PATHOGENESIS OF INFECTIOUS SALMON ANAEMIA VIRUS (ISAV) IN RAINBOW TROUT (*ONCORHYNCHUS MYKISS*)

A Thesis

Submitted to the Graduate Faculty

in Partial Fulfillment of the Requirements

for the Degree of

Master of Science

in the Department of Pathology and Microbiology

Faculty of Veterinary Medicine

University of Prince Edward Island

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Charlottetown, P. E. I.

2009

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ABSTRACT

The first objective of this thesis was to detail the pathology of ISAV-induced lesions in rainbow trout (*Oncorhynchus mykiss*) as compared to Atlantic salmon (*Salmo salar*) and to quantify the relative susceptibility of rainbow trout to clinical ISA disease. The virus infection in the two species demonstrated different mortality patterns and pathology characteristics. Atlantic salmon showed a typical acute mortality pattern peaking at 8-16 days post-infection (dpi) depending on virus dose, whereas in rainbow trout, only the highest virus dose ($10^{7.13-7.8}$ $TCID_{50}$/200µl) showed a similar pattern. The middle ($10^{4.13}$ $TCID_{50}$/200µl) and lowest virus doses ($10^{2.13}$ $TCID_{50}$/200µl) in rainbow trout induced only sporadic protracted mortality, lasting up to 46 dpi. Infected rainbow trout that were live-sampled and those that died, demonstrated increased erythrophagia, clusters of cellular degeneration in the haematopoietic portion of the kidney, and occasionally myocarditis. These lesions are very different from the typical necrosis in liver and kidney that occur in infected Atlantic salmon, and were indicative of an antiviral response by a host with some immunity to the ISAV infection.

The second objective was to assess the influence of cell line in the propagation of the viral inoculum. The pilot work had virus grown in the TO cell line, derived from Atlantic salmon head kidneys. It was hypothesized that propagating inoculum in a cell line derived from an *Oncorhynchus sp.* might increase viral pathogenicity in rainbow trout. Survival analysis failed to demonstrate a cell line influence on viral inoculum virulence.

The third objective of this research was to use an *in situ* hybridization protocol which had been previously optimized for ISAV-infected Atlantic salmon (Moneke *et al.*, 2003) and apply it to post-challenge rainbow trout tissues to determine the viral target cell and tissue distribution in this more resistant species. This work indicated a comparable tropism in both species, with endothelial cells, subendothelial macrophages and leucocytes being the predominant host cells.

The final objective was to use real time RT-PCR on the tissues of rainbow trout survivors of ISAV experimental infection to assess potential viral persistence and contribute to increasing knowledge of the pathogenesis of ISAV. Samples taken from rainbow trout 63 day after experimental infection with ISAV were found positive by this method, indicating the potential for a prolonged carrier state in rainbow trout.

ACKNOWLEDGEMENTS

I would like to acknowledge the contributions of my co-supervisors Dr. Gerald Johnson and Dr. Fred Kibenge. It was a privilege to work with these gentlemen. Their enthusiasm for research is unflagging and their patience toward the end was greatly appreciated. Further, I would like to acknowledge the financial support of an NSERC Strategic Grant awarded to Dr. Kibenge.

I would like to thank my Committee members Dr. David Groman, Dr. John Lumsden, Dr. Herman Barkema and Dr. Basil Ikede for their invaluable direction and constructive advice. This thesis is stronger because of their participation.

I would also like to offer thanks to Kathy Jones, Patty McKenna and Dr. Molly Kibenge, for technical support and friendship. Each could answer any question I asked and make me laugh while doing so. I might have gotten through this without them, but I wouldn't have enjoyed myself half as much. I must also thank Dr. Henrik Stryhn for his assistance with the survival analysis and Shelley Ebbett for her assistance with composite figures in this thesis and the DAO manuscript. I would also like to acknowledge Wayne Petley and the staff of AVC Aquatic Animal Resource Department for their excellent care of the research fish and facilities.

Finally, much of this work was done on the shoulders of my fellow graduate students, so I would like to acknowledge a debt of gratitude to Drs. Tomy Joseph, Emeke Moneke and Khalid Munir.

I wish to dedicate this thesis to my parents. I will never be able to thank them enough for their support and encouragement.

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1. GENERAL INTRODUCTION AND LITERATURE REVIEW

1.1. General introduction to infectious salmon anaemia virus

Infectious Salmon Anaemia (ISA), an economically important disease of marine farmed Atlantic salmon (*Salmo salar*), was first described in juvenile marine farmed Atlantic salmon in Norway in 1984 (Thorud & Djupvik, 1988). The disease is characterized by severe anaemia and a slowly increasing mortality rate culminating in high cumulative mortalities (Hammell & Dohoo, 2005a). Clinical signs of ISA include pale gills and hearts as a result of anaemia, exophthalmia, ascites and enlarged and congested liver and spleen. Histologically, haemorrhagic liver necrosis is considered pathognomonic (Thorud & Djupvik, 1988; Evensen & Thorud, 1991;Gattuso *et al.*, 2002). Other lesions may include renal haemorrhage and tubular necrosis (Simko *et al.*, 2000), filamental sinus congestion of the gill (Jones *et al.*, 1999), splenic congestion with concomitant erythrophagocytosis and congestion of the lamina propria of the foregut (Evensen & Thorud, 1991; Jones *et al.*, 1999).

Atlantic salmon are the only species of fish known to develop the disease following natural infection, however, wild fish surveys have detected asymptomatic infections of ISAV in feral salmonid species. It has been suggested that selective pressures related to intensive aquaculture operations may have influenced the evolution of isolate pathogenicity from resident nonpathogenic benign wild type ISAV strains (Rolland & Nylund, 1998; Plarre *et al.*, 2005).

Experimental challenges using ISAV have determined that the virus may survive and replicate in sea trout and brown trout (*Salmo trutta*), and rainbow trout (*Oncorhynchus mykiss* Walbaum) (Nylund *et al.*, 1994; Nylund & Jakobsen, 1995;Nylund *et al.*, 1997; Rolland & Nylund, 1998; Snow *et al.*, 2001). A Pacific Northwest laboratory performed a risk assessment of experimental ISAV infection of Pacific salmon species (Rolland & Winton, 2003). ISAV isolation was possible in randomly sampled chum (*O.*

keta), coho (*O. kisutch*) and steelhead trout (*O. mykiss*) at roughly 2 weeks post experimental infection at lower viral titrations than seen from the Atlantic salmon cohort given the same experimental infection protocol. No virus was isolated in the Pacific salmon species sampled after this time, and neither clinical signs nor mortalities occurred within the Pacific salmon species indicating disease resistance or viral clearance. Additionally, virus isolation was not possible from Chinook salmon (*O. tshawytscha*) at any point of the trial. These findings lead to the presumption that infection and replication of ISAV may take place in salmonids other than Atlantic salmon, however, the virus is insufficiently virulent in these species to cause clinical signs.

After contradictory results were seen in an injection trial using ISAV strains considered highly pathogenic by cell culture and previous challenge trial, it was noted that two ISAV isolates, NBISA01 and 810/9/99, were able to cause lethal infections in juvenile rainbow trout with clinical signs consistent with those expected in Atlantic salmon and subclinical infection of coho salmon (Kibenge *et al.*, 2004; Kibenge *et al.*, 2006). Identifying an ISAV isolate capable of infecting and causing disease in a species formerly considered resistant to the virus has enabled new directions of study to further characterize the pathogenesis of this important virus. Elucidating the disease expression in the resistant species may provide valuable insights into viral-host interaction which could then be exploited to help improve the antiviral immune response of the susceptible species.

The first research objective of this thesis was to compare the susceptibility of rainbow trout and Atlantic salmon to a highly pathogenic strain of ISAV and to perform a detailed comparison of the ISAV-induced lesions between the two species. The second objective was to assess the influence of cell line in the propagation of the viral inoculum. The pilot work demonstrating fatal infection of rainbow trout used ISAV grown in the TO cell line, derived from Atlantic salmon head kidneys. It was hypothesized that

propagating inoculum in a cell line derived from an *Oncorhynchus sp.* might increase viral pathogenicity in the more closely related rainbow trout. The third objective of this research was to use an *in situ* hybridization protocol which had been previously optimized for ISAV-infected Atlantic salmon (Moneke *et al.*, 2003), and apply it to post-challenge rainbow trout tissues to determine the viral target cell and tissue distribution in this more resistant species. The final objective was to use real time RT-PCR, a method of low-copy nucleic acid detection, on the tissues of the rainbow trout survivors to assess potential viral persistence, which has been demonstrated in Atlantic salmon and is a feature which has not been ascribed to an other member of the Orthomyxoviridae family.

1.2. Epidemiology of ISAV

1.2.1. History and Geographic distribution

Infectious Salmon Anaemia (ISA) Virus (ISAV), a teleost orthomyxovirus, is a commercially important viral pathogen of marine farmed Atlantic salmon (*Salmo salar*). First described in juvenile marine farmed Atlantic salmon in Norway in 1984 (Thorud & Djupvik, 1988), ISA has been diagnosed in New Brunswick, Canada in 1996, although evidence suggests that the disease may have been present in this area as early as 1995 (Byrne *et al.*, 1998). In 1998, it was identified in adult marine farmed Atlantic salmon in Scotland (Rodger *et al.*, 1998), in Nova Scotia, Canada in 2000 (Ritchie *et al.*, 2001a), in Maine, USA in 2001(Bouchard *et al.*, 2001), in the Faeroe Islands (Anonymous, 2000), and Ireland (Siggins, 2002). ISAV was first detected outside the North Atlantic Ocean in coho salmon, *Oncorhynchus kisutch*, in Chile in 2001. The coho salmon were diagnosed with Jaundice Syndrome, and the role of ISAV, either causative or incidental with regards to the clinical illness, was undetermined (Kibenge *et al.*, 2001a). In 2007, an outbreak of ISA occurred in Chilean farmed Atlantic salmon in 2007 (Godoy *et al.*, 2008) representing the first clinical case in the Southern hemisphere or the Pacific Ocean.

1.2.2. Host range

While Atlantic salmon is the only fish species known to develop the disease in a culture situation, experimental infections have demonstrated the ISAV may survive and replicate in brown trout (*Salmo trutta*), rainbow trout (*O. mykiss*) and Arctic char (*Salvelinus alpinus*) (Nylund *et al.*, 1994; Nylund & Jakobsen, 1995; Nylund *et al.*, 1997; Snow *et al.*, 2001). These species failed to exhibit clinical signs of disease, however a transient decrease in haematocrit was detected in each species. Brown trout may remain persistently infected, with detection of ISAV by reverse transcription – polymerase chain reaction (RT-PCR) seven months following experimental infection (Nylund *et al.*, 1994; Nylund & Jakobsen, 1995). In addition to viral persistence, experimentally infected brown trout have been shown to transmit the disease to naïve Atlantic salmon during cohabitation (Rolland & Nylund, 1998). Nonclinical ISAV infection has been detected by molecular methods in low numbers of wild salmonids (Atlantic salmon adults and parr, brown trout) during a wild fish survey in Scotland in 1999 and from apparently healthy seawater reared rainbow trout (*O. mykiss*) in Ireland (Raynard *et al.*, 2001; Siggins, 2002). These findings led to increased speculation that wild salmonids may act as a reservoir for ISAV in nature (Raynard *et al.*, 2001). ISAV has also been isolated from moribund coho salmon (*O. kisutch*) in Chile diagnosed with Icteric Syndrome (Kibenge *et al.*, 2001a). The virus did not produce cytopathic effects (CPE) in inoculated cell lines, however the supernatant was positive by RT-PCR for several ISAV gene segments, and transmission electron microscopy (TEM) demonstrated viral particles in the heart which were morphologically consistent with those of ISAV in other studies (Kibenge *et al.*, 2001a). Sequenced segments indicated homology with Canadian isolates (Kibenge *et al.*, 2001a). While the original detection of ISAV in Chile seemed to be an incidental finding of a nonpathogenic strain, recent ISA outbreaks have

been diagnosed in Chilean Atlantic salmon farms (Godoy *et al.*, 2008). Unlike the previous isolate, analysis of the highly polymorphic region (HPR) of the haemagglutinin gene of this isolate indicates it belongs to the European genotype and supports the argument that ISAV could be vertically or transgenerationally transmitted (Nylund *et al.*, 2007;Vike *et al.*, 2008).

It has been proposed that various wild salmonids may serve as ISAV carriers for an unknown duration of time following exposure to ISAV and serve as possible reservoirs of infection for Atlantic salmon in sea cages (Nylund *et al.*, 2003; Plarre *et al.*, 2005). Conversely, farmed Atlantic salmon sea cages may represent an ISAV transmission risk to native salmonid stocks. Initial studies on the potential risks to the health other salmonid species indicated that ISAV infection only results in subclinical infections (Nylund *et al.*, 1994; Nylund & Jakobsen, 1995; Nylund *et al.*, 1997; Snow *et al.*, 2001; Rolland & Winton, 2003), however, recently an experimental challenge with a virulent New Brunswick isolate of ISAV, NBISA01, caused the first reported rainbow trout mortalities with lesions characteristic of ISA (Kibenge *et al.*, 2004).

In addition to salmonids, work has been done on species of fish commonly found in the vicinity of Atlantic salmon farms, both in terms of the risk of disease development and the potential of being either a reservoir host or vector for ISAV. Blue mussels, *Mytilus edulis*, can take up the virus by filtering ISAV contaminated seawater, but rapidly inactivated the virus, making this an unlikely reservoir host or vector for ISAV (Skar & Mortensen, 2007). Saithe, *Pollachius virens*, are not susceptible to ISAV by either cohabitation or intraperitioneal infection challenge (Snow *et al.*, 2002). Further, cohabitating Pollack, *Pollachius virens,* have been sampled during a salvage harvest from an Atlantic salmon netpen experiencing an ISAV outbreak. The pollack kidney samples were negative for ISAV by RT-PCR (McClure *et al.*, 2004a). Other species are, however, capable of ISAV replication under natural or experimental conditions, including

herring (*Clupea harengus*) and Atlantic cod (*Gadus morhua*) (Nylund *et al.*, 2002; Grove *et al.*, 2007).

1.2.3. Transmission

ISAV is most often transmitted in marine waters via cohabitation with infected live salmon or via biological materials including animal wastes from a disease-positive aquaculture operation, waste water from slaughter facilities and processing plants, and contaminated fomites such as nets, divers, well boats or feed delivery boats traveling between sites or near sites (Murray *et al.*, 2002; McClure *et al.*, 2005). Infected fish shed virus within blood, feces, urine and epidermal mucus (Totland *et al.*, 1996). Within a site, identified risk factors for an outbreak at the fish level include group size, general health at seawater entry, stressful husbandry practices during growout and health and productivity during colder months (Hammell & Dohoo, 2005b). Farm level risk factors include depth of net pens, depth of water beneath a net pen, implementation of sea lice control measures, numbers of cohabitating pollack within the cages and failing to eradicate apparently uninfected cages at a site experiencing an outbreak (McClure et al., 2004b; McClure *et al.*, 2005). It has also been shown that the risk of disease transfer between an infected farm and uninfected farm was eight times greater if the farms were less than 5 km apart (Jarp & Karlsen, 1997). Additionally, farms within 5 km of a slaughter facility lacking wastewater disinfection had thirteen times higher risk of developing the disease (Jarp & Karlsen, 1997). The virus survives in nonsterile seawater for >1 day at 16°C and 14 days at 4°C. While ISAV genomic material has been detected by RT-PCR in seawater (Lovdal & Enger, 2002) and within 1.5 km of an infected farm during a natural infection, virus isolation to confirm infectious potential has not been possible (Giray *et al.*, 2005). A study of ISAV outbreak patterns found that new outbreaks were linked to outbreaks nearby 1 and 3 months previously, and new

outbreaks were likely to occur spatially within 1 tidal excursion of an outbreak (Gustafson *et al.*, 2007).

The gills are considered the most probable route of ISAV entry into the fish (Totland *et al.*, 1996; Mikalsen *et al.*, 2001). Alternative routes include the skin and lateral line. Oral entry is considered to have a lower infectious potential as the acidic environment of carnivorous fish stomach is capable of rendering the virus noninfective (Falk *et al.*, 1997).

A study on the embryos and progeny of females with ISAV-positive ovarian fluid failed to detect virus in eggs, alevins or parr or from naïve parr injected with homogenates from potentially infected eyed eggs, leading the authors to presume lack of vertical transmission of ISAV (Melville & Griffiths, 1999). However, there is growing suspicion of vertical or transgenerational transmission of the virus due to its detection in new locales following the translocation of iodophor surface disinfected eyed eggs (Nylund *et al.*, 2007; Vike *et al.*, 2008).

The outer surface of a fish serves as the first line of immune defense against pathogen invasion. The mucous layer is continually produced and sloughed and contains several important components of nonspecific immunity including anti-bacterial peptides, proteases, lectins and lysozyme. The skin serves as a physical barrier, with pluripotential epithelial cells capable of rapid wound healing (Roberts & Ellis, 2001). Sea lice are copepodid arthropods that infest fish worldwide, grazing on the mucus and epidermis of salmonids and causing superficial ulceration. During an ISA outbreak, the presence of sea lice within the affected netpen is considered a risk factor capable of exacerbating disease transmission due to sea lice-related breakdown in the integrity of the mucosal immunity. *Lepeophtheirus salmonis* and *Caligus elongates* are the sea lice species commonly found on marine farmed Atlantic salmon in the temperate regions of the Northern Hemisphere (Pike & Wadsworth, 1999). C. *rogercresseyi* is the

predominant species in the Southern Hemisphere (Zagmutt-Vergara *et al.*, 2005). In addition to mechanical damage, the adult and pre-adult stages of sea lice are motile and have been shown to travel between fish and may have an active role in the spread of ISAV, as has been demonstrated under experimental conditions (Nylund *et al.*, 1993).

1.3. Characterization of ISAV

1.3.1. Morphology and classification

The first electron micrographs of the putative agent of ISA were published in 1994, showing an enveloped spherical virus budding from endothelial cells (Hovland *et al.*, 1994). ISAV is approximately 100 nm in diameter with a buoyant density of 1.18 g/ml using both sucrose and cesium chloride gradients (Koren & Nylund, 1997; Mjaaland *et al.*, 1997). The genome of ISAV is similar to that of influenza A and B viruses in that it consists of eight segments of negative sense, single-stranded RNA ranging in size from 1.0 to 2.3 kb, with a total approximate size of 14.3 - 14.5 kb (Mjaaland *et al.*, 1997). Different researchers have attempted to sequence and assign viral proteins to each of these eight genomic sequences, relying on amino acid composition, protein size, computer-assisted motif identification and comparisons with the gene expression characterizations of other *Orthomyxoviridae*. The genome similarities plus the presence of conserved regions at the 3' and 5' termini of all ISAV segments and evidence that ISAV requires capped cellular mRNA for replication, all support the inclusion of ISAV in the *Orthomyxovirdae* family. Within this family, ISAV has been classified as the type species of a fifth genera, *Isavirus*, based on the natural host, replication temperature of <25°C and other characteristics distinguishing ISAV from other *Orthomyxoviridae* (Falk *et al.*, 1997; Kawaoka *et al.*, 2005).

1.3.2. ISAV target cells

Electron microscopy has revealed viral budding from the apical membrane of endothelial cells of both Atlantic salmon (Hovland *et al.*, 1994;Nylund *et al.*, 1995a;Koren & Nylund, 1997) and rainbow trout (Nylund *et al.*, 1997). The virus has also been shown within leucocytes, however in this cell type, viral budding has not been seen and it is thought that cell lysis after phagocytizing the virus may have a role in viral dissemination (Nylund *et al.*, 1995a). RT-PCR was used to demonstrate that ISAV mRNA is present in most organs within two weeks of experimental infection (Rimstad *et al.*, 1999). This widespread distribution is consistent with having endothelial cells as the primary target cell. *In situ* hybridization (ISH) exploits specifically designed nucleic acid probe sequences to detect complementary nucleic acid sequences. When applied to a complex tissue milieu, ISH elucidates the macroscopic distribution and cellular localization of targeted nucleic acid sequences, and therefore has value in the study of the pathogenesis of infectious agents. This technique was used on tissues sampled from farmed Atlantic salmon diagnosed with ISA during a natural outbreak to study the Atlantic salmon host cell distribution of ISAV gene expression. ISAV nucleic acids were found within endothelial cells with broad tissue distribution (Gregory, 2002). In order of declining signal strength, tissue signal localization was found to be highest in the heart, followed by the liver, kidney, spleen, intestine and pyloric caeca. A similar tissue distribution was seen following an experimental ISAV infection challenge (Moneke *et al.*, 2003). In addition to endothelial cells, ISH has detected ISAV mRNA in circulating leucocytes during the supposed period of viremia coinciding with the highest levels of mortalities (Gregory, 2002; Moneke *et al.*, 2005a; Moneke *et al.*, 2005b). However, hepatocytes and renal parenchymal cells do not demonstrate viral signal. Therefore, it is presumed that the lesions in the liver and renal parenchyma are the result of ischemic damage due to impaired blood flow secondary to a damaged endothelium.

An ISAV Segment 8 riboprobe was applied to TO and SHK-1 cell culture monolayers, demonstrating nuclear localization one day after ISAV inoculation, progressing to perinuclear three days later, and predominantly cytoplasmic localization one week following ISAV inoculation (Moneke *et al.*, 2003). This temporal distribution indicates a replication strategy consistent with that of members of the *Orthomyxoviridae* family. Further, ISH was able to detect replicating virus earlier than virus isolation, as CPE in the ISAV-inoculated TO cells was not evident till 3 days post inoculation. ISH has also been used to demonstrate a positive correlation between peak viremia (as indicated by presence of viral signal in both tissues and in circulating cells within blood vessel lumens), gross lesion development and onset of mortality (Moneke *et al.*, 2005b).

1.3.3. Haemagglutination

The clumping of red blood cells or haemagglutination involves the cross linking of erythrocytes by the simultaneous binding of the viral surface protein haemagglutinin to sialic acid residues on the carbohydrates of glycoproteins of multiple red blood cells. Previous studies showed that ISAV was able to haemagglutinate erythrocytes of several fish species, namely Atlantic salmon, rainbow trout, Atlantic cod (*Gadus morhua*), crucian carp (*Carassius carassius*) and wolfish (*Anarchichas lupus*) (Falk *et al.*, 1997; Kristiansen *et al.*, 2002). Brown trout was the only fish species whose red blood cells did not agglutinate with ISAV. Further, elution of the erythrocytes through receptor destroying enzyme activity of the acetylesterase component of the HE protein was noted within 3 to 4 hours for all fish species except Atlantic salmon, which failed to elute during the 24 hour period of observation. These findings were investigated in greater detail using transmission electron microscopy on Atlantic salmon and rainbow trout red blood cells to compare the time course of ISAV infectivity (Workenhe *et al.*, 2007). The study demonstrated apparent attachment and elution of rainbow trout erythrocytes with rare

intracellular virus particles evident at 4 to 18 hours post inoculation. This was in sharp contrast to the findings in Atlantic salmon erythrocytes, wherein numerous viral particles underwent attachment, endocytosis, with progression to late endosomes and cytoplasmic vesicles in the same time frame. As the initial determinant of microbial pathogenicity is attachment, it is presumed that the lack of elution seen in Atlantic salmon favours cellular infection of this species, contributing to the increased pathogenicity (Workenhe *et al.*, 2007). Further work revealed that Atlantic salmon erythrocytes challenged with a pathogenic strain of ISAV demonstrated virus uptake and productive infection which induced an innate immune response mediated via the Type 1 intereron system (Workenhe *et al.*, 2008).

1.3.4. Persistence

Viral persistence is not a feature associated with the *Orthomyxoviridae*. A strong potential for long term carrier status is, however, indicated by cohabitation trials demonstrating ISAV transmission from asymptomatic experimentally infected salmonids to naïve Atlantic salmon (Nylund & Jakobsen, 1995; Mjaaland *et al.*, 1997); ISAV-positive RT-PCR results detected as late as seven months after experimental infection (Nylund *et al.*, 1995b; Raynard *et al.*, 2001; Munir & Kibenge, 2004), and ISAV detection in asymptomatic, feral salmonids (Raynard *et al.*, 2001). The cell type and tissue localization of the latent, persistent virus remains unknown.

