Epidemiology of infectious salmon anemia in New Brunswick Atlantic salmon farms

A Thesis

Submitted to the Graduate Faculty

in Partial Fulfillment of the Requirements

for the Degree of

Doctor of Philosophy

in the Department of Health Management

Atlantic Veterinary College

University of Prince Edward Island

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Charlottetown, P.E.I.

September 2003

Your file Votre référence
ISBN: 0-612-93850-6
Our file Notre référence
ISBN: 0-612-93850-6

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Abstract

Infectious Salmon Anemia (ISA) is a recently described disease in farmed Atlantic Salmon, *Salmo salar*. A member of the newly identified Orthomyxovirus genus *Isavirus*, Infectious Salmon Anemia Virus (ISAV), has been identified as the causative agent. Virally infected Atlantic salmon experience lethargy, anorexia, severe anemia, exopthalmia, pale gills, ascites, and internal organ damage. Financial losses to the New Brunswick industry have been estimated to be $45M since 1997. The epidemiology of ISA in New Brunswick was investigated through five different studies.

Sensitivity and specificity of ISA diagnostic tests were evaluated as individual tests and in combinations, using data collected by the provincial government surveillance program. Depending on the test's cut-off value, the sensitivity and specificity for histopathology ranged from 30% to 73% and 72% to 99% respectively. IFAT had sensitivities and specificities in the range of 64% to 83% and 96% to 100% respectively. For the RT-PCR, sensitivity and specificity were 93% and 98% respectively. Test performances were also evaluated in series and parallel combinations. Sensitivities are maximized when tests are evaluated in parallel and ranged from 75% to 98%. Specificities are maximized when the tests are evaluated in series and ranged from 99% to 100%.

The prevalence of ISAV in cages experiencing an outbreak was compared with healthy cages from the same farm, neighboring farms, and distant farms. Salmon from five different groups were tested using an RT-PCR test. Groups included moribund fish from a cage experiencing an outbreak (A), healthy fish from an outbreak cage (B), healthy fish from a negative cage from a farm experiencing an outbreak in a different cage (C), healthy fish from a negative farm near an outbreak farm (D), and healthy fish sampled at a negative farm located in an area with only negative farms (E). Apparent prevalences (standard error) for the different groups (A-E) were 0.94 (.026), 0.41 (0.062), 0.29 (0.040), 0.08 (0.037), and 0.08 (0.037) respectively. All groups were significantly different ($p<0.002$) from each other except between groups B and C and between groups D and E.

Wild fish have been proposed as an ISAV reservoir because they are capable of close contact with farmed salmon. Pollock, *Pollachius virens*, are commonly found in and around salmon cages, and their close association with the salmon makes them an important potential viral reservoir to consider. Kidney tissue from 93 pollock that were living with ISA infected salmon in sea cages were tested with reverse transcription-polymerase chain reaction (RT-PCR) test. Results yielded the expected 193 bp product for positive controls, while no product was observed in any of the pollock samples resulting in an ISAV prevalence of 0%.

A risk factor study evaluated the associations between potential risk factors and ISA outbreaks in the Atlantic salmon sites in New Brunswick. The important factors identified by this study can be categorized as environmental, farmer controlled, or industry controlled according to the capacity to change or eliminate them. Environmental risk factors included shallow nets, deep water underneath the nets, and pollock in the salmon cages. Important farmer controlled risk factors included infrequent lice treatment, transferring large smolts into seawater, and high post-transfer mortalities. Industry controlled factors included boat traffic at the site and short distances to the closest neighbor with ISA.

Despite the introduction of an autogenous vaccine in 1999, Atlantic salmon in New Brunswick farms still suffer clinical ISA. Three vaccines were given to Atlantic salmon including Aquahealth Forte with autogenous inactivated ISAV and Bayoteck Multivacc4 with autogenous inactivated ISAV and the control, Aquahealth Forte with Renogen without ISAV. No ISAV specific antibodies were measured in the pre-vaccinated fish serum by enzyme-linked immunosorbent assay. There were only 6 of 235 post-vaccinated fish serum samples that had measurable levels of ISAV specific antibodies. This small number of fish with specific antibodies may be a result of a poor response to the vaccine, an ELISA test that was not analytically sensitive enough to measure small increases in specific antibodies, or an ELISA test that was not diagnostically sensitive resulting in many false negative results.

These epidemiological studies have resulted in new information regarding the disease. This information can be used to help reduce the impact ISAV has on the industry. Using the information from the diagnostic test evaluation combined with the information from the ISAV prevalence study, a better surveillance and control program could be designed to reduce ISA disease and its financial impact on the industry. Newly identified risk factors can be removed or modified to reduce the risk of disease for each farm. Following up the risk factor and vaccine studies, testing of particular risk factors such as ISAV vaccines by randomized clinical trials would answer the vaccine protection question.

Acknowledgements

After four years of work, it is hard to believe that this work has come to an end. Or maybe this is just the beginning?! This work could not have been accomplished without the help and support of many people and groups.

My co-supervisors. Dr. Larry Hammell and Dr. Ian Dohoo, have been extremely generous with the time that they have spent with me teaching me about epidemiology and aquaculture, but most importantly, teaching me how to conduct high quality research. Their time spent with me on this project has been a huge commitment on their behalf and invaluable to me. In the past four years I have been fortunate to see first hand how and why Larry has become the most respected aquaculture epidemiologist across Canada. I have also been equally privileged to have been instructed by the ultimate epidemiology teacher, Ian, who can make veterinary epidemiology understandable by his straightforward teaching style and unending veterinary examples.

Other members of my supervisory committee, Drs. Liz Spangler, Dr. Dan MacPhee, and Dr. Fred Kibenge have been excellent resources in current aquaculture and virology information as well as logistic support. Although not a member of my supervisory committee, Dr. Henrik Stryhn has provided many excellent statistical suggestions and deserves recognition for his significant contributions to this thesis.

Much credit for the completion of this program should be given to my fellow graduate students and technicians. Drs. Javier Sanchez and Aboubakar Mounchili have been my best friends as well as my epidemiology and STATA coaches. Graduate student life without their influence would not have been as fulfilling nor successful. Dr. Pascale Nerette and Jill Wescott have contributed so much to this aquaculture project and to every work day. Trips to St. George would have been cold and lonely without you both. Dr. Ane Nodtvedt may have contributed little to the academics of this project, but her friendship has been priceless. I also would like to recognize Holly Beaman for her significant contributions to sample collection and data management of this research and her companionship for all the years that I have been at the AVC.

Others who have contributed to the completion of this thesis have been the summer veterinary students who were beyond helpful in sample collection and keeping the summer fun. In particular I would like to acknowledge Natalie Keirstead, Mel Buote, and Pam Emdin for making my first summer sample collection successful, interesting, and enjoyable. I am much honored to have worked with you and am very proud of all your veterinary accomplishments thus far.

I am very grateful to the Natural Sciences and Engineering Research Council (NSERC), the New Brunswick Department of Agriculture, Fisheries, and Aquaculture (NBDAFA), the Atlantic Veterinary College, and the G. Murray and Hazel Hagerman Scholarship for their generous contributions for funding this project and my stipend.

Most importantly, I would like to express thanks to my family for supporting me through this program. My appreciation should be expressed to two women I admire and love, my mother, Judith Spradling and my mother-in-law, Gail McClure for encouragement, good advice, and being perfect role models. Finally, this thesis is dedicated to my husband, J, our daughter, Alexa, and to baby Trenton for any sacrifices they have made in order for me to complete this thesis and for all the love and joy they have shared with me since September 20, 1995.

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1 Epidemiology of infectious salmon anemia

1.1 Introduction

Infectious salmon anemia (ISA) is a recently described disease in farmed Atlantic salmon, *Salmo salar*. This disease has caused devastating losses in the Norwegian Atlantic salmon industry since the 1980's and is currently causing major mortalities in the Atlantic salmon farms in New Brunswick (NB), Canada[1]. In addition to Norway and Canada, the virus has caused disease in farmed Atlantic salmon in Scotland[2], the United States[3], and the Faroe Islands (Denmark)[4] as well as being isolated from Coho salmon (*Oncorhynchus kisutch*) in Chile[5]. Financial losses to the New Brunswick industry have been estimated to be $45M since 1997[6]. The newly identified Orthomyxovirus genus *Isavirus*, infectious salmon anemia virus (ISAV), has been identified as the causative agent[7].

Although the disease has been substantially controlled in Scotland and the United States, the disease continues to cause problems in the farmed salmon in New Brunswick. Despite continued husbandry and management changes in NB, the identification of ISAV in the salmon entered into seawater in 2002 has led to more fish being depopulated than from any previous year-class. The reasons for the continued losses are unknown, but resolution of some issues regarding the epidemiology of the disease may provide some insight to this continuing disease problem.

This introductory chapter contains some background information including the history of the disease as well as ISAV and its pathogenesis. The emphasis of the chapter is on epidemiological aspects of the virus and the disease. Individual testing methods are

described as are procedures to analyze how well these tests perform. Information regarding the spread of the virus passively through water, and actively on boats, through sea lice, and through wild fish is included. An examination of three published risk factor studies and how they have affected the current control programs for individual countries affected by the disease is featured. Finally, the current status of ISAV vaccinations is reviewed as these vaccines are new and continue to evolve.

1.2 History

In the Fall of 1984, a hatchery on the south west coast of Norway experienced a high level of mortalities in their Atlantic salmon parr[8]. Cumulative mortality levels reached 80% by the Spring of 1985[9]*****. The fish were all kept in tanks that were supplied a mixture of fresh water and untreated seawater[10]. The disease continued to spread from hatchery to hatchery and marine grow out sites due to the movement and sale of Atlantic salmon smolts. New cases were also seen in farms located near slaughterhouses or processing plants[11]. Mortality rates ranged from very low to moderate while the disease spread to different parts of Norway. Containment protocols[9] were instituted for the disease despite not knowing the identity of pathogen until 1995*****.

Until 1996, the virus had only been identified in Norway. In July of 1996, a newly identified disease was emerging in Atlantic salmon farms on the south east coast of New Brunswick, Canada[1;12]. The disease was causing increasing numbers of mortalities in several farms and was named Hemorrhagic Kidney Syndrome (HKS) due to the major pathologic sign[12]. Although the pathologic syndrome in New Brunswick salmon did not appear the same as in Norway, ISAV was eventually isolated from tissues of infected fish[13-15]. The 1996 year class was the first year class to be exposed at the time of entry

into seawater and the mortality patterns in affected cages were varied{425}. Out of 218 cages studied in the 1996 year class, 106 cages were considered ISA outbreak cages due to the length of time of increased mortalities or due to cumulative mortalities. The median peak daily mortality rate during outbreaks was 492 per 100000 fish per day, with 10% of cases experiencing greater than 5200 mortalities per 100000 fish per day. The median duration of outbreaks in cages for which the fish were not slaughtered during the outbreak was 33 days and the median total loss in those outbreaks was 6600 per 100000 fish. In 1998, clinically affected cages on 22 different salmon farms were depopulated to control the spread of ISA[1]. The Canadian Food Inspection Agency approved the use of an autogenous ISA vaccine for smolts that were placed into the cages in 1999. As fish in a cage become affected by an outbreak, all fish in the cage are slaughtered and the farmer is entitled to compensation when it becomes available. The current control program involves removal of infected fish on a cage-by-cage basis as directed by the Minister of Agriculture, Fisheries, and Aquaculture on advisement by the New Brunswick Fish Health Technical Committee (NBFHTC)[16].

ISA was identified in Atlantic salmon farms in Scotland in May 1998[2]. The clinical signs presented very similar to those reported in Norway. Among many control measures, Scottish officials also enforced ISA outbreak removal of entire farms, and only in special isolated situations, could cage-by-cage removal be considered. As of 2000, Scottish officials have declared ISA disease to be eradicated in Scotland[17]. The Faroe Islands had their first case of ISA in 2000[4] and five more in 2001. Regulatory officials in Faroe Islands have chosen to treat the disease in the same manner as in Norway[18]. The last new country to have an outbreak of ISA in farmed Atlantic salmon was the United

States, in the Cobscook Bay area in the state of Maine[3]. Although less than 2 km separates many Canadian sites from the US sites, the disease was not detected on US farms for many years. Unfortunately when established in Cobscook Bay, the disease was so extensive that to control the disease, regulators decided to depopulate all farms in the bay. Another country in which the virus had been identified in farmed fish is Chile where clinically affected Coho salmon have been observed[5]. Surveillance and control programs have not been reported for Chilean farms, and clinical ISA has not been an identifiable issue.

1.3 Virus

Many investigators tried to find the cause of the disease. The causative agent was presumed to be a virus because disease was experimentally created in Atlantic salmon by passing tissue homogenates from infected fish through a filter able to remove bacteria and then injecting the filtrates into healthy fish[8]. This virus was believed to be an enveloped because it did not retain its infectivity after it had been treated with chloroform[19]. The first direct observation of the virus was reported in 1994, when spherical viral particles of 100 nm in diameter were seen by electron microscopy in tissues of experimentally infected salmon[20;21]. The spherical enveloped virus was originally believed to have been an arenavirus. However, recent studies suggest that the ISA virus is actually an orthomxovirus[22;23]. *Orthomyxoviridae* is the family of virus that include Influenza virus.

Morphologically, the enveloped virus appears to be 100-120 nm in diameter and contains granules that are 10-20 nm in diameter[20;22;24;25]. There are mushroom shaped projections of 10 nm in length on the surface of the virus. Smaller defective virus

particles have been seen as well as large rod-shaped filamentous forms, which were up to 700 nm long. ISA virus is similar to the influenza virus in size, but the surface projections of the influenza virus are longer and rod shaped[22].

ISA virus has been described genomically by Mjaaland et al[23]. Specific DNA virus inhibitors and radioactive in vivo labeling of the virus that was cultured in Salmon Head Kidney (SHK-1) cells proved that the virus has an RNA genome. This negative sense RNA virus is single stranded and segmented. There are eight genomic segments that range from 1.0 to 2.3 kb with a total molecular size of 14.5 kb. Genomically, the ISA virus resembles other viruses in the *Orthomyxoviridae* family that have 6-8 segments in their genomes and 10-14.5 kb in molecular size.

All 8 segments have now been sequenced[7;23;26] and the nucleotide sequences for segments 2 and 8 have been used to distinguish between Norwegian and New Brunswick strains[27;28]. Although not associated with the Norwegian outbreaks, the strains of ISAV found in fish in Nova Scotia[29], Scotland[27], and the Faroe Islands were more like the Norwegian strain than the Canadian strain. The strain of ISAV isolated from the Coho salmon in Chile was more like the Canadian strain[5]. Strain differences are of interest because they offer epidemiologic clues to the virus origin and to the mode of virus transfer. Using evolutionary mutation rates on segment 2, it has been roughly estimated that the Norwegian and Canadian strains diverged around 1900[28]. Interestingly, this corresponds to the time at which the North Americans and Europeans had traded fish eggs extensively. Before the turn of the 20th century, North Americans sent Rainbow trout (*Oncorhynchus mykiss)* eggs to Europe and the Europeans sent sea trout (*Salmo trutta*) eggs to North America.

ISA virus has been characterized by its phenotypic qualities as well. The virus was first isolated in SHK-1 continuous cell line[24;30]. Cytopathic effect (CPE) was observed in this cell line 12-14 days after inoculation with kidney tissue from infected salmon. The infection of these cells was blocked by the addition of ammonium chloride, chloroquine, and bafilomycin A to the cultures indicating that entrance into the cells may require a low pH step[30]. Viral replication will occur at 15°C, but not at 25°C making homeothermic animals poor reservoir candidates. The virus loses its infectivity when exposed to chloroform, heat, and low pH. It also has the ability to hemagglutinate fish red blood cells.

1.4 Pathogenesis

Transmission of the virus from fish to fish has been studied using skin mucus, feces, urine, and blood from experimentally infected salmon[31]. All four biological products were able to produce the disease when injected intraperitoneally (IP) into naïve salmon, but were not capable of producing the disease when fed to the naïve salmon suggesting that coprophagy is not a likely route of entry. All four biological products were applied to gills of naïve salmon and blood and skin mucus showed high infectivity suggesting the gills were the natural route of entry.

ISAV was seen by electron microscopy (EM) after experimental IP challenge with sonicated blood derived from naturally infected farmed fish[21]. The virus was seen freely in the blood vessels and could be seen intracellularly in leukocytes and endothelial cells of all organs indicating that the target cells for the virus were leukocytes and endothelial cells and transport to the endothelial cells occurred through the leukocytes and freely in the blood. Confirmation of the target cells was obtained by observing naturally infected

farmed salmon that displayed budding of virus from endothelial cells, endocardial cells, and leukocytes on EM[32]. Eventually the virus was seen in endothelial cells of all organs.

Horizontal transmission is the common route of spread of the virus, but vertical transmission from broodstock to eggs is another possible route[33]. Nylund et al.[33] described a hatchery that had first-feeding fry die of ISA. The fry were kept in freshwater only, so saltwater contamination was not considered a possible route of entry. It was suggested that it was possible for eggs to be infected with ISAV, but it was more likely contamination from another unknown source. The possibility of vertical transmission was explored by testing fertilized eggs, alevins, and parr from ISAV infected female grilse with virus isolation and reverse transcription-polymerase chain reaction[34]. ISAV was not detected in any of the samples tested. Further testing included injecting homogenates of eyed eggs from the infected female grilse were into naïve parr. No mortalities occurred in the injected group. The results of these experiments indicate vertical transmission is not a likely route of ISAV infection.

1.5 Testing methods

1.5.1 History of testing

When the disease was first recognized in Norway in 1984, the etiologic agent was not known. Prior to identifying the viral cause, diagnosis of ISA was made by clinical signs of the affected fish and increased mortality rates. Gross pathology and histopathology were also used as diagnostic tests for ISA. The disease could be experimentally produced in Atlantic salmon by injecting tissue homogenates of infected fish into the healthy fish[8]. The ability to experimentally create the disease also led to the first visualization of the virus by electron microscopy[20]. It was not until 1995 that the

virus was first grown in Salmon Head Kidney (SHK-1) cells[24;30]. Virus isolation (VI) was the main diagnostic test until 1997 when Mjaaland et al. described a reverse transcription-polymerase chain reaction (RT-PCR) assay[23] and 1998 when Falk et al.[35] described a monoclonal antibody against the ISA virus that would be used in indirect immunofluorescent antibody test (IFAT) for the virus. Currently, SHK-1, Chinook salmon embryo (CHSE-214), TO, and Atlantic salmon kidney (ASK) cell lines are used for VI diagnostic testing[13;36;37].

1.5.2 Methods for testing for ISAV

1.5.2.1 Clinical signs, pathology, and mortality records

Before the causative virus was identified, diagnosis of ISA was made by post-mortem examination and evaluation of mortality patterns. Clinically ill fish usually have anemia and are anorexic and lethargic[38]. In Norway, the gross pathology of peracutely sick fish was said to include ascites and extreme pallor of gills and internal organs. If the disease was acute, signs of exopthalmia, petecchiae of the visceral fat, congestion of the gut, and congestion and enlargement of the liver and spleen were observed[8;38]. In New Brunswick, affected fish had gross pathologic changes that were very similar to the Norwegian disease except that New Brunswick fish occasionally had a patchy reddening within the kidney while liver congestion was rarely seen initially[2;12].

Microscopically, many affected Norwegian Atlantic salmon have a liver lesion considered to be pathognomonic. After 25 days post infection in experimentally infected salmon, there were focally extensive and bridging areas of congestion and hemorrhage with degeneration and necrosis of the hepatocytes[38;39]. Historically, liver lesions in New Brunswick salmon were varied and included vascular inflammation and cuffing by

leukocytes, multifocal to diffuse sinusoidal congestion and peliosis, and multifocal coagulative necrosis sometimes with hemorrhage. Interestingly, the large areas of hepatic necrosis seen in Norway but not common in New Brunswick are becoming more common in New Brunswick[9].

In New Brunswick, the pathognomonic lesion was not found in the liver, but rather in the caudal kidney[12;15;40]. Two changes in the caudal kidney are indicative of ISA infection. The first is interstitial renal congestion and hemorrhage which can be multifocal to diffuse, and the second is focal to multifocal coagulative tubular epithelial necrosis. Although originally not reported, histologic renal lesions similar to those in New Brunswick fish have been seen in archived tissues from affected fish in Norway[9].

Other histopathologic changes that may be seen in salmon with ISA, but are not pathognomonic include lesions in the gills, gut, and spleen[15;38]. The most common lesion of the gill is severe congestion of the central filamental sinus. Lesions of the gut included congestion of the lamina propria of the intestine and pyloric cecae as well as of the mesenteric vasculature. Severely infected fish often have sloughing of the tips of the intestinal mucosa. Erythrophagia and sinusoidal congestion characterize the spleen of affected fish.

Using clinical signs, pathology, and increasing mortality rates as a method of diagnosis is accurate when there is concurrent clinical disease. However, in experimental challenge with a strain of ISA virus that was isolated from sick fish from New Brunswick, the first histologic changes occurred after 20 days post infection (dpi) and pathognomonic changes occurred after 40 dpi[40]. Thus, using this test method early in the

infection for screening purposes is not recommended as its accuracy is based on the presence of clinical disease.

1.5.2.2 Electron microscopy (EM)

Initial electron microscopic studies on Atlantic Salmon in their freshwater phase that were experimentally infected with intraperitoneal (IP) injections of blood collected from salmon with natural ISA infections revealed that ISA was caused by a virus[20]. Electron microscopic diagnosis of the virus was described previously in the Virus, Section 1.3 above.

Although this test has been used in many research studies to identify the presence of the virus and its target cells, it is not a very practical diagnostic testing method[21;25;31;32;39]. A negative test result for ISAV would be very hard to declare with confidence because the virus may be in the tissue but not sufficiently concentrated to permit reliable visualization on the selected tissue sample. The virus also has different tissue selectivity depending on the stage of infection[31]. Early in the infection (<12 days post infection) virus can be found in pillar cells of the gills and endocardial cells. Between 13 and 19 days post infection, the virus is found in white blood cells and few red blood cells. Between days 20 and 22 post infection, the virus is found in leukocytes and endothelial cells of the spleen, head kidney, liver, and muscle. In the farm situation, it is impossible to identify the date of infection, thus selecting the appropriate tissues (e.g. gill, heart, spleen, kidney, liver, or muscle) for examined by electron microscopy is a difficulty in using this method as a diagnostic test.

1.5.2.3 Virus isolation (VI)

There have been six types of continuous fish cell lines (SHK-1, Atlantic salmon (AS), CHSE-214, ASK, TO, and Rainbow trout gill (Rtgill-W1)) that have been able to grow ISA virus. The commercially available, continuous cell line Atlantic salmon (AS) and the Rtgill-W1 will not be discussed because although the virus replicates in these cells, it does not cause cytopathic effect (CPE)[22;41].

The SHK-1 cell line was the first to be used to grow the virus. This long-term cell line was established from a culture of Atlantic salmon head kidney leukocytes that act like macrophages and is capable of propagating the ISA virus with the development of CPE[24;30;42]. Subculturing cells can be done every 10 to 14 days with the split ratio of 1:2 or higher when incubated at 20° C.

ISAV has been isolated from many tissues from infected Atlantic salmon including gill, heart, spleen, pyloric cecae, liver, and head, mid, and hind kidney[13;15;40;43]. The tissues are homogenized in PBS (pH 7.2) at a dilution of 1:10 (w/v) and then diluted with cell culture growth medium to a final dilution of 1:100 (v/v)[43]. Finally the solution is passed through a 0.45 μm filter to remove any bacteria. One-tenth of a milimeter of the filtered solution is inoculated onto SHK-1 cells after the culture medium has been removed from each well of a 24 well plate. The virus is allowed to adsorb for one hour at 15° C after which, 1 ml of culture medium is added and the plates are incubated at 15° C for 30 days while being monitored for CPE. At first CPE is identified by the presence of vacuolated cells[24]. As CPE progresses cells die and are then no longer adherent to the well. First signs of CPE are observed at 12-14 days post inoculation. CPE will develop earlier in subsequent passages and by the fourth passage, CPE can be observed as early as 3-4 dpi.

The CHSE-214 cell line is a continuous cell line that was isolated from Chinook salmon embryo cells and are used to isolate other salmonid viruses including infectious hematopoietic necrosis virus (IHNV) and viral hemorrhagic septicemia virus (VHSV)[44]. Subculturing of cells can be done every 7 days with the split ratio of 1:4 or higher when incubated at 15° C[45]. These cells are inoculated with tissue homogenates similar to that used with SHK-1 cells. As early as day 10 post inoculation, these cells showed plaques of refractile and necrotic cells indicating CPE[13]. This CPE will progress to include all cells of the monolayer appearing rounded, refractile, and necrotic.

In 2000, Devold et al.[37] introduced the Atlantic salmon head kidney cell line (ASK), which has distinct cell rounding and detachment from the substrate when incubated with ISAV. This CPE is evident within 7 to 8 days. In comparison to SHK-1, the ASK cell line had two distinct advantages. It was not as sensitive to variable growing conditions as the SHK-1 line and the distinct CPE improved the ability to quantify the amount of virus in the sample tested[46].

The TO cell line was established from Atlantic salmon head kidney leukocytes[36]. This cell line is extremely stable and cell culture morphology, growth, and viral yields did not change after 150 passages. The cell line has been shown to produce CPE 9 days post infection with ISAV. Although the viral yield for culture of ISAV on this cell line is very high, this cell line is not commercially available, due to patent issues.

There are some differences between the four cell lines. All identified ISA virus strains will grow on SHK-1 cells but only some of the strains will grow on CHSE-214 cells[45]. On the other hand, SHK-1 cells are not as easy to grow and subculture compared to the hardy CHSE-214 cells. The TO and ASK cell lines have two advantages in that it

is easy to grow the cell cultures and the presence of CPE is more rapid and distinct than the SHK-1 cell line.

One advantage of using VI as a diagnostic test for ISA virus is the resulting supernatant can be used to identify the strain of ISAV. Another advantage of VI is that only live virus capable of replication is detected. Unfortunately, there are many disadvantages to the VI technique. It is very expensive, tedious, and due to the long incubation period, results are often too long for management decisions. Once the test becomes positive for CPE, it requires confirmation with another test such as IFAT or RT-PCR to ensure the cells are dying from ISA virus and not another pathogen[47]. Another drawback is the inability to detect very small amounts of virus. The concentration of virions necessary to cause CPE is higher than the more analytically sensitive test, RT-PCR[43;48]. If tissues are not going to be processed directly, they should be placed on ice for no more than 24 hours until they can be stored at -80° C. Improper storage or freezing and thawing will result in the loss of live virus.

1.5.2.4 Indirect fluorescent antibody test (IFAT)

The use of monoclonal antibodies (MAb) against the hemagglutinin on the surface of ISAV techniques began in 1998[35]. The MAb was generated by injecting ISAV isolated from naturally infected fish into mice and using their resultant spleen cells for fusion with myeloma cells (SP2/0-Ag-14 cells) to create the MAb secreting hybridoma. Monoclonal antibodies can be collected directly from the supernatant of the hybridoma or from ascites from mice injected with these hybridomas.

This MAb has been used in a variety of types of tissue samples to diagnose ISA virus infection including identification of the ISAV in cell culture, in tissue sections, and

in tissue imprints. For the indirect fluorescent antibody technique (IFAT), tissue is fixed in acetone, blocked with 5% non-fat dry milk in PBS for 30 minutes, incubated with the anti-ISAV MAb solution for 1 hour, followed by incubation with diluted fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse immunoglobulin (1/100 in PBS) for one hour in order to detect bound antibodies. All incubations are at room temperature. Other types of fluorescence have been used including tetramethylrhodamine isothiocyanate (TRITC)[43].

The samples are viewed with a fluorescent microscope at 1000X magnification and are graded on a scale from 0 to ++++. Opitz et al.[43] gave some guidelines for interpreting the sample's fluorescence:

+	Sparse distribution of fluorescent grains or specks
++	More obvious distribution of grains (>10 per field) e.g.: around cell membranes
+++	Obvious distributions of grains e.g.: broken lines around cell membranes
++++	Broad fluorescence, no question that sample is highly infected: high confidence that tissue culture will yield virus

Unlike virus isolation, this test is very rapid and easy to run. Currently, this test is being performed as a screening test for the NB ISA surveillance program on imprints of the mid kidney from moribund salmon or fresh mortalities. Tissues from positive IFAT testing fish are tested using a different test (VI or RT-PCR) for confirmation. Although this test is rapid and done in the regional DAFA lab, there are two drawbacks. The first is that the analytical sensitivity of this test is unknown. Observations by veterinary practitioners indicate that clinical disease is necessary before there would be sufficient virions to get a positive test; meanwhile, negative tests are difficult to interpret. The

second disadvantage is the need for trained personnel to read the sample accurately and consistently.

1.5.2.5 Reverse transcription-polymerase chain reaction assay (RT-PCR)

The first reported use of RT-PCR was in 1997 when Mjaaland et al. described an RT-PCR assay for the diagnosis of ISAV in fish tissues as well as to confirm that the CPE in SHK-1 cell cultures was caused by the ISA virus[23]. Since that time, there have been many researchers and commercial laboratories that use RT-PCR for diagnosis and researching transmission[37;43;48-51].

Details vary from laboratory to laboratory, but the general procedure is the same. To identify ISA virus in a tissue or in cell culture, the viral RNA and cellular RNA is extracted and denatured[23]. cDNA is synthesized from the RNA by the addition of a solution containing deoxynucleoside triphosphates and primers, and the addition of Rnase inhibitors and reverse transcriptase. Polymerase chain reaction (PCR) is carried out on the cDNA to amplify the product. Deoxynucleoside triphosphates, two primers, and *Taq* DNA polymerase are added to the cDNA. The samples are cycled 35 times through 94, 55, and 72° C for separating DNA strands, annealing primers, and extension of primers respectively. Currently, there are one-step RT-PCR protocols using kits that are commercially available[37;50]. The DNA product is analyzed by gel electrophoresis to confirm it has the predicted base pair length. For further analysis such as strain specifications, the amplified product can be sequenced or tested by southern blot hybridization with a specific probe.

One advantage of this test is that it has been used on gill mucus and blood serum as a non-lethal test[52]. Non-lethal sampling could be used to test populations of fish in

tanks or sea cages to predict future outbreaks without sacrificing any animals. However, the greatest advantages to the RT-PCR diagnostic test are that the test is very rapid and highly analytically sensitive[37;48]. Modern thermocyclers can process thirty or more samples at one time and results are available in one day. Although it is unknown how much ISA viral RNA must be present in order for the RT-PCR test to amplify it, theoretically, it may only be one virion as PCR amplifies multiplicatively and after 32 cycles, there should be over one billion target DNA molecules for each virion.

Unfortunately the test's analytical sensitivity may also be one of its disadvantages. Sample processors must ensure that all equipment and receptacles are free of contamination. Another disadvantage of this test is tissue sample storage must be done properly. As tissue cells die, they release Rnase that will degrade any viral RNA if it is present in the tissues. Samples must be transported on ice and if not processed directly, should be stored at –80° C. Some laboratories prefer the sample to be saturated with RNA*later* (Ambion, Austin, Texas) an RNA preservative to protect viral RNA from degradation.

1.5.2.6 In situ hybridization

In situ hybridization[53] has been developed in order to identify the site of infection at the cellular level, to identify subclinical fish, and as a tool for epidemiological studies on archived tissue. Three probes were prepared, one from each segment 2, 6, and 8 of the ISAV. Under optimum conditions, the probes will hybridize with the nucleic acid of the virus that has infected specific organs. The test is *in situ* because the test is done on a prepared histologic slide of the fish tissues. Visualization of the hybridization is carried out using a microscope. All probes reportedly performed well but signals were strongest

using the probe for segment 8[53]. The strongest signals were seen in the endothelium of the heart, consistent with EM findings[20], but the liver, kidney, and spleen had medium intensity signals as well. There was no signal seen in the gills as might be expected from other diagnostic methods[21;48;51] but the viral nucleic acid may have been lost due to the decalcification process needed for the histological preparation of the gill tissue.

