris, EDTA buffer
3'	three prime
5'	five prime

Infectious salmon anemia virus (ISAV) isolates

NBC	NBISA01
NOR	Norway 810/9/99
RPC	RPC-01-0593-1
NSC	U5575-1
CH7	7833-1

CHAPTER 1

GENERAL INTRODUCTION AND LITERATURE REVIEW*

***Portions of this chapter appear in:**

KIBENGE FSB, MUNIR K, KIBENGE MJT, JOSEPH T, **MONEKE E** (2004) Infectious salmon anemia virus: the causative agent, pathogenesis and immunity. Ani Health Res Rev, in press.

1.1 GENERAL INTRODUCTION

Infectious salmon anemia virus (ISAV) is currently one of the most important viral pathogens threatening commercial Atlantic salmon mariculture in the Northern Hemisphere. ISAV is a member of the family *Orthomyxoviridae* belonging to the genus *Isavirus*. ISAV causes a disease, infectious salmon anemia (ISA), that is characterized by high morbidity and mortality in affected fish. Much of what is known about the pathogenesis of the virus is based on studies using fish cell lines that are permissive to the virus growth (Dannevig et al., 1995a,b; Bouchard et al., 1999; Kibenge et al., 2000b). Apart from the use of RT-PCR and IFAT to detect the viral RNA and antigen in fish tissues, little is known about the pathogenesis of the virus in both Atlantic salmon and other fish species. Recently, there have been increasing numbers of reports in the variation, and in some cases complete absence, of pathology observed in affected fish (Jones et al., 1999; Lovely et al., 1999; Mjaaland et al., 2002). Similarly, the paucity of information on the interaction of the virus and the host have made it more difficult to understand the mechanisms for spread and persistence of the virus in both asymptomatic and recovered fish.

This thesis describes the development and optimization of molecular methods that allowed for the study of direct virus interaction with the host. The methods included virus isolation, reverse transcriptase polymerase chain reaction (RT-PCR) and *in situ* hybridization. These techniques were used to visualize the progression and pattern of gene expression and viral pathogenesis using several different ISAV viral RNA segments. Histology was used to study the development of lesions as the infection progressed. This work has been presented as follows: Chapter 1, a literature review of the pathogenesis of

the virus; Chapter 2, the general materials and methods used to study the specific objectives of this thesis; Chapter 3, the development of the different riboprobes and the optimization of ISH conditions and ISAV gene expression in fish cell lines; Chapter 4, the optimization of ISH conditions and demonstration of viral mRNA in ISAV-infected fish tissues using different riboprobes; Chapter 5, the possible correlation between viral mRNA of different ISAV isolates and the lesions associated with the infection/disease, and attempts to identify the sites of ISAV persistence in recovered fish; Chapter 6, further characterization of the observable differences in hybridization signals of different ISAV isolates from different haemagglutinin genotypes in TO cells; Chapter 7, further studies on the differences in hybridization signals and the virulence of the different ISAV isolates in Atlantic salmon; Chapter 8, the blood cells associated with viremia of ISAV seen in fish with highly pathogenic isolates of ISAV; and Chapter 9, a general discussion of the results and a conclusion that will hopefully help the reader better understand the pathogenesis of ISAV.

1.2 LITERATURE REVIEW

1.2.1 History of Infectious salmon anemia (ISA) in Canada

Infectious salmon anemia virus (ISAV), is the causative agent of ISA, a highly infectious disease that affects farmed Atlantic salmon, *Salmo salar L.* ISA was first reported in a hatchery on the southwest coast of Norway in November 1984 (Thorud and Djupvik, 1988). Retrospective studies however, now show the presence of the disease to have been earlier than reported (Nylund et al., 1995a). There was no record of ISA outside Norway until July of 1996 when increasing numbers of moribund and dead Atlantic salmon

were reported on marine fish farms in the Bay of Fundy (Lime Kiln Bay, Bliss Harbor and Seal Cove), New Brunswick (Mullins et al., 1998; O'Halloran et al., 1999) and the virus was subsequently isolated from affected fish in 1998 (Bouchard et al., 1999; Lovely et al., 1999). Initially, the disease was diagnosed as hemorrhagic kidney syndrome (HKS) because of the predominant lesions present in kidneys (Byrne et al., 1998), although other lesions similar to ISA were present; the disease was later confirmed as the first recorded ISA case in North America. Epidemiological studies showed that more than 21 farms in New Brunswick were affected by ISAV during the 1997 outbreak and mortalities were high (Hammell and Dohoo, 1999). The spread of the virus continued in 1998 with new sites being affected such as Beaver Harbor, Back Bay, L' Etete Passage and a farm in Deer Island. Eleven sites tested positive for ISAV in January 1999. Recent control measures adopted to check the spread of the virus have helped in reducing the incidence of ISA in New Brunswick. ISA virus was also detected in farmed Atlantic salmon in Nova Scotia in 1999 (Hatt, 1999; Ritchie et al., 2001b) and a clinical outbreak identified in another farm in 2000 (Dr. Rolland Cusack personal communication). More than $70 million was lost in the first 3 years by the New Brunswick aquaculture industry alone due to ISA (Hasting et al., 1999).

1.2.2 Characteristics of ISAV

1.2.2.1 Morphology

ISAV is an enveloped virus that is pleomorphic in shape but appearing mostly spherical with a mean diameter of 100-140 nm (Hovland et al.,1994; Nylund et al., 1995b). It has

surface glycoprotein spikes or peplomers with a diameter of 10-13 nm radiating outward from the lipid envelope (Nylund et al., 1995b). The virus has a single-stranded RNA genome of eight segments of negative polarity ranging in size from 1 to 2.3 kilobases (kb) in size with a total molecular size of approximately14.5 kb (Mjaaland et al., 1997; Clouthier et al., 2002).

1.2.2.2 Classification

The morphological, biochemical and physicochemical properties of ISAV are closely related to the influenza viruses (Falk et al., 1997; Mjaaland et al., 1997; Krossøy et al., 1999; Eliassen et al., 2000; Sandvik et al., 2000). On this basis, the virus has been included in the family *Orthomyxoviridae*. The virus is distinct enough however, to be classified in a new genus *Isavirus* (Anon., 2001).

1.2.2.3 Viral genes and proteins

The initial assumption was that since the number and organization of RNA segments of ISAV were closely related to those of influenza viruses A and B, there was a possibility to draw a parallel on proteins encoded. All segments of ISAV genome have been fully sequenced (Clouthier et al., 2002) and this information is publicly available in the GenBank database. Several isolates of ISAV from different geographic regions have also been fully or partially sequenced. The genome information has shown that the gene order of ISAV has some important differences from that of influenza viruses (Tables 1.1).

Falk et al. (1997), separated the viral proteins of Norwegian ISAV isolate Glesvaer/2/90 by SDS-PAGE and used silver staining to show that it has four major polypeptides with estimated molecular sizes of 71, 53, 43 and 24 kDa. Kibenge et al. (2000a), using Coomassie blue staining and Western blotting, showed a similar protein profile for the Back Bay 98 and RPC/NB-049 ISAV isolates from New Brunswick, with molecular sizes 74, 53, 43, 26.5 kDa, and 71, 53, 46, 26.5 kDa for the Glesvaer isolate. In 2001, Kibenge et al. (2001a) using a more sensitive method of radiolabeling the viral proteins combined with immunoprecipitation by rabbit anti ISAV antibody detected twelve ISAV viral proteins of molecular sizes 80-94, 69, 38-41.5, 33.5-36, 30, 25, and 19-20 kDa from isolates 7833-1, Back Bay 98 and RPC/NB 970-877-2 suggesting that ISAV and influenza virus may have the same number of proteins. The exact functions of most of the ISAV proteins remain to be demonstrated. The putative viral proteins and their molecular sizes are summarized in Table 1.2.

Based on sequence analysis, ISAV genome segment 1 encodes the putative polymerase protein, PB2 (Clouthier et al., 2002; Snow et al., 2003a), segment 2 encodes the polymerase protein, PB1 (Krossøy et al., 1999; Blake et al., 1999; Clouthier et al., 2002). The putative ISAV PB1 has an amino acid sequence identity of 20.8 to 24.1% to those of other orthomyxoviruses (Krossøy et al., 1999) and is the most conserved protein (Lin et al., 1991). Segments 3 and 4 encode the nucleoprotein, NP (Snow and Cunningham 2001; Ritchie et al., 2001a; Clouthier et al., 2002), and putative polymerase protein, PA (Ritchie et al., 2001a; Clouthier et al., 2002), respectively.

The putative NP amino acid sequences of ISAV have 11.65-12.28% similarity to other orthomyxoviruses on pairwise comparison (Snow and Cunningham, 2001).

Table 1.1 Comparison of putative gene coding assignments of RNA segments of ISA virus and influenza A virus.

Gene	RNA segments		
	ISAV	Influenza A	Influenza B
PB2	1	2	2
PB1	2	1	1
NP	3	5	5
PA	4	3	3
NA (?)	5(?)	6 (NA)	6 (NA)
HA or HE	6 (HE)	4 (HA)	4 (HA)
NS1 & NS2 (?)	7 (?)	8 (NS1 & NS2)	8 (NS1 & NS2)
M1 & M2 (?)	8 (M and ?)	7 (M1 & M2)	7 (M1 & M2)

Table 1.2 The gene coding assignments and the putative viral polypeptides of ISAV

RNA Segment	Size (bp)	ORF (bp)	Putative protein	Molecular mass (KDa)	Accession #
1	2185	2127	PB2	80.5	AF404346
2	2205	2169	PB1	80	AJ514403
3	2046	1851	NP	72	AF40434
4	1085	1737	PA	65.3	AF306548
5	1504	1335	?	47	AF404343
6	1053-1261	1053-1168	HE	42	AF3020799 AF302803
7	1006	903/522	?/?	35.4/17.5	AY044132 AF404341
8	930	705/552	?/M	22/27	Y10404 AF404340

Secondary structure predictions and alignment of the putative ISAV NP revealed similar overall structures to those of influenza A and B viruses. Other features such as the phosphorylation site, hydrophobic regions and putative RNA binding sites with amino acids similar in physico-chemical characteristics to influenza viruses also provided further evidence that the putative ISAV NP might possess the same functional characteristics (Snow and Cunningham, 2001). Falk et al. (2004) recently analyzed metabolically radiolabeled inorganic ^{32}P ISAV Glesvaer/2/90 in infected cell lysates and revealed that the estimated 66 kDa protein was the only phosphorylated structural protein in the virion. They attributed the difference in molecular mass to the previously predicted 68 - 72 kDa for the protein (Krøssoy et al., 2001a; Biering et al., 2002; Clouthier et al., 2002) to a different molecular mass standard used. All the major viral structural proteins from ^{35}S methionine-labeled ISAV solublized when treated with lysis buffer containing Nonidet P40 (NP40) except the 66 kDa protein which remained complexed with RNA, prompting Falk et al. (2004) to conclude that it is the NP protein. The NP protein is a major structural protein that encapsidates viral RNA and is associated with the polymerase complex (PB1, PB2 and PA) to form viral ribonucleocapsid (vRNPs) complex (Lamb and Krug, 1996).

The genomic RNA segment 5 has been sequenced but the putative protein (P3) encoded is not yet identified (Clouthier et al., 2002). The genome segment 6 encodes the hemagglutinin protein (Rimstad et al., 2001; Krossøy et al., 2001b; Griffiths et al., 2001; Clouthier et al., 2002) and recently this protein was shown to also posses esterase activity (Falk et al., 2004). To date, only the segment 6 encoded protein has been functionally characterized as having hemagglutinin activity and was therefore initially designated HA protein (Krossøy et al., 2001b; Rimstad et al., 2001). The HA protein of ISAV like that of

influenza virus binds terminal sialic acid (glycoconjugates terminating in N-acetyl neuraminic acid) on host cell surfaces mediating the attachment of virus particle to the cell (Lentz, 1990; Eliassen et al., 2000) and the penetration of the virus into the cytoplasm via low pH-dependent fusion of the envelope of the endocytosed virus with the endosomal membrane (Tong et al., 1998; Eliassen et al., 2000). This allows the release of the viral nucleocapsid into the cytoplasm. In influenza virus, proteolytic cleavage of the HA into HA_1 and HA_2 is required to trigger the pH-dependent fusion (Huang et al., 1981; Kitame et al., 1982) which is essential for the virus to be infectious. The HA protein of ISAV has been suggested not to undergo proteolytic cleavage (Krossøy et al., 2001b). The orthomyxovirus HA protein is a major surface antigen against which neutralizing antibodies are produced in the host as well as, an important determinant for the host range of the virus. The hemagglutinin gene/protein of ISAV is highly variable and polymorphic (Rimstad et al., 2001; Krossøy et al., 2001a, Kibenge et al., 2001b), like that of influenza virus (reviewed by Webster et al., 1999). The variations in the influenza HA protein may contribute to the escape of influenza A virus strains from the immune response (Webster and Laver, 1980; Lambkin et al., 1995).

The surface protein projections of ISAV have sialidase activity (Falk et al., 1997). The sialidases remove sialic acids present on glycoproteins or glycolipids and are termed receptor destroying enzyme, RDE. RDE is required to keep the virus free from sialic acid receptors and stop the clumping or aggregation of released virus due to HA binding (Hofling et al., 1996). It also plays a role in the initiation of infection, by either promoting fusion activity (Huang et al., 1980; 1985) or removing sialic acid from oligosaccharides that might interfere with binding to cellular receptors near the receptor-binding site. Unlike

influenza virus which possesses neuraminidase as the RDE, ISAV has been shown to possess acetylesterase (Falk et al., 1997; Kristiansen et al., 2002) and not neuraminidase activity, suggesting the acetylesterase as the putative RDE. Falk et al. (2004) recently characterized the hemagglutination and esterase activity of ISAV with two serine hydrolase inhibitors, diisopropyl fluorophosphate (DFP) and 3,4-dichloroisocumarin (DCIC), and concluded that these inhibitors did not inhibit hemagglutination but they inhibited at very low concentrations the elution of the virus from horse, rabbit and rainbow trout erythrocytes by the viral esterases. Falk et al. (2004) located the esterase activity in ISAV to the heamagglutinin protein by incubating purified ISAV with serine hydrolase inhibitor 1, 3-^{3}H (DFP), followed by SDS-PAGE analysis of the labeled protein that comigrated with the 42-kDa hemagglutinin protein. They confirmed their findings by immunoprecipitation from infected cell lysate the HA protein with anti-ISAV HA monoclonal antibody 3H6F8 (Falk et al., 1998), and demonstrated acetylesterase activity in the precipitate by incubating them with p-nitrophenyl acetate (pNPA) (Falk et al., 2004). Therefore, they proposed that the hemagglutinin protein be renamed hemagglutinin-esterase (HE) protein even though the esterase activity has not yet been demonstrated in the heterologously expressed protein.

The coding assignment of genome segments 7 and 8 is still controversial. The two segments encode two proteins as in influenza A virus. However unlike in influenza A virus in which the second protein is produced via alternate splicing of the ORF1 mRNA, splicing seems to occur only in the segment 7 ORF1 mRNA of ISAV (Biering et al., 2002). Genome segment 7 has been reported to encode the putative membrane (matrix) proteins, M1 and M2 (Ritchie et al., 2002; Clouthier et al., 2002) and genome segment 8 to encode the putative non structural protein NS and nuclear export protein NEP (Blake et al., 1999;

Krossøy et al., 1999). A recent report by Biering et al. (2002) however, suggested that genome segment 7 encodes a non-structural or minor structural protein, while genome segment 8 encodes the 24 kDa major structural protein. Falk et al. (2004) recently used Western blotting with rabbit antibodies to the recombinant protein encoded by the large ORF of genome segment 8 (Biering et al., 2002) to show that the viral protein (vp22) found in pelleted and soluble fractions of infected cells was the same as the 24 kDa expressed by Biering et al. (2002). In addition, by staining of major ISA viral proteins with biotinylated concanavalin A and radioimmunoprecipitation assay (RIPA) of cell lysates labeled with ^{3}H mannose, Falk et al. (2004) revealed that the 42 and 50 kDa proteins were glycosylated, while the vp22 was non-glycosylated. The 42, 50 and a major part of the vp22 kDa proteins from purified ^{35}S methionine-labeled virus solubilized when treated with lysis buffer with NP-40 and detergent, while the minor part of the vp22 and the phosphorylated 66 kDa NP remained in the pelleted fraction, thus like other influenza viruses there is no complete separation of the vp22 protein from the ribonucleoprotein complex using nonionic detergents (Falk et al., 2004). They confirmed the identity of the vp22 as M protein by immunofluorescent staining of ISAV infected cells with anti-recombinant vp22 polyclonal antibody (Biering et al., 2002), then double stained with anti-HA monoclonal antibody 3H6F8 and showed that the vp22 appeared first in the nucleus as well as in the cytoplasm together with the HA protein at 24 hpi, suggesting they are both late proteins.

In influenza A virus, the M1 protein is the most highly conserved protein of influenza viruses indicating a high functional density (Brown, 2000). It functions as the matrix protein which underlies the viral lipid envelope and provides rigidity to the membrane and is also one of the most abundant polypeptides in the orthomyxovirus virion (Lamb and Krug,

1996). Falk et al. (2004) used densiometric analysis of autoradiograms of SDS-PAGE gel of radiolabeled purified ISAV Glesvaer/2/90 and calculated the relative amounts of the four viral major proteins as 40, 37, 11 and 12% for the 22-, 42-, 50-, and 66 kDa bands respectively, suggesting that the vp22 is the most abundant ISA virus structural protein.

The nonstructural protein of ISAV was initially thought to be encoded by genomic segment 8 RNA as in influenza viruses (Mjaaland et al., 1997). However, the gene coding of the nonstructural protein and its functional expression in ISAV are yet to be determined conclusively, since Biering et al., (2002) have suggested that segment 7 encodes non structural or minor structural proteins. Kibenge et al. (2003) studied the individual gene-coding assignments of ISAV by expressing the individual ORF in the eight RNA segments *in vitro* with rabbit reticulocyte lysates and analyzed the translation products by immunoprecipitation with rabbit antiserum to the purified virus. They reported that the *in vitro* products/transcripts of segments 1 -7 and ORF1 in segment 8 of ISAV are structural viral proteins. The NS1 protein is abundant in influenza virus-infected cells, and is not present in virions. It is the only nonstructural protein of influenza viruses (Knipe, 1996). NS1 is a phosphoprotein found in the nucleus early during infection and also in the cytoplasm at later times in the viral replication cycle (Egorov et al., 1998). The nuclear export protein (NEP) of influenza viruses was originally named NS2 (for nonstructural 2 protein) (Richardson and Akkina, 1992; Yasuda et al., 1993). It is responsible for nuclear export of vRNP for assembly via its interaction with M1 (O'Neill et al., 1998).

1.2.2.4 ISAV replication *in vitro*

Initial *in vitro* study of ISAV was hampered because it could not grow in the commonly available continuous fish cell lines like epithelium papillosum of carp (EPC), rainbow trout gill (RTG-2), fathead minnow (FH-10) and brown bullhead (BB) commonly used to isolate salmon viruses (Falk et al., 1997; Byrne et al., 1998). The virus was first grown in primary cultures of leucocytes from Atlantic salmon (Dannevig and Falk, 1994). The susceptibility of leucocytes was variable and the virus yield was low. Later, a continuous cell line, SHK-1, which allows the growth of the virus with development of CPE, was established from the culture of head kidney leucocytes of Atlantic salmon (Dannevig et al., 1995a,b; 1997). CPE was observed by 12 days post infection (dpi). When the virus was titrated in the cell line, moderate infectivity titers of 10^6 to 10^7 $TCID_{50}$/ml were obtained (Falk et al., 1997; Kibenge et al., 2000a). This cell line is widely used for primary isolation of ISAV. However, it is a very delicate cell line requiring very low split ratios and a complex growth medium. In addition, at higher passages this cell line loses sensitivity for ISAV resulting in poorly defined and slow developing CPE by some ISAV strains (Falk et al., 1998; Rolland et al., 2003). Furthermore, it has not always been possible to isolate virus from outbreaks using this cell line (Rimstad and Mjaaland, 2002).

Another cell line, Chinook salmon embryo (CHSE-214) cell line, has been used to isolate ISAV from clinical specimens (Bouchard et al., 1999). The CHSE-214 cell line has the advantage of a higher split ratio (1:4) and can be grown easily in the laboratory. Consequently, it is often used for harvesting the virus, such as in antigen production for use in vaccines or as a diagnostic reagent (Kibenge et al., 2000a). However, like SHK-1 cells, some ISAV isolates are noncytopathic in CHSE-214 cells (Kibenge et al., 2000b)

while others do not replicate in this cell line at all (Munir and Kibenge, 2004), thereby limiting its utility in virus isolation (Kibenge et al., 2000a). Another disadvantage of the CHSE-214 cells is that the CPE develops slowly, taking up to 17 dpi when cytopathic strains of ISAV are used to infect the cells, and the virus yields are lower ($10^{4.5}$ to $10^{6.5}$ $TCID_{50}$/ml) compared to SHK-1 cells (Kibenge et al., 2000a).

Newer cell lines derived from Atlantic salmon head kidney leucocytes such as TO (Wergeland and Jakobsen, 2001) and ASK-2 (Devold et al., 2000; Rolland et al., 2003) cell lines develop more discernible and quicker CPE (by 2-4 dpi) and also provide higher virus yields of up to $10^{9.1}$ $TCID_{50}$/ml (Wergeland and Jakobsen, 2001), and require less complex growth media. The cell lines, however, still have low split ratios, and may be unsuccessful in virus isolation from some ISAV RT-PCR positive samples (Kibenge et al., 2001a). ISAV also replicates but is noncytopathic in Atlantic salmon (AS) (Sanchez et al., 1993; Sommer and Mennen, 1997) and rainbow trout gill (RTgill-W1) cell lines (Bols et al., 1994; Falk et al., 1997). Virus replication can be detected by indirect fluorescent antibody test (IFAT), but the virus yield is very poor; the maximum viral titer reported on these cells was 10^3 $TCID_{50}$/ml (Falk et al., 1997). In summary, all the currently available fish cell lines are either not sensitive enough or not permissive for all ISAV strains (reviewed by Kibenge et al., 2004).

The replication strategy of ISAV seems to resemble that of influenza viruses (Hovland et al., 1994; Falk et al., 1997; Mjaaland et al., 1997; Sandvik et al., 2000; Eliassen et al., 2000; Rimstad and Mjaaland, 2002) although the biochemical events involved in ISAV replication have not been fully explored. The influenza virus attaches via its HA protein to host-cell receptors terminating in sialic acids (Zambon, 2001).

Hemagglutination assays with erythrocytes from several fish species have indicated that ISAV also uses the HA protein to bind sialic acid receptors on the cell surface (Falk et al., 1997; Eliassen et al., 2000; Kristiansen et al., 2002; Hellebø et al., 2004). Structurally diverse as well as certain unique sialoglycoproteins have been reported to occur in salmonid species (Iwasaki et al., 1990). Because ISAV agglutinates horse erythrocytes, and since these contain only *N*-glycol neuraminic acid, it has been suggested that this may be the cellular receptor for ISAV (Kristiansen et al., 2002).

Hellebø et al. (2004) recently analyzed the substrate specificity of the ISAV esterase on Atlantic salmon and horse erythrocytes by hemagglutination and hemagglutination inhibition assay of purified virus using different hemagglutination inhibitors that predominantly contain 9-O-acetylated sialic acids (bovine submaxillary mucin (BSM) and rat serum) or 4-O-acetylated sialic acids (guinea pig serum, horse serum and rabbit serum). They determined the viral esterase activity by hydrolysis with *p*-nitrophenyl actetate and used flurorimetric reverse-phase high-pressure liquid chromatography (HPLC) to analyze the sialic acids. Their results indicated that the purified virus hydrolyzed free and completely de-O-acetylated glycoprotein-bound 5-N-acetyl-4-O-acetyl neuraminic acid. Thus the enzymatic activity of HE of ISAV is comparable to that of the sialate-4-O-esterases of murine coronaviruses and related group 2 coronaviruses, unlike that of influenza C virus that hydrolyzes 9-O-acetylated sialic acid (Hellebø et al. 2004). They subsequently used solid-phase binding assay in which polystyrene enzyme-linked immunosorbent assay plates were coated with serum glycoproteins from BSM, horse and guinea pig followed by incubation with ISAV at 4°C and bound virus detected by incubation with 4-methyl umbelliferyl acetate. ISAV was found to specifically bind to glycoproteins

containing the 4-O-acetylated sialic acids as specific binding was observed only in serum glycoproteins from horse and guinea pigs. The 4-O-acetylated sialic acids were confirmed as the receptor determinant for ISAV, when recombinant esterase of sialodacryoadenitis virus (SDAV) specific for hydrolyzing 4-O-acetylated sialic acids was used to preincubate glycoproteins or erythrocytes from horse and rabbit resulted in almost complete inhibition of ISAV binding (Hellebø et al. 2004).

1.2.2.5 Gene regulation in orthomyxovirus-infected cells.

Little is known regarding both cellular and viral gene expressions in ISAV-infected cells. Most of our knowledge is based on work done with influenza viruses. Following the infection of host cell, viruses generally bypass the cellular antiviral defense mechanisms and effectively hijack the host cell protein synthetic machinery to their advantage. Studies in influenza virus infection have indicated that gene expression in orthomyxoviruses can be divided into an early and a late phase. During the early phase of virus infection, less than three hours post infection (hpi), the synthesis of cellular proteins is basically shut-off despite the presence of functional cellular mRNAs in the cytoplasm (Katze and Krug, 1984). In contrast, the synthesis of specific vRNAs, viral mRNAs and viral proteins are at high rates and are coupled (Smith and Hay, 1982). This means that in the early phase of influenza virus replication the rate of synthesis of a particular vRNA correlates with and therefore most likely determines, the rate of synthesis of the corresponding mRNA and of its encoded protein (Lamb and Krug, 1996). The early phase of influenza virus infection includes the expression of early genes (NS1, PB1, PB2, PA and NP). Although, the precise mechanism and factors involved in shut-off of infected host cell protein synthesis by the

virus are not yet fully known, the viral NS1 protein has been shown to affect different steps of the host cell metabolism.

The NS1 protein in influenza virus has been shown to accumulate in the largest amounts in the nucleus earlier in infection and has been implicated as a pleiotropic factor that inhibits splicing and polyadenylation of pre-mRNA (Fortes et al., 1994; Qiu and Krug, 1994; Lu et al., 1995; Nemeroff et al., 1998). It has also been implicated in retention of the cellular RNA polymerase II in the nucleus (Fortes et al., 1994) and it functions as a translational enhancer for mRNAs carrying the viral 5'-end sequences (Enami et al., 1994; de la Luna et al., 1995; Enami and Enami, 2000). The NS1 blocks the activation of double stranded RNA-activated protein kinase (PKR) and may inhibit interferon (IFN) response mediated by PKR (Lu et al., 1995; Tan and Katze, 1998). The IFN induced dsRNA-dependent PKR is activated during virus growth by dsRNA binding and phosphorylates the alpha subunit of eukaryotic translation initiation factor, *elf2,* leading to inhibition of translation of the host and virus mRNA, thus contributing to the IFN-mediated antiviral response (Hatada et al., 1999). Two nuclear localization signals have also been mapped on the NS1 protein (Greenspan et al., 1988). These different functions of NS1 protein have been suggested to provide the molecular basis for the role it plays in determining host range and virulence of influenza virus strains (Treanor et al., 1989). The broad functions of NS1 protein suggests that it undergoes multiple interactions with cellular and viral factors. However, this ability of NS1 protein to produce multiple effects in the infected cells has not yet been studied with ISAV in fish.

The NP protein is also synthesized early in the infection in influenza virus, however, its concentration in infected cells has not been quantified. It exerts a structural role in the

PB1, PB2 and PA complex, forming the ribonucleoprotein (RNP) complexes, which represent the minimum requirement for expression of influenza-like RNAs *in vivo* (Huang et al., 1990; de la Luna et al., 1993; Mena et al., 1994; O'Neill et al., 1995). The putative NP protein (Snow and Cunningham, 2001), and the NS1 proteins (Mjaaland et al., 1997) of ISAV are considered to be similar to those of influenza virus and therefore might have the same functions, however this is yet to be demonstrated in cells. Not much is known to date about the NS1 protein of ISAV. Most recently Kibenge et al. (2004) demonstrated that the protein encoded in segment 8 ORF1 was not immunoprecipitated with antibody to purified virus suggesting that it might be the nonstructural protein. It is presumed that the early synthesis of the NP protein in influenza virus is because it is needed for template RNA and vRNA synthesis, while the NS1 protein is needed for the control of both cellular and viral gene expressions.

The expression of the early genes in influenza virus is followed by intermediate levels of continuously expressed matrix proteins (M1 and M2), which accumulate to high levels during the late phase of the infection and have been shown to inhibit viral transcription (Hankins et al., 1989; Perez and Donis, 1998). In influenza virus, the M1 protein is known to interact with the vRNP, leading to shut off of transcription (Kretzschmar et al., 1996; Zhang and Lamb, 1996). It also interacts with RNA (Coleman, 1992), lipid membranes, NEP protein (Yasuda et al., 1993) and possibly the cytoplasmic tails of the membrane proteins HA, NA and M2 during viral assembly. Evidence indicates that M1 has several functions during virus replication. It seems to dissociate from the incoming vRNP during the uncoating process (Martin and Helenius, 1991), thereby allowing the transport of the vRNP complex into the nucleus (Bui et al., 1996), the site of viral transcription and

replication. Newly synthesized M1 is exported to the cytoplasm and transported to the plasma membrane for assembly into mature virions (Rey and Nayak, 1992). The nuclear localization signal sites have been mapped (Ye et al., 1995), and found to be pH dependent (Bui et al., 1996). Falk et al. (2004) recently showed the M protein and HA protein of ISAV as late protein synthesized from 24 hpi. The functional identity of M2 protein of ISAV is not known.

During the late phase (about 4 hpi), the HA and NA proteins of influenza virus are expressed. This phase is characterized by high rate of synthesis of all vRNAs and a drastic decline in viral mRNA concentration (Shapiro et al., 1987). Although no report on the expression of the hemagglutinin gene within the first cycle of ISAV replication in cell cultures is available to date, Falk et al. (1998) using fluorescent staining with the 3H6F8 monoclonal antibody to HA showed the presence of HA from 24 hpi in infected cells as a diffuse perinuclear staining which becomes more intense in the cytoplasm as the infection progressed. Rimstad et al. (2001) also did not find any staining in the nucleus of SHK-1 cells transfected with the hemagglutinin gene, although strong fluorescence/staining was seen in the cytoplasm, allowing these researchers to draw similarity to influenza virus HA protein which never enters the nucleus during replication. The demonstration of ISAV viral antigens with polyclonal antibody 8 hpi as weak perinuclear staining, and the detection of more prominent staining in the nucleus 24 hours later, coupled with the subsequent demonstration of viral antigen mostly in the cytoplasm as viral multiplication proceeded, strongly suggest that ISAV might have the same gene expression pattern as influenza virus in infected cells (Falk et al., 1997). During this late phase, vRNA and viral mRNA synthesis are not coupled; also vRNA and protein syntheses are not coupled as the synthesis of all

viral proteins continue at maximum levels (Lamb and Krug, 1996). The replication control system established by influenza virus in infected cells is directed at the preferential synthesis of the NP and NS1 proteins early and at delaying the synthesis of the M1 protein later (Lamb and Krug, 1996) but this is yet to be investigated for ISAV.

1.2.2.6 ISAV strain variation

The identification and characterization of the nucleotide sequences of the different segments of ISAV RNA have revealed the existence of nucleotide variations among different isolates (Snow and Cunningham, 2000; Krossøy et al., 2001a; Rimstad et al., 2001; Kibenge et al., 2001a; Devold et al., 2001; Griffiths et al., 2001; Ritchie et al., 2001b). The presence of significant differences between the partial genomic sequence of segments 2 and 8 of Canadian and Norwegian isolates of ISAV was first reported by Blake et al. (1999), although the variations were limited to one or two nucleotide substitutions.

The extent of the genetic variation of the different strains is not yet fully known. Using restriction fragment length polymorphism (RFLP) analysis and reverse transcriptase-polymerase chain reaction (RT-PCR) on RNA segment 2 of 13 ISAV isolates, Kibenge et al. (2000a) reported two different profiles for the RNA templates used. All the 11 Canadian isolates used had an RFLP profile distinct from those of Norwegian isolate Glesvaer/2/90 and Scottish isolate 390/98 further suggesting differences between the Canadian and European isolates. When the genomic sequences of segments 2 and 8 of ISAV isolates from Scotland were compared with those from Norway and Canada, the Scottish isolates were found to be more closely related to the Norwegian isolates phylogenetically and were grouped under the European genotype (Snow and Cunningham, 2000).

Using phylogenetic analysis on the segment 2 and 8, it was postulated that the different ISAV isolates from Europe and Canada diverged approximately 100 years ago (Krossøy et al., 2001b). However, Nylund et al. (2003) used the calculated mutation rate that clustered in the phylogenetic analysis of the segment 6 (hemagglutinin gene) of 70 ISAV isolates, to calculate the divergence date between the Norwegian and Canadian isolates, and determined that the divergence might have occurred around 200 years ago. But then Ritchie et al. (2001b) reported sequences from ISAV genome segment 8 amplified from Atlantic salmon in Nova Scotia that have greater sequence similarity to European than to New Brunswick isolates, suggesting that the distribution of the two virus genotypes are not restricted geographically.

It was initially suggested that ISAV isolates could be grouped into two phenotypes based on their infectivity and cytopathogenicity in CHSE-214 cells (Kibenge et al., 2000a; 2001b). Recently, Munir and Kibenge (2004) used real-time RT-PCR and virus isolation in CHSE-214 cells to show that isolates could be further grouped into three phenotypes thus; replicating cytopathic, replicating non-cytopathic and non-replicating phenotypes. However, the molecular basis for this phenotypic variation is not yet known.

Differences have been reported in the molecular sizes of the four major ISAV polypeptides among different ISAV strains (Kibenge et al., 2000a; 2001b), which like influenza viruses show strain variation in their polypeptide sizes (Kendal, 1975). Recently, using metabolic radiolabelling of the synthesized proteins coupled with immunoprecipitation, which allows the detection of structural and nonstructural viral proteins, Kibenge et al. (2001a) revealed that ISAV has up to 12 viral proteins, indicating that it has similar protein profile to influenza viruses (Cox et al., 2000). To date, there is no

information on whether the genetic variations of ISAV are associated with any phenotype of the virus based on antigenic, serotype, pathotype variations or virulence in the fish host (reviewed by Kibenge et al., 2004).

1.2.2.7 Antigenic variations

Orthomyxoviruses have the unique capacity to undergo a high degree of antigenic variation within a short period of time as exemplified by influenza A virus. These variations are a result of their ability to undergo genetic reassortment *in vivo* between members of the homotypic virus type. In nature, reassortments have only been detected with influenza A viruses, which might be either because influenza A virus is more studied or due to lack of circulating subtypes of influenza B and C viruses in humans and swine. Reassortments are mechanisms for the creation of a new combination of the eight RNA segments of two different strains of influenza virus when they infect the same cell. This in most cases will lead to antigenic shifts due to production of a different subtype of a virus with complete change in antigenic structure. This process of subtype switching, although not frequent, can result in severe influenza pandemics in the human population, since there is no prior immunity to these novel surface proteins (Murphy and Webster, 1996). Reassortment has not been demonstrated in ISAV, although the isolation of more than one ISAV isolate in the same salmon farm (Cunningham et al., 2002; Mjaaland et al., 2002) might indicate the possibility of its occurrence. Protective immune response against orthomyxoviruses is mediated by antibody to the HA and NA viral proteins. Antigenic changes however, circumvent prior immunity to allow for reinfection of the same host as a result of these

antigenic shifts.

Antigenic drift is a result of accumulation of a series of amino acid changes in the antigenically important regions of the HA molecule. Influenza virus epidemics are thought to be caused by viruses that have escaped the host defense mechanisms of acquired immunity by altering the antigenicity of HA protein. The rate of accumulation of these changes depends on the evolutionary rate and has been found to be lowest in avian influenza virus and highest in human influenza A viruses.

The hemagglutinin gene of ISAV is the most variable of all ISAV genes (Kibenge et al., 2001b, Rimstad et al., 2001; Krossøy et al., 2001a) and there are strong indications that antigenic drift occurs in ISAV (Mjaaland et al., 2002; Nylund et al., 2003). Sequence comparison of the hemagglutinin gene and alignments of encoded amino acids revealed that it has a highly polymorphic region (HPR) which could be useful for epidemiological studies (Krossøy et al., 2001a). The functional significance of the HPR is not fully known. However, it is located in close proximity to the predicted membrane spanning region and has been suggested to play a role in the pathogenic variation among the ISAV isolates (Devold et al., 2001; Griffiths et al., 2001). Devold et al. (2001) suggested homologous recombination as the possible mechanism for generating the large number of variants in the hemagglutinin sequence of ISAV. They analyzed the hemagglutinin sequence of 36 European and one Canadian isolates of ISAV and reported the presence of 11 HPR groups, out of which HPR 1 and 2 were the major groups. The authors also noted that the other HPR groups may be a result of possible recombination events within the HPR region with the HPR 2 group contributing sequences to the amino end and the HPR 1 group contributing sequences to the carboxyl end of the HPR region. Their assumptions were

further strengthened by demonstrating that fish from several ISA-affected farms harbored multiple ISAV HPR groups suggesting the existence of coinfection. Coinfections are absolute requirements in order for reassortment and/or recombination to occur, and they contribute to variability (Mjaaland et al., 2003), however, there is lack of firm evidence for homologous recombination in negative-strand RNA viruses (Webster, 1999).

Marshall (2003) analyzed 62 ISAV hemagglutinin sequences in the Genbank database and provided further support for earlier findings reported by Kibenge et al. (2001b), that there are two hemagglutinin genotypes, the North American and European genotypes. These two hemagglutinin genotypes have nucleotide sequence identities of less than 80.4% and amino acid sequence identities of less than 88.2% whereas within each genotype, both the nucleotide and the amino acid sequence identities are 90.7% or higher. The genotypes were shown to correlate to antigenic groupings leading Kibenge et al. (2001b) to suggest that they be designated as hemagglutinin subtypes. Marshall (2003) reported the presence of two major mutation sites between the European and the North American hemagglutinin subtypes. The first mutation site is at amino acid positions 320-323. All the isolates of the North American subtype contained ^{320}LEAQ ^{323} (leucine, glutamine, alanine, glutamic acid) while the isolates of the European subtype contain ^{320}VALH ^{323} (valine, alanine, leucine, histidine). The second mutation site is the HPR that occurs as an insertion/deletion and amino acid change spanning amino acid residues 339 to 367, relative to isolate RPC/NB-980-280-2 (Kibenge et al., 2001b).

Nylund et al. (2003) analyzed the hemagglutinin sequence of 70 ISAV isolates based on the 5' end flanking region to the HPR and showed that ISAV isolates clustered in 2 major groups. One group contained all the North American isolates with the exception

of the Nova Scotian isolate (Kibenge et al., 2001b; Ritchie et al., 2001b) and the other group consisted of all European isolates from Norway, Scotland, the Faeroe Islands and the single isolate from Nova Scotia, Canada. Comparison of the HPR groups with the HPR0 amplified from wild Atlantic salmon from Scotland (Cunningham et al., 2002), indicated that all the HPR groups could be suggested to occur as a result of deletion of amino acid segments from the full-length HPR0 sequence (Nylund et al., 2003). These authors therefore hypothesized that deletion from the wild type HPR0 rather than recombination accounted for the variability in the HPR of ISAV isolates.

1.2.3 Epidemiology

1.2.3.1 Geographic distribution of ISAV

ISA was first reported in Norway in 1984 (Hastein, 1997; Jarp and Karlsen, 1997) but retrospective studies suggest the presence of the disease and the virus in Norwegian aquaculture industry earlier (Nylund et al., 1995a; Devold et al., 2001). In 1996, ISA was diagnosed for the first time outside Norway in New Brunswick, Canada, amongst high mortalities in marine-farmed Atlantic salmon (Byrne et al., 1998; Mullins et al., 1998; Lovely et al., 1999). Anecdotal evidence indicates that the virus was present in the Bay of Fundy, New Brunswick, as early as 1995 (O' Halloran et al., 1999; Kibenge et al., 2004). The disease and the virus have since been described among market and premarket Atlantic salmon in Scotland, U.K., in 1998 and 1999 (Bricknell et al., 1998; Rodger et al., 1998; Murray et al., 2003), in the Faeroe Islands, Denmark since 2000 (Anonymous, 2000; Lyngøy, 2003) and Cobscook Bay, Maine, U.S.A in 2001 (Bouchard et al., 2001). The virus was detected in tissues and virus antibodies in sera from farmed coho salmon

Oncorhynchus kisutch, in Chile (Kibenge et al., 2001a; 2002). The virus was also detected in apparently healthy farmed rainbow trout *O. mykiss* in Ireland (Anonymous, 2002) and in randomly sampled apparent healthy wild Atlantic salmon, sea trout and brown trout *Salmo trutta* collected from the same geographic locations as Atlantic salmon farms with clinical ISA in Scotland (Raynard et al., 2001; Cunningham and Snow, 2003). Reports suggest that the loss caused by ISA outbreaks to the aquaculture industry in Norway in 1999 was about $11 million, and in Scotland in 1998-1999 was $32 million all in U.S. dollars (Hasting et al., 1999). The disease is a serious threat to the growing aquaculture industry and has been listed as one of the most dangerous fish diseases by the European Union.