1.3.5. Phylogenetic and phenotypic variations

Haemagglutinin-esterase (HE) is the ISAV virus protein with the most sequence variation, primarily concentrated within a small highly polymorphic region (HPR). Phylogenetic analysis of the 5'-end flanking region of the HPR of the HE gene allows the division of known ISAV isolates into 2 major groups, a North American and a European

group. The HPR region is characterized by the presence of gaps of multiple, as opposed to single nucleotide substitutions. Mutation rate studies of the HE gene among ISAV isolates have estimated the timing of the divergence between the two groups to be at least 100 years ago and possibly as long as 260 years ago (Krossoy *et al.*, 2001; Devold *et al.*, 2001; Kibenge *et al.*, 2001b; Nylund *et al.*, 2003; Kibenge *et al.*, 2007a). This timing is significant in that it pre-dates intensive salmon farming activities and suggests that natural reservoirs for the virus exist on both sides of the North Atlantic (Plarre *et al.*, 2005). It has been further theorized that the reservoir hosts for the virus are wild trout and salmon carrying an avirulent, wild-type full-length precursor strain (HPR0), and that differential deletions from the HE HPR of the archetypal ISAV isolate can explain the evolution of viral pathogenesis and ecology (Mjaaland *et al.*, 2002; Nylund *et al.*, 2003; Plarre *et al.*, 2005). A putative ancestral full-genome sequence has been reconstructed displaying the HPR0 genotype which shows agreement with nonpathogenic ISAV isolates cultured from wild salmonids (Markussen *et al.*, 2008). However, deletions within the HE-HPR are not the only determinant of ISAV virulence, as standardized experimental fish have shown variable survival following experimental infection with ISAV isolates with identical HE-HPR genotypes (Mjaaland *et al.*, 2005). Comparisons among 12 ISAV isolates of variable virulence indicated that reassortment and recombination between two different ISAV isolates within the same population of fish may play a role in the evolution of pathogenic ISAV strains. In particular, recombination in the F gene, contributing to an increased virulence, has been shown (Devold *et al.*, 2006; Markussen *et al.*, 2008). This is of particular interest, as it is analogous to the recombination events affecting the fusion protein of avian Influenza A virus, changing its host tissue tropism and transforming it from a respiratory pathogen to a virus capable of systemic infection and multi-organ failure in susceptible birds (Markussen *et al.*, 2008).

Phenotypic differences between ISAV strains in terms of virulence and ability to replicate in a variety of cell lines have been shown (Kibenge *et al.*, 2001b; Mjaaland *et al.*, 2002; Kibenge *et al.*, 2006). Viral neutralization of 24 ISAV isolates using rabbit antisera in the TO cell line and comparative sequence analysis of their haemagglutinin-esterase (HE) genes demonstrated that antigenic subtypes agree with phylogenetic ones, one American and one European (Kibenge *et al.*, 2001b).

1.3.6. ISAV and host immune response

Interferons are antiviral effector molecules that block the spread of viruses to uninfected cells. Interferons are cytokines, secreted from virus infected cells, that bind to a common cell surface receptor on infected and nearby cells. The receptor is coupled to JAK-STAT (Janus family tyrosine kinase and signal transducing activators of transcription (STATs)) which signal translocation to the nucleus and activate transcription of genes that induce a variety of host cell proteins that contribute to the inhibition of viral replication. Specifically, interferon induces endonucleases that degrade viral RNA, plus PI kinase and Mx proteins that inhibit viral replication. Interferon also upregulates cellular immune response to viruses by promoting major histocompatibility complex (MHC) Class I molecules on the cell surface, increasing antigen presentation for T cells (for review see Janeway *et al.* 2001). One mechanism of viral immune evasion used by other Orthomyxoviruses is to avoid MHC class I antigen presentation by antagonizing interferon expression (Hewitt, 2003). This is not the case in ISAV infected ASK cells which demonstrated comparable levels of type 1 interferon and MHC class 1 pathway induction when stimulated by polyinosinic polycytidylic (poly I:C), a synthetic analog of double stranded RNA which interacts with Toll-like receptor 3 (Jorgensen *et al.*, 2006).

Poly I:C is used experimentally to simulate viral infection. Atlantic salmon treated

with poly I:C, had increased Mx protein induction in the visceral organs (Jensen *et al.*, 2002). This increase was accompanied by reduced cumulative mortalities compared to untreated controls following ISAV intraperitoneal (ip) infection challenge, indicating that poly I:C can have a protective role against ISAV *in vivo*. However, this contrasts to other work that indicated that ISAV infection of Atlantic salmon derived cell lines, TO and SHK-1, induced Mx protein which conferred mild protection to cytotoxicity in SHK-1 cells, but did not inhibit viral replication in the TO cell line (Jensen & Robertsen, 2002). The differing response between the two cell lines is consistent with other work which demonstrated a cell-type specific mechanism of ISAV-induced cell death; with apoptosis induced in SHK-1 cells and necrosis induced in TO cells (Joseph *et al.*, 2004). Another study using CHSE-214 cells genetically engineered to continuously express Atlantic salmon Mx1 protein, had improved survival following infection with ISAV ; however viral mRNA levels were not altered, suggesting that MX1 protein antiviral effects on ISAV occur within the cytoplasm, possibly during post-translational modifications (Kibenge *et al.*, 2005).

Microarray analysis compared the gene expression responses of ISAV infected Atlantic salmon tissues from fish experiencing early mortality (sampled as moribund from among the first 10% of mortalities, beginning at 21 dpi by cohabitation challenge) versus fish experiencing late mortality (moribund fish from among the last 10% of mortalities, roughly 38 dpi). Results indicated that the early infections were characterized by high viral replication and dramatic activation of innate immune responses and marked cellular oxidative stress which did not confer protection to the virus. Alternatively, late infections were characterized by reduce viral loads with increased activation of adaptive cellular immunity and a decreased inflammatory response (Jorgensen *et al.*, 2008).

There is evidence that survivors of ISAV natural infection are less susceptible to re-infection by experimental challenge (Falk & Dannevig, 1995; Ritchie *et al.*, 2009). This

triggers questions about the duration of protection, the influence of isolate virulence on protection, and the type of host response stimulated, innate or acquired. It is unknown if these results can be extrapolated to a complex farm situation, due to multiple confounding variables (i.e. concurrent disease, predation, management practices, vaccination history, etc.).

Studies in our lab have identified possible Fc receptor-mediated antibody-dependent uptake and growth of ISAV in macrophage-like fish cell lines (Joseph *et al.*, 2003). Since ISAV in Atlantic salmon targets leucocytic cells and endothelial or endothelial-associated cells, it seems reasonable to speculate that Fc receptor-mediated antibody-dependent enhancement of ISAV infection may occur *in vivo*, potentially accelerating the disease process by enhancing delivery of the virus to target cells.

1.3.7. Genetic influence on susceptibility/resistance

Heritability refers to the proportion of phenotypic variation between individuals in a population that is attributable to genetic factors. Numerous studies have attempted to quantify the additive genetic contribution to disease resistance, as measured by survival, to ISAV experimental challenge models. A nonsignificant heritability of 0.13 was found based on data from a single year class at a broodstock farm (Gjoen *et al.*, 1997). A multi-year study of 11 year classes from three generations of an ongoing Atlantic salmon breeding program, however, found low but significant heritability estimates of 0.26 and 0.31 (Odegard *et al.*, 2007; Gjerde *et al.*, 2008). Further, a bivariate analysis was run on the same dataset to investigate (co)variance components of survival to challenge testing for furunculosis and ISAV; the genetic correlation between the two traits was estimated to be 0.15. Based on these results, the authors concluded that selective breeding programs have the potential to improve resistance in Atlantic salmon to both furunculosis and ISA (Odegard *et al.*, 2007).

The influence of genotype on susceptibility of juvenile rainbow trout to ISAV has also been examined (Biacchesi *et al.*, 2007). Homozygous strains, or clones, of juvenile rainbow trout were challenged by ISAV waterborne immersion challenge at <1g average size. The 6 strains demonstrated a full range of susceptibility, from relatively resistant to highly susceptible by timing of the acute phase of mortality, cumulative mortalities and presence of clinical signs typically associated with ISA. The challenge test was repeated on the most susceptible strain at a larger size (6.5 g average). Consistently high mortalities were seen. This finding was particularly interesting as the expected development of age/size related acquired immunocompetence did not confer improved survival. This study was the first to demonstrate that ISAV resistance in rainbow trout is influenced by genotype.

As discussed earlier, interferon is involved in the upregulation of the cellular immune response to viruses by promoting MHC Class I molecule transcription, thereby increasing antigen presentation for T cells. MHC genes encode cell-surface molecules capable of binding and presenting short peptides to T cells. MHC Class 1 molecules collect peptides derived from within the cytosol, including fragments of viral proteins, and display them on the cell surface for recognition by cytotoxic T cells. MHC Class II molecules bind peptides derived from intracellular vesicles of phagocytic cells like macrophages or from B cells. Helper T cells will activate macrophages displaying peptides in their MHC class II molecules to increase intracellular killing efficiency. B cells displaying peptides in their MHC class II molecules will be stimulated by helper T cells to proliferate or differentiate into antibody producing plasma cells (as reviewed by Janeway *et al.*, 2001).

The use of MHC compatible experimental fish is providing new information on disease resistance in Atlantic salmon. Using the parameters of prevalence of death and hazard ratio following ip injection or cohabitation challenge trials, a significant

association between genotype and resistance to ISAV was found for both single Class I and Class II alpha alleles and combinations thereof (Grimholt *et al.*, 2003; Kjoglum *et al.*, 2006). A number of single alleles associated with significantly increased susceptibility to ISAV was also identified. Three full sibling families with identical MHC class I and Class II genotypes demonstrated a significant variation in survival among families following ISAV ip injection challenge, with an estimated 7.5% contribution to prevalence of death made by nonMHC genes (Kjoglum *et al.*, 2005). In another experiment, half sibling Atlantic salmon with identical MHC genotypes were challenged with one of 11 different ISAV isolates via a cohabitation model. By minimizing the variability of the host immune response, the study was able to compare differences in disease susceptibility and virulence due to variations among viral genes, and additionally the study examined the relative contributions of cellular versus humoral response in ISAV protection. It was found that the ability to produce a strong proliferative cellular response correlated with survival and viral clearance while induction of a humoral response was less protective (Mjaaland *et al.*, 2005). Furthermore this study demonstrated that variability in the HE-HPR is not the only ISAV virulence marker, as isolates with identical HE-HPR genes induced a highly variable immune response and survival in the standardized experimental fish.

1.4. ISAV genes and proteins

ISAV Segment 1

Evidence indicates that segment 1 encodes a large nuclear protein, which is likely to have a function analogous to that of the influenza polymerase protein PB2 (Clouthier *et al.*, 2002; Snow *et al.*, 2003). Based on protein size and amino acid composition, this protein was shown to be similar to the PB2 protein of other orthomyxoviruses. Further, a nuclear localization signal was identified in the C-terminus

of the protein as is found on all the influenza virus polymerase proteins, and expression using green fluorescent marker fusion protein confirmed nuclear localization in a fish cell line, CHSE (Snow *et al.*, 2003).

ISAV Segment 2

This ISAV segment encodes the polymerase basic protein 1 (PB1), 80.5 kDa. This putative ISAV polymerase protein has a length of 708 amino acids, a charge of +22 at neutral pH and pI of 9.9; these characteristics are consistent with the properties of other orthomyxoviridae PB1 proteins. Pairwise comparison between the ISAV polymerase sequence and other orthomyxoviridae PB1 proteins revealed a 20.8 – 24.1 % range in amino acid identities and conserved core polymerase motifs characteristic of all RNA-dependent RNA polymerases (Krossoy *et al.*, 1999). Phylogenetic studies using this sequence indicate a distant relationship between ISAV and the members of the *Orthomyxoviridae*, with ISAV being more closely related to the influenza viruses than to the Thogoto viruses (Krossoy *et al.*, 1999). Previous work has indicated that PB1 is the most conserved orthomyxoviridae protein (Lin *et al.*, 1991) and therefore is a good candidate protein to evaluate the evolutionary relationship between ISAV and the members of the *Orthomyxoviridae* (Krossoy *et al.*, 1999).

ISAV Segment 3

Segment 3 has been suggested to encode the 66 kDa major structural protein, nucleoprotein (NP). Although ISAV NP sequences are dissimilar to those of other members of the *Orthomyxoviridae* (pairwise comparison between the ISAV and other orthomyxovirus NP amino acid sequences revealed a 11.65 – 12.88 % range in amino acid identities), the gene product has been identified as a nucleoprotein based on similarities to influenza NP with regards to its amino acid composition, location of putative phosphorylation sites, presence of a hydrophobic C-terminal, RNA binding activity and nuclear localization signals (Snow & Cunningham, 2001; Aspehaug *et al.*,

2004). Along with the polymerase proteins PB1, PB2 and PA and the viral RNA, the NP forms ribonucleoprotein complexes (vRNP) or nucleocapsids.

ISAV Segment 4

This segment is proposed to encode the acidic polymerase, PA 65.3 kDa, the negatively charged component of the vRNP. This designation is based on the predicted cytoplasmic location, presence of predicted transmembrane regions and putative phosphorylation sites. The function of this protein, however, remains unknown, as it doesn't contain expected polymerase motifs (Ritchie *et al.*, 2001b).

ISAV Segment 5

Hydrophobic amino acid sequence signal and potential glycosylation sites suggest Segment 5 encodes a surface glycoprotein. This 50 kDa protein has been proposed to be a proteolytic-dependent type 1 fusion protein, enabling fusion between viral and cellular membranes (Aspehaug *et al.*, 2005).

ISAV Segment 6

ISAV Segment 6 encodes the surface protein, haemagglutinatinin-esterase (HE). This is a 37 kDA protein which is glycolsylated and elicits virus neutralizing antibodies (Mikalsen *et al.*, 2005). HE is the major surface glycoprotein of ISAV and functions in host recognition by receptor attachment by specifically binding to and hydrolyzing 4-O-acetylated sialic acids (Falk *et al.*, 1997). The second function of HE is acetylesterase receptor-destroying activity, aiding in the dissemination of new virions (Falk *et al.*, 2004).

ISAV Segment 7

The gene coding assignments of Segment 7 have not been finalized. It was originally thought to encode two proteins, corresponding to the putative matrix proteins of Influenza A virus (Ritchie *et al.*, 2002). The use of ISAV antiserum, however, failed to immunoprecipitate the Segment 7 ORF1 protein, suggesting that the largest S7ORF1 gene encodes a nonstructural protein as opposed to a matrix protein (Bierin *et al.*, 2002).

Further work demonstrated that this protein is a putative interferon-signaling antagonist protein localized within the cytoplasm, which functions to block the host cell interferon signaling pathway and interfere with the transcription of Mx protein (McBeath *et al.*, 2006). Recently a revised gene expression model for ISAV Segment 7 has been proposed, suggesting this segment encodes 3 proteins: a 35kDa minor structural protein translated from ORF1 which was able to elicit a protective antibody response during a ISAV challenge trial; an 18 kDa protein based on the removal of an intron from the primary ORF1 transcript and continuation on the +2 reading frame which has been identified as the putative ISAV nuclear export protein and shown to bind caspase-8, an upstream initiator of apoptosis, in ISAV-infected SHK-1 and CHSE-214 cells (Joseph *et al.*, 2004); and a second spliced protein, a 9.5 kDa protein based on the removal of an intron from the primary ORF1 transcript and continuation on the +3 reading frame, of unknown function (Kibenge *et al.*, 2007b).

ISAV Segment 8

Segment 8 is the smallest ISAV segment. The segment sequence suggests that it encodes two ORFs which do not have significant homology with any other available nucleotide or amino acid sequences. Based on the genomic organization of the influenza viruses, it was originally assumed that this segment encoded two non structural proteins (Blake *et al.*, 1999). Subsequent evidence, however, suggests that ORF1 encodes the 23.5 kDa matrix protein (Bierin *et al.*, 2002; Falk *et al.*, 2004; Kibenge *et al.*, 2007b). The second protein, s8ORF2, has recently been characterized as an RNA binding structural protein with interferon antagonist properties (Garcia-Rosado *et al.*, 2008).

1.5. Replication of ISAV

1.5.1. Mechanisms of cellular entry

The initial determinant of viral pathogenicity is the ability of ligands, viral

attachment proteins, on the surface of the virus to bind to host cell surface receptors to initiate infection. The cellular receptor for ISAV is the terminal sialic acid on an oligosaccharide side chain of a host cell surface glycoprotein and/or glycolipid. Specifically, the ISAV surface protein, HE specifically binds to 4-O-acetylated sialic acids on host cell surface glycoproteins or glycolipids (Hellebo *et al.*, 2004).

Bound viral particles are internalized by receptor-mediated endocytosis and are trafficked along the endocytic pathway to acidic late endosomes. A low pH environment (5.5 – 5.6) has been shown to proteolytically cleave the precursor fusion protein, permitting fusion of the viral membranes with those of the endosome, and enabling the entry of viral nucleic acids into the cytoplasm (Aspehaug *et al.*, 2005). In addition to initiating membrane fusion, the low pH environment of the late endosome is thought to have a secondary role in virus entry via the viral transmembrane channel protein M2. The channel protein allows acidification of the virion, triggering the separation of the viral nucleocapsids from the viral matrix protein M1, uncoating the internal components. Thus, acidification culminates in the uncoating of the virus and the release of autonomous viral ribonucleoprotein (vRNP) complexes into the cytoplasm. The vRNPs are small enough to pass through the channel of the nuclear pore complex for entry into the nucleus. Nuclear localization signals on NP cooperate to mediate nuclear import of vRNPs for viral gene expression and replication (Ludwig *et al.*, 1999;Aspehaug *et al.*, 2004).

1.5.2. Replication and transcription

Once in the nucleus, vRNPs serve as templates for two forms of positive sense RNAs, viral messenger RNA (mRNA) and complementary RNA (cRNA). The synthesis of mRNA is catalyzed by the viral RNA-dependant RNA polymerase portion of the vRNP complex, specifically the three subunits PA, PB1 and PB2. Transcription involves a

phenomenon known as cap-snatching, whereby viral endonuclease PB2 cleaves the 5'-methylguanosine cap and adjacent 10 – 13 nucleotides from cellular RNAs for use as primers for the viral RNA polymerase PB1 to transcribe viral mRNA. These capped viral mRNAs are polyadenylated and are exported from the nucleus for translation by cytoplasmic ribosomes (Whittaker, 2001). For ISAV, transcripts for 6 of the 8 gene segments are translated directly, but the other two transcripts undergo splicing and are translated in different reading frames for a total of at least 10 proteins expressed (Bierin *et al.*, 2002; Falk *et al.*, 2004; Kibenge *et al.*, 2007b). Nuclear export of viral mRNA is controlled by the viral nonstructural nuclear export protein. PB1, PB2, PA and NP initiate the synthesis of complementary RNA (cRNA). The viral cRNA serves as a template for negative sense genomic RNA (vRNA) which assembles with newly formed translated viral proteins (NP, M1, NS2 and the polymerases) imported from the cytoplasm into progeny vRNPs. These vRNPs are then exported from the nucleus via nuclear pores (Ludwig *et al.*, 1999).

1.5.3. Assembly and budding of orthomyxoviruses

Virions are formed by budding at the apical surface of polarized cells. Transmembrane viral proteins are transported to the assembly site on the plasma membrane by cellular exocytic pathways. Once there, they utilize lipid rafts (glycolipid enriched domains) for assembly of viral components, cell surface transport and viral budding (Nayak *et al.*, 2004). M1 proteins serve as a bridge between the cytoplasmic tails of the transmembrane HE proteins of the future envelope and the vRNPs. Viral budding from the cell surface relies in the actions of acteylesterase component of the HE protein. This protein is a sialidase receptor-destroying enzyme. By removing the sialic acid receptor from both the surface of the host cell and newly formed virions, reattachment of the virus-infected cell and self-aggregation of viral particles are

prevented and dissemination of virions is encouraged (Kristiansen *et al.*, 2002; Nayak *et al.*, 2004; Hellebo *et al.*, 2004).

1.6. Clinical disease expression

1.6.1. Clinical signs and gross pathology

The disease is characterized by severe anaemia which is grossly evident as pale gills and often pale hearts. The infection spreads slowly and the virus is considered to be of relatively low virulence, however, high cumulative mortalities in marine-farmed Atlantic salmon can happen. On external examination, macroscopic lesions associated with disease include pale gills, ascites-distended abdomens, exophthalmia and epidermal haemorrhages. Internally, pale hearts, petechiation of the visceral adipose tissue and internal organs, enlargement and congestion of the spleen and kidney and darkening of the liver are common findings, although pale livers or yellow livers with haemorrhagic foci may also be present.

1.6.2. Histologic findings

Microscopic lesions include multifocal to coalescing areas of haemorrhagic liver necrosis and sinusoidal congestion (Thorud & Djupvik, 1988; Evensen & Thorud, 1991; Gattuso *et al.*, 2002), renal interstitial haemorrhage and tubular necrosis with eosinophilic casting (Simko *et al.*, 2000), filamental congestion of the gill (Jones *et al.*, 1999), splenic congestion with concomitant erythrophagia, and congestion of the lamina propria of the intestine (Evensen & Thorud, 1991; Jones *et al.*, 1999). Increased frequency of erythophagia has been noted in the kidney haematopoietic tissue and in areas of splenic sinusoidal congestion as an experimental infection sequelae in rainbow trout (Falk *et al.*, 1995; Byrne *et al.*, 1998; Moneke *et al.*, 2003; Kibenge *et al.*, 2006; MacWilliams *et al.*, 2007).

1.6.3. *Haematologic lesions*

Clinically affected fish may have haematocrits <10 (Thorud & Djupvik, 1988). Additionally, there will be a decreased ratio of leucocytes to erythrocytes, with lymphocyte and thrombocyte populations particularly reduced (Dannevig *et al.*, 1994). Erythrocytes may appear vacuolated and immature erythroblasts may be visible, identified by their irregular shaped nuclei. Increased serum glutathione can be attributed to hepatocyte damage and subsequent enzyme leakage (Hjeltnes *et al.*, 1992). Electrolyte abnormalities will also be common in fish in salt water unable to maintain osmoregulatory homeostasis. Differential diagnoses for population level single digit haematocrit values include trauma, ulcerations, toxins and erythrocytic inclusion body syndrome.

1.7. Justification of the study

ISAV is currently one of the most important viral pathogens threatening global commercial Atlantic salmon aquaculture. Highly pathogenic strains of ISAV, as demonstrated by cytopathology and pathology in Atlantic salmon, have been shown capable of causing fatal infection of rainbow trout with lesions characteristic of ISA (Kibenge *et al.*, 2004; Kibenge *et al.*, 2006). The present research project was devised to further examine the pathology of ISAV in rainbow trout, and to better understand virus-host interactions.

2. MORPHOLOGIC DESCRIPTION OF INFECTIOUS SALMON ANAEMIA VIRUS (ISAV)-INDUCED LESIONS IN RAINBOW TROUT *ONCORHYNCHUS MYKISS* COMPARED TO ATLANTIC SALMON *SALMO SALAR*

* portions of this chapter appear in:

MacWilliams, C., Johnson, G., Groman, D. & Kibenge, F. S. (2007). Morphologic description of infectious salmon anaemia virus (ISAV)-induced lesions in rainbow trout Oncorhynchus mykiss compared to Atlantic salmon Salmo salar. *Dis Aquat Organ* 78: 1-12.

2.1. Introduction

Infectious salmon anaemia virus (ISAV), a teleost orthomyxovirus, is a commercially important viral pathogen of marine-farmed Atlantic salmon (*Salmo salar*). ISA was first described in juvenile marine-farmed Atlantic salmon in Norway in 1984 (Thorud & Djupvik, 1988). Since then, it has occurred in marine-farmed Atlantic salmon in Scotland (OIE Wahid Event summary: infectious salmon anaemia, United Kingdom: http://www.oie.int/wahis/public.php?page=event_summary&this_country_code=GBR&reportid=7717; accessed March 2009)((Rodger *et al.*, 1998;OIE, 2009), New Brunswick (Byrne *et al.*, 1998) and Nova Scotia, Canada (Ritchie *et al.*, 2001a), in Maine, USA (Bouchard *et al.*, 2001) and in Chile (Godoy *et al.*, 2008). The clinical disease is thought to occur in wild free-ranging Atlantic salmon, but these fish are considered less susceptible than the farmed Atlantic salmon either due to genetic variation in the two groups or due to stress caused by management practices on the salmon farms (Nylund *et al.*, 1995b). The disease is characterized by severe anaemia and high cumulative mortalities in marine-farmed Atlantic salmon. Numerous associated gross lesions have been reported including ascites, exophthalmia, petechiation of the visceral adipose tissue, and enlargement and congestion of the liver and spleen. Microscopic lesions include haemorrhagic liver necrosis (Thorud & Djupvik, 1988; Evensen & Thorud, 1991), renal interstitial haemorrhage and tubular necrosis (Mullins *et al.*, 1998; Simko *et al.*, 2000), filamental arteriole congestion of the gill (Jones *et al.*, 1999), splenic congestion with concomitant erythrophagocytosis, and congestion of the lamina propria of the foregut (Evensen & Thorud, 1991; Jones *et al.*, 1999).

ISAV has also been isolated from jaundiced as well as apparently healthy coho salmon (*Oncorhynchus kisutch*) in Chile (Kibenge *et al.*, 2001a) and from seawater-reared rainbow trout (*O. mykiss*) in Ireland (Siggins, 2002). The virus may infect and

replicate in sea trout (*S. trutta* L.), brown trout (*S. trutta*), rainbow trout, eels (*Anguilla rostrata*), herring (*Clupea harengus*), and Arctic char (*Salvelinus alpinus*) resulting in asymptomatic, probably life-long, carriers of the virus (Nylund & Jakobsen, 1995; Nylund *et al.*, 1995b; Nylund *et al.*, 1997; Rolland & Nylund, 1998; Devold *et al.*, 2000; Snow *et al.*, 2001; Nylund *et al.*, 2002). The Pacific salmonid species, chum (*O. keta*), steelhead (*O. mykiss*), chinook (*O. tsawytscha*), and coho (*O. kisutch*) were also found to be resistant to experimental infection with ISAV even with doses as high as 10^8 TCID50/ml that induced 98% mortality in Atlantic salmon (Rolland & Winton, 2003). Although attempts to isolate the virus from some of these fishes have not been successful, the virus can be detected by RT-PCR (Devold *et al.*, 2000; Raynard *et al.*, 2001; Snow *et al.*, 2001; Nylund *et al.*, 2002). Such benign infections in the wild fishery are considered to be the source of virulent strains that cause clinical disease in marine-farmed Atlantic salmon (Murray *et al.*, 2002; Nylund *et al.*, 2003). Conversely, the sea cages with farmed Atlantic salmon may represent a source of transmission of ISAV to native, wild salmonid stocks. We recently observed that the most virulent ISAV strains also cause mortality in rainbow trout with lesions characteristic of ISAV (Kibenge *et al.*, 2004; Kibenge *et al.*, 2006).