1.5.2.7 Enzyme-linked immunosorbent assay (ELISA)

The ELISA is the first diagnostic test that has been developed to detect host antibodies to the ISA virus rather than identifying the presence of the virus itself. There are different types of ELISA tests. The competitive ELISA and two-site capture ELISA are capable of measuring viral antigen, but to date, these have not been developed for ISA virus. However, an indirect ELISA has been developed to test the level of antibodies against ISAV as a tool for diagnosis of ISAV exposure as well as for ISA surveillance purposes[54]. This test uses virus purified from cell culture on SHK-1 cells as the coating on the ELISA plate. Salmon serum is added followed by a commercial mouse monoclonal antibody against fish immunoglobulins. A goat anti-mouse IgG_1 alkaline phosphatase-conjugate is added to the wells of a 96 well ELISA plate and after incubation, an enzyme substrate *p*-nitrophenyl phosphate is added. Color change is read by an ELISA reader at 405 nm wavelength. Because of the potential of non-specific antibody binding to the ISAV coated plate, all results are compared to wells in which cell culture antigens without ISAV are applied to the plate. Only samples with a substantial OD difference for the two different antigens are considered positive for ISA exposure.

The advantages of this test are that it is relatively inexpensive, many samples can be tested at the same time, it is rapid, and does not require lethal tissue samples. On the

other hand, fish that are vaccinated against ISAV but are not infected by the virus potentially could have antibodies resulting in a false positive conclusion that the fish is infected or has been exposed to ISAV. Fish that are recently exposed may not have developed antibodies to the virus yet, resulting in a false negative diagnosis.

There is much optimism that this test will help reduce the cost of surveillance testing and provide information on vaccine efficacy by anti-ISAV antibody titers. Unfortunately, this is a novel test and because it has not had widespread use, there is little proven or anecdotal evidence on performance compared to other tests.

1.5.2.8 Serum neutralization (SN)

The serum neutralization test is the second diagnostic test that measures antibodies to the ISA virus rather than identifying the presence of the virus. Cells of a suitable cell culture are grown in wells of a plate. A known amount of virus is added to the cell culture wells. Serial dilutions of heat-inactivated serum are added to the wells and incubated for one hour at room temperature. Tissue culture medium is then added to the wells and the plates are incubated at 15° C for up to 21 days. Cells are monitored for CPE regularly. Results are measured as titres. If all wells have CPE, the results are negative and the fish does not have neutralizing antibodies to ISA virus. Positive titres are read as the last well not to have CPE[55].

Disadvantages to this test are similar to the ELISA test and also include having delayed results and being labor intensive. Because the test measures only neutralizing antibodies, if the virus is present and the fish only develops non-neutralizing antibodies results will appear negative. One advantage of this test is its ability to test for exposure to virus in fish that have recovered and were able to rid themselves from the virus.

1.5.3 Assessment of tests' performances

Test performances can be evaluated analytically or diagnostically. Analytical sensitivity measures the lowest level of detectable pathogen that the assay can identify[56]. Analytical specificity measures the ability to correctly identify the pathogen of interest as opposed to a different pathogen cross-reacting. As mentioned in the individual test descriptions, analytical sensitivity has usually been measured in the commonly used tests. Current knowledge about the tests indicate that the RT-PCR test is the most analytically sensitive test available[37;48] followed by VI[37], and then the least analytically sensitive test is the IFAT[43]. Analytical specificity has been evaluated for each test and there does not seem to be cross-reaction[35;37] to other important fish virus like VHS, IHN, and IPN except for VI in cell culture [44]. However, this does not preclude the possibility that the test is detecting a non-pathogenic strain or a novel virus.

Diagnostic sensitivity and specificity are two other test performance measurements[57] to be considered when interpreting tests in surveillance programs. Given that an animal is infected, diagnostic sensitivity is the proportion of animals that test positive. Given that an animal is not infected, diagnostic specificity is the proportion that test negative. Studies measuring the diagnostic sensitivities of fish pathogens including ISA are very limited[43;48]. ISA studies were relatively small in scale and used laboratory-induced infections as opposed to naturally infected fish. A limited number of experimentally infected fish were tested. Groups of ISA negative fish with natural exposure to other pathogens or healthy fish were not tested to evaluate how well the test can identify negative samples (diagnostic specificity).

Surveillance programs for ISA virus in Atlantic salmon farms in both southern New Brunswick and northern Maine have created the need for commercially available

diagnostic tests. The commercially available diagnostic tests include: (1) VI, (2) IFAT, (3) RT-PCR, and (4) histopathology on fish tissues. Many different laboratories will perform one or more of these tests. Although the different laboratories will run similar tests, each diagnostic laboratory will optimize conditions for each test and there are no standardized requirements to use shared protocols. The results from the same tissue sample submitted to different laboratories or for different tests often yield conflicting results.

Current NB industry control programs require ISA testing on moribund fish at least every six weeks for every farm. Such surveillance results in mandatory slaughter of a cage if there have been at least two positive tests on at least two fish and fish in the cage have clinical signs of ISA with mortalities $\geq 0.05\%$ per day (or less if authorities consider the ISA risk to be great). Currently there are no large scale studies evaluating the diagnostic sensitivity and specificity of the commercially available diagnostic tests. Without this information, aquaculture regulators, veterinarians, and farmers do not have the proper tools to make accurate interpretations of ISA test results. Large scale evaluations on testing data should be completed in order for the NB surveillance program to effectively accomplish its mandate.

1.6 Epidemiology studies

Clinical ISA disease continues to occur in NB and the 2002 year class has been the most severely affected to date. The reason the disease continues to spread remains an unanswered question. Dissemination of the virus through the water, on contaminated work objects, and in other living organisms are believed to be spreading the virus and are making control efforts difficult[58].

1.6.1 Studies on infectivity of ISAV in water

ISAV is infective in water alone in the absence of organic matter[59]. In an experimental study in which parr were immersed in purified virus which had been added to freshwater at varying concentrations ranging from $2.5x10^1$ $TCID_{50}$ ml^{-1} to $2.5x10^5$ $TCID_{50}$ ml^{-1} for four hours, an average of 80% of the parr died in the tanks with $2.5x10^3$ $TCID_{50}$ ml^{-1} or higher concentrations.

A second study was performed on transmitting ISAV in seawater after 10 minutes, 2.5 hours, and 20 hours after preparation[60]. Blood from infected salmon was sonicated, filtered, and then diluted 1 to 1 in seawater. After the allotted time period stored at 6°C, each solution was injected separately into 5 naïve smolts. All fifteen fish had clinical signs of ISA and were dead by 24 days post infection indicating that ISAV was still infective even after 20 hours in seawater at 6°C. These results indicate that non-disinfected wastewater from the processing plants can be highly infective since it is discharged along with organic matter derived from fish including blood.

A recent study using a novel testing protocol for the detection of ISAV in seawater demonstrated that seawater samples taken from ISA sites and from well boats contained ISAV[61]. The protocol used tangential flow filtration to concentrate the virus in the seawater. A nested RT-PCR was developed as a more analytically sensitive diagnostic test for ISAV to measure very low numbers of virus particles in the water. Despite a successful protocol when used on water sampled from one outbreak farm 5 hours prior to testing, there were no virus particles found in water that had been removed from a different ISA outbreak cage 15 hours before the water sample was tested. The authors believed that the extended length of time the water sample was held on ice resulted in decay of the virus and concluded the virus is unstable in seawater.

These studies indicate that salmon can be infected through the contact with water containing virus. What remains to be determined is how long the virus can remain active in seawater with and with out organic matter. This information is critical to researchers and government officials for recommendations regarding removal infected cages to protect other cages, duration of fallow periods, and the minimum distance between farms.

1.6.2 Study on boat traffic

The virus can be transmitted through water alone, but it can also be spread actively on fomites such as contaminated work objects. Processing boat traffic was identified as a major route of spread for ISAV in Scotland[62]. Using a quantitative analysis of the records kept in well boat logs, an association was found between ISAV detection in the area and the number of visits by a well boat to that area. The increased risk was only for areas that had well boats transporting fish to and from farms and visiting farms for harvest. There was no increased risk for areas that had well boats perform other functions not involving the movement of fish. The increased risk is believed to be from movement of contaminated ballast water filled while the well boat was at a processing plant or harvest station and discharged at a farm in exchange for filling the wells with fish. Improper disinfection of the wells after transporting infected fish was also implicated. In New Brunswick, processing boats travel with minimum regulations, and there is little information on the impact of disease spread by the movements of these boats and other boats that work in the area of the farms.

1.6.3 Studies on sea lice

Another mechanical vector of disease spread is sea lice (*Lepeophtheirus salmonis* and *Caligus elongatus*)[60;63]. Sea lice are a good candidate for ISAV vectors because the

pre-adult and the adult stages move easily over the fish and can transfer between fish[64]. In one study, sea lice were removed from fish with a known history of ISA at the farm and were placed onto naïve smolts[63]. These smolts died within 41 days with clinical signs of ISA. In the same study, a homogenate of gut contents of lice removed from clinically ill ISA infected salmon, was injected into naïve freshwater smolts. All of these fish died after 42 days with clinical signs of ISA. No ISA diagnostic tests were conducted on the dead or moribund fish from these two experiments because at the time of the experiments, ISAV had not been isolated. However, live transmission studies were conducted injecting the serum and supernatant from the red cells of the moribund fish from both groups into naïve freshwater smolts. All of these fish died of clinical signs of ISA after 23 days post infection indicating that ISAV had been transmitted from the sea lice or the sea lice guts into naïve salmon.

In a second study using saltwater smolts, ISA infected cohabitant smolts were placed into a tank with sea lice (*Lepeophtheirus salmonis*) infected, ISAV naïve smolts[60]. The control cage did not include sea lice, just ISAV naïve smolts. The risk of dying increased from 15% in the controls to 68% in the sea lice smolts after 30 days cohabitation and then from 58% to 100% after 70 days of cohabitation. These results were compared to ISAV-free lice infested salmon bathed in ISA contaminated seawater in which there was no increase in ISAV deaths due to the wounds created by the salmon lice. The increase in mortality was attributed to the more efficient transfer of ISAV by the ISAV infected sea lice from smolt to smolt beyond the passive spread in the water and the potential of spread by coprophagy.

Sea lice have been established to be transmitters of ISAV, but questions remain about the importance of sea lice to the probability of transmission under production situations. Lice are mechanical vectors taking ISAV from one fish to another, but how long does the virus remain infective in the sea lice and does the virus multiply inside the sea lice? Sea lice can transmit ISAV in laboratory studies, but do they pose a clinical risk in the farm setting? If so, do the current methods of controlling for sea lice have an impact on the amount of ISA disease that is experienced by the farms?

1.6.4 Studies on wild fish

Like sea lice, some wild fish would make good vectors for ISAV spread, and may also serve as a reservoir for the virus[58]. Many wild fish live in the vicinity of the sea cages or swim by or through the cages during their migration. These wild fish may have extended exposure to the farmed Atlantic salmon because they reside for long periods and eat the excess salmon feed. There have been numerous laboratory studies showing how ISAV can be transmitted from wild fish to farmed Atlantic salmon. These studies are summarized in tables 1.1-1.4.

Table 1.1: Summary of ISA studies using fresh water Brown trout, *Salmo trutta*

Study	Snow et al. (2001)[65]	Nylund et al. (1995b)[66]	Nylund et al. (1995)[67]
Fish type	Brown trout, *Salmo trutta*	Brown trout, *Salmo trutta*	Brown trout, *Salmo trutta*
Water type	freshwater	freshwater	freshwater
Infection method	IP injection of virus grown on cell culture	IP injection of ISAV-infected, filtered ascites from clinically ill Atlantic salmon	Two IP injections of ISAV-infected, filtered ascites from clinically ill Atlantic salmon given 71 days apart
Isolate of virus	Scottish 390/98	Norwegian	Norwegian
Clinical signs	none	In 2/60: Dark pigmentation, pale gills and heart, yellow livers, swollen spleen, ascites	none
Changes in hematocrit	Mild reduction at 22 dpi	N/A	Not significant
Mortality	none	2/60	none
Diagnostic test	4/10 RT-PCR + and 3/10 VI + at 28 dpi, 4/10 RT-PCR + and 0/10 VI + at 40 dpi	N/A	N/A
Ability to infect naïve Atlantic salmon	N/A	yes	yes
Infection method	N/A	IP injection of filtrate prepared form liver and kidney of trout 20 dpi, and IP injection of blood from a trout 198 dpi	IP injection of filtrate prepared from liver and kidney of trout 28 days after second challenge of ISAV
Ability to infect naïve Atlantic salmon by cohabitation	N/A	no	N/A
comment		The trout at 198 dpi was sexually maturing, perhaps lowering its immune system and allowing for propagation of ISAV	Authors propose that mortality of salmon was lower than if trout had been treated with only one challenge of ISA because trout responded immunologically better after second challenge.

N/A: results were not available or experiment was not conducted.
dpi: days post-infection

Table 1.2: Summary of ISA studies using sea water Sea trout, *Salmo trutta*

	Rolland and Nylund (1998)[68]	Nylund and Jakobsen(1995) [69]	Devold et al. (2000)[37]
Fish type	Sea trout, *Salmo trutta*	Sea trout, *Salmo trutta*	Sea trout, *Salmo trutta*
Water type	seawater	seawater	seawater
Infection method	IP injection of filtered sonicated blood from clinically ill Atlantic salmon	IP injection of ISAV-infected, filtered ascites from clinically ill Atlantic salmon	Two IP injections of ISAV-infected, filtered ascites clinically ill Atlantic salmon given 71 days apart
Isolate of virus	Norwegian	Norwegian	Norwegian
Clinical signs	none	In 2/200 had pale livers and enlarged spleens	none
Changes in hematocrit	N/A	N/A	Mild reduction at 119 and 135 dpi (16 d post pred injection)
Mortality	None due to ISA	2/200	none
Diagnostic test	N/A	N/A	Many RT-PCR positive trout. VI was done on ASK cells and was negative until 135 dpi which was 16 d after injection of prednisoloneacetate
Ability to infect naïve Atlantic salmon	yes	yes	yes
Infection method	IP injection of filtered sonicated blood from trout	IP injection of filtered sonicated blood from trout at 10, 15, and 20 dpi	IP injection of filtered sonicated blood from trout 21 dpi
Ability to infect naïve Atlantic salmon by cohabitation	yes	yes	N/A
comment	Experimented with sea lice on trout, but did not increase the any of the mortality percents compared to similar experiment without sea lice	Suggested the possibility that sea lice transferred the virus from the wild sea trout to the farmed salmon	Suggested that sea trout are long term carriers for virus even if not picked up by tests other than RT-PCR because they can produce infectious virus 135 dpi after latent carrier test (LCT) in which trout were injected with 2 mg prednisoloneacetate

N/A: results were not available or experiment was not conducted.
dpi: days post-infection

Table 1.3: Summary of ISA studies using wild salmonids other than *Salmo trutta*

Study	Nylund et al. (1995c)[70]	Snow et al. (2001)[65]	Snow et al. (2001)[65]	Nylund et al. (1997)[71]
Fish type	Atlantic salmon, *Salmo salar*	Arctic char, *Salvelinus alpinus*	Rainbow trout, *Oncorhynchus mykiss*	Rainbow trout, *Oncorhynchus mykiss*
Water type	freshwater	freshwater	freshwater	freshwater
Infection method	IP injection of thawed, filtered, sonicated blood from clinically ill Atlantic salmon	IP injection of virus grown on cell culture	IP injection of virus grown on cell culture	IP injection of ISAV-infected, filtered ascites from clinically ill Atlantic salmon
Isolate of virus	Norwegian	Scottish 390/98	Scottish 390/98	Norwegian
Clinical signs	Consistent with ISA	none	none	5/15 had petechiae on the liver
Changes in hematocrit	N/A	Mild reduction at 22 dpi	Mild reduction at 22 dpi	Mild reduction at days 10, 19, and 20 pi
Mortality	≥85% mortalities	none	none	none
Diagnostic test	N/A	9/10 RT-PCR + and 0/10 VI + at 28 dpi, 1/10 RT-PCR + and 0/10 VI + at 40 dpi	8/10 RT-PCR + and 1/10 VI + at 28 dpi, 10/10 RT-PCR + and 0/10 VI + at 40 dpi	Examination of heart on EM showed 100 nm in diameter virus particles budding from the endothelial cells of the compact layer of the heart ventricle
Ability to infect naïve Atlantic salmon	N/A	N/A	N/A	yes
Infection method	N/A	N/A	N/A	IP injection of filtered sonicated blood from Rainbow trout
Ability to infect naïve Atlantic salmon by cohabitation	yes	N/A	N/A	N/A
comment	Two wild stocks were used and were less susceptible to ISAV than the commercial stock.	Cleared virus rapidly. Not concerned that this is a major reservoir		Virus propogates in Rainbow trout as seen in heart endothelial cells.

N/A: results were not available or experiment was not conducted.
dpi: days post-infection

Table 1.4: Summary of ISA studies using wild non-salmonids

Study	Nylund et al. (2002)[72]	Snow et al. (2002)[73]
Fish type	Herring, *Clupea harengus*	Pollock, *Pollachius virens*
Water type	Seawater	Seawater
Infection method	IP injection of sonicated blood from clinically ill Atlantic salmon	IP injection of ISAV purified from cell culture on SHK-1 cells
Isolate of virus	Two Norwegian strains (ISA8 and ISA36)	Norwegian Glesvaer/2/90
Clinical signs	none	none
Changes in hematocrit	Average was 26.5% at 23 dpi for the first challenge study and 20% and 19% for 26 and 33 dpi respectively, but at other times, no difference	At 42 dpi, there was a mild drop in hematocrit (28.6% vs 31.6% in control pollock)
Mortality	yes	11/150
Diagnostic test	≥70% RT-PCR + at differing time periods, no + VI on ASK cells	No + RT-PCR, VI, or histopathology on any dead pollock
Ability to infect naïve Atlantic salmon	yes	no
Infection method	IP injection of filtrate prepared from heart, liver, and kidney from herring 26 dpi	Cohabitation of IP injected pollock or with pollock that were previously cohabitating with infected salmon
Ability to infect naïve Atlantic salmon by cohabitation	N/A	no
comment	Herring were able to infect naïve salmon (RT-PCR +), but only one salmon died with clinical signs of ISA. Also noted the possibility of infecting salmon as schools swim through cages and in feed.	Both pollock and salmon died during the course of the experiment, but there were no positive tests for ISA on any of the pollock or any of the salmon that cohabitated with salmon

N/A: results were not available or experiment was not conducted.
dpi: days post-infection

1.6.4.1 Summary of wild fish experiments

These experiments were designed to identify if there are wild fish that potentially could be reservoirs for the virus. If so, these reservoir fish could explain how farms that appear to be isolated and have no obvious exposure to other infected farms become infected with the virus. Sea trout and brown trout as well as wild Atlantic salmon and Rainbow trout appear to be excellent candidates for ISAV transfer to the farmed salmon. Although brown trout and wild Atlantic salmon are more common near European salmon farms[58], these are not common wild fish in the area of NB where the salmon are farmed (Personal communication, Gilles Oliver, Department of Fisheries and Oceans).

Arctic char and Rainbow trout were tested as potential reservoirs because they are fish that are commonly farmed in the same areas as Atlantic salmon and could potentially infect farmed Atlantic salmon or become infected by them. From the experiments it appears that ISA mortalities in these fish are unlikely.

However, Coho salmon are farmed in other parts of the world and in one case in Chile became clinically affected by the ISA virus[5]. These Coho salmon were held on the same farm as Rainbow trout and Atlantic salmon. Two months after seawater transfer, the Coho salmon had a sudden rise in mortality. These fish had an extreme anemia, on average <10% hematocrit. Externally, the fish had pale gills and yellow coloring of the abdomen and the base of the fins. Internally, the fish were jaundiced, had pale livers, and mild splenomegaly. The virus was identified by use of RT-PCR on tissues as well as RT-PCR and IFAT confirmation by VI on CHSE-214 cells. Sequencing segments 2 and 8, the virus strain was found to be similar to the strains found in New Brunswick.

Although, neither the rainbow trout nor the Atlantic salmon ever became clinically affected and Chile has not reported any other ISA outbreaks, finding the virus causing disease in a different species of fish implies that care must be taken when growing different types of fish in the vicinity of Atlantic salmon.

1.6.4.2 Wild fish surveys

In addition to individual species being tested for their ability to carry and infect Atlantic salmon, there have been three large ISAV surveys of wild fish caught in fresh water and in seawater[74-76]. The Scottish survey tested 1447 non-salmonids, and all were negative by RT-PCR and by VI[76]. There were 423 wild Atlantic salmon tested, and 9 individual fish and 5 pools were positive by RT-PCR but all were negative by VI. Brown trout were tested and of 134 individual fish, there were 5 pooled samples positive by RT-PCR and no positive by VI. There were 203 sea trout tested and 5 were positive by RT-PCR while there were 5 different fish that were positive by VI. This study is consistent with what was found in the experimental wild fish studies.

The North American surveys were performed in fresh and seawater in southern New Brunswick[74] and around Maine with minor efforts along the eastern coast of the USA[75]. New Brunswick's report was brief and stated that out of thousands of wild salmonids and non-salmonids, only a few farmed Atlantic salmon escapees were found to have the virus and a few salmonids were RT-PCR positive for ISAV. None of these fish had any clinical signs of ISA. The study from the United States tested almost 3000 wild fish caught in their natural environment. Fish were tested by both VI and RT-PCR and there were no positive test results. Wild fish were also sampled from within (16 pollock, 26 lumpfish, and 120 cod) and around (90 pollock and 60 winter flounder) ISA infected

sea cages. One pool of cod (5 fish per pool) out of 24 pools was positive by VI and confirmed by RT-PCR on the cell culture supernatant. Sequence of the product demonstrated 99% homology with the North American ISA strain. There were no positive RT-PCR tests for the individual fish that made up the positive pool. Two of the 16 pollock from within a sea cage had weak positive signals for the RT-PCR test but were negative by VI. The RT-PCR product was so small that sequencing was ineffective leaving the significance of these two positive RT-PCR tests ambiguous. Both the cod and pollock were collected from a cage with fish that had clinical signs of ISA. Because cod and pollock are commonly found living in the cages with the Atlantic salmon, further research is needed to decide if these fish pose a risk to Atlantic salmon.

The laboratory studies on ISAV in wild fish have shown that it is possible for wild fish to be carriers of the virus. However, the few wild fish surveys that have been conducted have shown that the chances of wild fish transmitting the virus to caged salmon are slim unless the fish has prolonged contact with the farmed fish. Cod, herring, lumpfish, mackerel, and pollock are commonly found in the cages with the salmon. In New Brunswick, pollock are the most common. Even though they eliminated the virus rapidly in experimental studies[73], a larger group of pollock from cages with ISA infected salmon should be tested for ISAV.

1.6.5 Risk factor studies

1.6.5.1 Norwegian risk factor studies

Factors that can increase the risk of a farm or a cage becoming positive for ISA can be analyzed by studies that evaluate one risk factor at a time like well boat traffic or

wild fish. A risk factor study (RFS) can evaluate all of these individual risk factors simultaneously, evaluating each individual factor taking into account the other factors.

This analytical observational type of study draws conclusions from measuring the association of a factor with the disease outcome not in an artificial setting but in the individual's normal production environment. There have been three important RFS for ISA. Two of them were performed in Norway and consisted of a case-control study using the farm as the unit of concern[10;77]. There were similar results from these studies. An increase in risk of ISA on a farm was highly associated with the farm's proximity to another farm with ISA or to a salmonid slaughterhouse that did not disinfect its wastewater. Vagsholm et al.[77] was not specific in distance to other ISA farms or to the slaughterhouse, but Jarp and Karlsen[10] used a distance of 5 km for both. Not removing mortalities at least once a day in the summer months also increased the risk of ISA[10]. The significance of these three factors suggests that the virus is spread passively through the water and to prevent this spread, proper disinfection of wastewater, mortality removal, and increasing the distance between sites and between sites and slaughter plants are necessary to reduce the spread of ISA. Having a work force that was shared by more than one site also increased the risk of ISA suggesting the virus is being moved from one site to another due to improper disinfection[77]. Other factors that increased the risk of a farm having ISA were the purchase of smolt (young fish adapted for transfer into sea water) from more than one hatchery[10;77], and purchasing smolt from a county other than the farm's county[10]. It is possible that risk increased because of the increased travel and chance that the smolts were infected by improperly disinfected transport vehicles.

1.6.5.2 New Brunswick risk factor study

The third ISA risk factor study was performed in New Brunswick, Canada in 1997[78]. This study differed from the Norwegian studies because it was multilevel, investigating both factors associated with the farm becoming an ISA problem site (if ≥50% of cages were ISA positive) and factors specifically associated with a cage becoming ISA positive (as defined by the mortality rate and clinical diagnosis). Some of the important risk factors for a cage becoming positive were having more than 12,000 smolt initially stocked in a cage, having a moderate fish density within a cage, a high cumulative mortality for the cage in the first year in seawater, and weight sampling the fish in the second year in seawater. A protective factor was increasing the number of times fish were treated for sea lice. Site level factors were more difficult to analyze due to the small number of sites, but unconditional associations included feed delivered by feed company, divers visiting multiple sites, sites belonging to companies that owned more than one site, and having at least one other cage at the site having ISA increased the risk of a site becoming a problem site for ISA. Having only one year class of fish on the farm and increasing the time the fish were fed moist feed reduced the risk of a site becoming a problem site for ISA. These risk factors were categorized into risk factors either associated with the transmission of the virus or associated with host resistance in order to provide recommendations for reducing the probability of transmission to unaffected farms (transmission) or for managing the health of fish on farms that have already had ISA diagnosed in at least one of their cages (resistance).

It is interesting to note the similarities of factors detected in these three studies. Under production situations, it appears that proximity to other sites with ISA, proximity

to slaughterhouses that do not disinfect the wastewater, and sharing equipment, boats, and personnel with other sites are most predictive of increased risk for ISA cases.

1.6.5.3 Changes made in response to the RFS

Studies regarding reasons for spread of ISA have been critical in making recommendations for disease reduction. As the virus can spread through the water, removing the source of the virus is essential. If the New Brunswick Fish Health Technical Committee (NBFHTC) has recommended depopulation of a cage due to ISA, the farmer must submit a harvest plan to the Department of Agriculture, Fisheries, and Aquaculture (DAFA) veterinarian within 72 hours[16]. Plans should include information regarding the number of fish in the cage and the expected date of harvest. This plan is to ensure that infected cages do not stay in the water any longer than necessary. Another important control measure that has been adopted in NB is proper disinfection to inactivate any ISA virus in the wastewater released by the processing plants. To reduce other sources of viral transmission, mortality divers should have a separate set of equipment for each site, and positive cages often have their own mortality bag for the diver and are commonly the last cage dived. Occurrence of sharing workforce amongst sites has been reduced, and biosecurity has been increased when travel to multiple sites is unavoidable. A third change that has been made is the method for removing sea lice. No longer are laborious and stressful bath treatments performed. The introduction of an in-feed ectoparasiticide, emamectin benzoate (SLICE™, Schering-Plough Animal Health, Quebec), has made controlling lice more effective and timely. Another adjustment the industry has made is keeping only one year class of fish on a farm as well as only one

year class of fish in a Bay Management Area (BMA). There are exceptions to this rule depending on ability of companies to coordinate harvesting and marketing constraints.

Despite the actions taken in the form of biosecurity, surveillance, and control, depopulation of cages due to detectable ISA has increased in the 2002 year class[6]. More research is needed to elucidate other risk factors that are particularly important to the current situation in the farmed Atlantic salmon farms in New Brunswick.

1.7 ISA control methods of different countries

The five countries that have had major problems with ISA in their salmon farms include: Norway, Canada, Scotland, Faroe Islands (Denmark), and the United States. Most of these countries have adopted or will be adopting specific control procedures as standard for their industry. These procedures include removing infected fish, limiting boat traffic, and other changes listed above in section 1.5.5.3. Despite these controls, Canada, Norway, and the Faroes are still experiencing significant problems. Most of the countries have had similar surveillance programs and protocols for elimination of the disease. There have been a few differences in protocols that may contribute to success or failure in the elimination of disease.

1.7.1 Norway

Norwegian farms have had disease since at least 1984[8]. In 1988 ISA became a notifiable disease in Norway because it was considered contagious[79]. Since that time, the disease has continued at various levels in the Norwegian farming industry, and Norway has chosen minimization of disease since eradication of the virus seemed unrealistic given the number of farms and the number of rivers with migratory salmonids[80]. If there is any suspicion of ISA at a farm, blood and tissue samples are taken from 5 suspect fish.

Histopathologic exam is performed on these tissues[80]. At the same time, kidney imprints and kidney tissue samples from at least 10 fish are tested by IFAT and VI at the National Veterinary Institute. Restrictions banning trade are placed on the suspect farm. Diagnosis of ISA in a cage is based on one of the following two designations: (1) fish must be consistent with clinical signs, gross pathology, histopathology, and clinical pathology (anemia) for ISA as based on OIE standards or (2) positive IFAT and suspicious findings on clinical pathology or histopathology. To remove the label of suspicion, sampling is carried out twice, one month apart, and results must be free of any indication of ISA. If daily mortalities rise over 0.05% attributed to ISA, fish are removed on a cage by cage basis. Once all of the fish on a positive farm have been harvested, the farm is disinfected and must remain fallow for at least six months. After diagnosis of a case of ISA, a combat zone is established for all sites within a 5 km radius of the affected farm. All farms within the combat zone are subject to increased surveillance and are not allowed to stock smolts.

The worst year for ISA disease in Norway was 1990 when over 95 farms were affected. The evolution in control procedures seemed to improve the situation, and in 1999 there were only 8 verified outbreaks[79]. However since 1999 the incidence of disease has increased, and in 2001 there were 20 new cases of disease. To combat this rise in new cases, the farmers and regulators have agreed to replace the guidelines with a contingency plan. When put into action, the plan will require depopulation of all fish on an ISA farm within 80 days of diagnosis. There will be tighter controls on boat traffic including no movement of healthy fish through a combat zone. Slaughterhouse and processing plant biosecurity regulations will also be more strictly enforced. In addition,

for the first time in Norway's history, limited vaccination against ISAV will be considered in buffer areas around positive sites. To improve the rate at which farmer's report early signs of disease, financial compensation for early harvest of fish due to official regulations is also being considered.

1.7.2 New Brunswick, Canada

Norway had been combating ISA for 12 years when disease emerged in Canada in 1996[1]. Once the virus had been identified as the problem in NB, a control strategy was put into effect. Since 1997, the NBDAFA has been operating a surveillance program to identify farms that have cages with ISA infected fish. Every farm has moribund fish or suitable samples from dead fish tested for ISA every six to eight weeks. If there are any positive RT-PCR or two positive IFAT on a farm, the farm is tested more frequently (every two to four weeks). If there are two positive tests on two fish from a cage but no increased mortalities, the farmer is encouraged to slaughter the cage early and is eligible for compensation through an industry fund. If the positive tests are associated with an increase in mortalities greater than 0.05% per day attributed to ISA then the cage is considered to have an ISA outbreak, and the farmer must slaughter the fish in the cage[16]. The provincial veterinary authorities are also able to consider mandatory depopulation for ISA cases with lower mortality rates when there is an increased risk of spread to other sites. Delays in depopulation of both high mortality and low mortality ISA outbreak cages may be increasing exposure over a prolonged period of time, contributing to New Brunswick's ISA problem.

As mentioned previously, a mandatory disinfection of the wastewater from the processing plants was implemented in 1999. Another change was the implementation of

single year class farming in which a farm will not be allowed to stock fish from two consecutive year classes so that older fish do not infect younger fish[81]. For the same reason, only a limited number of fish from the previous stocking will be allowed to be held over on the site for a limited amount of time after the new smolts are stocked. This practice of "holdovers" is only permitted under defined circumstances and is being phased out completely. Vaccination against ISAV started in 1998 with two autogenous vaccines, one made by Aquahealth, Ltd. and the other by Bayotek International Inc. (Saanichton, BC). In 2000, Aquahealth, Ltd. (Charlottetown, PE) released a conditionally licensed inactivated virus vaccine, Forte V1 for testing[82]. Although these vaccines have been available to the hatchery managers and there is some evidence for laboratory–based efficacy, there is little evidence that this vaccine is effective under farming conditions.