1.2.3.2 Host range of ISAV

The host range of the ISAV varies. The virus infects and replicates in sea trout, rainbow trout, brown trout, European eels *Anguilla anguilla* , Atlantic herring *Clupea harengus*, and Arctic char *Salvelinus alpinus*, without showing any serious gross clinical signs of disease, except for a drop in the hematocrit of rainbow trout during the first 3 weeks after infection (Nylund et al., 1994a ; 1995a). Brown trout produce neutralizing antibodies against ISAV within 45 days post primary infection, but the virus may still be present for months after the infection in rainbow trout (Nylund et al., 1994a). This fish specie might become asymptomatic life long carriers of the virus (Nylund et al., 1994a,b; 1995a, 1997; 2002; Nylund and Jakobsen, 1995; Rolland and Nylund, 1999; Snow et al., 2001; Devold et al., 2001) and might act as reservoir hosts.

Other fish species like the Pacific salmonid species, chum *O. keta*, steelhead *O.*

mykiss, chinook *O. tsawytscha*, and coho are resistant to ISAV experimentally (Rolland and Winton, 2003). However, recent studies show increased morbidities and mortalities in rainbow trout experimentally infected with selected strains of ISAV (Kibenge et al., 2004). A survey of 3000 non-salmonid fish belonging to nine different species reported the detection of ISAV only in pollock *Pollachius virens* and Atlantic cod *Gadus morhua* collected from cages with ISA-diseased Atlantic salmon (MacLean et al., 2003). ISAV appears to cause clinical and fatal systemic infection only in farmed and wild free-ranging Atlantic salmon. Wild Atlantic salmon have been reported to be less susceptible than the farmed Atlantic salmon to ISAV either as a result of genetic variations or management practices on fish farms (Nylund et al., 1995a).

1.2.3.3 Mode of transmission and route of infection of ISAV

ISAV can be transmitted to Atlantic salmon during experimental cohabitation or by intra-peritoneal injection of tissue homogenate of liver, kidney, spleen, gill, plasma, red blood cells and/or head kidney leucocytes from ISAV-infected Atlantic salmon (Dannevig et al., 1994). In a natural marine environment horizontal transmission occurs through cohabitation with infected live salmon or infected biological materials such as animal wastes or discharges from normal aquaculture operations such as slaughter houses (Vagsholm et al., 1994) and contaminated well boats (Murray et al., 2002). Totland and colleagues (1996) reported that ISAV can be transmitted horizontally from fish to fish, through natural secretions such as skin mucus, urine, gut excretions, and blood from infected smolts of Atlantic salmon during the presymptomatic phase. Moreover, fish that survive an experimental challenge/epizootic continue to shed the virus for more than one month into

the surrounding water and infect other healthy fish (Totland et al., 1996). The virus might replicate continuously in the surviving fish (Totland et al., 1996). Apparently healthy fish may be carriers as the virus was detected by RT-PCR in subclinically infected fish (Devold et al., 2000; Optiz et al., 2000), and could therefore act as reservoirs of the virus for a long period of time (Totland et al., 1996). The virus shed into the water column by fish that recovered from clinical infection may persist for only 20 hours at 6°C and up to 4 days in tissues at the same temperature (Nylund et al., 1994b). Contaminated biological materials therefore act as better reservoirs of the virus/infection than the water column. Blood and mucus contain large amounts of the virus and more efficiently transmit the virus/disease than feces, plankton, and salmon lice (Rolland and Nylund, 1999). Sea lice *(Lepeophtheirus salmonis* and *Caligus elongatus)* can function as mechanical vectors for the ISAV through their feeding habits (Nylund et al.,1994b). Local scavenging bird and fish populations can also act as vectors. ISAV propagates in other salmonid as stated earlier and can spread from them to Atlantic salmon causing disease (Nylund et al., 1997).

It has been suggested that the most important mechanism for the spread of the virus from a reservoir to a new host is by passive transmission in sea water as the virus has been detected in sea water within 5 km of an infected farm or slaughter house that handled infected fish (Jarp and Karlsen, 1997). Vertical transmission of the virus from the parent to the offspring through transovarian infection has not been demonstrated as part of the natural route of transmission of ISAV although passive transmission can occur from infected egg debris to the hatching fry (Melville and Griffiths,1999).

The gill is suggested as the main route of viral entry into fish, although the olfactory or lateral line cannot be excluded (Totland et al., 1996; Mikalsen et al., 2001). ISAV has

been shown not to be transmitted through the gastrointestinal tract and/or through coprophagy under controlled laboratory conditions (Totland et al., 1996).

1.2.4 Pathogenesis of ISAV

1.2.4.1 Early pathogenesis

ISAV replicates rapidly in infected fish and by 7 dpi is widely disseminated and present in large amounts in all fish tissues including mid-kidney, head kidney, liver, spleen, intestine, gills, blood, muscles and heart (Dannevig et al., 1994; Rimstad et al., 1999). Using RT-PCR to detect the viral load in tissues in an experimental infection, Mikalsen and others (2001) reported the presence of the virus in the gills two hours after the virus was added directly to the water, and after 24 hr virus could be detected in the heart, gills, mid-kidney and intestine of fish. They also reported that the viral load began to increase rapidly and became more dispersed to most tissues by 5 dpi in fish infected via excretion from experimentally ISAV-injected-cohabiting fish. Ultramicroscopic changes were observed from 4 dpi in the perisinusoidal macrophages in the liver of experimentally infected fish (Speilberg et al.,1995). These changes included large vacuolation, and accumulation of fine-granular material with low electron density in the cytoplasm, which persisted and became more severe as the infection progressed causing considerable increases in the size of the cell. By 14 dpi they reported degenerative changes in the sinusoidal endothelial cells which included marked swelling and vacuolation of the cytoplasm. By 18 dpi, some areas of the liver were devoid of endothelial lining bringing the hepatocytes in direct contact with blood cells (Speilberg et al., 1995). Hjeltnes et al. (1992), reported a decrease in the hepatic glutathione of up to 70 percent at the onset of clinical signs of ISA in diseased fish,

which may affect the capability of the liver to transform and excrete xenobiotics from the body.

The head kidney leucocytes from fish experimentally infected with ISAV responded to mitogens such as lipopolysaccharides (LPS) from *Eschericia coli* and phytohaemagglutinin (PHA) similarly in control and uninfected fish by 7 dpi (Dannevig and Falk, 1993). However, toward the onset of mortality from the disease, by 14 dpi, the LPS and PHA response of leucocytes of ISAV infected fish were significantly lower than those of control leucocytes. There is a marked reduction in hematocrit of infected fish beginning at 14 dpi suggesting that anemia is a late event in the course of ISAV infection (Dannevig and Falk, 1993) and a leucopoenia is suggested to develop concomitantly with anemia (Thorud, 1991). Dannevig and Falk (1993) found that the reduction in hematocrit/onset of anemia in most cases coincided with the onset of suppression of mitogenic response and mortality (Falk et al., 1995). They also reported that the two are independent events as suppression of immune response was observed in fish with hematocrits lower than10 and greater than 30. An increase in the plasma cortisol concentration and lactate level in ISAV experimentally infected fish was observed as the infection progressed, which correlated well with the severity of anemia as measured by fall in hematocrit values and development of disease conditions (Olsen et al., 1992).

In the spleen and head kidney, an increase in the reactivity of alkaline phosphatase with the presence of degradation products of hemoglobin in melanomacrophage and in sinusoidal macrophages of the red pulp, were observed at 4 dpi and became more pronounced by 7 dpi in ISAV infected fish suggesting an increased erythrophagocytosis (erythrophagia) due to increased erythrocyte destruction (Falk et al., 1995).

Clinical signs may be present from 2 - 4 weeks following natural infection and commonly include pale gills, ascites, enlargement of liver and spleen, petechia hemorrhages in the visceral fats, congestion of the gut, severe anemia, and mortality (Thorud and Djupvik,1988; Evensen et al.,1991 Hovland et al., 1994). Generalized internal hemorrhage associated with ISAV in clinically affected fish has been suggested to be caused by the destruction of endothelial cells as the virus buds after replication (Speilberg et al., 1995; Koren and Nylund, 1997).

1.2.4.2 Target cells of ISAV

Hepatocytes, immature erythrocytes and leucocytes were initially suggested as the target cells for the replication of the virus (Dannevig et al., 1994). Hovland et al. (1994), using electron microscopy reported that the virus buds off endothelial cells of infected fish suggesting that the virus propagates there. Further studies using electron microscopy showed that the virus replicates in leucocytes and endothelial cells lining blood vessels of the heart and supports the hypothesis that these cells may be the target cells (Nylund et al., 1995b; 1996). These authors however, could not show the presence of viral RNA or mRNA in these cells, and they could not demonstrate the presence of the virus in circulating leucocytes isolated from salmon with ISA. Recently, *in situ* hybridization using riboprobes from segments 2 and 8 vRNA was used to demonstrate the presence of viral mRNA in the endothelial cells of ISAV mortalities, further suggesting endothelial cells as the target cells (Gregory, 2002), and excluded the parenchyma and interstitial cells. It is still not fully known which blood cells support replication of the virus. Plasma, leucocytes and red blood cells separated by density gradients from an infected fish have been able

to transmit the virus on injection into naive fish (Dannevig et al., 1994).

1.2.4.3 ISAV virulence

ISAV causes clinical disease mainly in farmed Atlantic salmon. Infection of other fish species including rainbow trout, sea trout, herring, eel, and wild Atlantic salmon results in asymptomatic, probably life long carriers of the virus. It is assumed but not yet proven that there is variation in virulence among ISAV strains. Only clinical cases receive appropriate diagnostic and epidemiological attention, and natural avirulent ISAV strains are rarely isolated (reviewed by Kibenge et al., 2004). Also, virulence of the virus in other non clinical/asymptomatic hosts tends to be over looked. Routine sampling of apparently normal experimental Atlantic salmon (that were certified to be free of ISAV) has occasionally shown positive RT-PCR results with ISAV-specific primers (Kibenge et al., 2001b). Ritchie et al. (2001b) using RT-PCR detected ISAV in Atlantic salmon from Nova Scotia without any clinical signs of ISA, but could not isolate the virus using available fish cell lines. It has also not been possible to isolate the virus from every natural ISA outbreak (Mjaaland et al., 2002). More recently, the HPR0 sequence of an avirulent ISAV was amplified from wild salmon in Scotland. Although the virus could not be isolated (Cunningham et al., 2002), it was hypothesized to be the source of virulent ISAV isolates that contain various deletions in the HA gene (Nylund et al., 2003). It is not known whether fish cell lines are permissive to both virulent and avirulent ISAV strains or only to virulent strains. Kibenge et al. (2001b) reported that there are differences in ISAV strains susceptibility to CHSE-214 cell line and divided the studied ISAV isolates into two

phenotypes based on their ability to cause CPE in the cells. However, Munir and Kibenge (2004) recently showed that there are three ISAV CHSE-214 phenotypes thus, non-replicating, replicating cytopathic and replicating non cytopathic phenotypes. The difference in the cell culture susceptibility was suggested to be as a result of change in the receptor avidity due to a 3' insertion or deletion in the hemagglutinin gene in one strain (Griffiths et al., 2001). The cultivation/isolation of ISAV from different HPR variants in SHK-1 cells revealed differences in their ability to replicate as some did not replicate even when the HPR sequences were from farms with clinical diagnosis of ISA (Mjaaland et al., 2002). The failure to isolate virus using the SHK-1 and CHSE-214 cell lines indicates that these cell lines are not permissive to all ISAV strains (Kibenge et al., 2004).

The variation in the virulence of ISAV is further complicated by the possible variation in susceptibility of different genetic strains of farmed and wild Atlantic salmon to the virus (Raynard et al., 2001). Jones et al. (1999) were unable to reproduce all the microscopic lesions associated with ISA even when there was high mortality suggesting further studies for understanding the pathogenesis of ISA.

For avian influenza virus strains, the proteolytic cleavage of the nascent HA proteins (HA_0) into its biological active subunits HA_1 and HA_2 plays a central role in the virulence and pathogenicity of the virus in cell culture and animals (Lamb and Krug, 1996). The differentiation between virulent and avirulent strains correlates with the sequence of few amino acids at the HA_0 cleavage site, and the presence of the right proteases in the target tissue to carry out the cleavage. The presence of several basic amino acids at the cleavage sequence in avian influenza is associated with high pathogenicity in birds because it allows cleavage of the molecule by cellular protease widely distributed

throughout the body (Klenk and Garten, 1994). The ubiquitous proteases such as the proprotein convertase furin and PC6 in birds allow the cleavage activation of the HA_0 of highly pathogenic strains and their replication in virtually all cell types in the avian host. Virus strains lacking polybasic amino acids at the cleavage site are only activated by the protease expressed specifically in certain respective tissues and therefore can cause only localized infection (Klenk and Garten, 1994). However, avian influenza viruses containing polybasic amino acid cleavage sequences have also been isolated from internal organs of ducks which did not show any signs of disease (Kawaoka et al.,1987), suggesting that other factors also contribute to the virulence of the virus (Zambon, 2001).

In tissue culture, the cleavage of the HA of avian influenza virus isolates is assessed by the production of plaques in cell cultures that are permissive for virulent viruses but not permissive for avirulent viruses. The avirulent viruses require exogenously added trypsin for their multi-cycle replication (Klenk and Rott, 1988). The HA of ISAV does not need cleavage activation as the virus can replicate well in cell cultures without trypsin treatment (Falk et al., 1997; Kibenge et al., 2000a; Krossøy et al., 2001b; Rimstad et al., 2001; Rimstad and Mjaaland, 2002). Although the hemagglutinin sequence of more than 100 ISAV isolates have been deposited in the Genbank database to date, these sequences have only been used to confirm genetic relationships, as the molecular makers for virulence have not yet been identified (reviewed by Kibenge et al., 2004). In a recent study, Kibenge et al. (2001b) could not find any clear association between virulence of the virus in tissue culture and sequence variation in the HPR of the HA gene. It is still not known if virulent and avirulent strains of ISAV can be delineated successfully by molecular methods based on the hemagglutinin gene alone although other viral sequences like the

matrix protein and nucleoprotein have been shown to play a role in virulence in influenza viruses. Recent studies of more than 30 ISAV isolates on the RNA segment 5 encoding a putative surface protein revealed up to four distinct genotypes (Kibenge et al., 2003). This suggests that there might be significant differences in virulence and antigenic variations in ISAV similar to influenza viruses. However, to date no sequential studies have been undertaken to determine if there is correlation between the variations in virulence of ISAV and the pathology in the fish host.

1.2.4.4 Persistence of ISAV in fish

The recurrence of ISA outbreaks in Norway in farms where the virus was thought to have been eradicated led to the suspicion that the virus might be persisting in a reservoir host in the wild. For virus to survive in nature and remain in circulation, it requires continuous infection of susceptible individuals or establishment of long-term persistence within infected hosts (Knipe, 1996). ISAV persistence in fish (Devold et al., 2000; Optiz et al., 2000) is unusual for orthomyxoviruses known to date as there is no evidence of persistent influenza virus genetic material in any animal species. Influenza viruses are cleared from ducks in approximately 7 days post infection (Webster, 1999; Kibenge et al., 2004), however they can persist long-term in carrier cultures (Frielle et al., 1984; Urabe et al., 1992; 1993; Clavo et al., 1993). In influenza C virus, virus persistence in cell cultures is associated with a modified hemagglutinin/esterase sequence with altered activity and diminished receptor destroying enzymatic activity (Marschall et al., 1994). Marschall et al. (1999) have also shown that influenza C viral genes are expressed irregularly during persistent infection with

the vRNA being present predominantly at low levels and lasting over long inactive periods.

Nylund and Jakobsen (1995) reported the presence of ISAV in asymptomatic sea trout up to 7 months following challenge, suggesting that they could be life-long carriers. Asymptomatic ISAV infected sea trout and rainbow trout have been shown to transfer virus to disease-free Atlantic salmon on cohabitation (Nylund and Jakobsen, 1995). Devold et al. (2000) were able to amplify ISAV sequences from Atlantic salmon that recovered from ISAV challenge several months after the infection, although attempts at virus isolation were unsuccessful. Recently, the findings of ISAV hemagglutinin sequences in fish from farms without ISA outbreaks (Mjaaland et al., 2002) and in wild Atlantic salmon in Scotland without any sign of the disease (Cunningham et al., 2002), and the inability to isolate the virus, also suggest the persistence of the virus in infected fish at low levels.

The underlying cellular and molecular mechanisms of ISAV persistence in fish have not been studied and the tissue site of viral persistence is not known. The NS1 protein of non persistent influenza virus is synthesized in the early period of infection and declines steadily in the late phase of the infection, until there is almost complete loss of signals in infected cells (Zach et al., 1999). However, NS1 protein of the persistence variant of influenza C virus is produced continuously in detectable amounts with no reduction in protein synthesis even during late phase of infection (Marschall et al., 1999), suggesting that NS1- coding sequence plays a major role in viral persistence. Using the influenza C virus persistent model in cell culture, it has also been shown that the interaction between NP, M1 and cellular actin filament that is necessary for virion assembly, is affected in persistent infection (Hechtfischer et al., 1999). In ISAV infection, recovered farmed Atlantic salmon continue to shed virus for long periods of time, however the detection of ISAV in

carrier fish required use of RT-PCR (Devold et al., 2000), suggesting that the viral RNA is not abundant.

1.2.5 Clinical disease

1.2.5.1 Clinical signs and gross pathology

In Norway, clinical cases of ISA have been reported from spring or early summer up and until late autumn in farmed Atlantic salmon in sea cages (Thorud and Djupvik, 1988). Few salmon may exhibit clinical ISA during the winter (Thorud, 1991). Experimentally, clinical signs are seen as early as 5 dpi, with mortality starting at 12 days post infection (Jones et al., 1999). In some peracute cases, mortalities may occur without any prior signs of the disease (Vagsholm et al.,1994). Affected fish at the onset of the disease may go off feed and become darkened and lethargic. In the field, sick fish tend to swim close to the surface of the water (Vagsholm et al.,1994), sink to the bottom or rest near the edges of the cage (Traxler et al., 1998). The gross pathological changes observed in terminal stages of the disease include distended abdomen, exopthalmos, extreme pallor of the gills, skin edema and hemorrhage (Thorud and Djupvik, 1988). At necropsy, the disease is characterized by ascites, petechial hemorrhages of the visceral fat, congestion and enlargement of the liver, spleen, kidney and proximal part of the gut and extremely pale heart (Evensen et al., 1991).

Hematologically, there is a marked reduction in the number of circulating erythrocytes and leucocytes, and increased number of immature erythrocytes (Thorud and Djupvik, 1988). The hematocrit values may drop to less than 5% in the terminal stages of

the disease (Evensen et al., 1991). There is an inverse relationship between the ascites formation and hematocrit value during the course of the disease (Evensen et al.,1991). Infected Atlantic salmon had an overall increased level of plasma glutathione which was probably due to intracellular release of the gluthatione from lysed red blood cells and lower than normal level of liver glutathione due to hepatocellular damage (Hjeltnes et al., 1992). The plasma cortisol and lactate levels are also elevated, maybe as a result of liver damage and can be correlated with the severity of the anemia (Olsen et al.,1992). The increased plasma cortisol may be due to progressive cellular breakdown or reduction in the metabolic clearance of corticosteroids as disease develops and might trigger the stress response. The fish might adjust to the stress by swimming on the surface to enhance its oxygen uptake or sink to the bottom where it does not need to be disturbed.

Mortalities in sea farms and/or natural outbreaks vary considerably from insignificant to moderate (Jarp and Karlsen, 1997). Experimentally, mortalities vary from 0 to 100% depending on virus strain, infectious dose and route of infection (Jones et al., 1999; Optiz et al., 2000; Jones and Groman, 2001; Ritchie et al., 2001b). In recent natural outbreaks, mortalities could not be accounted for as most fish farms were depopulated as soon as ISA was suspected or the virus detected before the onset of the clinical signs. None of the clinical signs or gross lesions associated with this disease is pathognomonic, as other fish viruses cause similar signs and lesions. Recently, mortality was recorded in rainbow trout experimentally infected with ISAV and the gross lesions were similar to those in Atlantic salmon (Kibenge et al., 2004). Previously, the only clinical sign associated with experimental ISAV infection in rainbow and brown trout (Nylund and Jakobsen, 1995) and in Arctic char (Snow et al., 2001) was only a transient drop in hematocrit value. No

mortalities were recorded although ISA virus was recovered from these groups of experimentally infected fish.

1.2.5.2 Histopathology

Microscopic liver changes occur early in the infection and include marked congested and dilated sinusoids, followed by hepatocellular cytoplasmic vacuolation (Evensen et al., 1991; Simko et al., 2000). There is disorientation of the hepatocytic tubular arrangement and presence of blood filled cavities adjacent to the hepatocytes due to loss of the endothelial lining in some cases. The blood filled cavities coalesce to form large blood-filled areas (peliosis). Focal, multi-focal or confluent areas of hepatocellular degeneration and necrosis are present. They are often referred to as hemorrhagic necrosis and are characterized by cellular swelling, hypereosinophilia and loss of deferential staining of the cellular details (Evensen et al., 1991;Simko et al., 2000). In some cases, necrotic areas may give a bridging appearance (Evensen et al., 1991). Thrombi and often swollen degenerate endothelial cells may occur in hepatic blood vessels. The hepatocytic necrosis is suggested to be caused by a combination of factors which include the development of hypoxia from thrombi and/or congestion of the sinusoids resulting from blocked outflows of blood and/or direct destruction of the sinusoidal barrier all leading to ischemic necrosis (Evensen et al., 1991). A moderate edema may be found around blood vessels and biliary ducts. Evensen et al. (1991) reported the absence of inflammatory changes at any stage of the disease, but Jones et al. (1999) reported the presence of vasculitis with perivascular cuffing by mixed-leucocyte populations in the liver. The absence of inflammatory response in the Norwegian cases may be due to the acute nature of the infection and also to the fact

that the virus had been shown to target leucocytes causing their degeneration/necrosis and depletion.

In the posterior kidney, multi-focal, coalescing to diffuse interstitial congestion and hemorrhage and/or focal to multi-focal coagulation necrosis of renal tubular epithelium may be present (Byrne et al., 1998; Simko et al., 2000). Renal tubular eosinophilic casting has also been reported in natural outbreaks of ISA in Canada (Byrne et al., 1998). Major renal pathology was associated with initial ISA outbreaks in Canada hence the disease was called hemorrhagic kidney syndrome (Byrne et al.,1998). This difference contributed to the delay in the diagnosis of the disease as the European outbreaks showed more of the hepatic pathology (Thorud and Djupvik, 1988; Evensen et al., 1991). In the anterior kidney, there is congestion of the sinusoids and interstitial hemorrhages with individual hematopoietic cells showing degeneration (Evensen et al., 1991; Simko et al., 2000).

The histopathologic lesions in the spleen are characterized by congestion, hemorrhage and different levels of erythrophagocytosis (Evensen et al., 1991; Simko et al., 2000). The melanomacrophage accumulations/centers of the spleen are the major site for erythrocyte destruction (Agius and Agbede, 1984) in fish. Erythrophagocytosis is a normal process in fish for removal/destruction of expired mature erythrocytes. Increased erythrophagocytosis, therefore indicates increased destruction of erythrocytes and has been reported to occur in the spleen of ISAV infected fish as early as seven days post infection in the sinusoidal macrophage away from the melanomacrophage centers (Falk et al., 1995). Simko et al. (2000) however, reported that increased erythrophagocytosis occurs late in the disease. The effect of increased erythrophagocytosis and its contribution to the pathogenesis of ISA has not been investigated. It has been shown that the ISAV

can attach and agglutinate erythrocytes *in vitro* (Falk et al.,1997), but it has not been shown that any hemagglutinating virus causes erythrocytic agglutination *in vivo*, that might lead to increased erythrophagocytosis.

There is congestion and hemorrhage in the lamina propria and submucosal vasculature of the stomach, pyloric caeca and intestine (Evensen et al., 1991; Mullins et al.,1998; Simko et al., 2000; Jones and Groman, 2001) and of the mesenteric blood vessels. In some cases, degeneration and sloughing of epithelium/enterocytes have been reported in infected fish (Evensen et al., 1991; Mullins et al., 1998). Jones and Groman (2001) reported an apparent difference in the pathology between the upper and lower intestinal mucosa of experimentally infected fish and concluded that lesions were localized to areas above the intestinal valve, that is, to the upper intestine. This finding was overlooked in the early histological studies of the lesions associated with ISA.

No lesions were reported in other organs except the liver, kidney, spleen and gut in the initial report of ISA outbreaks in Norway (Evensen et al., 1991). Recent studies however, have shown presence of lesions in the gills and heart (Mullins et al., 1998; Simko et al., 2000). There may be severe congestion of the filamental and lamellar vasculature and in some cases swelling/hypertrophy of the lamellar epithelium and interstitial cellular necrosis involving the pillar cells. Distal lamella pavement and/or mucous cell hyperplasia may occur (Byrne et al., 1998; Simko et al., 2000).

Very minimal lesions have been reported in the heart (myocardium) of infected fish both in natural and experimental infections. Simko et al. (2000) reported minimal to mild changes in the junctional zone between the spongiform and compact myocardium. The myocytes were characterized by pyknosis of the nucleus, clumping of the chromatin

material and myofibrillar separation with coagulation and/or mild hypereosinophilia and hyalinization (Simko et al., 2000).

1.2.6 Diagnosis of ISA

Initial diagnosis of ISA was based on gross pathology and histopathology (Evensen et al., 1991; Falk et al., 1995; Speilberg et al., 1995; Byrne et al., 1998; McClure et al., 2003). Transmission electron microscopy was used to identify the causative agent as a virus that buds from leucocytes, macrophages and endothelial cells (Hovland et al., 1994; Nylund et al., 1995b). Virus characterization and laboratory diagnosis were improved when the SHK-1 cell line which supported the growth of ISAV was developed (Dannevig et al., 1994). Other cell lines such as ASK (Devold et al., 2000), CHSE-214 (Blake et al., 1998; Kibenge et al., 2000a), and TO (Wergeland and Jakobsen, 2001) that support the growth of ISAV with development of CPE have also been described.

An indirect fluorescence antibody test (IFAT) for the detection of viral antigens in both ISAV infected cell cultures and tissue section was introduced after Falk et al. (1995) produced polyclonal antibodies against ISAV. A 3H6F8 monoclonal antibody against the HA protein of ISAV has also been developed and used in both IFAT and enzyme linked immunosorbent assay (ELISA) for the detection of ISAV infected cell cultures (Falk et al.,1998). In addition, an indirect ELISA and indirect competitive ELISA that could detect ISAV-specific antibodies in the sera of infected and/or vaccinated fish have been described (Kibenge et al., 2002). Viral nucleic acid identification using RT-PCR has been developed for detection and diagnosis of ISAV in infected tissues (Mjaaland et al., 1997; Blake et al.,

1999; Rimstad et al., 1999; Kibenge et al., 2000b). Also, an *in situ* hybridization on both cell cultures and tissue from suspected and infected fish has been described (Gregory, 2002; Moneke et al., 2003). Recently, one-tube real-time RT-PCR using LightCycler technology (Roche Applied Science) and SYBR Green chemistry that quantitatively detects ISAV in biological samples was developed (Munir and Kibenge, 2004).

None of the above methods for the laboratory diagnosis of ISAV has been validated by the International Reference Laboratory like the National Veterinary Services Laboratories in Ames, Iowa, USA (Merrill, 2003). Optiz et al. (2000) and Kibenge et al. (2000a) have, however, shown that virus isolation techniques and RT-PCR (Snow et al., 2003b) to be the best methods for detecting ISAV in marine fish. Merrill (2003) on comparing the commercial diagnostic assays (histology, virus isolation, RT-PCR and IFAT) used for ISAV in the USA, also suggested virus isolation and RT-PCR on blood sample and kidney tissue as the most sensitive and specific methods. Initially the head kidney was suggested as the optimal organ for detection and virus isolation, however, the OIE publication on ISA for 2000 has suggested the inclusion of spleen, liver and heart for diagnosis. Snow et al. (2003b) have suggested that the spleen is unsuitable for ISAV, rather the heart and kidney should be the preferred organs for single organ samples. The heart has also been shown to contain the most viral mRNA in clinically affected fish (Koren and Nylund, 1997; Gregory, 2002; Moneke et al., 2003), making it the most ideal organ for virus detection and probably for virus isolation as well. It is still not known which organ supports the most viral replication at the early stages of the infection. The choice of the right organ during sampling would help in the early detection of the virus in infected and carrier fish.

1.2.7 Molecular methods for study of ISAV

1.2.7.1 *In situ* hybridization

Many molecular methods have been used to study ISAV. The development and optimization of RT-PCR technique for the detection of ISAV genomic sequences led to increase in the knowledge of the virus. RT-PCR, however, has some limitations in that it allowed only the study of the virus genetic material without recourse to impart of the viral activity on the host. This therefore leads to the need for development of other molecular methods for the study of this novel virus. *In situ* hybridization (ISH) also referred to as hybridization histochemistry or cytological hybridization, was introduced in 1969 (John et al., 1969; Buongiorno-Nardelli and Amaldi, 1970). It allows the detection of specific nucleic acid sequences directly within a cell or tissue (Singer et al., 1986; Stahl and Baskin, 1993; Murray and Ambinder, 1994), thereby allowing the direct morphological demonstration of specific DNA or RNA sequences in individual cells in tissue sections, single cells or chromosome. It is still the only method that allows one to study the cellular location of DNA and RNA sequences in a heterogenous cell population (Höfler, 1990).

The basic principle of nucleic acid hybridization utilizes the fact that DNA and RNA will undergo hydrogen bonding to complementary sequences of DNA or RNA under appropriate conditions. Therefore, labeled single stranded fragments of DNA or RNA of sufficient length containing complimentary sequence (probes) can be hybridized to particular sequences of DNA or RNA forming stable hybrids in a cell. The application of these probes to tissue sections allows DNA and RNA to be localized and also gives information on their distribution within tissue sections and cell types.

The success and sensitivity of the ISH depend on several variables. However, the main steps involve the preparation of the cell and/or tissues and labeling nucleic acids sequences to form the probe for hybridization. Several variables are considered when fixing tissues for *in situ* hybridization. This is because an optimum fixation of tissue is required for a balance, between the retention of maximum cellular target DNA and RNA, the optimal preservation of tissue morphology, and sufficient accessibility/penetration of the probes into the cells (Tecott et al., 1987; Höfler, 1990). DNA has been shown to be more stable than RNA. This is because mRNA is synthesized and degraded at high rates and also the RNA in tissues are susceptible to degradation on contamination by RNAses on skin or laboratory instruments (Simmons et al., 1989). Therefore when detecting mRNA levels, tissues should be fixed quickly and care taken to avoid contamination with RNAses. Different fixatives including acetic alcohol, glutaraldehyde, formalin and paraformaldehyde at different concentration and fixing time have been used to fix tissues for ISH without having any major influence on the DNA (Höfler, 1990). The optimum localization of RNA, depends on the type, time and concentration of the fixative used (Höfler, 1990). Glutaraldehyde provides the best RNA retention and tissue morphology but like formalin it causes an extensive protein cross-linking on fixed tissue, thereby reducing the penetration/accessibility of the probe to the target. Paraformaldehyde, though a cross-linking fixative like glutaraldehyde and formalin, does not cross-link protein extensively so as to prevent the penetration of probes (Brigati et al., 1983; Hafen et al., 1983; McAllister and Rock, 1985; Höfler et al., 1986). Therefore 4% paraformaldehyde is the most widely used fixative as it provides a compromise between tissue morphology, target retention, and probe penetration.

The penetration of the probe and accessibility to the target DNA especially in paraffin sections can be increased by the permeabilization of the fixed cells using detergent and/or proteases (proteinase K), acid (HCl) or heat denaturisation (Singer et al., 1986; Höfler, 1990; Murray and Ambinder, 1994; Guiot and Rahier, 1995). The permeabilization process also helps in the unmasking of the nucleic acids from the nucleohistones and other associated proteins, exposing the nucleic acid for efficient hybridization. Some researchers have reported that permeabilization of fixed cells with detergent or protease is not necessary, and concluded that fixation in paraformaldehyde is more important for optimal ISH (Brigati et al., 1983).

Radioactive isotopes (^{3}H, ^{32}P, ^{33}P, ^{35}S) (Stahl and Baskin, 1993; Wilcox, 1993) and non-radioactive (biotin, peroxidase or digoxigenin) (Singer et al., 1996; Murray and Ambinder, 1994) labels are commonly used for making the probes. The radioactive and non-radioactive labels have different basic attributes which can be exploited to an advantage. The advantages of radioisotope labels include the fact that they are readily incorporated into probes using many common enzymes. Also, they are more sensitive in detection than non-radioactive probes and can be analyzed quantitatively. The major disadvantages are that long exposure times are needed, and radioactive decay leads to a limited visualization window. Also the probes cannot be stored over long periods because of decay in radioactivity, coupled with safety and disposal problems associated with radioactive material. The non-radioactive probe/labels have the advantages of high sensitivity, safety, stability over long periods of time and results can be visualized quicker. The labeling process involves the incorporation of the label to the nucleic acid sequences of the probe. The commonly used labeling methods include, nick translation (Maniatis et

al., 1982), random priming (Feinberg and Vogelstein, 1983), *in vitro* transcription (Green et al., 1983), and PCR using labeled synthetic oligonucleotides (Stahl and Baskin, 1993).

Several different types of labeled DNA or RNA probes can be used for ISH each with advantages and disadvantages. The probes can be double stranded DNA (dsDNA), single stranded DNA (ssDNA), single stranded complementary RNA (sscRNA), and synthetic oligonucleotides. The major advantages of the dsDNA probe construction are the ease with which it could be produced. It has the advantage that many different labels can be attached to the DNA during labeling process, and the probe is stable over a long time. The main disadvantages of dsDNA are that the DNA probe must be denatured before the hybridization reaction and because of the presence of both strands in the hybridization solution during the reaction, the complementary strands compete with the target DNA or RNA for hybridization which can decrease sensitivity and increase background staining.

The sscRNA probe synthesis, has the disadvantage of requiring longer process which include subcloning of the DNA template into transcription vector before being synthesized by *in vitro* transcription. It has the same advantages as the dsDNA probe, and in addition the benefit that RNA-RNA interactions are more stable than DNA-DNA and DNA-RNA hybrids. Because of the stability of the RNA-RNA hybrid, more stringent washing conditions can be used after the hybridization to produce greater sensitivity and specificity (better signal to noise ratio). Also the signal to noise ratio can be further improved when RNA probes are used for hybridization since RNAses can be used to enzymatically destroy unhybridized ssRNA while sparing the hybridized RNA.

Another important consideration in ISH is the length or size of the probe, as it has a major influence in its diffusion into the cell. The optimal length of probe for effective

tissue penetration and high hybridization efficiency is 50 - 300 bases (Höfler, 1990), although probes of higher sizes can equally be used for special applications (Lawrence and Singer, 1985).

Hybridization of the probe to the target nucleic acid is performed by the application of a small amount of the hybridization buffer (containing the probe) to the tissue or cells under appropriate hybridization conditions. These conditions, also referred to as "hybridization stringency," include hybridization temperature, pH, formamide, and salt concentration of buffers, and they play an important role in the specificity of the hybridization. They are optimized/varied toward facilitating the hydrogen bonding of probes with high homology to the target sequence while at the same time preventing the matching of heterologous sequences. Under conditions of high stringency only probes with high homology to the target sequence form stable hybrids, while at low-stringency (i.e. reaction carried out at low temperature, or in high salt or low formamide concentrations) a probe may bind to sequences with < 70% homology, thus resulting in possible non-specific hybridization signals (Höfler et al., 1990). After hybridization, the tissues are washed under stringent conditions to remove unhybridized probes.

Hybridization in tissue sections or cells bound by radioisotopic labeled probes can be detected by autoradiography either by apposing the tissue section slides to X-ray film or by dipping the slides in photographic emulsion (Leitch, 1994; Murray and Ambinder, 1994). The non-radioactive labels can be detected by enzyme immunohistochemistry involving the use of antibodies (conjugated with either perioxidase or alkaline phosphatase) raised against the label, or by the use of avidin and streptavidin (both biotin-binding proteins). Alkaline phosphatase-conjugated antibodies are the most commonly used for

enzyme colorimetric detection of hybridization; upon incubation with a substrate 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and chromogen nitro blue tetrazolium (NBT) (Hölfer, 1990), a bluish purple product is obtained. The specificity and sensitivity of the isotopic and non-isotopic hybridization and detection are comparable (Höfler, 1990) but the non-isotopic probes last longer, allowing use on multiple samples (Table 1.3). The non-isotopic procedures in routine use can detect up to 20-30 copies of mRNA or viral DNA per cell (Höfler et al., 1986; Speel et al., 1995), and with amplification of the detection procedure, can increase the sensitivity up to five to 10 fold (Speel et al., 1999). The ultimate mRNA detection limit is difficult to determine, but it may reach the level of single mRNA molecules under most optimal test systems (Femino et al., 1998).

The ISH technique is a powerful and unique tool in research and diagnostics. It is widely used in pathology to study both normal nucleic acids like chromosomal mapping and abnormal nucleic acids especially in malignancy/cancers, and gene expressions under different conditions in the cells. In neuroscience, it is possible to use ISH to localize gene expression to specific cell types in specific regions and observe how changes in this distribution occur throughout development and correlate with behavioral manipulations (Montgomery, 2002). ISH has become a routine procedure in some research laboratories for the detection and diagnosis of viral, bacteria and protozoan infections (Murray and Ambinder, 1994). In virology, ISH has helped in detecting the presence of specific virus sequences and demonstrating the presence of the viral genome at the single cell level and correlating it in some cases to the pathology process (Herrington et al.,1990).

Table 1.3 Comparison of the merits of radioisotope and non-radioisotope labels used for probes in ISH (modified from Hölfer, 1990)

Label	Resolution	Sensitivity	Exposure (days)	Stability (weeks)
^{32}P	+	++	7	0.5
^{35}S	++	+++	10	6
^{3}H	+++	+++	14	> 30
Biotin	+++	++	<0.16	> 52
Digoxigenin	+++	+++	<0.16	> 52

1.2.8 Justification of study

ISAV is considered currently one of the most important viral pathogen threatening commercial aquaculture in the northern hemisphere. Much of our understanding of the aetiopathogenesis of ISAV infection comes from virus detection studies using virus isolation in SHK-1 cells (Dannevig et al., 1995a) and/or CHSE-214 cells (Bouchard et al., 1999; Kibenge et al., 2000a), RT-PCR (Mjaaland et al., 1997; Rimstad et al., 1999; Kibenge et al., 2000b), and IFAT on tissue samples from suspected fish (Falk et al., 1998), even though difficulties have been reported in reproducing all of the gross and microscopic lesions of ISA (Jones et al., 1999; Lovely et al., 1999). Moreover, not all infected Atlantic salmon develop gross lesions. Another remarkable feature of infected fish is that Atlantic salmon that survive the clinical disease and the asymptomatically infected salmonid become persistent carriers of the virus (Totland et al., 1996). Thus, it is not clear how well the presence of virus in tissues of fish with clinical disease correlates with the characteristic ISA lesions. In addition, the exact tissue(s) and cell(s) of virus persistence in infected fish are not yet known.

The phenomenon of persistent ISAV infection in fish (Devold et al., 2000) is unusual for orthomyxoviruses, as there is no evidence of persistence of influenza virus genetic material in any animal species. Influenza viruses are cleared from ducks in approximately seven days (Webster, 1999), although they can persist long-term in carrier cultures (Urabe et al., 1992; 1993; Clavo et al., 1993). In case of influenza C virus, virus persistence in cell culture is associated with a modified hemagglutinin/esterase sequence with altered activity and diminished RDE activity (Marschall et al., 1994). In case of ISAV, recovered farmed Atlantic salmon continuously shed virus for long periods of time, but detection of ISAV in carrier fish required use of RT-PCR (Devold et al., 2000) suggesting that the viral RNA is

not abundant. Such fish can however, transmit ISA to healthy Atlantic salmon upon cohabitation (Totland et al., 1996; Nylund et al., 1997; Optiz et al., 2000). The underlying cellular and molecular mechanisms of ISAV persistence in fish have not yet been studied.

In Atlantic salmon, the virus seems to target leucocytic cells (Falk et al., 1995) and endothelial cells (Falk and Dannevig, 1995) while *in vitro* it readily replicates in SHK-1 cell line, a macrophage-like cell line (Dannevig et al., 1995a; Koppang et al., 1999), although some of the ISAV isolates from Canada are also cytopathic in CHSE-214 cells (Kibenge et al., 2000a) while most, if not all, European isolates are noncytopathic in this cell line. Virus persistence in fish may be related to the ability of the virus to remain noncytopathic, particularly in fish species that are only subclinically infected. In Atlantic salmon, in addition to the probable presence of noncytocidal virus, pathology and virus persistence may also be due to induction of non neutralizing antibodies as a result of antigenic drift in the virus when it replicates in presence of antibody. In this case, lesions of ISA would be associated with deposition of virus-antibody and viral antigen-antibody complexes in various organs.

1.2.8.1. Hypotheses and Research Objectives

The main goal of this study was to identify the cells and tissues that harbor ISAV during the clinical and persistent phases of ISAV infection in Atlantic salmon, and to define the nature of the virus gene expression.

The rationale for the study is that understanding the gene expression of ISAV both *in vitro* and *in vivo* will allow for better understanding of why the virus is fatal to

marine-farmed Atlantic salmon when other fish species can be infected by ISAV and survive. These findings will not only extend our knowledge of the basic virus-host interactions at the cellular level, but will also be invaluable in formulating fish disease management and control strategies.

The following hypotheses were proposed in order to undertake this work:

a) Lesions in fish with clinical ISA disease are a direct result of multiplication of cytocidal virus in the cells of affected tissues;

b) ISAV persists in the host by causing a low-level noncytocidal infection of macrophage-like cells;

c) The difference in host response to ISAV is a consequence of strain variation of the virus and variation in host susceptibility.