Further investigations of the pathology associated with experimental ISAV infection in rainbow trout in contrast to the experimental disease in Atlantic salmon, has resulted in a more protracted infection. The lesions in rainbow trout are very different from the typical necrosis in liver and kidney that occur in infected Atlantic salmon, and some of them, like erythrophagia, may be indicative of an antiviral response by a resistant host to the ISAV infection.

2.2. Materials and methods

2.2.1. Virus and cell cultures

The ISAV isolate NBISA01 (Jones *et al.*, 1999) was propagated in CHSE-214 and TO cell lines as previously described (Kibenge *et al.*, 2000; Kibenge *et al.*, 2001). Serial 10-fold dilutions of viral stocks were inoculated on 24 h old cell monolayers in 48-well plates. The virus titre (expressed at TCID50/100µl) in each cell line was calculated using endpoint estimation (Reed & Muench, 1938).

2.2.2. Fish and rearing conditions

A total of 600 Atlantic salmon parr, Saint John River stock (mean weight 16 g) were obtained from the Cardigan Fish Hatchery, Prince Edward Island, Canada, and 600 rainbow trout, Kamloops strain (mean weight 11 g) certified as specific pathogen-free were obtained from Trout Lodge via the Dover Fish Hatchery, Prince Edward Island, Canada. The fish were maintained in the Aquatic Animal Facility of the Atlantic Veterinary College in 1 m diameter fibreglass-reinforced plastic tanks using a fresh water flow through system at a temperature of approximately 11°C. Rainbow trout were adipose fin-clipped on arrival for identification purposes. The fish were acclimatized for 7 days before use.

2.2.3. Experimental infection of fish with ISAV and fish sampling

The experimental procedures were performed in accordance with the guidelines of the Canadian Council of Animal Care (2005). The fish were removed from stock holding tanks and anaesthetized by immersion in an aerated solution of tricaine methanesulfonate (TMS) (100 mg/L). A combination of 80 rainbow trout and 80 Atlantic salmon were serially assigned to each of six experimental tanks and infected by intraperitoneal (ip) injection of one of six different ISAV dose/cell line combinations: TO cell line-propagated virus $10^{4.13}$, $10^{6.47}$, or $10^{7.8}$ $TCID_{50}$/200µl cell lysate per fish, and CHSE-214 cell line-propagated virus $10^{2.13}$, $10^{4.13}$, or $10^{7.13}$ $TCID_{50}$/200µl cell lysate per

fish, and returned to their individual tanks.

All fish were observed twice daily and mortalities were recorded and removed for the 8 week duration of the trial. During the three-week period coinciding with the majority of mortalities, fresh mortalities (collected within 1.5 hours of death or less) or severely moribund fish (euthanized by blunt cranial trauma) were removed from the tanks for postmortem examination and tissue sampling. Fish were considered to be moribund if they were swimming slowly at the surface, separate from the general population and were unresponsive, allowing easy netting. Other clinical signs observed included difficulty maintaining an upright body position and spiral swimming patterns. Of the 341 Atlantic salmon that died during the trial, 92 were collected within 1 h of death or while moribund. Of the 104 rainbow trout that died during the trial, 27 were collected promptly. In all cases gross lesions were recorded by one of two examiners, and tissues (head kidney, liver, heart and pyloric caeca) were fixed in 10% neutral-buffered formalin for histologic examination. Samples for virus analysis were not collected from these fish, as gross and microscopic lesions were expected to be sufficient to diagnose ISA.

Subject to the availability of fish, each week, five fish from each species from each tank were opportunistically sampled, euthanized using immersion in an aerated solution of tricaine methanesulfonate (TMS) (100 mg/L). Blood samples were collected from the caudal vein and microhaematocrit tubes were filled to 80% maximum, and centrifuged for 5 minutes at 12000g for packed cell volume (PCV) determination. The fish were then necropsied and all sampled tissues were bissected aseptically. Half of each tissue was fixed in 10% neutral-buffered formalin, and the other half was pooled by species (excluding the pyloric caeca) and placed in a sterile bag and stored at -80°C until used for viral analysis by RT-PCR. All formalin-fixed tissues were processed in an automatic tissue processor and embedded in paraffin wax. Serial 5-6 µm thick sections of the tissues were stained with haematoxylin and eosin (H & E) for light microscopy.

2.2.4. Reverse transcription polymerase chain reaction

Viral RNA was extracted from 300µl volumes of pooled tissue homogenates using TRIZOL reagent (Invitrogen Life Sciences, Carlsbad, California) following the manufacturer's protocol. The ISAV F5/R5 primer set and conditions used to detect ISAV by RT-PCR were as previously described (Kibenge *et al.*, 2000b). One-step RT-PCR reactions were carried out using Titan-One Tube RT-PCR System Kit (Roche Diagnostic Corporation, Indianapolis, Indiana) (Kibenge *et al.*, 2000b). Briefly, cycling was performed using the Peltier PTC-200 DNA Engine thermal cycler (MJ Research Inc., Waltham, MA, US). The conditions for cycling consisted of one cycle of cDNA sysnthesis at 55°C for 30 seconds and 94°C for 2 minutes, respectively, followed by 40 cycles each with denaturation at 94°C for 30 seconds, annealing at 61°C for 45 seconds and extension at 72°C for 90 seconds, with final extension at 72°C for 10 minutes. The RT-PCR products were resolved by electrophoresis on a 1% agarose gel, stained with ethidium bromide and visualized under 340nm UV light (Sambrook *et al.*, 1989).

2.2.5. Statistical analysis

Survival analysis is a statistical procedure that allows quantitative prediction of the time until an event (or failure) occurs. In this study, the outcome variable of interest was the death of the fish. The mean time to failure (MTTF) denotes the time at which there is a 50% probability of death of the study population. The MTTF for each species, in each cell line, for each viral dose was estimated by Kaplan-Meier survival curves. Between-group comparisons were done using a nonparametric log-rank test. All analyses were done using the Kaplan-Meier survival model of MINITAB version 13 statistical software.

2.3. Results

2.3.1. Mortality patterns

No signs of illness were observed in the fish during the 7 day acclimation period prior to the virus challenge. The total mortality for each species within each virus dose is shown in Figure 1. The general mortality pattern showed increased death with increasing virus dose, with the exception being the Atlantic salmon mortalities in the groups inoculated with CHSE-214-propagated virus. In this group the lowest virus dose group experienced higher mortality than the middle dose group. In all cases, the Atlantic salmon groups demonstrated considerably higher mortality then the rainbow trout groups at the same viral dose.

A fungal infection was seen in the Atlantic salmon population during the trial and formalin bath treatments were administered as indicated. Unknown to the researchers, it was later determined that these fish had been exposed to surface water prior to delivery to the AVC. No gross fungal lesions occurred on any of the rainbow trout during the experiment however, mild to moderate fin erosion began to develop in the rainbow trout in the later stages of the trial. This lesion was observed on 14 of the 90 rainbow trout sampled in the final three weeks. To better evaluate the effect of these secondary disease conditions, the statistical analysis of the mortality data was run twice, first using the original dataset and second using a dataset modified to exclude any fungus-positive fish that had no gross lesions suggestive of an ISAV infection. As no changes in significance between the two datasets were found, only the results from the original, unmodified data were discussed in this paper.

The cumulative daily mortality for the highest virus dose of the CHSE-214-propagated virus is depicted in Figure 2. Atlantic salmon showed a typical acute mortality pattern with the majority of mortalities occurring between 8 - 16 dpi and with the final mortality on day 21 pi. Of the 80 Atlantic salmon in this tank, 64 died and 16 were available for routine sampling. This can be compared with the rainbow trout mortalities, wherein 29 of the original population of 80 died during the trial, with the majority of

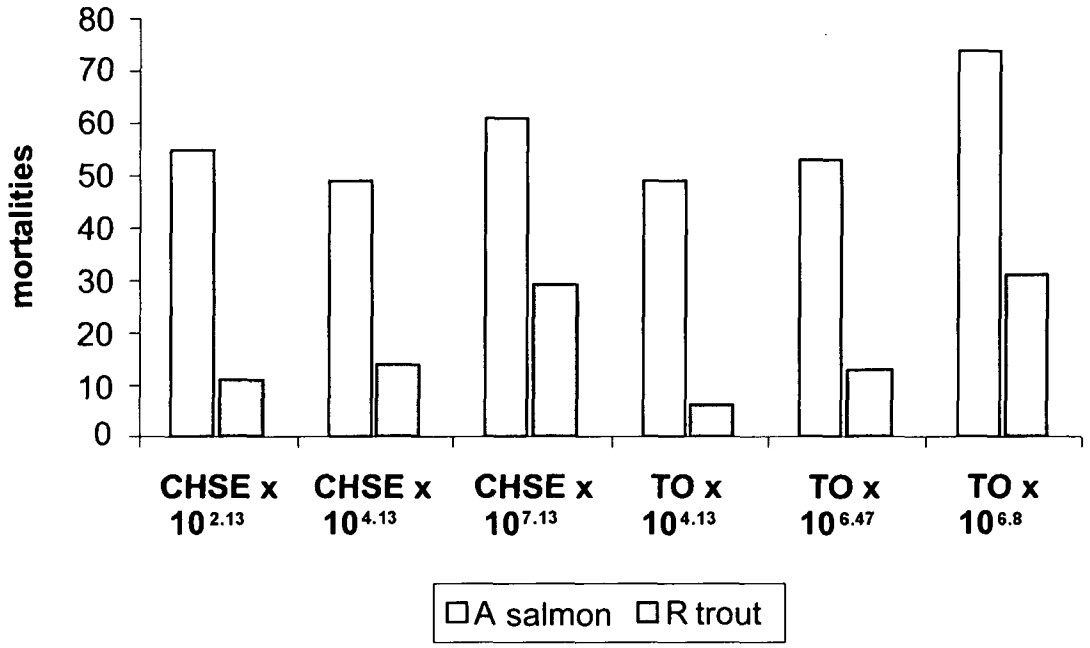

Figure 1. *Salmo salar* and *Oncorhynchus mykiss.* Total mortalities of Atlantic salmon and rainbow trout challenged with three viral doses of infectious salmon anaemia virus (ISAV) grown in two cell lines, CHSE-214 and TO.

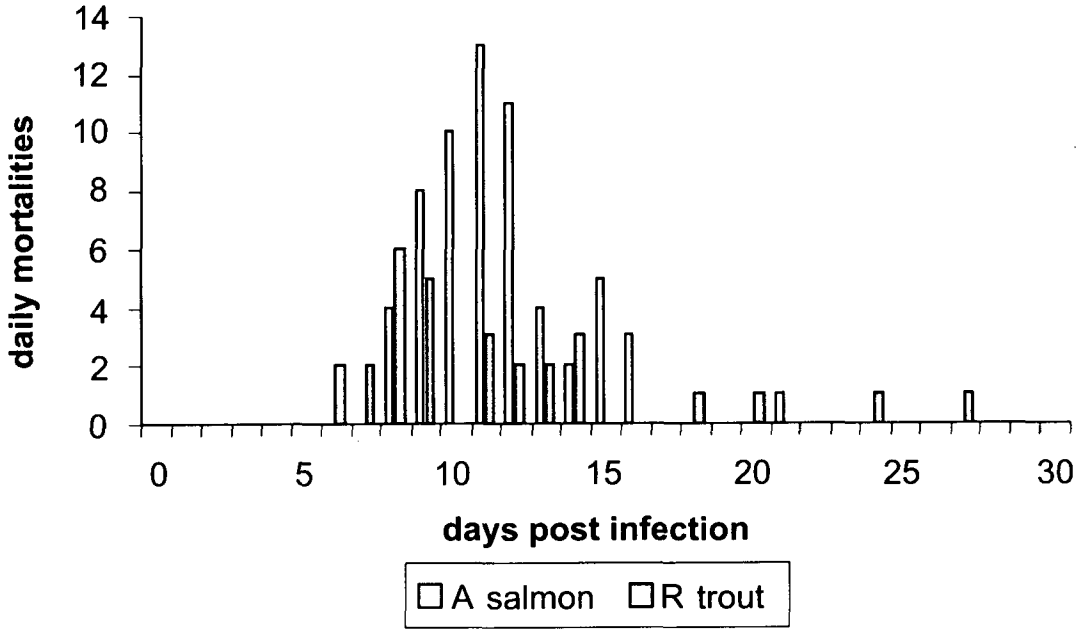

Figure 2. *Salmo salar* and *Oncorhynchus mykiss.* Mortality plot of Atlantic salmon and rainbow trout infected with the virus dose of $10^{7.13}$ $TCID_{50}$/200 µl per fish of CHSE-214-grown infectious salmon anaemia virus (ISAV).

deaths occurring between Days 6 - 14 pi and the final death occurring on Day 27 pi. This can be contrasted with the results observed in the tank infected with low dose CHSE-214-propagated virus. At the lower infective dose, 55 of the 82 Atlantic salmon died during the trial. The majority of deaths occurred between 12 - 22 dpi with the final death on Day 28 pi. For the rainbow trout given the lower infective dose, only 11 of 80 fish died during the trial. No mortality pattern for rainbow trout was discernable, with deaths occurring sporadically between 10 - 46 dpi. Similar patterns and numbers were generated in the tanks infected with TO-propagated virus.

In order to compare the cumulative mortalities between treatments and species, a survival analysis using MTTF was done. This method allows a comparison of the treatments that accounts for the presence of data censoring or rather the random removal of five living fish from each tank at weekly intervals. For example, for the tank with the highest virus dose of the CHSE-214-propagated virus (Figure 2), five rainbow trout were removed weekly for 7 consecutive weeks, totaling 35 fish that did not die, but might have had they not been removed prematurely. Alternatively, only 16 Atlantic salmon from the same tank were available for routine sampling, as the original population of 80 was exhaustively sampled due to high mortalities by 28 dpi.

Three comparisons are possible on data involving two species, two cell lines and three virus doses. Survival analysis was run for each species comparing each dose level of virus propagated in the two different cell lines. Tables 1 to 4 summarize the results of these analyses. The survival analysis for rainbow trout in the tanks inoculated with each of the three CHSE-214-propagated virus doses is presented in Table 1. The Kaplan-Meier Survival Plot corresponding to Table 1 is depicted in Figure 3 as being representative of the data in this study. Log-rank comparison demonstrated a significant difference between the survival curves of the highest virus dose ($10^{7.13}$ $TCID_{50}$) tank versus the middle virus dose $10^{4.13}$ $TCID_{50}$) tank ($p=0.0028$), versus the lowest virus

Table 1. *Oncorhynchus mykiss.* Mean time to failure (MTTF) for rainbow trout infected with three different doses of infectious salmon anaemia virus (ISAV) propagated in the CHSE-214 cell line.

Tank	MTTF	SEM	Lower 95% CI	Upper 95% CI
CHSE x $10^{7.13}$	21.0169[a]	0.9550	19.1453	22.8886
CHSE x $10^{4.13}$	34.1476[b]	1.1142	31.9638	36.3314
CHSE x $10^{2.13}$	42.3494[c]	1.1554	40.0849	44.6139

Significant differences between tanks are denoted alphabetically.

Table 2. *Salmo salar.* Mean time to failure (MTTF) for Atlantic salmon infected with three different doses of infectious salmon anaemia virus (ISAV) propagated in the CHSE-214 cell line.

Tank	MTTF	SEM	Lower 95% CI	Upper 95% CI
CHSE x $10^{7.13}$	12.8918[a]	0.4678	11.9749	13.8087
CHSE x $10^{4.13}$	18.2658[b]	0.6895	16.9145	19.6171
CHSE x $10^{2.13}$	19.7263[b]	0.6109	18.5290	20.9237

Significant differences between tanks are denoted alphabetically.

Table 3. *Oncorhynchus mykiss.* Mean time to failure (MTTF) for rainbow trout infected with three different doses of infectious salmon anaemia virus (ISAV) propagated in the TO cell line.

Tank	MTTF	SEM	Lower 95% CI	Upper 95% CI
TO x$10^{7.8}$	18.4506[a]	0.9649	16.5594	20.3419
TO x$10^{6.47}$	32.5817[b]	0.9736	30.6735	34.4899
TO x$10^{4.13}$	30.0899[b]	0.4610	29.1864	30.9933

Significant differences between tanks are denoted alphabetically.

Table 4. *Salmo salar.* Mean time to failure (MTTF) for Atlantic salmon infected with three different doses of infectious salmon anaemia virus (ISAV) propagated in the TO cell line.

Tank	MTTF	SEM	Lower 95% CI	Upper 95% CI
TO x$10^{7.8}$	9.9481[a]	0.2487	9.4607	10.4356
TO x$10^{6.47}$	15.0873[b]	0.3897	14.3236	15.8511
TO x$10^{4.13}$	19.5962[c]	0.5885	18.4429	20.7496

Significant differences between tanks are denoted alphabetically.

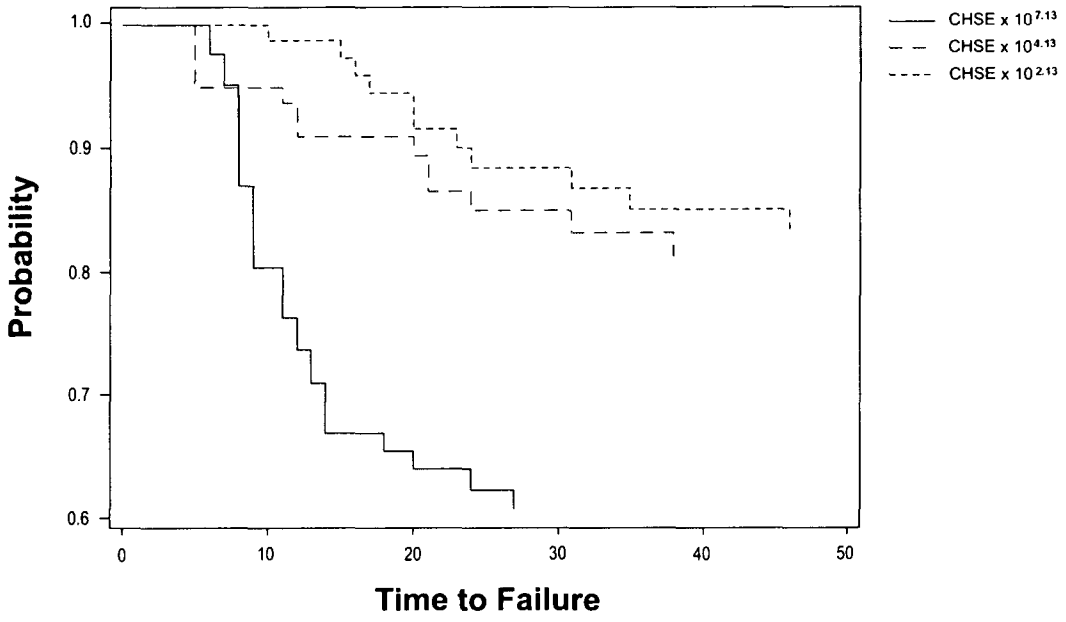

Figure 3. *Oncorhynchus mykiss*. Non-parametric Kaplan-Meier survival plot for mean time to failure (MTTF) of the fish for rainbow trout inoculated with three doses of infectious salmon anaemia virus (ISAV) grown in the CHSE-214 cell line.dpi: days post infection; Solid line: high-dose tank; large dashed line: mid-dose tank; small dashed line: low-dose tank.

dose ($10^{2.13}$ $TCID_{50}$) tank ($p < 0.001$). Similar results were seen for the MTTFs of the Atlantic salmon infected with three doses of ISAV propagated in the TO cell line (Table 3). However, for rainbow trout infected with three doses of ISAV propagated in the TO cell line and for Atlantic salmon infected with ISAV propagated in the CHSE-214 cell line, there were significant differences found between the high dose MTTF and other levels, but the differences found between the MTTFs of the low and mid doses were not significantly different and could be attributed to chance alone.

A second set of comparisons was run between the species within each tank at each infectious dose. Atlantic salmon had a significantly shorter MTTF when compared to rainbow trout ($p < 0.001$) at all treatment levels for both sources of inoculum (virus propagated in TO or CHSE-214 cell line).

Finally, comparison of the MTTFs of the two different virus preparations at $10^{4.13}$ TCID50 did not indicate a significant cell line associated change in virulence for either species of fish. The MTTF for rainbow trout in the TO-propagated virus was 30.09 as compared with the MTTF of 34.15 for rainbow trout inoculated with the CHSE-214-propagated virus ($p=0.071$). For Atlantic salmon, the difference between the MTTFs, 19.6 and 18.27 respectively, was even less ($p=0.205$).

2.3.2. Haematocrit values

Two-way ANOVA determined significant differences at the tank level ($F=14.42$; $df=2$; $p < 0.001$) and Bonferroni pairwise comparisons found that the control tank mean haematocrit value was significantly higher than those of each of the infected tanks. In the rainbow trout control group, the mean haematocrit was 44.78, range 28 to 60 ($n = 37$). The lowest mean haematocrit (Hct = 32.64, range 20 to 42, $n = 36$) was found in the fish inoculated with the high dose TO-propagated ISAV. No significant difference was noted in the haematocrit values taken over the 8 sampling periods, or in the interactions

between infected tanks and date of sampling.

2.3.3. Viral detection using RT-PCR

Table 5 summarizes the results of virus detection by RT-PCR in pooled tissue samples of fish from weekly live sampling fish. No obvious cell line-associated or dose-related patterns were discernable from this data. On Day 7 pi, positive RT-PCR results were obtained for Atlantic salmon in the low virus dose tanks of both cell lines and for rainbow trout infected with the low dose CHSE-214-propagated virus. Day 14 pi had the highest number of RT-PCR positive samples, with 3 of the 6 rainbow trout tissue pools and 2 of the 6 Atlantic salmon tissue pools being positive.

2.3.4. Gross pathology of recent mortalities or moribund fish

The descriptions of the terminal lesions of Atlantic salmon in this study are based on a subset of 12 recent mortalities or moribund fish that were collected for purposes of confirming pathogenicity of the virus and the compatibility of lesions with those characteristic of ISA (O'Halloran *et al.*, 1999). These fish were collected during the period when the majority of mortalities were occurring in the high dose tanks, between 9 and 13 dpi, and included fish inoculated with ISAV propagated in both TO and CHSE-214 cell lines. In contrast, all the recent moralities and moribund rainbow trout collected were examined (total of 27). The rainbow trout died between 6 and 36 dpi, with nine of the 27 fish dying after the initial three weeks of the trial, when the majority of mortalities had already occurred. The gross pathology lesions are summarized in Table 6. The most common lesions in Atlantic salmon were liver congestion, visceral adipose petechiation, ascites, periorbital petechiation, splenic congestion, and pale gills. These experimentally-induced ISA lesions mimic gross lesions that have been observed in Atlantic salmon retrieved from ISA-infected cage sites in the Bay of Fundy (Mullins *et al.*,

Table 5. *Oncorhynchus mykiss* and *Salmo salar.* Summary of RT-PCR results for detection (+/-) of infectious salmon anaemia virus (ISAV) in pooled samples of 5 fish per tank (CHSE-214- or TO-propagated virus doses) at each sampling interval.

dpi	CHSE-214 x $10^{2.13}$		CHSE-214 x $10^{4.13}$		CHSE-214 x $10^{7.13}$		TO x $10^{4.13}$		TO x $10^{6.47}$		TO x $10^{7.8}$	
	RT	AS	RT	AS	RT	AS	RT	AS	RT	AS	RT	AS
7	+	+	-	-	-	-	-	+	-	-	-	-
14	+	-	-	-	+	+	+	-	-	-	-	+
21	-	-	-	-	-	+	-	-	-	-	-	ND
28	-	-	-	-	-	-	+	-	-	-	-	ND
35	-	-	+	+	-	ND	-	-	+	ND	-	ND
42	-	ND	-	-	-	ND	-	-	-	ND	-	ND
49	-	ND	-	ND	+	-	-	ND	-	ND	-	ND
56	-	ND	-	ND	-	ND	-	ND	-	ND	-	ND

Dpi: days post infection; RT: rainbow trout; AS: Atlantic salmon; ND: not done

Table 6. *Salmo salar* and *Oncorhynchus mykiss*. Prevalence (Prev.) of gross lesions in recent mortalities or moribund fish experimentally infected with infectious salmon anaemia virus (ISAV).