Unregulated boat traffic has been a concern and the provincial government officials are currently working out a travel plan for processing boats to limit the spread of the virus and cross contamination at the wharves[6].

1.7.3 Scotland

After a severe outbreak of ISA in the Scottish farms that lasted from 1998 to 1999, eradication protocols dictated that the complete farms be depopulated unless they were in a remote location and had low mortalities. In this case, cage by cage depopulation was allowed[80]. The average time until the entire farm was harvested was extremely short, only 21 days[17], which was probably a big factor in successful eradication of the disease. Farms were confirmed to have ISA based on the following criteria: fish were diagnosed with ISA if they had clinical signs, histopathologic lesions consistent

with ISA, anemia, and were tested positive for the virus by VI, RT-PCR, or IFAT. Currently, legislation dictates that there must be isolation of virus in two samples from one or more fish at the farm sampled on two separate visits. Other important control measures instituted were the establishment of control and surveillance zones around infected farms, monthly surveillance site visits if ISA is suspected but not confirmed, single year class farming, and coordinated fallowing from 3 to 6 months depending on the farm situation. Since the removal of the last outbreak farm, Scotland has declared itself free of ISA and there have been no new cases. At this time, ISA vaccines are not used and compensation programs do not exist in Scotland.

1.7.4 The Faroe Islands, Denmark

The Faroe Islands had their first case of ISA in 2000[4]. There were five more outbreaks diagnosed in 2001[80] and in order to minimize the risk of more outbreaks, the Faroe Islands' regulators implemented policies and control measures similar to Norway[18]. As of 2003, not all of the hygienic practices that Norway employs have been put into place on the Faroes[18]. Because there is no compensation (Hans Jákup Mikkelsen, personal communication), and there are fewer farms with up to 2 to 3 million fish per farm, one outbreak can be devastating to the Faroese industry. To improve existing disease control, regulatory authorities in the Faroe Islands are reviewing current policies with the creation of a contingency policy that is similar to new policies put forth in Norway.

1.7.5 Maine, USA

The state of Maine in the United States had it first reported case of ISA in 2001[3]. The federal government rapidly introduced an indemnity package, and all farms in one

region (Cobscook Bay) were completely depopulated to ensure there were no farms misclassified as negative. In preparation for the disease identification in Maine, the farms had been subjected to third-party biosecurity audits and an ISA action plan that included a surveillance program. Once disease was identified, the Maine Aquaculture Association (MAA) developed a Bay Management Agreement that set minimum standards, practices, and protocols to be followed on farms to reduce the risk of disease[83]. Included in this legally binding agreement signed by all Maine farmers are sections addressing disinfection, biosecurity, waste management, sea lice control, and a communication plan. The communication plan outlines the farmer's responsibility to inform other farmers in the bay or in other bays of any potential increase in risk due to the ISA situation occuring. As of August 2003, Maine has had only two farms with fish that tested positive for ISAV since the depopulation of the farms occurred in Cobscook Bay in 2001.

1.7.6 NB surveillance program as a means to control viral spread

Salmon smolt transferred to seawater in 2002 have had significant positive testing which has lead to the highest depopulation in New Brunswick due to ISA to date. Despite the control methods put in place already, the disease continues to cause major economic hardship. One reason the disease may persist in New Brunswick is that infected cages may not be removed quickly enough and exposure of the virus to neighboring cages and sites occurs before the infected fish are removed. If this is the case, then either the surveillance program is not fulfilling its objective of identifying infected cages early, or depopulation delays are reducing the effectiveness of control practices. To evaluate if the current control program is identifying infected cages before

other cages are affected, the prevalence of the virus should be measured in different populations such as within the affected cage, other non-affected cages on the same site, and in neighboring and distant sites with no suspicion of ISA.

1.8 Vaccination

Vaccination against ISA as part of a control protocol has only been used in Canada since the1999 year class[84] and in the United States since the 2000 year class[3]. Other countries such as Scotland and the Faroe Islands are considering the use of vaccines, while in Norway's contingency plan, the use of vaccines will be considered only for restricted circumstances when several outbreaks occur in the same area[79]. Vaccines can be a very effective tool for the protection against a pathogen if they can stimulate a broad range of neutralizing antibodies and the vaccinated fish do not become carriers of the pathogen[84]. Currently there is little published data on how well the available vaccines and those vaccines in development accomplish the above requirements for a useful vaccine. There is historical proof that in the farming environment, ISAV vaccinated fish can become infected by the virus and many fish do become clinically ill[3].

There is also some evidence that salmon vaccinated with inactivated virus in an adjuvant are protected by the vaccine in laboratory studies[85-87]. Efficacies of vaccines from virus challenge studies are typically reported as relative percent survival (RPS) and can be calculated as RPS=[1-(% vaccinate mortalitity/ % control mortalitity)] x 100. In one study, a multivalent bacterial vaccine without ISA was shown to have the same efficacy as the multivalent bacterial vaccine with ISA after IP ISAV challenge suggesting the non-specific immune response was capable of decreasing the severity of the challenge[85]. However, if challenged by cohabitation with ISA infected salmon, the

multivalent bacterial vaccine without ISAV group had a RPS of 47% while the multivalent bacterial vaccine with ISAV group had one of 95% with 69% mortality in the controls signifying the adjuvant and bacterial antigens were somewhat protective in the passive cohabitational situation, but there was a better, more specific response when the ISA antigen was included in the vaccine.

In order for an inactivated vaccine to protect the fish it must promote the production of neutralizing antibodies. In many aspects, the ISA virus is still a mysterious pathogen because it is unknown why some salmon recover from infection and why some are incapable of recovering. Recovered salmon should have many specific neutralizing antibodies against some part of the ISAV. In laboratory experiments, Falk and Dannevig[88] showed that recovered fish did have a low level of humoral immunity when serum from recovered fish was passively given to naïve salmon. Sixteen of the 40 passively immunized salmon died after challenge with an IP injection of ISAV infected tissue homogenates as opposed to 28 of 40 of the control salmon. The reason for the low level of immunity in the convalescent antiserum is unknown, but an impaired immune response due to the virus replication in the immune cell populations is possible[89]. If impairment of B cell function is due to infection of these cells, this should not be a factor when using an inactivated virus and specific neutralizing antibodies should be produced in response to the vaccine.

Recently published work also suggests that certain antibodies produced during viral infection, may not be neutralizing, but may in fact, may aid in the process of viral entry into two macrophage-like cell lines, SHK-1 and TO cells[90]. If this is the case *in vivo*, an effective vaccine would result in the host producing neutralizing antibodies to the virus,

not antibodies that assist in the entry into the cell via the Fc receptor. If not there is always the potential that the vaccine would worsen disease by antibody enhanced infection of the virus into the fish leukocytes. More work is needed in this area to determine if certain antibodies resulting from vaccination or natural exposure facilitate infection rather than prevent it.

Inactivated ISA virus vaccine limited to one strain of virus is currently available to farmers. Because ISAV is an orthomyxovirus, like influenza virus, it may be subject to antigenic drifts (point mutation) and shifts (an exchange of the gene segment) of the hemagglutinin (HA) gene[84]. These drifts and shifts create multiple strains of the same virus. Vaccines for these viruses should contain either the strain of virus that would most likely affect the fish or a broad mixture of strains. Another difficulty encountered with the current vaccines is growing the virus in cell culture, which can be a very delicate and time-consuming process[24;91]. Advances in recombinant technology have made it possible to increase the production of an antigenic subunit of a virus for a subunit vaccine[84]. This would improve vaccine production and lead to a more specific antibody response. A further step is the creation of a DNA vaccine. DNA vaccines use plasmid DNA that encodes for the antigenic protein of the virus, injected into the host's cells ultimately expressing a low level of the viral antigen creating specific humoral (antibody) and cellular responses to protect against the virus. A useful ISAV DNA vaccine would immunize against a mixture of recombinant HAs or to a potentially highly conserved region of the virus' DNA.

The second important factor that a good ISAV vaccine requires is that if vaccinated fish are exposed to the virus, they should rapidly clear the virus and not become a carrier.

Little work has been done regarding this requirement. Six weeks after an IP ISAV challenge, Christie et al.[87] tested the vaccinated and unvaccinated fish that survived the challenge for virus using the RT-PCR test. Ten percent of the ISAV vaccinated fish and 80% of the ISAV unvaccinated fish were positive for ISAV. The groups of surviving fish were then mixed with naïve salmon and 5 weeks after mixing all fish were negative for ISAV by RT-PCR. There were no mortalities of the naïve fish maintained in the same tanks as the ISAV vaccinated post-challenge fish, but 40% of the naïve salmon cohabitating with the ISAV unvaccinated post-challenge salmon died indicating vaccinated fish cleared the virus while unvaccinated survivors were carriers of the virus at least for a short period. Thus, under laboratory conditions, vaccine reduced the risk of dying as well as reduced the risk of transmitting the virus to naïve salmon after exposure to the virus.

The issue of whether the nucleic acid within vaccines can give false positive results on RT-PCR testing remains unresolved. Currently this has not been reported, and it is believed that this phenomenon does not occur using the tests and the samples that are currently employed. During the recent USDA sponsored symposium "International response to infectious salmon anemia: prevention, control, and eradication" in New Orleans, LA, September 3-4, 2002, Dr. Peter Merrill from Micro Technologies, Inc., reported on a large-scale ISAV vaccine trial in which the fish were tested 1 day, 4 days, 7 days, 14 days, 28 days after vaccination, and monthly after that. There were no positive RT-PCR results and thus no evidence of vaccine interfering with the RT-PCR test.

For vaccine licensure within Canada, vaccine companies only have to prove efficacy based on laboratory vaccination-challenge models[92]. There is no need to prove

the vaccine is protective with large scale field trials or to quantitate antibody response. Recently, a small pilot study was performed to evaluate the efficacy of the vaccines under commercial conditions[93]. Fish were given different commercial vaccines from Aquahealth, Ltd. and Bayotek International Inc., some containing ISAV antigen and some without. Control fish were given saline instead of a vaccine. Fish were all tagged and mixed within one cage. Subsequently the farm became diseased with ISA and the dead and moribund fish from the study cage were collected and counted. Results from the study were not supportive of vaccine efficacy. During the ISA outbreak, ISAV vaccines did not significantly affect the time to dying, i.e. ISAV vaccinated fish did not die any later than non-vaccinated fish, leaving vaccine efficacy in doubt.

While others have had difficulty in developing an ELISA to measure antibodies against ISAV, Kibenge et al. verified that it is possible to measure antibody levels by ELISA, and this test may be useful in measuring antibody response to vaccines[54]. There is still more research to be done before ISA vaccines in North America can be recommended. Research addressing the question of current vaccines actually inducing any anti-ISAV antibodies in vaccinates that live in the farming environment is essential.

1.9 Overall objective

The overall objective of this thesis was to acquire a more complete appreciation of the epidemiology of ISA in farmed Atlantic salmon farms in New Brunswick. By answering important questions about the epidemiology of this disease, constructive recommendations could be made to the farmers and aquaculture regulators to minimize the amount and impact of the disease. There were 5 main issues that this course of study attempted to resolve: (1) The New Brunswick Department of Agriculture, Fisheries, and

Aquaculture (NBDAFA) operates an ISA surveillance program in cooperation with the veterinarians and farm owners. The mandate of the surveillance program is to identify cages with ISA infected fish and to remove them before the virus spreads to fish in another cage or to another farm. One challenge of the surveillance program is that the diagnostic tests on which depopulation decisions are based on have never been assessed for test performance making test result interpretation difficult. (2) Another concern with the surveillance program is that many cages are being removed to minimize the disease and this program has never been evaluated for how well cage removal works for protecting other cages or farms from the spread of the virus. (3) There is also no known virus reservoir in NB although wild fish including pollock have been suspected. Identification and removal of a viral reservoir would help reduce disease as well as keep fallowed farms from becoming infected once young salmon are stocked there. (4) Since the management of the control of the disease is continuously adapting to new scientific knowledge, unknown risk factors that contribute to the disease should be identified and modified. (5) Finally, ISA vaccines have been available since fish entered seawater in 1999 and yet these vaccines have not been rigorously evaluated under production conditions.

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2 Application of surveillance data in the evaluation of infectious salmon anemia diagnostic tests

2.1 Introduction

Infectious salmon anemia (ISA) virus has caused disease in farmed Atlantic salmon, *Salmo salar*, in New Brunswick (NB) since 1996[1]. This severe disease characterized by lethargy, anorexia, anemia, internal organ damage, and death[2;3] has occurred sporadically throughout New Brunswick fish farms in the Bay of Fundy, and in 1998, approximately 22 of the 83 salmon farms were completely depopulated for control purposes[1]. Costly control methods used on New Brunswick Atlantic salmon farms include a surveillance program, early slaughter of fish from test positive cages, and indemnity programs.

Current industry control programs require ISA testing on moribund fish at least every six weeks for every farm. Such surveillance results in mandatory slaughter of a cage if there have been at least two positive tests on at least two fish and fish in the cage have clinical signs of ISA. There are several commercial diagnostic tests including: (1) virus isolation (VI)[4;5], (2) indirect fluorescent antibody technique (IFAT)[6], (3) reverse transcription-polymerase chain reaction (RT-PCR)[7], and (4) histopathology on fish tissues[8;9]. Performance characteristics of these tests are not quantified, and test results from the same fish are often inconsistent. Although the ISA diagnostic tests have not been evaluated, results are used to make sizeable monetary decisions.

There are many different parameters to consider when describing test performance, but two of the most important are sensitivity and specificity[10]. Given that an animal is diseased, diagnostic sensitivity is the proportion of animals that test positive.

Given that an animal is not diseased, diagnostic specificity is the proportion that test negative. The computations of these two parameters in the simplest situation are shown in Table 2.1. Often diagnostic tests are used in combination on the same animal to improve the chances of a correct diagnosis, and the results can be interpreted in one of two ways. When the tests are combined in parallel, the interpretation is positive if one or both of the individual tests are positive and negative only when both of the individual tests are negative. When the tests are combined in series, the interpretation is positive only if both of the individual tests are positive and otherwise the interpretation is negative.

Analytical sensitivity differs from diagnostic sensitivity (described above) as it measures the lowest level of detectable pathogen the assay can identify[11]. Many studies have been performed to measure analytical sensitivities for important salmon pathogens including bacterial kidney disease (*Renibacterium salmoninarum*)[12], infectious hematopoietic necrosis virus[13], viral hemorrhagic septicemia virus, and infectious pancreatic necrosis virus[14]. Analytical specificity measures an assay's ability to correctly identify a specific pathogen minimizing the test's cross-reactivity with other pathogens. Studies measuring the diagnostic sensitivities and specificities of important salmon diseases are very limited[15]. A brief evaluation of ISA diagnostic tests from this current study's surveillance data has been reported previously[16] but not in the context of evaluating the surveillance program. An efficient surveillance program should be based on a quantitative assessment of test performance in order to make prudent decisions regarding cage removal due to disease.

Because performance reliabilities for each of the diagnostic tests were unknown, many tests were performed simultaneously on tissue from the same dead or moribund fish from 1998 to 2000 by the Provincial government as part of the early surveillance program. Those results were made available for evaluation of the diagnostic tests. The objectives of this study were two-fold. The first objective was to determine the diagnostic sensitivity and specificity of ISA diagnostic tests used in the surveillance program. The second objective was to evaluate the use of these tests in the current surveillance diagnostic protocol.

2.2 Materials and Methods

2.2.1 The data

In total, 30,255 test results were available from 8,167 fish that were sampled because they were dead or moribund. However, much of the data were not included here because the disease status of each fish's cage was available only from April 1999 to January 2000. All fish that had known diseases other than ISA were removed from the dataset. For the purpose of calculating sensitivity and specificity, our gold standards for disease status were defined as: ISA negative fish came from farms (sites) that had no outbreak of ISA in any cages during the period and ISA positive fish came from cages that were experiencing clinical disease (defined by greater than 0.05% mortalities per day at the time of sampling) which was attributed to ISA by regulatory officials.

Further, some of the laboratories and tests were dropped from the analysis because the numbers of samples were too small for statistical analysis. The tests and laboratories that were included in the study included the histopathology and VI performed at the Regional Diagnostic Virology Service and Aquatic Diagnostic Services

at the Atlantic Veterinary College (AVC) in Charlottetown, Prince Edward Island, the IFAT test performed at the New Brunswick Department of Agriculture, Fisheries, and Aquaculture laboratory (DAFA) in Blacks Harbour, New Brunswick, and the IFAT, RT-PCR, and VI tests performed at the Research and Productivity Council laboratory (RPC) in Fredericton, New Brunswick.

All test results were reported as dichotomous or ordinal results. Histopathology was reported on a scale of negative, suspect, and positive. For the sensitivity and specificity, histopathology data was analyzed two different ways, first with the suspect cases considered positive and second with the suspect cases considered negative. The IFAT results were reported as negative, 1+, 2+, 3+, or 4+ based on fluorescence intensity. The IFAT results were analyzed using two different cut points, first using 1+, 2+, 3+, or 4+ as a positive result (IFAT 1) and second using negative and 1+ as a negative result and 2+, 3+, and 4+ as a positive result (IFAT 2). The RT-PCR and VI have dichotomous results reported as positive or negative. Virus isolation on cell culture is positive if there is cytopathic effect on the culture confirmed by RT-PCR or IFAT to be caused by ISAV. Because of the expense of the VI test, pools of up to 5 fish were tested as one sample, and all fish in the pool were assigned a positive result if the pool was positive. Some fish were tested by VI individually (not in a pool) and these results were noted. The resulting data was used in the determination of sensitivity and specificity.

2.2.2 Estimation of sensitivity and specificity for individual tests

Sensitivities, specificities, and corresponding 95% confidence intervals (CI) were calculated in two different ways. Initially, test sensitivity, specificity, and 95% CI (exact based on the binomial distribution[17]) were calculated from a 2 X 2 table for all test results

and the gold standard classifications described above. For the VI tests, sensitivities, specificities, and 95% confidence intervals were calculated from the 2 X 2 table using the results from fish tested individually.

Secondly, to adjust for clustering of test results within a farm, Se and Sp were calculated using a series of steps for all tests except the VI due to the small number of test results available. Separate analyses were carried out for the gold standard positive and for the gold standard negative fish.

Step a: A logistic regression model was used to describe the probability of a positive or a negative test result conditional on the disease state of the fish. Because fish were sampled from cages and the results from fish from the same cage may not be independent from each other, a random effects logistic regression model with the farm as the random effect, the test result as the outcome, and maximum likelihood estimation of the model's parameters was used[18].

Step b: A random effects model yields "subject-specific" estimates but "population-averaged" estimates which give the average sensitivity across farms are more appropriate to describe test characteristics. To derive the "population-averaged" estimates of Se, the logit of the sensitivity (y) was calculated by the formula

$$y = \beta_0 \div \sqrt{1 + 0.346 \cdot \sigma^2} \qquad (1)$$

in which β_0 was the constant from the random effects logistic regression model for the ISA positive population and σ^2 was the estimated variance of farm random effects[19]. The logit of the specificity was calculated similarly.

Step c: The "population-averaged" estimates of the logit of the Se was converted to estimates of Se using the formula $Se = e^y/(1+e^y)$ with y from step b for the ISA positive population. Specificity was calculated using $Sp = 1-e^y/(1+e^y)$, with y from step b for the ISA negative population. Confidence intervals for sensitivity and specificity were calculated with the same formulas when substituting the limits of its CI for the constant, β_0.

Step d: To assess the degree to which the samples at the same farm were dependent[20], the intraclass correlation coefficient (ICC) was calculated as $\sigma^2/(\sigma^2+3.29)$.

Step e: To assess how the tests would perform if used on a new farm in New Brunswick, 90% prediction intervals for the farm's sensitivity and specificity were computed by the formulas in step c and using

$$y = \beta_0 \pm 1.645\sqrt{SE(\beta_0)^2 + \sigma^2} \quad . \qquad (2)$$

Prediction intervals are wider than normal confidence intervals because they include additional variation (σ) due to the random effects. The prediction intervals were relaxed to 90% because of the small number of farms in the study.

2.2.3 Estimate of sensitivity and specificity using two tests in combination

The sensitivity and specificity for each combined pair of tests was calculated by two methods: directly from the actual data, which accounts for any dependence between the two tests, and indirectly from the individual tests' sensitivity and specificity assuming independence between the two tests. If the tests' were dependent, test covariances were calculated to evaluate the degree of dependence.

2.2.3.1 Direct estimate

For the direct estimate, every diagnostic test was paired with each of the other diagnostic tests. The individual test results were evaluated in both series and parallel forming a new test result for the combined tests. These new results were then tabulated with the disease status of each fish in a 2 X 2 table, and sensitivity and specificity were estimated directly.

2.2.3.2 Indirect estimate assuming independence

Assuming independence between the two tests, the sensitivity and specificity of using two tests in parallel and in series were estimated using these formulas[10]: for parallel interpretation, sensitivity is $Se_p = [1-(1-Se_1)(1-Se_2)]$ where Se_p indicates parallel interpreted sensitivity and Se_1 and Se_2 indicate the sensitivity of the individual tests as calculated above. Specificity is $Sp_p = Sp_1Sp_2$ where Sp_p indicates parallel interpreted specificity and Sp_1 and Sp_2 indicate the specificity of the individual tests. For series interpretation, sensitivity is $Se_s = Se_1Se_2$ and specificity is $Sp_s = [1-(1-Sp_1)(1-Sp_2)]$.

2.2.3.3 Evaluating dependence between tests

If two tests evaluated in combination are independent, the direct estimate of the combined test properties should be the same as the indirect estimate if the same samples are used in each estimate. Because the two tests evaluated in combination may not be independent, the covariance can be estimated as a measure of their dependence. Independence of two tests, when fish were divided by their true disease status, was assessed by the Fisher's exact test[21]. If the p value for the Fisher's exact test was significant at $p<0.10$, the covariances of the test pairs were calculated.

The sensitivity covariance between the two tests used in combination is calculated as $\gamma_{Se} = p_{11} - Se_1Se_2$ where p_{11} is the probability of both tests being positive when the

animal is diseased, and Se_1 and Se_2 are the sensitivities of the two tests that are calculated from only the samples the two tests have in common[21]. The specificity covariance between the two tests used in combination is calculated as $\gamma_{Sp} = p_{00} - Sp_1Sp_2$ where p_{00} is the probability of both tests being negative when the animal is not diseased, and Sp_1 and Sp_2 are the specificities of the two tests calculated from only the samples the two tests have in common.

The upper and lower limits for possible values for the covariances were calculated according to Gardner et al., 2000. The covariances are directly affected by the values of Se and Sp, so the strength of the dependence was normalized by γ/maximal possible value of γ, as determined by the upper and lower limits.

2.3 Results

2.3.1 The data

The final dataset contained 3,721 test results from 1071 fish (807 negative and 264 positive). These fish came from 238 different cages and from 23 different farms.

2.3.2 Estimation of sensitivity and specificity for individual tests

Sensitivities and specificities with their associated confidence intervals for each test analyzed without (combined estimate) and with (population estimate) the random effect of the farm are shown in Table 2.2. In general, the sensitivity and specificity for histopathology ranged from 30% to 73% and 72% to 99% respectively depending on the cut-off value. IFAT had sensitivities and specificities in the range of 64% to 83% and 96% to 100% respectively. For RT-PCR, sensitivity and specificity were 93 % and 98% respectively and for VI, sensitivity was 67% and specificity was in the range of 99% to 100%. The small number of fish that were tested by VI made it impossible to evaluate

the random effects of the site with this test. Accounting for the within farm correlation altered the estimates for the other tests only slightly. There was significant within farm correlation for the sensitivity for histopathology with suspects considered negative and the DAFA IFAT 1 (cut-off between negative and 1+) as well as the specificity of histopathology with suspects considered positive, DAFA IFAT 1, and the RT-PCR.

2.3.3 Estimate of sensitivity and specificity using two tests in combination

Sensitivities and specificities calculated in series and parallel with their associated conditional covariance for each pair of tests are shown in Table 2.3 and Table 2.4 respectively. The test combinations with the virology tests from two different labs were not included in the analysis because of the limited number of samples these two tests had in common with the other tests. Sensitivities are maximized when tests are evaluated in parallel and ranged from 75% to 98% for the direct calculation from the actual data. Specificities are maximized when the tests are evaluated in series and ranged from 99% to 100%.

Also included in Table 2.3 and Table 2.4 are the sensitivities and specificities calculated in series and parallel assuming the tests are independent. These numbers were calculated from the individually calculated sensitivities and specificities presented in Table 2.2.

Except for the combinations of tests with significant dependence, all of the sensitivities and specificities changed only slightly compared to calculations using actual data when the two tests were both available. Gold standard positive fish for seven of the eighteen test combinations shown in Table 2.3 exhibited significant dependence. The covariances were all positive and ranged from 0.016 to 0.100. The sensitivity γ/maximal

possible value of γ ranged from 21% to 100%, or complete dependence. There were no significantly dependent tests for the gold standard negative fish.

2.4 Discussion

The farmed Atlantic salmon industry in New Brunswick is currently dealing with a diagnostic testing dilemma. The surveillance program tests many moribund fish from all of the farms in New Brunswick. If a cage is falsely diagnosed as negative for ISA, viral loads may increase and potentially spread to other cages or to neighboring farms. If a cage is falsely diagnosed as positive with ISA, the fish are harvested early resulting in high economic losses due to large volumes of non-market size fish going to market. As both of these scenarios are unacceptable, the identification of a diagnostic testing program with high sensitivity and specificity is imperative.

2.4.1 Evaluation of the individual tests

The results of our study showed the highest sensitivity in RT-PCR tests performed by the RPC lab. The RT-PCR test results are usually returned within a few days. Unfortunately this test's expensive price (>35 US$ per fish) may limit its adoption by the industry. The quickest and least expensive test available is the IFAT. Unfortunately, this test's sensitivity is at best 83%, leading to 17% of the truly positive fish testing as falsely negative. Histopathology did not perform very well as an ISA diagnostic test, but this test does have two advantages: it is relatively inexpensive and there is the potential to diagnosis concurrent diseases. Performance evaluation of VI was difficult in this dataset because most results were reported as pools, and the final evaluation was made only on fish that were tested individually. The pooled data was examined and there were very few pools of VI performed at the AVC lab. Those done in pools at the RPC lab were also

few, but the results from the pools would indicate perfect sensitivity and specificity if the samples were pooled. However, individually tested samples indicated that while the specificity of the VI was excellent, the sensitivity was poor for RPC's VI and not evaluated for AVC due to an insufficient number of samples. An advantage of VI is that a positive result indicates there is live virus in the sample. However, poor sensitivity, high expense, and long incubation periods restrict the use of this test[5].

2.4.2 Evaluation of surveillance program using performance of combined tests

Currently the New Brunswick Department of Agriculture, Fisheries, and Aquaculture (NBDAFA) uses a two test approach for its ISA surveillance program. Every six weeks a sample of suitable moribund or dead fish from every farm is tested for ISA. The fish are brought directly to the provincial laboratory on ice and a necropsy is completed or samples are taken on site, placed on ice, and brought to the laboratory. Samples are taken for all of the routine tests available. Kidney imprints are tested by IFAT at the DAFA lab immediately and all other tissue samples are stored for future testing. If the IFAT result is negative, the fish are considered ISA negative and no further testing is pursued. If the IFAT result is positive, the stored tissue is sent to the RPC laboratory for RT-PCR testing. If this test result is positive, then the fish is considered to be positive. If this result is negative, then the fish is considered to be negative. The results of this study suggest that this series interpretation has a sensitivity of 0.76 and a specificity of 1.00. Therefore, using this testing protocol approximately 24% of truly positive fish are falsely being labeled as negative for ISA.

An alternative approach would be to choose a testing strategy that maximizes sensitivity and specificity. The simplest approach would be to test all fish with the RT-

PCR test. This test has a high sensitivity, 0.93, and a high specificity, 0.98. However, due to its price, it is poorly suited for use on all fish tested in the surveillance program. Looking at the combined tests, the best performing pair of tests was the IFAT and RT-PCR with the tests interpreted in parallel. Unfortunately, this yielded only slightly improved sensitivities and specificities compared to the RT-PCR alone and would still require testing all fish with the pricey RT-PCR test. Another option is to test using both of the IFAT tests in parallel. If the DAFA IFAT is interpreted with the positive cut-off between 1+ and 2+ (DAFA IFAT 2) and the RPC IFAT is interpreted with the positive cut-off between negative and 1+ (RPC IFAT 1), then the resulting Se and Sp would be 0.87 and 0.97, respectively. This testing strategy, although not perfect, would offer improved sensitivity at a reasonable cost. Before testing strategies are changed, an in depth study using a larger disease positive pool, and an economic analysis using number of fish tested by the DAFA surveillance program and the cost of the individual tests should be completed.

2.4.3 Bias associated with data selection

Although we have estimated the sensitivities and specificities of these diagnostic tests, a critical review of the methods is important. Defining disease status on samples from perfectly healthy sites and highly diseased cages introduces bias that will cause tests to appear to perform better than they would if applied to all fish[22]. Fish that have just been infected and are not showing any signs of disease may not test positive on the available tests, resulting in a loss of sensitivity due to the analytical sensitivity of the test. Because the data was reduced significantly to identify obviously diseased and disease-free fish, test performance results in this study will appear better than they would be if the

tests had been assessed using the entire spectrum of disease states naturally present in production populations.

Conversely, the sensitivity of the VI test may have been falsely lowered. The DAFA lab pooled tissue samples from 1 to 5 fish. Fish in a pool usually came from the same cage with the number of fish in the pool varying with the severity of disease in the cage. If there were 5 fish in a pool, the cage probably had high mortalities and advanced ISA disease. These fish are likely to have abundant virus. If there was only one fish in a pool, there was probably fewer mortalities yielding only that one suitable sample from that cage. Therefore, the chance of the fish in that cage having advanced clinical illness due to ISA was lower. These fish may be infected, but may not have abundant live virus to easily create cytopathic effect on the cell culture, the endpoint of the VI test. If all fish in pools of 1-5 fish were used in this evaluation, the sensitivity may have been higher.

The gold standard positive fish were selected because they had clinical ISA. Gold standard negative fish were selected because they were from a farm that never had any history or suspicion of ISA. Because there was no way to be sure that the gold standard positive fish were 100% infected by ISAV and the gold standard negative were 0% infected by ISAV, it is possible that there were some misclassified fish resulting in a lowered sensitivity and specificity.

2.4.4 Accounting for clustering of results within farm

The random effects model was used to account for the fact that fish from one farm are potentially more alike than fish from different farms. Using steps b and c from the methods section, "population averaged" estimates of sensitivity and specificity were estimated from the results of the random effects logistic regression model. These

estimates would be considered more accurate than the combined estimates in tests where there was significant within farm correlation. In addition, the model provides prediction intervals (step e) for how the test would perform if it were used on a new farm in New Brunswick or a farm not included in this study. When variation between farms is present, these intervals are wider than the confidence intervals because they incorporate farm-to-farm variation. Possible reasons for extra variation between farms include genetic differences, geographic location, age of fish at the farm, and management factors (feed, handling, sea lice burdens, hygiene, etc.). A hypothetical scenario might involve a strain of Atlantic salmon with improved resistance to ISAV such that these fish rarely replicate enough virus to test positive on the IFAT test. This would result in an increase in false negative tests for fish from farms raising this strain of salmon and can explain the wide 90% predicted interval for the IFAT tests. A hypothetical geographical example may be that moribund fish that come from more remote farms are not processed as quickly as similar fish from nearby farms. As sampled fish decompose, the integrity of the viral RNA may be jeopardized. Fish from these farms are more likely to have false negatives on the RT-PCR test as a result of the increased time to processing and this can be seen by the very wide 90% predicted interval for the sensitivity for the RT-PCR test. The population estimate for the RT-PCR is 0.932 and the 90% predicted interval ranges from 0.828 to 0.981.