The specific objectives of this thesis research on the above hypotheses were:

1. To define the nature of viral gene expression in different fish cells infected with ISAV:

   a) This required the development of an ISH technique;

   b) The ISH method was used to investigate virus gene expression in different fish cell lines *in vitro* by using riboprobes prepared to different ISAV RNA segments to examine SHK-1, TO and CHSE-214 cells harvested at different times after inoculation with virus;

   c) The same riboprobes were then used on fish tissues collected from Atlantic salmon experimentally infected with ISAV in order to determine viral gene expression in clinical ISA disease.

2. To correlate the presence of ISAV with lesions of ISA in Atlantic salmon and identify the cells that harbor the virus in recovered and/or carrier fish:

a. The ISH technique developed in Objective 1 above was used with fish experiments to visualize the viral transcripts where virus was replicating in tissues and cells of infected fish.

b. Duplicate tissue sections/serial sections were used in a histolopathological study to establish the relationship between the virus and the ISA lesions

c. Using riboprobes from the ISAV RNA segments 6, 7 and 8, and correlating to lesions on the next serial section in fish tissues infected with different ISAV isolates it was possible to compare the sensitivity of the probes.

d. The ISH technique was used on tissue sections of Atlantic salmon and rainbow trout that survived an experimental ISAV infection in order to identify the site (s) of virus persistence in recovered fish.

e. Rainbow trout were used to study the site (s) of virus persistence in carrier fish.

f. ISH results were confirmed by RT-PCR

3. To determine if the two hemagglutinin genotypes of ISAV (North American and European) in cell culture react differently to segments 7 and 8 riboprobes using ISH.

a. The temporal reactivity of the two hemagglutinin genotypes of ISAV to ISH was determined in fish cell cultures.

b. The expression of both segments 7 and 8 genes by the two ISAV hemagglutinin genotypes was characterized in experimentally infected Atlantic salmon.

CHAPTER 2

GENERAL MATERIALS AND METHODS

2.1 VIRUSES AND CELLS

Five isolates of ISAV originally recovered from fish from Canada, Norway and Chile (Table 2.1) and one strain of infectious pancreatic necrosis virus (IPNV), strain FVX73, obtained from American Type Culture Collections (ATCC), Manassas, Virginia, were used for this study. The ISAV isolates NBISA01, U5575-1, RPC-01-0593-1, 7833-1 and Norway 810/9/99 will be referred to as NBC, NSC, RPC, CH7 and NOR, respectively, throughout this thesis. The ISAV isolate RPC/NB 98-0280-2 was used only in producing the riboprobes. ISAV isolates were propagated in three fish cell lines (TO, CHSE-214 and SHK-1) as previously described (Kibenge et al., 2000a; 2001a). Briefly, TO cells (Wergeland and Jakobsen 2001) were grown at room temperature (RT) (22°C) in Minimal Essential Medium containing Hanks' salts (HMEM) supplemented with 10% fetal bovine serum (FBS), 100µg ml^{-1} gentamycin, 1% non essential amino acids (NEAA), and 292 mM L-glutamine. The CHSE-214 cells (Fryer et al., 1965) were grown at 16°C in HMEM growth media, supplemented with 10% FBS, 100 units of penicillin, 100 µg ml^{-1} streptomycin and 0.25 µg ml^{-1} amphotericin B. The SHK-1 cells (Dannevig et al.,1995a) were grown at RT in Leibovitz's L-15 medium supplemented with 10% FBS, 4mM glutamine, 100 units penicillin, 100 µg streptomycin and 0.25 µg amphotericin B. All the fish cells were initially grown in 75 cm^2 flasks. For experimental infection, the cells were grown in 9 cm^2 chamber slide flasks (Nunc® Flaskette®, Nalge International, Naperzille, IL. USA) and allowed to monolayer overnight in the appropriate growth conditions. For all cell lines the FBS was reduced to 5% in the maintenance medium. The IPNV was propagated in TO cell line using the same procedures. One hundred micro liters of virus suspension in the

maintenance media were used to inoculate TO, CHSE-214 and SHK-1 cell monolayer in slide flasks. The virus was allowed to absorb at room temperature for one hour before the addition of the maintenance media. The flasks were incubated at 16°C and monitored daily under the microscope for development of cytopathetic effects (CPE). The monolayers of the different cell lines inoculated with ISAV were harvested daily. IPNV-infected cell mono layers were harvested 3 dpi when more than 60% of the cells were showing CPE.

2.2 VIRUS TITRATION

The virus was titrated on TO cell line to determine the titer of the stock virus and the amount of virus used for inoculation of cell cultures and fish. Virus titres were determined by serial 10 fold dilutions of each virus sample and was expressed as median tissue culture infective dose ($TCID_{50}$) equivalent to the amount of virus required to cause CPE in 50% of infected cell monolayer. To establish the 100 $TCID_{50}$/100 µl, the virus dilutions (100 µl) in maintenance media were added to cell monolayers on three wells of 48-well microtitre plate and allowed to adsorb for one hour at RT. Four hundred microliters of maintenance media were added to each well and the plates incubated at 16°C. The end point CPE was read until 12 dpi. The virus $TCID_{50}$ was calculated using the method of Reed and Muench (1938).

2.3 RT-PCR

Viral RNA were extracted from 300 µl of infected or non infected cell culture lysate and tissue homogenates using 700 µl Trizol LS reagent (Invitrogen Life Technologies,

Table 2.1 Geographic origin and hemagglutinin genotype of ISAV isolates used.

ISAV Isolate and References	Origin	Hemagglutinin Genotype (Kibenge et al., 2001b)
NBISA01(NBC) Jones et al., 1999	New Brunswick, Canada	North American
U5575-1(NSC) Kibenge et al., 2001b	Nova Scotia, Canada	European
RPC-01-0593-1 (RPC) (Kibenge, personal communication)	New Brunswick, Canada	North American
7833-1 (CH7) Kibenge et al., 2001a; 2001b	Chile	North American
Norway810/9/99 (NOR) Devold et al., 2000	Norway	European

Invitrogen Corporation, Carlsbad, CA. USA) following the manufacturer's protocol. The primer pairs used to generate the PCR products for use in preparation of the riboprobes for *in situ* hybridization are listed in Table 2.2. One-step RT-PCR reactions were carried out with the extracted RNA as described earlier (Kibenge et al., 2000a), using Titan-One tube RT-PCR Kit (Roche Molecular Biochemicals, Penzburg, Germany). Briefly, cycling was performed using the PTC-200 DNA Engine Peltier thermal cycler (MJ Research Inc., Waltham, MA. USA). The conditions for cycling consisted of one cycle of cDNA synthesis at 55°C for 30 min and 94°C for 2 min, respectively, followed by 40 cycles each with denaturation at 94°C for 30 sec, annealing at 61°C for 45 sec and extension at 72°C for 90 sec, with final extension at 72°C for 10 min. The RT-PCR products were resolved by electrophoresis on a 1% agarose gel and visualized under 340nm UV light staining with ethidium bromide (Sambrook et al., 1989).

For subcloning of the RT-PCR products, they were excised from the agarose gel and purified using High Pure PCR Purification Kit (Roche Molecular Biochemicals) following the manufacturer's protocols. The PCR products were precipitated in ethanol with high salt and stored at -80°C until used.

Table 2.2 RT-PCR primers

ISAV RNA Segment	Forward Primer	Reverse Primer	Genbank Accession #	Reference
6	5'-AAACTACCCTGACACC ACCTGG-3' (HA 1F) nucleotides 70-91	5'-ACAGAGCAATCCCA AAACCTGC-3' (HA 1R) nucleotides 1061-1082	AF220607	Rimstad et al., 2001
7	5'-ATGTCTGGATTTAACT CGAGG-3' (MF4) nucleotides 1-22	5'-CATAACAAGTTTTC AACCAATC3' (MR4 nucleotides 770-791	AX083264	Griffiths and Ritchie, 2001
8	5'-GAAGAGTCAGGTGCC AAGACG-3' (FA-3) nucleotides 342-363	5'-GAAGTCGATGATCT GCAGCGA-3' (RA-3) nucleotides 532-553	AF312317	Devold et al., 2000

2.4 RIBOPROBE SYNTHESIS

2.4.1 Molecular cloning

The PCR products from ISAV isolate RPC/NB 98-0280-2 were cloned into the pCR®II-TOPO® vector using the TOPO TA Cloning Kit (Invitrogen Life Technologies) according to the manufacturer's protocols. The recombinant plasmids were transformed in *Eschericia coli* DH5α competent cells supplied with the kit. Two microliters of the clone reaction was added to the *E.coli* DH5α competent cells, mixed gently and incubated on ice for 30 min. The bacteria were heat shocked at 42°C for 30 sec and placed on ice for 2 min. Bacteria were recovered by adding 250µl of SOC medium (2g tryptose, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10mM $MgSO_4$, 20 mM glucose) (Invitrogen Life technologies) placed in shaking water bath (225 rpm) at 37°C for 1 hour. One hundred microliters of cultures were plated onto YT plates containing X-gal and 100µg/ml ampicillin, at 37°C overnight. White colonies were transferred to fresh YT plates containing X-gal and ampicillin as Master plates and incubated at 37°C overnight. Transformants were screened and analyzed by isolating the plasmid DNA and *Eco* R1 digestion of inserts were visualized on 1% agarose gel electrophoresis.

2.4.2 Isolation of plasmid DNA

The rapid alkaline extraction method (Birnboim and Doly, 1979) was used for the isolation and screening of the recombinant plamids in pCRII and pGEM-3Z vectors (Promega Corporation, Madison, WI. USA). Briefly, white bacteria colonies from the master plate were inoculated in 5 ml of 2X YT broth containing 100µl (100µg/ml) of ampicillin and

incubated at 37°C with constant shaking at 250 rpm overnight. To isolate the plasmid DNA 1.5ml of the bacterial culture was centrifuged at 14000 rpm for 30 sec. The harvested bacteria pellet was resuspended in 110µl of solution #1 (50mM glucose, 10mM EDTA, pH 8, 25mM Tris-HCl, pH 8). To the resuspended bacterial pellet, 220µl of solution #2 (0.2N NaOH, 1% sodium dodecyl sulphate (SDS)) was added and mixed thoroughly by inverting the tube 10 times and the tube was incubated on ice for one minute. After the one minute incubation on ice, 165 µl of solution #3 (3M potassium/5M acetate) was added, vortexed and incubated for another 5 min on ice. Samples were centrifuged and the plasmid DNA precipitated from the supernatant by the addition of 1 ml chilled 100% ethanol, inverted several times and incubated on ice for 5 min. The plasmid DNA was pelleted by centrifugation at 14000 rpm for 5 minutes. The pellets were resuspended in 50µl of TE buffer containing 20µg/ml RNAse, vortexed vigorously and incubated at 37°C for 5 minutes. The plasmid DNA were precipitated by the addition of 200µl of 5 M ammonium sulphate and 400µl of cold 100% ethanol. The plasmid DNA pellets were washed with 70% ethanol, vacuum dried and resuspended in 20µl of TE buffer. The isolated plasmid DNA were screened and analyzed for inserts by restriction enzyme digestion.

2.4.3 Restriction enzyme analysis

To check for the presence of the inserts in the plasmid DNA, 1 µl of restriction enzyme buffer and 1µl of *Eco R1* restriction enzyme were added to eight microliters of the isolated plasmid DNA in a microfuge tube and incubated for 2hr in a water bath at 37°C. The restriction digestion products were analyzed by 1% agarose gel electrophoresis.

2.4.4 Subcloning and construction of an *in vitro* transcription vector

To produce the templates for preparation of the riboprobes from segment 6, 7 and 8 of ISAV, the DNA inserts were subcloned into pGEM-3Z vector having SP6 and T7 promoters (Promega) (Fig. 2.1). Briefly, the PCR products were cut out of the pCR®II-TOPO® recombinant plasmid using *Eco R1* and were subcloned into the *Eco R1* site of pGEM-3Z using the Rapid DNA Ligation Kit (Roche Molecular Biochemicals) according to manufacturer's protocol. Briefly, ligation was performed with 10µl of 2 X DNA T4 ligation buffer containing 1µl or 5 units of T4 DNA ligase and 10µl of DNA (9µl of PCR product + 1 µl of pGEM-3Z vector) in 1x concentration mixed thoroughly and incubated for 5 min at 25°C. The recombinant plamids were transformed in *E.coli* DH5α competent cells. The transformants were screened by *Eco R1* digestion and subsequent 1% agarose electrophoresis to visualize the size of the insert.

2.4.5 Production of templates for *in vitro* transcription

The recombinant pGEM-3Z plasmid for the *in vitro* transcription was purified using a midi-prep plasmid isolation method. The orientation of the DNA inserts in the plasmids were determined by restriction enzyme analysis. In order to check for the orientation of the DNA inserts with respect to the SP6 promoter which is the correct orientation, the recombinant pGEM-3Z with segments 6 and 7 insert was digested with *Kpn1* restriction enzyme, while segment 8 was digested with *Bam H1* restriction enzyme, both enzymes cut downstream of the T7 promoter. The digestion products were resolved in 1% agarose gel electrophoresis. To generate the linear templates needed for the preparation of riboprobes,

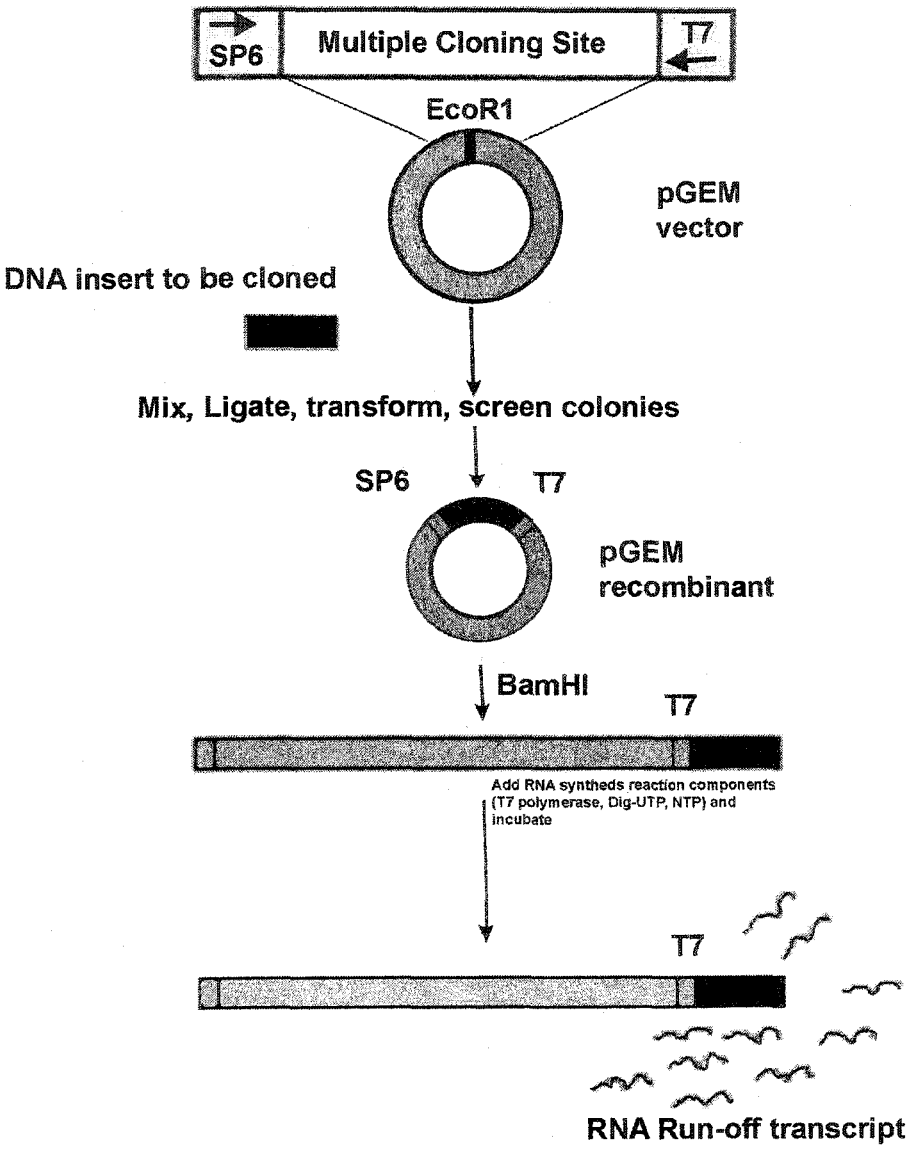

Figure 2.1 Diagram outlining the cloning of the ISAV RNA segment 8 cDNA in pGEM-3Z vector and subsequent *in vitro* transcription from the T7 promoter.

the plasmids DNA were digested with *Hinc I, Xba I and, Bam H1* for segments 6, 7 and 8 respectively. The digested DNA were purified by phenol/chloroform extraction and ethanol precipitation in high salt.

2.4.6 *In vitro* transcription

The *in vitro* transcription was carried out with the Riboprobe Combination System-SP6/T7 kit (Promega), following the manufacturer's protocol in presence of digoxigenin-11-deoxyuridine triphosphate (Digoxigenin -11-UTP, Roche Molecular Biochemicals). Briefly, the RNA synthesis reaction components, T7 polymerase and NTPs were added to the linearized DNA template with RNAse inhibitor (Roche Molecular Biochemicals) and incubated at 37°C for 2 hr. RNAse free RQ1 DNAse was added and incubated for further 15 min at 37°C to remove the DNA template. The transcription reaction products were visualized on 2% agarose gel electrophoresis. Five microliters of the transcription reaction product was added to 20µl of RNA sample buffer (10ml deionized formamide, 3.5ml of 37% formaldehyde, and 2 ml of MOPS buffer), and 5 µl of RNA loading buffer (50% glycerol, 1 mM EDTA, 0.4% bromophenol blue, 1 mg/ml ethidium bromide) and incubated for 10 min at 65°C prior to loading in 2% agarose gel in 1 X TAE. The incorporation of the digoxigenin into the probe was analyzed by Northern blot hybridization. The digoxigenin-labeled RNA transcripts were purified by ethanol precipitation in high salt and stored at -80°C until needed.

2.4.7 Northern blot hybridization

Total RNA was extracted as described above from infected and non infected CHSE-214 cells after infection with ISAV isolate NBISA01 or with IPNV strain FVX73 used as a CPE-positive, expected hybridization-negative control. Twenty-five microliters of total RNA in formamide were mixed with 5 µl of 10 X MOPS buffer (0.4 M MOPS, pH 7, 0.1 M sodium acetate, 0.01 M EDTA), 9 µl of 13.3 M formaldehyde and 11 µl of deionized sterile distilled water, and heated at 55°C for 15 min. Ten microliters of RNA loading buffer were added and 20 µl of the mixture loaded in a well on 1% agarose/formaldehyde gel. Electrophoresis was carried out in 1 X MOPS running buffer for 3 hours at 70 volts. The RNA was then blotted onto nylon membrane (Amersham Pharmacia Biotech, USA) using standard procedures (Sambrook et al., 1989). The RNA was immobilized by baking the nylon membrane at 80°C for 2 hr under vacuum. The membranes were pre-hybridized with 20 ml of prehybridization buffer (50% deionized formamide, 2 X standard saline citrate (SSC) (1 X SSC: 0.15 M NaCl, 0.015 M sodium citrate, pH 7), 1 X Denhardt's solution (2% Ficoll 400, 2% polyvinylpyrrolidine, 2% bovine serum albumin (BSA), 25 mM sodium phosphate buffer, pH 7,10mM EDTA, 250ng/µl calf thymus DNA, 500µg/l tRNA, and diethyl pyrocarbonate (DEPC) -treated water) incubated for 2 hours at 68°C in a water bath. Five microliters of the 40µg/ml riboprobe were added to 10 ml of fresh prehybridization buffer to give a final concentration of 200ng/ml of the hybridization buffer. After removing the prehybridization buffer, 5 ml of the hybridization buffer containing the respective riboprobes was added to each membrane and incubated in the water bath at 68°C overnight with shaking. The post hybridization washes of the membranes were under high stringency. Briefly, the membranes were washed twice with 2 X SSC in 0.1% SDS for 15 min at room

temperature, followed twice with 0.5 X SSC in 0.1% SDS for 15 min at 68°C. Positive hybridization was detected by colorimetric method using the DIG Nucleic Acid Detection Kit (Roche Molecular Biochemicals) following the manufacturer's protocol. Briefly, after stringency washes, membranes were rinsed briefly with washing buffer (maleic acid buffer: (0.1 M maleic acid and 0.15M NaCl), 0.3% v/v Tween 20) and blocked by gentle shaking in the blocking solution for 1 hr. Membranes were then incubated with alkaline phosphatase-conjugated anti-digoxigenin Fab fragments diluted 1:5000 in maleic acid buffer for 1 hr at room temperature. Membranes were washed twice 15 min each with the washing buffer. Hybridization signal was developed with 0.375mg/ml 5-bromo-4-chloro-3-indolyl phosphate (BCIP) as substrate and 0.188mg/ml nitroblue tetrazolium (NBT) salt as chromogen in detection buffer (0.1M Tris-HCl, 0.1M NaCl, pH9.5) overnight in the dark. Colour development was stopped by rinsing membrane in sterile distilled water. Positive hybridization was visualized as a purplish blue band on the membrane.

2.5 FISH EXPERIMENTS

2.5.1 Sample population

Atlantic salmon (AS) *Salmo salar L.*, rainbow trout (RbT) *Oncorhynchus mykiss*, and coho salmon (CS) *Oncorynchus kisutsh* were used for this study. The AS were obtained from the Cardigan Fish Hatchery and Atlantic Sea Smolt, P. E. Canada. The RbT and CS were obtained from Cardigan Fish hatchery and Aqua Health Ltd, P. E. Canada. The fish were without any history of disease and were certified to be ISAV free by RT-PCR and virus isolation on 5 fish pool of each species from the population before use for the experiment.

The fish were maintained in the Aquatic Animal Facility of the Atlantic Veterinary College. The mean weight and length of the fish at introduction into the facility were approximately 10-20g and 10 cm respectively. The fish were maintained in a fresh water flow through system at a temperature of approximately 11°C. The fish were acclimatized for 14 days during which period they were monitored for any diseases, before use for ISAV infectious study. The experimental procedures were performed in accordance with the guidelines of the Canadian Council of Animal Care (Olfert et al., 1993).

2.5.2 Experimental infection of fish and tissue sampling

The fish used for this study were removed from the stock holding tank, anaesthetized by immersion in an aerated solution of tricaine methane sulphonate (TMS-222) (100mg/l), and serially assigned to the experimental group. Each fish was challenged by intraperitoneal injection with the appropriate ISAV isolate in virus suspension and returned to the study tank in infected room. Uninfected fish used as control were housed in a separate clean room. The infected fish were observed daily for morbidity and mortality. Fish were necropised and the following tissues collected aseptically; heart, liver, spleen, gills, head and trunk kidney, and pyloric caeca. Portions of each tissue were fixed in 4% paraformaldehyde or 10% formalin or stored at -80°C in sterile plastic bags. After 24 hr, the formalin- and paraformaldehyde-fixed tissues were processed in an automatic tissue processor (Sakura, Tissue Tek® VIP, Torrance, CA) and were embedded in paraffin wax. Serial 5 µm thick sections of the fixed tissue were either stained with haematoxylin and eosin (H & E) stain for light microscopy or placed on silane coated DEPC-treated slides for ISH.

2.5.3 Assessment of histopathology in fish

The quality of the fixed tissues and the microscopic/histopathological lesions observed in organs of experimentally infected fish were qualitatively assessed and graded by modifying the method used by Jones and Groman (2001). For tissue conditions, 0, no significant autolysis, 1, mild-moderate autolysis, 2, too autolytic to interpret. For the liver, 1, mild to moderate local or diffuse sinusoidal congestion often distributed randomly, vasculitis or perivascular cuffing characterized by infiltration of mixed leucocyte population in the large and small vessel walls; 2, congestion, hemorrhages and hepatocellular degeneration and necrosis. In the kidney; 1, mild to moderate sinusoidal congestion, interstitial hemorrhages, intravascular hemolysis and increased erythrophagia, 2, marked congestion, diffuse interstitial hemorrhages, depletion and necrosis of the interstitial cells and tubular necrosis. In the heart; 1, mild to moderate swelling and hyperplasia of the endothelial cells, increased activated macrophages; 2, hydropic degeneration of myocytes. In the spleen, 1, mild to moderate sinusoidal congestion and/or increased erythrophagia, 2, congestion, hemorrhage, increased erythrophagia and depletion of the leucocytes. In the gill, 1, congestion of lamellar capillaries, 2, congestion of filamental arterioles, hemorrhages and necrosis of interstitial cells. In the pyloric caeca, 1, congestion of lamina propria vasculature, 2, congestion and necrosis of mucosa. In all tissues a score of 2 might include both lesions seen in mild to moderate conditions. In all tissues, a score of zero means that no microscopic changes were detected.

2.6 *IN-SITU* HYBRIDIZATION (ISH)

The cell monolayers fixed in 4% paraformaldehyde and fish tissues fixed in 4% paraformaldehyde and 10% formalin were prepared for ISH by a slight modification of the conditions described by Komminoth (1992) and Komminoth et al. (1992). Fish sections were deparaffinized by passing them through two changes of xylene at 5 min. Fish sections and/or cell monolayers were rehydrated in graded ethanol and rinsed finally in DEPC-treated deionzed distilled water. Slides (fish sections and cell monolayers), were moved to 0.2N HCl for 20 min at RT and then transferred into a 2 X SSC solution incubated for 15 min at 70°C. The cells were permeabilized with proteinase K in 50 mM Tris-HCl, pH 8.0, 5 mM EDTA for15 min at 37°C. The proteinase digestion was stopped by rinsing the slides with 2mg/ml glycine in phosphate buffered saline (PBS) for 2 min at RT. Slides were refixed in freshly prepared 4% paraformaldehyde for 5 min at RT. Slides were transferred to 3 X PBS for 5 min to stop fixing and washed twice with 1 X PBS, and acetylated with 0.5% acetic anhydride (Sigma, Mississauga, ON. Canada) in 0.1 M triethanolamine buffer (Sigma). The slides were blocked with 2 X SSC and dehydrated through graded ethanol and air dried. The slides were prehybridizied for 2 hr with prehybridization buffer and later hybridized overnight with hybridization buffer at 37°C in a moist chamber. The slides were washed at high stringency with 60% formamide and 2 X SSC twice at 37°C and once at RT for 30 min, and then rinsed twice with 1 X PBS at RT for five minutes. The slides were incubated with RNAse solution for 15 min at 37°C and finally washed twice in 2 X SSC for 5 min each at RT. Hybridization was detected using DIG-UTP-Detection Kit (Roche Molecular Biochemicals) following the manufacturer's protocol. Briefly, slides were incubated for 1 hr at 37°C with different concentrations of alkaline phosphatase-conjugated

anti-digoxigenin antibody diluted in maleic acid buffer (0.1 M maleic acid, 0.15 M NaCl, pH 7.5). Slides were washed twice in washing buffer (0.1 M maleic acid, 0.15 M NaCl, pH 7.5, 0.3% v/v Tween 20) for 15 min each at RT. Signal was developed with the substrate consisting of 0.375 mg/ml BCIP and the chromogen 0.188 mg/ml NBT salt in detection buffer (0.1 M Tris-HCl, 0.1 M NaCl), pH 9.5, for 16 hr in the dark. Colour development was terminated by dipping the slides in deionized distilled water for 5 min and counter stained with Nuclear Fast Red (Vector Laboratories Inc., Burlingame, CA. USA). The slides were mounted with Faramount aqeous mounting media (DAKO, Carpinteria, CA. USA) prior to microscopic examination. The hybridization signals were observed using a light microscope and images captured digitally. Digital images were enhanced using Adobe Photoshop 5.5™ (Adobe Systems Inc., San Jose, CA. USA).

2.7 DETERMINATION OF THE FREQUENCY OF HYBRIDIZATION SIGNALS

The intensity of the hybridization signals indicates the amount of the virus mRNA in an infected cell and was determined subjectively by looking at images of different fields in an infected cell layer or tissue section using Adobe Photoshop™. The frequency of signals indicates the number of cells showing signals in the preparation. In cell culture the frequency of signals was graded using digital images in 10 random fields acquired at X16 objective. Both negative and positive cells in five random fields out of the 10 fields were counted and the average of the cells showing signals calculated from the total cells in the fields. In fish tissues/organs, the frequency of hybridization signals was graded subjectively at X16 objective using a minimum of 10 fields as follows; 0, no signals, 1+, presence of signals in less than 4 fields, 2+, presence of signals in 4 to 7 fields, 3+, for presence of

signal in 8 to 10 fields.

2.8 STATISTICAL ANALYSIS

A *student t test* and one-way analysis of variance (ANOVA) were used to test for the difference in the frequency of hybridization signals in cell cultures between different isolates and segments of ISAV. The strength of the association between scores for lesions and ISH signals in tissues from infected fish was assessed by nonparametric Spearman correlation coefficient. A nonparametric Kruskal-Wallis test was used to test the differences in scores for ISH signals and lesions between groups infected with different isolates on a daily basis; p-value were adjusted for multiple-day testing by Bonferroni method (dividing the significance level by the number of tests carried out). The mortality in the groups was estimated by Kaplan-Meier survival curves and compared between the groups by a nonparametric log-rank test (Dohoo et al., 2003).

CHAPTER 3

DETERMINATION OF THE NATURE OF ISAV GENE EXPRESSION IN DIFFERENT FISH CELL LINES INFECTED WITH THE VIRUS*

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3.1 ABSTRACT

Infectious salmon anemia virus (ISAV) is a new member of the family *Orthomyxoviridae* recently assigned to the genus *Isavirus*. *In situ* hybridization conditions were established for the detection of mRNA transcripts of ISAV RNA segments 6 - 8 in TO, SHK-1, and CHSE-214 cell lines infected with ISAV. Hybridization signals first appeared in the nucleus, then in the cytoplasm of ISAV-infected cells. Transcription signals were intense and widespread for all three mRNA transcripts in TO cells as from 1 dpi. In contrast, the ISAV gene expression in the SHK-1 cells remained poor throughout the duration of the study. In CHSE-214 cells, maximum hybridization signal was seen in the nucleus by 2 dpi with the segment 8 riboprobe, at 4 dpi with segment 7 riboprobe, and 5 dpi with segment 6 riboprobe. With all three riboprobes, the hybridization signals in the ISAV infected CHSE-214 cells were initially focal but became multifocal by10 dpi, and started to decrease by 14 dpi. This result suggested that the slower replication of ISAV in CHSE-214 cells makes it more suitable for the identification of the temporal pattern of gene expression of ISAV. The earlier transcription of segment 8 and later transcription of segment 7 might suggest that there is a difference in the time requirement and the amount of proteins encoded by these segments. Our findings also confirm the nuclear transcription and replication of ISAV, which is consistent with other orthomyxoviruses.

3.2 INTRODUCTION

ISAV is currently one of the most important viral pathogens threatening commercial aquaculture in the northern hemisphere. It is the aetiological agent of ISA in marine-

farmed Atlantic salmon (Falk et al., 1997) which is characterized by exophthalmia, pale gills, ascites, severe hemorrhagic necrosis of internal organs, and high mortality (Thorud and Djupvik, 1988; Evensen et al., 1991; Byrne et al., 1998; Mullins et al., 1998). The virus has also been detected in diseased coho salmon in Chile (Kibenge et al., 2001a) and in apparently normal wild fish (sea trout, Atlantic salmon, and brown trout) from Scotland (Raynard et al., 2001). Brown trout, sea trout, rainbow trout (Nylund et al., 1994a and 1997; Nylund and Jakobsen, 1995), pollock, Atlantic cod (MacLean et al., 2003), Atlantic herring, and European eel have been shown to be asymptomatic carriers of the virus. Much of our understanding of the aetiopathogenesis of ISAV infection in fish comes from virus detection studies using virus isolation in SHK-1 cells (Dannevig et al., 1995a) and/or CHSE-214 cells (Bouchard et al., 1999; Kibenge et al., 2000a), RT-PCR (Mjaaland et al., 1997; Rimstad et al., 1999; Kibenge et al., 2000b), and IFAT on tissue samples from suspected fish (Falk et al., 1998).

The morphological, biochemical and replication properties of ISAV indicate strongly that it is a member of the virus family *Orthomyxoviridae* (Falk et al., 1997; Eliassen et al., 2000; Sandvik et al., 2000). As with the influenza A and B viruses, the genome of ISAV consists of eight single-stranded RNA segments of negative polarity. Unlike influenza A and B virus, information is still lacking on the gene expression in ISAV. Genomic organization and nucleotide sequence information of the 8 genomic RNA segments of ISAV is now publicly available in the gene bank (Mjaaland et al., 1997; Krossøy et al., 1999; Ritchie et al., 2001a; Rimstad et al., 2001; Kibenge et al., 2001b; Clouthier et al., 2002). Only the expression of the segment 6 gene that encodes the hemagglutinin protein has been described (Rimstad et al., 2001; Krossøy et al., 2001b). The gene-protein

assignments for segments 7 and 8 are controversial as each has two open reading frames (ORFs) capable of encoding proteins of sizes corresponding to membrane (M1 and M2) or nonstructural (NS) and nuclear export (NEP) proteins. The ORF 1 of RNA segment 8 of ISAV was reported to encode a 24KDa structural protein (Biering et al., 2002) which was designated the matrix protein (Falk et al., 2004). The identities of viral protein encoded on segment 7 and the second ORF in segment 8 are currently unknown.

The interest of this study was in identifying the tissues and/or cells that harbor ISAV in clinically infected and carrier fish. To investigate this phenomenon, an *in situ* hybridization technique was developed and used to detect expression of ISAV in infected fish cells. This technique was then used to determine the temporal appearance of mRNA transcripts of ISAV RNA segments 6, 7, and 8 in different permissive fish cell lines. RNA segment 6 was chosen since it is the only ISAV RNA segment that has been definitively correlated with its encoded protein product, hemagglutinin, a major glycoprotein on the virion envelope. ISAV RNA segments 7 and 8 were chosen since they were considered to encode both structural and nonstructural proteins. Moreover, we wished to determine if the temporal appearance of mRNA transcripts of these segments would clarify their gene-protein assignments.

3.3 MATERIALS AND METHODS

3.3.1 Cells and viruses

The ISAV isolate NBC (Jones et al., 1999) used in this study was grown and titrated in CHSE-214, SHK-1, and TO cell lines as previously described (Kibenge et al., 2000a; b).

IPNV strain FVX73 was used as the CPE-positive control.

3.3.2 Preparation of ISAV riboprobes

The materials and methods used to prepare the riboprobes from segment 6,7 and 8 of ISAV (isolate RPC/NB 98-0280-2) RNA used for the *in situ* hybridization have been described in Chapter 2, section 2.4.

3.3.3 Culture and experimental infection of fish cells.

The TO, CHSE-214 and SHK-1 cells were grown on Lab Tek® tissue chamber slide-flasks. Briefly, 3 ml of TO, CHSE-214 or SHK-1 cell suspension in growth media was added to each slide flask respectively and allowed to monolayer overnight. One hundred microliters of NBC ISAV suspension with a titer of $10^{4.77}$ $TCID_{50}$/ml was used to inoculate the cell monolayers. Three of the slide-flasks were left uninoculated to serve as uninfected controls while one flask of each cell line was inoculated with 100 µl of IPNV with a titer of $10^{7.5}$ $TCID_{50}$/ml, to check for specificity of the riboprobes. Presence of CPE was monitored daily by microscopic examination. The monolayers of the three different cell lines inoculated with ISAV were harvested daily. On 4 dpi, the additional ISAV infected TO cells were harvested and used for the optimization of ISH conditions. IPNV-infected monolayers were also monitored for CPE and were harvested 3 dpi when more than 60% of the monolayer had CPE. The harvested cell monolayers on the slide-flasks were fixed for 30 min in 4% paraformaldehyde, dehydrated with graded alcohol and stored at -70°C until used in ISH.

3.3.4 Optimization of ISH in fish cells

The slides of TO cells processed 4 dpi were used to optimize the ISH conditions. For this, the slides were each divided into 6 wells with hydrophobic marker (DAKO). The cell monolayers were prepared for ISH as already described in Chapter 2, section 2.6. The prehybridization buffers and conditions were as earlier described in Chapter 2, section 2.6. For the hybridization buffer, the following concentrations of the segment 8 riboprobe were applied to the different wells on the slides: 5ng/µl, 2.5ng/µl, 1.25ng/µl, 0.625ng/µl and 0ng/µl. Hybridization was carried out for 4, 8 or 16 hr at 37°C in a moist chamber. Post hybridization washes were as described earlier in Chapter 2, section 2.6. Hybridization was detected using the Dig-UTP-Detection Kit (Roche Molecular Biochemicals). Briefly, after blocking, slides were transferred to a slide incubation tray and were incubated with alkaline phosphatase-conjugated anti-digoxigenin Fab fragments diluted 1:2000, 1:1000, 1:750, or 1:500 in maleic acid buffer (0.1M maleic acid, 0.15M NaCl, pH 7.5). Slides were washed twice in washing buffer (maleic acid buffer, 0.3% v/v Tween 20) for 15 min each at room temperature. The signal was developed using 0.375mg/ml BCIP and 0.188mg/ml NBT salt in detection buffer (0.1M Tris-HCl, 0.1M NaCl), pH 9.5, for 16 hr in the dark. Color development was terminated by washing slides in distilled water for 5 min and was counter stained with nuclear fast red (Vector). The slides were then mounted with Faramount aqueous mounting media (DAKO) prior to microscopic examination.

3.3.5 ISH on different fish cell lines infected with ISAV

The harvested slides of ISAV infected and uninfected TO, SHK-1 and CHSE-214 cell

monolayers and IPNV infected TO cells were subjected to ISH using the optimized conditions. The cell monolayer on each slide was divided into 4 wells using the hydrophobic pen (DAKO) and hybridized with segments 6, 7 and 8 riboprobes, while the last well was used as a negative control. The hybridization signals were observed using a light microscope and imaged digitally. Digital images were enhanced using Adobe Photoshop 5.5™ (Adobe Systems Inc.).

3.4 RESULTS

3.4.1 Preparation of riboprobes

The RT-PCR primers used for ISAV RNA segments 6, 7 and 8 yielded PCR products of 967, 791, and 220 bps, respectively. These products were cloned using the TOPO TA cloning kit (Invitrogen Life Technologies) and were then moved to the transcription vector pGEM-3Z (Promega). Because the intent was to study the expression of the respective RNA segments that might lead to protein synthesis, the following antisense riboprobes were produced to detect mRNA transcripts: segment 6 antisense riboprobe of 387 nucleotides (nt), segment 7 antisense riboprobe of 405 nt, and segment 8 antisense riboprobe of 210 nt. A sense riboprobe of RNA segment 7 of 385 nt was also produced to see if there was any difference in the intensity of hybridization to vRNA transcripts of genome replication. The four riboprobes would produce hybrids with a calculated guanine and cytosine (GC) content of 47.0-48.3%. The integrity of RNA transcripts produced by *in vitro* transcription of the linearized constructs, and the polarity of the riboprobes and incorporation of the digoxigenin label were checked by electrophoresis on a 2% RNA gel (Figs. 3.1 and 3.2) and Northern blot hybridization (Fig. 3.3). Positive hybridization was

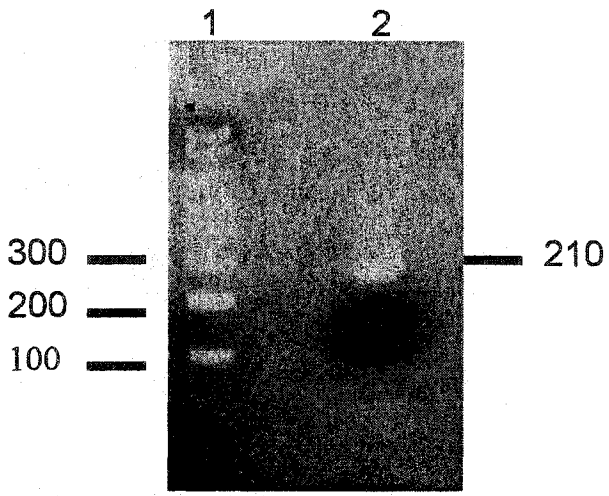


Figure 3.1 2% agarose RNA gel of segment 8 riboprobe; lane 1 contains the molecular size markers (1Kbp plus DNA ladder; Invitrogen Life Technologies), lane 2 contains 210 nucleotides Dig-UTP labeled antisense riboprobe of ISAV RNA segment 8.

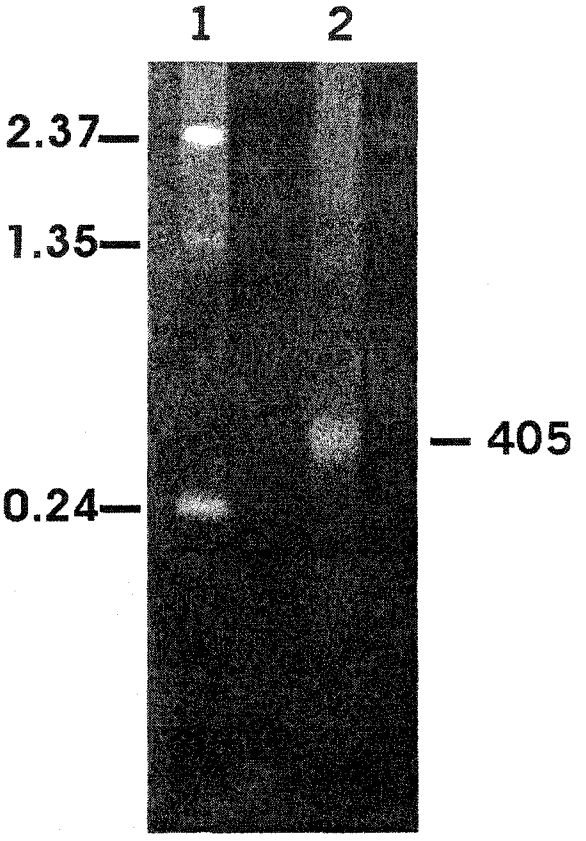


Figure 3.2 2% agarose RNA gel of segment 7 riboprobe; lane 1 contains the RNA molecular size markers (Invitrogen Life Technologies), lane 2 contains 405 nucleotides Dig-UTP labeled antisense riboprobe of ISAV RNA segment 7.