Gross pathologic lesion	Atlantic salmon (n = 12)		Rainbow trout (n = 27)	
	Prev.	%	Prev.	%
Liver congestion	12	100.0	6	22.2
Visceral adipose petechiation	11	91.7	12	44.4
Ascites	10	83.3	11	40.7
Periorbital petechiation	8	66.7	3	11.1
Splenic congestion	7	58.3	4	14.8
Pale gills	6	50.0	0	0.0
Exophthalmos	4	33.3	12	4.4
Fin base haemorrhage	1	8.3	14	51.9
Cardiac congestion	1	8.3	0	0.0
Pyloric caeca congestion	1	8.3	3	11.1

1998;O'Halloran *et al.*, 1999). Of these, only visceral adipose petechiation and ascites were commonly observed lesions among the rainbow trout mortalities. Conversely, the infrequent Atlantic salmon gross lesions of fin base haemorrhage and exophthalmos were common in the rainbow trout mortalities.

2.3.5. Histopathology of recent mortalities or moribund fish

Liver, anterior kidney, heart and pyloric caeca were examined by histopathology. The histopathologic lesions are summarized in Table 7. In contrast to Atlantic salmon, the rainbow trout very rarely (2 of 27) had liver necrosis. Figure 4 shows the most common microscopic lesion in the rainbow trout, which consisted of uni- to multi-cellular degeneration of an unknown cell type in the haematopoietic portion of the kidney. In contrast to Atlantic salmon which had no epicarditis/endocarditis, 5 of 27 rainbow trout had cardiac lesions ranging from mild epicarditis/endocarditis to severe endocarditis with adjacent degeneration of the compact myocardium of the ventricle. Figure 5 shows this myocardial involvement. The first cardiac lesion was evident in a fatally infected rainbow trout 6 dpi, but the most severe cardiac lesion was found in a mortality on Day 35 pi. However, from the microscopic evaluation of these myocardial lesions, the pathogenesis of lesion development could not be determined.

2.3.6. Gross pathology of fish from serial sampling

The presence of gross lesions in the live-sampled rainbow trout followed a similar pattern as the mortality curves. For example, visceral adipose petechiation and ascites were found in samples collected between 14 and 35 dpi; peaking at 14 and 21 dpi, then present in decreasing frequency in the surviving fish. Additionally, gross cardiac changes were noted only at 35 dpi, consistent with the chronic, progressive nature of cardiac lesions demonstrated in the fatally infected rainbow trout. The more nonspecific lesion of

Table 7. *Salmo salar* and *Oncorhynchus mykiss.* Prevalence (Prev.) of histopathologic lesions in recent mortalities or moribund fish experimentally infected with infectious salmon anaemia virus (ISAV).

Histopathologic lesion	Atlantic salmon (n = 12)		Rainbow trout (n = 27)	
	Prev.	%	Prev.	%
Liver congestion and haemorrhage	*10*	83.3	*11*	40.7
Liver necrosis	8	66.7	2	7.4
Renal haematopoietic cell degeneration	8	66.7	25	92.6
Increased erythrophagocytosis	8	66.7	22	77.8
Pyloric caeca congestion and haemorrhage	7	58.3	4	14.8
Renal congestion and haemorrhage	2	16.7	4	14.8
Cardiac endothelial cell hypertrophy	2	16.7	4	14.8
Epicarditis/endocarditis	0	0.0	5	18.5
Renal necrosis	0	0.0	1	3.7

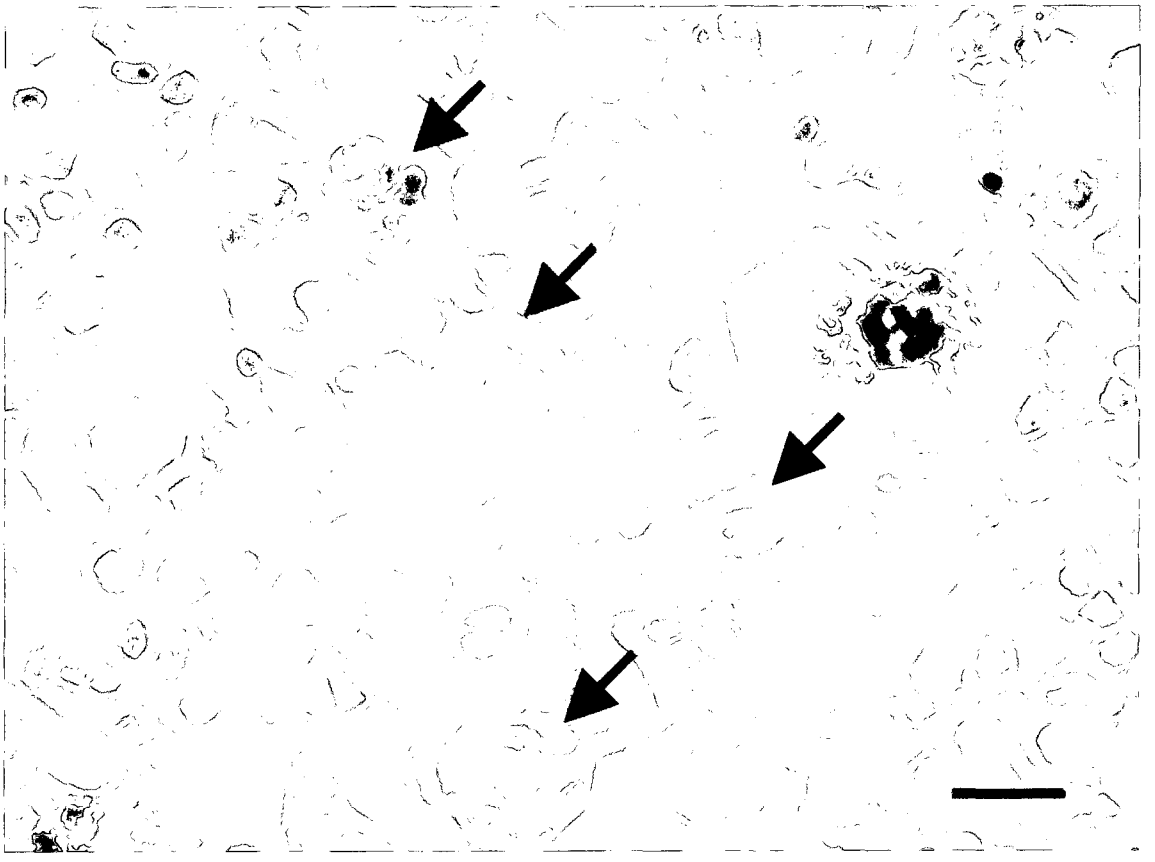

Figure 4. *Oncorhynchus mykiss*. Area of haematopoietic cellular degeneration (arrows) within the head kidney, with adjacent normal architecture; scale bar = 20 µm.

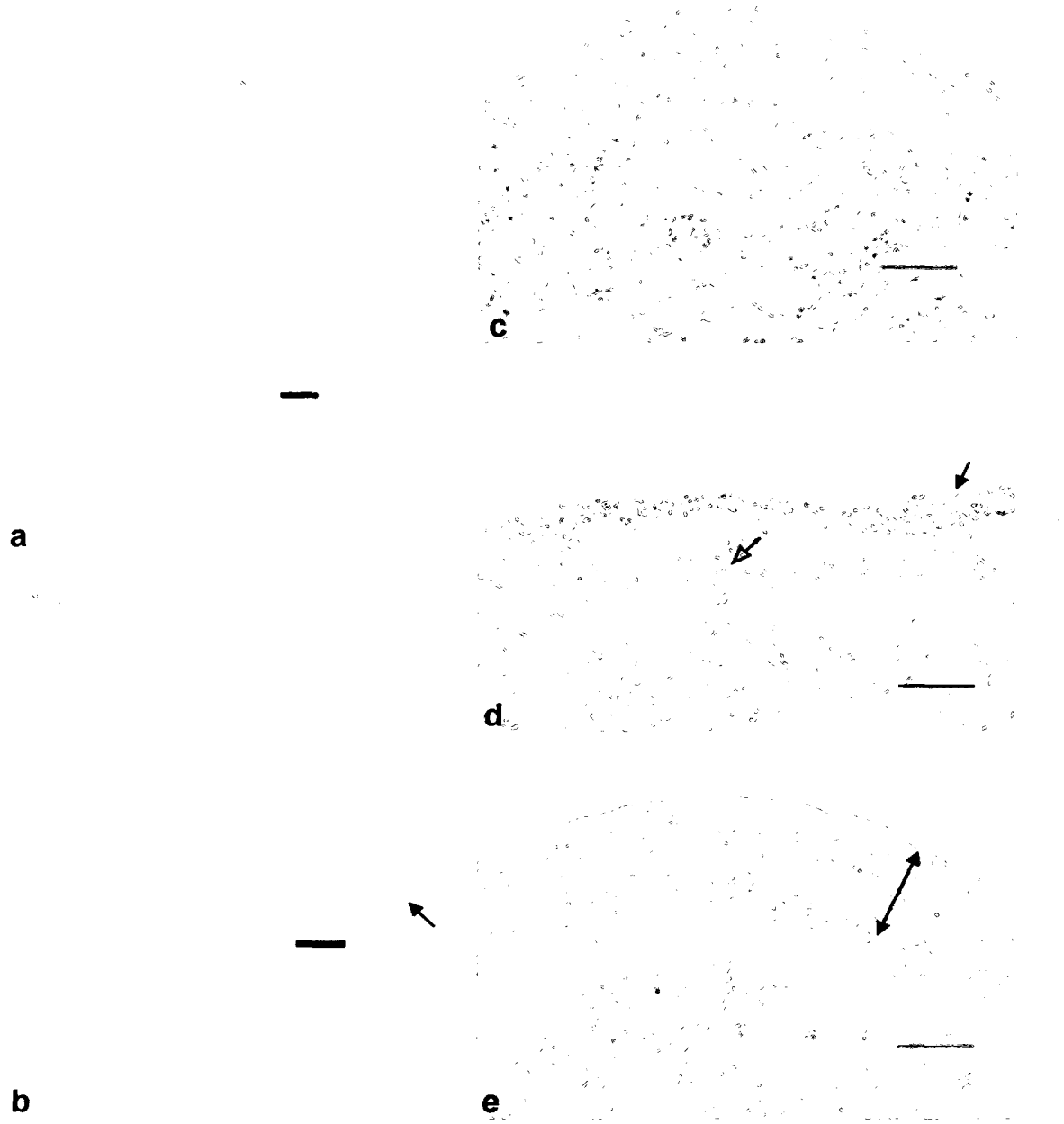

Figure 5. *Oncorhynchus mykiss*. (a) Ventricle from normal fish; bar = 200 µm. (b) Ventricle form infectious salmon anaemia virus (ISAV)-infected fish showing inflammatory cell infiltrate to the epicardium and apparent loss of the adjacent compact layer (arrow); scale bar = 200 µm. (c) Ventricle from normal fish at higher magnification, showing pericardium, compact and spongy layers; scale bar = 100 µm. (d) Ventricle from ISAV-infected fish showing mild inflammatory cell infiltration of the epicardium and area of myositis within the compact layer (open arrow); scale bar = 100 µm. (e) Ventricle from ISAV-infected fish showing epicarditis and endocarditis extending through the compact layer (double-headed arrow); scale bar = 100 µm.

fin base haemorrhage followed a different pattern, as it remained present at a relatively constant level throughout the weeks of sampling, but in the later weeks was accompanied by mild fin erosion suspected to be related to either a mild fungal infection or due to mechanical irritation of the small diameter fibreglass coated tanks.

2.3.7. Histopathology of fish from serial sampling

For microscopic examination, a rating scale (Moneke *et al.*, 2005b) for ISAV-related rainbow trout lesions was minimally revised (see Appendix 5.1) based on the findings of the recent mortalities or moribund fish summarized in Table 6, page 41. This rating scale was applied to the fish sampled weekly in an attempt to discern the development of the lesions. The histologic examination was performed on tissues from fish sacrificed at weekly intervals for the first 4 weeks of the infection (7, 14, 21 and 28 dpi). These times were chosen because they flanked the time of death of the majority of fish. Additionally, only tissues of fish inoculated with virus propagated in the CHSE-214 cell line were evaluated. These were chosen since the very high cumulative mortalities of Atlantic salmon in the highest virus dose of TO-propagated ISAV limited the number of survivors for sampling to the first 3 weeks post challenge. Limiting detailed histologic evaluation to the fish infected with CHSE-214-propagated ISAV was further justified since the survival analysis of the two different virus preparations at $10^{4.13}$ TCID50 did not indicate a significant cell line associated change in virulence for either species of fish (for rainbow trout p= 0.071; for Atlantic salmon p=0.205) as reported above. All slides were randomized and read blind.

The histopathologic lesions observed in the live-sampled fish are summarized in Table 8. A subsample of uninfected control tank fish from both species was included to assess pre-existing lesions. The lesions in Atlantic salmon were consistent with those previously reported for ISA, with the most prominent finding of focally extensive to

Table 8. *Salmo salar* and *Oncorhynchus mykiss*. Prevalence (Prev.) of histopathologic lesions in fish sampled at Days 7, 14, 21, and 28 days after infection with ISAV.

Histopathologic lesions	Atlantic salmon			Rainbow trout		
	Control (n = 8) Prev.	$10^{2.13}$ (n = 8) Prev.	$10^{7.13}$ (n = 8) Prev.	Control (n = 8) Prev.	$10^{2.13}$ (n = 8) Prev.	$10^{7.13}$ (n = 8) Prev.
Renal haematopoietic cell degeneration	0	3	4	2	7	12
Increased erythrophagocytosis	1	3	3	2	6	12
Cardiac endothelial cell hypertrophy	2	3	3	3	6	10
Liver congestion and haemorrhage	0	1	2	0	0	0
Liver necrosis	0	2	1	0	0	0
Renal congestion and haemorrhage	0	0	2	0	0	0
Pyloric caeca congestion and haemorrhage	0	2	1	0	0	0
Pericholangitis	0	0	0	10	10	7

bridging areas of acute haemorrhagic necrosis in the liver (Evensen & Thorud, 1991). In the rainbow trout, no liver lesions were observed and there was no evidence of congestion in the renal interstitium or the lamina propria of the foregut. The main microscopic lesions in the rainbow trout were renal haematopoietic cellular degeneration, increased erythrophagocytosis as assessed within the interstitium and vasculature of the anterior kidney, and cardiac endothelial cell hypertrophy/cellularity. These lesions were seen with an apparent virus dose-dependent incidence in the challenged fish. Mild pericholangitis was seen within the livers of rainbow trout regardless of sampling date or viral dose used. As this lesion was consistently mild and ubiquitous, it is considered an incidental finding.

2.4. Discussion

Although it has been widely reported that experimental ISAV infection of salmonids other than Atlantic salmon results in asymptomatic infection, those studies were based on single ISAV isolates that did not encompass the wide spectrum of ISAV virulence (Nylund *et al.*, 1994; Nylund & Jakobsen, 1995; Rolland & Nylund, 1998; Snow *et al.*, 2001; Nylund *et al.*, 2002; Rolland & Winton, 2003). In an attempt to identify and characterize the correlates of pathogenicity of ISAV, researchers in my lab have assessed the relative infectivity of 13 different ISAV isolates in 3 farmed fishes, Atlantic salmon, coho salmon, and rainbow trout. It was shown that the ISAV isolates that are highly pathogenic for Atlantic salmon also cause mortality in rainbow trout (Kibenge, 2004; Kibenge *et al.*, 2006). The experimental reproduction of ISAV-induced mortality in rainbow trout with ISAV isolate NBISA01 in the present study, conclusively demonstrates the risk of inter-species transmission and pathogenicity of this isolate. The present study describes investigations of the pathology associated with the experimental ISAV

infection of rainbow trout. The limited nature of the gross and microscopic pathology lesions seen in the infected rainbow trout indicates that this species is highly resistant to ISAV infection. As these lesions differ from the typical necrosis of the liver and kidney that occur in Atlantic salmon with ISA, if these lesions were viewed in rainbow trout in field conditions, it is unlikely that ISA would be considered as a differential diagnosis.

Regarding the deaths of the rainbow trout, there were insufficient numbers in the low- and mid-virus dose tanks from either cell line to discern any pattern other than sporadic, intermittent mortalities over time. This differs from the Atlantic salmon experimental infection model, where fish either die acutely or survive. While it is our interpretation that rainbow trout that are dying later than expected are succumbing to chronic disease processes, it could be argued that cohabitation with highly susceptible Atlantic salmon, presumably shedding substantial quantities of virus into the environment, provided a reservoir for re-infection. Virus detection using RT-PCR in the weekly live sampling showed the highest number of positive samples at Day 14 pi, which coincided with the timing of the majority of acute mortalities when viral replication would be expected to be high. However, it is of interest to note that the 8 RT-PCR positive rainbow trout samples were not clustered in the early weeks of the trial, indicating possible viral replication as late as Day 49 pi in the high virus dose CHSE-214 tank.

A subset of Atlantic salmon was chosen to demonstrate that the lesions seen in this trial following intraperitoneal injection of ISAV were consistent with those lesions described in the literature (Thorud & Djupvik, 1988; Evensen & Thorud, 1991; Speilberg *et al.*, 1995; Totland *et al.*, 1996). In other words, this species was included as a disease control to increase the validity of any findings in rainbow trout. The lesions seen in the Atlantic salmon subset were typical of ISAV lesions in previous experimental infections (Dannevig *et al.*, 1994; Moneke *et al.*, 2003). Similar gross lesions were seen in infected rainbow trout, but the distribution of the lesions was very different from those of Atlantic

salmon. Fin base haemorrhage (52%) was the most common finding, followed by visceral adipose petechiation (44%), exophthalmia (44%), ascites (41%), liver congestion (22%), splenic congestion (15%) and periorbital petechiation (11%). Additionally, the haemorrhagic lesions in rainbow trout were less severe than those seen in Atlantic salmon and generally had a more limited distribution.

The histopathologic lesions in rainbow trout were very different from the typical necrosis in liver and kidney that occurred in Atlantic salmon; liver disease in rainbow trout was seen at a relatively low level as compared to Atlantic salmon; liver disease in rainbow trout was seen at a relatively low level as compared to Atlantic salmon. Sinusoidal congestion was seen in 40% and necrosis in only 8% of the rainbow trout mortalities examined. However, 41% of these fish had ascites. It has been hypothesized that portal hypertension secondary to peliosis-like hepatic lesions in Atlantic salmon plays a role in the development of ascites (Evensen & Thorud, 1991; Speilberg *et al.*, 1995) but in the rainbow trout mortalities in the present study, there was a higher incidence of ascites than liver lesions. Cardiac dysfunction in Atlantic salmon, as demonstrated by a decreased Frank-Starling response, has been documented as part of the pathology of ISA, despite normal cardiac morphology (Gattuso *et al.*, 2002). It was found that cardiac endothelial damage effects on nitric oxide homeostasis resulted in decreased cardiac output and stroke volume in response to increased filling pressures. This endothelium-related response to ISAV would also be expected in rainbow trout, but additional cardiac dysfunction and vascular flow compromise secondary to the cardiac lesions in this species may account for the development of ascites in the absence of significant liver disease. Nineteen percent (5/27) of these rainbow trout had epicarditis of varying severity with adjacent myocarditis in the more severely affected fish. Surprisingly, this lesion was not seen in any of the live-sampled fish (rainbow trout or Atlantic salmon), and it is not a lesion previously associated with ISA in Atlantic salmon.

Differential diagnoses for epicarditis in salmonids include cardiomyopathy syndrome, heart and skeletal muscle inflammation and salmon pancreas disease. However, the signalment (rainbow trout parr) and lack of involvement of the exocrine pancreas, skeletal musculature and spongy layer of the ventricle are sufficient to conclude that the clinical signs demonstrated in this infection trial are inconsistent with these diseases.(Rodger & Turnbull, 2000; Desvignes *et al.*, 2002; Poppe & Seierstad, 2003; Kongtorp *et al.*, 2004) It is our considered opinion that this unique lesion is characteristic of fatal ISAV-infection of rainbow trout.

Within the kidney, interstitial congestion of the haematopoietic tissue of the head kidney (15%) and renal necrosis (4%) were of low incidence in the infected rainbow trout mortalities. Renal haematopoietic cellular degeneration, which has previously been reported in ISAV outbreak of a commercial Atlantic salmon farm (Evensen & Thorud, 1991), was a frequent finding in both species (92% of rainbow trout and 67% of Atlantic salmon) but its clinical relevance is presently unknown. Increased erythrophagia was also common in both species (80% of rainbow trout and 67% of Atlantic salmon). Based on counts of erythrophagocytosis per 5 high powered fields in the rainbow trout, the highest levels were seen in the fatally infected fish. But among the routine sampled fish, a pattern was evident in that there was an increase in severity of erythrophagocytosis, with higher levels seen in the survivors of the CHSE-214-propagated ISAV high dose tank as compared to the CHSE-214-propagated ISAV low dose tank as compared over time (data not shown). The prominence of this lesion in infected rainbow trout that did not die or took a long time to die has led us to speculate that it may be indicative of an antiviral response by a resistant host to the ISAV infection.

Haematocrit evaluations from the rainbow trout populations determined that the ISAV-infected tanks had mean haematocrit values that were significantly lower than those of the control rainbow trout. Among the infected rainbow trout groups, the reduced

haematocrit values persisted throughout the duration of the experiment without rebounding to normal levels as has been shown previously.(Nylund *et al.*, 1997; Snow *et al.*, 2001) While a significant difference was found between the reduced haematocrit values in infected rainbow trout as compared to the controls, it should be noted that none of the rainbow trout that died during this trial (between 6 and 36 dpi) demonstrated pale gills, supporting the absence of anaemia.

The ISAV isolate NBISA01 used in this study was highly pathogenic for Atlantic salmon as previously reported (Kibenge *et al.*, 2004; Kibenge *et al.*, 2006). In the present study, the virus produced a percent cumulative mortality of 88.09% (74/84) in the highest virus dose group of Atlantic salmon. Among the infected rainbow trout, the percent cumulative mortality in the highest virus dose group was 38.8% (31/80), slightly lower than what we previously observed (Kibenge *et al.*, 2004; Kibenge *et al.*, 2006), although the difference might simply be due to the larger number of fish used in the present study (i.e. 80 versus 10) or be related to the weekly sampling removing fish that might have died if left undisturbed. Survival analysis was used to compare the pathogenicity of different doses of ISAV, the effect of the cell line used to propagate the virus on the virulence of the virus, and the relative susceptibility of different fish species to ISA. The MTTF, the time at which there is a 50% probability of death of the study population, was significantly lower for both species in the higher virus dose than the lower virus dose tanks regardless of which cell line, TO or CHSE-214, the virus had been propagated in. Moreover, Atlantic salmon had a significantly shorter MTTF when compared to rainbow trout at all treatment levels for both sources of viral inoculum.

Not all ISAV isolates are cytopathic in the CHSE-214 cell line (Kibenge et al., 2000). As this cell line is derived from Chinook salmon belonging to the same genus, *Oncorhynchus*, as rainbow trout, we hypothesized that the cell line used to propagate ISAV could influence its virulence, and further that the resistance of rainbow trout to

ISAV infection reported in all previous experimental challenges might be overcome if the virus was grown in a closely related cell line. However, log-rank comparisons of the MTTFs for the virus propagated in TO or CHSE-214 cell line at the same virus dose ($10^{4.13}$ $TCID_{50}$ /200µl per fish) showed no significant cell line-associated difference in either fish species. In fact, the MTTF for rainbow trout was lower in the TO grown virus than in the CHSE-214 grown virus, MTTF = 30.09 and 34.15, respectively (p=0.071). For Atlantic salmon, the difference was even less, 19.6 and 18.27, respectively (p=0.205), probably because of their higher susceptibility to ISAV.

This is the first pathogenesis study detailing the pathology associated with experimental clinical ISAV infection in rainbow trout. In contrast to the acute experimental disease experienced in Atlantic salmon, in rainbow trout there is evidence of both acute and protracted pathogenesis. As the lesions in rainbow trout are very different from the typical necrosis in liver and kidney that occur in infected Atlantic salmon, if these lesions were viewed in rainbow trout under field conditions, it is unlikely that ISA would be considered as a differential diagnosis. Moreover, some of the lesions, like erythrophagia, may be indicative of an antiviral response by a resistant host to the ISAV infection.

3. USE OF *IN SITU* HYBRIDIZATION AND REAL TIME RT-PCR TO CHARACTERIZE THE PATHOGENESIS OF INFECTIOUS SALMON ANAEMIA VIRUS (ISAV)-INDUCED LESIONS IN RAINBOW TROUT, *ONCORHYNCHUS MYKISS*

* portions of this chapter appear in:

MacWilliams, C., Johnson, G., Groman, D. & Kibenge, F. S. (2007). Morphologic description of infectious salmon anaemia virus (ISAV)-induced lesions in rainbow trout Oncorhynchus mykiss compared to Atlantic salmon *Salmo salar*. *Dis Aquat Organ* 78: 1-12.