2.4.5 Evaluating independence of test results

A common assumption when interpreting tests in combination is that the two tests are conditionally independent[21]. In this study we were able to interpret the combinations both when assuming conditional independence and the more accurate interpretation when

we account for conditional dependence. Conditional dependence of test sensitivities occurs when the second test has a different sensitivity for diseased fish that test positive on the first test compared to those that test negative on the first test. Conditional dependence of test specificities occurs when the second test has a different specificity for non-diseased fish that test negative on the first test compared to those that test positive on the first test. The measures of dependence between two tests' sensitivities and specificities are measured by the covariances of the sensitivities and specificities and are gauged by the covariance/maximal covariance of the two tests' sensitivity and specificity. If interpreting two tests in parallel and the two tests have a high sensitivity covariance/maximal covariance, then using the second test will not yield much new information and the resulting sensitivity will be little more than just using the one test with the better sensitivity. The same is true for specificities when interpreting in series. It should be noted that two tests with a covariance/maximal covariance for Se (or Sp) equal to 1 are only in complete agreement on positive (negative) fish if the Se's (or Sp's) of the two tests are the same.

The best tests to use in combination may not always be the ones with the best individual sensitivities and specificities, but could possibly be the tests that have the lowest sensitivity and specificity covariance/maximal covariance. One example of this effect is using histopathology (with suspects being considered negative) and the DAFA IFAT 2. The sensitivity covariance/maximal covariance was very low at 0.133 and the sensitivity of the tests in parallel jumps from 0.302 for the histopathology and 0.644 for the IFAT to 0.746 for the combined sensitivity.

A positive dependence should be expected in tests that measure the same biological process[21] as can be seen when combining the IFAT with the positive cut-off between negative and 1+ from DAFA and RPC. Both tests measure the level of virus present in kidney imprints on glass slides. The covariance of the sensitivity is 0.100 and a sensitivity covariance/maximal covariance of 0.697 which was the highest positive dependence measured in this study that was not equal to 1. If these tests were independent, we would expect that sensitivity in parallel would be 96%, but because they are very dependent, the sensitivity in parallel is only 87%. This can be compared to the previous example in which histopathology was used in conjunction with IFAT. Positive diagnosis for ISA using histopathology is based on microscopic lesions in the fish tissues while the IFAT test is based on the presence of virus in the kidney. The biologic processes are different, thus the tests are less likely to be dependent.

Estimates for sensitivities and specificities for ISA diagnostic tests are helpful in choosing which test will most likely return a true result. However, each test measures something different about the disease. Virus isolation measures live virus[4;5], RT-PCR measures viral RNA[7], IFAT measures viral antigen[6], and histopathology assesses lesions[8;9]. If RT-PCR is positive, there is most likely ISA viral RNA in the fish, but this does not necessarily indicate that the fish is clinically ill or actively shedding virus. Until we are capable of predicting the clinical disease and increased mortalities of the fish in a cage using diagnostic tests, test results should be interpreted cautiously.

2.5 Acknowledgements

We thank the New Brunswick Department of Agriculture, Fisheries, and Aquaculture for providing the surveillance data. We are grateful for funding assistance from Natural Sciences and Engineering Research Council and the Atlantic Veterinary College for this study.

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Table 2.1: Estimation of Sensitivity (Se) and Specificity (Sp) of a diagnostic test using a 2 X 2 table.

		True disease status		
		Diseased	**Not Diseased**	
Test results	**Positive**	a	b	a+b
	Negative	c	d	c+d
		a+c	b+d	a+b+c+d=n

Sensitivity=a/a+c
Specificity=d/b+d

Table 2.2: The estimated sensitivities (Se) and specificities (Sp) for four infectious salmon anemia diagnostic tests in the New Brunswick Atlantic salmon farms

Test	Number tested	Parameter	Combined estimate (CI)	Population estimate (CI)	Random effect		90% Predicted interval
					estimated ICC	P value	
Histopathology (positive[a]) (AVC[δ])	674	Se	0.730 (0.653-0.797)	0.730 (0.655-0.793)	0.00	1.000	66.8-78.3
		Sp	0.725 (0.682-0.764)	0.721 (0.646-0.794)	0.07	0.000	52.8-86.6
Histopathology (negative[b]) (AVC[δ])	674	Se	0.302 (0.232-0.380)	0.299 (0.219-0.393)	0.34	0.000	4.7-75.3
		Sp	0.994 (0.982-0.999)	0.994 (0.981-0.998)	0.0	1.000	98.4-99.8
IFAT 1 (DAFA[α])	871	Se	0.791 (0.732-0.842)	0.794 (0.693-0.869)	0.14	0.011	54.5-94.0
		Sp	0.955 (0.936-0.970)	0.957 (0.924-0.976)	0.11	0.027	89.3-98.9
IFAT 2 (DAFA[α])	871	Se	0.644 (0.578-0.707)	0.644 (0.580-0.704)	0.00	1.000	59.0-69.5
		Sp	0.999 (0.991-1.000)	0.998 (0.989-1.000)	0.00	1.000	99.2-100.0
IFAT 1 (RPC[β])	473	Se	0.827 (0.697-0.918)	0.827 (0.700-0.907)	0.00	1.000	72.3-89.8
		Sp	0.983 (0.966-0.993)	0.980 (0.912-0.996)	0.31	0.070	90.3-99.9
IFAT 2 (RPC[β])	473	Se	0.731 (0.590-0.844)	0.736 (0.567-0.856)	0.05	0.313	52.4-88.2
		Sp	0.998 (0.987-1.000)	0.998 (0.983-1.000)	0.00	1.000	98.8-100.0
RT-PCR (RPC[β])	948	Se	0.926 (0.882-0.957)	0.932 (0.862-0.967)	0.10	0.103	82.8-98.1
		Sp	0.981 (0.968-0.990)	0.967 (0.910-0.988)	0.48	0.000	84.5-100.0
Virus Isolation (AVC[δ])	21	Se	No samples	N/A	N/A	N/A	N/A
		Sp	1.000 (0.839-1.000)	N/A	N/A	N/A	N/A
Virus Isolation (RPC[β])	72	Se	0.667 (.094-0.992)	N/A	N/A	N/A	N/A
		Sp	0.986 (0.922-1.000)	N/A	N/A	N/A	N/A

[a]Suspects were considered positive, [b]Suspects were considered negative, [α]New Brunswick Department of Agriculture, Fisheries, and Aquaculture; [β]Research and Productivity Council; [δ]Atlantic Veterinary College

Table 2.3: The estimated sensitivities (Se) for infectious salmon anemia diagnostic tests evaluated in series and in parallel.

Test 1	Test 2	Number Positive samples[Ψ]	$Se_{parallel}$		Se_{series}		p value[θ]	Covariance of Se	Cov_{Se}/Max
			actual	independent	actual	independent			
Histopathology (positive[a]) (AVC[δ])	IFAT 1 (DAFA[α])	130	0.923	0.944	0.608	0.577	0.146		
	IFAT 2 (DAFA[α])	130	0.869	0.904	0.515	0.470	0.061	0.038	0.213
	IFAT 1 (RPC[β])	52	0.904	0.953	0.635	0.604	0.100	0.046	0.375
	IFAT 2 (RPC[β]	52	0.865	0.927	0.577	0.534	0.081	0.057	0.297
	RT-PCR	135	0.978	0.995	0.719	0.716	0.021	0.016	1.000
Histopathlogy (negative[b]) (AVC[δ])	IFAT 1 (DAFA[α])	130	0.877	0.854	0.232	0.238	0.351		
	IFAT 2 (DAFA[α])	130	0.746	0.752	0.215	0.194	0.551		
	IFAT 1 (RPC[β])	52	0.827	0.879	0.192	0.250	0.178		
	IFAT 2 (RPC[β])	52	0.751	0.812	0.173	0.221	0.254		
	RT-PCR (RPC[β])	135	0.978	0.987	0.326	0.296	0.551		
IFAT 1 (DAFA[α])	IFAT 1 (RPC[β])	23	0.870	0.964	0.783	0.654	0.009	0.100	0.697
	IFAT 2 (RPC[β])	23	0.870	0.944	0.696	0.578	0.040	0.085	0.662
	RT-PCR (RPC[β])	176	0.949	0.996	0.761	0.776	<0.001	0.035	0.608
IFAT 2 (DAFA[α])	IFAT 1 (RPC[β])	23	0.870	0.938	0.652	0.533	0.067	0.078	0.641
	IFAT 2 (RPC[β])	23	0.826	0.904	0.609	0.471	0.045	0.095	0.521
	RT-PCR (RPC[β])	176	0.926	0.993	0.631	0.632	<0.001	0.047	1.000
IFAT 1 (RPC[β])	RT-PCR (RPC[β])	52	0.962	0.997	0.827	0.811	0.027	0.032	1.000
IFAT 2 (RPC[β])	RT-PCR (RPC[β])	52	0.962	0.995	0.731	0.717	0.069	0.028	1.000

[Ψ]Number of samples used in the calculation of the actual sensitivities

[θ]Fisher's exact test p value for significance of the dependence between tests

[a]Suspects were considered positive, [b]Suspects were considered negative

[α]New Brunswick Department of Agriculture, Fisheries, and Aquaculture; [β]Research and Productivity Council; [δ]Atlantic Veterinary College

Table 2.4: The estimated specificities (Sp) for infectious salmon anemia diagnostic tests evaluated in series and in parallel.

Test 1	Test 2	Number negative samples[ψ]	$Sp_{parallel}$		Sp_{series}	
			actual	independent	actual	independent
Histopathology (positive[a]) (AVC[δ])	IFAT 1 (DAFA[α])	341	0.686	0.692	0.988	0.988
	IFAT 2 (DAFA[α])	341	0.724	0.724	1.000	1.000
	IFAT 1 (RPC[β])	386	0.700	0.713	0.995	0.995
	IFAT 2 (RPC[β])	386	0.712	0.724	0.997	0.999
	RT-PCR (RPC[β])	478	0.713	0.671	0.998	0.980
Histopathlogy (negative[b]) (AVC[δ])	IFAT 1 (DAFA[α])	341	0.938	0.949	1.000	1.000
	IFAT 2 (DAFA[α])	341	0.988	0.993	1.000	1.000
	IFAT 1 (RPC[β])	386	0.974	0.977	1.000	1.000
	IFAT 2 (RPC[β])	386	0.990	0.992	1.000	1.000
	RT-PCR (RPC[β])	478	0.983	0.920	1.000	1.000
IFAT 1 (DAFA[α])	IFAT 1 (RPC[β])	268	0.944	0.939	0.996	0.999
	IFAT 2 (RPC[β])	268	0.963	0.953	1.000	1.000
	RT-PCR (RPC[β])	574	0.930	0.884	1.000	0.997
IFAT 2 (DAFA[α])	IFAT 1 (RPC[β])	268	0.974	0.982	1.000	1.000
	IFAT 2 (RPC[β])	268	0.996	0.997	1.000	1.000
	RT-PCR (RPC[β])	574	0.977	0.925	1.000	1.000
IFAT 1 (RPC[β])	RT-PCR (RPC[β])	420	0.957	0.910	1.000	0.999
IFAT 2 (RPC[β])	RT-PCR (RPC[β])	420	0.971	0.924	1.000	1.000

[ψ] Number of samples used in the calculation of the actual specificities

[a]Suspects were considered positive, [b]Suspects were considered negative

[α]New Brunswick Department of Agriculture, Fisheries, and Aquaculture; [β]Research and Productivity Council; [δ]Atlantic Veterinary College

3 Assessment of infectious salmon anemia prevalence for different groups of farmed Atlantic salmon, *Salmo salar*, in New Brunswick farms

3.1 Introduction

Infectious salmon anemia (ISA) is a disease that has been causing mortalities in Atlantic salmon (*Salmo salar* L.) farms in New Brunswick since 1996[1]. The disease is characterized by lethargy, anorexia, anemia, internal organ damage, and elevated mortalities[2;3]. The viral pathogen, the ISA virus (ISAV), has been classified as an orthomyxovirus[4], and is believed to be transferred from fish to fish directly through sea water[5], by infected fish mucus, blood, and tissues, and possibly even by fish feces[6;7]. The virus infects the naïve fish through the gills, and the infected fish then sheds virus to numerous other fish so that the infection continues on a logarithmic scale until the number of susceptible individuals becomes a limiting factor[6;8].

The ability of a disease to spread in the fish farm waters is based on the number of susceptible salmon in the population, the number of contacts the infected salmon has with susceptible salmon, and the infectivity of the pathogen[9]. The infectivity of the ISAV remains relatively constant in the population unless there is a genetic mutation of the ISAV. One of the greatest dangers with fish farming is that contact between individuals within a cage and to some extent, between cages is high. Containment of fish increases the number of susceptible salmon in close proximity and also increases the number of direct contacts the susceptibles can have with an infected salmon and its mucus, feces, tissues, and blood, resulting in fish to fish spread of the virus. Not only are fish clustered within cages, but cages are grouped in relatively close proximity on farms. This situation is unlike that of wild fish that have considerably less sustained contact with one another thus reducing the number of new infections from each sick fish.

ISA has infected Atlantic salmon farms in other countries including Norway and Scotland. Norway has been dealing with the disease since 1984, when the first reported case was in a hatchery on the south west coast[2]. Scotland had its first reported case of ISA in 1998[10]. Many control measures were initially adopted by Norway and Scotland to stop the spread of the disease, including depopulation of farms that were diagnosed with ISA. The disease appears to have been eliminated from Scotland since January 2000[11] however Norway has not been able to eliminate disease and has recently seen an increase in outbreaks[12].

In an effort to eliminate ISA in Canada, the New Brunswick Department of Agriculture, Fisheries, and Aquaculture (NBDAFA) has taken a depopulation approach. Since 1997, the NBDAFA has been operating a surveillance program to identify farms that have cages with ISA infected fish. Every farm has dead or moribund fish tested for ISA every six to eight weeks. If there are any positive reverse transcription-polymerase chain reaction tests (RT-PCR) or two positive indirect fluorescent antibody tests (IFAT) on a farm, the farm is tested more frequently (every two to four weeks). If there are two positive tests on two fish from a cage but no increased mortalities, the farmer is encouraged to slaughter the cage early and is eligible for compensation through an industry fund. If the positive tests are associated with an increase in mortalities greater than 0.05% per day attributed to ISA then the cage is considered to have an ISA outbreak, and the farmer must slaughter all of the fish in the cage[13].

Since 1997, New Brunswick has slaughtered over 7.5M salmon due to ISA and over $40M in compensation has been paid to farmers[14]. The 2002 year class of fish (i.e.

fish entered into seawater in the Spring of 2002) experienced greater depopulation events during the first half of the growing cycle than any previous year class.

In an attempt to protect non-outbreak cages, cages diagnosed with clinical ISA are depopulated. Due to their location at the site, other cages at the outbreak site may have a high prevalence of ISAV but remain undetected if mortality rates are not increasing. If prevalence of infection was high in these undetected cages, it may prove necessary to remove exposed cages in addition to outbreak cages to protect neighboring farms from the spread of the virus. It is difficult to evaluate the control program without some knowledge of the virus prevalence in fish from cages experiencing an outbreak and in non-outbreak cages on that farm, or at neighboring and distant farms. If similar viral infection prevalence occurs in different populations (i.e. in depopulated cages and in cages remaining in production), then depopulation of the outbreak cage is ineffective as a method of reducing exposure to the virus. The objective of this study was to compare the prevalences of ISAV in moribund and healthy fish from cages experiencing an ISA outbreak, in healthy fish from non-outbreak cages from the outbreak farms, in healthy fish from cages from farms neighboring the outbreak farms, and in healthy fish from cages from farms distant from the outbreak farms.

3.2 Materials and Methods

3.2.1 Sampling

When a cage experienced an outbreak of ISA, the provincial veterinarian contacted the farm owner to ask if they would participate in this prevalence study. If the farm owner agreed to participate, the veterinarian contacted the investigator and the arrangements were made with the owner to sample salmon as soon as possible. After

identifying an outbreak farm, the owner of the closest, ISA-negative, neighboring farm and the owner of a distant farm in a bay without any outbreaks were also asked to participate in the study. The sampling period was between April 2000 and February 2002.

There were five different populations of Atlantic salmon that were sampled. Three of the populations came from farms experiencing an ISA outbreak in at least one of its cages. The three populations from the ISA outbreak site were: moribund fish from the outbreak cage (A), healthy fish from an outbreak cage (B), and healthy fish from a non-outbreak cage (C). The fourth population was healthy fish from a non-outbreak cage from the nearest willing farm that did not have any suspicion of ISA disease (D). To reduce the financial loss to the farm owners, a convenience sample of cages from populations C and D were chosen. Usually these cages contained the largest fish so the best price per pound could be salvaged. The fifth population, (E), was healthy fish from a non-outbreak cage from a willing farm that was in a Bay Management Area (BMA) without suspicion of ISA. Figure 3.1 is a schematic of the different populations.

Because of time and financial constraints, between ten and twenty fish from a cage for each population were sampled at the time of an outbreak. The moribund salmon (A) were slowly swimming fish on the top of the water that were not schooling with the healthy salmon. A net was used to scoop all of the catchable moribund fish out of the cage. The fish were euthanatized by an overdose of the anesthetic, Tricaine methanesulphonate (Syndel International Inc., Vancouver, British Comumbia), in a saltwater solution and immediately transported on ice to the laboratory for tissue collection. As part of the ISA control program, healthy salmon from the outbreak cage

(B) were harvested by crowding the fish, removing them with nets, and severing the gill arches and then immediately placing them in ice water in a large plastic storage tubs. The tubs were transported to the processing plant where approximately ten salmon were removed from the tubs for organ sampling before returning the salmon to the processing line. On the day the outbreak cage was being harvested, the ten healthy salmon from a non-outbreak cage (C) were removed from their cage by using feed to attract them and then dipping them out with a net. The fish were killed in a similar manner as the healthy salmon from the outbreak cage and were placed into a separate storage tub in ice water. Organ samples were collected at the processing plant. The healthy salmon from a non-outbreak cage from a neighboring farm (D) were similarly sampled (as for group C). Group E, the healthy salmon from a distant farm without ISA, were sampled at the processing plant when the salmon were routinely harvested for marketing. Ten salmon were obtained from large storage tubs and the appropriate organ tissues were sampled at the processing plant.

All cages were sampled within three weeks of the outbreak. Due to financial and geographical restraints, not all outbreak cages have a complete set of controls (i.e. not every outbreak has a complete set of groups A-E). Table 3.1 shows the distribution of the number of cages sampled per outbreak.

The organ tissues were sampled aseptically. Multiple small samples of the mid-kidney (2-3 mm cubes) were placed into the RNA preservative, RNA*later* (Ambion, Inc., Austin, Texas), for the RT-PCR. In addition, samples of the liver, mid-kidney, spleen, gill, and heart were placed neat into individual tissue bags in case further testing was

required. Samples were transported on ice and then frozen at –80°C within 24 hours of collection.

3.2.2 Testing

Kidney samples in RNA*later* were sent in two batches to the Research and Productivity Council (RPC) laboratory in Fredericton, New Brunswick, Canada, the standard laboratory used for the NBDAFA surveillance program for RT-PCR testing. This test is based on the first report of successful RT-PCR for ISAV in Atlantic salmon[15]. Kidney tissue from 94 RT-PCR positive fish from group A, 50 RT-PCR positive fish from group B, 18 RT-PCR positive fish from group C, and all of the RT-PCR positive fish from groups D and E were tested by virus isolation in Salmon Head Kidney (SHK) cell culture by the Regional Diagnostic Virology Service at the Atlantic Veterinary College Diagnostic Laboratory (Charlottetown, Prince Edward Island, Canada) for confirmation[16].

3.2.3 Statistics

3.2.3.1 Estimating the apparent prevalences

The apparent prevalence (AP) of each group was calculated by computing the overall mean prevalence taking into account dependence of fish in a cage using linearization variance estimators[17] from the Stata7 software (College Station, Texas) to account for the clustering of fish within a cage. Fish collected from different cages but from the same population group at the same farm were considered to be from the same cage for statistical calculations. The standard error of the prevalence is estimated using the design effect[18] to account for the clustering of fish within a cage. Significant

differences between the prevalences of the groups were assessed using the same variance estimators from above using a p value <0.05 as significantly different.

Because cages were collected in groups according to the outbreak, between group variation was estimated using a generalized linear mixed model regression for a three levels (fish, cage, and group). Significance of the between group variation was evaluated using the likelihood ratio test assuming p value<0.05 as significant.

3.2.3.2 Estimating the unbiased prevalences

Two previous validation studies have reported a value for the diagnostic test performances for the RT-PCR diagnostic test[19;20;20]. Diagnostic sensitivity (Se) is the test's quantified ability to identify ISA infected fish in a population of ISA infected fish, and diagnostic specificity (Sp) is the test's quantified ability to identify ISA free fish in a population of ISA free fish. Unbiased estimates of prevalence, "true prevalence" (TP), were calculated as the

$$TP=(AP+Sp-1)/(Se+Sp-1) \quad (1)$$

using the Se and Sp from each of the validation studies and AP based on the test results[21]. If the values for TP were <0 or >1, they were truncated at 0 or at 1 respectively[22]. The 95% confidence intervals were calculated in a similar manner using the same formula but substituting the limits of confidence intervals for AP.

3.2.3.3 Estimating the range of TP using a sliding scale of specificity

The different estimates for specificities reported in the two previous validation studies[19;20;20] were 97% and 72%. Because the true specificity for the RT-PCR test is unknown but likely within the range of 72% to 97%, a sliding scale of the unbiased prevalences using the formula for TP and specificity range from 70% to 100% were calculated for each of the reported sensitivity estimates.

3.2.4 Outcome of non-outbreak cages (group C) from outbreak sites

There were seven cages that were included in Group C. The farm owners or managers were asked by telephone interview approximately one year after sampling if the cages had become ISA positive and if disease continued on the farm.

3.3 Results

3.3.1 RT-PCR testing

There were 463 fish from 34 different cages that were tested by RT-PCR. The apparent prevalences and their associated 95% confidence intervals (CI) are presented in Table 3.2. The moribund fish, group A had the highest AP and was significantly different from all the other groups ($p<0.001$). The neighboring farm and distant farm cages, groups D and E, had the lowest AP and were not significantly different from each other but significantly different from the other groups ($p\leq0.001$). Although the AP of group B was higher than group C, it was not significantly different ($p=0.115$), but these two groups were significantly different from the other groups ($p\leq0.001$). Due to the small p values, all significant differences remained significant after the application of Bonferroni's correction for multiple comparisons.

Between group variation was not significant and thus was not considered to effect apparent prevalences.

3.3.2 Virus Isolation testing for confirmation of positive RT-PCR samples

The test results for the confirmatory VI testing are presented in Table 3.3. The results from the VI testing of the RT-PCR positive samples are not all positive because the sensitivity of the VI and the specificity of the RT-PCR are not perfect. None of the RT-PCR positive fish from groups D and E were positive when tested by VI.

3.3.3 Estimation of true prevalences

The true prevalences and their associated 95% confidence intervals calculated using Se and Sp from previous validation studies are presented in Table 3.4. The TP estimated using test performance characteristics from McClure et al.[19] are not remarkably different from the AP. However, if estimated using Se and Sp from Dohoo et al.[19], the TP of groups B-E are very different from their AP and are equal to 0 or include 0 in their confidence intervals. .

3.3.4 Estimation of the range of TP using a sliding scale of specificity

The potential ranges for the TP for groups B-E using the Se from McClure et al.[19] and Dohoo et al.[19] are presented in figures 2 and 3 respectively. Group A was not included in the figures because the value of the TP was greater than 1 when estimated using Se and Sp from McClure et al.[19] and did not vary much (0.92- 0.95) when estimated using Se and Sp values from Dohoo et al.[19]

3.3.5 Outcome of non-outbreak cages (group C) from outbreak sites

One of the farms was ordered to harvest all of their fish prematurely because their bay was adopting a single age of fish to be stocked at the same time and this farm's remaining fish were out of production synchrony, having been stocked on the opposite year. Four of the remaining farms continued to have outbreaks in cages other than the ones included in this study until all of the cages were harvested. The sixth farm had four

cages removed for ISA and the progression of the disease stopped. All other cages from this farm including the study cage were harvested normally when they had reached market weight. The seventh farm had only one cage removed as an ISA outbreak but continued to have positive testing throughout the site. The site was harvested normally before the mortalities had become greater than 0.05% per day. None of the cages included in group C ever became ISA outbreak cages.

3.4 Discussion

3.4.1 RT-PCR testing

The apparent prevalences of ISAV in the different populations were measured using the RT-PCR test. There are other tests available for ISA testing including virus isolation on cell culture (VI)[23;24] and the indirect fluorescent antibody test (IFAT)[25]. These tests were not used for the prevalence evaluation for two reasons. First, the RT-PCR test unlike the VI and IFAT, has a very high analytical sensitivity signifying only a small amount of viral RNA needs to be present for this test to identify the sample as positive[8]. This high analytical sensitivity is necessary when the virus level may be low as in a fish that has recently been infected or is recovering from infection. The presence of viral RNA suggests that the fish had been infected and presumably at one point in time could have shed the virus even if the fish had not become clinically ill. Second, this ISA RT-PCR diagnostic test has been shown to have high diagnostic sensitivity on three validation studies[19;20;26] suggesting the test is able to detect most fish that are positive for the virus.

3.4.2 Standard error estimates

Because fish were sampled from individual cages, these fish should be more alike than if the fish were randomly selected from a list of all fish in all cages in the group. Because the fish were clustered in a cage, the fish from one cage are no longer independent from each other and should not be treated as such in the statistical analysis. If they were treated as independent, then the standard errors of the AP would have been smaller than expected. The design effect (Deff) is a measurement of the impact that the study design has on the variance estimates[17]. Usually Deff is greater than 1 when studies are designed with sampling within a cluster as seen in group B in which there was the largest Deff (2.6) due to the variation within each cage. However, the Deff for groups C and E were less than 1 indicating that the variation within the cage was not necessarily any less than the variation between cages of the group.

Because the fish were sampled from cages, the standard errors could have been more precise if more cages per group were sampled. Sampling more fish per cage would improve the precision of the SE somewhat, but because the fish in a cage are not independent from one another, sampling more cages per group rather than more fish in a cage would make a larger impact in the precision of the SE.

The estimates for standard errors for the TP were initially calculated by the formula given by Greiner and Gardner[19] to account for the variation in the AP, Se, and Sp of the tests, but due to the small sample sizes within the groups, the clustering of the data within cages, and the extreme values of the prevalences, these results were invalid. The 95% confidence intervals were estimated using formula (1). The limits of the CI are approximations and undoubtedly are underestimates because they do not account for the variation present in the estimates for AP, Se, and Sp. Unfortunately, methods to estimate

the standard errors for TP when the data used for the estimates of AP, Se, and Sp are clustered, are not available at this time.

3.4.3 Range of the TP

One assumption that is commonly made is that test performance, i.e. Se and Sp, remain constant in all different populations. Unfortunately, this is not necessarily true for all diagnostic tests[27]. From the two major validation studies, the ability of the tests to correctly identify an ISA positive sample (Se) were 0.93 and 1.00 and to correctly identify a negative sample (Sp) were 0.72 and 0.97[19;20;20]. These two validation studies have specificities that are extremely different. One explanation for their differences is the way the specificities were derived. Details of the McClure et al. study are given in Chapter 2. Sensitivities and specificities for the RT-PCR test were derived by Dohoo et al.[19] using three different populations of fish instead of two. These included moribund fish from an ISA outbreak cage (high prevalence group), healthy fish from an ISA outbreak site (medium prevalence group), and healthy fish from a site in an ISA-negative province (low prevalence group). Sensitivity and specificity were calculated using latent class modeling techniques[28] that use maximum likelihood procedures to arrive at the most likely estimate of sensitivity and specificity given the results. There are no gold standard positive and negative fish in this type of estimation.

There are many variables that may have resulted in such a large divergence between the specificities of the two studies. The time the RT-PCR testing took place for validation studies occurred in 1998-1999 for one study and in 2001 for the second. It is possible that the laboratory had slightly changed the protocol of the test or the strain of the virus had changed between those two testing times. Another possibility is the

different fish populations and the calculations used for the specificities resulted in the difference. In the first study, the two populations used were a high prevalence and a low prevalence. Using the high prevalence and low prevalence groups will result in estimations that appear better than if they came from three groups with high, medium, and low prevalence as in the second study. However, the second study does not make a direct calculation from the data but rather uses modeling techniques to arrive at estimations. There are assumptions that must be tested in order to validate the results from the second study and these assumptions have not been tested to date. Because there is still work that needs to be done in order to conclude the appropriate specificity to use when adjusting the apparent prevalence to a true prevalence, TP were calculated using the sensitivities and specificities for both test evaluation studies. Figures 2 and 3 present the potential TP of the different study groups if it was assumed that the specificity of the test for the different groups was somewhere in the range of 0.70 to 1.00.

If TP were estimated using a Se of 1 and a Sp of 0.72[20], it would be possible to believe that the TP of both groups B and C were not different from 0 (ie. CI included 0). However, this is unlikely because some of the fish from these groups were tested using the VI test for confirmation and although not all RT-PCR positive fish tested positive on VI, 27 of 50 (54%) in group B and 2 of 18 (11%) in group C did. The VI test is a unique ISA diagnostic test because the specificity may be assumed to be 1.00 and in addition a positive test result indicates that the virus is present in the fish and is still intact with the ability to replicate. One reason the confidence intervals for the TP for groups B and C may have included 0 is that the sample sizes were small and clustered within cages, 160 fish in 12 cages and 70 fish in 7 cages for the respective groups. The TP from groups D

and E were also 0 or had 0 included within the confidence intervals. Those fish testing positive by RT-PCR from these groups were also tested with VI and none of these RT-PCR positive samples were VI positive (0/11). Therefore it is likely that RT-PCR positive fish from this group were in fact falsely positive by the RT-PCR test and the actual prevalence for these groups is 0.

3.4.4 Outcome of non-outbreak cages (group C) from the outbreak sites

Of the seven cages in group C, none of them were ever diagnosed as having an ISA outbreak before they were harvested. The farms were sampled during the summer and were for the most part harvested within 8 months from the time they were sampled. However, on most of these farms, the virus continued to cause outbreaks in other cages on the farm. In 4 out of the 7 farms, the disease continued through the rest of the unharvested cages until the farm was completely harvested. Removal of the ISA outbreak cage that was tested in this study did not seem to have a beneficial effect on preventing disease in the other cages on the majority of these farms.

One possible reason the cages tested in group C did not eventually have disease was that for financial reasons, these cages were chosen because they had the largest fish on the site. They were the cages with healthy, fast growing fish that would be harvested earlier than any of the other non-outbreak cages on the farm.

3.4.5 Potential bias

Given that the cages in groups C and D were sampled because they had the largest fish on the farm potentially distorts the prevalence. The largest fish on the farm are likely to be the healthiest fish that are the least susceptible to the virus. Thus the prevalences may be systematically lower than if the cage was randomly sampled from the remaining

non-outbreak cages. Similarly, the systematic reduction of the prevalence may have occurred in group E because fish from group E were sampled from cages that were being harvested normally and thus it is highly likely that these fish were healthy market weight fish. If financial loss to the farmer was not a concern, choosing the cages to be tested would have preferably been conducted by random sampling to avoid any bias. Unfortunately, randomization was not possible in this circumstance.

Fish from groups C and D were also selected by feeding and then netting the fish that were attracted to the surface to eat. This method of fish selection also biases the results, lowering the prevelances because fish that are eating are less likely to be sick from an ISA viral infection. Unfortunately random sampling from live fish in a sea cage is impossible to do unless the fish are being harvested at the time of sampling[29].

Another potential source of bias in this study was contamination at the time of sampling or testing because the RT-PCR test is extremely analytically sensitive. Care was taken so that no tissue was sampled until the scalpel blade had been alcohol rinsed and passed through a flame. As well, all instruments were changed between each group of fish.