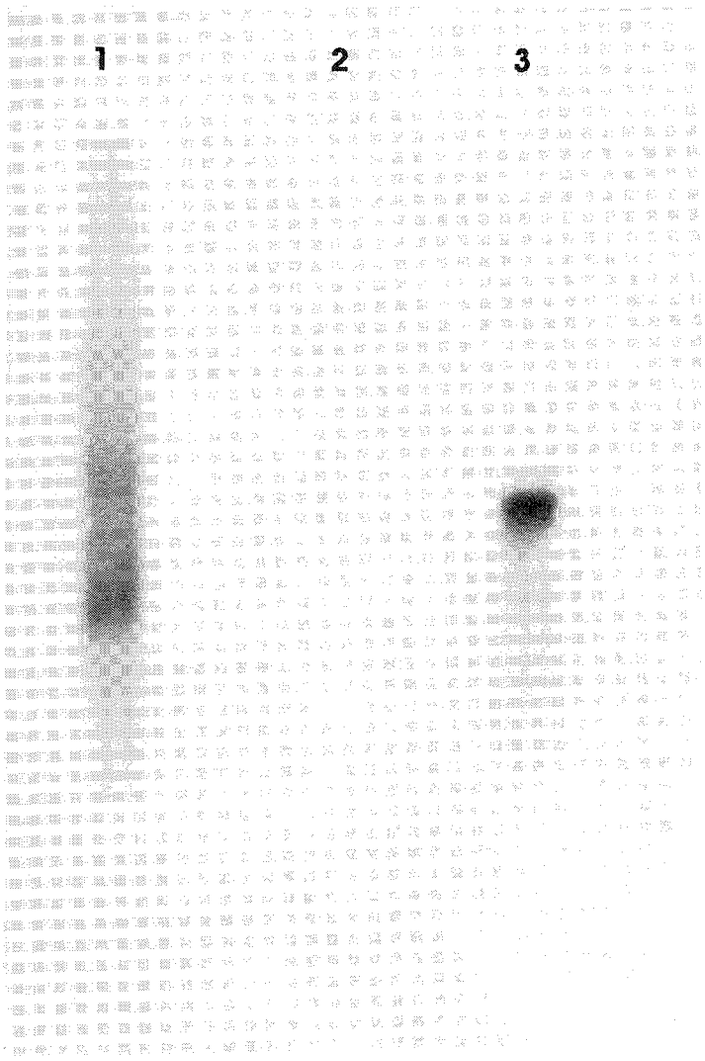


Fig. 3.3 Northern blot hybridization using ISAV RNA segment 8 antisense riboprobe; lane 1 contains total mRNA from uninfected CHSE-214 cells; lane 2, uniabeled RNA transcribed from the 210-bp cDNA fragment of ISAV segment 8 and therefore of the same polarity as the riboprobe; and lane 3, total mRNA from ISAV-infected CHSE-214 cells, showing a strong hybridization band.

detected only with total RNA extract from ISAV-infected cells. Absence of hybridization by segment 8 riboprobe to the unlabeled RNA transcript indicated that there was no self-hybridization with this riboprobe. All the other riboprobes were checked accordingly were subsequently used throughout the study in this thesis.

3.4.2 Optimization of ISH conditions on TO cells

The ISAV-infected TO cells used were harvested at 4 dpi when over 40% of the cell monolayer had CPE. The optimization utilized the RNA segment 8 riboprobe and chamber slides divided into 6 wells each using a hydrophobic pen. The results of the different hybridization conditions are summarized in Table 3.1. The hybridization signals at the 1:500 and 1:750 antibody dilution, although visible, were not sharp. The best hybridization signals (purple-blue color) were seen with a probe concentration of 5ng/µl, hybridization time of 16 hrs and antibody dilution of 1:1000. The length of color development with the BCIP/NBT substrate was also examined but no appreciable change was noted between 6 and 16 hr intervals studied. The uninfected cell samples showed no signal (Figs. 3.4; 3.5).

3.4.3 Development of ISAV-specific CPE in different fish cell lines

The ISAV-infected TO, CHSE-214 and SHK-1 monolayers were monitored daily for CPE prior to processing and storage. Briefly, in the TO and SHK-1 cells the CPE was characterized by vacuolation of individual cells and clusters, followed by lysis and lifting of the cells from the monolayer, while in the CHSE-214 cells CPE initially involved a limited number of cells appearing as a rounded refractile cluster, followed by lysis. The timing of the development of the CPE in the different cell lines is summarized in Table 3.2.

Table 3.1 Optimization of *in situ* hybridization (ISH) conditions for infectious salmon anaemia virus transcripts using a hybridization time of 16 hours.

Riboprobe concentration	Antibody dilution 1:500	1:750	1:1000	1:2000
5.0 ng	+++[a]	+++	++++	+
2.5 ng	+++	+++	+++	+
1.25 ng	+++	++	++	+
0.625 ng	+	+	+	-
0.0 ng	-	-	-	-

[a] Intensity of hybridization signals: 4+ = Very strongly positive; 3+ = strongly positive; 2+ = positive; + = weakly positive; - = no signal.

Table 3.2 Development of infectious salmon anaemia virus-induced cytopathic effects (CPE) in three different fish cell lines.

Days post infection	Intensity of CPE on the different cell lines CHSE-214	SHK-1	TO
1	-[a]	-	-
2	-	-	-
3	-	-	+
4	-	+	++
5	+	++	+++
6	+	++	++++
7	+	++	+++++
8	++	++	ND
9	++	++	ND
10	++	++	ND
11	++	++	ND
12	+++	ND	ND
13	+++	ND	ND
14	+++	ND	ND
22	++++	ND	ND
28	+++++	ND	ND

[a]- = no CPE observed; +, ++, +++, ++++, +++++ = increasing intensity of CPE; ND denotes not done.

Thus for the TO cells, visible onset of CPE was at 3 dpi with complete loss of the monolayer by 7dpi. In SHK-1 cell monolayers, the CPE was difficult to detect, and only slight detachment of the monolayer was observed by 11 dpi when the last SHK-1 infected cell was processed for ISH. The ISAV-infected CHSE-214 cells did not show CPE until 5 dpi. The CPE then increased steadily until14 dpi when it covered more than 50% of the cell monolayer as previously described (Kibenge et al., 2000a). The appearance of CPE on the TO, CHSE-214 and SHK-1 cells was as previously described by Wergeland and Jakobsen (2001), Bouchard et al. (1999) and Dannevig et al. (1995a) respectively. No CPE was observed in any of the uninfected control cell monolayers of the three different cell lines.

IPNV was used as the CPE-positive, hybridization-negative virus control for ISH. Over 50% of the CHSE-214 cell monolayer had CPE with detachment from the substrate by 3 dpi when the slides were processed for ISH. More than 50% of TO cells infected with IPNV had CPE by 3 dpi. Also, the IPNV-infected SHK-1 cells showed clear CPE with detachment of the monolayer by 3 dpi. The appearance of CPE on the CHSE-214 cells was as previously described (Kibenge et al., 2000a).

3.4.4 The patterns of ISAV gene expression in TO, CHSE-214 and SHK-1 cell lines

Because the intent was to study gene expression of the respective RNA segments leading to protein synthesis, antisense riboprobes were produced to detect mRNA transcripts of segments 6, 7, and 8. A sense riboprobe of RNA segment 7 was also produced in order to see if the intensity of hybridization to vRNA transcripts in genome replication was different from that of hybridization to mRNA transcripts of the same segment. The

riboprobes were applied to the different wells on the same slide. Table 3.3 summarizes the results of ISH using segments 6, 7, and 8 antisense riboprobes. Table 3.4 summarizes the distribution of hybridization signals in the nucleus and cytoplasm of different cell lines using only segment 8 riboprobe. Specific hybridization was initially detected exclusively in the nuclei and later in both nucleus and cytoplasm of ISAV-infected cells (Fig. 3.6). In ISAV-infected TO cells, the hybridization signals with segments 6 and 7 increased with progress of infection moving from nuclear to perinuclear cytoplasm at 4 dpi and becoming mostly cytoplasmic by 7 dpi, while that of segment 8 appeared to decrease with time. The ISAV-infected SHK-1 cells (Fig. 3.7) did not give a clear pattern with regard to the spread of the virus, and the overall number of positive cells was low. In the ISAV-infected CHSE-214 cells, no positive cells were observed at the first dpi with the three antisense riboprobes. Also, no hybridization signals were observed with uninfected controls (Fig 3.8). From 2-5 dpi, the segment 8 riboprobe showed slightly more intensity of signals than the other probes in CHSE-214 infected cells. From 5 dpi, the intensity of the hybridization signals was similar for all three antisense riboprobes (Fig 3.9 - 3.14), and remained so until 13 dpi when the intensity for segment 8 started to decrease (Table 3.3, Fig.3.15). The segment 7 sense riboprobe showed slightly weaker hybridization signals in the nucleus of infected cells first dpi, however, by 7 dpi the intensity of hybridization in the cytoplasm was comparable to that with the segment 7 antisense riboprobe (although in ISAV-infected SHK-1 cells the hybridization signal were confined to even fewer cells). No hybridization signals were observed in the uninfected cell controls nor in IPNV-infected TO, CHSE-214 and SHK-1 cells using the four ISAV riboprobes

Table 3.3 The development of ISAV induced CPE and detection of mRNA transcripts of ISAV RNA segments 6, 7 and 8 in TO, SHK-1 and CHSE-214 Cells.

	Days Post infection															
Cell line and ISAV RNA segment	1	2	3	4	5	6	7	8	9	10	11	12	13	14	22	28
TO cell line:																
Segment 6	+	2+	3+	4+	3+	2+	2+	nd	nd	nd	nd	nd	nd	nd	nd	nd
Segment 7	nd	+	3+	4+	4+	2+	2+	nd	nd	nd	nd	nd	nd	nd	nd	nd
Segment 8	+	3+	3+	3+	3+	2+	+	nd	nd	nd	nd	nd	nd	nd	nd	nd
SHK-1 cell line:																
Segment 6	+	+	+	nd	+	+	+	+	2+	nd	+	+	nd	nd	nd	nd
Segment 7	+	+	+	2+	+	2+	2+	+	+	2+	2+	2+	nd	nd	nd	nd
Segment 8	+	+	2+	nd	nd	+	+	3+	+	3+	+	+	nd	nd	nd	nd
CHSE-214 cell line:																
Segment 6	-	+	+	+	2+	2+	3+	3+	3+	4+	4+	5+	5+	5+	nd	nd
Segment 7	-	+	+	2+	2+	2+	2+	3+	2+	2+	4+	4+	5+	5+	nd	nd
Segment 8	-	2+	2+	+	3+	2+	2+	2+	nd	3+	3+	5+	4+	4+	nd	nd

+, Presence of CPE and/ or hybridization signal; 2+, 3+, 4+, and 5+ are increasing intensity of hybridization signals.
nd, denotes no data available.
-, no hybridization signals obtained.

Table 3.4 Distribution of hybridization signals during the course of ISAV infection in different fish cell lines using the segment 8 riboprobe

Cell		1	2	3	4	5	6	7	8	9	10	11	12	13	14
TO cells	Nucleus	++	++	++	++	+	+	-	nd	nd	nd	nd	nd	nd	nd
	Cytoplasm	+	+	++	++	++	++	++	nd	nd	nd	nd	nd	nd	nd
SHK-1	Nucleus	++	++	++	++	+	++	++	++	++	+	++	nd	nd	nd
	Cytoplasm	+	++	++	++	++	++	+	++	++	++	++	nd	nd	nd
CHSE-214	Nucleus	-	++	++	++	++	++	++	++	++	++	++	++	++	++
	Cytoplasm	-	-	+	+	++	++	++	+	++	++	+	+	+	+

++, very strong positive signal; +, positive signal; -, no hybridization signal obtained. nd, denotes not done.

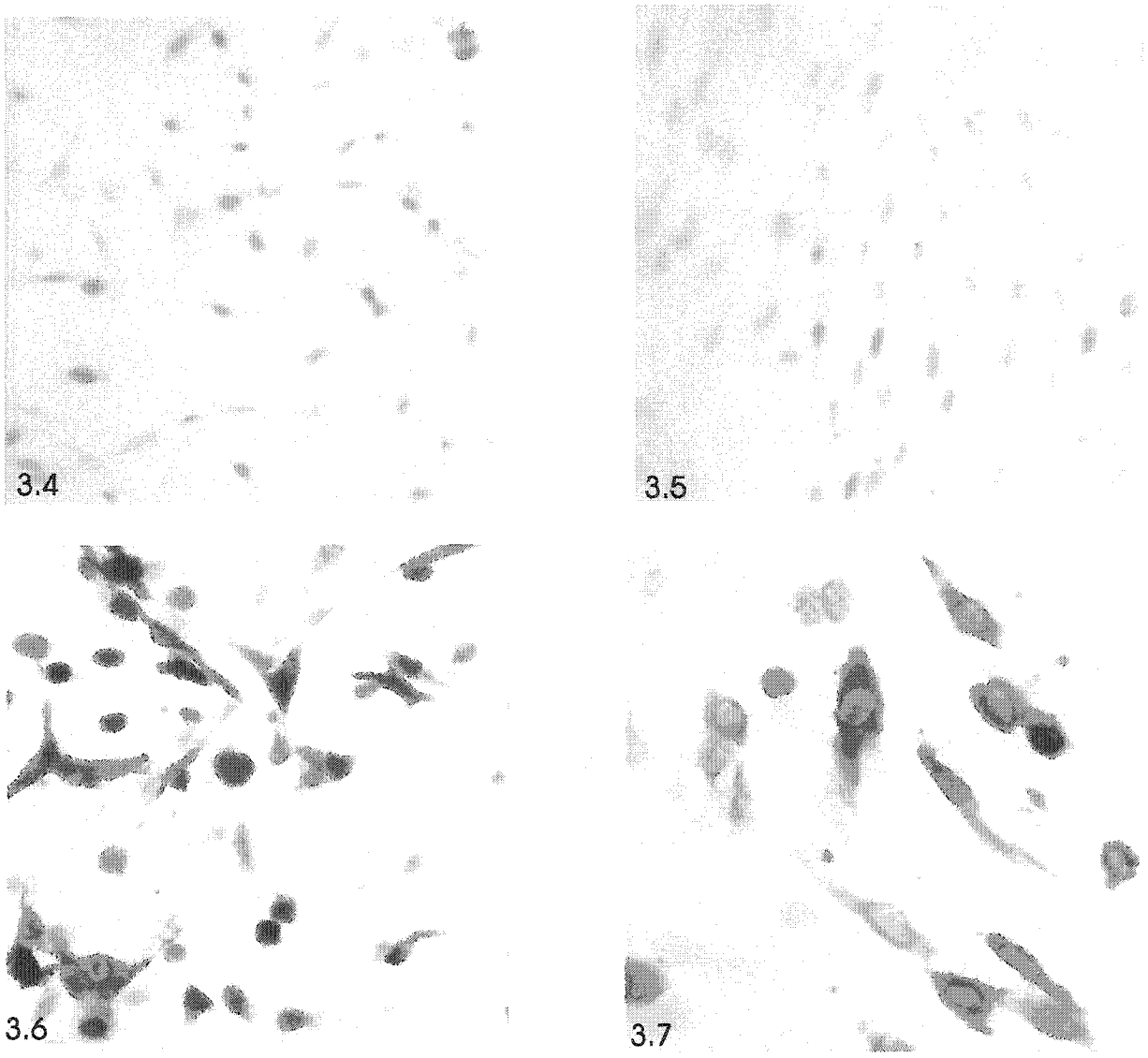
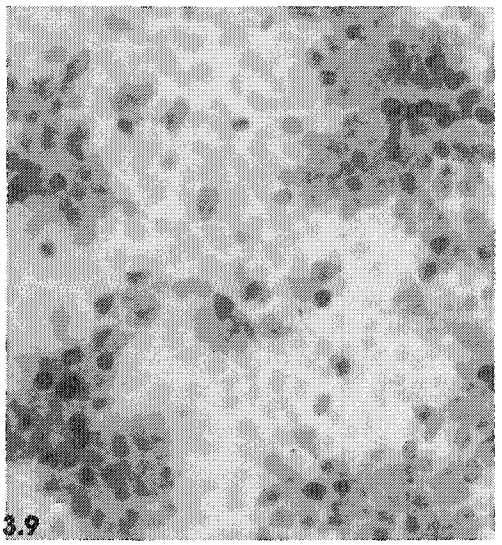


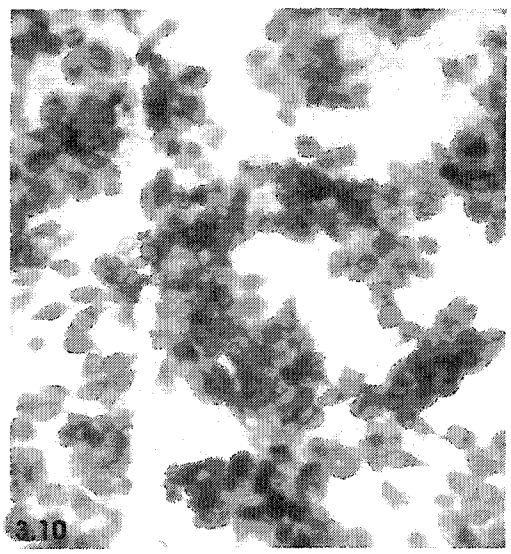
Fig. 3.4 TO cells uninfected. *In situ* hybridization using segment 8 antisense riboprobe, no hybridization signals observed.

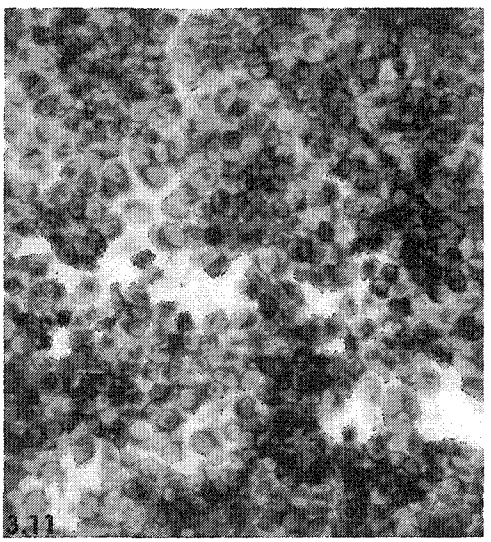
Fig. 3.5 SHK-1 cells uninfected control. *In situ* hybridization using segment 8 antisense riboprobe, no hybridization signals observed.

Fig 3.6 TO cells infected with ISAV NBC harvested at 1dpi. *In situ* hybridization using segment 8 antisense riboprobe resulted in strong signals in the nucleus and cytoplasm. The same signal was observed with the segments 6 and 7 antisense riboprobes.

Fig 3.7 SHK-1 cells infected with ISAV isolate NBC harvested at 2 dpi. *In situ* hybridization with segment 8 antisense riboprobe, showing strong hybridization signals in the nucleus and cytoplasm.



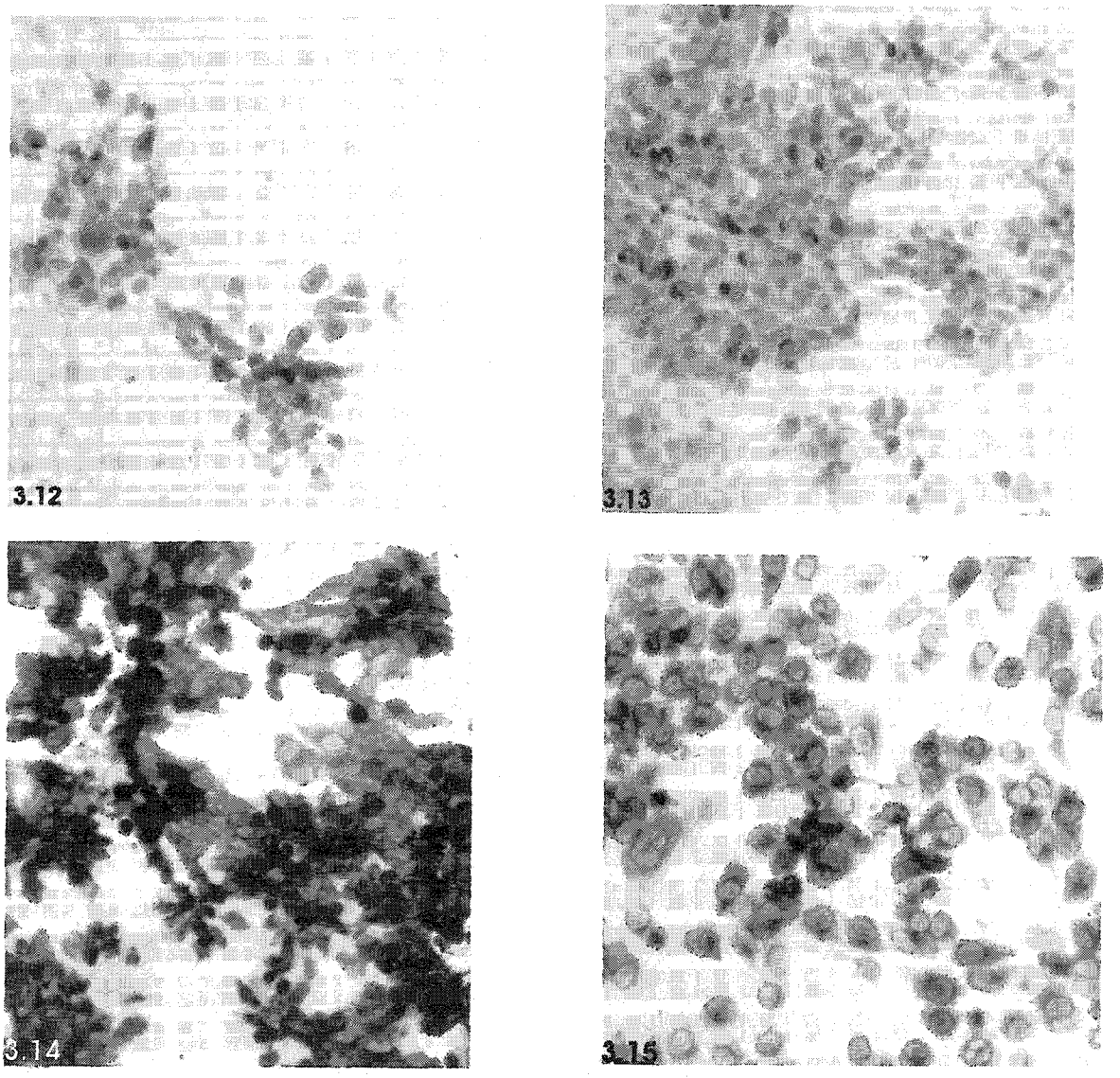




Figs. 3.8-3.11 CHSE-214 cells infected with ISAV isolate NBC.

Fig 3.8 Uninfected cells harvested at 14 dpi and hybridized with segment 8 riboprobe show no signal.

Figs. 3.9, 3.10, and 3.11 Infected cell harvested on 7 dpi, 10 dpi, and14 dpi respectively hybridized with segment 6 antisense riboprobe. The hybridization signals were first muti-focal at 7 dpi and later diffuse at 14 dpi.



Figs. 3.12, 3.13, 3.14, and 3.15 CHSE-214 cells infected with ISAV isolate NBC harvested on 2 dpi, 7 dpi, 10 dpi, and 14 dpi respectively, hybridized with segment 8 antisense riboprobe.The hybridization signals were multifocal at 2 dpi, almost diffuse at 7 dpi and started to wane at 14 dpi.

3.5 DISCUSSION

The present study describes the production of riboprobes specific for mRNA transcripts for RNA segments 6, 7, 8 and vRNA of segment 7 of ISAV. The riboprobes were used to study the temporal appearance and location of viral RNA transcripts in different fish cell lines infected with ISAV. The ISH technique is useful for the study of the kinetics of gene expression in cells and tissues. Recent advances in molecular biology have given rise to other widely used methods like serial analysis of gene expression (SAGE) (Velculescu et al., 1995), microarrays (Schena et al., 1995; Zhu et al., 1998; Hobbs and Deluca,1999), oligonucleotide chips (Lockhart et al.,1996), amplified restriction fragment length polymorphism (AFLP)-based technique for RNA fingerprinting, and gene calling (Shimkets et al., 1999) for large-scale study of gene expression. Although each of these methods is powerful in the study of genes that are differentially expressed either by viruses or infected cells, only the ISH technique gives a spatial localization or distribution of specific RNA or DNA in infected cells or tissues.

In this study the ISH conditions were optimized in three different ISAV-infected fish cell lines and the optimized conditions were used to study the patterns of gene expression of putative structural and nonstructural genes of ISAV. Northern blot hybridization and IPNV-infected cells were used to confirm the specificity of the probes for ISAV sequences. The IPNV-infected cells showed clear CPE with loss of the monolayer in all the three cell lines by 3 dpi when they were harvested, signifying that IPNV replicates in the fish cell lines used in the present study. However, no hybridization signal was observed when the four ISAV riboprobes were used for *in situ* hybridization on the slides with these samples.

This study shows that mRNA transcripts of segments 6-8 were initially located in the

nuclei of infected cells, which is consistent with the nuclear transcription of orthomyxoviruses. An interesting observation in the ISAV-infected CHSE-214 cells was that the hybridization signals with all riboprobes were initially focalized (Figs. 3. 9, 3.12), similar to what was previously described for the CPE (Kibenge et al., 2000a), and unlike in ISAV-infected TO and SHK-1 cells where the signals were dispersed throughout the cell monolayers. Because all the hybrids formed with the riboprobes used in the present study had a similar GC content (47-48%), the differences in kinetics of the three mRNA transcripts might be attributed to different times of expression of the three RNA segments and/or the viral requirements of the protein encoded by the different segments, but not to different sensitivities of the riboprobes. This is because, it is assumed that the three riboprobes have the same detection limit as they have the same GC content and concentrations in the hybridization buffers used.

Other reports have suggested that mRNA transcription in some cases is dependent on the extent of synthesis, requirement and turnover of a particular protein, therefore mRNA transcript is higher in cells for proteins that are in functionally high demand and with a high turnover rate than proteins that are stable over a certain time. In influenza A virus infected cells, it was reported that late in the infection cycle the amount of mRNA present in the cells does not represent the protein demand as after a certain level of mRNA transcript is reached, the protein synthesis will continue to increase without increased mRNA synthesis (Lamb and Krug, 1996). Thus in the CHSE-214 cell line, it was possible to identify a temporal pattern of ISAV gene expression whereby hybridization to segment 8 mRNA transcripts appeared first and was slightly intense at 2 dpi in contrast to hybridizations to mRNA transcripts of segments 6 and 7 which were maximum at 5 dpi.

This suggests that segment 8 might encode an early protein in transcription of ISAV in CHSE-214 cells. However, the absence of the same clear difference in hybridization signals in TO and SHK-1 cells from 1 dpi makes an obvious interpretation of this finding difficult. This is because, TO and SHK-1 cell lines are from the head kidney leucocytes of Atlantic salmon, the target cells for ISAV, while the CHSE-214 is from Chinook salmon embryo fibroblast. This might also account for the rapid replication and progressive CPE observed from ISAV infected TO and SHK-1 cell lines. The kinetics of gene expression in the CHSE-214 cells might therefore not represent, the absolute replication dynamics of the virus in target cells. However, there are possibilities that the replication requirements of the virus in the different cell lines might vary or are the same.

In ISAV, segment 6 encodes the HA protein, and it is the only segment for which a gene-protein assignment has been conclusively established (Rimstad et al., 2001; Krossøy et al., 2001b). The presence of intense cytoplasmic signals later in the infection suggests that it encodes a late protein that is required later in the infection. This finding supports the observations in influenza virus infected cells in which the HA gene is transcribed late in the replication cycle and remains high thereafter (Lamb and Krug, 1996). In influenza viruses, the HA protein is known to be synthesized poorly at early stages of infection but at high rates along with other structural proteins like M1 and M2 at late phases of the infection (Lamb and Krug, 1996). The NS1 protein of influenza viruses encoded by segment 8 RNA is synthesized in large amounts during the first few hours of replication as it functions to regulate translation of late viral mRNAs (Greenspan et al., 1988; Enami et al., 1994; de la Luna et al., 1995), while the M1 protein encoded by segment 7 is synthesized at a steady rate in early phase and increases late in the infection. The earlier appearance of ISAV

segment 8 mRNA transcripts and later appearance of segment 7 mRNA transcripts in CHSE-214 cells might suggest that segment 7 may encode structural proteins of ISAV. However, one might question hybridization signals as not being from a single replication cycle, but multiple cycles which is difficult to equate with what was observed with influenza virus. It was suggested, but not proven, that ISAV segment 7 codes for the putative membrane proteins (Griffith and Ritchie, 2001, GenBank Accession No. AX 083264.1). Most recently segments 7 and 8 were assigned to the putative membrane (28.6 kDa and 16.3 kDa) and NS (26.5 kDa and 20.3 kDa) proteins, respectively (Clouthier et al., 2002). Biering et al. (2002) expressed the ORF1 of the segment 8 that encodes a 24 kDa protein one of the major ISA viral proteins seen in the purified virus (Falk et al., 1997). Falk et al., (2004) recently demonstrated the 24 KDa protein expressed by Biering et al. (2002) to be a matrix protein. Kibenge et al. (2004) recently demonstrated that ORF1 and 2 of ISAV segment 8 encode structural and nonstructural proteins respectively.

In preparation for the present study, FASTA program was used to perform pairwise comparisons between influenza A virus M1, M2, NS1, NEP sequences and ISAV segments 7 and 8 sequences, and only the deduced amino acid sequence of ORF1 of ISAV segment 7 had sequence identity with influenza A virus M1 protein (F.S.B. Kibenge, unpublished data). This, together with the late transcription of segment 7 in ISAV-infected cells demonstrated in the present study, seem to support the contention that segment 7 encodes structural proteins.

Initial isolation of ISAV following the first outbreaks of ISA in Norway was difficult because the virus could not be propagated on available cell lines. The development of the SHK-1 cell line from Atlantic salmon head kidney in which the virus caused CPE allowed

the virus to be isolated (Dannevig et al., 1995a). Other cell lines like CHSE-214 cells (Bouchard et al., 1999), Atlantic salmon kidney (ASK) cells (Devold et al., 2000) and TO cells (Wergeland and Jakobsen, 2001) have now been used to isolate and propagate ISAV. Most work has been done on SHK-1 cells in which most ISAV isolates cause CPE, and on CHSE-214 cells in which only some isolates grow and cause CPE (Kibenge et al., 2000a; 2001a). In the present study, it was observed that while the CPE in the ISAV-infected CHSE-214 and TO cells progressed as previously described (Bouchard et al., 1999; Kibenge et al., 2001a; 20001b), the SHK-1 cell line did not show any clear pattern of CPE progression as in previous reports. Most authors have reported increased and visible CPE with almost complete loss of the monolayer in ISAV-infected SHK-1 cells by 10-14 dpi (Dannevig et al., 1995a). However, in this study such CPE development was not observed as the cell monolayer remained almost intact by 12 dpi and the CPE was difficult to read. Falk et al. (1998) reported slow and often weakly cytopathic growth of ISAV in SHK-1 cell cultures which made virus quantification difficult. Jones et al. (1999) using the same ISAV isolate as used in the present study observed occasional areas of cell detachment in ISAV-infected SHK-1 cultures, with the monolayer remaining partially intact throughout their 28 days' observation period. Kibenge et al. (2000a) reported that SHK-1 cells produced higher ISAV titres than CHSE-214 cells. In the present study, only few cells in ISAV-infected SHK-1 cell monolayers showed hybridization signals with the four riboprobes in contrast to the TO and CHSE-214 cells in which there was a clear increase in intensity of the hybridization signals as the infection progressed. Thus, the lack of obvious CPE in the SHK-1 cells was attributed to loss of sensitivity of this cell line for ISAV at higher cell passage levels (Rolland et al., 2003) such as was used in this study.

The detection of viral mRNA with the riboprobes by 1 dpi in the TO and SHK-1 cells and 2 dpi in the CHSE-214 cell lines, before the onset of visible CPE, demonstrates that this hybridization method is more sensitive for detection of the virus than the virus isolation method alone. The ISH can be combined with virus isolation for earlier detection of the virus in clinical samples. The ISH will also show the distribution of the viral genetic material in infected cells. In this particular case, ISH was positive 2-3 days before the appearance of CPE in the cell lines studied.

In conclusion, riboprobes specific for mRNA transcripts of RNA segments 6, 7 and 8 of ISAV were produced and used for ISH to detect viral RNA transcripts in different fish cell lines infected with this virus. These findings show that the slower replication rate of ISAV in CHSE-214 cells is suitable for the identification of the temporal pattern of gene expression of ISAV, and that the relative difference in the transcription of segment 7 and 8 would suggest that they encode for proteins required at different concentrations and time in ISAV infected cells. Moreover, these findings show for the first time the presence of viral RNA and mRNA in the nucleus of infected cells and further confirm nuclear transcription of ISAV which is consistent with orthomyxoviruses.

CHAPTER 4

DEMONSTRATIONS OF INFECTIOUS SALMON ANEMIA VIRUS RNA IN TISSUE SECTIONS OF ATLANTIC SALMON EXPERIMENTALLY INFECTED WITH ISAV*.

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MONEKE EE, KIBENGE MJT, GROMAN DB, JOHNSON GR, IKEDE BO, KIBENGE FSB (2003) Infectious salmon anemia virus RNA in fish cell cultures and in tissue sections of Atlantic salmon experimentally infected with infectious salmon anemia virus. *J Vet Diagn Invest*, 15, 407 - 417.

MONEKE EE, KIBENGE MJT, GROMAN DB, JOHNSON GR, IKEDE BO, KIBENGE FSB (2003) Localization of Infectious salmon anemia virus (ISAV) in experimentally infected fish by in situ hybridization using two riboprobes of the virus. *Aquacul Assoc Canada Spec Pub,* 6, 64 - 68.

4.1 ABSTRACT

The current understanding of the etiopathogenesis of ISAV infection in fish comes mostly from virus detection in homogenized tissues taken from ISA-suspected mortalities. This study combined ISH and histology to demonstrate viral RNA transcripts in tissues collected during the clinical phase of ISAV infection in Atlantic salmon. A riboprobe to mRNA transcripts of ISAV RNA segment 8 which was shown to be specific in ISAV-infected fish cell cultures was used for the ISH. In order to use the riboprobe in fish tissues fixed in paraformaldehyde or formalin, the conditions used to permeabilize tissues before ISH (proteinase K or Tween 20) were first optimized. Tissues were collected 15-20 days after challenge from 7 fresh mortalities of Atlantic salmon parr (~20g) showing severe gross and microscopic lesions, consistent with ISAV infection. RT-PCR on tissue pools confirmed the presence of ISAV in each of the 7 fish. Of the tissues examined in each fish, the heart and liver consistently showed the strongest hybridization signals and, therefore the most *in situ* virus replication, which was located in the endothelium and in macrophage-like cells.

4.2 INTRODUCTION

ISAV, a member of the Orthomyxoviridae family (Falk et al., 1997; Mjaaland et al.,1997; Krossøy et al.,1999; Eliassen et al., 2000; Sandvik et al., 2000), genus *Isavirus* (Anon. 2001), is currently one of the most important viral pathogen threatening Atlantic salmon *(Salmo salar)* aquaculture in the Northern Hemisphere. The single-stranded RNA genome of ISAV is comprised of 8 segments of negative polarity, ranging in size from 1.0 to 2.4 kb with a total molecular size of approximately 14.3kb (Clouthier et al., 2002). The smallest segment, segment 8, was the first part of the ISAV genome to be cloned and sequenced

(Mjaaland et al., 1997). Consequently, virus detection in clinical samples by RT-PCR is commonly based on detection of ISAV segment 8 sequences. This RNA contains 2 overlapping ORF estimated to encode proteins of 22 and 27.4 KD without splicing of transcripts (Rimstad and Mjaaland, 2002). Recently, the ORF1 of segment 8 was reported to encode the 24-KDa major structural protein of ISAV (Biering et al., 2002). This protein has been shown to be the ISAV matrix protein (Falk et al., 2004). Although the head kidney of ISAV-infected fish is generally considered as the organ of choice for virus isolation and the target organ that might harbor the most amount of virus (Dannevig et al., 1995a), different tissues from up to 5 fish suspected of ISA are usually pooled for the detection of the virus. Little effort has been made to substantiate the choice of tissues and whether virus presence in each individual tissue is correlated with lesions. The main goal of this study was to demonstrate the presence of viral RNA directly in fish tissues during the clinical phase of ISAV infection in Atlantic salmon. To do this, ISH technique was optimized for fish tissues and used to evaluate the distribution of virus in the affected tissues.

4.3 MATERIALS AND METHODS

4.3.1 Virus and cells

The ISAV NBC (Jones et al., 1999) used for experimental infection of fish was grown and titrated in the TO cell line (Wergeland and Jakobsen, 2001), as described in Chapter 2, section 2.1 and 2.2.

4.3.2. Preparation of ISAV Riboprobe

The methods used to prepare the segment 8 riboprobe used for the ISH have been

described in Chapter 2, section 2.4.

4.3.3 Fish experiment

4.3.3.1 Experimental infection and sampling of fish

The fish used in this study were part of the initial population of 300 Atlantic salmon parr, St. John River stock and without any history of disease, obtained from the Cardigan Fish Hatchery, P. E., Canada. The status of the salmon before the start of experimental infection and the housing of the fish were as described in Chapter 2, section 2.5.1.

For this study, the 50 challenge fish were removed from the stock-holding tank and anaesthetized by immersion in an aerated solution of tricaine methane sulphonate (MS-222) (100 mg l^{-1}). Each fish was challenged by intraperitoneal injection of NBC virus at a dose of $10^{4.8}$ $TCID_{50}$ in 0.2ml of virus suspension and was then returned to the study tank in the infected room. Sixty five uninfected control fish were kept in the holding tank in a separate "clean" room. All fish were observed twice daily for mortality; the fish were observed for abnormal behavior, and external lesions. All dead fish were necropsied and samples of heart, liver, spleen, gills, head and trunk kidney, and pyloric caeca were collected aseptically in triplicate. The triplicate samples were treated as follows: 1 set of tissues was fixed in 10% formalin, a 2nd set in 4% paraformaldehyde, and a 3rd set of tissues was pooled in a sterile plastic bag and stored at -80°C for later use in viral analysis by RT-PCR. Two uninfected control fish were sacrificed every 2 wk during the mortality phase of the ISA experimental infection, and tissues were collected as for the fish mortalities. After 24 hours, the formalin- and paraformaldehyde-fixed tissues were processed in an automatic tissue processor (Sakura, Tissue Tek$^{(R)}$ VIP) and were

embedded in paraffin wax. Serial 5µm thick sections of the fixed tissue were then stained with haematoxylin and eosin (H & E) for light microscopy or were processed for ISH.

4.3.3.2 Optimization of ISH conditions in fish tissues of ISAV experimentally infected fish.

For the fish tissues, 5µm thick sections of the paraffin-embedded formalin- or paraformaldehyde-fixed tissues from ISAV-infected and control uninfected fish were placed on glass slides pretreated with 3-aminopropyltriethoxysilane (Sigma), deparaffinized by immersion in two changes of xylene for 5 min followed by rehydration in decreasing concentrations of ethanol 5 min each and finally in DEPC-treated water for 5 min. The following permeabilization conditions of the tissues were examined to identify the one with the best ISH signal: incubation with 3% or 0.3% Tween 20 (Bio-Rad Laboratories, Hercules, CA. USA) in PBS (0.008 M sodium phosphate, 0.15 M NaCl, pH 7.2) or 200µg/ml or 20 µg/ml of proteinase K (Invitrogen Life Technologies) in 50 mM Tris.HCl/5mM EDTA, for 10, 20 or 30 min at room temperature or 37°C. Permeabilization was stopped by rinsing the slides with 2mg/ml of glycine (Bio-Rad Laboratories) in PBS for 30 sec. Other prehybridization, hybridization and post-hybridization conditions were based on the optimized conditions established using the ISAV-infected TO cells (Chapter 3, section 3.3.4).

Hybridization was detected in fish tissues as described in general materials and methods in Chapter 2, section 2.6 using a digoxigenin label detection kit (Dig-II-UTP Detection Kit, Roche Diagnostics). Slides were incubated for 1 hr at 37°C with alkaline phosphatase-conjugated anti-digoxigenin antibody diluted 1:500 in maleic acid buffer (0.1M

maleic acid, 0.15M NaCl, pH 7.5). Signals were developed with 0.375mg/ml BCIP and 0.188mg/ml NBT salt in detection buffer (0.1M Tris-HCl, 0.1M NaCl), pH 9.5, for 16 hr in the dark. Slides were counter stained with nuclear fast red (Vector) and mounted with Faramount aqueous mounting media (DAKO) prior to microscopic examination. Hybridization signals were observed using a light microscope and imaged digitally. Digital images were enhanced using Adobe Photoshop 5.5™ (Adobe Systems Inc) and analyzed.

4.4 RESULTS

4.4.1 Preparation of an ISAV-specific riboprobe

The antisense segment 8 riboprobe of 210 nt was produced by *in vitro* transcription as described in Chapter 3, sections 3.4.1.