3.1. Introduction

In situ hybridization (ISH) utilizes a specifically designed nucleic acid probe to detect complementary nucleic acid sequences. When applied to a complex tissue milieu, ISH elucidates the macroscopic distribution and cellular localization of targeted nucleic acid sequences, medically valuable in the study of the pathogenesis of infectious agents. ISH has been applied to Atlantic salmon tissues which had been either naturally or experimentally infected with ISAV (Gregory, 2002; Moneke *et al.*, 2003). In these studies, ISH was found to be very specific and allowed the localization of ISAV mRNA in the vascular endothelium and within macrophage-like cells, with the strongest hybridization signals seen in the endothelium of the heart and liver. The working hypothesis of our current experiment was that ISH would reveal a different or lower tissue tropism of ISAV mRNA within challenged rainbow trout as compared to Atlantic salmon. It was hoped that this could give insight into the lower morbidity evidenced by ISAV experimentally infected rainbow trout and the differing gross and histologic lesions characterized in Chapter 2. Secondarily, we wished to determine if there is a correlation between viral presence and lesions in this species. Specifically, we hoped to see if ISAV mRNA would be localized with the degenerating cells within the head kidney. Another key area of focus for this study was to determine the presence and distribution of ISAV mRNA within the heart, as the ISAV-associated cardiac lesions seen in Chapter 2 are thought to represent a unique disease expression in rainbow trout.

To accomplish these tasks, we constructed regions of each of three ISAV segments: 8, 6 and 2 for use as riboprobes. Signal intensity was highest for ISAV Segment 8 probe, with probe variability presumed to be related to the differences in probe length and the guanine:cytosine (G:C) content given the standardized hybridization conditions. In order of declining signal strength, tissue signal localization

was found to be highest in the heart, followed by the liver, kidney, spleen, intestine and pyloric caeca. A similar tissue and cellular distribution was seen following an experimental ISAV infection challenge of Atlantic salmon (Moneke *et al.*, 2003; Moneke *et al.*, 2005a).

In the TO and SHK-1 cell lines, ISAV Segment 8 riboprobe demonstrated nuclear localization one day after ISAV inoculation, progressing to a perinuclear localization three days later, and by predominantly cytoplasmic localization one week following ISAV inoculation (Moneke *et al.*, 2003). This temporal distribution indicates a replication strategy consistent with that of other members of the *Orthomyxoviridae*. Further, ISH was able to detect replicating virus earlier than virus isolation, as CPE in the ISAV-inoculated TO cells was not evident until 3 days post inoculation. ISH has also been used to demonstrate a positive correlation between peak viremia (as indicated by presence of viral signal in both tissues and in circulating cells within blood vessel lumens), gross lesion development and onset of mortality (Moneke *et al.*, 2005b).

Quantitative real time PCR is a sensitive detection method and valuable tool for the detection of nucleic acids in low copy number, as may be seen in subclinical or latent infections. Real time RT-PCR for ISAV has been shown to be at least 100x more sensitive than conventional one tube RT-PCR for the detection of ISAV mRNA from infected cell cultures (Munir & Kibenge, 2004). Further, this technique has been used to detect ISAV mRNA within various organs of rainbow trout surviving an experimental challenge with ISAV. At 76 days post infection, real time RT-PCR was able to detect ISAV mRNA in each of six tissues, including two (heart and gills) which had been found negative by conventional RT-PCR, leading the authors to conclude that this technique may be of use in detecting subclinically infected fish (Munir & Kibenge, 2004).

Real time PCR uses commercially available fluorescence-detecting thermocyclers to amplify specific nucleic acid sequences and simultaneously measure

the amplification product concentrations. In the LightCycler with glass capillary format (Roche Applied Science), amplification reactions are set up in sealed glass capillaries that are heated and cooled in an airstream, significantly reducing the time needed for each thermal cycle. The capillaries are rotated past a fluorometer each cycle. The resulting fluorescence data is then converted into an amplification plot for each of the reactions, documenting the rate of accumulation of DNA product over the whole course of PCR. This is an advantage of real time PCR. As quantification occurs during the exponential phase of amplification, when none of the reaction components should as yet be limited, the results should be more reliable than those based on end-point analysis.

The initial concentration of target sequences can be expressed as the PCR cycle time or threshold cycle (C_T) at which the PCR product's fluorescent signal exceeds the average background signal. The higher the amount of target nucleic acid within the sample, the lower number of PCR cycles will be needed for fluorescence to exceed background levels. The target sequences may then be quantified via comparison with a DNA standard of known copy number.

Several reporter systems are available for real time PCR. In this work, SYBR Green 1, a non-specific DNA binding dye will be utilized. SYBR Green 1 intercalates into any nucleic acid duplex and, once bound, emits greatly enhanced fluorescence, which is proportional to the total mass of DNA generated during PCR. However, SYBR Green 1 will also bind to any nonspecific amplification products and primer-dimers and therefore may generate significant errors in quantifying targets present in low copy number. To offset the uncertainty associated with use of a nonspecific reporting dye, melting point analysis can be used as an indicator of product specificity. The thermocycler/fluorometer can be programmed to generate a thermal denaturation curve (melting curve) of the PCR products at the completion of the PCR cycle. Further confirmation of the specificity of PCR product can be done by size-fractionation using agarose gel electrophoresis as

compared to a positive control reaction product.

The major advantages of real time PCR as compared to conventional PCR include the ability to precisely measure the concentrations of target nucleic acids in a relatively short period of time; high sensitivity; the capacity to process many samples simultaneously; information on reaction kinetics; and reduction of handling steps which in turn reduces the risk of inadvertent sample contamination. Our intention with real time RT-PCR was to determine if there is evidence of viral persistence following ISAV infection of rainbow trout.

3.2. Methods and materials

3.2.1. Virus and cell culture

The ISAV isolate NBISA01 (Jones *et al.*, 1999) was propagated in TO cell line as previously described (Kibenge *et al.*, 2000b) Virus titration was performed on 24 hour-old cell monolayers in 48-well plates and the virus titre (expressed as $TCID_{50}$/100µl) was determined from endpoint cytopathic effects (CPE) (Reed & Muench, 1938).

3.2.2. Fish and rearing conditions

A total of 115 rainbow trout, Kamloops strain (mean weight 10 g) certified as specific pathogen-free were obtained from Trout Lodge via the Dover Fish Hatchery, Prince Edward Island, Canada. The fish were maintained in the Aquatic Animal Facility of the Atlantic Veterinary College in a 1 m diameter fibreglass-reinforced plastic tank using a fresh water flow through system at a temperature of approximately 11°C. Fish were feed to satiation twice daily. The fish were acclimatized for 30 days before use. No signs of illness were detected during this period.

3.2.3. Experimental infection of rainbow trout with ISAV and sampling

The experimental procedures were performed in accordance with the guidelines of the

Canadian Council of Animal Care (1993; 2005). The fish were anaesthetized by immersion in an aerated solution of tricaine methanesulfonate (TMS) (100 mg/L). Five uninfected fish were randomly chosen as negative controls; these were euthanized by anaesthetic overdose, and necropsied: gross lesions were recorded and tissue samples were collected from the heart, head kidney, posterior kidney, liver and pyloric caeca. These tissue samples were then bisected aseptically. Half of each tissue was fixed in 4% neutral-buffered formaldehyde, and the other half was pooled (excluding the pyloric caeca), placed in a whirlpac bag and stored at -80°C until used for viral analysis by RT-PCR. The formalin-fixed tissues were processed in an automatic tissue processor (Sakura, TissueTekR VIP, Torrance, California) and embedded in paraffin wax within 12-24 hours of fixation. Serial 5-6 µm thick sections of the tissues were either stained with haematoxylin and eosin (H & E) and examined by light microscopy or placed on silane coated DEPC-treated slides in preparation for ISH. 106 rainbow trout were infected by intraperitoneal injection of $10^{6.05}$ $TCID_{50}$/200ul/fish.

All fish were observed hourly during the 14 hour period of 'daylight' after the onset of clinical signs of illness. Fresh mortalities (collected within 1 hour of death or less) or severely moribund fish (euthanized by blunt cranial trauma) were removed from the tanks for postmortem examination and tissue sampling. In all cases gross lesions were recorded by one of two examiners, and tissues (head kidney, liver, heart and pyloric caeca) were individually sampled. Samples for virus analysis were not collected from these fish, as the presence of post mortem lesions found in conjunction with clinical illness were expected to be sufficient to diagnose ISA. Additionally, starting on Day 13 pi, opportunistic sampling was done of three fish per day, three days a week to allow quantification of viral nucleic acids in the subclinically infected fish by real time RT-PCR. These fish were anaesthetized by immersion in an aerated solution of tricaine methanesulfonate (TMS) (100 mg/L), then sacrificed and subjected to the same

postmortem and tissue sampling protocol as the clinically infected fish. A subsample of these was chosen for real time RT-PCR viral quantification.

3.2.4. RNA extraction

Viral RNA was extracted from 300µl volumes of pooled tissue homogenates using Trizol LS reagent (Invitrogen Life Technologies, Carlsbad, California) following the manufacturer's protocol.

3.2.5. Reverse transcription polymerase chain reaction

The primer pair used to generate PCR product was ISAV F5 and ISAV R5 for ISAV Segment 8. RT-PCR reactions were carried out on the extracted RNA as previously described using Titan One Tube RT-PCR System Kit (Roche Diagnostic Corporation, Indianapolis, Indiana) (Kibenge *et al.*, 2000b). The Peltier PTC-200 DNA Engine thermal cycler (MJ Research Inc., Waltham, Massachusetts) was used. The conditions of cycling consisted of one cycle at 55°C for 30 minutes, then at 94°C for 2 minutes for cDNA synthesis; followed by 40 cycles of denaturation at 96°C for 30 seconds, annealing at 61°C for 45 seconds and extension at 72°C for 90 seconds; with a final extension at 72°C for 10 minutes. The RT-PCR products were resolved by electrophoresis in 1 x Tris acetate EDTA buffer (40mM Tris acetate and 1 mM EDTA) (Fisher Scientific) on a 1% agarose gel containing 1µg ethidium bromide and visualized under 304nm UV light (Sambrook *et al.*, 1989).

3.2.6. Riboprobe preparation

The pGEM-3Z vector carries a gene conferring ampicillin resistance and a second selection marker, the lacZα-peptide which has been engineered to contain a multiple cloning region flanked by both the SP6 and T7 RNA polymerase promoters. Therefore

this vector has two advantages: blue/white screening for transformants and the potential for *in vitro* transcription of RNA from either strand through the use of dual-opposed promoters and the appropriate RNA polymerase (T7 or SP6). The flanking promoters allow the production of both sense and antisense probes. Antisense sequences will be complementary to the target mRNA within the tissue, while sense probe can be utilized as a negative control having the same sequence as the mRNA. The correct orientation with respect to the SP6 promoter was identified by Moneke *et. al.* (2003) using restriction digestion analysis using the *Kpn* 1 restriction endonuclease (cuts at site 21, downstream of the T7 promoter). Agarose gel electrophoresis allowing visualization of the expected size of the DNA, was used to determined that the antisense riboprobe could be generated via the T7 promoter. Stock cultures of ISAV Segment 7 cloned in the correct orientation into the pGEM-3Z plasmid were preserved by mixing in a 1:1 ratio with gylcerol freezing solution in a cryovial and stored at -80°C. Plasmid DNA was isolated using the midi preparation technique (see Appendix for complete description). Briefly, 50ml of 2 x YT broth with 100µg/ml ampicillin were inoculated with *Escherichia coli* DH5α (GIBCO BRL/Life Technologies, Burlington, Ontario and Gaithersburg, MD) containing recombinant plasmid (pGEM-3Z and ISAV Segment 7 insert in the EcoR1 site) and incubated overnight on a shaker (180 rpm) at 37°C. Bacteria were harvested by centrifugation at 10 000 rpm for 5 minutes using a JA-14 rotor (Spinco, Division of Beckman Instruments, Palo Alto, CA). The pellet was resuspended in 2 ml of freshly prepared lysis buffer (0.5 gm lysozyme/10 ml of lysozyme buffer, vortexed and kept at room temperature for 5 minutes. 4 ml of freshly prepared alkaline solution (1% SDS in 0.2 N NaOH) was added and the tube was mixed by inverting 3-6 times and kept on ice for 5 minutes. 72 µl of 5 mg/ml RNase A stock was added followed by a 15 minute incubation on ice. 3 ml of 7.5 M ammonium acetate solution was added and the contents of the tube were mixed for a few seconds prior to 10 minute incubation on ice.

Centrifugation at 15,000 rpm for 30 minutes using the JA 14 rotor results in large insoluble pellet of bacterial proteins, cell wall fragments and denatured chromosomal DNA, leaving the plasmid DNA in suspension. The supernatant containing the plasmid DNA was carefully harvested and transferred to smaller tubes. 2 volumes of cold 100% ethanol were added and precipitation was allowed overnight at -80°C. The next day, centrifugation was performed at 15,000 rpm for 30 minutes using a JA-20 rotor (Spinco, Division of Beckman Instruments, Palo Alto, CA). The supernatant was discarded and the pellet resuspended in 1 ml of TE buffer, pH 8.0 and the contents were transferred to a microfuge tube on ice. 400 µl of 30% PEG 8000 in 1.8 M NaCl was added to allow the differential precipitation of the plasmid DNA and the tube was kept at 4°C overnight. Following centrifugation at 15,000 rpm for 30 minutes, the supernatant was discarded and the pellet resuspended in 200 µl of TE buffer. An equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) was added to the DNA solution to remove the protein contaminants. The tube was vortexed for 10 seconds and microcentrifuged for 5 minutes at maximum speed, room temperature. The top (aqueous) phase containing the DNA was carefully removed into a fresh tube. An equal volume of chloroform:isoamyl alcohol (24:1) was then added, followed by vortexing for 10 seconds and microcentrifuging for 5 minutes at maximum speed, room temperature. The top phase was again removed and transferred to a new tube. 1/10 volume of 3 M sodium acetate, pH 5.2 was added along with twice volume of 100% cold ethanol (calculated after the salt addition). The tube was then vortexed and placed in a -80°C freezer. Prior to use, the thawed tube was microcentrifuged at maximum speed for 30 minutes and the supernatant carefully removed. 1 ml of room temperature 70% ethanol was added; the tube was inverted to mix and microcentrifuged at maximum speed for 30 minutes. The supernatant was carefully discarded. The pellet was dried in a Savant SS1 Speedvac evaporator (Savant Instruments, Farmingdale, NY) for 8 minutes, then dissolved in 20 µl

RNase-free water. 5 µl of the resultant solution was mixed with 995 µl of RNase-free water and the amount of plasmid DNA isolated was quantified using a Pharmacio-Gene Quantitative Spectrophotometer (Pharmacia Biotech, Columbus, OH). The remaining sample was stored at -20°C until needed.

3.2.7. *Riboprobe transcription*

The following steps were used for transcription of the riboprobe. First, the plasmid was prepared by cutting with Xba1 restriction enzyme at site 32 downstream of the T7 promoter. This linearized the DNA, thereby limiting the size of the riboprobe being produced. Then the in vitro transcription reaction was carried out using the Riboprobe Combination System SP6/T7 kit (Promega) following the manufacture's protocol for synthesis of a large amount of RNA (Promega Technical Manual 016, section IV. F). Briefly, transcription reaction components were added to the linearized DNA template along with the T7 polymerase and the reaction was carried out at 37°C for 2 hours. The reaction components included equal amounts of dNTPs with 35% of the dUTP replaced with digoxigenin-11-UTP (Roche Molecular Biochemicals; cat# 11 209 256 910), transcription optimized 5X buffer, recombinant RNasin Ribonuclease Inhibitor and nuclease-free water. This is a non-isotopic method of riboprobe labeling. Transcription was terminated by 20 minute incubation at 65°C. Finally, RQ1 RNase-free DNase enzyme was used to remove the DNA template (15 minute incubation at 37°C) leaving the RNA 'run-off' transcripts in solution.

3.2.8. *In situ* hybridization

Paraffin-embedded tissue sections were mounted on glass slides pretreated with 3-aminopropyl triethoxysilane (Sigma Aldrich). Sections were de-waxed using 2 washes in xylene and then rehydrated using a graded series of washes using decreasing

concentrations of ethanol (100%, 95%, 70% and 50%), each for 2 minutes, followed by a diethyl pyrocarbonate (DEPC)-treated water rinse. Next, the slides underwent two denaturation steps; initial incubation with 0.2N HCl for 20 minutes to denature proteins and nick the DNA, and then a heat denature step using 2X saline sodium citrate (SSC) at 70°C for 15 minutes. The slides were then rinsed in 1 x phosphate buffered saline (PBS) in preparation for permeabilization. Slides were treated with 10 µg/ml proteinase K for 15 minutes at 37°C to digest proteins and dissociate nucleic acids. Permeabilization was terminated by 2 minute incubation with 2 mg/ml glycine. Prehybridization fixation was accomplished using 4% paraformaldehyde for 5 minutes to prevent/reduce the diffusion and loss of cellular nucleic acids and to stabilize chromosomes prior to the rigorous denaturation steps that follow. Fixation was stopped by a 3 x PBS 5 minute incubation, following by two 1 x PBS 30 second rinses. Acetylation followed to neutralize positive charged molecules and remove endogenous biotin to lower nonspecific binding. Initially, the slides were blocked using triethanolamine (TEA) buffer for 2 minutes, followed by two 5 minute incubations under continuous agitation using solutions of TEA buffer combined with increasing strength concentrations of acetic anhydride, 0.25% and 0.5%. Acetylation was concluded by a 2 X SSC 5 minute incubation and followed by dehydration for 2 minutes each in graded series of increasing concentrations of ethanol (50%, 70%, 95%, and 100%) to ensure that the probe was not diluted by any residual prehybridization solutions.

The slides were incubated for 2 hours at room temperature with the prehybridization buffer (60% formamide, 300mM sodium chloride, 30mM Sodium citrate, 10mM ethylenediaminetetraacetic acid, 25mM sodium phosphate, 5% dextran sulfate sodium salt, 0.25 mg/ml DNA calf thymus, 0.5 mg/ml tRNA from Brewer's yeast, 2% 50 x Denhardt's solution). The slides were then incubated overnight at 37°C with a hybridization solution consisting of prehybridization buffer supplemented with 5ng/ml

ISAV segment 7 antisense riboprobe incorporating digoxygenin-11-dUTP. The next day, post hybridization washes were performed to denature and remove any weakly bound probe. The initial wash step was in wash solution A (60% formamide, 300mm sodium chloride, 30mM sodium citrate, DEPC-treated water to final concentration) for 30 minutes at room temperature; followed twice with wash solution A for 15 minutes at 37°C, and a final rinse in 1X PBS for 5 minutes. Potential background signal was lowered further by a 15 minute incubation with RNase solution to remove single stranded, unhybridized RNA. This was followed by a second series of washes: Wash solution A for 30 minutes at room temperature; wash solution A for 30 minutes at 37°C, both on a shaking platform. And finally, two rinses were done using 2X SSC for 5 minutes at room temperature. Positive hybridization was detected using DIG Nucleic Acid Detection Kit (Roche Molecular Biochemicals; cat# 1 175 041; lot# 1201107(12)). The slides were rinsed in washing buffer (maleic acid buffer (0.1 maleic acid and 0.15M sodium chloride; pH = 7.5) and 0.3% v/v Tween 20) for 5 minutes at room temperature, then incubated for 30 minutes with blocking solution (commercial blocking reagent in 90% v/v maleic acid buffer) . A one hour incubation with 1:500 dilution of anti-DIG alkaline phosphatase-conjugated anti-digoxigenin Fab fragments in maleic acid buffer at 37°C was followed by two washes for 15 minutes each with washing buffer; equilibration in detection buffer for 5 minutes, then the colour reaction was allowed to proceed overnight at room temperature in the dark with fresh color substrate (0.375 mg/ml 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and 0.188 mg/ml nitroblue tetrazolium (NBT) salt in detection buffer (0.1M Tris-hydrochloride, 0.1M sodium chloride; pH=9.5). Colour development was terminated the next day by rinsing with ddH_2O. The slides were then counterstained with methylene green; dipped repeatedly in acetone with 0.25% acetic acid to remove excess stain and dehydrated through a graded series of increasing concentrations of ethanol as before, followed by a xylene wash for 2 minutes. The slides

were then mounted with Fisher Permount and examined using light microscopy.

3.2.9. Real time RT-PCR

ISAV real time RT-PCR was done using the LightCycler 1.2 instrument (Roche Applied Science) with RNA Amplification Kit SYBR Green 1 (Roche Applied Science) and PCR primers F5/R5 targeting a band size of 211bp on ISAV Segment 8. Samples consisted of pooled tissue samples (heart, head kidney, posterior kidney, and liver) from the rainbow trout challenged with ISAV by i.p injection, collected at various times after the first mortality occurred at day 11 post injection. The RNA samples were aliquoted and immediately frozen at -80°C. The RNA pellet was dissolved in 15 µl of RNase-free water. One microliter of this solution was added to 19 µl master mix containing 0.3 µM of the forward and reverse primers, 4 µl of the LC RT-PCR Reaction Mix SYBR Green dye, 0.4 µl of the LC RT-PCR Enzyme Mix, 3 µl of resolution solution, 1.6 µl of 25mM magnesium chloride, 9.4 µl of nuclease-free water. Assays were preformed on triplicate for each RNA sample. Thermal conditions were one cycle of reverse transcription at 55°C for 30 minutes, initial denaturation at 95°C for 30 seconds followed by 50 cycles of 95°C for 5 seconds, 59°C for 10 seconds, 72°C for 10 seconds, and data acquisition at 80°C for 2 seconds. Melting curve analysis was performed from 70°C to 95°C in 0.1°C per second increments to assess the specificity of the RT-PCR products. The quantitative (Ct values) and melting curve data were analyzed using LightCycler software version 3.5 (Roche Applied Science). The real time reaction products were also resolved by 1% agarose gel electrophoresis in 0.5 x TBE buffer, stained with ethidium bromide and photographed under 304 nm UV light.

3.3. Results

3.3.1. Mortality data

A total of 2 fish were found dead during the trial on Days 11 and 23 pi, and 6 fish were removed while moribund, for total cumulative mortalities of 8 of 106 (7.5%). No other fish had discernable clinical signs during routine hourly tank observation. No pattern or peak could be ascribed to the mortality plot.

3.3.2. Gross pathology

Gross lesions recorded during necropsy of the two mortalities and 6 moribund fish were consistent with those described in Chapter 2. Gross lesions are summarized in Table 9. No consistent pattern of clinical signs was evident, as all signs were either nonspecific or occurred in low frequency. Gross lesions within the 2 dead and 6 moribund fish included lack of ingesta/anorexia, exophthalmia, ascites, periorbital petechiation, kidney swelling and pale liver. Of the 24 behaviourally-normal opportunistically sampled 'survivor' fish sacrificed between Day 11 pi till Day 40 pi, grossly evident lesions included: ascites, exophthalmia, fin base haemorrhage, lack of ingesta/anorexia, periorbital petechiation and rare incidence of kidney swelling, cardiomegally, darkened skin, and swimbladder haemorrhage; only 8 of these fish had no visible gross lesions.

3.3.3. Microscopic pathology

Microscopic lesions of these ISAV-experimentally infected rainbow trout were consistent with those seen in Chapter 2, however the lesions were universally of lower severity. The heart remained the most consistently affected tissue. Cardiac lesions were confined to the endothelium, notably mild diffuse endocardial hypertrophy and hypercellularity with mild to moderate mononuclear cell infiltration, however there was no evidence of epicarditis or epicariditis with myocardial progression to the ventricular compact layer found within the dead or moribund fish of this challenge. The 4 fish with

Table 9. *Oncorhynchus mykiss*. Prevalence of gross lesions in recent mortalities and moribund rainbow trout as compared to opportunistically sampled rainbow trout experimentally infected with infectious salmon anaemia virus (ISAV).

Gross lesion	Mort/moribund (n=8)	(%)	Sampled (n=24)	(%)
Lack of ingesta/anorexia	4	50	0	0
Exophthalmos	2	25	6	25
Ascites	1	12.5	3	12.5
Periorbital petechiation	1	12.5	0	0
Kidney swelling	1	12.5	0	0
Pale liver	1	12.5	0	0
Fin base haemorrhages	0	0	2	8.3
Cardiomegally	0	0	1	4.2
Darkened skin	0	0	1	4.2
No abnormal findings	0	0	8	33.3

the most severe cardiac lesions were from among the apparent survivors sampled opportunistically between Days 27 and 31 pi. The cardiac lesions differed in this challenge, characterized by endothelial cell hyperplasia and hypertrophy, with a marked mononuclear leucocyte proliferation in the subendothelium of the spongy layer of the ventricle (Figure 6).

Mild congestion and focal haemorrhages were common in the head and posterior kidney interstitium, and less commonly within the lamina propria of the pyloric caeca and within the liver parenchyma. Additionally, degeneration of an unknown cell type within the haemotopoietic tisses of the head kidney was an infrequent finding and was again presumed to be ISAV-related. Increased levels of erythrophagia were detected within the haematopoietic regions of the kidney. For the dead/moribund group, erythrophagia peaked on Day 15 pi, with a mean erythrocyte count of 11 per high power field (hpf). On samples from this day, there was also abundant erythrophagia noted in the kidney blood vessel lumens, suggesting viremia. Erythrophagia also peaked on Day 15 pi within the 'survivor' group, with a mean of 16.6 per hpf. After Day 21 pi, erythrophagia in the 'survivor' group had returned to rare detections, with the exception of the fish with the cardiac lesion depicted in Figure 6, sampled on Day 27 pi with a mean erythrocyte count of 8.8 per hpf.