3.4.6 Assessment of the true prevalence in the different groups

Results from this study indicate that the TP of virus from cages from non-outbreak neighboring farms (D) and distant farms (E) were estimated to be 0 and were significantly different from fish from the outbreak farm. Using the test performances from McClure et al.[19], TP of healthy fish from non-outbreak cages (C) on the farm experiencing the ISA outbreak was significantly different than fish from all other groups except for the outbreak cage (B). The fish from group C had TP of 0.28. Knowing that

the fish from these cages are in such close proximity to the dying fish, an intermediate prevalence seems appropriate. Group B fish had a TP of 0.42 which was not surprising because there were fish from this cage that were dying from the virus. The TP from the moribund (A) fish from the outbreak cage was approaching 1 and was significantly different from all of the other groups. Because these fish were dying from the disease, a TP of 1 was to be expected.

3.4.7 Implications for disease control

Because the TP in the outbreak cages and the cages at neighboring sites were different, then there seems to be some protection provided to these farms by early removal of outbreak cages. This is supported by previous studies that have provided evidence of viral spread by infected tissues and blood, feces[6], sealice[7;30], and directly through the seawater[31]. Dying fish from outbreak cages will be shedding virus in excrement and body tissues and those healthy fish closest to these ailing ones will be at greatest risk for viral infection.

Cages belonging to the group of non-outbreak cages (C) on the farm experiencing the ISA outbreak should be assessed as to their role in disease spread. Knowing that this cage has a higher level of virus than the cages at neighboring farms, depopulation at the same time as the outbreak cage is necessary if the aim of the surveillance program is to remove all significant sources of viral exoposure from the farms. Adopting this strategy would require a farm that has one cage with positive ISA diagnostic testing and increased mortalities due to ISA, to slaughter all cages on the farm. Although useful for ISA control at the industry level, complete farm depopulation would be economically disastrous to the farm owner unless there was adequate financial compensation.

Unfortunately, the compensation program thus far has been under-committed and unpredictable.

Recent history has provided evidence that complete farm removal rather than cage-by-cage removal is likely more successful in elimination of the disease. After a severe outbreak of ISA in the Scottish farms that lasted from 1998 to 1999, eradication protocols dictated that the complete farms be slaughtered unless they were in a remote location with low mortalities, in which case, cage by cage eradication was allowed[32]. Since the removal of the last outbreak farm, Scotland has declared itself free of ISA and there have been no new cases. Similarly, the state of Maine in the United States had it first case in 2001[33]. The federal government rapidly introduced an indemnity package, and all ISA infected farms were completely depopulated. To ensure there were no misclassified farms (ISA outbreak farms that were not identified as such), the entire Cobscook Bay in which the outbreak took place was depopulated. As of August 2003, Maine has had only two farms with fish that have tested positive for ISAV since the depopulation of the farms occurred in Cobscook Bay in 2001. Currently, Norway and New Brunswick are depopulating outbreak cages when mortalities are high, rather than entire farms[13;34], and this may be one of the reasons why the disease continues to cause major problems in these areas.

If the aim of the surveillance program was not to eliminate the virus but to reduce disease by limiting the spread of the virus, early detection of viral infection in a cage as an alternative to complete farm depopulation may be useful. Currently, dead or moribund fish from all cages at all farms are being tested for ISA by use of the IFAT every six weeks. If the fish is positive on IFAT, it is tested by VI and RT-PCR. When

two fish are positive on two different tests and the mortalities due to ISA are above 0.05% per day or regulatory officials deem it to be an outbreak at lower mortality levels, the cage is diagnosed with an ISA outbreak. By the time the mortalities have attained greater than 0.05% per day, the virus has had more opportunity to spread to another cage and to potentially another farm. It is possible that earlier diagnosis of ISA in a cage could be accomplished by testing more frequently and not relying on increased mortality rates.

3.5 Conclusions

Prevalence of ISAV in apparently healthy fish within an ISA outbreak cage and in a non-outbreak cage on the ISA outbreak farm was much higher than the prevalence of virus in healthy neighboring and distant farms. There was little evidence that there was virus present in these healthy neighboring and distant farms. The results from this study indicate that removing outbreak cages in a timely fashion is important in reducing the exposure of ISA to other farms. Costly removal of non-outbreak cages at outbreak farms would also remove the virus from the farm. If virus eradication is not the goal, but rather disease minimization by reduction of viral spread, perhaps increasing the frequency of ISA testing and earlier removal of ISA positive cages is an acceptable alternative. Future work should include an in-depth analysis of the time points when ISA diagnostic tests became positive, the cage became an outbreak, the outbreak cage was removed, and the subsequent result of the rest of the cages on the outbreak farm and neighboring farms to help predict if a farm and its neighbors will be in danger of contracting ISAV if only the outbreak cage is removed and the other cages are allowed to remain on the farm.

3.6 Acknowledgements

We thank the New Brunswick Atlantic salmon farmers and the managers of the processing plants for participating in this study. We also thank the veterinary students who helped collect the samples. We are grateful for funding assistance from Natural Sciences and Engineering Research Council and the Atlantic Veterinary College for this study.

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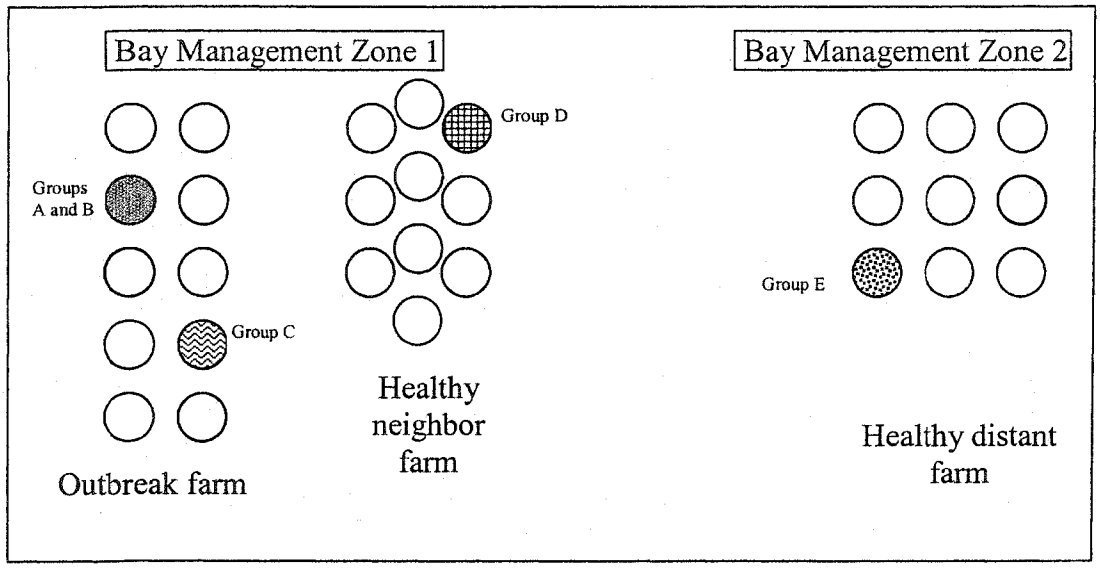
Figure 3.1: Schematic diagram of the sampled groups A-E



A: moribund fish from the outbreak cage
B: healthy fish from an outbreak cage
C: healthy fish from a non-outbreak cage on an outbreak farm
D: healthy fish from a non-outbreak cage from the non-outbreak neighbor farm
E: healthy fish from a non-outbreak cage at a distant non-outbreak farm

Table 3.1: Number of cages sampled per outbreak.

Case number	Cages type					
	A	B	C	D	E	Total cages per group
1		1		1		2
2		1	1	1		3
3		1	1			2
4		1	1			2
5	1	1	1	1	1	5
6		1	1	1		3
7				1		1
8	1	1				2
9	1	1		1		3
10				1	1	2
11					1	1
12	1	1	1		1	4
13	1				1	2
14	1	1	1			3
15	1	1		1		3
16		1				1
total	7	12	7	8	5	39

Table 3.2: Apparent prevalence of different populations of Atlantic salmon in New Brunswick farms.

Population	Apparent prevalence (95%CI)	Standard error	Deff (design effect)
A^{α}	0.940 (0.887, 0.993)	0.026	1.225
B^{β}	0.406 (0.279, 0.533)	0.063	2.588
C^{β}	0.286 (0.204, 0.368)	0.040	0.559
D^{γ}	0.084 (0.009, 0.160)	0.037	1.488
E^{γ}	0.080 (0.004, 0.156)	0.037	0.949

A moribund fish from the outbreak cage
B healthy fish from an outbreak cage
C healthy fish from a non-outbreak cage on an outbreak farm
D healthy fish from a non-outbreak cage from the non-outbreak neighbor farm
E healthy fish from a non-outbreak cage at a distant non-outbreak farm
Population with different superscripts (α, β, and γ) are significantly different from other groups ($p \leq 0.001$), but not from each other

Table 3.3: Confirmation of positive RT-PCR samples using the Virus Isolation test

Population	Total RT-PCR positive	Total VI tested	Total VI positive (%)
A	94	94	72 (77%)
B	65	50	27 (54%)
C	20	18	2 (11%)
D	7	7	0 (0%)
E	4	4	0 (0%)

A moribund fish from the outbreak cage
B healthy fish from an outbreak cage
C healthy fish from a non-outbreak cage on an outbreak farm
D healthy fish from a non-outbreak cage from the non-outbreak neighbor farm
E healthy fish from a non-outbreak cage at a distant non-outbreak farm

Table 3.4: The true prevalences after adjustment for the sensitivity and specificity of the RT-PCR diagnostic test

Population	TP[‡] (95% CI)	TP[ʃ] (95% CI)
A	1 (0.949, 1)	0.917 (0.842, 0.991)
B	0.415 (0.274, 0.556)	0.175 (0, 0.352)
C	0.281 (0.190, 0.372)	0.008 (0, 0.122)
D	0.057 (0, 0.141)	0 (0, 0)
E	0.052 (0, 0.134)	0 (0, 0)

[‡]Se=0.93 and Sp=0.97 from McClure et al.[19]

[ʃ]Se=1.00 and Sp=0.72 from Dohoo et al.[19]

A: moribund fish from the outbreak cage

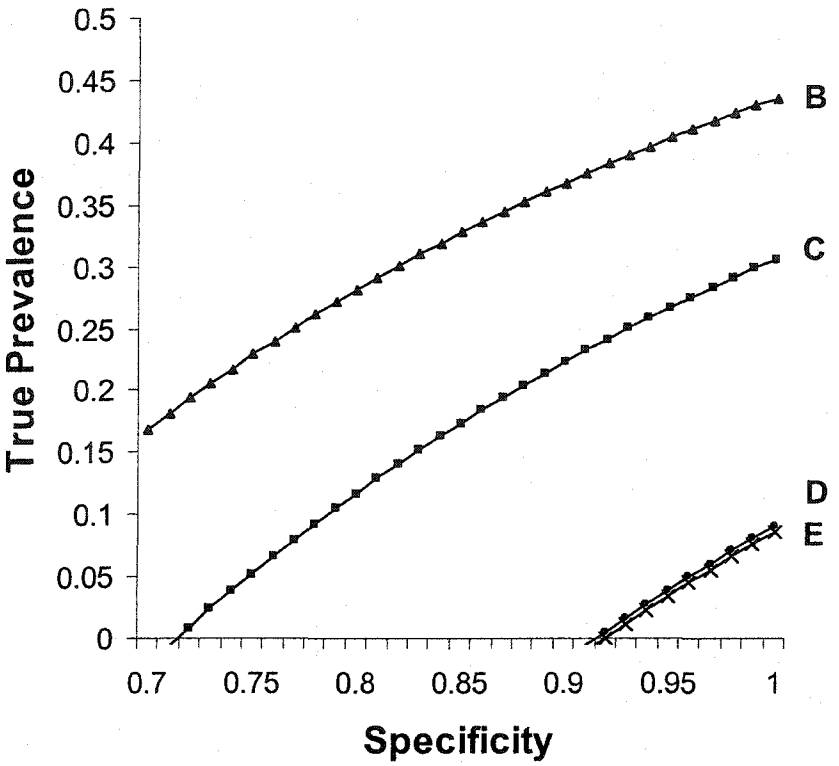
B: healthy fish from an outbreak cage

C: healthy fish from a non-outbreak cage on an outbreak farm

D: healthy fish from a non-outbreak cage from the non-outbreak neighbor farm

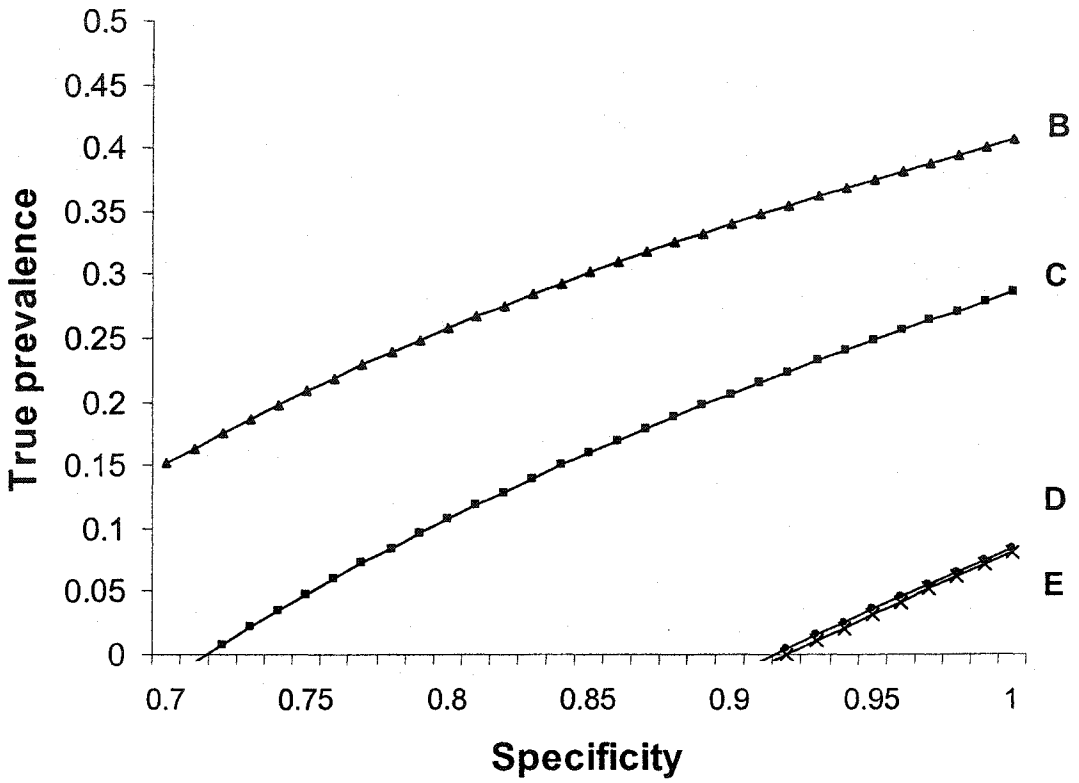
E: healthy fish from a non-outbreak cage at a distant non-outbreak farm

Figure 3.2: The true prevalence of the individual groups calculated by the sensitivity of 0.932 for the RT-PCR diagnostic test as reported by McClure et al.[19] and a sliding scale of specificity starting at 0.70 to 1.00.



▲ Group B, healthy fish from an outbreak cage
■ Group C, healthy fish from a non-outbreak cage on an outbreak farm
• Group D, healthy fish from a non-outbreak cage from the non-outbreak neighbor farm
× Group E, healthy fish from a non-outbreak cage at a distant non-outbreak farm

Figure 3.3: The true prevalence of the individual groups calculated by the sensitivity of 1.00 for the RT-PCR diagnostic test as reported by Dohoo et al.[20] and a sliding scale of specificity starting at 0.70 to 1.00.



▲ Group B, healthy fish from an outbreak cage
■ Group C, healthy fish from a non-outbreak cage on an outbreak farm
• Group D, healthy fish from a non-outbreak cage from the non-outbreak neighbor farm
× Group E, healthy fish from a non-outbreak cage at a distant non-outbreak farm

4 Absence of infectious salmon anemia virus in pollock, *Pollachius virens*, cohabitating with infected farmed Atlantic salmon, *Salmo salar*

4.1 Introduction

The infectious salmon anemia (ISA) virus (ISAV) causes a variety of problems in farmed Atlantic salmon including lethargy, anemia, hemorrhage of the internal organs, and death[1;2]. It has been identified as a cause of disease in Norwegian farmed Atlantic salmon (*Salmo salar*) since 1984[2] and has since been identified in salmon farms in Canada[3], Scotland[4], United States[5], Chile[6], and the Faroe Islands[7]. Despite aggressive control strategies this disease continues to cause mortalities in Norwegian and Canadian salmon farms. One possible reason the disease continues is the inability to identify and remove the reservoir for the virus.

Wild fish have been proposed as a viral reservoir because they are capable of having close contact with the farmed salmon. Although wild Atlantic salmon were less susceptible to ISAV, they were still able to infect naïve salmon in cohabitational studies[8]. Many laboratory studies have shown that other wild salmonid and non-salmonid fish including brown trout and sea trout (*Salmo trutta*)[9;10], rainbow trout (*Oncorhynchus mykiss*)[11;12], herring (*Clupea harengus*)[13], and possibly even Arctic char (*Salvelinus alpinus*)[12] tested positive for the virus weeks after intra-peritoneal injection. Brown trout and sea trout have been proposed as long term carriers. Sea trout tested positive by reverse transcription polymerase chain reaction test RT-PCR for the virus at 135 days post-infection[9], and blood taken from a brown trout seven months post-infection, that was injected intraperitoneally into Atlantic salmon, was able to cause disease in the naïve salmon[10].

Pollock (*Pollachius virens*) are commonly found in and around salmon cages, particularly in New Brunswick where wild salmonids are less common. Although pollock are not a salmonid, it is important to ensure this species is not a potential viral reservoir because of their close association with farmed Atlantic salmon. A recent laboratory study tested the ability of pollock to become infected with ISAV and to transfer the virus to Atlantic salmon[14]. The pollock were resistant to the virus, and the authors were unable to detect virus replication. Pollock that were infected by intraperitoneal injection were incapable of infecting naïve Atlantic salmon cohabitating in the same fish tank.

Laboratory studies are an excellent means to test hypotheses under controlled situations, but they cannot recreate the natural farming environment. The objective of this study was to identify the presence and if present, the prevalence of ISAV in pollock incidentally cohabitating with ISA-infected, farmed Atlantic salmon.

4.2 Materials and Methods

4.2.1 Pollock

Pollock that were living inside the salmon cages were collected either at the farm or at the processing plant when the cage was being slaughtered due to a provincially ordered depopulation for ISA. At harvest, the nets were lifted to one side of the cage to crowd the salmon such that it was possible to collect the salmon with a dip net. If collected during the harvest, the pollock were separated from the salmon as they were removed from the cages and then brought to the laboratory where tissues were sampled. If the pollock were collected at the abattoir, the pollock were removed from the large storage tubs that held the salmon and placed on ice until they were transported to the

Department of Fisheries and Oceans laboratory in Moncton, NB where the tissues were sampled. Up to 21 pollock were sampled from each cage. All samples were collected between February 2000 and July 2001.

The cranial and caudal kidney tissues were sampled aseptically. The combined cranial and caudal kidney sample was placed into a 1.6 ml microcentrifuge tube with the RNA preservative, RNA*later*™ (Ambion, Austin, Texas), for the RT-PCR test. Samples were transported on ice and then frozen at –80°C within 24 hours of collection.

4.2.2 RT-PCR testing for ISAV

RNA was extracted with a solution of phenol and guanidine isothiocyanate (TRI reagent®) according to supplier's instructions. Random hexamers primed cDNA was synthesized using M-MuLV (RevertAid™ First Strand cDNA synthesis kit, MBI Fermentas, Burlington, ON, Can.) according to supplier's instructions. PCR amplifications were performed in 20 ul volumes containing 2 ul cDNA, 50 mM KCl, 30 mM Tris-HCl pH 8.4, 2.5 mM $MgCl_2$, 0.01% Triton® X100, 1 ul DMSO, 0.025 U/ul of Taq DNA polymerase, and 10 pMol of each primer. Primers used were S6-321F (5'-ggacctgtacctgggagcat) and S6-513R (5'-agcaatgcagaccttgtagat). Cycling conditions were initial denaturation at 94°C for 2 minutes then 10 cycles of touchdown PCR (94°C for 30 seconds, 60°C dropping one degree per cycle for 30 seconds, and 72°C for 90 sec) and 35 more cycles at 94°C for 30 seconds, 50°C for 30 seconds, and 72°C for 90 seconds, followed by a final extension at 72°C for 5 minutes and holding at 20°C. PCR products were electrophoresed on 1.5% agarose at 110V and examined with ethidium bromide under UV light.

4.2.3 Statistics

The prevalence was calculated as the number of positive pollock divided by the number of pollock tested. The exact 95% confidence interval for binomial count data was calculated using the Stata7 (College Station, Texas) software.

4.3 Results

There were 93 pollock collected from six different sea cages from five different farms. The number of pollock sampled from each cage ranged from 7 to 21 with the average being 16. All of the sea cages were being slaughtered because they were experiencing increased mortalities due to an outbreak of ISA. Results from the RT-PCR tests yielded the expected 193 bp product for the positive controls, while no product was observed in any of the pollock samples resulting in an ISA viral prevalence of 0% and a 95% confidence interval of (0%, 3.9%).

4.4 Discussion

There have been isolated farms in New Brunswick that have become infected with ISA with no apparent link to other cases. There have also been certain bay areas in New Brunswick that have become infected with ISA disease in every salmon grow-out cycle. For the last 6 years, Limekiln Bay and Bliss Harbour have been dealing with repeated infections despite complete depopulation of all farms and fallow periods of 6 weeks or more. The reason these farms become infected may be their proximity to a viral reservoir that continuously exposes naïve fish. There has never been a definitive viral reservoir found, but three primary routes of re-infection are wild salmonids, wild non-salmonids, and invertebrate vectors such as sea lice. Wild salmonids are relatively uncommon in the area of New Brunswick fish farms (Gilles Olivier, personal communication) and therefore represent a less likely source of infection. A wild non-salmonid fish that can

transmit the virus but does not become clinically ill would be difficult to detect but important to control policies aimed at limiting spread between sites.

The wild fish species having the most contact with farmed Atlantic salmon in New Brunswick farms are pollock. The pollock are attracted to the salmon feed and stay in the vicinity of the cages in order to consume the feed that falls through the cage. If the pollock are small enough, they can enter the cage through the nets. If they stay in the cage for extended periods of time, their rapid growth may preclude them from exiting. Pollock may also enter the cages during net changes as new nets are pulled under the old nets incorporating the pollock near the cage into the population of salmon within the cage. In Eastern Canada for the last 7 to 10 years, there have been more Pollock in the cages since the industry has moved to larger circular cages that capture many more Pollock during net changes. When the farm managers and owners were asked about their perception of the number of pollock in their cages, 75% (59/79) answered that there were pollock in their salmon cages (McClure, unpublished data) reflecting the common frequency of pollock living in the farm cages. In a recent risk factor study, if greater than 1000 pollock were in the sea cages, the odds of an ISA outbreak in that cage were greater than three times indicating an association between the large number of pollock and ISA outbreaks (McClure, Ph.D. thesis, chapter 5).

The pollock tested in this study were collected for analysis prior to reports by Snow et al. (2002)[14] showing that pollock are not likely candidates for being ISA viral carriers because pollock injected with the virus were able to eliminate the virus within one week in a laboratory study. Unlike a laboratory setting, farmed fish are exposed to many more stressors including changes in weather, water temperature, oxygen content in the water,

harmful algal blooms, sea lice, and concurrent diseases. Due to the close proximity of fish within sea cages farmed salmon have increased contact between individuals making viral transmission more likely once the virus has successfully infected at least one fish in a cage. Because fish are managed differently in the farming situation, it was important to know if the wild pollock living inside the farm cages could be viral carriers.

Another reason for investigating pollocks' ability to become a virus carrier is that there are other fish from the Gadidae family including Atlantic cod (*Gadus morhua*), and haddock, (*Melanogrammus aeglefinus*), that are in early commercial stages of production and may be raised in close proximity to salmon farms in Atlantic Canada. If pollock were capable of carrying the virus, other members of the Gadidae family may be carriers and deserve further evaluation. There is some experimental evidence that haddock may survive infection and replicate the virus[15], but this has not been seen in wild fish surveys[16]. If these newly farmed species are capable of carrying the virus, then industry ISA control policies will need to incorporate surveillance and control to minimize the impact on salmon farms.

Although the results of this study indicated the prevalence of ISAV in wild pollock living in sea cages was zero, there are two assumptions that have been made. The ability of the RT-PCR test to identify a positive sample is the test's diagnostic sensitivity and must be fairly high so that there is confidence that the test did not falsely classify a positive pollock sample as negative. The sensitivity of this test has been identified as moderately high at 85% for salmon[17] but has not been evaluated for use in pollock. Assuming the test performs the same for pollock as it does for salmon, there is an

excellent chance (85%) that the test result would have found a single positive pollock if there was one in the samples tested.

Another assumption was that pollock kidney was the organ that would have the highest level of virus if the pollock was infected. A distribution study has been performed in Atlantic salmon to identify the organs that were RT-PCR positive after cohabitating with infected Atlantic salmon[18]. Atlantic salmon gill, liver, and spleen were also positive at differing times, but heart was most consistently positive from 5 to 70 days cohabitating with ISA infected salmon. If there was an infected pollock, performing the RT-PCR on combined heart, gills, liver, spleen, and kidney from the pollock may have increased the chance of identifying it as positive. However, the sampling of multiple tissues was beyond the scope of this initial evaluation of pollock.

Limiting the number of pollock in a salmon cage is recommended as they are carriers of other pathogens such as Caligus species of sea lice[19]. The presence of pollock may also act as a stressor because of the increase in total stocking density, the impact of water quality, and an unfamiliar species introduced into the cage.

The result of this study strengthens the evidence found by Snow et al. (2002)[14] that pollock are unlikely to be a viral reservoir for farmed Atlantic salmon. However, other wild fish commonly found in New Brunswick farm cages like cod, lumpfish (*Cyclopterus lumpus*), and Atlantic mackerel (*Scomber scombrus*) should be investigated to assess whether they can be viral reservoirs. Because herring swim freely in and out of the salmon cages and haddock may be farmed near salmon cages, they should be evaluated as to the likelihood of infecting farmed Atlantic salmon as there is some evidence that they are carriers of ISAV.

4.5 Acknowledgements

We thank the New Brunswick Atlantic salmon farmers and the managers of the processing plants for participating in this study. We also thank the veterinary students who helped collect the samples. We are grateful for funding assistance from Natural Sciences and Engineering Research Council and the Atlantic Veterinary College for this study.

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5 Risk factors associated with outbreaks of Infectious Salmon Anemia in farmed Atlantic salmon, *Salmo salar*

5.1 Introduction

Infectious Salmon Anemia (ISA) is a disease caused by the infectious salmon anemia virus (ISAV). The disease occurs in farmed Atlantic salmon, *Salmo salar*, and is characterized by lethargy, anorexia, anemia, and death[1]. The first case of ISA in New Brunswick Atlantic salmon sites occurred in 1996[2]. ISAV continued to cause disease sporadically through the Bay of Fundy farms, reaching a peak with the 1999 year class (fish that entered seawater in 1999) with 24 out of the 86 sites having clinical disease in this year class. Since the 1999 year class, the incidence of clinically diseased sites dropped to 9 affected sites out of approximately 85 sites in the 2000 year class and 15 affected sites in the 2001 year class[3]. Unfortunately, there was a resurgence of disease in the 2002 year class. This year class of fish has experienced greater depopulation events associated with ISA than any previous year class during the first half of the growing cycle (up to early 2003).

The economic impact from this disease has been very damaging to the industry. Financial losses to the farmers have been estimated to be between $4.8M to $5.5M per year. In an effort to control the disease, 7.5 million fish from outbreak cages have been destroyed since 1997[4]. Current detection and depopulation measures applied to fish from outbreak cages alone do not appear to be a solution to ISA in the New Brunswick fish sites.

Other control measures have been adopted to reduce the amount of disease. Many of these control measures have been suggested as a result of laboratory studies. Reduction of the ectoparasite, sea lice (*Lepeophtheirus salmonis*), on the skin of the

salmon has been recommended because sea lice have been shown in laboratory experiments to be mechanical transmitters of ISAV[5]. ISAV can be spread horizontally through blood and tissues of infected fish, thus frequent removal of dead fish from a cage has been recommended[6]. The virus has also been shown to remain infective after 20 hours in seawater, providing evidence that proper hygiene and disinfection should be maintained at all sites to reduce the spread of the virus[7].

Epidemiologic studies have also been important in identifying important control methods. Quantitative analysis of well-boat traffic in Scotland has identified an increased risk of ISA at the site if well-boats visited a site for the purpose of moving live fish from site to site or from site to the processing plant[8]. Other factors associated with an increase in risk for ISA have been identified by risk factor studies. There have been three important risk factor studies for ISA. Two of them were performed in Norway and consisted of case-control studies using the site as the unit of concern[9;10]. An increase in risk of ISA on a site was highly associated with the site's proximity to another site with ISA and to a salmonid slaughterhouse that did not disinfect its wastewater. Other factors that increased the risk of a site having ISA were the purchase of smolt (young fish adapted for transfer into sea water) from more than one hatchery, transportation of the smolt over a long distance, as well as removal of dead fish less than daily in the summer months.

The third ISA risk factor study was performed in New Brunswick, Canada in 1997[11]. This study differed from the Norwegian studies because it was multilevel, investigating both factors associated with the site becoming an ISA problem site (>50% of the cages at the site were ISA positive) and factors specifically associated with a cage

becoming ISA positive. Some of the important risk factors for a cage becoming positive were the initial number of smolt stocked in a cage, fish density within a cage, cumulative mortality for the cage in the first year in seawater, and whether the fish had been weight sampled in the second year in seawater. A protective factor was increasing the number of times that fish were treated for sea lice. Site level factors were harder to analyze due to the small number of sites, but using a Cox proportional hazards model, site factors such as feed delivered by feed company increased the risk of a site becoming positive. Having only one year class of fish on the site and increasing months feeding moist feed reduced the risk of a site becoming an ISA problem site.

The integration of ISA research, vaccine technology, and established farming principles has led to some significant changes in salmon farming in New Brunswick. Virtually all sites have only one year class of fish on a site and many areas have only one year class of fish in a Bay Management Area (BMA). This practice was established to minimize contact between older fish and young fish to reduce the risk of disease transmission. Another important control measure that has been implemented is proper disinfection of the wastewater released by the processing plants. A third change that has been made is the treatment to remove sea lice. Laborious and stressful bath treatments were performed until the introduction of an oral ectoparasiticide, emamectin benzoate (SLICE™, Schering-Plough Animal Health, Quebec), which is incorporated into the feed thus removing many of the stresses associated with bath treatments. Since 1999, inactivated ISAV vaccines have been available to the farmers. These vaccines have not been evaluated in large-scale clinical trials, but do offer some hope of protecting the salmon from the virus.

Despite the changes made by farmers, 2002 has been the worst year for clinical ISA disease in the New Brunswick sites resulting in the highest level of cage depopulation as a control measure[4]. This current risk factor study evaluates risk factors given the current situation in the Atlantic salmon sites in New Brunswick. The objective of the study was to identify host, environment, and management risk factors associated with a site or a cage experiencing an ISA outbreak.

5.2 Materials and Methods

5.2.1 Selection of sites and cages

5.2.1.1 Site selection

The New Brunswick Department of Agriculture, Fisheries, and Aquaculture (NBDAFA) provided a list of all farms in New Brunswick. Between June 2002 and September 2002, all farm owners that had stocked fish in the years 2000 and 2001 were asked to participate in the study. Upon agreeing, the owner was asked if the farm was ISA positive (case farm) or ISA negative (control farm). ISA positive farms were defined as having had at least one cage of the 2000 or 2001 year class of fish that had been removed because it had been diagnosed with clinical ISA prior to the start of the interview process (summer 2002). ISA negative farms were defined as farms that had not had any cages diagnosed with clinical ISA during this time period.