4.4.2 Optimization of ISH conditions for ISAV transcripts in fish tissues

Because the ISH signals in tissues are influenced primarily by the permeability of the tissue (Brigati et al., 1983), and conditions for permeabilization of fish tissues for ISAV studies have not been systematically investigated previously (Gregory, 2002), the fish tissues fixed either in formalin or paraformaldehyde in this study were initially optimized using two permeabilizing agents, a detergent (Tween 20) and a protease (proteinase K), when used at different concentrations, durations, and temperatures. The results of the different permeabilization conditions for proteinase K and Tween 20 are summarized in Tables 4.1 and 4.2, respectively. The best hybridization signal (++; strongly positive signal) in all the tissues fixed in either formalin or paraformaldehyde were seen when 20µg/ml proteinase K was used at 37°C for 20 min and with an antibody dilution of 1:500. The

length of color development with the BCIP/NBT substrate was also examined but no appreciable change was noted between the 6 and 16 hr intervals studied.

4.4.3 *In situ* hybridization in fish tissues

Seven fish from mortalities that occurred on days 15-20 post challenge were selected for histology and ISH on the basis of having gross lesions characteristic of ISAV infection (Thorud and Djupvik, 1988). The RT-PCR for ISAV was also positive for the seven fish mortalities and negative for the two uninfected fish used as control. Sections of the heart, liver, spleen, head and trunk kidney, gill, and pyloric caeca showed microscopic lesions consistent with ISAV (Thorud and Djupvik, 1988; Evensen et al., 1991; Speilberg et al., 1995), consisting mainly of hemorrhages, congestion and multifocal coagulative necrosis of the liver (Fig. 4.1). In addition, there was mild or low frequency widespread endothelial hyperplasia which was most marked in the heart (Fig. 4.2). Using the ISAV riboprobe for ISH on the fish tissue, hybridization signals were observed in all the tissues sampled. Signals were confined to the endothelial cells except in the heart where they were present also in what appeared to be subendothelial macrophage-like cells (Fig. 4.4), and in the spleen where they were present in the macrophages/leucocytes. No hybridization signals were seen in areas of coagulative necrosis in the liver (Fig. 4.1 and 4.3) nor in viable hepatocytes (Fig. 4.1). No hybridization signals were detected in any of the tissues from uninfected control fish (Figs. 4.5 and 4.6). Signals were present in gills within endothelial cells lining the vasculature of primary and secondary lamellae (Fig. 4.7).

Table 4.1 Optimization of permeabilization conditions with proteinase K for infectious salmon anaemia virus (ISAV) segment 8 riboprobe on tissues of fish experimentally infected with ISAV.

Fish#	Serial section#	Fixative	Prot. K (µg/ml)	Time(min)	Temp(^{0}C)	Intensity of *In situ* hybridization signals in fish tissues						
						Gill	Liver	AK	PK	Caeca	Spleen	Heart
AS-01	S4	PF	200	10	25	+	+	+	+	+	+	++
AS-03	S10	PF	200	10	25	+	++	+	+	++	+	++
AS-02	S8	F	200	10	25	++	++	+	++	++	++	++
AS-03	S2	F	200	10	25	++	++	nd	++	+	++	++
AS-03	S1	PF	200	10	37	++	++	+	+	+	++	++
AS-06	S3	PF	200	10	37	-	-	-	-	-	-	-
AS-03	S3	F	200	10	37	+	-	-	+	-	+	+
AS-06	S6	F	200	10	37	-	+	-	-	+	+	+
AS-02	S9	F	20	20	37	++	++	nd	+	++	++	++
AS-01	S11	F	20	20	37	nd	++	+	nd	++	++	++
AS-08	S8	PF	20	20	37	++	++	++	nd	++	++	++
AS-07	S5	PF	20	20	37	nd	++	+	+	+	++	++
AS-07	S4	PF	20	30	37	nd	++	+	nd	++	++	++
AS-09	S5	PF	20	30	37	++	++	-	nd	++	+	++
AS-03	S9	F	20	30	37	++	++	nd	+	++	+	++
AS-06	S11	F	20	30	37	-	-	-	-	-	-	-
AS-06	S8	PF	20	30	25	+	+	-	-	+	+	++

Anterior Kidney (AK), Posterior Kidney (PK)

-, no hybridization signals obtained; +, weakly positive hybridization signals; ++, strongly positive hybridization signals

nd, denotes no data available. PF, denotes 4% paraformaldehyde. F, denotes 10% formalin

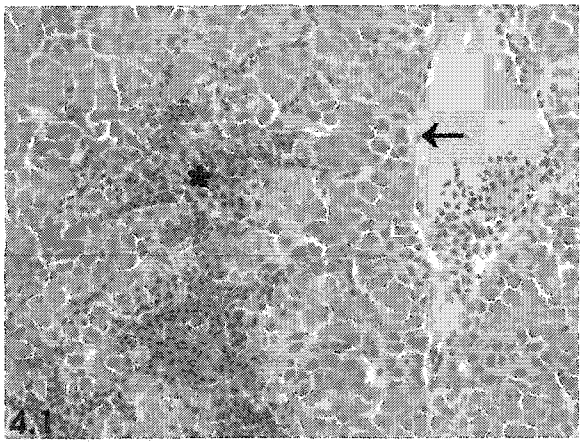
Table 4.2 Optimization of permeabilization conditions with Tween 20 for infectious salmon anaemia virus (ISAV) segment 8 riboprobe on tissue of fish experimentally infected with ISAV.

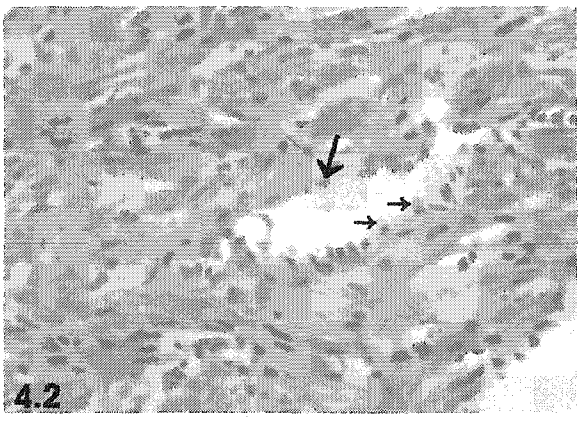
Fish#	Serial section#	Fixative	Tween 20 %	Time (min)	Temp(^{0}C)	Intensity of *in situ* hybridization signal in fish tissue						
						Gill	Liver	AK	PK	Caeca	Spleen	Heart
AS-02	S8	PF	3	10	25	+	++	+	+	+	+	++
AS-06	S9	PF	3	10	25	+	-	+	-	+	+	+
AS-03	S2	PF	3	10	37	+	+	+	-	+	++	++
AS-01	S3	F	3	10	37	-	-	-	-	-	-	-
AS-03	S4	F	3	10	37	++	+	+	+	++	++	++
AS-01	S10	F	3	10	25	nd	+	+	+	+	++	++
AS-03	S6	F	3	10	25	++	++	nd	+	++	+	++
AS-03	S5	F	0.3	20	25	+	+	-	+	+	++	++
AS-06	S8	F	0.3	20	25	nd	+	+	+	-	+	+
AS-03	S4	PF	0.3	20	25	+	++	+	-	+	++	++
AS-09	S4	PF	0.3	20	37	+	+	+	nd	+	+	++
AS-03	S8	F	0.3	20	37	++	+	nd	-	+	-	++
AS-01	S9	F	0.3	30	37	nd	++	nd	+	+	++	-
AS-02	S10	F	0.3	30	37	-	+	-	-	+	+	+
AS-09	S8	PF	0.3	30	37	-	-	-	-	-	+	+
AS-08	S5	PF	0.3	30	37	+	+	+	-	+	++	++
AS-02	S3	PF	0.3	30	25	+	+	-	-	++	+	++
AS-01	S5	PF	0.3	30	25	nd	+	nd	+	nd	+	+

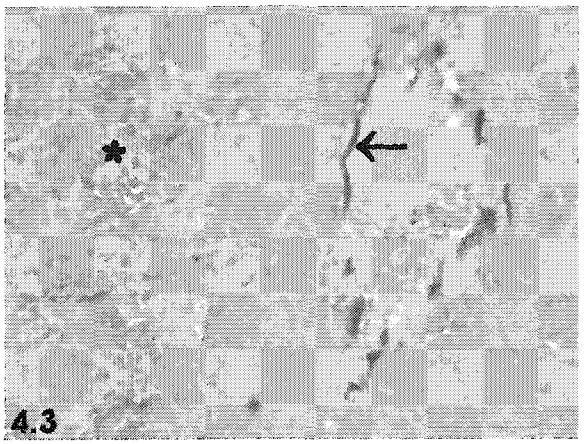
Anterior Kidney (AK), Posterior Kidney (PK)

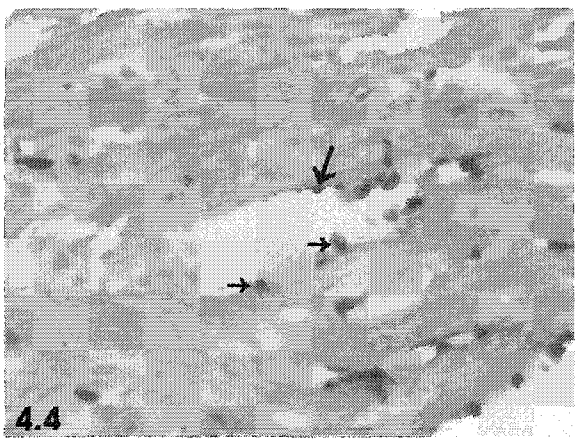
-, no hybridization signals obtained; +, weakly positive hybridization signals; ++, strongly positive hybridization signals

nd, denotes no data available. PF, denotes 4% paraformaldehyde. F, denotes 10% formalin









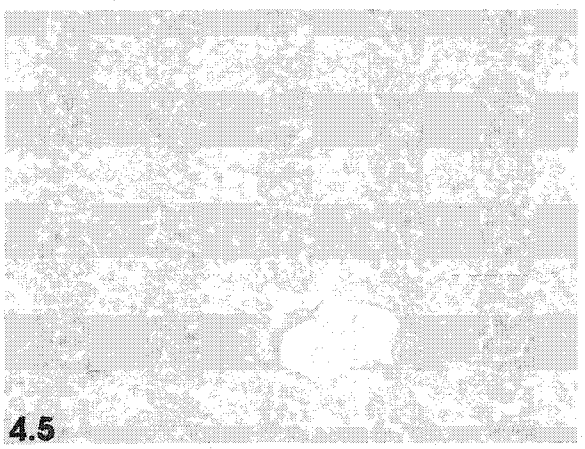


Fig. 4.1 H & E of liver from ISAV infected Atlantic salmon showing congestion and coagulative necrosis (*) in the hepatocytes and degeneration of the endothelial cells (arrow).
Fig. 4.2 H & E of heart from infected fish showing widespread hyperplasia of endocardial (appearing flat, large arrow) and possibly activated macrophage-like cells (small arrows) underlining the endothelium.
Fig. 4.3 Liver, showing hybridization signals in the endothelial cells (arrow) lining the blood vessels, but no signal in the area of necrosis (*).
Fig 4.4 Heart, showing numerous hybridization signals present in the endothelial cells (large arrow) and macrophage-like cells (small arrows).
Fig. 4.5 Liver of uninfected control fish, no signal observed.
Fig. 4.6 Heart of uninfected control fish, no signal observed.

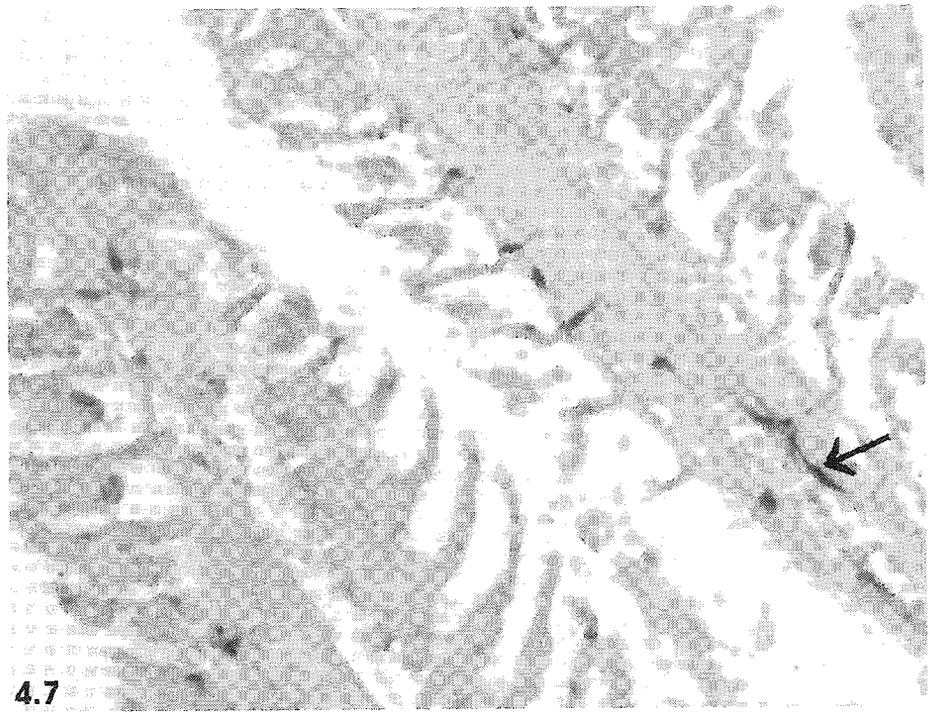


Fig. 4.7 ISH with ISAV RNA segment 8 riboprobe of negative sense to ISAV mRNA in the gills of infected fish, showing hybridization signals (arrow) in the endothelial cells lining the primary and secondary lamellar blood vessel.

4.5 DISCUSSION

The present study describes the optimization of ISH conditions using the segment 8 riboprobe and its application to the detection of viral RNA in tissues from Atlantic salmon experimentally infected with this virus. Histologic evaluations, RT-PCR, IFAT and virus isolation are commonly used to identify and diagnose the presence of ISA and ISAV in an infected tissue/fish. Electron microscopy has been used to show that the virus buds from endothelial cells in the heart and leucocytes (Hovland et al., 1994; Nylund et al., 1995b), none of these methods have been able to show the localization of the virus in tissues with histopathologic lesions. ISH gives a better spatial localization of the virus in the tissue than electron microscopy.

The optimal specific hybridization signals in tissue depends partly on the optimum permeabilization of the tissue/cells that harbor the viral genetic material (Brigati et al., 1983). In the present study, tissues were fixed in two different fixatives (10% formalin and 4% paraformaldehyde) because a permeabilization condition was needed that would give an optimum specific signal with either fixative. Moreover, most field samples of suspected cases of ISA are fixed in formalin, hence the need to achieve conditions that might give the best hybridization signals for such samples.

In the present study, Tween 20 did not give good hybridization signals when compared to the proteinase K (Tables 4.1, 4.2). In addition, positive signals were observed in at least one organ under all conditions when proteinase K was used. The best signals demonstrating almost the same intensity in both the paraformaldehyde and formalin fixed tissues were observed at 20µg/ml proteinase K at 37°C for 20 min. Paraformaldehyde fixed tissues are assumed to give better signals with *in situ* hybridization because

paraformaldehyde does not cross-link proteins extensively so as to prevent the penetration of probes (Lawrence and Singer, 1985). From the present study both paraformaldehyde and formalin fixed tissues gave reasonable good signals under all conditions studied with either of the permeabilizing agents. Moreover, in the present study both Tween 20 and proteinase K effectively permeabilized the gill tissue allowing it to be used in the ISH process. Others have reported difficulties with fish gill tissue, resorting to decalcification which results in acidic hydrolysis of RNA (Gregory, 2002).

In all fish tissues in the present study, the cells with the ISH signals, and therefore the target cells for the virus were endothelial cells and macrophage-like/mononuclear leucocytes. Surprisingly, the head and trunk kidney which are generally considered the target organ for ISAV (Dannevig et al., 1994) did not show as many hybridization signals as did the heart and liver. Earlier studies (Dannevig et al., 1994) used infectivity of different tissues from infected fish inoculated into naive fish to show that the head kidney is the most important site early in infection for the replication of the virus followed by the liver, although those studies did not include the heart. Recently it was shown that using nested RT-PCR on kidney tissues was the most sensitive method for the detection of the virus and that it correlated well with virus isolation and IFAT, as most fish studied were positive by all three methods (Optiz et al., 2000), but again the heart was not examined. From the ISH results of the present study, the heart appears to be another organ to include for virus detection since most of its endothelial cells showed signals at optimum permeabilization conditions. Also under "less ideal permeabilization conditions", positive signals were still picked up in the heart. This observation suggests the presence of more viral genetic material and/or replication of virus in the heart when compared to the head kidney.

Alternatively, the heart may retain more of the viral genetic material in dead or moribund fish. These findings support other reports that showed more pronounced reaction in the heart and kidney of infected fish by IFAT(Falk and Dannevig, 1995) and transmission electron microscopy (Hovland et al., 1994). With ISH, there might be some masking of the positive signal in the kidney by the brown pigments in melanomacrophages. The function of the melamanomacrophages with respect to the internalization, processing or replication of ISAV, and the ability of these cells to maintain the same receptors or phagocytic properties as macrophages are as yet not known. The absence of hybridization signals in any of the parenchyma cells is consistent with the targeting of endothelial cells and blood cells for virus replication.

In conclusion, ISH technique for the detection of ISAV in infected fish tissues has been optimized and has been applied to the detection of viral mRNA in mortalities from clinical ISA. The use of ISH with the riboprobe confirms that endothelial cells are the main target cells for ISAV in infected fish, followed by the macrophage-like/mononuclear leucocytes. In addition, the heart was found to have high amounts of viral genetic material in dead fish and might be a valuable organ to include during screening or study of suspected fish either by virus isolation or RT-PCR. The endothelial damage in the heart and elsewhere may also play some role in the pathogenesis of the disease as was previously suggested (Speilberg et al., 1995), for the liver.

CHAPTER 5

A CORRELATION BETWEEN VIRUS REPLICATION AND LESIONS IN ATLANTIC SALMON, AND ATTEMPTS IN THE IDENTIFICATION OF VIRAL RNA IN SURVIVED FISH AFTER AN EXPERIMENTAL CHALLENGE WITH ISAV.

5.1 ABSTRACT

Direct demonstration of ISAV in lesions associated with the infection has not been studied previously and the persistence of the virus in different fish species is unusual for orthomyxoviruses. Histology and ISH were used to study the replication of virus in tissues and development of lesions associated with ISAV infection in Atlantic salmon. RT-PCR and ISH were also used to study the persistence of the viral mRNA in tissues of fish that survived the infection. Two negative stranded riboprobes to ISAV segments 6, 7 and 8 mRNA transcripts were used for the ISH, while PCR primers targeting the segment 8 mRNA were used for RT-PCR, to detect viral RNA in sampled tissues. Rainbow trout (RbT), coho (CS) and Atlantic salmon (AS) mixed in three different tanks were infected with three different ISAV isolates, two belonging to North American (RPC and CH7) and one to European (NSC) hemagglutinin genotypes. Mortalities were observed only amongst AS in all infected groups. Liver, spleen, kidney, heart, gills and pyloric caeca collected from all dead fish were sampled. In the correlation study, seven fish mortalities with lesions consistent with ISA were selected from each group (n= 21). In the persistence study, two fish of each species that survived the experimental challenge were sampled at one week post natural mortality (pnm) in the NSC and CH7 infected group, while the RPC group was sampled at 2 wk pnm. All groups were subsequently sampled every 2 wk thereafter until the fourth week. The organs were sampled as in the mortalities. In the mortalities (n=21) lesions were observed in 95% of livers, 100% of spleen, 95% of pyloric caeca, 82% of hearts, 60% of gills and 27% of kidneys. Liver showed the most severe lesions followed by the spleen, pyloric caeca, gill, heart and the kidney. Hybridization signals were observed in the endothelial cells and macrophage-like cells in all tissues. Signals were observed in

100% of livers, 100% of hearts, 93% of gills, 84% of spleen, 79% of pyloric caeca, and 56% of kidneys. The most numerous signals were seen in the heart, followed by liver, pyloric caeca, spleen and gill, with the kidney showing the least signals. Viral RNA was not detected in parenchymatous cells undergoing degeneration and necrosis. These findings suggest that necrosis in parenchymatous cells in ISA may be a secondary lesion. No ISH signals were observed in the organs of any fish species at any sampling pnm. All infected sampled fish were RT-PCR positive at all sampling times pnm. The absence of signals by ISH, in the survived fish indicates that the viral RNA might be present at very low levels detectable only by very sensitive methods like RT-PCR.

5.2 INTRODUCTION

Difficulties have been reported in reproducing some or all the lesions associated with ISA under field conditions (Jones et al., 1999) and differences have also been reported in the pathology associated with the different isolates (Mullins et al., 1998; Jones et al., 1999; Kibenge et al., 2001a; Mjaaland et al., 2002). These observations led to the question as to whether the lesions seen in individual tissues of ISAV mortalities correlated with the presence of the virus. Also recent findings have shown that different isolates of the virus show different pathology when experimentally inoculated in cell cultures and fish (Kibenge et al. 2000a; Mjaaland et al., 2002). These differences in the pathology associated with the different isolates have led to the questioning of the role replication of the virus plays in the development of lesions seen in the host.

In most regions where the disease has been reported efforts to eradicate the virus has failed. Scotland announced the eradication of ISA from salmon aquaculture industry

in 2001, however, the virus was still present in the wild salmon population at that time (Raynard et al., 2001; Murray et al., 2002). After the initial disease out break in Norway, strict hygiene and control measures were introduced and led to a significant reduction in the number of outbreaks (Jarp and Karlsen, 1997). The re-emergence of new outbreaks however, have led to the suspicion that the virus might persist in a reservoir host in the wild. The persistence of ISAV in asymptomatic fish was reviewed in Chapter 1, sections 1.4.4.

Viruses can persist outside of their host or *in vivo*, however, for *in vivo* persistence, the virus must reside in a particular cell type or tissue. Virus persistence can be productive like the rabies virus that persists in the salivary glands of reservoir hosts, or non productive like the canine distemper virus that persists in the brain and can subsequently become productive when induced (Murphy et al., 1999). Some human viruses like Cytomegalovirus have been shown to persist in the kidney, salivary glands, and lymphocytes causing mild disease (reviewed by Ahmed et al, 1996). ISAV seems to persist *in vivo*, but the site of virus persistence is not known.

The main goal of this study was to determine if there is a correlation between the virus replication in tissues and histologic lesions found in fish mortalities from clinical ISA. ISH was also used in an attempt to identify the site (s) of ISAV persistence in fish that survived the experimental ISAV infection.

5.3 MATERIALS AND METHODS

5.3.1 Cells and viruses

The ISAV NSC, RPC and CH7 used for this study were from Nova Scotia, New Brunswick

and Chile respectively as previously described (Kibenge et al., 2001a;b). The isolates were grown and titrated in the TO cell line as described in Chapter 2, sections 2.1 and 2.2.

5.3.2 Riboprobe synthesis

The segment 6, 7 and 8 riboprobes used were synthesized as described in Chapter 2, section 2.4.

5.3.3 Experimental infection and sampling of fish

Atlantic salmon (AS) parr and rainbow trout (RbT) with no history of disease, were obtained from the Cardigan Fish Hatchery, while the disease free coho salmon (CS) was obtained from Aqua Health Inc., P. E. I., for this study. The condition and housing of fish were as described in Chapter 2,section 2.5.1.

At the start of the experiment, 150 AS, 30 RbT and 30 CS were individually removed from the holding tank and anaesthetized by immersion in an aerated solution of tricaine methane sulphonate (MS-222) (100 mg l^{-1}) and placed in three different tanks thus, 50 AS, 10 RbT and 10 CS for a total of 70 fish per tank. One virus isolate was used to challenge fish in each tank. The virus isolates used were NSC for tank # 1, RPC for tank # 2 and CH7 for tank # 3. Each fish was challenged by intraperitoneal injection of the virus at a dose of 10^6 $TCID_{50}$ in 0.2ml of virus suspension and was then returned to the study tank. Sixty-five AS, 55 RbT, and 41 CS were used as uninfected control, and were kept in one holding tank in a separate "clean" room. The fish were observed twice daily for abnormal behavior and external lesions. Mortalities were monitored and recorded. All dead

fish were necropsied and triplicate samples of heart, liver, spleen, gills, head and trunk kidney, and pyloric caeca were collected aseptically and disbursed as described in Chapter 4, section 4.3.3.1. For fish in tank # 1 and # 2 that survived the infection from one week after the last mortality two AS, RbT and CS per tank were sampled, while the fish in tank # 3 were sampled from 2 weeks pnm and subsequently every two weeks for 4 weeks in each tank. Triplicate samples of heart, liver, spleen, gills, head and trunk kidney, and pyloric caeca were collected and disbursed as in mortalities. The third set of tissues was individually placed in a sterile plastic bag and stored at -80°C for later use in viral analysis by RT-PCR. Two uninfected controls for AS, RbT and CS were also sacrificed every two weeks and tissues were collected as for the infected fish. Fixed tissues were processed as previously described in Chapter 4, section 4.3.3.1. Serial 5µm thick sections of the formalin fixed tissues were then stained with H & E stain for light microscopy or were generated for ISH.

5.3.4 ISH

Tissues from 7 dead AS per tank for a total of 21 fish (n=21) were selected and pooled for the correlation of lesion and ISH. Two fresh AS mortalities (with good histology) representing early, peak and decline mortalities periods/phases and an extra mortality within the mortality phases for a total of 7 fish per tank were selected for the study. Serial sections after the histology section were hybridized with segments 8 and 7 riboprobes. In survived fish that were sampled, the first, second and third sections after the histology section were hybridized with segments 8, 7 and 6 riboprobes respectively. ISH was

performed using conditions optimized previously in Chapter 4, section 4.3.3.2. Briefly, 5µm thick serial sections of paraffin embedded formalin fixed tissues from ISAV-infected and control fish were placed on glass slides pretreated with 3-aminopropyltriethoxysilane, deparaffinizied by immersion in two changes of xylene for 5 min followed by rehydration in decreasing concentration of ethanol 5 min each and finally in DEPC-treated water for 5 min. The tissues were permeabilized with 20µg/ml of protease K in 50 mM Tris.HCl/5mM EDTA, for 20 min at 37°C.

Prehybridization and hybridization buffers and conditions were as described in Chapter 4, section 4.3.3.2. Hybridization was carried out using segments 6, 7 and 8 negative sense riboprobes on serial sections. A total of 50µl of hybridization solution containing 25ng of the riboprobe was added to each slide. Hybridization was carried out for 16 hr at 37°C in a moist chamber. Post hybridization washes and detection of signals were as described in Chapter 4, section 4.3.3.2. Hybridization signals were observed using a light microscope and imaged digitally. Digital images were enhanced using Adobe Photoshop 5.5™ (Adobe Systems Inc.).

5.3.5 RT-PCR on tissue homogenate

Pooled tissues from AS mortalities in each tank, and pooled tissues from each fish species per sampling time pnm were used for RT-PCR. The segment 8 primer pairs of ISAV and the conditions used for RT-PCR were as described in Chapter 2, section 2.3.

5.4 RESULTS

5.4.1 Riboprobe synthesis

The specificity and sensitivity of the 387, 405 and 210 antisense segments 6, 7 and 8 riboprobe respectively synthesized by *in vitro* transcription were checked as described in Chapter 3, section 3.4.1.

5.4.2 Mortality and pathology

Two AS did not recover after inoculation with the RPC isolate and were removed from the experiment. The fish were considered to have died from the stress of handling. Morbidity and mortality were observed only among AS in all infected groups. Mortality started at 13 dpi in the CH7 group and peaked on 16 dpi and declined on 19 dpi. In the RPC group mortality started on 15 dpi, peaked on 18 dpi and declined from 21 dpi, while in the NSC group mortality started on 18 dpi, peaked on 21 dpi and declined from 26 dpi respectively. The mortality periods were 21 days for tank #1 (NSC group), 15 days for tank #2 (RPC) and 12 days for tank #3 (CH7). No mortalities were recorded from the uninfected control and in the infected RbT and CS. All dead fish were necropsied and gross lesions consistent with ISAV infection were observed in all mortalities. The histopathologic lesions observed in the organs (liver, spleen, kidney, gills, pyloric caeca and heart) were consistent with ISAV infection as described earlier (Thorud and Djupvik, 1988; Evensen et al. 1991). The grading of the lesions, a slight modification of that used by Jones and Groman (2001) described in Chapter 2, section 2.5.3, was adopted for assessment of the quality of the fixed tissues and histopathologic lesions in the different organs.

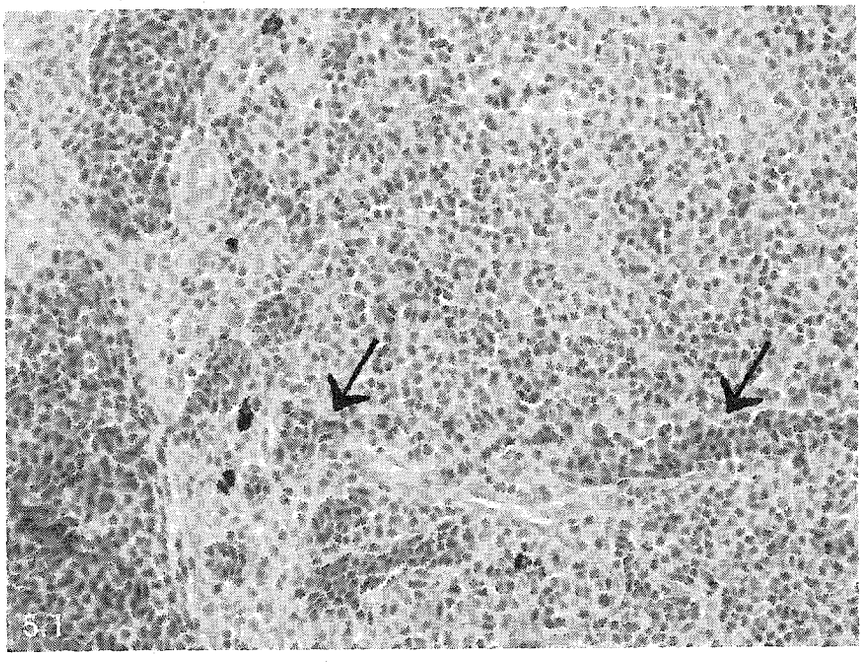
Since part of the intent of the study was to see if there was direct relationship between the lesions seen in individual cells or the organs and the presence of viral mRNAs, twenty-one mortalities (7 AS per tank) without evidence of autolysis were selected and pooled as stated in material and methods for the correlation studies. The total lesions seen in the different organs were calculated as a percentage of the total lesions in the organ sampled from the twenty-one fish. One hundred percent of spleen, 95% of liver and pyloric caeca sampled showed lesions, followed by 82% of the heart, 60% of the gills, and 27% of the kidney. The most severe lesions were observed in the liver, followed by the spleen, pyloric caeca, gills, heart and kidney the least.

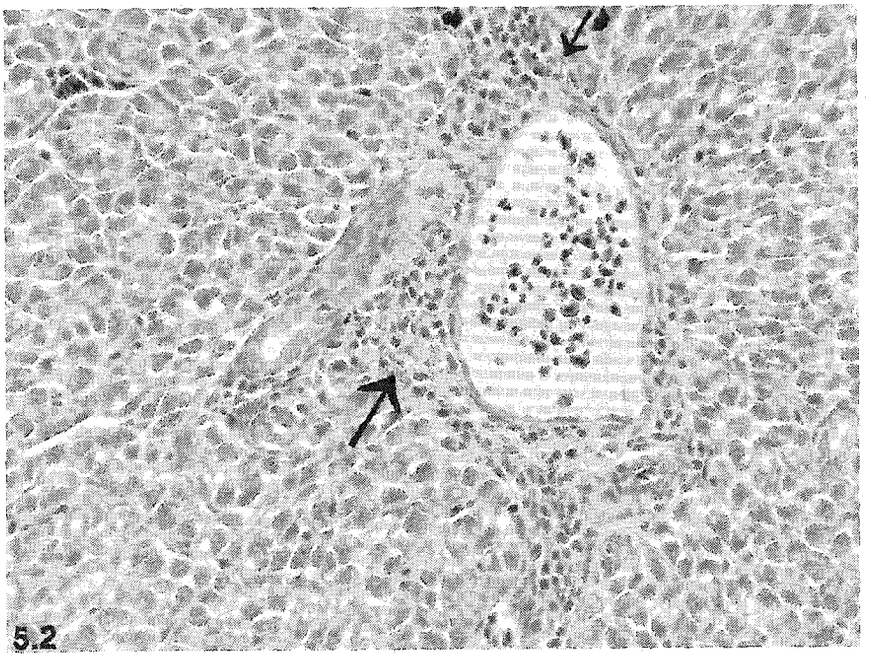
No gross lesions were observed in all the RbT and CS sampled pnm. Exopthalmia, few petechia hemorrhages in the visceral fats and slightly congested liver and kidney was observed in AS sampled at 7 days pnm from the CH7 group, while no gross lesions were observed in NSC and RPC groups.

No histopathological changes were observed in all the organs of RbT and CS sampled from all infected groups. In AS from NSC and CH7 groups, congestion was observed in the spleen (Fig. 5.1), while in the liver there was vasculitis (Fig. 5.2) in fish sampled at 7 days pnm. No lesions were observed in the other organs sampled. No histopathological lesions were observed in AS sampled thereafter. No histopathological lesions were observed in AS survivors sampled in the RPC group and in all the uninfected controls.

5.4.3 Correlation between lesions and hybridization signals in AS

Serial sections following the histologic sections were used for ISH. Segment 8 riboprobe





Figs. 5.1and 5.2 Tissues from Atlantic salmon infected with ISAV isolate NSC 7 days pnm.

Fig. 5.1 Spleen, H & E stain showing congestion of the sinusoids (arrows).
Fig. 5.2 Liver, H & E stain showing vasculitis and cuffing (arrows).

was used on the first section while segment 7 riboprobe was used on the next section of each slide. Bluish-purple hybridization signals depicting the presence of viral mRNA were seen in the endothelial cells of blood vessels in the organs sampled and also in leucocytes/macrophage-like cells in the heart, spleen (Fig. 5.3) and kidney (Fig 5.4). The intensity of hybridization signals indicate the amount of the virus mRNA in an infected cell, while the frequency of signals indicate the number of infected cells. Hybridization signals in organs were presented as a percentage of total signals in the twenty-one fish. Signals were observed in 100% of livers and hearts and in 93%, 84%, 79% and 56% of the gills, spleen, pyloric caeca and kidney, respectively (Tables 5.1; 5.2). The heart appeared to show the most intense signals followed by the liver, pyloric caeca, spleen, gill, and the kidney.

Hybridization signals were wide spread in degenerating endothelial cells of various organs examined, including within small foci of cellular degeneration and necrosis in the liver, kidney and spleen where the endothelium was still discernable (Figs 5.3; 5.4; Table 5.1). However, within larger foci of coagulative necrosis with complete loss of endothelial cells, signals were absent. Further more, hybridization signals were observed in some degenerating leucocytes and macrophage-like cells in the spleen (Figs. 5.3; 5.5), but not in degenerating hepatocytes, renal tubular epithelium (Figs 5.4; 5.6), interstitial cells of the gills (Figs 5.7; 5.8) or the mucosa of pyloric caeca (Figs 5.9; 5.10). At organ level, 95% of the livers showed hybridization signals and presence of lesion, followed by 84% of spleen, 79% of pyloric caeca, 70% of heart, 60% of gills and 16% of kidney (Table 5.2). Therefore, the presence of lesion and the presence of the viral mRNA is most correlated in the liver and least in the kidney. However, the kidney showed the most hybridization signals without

Table 5.1 Correlation between histopathologic lesions and presence of viral mRNA by ISH in ISAV-infected AS (n=21).

Tissue	Lesions	Presence of viral mRNA
Liver	Mild congestion, hemorrhage, swelling of the endothelium	+
	Severe hemorrhage, depletion of endothelium	-
	Hepatocellular degeneration, coagulation necrosis	-
Kidney	Mild interstitial congestion, hemorrhage	+
	Hematopoietic cell necrosis/depletion	+
	Coagulation necrosis of renal tubular epithelium	-
Spleen	Severe congestion, hemorrhages	+
	Degenerating leucocytes	+
	Increased erythrophagia	-
Heart	Endothelial hyperplasia	+
	Proliferation of macrophages/ activated macrophages	+
Gill	Congestion of the filamental arterioles and lamellar capillaries	+
	Interstitial cellular degeneration/lysis	-
Pyloric caeca	Mild to marked congestion of the lamina propria vasculature	+

lesion followed by the gills, heart, liver and none in the spleen (Table 5.2).

No appreciable difference was observed in the frequency of the hybridization signals between the segment 7 and 8 riboprobes in the tissues. The segment 7 riboprobes, however, appeared to show more intense signals. No difference was observed in the hybridization signals in organs from the different mortality phases.

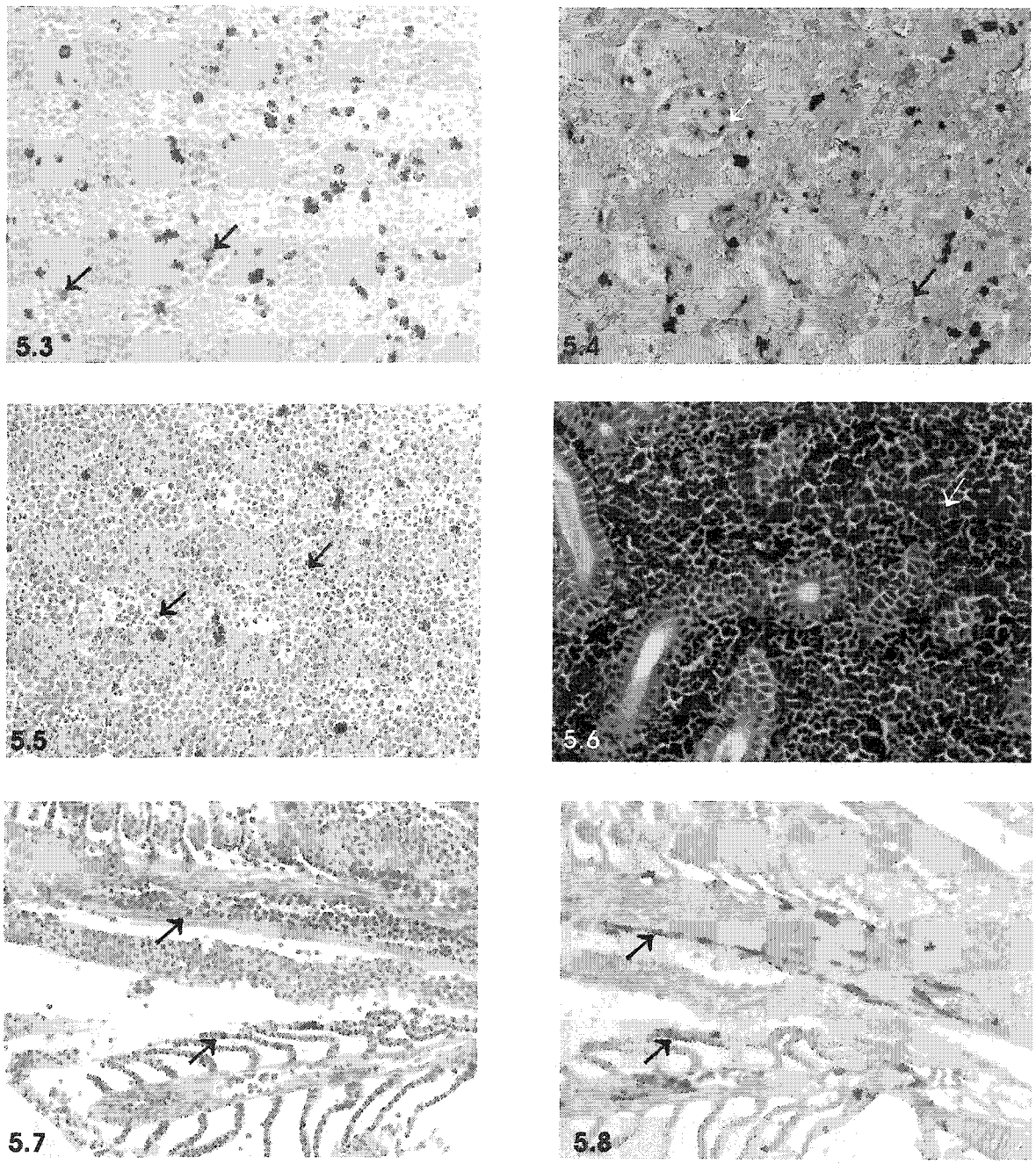
On subjective comparison of the frequency and intensity of hybridization signals from the different isolates, it was observed that hybridization signals appeared to be weak and in fewer cells in NSC group (Fig. 5.11) when compared to the RPC and CH7 groups (Fig. 5.12). There were no clear differences in the lesions observed with the three isolates in all the organs sampled.

5.4.4 ISH on fish tissues sampled pnm

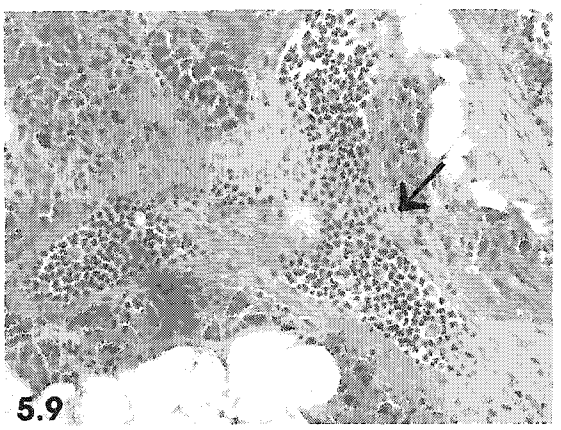
No hybridization signals were observed with segments 6, 7, and 8 riboprobes in organs from any infected AS, RbT and CS sampled from all groups at any sampling time pnm.