3.3.4. Viral detection using ISH

A riboprobe specific for mRNA transcripts of ISAV Segment 7 labeled with digoxigenin-11-dUTP was constructed as previously described (Moneke *et al.*, 2003) and used to demonstrate the cellular distribution of ISAV gene expression in putative ISAV lesions in experimentally infected rainbow trout. Figure 7 shows virus detection by

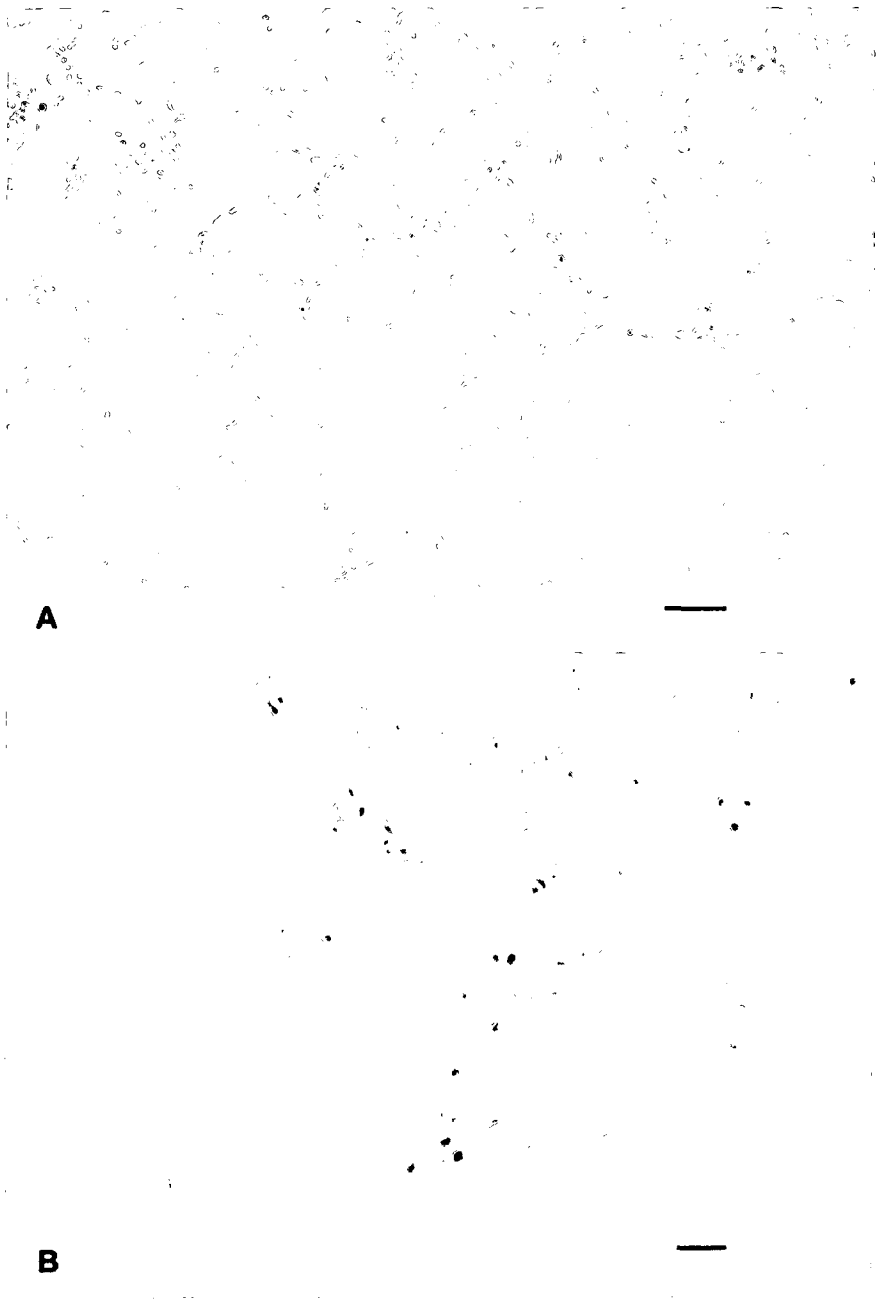

Figure 6. *Oncorhynchus mykiss*. (A) Ventricle from infectious salmon anaemia virus (ISAV)-infected rainbow trout showing endothelial hypercellularity and hypertrophy and subendothelial inflammatory cell infiltrate in the spongy layer; scale = 50 µm. (B) Ventricle at higher magnification; scale bar = 20 µm.

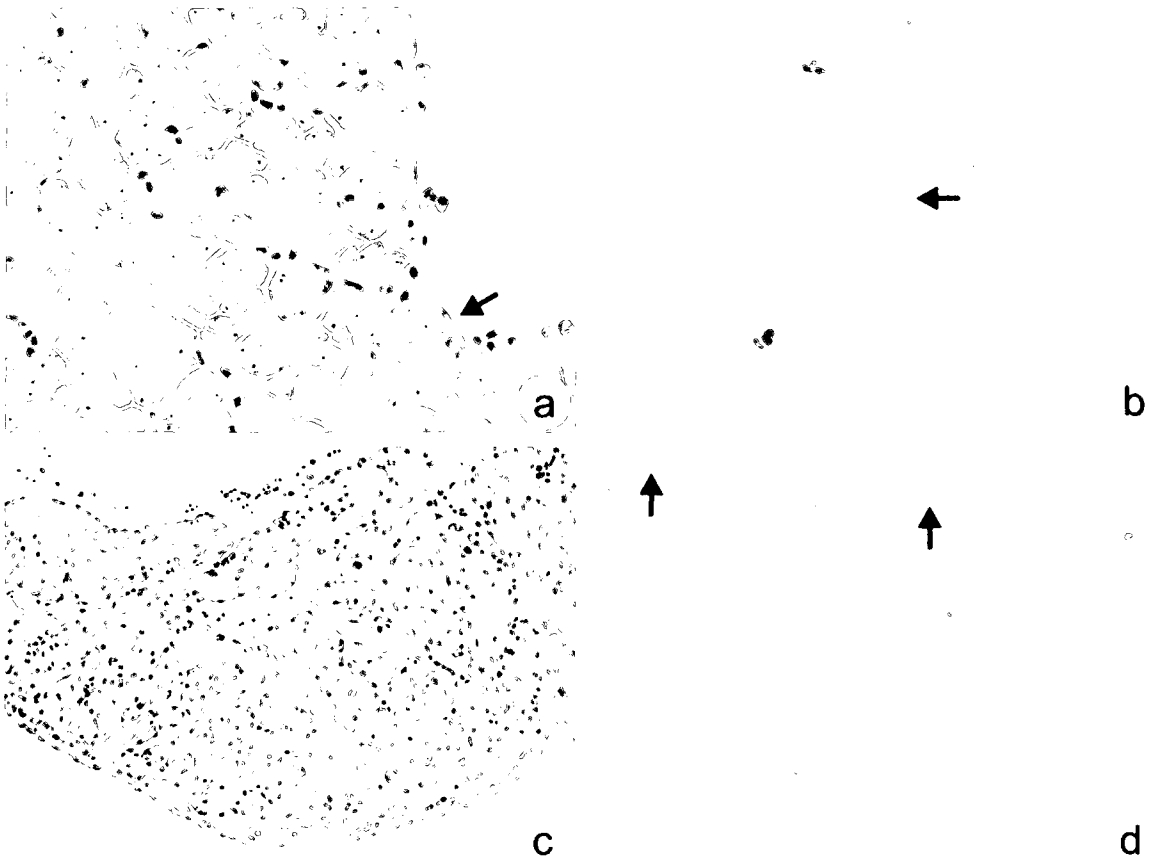

Figure 7. *Oncorhynchus mykiss.* Serial histologic sections from rainbow trout liver (a,b; 160 magnification) and atrium (c,d; 100 magnification) 12 d post-infection with infectious salmon anaemia virus (ISAV). (a,c) H&E staining, (b,d) *in situ* hybridization using antisense ISAV segment 7 riboprobe with no counter stain. Arrows indicate positive signal in endothelium.

ISH in liver and heart tissues from fish from serial live sampling. The blue colour seen in a positive reaction was apparent only within endothelium of the tissues, the primary target cell type of ISAV. The cellular distribution was demonstrated to be similar to that in Atlantic salmon; with the highest signal intensity and frequency was found in heart. Peak signal intensity and broad tissue distribution was evident in the samples from dead and moribund fish collected between Day 9 to 14 pi. Thereafter, signal intensity, frequency and distribution declined. Figure 8 depicts viral signal; in various tissues over time. No signal was detected in any tissues examined from fish sampled later than Day 23 pi. The heart showed the most intense and most abundant signal. The signal in the atrium was more abundant than that within the ventricle, which is expected due to the higher proportion of macrophage-like cells within the atrium. The signal in the ventricle was most frequently found in the spongy layer, in a location consistent with the endothelial cells lining the lumen. Infrequent signal was noted in the compact layer. ISH applied to serial sections of the slides demonstrating the mild-moderate cardiac lesions, failed to demonstrate a signal. These fish were opportunistically sampled on Days 27 and 31 pi. Lack of signal suggests that no viral replication is occurring and the lesions may be the result of a prolonged inflammatory response to the virus, continuing well after viral clearance. Signal was not found that could be definitively associated with individual cellular degeneration within the haematopoietic region of the head kidney; however, this lesion was relatively infrequent compared to the pilot study. The signal that was found within the posterior kidney was in a peritubular location, indicating endothelial as opposed to haematopoietic cell origin. Finally, there was little-to-no signal found within the lamina propria of the pyloric caeca, however associated lesions were mild in this challenge.

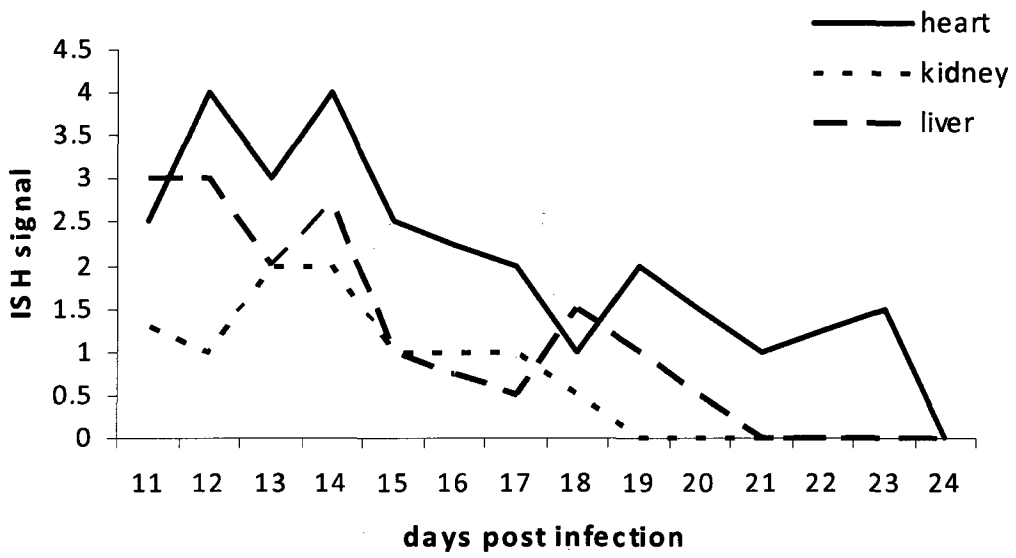

Figure 8. *Oncorhynchus mykiss.* Time course of Segment 7 riboprobe *in situ* hybridization signal detection in the heart, kidney and liver of infectious salmon anaemia virus (ISAV)-infected rainbow trout.

3.3.5. Viral detection using real time RT-PCR

A standard curve is used to allow quantification of ISAV target sequences in unknown samples based on the number of PCR cycles needed for product-specific fluorescence to exceed baseline levels. The ISAV standard curve for this work was generously provided by Dr. Khalid Munir, a fellow graduate student in our lab. The standard curve plotting fluorescence versus serial dilution of a known concentration of target nucleic acids was constructed, with the function determined to be:

threshold cycle number (C_T) = 15.0815 – 3.771 x log of template concentration.

Samples were run from surviving fish collected day 13 pi; day 19 pi; day 27 pi; day 35 pi. Only isolates collected before day 27 pi had C_T values in the reasonable range for quantification (Table 10). C_T values from samples collected from Day 27 through Day 63 pi had C_T values exceeding 49 and the associated fluorescence plots did not confirm to the expected shape of the log linear curve (Figure 8A). C_T values over 40 are considered unreliable for quantification. When the target sequence is in very low copy number, excessive nonspecific amplification or formation of primer-dimers can generate significant errors in quantification when a universal DNA binding dye like SYBR Green 1 is used. Despite being in too low a copy number to feasibly permit quantification, melting curve analysis was performed from 70 – 95°C in 0.1°C increments to verify the specificity of the amplicons to aid in interpretation of the SYBR green fluorescence data (Figure 8B). A single peak at 82.5°C was found to be consistent with that of ISAV Segment 8 positive control detection. The single peak also suggests the amplification products were specific and the virus population was uniform throughout the sampling times. Melting curve analysis indicated that fish sampled as late as Day 63 pi were positive by real time

Table 10. Transcript levels of infectious salmon anaemia virus (ISAV) Segment 8 by One-step RT-PCR with F5/R5 primer pair.

Sample id	Sampling days pi	Mean C_T value[†]
006	13	29.55 ± 0.15
007	13	31.78 ± 0.73
008	13	33.24 ± 0.76
018	19	36.69[‡]
019	19	39.31 ± 7.20
020	19	34.23 ± 0.86
Positive control	NA	24.39 ± 1.324

[†] Mean C_T values are averages ± standard deviation of triplicate observation
[‡] two broken capillary tubes - limited to single observation

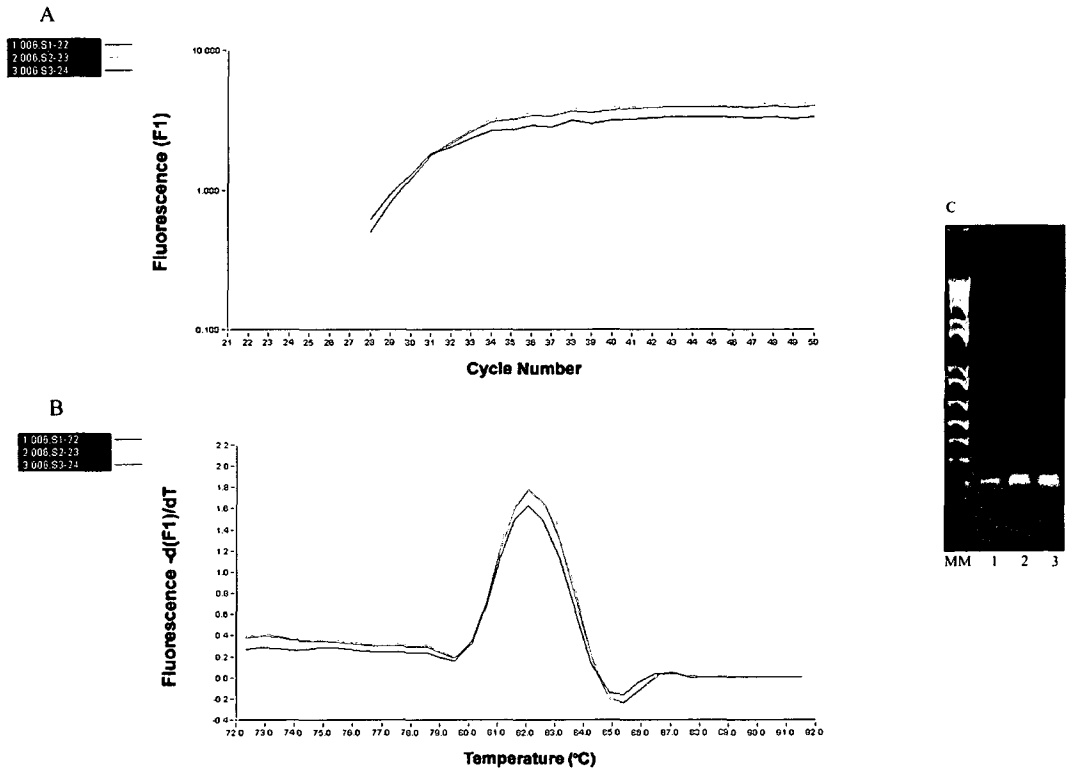

Figure 8. Real time amplification curve, melting curve and agarose gel electrophoresis of RT-PCR targeting a 220-bp product on ISAV segment 8 using total RNA from an apparently healthy rainbow trout sampled Day 13 pi. (A) Real time RT-PCR amplification curve in triplicate. (B) melting curve of real time RT-PCR of the run in (A). (C) RT-PCR products resolved on 1% agarose gel electrophoresis and visualized by ethidium bromide staining.

RT-PCR, and therefore had not cleared the virus and were potential long term viral carriers. This included one of the fish with the mild-moderate cardiac lesions noted during H&E examination (sample 032; sampled Day 27 pi); which had a C_T value too low for quantification, but was positive by melting curve analysis, indicating possible viral persistence.

The real time RT-PCR results were also confirmed by using agarose gel electrophoresis demonstrating an expected 220bp band (Figure 8C) indicating that the RT-PCR products are virus-specific. Samples positive by agarose gel were evident until Day 19 pi; after that sampling day, RT-PCR failed to detect bands of the expected size. This indicates the sensitivity of RT-PCR detection had been exceeded by the low copy number of ISAV segment 8 in the tissues.

3.4. Discussion

This challenge trial was characterized by a much lower degree of virulence and mortality than was expected based on the trial described in Chapter 2. From the pilot work (see Figure 1, page 33), it was anticipated that between 25 – 40 % of subjects would experience a fatal infection using an inoculum of $10^{6.05}$ $TCID_{50}$. Cumulative mortalities were, however, only 7.5% in this challenge. Genetic strain variation in rainbow trout (Biacchesi *et al.*, 2007) as well as size and age of Atlantic salmon (Glover *et al.*, 2006) have been shown to influence susceptibility to ISAV infections. But these factors were controlled to some degree by procuring rainbow trout from the same stock, albeit a different year class, and using similar size and age of fish. Timing of the trial might have also played a role in variable susceptibility to infection. The first challenge was started in May; the second, in October. There has been some anecdotal evidence of a seasonal (Devold *et al.*, 2000) or size-related (Glover *et al.*, 2006) effect with juvenile fish demonstrating increased susceptibility in the spring of the year, presumably

associated with the underlying physiologic process of smoltification (Mjaaland *et al.*, 2005). The increased disease expression in the pilot study, however, could have been a consequence of the unanticipated combination of factors which occurred during the trial. One factor which could have marked influence on virulence was the presence of infected cohabitating Atlantic salmon, presumably increasing the infectious load of the tank water and creating an environment of continuous waterborne exposure to infectious viral particles. This situation could have been exacerbated by the concurrent fungal infection: potentiating skin ulcerations and therefore increased osmoregulatory effort required to maintain homeostasis, diverting innate immune system capacity and/or causing mechanical damage to skin increasing horizontal transmission risk of ISAV shed from clinically ill cohabitants. Further, the formalin external bath treatments used on the fungal-infected fish could have also contributed to increased disease expression, due to mechanical damage to gills (considered a primary route of transmission in natural outbreaks (Totland *et al.*, 1996)) and the possible immunosuppressive corollary stresses of treatment: time off food, crowding, temporary water quality compromise, etc.

ISH applied to ISAV experimentally infected rainbow trout demonstrated the same ISAV nucleic acid tissue and host cell distribution as had been seen in Atlantic salmon (Gregory, 2002; Moneke *et al.*, 2003; Moneke *et al.*, 2005a; Moneke *et al.*, 2005b). This finding suggests that similar sialic acid moieties are available in the same target cells of both Atlantic salmon and rainbow trout. But viral-host interactions are much more complex than mere presence or absence of receptors. Influenza virus receptor specificity studies have shown that the host range depends not only on the affinity for the terminal sialic acid residues, but also on the number and accessibility of receptors on the cell surface, on the simultaneous binding of multiple HE spikes to multiple receptors on the cell, and the structure of the underlying glycoprotein or glycolipid (Klenk *et al.*, 2008).

Two pathways of cellular death are recognized, apoptosis and necrosis. Apoptosis is defined as the selective elimination of a single cell through activation of a genetically programmed, internally controlled "suicide" pathway. Characteristics of apoptosis include cell shrinkage, chromatin condensation, formation of cytoplasmic blebs and membrane bound apoptotic bodies, with clearance of apoptotic cell remnants through phagocytosis by macrophages and neighboring cells without stimulating an inflammatory response. This differs drastically from necrosis, which is a pathologic response to cellular injury characterized by severe cellular swelling, rapid cellular degeneration and coagulation of cellular proteins, breakdown of cellular organelles, disruption and loss of plasma membrane integrity, and leakage of cellular contents triggering an inflammatory response (as reviewed in Cotran *et al.*, 1999). Previously it has been shown that the ISAV-induced method of cell death is cell-type dependant, with epithelial-like cells like TO cells, undergoing necrosis and fibroblast-like cells like SHK-1 and CHSE-214, undergoing apoptosis (Joseph *et al.*, 2004).

It was hoped to find evidence of virus within the haematopoietic cells of the kidney to correlate with the single cell drop out notable in the rainbow trout during the pilot study. This lesion was an infrequent finding in the second challenge. Using ISH, no evidence of signal was seen within the renal parenchyma; riboprobe-associated signal was only seen in the peritubular subendothelial region. There are a number of possible explanations for lack of ISH signal within the haematopoietic cell population of the kidney. Single cell dropout may not prove to be a consistent clinical sign in productive rainbow trout infections with highly pathogenic ISAV isolates. Other explanations include: the lesion may not be related to ISAV infection, lack of optimization of the ISH technique for rainbow trout kidneys, or viral attachment or entry triggered the cell to enter apoptosis. Lack of an inflammatory response supports this final explanation however, the time course of inflammation in fish is temperature dependant and generally

less overt than in mammals. Consider the severe cardiac lesions from the first trial, the first evidence of cardiac inflammation was in a rainbow trout that died on Day 6 pi. However the most severe cardiac lesion was found in a fish that died on Day 35 pi indicating a chronic progressive response as opposed to an acute inflammatory one. However, lack of ISH signal in the areas of endothelial cell hypertrophy and hypercellularity with adjacent mononuclear cell infiltration, suggests that viral clearance has occurred and all that remains is an over exuberant, self propagating, inflammatory response. If this is true, this represents a different pathogenesis than seen in Atlantic salmon. Microarray analysis has been used to compare the gene expression responses of ISAV-infected Atlantic salmon tissues from fish experiencing early mortality (sampled as moribund from among the first 10% of mortalities, beginning at 21 dpi by cohabitation challenge) versus fish experiencing late mortality (moribund fish from among the last 10% of mortalities, roughly 38 dpi by cohabitation). Results indicated that the early infections were characterized by high viral replication and dramatic activation of innate immune responses and marked cellular oxidative stress which did not confer protection to the virus. Alternatively, late infections were characterized by reduced viral loads/clearance with increased activation of adaptive cellular immunity and a decreased inflammatory response (Jorgensen *et al.*, 2008).

Sampling survivors of infection began at Day 13 pi. Tissue sampling was initiated too late to demonstrate active viral replication; virus was detected from the chronological samples in descending quantities only. Therefore we make the assumption that viral replication did occur, based on the gross clinical signs and microscopic lesions found within the dead, moribund and opportunistically sampled fish, which were consistent with those found during the pilot work and other experimental infections of rainbow trout (Kibenge *et al.*, 2004; Kibenge *et al.*, 2006; MacWilliams *et al.*, 2007). Further, ISH clearly demonstrates a systemic ISAV Segment 7 distribution within the endothelium,

leaving little doubt that an active ISAV infection is occurring.

The innate immune system provides an immediate, nonspecific response against structurally conserved molecules associated with microorganisms, like dsRNA in viruses. This system is comprised of physical barriers, along with specialized destructive cells (i.e. macrophages and natural killer cells) and pathways (i.e. interferon and complement systems) to defend against pathogens. The adaptive or acquired immune system is slower to respond during infection. It is comprised of humoural and cell mediated immune responses, relying on B lymphocytes for antibody production and T cells to kill virus-infected cells. However, unlike the innate system, adaptive immunity is characterized by specificity and memory. A strong cell mediated response is considered vital in the clearance of acute viral infections. CD8+ T cells may act against viral infected cell through two methods: cell contact dependant lysis of infected target cells and via production of antiviral cytokines (as reviewed in Janeway *et al.*, 2001).

Persistence is not a recognized feature of the *Orthomyxoviridae*. Viruses that persist in their hosts must have a noncytopathic way to replicate to ensure survival of a critical number of host cells and avoid stimulating a host antiviral immune response. However, it has been long recognized that certain ISAV isolates are capable of replication in various cell lines without being cytopathic (Sommer & Mennen, 1997; Kibenge *et al.*, 2000a). Viruses that cause nonlytic productive infections are well suited for persistence and more likely to establish a chronic infection. Unlike DNA viruses which can establish latency by becoming integrated into the host chromosome or maintained as episomal circular molecules, it is unknown if there are mechanisms for long term propagation of RNA molecules in a host cell. It is assumed that RNA viruses persist by low-grade, chronic infection with continuous viral replication at a very low rate, insufficient to trigger a strong immune response. ref. An alternative would be for ISAV to infect cells that are not readily accessible to the immune system. Immunologically

privileged sites include the central nervous system, the excretory cells of the kidney or glands (excluded from general circulation by a basement membrane), epidermal cells and dendritic cells.