5.2.1.2 Cage selection

Once the owner had agreed to participate, either the owner or the site manager from the case farm was asked which of the cages on the farm had been removed due to ISA diagnosis. Currently in New Brunswick, moribund fish from every farm are tested for ISA every six weeks. ISA tests that are available include indirect fluorescent

antibody test (IFAT), reverse transcription-polymerase chain reaction test (RT-PCR), and virus isolation on cell culture (VI). A cage is considered ISA positive (case cage) if at least two fish are positive on two different tests, there are clinical signs consistent with ISA, and mortality rates were elevated (usually ≥0.05% per day). In a few farms in which the positive testing occurred in cages with very young fish or where there had already been cages with high mortalities due to ISA removed, cages were also considered ISA positive (case cage) even if they did not meet the requirement of ≥0.05% mortalities per day.

If a case farm had only one case cage, two control cages were selected randomly by drawing cage numbers out of a hat. If a case farm had more than one case cage, then two case cages and four control cages were selected randomly by drawing numbers out of a hat. For the control farms, three control cages were selected randomly in the same way.

5.2.2 Questionnaire

A questionnaire was developed for administration by personal interview of the site manager or the owner if the site manager was not available. The questionnaire (see appendix 8.1) was divided into site level questions and cage level questions. Sections for each level were further divided into information subsections such as area and site, health, feed, equipment and personnel, mortality removal, smolt history, holdovers, predators, weight sampling, wild fish, net care, sea lice, and harvest. Important predictors derived from the questionnaire which were ultimately found to have some association with ISA risk are listed in Table 5.1.

For control farms, information was collected covering the period from the time the fish were transferred to seawater until the time the cage was harvested or until the

time of the interview if the site/cage had not been harvested. For the case farms, information was requested for a case cage and two control cages from the time the fish entered sea water until the case cage was diagnosed with ISA.

The interviews were conducted from July 2002 through December 2002. By this time, all of the 2000 year class fish had been harvested and harvest of the 2001 year class fish had started. No data were collected on 2002 year class fish on any of these farms. Prior to the interviews, the questionnaire was tested with two farm managers and final amendments to the questionnaire were made.

5.2.3 Data Collection

Interviews took between 45 to 90 minutes depending on the site and were carried out by a team of five trained graduate students and veterinary students. At the completion of the interview, farmers were asked to provide farm records including cage mortality data, medication usage and disease diagnoses, and site maps. If there were any questions that the interviewee could not answer, then farm records were consulted. Questions referring to distances between farms were cross-checked using an official Bay of Fundy, marine aquaculture site map provided by the NBDAFA.

Hatchery information relevant for each site/cage including hatchery type, water source, number of smolts stocked in a cage, average weight of the smolt at the time of sea water stocking, condition of smolts at stocking, vaccines administered, and time since vaccination were collected directly from the hatchery managers by telephone interview, followed by a faxed questionnaire.

Data were entered into the Epidata[12] data management computer program twice and then validated to reduce data entry errors. When possible, interview answers were cross-checked with farm records.

5.2.4 Statistical analysis

5.2.4.1 Predictor variables

Continuous variables were kept as continuous variables if the relationship between the log odds of the outcome and the variable was approximately linear. If the graph evaluating this relationship was not approximately linear, the variable was categorized or dichotomized using cut points that represented natural distinctions between groups. Some categorical variables had the number of different categories reduced by combining categories if there were only a few sites in the group and it made biological sense. If there were a large number of related variables, a new variable was created in order to reduce the risk of multicollinearity[13]. An example of this was the variable for cage volume (CageVol). This variable incorporated the variables for cage type (CageType), size (CageSize), and depth (CageDeep) by using the shape of the cage (CageType) and the circumference or length of a side (CageSize) to estimate the surface area of the cage. The surface area was multiplied by the depth (CageDeep) to calculate the cage volume (CageVol). Some variables were dropped from the analysis because they were highly correlated to another factor that was kept in the analysis. An example of this was the variable for distance from the site to the processing boats traveling past the site (ProcBoatDist) and the variable for the number of processing boats that travel past the site (ProcBoatNum). The closer the site is to the processing plant, the closer the processing boats will travel by the site and the higher the number of processing boats that

travel past the site in order to get to the processing plant. The variable for the number of processing boats (ProcBoatNum) was dropped from further analysis.

5.2.4.2 Logistic Regression

5.2.4.2.1 Unconditional associations

Independent variables were first evaluated for unconditional associations with the outcome (case/control) using a χ^2 test for categorical data and a t-test for continuous data. Odds ratios (OR) were calculated using univariable logistic regression models for all variables with a p-value <0.20, and then these variables were tested in a multivariable logistic regression analysis.

5.2.4.2.2 Model building using site level predictors

Site level factors were analyzed to identify significant site level risk factors using a dataset with one record per site with the dependent variable being whether or not the site was a case farm. Initially all significant variables were fit and the least significant variable was removed one at a time until only significant variables (Wald-test p-value ≤0.05) were left in the model. The categorical variable CageMortNum had many missing values (farmer was unable to answer the question), and these missing values were coded with a new dummy value to include this predictor and all possible sites in the model building process.

Variables were evaluated for confounding by examining for a "clinically important" change in the magnitude of the coefficient of the variables in the model fit with and without the confounder[14]. Two-way interactions were assessed for any biologically reasonable interaction terms by adding the terms into the model and evaluating their significance by the Wald statistic.

The fit of all of the logisitic regression models was assessed by the Hosmer-Lemeshow Goodness-of-fit test. Pearson residuals and standardized Pearson residuals were inspected. The covariate patterns with the extreme values of the diagnostic parameters: leverage, delta-beta, delta-chi-square, and the delta deviance were examined for their influence on the model.

5.2.4.2.3 Model building using cage and hatchery level predictors

Cage and hatchery level factors were analyzed using a dataset that had one record per cage and the dependent variable was whether or not the cage was a case cage to identify significant cage risk factors and hatchery risk factors. In most cases, case cages were matched to two control cages from the same site. Initially a conditional logisitic regression model was attempted for the evaluation of cage and hatchery factors to account for matching of case and control cages. However, because of the lack of variation of cages within sites (cages did not vary in more than 5 different sites per predictor) use of the conditional logistic regression often produced unstable estimates. A random effects logistic regression model to account for variation between sites was not considered because for most cage-level predictors, there was little variation within the sites. Thus, a population averaged logistic regression model was used, to account for the clustering of cage within a site.

Cage level factors and hatchery factors were evaluated in a manner similar to site level factors. The significance of categorical variables that had many missing values (eg. the number of wild pollock, *Pollachius virens*, in the cage (Pollock) and the variable for the time between vaccination and seawater entry (DegreeDays)) was evaluated by coding

these missing values with a new dummy value to include these predictors and all possible cages in the model building process.

5.2.4.3 Survival analysis

The site data were modified for survival analysis using a Cox proportional hazard model to evaluate how the risk of ISA at a negative site changed as newly diagnosed ISA positive sites became progressively closer (in distance). The data were arranged such that it was possible to have multiple records per site. There was one record for the number of days from the time point that a site had a neighboring site with ISA in one distance category until the time point when there was a newly diagnosed site with ISA in a closer distance category. The start of the first time period at risk was defined as the point in time in which the smolts were transferred into the cage. ISA positive sites were classified as cases when they experienced an outbreak. Control sites were classified as censored at the time the fish were harvested, at the time of the interview if the fish were not harvested, or at 730 days (in most cases, maximum time for complete grow-out cycle) for control sites with missing harvest dates. The distance variable (ISANeigDist) was grouped into four categories (the closest site with ISA was within 0.5km, > 0.5km and ≤2km, >2km and ≤5km, and >5km). The failure event was set to whether or not the site was positive for ISA at the point in time that the disease came within a closer distance category. A backward elimination process removing the least significant variable until all remaining variables were statistically significant with Wald-test p value >0.05 was used to determine the final model. Potential confounders and interaction terms were evaluated in a similar manner as the logisitic regression models.

The assumption of proportional hazards was evaluated using the test for proportional hazards based on the Schoenfeld residuals. Further examination of the proportional hazard for individual variables was assessed graphically looking for parallel lines for the log-cumulative hazard plot, (log(-log S(t)) vs. log time. In addition, time dependent covariates were generated as interaction terms with individual variables. If the interaction terms were significant, then the proportional hazard assumption was violated.

Cox-Snell residuals were used to assess the overall fit of the model by graphing the cumulative hazard against the Cox-Snell residuals. If the model fits well, then the line should be straight at a 45° angle. Martingale and Deviance residuals were used to identify any outliers by graphing the residuals against the days the sites were at risk.

All data analysis was performed using Stata (version 7) software (College Station, TX).

5.3 Results

5.3.1 The data

Participation rate was 97.6% (83 of the 85 qualifying sites). There were 9 sites that did not qualify because they did not stock fish in the years 2000 and 2001. Of the 83 participating sites, 27 were case sites and 56 were control sites. From the 27 case sites, there were data from 41 case cages and 79 control cages. There was data from 151 control cages from the 56 control sites. For 7 sites, the cage data were not incorporated into the analysis due to many missing answers and the inability to verify given answers with site records.

5.3.1.1 Site-level data

There were 74 different variables tested. There were 26 unconditional associations (with $p<0.20$) between site level factors and the site being a case. Selected associations and their corresponding OR are presented in Table 5.2. The best logistic regression model using data from 81 sites with all significant variables having $p<0.05$ consisted of 3 risk factors: nearest neighbor site with ISA within 0.5km (ISANeigClose), distance to processing boats traveling past site being less than 1 km (ProcBoatDist), sites that dive for mortalities more than once a week in August (DiveAug), and sites that has at least one cage with post transfer mortalities greater than 5% in the first 30 days post seawater transfer (CageMortNum) which are presented with their corresponding OR in Table 5.3.

Evaluation for interactions among the variables presented in the site risk factor logistic regression models was attempted, but discontinued due to the small number of records in the analysis.

5.3.1.2 Cage-level data

There were 51 different cage-level variables tested that included cage and hatchery predictors. There were 13 unconditional associations (with $p<0.20$) between cage-level factors and the cage being a case. One significant continuous variable was the variable for cage volume, CageVol. For this variable, there were 41 case cages and 226 control cages. The average volume x 1000 for the case cages was 3,230 m^3 and for the control cages was 3,857 m^3. The odds for ISA in the cage were increased 1.18 times for every 1,000 m^3 less volume in the cage. The 95% confidence interval for the OR was (0.98,1.42) and the wald's test p-value for the logistic regression was 0.08. Unconditional associations for the categorical data and their corresponding OR are

presented in Table 5.4. The best logistic regression model with all significant variables having $p<0.05$ consisted of 4 risk factors: cages treated for sea lice less than three times (LiceTx), cages having more than 4 meters under the net at low tide (MeterUnder), cages with ≥ 1000 pollock (Pollock),and the average weight of the smolt at seawater transfer was ≥ 99g (SmoltWeight) which are presented in Table 5.5. This model incorporated data from 256 cages.

There were no significant interactions among the variables presented in the logistic regression models for the cage risk factors.

5.3.1.3 Testing the fit of all logistic regression models

The Hosmer-Lemeshow Goodness-of-fit testing provided evidence that the models did not fit poorly because all final models with more than one predictor had a p-value >0.05. The Pearson residuals and standardized Pearson residuals were inspected for all logistic regression models and gave no evidence that the data did not fit the model. The leverage and the extremes of the delta-beta, delta-chi-square, and the delta deviance were examined, and no influential observations were found.

5.3.2 Survival analysis of site data

The results from the best Cox proportional hazard model are presented in Table 5.6. There were 82 sites used in the data analysis, and the model identified 3 significant risk factors (ISANeigDist, ProcBoatDist, and CageDepth) with all significant variables having a $p<0.05$.

The proportional hazard assumption was validated by all methods described in the methods sections. The plot of cumulative hazard against the Cox-Snell residuals yielded a reasonably straight line indicating the model fit well. Graphs of the Martingale and

Deviance residuals against the time at risk identified one outlier. This site was an outlier because it was positive for all of the risk factors in the model, but it was not an ISA case site. Rerunning the model with this outlier removed, slightly changed the coefficients of the model, but did not affect their significance.

5.4 Discussion

5.4.1 Study type

This risk factor study was a retrospective cohort study of the entire of population (except for two sites) at the site level. At the cage level, the study was designed as a modified case-cohort design in that it incorporated ISA case cages from the entire industry and included data from a subset of controls that were followed retrospectively. Because the farmed Atlantic salmon industry in New Brunswick is small it was important to achieve a high rate of participation to increase the study's power. Surveying 83 of the 85 sites provided a much broader database than the earlier risk factor study in which only 14 sites were surveyed[11]. Expansion of the study to these sites was possible because ISA has now been identified in all regions of the NB industry, and therefore it is assumed that exposure to virus was possible in all areas. In earlier studies, it appeared that exposure was limited to three distinct regions. By increasing the power of the study, there is more chance that important risk factors will be identified[15].

As an analytical observational study, each factor is tested as it relates to the outcome in the animal's production environment. This is advantageous over a laboratory experiment in which all factors except one are held constant because even if the factor is significant in the laboratory, once the fish are in their production setting with the multitude of other factors, this one particular factor may no longer be significantly

associated with the disease outcome. An example of this is vaccinating the smolts against ISA. Small-scale laboratory studies have shown that Atlantic salmon have reduced mortalities after vaccination against ISAV compared with their non-vaccinated counterparts[16]. When tested in the farm setting and analyzed simultaneously with other variables, vaccination against ISAV did not appear to provide significant protection.

5.4.2 Significant risk factors

5.4.2.1 Site risk factors

There were three risk factors that remained in the final logistic regression model with ISA disease on the site as the outcome. Previous laboratory experiments have shown that the virus can survive in seawater[7;17] as well as be spread to naive Atlantic salmon by blood, infected tissues, and feces[6;7]. A site that has a neighboring site with ISA within 500 meters (ISANeigClose) has an increased risk of ISA disease. This is likely due to the spread of the virus through the water, but could also be indicative of increased boat traffic due to many sites in a small bay area and reflect the general increased risk of that geographical area.

Boat traffic by the site increased the risk. One study of well-boats in Scotland did find an association between boats carrying fish from site to site increasing the risk of disease[8]. It was not possible to measure the boat traffic at particular sites in this study, but the distance between the site and the processing boats traveling past the site on their way to the wharf (ProcBoatDist) was ascertained and if processing boats traveled within 1 km of the site then the site was at higher risk for disease. Currently members of the NBDAFA are reviewing boat traffic in the area of the sites and are making a plan to reduce the risk of ISA viral spread by boats.

The variable CageMortNum represents whether any of the cages on the site had experienced greater than 5% mortality in the first 30 days post seawater transfer. If any of the cages had, the risk of ISA at the site was increased. Similarly, Hammell and Dohoo[11] found an increased risk of disease for cages with high cumulative mortality rates during the salmon's first year in seawater. It was suggested that cumulative mortality rates during first year in seawater was an indicator for general fish health.

If the site had a diver removing dead and moribund fish more than twice a week (DiveAug), then the site had an increased risk for ISA. This is opposite to what was found in the Norweigan risk factor studies [9;10]in which the risk of ISA increased when dead fish were less often than daily. However, the importance of the time sequence for this predictor will be discussed in the section on Causality 5.4.5.

5.4.2.2 Cage risk factors

Having more than 3 meters between the bottom of the net and the ocean floor at low tide (MeterUnder) increased the risk of having disease in that cage. One possible explanation may be that greater water depth may allow for more distortion of the shape of the net enclosure. This may result in more stress on the fish or, in severe cases, the fish may have decreased room to maintain swimming patterns. Another possible reason for this increased risk is that cages that have more depth underneath the cages were in deeper water and thus experienced higher currents. If currents are high, fish may get pushed toward one side of the cage and if the currents are extreme, the fish may get pushed up against the nets causing skin and fin damage. This situation is not only stressful to the fish, but the wounds may allow for ISAV to enter the fish.

Sea lice have been confirmed to be ISA viral transmitters[5]. Previous reports have shown that the risk of ISA is less when sea lice are more frequently controlled[11]. Although sea lice are generally well controlled through the use of the in-feed product, SLICE™, sea lice remain an ongoing management issue and the number of times that the farmer treats for sea lice with SLICE™ remains a cage risk factor. If the farmer had treated a cage of fish for sea lice more than twice during the study period, the risk of becoming an ISA case cage was decreased.

One cage risk factor with many missing observations was whether the farmer perceived there were at least 1000 pollock in the cage with the farmed salmon (Pollock). Wild pollock are commonly found in Atlantic salmon cages in New Brunswick. Pollock can enter the salmon cage when they are small enough to swim through the net (tempted by the presence of salmon feed) and may become residents of the cage because they grow too large to swim out. They also get into the cages during net changes. Pollock are often found eating the feed that falls out of the cages. If they are close to the net at the time of net changing, as the clean net gets brought underneath the dirty net, the pollock may become trapped inside the clean net. Other wild fish such as sea trout[18], brown trout[19], and herring[20] have been shown to be carriers of ISAV and have been shown to infect naïve salmon in laboratory trials. There has been no proof that pollock can carry ISAV or infect salmon[21;22], but their presence in the cage may be stressful to the fish and will also increase the stocking density within the cage. This factor may be an indicator of the level of other wild fish that could be carriers of ISAV present inside or outside of the cage.

Transferring smolt greater than or equal to 99 g (SmoltWeight) increased the risk of the cage becoming diseased with ISA. The size of the smolt appears to be an important risk factor, perhaps due to better adaptation for seawater by the smaller smolts. Alternatively larger smolts are more likely to have problems such as conformational deformities due to the rapid growth. This factor could also be an indicator for the type of hatchery from where the smolt came. Recirculation hatcheries filter, and then reuse, the water that the fish live in. Usually this water is heated and is warmer than the water used in flow through hatcheries that do not reuse the water. Because the water is warmer, the smolt will grow faster and be larger at the time of seawater transfer. However, the variable for hatchery type (HatchType) was measured and was neither significant nor a confounder for SmoltWeight.

5.4.3 Survival analysis

The survival analysis was performed to take into account the time it takes to get ISA disease. In the site logistic regression model, if the nearest neighbor farm having ISA was within 0.5 km was an important risk factor. However in some cases, the ISA at the neighbor site occurred after the outbreak at the study site. In addition, it is likely that the risk of disease changes at a negative site over time as the nearest neighbor with ISA becomes closer. This change in risk is not accounted for in the logistic regression model. ISANeigDist was significant with increasing hazard ratios as the nearest neighbor with disease became closer to site of interest. The closest distance category was within 0.5 km and when the disease became this close, the risk of disease increased 5.5 times. Boat traffic was also an important factor with ProcBoatDist and DryFeedDel (having dry feed

delivered to the site by the feed company) increasing the hazard by 7.5 and 2.7 times respectively.

This study identified several risk factors as significant predictors of ISA disease. However, there is always the chance that there are other important risk factors for ISA, but that we did not have records to assess them. It is also possible that the risk factors that were significant in this study were actually indicators for other factors that we did not measure. An example of this is the variable for MeterUnder. This variable measured whether the meters under the net at low tide was greater than 3 meters but it could be an indicator for a factor not measured in this study such as the velocity of the current as mentioned above in the cage risk factor section.

5.4.4 Minimizing bias

Meticulous care must be taken when conducting an analytical observational study in order to reduce any systematic error (bias) in design, implementation, and analysis so as not to invalidate the results[23]. Selection bias for the site was minimized by selecting almost the entire population making it very representative of the population. Selection bias for cages was also minimized by randomly selecting the case and control cages.

Information bias can occur for either the outcome or the predictors. Outcome misclassification occurs when cases are classified as controls or vice versa. The first report of isolating the virus was in 1995[24]. Previous to this isolation, there was no definitive diagnostic test. Establishing case sites and cages in the previous studies had been made by the diagnosis of ISA from clinical signs with gross and histological pathology consistent with ISA in two studies[9;10]. In the third study, ISAV had not yet been diagnosed so case cages were defined by exclusion[11]. If a cage had seven or more

days with mortality rates greater than 0.1% per day or a cumulative mortality of greater than 5% after the 30 day post saltwater transfer period and there was no other disease diagnosed, then the cage was considered a case cage. Since 1998, there have been many ISA diagnostic tests available in New Brunswick including virus isolation on cell culture (VI), indirect fluorescent antibody testing (IFAT), and reverse transcriptase-polymerase chain reaction (RT-PCR). Cage diagnosis has been made by having at least two fish test positive by two tests and the presence of increased mortalities attributed to ISA. Although the sensitivity and specificity of the diagnostic test are not perfect, the use of a more definitive method of diagnosis and the requirement of two fish with two positive tests rather than one fish with one positive test improves the specificity of the diagnostic process and thus reduces the chance of having outcome misclassification bias.

Information bias can also be a problem when the questionnaire answers are answered inaccurately changing the influence of the predictors on the outcome. Information bias can occur if the site manager is informed about the purpose of the survey and answers questions not according to what occurred on the site, but according to what he believes to be the desired response. Another type of information bias is recall bias and it may be introduced if the interviewee is more likely to remember an answer because the site has had ISA or is dealing with ISA. Unfortunately this type of bias is differential, in that it would be an issue only for information from ISA positive sites or cages and its influence on estimates would have to be analyzed on an individual predictor basis. In general, it is difficult to remove information bias, but the survey avoided any leading questions and all surveyors were well trained not to unintentionally direct the site manager's responses (interviewer bias).

Another source of bias may arise due to confounders. If there were factors (confounders) not measured in the study that were associated with the ISA outcome of the site or a cage and were associated with one of the measured variables, but not on the causal pathway from measured variable to ISA outcome, then the variable would have been a confounder and the estimated OR of the predictor may have been incorrect, possibly resulting in a change of significance.

5.4.5 Causality

Finding an association between a factor and ISA disease does not necessarily prove that these factors are causes of the disease. It is possible that some of the associations are due to chance. This type I error is minimized by choosing α to be 0.05. As mentioned previously, it is also possible that some factors associated with disease are not actual causes but are indicators or markers for other risk factors. In order to prove that the association is causal for ISA, there must be a causal link between the risk factor and the disease. Hill (1965) proposed some guidelines for support of a causal relationship[25].

Time sequence: All factors except for one (DiveAug) precede the outcome of disease because they are constant events such as the shape of the cage or they were asked particular questions for the period up until the cage became diseased. The variable DiveAug did not conform to this time requirement. Clinical ISA is most common during the late spring until fall. Many of the case sites were experiencing ISA outbreaks during the month of August. Thus frequent mortality dives during the month of August should be excluded as a cause of increased risk of ISA because it is possible that the site already had disease and dive frequency was increased as a control method.

Consistency and reproducibility: If the important risk factors identified in this study are consistent with previous knowledge and are reproducible, then they are likely to be causal factors. Many of the significant risk factors were identified in previous laboratory and epidemiological studies. Laboratory studies have shown that the virus can spread through seawater[7;17] and be transferred from fish to fish by sea lice[5;7] increasing the evidence that risk factors such as ISANeigDist and LiceTx are causal. Other epidemiologic studies have shown the importance of boat traffic[8;11], proximity to a site with ISA[9;10], lice treatment[11], and healthy young fish[11], providing consistency and reproducibility of the risk factors ProcBoatDist, ISANeigDist, LiceTx, and CageMortNum. Newly identified risk factors such as CageDepth, MeterUnder, SmoltWeight, and Pollock should be tested either in the laboratory or with other epidemiologic studies to test for reproducibility.

Strength of statistical association: The higher the magnitude of the OR or hazard ratio of the risk factor, the more likely the risk factor is causative for ISA disease and the less likely it is due to a confounding factor. In the logistic regression and survival analyses, all risk factors increased the risk by at least two times. Care must be taken when evaluating this argument for causality as in certain cases when the number of records is small, the magnitude of the risk may be very large. For example in Table 5.3, if a site had at least one cage with post transfer mortalities greater than 5% (CageMortNum), then the odds of that site having ISA disease was 36 times a site that did not have any cages with greater than 5% mortalities. However, this large OR was very imprecise as demonstrated by the very wide 95% confidence interval (CI), ranging from 4 to 343. Because there were only a small number of site records available for this

factor (81 records), the statistical power was low and only variables with very large OR were significant.

Dose-response relationship: To provide more support that a risk factor is causal, the risk of disease should increase as the intensity of the exposure to the factor increases. In this study, a dose-response relationship may be difficult to appreciate because many of the variables were dichotomous. However, the risk factor for the distance to the closest neighbor with ISA (ISANeigDist) did adhere to this relationship. As the nearest neighbor with ISA progressed from site to site drawing closer to the site in question, the risk of ISA also increased as shown in Table 5.6.

Elimination or modification of the factor: Removal or reduction of the risk factor should lower the incidence of disease if the factor is causal. Removal of certain factors can be difficult due to financial and environmental complications. Changing the depth of the cage or the distance underneath the cage may not be possible for certain sites due to the depth of the water and tide conditions. With the availability of SLICE™ to control for lice and the implementation of processing boat traffic regulations, the factors LiceTx and ProcBoatDist may be tested for causality. If sea lice are controlled very well and processing boats are kept further away from sites, the likelihood that the site will become ISA diseased should be reduced.

Coherence with existing knowledge: Only one of the risk factors that was identified in this study was contradictory to a previous ISA risk factor studies[9-11] or any laboratory studies. DiveAug suggests that the more often mortalities are removed from cages in August, the more likely the site will become diseased. This is opposite of what was found by Jarp and Karlsen (1997) found. This is not much of a concern because this

variable was discounted as a causal risk factor because the factor does not necessarily precede the ISA disease.

As most of the variables that were significant in the logistic regression and survival analyses conform to most of these arguments, there is much evidence that these factors are likely to be causal for ISA disease. Therefore it is recommended that if sites can modify any of these factors in an economical manner or with a minimal logistical burden, they should do so to reduce the risk that the site will become diseased with ISA.

5.4.6 Statistical Analyses

Because the case cages were matched to control cages, estimates for individual predictors would have been more appropriate if the conditional logistic regression model could have been used for cage and hatchery dataset. Unfortunately, because cage and hatchery predictors often did not vary within a site, estimates for predictors were unstable and population-averaged logistic regression modeling was used to account for clustering of cages within a site.

Multivariable logistic regression is a helpful tool to identify important risk factors and their magnitude of increased risk. However, it is important to have many complete records to accurately assess a large group of variables[13]. For the site level factors, there were 74 variables and only 83 records. By removing variables that were not significant on unconditional associations, variables that had many missing records, and combining a group of variables into a new index variable, the number of important risk factors available for analysis was decreased considerably. Still, it was not possible to assess interaction in the site level data because of the small number of records.

When there were a small number of records in the analyses, the odds ratios for the significant factors were large but impercise. This was demonstrated above in the *Strength of statistical association* section using the site risk factor, CageMortNum. To improve the precision of the OR estimates, the power of the study should be increased. Making records more accessible for the study would have increased the number of complete records. However, because there were only 85 qualifying sites for the study, the precision of the OR estimates could only be improved modestly.

Knowledge of the farming industry and of the disease was used to formulate the initial extensive questionnaire that was reviewed by the local aquaculture veterinarians. Although the survey was meant to be inclusive, there is always the possibility that there were significant variables that were not included in the study. Likewise, there may be unmeasured variables (confounders) that are responsible for an inaccurate OR and possibly even making an insignificant variable appear statistically significant.

5.5 Conclusions

There were many important risk factors identified by this study. From a disease management position, by eliminating these risk factors, the disease should diminish and may even be eliminated. The important factors recognized by this study can be categorized as controlled by environment, farmer, or industry according to the capacity to change or eliminate them. Environmentally controlled risk factors such as the depth of the net and the depth of water underneath the net may be manipulated by the farmer in some circumstances, but for the most part these factors are dictated by site location. Wild pollock in the cage is another environmental factor that is not easily changeable as it is usually a reflection of the number of wild pollock that live in the vicinity of the site.

However, altering methods of changing nets may decrease pollock inclusion in salmon cages. Factors that are under site control are easier to change. These include improving lice control, transferring healthy smolt into seawater (possibly avoiding large smolt or investigating health of large smolt), and improving on the adaptation of smolt to seawater to reduce post transfer mortalities. Although these factors are changeable by the farmer, they may be very expensive changes. The third category, industry controlled factors, need to be addressed by the government officials and the industry representatives. Organizing boat travel to minimize the time and frequency of boats traveling to or by sites is currently being reviewed. Increasing the distance between sites to increase the distance to the nearest neighbor with ISA may be necessary if control of the disease is ever to be realized.

In addition to changing these factors, continued research and a coordinated effort by the government officials, veterinarians, site owners, site managers, and other members of the industry such as the hatchery owners and the processing plant managers are necessary to help improve the current ISA situation in New Brunswick.

5.6 Acknowledgements

The authors thank Jill Westcott, Karen MacDonald, David McRuer, Erin Wilson, and Holly Beaman for the help interviewing farmers and data management. They also appreciate the cooperation of the New Brunswick Salmon Growers Association and the Aquaculture Association of New Brunswick and the participation of the farmers and record keepers. They are grateful for the financial support provided by the New

Brunswick Department of Agriculture, Fisheries, and Aquaculture, the Natural Sciences and Engineering Research Council, and the University of Prince Edward Island.

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Table 5.1: List of important predictors and their description

Predictor Name	Group	description
ProcBoatDist	site	If processing boats travel within 1 km from the site
ProcBoatNum	site	Number of processing boats that travel past the site
CageMortNum	site	If ≥1 cages had >5% mortalities within 30 days post seawater transfer
CageMortPer	site	Percent of total fish that died in the first 30 days post seawater transfer
ISANeigDist	site	Categorical data, how close the nearest neighbor with ISA is to the site
ISANeighClose	site	If the nearest neighbor with ISA is within 0.5 km of the site
DryFeedDel	site	If the site had dry feed delivered by the feed company
DiveAug	site	If the site had mortalities removed more than once a week in August
CageVol	cage	Continous variable representing the volume of the cage in m^3
CageType	cage	Polar circle or square metal cages
CageSize	cage	Circumference of the circle cage or the length of a side of the square cage
CageDeep	cage	Depth of the net in meters
LiceTx	cage	If cage was treated for sealice less than 3 times with any product
MeterUnder	cage	If the cage had more than 4 m of water underneath the net at low tide
CageDepth	cage	If the net was less than 10 meters in depth
Pollock	cage	If the farmer perceived that there were at least 1000 pollock in the cage
SmoltWeight	hatchery	If the smolt weighed ≥99g at the time of seawater entry

Table 5.2: Selected unconditional associations (with p<0.20) between site level factors and the site's ISA status

Variable	level	Number of case sites (all sites)	Odds ratio	Lower 95% CI	Upper 95% CI	P-value (χ^2)
Distance to processing boats traveling past site (ProcBoatDist)	>1 km	1 (17)	1			
	≤1 km	26 (66)	10.4	1.41	452.51	0.009
Closest neighbor with ISA (ISANeighClose)	>0.5 km	16 (67)	1			
	≤0.5 km	11 (16)	7.01	1.86	29.01	0.001
Dry feed is delivered by feed company (DryFeedDel)	no	12 (56)	1			
	yes	15 (26)	5	1.63	15.43	0.001
Fish are fed by hand (FeedHand)	no	13 (53)	1			
	yes	14 (30)	2.69	0.93	7.75	0.039
Site allows visitors (Vistor)	no	6 (27)	1			
	yes	21 (56)	2.1	0.67	7.35	0.164
Number of mortality dives per week in August (DiveAug)	≤1/week	12 (58)	1			
	>1/week	15 (25)	5.75	1.85	18.11	0.001
Number of mortality dives per week during times of high mortalities (DiveHighMort)	≤2/week	7 (35)	1			
	>2/week	20 (47)	2.96	0.99	9.58	0.032
Smolts transferred to seawater site by a ferry (SmoltFerry)	no	8 (41)	1			
	yes	19 (42)	3.41	1.16	10.49	0.012
Percent of total mortalities during the first 30 days post seawater transfer (CageMortPer)	≤5%	10 (38)	1			
	>5%	10 (22)	2.33	0.67	8.11	0.130

Number of cages that had >5% mortalities during the first 30 days post seawater transfer (CageMortNum)	no cages	18 (40)	1			
	≥1 cage	3 (26)	6.27	1.48	36.87	0.004
Has site been attacked by seals (SealAttack)	yes	6 (27)	1			
	no	21 (56)	2.1	0.67	7.35	0.164
How often are fish weight sampled (WeightSample)	<1/month	9 (39)	1			
	≥1/month	18 (44)	2.31	.81	6.84	0.0835
Nets are cleaned in the water (CleanNets)	no	7 (34)	1			
	yes	20 (49)	2.66	0.89	8.59	0.053

Table 5.3: Logistic regression model for site level risk factors. Data from 81 sites.