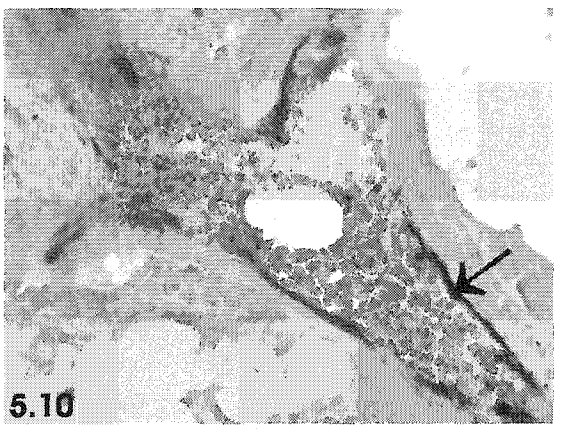
Table 5.2 Correlation between histopathological lesions and hybridization signals in organs of AS (n=21) infected with ISAV.

	Kidney	liver	spleen	pyloric caeca	gills	heart
Total number of organs	18	21	19	19	15	17
ISA lesion (%)	27	95	100	95	60	82
No lesions (%)	73	5	0	5	40	18
Hybridization signals (%)	**56**	**100**	**84**	**79**	**93**	**100**
No Hybridization (%)	44	0	16	21	7	0
Lesion & Hybridization signals (%)	**16**	**95**	**84**	**79**	**60**	**70**
No lesion & Hybridization signal (%)	56	4	0	0	33	18
Lesion & No hybridization (%)	11	0	16	16	0	12
No lesion & No hybridization (%)	16	1	0	5	7	0



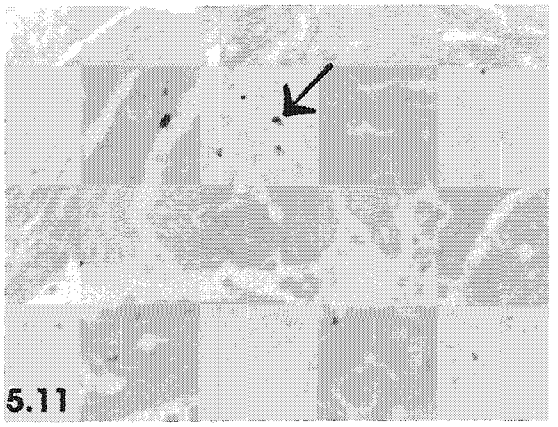
Figs. 5.3 - 5.8 Tissues from Atlantic salmon infected with ISAV. Fig. 5.3 Spleen showing hybridization signals with segment 8 riboprobe in individual leucocytes (arrows) undergoing degeneration. Fig. 5.4 Kidney showing hybridization signals in endothelial cells in the glomerulus (white arrow) and underlining the renal tubules (black arrow).
Fig. 5.5 Spleen, H & E stained showing congestion and degeneration of the leucocytes (arrows). Fig. 5.6 Kidney, H & E stained showing congestion and interstitial hemorrhages (arrow). Fig. 5.7 Gill, H & E stained showing congestion in the filamental blood vessels (arrows). Fig. 5.8 Gill showing hybridization signals in the endothelial cells lining the filamental blood vessels (arrows).





Figs. 5.9 - 5.10 Pyloric caeca from Atlantic salmon infected with ISAV.

Fig. 5.9 Note the congestion (arrow) in the major blood vessel around the pancreas.
Fig. 5.10 Hybridization signals to segment 8 riboprobe are present in the endothelial cells lining the congested blood vessel (arrow).



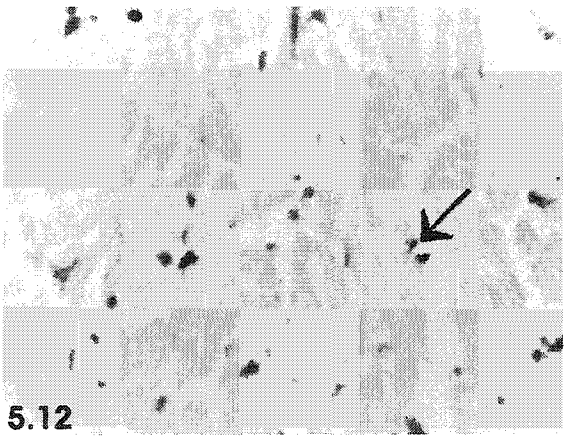


Fig. 5.11 Heart of Atlantic salmon infected with NSC isolate of ISAV showing hybridization signals to ISAV RNA segment 7 riboprobe in few endocardial endothelial cells (arrow).
Fig. 5.12 Heart of Atlantic salmon infected with ISAV RPC showing hybridization signals to ISAV RNA segment 7 riboprobe in more endocardial endothelial cells (arrow).

5.4.5 RT-PCR on tissue pools

Homogenates from organs pools from AS mortalities, and organ pools from two fish of the same species from the same infected groups, sacrificed the same day were used for RT-PCR using the ISAV segment 8 RNA primer pair. The RT-PCR products were analyzed on 1% agarose gel. The 220 bp amplification product was observed in AS mortalities and in all three species of fish infected with the three isolates (Fig. 5.13), throughout the sampling period pnm. Uninfected control fish were negative for ISAV RT-PCR.

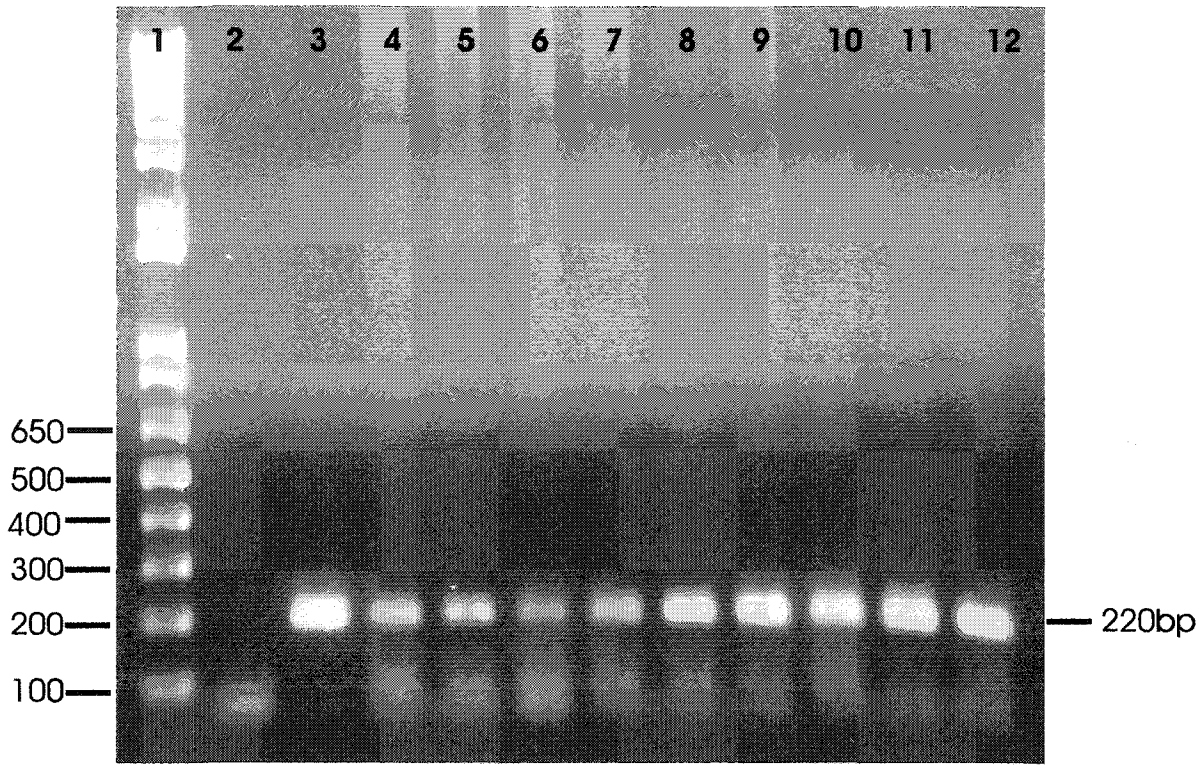


Fig. 5.13 Agarose gel of RT-PCR amplified cDNA of ISAV segment 8 from experimentally infected fish species. Lane 1: 1 Kb DNA ladder. Lane 2: water as negative control. Lane 3. NSC isolate positive control. Lanes 4-6, Pooled tissues from Atlantic salmon infected with ISAV isolate NSC sacrificed on 48, 62, 76 dpi. Lanes 7-9, Pooled tissues of rainbow trout infected with ISAV isolates NSC sacrificed on 48, 62 and 76 dpi. Lanes 10-12, Pooled tissues of coho salmon infected and sacrificed as in rainbow trout.

5.5 DISCUSSION

The pathologic changes observed in AS in this study are similar to those previously described for ISA (Evensen et al., 1991; Mullins et al., 1998). The disease was initially associated mostly with liver pathology in Norway (Thorud and Djupvik, 1988). However, mostly renal pathology was reported in the initial outbreaks in Canada (Byrne et al., 1998). Recent studies have shown that hepatic and renal lesions are present either singly or in combination in both natural and experimental ISA disease. Electron microscopy has been used to show that the virus buds from endothelial cells and leucocytes, hence the possible target cells of the virus (Hovland et al., 1994). The presence of hybridization signals in endothelial cells and leucocytes in AS mortalities indicates the presence of viral mRNA. The absence of hybridization signals in any of the parenchymatous cells in AS supports other findings that these cells are not the major target cells of this virus (Gregory, 2002).

Viral mRNA was observed in endothelial cells adjacent to foci of necrosis or degeneration of hepatocytes and renal tubular epithelium. However, where there was extensive necrosis and disruption of the general architecture of the liver, the sinusoidal endothelium was lost and viral mRNA was not detected. These findings support the view that the hepatocellular and renal tubular necrosis and other interstitial cellular degeneration observed during ISA in AS might be a result of the endothelial cell shut down by the virus leading to ischemic necrosis (Evensen et al., 1991; Speilberg et al., 1995).

In the spleen and head kidney rich in leucocytes, viral mRNA and degenerative changes co-exist *in situ* in AS. The replication and budding of the virus in both mature and progenitor leucocytes is believed to result in necrosis and cellular depletion observed in AS. Thorud (1991) reported leucopenia in AS with clinical ISA, but Dannevig and Falk

(1993) could not find any difference in the leucocyte numbers from the head kidney of ISAV infected and uninfected fish. However, the developmental stages and the particular leucocyte types that allow virus replication in AS are still not characterized, but the major cell type is assumed to be macrophage-like (Hovland et al., 1994; Nylund et al., 1996; Moneke et al., 2003). Dannevig and Falk (1994) showed that the virus can be experimentally transmitted to naive AS using leucocytes from head kidney of infected AS. They also showed that the virus can propagate in primary leucocyte cultures from AS (Dannevig et al. 1995a,b).

Hybridization signals and lesions were most correlated in the liver of AS mortalities which is consistent with the fact that the liver has many sinusoids lined by endothelial cells. It was observed that even when sinusoidal endothelial cells in affected areas were completely destroyed, endothelial cells at different stages of degeneration were present in major hepatic blood vessels and these showed hybridization signals. Speilberg et al. (1995) reported changes in the perisinusiodal macrophage of the liver as early as 4 dpi in AS. Other authors have reported that the absence of well developed resident macrophage system in the liver of teleosts (Ferguson, 1989) might account for the poor ability in trapping circulating pathogens in the liver. However, sinusoidal endothelial cells of teleosts exhibit potential phagocytic and digestive activity (Ferguson, 1984; Lamers and De Haas, 1985; Imagawa et al., 1994).

The low correlation between hybridization signals and lesions in the kidney of AS mortalities (even when more than 50% of the kidney sampled showed hybridization signals) was surprising since the fish kidney has a similar portal vascular system as the liver (Imagawa et al., 1990; 1994). However, the melanomacrophages, sinusoidal

macrophages, lymphoid cells, and sheath of reticular cells tend to line sinusoids of the portal veins of the kidney (Imagawa et al., 1991). Therefore, a virus selectively damaging only endothelial cells will have less severe vascular effects in the kidney when compared to the liver. Initial reports on ISA outbreaks mentioned few or no lesions in the kidney (Thorud and Djupvik, 1988; Evensen et al., 1991) and until recently, most authors reported mainly liver lesions in experimental ISAV infection (Jones et al., 1999; Simko et al., 2000; Jones and Groman 2001; Moneke et al., 2003). However, lesions might depend on the ISAV isolate (Evensen et al., 1991; Byrne et al., 1998; Simko et al., 2000; Mjalaand et al., 2002) and the route and duration of infection (Jones and Groman, 2001). Byrne et al. (1998) reported mostly renal pathology in one ISA outbreak and in some cases complete destruction of the sinusoidal endothelial cells in areas with severe lesions, just as in the liver. Also, the low correlation between lesions and hybridization signals in the kidney might depend on the portion of the kidney. Byrne et al. (1998) reported that it is not uncommon for sections of one kidney from different locations to show extreme variation in the severity of the histopathological changes. Also, the presence of melanomacrophages in the kidney might affect the amount of hybridization signals observed as discussed in Chapter 4, section 4.5.

In the RbT infected with the different isolates, no gross or histologic lesions or hybridization signals were observed in any organ sampled. The absence of lesions indicates that either the fish had fully recovered or never acquired any lesions from the infection as no morbidity or mortality was observed among the RbT during the experiment. Early studies reported only significant drop in hematocrit and petechia hemorrhages on the liver of few experimentally infected RbT sampled within peak infection period (Nylund et

al., 1997). Recently mortality was reported among RbT experimentally infected with ISAV, and weak and low frequency of hybridization signals were observed with the ISAV segment 8 riboprobe in the endothelial cells of tissues from those mortalities (Kibenge et al., 2004). This suggests that the virus in RbT replicates in the same cells as AS and to a detectable level with ISH during peak infection. The absence of ISH signals and the presence of viral sequences in the present study suggests that the virus probably replicated at very low levels, therefore requiring amplification by RT-PCR to be detected.

In CS as in RbT, no lesions or ISH signals were observed in any organ, however the amplification of viral RNA segment 8 indicates the presence of the virus in this fish species as well. A previous study reported the detection of the virus in tissues of diseased farm CS but isolation of the virus was difficult (Kibenge et al., 2001a). Rolland and Winton (2003) reported the isolation of the virus in experimentally infected CS randomly sampled at 13 dpi but these fish were non-clinical. In the present study it is not known if the virus caused any lesion at the onset of infection in this group of fish as sampling started several weeks after the last natural mortality in AS. Therefore, any lesions that would have developed within that period in the CS could have been missed. The presence of viral genetic material as shown by RT-PCR in infected fish throughout the sampling period indicates the presence of the virus at low levels suggesting the persistence of viral genetic material or the virus in CS as in RbT. Nylund and Jakobsen (1995), reported the presence of ISAV in experimentally challenged brown trout *Salmo trutta* for up to 7 months after infection, indicating the possibility of life-long carriers.

In AS that survived, except for the presence of few gross lesions and vasculitis in the liver of fish in CH7 group sacrificed on 31 dpi (7 day pnm), no lesions were observed

in the NSC and RPC infected groups sampled. The amplification of segment 8 viral mRNA by RT-PCR, and the absence of hybridization signals in the organs from the CH7 infected group that still showed slight lesions, indicates a sharp decline in the viral load in fish that survived the infection. The continued detection of the viral mRNA in AS infected with the three isolates throughout the sampling period also suggests the persistence of the virus or the viral genetic material. These findings support earlier reports that observed the viral mRNA for about 135 dpi in tissues of experimentally challenged AS (Devold et al., 2000). Totland et al. (1996) reported mortalities among naive AS cohabited with ISAV experimentally challenged AS that recovered from the infection several months, leading the authors to suggest that fish surviving ISA outbreaks may become persistently infected carriers that probably continuously shed the virus. The increasing reports of ISAV RT-PCR positives in asymptomatically farmed and wild AS might suggest that the virus persists, even though virus isolation in some cases has been difficult (Mjaaland et al., 2002; Nylund et al., 2003; Kibenge et al., 2004).

The survival of viruses in nature requires that they continuously either infect new susceptible hosts or establish a long term persistence within an infected host (reviewed by Ahmed et al., 1996). The survival of influenza viruses is hypothesized to be as a result of continuous transmission among wild aquatic birds which act as reservoirs without virus persistence (Murphy and Webster, 1996). This is unlikely in ISAV in fish where it appears that virus persistence and carrier status occur in some infected fish. Viruses can persist in a host by causing an acute infection followed by latent infection in which case the virus persists in a non infectious form with intermittent periods of viral reactivation and shedding, or by acute infection followed by a chronic infection in which case infectious virus is

continuously shed from or is present in infected tissue. Also viruses can persist by causing slow, chronic infection without ever progressing to acute infection (reviewed by Ahmed et al., 1996). From this study it seems that the persistence of ISAV in AS occurs as a result of acute infection followed by chronic infection, while in RbT and CS it persists as a result of slow chronic (non progressive) infection. This supports other suggestions that indicated the establishment of chronic infection and virus persistence in brown trout (Nylund and Jakobsen, 1995). It seems however, that this distinction cannot be clearly drawn as slow and chronic infections have also been reported in AS (Mjaaland et al., 2002; Nylund et al., 2003). It is therefore hypothesized that the pattern of persistence of ISAV depends on a combination of the virus isolate and fish species. More studies however, will be needed to fully understand the pattern of ISAV persistence in fish.

For a virus to persist in a host after infection it must have a mechanism for long term maintenance of its genome in host cells so as to avoid detection and elimination by the host immune system (reviewed by Ahmed et al., 1996). Viruses have several strategies in accomplishing this goal. Some of these strategies include the restriction of viral cytolytic effects, restriction of expression of viral genes and infection of immunologically privileged sites. The present study could not determine the tissue site or other strategies by which ISAV persists in fish. This report and those of other investigators, however, suggest that segments 6 and 8 gene products are involved in the ISAV persistence (Mjaaland et al., 2002; Nylund et al., 2003).

The sensitivity of the ISH depends on the method used in detecting the hybridization. The inability to detect ISH signals when the RT-PCR was positive means that the hybridization detection system used is not highly sensitive. ISH can detect up to 20-30

copies of mRNA per cell (Höfler, 1990), and with amplification of the visualization procedure, the detection limit can be increased to single mRNA levels (Femino et al., 1998). Devold et al. (2000) reported observing ISAV RT-PCR positive AS in only one male for several months after infection, and suggested that conventional RT-PCR might not be sensitive enough to pick out ISAV-carrying salmon. Recovered AS that were negative by conventional RT-PCR assay, were reported to still transmit ISAV to naive healthy AS on cohabitation (McAllister et al., 2003). Recently, Munir and Kibenge (2004), using the same tissues from recovered NSC infected AS and RbT as in the present study, sampled at 76 dpi (6 weeks pnm), showed that real time RT-PCR was 100 times more sensitive than conventional RT-PCR in the detection of ISAV in infected fish tissues. They were able to show presence of ISAV in all organs sampled from both fish species except in the kidney of RbT. Their result indicated that the most concentration of viral RNA was present in the heart and liver of recovered AS and RbT, respectively. Although this result might not be taken to mean that the virus persists more in these organs, the data in the present study and in Chapter 4 suggested that the heart has the most viral mRNA in clinical ISA and may be the site of persistence of ISAV. The presence of viral genetic material in all fish organs detectable by real time RT-PCR (Munir and Kibenge, 2004), however, might also indicate the involvement of the endothelial cells in ISAV persistence. More work is still needed to fully understand the mechanism of ISAV persistence in different fish.

The weak and low frequency of hybridization signals in some tissues from AS mortalities of NSC group even when lesions were severe seemed to be viral genotype-associated. Phylogenetic analysis using the segments 2 and 8 viral RNA of the three isolates in this study have grouped the virus along genetic lines (Kibenge et al., 2000a;

Ritchie et al., 2001b). The NSC, though isolated from Nova Scotia, Canada, is more closely related to the European genotype, while the RPC and CH7 are of North American genotype. Also, more recent phylogenetic analysis using the hemagglutinin gene grouped the NSC with the European hemagglutinin genotype while the RPC and CH7 are of North American hemagglutinin genotype (Kibenge et al., 2001b; Mjaaland et al., 2002; Nylund et al., 2003). Therefore, it seems that the difference in the hybridization signals observed with the single European isolate when compared with the two North American isolates might be genotype specific. It would also appear that the North American isolates are more virulent and replicate faster than the European isolate. Another possibility is that the European isolate may be more adapted to the Atlantic salmon and therefore does not require much replication to cause lesions in endothelial cells. Thirdly, the differences in the hybridization signals might be isolate-associated since variations occur within each genotype (Mjaaland et al., 2002). More work, however, is still needed to fully understand the pathogenesis of isolates from the different hemagglutinin genotypes.

In summary, this study has shown that there is no direct relationship between the presence of viral mRNA and lesions observed in hepatocytes and renal epithelium in AS mortalities from ISA. Instead, the lesions appear to be secondary to destruction of the endothelial cells lining blood vessels supplying these cells. Direct association could only be established between the viral mRNA and lesions in endothelial cells and leucocytes. This study also showed that RNA of different ISAV isolates persist in AS, RbT and CS up to 6 weeks after the last mortality in AS. The persistence of ISAV viral RNA is not dependent on the virus isolate or fish species, as all three isolates used in this study persisted in all three fish species. Although the mechanisms and site (s) of ISAV

persistence in these species are not definitely known, it seems that the endothelial cells might be involved.

CHAPTER 6

CHARACTERIZATION OF THE GENE EXPRESSION OF ISAV ISOLATES OF NORTH AMERICAN AND EUROPEAN GENOTYPES IN INFECTED FISH CELLS*.

*** This chapter will appear in:**

MONEKE E, JOSEPH T, IKEDE BO, KIBENGE FSB (2004) Molecular Pathology in ISAV infections. *Aquacul Assoc Canada Spec Pub (in press).*

6.1 ABSTRACT

The gene expression of one ISAV isolate (RPC) belonging to the North American hemagglutinin genotype and two isolates (NSC and NOR) belonging to European hemagglutinin genotypes was studied in the TO cell line. Infected cells were harvested at 4, 8, 12, 24, 48 and 96 hpi. Distinct hybridization signals were observed in the nucleus and cytoplasm from 24 hpi and CPE was observed from 48 hpi. At 24 hpi, signals were detected with segment 8 riboprobe in 48.65% (±9.89) of NOR, 28.41% (± 11.06) of NSC, and 21.39% (±5.22) of RPC-infected cells. Similar results were obtained with the segment 7 riboprobe except for NOR infected cells in which the frequency of signals were significantly lower (P = 0.001). At 96 hpi, 71.70% (±15.50) of NOR, 53.03% (±12.07) of NSC, and 64.16% (±6.39) of RPC-infected cells showed signals with segment 8 riboprobe, while 79.52% (± 8.01) of NOR, 54.14% (±8.13) of NSC, and 87.01 (±14.16) of RPC cells showed signals with segment 7 riboprobe. Signal intensity appeared to be higher with the segment 7 riboprobe but this was not quantified. The difference in the hybridization signals between NSC and the other two isolates was statistically significant (P = 0.004). No such difference was observed between NOR and RPC isolates (P = 3.44). The results suggest that the frequency and intensity of signals are dependent on the particular viral isolate rather than the hemagglutinin genotype of the virus, however, more isolates need to be examined in order to corroborate this claim.

6.2 INTRODUCTION

Earlier studies on the propagation of 13 ISAV isolates from Canada, Scotland and Norway in fish cell lines showed that they all replicated in SHK-1 cells, while only 6 out of 11 isolates from Canada and none of the European isolates replicated and caused CPE in CHSE-214 cells (Kibenge et al., 2000a). Based on this, the Canadian isolates were grouped into 2 phenotypes (Kibenge et al., 2000a). It was suggested that variation in the surface hemagglutinin protein (Griffins et al., 2001; Krossøy et al., 2001; Rimstad et al., 2001), could account for the difference in the virus phenotypes. Significant nucleotide and amino acid sequence differences in the hemagglutinin gene have however, been reported between the European and Canadian isolates (Kibenge et al., 2000a; 2001b; Rimstad et al., 2001). Differences have also been reported in the pathology associated with different isolates of ISAV (Byrne et al., 1998; Mullins et al., 1998; Jones et al., 1999).

The findings in Chapter 5 suggested that AS infected with ISAV isolates of the two hemagglutinin genotypes appeared to show differences in the frequency and intensity of hybridization signals even when lesions and cumulative mortalities were similar. Most studies on the variation in the virus have focused on the hemagglutinin genes, leading to the grouping of isolates based on hemagglutinin genotypes (Kibenge et al., 2001b; Mjaaland et al., 2002). The present study further characterizes the reactivity of segments 7 and 8 riboprobes and the pattern of expression of their mRNA in TO cells infected with three ISAV isolates in order to see if differences in hybridization signals could be genotype associated.

6.3 MATERIALS AND METHODS

6.3.1 Cells and viruses

The ISAV NSC, RPC, and NOR used for this study were originally from Nova Scotia, New Brunswick, and Norway respectively. The isolates were prepared as previously described in Chapter 2, sections 2.1 and 2.2.

6.3.2 Riboprobe synthesis

The segments 7 and 8 antisense riboprobes used are as described in Chapter 2, section 2.4.

6.3.3 ISH

In a preliminary experiment, two ISAV isolates (NSC and RPC) were used to infect TO cells grown to 80% confluent monolayer in 9 cm^2 Lab TeK tissue culture slide flasks as described in Chapter 2, section 2.1. A total of 15 slide flasks was used for this experiment; six flasks were each infected with 0.2ml of 1:100 dilution of the NSC isolate, and another six with same quantity of the RPC isolate. Three uninfected slide flasks served as control. The flasks were kept at 16°C and monitored for development of CPE. Infected slide flasks were harvested at 4, 8, 12, 24, 48 and 96 hpi, while the uninfected controls were harvested at 4, 24 and 96 hpi. Harvested cells were fixed in 4% paraformaldehyde and stored at -80°C until used for ISH as previously described.

In the main experiment, three ISAV isolates (NSC, RPC, and NOR) were used. The NOR isolate belongs to the European HA genotype as NSC and was used because of its

high virulence (Devold et al., 2001; Mjaaland et al., 2002), also because the initial interpretation of the difference in hybridization signals suggested it was based on the genotype. The NSC, RPC and NOR isolates had titers of $10^{6.5}$, $10^{7.83}$, $10^{7.83}$ $TCID_{50}$, on TO cells respectively. A total of 1.13 x 10^5 TO cells was allowed to monolayer on each slide chamber flask and twenty-two slide flasks were used. Six flasks were each infected one isolate and the monolayers were inoculated with 0.1ml of the virus at a multiplicity of infection (m.o.i.) of 10. Four flasks were left as uninfected control. Infected cells were checked for development of CPE and harvested as before.

The frequency of the hybridization signals was analyzed by taking pictures of cells in 10 random fields at X16 objective as described in Chapter 2, section 2.7. All cells with or without hybridization signals in five random fields were counted. The percent averages of cells with hybridization signals were calculated from the total cells in the five fields.

6.3.4 Statistical analysis

A *student t test* and one-way analysis of variance (ANOVA) in Minitab version 13.2 were used to test for the difference in the frequency of hybridization signals in cell cultures between different isolates and segments of ISAV.

6.4 RESULTS

6.4.1 ISAV isolates RPC, NSC and NOR differ in their hybridization signals in infected TO cells

Distinct hybridization signals were observed with segments 7 and 8 riboprobes from 24 hpi

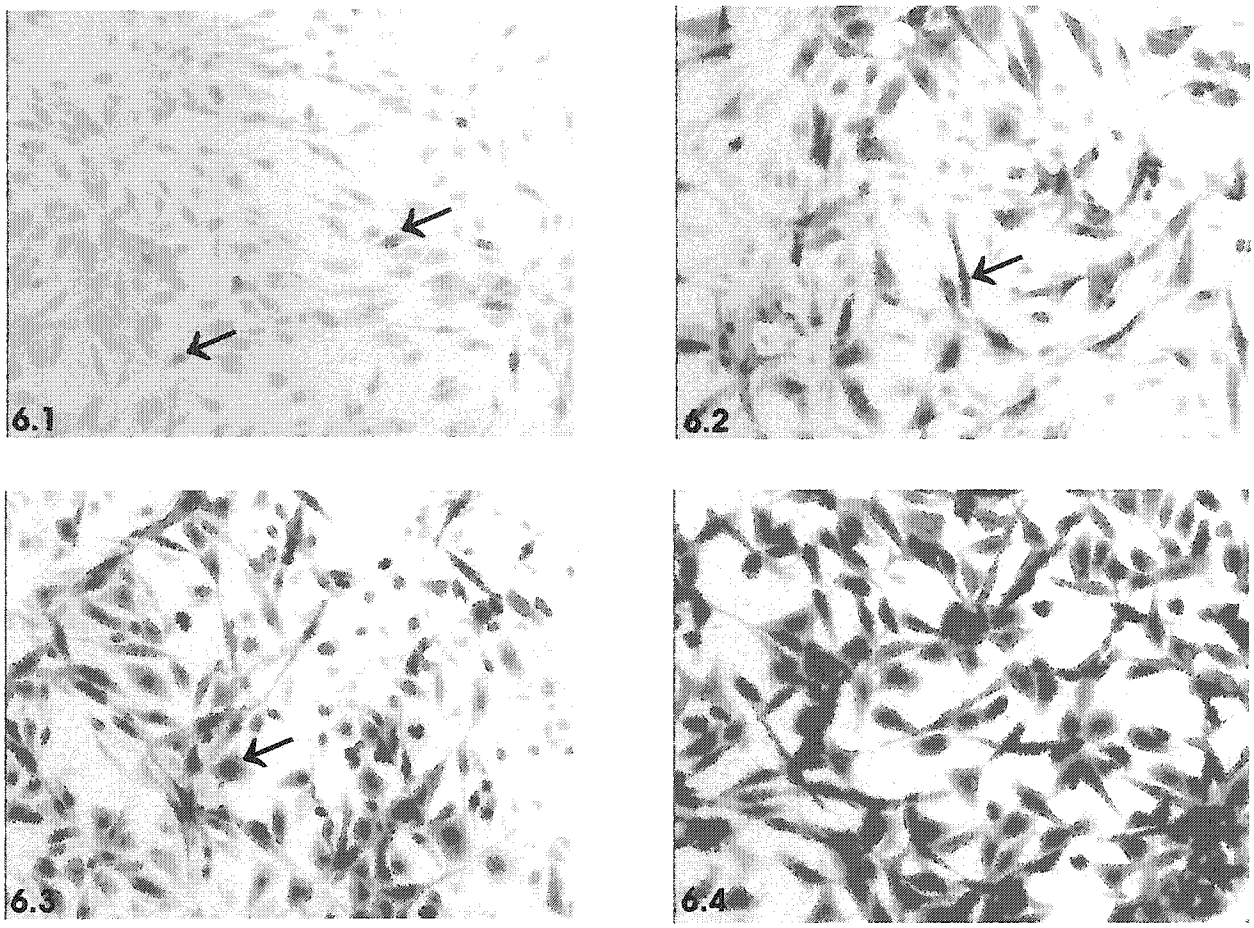
in the nuclei and cytoplasm of cells infected with each isolate (Fig 6.1) and onsets of CPE from 48 hpi. On subjective comparison of hybridization signals between the two isolates at 24 and 48 hpi, signals appeared stronger in cells infected with RPC (Fig 6.2) than with NSC (Fig 6.1). By 96 hpi hybridization signals were present in all infected cells (Fig 6.3 and 6.4). More intense signals were observed with segment 7 riboprobe at each sampling time as from 24 hpi as compared to segment 8.

In the main experiment a m.o.i of 10 was used to uniformly infect cells thus allowing for the quantifying of ISH frequency. CPE were observed from 48 hpi with all isolates as above. At 96 hpi, lifting of cellular monolayer was observed in RPC and NOR infected cells, while the monolayer of NSC-infected cells was intact.

Hybridization signals were observed from 24 hpi and the frequency summarized in Tables 6.1 (segment 7) and 6.2 (segment 8). In all isolates signals were first detected at 24 hpi and peaked at 96 hpi with both riboprobes. The signals at 48 hpi for NOR and NSC infected cells, and those of RPC at 24 hpi using segment 7 riboprobe could not be determined because most of the cells were lost during fixing.

6.4.2 Comparison of hybridization signals between the different isolates.

At 24 hpi there was no significant difference in the frequency of signals with segment 7 riboprobe in NOR and NSC -infected cells ($P = 0.677$). However, with segment 8 riboprobe a significant difference was observed with NOR infected cells showing more signals than other isolates ($P = 0.001$). At 48 hpi, the signal frequency increased rapidly and significantly in RPC infected cells and by 96 hpi, no significant differences were observed with the two riboprobes in NOR and RPC infected cells ($P = 3.44$). However, at 96 hpi



Figs. 6.1 - 6.4 *In situ* hybridization using a riboprobe to ISAV segment 7 in TO cells infected with different isolates (NSC and RPC) of ISAV.

Fig. 6.1 ISAV NSC infected cells 24 hpi showing signals in the nuclei of few cells (arrows).
Fig. 6.2 RPC infected cells 24 hpi showing signals in the nuclei and cytoplam of more than half of cells in the monolayer (arrow).
Fig. 6.3 NSC infected cells 48 hpi showing signals (arrow) in more cells similar to RPC infected cells at 24 hpi.
Fig. 6.4 All cells infected with RPC isolate showed signals at 48 hpi.

Table 6.1 Comparison of hybridization signals using segment 7 riboprobe on TO cells infected with three ISAV isolates at different time intervals.

Groups	% Average number (± standard deviation) of positive cells in five fields at different times					
	4hpi	8hpi	12hpi	24hpi	48hpi	96hpi
Uninfected	0	0	0	0	0	0
U5575-1 (NSC)	0	0	0	30.04 (±5.73)	-	54.44 (±8.13)
Norway 810/9/99 (NOR)	0	0	0	27.93 (±9.14)	-	79.52 (±8.01)
RPC/NB 01-0593-1 (RPC)	0	0	0	-	69.23 (±8.34)	87.01 (±14.16)

-, cells lost during fixation

Table 6.2 Comparison of hybridization signals using segment 8 riboprobe on TO cells infected with three ISAV isolates at different time intervals.

Group	% Average number (± standard deviation) of positive cells in five fields at different times					
	4hpi	8hpi	12hpi	24hpi	48hpi	96hpi
Uninfected	0	0	0	0	0	0
U5575-1 (NSC)	0	0	0	28.41 (±11.06)	-	53.03 (±12.07)
Norway 810/9/99 (NOR)	0	0	0	48.65 (±9.89)	-	71.70 (±15.50)
RPC/NB 01-0593-1 (RPC)	0	0	0	21.39 (±5.22)	54.80 (±15.33)	64.16 (±6.39)

-, cells lost during fixation

the NSC infected cells showed a significantly lower frequency of signals with both riboprobes when compared with other two isolates (P = 0.004). Subjectively, the signal intensity in the NSC infected cells appeared weaker compared to the other isolates. No difference was apparent in signal intensity between NOR and RPC infected cells.

6.4.3 Comparison of signals between the riboprobes.

At 24 hpi, significant difference was observed with segment 8 riboprobe showing more frequency in NOR infected cells (P = 0.011), even though signal intensity was more with segment 7. At 48 and 96 hpi, significantly more frequency of signals were observed with the segment 7 riboprobe in RPC infected cells than segment 8. No significant differences were observed at other times (P = 0.71 - 0.367). Subjectively, the intensity of signals appeared greater with segment 7 riboprobe than with segment 8 riboprobe overall.

6.5 DISCUSSION

This study further characterized the differences in hybridization signals in TO cells between ISAV belonging to the two hemagglutinin genotypes. TO cell line is derived from the head kidney leucocytes of Atlantic salmon (Wergeland and Jakobsen, 2001). The TO cell line was used in order to examine the frequency and intensity of hybridization signals from the different virus isolates in the observation reported in Chapter 5 away from interference by the host immune system which could have affected virus replication.

Initial results showed a clear difference in hybridization signals and invariably the replication and spread of NSC and RPC isolates in TO cells, further suggesting that the

difference might be based on the hemagglutinin genotype of the virus. The RPC showed more frequent and intense signals than NSC at the early stages of infection (Fig .6.1). However, by 96 hpi, no difference was observed in signals between the two isolates. This suggests that although the replication of NSC may be slow early in infection, as the infection progressed, its replication and spread picked-up to equal that of RPC.

In order to better understand the rationale for the difference in hybridization signals between the virus isolates, the NOR isolate that is genetically related to the NSC but is more pathogenic (Kibenge et al., 2001b; Devold et al., 2001; Mjaaland et al., 2002) was introduced into this study. The frequency and intensity of signals for NOR and RPC isolates were similar. Also, CPE and cellular detachments occurred at the same time with both isolates, indicating they might be similar in pathogenicity for TO cells. On the other hand, the NSC showed slower progression of CPE, lower frequency and weaker intensity of signals than the other two isolates, suggesting that it might be less pathogenic for the cell line. These findings indicate that the differences in ISH signals, replication, and the CPE observed are dependent on the particular virus isolate and not its hemagglutinin genotype. These results support other reports that showed different isolates of European genotype to replicate at different rates in SHK-1 cells (Mjaaland et al., 2002), and different isolates of North American genotype to replicate differently in CHSE-214 cells (Kibenge et al., 2000a).

It was suggested that sequence variations in the variable region of hemagglutinin gene/protein may be responsible for the variation in virus isolates (Devold et al., 2002; Mjaaland et al., 2002). The hemagglutinin protein of ISAV (Griffiths et al., 2001; Kibenge et al., 2001b; Rimstad et al., 2001), like in influenza viruses, has the function of host cell

recognition, attachment, and penetration into the cell (Eliassen et al., 2000). It is therefore a major antigenic determinant of the virus. The absence of association between hybridization signals and ISAV hemagglutinin genotypes in this study supports the report of Kibenge et al. (2001b) who found no clear association between the virulence of different isolates in tissue culture and sequence variations in the hemagglutinin gene.

The absence of significant difference in signal frequency of NOR and NSC infected cells with the two riboprobes at 24 hpi indicates the presence of ISH detectable mRNA in the same number of infected cells by the two isolates and might be as a result of the high initial m.o.i. However, the greater signal intensity observed with segment 7 riboprobe suggests that its mRNA is expressed in higher amounts than that of segment 8 at any given time. The higher frequency of signals observed at 24 hpi with segment 8 riboprobe in NOR infected cells might indicate that the segment 8 mRNA is expressed in more cells earlier in infection than the segment 7 mRNA. This observation supports the data in Chapter 3 in which at 24 hpi segment 8 riboprobe signals were more frequent than segments 6 and 7 riboprobes in NBC infected CHSE-214 cells. However, this result is difficult to extrapolate because of the multiple replication cycles of the virus by 24 hpi.

The weak intensity of signals observed from the segment 8 riboprobe, however, suggests that the amount of viral mRNA is low compared to the segment 7 mRNA which presented more intense signals. The signal intensity of segment 7 mRNA increased as infection progressed while that of segment 8 remained almost the same. The controversy surrounding the coding assignment of segments 7 and 8 of ISAV is not fully resolved (Clouthier et al., 2001; Biering et al., 2002; Ritchie et al., 2002), the segment 7 mRNA of ISAV, like influenza virus, has two open reading frames (ORF 1 and 2) and encodes two

proteins by a splicing action of the ORF1 mRNA (Ritchie et al., 2002). From current work on-going in Dr Kibenge's Laboratory, it is unlikely that these 2 proteins correspond to M1 and M2 proteins in ISAV. The membrane protein (M1) in influenza virus is a major structural and predominant protein in infected cells (Lamb and Krug, 1996). The ORF 1 and 2, in segment 7 of ISAV share the first 22 amino acids followed by a 526-nucleotide intron that is removed by the splicing of the ORF1 mRNA which then continues in a +1 reading frame to encode the second protein (Biering et al., 2002; Ritchie et al., 2002). From this study, the strong intensity of hybridization signals observed with the segment 7 riboprobe indicates that the segment 7 ORF1 mRNA is abundant and increases as infection progressed. Although, first hybridization signals were observed at 24hpi by which time virus replication must have gone multi cycle, the presence of the mRNA might still be correlated to the demand/function of the protein.

The segment 8 riboprobe of 210 bases was generated from position 346 to 553 of segment 8 mRNA and could be part of the sequences of ORF1 or 2 of segment 8 (Biering et al., 2002). Unlike the influenza virus segment 8, there appears to be no splicing for ISAV segment 8 transcripts (Biering et al., 2002). In influenza virus the ORF1 encodes the non structural protein NS1, while the ORF 2 encodes a structural protein (nuclear export protein). Kibenge et al.(2004) used an immunoprecipitation assay with rabbit antiserum to purified ISAV and were unable to precipitate the proteins from the ORF 2 of segment 8, suggesting it is a non-structural protein. Unlike in influenza viruses, the NS1 protein is the most abundant protein early in infected cells and therefore is expected to give more signals than any other segment (Lamb and Krug, 1996). However, the presence of more signals from the segment 7 riboprobe might be as a result of the difference in the coding pattern

of the different segments of the two virus.

In conclusion, this study shows that the difference in the hybridization signals and replication of the different ISAV isolates in TO cells is not dependent on the hemagglutinin genotypes but on the biological properties of the individual viral isolate. However, this finding might not apply to the definitive host of the virus, as the development of disease is complex involving the host and environment. More work therefore, needs to be done in order to understand the pathogenesis of the different isolates in the fish host.