Viral pathogens have evolved elaborate strategies to avoid the defenses of their hosts (as reviewed in Murphy 1999). A common method for a virus to evade immune surveillance is by altering its antigens to avoid antibody neutralization. Antigenic variation is a common immune system evasion mechanism of the *Orthomyxoviridae*. Influenza viruses have two distinct ways to change antigenic type. Antigenic drift refers to point mutations at occur frequently that alter binding sites for protective antibodies on surface glycoproteins. Cross reactivity with antibodies or T cells produced against other unaltered epitopes may afford some protection in previously infected individuals and only mild disease will occur. Antigenic shift refers to the exchange of viral segments between strains within a secondary host, leading to major changes in the viral surface glycoproteins. Therefore T cells and antibodies produced in earlier infections are not protective and severe pandemic infections may occur. This type of reassortment is much less frequent; occurring every 10 – 50 years (as reviewed in Janeway *et al.*, 1999).

There is strong evidence that ISAV isolates are evolving using reassortment and recombination, however recombination appears to occur more frequently in ISAV than in other *Orthomyxovirdae* (Devold *et al.*, 2006; Markussen *et al.*, 2008). Reassortment of the HE HPR has been shown to have a role in virulence, although not an exclusive role, as isolates with identical HE HPR demonstrate variable levels of virulence. A virus neutralization study allowed division of ISAV isolates into two major subtypes based on antigenic variation. These subtypes correlated with HE genomic variation (Kibenge *et al.*, 2001b). Recombination events leading to insertion upstream of the F protein cleavage site have been identified as a second virulence marker (Devold *et al.*, 2006; Kibenge *et al.*, 2007a; Markussen *et al.*, 2008). Further investigation of ISAV virulence markers

demonstrated that, sequence variations among ISAV isolates in the HE gene and the F gene correlate with geographic origin, cytopathogenicity in cell culture and pathogenicity in experimental infection of fish (Kibenge *et al.*, 2007a). It has been suggested that reassortment and recombination events might contribute to other potential ISAV virulence markers involving segments 7 and 8, as highly variable amino acid substitutions among ISAV isolates has been shown and the effector proteins of these segments are involved in host cell immune response through interferon antagonism (Markussen *et al.*, 2008).

Another common mechanism viruses use to evade the host immune system is the production of viral defense molecules that interfere with the function of antiviral cytokines. Orthomyxoviruses are known to antagonize the interferon (IFN) system through multiple mechanisms. For example, the influenza virus nonstructural (NS) protein binds to dsRNA to avoid recognition by intracellular receptors, antagonizing the type 1 interferon response pathway. ISAV S8ORF2 protein has been shown to have similar activity, binding ssRNA and dsRNA and downregulating type1 IFN promoter activity in poly I:C stimulated cells (Garcia-Rosado *et al.*, 2008). ISAV S7ORF1 also encodes a protein thought to be an interferon-signaling antagonist (Garcia-Rosado *et al.*, 2008), an action potentially mediated through the delayed expression of interferon regulatory factor (IRF)-7 (Kileng *et al.*, 2009). ISAV seems to delay the induction of IFN, as IFN transcripts appear much later than Mx and ISG15 transcripts in infected host cells (Kileng *et al.*, 2007) suggesting the ISAV stimulates the Mx promoter through a mechanism independent of the IFN pathway (Bergan *et al.*, 2006).

A cell line-dependant effect on Mx protein protection against ISAV has been identified. Atlantic salmon ASK cells were not protected despite an increase in Mx protein (Jensen & Robertsen, 2002), however, Chinook salmon embryo (CHSE-214) cells genetically engineered to continuously express Atlantic salmon Mx protein had

increased protection (as measured by time to cell death, lower severity of CPE and lower viral titres) (Kibenge *et al.*, 2005). This might represent a species-specific host response in post-transcriptional activity against ISAV, as the Mx protein did not influence the yield of ISAV transcripts. Additionally, a correlation between phenotypic response of ISAV isolate cytopathogenicity in a variety of cell lines and pathogenicity in various salmonids has been demonstrated (Kibenge *et al.*, 2000a; Kibenge *et al.*, 2007a).

Viruses can also hijack the immune responses of the host to contribute to increased pathogenicity. For example, antibody enhanced infection occurs when monocytes and macrophages are more efficiently infected by virus-antibody complexes via Fc receptor mediated endocytosis, than by virus alone. Studies in our lab have identified possible Fc receptor-mediated antibody-dependent uptake and growth of ISAV in the TO cell line, a macrophage-like cell line (Joseph *et al.*, 2003). Since ISAV in Atlantic salmon targets mononuclear leucocytes and endothelial cells or endothelial-associated cells of the reticuloendothelial system, it seems reasonable to speculate that Fc receptor-mediated antibody-dependent enhancement of ISAV infection may occur *in vivo*, potentially accelerating the disease process by enhancing delivery of the virus to target cells. A dire potential consequence of this is the risk of higher morbidity or mortality in survivors of previous infection or in post-vaccinated fish. Luckily, there is no evidence to suggest that this is more than a theoretical possibility, and reduced susceptibility to ISAV re-infection has been seen (Falk & Dannevig, 1995; Ritchie *et al.*, 2009).

Erythrophagocytosis seen in sinusoidal macrophages outside and within the melanomacrophage centers of the spleen and kidney is a common finding in ISAV-infected Atlantic salmon and rainbow trout (Falk *et al.*, 1995; Byrne *et al.*, 1998; Moneke *et al.*, 2003; Kibenge *et al.*, 2006). Erythrophagocytosis is the process of removal of senescent or malformed red blood cells, commonly by macrophage digestion (Ferguson,

1989). Viral receptor attachment to the vascular endothelium could also allow adsorption of circulating erythrocytes, especially in capillary beds or sinusoidal regions with a high blood volume to endothelial surface area ratio and slow blood flow rates. In Atlantic salmon, erythrocytes potentially bind irreversibly to ISAV haemagglutinin surface protein. Once bound, exposed viral antigens on the surface of the erythrocytes will trigger a host immune response. Red blood cells with antigen/antibody complexes are rapidly cleared by one of two methods; by interaction with Fc receptors of macrophages or through the complement cascade membrane attack complex (Janeway *et al.*, 2001).

Erythrophagocytosis can be a manifestation of a number of disease states including viral infection, neoplasia, immunodeficiency states, etc. Significant erythrophagia was observed in the kidneys of rainbow trout, both within sinusoidal macrophages in the endothelium and within the renal vascular lumen, in cells suspected to be circulating macrophages/monocytes. As erythrophagia is less commonly reported in Atlantic salmon with an acute ISAV infection, it has been suggested to occur in relation to either a low pathogenic ISAV strain or a resistant host. The frequency of its occurrence in rainbow trout is somewhat surprising, since viral elution occurs comparatively soon in this species as compared to Atlantic salmon. Perhaps the rapid, endocytosis and replication of viral particles in Atlantic salmon allows less opportunity for macrophage phagocytosis. Alternatively it could be argued that in a highly pathogenic strain in a susceptible species, T cell mediated clearance of virus infected cells would predominate as increased viral endocytosis would allow more viral peptides to be coupled to MHC class II molecules for presentation to macrophages or to MHC class I molecules for cytotoxic T cells. The situation would be different with either a low pathogenic strain of ISAV or a resistant host, and the innate system might be able to contain the infection without stimulating a strong cell mediated response.

Anaemia is a consistent endstage clinical finding in acutely fatal ISAV infections

of Atlantic salmon. Anaemia is reported in other fish species following experimental infection with ISAV, however it has been described as mild and transient. The contribution of erythrophagocytosis to anaemia is unknown. It has been proposed that erythrophagocytosis contributes to anaemia early in the course of an acute ISAV infection (Dale *et al.*, 2006), but release of red blood cells from splenic sequestration may compensate for the destruction. In later course of infection, anaemia can become severe, presumably related multisystemic endothelial disruption and circulatory failure, demonstrated by severe anaemia and the multi-organ congestion and haemorrhage associated with ISA.

ISH applied to ISAV-infected tissues in this trial and elsewhere (Moneke *et al.*, 2005b) demonstrated viremia, positive signal in blood vessel lumens of kidney. This detection correlated with the peak period of mortality. However, specifically examining whole blood fractions with ISH only demonstrated signal in the buffy coat layer, suggesting replication was only occurring in leucocytes, with no evidence of ISAV within erythrocytes (Moneke *et al.*, 2005b). Transmission electron microscopy has been used recently to demonstrate that highly cytopathic isolates of ISAV adsorb erythrocytes within 4 hours and demonstrate cytoplasmic vesicles within 18 hours of inoculation. Rainbow trout erythrocytes demonstrated infrequent intracellular virus particles over the same time course. Low cytopathogenic strains demonstrated elution (plasma membrane invagination without a virus particle) in both species (Workenhe *et al.*, 2007). Further work demonstrated both viral replication and induction of antiviral cytokines, indicating both the presence of an intact cellular machinery capable of nucleic acid transcription and stimulation of the interferon system (Workenhe *et al.*, 2008). Presumably the viral numbers within an erythrocyte are below the detection limit of ISH.

In conclusion, this trial has demonstrated a similar tissue tropism of ISAV for rainbow trout as found in Atlantic salmon. Viral replication in rainbow trout has been

demonstrated by two molecular techniques, ISH and real time RT-PCR. ISH signal was most intense in the heart and also was detectible later in this organ than elsewhere. This is consistent with what has been seen in Atlantic salmon, and lends to speculation that viral persistence might occur at a low level of replication within the heart (Moneke *et al.*, 2005a).

4. GENERAL DISCUSSION

The purpose of this thesis was to study the disease expression of a known pathogenic isolate of ISAV in rainbow trout. Atlantic salmon are the only species known to experience ISA in a natural setting. However, other salmonid species have been shown to support viral replication and persistence of pathogenic ISAV strains following experimental infection, and of avirulent strains of ISAV in asymptomatic feral fish. Gaining information on virus-host interactions in an alternate host species, with a focus on tissue tropism, pathology and persistence, may help contribute to disease management practices as the virus continues to evolve.

To achieve this purpose, we designed an experimental infection of rainbow trout with several aims. The primary objective was to use a highly pathogenic isolate of ISAV and to infect a large number of fish and, through frequent monitoring, cull moribund fish or recover mortalities soon after death to ensure sufficient number and quality of necropsy specimens to characterize disease expression in this species. A previous multi-isolate comparison of 13 geographically distinct ISAV isolates, confirmed the isolate used in this trial, NBISA01, is highly virulent in Atlantic salmon, as indicated by early onset mortality, rapid period of peak mortality and high cumulative mortalities (Kibenge *et al.*, 2006). A disease challenge trial lead to the observation that an isolate which is highly virulent in the susceptible species, Atlantic salmon, will cause subtle lesions with a similar distribution in the more resistant species, rainbow trout. Specifically, ISAV isolate NBISA01 infection caused mortality in 50% (5 of 10) rainbow trout with mild lesions consistent with clinical ISA.

The first objective was successfully met in the first challenge. Twenty-seven fatally infected rainbow trout were recovered for detailed gross and histologic examination. As expected the mortality pattern in rainbow trout followed a protracted duration, as compared to the rapid onset and high peak mortality seen in Atlantic

salmon. Table 6 page 39 summarizes gross lesions per species. Lesions associated with vascular disruption, i.e. fin base haemorrhage, periorbital petechial haemorrhage, liver congestion, pyloric caeca congestion, etc. were relatively common in rainbow trout, although the distribution differed from the gross clinical signs in the Atlantic salmon and the lesions were generally less severe. Histopathologic lesions were summarized in Table 7, page 43. In addition to histopathologic changes normally associated with ISA in Atlantic salmon, two previously unidentified lesions were seen which may represent unique disease expression in more resistant host species: epicarditis with or without progression to severe myocarditis of the compact layer of the ventricle and uni- to multi-cellular degeneration of an unknown cell type in the haematopoietic portion of the kidney.

The second objective involved the cell line used to propagate the viral inoculum. It was proposed that increased pathogenicity in alternate hosts might be achieved if the virus was propagated in an ontologically-related cell line. To explore this, we compared the virulence of virus grown in either of two cells lines. TO cells are a macrophage-like line derived from Atlantic salmon (genus *Salmo*) head kidney cells shown to support production of high viral titres (Wergeland & Jakobsen, 2001). CHSE-214 is a fibroblast-like cell line derived from Chinook salmon (genus *Oncorhynchus*) embryos, characterized by slow developing, focal CPE and low infective titres (Kibenge *et al.*, 2000). Further to this, CPE in ISAV-infected CHSE-214 cells has been identified as a reliable virulence marker to identify ISAV isolates with increased infectivity and pathogenicity in other salmonid species with species-specific presentations of disease (Kibenge *et al.*, 2006). Survival analysis comparing time to death and cumulative mortalities did not indicate that the cell line used for viral propagation influenced virulence. This was a bit surprising as it indicates that neither ontological relatedness nor *in vitro* mechanism of cellular death influence virulence as ISAV is cytolytic to TO cells

and induces apoptosis in CHSE-214 cells (Joseph *et al.*, 2004). In other words, comparably infectious virions are produced from these two ISAV-permissive cell lines suggesting that there is no cell-associated advantage in replication, post-translational modifications, and virus assembly and budding steps of virion production.

Having established that ISAV can cause morbidity and mortalities in rainbow trout, we then wanted to locate the virus within the lesions using *in situ* hybridization. This method has been used to demonstrate localization of ISAV genomic segments within infected Atlantic salmon tissues, specifically within endothelial cells in various organs throughout the body, with the highest distribution and intensity of signal found within the heart and liver, but also in the spleen, anterior and posterior kidney, intestines, pyloric caeca and gills (Gregory, 2002; Moneke *et al.*, 2003; Moneke *et al.*, 2005a). Localization by ISH was consistent with electron microscopic observations of virus budding from endothelial cells and leucocytes (Hovland *et al.*, 1994; Koren & Nylund, 1997; Bouchard *et al.*, 1999). We carried out a second rainbow trout infection trial limiting tissue fixation in 10% neutral buffered formalin to 24 to 36 hours due to concerns with protein cross linkages and target nucleic acid accessibility for the riboprobe. We adopted an ISH protocol formerly optimized for ISAV detection in Atlantic salmon tissues (Moneke *et al.*, 2003). ISH demonstrated ISAV nucleic acid segments within endothelial cells of all tissues examined and infrequently within intralumenal blood cells presumed to be leucocytes. The most intense, frequent signals were detected in the heart. This was also the last organ in which signal was seen, supporting the possibility that this organ may have a role in viral persistence. It should be noted that the brain and other immunoprivileged sites were not examined. Unfortunately, no signal was ever seen in the parenchyma of the kidney haematopoietic tissues to be able to indicate a relationship between ISAV and the uni- to multicellular drop out lesion.

The final objective was to investigate viral persistence in rainbow trout. RT-PCR

and agarose gel electrophoresis were unable to detect viral segments later than Day 19 pi, however, samples taken from apparent survivors on Day 63 post infection were found positive by real time RT-PCR melting point analysis, indicating the increased sensitivity of this method and the potential for long term carrier status in this species.

Cardiac involvement is a common feature of ISAV in Atlantic salmon, but this lesion is typically confined to the single layer endocardium, with cellular hypertrophy and hypercellularity of the fixed macrophage layer lining the atrial and ventricular lumens. A study of cardiac function in ISAV infected Atlantic salmon hearts demonstrated decreased function (as measured by the Frank Starling response, relating cardiac output and stroke volume to filling pressure) by Day 15 to 20 pi in hearts that were grossly normal (Gattuso et al., 2002). Cardiac lesions varied between the two rainbow trout challenges. Cardiac endothelial hypertrophy and hypercellularity were seen in both challenges, consistent with cardiac endothelial presentation in ISAV-infected Atlantic salmon. However in the first trial, 5 of 27 trout had epicarditis with progression to compact layer myocarditis of the ventricle in more prolonged infections, resulting in apparent constrictive epicarditis. The first cardiac lesion of note was evident in a fatally infected rainbow trout Day 6 pi, but the most severe cardiac lesion was found in a mortality recovered on Day 35 pi. In the second challenge trial, again, the most severe lesions were once again cardiac and seen long after infection, yet in this challenge the lesion presented as a moderate to severe mononuclear inflammation of the subendothelium of the ventricular spongy layer. Some of these cardiac lesions were evident grossly. Another potential consequence is related to blood supply to the heart muscles. The two areas of the ventricle have different blood supply. The compact layer is supplied with oxygenated blood from the coronary arteries, while the spongy layer relies on oxygen and metabolite exchange from the 'venous' blood in the ventricle lumen. The subendothelial hyperceullarity seen in the rainbow trout of the second

challenge could have important implications on cardiac function due to increased diffusion distance and resultant myocyte anoxia and acidosis. The chronic, progressive nature of both of these lesions suggests that unlike Atlantic salmon, wherein a fish which can survive the acute onset of high replication has a good chance of surviving infection; in rainbow trout, inability to rapidly clear the virus by innate mechanisms predisposes a fish to risk of a strong inflammatory response, which in itself may be detrimental to survival.

The reticuloendothelial system is a network of phagocytic cells widely dispersed throughout a fish, responsible for removing effete cells and particulate matter from the circulation. Tissue macrophages are derived from circulating monocytes. The macrophages lining the atrium of the heart are fixed, as well as some of the macrophages lining the kidney and splenic sinusoids. The inflammatory response of fish depends on tissue type, time scale, cause and environmental temperature, however a general pattern exists. Acute inflammation is mediated by vasoactive amines and cell breakdown products released by tissue damage on the microcirculation of the surrounding tissue. These cause capillary dilation, increased blood flow, increased endothelial permeability, which allows large serum molecules (i.e. fibrinogen and immunoglobulins) to enter tissues. Leucocytes actively migrate into damaged tissues with the degree of cellular migration depending on the stimulus (as reviewed in Cotran *et al.*, 1999b).

Variations in infectivity and clinical presentation in different species are not uncommon features of members of the *Orthomyxoviridae* family. For influenza viruses, pathogenicity and interspecies transmissibility are strongly influenced by the molecular composition of viral genes and gene products (Klenk *et al.*, 2008). Reassortment and recombination events among separate nonhomologous isolates in an unusual host species can alter Influenza HA receptor specificity. For example, it has been shown that

α-(2,6)-linked sialyloligosaccharides are detectible on the apical surface of human tracheal epithelial cells; whereas, in avian species α-(2,3)-linked sialyloligosaccharides are found on the intestinal cells (the natural site of influenza infection in this species). Pig tracheas contain both types of sialic acid linkages, and it has been proposed that pigs may play a role in initiating influenza pandemics by serving as a 'mixing vessel' for antigenic reassortment of avian and human influenza viruses during co-infection (as reviewed in Janeway *et al.*, 2001). ISAV HE HPR analysis has allowed the genotyping of isolates by region and the identification of HPR deletions associated with increased virulence, confirming that reassortment and recombination events are also occurring, potentially in less susceptible feral salmonid hosts (Mjaaland *et al.*, 2002; Devold *et al.*, 2006; Nylund *et al.*, 2007).

Virulence, in particular, appears to be a polygenic trait, wherein the extent of tissue damage and disease outcome of an infection can strongly be influenced by alterations of the viral genes. For example, the enzymes needed for cleavage activation of Influenza HA surface protein are provided by the host. Mutations at the cleavage site have been associated with both increased and attenuated viral pathogenicity, depending on the resulting type of proteolytic activation required for viral membrane fusion with the host cell (as reviewed in Klenk *et al.*, 2008). Influenza A viruses with a single arginine at the cleavage site can be activated by extracellular trypsin-like proteases secreted from respiratory or intestinal epithelial cells, thereby giving rise to local infections. Highly pathogenic avian influenza (HPAI) viruses have a cleavage site which can be activated by ubiquitous proteases, and are therefore capable of systemic infections. Studies in ISAV indicate that a conformational change needed to activate the fusion protein may be stimulated by low pH or trypsin and that upstream mutations can influence virulence (Aspehaug *et al.*, 2005; Devold *et al.*, 2006; Kibenge *et al.*, 2007a; Markussen *et al.*, 2008).

In addition to its role in viral replication, the Influenza virus NS1 protein has been shown to inhibit the innate antiviral immune response of the host by lowering induction of interferon and other pro-inflammatory cytokines in human antigen-presenting dendritic cells, but has also demonstrated evasion of the adaptive immune response through interfering with dendritic cell maturation, resulting in suboptimal T cell stimulation (Fernandez-Sesma *et al.*, 2006; Fernandez-Sesma, 2007). A similar immunomodulatory action against the type 1 interferon system has been demonstrated by ISAV NS1 protein (McBeath *et al.*, 2006; Garcia-Rosado *et al.*, 2008).

Viral polymerase catalyses the transcription of genomic RNA into two types of RNA species: cRNA for a complete copy of the genome for new virions, and mRNA to be translated into new viral proteins. However, the Influenza viral polymerase lacks a proofreading function, leading to a high rate of point mutations. Recombinant virus studies have shown that specific mutations to HPAI H5N1polymerase complex proteins can increase viral pathogenicity, trigger apoptosis of immune cells and mediate adaptation to a new host (as reviewed in (Korteweg & Gu, 2008)). The contribution of ISAV polymerase to virulence and interspecies pathogenicity is currently unknown.

The work detailed in this thesis increases the knowledge of ISAV disease expression in the more resistant species, rainbow trout. Our findings support the role of endothelial cells as the primary target cells. Systemic lesions related to vascular damage were consistent clinical findings as seen in Atlantic salmon. The use of increased experimental subject numbers also allowed identification of chronic, progressive cardiac lesions in rainbow trout, representing a lesion unique to this species.

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5.1 Appendix A: Histologic lesion rating system

Histologic analysis to be based on lesions predominantly expressed during the mortality phase of the trial. Only the head kidney, liver, heart and pyloric caeca were sampled for histology.

General Code:	sample was too autolytic to interpret or not provided for interpretation	
Condition:	(0)	sample shows no significant autolysis
	(1)	sample shows mild-moderate autolysis
	(2)	sample is too autolytic to interpret
Head kidney (haemaopoietic tissue)	(0)	sample shows no morphologic evidence of ISA
	(1)	sample shows single cell drop out
	(2)	sample shows multi-cell dropout
Head kidney (endothelial integrity)	(0)	sample shows no morphologic evidence of ISA
	(1)	sample shows vascular congestion suggestive of ISA
	(2)	sample shows interstitial haemorrhage consistent with ISA
	(3)	sample shows renal necrosis
Head kidney	(0)	sample shows no morphologic evidence of ISA
	(1)	sample shows increased erythrophagia
Heart	(0)	sample shows no morphologic evidence of ISA
	(1)	sample shows endothelial cell hypertrophy
Heart	(0)	sample shows no morphologic evidence of ISA
	(1)	sample shows mild epiicarditis
	(2)	sample shows severe epicarditis/endocarditis
Pyloric caeca	(0)	sample shows no morphologic evidence of ISA
	(1)	sample shows congestion of the lamina propria vasculature suggestive of ISA
	(2)	sample shows congestion and necrosis of mucosa consistent with ISA
Liver	(0)	sample shows no morphologic evidence of ISA
	(1)	sample shows mild sinusoidal congestion suggestive of ISA
	(2)	sample shows either sinusoidal congestion, coagulative necrosis and/or vasculitis suggestive of ISA

5.2 Appendix B: Riboprobe preparation - isolation of plasmid DNA from midi preparation

1. Harvest the bacteria by centrifuging at 10,000 rpm for 15 minutes (JA-14 rotor).
   Bacteria will be harvested from a 250 ml flask containing 50 ml 2X-YT broth plus 100 µg/ml ampicillin, which was inoculated with a white colony the previous day and left overnight on a shaking platform at 180 rpm at 37°C

2. Discard the supernatant and resuspend the pellet in 2 ml of freshly prepared lysis buffer (0.5 gm lysozyme/10 ml of lysozyme buffer (aka lysozyme stock solution). Vortex and keep at room temperature for 5 minutes.

3. Add 4 ml of freshly prepared alkaline solution (1% SDS in 0.2 N NaOH). Mix by inverting tube 3-6 times. The solution should become clear and viscous. Keep on ice for 5 minutes.
   Place the alkaline solution in the 37°C water bath for dissolution. The highly alkaline pH will lyse the bacterial cells, solubilize and denature cellular constituents and begin to denature DNA and RNA.

4. Add 72 µl of RNase A stock (5mg/ml) or 36 µl of 10mg/ml. Incubate on ice for 15 minutes.

5. Add 3 ml of 7.5 M ammonium acetate solution and mix contents of the tube for a few seconds. Keep on ice for 10 minutes.
   This will neutralize the solution, allowing most of the protein and chromosomal DNA to precipitate and the plasmid DNA base pairs to reanneal.

6. Spin at 15,000 rpm for 30 minutes (JA 14 rotor).
   The bacterial proteins, cell wall fragments and denatured chromosomal DNA will complex resulting in a large, insoluble mass following neutralization and centrifugation.

7. Carefully harvest the supernatant containing the plasmid DNA (about 9 ml) and transfer to small tubes

8. Add 2 volumes of cold 100% ethanol (about 18 ml) and precipitate at -80C for 2 hours or overnight.
   Ethanol induces a structural change in nucleic acids allowing aggregation and precipitation in the presence of monovalent cations.