Variable	level	Odds ratio	Lower 95% CI	Upper 95% CI	P-value
Distance to processing boats traveling past site (ProcBoatDist)	>1 km	1			
	≤1 km	23.13	1.15	463.32	0.040
Closest neighbor with ISA (ISANeighClose)	>0.5 km	1			
	≤0.5 km	7.73	1.64	36.33	0.010
Number of cages with post transfer mortalities greater than 5% (CageMortNum)	no cages	1			
	≥1 cage	36.29	3.84	342.64	0.002
	No response*	28.16	2.29	346.91	0.009
Number of mortality dives per week in August (DiveAug)	≤1/week	1			
	>1/week	17.66	3.30	94.48	0.001

*The no response reply is applied when the farmer is unable to provide an answer for the question

Table 5.4: Unconditional associations (with p<0.20) between cage level factors and the cage's ISA status.

Variable	level	Number of case cages (all cages)	Odds Ratio	Lower 95% CI	Upper 95% CI	P-value (χ^2)
Depth of cage (CageDepth)	>9 m	6 (75)	1			
	≤9 m	35(192)	2.56	1.00	7.78	0.037
Fish had gill parasites (GillParasites)	yes	1 (25)	1			
	no	40 (242)	4.75	0.73	200.22	0.098
Meters underneath net at low tide (MeterUnder)	≤3 m	31 (162)	1			
	>3 m	10 (98)	2.08	0.93	5.00	0.056
Number of lice treatments with SLICE™ (SliceTx)	>2 times	6 (70)	1			
	≤2 times	35(187)	2.46	0.96	7.48	0.048
Has the net been changed (NetChange)	no	27 (191)	1			
	yes	14 (61)	1.81	0.81	3.91	0.104
At seawater entry, did the smolts come in a wide range of sizes (SmoltUngraded)	yes	38 (219)	1			
	no	1 (27)	5.46	0.84	229.55	0.067
Number of pollock farmer perceived to be in the cage (Pollock)	<1000	28 (186)	1			
	≥1000	10 (27)	3.32	1.22	8.58	0.005
Vaccinated against ISA (VaxISA)	yes	21 (158)	1			
	no	19 (89)	1.77	0.84	3.71	0.099
Immersion vaccinated against *Aeromonas salmonicida* (VaxIM)	no	21 (158)	1			
	yes	16 (80)	1.63	0.74	3.53	0.177
Degree-days between intraperitoneal vaccination and seawater transfer (DegreeDays)	>700 degree-days	9 (79)	1			
	≤700 degree-days	18 (95)	1.82	0.72	4.89	0.171

Smolt weight (SmoltWeight)	<99 g	12 (117)	1			
	≥99 g	25 (116)	2.40	1.09	5.55	0.018
Number of smolts transferred into a cage (SmoltNum)	>16,000	15 (112)	1			
	≤16,000	24 (118)	1.65	0.77	3.60	0.161

[a] P value for t-test on a continuous predictor

Table 5.5: Logistic regression model for cage level risk factors. Data from 256 cages.

Variable	level	Odds ratio	Lower 95% CI	Upper 95% CI	P-value
Number of lice treatments with any product (LiceTx)	>2 times	1			
	≤2 times	3.80	1.37	10.49	0.010
Meters underneath net at low tide (MeterUnder)	≤3 m	1			
	>3 m	3.09	1.24	7.66	0.015
Number of pollock farmer perceived to be in the cage (Pollock)	<1000	1			
	≥1000	10.30	2.99	35.53	<0.001
	No response*	0.29	0.08	1.14	0.077
Smolt weight (SmoltWeight)	<99 g	1			
	≥99 g	2.81	1.23	6.42	0.014
	No response*	1.26	0.35	4.52	0.728

*The no response reply is applied when the farmer is unable to provide an answer for the question

Table 5.6: Survival analysis model for modified site risk factors. Data from 83 sites.

Variable	level	Hazard ratio	Lower 95% CI	Upper 95% CI	P-value
Nearest neighbor with ISA categorized (ISANeigDist)	≥5 km	1			
	≥2 km but <5 km	1.17	0.34	4.07	0.807
	≥0.5 km but <2km	2.01	0.63	6.46	0.240
	<0.5 km	5.50	2.03	14.95	0.001
Distance to processing boats traveling past site (ProcBoatDist)	>1 km	1			
	≤1 km	7.47	1.00	55.83	0.050
Dry feed is delivered by feed company (DryFeedDel)	no	1			
	yes	2.66	1.20	5.88	0.016

6 Specific antibody response produced by Atlantic salmon to infectious salmon anemia virus vaccines

6.1 Introduction

Infectious Salmon Anemia (ISA) is a recently described disease in farmed Atlantic salmon. ISA first emerged in a Norway in 1984[1]. Over the next 6 years, the disease spread throughout the farms along the southwest coast of Norway. This disease has caused devastating losses in the Norwegian Atlantic Salmon industry and is currently causing disease in the Atlantic salmon farms in New Brunswick (NB), Canada. The newly identified virus from the family *Orthomyxoviridae*, infectious salmon anemia virus (ISAV), has been identified as the causative agent[2] and now has also been detected in diseased salmon in Scotland[3], Faroe Islands[4], Chile[5], and the United States[6]. Virally infected Atlantic salmon experience lethargy and anorexia as well as pathologic lesions including severe anemia, exophthalmia, pale gills, ascites, and internal organ damage[1;7].

In Norway, the destruction caused by the virus was lessened by a strict eradication protocol established by the Norwegian government[8]. Years later in 1996, an Atlantic salmon farm off Grand Manan Island, New Brunswick, had what was believed to be the first case of ISA outside of Norway[7;9;10]. Since that time, NB Atlantic salmon farmers and NB aquaculture regulators have been trying to reduce the impact of this disease. An ISA surveillance program for early detection and removal of infected cages to limit the spread of the virus has been in place in NB since 1997[11]. Although the number of cases of ISA was beginning to decline, there has been a resurgence of the disease and major financial losses to the industry continue due to depopulation of affected cages. Despite making many changes to improve the way fish are managed to reduce exposure to the virus and despite the introduction of an autogenous vaccine in the fish stocked in 1999[9],

Atlantic salmon in NB farms continue to experience outbreaks of clinical disease attributed to the virus.

Vaccines are intended to protect the host from the pathogen against which they are vaccinated. Vaccines can be a very powerful tool for the protection against a pathogen if they can stimulate a broad range of neutralizing antibodies and the vaccinated fish do not become carriers of the pathogen[12]. Currently there are two different vaccines available for use in North America, one licensed vaccine produced by Aquahealth, Ltd. (Charlottetown, PE), and one autogenous vaccine produced by Bayotek International Inc. (Saanichton, BC). These vaccines were based on initial autogenous vaccines that were generated by inactivating virus that was originally isolated from farmed salmon in the Bay of Fundy, NB and then grown in cell culture with the SHK-1 cell line[13].

Efficacies of vaccines from virus challenge studies are reported as relative percent survival (RPS) and can be calculated as RPS=[1-(% vaccinate mortalitity/ % control mortalitity)] x 100. Documented proof of specific protection against ISA from the original vaccines is limited. Jones et al. (1999) reported laboratory trials in which the multivalent bacterial vaccine without ISAV had the same efficacy as multivalent bacterial vaccine with ISAV after intraperitoneal (IP) ISAV challenge suggesting the non-specific immune response was capable of decreasing the severity of the challenge[13]. However, when fish were challenged by cohabitation with ISAV infected salmon, the multivalent bacterial vaccine without ISAV group had a RPS of 47% while the multivalent bacterial vaccine with ISAV group had an RPS of 95% with 69% mortality in the controls signifying the adjuvant and bacterial antigens were somewhat protective in the passive

cohabitational situation, but there was a better, more specific response when the ISAV antigen was included in the vaccine.

There is also some additional evidence (provided in abstract form only) in which salmon vaccinated with inactivated virus in adjuvant were protected by the vaccine in laboratory challenge studies[14-17]. These vaccine experiments have all been done in a highly controlled setting. In the NB sea cages, many fish that experience clinical ISA outbreaks have been vaccinated against the virus. Clinical trials are important in measuring vaccine efficacy under production conditions. In another abstract, Hammell and Beamen reported on a small clinical trial performed to evaluate the efficacy of the vaccines in the farming environment in the sea cages[18]. Fish were given different commercial vaccines from Aquahealth, Ltd. and Bayotek International Inc., some containing ISAV and some without. Control fish were given saline instead of a vaccine. Fish were all tagged with transponders for individual identification and mixed within one cage. Subsequently the cage became diseased with ISAV (presumably through natural challenge) and the dead and moribund fish from the study cage were collected and counted. During the cage outbreak, ISAV vaccines did not significantly affect the time to mortality, i.e. ISAV vaccinated fish did not die any later than non-vaccinated fish, suggesting a lack of vaccine protection. The laboratory studies seemed promising, but the vaccine did not show any significant protection in the field trial, although this field study was limited in size and scope. More laboratory experiments and field trials are needed to resolve the efficacy issue of available commercial vaccines.

Another method to evaluate potential protection by a vaccine is to measure the anti-ISAV antibody levels in the serum after vaccination. . Enzyme-Linked

Immunosorbent Assay (ELISA) tests can be utilized to measure serum antibody responses to natural infection with ISAV[19;20] or to ISAV vaccines[19]. There have been no large-scale evaluations of the available ELISA tests that measure the anti-ISAV serum antibody levels of farmed Atlantic salmon using field samples. However, Norwegian researchers have repeatedly used an ELISA to monitor specific anti-ISAV antibodies in different populations following natural outbreaks (Knut Falk, personal communication). The objective of this study was to use the selected ELISA on ISAV vaccinated fish to determine if specific anti-ISAV antibodies could be detected in the serum of farmed Atlantic salmon under normal production conditions after vaccination and to assess the influence of water temperature and time after vaccination on the specific anti-ISAV antibody response in Atlantic salmon.

6.2 Materials and Methods

6.2.1 Fish populations and vaccine groups

In December 1999, fish tanks at a flow-through hatchery in New Brunswick were identified as to inclusion in the vaccine trial. The water source for the hatchery was a freshwater lake, and the water was distributed to multiple tanks that contained approximately 5,000 pre-smolt per tank.

There were three vaccines that were used including Aquahealth Forte with autogenous inactivated ISAV (A+ISAV) and Bayoteck Multivacc4 with autogenous inactivated ISAV (B+ISAV) and the control vaccine, Aquahealth Forte with Renogen (inactivated *Renibacterium salmoninarum*) without ISAV (A). One tank was chosen for each vaccine.

6.2.2 Serum collection

Ten to fifteen fish were removed from their tank by using feed to attract them and then dipping them out with a net. The fish were anesthetized by the anesthetic Tricaine methanesulphonate (Syndel International Inc., Vancouver, British Columbia) in a freshwater solution. The fish were weighed on a scale and then blood was collected by venipuncture of the caudal tail vein. The blood collection tubes were placed on ice until they were transported to the laboratory where the samples were centrifuged and serum harvested. Serum was stored at –80°C.

Fish were sampled prior to vaccination and approximately every three weeks after vaccination until seawater entry in April 2000. Sampling was discontinued until the salmon were acclimated to seawater. Blood samples were taken twice while in seawater, once in August 2000 and once in October 2000. For the samples taken from fish in sea cages, a similar protocol was used except that blood was collected from the fish immediately after they were euthanatized by an overdose of the anesthetic Tricaine methanesulphonate in a saltwater solution. No clinical cases of ISA had occurred at the site, nor was it suspected during the sampling period.

6.2.3 Enzyme-linked immunosorbent assay (ELISA)

The ELISA was performed at the National Veterinary Institute in Oslo, Norway in March 2003.

6.2.3.1 Preparation of viral antigen

ISAV isolate Glessvaer/2/90 was propagated in Atlantic salmon kidney (ASK) cells in 162 cm^2 flasks as previously described in Devold et al.[21] The infected cells were harvested following the identification of CPE at 3 to 4 days incubation at 15°C. First the cells were washed twice in 0.01 M PBS. Cells were then scraped off the bottom of the

well with a cell scraper and collected in cold PBS. Cells were washed again two times and then collected in a small volume of PBS. After the wash, the cells were subjected to three freeze-thaw cycles at -20°C and room temperature (RT) followed by sonification five times for 30 seconds while on ice at max energy without making bubbles. The debris was discarded following centrifugation at 4°C and 20,000 x *g* for 30 min. Protein concentrations in the supernatant were determined by the Coomasie blue based Bradford method using the standard procedure for microtiter wells from Bio-Rad (Bio-Rad Protein Assay Dye Reagent Concentrate, Bio-Rad, Oslo, Norway). Aliquots of the supernatant were frozen at -80°C until ELISA plate preparation.

Non-infected ASK cells were harvested similarly and were used as negative antigen to identify the level of non-ISAV-specific antibody binding.

6.2.3.2 Secondary antibody

Atlantic salmon Ig was purified from serum using gel filtration and ion exchange chromatography as described by Håvarstein et al.[22] The rabbits were immunized subcutaneously 4 times, at 4 week intervals, with 50 μg doses of Ig in Freund's complete adjuvant for the first immunization and Freund's incomplete adjuvant for the subsequent three immunizations. Rabbit anti-salmon serum IgG was then purified by affinity chromatography using a Protein G Sepharose FF column (Pharmacia, Uppsala, Sweden).

6.2.3.3 Indirect ELISA

The wash buffer for the ELISA was prepared with 0.01 M PBS with 0.1% Tween20 (PBST). Other buffers for the ELISA included the dilution buffer (PBST with 0.5% non-fat dry milk), and the blocking buffer (PBST with 5.0 % non-fat dry milk). At

each washing step the microtiter wells were washed 5 times using the Skatron ScanWasher300 96-plate washer (Skatron Instruments Inc, VA, USA).

Ninety-six well (Nunc Immunoplate Maxisorp, cert, 439454, Nunc A/S, Roskilde, Denmark) ELISA plates were coated with 100 µl/well of the antigen in 0.05 M Carbonate buffer with a pH of 9.6 (2.5 µg/ml determined by cross titration of each antigen batch). Plates were incubated overnight at 4°C. To ensure the ELISA was specific for anti-ISAV antibodies, the uninfected ASK cell lysate was used as the negative antigen at a similar protein concentration.

Plates were then washed followed by blocking of unbound sites with 150 µl/well of the blocking buffer for 60 min at room temperature (RT). The blocking buffer was discarded and 100 µl diluted salmon serum (1:200) in diluent was added to each well and allowed to incubate overnight at 4°C. Every plate also included wells that contained diluted serum from a known specific anti-ISAV antibody positive control salmon (1:200, 1:800, 1:3200 and 1:12800) produced by experimentally infecting Atlantic salmon, a known ISAV antibody negative control (1:200), and blank wells that received only dilution buffer. Plates were washed and 100 µl of the secondary antibody (rabbit anti-salmon Ig, 1:500) was added to each well and incubated for 60 minutes at RT. Plates were washed and then 100 µl of anti-rabbit horseradish peroxidase-conjugate (HRP) (Amersham Biosciences; NA934, Uppsala, Sweden) diluted 1:1000 was added to each well and allowed to incubate for 45 minutes at RT. Plates were washed and then 100 µl of OPD solution (3.5 mM O-phenylendiamine in 35 mM citrate and 150 mM phosphate, pH 5.0) with freshly added H_2O_2 (0.0012%) was added to each well and incubated for 20 minutes at RT. The enzyme reaction was stopped by the addition of 100 µl 2.3 M

H_2SO_4/well. The OD was read in an automated microtitre ELISA reader (Labsystems Multiscan RC, Helsinki, Finland) at 492 nm wavelength.

6.2.3.4 Testing of samples

The samples were coded in order to mask the tester when performing the ELISA. The results of each serum sample are given as the percentage of the positive control serum at 1:200 dilution. The positive control serum is calculated as the difference in the average absorbance (OD) from the two parallel wells of the infected cell culture antigen and the average OD from the two parallel wells of the non-infected cell culture antigen. The test serum is calculated similarly for the test sample wells. The difference in the average ODs from the test serum is then divided by the difference in the average ODs from the positive test serum wells and multiplied by 100 to get the percentage of the positive control serum. Sera with a response above 10% of the positive control serum are considered positive and sera with a response between 5 and 10% of the positive control are considered possible positives ("suspects").

6.3 Results

Serum samples from 265 fish were tested by ELISA for antibody to ISAV. No ISAV specific antibodies were measured in the pre-vaccination groups. There was one serum sample out of 15 that was anti-ISAV antibody positive in the B+ISAV group while there was one sample out of 15 that was suspect in the control group (A) in the first post-vaccination samples. There were no other anti-ISAV specific antibodies detected from the fish sampled during the next three hatchery visits. There was one fish positive for anti-ISAV antibody in each group and one suspect fish in groups A+ISAV and B+ISAV for the first time fish were sampled in seawater and one positive serum sample in groups

A and A+ISAV and one suspect in group A for the second time fish were sampled in seawater. The results from positive and suspect fish as well as the time of fish sampling and the fish weight averages are summarized in Table 6.1.

6.4 Discussion

As expected, no ISAV specific antibodies were measured in the pre-vaccination groups. The first post-vaccination serum samples were taken over one month later and there was very little antibody detected. There was one serum sample that was anti-ISAV antibody positive in the B+ISAV group while there was one sample that was suspect in the control group (A). These samples could be the first response of the fish to the vaccine, although very few fish had any detectable response. The water is very cold during the winter months in the flow-through hatchery and to stimulate much antibody response would be unlikely[23]. The one fish in the control group to respond may have had an immune reaction from the adjuvant and the bacterins in the multivalent vaccine that cross-reacted with the ISAV on the ELISA plate.

There were no other anti-ISAV specific antibodies detected from the fish sampled during the next three hatchery visits. Interpreting the lack of antibody response is difficult because it may be due to the continued cold water or it may be that the fish do not respond to the ISAV portion of the multivalent vaccine. It is also possible that this ELISA test is not sufficiently sensitive analytically to identify low levels of serum anti-ISAV antibody and that is why there were no positive or suspect results during these sampling times.

Another possibility for why there were minimal specific antibodies detected in general may be that this ELISA test has poor diagnostic sensitivity and a large percent of

serum samples with elevated specific antibodies are incorrectly identified as negative samples. No indirect anti-ISA antibody ELISA test has been evaluated in a large scale assessment of its test performance using field samples to identify diagnostic sensitivities and specificities of the test. Because there is no evaluated ELISA, this particular ELISA was selected on the basis that it has been used in Norway since 1994 for many different fish populations[20]. It has been used to test several thousand field samples in order to determine the use of the test both as a surveillance tool and as a diagnostic/prognostic tool. This includes both testing of diseased fish and diseased fish populations during outbreaks and after clinically affected cages had been removed. It also includes the testing of suspected infected fish populations in addition to several experimental infections at different times of the year and at different time points after challenge. Recently it has been used to test several hundreds of fish from the Faroe Islands ISA surveillance program (Knut Falk, personal communication). Because of its extensive use in Europe, monitoring antibodies in Canadian ISAV vaccinates was attempted.

After the hatchery samples were taken, there was a large gap of time (March 2000 through August 2000) in which the fish were not tested due to the reluctance of farm management to disturb the fish for sampling. Therefore it is difficult to say if there were any fish with measurable antibodies during that time and if the number of degree-days after vaccination during that time was important for antibody development.

There were minimal anti-ISAV antibody positive fish from the two times the fish were sampled in seawater. The fish were transferred from the hatchery to the seawater in late April of 2000. No samples were taken until August 2000 because for the first few months after saltwater transfer, the fish were adapting to the saltwater environment and

were very sensitive to handling. By the time the fish were sampled in August 2000, the water in the Bay of Fundy is relatively warm (approximately 14°C). The fish had adequate degree-days to respond to the vaccine. There was one fish positive for anti-ISAV antibody in each group and one suspect fish in groups A+ISAV and B+ISAV. There was less response in the October 2000 samples, with one positive serum sample in groups A and A+ISAV and one suspect in group A. These antibodies could be responses from three different possible mechanisms. The first possible mechanism is that they are specific antibody responses to the vaccines. The second possible mechanism is the antibodies could be responses to natural exposure to ISAV. The third possible mechanism is that they are false positive results due to an imperfect test.

The second possible mechanism (natural exposure) is more likely for two reasons. First, the salmon did not show much response to the vaccine while in the hatchery. Secondly, ISA disease was present in the Bay of Fundy during the sampling times. However, this farm was not diagnosed with clinical ISA until June 2001. The fish in the group A cage eventually became clinically ill and were removed one month later in July 2001. Although the samples were taken eight and ten months before the farm became clinically ill, it is possible that the virus was present at the site at very low levels.

There are other possible methods of vaccination that could create an anti-ISAV antibody response in the vaccinated salmon. Inactivated ISAV vaccine given intraperitoneally is currently available to farmers. Advances in recombinant technology have made it possible to increase the production of an antigenic subunit of a virus for a subunit vaccine[12]. This would reduce the amount of work to create the vaccine and potentially give a greater, more specific antibody response. One step further is the

creation of a DNA vaccine. DNA vaccines use a plasmid DNA that encodes for the antigenic protein of the virus. Once injected into the host's cells, the plasmid DNA will ultimately express a low level of the viral antigen creating specific humoral (antibody) and cellular responses to protect against the virus.

Although these vaccines did not produce any measurable anti-ISAV antibodies in all but a few fish that were sampled, the vaccines may have produced some level of cellular immune responses. However, the measurement of cellular responses was beyond the scope of this study but should be examined to help determine the potential protection provided by vaccination. The fact that a few of the fish had antibodies during August and October of 2000, many months before clinical disease was evident on the farm, indicates that there may be some benefit of using the ELISA test as part of an early surveillance program. Another reason this ELISA may be well suited for disease surveillance is that vaccination does not seem to result in antibodies that will interfere with the identification of specific anti-ISAV antibodies due to natural challenge. However, it may also be possible that these positive results from the saltwater fish represent false positive results that are not related to exposure of the vaccine or virus. More work on the use of this test to monitor disease progression over time is warranted, but before this is undertaken, a formal evaluation of test performance including diagnostic sensitivity and specificity should be conducted to ensure proper identification of serum with specific anti-ISAV antibodies.

6.5 Acknowledgements

The authors thank Heritage Salmon for their generosity and cooperation. We also wish to thank Larry Bradford, Allan Pineau, Natalie Kierstead, Mel Buote, and Pam Emdin for their technical assistance and Ole Bendik Dale and Knut Falk for their work with the ELISA. We are grateful for funding assistance from Natural Sciences and Engineering Research Council and the Atlantic Veterinary College for this study.

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Table 6.1: Results from positive and suspect fish as well as time and fish weight averages. ELISA results are reported as fish identification number:percentage of the positive control. ‡Indicates that the sample is positive for anti-ISAV antibodies. †Indicates that sample is a suspect for anti-ISAV antibodies. n is the number of fish tested in each group on particular days. A is the control vaccine, Aquahealth forte with Renogen. A+ISAV is the Aquahealth forte vaccine with inactivated ISAV. B+ISAV is the Bayoteck Multivacc4 vaccine with autogenous inactivated ISAV.

Group	Sampling date						
	December 8, 1999	January 13, 2000	February 2, 2000	February 23, 2000	March 17, 2000	August 23, 2000	October 4, 2000
Average days after vaccination (sd)	Pre-vaccination	30 (5)	50 (5)	71 (5)	94 (5)	253 (5)	295 (5)
Average degree days after vaccination (sd)	Pre-vaccination	78 (25)	86 (25)	93 (25)	119 (25)	>1000	>1400
Average fish weight (sd)	98.6 (8.2)	103.6 (7.2)	99.2 (11.2)	100.7 (3.2)	105.4 (11.1)	541.0 (16.4)	1115.2 (43.5)
A	none (n=10)	†6240: 8.08% (n=15)	none (n=15)	none (n=13)	none (n=15)	‡6292: 12.87% (n=10)	‡6077: 10.8% †6080: 6.01% (n=10)
A+ISAV	none (n=10)	none (n=15)	none (n=15)	none (n=14)	none (n=15)	‡6307: 11.73% †6309: 8.49% (n=10)	‡6069: 21.77% (n=10)
B+ISAV	none (n=10)	‡6215: 10.23% (n=14)	none (n=15)	none (n=14)	none (n=15)	‡6315: 10.3% †6318: 6.36% (n=10)	none (n=10)

7 Summary and Recommendations

7.1 Diagnostic test evaluation

Test methods used for regulatory decisions to remove infected cages include indirect fluorescent antibody test (IFAT), reverse transcription-polymerase chain reaction test (RT-PCR), and virus isolation (VI). However, no thorough evaluation of these diagnostic tests has been carried out on field samples. Sensitivity and specificity of ISA diagnostic tests were evaluated as individual tests and in combinations, using data collected by the provincial government surveillance program. Because a "gold standard" reference test for ISAV was not available, cage status was based on clinical disease records. A pool of dead or moribund fish from ISA negative farms that had never had clinical disease and a pool of dead or moribund fish from positive cages that were experiencing an outbreak of clinical ISA were obtained and assumed to be ISAV negative and positive respectively. A total of 1071 fish were used in this study.

The results of this study showed the highest sensitivity and a high specificity in RT-PCR tests performed by the RPC lab. For the RT-PCR, sensitivity and specificity were 93% and 98% respectively. The RT-PCR test results are usually returned within a few days. Unfortunately this test is expensive (>35 US$ per fish). The quickest and least expensive test available is the IFAT. Unfortunately, this test's sensitivity is at best 83%, leading to 17% of the truly positive fish testing as falsely negative. Depending on the test's cut-off value, the sensitivity and specificity for histopathology ranged from 30% to 73% and 72% to 99% respectively. Although histopathology did not perform very well as an ISA diagnostic test, this test does have two advantages: it is relatively inexpensive and there is the potential to diagnosis a concurrent disease. Performance evaluation of VI

was difficult in this dataset because most results were reported as pools, and the final evaluation was made only on fish that were tested individually. While the specificity of the VI is excellent (99%-100%), the sensitivity was poor for RPC's VI (67%) and not evaluated for AVC due to an insufficient number of samples. An advantage of VI is that a positive result indicates there is live virus in the sample. However, poor sensitivity, high expense, and long incubation periods restrict the use of this test.

Test performances were also evaluated in series and parallel combinations. Sensitivities are maximized when tests are evaluated in parallel and ranged from 75% to 98%. Specificities are maximized when the tests are evaluated in series and ranged from 99% to 100%. If a fish tested as part of the surveillance program has a positive IFAT result, the tissue is also sent to the RPC laboratory for RT-PCR testing. If this test result is positive, then the fish is considered to be positive. If this result is negative, then the fish is considered to be negative. The results of this study suggest that this series interpretation has a sensitivity of 76% and a specificity of 100%. Therefore, using this testing protocol approximately 24% of truly positive fish are falsely being labeled as negative for ISA. An alternative approach would be to choose a testing strategy that maximizes sensitivity and specificity. The simplest approach would be to test all fish with the RT-PCR test. This test has a high sensitivity, 93%, and a high specificity, 98%. However, before adopting new testing protocols, a complete economic analysis should be conducted accounting for the number of fish tested by the surveillance program, the cost of the tests, and the costs of the decision made using those test results.

7.2 Prevalence study

Evaluation of ISA control programs' success requires knowledge of the viral infection prevalence within outbreak cages compared to surrounding cages not showing signs of clinical disease. Prevalence was compared in cages experiencing an outbreak with healthy cages from the same farm, neighboring farms, and distant farms. Salmon from five different groups were tested using an RT-PCR test. Groups included moribund fish from a cage experiencing an outbreak (A), healthy fish from an outbreak cage (B), healthy fish from a negative cage from a farm experiencing an outbreak in a different cage (C), healthy fish from a negative farm near an outbreak farm (D), and healthy fish sampled at a negative farm located in an area with only negative farms (E). Survey data analysis techniques were used to evaluate the prevalence of ISAV. Apparent prevalences (standard error) for the different groups (A-E) were 0.94 (.026), 0.41 (0.062), 0.29 (0.040), 0.08 (0.037), and 0.08 (0.037) respectively. All groups were significantly different ($p<0.002$) from each other except between groups B and C and between groups D and E.

Because the prevalence of the virus was significantly higher in the outbreak cage (B) compared to other sites, early harvest of outbreak cages will remove one source of virus. Cages belonging to group of non-outbreak cages (C) on the farm experiencing the ISA outbreak should be assessed as to their role in disease spread. If the aim of the surveillance program was to eradicate the virus and knowing that this cage has a higher level of virus than the cages at neighboring farms, depopulation at the same time as the outbreak cage is necessary to completely remove the agent from the farm. Adopting this strategy would require a farm that has one cage with positive ISA diagnostic testing and increased mortalities due to ISA, to slaughter all cages on the farm. Although useful for

ISA control at the industry level, complete farm depopulation would be economically disastrous to the farm owner unless there was adequate financial compensation.

If the aim of the surveillance program was not to eliminate the virus but to reduce disease by limiting the spread of the virus, early detection of viral infection in a cage as an alternative to complete farm depopulation may be useful. Currently, dead or moribund fish from all cages at all farms are being tested for ISA by use of the IFAT every six weeks. If the fish is positive on IFAT, it is tested by VI and RT-PCR. When two fish are positive on two different tests and the mortalities due to ISA are above 0.05% per day, the cage is diagnosed with an ISA outbreak. By the time the mortalities have attained this level, the virus has had more opportunity to spread to another cage and to potentially another farm. It is possible that earlier diagnosis of ISA in a cage could be accomplished by testing more frequently and not relying on increased mortality rates.

Future work should include an in-depth analysis of the time points when ISA diagnostic tests became positive, the cage became an outbreak, the outbreak cage was eradicated, and the subsequent result of the rest of the cages on the outbreak farm and neighboring farms to help predict if a farm and its neighbors will be in danger of contracting ISAV if only the outbreak cage is removed and the other cages are allowed to remain on the farm.

7.3 Pollock study

Wild fish have been proposed as a viral reservoir because they are capable of close contact with farmed salmon. Laboratory studies have shown that brown trout and sea trout (*Salmo trutta*), rainbow trout (*Oncorhynchus mykiss*), and herring (*Clupea harengus*) tested positive for the virus weeks after intra-peritoneal injection. The wild

fish with the most contact with the farmed Atlantic salmon in New Brunswick farms are pollock (*Pollachius virens*). The Atlantic salmon in the sea cages are commonly overstocked, often greater than 30 kg/m^3 leading to increased stress and an increased contact rate between the salmon. Often there are more than a thousand pollock in the salmon cages increasing the already elevated stocking density and contact rate making viral spread highly likely once the virus has successfully infected a fish in the cage. Because of their close contact with the Atlantic salmon, it is important to know if the wild pollock living inside the farm cages are viral carriers.

Pollock cohabitating with ISA infected, farmed Atlantic salmon were tested for the presence or prevalence of ISAV to determine their potential as an ISAV reservoir. Kidney tissue from 93 pollock that were living with ISA infected salmon in five different sea cages were tested for ISAV with the reverse transcription-polymerase chain reaction (RT-PCR) test. Results yielded the expected 193 bp product for positive controls, while no product was observed in any of the pollock samples resulting in an ISA viral prevalence of 0%. The results of this study suggest that pollock are unlikely to be an ISAV reservoir for farmed Atlantic salmon. However, limiting the number of pollock in a salmon cage is recommended as they are carriers of other pathogens such as sea lice. The presence of pollock may also act as a stressor because of the increase in stocking density and an unfamiliar species introduced into the cage. Even though pollock are unlikely to be a viral reservoir for farmed Atlantic salmon, other wild fish commonly found in New Brunswick farm cages like cod, lumpfish (*Cyclopterus lumpus*), and Atlantic mackerel (*Scomber scombrus*) should be investigated to assess whether they can

be viral reservoirs as well as investigating the significance of the carrier state of herring and haddock to farmed Atlantic salmon.