CHAPTER 7

CHARACTERIZATION OF DIFFERENCES IN PATHOGENESIS AND PATHOLOGY OF DIFFERENT ISAV ISOLATES IN EXPERIMENTALLY INFECTED ATLANTIC SALMON*

*** Portions of this chapter appear in:**

MONEKE EE, GROMAN DB, WRIGHT GM, STRYHN H, JOHNSON GR, IKEDE BO, KIBENGE FSB (2004) Correlation of virus replication in tissues with histologic lesions in Atlantic salmon experimentally infected with infectious salmon anemia. *Vet Pathol* (in press)

7.1 ABSTRACT

This study investigated the replication of virus in tissues and development of lesions associated with ISAV infection in Atlantic salmon parr using ISH with a riboprobe targeting ISAV segment 7 mRNA and histologic examination of three major organs (heart, liver and kidney). Three ISAV isolates NSC, RPC and NOR were used to infect fish which were then sacrificed sequentially at 3, 6, 10 and 13 dpi and thereafter once a week for 8 weeks. Increased erythrophagocytosis was observed in sinusoidal macrophages from 3 dpi in the kidney of fish from all three isolates, suggesting an increased destruction of erythrocytes early in the infection. In the NSC infected fish, more appreciable increase in erythropagocytosis was also observed in both the sinusoidal macrophages and circulating macrophages/monocytes from 13 dpi up to 20 dpi, while the level of erythophagocytosis in the other groups remained lower. However, hybridization signals were weaker in the NSC group when compared to other groups. The high incidence of erythrophagia in the less virulent NSC group may suggest that erythrophagocytosis is linked to a host response strategy for control of ISAV infection. Severe histopathological lesions were observed in tissues from all groups beginning at the onset of mortality. The severe histopathological lesions correlated with maximum intensity and frequency of ISH signals ($P < 0.001$). There was a strong association between ISH signals and severity of lesions in the liver, kidney and heart (R = 0.81, 0.70, and 0.78 respectively, $P < 0.001$). The ISH signals were indicative of viremia as they were observed predominantly in individual blood cells and endothelial cells of the three tissues, but not in the necrotic hepatocytes or renal tubular epithelium. These findings suggest that while the endothelial lesions are a direct result of virus multiplication, the hepatocellular and renal tubular necrosis are possibly secondary

to ischemia. Of the three organs sampled, the heart, was the first and last to show ISH signals in each group. The NSC isolate appeared to be the least virulent isolate studied, compared to the other two (P = 0.001).

7.2 INTRODUCTION

The study in Chapter 6 showed a difference in the replication rate (and therefore virulence) of different isolates of ISAV in infected TO cells. Although, differences have been reported in the pathology associated with different isolates (Byrne et al., 1998; Mullins et al., 1998; Jones et al., 1999), efforts to reproduce all the lesions associated with the virus have proved difficult (Jones et al., 1999). Previous studies showed that histopathological lesions appeared at about 12-18 dpi in experimentally infected fish coinciding with the onset of mortality (Dannevig and Falk, 1994; Speilberg et al., 1995). However, these studies did not relate to the presence of the virus in these organs with either the lesion severity or mortality in infected fish.

In fish experimentally infected with ISAV, increased erythophagocytosis by sinusoidal macrophages outside and within the melanomacrophage centers have been shown to occur early in the spleen but not the kidney (Falk et al; 1995), indicating an increased rate of destruction of erythrocytes. Byrne et al. (1998) reported the presence of virus-like particles in erythrocytes of fish that had hemorrhagic kidney syndrome (HKS), a disease later confirmed as ISA (Bouchard et al., 1999; Lovely et al., 2000). They also reported increased erythrophagia by renal portal macrophages in diseased fish. Increased erythrophagocytosis by macrophages outside the melanomacrophage centers in both the spleen and kidney was observed in fish experimentally infected with three different isolates

of ISAV in Chapter 5. To date there has not been any studies on the possible significance of this lesion in the pathogenesis of ISAV.

ISAV genetic material is increasingly being detected by RT-PCR in wild Atlantic salmon that do not show signs of the disease, and in fish on some farms with clinical ISA disease, while the virus from such samples cannot be isolated in cell culture (Mjaaland et al., 2002). Moreover, no sequential studies have been performed on the relationship, if any, between the virus replication in tissues and the virus type (genotype and pathotype) and severity of lesions present.

The main goal in this study was to determine the tissue distribution of ISAV RNA segment 7 mRNA (as an indication of virus replication) of three ISAV isolates belonging to the North American and European hemagglutinin genotypes, in experimentally infected fish and correlate the pattern to the lesions observed. Also, the site(s) with the most *in situ* virus replication early in the infection and therefore the best tissue to sample during virus screening of suspected infected fish was identified.

7.3 MATERIALS AND METHODS

7.3.1 Cells and viruses

The NSC, RPC and NOR isolates used were prepared as described in Chapter 2, section 2.1 and 2.2.

7.3.2 Riboprobe synthesis

The segment 7 antisense riboprobe used were synthesized as described in Chapters 2,

section 2.4.

7.3.3 Experimental infection of fish with ISAV and tissue sampling

A total of 235 Atlantic salmon certified as specific pathogen-free and without any history of disease were obtained from the Atlantic Sea Smolts, PEI, Canada. The condition and housing of the fish were as described in Chapter 2, section 2.5.1. The fish were removed from stock holding tanks and anaesthetized by immersion in an aerated solution of tricaine methane sulphonate (TMS-222) (100 mg/L) and 70 fish were serially assigned to each of the three experimental groups in tanks # 1, 2 and 3 total 210 fish. Each fish was challenged by intraperitoneal injection of ISAV, (NSC for fish in tank #1, RPC in tank #2 and NOR in tank #3) at a virus dose of 10^6 $TCID_{50}$/0.2ml/fish and subsequently returned to their individual tanks. Twenty uninfected fish were kept in a control tank and used as negative control. All fish were observed twice daily and mortalities were recorded and removed. Three fish per tank from the infected group were sampled at 3, 6, 10 and 13 dpi and thereafter every week until 8 weeks post inoculation, while three fish from the uninfected control tank were similarly sampled at days 3, 13, 27 and after 8 weeks post infection. Duplicate samples of the heart, liver, kidney (head and trunk together) were collected aseptically and efforts were made to sample the same region of the tissue in each fish. One set of tissues was fixed in 10% buffered formalin for 24 hr and then processed in an automatic tissue processor and embedded in paraffin wax. The other set of tissues was pooled in a sterile bag and stored at -80°C until used for viral analysis by RT-PCR.

7.3.4 Histology and ISH

Serial 5-µm thick paraffin sections of formalin fixed tissues were stained with H&E for light microscopy or were generated for ISH. The tissues were hybridized with segment 7 riboprobe as described in Chapter 4, section 4.3.3.2. Lesions and hybridization signals were graded and analyzed as described in Chapter 2, section 2.5.3. Briefly, hybridization signals were observed using light microscopy. Digital images were captured and analyzed using Adobe Photoshop 5.5™ (Adobe Systems Inc.). The frequency of the hybridization signals was graded subjectively at X16 objective using a minimum of 10 fields as follows; 0 = no signals, 1+, = presence of signals in less than 4 fields, 2+, = presence of signals in 4 to 7 fields, 3+, = presence of signals in 8 to 10 fields indicating that almost all the endothelial cells and some individual blood cells showed strong signals. The maximum hybridization signal score was 27 per fish sampling time.

7.3.5 Statistical analysis

The strength of the association between scores for lesions and ISH signals in tissues from infected fish was assessed by nonparametric Spearman correlation coefficient. A nonparametric Kruskal-Wallis test was used to test the differences in scores for ISH signals and lesions between groups infected with different isolates on a daily basis; p-values were adjusted for multiple-day testing by Bonferroni method (dividing the significance level by the number of tests carried out). The mortality in the groups was estimated by Kaplan-Meier survival curves and compared between the groups by a nonparametric log-rank test.

7.4 RESULTS

7.4.1 Development of lesions and mortality

Liver, heart and kidney were sampled for this study because previous studies (Evensen et al., 1991; Hovland et al., 1994; Dannevig et al., 1995b; Spielberg et al., 1995) have shown that these organs are very important in the pathogenesis of ISAV. The mortality pattern from the three isolates is summarized in Table 7.1. The percent mortality could have been higher or lower than indicated as three fish were sequentially removed from each tank per sampling time. The first mortality occurred in tank # 1 on 17 dpi and the last on 38 dpi for a total mortality of 38 (54%). For tank # 2 group mortality started on 13 dpi and lasted until 28 dpi with a total mortality of 46 (66%). In the tank # 3 group, mortality started on 10 dpi and continued to 28 dpi, with a cumulative mortality of 53 (75%). Some or all the typical ISA gross pathology lesions (Evensen et al.,1991) including exophthalmia, petechia hemorrhages in the visceral fats, pale gills, congested liver and spleen, hemorrhage in the pyloric caeca, and hemorrhagic ascites were observed in fish in tanks # 2 and # 3 sampled from 10 dpi and in most mortalities of these groups (Fig. 7.1). In contrast, only exophthalmia and straw-colored ascites were observed in tank # 1 fish sampled from 20 dpi (Fig. 7.2). By 27 dpi, there were two fish remaining in tank #3, and these were sampled out. For tank # 2, sampling continued until 41 dpi when the last two fish were sampled. Sampling of fish continued in tank # 1 to the last sampling time of 8 weeks post infection. Neither mortality nor pathology was observed in the control group during the experiment. The histopathological findings in the kidney, liver and heart of fish sampled at different times post infection for the three groups are summarized in Table 7.2.

Table 7.1 Mortality records of fish infected with different isolates of ISAV (live fish sequentially removed for sampling not included in mortality).

Tank #	Isolate	1st Mort. DPI	Last Mort. DPI	Duration of Mort.	Total Mort.	Average Mort/day	% Mort.
1	U5575-1 (NSC)	17	38	21	38	1.8	54.3
2	RPC/NB 01-0593-1 (RPC)	13	28	15	46	3.1	65.7
3	Norway 810/9/99 (NOR)	10	28	18	53	2.9	75.7

Table 7.2 Total lesions score of ISAV infected fish at different days post infection.*

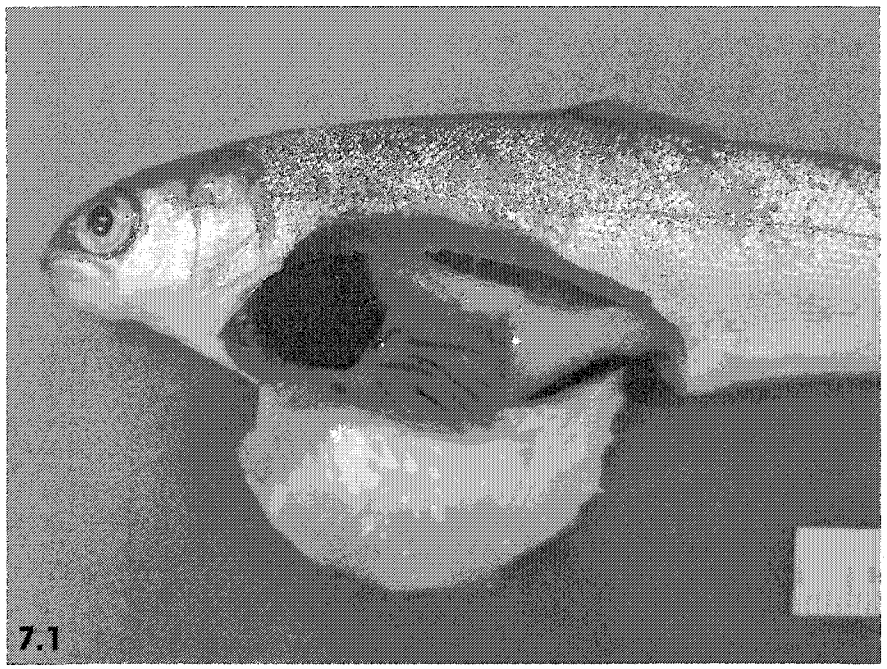
Tank #	ISAV Isolates	Days post infection (Dpi)									
		3 $^{p\Phi}$	6	10$^{p\Phi}$	13$^{p\Phi}$	20	27	34	41$^{p\Phi}$	48	55
1	U5575-1 (NSC)	2	4	6	6	10	6	3	5	2	0
2	RPC/NB 01-0593-1 (RPC)	2	5	10	15	13	4	1	2	nd	nd
3	Norway 810/9/99 (NOR)	7	4	15	15	13	5	nd	nd	nd	nd

* Based on 3 fish, 3 organs (heart, liver, kidney) per fish and maximum lesion grade of 2 (mild = 1, severe = 2), and a maximum score of 18.

^{p} Indicates days with statistically significant difference between infected groups ($P < 0.05$)

$^{\Phi}$ Indicates days with statistical significant difference between infected groups after adjustments for multiple day testing ($P < 0.05/8$)

nd, denotes not done because surviving fish were sampled out.



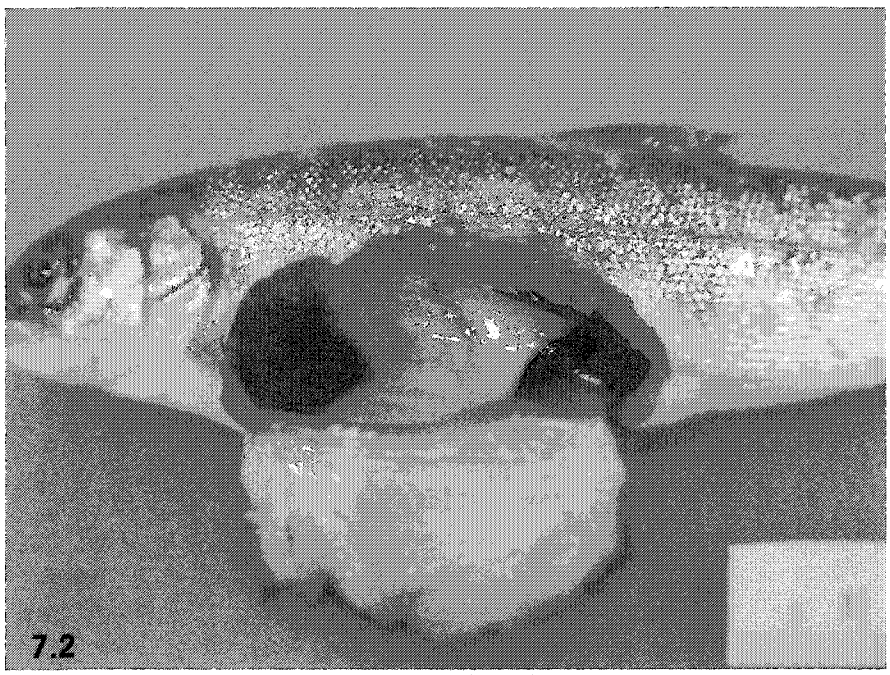
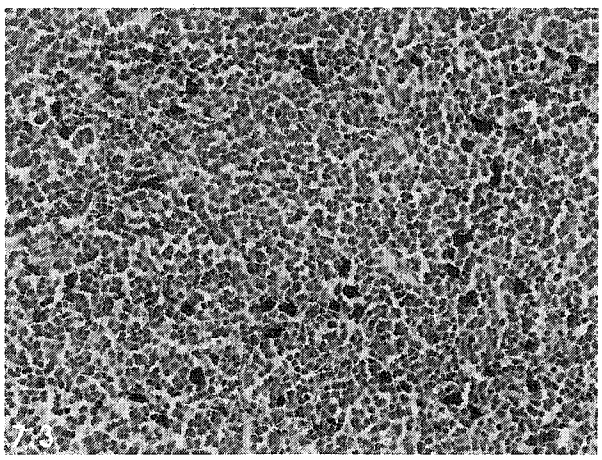


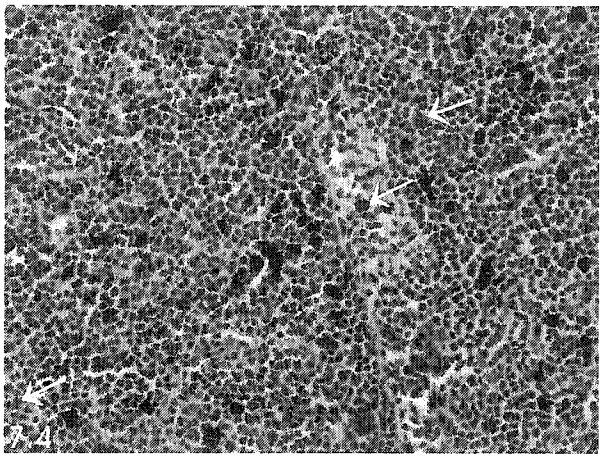
Fig. 7.1 Atlantic salmon, 13 dpi with ISAV isolate RPC, showing dark congested liver, congestion and hemorrhages in the abdominal cavity, ascites.
Fig. 7.2 Atlantic salmon, 20 dpi with ISAV isolate NSC, showing, straw colored ascites and absence of hemorrhages in the abdominal cavity.

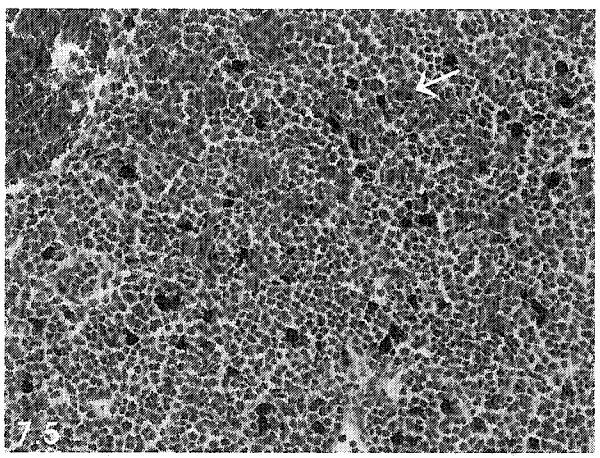
For the group 1 (tank # 1), mild changes were first seen in the kidney at 3 dpi and in one or two organs from 6 dpi onwards. By 20 dpi all three organs from the three fish per sampling time showed moderate to severe lesions, which coincided with the period of peak mortality. Mortality and lesions observed in sampled fish were few on 27 dpi and absent from 41 dpi onwards. In the group 2 (tank # 2), mild histopathological changes were seen in the heart and kidney of one fish at 3 dpi and from 6 dpi, either mild and/or severe lesions were seen in one or more organs of the three fish sampled. However, by 13 dpi, all three organs in the fish sampled showed severe lesions, which coincided with the start of mortality. The mortality increased and peaked a few days later, and by 20 dpi the severity of lesions in tissues had started to decrease, were few or absent by 27 dpi and absent from 34 dpi onwards. In group 3 (tank # 3), one organ per fish showed mild histopathological lesions from 3 dpi. Beginning at 10 dpi when mortality started, all three organs from all the three fish showed severe lesions. By 20 dpi severe lesions were still present in 2 or 3 organs of each fish when the mortality started to decrease. Lesions were few or absent in organs by 27dpi.

7.4.2 Erythrophagia in the kidney of infected fish.

Kidneys from sampled fish showed variation in the amount of erythrophagocytosis by macrophages outside the melanomacrophages centers. Such variations were absent in control fish sampled at 3, 13, 27 days and 8 weeks pi (Fig. 7.3). In tank # 1 an increase in erythrophagia was observed at 3 dpi mostly in the head kidney and at 13 dpi (Fig. 7.4) and 20 dpi there was an appreciable increases in erythrophagia in all parts of the kidney.







Figs. 7.3 - 7.5 Head kidney, Atlantic salmon.

Fig. 7.3 Uninfected control; H & E stain, no lesions. Fig 7.4 13 dpi with NSC isolate of ISAV; H & E stain. Vascular congestion, increased interstitial and vascular erythrophagocytosis (arrows). Fig. 7.5 10 dpi with NOR isolate of ISAV; H & E stain. Congestion, interstitial hemorrhage and very little erythrophagocytosis (arrow).

Also, at these times erythrophagia could be observed in blood vessels in the head kidney (Fig. 7.4). In tanks # 2 and 3, little erythrophagocytosis was observed on 3 dpi. At 10 dpi (Fig. 7.5) a slight increase in erythrophagia was observed in interstitial macrophages but not in the blood vessels of two fish from tank # 3 and after that no increase in erythrophagia was observed. However, no appreciable increase in erythrophagocytosis was observed in tank # 2 throughout the sampling time. The level of erythrophagocytosis observed in tank # 1 remained higher than the other groups throughout the study. No relationship was observed between erythrophagia and hybridization signals.

7.4.3 Relationship between *in situ* detection of ISAV mRNA and histopathologic lesions in tissues from fish infected with different virus isolates

The segment 7 antisense riboprobe was used for this study because previous experiments reported in Chapters 5 and 6 showed it to give more intense signals than other riboprobes. The intensity of ISH signals assessed qualitatively provided a good indication of the amount of virus mRNA in an infected cell, whereas the frequency of signals indicated the number of cells expressing the mRNA in infected organs. The frequencies of signals are summarized in Table 7.3. Signals were seen in endothelial and blood cells suspected as erythrocytes in the three organs sampled. Also, hybridization signals were observed in macrophage-like cells beneath endocardial endothelial cells (Fig 7.6). Some unidentified cells, possibly hematopoietic cells, in head kidney also showed hybridization signals (Fig. 7.7).

Table 7.3 Score of hybridization signals in fish organs (liver, kidney and heart) infected with different ISAV isolates at different days post infection.*

Tank #	ISAV Isolate	Days post infection (Dpi)									
		3	6[p]	10[p ÷]	13[p ÷]	20	27	34	41	48	55
1	U5575-1 (NSC)	0	0	5	8	18	2	0	0	0	0
2	RPC/NB 01-0593-1 (RPC)	0	7	27	27	10	4	0	0	nd	nd
3	Norway 810/9/99 (NOR)	2	4	27	24	20	1	nd	nd	nd	nd

* Hybridization signals were graded at X16 objective using a minimum of 10 fields as follows;

0, no signals

1+, presence of signals in <4 fields

2+, presence of signals in 4 - 7 fields,

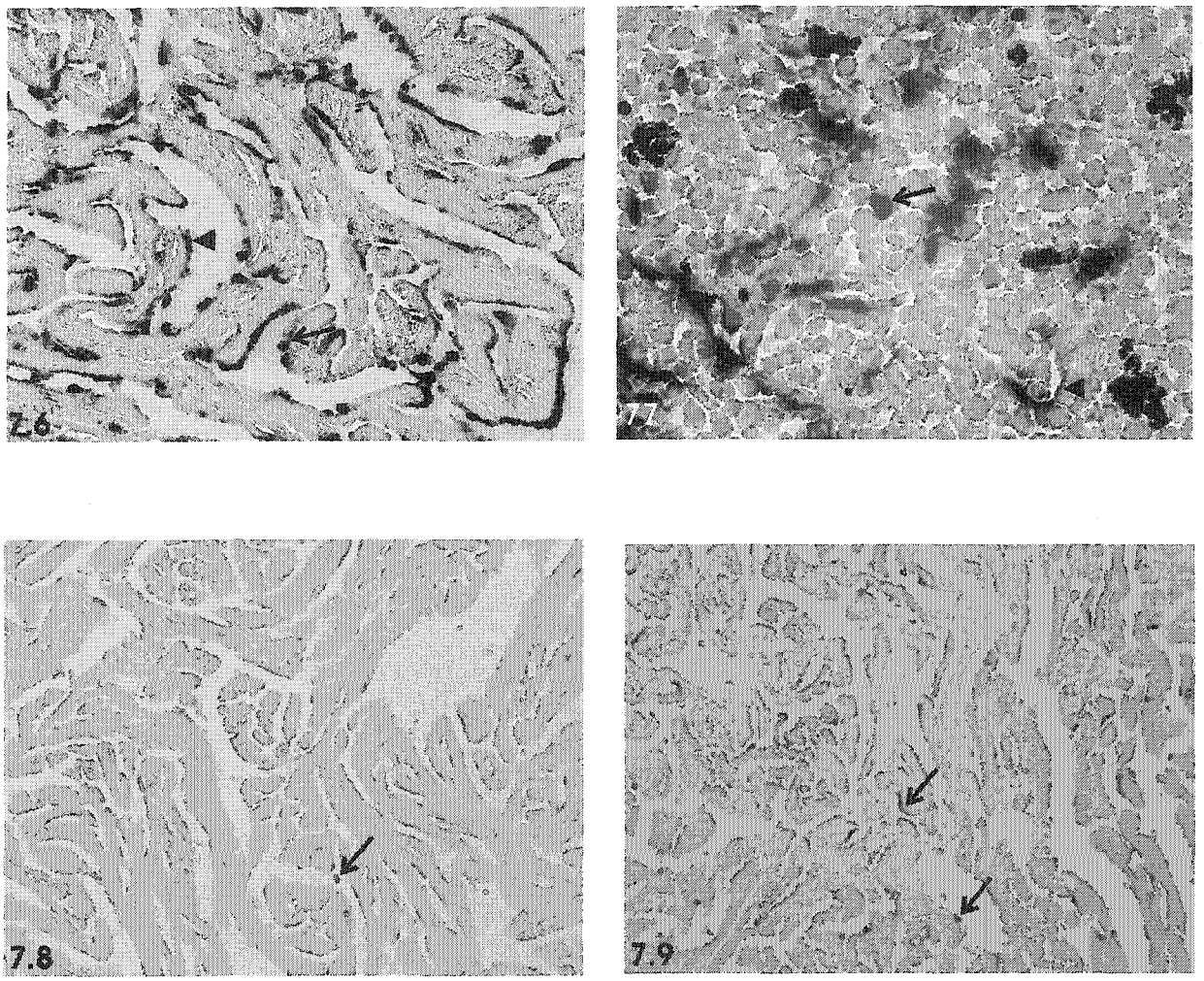
3+, presence of signals in 8 -10 fields.

The maximum ISH score = 27

[p] Indicates days with statistically significant difference between infected groups

÷ Indicates days with statistical significant difference between infected groups after adjustments for multiple day testing (P <0.05/6)

nd, denotes not done because surviving fish were sampled out.

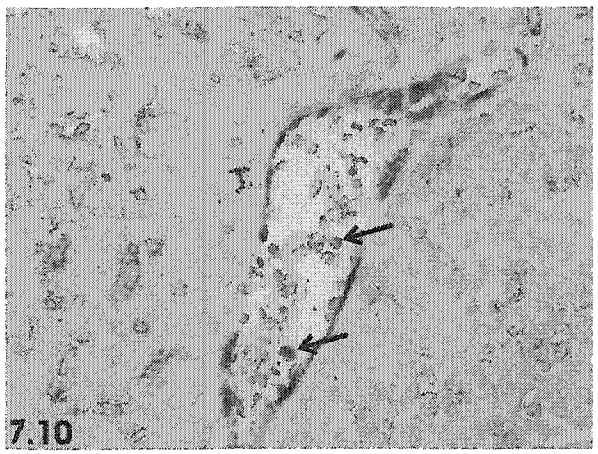


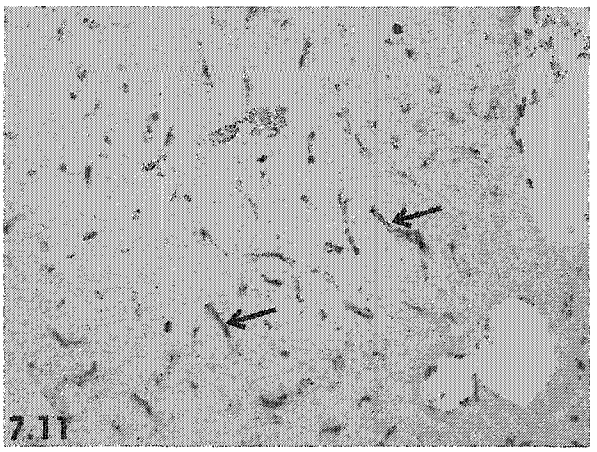
Figs. 7.6 - 7.9 Atlantic salmon tissues, ISH with segment 7 riboprobe.

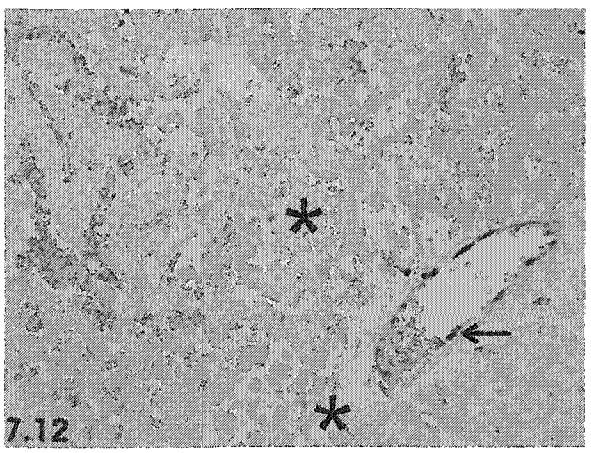
Fig. 7.6 Heart 13 dpi with ISAV isolate NOR. Hybridization signals are present in the endocardial endothelial cells (arrowhead) and macrophage-like cells (arrow). Fig. 7.7 Head kidney 13 dpi with ISAV isolate NOR. Signals are present in endothelial cells (arrowhead) and hematopoietic cells (arrow). Fig. 7.8 Heart 10 dpi with ISAV isolate NSC. Few signals are observed in endothelial cells (arrow). Fig. 7.9 Heart10 dpi with ISAV isolate RPC. Signals are intense and diffuse (higher frequency) in the endothelial cells (arrows).

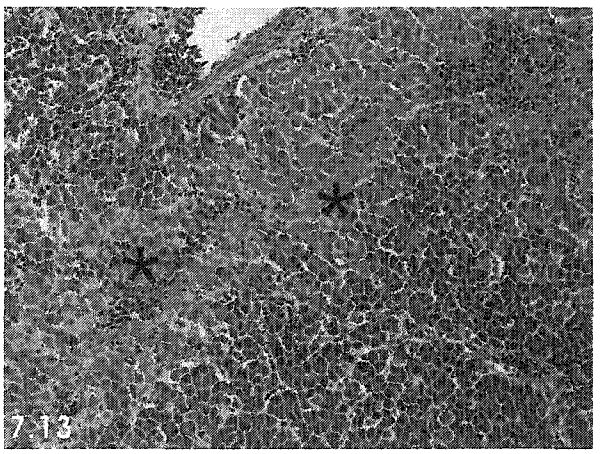
For tank # 1, signals were first seen in the heart at 10 dpi (Fig. 7.8) but were of weak intensity and low frequency. At 20 dpi, signals were of stronger intensity and were most frequent in all organs and also appeared in some blood cells, indicating peak viremia period. By 27 dpi, hybridization was seen mostly in the heart and after that ISH signals were not seen in any tissue. Hybridization was seen at 6 dpi in the heart of tank # 2 fish and in all three organs by 10 dpi. Increased frequency and more intense signals were observed by 10 dpi (Fig. 7.9), declining by 20 dpi, and only the heart showing less frequency and low intensity signals by 27 dpi. By 34 dpi, no ISH signals were observed in any organs. Tank # 3 fish showed the same intensity and frequency of ISH signals as in tank # 2 group, except that the first and last ISH signals were observed in the heart at 3 and 27 dpi respectively.

In all three groups, intense ISH was seen in blood cells suspected as erythroytes (Fig. 7.10) on 10 to 20 dpi respectively corresponding to peak viremia. In areas of maximum intensity and frequency of signals, virtually all endothelial cells were positive (Fig. 7.11), except where the intima was depleted of endothelial cells (Fig. 7.12). Degeneration and necrosis of hepatocytes (Fig. 7.13) and renal tubular epithelial cells were observed in all the mortalities sampled. However, no viral mRNA was detected within these cells. Hybridization was not observed in any of the uninfected control fish.









Figs. 7.10 - 7.13 Liver; Atlantic salmon. 13 dpi;

Fig 7.10 with ISAV isolate NOR; ISH with ISAV segment 7 riboprobe show signals in the endothelial and blood cells (arrows) in blood vessel.
Fig. 7. 11 with ISAV isolate RPC; ISH with ISAV segment 7 riboprobe show signals in most sinusoidal endothelial lining (arrows) and major blood vessels at peak viremia.
Fig. 7.12 with isolate NOR; ISH with ISAV segment 7 riboprobe show signals in the endothelial lining of major blood vessel (arrow) and not in the necrotic areas (*).
Fig. 7.13 with isolate NOR; H & E stain showing congestion and necrosis (*) of hepatocytes.

7.4.4 *In situ* detection of ISAV mRNA as an indicator of viral replication and relationship between viremia and histopathologic lesions in infected fish

The relationship between the frequency of ISH signals and histopathologic lesions observed as the infection progressed in fish infected with the different ISAV isolates is summarized in Figure 7.14. In the early stages of virus infection, few tissues had lesions, which correlated with the low frequency and weak intensity of ISH signals observed. As the infection progressed, more frequency and stronger ISH signals were seen with more severe histopathological lesions in all the organs sampled. There was a statistically significant relationship between the severity of lesions observed and the level of virus mRNA as depicted by the ISH signals in the three isolates used (R = 0.69, 0.72, 0.78, $P < 0.001$).

In the tank # 1 group the strongest intensity and highest frequency of ISH signals correlated with the severest lesions in all tissues by 20 dpi ($P < 0.001$). The tanks # 2 and 3 showed the strongest intensity and highest frequency of ISH signals to correlate with the severest lesions in all tissues by 13 and 10 dpi ($P, < 0.001$) respectively.

There was a strong association between the intensity and/or frequency of hybridization signals and the lesions observed in the liver, kidney and heart (R = 0.81, 0.70, and 0.78 respectively, $P < 0.001$) from infected fish sampled.

Mortality started at the peak lesions, when ISH signals were strongest and most frequent in all fish tissues infected by the different isolates. During this period, ISH signals were observed in blood cells in the blood vessels indicating a strong correlation between peak viremia, severity of lesion and onset of mortality. As the ISH signals decreased so

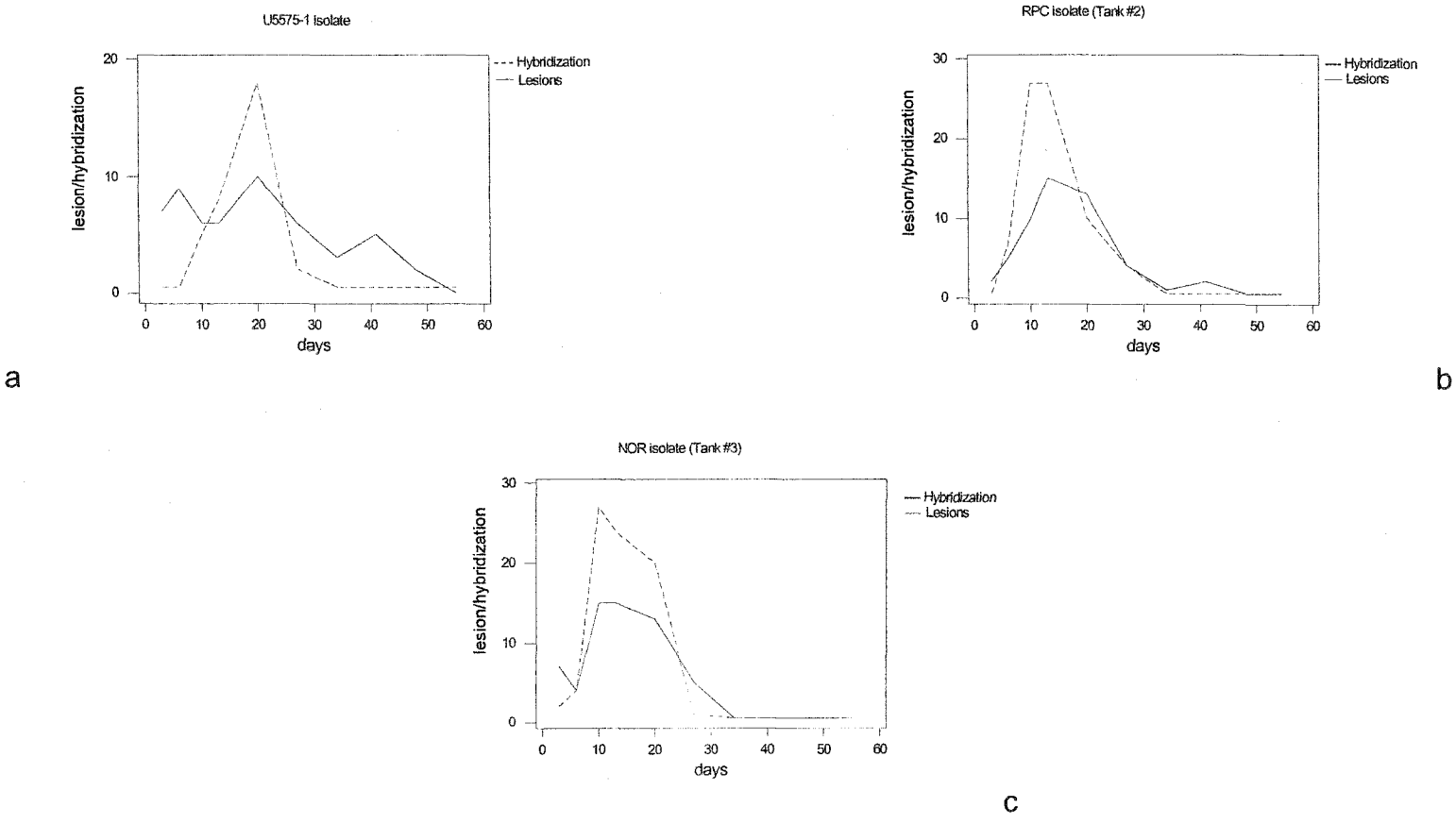


Figure 7.14 The total lesion and *in-situ* hybridization scores of fish infected with: (a) NSC, (b) RPC, (c) NOR isolates of ISAV per sampling day. As the hybridization signals increased, microscopic lesions became more severe in all three groups. Both the severity of lesions and hybridization signals peaked within the same period and started declining thereafter.

also did the severity of lesions and the mortality. By 34 dpi when mortality stopped in infected groups, ISH signals and lesions were absent in most, if not all the tissues of infected fish.

7.4.5 Comparison of histopathologic lesions and ISH signals between three different ISAV isolates

When the intensity and frequency of ISH signals and the lesions observed among the three isolates used were compared on a daily basis, isolates NOR and RPC showed the stronger and most frequent ISH signals, than NSC (uncorrected for 6, 10 and 13 dpi, $P \leq 0.001$). There was no significant difference in the daily intensity and frequency of signals and lesions between NOR and RPC isolates, although the NOR showed more signals and lesions (Fig.7.14).

7.4.6 Survival analysis of fish in the three different infected tanks

There was a significant difference in the mortality and survival rates of fish groups infected with the different isolates, with fish in tank # 1 (NSC) showing clearly the highest survival and lowest mortality rates while the tank # 3 (NOR) has the lowest survival rate and highest mortality ($P < 0.001$) (Fig. 7.15). There was a slightly significant difference in the mortality and survival rates of tank # 2 and 3 groups, with more mortality and less survival rates in tank # 3 ($P = 0.029$). These findings suggest that ISAV isolate NOR was the most pathogenic and NSC the least pathogenic of the three isolates used.

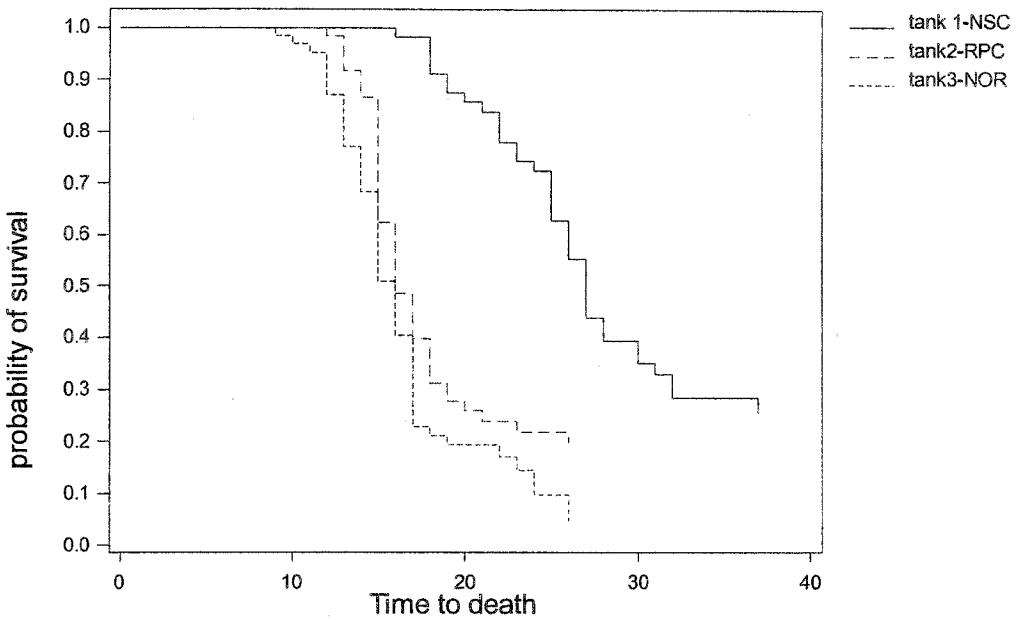


Fig 7.15 Analysis of the time to death (survival) of fish infected with the different isolates of ISAV. The NSC infected group (black line), had significantly higher probability of survival and therefore least time to mortality than the other two groups (dashed red line) and (dashed blue line). *(non parametric Kaplan-Meier survival analysis and log-rank tests)*

7.5 DISCUSSION

The development of an ISH protocol for the detection of ISAV in fish tissues (Gregory, 2002; Moneke et al., 2003), has provided a major tool to further study the pathogenesis of ISA. Other laboratory techniques like RT-PCR and IFAT have been used to study the pathogenesis of this virus. However, these methods have shown limitations even when an association could be established between the virus and the disease, and no direct relationship has ever been established with the histopathologic lesions observed. The ISH technique is unique because it allows for the detection of viral genetic material and its distribution directly within infected tissues, making it possible for lesions observed to be directly compared with the presence or absence of the viral nucleic acid *in situ*.