9. Spin at 15,000 rpm for 30 minutes (JA 20 rotor)

10. Discard the supernatant and resuspend the pellet in 1 ml of TE buffer, pH 8.0 and transfer contents to a microfuge tube on ice

11. Add 400 µl for 30% PEG 8000 in 1.8 M NaCl and keep at 4°C for at least 4 hours (overnight preferred)
    PEG allows the differential precipitation of the plasmid DNA

12. Spin at 15,000 rpm for 30 minutes - JA 20 rotor

13. Discard the supernatant and resuspend the pellet in 200 µl of TE buffer; keep on ice

    It can take up to an hour to soften the pellet enough to pass through a 200 µl pipette tip

14. Add an equal volume of phenol:chloroform:isoamyl alcohol to the DNA solution to be purified

    This step removes the protein contaminants

15. Vortex for 10 seconds and microcentrifuge for 5 minutes at max speed, room temperature

16. Carefully remove the top (aqueous) phase containing the DNA using a 200 µl-pipettor and transfer to a new tube. If a white precipitate is present at the aqueous/organic interface, repeat steps 14-16

17. Add an equal volume of chloroform:isoamyl alcohol to the DNA solution

    This step removes any residual phenol

18. Vortex for 10 seconds and microcentrifuge for 5 minutes at max speed, room temperature

19. Carefully remove the top (aqueous) phase containing the DNA using a 200 µl-pipettor and transfer to a new tube

20. Add 1/10 volume (~20 µl) of 3 M Na acetate, pH 5.2; mix by vortexing briefly or by flicking the tube several times with a finger

21. Add twice volume (~440 µl) of 100% cold ethanol (calculated after the salt addition). Mix by vortexing and precipitate in -80°C freezer for at least 15 minutes or store indefinitely.

    The remaining steps will be performed just before needed:

22. Microcentrifuge at maximum speed for 30 minutes and carefully remove the supernatant

23. Add 1 ml of 70% room temperature ethanol; invert the tube to mix and microcentrifuge at maximum speed for 30 minutes and carefully remove and discard the supernatant

    This wash will remove salts and small organic molecules. If the DNA molecules being ppted are very small (<200 bases), use 95% ethanol

24. Dry the pellet in a desiccator under vacuum or in a speedvac evaporator (8 minutes)

25. Dissolve the pellet in 50 µl TE buffer, pH 8.0

26. Quantitate 1:40 dilution and store at -20°C

5.3 Appendix C: Riboprobe preparation - restriction enzyme digestion

Linearize the DNA to limit probe length, using *Xba*1 restriction endonuclease to cut at pGEM-3Z site 32 downstream of the T7 promoter.

Reagent	Reaction Volume (µl)
DNA (0.345µg/µl)	30
REact 2 - 10 X buffer	10
Xba 1 endonuclease (10 units/µl)	5
ddH_20	54
total	100

The digested DNA must then be purified by phenol extraction and ethanol precipitation:

1. Add an equal volume of phenol (25): chloroform (24): isoamyl (1) to the DNA sample to be purified in a 1.5 ml microcentrifuge tube
2. Vortex for 10 seconds
3. Centrifuge at 14000 rpm for 5 minutes, room temperature
4. Carefully remove the upper aqueous phase containing the DNA using a 200 µl pipettor and transfer to a new tube. If a white precipitate is present at the aqueous/organic interface, repeat steps 1 to 4
5. Add an equal volume of chloroform (24): isoamyl (1) to the upper phase
6. Vortex 10 seconds
7. Centrifuge at 14000 rpm for 5 minutes, room temperature
   note: steps 5-7 will prevent carryover of residual phenol, however, these should not be necessary if the final pellet is washed well with 70% ethanol or if an additional ethanol precipitation step is added
8. Add 1/10 volume of 3 M sodium acetate, pH 5.2, to the solution of DNA
9. Finger flick to mix
10. Add 2 to 2.5 times the volume (calculated after the salt addition) of ice-cold 100% ethanol.
    Mix by vortexing and put at -80°C for at least 15 minutes or longer
11. Centrifuge at 14000 rpm for 15 minutes and carefully remove the supernatant
    note: pour off if a large pellet, or aspirate with a pipetting device on the opposite side against which the DNA precipitate was pelleted
12. Add 1 ml room temperature 70% ethanol. Invert the tube several times
13. Centrifuge at 14000 rpm for 15 minutes and carefully remove the supernatant
14. Dry the pellet in a dessicator under vacuum for 8 minutes
15. Resuspend the dry pellet in an appropriate volume of TE buffer, pH 8.0 for indefinite storage at -80°C

5.4 Appendix D: Riboprobe preparation – *in vitro* transcription and digoxigenin-11-dUTP labeling

The Roche digoxigenin-11-UTP (cat 3 11 209 256 910) technical manual states that dig-11-UTP is a substrate for T7, SP6 and T3 RNA polymerases and can replace UTP in *in vitro* transcription for DIG-labeling of RNA in a ratio of 35:65%. The volumes in the NTP/DIG mix required for the 100µl transcription reaction (see Section IV.F in Promega Technical Manual No. 016 - Synthesis of large amounts of RNA) are as follows:

NTP/DIG Mix:

concentration	volume (µl)
10mM ATP	5
10mM CTP	5
10mM GTP	5
6.5mM UTP	3.25
3.5mM DIG-11-UTP	1.75

Synthesis of RNA
note: this protocol typically yields 5 - 10 µg RNA/µg plasmid DNA

1. Add the following components to a sterile, RNase-free microcentrifuge tube at room temperature in the order listed:

Transcription optimized 5X buffer	20 µl
100mM DTT	10 µl
Recombinant RNasin Ribonuclease Inhibitor (40u/µl)	100u = 2.5 µl
NTP/DIG mix	20 µl
Linearized template DNA (goal: 5 µg in RNase-free water or TE buffer)	X µl
T7 polymerase (20u/µl)	150u = 7.5 µl
Nuclease-Free Water	to final volume of 100 µl

Gently vortex the tube to produce a homogeneous solution, then centrifuge briefly to collect sample at the bottom of the tube
2. Incubate for 2 hours at 37°C

3. Remove the DNA template: Add RQ1 RNase-Free DNase (1u/µl) to a concentration of 1u/µg of template DNA
4. Incubate 15 min at 37°C

The product may be visualized at this point:
Note: as the product is RNA, only RNase-free materials should be used. The gel equipment must be treated with 2% NaOH in DEPC-water for one hour and rinsed with DEPC-treated water and air-dried on paper towels. Additionally, all glassware and spatulas may be baked in advance.

a. Add a 5 µl aliquot of the upper, aqueous phase transcription product to 15 µl of RNA sample buffer (stored in 1 ml aliquots in -20°C freezer - 10 ml deionized formamide, 3.5 ml of 37% formaldehyde, 2 mls MOPs buffer)
b. Add 5 µl of RNA loading buffer (stored in 1 ml aliquots in -20$_o$C freezer - 50% glycerol, 1mM EDTA, 0.4% bromophenol blue, 1 mg/ml ethidium bromide)
c. Incubate the sample for 10 minutes at 65°C
d. Load in 2% agarose gel in 1 x TAE buffer and 0.5 µg/ml ethidium bromide; include RNA markers

5. Incubate at 65°C for 20 minutes to terminate the transcription reaction. (Alternatively, 2µl of 200mM EDTA, pH 8.0 could have been used to stop the reaction.)
6. Add 0.5 volume (50 ml) of 7.5 Ammonium acetate in DEPC water (finger flick to mix) and 2.5 volumes (375 ml) of 100% cold ethanol (invert to mix)
7. Precipitate in a -80°C freezer for at least 30 minutes or store indefinitely. Labeled probe will be stable at -20C or -70C for at least one year (Roche technical manual)

8. Centrifuge at 14000 rpm for 20 minutes
9. Carefully remove the supernatant and wash the pellet with 1 ml of 70% ethanol.
10. Dry the pellet under vacuum
11. Resuspend the RNA sample in 100 µl of sterile water (RNase and DNase-free). Use 8 ul of this for quantification. The expected riboprobe concentration will be ~40ug/ml. Store the rest of the riboprobe at -80°C for up to 12 months; alternatively, stock hybridization buffer containing 5ng/ml of the probe may be made and stored at -80°C for 6 months

5.5 Appendix E: *In Situ* Hybridization - Reagent Preparation

1 X PBS (R-01)
Reagents: Phosphate Buffered Saline 10 X solution. Fisher cat# BP399-1; lot# 027686-36 (RNase, DNase and Protease free; filtered; autoclaved; pH=7.4).

Prepare in RNase-free glassware; for a 1 L solution:

10 x PBS	100mls
DEPC-treated water (R-03) (or autoclaved ddH_2O)	900mls

store at 4°C

Note: keep 10 x stock sterile (open in a hood only)

DEPC - treated PBS (R-02)
Reagents: Phosphate Buffered Saline 10X solution. Fisher cat# BP399-1; lot# 027686-36
Diethyl pyrocarbonate, 97%NMR. Sigma cat# D-5758; lot# 053K3652

In a chemical fume hood, add DEPC at a 0.1% concentration in 1x PBS, leave in fume hood overnight, loosely capped. Autoclave in AM.

DEPC - treated H_2O (R-03)
Reagents: Diethyl pyrocarbonate, 97%NMR. Sigma cat# D-5758; lot# 053K3652

In a chemical fume hood, add DEPC to deionized water at a 0.1% concentration; shake vigorously to mix, leave in fume hood overnight, loosely capped. Autoclave in AM to inactivate the DEPC. Store at room temperature.

1N HCl Stock Solution (R-04)
Reagents: concentrated hydrochloric acid. Fisher cat# A-144-4

DEPC - treated dd H20 (R-03)	92mls
Concentrated HCl	8mls

Prepare in RNase-free glassware; add acid to water; mix by swirling; stable at room temperature.

0.2N HCl Working Solution (R-04a)
Reagents: concentrated hydrochloric acid. Fisher cat# A-144-4

DEPC - treated water	80 mls
1N HCl stock solution (R-04)	20 mls

Prepare in RNase-free glassware; add acid to water; mix by swirling; stable at room temperature

20 x SSC buffer (R-05)
Reagents: Sodium Chloride. Fisher cat# BP 358-1; lot# 035512; MW = 58.43
Sodium citrate (enzyme grade). Fisher cat# BP 327-500; lot# 037016

3 M NaCl	175.3g
300 mM sodium citrate	88.2g
DEPC - treated water (R-03)	800 ml

Prepare in RNase-free glassware; mix by swirling; stable at room temperature

2 x SSC Working Solution (R-05a)
Reagents: Sodium Chloride. Fisher cat# BP 358-1; lot# 035512
Sodium citrate (enzyme grade). Fisher cat# BP 327-500; lot# 037016

DEPC - treated water (R-03)	450 ml
20 x SSC (R-05)	50 ml

Prepare in RNase-free glassware; mix by swirling; stable at room temperature

Proteinase K Solution (R-06)
Reagents: Proteinase K. Sigma cat# P-2308; lot# 013K8601
Tris-HCl 1 M (pH 7.0) molecular biology. Fisher cat# BP 1756-100; lot# 994739
EDTA. Fisher cat# BP 2483-100; lot# 027445; FW = 372.24

for 10 ml at a final concentration of 10μg/ml:

Proteinase K	0.1 mg (0.0001g)
50 mM Tris-HCl	500 μl of a 1M stock (R-21)
5 mM EDTA	100 μl of a 0.5 M stock in DEPC-water (R-14)
DEPC - treated water	up to 10 ml

Add ingredients in the order listed above into a baked volumetric flask; with mixing

Note: Proteolysis is considered the most crucial step in *in situ hybridization*. In paraffin-embedded archival materials, optimal tissue permeabilization differs in each tissue, depending on the duration and type of fixation. Titration of the Proteinase K concentration from 5 - 20 μg/ml is recommended to find the highest concentration without compromising cellular morphology. Alternatively, incubation of slides for 20 - 30 minutes at 37°C with 1% pepsin in 0.2 M HCl may be attempted.

2 mg/ml Glycine in DEPC - treated PBS (R-07)
Reagents: Glycine. Fisher cat# BP 381-500; lot# 037392
Phosphate Buffered Saline 10X solution. Fisher cat# BP399-1; lot# 027686-36
Diethyl pyrocarbonate, 97%NMR. Sigma cat# D-5758; lot# 053K3652

Glycine	0.25 g
DEPC- treated PBS (R-02)	125 ml

Prepare in RNase-free glassware; mix by swirling; stable at room temperature

4% Paraformaldehyde (R-08)
Reagents: Paraformaldehyde. Sigma cat# P-6148; lot# 113K0657

PFA	4 g
DEPC-treated PBS (R-02)	100ml

Dissolve in baked glassware with a stir bar (stir bar may be pre-treated with 0.2% NAOH for 1 hour, then rinsed with DEPC-treated water); add 3 drops of 1N NaOH with a pasteur pipette; heat on a stir plate in a chemical fume hood to 60°C. Transfer to a cool stir plate, adjust pH to 7.4

TEA buffer 0.1M working solution (R-09)
Reagents: Triethanolamine hydrochloride. Sigma cat# T-1502; Lot# 122K5424; MW= 185.65

TEA	5.57g
DEPC - treated water (R-03)	300ml

Prepare fresh as needed in RNase-free glassware.

Note: 0.1M TEA buffer will be used with 0.25% acetic anhydride; acetic anhydride is highly unstable and should be added immediately prior to use.

3 x PBS (R-10)
Reagents: Phosphate buffered saline 10x solution. Fisher cat# BP399-1; lot# 027686-36

PBS	66.66 ml
DEPC - treated water (R-03)	133.34 ml

Prepare in RNase-free glassware; mix by swirling; store at 4°C.

1M NaCl (R-11)
Reagents: Sodium Chloride. Fisher cat# BP358-1; lot# 035512; MW = 58.44

NaCl	29.22g
DEPC - treated water (R-03)	up to 500 ml

Prepare in a 500 ml RNase-free volumetric flask; stir to dissolve; store in RNase-free glassware.

1N Na Citrate (R-12)
Reagents: Sodium citrate, enzyme grade. Fisher cat# BP327-500; lot#037016; MW=294.10.

Na Citrate	29.41g
DEPC - treated water (R-03)	up to 100 ml

Prepare in a 100 ml RNase-free volumetric flask; store in RNase-free glassware at room temperature.

1M NaH_2PO_4 (R-13)
Reagents: Sodium Phosphate Monobasic. Fisher cat# 5369-500; lot# 033858; FW=137.99.

NaH_2PO_4	13.8g
DEPC - treated water (R-03)	up to 100 ml

Prepare in a 100 ml RNase-free volumetric flask; store in RNase-free glassware at room temperature.

0.5 M EDTA in DEPC - treated water, pH = 8.0 (R-14)
Reagent: EDTA, Fisher cat# BP 2483-100; lot# 027445, FW =372.24
Diethyl pyrocarbonate, 97%NMR. Sigma cat# D-5758; lot# 053K3652
Sodium Hydroxide (molecular grade). Fisher cat# BP359-500; lot# 042654.

EDTA	18.61 g
NaOH pellets	2.0 g
DEPC - treated water	90 ml

Stir vigorously on a magnetic stirrer in RNase-free glassware. The disodium salt of EDTA will not go into solution until the pH of the solution is adjusted to 8.0 by the addition of NaOH pellets. Heat if needed to dissolve. Autoclave for 30 minutes.

Maleic Acid Buffer, pH = 7.5 (R-16)
Reagents: Maleic acid (cis-butenedoic acid). Fisher cat# 03417-500; lot# 037353; FW=116.1.
Tween 20 enzyme grade. Fisher cat# BP337-500; lot# 020436;
Sodium Chloride. Fisher cat# BP358-1; lot# 035512; MW = 58.44
Sodium hydroxide. Fisher cat# BP359-500; lot# 042654.

0.1M Maleic Acid	5.80g
0.15M NaCl	4.38g
DEPC - treated water (R-03)	up to 500 ml

Note: light sensitive (cover storage bottle with foil)

Measure dry ingredients above into an RNase-free volumetric flask, bring to volume with DEPC - treated water. Determine pH and adjust pH to 7.5 by adding NaOH (using NaOH pellets, 5 - 6 g or roughly 25 pellets will be needed).

Moist Chamber Solution (R-17)

Reagent: Formamide reagent grade. Fisher cat# F84-1; lot# 036353
Sodium Chloride Fisher cat# BP358-1; lot# 035512; MW = 58.44
Sodium Citrate, enzyme grade. Fisher cat# BP327-500; lot#037016; MW=294.10.
EDTA, Fisher cat# BP 2483-100; lot# 027445; FW = 372.24
Sodium Phosphate Monobasic. Fisher cat# 5369-500; lot# 033858; FW=137.99.

For a 300ml solution:

60% Formamide	180 ml
300 mM NaCl (R-11 - 1M stock)	90 ml
30 mM Na citrate (R-12 - 1M stock)	9 ml
10 mM EDTA (R-14 - 0.5M EDTA in DEPC -treated water)	6 ml
25 mM NaH_2PO_4 (R-13 - 1M stock)	7.5 ml
DEPC -treated water (R-03)	7.5 ml

Combine above reagents. Store 20 ml aliquots in blue capped 50 ml tubes at -20°C.

Prehybridization solution (R-18)

Reagents: Formamide reagent grade. Fisher cat# F84-1; lot# 036353
Sodium Chloride Fisher cat# BP358-1; lot# 035512; MW = 58.44
Sodium Citrate, enzyme grade. Fisher cat# BP327-500; lot#037016; MW=294.10.
EDTA, Fisher cat# BP 2483-100; lot# 027445; FW = 372.24
Sodium Phosphate Monobasic. Fisher cat# 5369-500; lot# 033858; FW=137.99.
Dextran Sulfate Sodium Salt. Fisher cat# BP1585-100; lot# 034876
Deoxyribonucleic Acid Calf Thymus. Sigma cat# D-1501' lot#091K7030
tRNA from Brewer's Yeast. Roche cat# 109 517; lot# 93159920
50 x Denhardt's solution. Fisher cat# BP515-5; lot# 034626

for a 50 ml solution:

60% Formamide	30 ml
300 mM NaCl (R-11 - 1M stock)	15 ml
30 mM Na citrate (R-12 - 1M stock)	1.5 ml
10 mM EDTA (R-14 - 0.5M EDTA in DEPC - treated water)	1.0 ml
25 mM NaH_2PO_4 (R-13 - 1M stock)	1.25 ml
5% Dextran Sulfate Sodium Salt	2.5 g
DNA Calf Thymus	0.0125 g
tRNA from Brewer's yeast	0.025 g
50 x Denhardt's solution	1 ml

Mix reagents with stir bar several hours or overnight, then aliquot into sterile, RNase-free microfuge tubes in 1 - 1.5 ml volumes and store at -20°C.

Ethanol series (R-19)
Reagent: Anhydrous Ethyl Alcohol (100% EtOH). Commercial Alcohols Ltd.; Brampton, Ontario.

95% EtOH	
100% EtOH	190 ml
DEPC - treated water	10 ml
70% EtOH	
100% EtOH	140 ml
DEPC - treated water	60 ml
50% EtOH	
100% EtOH	100 ml
DEPC - treated water	100 ml

Blocking Reagent (R-20)
Reagents: Blocking Reagent from DIG Nucleic Acid Detection Kit. Roche cat# 1 175 041; lot# 1201107(12).Maleic Acid Buffer (see R-16)

Blocking Reagent (Bottle 5)	25.0g
Maleic Acid Buffer (R-16)	200 ml

Dilute 10 x blocking solution 1:10 with Maleic Acid Buffer: Weigh blocking reagent into a 250 ml RNase-free volumetric flask. Add 200 ml maleic acid (R-16) and a baked or DEPC or 2% NaOH-treated stir bar. Stir until dissolved on a heating plate. Bring volume up to 250 ml with Maleic Acid Buffer. Transfer solution to a 1L RNase-free bottle, autoclave.

Prepare fresh (stable at 4°C).

Detection Buffer pH = 9.5 (R-21)
Reagents: Tris-HCl 1 M (pH 7.0) molecular biology. Fisher cat# BP 1756-100; lot# 994739
Sodium Chloride Fisher cat# BP358-1; lot# 035512; MW = 58.44

0.1 M Tris - HCl	25 ml
0.1 M NaCl (R-11 - 1M stock)	25 ml
DEPC - treated water (R-03)	Up to 250 ml

Prepare in an RNase-free volumetric flask; store in RNase-free glassware at room temperature. Add 1 N NaOH to adjust pH to 9.5.

Wash Solution A (R-22)

Reagents: Formamide reagent grade. Fisher cat# F84-1; lot# 036353
Sodium Chloride Fisher cat# BP358-1; lot# 035512; MW = 58.44
Sodium Citrate, enzyme grade. Fisher cat# BP327-500; lot#037016; MW=294.10.

60% Formamide	300 ml
300 mM NaCl (R-11 - 1M stock)	150 ml
30 mM Na citrate (R-12 - 1M stock)	15 ml
DEPC - treated water	35 ml

Prepare in a 500 ml RNase-free volumetric flask; store in RNase-free glassware at room temperature.

RNase Solution (R-23)

Reagents: Ribonuclease A (beef pancreas). Fisher cat# BP2539-100; lot# 030149.
Sodium Chloride. Fisher cat# BP 358-1; lot# 035512
Sodium citrate (enzyme grade). Fisher cat# BP 327-500; lot# 037016

Dilute with 2 x SSC for a 10 mg/ml concentration:

Ribonuclease A	20 mg (0.020 g)
2 x SSC (R-05a)	2 ml

Note: RNase is extremely stable and difficult to inactivate. Avoid contamination of any equipment or glassware which might be used for probe preparation or prehybridization procedures. Use separate glassware for RNase-contaminated solutions.

DIG Washing Buffer (R-24)

Reagents: Maleic Acid Buffer (see R-16).
Tween 20. Fisher cat# BP337-500; lot# 040294.

for a 500 ml solution:

Maleic Acid Buffer (mb 016)	498.5 ml
Tween 20 (0.3% v/v)	1.5 ml

Prepare in a 500 ml RNase-free volumetric flask; store in RNase-free glassware at room temperature.

Blocking solution (R-25)

Reagents: Blocking Reagent from DIG Nulceic Acid Detection Kit. Roche cat# 1 175 041; lot# 1201107(12).Maleic Acid Buffer (see R-16)

Blocking Reagent	10 ml
Maleic Acid Buffer (R-16)	90 ml

Dilute blocking reagent 1:10 with Maleic Acid Buffer. Store in RNase-free glassware.

Prepare and use fresh (Blocking solution is cloudy, but should not be filtered. It is stable at 4°C for 2 weeks if needed, but must be brought to room temperature before use).

Anti-DIG-AP Solution (R-26)

Reagents: α DIG-AP from DIG Nucleic Acid Detection Kit. Roche cat# 1 175 041; lot# 1201107(12).
Blocking solution (see R-25)

for a 1:500 dilution:

α DIG-AP (vial #3)	10 µl
Blocking Solution (R-25)	4.99 ml

Centrifuge vial #3 for 5 minutes at 10 000 rpm. Carefully remove 10 µl from the top of the vial; add this to 4.99 ml of blocking solution in an RNase-free container. Mix gently by inversion.

Prepare and use fresh (within 12 hours).

Note: Centrifugation is recommended as small antibody aggregates in the anti-DIG-AP may lead to background in the detection. After the first use it is sufficient to centrifuge the anti-DIG-AP for one minute directly before dilution.

NBT/BCIP Colour Substrate (R-27)

Reagents: NBT/NCIP from DIG Nucleic Acid Detection Kit. Roche cat# 1 175 041; lot# 1201107(12).
Blocking solution (see R-25)

NBT/NCIP (vial#4)	200 µl
Detection Buffer (R-21)	10 ml

Cover tube in foil (light - sensitive) and use fresh.

Decolourizer (R-28)

Reagents: Acetone from histology lab
Acetic acid, glacial "Baker Analyzed". JT Baker # 9508-03

Acetone	79.8 ml
Acetic acid	200 µl

Mix in RNase-free glassware and place in a labeled coplin jar.

0.5 M NaOH (R-29)

Reagents: Sodium Hydroxide (molecular grade). Fisher cat# BP359-500; lot# 042654, MW = 40.
Diethyl pyrocarbonate, 97%NMR. Sigma cat# D-5758; lot# 053K3652

Sodium Hydroxide	20.0 g
DEPC - treated water (R-03)	Up to 1000 ml

Mix to dissolve in RNase-free glassware and deliver to diagnostic histology laboratory for use during slide preparation for ISH.

5.6 Appendix F: Preparation of RNase-free glassware, coverslips and plastics

A. 2% NAOH

Reagents: Sodium Hydroxide (molecular grade). Fisher cat# BP359-500; lot# 042654.
Diethyl pyrocarbonate, 97%NMR. Sigma cat# D-5758; lot# 053K3652

Sodium Hydroxide (molecular grade)	20 g
ddH_2O	1000 ml

Dissolve with stirring. Soak glassware, lids, spatulas, etc for one hour; then rinse twice with DEPC - treated water and air dry on clean paper towels.

B. 3% Hydrogen Peroxide

Soak glassware, coverslips and plastics in 3% hydrogen peroxide for 10 minutes. Rinse in molecular biology grade (nuclease - free) water. Allow to dry and store in a dust-free environment.

C. Bake all glassware and/or heat tolerant items at 180 - 200°C for 4 hours or overnight.