7.4 Risk factor study

The integration of ISA research, vaccine technology, and established farming principles has led to some significant changes in salmon farming in New Brunswick. Despite the changes made by farmers, 2002 has been the worst year for depopulating farmed salmon due to ISA in the New Brunswick sites. This current risk factor study evaluated the associations between potential risk factors and ISA outbreaks given the current situation in the Atlantic salmon sites in New Brunswick. This was a multilevel study that evaluated both site and cage-level factors. The site level design was a retrospective cohort study of all sites that had fish stocked in the years 2000 and 2001, while the cage level design was a modified case-cohort study which included all case cages and a random cross-sectional sample of the cages. The questionnaire given to the producers was divided into site level questions, cage level questions, and hatchery information. Data was analyzed using logistic regression and survival analysis models.

The important factors identified by this study can be categorized as environmental, farmer controlled, or industry controlled according to the capacity to change or eliminate them. Environmental risk factors such as increasing the depth of the net (if nets were $\leq$9 m, odds ratio (OR) =3.34) and decreasing the depth of water underneath the net (if depth of water underneath the net >3 m, OR=3.34) are, for the most part, dictated by where the site is located. Having wild pollock, *Pollachius virens*, in the cage is usually a reflection of the number of wild pollock that live in the vicinity of the site and if there were $\geq$1000 pollock in the cage, the odds of disease in the cage increased 4.43 times. Important risk

factors that are under farm control are easier to change. These include increasing the number of times that the salmon are treated for sea lice (OR=3.31 if number of lice treatments are ≤2), transferring small smolts into seawater (OR=2.40 if smolts weighed>99g), and improving on the adaptation of smolts to seawater to reduce post transfer mortalities (OR=4.52 if there is one or more cages with post transfer mortalities >5%). The third category, industry controlled factors, needs to be addressed by the industry as a whole. Organizing boat travel to minimize the time and frequency of boats traveling to or by sites is currently being reviewed. This will be extremely important in controlling the spread of disease since the OR=9.43 if processing boats travel within 1 km of the site and the OR=4.03 if the site has dry feed delivered by the feed company. Because the hazard ratio increased stepwise from 1 if the nearest neighbor with ISA was ≥5 km up to 5.5 if the nearest site with ISA was within 0.5 km, increasing the distance between sites to increase the distance to the nearest neighbor with ISA may be necessary if elimination of the disease is ever to be realized. In addition to changing these factors, continued research potentially including randomized clinical trials to test the causal nature of these identified risk factors such as vaccination should be undertaken to help improve the current ISA situation in New Brunswick.

7.5 Antibody responses to vaccine study

Despite the introduction of an autogenous vaccine in the fish stocked in 1999, Atlantic salmon in New Brunswick farms are still suffering clinical disease attributed to the virus. Currently there are two different vaccines available for use in North America, one licensed produced by Aquahealth, Ltd. (Charlottetown, PE) and one autogenous produced by Bayotek International Inc. (Saanichton, BC). For this study, there were

three vaccines that were given including Aquahealth forte with autogenous inactivated ISAV (A+ISAV) and Bayoteck Multivacc4 with autogenous inactivated ISAV (B+ISAV) and the control vaccine, Aquahealth forte with Renogen (inactivated *Renibacterium salmoninarum*) without ISAV (A). Fish were blood sampled approximately every three weeks until seawater entry in April 2000. Sampling was discontinued until the salmon were acclimated to seawater. Blood samples were taken twice while in seawater, once in August 2000 and once in October 2000.

An enzyme-linked immunosorbent assay (ELISA) was used to assess the serum antibody levels. No ISAV specific antibodies were measured in the pre-vaccinated groups. The first post-vaccination serum samples were taken over one month later and there was very little antibody measured. There was one serum sample out of 15 that was anti-ISAV antibody positive in the B+ISAV group while there was one sample out of 15 that was suspect in the control group. There were no other antibodies detected from the fish sampled during the next three hatchery visits. The lack of response may be due to poor antibody responses when the hatchery water is cold or it is possible that the ELISA is not analytically sensitive enough to detect small increases in antibody levels. Another possibility is that the test has poor diagnostic sensitivity and is incapable of identifying serum samples with specific anti-ISAV antibodies.

There were minimal anti-ISAV antibody positive fish from the two times the fish were sampled in seawater. There was one anti-ISAV antibody positive fish in all groups and one suspect fish in groups A+ISAV and B+ISAV. There was less response in the October 2000 samples, with one positive serum sample in groups A and A+ISAV and one suspect in group A.

The few anti-ISAV antibody responses detected could be responses from three different possible mechanisms. The first possible mechanism is that they are specific antibody responses to the vaccines. The second possible mechanism is the antibodies could be responses to natural exposure to ISAV. The third possible mechanism is that they are false positive results due to an imperfect test.

Although these vaccines did not produce any measurable anti-ISAV antibodies in all but a few fish that were sampled, the vaccines may have produced some level of cellular immune responses. However, the measurement of cellular responses was beyond the scope of this study but should be examined to help determine the potential protection provided by vaccination. The fact that a few of the fish had antibodies during August and October of 2000, many months before clinical disease was evident on the farm, indicates that there may be some benefit of using the ELISA test as part of an early surveillance program. Another reason this ELISA may be well suited for disease surveillance is that vaccination does not seem to result in antibodies that will interfere with the identification of specific anti-ISAV antibodies due to natural challenge. However, it may also be possible that these positive results from the saltwater fish represent false positive results that are not related to exposure of the vaccine or virus. More work on the use of this test to monitor disease progression over time is warranted, but before this is undertaken, a formal evaluation of test performance including diagnostic sensitivity and specificity should be conducted to ensure proper identification of serum with specific anti-ISAV antibodies.

7.6 Conclusions and future research

Over the last 7 years, there has been much research on ISA in New Brunswick. There have also been many major changes to the way fish are farmed in New Brunswick in attempts to reduce the risk of disease. Despite the increase in our understanding of the disease and the changes that have been made, the disease is still a major cause of financial loss to the industry.

Epidemiological studies have resulted in new information regarding the disease. This information can be used to help reduce the impact ISAV has on the industry. Using the information from the diagnostic test evaluation combined with the information from the ISAV prevalence study, a better surveillance and control program could be designed to reduce ISA disease and its financial impact on the industry. Newly identified risk factors can be removed or modified to reduce the risk of disease for each farm.

Although our knowledge of the disease has expanded, there is still a need for more research to identify more ways of improving the disease situation. It is possible to take these studies one step further. For the evaluation of the diagnostic test, a retrospective dataset was used. An in depth evaluation of the tests using samples collected from known low prevalence, medium prevalence, and high prevalence populations and using traditional as well as latent class modeling techniques may provide more accurate estimates for the tests' performances. Following up the risk factor and vaccine studies, testing of particular risk factors such as ISAV vaccines by randomized clinical trials would answer the vaccine efficacy question. Another deficit in our ISA knowledge is cost-benefit analysis. Changing diagnostic testing protocols may improve virus detection and removal of cages may reduce the amount of the virus present, but whether or not they are cost effective should be analyzed. All of these efforts would be aided by the

establishment of an industry-wide record collection system. This would allow retrospective and prospective ISA studies without the extensive effort required for individual farm record collection. It would also provide some industry trends in which disease patterns and risk factors would be identifiable.

In addition to continued research, a coordinated effort with open communication by the government officials, veterinarians, site owners, site managers, and other members of the industry such as the hatchery owners and the processing plant managers are necessary to help improve the current ISA situation in New Brunswick.

8 Appendices

8.1 Appendix A: Risk Factor Study survey for ISA positive sites

2002 ISA Risk Factor Study for the New Brunswick Atlantic salmon farms

Instructions for the interviewer:

Please fill in the information below:

Name of person to be interviewed _____

Position of person to be interviewed _____

Office phone number of person to be interviewed _____

Cell phone number of person to be interviewed_____ Fax number _____

Date planned for interview _____

Time_____

Place to be interviewed _____

What is your current year class? 2001 or 2002

How many cages does your site have? (for the year class of interest) ____

What are/were the cage numbers? _____

This site is a (circle) a) an ISA positive site b) an ISA negative site (for the year class of interest)

If this is a positive site, which cages are positive? _____

Case cages # ____ and ____ or Not applicable

Control cage #'s (randomly picked out of a hat from ISA negative cages) _____ _____ _____ _____

(two cages if only one infected cage, three cages if an ISA negative site, four cages if two case cages)

Please inform the interviewer of these issues:

- ❑ This questionnaire is designed to identify potential factors that may increase the likelihood of a cage becoming infected by ISA. We feel that identifying risk factors will permit us to make recommendations that will benefit your site and the entire industry.

- ❑ This project is being supported by the NB Department of Agriculture, Fisheries, and Aquaculture and is being conducted by the Atlantic Veterinary College. We plan to survey all sites.
- ❑ If the site had 2000 year class fish, information up until harvest will collected. If the site has 2001 year class, information up until the time of the interview will be collected.
- ❑ All information will be used in a confidential manner that will keep your information from being identified as belonging to a particular company or site.
- ❑ This study will not cost you any money, just an hour of your time.
- ❑ Please be prepared to discuss many characteristics of the site of interest including cage types, feeding, smolt history, health concerns, and mortality records
- ❑ Records that you should bring to our meeting should include:
    - O Smolt history and transfer records
    - O Mortality records for the cages of concern
    - O Health records including medications used to treat fish
    - O Records on weight sampling
    - O Records on net changes
    - O Records on feeding
    - O Records from the processing plants

Information to be collected before the interview:

- ❑ Site map for the year class of interest
- ❑ Weekly mortality records for all cages from sea water transfer to harvest or current
- ❑ Smolt records including total number entered, first thirty day mortalities, and transfer dates for all cages
- ❑ Medication records (name of drug and dates given) from sea water transfer to harvest or current
- ❑ Harvest reports for cages of interest

Please fax this information to me at 902-566-0823 and have a copy available for the interviewer

A. Interview information (Please remind interviewee that all questions are for up to the time of harvest of the infected cage)	*Code*
Date _____	
Company _____	
Site Number MF_____	
Site name? _____	
Interviewer _____	
Person being interviewed _____	
Position _____	
DAFA bay management number ____	
I have collected weekly mortality records and available harvest records from all three cages [1]Yes or [0]No	
I have collected records on when drugs were administered and for what reason for all three or cages [1]Yes [0]No	
I have collected smolt records including total number entered, first thirty day mortalities, and transfer dates for all cages. [1]Yes [0]No	
I have collected a site map with all cages identified [1]Yes or [0]No	
B. Company information	
Records are mainly kept by? [1] Superior or a corporately designed or computer spreadsheet [2]Handwritten and placed into files [3]APHIN [4]Other _____	
How many total sites does your company have in New Brunswick? _____	
How many total sites does your company have in Nova Scotia? _____	
How many total sites does your company have in Maine? _____	
C. Area and site information	
Number of cages at the site? _____	
All cages at site:	

Type	12 m^2	15 m^2	50 m PC	70 m PC	90 m PC	Other: specify [030] ____
Total #	[018]	[020]	[022]	[024]	[026]	[028]

depth of nets (m)	019	021	023	025	027	029	

Seawater transfer occurred when? [1]S2000 or [2]F2000 or [3]S2001 or [4]F2001

How close do processing boats (harvest barges) travel past your site when headed to the wharf?

[0] They do not pass by our site
[1]Within 100 meters
[2]Within 500 meters
[3]Within 1 km
[4]Within 2 km

Description of boats carrying harvested fish (boat name, owner, type, etc.)?

Name of wharf or wharves to which processing boats are traveling?

_____

How close is your nearest neighbor with ISA?
<0.5 km[1] <1 km[2] <2 km[3] <5 km[4] >5 km[5] Don't know [-2]

D. Health Parameters

Site Veterinarian?

[1]Steve Backman
[2]Leighanne Hawkins
[3]Dan MacPhee
[4]Julia Mullins
[5]John O'Halloran

Does the veterinarian or veterinarian's assistant visit the site regularly? [1]Yes or [0]No

How often does the veterinarian or veterinarian's assistant visit the site?

[1]once a week
[2]once every two weeks
[3]once every month
[4]once every 6 weeks
[5]only when called
[6]other _____

Previous to the year class we are investigating, what was the last year the site was diagnosed with ISA?

[0]Never or [1]1997 or [2]1998 or [3]1999 or [4]2000

Has BKD (Bacterial Kidney Disease) been detected at this site in the 2000 or 2001 year class?

[1]Yes or [0]No

Have there ever been any algal blooms for this year class, if so how many? _____

How many periods of extreme low oxygen has this year class suffered (including algal blooms)? _____

E. Feed History

How are fish fed? [1]Feed Blowers or [2]Hand fed or [3]Combination blower and hand feeding or [4]Automatic feeder on timer

How many weeks were fish on moist feed after seawater transfer? _____

How was moist feed delivered to your site?

[1]Delivered by feed company by feed company boat/barge
[2]Delivered by farm owned feed delivery boat/barge
[3]Delivered by contract feed delivery boat/barge
[4]Picked up at wharf by farm boat

Name of boats that bring the moist feed to your site? _____

Did this boat visit more than your site on the days of delivery? [1]Yes or [0]No

Name of wharf that moist feed leaves from? _____

How was dry feed delivered to your site?

[1]Delivered by feed company by feed company boat/barge
[2]Delivered by farm owned feed delivery boat/barge
[3]Delivered by contract feed delivery boat/barge
[4]Picked up at wharf by farm boat

Name of boats that bring the dry feed to your site? _____

Did this boat visit more than your site on the days of delivery? [1]Yes or [0]No

Name of wharf that dry feed leaves from? _____

DATE STARTED	DATE ENDED	COMPANY	BRAND	TYPE (MOIST OR DRY)	SUPPLEMENT	REASON FOR CHANGE
043	044	045	046	047	048	049
050	051	052	053	054	055	056
057	058	059	060	061	062	063
064	065	066	067	068	069	070
071	072	073	074	075	076	077

If the feed chart is not convenient, please fill in feed history in space provided making sure that chart's information is included in your write-up.

F. Equipment and Personnel

Is there a policy about not sharing equipment with other sites? [1]Yes or [0]No	
Does this site share equipment with other sites? [1]Yes or [0]No	
Does it share boats? [1]Yes or [0]No	
Does it share a roller/crane barge? [1]Yes or [0]No	
What else does it share? _____	
Is there a policy about not sharing personnel with other sites? [1]Yes or [0]No	
Does this site share personnel with other sites? [1]Yes or [0]No	
How many staff would visit other sites in a typical month? _____	
What product is used for the foot baths? [1]Iodine based disinfectant [2]Vircon [3]Bleach [4]Other _____	
Is there a policy about no visitors allowed on the site? [1]Yes or [0]No	
Do visitors (not employed by company) visit the site? [1]Yes or [0]No	
G. Diver	
Does this site have a contract diver or a staff diver? [1]Contract or [2]Staff	
Does this diver dive at other sites? [1]Yes or [0]No	
Does the diver have a separate dry suit for each site? [1]Yes or [0]No	
Does the diver disinfect between cages? [1]Yes or [0]No	
How does the diver disinfect between cages? [0]He does not disinfect between cages [1]Complete submersion in disinfectant [2]Sprayed with disinfectant [3]Pours disinfectant on himself [4]Other _____	
Is there a separate mortality bag for each cage? [1]Yes or [0]No	
What are the mortality bags disinfected with? [1]Iodine based disinfectant [2]Vircon [3]Bleach [4]Other _____	
How often are mortality dives performed during January? [1]Twice a week [2]Once a week [3]Every two weeks [4]Once a month [5]Other _____	
How often are mortality dives performed during August? [1]Twice a week	

[2]Once a week [3]Every two weeks [4]Once a month [5]Other _____	
How often do dives occur in times of consistently elevated mortality? (>20 mortalities per dive in a cage) [1] Every day [2] Three times a week [3] Twice a week [4] Once a week [5] Every two weeks [6]Other _____	
How are mortalities disposed of? [1]Brought to Connors fish meal plant [2]Sent to a processing plant for rendering (used to feed other animals) Which one? _____ [3]Brought to compost facility in New Brunswick Which one? _____ [4] Brought to compost facility in Maine Which one? _____ [5]Other _____	
H. Smolt History	
What was the start date of transfer? _____	
What was the end date of transfer? _____	
How long was the fallow period? [0]There was no fallow period [1]Two weeks or less [2]One month or less [3]Two months or less [4]Two to four months [5]Greater than four months	
When the smolt left the hatchery, were the boxes oxygenated? [1]Yes or [0]No	
What was the method of smolt transfer? [1]Well boat or [2]Boxes on a barge or [3]Both	
Name(s) of Well boat or barges used for transfer? _____	
If the smolt were transferred by well boat, were they oxygenated? [1]Yes or [0]No or [2]N/A	
What was the name of the wharf the smolts left from? _____	
During well boat haul, where was the water taken for the holding tanks? [0]Not applicable (smolt were transferred in boxes)	

[1]Within 100 m of the wharf [2]Within 500 m of the wharf [3]Within 1 km of the wharf [4]Within 2 km of the wharf [5]Greater than 2 km of the wharf	
What type of barge was used during transfer? [0]Not applicable (smolt were transferred by well boat) [1]An on-site working barge/scow [2]A harvest barge [3]A feed barge [4]A ferry [5]Other _____	
In the two weeks prior to transfer, the barge was used for what other jobs?	
The barge was used to pick up feed? [1]Yes or [0]No or [-2]Don't know	
The barge was used for harvest? [1]Yes or [0]No or [-2]Don't know	
The barge was used at a different site? [1]Yes or [0]No or [-2]Don't know	
Other? _____	
What was the total % mortality during the immediate post transfer period (30 days) for this site? _____	
How many cages experienced more than 5% loss during the first 30 days of seawater entry? ___	
I. *Holdovers (fish from previous year class that remain on site after new smolt class have been entered on the site)*	
How many cages have/had fish heldover from the previous year class? _____	
What % of the total fish of that year class were heldover? _____	
What was the year class of fish that was held over? There were no holdovers[0] 1998 year class[1] 1999 year class[2] 2000 year class[3]	
How long were holdovers on the site after smolt transfer? [0]There were no holdovers [1]Two weeks or less [2]One month or less [3]Two months or less [4]Greater than two months	
J. Predators	
Have there been any known seal attacks at this site? [1]Yes or [0]No	

Which cages were more severely or more often affected? _____	
Have there been any otters seen in the cages on this site? [1]Yes or [0]No	
Which cages were affected? _____	
K. Weight Sampling	
How often are weight samples taken at your site? [0]never [1]once a month [2]once every two months [3]once every three months [4]other _____	
How are weight samples conducted when fish are <1 kg? [0]Not applicable (weight samples not done) [1]anesthesia and weighing on a scale [2]video camera and estimates [3]Infrared (AKVAsmart or VAKI) measurements and estimates [4]Other _____	
How many fish are usually weight sampled from each cage at this time? _____	
How are weight samples conducted when fish are > 1 kg? [0]Not applicable (weight samples not done on this site) [1]anesthesia and weighing on a scale [2]video camera and estimates [3]Infrared (AKVAsmart or VAKI) measurements and estimates [4]Other _____	
How many fish are usually weight sampled from each cage at this time? _____	
L. Wildfish	
When you harvest cages at this site, approximately how many Pollock are in each cage? [1]0-9 or [2]10-99 or [3]100-999 or [4]>999	
M. Net Care	
Are the nets treated with an anti-fouling agent? [1]Yes or [0]No	
Do you clean the nets in the water? [1]Yes or [0]No	
Do you use an Idema power washer (underwater disc remover) [1]Yes or [0]No	
Do you use manual removal by diver or staff [1]Yes or [0]No	
How else do you clean the nets while in the water? _____	

P. Site Practices Regarding Sea Lice Counting and Treatment	
What is your company policy regarding lice counts? [1] every cage [2] selected cages [3] other _____	
What is your company policy for frequency of lice counts conducted? [1] weekly [2] biweekly [3] every three weeks [4] once a month [5] every six weeks [6] other _____	
What is the number of fish sampled per cage when conducting lice counts? [1] 5-10 [2] 10-20 [3] 20-30 [4] 30-40 [5] 40-50 [6] 50+	
Are fish anesthetized when conducting lice counts? [1]Yes or [0]No	
If yes, what type of anesthetic is used? [1] TMS [Please circle : Definite or Probable] [2] Aquacalm (Metomidate) [3] Clove oil/Eugenol [4] other _____	
Are sea lice grouped into categories when sampling (ie. Chalimus, preadult, adult, gravid females)? [1]Yes or [0]No	
If No, Why not? Please explain _____	
Are there one or more persons at the site trained to identify chalimus vs. preadult stages? [1]Yes or [0]No	
If sea lice are grouped into categories when sampling which groups are used?	
Copepodid [1]Yes or [0]No	
Chalimus [1]Yes or [0]No	
Preadult [1]Yes or [0]No	
Adult [1]Yes or [0]No	
Gravid Female [1]Yes or [0]No	
Herring Lice (Caligus) [1]Yes or [0]No	
Other _____	
What is the decision to treat for sea lice based on? [1] lice counts [2] general fish appearance [3] recommendation by the site veterinarian [4] expected problem	

Explain: _____	
Who makes the final decision to treat for sea lice? [1] Site manager [2] Site Veterinarian [3] Site Owner [4] other _____	
Have there been any treatments for sea lice on your site in the past two years? [1]Yes or [0] No	
In the past 2 years, which treatment methods have been used at this site (choose all that apply) [1] not applicable (no treatment has been given) [2] tarp (full ensclosure) [3] skirted (open bottom) [4] no tarp [5] in-feed [6] other	
What is the method of sea lice control generally used on your site to treat <u>smolt</u>? [1] SLICE® (Emamectin Benzoate) [2] Salmosan®(Azamethiphos) [3] Calicide® (Teflubenzuron) [4] Hydrogen Peroxide [5] Ivermectin [6] Other _____	
What is the method of sea lice control generally used on your site to treat <u>pre-market salmon</u>? [1] SLICE® (Emamectin Benzoate) [2] Salmosan®(Azamethiphos) [3] Calicide® (Teflubenzuron) [4] Hydrogen Peroxide [5] Ivermectin [6] Other _____	
Site lice count dates: ☐ Check with site vet or applicable counter: _____	
1st _____	
2nd _____	
3rd _____	
4th _____	
5th _____	
6th _____	
7th _____	
8th _____	

9th _____	
10th _____	
11th _____	
12th _____	
other dates: _____	
What was the time to first treatment for sea lice on your site (time from smolt transfer to first treatment for sea lice)? _____	
O. Unusual circumstances and brief description of events before outbreak	
Unusual circumstances and brief description of events occurring before the outbreak	

Cage level data (please read "Now I am going to ask you questions regarding the specific cages) Please continually remind interviewee that information should be collected up until harvest time of the ISA infected cage.	***Code***		
	Case cage # ____	Con cage # ____	Con cage # ____
C. Area and site information			
Type of cage? [1]12 m^2 [2]15 m^2 [3]50 m PC [4]70 m PC [5]90 m PC [6]Other:_____			
How many meters are under the bottom of the net at low tide? _____			
How deep is the net (in meters)? _____			
D. Health Parameters ***("up until ISA infected cage removal...")***			
How many times had the fish been treated for lice since seawater entry?___			
How many times was Ivermectin used? _____			
How many times was Emamectin (Slice) used? _____			
How many times were bath treatments used? _____			

Had the fish in this cage ever been treated with antibiotics? [1]Yes or [0] No			
How many times had the fish been treated for skin sores? _____			
How many times did you treat the skin sores with TM Aqua (oxytetratcycline)? _____			
How many times did you treat the skin sores with Aquaflor (florfenicol)? _____			
How many times did you treat the skin sores with Romet 30 (sulphadimethoxine:ormetoprim)? _____			
How many times did you treat the skin sores with Tribrissen (sulphadiazine:trimethoprim)? _____			
How many times did you treat the skin sores with Amoxicillan? _____			
Had BKD been detected or diagnosed in this cage? [1]Yes or [0]No			
How many times had the fish been treated for BKD? _____			
Had there been any gill parasites since seawater entry? [1]Yes or [0]No			
How many times had the fish been treated for Gill disease since seawater entry? _____			
Had this cage been graded for grilse (sexual maturity)? [1]Yes or [0]No			
If yes, when was this cage graded for grilse (sexual maturity)? (dd/mm/yy) _____			
H. Smolt History			
Saltwater transfer year class? [1]S00 or [2]F00 or [3]S01 or [4]F01			
Total # smolts transferred into cage? _____			
Was this cage [1]Single stocked or [2]Double stocked or [3] Other (specify)_____?			
Date(s) transferred to sea? _____			
Hatchery source _____			
Hatchery Manager (or contact) and phone number _____			
Type of hatchery? [1]Flow through [2]Recirculation hatchery [3]Reuse (some water reused without a biofilter)			
If a flow through or reuse hatchery, what is the water source? [1]Lake/River water [2]Well water [3]Spring (Artesian well) water			

Average weight of smolt when stocked? _____ (in grams)			
Condition of smolts at transfer? Healthy, normal? [1]Yes or [0]No			
Unusually small? [1]Yes or [0]No			
Ungraded (wide range of sizes)? [1]Yes or [0]No			
Weak? [1]Yes or [0]No			
High degree of fin rot? [1]Yes or [0]No			
High degree of spinal or jaw deformities? [1]Yes or [0]No			
Other _____			
What was the last day the smolts were vaccinated? _____ (dd/mm/yy)			
What company supplied the vaccine? [1]Aqua Health [2]Bayotek (Microtek) [3]Other _____			
Was ISA virus included in the vaccine? [1]Yes or [0]No			
Full name of Vaccine? _____			
Was BKD bacterin (Renogen vaccine) included in the vaccine? [1]Yes or [0]No			
Number of degree days from the time of vaccination until the sea water entry? _____			
Were fish vaccinated by immersion as pre-smolt or fry? [1]Yes or [0]No or [-2]Don't know			
If Yes, which disease was the vaccine for? _____			
J. Predators **("up until ISA infected cage removal...")**			
Had there been any known seal attacks at this cage? [1]Yes or [0]No			
How many days were the seals a problem at this cage? _____			
Had there been any otters seen in the cages in this cage? [1]Yes or [0]No			
How many days were the otters a problem in this cage? _____			
K. Weight Sampling **("Prior to ISA infected cage removal...")**			
When was the last weight sample done on this cage? _____ (dd/mm/yy)			
How many fish were sampled? _____			
What was the average weight on that sample? _____			
What was the standard deviation on that sample? _____			

L. Net Care **("Prior to ISA infected cage removal...")**			
How many times were the nets changed on this cage?			
Was the net changed on this cage in last 90 days prior to disease outbreak of infected cage? [1]Yes or [0]No			
Was the net cleaned during the last 90 days prior to disease outbreak of infected cage? [1]Yes or [0]No			
M. Wild Fish ***("Prior to ISA infected cage removal...")***			
How many wild fish or were present in this cage:			
Pollock [1]0-9 or [2]10-99 or [3]100-999 or [4]>999			
Other (specify) _____			
[1]0-9 or [2]10-99 or [3]100-999 or [4]>999			
Had there been wild fish mortalities removed from this cage by the diver? [1]Yes or [0]No			
Pollock [1]0-9 or [2]10-99 or [3]100-999 or [4]>999			
Other (specify) _____			
[1]0-9 or [2]10-99 or [3]100-999 or [4]>999			
Which month had the most mortalities? _____			
Q: Information regarding sea lice treatment for specific cages			
Were these cages treated in the hatchery with emamectin prior to smolt transfer? [1]Yes or [0]No			
What was the date of the first sea lice treatment for these cages post transfer? (dd/mm/yy) _____			
What method(s) of treatment application have been used on these cages since smolt transfer? [1] tarp (full ensclosure) [2] skirted (open bottom) [3] no tarp [4] in-feed [5] other _____ [6] not applicable (no treatment has been applied)			

What method(s) of sea lice control have been used on these cages since smolt transfer? [1] SLICE® (Emamectin Benzoate) [2] Salmosan®(Azamethiphos) [3] Calicide® (Teflubenzuron) [4] Hydrogen Peroxide [5] Ivermectin [6] other _____ [7] not applicable (no treatment has been applied)			
What was the water temperature at the time(s) of application? _____			
What was the length of the treatment(s) applied (in minutes, hours, days)? _____			
R: Information regarding sea lice counts for specific cages			
NOTE: If the site does not have information regarding lice counts indicate whom we should contact to obtain such information: ☐ Site Veterinarian _____ ☐ Site Personnel _____ ☐ other _____			
What was the date of the first sea lice count for this cage?_____			
1st sea lice count information:			
# of Chalimus _____			
# of Pre-adults _____			
# of Adults _____			
# of Gravid Females _____			
# of Herring Lice (Caligus) _____			
What was the date of the second sea lice count for this cage?_____			
2nd sea lice count information:			
# of Chalimus _____			
# of Pre-adults _____			
# of Adults _____			
# of Gravid Females _____			
# of Herring Lice (Caligus) _____			
What was the date of the third sea lice count for this cage?_____			
3rd sea lice count information:			
# of Chalimus _____			
# of Pre-adults _____			
# of Adults _____			

# of Gravid Females _____			
# of Herring Lice (Caligus) _____			
What was the date of the fourth sea lice count for this cage?_____			
4th sea lice count information:			
# of Chalimus _____			
# of Pre-adults _____			
# of Adults _____			
# of Gravid Females _____			
# of Herring Lice (Caligus) _____			
If more than 4 counts per cage, please enter information on another piece of paper.			
N. Unusual circumstances and brief description of events before ISA outbreak			
Unusual circumstances and brief description of events occurring before the outbreak (ie: anything that distinguished this cage from others at site or anything happen at this site?)			
O. Final result of the cage			
Date cage diagnosed with ISA? _____		XXX	XX X
Date of letter from the Minister ordering slaughter?		XXX	XX X
Beginning date cage was harvested? _____ (dd/mm/yy)			
Ending date cage was harvested? _____ (dd/mm/yy)			
Where were the fish slaughtered? [1]On a barge specifically used for harvest [2]On a site working barge/scow [3]On a feed barge or boat used for feed delivery [4]On a boat used for fishing or non-farm activities [5]Other _____			
Who slaughtered the fish? [1]Site staff or [2]Contract company			
How were the fish slaughtered? [1]CO_2 and gilled [2]Chilled and gilled			

[3]Other _____

Was the blood water contained on the harvest barge? [1]Yes or [0]No

What percent of the blood water was actually contained? (estimate)
- [1]>95%
- [2]>90%
- [3]>75%
- [4]<75%

What was the name of the boat used for harvest? _____

Where were the fish processed?
- [1]Heritage Salmon in Black's Harbour
- [2]Limekiln fisheries (Ocean Legacy) in Limekiln
- [3]Stolt Sea Farms (Sterling) in St. George
- [4]Jail Island Salmon in St. George
- [5]Cooke Aquaculture (True North) in St. George
- [6]Atlantic Silver in St. George
- [7]Deer Island Salmon (DIS)
- [8]Other _____

Total number of fish harvested from this cage for each weight group?

Cage #	2-4 lbs.	4-6 lbs.	6-8 lbs.	8-10 lbs.	>10 lbs.
210	211	212	213	214	215
216	217	218	219	220	221
222	223	224	225	226	227

Average weight of fish at harvest from this cage?

_____

Total number of fish in each grade category?

Cage #	Top grade	Standard grade	Down grade
229	230	231	232
233	234	235	236
237	238	239	240

Total number of fish downgraded for being grilse or sexually mature?

_____

G. Mortality and fish movement data after transfer:
(please add additional information on back of this form)

Case Cage # _____

Code	Event
1	Mortalities
2	Additions due to split
3	Subtraction due to split
4	Normal harvest
5	Harvest due to disease
6	Other (describe)

Date of event	Code of event	Number fish +/-	Cumulative total	Comment (including testing data)