The onset, duration and total mortality recorded for the different ISAV isolates used in this study varied, with the first mortality recorded on 10 dpi in the NOR group (tank #3) and 17 dpi in the NSC group (tank #1). Some studies have shown that there is a relationship between the onset of mortality and the dose of ISAV inoculum used to infect the fish, in that those fish that received a higher viral dose started dying earlier than those given lower doses of the same virus (Raynard et al., 2001). In the present study, an equal virus dose was used to infect the fish with three different isolates, allowing us to speculate that differences in the mortality patterns can also be a result of differences in the virulence of the different isolates used.

The histopathological findings observed at the initial stages of the infection including mild congestion in the heart and kidney were not pathognomonic for ISAV infection. Although these lesions have been described as part of the early signs of the disease, they might also be a result of pooling of blood in these organs as observed in some uninfected

control fish during sacrifice as these organs are well vascularized. Previous reports have suggested changes in the function of leucocytes and macrophages in the head kidney of infected fish early in the infection (Falk et al., 1995) but not congestion as observed in this study. In the present study, no histopathologic changes were seen in the liver early in the infection, which supports an earlier report that lesions in the liver developed gradually over time (Evensen et al., 1991). However, early changes have been observed by transmission electron microscopy in the perivascular macrophages of the liver of infected fish by 4 dpi (Speilberg et al., 1995). In the present study, as the infection progressed, moderate to severe lesions were observed in other organs including the liver, with congestion and hemorrhages in most organs and degeneration of endothelial cells. Endothelial cell damage was most severe in liver sinusoids and corresponded to areas of mild to moderate hepatocellular necrosis at the onset of mortality in all three isolates. This observation supports other reports stating that degenerative changes in the sinusoidal endothelium from 14 dpi and in the hepatocytes by 18 dpi in ISAV-infected fish coincided with the onset of mortality (Speilberg et al., 1995). Evensen et al. (1991) also reported severe light microscopic changes in the liver of ISAV-infected fish as the infection progressed and hematocrit value decreased. Those studies were based on moribund or dead fish. This is the first sequential study to follow the progression of lesions in the different organs of fish infected with different isolates of ISAV.

Erythrophagia or erythrophagocytosis is the process in which macrophages engage in the digestion and destruction of red blood cells (erythrocytes). This is a normal physiologic process through which malformed and senescent red blood cells are removed from the circulation in vertebrates. In mammals, erythrophagocytosis can occur normally

in the Kupffer cells (liver macrophages) and spleen but also in the bone marrow (histiocytes) and in parenchymatous cells of other organs under different conditions (reviewed in Sheerin et al, 1999). In salmonids, normal removal of effete red blood cells occurs mainly in the melanomacrophage centers of the spleen (Ferguson et al., 1989) and in some cases the melanomacrophage centers of the kidney (reviewed by Agius and Roberts, 2003), although the major role of the head kidney is thought to be hematopoiesis (Zapata and Cooper, 1990).

Increased erythrophagocytosis as in pathological response to disease can lead to a fatal outcome in all animal species. In humans and animals, excessive erythrophagocytosis referred to as hemophagocytic syndrome, can be caused by neoplastic disease, immunologic disorders, infections (mainly viral), and immunodeficiency states (Potter et al., 1991; Sheerin et al., 1999). In most cases there is splenomegaly and in some cases erythrophagia in cells not normally involved in erythrophagocytosis in affected animals (Sheerin et al., 1999). In this study increased erythrophagocytosis was observed by macrophages outside the melanomacrophage centers in the kidney from 3 dpi in fish infected with different ISAV isolates. Earlier reports on the pathogenesis of this disease showed there was evidence of increased erythrophagocytosis by macrophages in the red pulp in addition to the usual melanomacrophage centers of the spleen by 7 dpi in infected fish (Falk et al., 1995). However, no signs of erythrophagocytosis or change in the enzyme reactivity of the diverse population of macrophages in the head or body portions of the kidney have been observed at the same period (Press et al., 1994). Although the spleen was not sampled at this time, this study suggests that kidney macrophages can also react in the same way as splenic macrophages early in ISAV

infection. Other viruses like IPNV and IHNV have also been shown to cause increased erythrophagocytosis in the spleen (Wolf, 1988). These viruses (including ISAV) also cause severe depletion and necrosis of the hematopoietic tissues in the head kidney (Roberts, 2001) leading to anemia.

As the infection progressed especially in the fish group infected with NSC, very appreciable erythophagocytosis was observed in sinusoidal macrophages in renal interstitium and by cells within the blood vessels suspected to be circulating macrophages/ monocytes (Agius and Roberts, 2003). Monocytes are found in small numbers in the circulating blood of fish (Roberts, 2001). The increased erythrophagocyosis by monocytes/circulating macrophages might be similar to that by histiocytes in human bone marrow induced as a result of virus infection (Zaharopoulos, 2001), as the head kidney is the major hematopoietic organ in fish.

The increased erythrophagocytosis by sinusoidal macrophages in the spleen early in ISAV infection has been suggested as an attempt by the fish to control ISA infection and initiate immune response as the hematocrit value was still within normal range (Falk et al., 1995). The significant increase in erythrophagocytosis observed in the head kidney of the NSC group when compared to other groups as the infection progressed in this study might support the findings of Falk et al. (1995). Therefore, it is possible that because of the low virulence of the NSC isolate, the host response of increased erythrophagocyosis might be one of the ways of controlling the infection. Recently, low mortality was reported amongst rainbow trout experimentally infected with a high dose of highly pathogenic strain of ISAV (Kibenge et al., 2004). The major histopathological finding was a significantly increased erythrophagocytosis in both spleen and kidney of mortalities (Chris MacWilliams, personal

communication). Previous studies have reported only transient drops in hematocrit value as the only clinical signs and pathology associated with ISAV infection in rainbow and brown trout (Nylund and Jakobsen, 1995) even when the virus was recovered from these fish. It is possible that the rainbow trout, an unusual and refractile host for ISAV, reacts to a high dose of highly pathogenic strain of ISAV through erythrophagocytosis just like the Atlantic salmon, a highly susceptible host for ISAV does to low pathogenic strain of ISAV. It can be hypothesized that the increased erythrophagocytosis observed in the kidney might be a mechanism for clearing the virus. Conversely, this might also account for the absence of increased erythrophagocytosis in the kidneys of ISAV-infected fish previously reported by Falk et al. (1995) as the virus strain used in that study was highly pathogenic, and could not be effectively cleared by the host.

In this study, the overall microscopic lesions observed in the heart were mild to moderate even at peak mortality. However, a recent report has shown evidence of cardiac dysfunction in ISAV infected fish from 15 to 16 dpi, even in the absence of histopathological lesions, which coincided with the onset of severe ISA clinical signs in infected fish (Gattuso et al., 2002). The appearance of ISH signals first in the heart from all the three groups in this study was unexpected, as the kidney and liver have previously been reported to be sites of early virus replication in infected fish (Dannevig et al., 1994; Speilberg et al., 1995; Rimstad et al., 1999). The observations in this study support other findings that used RT-PCR to show that the virus might be present and might replicate in the heart earlier than in other organs (Mikalsen et al., 2001). It was previously reported in Chapters 4 and 5 that the heart showed the most ISH signals when compared with other tissues in an experimental infection. The present study confirms those observations and

further shows that virus mRNA in the heart increases as evidenced by the intense ISH signals until peak viremia period followed by a decline. Moreover, the fact that the heart was the last organ to show ISH signals in surviving infected fish makes it an interesting organ to focus on as regards to pathogenesis and persistence of the virus. Although it cannot be hypothesized at this time as to the exact reason why the heart contains the most viral mRNA during early, peak and at the decline of the infection, it might be due to increased activity of the endocardial endothelial cells and the presence of underlining activated macrophages, which continuously trap and remove circulating virus.

The ISH signals were observed later in the kidney and liver, but as the infection progressed, both the intensity and frequency of ISH signals increased significantly, indicating increased replication of the virus in these organs. The data show that the ISH signals peaked in all three organs at the onset of mortality and declined in fish sampled after the peak mortality. This indicated that the virus had only a single peak viraemic period, and that fish that survived this period survive the infection. However, Mikalsen et al. (2001) reported a second peak viraemic period that resulted in 100% mortality within the same infected group. A bimodal peak of mortality is also thought by Mikalsen et al. (2001) to be due to a second round of infections from the high levels of virus shed by fish that were initially infected. This is more frequently seen with waterborne or cohabitation challenges. The difference in these two observations might be due either to virus isolates used (Mjaaland et al., 2002) or to differences in method of challenging the fish; cohabitation versus intraperitoneal inoculation with the virus. Another difference might be related to the techniques used to detect virus; studies detecting infectious virus (e.g., cultures) or viral genomic RNA (e.g., RT-PCR with only a sense primer in the initial

reaction) may not be directly comparable with the present study which measured virus mRNA expression. Moreover, although RT-PCR method used by Mikalsen et al. (2001) is very sensitive, quantifying the virus load without using real-time RT-PCR might be inaccurate (Munir and Kibenge, 2004). In the present study, the ISH technique allowed the detection of undiluted amounts of viral mRNA present in the tissue and there was no possibility of cross-contamination as might occur with RT-PCR.

Previous studies have provided an association between ISAV and the disease, but there has been no report on the relationship between level of virus replication, severity of lesions and mortality. Most studies have reported a relationship and/or association between decrease in the hematocrit value, onsets of anemia, liver lesions and mortality in infected fish (Evensen et al., 1991; Dannevig and Falk, 1993), but not with virus replication. In the present study, it was observed that both the frequency and intensity of ISH signals increased in tissues sampled from all infected groups as the infection progressed, and at the same time the lesions observed in these organs/tissues became more severe, indicating that as infection progressed, virus replication and reinfection of cells increased and leading to cellular degeneration and necrosis.

At peak ISH signals in most tissues, signals were observed in some individual blood cells suspected as erythrocytes in blood vessels indicating peak viremia. At this time most tissues showed severe histopathological lesions and the onset of mortality. This finding shows that there was a strong correlation between peak viremia, peak lesion development and onset of mortality in all three isolates.

This study demonstrated that most severe lesions were observed at peak viremia and suggests that the hepatocellular and renal tubular necrosis seen in ISA are secondary

to destruction of the endothelial cells by virus. No virus mRNA was detected in these parenchymatous cells at any stage of infection. The maximum ISH signals at which it could be comfortably assumed that mortality is inevitable could not be determined from this study. This is because the intensity and frequency of the ISH signals varied slightly amongst groups infected with RPC and NOR isolates and varied even more with the NSC isolate. Moreover, the ISH signals as an indicator of the amount of viral RNA in the tissues cannot be quantified numerically. However, it seems from the present study that the amount of virus replication in infected organ plays an important role in the pathogenesis of the disease.

In cell culture studies, Mjaaland et al. (2001) reported no clear correlation between replication properties in SHK-1 cells and the development of CPE, however, the isolates that replicated well showed CPE either early or later in the infection. It is believed that other factors also contribute to the pathology associated with ISAV in fish, as the development of disease is a complex process involving viral, host and environmental factors. This interpretation is supported by other studies that used ISH technique to show a significant correlation between the index of liver lesion and percentage of hepatocytes positive for the replicative-intermediate RNA of hepatitis C virus RNAs in infected tissues in man (Ming et al., 2000).

The higher mortality, coupled with higher intensity and more frequent ISH signals and lesions observed in the NOR (European genotype) infected group, followed by the RPC (North American genotype) infected group, compared with the NSC (European genotype) infected group (Fig.7.14) strongly support the hypothesis that the difference in the ISH signals and the virulence of the virus might depend on the particular isolate used

rather than on its hemagglutinin genotype or geographic region. Mjaaland et al. (2001) also reported difference in the rate of virus replication in the SHK-1 cell line and onset of CPE with different isolates of ISAV, even when the various isolates were closely related to each other at the nucleotide sequence level of the hemagglutinin gene.

In summary, this study demonstrates for the first time a direct relationship and/or association between virus replication (as in intensity and frequency of ISH signals) and severity of the pathology *in situ* in ISAV infected fish. There were strong correlations and associations between peak viremia, severe histopathological lesions, onset of mortality and peak mortality in ISAV infected fish. The findings in this study are consistent with those in the cell culture study in Chapter 6 and allow the conclusion that the virulence of the virus is based on the particular ISAV isolate and not on the hemagglutinin genotype or the geographic origin of the virus.

CHAPTER 8

VIREMIA IN ISA*

* **MONEKE EE,** IKEDE BO, KIBENGE FSB (2004) Viremia during Infectious salmon anemia virus infection of Atlantic salmon is associated with replicating virus in leucocytes. *Dis Aquat Organ* (submitted).

8.1 ABSTRACT

ISH was used to study the presence of viral mRNA in blood cells collected from ISAV infected fish at different times post infection. Fifteen Atlantic salmon were infected with ISAV NOR and blood samples collected from three fish and pooled on 6,8,10 and 12 dpi. Blood smears were made on clean glass slides, fixed and used for cytology and ISH. No hybridization signals were observed in blood cells collected at 6 dpi and in the erythrocytes collected throughout the sampling period. Hybridization signals were observed only in leucocytes collected from 8 - 12 dpi. This demonstrates that leucocytes are bonafide target cells of ISAV and also suggests 8 - 12 dpi to be within the peak viremic period for the ISAV isolate used. The presence of signals in the leucocytes at peak viremia suggests that the ISAV viremia in infected fish is most likely cell associated. The absence of hybridization signals in the erythrocytes indicates that they do not harbor the virus and therefore are not part of the viral target cells. Moreover, failure to demonstrate ISH signals in erythrocytes suggests that the increased erythrophagia in ISAV infected fish is unlikely a result of virus replication in fish erythrocytes.

8.2 INTRODUCTION

There have been conflicting reports on the blood types that harbor ISAV in infected fish, and to date there is no clear study demonstrating the specific blood cells harboring virus during viremia. Initial experimental studies of ISAV in fish used either tissue homogenates or blood cells collected from Atlantic salmon during a natural outbreak as inoculum to infect naive fish (Thorud and Djupvik, 1988; Christie et al., 1993; Hovland et al., 1994). Later, transmission electron microscopy was used to show that the virus targets and replicates

in endothelial cells and leucocytes in infected fish (Hovland et al., 1994). Using ISH, the presence of viral mRNA was shown in endothelial cells in the heart (Gregory, 2002; Moneke et al., 2003) but not in the circulating leucocytes of infected fish. However, Dannevig et al. (1994) reported that both the white blood cells (WBC) and red blood cells (RBC) from the head kidney of ISAV experimentally infected fish transmitted the virus to naive fish and caused disease, suggesting that the virus might replicate in all the different blood cells.

Recently, Cipriano and Miller (2003), reported that the development of anemia in ISA suggests that erythrocytes are among the target cells for the virus. The study in Chapter 7 using riboprobe targeting segment 7 showed hybridization signals in some circulating blood cells suspected as erythrocytes in blood vessels during peak lesions. Also, hybridization signals were observed in some individual cells in the head kidney suspected to be hematopoietic cells. The virus has been shown to agglutinate red blood cells of different fish species (Falk et al., 1997; Sommer and Mennen, 1997) suggesting that the virus might attach and replicate in the cell. Due to this confusing data, it was believed necessary to further characterize the particular blood cell(s) involved in the replication and spread of the virus in infected fish during viremia. The goal of this study was therefore to identify by ISH the blood cell (s) in the blood vessels that harbor the virus before or during viremia.

8.3 MATERIALS AND METHODS

8.3.1 Cells and viruses

The NOR isolate used was prepared as described in Chapter 2, section 2.1.

8.3.2 Riboprobe synthesis

The segment 7 antisense riboprobe used was synthesized as described in Chapter 2, section 2.4.

8.3.3 Fish experiment

A total of 23 Atlantic salmon were obtained from same source as those used in Chapter 7, and housed as described in Chapter 2, section 2.5.1. Seventeen fish were anesthetized as described in Chapter 2, section 2.5.2, and kept in one experimental tank. Each fish was challenged by intraperitoneal injection of 0.2 ml of the virus at a virus dose of10^6 $TCID_{50}$ and returned to the challenge tank. Six uninfected fish were kept in the control tank and used as negative control. All fish were observed twice daily for mortalities. Blood samples were collected in heparinized syringes from three infected fish and pooled on 6, 8, 10, and 12 dpi, and from three uninfected controls on 6 and 12 dpi, before sacrifice. Blood smears were made immediately on clean glass slides and allowed to dry before fixing in either 4% paraformaldehyde for 30 min or methanol for 1 min. Part of the blood was transferred into 1.5 ml heparinized microfuge tubes and centrifuged at 10,000 rpm. The other part of the blood was kept at 4°C to allow the red blood cells to sediment to the bottom of the tube. The different layers of the hematocrit (packed erythrocytes), buffy coat (leucocytes and platelets), plasma were carefully collected and used to make smears on clean glass slides. In some cases there was a mixture of the two adjoining layers in the sample used for smears. The slides were fixed in 4% paraformaldehyde for 30 min and stored at -80°C until used for ISH. The slides fixed in methanol were stained with Giemsa stain in order to identify the blood cell type.

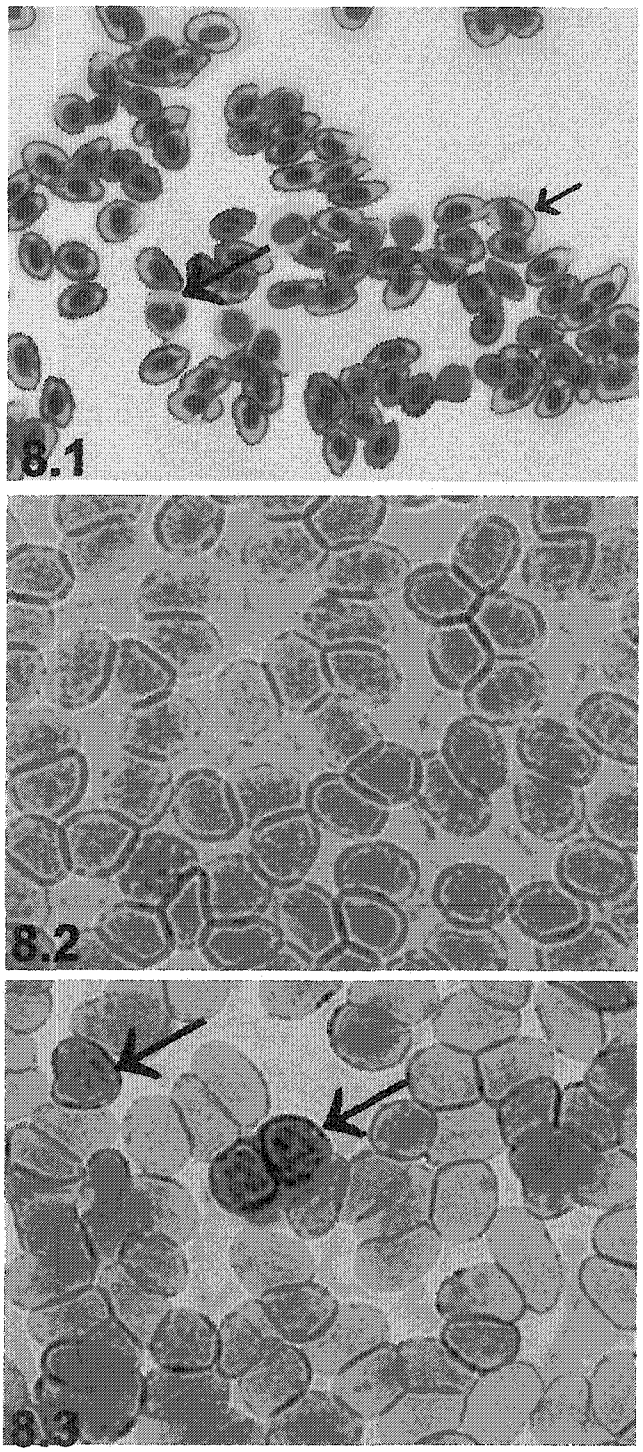
8.3.4 ISH on blood smears

ISH was performed on the blood smears fixed in 4% paraformaldehyde using segment 7 riboprobe as described in Chapter 2, section 2.6. The hybridization signals were observed under the microscope and images captured digitally. Digital images were enhanced and analyzed using Adobe Photoshop™.

8.4 RESULTS

8.4.1 Identification of blood cells that carry ISAV during viremia

The intent of this study was to identify the particular blood cells that carry ISAV during viremia. The NOR isolate was used because it is highly virulent to Atlantic salmon. No ISH signals were observed in the red blood cells (Figs 8.1 and 8.2) or in cell-free plasma at any time during the ISAV infection studied. ISH signals were also not observed in blood cells collected at 6 dpi (Fig 8.2). However, at 8, 10 and 12 dpi signals were observed in leucocytes in a smear of the buffy coat layers (Fig 8.3). No hybridization signals were observed in any of the blood cells from control fish.



Figs. 8.1- 8.3 Blood, Atlantic salmon. Fig. 8.1 8 dpi with ISAV isolate NOR. Giemsa stain showing nucleated red blood cells (small arrow) and leucocytes (large arrow). Fig. 8.2 6 dpi with ISAV isolate NOR. No hybridization signal was observed in any blood cells. Fig. 8.3 8 dpi with ISAV isolate NOR. Presence of hybridization signals in the leucocytes (large arrows) and no signal in the surrounding erythrocytes.

8.5 DISCUSSION

This study has identified the blood cells involved in the spread of ISAV during viremia in experimentally infected fish. For a virus to produce systemic disease it must be transported from the site of entry into the host to the target tissues (Tyler and Fields, 1996). The blood stream and nerves are two major pathways for virus spread. ISAV has been shown to be transmitted outside the host via blood from infected fish (Dannevig et al., 1994), and also to spread in the host via the blood (Hovland et al., 1994), producing systemic disease (Evensen et al., 1991). The initial inoculation of virus into the host might result in passive viremia as a result of direct spread of the viral inoculum due to limited replication in the primary site (primary viremia), before spreading to the target organs where more sustained replication and higher viral titers lead to secondary viremia (Tyler and Fields, 1996). Milkasen et al. (2001) reported that viremic spread of ISAV occurred rapidly after infection, but could not distinguish between passive transport of virus and active replicating virus, followed by a decrease in viral load. No study has been undertaken to determine the cells involved in the primary viremia of ISAV.

Viremia can be cell-associated as observed in neutrophil-associated influenza virus infection or cell free in the plasma as observed with togaviruses (Tyler and Fields, 1996).In this study hybridization signals were observed in the circulating leucocytes from 8 to 12 dpi in fish. The presence of signals in the leucocytes confirms reports by others (Hovland et al., 1994; Koren and Nylund, 1997) which showed that the virus replicates in leucocytes and that these cells are amongst the target cells for the virus. The presence of hybridization signals from 8 dpi coincided with the period of peak lesions in fish infected with this isolate as reported in Chapter 7, and might represent the peak viremia period. The

presence of signals also suggests that the viremia in ISAV infected fish is cell-associated. We cannot rule-out the possibility of cell-free viremia in these fish since ISH detected only viral mRNA.

The absence of hybridization signals in the leucocytes of fish collected at 6 dpi, might be as a result of low copy numbers of the virus present in the blood cells at that time or increased clearance of infected leucocytes from the blood. The magnitude of viremia in a virus infection may vary as a result of dynamic interrelationships between the amount of virus entering the blood compartment and the efficiency with which it is removed (Tyler and Fields, 1996).

In this study, absence of hybridization signals in the red blood cells would suggest that the virus does not replicate in erythrocytes. Previous reports indicated that red blood cells might harbor the virus and help in the spread of ISAV in infected fish (Dannevig et al., 1994). The increased erythrophagia observed in the spleen and kidney macrophages early in the infection, and the transmission of virus to naive fish inoculated with erythrocytes from moribund ISA fish (Christie et al., 1991; Dannevig et al., 1994; Falk et al., 1995), coupled with the earlier observation of hybridization signals in blood cells suspected as erythrocytes in Chapter 7, lead to suggestions that ISAV might be present and indeed replicate in red blood cells. Though fish and mammalian erythrocytes have general similarities, fish erythrocytes are nucleated and are of variable shapes depending on the fish species (Satchell,1991; Fange, 1997). Some authors have suggested that mature fish erythrocytes, as in mammalian erythrocytes may lack functional cytoplasmic organelles (Savage, 1983; Zuasti and Ferrer, 1989; Esteban et al., 2000) and therefore might not allow for virus replication. The absence of ISH signals in erythrocytes in the present study supports the

data of others (Dannevig and Falk, 1993) which showed that *in vitro* inoculation of salmon erythrocytes or reticulocytes with infective ISA material did not result in ISA-infective cells as judged by transmission trials. The data obtained in the present study also suggests that the increased erythrophagia observed early in the spleen and kidney of ISAV infected fish is not a result of virus replication in the erythrocytes.

In conclusion, this study confirms that leucocytes are among the target cells for ISAV. The viremia observed in ISAV-infected fish was cell-associated but did not involve erythrocytes.

9 GENERAL DISCUSSION

Commercial aquaculture is one of the fastest growing industries in countries with appropriate coastal areas. In the Northern Hemisphere, marine salmonid farming is the fastest industry and in Canada, Atlantic salmon production is the highest (Couturier, 2003). ISA is a highly fatal disease that is now a major threat not only to Atlantic salmon farming but to other farmed salmonids world wide. There is still a lack of information on the biology of this novel virus, its pathogenesis in Atlantic salmon, and why the virus causes clinical infection in certain fish species but only propagates in others. A better understanding of the viral-host interaction will help in the overall management of the disease.

The purpose of this thesis was to study the gene expression of the virus in the host as a way to better understand viral pathogenesis and persistence in Atlantic salmon and other salmonids. To achieve this, an ISH method was developed that allowed for the simultaneous study of viral gene expression and their impact on organ morphology and function in the host. Much of what was previously known on the etiopathogenesis of ISAV was based on *in vitro* studies. The development of a RT-PCR method (Mjalaand et al., 1997) increased knowledge on the viral genome organization and encoded proteins, however, it did not give any specific information about the cells infected.

ISH has been used to study the pathogenesis of other viruses like IPNV in fish (Alonso et al., 2004) and hepatopancreatic parvovirus (HPV) in penaeid shrimp (Pantoja and Lightner, 2001). This is the first study of the pathogenesis of ISAV, using ISH on different fish cell lines and fish species.

The genomic RNA of ISAV is composed of 8 segments that encode different proteins as described in Chapter 1, section 1.2.2.3. The functional products of all the ISAV

genes have not been fully characterized. Riboprobes from segments 6, 7 and 8 viral RNA were chosen to reflect the different stages of viral gene expression during virus replication based on what is known of other orthomyxoviruses (Lamb and Krug, 1996). The segment 6 of ISAV encodes the hemagglutinin protein (Krossøy et al., 2001; Rimstad et al., 2001), an important antigenic determinant that plays a major role in ISAV pathogenesis and pathogenicity (Krossøy et al., 1997; Eliassen et al., 2000; Kibenge et al., 2001b; Griffiths et al., 2001; Devold et al., 2002; Mjalaand et al., 2002; Nylund et al., 2003). Recently, this protein was shown to also have esterase activity (Falk et al., 2004) leading the authors to designate it the hemagglutinin-estrase (HE) protein. The coding assignments of segments 7 and 8 are still controversial (Ritchie et al., 2001a; Clouthier et al., 2002; Biering et al., 2002; Kibenge et al., 2004) and their inclusion as riboprobes contributed additional information about their expression in f ish cells. Reports in other orthomyxoviruses showed a clear difference in the amount and time of expression of the genes of the two segments during replication. The putative membrane (M1) and nonstructural (NS) proteins they encode play an important role in the early pathogenesis of orthomyxovirus infection (Lamb and Krug, 1996) and was expected to also be the case for ISAV.

The time, intensity, and frequency of the gene expression of segments 6, 7 and 8 mRNA of ISAV in CHSE-214, SHK-1 and TO cell lines were compared in Chapter 3. A temporal pattern of expression of the three segments was detected in infected CHSE-214 cells, in which segment 8 was weakly expressed first at 24 hpi followed later by more intense expression of segments 6 and 7 at 48 hpi. This observation seemed to agree with the differential expression of the different viral segments as in other orthomyxoviruses (Lamb and Krug, 1996). However, the CHSE-214 cell line was developed from Chinook

salmon embryo (Fryer et al., 1965), a species not considered to be a natural host for the virus, and might not represent the true gene expression of the virus in target cells.

The observation of signals beginning at 24 hpi when there would have been multiple replications could affect the interpretation of these results, since 4 hr has been suggested as the required time for a single replication cycle in other orthomyxoviruses (Lamb and Krug, 1996; Ludwig et al., 1999; Eliassen et al., 2000). There was, however, no difference in the onset of signals with the three riboprobes in TO and SHK-1 cells. The SHK-1 and TO cell lines are from Atlantic salmon leucocytes, the target of the virus (Falk and Dannevig, 1995; Koppang et al., 1999; Wergeland and Jakobsen, 2001). The absence of difference in the temporal expression of the different segments might be due to increased susceptibility and faster replication of the virus in target cells. The pathogenesis of most viruses is better studied in either non-target host or cells. Viral pathogenesis can also be studied by use of variant strains that allow for slower replication/expression of the viral genes (reviewed in Tyler and Fields, 1996). This therefore makes CHSE-214 cells a better cell line for the study of ISAV pathogenesis. The major draw back in its use however, is that most ISAV isolates especially of European hemagglutinin genotype do not replicate in the cell line (Kibenge et al., 2000a). Recently, Munir and Kibenge, (2004) used real time RT-PCR to show that ISAV isolates could further be classified into three CHSE-214 phenotypes based on their ability to replicate and cause CPE as follows: replicating cytopathic, replicating non-cytopathic and non-replicating CHSE-214 phenotypes of ISAV. Other reports have also shown that not all ISAV isolates replicate in TO and SHK-1 cells indicating that available cell lines might not be permissive to all isolates (Kibenge et al., 2001b; Mjaaland et al., 2002).

The ISH signal intensity from segments 6 and 7 riboprobes increased as the infection progressed suggesting that either their mRNA accumulates or are increasingly synthesized. The increased synthesis of the segments might be happening in ISAV, as previously reported in influenza virus (reviewed in Lamb and Krug, 1996). The presence of most intense signals with the segment 7 riboprobe is interesting since whereas in influenza virus segment 7 encodes the matrix protein (M1), the most abundant viral structural protein (Lamb and Krug, 1996), in ISAV, the matrix protein is encoded in segment 8 (Biering et al., 2002; Falk et al., 2004). The results in Chapter 6 also suggest that segment 7 may encode for an abundant protein as it appeared to show more intense signals than the segment 8 at any time in infected TO cells. However, it may not necessarily follow that the viral mRNA transcripts are all translated into protein.

The absence of ISH signals until 24 hpi with the three riboprobes in infected cell lines showed the difficulty in determining single replication or early gene expression pattern of the virus using ISH. The threshold for normal routine ISH detection is 10-20 copies of target nucleic acid sequence per cell (Nuovo et al., 1994; Speel et al., 1995), making it less sensitive than gene amplification methods like RT-PCR. However, with coupling of the amplification to the hybridization detection procedure, it is possible to increase the sensitivity of signals to that conventional for RT-PCR (Femino et al., 1998), making it a better tool than RT-PCR (Kim, 2003).

Although, the ISH technique is used routinely in some laboratories (Teo, 1990) for optimum hybridization and to avoid non-specific hybridization, several steps in the procedure need to be determined empirically. ISH has been used to study the pathogenesis of other fish diseases (Sanchez-Martinez, 2000; Gregory, 2002; Alonso et

al., 2004), however these studies used dsDNA probes for hybridization. In this study ISH conditions with riboprobes for detection of ISAV in fish tissues were optimized. At optimal permeabilization similar intensity of hybridization signals were detected in tissues fixed in either 10% formalin or 4% paraformaldehyde. Some authors have suggested that fixing tissues optimally plays a major role in the probe penetration, while permeabilization does not have much effect (Lawrence and Singer, 1985). Four percent paraformaldehyde has been recommended as the best fixative for ISH because it does not cross-link proteins as extensively as other formaldehdyde fixatives (Brigati et al., 1983; Höfler et al., 1986) therefore allowing better penetration of the riboprobe. It is therefore concluded that optimum fixation and permeabilization are important for the detection of target ISAV mRNA.

The presence of hybridization signals in endothelial cells and leucocytes and not in the epithelial cells in all organs sampled in Chapter 4, confirmed the reports of (Hovland et al., 1994; Nylund et al., 1995b; Gregory, 2002) that the endothelial cells and leucocytes are the primary target cells of the virus. However unlike Gregory (2002), the present study also detected hybridization signals in the endothelial cells of gills and blood cells of infected fish.

Because differences have been reported in the lesions observed in ISAV-infected fish (Byrne et al., 1998; Jones et al., 1999; Mjalaand et al., 2002), it is essential to understand the role of the virus in the lesions. This was addressed in Chapters 5 and 7, in which histology and ISH were used to study the correlation between the viral replication and progression of lesions in clinical ISA. Signals were present only in endothelial cells and leucocytes, suggesting a direct relationship between virus replication and degenerate

leucocytes and endothelial cells. On the other hand, no signals were detected in parenchymal cells of the gills, liver, kidney, gills, pyloric caeca and heart suggesting that the degeneration and necrosis in these organs are probably secondary to endothelial damage and hypoxia, as previously reported (Evensen et al., 1991). Viral mRNA was detected as early as 3 dpi in endothelial cells and before the onset of microscopic lesions. Evensen et al. (1991) suggested that thrombi formation in small venules of ISAV infected fish might be due to early damage of the sinusoidal endothelial lining not recognizable by light microscopy. The severe destruction of endothelial cells at the onset of mortality in the liver coupled with intravascular thrombi could explain pooling of blood in the organ.

Ascites is a prominent feature of ISA but its pathogenesis is not clear. Evensen et al. (1991) suggested that only changes in hepatic portal pressures contributed to the development of ascites, as they could not observe any lesion in the myocardium of infected fish. In this study, myocardial lesions were also not observed, but there were moderate to severe endothelial lesions in the heart, suggesting that there could be hypoxic damage in the heart, as in other organs. Recently, a significant impairment in the cardiac function was reported 15 dpi and the authors suggested that the mechanical performance of the heart could deteriorate even in the absence of microscopic lesions (Gattuso et al., 2002).

Previous reports have suggested that the kidney is the most important organ for the detection of the virus (Rimstad et al., 1999; Optiz et al., 2000; Merrill, 2003), also the 2000 edition of OIE report indicated the kidney as the preferred organ for diagnosis of ISA. However, the presence of more ISH signals in the heart (Chapters 4, 5 and 7) supports other reports (Hovland et al., 1994; Falk and Dannevig, 1995; Mikalsen et al., 2001; Gregory, 2002) that suggest the heart as the better organ (i.e., compared to kidney) to

sample for detection of ISAV. The low frequency of ISH signals in the kidney in this study could be attributed to the melano-macrophages that obscure ISH signals (Chapter 4). The paucity of lesions in the kidney can not be explained, however it might be related to the virus isolates used.

The differences in the ISH signals in fish infected with isolates of different hemagglutinin genotypes, might be based on virus genotypes. ISAV isolates have been grouped into North American and European hemagglutinin genotypes (Kibenge et al., 2000a; 2001b; Krossøy et al., 2001a,b; Devold et al., 2002; Mjaaland et al., 2003). The weak and low frequency of signals observed with NSC isolate initially suggested that the European hemagglutinin genotype might be replicating at slower rates than the North American hemagglutinin genotype even when they cause similar lesions. With the TO cell line, the NSC isolate replicated slower than the NOR isolate (of same European genotype) and the RPC isolate (of North American genotype). However, NOR and RPC isolates showed similar CPE and signals intensity/frequency. These observations indicated that the differences in ISH signals and onset of CPE were not dependent on the virus genotype but the virus isolate. This is the first report using ISH to show differences in the replication of different virus isolates and it agrees with other reports (Kibenge et al., 2000a; Mjalaand et al., 2002; Munir and Kibenge, 2004) based on CPE.

It was observed in the present study that the rate of virus replication in the host was related to the onset of microscopic lesions and mortality. Thus the NSC group had (i) the least cumulative mortality, (ii) the longest mortality period, (iii) the least lesions, and (iv) the least frequency and intensity of signals than other isolates. It can therefore be concluded that the lesions in infected fish depend in part on the rate of virus replication *in*

vivo, in addition to the route and duration of infection (Jones et al., 1999).

Initial studies on the pathogenesis of ISAV in fish focused mainly on mortalities, with little information on the relationship between virus replication and the onset of microscopic lesions. In the present study, ISH signals were observed in the heart endothelial cells and underlying macrophage first at 3, 6 and 10 dpi, before the onset of microscopic lesions in the NOR, RPC and NSC infected fish, respectively, confirming the report by Mikalsen et al. (2001). Subsequently, signals were observed in endothelial cells of other organs, which became more intense and frequent as the infections progressed. As the signal intensity and frequency increased, there was also an increase in the light microscopic lesions in the different organs. There was a strong correlation between intensity and frequency ISH signals, maximum lesions in the organs, and onset of mortality from the three isolates. It is probable that there is a threshold level of virus mRNA in cells before light microscopic lesions and mortality can ensue.

The observation of ISH signals in suspected red blood cells in Chapter 7 increased the suspicion of their involvement in the virus spread and within the infected host as target cells for the virus. There has not been any study to resolve this speculation in ISAV pathogenesis. In Chapter 8, it was shown that ISH signals were present only in circulating leucocytes during peak viremia. No signals were observed in red blood cells, suggesting that the leucocyte is the blood cell associated with ISAV viremia. This finding supports the report that identified the virus by ultramicroscopy only in leucocytes (Hovland et al., 1994). It is still not known whether ISAV is also spread by cell-free viremia in plasma.

The increased erythrophagia observed in the sinusoidal macrophage and melano-macrophage centers in the spleen and kidney early in ISAV infection previously suggested

the involvement of red blood cells in ISAV replication. Both organs are rich in melano-macrophage centers that are within the reticulo-endothelial supporting matrix of hemopoietic tissue (Agius and Roberts, 2003). In this study, significantly increased erythrophagia was present in the kidney of fish from the group infected with the low virulent (NSC) isolate when compared with the more virulent (RPC and NOR) isolates. A previous report suggested that the increased erythrophagocytosis in the spleen early in ISAV infection might be the host response in controlling the infection (Falk et al., 1995). The mechanism by which erythrophagocytosis occurs in ISA is still not known and its role in the pathogenesis of the disease is not clearly known. However, the previous suspicion of virus replication in the erythrocytes leading to their degeneration and subsequent phagocytosis is not supported by this study. Rimstad et al. (1999), suggested that the early increased erythrophagia in the spleen might be due to agglutination of ISAV bound to circulating erythrocytes since *in vitro,* bound virus does not elute spontaneously (Falk et al., 1997; Hellebø et al., 2004). Affected erythrocytes would then be identified as foreign and cleared from circulation by macrophages. However, *in vivo* hemagglutination of erythrocytes has not been observed with other orthomyxoviruses (Murphy and Webster, 1996). More work is needed to understand the significance of erythrophagocytosis in the pathogenesis of ISA.

Atlantic salmon that recover from clinical ISA and other asymptomatically infected salmonids shed the virus continuously for long periods of time (Totland et al., 1996). The carrier fish transmit the virus to naive Atlantic salmon on cohabitation, suggesting that the virus persists. However, the isolation of the virus in these carrier fish is difficult and virus detection requires the use of RT-PCR (Devold et al., 2000). The mechanisms and the site

of virus persistence is not yet known. Attempts were therefore made in this study to identify tissues that might harbor the virus in Atlantic salmon, rainbow trout and coho salmon that have recovered from experimental infection. There was no evidence of lesions associated with deposition of virus-antibody and viral antigen-antibody complexes in various organs that could contribute to viral persistence in survived fish. No ISH signals were observed in any tissues from the three fish species infected with NSC, RPC and CH7 isolates up to 6 weeks after the last mortality. However, RT-PCR products were amplified with segment 8 specific primers from pooled tissues of all fish species up to 6 weeks pnm. The absence of ISH signals in the tissues and the detection of viral genetic material only by RT-PCR further confirms that the viral RNA might not be abundant as the virus might be replicating at very low levels (Devold et al., 2000). The amplification of ISAV genetic material by real time RT-PCR in individual tissues from NSC infected Atlantic salmon and rainbow trout at 6 weeks pnm (Munir and Kibenge, 2004) suggests the involvement of endothelial cells in the persistence of ISAV. The last organ to show signals was the heart suggesting that this organ might play a role in ISAV persistence.

In conclusion, this study looked at the gene expression of ISAV in infected cells as a way to better understand viral pathogenesis and associated lesions in ISA. The use of ISH technique allowed the detection of viral RNA within the context of the effects of their replication in host cells. The target of segment 7 riboprobe is expressed the most in infected cells, followed by that of segment 6 and lastly, by that of segment 8. There was a strong correlation between virus replication and light microscopic lesions in Atlantic salmon. The heart harbored the most amount of replicating virus at any time in the infection and therefore represents the best organ to sample in ISAV surveillance and diagnosis. In

endothelial cells, replicating virus caused degeneration and necrosis which most likely led to hypoxic necrosis of the parenchymal cells. This study also revealed that the virulence and pathogenicity of ISAV isolates is dependent on the particular virus isolate and not the hemagglutinin genotype of the virus. The absence of ISH signals in erythrocytes indicated that they were not target cells of the virus and that viremia in ISA is leucocyte cell-associated. ISAV persists in different fish species, however the exact mechanism and sites of viral persistence remain unknown. From work in this thesis and elsewhere, there is however strong indication of the involvement of the endothelial cells and heart in ISAV persistence.

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